Title
Colorful niches enable *in situ* enrichment of surface-attached phototroph communities

Author
Sean Kearney
Department of Biological Engineering, Massachusetts Institute of Technology
skearney at mit dot edu
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Abstract
Phototrophic organisms span the tree of life: within the Bacteria, phototrophic organisms are found in multiple, phylogenetically disparate groups including the *Proteobacteria*, *Chlorobi*, *Cyanobacteria*, *Chloroflexi*, *Firmicutes*, and *Acidobacteria*. The phylogenetic dispersion of this functional trait indicate that phototrophy represents a robust, oft-used, and apparently convergent metabolic strategy for organisms living in the light. Each wavelength of solar radiation represents a potential niche for phototrophic bacteria, and it is possible to target enrichments based on specific light utilization profiles. Here, we used narrow-spectrum LED illumination to enrich for surface-attached phototrophic communities *in situ*. In particular, we show that *in situ* phototroph enrichment is a reliable strategy for enriching organisms optimized for living in particular wavelengths of light, and that we can differentiate these communities using topographical, spectral, and morphological features. This method is a simple and effective means to conduct culture-independent profiling of phototrophic communities as they assemble in the environment.

Background
In aquatic environments, light access for phototrophic organisms can be an important niche determinant. The accessible wavelengths of light depend on the particularities of any given environment, but a cursory examination would suggest that the absorbance spectra of liquid water, dissolved organic and inorganic matter, and phototrophic organisms themselves limit light as an available resource. Coexistence between phototrophic organisms competing for a limited resource entails partitioning of along the axis of this resource -- for light, available wavelengths enforce such resource use specialization (May RM, MacArthur RH, 1972).

The wavelengths of light available in different aquatic habitats can predict the kinds of phototrophic organisms that will be present in such a habitat. In clear waters, such as the open ocean where green light penetrates deeply, red cyanobacteria can dominate (Stomp et al, 2007). As water increases in turbidity, green cyanobacteria tend to dominate. Deep in sediments or in other anaerobic environments an additional niche axis comes in to play, where organisms adapted phototrophy in low light and anoxic conditions arise. Bacteria found in these groups include the green and purple sulfur and non-sulfur bacteria, which utilize light in the far red and infrared (Dutton PL, Prince, RC, 1978). The spectral niches available for phototrophic organisms, then, may be predictive of the community structure of phototrophic organisms found in the wild.

Thus, knowledge of the spectral niches available to phototrophic organisms in any given environment can inform selective enrichment strategies for particular groups of organisms, or offer testable predictions about the organisms that are likely to inhabit a given environment. As discussed previously, three features are predictive of the underwater light spectra available in any given environment, determined by Lambert-Beer's Law (Kirk 1994; Stompe M et al 2007):

$$I(\lambda,z) = Iin(\lambda)exp(-[KW(\lambda)+KGT(\lambda)+KPH(\lambda)]z)$$

with $I(\lambda,z)$ the light intensity of wavelength λ present at depth z, $Iin(\lambda)$ the solar irradiance spectrum, and $KW(\lambda)$, $KGT(\lambda)$, $KPH(\lambda)$ the absorbance spectra for pure water, gilvin (dissolved organic matter) and tripton (inaminate particulate matter), and phytoplankton, respectively. With slight modification, the exponential attenuation of solar irradiance in an aquatic habitat can be directly measured using the absorbance spectra of water collected from that environment, call it $KENV(\lambda)$. Using this approach, we can then predict the light availability as a function of depth, and use this information to determine the likely outcomes of an enrichment within an environment with defined spectral characteristics.

Here, we develop an experimental approach to enrich for phototrophic communities in their native environmental contexts. Using watertight boxes with embedded LEDs, we enriched for phototrophic biofilms on glass microscope slides across a range of wavelengths ranging from green (550 nm) to infrared (950 nm). We show that anoxygenic phototrophic communities develop quickly in environments with low dissolved oxygen concentration and high concentrations of anoxygenic phototrophs, and that this phenomenon depends critically on depth and ecology of the site in question, where sites expected to have few anoxygenic phototrophs do not permit rapid development of biofilms. Further, we examine the morphological, topographical, and spectral characteristics of biofilms grown *in situ*, and conclude with results indicating that wavelengths for *in situ* biofilm enrichment strongly determine wavelengths necessary for *in vivo* enrichment.

Results
Short term light box enrichments yield robust phototroph biofilms
As a preliminary evaluation of the ability of LED-containing light boxes (photoboxes) to enrich for phototrophic communities *in situ*, a single photobox containing LEDs with 550 and 650 nm peaks (Fig 1B) was incubated at 20 cm depth in a seagrass depression containing visible plumes of sulfate-oxidizing bacteria on July 30, 2015 in Trunk River (Falmouth, MA) (Fig 1A). After 24 hours of incubation, visible biofilms were present on the slides directly in contact with the top surface of the light box -- slides at 2, 4, and 6 cm above this slide had no visible growth at this time point. The slide showing visible biofilm growth was removed and replaced with a clean glass slide for an additional two days of incubation. Following this enrichment, the entire photobox was removed, biofilms were immediately examined spectroscopically, prepared for light microscopic examination, and fixed for topographic examination via scanning electron microscopy.

To examine the development of biofilm growth under different wavelengths, additional photoboxes were deployed to Trunk River containing LEDs with peaks at 850 nm or 950 nm. At two days, slides containing biofilms were handled as before to examine the diversity of *in situ* biofilms via morphological and spectral characteristics (Figure 2). Across the four light sources, the biomass accumulation (determined by diameter of the biofilms) was greatest under illumination at 850 nm, followed by lower but relatively similar biomass accumulation for 650 and 550 nm, and the lowest accumulation at 950 nm.

Habitat-specific light accessibility determines the speed of biofilm development
The success of short-term enrichments in relatively shallow waters (20 cm) in Trunk River prompted us to examine whether depth within the river basin led to differential biofilm development. Light boxes containing 850 nm and 650 nm LEDs were deployed at three different depths (15 cm, 20 cm, and 30 cm) in the river with different light availability, as determined by measurement of total luminescent intensity in the river (Fig 3A). Following four days of incubation, each photobox was retrieved and examined for the formation of biofilms.

The photobox incubated at 15 cm depth exhibited biofilm formation extending nearly to the edge of the slide in both directions (25 mm) under 850 nm illumination on the basal slide, with a smaller biofilm (15 mm) under 650 nm illumination (Figure 3B). By contrast, the basal slide from the photobox deployed at the 20 cm depth had comparatively smaller biofilms (15 mm and 10 mm for 850 nm and 650 nm, respectively). The photobox incubated at a 30 cm depth had no visible biofilm development, and was left in the river for an additional two days before retrieval. At six days, minimal biofilm development occurred (3-5 mm), but removal of the biofilms from the device and disruption during transport prevented further analysis of these communities.

In situ *enrichments depend on the ecology of the enrichment site*
Illumination in red and infrared light generally restricts growth to anoxygenic phototrophs -- we therefore examined the dependence of these tools for *in situ* enrichments on the physical and chemical characteristics of the aquatic enrichment site. In particular, we deployed photoboxes into four aquatic environments representing diverse environments with varying euphotic depths. Briefly, the four sites included Trunk River, as described previously, School Street Marsh, a freshwater marsh, Eel Pond, which has saltwater and freshwater inputs, and Cedar Swamp, an acidic (pH 5.5) freshwater marsh. All devices were placed at a 20 cm depth in the different habitats, and left for two days prior to retrieval. Of the four sites, only Trunk River and School Street Marsh exhibited visible biofilm development after four days, and the spectral signature for each were qualitatively more similar for a given site than for a given wavelength (Fig 4). Taken together, these data suggest that ecology of the site in question dictates the success of the enrichment approach rather than the quality of light available.

In situ *enrichments dictate light preference in* in vitro *enrichments*
As a preliminary examination of the selectivity of the light sources for particular phototroph communities, we inoculated low biomass scrapings from slides of two day incubations in Trunk River illuminated with 650 nm and 850 nm into saltwater sulfur phototroph medium to approximate mineral salt concentrations similar to those found at this depth (**Materials and Methods**). Liquid cultures were then incubated at both 650 nm and 850 nm for six days (with continuous examination) to observe the growth of bacteria under these conditions. Cultures grew to turbidity only when the *in vitro* light source matched the *in situ* LED wavelength, whereas cultures incubated with mismatched light conditions did not show such growth (Fig 5A).

We examined the spectral characteristics of these cultures to determine whether the original biofilm inoculum exhibited spectral maxima matching those in the enrichment cultures. While the 850 nm culture showed evidence of bacteriochlorophyll a (800 nm and 830 nm peaks) characteristic of purple sulfur and non-sulfur bacteria, there were additional peaks unaccounted for in existing literature on purple sulfur bacteria, including maxima at ~950 nm and ~1100 nm (Fig 5B). These peaks may represent novel

bacteriochlorophyll reaction centers, which may be uniquely adapted to the light available in Trunk River. The culture grown at 650 nm was dominated by green sulfur bacteria, in particular, those having bacteriochlorophyll c with absorbance maxima at 760 nm (Fig 5B). One of the most dominant phyla present in the sulfur plumes present in Trunk River is Chlorobi, of which most sequences come from the genus Prosthecochloris, members of which are known to be halotolerant and sulfur-oxidizing, consistent with their appearance in saltwater sulfur phototroph medium (Dutton PL, Prince RC, 1978). We suspect that these organisms contribute to the yellow color in the sulfur plumes, and that the purple sulfur phototrophs from the *Gammaproteobacteria*, and particularly those in the family *Chromatiaceae*, representing the other dominant group of sulfur-oxidizing bacteria in this environment, and likely the primary contributors to the bacteriochlorophyll a peaks present in both *in vitro* and *in situ* enrichments. The presence of conspicuous morphological characteristics, namely phase-bright sulfur granules, corroborates the presence of these organisms.

Discussion

Using custom-made photoboxes, we managed to enrich for phototrophic biofilms in the brackish Trunk River basin in under 24 hours. The abundance of anoxygenic phototrophs in this environment likely contributed to the success of this approach, as examination of phototroph growth in two environments did not permit such rapid assimilation of surface-attached phototroph communities. The wavelengths used to enrich in these environments (650 nm and 850 nm) would favor for the growth of anoxygenic phototrophs and not oxygenic cyanobacteria and eukaryotic algae, which are likely to dominate in the clear waters characteristic of Eel Pond. However, this explanation does not fit with the differential growth of biofilms in School Street Marsh and Cedar Swamp, the latter showing no visible biofilm growth at 48 hours. Cedar Swamp is acidic (pH 5.5) compared to circumneutral School Street Marsh -- this chemical constraint could slow the accumulation of phototrophic biofilms, suggesting that *in situ* phototroph enrichments using this technique should take into account these environmental parameters in determining the length of incubation, appropriate wavelengths, and depth of the devices in any particular aquatic environment.

In the short term incubations in Trunk River, each biofilm yielded qualitatively similar spectral characteristics, but morphologically distinct communities as determined by light and scanning electron microscopy. There were, however, subtle differences in the observed spectral characteristics, which may reflect the differential abundance of various members of the phototrophic community present in Trunk River -- where dominant purple and green sulfur bacteria may chemotax toward the light and dominate the spectral signal before less abundant communities of organisms optimized to use the particular wavelengths present have accumulated.

Incubation of the photoboxes at different depths in Trunk River revealed that light accessibility and, by extension, the abundance of phototrophic organisms, determine the speed with which biofilms form in response to the externally supplied light. Particularly in light-limited environments, chemotaxis of organisms to the light and accumulation by immigration (rather than growth) may be the primary means by which biofilms develop *in situ*. In the future, experimental designs that consider the relative importance of immigration and *in situ* growth could be useful for understanding ecological dictates of biofilm formation. Using metagenomic or single-cell sequencing approaches, it may be possible to determine whether any given biofilm represents primarily the clonal outgrowth of an early colonizer or constant immigration and emigration with continuous

remodeling of the biofilm over time. Obtaining a more highly resolved temporal structure, for instance, by simultaneously incubating multiple devices in the same site and retrieving biofilms at different intervals could provide a powerful approach for examining successional patterns in phototrophic communities *in situ*. Similarly, the device itself could be multiplexed to a 96-well or 384-well format, where each well carries an LED light source as a way to do long-term incubation experiments or tests for reproducibility.

Though not pursued here, preparing clone libraries and designing FISH probes based on the *in situ* enrichments will provide a ground-truth of the ability of different wavelengths to enrich for specific community compositions as well as informing whether communities enriched *in situ* differ substantially from those obtained under simulated conditions in the lab. How much diversity is lost from the bottle effect or in extracting a community from its native environment? Can we predict based on metagenomics and applications of ecological theory, the organisms that will likely occupy these *in situ* culture devices, or do we instead skew the community towards rare members unimportant in the native environment? Results with the *in vitro* enrichments of the *in situ* biofilms suggest that the wavelengths used *in situ* bottleneck the communities, winnowing the active fraction to only those particularly adapted to high-light and narrow-spectrum light utilization. Perhaps *in situ* enrichment provides a filter by which we can capture organisms particularly relevant in their native environments, with less of an effect from culturing biases. Additionally, the miniaturization of the LED source used for enrichment means that we can attempt to enrich bacteria that may have exotic or understudied, but ecologically meaningful spectral characteristics -- namely, by using a broad range of wavelengths to cultivate organisms *in situ* in a way that would be prohibitive in a closed system or when organisms are present at a very low abundance in a community. Generally speaking, the *knowingly* biased approach of *in situ* enrichment allows us to specifically calculate the niche that we're creating, and directly measure the effects of a targeted perturbation on the ecology of microbes in the wild.

Materials and Methods

In situ culturing devices

Devices were constructed using Toyogiken TIBOX (DS-AT-0609, WHD 65 x 95 x 55 mm) and AAA plastic battery cases housing four 1.5 V batteries. Briefly, holes to house LEDs were drilled 25 mm apart into clear plexiglass and mounted on the underside of the clear plastic lid. Circuits included one 100 Ohm resistor connected in series with two 5 mm LEDs, including Infrared 950 nm, Infrared 850 nm, Super Bright Red (650 nm) or Super Bright Green (550 nm) (sparkfun.com). Housing for glass slides was designed in Tinkercad (tinkercad.com) and printed on a MakerBot 3D printer with PLA filament.

Sampling Sites

Sites were visited during July and August 2015. Trunk River (41°32'06.2"N 70°38'28.0"W, Falmouth, MA), a brackish water basin with an active sulfur cycle was the primary site for enrichment of phototrophic bacteria. In particular, depressions in the seagress led to plumes of sulfur-oxidizing bacteria, a promising site for enrichment of purple and green sulfur and non-sulfur bacteria. For site comparison, Cedar Swamp (41.526894, -70.654151), School Street Marsh (41.525983, -70.666551), and Eel Pond (41.526201, -70.670370) were used as alternate environments for *in situ* enrichment of phototrophs. Slides were transported in site-specific water inside sealed glass slide containers.

Spectrophotometry
All spectra were obtained using the Spectral Evolution SR-4500 device and DARWin SP Data Acquisition software. Spectra thus obtained were analyzed using custom R scripts (http://cran.r-project.org). Inference of light availability as a function of depth were made using absorbance spectra of water obtained from different sites assuming exponential attenuation of light with depth.

Light Microscopy
Freshly obtained biofilms were imaged directly or by wet mount imaging of a biofilm scrape using a Zeiss Axio Imager M2. For visualizing nucleic acids, samples were incubated in DAPI for 20 minutes and viewed using the DAPI filter set (Ex/Em: 358/461 nm) using manufacturer's protocol. To examine cell wall material, biofilms were additionally incubated with Wheat Germ Agglutinin Alexa Fluor 647 Conjugate (Ex/Em: 650/665 nm) using manufacturer's protocol. Samples were scanned for autofluorescence under additional channels to identify cyanobacteria and eukaryotic algae present in samples. Images were processed using Fiji (http://fiji.sc/Fiji).

Enrichment Cultures
Biofilm inocula were scraped from glass slides following incubation and added under N2 stream into Pfennig bottles filled completely with anaerobic Saltwater Sulfur-Phototroph Medium (SI Appendix). Enrichment cultures were incubated 5 cm from light sources (650 nm red light and 850 nm infrared), and examined periodically for evidence of growth.

SEM
Glass slides recovered from the lightboxes were immersed in water from sampling site for transportation. In lab, slide were fixed by immersion in 1.5% (v/v) glutaraldehyde in PBS for 2 hours followed by dehydration in ethanol series (25%, 50%, 75%, 100%) for 20 minutes each step. Using a diamond knife, biofilms directly fixed to glass slides were cut into 1 cm x 1 cm squares and dried in a Tousimis Critical Point Dryer followed by sputter coating with a 10 nm coat of platinum in a Leica EM ACE Coater. Samples were visualized using a Zeiss Supra 40VP Scanning Electron Microscope.

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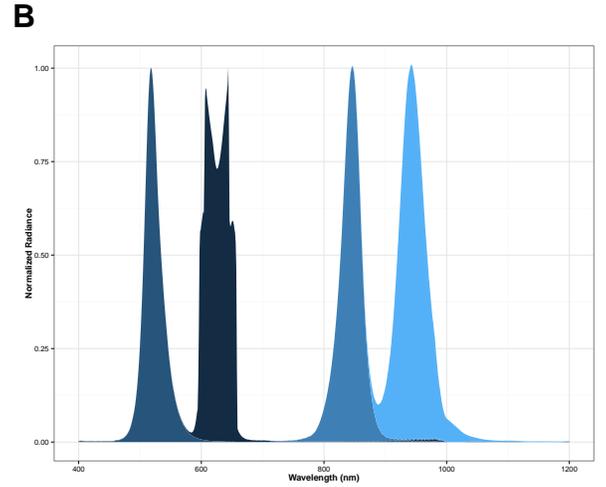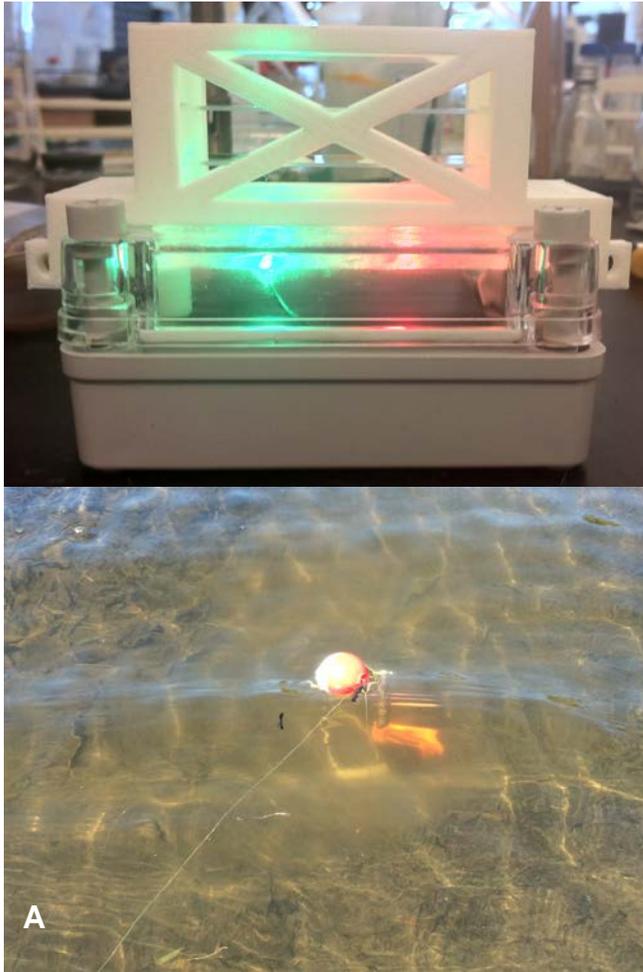
Appendix

Figure 1. Trunk River is the testing ground for *in situ* phototroph enrichments. (A) LED-containing photobox -- sideview (top) and sulfur plume in Trunk River for photobox incubation (bottom) (B) normalized irradiance for the four LEDs tested in photoboxes.
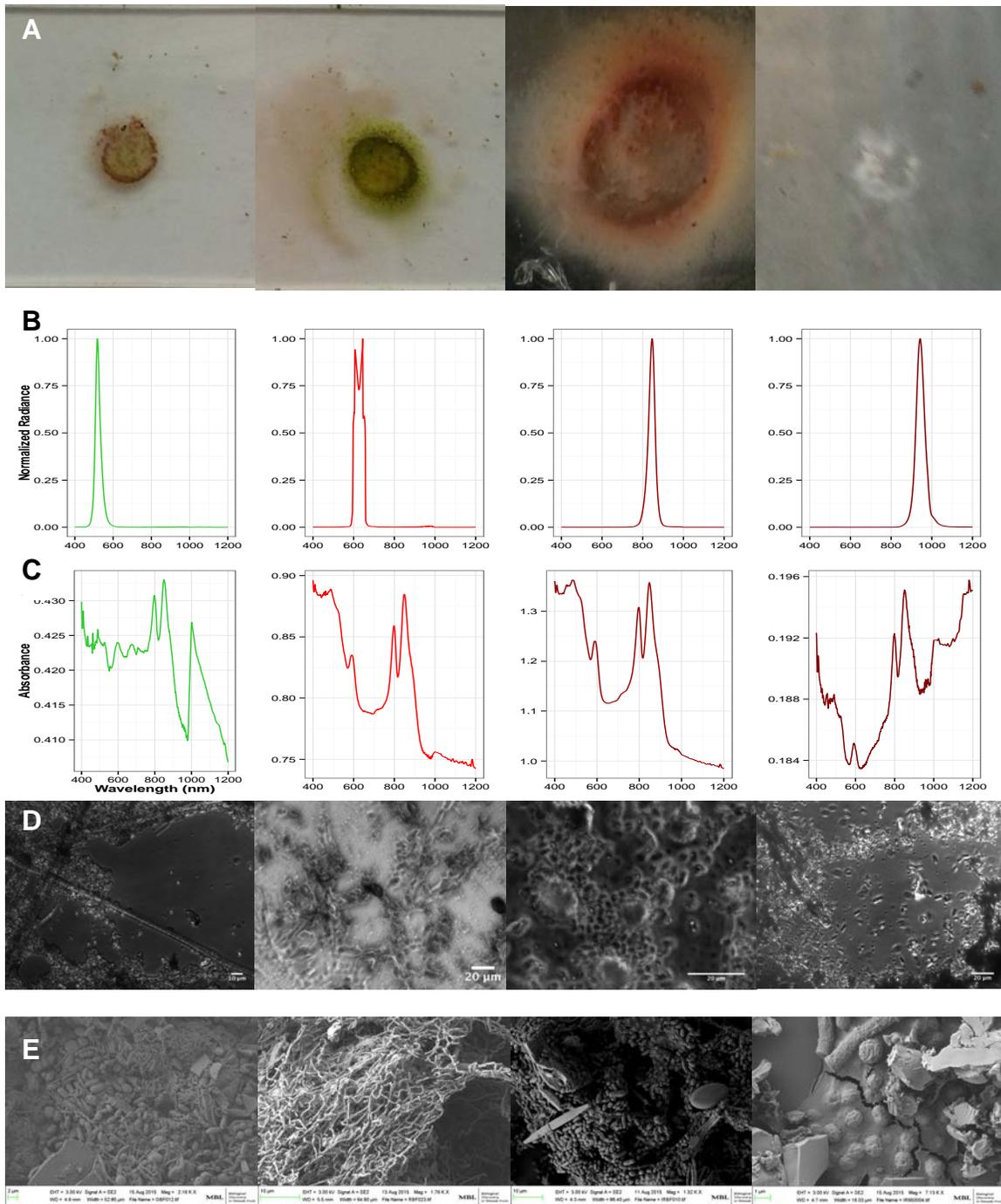
Figure 2. Phototrophic biofilm communities vary morphologically, spectrally, and topographically across wavelengths. (A) Two-day biofilms for each wavelength, (B) LED sources (green, red, IR850, IR950), (C) spectral characteristics of each biofilm, (D) selected phase images, and (E) selected SEM images.
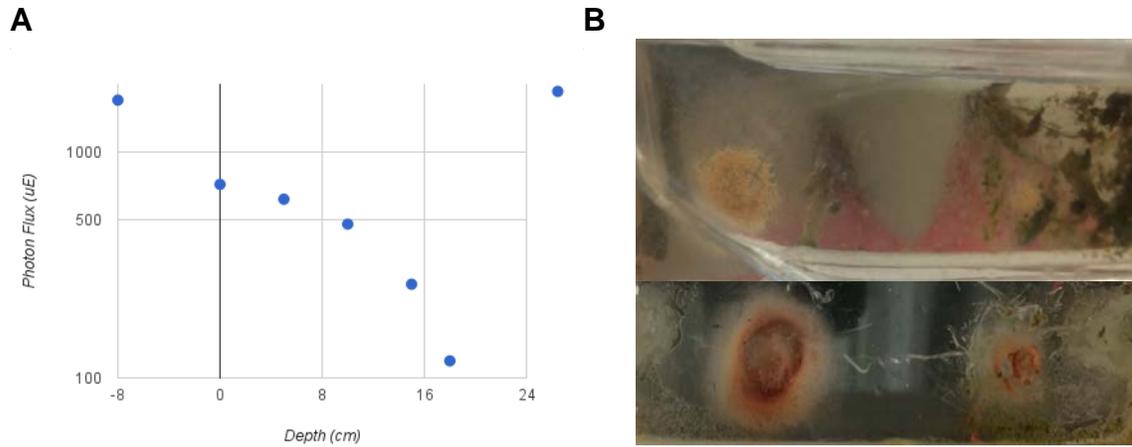
A

B

Figure 3. Luminescent intensity attenuates exponentially with depth in Trunk River. (A) Measured light intensity (µE) in different sites in Trunk River as a function of depth, (B) Biofilms obtained at 15 cm (top) and 20 cm (bottom) in Trunk River.
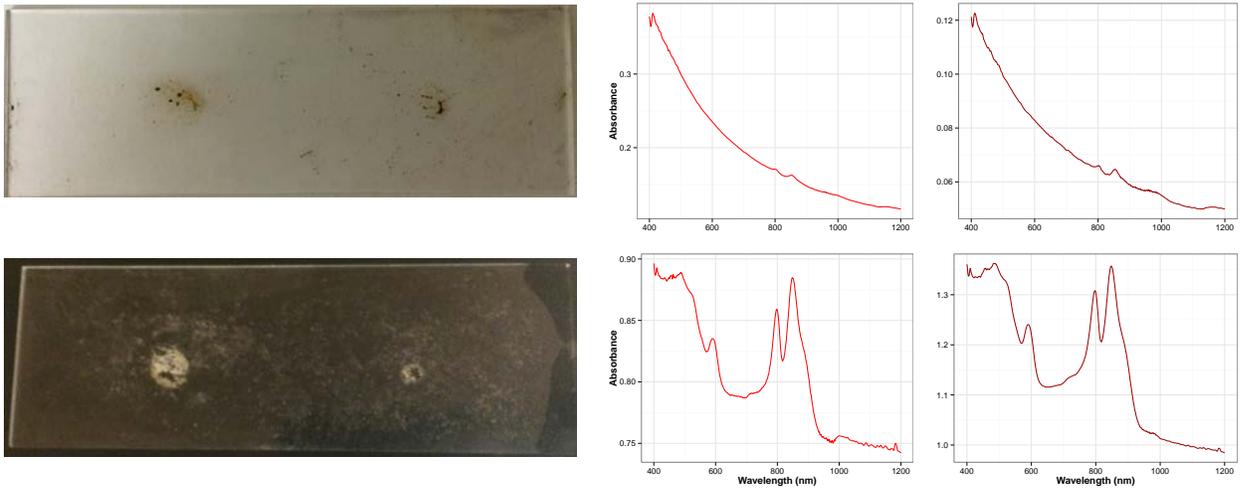
Figure 4. Spectral characteristics vary more by site ecology than by wavelength. (top) School Street Marsh biofilms (bottom) and Trunk River biofilms with accompanying absorbance spectra.
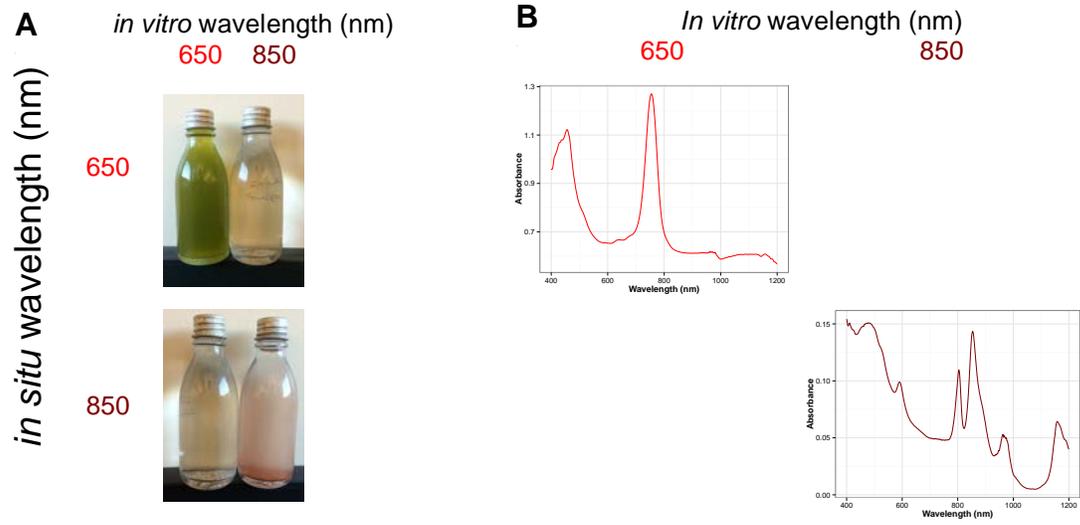
Figure 5. *In situ* enrichment wavelengths restrict *in vitro* enrichment cultivations. (A) Liquid cultures of enrichment cultures inoculated with *in situ* biofilm enrichments and (B) corresponding wavelengths.