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Shedding light on spatial structure and dynamics in phototrophic biofilms

Freddy Bunbury, Amanda N. Shelton and Devaki Bhaya

Abstract

Microbial phototrophic communities dominated early Earth and thrive to this day, particularly in extreme environments. We focus on the impact of diel oscillations on phototrophic biofilms, especially in hot springs, where oxygenic phototrophs are keystone species that use light energy to fix carbon and often nitrogen. They exhibit photo-motility and stratification, and alter the physicochemical environment by driving O_2, CO_2, and pH oscillations. Omics analyses reveal extensive genomic and functional diversity in biofilms, but linking this to a predictive understanding of their structure and dynamics remains challenging. This can be addressed by better spatiotemporal resolution of microbial interactions, improved tools for building and manipulating synthetic communities, and integration of empirical and theoretical approaches.

Addresses

Carnegie Institution for Science, Biosphere Sciences and Engineering, Stanford CA 94305, United States

Corresponding author: Bhaya, Devaki (dbhaya@carnegiescience.edu)

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Introduction

"These microscopic organisms form an entire world composed of species, families and varieties whose history, which has barely begun to be written, is already fertile in prospects and findings of the highest importance." Louis Pasteur In a paper read to the Académie de Médecine (March 1878).

Phototrophic (light-dependent) microbial communities were major life forms on early Earth [1]. Today, colorful, stratified phototrophic biofilms (often called microbial mats) thrive in extreme environments such as hot springs, desert crusts, and hypersaline habitats [2]. Phototrophs play a crucial role by fixing carbon and often nitrogen to support themselves and heterotrophs in the community [3].

Microbial mats in hot springs around the world, particularly in Yellowstone National Park, USA, have been studied extensively using traditional tools of environmental microbiology such as microscopy, 16S rRNA fingerprinting, and *in situ* measurements coupled with enrichment and isolation of species. These studies defined key and unique features of phototrophic biofilms and fueled the discovery of novel microbial phyla and enzymes [4]. High-throughput metagenomics tools have revealed the unprecedented genetic and functional diversity of the microbial world [2]. The ongoing challenge is to complement detailed profiles of communities ("*Who's there*?") with an understanding of the interplay between spatial architecture, dynamics of microbial activity, and their metabolic interactions ("*What are they doing?*").

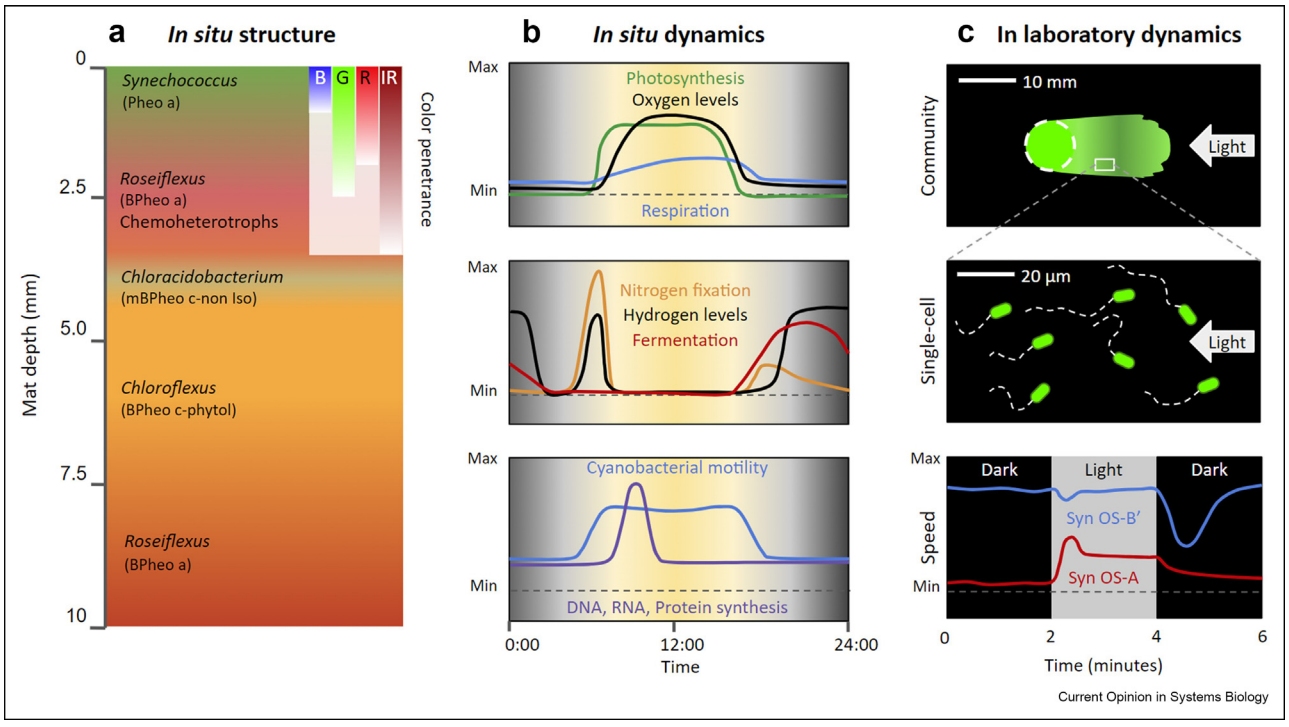
Phototrophic biofilms are subject to daily oscillations in light energy input resulting in dynamic microbial interactions. We need a systems-level and predictive understanding of how these microbial communities are maintained under fluctuating conditions. This is important from an ecological or evolutionary perspective and can also inform the emerging field of synthetic ecology. In this brief review, we focus on how diel light dynamics influence spatial stratification and community behaviors in phototrophic mats (primarily in hot springs). Integration of *in situ* microsensor data, metaomics analyses, and analyses of microbial isolates and synthetic communities are providing new insights into community function. We highlight how emerging techniques with increased spatial resolution, advances in laboratory manipulation of microbial communities, and predictive mathematical models have a role in the future of this field.

Dynamic features of phototrophic biofilms

Light controls biofilm structure

Light is a primary driver of spatial behaviors in phototrophic biofilms. Both light intensity and spectral quality change dramatically with depth in the biofilm due to absorption by pigments such as chlorophylls, which peak at 450 and 675 nm [5] (Figure 1a). Cyanobacteria that contain chlorophyll *a* are found in the top millimeters of biofilms in alkaline hot springs. At greater depths, they

Figure 1



Structure and dynamics of high-temperature phototrophic mats in Yellowstone National Park. (**a**) Depiction of a 1 cm deep microbial mat in a hot spring runoff channel at 65°C. The most abundant genera are labeled by depth with the major diagnostic chloropigment of the genus in brackets (Pheo = Pheophytin, BPheo = Bacteriopheophytin, mBPheo-non Iso = methylated Bacteriopheophytin esterified with non-isoprenoid alcohol, Bpheo c-phytol = Bacteriopheophytin esterified with phytol). Light attenuation of various wavelengths is indicated in the top right corner (B: blue, G: green, R: red, IR: near-infrared). Adapted from [9]. (**b**) Depiction of community activities and gas levels associated with them over a diel cycle in a Yellowstone hot spring mat using microsensor, metabolomic, and transcriptomic data [10,13,15,16] and Shelton et al. in prep. (**c**) Depiction of a colony of Synechococcus moving towards white light (top). A magnified view of individual cells with their recent movement history indicated by tracks (middle). Average speeds of phototactic cells from two species (Syn OS-A and Syn OS-B′) of Synechococcus under dynamic illumination conditions (from dark to light to dark) (bottom) [29].

have adapted to utilize near-infrared light by synthesizing chlorophyll *f* [6]. Anoxygenic phototrophs containing bacteriochlorophyll *a* (peak absorption at 800 and 870 nm) and heterotrophs also coexist at this depth [5,7,8]. Mass spectrometry (MS) imaging of pigments has been used to spatially map the transitions from oxygenic *Synechococcus* sp. to anoxygenic phototrophs such as *Roseiflexus* sp., *Chloracidobacterium* sp., and *Chloroflexus* sp. with increasing depth within an alkaline hot spring mat. Unlike hyperspectral imaging and fluorescence microscopy, this technique can identify a variety of biomolecules, and revealed that cyanobacteria nearest the surface synthesized more monounsaturated glycolipids to cope with high light [9].

Diel regulation of photosynthesis and metabolic processes

Optimal growth in phototrophic biofilms, where light intensity can fluctuate from limiting to damaging, requires regulation of the photosynthetic apparatus or movement into a suitable light environment (see motility section). Using RT-qPCR on time series samples from microbial mats has demonstrated that cyanobacteria regulate their gene expression such that transcripts for photosynthesis-related proteins rise at dawn, are maximal at midday, and decline over the afternoon [10]. Anoxygenic phototrophs, which can use infrared light for photosynthesis, transcribe light-harvesting genes at night [11,12]. Enzymes required for metabolic processes such as respiration and fermentation also exhibited diel oscillations with peaks in the afternoon and night, respectively [7,13]. Although a few studies have extended these gene-specific observations by conducting metatranscriptomic studies of phototrophic biofilms over a diel cycle [12], disentangling the interactions between community members and regulation of diel processes remains a challenge. Our knowledge of global transcriptional regulators has been primarily based on lab-based studies with model cyanobacteria. The cyanobacterial circadian clock controls entrainment by linking oscillations to photosynthesis through metabolic processes rather than direct

entrainment by light using sensory photoreceptors, as is more common in eukaryotic organisms [14]. Whether circadian clocks are common regulatory systems in other phototrophs has not been demonstrated, nor is there a complete understanding of how cell division and replication are regulated in these communities and if they are coordinated in response to diel oscillations in all taxa.

Light-driven O_2 dynamics

Oscillations in light intensity drive oscillations in photosynthetic O_2 production, which peaks around midday, creating hyperoxic conditions in the mat, low CO_2 levels, and resultant high pH [15] (Figure 1b). Hyperoxia increases photorespiration and glycolate excretion by cyanobacteria, which is taken up by filamentous anoxygenic phototrophs [16]. In the afternoon and night, the microbial mat becomes increasingly anoxic as O_2 consumption by aerobic respiration surpasses oxygenic photosynthesis. As O_2 drops further, cyanobacterial fermentation of stored glycogen releases ethanol and organic acids such as lactate, formate, and acetate [16]. Expanding on earlier work using oxygen microsensors, Kuhl et al. used a novel O_2-sensitive nanoparticle paint to record O_2 dynamics in a beach rock biofilm with high spatial resolution and calculate rates of respiration and photosynthesis [17]. Cyanobacteria also release organic carbon as extracellular polysaccharides, which can be available for uptake by other organisms. Still, nano-scale secondary ion mass spectrometry (Nano-SIMS) measurements have shown that a large proportion is re-assimilated [18].

Major anoxic/anoxygenic processes

Anoxygenic photosynthesis (AP) by Cyanobacteria and Chloroflexota also contributes to diel changes in phototrophic biofilms. Microsensor measurements of O_2 and H_2S and mathematical modeling in a cold sulfidic spring indicated that oxygenic and anoxygenic photosynthesis - in cyanobacteria that could do both - could regulate each other by converging on the plastoquinone pool [19]. Chloroflexota in hot springs perform AP and contribute to CO_2 fixation via the 3-hydroxypropionate (3-OHP) pathway during the early morning hours, as indicated by metatranscriptomics and ^{13}C bicarbonate incorporation in Chloroflexota 3-OHP enzymes [20,21].

Many Cyanobacteria in phototrophic mats can fix nitrogen, though the timing of peak activity varies between microbial mat types [3]. In hot springs dominated by filamentous cyanobacteria (*Mastigocladus* sp.) with specialized heterocysts that protect nitrogenase from oxygen, maximal N_2 fixation occurs even under hyperoxic conditions in the early afternoon [22]. In mats with abundant unicellular cyanobacteria, N_2 fixation peaks in the early morning or overnight when O_2 levels are low [13,23]. Measurements of active nitrogen uptake using Nano-SIMS and gene expression by RT-qPCR have revealed that genetic potential for N_2 fixation may not be correlated with *in situ* activity and that Cyanobacteria contributed more to N_2 fixation than sulfate-reducing bacteria in a hypersaline mat [23]. Thus, an accurate assessment of the dynamics of how microbial mats respond to their environment will require a combination of multiple techniques.

Light-driven motility *in situ* and in isolates

Phototrophic mats may be stratified, but they are not static. Manipulating light intensity in a coastal mat from 75 to 1000 $\mu mol/m^2/s$ resulted in the downward migration of filamentous cyanobacteria by 0.25 mm, as measured indirectly by oxygen evolution [24]. Cyanobacterial isolates from hot spring mats have different optimum light intensities for growth and are found at different depths [7,25]. Light-driven motility may alter their light exposure to optimize photosynthesis while avoiding high light stress. Our knowledge of photoreceptors and signal transduction cascades that control phototactic responses is based on the behaviors of a few model cyanobacteria [26]. However, recent exploration with wild isolates demonstrates the diversity of photoresponses [27]. For instance, motility analysis using single-cell tracking algorithms revealed that two dominant isolates (*Synechococcus* sp.) from Yellowstone hot springs differed in their directions of phototaxis at different light intensities and wavelengths (Figure 1c) [28,29]. When these species were mixed together, they developed reliable collective motility patterns that were not entirely predictable from their individual behaviors [29].

Current challenges and new approaches

Techniques to improve the spatial resolution of microbial interactions

Metagenomics provides abundant sequence data, but lacks spatial information. Metagenomic plot sampling by sequencing (MaPS-seq), which involves fixing a sample in a gel matrix, cryo-fracturing it, encapsulating microbes from fractured particles into barcoded microdroplets, and sequencing the recombined pool, allows spatial associations between species to be identified [30]. Traditional microscopy approaches, by contrast, have high spatial resolution but lack sequence-specific information; fluorescence *in situ* hybridization (FISH) partially overcomes this by using fluorescent DNA probes to locate microbes within complex biofilms. Improvements to FISH to increase the number of probes include 1) CLASI-FISH (combinatorial labeling and spectral imaging-FISH) where species are identified by unique combinations of probes [31], and 2) parseqFISH (parallel sequential FISH) which uses secondary probes with reversible binding to allow sequential imaging of many transcripts [32]. Environmental chemistry has been studied by mass spectrometry imaging to identify the locations of biomolecules and

species in mats [33], Nano-SIMS and Raman microspectroscopy with stable isotope probing (RMCS-SIP) to visualize nutrient uptake and transfer between individual cells [34,35], and luminescent sensor nanoparticles to provide spatiotemporal information of O_2 levels (Figure 2) [17].

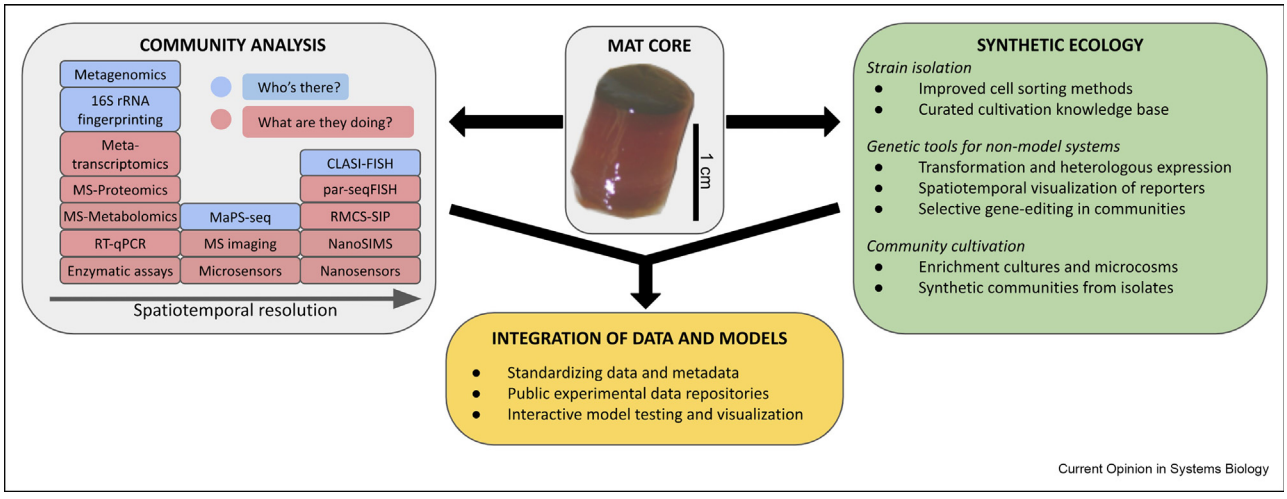
Bringing the mat community into the lab

A complementary approach to studying microbial communities *in situ* is to query synthetic communities under controlled conditions (synthetic ecology) (Figure 2). Strain isolation relies on selective growth conditions or physical separation [36]. Recent developments in growth-based selection include microfluidics-based or membrane-based culturing to increase throughput via miniaturization [36]. In terms of physical separation, live-FISH using fluorescent DNA probes and fluorescence-activated cell sorting (FACS) can separate cells based on 16S rRNA sequence, while Raman-activated cell sorting (RACS) of deuterium-labeled cells has been used to separate active from inactive cells [37,38]. Genome-informed media optimization techniques and curated online databases of media and culturing techniques can inform strain isolation and maintenance [39,40]. Certain microbes, such as anammox bacteria, are particularly difficult to isolate; however, stable enrichment cultures have proven valuable for uncovering their physiology [41]. Community gene editing tools such as species-targeted antibiotic resistance markers could improve isolation efforts by selection [42]. In simplified heterotrophic biofilms, fluorescent reporter genes have been used for understanding swarming, spatial segregation, and transcriptional responses to dynamic changes in pH [43,44]. Targeted gene knockouts and mutant screens have also been used to better understand polysaccharide secretion and motility, activities that impact community assembly and structure [45,46]. Progress is also being made in non-model organisms, such as thermophiles, using thermostable Cas-9 variants [47].

Integrating experiments and models

Developing mathematical models can help capture the dynamic interactions in phototrophic biofilms (Figure 2). This will be improved through data sharing by experimentalists (e.g., curating data on Github, Kaggle, or Figshare), developing universal data and metadata standards [48], and by modelers creating user-friendly interfaces for exploring model predictions (such as on Rshiny or Python Dash). Several mathematical models with applications in predicting phototrophic biofilm function have been developed. A genome-scale metabolic model and flux-balance analysis was used to predict growth and photosynthetic rates of two cyanobacteria exposed to different light spectra with reasonable accuracy and suggested how the changing activity of electron transport components would facilitate spectral acclimation [49]. Menon et al. (2021) compared the use of cellular automaton, reaction-diffusion, and active-matter models for modeling the movement of cyanobacterial populations [50]. They highlight the advantages of active-matter models in capturing both direct intercellular interactions via pili and indirect interactions through secreted compounds. Other types of individual-based models, such as rough surface patch models, have been used to predict the physical, chemical, and biological conditions in desert biocrusts over a diel cycle [51]. Despite needing to use multiple parameters from other

Figure 2



Evolving technologies for studying phototrophic biofilms. Left: various measurement methods used to interrogate what organisms are present (blue) and their activity *in situ* (red), with increasing spatiotemporal resolution from left to right. Right: methods used to cultivate and interrogate organisms enriched and isolated from biofilms. Bottom: recommendations for improving the use of mathematical models in biofilm systems.

microbial systems, the model predictions of O_2, CO_2, and pH dynamics as a function of time and depth agreed quite well with measurements from several independent studies on desert biocrusts [51]. We expect that the combination of data-rich omics and imaging technologies with mathematical models will increasingly be applied to microbial mat communities.

Conclusion

Our understanding of spatiotemporal dynamics and interactions in phototrophic biofilms has benefitted from observations and measurements *in situ*. To develop a systems-level understanding of these fascinating and environmentally important communities, deploying new imaging and omics technologies, developing synthetic communities, and combining these results with mathematical modeling will be critical. Coupling improved *in situ* understanding of diel dynamics in microbial mats to bulk genomics data could also lead to a greater appreciation of the role of microdiversity, horizontal gene transfer, and predators (including viruses) in microbial communities.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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