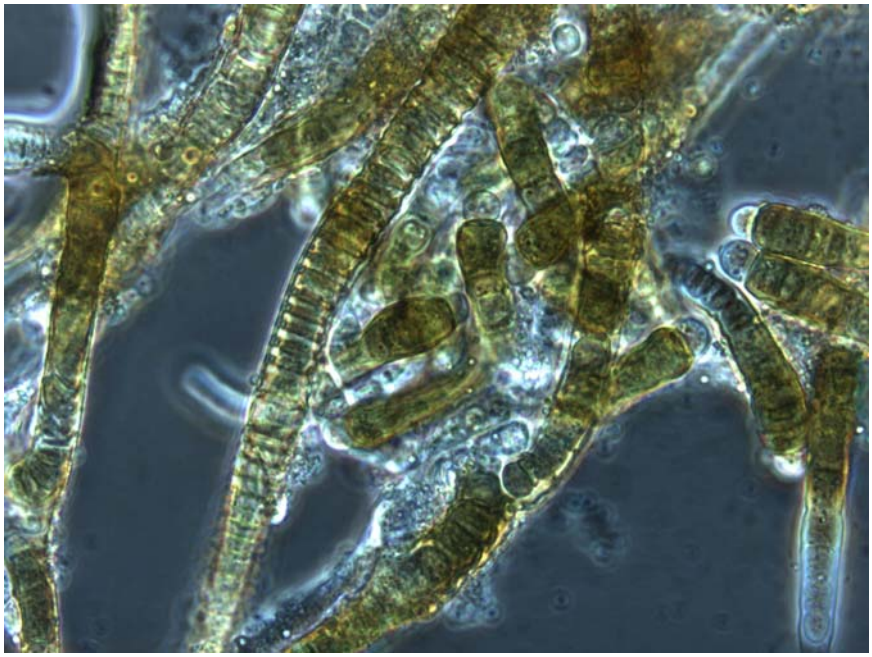
# The Effects of Sunscreen on Cyanobacteria



Kathryn M. Onesios
Department of Geography and Environmental Engineering
Johns Hopkins University
Baltimore, Maryland

Microbial Diversity Summer Course
Marine Biological Laboratory
Woods Hole, Massachusetts

July 29, 2008

**Abstract**

Ultraviolet-filters found in various manufactured cosmetic products have been detected recently as environmental contaminants at trace concentrations. The effects of these low levels of sunscreen products on cyanobacteria were investigated in this study. Average trends of triplicate conditions in this study suggest that sunscreen causes decreased scytonemin and chlorophyll a production, as well as decreased rates of photosynthesis, in *Calothrix* sp. (PCC 7102) over short time scales. At the community scale, clone library analyses indicate that cyanobacterial community composition was not significantly affected by the addition of sunscreen to the enrichment cultures.

**Introduction**

A variety of commercially-available cosmetic products contain ultraviolet-filters, which are compounds designed to absorb UV light (5). Due to the widespread use of sunscreen agents, researchers have begun studying the occurrence and fate of these compounds in the environment. UV-filters raise concerns because, unlike many other compounds in the pharmaceutical and personal care product (PPCP) category of trace pollutants, they are directly input into the aqueous environment in addition to input as wastewater treatment plant effluents. For example, recreational swimming can directly input UV-filters into the environment, thereby allowing the compounds to evade potential biodegradation or physical-chemical methods of removal during wastewater treatment (2). One occurrence study of two Swiss lakes used for recreational swimming found that the highest concentration detected was 125 ng/L for the compound benzophenone-3 (5). Another study examined Swiss lakes and a river and found concentrations ranging from <2 ng/L to 35 ng/L for UV-filters benzophenone-3, 4-methybenzylidene camphor, ethylhexyl methoxy cinnamate, and octocrylene (1). Compared to pharmaceuticals also in the PPCP category, relatively little is known about the effects of the sunscreens on nontarget organisms (2). The nontarget organisms of interest in this study are the cyanobacteria.

UV light has high energy levels that can damage DNA, proteins, and other important molecules in bacteria. Bacteria exposed to damaging UV rays have various mechanisms to overcome the damage that could lead to survival, growth, pigmentation, photosynthesis, and motility problems. In addition to repairing UV damage, some are also able to produce carotenoids, detoxifying enzymes, radical quenchers, antioxidants, and substances capable of absorbing or screening UV light. One of these UV-screeners and absorbers is scytonemin (7). Scytonemin is a pigment found in the sheath of more than 300 cyanobacteria and is yellowish-brown in color. This compound is thought to be unique to cyanobacteria (6) It is a proposed screening compound for UV-A and has shown to be induced by cyanobacterium exposure to UV-A (6, 7).

This study aimed to elucidate the effect of sunscreen at environmentally relevant concentrations on cyanobacteria. It was hypothesized that the presence of UV-filters in the waters surrounding cyanobacteria would benefit the bacteria by protecting them from harmful UV light, therefore allowing them to have increased photosynthesis rates and chlorophyll a production, and decreased need for scytonemin production. It was also predicted that environmental communities of cyanobacteria would shift after exposure to UV light depending on whether or not they were amended with sunscreen. Experiments

were conducted on both an individual cyanobacteria strain, *Calothrix* sp. (PCC 7102), and on environmental samples collected from beach sand.

## Materials and Methods

*Calothrix* Culture

*Cyanobacteria and sunscreen treatment*. A cyanobacterial culture of *Calothrix* sp. (PCC 7102) growing in GOX medium was obtained from J. Waterbury (Woods Hole Oceanographic Institution). This culture served as the inoculum for 27 cultures. A standard volume of culture (~1 mL) was added to each of 27 autoclaved test tubes with plastic caps containing 15 mL of BG-$11_0$ medium plus $NaHCO_3$ (5 mM). "Killed Control" cultures were autoclaved after being inoculated. Tubes were sacrificed at each time point for measurements of photosynthesis and pigment content.

The sunscreen used was Coppertone Oil Free Sunscreen Lotion, SPF 30. The active ingredients and their percent concentrations are: avobenzone 2%, homosalate 13%, octisalate 5%, octocrylene 2%, oxybenzone 4%. Three treatments were selected for study at 0 (pre-sunscreen spike), 1, and 5 day time points. The treatments were "Killed Control", "Sunscreen –", and "Sunscreen +". Each condition was set up in triplictate for each time point. Killed Control tubes were autoclaved, and then received an aliquot of sunscreen to raise the average active ingredient concentration to100 ng/L. Sunscreen + tubes also received 100 ng/L levels of sunscreen, whereas Sunscreen – tubes received no sunscreen. The cultures were all incubated on the roof of Loeb Hall, MBL, Woods Hole, Massachusetts.

*Respirometer measurements.* Measurements of oxygen production rates in the cyanobacteria cultures were made using a micro-respiration system (Unisense, Denmark). Approximately 5 mL of culture were added to the system's glass chambers, and measurements were made under applied light from a Dyonics Model 375 Fiberoptic Illuminator (Donsanto, Inc., Massachussetts) with a magnetic stirbar operating at 120 rpm. Glass chambers were sterilized in between uses by soaking in 70% ethanol. Photosynthesis rates were calculated as the slope of a best fit line on a plot of time (seconds) versus oxygen concentration (uM). When the rate of oxygen production was determined by calculating the slope of the line on the time versus oxygen concentration plot, points falling outside of the range of 95%-105% of the measured values for the immediately surrounding region of the line were omitted because they were vestiges of the unsteady baseline and would artificially decrease rates of oxygen production.

*Pigment Analysis.* Scytonemin and chlorophyll a concentrations were determined by a Cary 50 Scan UV-Vis spectrophotometer (Varian, Inc., California) after 5 mL samples were extracted following the procedure of Dillon and Castenholz (3). One change to the procedure is that correction of residual scatter was not taken into account as the absorbance was being used for relative comparison, not for determination of absolute pigment concentrations.

*Microscopy*. Still images and time-series images were photographed on the Zeiss Scope.A1 epifluorescent microscope with a Zeiss AxioCamMRc camera.

Community Study

*Samples.* Samples were collected from the beach southwest of the intersection of MBL St. and Water St. in Woods Hole, Mass. A sterile 15-mL Falcon tube was filled with moist sand from the intertidal region of the beach.

*T-RFLP*. Immediately upon collection of the beach sand samples, DNA was extracted from approximately 1 g of sand using an UltraClean™ Soil DNA kit (Mo Bio, Carlsbad, CA) following the kit's extraction protocol. A Polymerase Chain Reaction (PCR) was carried out with 46 uL of Mastermix containing a 6-carboxyfluorescein labeled 8F primer and an unlabeled 1492R primer, 46 uL mastermix, and 4 uL of a 1:10 dilution of the extracted DNA sample. A QIAQuick PCR Purification kit (Qiagen #28104) was used to clean up the PCR product. The kit's protocol was modified to include the following steps: (1) combine 5 volumes of Buffer PB with 1 volume of PCR product and vortex, (2) pipette the sample onto a QIAquick spin column and centrifuge for 30 seconds at 13,000 rpm, then discard the flow-through, (3)add 0.75 mL Buffer PE to the spin column and centrifuge for 30 seconds at 13,000 rpm, then discard the flow-through, (4) centrifuge for 1 minute at 13,000 rpm to dry, (5) transfer the column to a new 1.5 mL tube and elute by adding 30 uL water, let stand for 1 minute, then centrifuge for 1 minute at 13,000 rpm. The restriction digest of the sample occurred in a 10 uL reaction containing 3.3 uL PCR water, 1 uL Multicore 10x Buffer, 1 uL of 20,000 U/mL Msp1 enzyme (NEBioLabs, Ipswich, MA), and 5 uL of PCR product. This restriction digest was carried out at 37°C for 4 hours. Nucleotides were removed using a QIAquick Nucleotide Removal kit (Qiagen #28304). The kit's protocol was modified to include the following steps: (1) Combine 10 volums Buffer PN with 1 volume of the digested sample and vortex, (2) place the sample in a QIAquick spin column, centrifuge for 1 minute at 6,000 rpm, discard flow-through, (3) add 0.75 mL Buffer PE to the spin column and centrifuge for 1 minute at 6,000 rpm, (4) discard the flow-through and centrifuge for 1 minute at 13,000 to dry the colum, (5) transfer the colum to a 1.5 mL tube, elute with 30 uL water, let stand for 1 minute, centrifuge for 1 minute at 13,000 rpm. The product was concentrated by vacuum-centrifuge and resuspended in water for a final concentration of 24.3 ng/uL. This final product was then sequenced by the Bay Paul Center.

T-RFLP data was analyzed using Microbial Community Analysis III (MiCA3) software available from the University of Idaho (http://mica.ibest.uidaho.edu/runpat.php).

*Clone Libraries*. The same beach sand sample collected for T-RFLP was used to create two enrichment cultures of 5 g sand and 20 mL of BG-$11_0$ medium plus $NaHCO_3$ (5 mM) in 125 mL Erlenmeyer flasks each. One flask received 1 ug/L active sunscreen ingredients, while the other received none. Both cultures were exposed to UV light for 4 hours, then were kept in the dark until cloning began the following day.

PCR primers designed to amplify the 16S rRNA genes from cyanobacteria were used to prodcue clone libraries (4). The cloning was performed using a TOPO TA Clonging Kit (Invitrogen, Carlsbad, CA). The cloning reaction consisted of 1 uL salt solution, 2 uL fresh PCR product, 1 uL sterile water, and 1 uL pCR®2.1-TOPO vector, mixed on ice. These were gently mixed, then incubated for 5 minutes at room

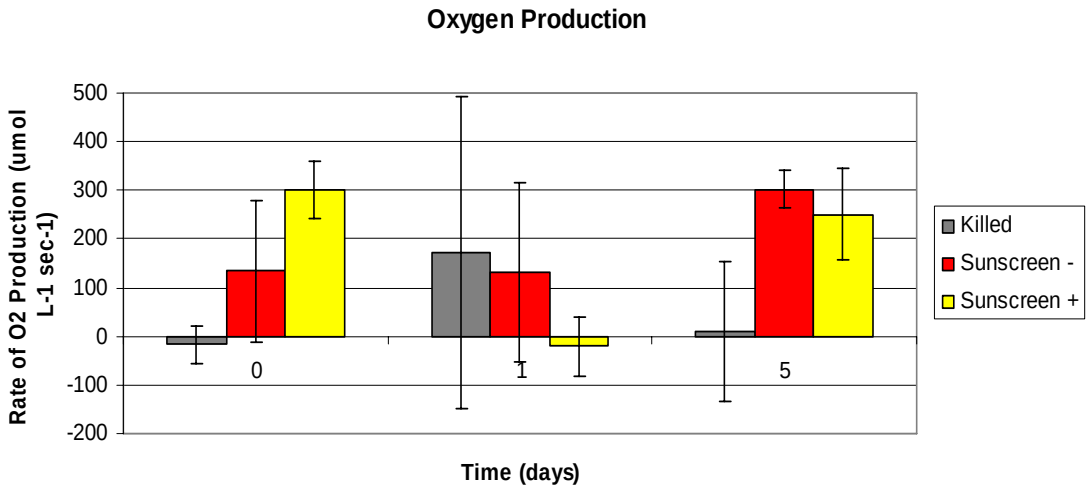
temperature. One Shot chemically competent cells were thawed on ice and gently mixed with 2 uL of the cloning reaction mix. After a 30-minute incubation on ice, the cells were placed in a 42°C water bath for 30 seconds. The tubes were then transferred to ice and combined with SOC medium. The tubes were shaken in an Erlenmeyer flask horizontally for 30 minutes at 37°C, after which they were placed on ice. Volumes of 40 uL and 80 uL were spread on two LB plates containing 50 ug/mL kanamycin. Plates were incubated overnight, and colonies were picked for sequencing at the Bay Paul Center the next afternoon.

## Results and Discussion

*Calothrix* Culture

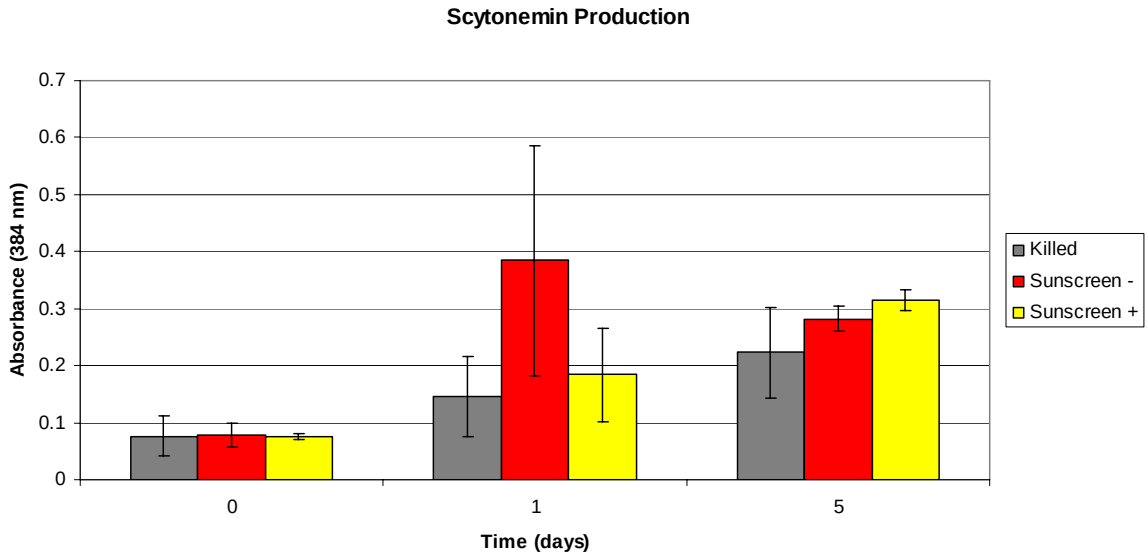
Photosynthesis rates, measured as oxygen production as a function of time, from five days are shown in Figure 1. Trends from triplicate tube analyses show that Sunscreen + oxygen production decreased with the addition of sunscreen (between day 0 and day 1 measurements), which would suggest that sunscreen has a negative effect on photosynthesis, and then the rate increases from day 1 to day 5, possibly as the sunscreen is degraded. The Sunscreen – tubes are also showing an increase in rate from day 1 to day 5 without the decrease in rate from day 0 to day 1 as seen in Sunscreen +. The Killed Control does not seem to have served as an effective control. Wide variation in triplicate values for all of the conditions makes meaningful comparisons of rates difficult. It can be said that at day 5, the Sunscreen – and Sunscreen + cultures had higher photosynthesis rates than the Killed Control, but distinction between the two non-killed cultures is not statistically valid. This was likely due to the drops in the baseline of the oxygen probe at random intervals.

Figure 1. Oxygen production by approximately 5 mL samples of *Calothrix* cultures was measured with a microrespirometer on day 0 (pre-sunscreen addition), day 1, and day 5. Bars represent the average rate for triplicate tubes with plus and minus one standard deviation error bars.



The average concentrations of scytonemin in the *Calothrix* cultures appears to have increased for all conditions at some point over the study time (Figure 2). Since this includes the Killed Controls, it is possible that there was some interference from other cellular components breaking degrading in this time period. The Sunscreen + and Sunscreen – cultures have higher average concentrations of scytonemin than the Killed Control cultures for both days 1 and 5, indicating that there was an increased production of these sunscreen compounds as the cultures were exposed to more UV rays over the experimental time. On day 1, the Sunscreen – cultures have on average produced more scytonemin than the Sunscreen + cultures have, which supports the hypothesis that addition of the commercial sunscreen results in less scytonemin production for Sunscreen + cultures as compared to Sunscreen – cultures. By day 5, scytonemin concentrations in the tubes have decreased for Sunscreen – and increased for Sunscreen + tubes to about the same absorbance, possibly suggesting that both the commercially available sunscreens and the natural sunscreens may have degraded to some extent, requiring the increased scytonemin production in Sunscreen -. Once again, there was high variability in the triplicate bottles for many of the conditions, so the statistical validity of these results is questionable.

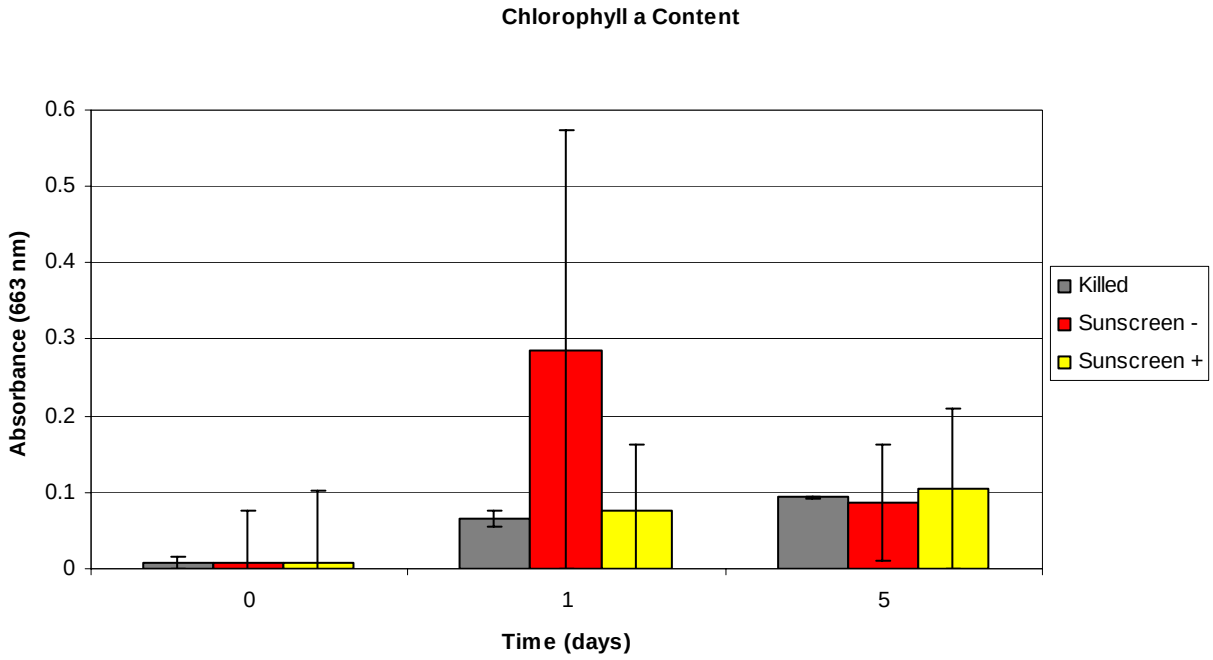
Figure 2. Scytonemin concentrations in *Calothrix* cultures were measured by spectrophometer following pigment extraction in acetone. Average values for triplicate bottles of every condition are shown with plus and minus one standard deviation error bars.




In Figure 3, chlorophyll a concentrations in *Calothrix* cultures are reported over 5 days. Chlorophyll a concentrations are often used as an indicator of cell growth for cyanobacteria, so this plot provides insight into which conditions (Killed Control, Sunscreen -, and Sunscreen +) contained the highest abuncance of cyanobacteria over time. The average chlorophyll a concentration for the Sunscreen – cultures on day 1 stand out as being much greater than all of the other conditions and days, which could

indicate that the addition of the commercial sunscreen impeded the growth of the *Calothrix* over the course of a day in Sunscreen +, and then as the sunscreen degraded over the next several days, the Sunscreen + cultures were able to increase their chlorophyll a concentrations as the Sunscreen – cultures decreased their concentrations due to the effect of UV light exposure over time. However, sizeable error bars prevent these trends observed on average from being statistically conclusive.

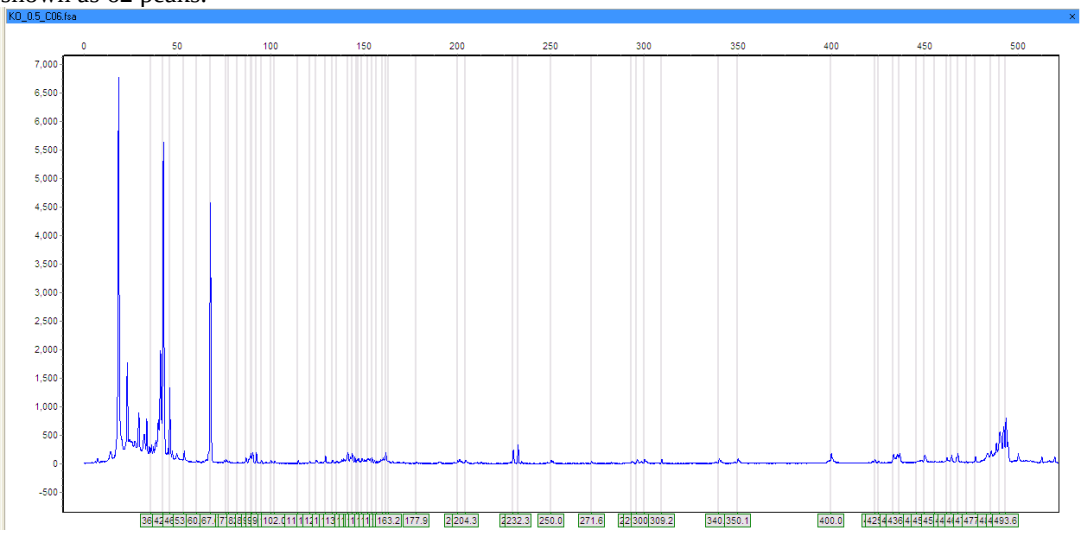
Figure 3. Chlorophyll a concentrations in *Calothrix* cultures were measured by spectrophotometer following pigment exctraction in acetone overnight. Average values for triplicate bottles of every condition are shown with plus and minus one standard deviation error bars.




Community

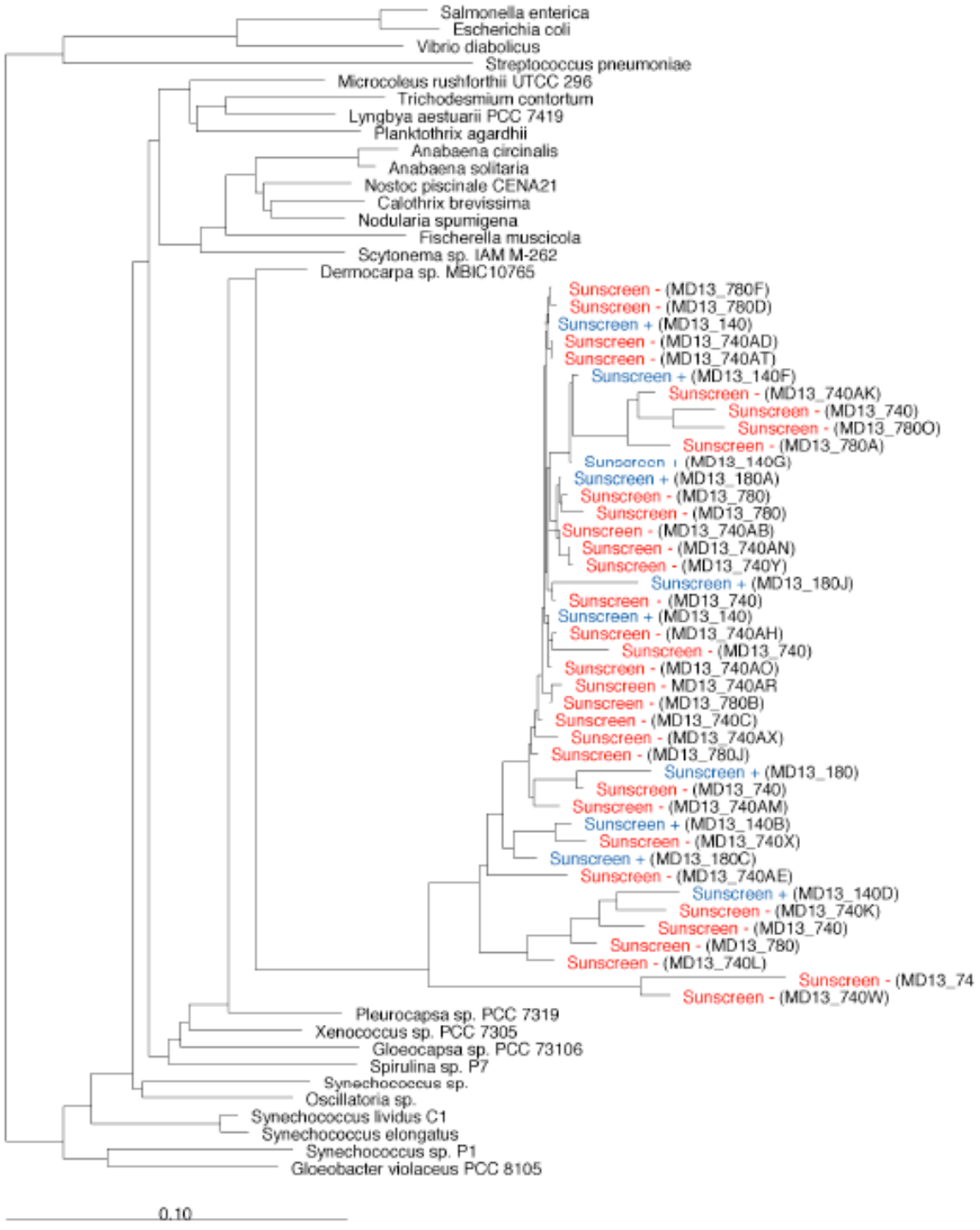
T-RFLP analysis of the beach sand sample yielded 62 operational taxonomic units (OTUs), which hints at the diversity of the bacteria present in the sample, as shown in Figure 4. It is difficult to make any quantitative statements, though, without having a clearer idea of what each OUT represents. The first most abundant fragment accounted for 28.9% of the overall abundance, and the second most abundant fragment accounted for 17.9% of the abundance, so it seems that these two OTUs were quite prevalent in the sample. Using MiCA III software, out of the 62 peaks, eight of them (129, 150, 152, 490, 491, 493, 494, and 495 were targeted as peaks that could possibly correspond to cyanobacteria fragments produced by various cyanobacteria.

Figure 4. T-RFLP chromatogram generated from beach sand sample with universal primers. OTUs are shown as 62 peaks.




The clone libraries made with cyanobacterial primers yielded only 20 pickable colonies for the Sunscreen + condition culture and 68 pickable colonies for the Sunscreen – condition culture. The phylogenetic tree created in Arb is shown in Figure 5. The tree shows that all of the clones clustered together, most closely to the Dermocarpa branch of cyanobacteria. An S-Libshuff analysis showed that there was no significant difference between the Sunscreen + cyanobacteria community and the Sunscreen – cyanobacteria sunscreen community, as might be expected from the lack of segregation of the clones from the different treatments on the tree. It is not surprising that there was no quantifiable shift in the communities as they were only exposed to natural sunlight under their treatment conditions for 4 hours. Since cyanobacteria do not have a very fast doubling time, this time period likely did not allow significant community shifting to manifest itself, as might have happened over a longer period of time.

Figure 5. This phylogenetic tree, generated in Arb, shows the clustering of all cyanobacterial clones with the other known cyanobacteria on the tree.



## Conclusions

Average trends suggest that sunscreen impedes photosynthesis in *Calothrix*, though not for a time period longer than 5 days after sunscreen addition. This study also indicates that sunscreen addition decreases the concentration of chlorophyll a and decreases the production of scytonemin over time periods less than 5 days in *Calothrix*. Due to high variation in triplicate tubes, these results can only be reported as observed trends, and not as statistically significant differences between the conditions. Due to the likely widespread occurrence of UV-filters in the environment already, the issue of sunscreens' effect on cyanobacteria merits further research, especially when potential impacts to global carbon cycling are taken into consideration.

## Acknowledgements

This work was supported by the MBL Associates Endowed Scholarship Fund, the Lola Ellison Robertson Endowed Scholarship, and STAR Research Assistance Agreement Numbers FP-916857, awarded by the U.S. Environmental Protection Agency. It has not been formally reviewed by the EPA. The views expressed in this document are solely those of the author, and the EPA does not endorse any products or commercial services mentioned in this publication.

The author would like to thank the 2008 MBL Microbial Diversity course directors William Metcalf and Thomas Schmidt, teaching assistants (especially Amy Apprill for her help with T-RFLP and use of the microrespirometer), and classmates for all of their assistance and support.

## References

1. **Balmer, M. E., H. R. Buser, M. D. Muller, and T. Poiger.** 2005. Occurrence of Some Organic UV Filters in Wastewater, in Surface Waters, and in Fish from Swiss Lakes, p. 953-962, vol. 39.
2. **Daughton, C. G., and T. A. Ternes.** 1999. Pharmaceuticals and Personal Care Products in the Environment: Agents of Subtle Change?, p. 907-938, vol. 107. The National Institute of Environmental Health Sciences (NIEHS).
3. **Dillon, J. G., and R. W. Castenholz.** 1999. Scytonemin, A Cyanobacterial Sheath Pigment, Protects Against UVC Radiation: Implications for Early Photosynthetic Life, p. 673-681, J. Phycol., vol. 35.
4. **Nubel, U., F. Garcia-Pichel, and G. Muyzer.** 1997. PCR primers to amplify 16S rRNA genes from cyanobacteria, p. 3327-3332, vol. 63.
5. **Poiger, T., H.-R. Buser, M. E. Balmer, P.-A. Bergqvist, and M. D. Müller.** 2004. Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere **55:**951-963.
6. **Sinha, R. P., and D.-P. Häder.** 2008. UV-protectants in cyanobacteria. Plant Science **174:**278-289.
7. **Sinha, R. P., M. Klisch, A. Gröniger, and D. P. Häder.** 1998. Ultraviolet-absorbing/screening substances in cyanobacteria, phytoplankton and macroalgae. Journal of Photochemistry and Photobiology B: Biology **47:**83-94.