Setting up a lemonade stand: all the juicy details
Lev Tsypin—Microbial Diversity 2017

Introduction

Trunk River forms a brackish pond of slow water near its entry into the ocean. Lemonade, comprising benthic turbid yellow pools in this pond, has been studied by the Microbial Diversity course over the past several years. These forays have characterized the maturation of new Lemonade, both in terms of chemistry and metagenome composition (Enalls, 2016), and have pursued the cultivation of some organisms inhabiting it (Buck, 2015; Mayr, 2016). Up to 9% of the matured Lemonade metagenome corresponds to the phylum Lentisphaeria (Fig. 1). Lentisphaeria is a rarely cultivated group of organisms that is sister to the Verrucomicrobia (Cho et al., 2004; Choi et al., 2013). This piqued our interest: It is not obvious that rarely cultivated or characterized organisms should be rare in the environment. If Lentisphaeria are indeed highly represented in Lemonade, and we have previous years' data at our disposal, then it is an excellent opportunity to try to cultivate a member(s) of this poorly understood clade.

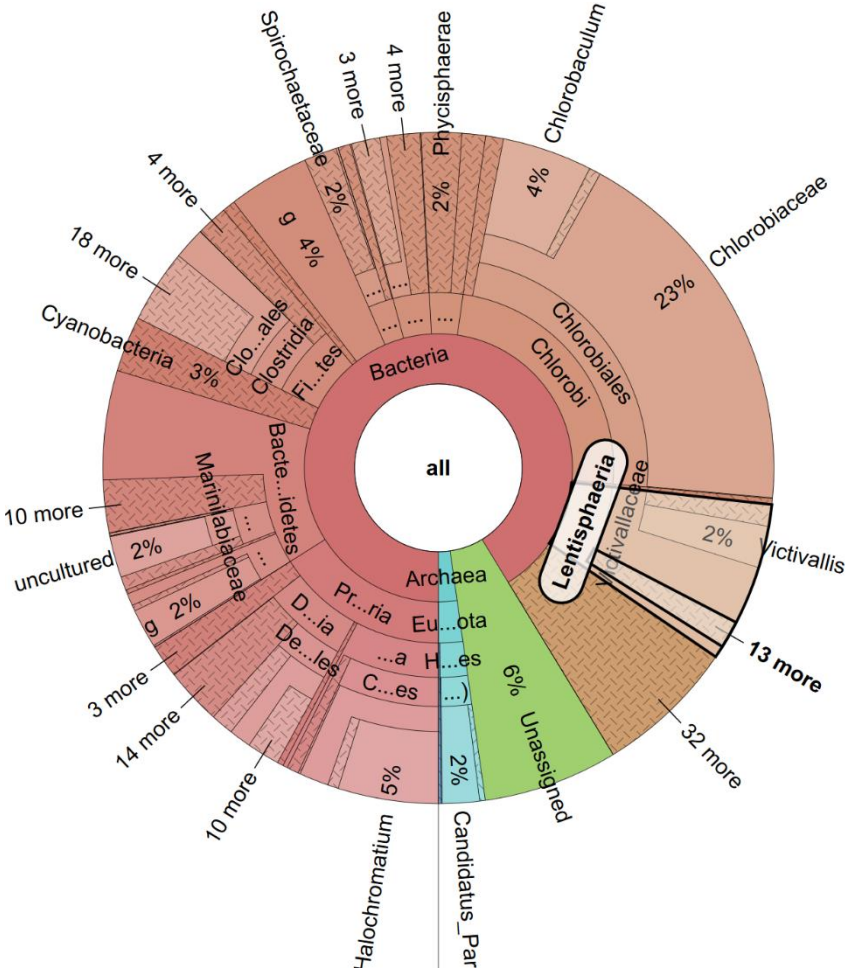

Figure 1. Lentisphaeral representation in matured Lemonade metagenome. A Krona plot of the Lemonade metagenome shows that the largest representation of the phylum Lentisphaeria (9% of total metagenome) was observed in Hole E at 35 cm depth after 15 days of maturation (Enalls, 2016). Lentisphaeral representation appears to increase as Lemonade matures.

I used Ion Chromatograph (IC) measurements of lemonade, as well as *in situ* YSI probe measurements, to inform an oligotrophic medium to culture Lentisphaerae. I made the assumption that it would be appropriate to use the same carbon sources as were used in previous Lentisphaera cultivations (Cho et al., 2004; Choi et al., 2013), and attempted to enrich for Lentisphaerae *via* dilution to extinction. I used a Fluorescence *In Situ* Hybridization (FISH) probe, which had been previously designed based on the Lemonade metagenome 16S sequences, to

detect Lentisphaerae (Scott Dawson, personal communication), as well as to design a PCR primer. I used PCR with this primer to get a better resolved understanding of organisms residing in Lemonade, as well as to screen for wells in my dilution series that maintained or grew a population of Lentisphaerae. While not definitive, this study merits further pursuit: For example, there was not enough time to successfully sequence a clone library from my enrichment. However, I was able to confirm that genomic DNA from lemonade contains poorly described organisms, including a couple from Lentisphaeria, and I was able to detect this PCR signal through my entire dilution series, indicating growth. And, while the FISH was ambiguous, we are now better equipped to design more specific probes and optimize the cultivation.

Materials and Methods

Chemical and physical analyses of Trunk River water

Different depths of Trunk River were analyzed with IC for various anions and cations, and *in situ* with a YSI probe, taking measurements for pH, conductivity, dissolved oxygen content, temperature, salinity, and oxygen reduction potential (ORP). The IC measurements were performed on two different days, August 1st and August 7th, which was the day when I collected the Lemonade for my inoculations. IC measurements were made on 1:100 and 1:1000 dilutions of sterile-filtered lemonade. The detected amounts of each anion and cation were averaged and extrapolated to the original concentration in Lemonade. The YSI probe measurements were also performed on August 7th.

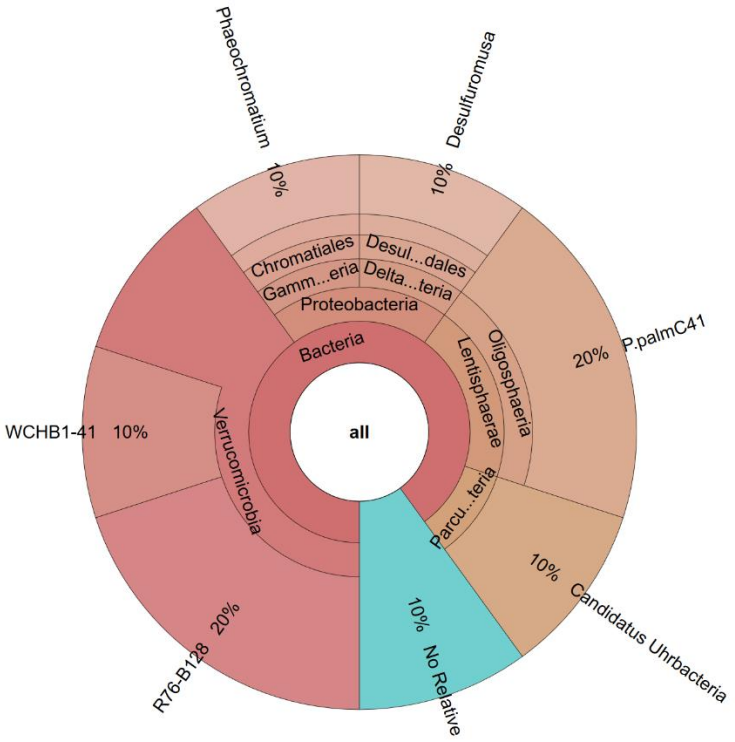

Figure 2. Krona plot of Lemonade gDNA 16S library, as generated by SILVAngs. The of the sequences are classified as Verrucomicrobia, two as proteobacteria, two as Lentisphaerae, one as Parcubacteria, and one was left unclassified.

Cultivation Medium:

Base medium composition:

- Sea water base
- 160 uM $NaNO_3$
- 100 uM NH_4Cl
- 100 uM K_2PO_4
- Trace Vitamins (prepared as a 1000x stock)
- Trace Metals (prepared as a 1000x stock)

- pH 6 (5 mM MES)

Carbons as in Cho et al., 2004 (prepared as a 1000x stock):

- 10 mg/L D-glucose (56 uM)
- 10 mg/L D-ribose (67 uM)
- 10 mg/L succinate (85 uM)
- 10 mg/L glycerol (110 uM)
- 10 mg/L N-acetyl D-glucosamine (45 uM)
- 20 uL/L EtOH (342 uM)

Medium Variant: Supplemented with 1x powdered lemonade extract

Powdered Lemonade Extract:
250 mL of Lemonade was pelleted at 1500g for 3 minutes. The supernatant was aspirated and the pellets were frozen at -20C. The pellets were then consolidated and ground with a mortar and pestle in liquid nitrogen. The powdered lemonade was resuspended in 50 mL cultivation medium base (excluding the vitamin solution). The extract suspension was centrifuged at 3000g for 10 minutes. The supernatant was filter-sterilized and used as a 5x lemonade extract to supplement the cultivation medium.

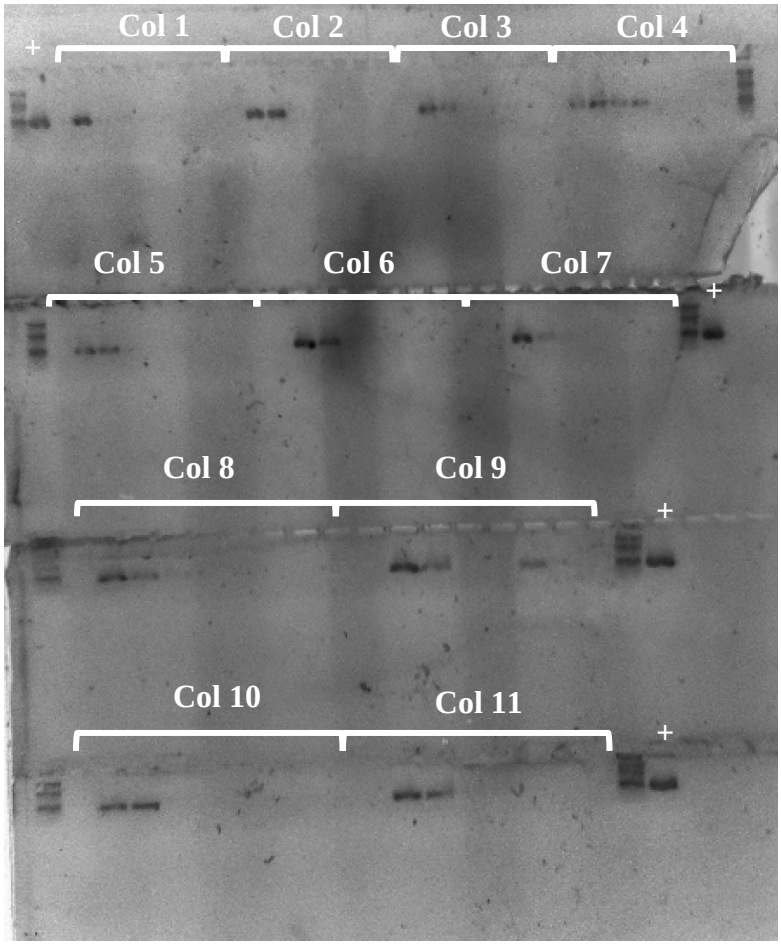

Figure 3. PCR screen of D1 plate. This PCR was performed on August 16th, nine days post inoculation. Pluses indicate positive control (amplification from Lemonade genomic DNA). Each column is represented as sequential sets of eight wells. The first well in each set corresponds to straight Lemonade inoculum. There is no amplification observed from the first well of any set. Col 9 shows a signal in the most dilute position relative to the other columns, corresponding to a dilution of 10^{-5}.

DAPI counting:
1 mL of Lemonade, same sample as was used to inoculate the dilution series, was fixed in 2% formaldehyde for an hour. 50 uL of fixed cells were filtered onto a 0.2 um nitrocellulose filter and stained with 1 ug/mL DAPI in Citifluor/Vectashield mounting medium. Cells were counted in four fields of view, using an objective-mounted counting grid (100 um x 100 um). Filter area was calculated based on the internal diameter of the vacuum column used, amounting to 226.9 mm^2. The calculation was then, $\frac{cells}{mL} = \frac{average\ cell\ number \times microscope\ factor}{volume\ filtered}$, where microscope factor is defined as $\frac{filter\ area}{grid\ area}$.

Inocula and dilution to extinction:

Enrichments were inoculated both with straight Lemonade and non-oxygenic phototroph enrichments that the class had previously set up, which had originally been inoculated with Lemonade. The non-sulfur and sulfur phototroph enrichment media compositions can be found in Table 1. The phototroph enrichments used were: (1) purple sulfur bacteria 7/21/2017 (first transfer) kept under 850 nm illumination; (2) non-sulfur bacteria switched from 660 nm to 850 nm on 7/24/2017; (3) purple non-sulfur bacteria 7/21/2017 (second transfer) kept under 850 nm illumination; (4) sulfur bacteria switched from 660 nm to 850 nm on 7/24/2017; (5) purple non-sulfur bacteria 7/17/2017 (second transfer); (6) green sulfur bacteria (no date—likely original enrichment) kept at 660 nm; (7) green non-sulfur bacteria (no date—likely original enrichment) kept at 660 nm; (8) green non-sulfur bacteria 7/11/2017 (original enrichment) kept at 660 nm; (9) green sulfur bacteria 7/11/2017 (original enrichment) kept at 660 nm; (10) green sulfur bacteria 7/11/2017 (original enrichment) kept at 660 nm. All cultures were inoculated for my enrichment on 8/7/2017.

Table 1. Non-oxygenic phototroph enrichment media that had been inoculated with lemonade on 7/11/2017

Non-sulfur medium	**Sulfur Medium**
2.9 L DI water	2.9 L DI water
30 mL 100x fresh water base	30 mL 100x fresh water base
15 mL 1 M NH_4Cl	30 mL 1 M NH_4Cl
3 mL 100 mM K_3PO_4, pH 7.2	30 mL 100 mM K_3PO_4, pH 7.2
0.3 mL 1 M Na_2SO_4	3 mL 1 M Na_2S
15 mL 1 M MES buffer, pH 6.15	15 mL 1M MES buffer, pH 6.15
2 mL 1,2 propandiol	30 mL 1 M $S_2O_3^{2-}$
3 mL Trace metals (1000x)	3 mL Trace metals (1000x)
3 mL Multivitamins (1000x)	3 mL Multivitamins (1000x)
10 mL 1 M $NaHCO_3$	50 mL 1 M $NaHCO_3$
50 mg DCMU (algicide)	50 mg DCMU (algicide)

Inocula were serially diluted down columns in Chicago-style (2 mL max well volume) 96-well plates. The first row contained 1 mL of the undiluted inoculum, each column being inoculated independently. 100 uL of each previous row was then added to 900 uL of sterile cultivation medium in the next row, resulting in steps of 10-fold dilutions to a maximum dilution of 10^{-7}. Four separate plates were prepared using this method. Plates L1 and D1, Lemonade extract supplemented and completely defined, respectively, were inoculated with Lemonade in each column. Plates L2 and D2, lemonade extract supplemented and completely defined, respectively, were inoculated with phototroph enrichments that were previously set up by the class from Lemonade. The phototrophs were inoculated into columns according to their enumeration above. I.e., in plates L2 and D2, columns 1-5 were inoculated with purple non-oxygenic phototroph enrichments and columns 6-10 were inoculated with green non-oxygenic phototroph enrichments. Columns 11 and 12 were inoculated with straight Lemonade. All plates were kept in Gas-pak chambers, with a standing solution of 25% sodium sulfide to create a sulfidic environment, at room temperature and out of direct light.

Genomic DNA (gDNA) extraction from Lemonade:

200 uL of pelleted lemonade (30 min at 3000g at 4 C), which had been previously used to inoculate the enrichments, was resuspended in 567 uL TE buffer (10 mM Tris, 1 mM EDTA, pH 8). 15 uL of 20% SDS and 3 uL of 20 mg/mL were added to the suspension, which was then mixed and incubated at 37 C for 1.5 hours. After the incubation, 100 uL of 5M NaCl was added to the suspension. The suspension was mixed, and 80 uL of a CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) were added, and the suspension was mixed again. Then, the aqueous phase was extracted with 24:1 chloroform/isoamyl alcohol, followed by an extraction with 25:24:1 phenol/chloroform/isoamyl alcohol, and isopropanol precipitation. The DNA pellet was resuspended in 100 uL TE buffer, its concentration quantified by a Promega Quantus fluorometer, and adjusted to ~50 ng/uL.

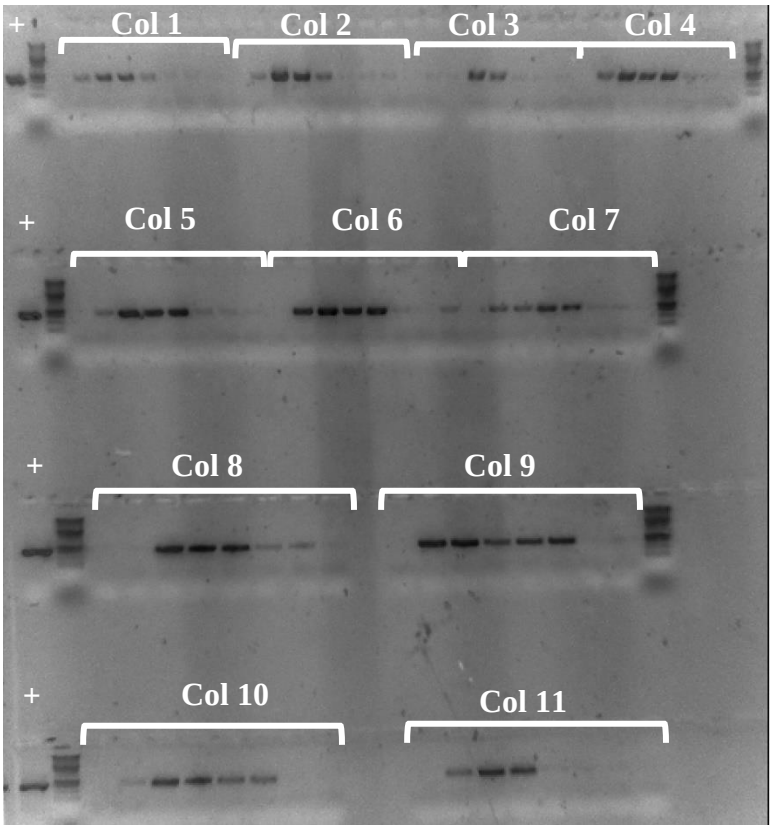

Figure 4. PCR screen of L1 plate on August 17th, ten days post inoculation. Pluses indicate positive control (Lemonade gDNA template). There is no amplification from the first well (straight lemonade) in any of the series. Overall, more signal is observed at greater dilutions relative to plate D1 (Fig. 3).

PCR primers and protocol:
Lenti 1342-rev: 5'-TGCTGATACGCCTTTACTAGCG-3'
Universal 515-fwd: 5'-GTGCCAGCMGCCGCGGTAA-3'

Reaction for lemonade gDNA:

- 25 uL GoTaq 2x buffer
- 1.25 uL Lenti 1342-rev (10 uM)
- 2.5 uL Universal 515-fwd (5 uM)
- 1 uL gDNA (~50 ng/uL)
- 20.25 uL H_2O

Reaction for enrichments:

1) Boil 1:1 mixture of alkaline phosphatase and enrichment culture at a final volume of 50 ul for 15 min at 95 C
2) PCR reaction
    a. 25 uL GoTaq 2x buffer

b. 1.25 uL Lenti 1342-rev (10 uM)
c. 2.5 uL Universal 515-fwd (5 uM)
d. 5 uL boiled template
e. 16.25 uL H_2O

Thermocycler program for PCR:

1) 10 min at 95 C
2) 30 sec at 95 C
3) 1 min anneal at 55 C
4) 2 min extend at 72 C
5) Go to (2) 29 times
6) 5 min extend at 72 C
7) Forever at 4 C

16S sequence quality control and analysis

Clone library sequences were processed for quality control *via* the RDP pipeline (https://rdp.cme.msu.edu/). The paramaters for trimming were: Base Score = 20; error probability = 0.01; 0.8 fraction > Q20 cutoff; 400 length cutoff. The analysis was performed against bacterial 16S sequences, using the pGEM-T-Easy vector. After quality control, the sequences were analyzed *via* BLAST against Bacterial and Archaeal 16S sequences, and SILVAngs.

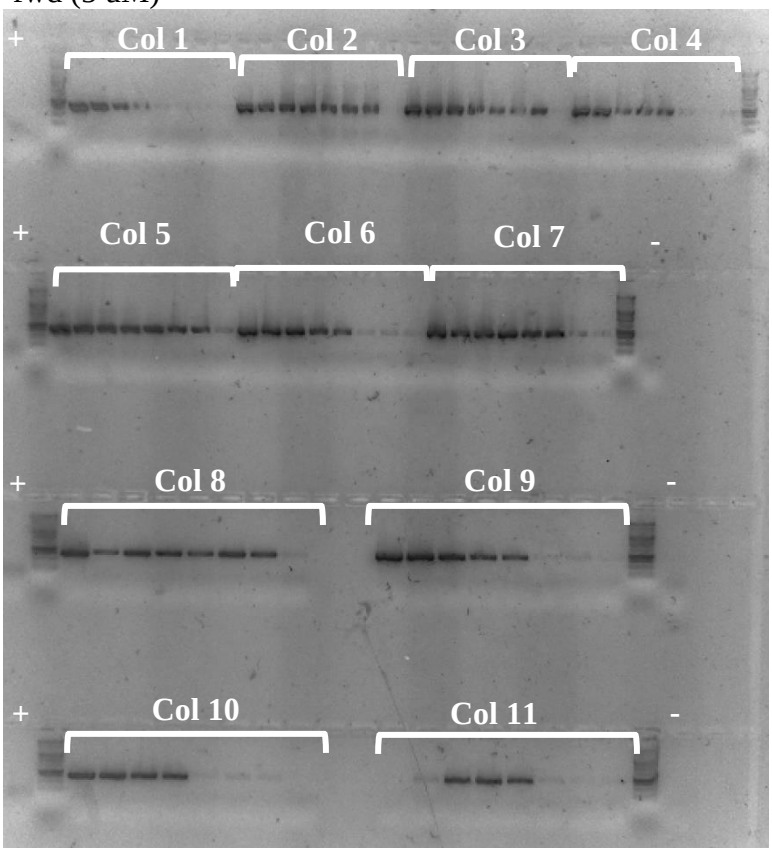

Figure 5. PCR screen of L2 plate performed on August 18th, 11 days after inoculation. Pluses indicate the positive control (Lemonade gDNA template), which did not work. Minuses indicate negative controls (no DNA template). Columns 2, 3, 5, 7, and 8 correspond to non-sulfur phototroph enrichment inocula; the other columns correspond to sulfur phototroph enrichment inocula. Columns 1-5 were purple enrichments. Columns 6-10 were green enrichments. Column 11 was inoculated with straight Lemonade. Unlike in plates D1 and L1, with the exception of column 11, the first wells in each set show a detectable PCR signal.

FISH

200 uL of the enrichment culture were fixed with 2% formaldehyde for an hour. The fixed cells were then filtered onto a 0.2 um nitrocellulose filter. The filters were hybridized to Eubacterial and Lentisphaeral probes for 3 hours at 46 C under a 30% formamide atmosphere. The hybridization buffer had 5 ng/uL of the respective probe, in: 900 mM NaCl, 20 mM Tris/HCl, 30-35% formamide, and 0.01% SDS. The filters were then incubated in a washing buffer (30% formamide, 112 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS) for 10 minutes at 48 C, rinsed and dried with pure water and 96% ethanol, and then mounted on with 1 ug/mL DAPI Citifluor/Vectashield. The Lenti FISH probe has the same sequence as the primer described above.

Results

Chemical and physical characterization of Lemonade

Different depths of Trunk River water were analyzed with a YSI probe *in situ*, and Lemonade was further analyzed by IC (Tables 2 and 3). No fluoride, nitrate, nitrite, or ammonium were detected. These data were used to help design the cultivation medium, particularly by implying that a sea water base would be appropriate.

Table 2. YSI probe measurements of Trunk River at different depths

	Surface	**~10 cm deep**	**~ 40 cm deep (lemonade)**	**~ 50 cm deep (sediment)**
pH	5.76	5.97	5.67	6
Conductivity (uS/cm)	10295	10598	44763	42204
Dissolved O_2 (%)	104.2	137.1	16	19.5
31.3	31.8	29.6	25.3	
Salinity (ppt)	5.11	5.08	27.1	27.5
Dissolved O_2 (ppm)	7.38	9.71	1	1.09
ORP (mV)	-286	-266	-276	-279

Table 3. IC analysis of Lemonade from two collection dates

Date	**August 1, 2017**	**August 7, 2017**
Chloride (mg/L)	3298.93	10102.03
Bromide (mg/L)	3.96	52.6
Phosphate (mg/L)	N/A	14.58
Sulfate (mg/L)	N/A	403.925
Lithium (mg/L)	N/A	8.87
Sodium (mg/L)	1981.475	4660.965
Potassium (mg/L)	549.395	334.655
Magnesium (mg/L)	193.775	771.775
Calcium (mg/L)	612.07	437.41

16S clone library from Lemonade gDNA using Lenti 1342-rev primer

10 out of 17 16S sequences from my clone library passed RDP quality control. BLAST analysis suggests that the Lenti 1342-rev primer amplifies 16S sequences from both proteobacteria and new phyla, which may be related to the PVC superphylum (Table 4). An analysis of the same sequences with SILVAngs gives a similar conclusion, despite the sequences being classified differently (Fig. 2).

Table 4. Top BLAST hits of 16S library from Lemonade gDNA

Organism(s)	**Clade**	**% sequence identity**
Lentisphaera aeronosa	*Lentisphaeria*	82
Victivallis vadensis (x2)	*Lentisphaeria*	86, 88

Kiritimatiella glycovorans (x3)	*Kiritimatiellaeota (Verrucomicrobia* subdivision 5)	87, 87 85
Geobacter metalloreducens	*Proteobacteria*	77
Desulfuromusa ferrireducens	*Proteobacteria*	91
Marichromatium purpuratum	*Proteobacteria*	97
Cerasicoccus frondis	*Verrucomicrobia*	80

Enrichment from dilution to extinction
The DAPI count resulted in 1.45 x 10^8 cells/mL in the sampled Lemonade that I used for my inocula. Hence, the final dilution in each column would be seeded with an average of 15 cells. Plates D1, L1, and L2 were screened by PCR with the Lenti 1342-rev and Universal 515-fwd primers (Figs. 3-5). Plate L1 showed better signal in the PCR screen than Plate D1. Plate L2 showed better signal still, particularly in column corresponding to inocula from non-sulfur non-oxygenic phototroph enrichments.

Microscopy
I looked at two wells (D1 column 9 row F; L1 column 9 row F) with strong PCR signal under phase contrast. D1 9F had largely enriched for motile rods, but I could also observe small non-motile cocci that had both phase-bright and -dark sub-cellular structures. These cells were coherent with previously described Lentispharae (Cho et al., 2003). L1 9F also had these non-motile cocci, but the culture did not clearly over-represent any single morphology. In other words, it appeared that slowly growing organisms were maintained, and no "weed" overran the population.

I followed up on these observations with FISH, but the results were not conclusive. I was not able to detect a signal with the Lenti probe in the sample from D1 9F, and found only several fields of view that had a potential signal from the L1 9F sample. The best L1 9F FISH field of view is presented in Fig. 6. The cell with a potential signal is, in fact, coccoidal.

Discussion
The 16S clone library results imply that the Lenti 1342-rev primer is not specific to the phylum Lentisphaeria. Nonetheless, both BLAST and SILVAngs classification show that the primer can also amplify Lentisphaeral sequences. Now that these 16S sequences are available, specific primers and FISH probes should be designed to detect all ten of the observed species. This will make it possible to better track which organisms are being enriched under which conditions, which is not possible now. As there was not enough time to successfully prepare a clone library from the enrichment itself, there is no way to interpret whether or not the enrichment was successful in cultivating Lentisphaerae specifically, or any of the other highly divergent organisms. Still, the fact that it was possible to detect PCR amplification down to the final dilution (Figs. 4 and 5), both in the L1 and L2 plates, implies that there must have been growth under these culturing conditions. It is not conceivable that a PCR signal could be detected from the original 15 cells in the final dilution from any of the series if those cells had not multiplied.

There were clear differences in growth between the D1, L1, and L2 plates. Seemingly, the lemonade extract supplement promoted growth of whatever organisms the Lenti 1342-rev primer can amplify. And the non-sulfur non-oxygenic phototroph enrichments from Lemonade may be bet-

ter inocula than Lemonade itself. Unlike in the D1 and L1 plates, the L2 plate showed PCR signal even in the undiluted wells. This implies that either Lemonade inhibits the PCR in a way that the phototroph enrichments do not, or that the organisms that the Lenti 1342-rev primer detects get out-competed in Lemonade, but not in the phototroph enrichments, under my growth conditions.

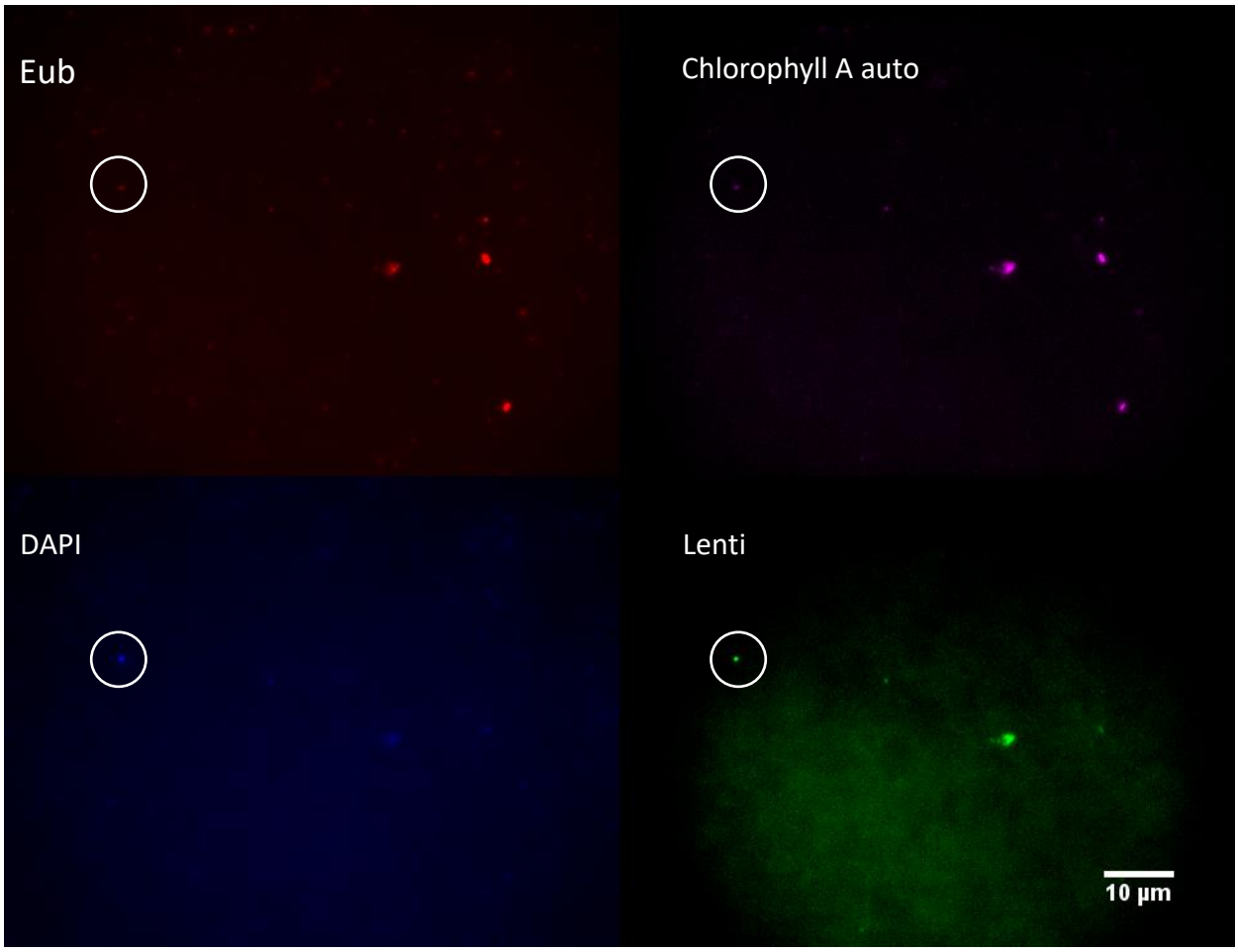

Figure 6. FISH with eubacterial and Lenti 1342 probes. The circle indicates a cell that is stained in all four channels, with relatively dim autofluorescence. Taken with an Axioplan ImagerA2 microscope at 63x magnification.

There are two potential complications to interpreting these data: First, it is possible that the different plates amount to batch effect. Second, it is possible that the differences between D1, L1, and L2 plates can be explained by their being analyzed on sequential days, giving L1 and L2 time to grow more than D1. However, it is unlikely that this is the case. Column 11 in the L2 plate is inoculated with straight Lemonade, and behaves in the same way as its replicates in plates D1 and L1 (Fig. 5). Hence, the differences among the enrichment conditions are probably not a matter of time or batch effect.

Since there was growth in the L1 plate, down to the final dilution, the organisms detected by the Lenti 1342-rev primer should be heterotrophic. If these organisms also thrive in the non-sulfur

non-oxygenic phototrophic enrichments, they likely make use of whatever metabolites these phototrophs produce. This makes sense, as a large proportion of the Lemonade metagenome consists of Chlorobi and Proteobacteria, which are the bacteria that are enriched as phototrophs under our conditions (Enalls, 2016). It is also interesting that the phototroph enrichment in based on a fresh water medium, while my cultivation medium is sea water based. In future enrichments, it will be important to compare fresh and sea water based media, as well as to try enriching for target Lemonade organisms in the presence of defined non-sulfur anoxygenic phototroph cultures.

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