PHYCOSOMAL DYNAMICS IN XENIC CULTURES OF THE ALKALITOLERANT GREEN MICROALGA CHLORELLA SP. SLA-04

by

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DEDICATION

I dedicate this dissertation to my family, who have been right by my side through all of the wonderful and horrible years that comprised my time in graduate school. They ask me to lean on them when I need it. And they give me the opportunity to grow on my own. Thank you so much for all of the help, pep talks, baked goods, bumper crops, mule deer, impromptu meals, and much more. My mom and dad are amazing and have never wavered in encouraging me to go for it. As my dad says, “If we had some ham we could have some ham and eggs if we had some eggs”. It makes sense and it doesn’t. To my sister Maria, who continuously shows me how to tackle the difficult things in life. We need to remember to celebrate, too. You and Zach bringing Zoe into the world has meant pure joy for all of us. And to my sister Morgan. In her life she taught me compassion and unconditional love. In her death I learned about the importance of balance. She was and is my role model.

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ABSTRACT

The production of microalgal biomass and biofuel is an important component of the transition away from a petroleum-based economy. Industrial scale microalgal cultures are often xenic, meaning they are comprised of microalgae as well as a phycosome (*i.e.*, microbiome). The microalgal field has begun to appreciate the ubiquity and potential influence of the phycosome, but there remains a critical need for comprehensive research to unravel the intricate metabolic and ecological relationships between microalgae and the respective phycosome that can be comprised of mainly bacteria but also other microorganisms (*i.e*., archaea, fungi, protists, viruses). Phycosome research is essential for potentially using these interactions to enhance the stability, productivity, and cost-efficiency of industrial microalgal cultivation. *Chlorella* sp. SLA-04 is an oleaginous, alkalitolerant microalga isolated from the alkaline Soap Lake (Washington, USA). Under alkaline conditions, SLA-04 can be grown to high biomass levels without reliance on the delivery of concentrated CO_2, an improvement in producing competitively priced biomass and biofuel. The high pH, high alkalinity systems are able to capture CO_2 directly from the air in open systems (*e.g*., raceway ponds) but the open systems can be dynamic in terms of stability and productivity. Despite growing knowledge of the importance of phycosomes in open production systems, little is known about how alterations to cultivation conditions can be used to maintain a xenic system with controllable outputs, especially under high pH, high alkalinity conditions. The work outlined in this dissertation employed long term temporal community studies, open outdoor raceway experiments, diel-cycle-resolved temporal sampling coupled with activity-based probing (bioorthogonal non-canonical amino acid tagging (BONCAT)), and quantitative measures of algal physiology to better understand the relationship between microalgal phenotype and the respective phycosomes. SLA-04 phycosome composition and culture physiology were consistent over time when maintained in xenic cultures under low and high alkalinity. When xenic cultures were used in successive open, outdoor raceway experiments, compositional community changes coincided with seasonal temperature and light shifts, providing evidence that abiotic and biological environmental stresses impact directly and indirectly SLA-04 productivity and phycosome composition. By employing temporally resolved sampling and probing the relationship between diel-cycle-dependent metabolism and the phycosome, we identified active bacterial populations that may play a role in culture productivity. Expanding beyond augmenting SLA-04 productivity, aggregation of xenic cultures was assessed as a quantifiable phenotype, uncovering a relationship between aggregation, taxonomic composition and algal growth conditions (*i.e.,* alkalinity level). All together, these results represent an initial description of the ecology (*e.g*., composition, succession, activity) of alkaline microalgae cultures and provide methodology and perspective for future phycosome studies.

INTRODUCTION

Between 2000 and 2015, substantial resources were dedicated to expanding microalgal biotechnology [1], yet the industry struggled to compete with established alternative feedstocks. Despite the waning enthusiasm of the early 2000s, microalgal biomass and biofuel research remains crucial. Over the past two decades, significant progress has been made in research and development, but scientific advancements alone likely cannot overcome an economy entrenched in petroleum. For microalgae to compete, policy changes were necessary, creating economic incentives to support green energy, enticing governments and industries historically reliant on oil to invest in sustainable alternatives. In recent years, a surge in public and private sector funding [1,2], motivated by either political gain or environmental necessity, has revitalized the field, ushering in another promising era for the microalgae industry.

Even though electric vehicles and sustainable electricity sources like wind, solar, and hydro power have dominated the recent expanse of green energy, high energy liquid fuels remain indispensable for aviation and large-scale transport [3]. In 2021, 300 airlines comprising 83% of global air traffic committed to achieving carbon neutrality by 2050, despite lacking a clear route to achieving this monumental goal [2]. To contribute to the shift away from petroleum-based jet fuel, the Bioenergy Technologies Office (BETO) of the Department of Energy outlined the Sustainable Aviation Fuel Grand Challenge Roadmap [2]. Herein ambitious targets were set: 3 billion gallons of sustainable aviation fuels (SAFs) produced by 2030 and 35 billion gallons by 2050. In 2021, the United States produced 5 million gallons of SAFs, a figure that tripled to 15 million in 2022, largely thanks to bioethanol production [2]. The incorporation of a range of feedstocks, including microalgae, is essential to continue this growth and to reach carbon neutrality

goals. Advances in technology such as reactor and media design, new strain development, and wastewater utilization necessitate a better understanding of the fundamental physiology and ecology of these systems.

The goal of this dissertation was to contribute to the understanding of how ecology impacts microalgal biomass productivity, focusing on ecology under high pH and/or high alkalinity conditions. The aims of the four primary chapters were: 1.) Chapter 2: characterize the phycosome of *Chlorella* sp. SLA-04 in low and high alkalinity maintenance cultures used as inoculum for experiments over multiple years; 2.) Chapter 3: assess how phycosome succession and environmental conditions impacted productivity in open outdoor raceway SLA-04 experiments; 3.) Chapter 4: characterize activity in SLA-04 cultures in relationship to algal light and dark cycle metabolism; 4.) Chapter 5: develop new workflows to screen co-culture growth and investigate aggregation as a phenotype in xenic SLA-04 cultures. While working to achieve these goals, many new directions emerged, encompassing both fundamental and applied science.

The SLA-04 maintenance cultures detailed in Chapter 2 (the first research chapter) served as xenic inoculum for the subsequent chapters. This allowed us to explore hypotheses concerning the influence of phycosome composition on culture productivity when exposed to various biological and abiotic environmental pressures. In Chapter 3, cultivation scale increased to 200 L in open, outdoor raceway experiments. Here, we illustrated how the phycosome community changed over time during seasonal weather changes, concluding that declining productivity resulted from the combined impact of multiple environmental stresses. In accordance with the scope of Chapter 2, we anticipated that an outdoor experiment initiated with axenic inoculum would undergo distinct processes of succession, assembly, stress responses, and, ultimately,

reduced productivity. In Chapter 4, we conducted growth experiments comparing LA and HA axenic and xenic culture physiology, as well as phycosome activity during the diel cycle using bioorthogonal non-canonical amino acid tagging and fluorescence activated cell sorting (BONCAT-FACS). These experiments were conducted using the UT LA and HA cultures described in Chapter 2. We went further than community taxonomic characterization by defining diel cycle-dependent trends in phycosome activity. Finally, in Chapter 5, we attempted to look beyond impacts on growth, assessing relationships between SLA-04 and the phycosome. By measuring the influence of single isolates and phycosome communities on SLA-04 aggregation, we established a new direction for designing, or selecting for, beneficial SLA-04 phycosome communities.

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CHAPTER ONE: PHYCOSOME ENGINEERING TO IMPROVE INDUSTRIAL MICROALGAE CULTIVATION AT HIGH PH, HIGH ALKALINITY

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Abstract

Algal biomass production for chemical feedstock and biofuel sources continues to grow as an active area of research in academia, government, and industrial sectors, and algal-bacterial interactions are ubiquitous to both natural and industrial systems. These microbial interactions can range from beneficial to commensal to antagonistic, with direct and indirect implications on stable and consistent bioprocessing. More recently, the characterization of these interactions has been re-invigorated with realized appreciation of microbiomes in general and the potential applications to bio-system health/productivity. However, research is needed to better understand the biosystems in terms of metabolic and ecological interactions between algae and associated microbiomes (*i.e.*, phycosome) and how these interactions can be controlled to significantly improve stability, productivity, and cost-effectiveness. Moreover, the use of high pH and/or high alkalinity to alleviate CO_2 limitations during industrial microalgal cultivation has garnered much attention, and the combination of high pH/alkalinity likely constrain microbial community structure and function in unique ways. Therefore, research is also needed to determine how the combinations of high pH and alkalinity constrain microbial interactions for maximal light-driven, carbon and nutrient processing, particularly in the context of optimal algal outputs and community stability. The review will highlight current knowledge and gaps as well as suggestions for near-term and longer-term improvements for large-scale cultivation and polyculture engineering.

Motivations and Challenges for the Microalgal Industry

Replacing petroleum-based transportation fuels with microalgae-based biofuels will increase energy security and reduce fuel cycle carbon emissions, and microalgae provide several

advantages over terrestrial plants as a renewable energy or chemical feedstock. First, high photosynthetic efficiency, lipid content, and fast growth rates can result in higher productivity per area relative to terrestrial plants [1]. Second, microalgae can be cultivated on marginal land and potentially marginal water without competing with food and feed crops [1]. Finally, microalgae cultivation for biofuel can be coupled with wastewater treatment, potentially reducing high-value fertilizer use [1-5].

However, despite years of research and development, microalgae-based biofuel production still faces techno-economic challenges. One main challenge for large scale microalgal cultivation is the typically used open ponds which are subject to numerous biotic and abiotic stresses that include infestation with grazers and pathogens, which can lead to significant yield loss [6,7]. In addition, fluctuations in light, temperature and nutrients threaten culture stability and consistent yields. While microalgal monocultures are well understood at the laboratory scale, significant perturbations are inherent to the open systems [3,8,9]. The use of closed bioreactors is possible but doing so at large-scale is costly in terms of capital investment as well as light and CO_2 delivery.

Industrial scale microalgal cultures are usually seeded with unialgal or co-algal inoculum, and colonization with other microorganisms is considered contamination that needs to be managed. However, not unlike human and plant microbiome research, ‘healthy’ microbial diversity is being appreciated and studied to better understand roles in ecosystem function, where algae, diatoms, and bacteria co-exist to form commensal and symbiotic associations [10-16]. In addition, consortia consisting of more than one microalgal species can result in higher culture stability and productivity [17,18], likely as a result of niche space occupation towards maximal carrying-

capacity. Therefore, creating pond ecosystems with mutually supportive microalgal and bacterial species may improve productivity and increase system resiliency.

The basic tenets of community ecology describe uninhabited, open systems as increasing in complexity over time. Monocultures will be invaded by organisms from neighboring environments until a stable state is reached, and this state is ultimately selected to be resilient to 'normal' changes [19,20]. Recent studies have provided evidence for advantages of polycultures over monocultures with respect to biomass productivity [21,22], stability [23], resource utilization [24], and resistance to invasive species (*e.g*., grazers) [25]. These studies also highlight potential trade-offs during the cultivation phase and in the downstream processing steps (harvesting, extraction, conversion) [18]. Previous research has evaluated the energy and environmental effects of polycultures compared to monocultures, with productivity models favoring polyculture assemblages over monocultures [26], and these findings coincide with observations for diverse, natural systems (*e.g.,* ocean cyanobacteria) [27]. Evidence thus far has supported the potential for polycultures and emphasized the value of engineering or selecting communities based upon maximizing synergies and minimizing competition in the context of targeted products [28,29].

Engineering microalgal cultures with mutualistic or commensal bacteria has been employed to enhance nutrient removal and bioremediation in wastewater sources [30] but less is understood how engineered polycultures at industrial scales will perform with regards to light-driven productivity and stability. Engineering microalgal cultures with bacteria could augment biofuel production by addressing biomass growth, nutrient recycle, harvesting, and biofuel extraction [3,31] as well as overall stability that could contribute to consistent harvests. In this review, we focus on the potential of using bacteria to improve high-pH algal cultivation through

comparison with productive natural systems as well as discuss the current understanding on how microalgal and bacterial interactions could alleviate system stress combined with new approaches to engineer productive, stable consortia.

Microalgae in Natural Systems

While microalgae can have significant impacts on human health and the economy (*e.g.*, harmful microalgal blooms), there are also many beneficial applications of microalgal biomass and metabolism (*e.g.*, biofuels, bioproducts and renewable food sources) [10]. Over 70,000 species of algae have been identified using a combination of morphological- and molecular-based techniques [32] and 26,860 algal species/strains were recorded in GenBank as of January 2020 [33]. While technological advancements in next-generation sequencing have allowed for exponential growth in genome sequencing efforts, the number of publicly available algal genomes (224 published genomes) [33] represents less than 0.1% of the known algal species. The gap in genome representation is in part due to the challenge of isolating and cultivating axenic algal strains, especially when it is presumed that many algal species rely on symbioses with bacteria and fungi [34,35, Moll, K., PhD thesis, Montana State University, 2021].

Unlike axenic laboratory cultures, natural aquatic habitats are diverse microbial communities that constantly exchange resources and respond to both abiotic and biotic fluctuations [15,36]. In a field study of a microalgal-bacterial community during a diatom-dominated bloom, Teeling et al. [37] identified almost 100 microalgal morphologies that co-occurred with bacterial clades. Bell et al. [22] surveyed community composition in a large wastewater treatment lagoon over a 1-year sampling period and detected extremely diverse communities (e.g., 445 eukaryotic OTUs) that included green microalgae and predatory protists. In another survey of wastewater,

Hena et al. [38] observed signatures of 20 microalgal and diatom species in dairy farm wastewater. A 4-year survey of the southern North Sea found that the overall species composition in plankton is balanced and remains consistent despite interannual variation [39]. It is likely that natural consortia have adapted to certain environments, fluctuations, and extremes, and insights into the role of each microalgal species in a certain habitat could inform strategies to design stable and productive microalgal consortia for industrial cultivation in different locales (*e.g.*, dry/sunny versus humid/cloudy). Microalgae in natural systems are typically observed in diverse communities and understanding relationships of microalgal species with each other and with non-microalgal partners could promote beneficial effects towards production and stability.

Many studies of microbial diversity in extreme environments have focused on low pH, high temperature environments (*e.g.*, hot springs). The ecology of alkaline systems, both natural and artificial, is poorly understood, though it has been observed that communities in alkaline environments are phylogenetically distinct from circumneutral [40,41]. Most phototrophic systems above approximately 45°C become predominated by cyanobacteria up to 75°C, whereas microalgae grow better between 10°C and 40°C [42] depending upon the environment. Alkaline lake systems have some of the highest primary productivity rates on the planet [43] and diverse and unique microalgae have been identified from alkaline systems [44-46]. However, surprisingly, few studies have systematically catalogued the extent of potential diversity and function of microalgae from temperate (20-40°C), alkaline systems. We hypothesize that given the high primary productivity rates and tolerance to high pH, a microalgal cultivation system at alkaline/high pH has the potential to achieve controllable inputs/outputs (*i.e.*, stable productivity with minimized inputs) via direct air CO_2 capture.

Microalgae in Industrial Systems

Microalgae require water, sunlight, and nutrients (*e.g.*, N, P, Fe) to grow via photosynthetic autotrophy (*i.e.*, carbon dioxide utilization), and the low-nutrient and low-quality water requirements coupled with rapid growth rates and high biomass yields make microalgal systems a target for biotechnology applications, including biofuel and bioproduct generation [3]. Most of the cultivation methods that have been developed for microalgae use autotrophic growth, where light energy is harvested via photo-pigments to convert CO_2 into biomass. While some microalgae can grow heterotrophically or mixotrophically with higher biomass yields [47,48], the use of additional carbon can increase overall energy demands as well as increase contamination with heterotrophic microorganisms that compete for N and P [49,50]. Therefore, the focus of this review is on photoautotrophic growth that uses CO_2 as the carbon source and sunlight as the energy-source.

Given the open-air nature of large-scale microalgal cultivation under photoautotrophic conditions, maintaining axenic cultures presents many challenges. Invasion or contamination is almost inevitable, resulting in a complicated ecosystem of bacteria, zooplankton, and microalgae as well as fungi and viruses [51-54]. Much research has focused on controlling infectious agents with strong chemicals and pesticides, as well as extensive sterilizing methods such as filtration. A more practical approach may be to select for less susceptible or invasion-resistant strains [55], well-structured polycultures that are tolerant to invaders, and/or cultivation conditions that limit invasion through selection (*i.e.*, high pH/high alkalinity). Moreover, naturally high pH and high alkalinity environments (*e.g.*, soda lakes) have some of the highest primary productivities on Earth [56], largely attributed to the increased availability of inorganic carbon in these environments. In recent years, high pH/high-alkalinity conditions have been used to increase microalgal biomass and lipid yields in shorter time periods, and these extreme conditions appear to reduce invasion by

harmful microorganisms and grazers [45,57-59]. While it is feasible to select and promote stable microalgae-bacterial consortia that can thrive at high pH/high alkalinity [45,60], little is known about the possible metabolic and/or ecological interactions specific to high pH and high alkalinity nor potential implications for long-term, repeated cultivations.

The Phycosphere - the Microalgal-Bacterial Interface

The term phycosphere refers to the immediate region surrounding a microalgal cell enriched in organic matter (*i.e.*, photosynthate) and inorganic nutrients [61] (Figure 1.1 A, B). The phycosphere is analogous to the rhizosphere in soils and refers to the thin fluid layer (diffusive boundary layer) that surrounds small aquatic microalgae (<100 μm). The size of the phycosphere is determined by the size and shape of the microalgal cell, growth and exudation rates, and the level of mixing in the bulk aqueous phase, with smaller, slower growing cells generally having thinner phycospheres [36]. The phycosphere includes the associated microorganisms to microalgal cell surface and/or algal aggregates [10] and includes both direct and indirect metabolic interactions, while the phycosome, building from the general concept of microbiome [62], is a microbial community occupying a defined habitat (*i.e.*, microalgal dominated environment) with distinct physio-chemical properties (metabolic potential/function) [63]. In extreme cases, such as in the interaction between Chlorophyta and Rickettsiales, bacteria are able to develop inside microalgal cells [64]. In natural habitats, there are examples of the following scenarios: (1) a clear partitioning of attached and free-living bacterial taxa [65,66], and (2) a large overlap of bacterial taxa in the attached and free-living fractions [67]. Interestingly, Eigemann et al. [68] reported a shift from scenario (1) to scenario (2) when culturing natural samples in laboratory conditions, and

the results suggested that the laboratory conditions that promote high microalgal biomass may also promote an expansion of attached bacteria into the free-living fraction (Eigemann et al., 2013).

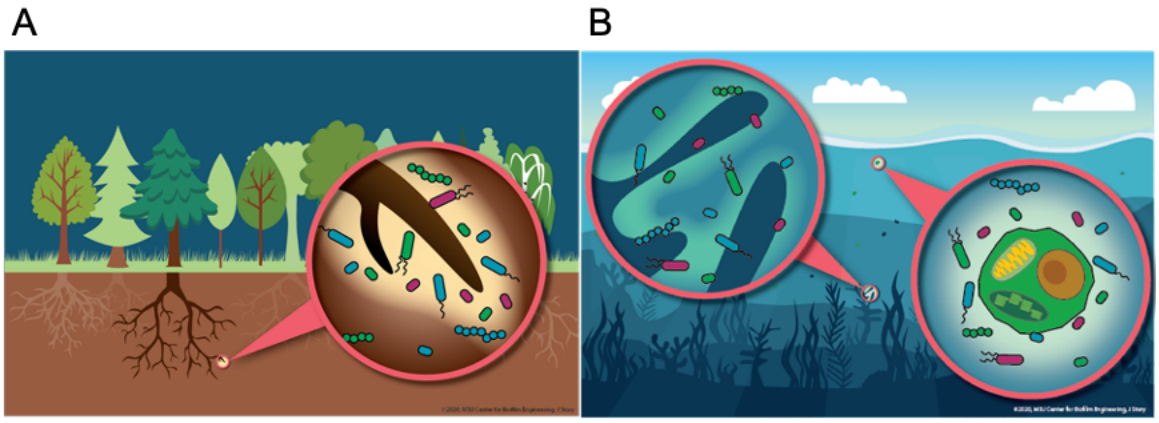

Figure 1.1. The algal phycosphere: extrapolating principles from the rhizosphere. (A) The rhizosphere is the key interface for plant-microorganism interactions in terrestrial habitats. (B) The phycosphere is the aquatic analogue of the rhizosphere and is defined as the region surrounding macroalgal blades or microalgal cells. Adapted and updated from a review defining the importance of the phycosphere in natural systems [36]

Although aquatic environments may seem to have a homogeneously low concentration of growth substrates, there is a "sea of gradients" available to planktonic bacteria [69] created through the release of dissolved organic carbon by microalgal cells into the surrounding water. Non-motile bacteria can encounter microalgal cells randomly, but the encounters are relatively rare [36]. Beyond random encounters, many marine bacteria may actively gain access to the phycosphere by chemotaxis to gain fitness advantages from the dissolved organic carbon [69-72]. Several microalgal exudates can elicit microbial chemotaxis including glycolate, acrylate, amino acids, and dimethylsulfoniopropionate (DMSP) [61,73-75], and differential DOC exudates have been shown to impact chemotaxis in phylogenetically distinct groups [71]. These results suggest that microorganisms can be attracted to algal cells and that some algae may actively recruit specific

microbial populations dependent upon direct or indirect metabolic interactions via different exudates.

Once within the phycosphere, bacteria may maintain association via attachment to microalgal cell surface [76], microalgal sheaths [31], or to the polymeric matrix [77] all of which could be potential sources of carbon (Figure 1.2 A-C). In some cases, bacteria colonize transparent exopolymer particles (TEPs), which are made of polysaccharides released by diatoms. Certain bacterial strains can modulate diatoms' TEP production, thereby promoting the aggregation of diatoms and promoting bacteria-diatom associations [78]. Bacteria themselves can also release exopolysaccharides in response to the presence of microalgae, which might facilitate interactions [79].

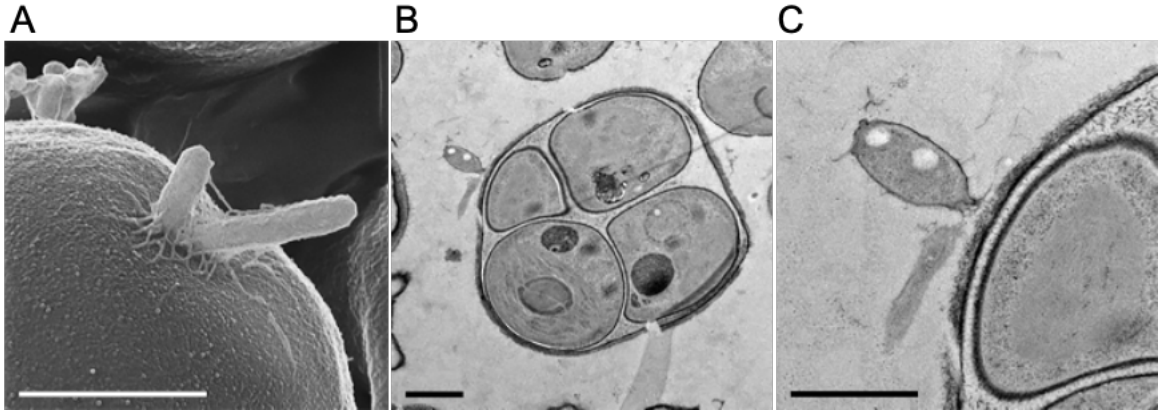

Figure 1.2. High resolution depictions of interactions between the alkalitolerant green microalga Chlorella sp. SLA-04 and associated microorganisms. Scanning helium ion microscopy image (A) and transmission electron microscopy images (B) and (C) depicting attachment of microorganisms to SLA-04 cells. Credit: Alice Dohnalkova and Shuttha Shutthanandan (Environmental Molecular Science Laboratory, Pacific Northwest National Laboratories) via a Facilities Integrating Collaborations for User Science (FICUS) grant. The scale bars for A, B and C represent 1 μm.

Natural phycosome communities are often more diverse and stable than those observed *ex situ* or in industrial settings. The selective forces acting on wild and industrial phycosomes are likely similar, yet the extent to which factors can influence structure and function is likely different. For example, marine microalgal phycosome diversity is correlated with latitudinal temperature gradients [80], but little is known if similar temperature effects could play a role in industrial cultures, which can experience large diurnal temperature shifts when maximizing sunlight exposure. Where a differentiation between free-living and directly attached bacteria has been observed in natural systems, the distinction is often less clear in laboratory or industrial cultures [10]. This is likely due to the homogeneity of cultures, relative to natural environments, that is achieved under typical mixing. Industry practices of mixing and nutrient delivery to maximize algal production ultimately minimize ecological niche partitioning and spatial differentiation that are likely important contributors to phycosome roles. Influential environmental parameters such as photosynthetically active radiation (PAR), pH, and oxygen concentration that might change under different industrial growth schemes likely impact both the microalgae and the phycosphere. Hence, research is needed to ascertain mechanistic phycosphere/phycosome roles that significantly impact algal culture productivity or stability under relevant industrial conditions.

Microalgal-Bacterial Interactions

Microalgal-microbial associations may provide selective advantages for microalgal health [81]; while conversely, axenic cultures of microalgae may be unstable and prone to perturbation [8]. Microalgae can release up to 50% of fixed carbon into the surrounding environment as excreted organic compounds (*e.g.*, carbohydrates) that is thought to directly and indirectly impact associated ecological partners, local photoinhibition, and aggregation [31,82,83]. An image of an

aggregate from a high pH, high alkalinity culture of the alkalitolerant *Chlorella* sp. SLA-04 is shown as an example of phycosome-microalgae spatial relationships (Figure 1.3).

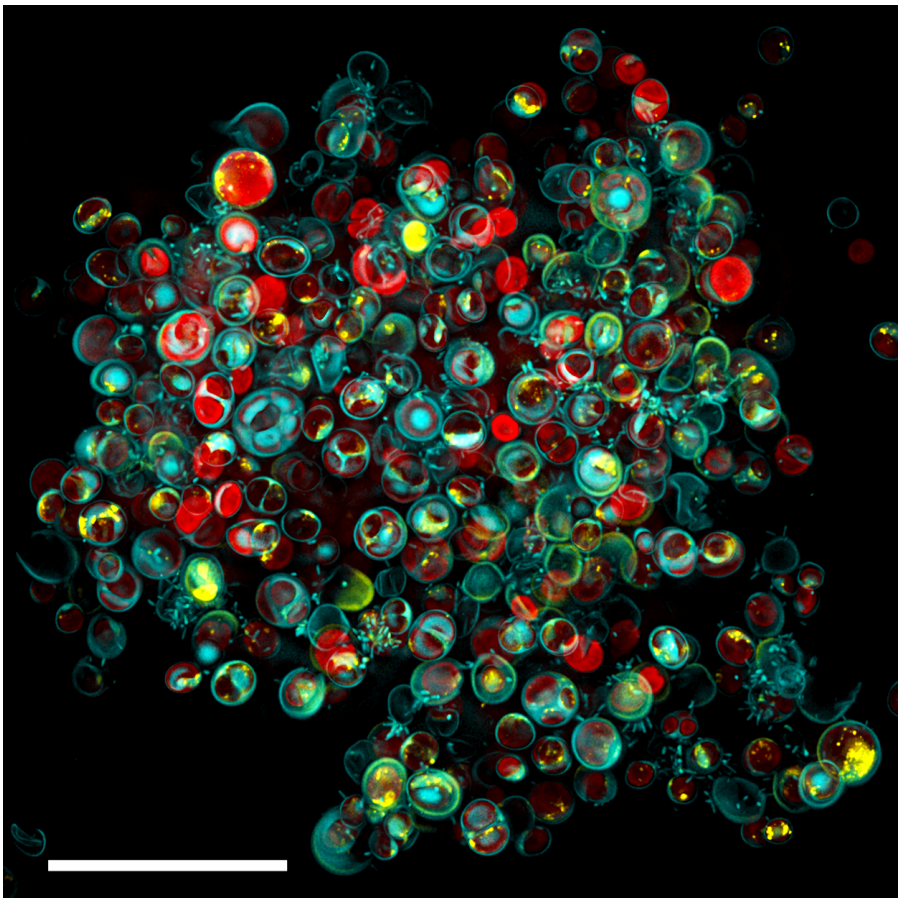

Figure 1.3. Aggregate of Chlorella sp. SLA-04 and associated phycosome from a xenic, high pH, high alkalinity lab culture. Chlorophyll autofluorescence is colored in red, DAPI (4',6-diamidino-2-phenylindole) (ThermoFisher) stained DNA in cyan, and Bodipy 505/515 (4,4-Difluoro-1,3,5,7-Tetramethyl-4-Bora-3a,4a-Diaza-s-Indacene) (ThermoFisher) stained lipids in yellow. The scale bar at bottom left represents 30 µm.

Microalgal-bacterial interactions are considered species- or even strain-specific, meaning that the phylogenetic composition of the bacterial community can depend on the microalgal host [66,84-86]. However, the level of specificity has been suggested to differ among microalgal genera [87] and the number of algal phycosomes studied for different genera are limited. Establishment

of long-term specific interactions likely correlate with metabolic dependencies as well as with long-term co-evolution [88-90]. Less is known about the drivers of short-term interactions (*i.e.*, diel cycle); however, preliminary studies have been conducted in freshwater environments, connecting abiotic and biotic diel patterns with bacterioplankton population dynamics [Papadopoulou, S., PhD thesis, Uppsala University 2021]. The species-specific nature of microalgal-bacterial interactions could be due to distinct exudates produced by a given microalgal species providing substrates that differentially attract bacteria based on metabolic potential [85,91]. Consistently, natural and industrial cultures containing multiple microalgal species have more diverse bacterial communities than uni-algal cultures [10]. When synthetic phycosome communities were established using a mixed bacterial starting community along with diatom and dinoflagellate host cells, direct relationships between host metabolism and community succession were uncovered based upon host-produced metabolites [92]. These findings suggest that specific host processes could select for specific phycosphere compositions.

Microalgal cell size could play a role in the interactions with bacteria. For example, microalgae with small cell size (*e.g.*, *Nannochloropsis salina*) may encounter bacteria at lower frequencies given lower surface area for bacterial attachment [30]. Larger cells might not only provide more surface for attachment but also release more exudates, resulting in wider diffusive boundary layers, hence being more likely to be detected and encountered by chemotactic microorganisms [36,86]. For this reason, the trade-off between costs associated with biomass production and surface area generation is an important consideration in understanding the selective pressures driving morphological diversity in microalgae. Smaller cells, however, may move around more, expanding the impacted area [36]. The growth mode or lifestyle of microalgae might

also be a determining factor. For example, in cultures enriched for microalgae-associated bacteria, the benthic marine diatom, *Phaeodactylum tricornutum*, appeared to have more bacterial cells attached on the cell surface than *N. salina* [30]. Changes in the environment likely lead to changes in the microalgal metabolism, which changes the chemical "profile" of the phycosphere and might result in microbiome shifts. Ultimately, it is likely that both host morphology and physiology can exert selective control over the composition of the associated microbial community and vice versa.

The Role(s) of Bacteria in Microalgal Cultures

Microalgal-bacterial interactions span a broad spectrum from mutualistic to commensal, competitive or algicidal [13,84,85,93,94] (Figure 1.4). For example, for the marine microalgae *Emiliania huxleyi* and the associated *Roseobacter* group, the interactions include distinct mutualistic and algicidal phases. In the first phase, the bacterium provides antibiotics that protect the microalgal cells from bacterial pathogens, and auxins, which promote microalgal growth. As the microalgal population ages, the bacterium switches to the algicidal phase in response to the increased level of *p*-coumaric acid, a breakdown product of dying microalgal cells. In this phase, *Roseobacter* can produce antialgal compounds, the roseobacticides, that kill microalgae [13,95-99]. This complex example suggests that at least some interactions will be condition- and time-dependent.

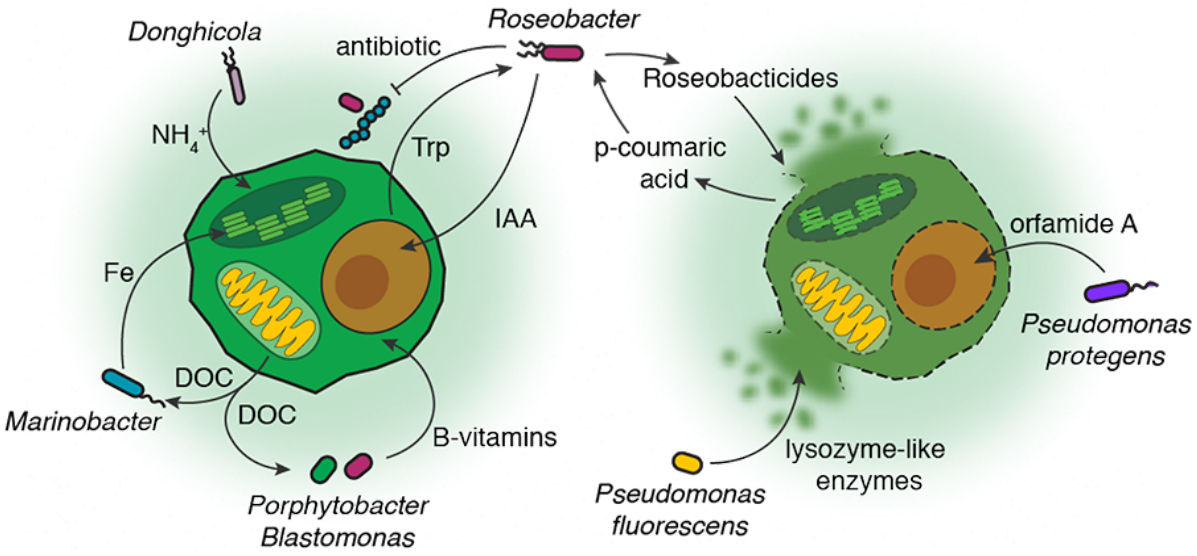

Figure 1.4. Mutualistic (left) and algicidal (right) interactions that occur in the phycosphere. A generic microalgal cell is portrayed to represent multiple species. Shading around microalgal cells represents gradients of metabolites that, through diffusion away from cells, comprise the phycosphere. Metabolite exchanges highlighted in this summary are ammonia (NH_4^+), iron (Fe), dissolved organic carbon (DOC), B-vitamins, indole-3-acetic acid (IAA), tryptophan (Trp), antibiotics, p-coumaric acid, enzymes, orfamide A, and roseobacticides.

Competitive or Antagonistic Interactions

In the relationship with microalgae, bacteria can be benefactors that can utilize DOM release by microalgae and compete for macro-nutrients (*e.g.*, N, P, Fe), resulting in decreased microalgal productivity [100]. In addition to utilization of microalgal exudates, some bacteria have adapted an algicidal lifestyle, in which bacteria actively attack microalgal cells to obtain nutrients [101]. While algicidal bacteria need to be avoided in microalgal cultivations, they could potentially be used for controlling harmful microalgal blooms [102,103]. Directly related to biofuel production, Lenneman et al. [104] demonstrated that two algicidal bacteria, *Pseudomonas pseudoalcaligenes* AD6 and *Aeromonas hydrophila* AD9, induced microalgal cell lysis leading to an at least six-fold improvement of lipid extraction from the microalgae *Neochloris oleoabundans*

and *Dunaliella tertiolecta* [105]. Bacterial algicides and the potential modes of action were reviewed extensively by Meyer and colleagues [106].

Just as bacteria have evolved to prey upon microalgae, microalgae have evolved strategies for self-defense. While released microalgal organic compounds serve as a food source for bacteria, larger exopolymers may also form a "mucus treadmill" that protects microalgal cells from excessive bacterial colonization [30,107]. Microalgae can also reduce bacterial growth and colonization by interfering with bacterial quorum-sensing systems [108-109]. Diatoms can secrete fatty acids and esters [110] that have been shown to act as signaling molecules to induce bacterial biofilm dispersal [111-112] or provide protection by serving as antibacterial compounds [113]. Polyunsaturated aldehydes (PUA) can suppress growth of some marine bacteria, but some bacteria in diatom phycospheres are resistant to PUA and indicate a potential selection mechanism [114]. In ways analogous to human gut microbiome roles [115], the presence of a diverse phycosphere may help reduce the occurrence/probability of antagonistic interactions (*i.e.*, niche exclusion).

Beneficial or Mutualistic Interactions

Mutualistic relationships are common between microalgae and bacteria, and bacteria can provide essential nutrients and exert growth effects as strong as those of light and temperature [116]. Capturing and maintaining these relationships in engineered settings is key for highly productive polycultures. There is ample evidence that bacteria can positively influence the productivity of microalgal systems via both direct and indirect routes [21,22,117,118]; and similar to other host-microbiome interactions these routes can include nutrient acquisition, growth effectors, and protection.

Nutrient Acquisition

In photo-aquatic ecosystems, as bacteria process carbon fixed by autotrophs, CO_2 is produced and inorganic nutrients are recycled (*e.g.*, N, P, Fe) [116]. While these nutrients directly benefit the bacteria, the occurrence of bacterial re-mineralization within the phycosphere may also provide microalgal cells with elevated local concentrations of nutrients [36]. Microalgae require iron for photosystem biosynthesis and function, and many bacteria produce siderophores for Fe-acquisition. For example, *Marinobacter* sp. can produce siderophores and contribute to iron chelation and iron uptake by microalgae [119,120]. In addition, bacteria like *Roseobacter* sp. have potential to regenerate iron hemoproteins that are released by lysed and decaying microalgal cells [121].

Bacteria also play critical roles in microalgal nitrogen uptake. Diazotrophic organisms such as cyanobacteria and other bacterial genera (*Rhizobium*, *Mesorhizobium* and *Azospirillum)* can fix N_2 into bioavailable forms [12]. Nitrogen fixation may be a more dominant process in the phycosome of microalgal cultures at times when oxygen levels are low *(e.g.*, during dark periods). Certain diatoms or prymnesiophytes have been shown to have obligate mutualistic interactions with nitrogen-fixing cyanobacteria [12,122]. Similarly, *Rhizobium* spp. can provide nitrogen for *Chlorella vulgaris* which has been shown to increase microalgal cell levels by ~72% [31,123]. Moreover, bacteria can facilitate microalgal nitrogen uptake by converting nitrogen containing compounds into more bioavailable or preferred forms for microalgal consumption. *Donghicola* spp. have been shown to degrade methylamine and release ammonium that can be utilized by photoautotrophs [124]. In another case, when cultured with the diatom *Pseudo-nitzschia multiseries*, *Sulfitobacter psuedonitzschiae* used nitrate from the medium and released ammonium,

which is the preferred nitrogen source for the diatom [125]. Even protists, which are often considered pests in microalgal systems, were found to facilitate nitrogen uptake in a closed system where ammonia released by *Paramecium caudatum* enhanced productivity in *Chlorella* [126].

Provision of Essential Vitamins and Growth Hormones

Stimulation of microalgal growth by bacteria can also occur via the production of vitamins [11,127-130]. Many microalgae are auxotrophic for essential vitamins such as B_{12}, thiamine and biotin [131-133], and these compounds are thought to be exchanged through mutualistic interactions with vitamin-producing microorganisms [11,134,135]. Using metagenomic and metatranscriptomic approaches, Krohn-Molt et al. [87] provided strong evidence that a-proteobacteria (*e.g.*, *Porphytobacter* and *Blastomonas*) were the main B-vitamin suppliers in the *Chlorella* and *Scenedesmus* microbiomes, while *Sphingobacteria* and Bacteroidetes species exhibited high expression of B_{12} biosynthetic genes in culture with *Micrasterias* [87].

Bacteria can also promote microalgal growth via the production and release of growth hormones such as indole-3-acetic acid (IAA) [30,130,136-138]. Although IAA has no clearly documented metabolic role in many bacteria, some bacteria can produce IAA from tryptophan. While the evidence for IAA involvement in microalgal physiology and development remains limited, IAA has been thought to be the driver for a number of intimate microalgal-bacterial interactions [139]. The coccolithophore *Emiliania huxleyi* exudes the amino acid tryptophan, which is utilized by the bacterium *Phaeobacter inhibens* to produce IAA [140]. A similar interaction was reported for a *Sulfitobacter* spp. and *Pseudo-nitzschia multiseries* symbiont [125]. IAA released from bacteria promotes algal/diatom growth that presumably results in more carbon available to support bacterial growth. Interestingly, IAA addition to culture medium resulted in reduced growth of the diatom

compared to IAA released by bacteria. One explanation is that bacteria-released IAA had higher local concentration in the phycosphere than the bulk concentration resulting from the addition of IAA to the medium [125].

Protection

Bacteria can provide a certain level of protection to microalgae beyond simple niche exclusion. Makridis et al. [141] tracked bacterial communities associated with green microalgae grown for aquafeed and observed that the presence of certain populations hindered the growth of pathogenic bacteria. For example, *Roseobacter* sp. produce tropodithietic acid (TDA), a dual sulfur-tropolone compound that inhibits a broad range of marine pathogens and may help to prevent harmful bacteria from colonizing the microalgal phycosphere [142]. Bacteria have also been found to infect and kill microalgal predators, which could result in increased microalgal growth. In particular, the bacterium *Pasteuria ramosa* is a parasite on the crustacean *Daphnia magna* [143], while *Holospora undulata* can infect the protozoan *Paramecium caudatum* [144]. In addition, many marine bacteria express antagonistic activity against other bacteria via antibiotics [145]. Finally, phycosphere bacteria can relieve microalgae from oxidative stress induced by reactive oxygen species. The epiphytic bacteria associated with the diatom *Amphiprora kufferathii* were shown to express catalase activity that reduced hydrogen peroxide levels in the local environment [146].

Learning from Nature: Ecological Engineering for the Laboratory and Industry

It is becoming clear that large scale, outdoor uni-algal cultivations are prone to infection, invasion, and contamination, resulting in communities of bacteria, zooplankton, fungi, viruses, and other microalgae [8]. Until recently, these contamination events have mostly been addressed

through the use of pesticides, as well as extensive sterilizing methods such as filtration [54]. However, a more sustainable approach may be to select for stable and resilient strains and communities [55]. The latter approach has gained increased attention recently as the application of bacteria to enhance microalgal growth continues to be a growing area of biofuels research [55].

However, studies examining inter-organismal interactions in engineered microalgal cultivation systems are still relatively rare, particularly at larger scale, and underscores our nascent understanding of metabolic cooperation within these microbial communities for biofuel production [125]. One of the main reasons is that up to this point, the laboratory practices for enriching and isolating microalgal strains are not optimal for maintaining the associated phycosome/phycosphere. As isolation protocols generally follow multiple rounds of streaking cultures on agar plates and antibiotic treatment, many phycosphere bacteria are likely eliminated. Since prolonged axenic cultivation of microalgal species in industrial scale systems is not practical and realizing that the absence of bacteria can negatively influence microalgal physiology and growth even in supportive media, engineering approaches should be pursued that promote beneficial interactions and minimize detrimental interactions.

In recent years, there has been increased interest in culturing microalgae with the associated microbial communities [26]. Although such studies have facilitated our understanding of the molecular mechanisms of interactions in microalgal-bacterial consortia, laboratory enriched cultures generally have a low diversity compared to *in situ* cultures [87]. Analogous to plant-microorganism interactions at plant roots and leaves [147,148], phycosphere/phycosome consortia could be designed to promote beneficial/protective interactions and/or limit negative interactions to improve the stability and resiliency of industrial cultures.

The critical questions to address gaps in knowledge between natural and engineered consortia in these settings are (i) whether enriched cultures capture the diversity present in natural microalgal phycospheres; (ii) whether polycultures in managed, open ponds have similar benefits that have been reported in natural habitats; and (iii) whether it is possible to reconstitute and/or design microbial consortia with consistently predictable and controllable outcomes. While the productivity of natural ecosystems is often measured by biomass production [149], productivity of a microalgal industrial cultivation is likely assessed by biomass quantity, compositional content (*e.g.*, lipids), culture stability/robustness, the net environmental impact (*e.g.*, water, nutrient, CO_2), and the overall costs [17]. Therefore, the phycosome and potential impacts should be considered during life cycle and techno-economic analyses [3].

Use of Algal Polycultures to Improve Productivity and Stability

In many environments, species form interactive consortia, in which they are more likely to interact with each other than with outside species [150,151]. Termed 'small world' networks, these consortia are common in natural and man-made systems in which micro-scale interactions impact overall productivity [152]. In addition, based on modeling clustered food webs [153,154], phycosphere communities can display increased stability compared to randomly assembled food webs and may display increased relative diversity because extinction rates are more gradual. Sinha and Sinha [154] showed that relationships between species can be independent of both the initial size and connectivity of the network, and that the number of interactive species is a fundamental property of network structure irrespective of biotic or abiotic conditions. The results also suggest that species that interact with too many other species are destabilizing to network persistence [155].

Two mechanisms believed to drive diversity-productivity relationships are the selection effect and the complementarity effect [24,28,156]. In the selection effect, the more species in a consortium, the higher the chance to have a species with a specific function. However, simply increasing the number of species does not always lead to increases in yield and stability if species are in the same functional group [18]. To maximize the potential for improving yield, polycultures could be designed based on specific traits/metabolic potential and the functional complementarity between species. Indeed, metabolic dependencies, interactions and exchanges are being increasingly suggested as major drivers of community structure and function in different systems. Metabolic modeling of >800 sub-communities with known species composition indicated that communities with high phylogenetic diversity tend to consist of species with a low degree of metabolic overlap [157]. These models emphasized metabolic dependencies as a key biotic force in determination of microbial communities in nature. Communities with high interaction potential among members are more likely to benefit from complementary biosynthetic capacities and require fewer resources [158]. Therefore, metabolite exchange could be a mechanism that stabilizes phycosphere interactions, and communities with metabolic synergy could therefore thrive in nutritionally poor habitats [159]. This principle can guide the design of polycultures through ecological engineering to maximize metabolic capacity and achieve target biomass composition in industrial microalgal cultivation, where the main aim is to maximize outputs (biomass, lipid content) using minimal inputs (*e.g.*, N, P, low quality water). To achieve this goal, it is necessary to identify the mechanisms behind diversity-productivity relationships so that design and control can be attempted.

Choosing Multiple Microalgae Strains as "Core" Biomass Producers

In the context of managed cultivation, consortia of microalgae with different traits might be more tolerant to changing environmental conditions (light, temperature) and more resistant to invaders [25]. When mono- and poly-algal cultures were evaluated, polycultures exhibited more stable production through time, higher biocrude yields over time, and were more resistant to invasion than monocultures [21,26]. The studies indicated that designing consortia requires characterization and selection of strains based on ecological principles to promote functional diversity [21,26]. Hena et al. [38] showed that consortia of multiple microalgae resulted in higher biomass production than those of mono-algal cultures when cultivated in wastewater. Interestingly, although four "standard" UTEX strains were included in the screen for the best consortia, the optimal consortium contained only native strains isolated from the wastewater sources used for cultivation. These native strains may have developed an optimal interaction network with one another and with other indigenous microorganisms. Therefore, identifying and promoting these natural relationships may be an effective strategy to design stable and productive microalgal consortia [38].

The positive diversity-productivity relationships could be explained by the efficient use of nutrients and light. Microalgae have different light preferences (wavelength, intensity), and microalgal consortia containing species with non-overlapping optimal wavelengths had higher lipid content and PAR absorbance than polycultures with overlapping light use [24]. In a study about algal nitrogen uptake, Mandal et al. [156] showed that algal species differed significantly in capacity to take up ammonium, urea, and nitrate, and as a result, co-cultures that differed in nitrogen preferences showed greater complementarity and higher productivity than monocultures

[156]. In addition, mixed microalgal communities were also shown to remove inorganic nutrients more rapidly than mono-algal cultures and exhibited increased growth rates [160].

Bacterial Consortia as Probiotic "Amendments"

Inoculating microalgal cultures with bacterial communities that confer health benefits could be used as a form of phycosphere engineering [30]. In photoreactor systems, microalgae and the accompanying bacterial flora were strongly positively correlated [118]. In a separate study, the growth of *Navicula veneta* was positively affected by *Halomonas* NC1, and diatom cells were 65% less productive without the bacterium [161]. When *Chlorella vulgaris* was grown in the presence of different bacteria, all important parameters for biofuel production were higher than those measured in corresponding axenic cultures [162]. More recently, Toyama (2018) [163] cultured each of three microalgal species *Chlamydomonas reinhardtii, Chlorella vulgaris*, and *Euglena gracilis* in the presence and absence of indigenous bacteria in wastewater effluent and for all three species, xenic cultures resulted in higher biomass yields (1.5-2.8-fold) compared to the respective axenic cultures.

The improved biomass production in polyculture may be explained in part by the higher rate of nutrient assimilation and uptake by both microalgal and bacterial members. For example, *P. tricornutum* cells in the presence of bacteria fixed 64% more carbon compared to axenic cells, while bacterial cells that attached to microalgal cells consumed more microalgal-fixed carbon than unattached bacteria [30]. In another study assessing the symbiotic relationship between cyanobacteria and diatoms (*e.g.*, *Climacodium*) in bulk seawater, cyanobacterial cells that attached to diatom cells showed higher nitrogen fixation rates (171–420-fold) compared to rates estimated for free-living cells, and the majority of the fixed nitrogen was transferred to the diatom [12]. In

addition, Ortiz-Marquez [164] was able to eliminate the need for providing inorganic nitrogen directly to the microalgal culture by adding *Azotobacter vinelandii*, a nitrogen fixing bacterium, that had been genetically engineered to secrete ammonium into the growth medium. In a recent study, *Janthinobacter* protected *Microchloropsis* from rotifer grazing pressure for short periods of time in outdoor cultures [165]. While these studies demonstrate both direct and indirect, positive impacts from added bacteria, the explicit use in promoting microalgal growth for biofuel production is still limited, owing to the knowledge gaps in interactions of bacteria with microalgae hosts, the potential tradeoff between yield and overall culture health, as well as challenges in maintaining stable healthy consortia. From the biofuel production standpoint, bacteria have potential use in cultivation (provide benefit on growth) [15,25,166]; reduction in invasion, harvesting (induce aggregation) [167,168]; and extraction (weakening of microalgal cell wall) [98,108]. However, the differences between different microalgal species and possible beneficial populations/communities in the respective phycosomes is largely unknown, particularly for sustained, industrial systems under different conditions (*e.g.*, high pH).

Toward Designing Microalgal Consortia with Controllable Outputs

Establishing a collection of microalgal strains together with the respective phycosphere community is a simple approach to designing consortia, and the characterization of microalgal consortia from extreme natural habitats could lead to cultures with higher productivity and consistent stability. Robust, resilient, adaptable, and productive communities have been established via simple enrichment of native consortia [10,30], or the assembly of novel synthetic consortia [26,169]. Habitats experiencing extreme environmental conditions hold potential for strains with unique traits.

In recent years, our group has been optimizing microalgal cultivation using microalgal consortia isolated from high pH-high alkalinity habitats. Alkaline aquatic systems have been shown to be among the most productive natural ecosystems in the world [56,174], and it is hypothesized that distinct but very well-developed metabolic interactions are at least partially responsible for these high productivities. Microalgae of diverse taxa including *Scenedesmus, Navicula, Chlorella*, and *Neospongiococcum* can thrive in high pH environments, and they are valuable resources for optimizing microalgal cultivation with atmospheric CO_2 [45,58, Vadlamani, A. PhD thesis, University of Toledo, 2016; Moll, K., PhD thesis, Montana State University, 2021). One of those strains, *Chlorella sorokiniana* SLA-04, achieved high biomass productivities (>20g/m^2/d) in open raceway ponds under high pH/high alkalinity without a culture crash over a two-year period [59, 170]. Given the adaptability and metabolic flexibility of such organisms, manipulating cultivation conditions may be a useful approach for selecting stable, beneficial consortia.

Consortium design will also benefit from a thorough understanding of the community composition and metabolic interactions between members, which is being facilitated by next-generation sequencing, next-generation physiology and imaging technology [171]. Metagenomic sequencing, for instance, will allow for the estimation of the metabolic potential of the community, and the prediction of possible metabolic relationships between community members. Advanced staining and imaging techniques may make it possible to elucidate how microalgal-bacterial interactions affect C, N, P and energy flow for maximum productivity. At the gene expression level, meta-transcriptomic analyses combined with BONCAT (biorthogonal non-canonical amino acid tagging), isotope-specific Raman confocal microspectroscopy and metabolite analysis can be

applied to help reveal how phycosome interactions may influence microalgal physiology and vice versa. These –omics, chemical, and imaging approaches will shed light on the temporal and spatial dynamics to inform consortia design. Furthermore, consortia design can be assisted by mathematic modeling including population-based modeling for prediction of interspecies dynamics [172], as well as metabolic network modeling to predict energy and material flows in a community [173,174]. Ultimately, the performance of consortia needs to be accessed at the industrial scale at the point-of-production. While studies so far suggest that engineering microalgal consortia could improve productivity and stability of large-scale cultivation, extensive life cycle and techno-economical assessments (LCAs and TEAs) are required to determine whether these improvements result in more sustainable operation in both economic and environmental terms during anticipated perturbations (*e.g.*, weather fluctuations).

In Silico Design of Phycosphere Communities

Synthetic microbial communities have traditionally been built using either top-down or bottom-up approaches. Top-down refers to breaking down complex systems into individual parts to simplify and understand function. Bottom-up refers to the integration of well-studied systems to form another, more complex system. Both approaches generally require extensive background observation, next-generation sequencing, and physiology work along with intensive experimentation [176,177]. The future of microbiome engineering has been suggested to hinge on the principles of design-build-test-learn (DBTL) [177], and recent advances in microfluidics, modeling, sequence-based technology, and bioinformatics can expedite the process of identifying, culturing, and applying synthetic communities (SynComs) to algal systems.

For phycosome applications, designing SynComs can be achieved using metabolic predictions of the host algal cell, a native phycosome, a desired microbial community, or a combination. With sufficient metabolic information about the host, exudate composition can be modeled and used to predict how microorganisms may be recruited to the host [72]. Additional approaches are being developed to predict and build systems based upon mathematical models of natural ecosystems [178]. Computational approaches have also been paired with high-throughput culturing techniques such as microfluidics to predict host processes that are integral in recruiting microbiomes [179] or how complementarity of host and microbial growth rate and substrate preferences can be used to train phycosome design algorithms [180]. Synthetic phycosomes have been designed by "letting the host decide" through swapping complex microbiomes from taxonomically distinct host species and letting the host recruit microbial species to a new microbiome [181]. Experimentation informed by *in silico* tools like these can strengthen or expand the potential of traditional bottom-up or top-down microbiome design.

Conclusion and Perspective

There is evidence that microalgal cultures can increase productivity, stability, and robustness through functional and phylogenetic diversification (both at the algal population and microbial community levels). Therefore, industrial scale microalgal culture productivity and stability might be improved through diversification and thoughtful design of microalgal phycosphere/phycosomes. Multiple microalgal species could provide high culture stability (*e.g.*, temperature and light intensity tolerance) and promote efficient resource utilization while the associated bacterial communities could provide essential nutrients, growth-promoting components, and protections against pathogens, grazers, *etc*.

Recently published literature has recognized the importance of microalgal microbiomes, but much research is needed to elucidate mechanistic understanding of phycosphere/phycosome interactions that directly promote productivity and stability. The challenge can become more complex when polyalgal cultures are considered for high pH/alkaline systems because of high but consistent pH with or without higher osmolarity. Yet, understanding these interactions is essential for controlling and optimizing the function of industrial ecosystems for maximal societal benefit in terms of direct air capture of CO_2 and the use of low-quality water and nutrients. In fact, natural systems typically operate with low-quality resources via recycling and a combination of functional redundancy and complementarity. An improved understanding of phototrophic biosystems in alkaline environments could inform the operation of alkaline biosystems at industrial scale for CO_2 capture by taking advantage of ecology and physiology that has evolved in highly productive and stable environments. Remaining challenges include the completion of in-depth physiological studies to understand the potential of organismal traits, the relationship between community members, selecting optimal consortia, and maintaining the consortia over industrially relevant time- and space-scales.

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CHAPTER TWO: TEMPORAL PHYCOSOME COMPOSITION IN CHLORELLA SP. SLA-04 MAINTENANCE CULTURES

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Abstract

Microalgal stock cultures used in industry and research are often xenic, meaning the cultures contain phycosomes (*i.e*., microbiomes) of varying diversity, that can fluctuate but are seldom tracked. In the present study, we report the phycosome composition of three maintenance cultures of the alkalitolerant green microalga *Chlorella* sp. SLA-04. By analyzing different initial SLA-04 maintenance culture sources, we show that stochastic assembly processes drive the formation of SLA-04 phycosome communities. A low alkalinity culture maintained at Montana State University (2018-2022) was predominated by ASVs closely related to *Paracoccus*, *Microbacterium*, *Brevundimonas*, and *Bosea* with sporadic appearance of *Gaiella*. A separate stock culture was received from the University of Toledo and maintained at low alkalinity and high alkalinity (2021-2022). Both the low and high alkalinity stocks were predominated by ASVs related to *Belliella* and *Aquimonas* with some *Halomonas* and *Salinariomonas* while the composition of the high alkalinity stock shifted following small increases in *Salinariomonas*, *Gaeilla*, and *Microcella*. The MSU stock appeared to have a more stable diversity while the newer UT stocks had increasing diversity (H' and 1/D) since being maintained in Montana. Principal components analysis demonstrated that the three stock cultures were distinct. Comparison of experiments started with axenic and xenic SLA-04 inocula over five years revealed similarities in time to nitrate depletion (TND) and maximum cell concentration despite the distinct phycosomes.

Introduction

Many hurdles have slowed the progress of microalgal, large-scale production, namely costs associated with supplying concentrated carbon dioxide, nitrogen and phosphorus nutrients, and

culture stability in both open and closed systems [1,2]. Cultivation at high pH and alkalinity can help alleviate carbon dioxide delivery as well as promote culture stability via niche exclusion and selection, but less is known about the long-term maintenance of xenic, algal cultures to be used for propagation and inoculation of large-scale cultivations, particularly at higher pH [3,4]. Historically, cryopreservation, a technique used in maintaining colonial cultures of bacteria or archaea for long periods of time (>5 years), has not been used in the maintenance of many microalgal cultivars of biotechnological interest mainly due to poor cell viability and unpredictability of genotype and phenotype post resuscitation [5]. Recently, methods for more effective cryopreservation of *Chlorella* [6], *Chlamydomonas* [7], and *Scenedesmus* [8] have been developed, yet long term maintenance of liquid cultures or colonies on agar plates is still common in microalgae research and industrial operations.

Growing axenic microalgal cultures at any scale larger than the benchtop is nearly impossible, and attempting to do so on an industrial scale is economically and environmentally expensive [9-12]. During the process of scaling up from benchtop to production scale, inoculum cultures will be challenged by the ecology of the water and built systems (*i.e.,* plumbing, reactors) [3,13]. In axenic cultures, temporally and spatially heterogenous niche space is open for invading microbes [14]. Conversely, xenic inoculum cultures that are accompanied by commensal populations may be less susceptible to invasion [15,16]. Thus, it may be advantageous to begin scaling up industrial cultivations with xenic cultures. Axenic cultures have, however, been shown to fare better under nutrient stress than xenic cultures, likely due to competition for limiting nutrients [17]. The possible dichotomy between commensal interactions and competition for resources underlines the importance of investigating the phycosome in each culture instead of

relying on inferences from other systems independent of phylogenetic or environmental similarity. Protection is not always conferred when comparing communities of similar diversity [1], thus the relationship between diversity and protection should be investigated on a case-by-case basis.

At present, there is no comprehensive theory explaining phycosome assembly that accounts for the wide range of deterministic, host-environment interactions that may shape these communities. Because microalgae are so diverse in metabolism and distribution, there is a wide range of possible drivers of community structure in each system, whether built or natural. In industrial cultures, it is unclear whether direct or indirect selective pressures, exacted on mixed communities by manipulating growth conditions, can be used to constrain phycosome assembly. Depending on conditions and the corresponding physiology of the host microalga, stochastic and/or deterministic processes may shape the composition of the developing phycosome community [18-20]. Deterministic processes are those forces imposed by the environment or the host, selecting for or against certain populations [21]. Stochastic processes describe those that are less predictable, leading to disparate succession and assembly outcomes [22]. Adopted from coral reef macroecology, the lottery hypothesis states that “lucky” populations randomly find and occupy niche space, lending less weight to host or environment determinism [23,24]. The goal of understanding the driving forces of phycosome assembly is to be able to control consortia with predictable phycosome community outcomes, but controlling consortia hinges on disentangling potentially coinciding deterministic and stochastic factors that shape community composition within a particular host system.

Metabolically diverse microalgal species exert diverse selective forces on respective phycosomes. Host-dependent deterministic processes persisted despite environmental disturbance

in outdoor cultivations of *Microchloropsis salina* [25]. Both environmental (*e.g.,* phosphorus, nitrogen limitation) and host-driven selective processes were found to dominate succession mechanisms in blooms of the toxin-producing diatom *Pseudo-nitzschia* [26]. In contrast, culture source was more important than host or environmental selection in *Nannochloroposis* cultivations [27], however stochastically formed communities were resilient, with cultures maintaining productivity following perturbation. Taking more of a synthetic biology approach, Duran and colleagues showed a "reciprocal complementation" between *Chlamydomonas* and *Arabidopsis* microbiomes, suggesting similar ecological principles govern assembly despite host evolutionary distance [28].

While phylogenetically unrelated microalgae have unique relationships with phycosome assembly, single host species have also been found to display a growth phase-dependent relationship with phycosome assembly. *Emiliana huxleyi* is a ubiquitous marine coccolithophore associated with vast coastal and pelagic blooms [29]. Bloom-forming communities displayed different mechanisms of community assembly than steady-state, low density systems [30]. Phycosome communities associated with *E. huxleyi* blooms from different sample sites were highly similar, indicative of deterministic processes with the host exerting control over available niche space, driving assembly by populations with a specific array of traits. In contrast, cultures obtained from non-bloom conditions displayed the influence of both deterministic processes and stochastic elements. This observation highlights the importance of time course community studies comparing multiple conditions and growth phases. There are likely parallels between algal cultivations and bloom dynamics as it pertains to competition for resources and control exerted over the associated ecosystem [31].

In the present study, we characterized the temporal phycosome composition and diversity in three maintenance cultures of the alkalitolerant, oleaginous *Chlorella* sp. SLA-04 for different stock cultures. We show that stochastic processes may shape SLA-04 phycosome community composition, identifying source and growth conditions (*e.g.*, alkalinity) as drivers of taxonomic composition. We compared the relationship between culture performance (*e.g.*, maximum cell concentration, time to nitrate depletion (TND)) and phycosome diversity by analyzing growth characteristics of experiments starting with inoculum from these three xenic cultures, as well as axenic cultures, over the course of five years.

Materials and Methods

History of Maintenance Cultures

Water samples from the alkaline soda lake, Soap Lake, in Washington (USA), were inoculated on sterile agar plates of Bolds Basal Medium (BBM). A rapidly growing algal colony was picked and streaked for further isolation, and the unialgal culture was confirmed using microscopic observations [32]. *Chlorella* sp. SLA-04 was isolated, and the full genome was recently published [33]. Xenic cultures of SLA-04, originating from the initial isolation, were maintained at the University of Toledo (UT) and Montana State University (MSU) in modified BBM under low alkalinity (LA) or high alkalinity (HA) for the past 12 years. The MSU xenic maintenance culture is hereafter referred to as MSU LA. In November 2020, a xenic SLA-04 culture was shipped from UT to MSU and split into two maintenance cultures, UT LA and UT HA. Samples from MSU LA were collected at nine time points from April 2018 - November 2022. Samples from the UT cultures were collected at four time points for each stock culture (LA and HA) from January 2021 – November 2022.

Algae Culture Preparation

A high alkalinity, xenic SLA-04 culture was collected from non-sterile bioreactor cultures at the University of Toledo, split into two separate cultures, and maintained in batch flasks on modified BBM under either low or high alkalinity conditions. The modified BBM was sterilized via autoclaving and had the following media composition: $NaNO_3$ (2.94 mM), K_2HPO_4 (1.43 mM), $MgSO_4 \cdot 7H_2O$ (0.30 mM), $CaCl_2 \cdot 2H_2O$ (0.17 mM), NaCl (0.42 mM), H_3BO_3 (0.18 mM), 1 mL/L S3 vitamin solution, 1 mL/L trace metal solution, 1 mL/L iron solution and 1 mL/L EDTA solution. The vitamin solution contained Inositol (2.78×10^{-3} M), Thymine (2.38×10^{-3} M) Thiamine·HCl (1.48×10^{-4} M) Nicotinic acid (8.12×10^{-5} M), Ca-pantothenate (4.20×10^{-5} M), *p*-Aminobenzoic acid (7.29×10^{-6} M), Biotin (4.09×10^{-7} M, and Folic acid (4.14×10^{-7} M). The trace metal solution contained $MnCl_2 \cdot 4H_2O$ (1.26 mM), $ZnCl_2$ (0.15 mM), $CuCl_2 \cdot 2H_2O$ (0.11 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.07 mM), $CoCl_2 \cdot 6H_2O$ (0.06 mM), $NiCl_2 \cdot 6H_2O$ (0.04 mM), V_2O_5 (0.01 mM) and KBr (0.08 mM). The iron solution contained 4.98 g/L $FeSO_4 \cdot 7H_2O$ and 1 mL 95% H_2SO_4. The EDTA solution contained 50 g/L Na_2EDTA and 31 g/L KOH. Low alkalinity (LA) conditions refer to modified BBM at an initial pH of 8.7 with 0 mM added alkalinity. High alkalinity (HA) conditions refer to modified BBM at an initial pH of 10.3 with 150 mM added total alkalinity ($NaHCO_3$ 50 mM, Na_2CO_3 50 mM). Cultures were grown at 20 °C in a temperature- and light-controlled photoincubator (VWR Model 2015-2), with a shaker set to 150 rpm, and a 14:10h light:dark cycle under 7500 lx cool fluorescent lights at an intensity of 175 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). Maintenance cultures were transferred every four weeks for the duration of the experiment by aseptically inoculating 180 mL of autoclaved BBM with 20 mL of stationary phase SLA-04 culture. Samples for community analysis were collected 120 hours post transfer.

Establishment and Maintenance of Axenic SLA-04 Culture

An axenic culture of SLA-04 was established by streaking a xenic culture onto an agar plate containing BBM agar with 100 µg/mL ampicillin and 100 µg/mL cefotaxime. A single colony was picked and re-streaked onto BBM agar with the same antibiotics two successive times. After that, a single colony was picked and grown in liquid BBM with 10 µg/mL kanamycin. To test for the presence of contaminants, samples were collected, DNA was extracted and 16S rRNA gene sequencing was conducted on the Illumina platform according to the aforementioned protocol. Axenic stock cultures were maintained under 10 µg/mL until the screening experiments. Physiological stress associated with antibiotics has been observed in Chlorophyte algae [35]. To ensure that antibiotic stress did not directly impact SLA-04 physiology, cell concentration, optical density and chlorophyll concentration were determined for the duration of one typical growth cycle and compared to an SLA-04 culture without antibiotics (unpublished data).

DNA Extraction, PCR and SSU rRNA Gene Sequencing

At each timepoint, 1 mL of culture was collected in a microcentrifuge tube and concentrated via centrifugation (15,000 x g for 10 minutes) and re-suspended into 50 µl molecular grade water and stored at –80 °C until DNA extraction. For extraction, samples were thawed and extracted using the FastDNA Spin Kit for Soil (MP Biomedicals). The V3-V4 region of the 16S rRNA gene was PCR amplified with the primers 341F-(CCTACGGGNBGCASCAG) and 805R-(GACTACNVGGGTATCTAATCC) [34]. The primers used also contained barcoded overhang sequences for application in Illumina MiSeq sequencing. PCR was performed in an Eppendorf Mastercycler Pro S with 25 µL reactions containing 0.2 M of each primer, 8 µL DNA extract, 1X KAPA HiFi Hotstart ReadyMix (Roche) and 2.5 µL bovine serum albumin. The amplification

protocol consisted of an initial denaturation at 98 °C for 10 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, extension at 68 °C for 30 s, and a final extension step at 72 °C for 1 min. Triplicate PCR reactions were pooled, and samples were purified using the Ampure XP Bead PCR Cleanup system (Beckman). The 16S rRNA amplicon libraries were prepared using the Nextera XT Index Kit (Illumina), quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher), and sequenced in separate v3 Illumina MiSeq lanes.

Illumina Data Processing and Analysis

The 16S amplicon sequencing reads were analyzed and assembled into amplicon sequence variants (ASVs) using Qiime2 following the Microbiome Helper workflow [36,37]. The 16S ASVs were classified to phylogenic groups using the RDP classifier5 with a bootstrap cutoff of 80%. The relative abundance of bacterial genera was calculated after filtering out chloroplast and mitochondrial 16S rRNA sequences. Alpha diversity indices were determined using the Past4 software package [38]. Rarefied ASV-level classifications were used to calculate Shannon H Index values and Inverse Simpson's Index values for each individual sample. Principal component analysis of log-transformed relative abundance data was performed using the prcomp function which is preloaded in the R stats package. The results were plotted using ggplot2 [39].

Determination of SLA-04 Physiology

Algal cell counts were performed on bulk samples using standard hemocytometer methods and a benchtop brightfield light microscope. Individual cell counts consisted of at least 300 cells to maintain statistical power. Dense samples were diluted in sterile phosphate buffered saline so that there was a maximum of 100 cells in one field of view. Time to nitrate depletion (TND) was determined by measuring extracellular nitrate concentration every 10, 14, or 24 hrs. The

Szechrome NAS assay was used to monitor nitrate concentrations in the culture medium according to previously established methods [40]. Samples were collected every 24 hours, at the end of the light cycle, for the duration of a growth cycle. Briefly, 1 mL of bulk sample was centrifuged at 14000 x g for 10 min and the supernatant was transferred to a clean tube and stored at –20 °C. Samples were diluted, and 20 µL total volume was added to a clean 96 well plate, and 200 µL NAS reagent was pipetted and mixed with the sample using a pipetter by aspirating and releasing the mixture 20 times. Plates were incubated in the dark at room temperature for 30 minutes before absorbance was measured at 570 nm using a plate reader (BioTek). Time to nitrate depletion (TND) values were reported by recording the time at which nitrate values were below the nitrate detection limit (~2.5 mg/L).

Results and Discussion

Industrial and laboratory microalgal cultures are often maintained under xenic conditions. While the importance of the microbiome for microalgal growth and productivity is well-accepted, the driving forces behind community assembly and the resulting structure/function relationships are often poorly understood for most microbial systems. It is also unknown how long-term xenic maintenance impacts the phenotype of highly productive algal cultures both under short- and long-term cycles of nutrient replete/deplete cycling. Phycosome community assembly likely differs for phylogenetically disparate microalgae, thus strains of interest that are maintained in liquid cultures should be examined by time course sequencing experiments and correlated with changes in culture productivity. Identifying whether stochastic or deterministic processes underpin phycosome assembly for a given microalga is the first step in designing productive xenic cultures in which stability and productivity can be maintained.

SLA-04 Phycosomes Show Temporal Consistency

The initial aim of the study was to identify trends in phycosome composition over time in xenic SLA-04 cultures. SSU rRNA gene sequencing was employed to define community composition at nine time points (04/13/18, 09/15/18, 09/09/19, 02/10/20, 07/17/20, 01/09/21, 06/25/21, 03/26/22, and 11/11/22) for MSU LA, and four time points each (01/09/21, 06/25/21, 03/26/22 and 11/11/22) for the UT LA and UT HA cultures. Genus-level relative abundance was plotted to illustrate phycosome community composition in MSU LA (Figure 2.1), UT LA (Figure 2.2A), and UT HA (Figure 2.2B) cultures. The relative abundance of bacterial populations compared to SLA-04 is approximated by including chloroplast sequences (Supplemental Figures 2.S1 and 2.S2), indicating that the MSU culture had lower bacterial cell concentrations than UT cultures, though exact quantitation with reads from chloroplast sources can lead to over- and underestimates [41].

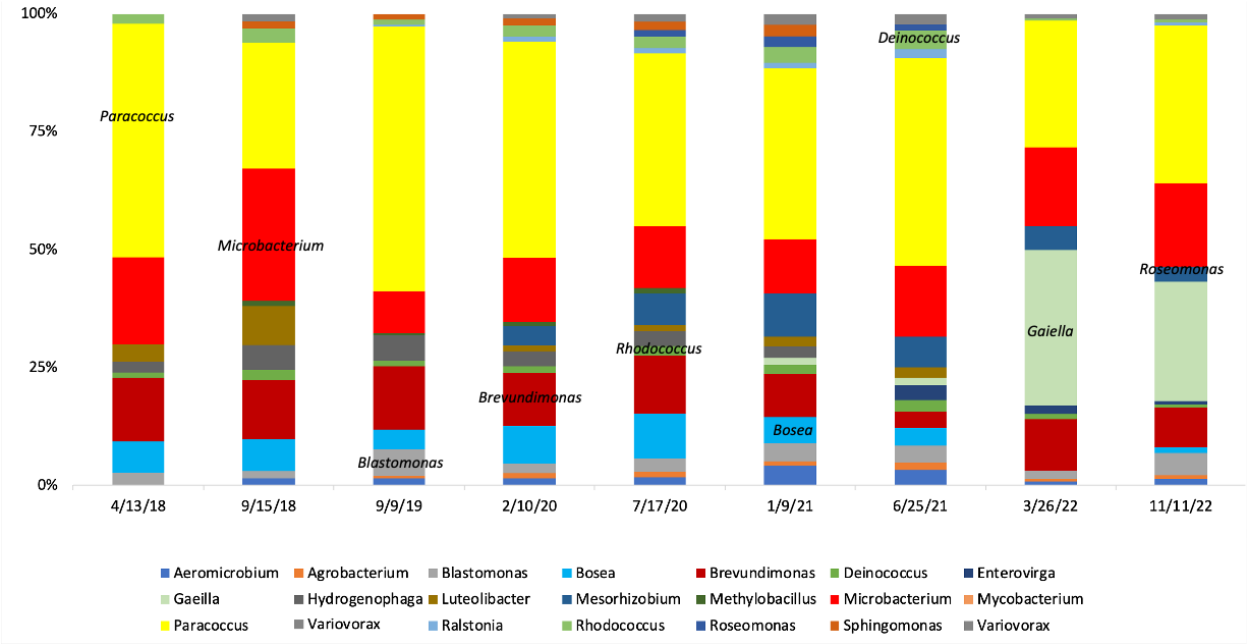


Figure 2.1. Relative abundance of bacterial genera in xenic SLA-04 cultures sourced from Montana State University (MSU). Maintenance culture samples spanning April 13, 2018, to November 11, 2022. Genera with a relative abundance > 0.5% are represented with colored bars.

The MSU LA samples (Figure 2.1) had 41 genera detected, and six of which were observed across all time points: *Blastomonas, Brevundimonas, Deinococcus, Microbacterium, Paracoccus,* and *Rhodococcus*. Given their consistent presence, these genera could be considered a core community of autochthonous populations over the tested time period. On the other hand, ASVs indicative of 16 genera were only observed at a single time point over the tested time period, and these results suggested that many populations do not permanently colonize the SLA-04 stock cultures. In the UT LA stock cultures, 14 of 21 genera were found across all four timepoints (Figure 2.2). In UT-HA samples, 20 of 34 genera were found across all four timepoints. There were nine genera present across all timepoints in both UT LA and UT HA cultures: *Belliella, Aquimonas, Salinarimonas, Rhodobaca, Gaiella, Porphyrobacter, Roseococcus, Lunatimonas, and Microcella*.

There were not genera common across all timepoints in the three cultures, though *Microbacterium* and *Paracoccus* ASVs matching those from MSU LA were observed in the later UT samples. The presence of a core community may suggest that a microbiome is in a relatively stable state, though it Is difficult to quantify stability in this case when community responses to perturbations, or substantial input of allochthonous microbial populations, were not monitored.

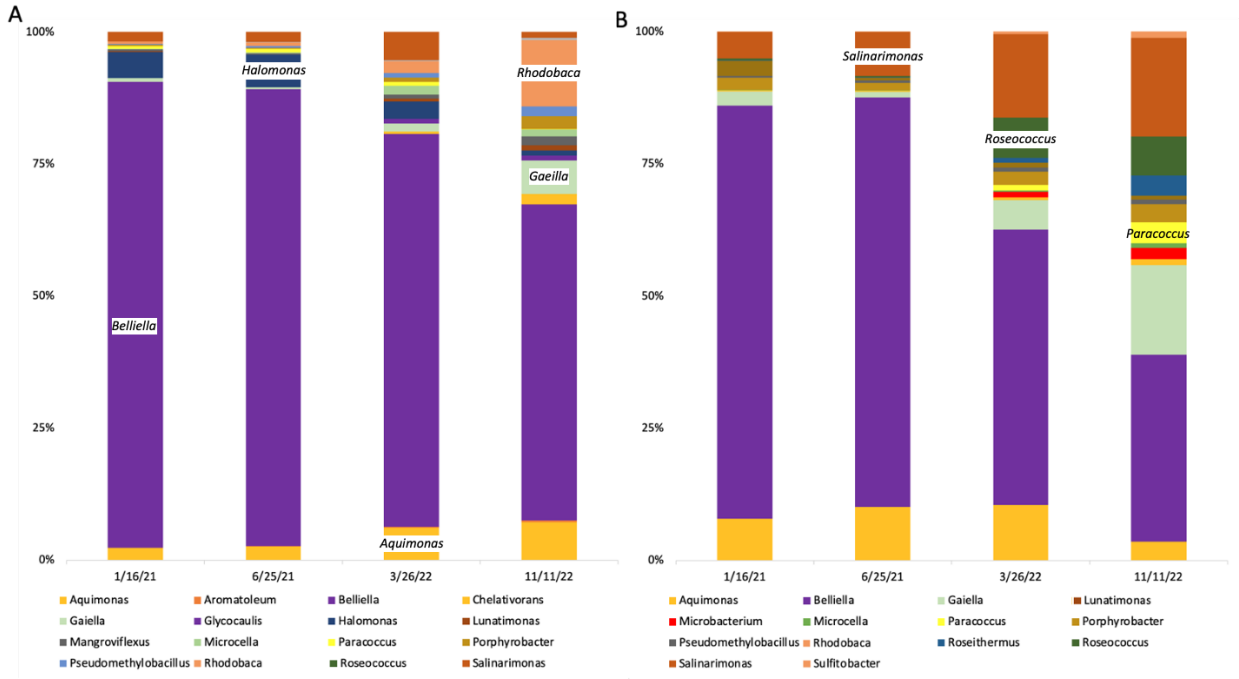


Figure 2.2. Relative abundance of bacterial genera in xenic SLA-04 cultures sourced from the University of Toledo (UT). (A) Low alkalinity and (B) high alkalinity maintenance culture samples spanning January 9, 2021, to November 11, 2022. Genera with a relative abundance > 0.5% are represented with colored bars.

When single timepoints or limited time ranges are used to assess microbiome community composition, there are often questions about whether the observed populations have a stable presence, and at which point can populations be considered allochthonous or autochthonous. Allochthonous populations are transient, often thought to pass through the system without impacting community function or long-term structure [42]. Autochthonous populations are those

which colonize a particular host or system and are more likely to influence community function [43]. Identifying the residence time of phycosome populations may be the first step in identifying function [44], potentially indicating which populations may be beneficial for the host. Investigations using larger time periods spanning successive generations and transfers, may be necessary to make such distinctions [31]. Drawing a parallel to more widely studied systems, epithelial colonization in gut microbiome studies is marked by distinct roles of allochthonous and autochthonous populations [45], with community structure often returning to a native state following disturbances. Similarly, autochthonous populations in soil communities have been shown to be resilient following disturbances [46]. In the gut and in soils, however, attachment is required for extended residence, as there is often continuous input, thus more closely resembling a continuous rather than batch cultivation system. In batch microalgae cultures like those In this study, competitive exclusion, rather than physical forces, could lead to the loss of populations [18,47]. In other types of algal cultivations, like continuously fed and harvested, as well as in attached biofilm systems, introduction of fresh media, along with potential contaminants or allochthonous populations, likely contributes to increased phycosome succession and perhaps stability [48].

Shannon's (H) and Inverse Simpson's ($1/D$) alpha diversity indices were determined for each sample to quantify amplicon sequence variant (ASV)-level composition of SLA-04 phycosomes over time (Figure 2.3). H and $1/D$ increased in each of the three cultures during this experiment. MSU LA H values increased from 1.56 to 1.85 across the duration of the experiment while the $1/D$ values increased from 0.7 to 0.78. UT LA and UT HA also increased starting with lower H values of 0.92 and 1.05 and increased to a larger extent (1.2 and 1.71), respectively. A

similar trend was observed for 1/D values, increasing from 0.30 and 0.54 to 0.60 and 0.72 for UT LA and UT HA, respectively. Significance of this increased diversity was not determined as alpha diversity was only calculated using a single biological replicate for each time point, thus further work with more replicates and more regular sampling is required. *H* values between 0.90 and 1.90, and 1/*D* values between 0.30 and 0.80 are lower than what is generally measured in microbiome studies [49,50], underlining the differences between maintaining a xenic culture with limited inputs and cultures in open or continuously fed systems. The range of observed ASVs in SLA-04 phycosome communities was 19 to 40, values that are, again, much lower than found across microbiome studies (*e.g.*, soil, human gut). The reduced taxonomic diversity of the SLA-04 phycosome may facilitate a more straightforward path toward engineering consortia cultures.

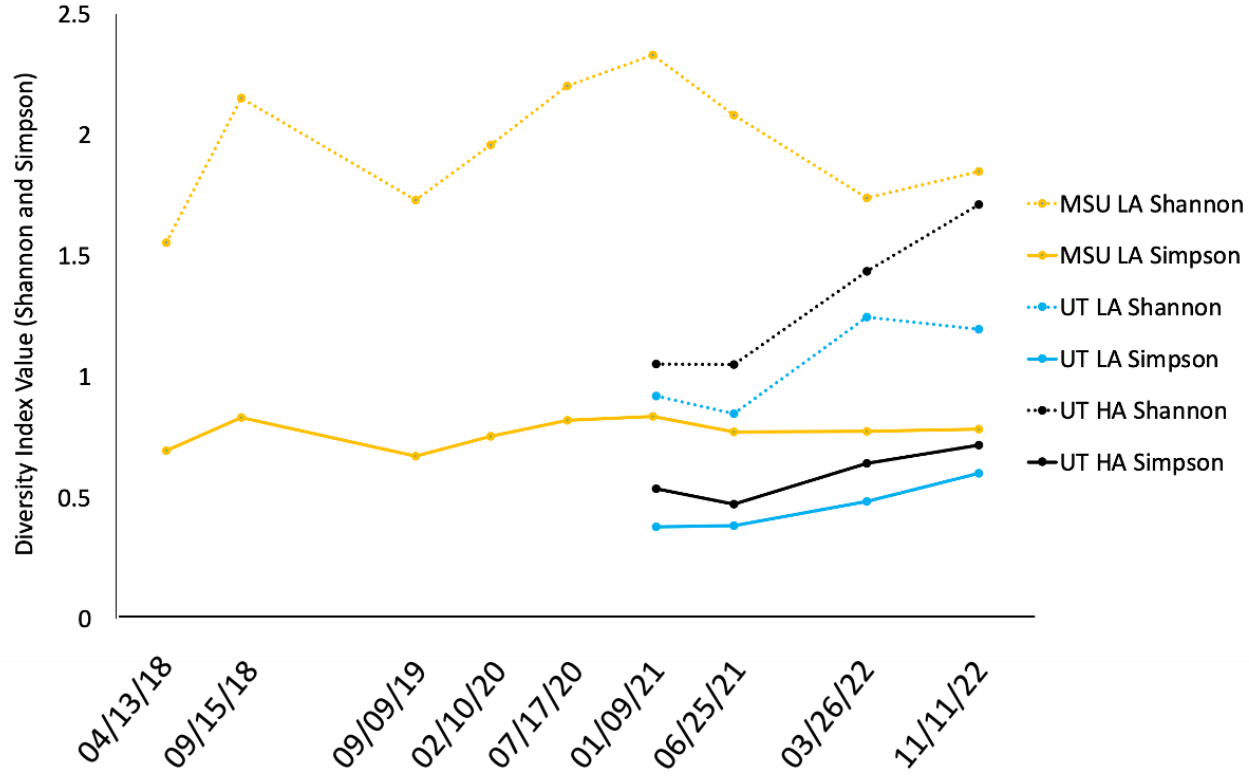


Figure 2.3. Alpha diversity of xenic SLA-04 maintenance cultures over time. Shannon's Index (*H*) (dashed lines) and Inverse Simpson's (*1/D*) (solid lines) values are plotted on the y-axis, with higher values corresponding to higher alpha diversity. Samples spanning April 13, 2018, to November 11, 2022, were collected for the Montana State University low alkalinity (MSU LA) culture (yellow). Samples spanning January 9, 2021, to November 11, 2022, were plotted for the University of Toledo low and high alkalinity (UT LA, UT HA) cultures (blue and black, respectively).

Stochastic Processes Underlie SLA-04 Phycosome Composition

A principal component analysis (PCA) was used to reduce dimensionality of the community data, assessing dissimilarity between samples in ordination space (Figure 2.4). As was expected from the relative abundance plots, temporally distinct samples from the same cultures grouped together. Separation was driven more strongly by source than growth condition, indicating that stochastic processes may govern the taxonomic composition of xenic SLA-04 cultures more strongly than host selection. Colonization or acquisition events in nascent environments (*e.g.*, axenic microalgal culture) are stochastic, and colonization success of different species in different contexts may provide ecological information about interactions [51]. In other words, colonization in the SLA-04 phycosome may be a lottery where the primary colonizers that occupy the niche space tend to persevere in the phycosome over time. Therefore, axenic cultures will acquire and build a respective phycosome dependent upon the local conditions that will likely then develop a community state that can be maintained relative to subsequent changing/stable conditions. Further work is needed with more sampling and different species to ascertain if phycosomes can be selected and maintained, not unlike hypotheses for the maintenance of healthy gut microbiomes in humans.

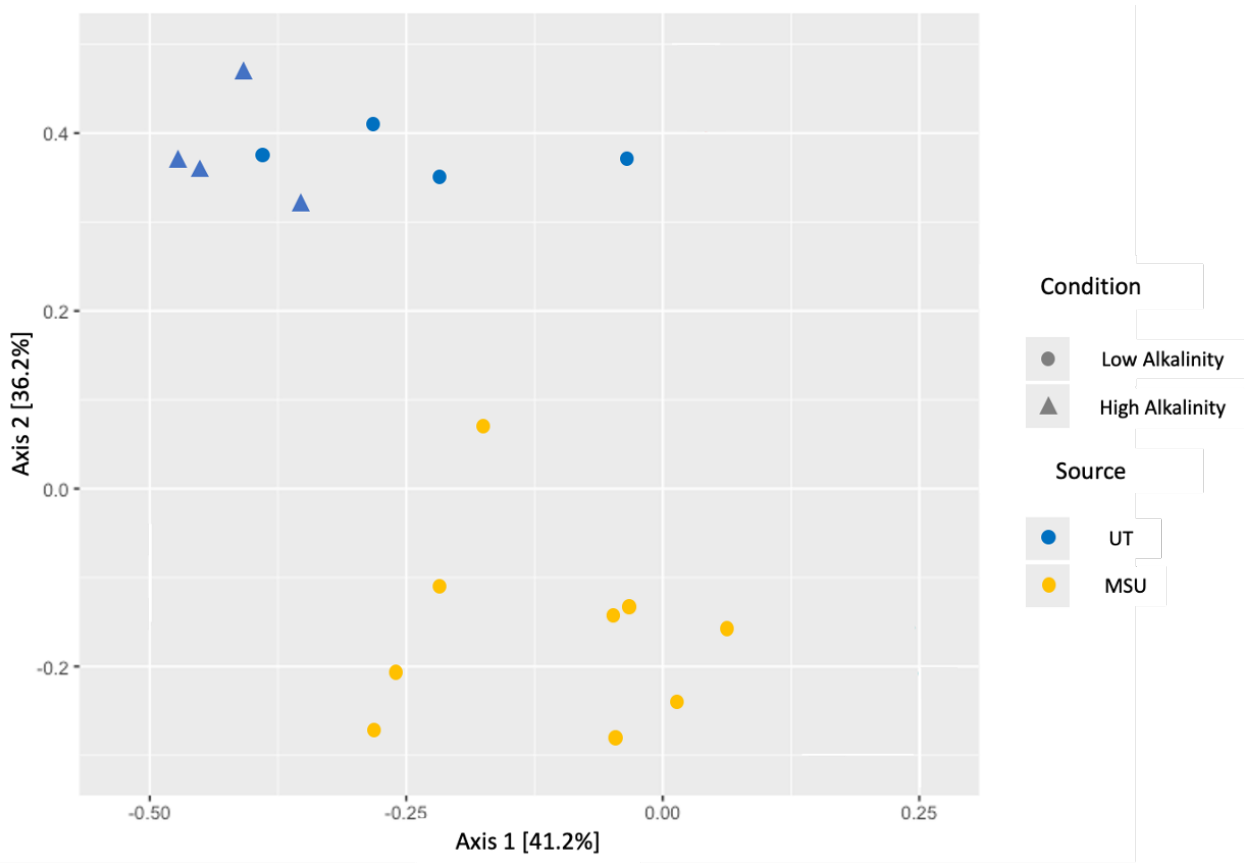


Figure 2.4: Principal component analysis (PCA) of xenic culture community samples. Data points are colored by source, with University of Toledo (UT) cultures in blue and Montana State University (MSU) in yellow. Circles represent low alkalinity cultures, and triangles represent high alkalinity cultures. The first two axes (PCA axis 1 and 2) together explain 77.4% of the variance between samples.

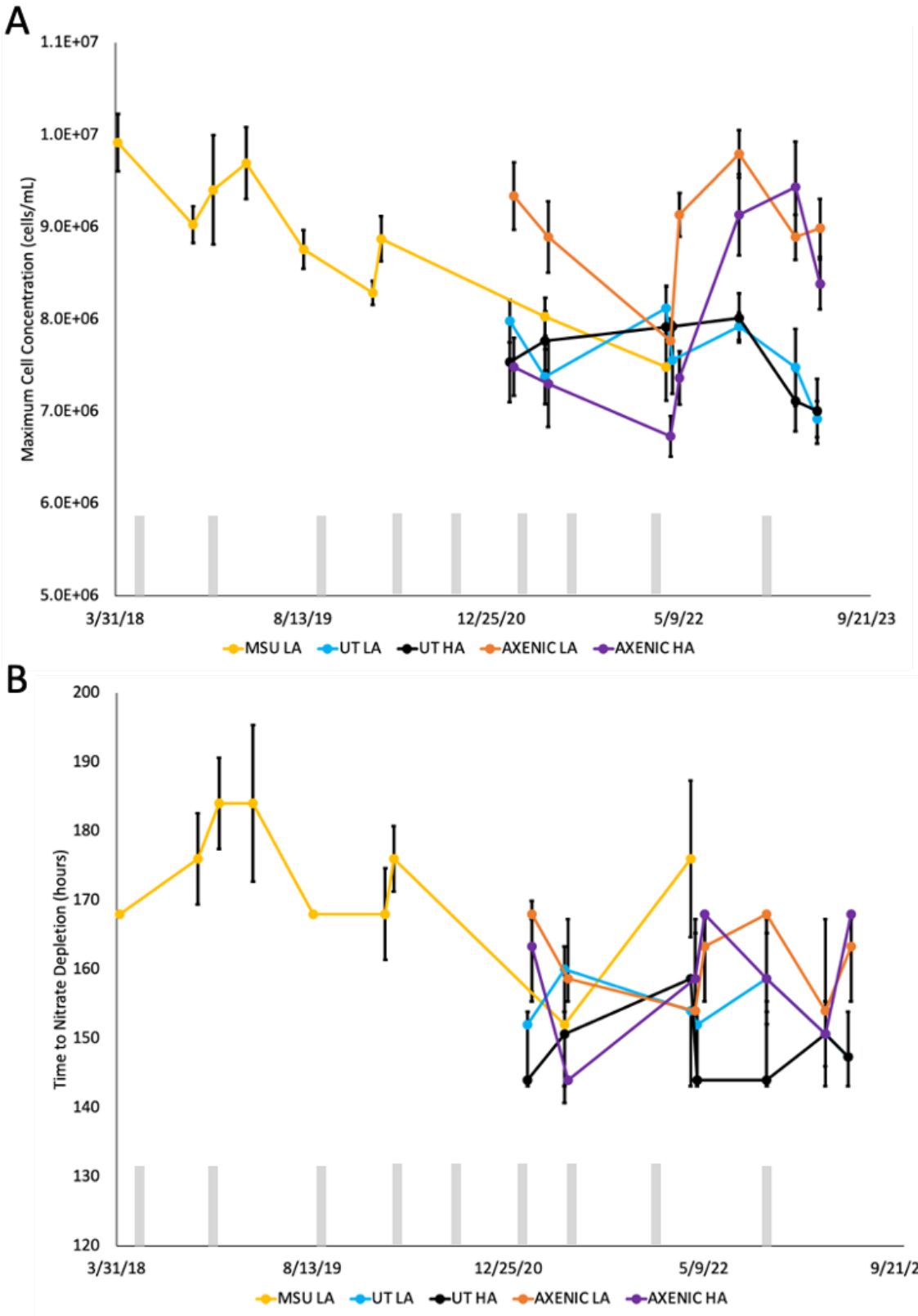
Given that SLA-04 was the only host species analyzed and there were a limited number of culture sources analyzed, we cannot definitively state that deterministic processes are not at play. We need to expand the scope of the study to include more hosts and initial community profiles [28]. For example, could species-specific and environment-dependent selections result in unique phycosome assemblies, and could these relationships relate to desired outcomes (*e.g.*, algal productivity and/or stability). As a comparison, community "types" have been identified that associate to healthy or disease states (*e.g.*, [52]), could similar profiles be identified for commercial

algal cultures? In parallel to experimental testing, predictions of assembly stochasticity should be assessed using statistical null model test or a nearest taxon index [21], but numerous samples are needed across given environments. Further, the presence of certain metabolisms and functional capabilities in the phycosome may be more important than phylogenetic similarity [53], so the outcome of competition and niche partitioning might not be defined by investigating taxonomy alone; and therefore, functional metagenomics would also be needed.

Temporally Consistent Growth of Axenic and Xenic Cultures

The final goal of the study was to determine whether culture productivity changed as a result of long-term cultivation in association with a phycosome. Because phycosome communities of the MSU and UT cultures had unique compositions, the growth characteristics of the different cultures were compared in terms of maximum cell yield (Figure 2.5A) and 'time to nitrate depletion' (TND, Figure 2.5B). These physiological markers of culture productivity are both industrially and ecologically relevant. The longest tracked culture, MSU LA, experienced a 1.3-fold decrease in maximum cell yield over the tested time surveillance, but the low value (approximately 7.5 x 10^6 cells/ml) was similar to the range for the UT-LA and UT-HA cultures (7 x 10^6 – 8 x 10^6 cells/ml). Interestingly, the maximum cell yields for axenic LA and HA cultures ranged between 7 x 10^6 to 10 x 10^6 cells/ml (Figure 2.5A). Likewise, all the tested cultures had TND values that ranged between 145 to 185 h. The results suggested that the different phycosomes for the same microalga did not result in significantly different cell yields or nitrate depletion, two important parameters for large-scale cultivation. Further work is needed to delineate population- and community-level impacts on algal production in open, challenged systems that also employ metagenomic sequencing to define the functional metabolic potential of green algal phycosomes.

Functional redundancy can contribute to stability following disturbances [52] and may be an important consideration in identifying and designing beneficial phycosome consortia.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8/13/19
12/25/20
5/9/22
9/21/23
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UT LA
UT HA
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130
120
Time to Nitrate Depletion (hours)
3/31/18
8/13/19
12/25/20
5/9/22
9/21/23
MSU LA
UT LA
UT HA
AXENIC LA
AXENIC HA

Figure 2.5. Physiology of SLA-04 cultures from experiments started using maintenance cultures from April 2018 to May 2023. (A) Maximum cell concentration (cells/mL) and (B) time to nitrate depletion (hours) in MSU LA (yellow), UT LA (blue), UT HA (black), axenic LA (orange), and axenic HA (purple). Grey lines correspond to the nine timepoints at which DNA was collected for 16S rRNA gene sequencing and community analysis (04/13/18, 09/15/18, 09/09/19, 02/10/20, 07/17/20, 01/09/21, 06/25/21, 03/26/22, and 11/11/22). Error bars represent one standard deviation from the mean (n=3) in the negative and positive directions.

In ecosystem-level ecological studies, environmental conditions are said to serve as filters, with selection against less competitive populations over time [56]. Aside from maintenance under nitrogen limitation, at high pH and HA conditions, and in successive dilutions, the cultures described in this experiment were not regularly exposed to abiotic or biological stresses. Thus, potential roles of the SLA-04 phycosome in culture stress response, as well as the assembly of the microbiome following disturbance, are yet to be defined. Introducing constraints through competition for micro- or macronutrients has also been connected with phycosome restructuring [27]. Theoretically, under a defined stress like iron limitation, SLA-04 may recruit organisms that help scavenge iron [57,58]. In a recruitment study like that, SLA-04 would need to be cultured under consistent or regular environmental input to provide the opportunity for invasion and assembly [28]. Future work should also assess resistance to pests, such as *Colpoda* sp. grazers [59] and the bacterium *Vampirovibrio chlorellavorus* [60], in axenic and xenic SLA-04 cultures. Interestingly, the UT LA and HA cultures both contain a population of *Colpoda* sp. ciliates, which, depending on conditions [59], may preferentially graze on microalgae or bacteria (Haberkorn et al., 2020). Preferential grazing activity could impact phycosome diversity, the ratio of microalgae to bacteria, and culture productivity.

Conclusion

Our work demonstrated that xenic SLA-04 stock cultures can maintain growth and productivity over the course of two to five years when the stock cultures are transferred in the same medium on a monthly time schedule. The manipulation of growth conditions did not impact SLA-04 phycosome composition as much as the history of the culture. Further work is needed to determine how axenic and xenic cultures compare in terms of exposure to invading populations during scale-up associated with outdoor, open systems. The use of the term stability as it relates to temporal microbiome composition is subjective, and studies often comment on the consistent presence of dominant taxa as being indicative of a stable community, but few studies have provided data over year-long time scales. When deterministic processes lead to the consistent return to a similar taxonomic or functional community composition following a disturbance, a community may be said to be in a stable state [55].

The present study represents an initial investigation of how alkalinity and culture history impact phycosome composition for the green microalga, *Chlorella* sp. SLA-04, and looking forward, selective pressures could be applied to test how long term xenic maintenance cultures may "direct" phycosome assembly for a desired characteristic (*e.g.,* tolerance to temperature change, pest resistance, *etc*.).

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Term	Definition	Reference
Allochthonous	A population that enters a community from an outside source; often referred to as a transient population	Barcina et al., 1997 [42]
Assembly	Given a species pool and the traits present in the species, which species and which traits will occur in a particular environmental niche	Keddy, 1992 [56]
Autochthonous	A population that is indigenous to a particular community, environment, or ecosystem	Savage, 1977 [43]
Axenic	A culture consisting of only one species	Gilbert, 1970 [62]
Deterministic	A factor influencing assembly that is imposed by the abiotic and biological environment	Stegen et al., 2012 [21]
Functional redundancy	The ability of one microbial population to carry out a process as another under the same environmental conditions	Allison and Martiny, 2008 [63]
Perturbation	An environmental change that causes a distinct selective pressure on the ecosystem, also called a disturbance.	Berg et al., 2020 [64]
Phycosome	The microbiome that is both directly attached to, and freely associated with, algal cells	Miller et al., unpublished
Phycosphere	The region immediately surrounding a phytoplankton cell that is enriched in organic molecules exuded by the cell into the surrounding water.	Seymour et al., 2017 [65]
Resilience	The rate at which microbial composition returns to its original composition after being disturbed	Allison and Martiny, 2008 [63]
Stability	Tendency of a given ecosystem to maintain a state of equilibrium and resist perturbations, or to show a return to original state follow perturbation	Lozupone et al., 2012 [55]
Stochastic	A factor influencing assembly that is unpredictable and random	Chase and Meyers, 2011 [22]
Succession	The changes in a community following a perturbation that open niche space	Connell and Slatyer, 1977 [66]
Xenic	A culture where there are multiple species	Gilbert, 1970 [62]

Table 2.1. Terminology used in this article with references in the rightmost column. Adapted and updated from Fassarella et al (2020) [67].

Supplemental Figures in This Manuscript

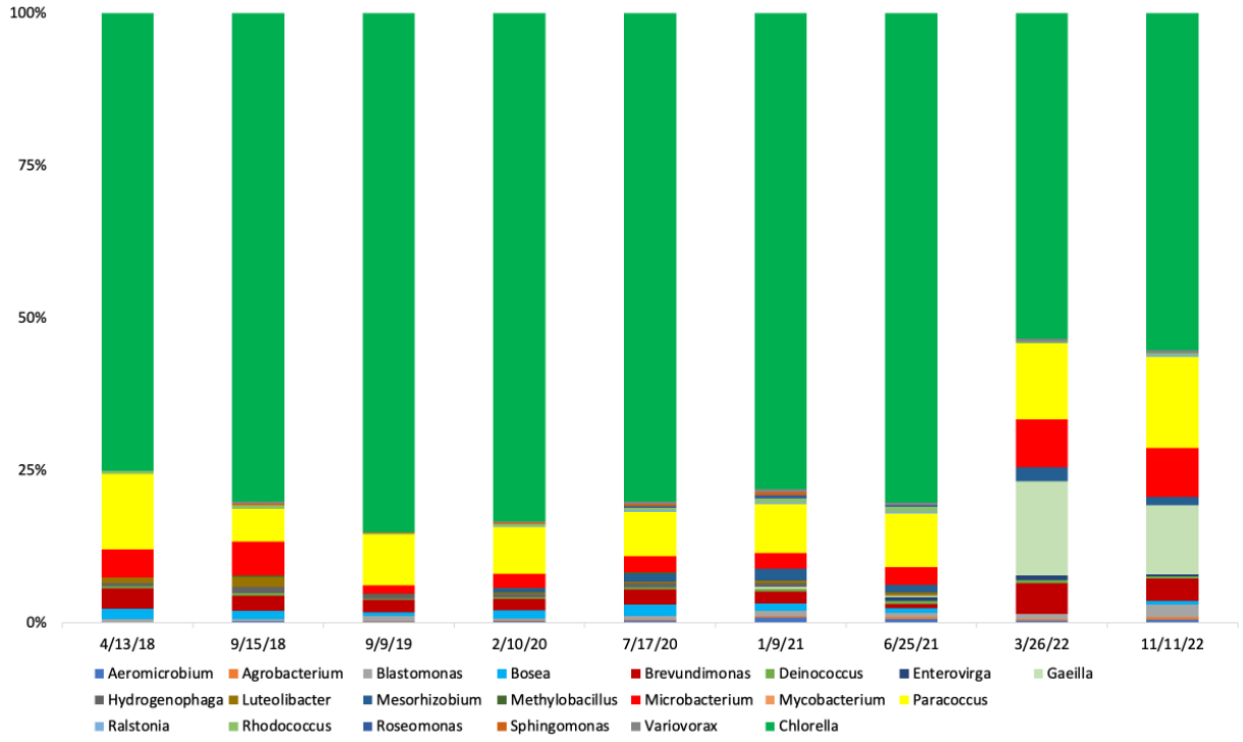

Figure 2.S1. Relative abundance of bacterial genera, and *Chlorella,* in xenic SLA-04 cultures sourced from Montana State University (MSU). Maintenance culture samples spanning April 13, 2018, to November 11, 2022. Genera with a relative abundance > 0.5% are represented with colored bars.

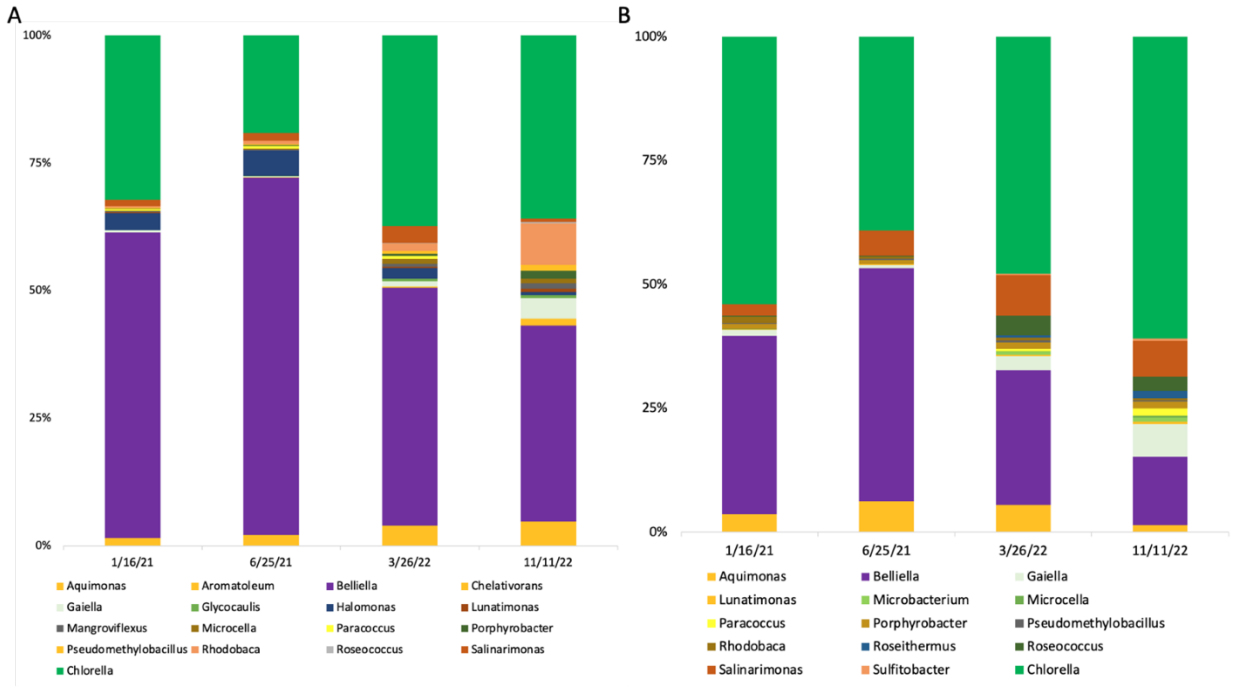

Figure 2.S2 Relative abundance of bacterial genera, and *Chlorella,* in xenic SLA-04 cultures sourced from the University of Toledo (UT). (A) Low alkalinity and (B) high alkalinity maintenance culture samples spanning January 9, 2021, to November 11, 2022. Genera with a relative abundance > 0.5% are represented with colored bars.

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CHAPTER THREE: PHYCOSOME DYNAMICS DURING SUCCESSIVE OUTDOOR MICROALGAE CULTIVATION FROM LATE SUMMER TO FALL

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Abstract

Phycosomes can impact algal productivity, and the influence of phycosome succession and turnover may be magnified during stresses such as low temperature, low solar irradiance, and/or high pH. The combined effects of abiotic (*e.g*., temperature, irradiance) and ecological factors on biomass productivity in outdoor, open raceway cultivation of the green algae were assessed on *Chlorella* SLA-04 in summer and fall conditions. Raceway ponds (200 L) were used to grow outdoor SLA-04 cultures under high pH conditions (*i.e*., pH 8 to pH 12) in five sequential runs. SLA-04 cultures had the highest biomass productivity in early runs (R1, R2, and R3) when maximum and minimum temperatures as well as radiative flux were higher while both algal dry weight and lipid levels declined with declining temperatures and light (R4 and R5). Despite lower algal biomass, chlorophyll did not decline to the same extent, and nitrate was still depleted albeit at a slower rate. The overall diversity of microbial communities (both bulk phase and algal associated) increased during the successive runs, and predominant bacterial groups shifted as temperature and light declined (R3). During R1 and R2, specific amplicon sequencing variants (ASVs) predominated (related to *Paracoccus*, *Brevundimonas*, *Rhodococcus*, and *Rhizobium*) and these ASVs declined in R3 as the light and temperature declined. These results are corroborated by decreased growth of a *Paracoccus* isolate at lower temperatures compared to SLA-04. As microbial growth slowed in R4 and R5, the following bacterial ASVs increased: *Roseivivax*, *Porphyrobacter*, unclassified GpV, *Gemmata*, *Rhizobacter*, and *Luteolibacter*. Overall, the results demonstrated that SLA-04 could tolerate transitions to lower temperatures (and light) compared to the present bacterial populations during outdoor cultivations, but the algal biomass productivity (ash free dry weight) and lipids (Nile Red fluorescence) decreased significantly. Coinciding with

these changes, *Flavobacteria* and *Deinococci* ASVs that were associated with higher algal biomass and higher temperatures declined and different ASVs (*Planctomycetes*, *Clostridia*, *Gemmatimonadetes*, *Chlamydia*) were enriched during lower algal productivity and lower temperatures. While changes in the microbial community are certainly related to the observed temperature shifts, some of the changes could be directly or indirectly related to SLA-04 growth dynamics over small or large temperature and/or light shifts. Weather (i.e., light and temperature) is predicted to have major Impacts on large-scale algal production, and the presented results provide insight into the relationships between declining light/temperature, algal growth, and phycosome composition during successive, outdoor cultivations.

Introduction

In a typical phototrophic culture system, the growth and productivity of microalgae can be impacted by light intensity, temperature, pH, gas exchange and nutrient composition [1-5], and these parameters can become more complicated in outdoor/greenhouse conditions. During typical conditions for algal biomass production, batch growth is a two-phase process dependent upon N- and P- utilization and depletion [6-8]. The first phase, where growth occurs in the presence of adequate nutrients, is designed to achieve elevated levels of cells/biomass while the second phase (nutrient deplete) is typically focused on bioproduct generation as fixed carbon (*e.g.*, lipids, starches) [9]. When photosynthesis-driven carbon fixation drives metabolism, inorganic carbon is often supplied continuously in the form of concentrated carbon dioxide, which can be costly [10]. When cultures are optimally mixed to enhance light, nutrient and gas exchange, biomass productivity reaches a maximum theoretical value [11]. However, observed experimental yields can deviate from expected yields due to abiotic and/or biotic disturbances such as temperature

shifts, daily and seasonal variation in light intensity, competition for nutrients, and predation. For example, Kleiman et al., 2021 [11] used techno-economic analyses to show that weather attributes such as solar irradiance and temperature can be significant sources of financial risk to algal production. Therefore, research is needed to elucidate algal system responses and resiliency to these anticipated disturbances, both at the algal and phycosome levels.

Algal research and algal industries face the challenge of replicating laboratory scale productivity at larger scales. Most large-scale algal cultivation is done in closed photobioreactors (PBRs) or a variation of an open system, and each method has a combination of advantages/disadvantages [8]. PBRs can provide increased productivity for desired products (*e.g*., biomass, bio-products) mainly due to easier control in terms of growth parameters and ecology [12,13], whereas open systems (*e.g.,* ponds, raceways) typically have lower productivities and less input control [14]. However, despite better control and productivities with PBRs, most economic assessments indicate that large-scale PBRs are cost-prohibitive, although some hybrid systems that use both closed and open systems have been proposed [8,11,15,16]. On the contrary, despite having less control than closed systems, comparable productivity can be achieved at a lower capital cost, making open systems a feasible option for algal biomass production [14]. However, open, large-scale cultivation can face multiple challenges, including maintaining productivity under variable changing environmental conditions (*e.g*., temperature, light, invasion). Fluctuations in temperature and solar irradiance have been shown to correlate with diminished biomass yield and biomass quality as well as a changing community composition [17], and these responses can differ for different algal species/consortia. Therefore, ongoing research and pilot large-scale production sites

continue to investigate crucial parameters for open systems, including CO_2 delivery, nutrient utilization, light, temperature, metabolic interactions, and ecology.

Spatial arrangement of microbial communities relative to host cells has long been known to impact growth and health of terrestrial plants (*e.g.*, rhizosphere assemblages) [18], however less is known about well-mixed, relatively homogeneous systems like algal raceways and reactors. While the physiological outcome of culture-scale ecology can be measured in terms of bulk productivity [19], metabolite exchange and physiological heterogeneity (*i.e.,* diffusion gradients) occurs on the micron scale in the phycosphere [20,21]. The phycosphere refers to the barrier region that limits the diffusion of algal-derived organic matter and inorganic nutrients, creating a microenvironment near the surface of the algal cell [22]. Community succession in open raceways has been documented [19] and the mechanisms explored [23], and desirable features (*e.g.*, resistance to invasion) is dependent upon the taxonomic and functional composition of phycosomes (*i.e.*, algal microbiomes) [24-25]. However, there is a lack of understanding of how these ecological shifts impact microalgal productivity over successive growth experiments, particularly during outdoor conditions. Past studies with natural systems have shown ecological relationships between algae, bacteria, and viruses [26-28], and these studies indicate that an ecological perspective must be considered for engineered systems to better optimize inputs and outputs. The described study tracked successive bacterial community changes during outdoor cultivation of *Chlorella* SLA-04 from late summer into early fall in open raceway ponds under non-sterile conditions.

Materials and Methods

Algae Culture Preparation

Experiments were conducted using a *Chlorella sorokiniana* SLA-04 that was isolated from the alkaline Soap Lake in Washington, USA in 2010, and the full genome of SLA-04 was recently published [29]. Initial cultures were started from single colonies and maintained in batch flasks on modified Bolds Basal Medium (BBM) with a starting pH of 8.7 and the following media composition: $NaNO_3$ (2.94 mM), K_2HPO_4 (1.43 mM), $MgSO_4.7H_2O$ (0.30 mM), $CaCl_2.2H_2O$ (0.17 mM), NaCl (0.42 mM), H_3BO_3 (0.18 mM), 1 mL/L S3 vitamin solution, 1 mL/L trace metal solution, 1 mL/L iron solution and 1 mL/L EDTA solution. The vitamin solution contained inositol (2.78×10^{-3} M), thymine (2.38×10^{-3} M) thiamine ·HCl (1.48×10^{-4} M) nicotinic (8.12×10^{-5} M) acid Ca-pantothenate (4.20×10^{-5} M), *p*-aminobenzoic acid (7.29×10^{-6} M), biotin (4.09×10^{-7} M, and folic acid (4.14×10^{-7} M). The trace metal solution contained $MnCl_2 \cdot 4H_2O$ (1.26 mM), $ZnCl_2$ (0.15 mM), $CuCl_2 \cdot 2H2O$ (0.11 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.07 mM), $CoCl_2 \cdot 6H_2O$ (0.06 mM), $NiCl_2 \cdot 6H_2O$ (0.04 mM), V_2O_5 (0.01 mM) and KBr (0.08 mM). The iron solution contained 4.98 g/L $FeSO_4 \cdot 7H_2O$ and 1 mL 95% H_2SO_4. The EDTA solution contained 50 g/L Na_2EDTA and 31 g/L KOH. Cultures were grown at 20°C in a temperature- and light-controlled photoincubator (VWR Model 2015-2), with a shaker set to 150 rpm, and a 14:10h light:dark cycle under 7500 lx cool fluorescent lights at an intensity of 175 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR). To grow raceway inoculum, 20 L glass carboys were aerated with 0.45 µm-filtered ambient air using aquarium air pumps. Indoor cultures (200 mL-20 L) were maintained under sterile media conditions. When cultures were prepared outdoors (200 L raceways), media conditions were unchanged aside from the use of unfiltered tap water (Bozeman, MT, USA).

Experimental Design and Raceway Cultivation

The raceway experiment was conducted in Bozeman, MT USA from July 26 to September 26, 2019, in open, uncovered outdoor raceways. Duplicate ~200 L raceways (total area 0.912 m^2), operated with paddlewheel speed of 20 rpm, were inoculated using 10% inoculum (*i.e.*, 180 L fresh, non-sterile BBM + 20 L stationary phase inoculum). Consistent with industry practices, algal cultures in each raceway were grown to nitrate depletion. After nitrate depletion, cultures were grown for an additional 24 hours, then 20 L of culture were retained, and the remaining 180 L were harvested. A fresh 180 L of pH adjusted BBM was added to the remaining culture (20 L). Air temperature and solar irradiance were monitored continuously throughout the duration of the experiment. These data were obtained from the NASA Langley Research Center (LaRC) POWER Project funded through the NASA Earth Science/Applied Science Program. Tap water was added periodically to compensate for evaporation losses as well as volume removed for periodic sampling.

Algae Physiology Assays

Each raceway was aseptically sampled every 24-72 hours for the duration of the experiment. OD_{600} was measured in 96 well plates on a plate reader (BioTek) using 200 µL of bulk sample, diluted in clean Bolds medium to attain readings under OD 1.5. pH was monitored using a standard benchtop meter (Hach) and a daily three-point calibration. Algal cell counts were performed on bulk samples using standard hemocytometer methods and a brightfield light microscope (AmScope). Chlorophyll concentrations were calculated and reported according to previously established methods [30]. Briefly, 1 mL of bulk sample was centrifuged at 14,000 x g for 10 minutes, and the supernatant was removed and used for other analyses. Cold 100% methanol

was added to the pellet and incubated in the dark at 4°C for 24 hours. After 24 hours, the sample was centrifuged at 14,000 x g for 10 min and 200 µL of supernatant was added to a 96 well plate and absorbances at 750nm, 665nm, 652nm and 635nm were measured (BioTek). Plate reader measurements were used to calculate chlorophyll *a* + *b* concentrations. Nile Red fluorescence was used to estimate total starch and lipid content of algal cultures according to previously established methods [31,32]. Nile Red dissolved in acetone at 250X concentration was added to bulk sample at a final concentration of 1 µg/mL, incubated in the dark for 10 minutes, and 200 µL was added to a 96 well plate and read in a plate reader (BioTek). The Szechrome NAS assay was used to monitor extracellular nitrate concentration according to previously established methods [32]. Briefly, 1 mL of bulk sample was centrifuged at 14,000 x g for 10 min and the supernatant was transferred to a clean tube and stored at –20 °C. Samples were diluted, added to a clean 96 well plate, and 200 µL NAS reagent was pipetted and mixed 20 times. Plates were incubated in the dark at room temperature for 30 minutes and then absorbance was measured at 570nm using a plate reader (BioTek), and nitrate concentration was calculated using a standard curve.

Moisture and ash contents of biomass (10 ± 0.5 mg) were estimated using SDT Q600 (TA Instruments, New Castle, DE). The following program was used for this analysis: under stream of N_2 gas (100 mL/min) the sample was heated to 105°C at the ramp rate of 20°C/min and then maintained for 15 min, the sample was then heated to 575°C at the rate of 20°C/min followed by an isothermal segment for 15 min, at 575 °C, the carrier gas was switched to air (100 mL/min) to completely volatilize carbon present in the sample, and the sample was then maintained at 575°C in air for 15 min.

$$moisture\ content = \frac{\text{weight}_{\text{initial}} - \text{weight}_{\text{at end of step (b)}}}{\text{weight}_{\text{initial}}} \times 100$$

$$ash\ content = \frac{\text{weight}_{\text{initial}} - \text{weight}_{\text{at end of step (e)}}}{\text{weight}_{\text{initial}}} \times 100$$

AFDW was calculated from TSS and ash content as follows

$$AFDW(\frac{g}{L}) = TSS \times \frac{100 - ash\ content}{100}$$

AFDW productivity was calculated as

$$AFDW\ productivity\ (\frac{g}{m^2 \cdot d}) = \left[\frac{(AFDW)_{final} - (AFDW)_{initial}}{Cultivation\ period, d} \times \frac{Volume,\ L}{Pond\ surface\ area,\ m^2}\right]$$

Community Analysis

Culture (10 ml) was collected in a sterile Falcon tube every 24 -72 h and then separated via slow-speed centrifugation to enrich for "closely attached to algae" and planktonic microorganisms. Samples were centrifuged at 500 x g for 5 min and the pellet (algae and closely attached microorganisms) was flash frozen and then stored at -20°C. The resulting supernatant was transferred to a new Falcon tube and centrifuged at 6,000 x g for 10 min, the supernatant discarded, the pellet flash-frozen, and then stored at -20°C until further analysis.

DNA Extraction, PCR and Sequencing

DNA was extracted from pellet and supernatant samples using the FastDNA Spin Kit for Soil (MP Biomedicals). The resulting DNA was quantified using the Qbit HS DNA quantification assay. The V3-V4 region of the SSU rRNA gene was PCR amplified with the primers 341F-

(CCTACGGGNBGCASCAG) and 805R-(GACTACNVGGGTATCTAATCC) [33]. Primers also contained barcoded overhang sequences for application in Illumina MiSeq sequencing. Duplicate 50 µL PCR reactions containing 0.2 M of each primer, 10 ng extracted DNA, 1X KAPA HiFi Hotstart ReadyMix (Roche) and 1X BSA. PCR conditions were designed according to previously established protocols. Duplicate PCR reactions were pooled, and samples were purified using the Ampure XP Bead PCR Cleanup system (Beckman). The SSU rRNA amplicon libraries were prepared using the Nextera XT Index Kit (Illumina), quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher), and sequenced in separate v3 Illumina MiSeq lanes.

Data Processing and Analysis

The SSU rRNA amplicon sequencing reads were analyzed and assembled into amplicon sequence variants (ASVs) using Qiime2 following the Microbiome Helper workflow [34-35]. The ASVs were classified to phylogenetic groups using the RDP classifier5 with a bootstrap cutoff of 80%. The relative abundance of bacterial phyla was calculated after filtering out chloroplast and mitochondrial SSU rRNA sequences. Alpha diversity indices were determined using the Past4 software package [36]. Rarefied ASV-level classifications were used to calculate Shannon H Index values (H) and Inverse Simpson's Index values (1/D) for each individual sample. Principal component analysis of log-transformed relative abundance data was performed using the prcomp function which is preloaded in the R stats package. The linear discriminant analysis (LDA) score chart, cladogram and relative abundance plots were generated according to standard methods accompanying the Huttenhower Lab Galaxy software [37]. Briefly, LDA effect size scores were calculated by comparing samples from runs that were grouped into binary designations based on a selected environmental or physiological parameter. Based upon Department of Energy targets,

runs that had an AFDW above 14 g/m^2/day were deemed to have "high" biomass productivity and runs with lower ash free dry weight were labeled "low". LDA effect size scores were assigned to taxonomic groups to determine significance in separating communities from high and low AFDW samples. The correlation in relative abundance of microbial phyla detected in our data was assessed using R packages recommended by the Bioinformatics Virtual Coordination Network (https://biovcnet.github.io/). Briefly, we calculated the Spearman correlations using the psych package [38], and the false discovery rate using the Benjamini & Hochberg method implemented in the dplyr package [39]. The results were plotted using ggplot2 [40].

Results and Discussion

Biomass Productivity Diminished During Successive Runs

The average maximum temperatures decreased over time with runs I 1, 2, and 3 having an average above 24°C, and the averages for R4 and R5 were 23.0°C and 16.2°C, respectively (Figure 3.1). The average minimum temperatures for R1 and R2 were above 12°C, approximately 9.5°C for R3 and R4, and 5.1°C for R5. The average solar irradiance from R1 to R5 was 7.86, 7.76, 7.56, 7.45, and 7.13 kW-h/m^2/d, respectively (Figure 3.1A). The change in conditions observed during the experiment is typical of a seasonal transition from Summer to Fall conditions in the Northern Rocky Mountain region. The observed decrease in temperature and solar irradiance correlated with decreasing biomass productivity (Figure 3.1B). Average biomass productivity, as measured by ash free dry weight was above 15 g/m^2/day during the first 3 runs, 9.3 g/m^2/day during R4, and 7.5 g/m^2/day during R5.

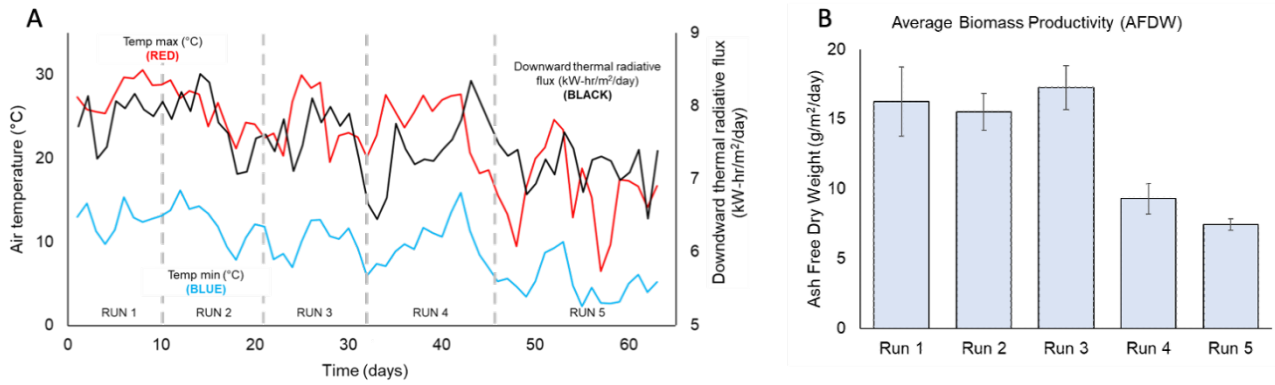

Figure 3.1. The relationship between environmental conditions and algal culture productivity was measured using temperature and light irradiance data (A) collected from NASA Langley Research Center (LaRC) POWER Project and biomass productivity data (B) from run 1 to run 5. Values plotted represent the mean of the two independent biological replicate raceways with error bars representing standard error of the mean.

SLA-04 culture conditions and composition, as measured by total lipid content, pH, chlorophyll concentration, and extracellular nitrate concentration, showed slower and lower growth levels when comparing the first 3 runs to R4 and R5 (Figure 3.2). Estimated lipid and starch content, reported in arbitrary fluorescence units (a.u.) from Nile Red staining, decreased from over 900 a.u. in R1 and R2 to 141 a.u. in R5 (Figure 3.2A). Changes in media pH were slower and less extreme when comparing early and late runs (Figure 3.2B). Increases in pH are linked to photosynthetic rates as well as nitrate utilization [41,42]. The pH started at about pH 8.25 for each run and increased to over pH 11 by day 8 in R1 and R2, by day 10 in R3 and did not reach a pH of 11 in R4 and R5. Maximum chlorophyll concentration averaged 12.6 µg/mL for R1, 14.9 µg/mL for R2, 13.2 µg/mL for R3, 9.2 µg/mL for R4 and 8.4 µg/mL for R5 (Figure 3.2C). SLA-04 cultures showed slower nitrate depletion in R1 and R2 (10 days) to R4 and R5 (15 days), although each run resulted in complete depletion of nitrate (Figure 3.2D). The declining trends in SLA-04 growth

represented a failed cultivation (*i.e.,* culture crash) for industrial scale requirements, as efficient growth, nutrient utilization, and lipid accumulation are essential for profitability.

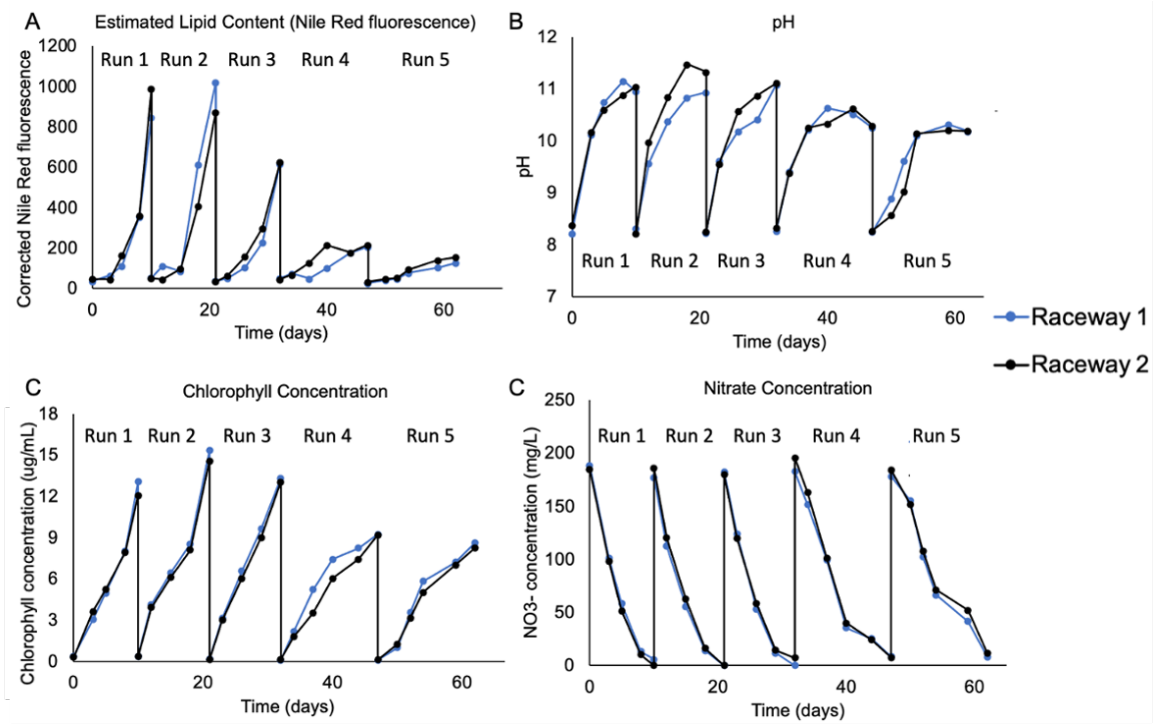

Figure 3.2. Continuous physiological data collected every 24-72 hours throughout the time course experiment. Values for each parameter are reported separately for the two independent biological replicates, raceway 1 in blue and raceway 2 in black. Values for corrected Nile Red fluorescence (A) are reported with arbitrary units corresponding to intensity of fluorescence. The pH measurements were taken from aliquots removed from the raceways (B). Chlorophyll concentration measured in μg/mL (C). Extracellular nitrate concentration (mg/L) measured using the Szechrome NAS assay (D).

Temporal Community Dynamics

Along with assessing the impacts of changing environmental conditions on culture health and productivity, temporally resolved sequencing was employed to characterize productivity responses to phycosome invasion and succession under changing conditions. Phycosome community analysis included a separation of communities based on the level of attachment to

SLA-04 cells. Samples were separated into pellet and supernatant fractions for this analysis and for the remaining analysis in this study (Supplemental Figure 3.S1). Alpha diversity metrics were used to assess community richness (*i.e.*, number of ASVs) and evenness (*i.e.*, counts of each ASV) in each sample, grouped by run (Figure 3.3). Communities in later runs were comprised of either more ASVs, a more even distribution of ASVs, or both. Communities that have higher alpha diversity may be more stable [43,44], which may suggest more resistance to invading pests or changes in conditions (*e.g.*, temperature). When the algal-associated and planktonic fractions were compared, significant differences were not observed between the two fractions (Figure 3.5A). These results suggest that the phycosphere, the microbiome within close proximity to algal cells, was not different from the associated planktonic phase. It is possible that the method did not adequately separate the fractions or there are stages of attachment and dispersal possibly driven by changes in SLA-04 physiology that were not captured. However, it is more likely that the similarity between the two fractions may indicate that consistent mixing at higher pH may promote more homogeneous distribution of phycosomes through decreased gradients (*e.g.*, dissolved carbon and nitrogen) although it is also possible that mixing does not allow adequate attachment and niche selection. Further work is needed to delineate phycosomal niche space under different growth conditions. Because large differences were not observed between the two fractions, the datasets were combined for between run comparisons.

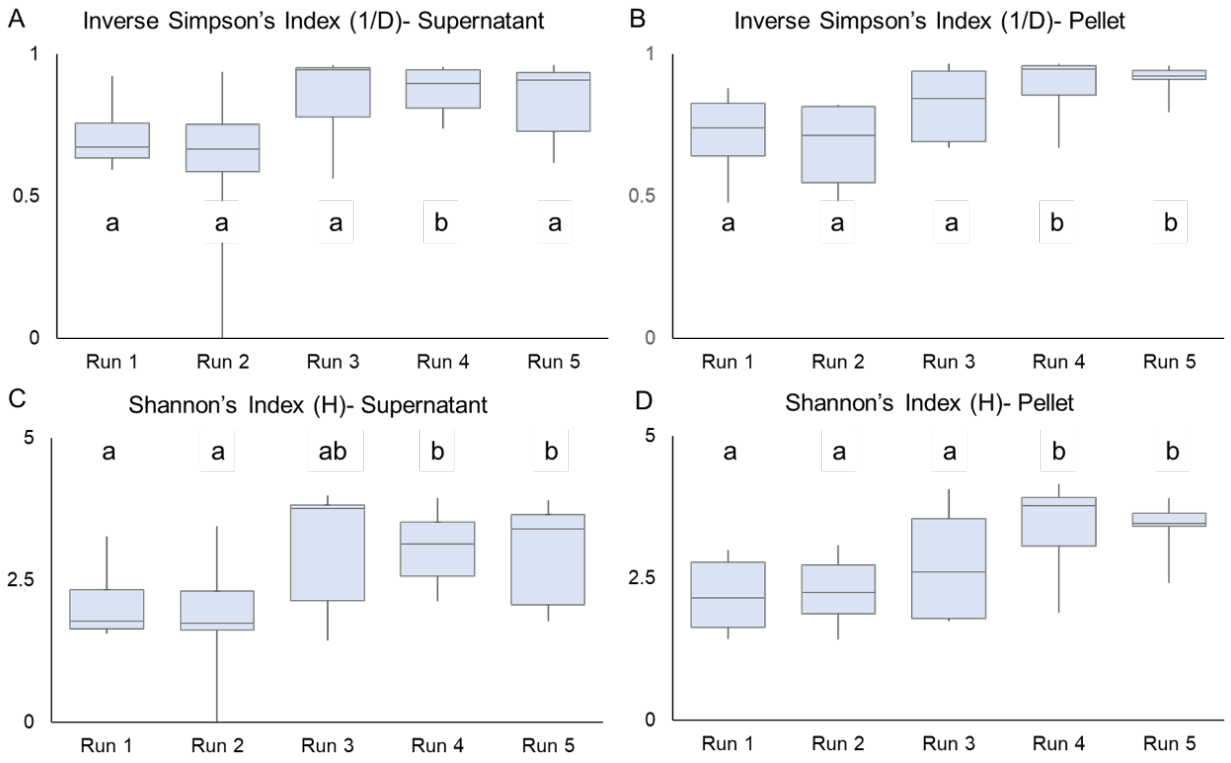

Figure 3.3. 16S ASV-level alpha diversity in samples across five runs and two replicate raceways and separated by supernatant or pellet fraction. Inverse Simpson's Indices for supernatant (A) and pellet (B) fraction samples. Shannon's Indices for supernatant (C) and pellet (D) fraction samples. The significance of differences between runs, indicated with letters above or below the box plots, were calculated using the two-way Mann-Whitney U-test with a p-value cutoff of 0.05.

The Inverse Simpson's Index (P) and Shannon's H-index (H) have been shown to yield different responses in calculating alpha diversity for complex communities [45]. Shannon's H-index gives more weight to evenness within a community whereas P is not as sensitive to a loss of dominant taxa, and similar trends were observed with both indices (Figure 3.3A and 3.3B). However, H showed larger differences between communities in early runs when compared to late runs (Figure 3.3C and 3.3D). Thus, the increase in evenness from the early to late run communities suggested that declining temperature promoted a more even increase in phycosome species

richness. The declining algal growth (temperature and light) may have also altered the fixed carbon available in the system and this may have also contributed to the observed changes (*e.g.*, r vs. K strategists).

Alpha diversity is generally correlated with metabolic diversity [46], although phenotype prediction can be difficult from SSU rRNA gene taxonomies [47]. While greater metabolic diversity in the phycosome may lead to competition with algae for macro- and micronutrients, it may also be associated with a more complete utilization of media components and greater overall biomass productivity as observed with plant systems [48]. The transition from simple consortia to a more diverse community is supported by observations in natural, phototroph-driven ecosystems [49,50] as well as in open cultures [51]. Algae and other phototrophs fix CO_2 into organic carbon compounds, which, due to the complexity of the fixed carbon, can support a wide range of microbial trophic groups. It is expected that, over time, the presence of the organic carbon pool (in addition to the added N) in an open raceway provides a diverse array of niches for colonization by autochthonous and allochthonous populations that can be advantageous or deleterious. Therefore, the extent of diversification and the relationship between phycosome diversity, biomass productivity, and nutrient recycling is important for larger-scale production.

Drivers of Community Shifts

R1 and R2 were predominated by a relatively low number of genera, and sequences indicative of *Paracoccus, Bosea and Deinococcus* were observed to be the most prevalent (Figure 3.4). A transition occurred from the initial community structure to a richer and more even community during R3. The observation that community evenness increased in later runs may indicate that the genera that dominated early communities, such as *Paracoccus,* lost a competitive

advantage. Disturbances such as changes in temperature, fixed carbon (indirectly light), or nitrogen may result in dynamic selective pressures on the phycosome. *Paracoccus* sp. are ubiquitous heterotrophic denitrifiers that have been observed in marine, freshwater, soil, plant and animal systems [52]. Given that the nitrogen source in BBM is nitrate, the presence of denitrifiers could lead to competition for resources [53] and thus diminished algal biomass productivity. However, recent studies have re-invigorated the notion that some algae can produce N_2O during nitrogen turnover [54], and thus, phycosome populations could be susceptible to changes in nitrogen pools beyond competition for nitrate dependent upon changes in algal growth (*e.g.*, NO/N_2O).

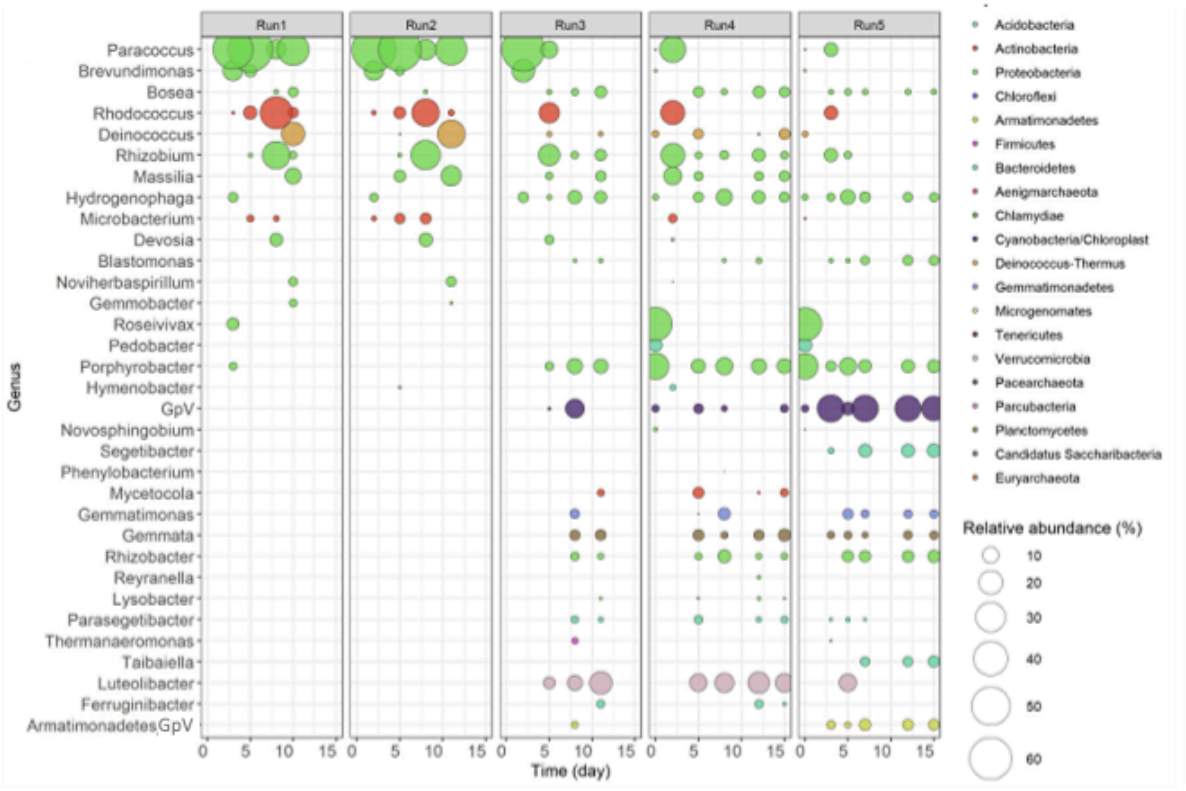

Figure 3.4. Genus-level relative abundance in communities from the pellet fraction from raceway 1 samples. Relative abundances of genera are indicated by size of bubble. Phylum-level characterizations are shown using bubble color. Additional bubble plots representing all samples are shown in Supplemental Figure 3.S2.

In addition to solar irradiance and subsequent carbon fixation effects, the changes in bacterial populations could be attributed to altered growth dynamics (both SLA-04 and bacteria). Given the impact of temperature on microbial growth kinetics and the observed changes in daily maximum/minimum temperature ranges in R3, R4, and R5, we compared growth between an axenic SLA-04 and a *Paracoccus* isolate at 4°C, 10°C, 20°C, and 30°C chosen to mimic the dynamic conditions present during the outdoor raceway experiments. The temperature-dependence of inorganic nitrogen incorporation and affinity for nitrate has been characterized in a number of bacteria and microalgae [55]. In the limited number of microorganisms that have been characterized, nitrate utilization decreases at lower temperature, with ammonium being favored, and sometimes required for growth [55]. Both SLA-04 and *Paracoccus* sp. had faster growth rates and higher maximum optical density at higher temperatures, hinting that elevated temperature could decrease time to overall maximum biomass productivity for the xenic culture. While SLA-04 had some growth at 10°C, the *Paracoccus* isolate growth was almost completely diminished (Supplemental Figure 3.S3). At 20°C, the impact was minimal for SLA-04 while the *Paracoccus* growth rate was almost 2-fold lower compared to 30°C (Supplemental Figure 3.S3). The results indicated that SLA-04 had a wider temperature range for growth (and better able to utilize nitrate at lower temperatures) that may bestow a selective advantage over bacteria at lower temperatures and/or broader temperature changes. These results also coincide with the more diverse, even bacterial communities in R4 and R5 despite a 10% inoculum transfer to initiate the next run with replete nutrients.

The relationship between samples based on run, timepoint and raceway replicate was also assessed (Figure 3.5B). There was a clear clustering along the first principal component (PC1),

driven largely by run time. Samples from R1/R2 and R4/R5 were clustered by a lower and higher PC1 value, respectively, while early R3 samples clustered with R1/R2 and late R3 samples clustered with R4/R5. Thus, samples from R3 represented a transition between the early and late runs as temperature and solar irradiance were changing significantly. Early samples, or samples from time points at or close to t=0 hours, clustered with high PC1 and low PC2 values, regardless of the run number, and these results indicated that the communities were more similar initially and then experienced strong selection during the run. However, SLA-04 dominated the culture and may have skewed the early time points for each run, and this may coincide with a vector representing chloroplasts impacting the PC2 value. Differential abundances of *Proteobacteria* and *Deinococcus* impacted PC1 values while a more even and species rich community (R4/R5) was associated with lower PC1 values.

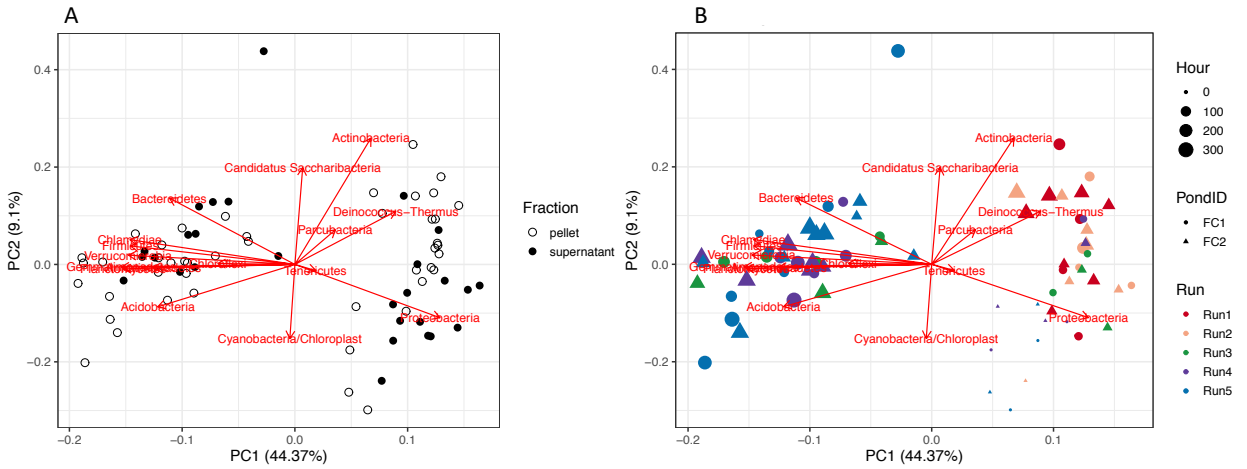

Figure 3.5. Principal coordinate analysis of 16S ASV communities across five runs in duplicate raceways, including all samples in supernatant and pellet fractions. The ordination space of both fraction type (A) and experimental conditions (B) were determined. Phylum level classifications are indicated by red text, with accompanying arrows indicating the location in ordination space. Pellet fraction samples are denoted by white circles and supernatant samples are denoted by black circles. Size of markers indicate the time of sample (hour) during each run, raceway one

(FC1) samples are represented by circles and raceway two (FC2) samples are represented by triangles. Experimental runs are separated by color of marker.

Abiotic and Biotic Influences on SLA-04 Productivity

To further investigate the impact of the observed diversification during successive algal cultivation cycles, we employed linear discriminant analysis effect size (LEfSe) analysis. LEfSe analysis indicated that several taxonomic groups were correlated with the difference in community composition between runs with high and low ash-free dry weight (AFDW) (Figure 3.6). LDA effect size scores were assigned to taxonomic groups to determine significance in delineating communities from high and low AFDW samples (Figure 3.6A), and the analyses identified specific taxonomic groups that were significantly associated with a high and low biomass productivity. The high and low was above or below the 14 g/m^2/day AFDW that was set as a target benchmark by the Department of Energy at the time of this study. For example, the genus *Deinococcus* and order *Flavobacteria* (phylum *Bacteroidetes*), were identified as contributing to a difference between runs with high and low AFDW. Run1 and R2 had high AFDW and had high relative abundance of *Deinococcus*, especially when compared to R4 and R5 (low AFDW).

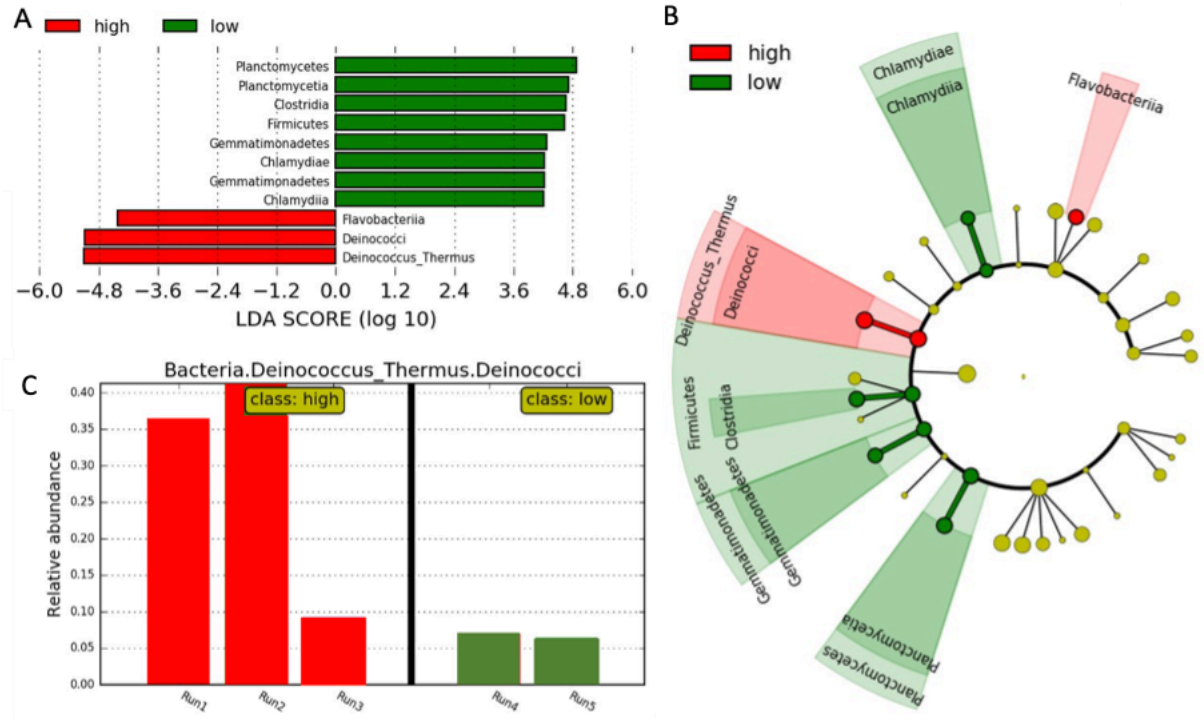

Figure 3.6. LEfSe analysis comparing communities present in runs with high and low biomass productivity (AFDW). This data is from the pellet fraction of samples collected from pond 1. A. taxonomic groups that differentiate samples that belong to the categories high and low biomass productivity. Panel B is a cladogram of the classes from panel A, showing phylogenetic relationships between the classes that are most significant in driving the difference between high and low biomass samples. Panel C shows the relative abundance of the selected genus *Deinococcus-Thermus* in each of the five runs.

Canonical correspondence analysis (CCA) was used to elucidate relationships between communities and environment (Figure 3.7) (*e.g.*, temperature and radiative flux), as it is likely that the changing environmental conditions in the late runs drove a change in community composition that impacted culture productivity. Algal biomass and biolipid productivity have been shown to vary greatly in response to changing temperature conditions [56,4]. Further, there is a direct, positive correlation between increasing temperature and bacterial growth and productivity in marine and freshwater algal cultures [50]. The CCA demonstrated that the temperature maxima

and minima as well as the radiative flux significantly impacted R1, R2, and somewhat R3 when radiative flux and temperatures were higher overall (and temperature changes were more drastic). In addition, the temperature minimum vector aligned more closely to the initial time points for R4 and R5, and later R4 and R5 time points appeared to be more driven by an overall lower temperature with less difference between the max and min (Figure 3.7). This observation also coincides with the lower CCA2 values for R5 samples and the lower temperatures and temperature differences (Figures 3.1 and 3.7). Future experiments using temperature control in open systems are necessary to disentangle the effects of overall temperature as well as temperature shifts that are likely experienced by algal cultures due to inherent diurnal as well as weather-related changes.

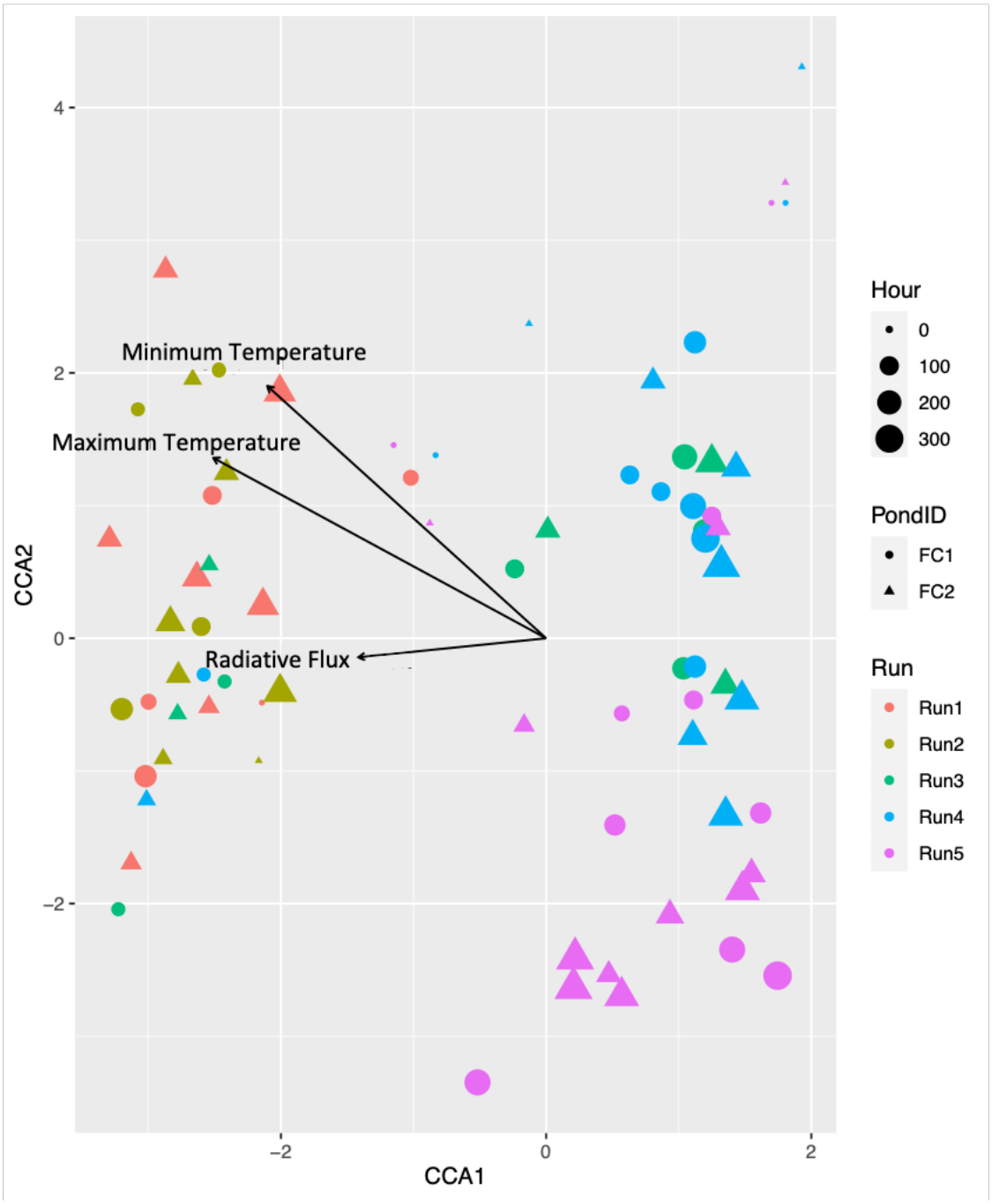

Figure 3.7. Canonical correspondence analysis (CCA) of all 16S ASV-level communities across five runs, two raceways and both supernatant and pellet fractions. The size of marker indicates time of sample (hour) during each run, raceway one (FC1) samples are represented by circles and raceway two (FC2) samples are represented by triangles. Experimental runs are separated by color of marker. The explanatory variables temperature maximum, temperature minimum and radiative flux are overlayed in ordination space as gradients that explain trends in the data.

Spearman correlation scores were used to estimate the potential relationships between phyla in all of the samples from replicate ponds (all five runs and all fractions, Figure 3.8). For example, *Bacteroidetes* have a strong positive association with many other phyla including *Planctomycetes* and *Verrucomicrobia, but Bacteroidetes* have a strong negative relationship with Proteobacteria. In fact, *Proteobacteria* and *Deinococcus-Thermus*, the two phyla identified in ordination analysis as contributing to differences between early and late samples, have negative relationships with a high number of other phyla. This complementary result provides additional indication that these phyla are correlated to differences in community composition between runs, and that direct and/or indirect relationships between phyla may be of interest as we learn more about these dynamic communities. Two sequence groups that became predominant in R5 were represented by *Parcubacteria* and *Saccharibacter*. Representative isolates of *Parcubacteria* are not available and only one isolate of *Saccharibacter* currently exist for these small-genome, small cell populations predicted to be ectosymbionts or parasites on other microorganisms [57,58], that may become more dominant in oligotrophic or slow-growth environments such as R5. These two groups were not detected until R5 and did not correlate to other bacterial groups. Additional runs may have been needed to identify possible populations that correlate with these groups, although it is also possible these small bacteria could associate with the algal cells (symbiotically or antagonistically).

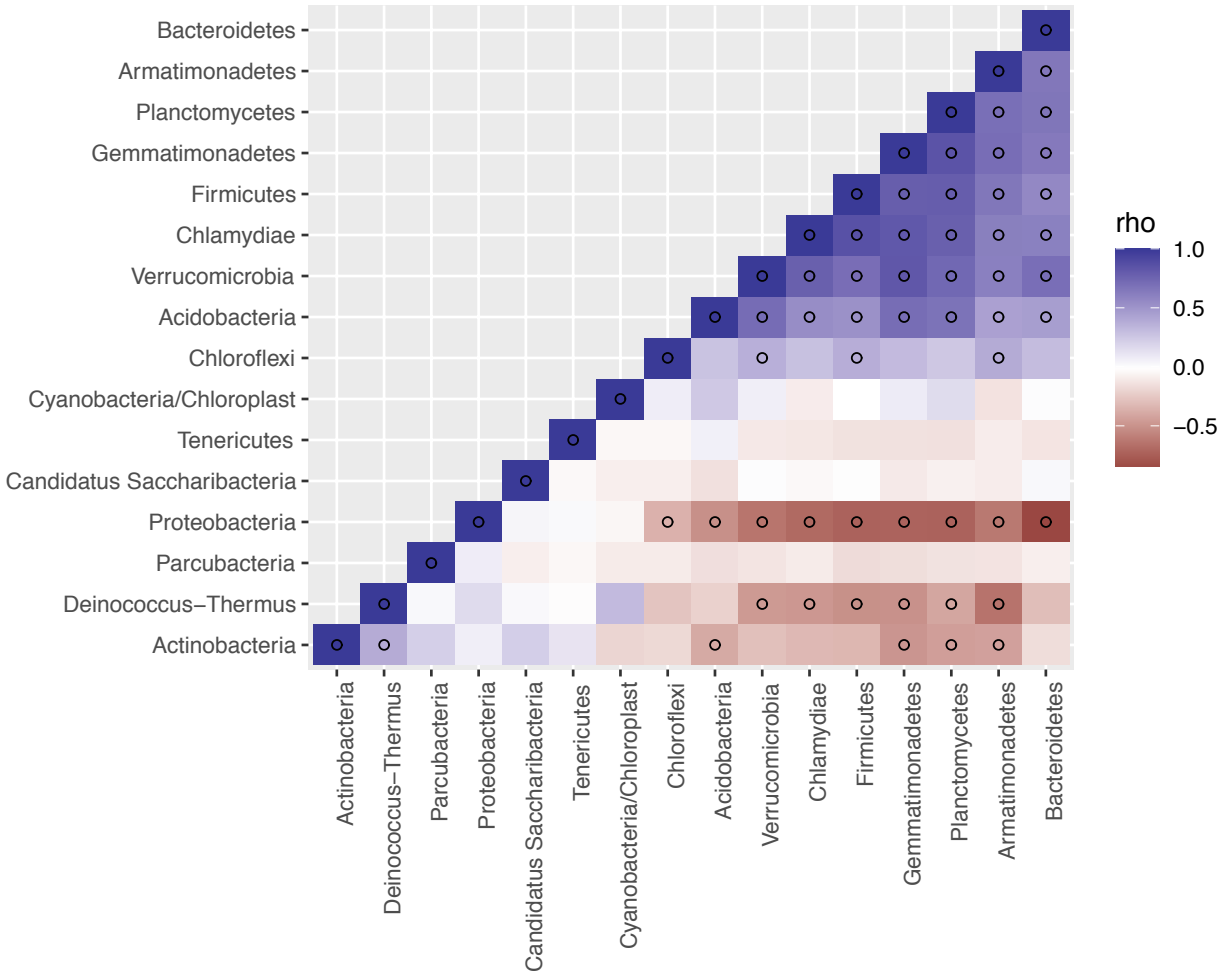

Figure 3.8. Co-occurrence network analysis. This plot represents all samples across ponds, runs and fractions. Spearman's correlation was used to investigate relationships between phyla across samples. Rho values were assigned to measure the strength of the association between two phyla. Rho values are high when observations are similar (blue color) and low when observations are dissimilar (red color). Small circles indicate statistical significance (false discovery rate < 0.01). Scores nearing one, indicated by a darker blue color, indicate that the two phyla are closely related in terms of trends in relative abundance.

Conclusion

Combined abiotic and biological variables impacted SLA-04 productivity in an open, outdoor time course experiment. In particular, diurnal cycles across the seasonal transition from summer to fall were captured, and the temperature and radiative flux changes strongly selected a

decline in the initially predominant ASVs to a more diverse and more even bacterial community. These phycosome changes were likely directly and/or indirectly related to the slower algal growth. It is still unclear exactly how phycosome diversity relates to biomass productivity and nutrient utilization. In open, outdoor raceway systems where temperature and light control is costly and difficult, changing conditions may act as selection events at the community level that ultimately contribute to biomass productivity. An improved understanding of the phycosome structure and function impacts on large, open microalgal cultivations could help optimize culture health, stability, carbon-use efficiency, and economic robustness. In particular, open, outdoor cultivations are susceptible to both seasonal and weather-related changes, particularly quick changes in temperature and light, and future work should include better resolved temporal sampling to examine finer scale changes in phycosome activity that can promote or hinder overall algal productivity.

Acknowledgements

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Supplemental Figures in this Manuscript:

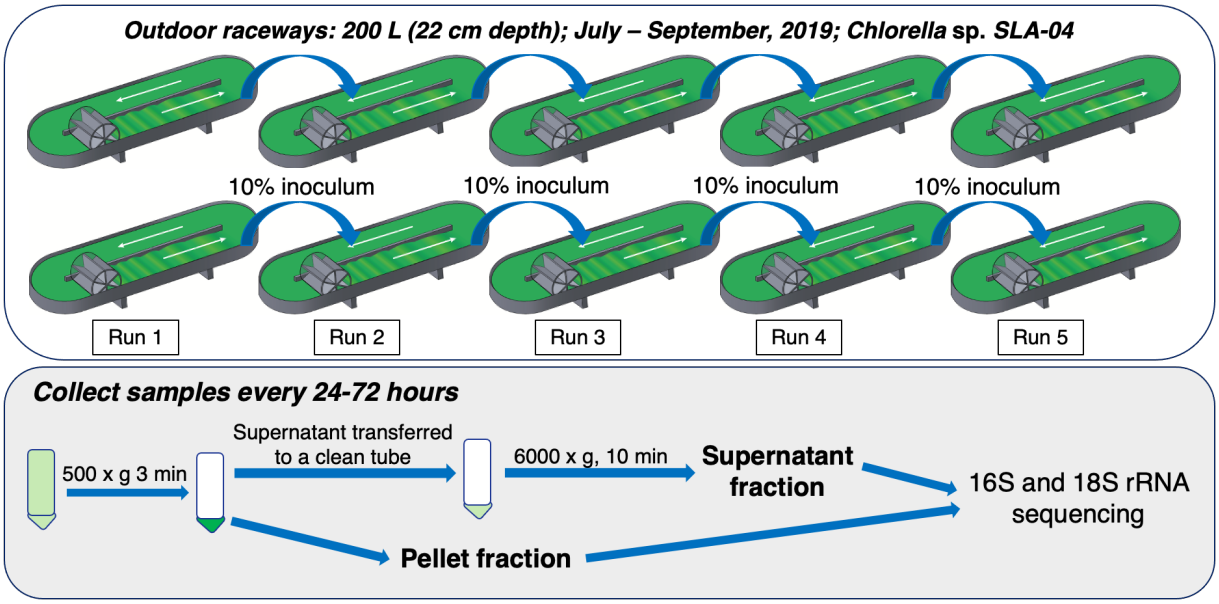

Figure 3.S1. Top: open, outdoor successive SLA-04 cultivation. Bottom: schematic depicting the method developed for the separation of pellet and supernatant fractions.

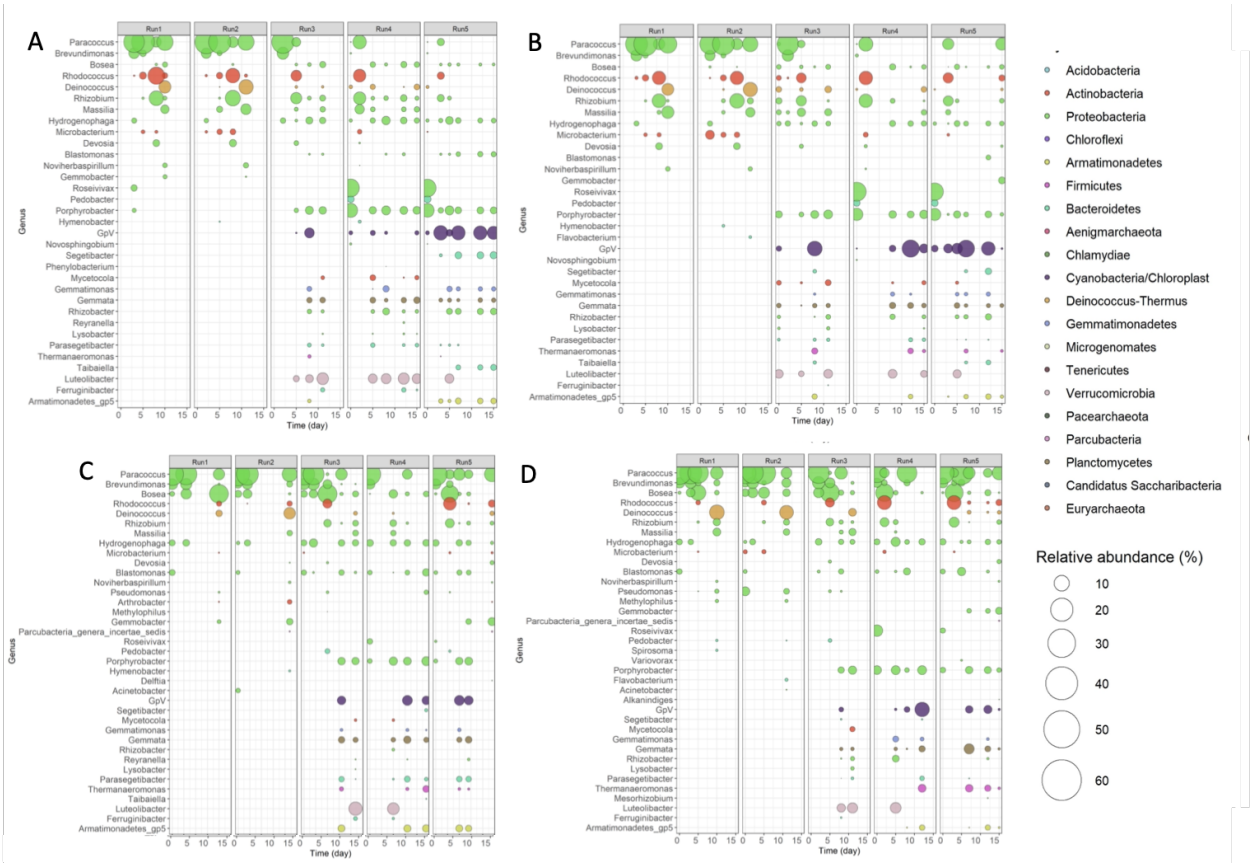

Figure 3.S2: Additional bubble plots showing the data from the entire array of samples taken during this experiment. A, B, C and D correspond to raceway 1 pellet, raceway 1 supernatant, raceway 2 pellet, and raceway 2 supernatant, respectively. Relative abundances of genera are indicated by size of bubble. Phylum-level characterizations are shown using bubble color.

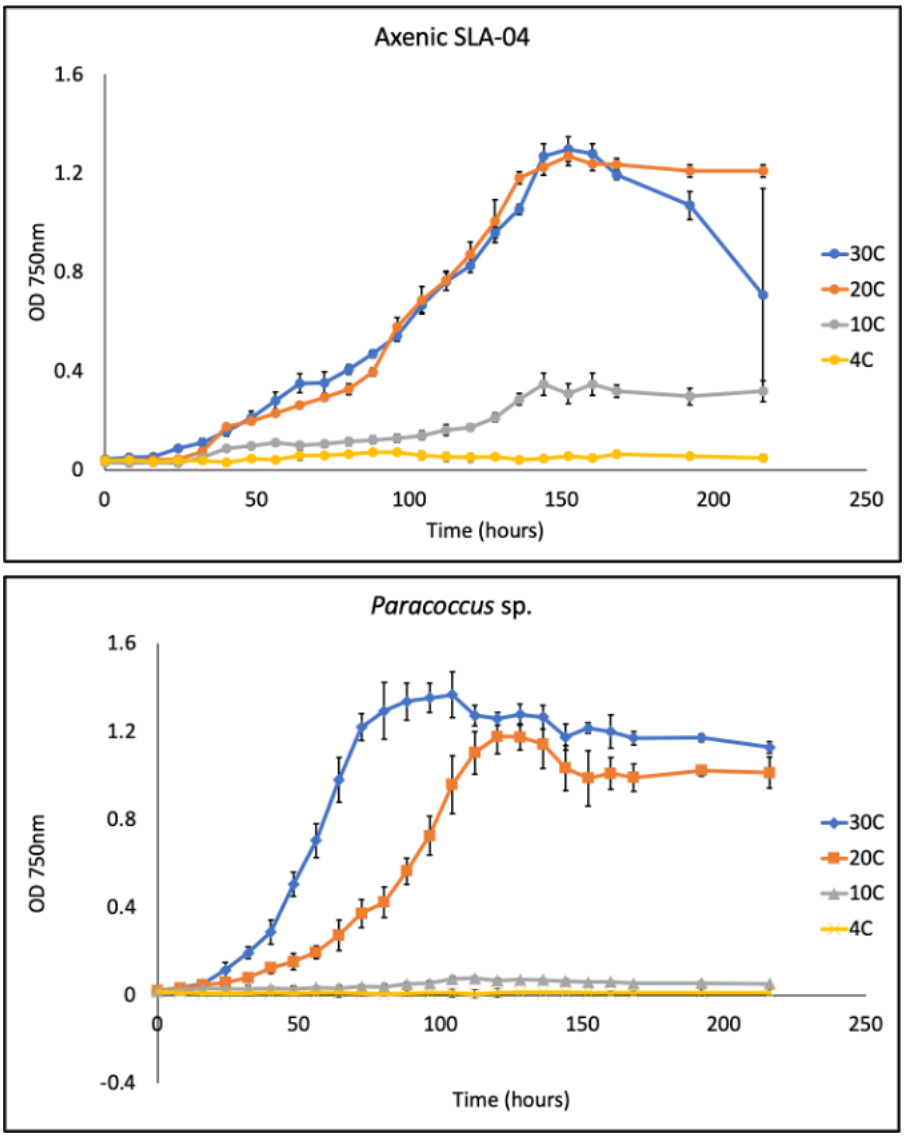

Figure 3.S3. Growth of axenic SLA-04 and a *Paracoccus* sp. isolate at 30 °C (blue), 20 °C (orange), 10 °C (grey) and 4 °C (yellow). Cultures were grown in triplicate and error bars represent one standard deviation in the positive and negative directions.

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CHAPTER FOUR: NIGHT AND DAY DIFFERENCES: DIEL PHYCOSOME ACTIVITY IN HIGH ALKALINITY MICROALGAL CULTURES

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Abstract

Phycosomes, or algae-associated microbiomes, can impact the growth, productivity and stability of algal cultures. Predictable diel changes (*i.e.,* light vs. dark cycle) in algal physiology influence nutrient cycling and local chemistry that likely impact phycosome activity and ecology and subsequently algal growth. Understanding this interplay is important for engineering productive and stable algal cultures with controllable outputs. In this study we compared the growth of axenic and xenic *Chlorella* sp. SLA-04, an alkalitolerant, green microalga, across light and dark periods within and across the diel cycle. Algal physiology, media chemistry, and extracellular organic carbon (EOC) levels were correlated with phycosomal activity and ultimately culture productivity. Bioorthogonal non-canonical amino acid tagging (BONCAT) was employed to determine the translationally active fractions of the phycosome during different points in the diel cycle under low (LA) and high alkalinity (HA) conditions. LA and HA cultivation resulted in similar cell numbers, but HA cultures exhibited higher optical density, chlorophyll, and Nile Red fluorescence (a measure of lipid and carbohydrate content) with smaller daily fluctuations in pH. Bacterial abundance and growth rate were approximately 3.5-fold and 2-fold higher, respectively, for HA conditions. Most of the bacterial growth under HA conditions occurred during the dark cycle, and this bacterial diurnal growth was not observed for the LA condition. The total LA and HA phycosomes were distinct from each other when sampled during the light period, and the BONCAT-active light samples were not only distinct from the respective total fraction but also distinctly different from the other condition (LA-active vs. HA-active). For the dark samples, LA total and BONCAT populations were more similar compared to the LA day samples, but the HA dark samples (total vs. BONCAT) were even more distinct. A *Salinarimonas* population was one

of the most active in the phycosome under the HA condition, particularly under dark conditions. Overall, LA conditions promoted lower phycosome abundance and activity compared to HA growth conditions, and the phycosomal activity in HA was more pronounced during the dark period. Despite lower levels of internal lipid and starch in the LA cultures, the LA and HA cultures had similar overall EOC levels, and these results suggest that different EOC and/or utilization patterns results coincide with the altered phycosome activity under the high alkalinity (high salt) conditions.

Introduction

Algal biofuels are a possible source for the production of sustainable transportation fuels, including aviation [1]. Continuing to pursue the development of diverse biofuel sources is essential for decreasing overall dependence on fossil fuels, even with the recent surge of renewable energy technologies such as solar and wind [2,3]. However, despite years of research, algal biofuels have not been widely implemented due to numerous challenges, namely the delivery of concentrated CO_2, culture crashes, and biomass de-watering [4,5]. Employing high pH, high alkalinity cultivation (*i.e.,* pH > 10, alkalinity > 50 mEq) may help overcome some of these hurdles by taking advantage of direct air capture [4,6] that simultaneously provides a selective barrier (i.e., high salt/pH) against culture invasion by pests [7], thus reducing costs and risks associated with the production of microalgal biomass and biofuel in open systems.

In typical batch microalgal cultivation systems there are two predominant physiological cycles: the light/dark diel cycle and a nutrient-deplete, biomass accumulation cycle. Diel cycling involves alternating light and dark periods on a consistent, repeating 24-hour pattern. During the light period, microalgae harvest light and use the energy to fix inorganic carbon through RuBisCO

into organic carbon [8], a process that results in a net accumulation of biomass [9]. During the dark period, algae no longer harvest energy from light, and instead metabolize internal carbon pools to maintain metabolic activity, in turn losing biomass to respiration [10]. In the nutrient-deplete, biomass accumulation cycle, the fate of fixed carbon depends on a changing level of macronutrients, most often nitrogen [11]. When nitrogen is present, much of the carbon is fixed into organic biomass [12]. As cell density increases and nitrogen is depleted, the fixed carbon is stored in either simple or complex forms such as sugar oligomers, starches, and lipids [13]. Increasing intracellular carbon stores could lead to higher extracellular carbon levels through passive diffusion [14], active transport [15], or, as cultures age, cell lysis and biomass turnover [16]. The exact relationship between internal carbon stores and extracellular organic carbon (EOC) flux has not been defined, but EOC concentration and composition in microalgal cultures likely changes in response to both the diel cycle and the biomass accumulation cycle.

In photoautotrophic systems where the only carbon sources are inorganic (CO_2, HCO_3^-, and CO_3^{2-}), heterotrophs such as invading bacteria and fungi, rely on photosynthate to support carbon and energy metabolism. Under high alkalinity (HA) conditions, there is an abundance of inorganic carbon, largely in the form of HCO_3^- and CO_3^{2-}, and these carbon species can be efficiently fixed by alkaliphilic or alkalitolerant microalgae [17]. The relationship between inorganic carbon concentration in the medium and EOC released from microalgae is poorly understood. An excessive release of organic carbon-rich photosynthate in open systems could leave algal cultures vulnerable to culture crashes by providing a niche for proliferation by invading populations [18-20]. Alternatively, consumption of EOC by stable, commensal phycosome communities could provide protection against opportunistic invaders [21]. Despite anecdotal observations that high

pH, HA conditions can be protective against pest/predator invasions [6,7], the relationship between local chemistry, culture productivity, and phycosome composition has not been defined.

Large-scale, open cultures are impossible to keep axenic (*i.e.,* comprised of one organism), and therefore, the phycosome will play an important role in industrial microalgal cultivation. Given the dynamic nature of xenic microalgal cultures, previous studies have had difficulty connecting growth phase, phycosome activity, and the resulting impact on culture productivity [22]. However, the response of microalgae to light and nutrient delivery in open systems means that manipulating media conditions, especially the delivery and form of inorganic carbon, may be a viable means of designing microbial consortia with controllable outputs [23,24]. Stable phycosome communities may offer protection against invaders and culture crashes [25,26], and, thus, maintaining healthy phycosomes may be vital for sustaining healthy, productive cultures [27].

Metabolic exchange between microalgal hosts and the phycosome is hypothesized to mirror the dynamic nature of diel cycle-dependent microalgal metabolism, we propose that a protective mechanism may be through controlling availability of nutrients *(e.g*., nitrogen and carbon). While dark cycle metabolism is important for maintaining microalgal viability, using carbon stores is not only counterproductive for biofuel accumulation [28] but also overall carbon-use efficiency, thus impacting symbioses within the phycosome [29,30]. Improved understanding of temporal dynamics across the diel cycle in phycosome activity might be essential for designing productive and stable xenic cultures. In the present study, we investigated the phycosomal community dynamics of the alkalitolerant, green microalga, *Chlorella* sp. SLA-04, over the first ten diel cycles during biomass accumulation. Algal physiology was assessed at the end of each light and dark period over 10 days to assess the role of the phycosome in culture growth and biomass productivity.

At nitrogen depletion, BONCAT was employed to identify active populations at distinct points in the diel cycle, correlating shifts in microalgal metabolism with phycosome activity and growth.

Materials and Methods

Algae Culture Preparation

Experiments were conducted using a *Chlorella* sp. SLA-04 (SLA-04) that was isolated from the alkaline Soap Lake in Washington, USA [6,31,32]. A high alkalinity, xenic SLA-04 culture was collected from non-sterile bioreactor cultures at the University of Toledo, split into two separate cultures, and maintained in batch flasks on modified Bolds Basal Medium (BBM) under either low or high alkalinity conditions at Montana State University. The modified BBM was sterilized via autoclaving and had the following media composition: $NaNO_3$ (2.94 mM), K_2HPO_4 (1.43 mM), $MgSO_4.7H_2O$ (0.30 mM), $CaCl_2.2H_2O$ (0.17 mM), NaCl (0.42 mM), H_3BO_3 (0.18 mM), 1 mL/L S3 vitamin solution, 1 mL/L trace metal solution, 1 mL/L iron solution and 1 mL/L EDTA solution. The vitamin solution contained Inositol (2.78×10^{-3} M), Thymine (2.38×10^{-3} M), Thiamine·HCl (1.48×10^{-4} M), Nicotinic acid (8.12×10^{-5} M), Ca-pantothenate (4.20×10^{-5} M), *p*-Aminobenzoic acid (7.29×10^{-6} M), Biotin (4.09×10^{-7} M, and Folic acid (4.14×10^{-7} M). The trace metal solution contained $MnCl_2 \cdot 4H_2O$ (1.26 mM), $ZnCl_2$ (0.15 mM), $CuCl_2 \cdot 2H2O$ (0.11 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.07 mM), $CoCl_2 \cdot 6H_2O$ (0.06 mM), $NiCl_2 \cdot 6H_2O$ (0.04 mM), V_2O_5 (0.01 mM) and KBr (0.08 mM). The iron solution contained 4.98 g/L $FeSO_4 \cdot 7H_2O$ and 1 mL 95% H_2SO_4. The EDTA solution contained 50 g/L Na_2EDTA and 31 g/L KOH. Low alkalinity (LA) conditions refer to modified BBM at an initial pH of 8.7 with 0 mM added alkalinity. High alkalinity (HA) conditions refer to modified BBM at an initial pH of 10.3 with 150 mM added total alkalinity ($NaHCO_3$ 50 mM, Na_2CO_3 50 mM). Cultures were grown at 20°C in a temperature-

and light-controlled photo incubator (VWR Model 2015-2), with a shaker set to 150 rpm, and a 14:10h light: dark cycle under 7500 lx cool fluorescent lights at an intensity of 175 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR).

Algae Physiology Assays

To assess the physiology of xenic SLA-04 cultures under LA and HA conditions, culture flasks were sampled and the OD_{600} was measured in 96-well plates (Biotech plate reader) using 200 μL of bulk sample diluted in clean BBM to attain readings under OD 1.5. The pH was monitored using a standard benchtop meter (Hach) and a daily three-point calibration. Algal cell counts were performed on bulk samples using standard hemocytometer methods and a benchtop brightfield light microscope (individual cell counts consisted of at least 300 cells). Chlorophyll concentrations were calculated and reported according to previously established methods [33]. Briefly, bulk sample (1 mL) was centrifuged at 14,800 x g for 10 minutes, and the supernatant was removed and used for other analyses. Cold 100% methanol was added to the pellet and incubated in the dark at 4°C for 24 hours. After 24 hours, the sample was centrifuged at 14,000 x g for 10 min and 200 μL of supernatant was added to a 96 well plate and absorbances at 750nm, 665nm, 652nm and 635nm were measured using a plate reader (Biotech). Plate reader measurements were used to calculate chlorophyll *a* + *b* concentrations. Nile Red fluorescence was used to estimate total starch and lipid content of algal cultures according to previously established methods [34,35]. Nile Red dissolved in acetone at 250X concentration was added to bulk sample at a final concentration of 1 μg/mL, incubated in the dark for 10 minutes, and 200 μL was added to a 96 well plate and read in a plate reader (Biotech). The Szechrome NAS assay was used to monitor extracellular nitrate concentration according to previously established methods [35]. Briefly, a bulk

sample (1 mL) was centrifuged at 14,000 x g for 10 min and the supernatant was transferred to a clean tube and stored at –20°C. Samples were diluted, added to a clean 96 well plate, and 200 µL NAS reagent was pipetted and mixed 20 times. Plates were incubated in the dark at room temperature for 30 minutes and then measured using a plate reader (BioTek).

Cell Counts to Determine Cell Ratios

Xenic culture (80 µL) was added to 20 µL 20% paraformaldehyde (PFA) for a final PFA concentration of 4% and incubated at 20°C for 2h before being washed and stored in PBS at 4°C until further analysis. Samples were vortexed in PBS containing 0.01% Tween 20 for five minutes and concentrated onto black membrane filters and flushed with ice cold MQ water twice. Samples were incubated in 4′,6-diamidino-2-phenylindole (DAPI) general nucleic acid stain at a concentration of 1 µg/mL, washed again, and then visualized using epifluorescence microscopy (Nikon Eclipse E-800). Bacterial and algal cells were counted in 10 randomly selected fields of view and reported as cell concentrations (cells/mL). At least 300 cells were counted for each field of view to ensure statistical power.

Determination of Total Extracellular Organic Carbon

The phycosomal dynamics were measured for xenic SLA-04 cultures grown under LA and HA conditions and compared to axenic cultures. Triplicate supernatant samples (4 mL) were collected at t=120 hours (pre-nitrate depletion) and t=240 (post nitrate depletion) by centrifuging at 14,000 x g for 15 minutes at 4°C and diluted 1:10 with 36 mL MQ water. Samples were stored at 4°C in washed glassware (acid washed, baked at 350°C for 3 hours) until further analysis. A Shimadzu TOC-CSH was used to measure non-purgeable organic carbon in water (NPOC) using the ASTM D7573 method with a sensitivity of +/- 0.1 ppm C. Background contamination arising

during the preparation process was assessed by processing MQ water in the same tubes, pipette tips, and glassware as the samples. This analysis was conducted by the Environmental Analysis Laboratory at Montana State University.

Diel Cycle BONCAT Labeling

The BONCAT experimental design (Figure 4.3B, C) was adapted from subsurface sediment and groundwater studies [36,37], for cultures with high growth rates. Xenic SLA-04 cultures were grown to log phase in modified Bold's Basal Medium (BBM) under LA and HA conditions. BONCAT labeling was conducted by adding 50 μM (final concentration) L-homopropargylglycine (HPG) (Click Chemistry Tools) into sub-volumes (10 mL) of the algal cultures that were maintained under constant shaking in the photoincubator. Subsampling was synchronized with the 14h:10h light:dark cycle so that hours t=1, t=2, and t=10 were sampled for both the light and dark period, with the addition of hour t=14 for the light period (Figure 4.3B). Immediately after an hour-long incubation with HPG, bulk samples were fractionated into two different fractions based on level of attachment. Samples were centrifuged at 500 x g for 5 minutes at 4°C to separate the pellet fraction containing algal cells and the bacteria that are tightly associated to the algal cells, and the supernatant was transferred to a clean tube (Figure 4.3C). Incorporation of HPG was terminated by centrifugation of both fractions at 14,000 x g for 10 minutes and washing twice with ice-cold PBS. Samples were stored in ice cold PBS at 4°C until further analysis. Bulk samples were also collected at t=0 and t=34 by centrifuging at 14,000 x g for 10 minutes and stored at -20°C for direct PCR and sequencing.

Click Chemistry Reaction

After all samples were collected, copper-catalyzed click chemistry was conducted; briefly, cells were resuspended in 100 µL and incubated in a mix of 100 µM copper sulfate pentahydrate, 500 µM tris-hydroxypropyltriazolylmethylamine (THTPA) (Click Chemistry Tools), 5 mM sodium *L*-ascorbate, 5 mM aminoguanidine hydrochloride, and 4 µM Cy3 picolyl-azide dye (Click Chemistry Tools) for 60 minutes, followed by a wash with PBS, and stored at 4°C. Each replicate sample (10 µL) was concentrated onto a glass slide and counterstained with the general nucleic acid stain DAPI (1 µg/mL). The slide was observed using epifluorescence microscopy (Nikon Eclipse E-800) and cells were counted to estimate the active fraction in each sample. The remaining click-labeled cells were stored at 4°C in PBS for fluorescence activated cell sorting (FACS).

Fluorescence-Activated Cell Sorting (FACS)

A Sony SH800S FACS was used to sort bacterial and algal cells from xenic SLA-04 cultures, and samples were diluted 1:10 in sterile PBS to improve sorting efficiency. Prior to sorting, diluted samples were vortexed in 0.01% Tween 20 for 5 minutes and then analyzed directly on the sorter. This step was added to disaggregate clusters of cells so that microfluidic droplets on the sorter contained single cells rather than cell aggregates. The sorter was operated with variable flow rate and sampling duration using a microfluidic chip with a channel size of 120 µm. Cells were sorted using the 488 nm laser for excitation of the Cy3 dye. Samples were gated manually so that sorted populations contained only BONCAT-labeled cells. To achieve this, an initial gating based on forward scatter area and back scatter area was drawn to select for bacterial and archaeal cells, which are distinct from algal cells based on size and shape. The next gate was drawn using

forward scatter width and forward scatter height to further select against erroneous inclusion of particles outside of the desired range of shape and size. The final gate, referred to as the 'BONCAT' gate, used forward scatter area and Cy3 fluorescence to capture BONCAT-labeled cells. Negative controls for each sample timepoint, prepared by omitting HPG during incubations, were used to draw the gate to ensure that only positively labeled cells would be collected. Fidelity of this method was confirmed using fluorescence microscopy as well as collecting events from the negative gate for 10 minutes to be loaded into PCR reactions. Each 'positive' sample was sorted until 100,000 events were recorded or until sample volume was depleted. A representative image of scatter plots representing unlabeled and labeled cells is depicted in Supplemental Figure 4.S1. The sorted fraction of the samples was collected in 1.7 mL Eppendorf tubes, vortexed to resuspend any cells that were deposited on the side of the tube and stored at 4°C until further analysis.

DNA Extraction, PCR and Sequencing

Following activity-based sorting, sorted cells were concentrated via centrifugation (15,000 x g for 10 minutes) and re-suspended into 50 µl of filter-sterilized PBS. PCR reactions were run in 8 µL triplicate reactions. The V3-V4 region of the SSU rRNA gene was PCR amplified with the primers 341F-(CCTACGGGNBGCASCAG) and 805R-(GACTACNVGGGTATCTAATCC) [38]. The primers used also contained barcoded overhang sequences for application in Illumina MiSeq sequencing. The 25 µL PCR reactions contained 0.2 M of each primer, 8 µL DNA, 1X KAPA HiFi Hotstart ReadyMix (Roche) and 2.5 µL bovine serum albumin. PCR was performed in an Eppendorf Mastercycler Pro S. The amplification protocol consisted of an initial denaturation at 98°C for 10 min (which also served as the cell lysis step), followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 15 s, extension at 68°C for 30 s, and a final extension step

at 72 °C for 1 min. Triplicate PCR reactions were pooled, and samples were purified using the Ampure XP Bead PCR Cleanup system (Beckman). Amplicon SSU rRNA gene libraries were prepared using the Nextera XT Index Kit (Illumina), quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher), and sequenced in separate v3 Illumina MiSeq lanes.

Data Processing and Analysis

The SSU rRNA gene amplicon sequencing reads were analyzed and assembled into amplicon sequence variants (ASVs) using Qiime2 following the Microbiome Helper workflow [39,40]. The ASVs were classified to phylogenic groups using the RDP classifier5 with a bootstrap cutoff of 80%. The relative abundance of bacterial phyla was calculated after filtering out chloroplast and mitochondrial SSU rRNA gene sequences. Alpha diversity indices were determined using the Past4 software package [41]. Rarefied ASV-level classifications were used to calculate Shannon H Index values and Inverse Simpson's Index values for each individual sample. Principal component analysis of log-transformed relative abundance data was performed using the prcomp function which is preloaded in the R stats package. The linear discriminant analysis (LDA) score chart, cladogram and relative abundance plots were generated according to standard methods accompanying the Huttenhower Lab Galaxy software [42]. Briefly, LDA effect size scores were calculated by comparing samples from runs that were grouped into binary designations based on a selected environmental or physiological parameter. LDA effect size scores were assigned to taxonomic groups to determine significance in separating communities from LA, HA, light period or dark period samples. Thus, this analysis identified specific taxonomic groups that were deemed to be significantly associated with a media condition or a diel cycle period. The correlation in relative abundance of microbial phyla detected in our data was assessed using R

packages recommended by the Bioinformatics Virtual Coordination Network (https://biovcnet.github.io/). Briefly, Spearman correlations were calculated using the psych package [43], and the false discovery rate using the Benjamini & Hochberg method implemented in the dplyr package [44]. The results were plotted using ggplot2 [45].

Results and Discussion

Axenic and Xenic SLA-04 Culture Productivity

To characterize the impact of (1) the phycosome and (2) cultivation conditions on SLA-04 growth and biomass accumulation, physiology was assessed for the duration of one 10-day growth cycle (10 diel cycles) in axenic and xenic SLA-04 cultures under LA and HA conditions. Algal cell concentration, chlorophyll concentration, optical density, extracellular nitrate concentration, Nile Red fluorescence (as an estimate for lipid and starch content), and pH were measured at the end of each dark and light period (Figure 4.1A-F). Despite similar cell concentrations between the four culture conditions, HA cultures had higher chlorophyll concentrations and optical densities than LA cultures. The higher OD measurements under the HA condition could be the result of larger cell sizes, which could be the result of (1) higher osmotic pressure and the resulting accumulation of osmolytes [46] or (2) an increased level of stored organic carbon (*e.g.*, lipids and starches) resulting from a higher concentration of bioavailable inorganic carbon [47,48]. Both of these scenarios could result in distinct metabolite production/utilization by SLA-04, potentially impacting interactions with the phycosome. Xenic LA and HA cultures reached nitrate depletion (t=144 hrs) faster than axenic cultures (t=168 hrs; Figure 4.1D), supporting biomass accumulation data (*e.g.*, chlorophyll, optical density). A 24 hr difference in reaching nitrate depletion has ecological implications, such as an elevated importance of organic nitrogen exchange, as well as

being of interest to the algal biomass and biofuel industry as increased time to nitrate depletion might lead to lower productivities [49,50].

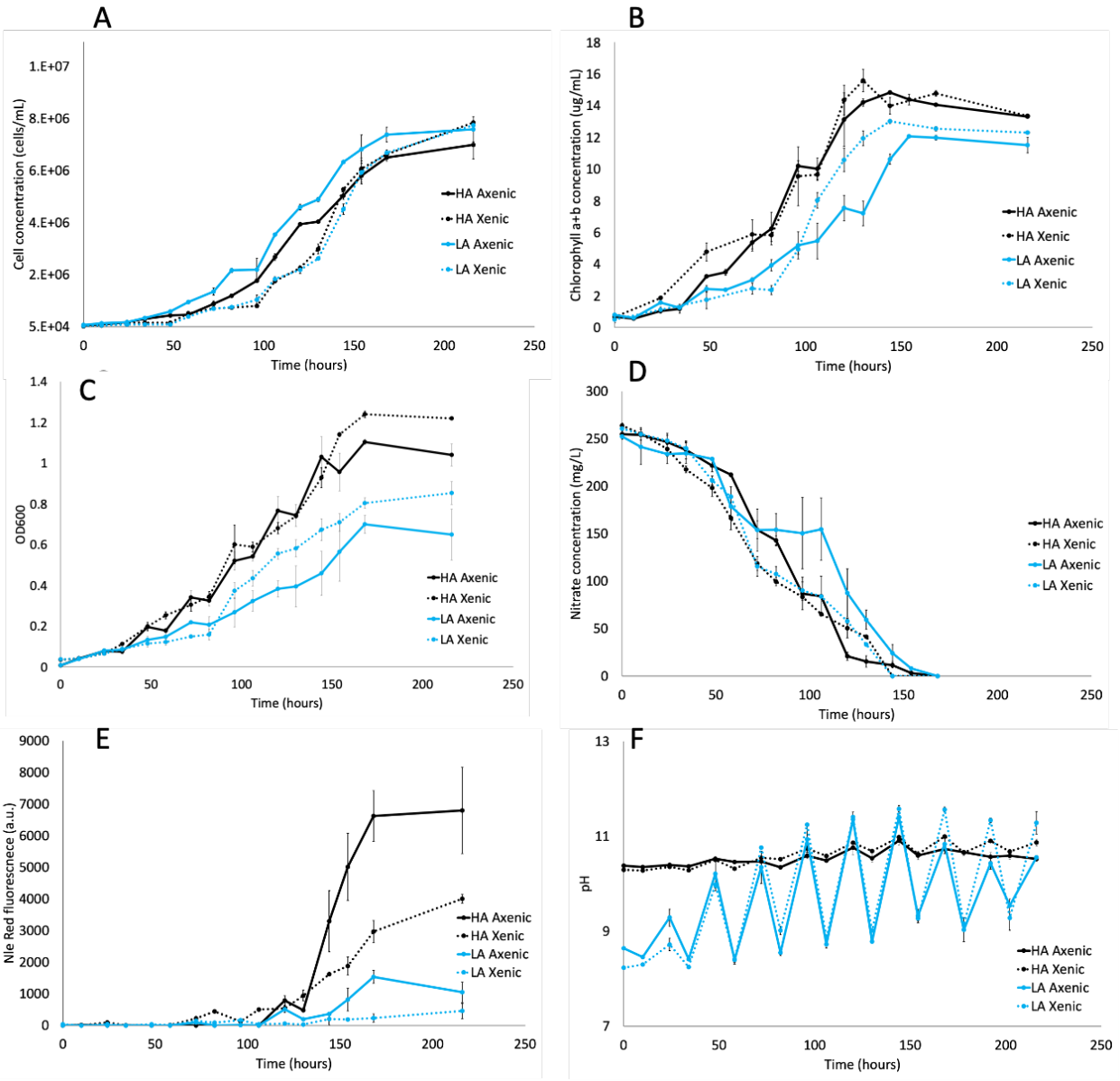

Figure 4.1. SLA-04 culture physiology over time under LA (blue) and HA (black) conditions (n=3). Xenic cultures are represented by dashed lines, axenic cultures are represented by solid lines. For plots A-C, E all measurements were taken at the end of each dark and light cycle for time points from t=0 hr to t=168 hr and then again at the end of the dark cycle at t=226 hr. (D) Nitrate concentration was measured at the end of each dark and light cycle until t=168 hr at which point

all cultures were below detection limits. (F) The pH was measured at the end of each dark and light cycle for the duration of the experiment (t=0 hr to t=226 hr). Error bars represent one standard deviation in the positive and negative direction.

Nile Red fluorescence (Fig 4.1E) and pH (Fig 4.1F) showed the greatest differences between LA and HA cultures. Nile Red fluorescence is a proxy for storage compound accumulation (*e.g.,* lipids and starches) [34,11]. As expected, the HA conditions had higher Nile Red fluorescence compared to LA conditions for both xenic and axenic cultures, but the HA-axenic culture had approximately 1.5-fold higher NR fluorescence than the HA-xenic culture (Figure 4.1E). The relationship between internal carbon stores and extracellular organic carbon concentration has been studied [51], but not when comparing axenic and xenic under LA and HA conditions. There was a marked difference in pH between LA and HA conditions as well, with LA cultures showing a daily fluctuation of up to pH 11.5 from pH 8.3. Condition-dependent, diel fluctuations in pH in phototrophic cultures has been reported [52], but the resulting impact on phycosome activity and nutrient cycling is not well understood.

Diel Cycle-Dependent Activity in the Phycosome

Previous studies have characterized growth of xenic cultures with respect to the diel cycle [53], finding a phylogenetic dependence with respect to timing of cell division [54,55] and nutrient uptake [56]. These observations are dependent on growth rate, generation time, and cultivation approach (*i.e.*, continuous vs. batch) [57]. To the best of our knowledge, this is the first study specifically quantifying phycosome growth patterns during the diel cycle. While algal cell concentrations were similar for LA and HA conditions, HA phycosome abundance was greater for the duration of the experiment, especially after nitrate depletion (t=144 hrs) (Figure 4.2A). Algal cell-bacterial cell ratios are illustrated in Supplemental Figure 4.S2. After nitrate depletion, there

was a diel cycle-dependent pattern of HA phycosome growth, where bacterial cell abundance increased during the dark period and did not show appreciable growth or even a decrease in cell concentration during the light period. The shift observed in HA cultures during the dark period could be driven by a change in competition for nutrients (*e.g.*, nitrogen, iron) as SLA-04 switches to metabolizing internal carbon stores, or an indication that dark-period-specific exometabolites (algal or bacterial origin) stimulate phycosome activity.

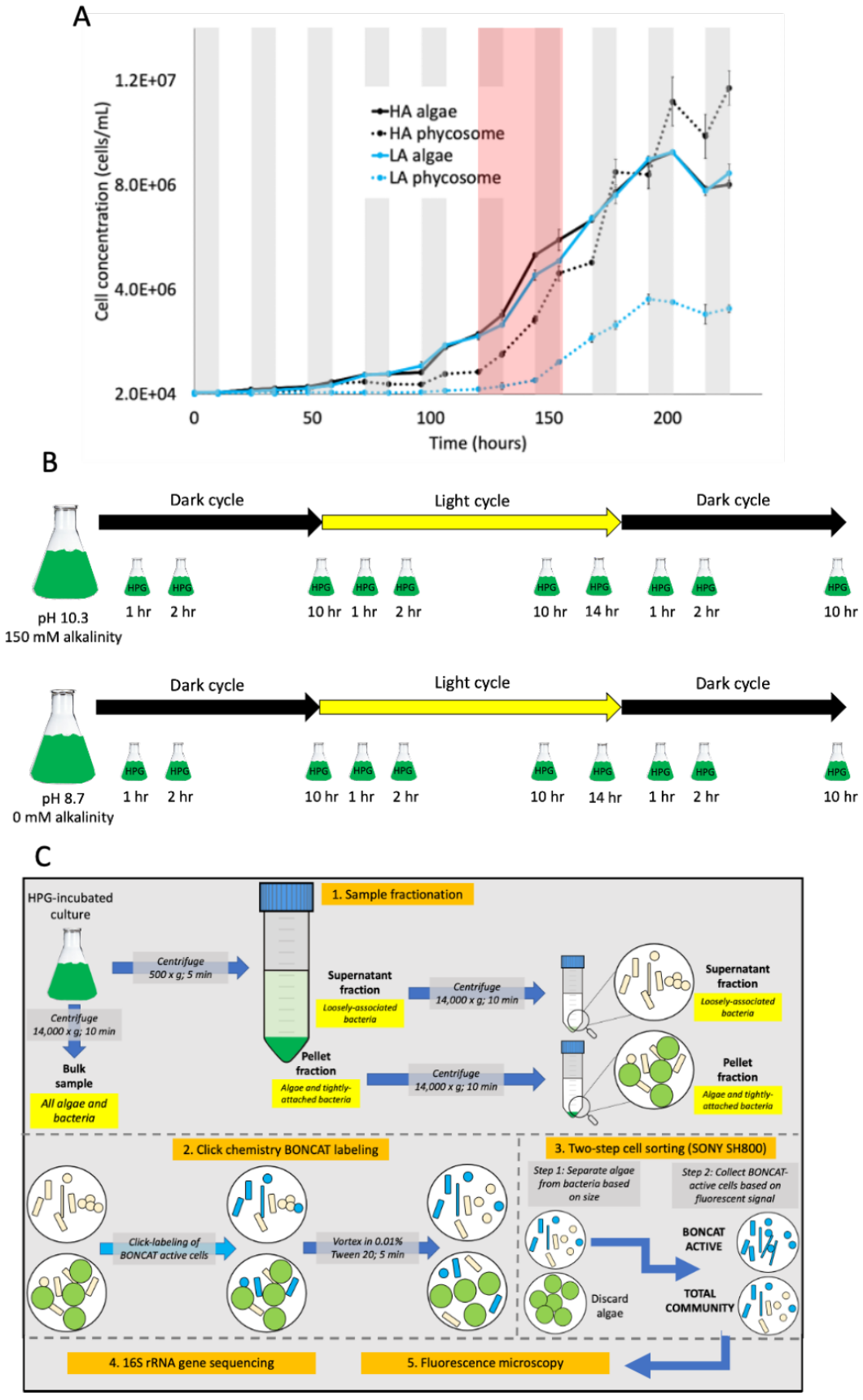
A
1.2E+07
8.0E+06
4.0E+06
2.0E+04
Cell concentration (cells/mL)
HA algae
HA phycosome
LA algae
LA phycosome
0
50
100
150
200
Time (hours)
B
Dark cycle
Light cycle
Dark cycle
pH 10.3
150 mM alkalinity
HPG
1 hr
2 hr
10 hr
1 hr
2 hr
10 hr
14 hr
1 hr
2 hr
10 hr
pH 8.7
0 mM alkalinity
C
HPG-incubated culture
Centrifuge 500 x g; 5 min
Centrifuge 14,000 x g; 10 min
Bulk sample
All algae and bacteria
1. Sample fractionation
Supernatant fraction
Loosely-associated bacteria
Centrifuge 14,000 x g; 10 min
Pellet fraction
Algae and tightly-attached bacteria
Centrifuge 14,000 x g; 10 min
2. Click chemistry BONCAT labeling
Click-labeling of BONCAT active cells
Vortex in 0.01% Tween 20; 5 min
3. Two-step cell sorting (SONY SH800)
Step 1: Separate algae from bacteria based on size
Step 2: Collect BONCAT-active cells based on fluorescent signal
BONCAT ACTIVE
TOTAL COMMUNITY
Discard algae
4. 16S rRNA gene sequencing
5. Fluorescence microscopy

Figure 4.2. Experimental design for employing BONCAT-FACS-Sequencing to identify active populations in the phycosome activity during the diel cycle in xenic SLA-04 cultures. (A) Bacterial and algal cell concentrations with the red shaded region demarcating the timing for the BONCAT experiment. (B) Schematic illustrating the timing of HPG incubations during a dark period (duration: 10 hrs), a light period (14 hrs), and another dark period (duration: 10 hrs). (C) Sample preparation workflow for separating supernatant and pellet fractions, BONCAT click chemistry labeling, FACS sorting of BONCAT active community fractions, and further microscopic and sequence-based analysis.

To identify which populations might be contributing to the growth pattern, we selected 34-hour period of the growth cycle leading up to nitrate depletion (t=120 hr to t =154 hr) to conduct the BONCAT-FACS experiment (Figure 4.2A). The temporal resolution needed to capture diel cycle growth dynamics was achieved by conducting successive HPG incubations of 1 hr each (Figure 4.2B) starting at various timepoints (t=120, 121, 130, 131, 134, 135, 144, 145, 154 and 155 hrs). Cultures were fractionated into attached and planktonic fractions prior to FACS analysis (Figure4.2C) to assess dependence of phycosome activity on physical association to SLA-04 cells. BONCAT-labeled samples were counted manually using epi-fluorescence microscopy prior to sorting to ascertain the fraction of phycosome cells tagged as BONCAT-active (Figure 4.3). When comparing activity across all sample points, planktonic phycosome populations *(i.e.,* supernatant fraction) showed significantly higher (p=0.001, two-tailed t-test) levels of activity (Figure 4.3B) than attached populations *(i.e.,* pellet fraction) (Figure 4.3A).

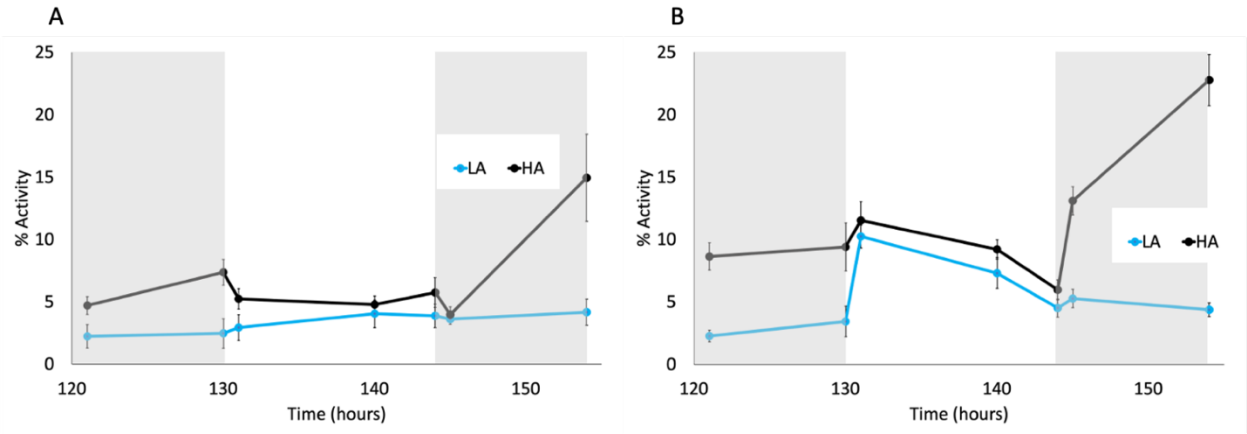

Figure 4.3. Percent BONCAT activity of phycosome communities in LA (blue) and HA (black) xenic cultures (n=3). Plots show average activity for all samples separated into pellet fraction samples (A) and supernatant fraction samples (B). Error bars represent one standard deviation in the positive and negative direction. Grey shaded regions represent dark cycle periods.

The dark-cycle growth of the phycosome that was previously observed (Fig 4.2A) was, in part, supported by BONCAT, where activity increased during the dark cycle in HA cultures. It is possible that some microbial populations are unable to incorporate BONCAT and/or respond to HPG itself, but previous work has shown that low HPG levels can capture a majority of populations in a substrate specific manner [37,58].

Amplicon SSU rRNA gene sequencing of BONCAT-FACS samples was used to characterize the BONCAT-active and total communities separately (Figure 4.2C). Relative abundance of samples from HA (Figure 4A) and LA (Figure 4.4B) cultures revealed condition-dependent compositional differences in total community fractions. Abundant ASVs in HA cultures such as *Belliella* and *Paracoccus* had lower relative abundances in LA cultures. Similarly, *Salinarimonas* and *Roseococcus* ASVs comprised a larger part of LA communities than HA communities, and a single *Salinarimonas* ASV dominated total and active communities in LA cultures. The same *Salinarimonas* ASV was enriched in the active fraction of HA phycosome

communities. *Salinarimonas* spp., like many of the other "active" populations, are halotolerant, alkaliphilic organisms [59] that likely grow under the high pH of both LA and HA conditions. *Belliella* ASVs made up a large proportion of the total community fractions in many HA samples but were not observed to be as abundant in the BONCAT-active populations, indicating that the *Belliella* ASVs likely grow at other times in the biomass accumulation cycle, or, alternatively, are not able to incorporate HPG at detectable levels. Across all samples, 91.4% of ASVs from the total community were found in at least one BONCAT-active sample, and 2.9% of BONCAT-active ASVs were not represented in a total community sample. In total, 42.6% of ASVs were found in HA and LA total community samples.

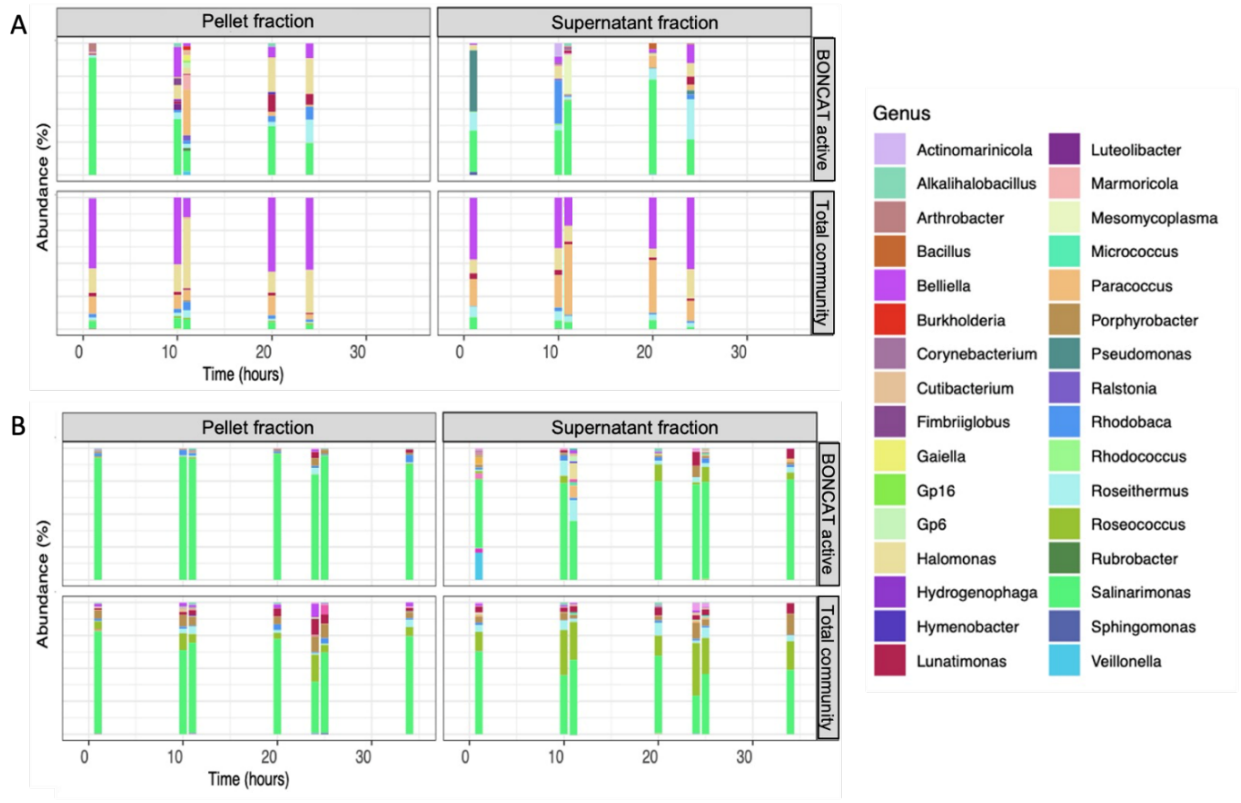

Figure 4.4. Composition of total and BONCAT active communities for the duration of the activity labeling experiment. Pellet and supernatant fractions were analyzed separately in cultures under

(A) HA and (B) LA conditions. The legend (right) indicates the genus-level classification for each of the taxonomic groups.

Principal component analysis (PCA) was used to further investigate relationships between active and total fractions in HA and LA conditions (Figure 4.5). By viewing samples in ordination space, we confirmed that the LA or HA condition could account for a significant portion of the observed differences for total communities. There was a general distinction between LA and HA samples taken during the light period for both total and active fractions (Figure 4.5A), and these results suggest that the total LA and HA phycosomes are different and therefore also have different active fractions. Interestingly, the LA total and active fractions were separated along PC2, while HA total and active fractions were separated more by PC1 (Figure 4.5A), and this result could be related to the different pH behavior of LA versus HA conditions. For the dark samples, clustering was much more distinct for the HA total and active fractions (Figure 4.5B), and this result suggests a predominance of certain ASVs at the selected time points that also coincided with the observed microbial growth during the dark period. For the LA conditions in the dark, the total and active fractions were more similar compared to the light period, and these results suggested less microbial activity during the dark period that may coincide with the drastic pH shifts encountered in the LA condition.

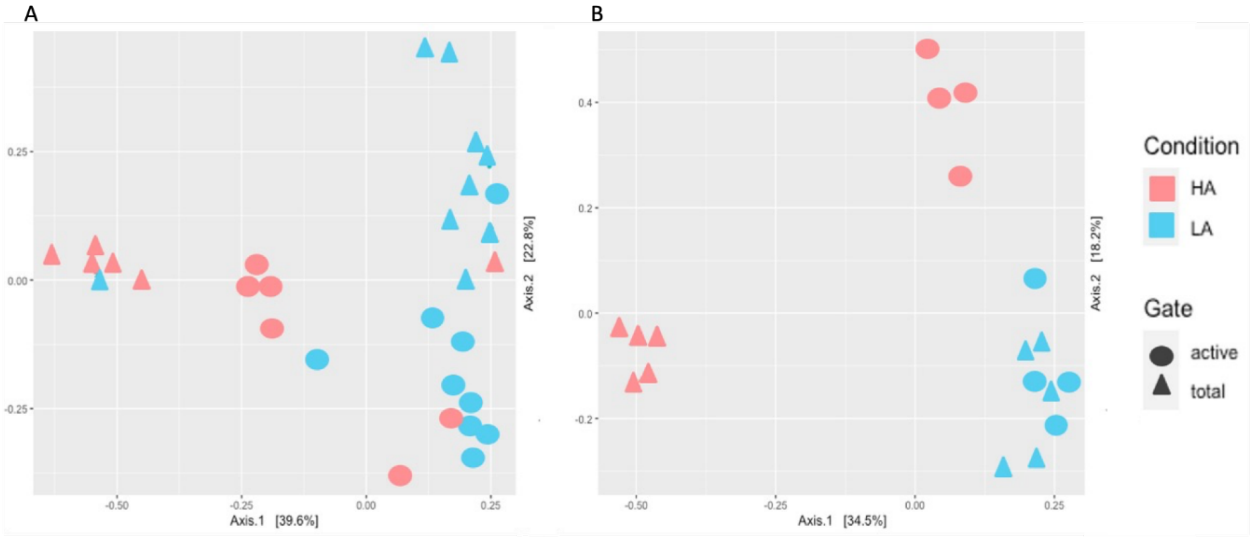

Figure 4.5: Principal component analysis illustrating dissimilarity between active (circle) and total (triangle) community fractions under low alkalinity (blue) and high alkalinity (red) conditions. Samples were taken during the light cycle (A) and dark cycle (B).

LEfSe (linear discriminant analysis effect size) was used to identify populations likely contributing to differences between defined conditions (HA total versus active) (Segata et al., 2011). Briefly, the LDA (linear discriminant analysis) score chart (Figure 4.6) reveals which ASVs contribute to differences in the HA active and total community fractions during the dark period. As opposed to the numerous ASVs correlated to the total phycosome community, the *Salinarimonas* ASV was the only group correlated to the active fraction during the HA dark period. The results suggested that this bacterial group could interact (*e.g.*, compete with or provide metabolites) with SLA-04 via metabolism of algal-based photosynthate during non-light conditions. Activity of organisms like *Salinarimonas* sp., which have been found to have a stable presence in these cultures, could provide dark period protection against invading opportunistic pathogens or provide novel osmolytes in the high salt conditions associated with HA cultures [19,60].

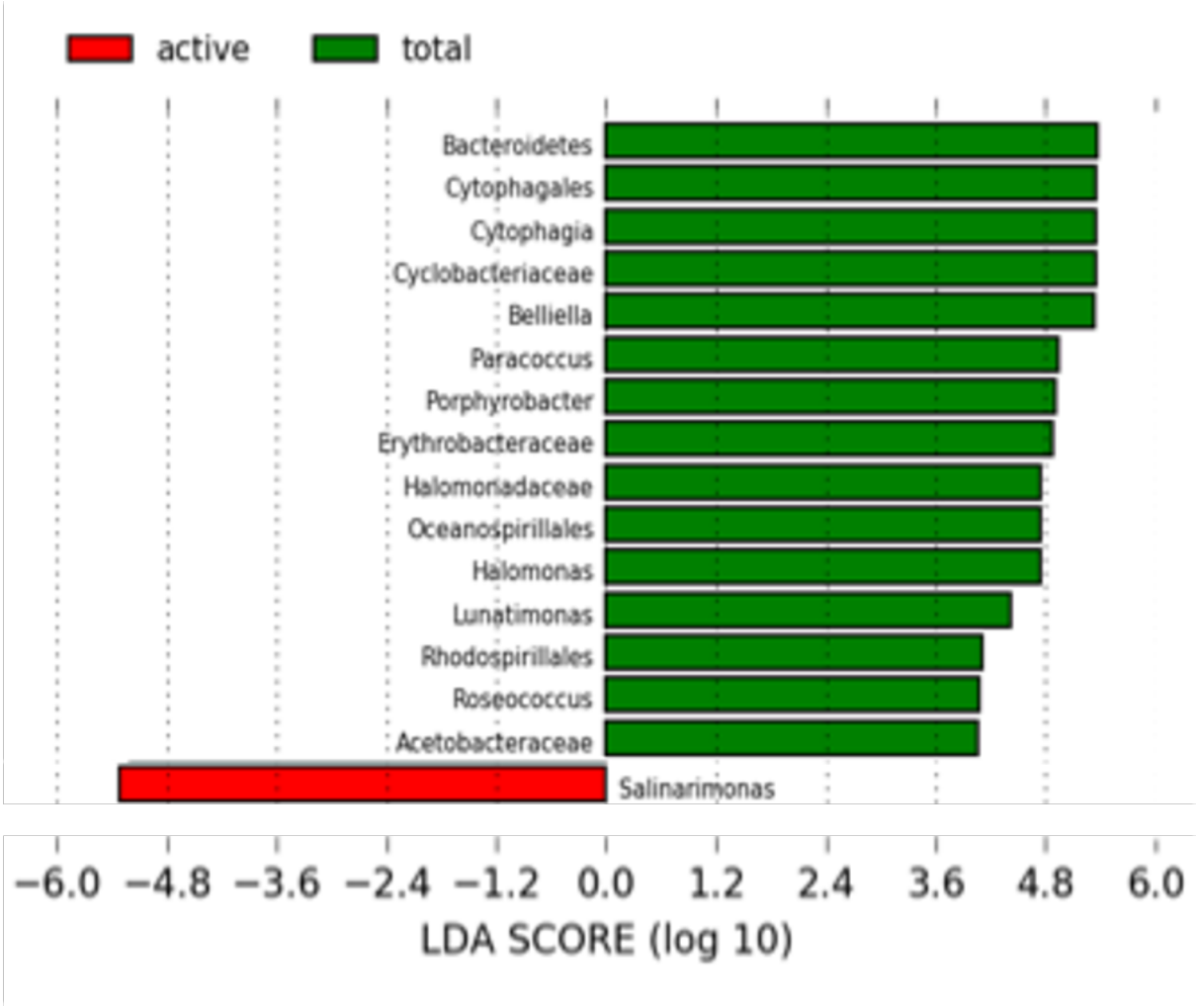

Figure 4.6: Linear discriminant analysis (LDA) score chart from LEfSe. Only HA, dark cycle samples were included in this analysis. Red bars represent groups that significantly contributed to differentiating the active samples from the total samples. Green bars represent effect size of groups that were abundant in total samples but not in active samples.

Condition-Dependent Constraints on Phycosome Activity

We hypothesized that the proliferation of bacterial populations relative to SLA-04 that occurs after nitrate depletion might be due to an increase in EOC concentration following nitrate depletion, particularly under the high inorganic carbon loads of the HA condition. It is well documented, in this system [6] and in other circumneutral [47] and alkaline systems [61], that

microalgal internal carbon stores significantly increase after nitrogen depletion. However, it is unclear from these foundational studies how internal lipid and starch accumulation correlates with EOC concentration. In a study on a close phylogenetic relative to SLA-04, *Chlorella vulgaris,* increased levels of inorganic carbon were associated with greater release of organic matter during the stationary phase [51]. The relationship between intra- and extracellular organic carbon likely varies between microalgal species and depends on local chemistry (*e.g.,* form and concentration of inorganic carbon) and available light and other nutrients. We measured EOC in axenic and xenic cultures to define how EOC is produced and consumed differentially in the presence or absence of a phycosome. Concentrations of EOC in xenic SLA-04 cultures were lower than axenic cultures under both LA and HA conditions, and these results suggest that there was EOC utilization by the phycosome (Figure 4.7A). Given that the bacterial populations present in these cultures are predominantly heterotrophic, carbon-rich photosynthate is a predictably important driver of phycosome activity and growth. If EOC flux is dictated, in part, by the form and concentration of inorganic carbon provided to the host microalga, manipulating delivery of inorganic carbon may be a sensible approach for designing xenic cultures with controllable outputs. For example, if HA is used to promote direct air CO_2 capture, phycosomes that can produce helpful osmolytes would be beneficial under the associated high salt conditions.

Despite approximately 100-fold higher inorganic carbon in HA cultures relative to LA cultures, EOC was not significantly elevated *(p*=0.251, two-tailed *t*-test). EOC might be limited in HA cultures given the higher osmotic pressure under conditions with elevated Na^+, HCO_3^- and H_2CO_3. Conditions with high ionic stress often lead to the accumulation of intracellular osmolytes, also called compatible solutes [46,62]. Compatible solutes common to *Chlorella* sp. are often

carbon-rich (*e.g.,* sucrose, glycerol), nitrogen rich (*e.g.,* glutamate, proline), and sulfur-rich (*e.g.,* dimethylsulfoniopropionate (DMSP)) and could act as potential sinks for nutrients in SLA-04 cultures when stored intracellularly [63,64]. When biomass is overturned, however, nutrient-rich osmolytes may be released, likely stimulating phycosome populations.

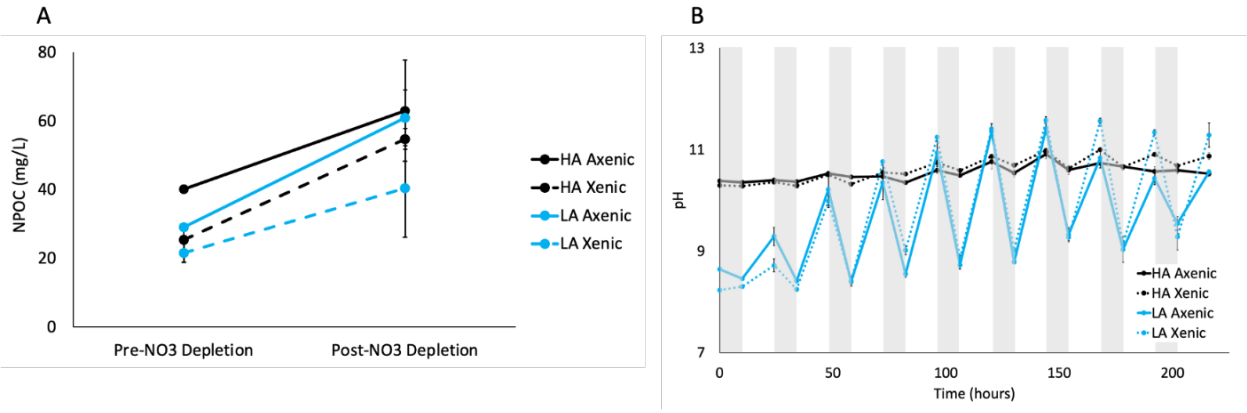

Figure 4.7. Extracellular chemistry in axenic (dashed lines) and xenic (solid lines) SLA-04 cultures under LA (blue) and HA (black) conditions (n=3). (A) Non-purgeable organic carbon (NPOC) concentration pre- and post-nitrate depletion (t=120 hr and t=168 hr, respectively). (B.) The pH measured at the end of each light and dark cycle for the duration of the experiment. Grey shaded regions represent dark periods. Error bars represent one standard deviation in the positive and negative direction.

Furthermore, the fluctuation in pH (Figure 4.7B) that was observed under LA conditions may select against activity in the phycosome. The assimilation of inorganic carbon in the form of HCO_3^- along with nitrate and phosphate consumption can result in the release of hydroxyl ions (OH^-) [65,66], driving an increase in pH. Conversely, CO_2 dissolution from the atmosphere, the lack of CO_2 consumption, and the production of CO_2 during respiration results in a decrease of the pH during the dark periods [52]. The HA condition has a strong carbonate-bicarbonate buffer capacity around the pKa value of the HCO_3^-/CO_3^{2-} pair (pKa 9.7 to 10.6), and thus, pH fluctuations are moderated. This difference in cyclic, environmental stress could indicate that the fate of

metabolite exchange and resulting phycosome growth is alkalinity dependent. Changing pH could impact proton-dependent transport across plasma membranes [67], altering metabolite exchange networks between SLA-04 and the phycosome, ultimately influencing biomass accumulation.

Diel pH fluxes could also impact SLA-04 carbon metabolism directly. The efficiency of the carbon concentrating mechanism in cyanobacteria has been shown to be sensitive to changes in pH [68]. However, it does not appear that SLA-04 growth is significantly impacted in the LA condition (Figure 4.1A). Alkalitolerant phototrophs (capable of growing from pH 7 to pH 12) cultivated under weakly buffered conditions may experience drastic pH changes during the diel cycle that is more extreme than that of any natural aquatic or soil environment, providing an inherent selective pressure that could be used to engineer xenic cultures.

Together our results suggest that phycosome growth and activity is different across the light/dark diel cycle in an alkalinity-dependent manner. Disparate responses among phycosome populations to changes in SLA-04 metabolism suggest that temporal resolution is essential in identifying causal relationships between phycosome populations and microalgal phenotypes. From the BONCAT activity data, the next step will be to test how the increasing activity during the dark cycle could be protective for microalgae cultures under different media conditions. Ideally, a *Salinarimonas* isolate could be grown in co-culture with SLA-04 to assess a predicted protective capacity in terms of competitive invasion and/or osmolytes. After multiple attempts, a representative *Salinarimonas* isolate has not been obtained, but other microorganisms that had differential activity during the diel cycle, such as isolates representing *Belliella* sp., *Paracoccus* sp. and *Roseococcus* sp., have been isolated.

While this study focused on temporal dynamics in phycosome activity and microalgal physiology, xenic cultures can also have a high level of spatial variability, especially in cultures where aggregation is prevalent [69-71]. The spatial relationship between phycosome populations and single cell microalgal physiology could be investigated using BONCAT coupled with high resolution techniques such as Raman microspectroscopy, stable isotope probing, or fluorescence *in-situ* hybridization nanoscale secondary ion mass spectrometry (FISH-NanoSIMS) [72]. By understanding metabolic interactions and the resulting impact on microalgal physiology on a single-cell level, it may be easier to identify important symbioses. For example, physical contact between microalgal cells and bacteria that recycle nitrogen could alleviate nitrogen stress and lead to diminished lipid production. This type of a metabolic exchange could be lost when assessing bulk-level physiology and may only be detected using single-cell physiology approaches involving stable isotope probing.

Supplemental Figures in this Manuscript

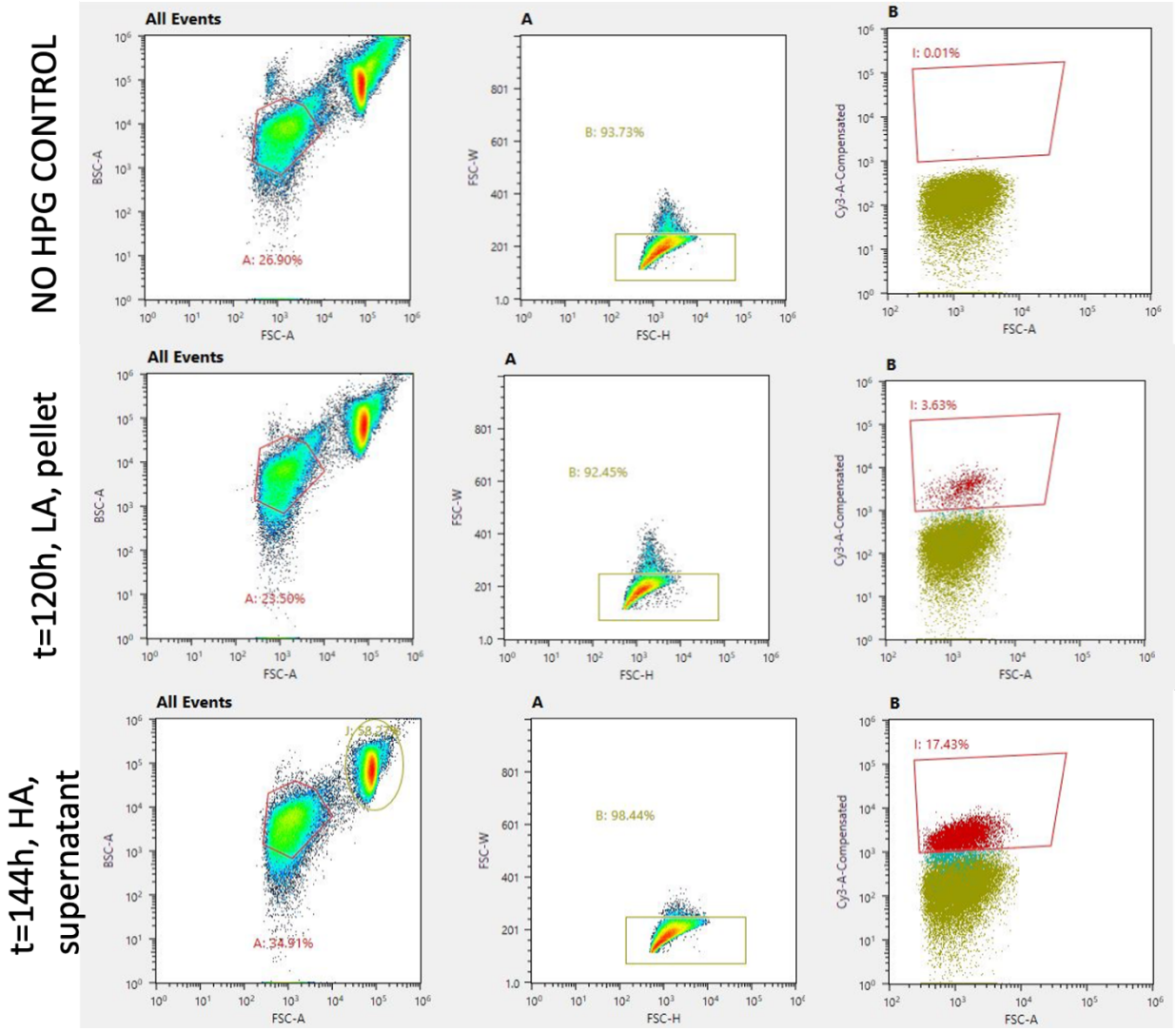

Figure 4.S1. Scatter plots representing xenic SLA-04 samples analyzed on the SONY SH800S FACS. (Top) No-HPG control from LA negative control; (middle) sample from LA culture pellet fraction at t=120h, representing lowly-labeled sample; (bottom) sample from HA culture supernatant fraction at t=144h, representing a highly labeled sample. The 'A' gate selects microbial cells based on size, away from SLA-04 cells. The 'B' gate further refines the selected sub-population based on size. The 'I' gate was drawn on the no-HPG control for each sample type to reduce the number of false positives collected. The 'I' gate setting was then transferred to all subsequent samples. The 'I' gates are the events considered BONCAT active. Values next to each gate correspond to the proportion of events falling into that gate. The gates are nested, meaning that gate 'A' selects events from all events, gate 'B' selects events from gate 'A', and gate 'I' selects events from gate 'B'.

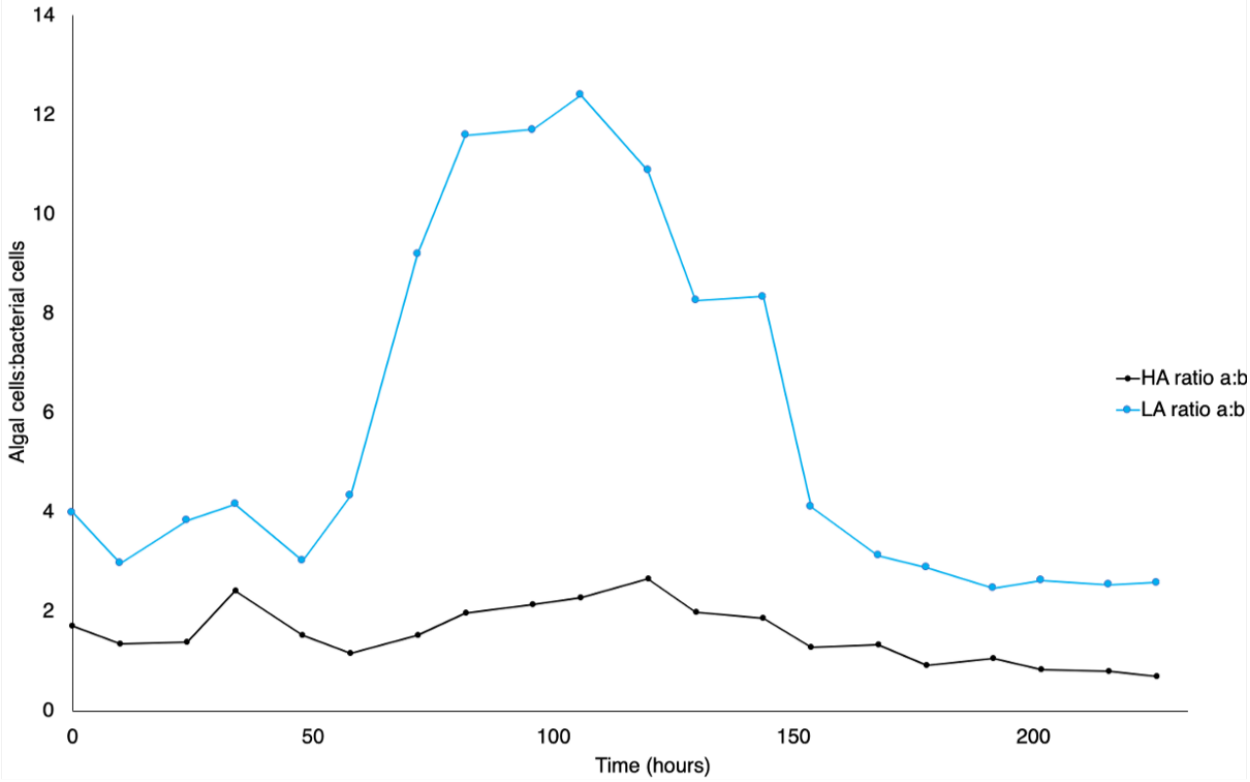

Figure 4.S2. Ratio of algal cells to bacterial cells in LA (blue) and HA (black) cultures. Each plotted value represents the average ratio from triplicate (n=3) cultures.

Acknowledgements

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CHAPTER FIVE: PHYCOSOMAL BIO-FLOCCULATION AS A XENIC CULTURE PHENOTYPE

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Abstract

In typical bottom-up phycosomal phenotype testing, single microbial strains are paired with the host to test the potential to improve microalgal growth rate, biomass accumulation, or culture resistance. In addition to single strain tests, multispecies consortia with increased diversity can be selected to better resist perturbations (*e.g.*, invasion). When co-culturing highly productive microalgae with single microbial isolates, it can be challenging to identify industrially relevant, temporally consistent phenotypes, and moreover, most industrial cultivations will be open systems that are not simple co-cultures. Cellular aggregation can be common in xenic microalgal cultures, yet the physiological significance of aggregate growth is poorly understood, nor has it been routinely quantified even though aggregation can contribute to biomass collection and dewatering. We show that aggregation is a quantifiable phenotype, and the extent of aggregation is dependent upon taxonomic composition that differs under low and high alkalinity. In addition to axenic and xenic cultures, different phycosomal isolates and their respective spent medium were compared for the ability to aggregate SLA-04. High alkalinity xenic cultures showed 87% aggregation efficiency (*i.e.*, 87% of biomass settled after 30 minutes) compared to 14% aggregation efficiency for high alkalinity axenic cultures or 74-84% efficiency for chitosan or $FeCl_3$ (commonly used chemical flocculants). The aggregation efficiency was 18-64% for the tested isolates and 12-44% for the respective spent media, and these results indicated that direct cellular interactions likely played a role in aggregation. FlowCam particle analysis was used to track aggregate size during SLA-04 growth, and the mean particle size ranged between 30 to 187 μm^2. The mean particle size (μm^2) followed the aggregation efficiency trend and was directly correlated to aggregation efficiency, R^2=0.7 and R^2=0.8, for low and high alkalinity, respectively. Despite the largest

aggregates in the xenic culture, cell numbers, growth rate, and chlorophyll levels were not significantly impacted. For the highly productive, alkalitolerant SLA-04, the results demonstrated that growth in aggregates does not impact productivity but could lead to improved harvesting outcomes via phycosomal bio-flocculation.

Introduction

The microalgae industry faces many challenges in being economically competitive with petroleum-based fuel or fuels derived from terrestrial plant feedstocks, namely inorganic carbon delivery, nutrient supply, and down-stream processing [1-4]. Recently, there has been a realization of the potential of manipulating culture ecology to overcome costs associated with large scale microalgal cultivation (*e.g*., culture crashes, nutrient requirements, dewatering) [5]. Efforts to identify and exploit beneficial ecological relationships show potential for enhancing culture productivity and stability through directly modulating microalgal growth [6], providing protection against pests [7,8], or improving quality of biomass [9]. Thus, there has been a broad acceptance of the advantages of maintaining xenic cultures with stable and diverse microbial communities. The term xenic refers to cultures that contain multiple species while axenic cultures contain a single species [10].

Interactions within the phycosphere, or the microenvironment surrounding microalgal cells, have been observed to influence fundamental processes such as nutrient provision and cycling, primary production, toxin biosynthesis, and biogeochemical cycling [11,12,13]. Phycosphere interactions, those occurring between microalgae and the respective phycosome (i.*e.,* microbiome), occur at the scale of individual microorganisms but have ecosystem- or culture- scale effects (Seymour et al., 2017). The chemical composition of the phycosphere may be dictated by

host physiology as well as phycosome metabolism through the exchange of metabolites, production of gases, and ion production (*e.g.,* H^+ or OH^-) [14,15].

Phycosphere interactions often result in the formation of multispecies aggregates as a direct or indirect consequence of forming consortia with associated phycosome communities [16-19]. Aggregation is often driven by algal or bacterial production of extracellular polymeric substances (EPS), which are often rich in organic carbon, nitrogen and other metabolites that are important for ecological exchange in systems driven by primary productivity [13,15,20,21]. Resistance to culture crash-inducing zooplankton by physically precluding pests through aggregate formation has long been understood to be a microalgal defense mechanism [22]. Aggregation in microalgal cultures can also be advantageous for dewatering and harvesting biomass, and effective aggregation-aided harvesting is described as reducing the biomass-containing volume by 80% or more, at which point dewatering processes are more economically viable [23,24].

Historically, chemical flocculants have been added to cultures after biomass growth and accumulation [25,26], and more recently, bioflocculation has been explored as an alternative to chemical application [27-31]. For the purposes of the present study, it is important to note that these “endpoint” applications of bacterial or fungal bioflocculants do not investigate productivity of flocculated cultures after aggregation is initiated. As the microalgal industry hunts for “probiotic” amendments that could improve culture phenotype through additions at different cultivation stages, the physiological tradeoff between aggregation and biomass growth and accumulation in xenic cultures remains unknown.

Unicellular microalgae form multicellular aggregates in response to abiotic (*e.g.,* high pH) and biological (*e.g.,* grazers, viruses) stresses [16,32]. Because keeping large scale cultures axenic

and free from environmental stresses (*e.g.*, diminished temperature and light) is impractical, aggregation in industrial cultures is likely common but seldom studied. Much of what is known about the triggers and mechanisms of aggregation has arisen from studies of *Chlamydomonas reinhardtii*, a model green microalga [33]. *Chlamydomonas* are flagellated, while other oleaginous, highly productive genera of biotechnological interest, such as *Chlorella* and *Nannochloropsis,* lack flagella, and likely use different mechanisms to sense and respond to stress. In *Chlorella*, aggregation is hypothesized to be driven by growth conditions, phycosphere composition, and phycosome activity.

Previous studies of aggregation have focused on flocculation at the end of a growth cycle rather than the role of aggregation formation during stress responses and growth. Lee et al. (2013) [17] explored the relationship between *Chlorella vulgaris* culture composition *(i.e.,* phycosome presence, media pH) and chemical flocculation. Aggregation was found to directly correlate with pH in xenic cultures, with aggregation increasing with increasing pH from pH 3 to pH 11. Axenic cultures did not aggregate and settle at any pH, indicating that phycosome activity was pH dependent. It is unclear whether the alga or the associated phycosome populations could grow across the entire range of pH tested. In *Nannochloropsis* cultures, pH-dependent, reversible aggregation following inoculation with various culture-sourced bacterial strains was proposed to be driven by the precipitation of magnesium and calcium [27].

While aggregation in cultures is advantageous for harvesting and protection against pests, aggregation under the wrong circumstances can be an early sign of a culture crash. When *Chlorella* cultures were infected with the algicidal bacterium *Vampirovibrio chlorellavorus,* significant clumping was followed by discoloration and complete loss of culture viability [34]. In this case,

aggregation of *Chlorella* in the presence of a predatory organism led to more efficient, direct lysis of microalgal cells [35]. It is hypothesized that aggregation itself is not detrimental to microalgal physiology in well-mixed cultivations, but the persistent presence of aggregates in cultures with stable phycosome communities and the resulting impact on culture productivity have not been explored.

One limitation in studying the impacts of aggregation on microalgal physiology and growth is the lack of accurate and robust methodology. Previous studies have used volume displacement [36] or reduction of OD after settling (*i.e.,* aggregation efficiency) [18] to characterize extent of aggregation. While these methods prove to be effective in heavily flocculated cultures, they lack the spatial and temporal resolution to characterize aggregation in cultures during active growth experiments. There is a need for more robust methodology to investigate how aggregation contributes to phenotypes of interest. Park et al. (2019) [37] used a FlowCam to characterize aggregate size of *Microcystis* colonies in low-density freshwater samples and Cruz and Neuer (2019) [38] used flow cytometry (BD Influx) to characterize aggregate size in axenic and xenic cultures of the marine picophytoplankton *Synechococcus* and *Prochlorococcus.* In both studies, cytometry was used as a diagnostic tool to characterize cultures of bloom-forming organisms at densities much lower than biomass productivity cultures. FlowCams have also been used for morphology-based characterization of the eukaryotic composition of production cultures [39]. Here, we employ FlowCam particle analysis to track aggregate size during growth experiments of the alkalitolerant, oleaginous microalga *Chlorella* sp. SLA-04 in high density cultures. The aims of this study were to outline improved methods for (1) screening the impact of bacterial isolates on the growth of SLA-04 and (2) characterizing aggregate formation and the resulting impacts on

culture productivity in axenic cultures, co-cultures, and xenic cultures with a diverse microbiome. It was hypothesized that aggregate size would be negatively correlated with SLA-04 growth. We show that the extent of aggregation in these cultures may depend on both the taxonomic composition of the phycosome as well as media conditions (*i.e.*, low vs. high alkalinity). We also demonstrate that there is not a strong correlation between aggregate size and growth.

Materials and Methods

History of Maintenance Cultures

Water samples from Soap Lake, an alkaline soda lake in Washington (USA), were inoculated on sterile agar plates of Bolds Basal Medium (BBM). A rapidly growing algal colony was picked and streaked for further isolation, and the unialgal culture was confirmed using microscopic observations [40]. Xenic cultures of *Chlorella* sp. SLA-04 all originated from the same isolation and same mother culture. Through maintenance in modified BBM over the course of 12 years at the University of Toledo and Montana State University, SLA-04 cultures recruited unique phycosome communities with varying levels of diversity (unpublished data).

Isolation of Bacteria and Preparation of Isolate Cultures

Agar plates containing Marine Agar 2216 (MA) (VWR, PA, USA), Reasoner's 2A Agar (R2A) (Becton Dickinson, NJ, USA), 0.5X Lysogeny Broth Agar (LB), or SLA-04-conditioned BBM with 1% w/v glucose (CBM) were inoculated with 100 µL xenic SLA-04 cultures grown in open, outdoor raceways, indoor shaking flasks, and indoor 3L bioreactors (University of Toledo). CBM was prepared by growing axenic SLA-04 in BBM for 96 hours, centrifuging to remove cells, adding 1% w/v D-glucose (Sigma Aldrich, MO, USA), and autoclaving with agar. Plates were

incubated in the dark at 22 °C until colonies formed. As colonies with unique morphologies emerged, they were picked and streaked onto clean agar plates. Individual colonies were then picked and streaked a total of four times before being transferred to liquid growth media; these cultures were centrifuged at 14,000 x g for 5 minutes and resuspended in 30% glycerol upon reaching stationary phase.

DNA Extraction, PCR, Sequencing

Isolate glycerol stocks were streaked onto the appropriate supportive medium (Supplemental Table 1) and single colonies of isolates were used for DNA extractions. DNA was extracted from isolates using PrepGEM (zyGEM, Charlottesville, VA, USA) in a standard freeze-thaw approach. The resulting supernatant was cleaned using Wizard SV Gels and PCR Clean-Up Kits (Promega, WI, USA). The V3-V4 region of the 16S rRNA gene was PCR amplified with the primers 341F-(CCTACGGGNBGCASCAG) and 805R-(GACTACNVGGGTATCTAATCC) [41]. The primers used also contained barcoded overhang sequences for application in Illumina MiSeq sequencing. PCR was performed in an Eppendorf Mastercycler Pro S with 25 µL reactions containing 0.2 M of each primer, 8 µL DNA, 1X KAPA HiFi Hotstart ReadyMix (Roche) and 2.5 µL bovine serum albumin. The amplification protocol consisted of an initial denaturation at 98 °C for 10 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, extension at 68 °C for 30 s, and a final extension step at 72 °C for 1 min. Triplicate PCR reactions were pooled, and samples were purified using the Ampure XP Bead PCR Cleanup system (Beckman Coulter, CA, USA). The 16S rRNA amplicon libraries were prepared using the Nextera XT Index Kit (Illumina, CA, USA), quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher, MA, USA), and sequenced in separate v3 Illumina MiSeq lanes.

Algae Culture Preparation

Experiments were conducted using a *Chlorella* sp. SLA-04 that was isolated from the alkaline Soap Lake in Washington, USA. The full genome of SLA-04 was recently published [42]. Initial axenic cultures were started from single colonies and maintained in batch flasks on modified BBM with a starting pH of 8.7 and the following media composition: $NaNO_3$ (2.94 mM), K_2HPO_4 (1.43 mM), $MgSO_4.7H_2O$ (0.30 mM), $CaCl_2.2H_2O$ (0.17 mM), NaCl (0.42 mM) H_3BO_3 (0.18 mM), 1 mL/L S3 vitamin solution, 1 mL/L trace metal solution, 1 mL/L iron solution and 1 mL/L EDTA solution. The vitamin solution contained Inositol (2.78×10^{-3} M), Thymine (2.38×10^{-3} M) Thiamine·HCl (1.48×10^{-4} M), Nicotinic acid (8.12×10^{-5} M), Ca-pantothenate (4.20×10^{-5} M), *p*-Aminobenzoic acid (7.29×10^{-6} M), Biotin (4.09×10^{-7} M, and Folic acid (4.14×10^{-7} M). The trace metal solution contained $MnCl_2 \cdot 4H_2O$ (1.26 mM), $ZnCl_2$ (0.15 mM), $CuCl_2 \cdot 2H2O$ (0.11 mM), $Na_2MoO_4 \cdot 2H_2O$ (0.07 mM), $CoCl_2 \cdot 6H_2O$ (0.06 mM), $NiCl_2 \cdot 6H_2O$ (0.04 mM), V_2O_5 (0.01 mM) and KBr (0.08 mM). The iron solution contained 4.98 g/L $FeSO_4 \cdot 7H_2O$ and 1 mL 95% H_2SO_4. The EDTA solution contained 50 g/L Na_2EDTA and 31 g/L KOH. Low alkalinity (LA) conditions refer to modified BBM at an initial pH of 8.7 without added carbonate species. High alkalinity (HA) conditions refer to modified BBM at an initial pH of 10.3 with 150 mM added total alkalinity ($NaHCO_3$ 50 mM, Na_2CO_3 50 mM). Cultures were grown at 20 °C in a temperature- and light-controlled photoincubator (VWR Model 2015-2), with a shaker set to 150 rpm, and a 14:10h light:dark cycle under 7500 lx cool fluorescent lights at an intensity of 175 μmol m^{-2} s^{-1} photosynthetically active radiation. Cultures were maintained under 10 mg/L kanamycin until the experiment, at which time antibiotic stress was relieved and cultures were transferred into the experimental treatments.

Establishment and Maintenance of Axenic SLA-04 Culture

An axenic culture of SLA-04 was established by streaking a xenic culture onto an agar plate containing BBM agar with 100 µg/mL ampicillin and 100 µg/mL cefotaxime. A single colony was picked and re-streaked onto BBM agar with the same antibiotics two successive times. After that, a single colony was picked and grown in liquid BBM with 10 µg/mL kanamycin. To test for the presence of microbial contaminants, samples were collected, DNA was extracted and 16S rRNA gene sequencing was conducted on the Illumina platform according to the aforementioned protocol. Axenic stock cultures were maintained under 10 µg/mL kanamycin until the screening experiments. Antibiotic stress on Chlorophyte algae has been observed [43]. To measure the impacts of antibiotic stress on SLA-04, cell concentration, optical density and chlorophyll concentration were determined for the duration of one typical growth cycle (Supplemental Figure 5.S1). Kanamycin did not impact cell concentration, chlorophyll concentration, pH, or OD750.

24-well Plate Screening Experiments

A semi-high throughput cultivation method was established to assess the impact of individual bacterial isolates on SLA-04 growth. Axenic SLA-04 cultures were grown to early log phase in modified BBM with an initial pH of 8.7. Cells were enumerated using a standard hemocytometer protocol, centrifuged at 5,000 x g for 10 minutes, and then resuspended in fresh BBM at pH 8.7. Bacterial isolates were streaked from glycerol stocks and single colonies were used to inoculate 10 mL of supportive media (Supplemental Table 1). Of 37 isolates obtained from a variety of SLA-04 cultures, 31 were screened for impact on axenic SLA-04 growth. Bacterial isolates were grown overnight in the supportive medium that was used for isolation and diluted samples were enumerated by measuring optical density at 600 nm (OD_{600}) and converting OD_{600}

to cell density using a conversion of OD_{600} 1 = $1x10^8$ cells/mL [44]. Bacterial cultures were centrifuged at 10,000 x g for 10 minutes, washed once, centrifuged again, and resuspended in fresh BBM at pH 8.7. Both bacterial and algal cell suspensions were adjusted to a cell density of $2x10^5$ cells/mL. Equal volumes of algal and bacterial suspensions (0.75 mL each) were added to each well of a 24-well plate to achieve a final density of $1x10^5$ cells/mL for both bacteria and algae (*i.e.*, 1:1 ratio of algae:bacteria) [27]. The starting SLA-04 concentration of $1x10^5$ cells/mL was chosen after testing concentrations ranging from $1x10^2$ to $1x10^7$ cells/mL, where the $1x10^5$ cells/mL concentration yielded the most consistent and informative growth curves (Supplemental Figure 5.S2). For the axenic control, 0.75 mL of the SLA-04 suspension were mixed with 0.75 mL of growth medium. Four blank samples (clean BBM without algae or bacteria) were placed in the corner wells where evaporation was expected to be the most significant, while other samples (n=3 per treatment) were randomly distributed in the remaining wells. The cultures in the clear 24-well Flat-Bottom Plate (Falcon) were sealed with a breathable microplate sealing tape (Corning) that should allow for the transmission of light and the exchange of gas while preventing evaporation and contamination. Plates were incubated at room temperature (~22°C) on a horizontal shaker with a speed of 300 rpm and a 14h:10h light:dark cycle. Over the course of seven-day experiments, readings were taken every 24 hours using a plate reader (BioTek). OD_{600} and OD_{750} measurements were taken to monitor optical density. Five consecutive *in-vivo* excitation/emission readings at 440nm/685nm designed to gauge chlorophyll content in each well. The average of five chlorophyll readings was reported as a single number. After determining that OD_{600}, OD_{750} and chlorophyll readings displayed the same trends, only OD_{750} was included in this manuscript for the sake of brevity.

Nitrogen, Iron, and Vitamin Stress Experiments

After screening 31 isolates for beneficial or detrimental impacts on SLA-04 growth, nutrient stress was applied to SLA-04 cultures to determine if physiological stress could be observed in axenic SLA-04 cultures, and then rescued by co-culture growth with single isolates. Serial dilutions of nitrate, iron, and vitamins from the concentration present in standard BBM to a 1000-fold dilution were tested (1X, 0.1X, 0.01X, 0.001X).

Aggregation Experiments

To assess the extent of aggregation in axenic, xenic and single isolate add-back cultures, four isolates, *Paracoccus* sp., *Pelagibacterium* sp., *Microbacterium* sp., and *Belliella* sp. were selected for aggregation experiments. Screened among the original library of 31, these four isolates were selected because of a stable presence in multiple experimental SLA-04 cultures, and sequenced genomes or metagenome assembled genomes (MAGs) are available (unpublished data). In addition to the bio-aggregation experiments, the common chemical flocculants ferric chloride ($FeCl_3$) (MilliporeSigma, MA, USA) and chitosan (Sigma Aldrich, MO, USA) were used to compare the extent of aggregation in SLA-04. To determine the appropriate experimental concentration of each chemical, serial dilutions spanning the highest known concentration for industrial flocculation, 1.00 g/L $FeCl_3$ and 0.15 g/L chitosan (Matter et al., 2019) down to chemical free were screened for impact on axenic SLA-04 growth (optical density and chlorophyll fluorescence) (*i.e.,* 1X, 0.1X, 0.01X, 0.001X, and chemical free). Aggregation experiments were designed following the same workflow as described in the screening experiments above. An additional step was added by screening bacteria-conditioned media (CM). CM was obtained by adding cell-free culture supernatants to the volume-adjusted media in the appropriate wells. After

taking daily OD and chlorophyll measurements, samples were removed to quantify aggregation on the FlowCam. Samples were fixed in 4% paraformaldehyde for 30 minutes to accommodate biosafety regulations and then analyzed directly on the FlowCam. Approximately 1,000 particle images were collected to avoid removing excessive volume from each experimental well.

FlowCam Preparation, Loading and Image Collection

A FlowCam 8100 (Yogogawa Fluid Imaging Technologies, ME, USA) was used for machine-learning-assisted particle analysis of SLA-04 cultures. The 10X objective lens was paired with the 100-µm flow cell at the suggested flow rate of 0.15 mL/min, yielding a particle diameter range from 2-100 µm [45]. Prior to data collection every day, the instrument was calibrated using 15 µm Duke Polystyrene Particle Counter Standards (Thermo Fisher, MA, USA). Samples were diluted in PBS to a final volume of 0.2 mL and samples were run until sample volume was used entirely. Between samples, 0.6 mL of PBS was passed through the instrument at an increased flow rate of 0.6 mL/min and then a fresh 1 mL pipette tip in the sample input port was loaded prior to running the next sample.

FlowCam Data Processing and Analysis

Particle image libraries were trimmed to 1000 particle images to standardize between samples. Libraries were constructed using a minimum edge gradient of 100 as a cutoff for image focus [45]. Particle image libraries were corrected by removing cellular debris, media precipitates and poorly focused images by filtering out any particle image collected that was reported to have an area of less than 2 μm^2. This filtering removed less than 5% of all images in any given library. The “Particle Edge Trace” feature of the VisualSpreadsheet 4 software (Yogogawa Fluid Imaging Technologies, ME, USA) was used to confirm that particle images, and area calculations, did not

contain multiple particles. The area based on diameter (ABD) function of the VisualSpreadsheet 4 software was used to characterize aggregate size. ABD is determined by measuring the number of pixels in a binary version of the particle image, and then converting to a circle with the same number of pixels, and an area is reported in μm^2 (Supplemental Figure 5.S3) [46]. The ABD algorithm has been shown to be the most effective for measuring diameter, area and biovolume [47].

Aggregation Efficiency Determination

To compare the potential for flocculation-aided dewatering and harvesting in xenic cultures to cultures where high concentration of flocculant is added after growth, aggregation efficiency (AE) was determined at the culmination of the aggregation experiments using an adaptation of previously established methods [18,26]. AE was determined using the following equation:

$$AE = \left(\frac{(OD_{t0} - OD_{t30})}{OD_{t0}} \cdot 100 \right)$$

where OD_{t0} is the optical density at 750 nm of the culture immediately after being removed from the shaker and pipetted into a cuvette and ODt_{30} is the optical density at 750 nm of the culture after settling for 30 minutes. AE was plotted in histograms as a percentage relative to no change in optical density.

Results and Discussion

This research took shape over three distinct steps: 1.) screening isolates for impacts on axenic SLA-04 growth under standard media conditions and under nutrient limitation; 2.) developing methods to characterize aggregation in growing SLA-04 cultures; 3.) measuring the

relationship between aggregate size, culture growth, and aggregation efficiency. This experimental workflow is illustrated in a schematic in Figure 5.1.

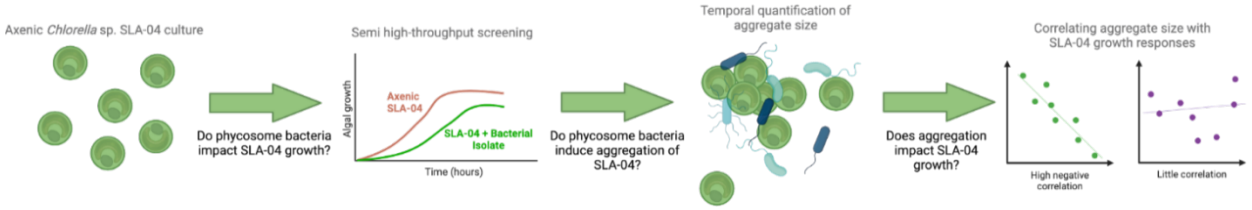

Figure 5.1. Schematic depicting the experimental workflow and research questions in the present study. This was a three-part study, motivated by the desire to identify causal relationships between SLA-04 growth and phycosome bacteria populations. After assessing the impact of the phycosome on growth-based phenotypes, aggregation was investigated as a phenotype that could be relevant for biomass productivity systems.

Co-culturing SLA-04 and Phycosome Isolates

Bacterial isolates obtained from four xenic SLA-04 cultures were screened in add-back co-culture experiments to determine their influence on SLA-04 growth under low alkalinity (LA) conditions (Supplemental Table 5.S1). None of the 31 co-cultures that were screened showed different growth patterns (*i.e.*, growth rate, maximum optical density) relative to the axenic culture. Under the tested conditions, SLA-04 showed consistent growth across axenic and co-culture conditions (Figure 5.2). SLA-04 is a robust and highly productive microalga that has been shown to grow with high growth rate and nutrient utilization efficiency under a wide range of pH, salinity, and alkalinity conditions [48,49]. Because axenic SLA-04 grows robustly under different conditions, beneficial impacts on growth may be difficult to detect, particularly in highly controlled, closed systems; and therefore, could lead to spurious conclusions about the ecological roles, or lack thereof, for each of the isolates. This observation underlines the potential challenge

of applying microbiome theory to short-term experiments in cultivation systems engineered for monocultures.

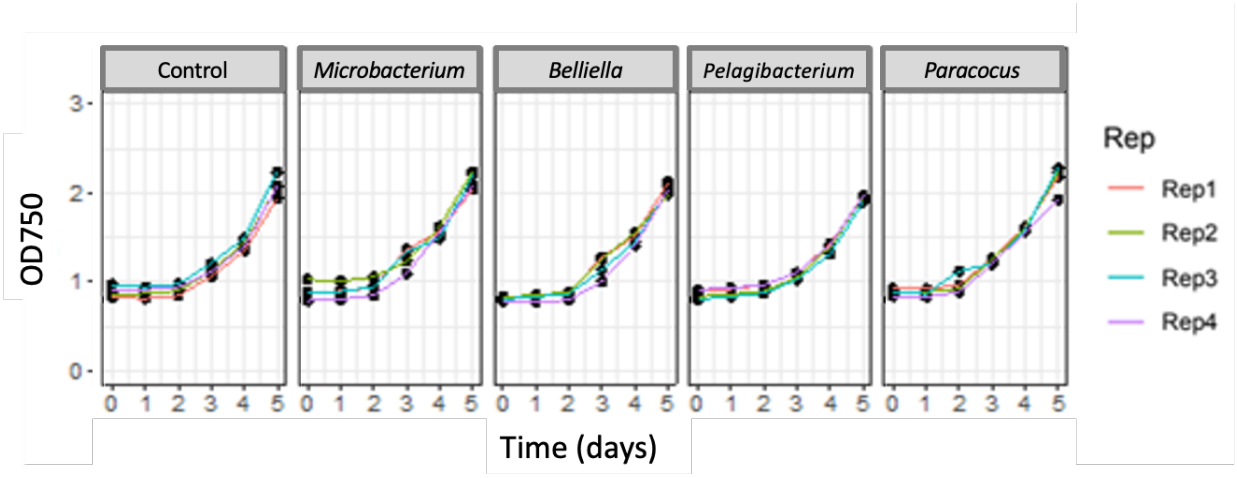

Figure 5.2. Growth of SLA-04 cultures over time (days) as reported by optical density at 750 nm (OD750). The control, left, is an axenic SLA-04 culture. The four panels to the right represent an axenic SLA-04 culture with the respective bacterial isolate added at a 1:1 ratio of algal cells to bacterial cells. The four colored lines represent individual replicates.

Axenic SLA-04 cultures were grown under serial dilutions of standard BBM vitamin, nitrate, and iron concentrations (Figure 5.3A, B, C) to assess whether sub-optimal media conditions could lead to diminished SLA-04 growth. While reducing vitamin concentration did not impact axenic SLA-04 cultures (Figure 5.3A), growth correlated directly with decreasing concentrations of nitrate (Figure 5.3B) and iron (Figure 5.3C). Eliminating vitamins from the medium did not impact SLA-04 growth despite expected vitamin auxotrophies (unpublished data), namely cobalamin, biotin, and thiamine [50,51]. This was likely due to the relatively low concentration of vitamins required to support growth, which could have been present in sufficient amounts in the inoculum. In the future, more effort to condition inoculum cultures to vitamin-free conditions may be necessary to test this hypothesis.

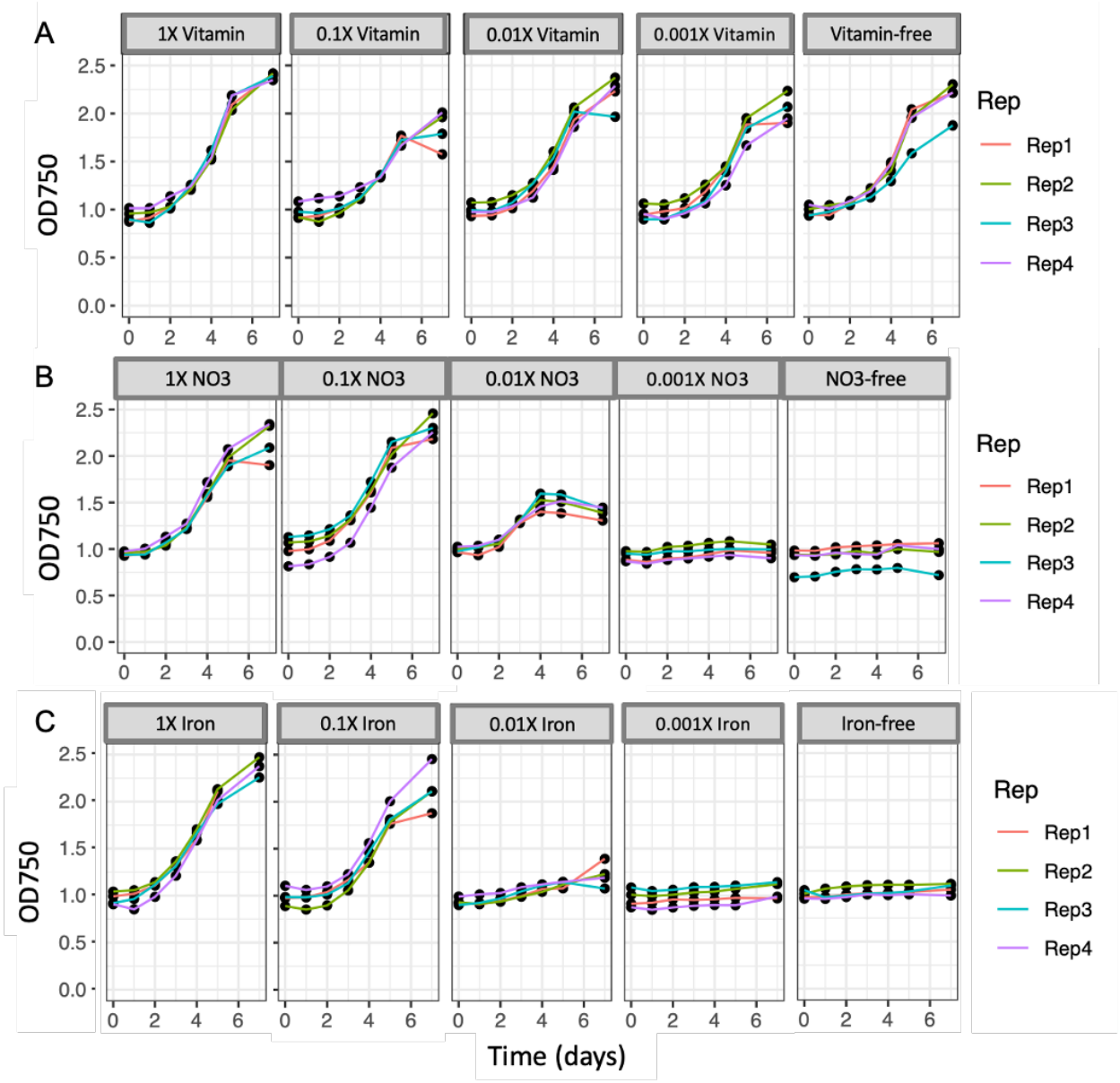

Figure 5.3. Growth of SLA-04 cultures over time (days) as reported by optical density at 750 nm (OD750). Axenic SLA-04 cultures were grown under serial dilutions of standard BBM concentrations of vitamins (A) nitrate (B) and iron (C). In each panel, the control (left) is an axenic SLA-04 culture grown under a 1X concentration of the respective nutrient. The four panels to the right represent the growth of the axenic SLA-04 culture under serial dilutions of each nutrient, 0.1X, 0.01X, 0.001X and -free, respectively. The four colored lines represent individual replicates.

Nitrate is the principal nitrogen source in the medium and reducing nitrate to 0.01X concentration and below did limit SLA-04 growth and yield. Similarly, removing iron correlated with a decrease in SLA-04 growth, which was not surprising given the requirement of iron for maintaining photosynthetic activity [52]. Iron limitation was investigated further as a stress condition that might be ameliorated by co-culturing SLA-04 with phycosome isolates. Many bacterial symbionts of green microalgae have been shown to possess siderophores [53,54] that can scavenge and concentrate iron thus increasing bioavailability when it is otherwise limited. Bacterial-microalgal symbioses based on iron exchange are well documented in nature [53] and in microalgal cultivation [55]. Co-culturing SLA-04 with a *Pelagibacterium* sp. isolate led to a recovery of SLA-04 growth, even under iron-free conditions (Figure 5.4). We increased scale from the 24-well plate to 200 mL cultures to confirm the recovery of SLA-04 growth by the *Pelagibacterium* sp. isolate under iron deplete conditions. However, we were not able to reproduce diminished growth under iron limitation as axenic SLA-04 under iron-free conditions grew normally at flask scale. In Figure 5.4 we demonstrated that measurable growth defects occur between 0.1 to 0.01X of standard iron concentration. At the flask scale there may have been trace amounts of iron present in glassware and media components, leading to challenges reproducing the result. Background iron contamination has been documented as a confounding variable in nutrient limitation studies [56,57]. Iron limitation experiments should be conducted using acid-washed glassware and using media components that are cleaned in iron binding columns. Given the difficulty maintaining iron-deficient conditions without the addition of a chelating agent, we concluded that iron stress might not be a relevant stress in the cultivation of highly productive

microalgae, though iron limitation is certainly an important factor in natural ecosystems [58], and in cultivation under sub-optimal conditions [55].

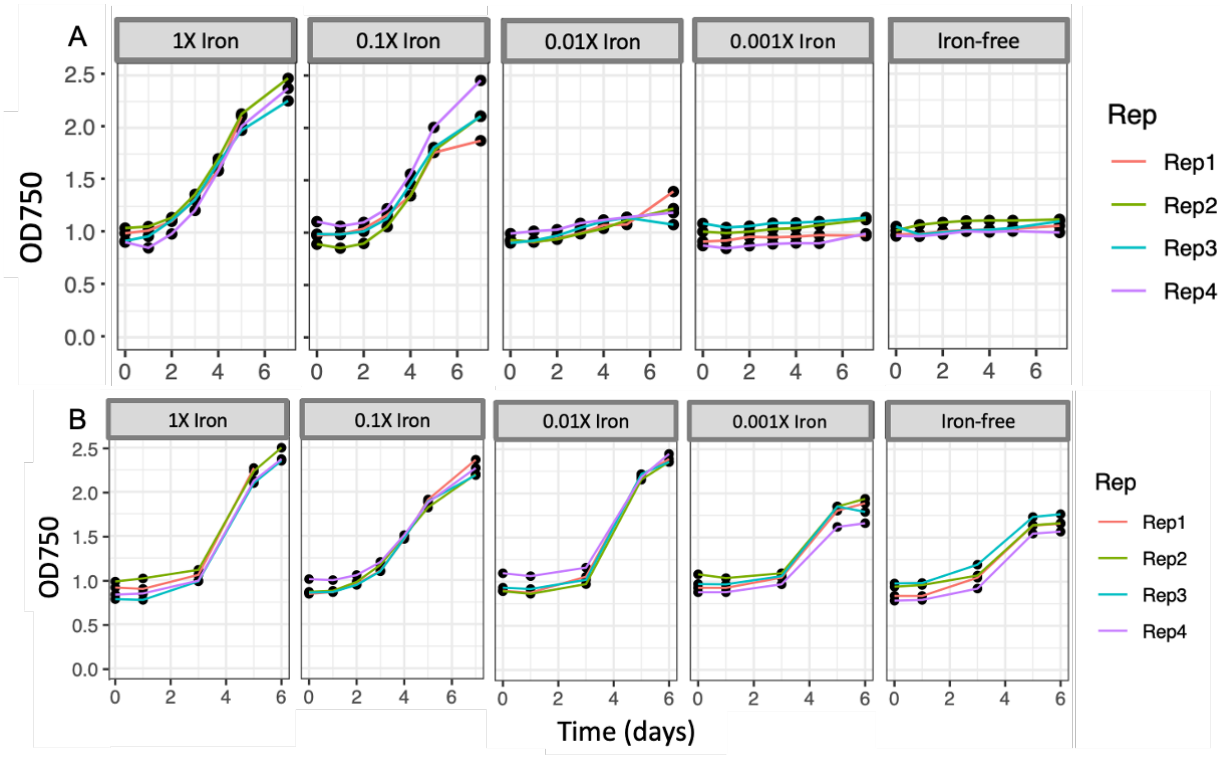

Figure 5.4. Growth of SLA-04 cultures over time (days) as reported by optical density at 750 nm (OD750). Axenic SLA-04 cultures were grown under serial dilutions of standard BBM concentrations of vitamins (A) (same as Figure 3C). Co-culture of SLA-04 and a bacterial isolate *Pelagibacterium* sp. grown under serial dilutions of standard BBM iron (B). The five panels represent the growth of the co-culture culture under serial dilutions of iron concentrations, 1X, 0.1X, 0.01X, 0.001X and iron-free, respectively. The four colored lines represent individual replicates.

Aggregation: A Desirable Phenotype or A Compensatory Effect?

Engineering microalgal co-cultures or mixed consortia to improve microalgal cultivation should expand in scope beyond the traditional approach of improving growth rate or nutrient utilization. Given the protective potential of aggregation along with the significant improvement

of harvesting planktonic cultures, engineering consortia that form aggregates could be a new frontier for sustainable microalgal cultivation. Through numerous cultivations across conditions and across scales, we noticed a relationship between phycosome composition and formation SLA-04 aggregates. Namely, axenic cultures of SLA-04 appeared to be comprised of an even distribution of non-aggregated single cells, while cultures containing phycosome communities of varying diversity are generally marked by aggregate formation. This observation has been made in other *Chlorella* cultures [17], as well as in *Nannochloropsis* [18,27], *Chlamydomonas* [16], *Tetraselmis* [27], and cyanobacterial cultures [38]. We hypothesized that there is a compensatory effect with aggregation in xenic cultures, and that there are physiological consequences of colony growth (*e.g.*, mass transfer limitation, diminished light exposure) [59]. Thus, we anticipated that there could be a negative correlation between aggregate size and growth, effectively nullifying benefits of diversity in these systems.

SLA-04 cultures were grown under LA and high alkalinity (HA) conditions (Figure 5.5A, C, E and 5.5B, D, F, respectively) to determine impacts on growth. The ratio of bacterial cells to microalgal cells was increased from 1:1, as was used in the first experiments, to 10:1 to mimic xenic SLA-04 culture composition when bacteria are at the maximum observed abundance. Axenic and xenic cultures showed similar growth under both the LA and HA conditions. SLA-04 was also grown with bacterial conditioned media, as previous studies have shown the potential for bacterial exometabolites to induce aggregation [17]. After increasing the concentration of bacteria to a 10:1 ratio (bacteria to algae), the only co-culture or conditioned media treatment that impacted axenic SLA-04 growth was the *Belliella* sp. isolate that had a negative effect in both the LA and HA condition (Figure 5.5). Interestingly, in the initial experiment assessing growth of the co-culture,

where the initial ratio of bacterial to algal cells was 1:1, the *Belliella* sp. isolate did not change growth relative to the axenic control (Figure 5.2).

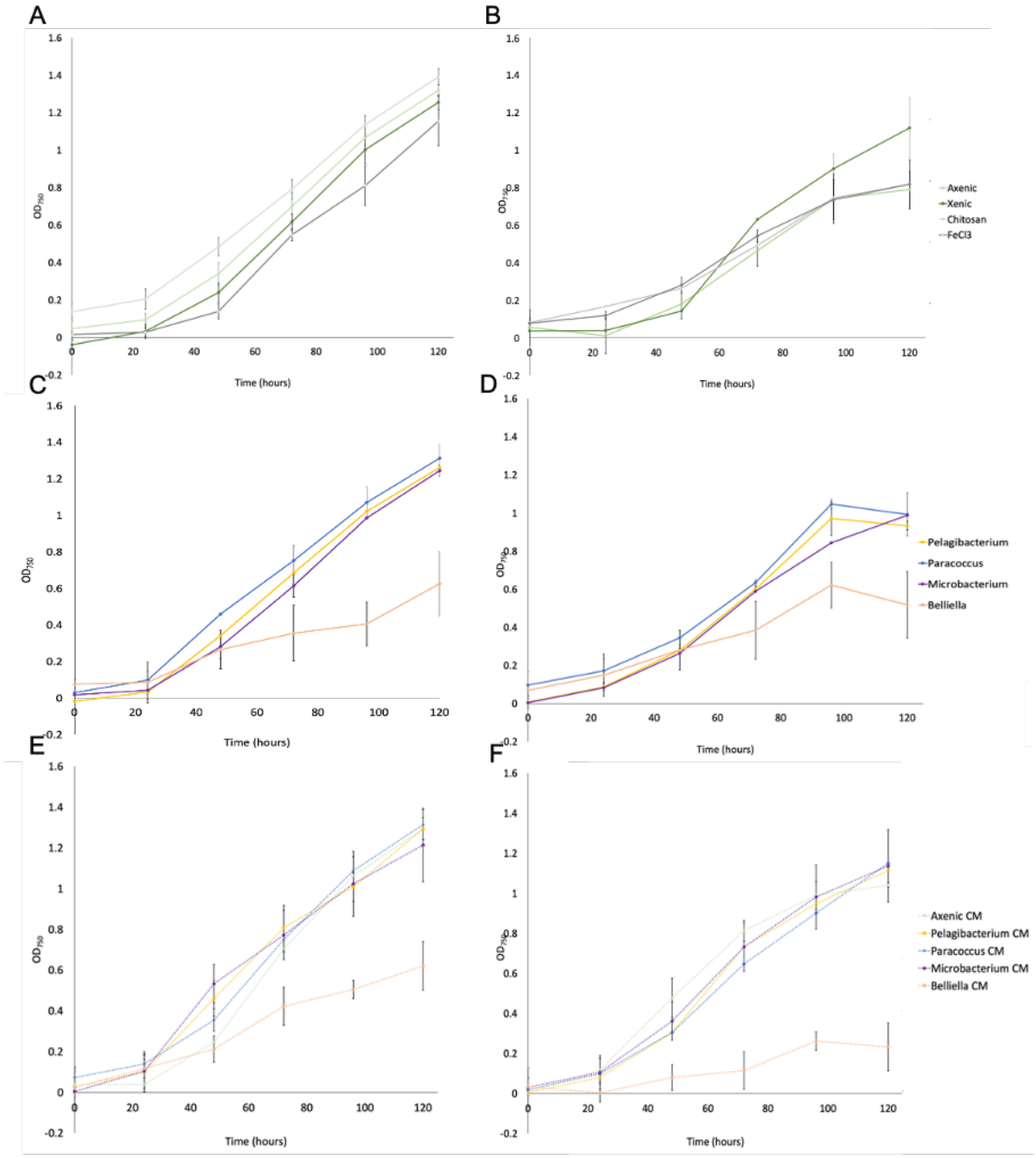

Figure 5.5. Growth of SLA-04 cultures under low alkalinity (A, C, E) and high alkalinity (B, D, F) conditions over time. Different colored lines indicate different treatments, from top to bottom: axenic (light green), xenic (dark green), $FeCl_3$ (dark grey), and chitosan (light grey) (A, B). *Pelagibacterium* sp. (yellow), *Paracoccus* sp. (blue), *Microbacterium* sp. (purple), and *Belliella* sp. (orange) (C, D). Conditioned media (CM) treatments are indicated with dashed lines of the same color (E, F). Error bars represent one standard deviation from the mean (n=3) in the positive and negative direction.

In addition, temporal sequencing of xenic maintenance cultures of SLA-04 revealed a highly abundant and stable presence of a *Belliella* sp. population (Dissertation Chapter 2). Cultured *Belliella* sp. representatives have originated from alkaline lakes [60,61] and microalgal enrichments [62], but the ecological role, in terms of contribution to algal productivity, has not yet been investigated. If antagonistic populations do not cause a culture crash, they may interfere with growth and lead to diminished productivity [19,63,64], ultimately causing an underperformance of xenic cultures, which, in-line with microbiome theory [65], were expected to be more productive than monocultures.

A novel approach was developed for characterizing particle size in growing microalgal cultures, bridging a gap between traditional growth studies and post-growth flocculant applications. By employing machine-learning driven particle analysis using FlowCam, particle size was measured from time of inoculation (t=0) to the end of the growth experiment (t=120 hours) with micron-level resolution and high particle throughput (10^3 particles/minute). At t=0 hours, axenic SLA-04 cultures were consistently composed of small particles, corresponding to an area of 16-20 μm^2, across co-cultures and treatments in LA and HA conditions (Figure 5.6 A, B). Xenic cultures started with larger average particle size at t=0 hours, 35 μm^2 under LA and 67 μm^2 under HA conditions. At t=72 hours, xenic cultures had an average particle size of 53 μm^2 under LA and 179 μm^2 under HA conditions. Additionally, the chemical flocculant ferric chloride

($FeCl_3$), induced aggregation under both LA and HA conditions, with chitosan inducing aggregation under only the HA condition. By the end of the experiment (t=120 hours), many treatments under HA conditions had greater particle size than the same treatment under LA conditions (Table 5.1). The *Belliella* treatments did not show greater particle size than the control, indicating that aggregation did not contribute to the diminished growth.

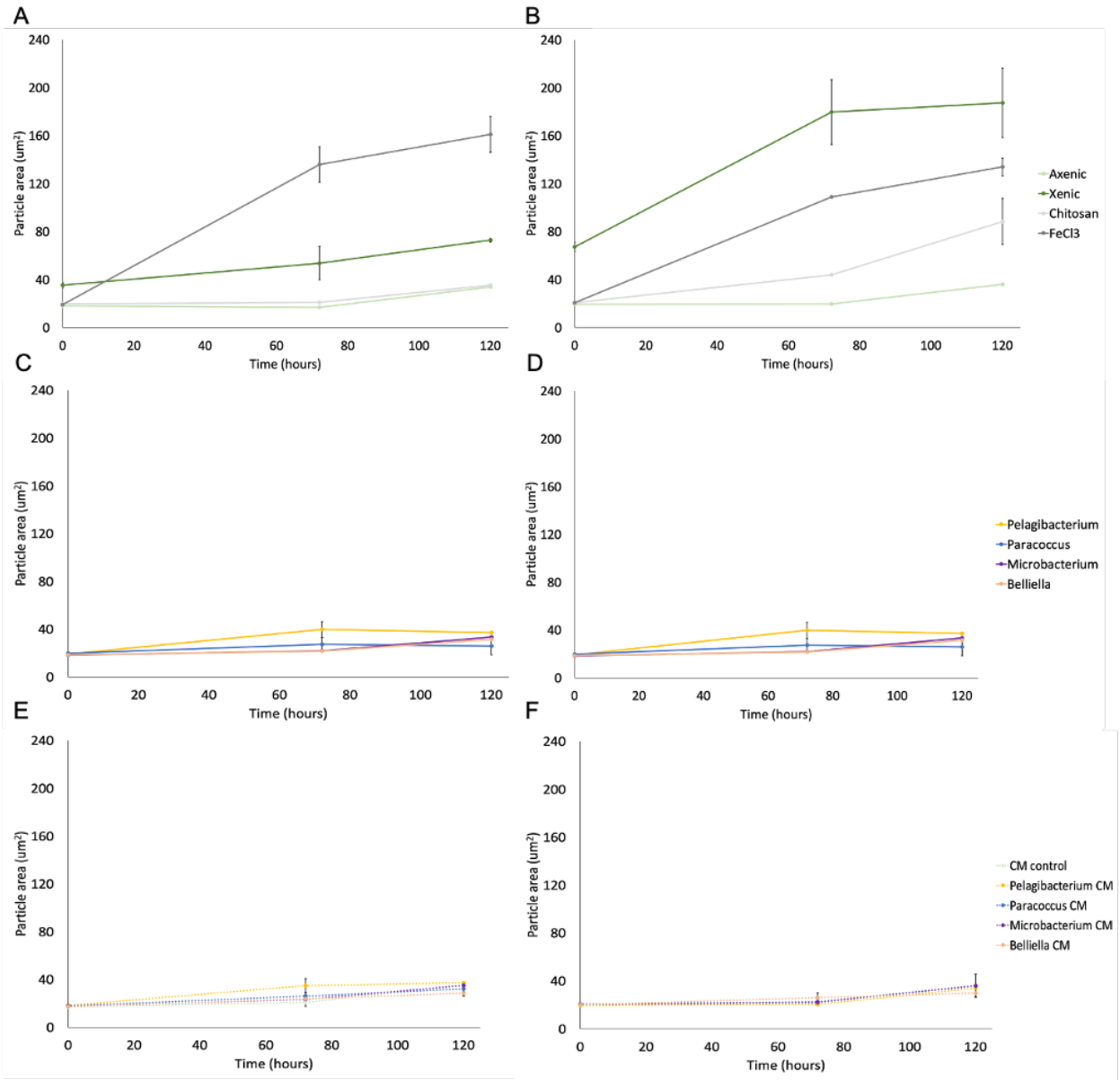

Figure 5.6. Particle size in SLA-04 cultures under low alkalinity (A, C, E) and high alkalinity (B, D, F) conditions over time. Different colored lines indicate different treatments, from top to bottom: axenic (light green), xenic (dark green), $FeCl_3$ (dark grey), and chitosan (light grey) (A, B). *Pelagibacterium* sp. (yellow), *Paracoccus* sp. (blue), *Microbacterium* sp. (purple), and *Belliella* sp. (orange) (C, D). Conditioned media (CM) treatments are indicated with dashed lines of the same color (E, F). Error bars represent one standard deviation from the mean (n=3) in the positive and negative direction.

Mean Particle Size (μm^2)			
	LA	HA	p-value
Axenic	**33.98**	**36.01**	**0.018**
Xenic	**72.86**	**187.48**	**0.028**
Pelagibacterium	**37.11**	**70.36**	**0.001**
Paracoccus	25.92	33.74	0.274
Microbacterium	33.28	39.87	0.280
Belliella	31.40	29.24	0.572
CM control	35.30	88.38	0.678
Pelagibacterium CM	37.40	48.05	0.268
Paracoccus CM	32.05	36.17	0.301
Microbacterium CM	34.98	35.89	0.280
Belliella CM	28.63	29.75	0.525
Chitosan	35.47	34.08	0.061
$FeCl_3$	160.98	134.09	0.187

Table 5.1: Mean particle size of SLA-04 cultures as measured by the FlowCam at t=120 hours. Values are calculated using the area based on diameter method and are reported in μm^2 for triplicate (n=3) measurements of 1000 particles per sample. Treatments are bolded if two-tailed, paired *t*-test comparison of means for LA and HA conditions have *p*-values < 0.05.

The difference in aggregate formation between taxonomically distinct co-cultures could result from differences in EPS production [66], and EPS could be produced by SLA-04 or the co-cultured bacteria. Growth rate, nutrient stress and the resulting changes in microalgal and bacterial physiology could impact EPS production [21]. Other studies have characterized the type and quantity of EPS produced in xenic microalgal aggregates [18] but using lower throughput microscopy methods than those described here.

High Alkalinity, Xenic Cultivation Improves Harvesting

To assess how aggregate-forming cultures compare to post-growth flocculated cultures in terms of improving harvesting and dewatering outcomes, settling experiments were conducted with common methodology from flocculation studies [18,26]. Axenic cultures, serving as a control, showed little settling (aggregation efficiency (AE) < 20%), indicating that it would be ineffective to use settling to aid in harvesting these cultures (Figure 5.7). Under both LA and HA conditions, 8 out of 12 treatments lead to significantly higher AE than the axenic control ($p < 0.05$). The only treatments that had AE approaching relevant levels for flocculation-assisted harvesting [24] were LA $FeCl_3$ (74%) and HA xenic (87%), HA $FeCl_3$ (84%) and HA chitosan (74%). HA conditions had higher mean AE when compared to LA across several treatments (Table 5.2), and the presence of larger aggregates under HA conditions may be due to abiotic or biotic factors. High alkalinity conditions could alleviate constraints in inorganic carbon diffusion into aggregates [67-69], allowing aggregates to grow to bigger sizes and/or providing more carbon for EPS production. HA conditions also have higher ionic strength that could increase flocculation efficiency due to the compression of the electrostatic double layer as outlined in the DLVO theory [70-71]. Alternatively, the LA and HA xenic cultures have different phycosome compositions (unpublished data), which could drive differences in aggregate size [17,18]. The significance of larger aggregates is shown in aggregation efficiency in these xenic cultures and may point to manipulating media or phycosome composition as targets for improving harvesting and dewatering processes.

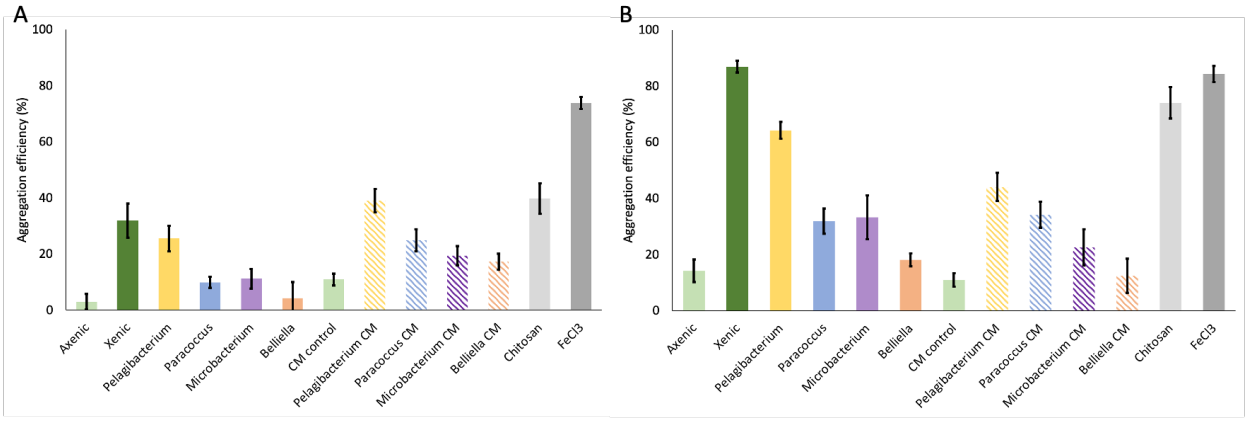

Figure 5.7. Aggregation efficiency in low alkalinity (A) and high alkalinity (B) cultures at t=120 hours. Different colored bars indicate different treatments, from left to right: axenic (light green), xenic (dark green), *Pelagibacterium* sp. (yellow), *Paracoccus* sp. (blue), *Microbacterium* sp. (purple), *Belliella* sp. (orange), $FeCl_3$ (dark grey), and chitosan (light grey). Conditioned media (CM) treatments are indicated with dashed bars of the same color. Error bars represent one standard deviation in the positive and negative direction. Asterisks above the bar denote treatments that have significantly higher mean aggregation efficiency ($p < 0.05$) than the axenic control.

Mean Aggregation Efficiency (%)			
	LA	HA	p-value
Axenic	2.97	14.26	0.123
Xenic	**31.90**	**86.92**	**0.011**
Pelagibacterium	**25.52**	**64.23**	**0.015**
Paracoccus	**9.95**	**31.88**	**0.038**
Microbacterium	**11.21**	**33.22**	**0.038**
Belliella	4.25	18.13	0.109
CM control	10.92	10.97	0.943
Pelagibacterium CM	39.01	44.09	0.168
Paracoccus CM	24.90	34.25	0.068
Microbacterium CM	19.44	22.56	0.684
Belliella CM	17.24	12.48	0.306
Chitosan	**39.77**	**74.07**	**0.040**
$FeCl_3$	73.77	84.28	0.069

Table 5.2. Mean aggregation efficiency at t=120 hours. Values are reported as percent reduction in OD750 after 30 minutes of settling. Treatments are bolded if two-tailed, paired *t*-test comparison of means for LA and HA conditions have *p*-values < 0.05.

Particle size did not correlate with maximum optical density in the LA and HA cultures (R^2=0.002, 0.043) (Figure 5.8 A, B), indicating that growth is not hindered by aggregation, and providing evidence to refute the original hypothesis. These results are supported by the observation that large marine diatom aggregates (> 1 cm) do not experience significant diffusion limitation until they encounter oxygen dead zones during sedimentation events (*i.e.,* marine snow) [72]. In these cultures, where mixing is vigorous and homogeneous, it is unlikely that gas exchange or light penetration is limited in aggregates. However, particle size was correlated with AE in LA and HA cultures (R^2=0.7, 0.8) (Figure 5.8 C, D), and these results demonstrated that the FlowCam methodology was supportive of the more traditional AE method but with increased quantification and throughput.

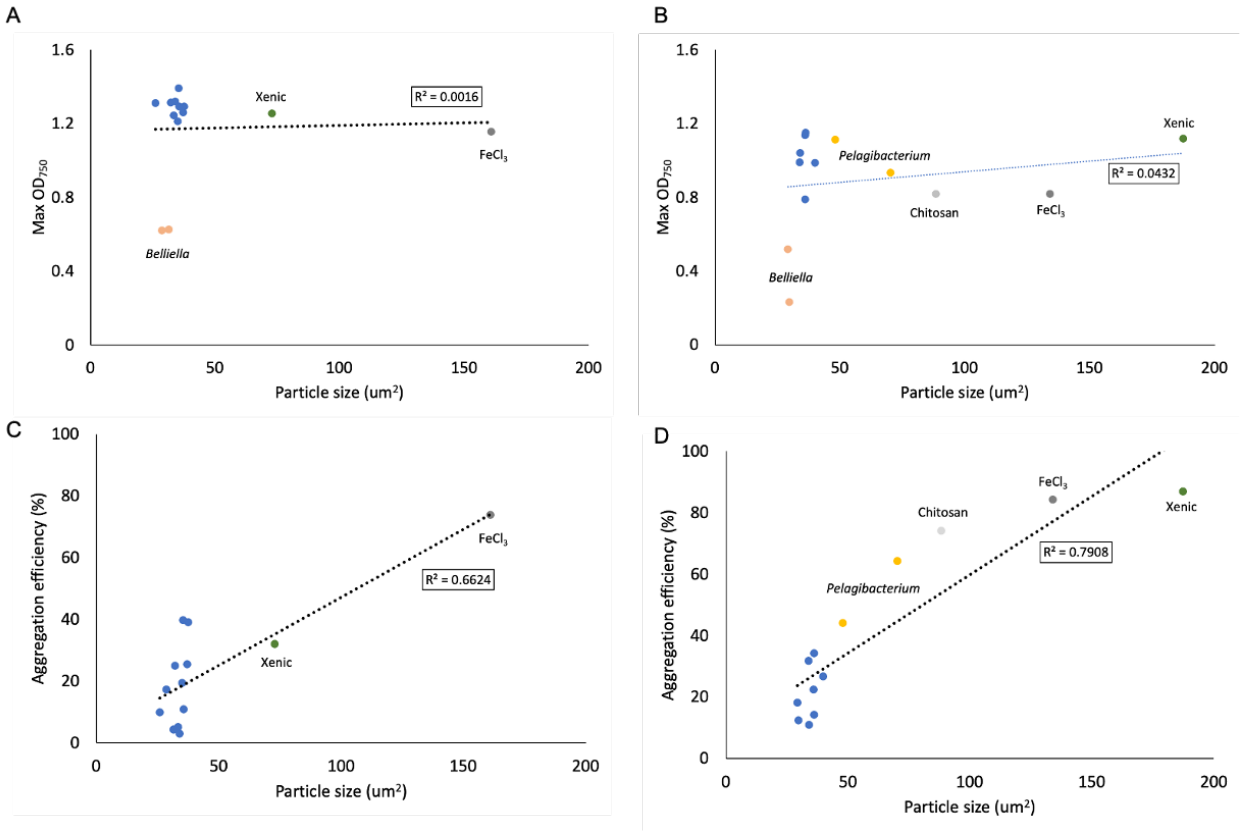

Figure 5.8. Scatter plots show correlation between maximum optical density and particle size for low alkalinity (A) and high alkalinity (B) treatments. Scatter plots show correlation between aggregation efficiency and particle size for low alkalinity (C) and high alkalinity (D) treatments. R-squared values are boxed in each plot.

Conclusion

These results are the first to show that xenic cultivation under HA conditions could lead to improved harvesting outcomes without the post-growth addition of external flocculants. Compared to a typical chemical flocculant such as $FeCl_3$, the xenic HA culture had a higher mean particle size and the aggregation efficiency was just as high (87% vs. 84%). This was achieved without a loss in productivity. In addition, the enhanced aggregation efficiency of *Pelagibacterium* co-cultures relative to the others tested suggests that engineering consortia to contain aggregate-inducing populations may be a promising direction for designing consortia to improve microalgal cultivation outcomes and economics. In addition to harvesting advantages, follow-up experiments challenging axenic, xenic, and co-cultures with culture crash-inducing organisms such as *Colpoda* grazers and *Vampirovibrio chlorellavorus* would demonstrate potential ecological role of aggregation that would provide culture stability over the course of cultivation. Preliminary results suggest that xenic cultures comprised of aggregates are more resistant to pests than axenic cultures (Wood et al., unpublished results). Additional work is needed to determine how manipulating environmental conditions, like temperature and light, impact aggregate formation. $FeCl_3$ and chitosan are widely used chemical flocculants that were added as positive controls for aggregation in this experiment. Each of the chemicals was only added at 1% of the typical post-growth flocculation concentration [23], indicating that long term growth in the presence of small quantities of the chemical flocculant could also be effective for efficient aggregation without leading to

detrimental growth outcomes. When applied as an end point treatment at standard working concentration in *Chlorella* cultures, $FeCl_3$ and chitosan have shown above 99% aggregation efficiency [23]. If successfully employed, this approach could significantly reduce the quantity of flocculant required, thereby lessening costs and environmental impact associated with algal biomass harvesting. Growth experiments at larger scales using diverse sets of microalgae and flocculants would be necessary to confirm that cultivation in the presence of low concentrations of these flocculants is not inhibiting growth of algae at larger scales.

Herron et al. (2019) [16] proposed two stress-dependent mechanisms for *Chlamydomonas* aggregate formation: (1) cells forming aggregates and then dividing, with new cells becoming part of a larger, growing aggregate; and (2) single planktonic cells coming together to form an aggregate, with more single cells being recruited to form a larger aggregate. In this study, the average size of SLA-04 aggregates in xenic cultures increased in size by up to 3-fold over 120 hours; however, more than 50% of particles were comparable in size to the axenic culture, indicating that even highly aggregated cultures of SLA-04 are comprised of many single planktonic cells. Thus, it is difficult to distinguish the mechanism driving aggregation in SLA-04 cultures. Monitoring single aggregates over time during the exposure to chemical or biological stressors will be necessary to elucidate the mechanism of SLA-04 aggregation and the potential role of individual bacterial populations.

Supplemental Figures in this Manuscript

Name	Source	Phylum	Genus	Isolation Media	Co-cultured w/ SLA-04
B1	LA flask	not sequenced	not sequenced	R2A	Y
B2	LA flask	Actinobacteria	*Agrococcus*	R2A	Y
IM1	LA flask	Actinobacteria	*Microbacterium*	LB	Y
IM13	LA raceway	Firmicutes	*Brevibacillus*	AE	Y
IM17	LA flask	Actinobacteria	*Rhodococcus*	ACM	Y
IM20	LA flask	Actinobacteria	*Rhodococcus*	ACM	Y
IM26	LA raceway	Proteobacteria	*Pseudomonas*	ACM	Y
IM27	LA raceway	Proteobacteria	*Pseudomonas*	ACM	Y
IM4	LA raceway	Actinobacteria	*Arthrobacter*	LB	Y
IM5	LA flask	Actinobacteria	*Rhodococcus*	BBM	Y
S13	LA flask	Proteobacteria	*Paracoccus*	R2A	Y
S2	LA flask	Proteobacteria	*Gemmobacter*	R2A	Y
U1	LA flask	not sequenced	not sequenced	LB	Y
U3	LA flask	not sequenced	not sequenced	LB	Y
U5	LA flask	not sequenced	not sequenced	LB	Y
W1	LA flask	Proteobacteria	*Rhizobium*	LB	Y
W2	LA flask	Proteobacteria	*Sphingomonas*	LB	Y
W3	LA flask	not sequenced	not sequenced	LB	Y
W4	LA flask	Proteobacteria	*Gemmobacter*	LB	Y
JS-1	LA flask	Actimomycetes	*Aeromicrobium*	MA 2216	N
JS-2	LA flask	Proteobacteria	*Paracoccus*	R2A	N
JS-11	HA flask	Proteobacteria	*Roseococcus*	R2A	N
JS-14	HA flask	Bacteroidetes	*Belliella*	MA 2216	N
JS-12	HA flask	Firmicutes	*Peribacillus*	MA 2216	N
N1-A	HA flask	not sequenced	not sequenced	R2A	Y
N1-B	HA flask	not sequenced	not sequenced	R2A	Y
N1-C	HA flask	not sequenced	not sequenced	MA 2216	Y
N3-B	HA flask	not sequenced	not sequenced	MA 2216	Y
N3-C	HA flask	Proteobacteria	*Pelagibacterium*	MA 2216	Y
N3-G	HA flask	Proteobacteria	*Paracoccus*	MA 2216	Y
N3-H	HA flask	Proteobacteria	*Porphyrobacter*	R2A	Y
N3-I	HA flask	Proteobacteria	*Aquimonas*	R2A	Y
N3-J	HA flask	Bacteroidetes	*Belliella*	R2A	Y
N3-K	HA flask	Proteobacteria	*Aquimonas*	R2A	Y
N3-L	HA flask	Proteobacteria	*Paracoccus*	R2A	Y
N3-O	HA flask	Proteobacteria	*Paracoccus*	R2A	Y
N3-P	HA flask	Proteobacteria	*Halomonas*	R2A	Y

Table 5.S1. Source culture, taxonomic identity, and isolation medium of all isolates obtained from xenic SLA-04 cultures. 31 of 37 of the isolates have been co-cultured with SLA-04. Highlighted isolates (n=4) were selected for discussion in the present study. Abbreviations: LA, low alkalinity; HA, high alkalinity; R2A, Reasoner's 2-Agar; LB, lysogeny broth; AE, algal extract; ACM, algal conditioned media; MA 2216, Marine Agar 2216; Y, yes, N, no.

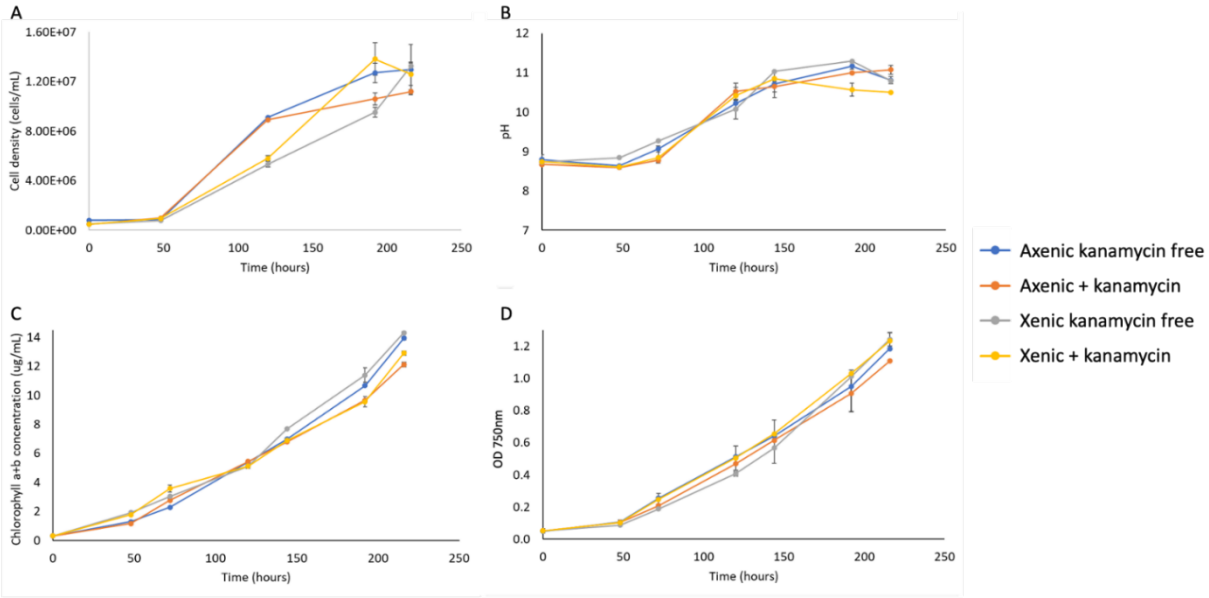

Figure 5.S1. SLA-04 physiology during growth with or without kanamycin. Cell density (A), pH (B), chlorophyll concentration (C), and OD 750nm (D) are reported at t=0, 48, 72, 120, 144, 192, and 216 hours. Four conditions were tested with triplicate flasks (n=3): axenic SLA-04 without kanamycin (blue); axenic SLA-04 with kanamycin (orange); xenic SLA-04 without kanamycin (grey); xenic SLA-04 with kanamycin (yellow). Error bars represent one standard deviation in both the positive and negative direction.

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CHAPTER SIX: EPILOGUE

Motivations for SLA-04 Phycosome Studies

The initial goals of my dissertation project were to grow microalgae in aquaculture effluent wastewater, use the resulting biomass to produce more sustainable aquafeed, and then study the impacts of diet on fish gut microbiome composition. Initial challenges in growing microalgae in aquaculture effluent led us to question whether native microbial communities in the effluent water were influencing microalgal growth (Appendix D). Elsewhere in the group, multiple projects working with the Chlorococcaceae-like microalga, PW95, identified hypotheses related to the role of the PW95 phycosome (Appendix A). As the effluent water experiments and the distinctive PW95 culture phenotype observations unfolded, the group's interest in the ecology of high pH, high alkalinity SLA-04 cultures grew.

Applying Ecological Theory to Microalgal Cultures

The research presented in this dissertation was framed around using ecology to enhance SLA-04 biomass productivity. From the perspective of the microbial ecologist, the motivation was the opportunity to study ecological interactions driven by a potentially dynamic organic carbon source (*i.e.*, the host algal cells). The nascent understanding of the relationship between the phycosome and microalgal biomass productivity aligned well with the exploratory nature of this research. One overarching theme was to determine if there is harmony between ecological theory and biomass productivity in engineered systems that are designed for unialgal biomass accumulation. There are many possible directions to take following this line of thinking. Namely, 1.) does an increase in community diversity correlate with improved stability, productivity, or

biomass accumulation? or 2.) does increased community diversity work antagonistically with the goals of a monoculture? The growth models, on which this research is based, account for the media components, reactor design, and presence of SLA-04 alone. Chapter 2 provided initial evidence that xenic cultures with disparate community structures maintain high levels of productivity. In Chapter 3, we summarized succession in xenic communities under changing environmental conditions. Still, questions remain regarding the relationship between diversity and productivity. It is also likely that studies of systems with different hosts, media conditions, and environmental constraints could produce different answers to these questions [4].

Identifying causal relationships between microbiome and host phenotype is a challenge in all host systems, from the human gut to microalgae. Many studies spanning a range of systems have commented on associations between the microbiome and host health. Koch's postulates highlight the importance of causative reasoning in making claims that a change in microbiome composition influenced the host. Although statistical inferences and 'omics-based predictions provide valuable insights, the presence of confounding variables in open systems and a vast microalgal metabolic diversity may limit the ability to assert causation based on the available data.

In SLA-04 cultures characterized by low diversity and a consistent composition over time, we encountered challenges in discerning noteworthy disparities in productivity between axenic and xenic cultures. Natural phycosomes generally have higher diversity than the SLA-04 phycosomes outlined in this dissertation [5]. It is possible that higher phycosome diversity in SLA-04 cultures would lead to different conclusions about the role of the phycosome in these studies. Periodically exposing xenic SLA-04 cultures to unfiltered water from Soap Lake would provide

the opportunity for recruitment of a more diverse phycosome, though it would be challenging to ensure that the culture remained unialgal.

There may be differences in phycosphere size and composition when comparing microalgae in well-mixed cultures to those in nature. Seymour et al. (2017) [5] modeled the influence of seawater turbulence on marine phycosome size and structure. We predict that constant, vigorous mixing in flasks, photobioreactors, or raceways, influences the size, shape, and composition of the phycosphere. In well-mixed cultures, to what extent do chemical gradients surround microalgal cells? Addressing this question is important for the future of phycosome studies and whether extrapolating findings from natural phycosome ecology studies into the laboratory is justified. We hypothesized that there would be taxonomic differences between communities that are directly attached to SLA-04 cells and freely associated. We were unable to find compositional differences through the methods described in Chapters 3 and 4. In Appendix A, where the aggregation of communities in unfiltered coal bed methane (CBM) production water was much more pronounced than in SLA-04 cultures, community differences between attached and planktonic communities were more obvious. Two key differences between the data in Appendix A and the data in Chapters 3 and 4 were 1.) CBM production water was unfiltered and 2.) the aggregates were large (>1 cm diameter), compared to the aggregates in the xenic SLA-04 cultures (~100 μm diameter). It may be interesting to analyze this data together to explore differences in diversity and taxa of interest in attached vs. planktonic communities.

The Importance of Scale in Phycosome Studies

Microalgal research faces difficulty in scaling benchtop findings to industrial scale cultures [6]. The research in this dissertation describes multiple scales: from 1.5 mL cultures in 24-well

plates, to 200 mL cultures in flasks, to 200 L cultures in raceways. In Chapter 5, when we attempted to confirm findings in 1.5 mL cultures by increasing volume to 200 mL, we experienced some of the challenges with scaling up. We predict that scalability could be an issue facing phycosome studies due to variability between experiments at different scales, specifically due to differences in mixing and environmental exposure. For reasons mentioned above, differences in mixing may lead to differences in phycosphere size and composition [5]. Culture mixing has been measured [7] and modeled [8] at large scales in raceways bioreactors, mostly to assess light delivery and gas exchange, but it may also be an important parameter to measure when scaling up bench top phycosome studies as well.

Temporal scale is another important consideration when designing phycosome studies. In Chapter 2, we tracked SLA-04 phycosome composition spanning up to five years by sampling every 4-10 months. We found consistent taxonomic composition and diversity as well as stable growth and productivity. In Chapter 3 we sampled and sequenced the phycosome every 24-72 hours during an open, outdoor raceway experiment. We showed a change in phycosome composition through five successive experiments over the course of 60 days. In Chapter 4 we sampled closed cultures every 10-14 hours during the BONCAT experiment, finding changes in phycosome activity with relationship to the diel cycle. When temporal resolution changed, so did the conclusions stemming from community analysis. For future studies assessing the physiological influence of the phycosome, experimental design should center on a temporal scale that best fits the objectives. For example, assessing phycosome recruitment and assembly might require a longer duration than an experiment defining the relationship between phycosome activity and extracellular nitrate concentration. Reflecting on the duration of the BONCAT experiment

(Chapter 4), collecting samples for more light and dark cycles might have better elucidated the relationship between internal microalgal carbon stores, extracellular organic carbon, and phycosome activity.

It is human nature to project human traits on cultures that contain billions of cells, viewing a culture as a single organism with a wholistic phenotype. And it is logical to follow that way of thinking in research that is motivated by large scale productivity values. Traditional microalgae productivity experiments reflect the cumulative or average phenotypes of more than 10^4 individual cells, yet single cell heterogeneity is known to persist [9-11]. When characterizing metabolic interaction in a xenic culture should phenotype be measured at a bulk level or at a single cell level? We have observed cell-to-cell heterogeneity but have yet to find a robust method of characterization. Using fluorescent microscopy and particle analysis (FlowCam) of cultures stained with lipid dyes, we have seen measurable differences in single cell lipid content (Figure 6.1 A-C). We sorted microalgae based on lipid content, separating single cells with low and high lipid content. After culturing for a growth cycle (t=240 hours), low and high lipid content cultures returned to a similar composition as the starting culture. Selective pressures such as nutrient stress may lead to phenotypic drift [12]. Phenotypic plasticity could pose a challenge for studying microalgae-phycosome interactions at the single-cell level where the goals would involve correlating microalgal phenotype with associations with specific populations in mixed consortia.

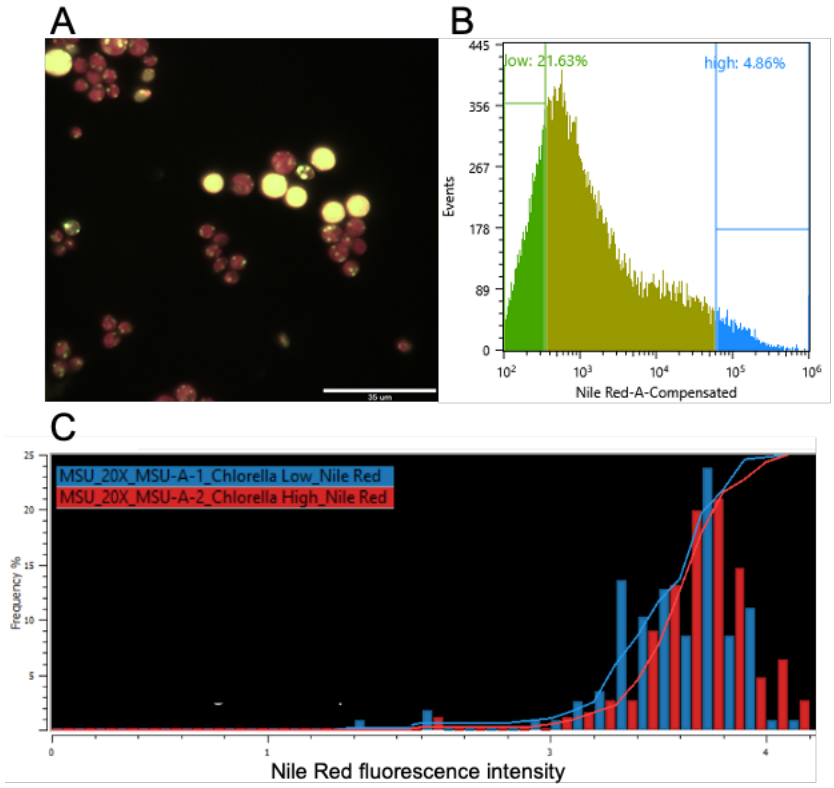

Figure 6.1. Nile Red staining of PW95 and SLA-04 cultures revealed heterogeneity in lipid content. (A) PW95 cells stained with the lipid stain Nile Red. Nile Red staining is visualized with the yellow color and chlorophyll autofluorescence is in red. The scale bar in the bottom right represents 35 µm. Image credit: Luisa Corredor-Arias. (B) Histogram illustrating the distribution of cells stained with Nile Red as measured on the Sony SH800. The arbitrary gates were drawn for cells with low lipid content (green) and high lipid content (blue). (C) Histogram showing the distribution of Nile Red stained SLA-04 cells from high lipid cultures (red) and low lipid cultures (blue) as measured using the FlowCam. This was part of a pilot project in collaboration with Nicole Sims at FluidImaging.

Over the past six years, we investigated SLA-04 physiology and ecology at the single cell level using Raman spectroscopy, nanoscale secondary ion mass spectrometry (nanoSIMS), and stereolithography 3D printing. The aim for each of these projects was to characterize how SLA-04 physiology changed in relationship with metabolite exchange between SLA-04 and a single isolate or mixed consortia. A summary of these efforts is provided in Appendix D. We would like to use

these techniques to study SLA-04 phycosome reactions at the single cell level over the duration of time course experiments.

Future Directions for SLA-04 Phycosome Studies

While the sequencing-based data presented in this dissertation consisted solely of amplicon SSU rRNA gene analyses, we have recently expanded our approach to include both metagenomic and whole genome sequencing. The gap between taxonomy and function was described in Chapters 2, 3, and 4. Through sequencing metagenomes we intend to assess how taxonomic differences relate to functional gene differences, especially in relationship to genes that are hypothesized to play important roles in our cultures. We hypothesize that despite taxonomic differences between the cultures, there may be more functional overlap. This would suggest that deterministic processes play a role in the functional assembly of the SLA-04 phycosome, an amended conclusion to Chapter 2. We also hypothesize that there will be functional differences between LA and HA metagenomes. For example, we expect that there may be an alkalinity-dependent relationship in prevalence of genes related to osmolyte synthesis and transport. Based on SLA-04 genomic analysis, trehalose, sucrose, and glycerol are predicted to be important osmolytes in SLA-04 systems. Because HA conditions also have higher salinity, the role of osmolytes likely differs between the two conditions. This work is being led by Adrienne Arnold and Dr. Ross Carlson.

Four bacterial isolates have been sequenced using PacBio whole genome sequencing (*Paracoccus* sp.*, Microbacterium* sp.*, Pelagibacterium* sp.*,* and *Belliella* sp.*)*. These isolates are representative of the organisms that were discussed in Chapter 5. Led by Adrienne Arnold and Dr. Ross Carlson, we have constructed metabolic models and begun to run metabolic flux analysis,

ultimately hoping to identify metabolic interactions between these organisms and SLA-04. It is hypothesized that using these approaches, we will be able to predict why certain populations (*e.g.*, *Belliella* vs. *Salinarimonas*) had differential activity during the BONCAT experiment in Chapter 4. Another interesting finding to probe would be the antagonistic interaction between the *Belliella* sp. isolate and SLA-04 (Chapter 5). *Belliella* is an abundant genus in UT SLA-04 cultures, thus an antagonistic relationship with SLA-04 could be an important consideration when comparing axenic and xenic culture physiology. We have also been working with nine different metagenome-assembled genomes (MAGs) from the MSU LA maintenance culture, with some of these closely related to those isolates for which genomes have been sequenced. We have also recently received metagenome sequences for the UT LA and HA cultures containing dozens of high-quality MAGs. Collaborating with a National Science Foundation Research Experience for Undergraduates (NSF REU) student Justus Smith, we grew *Microbacterium* and *Belliella* isolates on carbon and nitrogen Biolog plates (Biolog, DE, USA). We intend to use this data, coupled with data previously collected by Adrienne Arnold, to test predictions made using metabolic reconstructions of MAGs and genomes.

Grazing activity in *Colpoda*, which is the genus-level classification of the grazers in the UT SLA-04 LA and HA cultures, may be condition and composition dependent [13]. Using microscopic observations, grazers can be classified as bacterivorous, algivorous, or omnivorous [14]. We have attempted to conduct controlled experiments to identify conditions and prey sources that promote *Colpoda* grazing activity but have found difficulty separating the grazers from microbial intra- and extracellular communities. Future work will probe how alkalinity and phycosome composition impact *Colpoda* activity and grazing preference. Preliminary data suggest

that xenic cultures comprised of aggregates are more resistant to *Colpoda* grazing than axenic cultures (Wood et al., unpublished results). If these grazer populations have been in our cultures for years, why are cultures not crashing and why does phycosome composition remain stable? Does the phycosome provide protection by 1.) inducing aggregation; 2.) serving a food source for the grazer; or 3.) producing metabolites that inhibit grazing? When algae farmers detect grazers, they often use bleach or other chemicals to attempt to sterilize their cultures and reestablish a "healthy" monoculture [15]. We have already demonstrated why that practice is not supported by ecology. We would like to also demonstrate that, with the proper media and ecological constraints, the presence of grazers does not require the application of environmentally toxic chemicals.

In working with graduate student Bear Wood, we are probing the relationship between oxygen concentration, the diel cycle, and carbon metabolism. We have started to use the FireSting fiber optic system (PyroScience, Aachen, Germany) to make *in-situ* measurements of oxygen concentrations in axenic and xenic SLA-04 cultures. We hypothesize that xenic cultures will reach lower oxygen concentration during the dark cycle due to higher levels of respiration [16]. We predict the same for HA cultures, where inorganic and organic carbon fluxes are measurably higher which could lead to higher rates of dark cycle respiration [17]. If HA cultures show increased levels of respiration at night, it may be advantageous to use LA conditions prior to nitrogen depletion, such as adding alkalinity at nitrogen depletion, an approach explored by the MSU algal group for years (*i.e.*, bicarbonate trigger) [18-20]. We found that constantly shaking cultures keeps oxygen levels near oxygen saturation, so preliminary experiments have incorporated treatments where shaking is switched off during the dark cycle.

In Chapter 4 we discuss the role of extracellular organic carbon (EOC) as a potential driver of phycosome activity. By measuring EOC in these cultures, we showed increasing the concentration of inorganic carbon in the media does not lead to a proportional increase in EOC. However, this methodology is not specific enough to completely answer important questions about how SLA-04 and phycosome metabolite production might differ between LA and HA cultures, particularly with respect to nitrate concentration and diel cycling. To quantitatively assess the composition of the exometabolome, we began working with Gwen Cooper from the Bothner Lab at MSU to use liquid chromatography-mass spectrometry to characterize the exometabolome in axenic and xenic LA and HA cultures. We faced some difficulty with contamination and sample concentration, but this work is ongoing. We hypothesize that there will be unique exometabolomic profiles when comparing LA and HA cultures, potentially in type and concentration of osmolytes.

Finally, there are still many questions to answer about SLA-04 phycosome interactions with respect to pH and alkalinity. There are knowledge gaps in the understanding of energy metabolism in alkalitolerant and alkaliphilic organisms, particularly in relationship to how proton motive forces are used at high pH and at fluctuating pH levels [21]. When the SLA-04 growth medium includes a strong buffer, such as in the HA condition, there is a strong resistance to pH changes, and pH remains near the starting point of pH 10.3. When the systems are only weakly buffered, pH fluctuates with an amplitude related to growth rate, from pH 8 and pH 11.5 at the highest. As the HA condition has increased sodium concentrations, the role of sodium motive force should be investigated [22]. How change in pH change, rather than just stable, extreme pH, impacts microalgal and bacterial metabolism is still poorly understood and warrants future work.

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APPENDICES

APPENDIX A

APPENDIX A: TEMPORAL PHYCOSOME DYNAMICS OF A CHLOROCOCCACEAE-LIKE MICROALGAE IN STIMULATEED COAL BED METHANE PRODUCTION WATER

Contribution of Authors and Co-Authors

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Contributions: Experimental design; data collection; data analysis

Co-Author: Isaac R. Miller

Contributions: Data analysis; manuscript drafting and revising

Co-Author: Matthew W. Fields

Contributions: Experimental design; data analysis

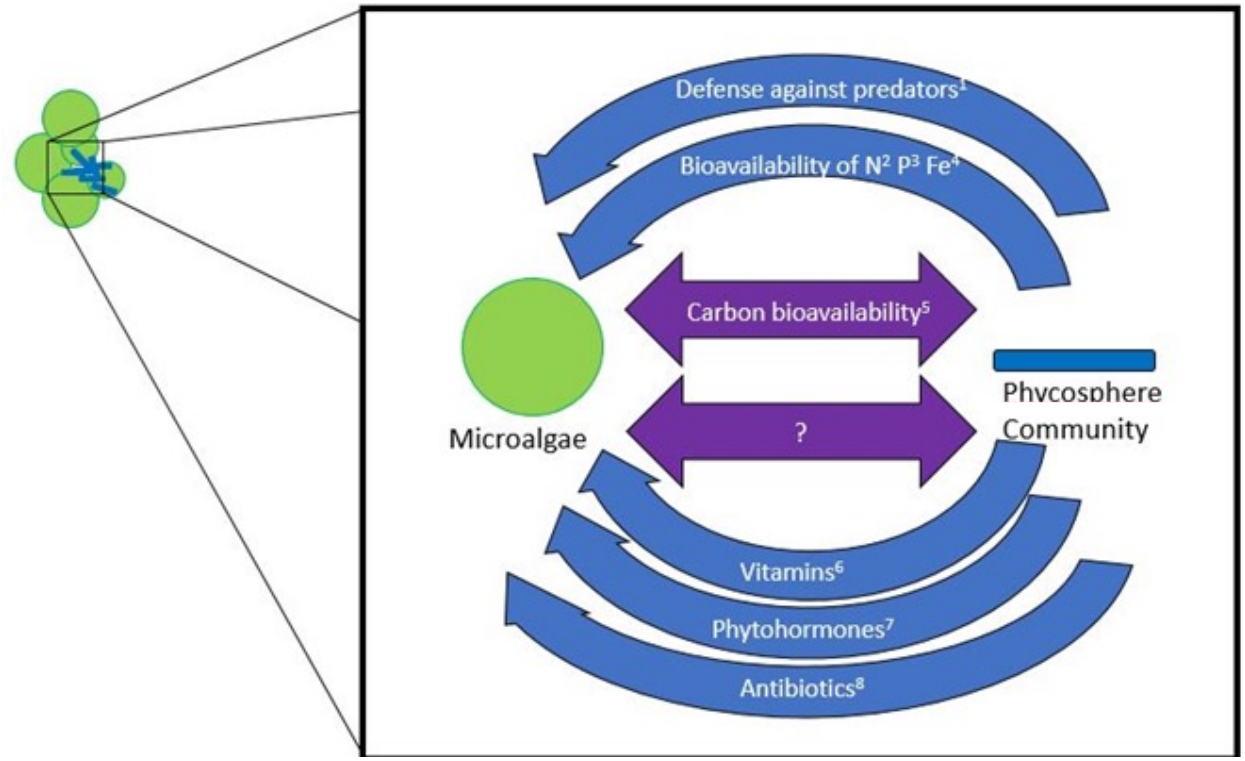


Figure A.1. Summary of selected literature characterizing interactions between microalgae and the phycosome. 1. Fels and Katz, 2008; 2. Ammerman and Azam, 1985; 3. Wheeler and Kirchman, 1986; 4. Amin et al., 2009; 5. Samo et al., 2018; 6. Croft et al., 2005; 7. De Bashan et al., 2008; 8. Long and Azam, 2005.

Materials and Methods

Algal Isolate PW95

The microalga, PW95, was isolated from a CBM production water pond in the Powder River Basin of northeastern Wyoming (44° 52.613′N 106° 54.700′W). PW95 was identified through 18S rRNA gene sequencing as a member of the Chlorococcaceae family (Hodgskiss et al., 2016).

Experimental Design

Three sets of triplicate flasks of unfiltered CBM production water were each inoculated with 10% inoculum of stationary phase PW95 culture. T=0 harvest occurred one hour after

inoculation. Three more flasks were harvested after one week and the last three were harvested after two weeks. Algae was allowed to settle out of the media and the supernatant was removed. The supernatant was concentrated on a 0.22 μm filter. Both the algal biomass and the dry filters were stored at -80 ºC. The community of the inoculum and the CBM water prior to inoculation were also sampled. The FastSpin DNA Extraction Kit (MP Biomedical, Solon, OH, USA) was used to extract DNA from the supernatant community and the algal phycosphere community separately.

16S rRNA Gene Sequencing

PCR was performed on extracted DNA with primers 9F (5' -GAGTTTGATCC TGGCTCAG) and 1492R (5'-GGTTACCTTGTTACGACTT). 16S rRNA gene was amplified in replicate 50 μL PCR reactions and contained extracted genomic DNA, 0.1 μM of each primer, a 1X final concentration of Bull's Eye Premium Taq 2X Mix (Midwest Scientific, St Louis, MO, USA), and nuclease free water. The thermocycler protocol consisted of an initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Next, amplified DNA was indexed for later application on the MiSeq. Primer complexes included the Illumina adaptor sequences followed by the universal primers 341F (5'acactctttccctacacgacgctcttccgatct CCTACGGGNGGCWGCAG-3') or 805R (5'gtgactggagttcagacgtgtgctcttccgatct GACTACHVGGGTATCTAATCC-3'). The 50 μL PCR reaction contained 2.5 μL of PCR amplicon and concentrations of PCR reagents as mentioned in the protocol above. The nested PCR amplification consisted of an initiation denaturation at 95 °C for 3 min followed by 12 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final

extension step at 72 °C for 5 min. PCR was performed in an Eppendorf Mastercycler pro S. Gel electrophoresis was used to visualize PCR products in a 1.0 % agarose Tris-acetate EDTA gel stained with GelRed. Blank sample controls for both the initial PCR amplification as well as the indexing of amplified DNA were included in the sequence libraries.

Sequence Processing and Analysis

Sequences were analyzed following a bidirectional MiSeq run utilizing the public access Mothur platform v.1.39.5 (Schloss et al., 2009). The maximum sequence length included in this analysis was 442 bp, with everything shorter than 406 bp being screened out. Sequences with homopolymers numbering greater than eight, ambiguous bases were also screened out. Finally, sequences with a quality score below 30 over a 60 bp range were discarded. The trimmed and screened sequences were then aligned with the SILVA 16S V3V4 database. Chimeric sequences were identified and removed using the VSEARCH tool in combination with the Mothur platform. Operational taxonomic units (OTUs) were assigned using the classifying tool in the Mothur package and were screened to include OTUs of 97% sequence identity against the database. Taxa which represented less than one percent of the community abundance across all of the treatments were filtered out. After the screening process there were 182,987 sequences remaining.

Statistical analysis was performed using the XLSTAT package as well as diversity metric tools in the Mothur package. Bray-curtis diversity metrics were calculated using the tools in the Mothur package. The data were normalized to relative abundance and then organized into bar charts using Microsoft Excel. Taxa at both the class and the genus levels were analyzed using a similarity percentage (SIMPER) analysis in the statistical package PAST (Hammer et al., 2001). The multivariate SIMPER statistics were generated using Bray-Curti's dissimilarity metrics.

Results

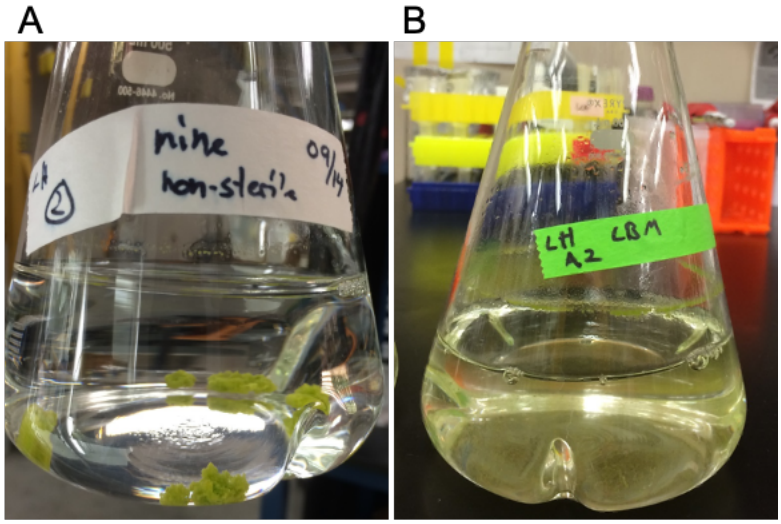
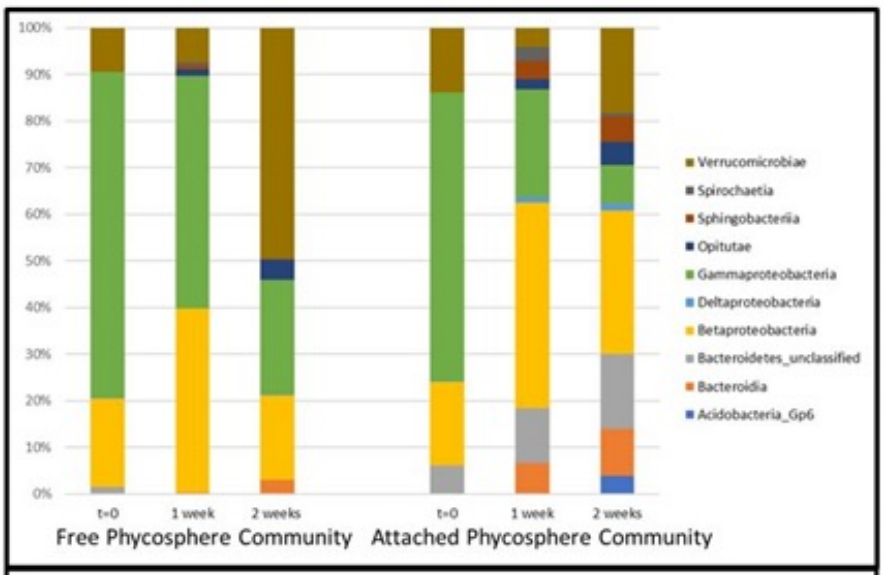


Figure A.2. Images of flasks containing PW95 in unfiltered CBM water (A) and axenic PW95 in filtered CBM water (B).

A



B

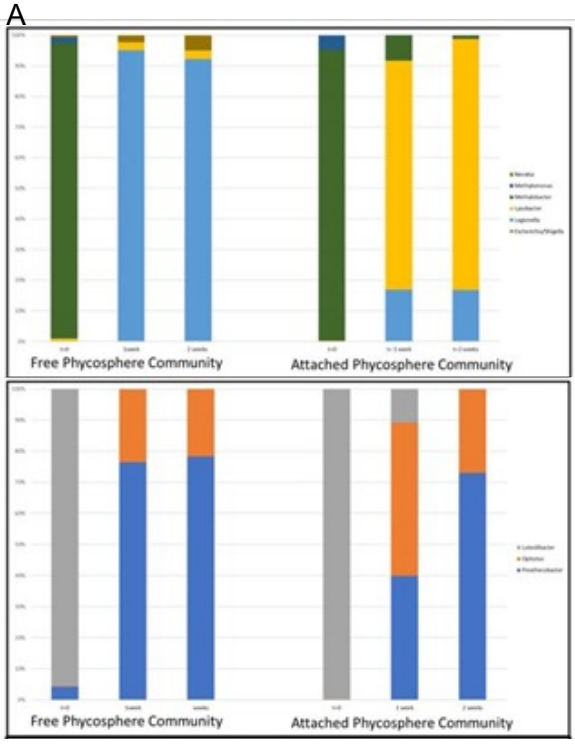
Free Phycosphere Community		
Group 1: t=0	Group 2: 2 weeks	Overall Average Dissimilarity : **50.00**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Gammaproteobacteria	**22.70**	**45.41**
Verrucomicrobiae	**20.00**	**40.00**
Betaproteobacteria	2.54	5.07
Opitutae	2.14	4.29
Bacteroidia	1.53	3.06
Bacteroidetes_unclassified	0.76	1.52
Spirochaetia	0.14	0.28
Sphingobacteriia	0.09	0.19
Deltaproteobacteria	0.08	0.17
Acidobacteria_Gp6	0.01	0.01

Attached Phycosphere Community		
Group 1: t=0	Group 2: 2 weeks	Overall Average Dissimilarity : **53.56**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Gammaproteobacteria	**7.41**	**24.11**
Verrucomicrobiae	**7.04**	**22.90**
Betaproteobacteria	6.69	21.77
Bacteroidetes_unclassified	2.14	6.95
Bacteroidia	1.92	6.24
Acidobacteria_Gp6	1.81	5.89
Opitutae	1.40	4.55
Spirochaetia	1.11	3.60
Sphingobacteriia	0.75	2.46
Deltaproteobacteria	0.47	1.53

Figure A.3. Class level composition of the PW95 phycosome in free and attached communities. (A) Relative abundance at the class level at t=0, 1 week, and 2 weeks. Similarity percentage

(SIMPER) analysis comparing the communities in the free and attached fractions at t=0 and t=2 weeks (B).

A



B

Free Phycosphere Community

Group 1: t=0	Group 2: 1 week	Overall Average Dissimilarity : **98.21**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Methylobacter	66.37	67.58
Legionella	29.84	30.38
Methylomonas	1.198	1.22
Nevskia	0.5464	0.5564
Lysobacter	0.2575	0.2622
Escherichia/Shigella	0	0

Attached Phycosphere Community

Group 1: t=0	Group 2: 1 week	Overall Average Dissimilarity : **97.14**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Methylobacter	80.29	82.65
Lysobacter	10.33	10.63
Methylomonas	4.151	4.275
Legionella	2.374	2.444
Nevskia	0	0
Escherichia/Shigella	0	0

Free Phycosphere Community

Group 1: t=0	Group 2: 2 weeks	Overall Average Dissimilarity : **98.63**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Methylobacter	83.65	84.82
Legionella	12.37	12.55
Methylomonas	1.51	1.533
Nevskia	0.5091	0.5166
Lysobacter	0.4848	0.4921
Escherichia/Shigella	0.01215	0.01233

Attached Phycosphere Community

Group 1: t=0	Group 2: 2 weeks	Overall Average Dissimilarity : **99.63**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Methylobacter	89.65	90.08
Methylomonas	4.576	4.598
Lysobacter	4.439	4.46
Legionella	0.8597	0.8637
Nevskia	0	0
Escherichia/Shigella	0	0

Free Phycosphere Community

Group 1: t=0	Group 2: 1 week	Overall Average Dissimilarity : **95.80**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Luteolibacter	65.34	68.21
Prosthecobacter	23.15	24.17
Opitutus	7.305	7.625

Attached Phycosphere Community

Group 1: t=0	Group 2: 1 week	Overall Average Dissimilarity : **94.39**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Luteolibacter	73.31	77.67
Opitutus	11.74	12.44
Prosthecobacter	9.337	9.892

Free Phycosphere Community

Group 1: t=0	Group 2: 2 weeks	Overall Average Dissimilarity : **96.75**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Prosthecobacter	45.35	46.88
Luteolibacter	35.36	36.55
Opitutus	16.05	16.57

Attached Phycosphere Community

Group 1: t=0	Group 2: 2 weeks	Overall Average Dissimilarity : **99.69**
Taxon	Average Dissimilarity	Contributed Dissimilarity (%)
Luteolibacter	42.59	42.72
Prosthecobacter	41.76	41.89
Opitutus	15.34	15.39

Figure A.4. Genus level composition within the Gammaproteobacteria (top) and Verrucomicrobiae (bottom) classes of the PW95 phycosome in free and attached communities. (A) Relative abundance at the class level at t=0, 1 week, and 2 weeks. Similarity percentage (SIMPER) analysis comparing the communities in thee free and attached fractions at t=0 and t=2 weeks (B).

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APPENDIX B

APPENDIX B: SEQUENCING PROGRESS FOR PHYCOSOME ISOLATES AND SLA-04 METAGENOMES

Sample name	Taxonomic ID	Original culture	Sequencing status	Notes
Paracoccus sp. S13E	*Paracoccus sp.*	MSU SLA-04 indoor xenic low alkalinity	Complete. PacBio whole genome.	Metabolic reconstructions, modeling and experimental follow-up in progress
Microbacterium sp. W1D	*Microbacterium* sp.	MSU SLA-04 indoor xenic low alkalinity	Complete. PacBio whole genome.	Metabolic reconstructions, modeling and experimental follow-up in progress
Neorhizobium sp. S13A1	*Bacillus* sp.	MSU SLA-04 indoor xenic low alkalinity	N/A	Abandoned; contaminated
Pelagibacterium sp. N3C-HB	*Pelagibacterium* sp.	UT SLA-04 N3 xenic high alkalinity	Complete. PacBio whole genome.	Contaminated with *Paracoccus* reads; correction on-going
Belliella sp. N3J	*Belliella* sp.	UT SLA-04 N3 xenic high alkalinity	Complete. PacBio whole genome.	Metabolic reconstructions, modeling and experimental follow-up in progress
AW6 co-culture	*Pelagibacteium* sp. *Chryseoglobus* sp. *Pseudonocardia* sp.	UT SLA-04 N3 xenic high alkalinity	Complete. Illumina metagenome 400 b.p.	
N3C-MT	*Pelagibacteium* sp. *Chryseoglobus* sp. *Pseudonocardia* sp.	UT SLA-04 N3 xenic high alkalinity	Complete. Illumina metagenome 400 b.p.	

Table B.1. Progress of whole genome sequencing of isolates and mini-metagenomes from SLA-04 phycosome.

Sample name	Original culture	Sequencing status	Notes
DNA-SLA04-IM Rep1 MG pilot	MSU SLA-04 indoor xenic low alkalinity	Complete. Illumina metagenome 270 b.p.	Metabolic reconstructions, modeling and experimental follow-up in progress
DNA-SLA04-IM Rep1 MG pilot	MSU SLA-04 indoor xenic low alkalinity	Complete. Illumina metagenome 270 b.p.	Metabolic reconstructions, modeling and experimental follow-up in progress
SSL1-WT (APEX)	PNNL cyanobacteria collection	Complete. Illumina metagenome 400 b.p.	Initial analyses
SSL1-Turbo (APEX)	PNNL cyanobacteria collection	Complete. Illumina metagenome 400 b.p.	Initial analyses
UT-N3 LA Pre-NO3	UT SLA-04 N3 xenic low alkalinity	Complete. Illumina metagenome 400 b.p.	Received on 09-26-23
UT-N3 HA Pre-NO3	UT SLA-04 N3 xenic high alkalinity	Complete. Illumina metagenome 400 b.p.	Received on 09-26-23
UT-N3 LA Post-NO3	UT SLA-04 N3 xenic low alkalinity	Complete. Illumina metagenome 400 b.p.	Received on 09-26-23
UT-N3 HA Post-NO3	UT SLA-04 N3 xenic high alkalinity	Complete. Illumina metagenome 400 b.p.	Received on 09-26-23

Table B.2. Progress of metagenomic sequencing of xenic cultures of *Chlorella* sp. SLA-04 and xenic SSL-1 (cyanobacteria).

APPENDIX C

APPENDIX C: PILOT STUDIES OUTLINING METHODS FOR INVESTIGATING SINGLE CELL PHYCOSOME INTERACTIONS

Contribution of Authors and Co-Authors

Author: Huyen Bui

Contributions: Experimental design; sample preparation; data collection; data analysis

Co-Author: Isaac R. Miller

Contributions: Experimental design; sample preparation; data collection; data analysis

Co-Author: Isaak Thornton

Contributions: Experimental design; sample preparation; data collection; data analysis

Co-Author: Kathryn Zimlich

Contributions: Experimental design; sample preparation; data collection; data analysis

Co-Author: Nathan Bowman

Contributions: Experimental design; sample preparation; data collection; data analysis

Co-Author: John Cliff

Contributions: Sample preparation; data collection; data analysis

Co-Author: Robin Gerlach

Contributions: Experimental design; data analysis

Co-Author: Matthew W. Fields

Contributions: Experimental design; data analysis

This appendix serves as a summary of the results from several pilot projects. We employed nanoscale secondary ion mass spectrometry (nanoSIMS), 3D stereolithography printing, and Raman microspectroscopy all with the goal of developing methods to study ecology and physiology in SLA-04 cultures with single cell or single aggregate resolution.

NanoSIMS Pilot Project:

This project was conducted in collaboration with John Cliff and Sarah Leichty at Pacific Northwest National Laboratory.

SLA-04 cultures were grown in BBM at an initial pH 8.75 to log phase and enumerated using a standard hemocytometer method. Cells were then resuspended in fresh media to a final concentration of $1x10^6$ cells/mL in two separate flasks. Flask A contained 3 mM NO_3^- and 0 mM HCO_3^- while flask B contained 0 mM NO_3^- 50 mM HCO_3^-. An isotopically labeled carbon substrate ^{13}C-HCO_3^- was added to flask A to a final concentration of 50mM. Similarly, an isotopically labeled nitrogen substrate ^{15}N-NO_3^- was added to flask B at a final concentration of 3mM. Flask A and B were incubated at 20 °C and a 14h:10h light:dark cycle. At selected timepoints (t=0, 1, 2, 4, 6, 12, 24 and 30 hours; t=0 was at the fourth hour of the dark period), 1 mL of each culture were immediately fixed in 4% paraformaldehyde and fixed samples were stored at 4°C until arriving at the NanoSIMS facility at PNNL (EMSL) for imaging.

The results of this experiment indicated that xenic SLA-04 cultures have low levels of inorganic carbon (Figure C.1) and nitrate (Figure C.2) incorporation during the dark cycle. The beginning of the light cycle (t=6 hrs) was marked by a noticeable shift to higher activity levels, as indicated by nitrogen and carbon incorporation.

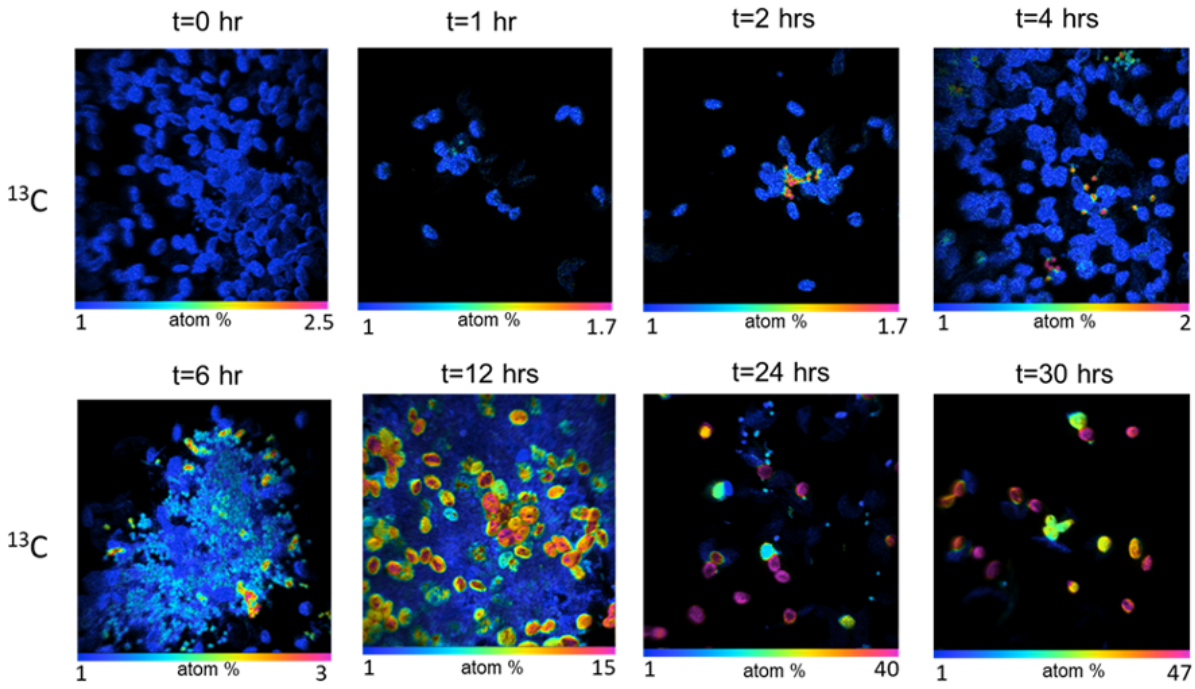

Figure C.1. NanoSIMS isotope ratio images from 13C-labeled HCO3- incubation experiments. The experiment was started (t=0 hr) during the dark cycle, and the light cycle began at t=6 hrs. The blue color of cells in the images indicate that the carbon in the sample is comprised largely of 12C carbon (~1% 13C, which is approximately the natural abundance of 13C). As the atom percentage shifts to more 13C, brighter green, yellow and red colors begin to appear in the images, corresponding to the atom percentage scale on the bottom of each image. Credit: John Cliff, Sarah Leichty (PNNL).

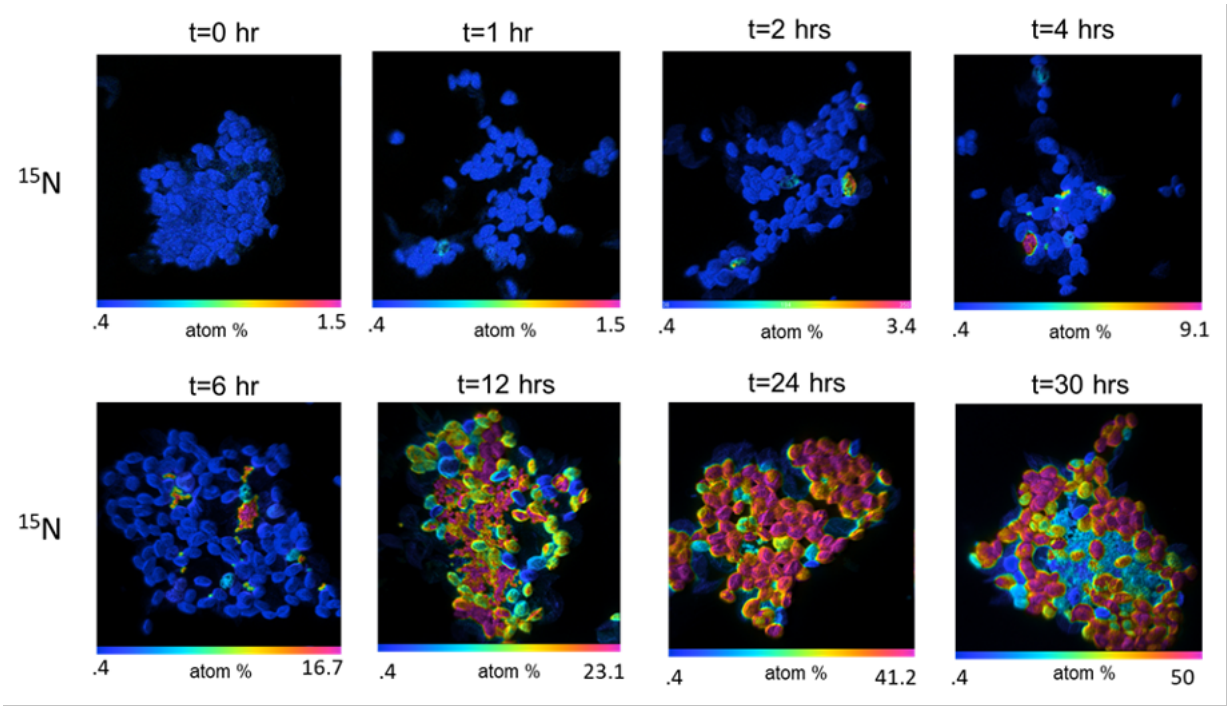

Figure C.2. NanoSIMS isotope ratio images from 15N-labeled NO3- incubation experiments. The experiment was started (t=0 hr) during the dark cycle, and the light cycle began at t=6 hrs. The blue color of cells in the images indicate that the nitrogen in the sample is comprised almost entirely of molecules containing 15N nitrogen. As the atom percentage shifts to being comprised of 15N nitrogen, brighter green, yellow and red colors begin to appear in the images, corresponding to the atom percentage scale on the bottom of each image. Credit: John Cliff, Sarah Leichty (PNNL).

The aim of this experiment was to determine how nitrogen and carbon were being exchanged between SLA-04 and the phycosome during the diel cycle. One potential issue facing this application of nanoSIMS is loss of native aggregate structure during the sample preparation process in which samples are dried onto

3D Stereolithography Printing SLA-04

This project started thanks to a collaborative effort between National Science Foundation Research Experience for Undergraduates (NSF REU) student Nathan Bowman and Isaak

Thornton, Wilking Lab. Together, we successfully printed axenic and xenic SLA-04 cultures in hydrogels. Figure 3 shows increase in biovolume from t=0 to t=168 hrs. This was the first time our group had successfully 3D printed microalgae. Figure A.4 depicts an aggregate of SLA-04 and phycosome bacteria in the hydrogel matrix at t=168 hrs. This represents proof and concept and the beginning of a project led by Isaak Thornton and Kathryn Zimlich

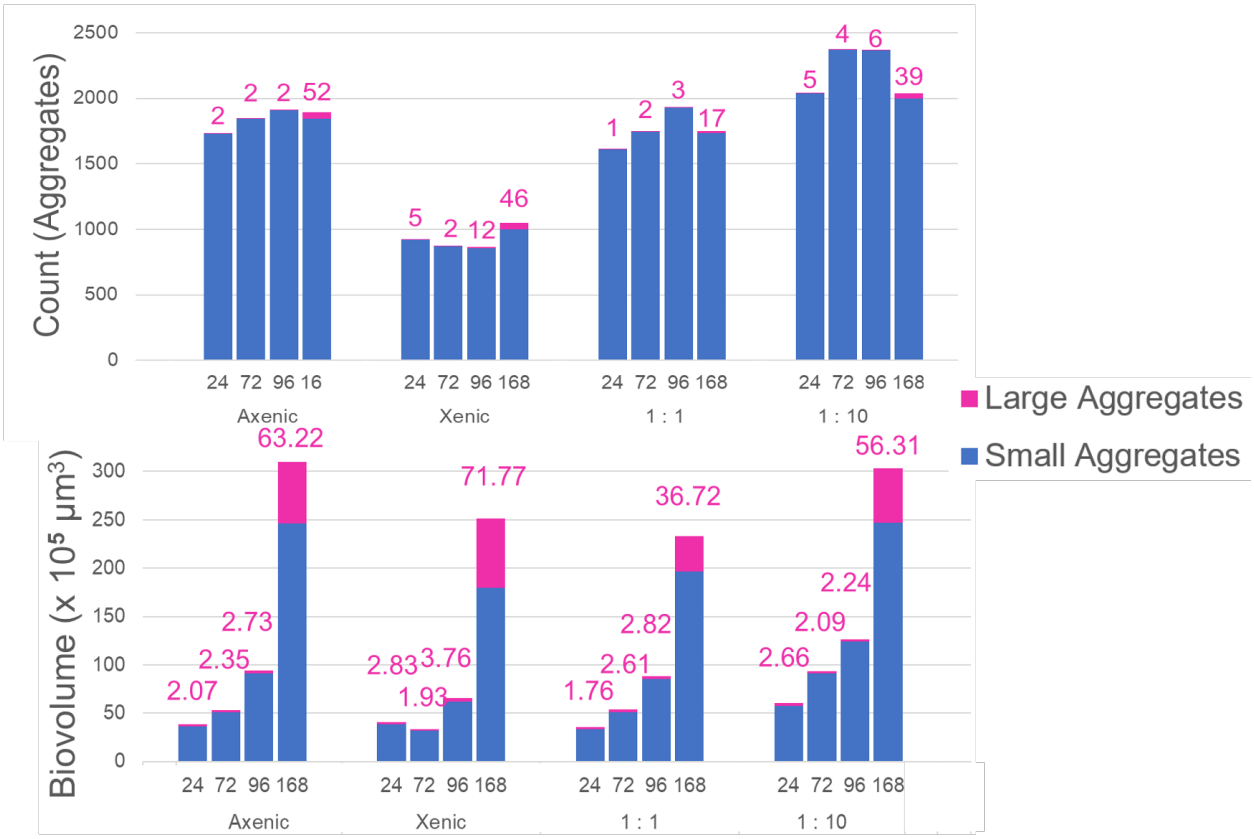

Figure C.3. Temporal growth data for 3D printed SLA-04 hydrogel experiments from t=24 to t=168 hrs. (Top) Aggregate counts were consistent for the duration of the experiment, with large aggregates (pink) showing a measurable increase in count, likely due to growth of individual aggregates. (Bottom) Biovolume in the hydrogel matrix as measured by chlorophyll autofluorescence.

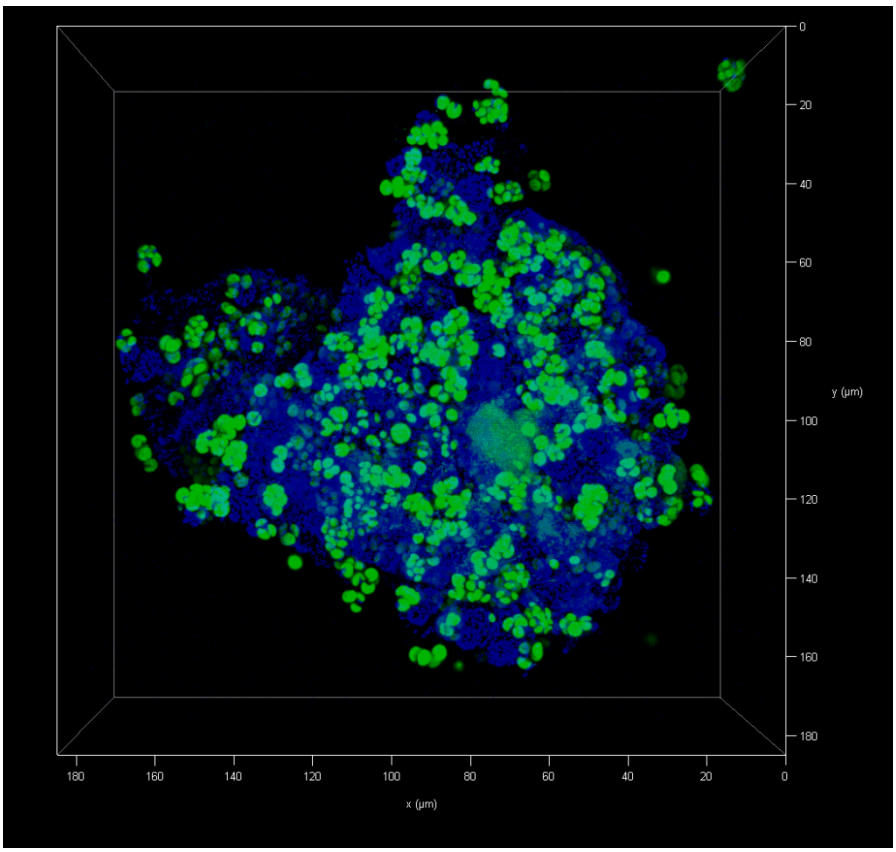

Figure C.4. Confocal image of a 3D printed aggregate from a HA xenic SLA-04 culture. Chlorophyll autofluorescence is visualized in green and DAPI stained bacteria are visualized in blue. Image credit: Isaak Thornton, Wilking Lab, MSU.

Raman Microspectroscopy

The motivation for employing Raman microspectroscopy was to understand how single cell interactions, especially attachment, impacted single cell SLA-04 physiology (*e.g.*, lipid content). We hypothesized that association with active bacterial populations would impact SLA-04 physiology. To investigate this, we attempted to measure Raman spectra of cells likely to have low and high lipid content. We also planned to use deuterium incorporation to assess activity in aggregates from xenic cultures.

The predominant challenge that we faced in using Raman microspectroscopy was the signal from chlorophyll autofluorescence. Several studies have documented the impact of pigment autofluorescence on Raman signal and various methods, including chemiphotobleaching and physical quenching with a laser, for suppressing autofluorescence [1,2]. We attempted to incorporate these methods but never found the balance necessary to suppress autofluorescence without damaging cellular molecular characteristics. Through corresponding with the authors of Moudrikova et al., 2016 [1] we determined that there may be strain-specific differences governing the successful application of pigment suppression methods. Physiological characteristics of SLA-04, such as a thick cell wall/membrane structure, could present challenges in the application of these methods.

It was challenging to maintain native spatial structures for analysis on Raman, a similar challenge observed during sample preparation in the nanoSIMS pilot project. Embedding samples in a low melting point agarose has been suggested as an improvement on preserving spatial structure but it has not yet been tested on these samples.

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APPENDIX D

APPENDIX D: GROWTH OF SLA-04 IN EFFLUENT WATER FROM THE BOZEMAN FISH TECHNOLOGY CENTER

Contribution of Authors and Co-Authors

Author: Isaac R. Miller

Contributions: Experimental design; sample preparation; data collection; data analysis

Co-Author: Jason Ilgen

Contributions: Sample collection

Co-Author: Matthew W. Fields

Contributions: Experimental design; data analysis

This appendix serves as a summary of the results from experiments conducted from 2017 to 2019 as part of a National Science Foundation Graduate Research Fellowship (NSF GRFP) funded project titled “Employing Microalgae as a Means of Improving Aquaculture Sustainability”. The goals of the project were 1.) grow microalgae in trout aquaculture effluent water, recycling nutrients through incorporation into biomass and cleaning water for reuse; 2.) using microalgal biomass to replace fish meal in aquafeed; and 3.) measuring the impact of microalgal-based aquafeed on trout gut community composition and function.

This project was a collaborative effort with the faculty and staff at the Bozeman Fish Technology Center (BFTC), namely Jason Ilgen and Dr. Wendy Sealey.

The first step was to grow SLA-04 in aquaculture wastewater, and this proved to be a challenge. The figures below document growth of SLA-04 and PW95 at different scales, the chemical composition of the effluent water, and the community present in the effluent water. When growth levels were low if amended effluent water, we hypothesized that there were chemical and/or biological inhibitors present. Ultimately, the experiments here inspired investigation of the role of the phycosome in SLA-04 growth and productivity.

The effluent water had lower nitrogen and phosphorous levels than we anticipated (Table 1), so we adjusted levels to mimic literature values for aquaculture effluent water (Goncalves et al., 2017).

Analyses	Result	Units	Method
INORGANICS	ND	mg/L	E300.0
Chloride	32	mg/L	E300.0
Sulfate			
AGGREGATE ORGANICS			
Organic Carbon, Dissolved (DOC)	1	mg/L	A5310 C
Organic Carbon, Total (TOC)	1	mg/L	A5310 C
NUTRIENTS			
Nitrogen, Ammonia as N	ND	mg/L	E350.1
Nitrogen, Nitrate + Nitrite as N	0.3	mg/L	E353.2
Nitrogen, Kjedahl, Total as N	ND	mg/L	E351.2
Nitrogen, Total	ND	mg/L	Calculation
Phosphorous, Total as P	0.015	mg/L	E365.1
METALS, TOTAL			
Calcium	25	mg/L	E200.7
Iron	ND	mg/L	E200.7
Magnesium	9	mg/L	E200.7
Manganese	ND	mg/L	E200.7
Potassium	ND	mg/L	E200.7
Sodium	ND	mg/L	E200.7

Table D.1. Results of chemical analyses of BFTC effluent water. ND = not detectable.

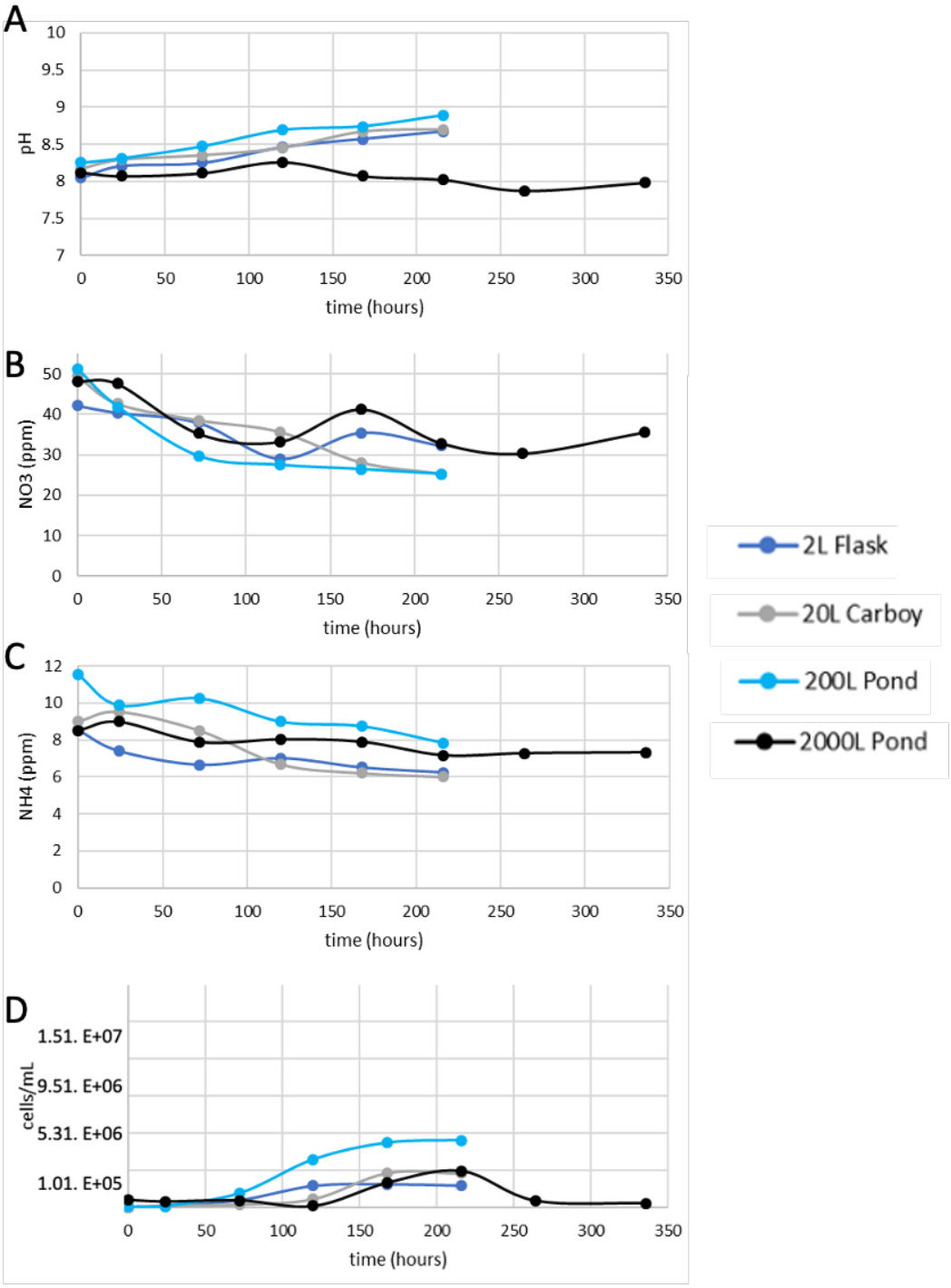

Figure D.1. Growth of SLA-04 on amended BFTC effluent water. Colored lines represent experiments conducted at 2 L (dark blue), 20 L (grey), 200 L (bright blue), and 2000 L (black). Physiological data plotted for the duration of the respective experiments includes pH (A), nitrate

concentration (ppm) (B), ammonium concentration (ppm) (C), and cell concentration (cells/mL) (D).

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