School of Physics and Astronomy

Proposed Project Title
First Year Report and Literature Review

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Abstract

The abstract is a short concise outline of your project area, **of no more than 100 words**.

Signature: Date:

Supervisor: Dr. Rosalind Allen

Contents

1 Background

Since their discovery in the early 1900s [1], antibiotics have shaped modern medicine, and indeed modern society as a whole. Yet despite their prevalence, with over 260,000,000 courses of antibiotics prescribed in the USA alone each year [2], very little is still known about the actual underlying pharmacodynamics. I.e., how these chemicals actually regulate the growth and death of bacteria. This lack of understanding is becoming increasingly significant with the rising emergence of antibiotic resistance. There were over 58,000 deaths in newborns under the age of a year in India in 2013 due to drug-resistant strains of bacteria [3], with experts predicting that the number of deaths from antibiotic-resistant bacteria will number in the millions by 2050 [4].

This project aims to shed further light on how the application of antibiotics causes bacterial populations to evolve and proliferate over time. In particular, in the cases of when the applied antibiotic concentration has the form of a gradient. A considerable portion of antibiotic research has been performed under ideal conditions with constant, uniform antibiotic concentration [5], however it has only recently been proposed that the effects of spatial heterogeneity may be a major factor in the emergence of resistance and the efficacy of drugs [6]. While these concentration gradients can simply arise due to scenarios such as diffusion throughout body tissue [7], the scenario which is most pertinent to this project is that of antibiotic concentration throughout biofilms.

Biofilms arise when microbial organisms adhere irreversibly to a surface and then begin to secrete various polymers which further aid in surface and inter-microbial attachment [8] and creates a "slimey" surface. These structures are particularly problematic as it is difficult to achieve sufficient drug penetration to adequately curtail microbial growth, which leads to an increase in the persistence of infections [9].

The ability to inhibit and even prevent biofilm growth and formation has not only a multitude of medical applications, but industrial as well. In the shipping industry it's estimated that around 10%, up to even 45%, of all fuel consumption of large shipping vessels arises from overcoming the hydrodynamic drag caused by biofilm formation on ship hulls below the water level [10]. This not only has economic influences, but also major environmental implications. When compared to other nations, the shipping industry is the 7th largest producer of CO_2 on the planet [11]. Therefore, research into how these marine biofilms form and develop is incredibly important.

Recently, several physical methods of reducing marine biofouling have been developed, which range from physical coatings that inhibit microbial attachment due to their topography [12] to usage of ultraviolet radiation [13]. However, the most widespread technique is that of anti-microbial paints which are applied to the boat hulls and then leach various antimicrobial compounds over time which inhibit biofouling [14]. It is this latter method which is of relevant interest to this project due to its analogous nature of applying antibiotics to bacterial biofilms.

This project will involve creating simulations of a range of scenarios where bacterial populations experience antibiotic gradients, and will investigate how these populations develop over time. Including but not limited to; the effects of bacterial evolution, differing types of growth-rate dependent antibiotics and populations with heterogeneous species and resistance distributions.

2 Review of Background Bibliography

- overview of antibiotics - how they work etc
- Causes of antibiotic crisis
- growth rate dependent antibiotics, β-lactams etc
- how biofilms form - the bacteria change when in a biofilm, why are they difficult to get rid of
- overview of biofouling, current ship hull anti-microbes
- difference of films and fouls
- brief mention of macrofouling
- surface colonisation
- opposite gradient directions for films and fouls
- how biocidal paint works, diffusion coefficients etc
- mention the differnt algorithms possible for the sims, gillespie etc - and give overview of what we used
- as in, mention the rates, algorithm and description of why used

2.1 Antibiotic gradients

The issue of antibiotic resistance is one of the key issues plaguing modern science as of today, and as such, the field commands a large volume of research dedicated to it with a wide range of methods involved, ranging from experimental to theoretical methods including both modelling and more in depth simulation [15, 16, 17]. While current research is investigating a wide variety of factors which contribute to the development of resistances, from mutational path lengths [18] to the synergistic effects of various antibiotics [19], the majority of these studies, including all studies referenced so far in this section, are performed with constant and uniform concentrations of antibiotics.

While this situation tends to be more convenient for idealised in vivo experiments, many real-world in vivo scenarios do not have these conditions. Many naturally occurring structures, typically the ones which the drugs are intended to target, do not allow said drugs to fully permeate throughout the region, creating drug gradients where the drug concentration can vary noticeable over space. These gradients can arise in a variety of situations, from tissue [20] to bacterial biofilms [21].

It is only in recent years that the effects of these spatial heterogeneities have been considered as a serious influencer on the evolution of resistance. In fact, models have been constructed which predict that drug gradients actually tend to accelerate the evolution of resistance [22]. To test this hypothesis, Zhang et al. [6] constructed an apparatus involving an array of several interconnected microhabitats with an antibiotic gradient of ciprofloxacin. This gradient ranged from no discernible antibiotic concentration at the

top of the array to a concentration of 10μg/ml at the bottom. This concentration is around 200 times the minimum inhibitory concentration (MIC) of ciprofloxacin [23]

The array was then inoculated in the central microhabitats with around 10^6 wild type *E. coli*. Chemotaxis due to nutrient consumption then drove the bacteria towards the perimeter microhabitats. Once resistant mutants had fixed, they then spread and propagated throughout the array, as shown in Figure 1.

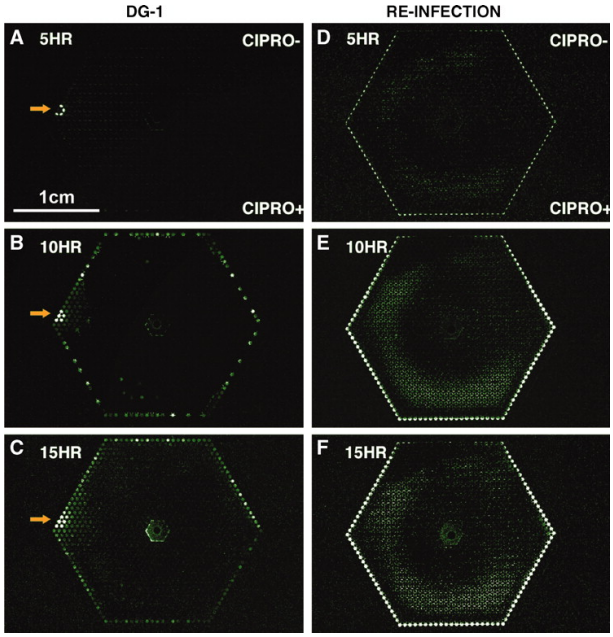

Figure 1: The proliferation of the bacterial population when exposed to an antibiotic gradient. The LHS shows the development of the population after the initial inoculation, and the RHS shows the development of an identical slide which was inoculated with the resistant mutants. Zhang et al., 2011

To confirm that it was indeed the gradient which allowed for this enhanced development of resistance, Zhang et al. conducted a range of further experiments. Firstly they eliminated the gradient by including ciprofloxacin at both ends of the array. This uniform antibiotic concentration resulted in no growth from the inoculated wild-type *E. coli*, as can be seen in Figure 2.

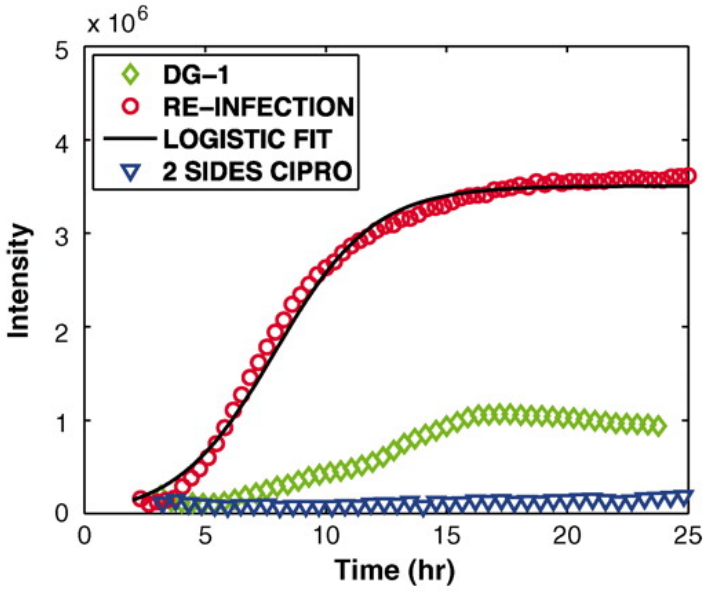

Figure 2: The summed growth over the entire array for various scenarios. The green diamonds are the initial experiment where the wild-type *E. coli* were placed in the antibiotic gradient. The red circles are the resistant mutants which were then used to re-inoculate an identically set up array. The blue triangles are the wild-type exposed to a uniform antibiotic concentration. Zhang et al., 2011

Zhang et al. then performed the experiment in a 96 well plate with a gradient present, but with the microhabitats now disconnected from one another, with discrete antibiotic concentrations in each well, ranging from low to high concentrations as in the previous array. This also resulted in no resistance being developed, as the growth of the bacterial colonies simply decreased as the concentration of ciprofloxacin increased, thereby implying that bacterial motility across the gradients is what is key to the emergence of resistance.

To confirm the effects of motility on resistance development, Zhang et al. then used a series of agar plates with the same gradients as the microhabitat arrays, but varied the initial population sizes. Once they reached an initial size of 10^8 bacteria, no motility was observed and as such growth only occurred in the regions of the plate where the antibiotic concentration was below the MIC, and no clear resistant mutants emerged.

Zhang et al. then proceeded to investigate what the source of the resistant mutants were, whether they were simply the descendants of already-present mutants, or if it was indeed legitimate de novo mutation as a response to the applied antibiotic stress. Zhang et al. reasoned that if resistance emerged due to preexisting, albeit rare, mutants, then either growth would have occurred above the MIC in the 96 well plate (which did not occur), or by serial dilution of the starting wild-type colony, they an initial population density would eventually be reached which contained no resistant mutants, and therefore no growth would occur.

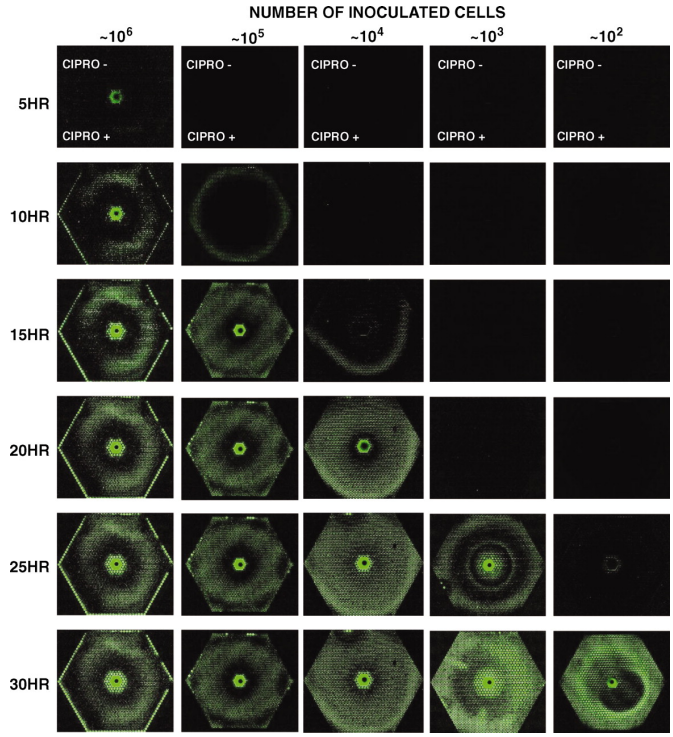

Figure 3: Bacterial growth over time in an antibiotic gradient up to 1 μg/ml of ciprofloxacin for various initial bacterial population sizes. The initial sizes range from the order of 10^6 to as low as 10^2. It can be seen that even at the lowest starting densities, resistance still emerges. Thus supporting the argument that resistance emerges due to de novo mutation and not preexisting mutants. Zhang et al., 2011

As can be seen in Figure 3, even the most diluted populations developed resistance when exposed to the antibiotic gradient. These results heavily support the proposal that the source of emergent resistance is due to de novo mutations and not from preexisting mutants distributed amongst the wild-type. Zhang et al. offered the following explanation as to why antibiotic gradients allow for such prevalent opportunities for resistance to emerge.

> A spatially complex environment may lead to an enhanced rate of evolution for two reasons. First, if a stress gradient is imposed on a connected network of populations, and if a mutant acquires some resistance to the local stress, the relative fitness of the mutant is increased if it moves to join a population exposed to even higher stress. Second, because there are fewer individuals in the region of higher stress, the mutant can fix more quickly in the smaller population.
>
> (Zhang et al., 2011)

2.2 Modelling the effects of antibiotic gradients

As seen, there is clear experimental evidence supporting the notion that non-uniform drug distributions can accelerate the emergence of resistant organisms. To gain a better understanding of whether this is always the case and also how the mutants emerge and

propagate, Greulich et al. (2012) [24] constructed a simple computational model which investigated how a bacterial population evolved along pathways in genotype space when exposed to both uniform and non-uniform antibiotic concentrations. It is this model which has also formed the basis for the other models constructed in this project.

The model consisted of L microhabitats interconnected in series with one another. Each of which had a carrying capacity K and a concentration of antibiotic present c_i, where i is the index of the microhabitat. Each bacteria had a numeric genotype m, which they could mutate between with a probability μ and which had a maximum value of M. This genotype described the level of resistance the bacteria had to the antibiotic. At each step in the simulation each bacteria could die or move to an adjacent microhabitat at constant rates d and b, or replicate at a rate given by

$$R_{rep} = \phi_m(c_i)(1 - \frac{N_i}{K}). \tag{1}$$

Where N_i is the total number of bacteria present in microhabitat i and $\phi_m(c_i)$ is the genotype and antibiotic dependent growth rate. This value decreases until the MIC for that particular genotype (β_m) is reached, after which that bacteria cannot replicate. The simulation is initialised by placing K bacteria of genotype $m = 1$ in the first microhabitat and then allowing them to proliferate throughout the system. To illustrate the effects of the antibiotic gradient, the simulations were performed under two different conditions; with a uniform antibiotic concentration ($c_i = c$) and with an exponentially increasing antibiotic concentration ($c_i = \exp(\alpha i) - 1$).

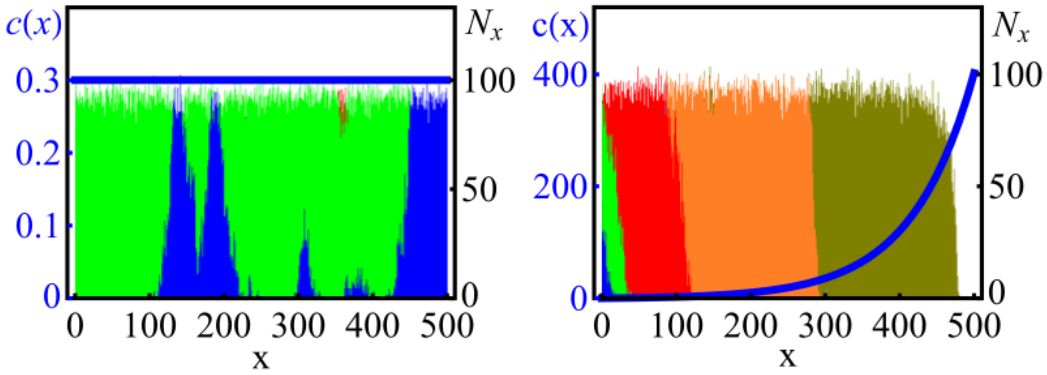

Figure 4: Distributions of bacterial genotypes throughout the series of interconnected microhabitats. The thick blue line is the concentration of antibiotic per microhabitat, and the colours represent the distributions of genotypes. $m = 1$ is blue, $m = 2$ green, with $m = 3, 4, 5$ shown by red, orange and olive respectively. For the uniform concentration, any mutations are sporadic and randomly distributed, however the exponential gradient shows clear emergence of resistance in a consistent manner. Greulich et al., 2012.

Once again, as shown in Figure 4, the dynamics of how evolution emerges varies greatly between the uniform and non-uniform drug distributions. For the uniform case, mutations arise sporadically and proliferate randomly, with the system as a whole generally evolving from one genotype to the other. However when exposed to the gradient, the genotypes tend to form "stationary fronts", with resistant mutants emerging at the tip of the colony,

then quickly spreading to fill the remaining space until the MIC for the current advancing genotype is reached, at which point the process repeats itself. As Figure 4 illustrates, where the snapshots were taken after an equal amount of time had passed, the presence of the gradient drastically reduced the time necessary for resistance to evolve. To further investigate the effects of the gradients, Greulich et al. ran a series of simulations varying the overall concentration of the antibiotic for the uniform case, and the steepness of the gradient for the non-uniform one and recorded the time taken ($\bar{\tau}$) for a fully resistant (i.e. $m = M$) mutant to arise.

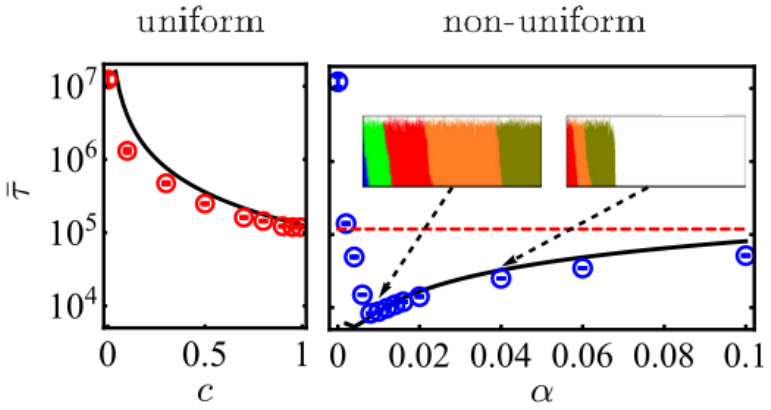

Figure 5: Time taken for a fully resistant ($m = M$) mutant to emerge for a variety of uniform drug concentrations c, and steepness of non-uniform drug gradient α. The red dashed line shows the minimum time taken for the uniformly concentrated system to reach resistance. Greulich et al., 2012.

This experiment produced an interesting result, as shown in Figure 6. $\bar{\tau}$ for the uniform case followed a simple inversely proportional relation, which makes intuitive sense. With a higher drug concentration present, there is also an increased pressure for resistance to emerge. However for the non-uniform case, the results are somewhat more involved. For the smallest values of α, it's seen that the presence of the gradient actually causes $\bar{\tau}$ to be larger than in the uniform case, as there is much less pressure on the system for resistance to evolve. Additionally, there seems to be an "optimal" value for α, above which $\bar{\tau}$ begins to increase. This is due to the fact that for the higher values of α, the regions which the lead mutants can fill get much narrower as α increases, as seen in the snapshots included in Figure 6. Thus reducing the available regions that the next generation of mutants can arise from. From this it appears that the contributions of antibiotic gradients on an evolving system are even more complex than first supposed.

2.3 Biofilms

- mention factors that affect formation, roughness etc
- changes undergone by bacteria in the biofilm - polymer secretions etc
- defense mechanisms from that paper - gradients caused by diffusions and changes in growth rates throughout the film

- this section will probs be the most involved

Bacteria which aggregate onto and around a surface forming a biofilm have a range of additional defences available to them which free-flowing planktonic bacterial colonies do not [25]. These defences can afford biofilm colonies resistances to antibiotics 10-1000 times greater than their individual counterparts [26], making treatment plans an immensely more complicated affair. Biofilms can form on almost any surface where there is a consistent influx of bacteria and other microorganisms, from medical devices [27] to the exterior of ship hulls [28].

The formation of biofilms is a complex and little-understood process. Whilst originally thought that biofilms were formed by single organisms attaching to a surface, leading to the formation of micro-colonies and then 3D structures [29], there is also now evidence that pre-existing bacterial aggregates can also seed the formation of biofilms [30], the differing shape and composition of which in turn can influence the final established biofilm [31], making the developmental paths of biofilms increasingly hard to predict.

3 Progress to Date

- make several sections giving brief overview of them - PRL replication, growth dependent, multispecies
- mention paper

3.1 Greulich et al. replication

Progress made so far in this project can be organised into several sections. The initial few weeks of the project were spent replicating the results and techniques found in Greulich et al., 2012 [24]. Discussion was had on the subject of which algorithm would be optimal for updating the system over time. Algorithms such as Gillespie [32] and τ-leaping [33] were proposed, but eventually a simple Monte-Carlo style selection process as detailed in the supplementary material of Greulich et al., was decided upon. The purpose of this was mainly as a "warm-up" project. Intended to increase fluency and familiarity in the techniques and background theory required for the modelling of biological systems. The results obtained from this body of work were rough, proof-of-concept illustrations, rather than the precise quantitative results obtained in the actual paper.

[INSERT SOME GRAPHS AND SCREENSHOTS]

3.2 Growth rate-dependant antibiotics

By far the majority of the time spent on this project has been on the modelling of growth rate-dependant antibiotics, based on the 2015 paper by Greulich et al. [34]. In this paper, Greulich et al. proposed that certain ribosome-targeting antibiotics were more effective on either fast-growing or slow-growing bacteria depending on how the antibiotic bound to the target ribosomes. It's been shown that the ribosome content of a cell correlates with the cell's growth rate [35], with fast-growing cells dedicating more of their resources

to ribosome production than their slow-growing counterparts [36]. For example, the antibiotic tetracycline binds reversibly to the bacteria's ribosomes and is more effective when the bacteria are fast-growing. In contrast, streptomycin binds irreversibly with the bacterial ribosomes and is more suited for bacteria which are growing slowly. Here we assume that the growth rate of bacteria is directly proportional to nutrient availability, with a rich nutrient supply corresponding to a high growth rate.

The foundation of this model was heavily borrowed from the one created in the previous section, but with the key addition of nutrients, which were used to modify the growth rate in lieu of the carrying capacity factor from the previous model. Two simple functional forms for the MIC of fast-growth and slow-growth targeting antibiotics were constructed as follows: for the fast-growth targeting antibiotics;

$$\beta = 10 - 9\frac{\mu(S)}{\mu_{\max}} \tag{2}$$

4 Proposal

Over the next year, the first step will be to complete writing the paper on the growth-rate dependent antibiotic model. This should not take longer than a few weeks, as all the results have now been collated, so a write-up and commentary, along with a discussion section is all that remains. Along with some mainly stylistic editing.

Following on from this, work will continue on the multispecies model. The current version is a simplistic toy model, similar to the one in the Greulich paper [34]. Ideally our industrial partners AkzoNobel will provide us with some more realistic parameters for factors such as growth and death rates for a variety of microorganisms, and their susceptibility to various biocides. When incorporated into the current model, this should provide us with some more accurate, if still overly simplified, results for how a system with multiple species compete with each other over time. Additionally, the current models only consider freely moving, independent bacteria. But as seen in the previous literature, there is a vast difference between the behaviour of these planktonic bacteria and those which have aggregated to form a biofilm. As such, it seems that the next logical step is to incorporate the formation and subsequent property-altering qualities of biofilms. I estimate that at least, but no more than a few months will be spent working on this model.

While these additions should certainly yield some interesting results, there is only so far that these simplistic models can take us in providing a clear picture of the mechanics involved in how biofilms respond to the application of antibiotics. Therefore the plan at present is to move on from this 1D "lattice" model and begin work on a more intricate continuum model. This model would allow us to incorporate key features of biofilms and the environment which surrounds them, such as surface roughness and flow around the biofilm. These models should allow us to perform research on both the medical and industrial scenarios where biofilms form, by varying the side of the biofilm that the drug gradient arises from, and discerning whether these two situations create differing outcomes. I predict that this will consume the majority of the second year of my PhD.

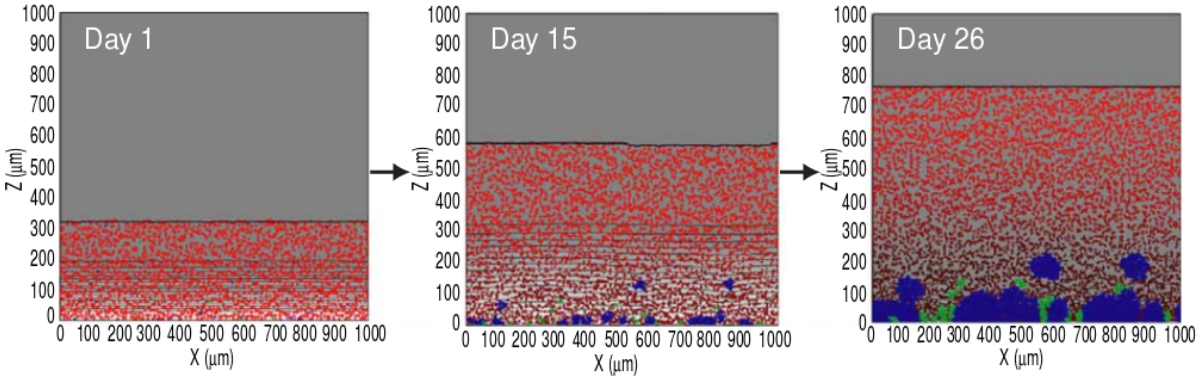

Figure 6: Screenshot taken from the paper by Matsumoto at al., 2007 [37] which contained several differing species of bacteria whose growth rates varied depending on the atmospheric composition of the biofilm. This is included for illustrative purposes as an idea of what the simulations constructed in the later stages of this PhD project might resemble.

In conjunction with AkzoNobel, some wet work will be undertaken at their laboratory in Newcastle. This would entail cultivating biofilms on various surfaces akin to industrial ship hulls and exposing them to a variety of biocidal paints and then examining the composition of the established biofilm to compare the model with reality. There might also be opportunity to undertake part of the metagenomic analysis of these cultivated biofilms. This aspect of the project is intended as more of an interesting aside, rather than as a basis of the overall PhD project. It should only require a month or so of involvement, and will likely be performed alongside my other commitments.

5 Summary

Simple models investigating the effects of antibiotic gradients have been constructed which demonstrate the relation between gradient steepness and time taken to evolve resistance, the differing inhibition properties of growth-rate dependent antibiotics and how colonies involving multiple species with differing levels of resistance develop over time. An article is currently being written on the findings of the growth-rate dependent simulations.

The aim for the next year or so is to finish writing the article regarding the work on the growth-dependent antibiotics, then to continue with the multispecies model and hopefully incorporate some realistic parameters contributed by AkzoNobel. Following on from this, work will begin on the proposed continuum model which could incorporate other factors such as surface texture and flow into the system. This will be accompanied by some wet work at the AkzoNobel laboratory at their Newcastle compound. This is mainly for interest and not intended to form a sizable component of the project. There has also been some discussion of my involvement in AkzoNobel's metagenomic analysis of organisms gathered from their laboratory to extract information about the growth rates and biocidal susceptibility from microorganisms taken from biofilms attached to industrial shipping vessels.

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