What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

Abstract:
Within the brackish Trunk River located in Falmouth, MA, periodic and persistent blooms primarily composed of sulfur-oxidizing bacteria nicknamed “lemonade” can be found (Cowley et al., 2015). However, little is known of how these blooms degrade or crash, both in the environment and in laboratory settings. Here, I sampled two liters from the lemonade at Trunk River and aliquoted it into two one-liter bottles, one of which was kept at room temperature and one that was stored at 4°C. When the room temperature sample was left in the light, filaments formed after about 2 days and slowly sunk to leave cleared liquid on top and mats at the bottom of the bottle. When they were kept in the dark, the liquid in the bottle never cleared, but did become more transparent, and eventually darkened in color. Viral abundance increased in the room temperature treatment relative to the 4°C bottle after day 7 and a couple different morphotypes of flagellates were observed starting by day 2. Thus, both viral lysis and grazing may have played a role in degrading the lemonade.

Introduction:
Trunk River is a shallow, brackish pond in Falmouth, MA, that is characterized by periodic yellow blooms nicknamed “lemonade”. These blooms can repeatedly be formed through disturbances in the seagrass and may persist for weeks (Cowley et al., 2015). While these communities have been investigated via sequencing and have been shown to be primarily dominated by Chlorobi and Proteobacteria (Cowley et al., 2015; Enalls, 2016), not much is known of how these blooms dissipate or crash. However, clearings have been observed in the lab multiple times when bottles of lemonade have been left at room temperature for 1-2 days, indicating that the community has shifted or crashed (Rachel Whitaker and Sarah Guest, personal communication, July 25, 2018). Bloom crashes and degradations can occur for many reasons. For example, viruses have been found to control blooms of the coccolithophore *Emiliania huxleyi* (Wilson et al., 2002). Similarly, grazers can act as a controlling force of blooms when the grazing rate becomes higher than the growth rate while nutrient availability can limit growth (Tiselius and Kuylenstierna, 1996; Gobler et al., 2007). Additionally, since all observations of lemonade clearings have been in bottles, bottle effects such as limited space or differing conditions from the environment may be stressing the microbial community, leading to a breakdown of community composition. Here, I will investigate the mechanism of bloom crash in a laboratory setting via a set of bottle experiments to attempt to find the mechanism by which the lemonade is clearing. I focused primarily on viruses, grazers, and nutrient utilization as the primary mechanisms of degradation.

Methods:
1. Sample Collection
   Preliminary experiment: An empty sediment core tube was used to collect water from a lemonade patch in the Trunk River. Lemonade could be attained by swimming out into the river on a boogie board and reaching down into the water and collecting from where there was a temperature difference (usually just above the sediments). The water was then transferred to a two-liter glass bottle which was filled to the brim via funnel. This generally required two to three rounds of sampling with the core tube. The water was then brought back to the lab as quickly as possible, homogenized by shaking, and sampled for the T0 time point. Two one-liter glass bottles were then filled with one liter of the remaining lemonade. One bottle was stored at 4°C and the other was stored on top of my bench at room temperature. Samples for 16S rRNA amplicon sequencing were
taken at 4 hours, 14.5 hours, 24 hours, 45.5 hours, and 6 days post sample collection.

**Secondary sampling:** Trunk River lemonade was collected in the same manner as before and brought back to the lab. After taking the T0 sample, the remaining water was aliquoted into one-liter bottles, where one was stored at 4°C and the other was kept in a drawer at room temperature. Both were kept in the dark with intermittent light. The bottles were sampled for 14 days with samples taken daily for ion chromatography, viral abundance, and wet mount imaging. Samples for 16S rRNA sequencing were also collected, but only during the first four days to conform to the sequencing deadline.

**Sediment Test:** A side 250mL bottle of Trunk River sediment plus lemonade was made with the remaining lemonade from the two-liter bottle. Sediment was first placed into a 250mL glass bottle using a scooper. The bottle was then filled to the brim with lemonade and then left in a drawer at room temperature along with the one-liter bottle of lemonade. This bottle was monitored by daily pictures to determine if having sediment present extended the lifetime of the lemonade.

2. **DNA extraction**
   **Collection:** 20mL of sample was spun down at 4,000 *g* for 15 minutes at 17°C to obtain a pellet. The supernatant was then poured off and the pellet was stored at -20°C until processing.
   **Processing:** DNA was extracted from pellets using the QIAamp PowerFecal DNA Kit and quantified via the BR dsDNA Qubit kit. The DNA was amplified for 16S rRNA sequencing using the GoTaq Green Master Mix.

3. **Viral enumeration**
   **Collection:** Four milliliter aliquots of sample were fixed in 0.5% glutaraldehyde at every time point and stored at 4°C until processing. When possible, samples were processed immediately.
   **Processing:** 750µl of sample was 0.2µm filtered and diluted 1:10 into 1mL of 0.2µm filtered MQ water. One milliliter of the dilution was then filtered onto a 0.02µm Whatman Anodisc filter and back-stained with SYBR Gold for 15 minutes as in Patel et al. (2007). Viral-like particles were counted via epifluorescence microscopy using a Zeiss Imager A2 at 100x magnification under the FITC channel. Twenty fields of view were counted with at least ten particles in each.

4. **Wet mount imaging**
   **Collection:** Samples collected for viral enumeration were also analyzed by wet mounts under the microscope.
   **Processing:** Ten microliters were pipetted onto a microscope slide and viewed under 100x magnification using the Zeiss Imager A2 microscope. Slides were viewed for 20-30 minutes with pictures being taken of anything new or interesting that was seen. Morphologies were categorized and the number of pictures containing each morphology were determined. These were then investigated for qualitative trends for morphologies that were observed only at room temperature, only at 4°C, or in both bottles.

5. **Amplicon pipeline**
   16S rRNA amplicon fastq files were processed using the Qiime2 pipeline from the Microbial Diversity genomics week Qiime lecture. Briefly, the fastq files were denoised using dada2, sequences were aligned using mafft, and taxonomy was
What happened to my lemonade? A look into bloom dynamics at Trunk River
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assigned using a classifier that was trained on the Greengenes 13_8 99% OTUs of the V4 region.

Results and Discussion:

Preliminary experiment:

In the preliminary experiment, one bottle was left at 4°C and the other was left at room temperature on my bench. Within the first 48 hours, large clumps were observed to be forming only in the room temperature bottle (Figure 1). By the sixth day, the room temperature bottle had formed filaments that were sinking as a group, leaving clear water on top and green and purple mats on the bottom (Figures 1 and 2). Throughout this time, the 4°C bottle did not lose any of its yellow color, though fine gray sediment did seem to be collecting on the bottom of the bottle.

Figure 1 (above). Images of the room temperature and the 4°C treatments over the sampling time course demonstrating the clearing of the room temperature bottle relative to the 4°C bottle.

Figure 2 (right). Image of the mat at the bottom of the room temperature bottle showing that there is some purple in the mat as well.

Wet mount images of the clear liquid and filaments from the room temperature bottle were viewed on day 5. In the cleared liquid, a large number of small rod-shaped bacteria were observed as well as some larger, rounder rods with what appeared to be phase bright sulfur granules on the ends. The mats or filaments also contained both of the described cell morphologies, but in much larger numbers and were observed more often in aggregates. Longer dark rods, spirochetes, and curved bacteria were also observed, indicating that the filaments are made up of a consortium of multiple bacterial types (Figure 3).

Figure 3. Wet mount images taken from the room temperature treatment on Day 5. Panel A shows a sample taken from the clear liquid on top, while B and C are from the mats or filaments on the bottom. A greater number of cell morphologies and aggregates were observed in the mats.
What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

16S rRNA amplicon sequences were analyzed for T0, T4, T14.5, T24, T45.5 and 6 days after sampling for both the room temperature and 4°C bottles. The phylum Chlorobi dominated in most samples, accounting for approximately 20–70% by relative abundance, which matches what has been observed in these lemonade consortia in the past (Figure 4; Cowley et al., 2015; Enalls, 2016). Proteobacteria was the next most abundant phyla, followed by Cyanobacteria, Bacteroidetes, and Spirochaetes. Distributions of relative abundance did not appear to differ throughout the first 48 hours. However, both the day 6 room temperature A sample, which was taken from the clear water, and the day 6 4°C B sample, which was from the sediment at the bottom of the bottle, have a lower proportion of Chlorobi and a higher relative abundance of Proteobacteria. In the room temperature sample, this could be due to a shift in community structure over time whereas in the 4°C sediment, this difference is likely due to the types of cells that have degraded. Additionally, the day 6 room temperature B sample taken from the filaments and the day 6 4°C A sample taken from the remaining lemonade both appear to be more similar to the earlier time points. Both are still dominated by a high proportion of Chlorobi, though at a slightly higher relative abundance (50 vs. 70%; Figure 4).

![Relative abundance taxa plots of samples from the preliminary experiments. Chlorobi, Proteobacteria, Cyanobacteria, and Bacteroidetes were the dominant phyla by relative abundance. Samples taken in the first two days appear to be similar, as do the Day 6 room temperature sample from the mats and the Day 6 4°C sample from the lemonade. However, the Day 6 sample from the cleared liquid has a higher relative abundance of Proteobacteria. Additionally, the Day 6 4°C sample from the sediment at the bottom appears more diverse and has a much lower proportion of Chlorobi.](https://example.com/image)

**Secondary experiment:**
In the secondary experiment, the room temperature bottle was kept in a drawer to match the intermittent light that was being received by the 4°C bottle in the fridge. In addition, the lemonade collected the second time around was much denser than in the
What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

preliminary experiment, so much so that you could no longer see through the bottle. This
was potentially caused by the disturbance of the river during the sampling done the
previous week for the preliminary experiment. Consequently, the timeline and
appearance of degradation was much different. Notably, instead of forming filaments
and sinking out to leave behind clear liquid, the room temperature bottle simply became
less dense over time, resulting in transparent yellow liquid between days 4 and 7 (Figure
5). Additionally, while these bottles were only sampled through day 14, by day 17, the
liquid in the room temperature bottle had darkened to a mustard yellow-brown color
compared to the more yellow-green liquid that remained in the 4°C treatment.
Consequently, if similar experiments are conducted in the future, I suggest tracking
spectra throughout the time series as well.

Figure 5. Images of the room
temperature and the 4°C treatments
over the sampling time course for
the secondary experiment
demonstrating the clearing of the
room temperature bottle relative to
the 4°C bottle.

Ion chromatography measurements were taken daily for both cations and anions.
Over time, there appeared to be a slight increase in sulfate concentration in the room
temperature treatment relative to the 4°C treatment (increase from ~400 to 800 mg/L vs.
steady at ~450 mg/L; Figure 6 A, B). This could potentially indicate that sulfide
concentrations were low, resulting in sulfur being oxidized all the way to sulfate more
often, but it is not a clear connection (Canfield et al., 2005). Ideally, sulfide would be
measured in the future to determine this with greater confidence. Among the cations,
there was a slight decrease in magnesium observed over time, though that trend is less
robust and observed in both bottles, indicating magnesium may just be a nutrient that is
slowly being utilized without replenishment (Figure 6 C, D).

Viral abundance was determined via epifluorescence microscopy. In all samples,
viral abundance was high (on the order of 10^8) relative to other surface seawater viral
abundances (Fuhrman, 1999). While both treatments have a slight increase in viruses
over time, at day 7 the number viruses in the room temperature treatment approximately
What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

Figure 6. Ion chromatography plots with room temperature samples plotted on the left (A and C) and 4°C samples plotted on the right (B and D). Anion data is plotted on top (A and B) and cation data on the bottom (C and D).

Figure 7. Viral abundance over the time series of 14 days. Both room temperature and 4°C had slight increases in concentration over time, but at day 7, the room temperature treatment approximately doubled in viral abundance.

doubled (Figure 7). This indicates that viruses could be partially responsible for the clearing observed in the room temperature treatments. However, since the increase in viruses in not as dramatic as one might expect and the clearing occurred between days 4 and 7, I do not believe it was the only cause. Additionally, samples for viral counts were fixed and stored at 4°C before processing. Days 8-14 were all processed within half an hour of sampling, but days 0-7 were processed between 1 and 13 days post collection. This may have resulted in viral degradation as storing in 4°C is not the ideal storage temperature and has been shown to have lower viral counts than freshly prepared samples or those flash frozen and stored at -80°C (Wen et al., 2004). However, since day 6 was prepped only one day later while days 4 and 5 were prepped 12 and 13 days later, it does not seem as if there were a significant amount of degradation that occurred, at least after the first day. Also, since day 7 is actually the highest count and it was counted one day late, there may not have been a lot of degradation occurring in the first 24 hours either.
What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

However, this is all speculative and future studies should ideally flash freeze and store their samples at -80°C to avoid any potential differences between samples. Wet mount images were taken and categorized by morphotype for all time points. Twenty-five different morphotypes were observed collectively between all the time points and treatments. These were then organized into three main categories: morphotypes observed in both treatments approximately equally, morphotypes observed more at 4°C, and morphotypes observed more at room temperature. Of these, the small rod-like bacteria seen in the preliminary experiment, and spirochetes were observed in all samples (Figure 8 A, B). While it is hard to determine based on morphotype alone, I believe these smaller rods might be within the phyla Chlorobi, as they appear similar to others seen before (Overmann, 2006). The larger rods with phase bright sulfur granules on either end and phase bright consortia were observed predominantly at 4°C (Figure 8 C, D). I believe the rods with bright spots to be a type of purple sulfur bacteria as similar looking cells have been observed in previous enrichments for purple sulfur bacteria from Trunk River (Wu, 2015; Liu, 2016).

Figure 8. Plots of time point against the number of pictures I observed a specific morphotype in. The morphotypes are displayed in the micrographs in the upper right corner of each graph. Panels A and B represent morphotypes seen in all samples, C and D are those that were seen more at 4°C, and E-H are those that were seen mostly or exclusively in the room temperature treatment. The green lines represent room temperature while the blue line is the 4°C treatment.
What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

Flagellates, cell aggregates, and larger black cells were observed most often or only at room temperature (Figure 8 E-H). The larger, round flagellates first appeared around day 2 and were seen most often from day 5 to day 10. As these flagellates are generally mixotrophic, this may represent a grazer that was predating on cells within the lemonade. Additionally, the longer skinny flagellate with bright spots, which may also be a type of purple sulfur bacteria, and the larger, slug shaped bacteria were much more common from day 8 to 14, which could be an indication in a shift in the community composition (Ruecker et al., 2011; Overmann et al., 2004). However, further tests would need to be conducted to verify this claim. Finally, more cell aggregates were observed in the room temperature treatments, which could be acting similarly to Chlorochromatium aggregatum, where it could be an aggregate of Chlorobi with sulfur reducing bacteria, or with a motile bacterium, that allow the Chlorobi to gain sulfide or move toward sulfide (Canfield et al., 2005). Thus, this may be a response to lower nutrient abundance, though again, further testing is required.

Finally, 16S rRNA amplicon sequences were analyzed for the first four days of sampling. Even though the room temperature bottle was not fully transparent by day 4, there was a clear difference between the room temperature and 4°C treatments after spinning them down for a pellet (Figure 9). However, this visible difference did not manifest itself in the sequence data where almost all samples look similar. The major exception to this are the D0 duplicates where one is dominated by the phyla Chlorobi by relative abundance and the other by Proteobacteria. Similarly, duplicates across the samples were not often entirely similar by relative abundance but they were also not as drastically different as the D0 samples. However, in most samples, Chlorobi is proportionately the dominant phyla, followed by Proteobacteria. Spirochaetes and Bacteroidetes are also present with relatively high sequence abundance (Figure 10).

Figure 9. Image of room temperature and 4°C samples post spin down for DNA pellet showing a clear difference between the samples.
What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

Figure 10 (above). Relative abundance taxa plots of samples from the secondary experiment. Chlorobi, Proteobacteria, and Bacteroidetes were the dominant phyla by relative abundance. There was a much higher proportion of Chlorobi in the second experiment than in the first, likely due to how dense the lemonade was.

Sediment Test:

The 250mL bottle of Trunk River sediment with the remaining lemonade from my second sampling day was kept in a drawer at room temperature and observed daily for any changes. While the lemonade began as dense as in the secondary test, it cleared within six days (Figure 11). This leads me to believe that volume may be a determining factor to how quickly lemonade clears. Additionally, we have a core with about 2 inches of sediment and 1.5L of lemonade that was collected on July 14, 2018. It was sealed at the top and bottom with electrical tape and has not yet cleared, even though it has been over a month. Consequently, I also believe oxygen input may play a role in lemonade degradation. Further studies investigating the presence of sediment and oxygen should be conducted to determine if either or both impact the rate or type of degradation observed. If the lemonade can survive with sediment, it may indicate that nutrient availability is more important to consider than predation by either grazers or viruses as the community may be maintained by a variety of organisms completing the sulfur cycle.

Conclusions

An increase in both viruses and grazers was observed in the room temperature treatment relative to the 4°C treatment. This indicates that predation may be playing a role in how blooms of lemonade crash, which could be expected to happen naturally at Trunk River. However, future studies with better measurements for more relevant nutrients should be conducted to determine that the disruption of the sulfur cycle is not playing more of a role. In addition, future experiments testing the presence of sediment, oxygen, and light vs. dark would also help to elucidate more of the trends occurring here. Also, it is important to remember that while lemonade is dominated by Chlorobi, there are many other microbe interactions to consider as well. For instance, sulfur reducing bacteria take up reduce sulfate to sulfide while the Chlorobi oxidize sulfide. Thus, if both groups are present in the lemonade, they may be able to support the survival of the other for without needing other nutrient inputs for a long period of time.

Acknowledgements

I would like to thank the Simons MD Scholarship for providing funding for me to attend this course. I would also like to thank both George O’Toole and Rachel Whitaker for organizing the course and always being willing to answer all of my random questions. Thank you to Kurt Hanselmann for being the best sampling buddy and keeping me on track when I was trying to think about sulfur cycling. Additional thanks to Gabriela Kovacikova, Sarah Guest, Rebecca Wipfler, José Vargas-Muñiz, and Elaina Graham for
What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018

all their help sampling and keeping me in Trunk River for the least amount of time possible. Also, a special thank you to Shannon Lynch, Sandra Rizt, and The Three Musketeers for keeping me sane and on track throughout all this wonderful craziness. And finally, thank you to all the participants of the 2018 Microbial Diversity course- you all are the best!!

References:


What happened to my lemonade? A look into bloom dynamics at Trunk River
Sarah Schwenck, Microbial Diversity 2018
