

Maintaining Microbial Diversity in Mixed Cultures

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Microbial Diversity 2009

Introduction:

Natural microbial communities are often highly diverse consortia of strains. Therefore to fully understand microbial communities it seems essential to have a fundamental understanding of how members within a microbial community interact. Studying interactions within these communities has been hindered by limited success in maintaining the full diversity of a community of natural isolates in the lab. In general, many of the isolate strains will be outcompeted by their neighbors in laboratory cultures, homogenizing the community to a few strains that survive best in the culturing setting of choice. Since stable communities of high diversity are difficult to maintain in the lab, traditional approaches have focused on either studying one or a few species at a time, or have relied on culture independent methods to measure community composition or chemical flux through natural systems in hopes that interactions within the community can be inferred. Some work has focused on maintaining mixed communities in the lab, most notably the work of Winogradsky in his examination of nutrient cycles through mixed communities in his Winogradsky columns. Further understanding of the community interactions that drive ecosystem function are likely to come from studies rooted in the use of mixed cultures. A major hurdle in studying mixed cultures is maintaining the natural diversity, both the richness and evenness, of a sample in the lab. What environmental factors help to maintain diverse communities? How can we replicate these factors in the lab? As

an initial step in developing mixed culturing methods that maintain diversity over time, here I examine how spatial and temporal heterogeneity in culturing conditions impact the diversity of microbial communities.

To explore the role of spatial and temporal heterogeneity in mixed microbial cultures, I have performed three types of cultures: 1) the growth of Sippewissett marsh isolates in agarose containing gradients of nutrients, 2) the growth of marine sponge isolates on porous surfaces, and 3) the growth of a defined consortium of fresh water lab isolates with temporally non-uniform nutrient availability.

Methods:

Gradient shake tubes containing Sippewissett salt marsh isolates:

Gradient tubes and jars were prepared in 16 mm diameter glass test tubes and 4 cm wide, 7 cm tall glass jars. The base agarose solution contained 1X seawater base, 10 mM NH₄Cl, 1 mM potassium phosphate, and 10 mM MOPS pH 7.2. After autoclaving, vitamin B12 and trace element solution was added. The nutrient containing agarose plug at the bottom of the culture additionally contained 3 wt% agarose, 5 mM succinate, and approximately 5 mM elemental sulfur. The sulfur was finely ground using a mortar and pestle. After the agarose in the nutrient containing plug had set, agarose solutions containing isolates from Little Sippewissett salt marsh were added to the tubes. Agarose solutions of 0.1, 0.3, 1.0, or 3.0 wt% were used.

Samples from Sippewissett salt marsh were taken on July 13, 2009, at approximately 8 pm. Two 6 inch deep cores of sand were taken from shallow water (samples 1 and 2). The surface of the

sand was purple in color. Another 6 inch deep core was taken from an area containing decomposing plant material at the edge of a pool of water several feet deep (sample 3). Isolates from the cores were transferred to the shake tubes within 6 hours of collection. Core samples were vortexed and filtered through 25 μm filter paper. Approximately 1 mL of the resulting cell solution was then added per 18 mL of agarose solution. After the gel had been poured, the tubes and jars were covered with foil. Foil covered the gel from the bottom of the tubes up to approximately 1 cm from the top of the gel. In this way, a gradient of sunlight was created during incubation, with the top 1 cm of gel receiving sunlight (see Figure 1A). The tubes were incubated at room temperature in indirect sunlight for more than 8 days.

During preparation of the gradient shake tubes in 4 cm wide jars, 1 inch by 3 inch glass slides were implanted in the gel. The slides were slightly roughened with sand paper and flame sterilized. Before the agarose set, the slides were inserted into the gel vertically, such that 3 inch side of the slide touched the bottom of the glass jar and extended past the top of the gel. Two jars were made in this way, the first containing Sippewissett sample #1 with 1 wt% agarose and the second containing Sippewissett sample #2 with 0.1% agarose.

Gradient shake tubes were analyzed by extracting whole colonies using a sterile glass pipette. Slides were made from the colonies and imaged using a 100X oil immersion objective. Portions of the colonies were also spread onto sea water complete (SWC) plates and incubated at 30°C overnight. DNA was extracted and amplified from pure colonies by boiling in the presence of the surfactant NP40 for 5 minutes and then performing PCR using bacteria 16S rRNA primers 8F and 1492R. The amplified DNA was then sequenced. The resulting 16S sequences from 12

such colonies were analyzed using the RDP database. A phylogenetic tree was made using the 16S sequences from the 12 isolates and the closest 16S matches in the RDP database with *Bacteroides fragilis* as the out group. The glass slides implanted in the jars were extracted after 8 days of culture and directly imaged on the microscope using the 100X objective.

Marine sponge isolates cultured on porous surfaces:

Marine sponge isolates were obtained the Atlantic Ocean near Woods Holes, MA. Sponges were collected from the sea floor and used within approximately 3 hours of collection. Microbial isolates were harvested from two species of sponges, a species resembling *Leucilla nuttingi* (sponge #1) and a species resembling *Microciona prolifera* (sponge #2). The sponges were dissected into small pieces and added to a tube containing 0.3 g of bead beater beads. The tubes were run in the bead beater for 1-2 seconds to partially lyse the sponge tissue. Three tubes were prepared in this way for each sponge, and the resulting isolates were pooled. The media used in incubation consisted of Seawater LTY media containing 0.1 mM succinate. 50 μ L of sponge isolates and 6 mL of media were added to each test tube. The tubes were incubated at room temperature.

Some of the tubes received porous materials during the incubation. For each sponge, 7 conditions were tested: 1) liquid (no added surfaces), 2) sand collected from Garbage Beach, 3) pieces of Nerf® football, 4) pumice stone, 5) a household sponge (wafer sponge), 6) a second type of household sponge (natural sponge), and 7) 0.66 wt% agarose. Each material was cut into sections small enough to fit into the bead beater tubes and autoclaved before use. For materials 3-6, the water level of media did not completely cover all of the material (see Figure 3A).

After 4 days of incubation, pieces of material were removed along with about 100 μ L of solution. DNA was extracted using the MO BIO Laboratories UltraClean Soil DNA extraction kit. Extracted DNA was amplified by PCR using bacteria 16S rRNA primers 8F and 1492R. The 8F primer had a fluorescent label to enable T-RFLP analysis. PCR product was purified using a QuickStep2 PCR Purification kit. Using the Nanodrop to measure the concentration of amplified 16S rRNA product, nucleic acid density was adjusted to approximately 200 ng nucleic acids per 40 μ L of enzyme solution. Two sets of enzymatic digestions were performed for T-RFLP analysis, *Sau* 96I and *Rsa*1.

T-RFLP data was analyzed using both GeneMapper software and T-Rex (trex.biohpc.org). In T-Rex, noise in each data set was reduced by removing peaks whose intensity was within 1 standard deviation of the background. TRFs less than 50 basepairs were also removed from the datasets before analysis. TRFs were aligned by rounding to the nearest whole basepair, and principle component analysis was used to compare the presence/absence of TRFs from the set of experimental conditions. Each condition had an n=1. 16S rRNA sequences from wafer sponge samples digested with *Rsa*1 did not yield sufficient TRFs for analysis.

Growth of a defined consortium with temporally non-uniform nutrient availability

A defined consortium was composed of five strains isolated from fresh water sources. The strains were identified through 16S rRNA sequences as: S1 *Bacillus aquimaris*, S2 *Massilia brevitalea*, S3 *Rhodococcus opacus*, S4 *Chryseobacterium* species, and S5 *Paenibacillus graminus*. The strains were chosen based on availability in the lab, their ability to form visible

colonies after overnight incubation at 30°C on nutrient agar plates, and that each strain formed a colony that could visually be distinguished from each other. Strains were isolated off of nutrient agar plates and diluted into fresh water base at pH 7.4 containing 10 mM NH₄Cl, 1 mM Na₂SO₄, 1 mM Na₂HPO₄, vitamin B12 solution and trace elements solution. The initial cell density of each strain was adjusted to approximately 10⁶ CFU/mL.

The consortia were cultured in 25 mL scale cultures in 125 mL Erlenmeyer flasks, in a shaker at 30°C. 9 time points were spaced approximately 6 hours apart. At each timepoint, 1 mL of culture was removed for plate counts and then carbon sources were added if needed. Plate counts were performed by diluting the culture 10² to 10⁶ fold in the same media, and spreading 30 µL of culture onto nutrient agar plates. For most timepoints, 1 plate with a 10³ dilution of cells and 1 plate with a 10⁵ cells were streaked per condition. The plates were incubated at 30°C overnight and then each colony type was counted.

The carbon sources used during incubation were succinate, cellobiose, sorbitol, and butyric acid. Four patterns of carbon source addition were tested. Each condition received the same total amount of each carbon source, but the time course of the addition of the carbon sources for each condition varied. For condition 1 (C1), all four carbon sources were added to 0.2 mM at time point 1 (tp1). For condition 2 (C2), 0.625 µmoles of each carbon sources was added at tp1 through tp8. For condition 3 (C3), the following pattern of carbon sources were added, 2.5 µmoles of succinate at tp1 and 5, 2.5 µmoles of cellobiose at tp 2 and 6, 2.5 µmoles of sorbitol at tp3 and tp7, and 2.5 µmoles of butyric acid at tp4 and tp8. For condition 4 (C4), 5 µmoles of

succinate was added at tp1, 5 μ moles of cellobiose was added at tp3, 5 μ moles of sorbitol was added at tp4, and 5 μ moles of butyric acid was added at tp7.

For analysis of community composition over time, colony forming units of each cell type were counted at each time point. The plate at each time point which yielded the most accurate count was used in data analysis. C3 at tp2, C4 at tp2, and C4 at tp8 did not yield any plates from which consortia composition could be determined. For Figure 4G, the Simpson's index was calculated using the following formula:

$$\text{Simpson's index} = 1 - \sum p_i^2 \quad (1)$$

In which p_i is the number of colonies of species i at a given time point divided by the total number of colonies counted at that time point. i is summed for consortium species S1 to S5.

Results and Discussion:

Three types of cultures were performed to explore the influence of increased spatial and temporal heterogeneity on the diversity of mixed cultures of cells over time: 1) the growth of Sippewissett marsh isolates in agarose containing gradients of nutrients 2) the growth of marine sponge isolates on porous surfaces and 3) the growth of a defined consortium of fresh water lab isolates with temporally non-uniform nutrient availability.

1) Gradient shake tubes

After several days of growth at room temperature, visible colonies emerged in the shake tubes. Some colony types appeared to be uniformly distributed throughout the tube (such as the small white colonies shown in Figure 1B), whereas others remained localized to specific regions of the tube (such as the medium sized colonies shown towards the top of Figure 1B). This indicates that some isolate strains may be specialized for growth in certain regions of the gradients formed throughout the tube. For example, bubbles, or pockets which appeared to be gaseous, often formed at the interface of the nutrient containing agarose plug (as shown in Figure 1C). These bubbles formed in the following tubes: #3 0.1%, #3 0.3%, #3 3%, #2 0.1%, #1 0.3%, #1 1%. These bubbles were 1-5 mm in diameter, and often seemed to have sulfur pieces at the bottom. It is unclear whether these bubbles were formed as the result of a microbial process, perhaps the reduction of sulfur to form hydrogen sulfide. Additionally several purple colonies a few 100 μm in diameter were observed a few mm below the top of the 0.3 wt% gel with sample #3. These colonies are both in the zone of the gel exposed to the sunlight and also slightly beneath the surface of the gel, which would make sense if the colonies are purple sulfur bacteria which prefer growth in anoxic zones with sunlight and hydrogen sulfide. The growth of purple bacteria also suggests that elemental sulfur was being reduced within the gel. Other samples such as #3 0.1% formed a faint purple/orange streak 22 mm below the surface of the gel, and an downward arch of growth presumably of other strains with its peak 5 mm below the streak. Sample #1 0.1% also formed 3 bands of dense growth, the first 12 mm below the top of the gel, just at the line of sunlight, another 29 mm below the top of the gel, and a final band 84 mm below the top of the gel. Although between bands 2 and 3 some cloudiness was observed, there was no cloudiness between bands 1 and 2. Also dense colonies of long, fibrous cells were observed at the top of sample #1 1% (Figure 1E). These types of cells were not observed in any other

location or in any other sample. Overall, the data suggests that the gradients of conditions did seem to support selective growth of certain colony types. Additionally, the lower weight percent agarose, 0.1 and 0.3%, seemed to have more interesting distributions of cells, including multiple bands of dense growth, arches of growth, large bubble colonies, purple colonies in the light zones, and ribbons of growth. Although the 1 and 3% gels had many colonies, many were very small and closely resembled each other.

Examination of isolated colonies and isolates attached to the inserted glass slide revealed possible symbiosis between cell types. As seen in Figure 1D, some colonies seemed to form around diatoms, whereas no free diatoms were observed in the culture. The cells shown in Figure 1F also suggests a symbiosis. It appears a colony was formed consisting of only 2 cells types. Half of colony consisted of one cell type and the other half of the colony consisted of another cell type. Another possible symbiotic pair of cell morphotypes was observed several times in the cultures. As seen in Figures 1G and H, these colonies consist of curved shaped cells that are motile and move similar to a spirillum and bean or egg-shaped cells. Groups of cell consisting mostly of these two cell types were observed in several samples and did not seem to be specific for a certain region of the gradient. It is possible that the curved species could be related to *Oceanospirillum* sp. that seemed abundant in sample #2 3%. Further examination and purification of these cell types into culture might reveal whether or not these are true symbiotes and the mechanisms through which the symbiosis is established.

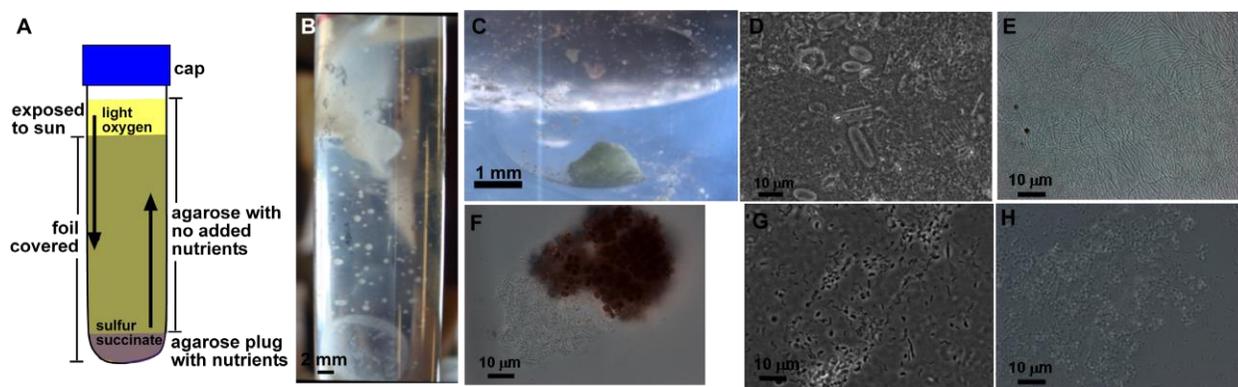


Figure 1: Gradient shake tubes. A) Samples from Little Sippewissett salt marsh were cultured in agarose shake tubes containing gradients of sunlight, oxygen, sulfur, and succinate. After 8 days of growth at room temperature, the tubes show visible growth. B) Sample #2 in 0.3% agarose shows a variety of colony types. C) Sample #2 in 0.1% agarose demonstrates a bubble that formed in the agarose at the interface of the agarose plug with nutrients. The yellow particle is likely a granule of elemental sulfur. D) A colony isolated 3 cm from the top of the gel containing sample #1 in 0.1% agarose, which shows microbes clustered around diatoms. E) Cells 7 cm from the bottom of the glass slide inserted into jar with 1% agarose containing sample #1. F) A colony 1 cm from the bottom of the glass slide inserted into jar with 0.1% agarose containing sample #2. G) Cell isolated from the large bubble colony seen at the bottom of panel B. H) Clustered cells 1 cm from the bottom of the slide removed from the jar containing 1% agarose and sample #1.

Although the community composition of the tubes was only partially defined (Figure 2), it was interesting to see that all of the strains identified from a given tube clustered closely together on a phylogenetic tree. This would suggest that each type of sample was dominated by at least one strain. It is unclear whether this is due to an uneven distribution of strains in the original sample, or whether each culturing conditioning enriched for specific strains.

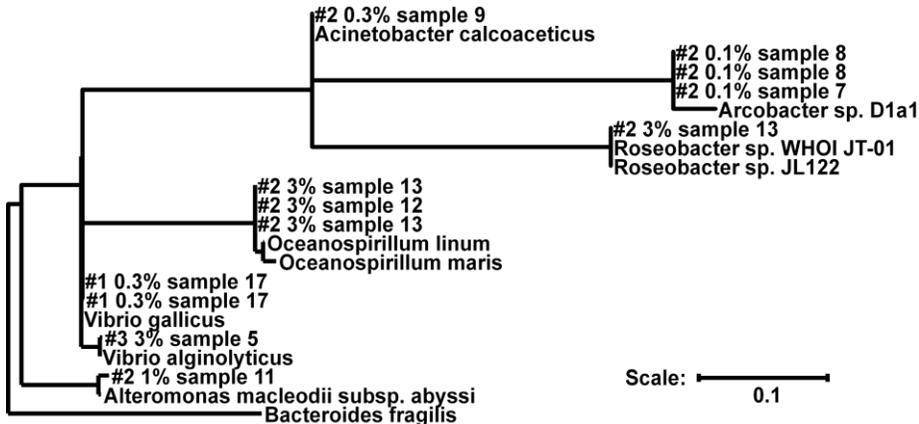


Figure 2: Phylogenetic tree made from the 16S rRNA sequences of strains isolated from Little Sippewissett salt marsh and cultured in gradient shake tubes.

2) Marine sponge isolates cultured on porous surfaces

T-RFLP analysis of the sponge isolate cultures after four days of incubation revealed that the diversity of community sampled was dependent on the culturing material. Digestion with *Rsa*1 or *Sau*96I each gave hundreds of TRFs (439 for *Rsa*1, 402 for *Sau*96I). The patterns of TRFs between samples revealed that the liquid culture with no additives and the “natural” sponge had the highest diversity (Figure 3C). Agarose, sand, the wafer sponge, and the Nerf material all had similar numbers of TRFs. Pumice stone seemed to support the least amount of diversity. It is unclear whether the diversity patterns observed are a result of the spatial structure of the added material (tortuosity, average pore size, surface roughness), or of the chemical composition of the materials (both the composition of the surfaces themselves and the chemicals that may leach out of the materials during culture). It would be interesting to separate these two possible influences by culturing isolates and adding the same material formed into different shapes (such as a solid block compared to a tortuous network of small pores).

In the *Sau*96I digestion, the TRFs seemed to partition nicely amongst sponge 1 and sponge 2 (Figure 3D). Besides the cultures with pumice stone as the additive, isolates from sponge 2 had

higher diversity of TRFs than sponge 1 after 4 days of culture. Since Sau96I seemed to better differentiate between sponge types, it was used to perform principle component analysis (PCA) on the TRFs from each culturing condition. PCA analysis revealed that the conditions clustered mostly by the species of sponge from which the community was isolated. The only outliers in this clustering appeared to be both the liquid cultures and the Nerf additive to sponge 2 (Figure 3D). This may indicate that although the liquid cultures had some of highest diversity, the liquid cultures and cultures with porous additives might be selecting for very different communities of cells. It would have been informative to also perform T-RFLP analysis on the cells isolated from seawater not associated with the sponges. If the porous materials are serving a similar role as the spatial architecture of sponge, it may be that the liquid cultures are selecting for the strains free living in the ocean, whereas the porous materials are selecting for strains requiring spatial complexity in the culture.

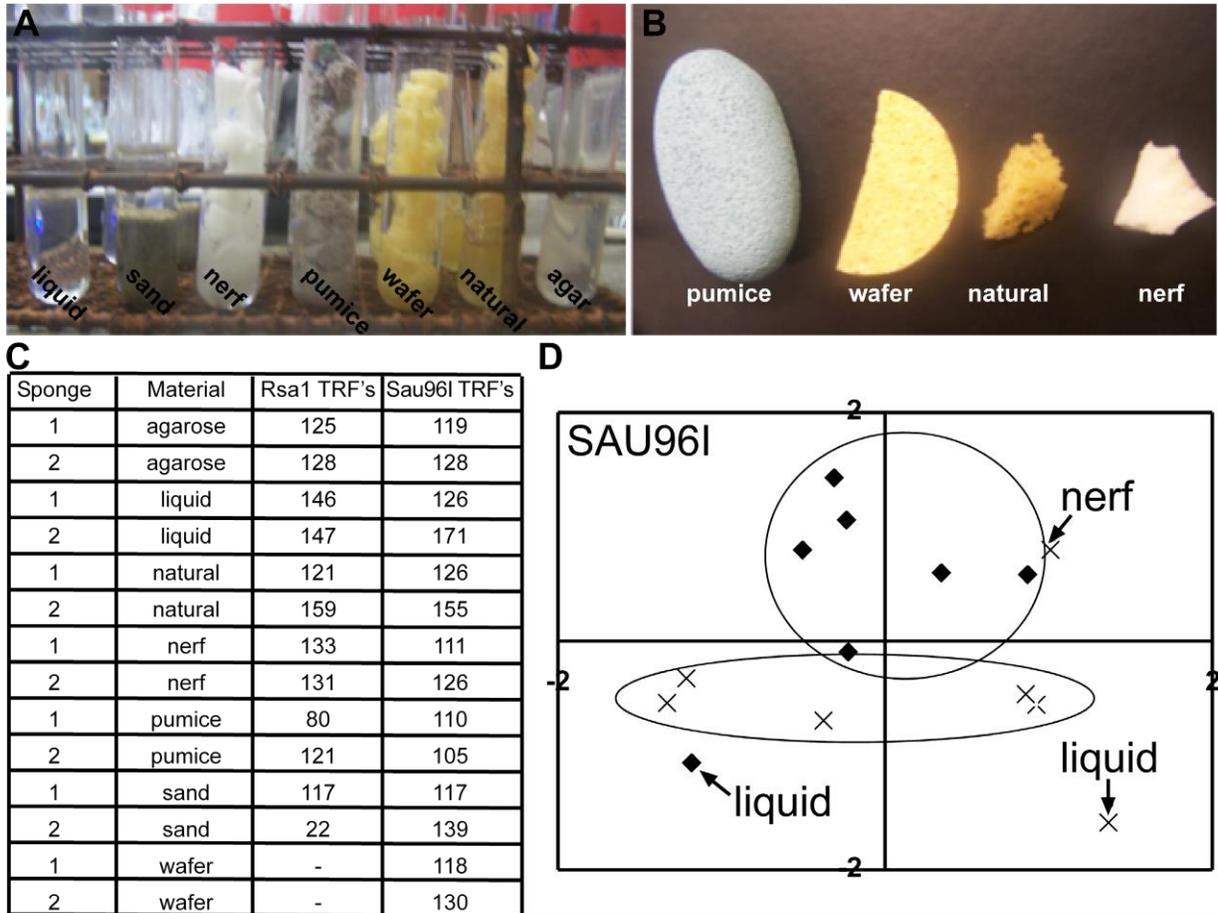


Figure 3: The culture of marine sponge isolates on porous surfaces. A,B) Microbes isolated from marine sponges were cultured in sea water media containing material additives such as household sponges, pumice stone, and pieces of a Nerf® football. C) After 4 days culture at room temperature, the community composition of each culture was analyzed using T-RFLP. D) Principle component analysis was performed based on the presence and absence of TRFs from each condition. Black diamonds represent conditions containing sponge1 isolates, Xs represent conditions containing Sponge 2 isolates. The circles indicate a rough grouping of cells showing that sponge 1 and sponge 2 isolates seem to be resolved by the principle components, with the exception of isolates from the liquid cultures (no material additive) which are outliers.

3) growth with temporally non-uniform nutrient availability

To explore the role of temporal heterogeneity in maintaining culture diversity, cultures were grown while implementing different patterns of carbon source additions. A defined consortium of 5 strains isolated from freshwater sources around Woods Hole, MA were used to evaluate how feeding patterns influenced community diversity patterns over time. These strains and carbon sources were not specifically chosen to balance each other, instead it was the hope that

oscillations of different types of carbon sources in a somewhat random consortium of cell types might have a stabilizing effect on the community composition. The total amount of each carbon source added during the experiment was kept constant for each treatment, so as not to encourage more growth in a particular condition.

The results showed that the overall growth in all four conditions was similar (Figure 4A). The communities from all conditions showed a 100 fold increase in cell number during the experiment. The increases and decreases of the portion of the population occupied by each cell type over time however was more variable (Figure 4B-F). The growth of many strains was dependent on the feeding pattern that was implemented. The proportion of *Massilia brevitalea* (S2) in the community oscillated drastically in all four conditions, suggesting that this strain may be heavily influenced by other strains in the community. Other strains however, such as *Bacillus aquimaris* (S1) and *Chryseobacterium* sp. (S4) seemed more consistent over time and less influenced by the different feeding conditions.

To quantify the change in community diversity over time, the Simpson's index was plotted for each condition (Figure 4G). The Simpson's index is a metric of community evenness. As defined in equation 1, the higher the value, the more even the community. Although the value appears to fluctuate significantly during the first half of the experiment, it seems that by around hour 30 that condition 2 is settling on a high index value (0.4) and condition 1 is settling on a lower index value (0.1). Perhaps this indicates that providing a lower concentration of food that is parceled out over time maintains a higher diversity than supplying the community with a large quantity of nutrient all at once. There are no clear trends in the Simpson's index for conditions 3

and 4 in terms of the Simpson's index approaching a definite value. Perhaps this indicates that when providing a feeding pattern whose period is longer (1 tp period for condition 2, 4 tp period for condition 3, 8 tp period for condition 4), it takes longer for the community of cells to adapt to the pattern. Another possibility is that some patterns of nutrient availability may be so heterogeneous, that the community cannot maintain a constant diversity over time but instead will undergo fluctuations of community composition over time.

It is unclear to what extent the fluctuations in community composition, particularly at early times, were driven by "startup" adjustments as a culture entered a new media, such as a lag phase. It would be of interest to follow cultures for more time points to see how the different feeding conditions influence the long term diversity of the cultures. It is also not clear if the influence of temporal heterogeneity in nutrient availability would be more important for more diverse (100s to 1000s of strains) mixed cultures. Perhaps in the experiments performed here, temporal heterogeneity was not needed to maintain diversity because the number of carbon sources used was approximately the same as the number of strains in the community. Experiments which answer these types of questions are likely to require more sophisticated and automated methods of community composition.

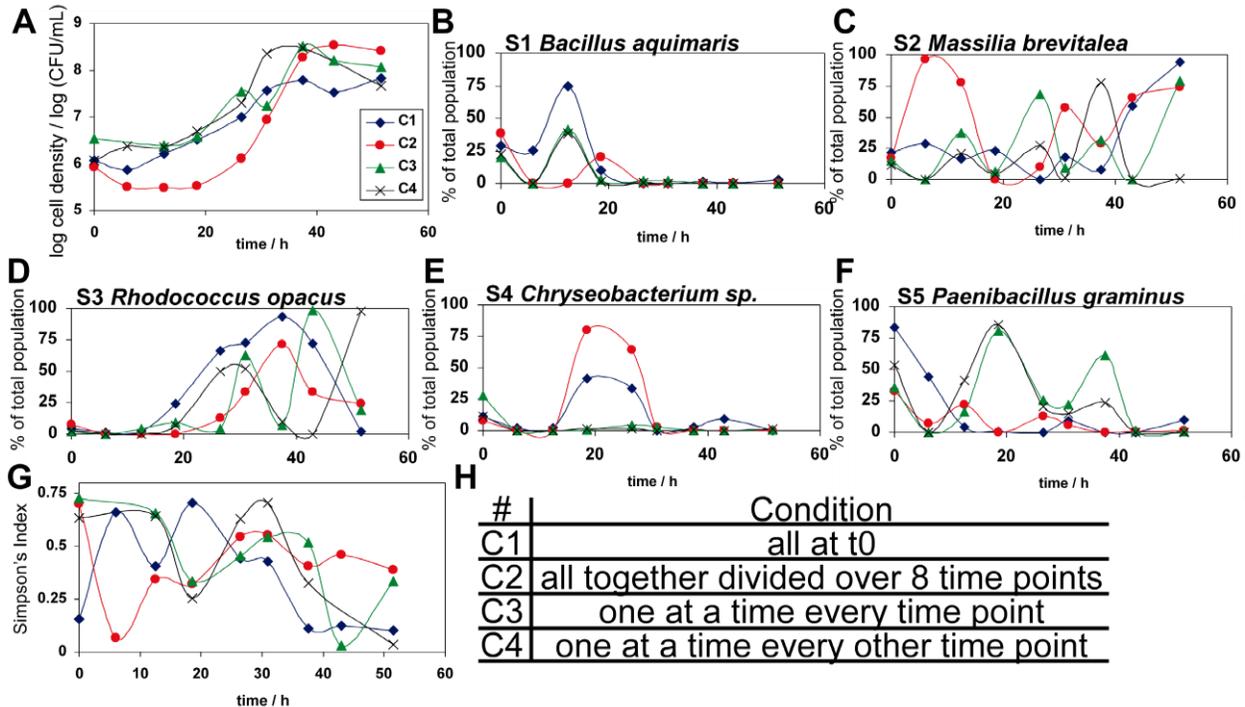


Figure 4: Temporal variations of nutrient availability influenced changes in the diversity of a defined consortium of five strains. A) Over the course of the experiment, the total density of cells in all conditions increased by approximately the same amount. B-F) The populations of individual strains fluctuated over time. The fluctuations of individual populations were dependent on the temporal variations of nutrient availability. G) The Simpson's index was used to quantify the diversity of the consortium under each treatment (see H for definition of treatments C1-C4), with 0 representing an uneven population dominated by a single strain and 0.8 representing an even distribution of all 5 strains.

Conclusion:

Maintaining the diversity of mixed cultures in the lab remains a challenge. Here I have presented and tested three strategies for maintaining mixed cultures: isolates from Sippewissett salt marsh were incubated in shake tubes with nutritional gradients, isolates from marine sponges were cultured on porous surfaces, and a defined consortium of 5 lab isolates was incubated under a variety of temporal patterns of nutrient additions. While the results reported here do not indicate that the problem of diversity loss from mix cultures has been solved, the results do suggest that adding spatial and temporal complexity to culturing conditions does impact the overall diversity of microbial communities. Isolates did respond to the gradient of nutrients in

the shake tubes, and it appeared that lower weight percentages of agarose lead to more interesting growth forms. Gradient shake tubes also appear to be an experimental technique capable of identifying possible symbiotic interactions within microbial communities, which should be essential work for developing our understanding of microbial communities and ecosystems. Although it was not clear that the availability of porous surfaces increased the diversity of a community of marine sponge isolates, the data do suggest that the presence of spatially complexity did cause a shift in community composition. Finally, temporal complexity in nutrient availability influenced the changes in evenness of a defined consortium. The data suggest that a prolonged addition of dilute nutrients would lead to a more diverse community, although it still remains to be determined how the patterns of nutrient addition affected diversity over long time periods, which pattern of nutrient additions would be optimal for maximizing community diversity, and how communities with higher species richness would respond to such treatments.

There still remains much to be done in the area of high diversity mixed cultures. Although challenging, the study of mixed cultures in the lab should be essential to developing our understanding of microbial community interactions and functions. Most natural communities do not seem to exist as monocultures in well mixed flask, on lawns of solid media, or in constant flow chemostats. By increasing the complexity of our cultures in the lab, it is hopeful that we will better understand interactions between strains and begin to develop a more predictive form of microbial ecology

References:

There certainly exists a large amount of literature on: mixed cultures, mixed species chemostats, competitive and symbiotic interactions amongst mixed communities, multispecies signaling, the spatial distribution of microbes in natural settings, nutrient fluctuations in natural settings, etc. Because I could not give the reader an adequate assessment of this vast and scattered literature and because no specific references were use as background for the design and execution of the experiments presented here, references have purposefully been omitted from this manuscript.