Bacterial community changes in an industrial algae production system

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SUMMARY

While microalgae are a promising feedstock for production of fuels and other chemicals, a challenge for the algal bioproducts 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. To overcome bacterial contamination, algae growers may use closed photobioreactors designed to reduce the number of contaminant organisms. Even with closed systems, bacteria are known to enter and cohabitate, but little is known about these communities. Therefore, the richness, structure, and composition of bacterial communities were characterized in closed photobioreactor cultivations of *Nannochloropsis salina* in F/2 medium at different scales, across nine months spanning late summer – early spring, and during a sequence of serially inoculated cultivations. Using *16S rRNA* sequence data from 275 samples, bacterial communities in small, medium, and large cultures were shown to be significantly different. Larger systems contained richer bacterial communities compared to smaller systems. Relationships between bacterial communities and algae growth were complex. On one hand, blooms of a specific bacterial type were observed in three abnormal, poorly performing replicate cultivations, while on the other, notable changes in the bacterial community structures were observed in a series of serial large-scale batch cultivations that had similar growth rates. 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 important information for crop protection in algae systems, and demonstrates the complex ecosystems that need to be understood for consistent, successful industrial algae cultivation. This is the first study to profile bacterial communities during the scale-up process of industrial algae systems.

INTRODUCTION

Microalgae (herein, “algae”) are photosynthetic unicellular eukaryotes that grow in aquatic or marine environments. For reasons including rapid growth and high lipid content, certain varieties of algae are considered promising biofuels feedstocks (Chen et al., 2011; Mata et al., 2010). 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 (Shurin et al., 2013). 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 confined in bags or tubes that reduce exposure to the environment. Closed systems have higher capital costs but allow greater control over parameters such as CO_2 and nutrient concentrations while limiting the potential for invasion by unwanted organisms (Grobbelaar, 2009; Slade and Bauen, 2013).

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) (Shurin et al., 2013). 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% (Griffiths et al., 2012; Rodolfi et al., 2009). Algae growth parameters are often studied and optimized using laboratory conditions including small-volume cultures, aseptic conditions, and precisely controlled light, temperature and nutrient regimes. Since elite algae have not historically been grown at the large volumes required by the biofuels industry (Fishman et al., 2010), a challenge is translating the productivity of elite strains optimized under highly controlled lab environments to consistent outdoor culture productivity at large scales.

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 (Fulbright et al., 2014). Zooplankton grazers prey on small algae (Smith and Crews, 2014) such as *Nannochloropsis*. Fungi and bacteria also affect algae productivity (Smith and Crews, 2014; Lakaniemi et al., 2012); however, there is little understanding of the interactions among elite algae and 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 (Letcher et al., 2013,

Fulbright et al., 2016).

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 (Wang et al., 2016). Although bacteria are often considered contaminants that can inhibit algae productivity or terminate algae populations, bacteria-algae interactions have a range of potential outcomes (Lakaniemi et al., 2012; Skerrat et al., 2002; Lee et al., 2000; Mayali and Azam, 2004). Algae support bacterial growth by releasing 25% of the total organic carbon fixed by photosynthesis (Rooney-Varga et al., 2005; Lakaniemi et al., 2012). Reciprocally, of hundreds of algae varieties surveyed, over half do not endogenously produce vitamin B12 and therefore require bacteria-produced vitamin B12 for growth (Croft et al., 2005). Additionally, specific bacteria may stimulate algae growth through activities including regulation of the amount of available nutrients such as iron, nitrogen, and phosphates (Amin et al., 2009; Foster et al., 2011; Reijnders, 2008), or by releasing phytohormones such as indole-3-acetic acid into the growth environment (De-Bashan et al., 2008). In some instances, bacteria reduce algae productivity by competing for these same nutrients (Cole, 1982; Kazamia et al., 2012). In addition to nutrient competition, non-lethal bacterial pathogens may inhibit algae productivity by diverting algal cellular resources from growth to defense. Finally, some bacteria can directly kill algae, causing cultures to collapse (Wang et al., 2012; Lewin, 1997). 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 bioproducts 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 200-L cultures grown in closed, but not aseptic, photobioreactors. 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, including sterilized glassware and media, with all handling of open containers occurring in a laminar flow hood. These small cultures are grown under artificial light sources in shaking incubators or on shaking platforms. Medium cultures (20 to 60 L) are grown in flat-panel bioreactors under ambient light in a greenhouse, whereas large cultures (200 L) are grown in closed photobioreactors in an outdoor water basin under natural light. Though medium and large cultivations are grown in closed systems, 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.

It was hypothesized that bacterial communities would differ across growth system scales, across seasonal changes in environmental conditions, and in algae cultivations exhibiting different algae growth rates. To monitor bacterial communities in these *N. salina* cultivation systems, 275 samples were collected from small, medium, and large cultivations over 244 days. 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 contained 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-scale cultivations supporting different algae growth rates. Relationships between bacterial community structure and algae growth rates were evaluated.

MATERIALS AND METHODS

Algae growth systems

All samples were collected from cultivations of *Nannochloropsis salina* at a single growth facility operated by 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 (Quinn et al., 2012). To scale up the culture volume (Fig. 1A), 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 (Fulbright et al., 2014). 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 and not all systems were sampled on every sampling date, but the overall system was sampled at least once per calendar month from July, 2011 to March, 2012. Whenever a particular system scale was sampled, between 2 – 16 samples were isolated from cultivations within that growth scale. 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 (Fulbright et al., 2014).

Extraction and sequencing of nucleic acids

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/) (Caporaso et al., 2010). 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. Amplification reactions were done in triplicate, and PCR reaction products were pooled prior to sequencing 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.

Data processing

QIIME version 1.8.0 was used for all sequence analyses (Caporaso et al., 2010). 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 (McDonald et al., 2012). Since community DNA extracted from archived samples included 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 [27]. 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 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.

Comparisons of communities across system scales included 17, 81, and 177 samples from small, medium and large cultures, respectively**.** The QIIME pipeline was used to identify and count OTUs, to compare relative abundances across scales, and to contrast phylogenetic composition of samples. To ensure even representation across system scales, 1000 amplicon sequences were randomly selected and analyzed for every sample. Using PYNAST, amplicon sequences that met a 97% similarity threshold were clustered together as an OTU (Caporaso, 2010), total numbers of OTUs were quantified, and relative abundance of each OTU was determined for each sample. To summarize the data by system size, relative abundances were averaged for all samples within each scale, resulting in a single relative abundance for each system. To calculate phylogenetic diversity represented within each sample, Faith's Phylogentic Diversity was used (Faith et al. 1992, Peiffer et al., 2013). Essentially, this measures diversity by adding up all the branch lengths of OTUs found in

samples. UniFrac was used to further clarify relationships between samples and systems. UniFrac takes taxa in each sample and places them on a phylogenetic tree. The phylogenetic trees produced from each sample are compared in a pairwise fashion. Taxa found in both samples are considered “shared”, whereas taxa found only in one sample are considered “unshared” (Lozupone & Knight, 2005). The fraction of unshared branch lengths relative to total branch lengths is used as a summary statistic for comparisons (Lozupone et al. 2007). To compare samples with principal coordinates analysis (PCoA), a multivariate statistical test principle coordinate analysis was used (Lozupone et al. 2011, Kuczynski et al., 2011). Computations were done on the Pando supercomputer. Data were deposited in the European Bioinformatics Institute with accession number ERP010414.

For comparisons that only involved samples from large-scale cultivations, 2740 amplicon sequences were randomly selected and analyzed for each sample. Further aspects of these community analyses were done as described above. To compare communities in outdoor, large-scale cultivations across nine months (Fig. 1D), 177 samples were used. Analysis of five large-scale cultivations inoculated using the serial batch strategy (Fig. 2) included five biological replicates (derived from a single inoculum source) per sampling date, with two slower growing cultivations being sampled on two dates each, resulting in comparison of 35 samples. Finally, the comparison of communities in healthy and stagnant large-scale cultivations (Fig. 3) included 13 healthy replicates and 3 stagnant replicates, all derived from the same batch inoculum.

RESULTS AND DISCUSSION

Bacterial communities differed across cultivation scales

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) (Fig. 1A). 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.

Bacterial communities were characterized in small, medium, and large industrial cultivations of *N. salina* algae. The composition of these bacterial communities was compared across all samples using unweighted UniFrac as a distance metric. In the PCoA plot in Fig. 1B, the distance separating sample points represents differences among bacterial communities, measured as the fraction of evolutionary history in a phylogenetic tree that is unique to one of the samples (Peiffer et al, 2013). Three primary clusters were observed, corresponding to samples from small, medium, and large growth scales (Fig. 1B). Thus, algae cultivations at different scales contained bacterial communities that were distinct in terms of phylogenetic structure.

Since manipulations of small-scale cultures were done under sterile conditions, it is probable that bacterial communities in these cultures represent bacteria that were associated with the initial algal inoculum or introduced to stock cultivations in an early stage of sub-culturing. Beyond those initial cultures, 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. Some of these factors would directly influence bacteria (e.g., temperature management), while others (e.g., the ratio of surface area to volume, light source intensity, illumination period) have impacts on the growth of *N. salina*, which in turn would influence bacterial growth.

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 (Figs. 1A & 1C); 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 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.

The bacterial community structures in different algae growth systems were analyzed. At the phylum level, *Bacteroidetes* and *Proteobacteria* dominated communities from all system scales (Table 1). The total abundance of *Bacteroidetes* and *Proteobacteria* was constant across all systems, respectively comprising 91.8%, 89.9%, and 90.6% of bacteria in small, medium, and large cultivations. Considering all samples, *Bacteroidetes* increased in relative abundance as system scale increased, from 48.5% abundance in small-scale cultivations to 63.3% in medium-scale and 70.7% in large-scale growth environments (Table 1). *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. *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 (Kazamia et al., 2012). This finding is also consistent with results of previous studies of *Nannochloropsis* laboratory cultivations (Wang et al., 2016). Within both *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 (Tables 1 and S1).

Within each cultivation system size, bacteria were ranked by relative abundance and rankings were compared across systems (Tables 2 and S2). The 10 orders most abundant in small systems accounted for 94.9% of the bacterial communities (Table 2A). All ten of these orders were also identified in medium and large systems, although they totaled only 74.0% and 75.4% of the respective bacterial populations at these larger scales (Table 2A). In large systems, the ten most abundant bacterial orders represented, on average, 87.2% of the bacterial community (Table 2B). All these large-system OTUs were identified in medium systems, but four were not identified in small systems, indicating these OTUs may have entered the growth environment during non-sterile handling. With respect to specific orders, small systems had much higher abundance of *Flavobacteriales* and *Rhizobiales* than was observed at larger scales. Conversely, medium and large systems contained a higher relative abundance of *Cytophagales* than small systems.

Since the handling of cultures at medium and large scales was not sterile, each handling was an

opportunity for bacteria and other microbes to enter the community and increase species richness and phylogenetic diversity. Average species richness within each growth scale was compared using OTU counts (Fig. S1). Overall, species richness increased as the size of the system increased. Small cultures averaged 88.0 ± 8.1 OTUs (N = 17), medium cultures contained 108 ± 22.8 OTUs (N = 81), and large cultures contained 132 ± 19 OTUs (N = 177). Furthermore, an increase in diversity across growth scales was also observed when assessed using phylogenetic distance (Fig. S2), which quantifies diversity based on total branch length of bacterial *16S rRNA* phylogeny represented in a sample (Peiffer et al., 2013).

Bacteria prevalent in *N. salina* cultivations

To determine which bacteria were associated with *N. salina* across the majority of culture conditions, OTUs were identified that were present in 90%, 95%, or 100% of all samples (at least 0.01% abundance). There were 16 OTUs detected in at least 90% of cultivations (Table 3). These OTUs together averaged 63% of the relative abundance of bacterial communities across all systems. Of these, seven OTUs were identified in at least 95% of samples. Together, these seven OTUs were present at 47% relative abundance across all samples. A single OTU was present in 100% of samples (Table 3). This OTU is of the phylum *Bacteroidetes*, and is identified as *Saprospiraceae* at the family level. However, its classification at the Class and Order levels ([*Saprospirae*] and [*Saprospirales*]) is disputed within the Greengenes reference database (DeSantis et al., 2006). In addition to being in every sample, *Saprospiraceae* was the most abundant OTU on average, comprising 34.7% ± 14.3% of bacterial communities, and its average abundance increased in larger growth systems (Table 2B). *Saprospiraceae* abundance varied in individual samples from 0.3 – 67.0%, with the lowest and highest abundances both observed in large cultivations. Of 275 cultivations profiled, only 16 bacterial communities contained less than 5% *Saprospiraceae*. No correlation was observed between *Saprospiraceae* abundance and *N. salina* growth performance. Nonetheless, the presence of *Saprospiraceae* in every sample suggests that there are important interactions between this bacterium and *N. salina*, and makes *Saprospiraceae* a clear candidate for further study. While the activity of *Saprospiraceae* in this system is unknown, a strain of *Saprospirales* was shown to be capable of lysing the microalgae diatom *Chaetoceros ceratosporum* (Gou et al., 2003).

In a previous study of bacteria associated with *Nannochloropsis oceanica* algae (Wang et al., 2012), several bacteria were isolated with taxonomy similar to bacteria prevalent in *N. salina* cultivations (Table S3). Members of the genus *Marinobacter*; the families *Cytophagaceae*, *Phyllobacteriaceae*, *Hyphomonadaceae*, and *Erythrobacteraceae*; and the orders *Flavobacteriales*, *Oceanospirillales*, *Planctomycetales*, and *Pseudomonadales* were identified in *N. salina* and *N. oceanica* cultures

(Tables 3 and S3). Association of these bacteria with both *N. salina* and *N. oceanica* in distinct environments suggests these bacterial types may have specific relationships with *Nannochloropsis* species in general.

In experiments involving a third species of *Nannochloropsis* algae, bacteria from laboratory *N. gaditana* cultures were plated on marine agar, and a representative of the family *Phyllobacteriaceae* was recovered (SPF, KFR, unpublished). Since *Phyllobacteriaceae* was also identified in 95% of samples in this study and two *Phyllobacteriaceae* members were isolated from *N. oceanica* cultures (Wang, et al. 2012), there may be an 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 (Grant et al., 2014). Separately, *Mesorhizobium* was shown to be one of several nitrogen-fixing species associated with growth promotion of four different green algae (Kim et al., 2014).

Although there are limited studies related to influences of bacteria on *N. salina* health, general ecological activities are known for some of the bacteria that were common across systems. The second most abundant bacterial order in larger systems was *Cytophagales*, of the class *Cytophagia*. "Cytophagia" roughly translates to "eats cells" and, like *Saprospirales*, members of *Cytophagia* are capable of lysing a variety of microalgae (Cole, 1982). The combined abundance of *Saprospirales* and *Cytophagales* averaged 50.9% and 59.6% in medium and large systems, respectively, whereas they totaled only 28.2% of the average bacterial communities in small cultivations (Table 2B). Given their common association with healthy *N. salina* cultivations, potential lytic activities of these bacterial orders may relate to desired processes like nutrient recycling. As such, they might represent "neutral" community members rather than pathogenic or otherwise negative organisms. Considering bacteria that might positively contribute to algal growth, four distinct bacterial families within the order *Rhizobiales* were conserved across 95% of sampled *N. salina* cultivations. Members of *Rhizobiales* are known to fix nitrogen and increase the growth of algae (Carney et al., 2014; Kim et al., 2014). In one instance, a *Rhizobium sp.* increased the growth of the algae *Botryococcus brauni* by 50% compared an axenic algal culture (Rivas et al., 2010). In addition, a *Mesorizobium sp.*, a type of *Rhizobiales*, was found to provide vitamin B12 to algae (Grant et al., 2014). As such, members of the order *Rhizobiales* represent bacteria that algae producers might use to supplement growth media in order to maximize algal productivity. Another way to improve algal productivity would be to minimize the level of bacteria that have strictly negative impacts on algae. Bacteria of the order *Sphingobacteriales* can cause flocculation of some microalgae (Lee et al., 2000).

Sphingobacteriales was found in over 90% of all samples in this study and was one of the ten most abundant Orders in medium and large cultivations (Tables S1 and 2B), but was not detected in small-scale cultivations. Since small-scale cultivations typically lacked *Sphingobacteriales*, the bacteria could be added to laboratory-scale *N. salina* cultures to determine whether it has a direct negative impact on algal productivity.

Relationships among bacterial communities in replicate 200-L *N. salina* cultivations grown using the serial batch inoculation strategy

In large cultivations grown using a serial batch inoculation strategy (Fig. 1C), a series of replicate 200-L cultivations are simultaneously inoculated from a single source. Once cultivations are mature, biomass from healthy panels is harvested in a batch. The majority of the harvest is used toward product, while a fraction is diluted as inoculum for the next batch of replicate 200-L panels. In addition to algal biomass, this inoculum includes bacteria present in the preceding batch harvest.

To reveal relationships among bacterial communities in large cultivations spanning 9 calendar months (July 2011 – March 2012), unconstrained, unweighted UniFrac was used as a distance metric. In the resulting PCoA plot (Fig. 1D), points representing bacterial communities sampled on a single day form a cluster; this is expected since such points represent bacterial communities from replicate algal cultivations. Furthermore, the overall arrangement of communities within the plot roughly corresponds with time from the start of monitoring (white data points, see legend) to the conclusion (purple data points). Presumably, a major factor governing this progression of data points relates directly to the serial batch inoculation strategy, specifically the inherent relatedness between the bacterial community in one batch at harvest and the community in the subsequent batch at inoculation. Since these large cultivations were grown outdoors, additional factors (e.g., day length) inherently varied along with the progression of time.

To further examine the relationship among bacterial communities in serially inoculated large-scale cultivations, five consecutive *N. salina* batches were characterized in more detail. These cultivations spanned 77 days from July – October 2011. Five replicate 200-L cultivations from each batch were characterized. These large-scale batches were grown outdoors under sunlight, and later batches grew more slowly than earlier ones as the day length shortened (Fig. 2A & 2C). For this reason, replicates in the first three batches were sampled only once, while the final batches were each sampled on two dates, meaning a total of 35 bacterial communities are represented. All batches in this analysis exhibited generally healthy growth, assessed visually by density and green color, and confirmed by OD_{750} (Fig. 2A). Relative abundances of the 16 OTU identified in at least 90% of all 275 samples in this study (Fig. 2B and Table 3) are shown as a stacked bar graph (Fig. 2C, middle

panel), while their normalized abundances are represented in a heat map (Fig. 2C, bottom panel). Generally, bacterial communities from the five replicates sampled on a particular day appear similar to each other, even though individual replicates may have had different rates of increase in algal density over the entire batch growth period (Fig. 2C, “Total Increase”) or in the approximately 24 h period prior to sampling (Fig. 2C, “24h Increase”). An exception to this generalization is data from the second sampling date of *N. salina* batch 938 (Fig. 2C, “938-B”). The bacterial communities in these five replicates appear distinct from one another when compared by relative or normalized OTU abundances. However, the algal growth rates of the replicates are broadly similar to one another, whether calculated across the entire batch growth period or for the 24 h preceding sampling. Four of these replicates have elevated levels of the *Sphingobacteriales*, which is known to induce flocculation of some algae (Zhou et al., 2015). The data from the fifth replicate (denoted with an asterisk in Fig. 2C) indicate an extremely high amount of *Saprospirales* and a complete absence any of the other OTU conserved across 90% of samples in this study. As this culture replicate had no aberrant growth phenotypes and grew at a rate similar to the other four replicates presented, these statistics likely result from a sample handling or processing error that resulted in incorrect data and do not accurately indicate bacterial abundances in this cultivation.

As noted above, algae in the batches and individual replicates represented in Fig. 2 appeared generally healthy and algal growth rates were within the producer’s expectations. Nonetheless, average growth rates of batches varied approximately twofold across the course of the experiment (Fig. 2C, “Growth/d”). Since those batches spanned July – October, external factors such as day length presumably strongly influenced growth rates and, therefore, limited the ability to identify relationships between bacterial communities and algal productivity. To maximize the potential to identify bacterial community members that affect productivity, 16 replicate 200-L *N. salina* cultivations were analyzed (Fig. 3). In the algae batch presented in Fig. 3, three replicates exhibited stagnant growth, while the remaining 13 were generally healthy, though the growth rates of the healthy replicates varied twofold (Fig 3A, top). When comparing average OTU abundances in the 13 healthy and 3 stagnant cultivations, stagnant cultivations have elevated relative abundances of *Spirobacillales* (Fig 3A, upper heat map). Abundances of OTUs in the 13 healthy and 3 stagnant replicates were also normalized to their abundances in the 35 healthy serial batch communities in Fig. 2A – 2C (Fig. 3A, lower heat map). This reveals that in the healthy and stagnant cultivations in Fig. 3, levels of *Spirobacillales* are, on average, respectively elevated to 11.3× and 133× their abundance in the serial batches. Therefore, it is possible that the batch of inoculum used to start these 16 replicate cultivations already contained an elevated level of *Spirobacillales* and that conditions in the stagnant cultivations lead to its dominance of those communities. *Spirobacillales* is generally uncharacterized, and 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.

Implications for industrial algal cultivations

As demonstrated in this study, bacteria were abundant in closed phototrophic algal production systems, and differences in community composition were found across growth conditions. Since all samples characterized in this study were obtained from a single facility, some members of the associated bacterial communities may be unique to this particular growth environment or geographic location. Growers at different locations might observe distinct populations of common bacteria. To identify bacteria or bacterial functions required for (or detrimental to) efficient *Nannochloropsis* growth across multiple environments, future studies could include cultivations grown in different cultivation systems and from around the world.

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 associations 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. Although complete evaluation of the influences of the bacteria in these cultures was beyond the scope of this study, a limited study demonstrated that at least one isolate had the potential to have a detrimental impact on *N. salina*. 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* 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 (Fig. 3). 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 sterile 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 major shifts in the composition 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 (Kazamia et al., 2012). 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. These targeted efforts will be facilitated by isolation and cultivation of highly conserved bacterial strains or, conversely, by removal of bacterial types from algal cultures through use of antibiotics or dilution strategies. Additional molecular procedures – such as monitoring algal growth rates, transcriptomes, and proteomes – can be used to define the effects of these bacteria on algal phenotypes.

SUPPLEMENTAL INFORMATION

Supplemental Information consists of additional data presented in three tables and two 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.

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STATEMENTS REGARDING CONFLICTS, CONSENT, AND HUMAN/ANIMAL RIGHTS

No conflicts, informed consent, human or animal rights applicable.

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Figure captions

Figure 1. Overview of bacterial communities in *N. salina* growth systems.

(A & B) Bacterial communities differ across growth scales

(A) Serial batch inoculation strategy of *N. salina* cultivation systems contrasted with a progressive inoculation strategy. Growth systems are categorized as small, medium or large as illustrated and further described in the text. Shading of system sizes corresponds to coloring in Fig. 1B. Arrow intensities indicate relative movement of inoculum biomass within and between systems.

(B) Principal 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.

(C & D) Relationships among bacterial communities in replicate 200-L *N. salina* cultivations grown in outdoor systems using a serial batch inoculation strategy

(C) Schematic representation of serial batch inoculation strategy for 200-L cultivations in large growth systems. Algae were grown to maturity in individual 200-L cultivations before being harvested in batch. The majority of the harvest was used for product extraction. A portion of the batch harvest was diluted and used to inoculate subsequent replicate panels of algae cultivation.

(D) Principal coordinates analysis plot showing 135 bacterial communities isolated from large *N. salina* growth systems during a 9-month period. Each point represents the bacterial community in a single sample. Day, day relative to start of experiment; Month, month of sampling day; #, number of large system samples analyzed

Figure 2. Analysis of five serially inoculated batches of replicate *N. salina* 200-L cultivations. Each batch was cultivated between 13 and 21 days before being harvested and used to inoculate the next batch of panels. In total, these batches spanned 77 days. For each sample day, data are presented for five replicate cultivations.

(A) Average algae growth. *N. salina* density was monitored using OD_{750}. Green line indicates OD_{750} averaged across five replicate cultivations in each batch; light gray bars indicate one standard deviation. Day, day of batch harvest / inoculation relative to start of experiment; Batch, batch number; Time, length of batch cultivation (in days). Dotted orange vertical lines indicate sampling days; batches 938 and 949 were each sampled twice. Intensity of green shading near base of orange lines represents relative algae density at sampling, as in Fig. 2C. Error bars represent ± 1 standard deviation.

(B) Consensus taxonomy for 16 OTUs conserved among at least 90% of the 275 samples in this study, listed in order of relative abundance in the 35 samples in Fig. 2C. ID, indicates relative rank of abundance and color-coding for bar chart and heat map in Fig. 2C. Ph, Phylum (see Table 1). %, indicates OTUs found in 90%, 95% or 100% of the 275 samples in the entire study; gray and dark gray shading highlight OTUs conserved among 95% and 100% of communities, respectively.

(C) Relative bacterial abundance in replicates of sequentially inoculated large cultivations. Batches are labeled at the top and bottom, and are separated by dotted light-green vertical lines. Order and color-coding of OTU corresponds to Fig. 2B. Top, “Cultivations”: Growth metrics for cultivations in sampled panels. Batch Day, days since inoculation; [Bacteria], cells / mL of bacteria on sampling

date, as measured using flow cytometry; [Algae], OD_{750} at sampling; Growth / d, ΔOD_{750} averaged across 5 panels and normalized to days of batch growth (Fig. 2A); Total Increase, percent increase in OD_{750} for each cultivation at harvest relative to inoculation; 24h Increase, percent increase in ΔOD_{750} for each panel during approximately 24 h preceding sampling. Heat map for "24h Increase" is formatted separately within each batch; other heat maps are formatted across all batches. Middle, "OTU Relative Abundance (%)": stacked bar graph showing relative abundances; OTU order and color-coding correspond to Fig. 2B; white bars represent the category "Other" (top of stack, regardless of abundance). Bottom, "OTU Abundance, Normalized to Average": ID, rank and color-coding correspond to Fig. 2B; [OTU], average OTU abundance across the 35 represented cultivations; a heat map is formatted within the [OTU] column (white = 0; orange = maximum). Remaining columns indicate OTU abundance in the sampled cultivation relative to the average abundance (i.e., [OTU]). To highlight OTU variability across cultivations and batches, heat maps are formatted separately for each OTU (blue = minimum; white = 1; yellow = maximum). Asterisk denotes community profiling data from a replicate of Batch 938 for which the statistics likely result from a sample handling or processing error and do not reflect the actual bacterial abundances in this cultivation.

Figure 3. Bacterial communities in healthy and stagnant replicate large cultivations of *N. salina*

(A) Abundance of bacterial OTU in 16 replicate 200-L *N. salina* cultivations. Panels are labeled at far left. "Δ[Algae]": algae growth rates (ΔOD_{750nm}) for replicate cultivations are ranked from highest to lowest (left to right); light-green dashed vertical bar separates 13 healthy replicates from 3 replicates with stagnant growth; dark-green dashed vertical bar separates values for individual replicates from average values for healthy (+) and stagnant (-) cultivations. OTU Relative Abundance: stacked bar chart showing relative abundances of the 16 OTU present in at least 90% of samples in this study; these OTU are stacked based on average abundance in the 13 healthy replicates, from most to least abundant; all other OTU are represented at the top of the bar chart as single category (Other, O); consensus taxonomy and color-coding is given in Fig. 3B. "OTU Abundance, Normalized to (+)": abundances were normalized to average abundances in the 13 healthy (+) replicate cultivations included in this figure; a single heat map is formatted across this panel (blue = minimum value, white = 1, yellow = highest value); column to left of heat map corresponds to consensus taxonomy in Fig. 3B. "OTU Abundance, Normalized to Serial Batches": abundances were normalized to average abundances in the 35 communities shown in Fig. 2; heat map formatted as above.

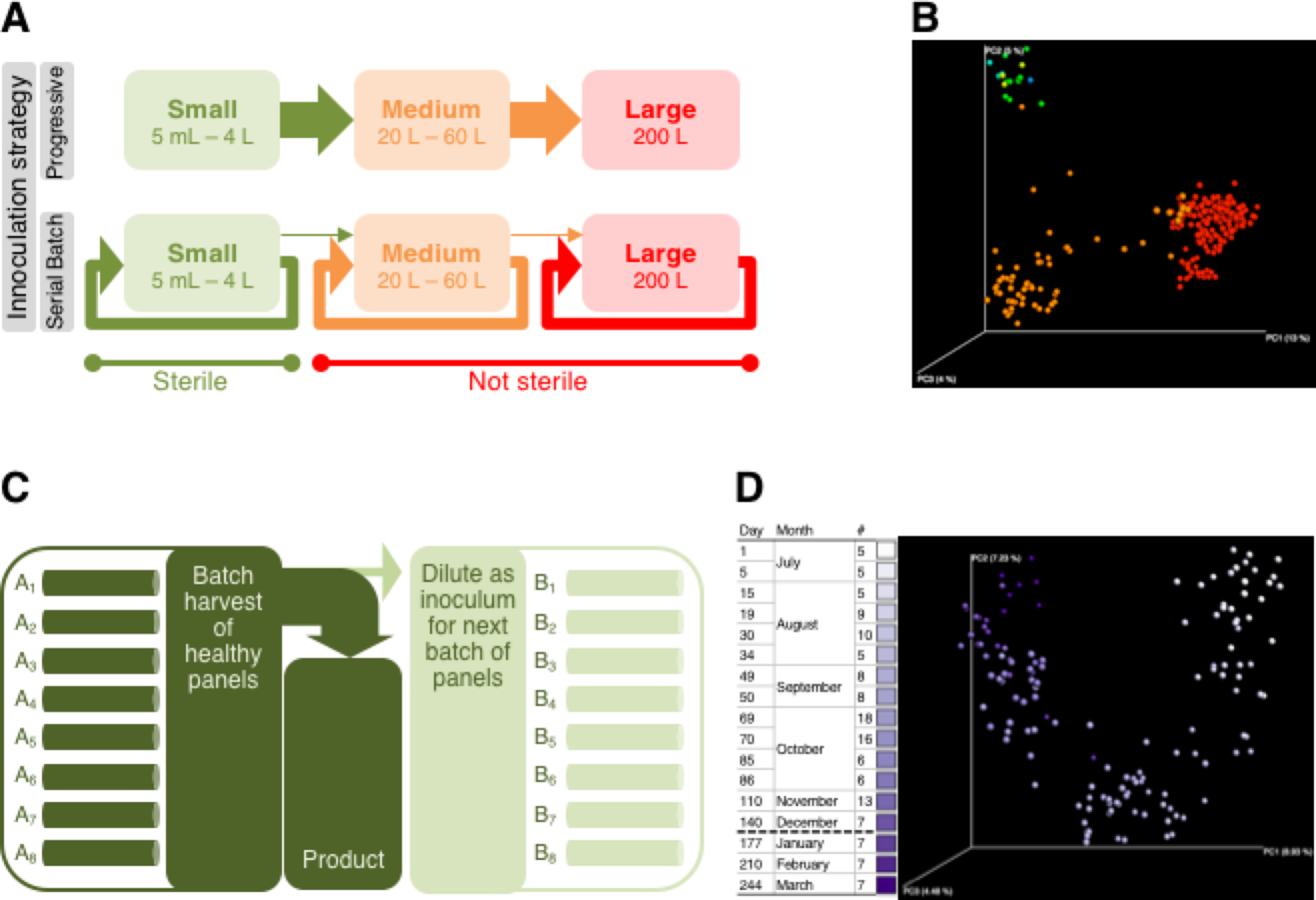
(B) Comparison of OTU abundances in replicate healthy and stagnant cultivations in Fig. 3A with average abundances in serial batch cultivations in Fig. 2. Consensus taxonomy is given for 16 OTU conserved in at least 90% of samples in this study; OTU are listed by average abundance in healthy (+) cultivations shown in Fig. 3A. Ph, phylum (see Table 1); Cl, class: A, Alphaproteobacteria; C, Cytophagia; D, Deltaproteobacteria; G, Gammaproteobacteria; Sp, Sphingobacteria; S, [Saprospirae]. ID: rank abundance for each of the 16 OTU in healthy cultivations shown in Fig. 3A (+), and in 35 communities included in Fig. 2 (SB); color-coding for (+) corresponds to Fig. 3A. %: Indicates OTU found in 90%, 95% or 100% of the 275 samples in this study; gray and dark gray shading indicate OTU conserved among 95% and 100% of communities, respectively. $[OTU]_{AVG}$: Average relative abundance for OTU in 13 healthy (+) and 3 stagnant (-) replicate cultivations in Fig. 3A, plus 35 communities analyzed in Fig. 2 (SB); heat maps are formatted separately within each column (lowest value = white; highest value = orange).

Table captions

Table 1. *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 Tables. 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; $\Sigma_{(B+Pr)}$ (bottom) indicates total abundance of *Bacteroidetes* and *Proteobacteria* in each system.

Table 2 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 Table 1). Heat maps are formatted separately for each growth system as in Table 1. Σ, total abundance of these ten orders in each system. $\Sigma_{(Saprospirales + Cytophagales)}$, combined abundance of the orders *Saprospirales* and *Cytophagales* in each system. $\Sigma_{(Cytophagales + Flavobacteriales)}$, combined abundance of the orders *Cytophagales* and *Flavobacteriales* in each system.

Table 3. 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 Table 1).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Medium
20 L – 60 L
Large
200 L
Serial Batch
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5 mL – 4 L
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Not sterile
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Batch harvest of healthy panels
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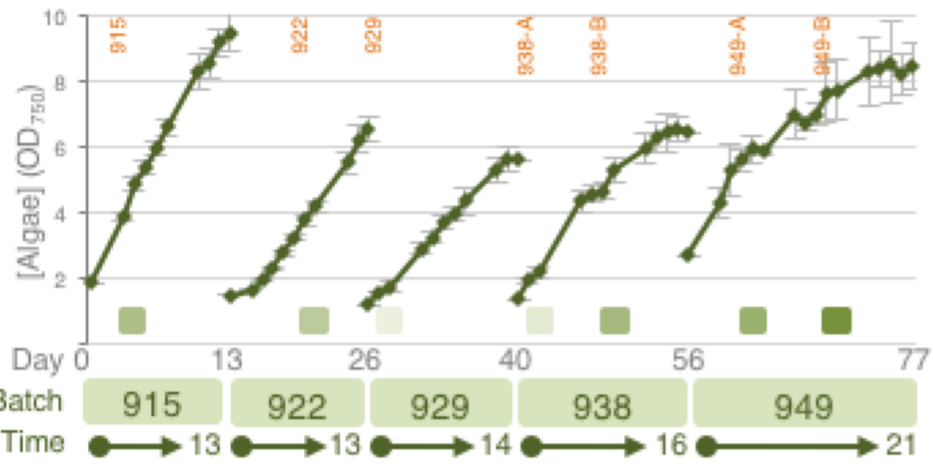
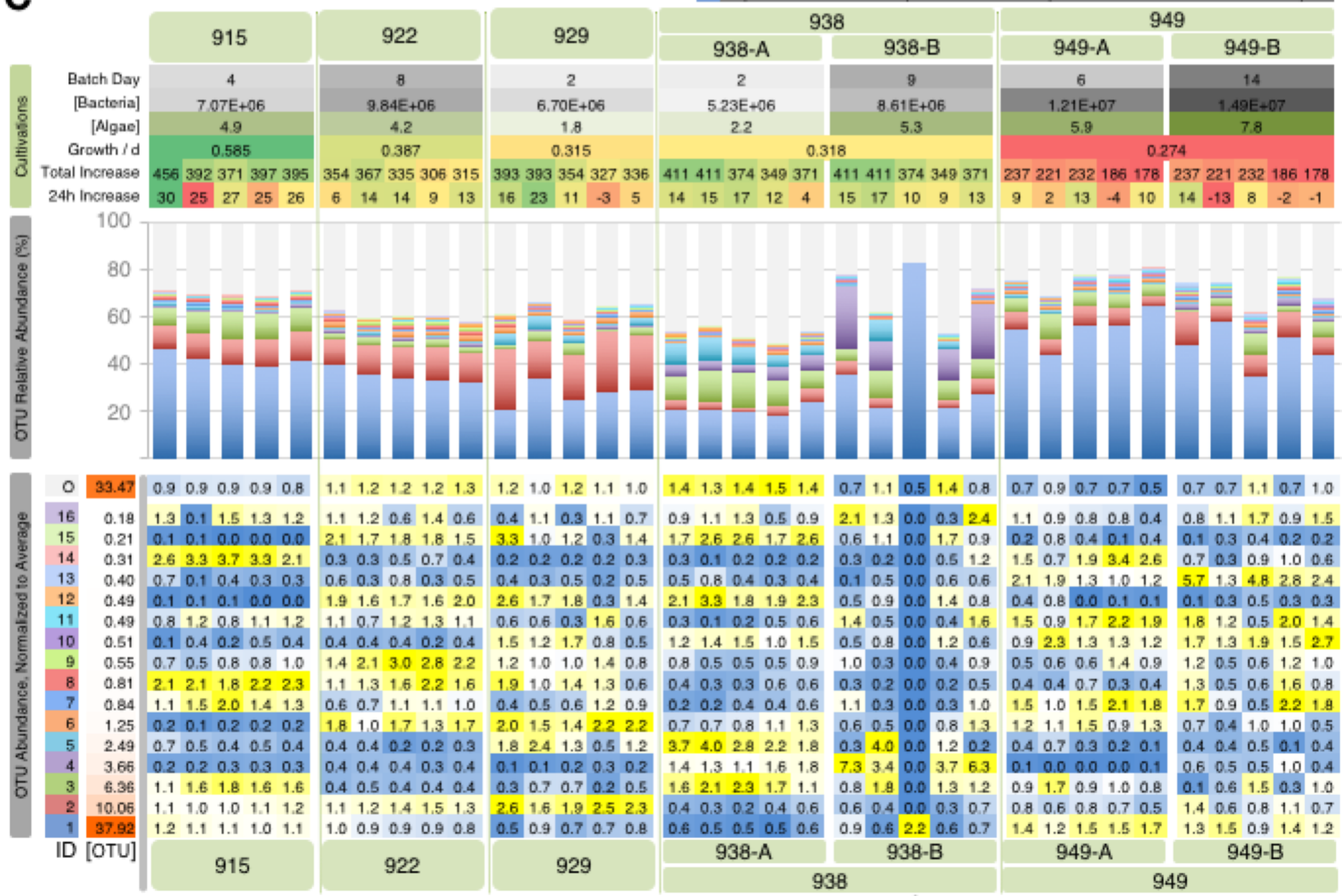
A



B

ID	Ph	Class	Order	Family	Genus	%
16	Unassigned (NR142)					90
15	Pr	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae (NR284)		90
14	Pr	Deltaproteobacteria	Spirobacillales			90
13	B	[Saprospirae]	[Saprospirales]	Chitinophagaceae		90
12	Pr	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae (95991)		90
11	Unassigned (NR178)					95
10	Pr	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae (917635)		95
9	Pr	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae		90
8	Pr	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae		95
7	Unassigned (NR242)					95
6	Pr	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Parvibaculum	95
5	Pr	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	Marinobacter	90
4	B	Sphingobacteriia	Sphingobacteriales			90
3	B	Cytophagia	Cytophagales	Cyclobacteriaceae		90
2	B	Cytophagia	Cytophagales	Cytophagaceae	Leadbetterella	95
1	B	[Saprospirae]	[Saprospirales]	Saprospiraceae		100

C



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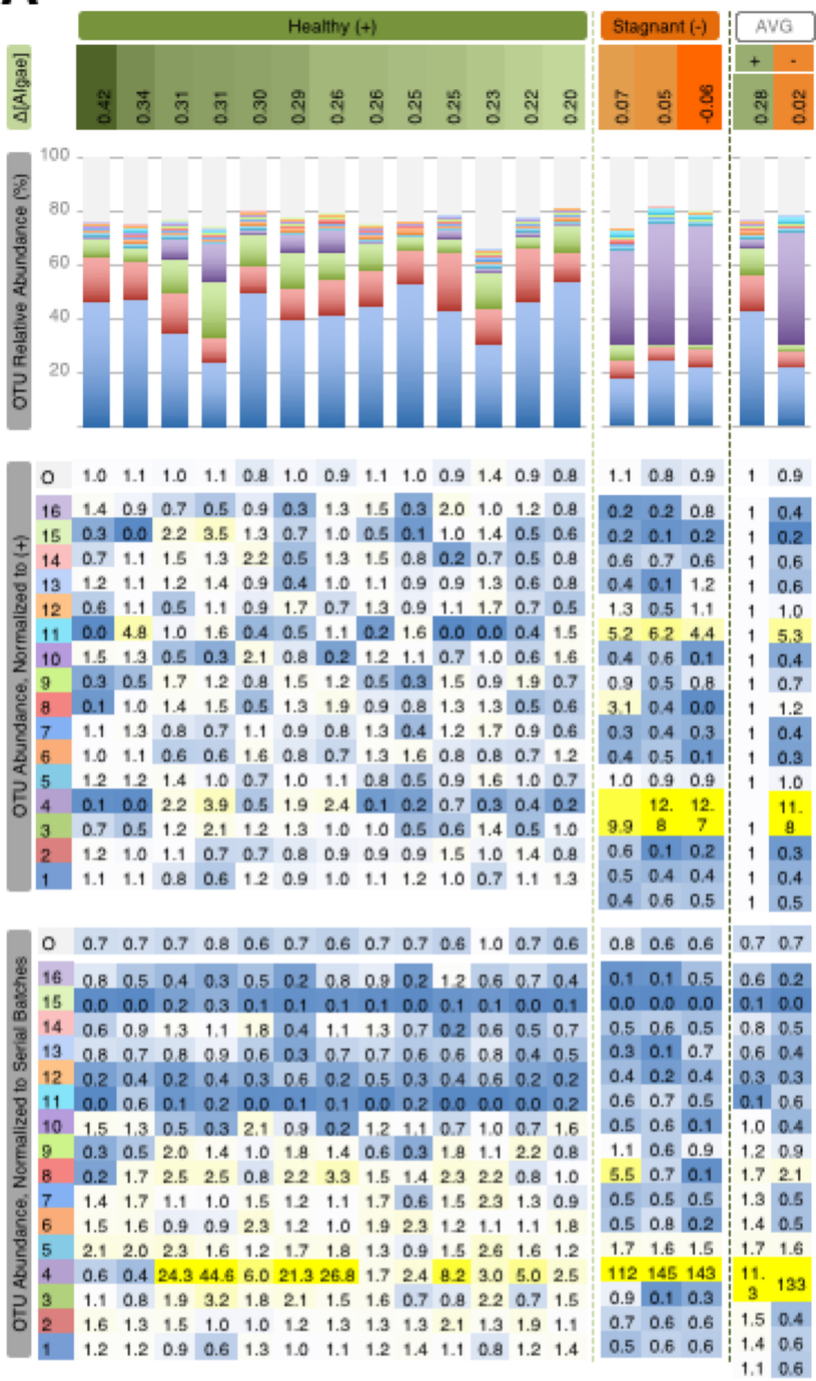
A



B

ID		Consensus Taxonomy					%	[OTU]AVG		
SB	+	Ph	Cl	Order	Family	Genus		SB	+	-
O	O	Other						33.5	23.5	21.8
15	16	Pr	A	Rhodobacterales	Hyphomonadaceae (NR284)		90	0.2	0.1	0.0
5	15	Pr	G	Alteromonadales	Alteromonadaceae	Marinobacter	90	2.5	0.2	0.0
16	14	Unassigned (NR142)					90	0.2	0.2	0.1
12	13	Pr	A	Rhodobacterales	Hyphomonadaceae (95991)		90	0.5	0.3	0.2
6	12	Pr	A	Rhizobiales	Hyphomicrobiaceae	Parvibaculum	95	1.2	0.4	0.4
4	11	B	Sp	Sphingobacteriales			90	3.7	0.4	2.3
11	10	Unassigned (NR178)					95	0.5	0.5	0.2
10	9	Pr	A	Rhizobiales	Hyphomicrobiaceae (917635)		95	0.5	0.6	0.4
13	8	B	S	[Saprospirales]	Chitinophagaceae		90	0.4	0.7	0.8
9	7	Pr	A	Sphingomonadales	Erythrobacteraceae		90	0.6	0.7	0.3
7	6	Unassigned (NR242)					95	0.8	1.2	0.4
8	5	Pr	A	Rhizobiales	Phyllobacteriaceae		95	0.8	1.4	1.3
14	4	Pr	D	Spirobacillales			90	0.3	3.5	41.3
3	3	B	C	Cytophagales	Cyclobacteriaceae		90	6.4	9.8	2.7
2	2	B	C	Cytophagales	Cytophagaceae	Leadbetterella	95	10.1	13.9	6.1
1	1	B	A	[Saprospirales]	Saprospiraceae		100	37.9	42.6	21.6

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Table 1

Phylum		Class	Abundance		
			Sml	Med	Lrg
B	Bacteroidetes	Bacteroidia	0.0	0.0	0.1
		Cytophagia	5.0	18.9	22.3
		Flavobacteriia	20.1	4.6	3.4
		Sphingobacteriia	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	Acidimicrobiia	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
		Σ =	99.9	99.6	98.8
		$\Sigma_{(B+Pr)}$ =	91.8	89.9	90.6

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Table 2A

			Abundance		
Ph	Class	Order	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
Cy	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

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Table 2B

			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	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
		Σ (*Saprospirales* + *Cytophagales*) =	28.2	50.9	59.6
		Σ (*Cytophagales* + *Flavobacteriales*) =	25.1	23.5	25.7

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Table 3

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)				
			B	**Cytophagia**	**Cytophagales**	Cyclobacteriaceae	
				[Saprospirae]	[Saprospirales]	Chitinophagaceae	
				Sphingobacteriia	Sphingobacteriales		
			Pr	**Alphaproteobacteria**	**Sphingomonadales**	**Erythrobacteraceae**	
					Rhodobacterales	**Hyphomonadaceae (90a)**	
						Hyphomonadaceae (90b)	
				Gammaproteobacteria	**Alteromonadales**	**Alteromonadaceae**	**Marinobacter**
				Deltaproteobacteria	Spirobacillales		

* Bold text indicates taxonomy shared with bacteria isolated from *N. oceanica* cultures by Wang, et al. (2012). For more information, see Supplemental Table S3