DISSERTATION

CROP PROTECTION IN INDUSTRIAL ALGAE FARMING: DETECTING WEEDY

ALGAE AND CHARACTERIZING BACTERIAL COMMUNITIES

Submitted by

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ABSTRACT

CROP PROTECTION IN INDUSTRIAL ALGAE FARMING: DETECTING WEEDY

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Microalgae are a promising source of feedstock for biofuel and bioproducts. Algae have higher rates of biomass production than terrestrial crops, and therefore can use less land for producing equivalent energy compared to other biofuels. Elite algae strains are chosen based on traits such as fast and robust growth, and rapid production of desired biochemical products, including fatty acids and other high-energy compounds. Monocultures of elite strains are grown in large algae production systems. A major challenge algae growers face is consistently growing robust cultures of elite algae. This is due to unwanted organisms invading cultures such as weedy algae that contain less desirable biochemical products, and bacteria that can detract from algae growth, thereby reducing overall system productivity. Historically, algae have not been grown at scales required for biofuels and bioproducts, and thus there is a lack of fundamental pest management knowledge and developed tools. In this work, we developed three polymerase chain reaction (PCR)-based tools for detecting and quantifying weedy and elite algae. We developed a simple and inexpensive CAPS (cleaved amplified polymorphic sequence) assay that can determine the presence of dominant algae species in cultures. Also, we developed and validated qPCR primers were able to detect one weedy algae cell in 10^8 cells in a culture. Compared to flow cytometry, the qPCR primers were 10^4 times more sensitive for detecting weedy algae. We validated tools by

monitoring industrial algae systems, and exhibited their utility for assisting in culture management decisions.

Bacteria are also prevalent in industrial algae cultures yet little is understood about their dynamics or role in the ecosystem of elite algae cultures. We sampled small, medium and large cultures from an industrial algae system growing elite algae *Nannochloropsis salina*, and sequenced the 16S rDNA gene and used QIIME bioinformatics program to analyze data. In this study, we characterized bacterial communities diversity, richness, and composition in industrial algae bioreactors during the scale-up process, through time and during various algae growth rates. We demonstrate that bacterial diversity richness increases as the size of the algae production system increases in the scale-up process. Therefore, larger cultures are comprised of more complex communities than smaller cultures, thus increasing the probability of detrimental algae-bacteria interactions. We identified a single core bacterium *Saprospiraceae* that was present in 100% of samples, and was on average the most abundant bacterium in all systems. Further, we identified a *Deltaproteobacterium* that was detected at abnormally high relative abundances in poorly growing algae cultures.

Identifying pest bacteria that can detract from elite algae growth is an important step in developing crop protection strategies. We isolated bacteria from a poorly performing algae system and determined their influence on algae growth. We identified a single isolate, S7 as a growth inhibiting bacteria that was capable of completely inhibiting *Nannochloropsis gaditana* and *N. salina* growth. The bacterium was characterized as *Bacillus pumilus*. Additionally, we identified nutrients and cell

concentrations required for inhibition of *N. gaditana* and *N. salina*. *B. pumilus* inhibition effect is species-specific as it did not inhibit weedy algae, *Chlorella vulgaris* and *Tetraselmis striata*. Due to this, *B. pumilus* is capable of manipulating algae population composition and reducing productivity. Contaminating organisms such as bacteria will often be prevalent in algae systems and understanding their influence on culture productivity is essential for successful large-scale cultivation of algae.

   In summary, we 1) developed molecular tools to monitor weedy algae that can be used by growers, 2) characterized bacterial communities in industrial algae system cultures, and 3) identified a novel pest for elite algae, *N. gaditana* and *N. salina*.

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CHAPTER 1

INTRODUCTION

1.1. MICROALGAE HISTORY AS A SOURCE FOR BIOFUELS AND BIOPRODUCTS

Microalgae are a diverse group of single-celled photosynthetic eukaryotic organisms that convert sunlight, CO_2, water and nutrients to produce biomass. Algae are ubiquitous and grow in fresh, brackish and saline environments. Select species of algae are known to have relatively fast growth rates and high oil productivity compared to traditional agricultural crops. Therefore, since the 1950's, researchers have considered algae a potential energy and food source. This objective was significantly researched during the 1970's energy crisis when the Department of Energy began a twenty-year ($25 million dollar) project called the Aquatic Species Program (ASP). Much of this research was dedicated to bioprospecting, genetic engineering and outdoor mass cultivation (1). Though the ASP concluded in 1995, it is still considered the foundation of algae cultivation knowledge with relatively few research programs focused on algae cultivation during the following decade. Renewed interest in algae as an energy source has developed as record oil prices and environmental concerns led governments and industry to invest billions of dollars into basic and applied research with the hope of developing algae into a sustainable biofuels and bioproducts source. The four general steps for using algae to produce biofuels are: 1) grow algae, 2) harvest biomass, 3) extract oils and 4) convert oils to fuel. Every step must be improved in order to increase efficiency, while lowering capital and operating costs for algae biofuels to be successful and profitable at large-scale. Much of this research and development effort is directed at

designing growth systems, developing strains and extracting lipids. Additionally, numerous companies are attempting to grow monocultures of elite strains in open, closed or hybrid growth systems in hope of scaling beyond several million liters per system. Elite strains are specific algae selected for either high lipid or carbohydrate production for particular fuels such as biodiesel and ethanol, respectively. However, stable cultivation of elite strains in growth systems is challenging because of culture failures due to interactions with eukaryotic pest organisms such as weedy algae species, predators and pathogens (1). These pests are often controlled in laboratory settings, but as the scale increases during mass algae cultivation this problem is exacerbated. Compared to traditional crops, algae pest management strategies are extremely undeveloped. Therefore, one of our research aims is to develop molecular tools to detect and monitor unwanted weedy algae species, which will assist crop management strategies.

In addition to eukaryotic pests, bacteria are known to exist in and be dynamic in algae cultures, but limited knowledge exists about algae-bacteria interactions. Past research has revealed that many bacteria have symbiotic and pathogenic relationships with plants that greatly affect growth and/or productivity. Therefore, it is not unreasonable to assume that bacteria have direct or indirect interactions with algae that could potentially influence algae productivity. To better understand the role bacterial populations have on algae, we will use molecular community profiling in algae cultures to identify bacteria biodiversity and influential bacteria necessary for robust algae growth. Identification of influential bacteria could be used to develop crop management strategies that stabilize and increase elite strain algae growth.

1.2. ALGAE PRODUCTION SYSTEMS ARE SIMILAR TO TRADITIONAL CROPPING SYSTEMS

Algae production systems can be divided into open and closed systems. Currently there is a debate in the industry about which system should be adapted for commercialization. Open systems are most commonly raceway ponds approximately 20 cm deep and use a paddle wheel for culture mixing (figure 1). Raceways are often considered to have cheaper capital and operating costs, however the culture is exposed to the open environment, which increases the risk of unwanted organisms entering and reducing productivity and even causing cultures to fail. In comparison to open systems, closed systems are often made from transparent materials in the form of tubes or bags (figure 2). They have the potential for high growth rates due to increased photosynthetic efficiency due to greater surface area to volume ratios. Unwanted organisms entering the system are less of a threat, although some closed system operators have reported major contamination problems. Currently, companies around the world are scaling both open and closed systems. This often involves a small milliliter culture used to inoculate several liter cultures that are used to inoculate several 100-liter systems and continuing up to millions of liters per system. Open system demonstration plants may contain single cultures up to 3,750,000 liters that combine to be approximately 15,000,000 liters on 12 acres (2). In comparison, closed system demonstration plants may contain hundreds of 400-liter single cultures that total 150,000 liters on ¾ acres (3). It has been estimated that 5.5% of the United States is suitable for algae cultivation (4). Based on this, algae systems could be grown on 100 million acres with a total volume of 145

trillion liters. To put this in prospective, corn production in the United States is currently produced by 400,000 farms and occupies 80 million acres of land (5)

Algae cultivation is similar to traditional cropping systems in that weeds, insects and pathogens are serious threats that can substantially reduce crop yields. For example, nearly 2000 weeds have been identified in agriculture and hundreds of herbivorous insects have been characterized and many pathogenic bacteria, fungi and viruses exist that can infect and cause significant damage to crops (6). For millennia, farmers have developed crop management strategies such as tilling, crop rotations and crop variety selection. More advanced strategies include chemical input of pesticides, insecticides and fungicides. Traditional agricultural has developed diagnostics and strategies to control specific pests, whereas these methods are immature for algae agriculture. Successful algae cultivation requires the development of diagnostic techniques and management strategies to eliminate pests, while maintaining beneficial organisms, such as potential bacteria symbionts.

1.3. WEEDY ALGAE SPECIES REDUCE SYSTEM PRODUCTIVITY

Different microalgae have unique oil compositions and growth characteristics. Elite algae strains are selected for high oil content (~50% oil by weight), whereas undesired algae contaminants of growth systems are considered weedy species, which often have low oil content. Weedy species reduce system oil productivity because they are harvested with elite strains, but contribute little to no oil to the extraction process. Weedy species also compete for nutrients (Nitrogen, Phosphorus and sunlight) that otherwise would be used by elite strains. Additionally, weedy species may adhere to closed system materials and blocks light from entering the system.

1.4. MICROBIOTA HAVE CRITICAL ROLES IN ECOSYSTEMS

The microbiome is the total bacteria that live in or on an environment. For example, the human microbiome is extremely dynamic and includes, but is not limited to, thousands of bacteria types living in nasal, oral, skin, gastro-intestinal and urogenital environments. Each area has bacterial communities with metabolic capabilities that are not encoded in the human genome, such as specific mechanisms for digestion and defense. It is estimated that the human microbiome has at least 200-times more genes than the human genome (7). A study of the microbiome's influence on mice and humans found that obesity correlates to changes in abundance of two dominant bacterial divisions, *Bacteriodetes* and *Firmicutes*. Subsequently, biochemical studies revealed that this shift in abundance toward the obese microbiome increases the efficiency of energy harvested from the diet, leading to weight gain by the host (8). In another example, microbial communities identified in acid mine drainage (AMD) stream biofilms had bacteria stratification based on specific function and interaction. Because of the harsh environment, AMD communities have relatively low species diversity, which in this example the majority species was *Leptospirillium* group II known for having a cytochrome for iron oxidation. This bacterium grows on the AMD substrate that has high concentrations of iron and therefore the majority of iron oxidation takes place at the substrate-biofilm interface. Additionally, the top layer of the biofilm was archaea (*thermoplasmatales*) that most likely relies on the organic carbon produced by *Leptospirillum* group II. In return, archaea reduce organic carbon that acts as an autotoxin and produces metabolites used by *Letpospirillium* group II (9). Stratification

based on function demonstrates that bacteria don't only interact with eukaryotes, but are known to function with archaea and other bacteria.

1.5. ALGAE COULD HAVE SPECIFIC AND IMPORTANT MICROBIOMES

The zone surrounding the algal cell is called the "phycosphere" and is known to have high concentrations of algae-derived organic matter that bacteria thrive on. In fact, some bacteria have been observed using chemotaxis to track algal cells' movement, which is most likely chasing after the organic matter (10). It is broadly accepted that bacteria have a range of interactions with algae that includes stimulation, inhibition and termination of algae populations. However, research to date is relatively limited because assays used in the past to study complex communities relied on culturing techniques and non-comprehensive molecular methods leaving many unanswered questions. There is evidence to suggest that 1) distinct bacterial communities affect the physiology of algae and 2) unique bacterial communities associate with algae based on physiological growth. This is demonstrated by evaluating the microbiomes of two closely related diatoms, *Thalassiosira rotula* and *Skeletonema costatum* using denaturing gradient gel electrophoresis (DGGE), microscopy and fluorescence *in situ* hybridization (FISH). The algae strains were made axenic and at different growth stages were inoculated with the natural bacterial assemblages from a saline environment. First, when the bacteria assemblages were added to an axenic *T. rotula* culture in stationary phase, the algae cells died within two days. On the other hand, during *T. rotula* exponential growth the bacterial abundance was extremely low. It is reasonable to assume that the addition of bacteria creates a harmful interaction with the stationary algae cultures. Secondly, both strains of algae had the majority of attached bacteria

from the *Flavobacteria-Spingobacteria* group, but at different physiological conditions this changed. The conclusion is that attached bacterial communities were completely dependent on algal species and physiological growth stage (11), suggesting that algae have species-specific bacteria that make up distinct microbiomes. Additional studies have defined functional relationships between bacteria and algae, suggesting some algae require a microbiome to grow. When surveying the genomes of 326 algae species, it was found that 171 require vitamin B_{12} from outside sources, such as bacteria. Vitamin B_{12} auxotrophy in some algae most likely occurred because bacteria are major producers of the vitamin, which takes 19 energy intensive enzymatic steps to make (12). By developing an intimate relationship with bacteria, algae do not need to expend energy to make this nutrient. Numerous studies have focused on the bacteria-algae relationship, but many of the studies do not draw strong functional conclusions. Below are examples of various relationships.

- The bacterium *Silicibacter sp.* TM 1040 forms a biofilm on the outside of a dinoflagellate, *P. piscidcida.* When the bacterium is removed from the *P. piscidcida* culture, the dinoflagallate struggles to stay alive, suggesting that this bacterium provides a function that is not encoded or active in *P. piscidcida* genome and therefore is a necessary component of the microbiome for algae survival (13).

- Bacteria were isolated from a biofilm on the alga *Botryococcus brauni* and added to an axenic culture of *B. bruani*. It was found that *Rhizobium* sp increased growth of oil-producing algae by 50%, whereas an *Acinetobacter sp.* decreased *B. brauni* growth (14).

- Eight bacteria were isolated from a *Chlorella* culture and all eight promoted *Chlorella* growth when individually co-cultivated with the alga. In particular, *Brevundimonas* directly adhered to the *Chlorella* cell and increased growth by three times (15).

- Iron is a limiting nutrient for algae in the ocean and is crucial for photosynthesis and respiration. To overcome this, algae associate with marine bacteria that have developed siderophores, or organic molecules that bind iron and enhances the solubility of iron (and therefore its availability to algae). When *Marinobacter* are present, there is a 20×increase in iron assimilation in the dinoflagallate *Scrippsiella trochoidea*. Moreover, *Marinobacter* only grew when the dinoflagallate was present, which suggests a mutualistic relationship (16).

- *Roseobacter* is a common clade of bacteria found throughout marine systems that when sessile, releases TDA (a novel sulfur tropolone compound known to inhibit pathogens). *Roseobacter* may settle on algae cells, enter sessile phase and release TDA, which indirectly promotes algae growth by killing off other bacteria that could harm or inhibit the dominant algae strain's growth (13).

- A probiotic effect was produced by *Flavobacterium sp.*, which is known to promote the growth of diatom *Chaetoceros gracilis*. *Flavobacterium sp.* were grown up and artificially added to the diatom culture. In the presence of Flavobacteria, the diatom grew faster and was significantly more stable while in stationary phase (17).

1.6. METAGENOMICS ALLOWS GENOMIC STUDY OF UNCULTURED MICROBIAL COMMUNITIES

Metagenomics is a relatively new culture-independent technology that uses nucleic acid analysis to study communities of organisms. High throughput second-generation sequencing technologies are used for two different metagenomics approaches: 1) rapid tracking of complex systems by cataloguing community structure, or 2) shotgun sequencing of complete bacteria genomes to understand biochemical pathways. The former provides the most appropriate approach when characterizing communities' biodiversity and relative abundance. To do this, an environmental sample collects all organisms in a community and a complete DNA extraction is done on all organisms within the sample. Polymerase chain reaction (PCR) amplifies the 16S rRNA gene (or other diagnostic genes), which is used as a diagnostic gene because it has conserved regions used for universal primers that flank hypervariable regions. Amplicons are sequenced by second-generation sequencing technologies. Data analysis programs cluster sequences into groups that are classified by comparing information with DNA sequence databases.

Metagenomics enables researchers to identify and understand microbiomes that live in or on different environments. Pioneering studies focused on human-bacteria interactions, but we intend to use metagenomics to study algae growth system cultures. Studying bacterial communities in production systems will lead researchers to better understand fundamental biological interactions while also providing strategies for improving growth, stability and lipid production in cultivation systems.

1.7. SCOPE OF DISSERATATION

A major challenge to overcome for cultivation of algae for biofuels and bioproducts is developing culture management strategies to increase stability, consistency and productivity of elite algae cultures. It is starting to become widely accepted that an integrated pest management solution is necessary. This includes identifying pest organisms, developing tools to track abundance, and develop management strategies. In this work, molecular diagnostics were developed to track weedy algae contaminants in algae production systems. Additionally, bacteria are likely to play a role in system community dynamics and productivity. Therefore, molecular techniques were used to characterize and better understand bacterial dynamics in industrial algae productions systems. Lastly, we identified a pest bacterium that inhibits *N. salina* and *N. gaditana* growth. We accomplished his by isolating bacteria from a poorly performing algae cultivation system and added them directly to algae cultures to observe their influence on algae growth.

1.8. RESEARCH GOALS

1) Develop DNA diagnostics to identify and quantify unwanted weedy algae species and track weedy algae in production systems.

2) Comprehensively characterize bacterial communities in the scale-up process and seasonally in industrial algae cultivation systems

3) Isolate bacteria and screen for inhibition phenotype on elite algae and weedy algae

A.   B.

Figure 1. A) A closed algae system using plastic bags to protect culture from the outside environment. B) An open pond system exposed to the environment.

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CHAPTER 2

MOLECULAR DIAGNOSTICS FOR MONITORING CONTAMINANTS IN ALGAL

CULTIVATION[1]

SUMMARY

There is currently great interest in mass cultivation of microalgae for production

of fuels and other high value products. Since algae have not previously been grown at

the scales and with the precision required for these endeavors. Sensitive methods are

needed for enumeration of elite algal varieties relative to "weedy" invader strains that

are ubiquitous in the environment and a common issue with culture management. The

ideal monitoring strategy would be inexpensive and identify weedy algae long before

they become prominent in cultures of elite varieties. Herein, multiple polymerase chain

reaction (PCR)-based tools for monitoring contaminants are presented. These include

resources to identify unknown strains, to routinely monitor dominant constituents in

cultures, and to detect contaminants constituting as little as one in 10^8 cells in a culture.

Quantitative PCR was shown to be 104 times more sensitive for detecting weeds than

flow cytometry. During characterization of these tools, it was demonstrated that

contamination is a common phenomenon and that early detection is necessary for

informed decision making during culture selection for subculturing or scale-up. Thus,

implementation of strategies for monitoring contaminants in algal cultivation is a critical

component of culture management for optimal productivity.

2.1 INTRODUCTION

Microalgae (herein, "algae") comprise a highly diverse set of photosynthetic eukaryotes that arose via independent endosymbiotic events [1,2]. Because strains from divergent taxa produce oils appropriate for use in production of renewable biofuel, general interest in algae has increased significantly [3]. Oil productivity in some algal varieties is significantly greater than even the most robust oil-producing traditional crops [4], and genetic modification is now common in multiple relevant algal strains and thus may be used to further enhance high-oil-productivity strains [5–8]. Following agricultural convention, these desired algal varieties with high oil productivity and other inherent or engineered qualities that make them suitable crops for commercial production may be generically referred to as "elite" lines. Algae have not historically been cultivated at the scales nor with the technical precision required for affordable, reliable mass cultivation and quality-controlled fuel production. Major barriers that currently limit the potential of algal biofuels include proven, stable, large-scale (1000 ha) cultivation methods for appropriate high-oil-content algal strains and an understanding of culture maintenance and pest management strategies [9].

Because algae are ubiquitous in the environment, there are constant opportunities for low oil content algae to contaminate cultures and compete with elite strains for sunlight and nutrients. Such contaminants are appropriately referred to as "weeds" and must be managed as such to minimize their impact on crop productivity and resulting fuel quality. Because lipids are more reduced than carbohydrates and proteins, high-oil elite algae require more photosynthetically derived reductant per unit biomass than weedy strains containing less oil. Thus, weedy algae may grow faster

than elite strains and have the potential to become abundant or dominant in a culture [10]. Both open pond and closed photobioreactor systems are known to be invaded by weedy species, grazers and pathogens [11–13], so such invasions must be expected regardless of the cultivation system. Clearly, algal culture monitoring methods will be needed along with pest management programs for algae-based biofuel production, and culture monitoring is equally important for production facilities, research laboratories and culture collections [14]. To be included as part of a routine culture monitoring regime, these tools and related protocols should be of low or moderate cost, versatile for adaptation to various algal communities, able to be implemented immediately, require only limited technical expertise, and be informative.

Current culture monitoring methods vary in throughput, instrumentation, degree of experience required and cost. Growers may use microscopy to manually observe cultures and identify algae based on morphology and pigmentation. This methodology is low throughput and requires considerable expertise distinguishing strains. Microalgae are small (1–100 µmin diameter), and distinct genera may have nearly identical overall appearances [15,16]. Furthermore, algal strains of the same species may be morphologically indistinguishable, yet harbor cryptic genetic diversity that affects crop value [17]. In addition to standard microscopy, it is common to use flow cytometry and imaging flow cytometry to group cells based on phenotypes such as size and chlorophyll content [18]. Despite its increased throughput, flowcytometry has limited ability to identify algae with certainty or to distinguish strains with similar phenotypes.

Nucleic-acid-based methods may be used to unambiguously identify algae, for example by sequencing or otherwise characterizing a portion of algal genomes. Genes

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encoding RNA subunits of prokaryotic or eukaryotic ribosomes are commonly characterized for taxonomic and phylogeny purposes. Relevant to the work presented herein, there are evolutionarily constrained regions of rRNA genes ideal for design of PCR primers of broad specificity or for comparison of distantly related organisms, as well as interspersed variable regions that may be used to distinguish more closely related organisms [19], [20] and [21]. Additionally, there are millions of rRNA sequences deposited in general nucleotide databases (i.e., Genbank, http://www.ncbi.nlm.nih.gov/genbank/) and specialized rRNA databases (i.e., SILVA,http://www.arb-silva.de).

In this work, molecular tools were developed for routine monitoring of elite and weedy algae in laboratory and production cultures. The various tools and procedures involved characterization of 18S rRNA genes. In the analyses presented, the polymorphism among algal 18S rRNA genes was sufficient to distinguish different genera, species of the same genus, and geographic isolates seemingly of a single species. Specifically, PCR primers were designed to amplify an approximately 1500 nt region of 18S rRNA genes from three classes of algae: *Bacillariophyceae*, *Eustigmatophyceae*, and *Chlorophyceae* (herein referred to as "BEC"). These amplicons can be sequenced for definitive identification of strains, or they can be digested with a restriction enzyme to generate allele-specific fragmentation patterns for rapid, inexpensive characterization of strains and cultures (Fig. 1, left panel). Two strategies for culture monitoring based on quantitative PCR (QPCR) were also compared for their ability to detect weedy algae at low abundance in elite cultures (i.e., allele-specific QPCR probes and allele-specific QPCR primers; Fig. 1, middle and right

panels, respectively). We chose the more promising allele-specific QPCR primer method and compared its sensitivity and specificity to that of flow cytometry for detecting weedy algae at low abundance in cultures. In addition to clarifying the utility and limitations of these tools, we demonstrate the importance of sensitive and accurate weed detection during selection of potential innocula for scale-up or subculturing.

2.3 MATERIALS AND METHODS

SAMPLING

Samples were collected (Solix Biosystems; [11]). Approximately 1.5 mL of culture was sampled from cultures ranging in biomass density between 0.5 and 5 g(dry weight)/L, equivalent to 9×10^7 and 1×10^9 cells/mL, respectively. Other samples came from agar plates where single colonies or numerous colonies were picked using a pipette tip and placed into F/2 media. The samples were centrifuged at 6000 $\times g$ for 10 min at room temperature and the supernatant was decanted. Cell pellets were less than 100 mg and were stored at -20 °C until DNA extraction.

FLOW CYTOMETER

Samples were analyzed using a guava easyCyte HT + flow cytometer (EMD Millipore) equipped with an argon laser (488 nm) and 680/30 nm bandpass filter. For each sample, 20,000 events (i.e., cells) were scored for red fluorescence to identify chlorophyll-positive cells and for low-angle forward scatter to determine approximate diameter. Algal cells were identified as chlorophyll-positive events, and populations of algal genera were distinguished by size.

DNA EXTRACTION

Total DNA was isolated from frozen cell pellets. Cells were disrupted by grinding in liquid nitrogen with a mortar and pestle for 5 min or by mechanical disruption using a bead beater (BioSpec Products) or paint shaker (Fluid Management). Frozen cell pellets in microcentrifuge tubes were shaken 3 × 1 min in the presence of 0.5 mm zirconia/silica beads (BioSpec Products Inc.). Prior to and between each round of shaking, the biomass was flash frozen in liquid nitrogen. Following cell disruption, genomic DNA extraction was done using the Easy-DNA kit (Invitrogen) or DNeasy Plant Mini kit (Qiagen), according to manufacturer's instructions. Isolated DNA concentration was determined using a spectrophotometer (ND-1000 Thermo Scientific).

18S rRNA SEQUENCE ALIGNMENTS AND BEC 18S PRIMER DESIGN

The 18S rRNA gene sequences of representative members of algal classes *Bacillariophyceae*,*Eustigmatophyceae* and *Chlorophyceae* were retrieved from GenBank. A total of 117 unique sequences (42 *Bacillariophyceae*, 19 *Eustigmatophyceae*, 56 *Chlorophyceae*) larger than 1000 nt were aligned using ClustalW. Primers (BEC 18S Forward & Reverse; Table 1) were designed to anneal to highly conserved regions (Supplemental Fig. 1) and to generate amplicons of approximately 1500 bp.

BEC 18S PCR

The PCR of 18S rRNA genes was done using 50 μL reactions containing a final concentration of 10 ng template DNA, 0.5 μM each BEC 18S Forward and Reverse primers (Integrated DNA Technologies;Table 1), 1 U High Fidelity Phusion DNA Polymerase (New England Biolabs), 1 × HF buffer and 0.2 mM dNTPs (Fisher

Scientific), Thermal cycling consisted of initial denaturation at 98 °C for 2 min; 40 cycles

of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for

1 min; and a final extension at 72 °C for 10 min. Amplicons were resolved using

agarose gel electrophoresis and visualized following ethidium bromide staining.

CLONING

As needed, PCR products were either cloned directly or following gel purification

using a Geneclean (Qbiogene) or QIAquick PCR purification kit (Qiagen). Amplicons

were ligated into the pSC-B vector and transformed into *Escherichia coli* cells using

StrataClone Blunt PCR cloning kit (Agilent) according to manufacturer's instructions.

Transformations were plated on selective LB agar and incubated overnight at 37 °C.

Colonies of putative transformants were isolated, used to inoculate 5 mL of selective LB

media and grown overnight at 37 °C with agitation. Plasmids were extracted using a

Gene JET Plasmid Miniprep Kit (Fermentes) following manufacturer's instructions.

Plasmids were eluted in 200 µL water.

SEQUENCING AND ANALYSIS

Purified PCR products or plasmids were sequenced using ABI BigDye

Terminator v3.1 chemistry and an ABI 3130xL Genetic Analyzer at the Colorado State

University Proteomics and Metabolomics Facility. Primers for sequencing included BEC

18S primers (Table 1) and standard M13 primers. To determine algal strain identity,

DNA sequences were queried against GenBank using BLASTn.

CLEAVED AMPLIFIED POLYMORPHIC SEQUENCES (CAPS)

BEC 18S amplicon sequences were aligned to identify polymorphisms

within *Hae*III restriction enzyme cut sites. PCR products were generated with BEC 18S

primers. Restriction digests were done using 20 μL reaction volumes containing 10 μL

PCR product, 1 U *Hae*III (New England Biolabs), 1 × BSA, 1 × NEB Buffer 4. Reactions

were incubated overnight at 37 °C followed by inactivation at 80 °C for 20 min. Digest

products were resolved by gel electrophoresis using 1%–2.5% agarose or 4% metaphor

agarose (Lonza) and visualized following ethidium bromide staining. For high resolution,

1 μL of restriction digest was prepared with the DNA 1000 kit (Agilent) and assayed

using a Bioanalyzer 2100 (Agilent) according to manufacturer's instructions.

REAL-TIME QPCR AND PROBE THRESHOLD CYCLE ANALYSIS

Real-time QPCR assays used a CFX96 Real-Time System (BioRad). Threshold

cycles (C_t) were identified using single threshold determination and baseline-subtracted

analysis. For all QCPR amplicons, amplification efficiencies (Table 1) were calculated

using C_t values from a series of reactions in which templates were serial dilutions of

linearized plasmid DNA containing the target sequence [19]. To establish specificity,

primers were used in QPCR reactions in which template was linearized plasmid DNA

containing a non-target 18S rRNA gene sequence.

ALLELE- SPECIFIC FLURESCENT QPCR PROBES GENERA *TETRASELMIS*

A total of 81 gene sequences of the 18S rRNA gene from the

genera *Tetraselmis* and *Nannochloropsis* were retrieved from GenBank, aligned using

ClustalW or ClustalOmega, and viewed with JalView. Primers (NT Forward &

Reverse; Table 1) were designed in conserved regions. Probes

(*Nannochloropsis* & *Tetraselmis* probes; Table 1 and Supplemental Fig. 2) were

designed"molecular beacons" [20] that are stem-loop structures with 5′ fluorophores and

3′ quenchers. Primers were used in QPCR with a reaction volume of 10 μL, containing a

final concentration of 10 ng template DNA, 300 nM each NT Forward and Reverse primer, 150 nM probe and 1 × SsoFast Probes Supermix (BioRad). Multiplexed reactions contained two probes with unique fluorophores each at 150 μM. Thermal cycling consisted of initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 5 s, annealing and extension at 60 °C for 4 s; followed by melt curve analysis from 65 °C to 95 °C in 0.5 °C increments. Fluorescence was measured using the HEX and FAM channels during annealing/extension steps and the melt curve. The same methodology was used to design primers (NsNo Forward & Reverse; Table 1) and probes (*salina* & *oculata* probes; Table 1) for discrimination of QPCR amplicons produced from 18S rRNA genes of*Nannochloropsis salina* (GenBank accession AF045048.1) and *Nannochloropsis oculata* (GenBank accession U38902.1) (Supplemental Fig. 3).

ALLELE-SPECIFIC QPCR PRIMERS

The alignment of 81 *Tetraselmis* and *Nannochloropsis* 18S rRNA gene records was used to identify regions highly conserved within each individual genus but polymorphic between the genera (Supplemental Fig. 4). Primers (Table 1) were designed to specifically amplify *Nannochloropsis* (Nanno Forward & Reverse) or *Tetraselmis* (Tetra Forward & Reverse) strains (Supplemental Fig. 4). Primers were used in 10 μL QPCR reactions containing a final concentration of 10 ng template DNA, 300 nM each forward and reverse primer, and 1 × SsoAdvanced SYBR Green Supermix (BioRad). Thermal cycling consisted of initial denaturation at 98 °C for 2 min; 40 cycles of denaturation at 98 °C for 30 s, annealing and extension at 67 °C for 30 s; followed by

melt curve analysis from 65 °C to 95 °C in 0.5 °C increments. Fluorescence was

measured using the SYBR channel during annealing/extension steps and the melt curve.

2.3 RESULTS AND DISCUSSION

BEC PRIMERS FOR IDENTIFICATION OF ALGAE

To facilitate identification of algae in laboratory and production cultures, we

designed primers to amplify the 18S rRNA gene of three major algal classes. The 18S

rRNA gene sequences of algae from the BEC classes were retrieved from GenBank. A

total of 117 unique 18S rRNA gene records containing sequences larger than 1000 nt

were aligned using ClustalW or ClustalOmega and regions highly conserved among all

sequences were identified (Supplemental Fig. 1). Within such conserved regions,

primers were designed to generate amplicons of approximately 1500 bp (Table 1, BEC

18S Forward & Reverse), including multiple variable 18S rRNA gene regions. This

primer set produced specific amplicons ("BEC 18S amplicons") from representatives of

BEC classes (Fig. 2A). These amplicons may be cloned using standard procedures and

subsequently sequenced to definitively identify algae by querying 18S rRNA databases

using BLAST. Nucleotide sequencing of cloned amplicons provided information

sufficient to unambiguously identify the corresponding algal species, even for related

species such as *N. salina* and *N.oculata*. Compared to using microscopy or flow

cytometry for algal identification, there are numerous advantages to these amplicons.

Researchers without expertise in algal morphology may identify algae they have not

previously encountered and they may have a high degree of confidence in the

identification. Furthermore, sequence comparisons may discriminate algae such as *N.*

salina and *N. oculata* that are morphologically indistinguishable even to experienced

researchers. This method of strain identification is only limited by the length of accurate sequence recovered and the population of properly annotated 18S rRNA gene sequences in GenBank and other databases.

CAPS ANALYSIS DISCRIMINATES ALGAL STRAINS

We anticipate growers will commonly work with a small number of elite algae and for any particular location and production environment – will encounter a finite set of weeds. Using sequencing to routinely characterize algal populations of limited diversity would be inefficient. Therefore, we developed a cleaved amplified polymorphic sequences (CAPS) assay [21] for discrimination of strains based on nucleotide polymorphisms in restriction enzyme recognition sequences within BEC 18S amplicons (Fig. 1, left panel). Based on alignments of BEC 18S amplicon sequences from representative algal strains, there are numerous nucleotide polymorphisms among these amplicons. Some of these polymorphisms produce or eliminate restriction endonuclease recognition sequences and are specific to particular genera, species or strains (e.g., Fig. 2B). As a result, strain-specific restriction fragmentation patterns are produced after BEC 18S amplicons are digested with an appropriate restriction enzyme. These unique fragmentation patterns may be readily distinguished using gel electrophoresis and used to putatively identify organisms. For example, the BEC 18S amplicons of *N. salina*, *N. oculata* and *Tetraselmis striata* (a prevalent weed in saline cultures) contain 8, 8 and 6 predicted *Hae*III sites, respectively. The predicted restriction fragmentation patterns should be unique to each organism and therefore useful for identification purposes. Specifically, *N. salina*, *N. oculata* and *T. striata* digest products are predicted to include unique fragments of 449, 333 and 600 nt, respectively.

To confirm these and other algae can be distinguished using restriction fragmentation patterns, genomic DNA was extracted from presumed unialgal cultures, BEC 18S amplicons were produced by PCR, amplicons were digested with *Hae*III, and the resulting restriction fragments were resolved using electrophoresis. Indeed, fragmentation patterns for representative BEC algal strains could be differentiated following this procedure (Fig. 2C). The assay even discriminated the related species *N. salina* and *N. oculata* that have only 31 nucleotide polymorphisms between their full-length (1790 nt) 18S rRNA genes and are indistinguishable by microscopy or flow cytometry. It is evident the CAPS procedure is an effective tool for rapid and inexpensive routine characterization of cultures. To compare separation and visualization technologies, restriction digest products were resolved using 2.5% agarose (Fig. 2C) and an Experion automated electrophoresis system (Bio-Rad) (Fig. 2D). Electrophoretic separation of restriction fragments using a 2.5% agarose gel commonly resolves fragments between 200–1000 nucleotides and therefore provides resolution sufficient to discriminate the selected algae. For the Experion capillary system, Bio-Rad 1K LabChips, which separate DNA fragments between 15–1500 nucleotides, were used. As seen inFig. 2C & 2D, both technologies sufficiently resolved *Hae*III-digested BEC amplicons to allow discrimination of algae based on restriction fragmentation patterns. In Fig. 2D, lanes 3 and 9 contain previously unreported isolates of *T. striata* ("Ute" and "Poudre" isolates) recovered from southwestern and northern central Colorado, respectively. Following observation of their distinct restriction fragmentation patterns, the BEC amplicons from each isolate were sequenced. The isolates have unique 18S sequences and are therefore distinct. Queries of GenBank revealed the 18S sequences

from both isolates are most similar to *T. striata* strain SAG 41.85 (GenBank

recordJN904000.1). In Fig. 2C, it was shown that the CAPS analysis was able to

distinguish species of the same genus, *N. salina* and *N. oculata*. As shown in Fig. 2D,

even these two geographical isolates of the same weedy algal species have distinct

fragmentation patterns resulting from our standard CAPS analysis, with the Poudre

isolate having a distinguishing restriction fragment of approximately 320 nt. In addition

to demonstrating the versatility of the CAPS procedure, this result indicates that

different geographic locations will have genetically distinct populations of weeds, even

though those weeds may belong to the same genus or species and further

demonstrates CAPS analysis may distinguish algae that appear identical when

observed by microscopy.

Another sample examined by CAPS analysis was a stock culture of *Dunaliella*

salina. The observed fragmentation pattern (Fig. 2C, lane 9) for this culture did not

match the fragmentation pattern predicted based on sequence of the *D. salina* BEC

amplicon. The observed fragmentation pattern lacked a 994 nt fragment predicted

based on the *D. salina* 18S rDNA sequence and was seemingly identical to that of *N.*

oculata (Fig. 2C, lane 4), with a distinguishing band of approximately 333 nt.

Sequencing of cloned BEC 18S amplicon from this sample confirmed the algae to be *N.*

oculata, indicating the supposed *D. salina*culture had at some point been mislabeled or

contaminated with – and eventually dominated by – *N. oculata*. This demonstrates the

practical use of this simple and rapid CAPS procedure for monitoring dominant algae in

cultures.

CAPS ANALYSIS DISTINGUISHES ABUDANT SPECIES IN POLYALGAL CULTURES

We next determined whether the CAPS procedure could recognize two distinct algal strains in a culture if those algae are present at similar levels. To mimic a mixed culture but allow more precise control of DNA ratios, genomic DNA was extracted from *T. striata* and *N. salina* cultures, combined at different ratios, and used as templates in BEC CAPS analyses. As shown in Fig. 3A, restriction fragments indicative of both algal strains were visible in each of the three reactions using mixed templates. We did not determine the limits for detection of a less-abundant algal strain in a culture dominated by another algal strain. Even if BEC amplicons were produced in a quantitative fashion (amplicons of this size are not), the dynamic range of technologies used to observe fragmentation patterns following *Hae*III digestion and electrophoretic separation limit the potential to visualize the fragmentation pattern from a weed at low abundance in an elite culture. In the course of this work, we used CAPS analyses to identify numerous cultures as containing multiple algal strains, some of which are represented in Fig. 3B. Each of these cultures was intended to contain *N. salina*, and the known *N. salina* fragmentation pattern was present in all lanes. However in lanes 2, 3, 4, 9 and 10, additional restriction fragments were visible, indicated unwanted algal strains contaminated these cultures.

It is easy to envision situations in which culture components may not be confidently identified based on fragmentation patterns. Nevertheless, if a novel algal strain begins to dominate a culture, its fragmentation pattern will likely be distinct from those of the targeted elite algae strain or contaminants encountered previously. Additionally, the presence of multiple organisms in a culture may result in a

fragmentation pattern too complex to deconvolute with confidence. These two scenarios are readily distinguished. In the case of a single organism of unknown identity, the sum of the individual restriction fragments should total approximately 1500 nt (the size of BEC amplicons). If multiple organisms are present, the sum of the individual restriction fragments should clearly exceed 1500 nt. In either case, a simple solution is to clone an aliquot of the same BEC amplicons that were used as input for CAPS analysis. In the case of a single novel organism, sequencing a clone should be sufficient to identify the algal strain. In the case of a complex mixture of algae, multiple individual clones may be used as templates for CAPS analysis. Each clone will produce a fragmentation pattern corresponding to a single algal strain from the mixed culture. Such an approach was used to confirm identities of algae present in a polyalgal culture. Initial CAPS analysis of the culture indicated at least two algal strains were present, but their respective identities could not be confidently determined based on the fragmentation pattern (data not shown). Subsequently, an aliquot of the relevant BEC 18S amplicons was cloned into a standard PCR-cloning plasmid, plasmid DNA was extracted individually from ten clones and used as template in PCR with BEC 18S primers. Products were digested with *Hae*III and restriction fragments were resolved using 2.5% agarose and 4% Metaphor agarose (Fig. 3C). Based on the fragmentation patterns, two clones were *T. striata* and the remaining eight were *Chlorella vulgaris* (Fig. 3C). Individual clones were subsequently sequenced to confirm the CAPS-based identification (data not shown). It is possible the ratio of *T. striata*:*C. vulgaris* clones approximated relative abundance of these algae in the initial culture. However, since this CAPS procedure used 1500 nt

amplicons from standard PCR as inputs for the *Hae*III digest, the results should not be

interpreted in a quantitative manner.

ALLELE-SPECIFIC QPCR PROBES FOR MONITORING LOW-ABUNDANCE
ORGANISMS

Given the potential impact of contamination by weedy algae, it would be optimal

to detect weeds when they are at low levels relative to elite strains so that remediation

strategies may be pursued to salvage the culture. The CAPS procedure is suitable for

discriminating algal species and identifying dominant culture constituents, but is not

practical for detection of a weed at low abundance in a culture dominated by an elite

algal strain. We compared the capabilities of two QPCR-based procedures for early

detection of weedy algae (Fig. 1, middle and right panels). Both QPCR assays amplify

portions of the 18S rRNA gene and must be customized to detect algae of interest (i.e.,

elite strains and common weeds). One assay uses a single set of primers to amplify the

same 18S region from different algae, and amplicons are then distinguished using

multiplexed allele-specific fluorescent probes. The second assay uses allele-specific

QPCR primers to produce amplicons from the 18S rRNA genes of different algae.

The fluorescent probe assay (Fig. 1, middle panel) uses "molecular beacons", which are

stem-loop structures in which the loop corresponds to the allele-specific sequence [20].

One end of the self-complimentary stem is fused to a fluorophore, the other to a

quencher. When the probe is not bound to a target amplicon, it assumes the stem-loop

conformation, bringing the quencher into proximity with the fluorophore, thereby

suppressing probe fluorescence. During each cycle of QPCR, probes anneal to target

amplicons and fluoresce; this fluorescence level is used to quantify amplicon production

per cycle. Probes with distinct fluorophores may be multiplexed to detect multiple alleles

in a single QPCR reaction. In theory, these probes may be designed with enough

specificity to distinguish amplicons differing by as little as a single nucleotide. To

establish the ability of fluorescent QPCR probes to distinguish sequences with limited

polymorphism, a probe set was designed to distinguish *N. salina* and *N. oculata*. We

aligned 18S rRNA sequences from *N. salina* and *N. oculata* and identified conserved

regions for QPCR primers (Supplemental Fig. 3). We designed primers (Table 1, NsNo

Forward & Reverse) to produce a 135 nt amplicon spanning 15 polymorphic positions

and developed allele-specific probes to distinguish amplicons derived from *N.*

salina and *N. oculata* (Table 1, *salina* and *oculata* probes). To test whether the QPCR

primers amplify *N. salina* and *N. oculata* templates with similar efficiencies and produce

a single amplicon, these primers were analyzed in QPCR reactions in which the

template was linearized plasmid containing cloned BEC 18S amplicons derived from *N.*

salina or *N. oculata*. These QPCR primers amplified *N. salina*and *N. oculata* templates

with efficiencies of 93.0% and 103%, respectively (Table 1; Supplemental Fig. 5).

To determine the specificity of probes for their intended targets, *N. salina* and *N.*

oculata sequences were individually used as templates for QPCR in which

the *salina* and *oculata* probes were both present. When*N. salina* DNA was used as

template in a QPCR reaction containing *salina* and *oculata* probes, only the*salina* probe

effectively detected amplicons (Fig. 4A). Similarly, when *N. oculata* DNA was used as

template with both probes, only the *oculata* probe effectively detected amplicons

(Fig. 4A). Additionally, the*salina* and *oculata* probes each produced specific signal

above background levels with as little as 0.001 pg template DNA. Similar results were

observed when *N. salina* and *N. oculata* genomic DNA were used as template in the

QPCR reactions (data not shown). In summary, the *salina* and *oculata* QPCR probes were efficient, specific and sensitive when used in QPCR reactions with template derived from a single organism.

Nannochloropsis and *Tetraselmis* were then used to demonstrate the ability of QPCR probes to discriminate elite and weedy algae. We aligned an approximately 650 nt region from 81 GenBank records of 18S rRNA genes from strains of the genera *Nannochloropsis* and *Tetraselmis* (Supplemental Fig. 2). We anticipated the diversity within and between these genera would present challenges in designing primers to produce a single amplicon with similar efficiencies from different strains. Nonetheless, we identified regions highly conserved among *Nannochloropsis* and *Tetraselmis* strains and designed QPCR primers (Table 1, NT Forward & Reverse) to anneal within these regions (Supplemental Fig. 2). Though the primer binding sites are conserved among *Nannochloropsis* and *Tetraselmis* 18S genes, there are 25 genus-discriminating positions within these amplicons and allele-specific probes were designed based on these polymorphisms (Supplemental Fig. 2 and Table 1, *Nannochloropsis* and *Tetraselmis* probes). As described above for the *salina* and *oculata* probes, the primers and probes designed to monitor *Nannochloropsis* and *Tetraselmis* were shown to efficiently produce amplicons from both templates (Table 1; Supplemental Fig. 6) and to be highly specific for their intended targets (Fig. 4B), respectively.

LOSS OF ALLELE-SPECIFIC PROBE SIGNAL WITH COMPLEX TEMPLATES

Since the intended use of these probes was to detect unwanted algae at low abundance within a culture dominated by an elite strain, we determined the sensitivity of

QPCR probes when the template was serial dilutions of DNA from one algae made in a background of DNA from another algae. To test the *salina* and *oculata* probes, template DNAs were linearized plasmids containing cloned *N. salina* or *N. oculata* 18S BEC amplicons. The concentration of *N. oculata* template was held constant in the reactions at 5 pg, 0.05 pg or 0.0005 pg, while the *N. salina* template ranged from 50 pg to 0.00005 pg per reaction. The *salina* probe performed as expected when the *N. oculata* template was less abundant or nearly equal in concentration to the *N. salina* template. However, when the ratio of *N. oculata*:*N. salina* template was 1000:1 or greater, there was strong interference with detection of the *N. salina* allele (Fig. 4C). A similar and more extreme interference was observed in reciprocal experiments in which serial dilutions of *N. oculata* DNA were made in a background of *N. salina* DNA (Fig. 4D). In this case, there was strong interference with and high variability of the *oculata* probe signal when the ratio of *N. salina*:*N. oculata* is 10:1.

This interference phenomenon occurs when using the corresponding primers and probes in QPCR reactions with mixed *Tetraselmis* and *Nannochloropsis* templates. As shown in Fig. 4E, there was interference with detection of the weed *Tetraselmis* when the ratio of *Nannochloropsis*:*Tetraselmis*template was 10:1 or greater. Varying QPCR primer or $MgCl_2$ concentrations did not alleviate this interference (data not shown). We did not determine the molecular basis for the observed interference with probe signal when multiple 18S rDNA alleles were present, though a similar phenomenon was previously reported in experiments using QPCR to detect multiple organisms with concentration differences greater than three orders of magnitude [22]. Fluorescent QPCR probes are extremely sensitive and specific to their targets; however, due to the

signal interference phenomenon they are not appropriate for detecting a small amount of weedy algae in a culture dominated by an elite strain.

ALLELE-SPECIFIC PRIMERS DETECT MINORITY ALGAE IN POLYALGAL CULTURES

As an alternative to multiplexed probes, we designed allele-specific primers to detect either *Tetraselmis* or *Nannochloropsis* in polyalgal cultures (Fig. 1, right panel). Using the same alignment of 81 *Tetraselmis* and *Nannochloropsis* 18S rRNA gene records from above (Section 3.4), regions polymorphic between genera but conserved within each genus were identified (Supplemental Fig. 4) and used to design allele-specific QPCR primers (Table 1, *Nannochloropsis* and *Tetraselmis* diagnostics). In this assay, accumulation of QPCR products is monitored by incorporation of a fluorescent dsDNA-binding dye that is not specific for any particular amplicon. To determine whether the genus-discriminating primers would amplify the non-target genus, *Tetraselmis* primers were included in QPCR reactions with 10 ng purified plasmid DNA containing a cloned fragment of the *N. oculata* 18S rRNA gene. In reactions containing 10 ng *N. oculata* plasmid but lacking *Tetraselmis*, there was not significant accumulation of product within 40 QPCR cycles (Fig. 5A), indicating that the *Tetraselmis* primers do not efficiently amplify *Nannochloropsis* 18S rRNA gene templates. Similarly, in the absence of *N. oculata* template, the *Nannochloropsis* primers did not effectively amplify *T. striata* plasmid template within 35 QPCR cycles (Fig. 5B). Therefore, both sets of genus-discriminating primers efficiently amplified their intended targets, but not the non-target template. Throughout the course of this work, negative controls using these primers sets produced results similar to those described here, such

that C_t values greater than 35 were considered non-specific amplification and C_t values less than 35 were regarded as specific amplification.

The sensitivity of primer-based QPCR assays was determined using reactions in which the template was serial dilutions of DNA from one algae made in a background of DNA from another algae. Dilutions of *T. striata* plasmid (ranging from 50 pg to 0.000005 pg) were made in a background of 10 ng *N. oculata* gDNA, and these mixed DNAs were used as templates in QPCR reactions with the *Tetraselmis* primers. Even when the ratio of *N. oculata*:*T. striata* template was 2×10^8:1, C_t values were nearly identical to those observed when no *Nannochloropsis* template was included (Fig. 5A). In similar experiments, *N. oculata* plasmid was serially diluted in 10 ng *T. striata* template, and the mixed templates were included in QPCR reactions with the *Nannochloropsis* primers. The *Nannochloropsis* primers effectively detected *N. oculata* even when the ratio of *T. striata*:*N. oculata* was 2×10^7:1 (Fig. 5B). The allele-specific primer assay is very effective at detecting a weed at an early point, however the assay is not without limitations. Firstly, it is challenging to design allele-specific primers that eliminate the potential of amplifying targets other than the organism of interest. For this reason, we refer to these assays as "allele-specific" rather than "organism-specific". For example, the allele-specific primers we use to detect *Tetraselmis* also amplify the 18S rRNA gene of *Chlorella vulgaris* (data not shown). While *C. vulgaris* is often considered an unwanted weed and it is useful to have tools to detect it a low abundance, if amplicons are produced and detected with these primers a researcher would not immediately be sure whether the contaminant was *Tetraselmis* or *Chlorella*, though sequencing the QPCR amplicons would reconcile this. Additionally, there is limited

potential for multiplexing of these reactions, so a separate QPCR reaction would likely be needed to assay for each organism of interest.

QPCR OUTPERFORMS FLOW CYTOMETRY FOR EARLY DETECTION OF WEEDS

During scale-up, biomass from smaller cultures is used to inoculate larger cultures. Even at small scales, algal cultures are often maintained by subculturing. Thus, many cultures – regardless of scale or cell density – are old and many generations removed from starter material of confirmed identity [10]. A logical point for weed monitoring is during selection of inocula for subculturing or scale-up. By analyzing potential sources of inoculum in parallel, researchers may identify the sample with the least relative contamination and select this for use.

Presently, flow cytometry is commonly used to monitor algal cultures. To compare the ability of allele-specific QPCR primers to detect weeds in a mixed cell population with that of a flow cytometer, known ratios of *T. striata* and *N. salina* cells were analyzed using both technologies. Cell densities of unialgal cultures were determined and serial dilutions of *T. striata* cells were made in a background of 1×10^8 *N. salina* cells mL^{-1}, such that the final concentration of *T. striata* ranged from 10% to 0.000001% of cells. For each aliquot of the dilution series, *T. striata* and *N. salina* cells were counted using flow cytometry.

Both expected and observed (counted in triplicate) flow cytometry results are shown in Fig. 6A (left panel, dashed gray and solid black lines, respectively). Flow cytometry accurately detected *T. striata* cells when they were 10% — 0.01% of the population. In the four dilutions for which *T. striata* comprised less than 0.01% of the population, flow cytometry overestimated abundance. Not only are the flow cytometry

results inaccurate estimations of *T. striata* abundance, these four samples that varied in *T. striata* abundance by a factor of 10^4 were scored as having similar levels of weedy cells (0.001%–0.008%). If these samples represented potential sources of inocula for subculturing or scale-up, a grower using this data would be unaware of the differences in *Tetraselmis* concentration among the cultures and therefore would likely make suboptimal culture management decisions.

The inaccuracy of flow cytometry for detecting *Tetraselmis* cells when they are at low abundance in populations may be due to factors such as sampling error and technical limitations of the instrument. For example, differences in buoyant densities across algal species can result in settling artifacts. Even with conscientious mixing, this can preclude injection of a representative portion of cells of rapidly settling organisms like *Tetraselmis* into the flow cytometer. In addition to potential false negatives, flow cytometry results may include false positives, in that debris or aggregates of smaller cells may be scored as a single event within the size range of (in this case) *Tetraselmis* cells.

Following analysis by flow cytometry, the remaining cells in each serial dilution were pelleted and gDNA was extracted for use as template in QPCR with the *Tetraselmis* allele-specific primers. In Fig. 6A (right panel), the expected and observed data are shown (dashed gray and solid black lines, respectively). The*Tetraselmis* allele-specific primers effectively detected *Tetraselmis* cells at all dilutions tested. Importantly, C_t values correlated strongly with relative abundance of *T. striata* cells across the dilution series (R^2 = 0.9982). In QPCR reactions of 100% efficiency, the C_t values for 10-fold dilutions should differ by 3.32[19]. Thus, the

expected curve was plotted by extending a line with a slope of -3.32 from the observed data point for the most concentrated sample (10% *T. striata* cells; C_t = 14.41 ± 0.178). As seen in Fig. 6A, the observed data approaches the expected values across all dilutions tested.

The C_t values from the QPCR assay correspond with relative abundance of (in this case) *Tetraselmis* cells in samples, though not necessarily to an absolute number of cells. By comparing C_t values from samples analyzed in parallel, researchers may establish the relative levels of weeds in cultures and make informed decisions regarding which cultures to use as inocula for scale-up or subculturing. Thus, the greater dynamic range of the QPCR assay provides a measure of certainty that flow cytometry does not.

Early weed detection enables informed culture management decision-making

To demonstrate the utility of the QPCR primer assay for culture monitoring when selecting inoculum for scale-up or subculturing, 20 *N. salina* cultures (ranging from 200 mL to 200 L) were analyzed using QPCR and flow cytometry. Flow cytometry was used to characterize each sample, and the number of *Tetraselmis*-like events identified is indicated in Fig. 6B. The remaining cells in each aliquot were used for DNA extraction and analysis by QPCR. To confirm reproducibility of the results, QPCR was done in triplicate on two occasions (Fig. 6B, gray and black bars). The QPCR assay resulted in strong *Tetraselmis* signals in three samples for which flow cytometry detected no *Tetraselmis* (Fig. 6B, single asterisks). Further, strong QPCR signals indicating *Tetraselmis* resulted from analysis of 4 additional samples for which flow cytometry identified between only 1 and 3 *Tetraselmis* cells (Fig. 6B, double asterisks). For four samples in which one or two *Tetraselmis*-like events were detected by flow

cytometry, the QPCR assay produced no signal or inconsistent weak signals across triplicates from the two repetitions of the experiment (Fig. 6B, triple asterisks).

The quantity of *Tetraselmis* cells in these cultures was not determined using an independently validated method, so it is not feasible to definitively conclude which technology more accurately quantified*Tetraselmis* in the samples. Among other potential sources of error, all PCR-based procedures are susceptible to false positives resulting from contamination of DNA preparations or other reagents with target sequences, or false negatives resulting inefficient primer binding to potential targets. Nonetheless, results from experimental controls (Fig. 6B), the relative accuracy of the technologies in quantifying the*Tetraselmis* dilution series (Fig. 6A), as well as quality control experiments in preceding sections, all indicate the QPCR assay effectively detects *T. striata* DNA at 0.0000005% of a mixed template (Fig. 5A) or*Tetraselmis* cells when they constitute as little as 0.000001% of a culture (Fig. 6A), yet does not give positive signal in the presence of *Nannochloropsis* cells or DNA, or in the absence of template (e.g., Fig. 6B, QPCR controls). Furthermore, the data in Fig. 6A (left panel) demonstrate flow cytometry is inaccurate for quantification of *T. striata* at low levels. Data from the 20 samples analyzed above illustrate that culture contamination becomes more prominent in aging cultures during the scale-up process (Fig. 6B) and that informed decisions regarding culture selection may minimize this. The six samples from 200 mL cultures had little (if any) *Tetraselmis*, as detected using QPCR diagnostics. Samples from four 40 L cultures varied greatly in the amount of *Tetraselmis*, and all samples from 200 L cultures had significant quantities of *Tetraslemis* detected by QPCR.

In the absence of additional culture remediation strategies, it would be practical

to discard significantly contaminated cultures as early as possible in the scale-up

process and to preferentially use non- or less-contaminated cultures as inoculum. The

QPCR assay allows determination of relative amounts of *Tetraselmis* in samples

analyzed in parallel. With respect to inoculum selection from the 40 L cultures inFig. 6B,

the QPCR data is more informative than flow cytometry. Flow cytometry indicated there

were between zero and three *Tetraselmis* cells in each of these cultures. The QPCR

assay established relative levels of contamination among these samples, clarifying

which 40 L culture would be the most appropriate for use as inoculum. In fact, the

culture that QPCR data suggested was the most contaminated with *Tetraselmis* was the

culture flow cytometry data indicated was free of *Tetraselmis* (Fig. 6B, black arrow). If

growers relied on flow cytometry data for culture characterization and sample selection,

they would have likely chosen to use the most contaminated 40 L culture as inoculum

for scale-up or subculturing. Therefore, the superior accuracy and sensitivity of the

QPCR assay for the detection of weeds at low abundance provide critical information for

culture management and selection of inoculum.

2.4 TECHNOLOGY OVERVIEW

Given the ubiquitous nature of weedy algae, routine validation of algal cultures is

an essential element of weed management and quality control. Depending on the

growth characteristics of elite strains, it may take many months to scale up from a small

maintenance culture to production cultures covering hundreds to thousands of acres. In

laboratory settings, cultures may be maintained for years by subculturing. If weeds or

other undesired algae grow undetected during this time, they may easily render cultures unusable.

The unique characteristics of 18S rRNA genes make them particularly well suited for diagnostic purposes. The presence of both highly conserved and hypervariable regions within these genes allow the production of various PCR-based monitoring tools. As demonstrated throughout this report, 18S rRNA genes contained polymorphisms sufficient to discriminate algal genera and species. The BEC primers are useful for initial characterization of algal strains, in that they amplify templates from a broad range of algae, providing enough sequence information to unambiguously identify strains. Once BEC sequences are known for strains, restriction fragmentation patterns may be predicted for development of CAPS assays. In all cases herein, *Hae*III digestion of BEC 18S amplicons distinguished strains and was useful for rapid routine monitoring of major culture constituents. The BEC 18S CAPS procedure – coupled with sequencing of BEC 18S amplicons, as needed – is a practical qualitative strategy for monitoring algae that are abundant in cultures.

In the results presented, even two geographic isolates seemingly of the same species were discriminated using CAPS analysis of BEC amplicons. Nonetheless, it is anticipated that *Hae*III digestion of BEC 18S amplicons will not discriminate all organisms. In that case, researchers may wish to digest BEC 18S amplicons using alternate restriction enzymes or to develop similar resources to examine additional loci. Loci encoding large ribosomal subunits (eg, 28S rRNA), internal transcribed spacers between adjacent rRNA genes, and the chloroplast-encoded large subunit of RuBisCo

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(*rbcL*) are commonly used for taxonomic studies and such sequences from numerous organisms are contained in standard online databases [23].

Relative to the qualitative CAPS analyses, quantitative PCR approaches provide vastly increased sensitivity for early detection of unwanted organisms. Though the allele-specific probes described herein did not effectively detect minority organisms, allele-specific primers accurately detected and established relative concentrations of weedy cells comprising as little as 0.000001% of a culture. In contrast, flow cytometry may not always be able to distinguish weedy and elite algae, and it gives inaccurate results when weedy cells constitute less than 0.01% of a population. Thus, incorporating an allele-specific primer assay into current weed monitoring practices will allow growers to identify weeds at low abundance and to make informed decisions regarding culture management and inoculum selection.

Recently, taxonomic surveys of microbial environments – in particular, 16S rRNA profiling of prokaryotic communities – have taken advantage of high throughput sequencing technologies [24]. This is particularly useful for characterization of complex communities that may contain hundreds to thousands of distinct taxonomic units of bacteria with relative abundances that vary by orders of magnitude. Such technologies seem well suited for identification of all algae in a culture population, and to confidently detect weeds at extremely low abundance. At present, several factors preclude implementation of these technologies as part of a standard culture monitoring regime. There are high costs associated with instrumentation, sample preparation, and sequencing. The resulting datasets are massive and require considerable time and expertise to properly analyze and interpret. Most current technologies balance

sequence read length, accuracy, and throughput. To distinguish closely related organisms, it would be ideal to have long sequences of high accuracy; to detect an organism of low abundance (e.g., a weed), it would be ideal to maximize throughput. It is likely at some point the associated direct costs will decrease, data analysis will require less expertise, and the timeframe for sequencing and analysis will be reduced, such that eukaryotic community profiling by advanced sequencing will be a practical component of routine culture monitoring.

Throughout this work it was demonstrated that culture contamination is common and likely increases throughout the culture scale-up process. Therefore, monitoring cultures for contaminants is essential for efficient cultivation of elite algal strains. We anticipate advances in high-throughput sequencing, flow cytometry and additional technologies will eventually provide efficient and cost effective alternatives to the PCR-based monitoring described in this report. However, the technologies presented may be implemented immediately at little to moderate cost and involve procedures accessible to most researchers possessing a general familiarity with PCR. We urge industrial and academic growers of algae to implement such monitoring strategies as part of a their standard quality control procedures.
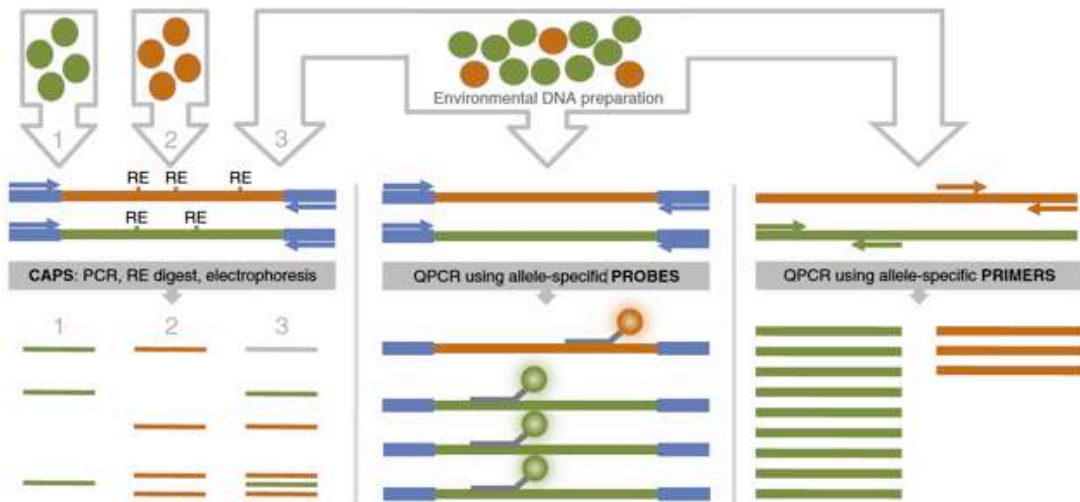
Figure 2 (1). Nucleic acid-based diagnostics for monitoring algal cultures. Schematic overview contrasting three strategies for monitoring algal cultures. Left panel: Using cleaved amplified polymorphic sequences (CAPS), a portion of the 18S rRNA gene is amplified from different algae (represented as green or orange cells) using a single set of primers with broad specificity (blue arrows). Amplicons are digested with an appropriate restriction enzyme (RE, restriction enzyme cut sites) and restriction fragments are resolved by electrophoresis. Allele-specific fragmentation Patterns may be used to identify algae in unialgal cultures (e.g., inputs 1 & 2) or mixed cultures (input 3). Some restriction fragments may be shared by multiple organisms and are not useful for diagnostic purposes (e.g., gray fragment in restriction pattern 3). Middle panel: fluorescent probes in QPCR reactions detect allele-specific polymorphisms within 18S rRNA amplicons produced using primers with broad specificity (blue arrows). Relative fluorescence intensity from multiplexed probes with distinct fluorophores may be used to estimate relative abundances of organisms in cultures. Right panel: allele-specific QPCR primers amplify 18S rRNA gene regions from specific organisms in a culture and estimate their relative abundances.

Table 1.

[1] For primers designed to amplify multiple alleles, amplification efficiency for each allele is given.

[2] Relative to non-target allele.

[3] Uppercase: target sequence; lowercase, self-complementary stem sequences of probes; FAM, 6-fuorescein amidite; HEX, hexachloro fluroscein; BHQ, black hole quencher; IBQ, Iowa black quencher.

[4] Precise amplicon sizes vary by organism.

PCR amplicons, primers and probes.

	Amplicon length (nt)	Amplification efficiency[1]	Primer/Probe	SNPs[2]	Sequence[3]
BEC 18S amplicon					
BEC 18S	~1500[4]	n/a	BEC 18S Forward	n/a	CCGTAGTAATTCTAGAGCTAATAC
			BEC 18S Reverse	n/a	CGGTGTGTACAAAGGGCAGGGACGTAATC
Allele-specific QPCR probes					
Nannochloropsis v. Tetraselmis	Nannochloropsis: 181	N. salina: 94.7%	NT Forward	n/a	GGATGTTTTCATTAATCAAGAAC
			NT Reverse	n/a	GTTTCAGCCTTGCGACCATA
	Tetraselmis: 183	T. striata: 93.3%	Nannochloropsis probe	7	FAM-cgggcgTGCCGACTAGGGATCGGTGGGTGCATcgcccg-BHQ
			Tetraselmis probe	16	HEX-ccggccCAGACGTTTTTTTGATGACTCTGCCAGCAggccgg-BHQ
N. salina v. N. oculata	135	N. salina: 93%	NsNo Forward	n/a	GCTCGTAGTTGGATTTCTGG
			NsNo Reverse	n/a	CACAGTAAAAGATAGGGATCC
		N. oculata:103%	salina probe	9	FAM-ccggggTCGGTTCCGATAAGGGGCCGTACTGTTGcccgg-BHQ
			oculata probe	4	HEX-cccggcGCGGCTCTTACATTAAGTTGTCGGCGTgccggg-IBQ
Allele-specific QPCR primers					
Nannochloropsis diagnostic	104	N. salina: 92.6%	Nanno Forward	14	CGTCGGGATCCCTATCTTTT
			Nanno Reverse	10	AGACCACCAAGGTCGTATCTTA
Tetraselmis diagnostic	140	T. striata: 92%	Tetra Forward	13	TAGCTCCTGGGCTTCACTGT
			Tetra Reverse	9	CCAACAAGATAAGCCAGAGTCC
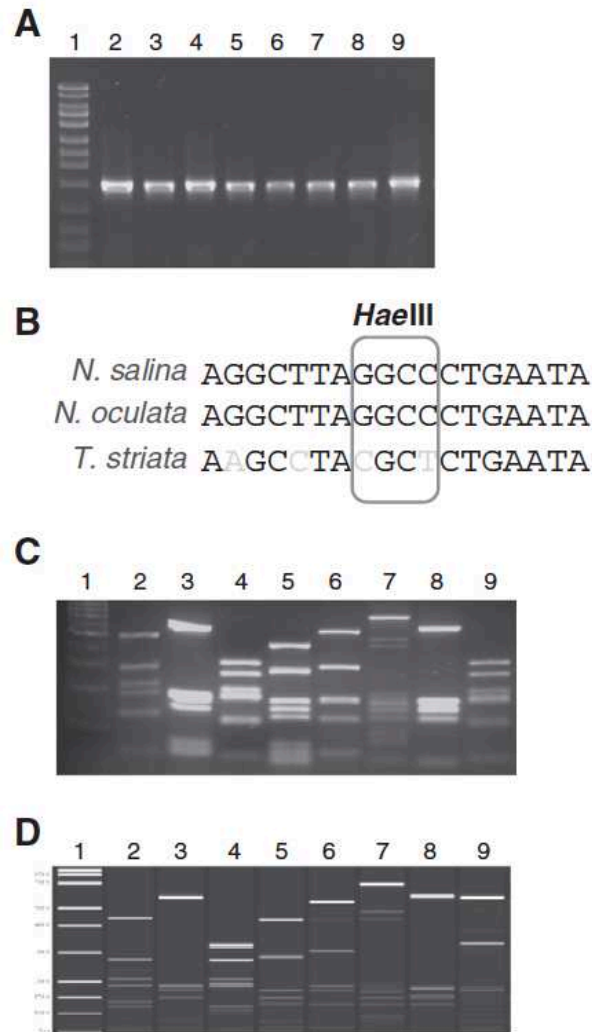
Figure 3 (2). BEC amplicon production and CAPS analysis. A) BEC amplicons were produced from gDNA templates and resolved by electrophoresis. Lanes: 1. size standard, 2. & 3. *N. salina,* 4. *N. oculata*, 5. & 6. *T. striata* (Ute isolate), 7. *Prymnesium parvum*, 8. *Phaeodactylum tricornutum*, 9. *Chlorella vulgaris*. B) A representative 18 nt region within the BEC amplicon containing a *Hae*III restriction site polymorphism. In the example shown, *Hae*III should cut the *N. salina* and *N. oculata* BEC amplicons at this position, but not the *T. striata* amplicon. C) *Hae*III-digested BEC 18S amplicons were resolved using a 2.5% agarose gel. Lanes: 1. Size standard, 2. *N. salina*, 3. *T. striata* (Ute isolate), 4. *N. oculata*, 5. *Chlorella vulgaris*, 6. *Prymnesium parvum*, 7. *Pavlova lutheri*, 8. *Phaeodactylum tricornutum*, 9. *N. oculata*. D) *Hae*III-degested BCEP 18S amplicons were resolved using an Experion automated electrophoresis system. Lanes: 1. Size standard, 2. *N. salina*, 3. *T. striata* (Ute isolate), 4. *N. oculata*, 5. *Chlorella vulgaris*, 6. *Prymnesium parvum*, 7. *Pavlova lutheri*, 8. *Phaeodactylum tricornutum*, 9. *T. striata* (Poudre isolate).

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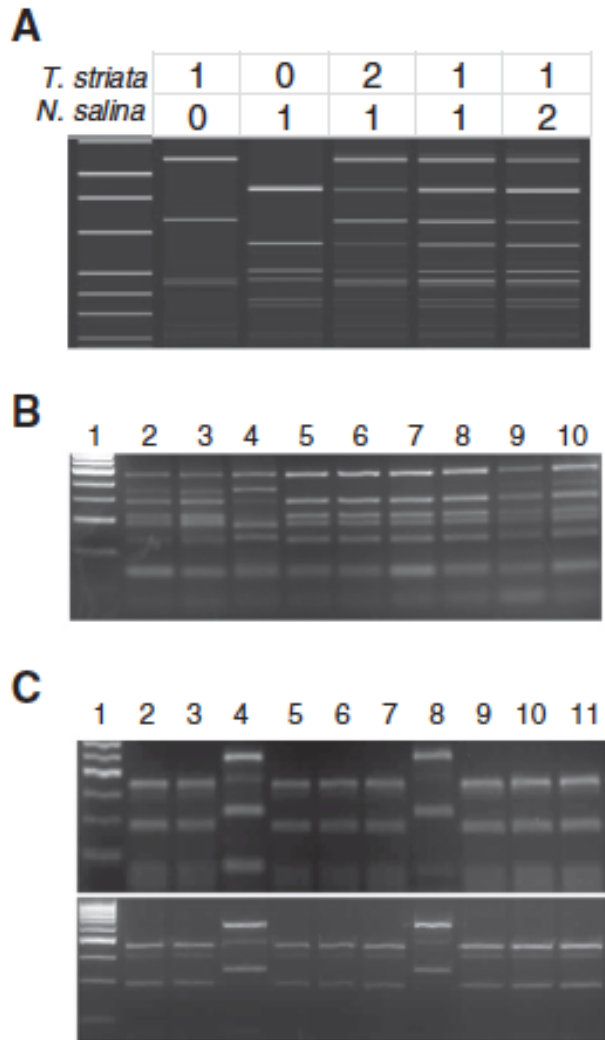
Figure 4 (3). CAPS analyses of polyalgal cultures. A) Genomic DNA from *T. striata* and *N. salina* were mixed at the indicated ratios and used as template for BEC 18S CAPS analysis. *Hae*III restriction fragments were resolved using Experion capillary electrophoresis. Leftmost lane: size standard. B) Nine *N. salina* cultures were analyzed by the *Hae*III CAPS procedure. Resulting restriction fragments were resolved using a 2.5% agarose gel. Lane 1, size standard; lanes 2–10, individual cultures. C) BEC amplicons from a contaminated culture were cloned and individual clones were analyzed using the BEC *Hae*III CAPS analysis. Resulting fragmentation patterns from 10 clones are shown following separation using a 2.5% agarose gel (top panel) or 4% Metaphor agarose (bottom panel). Lane 1, size standard; lanes 2–11, individual clones.
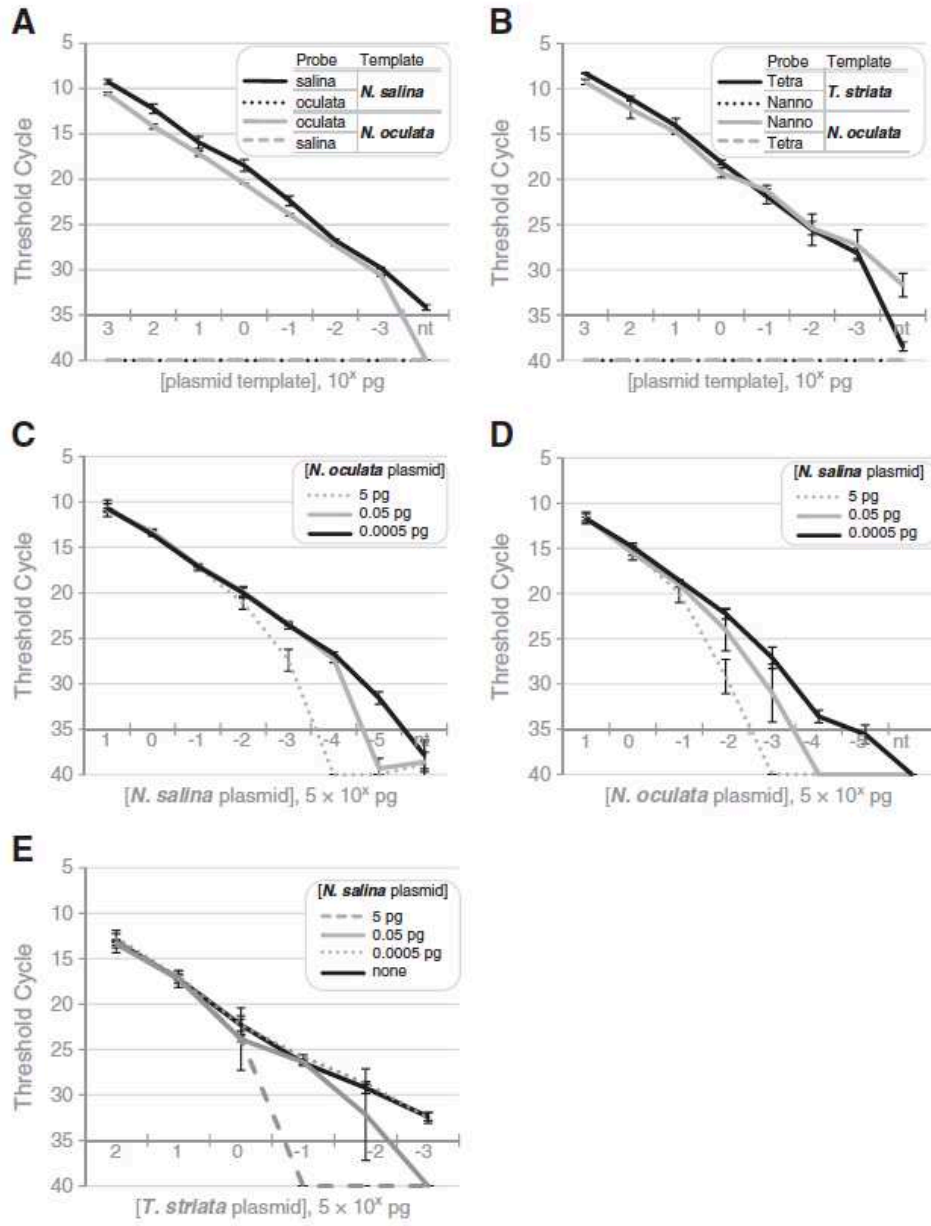
Fig. 5 (4). Fluorescent probes are specific and sensitive, but do not accurately detect minority organisms in polyalgal samples. For all panels, serial dilutions of plasmid DNA templates containing cloned 18S BEC amplicons from the specific target were made alone or in the presence of the non-target templates indicated in inset graphical legends for individual panels. Forty or 45 cycles of QPCR were done for all experiments; if no fluorescence above background was detected, a Ct value of 40was assigned. A) Fluorescent probes to discriminate *N. salina* (*salina*) and N. oculata (*oculata*) efficiently detect as little as 10−3 pg target template, but do not produce fluorescence above background levels in reactions containing only non-target templates. B) Fluorescent probes to discriminate *Nannochloropsis* (*Nanno*) and *Tetraselmis* (*Tetra*) efficiently detect as little as 10−3 pg target template, but do not produce fluorescence above background levels in reactions containing only non-target templates. C) Fluorescent

probes inefficiently detect specific targets diluted in an excess of non-target template. Plasmid templates containing cloned N. salina 18S BEC amplicon was serially diluted in 5 pg, 0.05 pg or 0.0005 pg plasmid containing cloned *N. oculata* BEC 18S amplicon.When the ratio of *N. salina:N. oculata* was 1:1000 or greater, the *salina* probe did not efficiently detect *N. salina*, as indicated by higher Ct values than when the *N. salina* is more concentrated than or approximately equal in concentration to *N. oculata*. D) Fluorescent probes inefficiently detect specific targets diluted in an excess of non-target template. Plasmid templates containing cloned *N. oculata* 18S BEC amplicon was serially diluted in 5 pg, 0.05 pg or 0.0005 pg plasmid containing cloned N. salina BEC 18S amplicon. When the ratio of *N. oculata:N. salina* was 1:100 or greater, the *oculata* probe did not efficiently detect N. oculata, as indicated by higher Ct values than when the *N. oculata* is more concentrated than or approximately equal in concentration to *N. salina*. E) Fluorescent probes inefficiently detect specific targets diluted in an excess of non-target template. Plasmid templates containing cloned *N. oculata* 18S BEC amplicon was serially diluted in 100 ng, 1 ng or 10 pg *N. salina* genomic DNA. When the ratio of *N. oculata* plasmid:*N. salina* gDNA was 1:20,000 or greater, the *oculata* probe did not efficiently detect *N. oculata*. F) Fluorescent probes inefficiently detect specific targets diluted in an excess of nontarget template. Plasmid templates containing cloned *T. striata* 18S BEC amplicon was serially diluted in 5 pg, 0.05 pg, 0.0005 pg or no plasmid containing cloned *N. salina* BEC 18S amplicon. When the ratio of T. *striata:N. salina* was 1:10 or greater, the *Tetraselmis* probe did not efficiently detect *T. striata*, as indicated by higher Ct values than observed in the absence of *N. salina* or when the *T. striata* is more concentrated than or approximately equal in concentration to *N. salina*.
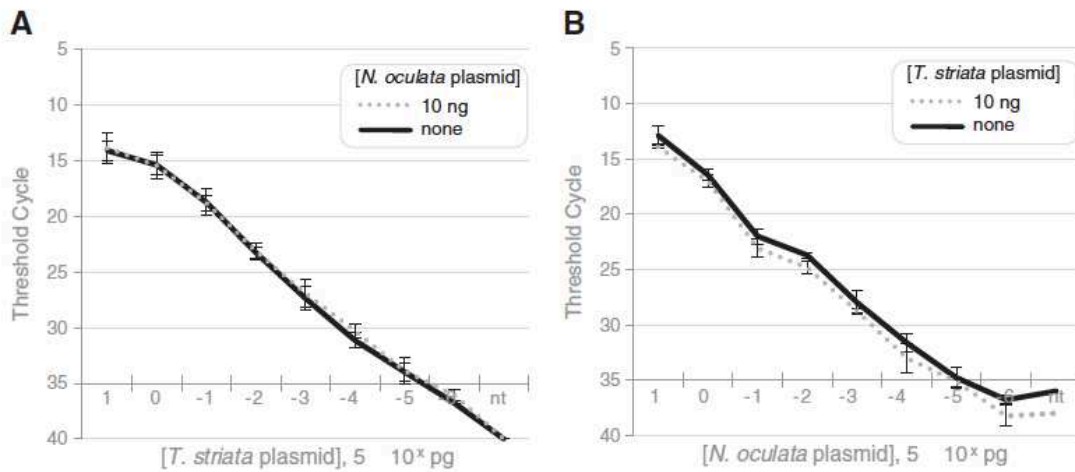
Figure. 6 (5). Allele-specific QPCR primers are specific, sensitive and detect minority organisms in polyalgal samples. A) Allele-specific primers were used to amplify *Tetraselmis* templates alone (solid line) or diluted in a background of 10 ng *Nannochloropsis* DNA (dotted line). Data from three replicates is shown and standard deviations indicated. The primers detect *Tetraselmis* with nearly identical efficiencies in the absence or presence of an excess of *Nannochloropsis* template, indicating the primers do not amplify *Nannochloropsis* template and that the presence of a massive excess of *Nannochloropsis* template does not interfere with the primers' ability to detect *Tetraselmis*. nt, no template. B) A distinct set of allele-specific primers were used to amplify Nannochloropsis templates alone (solid line) or diluted in a background of 10 ng *Tetraselmis* DNA (dotted line). Data from three replicates is shown and standard deviations indicated. The primers detect *Nannochloropsis* with nearly identical efficiencies in the absence or presence of an excess of *Tetraselmis* template, indicating the primers do not amplify *Tetraselmis* template and that the presence of a massive excess of *Tetraselmis* template does not interfere with the primers' ability to detect *Nannochloropsis*. nt, no template.
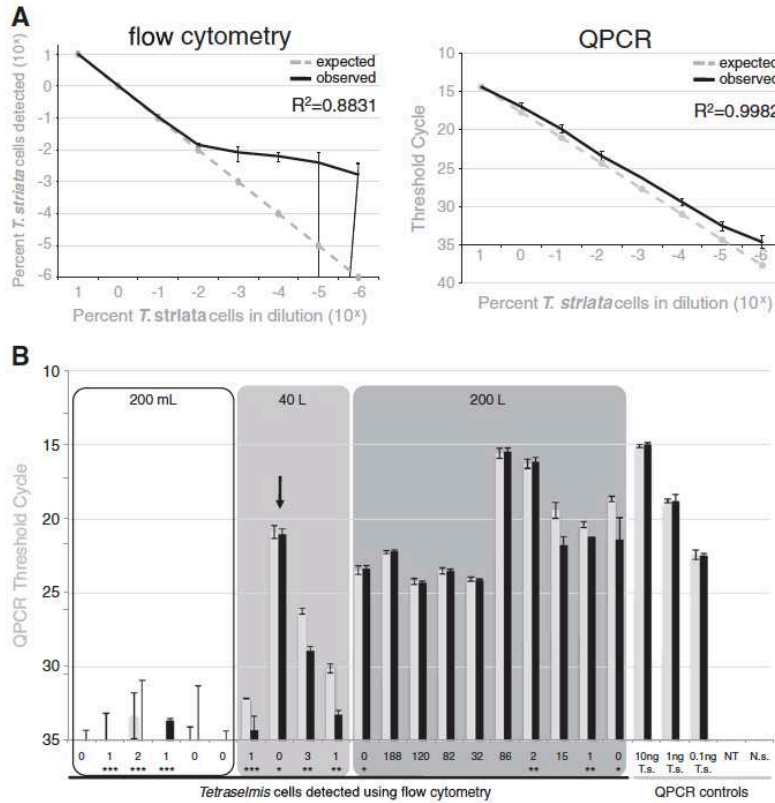
Figure 7 (6). QPCR has greater sensitivity, accuracy and dynamic range than flow cytometry. A) Comparison of flow cytometry and allele-specific QPCR primers for detection of *T. striata* cells serially diluted in *N. salina* cells. Left panel: Flow cytometry was used to score 20,000 events, and percent *Tetraselmis* cells detected and standard deviations across three technical replicates are shown (black line). The expected curve (dashed gray line) indicates actual percentages of *T. striata* cells in the dilution series. Right panel: Triplicate QPCR reactions were done using DNA extracted from the serial dilutions. Observed data and standard deviations for triplicate reactions are shown (black line). The expected curve (dashed gray line) was plotted by extending a line with a slope of−3.32 starting from the observed data point for the most concentrated sample (see accompanying text). For both panels, standard deviations across three replicates are shown, and the correlation of determination (R^2) between the observed data and the amount of *T. striata* cells in each dilution is given. B) Comparison of allele specific QPCR primers and flow cytometry for detection of *T. striata* cells in 20 *N. salina* cultures between 200 mL and 200 L. Flow cytometry was used to score 20,000 events. The number of events identified as *Tetraselmis* is indicated on the x-axis for each sample. Allele-specific QPCR primers were used to detect *Tetraselmis*. Threshold cycles and standard deviations from triplicate reactions in repeated experiments (gray and black columns) are plotted on the y-axis. Black arrow, a 40 L culture flow cytometry indicated was free of *Tetraselmis* was shown using QPCR to be heavily contaminated with *Tetraselmis*. T.s., *T. striata* plasmid; NT, no template; N.s., *N. salina* plasmid.

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CHAPTER 3

BACTERIAL COMMUNITY DYANAMICS IN INDUSTRIAL ALGAE PRODUCTION
SYSTEMS

SUMMARY

Microalgae are a promising feedstock for production of fuels and other chemicals.
A challenge for the algal industry is obtaining consistent, robust algae growth. Algal
cultures include complex bacterial communities and can be difficult to manage because
specific bacteria can promote or reduce algae growth. The richness, structure, and
composition of bacterial communities were characterized in closed photobioreactor
cultivations of *Nannochloropsis salina* at different scales and in different seasons. Using
16S rRNA sequence data from 275 samples, bacterial communities in small, medium,
and large cultures were shown to be significantly different. Large systems contained
richer bacterial communities compared to small systems. Bacteria common to the
majority of samples were identified, including a single OTU within the class *Saprospirae*
that was found in all samples. This study contributes critical information for crop
protection in algae systems, and demonstrates that successful industrial algae
cultivation requires understanding the ecology of algal growth systems.

3.1 INTRODUCTION

Microalgae (herein, "algae") are photosynthetic unicellular eukaryotes that grow
in aquatic or marine environments, converting CO_2 to biomass. For reasons including
rapid growth and high lipid content, certain varieties of algae are considered promising
biofuels feedstocks (1, 2). Algae may be cultivated on otherwise non-arable land in
growth systems that use salt water or wastewater, so production of algae biomass does

not necessarily divert land and fresh water from production of traditional agricultural crops (3). Generally, large-scale industrial growth systems circulate algae, nutrients, and water around open ponds or within closed photobioreactors. Open ponds use a paddle wheel to circulate algae around a constantly exposed raceway. In closed systems, algae cultures are grown in bags or tubes that reduce exposure to the outdoor environment. Compared to open raceways, closed systems are more expensive to build and operate (4), but allow greater control over parameters such as CO_2 and nutrient concentrations while limiting the potential for invasion by unwanted organisms.

Growers typically desire to cultivate monocultures of algae selected or engineered for traits such as robust growth and accumulation of desired biochemical products (e.g., lipids or other high-value compounds) (3). Following conventions used with traditional agricultural crops, these high performance algae varieties may be referred to as "elite". For production of lipids, several commonly used elite strains are members of *Nannochloropsis*, a genus of marine algae with doubling times on the order of 30 h and lipid contents ranging from 30–60% (5,6). Algae growth parameters are often studied and optimized using small laboratory cultures grown in aseptic conditions using precisely controlled light, temperature, and nutrient regimes. Since elite algae have not historically been grown at the large volumes required by the biofuels industry (7), a challenge is translating the productivity of elite strains optimized under highly controlled lab environments to consistent culture productivity at large scales. Therefore, researchers must understand the variables associated between small versus large scale cultivation.

Much like terrestrial crops, algae productivity may be modulated by biotic factors such as weeds, predators, and other microbes. For example, algae with low lipid content that contaminate elite cultures – and compete for resources such as light and nutrients – are considered weeds (8). Zooplankton grazers prey on small algae (9) such as *Nannochloropsis*. Fungi and bacteria also affect algae productivity (9,10); however there is little understanding of the interactions among elite algae and these co-resident microbes. The majority of algae pathogens and pests have not been identified and industry pest management standards are at an early stage of development (11).

Bacteria are abundant and dynamic in algae cultures, and bacterial counts commonly reach 1 x 10^9 cells/mL, outnumbering algae cells 10- to 100-fold (4). Although bacteria are often considered contaminants that can inhibit algae productivity or terminate algae populations, bacteria-algae interactions have a range of potential outcomes (10,12,13,14) Algae support bacterial growth by releasing 25% of the total organic carbon fixed by photosynthesis (15,10). Reciprocally, of hundreds of algae varieties surveyed, over half do not endogenously produce vitamin B12 and therefore require bacteria-produced vitamin B12 to promote algae growth (16). Other studies have determined specific bacteria that may stimulate algae growth through activities including regulation of the amount of available nutrients such as iron, nitrogen, and phosphates (17,18,19), or by releasing phytohormones such as indole-3-acetic acid into the growth environment (20). In some instances, bacteria reduce algae productivity by competing for these same nutrients (21,22). In addition to nutrient competition, non-lethal bacterial pathogens may inhibit algae productivity by diverting cellular resources from algal growth to cell defense. Finally, some bacteria can directly kill algae, causing

cultures to collapse (4, 23). Much of this knowledge of algae-bacteria interactions derives from ecological studies of harmful algal blooms in natural environments, with the general aims of identifying bacteria or specific bacterial functions that promote or inhibit such blooms. Of immediate need for the algae biofuels industry is an understanding of the relationships among elite algae and co-resident bacteria in engineered cultivation systems containing high concentrations of cells and nutrients.

In this study, bacterial communities were monitored during industrial algae production at Solix Biosystems (Fort Collins, CO). At this facility, production involves scale-up from 5 mL algae cultures grown under aseptic conditions to growth of 200 L cultures in closed, but not aseptic, systems (Figure 8A). Smaller cultures are used to inoculate larger ones until the 200-L scale is reached. Small cultures of 4 L or less are kept under aseptic laboratory conditions using sterilized glassware and media, with the handling of open containers occurring in a laminar flow hood. These small cultures are grown under light sources in shaking incubators or on shaking platforms. Medium (20 – 60 L) and large (200L) cultivations are grown in closed systems, but handling of these cultures involves system components that are not sterile. In addition to opportunities for microbe entry during culture handling, the medium and large closed growth systems are technically more difficult to isolate from microbes in their environment. Medium cultures are grown in flat-panel bioreactors under ambient light in a greenhouse, whereas large cultures are grown in closed photobioreactors in an outdoor water basin under natural light.

To monitor bacterial communities in these *N. salina* cultivation systems over time, we collected samples from small, medium, and large cultivations across eight months.

From these samples, a region of bacterial *16S rRNA* was amplified and sequenced, and

the composition, structure, and richness of bacterial communities associated with *N.*

salina were determined. Although different growth systems contain distinct bacterial

communities, 16 bacterial OTU were identified in 90% of *N. salina* cultivations, including

a single OTU found in all samples. Differences in community composition were

observed across *N. salina* growth systems, across the duration of the experiment, and

among replicate large systems supporting different algae growth rates.

3.2 RESULTS

BACTERIAL COMMUNITY DIFFERENCES ACROSS CULTIVATION SYSTEMS SIZE

Community DNA was extracted from archived biomass samples collected over

an 8-month period from small, medium, and large industrial algae cultivation systems at

Solix Biosystems (Fort Collins, CO) (Figure 8A). In total, 275 samples were processed.

The V3 region of *16S rRNA* genes was amplified and sequenced, generating 10.9

million sequenced amplicons. Following filtering steps that removed algae-derived

chloroplast and mitochondrial sequences along with extremely rare sequences and

other potential sources of error, 2 million bacterial reads were used for further analyses.

The composition of these bacterial communities was compared across all

samples using unweighted UniFrac as a distance metric. As seen in a resulting principal

coordinates analysis (PCoA) plot (Figure 8 B), the data formed three major clusters

corresponding to communities in samples from large, medium and small growth

systems, respectively.

To compare the alpha diversity of bacterial communities from different system

scales, we used OTU counts to calculate average species richness within each growth

scale (Figure S1). Overall, alpha diversity increased as the size of the system increased. The large cultures contained 132 ± 19 OTUs, medium cultures contained 108 ± 22.8, and small cultures averaged 88.0 ± 8.1 OTUs. This same trend was observed when alpha diversity was determined as phylogenetic distance (Figure S2).

The bacterial community structures in different algae growth systems were analyzed. At the phylum level, *Bacteroidetes* and *Proteobacteria* dominated communities from all system scales. On average, *Bacteroidetes* increased in relative abundance as system scale increased, from 48.5% abundance at small scale to 63.3% at medium scale and 70.7% in large-scale growth environments (Figure 9). *Proteobacteria* became less prevalent as the system size increased, having relative abundances of 43.6%, 28.6%, and 25.7% in cultivations grown at small, medium, and large scales, respectively. The total abundance of *Bacteroidetes* and *Proteobacteria* was constant across all systems, respectively representing approximately 91.8, 89.9, and 90.6% of bacteria at small, medium, and large systems (Figure 9). In addition, the relative abundance of unassigned phyla increased with system size (2.4, 3.4, and 4.4% in small, medium, and large cultivations, respectively).

Within *Bacteroidetes* and *Proteobacteria*, the total number of distinct taxa identified at the class and order levels (and comprising at least 0.1% relative abundance) increased as culture scale increased (Figures 2 and 3). Three classes of *Bacteroidetes* were identified in small cultures, five in medium cultures, and six in large cultures. At all scales, *Bacteroidetes* [*Saprospirae*] was the most abundant *Bacteroidetes* class. Within *Proteobacteria*, two, four, and five classes were detected in small, medium, and large systems, respectively. *Alphaproteobacteria* was the most

abundant class of *Proteobacteria* in all production systems. In small-scale cultures, three orders of *Bacteroidetes* were observed, while five and six orders were found in medium and large cultivations, respectively. At all system scales, *Bacteroidetes* [*Saprospirae*] [*Saprospirales*] was the most abundant order of *Bacteroidetes* (Figure 11). The small, medium, and large cultures included 8, 17, and 19 orders of *Proteobacteria*, respectively. Distinct combinations of *Proteobacteria* were identified in each system scale, with large cultures containing the most unique taxa (five) at the order level (Figure 3). Nonetheless, *Rhizobiales* (*Alphaproteobacteria*) was the most abundant order of *Proteobacteria* in small and medium cultures, and was the second most abundant *Proteobacteria* in large cultures.

MOST ABUNDANT BACTERIA FOR EACH CULTIVATION SIZE

Within each cultivation system size, bacteria were ranked by relative abundance and rankings were compared across systems (Figures 11 and S3). The 10 orders most abundant in small systems accounted for 94.9% of the members of the bacteria communities (Figure 11A). All ten of these orders were identified in the medium and large systems. *Saprospirales* was the most abundant order in small systems (23.2%), and was also the most abundant in medium and large cultivations (32.0% and 37.3%, respectively). *Flavobacteriales* and *Rhizobiales* were the second and third most abundant orders in small systems. These orders represent 20.1% and 19.7% of the bacteria in small cultures, but their average abundances declined to between 3%-5% (*Flavobacteriales*) and 6%-8% (*Rhizobiales*) in medium and large systems. The ten bacterial orders most abundant in large systems (Figure 11B) represented, on average, 87.2% of the bacteria in that system. All these bacteria were identified in medium

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systems, but four of the bacteria were not identified in small systems (at 0.05% or above). *Cytophagales* was the second most abundant order in large systems, where it averaged 22.3% of bacterial communities. Although it was also the second most abundant order in medium cultivations (Figure S3) and similarly comprised 18.9% bacterial abundance in those systems, *Cytophagales* was only present at 5.0% abundance in small systems.

BACTERIAL COMMUNITY DIFFERENCES ACROSS SEASONS

Unconstrained, unweighted UniFrac (sensitive to rare taxa) and PCoA were used to show that bacterial communities in large systems were different across the 8-month study period, July 2011 – March 2012 (Figure 12). Comparisons of alpha diversity revealed that the final study month, March 2012, contained highest richness based on phylogenetic distance, whereas all other months had no significant difference (data not shown).

The average relative abundances of several bacteria types from classes dominant in large systems (Table 3B) were plotted across months (Figure S4). *Saprospirae* remained the predominant bacterial class during the course of sampling. *Cytophagia* were dominated by *Leadbetterella (Cytophagaceae)* and members of the genus *Cyclobacteriaceae*, which peaked in the summer to fall months (July – October; Figure S2). The most abundant *Alphaproteobacteria* in large systems were members of the *Kiloniellales* and *Rhizobiales* classes, with *Kiloniellales* reaching maximum abundance in winter months (January and February).

BACTERIA PREVALENT IN ALL SAMPLES

To determine which bacteria were associated with *N. salina* across the majority of culture conditions, OTUs were identified that were present in 90% of all samples (at least 0.01% abundance). This consisted of 16 OTUs that together totaled 63% of the relative abundance of bacterial communities averaged across all systems. This grouping included eight *Proteobacteria*, five *Bacteroidetes*, and three bacteria unassigned to any taxa (Figure 13). The bacterial community present in 95% of samples consisted of seven OTUs that together averaged 47% relative abundance across all samples, and ranged from 2.9% to 83.3% relative abundance in individual samples. Of the seven OTUs, three were *Alphaproteobacteria,* two were *Bacteroidetes* and two were unassigned (Table 1).

A single OTU was present in 100% of samples (Figure 13). This OTU is of the phylum *Bacteroidetes*, and at the class level is [*Saprospirales*], a group that is contested in the Greengenes reference database. In addition to being in every sample, *Saprospirales* was the most abundant OTU overall, comprising 34.7% ± 14.6% of bacterial communities across all cultivations. Within small, medium, and large systems, *Saprospirales* had average relative abundances of 21.4% ± 8.4%, 31.9% ± 18.3%, and 37.6% ± 11%, respectively. *Saprospirales* varied in individual samples from 0.3 – 83.3% relative abundance, with the low and high values each observed in samples from large cultivations. Although only 16 of 275 total samples contained less than 5% *Saprospirales*, no correlation was observed between *Saprospirales* abundance and *N. salina* growth performance.

One OTU of the bacteria found in 90% of samples was *Spirobacillales* (*Deltaprotebacteria*), present in large systems at an average relative abundance of 1.2% ± 5.2%. In three abnormal, stagnant cultivations, *Spirobacillales* bloomed to 35% – 45% of the total bacteria population (Figure 14). Algae concentrations in these three cultivations declined by 92.0% compared to 13 replicate large cultures sampled on the same day (Figure 14).

3.3 DISCUSSION

BACTERIAL COMMUNITIES DIFFERED ACROSS CULTIVATION SCALES

Bacterial communities were characterized in small, medium, and large growth systems for industrial *N. salina* production. It was hypothesized that bacterial communities would differ across growth system scales and seasons, and in algae cultivations exhibiting different algae growth rates. Unweighted UniFrac and PCoA were used to compare a total of 275 samples from all *N. salina* growth scales. In Figure 8, the distance separating samples represents differences among bacterial communities, measured as the fraction of evolutionary history in a phylogenetic tree that is unique to one of the samples (24). Three primary clusters were observed, corresponding to samples from small, medium, and large growth scales (Figure 8B). Thus, algae cultivations at different scales contained bacterial communities that were distinct in terms of phylogenetic structure. Bacterial communities from algae cultivations of the same system scale were similar in phylogenetic structure, as indicated by the tight clustering of samples from each growth system.

There are numerous environmental differences during cultivation at small, medium, and large scales that might affect bacterial populations and cause distinct

communities to dominate different growth systems. These include culture volume, the ratio of surface area to volume, light source intensity, illumination period, environment sterility, method of mixing, method of temperature management, and others (Figure 8A).

In addition to differences in environmental parameters, the serial batch strategy used for these cultivations may affect bacterial community composition across different scales. In the serial batch mode used here, biomass from dense *N. salina* cultures of a particular scale was harvested and additional cultures at that scale were inoculated using a portion of this harvest; occasionally, biomass harvested at one scale was used to inoculate cultivations in a larger growth system. Because culture communities (*N. salina*, bacteria, and other constituents) were repeatedly reused for cultivation at a particular scale, this inoculation strategy provides additional generations within which communities may have been affected by the environmental conditions of that system scale and therefore became increasingly distinct from communities grown at different scales. It is conceivable that the community structure associated with productive *N. salina* cultivations at one growth scale could be less optimal at other scales.

As measured using phylogenetic distance, bacterial communities in large cultivation systems were more diverse than those from small cultures. Phylogenetic distance quantifies diversity based on total branch length of bacterial *16S rRNA* phylogeny captured in a sample (24). Communities sampled from large systems contained higher levels of phylogenetic diversity (Figures S1 and S2). Quantified by OTU counts, bacterial richness also increased from small to large systems. Although most large cultivations were performed in serial batch mode during this study, all of the cultures in large systems passed through small and medium systems at some point in

their history. The 10 orders most common in small systems accounted for 94.9% of all bacteria at that scale, whereas they totaled 74.0% and 75.4% of the bacterial populations in medium and large systems (Figure 11A). Since the handling of cultures at medium and large scales was not aseptic, each handling was an opportunity for bacteria and other microbes to enter the community and increase species richness and phylogenetic diversity. The ten bacterial orders most prevalent in large systems accounted for 87.2% of the bacteria in those communities (Figure 11B). Only six of these orders were identified in small systems, in which they cumulatively constituted 77.8% of bacteria. Nonetheless, bacteria detected only in large systems may also have been present in small and medium systems, but below the detection threshold of this study (0.05% for data presented in Figure 11B).

Large systems of *N. salina* cultivation contained the highest number of unique bacteria at each taxonomic level. However, unique bacteria were found in the bacterial communities at each scale. For example, the order *Pseudomonodales* (*Gammaproteobacteria*) was identified in cultivations of medium scale, but not in small or large cultivations (Figure 3). A species of *Pseudomonas (Pseudomonadales)* bacteria was reported to reduce growth of the alga *Ankistrodesmus* under phosphate-limited conditions (11). If the *Pseudomonodales* bacteria in the cultivations studied here similarly reduced growth of *Nannochloropsis* in phosphate-limited environments, cultivations in medium systems would be more susceptible to phosphate limitation than cultures grown in small or large systems using the same nutrient composition.

BACTERIAL COMMUNITIES DIFFERED ACROSS SEASONS

Large-scale *N. salina* growth systems used in this study were enclosed photobioreactors submerged in an outdoor water basin in natural light. Thus, day length and light intensity varied across seasons. Light and other seasonal variables directly affect algae growth, which, in turn, affects the amount of organic carbon algae released to the system. At the study location, algae growth rates were higher during the summer and fall, and lower during winter. Seasonal variables of outdoor systems may also directly or indirectly influence bacterial communities. Day length is known to contribute to bacterial community composition in natural marine environments (25). Samples for this study were collected from July 2011 – March 2012, including 135 samples from cultivations in large outdoor growth systems under natural light. Analysis of these 135 samples revealed a continuum of differences among bacterial communities over the course of the experiment (Figure 12). The abundance of some bacteria, including the genera *Leadbetterella* and *Cyclobacteriaceae*, peaked in the summer and fall, seasons of high algae growth. In contrast, *Kiloniellales* abundance was greatest during the winter when algae generally grew more slowly. Since this study looked at a single continuous 8-month period, differences in bacterial community composition through time are not necessarily related to seasonality. Continued monitoring across years will allow prediction of potential seasonal effects on bacterial community composition, as well as clarification of the nature of any such correlations.

CERTAIN BACTERIA WERE HIGHLY PREVAENT IN *N. SALINA* CULTIVATIONS

Across all characterized *N. salina* cultivations, the phyla *Bacteroidetes* and *Proteobacteria* dominated bacterial communities. *Bacteroidetes* and *Proteobacteria*

previously have been shown to be the most abundant bacteria in marine environments, with *Alphaproteobacteria* and *Gammaproteobacteria* typically dominating the *Proteobacteria* in marine systems (26). This finding is also consistent with results of previous studies of *Nannochloropsis* cultivations (4). Members of these phyla may generally contribute functions that promote the growth of *Nannochloropsis* species in various environments.

To identify bacteria associated with *N. salina* under the majority of conditions in this study, the OTUs present in 90% or more of all samples were determined (Figure 13). This group consisted of 16 OTUs that together averaged 63% relative abundance of the bacterial communities in this work. Half of these OTUs were *Proteobacteria*. One type of bacteria associated with 90% of *N. salina* cultivations belonged to the order *Spirobacillales (Deltaproteobacteria)*. This OTU was present at unusually high levels in three replicate large algae cultures with stagnant growth, compared to the abundance of this OTU in 13 other replicate cultures that grew at normal rates (Figure 14). It is unknown whether the higher *Spirobacillales* abundance limited *N. salina* growth, or itself was a result of culture stagnation. Similarly high levels of *Spirobacillales* were not observed in other large cultivations with slow or stagnant growth.

The group of bacteria associated with *N. salina* across 95% of samples consisted of seven OTUs that averaged 47% relative abundance across all samples. Of these, four were identified in a separate study of *N. oceanica* that was limited to analysis of 510 *16S rRNA* sequences representing 39 total OTUs (4). Thus, 10% of the OTUs identified in the *N. oceanica* study belong to the group associated with *N. salina* in 95% of the samples in this study. Association of these four OTUs with both *N. salina* and *N.*

oceanica in distinct environments suggests that these OTUs may have specific relationships with *Nannochloropsis* species and merit further study. In a separate investigation, laboratory *N. gaditana* cultures were treated with multiple rounds of antibiotics to eliminate bacteria non-essential for algae growth [SPF, KFR, unpublished results]. Although it is not known how many OTUs remained in the *N. gaditana* cultures following these treatments, only one bacterial species was recovered when the algae cultures were plated on marine agar. This was a representative of the family *Phyllobacteriaceae*, which was also identified in 95% of samples in this study. This indicates intimate association of this bacterial family with several species of *Nannochloropsis*. In fact, members of the family *Phyllobacteriaceae* have been identified as supporting algae growth in additional studies. *Mesorhizobium loti* (of the *Phyllobacteriaceae*) was found to supply vitamin B to the alga *Lobomonas rostrata*, with this interaction optimized at a 1:30 (algae:bacteria) cellular ratio under the examined conditions (27). Separately, *Mesorhizobium* was shown to be one of several nitrogen-fixing species associated with growth promotion of four different green algae (28).

A single OTU of the class *Saprospirales* (a contested grouping in Greengenes) was found in all of the 275 *N. salina* samples. This OTU was also the most abundant, on average comprising 34.7% ± 14.6% of bacterial communities in all cultivations. In the small, medium, and large growth systems, *Saprospirales* averaged 21.4% ± 8.4%, 31.9% ± 18.3%, and 37.6% ± 11% abundance, respectively. In individual samples, the relative abundance of *Saprospirales* varied from 0.3% – 83.3%, with both extremes observed in samples grown in large systems. No correlation between *Saprospirales* abundance and *N. salina* growth was observed. While the activity of *Saprospirales* in

this system is unknown, a strain of *Saprospirales* was shown to be capable of lysing the microalgae diatom *Chaetoceros ceratosporum* (29). The presence of *Saprospirales* in every sample suggests that there are important interactions between this bacterium and *N. salina*, and makes *Saprospirales* a clear candidate for further study. Determining the functions contributed by highly conserved bacterial community members such as *Saprospirales* will facilitate efforts to optimize bacterial communities in *Nannochloropsis* production systems.

Although studies specifically related to bacterial influences on *N. salina* health are limited, ecological activities are known for some of the bacteria that were common in large and small systems. The second most abundant bacteria in the large systems were *Cytophagia*, which had a much lower relative abundance in small cultures. Like *Saprospirales*, members of *Cytophagia* are capable of lysing a variety of microalgae, demonstrating how differences in bacterial composition among systems could affect algae health and productivity (30). *Rhizobiales* were three times as abundant in small systems as in large ones. Other studies have shown that members of *Rhizobiales* fix nitrogen and increase the growth of algae (31,29). In one instance, a *Rhizobium sp.* increased the growth of *Botryococcus brauni* by 50% compared the axenic culture (32). In addition, a *Mesorizobium sp.*, a type of *Rhizobiales*, was found to provide vitamin B12 to algae (27). *Sphingobacteriales* comprise 3.0% of the bacterial abundance in large systems and 5.6% of medium systems, but were not detected in small systems. This group of bacteria can cause flocculation of some microalgae (33).

IMPLICATIONS FOR INDUSTRIAL ALGAL CULTIVATIONS

As demonstrated in this study, bacteria were abundant in a closed phototrophic algal production system, and differences in community composition were found across growth conditions. Ultimately, algae producers will benefit from detailed molecular understanding of mechanisms underlying bacterial impacts on algal culture performance. In the near-term, however, culture management strategies may be best informed by determining correlations between system constituents and algal culture performance. The profiling of *16S rRNA* sequences presently allows a detailed systems-level characterization of bacterial communities during algal cultivation. The presence or absence of specific community members may be correlated with algal performance metrics such as growth rate and lipid productivity. Whether such bacteria directly impact algal productivity or merely serve as predictors of culture performance, diagnostics may be developed to routinely monitor for presence or abundance of these specific community members. For example, 16 bacterial OTU were identified in 90% of all samples in this study, seven OTU were in 95% of samples, and a single OTU was found in every sample. To favor stable *N. salina* growth in large systems, potential sources of inoculum could be screened to confirm that they contain the bacterial community found in 90%, 95%, or 100% of samples in this study. In some instances, it may not be sufficient to monitor for the presence or absence of specific organisms. In this study, *Spirobacillales (Deltaproteobacteria)* was one of the OTUs observed in more than 90% of samples. This conservation across samples suggests it is beneficial to monitor for retention of *Spirobacillales* in cultivations and potential inoculum. However, of 16 replicate large system cultivations of *N. salina*, *Spirobacillales* was present at unusually

high abundances in three cultures undergoing stagnant growth, but was found at lower levels in the remaining 13 cultures growing at normal rates (Figure 14). Thus, it may be desirable to monitor for its abundance of *Spirobacillales* relative to some standard across cultivations (such as *N. salina* abundance or total bacterial abundance).

Small cultivations grown under aseptic conditions contained less bacterial diversity than cultivations grown in medium and large systems. As a practical matter, experiments to determine optimal conditions for algae productivity often use small cultivation systems. The different bacterial community composition and reduced diversity of small cultivations may impact the ability of researchers and producers to translate *N. salina* productivity levels observed in small laboratory systems to performance in large systems.

This study revealed differences in the composition and dynamics of bacterial communities in *N. salina* algae cultivation systems. Understanding bacterial functions in algae cultures is critical for successful large-scale algae cultivation. Bacteria that are detrimental to algae growth must be identified, tracked, and minimized. Bacterial communities that promote algae growth and stability could be included in a probiotic cultivation supplement (16). In addition to systems-level monitoring of community constituents, targeted experiments are necessary to determine specific bacterial functions that promote or inhibit algae productivity. Candidates for further characterization include bacteria associated with the majority of all cultivations, with specific growth scales, or with cultures exhibiting extreme growth rates.

3.4 EXPERIMENTAL PROCEDURES

ALGAE GROWTH SYSTEMS

Samples were collected from small, medium, and large cultivations of *Nannochloropsis salina* at Solix Biosystems (Fort Collins, CO). *N. salina* was originally obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (formerly, Center for Culture of Marine Phytoplankton, CCMP) (Bigelow Laboratory for Ocean Sciences, East Boothbay, ME). All algae cultures were grown in F/2 medium (34). To scale up the culture volume (Figure 8A), a single *N. salina* colony was isolated from an F/2 agar plate and grown to high density in 5 mL liquid culture. Cultures were primarily grown in a serial batch mode with a portion of each harvest used to inoculate the subsequent cultivations in the same-volume system, or used to start a new cultivation in larger systems. For this study, culture volumes of 5 mL, 1 L, 2 L, and 4 L are all designated as "small". Sterile technique was used with all small cultures, including growth in sterilized containers and F/2 medium, as well as use of a laminar flow hood during culture handling. Small cultures were maintained on a shaker table rotating at 200 rpm and supplemented under 24-hour artificial light at 50 μE. Cultivations designated as "medium" were grown in variable volume (20 – 60 L) flat-panel bioreactors aerated with 2% CO_2 at 2.5 vvm (volume gas per volume liquid per minute) in a greenhouse under ambient light. Cultivations designated as "large" were approximately 200 L and grown in enclosed photobioreactors located outdoors in a water basin in which the temperature was maintained between 19 and 26 °C, and pH was maintained at approximately 7.3. System specifics are provided elsewhere (8) Flow cytometry was used to evaluate the purity of the algal population, and specifically the

presence of a *Tetraselmis* sp. that had previously been observed at this site. This analysis revealed that the cultivations contained only low levels of this weedy species: 89.9% of the samples had less than 1% of *Tetraselmis*, 95.3% contained less than 2% of *Tetraselmis*, and 98.9% (3 samples) contained less than 5% of *Tetraselmis* (data not shown).

ALGAE CULTIVATION SAMPLING AND GROWTH MONITORING

A total of 17, 81, and 177 samples were obtained from small, medium and large cultures, respectively. The frequency of sampling varied, but the production system was sampled at least once per calendar month from July, 2011 to March, 2012. For samples from small cultures, an adjustable pipette was used to transfer 1 mL culture to a microcentrifuge tube in a laminar flow hood. Samples from medium and large systems were drawn using a sterile 10-mL needleless syringe through a non-sterile plastic sample line connected to sample ports at one end of the photobioreactor. To ensure that sample lines and ports were clear of waste material, a 20-mL volume of culture was drawn and discarded. Subsequently, 10 mL of culture were drawn and mixed by inversion, and 1 mL of mixed sample was transferred to a microcentrifuge tube. Sample biomass was pelleted using centrifugation at 15,000 x g. The supernatant was discarded, and the biomass was stored at –80 °C.

Algae culture density was monitored by optical density measured at 750 nm (OD_{750}) using a Hach DR5000 spectrophotometer. Algae growth was estimated using $\Delta(OD_{750nm}) = OD_{750(t2)} - OD_{750(t1)}$, where *t1* and *t2* represent adjacent time points. Additionally, a Guava easyCyte HT+ flow cytometer (EMD Millipore) equipped with an

argon laser (488 nm) and 680/30 nm bandpass filter was used to directly count cells in a

given volume, identifying algae cells based on size and chlorophyll fluorescence (8).

MOLECULAR TECHNIQUES AND SEQUENCE ANALYSIS

DNA extractions and *16S rRNA* amplification were done according to protocols

standardized for the Earth Microbiome Project (EMP;

http://www.earthmicrobiome.org/emp-standard-protocols/) (35). Briefly, community DNA

(including algae and bacteria DNA) was extracted from archived biomass using

PowerSoil®-htp 96 Well Soil DNA Isolation Kits (MoBio; Carlsbad, CA), and 300-350-bp

amplicons from the V3-V4 regions of included *16S rRNA* genes were generated by PCR

using primers 515f and 806r. Amplicons were sequenced at the BioFrontiers Institute

(University of Colorado, Boulder) using an Illumina MiSeq, resulting in 10.9 million 150-

bp reads derived from the V3 region of amplicons. QIIME version 1.8.0 was used for all

sequence analyses (32). Sequences were quality filtered and demultiplexed using

default settings of the split_libraries_fastq.py QIIME script. Greengenes version 13_5

was used as the reference database for all OTU picking steps (25). Since community

DNA extracted from archived samples includes significant amounts of algae DNA,

sequences were filtered to eliminate reads of chloroplast or mitochondrial origin in two

steps: one prior to the main OTU picking step, and one following. For the first filtering

procedure, a subset of the Greengenes reference was generated that contained

representatives from only mitochondrial and chloroplast clusters (using the 97%

similarity Greengenes clusters and associated taxonomy assignments); all 10.9 million

sample-derived sequences were assigned to OTUs at 97% similarity using the closed-

reference protocol with this reduced reference database of chloroplast and mitochondria

sequences; 5.6 million sample sequences that hit were assumed to be derived from algae chloroplasts or mitochondria and were eliminated from analysis. The main OTU picking step used the subsampling open-reference protocol to assign approximately 3.1 million of the remaining 5.3 million sequences to OTUs, using Greengenes 97% clusters and 97% similarity threshold. Approximately 200,000 sequences belonged to OTUs containing fewer than two sequences and were eliminated from further analyses, and a further 2 million sequences that did not align to reference *16S rRNA* sequences using PyNAST was used (36). Some of the new (i.e., non-reference) OTUs were assigned chloroplast or mitochondrial taxonomy; the second filtering step eliminated these OTUs, reducing the sequence count for downstream analyses to 2 million out of the initial 10.9 million. An additional filtering step eliminated low abundance OTUs comprising less than 0.005% of the total sequence count. To compare samples with PCoA when data from all growth systems were in the analysis, samples were rarefied at 1000 sequences. For large system-specific plots data were rarefied at 2740 sequences. Computations were done on the Pando supercomputer. Data were deposited in the European Bioinformatics Institute with accession number ERP010414.

SUPPLEMENTAL INFORMATION

Supplemental Information includes additional data presented in one table and three figures.

AUTHOR CONTRIBUTIONS

S.P.F., K.F.R., and S.T.C conceptualized the study; A.R.P. and S.P.F. performed the formal analysis; S.P.F., D.B.-L., and A.R.P. conducted the investigation; S.T.C, KFR, and R.K. provided resources; S.P.F. and A.R.P. performed data curation; S.P.F. wrote

the original manuscript draft; K.F.R, S.T.C, R.K., and A.R.P reviewed and edited the

manuscript; S.P.F. and S.T.C. developed the data visualization; and K.F.R., S.T.C., and

R.K. supervised the project.
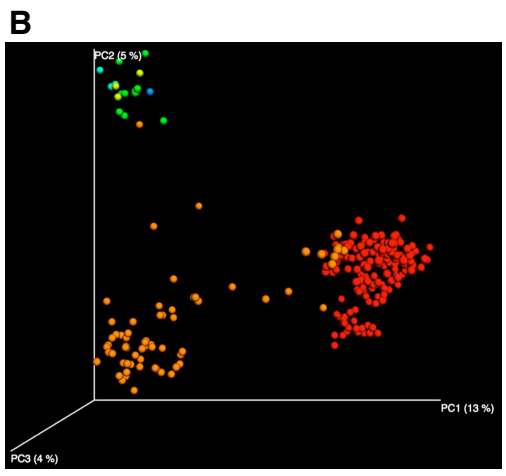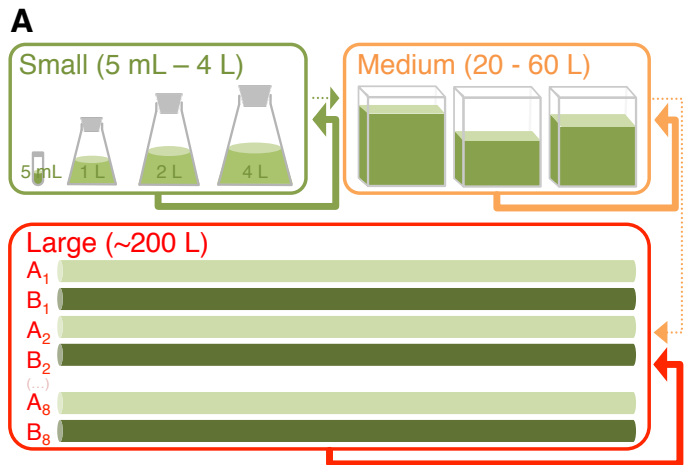
Figure 8. Bacterial communities in *N. salina* growth systems. A) *N. salina* cultivation systems and inoculation strategy. Growth systems are categorized as small, medium or large as illustrated and further described in the text. Arrow intensities indicate relative movement of inoculum biomass within and between systems (solid and dashed arrows, respectively). B) Principle coordinates analysis plot showing relationships among bacterial communities isolated from algae growth systems. Each point represents the bacterial community isolated in a single sample. Colors indicate samples from small (multi-color, blue – yellow spectrum), medium (orange), and large (red) cultivations.

			Abundance		
Phylum		Class	Sml	Med	Lrg
B	Bacteroidetes	Bacteroidia	0.0	0.0	0.1
		Cytophagia	5.0	18.9	22.3
		Flavobacteria	20.1	4.6	3.4
		Sphingobacteria	0.0	5.6	3.0
		[Rhodothermi]	0.0	0.2	0.1
		[Saprospirae]	23.2	32.0	37.3
Pr	Proteobacteria	Alphaproteobacteria	35.0	18.6	21.7
		Betaproteobacteria	0.0	1.4	0.4
		Deltaproteobacteria	0.0	5.1	2.1
		Epsilonproteobacteria	0.0	0.0	0.1
		Gammaproteobacteria	8.5	3.5	0.1
A	Acidobacteria	Solibacteres	0.0	0.1	0.1
Ac	Actinobacteria	Acidimicrobidae	0.2	0.1	0.0
		Actinobacteria	0.2	0.1	0.0
Ar	Armatimonadetes	[Fimbriimonadia]	0.0	0.1	0.0
Ch	Chloroflexi	Anaerolineae	0.0	0.0	0.1
Cy	Cyanobacteria	4C0d-2	0.0	0.2	0.0
		ML635J-21	2.8	0.3	0.1
		Synechococcophycideae	0.0	2.2	0.0
F	Firmicutes	Clostridia	0.0	0.0	0.2
P	Planctomycetes	Planctomycetia	1.4	1.8	0.9
T	TM7	SC3	0.0	0.3	0.0
V	Verrucomicrobia	Opitutae	0.0	0.6	0.9
		Verrucomicrobiae	1.1	0.3	1.2
cd	BRC1	PRR-11	0.0	0.0	0.1
	OD1	ZB2	0.0	0.2	0.2
Unassigned			2.4	3.4	4.4

$$\Sigma = 99.9 \quad 99.6 \quad 98.8$$

$$\Sigma_{(B+Pr)} = 91.8 \quad 89.9 \quad 90.6$$

Figure 9. *Bacteroidetes* and *Proteobacteria* dominate communities across *N. salina* growth systems. Relative abundances of bacterial classes identified in *N. salina* growth systems are represented. Phylum abbreviations given herein are used in subsequent Figures and Table 1. cd, Candidate division. Heat maps are formatted separately for each scale growth system, ranging from zero to the maximum value in that system (white to black); for contrast, relative abundances above 18 are listed in white font. Since OTU counts are rounded to the nearest tenth, zero values represent relative abundances less than 0.05%. Σ (bottom) is the sum of all values, and reveals rounding errors; Σ$_{(B+Pr)}$ (bottom) indicates total abundance of *Bacteroidetes* and *Proteobacteria* in each system.

Ph	Class	Order	Abundance		
			Sml	Med	Lrg
B	Bacteroidia	Bacteroidales	0.0	0.0	0.1
	Cytophagia	Cytophagales	5.0	18.9	22.3
	Flavobacteria	Flavobacteriales	20.1	4.6	3.4
	Sphingobacteria	Sphingobacteriales	0.0	5.6	3.0
	[Rhodothermi]	[Rhodothermales]	0.0	0.2	0.1
	[Saprospirae]	[Saprospirales]	23.2	32.0	37.3
Pr	Alphaproteobacteria		0.1	0.4	0.2
		BD7-3	0.0	1.8	1.5
		Caulobacterales	0.0	0.1	0.1
		Kiloniellales	0.0	1.1	7.1
		Rhizobiales	19.7	8.0	6.2
		Rhodobacterales	8.7	3.6	2.9
		Rhodospirillales	1.1	1.3	2.0
		Rickettsiales	0.0	0.2	0.3
		Sphingomonadales	5.5	2.1	1.4
	Betaproteobacteria	Burkholderiales	0.0	0.0	0.1
		Rhodocyclales	0.0	1.3	0.3
	Deltaproteobacteria	Desulfovibrionales	0.0	0.0	0.4
		Myxococcales	0.0	0.0	0.2
		Spirobacillales	0.0	5.0	1.5
	Epsilonproteobacteria	Campylobacterales	0.0	0.0	0.1
	Gammaproteobacteria	Alteromonadales	6.9	2.2	1.0
		Chromatiales	0.0	0.2	0.1
		Legionellales	0.0	0.1	0.0
		Oceanospirillales	1.6	0.8	0.1
		Vibrionales	0.0	0.0	0.1
		Pseudomonadales	0.0	0.1	0.0
		Thiotrichales	0.0	0.1	0.0
		Vibrionales	0.0	0.0	0.1
		Other	0.1	0.0	0.0
A	Solibacteres	Solibacterales	0.0	0.1	0.1
Ac	Acidimicrobidae	Acidimicrobiales	0.2	0.1	0.0
	Actinobacteria	Actinomycetales	0.2	0.1	0.0
Ar	[Fimbriimonadia]	[Fimbriimonadales]	0.0	0.1	0.0
Ch	Anaerolineae	SBR1031	0.0	0.1	0.1
Cy	4C0d-2	SM1D11	0.0	0.2	0.0
	ML635J-21		2.8	0.3	0.1
	Synechococcophycideae	Pseudanabaenales	0.0	2.2	0.0
F	Clostridia	Clostridiales	0.0	0.0	0.2
P	Planctomycetia	Pirellulales	1.4	1.5	0.7
		Planctomycetales	0.0	0.3	0.1
T	SC3		0.0	0.3	0.0
V	Opitutae	Opitutales	1.1	0.5	0.7
		Puniceicoccales	0.0	0.1	0.2
	Verrucomicrobiae	Verrucomicrobiales	0.0	0.3	1.2
cd	PRR-11 (BRC1)		0.0	0.0	0.1
	ZB2 (OD1)		0.0	0.2	0.2
	Unassigned		2.4	3.4	4.4

Σ = 100.1   99.5   100.0

$\Sigma_{(B+Pr)}$ = 92.0   89.7   91.9

Figure 10. Abundance of bacterial orders across *N. salina* growth systems. Relative abundances of bacterial orders identified in *N. salina* growth systems are represented. Data are formatted as in Figure 9. Ph, Phylum (for phylum abbreviations, see Figure 9).

A

Ph	Class	Order	Abundance		
			Sml	Med	Lrg
B	[Saprospirae]	[Saprospirales]	23.2	32.0	37.3
B	Flavobacteria	Flavobacteriales	20.1	4.6	3.4
Pr	Alphaproteobacteria	Rhizobiales	19.7	8.0	6.2
Pr	Alphaproteobacteria	Rhodobacterales	8.7	3.6	2.9
Pr	Gammaproteobacteria	Alteromonadales	6.9	2.2	1.0
Pr	Alphaproteobacteria	Sphingomonadales	5.5	2.1	1.4
B	Cytophagia	Cytophagales	5.0	18.9	22.3
C	ML635J-21		2.8	0.3	0.1
Pr	Gammaproteobacteria	Oceanospirillales	1.6	0.8	0.1
Pl	Planctomycetia	Pirellulales	1.4	1.5	0.7
			Σ = 94.9	74.0	75.4

B

Ph	Class	Order	Abundance		
			Sml	Med	Lrg
B	[Saprospirae]	[Saprospirales]	23.2	32.0	37.3
B	Cytophagia	Cytophagales	5.0	18.9	22.3
Pr	Alphaproteobacteria	Kiloniellales	0.0	1.1	7.1
Pr	Alphaproteobacteria	Rhizobiales	19.7	8.0	6.2
B	Flavobacteria	Flavobacteriales	20.1	4.6	3.4
B	Sphingobacteriia	Sphingobacteriales	0.0	5.6	3.0
Pr	Alphaproteobacteria	Rhodobacterales	8.7	3.6	2.9
Pr	Alphaproteobacteria	Rhodospirillales	1.1	1.3	2.0
Pr	Deltaproteobacteria	Spirobacillales	0.0	5.0	1.5
Pr	Alphaproteobacteria	BD7-3	0.0	1.8	1.5
			Σ = 77.8	81.9	87.2

Figure 11. Bacteria predominant in small and large *N. salina* cultivation systems. A) Ten most abundant bacterial orders identified in small growth systems. B) Ten most abundant bacterial orders identified in large growth systems. Zero values represent relative abundances less than 0.05%. Ph, Phylum (for phylum abbreviations, see Figure 2). Heat maps are formatted separately for each growth system as in Figure 2. Σ, total abundance of these ten orders in each system.

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Day	Calendar	#
1	July 26, 2011	5
5	July 30, 2011	5
15	August 9, 2011	5
19	August 13, 2011	9
30	August 24, 2011	10
34	August 28, 2011	5
49	September 12, 2011	8
50	September 13, 2011	8
69	October 2, 2011	18
70	October 3, 2011	16
85	October 18, 2011	6
86	October 19, 2011	6
110	November 12, 2011	13
140	December 12, 2011	7
177	January 18, 2012	7
210	February 20, 2012	7
244	March 25, 2012	7

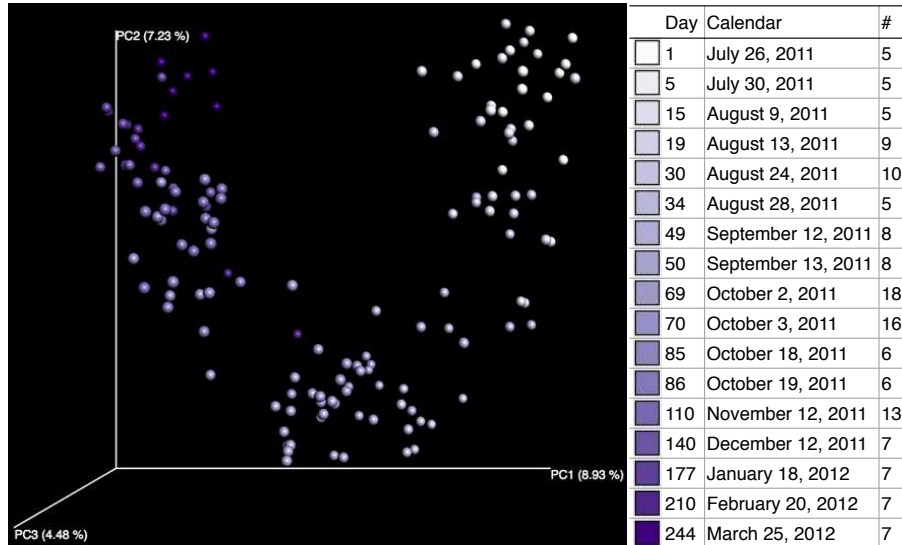Figure 12. Bacterial communities in large outdoor *N. salina* systems differ across eight months. Principle coordinates analysis plot showing 135 bacterial communities isolated from large *N. salina* growth systems. Each point represents the bacterial community isolated in a single sample. Day, day relative to start of experiment; Calendar, calendar date; #, number of large system samples analyzed

| Community				Consensus Taxonomy			
100%	95%	90%	Ph	Class	Order	Family	Genus
■			B	[Saprospirae]	[Saprospirales]	Saprospiraceae	
			Unassigned (95a)				
			Unassigned (95b)				
			B	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella
			Pr	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	
						Hyphomicrobiaceae	Parvibaculum
						Hyphomicrobiaceae (95a)	
			Unassigned (90)				
				Cytophagia	Cytophagales	Cyclobacteriaceae	
			B	[Saprospirae]	[Saprospirales]	Chitinophagaceae	
				Sphingobacteria	Sphingobacteriales		
				Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	
					Rhodobacterales	Hyphomonadaceae (90a)	
			Pr			Hyphomonadaceae (90b)	
				Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter
				Deltaproteobacteria	Spirobacillales		

Figure 13. Bacterial communities identified in greater than 90% of samples. Bacteria identified in 100%, 95% and 90% of the 275 samples included in this study are indicated. Ph, Phylum (for phylum abbreviations, see Figure 2)

	Consensus Lineage				Algae growth (ΔOD750)																Average	
					Normal (+)													Stagnant (-)			+	-
					0.42	0.34	0.31	0.31	0.30	0.29	0.26	0.26	0.25	0.25	0.23	0.22	0.20	0.07	0.05	-0.06	0.28	0.02
Ph	Class	Order	Family	Genus	OTU Relative Abundance (%)																	
Pr	Deltaproteobacteria	Spirobacillales			0.2	0.1	7.5	13.8	1.9	6.6	8.3	0.5	0.7	2.5	0.9	1.5	0.8	34.7	44.9	44.4	3.5	41.3
B	Sphingobacteriia	Sphingobacteriales			0.0	2.1	0.4	0.7	0.2	0.2	0.5	0.1	0.7	0.0	0.0	0.2	0.6	2.3	2.7	1.9	0.4	2.3
B	[Saprospirae]	[Saprospirales]	Chitinophagaceae		0.1	0.7	1.0	1.0	0.3	0.9	1.3	0.6	0.6	0.9	0.9	0.3	0.4	2.2	0.3	0.0	0.7	0.8
Pr	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae (917635)		0.2	0.3	1.0	0.7	0.5	0.9	0.7	0.3	0.2	0.9	0.6	1.1	0.4	0.5	0.3	0.5	0.6	0.4
Pr	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae (95991)		0.4	0.4	0.4	0.4	0.3	0.1	0.3	0.4	0.3	0.3	0.4	0.2	0.2	0.1	0.0	0.4	0.3	0.2
Pr	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae		1.7	1.6	1.9	1.3	1.0	1.4	1.4	1.1	0.7	1.2	2.1	1.3	1.0	1.4	1.3	1.2	1.4	1.3
Pr	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Parvibaculum	0.3	0.5	0.2	0.4	0.4	0.7	0.3	0.6	0.4	0.5	0.7	0.3	0.2	0.5	0.2	0.5	0.4	0.4
Pr	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae (NR284)		0.2	0.1	0.1	0.1	0.1	0.0	0.2	0.2	0.0	0.2	0.1	0.1	0.1	0.0	0.0	0.1	0.1	0.0
Pr	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae		0.8	1.0	0.6	0.5	0.8	0.7	0.6	1.0	0.3	0.8	1.2	0.7	0.5	0.3	0.3	0.3	0.7	0.3
Pr	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	0.1	0.0	0.5	0.8	0.3	0.2	0.2	0.1	0.0	0.2	0.3	0.1	0.1	0.1	0.0	0.0	0.2	0.0
Unassigned (NR178)					0.7	0.7	0.2	0.2	1.0	0.4	0.1	0.6	0.6	0.3	0.5	0.3	0.8	0.2	0.3	0.1	0.5	0.2
Unassigned (NR142)					0.1	0.2	0.2	0.2	0.3	0.1	0.2	0.2	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1
Unassigned (NR242)					1.3	1.3	0.7	0.8	1.9	1.0	0.8	1.6	1.9	1.0	0.9	0.9	1.5	0.4	0.6	0.2	1.2	0.4
B	Cytophagia	Cytophagales	Cyclobacteriaceae		7.0	5.3	12.0	20.3	11.7	13.1	9.7	10.0	4.6	5.4	13.7	4.6	9.6	5.7	0.6	1.8	9.8	2.7
B	[Saprospirae]	[Saprospirales]	Saprospiraceae		46.2	47.3	35.0	23.6	49.8	39.3	41.5	44.8	52.4	43.0	30.4	46.4	53.3	18.2	24.2	22.3	42.6	21.6
B	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	16.5	13.5	14.8	9.6	9.6	11.7	12.9	13.1	12.8	21.4	13.2	19.5	11.4	6.7	5.6	6.2	13.9	6.1
				Σ =	76	75	77	75	80	77	79	75	76	79	66	78	81	73	81	80	76	78

Figure 14. Bacterial communities in replicate large *N. salina* cultivations with distinct growth rates. Growth rates (DOD$_{750nm}$, top) for sixteen replicate large *N. salina* cultivations are ranked from highest to lowest (left to right). Vertical bar separates 13 replicates of normal growth from three replicates with stagnant growth. Relative abundances of OTU identified in 90% or more of all *N. salina* cultivations in this study are represented in heat maps. Zero values indicate relative abundances less than 0.05%. Heat maps are formatted separately based on abundances in each replicate culture. Ph, Phylum (for phylum abbreviations, see Figure 2). Average OTU abundances in normal-growth (+) and stagnant (-) cultures are given (far right). Σ, total abundance of selected OTU in each sample or sample average.

83
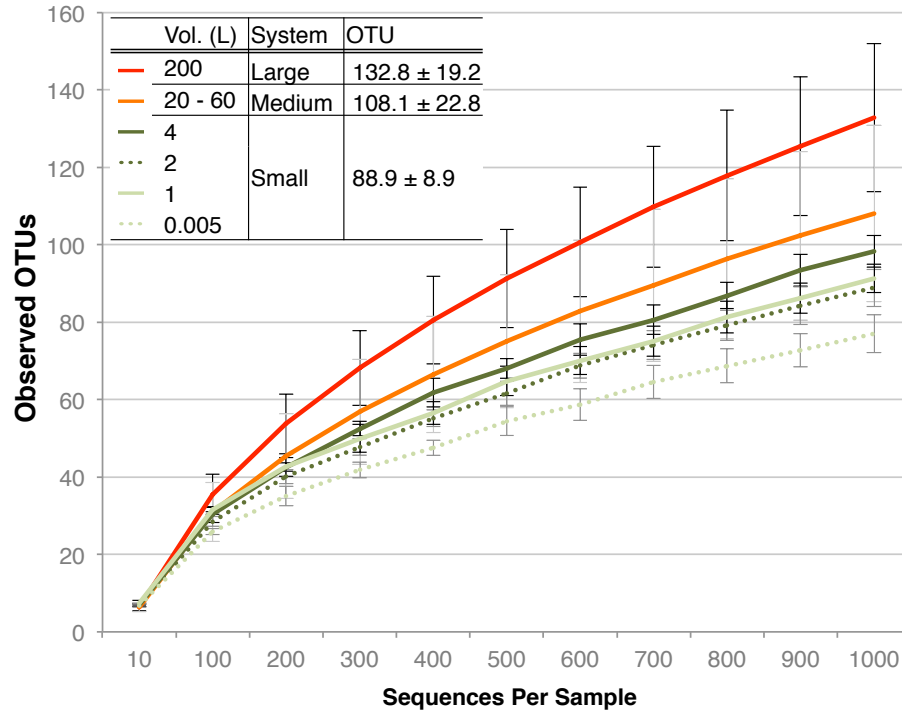
Supplemental Figure 1. Observed OTUs increase with system scale. Rarefaction curve showing, on average, that there are increased OTUs at larger system scales than smaller ones. Small systems include volumes ranging from 5 mL – 4 L, plotted separately. Average observed OTUs for each system are listed in sample legend.

| Vol. (L)	System	PD
200	Large	18.2 ± 2.3
20 - 60	Medium	15.6 ± 3.1
4		
2	Small	10.6 ± 1.0
1		
0.005		
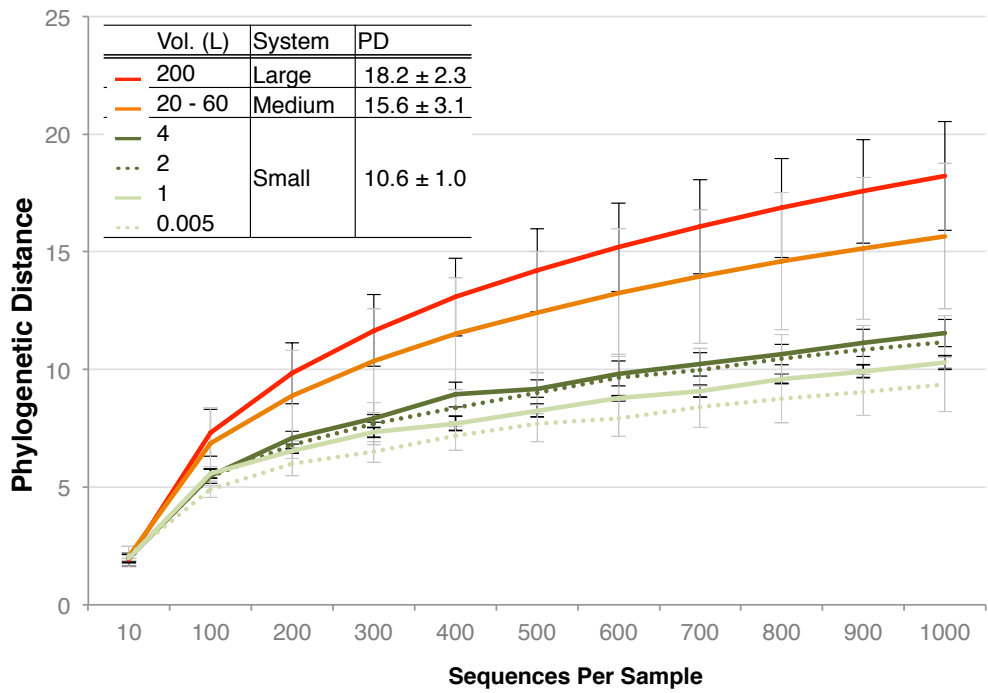
Supplemental Figure 2. Phylogenetic Distance increases among communities in medium and large systems. Rarefaction curve showing phylogenetic distance (PD) increases on average at larger system scales. Small systems include volumes ranging from 5 mL – 4 L, plotted separately. Average PD for each system is listed in sample legend.

| | | | Abundance | | |
Ph	Class	Order	Sml	Med	Lrg
B	[Saprospirae]	[Saprospirales]	23.2	32.0	37.3
B	Cytophagia	Cytophagales	5.0	18.9	22.3
Pr	Alphaproteobacteria	Rhizobiales	19.7	8.0	6.2
B	Sphingobacteria	Sphingobacteriales	0.0	5.6	3.0
Pr	Deltaproteobacteria	Spirobacillales	0.0	5.0	1.5
B	Flavobacteria	Flavobacteriales	20.1	4.6	3.4
Pr	Alphaproteobacteria	Rhodobacterales	8.7	3.6	2.9
Pr	Gammaproteobacteria	Alteromonadales	6.9	2.2	1.0
C	Synechococcophycideae	Pseudanabaenales	0.0	2.2	0.0
Pr	Alphaproteobacteria	Sphingomonadales	5.5	2.1	1.4

Σ = 89.1 84.2 79.0

Supplemental Figure 3. Bacteria predominant in medium *N. salina* cultivation systems. The ten most abundant bacterial orders identified in medium growth systems are shown. Zero values represent relative abundances less than 0.05%. Ph, Phylum (for phylum abbreviations, see Figure 2). Heat maps are formatted separately for each growth system as in Figure 2. Σ, total abundance of these ten orders in each system.
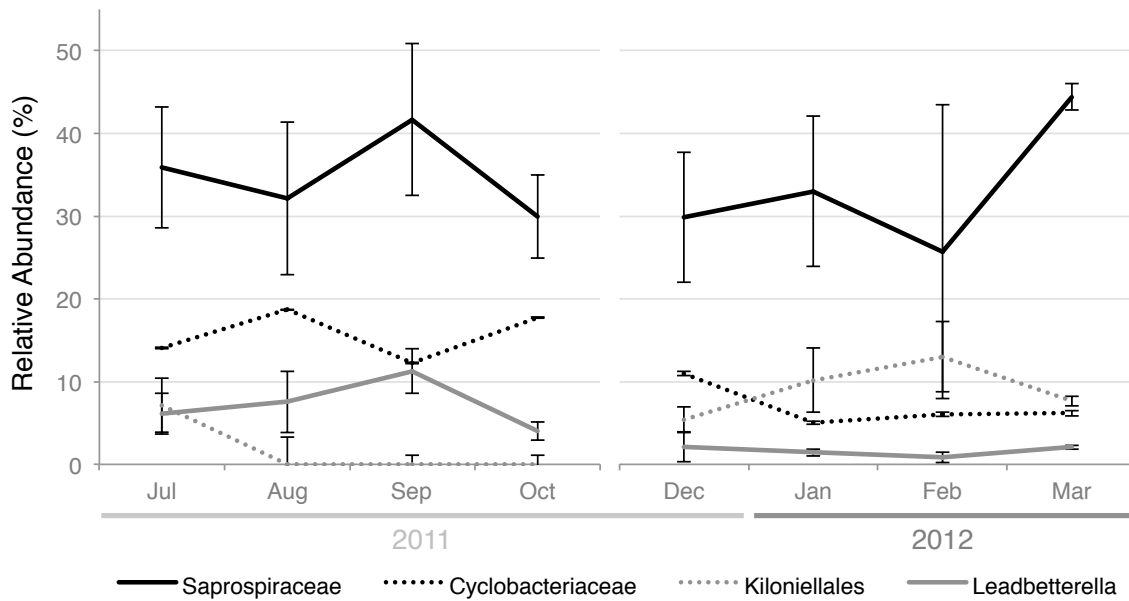
Supplemental Figure 4. Abundance of orders *Saprospiraceae*, *Cyclobacteriaceae*, *Kiloniellales*, and *Leadbetterella* in large outdoor *N. salina* systems across months. Relative abundances of selected bacterial orders were averaged within calendar months. Due to insufficient data, November 2011 was not included. Error bars indicate standard deviation.

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CHAPTER 4

GROWTH INHIBITION OF NANNOCHLOROPSIS BY THE BACTERIUM BACILLUS
PUMILUS ISOLATED FROM AN INDUSTRIAL ALGAE CULTIVATION

SUMMARY

Consistent cultivation of robust algae cultures is a major challenge at large-scales due to pests such as bacteria that can severely detract from algae productivity. To optimize algae productivity, understanding algae culture ecology will need significant attention to advance pest management practices. To date, there is limited knowledge about pests that affect commercially relevant algal strains. In this study, bacteria were isolated from a poorly performing 200-L industrial algae growth system that was growing *Nannochloropsis salina* with the goal of determining whether growth-inhibiting bacteria of *Nannochloropsis sp.* were present in the culture. Isolated bacteria were grown in liquid culture and re-inoculated *Nannochloropsis sp.* cultures. A single isolate was determined to inhibit algal growth, which was later characterized as *Bacillus pumilus*. Bacterial inhibition of algal growth was only observed when marine broth medium was present in the cultures. It was determined that *B. pumilus* culture filtrate was capable of inhibiting *Nannochloropsis sp.*, suggesting an active molecule is released into the culture. Further, *B. pumilus* does not affect the growth of weedy algal genera, *Chlorella vulgaris* and *Tetraselmis striata.* Therefore, *B. pumilus* can significantly alter the algal species composition within algal co-cultures, allowing weedy algae to bloom. Contaminating organisms such as bacteria will often be prevalent in algae systems and understanding their influence on culture productivity is essential for successful large-

scale cultivation of algae. This study is the first to report a bacterium that significantly

detracts from the commercially important genus, *Nannnochloropsis*.

4.1 INTRODUCTION

Photosynthetic microalgae (herein, "algae") are a promising source of bioenergy

with the potential to produce a significant portion of the world's liquid transportation fuel.

Since algae can be cultivated on non-arable land with wastewater or high saline water,

algae represent a feedstock that will not compete with land or water allocated for

traditional food crops. In addition, oil productivity by some microalgae varieties has the

potential to exceed that of terrestrial oil crops [1]. Algal strains that have high oil

productivity combined with other traits suitable for large-scale algae production are

referred to as "elite" strains. A considerable amount of resources have been spent on

discovering, screening, and genetically manipulating elite algae for increased oil content.

Algae growers typically have the goal to cultivate monocultures of elite strains to

relatively high densities in open raceways or closed photobioreactors. In both open and

closed systems, non-elite organisms such as predators, weedy algae, viruses, fungi,

and bacteria enter the systems, posing a major challenge to consistently growing robust,

large-scale elite cultures. These organisms make up complex and variable communities

that can have a neutral, negative, or positive influence on algae productivity [2]. For

example, Rivas et al. isolated and screened bacteria to identify specific bacteria that

influenced growth of the alga *Botryococcus braunii* [3]. They isolated three bacteria,

Rhizobium sp., *Acinetobacter sp.* 1, and *Pseudomonas sp. 1*, that increased, reduced,

and had no influence on *B. braunii* growth rate, respectively. This demonstrates the

diverse impacts specific bacteria can have on algal growth. A major problem for algae

growers is the lack of understanding of growth system ecology, specifically the subset of non-elite organisms that can negatively impact algae productivity [4]. It is unlikely that large-scale algae production will reach commercial viability unless destructive, non-elite organisms are identified and management strategies are developed [5.6].

Bacteria are abundant and diverse in algal production systems, and particular strains of bacteria pose a significant threat to reducing algal productivity [7,8,9], These bacteria are considered to be algicidal because they have the ability to kill or inhibit algal growth. Broadly, there are two scenarios in which bacteria can detract from algal production systems: 1) gradual reduction in productivity over extended periods of time, and 2) sudden impact causing cultures to "crash". Conceptually, bacteria can exert detrimental effects either by directly attaching to algal cells or by releasing algicidal or inhibitory molecules into the surrounding environment [10,11,12,13]. Prior to the recent expansion of the algal bioproducts industry, the majority of the previous bacteria-algae interaction research was done to investigate harmful algal bloom cycles. Therefore, few studies have been performed to understand the relationships between bacteria and elite algae species, especially in the high cell concentration, nutrient-rich conditions that exist in engineered production systems. Since it is well established that bacteria can negatively impact algal growth, it is necessary that researchers isolate bacteria and screen their influence on elite algae performance.

The goal of this work was to determine whether growth-inhibiting bacteria were present in industrial cultures, and to characterize conditions that caused bacteria to impact algae growth. To accomplish this goal, we isolated bacteria from a poorly performing algae production system and screened these bacteria for the capability of

inhibiting *N. gaditana* growth. We identified *Bacillus pumilus* as an inhibiting bacterium and characterized its influence on *Nannochloropsis gaditana, Nannochloropsis salina*, *Chlorella vulgaris* and *Tetraselmis striata* growth.

4.2 MATERIALS AND METHODS

ISOLATION OF BACTERIA

A 10-mL sample was collected from an outdoor 200-L industrial bioreactor in which *N. salina* was growing [14]. A 50-µL aliquot was spread onto a petri dish that contained marine agar (BD, Franklin Lakes, NJ). Phenotypically distinct colonies were isolated onto different marine agar plates and stored at 4°C.

Screening process

Isolated bacterial colonies were picked with sterile pipette tips from the marine agar plates and placed into test tubes containing 6 mL of marine broth. Cultures were grown for 12 h, at which time a 250-µL aliquot of each culture was added to separate 750-µL cultures of *N. gaditana, Tetraselmis striata* and *Chlorella vulgaris* at 0.5 OD_{750} in a 24-well plate (Corning, Corning, NY). All algal cultures were grown in artificial seawater unless stated otherwise. The 24-well plate cultures were grown at room temperature shaking at 120 rpm for 7 d. Daily growth was monitored by eye for cell density and green color (chlorophyll content).

BACTERIUM DNA EXTRACTION, PURIFICATION AND PCR AMPLIFICATION

To characterize bacterial isolates inhibiting *N. gaditana* growth, a colony was picked, added to 6 mL tube of marine broth, and grown for 24 h. Two mL of this culture were pelleted at 12,000 x g. Total DNA was isolated from the flash frozen pellets using liquid nitrogen. Cells were disrupted by shaking in a bead beater (BioSpec Products

Inc.) for three-1 min intervals in the presence of 0.5 mm zirconia/silica beads (Biospec

Products Inc.). Following cell disruption, DNA was isolated using a PowerSoil DNA

Isolation Kit (MO BIO laboratories, Carlsbad CA) according to the manufacturer's

instructions. The concentration of the isolated DNA was determined using a

spectrophotometer (ND-1000 Thermo Scientific) at wavelength ratio 260/280 nm.

Subsequently, 16S rDNA genes were amplified using PCR in 50 μL reactions containing

a final concentration of 10 ng of template DNA, 0.5 μM of each primer forward (5'-

TGGAGAGTTTGATCCTGGCTCAG-3'), and reverse (5'-TACCGCGGCTGCTGGCAC-

3') primers (Integrated DNA Technologies), 1 U High Fidelity Phusion DNA Polymerase

(New England Biolabs), HF buffer and 0.2 mM dNTPs (Fisher Scientific), Thermal

cycling consisted of initial denaturation at 98 °C for 2 min; 40 cycles of denaturation at

98 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; and a final

extension at 72 °C for 10 min. Amplicons were resolved using agarose gel

electrophoresis and visualized following ethidium bromide staining.

SEQUENCING AND ANALYSIS

Purified PCR products were sequenced using ABI BigDye Terminator v3.1

chemistry and an ABI 3130xL Genetic Analyzer at the Colorado State University

Proteomics and Metabolomics Facility. Primers for sequencing were the same primers

used in the PCR reaction. To determine algal strain identity, DNA sequences were

queried against GenBank using BLASTn.

CULTURE CONDITIONS FOR ALGAE AND BATERIA

Liquid cultures of bacterial isolates were grown in marine broth 2216 (BD) at

30°C in 250 mL Erlenmeyer baffled flasks or test tubes shaking at 200 rpm. Marine agar

was used for solid medium (BD). *Nannochloropsis salina* CCMP 1776, *Nannochloropsis gaditana* CCMP 526 (NCMA), *Chlorella vulgaris* (Algae Analytics, Las Cruces, NM), and *Tetraselmis striata* (Ute isolate) were grown in unbuffered artificial seawater medium (ASW) in an incubator of 24 h light at 50 uE, at 21 °C with shaking at 120 rpm [15]. ASW consisted of 15 g L^{-1} NaCl, 1.45 g L^{-1} KNO$_3$, 0.12 g L^{-1} KH$_2$PO$_4$, 0.04 g L^{-1} NaHCO$_3$, 0.01 g L^{-1} FeCl$_3$•6H$_2$O, 0.035 g L^{-1} Na$_2$-EDTA, 0.25 mL of 0.91 mM MnCl$_2$•4H$_2$O, 0.5 g L^{-1} CaCl$_2$•2H2O, 6.6g L^{-1} MgSO$_4$•7H$_2$O, 5.6 g L^{-1} MgCl$_2$•6H$_2$O, and 500 mL trace metals (7.8 g L^{-1} Na$_2$-EDTA, 20 mg L^{-1} CoCl$_2$·6H$_2$O, 12 mg L^{-1} Na$_2$MoO$_4$·2H$_2$O, 44 mg L^{-1} ZnSO$_4$·7H$_2$O, 20 mg L^{-1} CuSO$_4$·5H$_2$O). Parent algae cultures were grown at volumes of 100 mL in 250 mL Erlenmeyer flasks, and inhibition experiments were done with 5 mL of volume in CytoOne T25 flasks (USA scientific, Ocala, FL) or 6mL of volume in 150 mL Erlenmeyer flasks. Marine broth and agar support bacterial growth through the presence of yeast extract and peptone, whereas the algae media, ASW does not contain a carbon source.

ALGAE AND BACTERIA CONCENTRATION

Bacteria and algae growth were monitored using the optical density at 600 nm and 750 nm, respectively; measured with a DU 730 spectrophotometer (Beckman Coulter). A Guava easyCyte HT + flow cytometer (EMD Millipore) equipped with an argon laser (488nm) and 680/30 nm bandpass filter was used to monitor algae cell number based on size and chlorophyll fluorescence.

EVALUATING THE EFFECT OF ADDITIONAL NUTRIENT MIXES, PHOSPHATE, IRON AND PH RANGES FOR B. PUMILUS INHIBITING N. GADITANA GROWTH

N. gaditana cultures were harvested at OD_{750} 0.7 to 1.0, centrifuged at 2000 x g for 10 min, and resuspended in ASW to a final OD_{750} of approximately 0.3-0.5. Four-mL aliquots of resuspended culture were added to each T25 flask. One mL of *B. pumilus* culture were centrifuged at 2000 x g for 5 min and resuspended in separate tubes using marine broth or ASW. Each treatment of 1 mL of *B. pumilus* culture were added to independent *N. gaditana*-containing T25 flasks and algae growth was monitored using OD_{750}. Control cultures lacked *B. pumilus*; instead, 1 mL of marine broth or ASW was added.

To test the influence of higher phosphate and iron concentrations, *B. pumilus* was grown in liquid culture for 12 h, and then cells were pelleted, resuspended in marine broth, and added to *N. gaditana* cultures in T-25 flasks. Subsequently, 0.88 mM of KH_2PO_4 and 0.37 mM of $FeCl_3$-6H20 were added daily to the co-cultures.

To test the effect of pH, *B. pumilus* cells were grown in pure culture in marine broth for 12 h, pelleted and resuspended in marine broth, added to *N. gaditana* cultures, and the pH was adjusted twice daily and maintained at 7 and 10 using concentrated 5M NaOH and 5M HCl. These two values were chosen because they represent the range of typical algae production systems.

EFFECT OF B. PUMILUS GROWTH PHASE AND CELL CONCENTRATION ON N. GADITANA GROWTH

To test for the effects of bacterial growth phase on *N. gaditana* growth, *B. pumilus* was grown for 7, 12, or 24 h, corresponding to log, late log, and stationary

phases, respectively. One mL of *B. pumilus* culture was sampled during each phase, pelleted, resuspended in 1 mL of marine broth and added to 4 mL of freshly resuspended *N. gaditana* culture. Growth was monitored with by OD_{750}.

To determine the concentration of *B. pumilus* cells required for inhibiting *N. gaditana* growth, a 50-mL culture of *B. pumilus* was grown for 12 h. To determine the concentration of cells in this culture, ten-fold serial dilutions were prepared, cells were plated on marine agar plates, incubated for 24 h, and colony-forming units (CFUs) were counted. One ml of these same dilutions were centrifuged, resuspended in 1 mL of marine broth, and added to 4 mL of freshly resuspended *N. gaditana* culture. Growth of the co-cultures was monitored by cell count using flow cytometry.

DETERMINING CELL-FREE BACTERIAL FILTRATE EFFECT ON N. GADITANA GROWTH

B. pumilus was grown in 250-mL baffled flasks for 7 h and 32 h. To obtain cell-free filtrate, cultures were filtered through 0.22 um sterile polyethersulfone (PES) filters. To test the effect of the filtrate, algal cultures were pelleted at 2000 x g for 10 min and the pellets were resuspended in T25 flasks in a total of 5 mL of liquid, which was composed of different ratios of *B. pumilus* filtrate and ASW: 100% ASW, 80 % ASW and 50% ASW. Algal growth was monitored by OD_{750} over 7 d.

DETERMINING THE EFFECT OF B. PUMILUS ON ALGAE COMMUNITY DYNAMICS

Separate, exponentially growing *N. gaditana* and *T. striata* cultures were diluted to OD_{750} = 0.5. These cultures were used to inoculate 150 mL Erlenmeyer flasks in 6 mL portions. Three different algae cultures were tested, consisting of *N. gaditana*, *T. striata*, and a mixture of *N. gaditana* and *T. striata* at 95% and 5% by cell count, respectively.

One set of these cultures were the control, to which 1 mL of marine broth was added, while another set had 1 mL log phase *B. pumilus* resuspended in fresh marine broth. Algal growth was monitored by flow cytometry over 7 d.

4.3 RESULTS AND DISCUSSION

BACTERIAL ISOLATE SCREEN

To determine if any bacteria strains have the potential to inhibit *N. gaditana* growth, we sampled a poorly performing 200-L industrial algal production system that exhibited stagnant growth, flocculation, and foaming. We then isolated bacteria on marine agar from this sample. In total, 20 visually distinct isolates based on phenotype were obtained. These individual isolates were used to inoculate liquid cultures, which were grown for 12 h and in turn used to inoculate cultures of *N. gaditana* in 24-well plates at a 1:4 volumetric ratio (bacteria culture: algae culture). The bacteria-*N. gaditana* co-cultures were grown for 7 d and algae culture health was visually scored by 1) turbidity, indicating cell growth and 2) green color, indicating that the growth included a significant portion of algal cells with chlorophyll. Of the 20 isolates screened, one isolate, S7 appeared to prevent both *N. gaditana* and *N. salina* growth. The co-cultures that contained this bacterial isolate were colorless and contained no visible turbidity, while the co-cultures containing the other 19 isolates were green and had visible turbidity (Fig 15).

To determine the phylogeny of the bacterial isolate S7, an 873-nt portion of the 16S rDNA gene was PCR amplified and sequenced. The resulting nucleotide sequence had 99% (502/506 nt) identity with *Bacillus pumilus* S10 according to BLASTn, and the top five results were *B. pumilus.* To confirm that other *B. pumilus* isolates inhibit *N.*

gaditana and N. salina growth, we obtained two additional *B. pumilus* strains, isolates

C6 and C12, both originally isolated from a rainforest soil in Peru [16]. These strains

were grown in liquid culture, and added to *N. gaditana* and *N. salina* cultures within a

24-well plate. Both strains inhibited *N. gaditana* and *N. salina* growth as determined by

the lack of green color and low turbidity (Fig 15).

Since algae cultures are complex microbial communities, we hypothesized that a

biological contaminant, and specifically a bacterium, was causing the poor algae growth

in the bioreactor. We identified S7 as an inhibitory bacterium using a simple and

efficient 24-well plate screening protocol. While other bacteria may have contributed to

the poor growth of the original *N. salina* culture, we focused on the effects of the *B.*

pumilus isolate on *N. gaditana* growth. To our knowledge, this is the first study where *B.*

pumilus has been isolated and screened against *N. gaditana*.

It is not surprising that *B. pumilus* was present in the algae culture we sampled,

as *B. pumilus* strains are known to be ubiquitous, tolerant of high salinity, and resistant

to oxidizers [17,18]. We isolated *B. pumlius* in a photobioreactor in Fort Collins,

Colorado, under high salt conditions, in which hydrogen peroxide and ozone are used to

sterilize the culture system. Further, it is not unexpected that *B. pumilus* has the

potential to inhibit algae growth, as members of the *Bacillus* genus have been reported

to inhibit the growth of bacteria, fungi and oomycetes [19,20]. In addition, *Bacillus*

strains have been isolated and shown to lyse algae and cyanobacteria under specific

conditions. In a previous study, *Bacillus subtilis* was shown to completely inhibit the

growth of *Microcystis aeruginosa*, a bloom forming cyanobacterium [21]. Upon further

investigation, a surfactin produced by *M. aeruginosa* at 10 mg L^{-}1 was identified as the

inhibitory compound. In another study, *Bacillus sp.* was found to release extracellular molecules that lysed, *M. aeruginosa* and *Chlorella sp.* [9].

EFFECT OF NUTRIENTS, MEDIA AND PH ON BACTERIAL INHIBITION

One series of *B. pumilus-N. gaditana* co-cultures was supplemented with ASW medium, which is used to grow microalgae, while the other series were supplemented with marine broth, which supports bacterial growth and contains yeast extract and peptone. *N. gaditana-B. pumilus* co-cultures with the addition of marine broth decreased in total cell concentration by 27.3% over 7 d, and appeared visually chlorotic. In contrast, the control *N. gaditana* culture with marine broth increased in OD_{750} by 625% over the same time period (Fig 16). The OD_{750} of *N. gaditana-B. pumilus* co-cultures with additional ASW increased by 393%, a similar extent as the control *N. gaditana* culture, which increased by 395% over 7 d.

Our results show that *B. pumilus* does not cause *N. gaditana* growth inhibition with additional ASW, whereas as the addition of marine broth medium causes *B. pumilus* to inhibit *N. gaditana* growth. The major differences between ASW and marine broth supports bacterial growth. Two hypotheses for why marine broth causes *B. pumilus* to inhibit *N. gaditana* are: 1) marine broth supports *B. pumilus* growth to a high density, and each cell releases metabolites that inhibit *N. gaditana* growth, or 2) marine broth contains a chemical that stimulates *B. pumilus* to produce one or more compounds that inhibit *N. gaditana* growth. Importantly, *B. pumilus* does not always inhibit *N. gaditana*, but requires one or more components of marine broth to exert this effect. To test whether iron or phosphate affected this bacterial-algal interaction, we added excess iron or phosphate daily to *N. gaditana-B. pumilus* co-cultures. The

addition of excess iron showed had no influence on reducing the inhibition of *N. gaditana* growth. Whereas, after 3 d, *N. gaditana-B. pumilus* co-cultures that were fed phosphate increased by an average of 67%. The *N. gaditana* control cultures that were fed phosphate grew much more, with cell density increases of an average of 345%.

Phosphorous and iron are two of the frequently limiting nutrients in aquatic ecosystems. For example, Guerrini et al. demonstrated that bacteria were more effective than algae at scavenging for phosphate and thus bacteria caused reduced algae growth in phosphate-limited conditions [22]. Merrell et al. determined that iron regulates the virulence of some bacteria [23]. Therefore, we hypothesized that phosphorus and iron might have an impact on the *B. pumilus-N. gaditana* relationship. Our results demonstrate that *B. pumilus* significantly inhibits *N. gaditana* growth with additional iron, and with base levels, while the addition of excess phosphate led to only minimal increases in *N. gaditana* growth. This suggests that phosphate or iron competition is not the cause of *B. pumilus* inhibiting *N. gaditana* (Sup 1).

We determined the role pH has on *B. pumilus* inhibition activity on *N. gaditana* by culturing the algae in the presence and absence of bacterium at pH 7 or 10. At both pH 7 and 10, *N. gaditana-B. pumilus* co-cultures that contained *B. pumilus* cells did not grow significantly by 3 d. As controls, *N. gaditana* cultures that lacked *B. pumilus* increased in cell density by an average of 280% and 380% for pH 7 and 10, respectively.

pH is another factor that can strongly affect microbial-host interactions by directly altering cellular structure, affecting cell-cell interactions, altering cellular physiology by changing gene expression, and inducing specific virulence genes [24]. In a previous study, *Bacillus sp*. caused flocculation of *Nannochloropsis sp*. and the effect was

dependent on pH [25]. We chose to study pH 7 and 10 because this is the common range for industrial growth systems. However the *B. pumilus* strain isolated in this study caused *N. gaditana* growth inhibition at both pH 7 and 10 (Sup 3).

EFFECT OF B. PUMILUS GROWTH PHASE AND CELL CONCENTRATION

We tested the ability of *B. pumilus* during log, late log, and stationary phase to inhibit *N. gaditana* growth (Sup 2). The *N. gaditana* control culture with marine broth grew by 480%. In contrast, *N. gaditana* cultures where *B. pumilus* cells were resuspended in marine broth showed significant growth inhibition with OD_{750} of cultures changing on average by -27.3%, 22.7% and 48.6% for log, late log and stationary phase growth, respectively (Fig 16).

Bacteria use quorum-sensing molecules to coordinate growth during log, late log, and stationary growth phases [26]. During these growth phases, bacterial cells display different physiological characteristics. For example, Mitsutani et al. determined that the bacterium *Pseudoalteromonas* sp. could lyse the alga *Skeletonema costatum* in 2 d, but only when the bacteria was in stationary growth phase [27]. Further, using two-dimensional electrophoresis they identified proteins in the stationary phase that were not observed in late-logarithmic phase, suggesting that one or more of these proteins in stationary phase may lyse the alga. Our results suggest that *B. pumilus* cells in log, late log, and stationary phases inhibit *N.* gaditana when supplemented with marine broth. Of these, the *B. pumilus* in log phase caused the most severe inhibition effect.

To determine the influence *B. pumilus* cell concentration had on *N. gaditana*, different concentrations of *B. pumilus* in marine broth were added to *N. gaditana* cultures with an initial concentration of 2.7 x 10^7 *N. gaditana* cells/mL, as measured by

flow cytometry (Fig 17). *N. gaditana* growth was only inhibited by the highest concentration (2 x 10^9 cells/mL) of *B. pumilus*. After 8 d, *N. gaditana* cell concentration decreased by 90%. Conversely, *N. gaditana* cultures with *B. pumilus* cell concentrations of 2 x 10^8, 2 x 10^7 and 2 x 10^6 cells/mL increased on average by 3450%, 2620%, 3820%, respectively (Fig 17).

Bacterial cell concentrations can be a major factor for inducing the secretion of secondary metabolites that can inhibit algae. Paul and Pohnert determined that the bacterium *Kordia algicida* released a protease that lysed *Phaeodactylum tricornutum, Thalassiorsira weissflogii*, and *Skeletonema costatum*, but the protease was only released when the cell density was above a specific cell concentration, suggesting that quorum sensing played a role [28]. We hypothesized that cell concentration would be an important factor for inhibiting *N. gaditana* growth, which our results demonstrate to be correct. No influence was observed on *N. gaditana* growth if the *B. pumilus* cell concentration was 2 x 10^8 cells/mL or less. The ratio of cells might be important for this interaction. For example, we saw inhibition of *N. gaditana* when the ratio was 1:100 (*N. gaditana*: *B. pumilus).* Previous studies reported *Bacillus* inhibiting other strains of algae growth at a ratio of 1:65 (bacteria: algae) [30].

EFFECT OF B. PUMILUS CELL-FREE FILTRATES ON N. GADITANA

Since *B. pumilus* is grown in marine broth and *N. gaditana* is grown in ASW, the *B. pumilus* filtrate from log phase and stationary phase was mixed with fresh ASW at ratios of 1:0 (undiluted), 1:1 and 1:4, and used to resuspend *N. gaditana* cells. Filtrate from *B. pumilus* log phase cultures did not significantly affect *N. gaditana* growth in mixtures of 1:1 and 1:4. However, significant *N. gaditana* growth inhibition was

observed for the 1:0 ratio by 7 d, with an average OD_{750} decrease of 26% compared to control (Fig 18). Filtrate from *B. pumilus* stationary phase cultures significantly inhibited *N. gaditana* for the three ratio treatments. By 7 d, the 1:0, 1:1, and 1:4 treatments had OD_{750} values by 414%, 364% and 44% lower than the controls, respectively.

The majority of known algicidal bacteria inhibit algae growth by releasing molecules into the local aquatic environment [31,32]. Our results suggest that *B. pumilus* releases a *N. gaditana*-inhibiting molecule during stationary phase, and only minimally during exponential phase. It is possible that *B. pumilus* only releases the inhibitory molecule in stationary phase, or that *B. pumilus* always releases the molecule but the cells are not concentrated enough in exponential phase to inhibit *N. gaditana* growth. It is interesting that the inhibitory molecule in stationary phase inhibits *N. gaditana* growth through 4 d, but that by 8 d *N. gaditana* began to increase in cell concentration (OD_{750}). This is in contrast to when the *B. pumilus* cells are physically present in the *N. gaditana* culture, where the algae growth is completely inhibited through 7 d. Two hypotheses for this are 1) the effect of the molecule in the filtrate is concentration dependent and is limited in its ability to kill all cells, and thus some algal cells survive and continue to be viable, and 2) the inhibitory molecules are labile and degrade over the cultivation period. Few previous studies have characterized algae-inhibiting molecules. However, some researchers are starting to use high performance liquid chromatography and mass spectrometry to identify molecules released by *Bacillus* sp. that inhibit algae growth [32] Future work could use -omics methods to identify genes, and proteins active in producing inhibiting molecules, or directly identify the inhibiting molecules.

DETERMINING B. PUMILUS IMPACT ON ALGAE COMMUNITY DYNAMICS

We evaluated whether *B. pumilus* activity is species-specific by testing its influence of the common weedy algae, *T. striata* and *C. vulgaris*. After 7 d, both cultures of algae were green with increased turbidity, indicating they were not significantly inhibited (Fig 15). To determine the impact *B. pumilus* has on algae growth and dynamics in a co-culture of *N. gaditana-T. striata*, we inoculated co-cultures with 95% *N. gaditana* cells and 5% *T. striata* cells as determined by flow cytometry. To determine *B. pumilus* inhibitory influence, we grew the co-cultures in the presence and absence of the bacterial cells. In the algae co-cultures with *B. pumilus*, the *N. gaditana* concentration decreased from 95% to 10% by 7 d. In the same co-cultures, the *T. striata* cell concentration increased from 5% to 90% (Fig 19). In contrast, the series of algal co-cultures that lacked *B. pumilus* started with 95% *N. gaditana* cells and 5% *T. striata* remained unchanged in population composition by 7 d.

Bacteria-algae interactions are known to be species-specific. In some instances, specific bacteria have been found to lyse a specific algae species, but have no influence on the growth of other algae species. Wang et al. determined that *Pseudomonas aeruginosa* releases a biosurfactant that lyses *Heterosigma akashiwo*, but does not inhibit *Gymnodinium* sp [32]. In our study, we determined that *B. pumilus* specifically inhibits *N. gaditana* and does not influence *T.striata* growth, and thus the presence of *B. pumilus* can alter the dynamics in a co-culture of these algae. To simulate a common situation observed in production systems, we inoculated a culture with *N. gadiata* and *T. striata* at 95% and 5% respectively. In the absence of *B. pumilus, N. gadiatana* remained the dominant algae throughout the study. However, by adding *B. pumilus* to

this co-culture, *T. striata* became the dominant algae. This example confirms that bacterial activity can significantly alter of the algal community composition.

PRACTICAL APPLICATIONS

Much of the success of conventional agriculture is due to the considerable development and accumulation of knowledge of crop pests to help inform management strategies. In comparison, the nascent algae bioproducts industry lacks known pests and established pest management strategies. In many instances, algae cultures will crash, and the causative organisms are never identified [2]. In the absence of appropriate knowledge the pest organisms cannot be monitored and management strategies are not developed.

Identifying a pest bacterium was a major aim of this study, while we will never be able to discern whether *B. pumilus* played a role in the unhealthy bioreactor that we initially sampled. In future studies, researchers should continue to search for bacteria that are detrimental to elite algae growth by isolating and screening bacteria from diverse algae cultures and environmental samples. However, less than 1% of bacteria are cultivable, making it difficult to isolate the majority of bacteria [33]. In the future, researchers will study microbial communities in algae production systems using non-culture based methods, such as metagenomics, which can be used to comprehensively characterize the bacterial communities using DNA sequencing technologies [34]. The metagenomics approach is promising and necessary, but often leads to correlations, making it difficult to determine distinct algae-bacteria interactions. Using isolates of bacteria allows for direct studies to conclusively understand the biochemical

mechanisms involved in algae growth promotion or inhibition of algae-bacteria cultures [35, 4, 36].

Combining isolation methods and next-generation sequencing methods will assist in building an extensive catalog of pest organisms so that diagnostics can be developed and used for monitoring pests in the field [5]. Further, understanding the environmental conditions that induce algicidal behavior from pests will be significant. For example, events such as lysed algae cells that release dissolved organic carbon (DOC) could cause pathogenic bacterial bloom and therefore cause a decline in algae performance. If this were the case, monitoring the relative abundance of bacteria in parallel with system conditions such DOC may be critical for culture management.

Our results demonstrate that bacteria can inhibit elite algae and contribute to shifts in algae community composition. This demonstrates that algae cultures are complex microbial communities in which microbes can form symbiotic, mutualistic, and pathogenic relationships with elite algae. For example, there are microbes in algae production systems that promote algae growth. Specifically, some bacteria provide vitamin B or iron to algae [37, 38]. For this reason, using non-targeted treatments that kill the majority of bacteria is not appropriate since some species are critical for algae growth. By understanding these communities better, researchers will have the potential to build and control stable and productive ecosystems by exploiting growth promoting bacteria and reducing pathogens influence.

Another strategy to overcome pests is to grow polycultures, which have been shown to be more productive and resilient than monocultures [39]. In a monoculture of *N. gaditana, B. pumilus* can significantly reduce culture productivity over days. With

several elite strains growing, there is an increased chance that some of the elite algae

species will be unaffected by the pest bacterium and the system productivity could

remain steady.

4.4 CONCLUSION

Contaminating organisms will always be present in algae production systems and

some may negatively impact elite algae growth. The first step for culture management

requires the isolation and identification of pest organisms that inhibit or kill elite algae. In

this study, we isolated bacteria from a poorly performing industrial 200-L algae

production system and identified a single isolate, *B. pumilus*, as detrimental to the

growth of the elite algae *N. gaditana and N. salina*. Subsequently, we determined that *B.*

pumilus releases a molecule that causes *Nannochloropsis sp*. growth inhibition. To our

knowledge, this is the first study to isolate a bacterium from an industrial algae

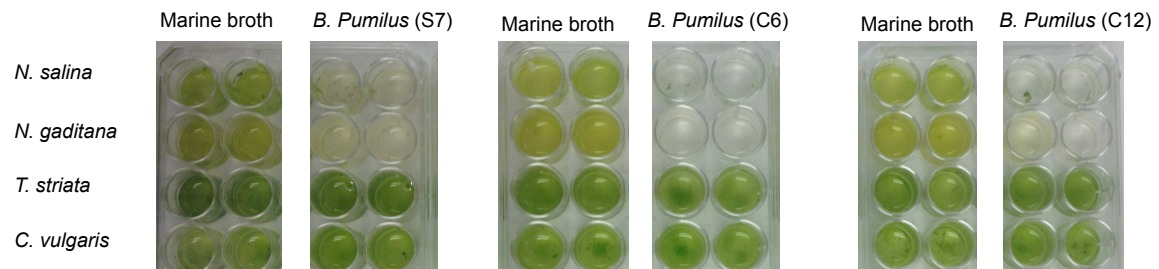production system and demonstrate its ability to inhibit *N. gaditana* and *N. salina* growth.

Figure 15. 24-well plate screening protocol where the wells with *B. pumilus* inhibited *N. salina* and *N. gaditana*, which was determined by the lack of chlorolphyll color and turbidity. In contrast, *T. striata* and *C. vulgaris* both increased in chlorophyll color and turbidity in the presence of *B. pumilus*.
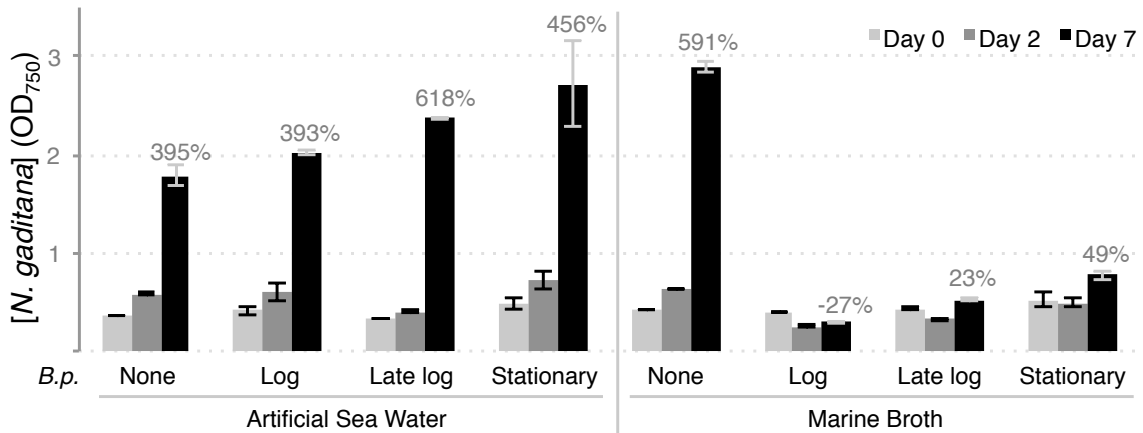
Figure 16. *N. gaditana* growth in the presence (+Bp) or absence (-Bp) of *B. pumilus*, and tested with two different media supplements, marine broth and ASW. The *B. pumilus* cells that were used to inoculate *N. gaditana* were in different growth phases including log, late log, and stationary. *B. pumilus* in log phase with marine broth demonstrated the most inhibition activity.
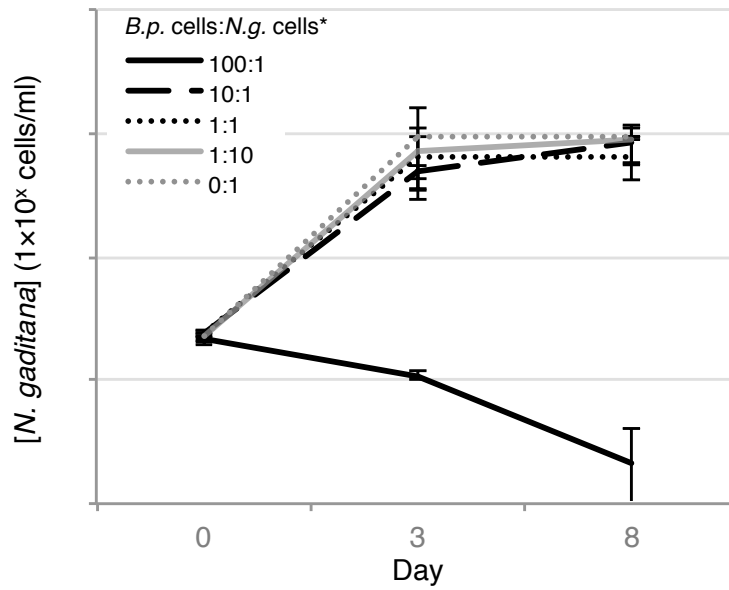
114

Figure 17. Different concentrations of *B. pumilus* were added to *N. gaditana* cultures and *N. gaditana* cells/mL were evaluated with flow cytometry. The highest concentration of *B. pumilus* ($2e^9$) was the only concentration that inhibited *N. gaditana* growth.
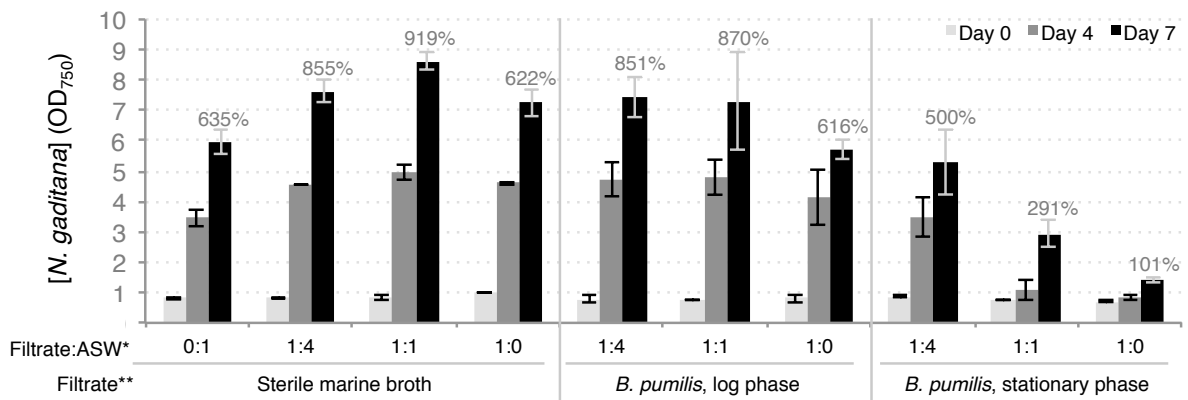
Figure 18. *N. gaditana* was grown in mixtures of cell-free *B. pumilus* filtrate and ASW. The *B. pumilus* filtrate was generated from log phase (7 h) and stationary (32 h) cultures. Filtrate from stationary phase cultures at 1:0 and 1:1 ratios demonstrated the most severe *N. gadtiana* growth inhibition.
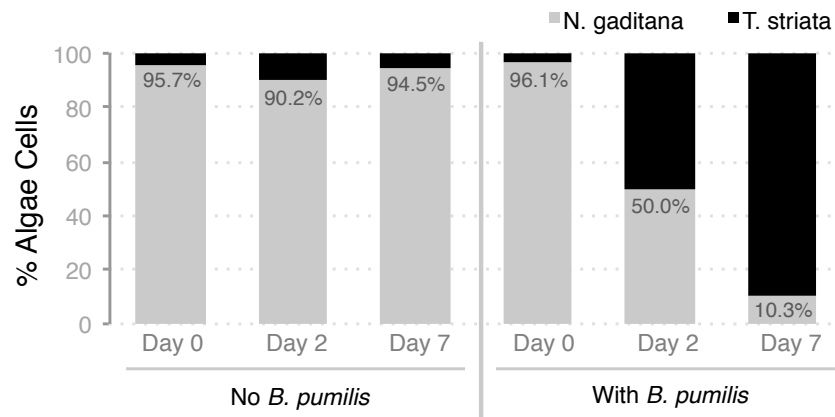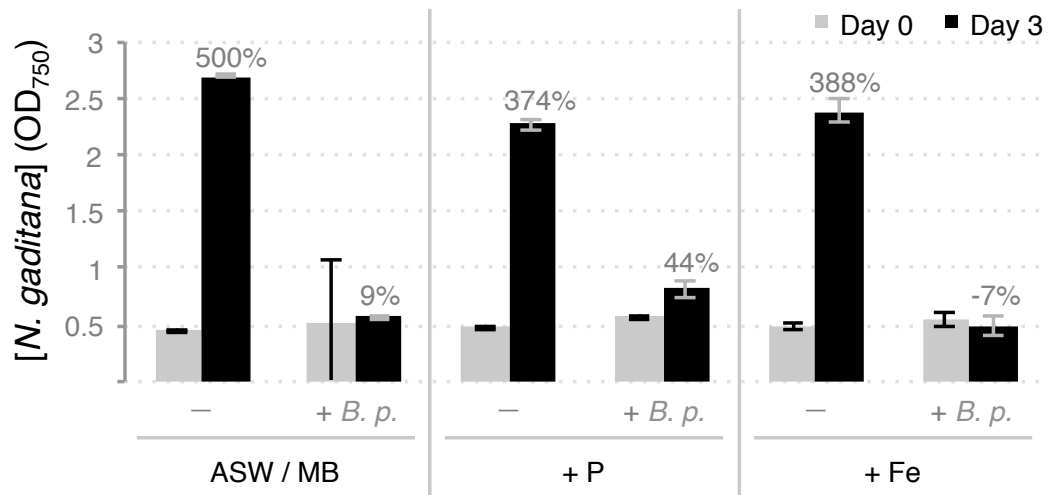
Figure 19. Co-cultures of elite algae, *N. gaditana* (green) and weedy algae (orange), *T. striata* were grown in the absence and presence of *B. pumilus*. A) In the absence of *B. pumilus*, *N. gaditana* remains above 90% of total algae cells. B) In the presence of *B. pumilus*, *N. gaditana* decreased from 95% of total algae cells on day 0 to less than 10% by day 7.
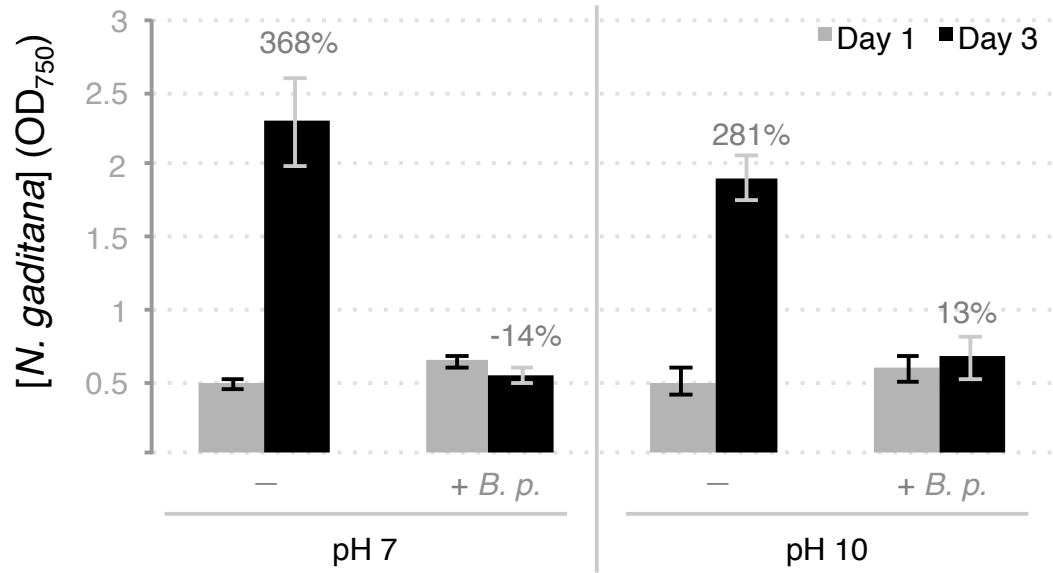
Supplemental Figure 1. *N. gaditana* grown with supplemental phosphate and iron, and in presence(+Bp) and absence (-Bp) of *B. pumilus*. Compared to the controls, *B. pumilus* significantly inhibits *N. gaditana* growth even with the additional nutrients.
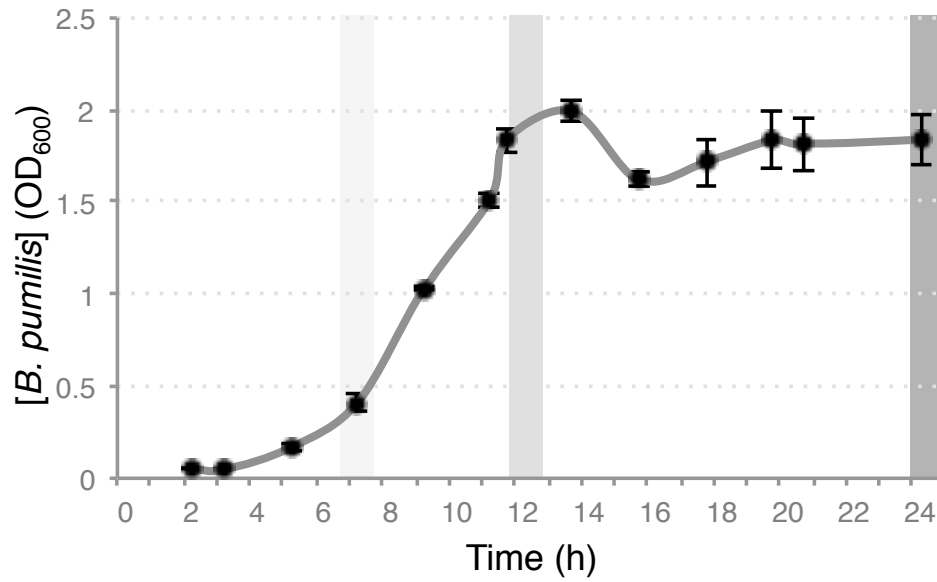
Supplemental Figure 2. Growth of *B. pumilus* in liquid culture that monitored was monitored for 24 hours. The medium used was marine broth. Vertical shadings represent where bacteria cultures were sampled for experiments.

Supplemental Figures 3. *N. gaditana* grown at pH 7 and pH 10 in the (+Bp) presence and absence (-Bp) of *B. pumilus. B. pumilus* caused inhibition of *N. gaditana* at both pH values.

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CHAPTER 5

CONCULSIONS AND FUTURE PERSPECTIVES

Growing algae for bioproducts and biofuels has a promising future, but will require significant advances in cultivation to reach commercial scale. The body of work presented in this dissertation represents incremental advancements in developing crop management strategies in industrial algae systems.

Weedy algae are ubiquitous in the environment and therefore a common challenge is reducing their presence in algae production systems. The first step in mitigating weedy algae impact is detecting their presence and relative abundance. In Chapter 2, we highlight the challenge of maintaining monocultures due to invasion of weedy algae with a low oil content. We developed a quick and simple non-quantitative PCR-based assay using CAPS markers to differentiate algae strains. This method should be adopted by the industry as a routine quality control test to ensure that the expected elite algae strain is the dominant strain. This inexpensive upstream assay could save a significant amount of time and money for algae researchers. Future work should combine more than one restriction enzyme, which will increase the total number of cut sites and increase the probability of differentiating strains in a culture that contains complex algal populations. In addition, T-RFLP (terminal restriction fragment length polymorphism) should be used to profile algae communities by detecting specific terminal fragments using a DNA sequencer. T-RFLP has a greater potential for higher throughput assays than using CAPS markers (1). To improve T-RFLP methods, high-throughput sequencing can be used to initially characterize the weedy algae present in

cultures. A database can than be developed that contains the 18S rRNA gene of common weeds, and thereafter T-RFLP data can be used to characterize diversity while determining the identities and relative abundances of weedy algae in cultures.

We also developed *quantitative* PCR methods to monitor weedy algae abundance in algae systems (Chapter 2). This method is 10^4 times more sensitive than flow cytometry, which is commonly used in the algae farming industry. Using the qPCR method we developed, algae growers can monitor weedy algae and make informed culture management decisions. However, this method requires weedy algae to have been characterized before primers can be designed. Future work for monitoring weedy algae requires comprehensive knowledge of all weedy algae strains present in a culture. After weedy algae are identified, specific diagnostics can be developed to track them. Using 18S rRNA primers for PCR and sequencing amplicons is the future of characterizing algal populations in complex cultures. An additional method to identify all weedy algae could be performed using a sorting flow cytometer, or a microfluidics device that can physically separate and bin cells based on size, shape, pigment types and content. All binned species would require DNA extracted and the 18S rRNA gene would be characterized. Once the gene sequence is confirmed probes can be designed for each weedy algae strain, including fluorescence in situ hybridization (FISH) probes. Subsequently, FISH probes can be combined with flow cytometry. Thus, a culture sample could be analyzed with a flow cytometer and the probes would allow specific strains to be monitored and characterized with high confidence. An additional advantage to physically separating the cells in the first step is that the cells remain viable. Thus, binned cells can be used to inoculate individual cultures and future studies

such as growth characteristics, lipid content or sensitivity to culture management strategies can be studied.

To improve molecular diagnostics such as qPCR primers, other loci besides the 18S rRNA gene should be used. For example, the large ribosomal subunit including the 28S rRNA gene, internal transcribed spacers between rRNA genes, and RuBisCo (*rbcL*) genes should be used. These genes may provide more variability compared to the 18S rRNA gene, thus providing a greater capacity to differentiate algal strains. Depending on the variability of these non-18S rRNA genes among organisms, multiplex PCR might be possible so that multiple primers can amplify more than one algal strain in a single PCR reaction, significantly reducing time and resources.

Algal growers producing biofuels have historically targeted production of lipids that are converted into biodiesel. However, recent improvements in downstream processing technologies such as hydrothermal liquefaction (HTL) have been developed that require biomass regardless of the lipid content. HTL uses high pressure and temperature to convert biomass into biocrude oil (2). This method allows researchers to focus on growing biomass rather than specific strains of elite algae, and thus weedy algae contamination would not be a significant concern.

Eventually, next-generation shotgun sequencing will be done to comprehensively characterize all organisms including weedy algae, predators, fungi, and bacteria. This method will also characterize entire genomes of organisms present in cultures, thus capturing genes of specific organisms will allow researchers to better understand community interactions. A trade-off with this method is that much of the findings are

correlative, where the genetic foundation of the community is elucidated; however, the physical interactions among organisms in complex cultures can be difficult to tease apart using shotgun sequencing methods.

Chapter 3 begins to lay a foundation of understanding of bacterial communities in algae production systems. The major challenge for algae cultivation is scaling to large volumes. Small-scale cultivation, in the laboratory under aseptic conditions, is typically easy and consistent growth rates are observed. However, algae cultivated in large systems are not grown in aseptic conditions, and thus unwanted organisms enter the cultures. Organisms such as bacteria are thought to play a major role in algal growth system productivity. In Chapter 3, we characterized bacterial communities during the scale-up process of an industrial algae system. We sequenced the 16S rDNA genes in those communities, and analyzed the data to compare the bacterial communities among systems. Results confirmed that as the scale of the system increases, the bacterial richness in terms of number of different types of bacteria identified increases, demonstrating that bacterial communities in small, medium, and large cultures are significantly different. This is a significant finding because many researchers perform experiments at lab scale, and extrapolate these results to estimate large-scale productivity. Future work should include isolating bacterial communities from different size systems and use them to inoculate axenic strains of elite algae. This will provide support to determine the effect bacterial communities from each size system have on elite algae growth.

In Chapter 3, we demonstrate that specific bacteria, including *Spirobacillales*, were present in abnormally low growth elite algae cultures. We did not determine if this

bacterium caused lower growth, or was an effect of slow algal growth. Isolating bacteria such as this *Spirobacillales* to perform one-on-one studies to clarify the effects bacteria have on algae growth should be a high priority. One-on-one studies could be done in combination with proteomics, metabolomics, and transcriptomics analyses to characterize the functional mechanisms that cause inhibition or promotion of algal growth. Similar to conventional crops such as corn and soybean, bacteria should be isolated and tested against elite algae stains. Understanding these interactions and mechanisms would provide information that would assist algae cultivation. Results should be placed into a database for algae growers and researchers to use for developing pest management strategies.

In our study, we found that *Proteobacteria* and *Baceteroidetes* were the most dominant phyla in all size cultures. Within *Bacteroidetes* was a class of bacteria, *Saprospirales* that was present in every sample, and therefore is considered to be a highly associated bacterium to *N. salina* in the algal production systems we sampled. This finding suggests that this bacterium could potentially have functions that support algae viability or productivity. Future work should attempt to isolate this strain of bacteria and add it back to algae cultures at various concentrations to determine if it affects growth rate or stability of algae. Experiments could determine if relative abundance of this particular bacterium plays a role in culture productivity. It would be interesting to clarify the *Saprospirales* interaction with other algae species, such as weedy algae. To better understand *Saprospirales* association with *N. salina*, bacterial communities `from *N. salina* cultures from culture collections and other production systems from around the world should be investigated. This would determine if this bacterium is always present

with this elite strain of algae, or if our finding was algae growth system specific. To do this, a qPCR primer or probe set could be designed to track *Saprospirales*.

Future work should follow the 16S rRNA protocols established in our work, to continue to sample and analyze bacterial communities in algal production systems. Ideally, projects will closely monitor environmental parameters closely such as pH, temperature, length of day and elite algae growth rate. This monitoring should be carried out over years to see if there are seasonal differences, and strengthen correlations of the role specific bacteria have on production. Throughout these experiments samples can be obtained and preserved in glycol, or used instantly to inoculate axenic strains of elite algae. This will enable clear conclusions of the effect bacteria have on algae growth. These co-cultures could be grown in incubators that can simulate various times of the year including length of day, light intensity, and temperature. For example, a sample obtained from a poorly growing algae culture could be used to inoculate an axenic culture that would allow researchers to determine if bacterial community played a role in poor growth. This same concept can be used to identify bacterial communities causing higher than normal elite algae growth. Upon finding bacteria or bacterial communities that impact algae growth, communities can be applied to crop management strategies to optimize large-scale algae cultivation.

In Chapter 4, we sampled a poorly performing algae system and isolated bacteria to test if inhibiting bacteria were present in the culture. We identified *B. pumilus* as inhibitory for the elite algae, *N. gaditana* and *N. salina*, but not inhibitory for the weedy algae, *C. vulgaris* and *T. striata*. We determine that marine broth, a medium that supports bacterial growth, is required for the inhibitory *B. pumilus* activity. Further, we

determined that *B. pumilus* filtrate inhibits *N. gaditana* and thus conclude that a molecule released by the bacterium confers the inhibition. Since *B. pumilus* has species-specific algal inhibitory activity, we revealed that it could manipulate the composition of algae populations. The presence of *B. pumilus* significantly inhibits the elite algae, *N. gaditana* and has no effect on the weedy algae, *T. striata*, and thus the weedy algae becomes dominant with in days of *B. pumilus* inoculation. In this work, we established that bacteria are pests that can significantly reduce productivity of algal growth systems and should be taken into consideration by algae growers.

Future work to improve detrimental bacteria-algae interactions should involve understanding molecules that inhibit algae. A next step for understanding the behavior of *B. pumilus* in algal cultivations could use mutagenesis techniques to create mutations in the bacterium's genome, and then use the high throughout algae screening method to identify whether any of the *B. pumilus* mutants lost the ability to inhibit *N. salina*. When such a mutant bacterium is identified, the genome could be sequenced and compared to the original (inhibitory) *B. pumilus* genome. This would be the first step in identifying the gene networks involved in releasing molecules that inhibit *N. salina*. Beyond genomics, researchers should use high performance liquid chromatography and mass spectrometry to identify molecules released from the mutant *B. pumilus* compared to the *B. pumilus* that inhibits *N. salina*.

A promising future direction for algae cultivation is to use a probiotic strategy to increase elite algae growth rate and stability. Bacterial pathogens could be mitigated by identifying growth-promoting bacteria to culture independently and supplemented into large-scale algae cultures. Additionally, Jousset et al. discovered that by increasing the

genetic diversity of a bacterial community within the rhizosphere of plants, pathogens could be kept away (Jousett et al. 2011). The hypothesis is that certain bacteria can directly inhibit pathogenic bacteria. Also, a diverse bacterial community limits the amount of available nutrients to pathogens thereby reducing their ability to thrive in the culture. Understanding the ideal genetic makeup of a bacterial community could allow algae growers to inoculate bacterial communities with algae, and supplement during episodes of poor algae growth. Our work demonstrated the potential of increasing bacterial biodiversity to overcome the inhibitory effect of *B. pumilus*. In a preliminary experiment, we grew naturally occurring bacterial communities from local soils, ponds, and rivers in marine broth bacterial liquid media, and added these communities to *B. pumilus-N. gaditana* cultures. Preliminary results show that several of these communities could "protect" *N. gaditana* from *B. pumilus*. Further work is necessary to understand which bacterial communities protect *N. gadiana*, and the mechanisms involved.

Another intriguing avenue to explore is artificially selecting for bacterial communities that stimulate elite algae growth. Historically, researchers have used artificial selection to select for specific properties from individual organisms (Swenson). This same concept applied at the community level. For example, hundreds of algae cultures can be grown and the top five cultures are combined, and this is repeated hundreds of times. At the end of the experiment, the community that supports algae growth could be selected for and applied to large-scale cultivation. During this experiment it would be interesting to characterize and compare the starting and final bacterial communities.

Naturally occurring bacteria can be used to build growth-enhancing communities, and specific bacteria can be engineered to promote algae growth. For example, *Azotobacter vinelandii* was genetically modified to secrete ammonium to supplement the media and allow the algae to grow (3). This example demonstrates that bacteria in the future of algae cultivation could play a major role in feeding and protecting elite algae.

In summary, this work established molecular tools and protocols for characterizing weedy algae and bacteria within algae cultures. We demonstrate for the fist time that small, medium, and large cultures are comprised of distinctly different bacterial communities, which must be taken into consideration when pursuing research and development. Using our protocols, controlled experiments can be done to better understand how bacterial communities influence algae performance. We demonstrate the usefulness of isolating bacteria and screening their influence on elite algae growth, which should be performed often by industrial algae growers to identify novel pests. Since there are few identified pests of elite algae, crop protection strategies are currently difficult to develop. However, as researchers identify pests, strategies will be developed allowing for an increased chance of successful algae cultivation at large-scale where biofuels and bioproducts will be prevalent.

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