Factors that enhance the ability of *Pseudomonas aeruginosa* to resist the action of antibiotics

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Declaration

I declare that this thesis entitled Factors that enhance the ability of *Pseudomonas aeruginosa* to resist the action of antibiotics is my own work, and that neither the whole nor part has been submitted to another university.

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July 2016

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Abstract

P.aeruginosa is one of the most important pathogens in nosocomial infections and fails to respond to standard treatment, particularly in the case of patients subjected to prolong antibiotic treatment. To generate a more comprehensive understanding of the failure of antimicrobial treatment, focusing especially on the adaptive resistance could be the key area that the bacterium develops in this phenomenon. Most studies on antibiotic resistance in *P.aeruginosa* have focused upon genotypic studies. This study set out to develop an *in vitro* model to examine the effect of continual exposure of *P.aeruginosa* PA01 to the antibiotics studied. Experiments were initially conducted to consider the factors that having a significant influence on antibiotic susceptibility using a novel fluorescence based assay (OxoPlate® system). *P.aeruginosa* was subjected to the action of tobramycin, amikacin and colistin under various environmental factors. The results of the *in vitro* analysis showed that, from among the three antibiotics used, amikacin was the antibiotic where resistance was most readily developed. From these results, chemostat studies were designed to examine prolonged exposure of the antibiotic to planktonic cells. Chemostat cultures were exposed to amikacin at sub-inhibitory concentrations using Evans defined synthetic medium at different dilution rates (*D*) under glucose limitation. Both cultures grown at $0.025h^{-1}$ and $0.06h^{-1}$ developed the following characteristics i. low-level amikacin resistance, which exhibited an increase in the MIC 4-fold. ii. a clear development of phenotypic resistance and this resistance was not acquired as evidenced by the loss of resistance on culture into fresh medium lacking antibiotic. iii. adaptive resistance to amikacin conferred low-level resistance to other aminoglycosides such as tobramycin and antibiotics with different modes of action such as colistin. Low oxygen availability was seen in the cultures grown at 0.099 h^{-1} and 0.125 h^{-1}, which lead to i. the appearance of the so called "persister" phenotype. These persisters are sub populations of cells that showed a reduction in bacterial cell size as evidenced from the flow cytometry output as well as being slow growing and resistant ii. extracellular polymeric fibrils were produced in the cells derived after 72h incubation time. In all cases, continual exposure resulted in phenotypically distinct mucoid and non- mucoid colony morphotypes, which were clearly observed on amikacin-free nutrient agar. Some of these selected morphotypes showed from the MIC and MBC data a high-level resistance to the antibiotic when left without

antibiotics. The biological responses resulting from these studies offer valuable clues underlying unsuccessful treatment. Conducting experiments using robust systems renders this project extremely novel in the field of microbiology and this will contribute to the development of viable treatment options and ultimately the reduction of the emergence of antibiotic resistance.

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Abbreviations

AGPs	Antibiotic Growth Promoters
APUA	Alliance for the Prudent Use of Antibiotics
Appx.	Appendix
AST	Antimicrobial susceptibility tests
CF	Cystic fibrosis
CFUs	Colony forming unites
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
dO_2	Dissolved Oxygen
EU	European Union
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
E-test	Epsilometer test
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular Polymeric Substance
FSC	Forward Scatter
h	Hours
ICAAC	Interscience Conference on Antimicrobial Agent and Chemotherapy
LPS	Lipopolysaccharide
MBC	Minimum Bactericidal Concentration
MHA	Muller-Hinton Agar
MHB	Muller-Hinton Broth
MIC	Minimum Inhibitory Concentration
Min.	Minute
NA	Nutrient Agar
NB	Nutrient Broth
OD	Optical Density
PCR	Polymerase Chain Reaction
pO_2	Oxygen partial pressure
QS	Quorum Sensing
rpm	Revolutions Per Minute
RMS	Resistant Mutant Subpopulations
RSCVs	Rugose Small Colony Variants
SCVs	Small Colony Variants

SEM	Scanning Electron Microscopy
T	Temperature
TAE	Tris base, Acetic acid and EDTA
TE	Tris and EDTA
USA	United Stands of America
UK	United Kingdom
UV	Ultra violet
V	Volt
V/V	Volume/Volume

Nomenclature

D	Dilution rate (h^{-1})
D_c	Critical dilution rate
F	Flow rate (h^{-1})
Ks	half-rate saturation constant
S	Substrate concentration
S_r	Limiting nutrient concentration in the feed container
td	Doubling time
V	Volume
x	Biomass concentration in the fermenter
μ	Specific growth rate
μ_{max}	Maximum specific growth rate

Chapter 1
Literature Review

1.1. Introduction

It was only by chance that, in 1928, whilst studying bacteria in his laboratory at St Mary's Hospital in London Alexander Fleming discovered that bacteria could be destroyed by the mould-produced substance penicillin, due to the accidental contamination of his petri dish with mould. In a little over a decade after this discovery, drugs containing penicillin were prepared by Fleming and other researchers for administration in the treatment of bacterial infections. Subsequently, other antibiotics were created, paving the way for the unprecedented advancements made in healthcare and medicine during the 20th century. The discovery of antibiotics improved the efficiency of treatment of diseases associated with a high prevalence and high mortality rate, including pneumonia and tuberculosis (Cars et al., 2011, Stover et al., 2000). Recently, however, many strains of bacterial pathogens which could previously be killed with effective antibiotics have become resistant to these drugs (Canton and Morosini, 2011). In fact, the problem with antibiotic resistance has globally increased since the 1940s (Partridge, 2011). However, the widespread use of antibiotics led to the appearance of bacterial strains that could resist the drug effects. Therefore, antibiotic resistance is considered to be the greatest problem faced by the healthcare sector in the present modern era (Cars et al., 2011). From a microbiological point of view, this resistance refers to bacterial pathogens which have a resistance mechanism, making them less susceptible to antibiotics. Clinically, resistance is viewed as a clinical criterion of treatment in patients who are infected by specific bacterial pathogens which are difficult to treat (Turnidge and Paterson, 2007).

In more recent times, antibiotic resistance has become more extensive, and is now considered to be among the gravest threats to healthcare in communities as well as in hospitals (Rice, 2009, Witte, 1998, Aarestrup, 2005). Human activities, such as agricultural practice and frequent use and abuse of antibiotics in the treatment of

human and animal conditions, are deemed to be the main cause for the development of antibiotic resistance at a global level (Witte, 1998, Aarestrup, 2005, Ferber, 2000, McManus et al., 2002, Cabello, 2006). The selection of resistant mutants in bacterial strains is a logical consequence of the use of antibiotics at high concentrations. However, it is not clear to what extent mutant selection in a polluting environment is fostered by low antibiotics concentrations. Such antibiotics are naturally produced by microorganisms or introduced by human or animals during use of antibiotics for therapeutic or growth stimulation purposes (Kummerer, 2009, Thiele-Bruhn, 2003, Chander et al., 2005).

Due to its complexity, the issue of antibiotic resistance must be addressed from different angles (Levy and Marshall, 2004). The increase in emergence of antibiotic resistance is believed to be promoted first and foremost by exposure to antibiotics. Thus, there are major concerns regarding the dangers of microbes being 'trained' to become highly resistant to drugs (Singer et al., 2003). It has been argued that the extensive presence of antibiotics in the environment enables bacteria to acquire antibiotic resistance (Guilfoile, 2007). This issue has been particularly observed in animal husbandry, where antibiotics are routinely used for promoting growth. In a review by Phillips in 2007 it was reported that in Denmark the resistance to the antibiotic streptogramin had dropped from 40–60% in the late 1990s to 16% in pigs and 13% in chickens after antibiotic growth promoters had discontinued in this country. A considerable quantity of antibiotics are naturally eliminated by humans and animals after ingestion, ending up in the sewage treatment centres and finally in rivers or lakes (Guilfoile, 2007). There is important evidence that support prolonged drug exposure may lead to the development of antibiotic resistance in bacteria present in the environment. The US geological survey revealed that antibiotics were found in 22% of the rivers that were analysed (Kolpin et al., 2002). What is more, alarming is that bacteria can develop resistance even when exposed to low concentrations of antibiotics. The subpopulations of cells that survive under these conditions are selected for because they have mutations that give the cells an advantage over the general population. Because of this advantage they dominate and essentially become selected (Canton and Morosini, 2011). This process facilitates the exposure of an immense number of bacteria to the variety of different types of antibiotics (Guilfoile, 2007). Thus, resistant bacteria may arise and find themselves

in a human clinical setting as a result of using antibiotics in this way, leading to antibiotics being banned in some countries for use in animal husbandry (Singer et al., 2003). The resolution of the European Union to forgo the practice of accelerating the growth of food animals with the use of antimicrobials was revised in May 2010 by the Alliance for the Prudent Use of Antibiotics (APUA). This committee was composed of 16 representatives from 10 European Union nations and overseen by Herman Goossens from the University of Antwerp, Belgium and by Christian Greko from the National Veterinary Institute in Uppsala, Sweden. A number of European Union (EU) member nations have interdicted the use of antibiotic growth promoters (AGPs) in food animals, including Sweden, Denmark and Great Britain (Cogliani et al., 2011). In a review by Phillips in 2007 it was reported that since 1986, antibiotic growth promoters had been withdrawn from Sweden. In 1995, the rest of the EU banned the use of growth promoting antibiotics. Based on the Precautionary Principle basis, other growth promoters such as bacitracin, spiramycin, tylosin and virginiamycin were also discontinued from the middle of 1999 in the whole of the E U. The frequency of antibiotic resistance among bacterial pathogens is high, and is on the increase due to the reportedly intensive use of antibiotics (Partridge, 2011). Considering the connection between the use of antibiotics and acquisition of resistance, bacteria can prove to be seriously dangerous to human life. The results of two European studies proved particularly enlightening with regards to the link between the exposure of bacteria to antibiotics and the development of resistance. The first study, conducted on wild mice, discovered that a large number of antibiotic resistant bacteria could be isolated from these rodents living in wooded areas in England (Gilliver et al. 1999 cited in Guilfoile, 2007). The second study, carried out in Finland, examined the bacteria found in the intestines of a number of species of mammals and revealed that the level of antibiotic resistance of the bacteria was low (Osterblad et al. 2001 cited in Guilfoile, 2007). It was concluded that the factor which determines the level of antibiotic resistance of bacteria is related to the proximity to people (Guilfoile, 2007). Undoubtedly, there is a strong relationship between the emergence of resistance and antibiotic use, as observed in community environments and hospitals (Canton and Morosini, 2011).

The global spread of antimicrobial resistance has resulted from the use of several million tons of antibiotics in both clinical and agriculture sectors (Chait et al., 2012).

Nearly twelve tons of antibiotics are globally used every hour, with many being administrated without proper regulations or prescriptions (Harbarth et al., 2015). The rapid speed of resistance evolving throughout the bacterial population is the greatest risk to effective bacterial infection treatment (Cars et al., 2011). In fact, an increase in antimicrobial resistance worldwide is a significant source of global health concern. Antibiotic resistant microorganisms are considered to be one of the most significant life-threatening issues for public health, which must be experimentally addressed (Chait et al., 2012). Many of these links between antibiotic use and resistance have been deduced from *in vivo* studies. What is lacking is an *in vitro* approach to link the overuse of drugs to resistance. In addition, a robust model system would allow us to investigate other contributory factors resulting in resistance.

1.2. An overview of antibiotic resistance

For all of scientists, antibiotic resistance has been an essential issue of concern since the very beginning of antibiotic use. Around 162512 publish papers were listed by PubMed in June 2016, those which had the expression “antibiotic resistance” iterated under “any field”. Genetic elements and/or chromosomal genes were the focus of concern in the majority of these publications. A number of viable reasons are present to justify the considerable amount of attention that has been devoted to inherited antibiotic resistance. Alongside being a significant health issue, it has shown no signs of improving with the passage of time (Rubin et al., 1999, Lipsitch, 2001, Levy and Marshall, 2004, Fuller and Low, 2005, Cosgrove, 2006). Researchers find inherited antibiotic resistance to be particularly interesting as it has a number of special characteristics. Various studies such as those regarding mathematical modelling, biochemistry, physiology, molecular biology, and bacterial genetics have been conducted. The hereditary resistance, which resist the action of the most of antibiotics, has proved to be an important influence of motivation for the pharmaceutical industry to discover and develop enhanced and advanced medicines (Andriole, 2005), that have the characteristics for defeating the resistance mechanisms (Lomovskaya et al., 2001, Lomovskaya and Bostian, 2006).

In spite of significant progress being made in term of treatment options and the improvement of available antibiotics, it has shown no long term solution to this issue (Sandoval-Motta and Aldana, 2016).

Even though such inherited resistance is particularly important for clinical reductionism, it is not considered to be the only route to resistance. This is supported by the recent study by Feng et al. (2016), who showed that a minimal fitness cost was associated when *P.aeruginosa* cultures were subjected to a week-long course of high-dose ceftazidime and meropenem using chemostat cultures. The study suggested that resistance in patient treatment is not necessarily linked to genetic resistance. In the case of *P.aeruginosa*, reports by Lee et al. (2016) have pointed to the phenomenon of phenotypic resistance where cells do not inherit resistance rather they become 'fitter' due to exposure to colistin resulting from altered gene expression. The susceptibility of the bacterial population to colistin returned to its previous levels when the antibiotic was removed.

The effectiveness of antibiotic treatment is related to host factors (Sorensen et al., 1988). Factors such as the health condition, age, and genetics are the major factors upon which the path of an infection directly depends. In some cases the infection may appear to be cleared but following the removal of chemotherapy the infection may re-appear. Such manifestations can arise due to subpopulations that have the ability to survive. Their survival may not be simply explained by mutations in these populations. Their survival could be due to their physiological state at the point of antibiotic treatment (Janeway and Travers, 1996). Such survival can be attributed to non-inherited resistance. Non-inherited resistance is often classified as were being phenotypic which is in contract to inherited resistance. The hereditary susceptibility remains unchanged when the population is being treated with the antibiotics (Levin and Rozen, 2006).

Thus, there are two types of antibiotic resistance – inherited and phenotypic resistance. The former arises from genetic transformations and has received the most attention from researchers. The latter refers to the expression of a phenotype of a bacterium those results in it being less susceptible to an antibiotic with no resistant underlying genetic change. In all phenotypic resistance situations, considerable attention has been devoted to drug indifference as well as bacteria grown in biofilms.

Bacterial permeability changes are also researched thoroughly alongside working on persistence and its entire construction. Nevertheless, there are still situations in which the vulnerability to antibiotics depends on the state of metabolism. While in certain occasions, resistance phenotype to antibiotics is involved to a condition of growth arrest that inhibits the action of certain bactericidal antibiotics such as *β*-lactams. There are a number of researches that have highlighted how a prominent connection is apparent between the susceptibility phenotype and the bacterial population's metabolic state. This has proven to be rather complex (Corona and Martinez, 2013). This section was conducted of numerous scenarios where non-inherited and transient antibiotic resistance states are formed.

1.3. Non-inherited antibiotic resistance (phenotypic variation)

In 1985, the first phenotypic resistance switch was characterised in the fungal pathogen *Candida albicans*. In the case of the pathogenic *C. albicans* reports by Slutsky et al. (1985) have pointed to seven general phenotypes recognized by variant colony morphology on agar Fig.1. Other fungi for instance *Crytococcus neoformans* were introduced later. These increments also incorporated other *Candida* species (Fries et al., 2002). Afterwards, it was further identified in the microorganisms as *Neisseria* spp, *Salmonella* spp, *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. In a review by Proctor et al. (2006), it was reported that the first phenotypic switch termed bacterial small colony variants (SCVs) was identified in 1910 in *Salmonella enterica* and has since been observed in a wide range of microorganisms. Thus, for both non-pathogenic as well as pathogenic species, phenotypic switching is regarded as commonplace.

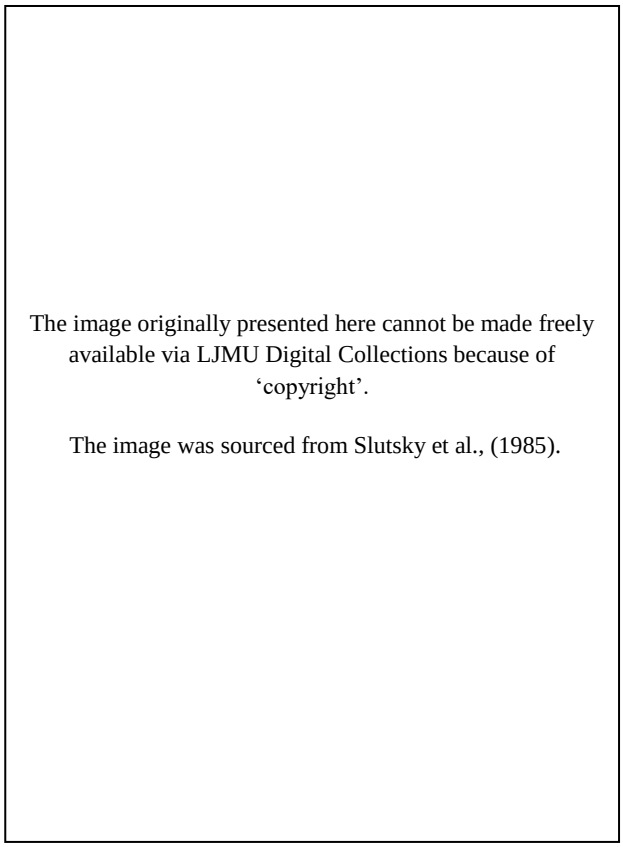


Figure 1: Colony morphology switch in the fungal pathogen *C. albicans* 3153A. (A) Original smooth; (B) star; (C) ring; (D) irregular, wrinkle; (E) stippled; (F) hat; (G) fuzzy (note aerial mycelia on colony dome); (H) revertant smooth (Slutsky et al., 1985).

1.3.1. Bacterial persistence

Since the antibiotics were developed, it was well understood that antibiotics that possess bactericidal activity are ineffective in killing all the bacterial population (Bigger, 1944). With the passage of time, the killing rate significantly decreases and certain bacterial population fractions survive as a result of being less susceptible to the action of antibiotics. This phenomenon was first documented in 1942, in cases of Staphyloccocal infections which reappeared even after intensive administration of penicillin. Despite the ability of penicillin to destroy bacteria, it was found that it was difficult to carry out complete eradication of the bacterial population. This particular aspect is referred to by a number of names, for instance 'bacterial persistence' (Bigger, 1944, Balaban et al., 2004), 'adaptive resistance' (Barclay et al., 1992), and 'phenotypic tolerance' (Wiuff, 2005, Levin, 2004). Persistent cells refer to the fact that the same cultures of bacteria react in different ways when exposed to antimicrobial agents. Persistence represents a temporary phenotype

which manifests when a bacterial culture is subjected to the action of antimicrobial agents and the number of surviving bacteria is recorded. The surviving bacteria do not possess intrinsic resistance mechanisms and they regenerate a culture that is susceptible to the effects of antibiotics. Microbial persisters are produced by bacterial populations to ensure population survival, and can be viewed as an important example of a high tolerance to antibiotics (Keren et al., 2004a). A small fraction of the population is made up of microbial persisters. One of the most common characteristics is that microbial persisters have the ability to survive antimicrobial treatment with a high concentration of bactericidal antibiotics, because these cells are in an inactive state. By removing the antimicrobial agent and culturing persister cells in fresh media, not only will the sensitivity to antibiotics be acquired again by the progeny of "persister cells", but microbial persisters will also resume growth (Dawson et al., 2011). It is easy to repopulate the majority of bacterial infections after finishing off a course of antibiotics, because bacterial persisters are present in a dormant state in a bacterial population, which means that there has been no growth or death. These cells are highly resistant to antibiotics, because antibiotics are unable to destroy non-growing bacterial cells such as "persister cells" (Martínez and Rojo, 2011). The reason why the persistent subpopulation is in a non-dividing is that the production of toxins is responsible for stopping essential cellular functions which put bacteria in a dormant stat (Lewis, 2005). Thus, "persister cells" are considered to be the best example for studying phenotypic change among bacterial pathogens. Experimentally however, it has been quite difficult to study persistence as a phenotype. This is because the fraction is comparatively small in contrast to the overall bacteria population: only 1 in 10^5–10^6 cells of the wild-type *E.coli* are classified as persister cells (Moyed and Bertrand, 1983). *E.coli* has so far been utilized for characterizing the persister formation, (Lewis, 2007), and considerably less is known in regard to better understanding such cells of *P. aeruginosa*.

No explanations were provided for this phenomenon and the aspect was comparatively ignored until such persistent cells were extensively studied by Harris and Moyed in the 1980s (Moyed and Bertrand, 1983, Moyed and Broderick, 1989). There are currently a number of hypotheses that have been formulated to provide an explanation for the differences in reaction to the effects of antibiotics that have been observed in the same bacterial culture (Gefen and Balaban, 2009). The reason why

this aspect was disregarded for a long time is due to the fact that the immune system disposes of surviving bacteria in cases of bacterial infections. Nevertheless, pathological conditions such as tuberculosis has made it clear that persistence represents the principal issue, a single surviving bacterial organisms being enough to trigger the infection, thus rendering the immune system powerless.

1.3.1.1.Persistent cells type

Persistent cells can be classified into two types. Starvation is the major factor resulting in generating the Type I persistence. This phenomenon is not directly a result of bacterial cells that are not growing rather it is thought to be linked to a metabolic shift that is present once the end of stationary phase is reached. The cells eventually become fully sensitive to antibiotic after the whole population is re-grown in replete media (Gefen et al., 2008). The stringent responses is considered to be crucial in Type I persistence development. This particularly pertains to *E. coli* persistence (Korch et al., 2003).

On the other hand, Type II persister cells are created as a result of a phenotype-switching mechanism in which normal cells eventually evolve to become such persister cells type II. The type II also possesses the ability to return back to normal phenotype. However, there seems to be no apparent relationship between the stationary phase, stringent response and the switch (Kussell et al., 2005).

1.3.1.2.Mechanisms of persistence

All bacterial species appear to have persistence as one of their primary characteristics (Cohen et al., 2013). A number of biological mechanisms have been put forward over the years for highlighting how certain sets of dividing bacterial population can stop replicating and thus end up adopting the persistent phenotype. The reason behind this is that a number of a subset of cells that have not started the replication procedure would be included in growing populations that exists during the exponential phase (Balaban et al., 2004, Metris et al., 2005). Cells currently conducting DNA repair would also be present during the growth phase. Such cells would have stopped division while the repair is being carried out (Debbia et al., 2001). Certainly, the SOS system is induced by various antibiotics that lead to a halt in the division of cells (Pérez-Capilla et al., 2004). Lastly, based on the study of molecular genetics and genome-wide mutant screening, there are two major classes

of genes which contribute to the development of persister cells. The expression of toxin or antitoxin (TA) genes lead to a direct generation of persistent populations (Cohen et al., 2013, Gerdes and Maisonneuve, 2012). The initial linking of persistence and TA systems was conducted in the 1980s, (Moyed and Bertrand, 1983) via ethylmethane sulfate mutagenesis of *E. coli.* Such factors helped to identify high persistence (*hip*) mutants in *E. coli* (Korch et al., 2003). Bertrand and Moyed were the first to identify the hipA mutants of *E. coli*, and how these were able to result in considerably higher persistent cell frequencies pertaining to this mechanism (Moyed and Bertrand, 1983, Lewis, 2005). However, a significant number of persister cells in *E. coli* were derived due to over expression of the toxin element present in the TA systems called ReIE, which leads to bacterial growth arrest (Keren et al., 2004b). These modules are considered to be comparatively common in microbial genomes and thus could be seen to be representing general mechanisms that allow persistent population to be generated (Levin and Rozen, 2006). This mechanism is affected by changes or modifications in the concentration of the proteins (Cohen et al., 2013, Gerdes and Maisonneuve, 2012).

The knowledge regarding persistence mechanisms has been significantly increased over the years leading to various approaches to eliminate the subpopulations of such bacteria that are rather resisting to the action of drugs. The response of Quorum Sensing (QS) in *P. aeruginosa* can be related to persister cells (Moker et al., 2010). Moreover, infection caused by *P.aeruginosa* could be tackled as a result of inhibition of QS, which constitutes an anti-virulence approach (Hentzer et al., 2003, Wu et al., 2004). This type of inhibitors lead to reduce the proportion of persistent cells or on the other hand, increase how susceptible they are to such drugs, which is considerably crucial for treating infections pertaining to *P. aeruginosa.* Various recent studies have highlighted how the QS inhibitor (*Z*)-4-bromo-5-(bromomethylene)-3-methylfuran-2 (5*H*)-one revives the susceptibility of persister cells in *P. aeruginosa* PAO1. Still, such results cannot be solely attributed to QS inhibition and the various mechanisms involved in determination of such activities (Pan et al., 2012). Some studies conducted in this regard have indicated knockout of such genes coding for metabolic enzymes, for instance *yigB,* which encodes a flavin mononucleotide phosphatase or *ygfA*, also encoding enzymes that are utilized in folate biosynthesis which decreases the level of persistence (Hansen et al., 2008),

while over expression of these genes lead to enhance the tolerance levels. Shifting of the microbial metabolism is a rather unique approach for the eradication of such persistent cells. Various articles published recently have showed the feasibility and viability of using such an approach. For instance, a researcher indicated how persister cells were killed used aminoglycoside antibiotics as a result of simply adding various specific metabolic substrates, which permit recoveries of the bacterial proton motive force alongside ensuring that growth is not resumed (Allison et al., 2011). In line with the same procedure, it was also subsequently discovered that adding 3-[4-piperazin-1-yl]piperidin-4-yl biphenyl-4-carboxylate to the bacterial cultures results in reversing the persistent cells to become sensitive to the action of antibiotics (Kim et al., 2011).

1.3.2. Biofilms

Another common type of phenotypic change is the bacterial biofilm. Communities of microorganisms binding to the surface are called biofilms. Every year, millions of lives are being threatened by persistent bacterial infections caused by cells living in biofilms. Developing a biofilm is a phenotypic strategy employed by microbes, so that they are able to survive in the host (Chen and Wen, 2011). Anaerobic growth takes place in the centre of biofilms, exhibiting a slow growth rate and little metabolic activity when compared with bacteria living on the surface of biofilms which grow aerobically, allowing a faster growth rate and high metabolic activity (Høiby et al., 2011). It is important to note that resistance mechanisms in planktonic bacteria seem to be different to those of bacteria in a biofilm, mainly due to the fact that bacteria in biofilms are highly resistant to antimicrobial agents compared with stationary phase planktonic bacteria (Drenkard, 2003). One of the most important properties affecting bacteria living in biofilms is a slow growth rate in the centre of biofilms, which enhances the resistance to antimicrobial agents. Gene expression in biofilms is another property which gives bacteria the opportunity to become more adaptive to physiological and biochemical conditions. Up-regulation of specific gene expression occurs in the bacteria living in the centre of biofilms, making them able to adapt to anaerobic growth (Høiby et al., 2011). A host of genes are up-regulated and downregulated during in response to a biofilm lifestyle and a study by Hentzer et al. (2003) have characterised the response at the level of the transcriptome. In addition, the factor which plays the most significant role in restricting the diffusion

of chemical components of antibiotics is the physical and chemical structure of the exopolysaccharide matrix, which surrounds the bacterial population in biofilms. A biopolymeric matrix consists of proteins, polysaccharides and extracellular DNA. This factor has a serious impact on chronically ill patients infected by bacterial biofilms, as it is extremely difficult to eradicate from the host. Bacterial growing in biofilms exhibit higher Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC) when compared with planktonic bacteria. Indeed, biofilms have a high resistance both to host immune system components and to antimicrobial agents (Drenkard, 2003, Hoiby et al., 2011). A mature biofilm of *P. aeruginosa* can be produced very rapidly, usually in 5 to 7 days (Fig.2) (Høiby et al., 2011). Planktonic cells (free living) are involved in the first step of biofilm formation, when they attach to a surface reversibly. In this stage, such cells are sensitive to antibiotics. Irreversible binding to the surface takes place in the next step of the formation of biofilm. In this stage bacteria continue growing and form a microcolony surrounded by a polymer matrix. The thickness of biofilm is up to 50μm and it looks like a mushroom. A mature biofilm is the final stage of formation which involves focal dissolution to ensure formation of a new biofilm to take place in another location caused by spreading of liberated bacterial cells. The conventional resistance mechanisms can be clearly studied in *P. aeruginosa* biofilms which are exposed to *β*- lactam antibiotics and colistin (Høiby et al., 2011). Studies have shown that there is a strong relationship between survival strategies, including the formation of a biofilm or small-colony variants, and the response of the bacterial population of *P. aeruginosa* to treatment with antibiotics (Surhone et al., 2010).

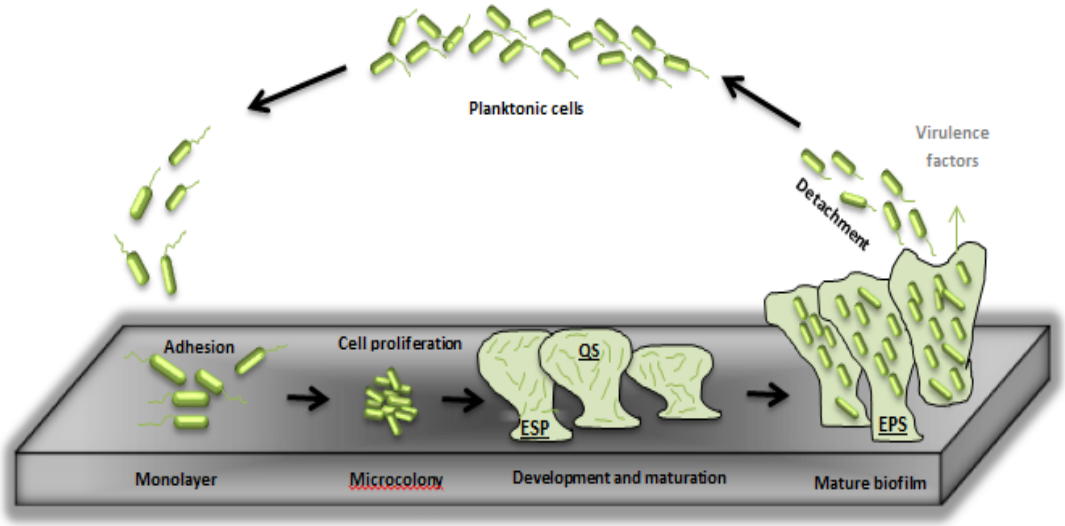


Figure 2: An *in vitro* experiment of biofilm formation of *P. aeruginosa* with *green fluorescent protein*-tagged. Cells grown in a flow-cell and confocal scanning laser microscope used to exam the structure of biofilm. A cell-to-cell communication results from Quorum Sensing (QS) and Extracellular Polymeric Substance (EPS) is hydrated extra-cellular biofilm matrix. The biofilm become thick between 25–100 μm. Adapted from Hoiby et al. (2011).

1.3.3. Drug indifference

Very soon after discovering that penicillin could be utilized for clinical purposes, it was also discovered that there was a direct relationship between the growth rate and the rate at which these microorganisms are being killed as a result of the action of antibiotic (Lee et al., 1944). The antibiotic has no effect on those bacteria, which were not dividing. This phenomenon was termed as ‘drug indifference’ by Walsh McDermott, when he reviewed the subject. This was also not limited to a particular antibiotic class, for instance the β-lactam class (McDermott, 1958, Paramasivan et al., 2005, Herbert et al., 1996). Those bacteria that have inadequate nutrients or are not dividing appear to be refractory to being killed by the antibiotics.

The bacteria can be effectively killed in the exponential phase of growth by the major classes of antibiotics that are available. Persister cells can be found in the stationary phase of antibiotic free media. However, if cultures are exposed to antibiotics during the growth phase the prevalence of persister cells in the stationary phase is much greater. Even though ciprofloxacin and streptomycin contribute towards the killing of bacteria in the stationary-phase, death rates are comparatively lower in contrast to the incidence of death when the antibiotics are present during the

exponential phase. The persister phenomenon is not just found in *in vitro* situations. Using mouse and rabbit models eagle demonstrated that infection became difficult to eradicate as the infection progressed and the bacteria became nutrient depleted. There was also a clear correlation between the inoculum density and the resistance of the population to eradication by antibiotics (Eagle, 1952, Eagle et al., 1950, Eagle, 1949, Darnell et al., 1955, Wood and Smith, 1956). The results indicated that the bacteria tended to become progressively less susceptible to antibiotic treatment as the infection period progresses (Bull et al., 2002). This change in susceptibility cannot be attributed to heritage and rather are considered to be a result of alternate changes that occur in bacterial physiology. Moreover, it was recently indicated that drug indifference can be observed when the bacteria are in "resting state", after the infection has taken place (Levin and Rozen, 2006).

1.3.4. Colony morphology switches

Various dynamic and different populations result due to phenotypic switching. Such populations also possess the capability to tackle different challenges. Colony morphology variation is considered to be one of the extremely prominent characteristic of phenotypic switching which is also known as "dissociative attitude". This term was first illustrated by Zierdt et al, back in 1964. Changed colony manifestations were established for quite a few bacteria such as *Streptococcus pneumonia* (Allegrucci and Sauer, 2007), *Haemophilus influenza* (Roche and Moxon, 1995), *Enterococcus faecalis* (Wellinghausen et al., 2009), *Burkholderia pseudomallei* (Chantratita et al., 2007) , and *P.aeruginosa* (Haussler, 2003).

Any sort of change in colony morphology might be an indicator of transformed appearance of the traits of the bacteria. These modifications may be in the form of changes in texture, colour, or opacity. A number of hospitals and laboratories are actively utilizing colony morphology for identification of microorganisms as well as isolation. Less information is available regarding the relationship between colony morphologies and bacterial features, irrespective of the importance of this aspect. For predicting and identifying the altered bacterial traits, colony visualization might be utilized. External factor relationships would also be highlighted. Coupled with it, the examination of colonies can play an important role to create connection among factors such as virulence, variations in morphological colony, persistence and

antimicrobial resistance. The relationships between the above mentioned can prove to be tremendously constructive to devise curative and biotechnological techniques (Sousa et al., 2011). For many microorganisms, quite a few colony morphotypes are recognized and further explained out of which the finest ones are the rugose small colony variants (RSCV) (Starkey et al., 2009), the mucoid morphotypes (Kirov et al., 2007, Govan and Deretic, 1996), and the small colony variants (SCV) (Singh et al., 2009, Malone et al., 2010). Few of the aforementioned morphotypes can be linked with decreased immunogenicity, resistance of antibiotic, and changed metabolism. All these factors may result in an augmented ability of an organism to cause disease and persistence.

1.3.5. Bacterial permeability

A particularly crucial reason that enhances susceptibility of an antibiotic against bacteria is that they penetrate inside the targeted cells. This also implies that bacterial envelopes are thus the first obstacle encountered by antibiotics. As a result, antibiotic effectiveness may be affected as a result of bacterial permeability changes (Fernandez and Hancock, 2012, Pages et al., 2008). Three differing mechanisms can be utilized for modulating the permeability of bacteria to antibiotics. **Firstly,** the surface of bacteria might be altered through the change in lipopolysaccharide which subsequently decreases the interactions of the antibiotic with this molecule and decreasing the penetration of the drug. **Secondly,** altering the antibiotic transporters or the porin type proteins can also have a significantly influence antibiotics susceptibility. **Thirdly,** once the cytosol is reached by the antibiotic, active pumping can be carried out by efflux pumps. The outer membrane is considered to be the foremost barrier encountered by the antibiotic penetration in gram-negative bacteria. This is comprised of lipopolysaccharide (LPS), which indicates particular anionic groups in which there is a binding of the cationic antibiotics. This also represents the initial step that needs to be taken in adsorbing antibiotics (Peterson et al., 1985). It was additionally described in *P. aeruginosa* that altering the negative charges possessed by the lipid A of the LPS through adding 4-aminoarabinose would lead to an increase in the resistance against cationic antimicrobial peptides, aminoglycosides, and polymyxin. Such responses can be particularly induced through concentration limitations of Ca^{2+} as well as Mg^{2+}. This usually pertains to

the antimicrobial peptides and polymyxin. Three particular component systems have been identified in *P. aeruginosa*, these are PhoP-PhoQ (Macfarlane et al., 2000, Macfarlane et al., 1999), PmrA-PmrB (McPhee et al., 2003), and ParS-ParR (Fernández et al., 2010), which up-regulated the arnBCADTEF operon. The products of these systems possessed the capability to modify the LPS. Inducing antibiotic resistance to the various cationic antibiotics by the lowering of concentrations of magnesium had been highlighted by work on *Salmonella typhimurium* (Groisman et al., 1997*). Stenotrophomonas maltophilia* highlighted a much similar phenomenon of such LPS alterations that eventually led to aminoglycosides' transient resistance (Rahmati-Bahram et al., 1996).

A correlation was found to exist between the increase in the resistance of the aminoglycosides antibiotics and the composition of LPS. This was particularly applicable when the bacteria were incubated at the temperature of 30°C resulting in a change in LPS content. Particularly important in this overall phenomenon is antibiotic resistance induced by the host-defence peptides that belonged to the innate human defence (Gellatly et al., 2012). This is an area that is being exploited to develop novel active peptides as new antimicrobials (Hancock and Sahl, 2006).

1.4. Selective pressure

1.4.1. External factors

External factors continuously pose a threat to microorganisms in natural environments. Adapting to the environment thus becomes essential in order to survive. A number of stressful conditions might also be encountered by such organisms which include immune host defences, the action of a wide range of antimicrobial agents, predation, UV exposure, anoxia or hypoxia conditions, pH changes, temperature shocks, and even starvation (Costerton et al., 1987). Adaptive response refers to the capability of bacteria to modify itself based on fluctuations in the environment. Various complex and detailed regulatory networks are triggered by the microorganisms in response to changes in the external environment. Such actions ensure that they survive. The genetic variations along with behavioural changes and physiological changes are a part of the process of adaptation of microorganism. This adaptation can take place by various mechanisms, including phenotypic switching.

The reversible switches that are present in states of phenotype are referred to as phase variations or phenotypic switching. Phenotypic switching in a population of cells is believed to be less prevalent than spontaneous mutations. The result of such phenotypic switching is a dual sub populations in which the entire genes profile is showed by one, while a certain modified expression of certain variable gene phases are expressed by the other (van der Woude, 2006). The ON/ OFF mechanism is one of the most interesting characteristics in the study of phenotypic switching. This allows the bacteria to take preference in the process of state interchange. Repetitive switching capabilities have been identified in microorganisms that possess a particular phenotypic state. This is usually done in response to new environmental factors, and switching back to the prior state might also occur as a result of conditions returning to normal.

As identified earlier, a population that is hereditarily alike (also referred to as isogenic population), usually can contain more than one phenotypes. This may happen in an environment that is homogenous. This characteristic is known as the inherent phenotype heterogeneity or at times referred as individuality. This particular microbial phenotype heterogeneity does not engage variations within the genome, and is considered to be a non-genetic disparity. This may occur because of the noise factor; that is the result of variations occurring from the stochasticity of responses of chemicals at the level of DNA. For example, the casual modifications in the degradation rate and the rates of protein synthesis (Veening et al., 2008 & Davidson and Surette, 2008). Different stress responses highlight the inherent phenotype diversity that is present in a particular homogenous population (Avery, 2006). This is very essential for microbial development and fitness. A number of studies highlighted that external factor (responsive switching) influence the switching that is carried out, even though random events (stochastic switching) might trigger phenotypic switching.

Phenotypic switching creates microbial heterogeneity which produces variants of phenotype. These variants are considered to be “healthier” and “better” , this part of the population manages to become accustomed in the new surrounding better than the rest of overall population (Bayliss, 2009). Conversely, those parts of the population have the capacity to prevail over all possible future and new

consequences which may arise. The “insurance hypothesis” of the model of ecology suggests that a greater amount of biodiversity makes it more probable that ecosystems will be better able to uphold or further develop their performance to deal with the fluctuations occurring in the environment (Yachi and Loreau, 1999). This supposition is supported by quite a few reports based on microbial diversity (Goerke et al., 2007, Boles and Singh, 2008, Boles et al., 2004).

1.4.2. Antimicrobial agents

Apart from helping in the avoidance of immune defence, phenotypic switching also has a remarkable effect on a number of biological aspects. As mentioned earlier, there is already a connection recognized among microbial virulence and phenotypic switching for certain bacteria. Nevertheless the reconsideration of the process of phenotypic switching holds vital importance considering that it may affect certain other biological processes like antimicrobial resistance (Sousa et al., 2011). It has been demonstrated that bacterial fitness costs are mainly associated with bacterial antibiotic resistance. This correlation, however, has been negated by Massey et al. (2001) when he and other researchers examined the resistance mechanism of *S. aureus.* The results revealed that infection caused by *S. aureus* in both *in vivo* and *in vitro* models lead to the generation of SCV when the population was treated with gentamicin. Phenotypically, SCV possesses important features such as pigmentation, high resistance to antibiotics, small size, and slow growth rate compared with the normal phenotype. Such SCV can revert to the parental phenotype when grown in antibiotic free media. It can be concluded that SCV established by Staphyloccocal infections represents a temporary phenotype which manifests when a bacterial culture is subjected to the action of gentamicin but resume growth characteristics as a wild-type when selective pressure is being taken off.

1.5. Background of the genus *Pseudomonas*

The group of bacteria known as *Pseudomonas* is highly adaptable and, as a result, it can proliferate and thrive in a great variety of environments, both terrestrial and aquatic. In addition, *Pseudomonas* can attach themselves to plants as well as animals. The wide ranging prevalence of this type of bacteria has been attributed to genomic heterogeneity and genetic versatility (Rehm, 2008). The description of the

genus *Pseudomonas* was conducted in 1894 by Migula, and then became a part of bacteriology history (Peix et al., 2009). This genus can be easily isolated from a wild range of natural materials, because members of the genus *Pseudomonas* have been widely observed in all types of environments (Goldman and Green, 2009, Rehm, 2008).

1.5.1. Taxonomy

The class *Pseudomonas* forms Genus I of the family of bacteria *Pseudomonadaceae.* The common functional characteristics of this family include the chemoorganotrophic metabolism which requires oxygen to function, the lack of fermentation and photosynthesis, as well as the ability to use a wide range of organic substances to develop (Moore et al., 2006).

1.5.2. Habitat

These characteristics enable *Pseudomonas* to adjust to any type of environment, from dry and aquatic environments to animal and plant organisms. In addition, it uses other types of bacteria, which it lives in conjunction with, as sources of carbon for development (Moore et al., 2006). *Pseudomonas* pose significant problems in the food industry as they cause meat, whether kept in refrigerators or not, to decay. What is more, the bacteria were discovered even in tap water and saline solutions, which are considered to lack the nutrients needed by the bacteria. Oligotrophic mineral water sources were also found to contain new types of *Pseudomonas.* Furthermore, the bacteria contribute to the creation of biofilms on the interior surface of pipelines (Moore et al., 2006). Cultures of broth media have been commonly used to study *Pseudomonas* species as ‘planktonic forms’. Nevertheless, the types of bacteria which proliferate in natural ecosystems have been noticed to live in biofilm environments of either small or large dimensions. The system of QS has been shown to have a significant influence on the creation of biofilms, due to the fact that it enables the detection of culture density and it dictates which genes included in the different phases of the creation of biofilm should be manifested (Moore et al., 2006).

1.5.3. Isolation

The nutritional preferences of *Pseudomonas* are not complex and the bacteria can be sampled from a wide range of environments without difficulty. In artificial

laboratory environments, the bacteria favour solutions which contain organic material, have a neutral pH and moderate temperatures of between 28 and 30°C. For instance, *P. aeruginosa*, which is a type of *Pseudomonas* most frequently used in laboratory environments, requires a temperature of 37°C to proliferate. The ideal environment for the majority of *Pseudomonas* is one which contains a substantial concentration of peptides, such as Nutrient Broth and Agar or Tryptic Soy Broth and Agar (Moore et al., 2006).

1.5.4. Identification

Pseudomonas can be identified based on its straight or somewhat curved rod shape. The rods are gram-negative and can move with the help of one or more flagella situated at the both extremities. Certain types of *Pseudomonas* also have short lateral flagella. In general, the metabolic functions of *Pseudomonas* require oxygen, but sometimes it can be replaced with nitrate, thus enabling development in the absence of oxygen. Furthermore, it can only live in an environment with a pH higher than 4.5, and needs an organic source of carbon and metabolic energy, can be oxidase positive or negative, and are catalase positive.

Pseudomonas spp. can cause disease in animal or vegetal organisms. Some types of *Pseudomonas* have been studied more than others and, consequently, the information known about them differs. *P. aeruginosa* is one example of a thoroughly studied *Pseudomonas*, mainly due to the fact that it is an opportunistic pathogen and, thus, is of clinical significance. Moreover, unlike other types of *Pseudomonas*, *P. aeruginosa* is a homogenous taxonomic organism that can be readily distinguished (Moore et al., 2006).

1.5.5. Pigment production

In order to classify *Pseudomonas*, preliminary studies of *Pseudomonas* relied heavily on the ability of this type of bacteria to generate pigments. Subsequently, it was discovered that there are also types of *Pseudomonas* which do not produce pigments; nevertheless, the feature of pigmentation remained a significant indicator in the identification of certain varieties (Goldman and Green, 2009).

1.5.6. Nucleotide Base

The nucleotide base composition of genomic DNA has been the first such genotypic examination to be used in studies of bacteria and is designed to measure the concentration of guanine and cytosine nucleotides from the overall concentration of nucleotide bases. The G+C concentration of *Pseudomonas* is approximately 59-68 mol% (Moore et al., 2006).

1.5.7. Ribosomal RNA Gene Sequences

According to Moore et al. (2006) and Brogden et al. (1976), in order to determine the intergenetic connections between bacteria, a comparison of the rRNA-rDNA sequences is necessary. With regard to new species of bacteria, identification of sequences and investigation of 16S rRNA are required.

1.6. General characteristics of *P.aeruginosa*

According to Goldman and Green (2009), all types of *P.aeruginosa* are characterised by a motile bacterium with straight rod shape and one or more flagella at the extremities. They do not produce spores, nor do they undergo fermentation. The ideal temperature for the proliferation of *P.aeruginosa* is 37°C, but it can withstand a large variety of temperatures, from normal environmental temperatures to 42°C. The resistant nature of *P.aeruginosa* is given by its highly adaptable metabolism, which enables the bacterium to extract the necessary carbon from both simple as well as complex organic composites. In addition, the fact that it can use nitrates and amino-acids, such as arginine, as respiratory electron receptors, signifies that *P.aeruginosa* can reproduce in environments both with and without oxygen (Rehm, 2008). Furthermore, *P.aeruginosa* is an opportunistic bacterium, which has clinically become the most prevalent bacterial pathogen in nosocomial infections, due to the fact that it is capable of resisting the effect of a wide range of antimicrobial agents (Tenover, 2006, Rehm, 2008). It has the ability to cause diseases in mammals, including humans. Patients who suffer from AIDS, cancer, burn injuries and cystic fibrosis are particularly prone to becoming infected with *P.aeruginosa*, due to their weak immune system. Once infected with *P. aeruginosa*, people are likely to carry it forever, as an efficient treatment is yet to be developed (Rehm, 2008).

The main identification mark of the bacterium *P.aeruginosa* is the blue water-soluble phenazine pigment that it produces, known as pyocyanin. The pyocyanin generates what is known as 'blue pus' in infections with *P. aeruginosa*. This pigment has been the focus of numerous studies, particularly regarding its toxic effect on other bacteria and the part that it plays in eukaryotic infection. Nevertheless, the physiological effect that pyocyanin has on the bacterium that produces it is yet to be fully elucidated (Price-Whelan et al., 2007).

As explained by Lau et al. (2004), the redox-active blue pigment pyocyanin is produced by *P. aeruginosa,* based on two novel phenazine-modifying genes, namely, *phzM*, which encodes phenazine-specific methyltransferase, and *phzS*, which encodes flavin-containing monooxygenase. Phenazines generate significant biological activity as they go through redox cycling when exposed to different reducing agents as well as molecular oxygen. This causes toxic superoxide (O_2^-) and hydrogen peroxide (H_2O_2) to accumulate in the cell, which can die as a result (Mavrodi et al., 2001).

As explained by Goldman and Green (2009), particular culture environments are capable of activating or increasing the production of pigments. However, recurrent transfers of strains conducted in the laboratory frequently led to the complete loss of the ability of *P. aeruginosa* to generate pigments. Such experiments have indicated that, among all the phenotypic characteristics, pigmentation is the most unpredictable.

1.6.1. Genome diversity of *P.aeruginosa*

P.aeruginosa PA01 has a high concentration of G+C 66.6% (Stover et al. 2000). The genome of *P.aeruginosa* consists of one chromosome spheroid in shape and an irregular number of plasmids. The chromosome is composed of a central core and an auxiliary part, which determines fluctuations in the dimensions of the genome of between 5.2 and 7 Mbp. The main strain of *P.aeruginosa*, PA01, is characterised by a coding density of 89.35% and contains 5570 protein coding sequences (Cornelis, 2008). In 2000, a breakthrough was made when a wild-type *P.aeruginosa* PA01 strain was completely sequenced (Stover et al., 2000), see Fig.3, enabling the creation of a more comprehensive picture not only about its ability to cause disease,

but also about its ability to resist the action of antimicrobial agents (Mesaros et al., 2007).

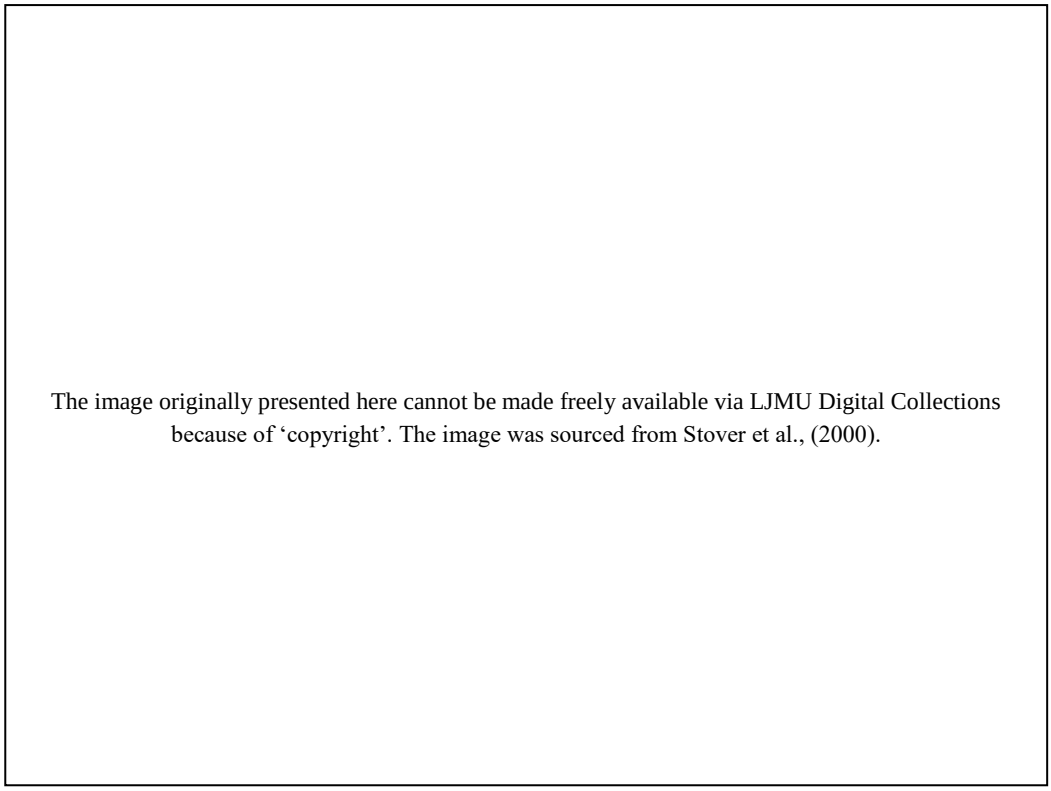


Figure 3: The genome of *P. aeruginosa.* It has been represented as a circular structure. The situation of base pairs of chromosomes is in the outermost circle. Different coloured boxes are used to indicate the location of genes of different functions and direction of transcription. The position and direction of transcription of ribosomal RNA genes is indicated by the red arrows. The process of homologous recombination between *rrnA* and *rrnB* generates a reversed area indicated by the green arrow. The areas consisting of potential bacteriophages are indicated by the blue arrows. The percentage G+C content is represented as a black central plot, the average for non-overlapping 1-kb windows covering a single strand of the whole genome. The areas of ≥3.0 kb with G+C content of two standard deviations of 58.8% below the average of 66.6% are indicated by the yellow bars (Stover et al., 2000)

Prokaryotic genomes of pathogenic bacteria compared with that of *P. aeruginosa* are shown in Fig.4. This genome is composed of an inordinate number of proteins, which are in charge of activities of regulation, transport and infectiousness. Given the fact that 0.3% of the entire gene code for proteins geared towards withstanding the antimicrobial agents, the resiliency and adaptability of the bacterium may be attributed to this protein composition. What is more, the genome is characterised by a high degree of flexibility, 10% of the genes with coding for virulence factors being structured into 'pathogenicity islands'; such structures are also capable of amassing mobile genetic elements of large dimensions known as integrons, which contain resistance genes (Stover et al., 2000). The ability of *P. aeruginosa* to develop in a

variety of different environments, as well as to cause extensive damage to different types of tissues and to withstand the action of the majority of available antibiotics, can be attributed to its complex genome (Mesaros et al., 2007).

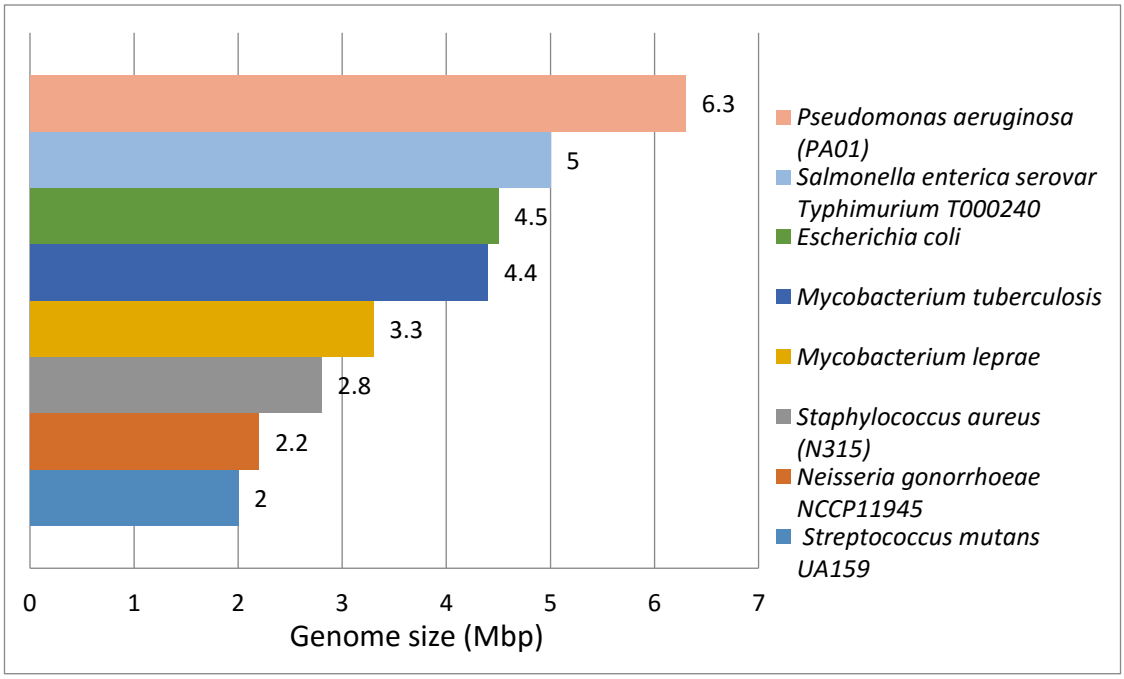


Figure 4: Prokaryotic genomes of pathogenic bacteria compared with that of *P. aeruginosa* (PA01) (Stover et al., 2000). *Staphylococcus aureus* (N315) (Baba *et al*., 2008), *Neisseria gonorrhoeae* NCCP11945 (Chung et al., 2008), *Streptococcus mutans* UA159 (Ajdic et al., 2002), *Salmonella enterica* T000240 (Izumiya et al., 2011), *E.coli*, *Mycobacterium leprae* and *Mycobacterium tuberculosis* (Sandy and Primrose, 2003)..

1.6.2. Emergence of antibiotic-resistant of *P.aeruginosa*

As antibiotic treatments have become increasingly specialised, bacteria have become more and more resistant. *P. aeruginosa* has joined the level of 'super-bugs', having developed resistance to multiple antimicrobial agents (Breidenstein et al., 2011). The resistance of these super-bugs is the result of its ability to develop an intrinsic and acquired resistance mechanism against a wide range of antibiotics, including the fact that the outer membrane has low permeability (Tenover, 2006). Mutations in chromosomal genes are responsible for regulating resistance genes, such as aminoglycoside-modifying enzymes, and other organisms can pass additional resistance genes through transposons, plasmids and bacteriophages (Chen and Wen, 2011). The resistance of bacteria to the effect of antimicrobial agents is enhanced even further due to the fact that these processes frequently occur at the same time

(Mesaros et al., 2007). Infections caused by *P. aeruginosa* are becoming increasingly difficult to treat as the organism is becoming more resistant to a significant range of commonly used antibiotics (Ding et al., 2016) .

For a long time, *P. aeruginosa* was considered to affect tissues in the aftermath of a different infection, causing extensive damage. Only recently has it been demonstrated that *P. aeruginosa* develops in people with weak immune systems independent of other pathogens that initially affected the health of those people. The high resistance of *P. aeruginosa* to antibiotic treatments is a well-known fact. There are a number of internal factors which enhance the resistance of *P. aeruginosa* to the effects of antibiotics, including its almost impermeable external membrane, the activity of the efflux pumps, and the generation of enzymes which inhibit the action of antibiotics. In addition, *P. aeruginosa* has the ability to generate new mechanisms of withstanding the effect of antimicrobial agents due to the complexity and dimensions of its genome, as well as to its proliferation in aquatic environments, where it can acquire resistance genes from other bacteria. Resistant strains determine infections which are three times more likely to result in death, the patients being nine times more likely to develop secondary bacteremia and extend their period of hospitalisation, which contributes to a substantial increase in the cost of treatment (Mesaros et al., 2007).

Given their variety, likelihood of generating epidemic isolates and their ability to withstand most antibiotics, the treatment of infections with *P. aeruginosa* should be geared towards recognizing the pathogen and determine the extent to which it is affected by antimicrobial agents. This can be done by creating microbiological growth of cultures of *P. aeruginosa* and observing their cultural and phenotypic features (Mesaros et al., 2007). What makes *P. aeruginosa* stand out from other pathogens is its ability to resist an extensive range of antibiotics, such as aminoglycosides, fluoroquinolones, and β-lactams, given by its external membrane which is almost impermeable, as well as by its ineffectual porins, thus making it difficult for antibiotics to gain access. The development of secondary mechanisms of resistance is also favored by these characteristics; among such mechanisms are the increased efflux and the enzymatic antibiotic transformations, such as β-lactamase activity. Moreover, the resistance mechanisms determine an extremely high minimal inhibitory concentration (Høiby et al., 2011), basically suppressing the effect of

antibiotics on the bacterium. Genetic mutations balance or increase the efficiency of the resistance mechanisms. Furthermore, *P. aeruginosa* gains additional adaptive resistance mechanisms due to its capability of thriving in different environments and modified growth conditions, as well as withstanding exposure to the action of antimicrobial agents. Despite having been discovered a while ago, research on the adaptive resistance mechanisms and their relevance from a clinical point of view, has only just begun. It is small wonder that the resistance of *P. aeruginosa* has been recorded with all existing antibiotics that target it, given the numerous ways in which the bacterium acquires resistance. There are a number of antibiotics which can be used to treat infections with *P.aeruginosa*; nevertheless, their efficiency is diminished due to the constant increase in the resistance exhibited by the bacterium, which makes the development of new antibiotics ever more difficult. Hence, it is necessary to gain a comprehensive picture of the resistance mechanisms of *P. aeruginosa* (Breidenstein et al., 2011).

Genetic or phenotypic changes are responsible for the inefficiency of antibiotics. The present study will focus particularly on phenotypic modifications.

1.7. The mode of antibiotics action

Antibiotics are organic compounds which form naturally and have bacterial action which makes it possible to employ them as chemotherapeutic agents to combat infections (Russell and Chopra, 1990). Terms such as ‘bacteriostatic’ or ‘bactericidal’ are frequently used when referring to antibiotics. Bacteriostatic antibiotics momentarily suppress the development of bacteria, which continues after the administration of the antibiotics ceases. Bactericidal antibiotics completely destroy bacteria; as such, they are frequently used in the treatment of patients with a weak immune system or those with bacterial infections which cannot be destroyed by the host organism (Schwalbe et al., 2007). Thus, antibiotics aim to suppress the development of bacteria by eradicating them or diminishing their proliferation in order for the defence systems of the organism to be able to eliminate the infection. Antimicrobial agents have been categorized differently, according to the manner in which they act, their chemical composition, and targets (Russell and Chopra, 1990). More specifically, this entails preventing protein and nucleic acid synthesis,

peptidoglycan synthesis, or destroying the cell wall/membrane (Schwalbe et al., 2007) (Fig. 5)

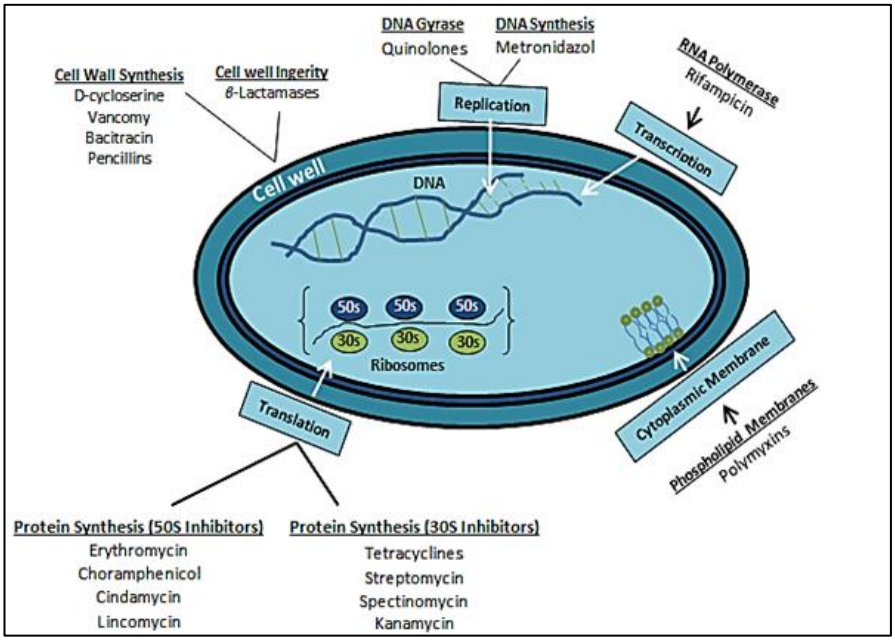


Figure 5: Schematic diagram of the mode of antibiotics action. Adapted from Essential Biochemistry (2004).

1.7.1. Inhibits of protein synthesis

There are a number of antibiotics which target bacterial protein synthesis by employing selective toxicity such as aminoglycosides. Aminoglycoside antibiotics are produced from *Streptomyces* (Brown and Stoudemire, 1998). The first aminoglycoside was produced by Selman Waksman in 1944 from *Streptomyces griseus*. Other types of *Streptomyces* were used to extract neomycin, kanamycin, tobramycin, and paromomycin. Gentamicin was first extracted from *Micromonospora* spp. by Weinstein in 1980, amikacin and netilimicin being semi-synthetic subsidiaries. The names of aminoglycosides are an indication of the sources from which they derived. Those which terminate in *mycin* are sourced from *Streptomyces*, whilst those which terminate in *micin* are sourced from *Micromonospora* spp (Schwalbe et al., 2007).

Aminoglycosides suppress protein synthesis by attaching themselves to the bacterial ribosome. The wall of the bacterial cell must firstly be penetrated, which is achieved

through active transport and passive dissemination. When it penetrates the core of the cell, the aminoglycoside attaches to the 30S subunit of the ribosome, hinders protein synthesis and finally destroys the cell by elimination of its components (Schwalbe et al., 2007).

Bacterial resistance to aminoglycosides manifests in three different ways. Disruptions to active transport or passive dissemination can render the antibiotic incapable of infiltrating the cell wall. The antibiotic may not be able to attach itself to the 30S subunit of the ribosome due to chromosomal alterations. Finally, the bacterium can generate enzymes which neutralize the effect of the antibiotic (Schwalbe et al., 2007). Antibiotics such as gentamicin, tobramycin and amikacin target enteric gram-negative bacilli. As such, they are used to treat people with febrile neutropenia or nosocomial infections. However, in cases when bacteria exhibit high levels of drug resistance, tobramycin or amikacin are administered. In the case of severe gram-negative infections, such as those engendered by *P. aeruginosa*, aminoglycosides are usually administered in combination so as to hinder the build-up of resistance (Schwalbe et al., 2007).

1.7.1.1.Tobramycin

Tobramycin is an aminoglycoside , which can be produced by *Streptomyces tenebrarius* (Funk, A et al. 1959 cited in Neu, 1976). Tobramycin is chemically represented as $C_{18}H_{37}N_5O_9$ and has a molecular weight of 467.52. As regards its structure, tobramycin is similar to the kanamycin aminoglycosides.

The bacterial actions of tobramycin include suppression of protein synthesis which determines increased porosity of the cell membrane and its gradual deterioration, finally causing the cell to die. Tobramycin suppresses protein synthesis in a bactericidal way by attaching itself to the 30S subunit of the ribosome, which is thus displaced from the polysome pool. This is the main *in vivo* activity not only of tobramycin but of other aminoglycosides as well. However, *in vitro* misconstructions of polypeptide synthesis can happen. Tobramycin can act *in vitro* upon a variety of gram-negative bacteria, among them *P.aeruginosa*. When it has a concentration which is equal or higher than the inhibitory concentration, the antibiotic is bactericidal in nature. A low concentration of tobramycin is enough to suppress

gram-negative bacilli. Similarly, most types of *Escherichia coli*, *Klebsiella*, *Enterobacter*, *Proteus mirabilis*, and *P. aeruginosa*, are inhibited by a tobramycin concentration of < 1.5 µg/ml (Neu 1976).

1.7.1.2. Amikacin

Amikacin is a semi-synthetic derivative of kanamycin which has a strong effect against the majority of gram-negative bacteria, even against strains which are resistant to gentamicin and tobramycin, as numerous studies have revealed. The molecular formula of the antibiotic is $C_{22}H_{43}N_5O_{13}$ (Ristuccia and Cunha, 1985).

Amikacin is an aminoglycoside, which means that it has a bactericidal effect on the 30S and 50S ribosomal subunits of bacteria, preventing protein synthesis. Within the cell membrane, amikacin is conveyed in two different transport stages, due to the fact that it is extremely polar. Amikacin attaches itself irrevocably to the ribosomes of the bacteria, thus disrupting the protein synthesis and the reading of the genetic code, which determines the formation of impaired proteins. Subsequently, the ribosomes divide from mRNA too early, causing the cell to die (Ristuccia and Cunha, 1985).

It has been observed that both *in vitro* and *in vivo*, amikacin has an effect on an extensive range of gram-negative bacteria, in particular *P. aeruginosa*, which can cause a variety of infections. However, in contrast to other aminoglycosides, such as gentamicin or tobramycin, amikacin has a less powerful effect, on a weight basis. Nevertheless, the selection of an aminoglycoside should take into consideration the attainable concentration and not the weight basis. In spite of this, comparative analyses have indicated that amikacin generated higher serum concentrations than gentamicin or tobramycin. The extent of the impact that aminoglycosides have on strains of *Pseudomonas* at clinically attainable concentrations is an important consideration. In particular, amikacin has a strong effect on certain isolates of *Enterobacteriaceae* and *Pseudomonas*, which are resistant to the action of gentamicin and tobramycin (Ristuccia and Cunha, 1985).
This represents the main benefit of amikacin and has been demonstrated by the successful administration of amikacin to patients with infections caused by resistant

bacteria as well as to patients who had received earlier aminoglycoside treatment which had been unsuccessful.

In contrast to gentamicin and tobramycin, which can be inhibited by eight different enzymes, there are only two enzymes which can inhibit amikacin. Among these enzymes, plasmid-mediated production of enzymes is the most common one, and alters tobramycin via acetylation, adenylylation or phosphorylation (Ristuccia and Cunha, 1985). Some strains may have common enzymes, like an aminoglycoside adenylyltransferase AAD(2") and AAD(3"), in an inactive state on amikacin (Ristuccia and Cunha, 1985).

This makes amikacin superior to gentamicin and tobramycin, particularly as it effectively suppresses the action of bacteria which are resistant to the other two aminoglycosides (Ristuccia and Cunha, 1985), due to its chemical structure (Chambers and Sande, 1996).

When penetration of the antibiotic depends upon a transporter protein a lack of this protein can result in an increase in resistance to the antibiotic attributed to adaptive resistance to aminoglycosides in the case of *P. aeruginosa.* Such adaptive resistance can result in an *in vivo* in spite of the sensitivity recorded in *in vitro* appraisals (Karlowsky et al., 1997).

1.7.1.3.Divalent cations affect the activity of aminoglycosides

Falagas and Kasiakou (2005) have discovered that the use of calcium and magnesium determined an increase in the MIC value of *P. aeruginosa*, which means that calcium has the potential to overturn the bactericidal effect of aminoglycosides against *P. aeruginosa*, but not against other types of enteric bacteria. The location of interaction between *P. aeruginosa* and magnesium or calcium, which builds resistance to the effect of antibiotics, is not only the cell wall, but also at the cytoplasmic membrane (Jorgensen and Ferraro, 2009). In addition, calcium can diminish the synergistic activity of aminoglycosides and carbenicillin only to a certain extent. According to Brogden et al. (1976), considerable discrepancies in the ion concentrations can generate different MIC values, which could determine the use of an inadequate antibiotic against infections with *Pseudomonas*.

Divalent cations, particularly Ca^{2+}, in high concentrations inhibit aminoglycoside activity and therefore are conducive to bacterial survival. Ca^{2+} is more efficient than Mg^{2+} in protecting bacterial cells due to often present this element in the structural components of bacteria (Verkhovskaya et al., 2011). The outer membrane of *P. aeruginosa* was found to contain an Mg^{2+} binding site with involvement in cation antagonism (Nicas and Hancock, 1980). This explains why cations have such a marked effect in *P. aeruginosa* which, by comparison to other gram-negative bacteria, has a high concentration of lipopolysaccharide phosphate residues. Hence, in *P. aeruginosa*, MIC is increased by even a small cation concentration (Zimelis and Jackson, 1973, Garrod and Waterworth, 1969).

1.7.2. Inhibits of membrane integrity

Polymyxins is the collective name for a group of polypeptide antibiotics composed of five chemical compounds, first produced in 1947 (Falagas and Kasiakou, 2005). As noted by Russell and Chopra (1990), apart from polymyxin B and polymyxin E, polymyxins have not received a great amount of medical attention as they act only upon mammalian membranes. The most frequently applications of polymyxins have been in ear and eye solutions (Falagas and Kasiakou, 2005).

The interaction with the cytoplasmic membrane of the bacterial cell provides polymyxins with a bactericidal effect, which generates disruption to the structure of the cell membrane. Gram-negative bacteria are more affected by polymyxins than gram-positive ones due to the fact that the membranes of the former include phospholipid phosphatidylethanolamine, which is highly susceptible to polymyxins (Russell and Chopra, 1990). Polymyxins have to penetrate the external membrane of the bacterial cell to arrive at the target location within the gram-negative bacteria. As explained by Russell and Chopra (1990), in order to do this, a self-promoted uptake of polymyxins is undertaken which entails the dislocation of divalent cations which, in turn, causes disruptions to the membrane, enabling the infiltration of antibiotic molecules.

1.7.2.1.Colistin

Among the polymyxins, colistin is a cationic cyclic polypeptide extracted from *Bacillus polymyxa var colistinus*. It has the same structure as polymyxin E and a similar one to polymyxin B (Falagas and Kasiakou, 2005).

Colistin was first synthesised in 1949 from *colistinus Koyama*, a subspecies of *Bacillus polymyxa*. During the 1950s, colistin was used in therapeutic treatments in Japan and Europe and it began to be used in the United States in 1959 as colistimethate sodium. By the early 1980s, the intravenous administration of combinations of colistin and polymyxin B ceased due to the high levels of nephrotoxicity documented. Since then, colistin was administered intravenously only in cases of lung infections with gram-negative resistant bacteria (Falagas and Kasiakou, 2005). Polymyxins have received a great amount of attention in the face of development of multi-resistant bacteria and scarcity of antibiotics which can effectively target gram-negative bacteria. The molecular weight of colistin is 1750 Da (Falagas and Kasiakou, 2005).

Colistin has a more efficient action than polymyxin B against the majority of gram-negative aerobic bacilli, including *P. aeruginosa*, despite the fact that resistance has been recorded in the case of the latter. On the other hand, colistin does not have any influence on parasites, fungi, gram-positive aerobic bacilli, anaerobic bacteria, gram-negative and gram-positive aerobic cocci. *In vitro* susceptibility analyses have to take into consideration the concentration of magnesium and calcium in the culture environment (Falagas and Kasiakou, 2005).

Colistin targets the cell membrane of the bacteria. This activity entails electrostatic interactions between the antibiotic and the anionic lipopolysaccharide (LPS) molecules in the bacterial external membrane, colistin removing the magnesium and calcium required by the LPS molecules, which causes disruptions to the structure of the cell membrane. Consequently, the membrane has increased porosity which enables the removal of cell contents, which the cell dies (Falagas and Kasiakou, 2005). Mechanisms of genetic mutation or adjustment enable gram-negative bacteria to become resistant to colistin. Research related to the resistance of *P. aeruginosa* to polymyxin has indicated that the changes that the external cell membrane undergoes,

such as decrease in concentrations of LPS, proteins, magnesium, calcium and lipids, are the result of the build-up of bacterial resistance (Falagas and Kasiakoul, 2005).

1.8. Antibiotic Susceptibility testing

Laboratory tests are mainly focused on observing the reaction of *P. aeruginosa* to antimicrobial agents, as well as on recognizing its resistance mechanisms. In order to assess the susceptibility of *Pseudomonas* to the effects of antibiotics, it is important to employ distinct methods at the same time, which should also comprise the determination of the minimal inhibitory concentration of the antibiotics. Furthermore, it is important to carefully choose the antibiotics to be tested, with emphasis on efficient phenotypic indicators of resistance mechanisms. Nevertheless, *P. aeruginosa* poses considerable difficulties with regard to the examination of susceptibility tests and identification of resistance mechanisms dependent on phenotypic information for a number of reasons: (i) several resistance mechanisms often act simultaneously on the same antibiotics (Baba et al.) and Keren et al. (2004a); (ii) indicate variations in the efficiency of the resistance mechanisms in different strains; (iii) the methods employed to determine low-level resistance are inadequate (Mesaros et al., 2007).

Antimicrobial susceptibility tests generate *in vitro* data, which can be used to ensure a suitable and efficient antimicrobial treatment (Goldman and Green, 2009). As indicated by Goldman and Green (2009), the empiric therapy can be altered or amended based on the results of the antimicrobial susceptibility testing (AST). It can employ either qualitative or quantitative methods. The latter generate MICs and qualitative method refers to MBCs. MICs represent the smallest dose of antibiotics, which can inhibit the growth of bacteria after 24 hours incubation. MICs are employed to confirm resistance, as well as to examine the *in vitro* activity of newly developed antibiotics (Andrews, 2001). The main applications of quantitative methods are related to the treatment of severe infections, including endocarditis and osteomyelitis, or infections associated with a weak immune system, as in the case of transplant or gravely ill patients (Goldman and Green, 2009). According to Andrews (2001), MICs are the ‘gold standard’ for assessing the extent of the effect of antibiotics on bacteria and, as such, the efficiency of all the other methods of

sensitivity testing is evaluated against the MICs. The standard doses of antibiotics employed in the MIC tests are doubly diluted and lower than 1mg/l. MBCs refer to the smallest dose of antibiotics which can kill the microorganism (Andrews, 2001).
Among the analyses carried out by the medical microbiology laboratory, testing the sensitivity of certain bacterial cultures to antibiotics is particularly important. The main objectives are identifying potential antimicrobial resistance in ordinary pathogens and ensuring the efficacy of antibiotics which target specific infections. The testing methods which are frequently employed are broth dilution and rapid automated instrument methods which rely on a variety of materials and devices available on the market (Jorgensen and Ferraro, 2009). The activity of antibiotics was measured by determining MIC and MBC. The determination of the MIC value of antibiotics used against human pathogens is a very useful way for identifying the lowest concentration of antibiotics, which have the ability to completely inhibit growth of the bacteria. The determination of the MBC value is also important to test the bactericidal activities of the antibiotics.

1.8.1. Microtiter plates (OxoPlates).

The OxoPlates are micro-plates with 96 wells, each equipped with optical oxygen sensors. Two types of OxoPlate are available, one called OxoPlate OP96U, which has a round bottom, whereas; OxoPlate OP96C has a flat bottom (Precision Sensing GmbH, 2012). The OxoPlate OP96U is the most widely used type as the signal it generates is extremely powerful.

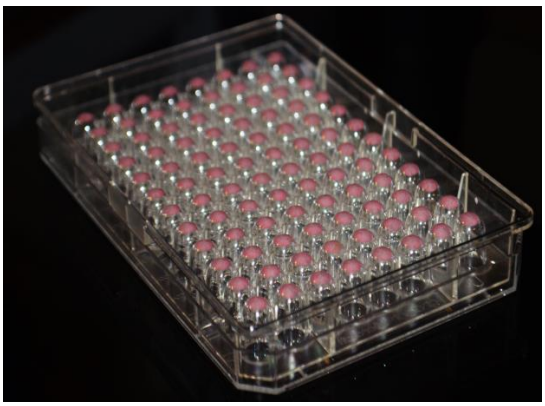


Figure 6: Photograph of the OxoPlate.
It contains 96 wells with integrated sensors on its bottom.

It has the ability to determine in near-real time the oxygen concentration in all the 96 wells of a well plate, due to the its oxygen sensor incorporated at the bottom of the plate. Each sensor is composed of a thin polymer film about 10μm consisting of two distinct dyes, namely, the indicator dye and the reference dye. Owing to the thin sensor polymer film (10μm), the OxoPlate's response time does not exceed 10 seconds. The volume of oxygen contained by the sample dictates the phosphorescence potency of the indicator dye ($I\text{-}_{indicator}$). On the other hand, the fluorescence potency of the reference dye ($I\text{-}_{reference}$) is not influenced by the volume of oxygen ratio I_R, which indicates the concentration of oxygen. Any fluorescence intensity reader which can scan from the base can be used to read the OxoPlate®. Due to the fact that the sensor consists of two distinct dyes, the reader can measure *the dual kinetic mode* by two different readings repeatedly taken from every well, which represents the measuring point, with the help of two different filter pairs. The concentration of oxygen in the OxoPlate is measured with the use of two filter pairs. The first filter pair is 540 / 650 nm and the second filter pair is 540 / 590 nm. The first pair is designed to determine the indicator dye, I-indicator, whereas the second filter pair determines the reference dye, I-reference (Precision Sensing GmbH, 2012). Kinetic parameters like enzyme or respiratory processes can be accurately detected resulting from the rapid response time and extensive wavelength of the OxoPlate indicator dye.

1.8.1.1.Composition of the OxoPlate Sensor

The OxoPlate sensor configuration is generally made up of luminescent dye with optical qualities susceptible to temporary alteration when exposed to molecular oxygen. Typically the dye is present in a polymeric matrix and placed on a solid support such as a microtiter plate. As numerous key metabolic activities of both prokaryotes and eukaryotes (e.g. respiration, biological oxidation, and photosynthesis) occur with the participation of porphyrins, the oxygen indicator is equipped with Pt(II)meso-tetra (pentafluorophenyl) porphyrine that affords the extensive assimilation and powerful emission properties in the visible area. Meanwhile, oxygen-permeable particles that display hydrophobicity also contain the reference dye sulphorhodamine B (chloride) (Fig.7). A hydrogel matrix contains the scatter of microspheres with oxygen sensitivity (Fig.8). The constancy of ratios and

internal referencing of the dyes' fluorescence intensities are made possible by the fact that the same particle includes both luminophores (Arain, 2006).

The image originally presented here cannot be made freely available via LJMU Digital Collections because of 'copyright'. The image was sourced from Arain (2000).

Figure 7: Structure of two dyes that use in the oxygen sensor of the OxoPlate. Indicator dye Pt(II)meso-tetra(pentafluorophenyl) porphyrine and reference dye sulphorhodamine B (chloride) (Arain, 2006).

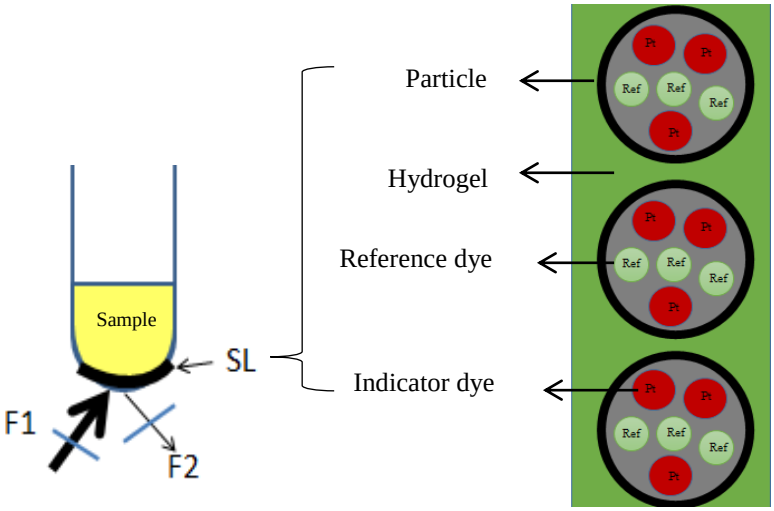


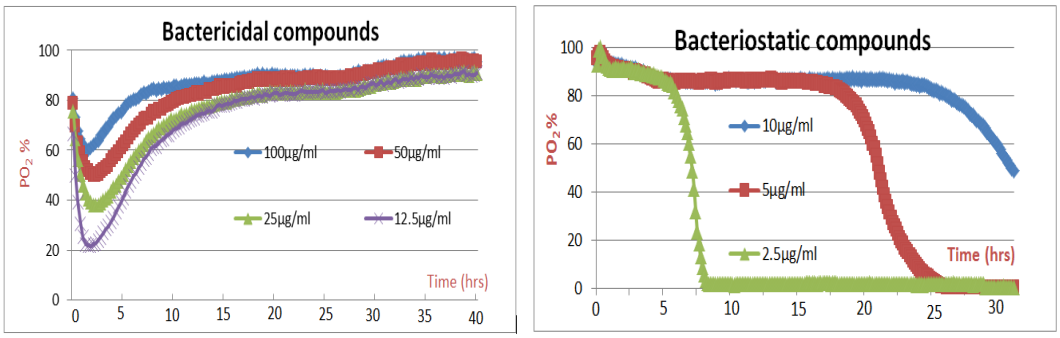
Figure 8: Diagram of the optical sensors located at the bottom of the wells. 'F1 is for excitation filter 540/650 nm F2 is for emission filter 540/590 nm, and Sensor layer'. Oxygen-permeable particles that display hydrophobicity contain the indicator dye and reference dye embedded in a hydrogel matrix Adapted from Arain (2006).

1.8.1.2.The principle of an optical oxygen sensor of the OxoPlate

In terms of the operational processes, air pressure is measured on the basis of the OxoPlate optical oxygen sensing underpinned by phosphorescence measurements. The manner in which oxygen concentration and air pressure are correlated can be summarised as follows: since the rise in oxygen concentration is directly proportional to rise in oxygen partial pressure, painting of porphyrin on a solid surface causes a change in the surface oxygen concentration according to the alteration in air pressure and phosphorescence intensity reaction to alteration (Asakura and Okura, 2009). The emission intensity of the first dye can be increased

by the decrease in level of oxygen caused by the decline in partial pressure in the well solution, leaving the emission intensity of the second dye unchanged. The decrease in the oxygen level has a negative impact on cell development, which stops completely when the oxygen partial pressure is at or near-zero. The sigmoidal curve of the fluorescence emission intensity proportion of the two dyes indicates this pattern of development. On the other hand, the oxygen level remains unaltered if the well contains medium that does not stimulate growth, reflected by the fluorescence emission intensity line (Becker, 2011). The fluorescence response of the sensor is transmitted by a polymer optical fibre to the instrument (PreSens) to be measured. The calculation of the oxygen partial pressure (pO_2) can subsequently be undertaken. The obtained oxygen concentration is expressed as air saturation percentage (% air saturation), which represents a relative measure that indicates how much oxygen has been dissolved in a medium associated with ambient air oxygen concentration. A medium with full air saturation is considered to be 100% (Asakura and Okura, 2009).

The growth of microorganism is assessed based on the analysis of the amount of oxygen dissolved in the culture environment. The fluctuation exhibited by the concentration of dissolved oxygen over time is associated with bacterial growth and metabolic action. The dynamics of oxygen consumption are affected by the inhibitory action of the antibiotics on the metabolic performance. The use of the OxoPlate enabled the straightforward estimation of the minimum inhibitory concentration from the oxygen consumption curves, as well as the differentiation of bactericidal and bacteriostatic capacities. Bactericidal antibiotics show a decrease in the levels of oxygen in the cultures, followed by an increase when the cells had been destroyed (Fig.9a). Bacteriostatic antibiotics, on the other hand, did not show any increase in the levels of oxygen after the initial growth phase (Fig. 9b) (Hutter and John, 2004). The use of the OxoPlate for the incipient assessment of antibacterial agents is highly advantageous. Temperature has a considerable impact on the optical oxygen sensors signal. As noted by Arain (2006), temperature influences the attributes of the sensor, as well as the degree of the oxygen solubility in the sample



(a) (b)

Figure 9: Diagram of the differentiation between bactericidal (a), and bacteriostatic compounds (b), shown curves of the dissolved oxygen concentration in air saturation percentage against time.

1.8.2. Antibiotic plate assay (agar well diffusion method)

Microbial or microbiological assay represents a category of bioassay geared towards the examination of the chemical substances which bacteria are susceptible to. The microbiological assay aims to determine the antibiotic concentration or potency based on the measurement of the zone of inhibition generated by the test sample and its comparison to an accepted standard concentration. The microbiological assay is generally employed to determine the efficiency of antibiotics. As such, it is helpful in establishing optimal antibiotic concentrations necessary to effectively treat pathogenic infections (Hewitt, 2004). In order to determine the susceptibility of *P. aeruginosa* to antibiotics, an antibiotic plate assay has been conducted. This assay is generally considered as the "gold standard" method.

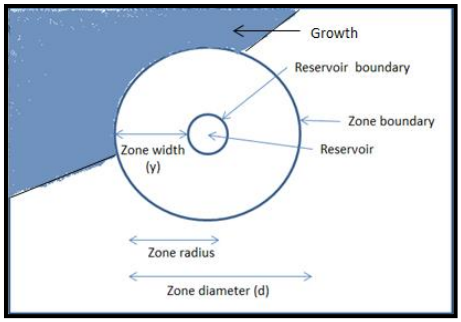


Figure 10: Diagram of inhibition zone. Adapted from Dart (1996).

1.8.3. Epsilometer test (E-test)

In 2007, Schwalbe reported that the E-test or Gradient Diffusion Method was introduced in 1988 at the Interscience Conference on Antimicrobial Agent and Chemotherapy (ICAAC) in Sweden. The principle of E-test is underpinned by both methods of disk diffusion and agar dilution, this method is the standard approach applied to assess quantitative antimicrobial susceptibility testing. It is preformed from the predefined antibiotic concentration gradient on a plastic-covered strip. The calibration of the continuous concentration gradient covers 15 two fold dilution, which included a wide range of MIC. After E-test strip is introduced on the surface of an inoculated agar plate, the antibiotic on the E-test strip undergoes immediate transfer to the agar. The MIC is determined after one day incubation, which can be read straight from the strip scale at a point where. The inhibition ellipse is observed on the top of the strip intersection with calibrated strip (Fig. 11).

The E-test is similar to the disk diffusion technique, with the exception that the suitability time for the antibacterial gradient is extended. Furthermore, unlike the disk diffusion method which is appropriate solely for aerobic bacteria with fast growth, the E-test remains stable for between eighteen and twenty hours, thus spanning the growth intervals of various different pathogens with both rapid and slow growth. What is more, the stable gradient permits the application of a macro-method with heavy inoculum in order to detect low-level resistance, hetero-resistance and resistant subpopulations. In addition to susceptibility aims, the E-test is also applicable in the identification of hypermutable strains of *P. aeruginosa* which is often accompanied by the hypermutator phenotype and Resistant Mutant Subpopulations (RMS) which are typically present in CF patients with chronic colonisation (Waine et al., 2008).

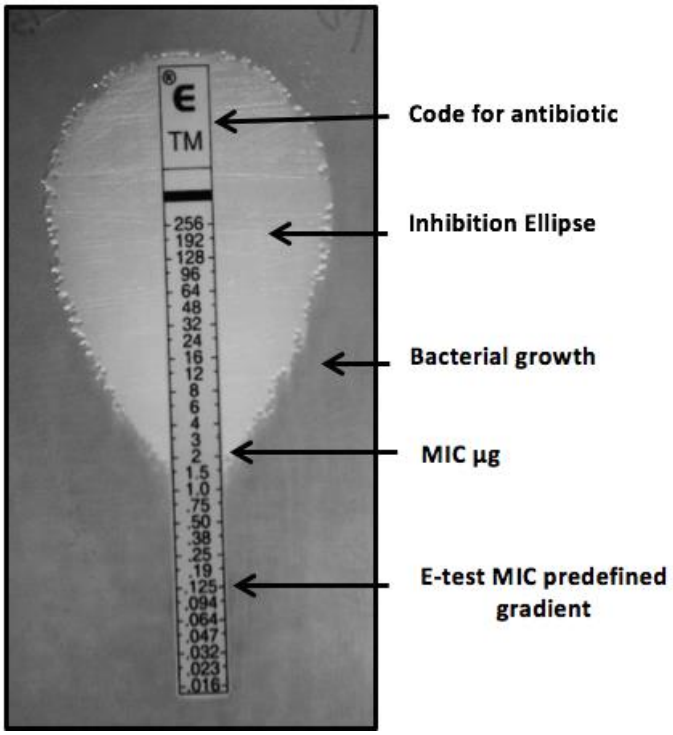


Figure 11: The E-test strip. The E-test inhibition ellipse illustrates the MIC at the point where organism growth and calibrated strip intersection.

1.9. Background of chemostat

A microorganism culturing technique which involved maintaining a liquid medium at a constant volume to promote ongoing growth of the cells within it was developed by Jacques Monod in 1950. Concomitantly, this method was employed by Szilard and Novick to create a cell culturing device which has been since known as a chemostat (Novick and Szilard, 1950a,b). This device is a bioreactor characterised by a state of equilibrium that fosters the constancy of the chemical properties of pH, cell density and such equilibrium is reached by constantly adding medium with single growth-limiting nutrient and at the same rate removing culture. Under these circumstances, the rate of cell population growth and the rate of culture dilution are the same (Gresham and Hong, 2014). Although this steady state is generally reached following cell growth that spans 10-15 generations, this state can be occasionally achieved quicker if the physiological status of the microorganism allows it. Thus, the chemostat is advantageous because it offers a state of equilibrium in a stable environment and it enables regulation of the rate at which cells grow through control of the rate at which the culture is diluted. However, to be functional, a chemostat needs ample quantities of media in keeping with the fermenter size and rate of dilution.

Due to their practical benefits, chemostats have been widely incorporated in the formulation of general ecological theories. The first benefit of chemostats is that they promote the proliferation of microorganisms in a physiologically steady state, thus facilitating the analysis of multiple generations in a short period of time. The second benefit is that chemostats permit the simulation of various environmental factors under closely regulated laboratory conditions. The third benefit is that chemostats provide the spatial uniformity needed for the assessment of a wide range of ecological models. Finally, chemostat experiments are so accurate that they afford unparalleled replication exactness (Huisman et al., 2002). As noted by the chemostat creators, these singular properties make the device particularly useful in cell growth research and analyses of evolution by mutation and selection. Six decades after its invention, the chemostat has attracted different attention due to the development of genome-scale experimental techniques and the resuscitation of a quantitative approach to biology (Bull, 2010, Hoskisson and Hobbs, 2005, Ziv N et al., 2013a).

Chemostats continue to be used mainly in research on cell growth and adaptive evolution which are essential study topics due to their significance in cell and evolutionary biology (Gresham and Hong, 2014).

1.9.1. The environment of the chemostat and its uses for bacterial research

Chemostats can have different configurations. Apart from chemostats intended for commercial purposes, a variety of chemostat were designed to be a suitable in research. It is very useful to use this technique to ensure that microorganisms grow in a physiologically steady state. There are some factors which need to be constantly maintained during growth, namely pH, oxygen, and the concentration of nutrient and metabolic products. The technique is considered to be a valuable research tool, because it offers the advantage of controlling the growth rate of cells, allowing the monitoring of dissolved O_2, CO_2 and biomass. The fermenter is the heart of the system, and a pump is used to feed the culture and probes are the monitoring devices. It has a straightforward process which allows the reservoir to enter a sterile medium into the culture vessel at the same rate as the efflux of a spent medium, ensuring that the growth rate becomes balanced and that new cell production is at the same rate as lost cells. In this system, a fresh sterile nutrient medium feeds cultures at a suitable rate to keep the growth of the bacterial population at a constant rate Fig.12 (Hoskisson and Hobbs, 2005). The volume of a fermenter determines the rate of medium flow rate, which is denoted by the dilution rate (D) with F and V representing the flow rate and culture volume, respectively.

$$\boldsymbol{D=F/V} \quad (1)$$

During stable growth, the biomass change (x) over time has the following expression:

$$\mathbf{dx/d}\boldsymbol{t=\mu x-Dx} \quad (2).$$

The concentration of biomass remains the same during stable growth:

$$\mathbf{dx/dt = 0, thus}\ \boldsymbol{\mu x-Dx}\mathbf{= 0\ or}\ \boldsymbol{\mu=D} \quad (3).$$

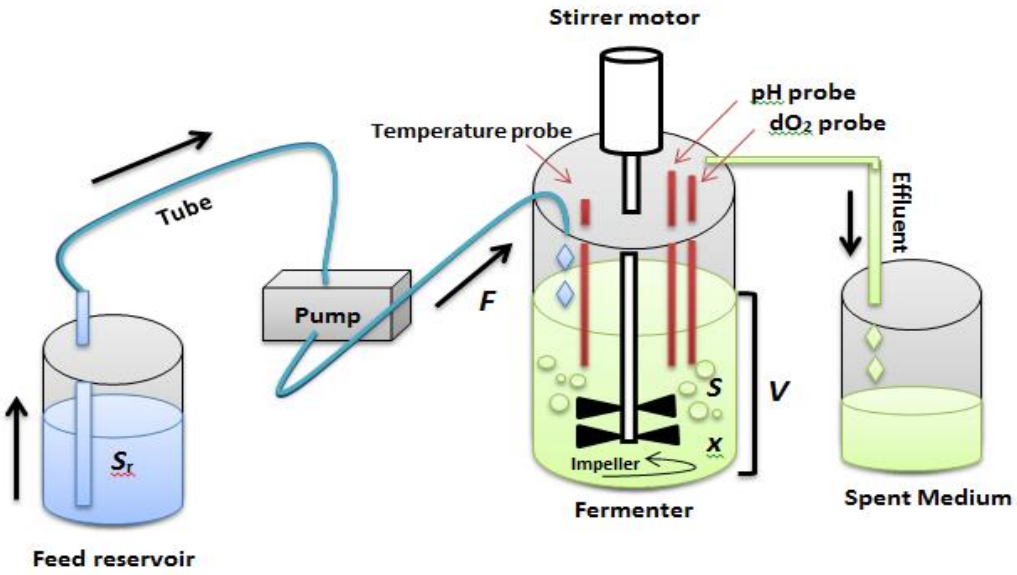


Figure 12: Schematic illustration of a simplified chemostat. The direction of the medium flow is shown by the arrows. To establish the dilution rate (*D*) and therefore to determine the rate at which the specific culture grows (μ), the medium flow rate (*F*) is regulated by the pump as a function of the working volume (*V*) of the vessel. S_r, *x* and *s* refer to the limiting nutrient concentration in the feed container, the biomass concentration in the fermenter, and the residual limiting nutrient concentration in the fermenter, respectively. Adapted from Hoskisson and Hobbs (2005).

As such, under stable conditions, as long as the growth rate does not exceed the critical dilution rate D_c (i.e. the rate at which the steady-state biomass concentration is zero), it is possible to regulate the growth rate in relation to the dilution rate. More specifically, under the set conditions, D_c exceeds the maximum specific growth rate (μ_{max}) of the organism and the culture is washed out. The straightforward correlation between growth and substrate use was validated by Monod (1942). Therefore, the rate at which a growth limiting nutrient is supplied represents the limiting factor, since the dilution rate determines microorganism growth in steady state culture. Monod (1942) established that the biomass of a stable culture was related to the concentration of residual limiting substrate and the dilution rate based on the following equation: $\boldsymbol{\mu=\mu_{max}\, S/\,(Ks+\,S)}$ (4)In which *S* and *K*s represent the residual substrate concentration and the half-rate saturation constant that is equivalent to *S* when the rate of growth is 0.5 μ_{max}. In the context of continuous culture, this is expressed as $\boldsymbol{D=D_c\, S/(Ks+S)}$ (5).

The outcome of experimental observation of how growth rate and substrate concentration are correlated, the chemostat behaviour model proposed by Monod is not optimal. Thus, the model has been subjected to numerous amendments such as

Droop (1968, 1973, 1974), Fredrickson et al. (1970) and Goldman and McCarthy (1978). Nevertheless, due to its simplicity, the Monod model is still popular and is appropriate for use in the majority of kinetic studies. However, regardless of the chemostat design, the researcher has the same experimental options, as follows:

(a) Organism-related options: the choice of strain is determined by the researcher's objectives; however, there is a number of variables that can effect experiments course should be taken into account (Ferenci, 2008). The behaviour of bacterial strain is one of variables that influences when cultivation in continuous culture. A study was conducted by Mikkola and Kurland (1991) showed that a slow adaptive respond was seen in 70 strains of *E. coli* isolated from natural habitats and cultivation immediately in glucose-limited chemostat.

(b) Options related to limiting nutrient: although the rate of growth in a chemostat is the same whether carbon, nitrogen or phosphorus sources are limited, there are clear discrepancies in the patterns of gene expression (Hua et al., 2004).

(c) Options related to limiting nutrient concentration and medium choice: cell density in the continuous culture depends on the pump of the nutrient concentration into the chemostat. Culture density affects quorum sensing aspects, (Liu et al., 2000) and a high density can also reduce the amount of oxygen available or trace elements requirement. To prevent these secondary limitations of high density, cultures of approximately 10^8 cells/mL will be sufficient.

(d) The dilution rate for chemostat functioning: the rate of growth in a continuous culture is determined by the dilution rate (D) (Monod, 1950).

(e) Sampling times for gene expression or mutations: rather than being merely a shift between two states (batch and continuous), the time-course of activities in a chemostat is characterised by great complexity compared with a batch-culture (Ferenci, 2008).

Microbial growth is an essential factor in the study of bacteria physiology and biochemistry. The contribution of rate of growth to bacterial resistance to antibiotics has been highlighted by several chemostat studies (Brown et al., 1990). A key aspect

of fundamental microbiology knowledge, bacterial growth kinetics, representing the correlation between bacterial specific growth rate (μ) and substrate concentration (s), plays an important role the microbiology branches of physiology, genetics, ecology and biotechnology (Kova´Rova´-Kovar and Egli, 1998). It has been found that bacteria with a rapid growth rate are more susceptible to antibiotics than bacteria growing at slow rates of dilution (Brown et al., 1990). When bacteria grow slowly, the chance is that they become more resistant, and this is a big problem in microbial pathogens. However, this observation does not apply to every form of limitation. Notably, chlorhexidine susceptibility is influenced differently by distinct limitations, increasing and decreasing with carbon and phosphorus limitation, respectively (Brown et al., 1990). Similar to polymyxin susceptibility, this discrepancy in sensitivity has been attributed in certain cases to variation in the content of lipopolysaccharide at different rates of dilution (Wright and Gilbert, 1987). Furthermore, support also exists for the notion that antibiotic susceptibility through efflux is dependent on the rate of growth. At low dilution rate, there was elevated expression of the *E. coli* acrAB efflux system, which increased even more at low glucose levels (Rand et al., 2002), denoting a likely involvement in resistance at slow dilution rates. In addition, a direct correlation has been established between a greater population of persister cells and increased resistance to ciprofloxacin and tetracycline at slow growth rates (Sufya et al., 2003). Persister cells find themselves implicated in the human clinical setting due to their lower susceptibility to antibiotics compared to other bacterial cells (Balaban et al., 2004, Kussell et al., 2005). The variables influencing the growth and distribution of persister cells within a bacterial population could be comprehensively investigated with the help of chemostats (Ferenci, 2008).

1.9.2. The chemostat underlying principle

There are some important distinctions between microorganism culturing in a chemostat and the batch- culture growth technique that microbiologists usually use to obtain continuous cultures.

1.9.2.1.Bacterial growth in batch mode

Bacteria undergo a distinctive series of transformations upon introduction in a nutrient medium and incubation at an appropriate temperature. Following a “lag

growth phase", bacteria grow in mass and subsequently divide. During the process of growth, known as the exponential growth phase, bacteria assimilate nutrients from the medium and eliminate metabolic products into it. In general, metabolic activity determines a change in pH and, in the case of aerobic organism cultures, the rate of oxygen solution may not be enough to sustain appropriate aeration of the culture. Nutrient depletion occurs when the density of a population becomes relatively high, at which point the cells stop growing and a quiescent state is established. The culture reaches the stationary phase when the environment is modified to such an extent by the growth process that it becomes incapable of sustaining growth (Gresham and Hong, 2014, Novick and Szilard, 1950a).

1.9.2.2.Bacterial growth in a continuous culture

All continuous cultures are initially batch cultures, in which organisms are inoculated in the medium where they undergo growth and division as early explained. The difference is that, unlike in a batch culture, growth goes on continuously if the culture is fed with new medium during the exponential growth phase at a rate at which the density of the culture population can be preserved at a stable, less-than-maximum value. The process by which new medium is added and culture is removed on a constant basis is known as chemostat (Gresham and Hong, 2014, Novick and Szilard, 1950a). However, if the culture is not constantly removed at the same rate as that of the addition of medium, there will be an exponential increase in the medium input rate and in the concentration of microorganisms.

The researcher can choose any of the nutrients that play a crucial role in cell growth as the nutrient that will suppress growth. For instance, a chemostat in which glucose is the limiting nutrient is defined as 'glucose-limited'. Based on experimental measurements of the growth rates of *E. coli* in various concentrations of glucose, Monod was the first to suggest this correlation between growth rate and nutrient concentration (Monod, 1949). Furthermore, the correlation has also been noted by Novick and Szilard (1950b) when varying tryptophan concentrations were used to grow a strain of *E. coli*.

The concentration of microorganisms (x) and the concentration of the growth limiting nutrient (s) are the key dynamic variables in a chemostat, which influence

by dilution rate. Dilution rate variation affords a range of stable conditions characterised by the fact that the growth rate and dilution rate are identical, provided that the dilution rate does not exceed the maximum rate of growth of the cells (Gresham and Hong, 2014). However, when dilution rate sets at or exceed $\mu_{Max,}$ the bacterial biomass will be increased rapidly resulting in depleting in the growth-limiting nutrient concentration due to use by the cells within the chemostat culture. From this model, it can be inferred that, under stable conditions, the exponential growth rate constant, in other words, the specific growth rate (μ), and the rate of dilution (D) are directly equivalent. Therefore, the doubling time (td) of growing microorganism in exponential phase is basically calculated using

$$\mathbf{td} = \boldsymbol{ln}\,\mathbf{(2)}/\boldsymbol{D} \qquad (6)$$

1.10. Biomass Monitoring System

The BE2100 is a commercially distinguished technology directed at the provision of rapid and efficient biomass monitors of both microbial and animal cell cultures. The system offers the added advantage of being non-intrusive and is, thus, increasingly protected from contaminations in comparison to conventional immersive technologies. The method functions by means of attaching a sensor to the exterior of a bioreactor (various sizes and materials) and tracks the biomass in real time. Furthermore, the system is applicable to both laboratory and commercial research.

In terms of the operational processes, this unique system is capable of generating constant and instantaneous biomass monitoring through the employment of a BE2100 Base Unit with an individual sensor in conjunction with BugLab software. Additionally, in contrast to previously employed optical biomass probes that are dependent on exclusive emission and detection combinations that are limited in biomass range, the BE2100 monitoring system measures a broader linear biomass OD unit range (≤ 0.1 to ≥ 300), eliminating the necessity for sample dilutions. Such a benefit is conferred through the utilisation of multiple infrared laser and detector combinations, the former of which encounters the bacterial cells in culture to generate light scatter patterns that are measured by the laser. Importantly, each of these combinations measure distinctive biomass alterations. The OD value is directly proportional to the concentration of the reflected light. As such, the Bug Lab software is able to generate increasingly reliable, broadly-ranging and linearised

biomass changes through the amalgamated evaluation of independent laser and detection readings (Applikon Biotechnolog, n.d).

1.11. Aims of the investigation

The aim of conducting this project is to consider the factors that influence the susceptibility and growth behaviour of the organism to antimicrobials and then to set the stage for developing an *in vitro* model to investigate the effects of long term exposure of *P.aeruginosa* to the antibiotics studied and to explore other contributory factors resulting in the evolution of antimicrobial resistance.

1.12. Research objectives

Several objectives have been established to attain the research aim, as follows:

- To investigate the dynamics of *P. aeruginosa* growth suppression in the presence of stress factors using a novel fluorescence based assay (**OxoPlate® system**)
- To determine antimicrobial activities of antibiotics against planktonic cells of *P.aeruginosa* using OxoPlate® system.
- To analyse the growth behaviour of wild-type strain of *P.aeruginosa* to be able to determine the rate at which the cell population is growing.
- To determine if over exposure to antibiotics at very low antibiotic concentrations below MIC can lead to phenotypic resistance, using a continuous culture (Chemostat system).
- To evaluate whether prolonged exposure to antibiotics resulted in colony alterations.
- To control the growth rate to see the effects of antibiotics at different growth rates and monitor dissolved O_2 and biomass.
- To sort cells to be able to identify subpopulation of “persister cells” from the whole population.
- To measure how quickly antibiotics act on bacterial cell populations using appropriate techniques, such as the *Bac*Light stain (Molecular Probes), to assess cells viability.

Chapter 2

Material and methods

2.1. Bacterial strain

A reference strain of *Pseudomonas aeruginosa* PA01 (ATCC 15692) was used throughout the study provided by Liverpool John Moores University (LJMU).

2.2. Culture media

Mueller Hinton agar, Nutrient agar, Nutrient broth, and Mueller Hinton broth were used throughout the study. Media were obtained from OXOID LTD, UK and preparation were carried out in accordance with the instructions provided by the manufacturer.

2.2.1. Nutrient Agar (NA)

The process entailed mixing 28g of **NA** with one litre of distilled water and the mixture was poured into Duran bottles and sterilised for 15 minutes at a temperature of 121°C. Afterwards, the medium was poured into 90 mm sterile agar plates and left to solidify and then stored in a refrigerator at 4 °C.

2.2.2. Mueller Hinton Agar (MHA)

Mueller-Hinton Agar was prepared by weighing (38g) of MHA base powder and dissolved it in one litre of distilled water and the mixture was poured in Duran bottle and sterilised for 15 minutes at a temperature of 121°C. The medium was poured into 90 mm sterile agar plates and left to solidify and then stored in a refrigerator at 4°C.

2.2.3. Nutrient Broth (NB) and Mueller-Hinton Broth (MHB)

Preparation of the Nutrient Broth and Mueller-Hinton Broth were conducted in the same way as the agar, the only difference being that only 13g of **Nutrient Broth** was dissolved in one litre of distilled water and 21g of **Mueller-Hinton Broth** was dissolved in one litre of distilled water.

2.2.4. Evan's defined synthetic medium

The medium composition was $NaH_2PO_4.2H_2O$ (0.62g/l) (BDH, England), KCl (0.75g/l) (Fluka), $NaNO_3$ (0.849g/l) (Sigma-Aldrich, United Kingdom (UK)), Na_2SO_4 (0.28g/l) (Sigma, Japan), citric acid (0.38g/l) 0.02% (v/v) (The British Drug Houses (BDH), England) and (1.8g/l) D-Glucose anhydrous (Fisher Scientific, UK), $CaCl_2.2H_2O$ (0.037g/l) (BDH, England) and $MgCl_2$ (0.11g/l) (Amresco, United States American (USA)) were added for defined medium used for testing and the pH was adjusted to 7.2 using1.5M NaOH (Fisher Scientific, UK) and autoclaved at 121°C for 15 min. Furthermore, the autoclaving of glucose took place separately. Following autoclaving, for each litre of defined medium, one millilitre of trace elements was introduced in the sterile defined medium with the help of a disposable sterile syringe filter (0.2 µm, Corning) (Life Sciences, UK).

2.2.5. Trace elements

The trace elements solution had the following composition; ZnO (4.1 g/l), $FeCl_3$ (3.2 g/l) (Sigma-Aldrich, UK), $MnCl_2.4H_2O$ (2.0g/l) (BDH, England), $CuCl_2$ (1.4 g/l) (BDH, England), $CoCl_2.6H_2O$ (4.8 g/l) (BDH, England), $Na_2MoO_4.2H_2O$ (0.0048 g/l) (BDH, England), $NOB_4O_7.10\ H_2O$ (3.82 g /l) (Sigma, Japan) and 80ml of concentrated HCl (Sigma-Aldrich, UK).

2.3. Selection of antibiotics

In vitro antibiotic susceptibility testing was conducted with *P.aeruginosa* by using these antibiotics, which were obtained from Sigma-Aldrich, UK (Table 1).

Table 1: The most common agents to treat *P.aeruginosa*

Antibiotic	Potency	Molecular Weight
Tobramycin sulphate salt	701µg/mg	467.51
Amikacin disulphate salt	674-786µg/mg	781.76
Colistin sulphate salt	≥15000UI/mg	1750

2.4. Broth methods

2.4.1. The broth microdilution method (Standard)

The broth microdilution method was one of the first methods used to test bacterial sensitivity to antibiotics. The process entails producing two-fold dilutions of antibiotics in Muller – Hinton Broth medium. For the preparation of a stock solution, 20mg of amikacin and 32mg of tobramycin and colistin were dissolved in I ml of distilled water. Subsequently, the antibiotics were further diluted to obtain the final concentration ranges in the test medium. The preparation of the standardized bacterial suspension entailed the selection of a colony of *P.aeruginosa* with the use of a sterile plastic loop, followed by its suspension in 50 ml of MHB which was then left to incubate in an orbital shaking incubator (Sartorius, Germany) for 24 hours at a temperature of 37°C and a speed of 250 rpm. A sample of an overnight culture of *P. aeruginosa* was adjusted to equale the 0.5 MacFarland's standard. The purpose of this experiment was to establish the MIC value of the antibiotics used, as well as to differentiate between bactericidal and bacteriostatic antibiotics.

The wells were divided into samples and controls. The samples consisting of 180 µl of media amended with 10 µl of antibiotic to reach the required concentrations and then inoculated with 10 µl of an overnight culture. Replicates of each sample (3or 4 each) were employed. The positive and negative controls consisted of 12 wells each, containing 180 µl of broth injected with 10 µl of water inoculated with 10 µl of an overnight culture. In addition, a 10 µl solution of sodium hypochlorite (reagent grad chlorine 10-15%), obtained from Sigma-Aldrich, UK, was added to the negative control wells. The microplate then left to incubate at a temperature of 37°C for 24 hours. Subsequently, the wells were inspected for signs of bacterial development with sub-inhibitory concentrations, such as turbidity. However, with inhibitory concentrations, no growth is visually observed in the media because the growth of bacteria is inhibited by antibiotics used.

2.4.2. OxoPlate®

Antibiotic susceptibility assay was prepared as previously mentioned in the broth microdilution method (see section 2.4.1). The OxoPlate OP96U (Precision Sensing GmbH, Germany) with a round bottom was used. The plate was covered with a polystyrene lid, the adhesive membrane of which permits gas to go through (Thermo

Scientific AB-0718, UK). The time-resolved fluorescence measurement technique and the plate mode reading for slow dynamics were employed to carry out the test. The wells represented in the layout were read once every plate cycle, which amounted to 250 cycles in total. Prior to and after every cycle, the plate was agitated for 3 seconds. The optical sensors at the bottom of the OxoPlate wells were scanned with the FLUOstar OPTIMA fluorescent plate reader (BMG LABTECH GmbH, Germany). To identify the indicator dye and the reference dye, excitation filter 540/650 nm and emission filter 540/590 nm were used respectively to measure the plate.

The plate carrier was able to move from left to right across consecutive columns based on horizontal reading direction. The temperature was set at the desired temperature. The well with the untreated positive control sample was chosen to conduct the gain adjustment, as it had the highest intensity. Gain adjustment maximises signal intensification to enhance sensitivity and the dynamic range as much as possible. The reader measured the minimum times intervals. The test was carried out over a period of 48-72 hours and the results were recorded automatically in the MARS data analysis software version 2.20 and represented as curves. The data was analysed using Microsoft Excel, 2010 to calculate means, the fluorescence intensity ratio and the oxygen concentration. Then, data was presented graphically using Sigma Plot v.12.

Calculation of oxygen concentration

The equation used to calculate the fluorescence intensity ratio at the oxygen concentration was: $\mathbf{I_R = I_{indicator}/I_{reference}}$ (7)

The equation used to calculate the oxygen concentration as percentage air saturation in each well was: $\mathbf{pO_2 = 100.\ (K0/I_R - 1)\ /\ (k0/k100 - 1)}$ (8)

2.5. Diffusion methods

2.5.1. Agar well diffusion method

A sample of an overnight culture of *P. aeruginosa* was adjusted to equal the 0.5 MacFarland's standard. The accepted absorbance range for 0.5 MacFarland's standard at wavelength of 625nm is between 0.08 and 0.13 (corresponds to

approximately 1.5 X 10^8 CFU/ml) and in all cases a spectrophotometer with a 1 cm light path was used and adjustments made by the addition of distilled water.

The antimicrobial bioassay was performed in square agar plates obtained from Thermo Scientific (Nunc, UK). In this method, the standard dilution of antibiotic from stock solution was prepared to reach the final concentration required by using the twofold dilution method using sterile distilled water as diluent. The suspension (10ml) was poured over the entire surface of the square agar plate and rotated to completely cover the plate. The surplus liquid was disposed of. To expose the antibiotic on the plates, 10 mm diameter reservoirs were cut into the agar plates with a sterile cork borer. Standard antibiotic concentrations (100 µl) were introduced into the reservoirs. It is necessary for all doses of antibiotic to be introduced at the same time, particularly in the case of large plates with more reservoirs to avoid discrepancies in antibiotic diffusion between the first and last reservoirs. To this end, it is helpful to use a template sheet to create a pattern of sample distribution, which enables the pre-determined application of a high number of samples into a square agar plate. In order to permit the applied antibiotic to diffuse, the plates were incubated for 18 hours at a temperature of 4°C to minimize growth of bacteria. To increase the inhibition zone size and the dose response curve, the period of diffusion of the antibiotic should be extended. In addition, diffusion must occur at a temperature lower than the room temperature to prevent bacterial growth and an indeterminate zone. However, a low temperature slows down the process of diffusion and so it is necessary to assess the most suitable conditions by trial and error. It is considered that a period of several hours at 4°C is suitable for the process of diffusion to occur (Dart, 1996). Subsequently, the plates were incubated over a period of one day at a required temperature. Plug assays were carried out and evaluated based on the dimensions of the inhibition zones. Inhibition zone with diameter of 10mm was considered as having no antimicrobial activity. In order to determine the zones measurement, a ruler positioned underneath the inverted plate was used and had a standard error of ± 0.5 mm. The area without any apparent growth visible with the unaided eye was established to be the zone edge. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines were consulted to define the inhibition zones sizes, being classified as susceptible, intermediate or resistant to the antibiotics used. The guidelines of the EUCAST were followed to carry out the tests.

2.5.2. E-test

E-Test gradient strips containing amikacin, colistin and tobramycin provided by manufacturer (Thermo Scientific, UK). The antimicrobial concentration ranges tested were 0.016 to 256 µg for amikacin, tobramycin and colistin. These strips were stored at a temperature of -20°C and, upon removal from the freezer, they were not immediately opened but kept for about half an hour at room temperature to allow for the complete evaporation of the condensed moisture on the external surface of the package containing the strips. The strips were used after the package reached room temperature.

A molten MHA (25ml) was poured into 90mm diameter of sterile Petri dishes to give 4 mm ± 0.5 mm depth and left to solidify. The plates were left to reach ambient temperature and they were put into refrigerated storage at 2-8°C if they were not used in the same day. All plates were employed within a week after they were prepared. For purposes of sterility assessment, every plate before use was incubated to one day at a temperature of 37°C.

To attain the turbidity of a 0.5 McFarland standard (corresponds to approximately 1.5 X 10^8 CFU/ml), distilled water was used to dilute an overnight *P. aeruginosa* culture. Subsequently, the inoculum was spread out onto a MH agar plate using a serial cotton swab. Within 15 minutes following the adjustment of the inoculum suspension turbidity, a sterile cotton swab was dipped into the adjusted suspension. In order to eliminate excess inoculum, the swab was squeezed on the internal tube wall above the liquid level. The swab was then spread over the whole dried surface of the sterile Mueller-Hinton agar plate to inoculate it. The plate was rotated about 60° to make sure that the inoculum was uniformly spread including the agar rim. Before the E-test strip was applied, the lid was left slightly open for a period of between three and five minutes for the absorption of any excess surface moisture. After drying, the plate had an E-test strip applied to it and was subjected to incubation at the required temperature.

The guidelines specified by the manufacturer were followed to interpret the results. In the case of measuring MIC for wild-type susceptibility, the reading of the plates was done upon observation of enough growth after the one-day incubation period,

while in the case of the subpopulation and resistance subpopulation, the plates were read after that period.

2.6. The measurement of *P. aeruginosa* growth

The SPECTRO $_{star}$ Nano (BMG LABTECH, Germany) was employed to monitor the growth/inhibition of *P. aeruginosa*. To monitor bacterial growth, the optical densities in every well are measured by the plate reader. Having a sophisticated photometrical technology in this plate reader was useful to conduct kinetic measurements of modifications in optical densities caused by bacterial growth or inhibition. In each experiment, the measurement of the optical densities was undertaken based on a wide-band filter associated with a spectrum range of between 420 and 700nm.

Threefold repetition of every growth curve was carried out, while the SPECTRO $_{star}$ Nano software automatically recorded at every hour the optical density values across 48h. These values were then transferred to Microsoft Excel 2010 spreadsheet to calculate means and represented graphically using Sigma Plot v.12.

2.7. The dynamics of growth inhibition in the presence of stress factors

2.7.1. Temperature

The antimicrobial activity of the antibiotics was determined under a range of temperatures (25, 30, 37, 42^{o}C) using standard methods (E-test and Agar Well Diffusion method) and innovative method (OxoPlate). The assays were prepared as previously mentioned in the sections (2.4.2, 2.5.1& 2.5.2).

2.7.2. Divalent cation

The following procedure was undertaken to observe how the dynamics of *P. aeruginosa* growth suppression responded to the effect of Ca^{2+} and Mg^{2+} using OxoPlate. Different concentrations of Ca^{2+} and Mg^{2+} (1-5mM) were added to the medium both on their own and together, alongside bactericidal doses of the antibiotics employed. The 160 μl of media in every well contained 10 μl of antibiotic and 20μl ions to attain the necessary concentrations, to which 10 μl of an overnight

culture was added. Each sample was associated with three replicates. Incubation lasted for 72h, at a temperature of 30°C.

2.7.3. Inoculum size

To assess the effect of three antibiotics used on the bacterium at different inoculum densities, experiments were run using OxoPlate method inoculated with high and low inoculum sizes. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml. The rest of the assay was prepared as previously mentioned in section (2.4.2).

2.8. Determination of limiting nutrient

Growth was regulated by the medium through limitation of one constituent element, which allows growth of *P.aeruginosa* to be limited. In the case of synthetic medium with just one vital nutrient in a concentration less than that usually employed in batch culture, the chemostat is referred to as a nutrient-limited chemostat because growth is limited by depleting a specific concentration of nutrient, whilst the rest of the nutrients exist in excess concentration.

2.8.1. Glucose-limited growth

Different concentrations of D-Glucose anhydrous (Fisher Scientific, UK) were prepared to determine the concentration that gave carbon limitation in continuous culture. The concentrations were 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50mM. In shake flasks, each concentration was added into Evans' defined synthetic medium containing (10 mM sodium nitrate as sole nitrogen source). The defined synthetic medium (100ml) was inoculated with 10% (V/V) solution (10 ml) of cell culture in the exponential growth phase of *P.aeruginosa* PA01 1.5 X 10^8 CFU/ml. It was then left to incubate in an orbital shaking incubator for 24 hours at a temperature of 30°C and a speed of 250 rpm. The measurement of the Optical Density (OD) after 24h incubation was conducted at A625 using the SPECTRO $_{star}$ Nano.

2.8.2. Nitrate- Limited growth

The initial sodium nitrate concentration was also determined in order to use only one nutrient limitation in the glucose limited chemostat culture. Different concentrations of $NaNO_3$ were prepared to determine the nitrogen limited concentration, which ranged from 1 to 20mM. In shaking flask each concentration

was added into Evans' defined synthetic medium supplemented with 1.8g/l of glucose. The rest of the preparation as mentioned in glucose limited.

2.9. Setting up the chemostat

The confirmation of chemostat is illustrated in Fig.13. The components of chemostat are shown in Fig. 14.

2.10. Cell culture preparation and conditions of chemostat

A new applikon chemostat from Holland was used in this study. The bacterial growth was undertaken in a vessel with a capacity of 2.8L and a working volume of 2L and airflow rate was 1.75 l/h. The system was initially run as a batch mode at different stirring speeds 150, 250 and 350 rpm in order to gain a homogenous culture and ensure that the continuous culture was running under optimum conditions. Dissolved oxygen concentrations were monitored using a poloragraphic dissolved oxygen probe (Applikon, Holland) (Fig.14B). CO_2 and biomass were also monitored using a CO_2 gas analyser (Electrolab, UK) and BagLab system (BE/2100) (Applikon, Holland) respectively (Fig.14 G and H). The pH was controlled at 7.2 using solutions of 2.5M NaOH and 2.5M H_2SO_4. The temperature was controlled at 30ºC using a chiller and heating jacket (Applikon, Holland). An anti-grow back tube (Applikon, Holland) was used to minimise the growth of cells into the medium feed line (Fig. 14 D). Silicone antifoam (1g/l) (BDH, England) was prepared to control excessive foaming by injecting 1ml of diluted antifoam into the culture every 6h using timed addition.

The 2L volume of chemostat Evans' defined synthetic medium was inoculated with a 10% (V/V) solution (200 ml) of cell culture. The bacterial inoculation was prepared as follows: a pure colony of *P.aeruginosa* PA01 (ATCC 15692) was selected using a sterile plastic loop, followed by its suspension into separate flasks containing 100 ml of defined medium and 100µl of filtrated trace elements solution which was then left to incubate in an orbital shaking incubator for 24 hours at a temperature of 30°C and a speed of 250 rpm. The OD was measured after 24h incubation using The SPECTRO $_{star}$ Nano. Then, the overnight culture of *P. aeruginosa* was adjusted to equal the 0.5 MacFarland's standard. When the exponential growth phase was entered after 20-24 h, the pump-323 (Watson Marlow Fluid Technology Group, UK) was activated to permit a steady stead culture to

begin. Different dilution rates were set up (from the slowest to the fastest) as following 0.025h^{-1}, 0.06 h^{-1}, 0.099 h^{-1} and 0.12 h^{-1}. In order to obtain the growth of the bacterial population at a constant rate, fresh sterile nutrient medium was fed at an appropriate flow rate. When the reactor volume reached or exceeded the 2L volume, effluent was eliminated via the weir. After growth was stabilized, amikacin at sub inhibitory concentration was then introduced into a 10L or 20L bottle and fed to the culture. The results were automatically recorded in Bioexpert software and represented as data plots. The data was statistically analysed using Microsoft Excel 2010 and SPSS v.21. Data was presented graphically using Sigma Plot v.12.

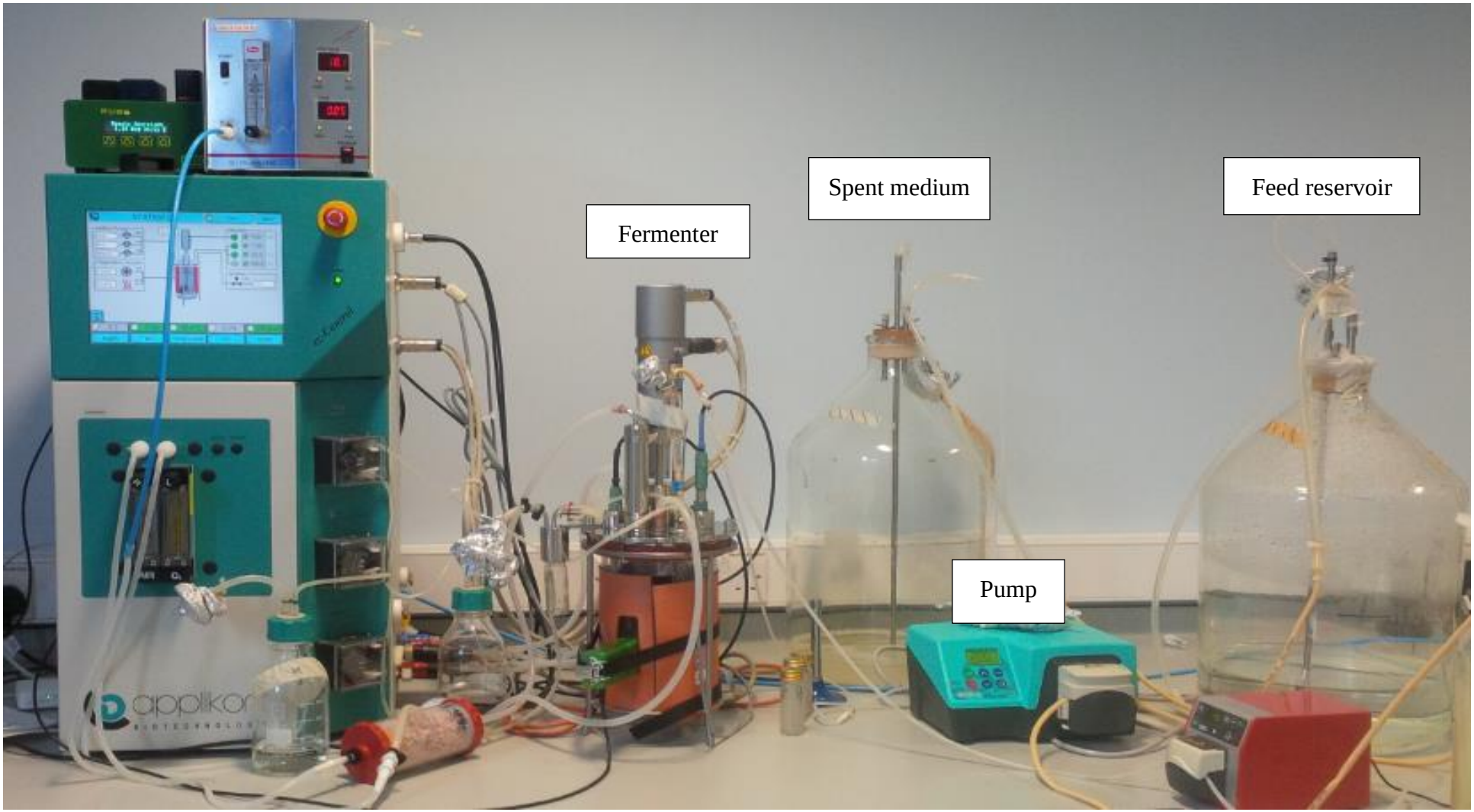


Figure13: Photograph of chemostat system

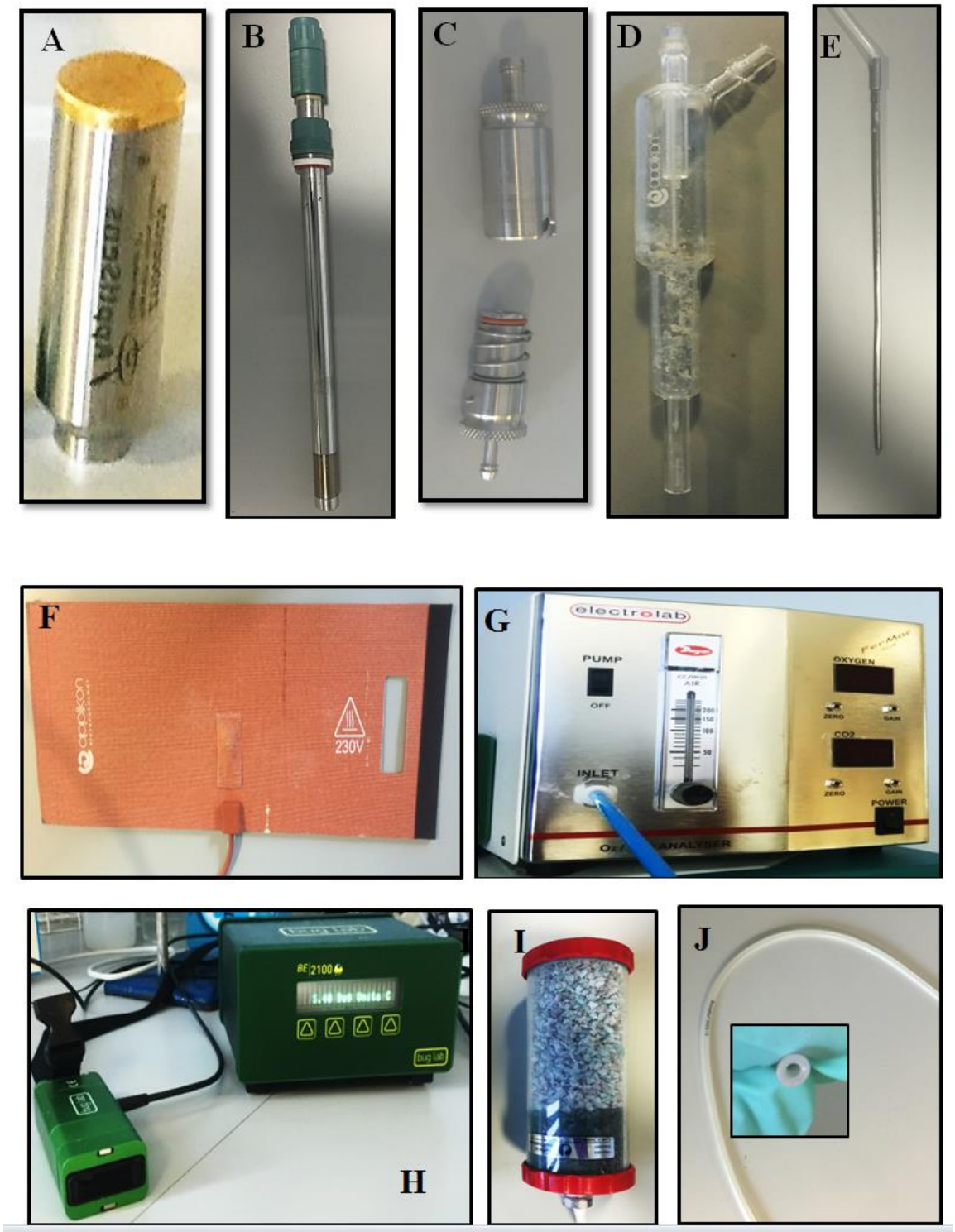


Figure 14: The components of the chemostat. Polarizer (Applikon, Holland) (A), dO_2 probe (B), An aluminum blocks (24 x 1.5ml) (Appleton wood HE1513/000205, UK) (C), Anti-growth back tube (D), Weir (E), Heating jacket (F), CO_2 gas analyser (G), BagLab system (H), Filter with blue stone (I) and Feeding tube made from peroxide-cured silicone with masterflex (96400-14) (J).

2.11. Analytical methods

A 10-20ml of sample was collected during the steady state culture for each experiment to not only determine the cell viability, glucose concentration, antibiotic susceptibility testing and bacterial optical density but also to analysis the cell population and morphology of colony and to check the purity of culture.

2.11.1. The cell viability assessment

A sample was taken both before and after treating with antibiotic to determine the cell viability, using the Miles and Misra assay and LIVE/DEAD® *Bac*Light™ strain.

2.11.1.1. The Miles and Misra method

For the viable bacterial cell count, the culture sample was diluted in a series of tenfold dilutions using sterile distilled water. The values were represented a mean of 3 repetitions. The mean number of Colony Forming Units (CFUs) was calculated using the following formula:

Number of CFU/ml = average of colony counts x 50 x dilution factor. (9)

The results were represented graphically as the time of exposure against the log of surviving' colonies.

2.11.1.2. Fluorescence microscopy

The live/dead cells were also assessed using the *Bac*Light™ stain (Life Technology L7007, USA). The *Bac*Light stain is a mixture of two coloured fluorescent dyes the green fluorescent nucleic acid stain is SYTO 9 to stain cells with undamaged membranes (live cells) and the red fluorescent nucleic acid stain is propidium iodide to stain cells with damaged membranes. The dyes are assessed with the use of two filter pairs. The first filter pair of excitation/emission is 480/500 nm and the second filter pair is 490/635 nm. The first pair is designed for SYTO 9, and the second filter pair for propidium iodide. The sample and staining optimization were prepared according to the instructions provided by the manufacturer. Then the stained sample was observed under 100x lens using fluorescence microscope (Olympus, UK). The images were observed in an image analysis program Volocity software version 6.3.

2.11.2. Glucose concentration assay

The glucose concentration was measured using enzymatic colourimeteric method (Gulcose oxidase /PAP) from Randox, UK. The measurement of glucose concentration of each steady stead experiment was conducted as following:

In a cuvette of 1cm light path, bacterial culture (10µl) was diluted into 1ml of reagent blank. The preparation of glucose standard was conducted by diluting 10 µl into 1ml of reagent blank. The blank consisting of 1ml of reagent with 10 µl of dH_2O. The sample and glucose standard were mixed well and incubated at 37°C for 20minutes. After this period, the absorbance of sample and glucose standard were measured at 500nm and the concentration of glucose was determined by applying the following equation:

$$\textbf{Concentration of glucose} = \textbf{Standard concentration} \times \frac{\Delta \mathbf{A}_{\textbf{Sample}}}{\Delta \mathbf{A}_{\textbf{Standard}}} \ \textbf{(g/l)} \quad (10)$$

2.11.3. Determination of *K*s

The nutrient is considered to an essential factor to limit the growth of the organisms. The correlation between the initial glucose concentration and specific growth rate were determined. From this correlation, the maximum specific growth rate μ_{max} can be also determined. Based on the values of specific growth rate and initial concentration of glucose, K_s for glucose was also measured using the following equation:

$$\frac{1}{\mu} = \frac{Ks}{s.\,\mu_{max}} + \frac{1}{\mu} \qquad (11)$$

Where ***K*s** is the substrate affinity constant (g/l), **µ** is the specific growth rate (h^{-1}), **s** is the initial concentration of glucose (g/l) and $\boldsymbol{\mu_{max}}$ is the maximum specific growth rate (h^{-1}).

The inverse of the initial concentration of glucose (1/ s) was plotted against the inverse specific growth rate (1/µ) to determined K_s. Based on the values obtained, the straight line will crossed the X axis and the intercept gives the value $-1/K_s$.

2.11.4. Colony morphology

In order to detect any phenotypic switch, the bacterial cells were grown on nutrient agar plates (antibiotic free). The plates inoculated with 10µl of bacterial cells from a steady state cultures and incubation between 24 h and 72h at 30°C. Any colony morphology changes were observed and evaluated based on the antibiotic susceptibility testing using OxoPlate method.

2.11.5. Cells sorting

The cells of 3 and 6 days age were taken from the centre and the edge of any mutant colonies and re-suspended in filter sterilized dH_2O to reach the final concentration of $1x10^8$ CFU/ml (~0.03 OD_{670}). In an Eppendorf tube, 1 ml of the bacterial suspension was stained with 3µl of the staining reagent mixture of the LIVE/DEAD *Bac*Light dye. The samples were covered with foil then vortexed and incubated between 10-15 minutes at room temperature in a dark place. After this period, the BD Accuri® C6 Flow Cytometer (Biosciences, UK) was used to sort the cells. In order to analyse the cells, the appropriate threshold value was set up at 10,000 and the sample was run with a limit of 90,000 cells at a slow flow rate. The results were recorded automatically in the BD Accuri® C6 software version 1.0.264.21 and represented as data plots.

2.11.6. Antibiotic susceptibility testing

The susceptibility of *P.aeruginosa* cultures that prolonged exposure to sub-inhibitory concentrations of amikacin in the glucose limited chemostat was determined in MHB by using OxoPlate method. Antibiotic susceptibility assay was prepared as previously mentioned in section (2.4.2). The susceptibility of *P.aeruginosa* was classified as susceptible, intermediate or resistant to the antibiotics use by referring to the EUCAST guidelines**.**

2.11.7. Scanning electron microscopy (SEM)

Following 3days incubation in absence of amikacin, bacterial cells presented in the centre of colony of culture exposed to amikacin in the chemostat grown at $0.125h^{-1}$ were harvested and fixed with 2.5% glutaraldehyde (Agar Scientific Ltd, UK) at 4°C

overnight. An overnight culture was re-suspended in 1.5 ml micro-centrifuge tube and centrifuged at 4000x*g* for 5 minutes. The supernatant was discarded and the pellet was washed three times with dH_2O. A loop of fixed bacterial cells was placed on a circular coverslip and gold-coated (15nm thickness) for 25 minutes at 25mA using K550X Sutter Coater (EMITECH, UK).

The colony of treated *P.aeruginosa* had been cultured for 3days and the centre was cut and fixed by freezing in liquid nitrogen (BDH, England). A liquid N_2-cooled brass metal holder was used to place the sample into a cold trap and allowed to dry overnight before examined under Inspect S Electron Scanning Microscope (FEI, USA). The images were observed in an image analysis program FEI software version 4-1.10.2127.

2.12. Bacterial DNA extraction for 16S ribosomal RNA

The identification of species of bacteria and the formation of taxonomic classifications have relied on the sequence analysis of the 16S ribosomal RNA gene. Chakravorty et al. (2007) explained that the 16S rRNA genes of bacteria consist of nine 'hypervariable regions', which can be employed to distinguish between species as their sequence varies significantly among the wide range of species of bacteria.

2.12.1. Extraction of genomic DNA

Genomic DNA was extracted from cells using E.Z.N.A. Bacterial DNA Kit obtained from Omega, USA and instructions were followed according to the manufacturer.
NB medium (100ml) in a 250ml shaking flask was inoculated with *P.aeruginosa* and incubated in an orbital shaking incubator for 24 hours at a temperature of 30°C at a speed of 250 rpm. An overnight culture (1.5 ml) was re-suspended in 1.5 ml micro-centrifuge tube and centrifuged at 4000x*g* for 10 minutes using centrifuge 5418, Germany. The media was discarded and the pellet retained. Lysozyme (10µl) was added to the pellets in the tubes and following vortexing the tubes were incubated at 37ºC for 10 minutes. BTL buffer (100µl) and proteinase (20µl) were added and the tubes vortexed to mix thoroughly. The samples were incubated at 55ºC in a shaking water bath for 1 hour. RNase (5µl) was added and the samples inverted up and down in order to mix the samples were then incubated at room temperature for 5 minutes.

The samples were centrifuged for 2 minutes at 10,000x*g* to pellet any undigested material. The supernatant of each tube was transferred to a new 1.5 ml micro-centrifuge tube without disturbing the pellet. BDL buffer (220µl) was added and samples were vortexed to mix. The samples were then incubated at 65°C for 10 minutes. Ethanol (96-100%) (220 µl) was added in each tube and vortexed for 20 seconds at a high speed. The samples were then transferred into HiBind®DNA Min columns and centrifuged at 10,000x*g* for 1 minute. The filtrate in the collection tube was discarded. The column was then inserted into a new 2ml collection tube. DNA wash buffer (700µl) was added then the tubes centrifuged at 10,000x*g* for 1 minute. The filtrate was discarded and the collection tube was reused. A second DNA wash buffer was also applied. The empty HiBind®DNA Min column was then centrifuged at maximum speed >10,000x*g* for 2 minutes to dry the column. HiBind®DNA Min column was then inserted into a new 1.5 ml micro-centrifuge tube. Preheated Elution buffer (65C°) (50 µl) was added to the HiBind®DNA Min column. The HiBind®DNA Min columns were incubated at room temperature for 3 to 5 minutes. The samples were then centrifuged at 10,000x*g* for 1 minute to elute the DNA. A second elution step was repeated. Eluted DNA was stored at -20C°.

2.12.2. 16S rRNA gene amplification and detection of PCR products

The 50µl PCR reaction mixtures contained 45µl of MasterMix (1.5 mM $MgCl_2$) (Qiagen), 2µl of DMSO, 1µl of genomic DNA, 1µl of sterile dH_2O and 0.5 of each primer (forward and reverse) (Table 2). Amplification of the 16S rRNA sequences was performed using thermal cycler 2720 (Applied Biosystems, UK). The 35 cycles of the amplification conditions was conducted including a denaturing step at 95°C for 1 min followed by an annealing step at 52°C for 1 min, and the final elongation step was performed at 72°C for 2 min.

Table 2: Eubacterial universal primers were used to amplify the 16S rRNA gene.

Primer name	Sequence (5'to 3')	Positions
27f	AGA GTT TGA TCM TGG CTC AG	8-27
1492r	TAC GGY TAC CTT GTT ACG ACT T	1510-1492

2.12.3. Purification

In accordance with the guidelines provided by the manufacturer, a QIAquick kit (QIAGEN, UK) was used to purify the PCR products. pH indicator (4µl) was added into 1000µl of buffer PB. Buffer PB (100µl) was added into 20µl of PCR products. 3M sodium acetate pH 5.5 obtained from Ambion the RNA Company (10µl) was added to each sample. The colour of the mixture turned yellow. Samples were applied to the QlAquick column and centrifuged at 13, 000 rpm for 30-60 seconds. The flow through was discarded and the QlAquick column was placed back in the same tube. Buffer PE (750µl) was added into the QlAquick column and centrifuged at 13,000 rpm for 30-60 seconds. The flow through was discarded again and the QlAquick column placed back in the same tube. The QlAquick columns were centrifuged once more in 2 ml collection tubes for 1 minute to remove residual wash buffer. Each of the QlAquick columns was placed in a 1.5 ml micro centrifuge tube. Elution buffer (30µl) was added to the centre of the QlAquick membrane and the column was allowed to stand for 1 minute and then centrifuged at 13,000 rpm for 30-60 seconds. The presence of PCR products was confirmed by agarose gel electrophoresis.

2.12.4. Agarose gel electrophoresis

EDTA (0.5M): Distilled water (88 ml) was used to dissolve 18.6g of 0.5M EDTA (Sigma, USA) and sterile distilled water was added to increase the volume to 100ml, whilst the pH was adjusted to 8.0 using 1.0 M NaOH (Fisher Scientific, UK).

TAE: Distilled water (750 ml) was used to dissolve 242g of Tris base (Severn Biotech ltd, England). Furthermore, the mixture contained 57.1ml of glacial acetic acid (20mM) (BDH, England) and 100ml of 0.5 M EDTA. Sterile distilled water was added to increase the volume to 1L, and the pH was adjusted at 8.5 using 1.0 M NaOH (Fisher Scientific, UK).

Electrophoresis running buffer: TAE (6 ml) was used to dissolve 0.6g of Agarose (Molecular Grand) obtained from Bioline, UK and distilled water was added to increase the volume to 60 ml. Upon attaining a hazy appearance, the solution was introduced in the microwave and heated for no more than 3 minutes on medium heat. Afterwards it was gently mixed to avoid the formation of bubbles and when it

became clear it was mixed with a solution of 3 µl of ethidium bromide obtained from Sigma, Germany. The solution was introduced into a horizontal tank containing 10 ml of 50x TAE and distilled water was added to increase the volume to 500 ml.

2.12.5. Analysis of the purified DNA on the gel

DNA loading buffer (2 µl) (Bioline, UK) was added into 5 µl of purified DNA and mixed well by pipetting up and down before loading it into the gel. HyperLadder ™ I is a molecular weight marker obtained from Bioline was loaded into gel. The gel was then run at 80 mV until the dye extended through the gel using runVIEW-B (Geneflow, UK). Then, the gel was visualised under gel imaging system (Gel Doc™ XR) (Bio-Rad, UK).

Chapter 3

Results

3.1. Antimicrobial susceptibility testing

The tests were carried out to assess *in vitro* the extent of the effect of antibiotics on planktonic cells of the wild-type of *P.aeruginosa*. A number of standard methods such as Broth Micro-Dilution, Agar Well Diffusion and innovative method (OxoPlate) were applied to evaluate the effect of the antibiotics tobramycin, amikacin and colistin against *P. aeruginosa*. Clinically, aminoglycoside antibiotics are considered to be an important group of antimicrobial agents. However, due to the limited therapeutic options, the use of colistin has received greatest attention in treating multidrug-resistant gram-negative bacteria (Lee et al., 2016). Aminoglycoside antibiotics are still used clinically in spite of their well-known toxicity due to their limited resistance development during treatment of infections (Loffhagen et al., 2004). The recent study showed that only 5.7% of *P.aeruginosa* strains exhibited resistance against amikacin in the health care setting (Özyürek and Bilkay, 2015).

3.1.1. Determination of MIC and MBC

The OxoPlate reader was employed to determine MIC and MBC based on the oxygen depletion levels. The MIC represents the lowest concentration which exhibits visual discrepancies from the untreated positive control sample at any given point in the first 10 hours following drug treatment. This visual discrepancy is an increase in the oxygen curve which lasts longer than 15 minutes in contrast to the untreated control sample (Fig.9b).

This is in sharp contrast to the MBC, whose equivalent to the lowest concentration induces an increase in the oxygen concentration as a result of the diffusion of atmospheric oxygen into the wells following cell death which indicates that the cells are no longer respiring and hence this represents the MBC (Fig.9a). Oxygen

depletion curves were generated for all the samples of bacterial culture treated with antibiotics. A connection between the MIC value and MBC that influences oxygen depletion in the OxoPlate was noticed in the case of all the antibiotics used. By plotting the oxygen concentration in relation to time, it is possible to obtain the lowest concentration of antibiotic that gave a MIC and MBC.

In addition, a comparison was carried out between the MIC values obtained in the test and the established guidelines of the EUCAST, in order to verify the resistance or susceptibility of the organism. The lowest concentration of antibiotic that inhibited the visible growth of *P.aeruginosa* after 24h incubation at 37C˚ for amikacin was 1.25µg/ml (Fig.15A). The MIC value obtained, however was twofold lower than the conventional method (2.5 µg/ml) (Fig.16). According to the EUCAST guidelines, *P.aeruginosa* was considered a sensitive strain to the antibiotic tested. In the case of tobramycin and colistin, the MIC values were 0.25µg/ml and 0.5µg/ml respectively (Fig.15 B-C). The MIC value obtained was similar to traditional MIC values for colistin, whereas, tobramycin was twofold lower than the conventional method (0.5 µg/ml) (Fig.16).

The lowest bactericidal concentration of tobramycin and colistin was >1µg/ml. both antibiotics proved to have highest activity against to *P.aeruginosa*, in contrast to the MBC of the antibiotic amikacin was found to be four folds higher than the MIC. This was clearly observed in the Fig.15 at concentration of 5 µg/ml, which did not reveal any indications of bacterial development after being left incubated for 72h at 37ºC. It was thus possible to conclude that these concentrations inhibited the growth of *P. aeruginosa* during the exponential and stationary stages of the growth curve.

Observations revealed that tobramycin and colistin displayed nearly identical spectrums of activity and had a considerable effect against *P. aeruginosa*, which indicated it to be more susceptible to tobramycin and colistin at a low concentration compared with amikacin.

3.1.2. Kinetics of growth inhibition

The oxygen depletion curves provide information not only with regard to the MIC and MBC values, but also for the kinetics of bacterial growth inhibition. The antibiotics used have been proven to be particularly effective against the test strain. Figure15A shows that the presence of amikacin at 1.25-2.5 µg/ml resulted in a delay in oxygen depletion followed by a gradual decrease in the oxygen level in the wells to 0%. On the other hand, amikacin efficiently prevented bacterial development at ≥ 5 µg/ml during the period of experiment. Tobramycin had a bactericidal effect on the wild-type, which showed a decrease in the amounts of oxygen at the beginning of the run, followed by a steady state of 100% pO_2 when the cells had died at the concentrations of ≥1µg/ml. However, it showed a bacteriostatic effect at a concentration of 0.25-0.5µg/ml (Fig.15B). Based on the level of oxygen, colistin had a bacteriostatic effect on the strain at a concentration of 0.5µg/ml. However, no oxygen consumption was observed to occur when colistin was applied to *P.aeruginosa* at a concentration of ≥1 µg/ml (Fig. 15C).

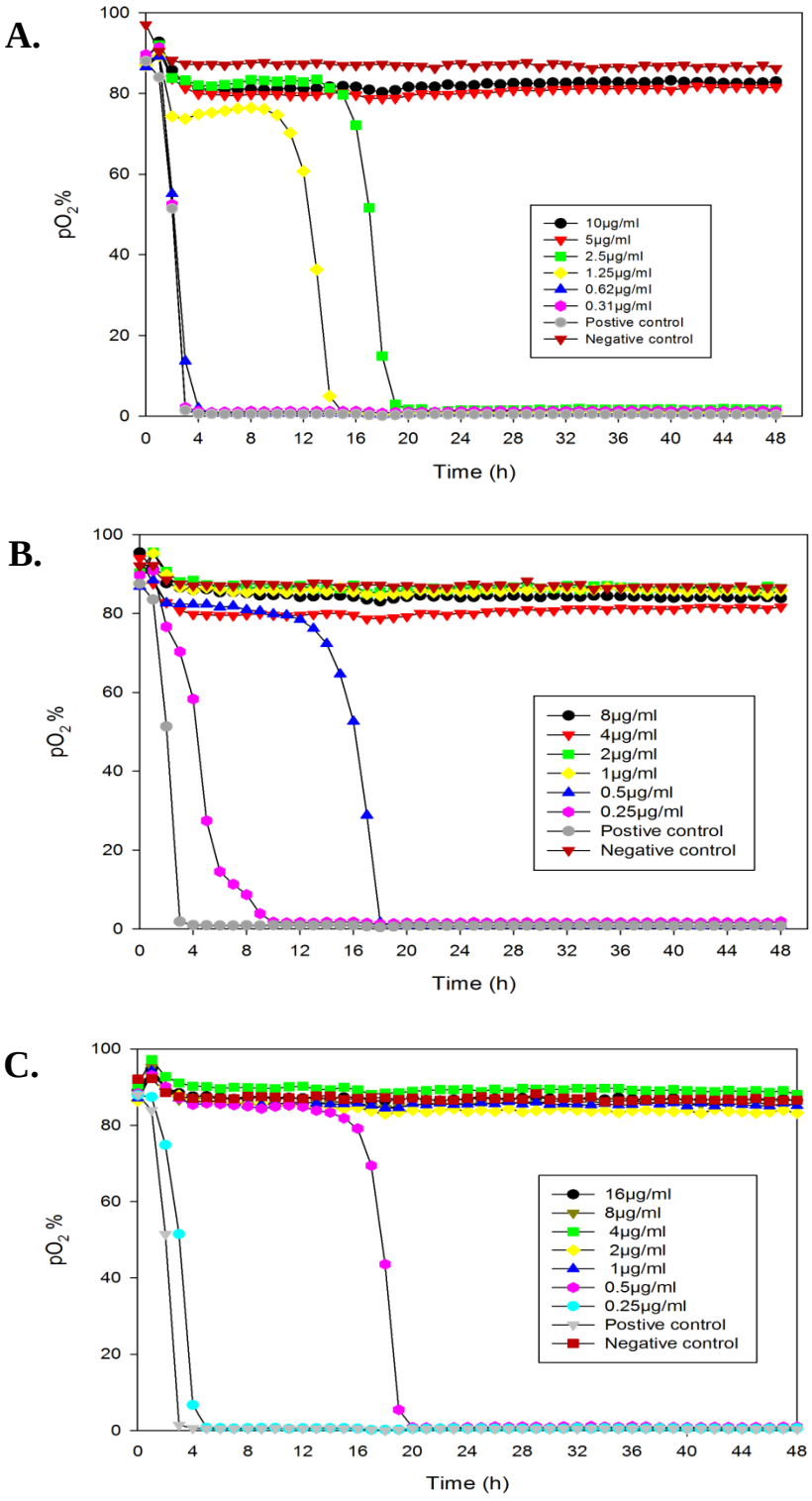


Figure 15: MIC and MBC of planktonic cells of *P.aeruginosa* treated with (A) amikacin; (B) tobramycin and (C) colistin at 37°C for 48h. It was assessed using a fluorescent plate reader, the lines represent the means of three replicates. Antibiotic concentrations have been indicated. The positive control presents an overnight culture of *P.aeruginosa* (untreated). The negative control represents an overnight culture injected with solution of sodium hypochlorite.

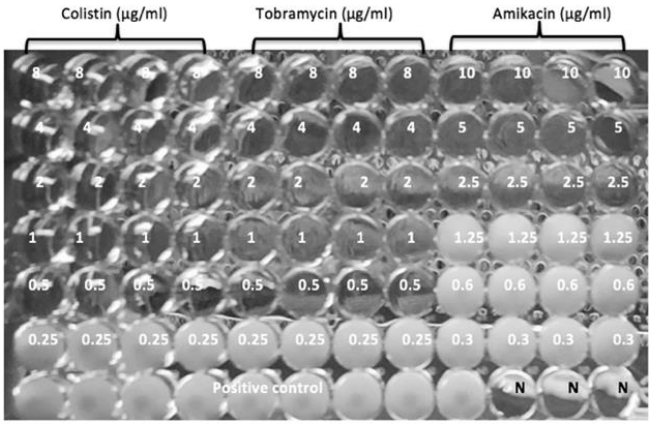


Figure 16: Conventional assay of the MIC for colistin, tobramycin and amikacin against "wild type" *P.aeruginosa* at 37°C for 24h. *P.aeruginosa* was tested for susceptibility at different concentrations ranges from 10-0.3µg/ml for amikacin and 8-0.25µg/ml for both colistin and tobramycin. The positive control represents an overnight culture of *P.aeruginosa* (untreated). The negative control represents an overnight culture injected with solution of sodium hypochlorite.

3.1.3. Measurement of antimicrobial activity using agar well diffusion method

A conventional bioassay method was employed *in-vitro* to ascertain the antibacterial activities of all three antibiotics on the bacterium *P.aeruginosa*. The results indicated that all three antibiotics had a pronounced effect on *P.aeruginosa*, generating extensive average zones of inhibition. These were identified by taking measurements of the zone diameter. For every antibiotic at each concentration, the test was carried out four times and the average values were recorded. According to the breakpoint interpretation by the EUCAST, *P.aeruginosa* was found to be active against three antibiotics.

Figure 17, shows that *P. aeruginosa* was particularly susceptible to tobramycin and colistin, as indicated by the inhibition zone size. Tobramycin at the concentration of 10µg/ml showed a zone of inhibition of 19.25 mm and the zone size of inhibition exhibited by colistin was 20.25 mm at the concentration of 25µg/ml. However, amikacin at 30µg/ml showed intermediate performance since the size of the inhibition zone was <18mm.

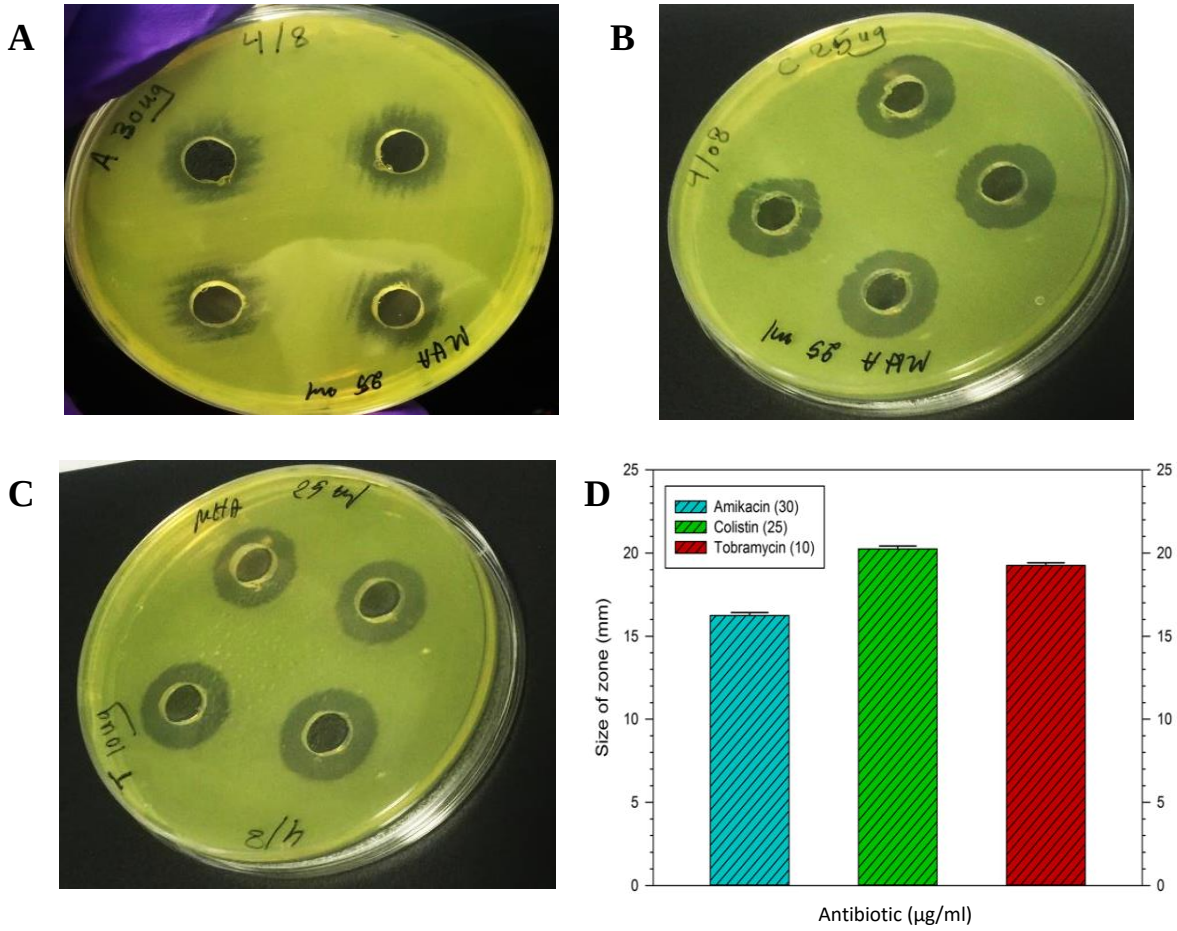


Figure 17: Growth inhibition zones of the planktonic cells of *P.aeruginosa* using the agar well diffusion method. (A) Agar size of plugs for amikacin at 30µg/ml; (B) Agar size of plugs for colistin at 25µg/ml; (C) Agar size of plugs for tobramycin at 10µg/ml. The Plates were incubated for 24 h at 4°C, followed by incubation at 37°C for another 24h. The clear zones around the well are the degree of the activity of antibiotics to inhibit growth of bacterium. (D) The means of four replications of growth inhibition zone size. The bars indicate the standard errors of the means.

3.2. Effect of selected antibiotics on *P.aeruginosa* growth dynamics

An automated plate reader called the SPECTRO $_{star}$ Nano was used in order to assess the growth response of wild-type of *P.aeruginosa* to a selection of antibiotics. The process of the automated cultivation based assay is a high throughput method and allows for on-line analysis of the bacteria that develop. Observing the OD during antibiotic treatment helps to obtain more detail in how bacterial cell populations behaved, the overall productivity of bacterial density during time of the experiment and the growth rate of the cell population. This method is important to perform because the activity of antimicrobial agents known to be influenced by the growth rate of microorganisms, which displayed diverse susceptibility to antibiotics depending on the different growth phases.

The results of this analysis help to determine which antibiotic concentrations impact on the extent to which *P.aeruginosa* grows. The control culture that has not been exposed to an antibiotic indicates the natural development of *P.aeruginosa.* It is apparent that after a 4-hour cultivation period (lag phase) this control culture demonstrated a significant increase in the OD. The remaining cultivation period showed a subsequent decline in the OD levels. However, the concentration of the antibiotic was directly influenced the lag phase period. For example, colistin (concentration 0.5 μg/ mL) treated *P.aeruginosa* experienced a lag phase duration of 11 h, whilst amikacin (1.25μg/mL) exposed to bacterial cells experienced a lag of 20 hours. For the higher amikacin concentration 2.5 μg/mL, there was a delayed up to 34 hours before the *P.aeruginosa* commenced growth. Thus the higher the concentration, the greater the lag phase. Furthermore, the duration of the lag phase in the culture exposed to tobramycin at a concentration of 0.25μg/mL was 10 hours whereas, a longer lag phase was observed of 26 hours in the presence of 0.5 μg/ mL of tobramycin. These findings are illustrated in Figure18. Based on these results, the detection of the bacterial growth inhibition treated with aminoglycoside antibiotics using the SPECTRO $_{star}$ Nano was not as high in terms of temporal resolution as the OxoPlate outputs.

During the exponential phase, however, the final OD values of the *P.aeruginosa* treated with amikacin and colistin (inhibitory concentrations) were comparable with

that of the control culture which was devoid of any antibiotics (Fig. 18A&C). The remaining antibiotic, culture had significantly lower OD values than those of the control culture when cultures exposed to inhibitory concentrations (Fig.18 B). Within the logarithmic phase, the OD values of *P.aeruginosa* treated with colistin at sub-inhibitory concentrations was noticeably higher than that of the control antibiotic free culture. This indicates that colistin is capable of acting as a growth stimulant when administered at sub-inhibitory concentrations such as 0.125-0.25 μg/mL (Fig.18C). After 18h, however, the OD values showed a gradual decreased in the colistin-exposed culture (sub-inhibitory concentrations) compared with control culture, which showed an extended period of the stationary phase. Furthermore, when the concentration of colistin was increased ≥1μg/mL, the treated culture becomes under stress and thus prevents the growth of *P.aeruginosa* entirely. For the remaining antibiotics that were tested, it is clear that treating *P.aeruginosa* with a high concentration affected the growth dynamic of cells.

This finding brings in to question which factors can be meaningful in predicting how an antibiotic will affect microbial growth. The final OD value, the lag phase duration and the growth of *P.aeruginosa* were all affected through concentration of antibiotics either sub- inhibitory or inhibitory during the course of treatment.

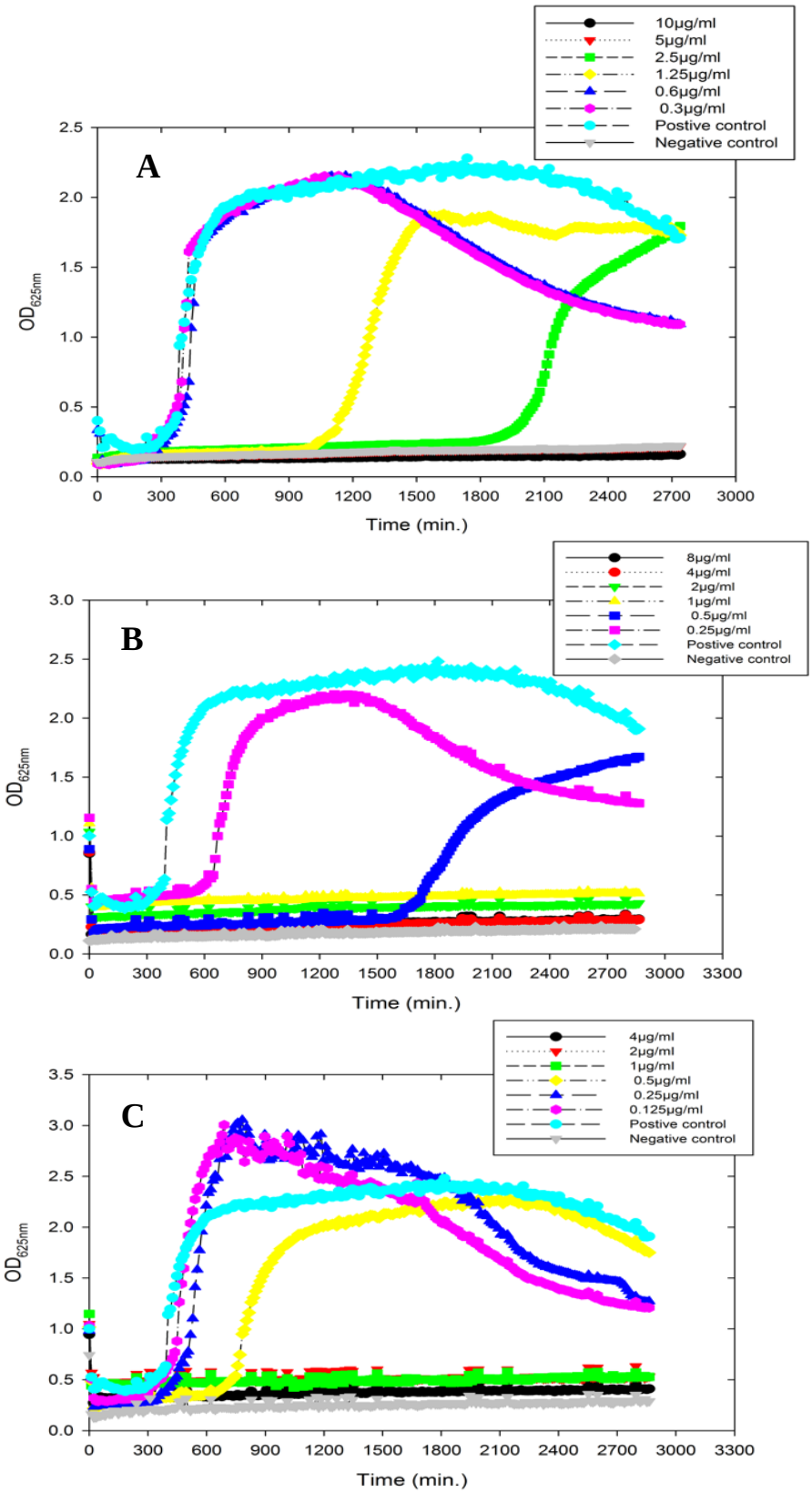


Figure 18: Effects of selected antibiotics on the growth dynamics of *P.aeruginosa* at 37°C. The individual data points represented in the above figure, over a 48 h period, reflect the progress of each of the cultures, which have been subsequently normalized by the control culture that was devoid of any antibiotic. In conjunction with a negative control culture represents growth medium- lacking bacterial cells. A range of antibacterial concentrations were tested to determine *P.aeruginosa*'s susceptibility. These concentrations ranged from 10-0.3µg/ml for amikacin (A) and 8-0.25µg/ml for tobramycin (B), and 4-0.125 µg/ml for colistin (C).

3.3. Environmental Factors

A range of environmental cues can affect the outcome of susceptibility testing, including the size of the bacterial inoculum, the chosen culture media and their pH, as well as incubation length and temperature (Gilbert et al., 1971).

3.3.1. The effect of temperature on the antimicrobial susceptibility of PA01

The classical antimicrobial techniques have been usually used to determine the activity of antibiotics by observing the growth inhibition of the test organisms. Thus, the *in vitro* susceptibility testing has to be reliable in order for the antibiotics to be used rationally. In this study, the antimicrobial activity of the antibiotics was determined under a range of temperatures using standard and innovative methods. The OxoPlate method was used in order to assess the antimicrobial action based on the oxygen depletion levels. The growth inhibition was also determined in conjunction with the MIC. To determine the inhibition that the antibiotics caused to the growth of *P.aeruginosa*, the treated cultures were compared against the untreated culture (control). The MIC and MBC values obtained by method of OxoPate for the three antibiotics against the strain PA01 is shown Fig.19, 20 &21. Figures 15 and19 show that, a considerable difference in the susceptibility to amikacin was observed at the different incubation temperatures 42°C, 37°C and 30°C. At 42°C, PA01 became more sensitive to amikacin, which exhibited a 4- fold decreased in the MIC value compared with those obtained at 25°C or 30°C. A similar result for the MIC value (2.5μg/ml) was obtained at both temperatures 25°C and 30°C, in spite of the different incubation temperatures of amikacin. However, when the bacterial culture was incubated for another 24h the activity of amikacin at 10 μg/ml switched from bactericidal to bacteriostatic when incubation temperature was decreased from 30°C to 25°C. Through the use of the E-test (Fig. 22), the presence of surviving colonies was indicated in association with an amikacin at 25°C. It has been suggested that the surviving colonies may represent persistent bacterial cells that respond heterogeneously to the action of the antimicrobial agent.

The activity of tobramycin remained the same (no change in the MIC value) when incubated at the temperature of 42°C compared with control sample. Tobramycin however, had the ability to inhibit the growth of PA01 for a long period when

incubated at 42°C compared with control sample at optimum temperature Fig.15B and 20. The length of the antibiotic action was diminished from11h to 6h when the temperature decreased from 42°C to 37°C in which bacteria were treated with 0.25µg/ml of tobramycin. From this analysis (Fig.21), the OxoPlate results showed that at a temperature of 25°C and 30ºC there was no observable change in the MIC value of colistin to PA01 compared to control culture at optimum incubation temperature (37°C). At these temperatures, however the activity of colistin was determined through detecting the time taken to observe the initial decrease in oxygen (time to detect). For colistin the time to detect was delayed for 19h at 37°C while at 25 °C and 30 °C the time to detect was shortened to 8h and 14h respectively.

The MBC value for amikacin showed a decrease when the temperature was increased from 20 µg/ml at 25 °C to 2.5 µg/ml at 42ºC. Decreasing the temperature to 25ºC during incubation with colistin led to an increase in the MBC value to 2µg/ml compared with the control sample at 37°C. However, the reduction of the MBC value (0.5 µg/ml) was enhanced at 42°C for colistin. The result showed that a notable difference was exhibited by the action of tobramycin; at the low incubation temperatures (25°C and 30°C). Tobramycin exhibited the same MBC (2µg/ml) and there was no observable change. In addition, at the high temperatures (37°C and 42°C), tobramycin also displayed identical MBC values (1µg/ml) for both temperatures. It was observed that, in comparison to the MBC of colistin and tobramycin, amikacin MBC values were more affected by temperature.

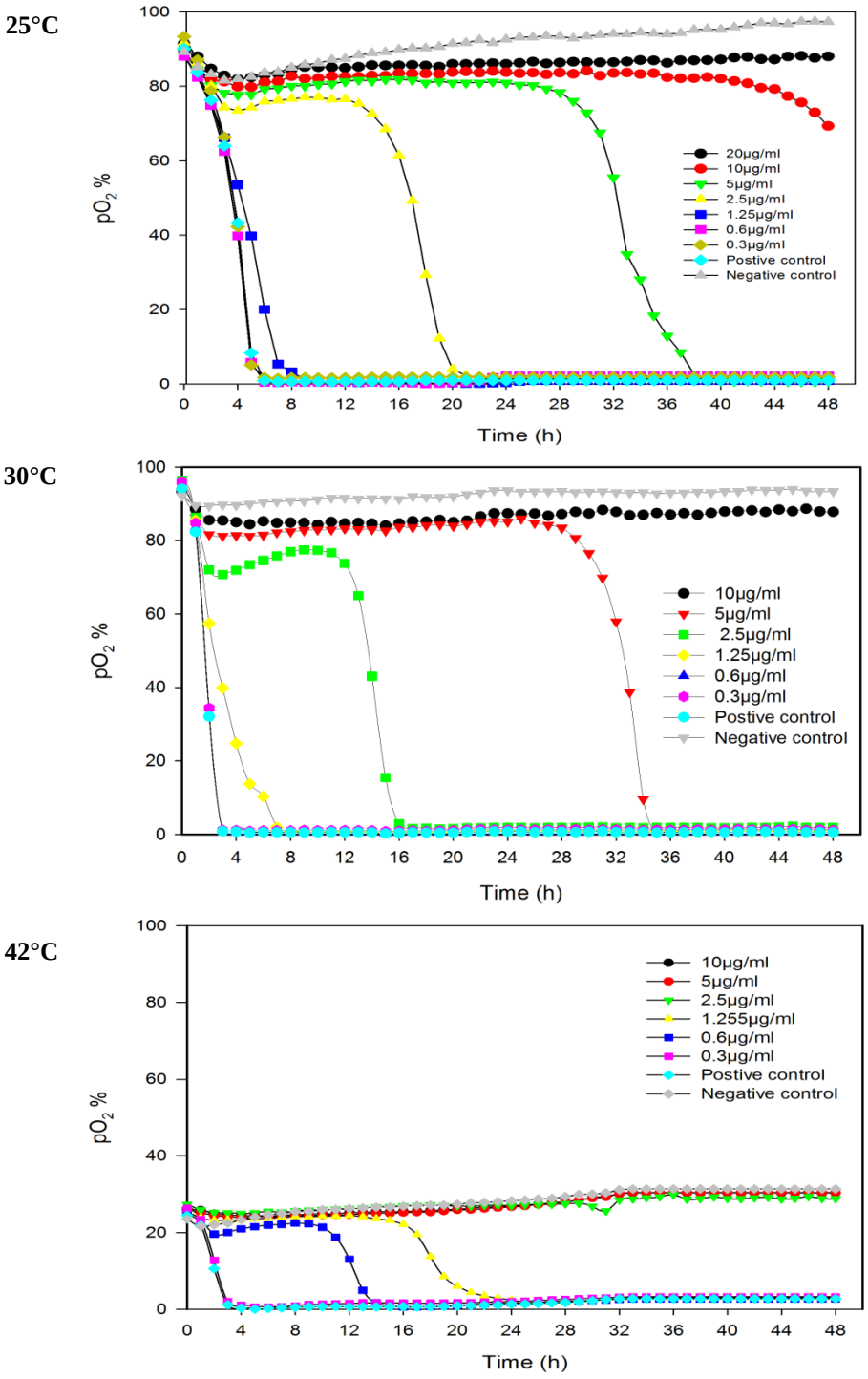


Figure 19: Effect of temperatures on the susceptibility of amikacin against PA01. It was assessed based on the depletion of the oxygen using the OxoPlate OP96U. The lines represent the means of three replicates. Antibiotic concentrations have been indicated. The positive control presents an overnight culture of *P.aeruginosa* (untreated). The negative control represents an overnight culture injected with solution of sodium hypochlorite (10% v/v). The MIC represents the lowest concentration that exhibits visual discrepancies from the untreated positive control sample at any given point in the first 10 hours following drug treatment. The MBC, which is the equivalent to the lowest concentration that induces an increase in the oxygen concentration.

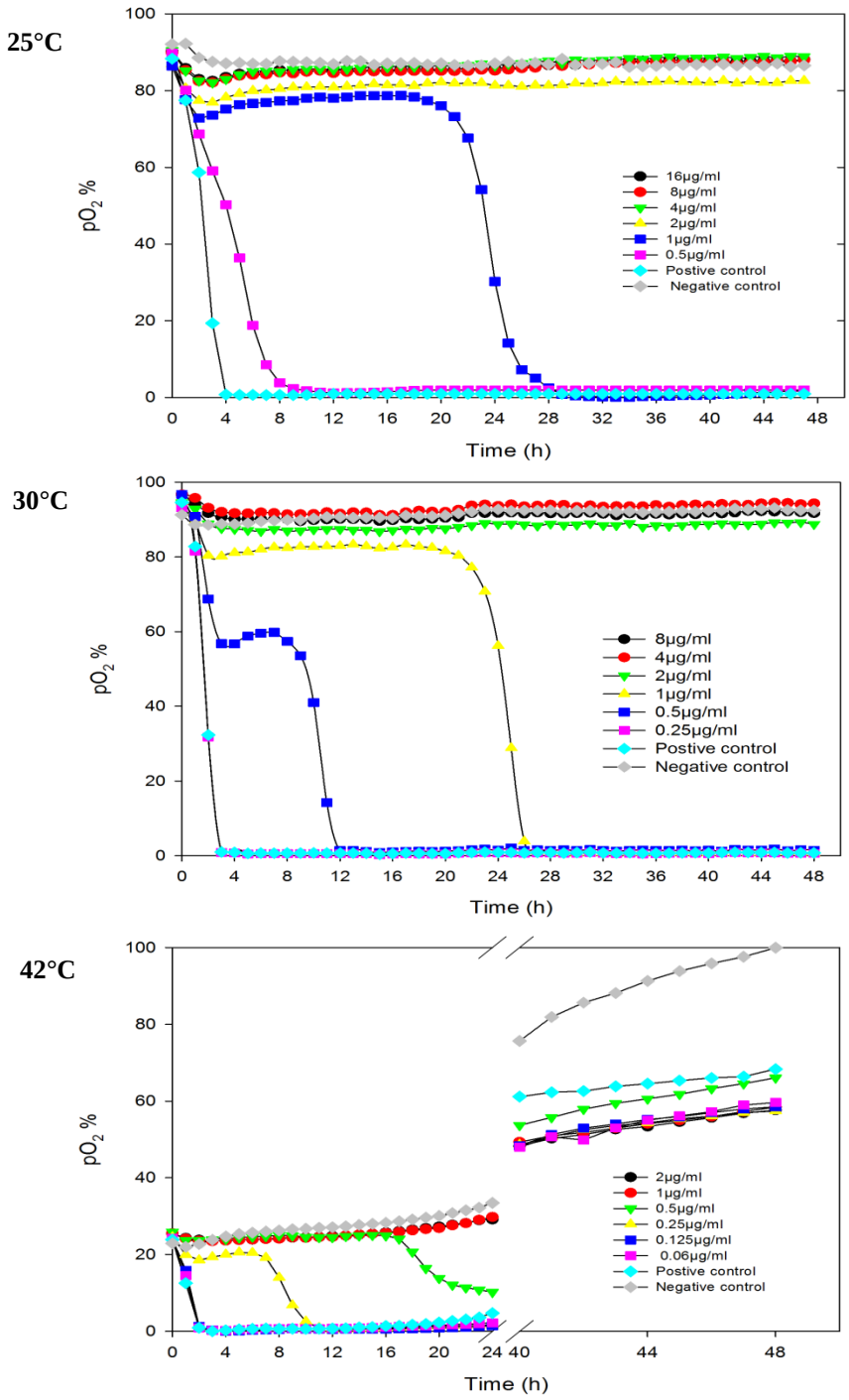


Figure 20: Effect of temperatures on the susceptibility of tobramycin against PA01. It was assessed based on the depletion of the oxygen using the OxoPlate OP96U. The lines represent the means of three replicates. Antibiotic concentrations have been indicated. The positive control presents an overnight culture of *P.aeruginosa* (untreated). The negative control represents an overnight culture injected with solution of sodium hypochlorite (10% v/v). The MIC represents the lowest concentration that exhibits visual discrepancies from the untreated positive control sample at any given point in the first 10 hours following drug treatment. The MBC, which is the equivalent to the lowest concentration that induces an increase in the oxygen concentration.

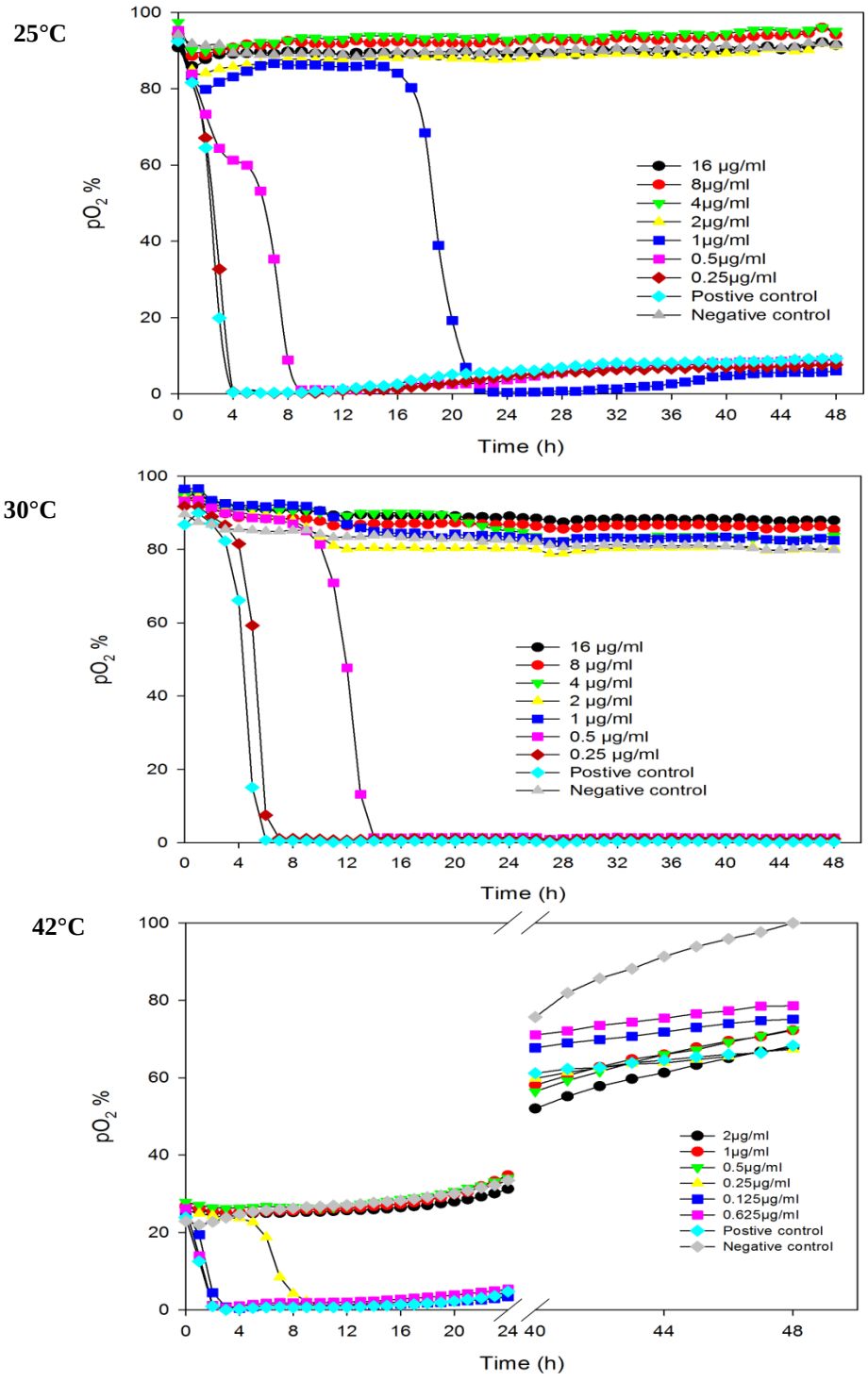


Figure 21: Effect of temperatures on the susceptibility of colistin against PA01. It was assessed based on the depletion of the oxygen using the OxoPlate OP96U. The lines represent the means of three replicates. Antibiotic concentrations have been indicated. The positive control presents an overnight culture of *P.aeruginosa* (untreated). The negative control represents an overnight culture injected with solution of sodium hypochlorite (10% v/v). The MIC represents the lowest concentration that exhibits visual discrepancies from the untreated positive control sample at any given point in the first 10 hours following drug treatment. The MBC, which is the equivalent to the lowest concentration that induces an increase in the oxygen concentration.

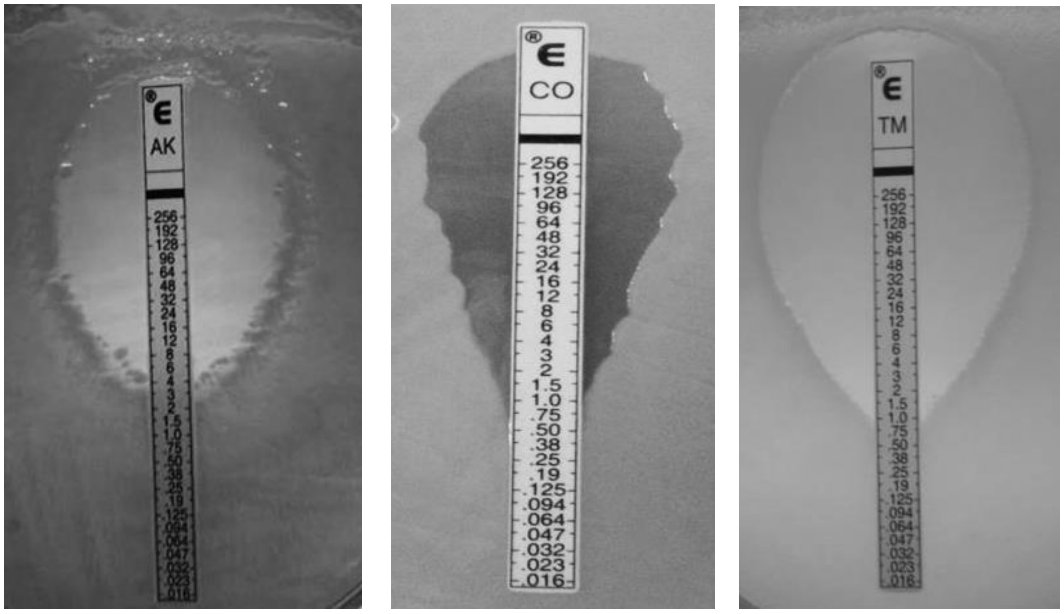
The distribution of the MICs by the Agar Well Diffusion and the E-test of the three antibiotics for PA01 is shown in Table 3. The MIC values obtained by the E-test and Agar Well Diffusion were higher than the OxoPlate's MICs except the MIC obtained by E-test at 42°C. However, the E-test showed that test organism was strongly influenced by temperature. The MIC values of colistin declined as the temperature increased, from 1µg at 25°C to 0.01µg at 42°C (Fig.22). Yet again, the E-test MIC values for amikacin increased with a decrease in the incubation temperature, from 0.12µg at 42°C to 4µg at 25°C (Fig.22). The results obtained from the Agar Well Diffusion method showed that at a temperature of 30°C and 37ºC there was no observable change in the MIC value for all antibiotics tested (Table 3). However, when the temperature was increased from 25°C to 42°C, the MIC of colistin showed a decrease from 8 µg/ml to 2 µg/ml. The activity of tobramycin became less when temperature decreased to 25ºC.

Table 3: The MICs values of antibiotics over a rage of temperatures against *P.aeruginosa* using the Agar Well Diffusion and the E-test

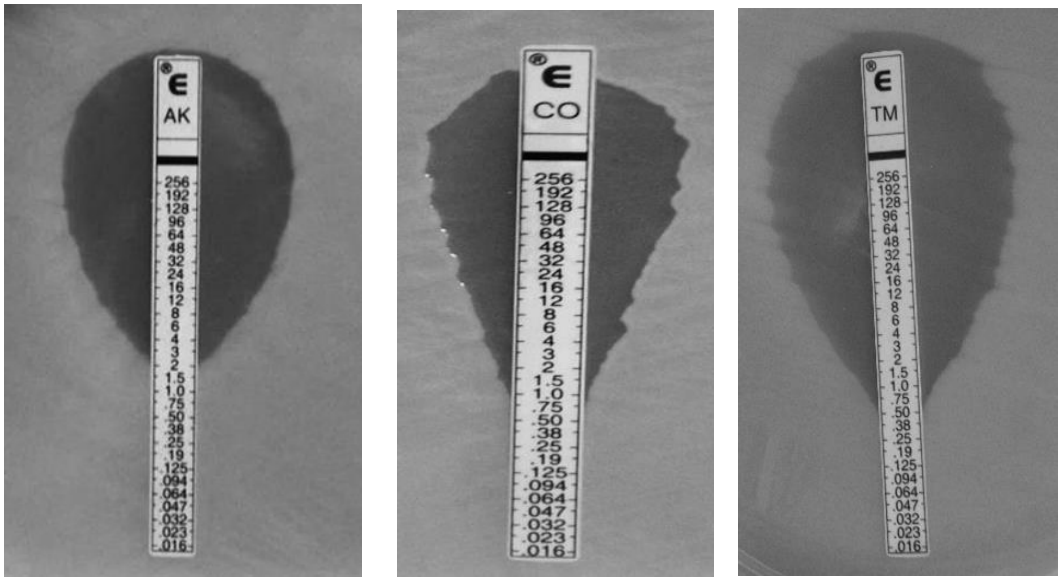
	E-test				**Agar Well Diffusion**			
Antibiotic code	MIC (µg)				MIC (µg/ml)			
	37°C Con.	**25°C**	**30°C**	**42°C**	**37°C Con.**	**25°C**	**30°C**	**42°C**
AM	1	4	2	0.12	10	10	10	5
TM	0.38	1	0.5	0.32	2	8	2	2
CO	0.5	1	0.75	0.01	4	8	4	2

Con: Control; **AM**: Amikacin; **TM**: Tobramycin; **CO**: colistin

25°C



30°C



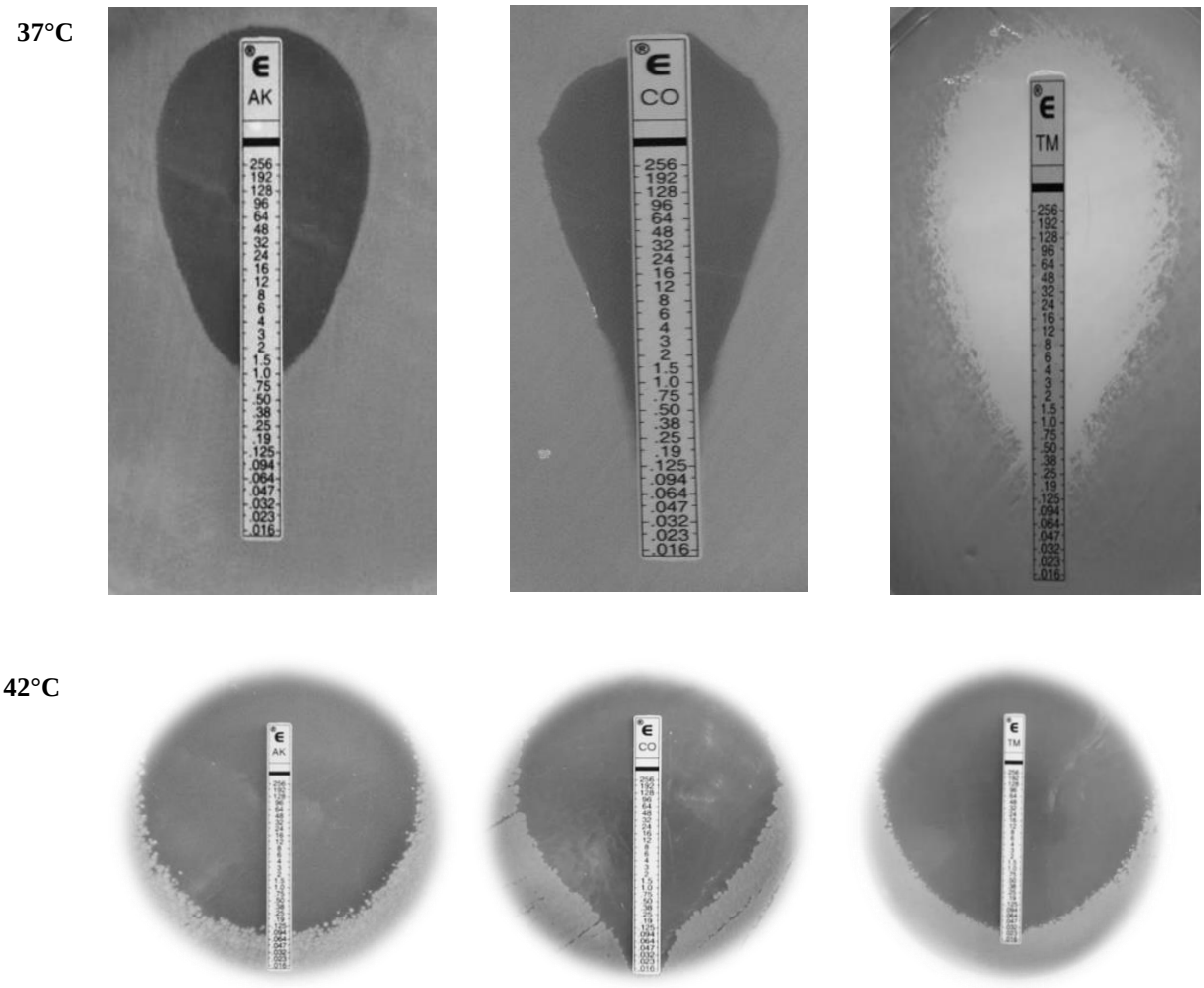


Figure 22: The MIC of amikacin (AK), colistin (CO) and tobramycin (TM) generated by E-test. The MIC determined after 24h at different incubation temperature. It was measured based on a point where the inhibition ellipse is observed on the top of the strip intersection with strip.

3.3.2. The relationship between the growth rate of *P.aeruginosa* and incubation temperature

An automated 96-well microtiter plate assay (SPECTRO $_{star}$ Nano) was used to monitor the effect of temperature on the growth dynamics of *P.aeruginosa*. The growth rate curves of *P.aeruginosa* versus temperature are shown in Fig. 23. The temperature was in the range from 25°C to 42°C. The impact of the temperature on the rate of bacterial growth over 24-h incubation became evident, when the growth dynamics was examined in real time using the automated assay. Figure 23 shows that cultures exposed to low temperature exhibited a longer lag phase compared with other cultures. The growth rate was measured using the following equation

$$\mu = \text{In Nt-In N0/t} \quad (12).$$

μ: represents the specific growth rate, **Nt:** represents the final optical density value during the log phase**, N0**: represents the initial value of bacterial density and **t:** represents time**.**

The growth rate of bacterial cells was increased as the temperature increased (Fig.23). At 25ºC, bacterial population exhibited lower overall productivity compared to the other cultures. Thus, temperture had a significant effect in the final OD value, lag phase duration and specific growth rate of *P.aeruginosa*.

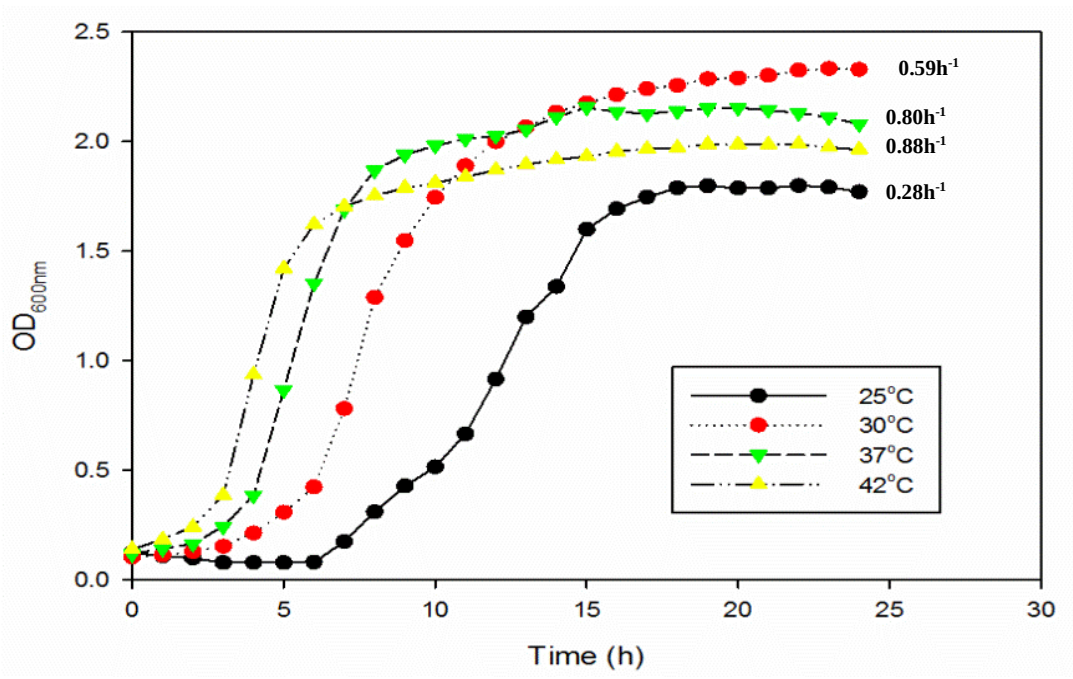


Figure 23: Effect of incubation temperature on the growth of *P.aeruginosa* using the SPECTRO $_{star}$ Nano. (A) The growth rate of tested bacterium. It was assessed based on ΔOD at 600 nm. The lines represent the mean of three replicates. The growth rate of *P.aeruginosa* at different incubation temperatures was also measured.

3.3.3. The effect of divalent cations in a defined synthetic medium on the antimicrobial susceptibility of PA01

Divalent cations are known to influence the measurement of MBC values when studying the effects of aminoglycoside antibiotics and colistin on gram negative bacteria. In this study we have used a defined synthetic medium and investigated the effect of the levels of Ca^{2+} and Mg^{2+} on the metabolism of *P.aeruginosa* in the presence of bactericidal concentrations using an OxoPlate system.

3.3.3.1.Deducing the MBC from OxoPlate measurements

The MBC was determined using the OxoPlate reader on the basis of levels of oxygen depletion. The minimum antibiotic concentration reflecting the MBC was attained by plotting the oxygen concentration alongside time. The MBC was determined based on the re-introduction of atmospheric oxygen into the well once the cells had died, which caused the MBC correspondent to the minimum concentration to trigger a rise in the oxygen concentration. Every bacterial culture sample that antibiotics were applied to was characterised by an oxygen depletion curve. Figure 24 indicates the MBCs of the strain that was tested. Amikacin had an MBC of 2.5µg/ ml, whereas tobramycin had an MBC of 0.5µg/ ml. Colistin had a bactericidal effect on PA01 at a concentration of 2µg/ ml, which represents the MBC value.

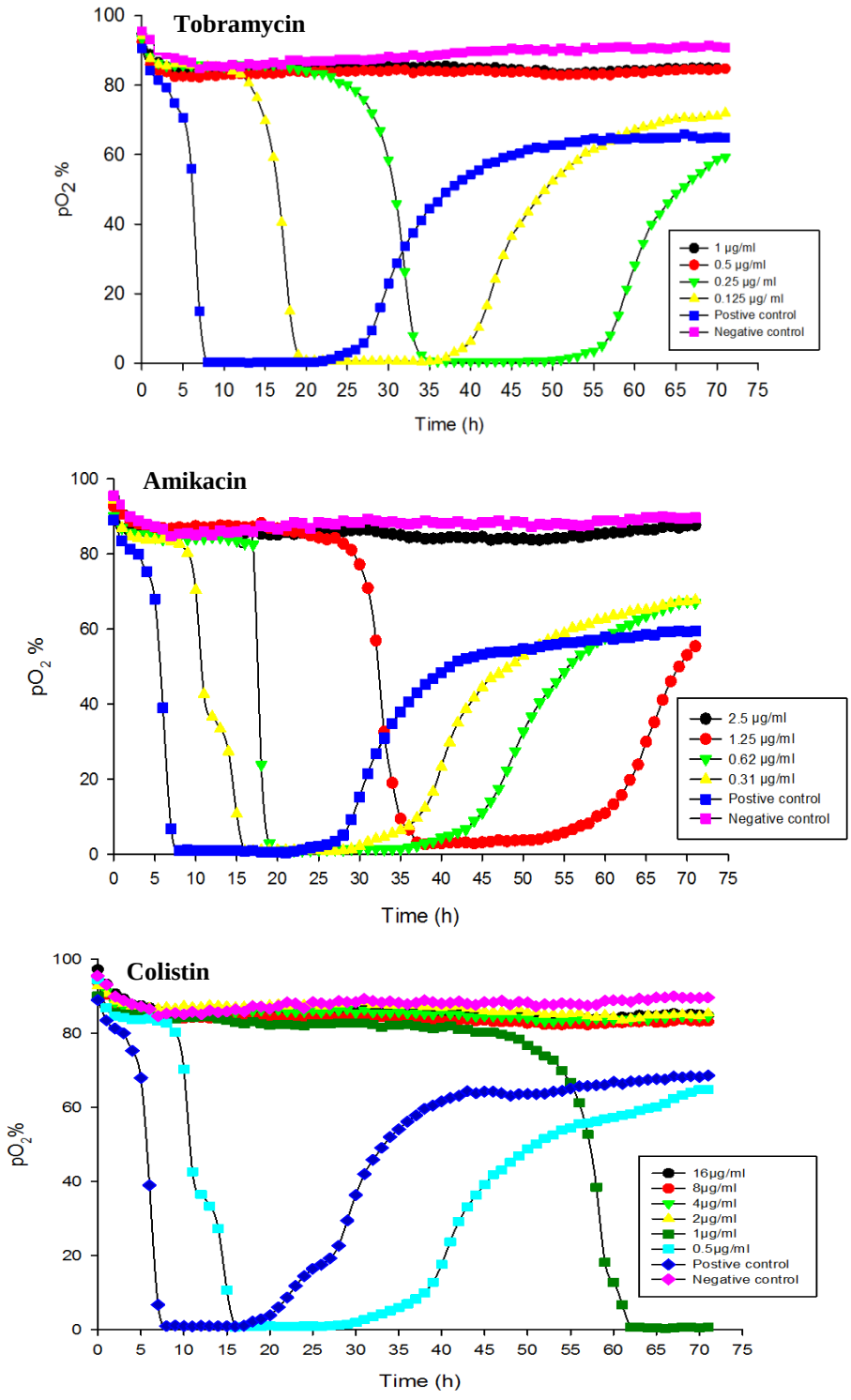


Figure 24: The MBC of classes of antimicrobial agents in a defined synthetic medium against *P.aeruginosa.* The oxygen consumption of an overnight culture of planktonic cells of *P.aeruginosa* treated with amikacin, tobramycin and colistins using a fluorescent plate reader incubated at 30°C for 72 h. Antibiotic concentrations have been indicated. The MBC represents the lowest concentration that induces an increase in the oxygen concentration as a result of oxygen diffusion back into the wells following cell death. In contrast to the untreated positive control sample, the negative control represents an overnight culture injected with a solution of sodium hypochlorite (10% v/v).

3.3.3.2.Effect of different divalent cation individually on the susceptibility of *P. aeruginosa* to antibiotics

The effect of the various concentrations of Mg^{2+}or Ca^{2+} on the susceptibility of *P.aeruginosa* to lethal doses of the antibiotics is shown in Figures 25 and 26. The level of kinetics of bacterial growth suppression is represented by the oxygen curves. A decline in these curves over a short time indicated accelerated cell growth, promoted by the higher concentration of ions. *P.aeruginosa* showed a change in the growth inhibition when the concentration of Ca^{2+} or Mg^{2+} was increased. The consumption of oxygen was observed immediately at a high cation concentration whereas; oxygen consumption occurred at a slower rate at low cation concentrations.

No oxygen consumption was observed to occur when a bactericidal dose of aminoglycoside and colistin was applied to *P.aeruginosa* in the absence of divalent cations. The study results corroborate that, in comparison to tobramycin and colistin, amikacin had a weaker effect on *P. aeruginosa* when Mg^{2+}were individually added in defined medium. The action of amikacin was gradually obliterated, shown by a decline in the duration of oxygen depletion as the concentration of Ca^{2+} or Mg^{2+} were increased from 2 to 5mM. For tobramycin at concentration of Mg^{2+} up to 3mM no oxygen consumption was detected. The susceptibility of *P. aeruginosa* to the action of colistin was low in medium that contained Ca^{2+} ranging from 1mM to 5mM, which showed a large change in the period of growth inhibition. Even at a low concentration of Ca^{2+}, the level of the oxygen was reduced following a period of incubation of 56 h in the presence of colistin (Fig. 26). Ca^{2+} was found to provide greater protection to bacterial cells over that provided by Mg^{2+}.

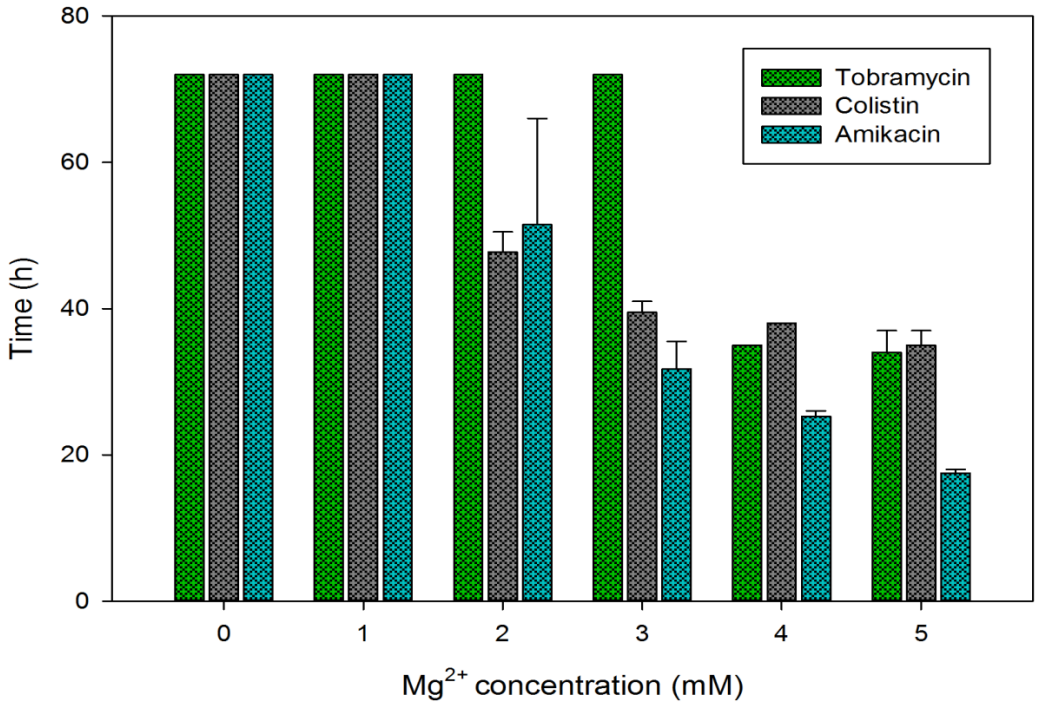


Figure 25: The effect of various concentrations of Mg^{2+} on PA01 treated with amikacin at 2.5µg/ ml, tobramycin at 0.5µg/ ml and colistin at 2µg/ ml. The amount of dissolved oxygen was followed over 72h. The time represents the initial decrease of oxygen level "time to detect". The values shown are the means of three replicates. Note: Where time to detect values is shown as 72 h this indicates that no oxygen consumption was detected in the duration of the experiment.

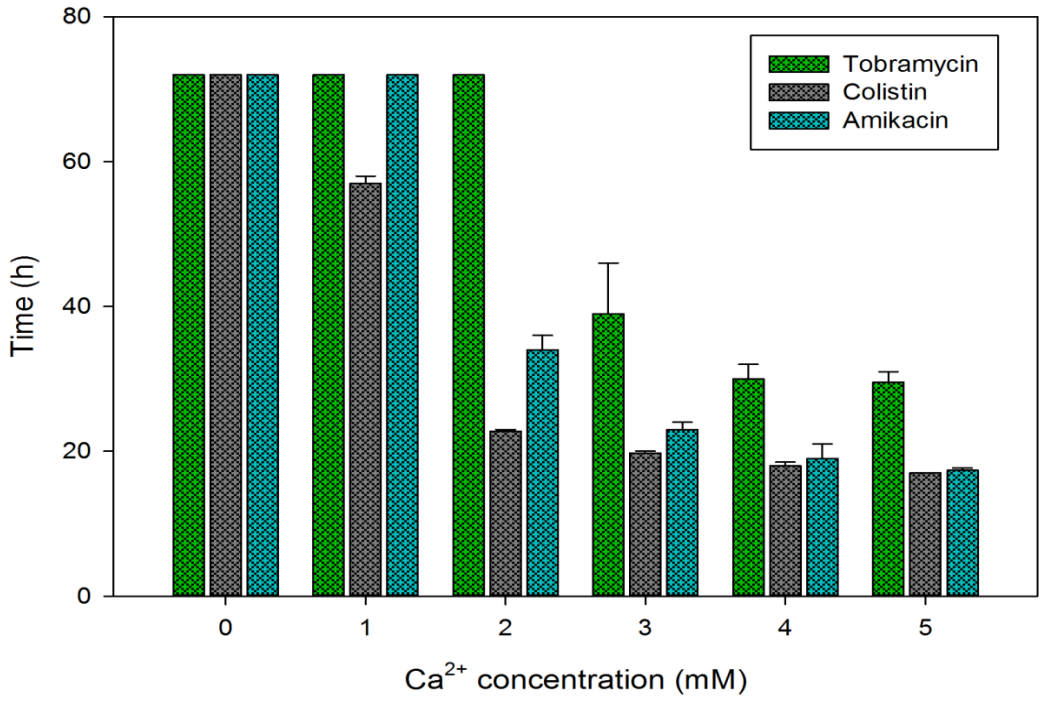


Figure 26: The effect of various concentrations of Ca^{2+} on PA01 treated with amikacin at 2.5µg/ ml, tobramycin at 0.5µg/ ml and colistin at 2µg/ ml. The amount of dissolved oxygen was followed over 72h. The time represents the initial decrease of oxygen level "time to detect". The values shown are the means of three replicates. Note: Where time to detect values is shown as 72 h this indicates that no oxygen consumption was detected in the duration of the experiment.

3.3.3.3.Effect of Mg^{2+} in combination with Ca^{2+} on the *in-vitro* susceptibility of *P. aeruginosa* to antibiotics

The impact of combining Mg^{2+} and Ca^{2+} on the *in vitro* action of amikacin, tobramycin and colistin on *P. aeruginosa* was also investigated using the OxoPlate reader, which was based on the depletion of dissolved oxygen (see Fig. 27, 28 and 29). It was observed that the combined concentrations of Mg^{2+} and Ca^{2+} had greater influence on the effect of amikacin than that of tobramycin. The development of *P. aeruginosa* was suppressed by tobramycin for a long period time when 1mM of Ca^{2+} was added in the defined medium in the presence of increasing concentrations of Mg^{2+}. The duration of dissolved oxygen declined when the Mg^{2+} concentration was increased, from 72 h at 1mM to 21 h at 5mM (see Figure 28). In contrast, the *in vitro* efficacy of amikacin against PA01 was reduced by the combined effects of Ca^{2+} and Mg^{2+}supplementation. Figure 27 shows that the period of growth inhibition declined when it was in the presence of a bactericidal concentration of amikacin at increasing concentrations of Mg^{2+} combined with a constant concentration of Ca^{2+} (1mM), from 34 h at 1mM to 12 h at 5mM. The bacterial cells treated with colistin showed that the addition of ≤ 3mM Mg^{2+} in the presence of low concentrations of Ca^{2+} (1-2mM) resulted in a short period of the growth inhibition (Figure 29).

The protection of bacterial cells against the action of antibiotics, provided by divalent cations, was evident. High concentrations of Ca^{2+} provided a greater level of protection when it was combined with Mg^{2+}. The bactericidal activity of colistin was sharply reduced when 5mM of Ca^{2+} were combined with Mg^{2+} (5mM) (Figure 29). The oxygen consumption was detected following a period of incubation of 5 h. Figure 25 shows that when the medium was treated with tobramycin in the presence of Mg^{2+} concentrations, ranging from 1 to 3mM, no oxygen consumption occurred. This effect was seen in the medium where calcium was absent. The results show that the effects of Mg^{2+} were removed when combined with Ca^{2+} (5mM) in the presence of tobramycin. *P. aeruginosa*'s susceptibility to tobramycin was constantly responded to by the addition of 5mM of Ca^{2+} combined with Mg^{2+}concentrations of between 1 and 5mM. The oxygen consumption was detected following a period of incubation of 14 h (see Figure 28). The bactericidal activity of amikacin was progressively reduced when concentrations of 4 or 5mM of Ca^{2+} were added alongside increased concentrations of Mg^{2+} (see Figure 27).

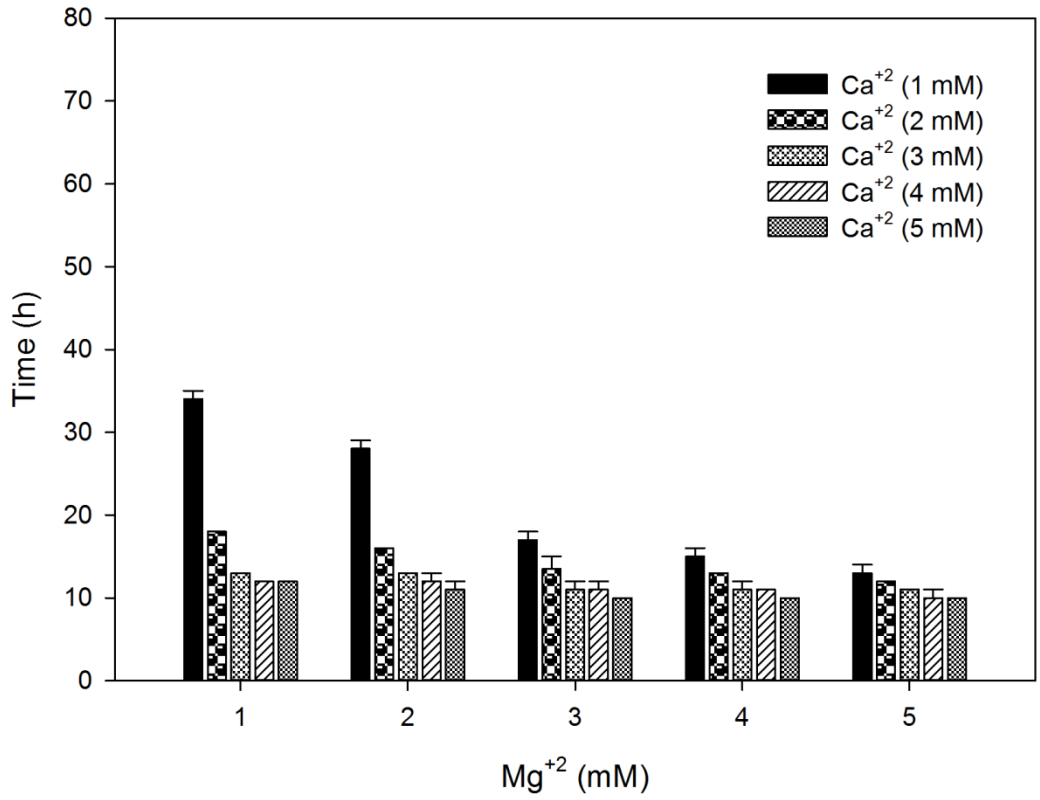


Figure 27: Effect of combinations of Mg^{2+} and Ca^{2+} on the rate of dissolved oxygen depletion in culture medium treated with amikacin at 2.5µg/ ml . The time represents the "time to detect" the start of oxygen depletion. The values shown are the means of two replicates and the vertical bars represent the standard errors of the values.

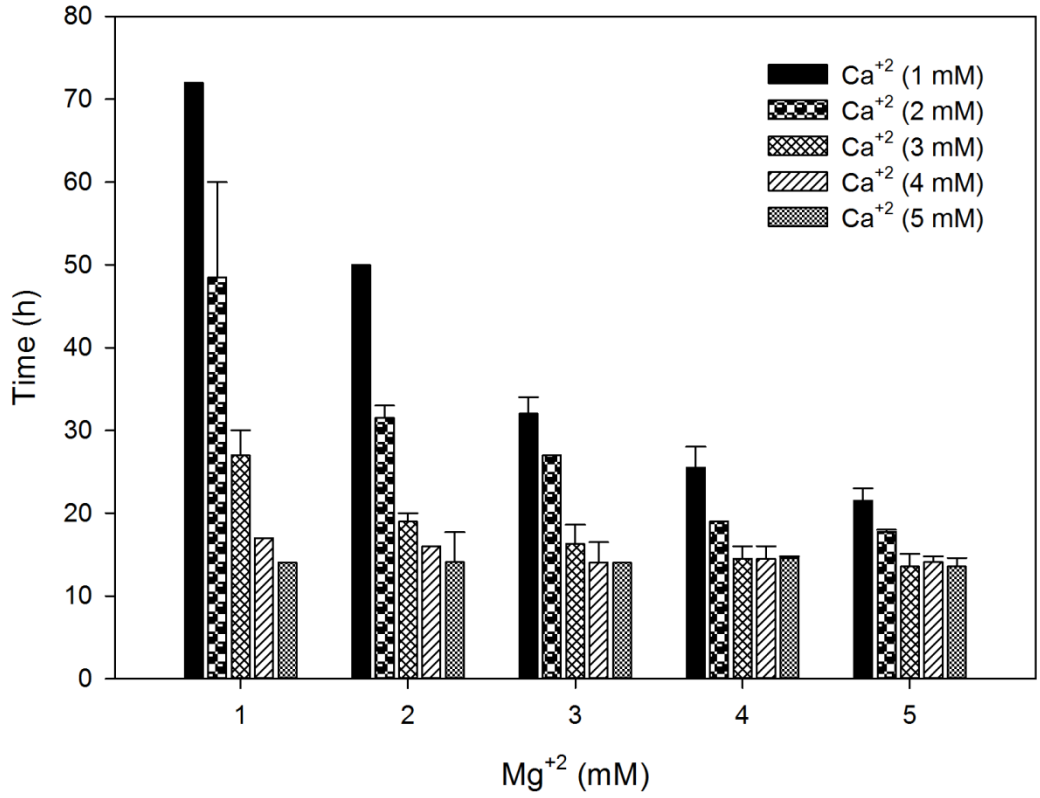


Figure 28: Effect of combinations of Mg^{2+} and Ca^{2+} on the rate of dissolved oxygen depletion in culture medium treated with tobramycin at 0.5µg/ ml. The time represents the "time to detect" the start of oxygen depletion. The values shown are the means of two replicates and the vertical bars represent the standard errors of the values.

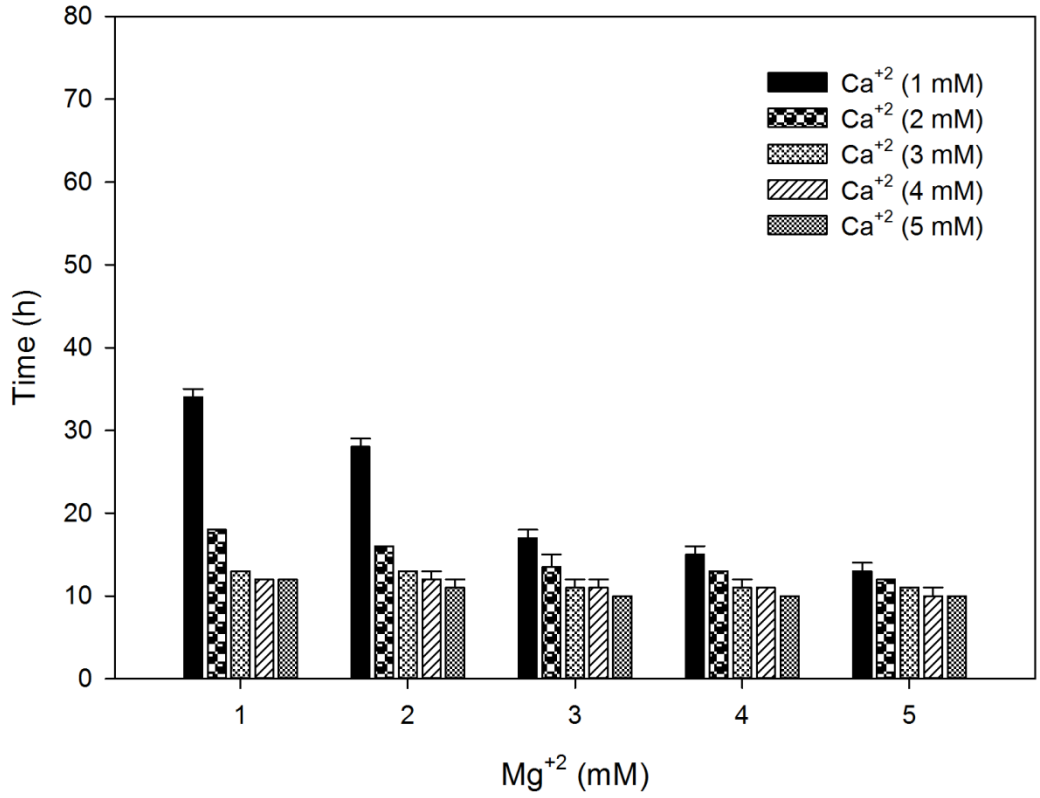


Figure 29: Effect of combinations of Mg^{2+} and Ca^{2+} on the rate of dissolved oxygen depletion in culture medium treated with colistin at 2µg/ ml. The time represents the "time to detect" the start of oxygen depletion. The values shown are the means of two replicates and the vertical bars represent the standard errors of the values.

3.3.4. Influence of divalent cations on the growth of *P.aeruginosa*

Divalent cations are well known ions that influence a number of fundamental processes in bacteria. In this study a defined synthetic medium was used to investigate the effect of Ca^{2+} and Mg^{2+} in the presence of antibiotics on the growth kinetics of *P. aeruginosa* using the SPECTRO $_{star}$ Nano system.

3.3.4.1.Response of *P.aeruginosa* to Ca^{2+} and Mg^{2+} when individually added into defined medium containing antibiotics

Effects of Ca^{2+} concentrations: Based on the growth of *P.aeruginosa*, the activity of all antibiotics used was clearly affected by the concentrations of Ca^{2+} (Fig. 30). Based on ΔOD at 600 nm during the time of the experiment, growth was clearly inhibited when Ca^{2+} (1-5 mM) was individually added in the cultures lacking antibiotic (Fig.30.A). The growth curves show that in comparison to colistin, aminoglycoside agents had a higher OD values when Ca^{2+} was individually added in the defined medium. The bacterial density reached 0.72, 0.69 and 0.56 when the cultures were treated with amikacin supplemented with Ca^{2+} at a concentration of 3, 4 and 5mM respectively (Fig.30 B). In the case of colistin, however the OD of the cells was reduced when the Ca^{2+} concentration was increased, from 0.51 at 3mM to 0.39 at 5mM (Fig.30.D). The study indicated that as Ca^{2+} concentration increased, bacterial densities were decreased. Within the logarithmic phase, the growth dynamic of *P.aeruginosa* treated with colistin in the presence of 1mM of Ca^{2+} had higher OD values than culture exposed to ≥ 2mM of Ca^{2+}.

To investigate the effect of divalent cations on the growth rate of *P.aeruginosa*, Eq. 12 was taken into account. The results of the influence of Ca^{2+} on the growth *(μ)* of *P.aeruginosa* presents in Table 4. The data indicated that the presence of Ca^{2+} ions in the antibiotic free medium caused a gradual decreased in the growth rates. The addition of increasing concentrations of Ca^{2+} from 3 to 5mM showed a large difference on the growth rates of bacterial cultures exposed to antibiotics compared with the control cultures. The growth rates show that in comparison to colistin and tobramycin, amikacin had a faster growth rates when Ca^{2+} was individually added in

the defined medium. Based on the growth rate of *P.aeruginosa*, the culture treated with amikacin in the presence of 3mM of Ca^{2+}exhibited an increase in the growth rate to 0.14 h^{-1} compared with other cultures.

Table 4: Effect of Ca^{2+} concentrations on the growth rate of *P.aeruginosa*

Antibiotic concentration ug/ml shown in parenthesis and growth rate (*u*) h^{-1}				
Ca^{2+}	Control	Amikacin (2.5)	Tobramycin (0.5)	Colistin (2)
0mM	0.02	–	–	–
1mM	0.06	–	–	0.07
2mM	0.06	–	–	0.06
3mM	0.03	0.14	0.11	0.07
4mM	0.03	0.09	0.06	0.07
5mM	0.02	0.09	0.04	0.03

Effects of Mg^{2+} concentrations: Based on the growth curves (Fig.30), Mg^{2+} had a strong effect on the cells of *P.aeruginosa* when it was individually added in the antibiotic free medium, which resulted in a 6-fold increase in the OD values compared with Ca^{2+}. Observations revealed that the addition of divalent cations inhibited the activity of antibiotics used but the inhibition potency of Ca^{2+} was higher than that of Mg^{2+}. With Mg^{2+} as the concentration was increased final bacterial densities also increased. This was observed in all stages of growth when the bacterial culture treated with amikacin in the presence of different concentrations of Mg^{2+} (Fig. 30.B). For the remaining two antibiotics, cultures had lower final OD values when cultures were exposed to high concentrations of Mg^{2+} compared with amikacin. For tobramycin, the culture of bacterial cells reached a lower OD value when 5mM of Mg^{2+} was added individually into defined synthetic medium compared with lower concentrations (Fig.30. C). However, a lowering of Mg^{2+} concentration to 2mM resulted in an increase in the cell density when the culture was treated with colistin (Fig.30. D).

The effect of Mg^{2+} concentrations on the growth rate of *P.aeruginosa* is shown in Table 5. The presence of Mg^{2+} ions in the synthetic medium treated with colistin caused a decrease in the specific growth rates as the concentrations of Mg^{2+} increased from 0.12h^{-1} at 3mM to 0.08h^{-1} at 5mM. However, addition of Mg^{2+} (2mM) resulted in an increase in the growth of *P.aeruginosa* when the bacterial

culture was treated with colistin. The addition of increasing concentrations of Mg^{2+} did not show a significant difference on the growth rate of bacterial cultures exposed to aminoglycoside agents.

Table 5: Effect of Mg^{2+} concentrations on the growth rate of *P.aeruginosa*

Antibiotic concentration ug/ml shown in parenthesis and growth rate (u) h^{-1}				
Mg^{2+}	Control	Amikacin (2.5)	Tobramycin (0.5)	Colistin (2)
0mM	0.021	–	–	–
1mM	0.13	–	–	–
2mM	0.14	0.11	–	0.17
3mM	0.14	0.11	–	0.12
4mM	0.14	0.14	0.15	0.09
5mM	0.13	0.11	0.15	0.08

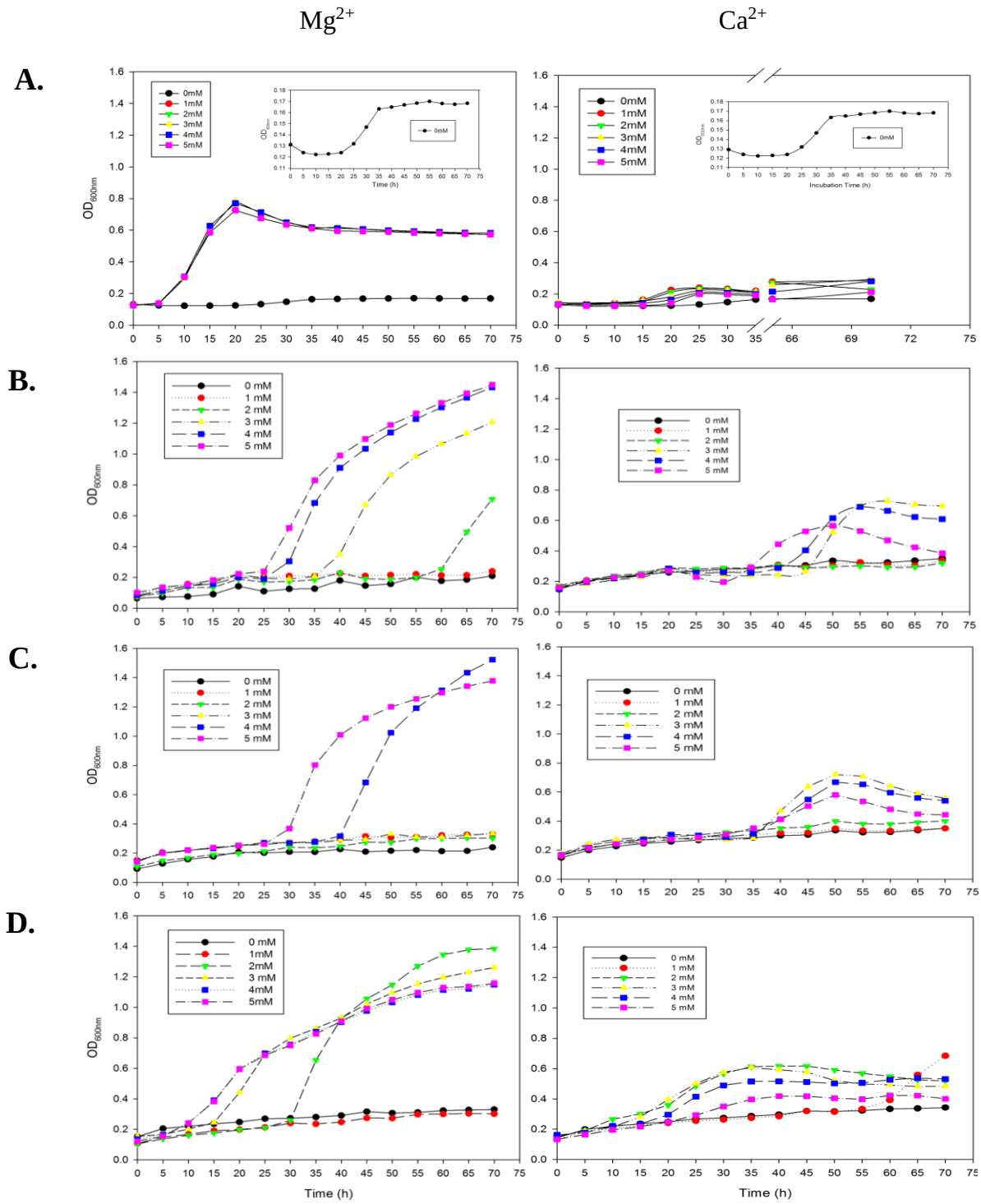


Figure 30: Effect of Mg^{2+} and Ca^{2+} when individually added into defined medium on the growth of PA01. The concentrations of Mg^{2+} and Ca^{2+} supplemented were from 1mM to 5mM. The bacterial cultures incubated at 30ºC for 72h without antibiotics (control) (A), with bactericidal concentrations of 2.5ug/ml of amikacin (B), 0.5ug/ml of tobramycin (C), and 2ug/ml of colistin (D). The values shown are the means of two replicates.

3.3.4.2.Influence of combinations of Ca^{2+} and Mg^{2+}on the response of *P.aeruginosa*

Our early study confirmed that the bacterial densities were dramatically inhibited by increasing concentrations of Ca^{2+} compared with Mg^{2+} (Fig.30 A). Those densities however; had significantly increased when Mg^{2+} was added into the defined synthetic medium with supplementation of Ca^{2+}. This result was observed in the medium lacking antibiotic (Fig.31). The impact of combining Mg^{2+} and Ca^{2+} on the behaviour of *P.aeruginosa* in the presence of antibiotics was also investigated using the SPECTRO $_{star}$ Nano system (Fig. 32). As a result of this experiment, the growth of *P.aeruginosa* exhibited an increase in the final cell densities when the Mg^{2+} added into defined synthetic medium treated with aminoglycoside agents and colistin in the presence of Ca^{2+}. The result indicates that the final optical densities had significantly increased to double the value when Mg^{2+} was added into the defined synthetic medium with supplementation of low concentration of Ca^{2+} (1mM) when the cells were treated with colistin. What does appear from this result is that increasing concentrations of Mg^{2+} resulted in higher final optical densities suggesting growth stimulation by this ion in the presence of 1mM of Ca^{2+}. The combination of 1mM of Ca^{2+} with low concentrations of Mg^{2+} (1mM) resulted in an increase in the cells density to the highest value (from 0.6 to1.09) when cells were treated with amikacin. Yet again the final densities of bacterial cells treated with tobramycin or amikacin showed that the addition of ≥ 2mM Mg^{2+} in the presence of low concentrations of Ca^{2+} (1-2mM) slightly increased in the optical density values compared with the control cultures (Fig.31 & 32). However, the combination of 5 mM of Ca^{2+} and ≥ 1mM of Mg^{2+} gradually exhibited a reduction of the final bacterial density from 0.8 to 0.6. What does appear from these results is that increasing concentrations of Ca^{2+} resulted in lower final optical densities suggesting growth inhibition by this ion in the presence of Mg^{2+}. This result was observed when the medium treated with 2.5µg/ml of amikacin or 2µg/ml of colistin.

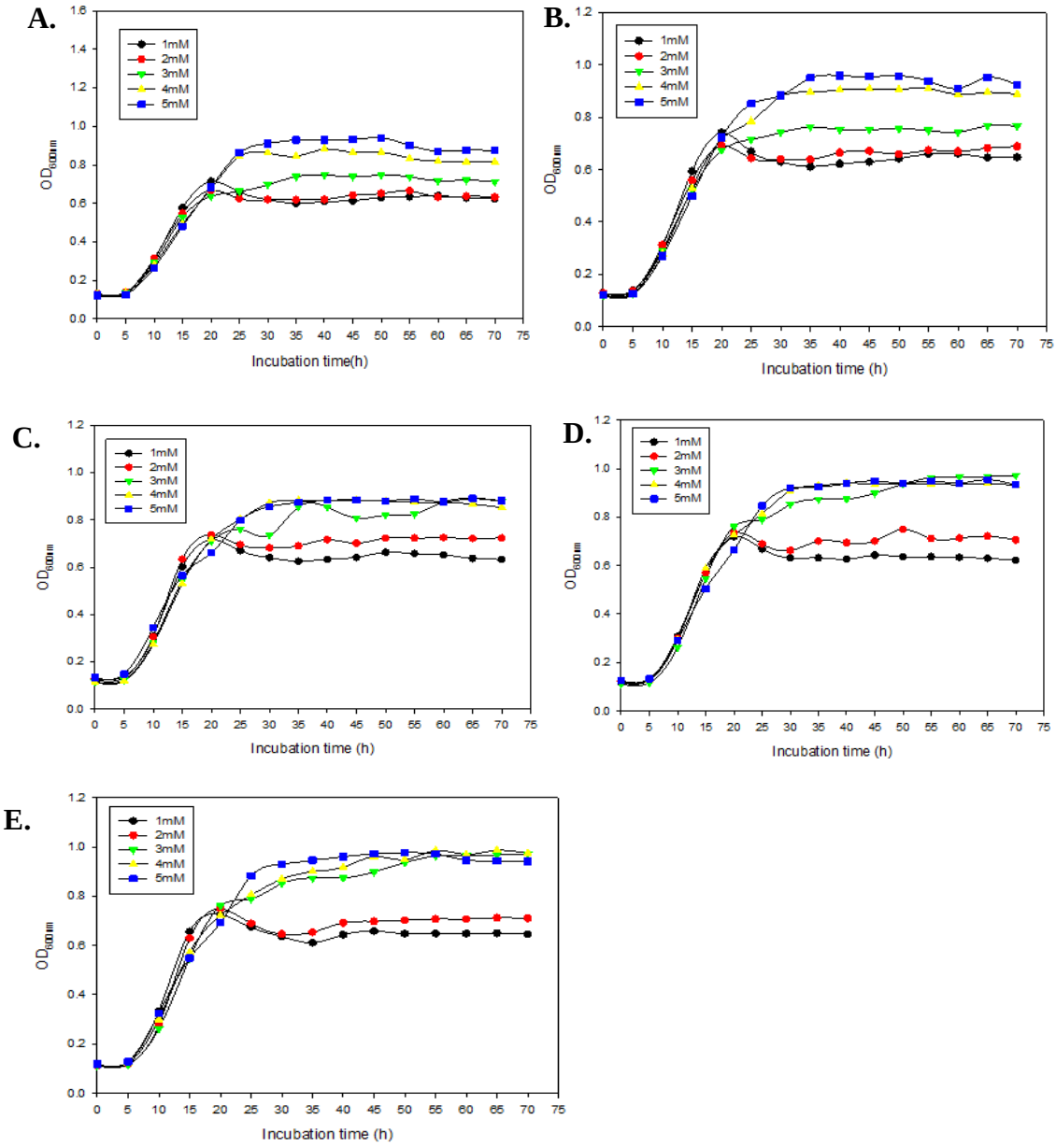


Figure 31: Control cultures of the effect of various concentration of Ca^{2+} combined with a constant concentration of Mg^{2+} A. 1mM, B. 2mM, C. 3mM, D. 4mM and E. 5mM on the growth of PA01 at 600nm. The bacterial cultures incubated at 30C° for 72h in the absence of antibiotics.The values shown are the means of two replicates.

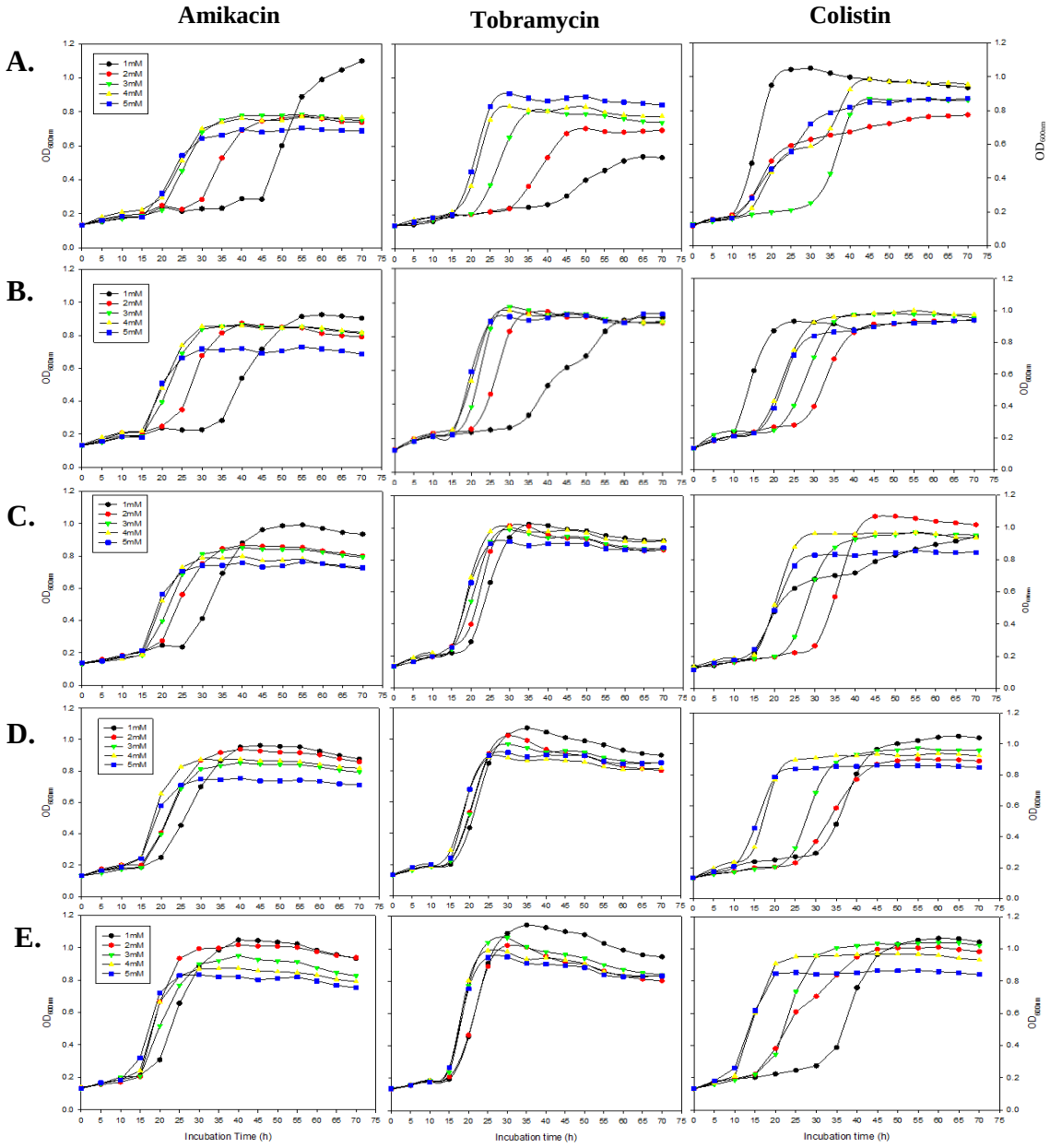


Figure 32: Effect of various concentration of Ca^{2+} combined with a constant concentration of Mg^{2+} A. 1mM, B. 2mM, C. 3mM, D. 4mM and E. 5mM on the growth of PA01 at 600nm. The bacterial cultures incubated at 30C° for 72h with 2.5 ug/ml of amikacin, 0.5 ug/ml of tobramycin, and 2 ug/ml of colistin. The values shown are the means of two replicates.

3.3.5. Effect of inoculum size on the susceptibility of antibiotics against *P.aeruginosa*

To assess the effect of three antibiotics used on the bacterium at different inoculum sizes, experiments were run using 96-well microtiter plate (OxoPlate) inoculated with high and low inoculum sizes. The effect of inoculum size on the activity of tobramycin is shown in Fig. 33. The results indicate that at the high inoculated size ($6x10^8$ cfu/ml); the ability of tobramycin to suppress the growth of *P. aeruginosa* was considerably reduced. The oxygen concentration decreased in the presence of tobramycin at 8µg/ml, due to the high metabolic activity of the high inoculum size of *P.aeruginosa.* The MIC and MBC value of the high inoculum ($6x10^8$ cfu/ml) was 4µg/ml and 8µg/ml respectively. *P.aeruginosa* displayed similar MIC and MBC value when initial inoculum size was diluted two-fold. Based on the MIC value, tobramycin was more active against *P.aeruginosa* when the culture was inoculated with $1.5x10^8$ cfm/ml, but the MBC value had no further effect at this inoculum size, when compared with the inocula of $6x10^8$ and $3x10^8$ cfu/ml. The activity of tobramycin to suppress the growth of *P.aeruginosa* gradually increased when the inoculum size was further diluted. This result was observed when inoculum size was decreased to$1.9x10^6$ cfu/ml and the bacterial growth was inhibited at a lower concentration of tobramycin (1µg/ml). However, tobramycin efficiently inhibited bacterial development at the low inoculum sizes of $2.3x10^5$ and $1.2x10^5$ cfu/ml. As a result of this the MIC value was reduced eightfold (0.25µg/ml) and MBC value also reduced 4-fold (2µg/ml).

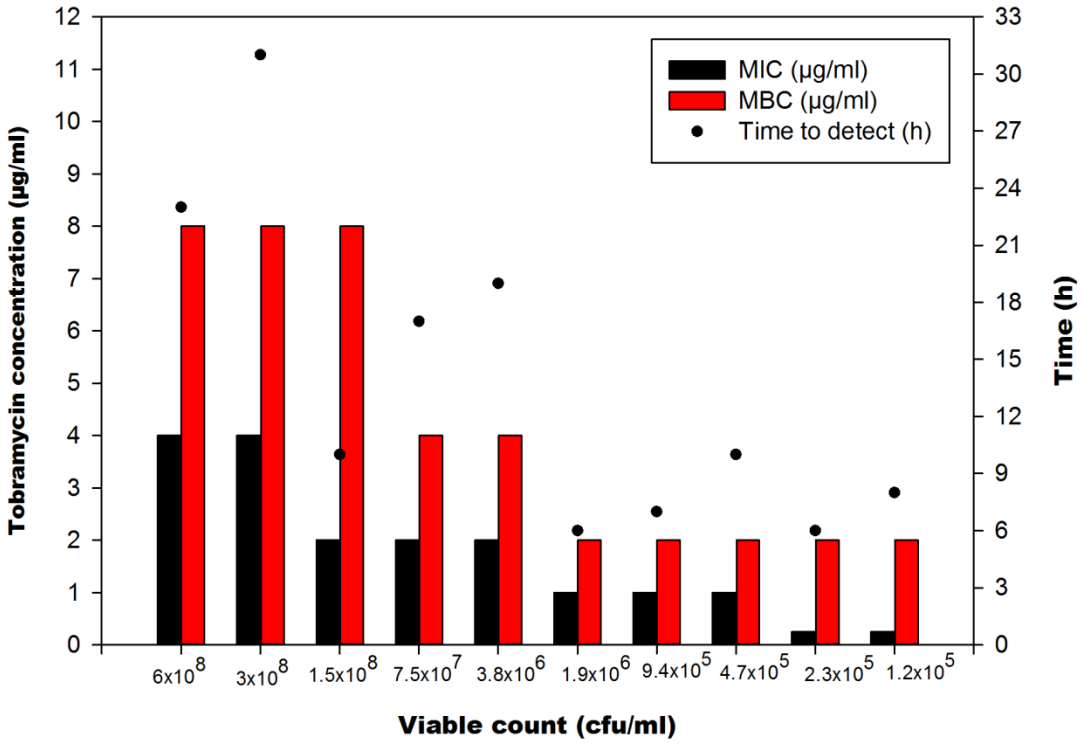


Figure 33: Inoculum effect on MIC and MBC of tobramycin. Planktonic cells of *P.aeruginosa* were treated with tobramycin at different concentrations from 16-0.25µg/ml and incubated at 30°C for 48 h using Oxo Plate reader. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml.

The effect of inoculum sizes on the antimicrobial activity of amikacin was also determined. The data given in the Figure 34 revealed that when the inoculum size increased from $1.5x10^8$ cfu/ml to $6x10^8$ cfu/ml, the activity of amikacin exhibited an increase in the MIC to 5µg/ml and the MBC to >10µg/ml. Based on the MIC results, the activity of amikacin was the same at the inoculum of $1.5x10^8$ cfu/ml and $7.5x10^7$ cfu/ml. As shown in Fig. 34, amikacin exhibited greater activity against *P.aeruginosa*, which inhibited the bacterial growth at 2.5µg/ml compared with initial inoculum size. Cultures treated with amikacin inoculated with ($3.8x10^6$ cfu/ml-$9.4x10^5$ cfu/ml), resulted in significantly decreased in the MIC and MBC to 0.6 and 2.5µg/ml respectively. This reduction was further observed in the low inoculum sizes ($4.7x10^5$, $2.3x10^5$ and $1.2x10^5$cfu/ml.), where the sensitivity of *P.aeruginosa* to amikacin was strongly increased (MIC 0.3µg/ml and MBC 1.25µg/ml) compared with high inoculum size.

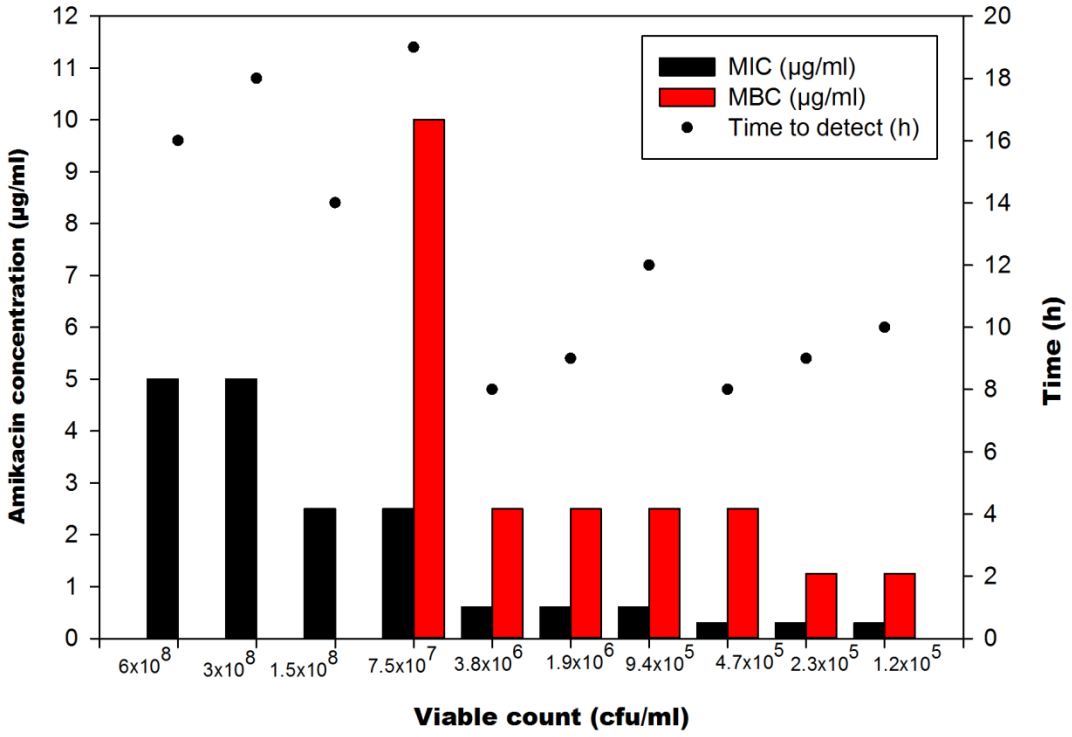


Figure 34: Inoculum effect on MIC and MBC of amikacin. Planktonic cells of *P.aeruginosa* were treated with amikacin at different concentrations from 10-0.31µg/ml and incubated at 30°C for 48 h using OxoPlate reader. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml .

In the high inoculum size ≥ $1.5x10^8$ cfu/ml, there was no marked difference in the MIC values (0.5µg/ml) and this was observed when cultures were treated with colistin. For those inocula, however the MBC values showed a gradual decrease as the inoculum size was lowered from 4µg/ml at $6x10^8$ cfu/ml to 1 µg/ml at $1.5x10^8$ cfu/ml (Fig. 35). Decreasing inoculum size ranging between ($7.5x10^7$ cfu/ml – $1.9x10^6$ cfu/ml) and ($9.4x10^5$ cfu/ml-$1.2x10^5$cfu/ml) resulted in identical MBCs, in spite of difference in MICs. At ≤ $2.3x10^5$ cfu/ml, the MIC and MBC of this experiment were 0.03 and 0.5µg/ml respectively. Based on the susceptibility test, the antibacterial activity of colistin was significantly higher (Fig.35) than aminoglycoside agents (Fig.33 and 34) against *P.aeruginosa*. As can be seen in the Fig. 33, 34 and 35, the novel assay based on the OxoPlate system indicates that suppression of growth following treatment with three antibiotics was found to be less marked with high inoculum of *P. aeruginosa.*

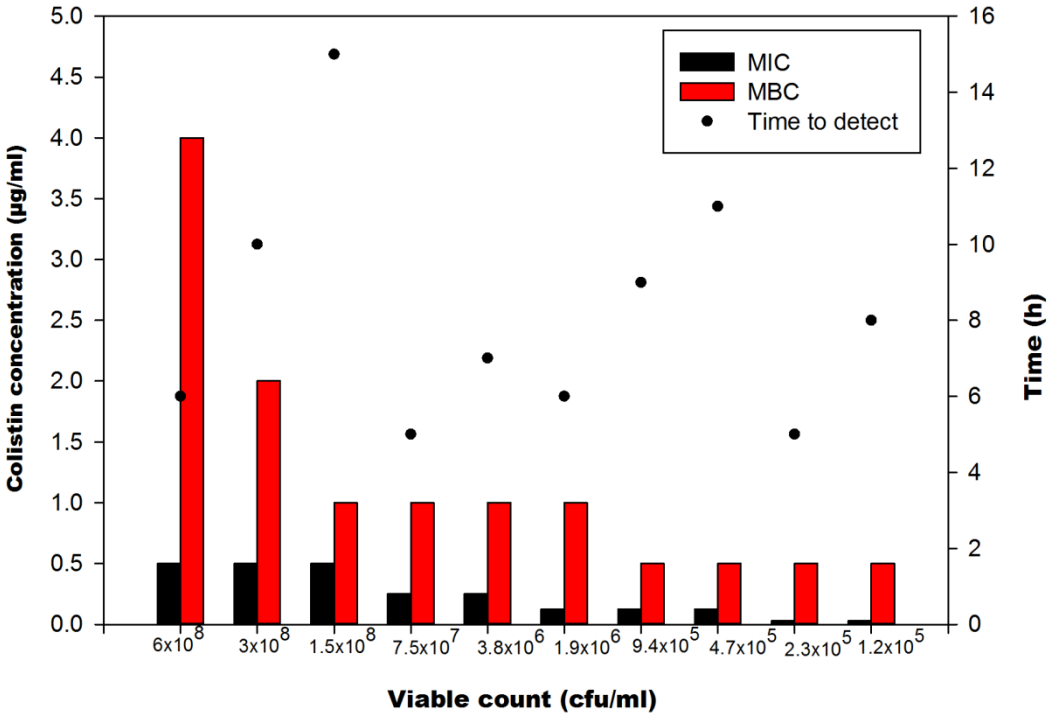


Figure 35: Inoculum effect on MIC and MBC of colistin. Planktonic cells of *P.aeruginosa* culture treated with colistin at different concentrations ranges from 16-0.031µg/ml incubated at 30°C for 48 h using OxoPlate reader. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml .

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3.3.6. Impact of inoculum size on the duration of lag phase of *P.aeruginosa*

To assess the effect of different inocula on the duration of lag phase, experiments were conducted using the SPECTRO $_{star}$ Nano system. Based on the OD obtained by this method, the duration of lag phase was influenced by the inocula. Figure 36 shows the effect of inoculum sizes on the duration of the lag phase. The results demonstrated that the duration of lag phase was gradually increased as the size of inoculum was decreased. Decreasing the inoculum size from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml resulted in an extended lag phase from 2 to 8h.

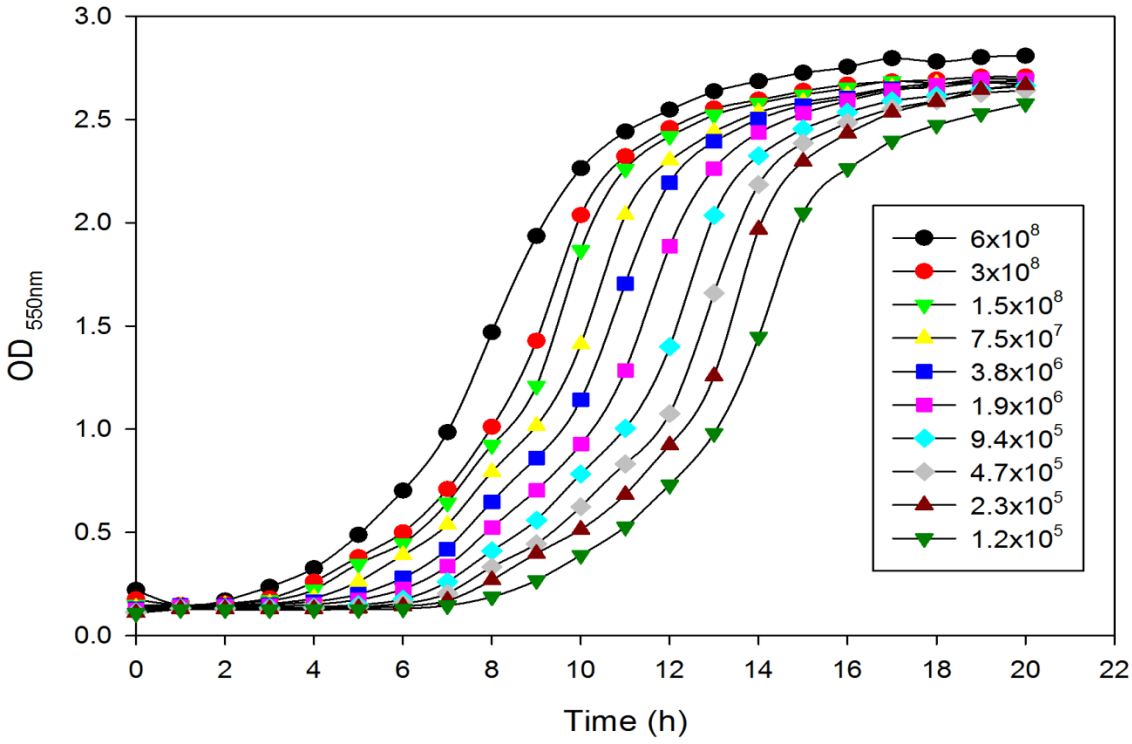


Figure 36: Effect of inoculum size on the duration of lag phase of *P.aeruginosa*. Planktonic cells of *P.aeruginosa* culture incubated at 30°C for 20 h using the SPECTRO $_{star}$ Nano. The test bacterium was used at various inoculum sizes from $6x10^8$ cfu/ml to $1.2x10^5$ cfu/ml.

3.4. The influence of prolonged exposure of sub-inhibitory concentrations of amikacin in glucose limited chemostat cultures of *P.aeruginosa*

3.4.1. Determination of nutrient limited growth

The limiting nutrient can be determined, once the final density of the bacterial cell is a linear function of the initial concentration of selected the nutrient. A chemostat in which glucose is the limiting nutrient is defined as 'glucose-limited' Glucose and sodium nitrate were used as a carbon and a nitrogen source respectively in this experiment. From statistically analysis (SPPS, v.21), there was a significant correlation between the concentration of glucose and the bacterial cells density over the range 5-20mM (P <0.01) (see appx. Table E.1). This correlation is considered to be a positive. Similarly, based on the R^2 value obtained, the values were linear. As shown in Fig. 37, the optical density of bacteria was dependent on the concentration of glucose at these ranges, which showed a straight proportionality. At a concentration of 25mM of glucose there was a slight increase in the optical density. However, increased concentration of glucose from 30-50mM resulted on the growth of bacterial cells was significantly inhibited over the time of experiment. The half-sturation constant (Ks) for glucose uptake was 0.5g/l (see appx. Fig.E.1)

In the nitrate limitation study, at a low concentration of nitrate, there was a significant relationship between optical density and concentration of nitrate (P <0.01). The concentration of nitrate ranged from 1-5mM showed a well-fitting regression line (R^2=0.96) (Appx. Table E.2). When the concentration of nitrate was increased above 5mM, the final bacterial cells density steadily decreased, demonstrating that bacterial growth was inhibited by such high concentrations (Fig. 38).

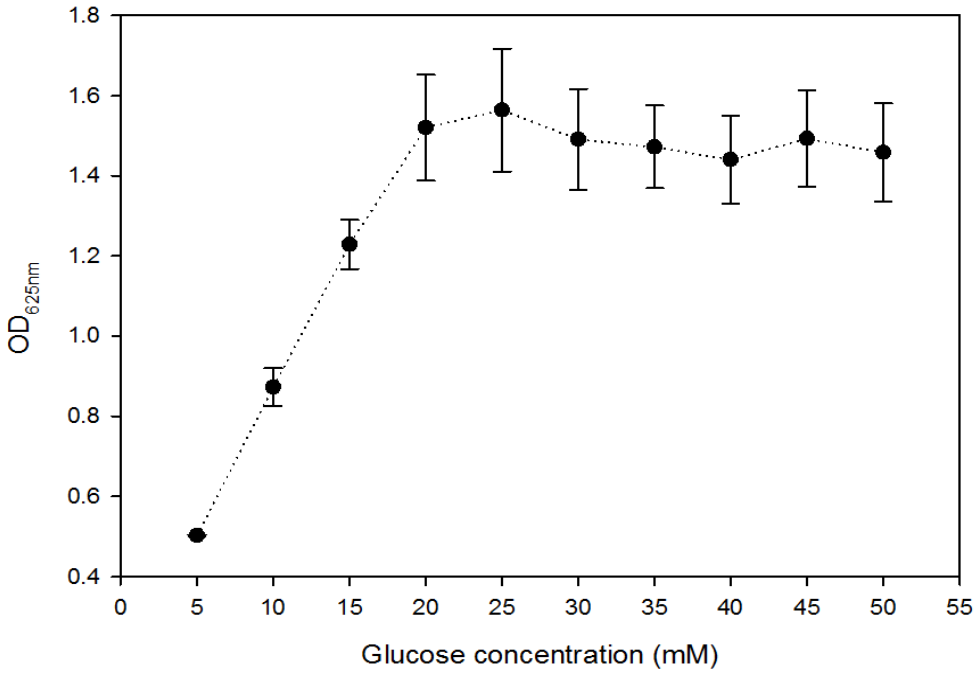


Figure 37: Determination of glucose-limited growth. Different concentrations of glucose were prepared in a series of cultures ranging from 5 to 50mM. The concentration of sodium nitrate was initially used in the medium at 10mM. The values shown are the means of two replicates and the vertical bars represent the standard errors of the values.

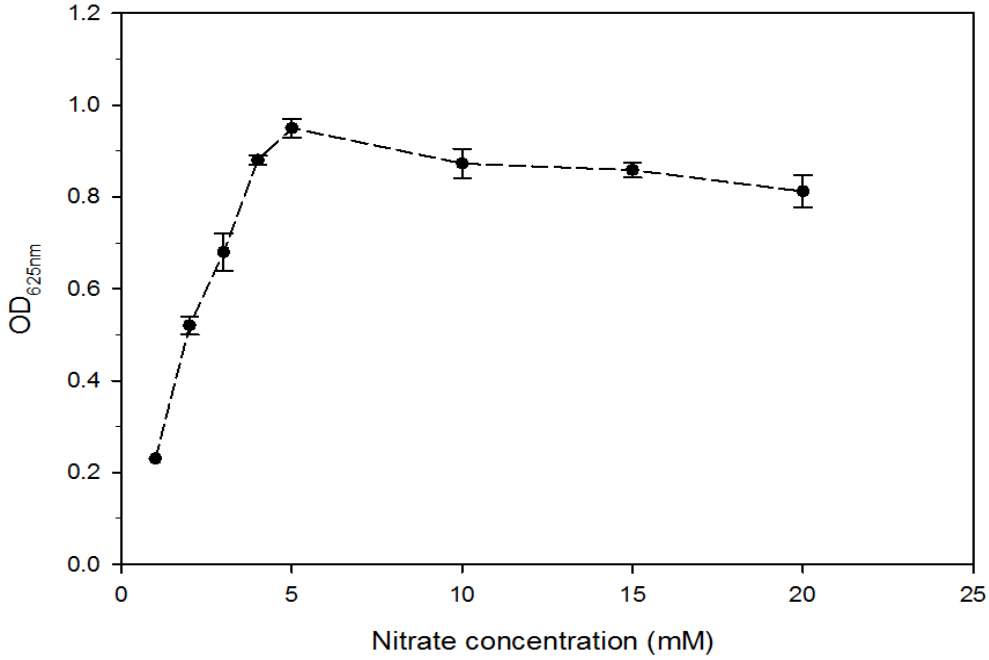


Figure 38: Determination of nitrate-limited growth. Different concentrations of sodium nitrate were prepared in a series of cultures ranging from 1 to 20 mM. The concentration of glucose was initially used in the medium at 10mM. The values shown are the means of two replicates and the vertical bars represent the standard errors of the values.

3.4.2. The growth behaviour of *P.aeruginosa* in the batch culture:

The *P.aeruginosa* culture was initially grown as a batch culture and the results of the fermentation profile of bacterial cell growth and oxygen consumption are illustrated in Fig. 39. During the batch culture, *P.aeruginosa* entered a logarithmic phase after 4h cultivation. During this phase, the concentration of biomass gradually increased along with a decrease in the dissolved oxygen concentration over the period (4-9h). Based on these results, oxygen was completely depleted at 9h when bacterial biomass reached 0.5 Buglab units. Interestingly the majority of the biomass accretion occurred over periods (9-15h) where the dissolved oxygen level was at 0%. During this period, a sharp slope was observed during a second biomass accretion phase. From cell mass data obtained, two growth rates were observed when bacteria were growing exponentially. For cells grown in the presence of oxygen, the growth rate was found to be $0.26h^{-1}$. However, the rate of bacterial growth of the second biomass phase of which was associated with oxygen limitation; was increased to $0.36h^{-1}$. This was followed by a stationary phase, where the concentration of dissolved oxygen gradually increased to 100% and bacterial mass remained fairly constant (15-19h).

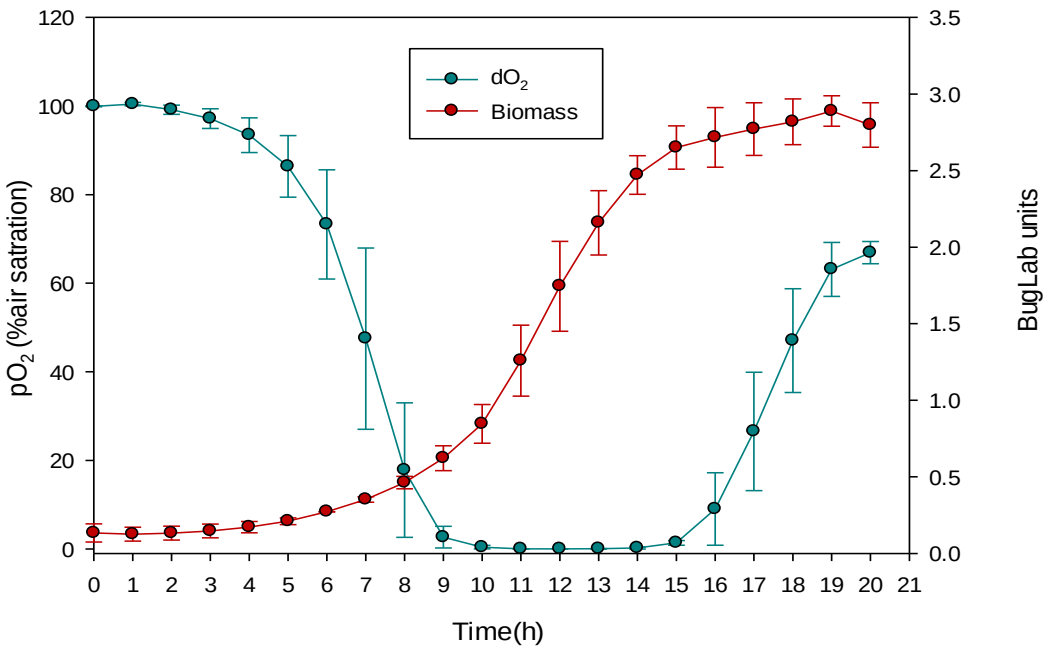


Figure 39: Batch culture of *P.aeruginosa*. During the process of growth, dissolved oxygen and biomass showed a significant change in its levels. The values shown are the means of two independent experiments and the vertical bars represent the standard errors of the values. pH of the fermentation was controlled throughout at pH 7.2.

3.4.3. A glucose-limited chemostat culture of *P.aeruginosa*

After the exponential growth phase was entered, the feed pump was activated to permit a constant flow of fresh medium into the vessel. For all subsequent experiments, the growth rate of the organism in the chemostat was regulated by the flow rate of the incoming medium. A steady state was deemed to be reached when the biomass levels stayed constant as evidenced from the Buglab output. Different dilution rates (*D*) were controlled (from the slowest to the fastest) as following $0.025h^{-1}$, 0.06 h^{-1}, 0.099 h^{-1} and 0.125 h^{-1} under a glucose limited chemostat culture (Fig.40 and 42). Glucose was measured off-line and found to be zero during the steady state (Appx. F. Table F.1). During initial feeding, the viability of bacterial cells grown in the absence of amikacin exhibited 100% of live cells (Fig.41A).

After 5 to 10 generations, cultures were exposed to amikacin by introducing the antibiotic into the feed reservoir at the concentrations indicated. The growth rate of *P.aeruginosa* was initially controlled using slow dilution rates D= $0.025h^{-1}$ and $0.06h^{-1}$. The bacterial cultures were maintained over 72 generations; introducing sub-MIC concentrations of antibiotic at day 7. The MIC of amikacin against *P.aeruginosa* was previously measured during batch fermentation using MHB (1.25 ug/ml) (Appx. Fig.E.3). The MIC of bacterial cells in chemostat medium before antibiotic introduced was 5ug/ml (Appx. Fig.F.1). In the culture grown at $0.025h^{-1}$, the level of biomass was slightly decreased for nearly10 h after initial exposed to amikacin (0.625µg/ml) as evidenced from the BugLab output (Fig.40-arrow A). It was immediately linked to a decrease in the bacterial cells number from 4.9 to 4.5 $\log_{10}$ cfu/ml. After 24h treatment, the bacterial mass stopped decreasing and remained constant at this level as evidenced from the BugLab monitor. Over the first 4 days of treatment, the cell population started to became less susceptibility to amikacin as evidenced from OxoPlate data output, which showed an increase in the MBC value. After 4 days exposure, the MIC value was increased 4-fold (Appx.F.2). After this point, the MIC values remained constant.

When the dilution rate increased to $0.06h^{-1}$, the culture appeared to be more sensitive to antibiotic action compared with culture exposed to the same concentration (0.625 µg/ml) at low dilution rate ($0.025h^{-1}$) (Fig.40 arrow B). This was evident from the BugLab data and viability of cells, which showed a decrease in the biomass level

(Fig.40 arrow B) and the cells viability (Fig. 41C) following a recovery (816h) (Fig. 41D). At this concentration of antibiotic, it took 4 days for the culture to adapt to the antibiotic. However, during the length of treatment, both cultures grown at $0.025h^{-1}$ and $0.06h^{-1}$ exhibited slower and smaller increases in the MIC value (as measured using the OxoPlate method). For both cultures exposure to the antibiotic resulted in an increase in the MIC 4-fold. The final MIC value after prolonged exposure to sub-inhibitory concentrations of amikacin was 5µg/ml (Appx. F.4). For both cultures exposure, adaptive resistance to amikacin conferred low-level resistance to other aminoglycosides such as tobramycin and antibiotics with different modes of action such as colistin (Appx.F.3 &5).

An analysis of the stability of the adapted cultures to amikacin were carried out following passaging of the cultures every 24h through drug-free medium (Nutrient agar) and testing the MIC using OxoPlate technique. The MIC of bacterial cells grown at $0.025h^{-1}$ was stable for the first nine subcultures. However, over the next one subculture, the MIC was decreased from 5 to 1.25µg/ml. The MIC value of *P.aeruginosa* grown at $0.025h^{-1}$ under limiting nutrient did return to its original sensitivity level after ten subcultures. In the case of the MIC of the bacterial population grown in the chemostat at $0.06h^{-1}$ it took 8 subcultures before the organism returned to its previous MIC for the antibiotic.

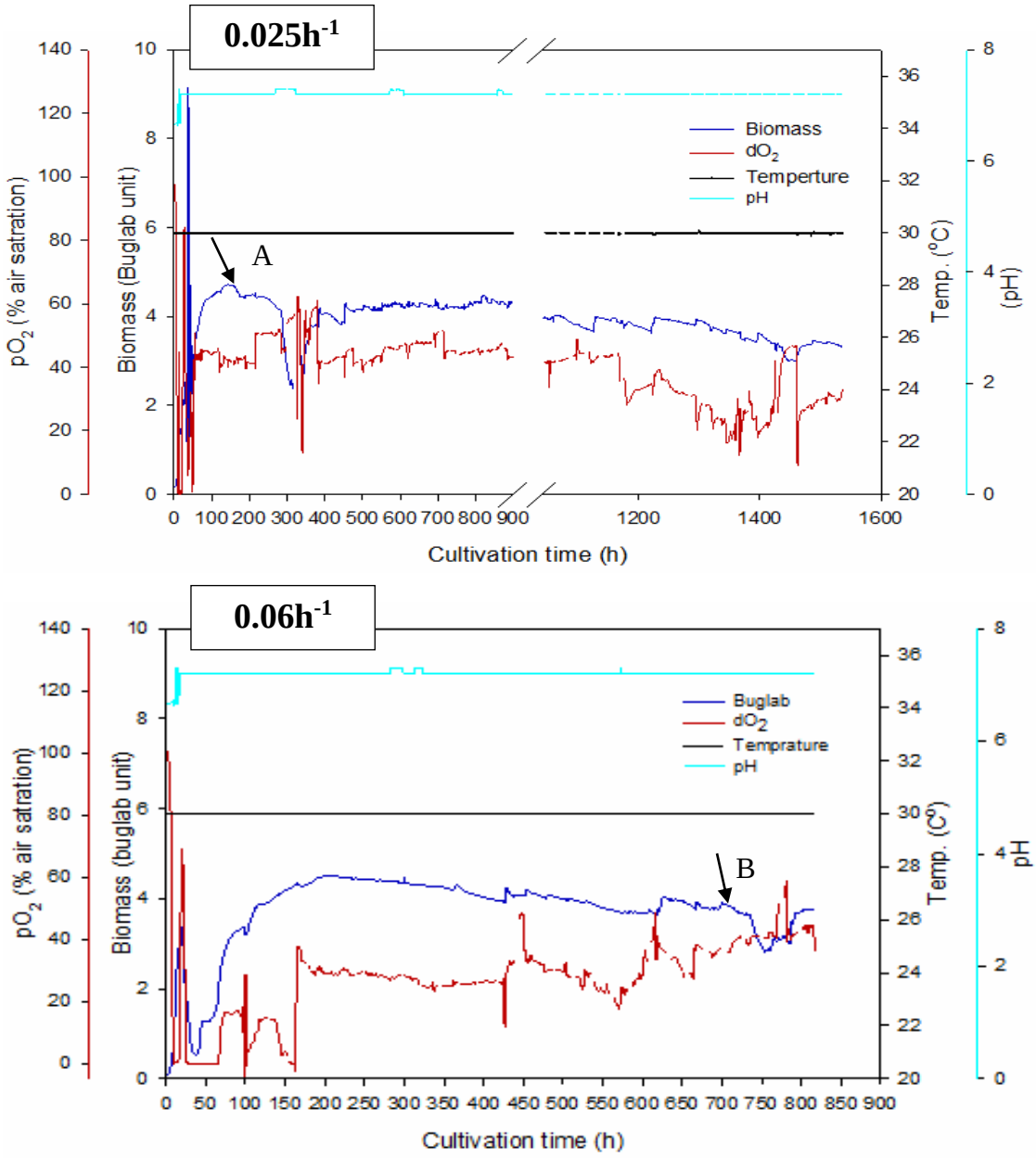


Figure 40: A glucose-limited chemostat culture of *P.aeruginosa* under stress of amikacin. The conditions of the steady-state experiments were as following: T (30◦C), pH (7.2), agitation rate (150 rpm), glucose (1.8 g/l) and air flow rate (1.75 l/h). Dilution rates have been indicated in the above diagrams. The arrow represents the time of the feed change to antibiotic containing medium at 0.625μg/ml.

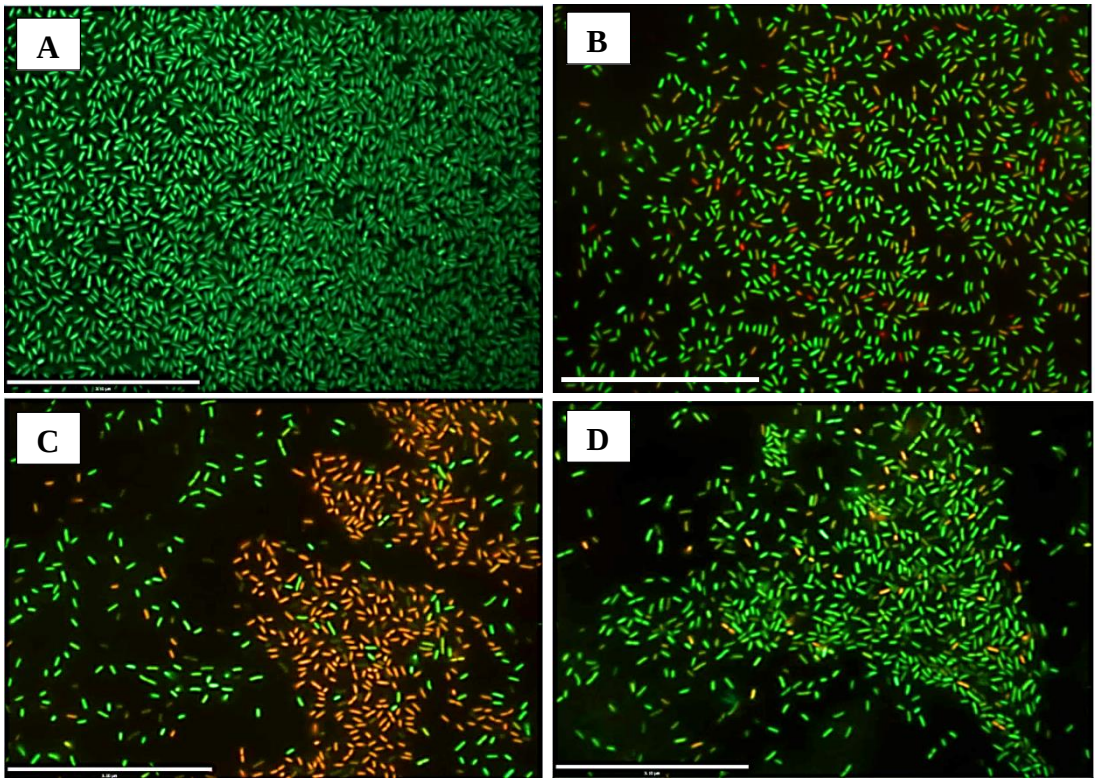


Figure 41: Fluorescent microscopy images at 100X showed the viability of *P.aeruginosa* culture grown in chemostat at 0.06h^{-1} stained with *Bac*Light dye. Viability of bacterial cells that grown during initial feeding in the absence of antibiotic stress (control) (A).Viability of bacterial cells after three-day exposure to amikacin at 0.3µg/ml (B). Viability of bacterial cells after increased the concentration of amikacin to 0.625 µg/m, the cells examined after two-day treatment (C) and recovered culture (4days after exposure) at 816h cultivation time (D). The green fluorescent nucleic acid dye stains live cells and the red fluorescent nucleic acid dye stains dead cells. Scale bar =0.5-2µm.

Cells were grown at "rapid" dilution rates (D=0.099 and 0.125h^{-1}) were maintained over 50 generations (Fig.42). Cell death was faster as evidenced by cell counts and the BugLab data in these fermentations when the culture was exposed to 0.625µg/ml amikacin compared with cells grown at the slower growth rates. Following an initial 4-h amikacin exposure at a concentration of 0.625µg/ml to a culture grown at 0.099h^{-1}, a decrease in the viability cells from 4.45 to 3.73 $\log_{10}$ cfu/ml was observed. After 24-h exposure to the antibiotic, bacterial cells showed a further decrease to 3.25 $\log_{10}$ cfu/ml. Based on the viability test, the number of viable cells increasing to 3.74 $\log_{10}$ cfu/ml. The cell viability then remained constant.

At D=0.125 h^{-1} when the culture was exposed to 0.625 µg/ml amikacin, there was a decrease in biomass level from 3.5 to 1.8 biomass units over the initial 4- days followed by a recovery in biomass levels after 300h cultivation time (Fig.42). Using

the *Bac*Light stain on the cultures it was evident that the number of viable cells decreased immediately after exposure to the antibiotic (Fig.43) and that the culture then regained viability with prolonged exposure to the antibiotic after 300h.

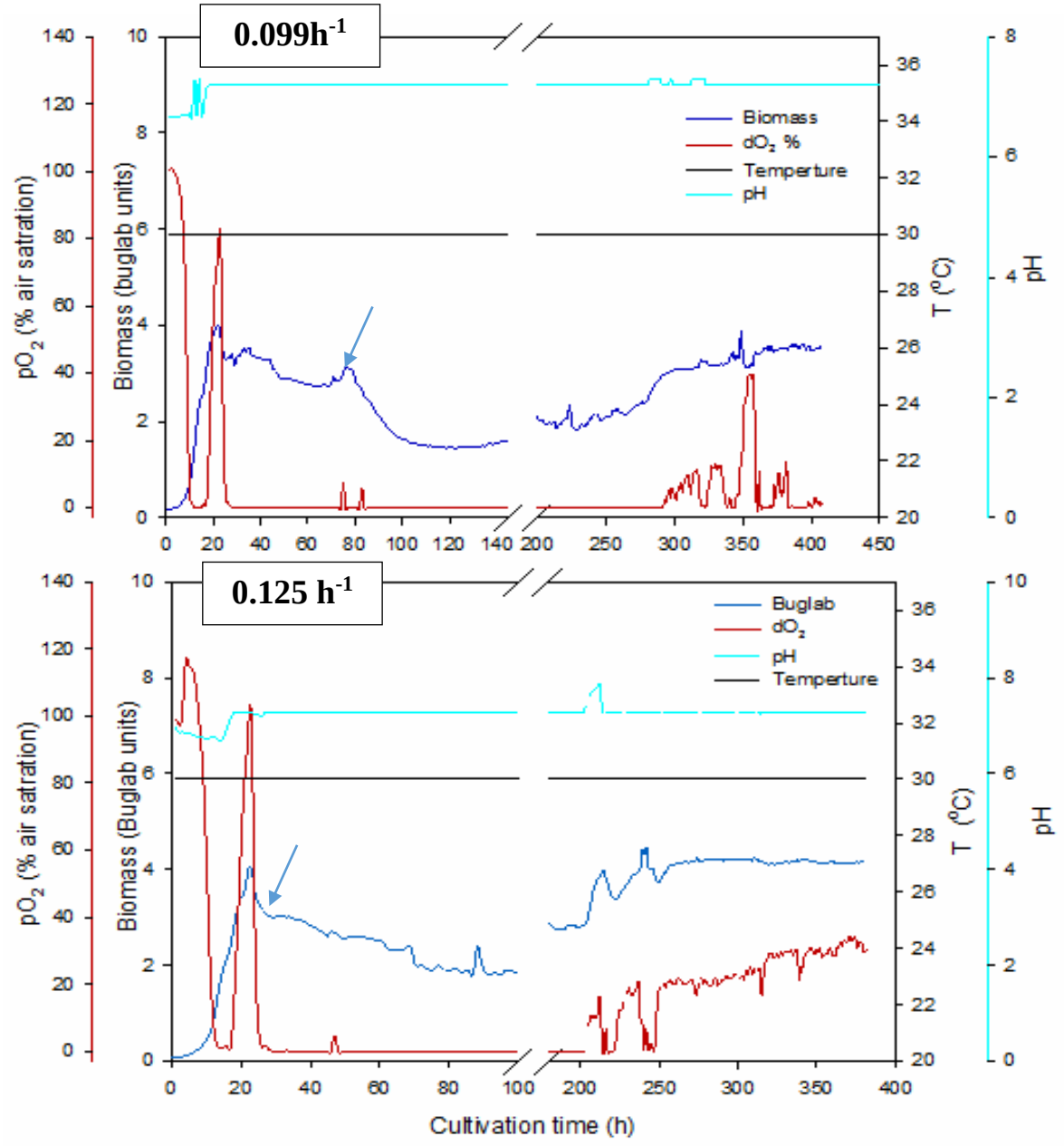


Figure 42: A glucose-limited chemostat culture of *P.aeruginosa* exposed to amikacin. The conditions of the steady-state experiments were as following: T (30◦C), pH (7.2), agitation rate (150 rpm), glucose (1.8 g/l) and air flow rate (1.75 l/h). Dilution rates have been indicated in the above diagrams. The arrow represents the time of the feed change to antibiotic containing medium.

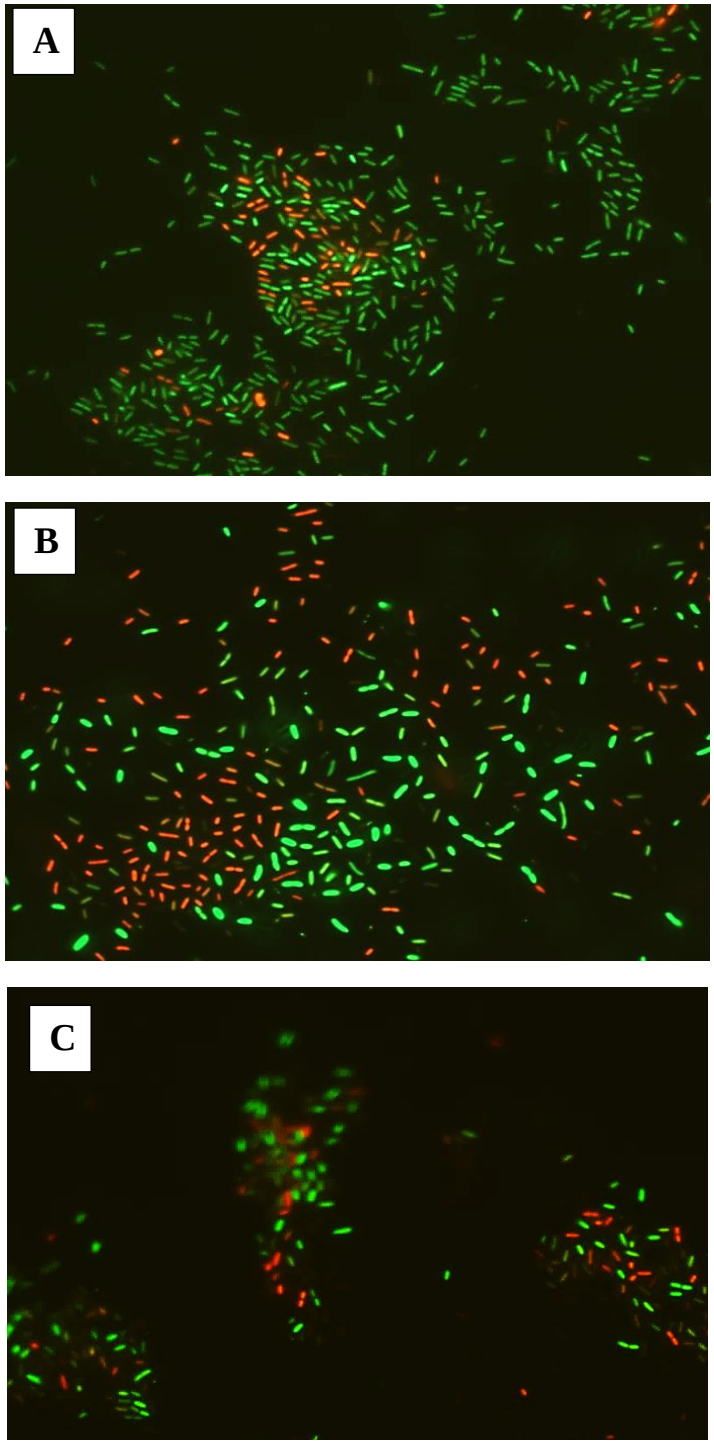


Figure 43: Fluorescent microscopy images at 100x showed the viability of *P.aeruginosa* culture grown in chemostat at 0.125h^{-1} stained with *Bac*Light dye. Viability of bacterial cells after 2-h exposure to amikacin at 0.625 µg/ml (A). The cells examined after 24-h treatment (B). A decrease in cells number within 4- days treatment (C). The green fluorescent nucleic acid dye stains live cells and the red fluorescent nucleic acid dye stains dead cells. Scale bar =0.5-2µm.

The OxoPlate was used to test the susceptibility of faster-growing cells during the treatment period. Based on the susceptibility test, the continued presence of amikacin at a sub-inhibitory concentration in cultures grown at high dilution rates showed no change in MIC value in the both cultures during the period of treatment. However, an increase was observed in the MBC value from 10 to 40µg/ml in both cultures compared with the untreated control culture (Fig.44& appx.E.3). The increase in the MBC was transient and the MBC returned to control levels after three subcultures in amikacin-free nutrient agar. It has been suggested that the surviving cells up to a concentration of 20µg/ml may represent persister bacterial cells that respond heterogeneously to the action of the antimicrobial agent.

This observation was confirmed by E-test. As illustrated in Fig.45, survival colonies were clearly observed after 72h incubation time in treated culture (Fig.45-A). Survival colonies were tested for its susceptibility to amikacin using the E-test. The MIC value of surviving colonies obtained from the E-test was 64µg for the treated culture (Fig.45-B). This result was further investigated by analysing the bacterial cell size distribution during the incubation time of 24 and 72h using flow-cytometry after staining with the *Bac*light dye. After bacterial cells were sorted as live and dead, the flow-cytometry analysis demonstrated that a small subpopulation showed a reduction in bacterial cell size see (Fig.45-D). This distinct in the size could potentially account for why cells persistence the action of amikacin.

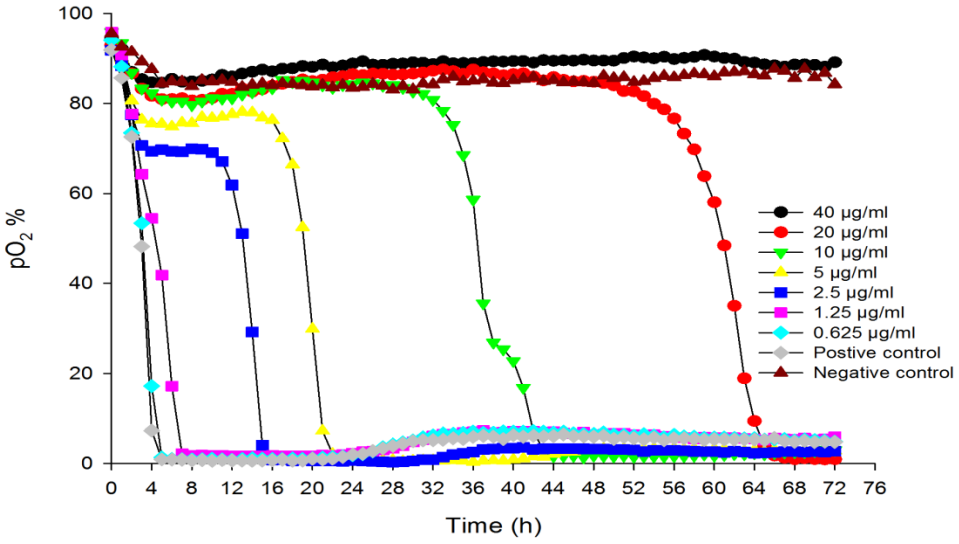


Figure 44: MIC and MBC of of *P.aeruginosa* after prolonged exposure to amikacin at 0.099 and 0.125h^{-1}. It was assessed using a fluorescent plate reader, the lines represent the means of three replicates. Antibiotic concentrations have been indicated. The positive control presents an overnight culture of *P.aeruginosa* (untreated). The negative control represents an overnight culture injected with solution of sodium hypochlorite (10% v/v).

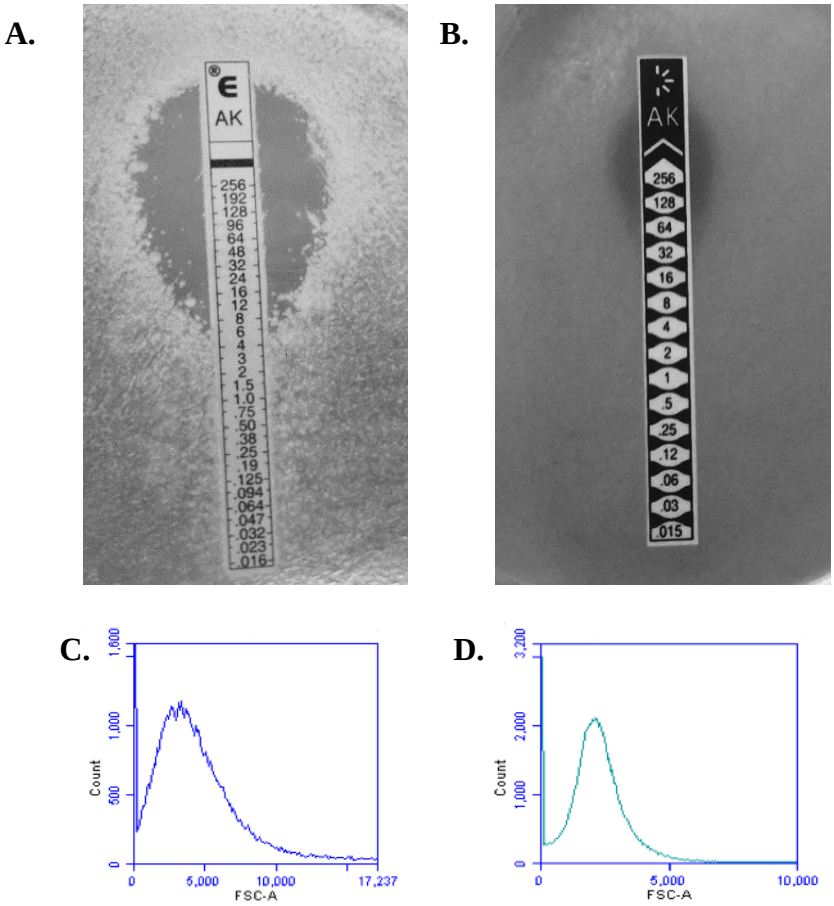


Figure 45: E-test represents survival colonies after 3days incubation (A) and the MIC of survival colonies (B). Flowcytometry analysis of treated culture grown at the highest dilution rate. Histograms show FSC-A, which represent the size distribution of live cells. Analysis of treated culture after 24h (C) and 72h (D) incubation at 30ºC.

3.4.4. Effects of dilution rate on the colony morphology of the population

During the first few days of the chemostat culture samples were taken and plated out onto nutrient agar without antibiotic. After 24 h the resultant colonies were homogeneous and identical to the wild type. With further cultivation in the fermenter in the presence of antibiotic in the feed the colonies became distinctly different taking on shapes that had irregular margins. To establish that these colonies were not contaminants random colonies were sub-cultured into nutrient broth with no antibiotic and the DNA was extracted, and the 16S rRNA was sequenced, the result indicated that the culture remained pure and indeed was still *P. aeruginosa*.

The results indicated that the colony morphology of *P. aeruginosa* was clearly affected by controlling the dilution rate under antibiotic stress (Fig.46). Each morphology described in this study was apparent in a minimum of three of the four replicates. It was observed that colonies of planktonic *P. aeruginosa* that were not subjected to antibiotics exhibited a circular form, smooth texture, flat elevation, entire margin, and large size (Fig. 46-A). However, distant colonies of treated cells at growth rate of 0.125 and 0.099 h^{-1} exhibited irregular form, lobate margin (Fig. 46-B1- 2), wrinkled texture and convex elevation (Fig.46-B3). When grown at a lower growth rate of 0.025 and 0.06h^{-1}, colonies displayed an irregular form and rough/ dry surface appearance (Fig.46-C).

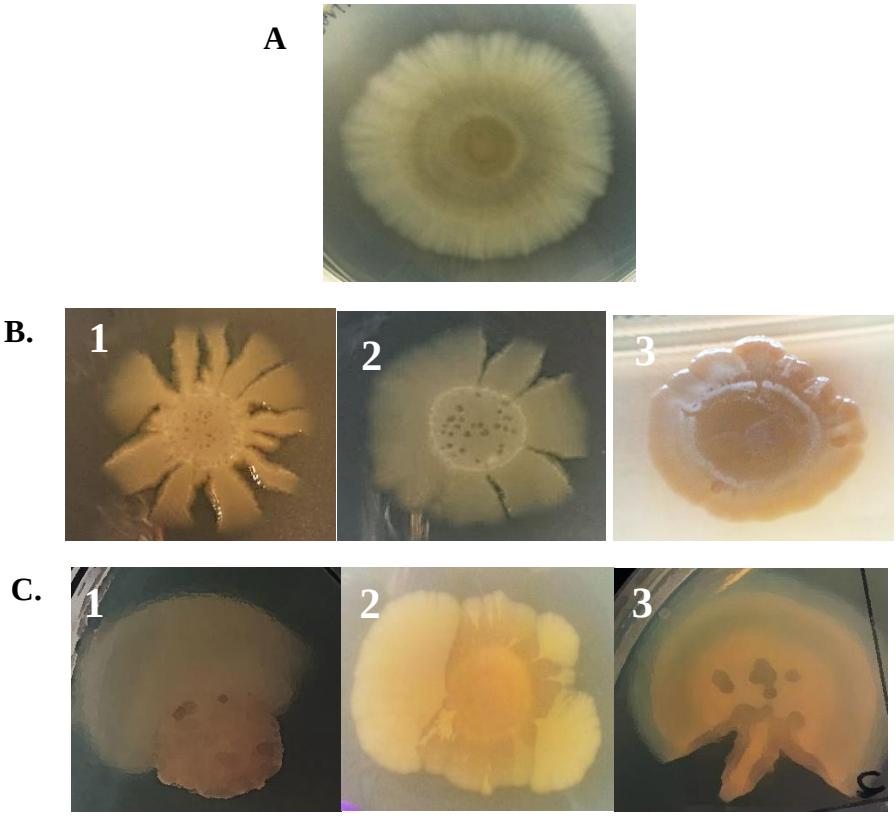


Figure 46: The diverse of colony morphology of *P.aeruginosa* exposed to amikacin in chemostat at different dilution rates. The morphological change was observed on NA media after 6days incubation at 30°C. (A). Control colony morphology of *P.aerugionsa* was not treated. Row B represents the colony morphology changed of culture at dilution rate 0.125and 0.099h^{-1}. Row C represents the colony morphology changed of culture at dilution rate 0.025 and 0.06h^{-1}.

3.4.5. The effects of colony age on colony morphological characteristics and on the susceptibility to antibiotic

In order to detect any colony alterations, samples were taken from the chemostat at high dilution rates during the treatment period and plated out onto antibiotic-free medium (Nutrient agar) and incubated over the period of 6 days. During this process, colonies were analysed at different stages of colony growth (one, three and six days). As illustrated in Fig. 47, a morphological change was noted, such as the size and texture of colonies after the first day of growth on the agar plate. As shown in Fig.48, morphological differences were observed in the colonies incubated for 3 and 6 days growth on the ager plates compared with the control colony (Fig.48-A).

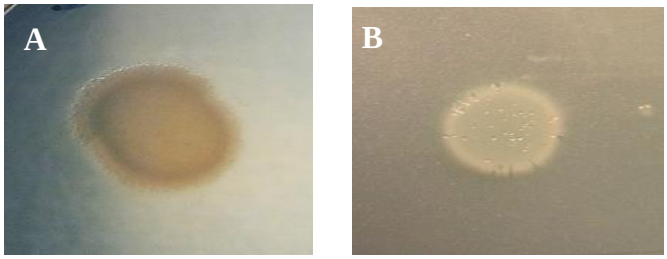


Figure 47: Colony morphology of planktonic cells of *P.aeruginosa* observed in amikacin-free nutrient agar after 24h incubation at 30°C. The colony morphology development of the control culture (A) and treated bacterial cells growing in continues culture (B).

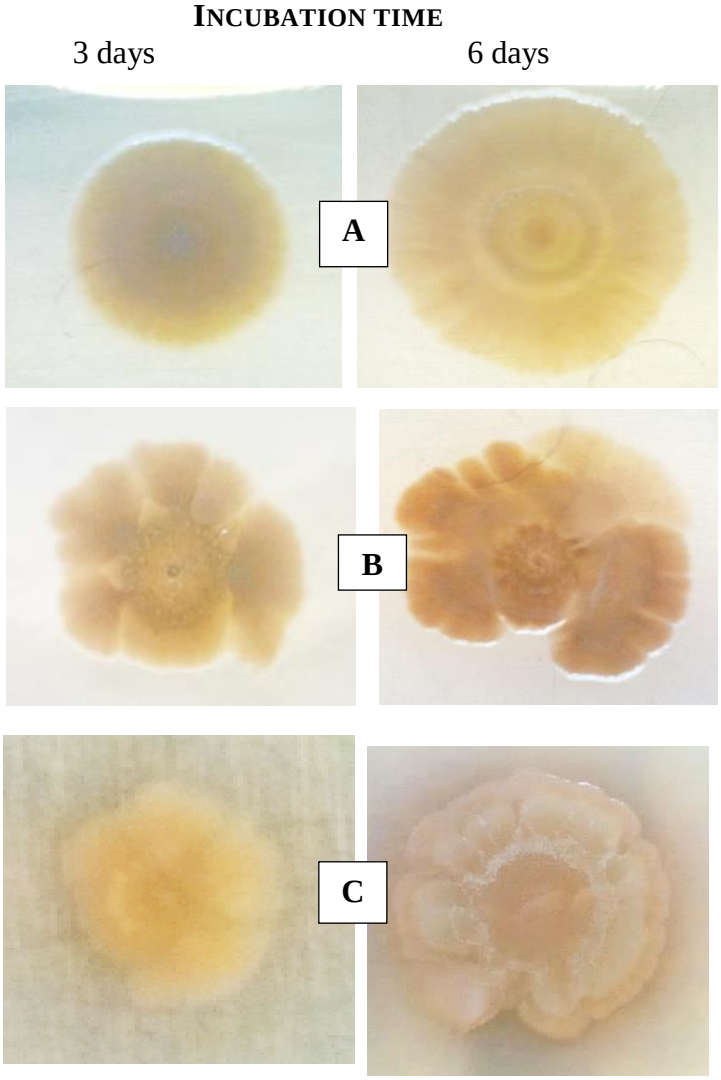
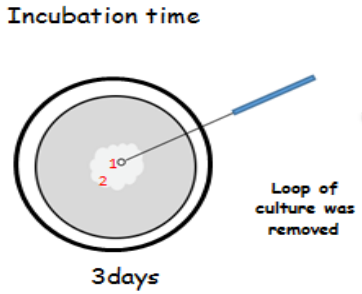


Figure 48: Evolution of morphology change of *P.aeruginosa* colonies observed in NA media after 3 and 6 days incubation at 30°C. A) Represents the development of colony morphology of untreated cells (control). B and C represent the colony morphology variation during incubation time detected on bacterial cells subjected to amikacin in continues culture at dilution rate 0.099 and 0.125h^{-1}.

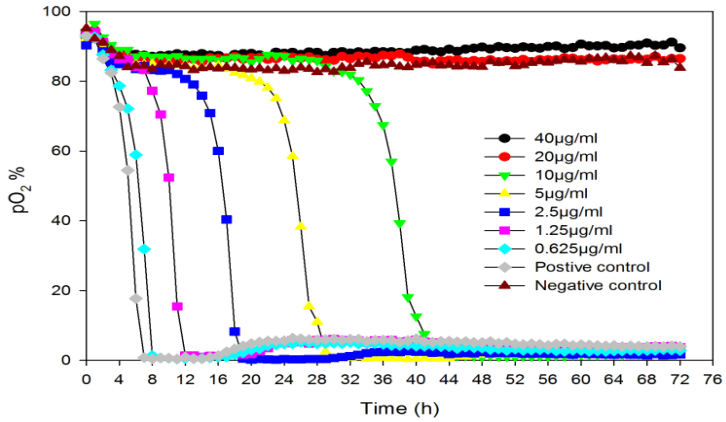
To assess the susceptibility of these colonies at different incubation times, a loop of culture was removed from different positions in the colony (centre and edge) see Figure 49. Based on the susceptibility testing, no difference was observed in the MIC values (1.25 µg/ml) of cells located at the centre and the edge of colony after 3 days growth, although the MBC value of cells at the centre of colony was increased from 10 to 20 µg/ml (Fig.49). The increase in the MBC value was due to development of a small subpopulation as confirmed by Flowcytometry analysis (Fig. 49 (FL1-A)). The flow-cytometry analysis also showed that cells size was distinctly different in the two colonial positions (Fig.49 (FSC-A)). The size of bacterial cells located at the edge of colony was greater than the cells size located at the center of the colony. Scanning electronic microscopy confirmed the ability of the *P.aeruginosa* cells that were located in the centre of colony to form fibers (Fig.50) and cells become encapsulated with membranous extracellular matrix components after 3-days incubation (Fig.51).

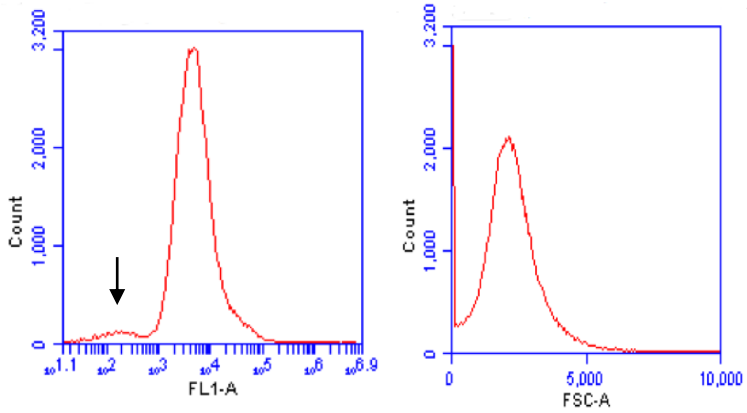
With colonies that had been cultured for 6 days, visible differences were observed in *P. aeruginosa* colonies compared with earlier time points (3days) (Fig.48), and greater resistance to amikacin was also noted. The data presented in Fig. 49 indicates that, when colony incubation time was increased to 6 days, the susceptibility of bacterial cells located at centre of colony showed a mark increased in MIC value to 40 µg/ml. At this point, the level of the changes in the susceptibility test was dependent on the results obtained from FL1-A, which showed an increase in the formation of the subpopulation and the cell size remained constant at this stage compared with younger colonies. No change in the MIC value was observed for the cells located at the edge of colony but the MBC was increased to >40 µg/ml. This observation was further examined by sorting the bacterial cells population using flow-cytometry. The results showed that increasing the incubation time to 6 days led to decrease in the cell size located at the edge of colony and the formation of two populations was also noted compared with early period (3days).



OxoPlate **Flowcytometery**

1.





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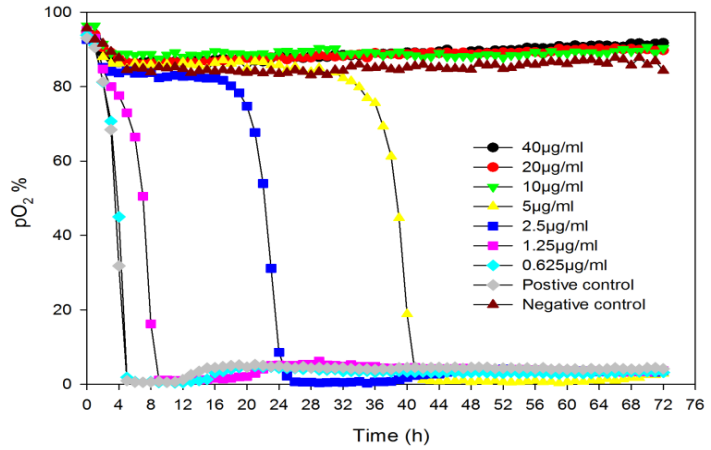

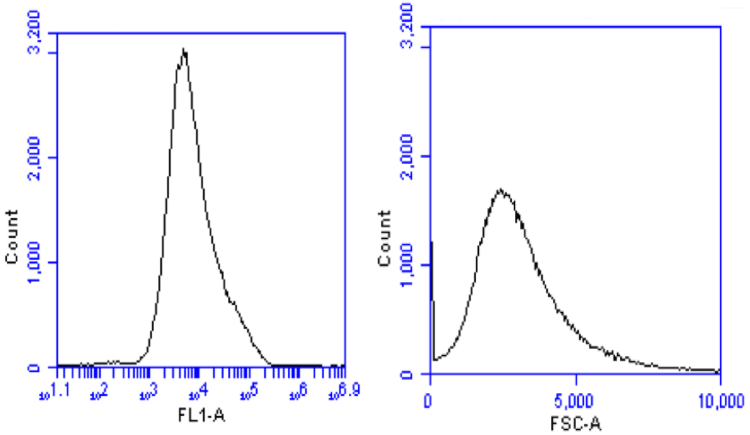

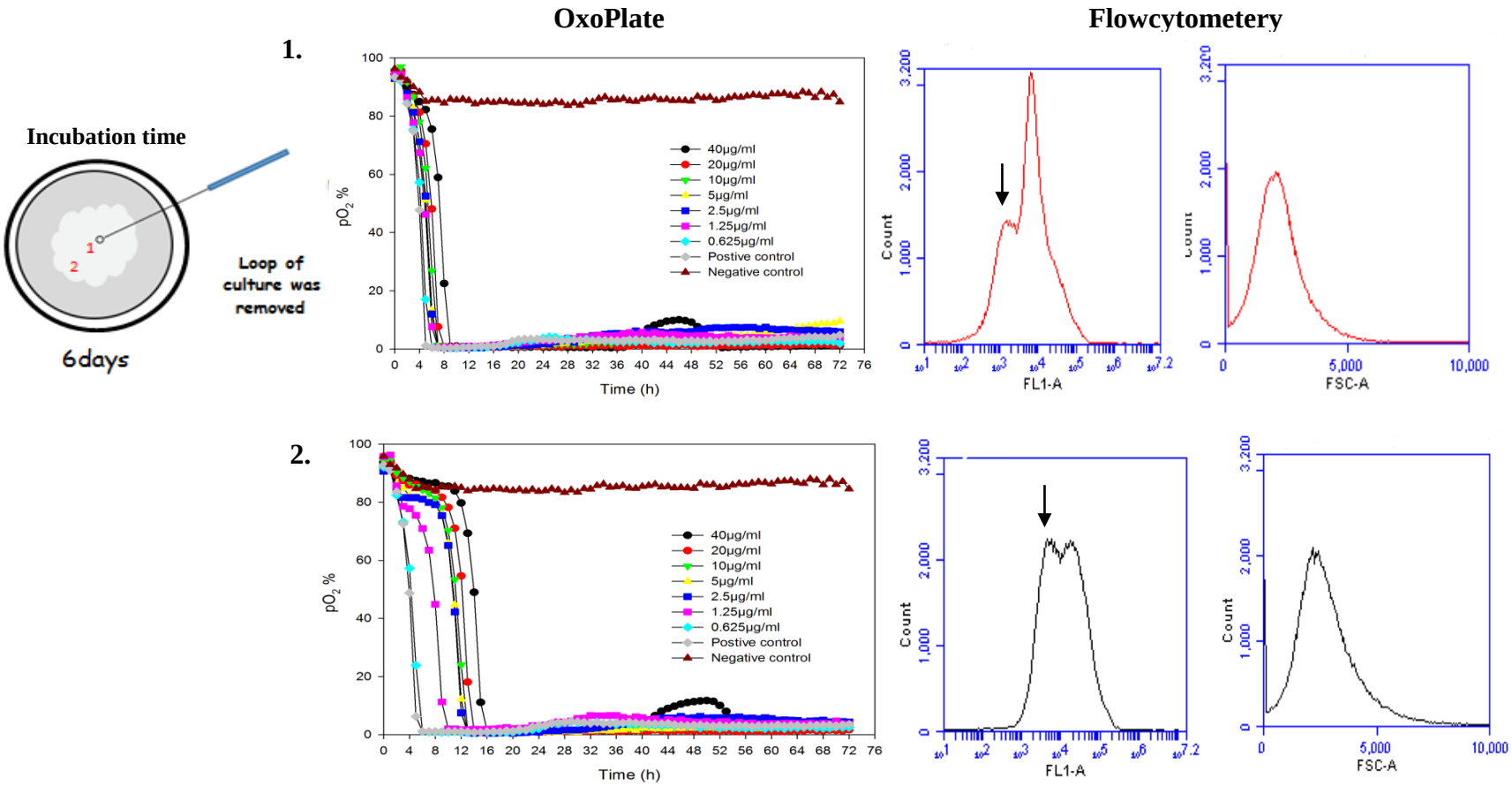

Figure 49: Effects of growth time of adapted colonies of ***P.aeruginosa*** **that grown at high dilution rate in chemostat on the susceptibility to amikacin.** It had been isolated from chemostat in the presence of amikacin at 0.125$h^{-1}$and incubated at 30ºC in amikacin-free nutrient agar. Oxoplate output represents the MIC and MBC of colonies incubated for 3 and 6 days. Flowcytometry analysis of different stages of colony growth at centre (1) represents a red curve and at edge (2) represents a black curve. Histograms show FL1-A and FSC-A which represent the fluorescent parameter of live cells and cells size respectively. The analysis of the bacterial cell size distribution represented by FSC-A. The arrow represents the development of subpopulations.

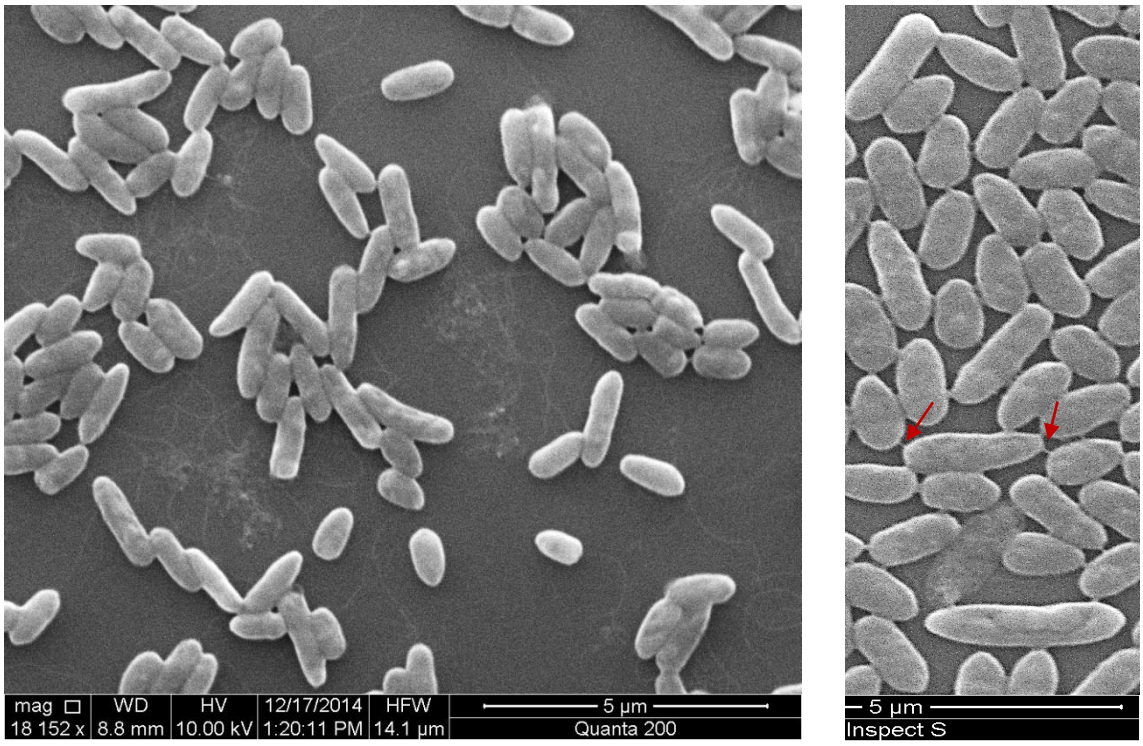

Figure 50: SEM image showing a great number of extracellular polymeric fibrils in the cells derived from the centre of colony of *P.aeruginosa*. It had been isolated from chemostat in the presence of amikacin at 0.125$h^{-1}$and incubated at 30ºC for 3 days in amikacin-free nutrient agar. The arrow represents the cells linking with fibrils.

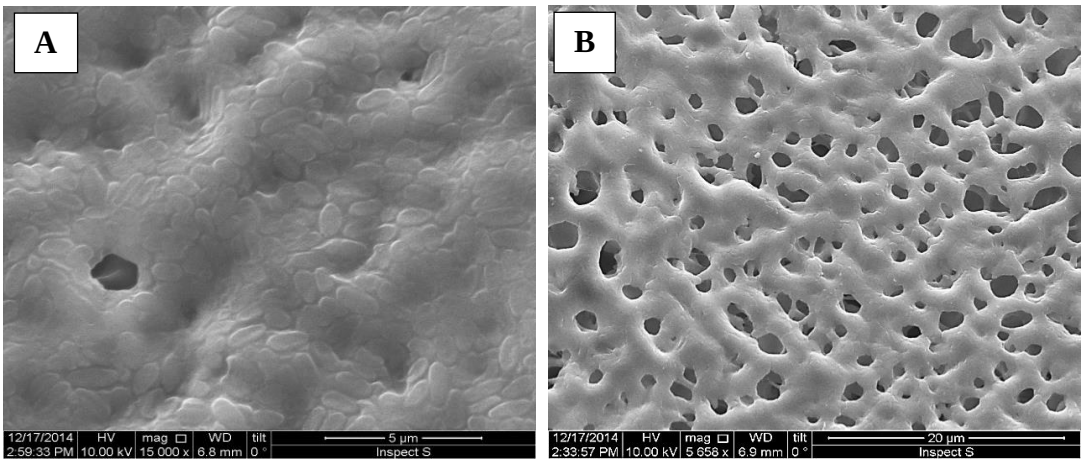

Figure 51: SEM image showing the *P.aeruginosa* cells encapsulated with the extracellular polymeric components. A) The clonal populations of *P.aeruginosa* located at the centre after exposed to amikacin in chemostat at 0.125$h^{-1}$and incubated at 30ºC for 3 days in amikacin-free nutrient agar. B) View of the surface of the colony at the centre showing a porous surface.

3.5. Confirmation that the cells exhibiting increased resistance following prolonged antibiotic exposure were not contaminants

One possible scenario with the experiments involving continuous culture is that during this process we may have inadvertently introduced a contaminating bacterium. This contaminant could then be maintained on subculture and could in part explain our phenotypic resistance. To eliminate this scenario, on a routine basis samples were taken from the chemostat to determine that the culture remained pure. DNA for 16S ribosomal RNA was extracted (Fig. 52). The strands of the PCR product were sent to be sequenced by Source Bioscience LifeSciences Company. DNA sequence traces were conducted using the windows software program called Geospiza's FinchTV, version 1.4, which is a chromatogram viewer capable of exhibiting a complete trace in a multi-pane view with scale options. In addition, the program is able to communicate with BLAST searching in the NCBI website for correlating sequences and traces. A comparison was conducted between the 16S rRNA gene sequence of *P. aeruginosa* and with those in the NCBI nucleotide sequence database. The result of the 16S rRNA gene sequence analysis revealed that the strain used is, indeed, *P. aeruginosa,* a type of bacterium of the genus *Pseudomonas* and exhibited maximum similarity with the 16S rRNA sequence of *P. aeruginosa* (99% sequence similarity).

Figure 52: *P.aeruginosa* template DNA for 16S rDNA PCR assay, PCR amplification products targeting the 16S rRNA gene using 27f and 1492r primers. Lane 1 represents the negative control (a sterile distilled water, Lane2 represents the positive control (purified *P.aeruginosa* DNA), lanes 3-6 represent *P.aeruginosa* grown under a glucose limited chemostat culture and lane 7 represents Hyper ladder I.

Chapter 4

Discussion

4.1. Susceptibility of *P.aeruginosa* to antibiotics

In this study, a number of antimicrobial susceptibility test methods were employed to assess the *in vitro* sensitivity of culture of *P. aeruginosa* to antibiotics. Susceptibility testing was carried out with the use of the conventional technique geared towards estimating the MIC and MBC as well as a novel technique OxoPlate. This study made use of three of the most commonly used anti- Pseudomonal drugs to treat *P. aeruginosa* infections, namely, tobramycin, amikacin and colistin. Determination of MIC and MBC values were carried out. The results of the present study revealed that *P.aeruginosa* was highly sensitive to the effect of the antibiotics selected. Our data shows in general that, tobramycin and colistin had the most extensive effect on the wild type (PA01) of *P. aeruginosa*, followed by amikacin. This result supports data previously reported by others and suggest that amikacin has a more reduced bactericidal effect compared with tobramycin (Burton et al., 2006). It has also been reported that among seven of the antibiotics, ceftazidime, aztreonam, amikacin, ticarcillin, gentamicin, ciprofloxacin, and tobramycin. It was tobramycin that had the most efficient bactericidal effect against isolated strains of *P. aeruginosa* found in individuals suffering from cystic fibrosis (Shawar et al., 1999).

The effect of antimicrobial agents on bacterial development, as well as the dynamics of this process, can be inferred from the oxygen concentration. The measurement of oxygen concentration is considered to be a central to numerous research areas since this substance is inevitable fundamental to behaviour and growth of aerobic cells. In antibacterial research, the use of the OxoPlate could prove particularly advantageous as it can readily determine the MIC value by measuring the rate oxygen depletion. The measurement of oxygen depletion indirectly indicates the level of bacterial development, which alongside bacterial metabolic activity, alters the oxygen concentration with time. This study has evaluated the use of a fluorescence-based assay to determine the oxygen concentration in the growth environment. The

OxoPlate method has proven to be an accurate, real time method to test the susceptibility of *P.aeruginosa* to antimicrobial agents. In the case of aminoglycoside antibiotics used, the OxoPlate technique has been observed to show a higher sensitivity to the antibiotics than the broth dilution technique, the broth dilution method led to a two-fold higher MIC (Fig.15 A&B and 16). The MIC values obtained by way of this method were more accurate to those obtained through the standard methods because these methods rely on measurements at specific time points normally between 18 and 24h. The accuracy of the OxoPlate is related to analysing samples with two fluorescent dyes, one being receptive to oxygen and the other emitting a regular signal. Any alterations in the otherwise constant ratio between these two dyes are recorded in real time with high temporal resolution.

The reliability of the susceptibility tests used to evaluate the antimicrobial activities of antibiotics against bacteria is considered an important parameter of the sustainable use of antimicrobials. At present, bacterial development, in the presence or absence of antibiotics, can be examined through a number of methods. Conventional susceptibility tests (endpoint or off-line measurement) are usually used to assess the activity of antibiotics, including microscopy, turbidity measurements, indirect staining methods, a viable count and a agar well diffusion method based on inhibition zone measurements (Jorgensen and Ferraro, 2009). In addition, standard methods (known as 'applied methods') include broth micro-dilution, which is geared towards the determination of MIC, disc diffusion and the E-test, are used on a regular basis as per the recommendations of CLSI (2013) and EUCAST (2014). One major drawback of these methods is that they cannot be automated, which means that it is difficult to apply them to higher throughput applications. By using the above methods, the kinetic of microbial growth in the presence of antibiotics cannot be monitored with high temporal resolution because it is only reliant upon the discrete intervals. Cultivation-dependent analyses are still fundamental for routine examinations despite the increasing use of molecular methods in research.

Thus, an alternative assay able to detect or quantify the growth inhibition of bacterial cells when exposed to antibiotics is the OxoPlate. This method is advantageous as it does not need calibration, it is not difficult to use and the process can be automated. The OxoPlate represents a viable, flexible and straightforward option for undertaking

bacterial susceptibility assessment in broth. Despite the fact that there have been previous studies that have analysed oxygen depletion with the use of OxoPlates, no studies prior to this have investigated the ability of such a system to distinguish between bactericidal and bacteriostatic antibiotics. The OxoPlate system can be adjusted to suit the development pattern of a certain bacterium (Hutter and John, 2004). The microbiological assay system is highly sensitive and has a wide dynamic range.

4.2. *P.aeruginosa* growth dynamics in the presence of antibiotics

In this study, an automated cultivation based assay (the SPECTRO $_{star}$ Nano) was used in order to assess the growth response of a wild-type *P.aeruginosa* to a selection of antibiotics. The results of this analysis indicate which antibiotic concentrations affect the extent to which *P.aeruginosa* grows. Based on these findings, the growth rate in the culture exposed to colistin exceeded the final OD values of the control culture (absence of antibiotic) during log phase. This indicates that colistin is capable of acting as a growth stimulant when administered at sub-inhibitory concentrations such as 0.125-0.25 μg/ml. If a phenomenon does *in vivo,* it is difficult to achieve antibiotic action. It is also difficult to ensure that the antibiotic level is above the threshold during long-term treatment. During chronic infection, the antibiotic concentration may decrease to sub-inhibitory levels in some parts of the body. The consequences of this over the course of treatment could enhance the protection of microorganism from the action of antibiotics and lead to selection mechanisms including SOS response, direct mutagenic effects, presister cells and hypermutation (Rodriguez-Rojas et al., 2013). In our study, growth promotion occurred as a result of treating *P.aeruginosa* at sub-inhibitory concentration of colistin, which may go along with or be enhancing by other mechanisms. In addition, the effect of sulfate salt present in the colistin components might have induced the growth promotion of *P.aeruginosa* by colistin.

4.3. Effect of inoculum size on susceptibility test

A number of reports have described that bacterial inoculum size is a critical factor in determining the level of activity of antimicrobial agents (Brook, 1989, Soriano et al., 1990, Reguera et al., 1991, Reguera et al., 1988). The activity of antimicrobial agents can be assessed based on the MIC value. The MIC of bacterial populations in infections tends to rise when bacterial loads increase. This issue may explain why such infection is not successfully treated when more cells are present, because the MIC value obtained from the classic laboratory tests use the standard bacterial inoculum size is lower than the actual MIC of those treated *in vivo* (Soriano et al., 1990).

The impact of bacterial inoculum size has been primarily considered in microbes producing enzymes that inactivate antimicrobial agents, including the staphylococci species. Almost every class of antibiotics is influenced by the microbial inoculum size, particularly *β*-lactam antibiotics. Therefore, it is believed that the primary cause of the inoculum effect is the loss of antibiotic activity that occurs as a result of *β*-lactamase enzyme production. This causes a high antibiotic degradation rate when a large number of bacterial cells are treated with the *β*-lactam antibiotics (Eliopoulos and Moellering, 1996, Reguera et al., 1988, Reguera et al., 1991). However, the correlation between this effect and bacteria (where resistance mechanisms do not involve the degradation of antibiotics) is less apparent (Corona and Martinez, 2013). Aminoglycosides (for example, tobramycin) do not usually trigger the inoculum effect when treating the Pseudomonas species (Brown et al., 1990).

It is well- documented that the MIC value can be affected by the size of bacterial inoculum, and this effect has been extensively examined using only the survival-time method (Bulitta et al., 2015, Chan et al., 2006). This research has employed alternative evaluation techniques from those used in the studies mentioned above, particularly in terms of the range of inoculum sizes used and the types of microbiological tests performed. This study uses the OxoPlate system to carry out an *in vitro* study of *P. aeruginosa* (a reference strain PA01), in order to derive a new understanding of the inoculum effect. No previous studies have documented the use of the Oxoplate system to determine the impact of inoculum size. However, this

analysis showed not only excellent replication but also precision, sensitivity and accuracy.

In our study, the novel assay based on the OxoPlate system indications that suppression of growth following treatment with tobramycin, amikacin and colistin was found to be less marked when the size of inoculum of *P. aeruginosa* was increased. The fact that colistin exhibits an inoculum effect has been well reported. However, previous studies have concluded that the phenomenon is not typically associated with aminoglycosides, meaning that our findings contradict the existing literature. It is important to note that, our results are in agreement with only one recent study has linking aminoglycosides, such as tobramycin with an inoculum effect when multiple inocula was used (Bulitta et al., 2015) and another study also showed this effect only when amikacin was combined with piperacillin (Chan et al., 2006). The results showed that minor changes in the total initial cell of *P.aeruginosa* treated with tobramycin and amikacin can account for the increase in the MIC values recorded, as explained in the results section (Fig. 33-34).

One possible explanation for the detected inoculum effect might be that our work used a wide range of inoculum sizes (above and below standard inoculum) in order to perform a full and broad evaluation of this effect, yet earlier work only focused on single inoculum size (above standard inoculum). In light of this investigation, as other situations can be associated with inoculum dependent antibiotic including mathematical modelling and experimental approaches, other processes are also possible to effect this phenomenon (Udekwu et al., 2009). It is suggested that when the bacterial cell is loaded in large numbers, the commensurate reduction of its capacity to bind to cell envelopes and debris from live and dead cells may cause it to become less vulnerable to the effects of aminoglycoside. The reduced accumulation of antibiotic molecules in each cell at high bacterial cellular mass may provide an alternative explanation for the occurrence of the inoculum effect with both tobramycin and amikacin. The fact that high bacterial cell loades are able to activate the response of QS, together with the suggestion that QS might have an impact on the susceptibility of bacteria to antimicrobial agents, may indicate that QS is significant in controlling the susceptibility of inoculum-dependent

antibiotics to the populations of microorganism. So far, no examination of this theory has been recorded (Corona and Martinez, 2013).

The anti-endotoxin action of colistin in counteracting bacterial lipopolysaccharide (LPS) was tentatively identified as a possible cause for the inoculum effect of colistin. In conjunction with phospholipids, colistin creates combined monolayers encompassed in micelles *in vitro*. (Mestres et al. 1998 cited in Bulitta et al., 2010). The inoculum effect may be brought about through the attachment of colistin to the LPS fragments of destroyed bacteria which can determine the reduction of the concentration of unattached colistin *in vitro* (Bulitta et al., 2010). The reduced effect of colistin on bacterial development may be due to a decrease in the negative charge of LPS as well as by transformations in the hydrophobic side chain located in the external membrane of the bacterial cell induced by the cells sensing as they react to their medium (Bulitta et al., 2010).

4.4. Phenotypic variation in *P.aeruginosa* isolated from continuous culture

4.4.1. Adaptive phenotypes

In the current study, which explored the prolonged exposure of amikacin in glucose-limited chemostat cultures, it was revealed that *P. aeruginosa* survived and subsequently developed temporary adaptive resistance to amikacin. When growth without antibiotic presence, however, this phenomenon was not observed. Adaptive resistance to amikacin conferred low-level resistance to other aminoglycosides such as tobramycin and antibiotics with different modes of action such as colistin when cultures grown at $0.025h^{-1}$ and $0.06h^{-1}$. This finding is supported by the recent work carried out by Amabile-Cuevas (2016) where resistant strains of *P.aeruginosa* exposed to graded concentrations of amikacin resulted in a rise in the MIC and this resistance was not associated with genetic resistance. Altered susceptibility of *P.aeruginosa* to other antibiotics is also occurring.

Gilleland et al. (1989) reported that adaptive resistance has been shown to be associated with the prolonged administration of aminoglycoside antibiotics. It is common to observe aminoglycoside-resistant bacteria instability in adaptively

resistant bacteria. This results in a return to the initial susceptibility level, upon several passes through a drug-free medium (Poole, 2005).

It has been previously demonstrated that the concentration of aminoglycoside and the length of exposure of aminoglycoside therapy are the main factors that impact the level of adaptive resistance (Daikos et al., 1990). Our results suggest that another factor that affects this phenomenon is the growth rate of bacteria, which showed that the time it took for the culture to become adaptive to amikacin was longer with culture exposed to amikacin at fast dilution rate (Fig. 40&42). Resistant subpopulations that normally developed as a result of continued aminoglycoside therapy in hospitalised patients became undetectable in the absence of antibiotics, as reported by epidemiological surveys. On the other hand, since the phenotype may immediately revert to its original state during the initial isolation (Daikos et al., 1990), it may be difficult to detect aminoglycoside adaptive resistance in immunocompetent patients and clinical microbiological laboratories (Price et al., 1981, Weinstein et al., 1980) . The presence of resistant subpopulations during infection may be linked to the failure to treat chronic infections, including endocarditis, cystic fibrosis or other immunity issues in patients who were subjected to a continued dose of aminoglycoside (Gilbert, 1995).

Adaptive resistance has been well associated with *P.aeruginosa* because it is one of the most important pathogens in nosocomial infections and fails to respond to standard treatment (Korvick and Yu, 1991). Due to continuing use of aminoglycoside therapy during chronical infections caused by *P.aeruginosa*, it is clinically essential that the nature of adaptive resistance of *P. aeruginosa* to aminoglycoside antibiotics is understood to improve the usage of these antibiotics.

4.4.2. Colony morphotypes

The observations of morphological changes in bacterial colony are becoming increasingly significant as these are believed to be caused by adaptation to survive the stressful challenge of a new environmental condition, which become an obstacle issue in clinical diagnosis to identify pathogens because it adds variation to morphological trials. The accuracy of diagnoses may be compromised by the

occurrence of alteration in colony morphotypes. This observation has been previously reported in cystic fibrosis patients infected by *P.aeruginosa*, which regularly converts the structure of the cell surface from a non-mucoid to a mucoid phenotype (Ogle et al., 1987). The effects of experimental conditions under which results were obtained, resulting in alteration of colony morphology and are considered to be an important factor to exam the phenomena of bacterial adaptation. By way of example, the characterisation of *P. aeruginosa* colonies has been accomplished using various agar growth media types and with a number of supplements, each conducted within different time scales (Hay et al., 2009, Starkey et al., 2009). The following species have been similarly examined for their colony morphologies: *Staphylococcus aureus*, (Norstrom et al., 2007, Schneider et al., 2008), *Streptococcus pneumoniae* (Allegrucci and Sauer, 2007) and *Enterococcus faecalis* (Wellinghausen et al., 2009, Qamer et al., 2003).

Under all growth conditions used, different colony morphological types were clearly detected in a single sample isolated from chemostat. The results of the *in vitro* study of *P.aeruginosa* exposed to amikacin in chemostat at different dilution rates showed that morphological alterations were clearly observed in the colony growth on amikacin-free nutrient agar compared with the untreated-control colony (Fig.46). Visible differences were also observed in *P. aeruginosa* colonies that had been cultured for 6 days, which leads to the introduction of subpopulation, resulting in development of drug resistance compared with earlier time points (3days) (Fig.49). Since the effect of experimental factors into colony morphology trials is as yet unquantified, the validity of apparent positive outcomes in respect of research into bacterial adaptation such as bacterial persistence and phenotypic switching may be affected. Additionally, changes in characteristic morphologies of colonies may be suggested to be a change arising as a result of phenotypic switching. The binary alternation between phenotypic states in response to situational challenges is the same as ON/Off mechanism that do not engender a permanent mutation that may result in bacteria becoming more virulent, less vulnerable to microbial effects or exhibiting of bacterial persistence phenotype (Massey et al., 2001, Sousa et al., 2011). The phenotypic switching phenomenon has been considered in respect of

colony morphology (Be'er et al., 2011, Sousa et al., 2013). Colony morphology is considered an important tool to identify bacterial type; nowadays this tool should be used with caution because of increasing observation of a phenotypic change in bacteria involved in cystic fibrosis and other chronic lung infections.

4.5. Is the behavioural response of bacterial cells within colonies grown on an agar plate equal to that of a biofilm?

The relationships between planktonic cells and biofilms have been extensively investigated, with significant differences being noted between the growth modes of the two states. However, few of these investigations have analysed the growth of colonies on agar plates. In this study, colonies were grown on an agar plate over six days to obtain a clear overview of the development of bacterial colonies over the time period as well as their susceptibility to antibiotics. Our results showed that the comparison of *P. aeruginosa* colonies grown on agar plates (isolated chemostat) for three days and six days presented visible differences with respect to morphology and susceptibility (Fig.48). *P.aeruginosa* colonies that had been cultured for six days exhibited higher amikacin resistance than cells derived from early agar-grown colonies (three days old). In our study, an important indicator of colony morphology was colony age. This finding is in agreement with the study conducted by Pipe (2014) who found that colony growth resulted in differentiation. In addition, SEM analysis revealed that cells derived from a colony that was isolated from chemostat incubation for 3 days led to the development of an extracellular matrix, unlike with control (untreated) sample. Thus, a direct effect on the ability of amikacin to form a biofilm in agar when antibiotic was removed. This phenomenon may explain the alteration that was observed in bacterial susceptibility. It has been indicated that biofilm formation can directly be influenced by manipulating cell morphology via exposure of planktonic cells to antibiotics at a concentration below the lethal level (Song et al., 2016). It has been found that film formation occurs in bacterial cells of *E.coli* when treated by either piperacillin or a pipercillin-tazobactam at a sub-inhibitory concentration (De Andrade et al., 2016). The recent study conducted by Amabile-Cuevas (2016) demonstrated the ability of *P.aeruginosa* to generate a biofilm when amikacin was applied as a graded exposure to resistant strains of

P.aeruginosa .

It has been shown that the bacterial cell growth in colonies in agar plates possess similar features to that of biofilms. This includes the development of communities with high cell densities, encapsulated with a polysaccharide matrix and coordinating cellular behaviour. Therefore, the behavioural responses of both models are expected to be the same (Shapiro, 1998). Bacterial populations prefer to organise an extensive communication between bacterial cells, as they provide defence against stressors such as biotic, chemical and physical factors (John et al., 2003). Kolter and Greenberg (2006) likened *P. aeruginosa* colonies to air-exposed biofilms, and both structures showed bacterial cells entrapped within an exopolymer matrix (Asakura and Okura, 2009). In addition, both biofilms and cells within colonies created heterogeneously-temporary environments (e.g. gradients of nutrients, oxygen, ionic strength, pH, redox potential, and waste products) (Shapiro, 1998). However, mutagenesis was extensively increased in both structures. Thus, the growth of colonies on agar plates is associated with genetic or morpohotype change (Be'er et al., 2011)

4.6. Interaction of bacterial cells within colony grown an ager plate

The ways by which cells interact within colonies are extremely coordinated and complex. Detailed study has revealed that cells in colonies have developed differential morphological and physiological characteristics, which are location-specific.

The length of individual microbe cell within a colony differs depending on their position. Thus, the length of bacterial cells grows in the internal colony area maybe shorter compared with those located in the edge of colony. For this reason, the ability of microbial cells to build up a tolerance to the deadly effect of antimicrobial agents is increasingly being seen in the cells located at the heart of the colony. This theory may explain our finding in which cells derived from the central colony area gained higher resistance to amikacin than those located in the peripheral area. An increase in MIC value (40µg/ml) was observed at the centre of the colony compared

with cells at the colony margin (1.25µg/ml) (Fig.49). The reduction in the cell size was also observed in the cells located on the interior structure of colony. In this stage, the formation of subpopulation was also increased. It is possible that their drug targets were inactivated due to the physiological state rather than genetic mutations in a subpopulation of bacteria.

Another possible reason may be due to an increase of nutrient limitation in the centre when the colony becomes large, resulting in successfully adaptation to a new condition. The physiological status of *P. aeruginosa* would be negatively impacted by depleted nutrient supply, especially intracellular energy metabolism. Then, utilization of nutrients would have to be improved and the growth rate of bacterial cells within colony would be reduced (Terry et al., 1991). Therefore, the colony has protected itself by evolving; stationary-phase cells are located in the centre of the community, while the cells at the edge have developed the ability to divide and grow outwards to reach nutrient sources far away from the core of the structure (Lovitt and Wright, 2014).

Other factors that influence the physiological response of cells within colony including enzyme composition (Saier, 2000) and the gene expression. (Lovitt and Wright, 2014). The variations of both enzyme composition and the gene expression within the colony are responsible to control their role depending on their site inside the colony. Hence, the complexity of construction of a colony during growth relies on the interaction with external influences, such as diffusion of nutrients and gases across the structure, and innate factors such as size and shape of bacterial cell, method of reproduction, bacterial motility, and the ability to produce extracellular molecules (Lovitt and Wright, 2014). Colonies are an excellent model system for understanding the complexity that is emerged at the dawn of life of microorganisms. The use of bacterial colonies as models for understanding the emergence and evolution of complex life forms from bio-complex micro-organisms is gradually being appreciated.

4.7. Effect of growth rates on the susceptibility to antibiotic

Based on the MIC values, the continued presence of amikacin in cultures grown at slow dilution rates showed less susceptible to amikacin compared with those grown at high dilution rates (Fig.44, appx. F 2&4). The reason they persist being thought to be growth rate related. There is a well-documented relationship between growth rate and antibiotic resistance. Work carried out using chemostat cultures by Wu and Lfrettnore (1990) where the growth rate of the bacteria was regulated by the nutrient feed rate showed that at low growth rates the organisms were less susceptible to the antibiotics than at faster growth rates. This study was conducted to exam the effect of several antibiotics – ceftazidime, imipenem, piperacillin and meropenem – against different growth rate of *P. aeruginosa* developed in a chemostat. Thus, a slower rate of development enables bacteria to acquire resistance against antibiotics, which represents a significant issue in microbial pathogens. The resistance can be acquired as a result of transformation, conjugation and transduction (Tenover, 2006).

A small fraction of less-susceptible cells was observed after 72h incubation time in the *P. aeruginosa* exposed to amikacin in the chemostat at high dilution rates using the OxoPlate (Fig.44), which demonstrated that exposure to amikacin resulted in some surviving bacterial cells that escaped the lethal action of the antibiotic. These surviving cells may well be the so called “persister” cells described in the literature (Gefen and Balaban, 2009). Persister cells are often considered to be slow growing variants within a population and respond heterogeneously to the action of the antimicrobial agent.

4.8. Effect of glucose concentration on the growth of *P.aeruginosa*

An increase in the concentration of glucose from 30-50mM resulted on the growth of bacterial cells was significantly inhibited over the time of experiment (Fig.37). This finding indicated that increasing glucose above 25mM had no further increase on the density of *P.aeruginosa*, which indicating that bacterial densities were noticeably independent on presence of theses concentrations of glucose. Based on the study published previously on the growth rate of *E.coli* in the presence of different

concentration of glucose, the reason for inhibition of bacterial growth was due to acetate production (Vemuri, et al., 2006).

4.9. Response of *P.aeruginosa* to the availability of O_2 concentration during fermentation process

The results presented in this study reveal that *P. aeruginosa* exhibited significant physiological responses to oxygen level upon its growth rate during batch mode. During batch culture, *P.aeruginosa* showed a strong preference for a microaerophilic growth environment, achieving its fastest growth rate ($0.36h^{-1}$) when the concentration of O_2 was low. However, a high pO_2 value in the growth medium caused a dropped in growth rate to $0.26h^{-1}$ (Fig.40). This finding was also observed during continuous cultures, where a reduction in the concentration of oxygen was exhibited when dilution rates were increased to 0.099 and $0.125h^{-1}$ (Fig.42). This finding is supported by the previous work carried out by Sabra et al. (2002), where the pO_2 value was controlled to 1% air saturation, revealing a greater increase in the growth rate of *P. aeruginosa* (PA01) under low oxygen concentration than under a high concentration of oxygen. Oxygen limitation was only observed in the cultures at fast dilution rates (Fig.42) and not with slow cell growth (Fig.40). As a result of reducing the oxygen concentration at high dilution rates, the cells adapted a mucoid phenotype when colonies were grown in an agar plates for 3 days. Thus, oxidative stress observed during continuous cultures may explain the extraordinary physiological reaction of *P.aeruginosa.*

The formation of reactive oxygen and nitrogen intermediates is a typical immunological response against pathogenic bacteria that attacks the host cells. Consequently, pathogenic bacteria such as *P. aeruginosa* protect themselves against these poisonous reactive intermediates by developing defence mechanisms. One of the defence mechanisms in which pathogenic bacteria use against oxidants is that the transportation of oxygen to the culture media is reduced by blocking the formation of the oxygen reactive intermediate. Other pathogens have evolved with a number of different responses, not only enzymic and non-enzymic but also the polysaccharide coat that serves to eliminate these intermediates from their environment (Bogdan et al., 2000). Oxidant inactivation in *P. aeruginosa* has been identified through specific

enzymic pathways such as enzymes catalysed by peroxidase, catalase and superoxide dismutase. (Sabra et al., 2002).

4.10. Effect of ions on the susceptibility to antibiotic

To evaluate the correlation between the experimental results and studies published previously, the dissolved oxygen curves revealed that the extent to which *P. aeruginosa* was susceptible to antibiotics depends on the concentration of divalent cations in the test medium. Similar results have been published previously based on the susceptibility of *P. aeruginosa* to both aminoglycosides and colistin in the presence of the cations in broth as well as agar (Jeffrey et al., 1982). Posing a major issue in aminoglycoside therapy, aminoglycoside antagonism by divalent cations has been comprehensively explored in the case of *P. aeruginosa* (Medeiros et al., 1971, Zimelis and Jackson, 1973, D'Amato et al., 1975). As confirmed by many studies, this antagonism is caused by the fact that aminoglycoside assimilation at sites on the external and internal membrane is disrupted by cations (Bryan and Van De Elzen, 1977, Campbell and Kadner, 1980, Zimelis and Jackson, 1973). Additional evidence in support of the fact that the internal membrane is a site for cation antagonism was provided by Mao et al. (2001), who demonstrated that aminoglycoside assimilation in *Staphylococcus aureus* and in spheroplasts of *Escherichia coli* seemed to be suppressed by cations. Furthermore, according to the findings of Mao et al. (2001), a direct correlation exists between the functionality of the MexXY-OprM efflux pump and antagonism of aminoglycoside by divalent cations.

Based on the oxygen depletion curves, Mg^{2+} was not as effective as Ca^{2+} in suppressing the effect of aminoglycoside agents against *P.aeruginosa.* Similar results were found in resent study for gram negative bacteria (Sahalan et al., 2013), where the activity of Polymyxin B was significantly inhibited in the present of Ca^{2+} in defined medium, which lead to survival bacteria. The experimental findings suggest that the greater efficiency of calcium can be explained in terms of specific cations adapted to particular functions. In fact, calcium is rather an element of the cell structural components, and less frequently a co-factor of active proteins as magnesium in *E-coli* (Verkhovskaya et al., 2011). The reliability of the observation of growth inhibitors is enhanced by the oxygen consumption measurements.

4.11. Effect of temperature in the growth rate and antibiotic susceptibility of *P.aeruginosa*

Temperature is one of the most important environmental factors that influence the growth behaviour of microorganisms and their susceptibility to antibiotics (Estrela et al., 1995, Segun, 2004). Only a small number of research papers reveal the way in which *P. aeruginosa* responds to growth temperature. In addition, very little evidence exists in the literature for the behavior of *P. aeruginosa* at temperatures below and above the optimal level despite a high number of studies having investigated the way in which *P. aeruginosa* behaves at 37°C (optimal).

A study on *P. aeruginosa* strain PAO1 revealed that temperature-based adaptation occurred in its outer membrane protein, lipopolysaccharide and phospholipid (Kropinski et al., 1987). Research also reveals that temperature changes in the environment cause the channel size of *P. aeruginosa* porin (OprF) to fluctuate, with suboptimal temperatures resulting in a small pore size (Jaouen et al., 2004). It is probable that these adaptations protect the bacterium by helping it to hold essential metabolites. This also likely protects the bacterial cell from the invasion of toxic substances. A study conducted by Jaouen et al. (2004) showed that temperature regulation has also been found in gene PA0011 in *P.aeruginosa* PAO1. Here, it was found that temperature impacts the bacterium's resistance to carbapenem, with a temperature of 21^{o}C resulting in a higher PA0011 expression than a temperature of 37^{o}C (body temperature). It is proposed that in order for *P. aeruginosa* to grow or survive at suboptimal temperatures, up-regulated PA0011 is essential. It is probable that growth temperature fluctuations have an impact given the likelihood that phospholipids and other lipids within the cell wall protect the cell from toxic substances (Farrell and Rose, 1967).

In light of thermal adaptation, *P.aeruginosa* is able to thrive in many different environmental conditions due to its complexity and large genome size. Metabolic flexibility and strong adaptation to new environments are the two abilities for which the ubiquitous environmental bacterium *P.aeruginosa* is most known. Given this, the

opportunistic behaviour of the pathogen is likely prompted by the facilitation of these very factors.

In the study of pathogenic bacteria, the determination of microbial growth rates is essential, particularly in relation to temperature. The effect of temperature on the growth rate of bacteria and on its susceptibility to antibiotics has been investigated in this study. Three independent methods were used in this study to evaluate temperature-based fluctuations of *P. aeruginosa* susceptibility. The OxoPlate revealed that susceptibility to aminoglycosides fluctuated significantly between 30°C and 37°C, where in susceptibility doubled at 37°C compared to 30°C. When exposed to colistin at these temperatures, however, the same MIC was shown and no change was noted. However, no difference in the MIC breakpoints was observed in all antibiotics when the agar well diffusion method was used at these temperatures. This finding is in agreement with a study conducted in 1985, exists regarding the way in which *P.aeruginosa* reacts to aminoglycoside at certain temperatures. In this study, the disk diffusion method was used to test the behaviour of *P. aeruginosa* with aminoglycoside antibiotics at two different temperatures: 30^{o}C and 37^{o}C. The study revealed that neither temperature altered the susceptibility of *P. aeruginosa* strains (Wheat et al., 1985). On the other hand, *Pseudomonas maltophilia* was found to exhibit significantly varied susceptibility to aminoglycosides, as well as to other antibiotics. Furthermore, *Stenotrophomonas (Xanthomonas) maltophilia* strains were exposed to polymixin B and aminoglycosides and found to exhibit susceptibility to these antibiotics at a temperature of 37°C but resistance at 30°C (Wheat et al., 1985, Rahmati-Bahram et al., 1995).

At 25^{o}C, *P.aeruginosa* became less sensitive to aminoglycoside because the growth rate of bacterial cells was lower. In most cases, phospholipid composition appears to vary at lower temperatures, whilst lipids also show a higher level of unsaturated fatty acid residues at the lower end of the growth temperature range. This effect was reported in some *Pseduomonas* species such as *P.putida* (Loffhagen et al., 2004). However, the susceptibility of *P.aeruginosa* to colistin and amikacin was also tested at 42^{o}C (above optimal) and the result indicated that, bacterial cells became more sensitive to antibiotics. Based on the measurement of the growth rate, this study

found that temperature was influenced the growth of cells, resulting in a fast growth rate when the temperature was increased. Worthy of note is the fact that many researchers have found that as temperature rises, various antimicrobial agents become more active against both gram-negative bacteria and gram-positive cocci (Mackowiak et al., 1982).

The susceptibility of *P.aeruginosa* to all antibiotics tested at 25, 30, 37 and 42^{o}C, showed a significant variation in its MICs when an E-test was used. It is important to note that E-test (diffusion technique) has observed to show a higher sensitivity to the antibiotics at 42^{o}C. Due to the fact that the agar diffusion technique depends on several factors, including agar depth, antibiotic dispersal rate, bacterial growth rate, the temperature and period of incubation (Mesaros et al., 2007). These factors can affect the outcome of the results. Moreover, the broth dilution method relies on the manual preparation of antibiotic dilutions; the accuracy of the method was reported to be +/- a two-fold concentration compared with E-test that has a precise known gradient of antibiotic concentrations. Apart from the manual preparation of the antibiotic solution, this can result in errors (Jorgensen and Ferraro, 2009).

Chapter 5

Conclusion and future work

While antimicrobial medicine has been in existence for almost one hundred years, it is now necessary to review its usage in light of the widespread antibiotic resistance. Nowadays, antibiotics are regularly prescribed to remedy infectious conditions in both human and animal species, along with additional areas such as in farm-related activities. Notwithstanding this, an entire body of research attests that in reality a paradoxical reaction may occur as a result of its usage, thus contributing to the development of antibiotic resistance.

Infections caused by *P. aeruginosa* are becoming increasingly difficult to treat as the bacterium is becoming more and more resistant to a significant range of commonly used antibiotics. With the extensive use of antibiotics, *P. aeruginosa* is responsible for the greatest number of gram negative bacterial infections, particularly in the case of patients subjected to prolong antibiotic treatment. By developing and growing bacteria in the presence of antibacterial agents, bacteria acquire/develop resistance to them and seem to have a 'life of their own'. This study has aimed to develop an *in vitro* model to examine the effect of continual expose of *P.aeruginosa* PA01 to amikacin as a model antibiotic. Experiments were initially conducted to consider the factors that having a significant influence on the susceptibility and growth behaviors of the cells population using an automated cultivation based assay (the SPECTRO $_{star}$ Nano and OxoPlate system). To the best of our knowledge, the development of antibiotics resistance in planktonic cells has not been studied as extensively as that in biofilms. Planktonic cells use in this thesis as stepping stones to understand the complexity that is emerged at the dawn of life of microorganisms. This point is to the potential to substantially increasing the likelihood of development of phenotypic resistance in the case of planktonic cells of *P. aeruginosa.*

The results of the *in vitro* analysis showed that, from among the three antibiotics used in this study, amikacin posed a more serious risk to develop resistance in *P. aeruginosa* , which was a more effect by selecting stress including:

I. The least effective antibiotic against *P.aeruginosa* compared with other antibiotics used was amikacin as evidenced from OxoPlate output.

II. *P.aeruginosa* displayed intermediate susceptibility to amikacin as observed in the agar well diffusion method.

III. Activity was influenced by temperature, which resulted in cultures that showed from the MIC and MBC data, less susceptibility to amikacin when temperature was decreased from 42 to 25^{o}C.

IV. The presence of surviving colonies was indicated in association with amikacin at low tempertures 25 and 30°C.

V. In comparison to colistin and tobramycin, amikacin had a weaker effect on *P. aeruginosa* when Mg^{2+}were individually added in defined medium.

VI. The highest optical density (OD_{600nm}) values were detected when Mg^{2+} or Ca^{+2} was individually added in the defined medium in the presence of lethal concentration of amikacin that means the action of amikacin was obliterated and provided a greater level of protection to the cells.

VII. The efficacy of amikacin against PA01 was reduced markedly by the combined effects of Ca^{2+} and Mg^{2+}supplementation compared with tobramycin.

VIII. It was investigated that amikacin with an inoculum effect when multiple inocula were used, this finding contradict the existing literature.

IX. Bacterial cells had faster growth rates when Ca^{2+} was added individual into defined medium in the presence of amikacin.

Consequences of these, chemostat studies were designed to examine prolonged exposure of the antibiotic to the planktonic cells. The issue of adaptive resistance may emerge in a clinical context, culminating in a lack of response to treatment. In addition, it may arise in situations where an insufficient quantity of medication was administered or where the recipient did not comply with the recommended treatment plan. Sub-inhibitory concentrations of medication may occur in every one of these conditions. The consequences of this over the course of treatment could enhance the protection of microorganism from the action of antibiotics and lead to developing resistance. Sub-lethal concentrations may play an important role in developing resistance in patients who requiring prolonged therapy.

Thus, *in vitro* studies were designed using chemostat systems to examine the effect of prolonged exposure of antibiotic at sub leathal concentrations at different growth rates. By regulating the growth rates it will be possible to also investigate whether or not a continuous prescription of antibiotics for a human will be a greater threat than the environment, where antibiotics at sub-inhibitory concentrations are routinely used as additives in food for animal husbandry. Based on the results, *P. aeruginosa* exhibited low-level resistance to more recently developed antibiotics with broader spectra of antibacterial activity such as amikacin, tobramycin and colistin. The results of the tests conducted during this study revealed that the phenotypic transformations exhibited by the resistant strains of *P. aeruginosa* are temporary; returning to normal after the strain was left to develop over a number of days in a medium that does not contain antibiotics, thus becoming once again susceptible to the effect of antibiotics. These findings indicate a clear phenotypic variation during long term therapy to the most common antibiotics used to treat *P.aeruginosa*. Low oxygen availability was seen in the cultures grown at 0.099 h^{-1} and 0.125 h^{-1}, which leading to the development of the so called "persister" phenotype that showed a reduction in bacterial cell size and resistance to the action of antibiotics. The *P.aeruginosa* cells encapsulated with the extracellular polymeric components in the cells derived after 72h incubation time when antibiotic was removed. In all cases, continual exposure resulted in phenotypically distinct mucoid or non- mucoid colony morphotypes, which were clearly observed on absence of antibiotics. Some of these selected morphotypes showed from the MIC and MBC data a high-level resistance to the antibiotic in spite of media lacking antibiotics.

Based on the results obtained, the major response to the action of amikacin was attributed to persisters cells. This increases the challenge of distinguishing a direct relationship between antibiotic use and development of resistance. Clearly antibiotic resistance can be induced in situations where an organism is exposed continually to an antibiotic, as may be the case in some clinical settings, for example cystic fibrosis patients. Based on the results of this study, it can be concurred with that failure to eradicate *P. aeruginosa* in patients suffering from cystic fibrosis can be attributed to temporary bacterial resistance to an antimicrobial agent used to treat the patients. The biological responses resulting from these studies offer valuable clues underlying unsuccessful treatment and this will contribute to the development of viable

treatment options and help to reduce of the emergence of high antibiotic resistance. The response of *P.aeruginosa* to fast growth rates (expected in human) showed a greater threat when left without antibiotic (stress was removed) than cultures grown at slow growth rates (expected in environment). This means misuse of antibiotics leads to the development of much more aggressive strains of *P.aeruginosa.* Based on the results obtained from divalent cation experiments, one of the most important factors that contributed to increase in growth rates of *P.aeruginosa* when treated with amikacin was the presence of ≥ 3mM of Ca^{2+}.

As far as conclusions based on the research conducted on the planktonic cells of the bacterium *P. aeruginosa*, using an *in vitro* procedure treated with a single antibiotic, it was found that amikacin typically performed a crucial function in the development of resistant variants. This project indicates the way in which the extensive utilisation of antimicrobial medicines resulted in alterations occurring in bacterial cells, resulting in a reduction in the effectiveness of these antibiotics. Furthermore, it demonstrates the manner in which bacteria may become less receptive to medicine, in particular circumstances or physical conditions. It is worth to note that, the more bacteria are exposed to antibiotics, the more reduced the number of susceptible bacteria will become and the higher the probability of selecting resistant bacteria. It would be advantageous if research examining the change process of antibiotic resistance would focus more closely on the part limiting nutrient and growth rates can play on the consequences and selection of adapted strains.

In summary, the phenomenon of adaptive drug resistance needs to be cognisant of advances in new antimicrobial agents, as well as the analysis and correction of any difficulties arising from this process. Therefore, in introducing new pharmaceutical products to the market, clearly not only must their capability to destroy bacteria and toxicity be examined, but also how they stimulate adaptive resistance must take into account. Gaining a more in-depth knowledge of the processes by which adaptive resistance to antibiotics occurs will assist in devising more successful interventions. In addition, it will support the introduction of new drugs, with a reduced likelihood of them becoming resistant to disease, but producing equally beneficial medical outcomes. These approaches should be established at the initial drug development phase, so as to modify the therapeutic intervention (for example, quantity of

medication) and reduce the persistence of resistance variables at low levels, as they often occur by choosing concentrations at specific levels.

Furthermore, greater emphasis should be placed on the slight rise in drug resistance emerging from ongoing clinical reviews. This could be suggestive of adaptive resistance occurring throughout an illness, and could also determine indicators of high resistance in the future. At the same time, the reliability of the susceptibility tests used to detect low-level resistance is considered an important parameter of the sustainable use of antimicrobials and prevents the development of high level resistance. Thus, use of OxoPlate system as an alternative method to existing techniques (conventional methods) would allow monitoring the metabolic activity of microorganism in the presence of antibiotics with high temporal resolution. The study results underscore that the reliability of the observation of growth inhibition is enhanced by the oxygen consumption measurement. With respect to standard methods, the OxoPlate method has proven to be an accurate method to detect survival subpopulations that resist the action of antibiotics and to test the factors that influence the susceptibility of *P. aeruginosa* to antimicrobials.

It is imperative that we remain alert to the significant directional changes microorganisms are undergoing. In this context, it is necessary to gain a more comprehensive understanding of living organisms, as well as an awareness of the incidence and management of low levels of antibacterial resistance. A number of measures must be taken in an effort to ensure that antibiotics are utilised in a more appropriate manner, and to reduce environmental pollution by the drug industry. These include providing advice and training, in addition to circulating details on the correct enactment and imposition of the law, in relation to the use and discarding of medication. Sub-inhibitory antimicrobial levels will play one key function in the coming years by comprehensively outlining how these agents can produce antibacterial properties, from a molecular biological perspective.

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Appendices

Appendix A: Statistical analysis of the effect of temperature on the susceptibility of *P.aruginosa*

Appendix B: Susceptibility of *P.aeruginosa* in the presence of divalent cation

Appendix C. Growth rate of *P.aeruginosa* in the presence of divalent cation

Appendix D: Effect of inoculum size on the susceptibility of *P.aeruginosa*

Appendix E: Nutrient limitation and batch mode

Appendix F: Continues cultures

Appendix G: Poster presentation

The data of the appendices is found in the supplementation material.