Modelling Competition and Signalling Between Microbial Cultures Growing on Solid Agar Surfaces

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1 AIMS

Quantitative fitness analysis (QFA) is a method for inferring the fitness of microbial cultures from their growth curves and can be used for genome-wide screening for genetic interactions or drug responses (see Addinall *et al.* (2008, 2011); Lawless *et al.* (2010); Banks *et al.* (2012); Andrew *et al.* (2013)). In QFA, growth curves are automatically captured for arrays of typically 384 cultures grown on solid agar, with each culture containing an individual genetic strain. Plates can contain any combination of different strains or repeats. Fitness is a good phenotype for measuring GI because...

Usually QFA cultures are assumed to grow independently (e.g. Addinall *et al.* (2011)). We aim to test the validity of this assumption in a range of different QFA experiments. We propose a new model of population growth which includes competition between cultures. We expect that accounting explicitly for competition in analysis of QFA data will increase the reproducibility of fitness estimates and ultimately increase the statistical power of QFA in screens for genetic interactions and drug responses.

An analysis by Baryshnikova *et al.* (2010a), of data obtained using 1536-pin synthetic genetic array (SGA), an alternative procedure also using an array of cultures on solid agar, attempts to normalise for systematic variation in growth observations, which may include variation from competition and signalling effects, using statistical techniques aimed at improving the correlation between repeats of identical mutant strains at different locations. In order to learn more about the sources of systematic variation in both QFA and SGA experiments, and to develop analyses and experimental designs for dealing with them, this project aims to account for competition and signalling mechanistically. We then aim to determine what effect accounting for competition and signalling has on variability and rank order of fitness estimates. We will study unpublished QFA data for the model organism *Saccharomyces cerevisiae*. We will package all models and analysis tools so that they may be used in future studies or to reanalyse data from past studies.

1.1 Hypotheses

This project aims to test the following hypotheses:

- Growth of cultures in fitness screening experiments using solid agar is affected by competition for nutrients and/or signalling.
- By accounting mechanistically for competition effects, it will be possible to design analyses and experiments which minimise these effects.
- Explicitly accounting for competition effects in data analysis will improve the power to infer genetic interactions and drug responses.

2 OBJECTIVES

The project can be broken down into the following set of objectives:

- Develop models of culture growth on solid agar which account for competition for nutrients and signalling between cultures.
- Analyse experimental data to determine whether competition for nutrients and signalling affect colony growth. If so, determine the size of the effect on different fitness measures.
- Compare competition and signalling effects in different experimental designs in order to design experiments which minimise these effects. For instance, compare culture and agar geometries, initial culture and nutrient densities, and the effect of randomisation. Also look at miniQFA data to investigate the effect of diffusion across agar height.
- Compare the reproducibility of fitness estimates between analyses using the competition and independence models and assess the likely effect on screens for genetic interactions and drug responses.
- Continuously package SBML models and Python analysis tools so that they may be used in subsequent stages of the project and eventually by

other researchers. Conform to minimum information standards so that SBML models may be published in the BioModels database.

3 BACKGROUND OF RESEARCH

3.1 A Comparison of Methods for Screening Strain Fitness

QFA and SGA are methods for high-throughput fitness screening using cultures grown on solid agar (Baryshnikova *et al.*, 2010b; Banks *et al.*, 2012). SGA uses a single measurement of culture size, taken at some midpoint in the growth curve, as a surrogate for growth rate (Baryshnikova *et al.*, 2010b). QFA collects more information about growth by taking images of plates at points throughout the growth curve. QFA can be performed using either the pinned cultures used in SGA or dilute liquid cultures ("spots") of lower initial cell density. Although pinned QFA allows for more cultures per plate (1536 vs 384 in spotted), spotted QFA allows for more accurate fitting of growth models as growth curves are more complete (see Figure 1) (Lawless *et al.*, 2010). Comparison of spotted and pinned QFA cultures in Figure 1c shows how spotted cultures are composed of many individual colonies which increase in size and thickness, whereas pinned cultures are composed of a single uniform colony which grows radially. The number of individual colonies in a spotted culture is high enough that lag and other stochastic effects should average out.

Colonyzer (Lawless *et al.*, 2010), a QFA tool for automatic capture of growth curves, from images of both spotted and pinned plates, uses integrated optical density (IOD) as a proxy for cell density. Figure 2 (Banks *et al.*, 2012), shows an example of 308 growth curves from a spotted QFA plate captured by Colonyzer and assembled by the QFA R package (Lawless *et al.*, 2016). In SGA, culture area, rather than IOD, is used as a proxy for culture density (Baryshnikova *et al.*, 2010b). Lawless *et al.* (2010) find that direct area measurements are more noisy than IOD measurements and provide a worse fit to the logistic model. To prevent bias in comparison of experimental designs, we will use Colonyzer to generate more accurate IOD measurements from spotted and pinned QFA images.

Fitness screening can also be performed using independent liquid cultures which are not susceptible to competition or signalling effects (Jasnos and Korona, 2007). However, this approach has lower throughput.

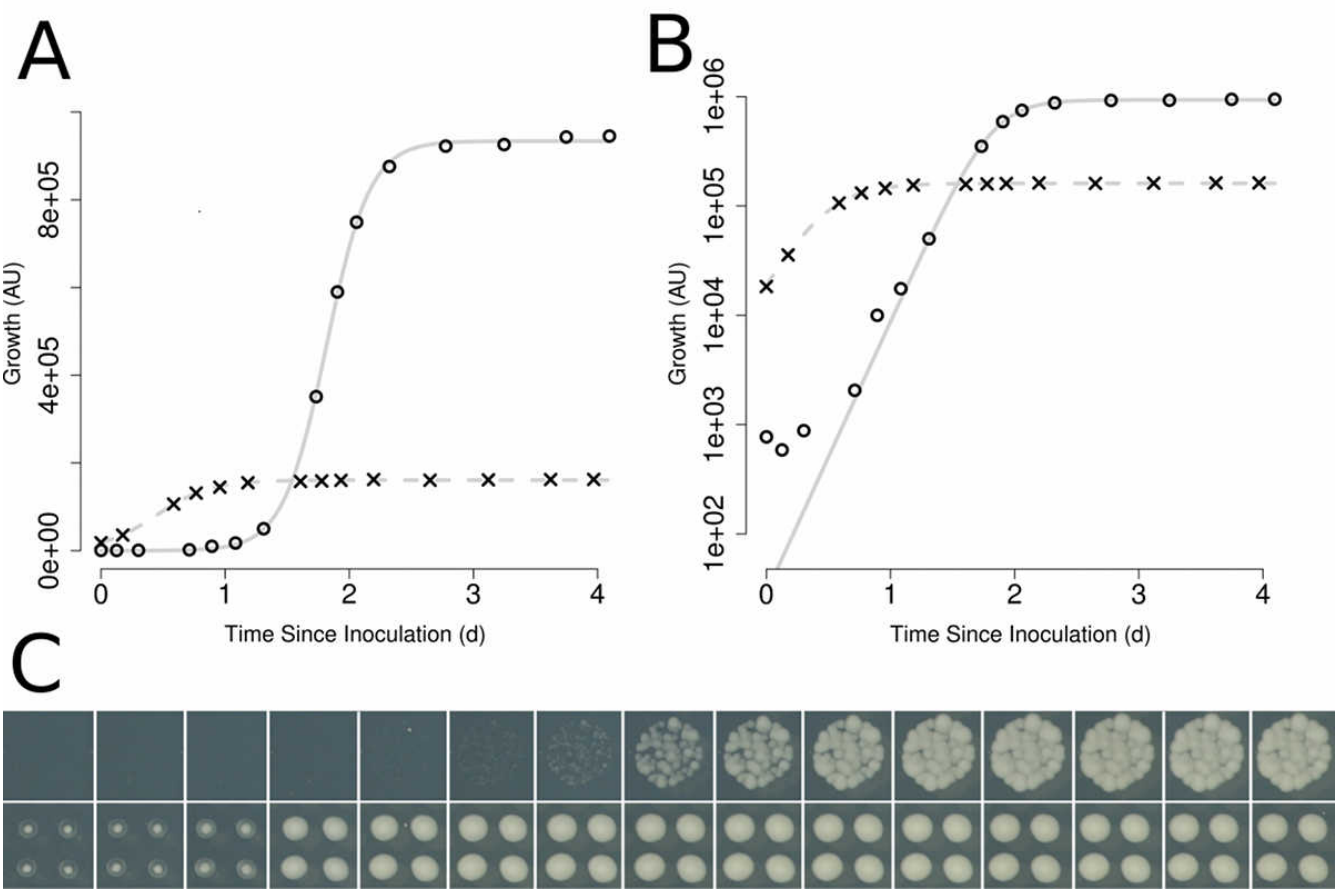

Figure 1: Fits of the logistic growth model, to cell density observations of yeast growing on solid agar, for spotted (circles) and pinned (crosses) QFA cultures. In A) cell density is plotted on a linear scale. In B) cell density is plotted on a logarithmic scale. C) shows images of the spotted and pinned cultures corresponding to the data in A) & B) (Lawless *et al.*, 2010).

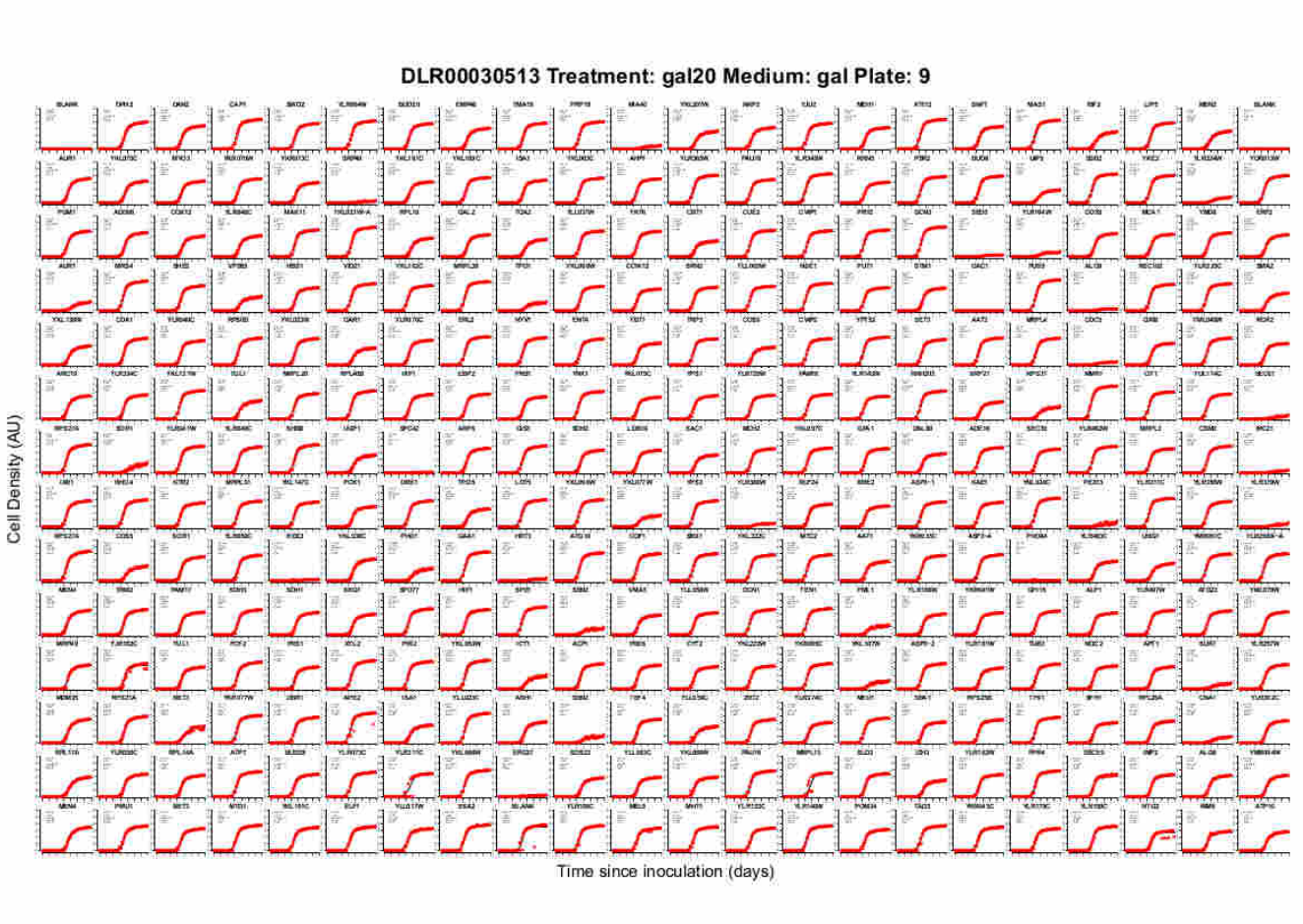

Figure 2: 308 growth curves from a spotted QFA plate captured by Colonyzer (Lawless *et al.*, 2010) and assembled by the QFA R package (Lawless *et al.*, 2016) (Banks *et al.*, 2012).

3.2 Competition and Signalling

At the beginning of QFA, nutrients are distributed uniformly throughout the agar. During incubation, cultures grow, consuming nutrients and creating gradients in nutrient density. A QFA plate taken partway through incubation can be seen in Figure 3. As some cultures grow much faster than their neighbours, using more nutrients, we believe that diffusion of nutrients along gradients between cultures might cause a significant competition effect. Growth of yeast cultures may also be affected by signal molecules produced by cells, which diffuse through the agar or travel across its surface. Candidates are ethanol (Fujita *et al.*, 2006), a poison produced by fermentation, and ammonia which is involved in regulatory response to changes in cell density (quorum sensing) (Sprague and Winans, 2006; Honigberg, 2011).

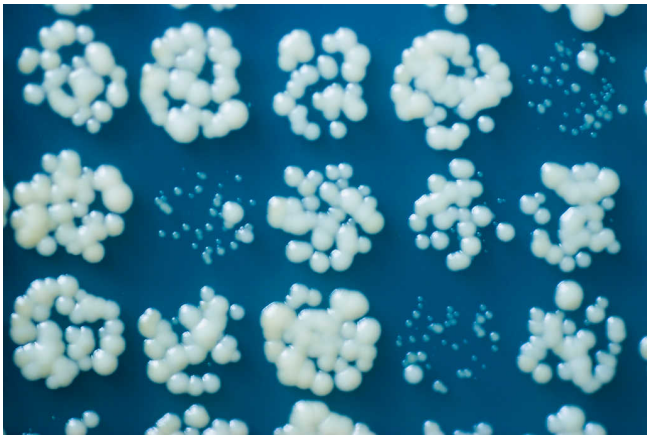

Figure 3: An image of 15 cultures on a section of solid agar from a QFA procedure. (Lawless *et al.*, 2010) https://github.com/CnrLwlss/Colonyzer

3.3 Modelling Approaches

3.3.1 Mass Action Kinetics

Chemical reactions are commonly modelled using mass action kinetics, where the rate of a reaction in a well

stirred mixture is proportional to the product of the concentrations of the reactants. This accurately reflects the probabilistic nature of reactions in the gas phase, which arise from collisions between reactants travelling at high velocity in random directions. If species numbers are large enough, reactions can be modelled continuously and deterministicly using ordinary differential equations (ODEs). Microbial population growth, with cell division dependent on nutrient availability, can also be modelled using mass action kinetics. We can use the following reaction equation for a culture i growing on the agar,

$$N + C \xrightarrow{r_i} 2C, \tag{1a}$$

$$rate = r_i[N][C] \tag{1b}$$

where, r_i is a growth constant (units $M^{-1}s^{-1}$), C is a cell, N is an amount of nutrients required for a cell to divide, and [ ] are concentrations. In ODE form,

$$\frac{dC_i}{dt} = r_i N_i C_i \tag{2}$$

where C_i and N_i are the amount of cells and nutrients for culture i. The assumption of a well stirred reaction mixture is perhaps not valid for a culture growing on agar. However, a mass action approximation has been used with some success to model predator-prey dynamics of animal populations (Berryman, 1992) and signalling and reactions in the spatially heterogeneous environment inside cells (Aldridge *et al.*, 2006; Chen *et al.*, 2010) where this assumption is also questionable. If nutrient gradients within cultures are too large then a spatial PDE model may be required. If nutrients are in some way dimensionally confined inside the culture medium, perhaps within a channel or on a surface, then a fractal kinetics model may be more appropriate (Savageau, 1995).

3.3.2 The Logistic Growth Model

The logistic growth model,

$$\dot{x} = rx\left(1 - \frac{x}{K}\right) \tag{3}$$

(Verhulst, 1845), for a population of density x, with parameters, r, growth rate and K, carrying capacity, describes self-limiting growth and is commonly used to fit QFA data with an assumption of independence between cultures. It has the following analytical solution:

$$x(t) = \frac{KPe^{rt}}{K + P(e^{rt} - 1)}, \tag{4}$$

where P is the initial population density. Measures of fitness are commonly defined in terms of the parameters r, K, and P (Addinall *et al.*, 2011).

3.4 Fitness Screening in Functional Genomics

Fitness screening using cultures grown on solid agar has important uses in functional genomics for inferring genetic interactions, response to environmental conditions drug responses, and response to environmental conditions (Addinall *et al.*, 2011; Andrew *et al.*, 2013) and can be carried out on a genome wide scale. For example, Costanzo *et al.* (2010) use SGA data to construct a genetic interaction map for ~75% of the *S. cerevisiae* genome. The statistacal power to predict genetic interactions may be improved by accounting for competition and signalling in reanalysis of data from such studies. If competition and signalling has an affect in yeast this is also likely to be relevant for fitness screening using other types of microbial organisms.

4 APPROACH

4.1 Develop an Initial Model

Figure 4 shows a schematic of our initial approach to constructing a competition for nutrients and signalling (CANS) model. Each culture at location, i, is associated with three variables: one observed variable, C_i, the amount of cells, and two hidden variables, N_i and S_i, the amount of nutrients, and signal. Cultures in QFA and SGA agars are arranged in a rectangular array, each culture having eight neighbours with which they could conceivably interact directly (except at the boundaries). Initially, we will model diffusion between only the four closest neighbours, indicated by the darker blue circles in Figure 4. Assuming a well stirred mixture, we will begin by describing nutrient dependent growth at each location using mass action kinetics and the reaction equation 1a. As a first approach, assuming that the number of cells is continuous, we will incorporate the effect of signal molecules on growth and the diffusion of both signal molecules and nutrients using the following ODEs (CANS model):

$$\frac{dC_i}{dt} = r_i N_i C_i - \beta S_i, \tag{5a}$$

$$\frac{dN_i}{dt} = -r_i N_i C_i - k_n \sum_{j \epsilon \delta_i} (N_i - N_j), \tag{5b}$$

$$\frac{dS_i}{dt} = \alpha C_i - k_s \sum_{j \epsilon \delta_i} (S_i - S_j), \tag{5c}$$

where, δ_i indicates the set of closest neighbours, k_n and k_s are nutrient and signal diffusion constants, α is a rate constant for signal secretion, and β is a constant for the effect of signal on culture population. ?do we assume no cell death detect dead cells on the agar/ times

are short? Alternative models of signalling effect could be used, depending on the mechanism of signalling under investigation (see background section); initially, we have chosen to use the simplest modelling approach. Setting diffusion constants to zero reduces 5 to the independence model. We may also study the effects of signalling and competition for nutrients separately by setting other parameters to zero. If necessary, diagonal neighbours could be incorporated by adding or scaling diffusion constants. In section 4.3, we discuss the possibility of using finer-grain spatially-discretised or continuous models of diffusion which would also achieve this.

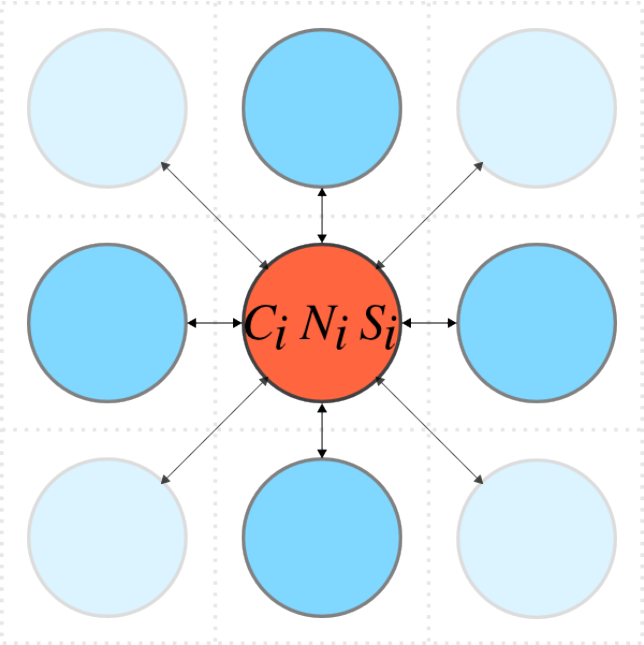

Figure 4: Schematic of simple modelling approach.

Models will be written in SBML (Hucka *et al.*, 2003), using SBML shorthand (Wilkinson, 2011), so that they may eventually be published in the BioModels database (Juty *et al.*, 2015) and available publicly. This will require conformation to the minimum information standard MIRIAM (Le Novère *et al.*, 2005). (Simulation experiments should also conform to MIASE Waltemath *et al.* (2011).) We will write code for the rest of the project in Python as this has several advantages: it is used widely by the scientific community, has libraries which will be of use to us, and related tools such as Colonyzer (Lawless *et al.*, 2010) are already written in Python. To interface SBML models with Python we will use the libSBMLO library (Bornstein *et al.*, 2008). ODEs will be solved using odeint from the SciPy library (Jones *et al.*, 01). We will use git as version control and GitHub as a remote repository where packages will eventually be released publicly.

It is anticipated that solving sets of ODEs for typically 384 cultures in QFA and 1536 cultures in SGA will be take a long time. Therefore, in the testing stage, we will simulate smaller sets of artificial data from the ODE models and attempt to fit these. We will also incorporate unit-testing into the development to try to ensure that code will still work when scaled up to larger arrays used in experiments.

When dealing with experimental data we must also consider how to treat cultures around the edge of the agar. These have access to proportionally more nutrients because there is a relatively large area beyond them which is unoccupied by cultures. In photographic images, these sites are also affected by reflections from plate walls which cause errors in integrated optical density (IOD) measurements, a proxy for cell density, which cannot be fully corrected for by Colonyzer (Lawless *et al.*, 2010). In experiments, an identical culture is grown in edge locations and the results are discarded (see refs). We may choose to adjust for the increased nutrient access and include edge cultures in our model and analysis. This would introduce a systematic error for sites close to the plate edges and is an argument for the use of repeats and randomisation of location (see 4.4). A simpler modelling approach is to discard the first outer layer entirely from the model and the second outer layer from the final results. However, this is undesirable as it reduces the amount of information that is gathered from each plate and may not account for the systematic error any better than the more complicated approach, possibly allowing it to propagate further.

4.2 Analyse Experimental Data

List of strains of known fitness??
Specifically what machines do we use for computation? Can use yzer.ncl.ac.uk for computation: 56 cores, 256Gb RAM, massive GPU.

We will investigate unpublished QFA and SGA data for the model organism *S. Cerevisiae*. Some data (Figure 5), with vacant gaps, is specifically designed for the study of competition. Figure 5 shows such an agar where each culture is also an identical strain. We plan to develop a method for Bayesian inference using the CANS model which will estimate distributions of parameter values accounting for our prior belief and allow us to conduct model comparison. For this, we will use either of the Python libraries pyMC (Patil *et al.*, 2010) or pySTAN (Stan-Development-Team, 2016). For pySTAN we would have to rewrite/parse the ODE model in Stan but it could be faster.

In an analysis of high-throughput QFA data from a genetic interaction study by Addinall *et al.* (2011), Heydari *et al.* (2016) use a Bayesian hierarchical model which mirrors the experimental structure (from the time-point to population level) and simultaneously estimates growth parameters and genetic interaction strength by sharing information between levels. This approach accounts for differences in replicate fitness variances between different mutant strains which cannot be efficiently factored into statistical analyses (Heydari *et al.*, 2016). When looking at the most significant genetic interactions, for the increased computational time that it takes (4 weeks vs 3 hours), this analysis does not offer a significant advantage over the original statistical analysis carried out by Addinall *et al.* (2011). However, Heydari *et al.* (2016) do identify weakly interacting genes for which there is no previous evidence, and a hierarchical model which only modelled population dynamics took significantly less time (1 week). As we are only studying single plates, and not going on to infer genetic interaction strength, there will be fewer levels in our hierarchy allowing for a faster computational time, and this type of analysis may prove more effective in identifying competition and signalling effects than it is in identifying genetic interactions.

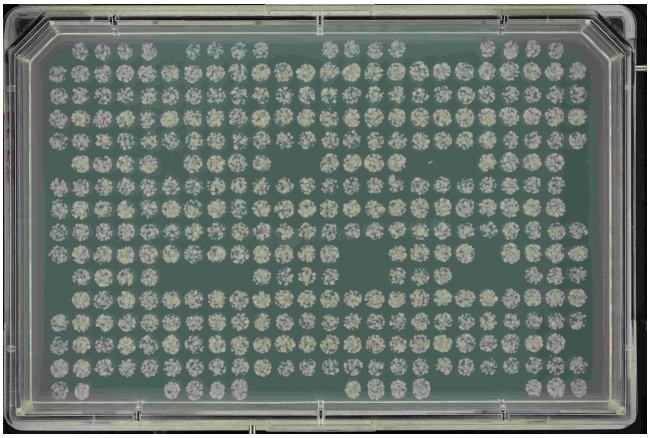

Figure 5: An agar with locations left empty ("gaps") designed to exhibit effects of competition and signalling.

If we fail to discover competition or signalling effects by fitting the CANS model, we may need to improve aspects of our modelling approach (see section 4.3), study different data, or consider that the data is not subject to these effects. Otherwise, if we find evidence of a significant competition and/or signalling effect, we have proved our main hypothesis and can move on to comparing experimental designs (section 4.4).

4.3 Develop Model Further

If we find that our initial CANS model offers no improvement over the independent model, or is in some other way inaccurate, we could consider improving the model in several ways. For instance, we may propose a different model of signalling effect and instead model culture growth rate as,

$$\frac{dC_i}{dt} = r_i N_i C_i \left(1 - \frac{S_i}{S_{crit}}\right), \tag{6}$$

where S_{crit} is some critical concentration of signal above which cultures do not grow. We must, however, be careful to avoid adding too much complexity to models when we are unsure of the underlying mechanism as this could result in over-fitting.

We may also model diffusion more realistically, and incorporate interactions between diagonal neighbours, using a spatially-discretised model of diffusion with a finer grid (see Figure 6). We would begin using a two-dimensional model as this is simpler. Although variance between cultures on the same line will still be an issue, we may attempt to validate against data from miniQFA (Figure 7), and remain in two-dimensions, to investigate how diffusion varies across agar height (see Figure 8 middle). We should do this for different agar geometries, varying d and w in Figure 9, to represent QFA and SGA experiments. If height dependence is found to be unimportant, we may apply a two-dimensional grid in the plane of the agar surface as in Figure 6. Otherwise, we would have to use a three dimensional model of diffusion. It may suffice to model nutrient diffusion in three-dimensions and signal diffusion in only two-dimensions, as nutrients are assumed to be distributed evenly throughout the agar at time zero, whereas signal molecules will be secreted by cultures growing at the agar surface. We could also consider using a continuous PDE model of diffusion, although this is likely to take more computational time and be more difficult to implement. Because of the generality of the problem, it is possible that computational models of diffusion already exist publicly, and we would explore the possibility of using them.

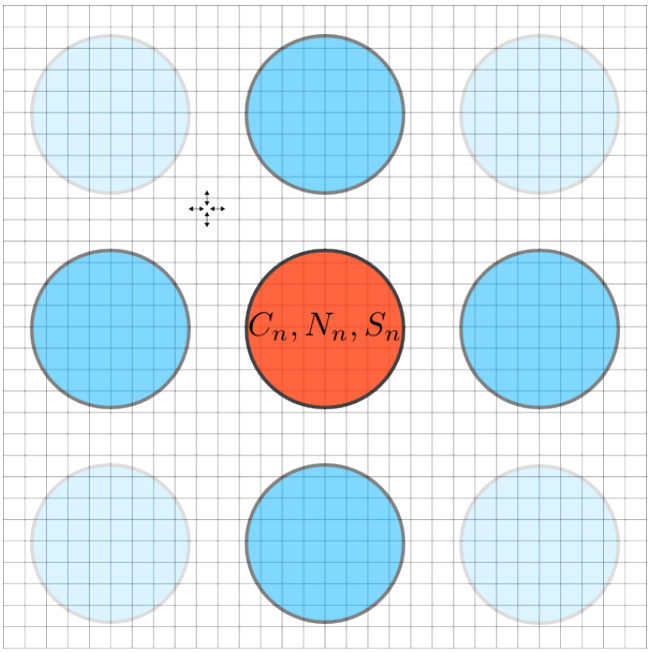

Figure 6: Schematic of a spatially-discretised two-dimensional model.

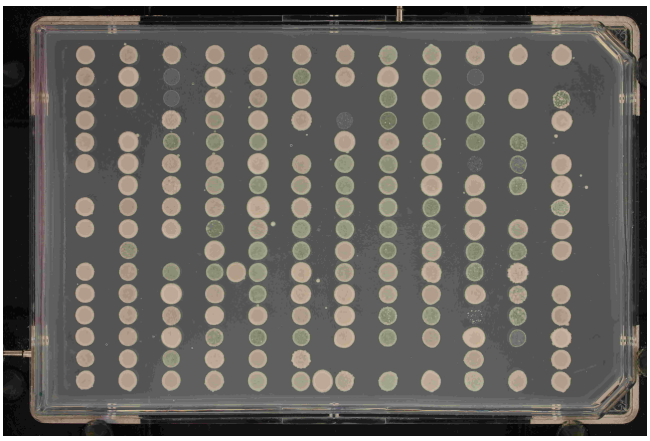

Figure 7: A miniQFA agar in which lines of locations ("stripes") are left vacant. We can use a similar experimental setup, with uniform cultures in one dimension, to study diffusion at different agar heights in only two dimensions.

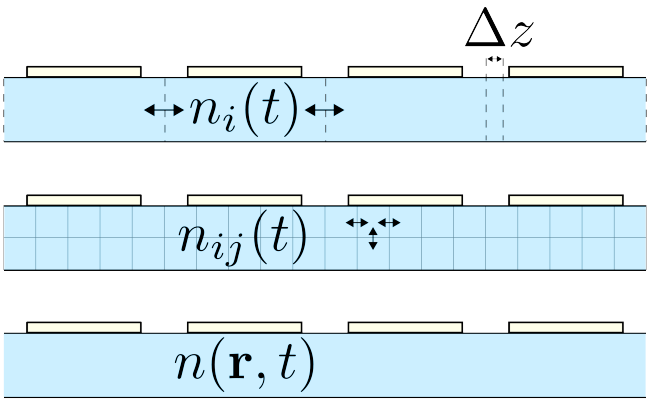

Figure 8: Schematics of diffusion across agar height.

If no effect is found, we may remove either signalling or competition for nutrients from our model entirely. Ultimately, if neither effect is discovered we may reject our initial hypothesis and accept that growth is independent.

We also intend to determine the effect of competition on different measures of fitness (see section 3.3.2). In previous studies, which use fits of the independent logistic growth model (e.g. Addinall *et al.* (2011)), fitness estimates based on carrying capacity, Z, might be more affected by competition and signalling than those based on rate constant, r, because gradients in nutrient and signal density will likely be greater towards the end of growth curves, after the exponential growth phase, when cultures are larger. However, we also expect modelling competition to improve our confidence in estimates of r by using information the beginning and end of growth curve to inform our estimates. If it is possible to relate parameters in our model to those of the independent logistic model (3) we will make this comparison.

4.4 Compare Experimental Designs

If competition is present, without randomisation of culture location, repeat observations may lead to overconfidence in fitness estimates. As discussed in section 4.1, reflections from plate edges at agar boundaries introduce a further systematic error which persists even when we are trying to correct for competition. We will compare growth parameters and fitness measures estimated from fits of the CANS and independence models, for repeats with and without randomisation, and expect to find closer agreement between models when randomisation is used. In the case that the CANS model is too slow to use in analysis of large sets of data from high-throughput experiments, we will still be able to determine how large a reduction in error can be achieved through randomisation when using the independence model.

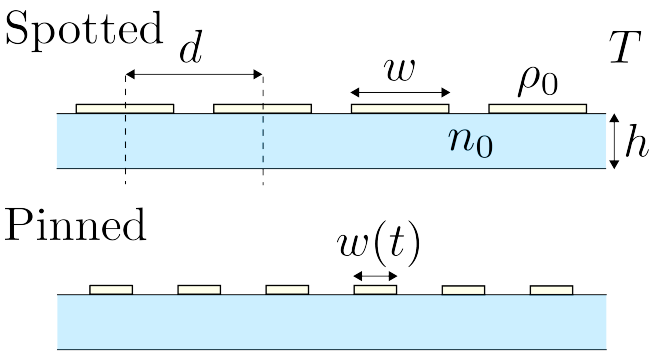

Figure 9: Spotted and pinned agars.

If time allows, we wish to compare differences in CANS effects between spotted and pinned QFA designs. To do this we change the following variables: ρ_0, the initial concentration of cells, d, the distance between cultures, and w, the diameter of cultures (see Figure 9). For pinned cultures, we also have to consider that the diameter/area of cultures changes with time and account for this in our models. We should also account for the statistical advantage of SGA, in that roughly five times more cultures can be grown on SGA plates, allowing for more repeats. Simulations may be of use to study the effect of one variable at a time. However, it is of greater priority to compare SGA and QFA as they are currently performed, because data and machinery already exists for these designs.

It is cheaper to use YEPD agar than CSM agar, but YEPD agars have a higher initial nutrient density, n_0, and this reduces the time available to observe culture growth. We may study this trade-off and see if there is any reduction in CANS effects due to there being a shorter time for diffusion to occur. However, we expect that CANS effects would be small compared to the error from shorter observation time and that a better approach would be to correct for CANS in CSM data either directly or using randomisation.

4.5 Package and Distribute

Already mentioned much of this in other parts of the approach. Anything missing?

5 PLAN

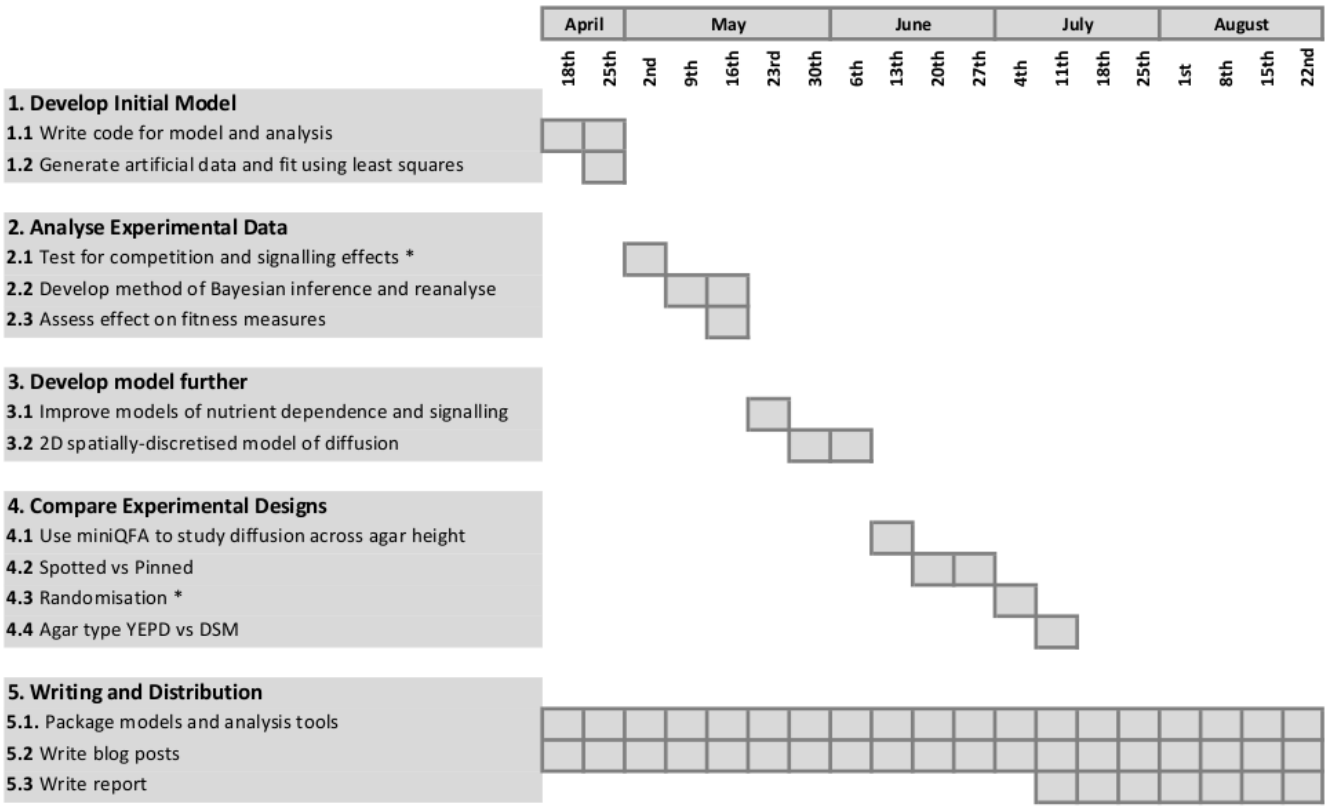

Figure 10: Gantt chart showing project plan. * marks important events.

flow chart showing cyclical nature of investigation

$$\begin{aligned} &C_i - Cells \\ &N_i - Nutrients \\ &S_i - Signal \end{aligned} \tag{7}$$

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