Investigating Phage-Antibiotic Synergy (PAS) using T4 Bacteriophage in *Escherichia coli*.

Abstract

Antibiotic-resistant bacterial strains pose an ever-present problem for human healthcare and other areas such as livestock food production. This problem is only getting worse with time, and other strategies for controlling pathogenic bacterial growth are essential. Bacteriophages have shown promise in recent years, particularly in combination with antibiotics to produce a synergistic response – Phage Antibiotic Synergy (PAS). Many studies have looked at PAS, but few have run trials that include analysis over several varying concentrations of phages in addition to varying antibiotics levels, and the methods often require time-consuming assays to gather data. In this study a novel approach is proposed, using optical density (OD) measurements to track the growth curves of many populations simultaneously in a 96-well plate over a period of 6 hours. Detailed analysis of the resulting data is carried out, and the detection of synergy via a grid system and smile-frown curves (Pena-Miller et al. 2013) undertaken, with great potential but mixed results with the data obtained here.

Introduction

Bacterial infection and the resulting diseases are still a major health issue, despite dramatic improvements in the last 300 years in hygiene, living conditions and available treatments such as antibiotics. Some would argue that the situation is in fact worsening, due to the increased prevalence of multi-antibiotic resistant bacteria, particularly in hospitals and animal agriculture where antibiotic use is more frequent. Clearly new approaches are needed, and one such avenue gaining traction at the moment is phage therapy.

Therapeutic application of phages to treat bacterial infection is a fairly old practice, but it had fallen out interest in the west, with the discovery and popularisation of antibiotics, until recently. In the last 15 years, increased focus on phages has seen many new studies carried out, investigating phages as a standalone treatment but also in combination therapy, with application of both phages and antibiotics together. This combined effect has the potential to provide benefits beyond simply adding the killing effects; if the action together is more than the sum of its parts, it is known as Phage-Antibiotic Synergy (PAS), and studies in this area have seen promising results.

Escherichia coli is one of the most common pathogenic bacteria, causing many nosocomial infections (Tabasi et al., 2015). Several PAS studies carried out on *E. coli* have shown promise far: phages tested include T4 and T4-like viruses (Ryan et al, 2012; Comeau et al., 2007; Lopes, Pereira and Almeida, 2018), as well as ΦMFP (Comeau et al., 2007), ECA2 (Valério et al., 2017) and others. Papers tend to focus primarily on the effect of different antibiotics at varying concentrations, with a one type of phage facilitating the antibacterial effect. In studies (primarily following this structure), PAS against *E. coli* has been found multiple times with cefotaxime and ciprofloxacin, but for the most part has been lacking with other antibiotics, including a lack of PAS effects with antibiotics that the bacteria had *a priory* resistance to.

It is obvious that the effects of combined antibiotic and bacteriophage effects are not simple. Sometimes PAS is only found at sub-lethal doses of antibiotic (Valério et al., 2017), other times over all concentrations tested. The length of delay before adding substances can change the result: Lopes, Pereira and Almeida (2018) found that antibiotic added at t = 0h exhibited no synergy, whereas if it was instead added at t = 6h, PAS was observed. One of the biggest areas still underexplored seems to be investigating the effects of varying phage MOI, rather than just antibiotic concentrations. One study has been carried out to investigate this, using multi-resistant *Acinetobacter baumannii* (Jansen et al., 2018). Strong PAS was

reported at very low MOIs (10^{-7}) but possible antagonism at higher antibiotic concentrations. This might offer a more realistic model of the situation *in vivo*, where initial phage concentration at the site of infection would likely be very low.

In light of these findings, a more exhaustive exploration of PAS is proposed in this study. Cultures grown in parallel in a 96-well plate allow many combinations of concentrations of both types of antibacterial to be explored at once, with bacterial load recorded at regular timepoints through OD measurements. Data analysis from this investigation showed antibiotic efficacy at varying phage concentrations, and differences in growth curve patterns were displayed across the plate for both Ampicillin and Streptomycin. Finally, an attempt was made to identify PAS mathematically, using the 'smile-frown' method described by Pena-Miller et al. (2013) in their paper on synergy/antagonism between two different antibiotics, with mixed results.

Materials and Methods

Bacterial culture preparation

E. Coli strain BW25113 was streak-plated from lab cryo-store. For each plate run, a single colony was removed and cultured for 14h in 200ml LB broth. 1ml aliquot of suspension was added to 200ml fresh LB, then cultured for 3h to reach mid-exponential phase (Smith et al., 2018). An aliquot was then taken and diluted to a target final optical density of around 0.03, within the well plate wells, corresponding to ~10^7 CFU ml^{-1}. Old streak plates were replaced with freshly created plates from cryo-store bacterial stock every 2-3 weeks.

All cultures with bacteria (and with phage) including 96-well plates grown in incubating conditions at 37°C. Liquid colonies cultured on a shaker plate. All growth media and dilutions were made using LB broth with NaCl concentration 10g L^{-1}.

Phage propagation

T4 *E.* coli bacteriophage was used in this trial, propagated from a sample of lab stock. Original T4 phage (several repropagations previous to this) was a product of ATCC, product code ATCC® 11303-B4™. An *E. coli* culture was prepared: streak plate colony to 14h overnight growth, 500µl aliquot of suspension added to fresh LB and grown for 6 hours to reach late exponential phase. 500µl phage stock (at 5x10^9 pfu ml^{-1}) was used to infect 6h *E. coli* culture and left to propagate for 18h.

Phage separation: 25ml aliquot of 18h infection flask was taken, and centrifuged 30mins at 4000rpm. Supernatant was twice filtered with separate .22µm micropore filters, then filtrate was refrigerated at 5°C and used for the duration of the study.

Spot assays on *E. coli* lawn confirmed presence of phage in created stock, estimated concentration ~6x10^8 pfu ml^{-1}. Assumed no significant degradation over period of study (~ 3 months).

Antibiotic preparation

Antibiotics used in this study were ampicillin and streptomycin, solutions made up from Sigma-Aldrich ampicillin sodium salt powder and streptomycin sulphate powder respectively. Stock solution of concentration 10 mg ml^{-1} was made up for both antibiotics, by dissolving in Milli-Q water then filtering through .22µm micropore filter under aseptic conditions. This stock was frozen at -20°C in 100µl aliquots, then defrosted and diluted in LB as needed throughout the study. Fresh stocks were made up every two weeks.

Well-plate filling

This study was carried out using 96-well Greigner flat-bottomed plates and 96-well fluorescence plates, on the well-plate readers clarioSTAR and fluoroSTAR. Plate layout is shown in figure 2A, with an arranged grid of combinations of concentrations of antibiotic and bacteriophage, and *E. coli* bacteria present in all wells

at a uniform concentration as described above (except those marked LB, which contained LB only). Separate plates were made with Ampicillin and Streptomycin.

Plate filling order was as follows. Wells were pre-filled with specific volumes of LB. Antibiotic was added to the highest concentration column and diluted along the plate using a multi-channel pipette. Next bacteriophage was added at pre-diluted concentrations, then bacterial suspension added last to make a final volume of 100µl in each well.

Plate reading protocol

Automatic time-interval optical density (OD) reading was set up on the plate readers. Parameters were: 600nm wavelength absorption read, 10 minute read intervals, single centre reading per well, 6h total run time. 200rpm orbital shaking in between reads.

Analysis

The OD data from the plate readers was exported into Microsoft Excel files, then processed and analysed using MATLAB. The data in the files was aligned and zeroed using the negative control data. From there, 'growth score' (fig.1, also see results section) (proportional to the integral of the growth curve for each well), was calculated over the whole time period, then zeroed and normalised. The main script to plot the data offers various options for how to process the data, and which graph(s) to plot. The full suite of functions and scripts created for the project are available free to use (with credit) on GitHub, at https://github.com/finnmerlett/bacteriophage-project

Results

Data from the plate read over time was plotted in a grid of graphs to give the best overall feel of the information collected (fig.2B, combined data for Ampicillin). Each graph represents one well, with a black or white line for each average growth curve (OD over time) at that well location, over all plate runs for this antibiotic. The red band indicates ± 1×SD.

Interestingly, all wells containing phage at any concentration had reached an OD ≤ 0 by the 6h end time, indicating the majority of bacteria had been killed. This made it difficult to discriminate between wells on basis of MIC, as all wells with phage were above the effective MIC for the combined antibacterial effects (bacteria were always inhibited by t = 6h). To discriminate effectively, the area under each growth curve was used as a 'growth score' value for that well, and the values were normalised across all wells containing phage (blue box, figure 2A) to avoid huge growth scores on the bottom row (no phage) dwarfing the other data. The normalisation process linearly scaled all growth score values, to align the lowest integral well in the blue box (fig.2A) with 0 and the highest inside the box with 1.

The graph background shade of green in figure 2B represents the growth score, with lightest in the top left where antibacterials were the strongest, to the darkest where exponential growth was not impeded at all. The bottom row of the plate shows the MIC of ampicillin to be around 3.54 µg ml^{-1}, where exponential growth begins unchecked. The lab MIC value is recorded at 5µg ml^{-1} (Ashley et al. 2018).

Normalised growth score plotted against antibiotic concentration, for various phage concentrations behaves as expected (figure 3). As the phage concentration increases, the growth score lowers more at lower antibiotic concentrations. The growth score appears to reach a minimum of ~0.2, even with phage concentration at 5000 pfu ml^{-1}. With less phage (0, 50 pfu ml^{-1}), a growth spurt occurs from low to medium growth score with falling antibiotic concentration. Higher phage levels cause the growth spurt to be delayed to an even lower antibiotic concentration, or not occur at all. Results for both Ampicillin (figure 3A) and Streptomycin (figure 3B) are similar, although the growth spurt occurs at a lower antibiotic concentration in Streptomycin. Standard deviation (SD) in the graphs is larger than ideal, however, and so interpretation must be tenuous. The spread of the SD interestingly increases as phage counts decrease, maxing out at 50 pfu ml^{-1} with the largest uncertainty present here in both antibiotic plates.

Patterns in 2D within the blue box area of the plate were difficult to analyse by eye from figure 2B, so the next step was to treat the growth score at each well position as a height value, and then fit a smoothed surface through the points to help ignore noise and anomalies and attempt to identify synergy. A regular scale was needed for the latter goal, and so a further subsection was decided upon, marked by a yellow box on figure 2A. This simply removed any wells with 0 of either phage and/or antibiotic, as 0 concentration would not work with the logarithmic concentration sequences (present over the rest of the well-plate). A smoothing value of 25% was selected to balance feature preservation and smoothness, and coloured with contoured bands to show the height of different areas (figure 4).

Discussion

Both antibiotics demonstrated simple facilitation: for the most part there was an increase in the antibacterial effects when a combination of bacteriophage and antibiotic were used together (fig.3). This supports the idea that combination therapy could be useful to increase the bacterial clearing speed, as the growth score used in this study represents bacteria minutes, i.e. bacteria × length of time alive. Although bacteria were always cleared if phage was present, a lower growth score indicated a faster clearance time, and if applied to a situation *in vivo* could imply a lighter bacterial infection for the patient's immune system to handle more easily.

This kind of near total clearance even at MOIs as low as 5×10^{-6} (phage at 50pfu ml^{-1}, *E. coli* at 10^7 cfu ml^{-1}) is not unheard of (Jansen, 2018), but it is unusual and wasn't apparent in the other studies carried out on *E. coli* previously. It is possible that part of the reason is the mild nature of the strain used in this study – with no *a priory* antibiotic or phage resistance, and non-pathogenicity, the *E. coli* might just have been killed too easily by the bacteriophage. This is still useful though, as it represents the impressive potential of bacteriophage's effects, even at low doses more representative of *in vivo*.

The slightly greater bactericidal efficacy of streptomycin was expected, as the MIC is slightly lower than for Ampicillin. Both antibiotics appeared to cause similar responses in combination therapy. Streptomycin has had mixed reports with regard to PAS, with papers saying PAS was found (Torres-Barceló et al., 2014), and others demonstrating antagonism (Cairns et al., 2016), so this result is inconclusive.

Several issues brought unwanted random noise into the data, particularly working with such low numbers of phages per well – variations in the actual numbers of phages carried across due to the stochastic nature of serial dilutions, would have lead to large percentages differences. Just 2 phages more or less would equal a ±40% initial phage presence in the well, leading to random variation in growth score. You can see this in the large increase in size of the red standard deviation bands in figure 2B towards the lower phage concentrations (3rd, 4th and 5th rows of the plate). Solutions to this could be to either work with a hardier bacteria species, or to introduce an artificial phage degradation chemical to the wells, to mimic the loss of phage that would occur naturally *in vivo*. Another source of noise appeared to be a random product of the OD reader – if further experimentation is done, at least 3x repeat readings at each timepoint would be incredibly useful to help identify and eliminate this noise.

Attempting to determine synergy in a similar style to that utilised by Pena-Miller et al. proved to be more of a challenge than anticipated, due to a multitude of reasons. The definition of what specifically classes as synergy is not rigorously defined, with some papers stating that 'greater than multiplicative effects' constitutes synergy, whilst others use the definition 'greater than additive effects'. The situation here is also more complicated than Pena-Miller's antibiotic synergy, as phages behave in a fundamentally different way to antibiotics. Antibiotics concentration will remain constant from the point of addition, perhaps decrease slightly through degradation, but phages will increase in number exponentially.

The 96-well plate was set up with logarithmically decreasing concentrations of both antibiotic and phage along the two axes, and when contour-plotted with no axis adjustment, the shape indicates antagonism

rather than synergy (fig.4). However, when the antibiotic axis (horizontal) is adjusted to counter the logarithmic scale, the pattern looks more like facilitation (no positive or negative synergistic effects) (fig.5).

Any attempts to draw conclusions about synergy in the systems investigated here should only be considered as rough guidelines for future work. With the code setup to process well-plate reads effectively, and plotting and analysis tools on hand, the way is paved for future work to increase the body of data and eliminate some of the random error through averaging over more datasets.

Conclusion

Although this investigation was a promising start into a more thorough look at PAS, there is still more work to be done before this method can be relied upon fully. Evidence of facilitative interactions between bacteriophages and antibiotic was found, and together have a greater antibacterial effect than either alone even if not greater than the sum of their respective effects, for both Ampicillin and Streptomycin. T4 bacteriophages were found to be incredibly effective against this strain of *E. coli*, usually completely clearing the bacterial population within 6 hours with or without antibiotics, and more research into the cause of this efficacy would be interesting, in an effort to emulate it in other phages. The antibiotics did have an effect though, even at quite low concentrations, contributing clearing speed and limiting the maximum bacterial load when added in combination to phage.

While hampered by several issues, I think this method shows potential for a wider look at the effects of PAS with various different antibacterial combinations, given more time and research to standardise the process. In particular, I feel the process can offer a useful way of investigating the effects of phages in combination therapy at low MOIs, with ease and autonomy of trials of many populations in parallel.

Figures

Figure 1

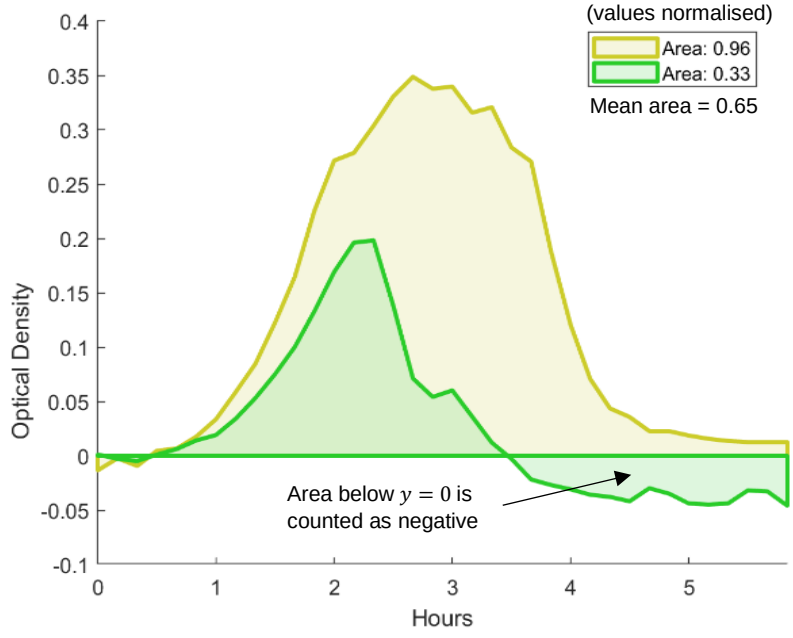

Figure 1 Illustration of the method used to calculate 'growth score' values for each well in the 96-well plate. Green and yellow lines are the growth curves from the same well in two different datasets. The area under each curve is calculated, all values normalised and linearly scaled, so the minimum and maximum values found in the blue boxed area (fig.2) of the well plate correspond to values 0 and 1 respectively. The normalised area values are then averaged in each well to give the mean dataset used to display growth score for that setup.

Figure 2A

Antibiotic concentration / μg ml^{-1}

Bacteriophage concentration / pfu ml^{-1}

5000 →	5.00	3.536	2.500	1.768	1.250	0.884	0.625	0.442	0	LB
1581 →	↓	↓	↓	↓	↓	↓	↓	↓	↓	LB
500 →										LB
158 →										E.coli
50 →										E.coli
0 →										E.coli

Wells used in contour plot

Wells used to calibrate 'growth score' normalisation

Figure 2B

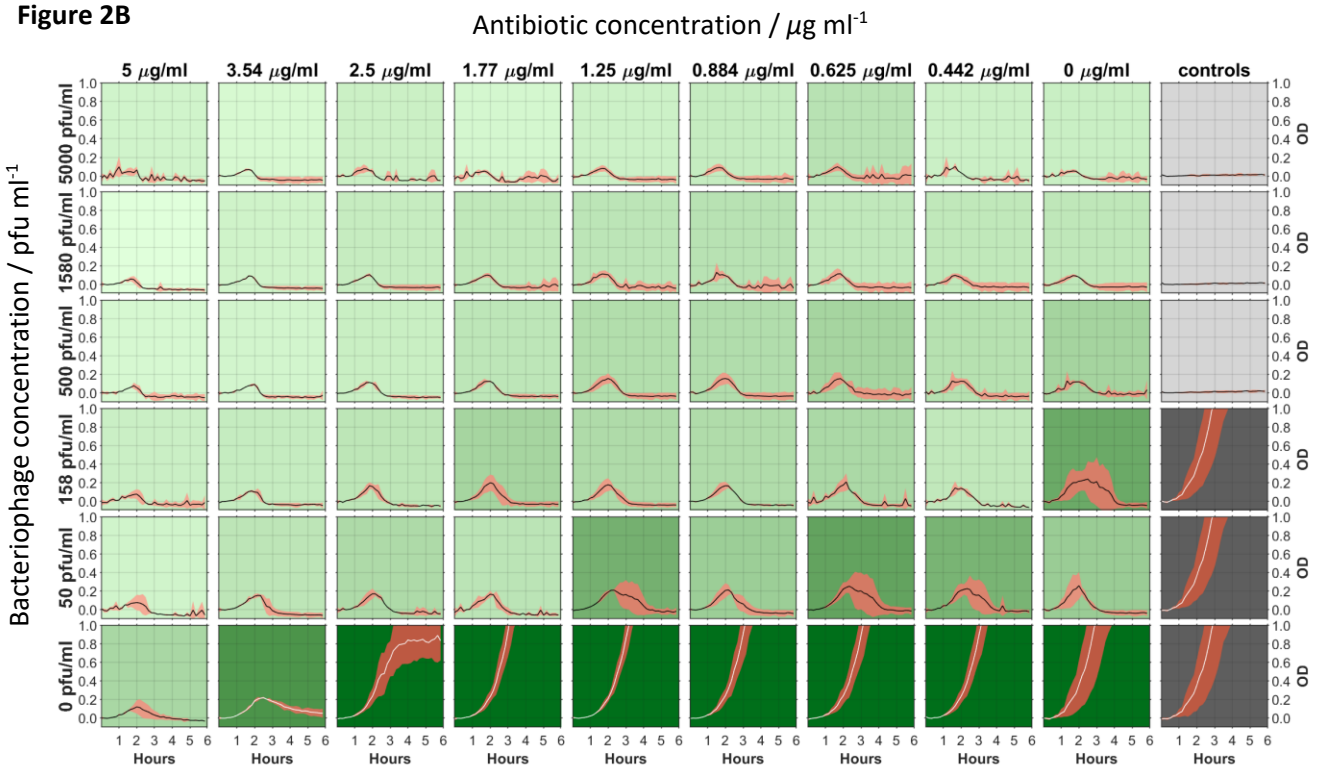

Figure 2A 96-well plate layout schematic of antibacterial concentrations. A boundary one well wide is left empty to avoid potential temperature inconsistencies near the edge of the plate, hence the layout dimensions are 6×10 (rather than the expected 8×12). Arrows next to a number on the layout indicate that concentration of that antibacterial substance in every well along the row or column in the direction of the arrow. The final column contains positive and negative controls, with no antibacterial substances. Values in the left-hand column starting at 5000 are phage concentration / pfu ml^{-1}, values along the top row starting at 5.00 are antibiotic concentration / cfu ml^{-1}. Enclosed in yellow and blue boxes respectively are the wells used in the contour plot, and wells included in calculating the normalisation of growth score values.

Figure 2B Plate view as in figure 2A, but with the average growth curve (black or white line in each cell) for each well plotted in place. Red band is average curve ±1×SD. Intensity of green background in each graph corresponds to the averaged normalised growth scores at each well, as described in figure 1. Units are arbitrary.

Figure 3A

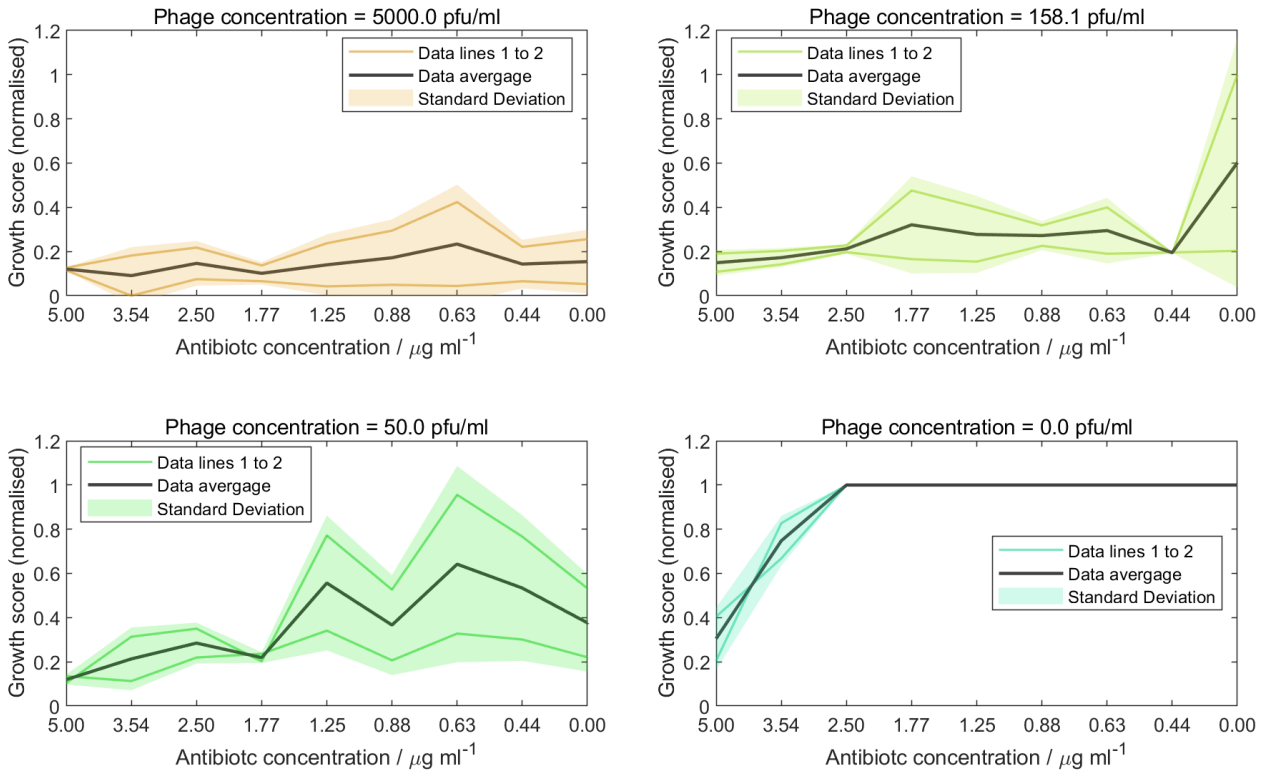

Figure 3B

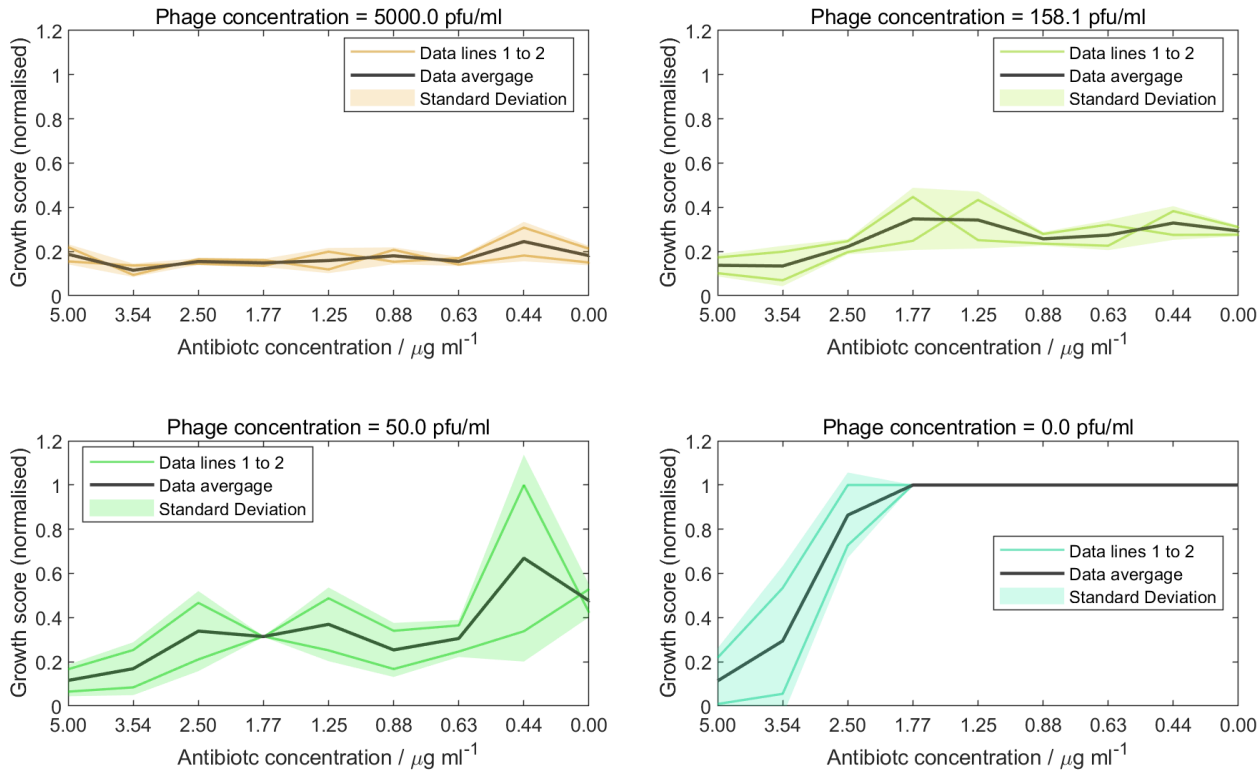

Figure 3A Growth scores across entire Ampicillin concentration range, at four selected phage concentrations. Black lines are the average over the two datasets, coloured lines are the individual datasets, light coloured fills are ± 1×SD from the data average. The phage concentrations between 5000 and 158.1 pfu ml^{-1} were left out as the data they showed was very similar to the 5000 pfu ml^{-1} set.

Figure 3B Growth scores as above, but across entire Streptomycin range instead. Everything else is the same.

Figure 4A Smoothed contour plot of 'growth score' for Ampicillin – Bacteriophage combination

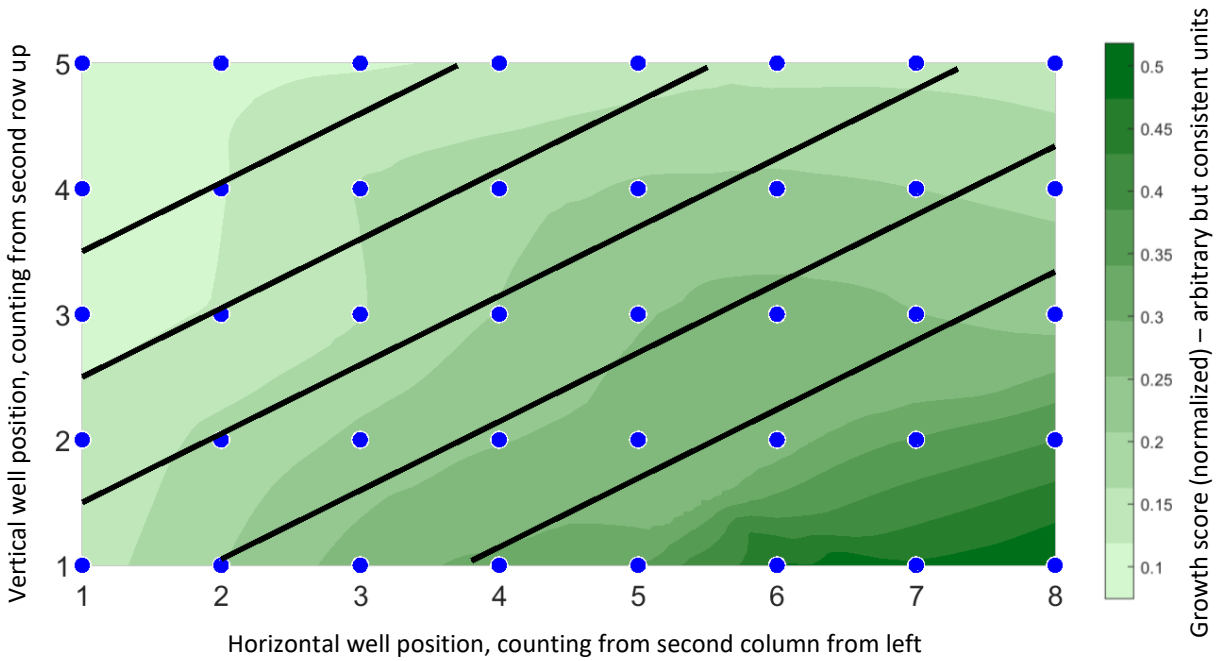

Figure 4B

Diagonal transects across smoothed 'growth score' for Ampicillin – Bacteriophage combination

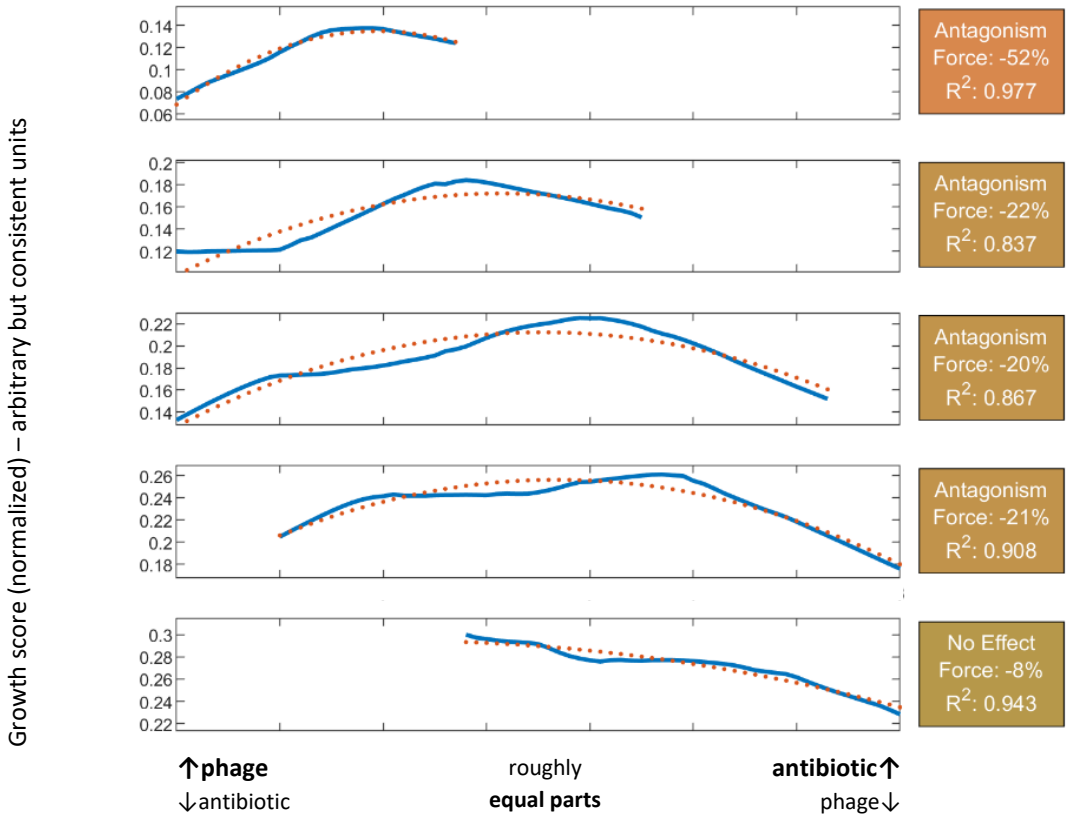

Figure 4A Growth scores for Ampicillin – Bacteriophage combinations over yellow boxed subsection of well plate (see figure 2A). Smoothed surface has been fitted to the growth score values and displayed as a contour plot with shades of green corresponding to smoothed growth score (see colour bar). Blue dots represent the original well locations, black diagonal lines are transects across the surface plotted below.

Figure 4B Transects diagonally across the smoothed 'growth score' surface. No interaction is assumed to give a straight line, synergy would be expected to be a 'smile' shaped curve as growth score would dip when a combination of phage and antibiotic were used, and antagonism a 'frown' curve. This curve was estimated by fitting a 2nd degree polynomial to the transect, and then multiplying the x^2 coefficient by a set constant to give the 'force' score corresponding to the strength of interaction. R^2 score is confidence value, if < 0.85 → 'Unknown'

Figure 5A

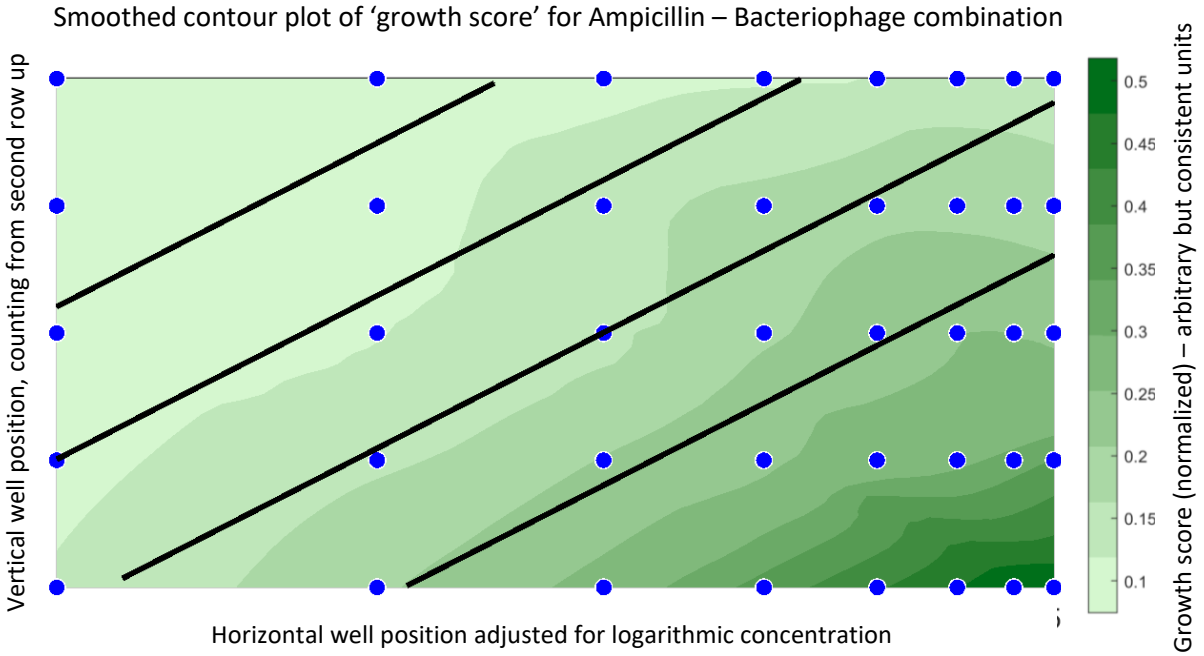

Figure 5B

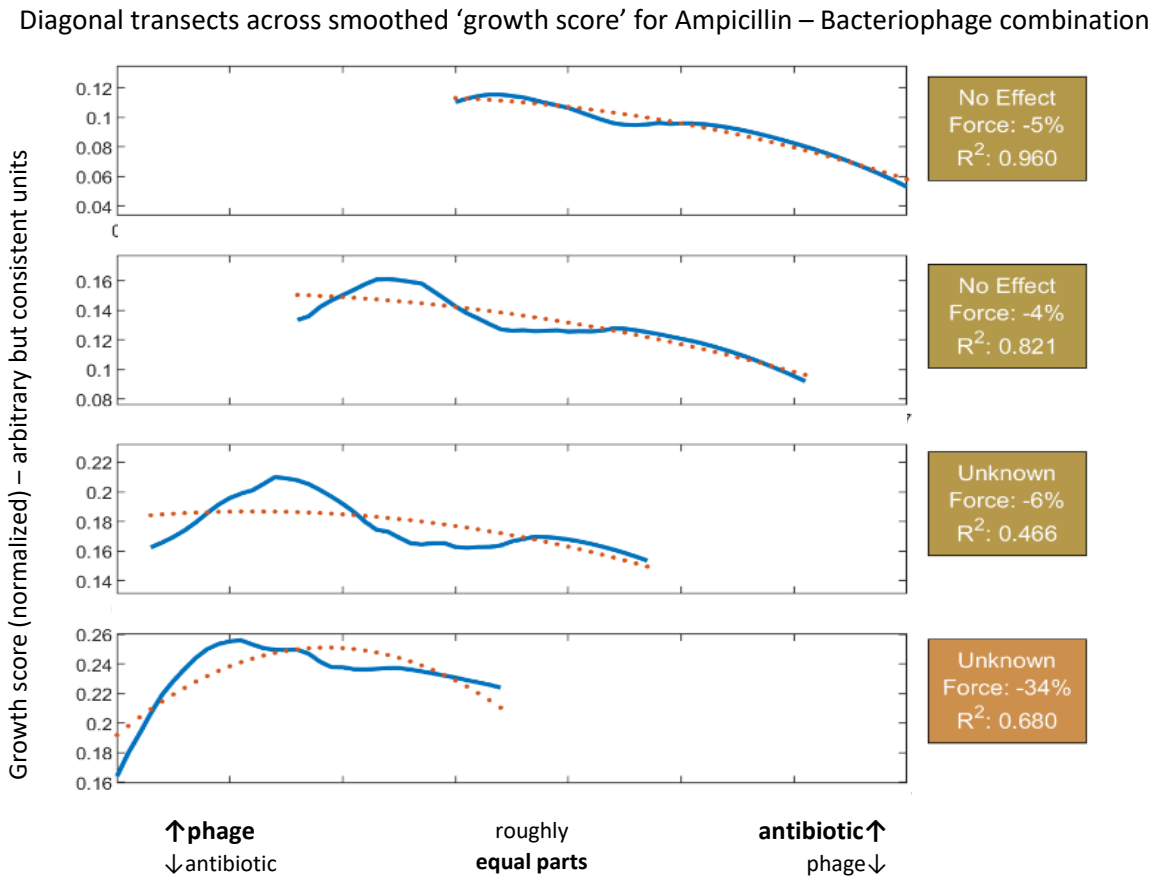

Figure 5A Smoothed growth scores for Ampicillin – Bacteriophage combinations over yellow boxed subsection of well plate (see figure 2A). Blue dots represent the original well locations, adjusted on the x axis to take into account the logarithmic dilution scheme. Phages left linear due to their exponential growth nature over the course of the experiment. Black diagonal lines are transects across the surface plotted below.

Figure 5B Transects diagonally across the smoothed 'growth score' surface. No interaction is assumed to give a straight line, synergy would be expected to be a 'smile' shaped curve as growth score would dip when a combination of phage and antibiotic were used, and antagonism a 'frown' curve. x^2 coefficient of fitted polynomial gives the 'force' score corresponding to the strength of interaction. R^2 score is confidence value, if < 0.85 → 'Unknown'. These transects do not display clear synergy or antagonism.

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Acknowledgments

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