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Quantification of heterotrophic bacteria during the growth of *Synechocystis* sp. PCC 6803 using fluorescence activated cell sorting and microscopy

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ABSTRACT

The presence of heterotrophic bacteria in microalgal cultures can dilute the microalgal content of the harvested biomass, compete for nutrients, and be associated with culture crashes. Being able to detect and quantify heterotrophic bacteria would be of high value for monitoring culture health and reducing deleterious effects. Here, we developed and applied a new method that combines flow cytometry (FC) and fluorescence activated cell sorting (FACS) for the quantification of heterotrophic bacteria in cultures of the cyanobacterium *Synechocystis* sp. PCC 6803. Particles not containing chlorophyll – heterotrophic bacteria and cell debris – were separated from mixed cultures using FACS based on autofluorescence of *Synechocystis*. Heterotrophic bacteria were differentiated from cell debris using FC with SYTOX green fluorescence. Using microscopy, we verified that FACS was able to quantify heterotrophic bacteria in *Synechocystis* cultures effectively. Applying these methods to batch cultures of *Synechocystis* showed that the count proportions of heterotrophic bacteria were significant (3–13%) and that depletion of inorganic P in the culture favored *Synechocystis* over heterotrophic bacteria, but led to more cell lysis.

1. Introduction

Cyanobacteria are valuable sources of bioproducts that span energy feedstock, cosmetics, and nutraceuticals [1–3]. One of the largest concerns in scaling microalgal cultures is the presence of microbial "contaminants" that can reduce bioproduct productivity or quality [4, 5]. Heterotrophic bacteria are one type of "contaminant" of concern, because they can dilute the cyanobacterial content of the harvested biomass, compete for nutrients, or be associated with culture crashes [4, 6]. Cyanobacteria are the primary producers during cultivation, while the heterotrophic bacteria are secondary consumers of materials generated by the cyanobacteria. The cyanobacteria contain the valuable outputs, such as lipids and pigments.

All microorganisms produce extracellular polymeric substances (EPS) and soluble microbial products (SMP), which are sources of organic carbon and electrons. During phototrophic cultivation of cyanobacteria, the cyanobacteria generate EPS and SMP, which become food for heterotrophic bacteria [3, 7, 8]. While most heterotrophic bacteria have little effect on cyanobacterial growth [4, 9], they still compete for nutrients and can change the quality of the harvested biomass [5, 10]. Stress to the phototrophs can lead to increased release of microbial products [7, 11], and increases in heterotrophs are expected. Additionally, some heterotrophic bacteria can cause lysis of cyanobacteria cells through enzymatic or antibiotic mechanisms leading to drastic declines in yield or "culture crashes" [3, 12]. Quantification of heterotrophic bacteria during the culturing of cyanobacteria is, therefore, valuable for assessing culture health.

Traditionally, microscopy has been applied for counting heterotrophic bacteria [13, 14], but it is reliable only when the heterotrophic bacteria are phenotypically distinct from the phototrophs [15]. While this approach may work with eukaryotic algae, it is not as effective for cyanobacteria. In any case, microscopy is tedious and time-consuming.

The limitations of microscopy can be overcome by cell sorting, which permits differentiation and subsequent isolation of single cells based upon their cell size and autofluorescence [16]. Flow cytometry (FC) and fluorescence activated cell sorting (FACS) are powerful tools that enable rapid detection of differences among cells according to size and physiological characteristics [14], and this includes differentiating intact cells from cellular debris after lysis [17, 18].

FC sorts particles according to the characteristics detectable by multi-dimensional and quantitative measurement of light scattering and fluorescence emission [14]. In microbial cultures, it can be used to

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achieve two goals [19–21]: (1) characterizing cell features, such as cell size, granularity, and membrane integrity; and (2) cell sorting according to size or metabolic features that can be identified by staining. FC can also be used to differentiate heterotrophic bacteria from eukaryotic algae based on the significant difference in cell size [14].

FACS separates cells based on fluorescence derived from photosynthetic pigments (autofluorescence) or applied fluorescent probes [14]. Photosynthetic pigments in cyanobacteria include chlorophylls, carotenoids, and phycobilins [22–24], which autofluorescence with an intensity that is linearly related to the pigment concentration [25, 26]. Being the primary photosynthetic pigment, chlorophyll *a* is the only pigment present in all photosynthetic microalgae, and it emits UV-blue light (< 450 nm) and far-red light (> 650 nm) [27, 28]. Thus, FACS can be used to differentiate cyanobacteria and heterotrophic bacteria based on cyanobacteria's autofluorescence, which the heterotrophic bacteria do not have. FACS also has been used to separate heterotrophic bacteria from a mixed culture to provide an axenic culture of *Chlorella vulgaris* [14]. It should be noted, however, that the cell size for *Chlorella* is larger than 5 μm, and the authors did not attempt to quantify and detect low levels of heterotrophs. The average cell size of *Synechocystis* is approximately 3 μm, and the fraction of the biomass that is heterotrophic can be substantial [29, 30], making their differentiation from heterotrophic bacteria using FACS a much greater challenge. Another challenge is differentiating cell debris due to cell lysis from heterotrophic bacteria in a mixed culture because neither autofluorescence. Cell debris does not have the same potential deleterious effects as heterotrophic bacteria.

In this study, we develop and apply a fluorescence method for the quantification of heterotrophic bacteria in a culture of *Synechocystis* sp. PCC 6803 (*Synechocystis* from here). FACS is used to separate particles not containing chlorophyll (the heterotrophic bacteria and cell debris) from autofluorescencing *Synechocystis* cells. The non-fluorescing particles are then separated into heterotrophic bacteria and cell debris using a fluorescence stain, SYTOX green, that is better absorbed by the heterotrophs. We use light microscopy to verify the accuracy of the divisions using FC and FACS. Finally, we apply the method to evaluate how the availability of phosphorus (P) in *Synechocystis* cultures affects the quantity of heterotrophic bacteria present at the end of batch experiments.

2. Materials and methods

2.1. Synechocystis sp. PCC 6803 cultures and growth experiments

Wild-type *Synechocystis* sp. PCC 6803 was maintained in 500-mL Erlenmeyer flasks with a working volume of 300 mL. The medium was standard BG-11 [31] bubbled with air filtered through a 1.0-μm air filter (Pall, Port Washington, NY, U.S.). An aliquot from a flask culture was diluted to an optical density (OD) of 0.6 ± 0.02 to initiate each batch growth experiment.

1-L Erlenmeyer flasks with working volumes of 700 mL were used for batch growth experiments. *Synechocystis* was cultivated at a constant temperature of 30 ± 0.8 °C maintained by 3 12-W automated-air fans (Minebea-Matsushita Motor Corp., Japan) [32], and sparging with humidified air (bubbled through deionized water) filtered through a 1.0-μm air filter (Pall, Port Washington, NY, USA). The incident light intensity was 276 μmol photons $m^{-2} s^{-1}$ using T5 fluorescent plant-grow lamps (Envirogro Hydrofarm, USA). The pH of the culture was maintained at 8.0 using a pH-Stat that initiated pure CO_2 sparging when the pH rose above 8.01 [33].

In batch growth experiments, NO_3-N and PO_4-P concentrations of 120 mg N/L (8.6 mM) and 12 mg P/L (0.39 mM) served as the baseline concentrations, consistent with the stoichiometric ratio of 22 mol N/mol P in standard BG-11 medium [11], although the N concentration we used was lower than in standard BG-11. To evaluate the effects of P limitation, we decreased the starting concentrations of P from 0.39 mM (High P) to 0.055 mM (Low P) while keeping the NO_3-N concentration at 8.6 mM. We augmented the starting alkalinity by adding 6.0 mM of bicarbonate (as $NaHCO_3$). All constituents other than N, P, and alkalinity were the same as standard BG-11. Prior to inoculation, the flasks and the BG-11 medium were sterilized by autoclaving, and the pH probe was sterilized using 75% ethanol.

2.2. Autofluorescence detection

Autofluorescence, a unique biomarker for photosynthetic organisms, enabled us to set a FC gate that separated *Synechocystis* from non-fluorescing particles [34]. For autofluorescence analysis, we withdrew a 2-mL sample and diluted it to a particle concentration with the OD_{730} of about 0.08, which is suitable for counting in the FACSAria flow cytometer (BD Biosciences, CA, U.S.), and the FC had an air-cooled 20-mW argon ion laser. FC settings were: counting speed, 300 to 400 events/s; counted events for each sample, 10,000; excitation and emission wavelengths, 488 nm and > 650 nm, respectively, to detect the autofluorescence from chlorophyll [27, 28].

2.3. SYTOX Green staining and fluorescence detection

We adapted SYTOX Green (SG) (Invitrogen, Carlsbad, CA) staining and flow cytometry (FC) to differentiate heterotrophic bacteria from cell debris in the non-chlorophyll particles. At the noted time, we withdrew a second 2-mL sample, mixed it with 1 μL SG, and then allowed them to react in a rocker mixer (Lab-Line, TX, U.S.) for 15 min in the dark. We used non-chlorophyll particles directly (without SG) to zero the fluorescent intensity (FI). After staining, we detected the biomass's fluorescence using FC. The FC settings were the same as for FAC, except that we changed the emission wavelength to a band of 510–550 nm to detect SG's emission. We performed data analysis and generated graphical outputs using FlowJo 7.6.1 software (Treestar, Inc., San Carlos, CA, U.S.).

2.4. Procedures to develop and verify the method

Fig. 1 illustrates the fluorescence-based protocol for developing and verifying the method to detect heterotrophic bacteria in a culture of *Synechocystis*. In Step I, we applied FC to characterize the autofluorescence spectrum of a non-axenic culture of *Synechocystis* withdrawn from the 500-mL Erlenmeyer flasks after incubation for 3 days. Based on the spectrum from Step I, we used Step II to separate particles having low Fluorescence Intensity (FI) (i.e., putative heterotrophs) based on autofluorescence. We set the FACS cutoff at the following cumulative percentages from the low-FI end of the spectrum: 40%, 35%, 30%, 25%, 20%, 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2% and 1% of the total distribution.

In Step III, we used light microscopy to observe the morphology of particles for each of the low-FI fractions. Large particles containing pigmentation were *Synechocystis*, while smaller non-pigmented particles were heterotrophic bacteria or cell debris. One goal was to identify the fraction below which *Synechocystis* cells were no longer detectable, which provided the threshold for detecting *Synechocystis*. Fractions below this point contain only heterotrophic bacteria or cell debris. We define the fraction of non-chlorophyll particles as R_{LP} (%). Using sorting results from a higher-FI fraction, we verified that the proportion of the non-pigmented particles in the mixed culture (i.e., R_{LP}).

In Step IV, we differentiated heterotrophic bacteria from cell debris using SG and FC. SG binds to nucleic acids (NA). The extracellular polymeric substances (EPS) of intact heterotrophic bacteria contain a small amount of DNA that binds with SG and emits enough fluorescence that they can be detected by FC [17, 18]. The DNA content of cell debris was much lower than that of intact bacteria [35], which led to a much lower fluorescence intensity from cell debris. Thus, after staining with SG dye, the high-fluorescence peak in the fluorescence spectra

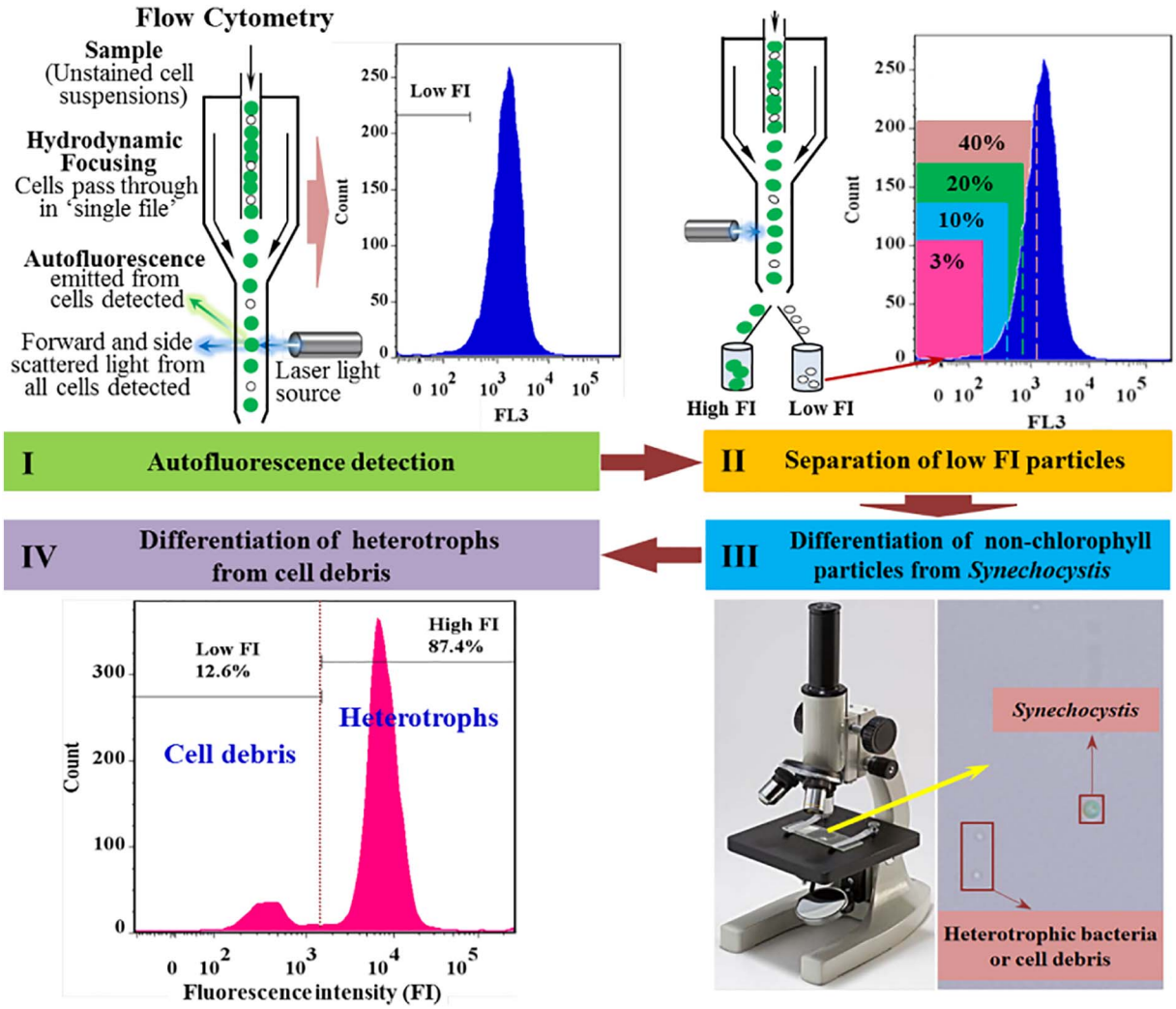

Fig. 1. The fluorescence- and morphology-based separation protocol for the quantification of heterotrophic bacteria in a culture of *Synechocystis*.

represented heterotrophic bacteria, while the low-fluorescence peak represented cell debris; we obtained their proportions directly from the fluorescence spectra. We define the fraction of non-chlorophyll particles that are heterotrophic bacteria as $R_{H\text{-}LP}$ (%).

Based on the fraction of non-chlorophyll particles in the mixed culture and the proportion of this fraction being heterotrophic bacteria, we estimated the quantity of heterotrophic bacteria in the mixed culture (R_H, %) using Eq. (1).

$$R_H = R_{LP} \cdot R_{H-LP} \quad (1)$$

2.5. Analytical methods

Sample OD was measured using a UV–vis BioSpec-mini spectrometer at 730 nm (Shimadzu Corp., Japan), and dry weight (DW) was assayed as total suspended solids, Method 2540D in *Standard Method* [36]. We carried out triplicate assays of OD and DW, and results are expressed as the mean and standard deviation of the three measured samples (mean ± SD). We separated the bulk solution (BS) from the mixed culture by centrifuged the sample at 4000 rpm and 4 °C for 15 min and further centrifuged the supernatant at 12000 rpm (Microfuge® 22R Centrifuge, Beckman Coulter, CA, USA) and 4 °C for 10 min to remove all particles, and we measured the residual phosphorus in the BS using HACH TNT843 kits. The morphology of particles was observed using an optical microscope (BX61, Olympus Corporation, Germany), with images captured by a digital camera (DP70, Olympus Corporation, Germany) with 2000× magnification. When presenting the results of *Synechocystis* cell morphology, we show one representative result for each sample, although we evaluated two microscopic images for each fraction proportion. The images were nearly identical.

3. Results and discussion

3.1. Autofluorescence characteristics of Synechocystis

Presenting the results of Step I, Fig. 2 shows the FC autofluorescence characteristics and the corresponding intensity for a *Synechocystis* culture. Most of the counts concentrated in the region with autofluorescence intensity (AFI) of 300 to 4000 Fluorescence Intensity Units (FIU) (panel (a)). This means that most of the particles had similar cell granularity and autofluorescence characteristics. Panel (b) presents the corresponding autofluorescence spectra and various cutoff fractions based on the auto-emission FI (AFI) for autofluorescing *Synechocystis*. Only 3% of particles had < 150 FIU, 8% < 300 FU, and 10% < 550 FIU.

In Step II, we employed FACS to distinguish when the low-AFI particles no longer contained any *Synechocystis*. The cut offs were the AFIs that contained the lowest 40%, 35%, 30%, 25%, 20%, 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2% or 1% of the particles (Fig. 2(b)). These fractions were then evaluated using microscopy to differentiate particles that contained chlorophyll versus those without chlorophyll (Step III). The goal was to find the threshold AFI having no *Synechocystis*.

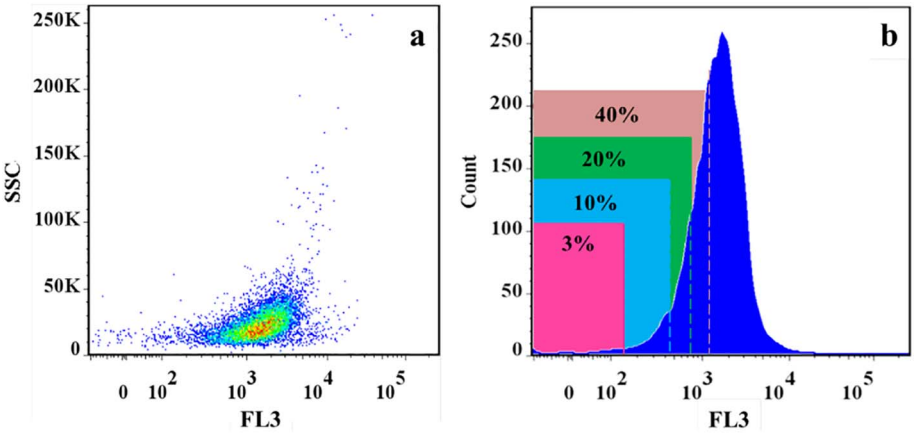

Fig. 2. FC results for the (a) autofluorescence characteristics and (b) corresponding autofluorescence spectra of *Synechocystis* (without staining by SYTOX Green (SG) dye). The side scatter (SSC) refer to the cell granularity; FL3 is detected autofluorescence at > 650 nm, representing chlorophyll in *Synechocystis*; count means the number of particles counted at specific autofluorescence intensity; and the square area with different colors designates the low AFI particles and was the criteria set for gating at different fractions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

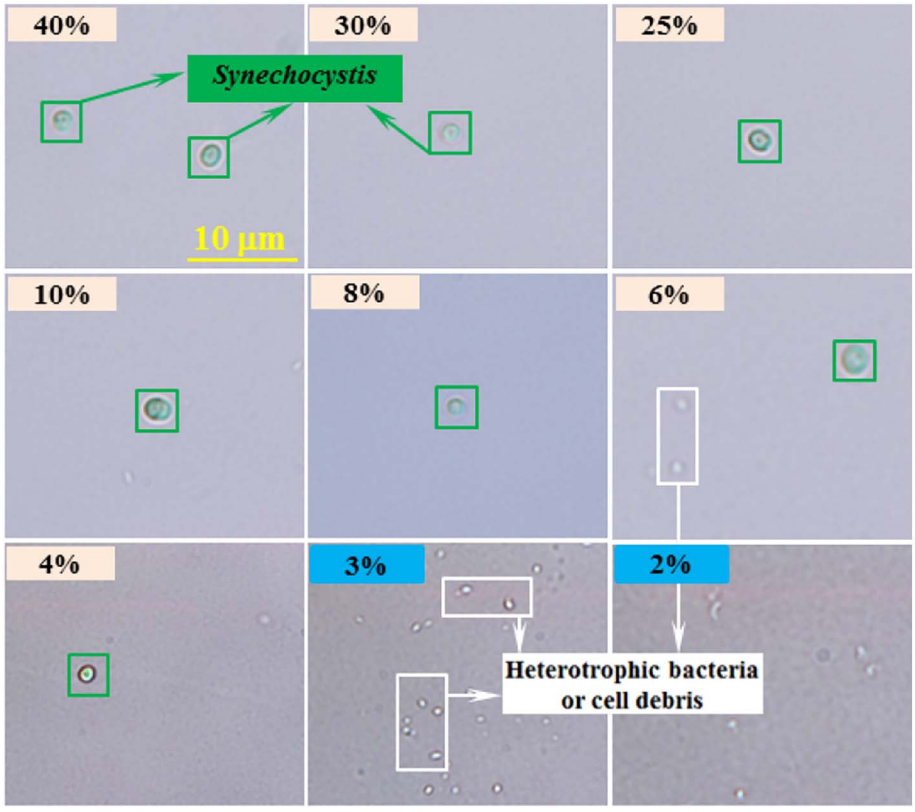

Fig. 3. Microscopic images of the separated culture with various proportions of low autofluorescence intensity (AFI) particles. The scale bars throughout the images are the same, 10 μm. The representative result is shown in Fig. S2.

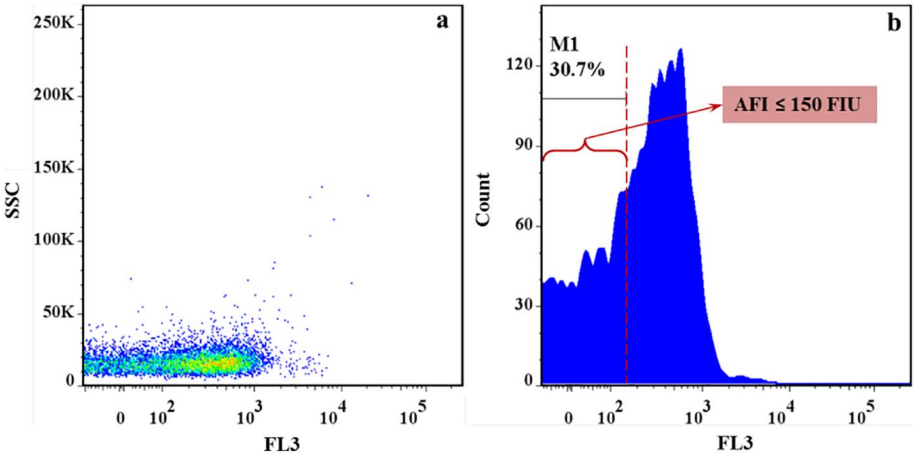

Fig. 4. FC results for the (a) autofluorescence characteristics and (b) corresponding autofluorescence spectra of the separated particles with the total fraction lower than 10%. FL3 is detected autofluorescence at > 650 nm, representing chlorophyll in *Synechocystis*; count means the number of particles counted at specific autofluorescence intensity.

3.2. Threshold for the low-AFI particles

Fig. 3 (representing the results of step III) shows microscopic images of the cells separated into the low-AFI fractions, and the other set of results is shown in Fig. S2. *Synechocystis* are green-colored cocci having a diameter of 1 to 2 μm, and heterotrophic bacteria and cell debris are smaller particles without color [25]. The fraction of *Synechocystis* cocci clearly decreased as the AFI fraction declined, and the fraction of heterotrophic bacteria or cell debris increased. *Synechocystis* cocci were no longer detectable when the fraction captured was lower than 3% (i.e., AFI ≤ 150 FIU); only non-pigmented particles existed in the microscopic images. Thus, we established ≤150 FIU as the threshold for detecting only heterotrophic bacteria or cell debris. This threshold provides an estimate of the fraction of particle that are not *Synechocystis*: R_{LP} = 3%.

In order to confirm the estimate of R_{LP} = 3%, we retested the autofluorescence of particles separated into the fraction containing the lowest 10% (Fig. 2(B)), which increased the resolution of the results at lower FIU. Fig. 4 shows the autofluorescence characteristics and the corresponding autofluorescence spectra. 31% of the separated particles had an AFI lower than 150 FIU. Since we retested the 10% fraction, its having 31% non-chlorophyll particles confirms that the proportion of particles with the AFI lower than 150 FIU in the mixed culture was ~3%.

3.3. Differentiation of heterotrophic bacteria and cell debris in low AFI particles

Fig. 5 (representing step IV) shows the SG-fluorescence characteristics of particles with an AFI lower than 150 FIU from the separated culture. Fig. 5(a) shows two fluorescence regions: low fluorescence (FI < 1600 FIU) and high fluorescence (FI > 1600 FIU). SG bound with nucleic acid (NA) in the EPS bound to heterotrophs [19] resulted in the higher intensity SG fluorescence, but cell debris had such low NA content that fluorescence intensity from SG binding was small [35]. Thus, the low-fluorescence region represents cell debris, and the high-fluorescence region represents intact heterotrophic bacteria. For the separated culture with AFI ≤ 150 FIU, the fractions of heterotrophic bacteria and cell debris were 87% and 13%, respectively. Since particles with an AFI lower than 150 FIU were 3.1% of the total counts, heterotrophic bacteria and cell debris accounted for 2.7% and 0.4% of the particles in the *Synechocystis* culture, respectively.

In our study, autofluorescence intensity ≤150 FIU was the threshold for detecting only heterotrophs or cell debris, while and autofluorescence intensity > 1600 FIU was the threshold to differentiate heterotrophs from the non-chlorophyll particles for the quantification of heterotrophs. These thresholds may not be optimal for other species of microalga, as the concentrations of chlorophyll and DNA differ.

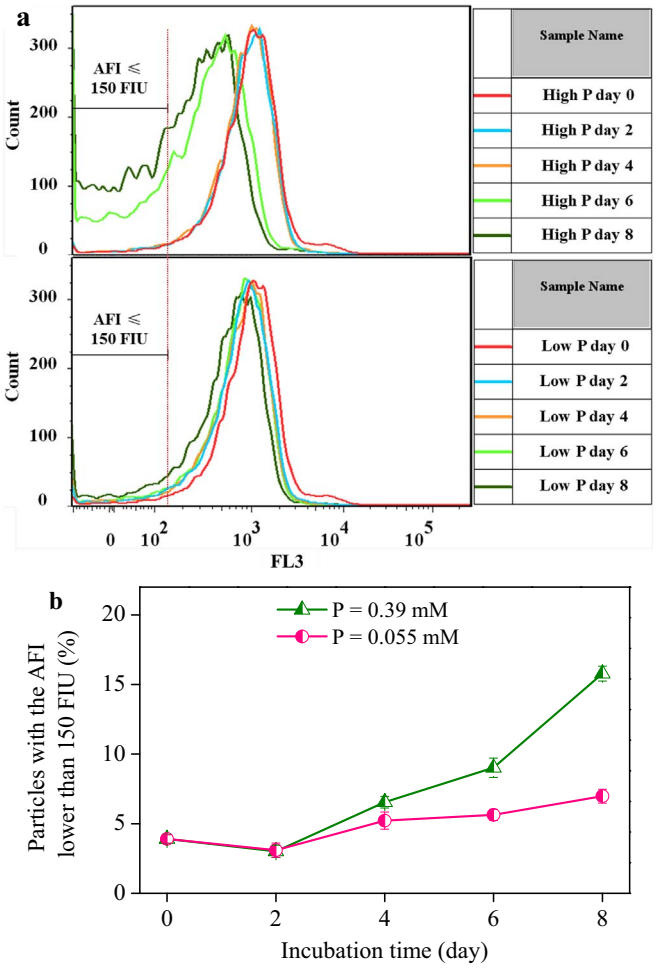

Fig. 6. (a) Fluorescence spectra of *Synechocystis* over time and (b) the proportion of particles with the AFI lower than 150 FIU for the two starting P concentrations at the noted incubation times.

Recognizing that the threshold value likely will vary, we emphasize the importance of our method for finding the threshold value over the particular threshold values we obtained here. Using our method, researchers can define thresholds for their own microalgae species. Thus, we lay the foundation for anyone to quantify the heterotrophs in any bacteria-algae symbiotic system.

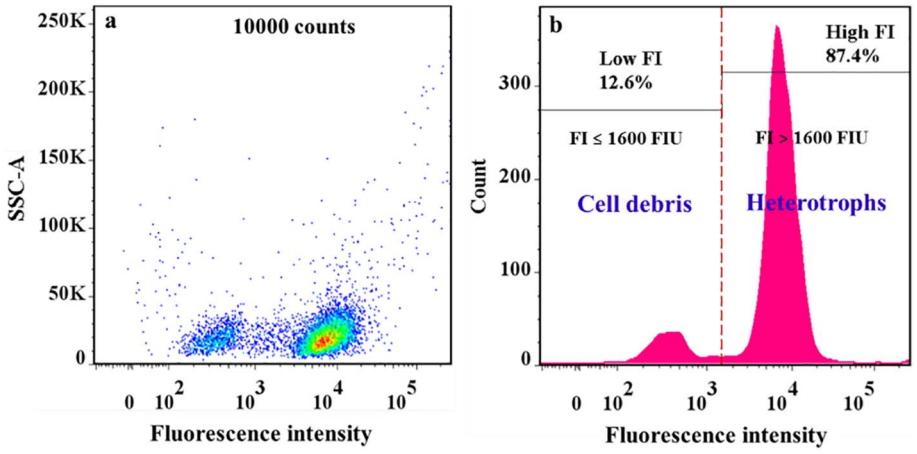

Fig. 5. FC results for (a) fluorescence characteristics and (b) the corresponding spectrum of NA-complexed SG of the particles with AFI lower than 150 FIU in the separated culture. The dashed line in panel b signifies the fluorescence intensity boundary for heterotrophic bacteria and cell debris.

3.4. Autofluorescence of Synechocystis growth under various P concentrations

Fig. 6 shows the autofluorescence spectra of *Synechocystis* cultures every 2 days during batch growth with high or low P concentrations; Fig. S3 in SI shows the corresponding fluorescence characteristics of *Synechocystis*; Figs. S4 and S5 in SI represent the biomass dry weight and the residual P concentration in the bulk solution for the two initial P concentrations during batch-growth experiments, respectively. Starting P concentration had little effect on the final dry weight (DW) of the cultures, both of which increased from 165 mg/L to 2280 mg/L over the eight days (Fig. S4). For the high-P culture, the residual P gradually declined, but was not depleted; it reached its minimum concentration of 1.4 mg/L after 8 d. In contrast, the residual P concentration of the low-P culture approached zero by day 2 (Fig. S5).

For the high-P culture, the AFI distribution remained stable and similar to that of the low-P culture during the first 4 days, but it shifted towards lower FIU after 4 days (Figs. 6 and S3). The fraction of the non-chlorophyll particles (≤150 FIU) increased and reached about 16% by 8 days (Fig. 6(b)).

For the low-P culture, most of the particles were in the region with FI of 300–4000 FIU throughout the experiment, and the peak of the region shifted only slightly to the left on day 8 (Figs. 6 and S3). The fraction of non-chlorophyll particles (≤150 FIU) slightly decreased during the initial 2 days, slowly increased thereafter, but reached only ~8% at 8 days (Fig. 6(b)). Thus, depletion of residual inorganic P minimized the accumulation of heterotrophic bacteria and cell debris. This difference suggests that *Synechocystis* was able to out-compete the heterotrophic bacteria for inorganic P, possibly due to their capability for rapid uptake and sequestration of inorganic P [11, 37, 38].

3.5. Flow cytometry analysis of non-chlorophyll particles staining with SG dye

Fig. 7 shows the fluorescence spectra of NA-complexed SG of the non-chlorophyll particles (≤150 FIU) and the fractions of heterotrophic bacteria and cell debris in *Synechocystis* cultures with high and low P concentrations; Fig. S6 presents the corresponding SG-fluorescence characteristics. During growth of *Synechocystis*, the distribution of particles shifted from the high-fluorescence region (FI > 1600 FIU) to the low-fluorescence region (FI < 1600 FIU) for both P concentrations, indicating an increase in cell debris due to cell lysis. The fractions of cell debris were similar in the initial 4 days, but cell debris became relatively more important in the low-P culture after 4 days (Fig. 7(b)). This trend suggests that P depletion (after day 2 in the low-P culture) accentuated cell lysis.

3.6. Quantification of heterotrophic bacteria and cell debris

Fig. 8 shows the fraction of the heterotrophic bacteria calculated from the fluorescence-based method over time for the two P concentrations. Due to the strong growth of *Synechocystis* from day 0 to day 2, the fraction of heterotrophic bacteria decreased from day 0 to day 2, and only minimal cell debris existed in this stage. After day 2, heterotrophic bacteria and cell debris increased. Although heterotrophs continued to increase, up to 12.6%, with high P, they stabilized at ~5% with low P. Cell debris kept increasing in both cultures. These trends reinforce the interpretations that low P favored *Synechocystis* over heterotrophs, but also accelerated cell lysis.

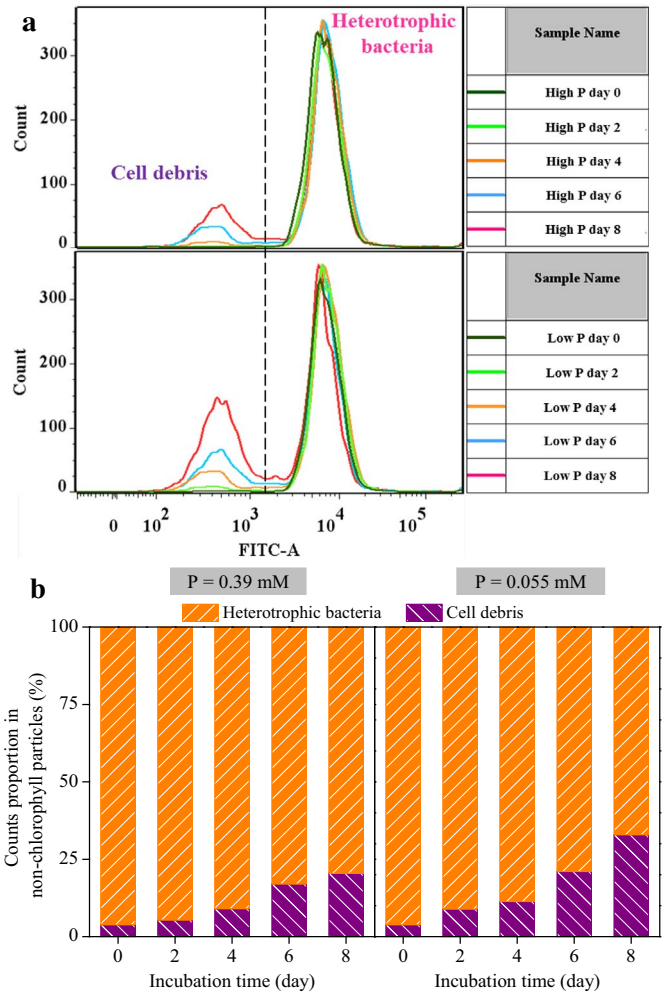

Fig. 7. (a) Fluorescence spectra of NA-complexed SG of the particles (AFI lower than 150 FIU) and (b) the proportion of heterotrophic bacteria and cell debris in the non-chlorophyll particles with various P concentrations.

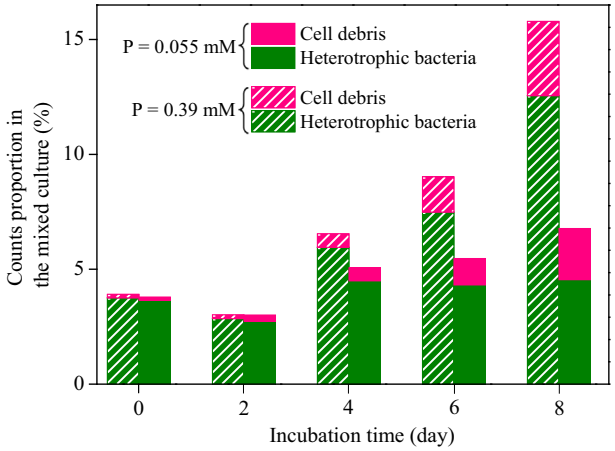

Fig. 8. Counts proportion of heterotrophic bacteria calculated based on the fluorescence method in the growth of *Synechocystis* under various P concentrations at the noted incubation time.

4. Conclusions

During the growth of microalgal biomass, heterotrophic bacteria can dilute the cyanobacterial content of the harvested biomass, compete for nutrients, or be associated with culture crashes. Being able to detect and quantify heterotrophic bacteria would be of high value for monitoring culture health and reducing deleterious effects. In this study, we developed and applied a new method that combines FC and FACS for the quantification of heterotrophic bacteria in cultures of cyanobacteria. Particles not containing chlorophyll – heterotrophic bacteria and cell debris – were separated from mixed cultures using

FACS based on autofluorescence of *Synechocystis*. Heterotrophic bacteria were differentiated from cell debris using FC with SYTOX green fluorescence. Using microscopy, we verified that FACS was able to quantify heterotrophic bacteria in *Synechocystis* cultures effectively. Applying these methods to batch cultures of *Synechocystis* showed that the count proportions of heterotrophic bacteria were significant (3–13%) and that depletion of inorganic P in the culture favored *Synechocystis* over heterotrophic bacteria, but led to more cell lysis.

Author contributions statement

Yun Zhou contributed to conception of the experiments, performing experiments, analysis and interpretation of the data, and writing the manuscript. Everett Eustance contributed to interpretation of the performing experiments, interpretation of the data and revising the manuscript. Levi Straka contributed to analysis and interpretation of the data and revising the manuscript. YenJung Sean Lai contributed to analysis and interpretation of the data and revising the manuscript. Siqing Xia contributed to conception and design of experiments, interpretation of the data and writing the manuscript. Bruce E. Rittmann contributed to conception of the experiments, interpretation of the data, and writing the manuscript. Funding information was provided in Acknowledgement.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2018.01.006.

References

[1] D. Bilanovic, A. Andargatchew, T. Kroeger, G. Shelef, Freshwater and marine microalgae sequestering of CO_2 at different C and N concentrations – response surface methodology analysis, Energy Convers. Manag. 50 (2009) 262–267.
[2] H.W. Kim, A.K. Marcus, J.H. Shin, B.E. Rittmann, Advanced control for photoautotrophic growth and CO_2-utilization efficiency using a membrane carbonation photobioreactor (MCPBR), Environ. Sci. Technol. 45 (2011) 5032–5038.
[3] A.S. Zevin, T. Nam, B. Rittmann, R. Krajmalnik-Brown, Effects of phosphate limitation on soluble microbial products and microbial community structure in semi-continuous Synechocystis-based photobioreactors, Biotechnol. Bioeng. 112 (2015) 1761–1769.
[4] L. Straka, B.E. Rittmann, The role of heterotrophic bacteria in assessing phosphorus stress to *Synechocystis* sp. PCC6803, J. Appl. Phycol. (2017) 1–6.
[5] H. Wang, W. Zhang, L. Chen, J. Wang, T. Liu, The contamination and control of biological pollutants in mass cultivation of microalgae, Bioresour. Technol. 128 (2013) 745–750.
[6] P.J. Lammers, M. Huesemann, W. Boeing, D.B. Anderson, R.G. Arnold, X. Bai, M. Bhole, Y. Brhanavan, L. Brown, J. Brown, Review of the cultivation program within the National Alliance for Advanced Biofuels and Bioproducts, Algal Res. 22 (2017) 166–186.
[7] B.T. Nguyen, B.E. Rittmann, Electron partitioning in soluble organic products by wild-type and modified *Synechocystis* sp. PCC 6803, Biomass Bioenergy 90 (2016) 237–242.
[8] C.S. Laspidou, B.E. Rittmann, A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass, Water Res. 36 (2002) 2711–2720.
[9] K.A. Berg, C. Lyra, K. Sivonen, L. Paulin, S. Suomalainen, P. Tuomi, J. Rapala, High diversity of cultivable heterotrophic bacteria in association with cyanobacterial water blooms, ISME J. 3 (2009) 314.
[10] M. Danger, C. Oumarou, D. Benest, G. Lacroix, Bacteria can control stoichiometry and nutrient limitation of phytoplankton, Funct. Ecol. 21 (2007) 202–210.
[11] Y. Zhou, B.T. Nguyen, C. Zhou, L. Straka, Y.S. Lai, S. Xia, B.E. Rittmann, The distribution of phosphorus and its transformations during batch growth of *Synechocystis*, Water Res. 122 (2017) 355–362.
[12] K.K. Rashidan, D.F. Bird, Role of predatory bacteria in the termination of a cyanobacterial bloom, Microb. Ecol. 41 (2001) 97–105.
[13] W.K.W. Li, Macroecological patterns of phytoplankton in the northwestern North Atlantic Ocean, Nature 419 (2002) 154–157.
[14] P. Hyka, S. Lickova, P. Přibyl, K. Melzoch, K. Kovar, Flow cytometry for the development of biotechnological processes with microalgae, Biotechnol. Adv. 31 (2013) 2–16.
[15] C.D. Sinigalliano, J. Winshell, M.A. Guerrero, G. Scorzetti, J.W. Fell, R.W. Eaton, L. Brand, K.S. Rein, Viable cell sorting of dinoflagellates by multiparametric flow cytometry, Phycologia 48 (2009) 249–257.
[16] M. Cellamare, A. Rolland, S. Jacquet, Flow cytometry sorting of freshwater phytoplankton, J. Appl. Phycol. 22 (2010) 87–100.
[17] J.L. Collier, Flow cytometry and the single cell in phycology, J. Phycol. 36 (2000) 628–644.
[18] I. Vermes, C. Haanen, C. Reutelingsperger, Flow cytometry of apoptotic cell death, J. Immunol. Methods 243 (2000) 167–190.
[19] Y. Zhou, B.T. Nguyen, Y.S. Lai, C. Zhou, S. Xia, B.E. Rittmann, Using flow cytometry to evaluate thermal extraction of EPS from *Synechocystis* sp. PCC 6803, Algal Res. 20 (2016) 276–281.
[20] H. Zipper, H. Brunner, J. Bernhagen, F. Vitzthum, Investigations on DNA intercalation and surface binding by SYBR green I, its structure determination and methodological implications, Nucleic Acids Res. 32 (2004) e103.
[21] J. Sheng, R. Vannela, B.E. Rittmann, Evaluation of cell-disruption effects of pulsed-electric-field treatment of *Synechocystis* PCC 6803, Environ. Sci. Technol. 45 (2011) 3795–3802.
[22] B.-H. Liu, Y.-K. Lee, Secondary carotenoids formation by the green alga *Chlorococcum* sp, J. Appl. Phycol. 12 (2000) 301–307.
[23] J. Toepel, C. Wilhelm, A. Meister, A. Becker, M. del Carmen Martinez-Ballesta, Cytometry of freshwater phytoplankton, Methods Cell Biol. 75 (2004) 375–407.
[24] T. Leya, A. Rahn, C. Lütz, D. Remias, Response of arctic snow and permafrost algae to high light and nitrogen stress by changes in pigment composition and applied aspects for biotechnology, FEMS Microbiol. Ecol. 67 (2009) 432–443.
[25] M.J.W. Veldhuis, G.W. Kraay, Application of flow cytometry in marine phytoplankton research: current applications and future perspectives, Sci. Mar. 64 (2000) 121–134.
[26] C.S. Yentsch, C.M. Yentsch, Single cell analysis in biological oceanography and its evolutionary implications, J. Plankton Res. 30 (2007) 107–117.
[27] G.H. Krause, E. Weis, Chlorophyll fluorescence and photosynthesis: the basics, Annu. Rev. Plant Biol. 42 (1991) 313–349.
[28] G.C. Papageorgiou, Chlorophyll a Fluorescence: A Signature of Photosynthesis, Springer Science & Business Media, 2007.
[29] U. Schreiber, W. Bilger, C. Neubauer, Chlorophyll fluorescence as a nonintrusive indicator for rapid assessment of in vivo photosynthesis, Ecophysiology of Photosynthesis, Springer, 1995, pp. 49–70.
[30] V. Dashkova, E. Segev, D. Malashenkov, R. Kolter, I. Vorobjev, N.S. Barteneva, Microalgal cytometric analysis in the presence of endogenous autofluorescent pigments, Algal Res. 19 (2016) 370–380.
[31] R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdman, R.Y. Stanier, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, Microbiology 111 (1979) 1–61.
[32] B.T. Nguyen, B.E. Rittmann, Effects of inorganic carbon and pH on growth kinetics of *Synechocystis* sp. PCC 6803, Algal Res. 19 (2016) 363–369.
[33] B.T. Nguyen, B.E. Rittmann, Predicting dissolved inorganic carbon in photoautotrophic microalgae culture via the nitrogen source, Environ. Sci. Technol. 49 (2015) 9826–9831.
[34] P.H. Burkill, R.F.C. Mantoura, M. Cresser, The rapid analysis of single marine cells by flow cytometry [and discussion], Philos. Trans. R. Soc. Lond. A Math. Phys. Eng. Sci. 333 (1990) 99–112.
[35] K. Shortman, N. Williams, P. Adams, The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell debris, damaged cells and erythroid cells from lymphoid cell suspensions, J. Immunol. Methods 1 (1972) 273–287.
[36] A.P.H. Association, Standard Methods for the Examination of Water and Wastewater, 1268 American Public Health Association, Washington, DC, 1998.
[37] B. Yao, B. Xi, C. Hu, S. Huo, J. Su, H. Liu, A model and experimental study of phosphate uptake kinetics in algae: considering surface adsorption and P-stress, J. Environ. Sci. 23 (2011) 189–198.
[38] Z. Yuan, S. Pratt, D.J. Batstone, Phosphorus recovery from wastewater through microbial processes, Curr. Opin. Biotechnol. 23 (2012) 878–883.