**Nutrient fluctuations characteristic of microscale heterogeneity**

**reduce bacterial growth rates**

Hypotheses:

1. Compared to steady environments, nutrient fluctuations are a growth cost to bacteria because bacteria must first expend energy adapting to an environment before growing optimally.
2. The cost in growth depends on the timescale of nutrient fluctuations, due to the interplay between the timescale of fluctuations and the timescale of bacterial adaptations.

Open questions:

1. Are bacterial continually remodeling themselves to their current environment?
2. Is there a fluctuation-adapted physiology? If so, how is this advantageous?

Results:

1. Generation of precise and automated nutrient fluctuations
2. Fluctuations consistently reduce growth compared to steady environment
3. Growth costs are timescale dependent
4. Evidence for fluctuation-adapted physiology.
   1. Different responses to upshift between repeated shifts and single.
   2. Does this physiology vary with timescale? How stable is it?

Abstract

How do such rapid environmental fluctuations affect bacterial behavior? Because bacteria coordinate growth with nutrient availability, frequent nutrient fluctuations an exhausting challenge. Is growth in dynamic environments an integration of efforts to tune physiologies to immediate cues? Or could fluctuation-specific strategies exist? Using microfluidics to control the magnitude and period of nutrient cycles, we observed the effects of minute-scale fluctuations on the growth of individual *Escherichia coli*. Consistently, fluctuations reduced growth compared to stable environments delivering equal time-averaged amounts of nutrient. These reductions varied with nutrient timescale and arise from the differences between timescales in the dynamic growth responses to nutrient shifts.

Introduction

**take this and flesh it out**: sprinkle some physiology and curiosity to it!

* Fluctuations in the environment can be sub-cell cycle timescales: seconds or minutes
* The knowns from steady environments
* The unknowns surrounding fluctuations and physiology (link to biogeochem cycles)
* The unknowns that are now known to us (haha!) thanks to our cool technique and first study ☺

From the ocean to soils to wastewater, bacteria are key players in the cycling of all the major elements. Underlying these large-scale effects are the growth and activities of individual bacteria, which often unfold in a nutrient landscape that is highly heterogeneous at sub-millimeter scales (Stocker, 2012). A lysing cell, for instance, is a local hotspot of nutrient orders of magnitude richer than background concentrations, creating a gradient up which chemotactic bacteria can traverse within a minute (Smriga, Fernandez et al., 2016). Diffusion would dissipate such a nutrient patch within 10-20 min. Fluid mixing would stir the patch into thinner sheets and filaments, which would then dissipate on timescales of a few minutes or even seconds (Taylor & Stocker, 2012). This ephemeral microscopic heterogeneity, a major feature of many microbial habitats, implies that individual bacteria experience not a steady nutrient concentration, but one that frequently fluctuates over timescales often shorter than the division time.

For decades, studies have alluded to the relevance of environmental fluctuations by characterizing how bacteria coordinate growth and gene expression with the environment. For example, steady-state studies have well established a positive correlation between cell mass and growth rate (Schaechter et al., 1958; Godin et al., 2010; Taheri-Araghi et al., 2014) – a so-called growth law, suggesting that bacteria prioritize biomass synthesis when nutrient is abundant. Rapid protein synthesis is less valued during slow growth, when expression is focused on genes that work to endure nutrient deplete conditions, such nutrient uptake (ppGpp ref; decreased ribosomal expression ref; Scott et al., 2010).

Yet, it is unclear how trends derived from steady-state growth conditions can apply to realistic environments that fluctuate. One might expect monod or jensens. But what happens when we fluctuate on timescales faster than adaptation? Switching between two steady-states requires energy. Are cells continuously switching between these two states? If so, we expect losses in growth that depend on the timescale of nutrient fluctuations, due to the interplay between the timescale of fluctuations and the timescale of bacterial adaptation.

We propose that fluctuations can considerably affect bacterial growth and aim to address this knowledge gap. We hypothesize that the fine structure of the nutrient landscape, not just the average nutrient concentration, determine bacterial growth.

When and to what extent do high-frequency fluctuations affect bacterial growth? To test this hypothesis, we developed a novel microfluidic device that enabled us to observe and quantify single cell growth dynamics while precisely delivering automated nutrient signals. With this new approach, we show that fluctuations consistently reduce growth when compared to a steady environment delivering the same total nutrient. The growth costs are dependent on the timescale of nutrient fluctuation: (what does this tell us about the physiology?).

* Enticing lead up to results!

Results

**Generation of precise and automated nutrient fluctuations.**

Experimental mimicry of a heterogeneous nutrient landscape involves both addition and removal of nutrient. As our timescales of interest required rapid yet precise concentration switches, we engineered a microfluidic device to vary the strength, duration, and frequency of nutrient pulses while simultaneously observing individual *E. coli* cells with time-lapse phase microscopy (**Figure IA**). In this study, all delivered signals switch between the same two nutrient concentrations: a lower and higher dilution of LB (**Figure IB**). With automated control over the rate at which each LB solution is driven into the device (**Methods I; Supplementary Methods 1**), the cells – attached to the glass surface of the device, downstream of the switching junction – reliably experience sharp switches between high and low LB within 2-3 seconds, with hardly any smoothing (**Figure 1C & 1D**; **Supplementary Methods 2**). Cells from an exponentially growing batch culture of E. coli NCM3722 ∆*motA* attach within the microchannel in greater numbers when the channel is treated with poly-L-lysine, which in our system has no treatment-specific effects on growth rate (**Supplementary Methods 2**). Poly-L-lysine treatment also extends attachment duration, allowing for longer observations of single cells as they grow and divide **(Figure 1E**; **Supplementary Methods 3**).

Each fluctuating environment was performed in parallel with three control conditions: (1 and 2) steady low LB and steady high LB, to measure the expected “boundary” physiologies we could expect to see; and (3) steady averaged LB, the time-averaged nutrient environment, representing a “bulk sampled” environment that may actually be heterogeneous (**Figure 1A**; **Supplementary Methods 4**). From each of these environments, we have tracked thousands of single cell lineages (**Figure 1F**; **Supplementary Methods 5**) with which we ask: how do laboratory-observed behaviors translate to other environments? E.g., can we assume that a growth rate observed in a liter of chemostat culture to apply in a liter of seawater with an equivalent total amount of nutrient? Macroscopically, at the liter-scale, these environments contain identical nutrient concentrations. But microscopically, the chemostat is well mixed whereas seawater is not (**REF 1 & 2**). We hypothesize that the microscopic heterogeneity of seawater, like many microbial environments, would lead to growth rates that differ from those in a well-mixed chemostat, due to temporal fluctuations in nutrient concentration inherent to patchy environmental landscapes.

**Fluctuations reduce growth in a timescale-dependent manner.**

To ask how the timing of nutrient availability influences growth, we first determined a concentration range in which to fluctuate. We experimentally measured steady-state growth rates across a range of fixed nutrient concentrations, spanning five orders of magnitude (**Supplementary Methods 5**). As expected, the cells require about 2-3 hours to adapt to their new microfluidic environment (**Figure 2A**). Due to the high flow rates used in this study, no nutrient depletion or metabolite excretion is expected to alter the original composition of the nutrient media. (**Supplementary Methods 6**). The steadiness of the nutrient environment is apparent in the saturated growth rates that remain steady for several hours after the initial adaptation (**Figure 2A**). As expected, the average growth rate during this steady phase of the growth curve – i.e. the steady-state growth rate – varies with nutrient concentration, displaying a saturating Monod curve that plateaus at a growth rate of 2 hr-1 at 3% LB or higher (**Figure 2B**). We avoid this saturated regime by designating 2% LB, with a steady-state growth rate (Ghigh) of 1.87 ± 0.10 hr-1, as the “high nutrient” phase of all fluctuating signals (**Figure 2B**). The “low nutrient” phase (0.1% LB; Glow = 0.67 ± 0.15 hr-1) was selected such that the “average nutrient” condition (1.05% LB; Gave = 1.51 ± 0.14 hr-1) would be significantly distinct from the high (**Figure 2B**).

After defining the high and low nutrient phases, we measured average growth rates from E. coli experiencing fluctuating nutrient environments. Complete switching between high and low occurs so quickly that the cells effectively experience an oscillating square wave with equal amounts of time in both phases (**Figure 1B**; **Supplementary Methods 2**). Across all nutrient signals, growth rates oscillated on the same timescale as nutrient (**Figure 2C**; **Supplementary Methods 7**). However, while the nutrient signal amplitude saturates within seconds of a switch, growth rate transitions occurred over minutes and amplitudes often less than matched those observed in steady-state (**Figure 2C**). In fact, time-averaged growth rates from fluctuating environments consistently fell lower than growth rates measured from the equivalent time-averaged steady environment (Gave), reduced by up to 40% (**Figure 2B**).

Intriguingly, the average growth rate under fluctuations was dependent on the timescale of nutrient fluctuation (**Figure 2B & 2D**). Of the nutrient timescales tested – 30 sec, 5 min, 15 min and 60 min periods – the second-scale fluctuations were less detrimental than minute-scale ones (**Figure 2D**). These costs in growth rate, as a percent of Gave from the steady average LB environment, were 15, 30, 40 and 40% respectively (**Figure 2D**). We hypothesize that this difference in average growth rate between fluctuating timescales is linked to how each timescale relates to the timing of bacterial responses to shifts in nutrient availability. For example, fluctuations on timescales that are faster than bacterial response times (i.e. milliseconds (**REF 3**)) should lead to cellular averaging of the external nutrient environment and thus growth as if the nutrient environment were steady (**Figure 2D**). Similarly, fluctuations on timescales so great that bacterial adaptation times are negligible (i.e. tens of hours, days) should have an average growth rate equal to the average of the two steady-state rates, Ghigh and Glow (**Figure 2D**).

Between these two extremes are timescales of many plausible categories, including: first, timescales in which physiological adaptation moves towards that of the steady average and any growth rate deviations are plastic responses of that Gave physiology to brief fluctuations in nutrient concentration (**Figure 2E**). Second, there may be intermediate timescales at which cells begin to adapt towards the high or low physiology but do not have sufficient time in either phase to fully reach steady-state (**Figure 2E**). Third, some timescales are likely long enough for cells to reach steady-state in both high and low nutrient phases, but short enough for the sub-steady-state adaptation growth rates to manifest as a cost to the overall time-averaged growth rate (**Figure 2E**). Finally, there may be timescales that induce bacterial to stabilize around a fluctuation-adapted physiology distinct from the steady-state physiologies of Gave, Ghigh and Glow. Which, if any, of these behavioral classifications apply to the timescales we observe here?

**Single perturbations do not capture dynamics of rapidly fluctuating environments.**

To better understand the growth behaviors observed from our fluctuating environments, we performed a series of single nutrient shift experiments in which *E. coli*, once adapted to one environment, were permitted to fully adapt to another (**Figure 3A & 3B**). These data give us the plasticity of growth rate for a given physiology (e.g. steady-state growth at average LB) as well as the expected behavioral trajectory during adaptation between steady-states. Let’s first consider a single upshift from low to high nutrient, where the switch occurs after the cells reach steady-state growth in low LB (**Figure 1B**; **Figure 3A**; **Supplementary Methods 8**). Low-adapted cells transitioned to steady-state growth at high LB over roughly an hour (**Figure 3A**; **Supplementary Methods 8**). Immediately after the upshift, we observed an initial jump in growth rate (**Figure 3A**), consistent with previous reports (**REF 4 & 5**) that attribute such rapid growth rate responses to unoccupied ribosomes in low nutrient ready to take advantage of increased influxes of nutrient upon upshifts. This initial jump is then followed by a gradual adaptation to the new steady-state growth rate Ghigh (**Figure 3A**). Were cells in nutrient fluctuations physiologically adapted to the low, we should expect to see a similar response to the several upshifts delivered in each fluctuating environment – we do not. In all minute-scale fluctuations, growth rates increase rapidly for 2-3 min before saturating instead of the hour needed for low-adapted cells in the single upshift controls to stabilize at Ghigh (**Figure 3C**). This exceedingly quick response time suggests that cells experiencing repeated nutrients may contain an even greater number of ribosomes (or transporters, *etc.*) than cells adapted to a steady background concentration of nutrient. Overall, single upshifts in nutrient concentration clearly do not capture the growth rate dynamics occurring in rapidly fluctuating environments.

Likewise, single downshifts in nutrient concentration revealed disparate dynamics from environments with rapid repetitive nutrient fluctuations. While adaptation from steady-state Ghigh takes hours (**Figure 3B**), cells experiencing minute-scale fluctuations returned to growth at Glow within minutes (**Figure 3D**).

*[insert description of simulation, as if cells were adapted to average]*

Class 3 timescales are longer than those tested in this study; we do not observe steady-state growth rates achieved in our fluctuating environments, as noted previously (**Figure 2C**).

Apparent growth physiology: stability in phenotype after immediate response is suggestive of a fluctuation-adapted physiology. Increased speed of response suggests that, under fluctuations, cells prepare for rapid growth as enabled by their current nutrient environment.

**Supplementary Methods 9:** text

**REF 6**: text

Discussion

Fluctuations are a major feature of many microbial habitats, yet the current knowledge on bacterial growth is largely based from steady nutrient conditions. This study is to our knowledge the first attempt to connect the existing literature on single cell growth to applications for realistic environments.

**Bulk assessments of nutrient environment substantially overestimate bacterial growth.**

Fluctuations that oscillate between the same two nutrient concentrations can substantially reduce single cell growth rates. Overall, we found that, between environments delivering equal net nutrient, stable and fluctuating environments produce unequal growth yields and that these differences, even those between fluctuating timescales, can be explained by a careful look at the minute-scale response of growth to shifts in nutrient availability.

**Repetitive environmental change induces a fluctuation-adapted physiology.**

Text

Final statements should zoom out and consider not only biogeochemistry but other processes (population dynamics, ecology, etc) as well.

This work represents an ambitious step towards understanding bacterial growth in realistic environments.

the fact that single cell mass increases with increasing growth rate (Schaechter or Kjeldgaard et al., 1958; Taheri-Araghi et al., 2014) supports the hypothesis that the production of biomass – namely ribosomes, which contribute x-y% of dry cell mass (REF) – is prioritized under conditions in which nutrient is abundant. Rapid protein synthesis is less valued during slow growth, when expression focuses rather on genes that work to endure nutrient deplete conditions, such nutrient uptake (Scott et al., 2010; is nutrient transport upregulated with ppGpp?).

Could the average concentration, *i.e.* the bulk C, N and P measured from a liter of seawater, be sufficient to predict growth? Likely not.

Single shifts between high and low growth rates have followed the hours-long progression between physiological steady-states and show minute-timescale responses that require energy. Do cells continuously move between these two steady-states?

What internal sensing signals to cells that the external environment is abundant or deplete (ppGpp)? Surely, the timescale matters in determining whether to average versus not.

Figures

**Figure 1. The Microfluidic Pulse Generator enables simultaneous generation of dynamic nutrient signals and single-cell microscopy.** (A)

**Figure IA:** visual of MPG structure, noting regions of switching and imaging, parallel steady channels.

**Figure IB:** visual list of signals, representing all experiment types presented here

* *Would be cool to generate a stochastic signal and predict the growth rate from that. Ultimately coming back to the environment.*

**Figure 1C**: switching in microfluidic junction

**Figure 1D**: fluorescein signal in junction and at cell XY position looks square

**Figure 1E**: images of cell growing and dividing in device

**Figure 1F**: single cell trajectories in fluctuating and steady environments

**Methods I:** pressure control of MPG, fluorescein calibration

**Supplementary Methods 1**: scripts for controlling MPG

**Supplementary Methods 2:** comparison of fluorescein signal at junction and at imaging XYs, signal does not suffer any smoothing.

**Supplementary Methods 3**: poly-lysine does not affect growth rates as measured in MPG. Rather it enhances attachment, increasing the number of attached cells and extending the amount of time for which they are attached (boosting observation)

**Supplementary Methods 4**: fluctuating between ave and ave is the same as steady ave in parallel channel; fluorescein does not affect growth rate (2017-11-09 experiment)

**Supplementary Methods 5**: description of particle tracking

**Figure 2. The magnitude of growth reductions varies with the timescale of nutrient switching**. (**A**) Time-resolved evolution of population growth rate in steady and fluctuating nutrient environments. The start of image acquisition occurs at t=0, which also denotes the start of nutrient fluctuation. Parallel to each fluctuating environment (blue), three steady environments reveal the expected steady-state growth rates for cells physiologically adapted to growth at the low (purple), average (gold), and high (red) nutrient concentrations. Each point time-averages all instantaneous, single-cell growth rates within a 2-min time bin. (**B**) Time-averaged growth rate versus time-averaged nutrient concentration. Each point represents the mean growth rate of all individual growth rates measured after 3 hours into each technical replicate. Because nutrient concentration is time-averaged, data from steady average LB environments are vertically aligned with data from fluctuating environments with nutrient oscillations of varying period lengths: 30 sec (red), 5 min (yellow), 15 min (green), 60 min (lavender). (**A-B**) Error bars report standard error of the mean.

(**C**)

(**D**) Growth rate under fluctuations as a fraction of growth rate in the steady average nutrient environment. Each point represents the average mean growth rate between experimental replicates. Error bars report standard deviation.

**Figure 2A**: mean growth rate of populations over time to illustrate approach toward steady-state, stable and fluctuating

**Figure 2B**: Monod plot with colorful fluctuating conditions

**Figure 2C**: under all fluctuating timescales, growth rate oscillates with nutrient concentration

**Figure 2D**: growth in fluctuations relative to expected growth vs. timescale

* *The 5-min period represents a cell surfing stirred filaments of nutrient, produced from a patch in turbulence. Again, would be great to have a bulk test that takes advantage of this set-up.*

**Figure 2E**: schematic depicting plausible classifications of timescale based on hypothesized implications on cell physiology.

**Supplementary Methods 5**: measuring growth rate from image data

**Supplementary Methods 6**: discussion on flow rates and channel volume, arguments for steady environment

**Supplementary Methods 7**: replicate growth rate vs time plots for one example timescale, to illustrate consistency

**REF 3**: millisecond timescales are faster than bacterial response times

**Figure 3. Single nutrient upshifts and downshifts are not representative models for fluctuating environments with frequent shifts**. (**A**) Time-resolved

**Figure 3A:** growth rate vs time for single upshift from low to high, include stable controls

**Figure 3B:** growth rate vs time for single downshift from high to low, include stable controls

**Figure 3C**: single upshifts overlays with fluctuating

**Figure 3D:**

**Figure 3E:** single downshifts overlaid with fluctuating

**Supplementary Methods 8:** additional markers that the cells have reached steady-state growth, i.e. size (probably not enough time to assess inter-division times). Note: this may not work… perhaps size and growth rate adapt at different timescales….

**REF 4** & **5**: Uri Alon and Terry Hwa group papers on single upshifts

**Figure 3X**: growth rate vs time for single upshift from ave to high, include stable controls

**Figure 3X**: growth rate vs time for single downshift from ave to low, include stable controls

**Figure 3X**: simulated mean growth rate were cells adapted to average condition and plastically respond or begin to adapt to switches across different timescales

**Figure 3X**: simulated mean growth rate were cells adapted to previous (high or low) condition before switch across different timescales

Methods

**Growth rate calculations**

One widely used method to calculate growth rate is to consider the growth curve exponential (Godin et al., 2010) and solve for growth rate from Vf = Vi \* 2µt. We take this same approach between neighboring timepoints, by measuring the rate of change between the natural log of volume.

**References that I want or need:**

Russell & Cook (1995): 50% energy consumed by translation machinery

\_\_\_\_( ): Translational capacity is determined by ribosome content

Bacteria play a fundamental role in the environment. In the ocean, for example, bacteria are key players in the cycling of all the major elements. Underpinning these large-scale effects are the growth and activities of individual bacteria, which often unfold in a nutrient landscape that this highly heterogeneous down to sub-millimeter scales. The result is that individual bacteria experience not a steady nutrient concentration, but one that fluctuates over timescales often shorter than the division time: we call these “high-frequency fluctuations”. Despite these fluctuations being a major feature of many microbial habitats, the vast majority of current knowledge on bacterial growth is based on steady nutrient conditions. We propose that fluctuations can considerably affect bacterial growth and aim to address this knowledge gap. When and to what extent do high-frequency fluctuations affect bacterial growth? We hypothesize that the fine structure of the nutrient landscape, not just the average nutrient concentration, determine bacterial growth. To test this hypothesis, we precisely quantify bacterial growth dynamics under nutrient fluctuations with different frequencies using a new approach we recently developed.

Bacteria are ubiquitous and are responsible for a broad range of processes in nature, industry and health. Perhaps the most striking contrast between the scale of individual bacteria and the scale at which their effect is realized occurs in the ocean, where these micrometer-sized organisms drive biogeochemical dynamics at scales of hundreds of kilometers (REF 1-3). For example, organic matter tends to sink in the ocean in the form of particles, resulting from the death and coagulation of photosynthetic microorganisms (phytoplankton), removing carbon from the upper ocean and burying it at depth for hundreds of years (REF 4). The magnitude of this vertical flux, also called “the biological pump”, is shaped by bacteria: the more particles they remineralize, the less material reaches the bottom. How microbes access and grow on this organic matter is thus important for the physiology of the bacteria, but also for the biogeochemistry of the oceans.

Our understanding of how bacteria access nutrients in aquatic environments has to-date remained rather simplified, owing to both the technical challenges of replicating key features of the natural nutrient landscape and to a diminishing focus in the field on physiological studies in favor of genomic ones. The realization that the natural environment of aquatic bacteria is rarely the steady, homogenous habitat has not yet translated into systematic experiments to determine how microbial growth is affected by spatially and temporally variable conditions. To date, studies of microbial processes in the ocean still typically examine large-volume water samples (>liters) collected across large spatial scales and at low temporal resolution (REF 8-10). Yet, over the past two decades it has become clear that the nutrient landscape of marine bacterial is characterized by a plethora of microscale sources and hotspots, often sub-millimeter in size, including the lysis of other microorganisms such as phytoplankton cells, excretions by larger organisms such as copepods, marine particles, fecal pellets, and sloppy feeding events (REF 11-15). Further variability is introduced by turbulence, which is nearly always present in aquatic environments ad stirs larger nutrient sources into filaments on the scale of 30-300 micrometers. The nutrient landscape is thus characterized by hotspots of high nutrient concentration amidst an otherwise nutrient poor ocean. From the perspective of individual bacteria, this microscale heterogeneity results in strong temporal fluctuations in nutrient concentration, with characteristic timescales of tens of seconds to several minutes (REF 6). We refer to these fluctuations as “high-frequency fluctuations”, to distinguish them from other slower fluctuations, such as diurnal or seasonal ones. Here, we consider “high-frequency” as any fluctuation occurring on a timescale shorter than the cell doubling time, typically 30-60 min in the laboratory and several hours in the ocean. These scales are currently inaccessible *in situ*.

In terms of nutrient acquisition strategies, marine microorganisms have largely been categorized into two groups (REF 18): copiotrophs, which are often motile and have large genomes, likely to exploit rapid changes in nutrient availability; and oligotrophs, which have small genomes and low growth rates, and thrive on the very low concentrations of organic matter characteristic of most of the ocean. Beyond this coarse classification, and the recognition that some bacteria can exploit fleeting nutrient concentrations, we still largely ignore how temporal changes in nutrient concentration impact the physiology, growth and ultimately the biogeochemical effects of marine bacteria, compared to the classic picture of growth in steady conditions. In fact, microorganisms have traditionally been studied in steady or quasi-steady environments: in chemostats the environment is imposed to be steady, while changes in batch cultures are slow and gradual. Realization of controlled, high-frequency nutrient fluctuations in the laboratory requires rapid and accurate temporal control of the cells’ environment, which is often beyond traditional culture approaches.

If we find that high-frequency fluctuations affect the growth rate of bacteria, it will show that the bacterial biomass produced from a given amount of nutrients – the growth yield – is a function of the spatial structure of the nutrients, not just their average nutrient concentration, as implicitly assumed in much of microbial ecology to date. This project also builds upon classic experiments on growth adaptations to single up- or down-shifts in nutrients (REF 25-27). Such studies have identified multiple factors (DNA-binding proteins, metabolic enzymes, transporters, non-coding RNAs) as regulated in response to single nutrient shifts (REF 27), and have characterized the molecular adaptations to such shifts including the timescales over which DNA, RNA, protein and biomass synthesis rates change, and the gene-specific dynamics of transcript and protein abundance (REF 28). Here, instead of single shifts, we will investigate whether bacteria have specific adaptations to repeated changes in nutrient concentration – to fluctuations.

^ End of 2.1 in SNSF research proposal.