Dry Weight and Cell Density of Individual Algal and Cyanobacterial Cells for Algae Research and Development

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

Dry Weight and Cell Density of Individual Algal and Cyanobacterial Cells for Algae Research and Development

presented by Wenna Hu,

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and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Zhiqiang Hu

Professor Enos C. Inniss

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DEDICATION

I dedicate this thesis to my beloved parents, whose moral encouragement and support help me earn my Master's degree.

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List of Abbreviations

OD	Optical Density
SPC	Solid Phase Cytometry
q-PCR	Quantitative Polymerase Chain Reaction
SRT	Solids Retention Time
ADP	Adenosine Diphosphate
A- MBR	Algal-based Membrane Bioreactor
TOC	Total Organic Carbon
CSTR	Continuous Flow Stirred-tank Reactor
FS	Forward Light Scatter
SS	Side Light Scatter
BBM	Bold's Basic Medium
R2A	Reasoner's 2A Agar
CFU	Colony Forming Unit

Abstract

Phototrophs have received more and more attention due to their nutrient removal, biomass production as well as the potential for biofuel feedstock. This research determined the dry weight of algal and cyanobacterial cells and compared with that of regular bacteria. For microbes growing during exponential phase in batch studies, the average dry weights of green alga *Chlorella vulgaris*, cyanobacterium *Microcystis aeruginosa* and facultative bacterium *E.coli* were $(2.24 \pm 0.16) \times 10^{-11}$ g/cell, $(1.21 \pm 0.09) \times 10^{-11}$ g/cell and $(1.69 \pm 0.36) \times 10^{-13}$ g/cell, *respectively*. By using fluorescent microscopy, the average sizes of *Chlorella vulgaris* and *Microcystis aeruginosa* were (4.34 ± 0.90) μm and (2.29 ± 0.61) μm, respectivily. It is obvious that the dry weight of individual cells increases as cell size increase. Correspondingly, the average density of single cells were (0.57 ± 0.04) g/cm^3 and (0.87 ± 0.02) g/cm^3 for algae and cyanobacteria, respectively. In a continuous flow algal cultivation system, individual algal cell weight was $(3.91 \pm 0.26) \times 10^{-11}$ g/cell and cell density was (0.51 ± 0.07) g/cm^3 with an average cell size of (5.28 ± 1.33) μm under steady-state conditions. The results could be used for the estimation of algal biomass production for algae research and development.

1. Introduction

1.1 Microalgae and Cyanobacteria

Microalgae and cyanobacteria are the primary producers of organic matter in aquatic environments due to CO_2 fixation during photosynthesis. They are mostly found in oceans, lakes, rivers and salt ponds and are able to grow and live in terrestrial environments and harsh conditions as well (Rodríguez-Palacio, Cristóstomo-Vázquez, Álvarez-Hernández, & Lozano-Ramírez, 2012). Microalgae are unicellular eukaryotic organisms, some of which form colonies resulting in green colonies that are visible to naked eye. Cyanobacteria, often known as blue-green algae, are oxygenic photosynthetic bacteria which contain blue-green and green pigments (WHO, 2009). Cyanobacteria lack phycobilins but have chlorophyll a and b, giving them a more green than blue color (Parmar, Singh, Pandey, Gnansounou, & Madamwar, 2011).

Microalgae and cyanobacteria are the only known organisms that are capable of both oxygenic photosynthesis and hydrogen production. Thus, these organisms have the potential to produce renewable energy and serve as alternative sources for biodiesel from nature's most plentiful resources: water and solar energy (Parmar et al., 2011). Nevertheless, a series of problems have occurred worldwide due to water pollution

associated with algae and cyanobacteria bloom, such as in lakes and reservoirs, and drinking water supplies, which are harmful to human health.

1.2 Classification

1.2.1 Microalgae Classification

Algae have been classified in terms of various parameters such as pigments, flagella, reserve material, shape and cell wall composition. Based on the different chlorophylls and photosynthetic pigments they contain, algae can be classified into seven taxonomic groups (Rittmann, 2001). Table 1.1 summarizes the groups of algae and their characteristics, such as cell covering and habitat (Sze, 1998).

Table 1.1: Algae classification and characteristics

Algae group	Chlorophylls	Outer chloroplast membranes	Habitat
Cyanophyta	a,b	0	Marine, freshwater, soil
Chlorophyta	a,b	2	Marine, freshwater, soil
Chrysophyta	a,c,e	4	Marine, freshwater, soil
Euglenophyta	a,b	3	Mostly freshwater
Phaeophyta	a,c	4	Marine
Pyrrophyta	a,c	3	Marine, freshwater
Rhodophyta	a,d	2	Marine

1.2.2 Classification for Cyanobacteria

Cyanobacteria are classified into five groups by morphology. The first three, *Chroococcales*, *Pleurocapsales* and *Oscillatoriales*, are not supported by phylogenetic studies. However, the latter two, *Nostocales* and *Stigonematales*, are monophyletic and make up the heterocystous cyanobacteria which are nitrogen-fixing bacteria formed during N_2 starvation (Lele, 2007). Heterocystous species dominate N_2 fixation in

freshwater and brackish environments. By protecting nitrogenase from being inactivated by O_2, heterocystous cyanobacteria fix N_2 and perform photosynthesis simultaneously (Miller, Cloutier, & Turner, 2003).

1.3 Cell Morphology

Individual size, is a fundamental indicator of cell state, affecting most aspects of a cell since allometric relationships link cell size to processes, such as nutrient uptake, photosynthesis and respiration, settling rates and physical/chemical transport. Hence, cell morphology and size are potentially suitable for environmental monitoring (Jacson, 1989).

1.3.1 Microalgae

Most microalgae are in micrometer size. For instance, *Chlorella vulgaris*, a green microalgae with spherical shape, is 2 to 10 μm in diameter (Havlik et al., 2013; VanderGheynst, Guo, Cheng, & Scher, 2013). In terms of cell morphology, microalgae may be spherical (Figure 1.1, left panel), rod-shaped, spindle-shaped or club-shaped. They may be in groups like filamentous clusters or as individual strands that are either branched (Figure 1.1, right panel) or unbranched (Mata, Martins, & Caetano, 2010).

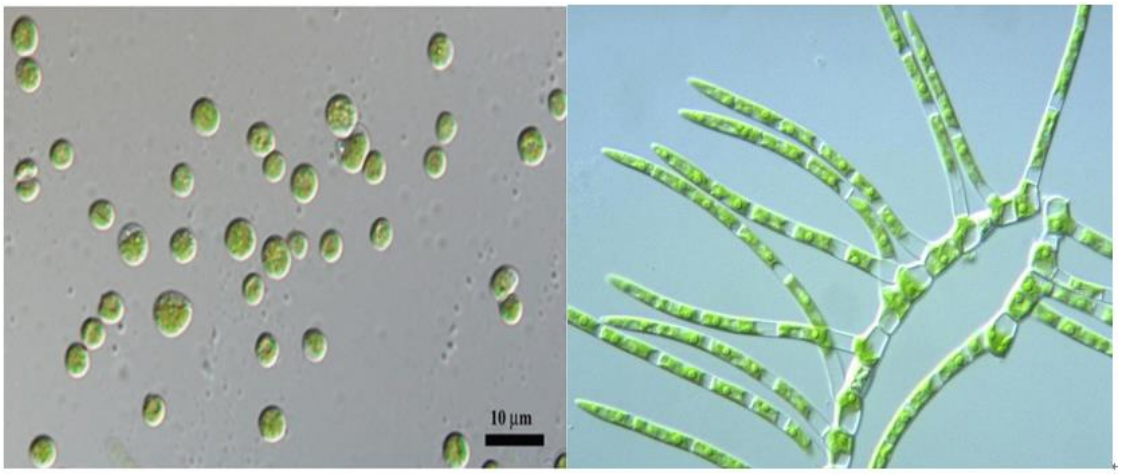


Figure 1.1 Images of unicellular (left) and multicellular (right) microalgae (Held, 2011; Rumora, 2011)

As photosynthetic eukaryotes, microalgae typically contain flagellum, cell wall, cell membrane, nucleus, nucleolus, cytoplasm, mitochondrion, Golgi apparatus, centrioles, ribosomes, starch vacuole and a chloroplast (Figure 1.2) (Johnson et al., 2013). The chloroplast contains circular DNA and the specific chlorophylls responsible for photosynthesis. All microalgae contain chlorophyll *a* but some contain other types of chlorophyll (Table 1.1). Each type of chlorophyll is efficient in absorbing a certain range of wavelengths in the light spectrum (Mata et al., 2010). Chloroplast contains two membranes: outer membrane, which is permeable, and an inner one, which is less permeable (Figure 1.2 right panel). In green algae, thylakoids are staked into discrete structural units called granum. Stroma is a connective tissue found in the loosely connected area (Madigan, 2005).

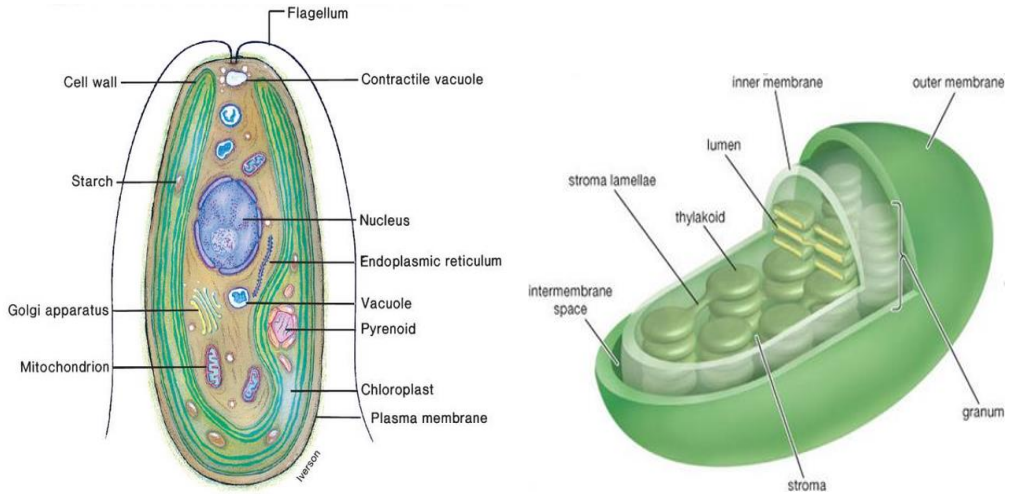


Figure 1.2 Microalgal cell (left) and chloroplast (right) structure (Madigan, 2005; Rittmann, 2001)

1.3.2 Cyanobacteria

Cyanobacteria include unicellular (Figure 1.3, left panel) and filamentous forms (Figure 1.3, right panel) and vary from spherical, oval, fusiform and rod-like, to irregular in shape (Lele, 2007). Cell size ranges from 0.5 to 60 μm (Srivastava, 2013).

Figure 1.3 Images of unicellular (left) and filamentous (right) cyanobacteria (NIES, 2013; Tsukki, 2013)

A cyanobacterial cell has a peptidoglycan cell wall and sandwiched between cytoplasmic and outer membranes (Figure 1.4) (Srivastava, 2013). As prokaryotes, cyanobacteria do not have nuclear envelope or a true nucleus, but rather have a nucleoid.

The cytoplasm contains the photosynthetic apparatus, called thylakoids, which contains the phycobillisomes (Khan, 2013).

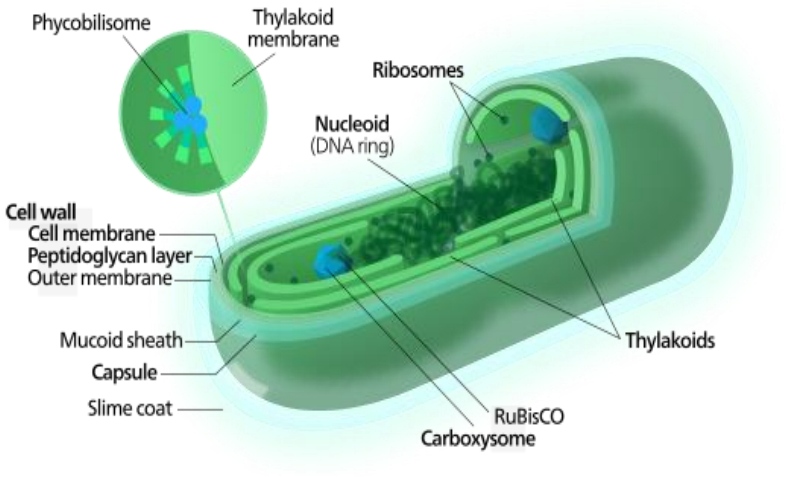


Figure 1.4 Intracellular membranes and compartments in a cyanobacterial cell (Kelvinsong, 2013)

1.4 Methods for Microbial Cell Counting

Microbial cell counting, or cell enumeration, is necessary to determine the number of cells in a milliliter (mL) of liquid or in a gram (g) of solid material (Tortora, 2007). Counting cells in culture has two major applications. The first is to estimate the size of the cultured algal and cyanobacterial population. Although many alternative estimates of population size are available, such as biomass, wet or dry weight, chlorophyll content and organic nitrogen content, these indirect measurements do not determine the number of individual cells in a population (Andersen, 2005). For instance, cell counting is an essential component of cell quota which gives the average single cellular content of some constituents, such as nitrogen and phosphorous (Andersen, 2005). The second application of cell counting is to estimate the microbial growth rate. For instance, the

early detection of increasing cell numbers of microalgae and cyanobacteria would allow mitigation measures to be taken in time to prevent a bloom and subsequent ecological damage. Counting microscopic cells is challenging since 1 mL of liquid contains millions of cells (Katase, Tsuboi, & Tsumura, 2013). Several direct and indirect methods for cell enumeration are summarized in the following sections:

1.4.1 Spectrophotometry

Spectrophotometry is a convenient indirect method to record the optical density (OD) value at a specific wavelength. OD is represented in terms of transmittance, which can be determined by Beer-Lambert Law of Absorbance (Adrien, 1998). The Beer-Lambert law relates the concentration of a sample to the attenuation of light as it passes through the sample, so that it is capable of proportionaly correlating cell concentration ([x], cells/mL) with OD (Myers, 2013).

$$[X] = k \times OD$$

For a given culture sample, a good spectrophotometer should yield a linear relationship between cell number and the absorbance.

Both microalgae and cyanobacteria have chlorophyll a, so spectrophotometry is widely applied in phototrophic research. Chlorophyll a absorbs the light with wavelength of 680 nm through the suspension of cells, and the amount of absorbed light is proportional to the cell density or cell number in suspension by using a standard curve (M. J. Griffiths,

C. Garcin, R. P. van Hille, & S. T. Harrison, 2011). In addition to cell number, the amount of light absorbed is also a function of cell morphology. Consequently, an independent calibration curve is required for each phototrophic study, as the cell size and shape are related to the specific growth rate and the nutrient composition (Biesta-Peters, Reij, Joosten, Gorris, & Zwietering, 2010).

Although counting microbial cells by spectrophotometry is rapid and nondestructive, it is not an ideal method since sensitivity is a drawback. For instance, the sensitivity is limited to about 10^7 cells per mL for most bacteria (Nair, 2005).

1.4.2 Hemocytometry

A hymocytometer is a special type of microscopic slide consisting of two chambers, which is divided into nine (1.0 mm × 1.0 mm) large squares with each area of 1 mm^2. The central counting area of the hemocytometer contains 25 large squares and each large square has 16 smaller squares (Figure 1.5). The chambers is 0.1 mm in height so that each square corresponds to a given volume, applied to count cells with a size of 2-30 μm and concentration of 10^4-10^7 cells/mL (Andersen, 2005). Suspensions should be dilute enough to ensure the cells do not overlap each other on the grid and distribute uniformly (Grigoryve, 2013). To avoid counting cell twice, only those cells on the lines of two sides of the large squares are counted with the microscope.

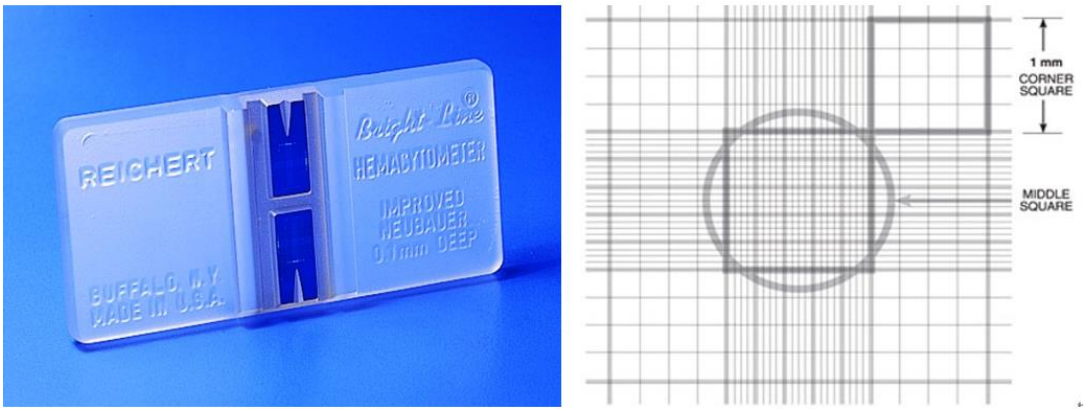


Figure 1.5 A hemocytometer (left) and the hemocytometer image at 100 × microscopic magnification (right) (SIGMA company)

Although hemocytometry is widely used due to its low cost and relatively easy operation, it is time consuming and labor intensive. Meanwhile, a significant amount of error can come from improper loading of the hemocytometer, inadequate dilution of the samples, and human error in counting the cells. Moreover, the measured counts are total cells since it is impossible to differentiate between live and dead cells.

In order to enumerate viable cells by hemocytometry, the LIVE/DEAD *Bac*Light viability assay kit has been widely employed for bacteria, composed of a mixture of two nucleic acid-binding stains: SYTO 9 and propidium iodide (PI) (Boulos, 1999). SYTO 9 is membrane permeable and stains all cells green, while PI penetrates cells whose cell membrane has been damaged, staining them red. However, this method cannot be used for microalgae and cyanobacteria since the autofluorescence of pigments such as chlorophyll obscures the PI signal. An alternative viability assay using

SYTOX Green may be applied to differentiate live and dead phototrophic cells. SYTOX Green is a fluorescent stain with a high affinity for nucleic acids that only penetrates damaged cell membranes. The three positive charges carried by the stain cause it to be excluded completely from living cells (Knowles & Castenholz, 2008). Once the stain binds with nucleic acids it fluoresces bright green, with excitation and emission maxima at 502 and 523 nm, respectively (Roth, 1997). A red fluorescence signal emitted by chlorophyll a with a maximum wavelength near 680 nm, often persists after a cell has been killed by membrane permeabilization. However, the strong green fluorescence of SYTOX Green bound to intact nucleic acids remaining in the cell overpowers the weaker red signal. As a result, the dead cell will appear bright green (Figure 1.6) (Knowles & Castenholz, 2008). Therefore, SYTOX Green fluorescence and autofluorescence can be used simultaneously as markers to count live and dead cells of microalgae and cyanobacteria.

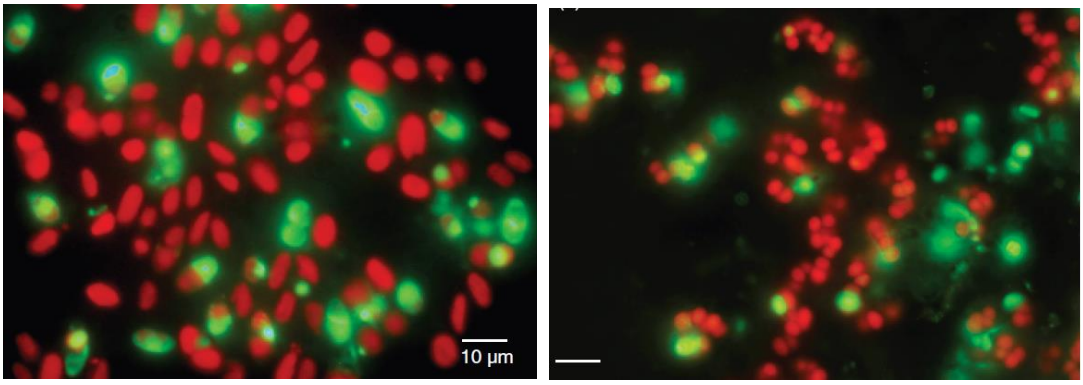


Figure 1.6 Epifluorescence images of cells showing SYTOX Green fluorescence (green) and autofluorescence (red) for green algae: *Chlorella* sp. (left) and cyanobacteria: *Chroococcidiopsis* sp. (right) (Knowles & Castenholz, 2008)

1.4.3 Solid Phase Cytometry (SPC)

SPC is a novel technique that detects and enumerates microorganisms at low cell concentrations (Haese, 2002). Prior to scanning, microbes are collected following membrane filtration and fluorescently labelled with an argon laser excitable dye. Next, the entire membrane filter surface is scanned and the fluorescent light emitted by labeled cells is detected by two photo multiplier tubes with wavelength 500-530 nm and 540-585 nm, respectively (Mandal, 2011). Meanwhile, fluorescent events are automatically counted by a laser scanning device. A set of discriminants are applied by software to differentiate viable microorganisms from electronic noise or non-target autofluorescent particles (Tobe, 2006). The technique therefore allows an accurate enumeration down to a detection limit of one cell per filter. After scanning, cells can be visualized by epifluorescence microscopy.

SPC is capable of rapid and accurately enumerating rare evens with similar precision to flow cytometry and is an alternative to traditional plate counting. By allowing analysis of the spatial and temporal dynamics of specific populations, SPC is widely applied in toxic algal quantification. For instance, cell counts of algal *P. parvum* are 2-8 $\times 10^3$ cells/mL by SPC after fluorescent in situ hybridization, close to the light microscopic cell counts of 6-9 $\times 10^3$ cells/mL. (Tobe, 2006).

1.4.4 Flow cytometry

Flow cytometry is a powerful tool for the analysis of multiple biological parameters of individual cells or particles within heterogeneous cell populations. It has been widely used to perform cell counting and for numerous cell function assessments (De Graaf et al., 2011). Flow cytometry may be considered as the form of automated fluorescence microscopy in which a sample is injected into a fluid (dye) that passes through a sensing medium of flow cell for recording. In flow cytometry, the cells are carried in a laminar flow through a focus of light the wavelength of which matches the absorption spectrum of autofluorescence or the dye with which the cells have been stained (Figure 1.7). On passing through the focus each cell emits a pulse of fluorescence and the scattered light is collected by lenses and directed on to selective detectors. These detectors transform the light pulses into an equivalent electrical signal. The light scattering of the cells gives information on their size, shape and structure (Su, Mennerich, & Urban, 2012). Therefore, a flow cytometer is highly effective in rapid analyzing individual cells at the rate of thousand cells per second.

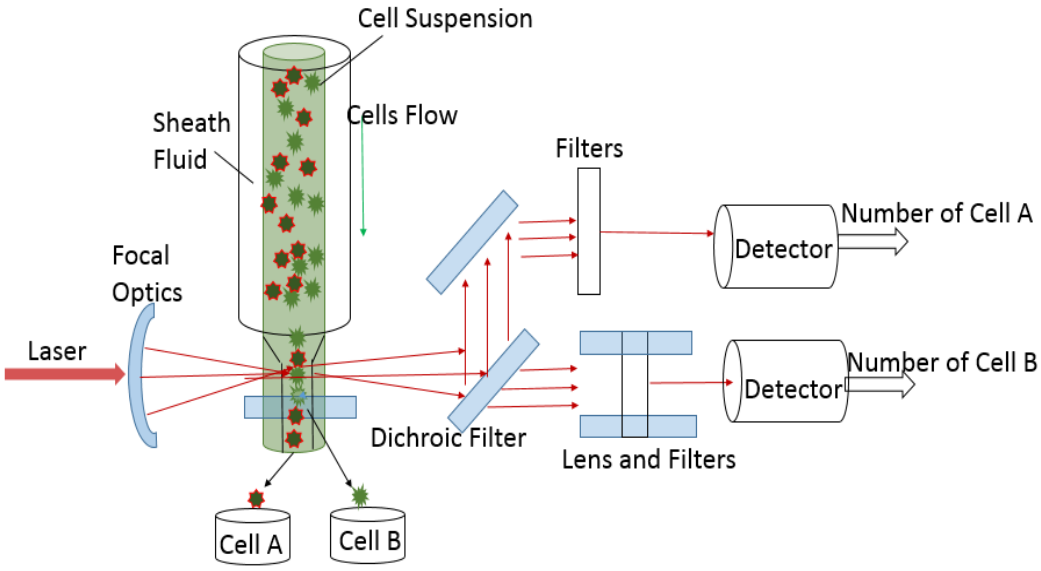


Figure 1.7 A schematic of flow cytometry (WIKIPEDIA)

One important feature of flow cytometry is that measurements are made separately on each particle within the suspension in turn, and not just as average values for whole population. For instance, with two typical strains in China waters, flow cytometry protocols for algal cell counting and for algal live/dead cell assay were established by using *Microcysis aeruginosa* and *Chlorella ellipsoideac* (Chen et al., 2013). The protocols were adopted for cell counting and live/dead assay of the algal cells with UV-C disinfection treatment at 0-200 $mJ.cm^{-2}$. The results indicate that the cell counting data based on flow cytometry analysis are well correlated with that of microscopic analysis (R^2=0.98) while reducing the detection time to less than 5 min (Chen et al., 2013).

One advantage of using flow cytometry to analyze microalgae is the autofluorescence of naturally occurring intracellular pigments, which can be employed to distinguish between different species or between microalgae and other microorganisms without applying fluorescent probes (Hyka, Lickova, Přibyl, Melzoch, & Kovar, 2013). On the other hand, the autofluorescence spectrum can overlap with the red spectral range of commonly used dyes. Therefore, flow cytometry established with bacteria or yeasts which use, for instance, propidium iodide or Nile red are not applicable for microalgae (Veldhuis & Kraay, 2000). For this reason, careful attention should be paid to selection of an appropriate fluorescent dye with respect to the particular microalgal strain being studied and the lasers available with the flow cytometry device being used. Algae possess pigments (chlorophylls, carotenoids, sometimes also phycobilins), which are detectable by flow cytometry as strong red or orange autofluorescence (>600 nm). The strong pigment autofluorescence can cause interference or quenching of a dye if it fluoresces within the same spectrum. This affects the quantitative signal information from the fluorescent dye. Therefore, ideal fluorescent dyes/probes chosen for the investigation of algae should: (1) have maximal fluorescence intensities in the range of 500-600 nm, at which there is almost no interference with the autofluorescence of the pigments (mainly chlorophyll a); (2) be non-toxic and sensitive at low concentrations of the dye to achieve better recovery of the cells after sorting; (3) show excitation at

488 nm, since affordable flow cytometry devices used in industry are only with an air cooled single-line argon blue laser tuned to this wavelength (Veldhuis & Kraay, 2000).

Flow cytometric analyses of *C. vulgaris* cells are performed in a Coulter Epics XL4 flow cytometer equipped with an argon-ion excitation laser (488 nm), detectors of forward and side light scatter and four fluorescence detectors corresponding to four different wavelength intervals: 505-550, 550-600, 600-645, and >645 nm. For each analyzed parameter, data are recorded in a logarithmic scale and results are expressed as a mean value obtained from histograms in arbitrary units. Fluorescence of chlorophyll a (>645 nm) is used as a flow cytometry gate to exclude non-microalgal particles. At least 100,000 cells per culture are analyzed. Cell size and morphology are determined since the forward light scatter (FS) is correlated with the size or volume of a cell or particle and the side light scatter (SS) is correlated with the granularity. (Rioboo, O'Connor, Prado, Herrero, & Cid, 2009).

Flow cytometry has been also used to determine the growth of *C. vulgaris*. Photosynthetic single-cell microorganisms generally show very strong autofluorescence due to the presence of chlorophyll and/or other pigments like carotenoids or phycobiliproteins. In *C. vulgaris* cells, autofluorescence corresponds to the signal gathered in the FL4 detector (660-700 nm), emitted by the chlorophyll a and

related pigments after being excited at 488 nm. This autofluorescence is used to exclusively analyze intact algal cells and to exclude non-microalgal particles (Chioccioli, Hankamer, & Ross, 2014).

1.4.5 Quantitative polymerase chain reaction (q-PCR)

Quantitative polymerase chain reaction (q-PCR) is an in-vitro method used to increase number of specific DNA sequence in a sample. PCR is used increasingly in research because of its high sensitivity and specificity. A specific DNA fragment is amplified during a 4-step PCR process (Katase et al., 2013). The target DNA is first denatured at high temperature and two synthetic oligonucleotides primers flanking the target DNA are present in excess. DNA polymerase then extends the primers using the original DNA as the template. After an appropriate incubation period, the mixture is heated again to separate the strands. The mixture is then cooled to allow the primers to hybridize with complementary regions of newly synthesized DNA, and the whole process is repeated.

When this is performed over and over with newly synthesized DNA as template in addition to original target DNA, an exponential amplification of the DNA fragment between two primers is obtained. Theoretically, PCR can amplify a single copy of DNA a million fold in less than 2 h, hence, it has the potential to disseminate or greatly reduce the dependence on cultural enrichment (Boyer & Combrisson, 2013).

Because q-PCR quantifies the number of copies of a targeted sequence and there are potentially multiple copies of that sequence in a cell, we need to determine the copy number of the sequence that we targeted for a cell before we translate gene quantities into meaningful cell densities (Ata, Nalcaci, & Ovez, 2012).

Culture sample cell densities are determined via flow cytometry while q-PCR was performed after sample filtration. Copy number is then defined as the slope of simple linear regression of copies detected by q-PCR vs. abundance detected with flow cytometry. The copy number regression is forced through the origin, as copy number should directly correspond to cell number (Zamor, Glenn, & Hambright, 2012).

To estimate the number of DNA copies per genome, the plasmid standard with a known copy number per microliter is plotted against the number of DNA copies per microliter of DNA extract and thereafter divided by the cell density representing 1 µL of DNA extract. Thus, the regression slope represents an estimate of the number of DNA copies per cell for each cultured strain. The gene copy number per cell is subsequently analyzed against the length and biovolume of the cells, in turn, to establish a relationship between cell size and number of gene copies for the different species of cultured cells (Godhe et al., 2008).

Although q-PCR is a powerful technology, the reactions can be dramatically affected by presence of inhibitory compounds and selective microbiological media. A problem for routine use of q-PCR in lab is that the procedures are rather complicated and very clean environment is needed to perform the tests. Further, q-PCR cannot distinguish between live and dead cells and hence may provide false negative results (Sanzani, Li Destri Nicosia, Faedda, Cacciola, & Schena, 2014)

1.5 Microalgae cultivation

The current algae cultivation systems can be categorized into three groups according to their reactor design: open systems, closed systems, and hybrid systems. The major difference among these systems is whether the algae are exposed to the surrounding environment. A hetero-photoautotrophic two-stage cultivation process is also an option for nutrients removal, wastewater recycling and algal lipid accumulation (Zhou et al., 2012).

1.5.1 Open systems

A raceway pond (Figure 1.8), an open system for algae cultivation, has been extensively used since the 1950s. It is usually about 0.3 m deep to provide sufficient sunlight to allow photosynthesis by microalgae cells (Chisti, 2007).

Figure 1.8 Image of an open pond system (Wen, 2014)

Currently, most commercial scale algae cultivation systems are open ponds because they are relatively inexpensive to build. Some are built near wastewater treatment plants to easily access nutrients available in wastewater. Despite the economic advantage of open ponds, there are disadvantages. Firstly, since the system is exposed to the atmosphere, water loss by evaporation increases considerably as water temperature increases. Secondly, contamination is another inevitable issue with this system (Mata et al., 2010). Finally, open ponds require large land areas.

1.5.2 Closed systems

Typical closed reactors include flat plate reactors, tubular photobioreactors (PBRs) and bag systems (Wang et al., 2010). Compared with open ponds, the closed reactors minimize water evaporation and contamination, and increase photosynthesis efficiency due to culture conditions and operating parameters such as light intensity, temperature, and CO_2 that can be controlled better in PBRs (Mata et al., 2010). However, PBRs have

several drawbacks including overheating, oxygen accumulation, scale up difficulties and high capital and operating costs (Mata et al., 2010).

1.5.3 Hybrid systems

Hybrid systems combine the merits of open and closed systems in a two-stage cultivation system. The first stage uses closed PBRs to culture the inoculum for the second stage where algae are cultivated in open ponds. In this way, culture is exposed to minimal contamination before being fed to large scale ponds (Chisti, 2007). However, large-scale applications of this approach has been limited by the high cost of the first stage.

1.5.4 A hetero-photoautotrophic two-stage cultivation process

Some algae species can grow in a photoautotrophic mode under light, or in a heterotrophic mode in the presence of organic carbon or in a mixotrophic culture mode when supplied with both organic and inorganic carbon under light/dark conditions (Das, Aziz, & Obbard, 2011). A hetero-photoautotrophic two-stage cultivation process is a way of sequential heterotrophic and photoautotrophic growth for improved nutrient removal, wastewater recycling and enhanced algal lipid accumulation as well as CO_2 fixation for cost-effective and environmentally-friendly production of algae-based biofuel (Zhou et al., 2012).

Growing algae in heterotrophic mode is a way to obtain high cell density biomass with high lipid content for high-quality biodiesel feedstock (Miao and Wu, 2006). The organic carbon in wastewater is not sufficient to sustain continuous growth of algae if complete utilization of nutrients in the wastewater is desirable. A hetero-photoautotrophic mode is therefore an option to solve this problem. Since the accumulation of neutral lipids trends to occur in nitrogen-limited environments (Richardson et al. 1969), the presence of nitrogen compounds may lead to the biomass with low lipid content. Therefore, the culture process is to be separated into two independent but sequential parts, a heterotrophic dominated stage using organic carbon in wastewater for maximal cell density and lipid accumulation and an autotrophic dominated stage to further remove nutrients in the recycled wastewater and accumulate lipid in the nitrogen-deficient environment (Zhou et al., 2012).

1.6 Environmental factors affecting algal growth

A number of environmental factors that affect the growth of microalgae includes light intensity, temperature, nutrient concentration, O_2, CO_2, pH, solids retention time (SRT) and toxic chemicals. Several important factors are discussed below.

1.6.1 Light

As algae are photosynthetic organisms, light intensity is a controlling parameter in algal growth; however, excessive light can inhibit photosynthesis. Algae absorb photosythetically active part of solar radiation, which ranges from 400-700 nm

(Červený, Šetlík, Trtílek, & Nedbal, 2009). In laboratory-scale studies, PBRs are internally or externally illuminated with fluorescent lamps or other light distributors. To successfully use artificial light for photosynthesis, photons with wavelengths between 600 and 700 nm must be generated (Bitog et al., 2011). It is reported that both the CO_2 fixation rate and O_2 evolution rate of *C. vulgaris* were directly proportional to the light intensity less than 97.2 μmol/m²/s (Fan et al., 2007). As soon as the intensity exceeded 97.2 μmol/m²/s, the CO_2 fixation rate and O_2 evolution rate both increased slightly and then remained constant after 145.8 μmol/m²/s. Thus, the light intensity value of 145.8 μmol/m²/s was the saturation point, which should be the optimum value for *C. vulgaris* (Fan et al., 2007). The outer layer of algae in the reactor can sometimes absorb too much light, inhibiting algal growth. Under such conditions, toxic photocatalytic products like hydrogen peroxide and superoxide can accumulate. Triplet chlorophyll can be formed and the photosystem II reaction centers are damaged (L. Xu, Weathers, Xiong, & Liu, 2009). On the other hand, the inner layers of algae may not get enough light because of self-shading, so the volumetric biomass productivity per unit of incident light will reduce. Therefore, efficient light intensity in the culture should always be maintained.

1.6.2 Temperature

Algal growth is also temperature dependent, requiring an optimal value for maximal growth. Temperature is also important for the dissociation of carbon-containing

molecules, making carbon available for photosynthesis. Temperature influences respiration and photorespiration more strongly than photosynthesis ((Necchi, 2004). However, if CO_2 or light is a limiting factor for photosynthesis, the influence of temperature can be insignificant (Pulz, 2001). The optimal temperature for algae cultures is generally between 20 to 24 ℃, although this may vary with the composition of the culture medium, the species and strain cultured. Most commonly cultured species of algae tolerate temperatures between 16 and 27 ℃. Temperatures lower than 16 ℃ will slow down algal growth, whereas those higher than 35 ℃ are lethal for a number of species (Bitog et al., 2011).

1.6.3 Nutrients

The essential elements for algal growth, such as carbon, nitrogen and phosphorous are abundant in wastewater. Hence, microalgae have the potential to clean wastewater by removing nitrogen, phosphorus and organic carbon from water, which helps reducing the eutrophication in the environment.

Inorganic nitrogen such as NH_4^+ or NO_3^- is an essential nutrient for amino acid synthesis in algae. Availability of the nitrogen source affects cell content or composition of proteins, carbohydrates, pigments lipids and fatty acids (Harrison, Thompson, & Calderwood, 1990). Typically, NO_3^- concentrations in natural waters are higher than NH_4^+ concentrations, and NO_3^- is therefore the dominant nitrogen source for algae in

natural water. However, to assimilate NO_3^-, algae need to reduce NO_3^- to NH_4^+, a process that requires energy. As a result, NH_4^+ is a more energy-efficient nitrogen source for algae (Harper, 1992). Ammonium, at concentrations of 100 to 250 μM, may be inhibitory to some algae species, but most algae species tolerate concentrations as high as 1,000 μM (Ross, 1973).

Apart from nitrogen and phosphorus, both iron and manganese are required for electron transport, energy transfer, nitrogen assimilation, oxygen metabolism and enzymatic processes, as well as for DNA, RNA, or chlorophyll synthesis (Kuma & Matsunaga, 1995). Iron and manganese can limit algal growth due to their low concentrations in most waters (Coale et al., 1996).

Zinc is another important trace metal involved in algal growth. At the concentrations of 10-100 μmol/L Zn^{2+} is essential for optimal growth of *Isochrysis galbana* but higher concentrations of Zn^{2+} (≥ 1,000 μmol/L) reduce the growth (Sun & Wang, 2009).

1.6.4 Carbon dioxide and pH

During photosynthesis, algae utilize carbon dioxide as the carbon source for cell growth. Since algae live on a high concentration of carbon dioxide, greenhouse gases, nitrogen dioxide and pollutants in the atmosphere from different sources can serve as the algal nutrients. Algae production facilities can thus be fed with exhaust gases from

combustion of fossil fuels to significantly increase productivity (Bitog et al., 2011). Moreover, algae extract carbon dioxide from water and convert it into organic compounds. The amount of CO_2 needed for growth varies according to algal species and PBRs. The specific CO_2 requirements of algae is likely influenced by PBRs type, although this remains to be investigated. In terms of maximum CO_2 tolerance, some species can survive with very high CO_2 concentration, but usually lower CO_2 concentration is required for their maximum growth which can be shown in Table 1.2 (Merchuk, Gluz, & Mukmenev, 2000).

Table 1.2 CO_2 tolerance and optimum CO_2 concentration of various microalgae species

Speccies	Maximum tolerance of CO_2 concentration	Operating concentration
Cyanidium caldarium	100%	
Scenedesmum sp.	80%	20-20%
Chlorococcum litoorale	60%	
Synechococcus elongatus	60%	
Euglena sp.	45%	5%
Chlprella sp.	40%	10%
Eudorina sp.	20%	
Dunaliella terialecta	15%	
Nannochloris sp.	15%	
Chlamydomonas sp.	15%	
Tetraselmis sp.	14%	

High concentrations of CO_2 decreases water pH because unutilized CO_2 will be converted to H_2CO_3. Alternatively, using bicarbonate instead of gaseous CO_2 will result in pH increase (Widjaja, Chien, & Ju, 2009).

Algae generally prefer a near neutral to alkaline pH (7.5). A pH value greater than 8.5 to 9 starts to affect algal growth (Richmond, 2004). But in general, different pH values seem not to have a significant effect on algal growth (Richmond, 2004).

1.7 Applications of algae for wastewater treatment and biofuel production

1.7.1 Wastewater treatment

Culturing phototrophs in nutrient-rich water is a new attempt to trap nitrogen, phosphorus and toxic metals from the polluted water while producing phototrophic biomass for biofuel applications.

Compared to other biofuel feedstock, phototrophs have the following advantages: First, phototrophs can grow rapidly and have high oil contents of 20-50% on a dry weight basis. Algae do not compete with crops for arable land and freshwater since they can be cultivated in brackish water and on non-arable land. Second, phototrophs have the ability to utilize nutrients from wastewater and to fix carbon dioxide, thus providing an alternative method for wastewater treatment and reducing greenhouse gas emissions to improve air quality. Moreover, byproducts of phototrophs cultivation after lipid

extraction, namely algal biomass residue, can be used as a nitrogen source, such as a protein-rich animal feed or fertilizer for crops.

1.7.1.1 Nitrogen removal

Nitrogen is a critical nutrient required for the growth of all organisms. Organic nitrogen is found in a variety of biological substances, such as peptides, proteins, enzymes, chlorophylls, energy transfer molecules (ADP, ATP), and genetic materials such as RNA and DNA (Harrison et al., 1990). Algae play a key role in converting inorganic nitrogen to its organic form through assimilation. As shown in Figure 1.9, nitrate and nitrite undergo reduction with the assistance of nitrate reductase and nitrite reductase, respectively. Thus, all forms of inorganic nitrogen are ultimately reduced to ammonium. Finally, using glutamate (GLU) and adenosine triphosphate (ATP), glutamine synthase facilitates the incorporation ammonium into the amino acid glutamine (Infante et al., 2013).

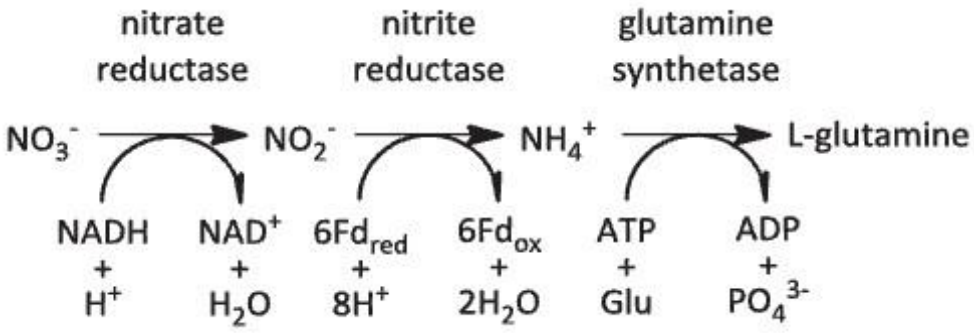


Figure 1.9 A simplified schematic of the assimilation of inorganic nitrogen by algae (Infante et al., 2013)

Ammonium is thought to be the preferred form of inorganic nitrogen because a redox reaction is not involved in its assimilation; thus, it requires less energy. Studies have shown that, in general, algae tend to prefer ammonium over nitrate, and nitrate consumption does not occur until the ammonium is almost completely consumed (Harrison et al., 1990). Therefore, wastewater with high ammonium concentrations can be effectively used to rapidly grow microalgae. It has been reported that *Chlorella sp.* is tolerant to NH_4^+-N at a high concentration ranging from 81 to178 mg/L (Wang et al., 2010).

1.7.1.2 Phosphorus removal

Phosphorus is also a key factor in the metabolism of algae. During algae metabolism, phosphorus, preferably in the forms of $H_2PO_4^-$ and HPO_4^{2-}, is incorporated into organic compounds through phosphorylation, much of which involves the generation of ATP from adenosine diphosphate (ADP), accompanied by a form of energy input (T. Cai, Park, & Li, 2013). Energy input can come from the oxidation of respiratory substrates, the electron transport system of the mitochondria, or in the case of photosynthesis, from light. Phosphates are transferred by energized transport across the plasma membrane of the algal cell. Not only are inorganic forms of phosphorus utilized by microalgae, but some varieties of algae are able to use the phosphorus found in organic esters for growth (Martin et al., 1994). Phosphorus removal is not only governed by the uptake into the cell, but also by external conditions such as pH and algal biomass concentrations.

Under phosphorus limitation, more phosphorous is taken up than required by algae for their growth, known as "luxury uptake" (Riegman, Stolte, Noordeloos, & Slezak, 2000). Furthermore, the induced flocculation of microalgae can facilitate chemical phosphate precipitation. Therefore, phosphorous can be removed by algae through a combination of adsorption and algae-induced chemical precipitation(Sañudo-Wilhelmy et al., 2004). It is reported that the intracellular phosphorus is 0.4% in an algae-based membrane bioreactor (A-MBR), indicating there is no luxury uptake occurring due to relative high influent phosphorus concentration (M. Xu, Bernards, & Hu, 2014). Meanwhile, the overall average extracellular phosphorus content is 7.5%, which accounts for more than 90% of the total phosphorus in the algae biomass.

1.7.1.3 CO_2 sequestration and organic carbon removal

CO_2 from the atmosphere and industrial exhaust gases can be fixed through the photosynthetic activity of autotrophic microalgae. In addition, high levels of total organic carbon (TOC) in the wastewater can be utilized by some algae species for fast growth under photoheterotrophic or mixotrophic mode in light or under chemoheterotrophic mode in dark (Wang et al., 2010). Carbon can be also utilized in the form of soluble carbonates for cell growth, either by direct uptake or conversion of carbonate to free carbon dioxide through carboanhydrase activity. For instance, mixotrophic algae can assimilate CO_2 and organic carbon simultaneously. The

assimilation of organic carbon can be through either phagotrophy or osmotrophy (Subashchandrabose, Ramakrishnan, Megharaj, Venkateswarlu, & Naidu, 2013). The mechanism of converting CO_2 to biomass is shown in Figure 1.10 (Widjaja et al., 2009). Therefore, the use of algae to mitigate CO_2 from flue gases is an emerging research area and, if effective, could benefit both the environment and biofuel production.

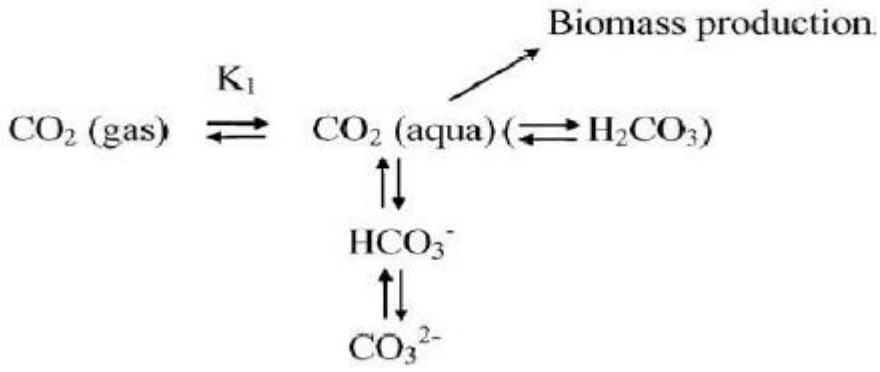


Figure 1.10. Schematic of the mechanism of converting CO_2 to algal biomass (Widjaja et al., 2009)

1.7.1.4 Toxic metal removal

Wastewater from industries such as leather, steel and electroplating, contain high levels of toxic heavy metals such as chromium, silver, copper, zinc and iron. Biosorption is considered as an effective technique to remove metals from industrial wastewater because of obvious benefits of high efficiency and selectivity for absorbing metals in low concentrations, due to wide operational range of pH and temperature, energy-saving, easy retrieval of metal and easy recycling of the biosorbent (Lam, Wut, Chan, & Wu, 1999).

Among easily available biomasses groups, algae may provide a sustainable solution for the treatment of municipal and industrial wastewater. For example, *C. minutissima* can be used as a biosorbent to remove Cr (VI) (Ata et al., 2012). Metal cations and anions can be adsorbed onto the biomass materials through physical and chemical adsorption by van der Waals forces, electrostatic interactions, ion-exchange reaction, complexation, surface precipitation, or their combinations. Microalgae, either in original or modified forms, have been found to possess the ability to bind metals in solution and be able to be utilized as adsorbents for metals (Monteiro, Castro, & Malcata, 2012).

1.7.2 Biofuel production

Many species of microalgae are known to have high content of lipids, which have the potential to be converted to biofuel, mainly biodiesel, via the transesterification of intracellular accumulated oils. Due to their rapid growth and ability to accumulate oil in concentrations of up to 50% of their dry weight, together with the possibility of year-round production, microalgae can enable higher oil yield than oilseed crops (Widjaja et al., 2009). Some algae species have high lipid contents when grown in wastewater. *C. vulgaris* grown in an artificial medium resulted in 20-42% (dry basis) lipids with removal efficiencies of 97% for ammonium and 96% for total phosphorus (Johnson et al., 2013)

1.8 Research Objectives

Although microalgae and cyanobacteria are capable of treating wastewater and extracting biofuel, algal blooms occur in the world. Therefore, algae harvesting is important to both biofuel extraction and algal bloom control. Fundamental research on microalgae and cyanobacteria based on cell morphology, concentration and density has potential applications to estimate harvesting efficiency and lipid content in a given volume of algal culture. For instance, cell density (g/cm^3), determines whether microbes float on the surface of water, suspend in water or sink to the bottom. Furthermore, density is an essential factor to determine the appropriate methods and anticipate the degree of difficulty for cell harvesting. Notably, cells adjust their density during important processes such as cell cycle progression, apoptosis, differentiation and malignant transformation even when volume and mass appear to remain constant (Bryan et al., 2014; Grover et al., 2011). Moreover, combining concentration (cells/mL) and weight of individual cells (g/cell) as well as theoretical lipid content of species, biofuel productivity can be estimated.

The main objective of this study was to determine the dry weight of individual algal and cyanobacterial cells and compared with that of nonphotosynthetic bacteria. Moreover, we compared cell size and density of microalgae (*chlorella vulgaris*) and cyanobacteria (*Microcystis aeruginosa*) during exponential phase of a batch study. In

particular, the dry weight of microalgae (*Chlorella vulgaris*) was compared between batch study and continuous flow stirred-tank reactor (CSTR) operation.

2. Materials and Methods

2.1 Algal and Cyanobacterial Cultivation

Pure algal species *Chlorella vulgaris* (Carolina Biological Supply Company, Burlington, NC) and pure cyanobacteria species *Microcystis aeruginosa* (UTEX 2385) were grown individually in 250 mL Erlenmeyer flasks containing 125 mL of sterilized Bold's basal medium (BBM). The BBM contained the following key nutrients : 2.94 mM $NaNO_3$, 0.2mM $MgSO_4.7H_2O$, 0.58 mM K_2HPO_4, 1.1 mM KH_2PO_4, 0.23mM $CaCl_2$, 0.43 mM NaCl, 0.13 mM EDTA, 0.47 mM KOH, 0018 Mm $FeSO_4.7H_2O$, 0.18 mM H_3BO_3, 0.055 mM $ZnSO_4$, 0.005 mM $Na_2MoO_4.H_2O$, 0.004 mM $CoCl_2$, 0.01 mM $MnCl_2$ and 0.06 mM $CuSO_4.5H_2O$ (Simmons & Wallschlager, 2011). CO_2 available in the air was used as carbon source for photosynthesis.

2.2 Cell Concentration Determined by Spectrophotometry, Hemocytometry and Flow Cytometry

Indirect measurements of concentrations of *Chlorella vulgaris* and *Microcystis aeruginosa* were determined by UV-Vis spectrophotometer (Thermo Scientific, GENESYS 20) at 680 nm (M. J. Griffiths, C. Garcin, R. P. van Hille, & S. T. L. Harrison, 2011). Hemocytometry was applied for direct cell enumeration after a series of 10-fold dilutions.

A linear relationship between cell number based on hemocytometry and flow cytometric measurement was established.

To differentiate algal and cyanobacterial cells, a phototrophic suspension was prepared by mixing *Chlorella vulgaris* and *Microcystis aeruginosa* at the ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 2:1, 3:1, 4:1 and 5:1, respectively. A linear relationship between the ratio of algal to cyanobacteria based on hemocytometry and flow cytometry was also established.

2.3 Determination of Cell Dry Weight of Algae and Cyanobacteria at Exponential Growth Phase

Studies of phototrophic growth were set up to determine individual cell dry weight, size and cell density during exponential phase. Triplicate samples of *Chlorella vulgaris* and *Microcystis aeruginosa* were collected on days 4, 8, 12, 16 during their exponential growth phase. The initial cell concentration in a 200-mL cultivation was maintained the same at an OD of 0.20 for both algae and cyanobacteria using modified BBM which contained a low phosphorous concentration (2 mg/L as PO_4^--P) to minimize P adsorption/precipitation on the cell surface.

Both *Chlorella vulgaris* and *Microcystis aeruginosa* were cultured at 24 ± 1 ℃ on a shaker (Eppendorf, Innova 2000). The culture was mixed at 160 rpm and grown for 16

days (Figure 2.1). The batch cultivation went through 12h light/12h dark (L/D) cycle on a daily basis to mimic natural conditions. The algal or cyanobacterial suspension was exposed to fluorescent light at an average light intensity of 63.5 μmol/m²/s measured by a light meter ((LI-250A, Biosciences). During the sampling period, water quality parameters such as pH, and orthophosphate-P concentration were determined following the standard methods (APHA, 2005).

Figure 2.1 An experimental setup of batch study of phototrophic growth

Hemocytometry and flow cytometry were applied to determine the algal or cyanobacterial cell concentrations. The hemocytometer is composed of nine equally sized large squares. The central one is divided into 25 small squares with each volume of 4×10^{-6} mL. *Chlorella vulgaris* and *Microcystis aeruginosa* were counted in small squares since their sizes are less than 10 μm. The cell concentration was calculated as follows:

$$\frac{cell\ number}{mL} = \frac{cell\ number\ per\ small\ square}{4 \times 10^{-6}\ mL}$$

Flow cytometric analysis was carried out for cell counting. Fluorescent beads of known size and concentration were added to samples to provide an internal standard. To differentiate pure phototropic species from the beads, the differences should exist based on their size, morphology and their fluorescence. In this study, fluorescent counting beads (7.7 μm, SPHERO AccuCount) were applied at a final concentration of 1×10^5 particles/mL during phototrophic cell counting by a flow cytometer (CyAn ADP, Beckman Coulter Inc., Brea, CA). Briefly, an aliquot (100 μL) of fluorescent bead stock was added to a 1 mL cell culture sample and was vortexed for 10 s for complete mixing before analysis. Both *Chlorella vulgaris* and *Microcystis aeruginosa* were well differentiated from the fluorescent beads by recording the autofluorescent signals of FL1 (530/40 nm) and FL4 (680/30 nm) band pass filters, which allow transmission of photons that have wavelengths within a narrow range. Each flow cytometric analysis required a total of 500,000 counting events (Figure 2.2). Summit V5 software was applied to the analysis of flow cytometric images.

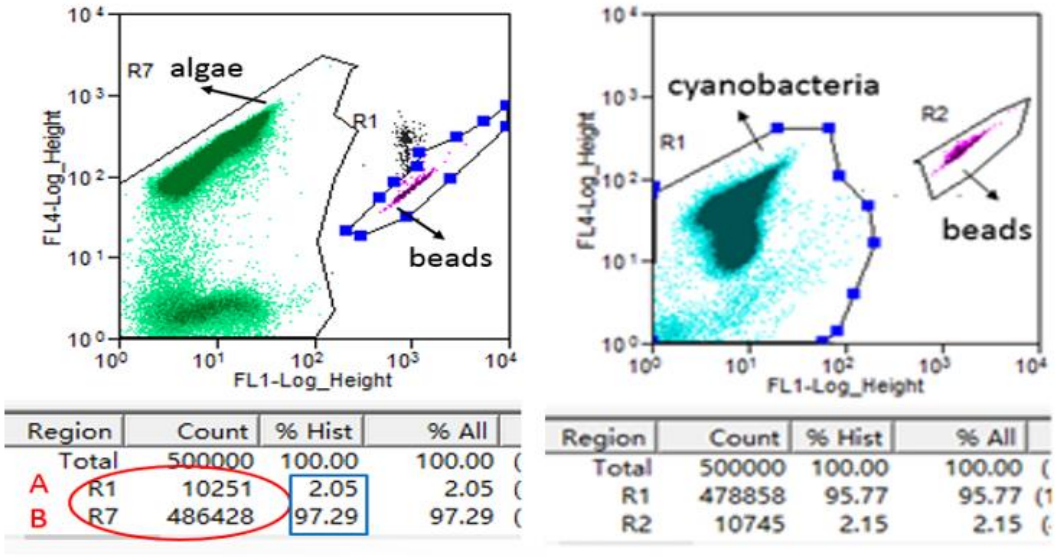


Figure 2.2 Flow cytometric image for algae (left) and cyanobacteria (right)

The cell concentration was calculated in the following:

$$\frac{cell\ number}{mL} = \frac{cell\ number\ counted\ by\ FCM}{beads\ number\ counted\ by\ FCM} \times \frac{1 \times 10^5}{1\ mL}$$

For the dry weight of biomass per volume of the suspension of *Chlorella vulgaris* or *Microcystis aeruginosa*, initial mass of a 0.45 μm filter membrane was measured after heating in a furnace at 105 ℃ for 2 hours. An aliquot (140 mL) of biomass sample was collected on each sampling day during exponential phase and was vacuum filtered through the filter membrane. DI water was used to wash sample on the filter for 3 times to wash out chemicals attached on the cell surface. The filter membrane along with biomass was dried for 2 hours at 105 ℃ and the dry weight of biomass per volume was calculated as follows：

$$\frac{g}{mL} = \frac{net\ weight\ of\ membrane\ before\ and\ after\ filtration}{filtration\ volume}$$

Finally the dry weight of individual algal or cyanobacterial cells was calculated based on cell concentration and dry weight of biomass per volume in the following:

$$\frac{g}{cell} = \frac{dry\ weight\ (g/mL)}{cell\ concentration\ (cell/mL)}$$

2.4 Determination of Cell Dry Weight of Nonphotosynthetic Bacteria at Exponential Growth Phase

Escherichia. coli (TOP 10) was inoculated in sterilized Reasoner's 2A agar (R2A) medium with 5-fold dilution since original R2A medium easily caused biofilm formation. The diluted R2A medium contains the following nutrients per liter: 0.1g yeast extract, 0.1 g pepteose peptone, 0.1 g casamino acids, 0.1 g dextrose, and 0.1 g soluble starch. In addition, the medium contained 0.06 g/L sodium pyruvate, 0.06 g/L dipotassium sulfate and 0.01 g/L magnesium sulfate (H. Cai et al., 2014). *E.coli* was cultivated on a shaker (180 rpm) in an incubator at 37 ℃ for 6 hours. The cell concentration and cell size were determined at the end of cultivation.

To count *E.coli* cell number of a sample at the exponential phase, a standard plate count method was applied for bacterial enumeration. By using PBS buffer, serial dilution was performed and inoculated agar plates were stored for 10 h at 37℃ in incubator. The bacterial cell numbers in colony forming units (CFU) were determined after the cultivation by taken into account the dilution factor.

The dry weight per volume of *E.coli* sample was determined similarly to that of algae or cyanobacteria with minor modification. An aliquot (100 mL) of bacterial suspension was centrifuged at 30,000 rpm for 10 min to remove the upper layer of supernatant (about 90 mL). The remaining cell suspension was filtered through 0.22 μm filter for bacterial mass determination.

2.5 Determination of Cell Dry Weight of the Algae in Continuous Flow Bioreactor

Two bench-scale continuous stirred tank reactor (CSTRs) were operated for *Chlorella vulgaris* cell size, cell dry weight and cell density calculation (Figure 2.3).

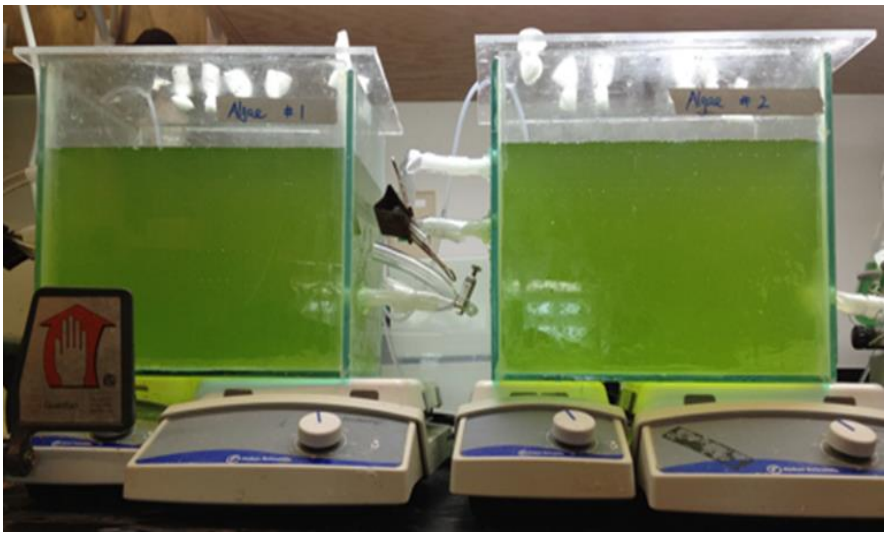


Figure 2.3 Experimental setup of two identical bench-scale CSTR systems

Each CSTR system had a total volume of 7.7 L, which was operated at a SRT of 7 d. Because of complete mixing and with no biomass recycling, SRT and HRT were the same in such a system. During the start-up operation, 1.2 L *Chlorella vulgaris* was inoculated with the modified BBM medium. The algal CSTR system was operated at 24 ±1 ℃ with a 12/12 L/D cycle until steady state (> 3 SRTs) was reached. The cell

concentration, cell size, OD, influent and effluent water quality parameters, such as pH, orthophosphate-P, and biomass COD concentrations were determined when the CSTR system reached steady-state. To determine the effect of phosphorous adsorption on cell dry weight measurement, intercellular and extracellular phosphorous contents of algal biomass in the algal CSTRs were determined according to the method described elsewhere through oxalic acid washing (Sañudo-Wilhelmy et al., 2004)

2.6 Cell Size of Algae and Cyanobacteria

To measure phototrophic cell size, fluorescent microscopy is an appropriate method based on autofluoresence of phototrophs. Fluorescent microscopy (Olympus I×70) with Cy5HQ filter with excitation wavelength of 590-650 nm and emission wavelength in the rage of 670-735 nm was applied to determine the size of *Chlorella vulgaris* and *Microcystis aeruginosa*. MetaMorph software was applied for image analysis. For fluorescent microcopy, cell size was averaged from the measurements of 30 cells measured for each sample. With triplicate samples, a total of 90 cells were measured for size on each sampling day.

2.7 Cell Density of Algae and Cyanobacteria

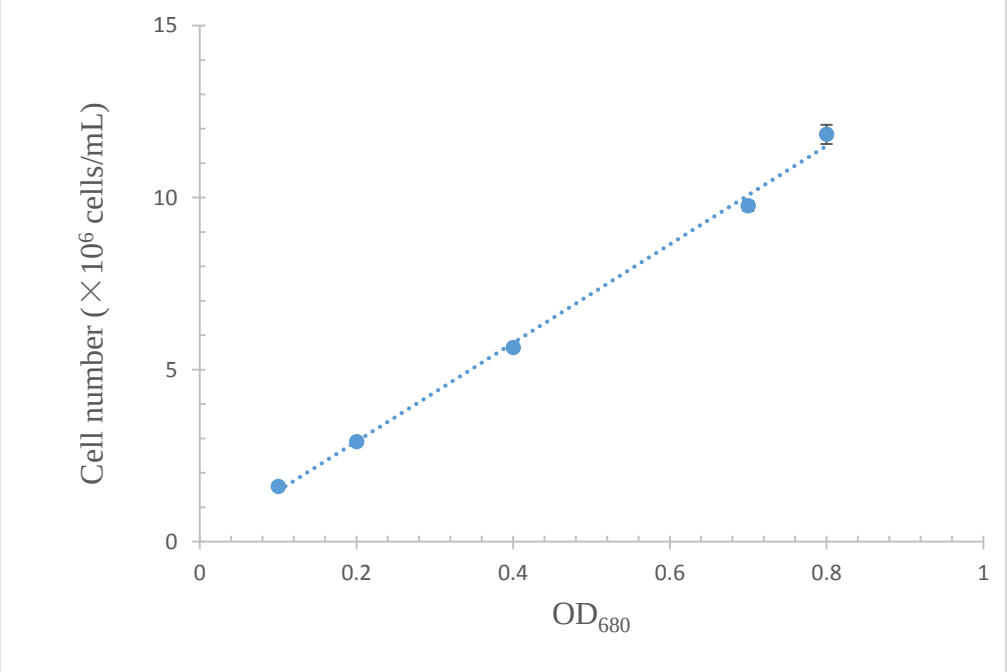
Based on cell size data collected by fluorescent microscopy, individual cell volume was calculated since cells are spherical. Associated with cell concentration measured by flow cytometry and hemocytometry and dry weight biomass concentration, cell density was calculated as follows:

$$\text{cell density } (g/cm^3) = \frac{dry\ weight\ (g/cell)}{individual\ cell\ volume\ (cm^3)}$$

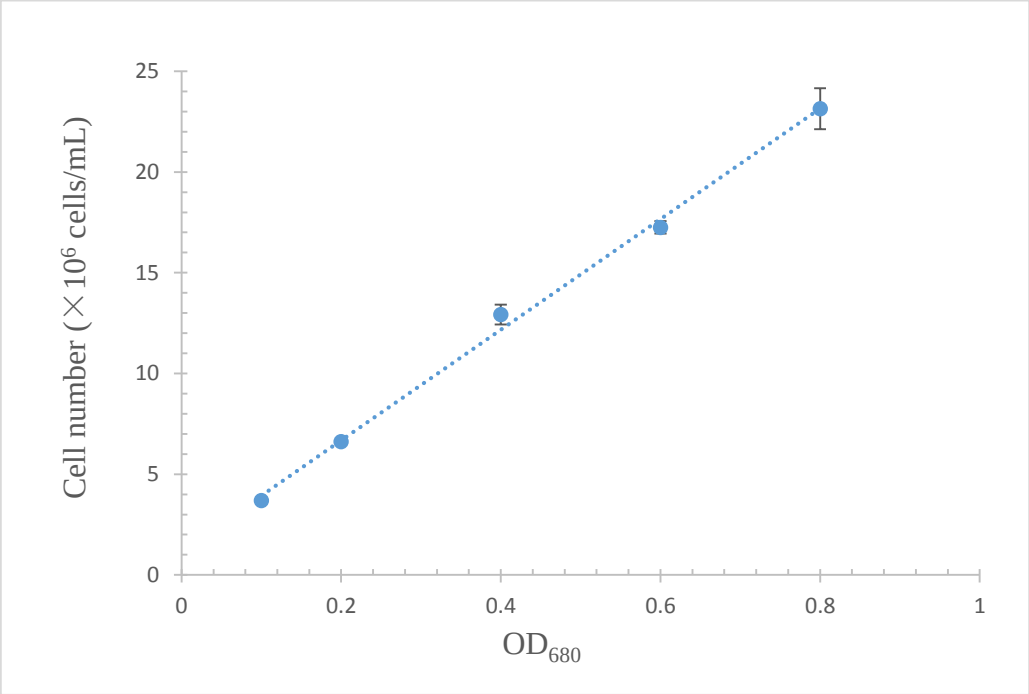
3. Results and Discussion

3.1 Cell Concentration Determined by Spectrophotometer, Hemocytometry and Flow Cytometry

Pure *Chlorella vulgaris* and *Microcystis aeruginosa* cultures were diluted in series to have different OD values before the cell concentration was correlated and determined by hemocytometry, spectrophotometry, and flow cytometry, respectively, as shown in Figure 3.1 and Figure 3.2.

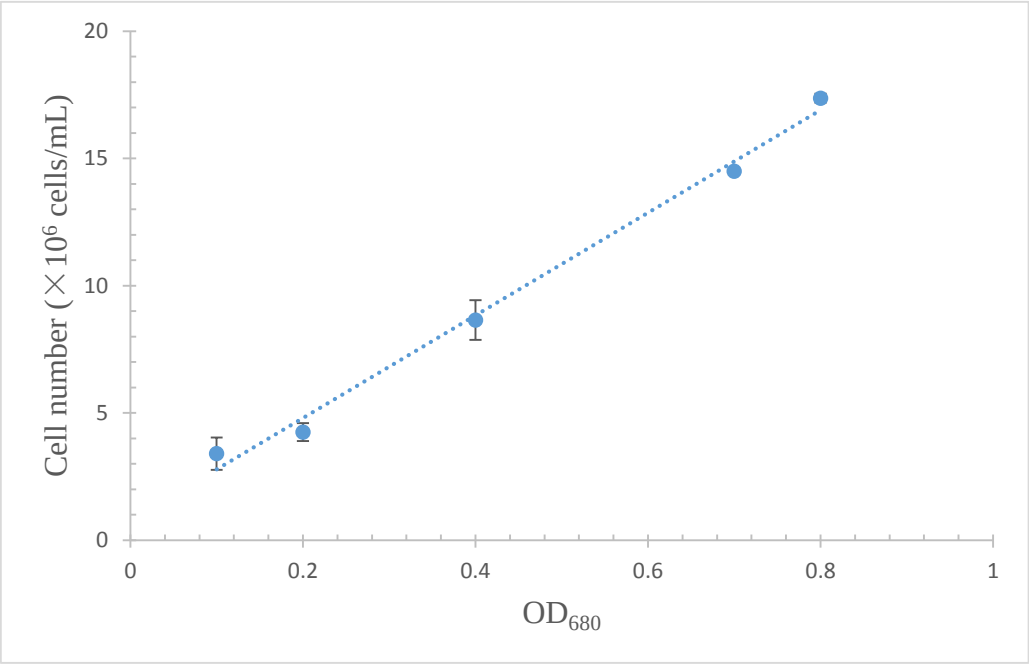


(a)

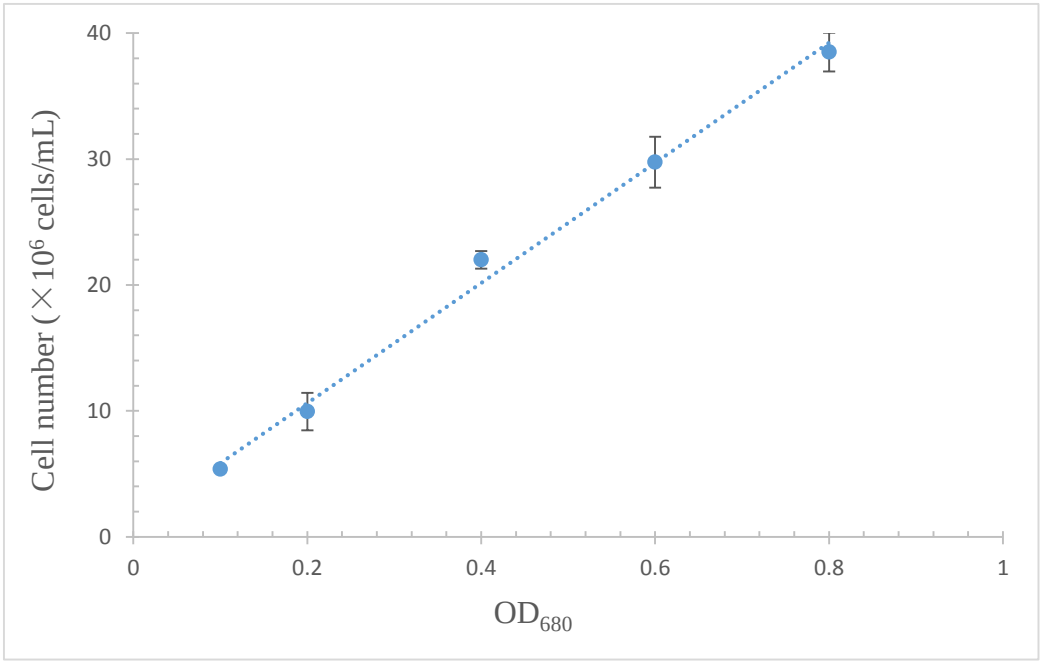


(b)

Figure 3.1 A standard curve of cell concentration determined by flow cytometry for algae (a, R^2=0.997) and cyanobacteria (b, R^2=0. 997)



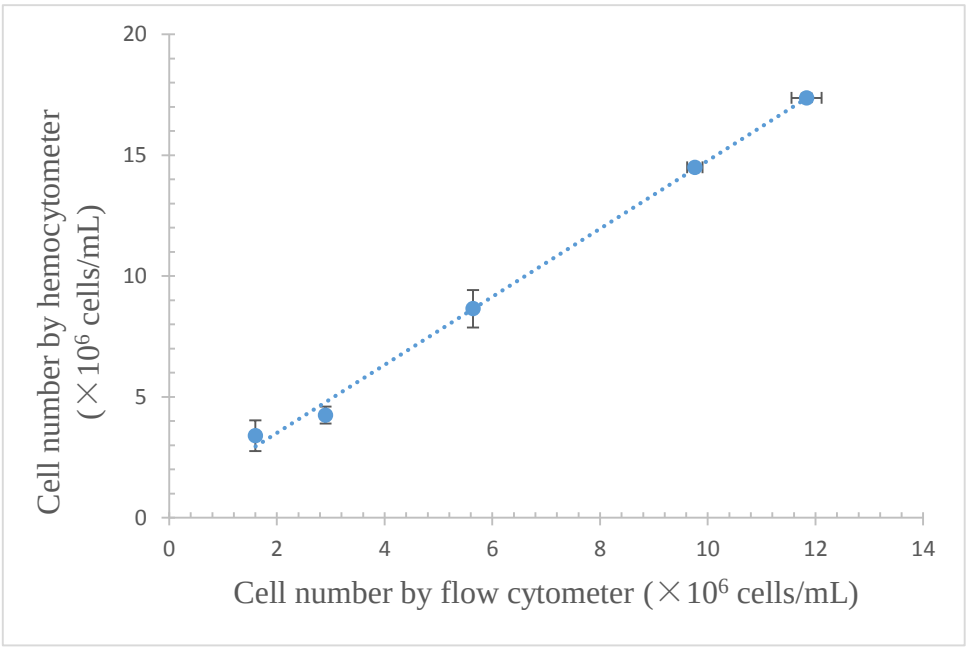
(a)



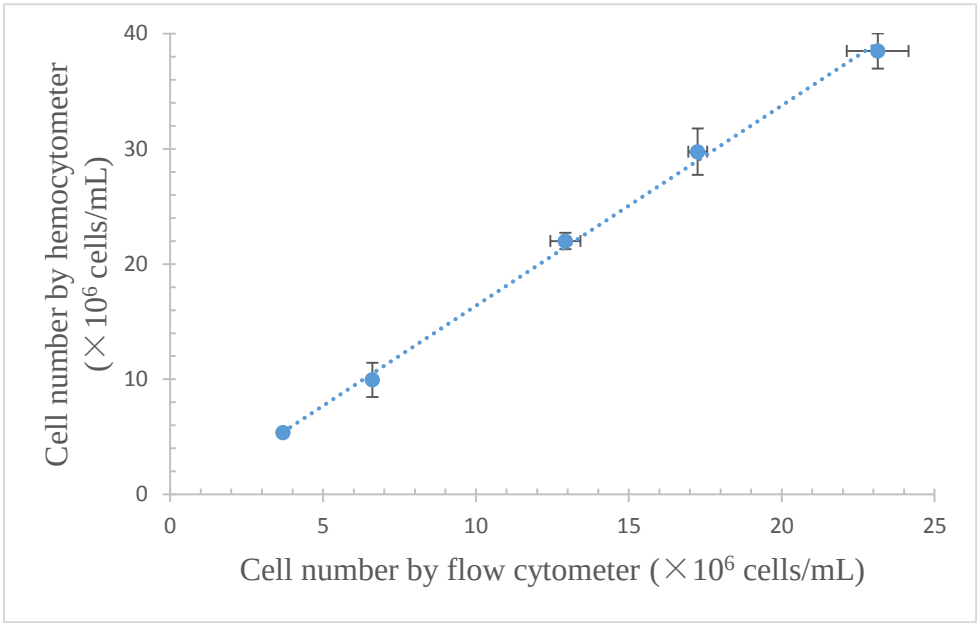
(b)

Figure 3.2 A standard curve of cell concentration determined by hemocytometry for algae (a, R^2=0.993) and cyanobacteria (b, R^2=0. 997)

The two methods used for cell counting, has a good linear relationship (Figure 3.3). However, hemocytometric counting results were always greater than those of flow cytometry. For algae, cell numbers counted by hemocytometry counts are about 1.4 times more than flow cytometer. While for cyanobacteria, hemocytometry counts are about 1.7 times greater than flow cytometry. Flow cytometry may underestimate cell numbers due to cell aggregation during cell counting while hemocytometry could include more human errors.



(a)

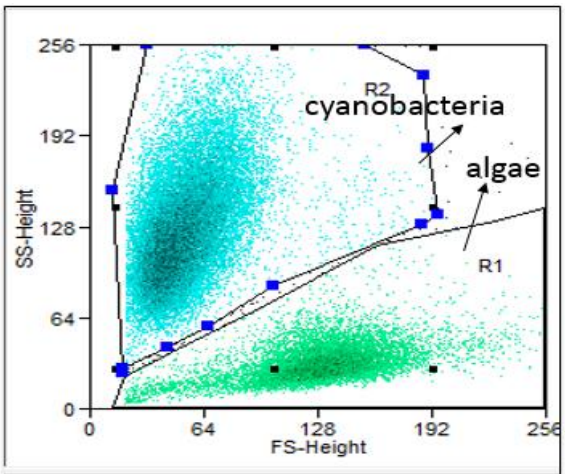


(b)

Figure 3.3 A correlation of cell concentration determined by hemocytometry and flow cytometry for algae (a, R^2=0.997) and cyanobacteria (b, R^2=0. 998)

3.2 Cell Counting for Mixed Phototrophic Samples

The phototrophic samples were prepared as mixed cultures with ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 2:1, 3:1, 4:1 and 5:1 between *Chlorella vulgaris* and *Microcystis aeruginosa* based on hemocytomotry. Flow cytometry was applied to differentiate phototrophs and determine their fractions (Figure 3.4). A standard curve of cell ratio between *Chlorella vulgaris* and *Microcystis aeruginosa* determined by flow cytometry versus that by hemocytometry are shown in Figure 3.5.



Region	Count	% Hist	% All
Total	50000	100.00	100.00
R1	13939	27.88	27.88
R2	35981	71.96	71.96

Figure 3.4 Difference in size and morphology based flow cytometry (algae: cyanobacteria=1:1 counted by hemocytometry)

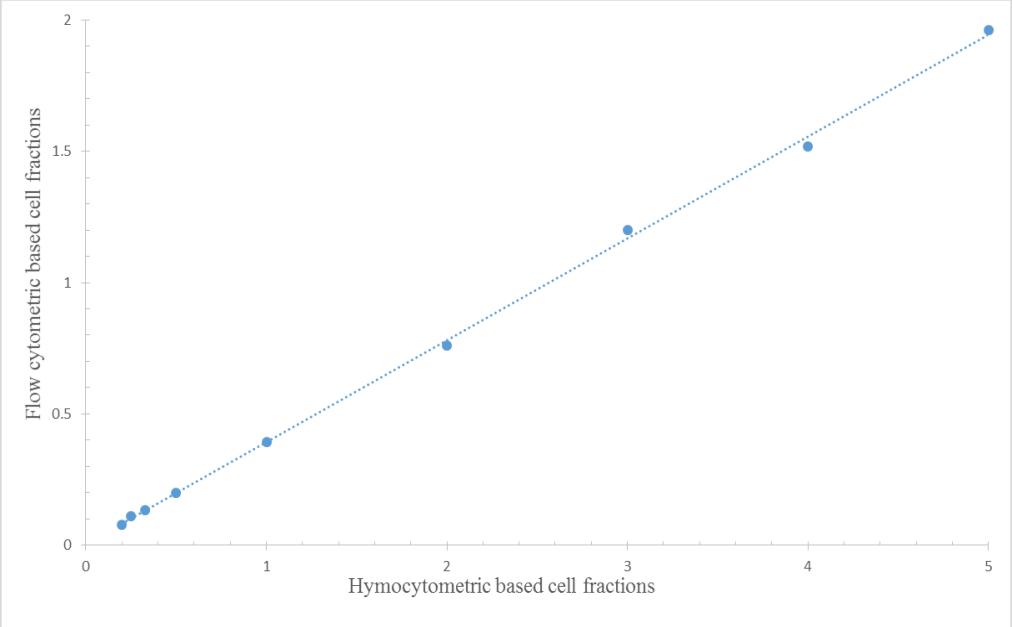


Figure 3.5 A standard curve of the ratio of algae to cyanobacteria determined by flow cytometry verse that by hemocytometry (R^2=0.999)

The results demonstrated that flow cytometry is capable of differentiating algae from cyanobacteria based on their difference in cell size and morphology. A good linear relationship between the two ratios existed. However, the ratio determined by hymocytometry was about 2.5 times of that by flow cytometry. This might be due to underestimation of cyanobacterial cell numbers by hemocytometer as cell sizes of cyanobaceteria were too small to differentiate under microscopy. Moreover, aggregation of cyanobacteria could affect cell counting.

3.3 Dry Weight of Individual Algal, Cyanobacterial and *E. coli* Cells at Exponential Growth Phase

From the batch growth curve of *Chlorella vulgaris*, algal cells continued to grow from day 0 to day 16 (Figure 3.6). Algal samples were taken on days 4, 8, 12, and 16.

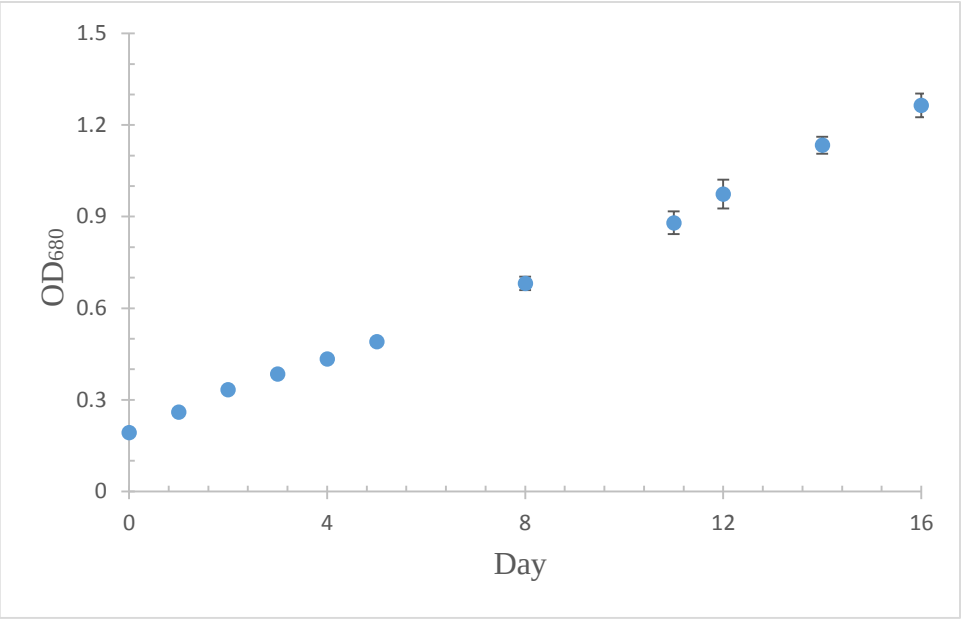


Figure 3.6 *Chlorella vulgaris* growth curve

The OD of algal samples was adjusted to 0.433 before analysis by flow cytometry and hemocytometry. Then combined with dry weight biomass concentration, individual cell weight was determined (Table 3.1).

Table 3.1 Dry weight of individual *Cholorella Vulgaris* cells at exponential phase

Sampling day	OD	Individual cell weight (g/cell)	
		Flow cytometry	Hemocytometry
Day 4	0.433	$(3.08 \pm 0.03)\times10^{-11}$	$(1.76 \pm 0.04)\times10^{-11}$
Day 8	0.433	$(3.32 \pm 0.01)\times10^{-11}$	$(1.54 \pm 0.06)\times10^{-11}$
Day 12	0.433	$(3.35 \pm 0.71)\times10^{-11}$	$(1.70 \pm 0.07)\times10^{-11}$
Day 16	0.433	$(3.01 \pm 0.33)\times10^{-11}$	$(1.73 \pm 0.02)\times10^{-11}$

At exponential growth phase, the dry weight of individual cells of *Chlorella vulgaris* ranged from $(1.54 \pm 0.06) \times 10^{-11}$ g/cell to $(3.35 \pm 0.71) \times 10^{-11}$ g/cell, with an average

of (2.24 ± 0.16) × 10^{-11} g/cell (n = 12). Although the modified BBM contained sufficient phosphorous for *C. vulgaris* growth, the P concentration only decreased from 6.1 mg/L to 4.6 mg/L (Figure 3.7), suggesting that phosphorous uptake has little effect on dry weight measurements in batch studies

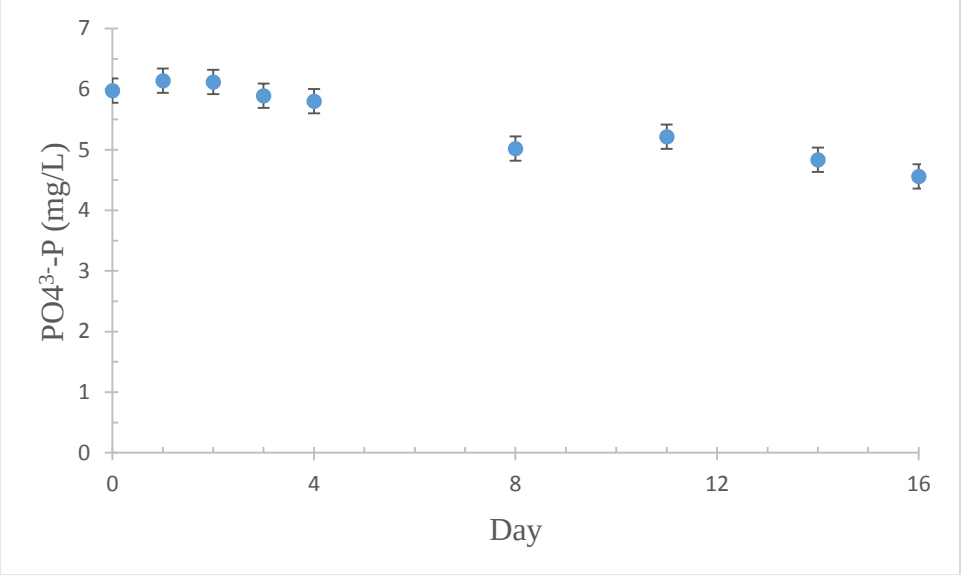


Figure 3.7 Change in the orthophosphate-P concentration during the growth of *chlorella vulgaris*

Similar to algae, cyanobacteria continued to grow from day 0 to day 16 (Figure3.8). The OD of cyanobacterial samples was fixed at 0.423 before flow cytometric and hemocytometric measurements (Table 3.2).

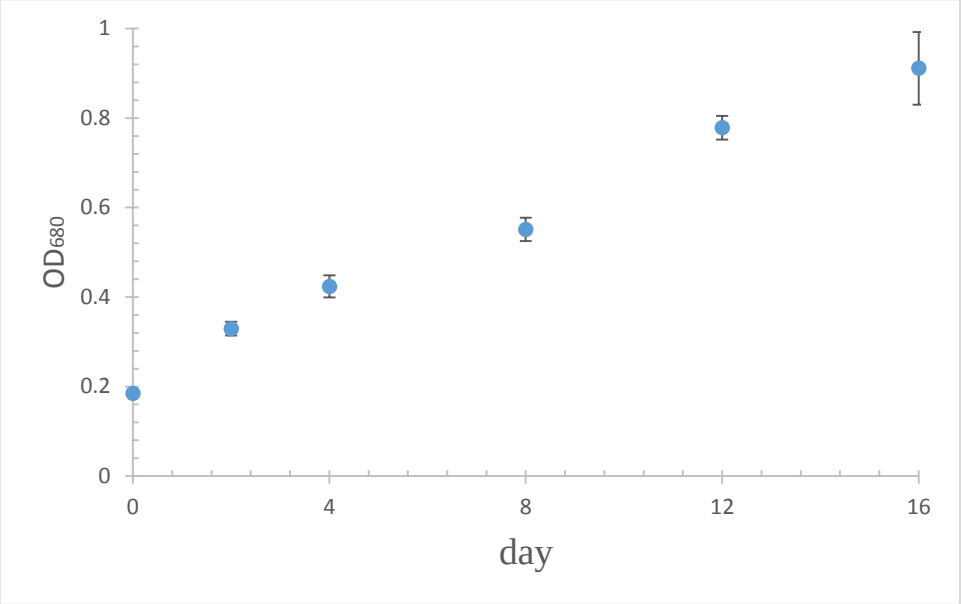


Figure 3.8 Growth curve of *Microcystis aeruginosa*

Table 3.2 Cell concentration and individual cell weight of *Microcystis aeruginosa* in exponential phase

`Sampling day	OD	Individual cell weight (g/cell)	
		Flow cytometry	Hemocytometry
Day 4	0.423	$(1.43 \pm 0.24) \times 10^{-11}$	$(6.02 \pm 0.01) \times 10^{-12}$
Day 8	0.423	$(1.87 \pm 0.05) \times 10^{-11}$	$(7.33 \pm 0.11) \times 10^{-12}$
Day 12	0.423	$(1.86 \pm 0.02) \times 10^{-11}$	$(6.38 \pm 0.05) \times 10^{-12}$
Day 16	0.423	$(1.72 \pm 0.03) \times 10^{-11}$	$(7.99 \pm 0.24) \times 10^{-12}$

At exponential phase, individual cell weight varies from $(6.02 \pm 0.01) \times 10^{-12}$ to $(1.87 \pm 0.05) \times 10^{-11}$ g/cell, with an average of $(1.21 \pm 0.09) \times 10^{-11}$. Compared to algae, at the same OD value, cell concentration of cyanobacteria is much greater, which results from the cell size of cyanobacteria being smaller than algae. No phosphorous aggregation was observed in the exponential phase of cyanobacteria (Figure 3.9).

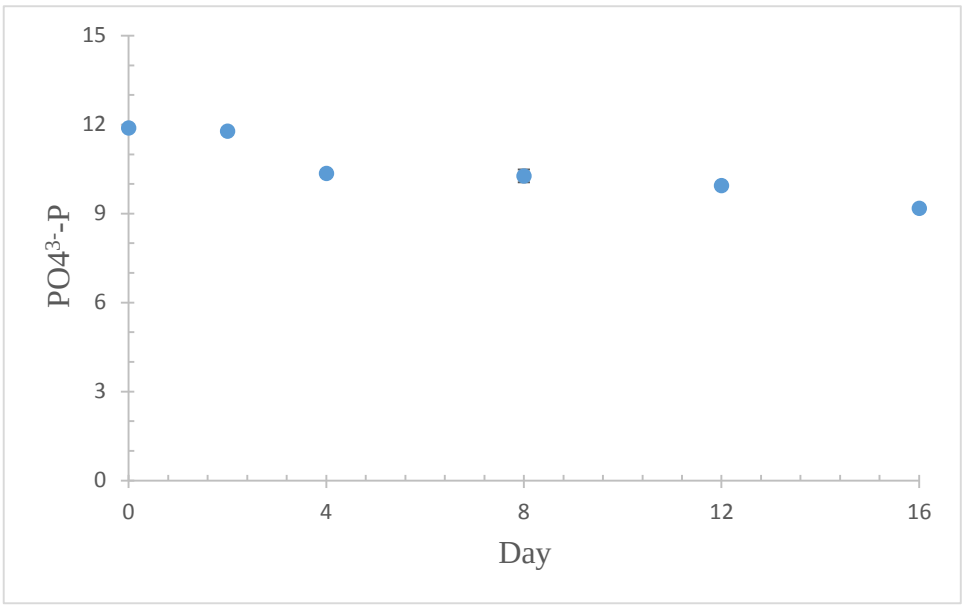


Figure 3.9 Orthophosphate-P concentration for *Microcystis aeruginosa* in exponential phase

E.coli individual dry weight determined by flow cytometry was $(4.28 \pm 0.43) \times 10^{-7}$ g/cell, indicating flow cytometry was not successful in counting *E. coli* cells due to cell aggregation and floc formation during analysis. Hemocytometry was also not successful in bacterial cell counting due to resolution issues. Alternatively, standard agar plate counting was applied. The average dry weight of individual *E. coli* cells was $(1.69 \pm 0.36) \times 10^{-13}$ g/cell (n = 6).

3.4 Dry Weight of Individual Algal Cells in a Continuous Flow Bioreactor

An algal CSTR system was operated for more than 30 days (Figure 3.10). Algal samples were collected on day 21 and day 26. Individual cell dry weight is shown in Table 3.3.

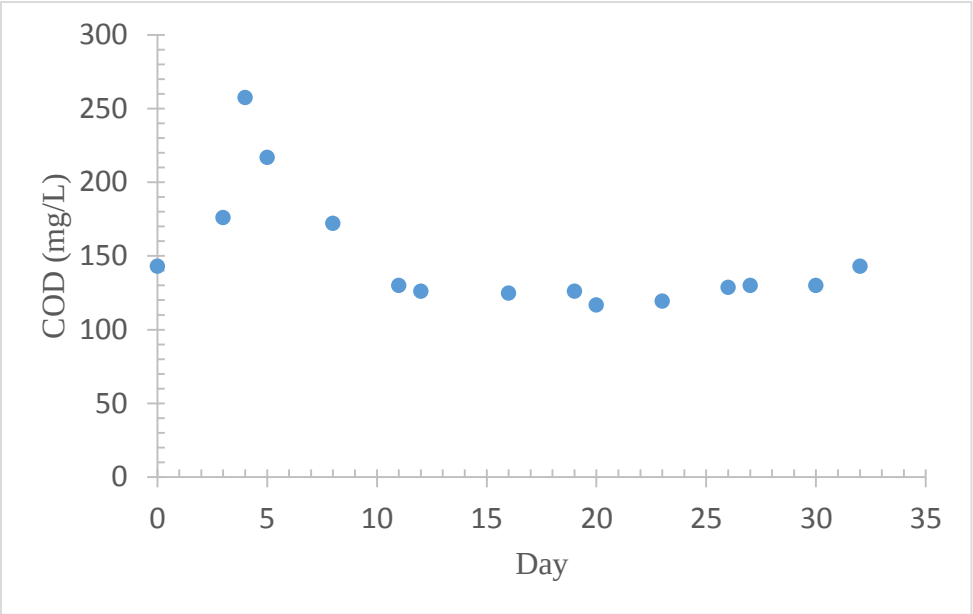


Figure 3.10 Change in the concentration of *Chlorella vulgaris* of the CSTR at the SRT of 7 d

Table 3.3 Individual cell dry weight of Cholorella vulgaris in CSTR

Sampling day	Individual cell weight (g/cell)	
	Flow cytometry	Hemocytometry
Day 21	$(5.87 \pm 0.27) \times 10^{-11}$	$(2.58 \pm 0.13) \times 10^{-11}$
Day 26	$(5.02 \pm 0.54) \times 10^{-11}$	$(2.16 \pm 0.09) \times 10^{-11}$

The average dry weight of individual *C. vulgaris* cells of the CSTR system ranged from $(2.16 \pm 0.09) \times 10^{-11}$ g/cell to $(5.87 \pm 0.27) \times 10^{-11}$ g/cell with an average of $(3.91 \pm 0.26) \times 10^{-11}$ g/cell (n = 8), which almost double compared to that from the batch study. However, orthophosphate-P concentration decreased rapidly in the first 4 days due to the presence of phosphorus in the seed culture (Figure 3.11).

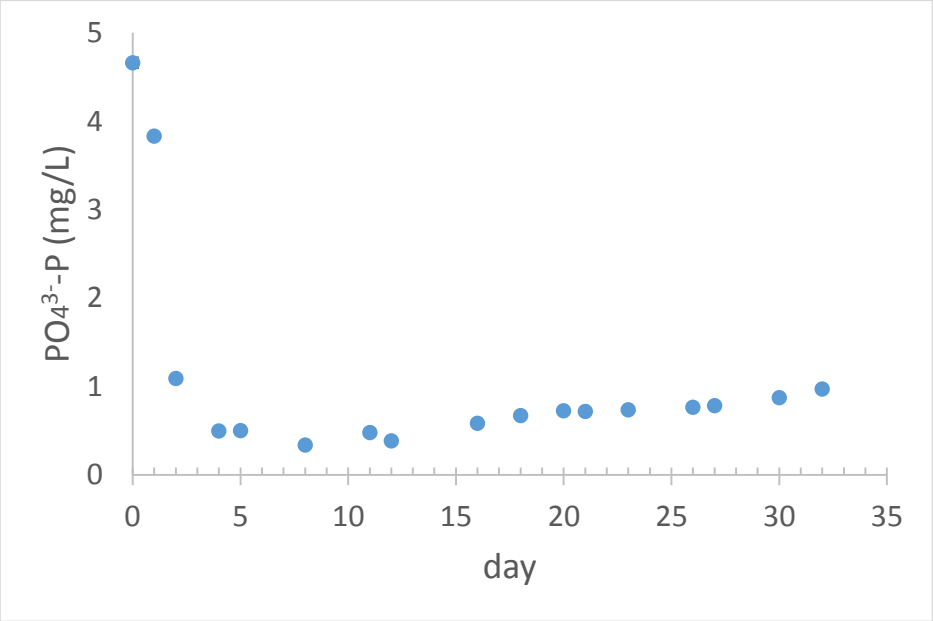


Figure 3.11 Change in the orthophosphate-P concentration of the CSTR for the growth of *Cholorella Vulgaris*

To determine whether phosphorous adsorption or coprecipitation on the cell surface has a significant effect on individual cell weight, intercellular and extracellular P contents were determined to be 7.18×10^{-13} and 4.23×10^{-13} g P/cell, respectively. Compared to the average dry weight of an algal cell, the effect of phosphorous adsorption/precipitation is neglected, which means most phosphorous decreased can be attributed to the use orthophosphate-P for growth by the algae for cell formation. Indeed, given the known of cell concentrations, the intracellular P content of algae in the CSTR was 1.30 %, which is very close to the empirical value 1.35% of algae from the formula $C_{106}H_{181}O_{45}N_6P$. These data suggest that individual algal cell weight in CSTR is larger than batch study.

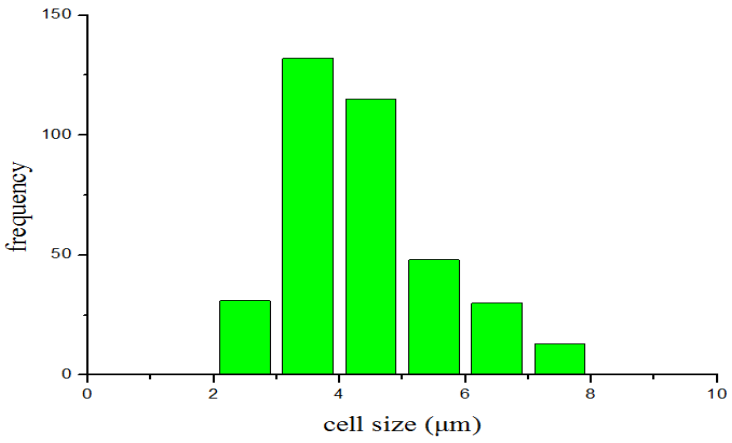
3.5 Cell Size and Cell Density of Algae and Cyanobacteria

3.5.1 Cell Size of Algae and Cyanobacteria

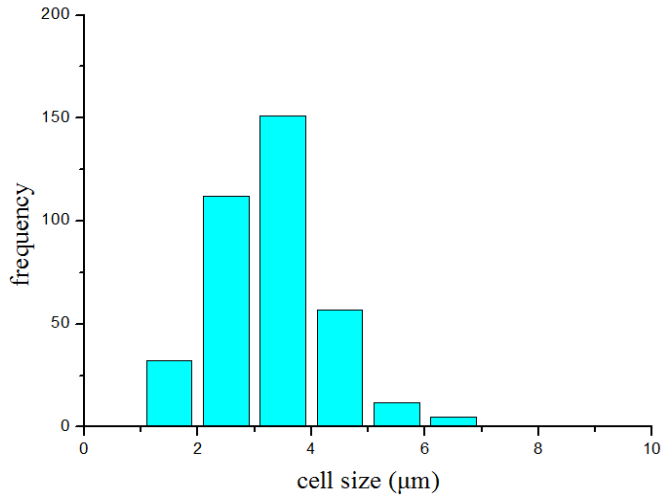
The cell size in micrometers of algae and cyanobacteria were measured by fluorescent microscopy. Cell sizes for *Cholorella Vulgaris* and *Microcystis aeruginosa* in batch study are shown in Table 3.4 and Figure 3.12.

Table 3.4 Cells size in batch of *Cholorella vulg*aris and *Microcystis aeruginosa*

	Algae diameter (µm)			Cyanobacteria diameter (µm)		
Sampling day	Min	Max	Mean	Min	Max	Mean
4	2.66	5.72	3.79 ±0.61	1.47	7.20	2.62 ±0.46
8	2.90	7.99	4.72 ±1.20	2.64	4.45	3.33 ±0.38
12	2.43	8.12	5.32 ±1.27	1.40	6.68	3.30 ±0.91
16	2.62	5.13	3.54 ±0.51	1.35	4.19	2.74 ±0.68



(a)



(b)

Figure 3.12 Cell size measurement for algae (a) and cyanobacteria (b) with fluorescent microscopy

Cell size of *Cholorella vulgaris* is in the range of 2.43-8.12 μm while *Microcystis aeruginosa* is in the range of 1.35-7.20 μm. Nevertheless, for microalgae, most cell size spread in 3-8 μm with an average diameter of (4.34 ± 0. 90) μm and no cell are less than 2 μm. However, most cell size of cyanobacteria are in 1-6 μm with an average size of (2.99 ± 0. 61) μm, which means majority of cyanobacteria cells are smaller than algae. Therefore, the expectation is that cell concentration of cyanobacteria is greater than algae in the same OD value.

Cell size was determined in the algal CSTR system as well, as shown in Figure 3.13. The maximum cell size was 8.70 μm with an average of (5.28±1.33) μm. In addition, many large cells existed in the CSTR system with 53% of cell size greater than 5 μm while only 27% in batch study, resulting in cell dry weight in CSTR almost double than

that of batch study. These larger cells were attributed to cell aggregation and floc formation in the long-term CSTR operation.

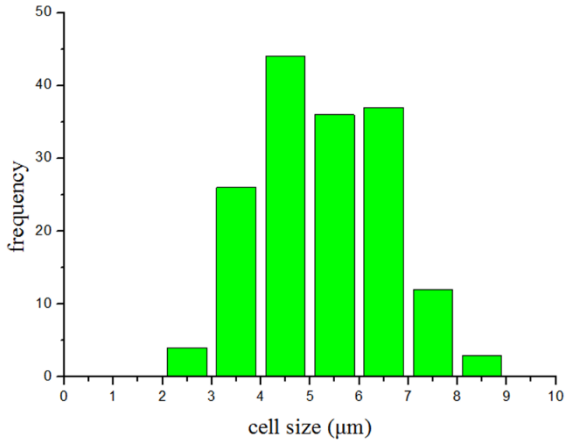


Figure 3.13 Cell size measurement in algal CSTR with fluorescent microscopy

3.5.2 Cell Density of Algae and Cyanobacteria

Combined individual cell dry weight and size, cell density was determined. Cell density of *Chlorella vulgaris* and *Microcystis aeruginosa* were (0.57 ± 0.04) g/cm^3 and (0.87 ± 0.02) g/cm^3, respectively. In the algal CSTR system, cell density of *Chlorella vulgaris* was (0.51 ± 0.07) g/cm^3.

4. Conclusions

This research determined the cell dry weight, cell size and cell density of algae *Chlorella vulgaris* and cyanobacteria *Microcystis aeruginosa* at exponential phase. The results were also compared with those of an algal CSTR system under continuous flow operation.

For phototrophs cultivated in batch systems, the dry cell weight of individual cells of *Chlorella vulgaris* and *Microcystis aeruginosa* were $(2.24 \pm 0.16) \times 10^{-11}$ g/cell and $(1.21 \pm 0.09) \times 10^{-11}$ g/cell, respectively. For comparison, a regular bacterium *E.coli* has a cell weight of $(1.69 \pm 0.36) \times 10^{-13}$ g/cell. Compared to that of batch growth result, in the algal CSTR system, the average cell weight increased to $(3.91 \pm 0.26) \times 10^{-11}$ g/cell, which was attributed to cell aggregation or colony formation through long-term continuous flow operation.

The dry weight of individual algal or cyanobacterial cells depends on cell size. The cell size of algae ranged from 2.43 to 8.12 μm, with an average of (4.34 ± 0.90) μm. For comparison, the cell size of cyanobacteria ranged from 1.35 to 7.20 μm with an average of (2.29 ± 0.61) μm. Cell densities for algae and cyanobacteria were (0.57 ± 0.04) g/cm^3 and (0.87 ± 0.02) g/cm^3, respectively. While in algal CSTR systems, average algal cell size increased to (5.28 ± 1.33) μm and the cell density was (0.51 ± 0.07) g/cm^3.

5. Future Study

Individual cell weight may be affected by lipid content in phototrophs. Light intensity, nutrients concentration, nitrogen and phosphorous ratio and other factors may change intracellular composition including lipid content. Future research can be explored to determine the effect of these factors.

Individual cell weight and cell density may change when algae are cultivated under P limited conditions, which often results in luxury P uptake (Riegman et al., 2000). Future research is needed to determine these physical properties of the cells under environmental stress conditions.

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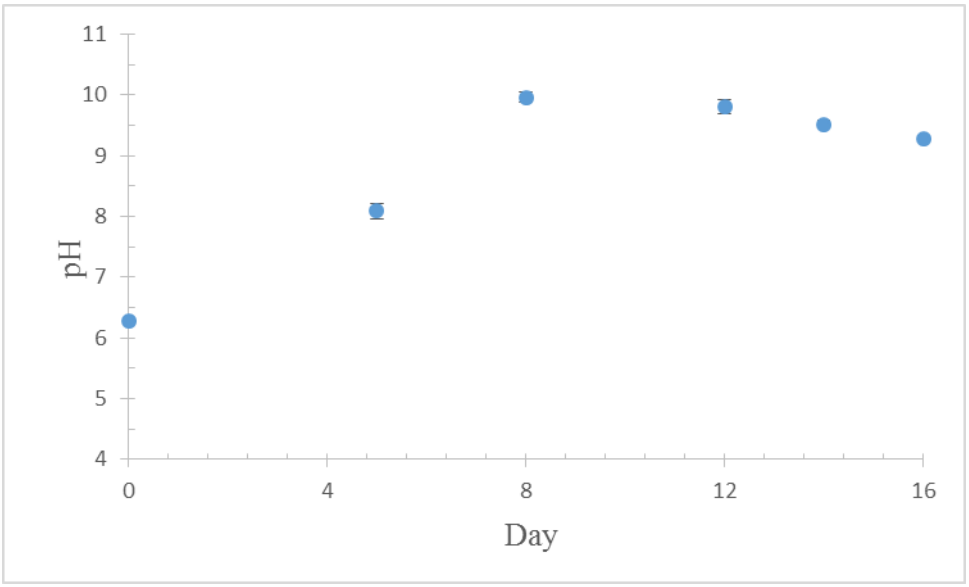
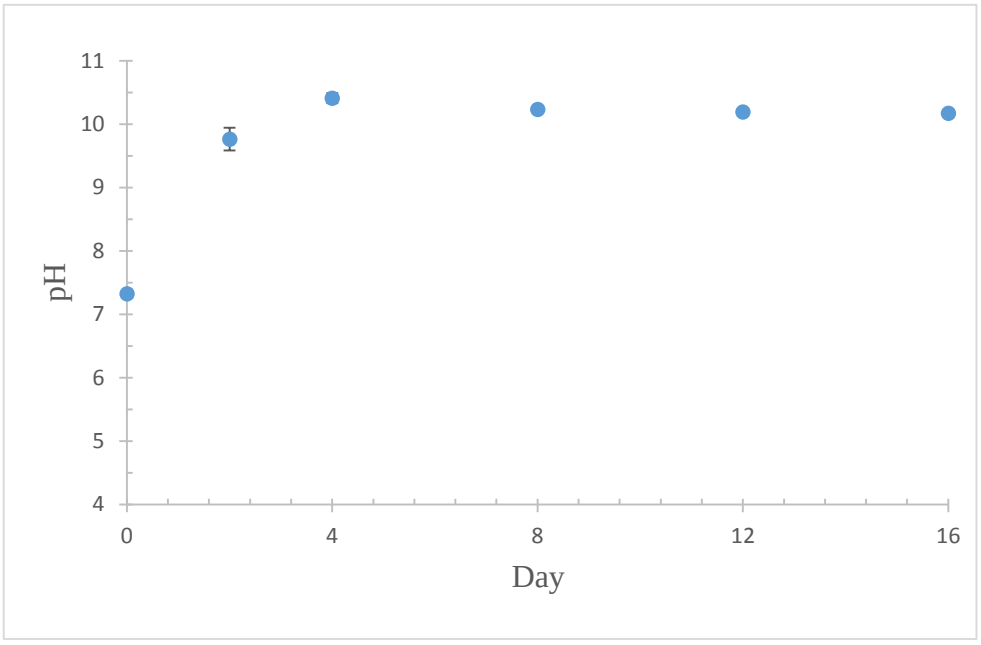
Appendixes



(a)



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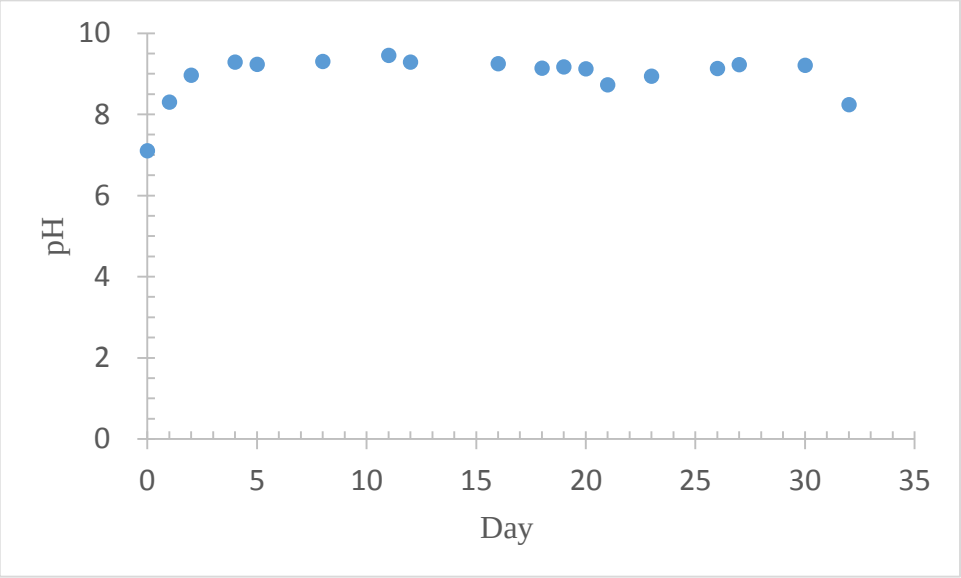

(c)

Figure S1 Changes in mixed liquor pH for the growth of *Chlorella vulgaris* (a) and *Microcystis aeruginosa* (b) in batch and CSTR systems (c)

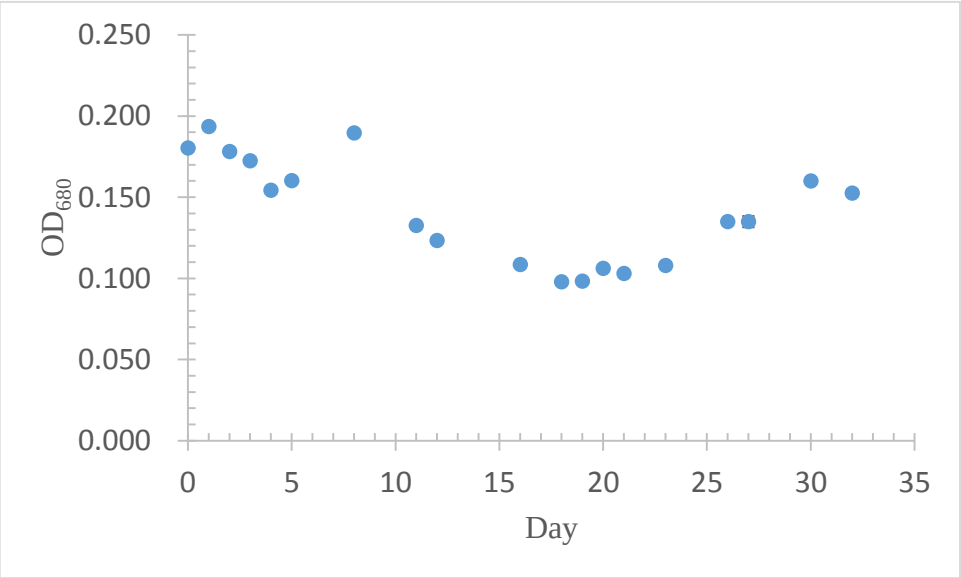

Figure S2 Changes in OD in an algal CSTR

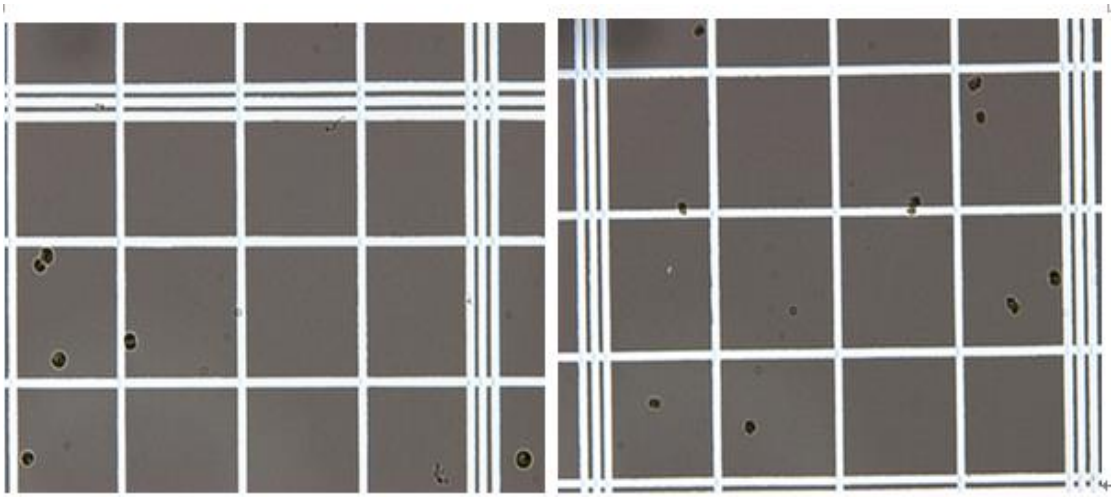

Figure S3 Hemocytometric images of algae (left) and cyanobacteria (right)

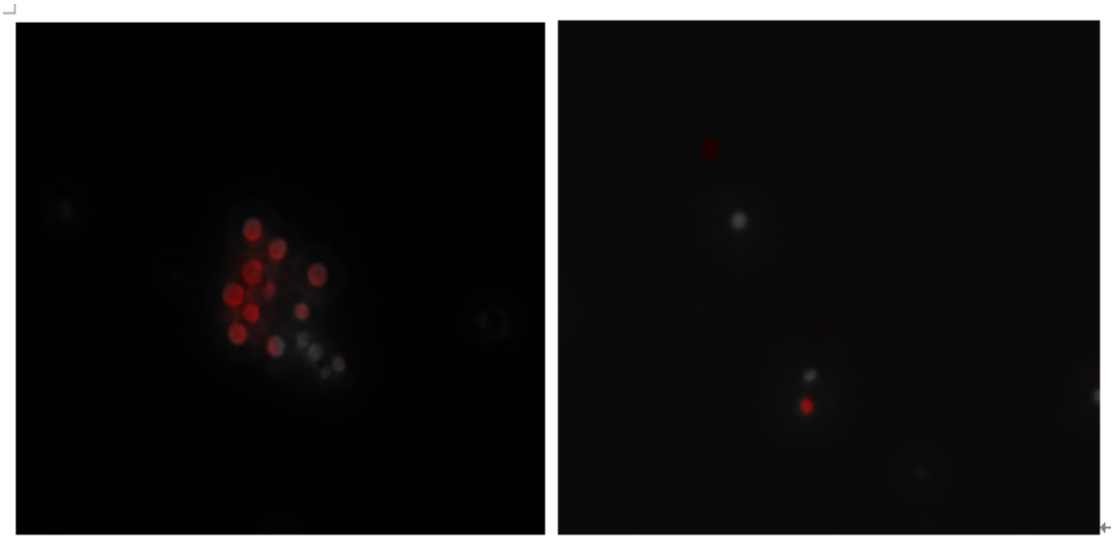

Figure S4 Overlay images in bright field and fluorescent field of algae (left) and cyanobacteria (right)