**Response to reviewers**

**PNAS #2019-02217P**

April 15, 2019

In the following, we provide the reviewers’ comments as they are, and reply with a different font and marked by bullets. When quoting text, we use italics, and underline newly added text.

**Reviewer #1**

In microbiological work, growth assays, in which a population of microbes is tracked over time using optical density in a spectrophotometer, are popular. This is because they are relatively high throughput and easy to conduct. However, the OD trace is often boiled down to one parameter (such as maximal growth rate) meant to summarize fitness of the relevant strain. Not surprisingly, such partial information will often not suffice in making predictions about how different strains fare in competition for limited resources. This is because other elements of a strain’s growth profile (e.g., its lag phase, the transition dynamics out of lag, the carrying capacity, etc.) are often important to overall competitive ability. For researchers interested in competitive dynamics, pairwise competition experiments are consequently embraced as they directly assess competitive outcomes. However, these assays are often lower throughput and come with a series of different caveats.  
  
This is where this current manuscript enters the scene. The authors introduce a framework based on growth on a limiting resource, in which the dynamics of a microbial population is described by a multi-parameter model. Mono-culture growth data can be used to estimate the parameters of this model for different strains. Then mixed culture growth data (where total population, but not individual types, is tracked) can be used to estimate coefficients in a competition model with both strains. The parameterized competition model can then be used to predict competitive success of each strain in mixed culture scenarios. The authors validate their approach with data from bacterial experiments, and show that the approach can work quite well.  
  
Overall, I found this to be an interesting and useful manuscript. A genuine problem was identified and a nice solution was advanced. I think that researchers working with microbial populations in liquid culture and using a spectrophotometer to understand fitness will find this paper immensely satisfying and extremely helpful. I applaud the authors for making this framework available as an open-source package. I imagine that researchers in microbiology, microbial evolution and ecology, and theoretical biology will enjoy reading this manuscript. Overall, I think the paper is clear and well organized. I include a few specific comments below, but these are mostly minor (and some are beyond the purview of the current study).  
  
Specific comments  
  
1. Pg. 8, Figure 3: A minor issue–the scale on the x-axis is different for each experiment. Were these experiments run for different amounts of time, or are the authors focusing on different portions of the same growth period?

* The experiments were all run for 24 hours, but the figure omits the stationary phase growth.
* We have added a comment to Figure 3 caption (line 177): “*The figure omits the stationary phase.*”

2. Pg. 9, Table 1: Given that the same strains were used in experiment A and experiment B, it is interesting that some of the estimated parameters, such as K, were different in these experiments. Do the authors have ideas about why this might be?

* We suspect the differences between experiment A and B are related to day-to-day variation (Hall et al 2014), as well as possible sensitivity to assumptions of the Baranyi-Roberts model on the adjustment to novel growth conditions (Baranyi 1997).

Also, using the parameters from experiment A, does the N\_0 for each strain for experiment B lie between N(4) and N(6) for each strain as predicted from the model fit from experiment A data? (Given that the initial densities for experiment B followed 4-6 hours of growth of the same strains.)

* Yes. The growth curves of the red strain from experiment A overlap with those of experiment B if the latter is offset by 3 hours. Similarly, the curves of the green strain from A overlap with those from B if the latter is offset by 6 hours (due to a longer lag phase), except that in experiment B the green strain grows to a higher maximum density (as noted above).
* We changed the text to read “*3-6 hr*” instead of “*4-6 hr*” (line 412).

3. Pg. 10, line 215. Are the parameters r\_1 and r\_2 coming from mono-culture growth models?

* Yes. To clarify, we added this text to the caption of Figure 4, line 217: *“Dashed black lines show the prediction of an exponential model with , where are estimated by fitting an exponential model to mono-culture growth curves (see Figure 1).”*

4. Pg. 11, line 236. For the f\_1(t) equation, aren’t initial densities needed in the equation if strain 1 and strain 2 start at different initial densities? That is, something like: f\_1(t) = [N\_0,1]\*e^(r\_1\*t) / {[N\_0,1]\*e^(r\_1\*t) + [N\_0,2]\*e^(r\_2\*t) } = 1 / {1 + ([N\_0,2]/[N\_0,1])\*e^([r\_2-r\_1]\*t)}. Also is there a t missing in the exponent?

* Thank you for identifying this mistake! We have corrected the equation accordingly (Figure 5 caption, line 241).

5. Pg. 12, lines 251-253. It might be worth mentioning that the good fit between the model and data also suggests that c\_i=1 was a reasonable assumption in this case.

* We have added such a remark in line 257: *“This suggests it was reasonable to fix the competitions parameters at ci=1.”*

6. Pg. 12, line 258. The filled circle icon looks like an open square on my version.

* We changed both icons to their Unicode versions – ○ and ● (line 263). We hope this solves the issue. We will monitor this at the proofing stage.

7. Pg. 13, Fig. 7. When competing strains of interest can’t grow in the same environment, must we assume something about c\_i coefficients (e.g., c\_1=c\_2=1)?

* That’s a good question. We must assume something, and this assumption seems like a good null model.

8. Pg. 14, lines 298-299. Although likely beyond the purview of the framework developed here, it would be interesting to extend this approach to address cases of public good production (where a resource was generated by one or more strains).

* We agree. This is part of our future research plan. We discuss how our model currently performs in such cases in a new paragraph, line 303.

9. Pg. 14, lines 318-321. As I understand this, certain assumptions (such as c\_1=c\_2=1) had to be made for this analysis. Thus, in future studies concerning the costs of different expression levels, it might be interesting to test some of the predictions by using two strains with the same gene/operon under distinct inducer control. This way, mixed culture competitions with distinct control of expression of each strain could be executed and some of these assumptions could be checked.

* This is a very good idea, thanks.

10. Pg. 15, lines 328-330. I think the authors could go even further here. Suppose a given mutation will move a strain by epsilon in one of its parameters. Further, suppose that every parameter is equally likely to move by epsilon via mutation (which is, of course, an extreme simplifying assumption). Now the framework here could be used to predict which mutation has the greatest selective potential (or, the parameter for which change gives the greatest payoff).

* We agree. This is part of our future research plan. We made a small change in line 364: *“one can change specific growth parameters and simulate competition, thereby predicting ~~the effects~~ adaptive potential of such changes ~~on competition~~.”*

11. Pg. 16, lines 372-375. Just out of curiosity, were there edge or corner effects on growth trajectories of cultures within the microtiter plates?

* We did not see any edge/corner effects.

That’s all the comments I have. Thank you for letting me read this interesting paper!

* Thank you for taking the time to read it and for your helpful comments!

**Reviewer #2**

In the paper, the authors describe an approach to predict fitness estimates without the necessity of a “gold standard” competition experiment and then validate the approach using a set of experiments. There has been an explosion of interest in microbes and comparative estimates of fitness, particularly following experimental evolution studies. Competition assays are valuable in estimating fitness, but there are laborious even with recent technological advances. The procedure described by the authors substantially reduces the burden of a conventional fitness assay while providing estimates that are similar to those found using the standard approaches.   
  
The procedure described is interesting and has merit. The combination of a computational approach, and growth curves of mono- and mixed cultures is appealing. At the same time, the paper has a few deficiencies.

The authors argue that their approach provides an interpretation of the demographic basis of microbial fitness, and yet there is relatively little development of this idea.

* To de-emphasize this argument, we removed the last sentence from the abstract (line 35), which argued that our approach provides an interpretation of the demographic basis of microbial fitness.

The authors correctly note that the approach described would be used inappropriately if the interactions between the strains are not solely due to resource competition, and yet there is little discussion on how the results would be affected by non-resource based competition. Given the general likelihood of non-resource mediated competition among microbes, the absence of experimental data is unfortunate.

* The way our competition model and approach is set up, we assume density-dependent interactions between competing strains; resource competition is a good example of density-dependence.
* The text that introduces the competition model was changed (line 186): *“To model growth in a mixed culture, we assume that interactions between strains are ~~solely~~ density-dependent, for example due to resource competition. ~~(excluding, for example,~~ This excludes frequency-dependent interactions, which can arise due to production of toxins (18) or public goods (19)~~)~~ . Therefore, all interactions are described by the deceleration of the growth rate of each strain in response to the ~~depletion of resources due to growth~~ increased density of both strains.”*
* We added a new paragraph and figure to the Discussion to demonstrate how our model is expected to perform if growth is frequency-dependent (line 303, Figure 8): *“Our approach assumes that the assayed strains will grow in accordance with the density-dependent growth and competition models, which can occur when growth depends on the availability of a limiting resource (see* ***Appendices A*** *and* ***B****). Therefore, this approach can be applied to data from a variety of organisms, experiments, and conditions. However, our approach is not appropriate if growth is frequency-dependent, for example due to production of public goods (22–24) and toxins (18) or due to cross-feeding (25).* ***Figure 8*** *demonstrates the applicability of our model to simulated experimental results from four different frequency-dependent dynamics (1). When density- and frequency-dependent interactions work in the same direction, e.g. due to exploitation of the slow-growing strain (green) by the fast-growing strain (red), our approach is consistent with the simulated experiments: the competition model fits the total density in mixed culture quite well (****Figure 8A****), and its mixed growth prediction is consistent with the final outcome after 10 hours, but not with the full frequency trajectories (****Figure 8E****). However, this is not the case when density- and frequency-dependent interactions do not agree so that the slow-growing strain benefits from the presence of the fast-growing strain, e.g. due to mutualism, competition, or exploitation by the slow-growing strain. In these cases, the fit of our competition model to total density in a mixed culture is poor (****Figure 8B-D****), and the model can fail to predict even the final outcome of the pairwise competitions (****Figure 8H****). Future work will determine if such divergences between experimental results and model predictions could be used to detect frequency-dependent interactions.”*

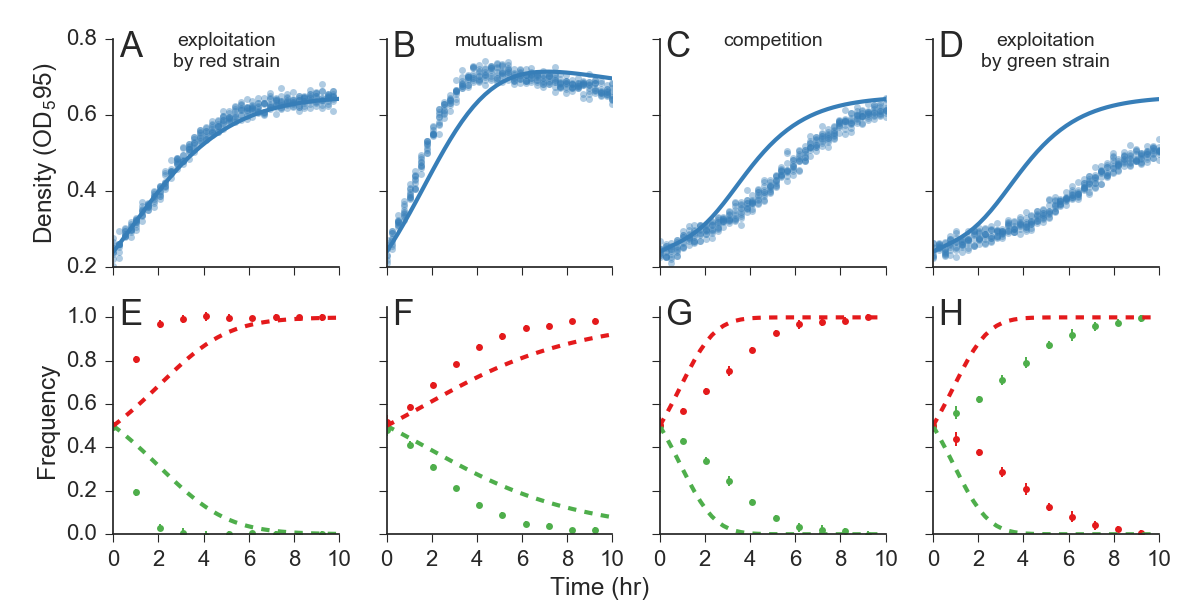


Figure 8. Frequency-dependent growth. Results from simulated experiments, which include frequency-dependent interactions, analyzed using our competition model, which assumes density-dependent growth. Top row shows the fit of competition models (solid lines) to total densities from mixed growth (markers), as in Figure 4. Bottom row shows actual (markers) and predicted (dashed lines) relative strain frequencies, as in Figure 5. Each column corresponds to a different type of frequency-dependence. Growth of strain *i* in the presence of strain *j* follows , with for mutualism (panels A and E); for exploitation of the green strain by the red strain (B and F); for competition (C and G); and for exploitation of the red strain by the green strain (D and H); growth parameters as estimated from experiment A; competition coefficients . 10 replications per pair of and .

* In the future we plan to use our model as a null model for detection and estimation of frequency dependent interactions, but such work is out of the scope of the current paper.

The biggest deficiency is the perspective described, more as a methods paper rather than a conceptual advance in understanding the nature of competitive fitness.

* To emphasize the conceptual advancement in our new approach, we added the following text to line 336: *“Current approaches to estimate fitness from growth curves only incorporate measurements from mono-culture experiments. In contrast, our new approach infers actual competition by directly incorporating measurements from mixed culture experiments.”*
* We also modified the our “Conclusions” paragraph to offer a perspective as a conceptual advance in understanding competitive fitness (line 380): *“We developed and tested a new approach to analyzing growth curve data, and applied it to predict the relative growth and fitness of individual strains within a mixed culture. Competitive fitness is defined as the relative change in frequency during growth in mixed culture. Therefore, any process that affects relative growth in a mixed culture might affect competitive fitness. Current approaches use growth curve experiments because they are easy to obtain, and despite their clear deficiencies. Our approach allows the use of such growth curve data, incorporating growth curves measured in a mixed culture, and thus incorporates various processes that occur in a mixed culture, including actual competition dynamics. By predicting growth in mixed culture and estimating competitive fitness, our approach can improve our understanding of the nature of competitive fitness in microbes.”*

**Reviewer #3**  
The authors describe a method to infer growth dynamics of competing microbes from single strain growth curves. Given the pain involved in measurements of competitive fitness involving mixed culture the single growth curve approach will find ready adherents. The basic idea is straightforward: there is more information in a standard growth curve than typically used, so why not put this additional information to use.   
  
Overall the work makes a useful contribution. I do though have one reservations and this concerns the likely highly restrictive set of conditions under which the method can be validly used (where competitive interactions are determined solely by a single limiting resource). While this in itself is not a problem, it does present as a very significant problem to an unwary adopter. My concern is that the ease with which the method can be applied to data that spews without thought from plate reader devices could easily result in inappropriate application.   
  
This is not reason to hold back publication, but I think the authors might go further than they have and make very clear the fact that it is inappropriate to apply their method in situations where interactions are mediated by anything other than direct completion for a limiting resource – which will very often be the case. This rules out competitive interactions mediated by, for example, cross feeding, allelopathy or any thing else that provokes frequency effects.

* The way our competition model and approach is set up, we assume density-dependent interactions between competing strains; resource competition is a good example of density-dependence.
* The text that introduces the competition model was changed (line 186): *“To model growth in a mixed culture, we assume that interactions between strains are ~~solely~~ density-dependent, for example due to resource competition. ~~(excluding, for example,~~ This excludes frequency-dependent interactions, which can arise due to production of toxins (18) or public goods (19)~~)~~ . Therefore, all interactions are described by the deceleration of the growth rate of each strain in response to the ~~depletion of resources due to growth~~ increased density of both strains.”*
* We added a new paragraph and figure to the Discussion to demonstrate how our model is expected to perform if growth is frequency-dependent (line 303, Figure 8): *“Our approach assumes that the assayed strains will grow in accordance with the density-dependent growth and competition models, which can occur when growth depends on the availability of a limiting resource (see* ***Appendices A*** *and* ***B****). Therefore, this approach can be applied to data from a variety of organisms, experiments, and conditions. However, our approach is not appropriate if growth is frequency-dependent, for example due to production of public goods (22–24) and toxins (18) or due to cross-feeding (25).* ***Figure 8*** *demonstrates the applicability of our model to simulated experimental results from four different frequency-dependent dynamics (1). When density- and frequency-dependent interactions work in the same direction, e.g. due to exploitation of the slow-growing strain (green) by the fast-growing strain (red), our approach is consistent with the simulated experiments: the competition model fits the total density in mixed culture quite well (****Figure 8A****), and its mixed growth prediction is consistent with the final outcome after 10 hours, but not with the full frequency trajectories (****Figure 8E****). However, this is not the case when density- and frequency-dependent interactions do not agree so that the slow-growing strain benefits from the presence of the fast-growing strain, e.g. due to mutualism, competition, or exploitation by the slow-growing strain. In these cases, the fit of our competition model to total density in a mixed culture is poor (****Figure 8B-D****), and the model can fail to predict even the final outcome of the pairwise competitions (****Figure 8H****). Future work will determine if such divergences between experimental results and model predictions could be used to detect frequency-dependent interactions.”*

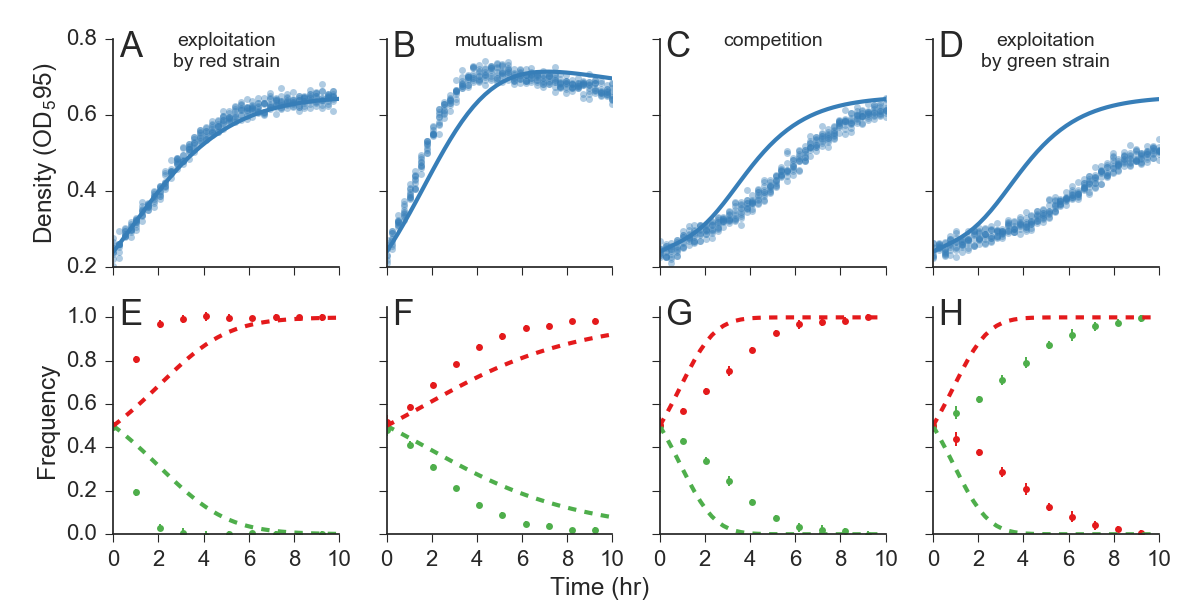


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Now, knowing whether such effects are ever at play is a challenge. This thought did lead me to wonder whether a useful extension of the authors’ method might be as a null hypothesis against which straight forward resource competition might be rejected. I realise this would take more work and might end up going nowhere, but I could imagine confidence limits around the outcome of competition assuming nothing other than competition for a resource.

* In the future we plan to use our model as a null model for detection and estimation of frequency dependent interactions, but such work is out of the scope of the current paper.

In a similar vein, I would go easy downplaying the value of flow cytometry: it is true that it requires at least one fluorescent maker (and preferably two), but beyond this, the continuing improvement in technology makes achieving high quality (time-resolved) measures of competitive fitness via co-culture reasonably painless.

* We agree that in some cases competition experiments can be painless. However, in our experience it can be “painful” in laboratories that have never carried such experiments before, and we have changed the text accordingly (line 66): *“Competition experiments are often more demanding and expensive than simple growth curve experiments, especially in labs where they are not routinely performed.”*

Additionally, there are not many organisms into which a fluorescent marker cannot be introduced.

* We changed the text accordingly in line 67: “*Competition experiments … require the strains of interest to be engineered with genetic or phenotypic markers which is ~~impractical~~ difficult or impossible in some non-model organisms.”*, and in line 358: *“even if it is very hard or impossible to insert phenotypic or genetic markers into the strains in question, e.g. with some non-model organisms”*

And of course it doesn’t suffer from the same limitations as the growth curve method

* Of course. This is already emphasized in the text.

Some additional comments:  
  
In the caption to Figure 1 it is said that the solid line is a polynomial fitted to the data: I wondered firstly, what kind of polynomial and secondly, why not a logistic curve?

* In Figure 1 a logistic curve doesn’t provide a good fit to this curve; this is why we use the Baranyi-Roberts model in our approach.
* We use polynomial as a smoothing technique so that we can calculate the derivative and take the maximum of the derivative. The polynomial is of degree 3, and we modified the text to reflect this (Figure 1 caption, line 87).

Figure 2 got me confused. Listed in A are six growth, but once I got to the Results the authors seem to deal with five parameters and have ditched the declaration parameter, or maybe it is subsumed within the adjustment function?

* Equation 2 includes six parameters, as in Figure 2 (line 153): N0, K, r, ν, q­0, and m. Note that q­0 and m appear in A(t) (eq. 2b), which appears in eq. 2a. The deceleration parameter ν (nu) appears in eq. 2a twice: in the exponent as a product with r and A(t), and as the root of the denominator (i.e. power of 1/ν).

The first part of the Results should really be in the Materials and Methods section.

* We tried different layouts for the manuscript. As suggested, we tried moving the subsection titled “Experimental validation design” to the “Materials and Methods” section. We think that to understand our experimental results and how they correspond to our model, a short overview of the experimental design is required at the beginning of the “Results” section, especially because the “Materials and Methods” section appears at the end of the manuscript.

I did appreciate that the authors have extended their work to the experimental realm. However, harking back to the point above, I wonder if the authors might consider performing a similar experiment in which interactions between two genotypes are known to frequency dependent. Take for example acetate cross-feeding E. coli. It would be valuable – possibly even salutary – to see to what extent estimates of fitness depart from those predicted by curveball in cases where the method is incorrectly applied.

* As noted above, we include a new paragraph and Figure 8 that demonstrate how the growth predictions depart from simulated experiments given frequency-dependent growth.
* In the future we plan to use our model as a null model for detection and estimation of frequency dependent interactions. We therefore thank the reviewer for this valuable suggestion and encouragement. However, we think that such work is out of the scope of the current paper.

**Other changes**

The current address of one of the authors, Uri Obolski, has changed.