

# COMMUNITY-LEVEL METABOLIC CHARACTERIZATION OF A CONSORTIUM

Research project report

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## ABSTRACT

Which organisms are present in a given consortium is determined by a large number of factors, including history of exposure to different organisms and their unique features, which determine interactions in the community and shape its structure. Despite this complexity, certain community-wide characteristics can often be predicted with a high degree of confidence, because microbial metabolism is subject to constraints set by thermodynamics, biochemistry and resource availability. In this work, I investigated the interplay between these predictable and unpredictable features of community dynamics. The data I collected provides an example of two consortia that follow highly similar “metabolic trajectories” while being very distinct in composition.

# INTRODUCTION

In the era of metagenomics analyses and other community-wide investigations, more and more studies advocate for shifting the focus from the question “Who is there?” to “What are they doing?”. If this question is asked in the metabolic sense (what metabolites is the community consuming / excreting?), then a large part of the answer can be predicted based on general thermodynamic and biochemical principles. One expects, therefore, that consortia of bacteria inhabiting similar niches may exhibit predictable and perhaps reproducible behavior, even if the details of precisely which organisms are present and which of them performs which function might perhaps vary dramatically, and be less reproducible or predictable.

In this project, I focused on investigating this idea by following the metabolic activity of two cultures inoculated by a consortium consisting (inasmuch as possible) of the same organisms, but present at different relative abundances. This difference was created by “preconditioning” the same inoculum in two different media for a period of 5 days. The goals of the project were to:

- 1) Focus on a consortium rather than an artificial situation of a pure culture of any one organism.
- 2) Monitor the metabolic activity of the community by measuring major metabolites expected to be important.
- 3) Monitor culture composition by examining morphologies of community members under a microscope.
- 4) Determine to what degree the two trajectories (metabolic and compositional) were reproducible in the two cases.

For the consortium in question, I chose to use one of the enrichments our group has performed at the beginning on the course as part of the group exercises, specifically the enrichment for succinate-degrading “green non-sulfur” anoxygenic phototrophs. There were two reasons for this choice. First, based on the experience of Microbial Diversity students from previous years, this enrichment gave rise to an association between green sulfur phototrophs and sulfate reducing bacteria, rather than promoting a single type of phototrophic bacterium utilizing sulfate directly. Second, I was intrigued by the fact that other groups’ enrichments yielded deeply green cultures, while ours had a distinctly different yellow-green color.

# MATERIALS AND METHODS

## STRAIN COLLECTION

For the original inoculum I chose a water sample from Trunk River, collected approximately 10cm below the surface in a sulfur-rich (yellow) area, whose location is indicated on the figure to the right. The turbid, pale-yellow color of the sample earned it the designation of “swamp lemonade”; a substantial number of enrichment studies performed at the early stages of the course use the same sample for inoculation and could be used to gain additional insight into its composition.



FIGURE 1. SAMPLING LOCATION.

## MEDIA FOR ENRICHMENT AND GROWTH

The succinate-degrading “green non-sulfur consortium” was grown anaerobically in “non-sulfur medium” from the course manual, supplemented with extra sulfate. Specifically:

Deionized water	3.9 liter
100X FW Base (see below)	40 mL
Sodium Succinate	22 g
1 M NH <sub>4</sub> Cl solution	20 mL
1 M Sodium sulfate	<b>40 mL</b>
100 mM K phosphate, pH 7.2	40 mL
1 M MES Buffer, pH 5.5	5 mL
Trace Elements	4 mL

*-Autoclave in Widdel vessel*

*-Cool under stream of N<sub>2</sub>/CO<sub>2</sub> (80%:20%) gas*

*-After cooling, add:*

Multivitamin solution	4 mL
1 M Sodium bicarbonate	10 mL
DCMU	50 mg

### **100X Freshwater (“FW”) Base (per liter)**

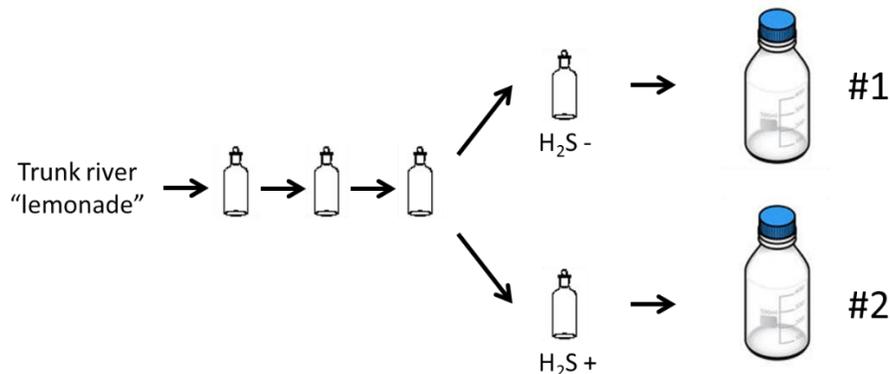
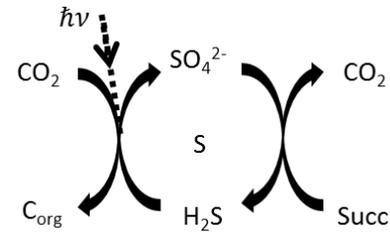
<b>Component</b>	<b>Amount</b>	<b>FW</b>	<b>100X Conc.</b>	<b>Final Conc.</b>
NaCl	100 g	58.44	1711 mM	17.1 mM
MgCl <sub>2</sub> ·6H <sub>2</sub> O	40 g	203.30	197 mM	1.97 mM
CaCl <sub>2</sub> ·2H <sub>2</sub> O	10 g	147.02	68 mM	0.68 mM
KCl	50 g	74.56	671 mM	6.71 mM

Most incubations were performed in 50mL Pfenning bottles in a cabinet illuminated with 650nm LED arrays. The bottles were placed approximately 10cm away from light source. Initial inoculation and first transfer used 1mL of inoculum and took a week to grow. For the second transfer, I used 5mL inoculum from the first transfer to generate 1:10 and 1:100 dilutions. 5mL of culture from this 1:100 dilution (once it reached a deep yellow-green color) was the inoculation source for each of the “pre-conditioning” bottles below (see below). All of these bottles remained clear for 3-4 days, after which clear flakes could be seen at the bottom (“stage 1”). Once the bottles reached this stage, they grew pale-green overnight and turbidity increased quickly (“stage 2”).

After any significant exposure to oxygen (at the initial inoculation and transfers, but not after sampling), the cultures were left in the dark for 2h prior to exposure to light. Generally, however, I did not find the culture to be particularly oxygen-sensitive. Comparing wet mounts of fresh samples and those left overnight in closed Falcon tubes showed vigorous motility of all the same community members (despite Falcon tube plastic being somewhat oxygen-permeable).

## “PRE-CONDITIONING” OF THE CONSORTIA

I hypothesized that the consortium targeted by the enrichment consisted of an association between sulfate-reducing microorganisms (reducing sulfate while oxidizing succinate) and green sulfur phototrophs (consuming sulfide and fixing CO<sub>2</sub> with light; see diagram). I further hypothesized that the “stage 1” growth of colorless flakes corresponded to the sulfate reducers, and that the relatively slow growth of the consortium (1 week to high turbidity of phototrophs in stage 2) was due to the time required for the sulfide to accumulate. I therefore used the 1:100 dilution to inoculate two new Pfenning bottles. In one of these (“sulfide -”) the medium was as above. In the other (“sulfide +”), the same medium was supplemented with 50 μL 1M sulfide. The idea was to selectively promote the phototroph branch of the cycle, while repressing the sulfate-reducing branch. This experimental strategy is on the diagram:



Simultaneously, I used 2.5mL of the same culture to inoculate an enrichment experiment for sulfate-reducing bacteria (25 mL of SRB medium in a 130 mL anoxic serum bottle; the SRB medium was as described in the course manual, supplemented (per bottle) with 1mL acetate 1M, 0.5mL sulfate 1M and 0.25 bicarbonate 1M). These were incubated in the dark and took over 10 days to grow; at which point they showed visible growth in the form of colorless flakes at the bottom. These seemed morphologically similar to the putative sulfate-reducing organisms in the 600mL cultures, but I did not perform any subsequent analysis on them.

## TIME SERIES SAMPLING

The entire contents of the two “pre-conditioned” consortia were used as inocula for two 600mL bottles of freshly prepared medium (Sample #1 and #2 from the “sulfide -” and “sulfide +” inocula, respectively). The large volume of these two cultures allowed for daily removal of material for sampling. These two 600mL cultures were placed approx. 50cm equidistant from two light sources illuminating both sides, each on a stirrer. The stirrer was set to low speed (so that the dimple from rotating medium fluid was just apparent). This was done to prevent accumulation at the bottom (observed in all Pfenning bottles), while still allowing associations between organisms to form in the medium.

Sampling was performed daily under stream of N<sub>2</sub>:CO<sub>2</sub> (80:20) gas. 2.6 mL of culture were retrieved. Of these, 0.1 mL were set aside for inspection under microscope, and the remaining volume placed in a thin glass test tube to measure absorption at 600 nm and 750nm. The first was used as a measure of optical

density, while the other corresponded to the peak of the phototroph absorption spectrum (bacterial chlorophyll C); the ratio of the two therefore constituted a measure of pigment content per biomass (i.e. how much of the OD is due to pigmented phototrophs).

After OD measurement, the culture was filtered with a 0.2 micron filter. Instead of disposable syringe filters, reusable filter holders were used; these allowed the filter to be extracted, dried and saved to reflect culture density and pigment. If desired, these filters could have been used for a FISH assay as well; however, I did not do this.

0.5mL of the filtrate was mixed with 0.15mL of Zn acetate to precipitate sulfide so its concentration could later be measured in a standard colorimetric assay with methylene blue. These precipitated samples were stored at 4C for a few days so the accumulated samples could be processed together. The sulfide assay measurements were performed in a 96-well plate using a plate reader, with 200  $\mu$ L of mixture per well, in triplicates of dilutions 1:1 or 1:10 based on the color intensity; the data from 1:1 dilution was used preferentially, whenever it was within linear range of the 4-point calibration curve with 0, 1, 10 and 100 mM sulfide. To reduce loss of sulfide, every effort was made to reduce time between the drawing of the sample and the mixing with Zn acetate. To preserve all of the sulfide, I could have used 0.5mL of the initial sample rather than of the filtrate; however, once phototrophs had grown, such samples became visibly green, which affects the absorption measurement in the colorimetric assay. Using the filtered sample improved accuracy, and if done quickly, the sulfide concentration loss can be expected to be insignificant.

0.6mL of the filtrate was used to generate 5mL of 1:10 and 1:100 dilutions for measuring sulfate concentrations with a Dionex ion chromatographer. I later found the measurements of the 1:100 dilution to be redundant, as the 1:10 dilution was within well-calibrated range of the instrument (below 250 mg/L) for all samples.

Finally, 0.7mL of the filtrate was mixed with 78 $\mu$ L of sulfuric acid 5M, spun down at 15000g for 3 min and 500  $\mu$ L of the supernatant used as the sample for HPLC to measure organic acids.

The extra volume of the culture allowed for loss during filtration, which became very significant once phototroph density became large.

## DATA ANALYSIS

The chloride concentration reported by ion chromatography was used as internal standard and typically exhibited very little variability. I did, however, have some trouble with a "leaky" pipette and used chloride normalization to correct the measurements from a few samples where an abnormally low concentration of chloride was reported, corresponding to an insufficient volume of sample.

In the HPLC data, one of the peaks with an extremely stable area was identified as the MES buffer and was similarly used as an internal control.

## MIXED INOCULA

After both the 600mL cultures reached an optical density that saturated the OD detector, I attempted an experiment whereby samples sourced from the two bottles were mixed in a varying proportion (5 bottles with a 100:0, 75:25, 50:50, 25:75 and 0:100 proportions of the 5mL total) and used to inoculate Pfennig bottles with fresh medium.

My initial intention was to use “washed” inocula to reduce the effect of differing media compositions at the final timepoints (so that the difference between different inocula would be in the organisms only, not in metabolite concentrations). For this, I attempted to gently spin down 50 mL of sample, replace the supernatant by fresh medium, resuspend and repeat. Unfortunately, spinning at 3000g for 20 minutes failed to generate a clear supernatant. In fact, retrieving this supernatant and spinning it down even at 7000g for 20 minutes failed to pellet the organisms that remained in suspension. As a result, rather than generating a “washed” inoculum harboring the same organisms in a fresh medium, the procedure yielded four fractions that all contained microorganisms. Ideally this experiment should have been repeated using the original inocula rather than these spun-down fractions; however, due to the shortage of available time, I only used the spun-down fractions as my inocula, mixing them in varying proportions, as originally planned. As a consequence, the results of this mixing experiment are not interpretable except in a very qualitative sense. Still, the interesting qualitative result (see below) could inspire a follow-up experiment.

Interestingly, the two fractions per bottle generated by the spin-down procedure (the resuspended pellet from a gentle 3000g spin-down, and the supernatant from a second, harsher, 7000g spin-down of the first supernatant) were readily distinguishable under the microscope by the morphology of the different dominating organism types. In particular, the “supernatant fraction” had a viscous appearance under the microscope and appeared enriched for matrix-secreting types. Together with the fact that the spun-down fraction successfully grew after re-inoculation (and therefore remained viable) suggests that density gradient separation, coupled with a dilution to extinction of the resulting fractions, could be successfully used as a technique to enrich for specific members of the consortium.

## ABSORPTION SPECTRUM MEASUREMENTS

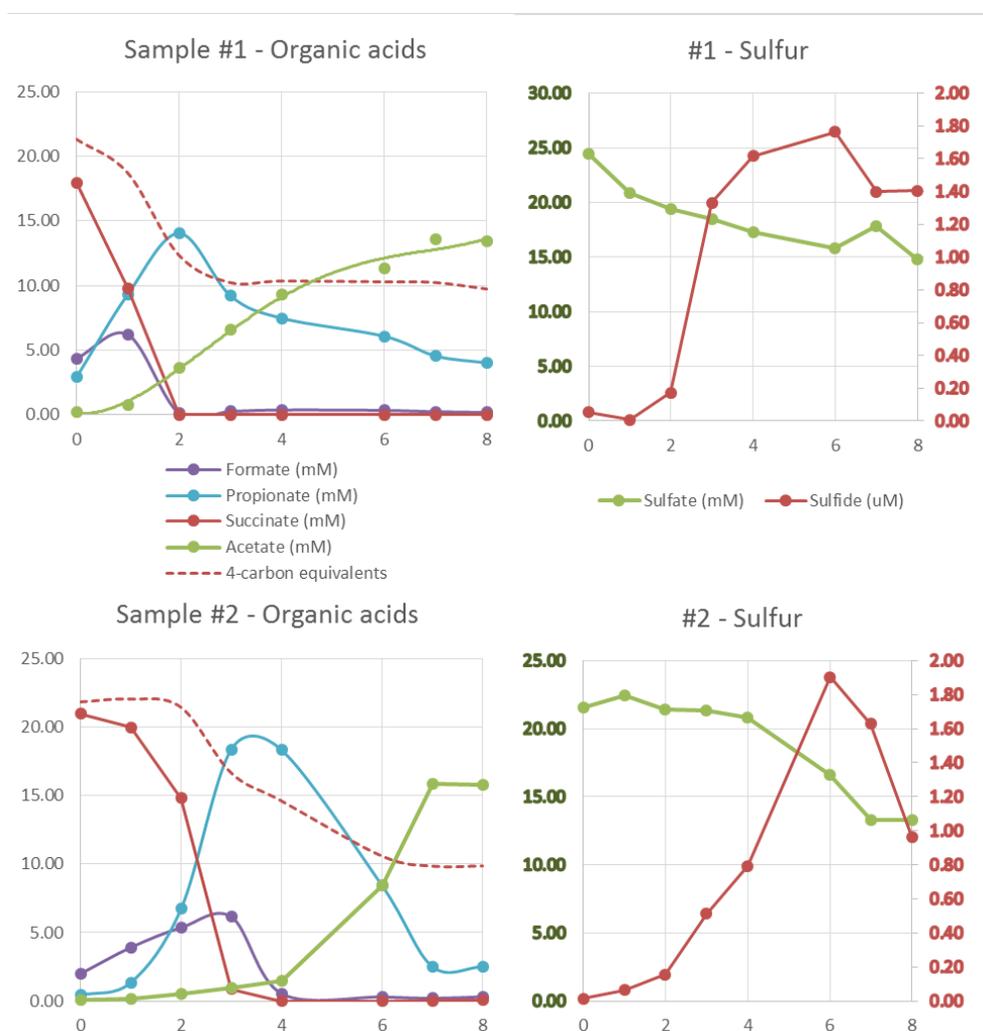
Absorption spectra of the culture (presumably dominated by the chlorophyll of the phototrophs) were measured with a spectral photometer. The locations of the absorption peaks identified the dominating pigment as bacterial chlorophyll C; however, I was unable to extract any additional information from these spectra for quantitative analysis of culture composition. In particular, in the experiment using mixed inocula (whose colors could be readily distinguished by eye), I had hoped to use the varying relative heights (areas) of the two dominating absorption peaks as indication of changing community composition. However, the initial measurements immediately after inoculation failed to show a consistent association between this ratio and the relative fraction of the two inocula. If this is re-attempted, I would recommend collecting and averaging several spectra (rotating the bottle in front of the spectrometer): although replicate spectra are highly reproducible if the bottle is not moved, the spectrum distortion by the glass may be obscuring the relatively subtle differences that are due to true color differences of the culture itself.

# RESULTS AND DISCUSSION

## COMMUNITY-WIDE METABOLISM IS REPRODUCIBLE

The pre-conditioning of the two consortia under different conditions (with and without added sulfide) were expected to cause shifts in the relative abundance of different community members. As a result, the two 600mL culture bottles with identical media were inoculated by communities with (presumably) the same organisms present at different relative abundances. It was therefore intriguing to ask: to what degree are the “metabolic trajectories” of these communities similar or different?

The measurements of the organic acids and sulfur compounds (sulfide and sulfate) are presented in Fig. 2. With exception of a “delayed start” of the community #2 by approximately 1.5 days, the metabolic trajectories of the two samples exhibit remarkable reproducibility (for example, compare the concentrations of the organic acids in the two samples at the “final state” after 8 days of incubation).



**FIGURE 2. METABOLIC TRAJECTORIES OF THE TWO SAMPLES ARE REMARKABLY SIMILAR, EXCEPT FOR A 1.5-DAY DELAYED START FOR SAMPLE #2.**

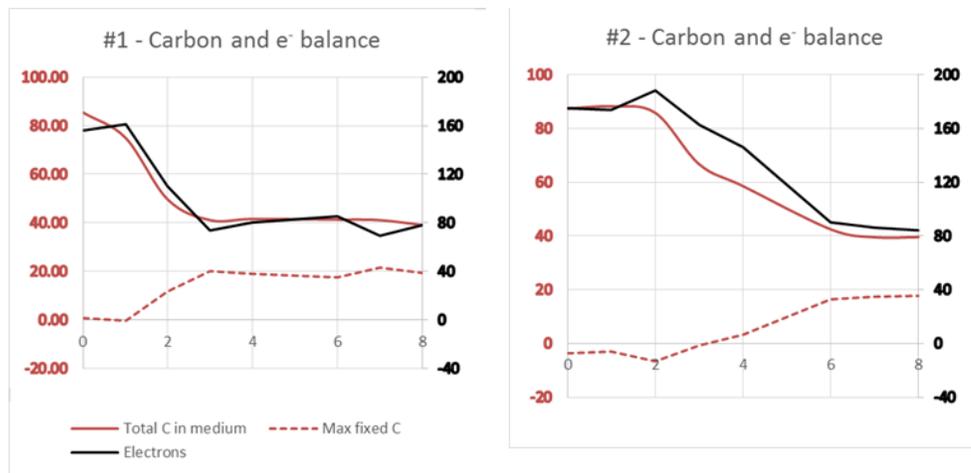


FIGURE 3. ELECTRON BALANCE CALCULATION ALLOWS ESTIMATING THE AMOUNT OF FIXED CARBON.

### THE OBSERVED METABOLIC ACTIVITY IS THERMODYNAMICALLY REASONABLE

Does the data presented in Fig. 2 “make sense” given our expectations about community metabolism?

Fig. 3 presents the curves of total carbon content in the medium (red solid) and the “total electron content” of the compounds present in medium. Here, the “electron content” is defined as the number of electrons the compounds can donate in the process of conversion to the reference compound, i.e. CO<sub>2</sub> for carbon compounds and elemental sulfur for sulfur compounds. This choice of the reference compounds differs from the standard choice of the most oxidized form of the respective element (in particular, with my convention the “electron content” of sulfate is negative 6). However, the choice of reference compound is arbitrary, and the one I made is particularly convenient here, given that CO<sub>2</sub> and elemental sulfur are both used by the community, but are not tracked by my measurements.

The curves in Fig. 3 show a clear “drop” in the electron content (again, identical in the two cultures). This drop can be used to estimate the amount of carbon that could have been fixed by the consortium (note that this effectively assumes that the sulfur fixed in the biomass is negligible, so that the dominant form of the unaccounted sulfur is the elemental sulfur, for which the electrons content is zero by definition; only under this (approximate) assumption does carbon fixation become the unique sink for electrons). Indeed, since the oxidation state of carbon in CO<sub>2</sub> is +4, all we need to know is the average oxidation state of organic carbon. The different biomass composition estimates in the literature correspond to an average carbon oxidation state ranging from zero (Redfield et al., 1963) to -0.45 (Anderson, 1995). For our purposes, however, the most relevant work is that of DelDon et al. (1994) which studied both sulfate-reducers and green phototrophs and which estimated the average redox state of carbon to be -0.23. The “fixed CO<sub>2</sub>” curve in Fig. 3 is calculated using this value. We find that both communities can be expected to have fixed 40 mmoles, or 0.5g of carbon. In the same paper, DelDon et al. estimated carbon to constitute 45% of dry mass, and we find therefore that the new biomass corresponds to approximately 1g of dry mass, which is a very reasonable ballpark. I conclude that the metabolic activity of the community is quantitatively reasonable.

Another way to verify that community metabolic function corresponds to one’s expectations is to place the culture in the dark for 12 hours, and compare sulfide concentrations before the dark period, immediately after, and after another 12 hours in the light. I did this experiment and found that,

predictably, after 12 hours of darkness the sulfide concentration exhibits a sharp increase, and drops again after exposure to light. This is the expected result due to modulation in the activity of sulfide-oxidizing phototrophs. (Data not shown.)

#### COMMUNITY COMPOSITION OF THE TWO SAMPLES IS STRONGLY DIFFERENT

Based on the data in Fig. 2, one might expect that, except for the “delayed start” of sample #2, the two communities followed the same succession pattern in terms of organism presence or dominance. This, however, was not at all the case. Fig. 4 shows a photograph of the filters from the daily sampling of the two cultures. It clearly indicates not only that the trajectories followed by the two communities were strongly different, but also suggests that the final states are compositionally distinct (as indicated by the difference in color: yellow-green for Sample #1 and deep green for Sample #2). This is confirmed by both OD measurements and examination of a sample from the final state under the microscope (a wet mount) as shown in Fig. 5. The “pigment” traces correspond to the ratio between absorption at wavelength 600 and 750 and characterizes the prevalence of phototrophs (see methods).

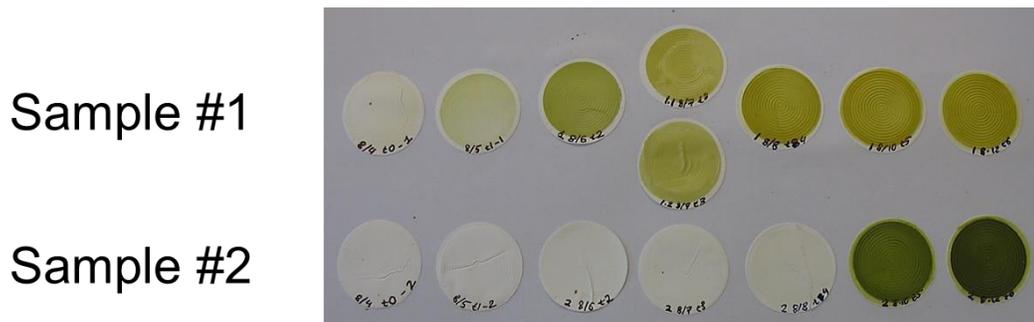


FIGURE 4. THE COMPOSITION OF THE TWO CONSORTIA WAS STRONGLY DISTINCT.

In fact, daily examination of wet mounts from the two cultures under the microscope revealed that after initial enrichment for a small number of bacterial types, the communities continued to diversify in composition and exhibited inter-species aggregates of ever-changing sizes and morphologies; the community composition trajectories were remarkably complex. I did not attempt to catalog or identify dominant types, since for my purposes I was quite content to simply observe in the microscope the staggering diversity of organisms and their behaviors. I found that one commonly observed association occurred between the pigmented phototrophs and a spiral-shaped bacterium (which I conjectured to be a sulfate-reducing *Desulfovibrio*). The two types were held together by what appeared to be extracellular matrix secreted by one of them; this matrix would be stable under visible light but would quickly dissolve/break after exposure to UV (I used the lamp intended to excite fluorescence from DAPI). This allowed me to examine the morphologies of organisms participating in such aggregates as they drifted apart, no longer held together.

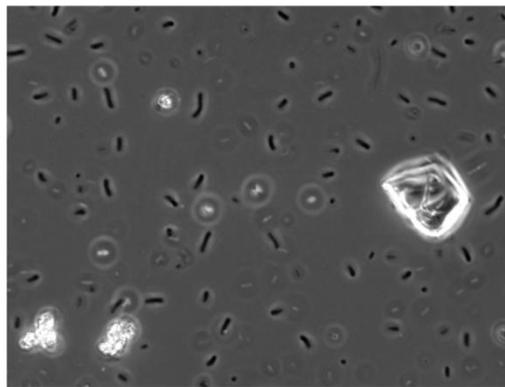
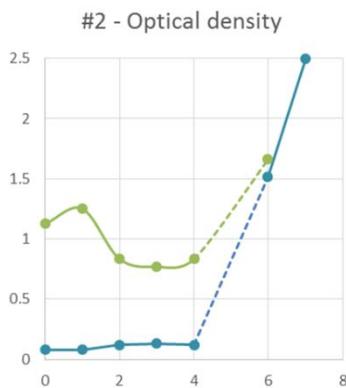
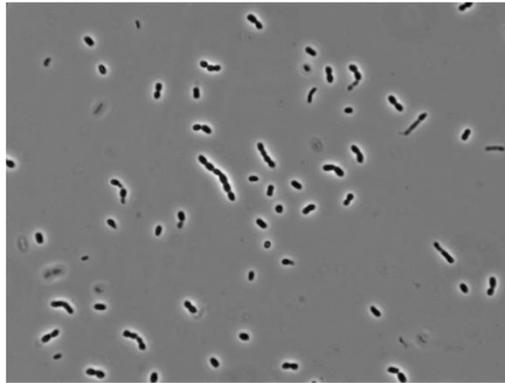
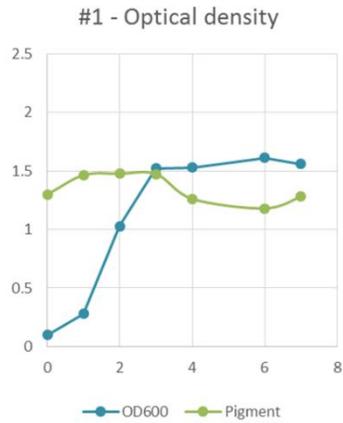


FIGURE 5. OD MEASUREMENTS AND THE MORPHOLOGIES OF THE DOMINATING PHOTOTROPHS IN THE TWO SAMPLES (#1, TOP; #2, BOTTOM) CONFIRM THE DIFFERENCE OF THEIR COMPOSITION.

## MIXED INOCULA

After 8 days of incubation my two 600mL bottles, the concentration of all the metabolites I could measure were highly similar, yet the communities that established themselves were clearly distinguishable (both by color and the morphology of the dominating phototroph). I hypothesized that these two communities constituted alternative states that were both well adapted to this medium concentration; if so, it is intriguing to ask what might be the outcome of “community competition” experiments whereby a fresh bottle of medium would be inoculated by a mixture of the two communities. Would the resulting community resemble one source more than the other? For example, which of the two phototrophs (if any) would become dominant, and how would this outcome depend on the proportion of the two communities in the inoculum?

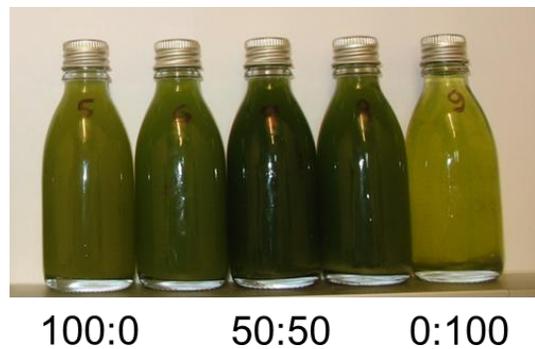


FIGURE 6. THE 50:50 INOCULUM MIX APPEARS TO EXHIBIT THE MOST EFFICIENT GROWTH.

In an attempt to address this question, in the final few days of the course I set up 5 bottles inoculated with varying fractions of two suspensions derived from the two consortia (sampled at day 8); these mixtures were 100:0, 75:25, 50:50, 25:75 and 0:100. For reasons I describe in the Methods, the version of this experiment that I performed is hard to interpret and should ideally be re-done: briefly, rather than mixing the original inocula themselves, I used the spun-down-and-resuspended fractions that were not in fact representative of the full composition of the original inocula. Nevertheless, the results of 48 hours of growth (presented in Fig. 6) are qualitatively interesting and suggest this experiment might be worth doing carefully. Qualitatively, the bottle with qualitatively highest growth was the 50:50 mix, while both 100:0 and 0:100 mixtures exhibit significantly lower turbidity. It is tempting to interpret these results as evidence of some “synergistic” interactions occurring in the more diverse community; it is my hope that a more careful investigation could confirm or nuance these preliminary observations.

## CONCLUSIONS AND OUTLOOK

Monitoring of whole-community metabolism of a diverse consortium revealed that a highly reproducible pattern in the succession of metabolites can contrast with a strongly divergent trajectory of community composition. These findings further suggest that a carefully planned and implemented “community competition” experiment might be an enterprise worthy of another project for a Microbial Diversity course in the future.

## ACKNOWLEDGMENTS

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