Using bacteriophages and antibiotics in combination to control pathogenic bacteria

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1.Abstract

Multidrug resistance (MDR) is becoming an increasing threat to the global public health and *Pseudomonas aeruginosa* is one of the leading causes of nosocomial infections as it can quickly develop resistance to clinical antibiotics. The rise of MDR has rejuvenated interest in alternative therapies where, for example, pathogen-specific phages could be used on their own or alongside antibiotics to improve treatment efficacy. Benefits of phages include high pathogen specificity, efficacy against antibiotic-resistant bacterial genotypes and their ability to co-evolve to overcome evolution of phage resistance. Phages also have low toxicity to the patients, minimal disruption of the patient's microbiota, and can auto- "dose" themselves by replicating in their host pathogens. This research investigates using phages and antibiotics in combination therapy *in vitro* against the *Pseudomonas aeruginosa* bacterial pathogen. The main aim was to identify effective phage-antibiotic combinations using various commonly used clinical antibiotics and LPS and pilus-targeting phages that have been shown to be effective against *P. aeruginosa* previously. Results show that certain combinations of antibiotics and bacteriophages are more efficient at suppressing bacterial growth than the others. Specifically, it was found that antibiotic combinations retain their synergies, antagonistic or not, even when phages are added. Furthermore, we show that two bacteriophages alongside one antibiotic is likely to be the best combination for designing efficient phage-antibiotic therapy cocktails. Interestingly, antibiotic-phage synergies were different when applied against clinical *P. aeruginosa* strains. Together, these results suggest further research on combination therapies aimed to suppress bacterial growth is needed, and specifically, combinations therapies need to be tested not only against laboratory but also against clinical strains to obtain effective outcomes.

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4. Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Edouard Depret.

5.Introduction

Antibiotic resistance is on the rise in the modern world (Fair *et al* , 2014) and this has a major burden on society. Multidrug resistant (MDR) bacterial infections are predicted to cause ten million deaths per year by 2050 (O'Neill, 2014) and pharmaceutical companies have dropped out from the antibiotic market due to the lack of return on investment, primarily due to the rise of antibiotic resistance in bacteria. Alternatives to antibiotic treatments exist including pathogen-specific, vaccines and prevention techniques, antisense therapies (Penn State, 2018), which is a type of antibiotic therapy targeting specific sequences in the bacterium's mRNA, and recently, many new techniques to reduce virulence factors, such as quorum sensing quenching (Utari *et al* , 2018), which aims at manipulating sensorial molecules to disrupt bacterial quorum sensing. These techniques and treatments can also be used in combinations with already existing therapies and antibiotics. This in turn, would help to make the most of the antibiotics we currently have available to treat patients while on-going research is discovering novel and alternative ways to treat infections.

5.1 *Pseudomonas aeruginosa* pathogenesis

Pseudomonas aeruginosa is an opportunistic, biofilm forming, Gram-negative bacterium that typically inhabits soils and aqueous environments. It has attracted a lot of interest due to its high intrinsic resistance and how quickly it can evolve resistance to antimicrobials and is in fact on the WHO priority 1 pathogens list for R&D of new antibiotics (World Health Organisation, 2017). This pathogen is a growing issue in the clinic as it is estimated 10% of all nosocomial infections arise from *P. aeruginosa*, as well as being a major issue for immunocompromised individuals and lung-defect patients such as cystic fibrosis (CF) patients. It has a large genome (5.5-7 Mbp) (Pang *et al*, 2019) compared to other common bacteria (for reference, *E.coli* has a genome of 4.6 Mbp) and most of the genome are regulatory enzymes which allows the bacteria to rapidly and efficiently adapt to environmental changes (Klockgether *et al*, 2011). This bacterium most commonly causes respiratory infections, skin infections, urinary tract infections, sepsis and bacteraemia, and otitis. Most importantly, since *P. aeruginosa* is an opportunistic pathogen, patients suffering from auto-immune diseases such as cystic fibrosis, or patients with burned skins, are the most at risk from this pathogen.

There are several virulence factors in *P. aeruginosa*, but the most notable ones, and the ones that have been considered as viable, important targets for therapy, are the type III secretion (TTS) system, the lipopolysaccharide (LPS), the quorum-sensing (QS), and the type IV pili of the bacterium (Hauser, 2011). Those virulence factors underline how complex *P. aeruginosa* is as a pathogen. The QS signalling leads to two of *P. aeruginosa*'s toxins which are pyocyanin and elastase (Le Berre *et al*, 2008), the pyocyanin being an important factor biofilm formation (Das *et al*, 2016) and elastase causing important tissue damage *in vivo* (Kamath, 1998). The TTS, being a secretory system, allows for the injection of the ExoU and ExoS toxins directly into the target cells (Shaver & Hauser, 2004) causing a disruption of the cell's cytoskeleton (Soong *et al*, 2008). The lipopolysaccharide, just like many Gram-negative bacterium, is a virulence factor due to the O-antigen, present on the outer surface of the bacterium. Another virulence factor from this pathogen is the type 4 pili (Winstanley *et al,* 2016) which is linked to motility and sensing of the surfaces it meets. Lastly, *P.* aeruginosa being a biofilm-forming pathogen, it can hide some virulence factors when switching to a more sessile lifestyle rather than a motile lifestyle (Moradali *et al*, 2017). Due to the high number of serotypes for *P.* aeruginosa, each of those virulence factors have varying expressions and effects which underlines and confirms how difficult *P. aeruginosa* is to eradicate in patients, as well as how damaging it can be, both to healthy individuals and patients suffering from cystic fibrosis or any other underlying lung defects.

In the clinic, this pathogen can be presented in two forms, chronic and intermittent. Chronic infections typically are infections which can evade the immune system and are a lot more challenging to treat as they typically get resistant to antibiotic courses in a short amount of time (Grant & Hung, 2013). Meanwhile intermittent strains of the bacterium typically are acute infections, also known as one-time infections. This is of dire importance in the context of cystic fibrosis (CF) patients, where CF patients go through intermittent phases of bacterial colonization until the infection and colonization of the airways becomes chronic, despite the best efforts in the clinic to use intensive antibiotic treatments (Johansen & Høiby, 1992). CF patients being a lot more susceptible to airways infections, due to their mutation in a regulatory gene, which interferes with the correct mechanism of cyclic AMP-regulated chloride ion channel (Folkesson *et al*, 2012). The treatments to deal with bacterial infections, and most specifically, *P, aeruginosa* infections, is a lot more intensive and aggressive as the end goal is preventing the long-term colonization of the airways, which is a challenge in the clinic since the large genome of this bacterium encodes for a wide range of antibiotic resistance genes.

5.2 Antibiotic resistance mechanisms in *Pseudomonas aeruginosa*

The most notable antibiotic resistance mechanism that *P. aeruginosa* has are its efflux pumps. Four multidrug efflux pumps systems have been well characterized and identified as MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexX-MexY-OprM systems (Aeschlimann, 2003). Off those four efflux pumps, the MexAB-OprM is considered to be the most important pump as it is constitutively expressed and is the main pump providing intrinsic resistance to multiple antibiotics (Rampioni *et al*, 2017). The second system, the MexXY-OprM is another efflux pump that is a major component of antibiotic resistance, and specifically effective against aminoglycosides, such as gentamicin (Morita, Y *et al*, 2012). Those two pumps alone can explain the resistance of *P. aeruginosa* to many antibiotics, for example aminoglycosides triggers overexpression of the MexXY gene, which in turn provokes the overexpression of the MexXY system which leads to decreased sensitivity to aminoglycosides and tetracyclines (Guénard *et al*, 2014). Another such example of an antibiotic that is resisted by those efflux pumps is chloramphenicol, a 50S subunit inhibitor, for which the bacterium is intrinsically resistant thanks to the MexAB efflux system. Interestingly, sub-inhibitory concentration of chloramphenicol will also trigger the expression of the MexXY system (Morita *et al*, 2014), which further helps *P. aeruginosa* resist chloramphenicol. Furthermore, those efflux pumps are found to be the first point of resistance mechanism when developing new antibiotics, for example the trimethoprim (TMP) - sulfamethoxazole (SMZ) combination. Trimethoprim (TMP) is a folic acid inhibitor which is more efficient when used alongside sulphonamides, and the most effective and the currently most used sulphonamide in the clinic is sulfamethoxazole (SMZ). This TMP-SMZ combination was used for only 4 years before resistant genes have been discovered, and in the case of *P. aeruginosa*, a single efflux pump was discovered to be responsible, the MexAB efflux system that provided intrinsic resistance to the bacterium (Huovinen, 2001). The importance of those efflux pumps lies in their porins, which is where some antibiotics will bind. For example, OprD is a porin which is the binding site for most carbapenems (Li *et al*, 2012). This binding site being next, or sometimes coupled, with the efflux pumps means that a lot of antibiotics will simply be pumped out without reaching its target. However, efflux pumps are not the only defence tool this bacterium has.

The main mechanism of defence against fluoroquinolones, such as ciprofloxacin is its ability to mutate and make missenses on the gyrase *gyrA* and/or *gyrB* and on the topoisomerase IV *parC* and *parE* which leads to the antibiotic not binding to its target anymore (López-Causapé *et al*, 2018). *Pseudomonas aeruginosa* is also intrinsically equipped with AmpC, a β-lactamase induced by any β-lactam present in the cell, that provides the bacterium with a mean of resistance against a wide range of penicillin antibiotics (Berrazeg *et al*, 2015), such as ampicillin. However, while this bacterium is intrinsically

ampicillin-resistant, it was hypothesised that ampicillin could still be used in combination with other antimicrobials due to ampicillin disrupting and damaging the cell wall (Alam *et al*, 2019). Membrane permeability is also a major factor in antibiotic resistance. Most of the antibiotics that are commonly used need to penetrate the outer membrane (Lambert, 2002), and *P. aeruginosa* being a Gram-negative bacterium, means that it has very low permeability barriers (Zgurskaya,2015).

All in all, this makes *Pseudomonas aeruginosa* one of the most difficult extracellular bacteria to remove in the clinic and one of the major reasons why it appears on the WHO health issue list.

5.3 Antimicrobial therapies against *Pseudomonas aeruginosa*

Typically, patients suffering from a *P. aeruginosa* go through intensive antibiotic treatments to clear the infection, such as treatments with aminoglycosides. Recently, more and more of those therapies implies some sort of combinations with multiple antibiotics, and the most effective one is a combination of aminoglycoside and beta-lactam antibiotics, for example gentamicin and cephalosporin. This approach of combining antibiotics serves a purpose of increasing the longevity and efficacy of the antibiotics we currently have. Studies of those combinations led to the discovery that some antibiotics work well with each other (Collateral sensitivity) or sometimes, have antagonistic effects with each other (Collateral resistance). These correlative effects have been visualised using network maps and one such example has been made by Pál in 2015 ((Pál *et al*, 2015) and is shown below in Figure.1.

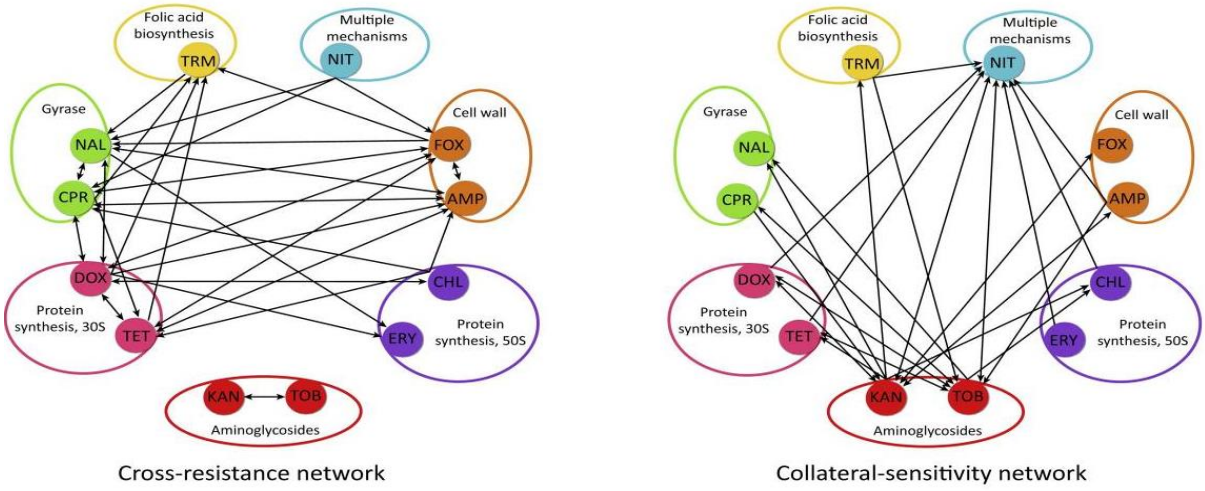


Fig.1 Network of cross-resistance interactions (left) and collateral-sensitivity (right) between different antibiotics. Antibiotics are grouped according to their mode of action. On the left, an arrow from antibiotic A to antibiotic B indicates that adaptation to A decreased sensitivity to B in at least 50% of the evolved populations. On the right, an arrow from antibiotic A to antibiotic B indicates that adaptation to A increased sensitivity to B in at least 50% of the evolved population (Pál *et al*, 2015). Abbreviations in those figures stand for names of antibiotics: TRM = Trimethoprim, NIT = Nitrofurantoin, FOX = Cefoxitin, AMP = Ampicillin, CHL = Chloramphenicol, ERY = Erythromycin, TOB = Tobramycin, KAN = Kanamycin, TET = Tetracyclin, DOX = Doxycycline, CPR = Ciprofloxacin, NAL = Quinolone

However, since CF patients are a lot more susceptible to airways infections, they will need a more intensive and aggressive form of antibiotic treatments, but those intensive treatments very commonly lead to antibiotic resistance. This leads back to how efficient *P. aeruginosa* is at evolving resistance to antibiotics. Not only do antibiotics have to be used in a synergistic context, as shown above, the

environment and the serotype of the invasive *P. aeruginosa* affects how it is treated. Firstly, the environment in the CF lung is an antimicrobial of its own. Due to how CF lungs operate with their deficient channels, bacteria colonizing the airways are under oxidative, nitrosative and cell envelope stress, because there is little to no clearance of debris, inhaled micro-organism (Folkesson *et al*, 2012). Ultimately, this lack of clearance leads to upregulated recruitment of leukocytes and antibodies. Those cells in turn provoke an oxidative and nitrosative stress as it is part of the inflammatory response they will cause. For the bacteria, this means that colonizing the CF airways will be challenging as there will be reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) (Folkesson *et al*, 2012). Normally, the efflux pumps of the bacteria are enough to get rid of those molecules, however, when it becomes too much, it leads to selection for the bacteria that can grow under those conditions, due to DNA mutations, ultimately leading to very resistant forms of *P. aeruginosa* which would normally not arise in non-CF patients.

An alternative to using antibiotics in combinations or in cycles is to combine them with other type of antimicrobials. For example, bacteria-specific parasites and bacteriophages are still used in Georgia, Russia, and in the Eastern world to treat bacterial infections. This form of therapy has gained renewed interest over the last decade as a new means to control and contain bacterial infection in the Western world. Many clinical fields are using bacteriophages, ranging from treating addiction to drugs with viruses' therapies (Carrera *et al*, 2004) to CRISPR/CAS9 bioengineering to create nano-carriers capable of delivering vectors to the selected host (DePorter *et al*, 2014). Since bacteriophages are so specialized to their host cells, this would have beneficial effects on the surrounding micro-environment if it was to be used in clinical settings, because antibiotics causes collateral damage to commensal and mutualistic bacteria, but phages being specific to their target, they do not cause the collateral damage antibiotics do. There have already been several clinical trials with bacteriophages as a way to treat bacterial infections (Wright *et al*, 2009) and there are also bacteriophage therapies in clinical trials. Authors of clinical trials involving bacteriophages (Wright *et al*, 2009; Sivera *et al*, 2006) reported successful therapies using bacteriophages on MDR *P. aeruginosa* on both patients with burn wounds prior to a skin graft and to MDR *P. aeruginosa* in cases of bacterial otitis. However, just like in the case of antibiotics, *P. aeruginosa* can evolve resistance to bacteriophages. Consequently, phage cocktails have been developed to improve and extend the longevity of phages in a clinical setting (Goodridge, 2010), similar to increasing the efficacy of antibiotics in combinations (Forti *et al*, 2018). Cross-resistance studies have also been conducted, where two bacteriophages in combination from two modules provides improved efficiency. This was because phages from the same module target the same receptor (either LPS or type IV pilus, Figure.3), therefore having phages from different modules leads to targeting different receptors and this has an improved effect on controlling the pathogen. As a result, it was shown that combining both module 1 and module 2 bacteriophages had the biggest impact on the growth of *P. aeruginosa* likely due to constrained resistance evolution (Wright *et al*, 2018). Phage combinations work thus better if the aim of the therapy is to target different receptors at the same time, so that if the bacterium evolves resistance to one phage, it doesn't lead to cross-resistance due to the high cost of adaptation, as phage-resistance mutations are costly when counted in terms of pathogen growth and competitive colonizing between the evolved strain versus the ancestral strain (Wang *et al*, 2019).

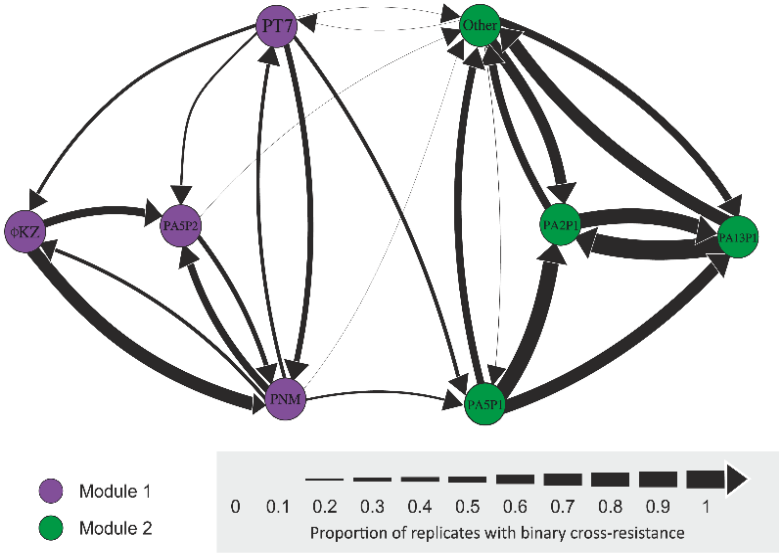


Fig.2 Network of cross-resistance. The arrow represents the proportion of mutants selected against the origin bacteriophage that have resistance to the target bacteriophage. Module 1 bacteriophages affect the Type IV pilus while module 2 bacteriophages affect the LPS. (Wright *et al*, 2018)

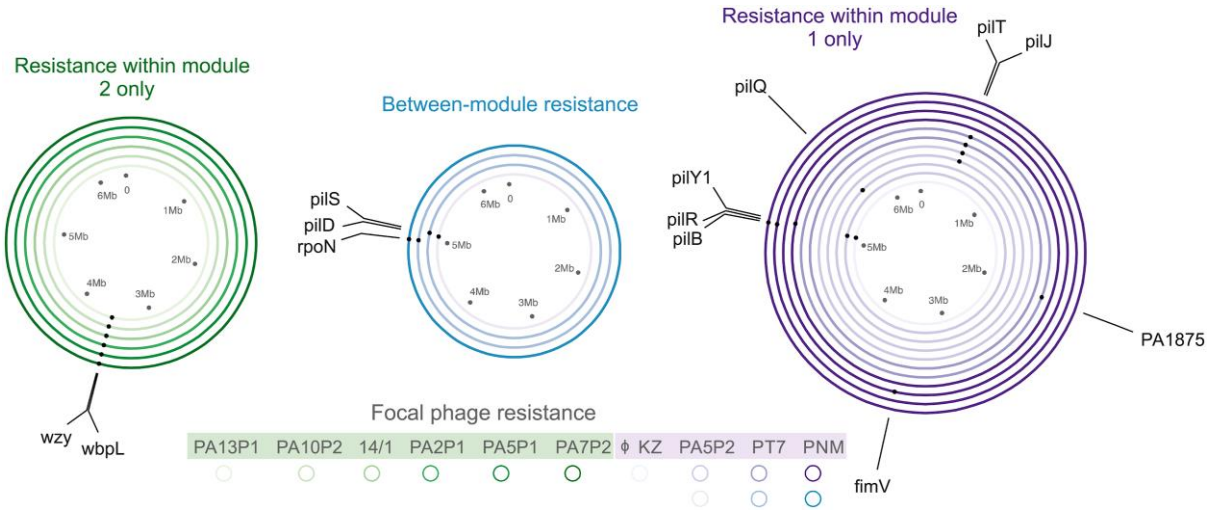


Fig 3. Genetic basis of phage resistance. Circles represent different phage-resistant mutants selected against different focal phages (indicated by the colour shade; see key), and dots on each circle show the position of mutated genes. Colour represents the cross-resistance profile of each sequenced resistant mutant: resistance within module 1 (purple), within module 2 (green), and between modules (generalist resistance, blue). (Wright *et al*, 2018)

Lastly, instead of replacing old antibiotics with new ones, it has been proposed that bacteriophages could be used alongside antibiotics as combinations. The very first proposition to using bacteriophages to deal with bacterial infections goes all the way back to Felix D'Herelle (Chanishvili, 2012). Since then, phage therapies have raised interest as an alternative way to combat bacterial infections, where phage cocktails were prepared and used, and in several cases with some success in the treatment of drug-resistant *Staphylococcus*, *Pseudomonas, Klebsiella*, and *E. coli (*Weber-Dabrowska *et al*, 2000; Carlton, 1999). As a result, combinations of phages and antibiotics have been investigated as another viable way to deal with bacterial infections (Kim *et al*, 2018; Chaudhry *et al*, 2017). Current research

is turning toward those phage-antibiotic synergies and how to best maximize their effect, calling this the phage-antibiotic synergy (PAS). The effect of phage-antibiotic synergies (PAS) effect is sensitive to the specific combinations of antibiotics and phages. Research which targeted phage-antibiotic synergies found that the concentration of antibiotics required to kill the bacterium is lower when used alongside bacteriophages rather than used on its own, but also that this also works when the bacterium is in biofilms (Chaudhry *et al*, 2017). An experiment from Chan in 2016 (Chan *et al*, 2016) further showed that phage attacks, and in this one example, the OMKO1 phage, changes the efflux pumps mechanisms, which then in turns decreases antibiotic resistance. For example, *P. aeruginosa* strains PAPS has its ciprofloxacin sensitivity increased 12 folds (Chan *et al*, 2016). This shows that using phages and antibiotics in synergy is an efficient way to control the evolution of antibiotic resistance in the bacterium. Phage-antibiotic synergy is originally described by using one antibiotic and one phage (Comeau *et al*, 2007), however here I will mention the effect of phage on several antibiotics at the same time.

5.4 Aims and hypothesis

This MSc thesis tests PAS synergies *in vitro* using multiple phages and antibiotics in an attempt to identify potentially useful combinations in the clinical context. Of particular interest, there are four main questions that will be addressed in this thesis.

Question 1: Does pathogen suppression increase by using antibiotics and phages together as a combination?

This hypothesis will be tested by using a range of clinically relevant antibiotics and phages that have different methods of actions, namely trimethoprim, ampicillin, gentamicin, sulfamethoxazole, ciprofloxacin and chloramphenicol for the antibiotics, PA10P2, 14/1 and ΦKZ for the bacteriophages. Each of those antibiotics target a different receptor and have various effects. Trimethoprim prevents the synthesis of folic acid by blocking the reduction of dihydrofolate to tetrahydrofolate (Gleckman *et al*,1981). Ampicillin is an irreversible inhibitor of the enzyme transpeptidase, which is an enzyme required for the synthesis of the cell wall. Gentamicin binds to the 30S subunit of the bacterial ribosome, which affects the protein synthesis of the bacterium. Sulfamethoxazole is an analogue of para-aminobenzoic acid and binds to dihydropteroate synthetase, the enzyme responsible for the conversion of dihydrofolate to tetrahydrofolate (Hong *et al*, 1995). Ciprofloxacin inhibits DNA gyrase, most specifically the topo-isomerase II and topo-isomerase IV (LeBel, 1988). Chloramphenicol binds to the 50S subunit, which affects the integration of tRNA in the protein synthesis mechanism (Inkling, Infectious Diseases by Jonathan Cohen). For the phages, PA10P2, 14/1, and ΦKZ were selected. Module 1 bacteriophages affects the Pil gene family (E.g ΦKZ affects *pilR*, while other phages such as PT7 affects *pilS, pilT* and *pilJ*) while module 2 bacteriophage affect the LPS formation. It would be expected that using antibiotics affecting different pathways, alongside using bacteria from different modules would give a higher reduction in bacterial densities. Many resistance mechanisms are transcriptional, where, as an example, as described in Chan's paper, 2016 (Chan *et al*, 2016), bacteriophages can disturb the efflux pump upregulation, which in turns allow the antibiotic to enter the cell to act upon it.

Question 2 Identifying specific antibiotics that work efficiently alongside certain bacteriophages, and vice-versa.

Two, three- and four-way combinations will be performed as part of this thesis to identify various combinations of antibiotics and bacteriophages that had synergistic, antagonistic or no effects with each other. Each of the antimicrobial used will be combined with one or more other antimicrobials and then applied on *P. aeruginosa* to identify which combination worked best to reduce and restrict bacterial growth. It was hypothesised that the more antimicrobials are used, the less bacteria would grow resistance to it, as it is a massive burden to evolve resistance to many antimicrobials at once. In light of the recent studies, it would be expected that antibiotics used in combination with bacteriophages would have a greater effect than antibiotics used together or phages used together, but some conflicts and loss of synergy will be observed due to the already known synergy and counter-synergy of the antibiotics and phages.

Question 3: Determining if efficient phage-antibiotic combinations also suppress clinical *P. aeruginosa* strains

After the previous test is performed, a subset of identified combinations that had either a synergistic, antagonistic or no effect will be tested on clinically relevant strains of *P. aeruginosa* in an attempt to translate the findings observed with the PAO1 lab-strain to clinical strains originating from CF lungs. It was hypothesised that chronic strains are usually more antibiotic resistant due to the permanent colonisation of the airways they cause, and as such, successful combinations might be less effective on those strains. Meanwhile, intermittent strains are likely less exposed to antibiotics and as such, combinations might have more of an effect on those particular strains.

6.Materials and methods

6.1 Bacterial strains, antibiotics, phage strains and media

Laboratory strains used in this study were derived from Pseudomonas *aeruginosa* PAO1 type strain (ATCC15692). The antibiotics were diluted from a powder form to a concentration of 10 000 µg/ml. Different antibiotics were dissolved in different solvents as follows: gentamicin was diluted with pure water, ciprofloxacin was diluted with 0.1 M HCl, chloramphenicol with 70% ethanol, ampicillin with pure water, trimethoprim with 0.1 M HCl, and sulfamethoxazole with water. In the case of the first experiment, where I investigated the effect of solvents on *P. aeruginosa* growth, the same solvent concentrations were used but no antibiotics were used. These final prepared antibiotic solutions were then used as stocks and diluted down to the relevant concentration during the experiment, typically ranging from 10 000 µg/ml to 0.1 µg/ml. The antibiotics were stored at + 4°C.

All experiments were conducted in LB medium [tryptone (10 g/l), yeast extract (5 g/l), and NaCl (5 g/l)] which was prepared from a powder stock (Sigma L3022-1KG) suspended in a total of 1 litre of deionized water and autoclaved at 121°C then stored at room temperature. The phage strains used were pseudomonas phage 14/1, ΦKZ, and PA5P10 bacteriophages (Wright et al. 2018). These were stored at + 4°C. Chronic and intermittent bacteria strains of *P. aeruginosa* were obtained from the Copenhagen CF Center Rigshospitalet (Fig S.1) and were categorized into two categories, the intermittent and chronic strains. Soft agar was prepared by using a 1:2 w:v ratio of agar:LB, suspended in a total of 1 litre with deionized water and autoclaved at 121°C and stored at room temperature. LB - agar was prepared using a formula from Formedium (Ref No: LMM0204) by mixing 40 g of LB - agar powder mix in 1 L of distilled water and autoclaved at 121°C and stored at room temperature.

6.2 Determining antibiotic resistance by measuring minimum inhibitory concentrations (MIC)

The MIC of the bacteria was measured using 96 well plates, liquid LB media mixed with antibiotics. 10 µl of bacteria were added to 100 µl of LB media in 96well plates. The concentration of antibiotics across the plate ranged from 10 000 µg/ml to 0.1 µg/ml using serial dilutions (0.15, 0.3, 0.6, 1.22, 2.44, 4.8, 9.7, 19, 39, 78, 156, 312, 625, 1250, 2500, 5000, 10 000 µg/ml) After a 24 h incubation at 37°C, bacterial densities were measured as optical density at 600 nm wavelength using a Tecan Infinite M200 pro plate reader.

6.3 Cryopreservation of bacterial samples

For the preservation of the strains, a solution of 1:1 v:v of glycerol and water was prepared to make a final solution of 50% glycerol and autoclaved. Then, 600 µl of the strains are mixed with 400 µl of glycerol to obtain 20% of final concentration of glycerol before preserving in the -80°C freezer.

6.4 Phage culturing and isolation from bacteria

Phages were grown alongside bacteria in LB in 50 ml tubes for 48 h at 37°C (150 rpm shaking). Each tube was then divided in 1.5 ml eppendorf tubes, each with 100 µl of chloroform and 900 µl of phage + bacteria mix to kill the bacteria after vortexing and centrifuging at 17 G for 5 minutes. Phages were then filtered through a 0.2 µm pore size filter into new eppendorf tubes to get rid of the chloroform and stored at + 4°C

6.5 Phage density measurements

Phage densities were measured as plaque forming units (PFU) per mL on *P. aeruginosa* soft agar overlays as follows (Kaufmann, S.H. 2002). Phages were serial diluted from stock (10^0 cells per mL) 8 times (to 10^{-7} cells per mL) in eppendorf tubes using a 1 in 10 dilution each time. 400 µl of *Pseudomonas aeruginosa* was mixed with 40 ml of soft agar, mixed using a vortex and poured on top of agar in a petri dish, as an overlay. Each petri dish was divided in 8 section, one for each dilution. 10 µl of phage were spotted in each section. After a 24 h incubation at 37°C, plaques were counted to calculate a PFU.

The PFU's have been determined from the experiment to be:

PA10P2	$1.2x10^7$ PFU/ml
14/1	$5.7x10^{10}$ PFU/ml
ΦKZ	$8.5x10^{10}$ PFU/ml

As a result of this preliminary experiment, the phages were diluted down to $5x10^6$ PFU/ml before all the experiments.

6.6 Bacterial density measurements

Bacterial densities were measured as colony forming units (CFU) per mL on an agar dish as follows (Goldman, 2008): Bacteria were diluted to 0.5 OD (OD600) after a 24 h incubation at 37°C, then serial diluted 1 in 10 to make 8 different dilutions. Then, on a LB-agar plate, 20 µl of bacteria and 10 plastic beads were inoculated in the petri dish and spread evenly on plates by shaking. After removing the beads, plates were incubated at 37°C for 24 h. Based on these results, bacterial CFU's were adjusted to $6x10^8$ CFU per mL for all initial starting cultures.

6.7 Phage-antibiotic synergy method

Phages and antibiotics are used in combinations to test their efficacy in terms of reduced bacterial growth. To perform this test selected antibiotics and bacteriophages were first made up to the required concentration based on the minimum inhibitory concentration in case of antibiotics (3.2) and

standardised phage densities based on the plaque forming unit experiments (3.5). Combinations were created by combining a maximum of two antibiotics and two bacteriophages simultaneously. Therefore, the two-way combination treatments contained either two antibiotics, one antibiotic and one phage, or two phages. The three-way combination treatments contained either two antibiotics and a phage or one antibiotic and two phages. The four-way combination treatments always contained two antibiotics and two phages. Experiments were conducted in 96 well plates and each well contained 10 µl of bacterial stock cultures, 10 µl of phages stock cultures or 10 µl or a mixture of both and 180 µl of LB. More specifically, in mono-treatments, each well contained 10 µl of bacterial stock culture, 10 µl of phage culture or 10 µl of LB if it is a mono-treatment without phages, 90 µl of antibiotic (or 90 µl of LB if it is a mono-treatment without antibiotics), and 90 µl of LB. Two-way treatments with only antibiotics included 90 µl of antibiotic A + 90 µl of antibiotic B alongside the 10 µl of bacterial stock, two-way with antibiotic and phage included 90 µl of antibiotic A + 90 µl of LB + 10 µl of phage alongside the 10 µl of bacterial stock, two-way phages only included 180 µl of LB + 5 µl of phage 1 + 5 µl of phage 2 alongside the 10 µl of bacterial stock. Three-way with two antibiotics and one phage included 90 µl of antibiotic A + 90 µl of antibiotic B + 10 µL of phage + 10 µl of bacterial stock, while three-way with one antibiotic and two phages included 90 µl of antibiotic + 90 µl of LB + 5 µl of phage 1 + 5 µl of phage 2 + 10 µl of bacterial stocks. Four-way were 90 µl of antibiotic A + 90 µl of antibiotic B + 5 µl of phage 1 + 5 µl of phage 2 + 10 µl of bacterial stock. Bacterial densities were measured as optical density at 600 nm wavelength at 24 h, 48 h and 72 h timepoints with a Tecan Infinite M200 pro plate reader.

6.8 Statistical analysis

Numerical data obtained from the first solvent experiment, section 4.1, were analysed by a two-way factorial ANOVA alongside a Dunnett's multiple comparison, and the numerical data obtained by most of the PAS experiments, section 4.3 and 4.4, were analysed by a two-way factorial ANOVA, where bacterial density is used as a dependant variable, and the variations were explained with time and different combination treatments as the independent variables. When analysing the effects of solvents or the effects of different phage-antibiotic treatments on bacterial density, we explained the variation in bacterial growth by solvent treatment, number of antimicrobials included within treatment combinations and antibiotic-phage treatment identities. Differences in broad patterns were analysed first after multiple simpler models were constructed to analyse antibiotic-phage combinations in more detail.

The observed vs predicted graphs were performed to see if antimicrobial combinations can predict higher order interactions, as it will indicate if the effects between different antimicrobials is additive, multiplicative, and underlines possible synergies and anti-synergy. The observed vs predicted graphs were performed using the following method:

- The observed value was calculated as the difference between the control OD and the OD reading following the treatment during the experiment.

The effect of antibiotics and phages on bacterium was estimated as a reduction in growth by antimicrobial treatments relative to no-treatment control where it is bacterial optical density alone minus bacterial optical density in the presence of treatment containing antimicrobials.

To predict the combined effects of phages and antibiotics, we used a summative model where the individual effects of mono antibiotic or phage treatments were added up and divided by the total number of individual treatments.

For example, the predicted value for Phage1 - Phage2 - AB1 combination treatment was counted as (Phage1 mono + Phage2 mono + AB1 mono)/3.

A diagonal line was drawn on those graphs to show a 1:1 ratio between the observed vs predicted effect, where values above the line means the combination performs better than predicted (synergy, multiplicative effect), and values below the line perform worse than predicted (no synergy). Values directly on the line implies no synergy, and it is simply the additive effect of the antibiotic on their own that is at work.

All statistical analysis was performed using GraphPad Prism 6.

7. Results

7.1 Preliminary experiment 1: Solvent assay

As not all antibiotics could be dissolved in water, the first experiment was performed to determine if the used solvents (Water, 0.1 M HCl, and 70% ethanol) had any effect on bacterial growth in the absence of antibiotics. The results from this test show that there are no statistical differences between the control and the other solvents (Kruskall-Wallis, P = 0.0797), which means that solvents alone had no effect on bacterial growth and all effects were mediated by antibiotics and phages.

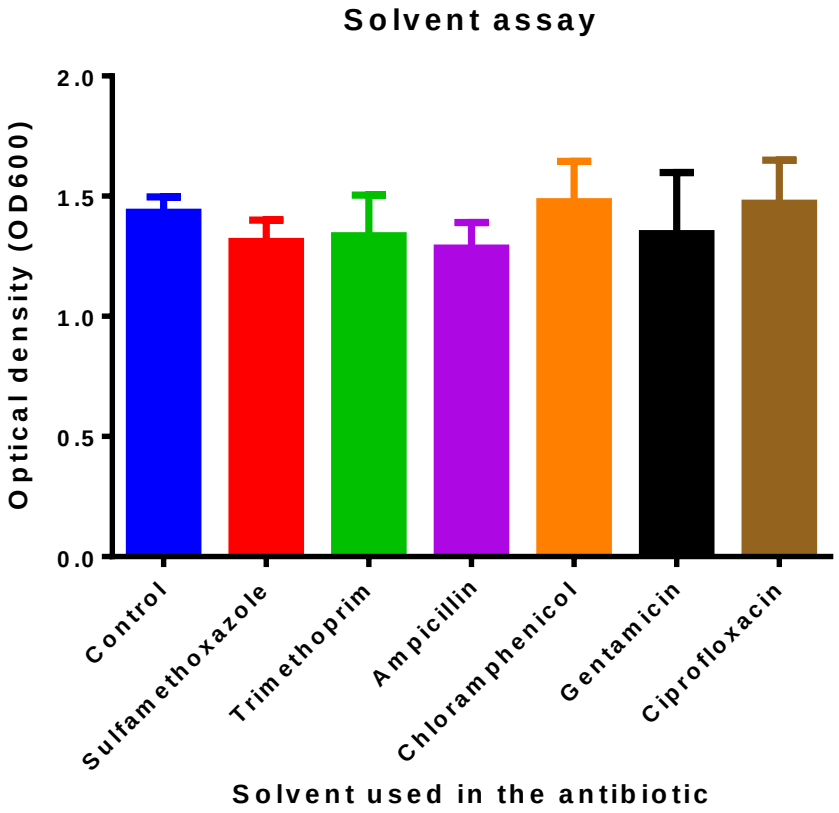


Fig.4 Solvent assay. Bar graph of *P. aeruginosa* growing in different solvents used in each of the antibiotics alone for a duration of 24 h at 37°C. In each treatment, N = 8. The dilutions were made similar to the following experiment, Ampicillin: 1180 µg/ml, Gentamicin: 10 µg/ml, Sulfamethoxazole: 2000 µg/ml, Ciprofloxacin: 0.1 µg/ml, Chloramphenicol: 50 µg/ml, Trimethoprim: 275 µg/ml, and the solvent used in each antibiotic is Sulfamethoxazole: water, Trimethoprim: 0.1 M HCl, Ampicillin: water, Chloramphenicol: 70% Ethanol, Gentamicin: Water, Ciprofloxacin: 0.1 M HCl.

7.2.Preliminary experiment 2: determining Minimum Inhibitory Concentration (MIC) of different antibiotics.

In order to determine sub-inhibitory concentrations of antibiotics that would not kill all the bacteria, minimum inhibitory concentrations of different antibiotics were measured in short-term growth assays. Antibiotic concentrations ranged from 10 000 µg/ml to 0.01 µg/ml for all six different antibiotics: trimethoprim, sulfamethoxazole, gentamicin, ampicillin, chloramphenicol, and ciprofloxacin. While the MIC test serves to find the MIC, in this particular case it was more important to identify a suitable sub-lethal MIC for the purpose of the following experiments. As such, the sub-lethal MIC, and the concentrations that are going to be used in future experiments, were selected as approximately half-MIC, and chosen to be:

Ampicillin	1180 µg/ml
Gentamicin	10 µg/ml
Sulfamethoxazole	2000 µg/ml
Ciprofloxacin	0.1 µg/ml
Chloramphenicol	50 µg/ml
Trimethoprim	275 µg/ml

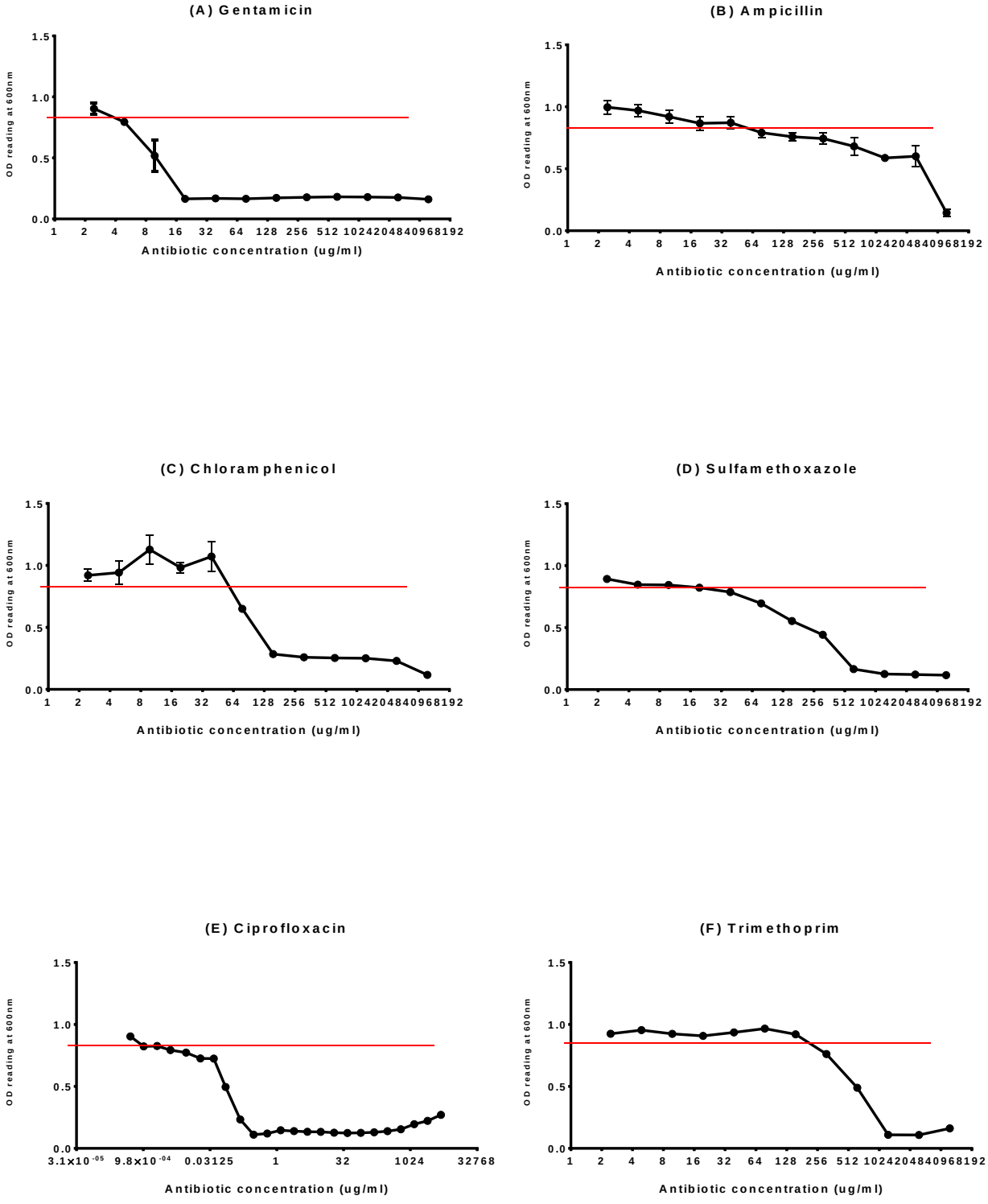


Fig.5 MIC experiments for 6 different antibiotics. Log 2 antilog graph of the MIC determination of antibiotics ranging from 10 000 µg/ml to 1 µg/ml, incubated for 24 h at 37°C. The red line serves as a visual guide, it represents the OD of the control *P. aeruginosa* growing without any antibiotics. In all tested concentrations, N = 8.

7.3 Experiment 1: Investigating phage and antibiotics synergies

Phage-antibiotic synergy is a term that defines the interaction between antibiotics and bacteriophages in restricting bacterial growth. These can vary from positive to neutral and negative. In this experiment, antibiotics and phages were mixed together in two, three or four-way treatments. The different treatments included in this experiment are:

2-way combinations: phage – phage; antibiotic - phage; antibiotic – antibiotic
3-way combinations: phage – phage – antibiotic; antibiotic – antibiotic – phage
4-way combinations: phage – phage – antibiotic – antibiotic

Data was visualised using box and whiskers box plots showing medians, quartiles, highest and lowest values for every combination in relationship to the other combinations of the same order (Fig.6 to Fig.14).

7.3.1 General overview of the data and the effect of time

The overview graph (Fig.6) shows the effect of time and number of antimicrobials included with each treatment combinations on PAO1 growth. The overall analysis from this overview result leads to the finding that bacterial growth varied between different antimicrobial combination treatments ($F_{3,1228}$ = 309.5, $p < 0.001$): highest growth was achieved in the absence of antimicrobials, second highest in mono treatments, third highest in two-way treatments and fourth highest in three-way and four-way treatments, which did not differ from each other. Second to this test, a test was performed to compare all the types of combinations, namely Control, Antibiotics, Phage, Ab + Ab, Ph + Ph, Ab + Ph, Ab + Ph + Ph, Ab + Ab + Ph, Ab + Ab + Ph + Ph. It was therefore found that all the different combinations are statistically different from each other ($F_{6,1225}$ = 219.3, $p < 0.001$) and that over time, the growth also increased (Time: $F_{2,1225}$ = 67.2 $p < 0.001$) and that the effect varied very slightly between the different combination treatments ($F_{12,1225}$ = 10.7 $p < 0.001$). In general, bacterial growth increased in time during this assay ($F_{2,1228}$ = 120.5, $p < 0.001$) and this effect varied only slightly between different combination treatments ($F_{2,1228}$ = 120.5, $p < 0.001$). The control shows that the bacterial growth dynamics in the absence of antibiotics peaked at 48 h but drops at 72 h time point. This suggests that bacterial cell death started to occur after 48 h of growth. However, for the rest of the combinations, mono, two-, three-, and four-way treatments, the pattern is that over time, bacterial growth dynamics tends to increase in density, showing a different dynamic than previously observed with the control group. While only a couple of data points are responsible for this increase over time, it could imply that some colonies within the replicates are managing to evolve some sort of resistance mechanisms. Therefore, it was found that, as a whole, datapoints are all statistically different ($F_{153,1079}$ = 60 $p < 0.001$).

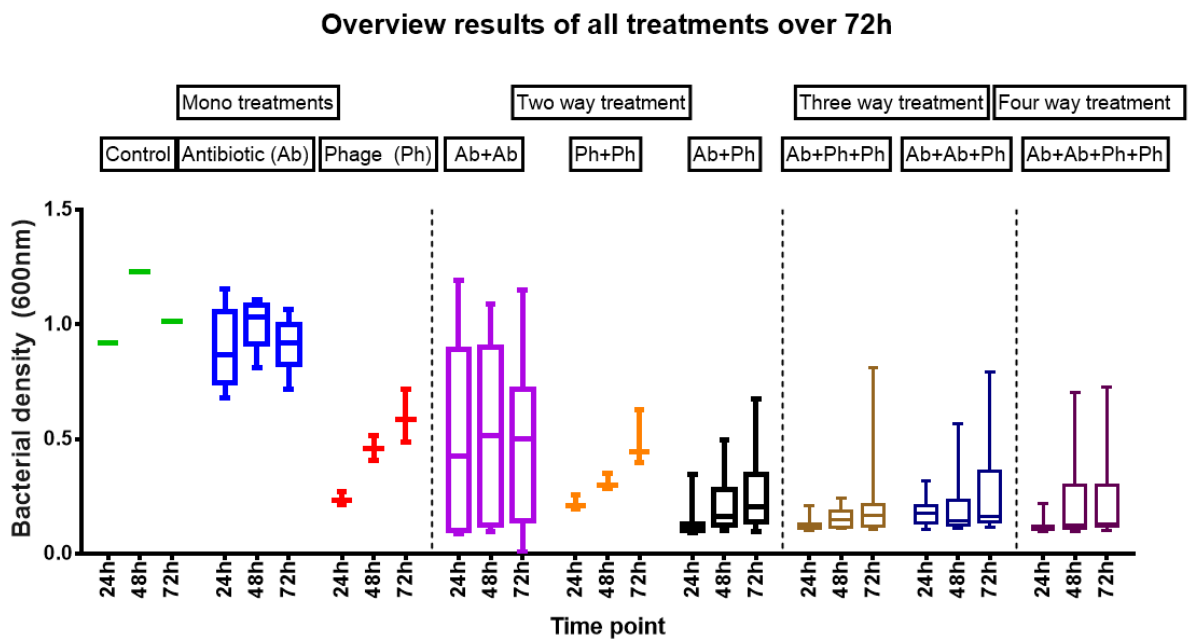


Fig.6: Overview of all the treatments after 72 h of growth. Box and whisker graph of every combination of treatments, mono, two, three and four way treatments at 3 time point intervals, 24 h, 48 h and 72 h. Ph = Phage, Ab = Antibiotic. The results of all treatments within a category has been added together and plotted in a box and whisker graph. For example, all two-way antibiotic + antibiotic have been added together in the same box. Control N = 3, Antibiotics N = 6, Phage N = 3, Antibiotic + Antibiotic N = 15, Phage + phage N = 3, Antibiotic + phage N = 18, Antibiotic + Phage + Phage N = 18, Antibiotic + Antibiotic + Phage N = 45,

7.3.2 Mono-treatment effects

Mono-treatment results are shown in Fig.7 using a box and whisker graph. The effects observed on the mono-treatments indicates that phages mono-treatments have a bigger impact at reducing bacterial growth than antibiotics do, when comparing antibiotics vs bacteriophages across all three time points ($_{F2,56}$ = 19.54 $p < 0.0001$). Phages as a mono-treatment have a very impactful effect at 24 h to reduce bacterial growth, but over time, the effect weakens, and bacterial density increases. The antibiotics on their own had a much more varied effect, ranging from no significant effects on the bacteria (for example trimethoprim) to some significant effects on the bacteria (for example sulfamethoxazole) ($F_{2,28} = 3.383$, $p < 0.05$). Of all the antibiotics, only three antibiotics were different than the control, and they are gentamicin (q = 2.763 DF = 210), and ampicillin at 48 h (q = 4.143 DF = 210), and gentamicin at 72 h (q = 2.951 DF = 210). Other antibiotics at other time points were not different to the control but all the phages were different to the control over all three time points. Time had a significant effect on those treatments, as a whole ($F_{2,56} = 19.54$, $p < 0.0001$), and this effect is even more noticeable when dealing with bacteriophages ($F_{2,42} = 45.74$, $p < 0.0001$). This shows a sharp decrease in bacterial density at 24 h, and a sharp increase followed at 48 h and 72 h. This effect is noticeable on the antibiotic treatments, though not to the extent similar to bacteriophages ($F_{2,84} = 3.651$ $p < 0.05$). The ΦKZ bacteriophage had the most significant effect amongst the three bacteriophages used, followed by the PA10P2 and then the 14/1 phage. For the antibiotics, ampicillin had the most significant effect amongst the 6 antibiotics used, surprisingly due to its intrinsic resistance by AmpC, followed by gentamicin, ciprofloxacin, trimethoprim, chloramphenicol, and sulfamethoxazole.

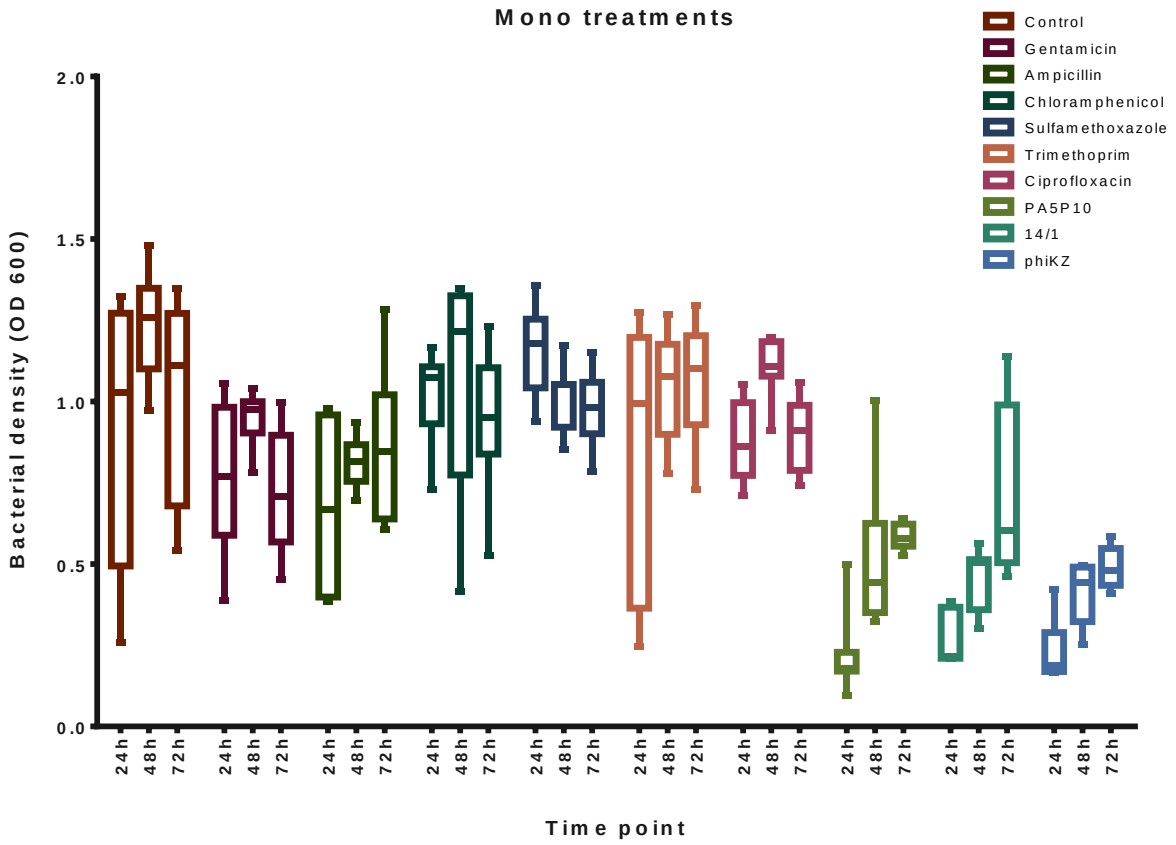


Fig.7 Reduction in PAO1 growth by mono antibiotic and phage treatments. Box and whisker graph of every combination of the mono-treatments used in the study at all 3 different time points, namely 24 h, 48 h and 72 h. GEN = Gentamicin, AMP = Ampicillin, CHL = Chloramphenicol, SFX = Sulfamethoxazole, TRI = Trimethoprim, CIP = Ciprofloxacin. In each case, N = 8

7.3.3 Two-way treatments effects:

The two-way treatments have been split into 3 figures, Fig.8, Fig.9 and Fig.10, for the clarity of the results. Fig.8 shows the antibiotic-antibiotic effect, Fig.9 the antibiotic-phage effect, and Fig.10 the phage-phage effect.

The antibiotic-antibiotic graph, Fig.8, serves mainly as a way to research and confirm a concept that has already been researched, as it depicts antibiotic synergies, and the results found here shows some collateral sensitivity as well as collateral resistance. As such, it is clear to see that combinations with ampicillin yield the strongest reduction in bacterial density, as on its own, ampicillin is not an efficient antibiotic (Fig.7) against *P. aeruginosa* but it has been shown several times that combinations with ampicillin yield good results in terms of bacterial density reduction, while combinations using sulfamethoxazole, at the exception of the trimethoprim – sulfamethoxazole combo, which is a currently used combo in the clinic, did not yield strong effects at reducing bacterial growth, as on its own, it was the least efficient antibiotic (Fig.7). The most efficient two-way antibiotic combination is the ampicillin – sulfamethoxazole combination (column statistics, sum = 0.3394), followed by the gentamicin – ampicillin combination (Column statistics, sum = 0.3486). This contrasts to the mono-treatments were ampicillin is also the most efficient antibiotic, followed shortly after by gentamicin, it is therefore little to no surprise that combining those two yields the highest effect as well. The least efficient combinations were chloramphenicol – sulfamethoxazole (column statistics, sum = 3.127), and chloramphenicol – ciprofloxacin (column statistics, sum = 2.683). Chloramphenicol and

sulfamethoxazole were the least efficient mono-treatment antibiotics, and therefore, the result of combining those two antibiotics can be predicted by those mono-effects alone. Trimethoprim was the third least efficient antibiotic and sulfamethoxazole the second least efficient antibiotic, and the combination of those two led to the second least efficient combination. There are massive variations in the combinations dependant on time alone ($F_{2,224} = 23.87$ $p < 0.0001$), the effect are either a massive initial drop in optical density, as it is the case in the trimethoprim – sulfamethoxazole combo, or the gentamicin - sulfamethoxazole combo, the effect can also be no visible effect at 24 h then followed by a drop in bacterial density at 48 h or 72 h, as it is the case in the gentamicin – ciprofloxacin combo, or the gentamicin – chloramphenicol combo. Therefore, as a whole, combinations work a lot better than on their own when a good combination is picked, as for example sulfamethoxazole and trimethoprim, on their own (Fig.7) yield the lowest growth reduction, but together, they are an efficient combination to use.

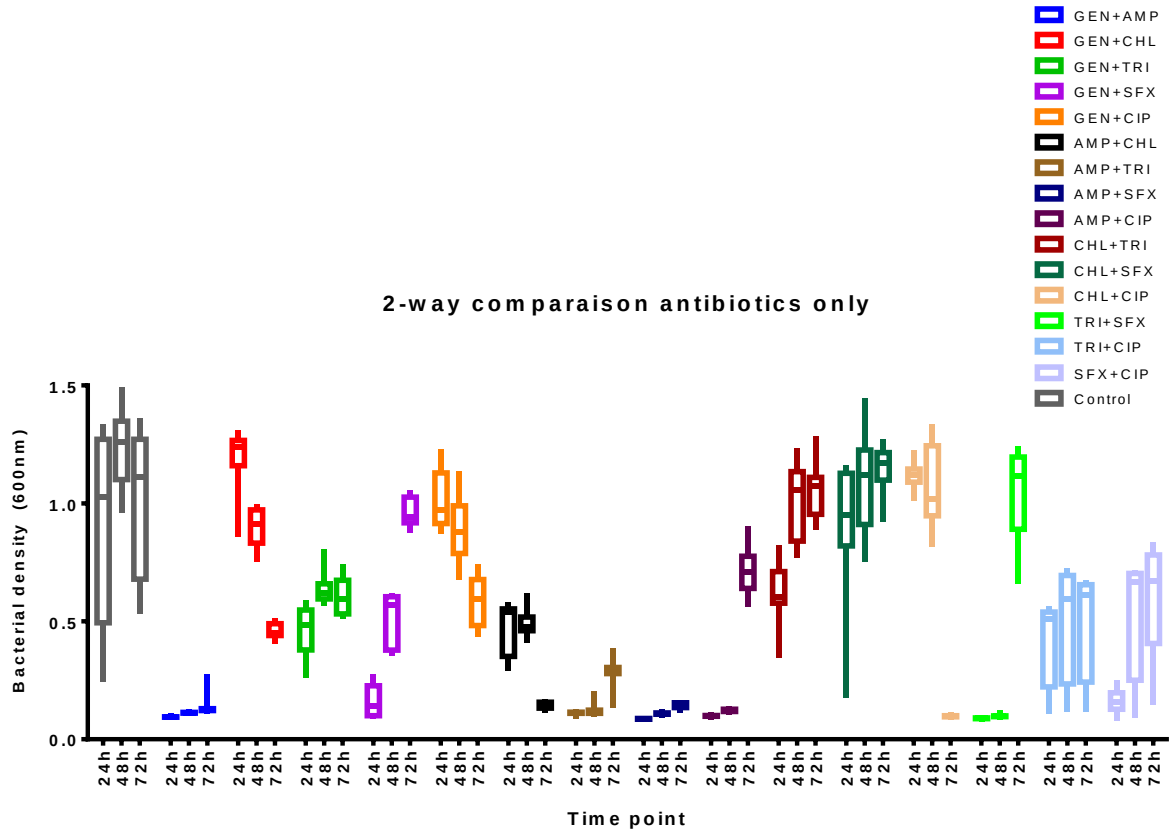


Fig.8: Reduction in growth by antibiotics in a two-way treatment
Box and whisker graph of every combination of two-way treatments featuring only the 6 antibiotics used in the study at all 3 different time points, namely 24 h, 48 h and 72 h. GEN = Gentamicin, AMP = Ampicillin, CHL = Chloramphenicol, SFX = Sulfamethoxazole, TRI = Trimethoprim, CIP = Ciprofloxacin. In each case, N = 8

Fig.9 shows the antibiotic-phage combinations that were performed as part of this phage - antibiotic synergy experiment (PAS). the most efficient combinations were the Sulfamethoxazole – 14/1 and the ampicillin – PA10P2 which restricted bacterial growth the best, while Trimethoprim – ΦKZ was the least efficient combo. The general observed trendline was that phage combined with ampicillin were the most efficient combinations while phages combined with chloramphenicol or trimethoprim were the least efficient combination. As it can be seen, as opposed to the antibiotic - antibiotic, Fig.9, graph, all of the antibiotic-phage combinations had a very low initial optical density at 24 h, which did or did not increase as time went on. Time had a great effect on the efficacy of those combinations, ($F_{2,266} =$

33.03 p < 0.0001), which indicates that, in this particular case, those antibiotic – phage combinations had a great initial effect, and over time, some combinations successfully restricted bacterial growth, which are combinations of ampicillin with any phage, sulfamethoxazole with PA10P2 or 14/1, while other were not as successful to restrict growth such as the trimethoprim – ΦKZ combo which had a great initial bacterial density, but after 72 h, it reached levels of optical density akin to the control measurement. Another example is gentamicin with 14/1 or ΦKZ, where after a very low starting density, there is an important bacterial growth at 48 h and 72 h.

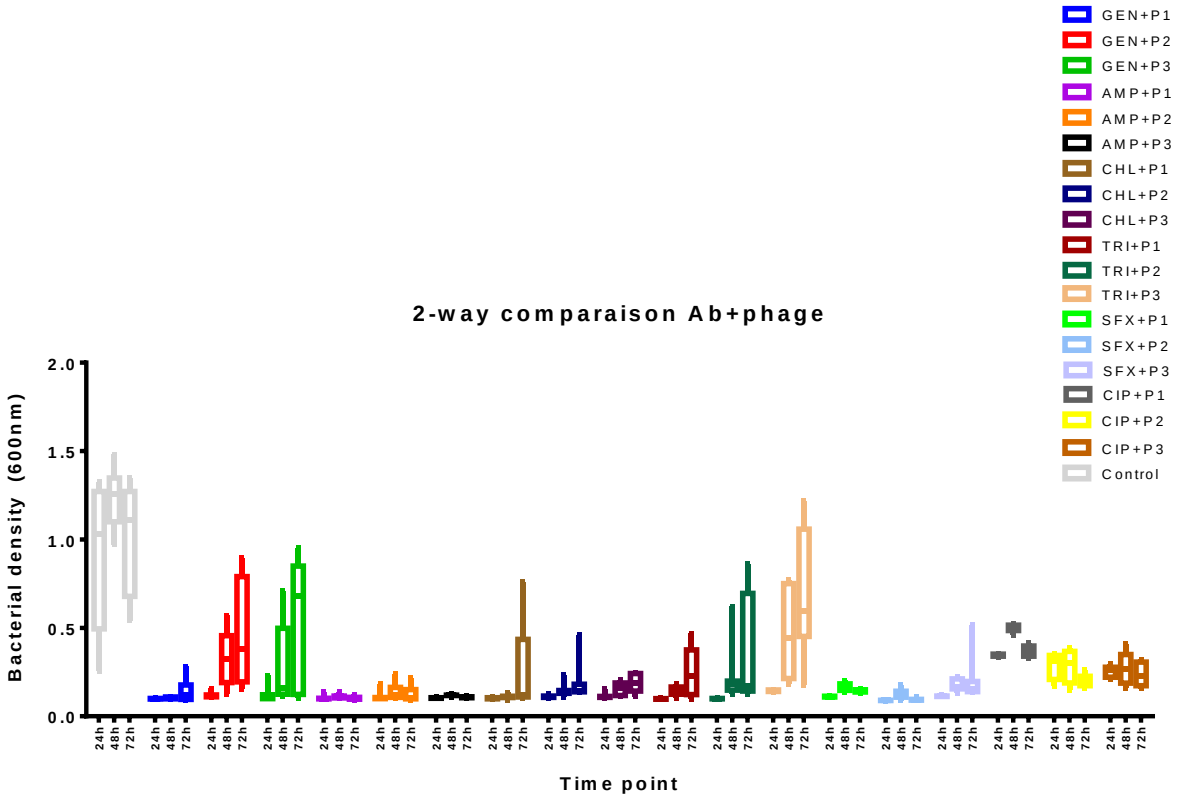


Fig.9: Reduction in growth by antibiotics and phages in a two-way treatment Box and whisker graph of every combination of two-way treatments featuring both the antibiotics and the bacteriophages used in the study at all 3 different time points, namely 24 h, 48 h and 72 h. GEN = Gentamicin, AMP = Ampicillin, CHL = Chloramphenicol, SFX = Sulfamethoxazole, TRI = Trimethoprim, CIP = Ciprofloxacin, P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

Lastly, Fig.10 shows the phage - phage two-way combinations, which serves as a follow up to a research performed in 2018 by Wright (Wright, R.C.T. et al., 2018) which shows the collateral sensitivity and resistance of phages from different modules. Here, however, it shows that there are no differences between all three phage combinations used ($F_{4,42} = 1.662$, $p = 0.1767$), which means no matter which phage combinations we used here, they all worked in the same way. Time had a significant effect on the efficacy of the treatments ($F_{2,21} = 34.12$ $p < 0.0001$), which here shows that there is a significant reduction in optical density at the 24 h timepoint which then, as time went on, increased, in some colonies, to levels similar to the control value. However, despite no significant difference between the three different treatments, the most efficient phage combination was the PA10P2 – 14/1 and the least efficient was the PA10P2 – ΦKZ.

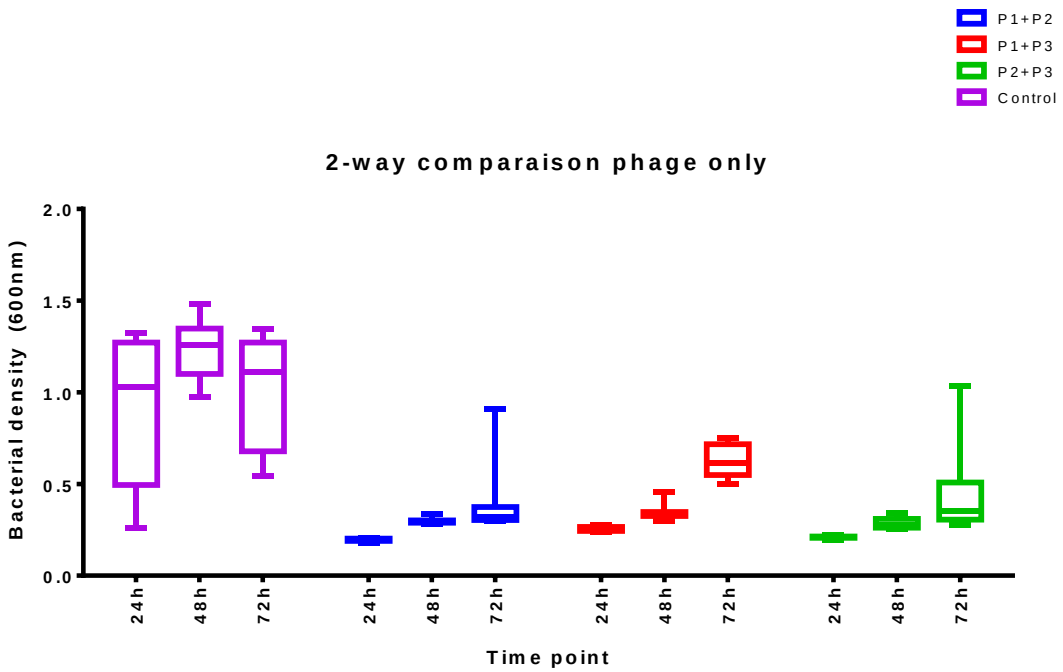


Fig.10: Reduction in growth by phages in a two-way treatment
Box and whisker graph of every combination of two-way treatments featuring only the bacteriophages used in the study at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2= 14/1 and P3 = ΦKZ In each case, N = 8

Overall, the main result that is found from the two-way combinations are that antibiotic - antibiotic combinations work better than antibiotics on their own, but this depends on the antibiotic synergy that exists between them, which has already been documented several times in research. To note that concentrations used here are sub-lethal MICs and as such, combining two efficient antibiotics at sub-lethal MIC can reduce and control bacterial growth significantly compared to their mono-effect counterpart. For example, ampicillin + sulfamethoxazole is a good combination that reduced bacterial growth more than if they are used on their own, while chloramphenicol with sulfamethoxazole had barely any effect compared to if they were used on their own, underlying that the synergies between the antibiotics is important and significant. Secondly, while combining antibiotics together can yield improved results compared to when used on their own, combining antibiotics with bacteriophages had a bigger impact on the efficacy of the treatment compared to the antibiotic-antibiotic combinations. Antibiotic-phage combinations improved on the efficacy of the mono-antibiotic effect. As an example, ampicillin combined with phages efficiently reduced bacterial growth, and ampicillin on its own, even at sub-lethal MIC, can significantly reduce bacterial growth below that of the control though it should be noted that a resistant strain of *P. aeruginosa* can withstand concentrations of ampicillin way greater than a sensitive bacteria, and as such, ampicillin-related results are to be taken lightly.

Importantly, time was the biggest factor in the efficacy of the antibiotic-phage combination, as some combinations worked efficiently throughout the three time points, such as the three different ampicillin-phage combinations, sulfamethoxazole + PA10P2 and the sulfamethoxazole + 14/1. Those combinations did not lose in efficacy over time, meanwhile the different trimethoprim + phage combos, the gentamicin + 14/1 and gentamicin + ΦKZ lost in efficacy over 48 h and 72 h. Phage + phage combinations were efficient in reducing and keeping the bacterial density low, but here again,

time was the biggest factor in the efficacy of the combinations, where there is a slight loss of efficacy over time.

As a whole, the results from the two-way combinations show that the antibiotic-phage combination had, in general, a greater effect at reducing and inhibiting bacterial growth than their antibiotic - antibiotic and phage - phage counterparts.

7.3.4 Three-way treatments effects

The three-way treatments have been split into 6 graphs due to the large amounts of data present, and they have been split depending on the phage used in the treatments. As such, Fig.11 and Fig.14 show the antibiotic – antibiotic - phage and the antibiotic – phage - phage, respectively, with a focus on PA10P2. Fig.12 and Fig.15 show the antibiotic – antibiotic - phage and the antibiotic – phage - phage, respectively, with a focus on 14/1 and lastly, Fig.13 and Fig.16 show the antibiotic – antibiotic - phage and the antibiotic - phage - phage with a focus on ΦKZ.

It was found that all the three-way combinations are significantly different ($F_{124,882} = 11.76$ $p < 0.0001$) and bacterial density significantly slightly increased over time ($F_{2,882} = 135.4$ $p < 0.0001$). Splitting the data set based on a particular phage mean that some results will figure more than once, but the general trendline remains the same, bacterial growth is massively reduced when combining two phages with one antibiotic, except for a couple of specific points (notably sulfamethoxazole and trimethoprim, Fig.14, Fig.15, and Fig.16). As opposed to two phages + one antibiotic, the two antibiotic + one phage had more variable results where the main trendline is that the synergy between the antibiotics is kept when using it in combination with phages (e.g. chloramphenicol + Sulfamethoxazole on their own did not reduce bacterial OD massively, and neither did it when adding a phage on top of it, Fig.7 and Fig.12) but the type of phage did have an effect on the efficiency of the synergy (e.g. comparing CHL + SFX + P1/P2/P3 on Fig.11, Fig.12 and Fig.13). Where it was found that ampicillin was a good antibiotic to use in combination with phages (Fig.9), here it is still the case where ampicillin + another antibiotic + phage still had a great efficacy at reducing bacterial growth. The two-way combinations that did not work great, such as the trimethoprim + ΦKZ combination did see an improve in efficacy by the addition of a second antibiotic to the antibiotic – phage combination (comparing Fig.9 with Fig.13). Chloramphenicol, when combined with another antibiotic (Fig.8) did not yield efficient results, barely reducing bacterial growth, and when it is added to a phage (Fig.11,12 and 13) did not change anything, the combination is still not efficient, but not worse. This shows that some combinations can be improved by adding a phage or another antibiotic on top of the combination, but the results are dependant and variable on the inner mechanics of the antibiotic.

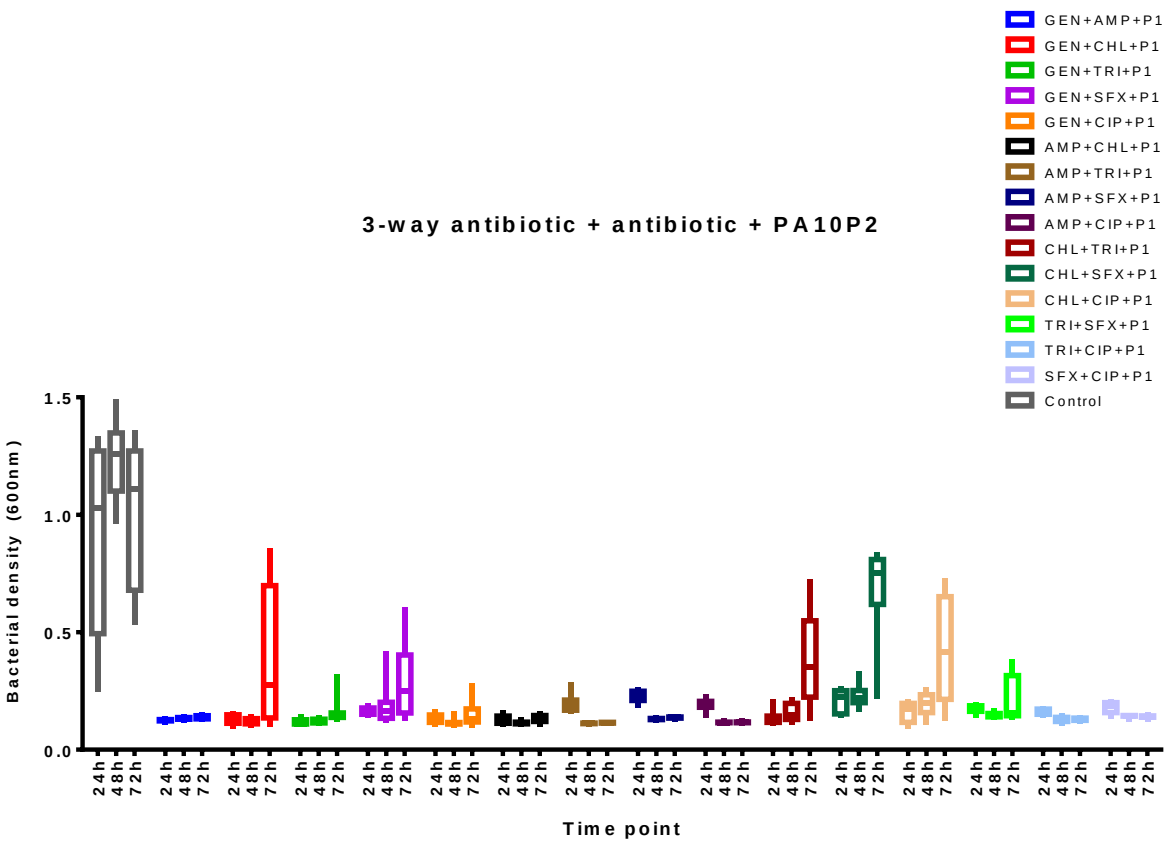


Fig.11 Reduction in growth by 2 antibiotics and PA10P2 in a three-way treatment
Box and whisker graph of every combination in the 3-way treatment that comprises 2 antibiotics and one phage, the phage being PA10P2, at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

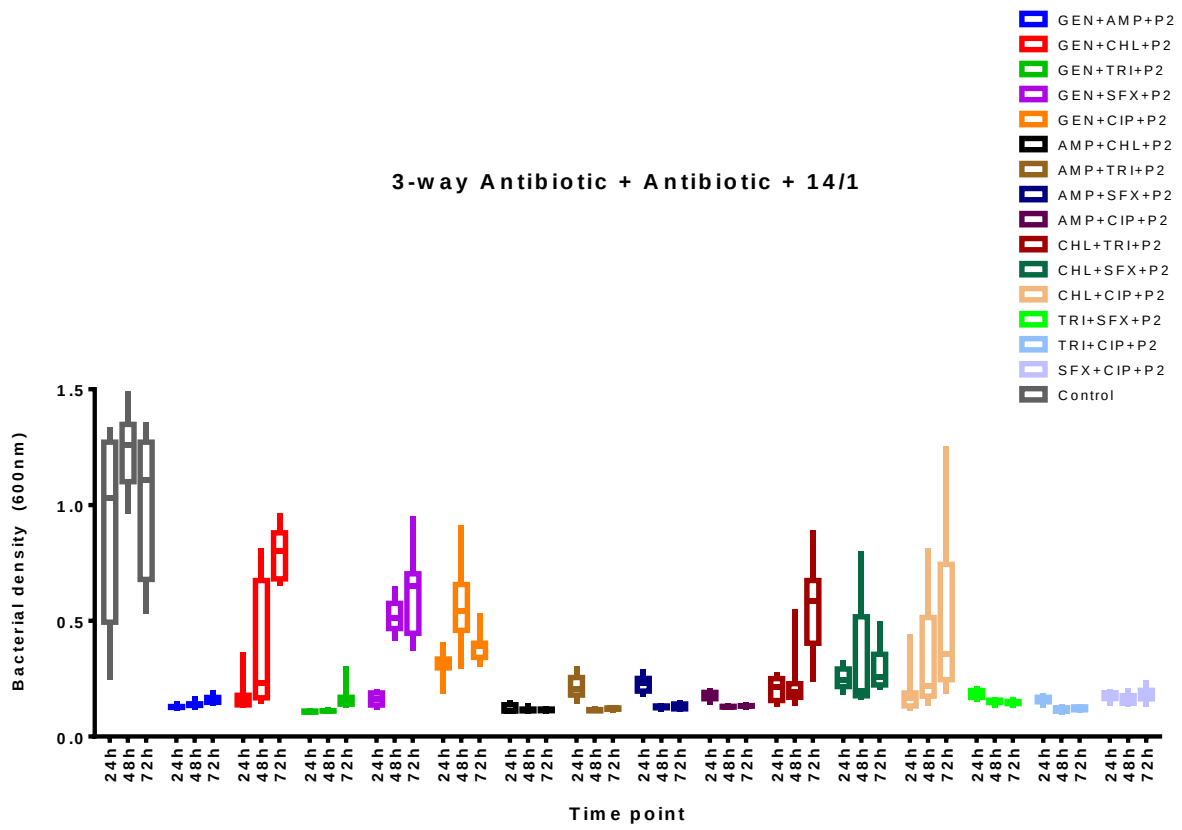


Fig.12 Reduction in growth by 2 antibiotics and 14/1 in a three-way treatment
Box and whisker graph of every combination in the 3-way treatment that comprises 2 antibiotics and one phage, the phage being 14/1, at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

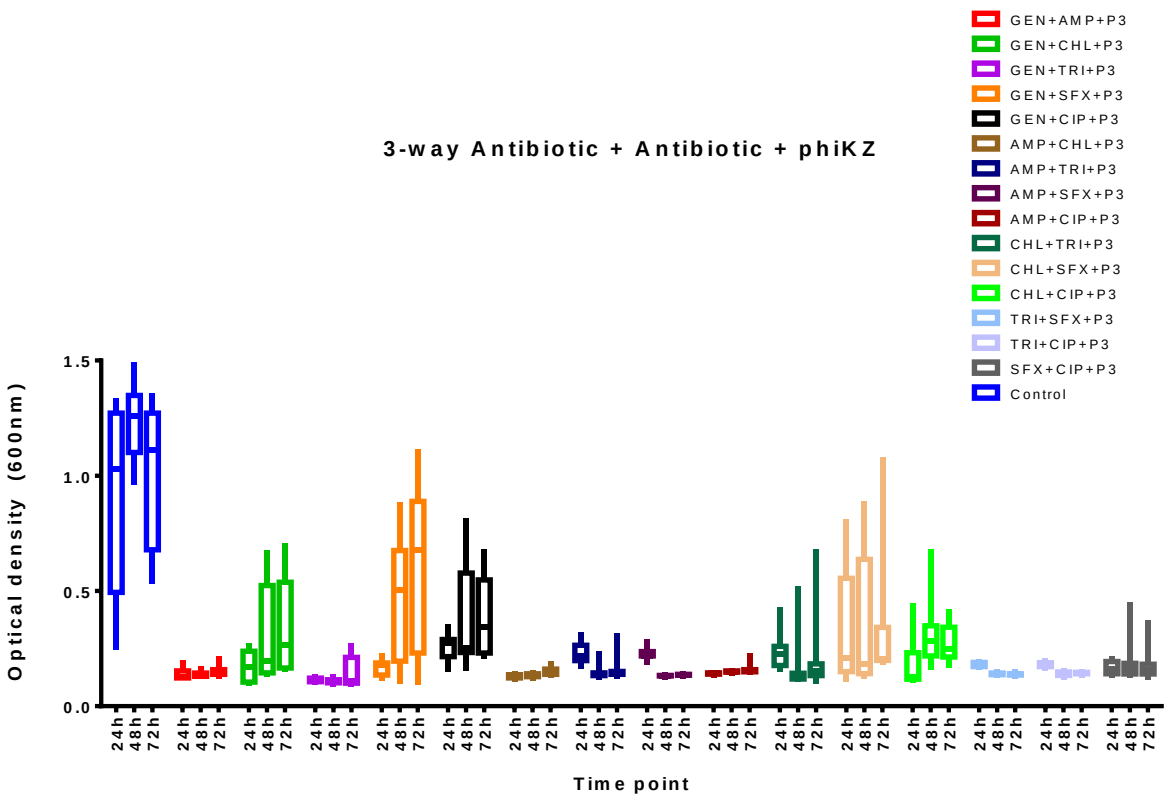


Fig.13 Reduction in growth by 2 antibiotics and ΦKZ
Box and whisker graph of every combination in the 3-way treatment that comprises 2 antibiotics and one phage, the phage being ΦKZ at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

The most notable key point from Fig.10 was that phage combinations are efficient but lose in efficacy over time. This pattern can still be observed when an antibiotic is added on top of the phage – phage combination, but this effect is lessened (Fig.14, Fig.15 and Fig.16). Here, just like the phage – phage combination (Fig.10), shows a very low bacterial density at 24 h which, in some combinations, increased at 72 h. Only a select few antibiotics caused that increase in bacterial density, which are sulfamethoxazole and trimethoprim. Any other antibiotic on their own combined with two phages from whichever module all gave a significant reduction in bacterial density compared to their two-way counterpart.

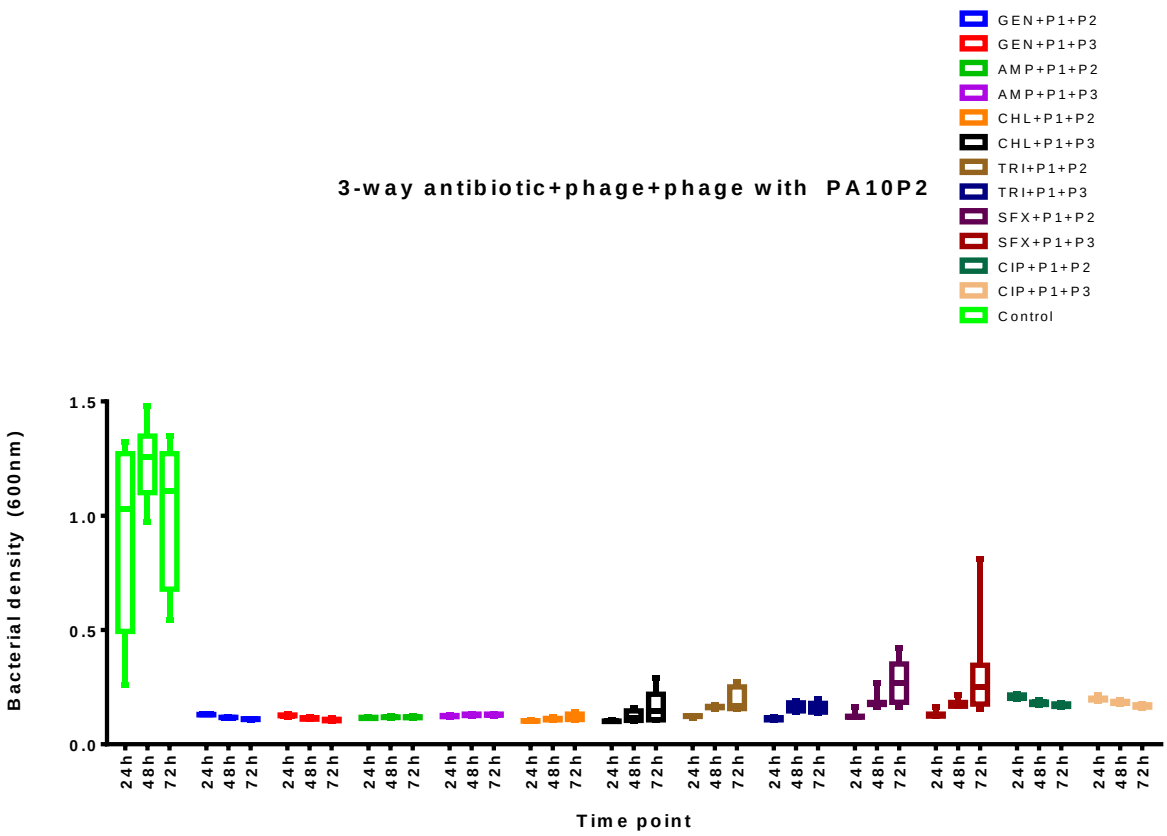


Fig.14 Reduction in growth by 1 antibiotic and 2 phages in a three-way treatment with a focus on PA10P2
Box and whisker graph of every combination in the 3-way treatment that comprises one antibiotic and two phages, one of which is PA10P2 at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

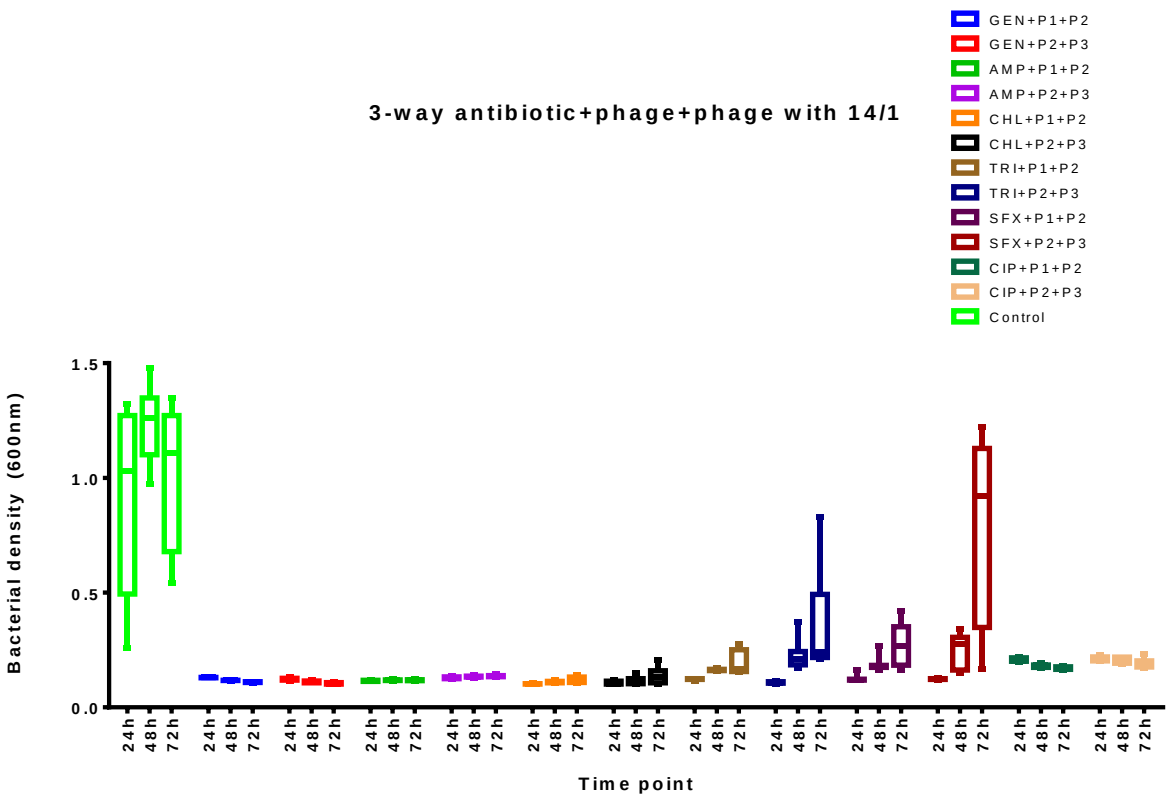


Fig.15 Reduction in growth by 1 antibiotic and 2 phages in a three-way treatment with a focus on 14/1
Box and whisker graph of every combination in the 3-way treatment that comprises one antibiotic and two phages, one of which is 14/1 at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

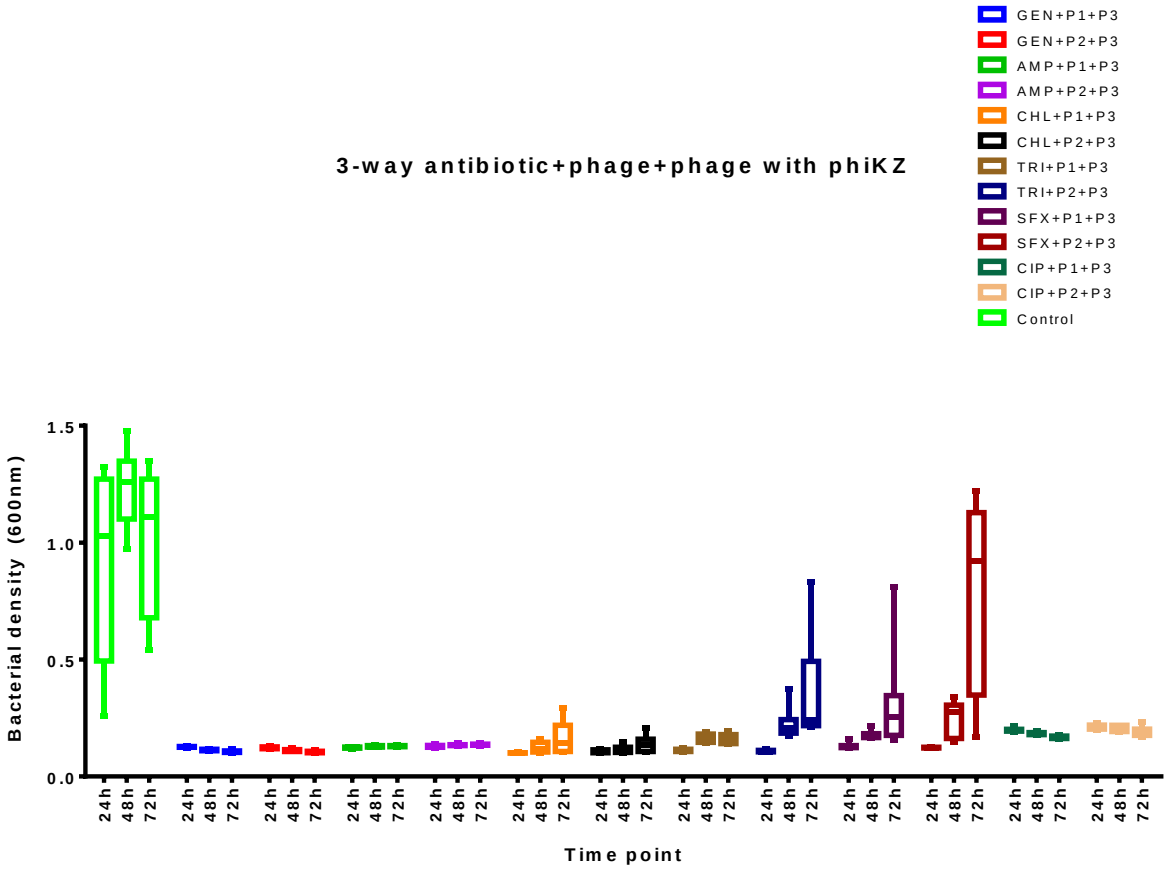


Fig.16 Reduction in growth by 1 antibiotic and 2 phages in a three-way treatment with a focus on ΦKZ
Box and whisker graph of every combination in the 3-way treatment that comprises one antibiotic and two phages, one of which is ΦKZ at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

As such, time have a great effect on the efficacy of those combinations, ($F_{2,896} = 106.9$ $p < 0.0001$), it is clear that over time, even with three different antimicrobials, those combinations have a great starting effect. Notably, the combination that had the greatest effect amongst the three-way combinations was the chloramphenicol – PA10P2 – 14/1 combination, and the least successful combination was the gentamicin – sulfamethoxazole – 14/1 combination. As a general trendline, antibiotic – antibiotic - phage combinations had a worse effect at reducing bacterial growth than the antibiotic – phage - phage combinations.

Overall, the main finding from the three-way combinations is that, for the antibiotic – antibiotic – phage combinations, the effect of adding a phage on top of an antibiotic-antibiotic combination can affect, positively or negatively, the synergy between the two antibiotics. Some combinations that worked great such as the ampicillin – sulfamethoxazole saw no additional benefits to having a phage added. Some combinations which did not work that well such as the chloramphenicol – ciprofloxacin did not see their effect improved either by adding a phage on top of it. However, for combinations that had an average effect, and that no particular synergy has been found between those antibiotics, such as the chloramphenicol – trimethoprim, having a bacteriophage helped reduce bacterial density down. Concerning the phage – phage – antibiotic combinations, at the exception of sulfamethoxazole and trimethoprim, the synergy between phages is improved by the addition of antibiotics, as most of the antibiotic – phage – phage combinations kept the bacterial density at a very low point.

7.3.5 Four-way treatments effects:

Similar to the three-way graphs, the four-way data has been split into three main graphs, each of them featuring a different phage (Fig.17 to Fig.19). However, since all the combinations are antibiotic - antibiotic – phage - phage, the division into three graphs has only been done for the clarity of understanding. Similarly to every other graphs, time had a great effect on the efficacy of the treatments ($F_{2,664}$ = 142.8 p < 0.0001), which here is better seen as almost every combination started with a very low bacterial concentration at 24h, but some of them could not inhibit bacterial growth beyond 24 h. One such example would be the trimethoprim - ciprofloxacin - 14/1 - ΦKZ combination.

Of importance, it appears that the four-way treatments are not significantly different than the two different three-way treatments. It is not significantly different than the antibiotic – phage - phage treatments ($F_{2,122}$ = 1.29 p = 0.2963), and it is just short of being significantly different to the antibiotic – antibiotic - phage treatments ($F_{2,176}$ = 2.546 p = 0.0813). Very similarly to the three-way combinations, there are some combinations that saw either an improvement or no effect of the combination due to the addition of another antimicrobial, but no worsening. For example, in the three-way combinations, the notably bad combinations such as chloramphenicol – trimethoprim – PA10P2 is still as inefficient when adding another phage, for example chloramphenicol – trimethoprim – PA10P2 – 14/1 is not more efficient than the former.

Ampicillin still remains an efficient antibiotic used in combinations of antibiotics and phages in four-way combinations. When it comes to the average combination, such as the gentamicin – chloramphenicol combination, adding one phage helped reduce the bacterial growth (Fig.11,12 and 13), adding a second phage on top of it reduces bacterial growth even more (Fig.17,18 and 19). This means that when using combinations of antimicrobials that have no obvious synergies between them, the addition of a fourth antimicrobial helps reduce the bacterial density.

As a whole, the main result from all those treatments is that three antimicrobials are just as efficient to use as four antimicrobials at once, and that two phages + one antibiotic will be the preferred focus for the treatments variety, as the aim would be to use already efficient combinations of antimicrobials together and to improve on them.

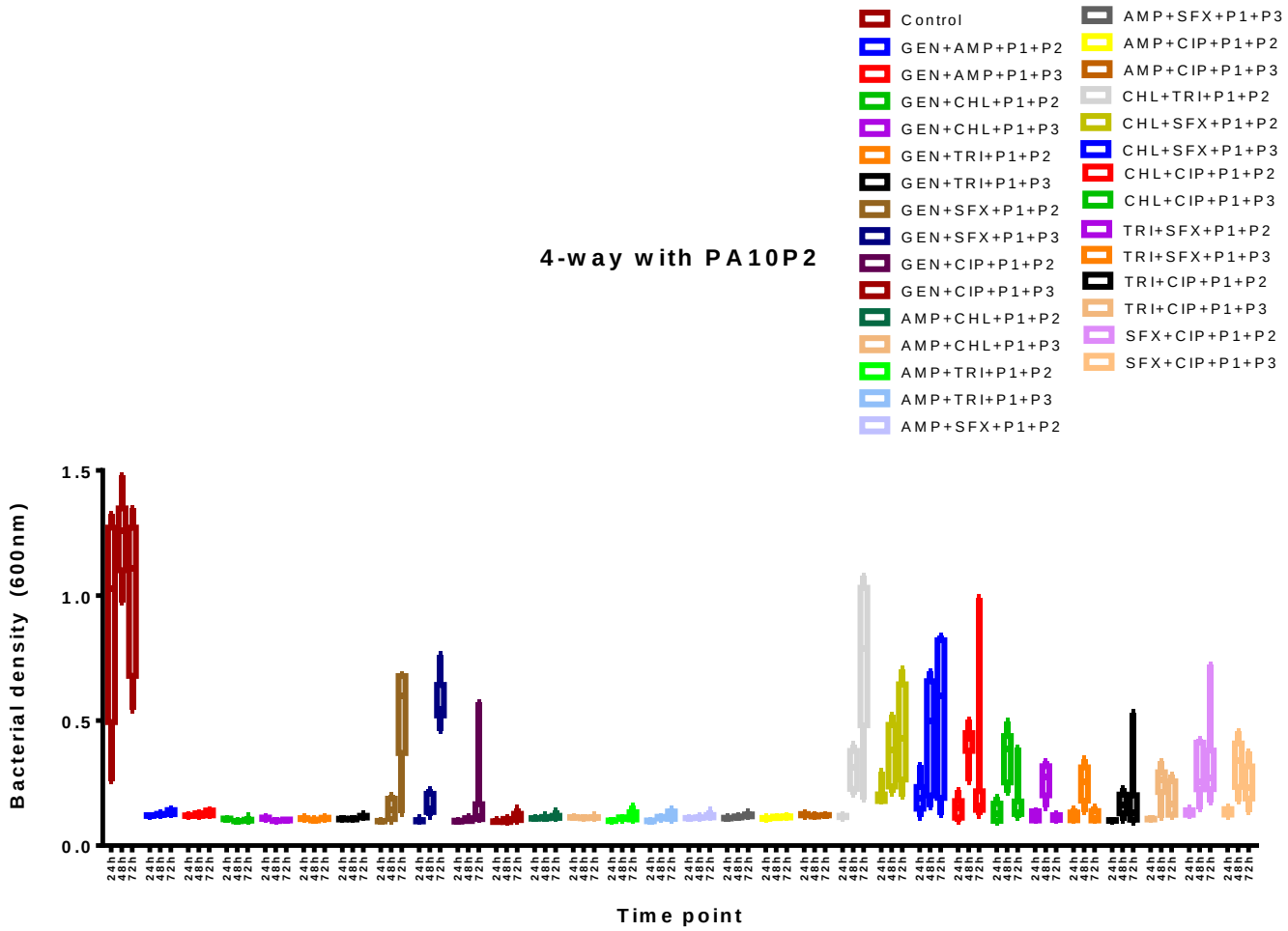


Fig.17 Reduction in growth by 2 antibiotic and 2 phages in a four-way treatment with a focus on PA10P2
Box and whisker graph of every combination in the 4-way treatment that comprises two antibiotics and two phages, one of which is PA10P2 at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

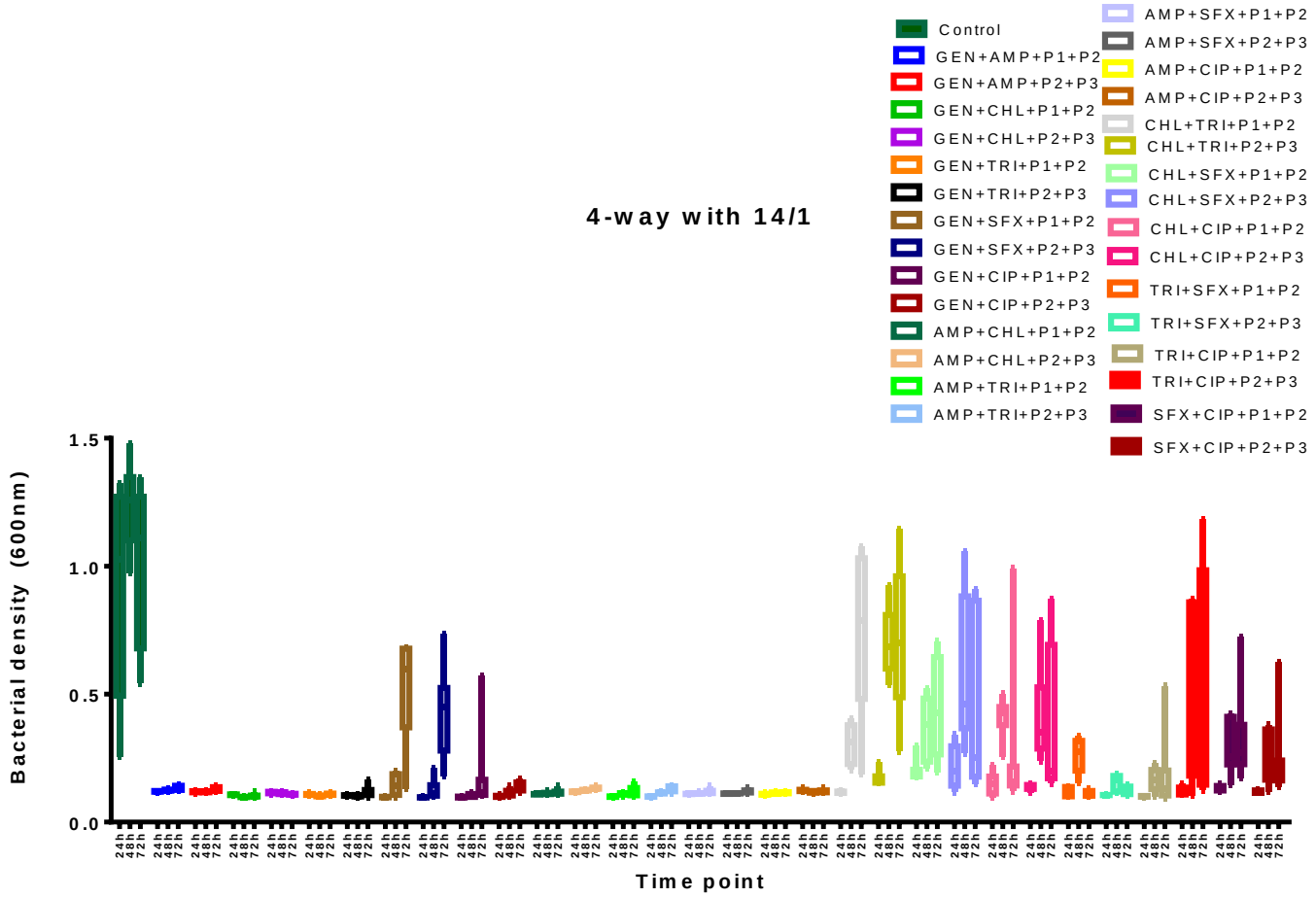


Fig.18 Reduction in growth by 2 antibiotic and 2 phages in a four-way treatment with a focus on 14/1
Box and whisker graph of every combination in the 4-way treatment that comprises two antibiotics and two phages, one of which is 14/1 at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

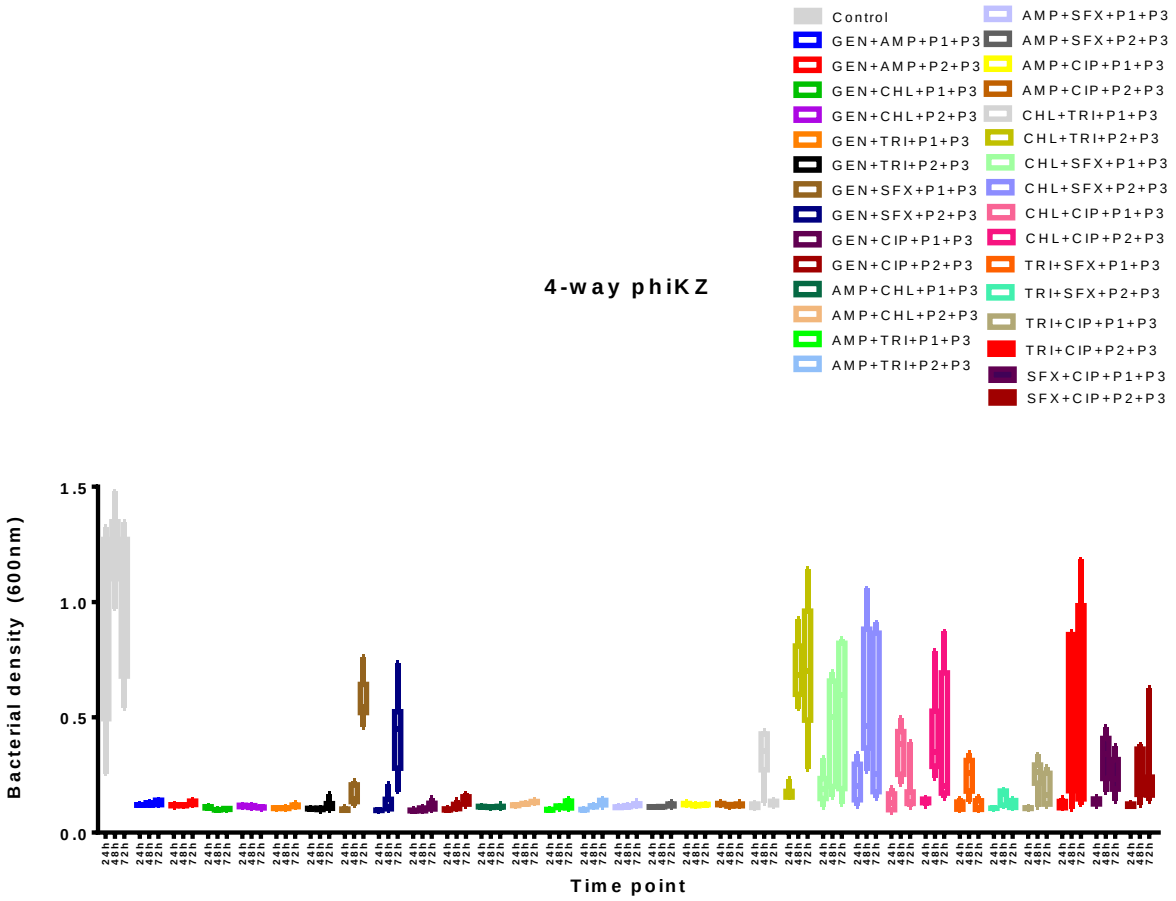


Fig.19 Reduction in growth by 2 antibiotic and 2 phages in a four-way treatment with a focus on ΦKZ
Box and whisker graph of every combination in the 4-way treatment that comprises two antibiotics and two phages, one of which is ΦKZ at all 3 different time points, namely 24 h, 48 h and 72 h. P1 = PA10P2, P2 = 14/1 and P3 = ΦKZ In each case, N = 8

7.3.6 Are phage-antibiotic synergies additive?

To explore if combinations effects are synergistic or combined sum of their individual effects, antibiotics and phage monocultures were used to predict their observed effects in antibiotic - phage combinations treatments Fig.20 to Fig.22). The whole dataset is split between the two-way, three-way, and four-way treatments, in all three different time points show effects after 24 h, 48 h and 72 h of growth, respectively. The format of graphs used is an expected vs observed graph which compares the mono-treatment results and compares it to the observed values for the higher order treatments. As such, if we expect one treatment to reduce the bacterial growth but we observe a lower than expected bacterial growth, it means the combination did not perform as well as we had expected, and as such, while it might still be efficient and reduce bacterial growth, is not performing synergistically but rather additively, or independently from one another. As a visual guide, a bold, diagonal line has been drawn that represents a 1:1 ratio between the observed vs expected values, and as such, points that are below the line (High expected, low observed) are performing badly, while points that are above the line (Low expected, high observed) are performing well and above expectations, and as such, probably have a synergistic or additive effect. On the two-way and three-way, the colour coding serves as a quick visual guide to compare how the different two-way and three-way combinations (where you have either 1 phage, 2 phages, 1 antibiotics, or 2 antibiotics)

performed and allow for quick comparison between them. On the four-way graphs, however, all the points are two antibiotics + two phages. The two-way combinations show varied results. When it comes to antibiotic - antibiotic combinations, the results are scattered all around the graph (Fig.20, green) where the combination either performed well (Above the line), showing a synergy, for example the gentamicin + ampicillin combination, or performed poorly (Below the line), showing a lack of synergy, for example the chloramphenicol + ciprofloxacin combination ($F_{1,14} = 17.91$ $p = 0.0008$). Phage - phage combinations (Fig.20, red) mainly showed no synergy between each other, but their effects can be calculated additively ($F_{1,2} = 1.672$ $p = 0.352$). When it comes to the antibiotic - phage combinations (Fig.20, black), the results are always above the 1:1 ratio line ($F_{1,23} = 255.4$ $p < 0.0001$), which indicates that there is always some form of positive synergy between antibiotics and phages when compared to antibiotics - antibiotics and phage - phages. Time had an effect on the antibiotic - antibiotic combinations ($F_{5,70} = 17.51$ $p < 0.0001$), but the results being still scattered over the graph, it shows that the efficacy of the antibiotic - antibiotics combination is still very dependant on their synergies, some values being above, under, or on the line still (Fig.20, green). Time significantly affected the phage - phage combination ($F_{2,8} = 146.4$ $p < 0.0001$), but the values still being on the line ($F_{1,8} = 13.86$ $p = 0.0059$) shows that while time had an effect on how efficient the combination was, the values are still close to a 1:1 ratio and therefore denotes no obvious or clear synergy.

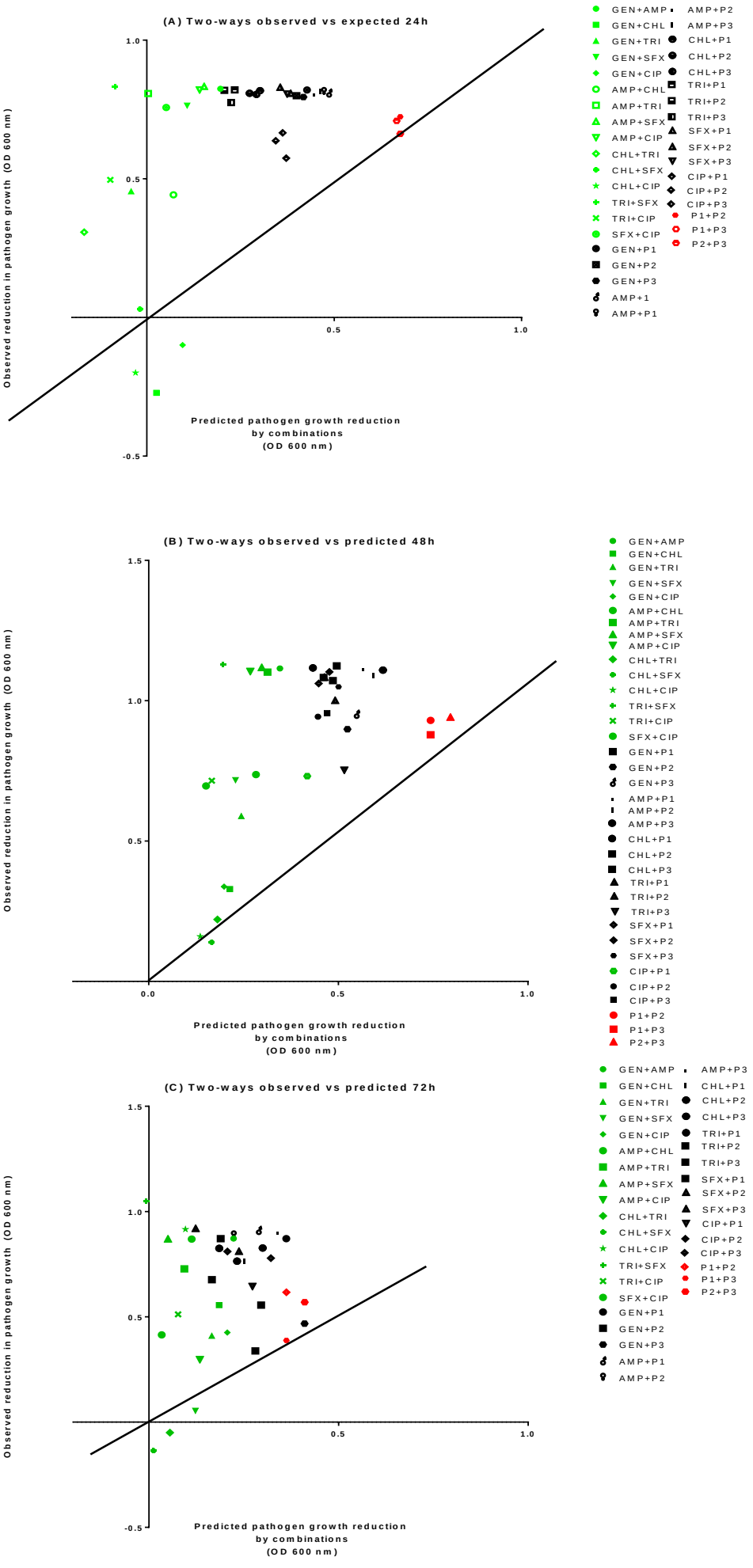
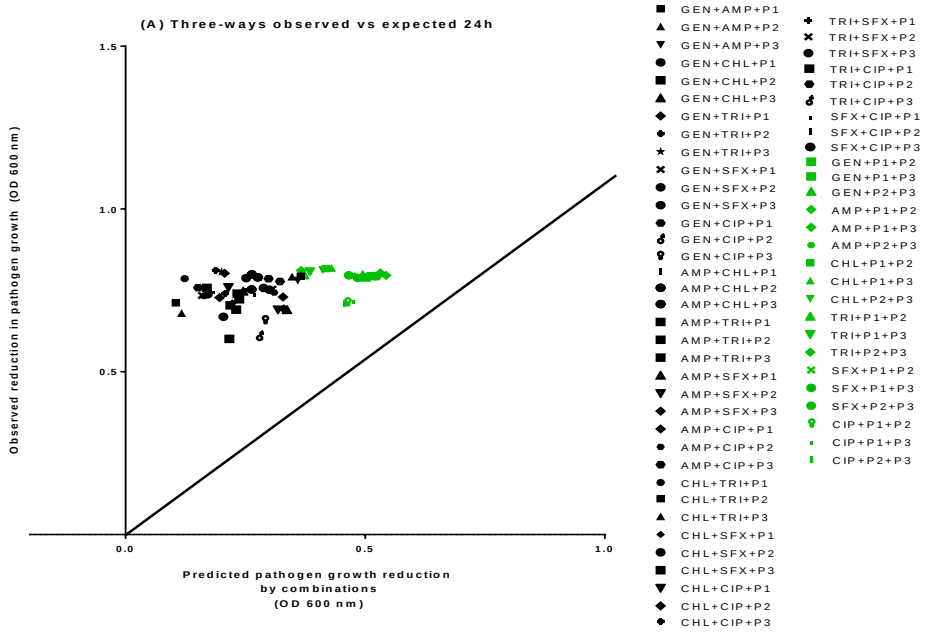
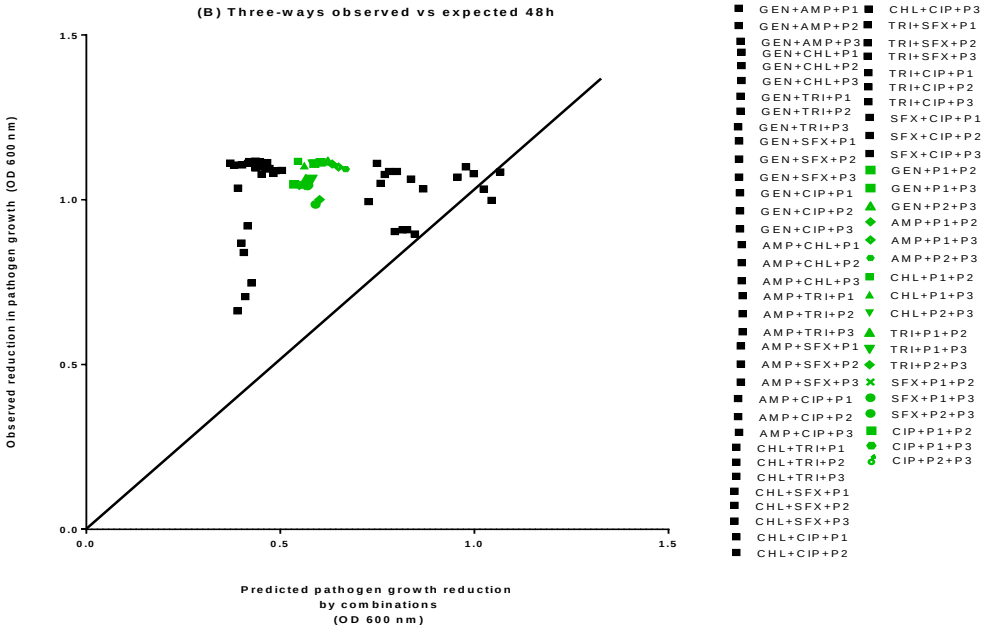


Fig.20 All two-way observed vs predicted graphs.
(A) shows the 24 h Observed vs predicted two-way graphs, (B) for 48 h and (C) for 72 h. The combinations are colour-coded green for two antibiotics, black for antibiotic - phage, and red for two phages. The diagonal line represents a 1:1 ratio between observed and expected, which serves as a visual line to indicate if each combination is performing better (above the line) or worse (under the line) than expected.

The three-way combinations observed vs expected graphs (Fig.21) shows an observable divide over time (Panel A to C). The main result from this particular panel is that antibiotic-antibiotic-phage combinations (black) lose in efficacy over time and leads to results shifting under the 1:1 ratio line, also shifting from left to right, indicating an increase in predicted efficacy, but no change in observed efficacy. This shows that time is an important factor in the efficacy of the antibiotic – antibiotic - phage combination, but in the antibiotic – phage - phage combination, time barely affected the results, as they stayed mainly in the same spot, except for the chloramphenicol – 14/1 – ΦKZ, which has been shown in previous experiments to not be a viable combination of antibiotics and phages (4.3.4). This indicates that, in conjunction with the previous experiments where I show that over time, some combinations lose in efficacy in the antibiotic – phage - phage treatments, it stays, as a whole, more effective than some antibiotic - antibiotic combinations, and that the antibiotic – phage - phage combinations tends to lose less in efficacy over time than the antibiotic – antibiotic - phage combinations do.





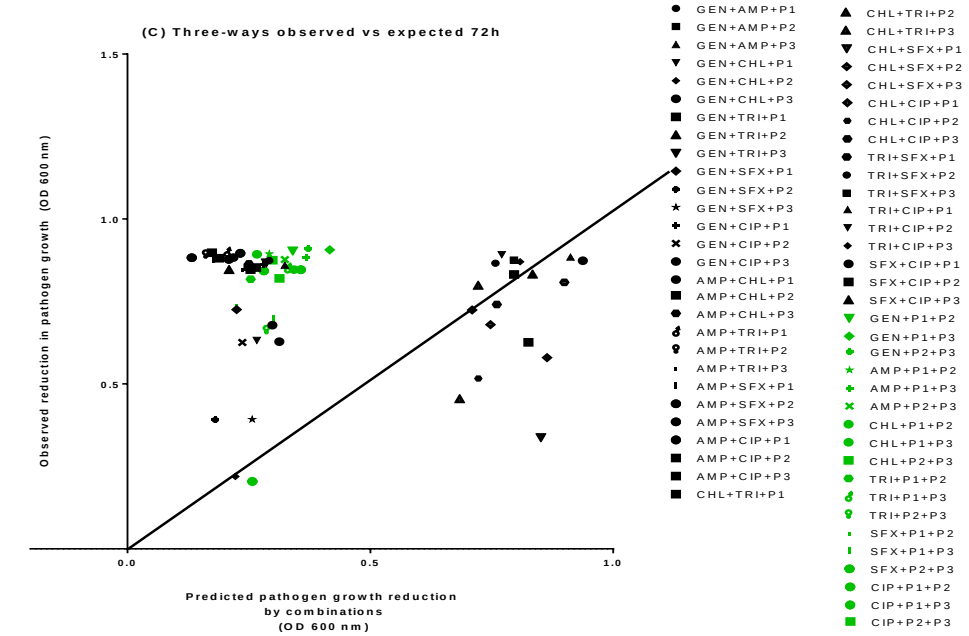
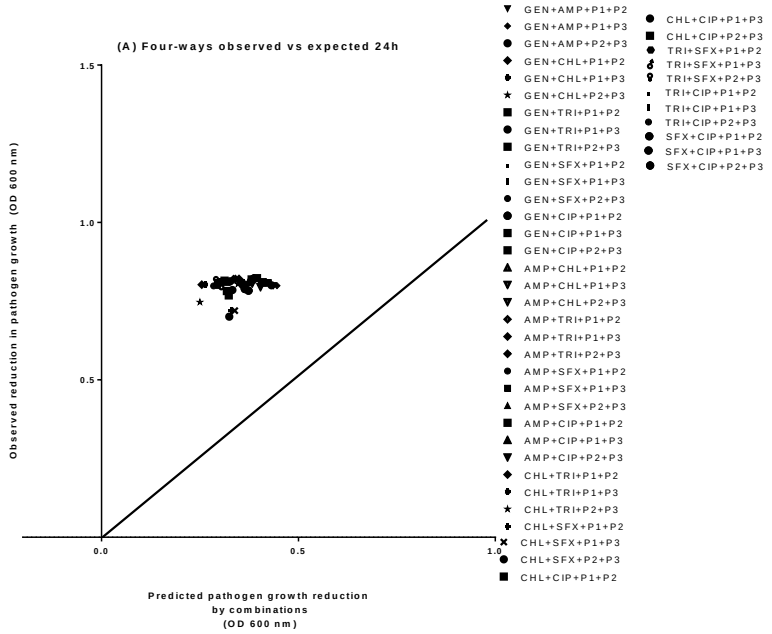
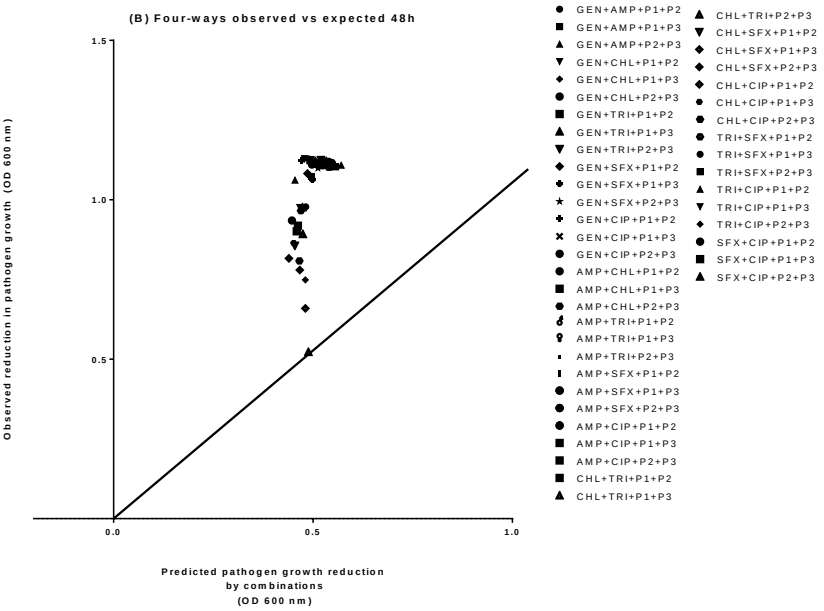


Fig.21 All three-way observed vs predicted graphs.

(A) shows the 24 h Observed vs predicted three-way graphs, (B) for 48 h and (C) for 72 h. The combinations are colour-coded green for two phages + one antibiotic, and black for two antibiotics - one phage. The diagonal line represents a 1:1 ratio between observed and expected, which serves as a visual line to indicate if each combination is performing better (above the line) or worse (under the line) than expected.

When it comes to the 4-ways treatments, all of the values are above the line. This shows that 4-way combinations are all efficient in controlling bacterial growth, but just like the three-way antibiotic – antibiotic - phage, over time, it loses in efficacy and it is observable (Fig.22) as a "leakage" toward the 1:1 ratio line. This drops in observed value compared to the predicted values clearly indicates that the combination was not as efficient at keeping the bacterial density down over time. Combined with the previous experiments, this can be observed (Fig.17 to Fig.19) by some antibiotic – antibiotic – phage - phage combinations that were not efficient over time, such as the trimethoprim – ciprofloxacin - 14/1 - ΦKZ combination. As a whole, the four-way treatments show that adding more antimicrobials might not be an efficient solution as it appears to be time-dependant most of the time.





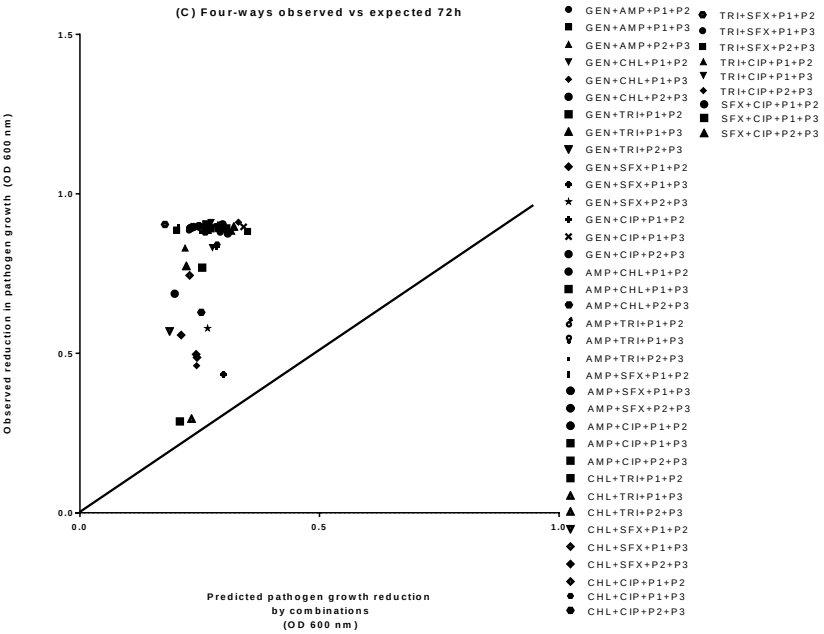


Fig.22 All four-way observed vs predicted graphs. (A) shows the 24 h Observed vs predicted four-way graphs, (B) for 48 h and (C) for 72 h. The diagonal line represents a 1:1 ratio between observed and expected, which serves as a visual line to indicate if each combination is performing better (above the line) or worse (under the line) than expected.

7.4. Experiment 2: Investigating the effect of PAS on clinical strains

Various treatments and combinations were used on various clinical strains of *P. aeruginosa* (Fig S.1) that are classified as either chronic or intermittent strains. Those combinations arose from a selection of combinations that had a range of efficient and not efficient effects on the PAO1 *P. aeruginosa* strain as seen in experiment 1 (section 4.3.3). Those combinations therefore have an history of being either successful against the pathogen, such as ampicillin – PA10P2, or unsuccessful, such as the sulfamethoxazole – gentamicin. The combinations which were selected were comprised of three different two-way combinations. Antibiotic – antibiotic combinations were the ampicillin – gentamicin, ampicillin – sulfamethoxazole, and gentamicin – sulfamethoxazole; the phage - phage combination was the PA10P2 - ΦKZ, and the antibiotic – phage combinations were the gentamicin – PA10P2, gentamicin – ΦKZ, sulfamethoxazole – PA10P2, sulfamethoxazole – ΦKZ, ampicillin – PA10P2, and ampicillin – ΦKZ. Combinations used are either two antibiotics, two phages, or one antibiotic and one phage. Each combination was replicated 8 times and the experiment ran for 72 h, with data points taken at 24 h, 48 h and 72 h. The aim of this experiment is to test various strains that had an observable effect on PAO1 on clinically relevant strains of *P. aeruginosa*. As such, the following figures (Fig.23 and Fig.24) are layouts of all 5 chronic strains (Fig.23) and all 5 intermittent strains (Fig.24), graphed after 24 h and 72 h for a side-by-side comparison of both the effect of the treatment over time, and the effects based on the strain used. The next figures (Fig.25) show the average effect of all 5 chronic strains at 24 h vs all 5 chronic strains at 72 h (top) and the average effect of all 5 intermittent strains at 24 h vs all 5 intermittent strains at 72 h (bottom). On average, the OD readings of the 72 h timepoints for the chronic bacterial strains (Fig.25, top panel) are higher than the OD readings of their respective 24 h timepoints, except for the control measurement which has non-significant variation within the values ($t = 1.395$, $p > 0.05$). All in all, all treatments had various effects on each of the chronic strains and no pattern can be found within all of them. Some treatments are more efficient on one strain than on another. One notable treatment for the chronic bacteria (Fig.23, all panels) is the ΦKZ + Ampicillin (pA) treatment that did not yield any significant result overtime for any of the five chronic strains tested, despite results from previous experiments showing that ampicillin is an efficient antibiotic when combined with any phages. Compared to the control, the treatments tested on the chronic strains had a significant effect at reducing the bacterial growth. In the case of the intermittent strains, the control had some significant variation within each of the intermittent strains, which could be a sign of desiccation or fitness cost for growth. Treatment-wise, just like the chronic strains, there is a wide range of variation, both significant and non-significant, for each of the treatments, but most notably is the intermittent 2 strain which, apart from the ampicillin + sulfamethoxazole treatment (AS), showed significant variations, both positively and negatively, between all of the treatments. All in all, on average for the chronic strains, only the PA10P2 + gentamicin (1G) and the ΦKZ + gentamicin (pG) did not yield significant differences between the 24 h and the 72 h timepoints. However, on average, for the intermittent strains, only the PA10P2 + Sulfamethoxazole showed a significant change across all the intermittent strains. When taken on a case per case basis, however, those significant differences between the treatment can mean various factors are at play, as the change denotes a low initial OD to a high OD, or vice-versa, but there is again no pattern between those significant differences. This means that each strain has a different defence mechanism and more importantly, that mechanism is slightly different to show such a difference in the efficacy of the antibiotics tested, but the general trendline is still the same as for the PAO1 strains, ampicillin with an antibiotic or a bacteriophage is still a very efficient way to reduce bacterial growth, but this is a two-way combination and as such, is not representative of the three- and four-way combinations that implies ampicillin as one of the antibiotics used as well. Antibiotic - phages combinations, in general, are more efficient than the antibiotic - antibiotic or the phage - phage combinations.

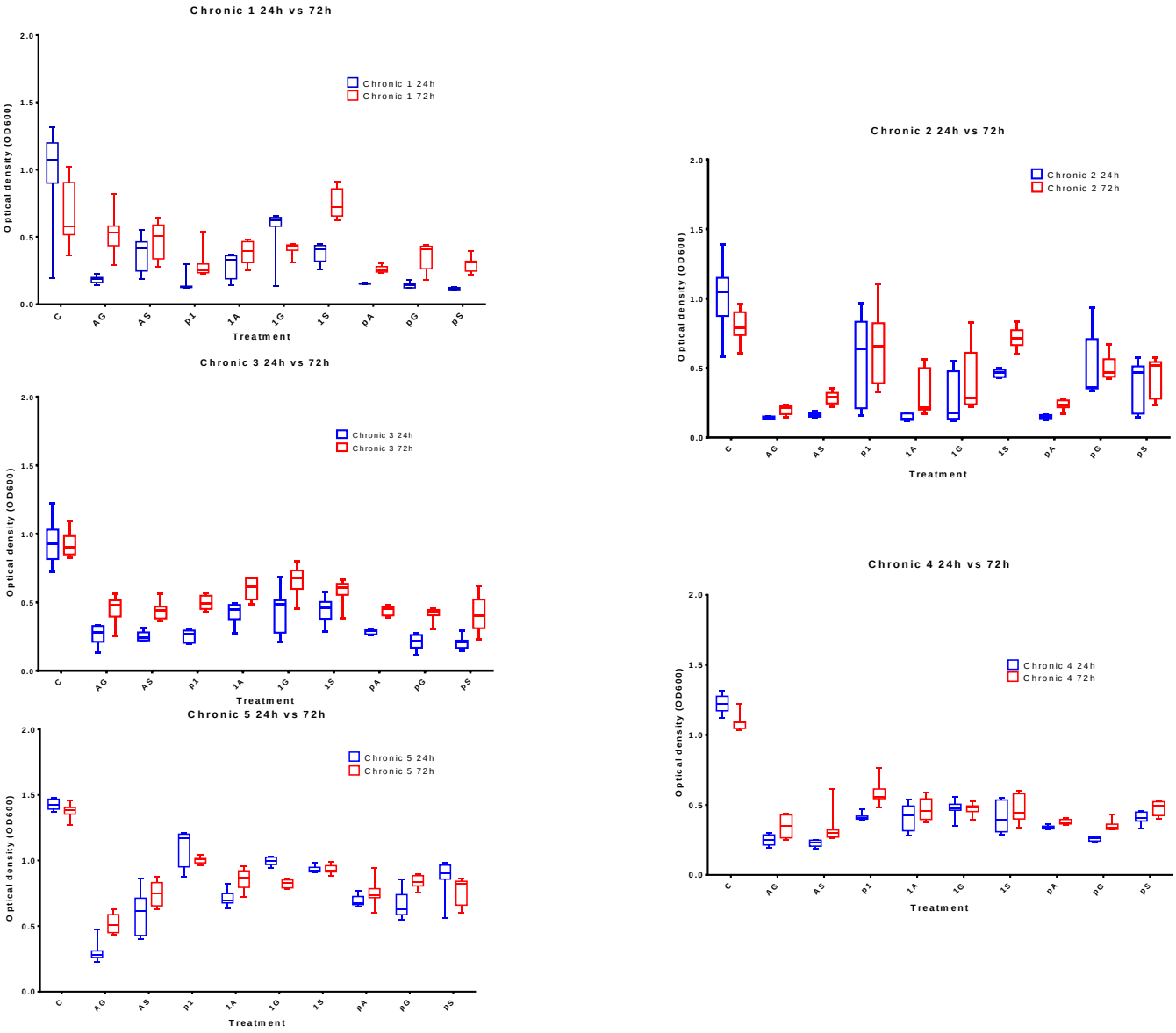


Fig.23 Box and whisker layout of all 5 chronic strains Layout based on the treatment at 24 h (blue bar) vs 72 h (red bar) for all 5 different chronic strains of *P. aeruginosa*. The treatments, apart from the first treatment which is the Control (C), the other combinations are A = Ampicillin, G = Gentamicin, S = Sulfamethoxazole, p = ΦKZ, 1 = PA10P2. A combination with two letters or one letter and one number therefore mean both antimicrobials are present in the solution.

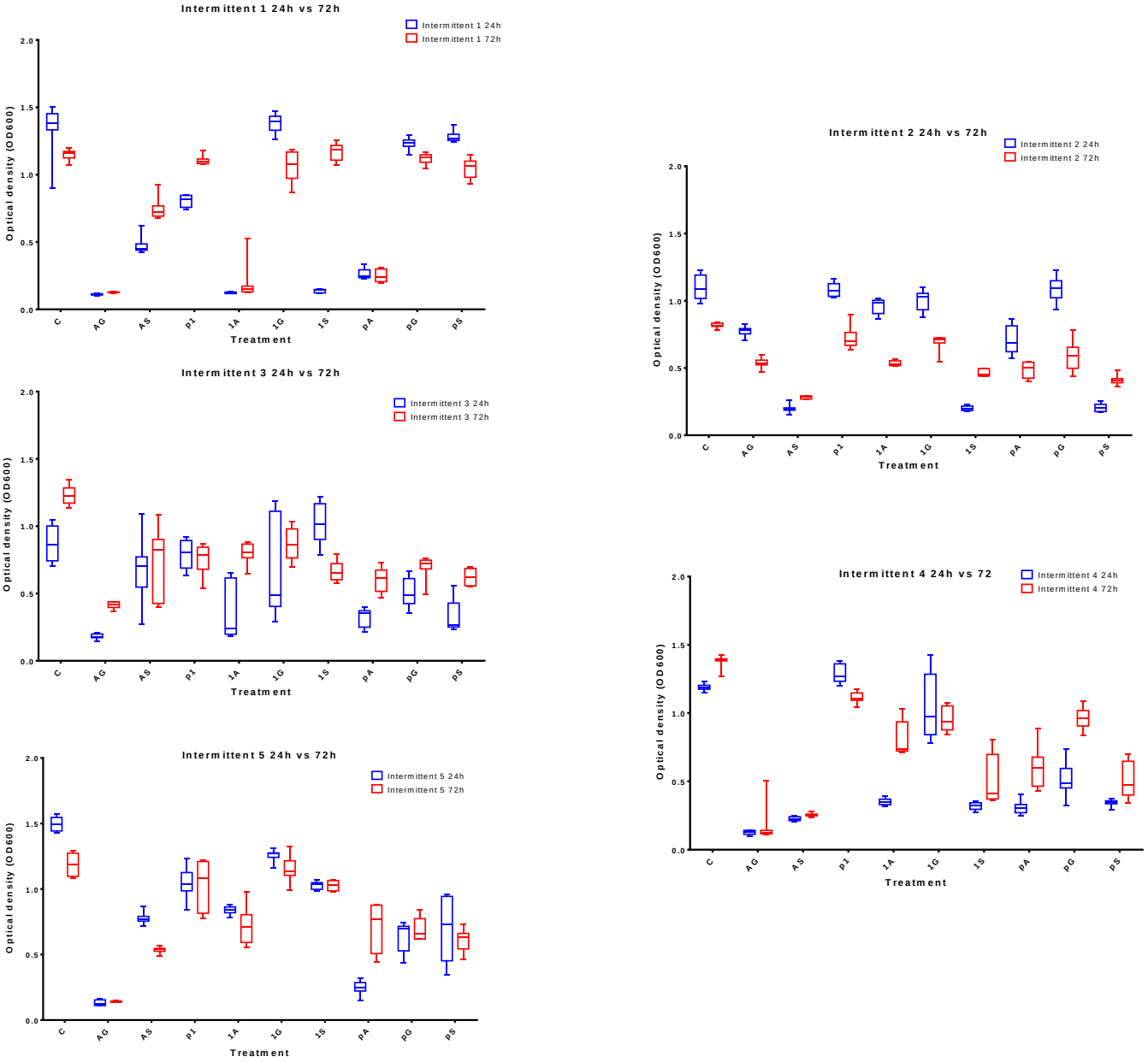


Fig.24 Box and whisker layout of all 5 intermittent strains
Layout based on the treatment at 24 h (blue bar) vs 72 h (red bar) for all 5 different intermittent strains of *P. aeruginosa*. The treatments, apart from the first treatment which is the Control (C), the other combinations are A = Ampicillin, G = Gentamicin, S = Sulfamethoxazole, p = ΦKZ, 1 = PA10P2. A combination with two letters or a letter with a number therefore means both antimicrobials are present in the solution.

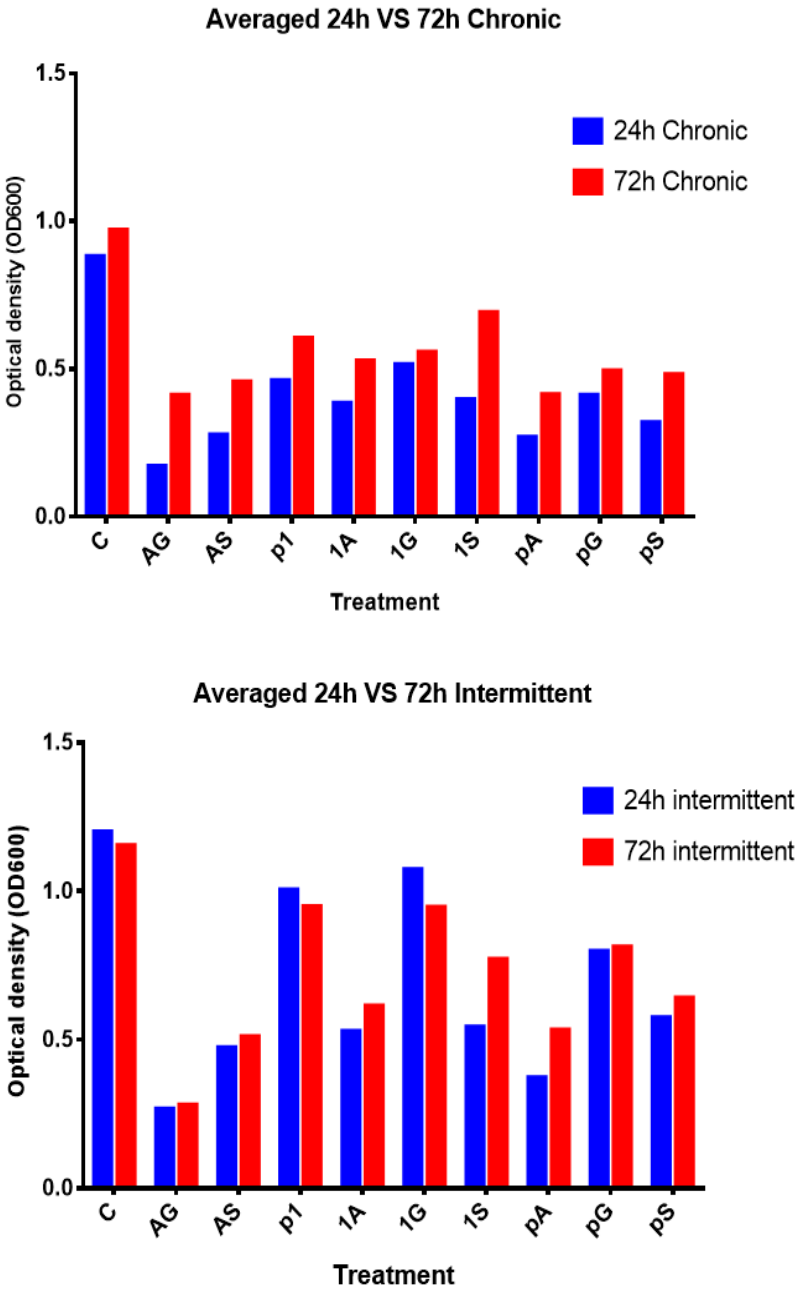


Fig.25 Bar graph layout of the average values of all chronic and intermittent strains Average of all 5 chronic strains (Top panel) and all 5 intermittent strains (bottom panel) of *P. aeruginosa* for the 24 h (blue bar) and 72 h (red bar) time points. The treatments, apart from the first treatment which is the Control (C), the other combinations are A = Ampicillin, G = Gentamicin, S = Sulfamethoxazole, p = ΦKZ, 1 = PA10P2. A combination with two letters or a letter with a number therefore means both antimicrobials are present in the solution.

8.Discussion

With the rise of antibiotic resistance and antimicrobial resistance, *P. aeruginosa* has become one of the most dangerous bacterial pathogens listed by the WHO (WHO, World Health Organization, 2017). It was therefore important to not only assess the antibiotic resistance, but also to find out new means of controlling bacterial growth that could also be applied in the clinic. Bacteriophage therapies and antibiotics being the latest two hot topics the clinic, using antibiotics and phages in combination rather than separately could prove to be important. I interpret the results found in this thesis as additional

information for the phage - antibiotic synergy research. To note is that the sulfamethoxazole MIC and concentrations used in this research do not match, and that is due to many inconsistencies and incoherence received between the MIC results and the results received in phage - antibiotic synergy, where the same concentration led to different OD values in the MIC and the phage - antibiotic synergy. As such, results with sulfamethoxazole included should be cautiously looked at, as this should be re-done.

There are four results of relevance found in this thesis, which could prove to be of importance when designing a phage-antibiotic combination therapy:

1) Phages are very potent inhibitors of bacterial growth on their own, and just like antibiotics, have two different modules (Wright *et al*, 2018) that implies there is an importance in the selection process for phage cocktails, though in this research, only three phages have been used and are not representative of the large bacteriophage pool that is available against *P. aeruginosa.*

2) Antibiotic - antibiotic combinations keep their synergies even when bacteriophages are added.

3) When bacteriophages are added in the mix, lower concentrations of antibiotics below MIC levels are sufficient to inhibit bacteria.

4) Three-way combinations are as potent as four-way combinations, there would be no need to add more antimicrobials to the mix. This makes manufacturing of commercial phage cocktails easier.

8.1 Mono effects of antimicrobials

The 6 antibiotics used were ampicillin, gentamicin, sulfamethoxazole, trimethoprim, chloramphenicol, and ciprofloxacin. Those 6 antibiotics were chosen based on their mechanism of action. Ampicillin belongs to the penicillin class of antibiotics and *Pseudomonas aeruginosa* is innately resistant to penicillins due to its β-lactamase AmpC, but recent papers (Alam *et al*, 2019) tend to show that ampicillin can still find use in ampicillin-resistant *P. aeruginosa* in combination with other antimicrobials, which is exactly what was found in the two- three- and four-way combinations. However, while ampicillin is commonly used with other antimicrobials, the values used here are high and therefore may not be suited for use in the clinics as, unless used intravenously at high dosage for an aggressive antibiotic treatment, and even then the side-effects are varied and severe, ampicillin is used in a much lower dosage. Gentamicin, chloramphenicol, sulfamethoxazole, trimethoprim are all four antibiotics that target different pathways in the bacterium, the 30S ribosomal subunit, the elongation chain and two different molecules on the folic-acid biosynthesis pathway respectively, and *P. aeruginosa* deals with this antibiotic through its efflux pumps, and more precisely, the MexAB-OprM efflux system. Ciprofloxacin is a fluoroquinolone which is currently used in the clinic as a last-resort treatment, and the defence mechanism the bacteria has against this specific antibiotic is by mutational changes to the fluoroquinolone targets, such as mutational changes to gyrase *gyrA* and *gyrB* as well as changes to the topoisomerase *parC* and *parE* (Lister, 2009). This means that out of all the antibiotics that are used in this experiment, *P. aeruginosa* has at least one way to protect itself, and for most of it, it implies the use of its efflux pump, but as a general pattern, it relies on preventing the antibiotic from entering the cell, at the exception of the penicillin-class of antibiotic. As such, the mono-effects that were observed as part of this experiment are explained by the sub-lethal MIC concentrations used, which in most case actually upregulates the efflux pumps genes (Morita *et al*, 2006). This explains the high bacterial density found in the mono-results (Fig.7), and why sub-lethal MIC does not reduce bacterial density that great.

There were 3 phages that were used in this experiment, PA10P2, 14/1 and ΦKZ. ΦKZ belongs in module 1 and affects the pilus, in particular, *pilR*, of which mutants with a mutated *pilR* will be resistant to ΦKZ (Wright *et al*, 2019). Meanwhile, PA10P2 and 14/1 affects the LPS, and *P. aeruginosa* defends itself by mutating the *wzy* gene, changing the structure of the O-antigen (Wright *et al*, 2019). The

results from the mono-treatments concerning the phages seems to indicate it takes longer to adapt to a phage attack than it takes to adapt to an antibiotic attack. This can be due to the "auto-dosing" effect of the bacteriophages which will see more hosts, and therefore more ways to replicate itself, the more bacteria there is available in its vicinity. However, since the mutations that are required to resist a phage attack are single point mutations, in this particular case, a mutation in the *wzy* and the *pilR* gene, the population density of the bacterium picks up again at 48 h and 72 h. As such, on their own, none of those antimicrobials seems to be efficient on the long term at sub-lethal MIC.

8.2 Phages in antibiotic-antibiotic combinations

Antibiotics have an already well defined map of collateral resistance or sensitivity, and results found in this thesis only serve to reinforce what was already known, and as such, results obtained from the PAS experiment (4.3) show that some antibiotics synergies, even when it is an antagonistic synergy, are not affected by the addition of a bacteriophage. It is not exactly clear why, as some of the recent work done with phages and antibiotics tend to show that antibiotic sensitivity is restored when the bacterium is subject to bacteriophages (Chan *et al*, 2016), but it would imply that the core problem lies within the antibiotic mix rather than the effect it has on the bacterium itself. As such, in this experiment, and in order to test those combinations, the concentration of antibiotics used in the following experiments, section 4.3 was picked as a sub-inhibitory concentration in order to not kill the bacteria when antibiotics was used, and the same was done with phages. The reasoning behind it is that I'll use antimicrobials in combination with each other and as such, I would not want the antibiotic to fully inhibit growth on its own, and to actually see the combined effect of both antimicrobials together, which is different to most studies that have been performed on the same subject, where they tend to use MIC rather than sub-MIC. Researchers typically use MIC levels of antibiotics in combination with phages then decrease the concentrations or increase it to see the phage's effect, when used in combination with antibiotics, and if it would allow to use lower concentrations of antibiotics (Chaudhry *et al*, 2017; Akturk *et al*, 2019). As such, it was found that in the two-way combinations featuring two antibiotics, the majority of the effect was caused by the antibiotic synergies on their own, for example ciprofloxacin and chloramphenicol have cross resistance (Fig.1, and Fig.8) while ampicillin and gentamicin have cross-sensitivity (Fig.1 and Fig.8). Then, when testing with bacteriophages in the phage , using the former two as an example, in a combination that showed to be effective, ampicillin + gentamicin, adding a phage on top of it whether it is PA10P2, 14/1 or ΦKZ (Fig.11, Fig.12 and Fig.13), did not affect the inhibition, as there was no visible growth. In contrast, using a bacteriophage alongside a combination that was not effective, such as ciprofloxacin and chloramphenicol, depending on the nature of the phage it had some varying effect. Again, this is in line with what was found in other research papers, except here we show that phages belonging in different modules can have varying effects as well. When used with PA10P2 (Fig.11), growth was inhibited in the first 24 h but the bacteria quickly evolved resistance to it and by 72 h, the growth boomed. When used with 14/1 (Fig.12), there was simply no inhibition of the bacteria. When used with ΦKZ, there is some visible growth, but it was as a much lower density than the two antibiotics on their own. As such, it can be concluded that antibiotics keep their synergies when used alongside a bacteriophage, whether it is a synergistic or antagonistic synergy. The only two combination that did not follow this conclusion were sulfamethoxazole and trimethoprim, and sulfamethoxazole and ciprofloxacin, for which the addition of a bacteriophage significantly improved the synergy that exists between them.

8.3 Phage-phage synergies with antibiotics

When it comes to the one antibiotic and two phages combinations (Fig.14, Fig.15 and Fig.16), every combination was effective at significantly reducing bacterial growth except for sulfamethoxazole and trimethoprim. This can be explained by trimethoprim needing a sulphonamide to be truly effective, so both of those antibiotics when used on their own did not reduce bacterial growth to the same level as other antibiotics alone with two bacteriophages. Regardless of which two phages you use, all the

combinations are at least more effective than their two-way counterparts, if not more. Bacteriophages belonging to different modules have different synergies, but in my experiment, which in some ways support the already existing research (Chaudhry *et al*, 2017; Akturk *et al*, 2019), shows that whichever phage is used alongside a suitable antibiotic, will yield significantly higher results, and there is, for the phage I tested, no significant difference between any of the three. This particular effect could be explained by the duration of the experiment, since the experiment ran for 72 h, it might not have been sufficient time for the *Pseudomonas aeruginosa* to evolve. Since evolution might not have risen in this system, the effect of providing phages binding to different receptors could have been missed.

Four way combinations (Fig. 17, Fig.18 and Fig.19) follow the same as the three-way antibiotic + antibiotic + phage (Fig.11, Fig.12 and Fig.13), where the synergy between the antibiotics is kept even when two phages are added on top of it, which indicates that no matter how many phages you add, the main, underlying property of the combination is the synergy between the antibiotics, and adding the viruses just deepens that synergy.

8.4 Comparing the degree of antibiotic-phage synergy

The observed vs predicted results that arose from Fig.20 to Fig.22 mention about the synergy between the combinations. The two-ways combinations panels (Fig.20) show rather clearly that antibiotic - antibiotic synergies (green) are varied between synergy, no synergy, and antagonists. Phage - phage synergies are predictable, and are currently predicted through an additive model, and antibiotic - phage are all synergistic. This pattern remains throughout the experiment and with the various antimicrobials added to those combinations. Where we had an antibiotic - antibiotic combination, adding a bacteriophage to make a three-way combination (Fig.21, black) affect positively the effectiveness of the combination at first, for the first 24 h. Over time, however, some combinations lose in efficacy and there is a migration of results (as seen in Fig.21 panel B and C) showing that the initial result at 24 h probably arose from the intensity of the treatment, having 3 antimicrobials. For some combinations, there was no obvious or stable synergy which led to that loss of efficacy over time. This same pattern is also seen in Fig.22, in the four-way treatments, where adding yet another bacteriophage, thus combining an antibiotic - antibiotic synergy alongside a phage - phage synergy, for some combinations, still does not resist the effect of time. This means that more antimicrobials to combat *P. aeruginosa* is probably not the solution, and that synergies are likely not improved by the addition of more antimicrobials. As such, the clear main result from this experiment would be that using successful synergies weights more than more antimicrobials and is likely to resist the effect of time.

8.5 Could resistance evolution have explained reduced treatment efficacy in time?

This is no surprise that *P. aeruginosa* has many defence mechanisms against many of the mainstream class of antibiotics (Morita *et al*, 2014), and while most of it is innate resistance, the effect of using several antimicrobials at the same time throws the bacteria in an arms race where it will require upregulation of its efflux pumps, and point mutations to specific genes to resist phage attacks, and this pathogen is efficient at it. As such, it is resistance to evolve and to be selected against specific mechanisms of defence. For example, when exposed against ampicillin, chloramphenicol and ΦKZ, we can predict there will be an upregulation of AmpC (Morita *et al*, 2006), an upregulation of efflux pumps (Morita *et al*, 2006) and a point mutation on the *pilR* gene (Wright *et al*, 2019). So over time, if it is not a combination that can eradicate the bacterium in a short amount of time, it is not impossible for the pathogen to evolve resistance by acquiring those specific traits, which will allow it to remain alive, but probably not in a viable way as the pathogen when pushed to that extent will not be fit. However, efflux pumps and point mutations are not the only mechanism of defence *P. aeruginosa* has, it also has mechanisms of tolerance. The most striking feature is that *P. aeruginosa* is a biofilm forming pathogen, and as such, biofilms can act as an additional barrier of resistance against antimicrobials. In this research, everything was done *in-vitro*, and therefore it does not represent the biofilm formation

that can be observed *in-vivo*. Biofilms are in fact one of the major reasons why patients with chronic conditions relapse after what appears to be an eradication of the pathogen (Fernandez-Barat *et al*, 2017). It has also been found recently (Fong *et al*, 2017) that bacteriophages can reduce the biofilm formation, and as such, leads to the belief that cocktails combining antibiotics and phages could in fact help eradicate *P. aeruginosa* infections. However, since this is still an *in-vitro* experiment, this research will need to be taken onto a model investigating the biofilm formation and structure after different treatments of antibiotics, phages, or a combination of both, in both an agar plate and on a model mimicking CF-lungs to really see the extend on which antimicrobials can affect this pathogen.

8.6 Clinically relevant strains of *P. aeruginosa*

The second experiment is based on chronic and intermittent strains of *P. aeruginosa* and was performed as a preliminary test for the conversion of the first experiment (PAS synergy) onto clinically relevant strains of *P. aeruginosa*, precisely five different chronically occurring strains of *P. aeruginosa* and five different clinically occurring strains of acute strains of *P. aeruginosa*. The results that arose from those tests (Fig. 23 to Fig.25) show massive variations in the efficiency of all the combinations tested and unfortunately there were no patterns to observe. Interestingly, the control of each of the chronic strains tested (Fig.23) showed no differences between the 24 h and 72 h time point while the control for each of the intermittent strains showed variations in the OD at 24 h and 72 h (Fig.24), which would confirm the theory that chronically occurring strains of *P. aeruginosa* can survive for longer periods of time without any assistance, as they are specialised into colonising a host for a long period of time. The same is not true in this case for the intermittent strains, as in the case of the intermittent 2 and intermittent 5 (Fig.24) had a significant drop in optical density after 72 h, which would underline either the desiccation process, or an original colonisation process that boomed the optical density high at 24 h, but over time, the death phase of the bacterium arose quickly. In the case of the intermittent 3, there is actually a rise in the optical density in the control after 72 h, which would underlie a slow set up phase, which looks strange for an acute bacterium, as you would expect acute infections to have a quick colonization process (Folkesson *et al*, 2012). As a general pattern from what is observable, it is that the chronic strains of *P. aeruginosa* seem a lot more resistant compared to the intermittent strains, as the variations within all chronic strains are a lot less significant than the variations in the intermittent strains (Fig.25), which would underlie that chronic strains are naturally more resistant to antimicrobials than acute strains, which makes sense seeing that chronic strains are specialized into colonizing a host for long periods of time (Folkesson *et al*, 2012), and therefore specialize into bacterial mechanisms of resistance. Meanwhile, intermittent strains specialize in colonizing a host as quickly as possible, and it is actually observable on the Fig.36 as the mean OD for the controls at both 24 h and 72 h is a lot higher than the chronic bacteria, the mean OD for all the treatments on the chronic strains are also, on average, smaller than the mean OD for intermittent bacteria, though what is to note is that also, on average, the 72 h average for the chronic strains rises compared to the intermittent strains that barely vary. This shows that chronic strains typically have a slow colonization process but specialize into their resistance mechanisms and are therefore able to colonize, grow and strive in a hostile environment while the intermittent strains have a sudden surge of colonization very quickly, but falls off once antimicrobials are in use. Those tests underlie the differences between chronic and acute strains, though no treatments could stand out as being efficient in all strains, and more importantly, only two-way treatments have been tested as part of this experiment, or even most strains, as all the variations within all the strains and treatment were so massive that further testing should be done on a one-on-one basis.

9. Future work

In the future, there are many more combinations that can be tested on the PAO1 strains, such as various other antibiotics from other antibiotic classes, as well as other phages from both modules. The sulfamethoxazole part of this research should also be re-done, as the concentration used for this research does not reflect on the MIC that was performed, as there was a massive incoherence

between the values received from both the MIC test and the phage - antibiotic synergy experiments. Molecular analysis of the strains after being subjected to various antimicrobials could also potentially reveal the resistance mechanism that can arise from being attacked by various antimicrobials at once, on either a molecular or even on a genetic level.

Combinations of a higher order could also be tested on the chronic and intermittent strains as the experiment done with both those classes of *P. aeruginosa* only tested up to two antimicrobials at once, while we could experiment with three or four antimicrobials at once just like the first experiment, which could indicate another level of complexity in the resistance mechanism of *P. aeruginosa* as such magnitude of treatment, on a clinically relevant strain of *P. aeruginosa*, could indicate just how potent *P. aeruginosa* can be as a pathogenic bacterium. Another major aspect in the resistance mechanisms of *P. aeruginosa* is the fitness cost of evolving resistance to one or several antimicrobials. Preliminary experiments on the chronic strains of *Pseudomonas aeruginosa* compared to the intermittent strains of the bacterium shows that while they are both the same species, they grow differently, which underlies the fitness cost of being able to strive in a hostile environment. Along with the fitness cost, biofilm assays could also be performed to see if there is a major difference between the chronic and intermittent strains, as since the chronic strains specialised in being a persistent pathogen in the host, it would be expected that the chronic strain's biofilm would be denser, or less permeable to antimicrobials. Then, to translate the research and findings into a more clinically oriented context, an investigation on the immunological aspect of a chronic and persistent infection could be carried out, with an emphasis on the interactions between the host's immunological system and antibiotics and bacteriophages on the survival of *P. aeruginosa*. Then, translating that research onto a cystic fibrosis-oriented context, as CF patients typically show a deficient immunological system (Warner, 1992). One of the biggest constrain would be that antibiotics are still highly toxic for the patients as it tends to cause collateral damage on the surrounding microbiota. As such, combining several antibiotics might lead to a higher burden, and therefore I would advise on concentrating on three-ways antimicrobials with two phages and one antibiotic, as phages do not tend to cause that type of collateral damage. However, due to the already existing microbiota, especially in a CF-lung, where the microbiota tends to be a lot more populated, there could be interferences, even though it would be predicted that bacteriophages are specific enough not to be perturbed in that aspect. Antibiotics were not previously successful, even at high dosage, to treat CF patients, because it could not clear out the biofilm, and therefore the addition of phages might solve this issue. All in all, there is still a lot to test on *P. aeruginosa* and its evolutionary and pathogenic effects on humans, as well as the ways we must control that bacterium's infections.

10. Supplementary material

Named in the experiments	Bacterial strains	Classification
	PAO1	Natural
Intermittent 1 (I1)	B3-0	Intermittent
Intermittent 2 (I2)	B6-2	Intermittent
Intermittent 3 (I3)	B12-0	Intermittent
Intermittent 4 (I4)	B13-2	Intermittent
Intermittent 5 (I5)	B28-1	Intermittent
Chronic 1 (C1)	2-4	Chronic
Chronic 2 (C2)	6-11	Chronic
Chronic 3 (C3)	10-13	Chronic
Chronic 4 (C4)	11-10	Chronic
Chronic 5 (C5)	16-3	Chronic

Fig. S.1: Table of the various intermittent and chronic strains used in the second experiment (Section 4.4)

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Using the reference style of Harvard Reference Format

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