Title: Effects of inoculum source on the structure of bacterial communities in
Synechocystis sp. PCC6803-based photobioreactors

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Abstract

Cyanobacteria, which grow using solar energy and carbon dioxide, provide an attractive avenue for sustainable production of biomass and biofuel feedstock. Heterotrophic bacteria can colonize photobioreactor (PBR) cultures of cyanobacteria and may affect the productivity of the system. However, little is known about the types of heterotrophic bacteria associated with PBR cultures of cyanobacteria. The objective of this work was to evaluate the heterotrophic communities in PBR cultures of the cyanobacterium *Synechocystis* sp. PCC6803 using terminal-restriction fragment length polymorphism (T-RFLP) and high-throughput sequencing to compare the structure of heterotrophic communities in PBR cultures. To improve the resolution of the heterotrophic bacterial genomic signatures in the PBR communities, we utilized a targeted T-RFLP strategy to remove the T-RFLP signal of *Synechocystis* sp. PCC6803 from the analysis. For all experiments, the results of high-throughput sequencing were consistent with T-RFLP results. In particular, the inocula contained heterotrophic bacteria despite appearing to be pure cultures by light microscopy or non-targeted T-RFLP. Furthermore, the heterotrophic communities in the PBRs were strongly influenced by the microbial community in the inoculum used to start the PBR.

1. Introduction

Cyanobacteria are promising candidates for large-scale production of renewable biofuels to replace petroleum resources, including biodiesel made from lipids extracted from biomass [1,2]. Cyanobacteria perform oxygenic photosynthesis, gaining energy from sunlight and carbon from carbon dioxide. Thus, biofuels derived from cyanobacteria represent a renewable fuel source that can have a reduced carbon footprint compared to conventional diesel. Cyanobacteria have many advantages over terrestrial plants, including higher areal yields, superior photosynthetic efficiencies, faster growth rates, and no requirement for arable land for cultivation [3–5].

Heterotrophic bacteria grow by oxidizing organic molecules produced by cyanobacteria, including proteins, lipids, nucleic acids, and sugars [6]. Associations between cyanobacteria and heterotrophic bacteria are common, and a number of studies have examined the heterotrophic bacteria associated with cyanobacteria in natural settings [7–10]. Yet, few studies to date have addressed the structure and function of heterotrophic communities in photobioreactor (PBR) cultures of cyanobacteria [11]. In large-scale PBRs, sterilizing the culture medium and maintaining axenic culture conditions are economically and practically prohibitive [12]. Thus, a complete understanding of PBRs as engineered biological systems demands knowledge of the structure and dynamics of the microbial community, including the heterotrophic bacteria.

While the functions of the heterotrophic bacteria that associate with cyanobacteria are not fully understood, several important beneficial interactions have been documented.

Primarily, heterotrophic bacteria can provide CO₂ to the cyanobacteria by oxidizing organic compounds released by the cyanobacteria. Additionally, heterotrophic bacteria can recycle macronutrients, such as nitrogen (N) and phosphorus (P), or increase the availability of micronutrients, such as iron, to the cyanobacteria with which they associate [13]. Heterotrophic metabolism also may reduce O₂ super-saturation in cyanobacterial cultures [14]. Heterotrophic bacteria also can be detrimental to PBR operations by causing lysis of cyanobacterial cells [15], competing for key macronutrients, or consuming a desired product produced by the cyanobacteria such as excreted fatty acids [16]. Since the purpose of the PBR is to produce cyanobacteria biomass, exclusion of specific heterotrophic bacteria may be difficult, as the best growth conditions for the cyanobacteria also will be suitable for an array of heterotrophic bacteria. Thus, elucidating the common heterotrophs and key microbial interactions is critical for successful photobioreactor operation.

Synechocystis sp. PCC6803 (hereafter referred to as *Synechocystis*) is one of the most extensively studied cyanobacteria and was the first phototrophic organism to have a completely sequenced genome, making it an ideal model organism for further studies leading to large-scale use of cyanobacteria to produce renewable feedstock [5,17,18]. However, little is known about the types and temporal dynamics of the heterotrophic bacteria that can associate with *Synechocystis*-based PBRs. Molecular methods targeting the 16S rRNA gene are useful to uncover the structure of microbial communities associated with PBR cultures of *Synechocystis*, and to monitor these communities through time [19]. Terminal-restriction fragment length polymorphism (T-

RFLP) is a rapid, robust, and cost-effective method that is widely used to analyze microbial communities [20–22] and provides a useful avenue to compare and contrast the structures of different PBR microbial communities. Similarly, high-throughput sequencing of 16S rRNA gene libraries can be used to assess the structures of microbial communities [23]. High-throughput sequencing is beneficial because it is highly sensitive and provides phylogenetic information not afforded by T-RFLP; however, it is not accessible to every researcher and requires significant time for sequencing and data analysis.

Here, we used T-RFLP and high-throughput sequencing of 16S rRNA gene libraries to monitor the microbial communities in PBR cultures of *Synechocystis*. We demonstrate that communities of heterotrophic bacteria exist in the PBRs and show that the structure of the PBR microbial communities can be different for each PBR. We also provide evidence that the starter cultures used to inoculate the various PBR experiments play an important role in determining the structure of the resulting PBR microbial communities. Finally, we demonstrate strong agreement between T-RFLP and high-throughput sequencing data, showing that these techniques can be used in tandem or separately to provide a detailed understanding of PBR microbial communities.

2. Materials and Methods

2.1 Cell cultures, strains, media and preparation of inocula

The laboratory of Dr. Willem Vermaas (School of Life Sciences, Arizona State University) provided stock cultures of *Synechocystis* sp. PCC6803, which were

maintained in BG-11 medium [24]. To make freezer stocks, 0.5 mL of a *Synechocystis* sp. PCC6803 stock culture was mixed with 0.5 mL of sterile 40% glycerol and stored at -80°C. To prepare fresh PBR inoculum, we spread a small amount of the freezer stock on a sterile BG-11 plate with 1.5% Bacto Agar (BD, Sparks, MD) using a sterile inoculation loop. We grew the inoculated plates at 30°C under 200 $\mu\text{E m}^{-2} \text{sec}^{-1}$ constant incident light intensity until a lawn of cells was evident (5-7 days). We then scraped cells from the lawn using a 10 μL inoculation loop and transferred the cells into 100 mL sterile liquid BG-11 medium in a 250 mL Erlenmeyer flask bubbled with air filtered through a 0.2 μm filter (Pall). We grew this starter culture at 30°C under 200 $\mu\text{E m}^{-2} \text{sec}^{-1}$ constant incident light intensity until cells grew to an optical density at a wavelength of 730 nm (OD_{730}) of 0.5-1 (3-5 days). For the purposes of this work, each separate starter culture can be considered as originating from a separate BG-11 agar plate.

2.2 PBR operational parameters

We used a Photobioreactor FMT-150 (Photon Systems Instruments, Czech Republic) equipped with a temperature/pH probe and bubble interrupter for all experiments. We autoclaved the cultivation chamber to ensure that it was sterile prior to inoculation. All PBR experiments were operated with a constant incident light intensity of 200 $\mu\text{E m}^{-2} \text{sec}^{-1}$, maintained at 30°C, and bubbled with air that was humidified by passing through sterile water and filtered through a 0.2- μm filter (Pall, Ann Arbor, MI). We measured optical density (OD) at a wavelength of 730 nm using a Cary-50-Bio UV-Visible spectrophotometer (Varian, Palo Alto, CA) and pH directly using a pH probe integrated

with the Photobioreactor FMT-150 and calibrated according to the manufacturer's directions. We inoculated four PBRs at a starting OD₇₃₀ of 0.1 and ran each experiment for a total of 168 hours (7 days). Two of the PBRs (PBR-A and PBR-A2) were inoculated from the same starter culture originating from the same agar plate and culture flask, and the other two PBRs (PBR-B and PBR-C) were inoculated using different starter cultures, which originated from different agar plates and liquid culture flasks.

2.3 Light Microscopy

For light microscopy, we took 1-mL samples of PBR cultures daily, added 0.2 mL 37.5% formaldehyde (Sigma Aldrich) to fix the cells, and stored these samples at 4°C. Cells were imaged later by light microscopy using an Olympus BX61 light microscope (Olympus Inc., Center Valley, PA) equipped with differential interference contrast (DIC) using a 100X oil-immersion objective. To evaluate the presence or absence of heterotrophic bacteria, we counted at least 200 total cells. Images were captured with an Olympus DP72 color camera.

2.4 DNA extraction

For DNA extraction we used a previously described method [25]. Briefly, 1-mL samples of the PBR culture were taken daily with a sterile syringe and transferred to a sterile microcentrifuge tube and centrifuged (13 g, 3 minutes) to concentrate the biomass, which was stored at -80°C prior to DNA extraction. We extracted total genomic DNA from PBR samples using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with

the following modifications to enhance lysis. We resuspended cell pellets in 200 μ L lysis buffer (30 mM Tris·HCl, 10 mM EDTA, 200 mM sucrose, pH 8.2) and incubated the mixture at 65°C for 10 minutes. We then added chicken egg white lysozyme (Sigma Aldrich, St. Louis, MO) to a final concentration 10 mg/mL and incubated the samples for 1 hour at 37°C. Next, we added SDS at 1% (w/v) and incubated the samples at 56°C for 10 minutes. Finally, we added 25 μ L proteinase K and 200 μ L buffer AL (Qiagen) and incubated that mixture at 56°C for 30 minutes. After these additional lysis steps, we completed the DNA extraction according to the manufacturer's (Qiagen) instructions.

2.5 Terminal restriction fragment length polymorphism (T-RFLP)

To improve the resolution of the heterotrophic bacteria, we applied a strategy to remove *Synechocystis* from the T-RFLP analyses. We achieved this goal by choosing a primer and restriction enzyme combination that produced a *Synechocystis* terminal-restriction fragment (T-RF) outside the detectable range of the assay, thereby excluding the *Synechocystis* signal from the analysis and increasing sensitivity towards 16S rRNA gene fragments belonging to other bacteria present in the PBR. We performed T-RFLP analysis using a previously described method [25]. Briefly, we amplified 16S rRNA genes using the universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') with a 5'-HEX fluorescent dye and 1392R (5'-ACACACCGCCCGT-3') [21,22,25]. PCR conditions were as follows: 1x *Taq* PCR Master Mix (Qiagen), 250 nmol L⁻¹ each primer, 1 mmol L⁻¹ Mg²⁺, and 10 ng template DNA in a total of 50 μ L. PCR reaction temperature profiles were the following: 94°C for 6 minutes, followed by 30 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 2 minutes and a final extension

period at 72°C for 10 minutes. Following PCR, we checked for amplicons of the proper size (approximately 1400 bp) on a 1% (w/v) agarose gel. We then digested the amplified 16S rRNA genes using the restriction enzymes *HhaI*, *MseI*, *HaeIII*, or *HpaII* (New England Biolabs, Ipswich, MA). Next, we analyzed the sizes of the T-RFs produced on an ABI 3730 DNA Analyzer using the GeneScan500 ROX Size Standard (Applied Biosystems).

2.6 Analysis of T-RFLP data

We gathered raw T-RFLP data using the free Peak Scanner Software v1.0 (Applied Biosystems) and analyzed the data using previously described methods [26]. We determined total fluorescence intensity within each individual sample by summing the fluorescence intensity of all detected T-RFs for that sample. We normalized T-RFLP data by removing peaks that contained less than 1% of the total fluorescence intensity and calculated a new total fluorescence intensity using only T-RFs detected as above background. We then determined true T-RFs as any T-RF that contained more than 5% of the new total fluorescence intensity. To compensate for T-RF drift, all T-RFs were aligned manually. T-RFs smaller than 50 base pairs (bp) and larger than 600 bp were omitted from the analyses, as fragments of these sizes are not supported by the size standard we used. We performed all analyses of raw T-RFLP data using original Perl scripts and Microsoft Excel spreadsheets. When appropriate, we removed the *Synechocystis* T-RFs from the raw data prior to downstream analyses. All T-RFs determined as above background were plotted in GraphPad Prism (GraphPad, La Jolla, CA). We used the QIIME software [27] for principal coordinate analysis (PCoA) of T-

RFLP data. Further explanation of these analyses is provided in the **Supplementary Information**.

2.7 Prediction of *Synechocystis* T-RF sizes

Ideally, each species generates a unique T-RF size. We predicted the size of the *Synechocystis* T-RF generated with each enzyme used by submitting the *Synechocystis* 16S rRNA gene sequence (NCBI accession number NR_074311.1) to *in silico* restriction digests. We determined T-RF sizes of 1048, 546, 293, and 490 bp for the enzymes *HhaI*, *MseI*, *HaeIII*, and *HpaII*, respectively. We validated the predictions by performing T-RFLP on pure cultures of *Synechocystis* (**Supplementary Information Figure S1**).

2.8 High throughput 16S rRNA gene sequencing and data analysis

We sequenced 16S rRNA genes from the enriched consortia of heterotrophic bacteria and from representative PBR experiments using previously described methods [28]. We amplified the V4 region of bacterial 16S rRNA genes using Golay barcoded primer set 515F/806R [29]. We then pooled the triplicate PCR reactions and quantified them using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies). Next, we pooled 240 ng of each sample in and cleaned the final pool using the QiaQuick PCR Cleanup Kit (Qiagen). For loading the samples onto the Illumina MiSeq, we quantified the PCR library using the KAPA SYBR FAST Universal qPCR Kit for Illumina (KAPA Biosystems). We then sent the prepared library to the Microbiome Analysis Laboratory at Arizona State University for sequencing on the Illumina MiSeq. We analyzed all 16S

rRNA gene sequencing data using the QIIME v1.8.0 software [23,27]. OTUs were assigned at 97% similarity using Greengenes v 13.5. To normalize the data set from each sample, we randomly sampled 40,000 sequences from each sample prior to analysis. All analyses are of 150 bp forward reads.

2.9 Inoculum effect experiments

To determine the impact of the inoculum on the structure of PBR microbial communities, we conducted a series of batch tests. We collected three different *Synechocystis* cultures, each of which was grown in a different building on the Tempe campus of Arizona State University. We then used these flasks to inoculate three identical experimental flasks, giving a total of nine experimental flasks. All experimental flasks were grown in the same light incubator under identical conditions (30°C, 85 $\mu\text{E m}^{-2} \text{ sec}^{-1}$) for 96 hours and were bubbled with air filtered through a 0.2 μm filter (Pall). Samples were collected from each inoculum at the time of inoculation and from the experimental flasks after the 96-hour incubation period. For these experiments, we performed T-RFLP using all four enzymes and evaluated the structure of the microbial communities based on the presence or absence of the different T-RFs detected. If a T-RF was detected in a specific sample, it was assigned a value of 1, and if it was not detected in that sample, it was assigned a value of 0.

3. Results and Discussion

3.1 PBRs operated under identical conditions show different performances

Figure 1 shows the OD₇₃₀ and pH for the four PBR experiments. PBR-A and PBR-A2 (inoculated from the same starter culture) achieved maximum OD₇₃₀ values of 3.0 and had average pH values of 11.8 and 9.2, respectively. PBR-B and PBR-C (different starter cultures) attained maximum OD₇₃₀ values of 2.8 and 2.0 and had average pH values of 9.7 and 10, respectively. The lower OD₇₃₀ for PBR-C correlated with a 2-day lag period not seen in the other experiments. The high pH values in all the PBR experiments indicated that *Synechocystis* was photosynthetically active and utilized available inorganic carbon efficiently (Kim et al. 2010a). Thus, despite being operated under identical conditions with the same culture medium, each PBR produced a different amount of biomass and had different pH values.

Figure 2 shows light microscopy images from PBR-A, which showed similar patterns to the other PBR experiments (shown in **Supplementary Information Figure S2**). On Day 0 (inoculation), only green *Synechocystis* cocci were detected in the PBR culture after counting at least 200 cells per sample, indicating that heterotrophic bacteria comprised $\geq 0.5\%$ of the microbial community. Rod-shaped heterotrophic bacteria became detectable by microscopy in PBR-A starting on Day 4. For the other PBR experiments, rod-shaped heterotrophic bacteria were observable by light microscopy starting on or around Day 4. Once the heterotrophic bacteria became detectable in the PBR, they persisted for the duration of the experiment (Day 7). This was expected, as the association of heterotrophic bacteria with cyanobacterial blooms and cultures is

ubiquitous in natural conditions [10] and has been observed and predicted to have impacts in other engineered PBR systems [31,32].

3.2 Removal of *Synechocystis* signal improves detection of heterotrophic bacteria with T-RFLP analyses

Table 1 shows the number of unique T-RFs detected using the restriction enzymes *MseI*, *HaeIII*, *HpaII*, or *HhaI* for the PBR-A, PBR-A2, PBR-B, and PBR-C at the end of each experiment. As predicted, eliminating the *Synechocystis* T-RF via the *HhaI* digest increased the resolution of the rest of the PBR microbial community. This was especially evident for PBR-B, where the *HhaI* digest yielded one unique non-*Synechocystis* T-RF even though no non-*Synechocystis* T-RFs were detected in any of the other digests. Even for PBR-A and PBR-C, where one non-*Synechocystis* T-RF was detected with the *MseI*, *HaeIII*, and *HpaII* digests, the *HhaI* digest still improved the resolution of the non-*Synechocystis* members of the PBR microbial communities. This shows that using the *HhaI* restriction enzyme provided best resolution of the heterotrophic bacteria in the PBRs.

3.3 T-RFLP and high-throughput sequencing analysis of PBR microbial communities showed similar trends

In order to understand better the heterotrophic bacteria present in our PBR, we employed T-RFLP using the *HhaI* restriction enzyme to target the heterotrophic bacteria in the microbial communities and high-throughput sequencing. **Table 2** shows the results of T-RFLP analysis of the microbial communities from PBR-A, PBR-A2, PBR-B,

and PBR-C using the *HhaI* restriction enzyme on Day 0, Day 3, and Day 7 of each experiment. **Figure 3** shows genus-level taxonomic classification determined by high-throughput sequencing of 16S rRNA gene libraries from PBR-A, PBR-A2, PBR-B, and PBR-C from Day 0, Day 3, and Day 7 of each experiment.

According to 16S rRNA gene sequencing, on Day 0, PBR-A and PBR-A2 had very high abundances of *Synechocystis* and only small proportions of heterotrophic bacteria. In contrast, the T-RFLP method did not detect non-*Synechocystis* T-RFs for these PBRs on that day. Thus, high-throughput 16S rRNA gene sequencing was more sensitive to the presence of heterotrophic bacteria than was the *HhaI* T-RFLP. On Days 3 and 7, PBR-A and PBR-A2 showed a large proportion of heterotrophic bacterial phylotypes from the genus *Bacillus* that corresponded to a large 242-bp T-RF detected in both PBRs. The smaller 582-bp T-RF may correspond to the bacteria assigned to the genus *Stenotrophomonas*. These data demonstrate that the same species of heterotrophic bacteria were present in the microbial communities of PBR-A and PBR-A2. A *Pseudomonas* sp. was detected on the last day of PBR-A while one *Sphingomonas* sp. was detected on the last day of PBR-A2, but no corresponding T-RFs for either species were detected. Thus, while the heterotrophic communities in these PBRs were dominated by a *Bacillus* sp., the structure of the final microbial communities also had some variability.

On Day 0 of PBR-B, we detected heterotrophic bacterial phylotypes assigned to *Comamonadaceae*, *Pseudomonadaceae*, and *Chitinophagaceae*. By Day 7,

Chitinophagaceae and a *Bacillus* sp. became the most dominant phylotypes. This shift in the structure of the microbial community also was seen with the *HhaI* T-RFLP. A 570-bp T-RF, possibly corresponding to the detected *Pseudomonadaceae* sp., disappeared between Day 0 and Day 3. The 204-bp T-RF showed a similar trend as the *Comamonadaceae* sp., showing a reduction in abundance from Day 0 to Day 3 before disappearing on Day 7. A 135-bp T-RF was detected on Day 3 and Day 7 and may correspond to the *Chitinophagaceae* sp. detected with high-throughput sequencing. Finally, a 240-bp T-RF appeared on Day 7 and may correspond to the *Bacillus* sp. detected with high-throughput sequencing, which increased greatly in abundance from Day 3 to Day 7.

According to 16S rRNA gene sequencing, heterotrophic bacteria assigned to *Xanthomonadaceae*, *Flavobacterium*, and *Rhizobaceae* were most abundant on Day 0 of PBR-C. The lag period at the beginning of this PBR was correlated with the presence of a *Flavobacterium* sp. *Flavobacteria* associated with marine phytoplankton typically are most abundant during the decay phase [33]. As the culture entered exponential growth, the *Flavobacterium* sp. was no longer detected, and the relative proportion of *Synechocystis* increased. By Day 7, the most predominant non-*Synechocystis* phylotypes were *Xanthomonadaceae*, *Pseudomonaceae*, and *Rhizobiaceae*. T-RFLP also detected the shift that occurred in microbial community of PBR-C between Day 0 and Day 3 through the disappearance of the 365-bp T-RF, which likely corresponded to the *Flavobacterium* sp., and the appearance of a 342-bp T-RF, which may correspond to the *Pseudomonadaceae* sp. Thus, the 212 and 372-bp T-RFs

that were detected throughout the experiment can be assigned, based on the relative abundance of the T-RFs, to the *Xanthomonadaceae* sp. and *Rhizobaceae* sp. detected with high throughput sequencing, respectively.

Our data demonstrated strong agreement between the high-throughput sequencing and T-RFLP analysis of the different PBR microbial communities. While high-throughput sequencing usually detected more unique taxa of heterotrophic bacteria (more non-*Synechocystis* OTUs) than did the *HhaI* T-RFLP, T-RFLP usually detected the major heterotrophic phylotypes in the PBRs. A likely explanation for this discrepancy may be that not all bacterial species may produce a T-RF of 50-600 bp when the *HhaI* restriction enzyme is used [34]. Thus, certain heterotrophic bacteria are excluded along with *Synechocystis* when using *HhaI*. However, previous studies have demonstrated that the majority of bacteria produce a T-RF in this range with the *HhaI* restriction digest [22]. Furthermore, not all bacteria may have been abundant enough to be detected above background using T-RFLP. This effect can be attributed to greater sensitivity of the sequencing technologies. Thus, high-throughput sequencing was more informative than T-RFLP for determining microbial community structure, but T-RFLP was more suitable for routine monitoring of the structures of different PBR microbial communities due to its low cost and rapid turnaround time.

3.4 Inoculum source strongly influences PBR microbial community structure

The two PBRs inoculated with the same starter culture (PBR-A and PBR-A2) had similar microbial communities, while the two PBRs inoculated with different starter

cultures (PBR-B and PBR-C) had unique microbial communities. We conducted a separate set of experiments to examine more closely the effects of using different inocula on the structure of PBR microbial communities. Here, three unique inocula (labeled 1, 2, and 3) were used to inoculate three experimental flasks (nine flasks in total), which were then grown under identical conditions. **Figure 4** shows high-throughput sequencing data from the microbial communities from the three unique *Synechocystis* inocula and from the experimental cultures derived from the inocula. The microbial communities in the respective experimental flasks were most similar to the microbial community from that inoculum, even though the culture conditions were identical. The microbial communities within a set of experimental flasks also were more similar to one another than to any of the other experimental or inoculum flasks. The heterotrophic bacteria in the first set of flasks (1Inoc, 1A, 1B, and 1C) contained mostly phylotypes from the order *Cytophagales*, while those in the second set (2Inoc, 2A, 2B, and 2C) mostly contained phylotypes from the orders *Burkholderiales* and *Sphingobacteriales*. All of the flasks in the third set (3Inoc, 3A, 3B, and 3C) were composed almost entirely of *Synechocystis*. However, the microbial communities exhibited slight difference between the flasks within each set. Most notable was the second set (2Inoc, 2A, 2B, and 2C), where each of the experimental flasks contained bacteria from the order *Sphingobacteriales*, even though those bacteria were not detected in the inoculum flask.

Figure 5 shows PCoA biplots of the high-throughput 16S rRNA gene sequencing data and T-RFLP data generated using the *MseI*, *HaeIII*, *HpaII*, or *HhaI* restriction enzymes.

As expected from the 16S rRNA gene sequencing taxonomic data, PCoA showed that the microbial communities in each set of experimental flasks grouped with the inoculum flask from that set, indicating that those microbial communities were more similar to one another than they were to the microbial communities in the other sets of flasks. PCoA generated from the T-RFLP showed the same trends as the PCoA generated from the high-throughput sequencing data in terms of the grouping of the different microbial communities. Taken together, these data show that the inoculum source strongly influenced the structure of microbial communities of the PBRs, an effect that has been demonstrated in other biotechnological applications [35]. This further explains the similar structure of the microbial communities in PBR-A and PBR-A2, for which we also showed strong agreement between the T-RFLP and high-throughput sequencing data.

Heterotrophic bacteria associated with large-scale PBR cultures and may enhance the growth and health of cyanobacterial cultures by recycling carbon, nitrogen, and phosphorous from decaying biomass and cyanobacterial exudates [33], or by consuming oxygen, thereby promoting photosynthesis [14]. Conversely, certain heterotrophic bacteria can induce lysis of cyanobacteria by secretion of enzymes capable of hydrolyzing the cell walls of cyanobacteria [15]. Thus, there is a need to develop methods to monitor and manage the microbial communities in PBRs in order to maximize productivity of these systems and enhance the economic viability of biofuels derived from cyanobacterial biomass.

413 We operated four PBRs under identical conditions and detected heterotrophic bacteria
414 in the inoculum cultures with either T-RFLP, high-throughput sequencing, or both
415 techniques, even though light microscopy indicated that these cultures were free of
416 heterotrophic bacteria. This finding points out the value of using sensitive, genome-
417 based methods to assess the purity of starter cultures, which seem to control the
418 heterotrophic community structure. We demonstrated that T-RFLP (a quick and
419 inexpensive method) was useful for comparing and tracking the structures of different
420 PBR microbial communities, although it does not provide phylogenetic information and
421 can be limited by phylogenetically disparate strains of heterotrophic bacteria producing
422 the same size T-RF [22]. This limitation is especially important here where only one
423 restriction enzyme (*HhaI*) provides appropriate resolution of the heterotrophic bacteria in
424 the PBR. Phylogenetic information can be obtained through high-throughput
425 sequencing and compared with T-RFLP data to enhance the ability of T-RFLP to track
426 specific heterotrophic bacteria in *Synechocystis*-based PBRs. Once the system is well
427 understood, these two approaches can be combined and T-RFLP with optimized
428 enzyme choices can be used for quick and routine quality control assessments.

429

430 We showed that using the same inoculum source preserved PBR microbial
431 communities. Thus, in order to truly appreciate the effects of different operational or
432 culturing conditions on the performance of PBRs and on the structure of the PBR
433 microbial communities, it is important to use the same inoculum source. This would
434 provide some control over the structure of the PBR microbial community and allow for
435 more meaningful comparisons between differing cultivation conditions.

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6. Tables

Table 1 Number of unique T-RFs detected with each restriction enzyme. *Synechocystis* T-RFs were detected for all restriction enzymes except for *HhaI*, where the *Synechocystis* T-RF is too large and is excluded from the analysis. In all of the PBRs, the number of non-*Synechocystis* T-RFs detected with *HhaI* was greater than the number of non-*Synechocystis* T-RFs detected with any of the other restriction enzymes.

	PBR-A	PBR-A2	PBR-B	PBR-C
<i>MseI</i>	1	2	1	2
<i>HaeIII</i>	2	2	1	1
<i>HpaII</i>	2	2	1	2 ⁵⁷⁴
<i>HhaI</i>	2	2	2	3

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Table 2 T-RFs detected in each PBR using the *HhaI* restriction enzyme. PBR-A and PBR-A2 had nearly identical T-RF patterns while PBR-B and PBR-C had unique T-RF patterns.

	Day 0		Day 3		Day 7	
	T-RF Size (bp)	% Total Area	T-RF Size (bp)	% Total Area	T-RF Size (bp)	% Total Area
PBR-A	ND	ND	242, 582	81.6, 5.9	242, 582	87, 11.6
PBR-A2	ND	ND	242, 582	79.3 11.9	242, 582	68.7, 12.1
PBR-B	204, 570	56, 38.5	135, 204	31.9, 17.8	135, 240	28.2, 71.8
PBR-C	212, 365, 372	65.8, 19.1, 6.6	212, 342, 372	75.1, 5.5, 9.4	212, 342, 372	74.8, 8, 9.1

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Figure 1 OD₇₃₀ and pH from four PBRs operated under identical conditions. All PBRs were inoculated at OD₇₃₀ = 0.1 with 200 $\mu\text{E}/\text{m}^2/\text{sec}$ constant light intensity, held at 30°C, and bubbled with filter-sterilized air. A, B, or C indicates inoculation with different starter cultures; PBR-A and PBR-A2 were inoculated with the same starter culture. Despite identical operating parameters, each PBR had unique OD₇₃₀ (green) and pH (black) profiles, and PBR-C did not grow as well as the others.

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Figure 2 Light microscopy images from PBR-A. All images were taken with a 100X oil-immersion DIC objective. Upon inoculation (Day 0), only *Synechocystis* (black arrowheads) was detected. By Day 4, rod-shaped heterotrophic bacteria (white arrows) were detectable in the PBR. Heterotrophic bacteria grew in the PBR until the end of the experiment (Day 7). Scale bars are 10 μm.

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Figure 3 Bacteria detected in the four PBR experiments through high-throughput sequencing. All taxa are classified at the genus-level unless otherwise indicated.

*OTU classified at family-level

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Figure 4 Microbial communities in experimental flasks are similar to the microbial communities in inocula. For each different inoculum (1, 2, 3), the microbial communities in the experimental flasks (A, B, C) were similar to the microbial communities in the respective inoculum (Inoc) flasks.

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Figure 5 PCoA of high-throughput 16S rRNA gene sequencing (Left) and T-RFLP (Right) data. The T-RFLP data represent a jackknifed PCoA analysis of data from the four restriction enzymes used. Each set of four flasks (inoculum and three experimental flasks) is shown in a different color. Both techniques show that the microbial community in each flask was more similar to those in the flasks within that set than to the microbial communities in the flasks from the other sets.

8. Table Captions

Table 1 Number of unique T-RFs detected with each different restriction enzyme.

Synechocystis T-RFs were detected for all restriction enzymes except for *HhaI*, where the *Synechocystis* T-RF is too large and is excluded from the analysis. In all of the PBRs, the number of non-*Synechocystis* T-RFs detected with *HhaI* was greater than the number of non-*Synechocystis* T-RFs detected with any of the other restriction enzymes.

Table 2 T-RFs detected in each PBR using the *HhaI* restriction enzyme. PBR-A and PBR-A2 had nearly identical T-RF patterns while PBR-B and PBR-C had unique T-RF patterns.

9. Figure Captions

Figure 1 OD₇₃₀ and pH from four PBRs operated under identical conditions. All PBRs were inoculated at OD₇₃₀ = 0.1 with 200 µE/m²/sec constant light intensity, held at 30°C, and bubbled with filter-sterilized air. A, B, or C indicates inoculation with different starter cultures; PBR-A and PBR-A2 were inoculated with the same starter culture. Despite identical operating parameters, each PBR had unique OD₇₃₀ (green) and pH (black) profiles, and PBR-C did not grow as well as the others.

Figure 2 Light microscopy images from PBR-A. All images were taken with a 100X oil-immersion DIC objective. Upon inoculation (Day 0), only *Synechocystis* (black arrowheads) was detected. By Day 4, rod-shaped heterotrophic bacteria (white arrows) were detectable in the PBR. Heterotrophic bacteria grew in the PBR until the end of the experiment (Day 7). Scale bars are 10 µm.

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Figure 4 Microbial communities in experimental flasks are similar to the microbial communities in inocula. For each different inoculum (1, 2, 3), the microbial communities in the experimental flasks (A, B, C) were similar to the microbial communities in the respective inoculum (Inoc) flasks.

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665 **Figure 5 Jackknifed PCoA of T-RFs detected with all four restriction enzymes.**

666 Each color represents a different inoculum. The microbial communities from the

667 experimental flasks were most similar to the inoculum flask from that set.

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