GROWTH OF CHLAMYDOMONAS REINHARDTII UNDER NUTRIENT-LIMITED CONDITIONS IN STEADY-STATE BIOREACTORS

by Prayut Mahendrakumar Bhamawat

GROWTH OF *CHLAMYDOMONAS REINHARDTII* UNDER NUTRIENT-LIMITED CONDITIONS IN STEADY-STATE BIOREACTORS

A Thesis

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by

Prayut Mahendrakumar Bhamawat

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ABSTRACT

In recent years, massive mobilization of researchers is mounting regarding the development of more sustainable energy supplies, driven by factors such as oil price spikes and the need for increased energy security. Biofuel production is, at its purest, an attempt to wean the world's addiction to fossil fuels. Large-scale algae production has the potential to provide a significant fraction of the liquid fuel market, due to its high productivity per area. In order to realize the great potential of algae for biofuel, economical production systems must be developed that minimize inputs, such as nutrients and water. It should be made more robust, productive and predictable in order to ensure successful scale-up. Using traditional reactor engineering we propose to further the understanding of algae reactor systems by investigating the effects of various bioreactor operating conditions on growth rate, cell composition, and storage of neutral lipids.

Chlamydomonas reinhardtii is a model green algae species, widely grown in the laboratory for research in cell and molecular biology due to its fast generation time and ability to grow photo-, mixo-, or heterotropically while maintaining a functional photosynthetic apparatus. The productivity of *C. reinhardtii* was tested in tubular flow-through photo-bioreactors under steady-state conditions. Reactors were continuously fed media containing different nitrogen, phosphorus and sulfur concentrations. The cell mass density, chlorophyll a and pH were monitored during growth. Algae were harvested and the carbon, nitrogen, phosphorus and lipid content of the cells were measured under conditions of replete and deficient nitrogen, phosphorus and sulfur. The nutrient consumption was calculated by analyzing influent and effluent nutrients concentrations.

In experiments where nitrogen inputs were varied from sufficient to limiting, cellular composition changed significantly showing 3-fold higher C:N ratio in nitrogen-deplete cells, and higher carbon content is also supported by four-fold higher relative lipid content. For phosphorus limiting conditions, cells showed slightly higher C:N ratio but no significant increase in carbon content or relative lipid content was observed. Unfortunately for sulfur limitation, cellular composition was not determined so far and thus nothing certain could be said for carbon accumulation in sulfur-deplete cells. Also, no significant change was observed in relative lipid content under this condition. This work also confirms the decline in growth rate affecting final biomass yield (g/L-day) and decrease in cell quota of non-limiting nutrients under limiting nutrient supply. Also, of all the nutrients evaluated, nitrogen was observed to be the most critical nutrient affecting growth rate, cellular composition and neutral lipid storage.

BIOGRAPHICAL SKETCH

Prayut Bhamawat was born in Dungarpur, Rajasthan in India. He was brought up in Vadodara, Gujarat in a business family and attended the public school system, eventually graduating from D.A.V public school in 2003. He attended Institute of Technology, Banaras Hindu University in Varanasi, Uttar Pradesh, and obtained a Bachelors of Technology degree in civil engineering with honors in 2008. In college, apart from maintaining a good academic record, he actively participated in lots of extra-curricular activities. He organized a national technical festival at his university in 2007 and also served as the General Secretary of IT-Gymkhana (Student Activity Center) the same year. In May 2007, he was accepted as a Summer Scholar at the Indian Institute of Technology, Kanpur (IIT-K) in Civil and Environmental engineering, where he developed his interest for higher studies in environmental engineering. Just after finishing his bachelor's, Prayut entered Cornell University in Fall 2008 to pursue a Master of Science degree in the Civil and Environmental Engineering department with a specialization in Environmental Processes which this work is the culmination.

Dedicated to those closest to my heart

Mom, Dad, my younger brother Milind

and all of my friends

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LIST OF ABBREVIATIONS

”…… ……Inch

^{o}C……..….Degree Celsius

µ…...…….Growth Rate

µL…….....…Microliter

µM……..……Micromolar

µm……..Micrometer

B…….…….Boron

C. reinhardtii……..….*Chlamydomonas reinhardtii*

C……....Carbon

Ca……..…Calcium

$CaCl_2.2H_2O$…….…Calcium chloride dehydrate

Cl……...……..Chlorine

Co……...Cobalt

CO_2……...……Carbon dioxide

$CoCl_2.6H_2O$…….……Cobalt chloride hexahydrate

Cu……..Copper

$CuSO_4.5H_2O$……...……..Copper sulfate pentahydrate

DCW……...……Dry Cell Weight

DNA……..……...Deoxyribonucleic Acid

dw…….dry weight

EDTA-Na_2……...……Ethylene Diamine Tetraacetic Acid Disodium

EPA……Environmental Protection Agency

Fe……...Iron

$FeSO_4.7H_2O$……Ferrous sulfate heptahydrate

FU……....Fluorescence Unit

g……..Grams

H_2O_2…….....Hydrogen peroxide

H_3BO_3……...……..…….Boric Acid

HNO_3……Nitric acid

hr……..….Hour(s)

K……...…Potassium

K_2HPO_4……..Dipotassium hydrogen phosphate

KH_2PO_4.... …….....Potassium dihydrogen phosphate

KNO_3……..……..Potassium nitrate

K_{SN}……..……Half-saturation constant for nitrogen

K_{SP}……..……Half-saturation constant for phosphorus

L……...Liters

Mg……..…Magnesium

mg…….Milligram

$MgSO_4.7H_2O$……...Magnesium sulfate heptahydrate

min……..…….Minutes

mL……...…Milliliter

mM……...……Millimolar

mmoles……..….…….Millimoles

Mn……...Manganese

$MnCl_2.4H_2O$……..……Manganese chloride tetrahydride

Mo……..……..Molybdenum

N……...……...Nitrogen

Na……..……Sodium

NA……..…….Not Available

Na_2SeO_4……..……Sodium selenate

NH^{4+}……..Ammonia

NH_4Cl……..Ammonium chloride

$(NH_4)_6Mo_7O_{24}.4H_2O$…….……..Ammonium Molybdate

nm……...…….Nanometer

NO_3^-……..……..Nitrate

P……..……Phosphorus

PBR……..…….……Photobioreactor

PIPES……..……Piperazine-N,N’-bis(2-ethanesulfonic acid)

PO_4^{3-}……..Phosphate

PTFE……Polytetrafluoroethylene

S……..…….Sulfur

Se…….……...Selenium

SO_4^{2-}…….……..Sulfate

TAG……..….Triacylglycerol

V……...….Vanadium

Zn……..……..Zinc

CHAPTER 1

INTRODUCTION

1.1. Algal Biofuel

Microalgae, sunlight-driven cell factories, have recently received a lot of attention as a source of sustainable energy production. The main characteristics which set algae apart from other biomass sources are that they can grow fast, in a variety of environmental conditions, requiring just sunlight and basic nutrients. Microalgae can grow by converting solar energy into chemical energy using photosynthesis, and have doubling times as short as half a day. Compared to other agriculturally produced biodiesel feedstocks (e.g. corn, soybean, jatropha, palm oil), microalgae require much less land area and they seem more capable of displacing fossil fuels (Chisti 2007). But there are challenges involving high capital and operating costs especially with harvesting, developing effective measures to prevent contamination and selecting strains that provide high oil production.

Lipid content in microalgal cells varies from 2-60 percent of total cell dry matter depending on strain and growth conditions (Becker 1994; Sheehan et al. 1998; Hu et al. 2008). A current focus in microalgae research is the selection of the strains with rapid biomass doubling times and naturally high lipid content. It has been reported that under unfavorable environmental conditions or under stress conditions for growth (nutrient-limiting conditions), many microalgae accumulate neutral lipids (Hu et al. 2008). However, nutrient limitations can reduce total biomass growth. Thus, optimizing biomass production under nutrient-limited conditions while still inducing the storage of carbon-rich lipids is another interest of research. Efforts are also being made on a different approach of genetically engineering microalgae to increase the productivity. Contribution to this approach of manipulating of microalgae lipid production by genetic engineering was first reported by Dunahay and colleagues

(Dunahay et al. 1996). Genetically engineering species that could be contamination resistant while providing consistently high lipid content under optimal nutrient intake seems to be most promising for meeting the challenges for algal biofuels. However, genetic modification is considered very controversial and there are many underlying ethical, legal and even environmental issues to be addressed. Thus, very little progress has been made so far with genetic engineering and major work has been done with wild-type of algae.

1.2. Research Objective

The main objective of this thesis research was to grow the microalgae *C. reinhardtii* under nutrient-limited conditions such that cellular composition is changed reproducibly. A replicable chemostat PBR (photo-bioreactor) system was designed and created for growing microalgae cells in steady-state condition that can be use for evaluating their physiology and storage of neutral lipids in response to controlled environments.

Little information is available concerning the effects of various nutrient limitations on the autotrophic growth of *C. reinhardtii*, and this research will help to investigate the effect of growth-limiting substrate influencing cell composition and cell metabolism. Moreover, it also provides information on the variation of the growth rate of a culture with limiting concentrations of nutrients which can be use to develop a mathematical model to simulate a substrate limiting system to assist the design and scale-up of the cultivation process. Also, it can be used for optimizing the biomass production for nutrient availability.

Using chemostats ensures large volumes of uniform cells and provides reproducible conditions with a precision unobtainable in batch culture. It helps to

maintain substrate-limited growth while at the same time maintaining a constant environment.

Parameters used for monitoring growth rate were mass density and fluorescence (chlorophyll-a content). To study the cells physiological response, C, N and P content in cell were measured. Cell composition is a useful indicator of nutrient limitation because of the significant responses to the nutrient-limited growth rates. Finally a mass balance was performed on N and P by accounting for nutrient entering (influent) and leaving (effluent) a system and that accumulated in cells (cell composition), to check for the conservation of mass of each nutrient.

CHAPTER 2

BACKGROUND

2.1. Chlamydomonas reinhardtii

Chlamydomonas reinhardtii is a ~10-µm diameter, motile unicellular eukaryotic green alga that swims with two flagella. It has long been used as a model system for genetic and biochemical studies of various cellular processes and for the study of photosynthesis (Lemaire et al. 2003). Because of the availability of its complete genome sequence, it has developed into the principal genetic model organism. It is the only eukaryotic alga that has been genetically modified to produce high value proteins (Mayfield et al. 2007).

C. reinhardtii is also a model organism for response mechanism studies with the change of environmental conditions. It is highly adaptable and can live in many different environments; e.g., in presence of alternative carbon source it can also thrive in total darkness (Merchant et al. 2007). Its adaptation to environmental constraints is robust and rapid which makes it ideal for research. Also, it is easy and fast to grow and requires inexpensive medium. Because of its known genome sequence, fast generation time, and relative adaptability, we considered *C. reinhardtii* as an important model organism to study responses to nutrient deprivation.

2.1.1 C. reinhardtii Nutrient Requirements

Nutrient requirements for any algae depend on many factors. Requirements for developing nutrient recipes for algal cultivation can be summarized on the basis of total salt content, cellular nutrient content, nitrogen source, carbon source, pH, trace elements and some chelating agent and vitamins (Vonshak 1986). Other factors that can be considered are the purpose for which the algae will be cultured (e.g., for

optimal biosynthesis of valued biofuel feedstocks, for nutriceuticals, or for aquaculture feed).

The most important nutrients for autotrophic growth of algae are C, N and P, and their supply is central to algal biotechnology. Other macronutrients of importance are S, K, Na, Fe, Mg, Ca and trace elements B, Cu, Mn, Zn, Mo, Co, V and Se. Most of these nutrients are usually present in sufficient amounts, relative to the algae's needs, so as not to be potential limiting factors for growth. Many of the trace elements are important as enzyme cofactors. P and S are two macronutrients that photosynthetic organisms require in relatively large amounts. For the purpose of this research, focus is on the N, P and S content under replete and deplete conditions.

2.1.2 Nutrient-limited Growth

Nutrient supply can affect the growth rate of algae in addition to affecting its final biomass and lipid yield. The growth rate of algae will decline if the concentration of a given nutrient drops below that supporting an uptake rate sufficient to maintain the existing growth rate. Such nutrient-limited growth is best studied in chemostat cultures, where the growth rate of cells is a function of the concentration of limiting nutrients in the fresh medium, the illumination and of the dilution rate only. Studies have looked at the effects on cellular nutrient change following the transfer of cells from nutrient-replete conditions to nutrient-starved conditions and also, to link cellular growth phase, internal P quota and cell volume to carbon allocation amongst macromolecule classes and storage products (Dean et al. 2008). One of the most common responses to nutrient limitation is a decrease in the cell quota of that nutrient, where cell quota can be defined as the content of a chemical element in the cells (Smirnov and Revkova 2002), and it was first introduced by Droop (1968) for the species *Monochrysis luthery*. It has been also found that under limiting condition, cell

quotas of non-limiting nutrients also may decline, though not as much as the limiting nutrient (Darley 1982). Apart from that, cell composition is also considered to be a useful indicator of nutrient limitation because of the significant responses to the nutrient-limited growth rates. For natural phytoplankton, cells growing near nutrient-saturated growth rates typically contain C, N and P in the atomic ratio of 106: 16: 1, also known as the Redfield ratio (Goldman et al. 1979). Hence, variation in biomass C: N: P ratio is also widely used to quantify possible nutrient limitation.

It has been also found that under unfavorable environmental or stress conditions for growth including nutrient limiting conditions, many algae revise their lipid biosynthetic pathways in the direction of accumulating neutral lipids (20-50% DCW (Dry Cell Weight)), mostly in the form of triacylglycerols (TAG), and hence microalgae have been postulated as cell factories to produce oils and other lipids for biofuels and other biomaterials (Hu et al. 2008). So far for *Chlamydomonas*, stress response has been well studied and established (Grossman 2000) but few studies have focused on the lipid biosynthesis pathways under such conditions.

2.1.2.1 Effect of Nitrogen

Nitrogen is among the most important nutrients contributing to the biomass produced. It generally accounts for about 7-10% cell dry weight and is an essential constituent of all structural and functional proteins in algal cells. Nitrogen is mostly supplied as nitrate (NO_3^-), ammonia (NH_4^+) or urea. Ammonia nitrogen is reported to be the preferred N-source for microorganisms (Richmond 2004) which can be because assimilation of ammonium is energetically less demanding than that of other prevalent nitrogen sources such as nitrate and nitrite (Grossman 2000). Also, assimilation of either NO_3^- or NH_4^+ is found to be related to the pH of the growth

media, pH drops significantly; during active growth when ammonia is used as the sole source of N (Richmond 2007).

Chlamydomonas does not have the ability to fix atmospheric nitrogen, but can assimilate N from number of different nitrogen sources. Typical responses to nitrogen limitation are discoloration of the cells (decrease in chlorophylls and an increase in the carotenoids) (Richmond 2004) and accumulation of organic carbon compounds such as carbohydrate and lipids (Dean et al. 2010). Of all the nutrients evaluated, it has been reported that nitrogen limitation is the most critical nutrient affecting lipid metabolism in algae generally (Hu et al. 2008).

C. reinhardtii under N-limitation, they demonstrated restricted cell division and increased cell size following N-limitation and also elicited rapid and distinctive changes in cellular carbon allocation with growth stage and nutrient availability (Dean et al. 2010). It has been also reported that N-deprived *C. reinhardtii* cells will over-accumulate starch and lipids which can be used for formate, alcohol and biodiesel production (Mus et al. 2007). Wang (2009) has reported increases in lipid bodies by 15 times under N-limiting condition in presence of acetate.

2.1.2.2 Effect of Phosphorus

Phosphorus is another major macronutrient that plays an important role in cellular metabolic processes by forming many structural and functional components required for normal growth and development of microalgae (especially nucleic acids and phospholipids). It is essential for many cellular processes such as energy transfer, biosynthesis of nucleic acids, DNA, etc. In non-limiting conditions, cellular phosphorus content is 1-2% dry weight (Goldman 1980). Orthophosphate, PO_4^{3-}, is the main inorganic phosphorus source for algae, although most can obtain the element from various organic phosphates. Also important is the ratio of N:P in the growth

media as this is also responsible for the potential productivity and can be important in maintaining the dominance of the candidate species in culture (Kaplan et al. 1986).

Some of the symptoms of phosphorus limitation in algae are the same as those observed in nitrogen-deficient cultures; the chlorophyll-a content decreases and carbohydrate content increases (Richmond 2004). In this case, polyphosphate (which acts as a P reservoir in P-replete conditions and can store excess P (luxury uptake)), also decreases in cells as a function of P-limited growth rate along with the decline in total cell quota of P (Darley 1982). For a few algal species, P-limitation has been reported to increase lipid content in cells, mainly TAG (Hu et al. 2008).

In addition to general nutrient stress responses, *Chlamydomonas* cells also exhibit specific P deprivation responses, including changes in the pattern of gene expression, as well as mobilization and transport of P (Irihimovitch and Yehudai-Resheff 2008).

2.1.2.3 Effect of Sulfur

Sulfur (S) is an essential element present predominantly in proteins and various coenzymes. It is critical for the association of metal ions to proteins (electron carriers and redox controllers) and is a component of metabolites that function in photoprotection and signal transduction. The majority of accessible S is in the form of sulfate, SO_4^{2-}. Like most organisms, *Chlamydomonas* has the capacity to assimilate S as a sulfate anion (SO_4^{2-}). They translocate it to the plastid, where primary S metabolism takes place (Irihimovitch and Yehudai-Resheff 2008)

Responses to sulfur starvation for photosynthetic organisms include increasing the capacity of the cell for transporting and/or assimilating exogenous sulfate, restructuring cellular features to conserve sulfur resources and modulating metabolic processes and rates of cell growth and division (Zhang et al. 2004).

Studies in *Chlamydomonas* have shown that limiting P and S conditions elicits similar responses. Because of the absence of a storage compound for S, *Chlamydomonas* requires a continuous supply of S (Irihimovitch and Yehudai-Resheff 2008). Limiting S conditions in *Chlamydomonas* has been reported to induce a rapid decrease in photosynthesis activity, specifically a decline in photosystem II electron transport (Wykoff et al. 1998). The patterns of gene expression in *C. reinhardtii* cells following S deprivation as obtained by Zhang, Shrager et al. (2004) reveal both the general and specific responses that enable a cell to survive extended periods of time under specific deprivation conditions. Studies have also shown that total lipid content is enhanced by 12% in sulfur deprivation in the green algae *C. reinhardtii* (Hu et al. 2008; Sato et al. 2000).

2.2. Photobioreactors

Algae are mostly cultivated in either open ponds or in photobioreactors (PBRs). According to Tredici (2004), PBRs are illuminated systems that do not allow direct exchange of gases or contaminants between the culture and the atmosphere. These are closed, illuminated vessels where algae are cultured under controlled conditions for biomass production. They help minimize the threat of contamination from competitive species, permitting cultivation of a monoculture and preventing culture collapse from predation which are some of the significant problems in open ponds. Microalgal biomass production is limited to a few thousand tons per year with only few hundred tons produced in closed PBRs and the balance of the production in open ponds (Borowitzka 1999). The critical issues with PBRs are high construction cost and operating cost for converting it to commercial scale, but they have high rates of productivity, better control over biocultural conditions (pH, light, carbon dioxide,

temperature), prevent water evaporation, reduce carbon dioxide losses and permit higher cell concentrations (Richmond 2004).

In some cases, PBRs serve only as the first step in an algal production system, where they are used to create a monoculture under near-ideal conditions, with the mature algae culture then being discharged to open ponds. Such systems are also known as hybrid systems. This system reduces the operating cost of PBRs and also limits the chances of contamination in open pond systems.

Due to current interest in microalgal biotechnology, the topic of PBRs has been revisited with respect to scale up, reduced cost, and increased life span. So far in the PBRs studied, the auxiliary power required is too high, while the obtained biomass concentrations are too low. Thus, higher production for algal biomass in PBRs has been major focus and has been reviewed by a number of authors (Borowitzka 1999; Pulz 2001; Richmond et al. 2003; Tredici and Richmond 2004; Rodolfi et al. 2009). Different geometries and operating systems developed depend on the product to be produced and economic constraints to bring the performance close to theoretical maxima. They has been explored for obtaining high-density culture by optimizing environmental factors like light delivery, CO_2 transfer, medium supply, mixing and temperature (Suh and Lee 2003). Because of intense interest in this field, the design of PBRs in commercial scale has made good progress in the last decade. Posten (2009) discussed the basic trends emerging in research to enhance bioreactor performance and to reduce capital costs.

2.2.1 Photobioreactor Design

Standard design reactors that are commonly used are flat plate reactors, bubble column reactors and tubular reactors. Depending on the different growth conditions, product formation kinetics and type of product required, reactors are developed from

these standard designs. Flat plate reactors are the most robust design (Ratchford and Fallowfield 1992), but bubble columns are the ones which are frequently used in lab scale for indoor experiments (Posten 2009).

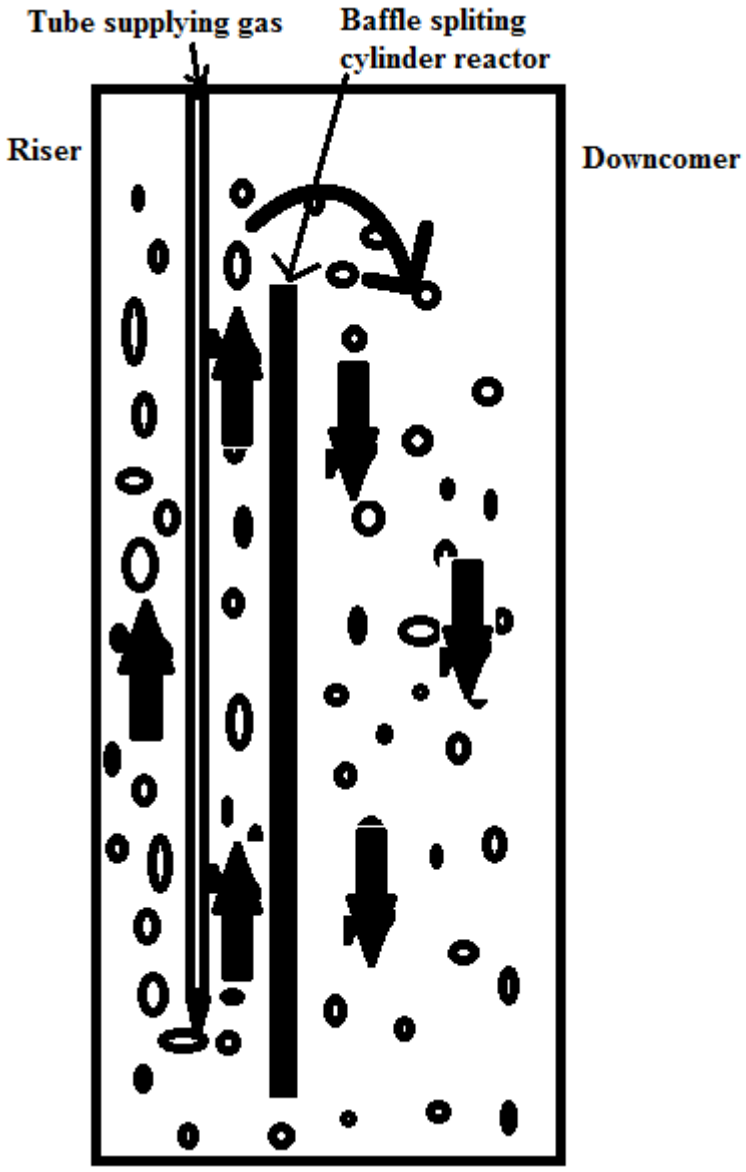


Figure 2.1 Diagram of split cylinder reactor showing direction of flow of gas bubbles and cells in riser and downcomer

For bubble column reactors, airlift principles are used to increase the axial transport (Mirón et al. 2000). The rising gas bubbles induce liquid circulation via sheer forces as they rise up through the riser. Thus, the cells flow through the riser and downcomer (downcomer is a section of the entire cross-section in a split cylinder reactor or a coaxial inner cylinder in draft tube/annualar column reactor) intermittently (Figure 2.1), resulting in an additional light/dark cycle in the range of 1-100 seconds (Barbosa et al. 2003; Janssen et al. 2000). Design considerations for bubble column airlift bioreactors have been described extensively (Acién Fernández et al. 2001; Chisti et al. 1988; Merchuk and Gluz 1999; Kantarci et al. 2005).

CHAPTER 3

MATERIALS AND METHODS

3.1 Microalgae and Culture Media

The microalgae *Chlamydomonas reinhardtii* wild type (to be referred to as *C. reinhardtii*) was used for all experiments. The inoculum for the experiments was grown inside a growth chamber in 10 mL tubes at low irradiance, room temperature of 20 ± 1^{o}C and transferred once every two weeks to fresh media at a 1:5 dilution. Cultures were used to innoculate experimental vessels in late log-phase of growth around 10th or 11th day. Culture vessels were maintained in artificial 24-hour light provided by sodium lamps, measured at 600 µmol photons m^{-2} s^{-1} photosynthetically active radiation at the vessel's surface and at constant room temperature of 20 ± 1^{o}C in a separate growth chamber.

A new minimal medium was generated for these experiments which we called NAPK media with PIPES as an external pH buffer, is a modification of the popular TAP media using Khul's trace metal solution (Harris 1989). Table 3.1 gives the chemical composition of the NAPK media compared to TAP media. The pre-culture medium was the same used for experimental cultivation in nutrient-replete conditions. Media was sterilized before use by autoclaving.

Table 3.1 NAPK media for cultivation of *C. reinhardtii* and comparison with TAP media

Chemicals	**TAP media**		**NAPK media**	
NH_4Cl	0.40 g/L	7.5 mM	0.20 g/L	3.73 mM
$CaCl_2.2H_2O$	0.05 g/L	0.34 mM	0.05 g/L	0.34 mM
$MgSO_4.7H_2O$	0.10 g/L	0.4 mM	0.10 g/L	0.40 mM
K_2HPO_4	0.12 g/L	0.68 mM	0.12 g/L	0.68 mM
KH_2PO_4	0.06 g/L	0.45 mM	0.06 g/L	0.45 mM
KNO_3	-	-	0.38 g/L	3.75 mM
Tris Base	2.42 g/L	20 mM	-	-
PIPES			0.04 g/L	1.29 mM
Glacial Acetic Acid	1mL/L	-	-	-
Trace Elements[a]	1 mL/L	-	1mL/L	-

a→TAP media includes Hutner's trace metal solution (Harris 1989) whereas NAPK media includes Kuhl's trace metal solution (Harris 1989)

Chemicals	Kuhl Trace Metal Solution after dilution into media	
$EDTA\text{-}Na_2$	9.300 mg/L	24.98 µM
H_3BO_3 (boric acid)	0.061 mg/L	0.99 µM
$ZnSO_4.7H_2O$	0.288 mg/L	1.00 µM
$MnCl_2.4H_2O$	0.198 mg/L	1.00 µM
$FeSO_4.7H_2O$	6.950 mg/L	25.00 µM
$CoCl_2.6H_2O$	1.610 mg/L	6.76 µM
$CuSO_4.5H_2O$	0.003 mg/L	0.01 µM
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.012 mg/L	0.01 µM
Na_2SeO_4	0.002 mg/L	0.01 µM

3.2 Experimental Setup

Twelve identical mini-airlifts of 550 mL working volume were constructed based on the design of (Vega-Estrada et al. 2005) (Figure 3.1).

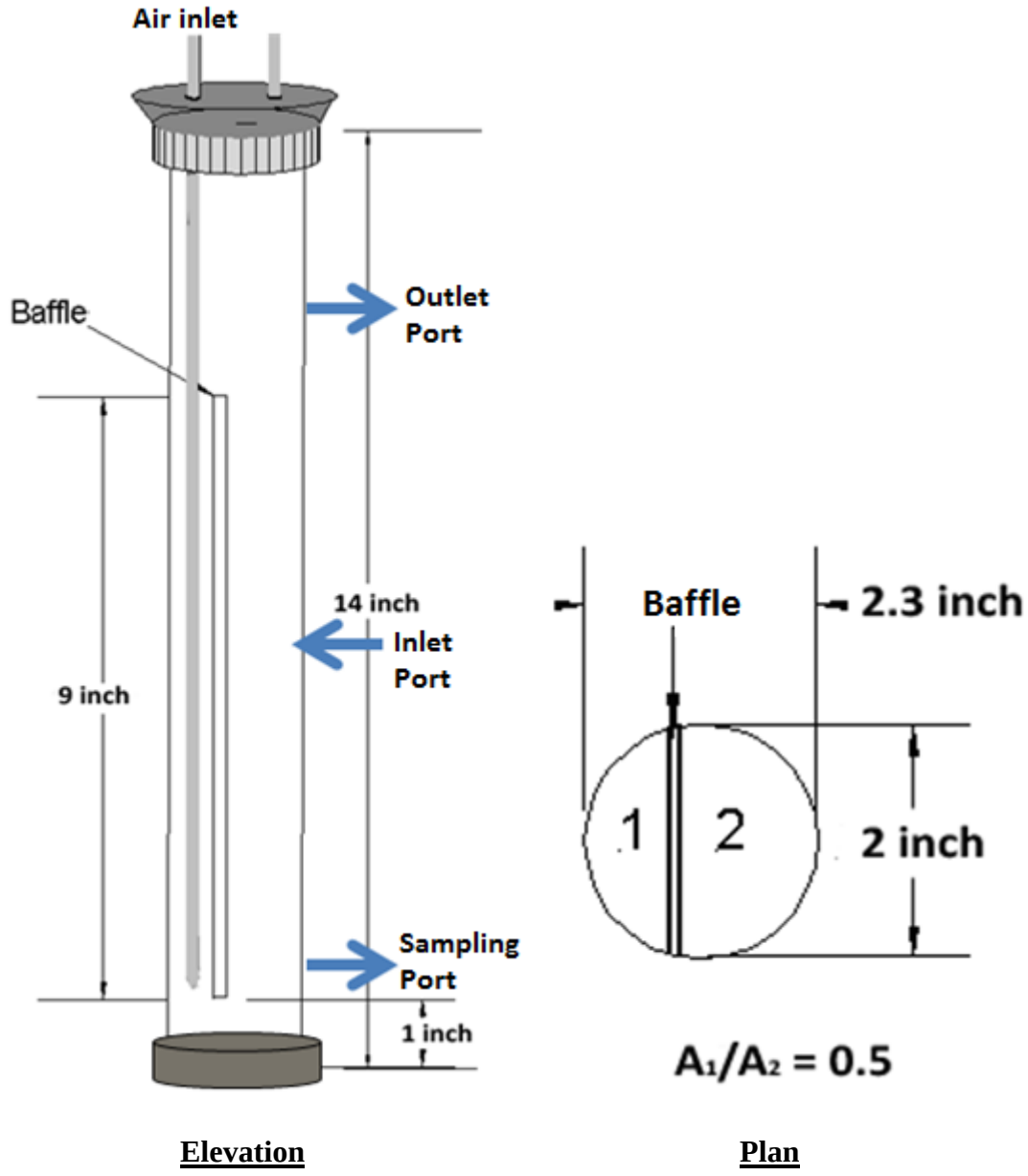


Figure 3.1 Schematic drawing of the airlift bench-scale photobioreactor. In the plan view, (1) represents the riser and (2) represents the downcomer.

The 14.0” long cylinder was constructed of clear polycarbonate with an inside diameter of 2.0” and with thickness of 0.3”. To close the cylinder it had a clamp-tight pipe-end cap and a rubber stopper (size 13) with two holes. A vertical clear acrylic baffle (9.00” x 1.97” x 0.13”) split the riser from the downcomer while allowing light to pass through. The riser/downcomer cross-sectional area ratio (A_r/A_d) was 0.5 and the bottom clearance was 1.0”. The cylinder had 3 ports, namely sampling port, inlet port and outlet port. Each airlift was able to be disassembled to be disinfected separately, with the exception of baffle, which was permanently attached to the cylinder.

Complete setup is shown in a diagram and photos below (Figures 3.2 and 3.3). Reactors were filled with a total volume of 550 mL consisting of media and inoculum. The liquid was mixed by air bubbled into the reactor bottom through the end of a glass pipet that passed through one of the holes in the rubber stopper. Room air was supplied using an aquarium pump (Dolphin Three Star air pump) which had an adjustable air flow-rate. Air from this pump is first passed through a water container to convert dry air to moist air to reduce evaporation loss inside the reactors. Moist air is then passed through 0.2 μm Pall acrodisc air filter before going into the reactor. CO_2 from the air (~0.03%) is the only source of carbon for the algae. For running reactors in continuous culture mode an Ismatec peristaltic pump (IPC 8) was used which drew fresh sterile media from the reservoir and delivered it to the reactor through the inlet port. Circulation within a reactor because of air bubbling ensured proper mixing of fresh media and algal culture. The effluent exits through the outlet port via gravity and was collected in an effluent tank.

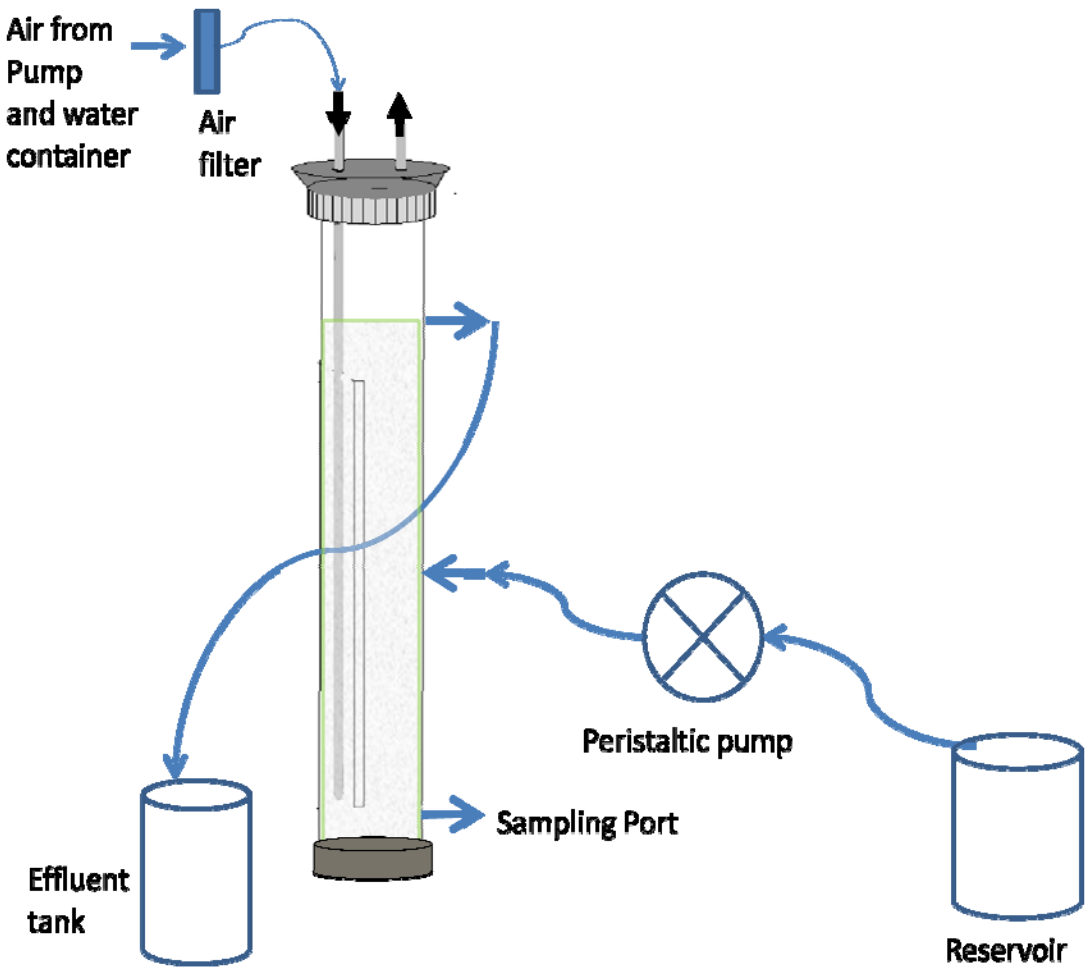


Figure 3.2 Schematic diagram of experimental setup

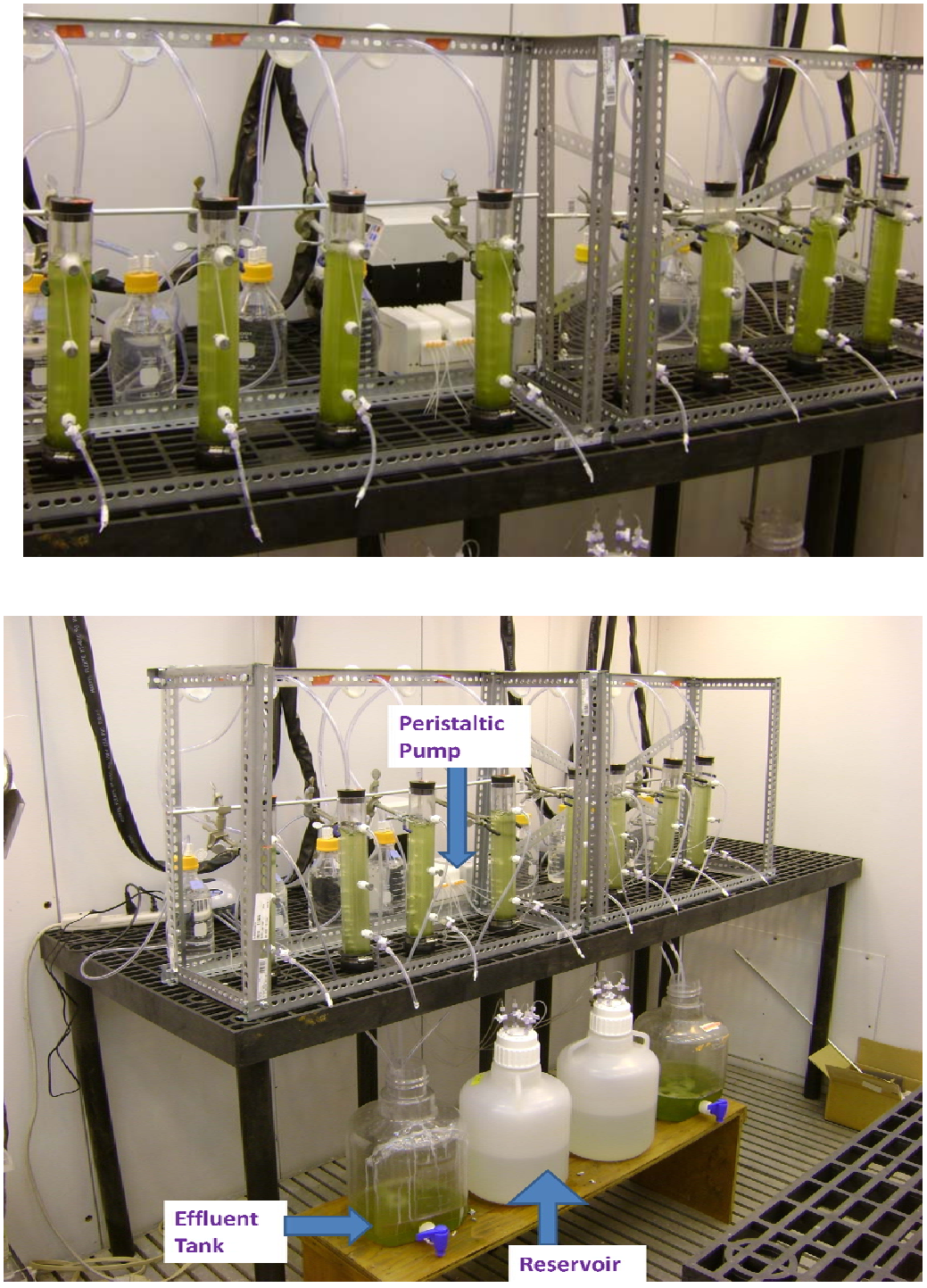


Figure 3.3 Pictures of the experimental setup with the top picture running in batch mode and bottom picture in continuous flow mode.

After each experiment, the reactors were disassembled and the entire setup, including the air filters, was sterilized by autoclaving at 121^{o}C for 15 minutes.

3.3 Growth Rate in Batch Culture

For batch culture, the cell growth rate can be described by Monod kinetics:

$$\frac{dx}{dt} = \mu x - bx$$

where x is the final biomass, b is the decay rate (hr^{-1}), t is time (hr) and μ is the specific growth rate (hr^{-1}). Ignoring the decay term for exponential growth phase, it reduces to

$$\frac{dx}{dt} = \mu x$$
$$\Rightarrow x = x_0 e^{\mu t}$$
$$\Rightarrow \ln x = \mu t + \ln x_0$$
$$\Rightarrow \mu = \frac{\ln x - \ln x_0}{t}$$

where x_o is initial biomass. Biomass concentration was monitored as chlorophyll-a fluorescence. Thus, the specific growth rate was determined by plotting the natural logarithm of fluorescence against time and calculating the slope of the growth curve of cells within the exponential phase. The maximum growth rate (μ_{max}) was calculated from the first 36 to 48 hours, when the growth rate was highest throughout all experimental conditions and trials.

3.4 Growth Rate in Continuous Culture

In continuous-flow cultures, fresh culture media was supplied to the homogeneously mixed culture and excess culture was removed continuously. The cell growth for continuous culture can be described as (ignoring the decay rate)

$$\frac{dx}{dt} = (\mu - D)x$$
$$\Rightarrow x = x_0 e^{(\mu - D)t}$$
$$\Rightarrow \ln x = (\mu - D)t + \ln x_0$$

where x, x_o, t and μare the same as described above and D is the dilution rate (hr^{-1}). This equation suggests that at steady-state, the specific growth rate equals the dilution rate (μ-D = 0); that is, no net change in biomass concentration takes place. This steady-state condition was attained in experiments, judging by fluorescence readings. Thus, under nutrient limiting condition, growth rate at steady-state in continuous culture was calculated by fixing the dilution rate. Also, specific growth rate was related to concentration of growth-limiting nutrient by the Monod equation.

3.5 Experiments

All cultures were started by inoculating with a 2% inoculum in NAPK media with PIPES. Eight reactors were started at a time, all in similar conditions in batch mode with an air-bubbling rate of 1 L min^{-1}. Growth of cells was monitored by measuring fluorescence (10-AU Fluorometer, Turner designs) of culture twice a day at a regular interval and the pH of the culture media was monitored to ensure favorable growth conditions (pH value should be between 5.5 and 8) (Lustigman et al. 1995). Once fluorescence reached its peak - i.e. late exponential growth phase (explained

more in result and discussion),- the peristaltic pump was turned on to switch the reactors from batch mode to continuous mode. The pumping rate of influent sterile media (flow-rate) was decided on the basis of growth rate of culture calculated from exponential growth curve (μ_{max}) so as to grow the culture at maximum growth rate in steady-state condition. Thus, the dilution rate was same as μ observed during the exponential phase. The system was allowed to run for few days until it reached steady-state condition, where the fluorescence readings seemed constant. At this steady-state condition, four out of eight reactors (for second run of each experiment, two out of eight reactors) were harvested for analyzing various parameters for nutrient-replete conditions (also referred to as control reactors) and the remaining four (or six) reactors were subjected to specific nutrient limiting conditions with a reduced nutrient concentration of either nitrogen, phosphorous or sulfur.

Each experiment was performed twice (except the one with sulfur limitation which was performed only once) and a minimum of three replicates of each culture were monitored in each trial.

3.5.1 Nitrogen Limitation

After steady-state conditions were reached, the nitrogen concentration in the influent media was reduced from 7.5 mM to 0.9 mM (theoretical value) (reduced to $1/8^{th}$, shift 1 (1^{st} change in nutrient concentration in influent media)) reducing both the ammonia and nitrate concentrations by an equal amount. The flow rate of influent media was adjusted (reduced to 75%) in order to avoid wash-out because of reduced growth rate (as observed by decrease in fluorescence reading). The system was again allowed to run for few days until a new steady-state condition was reached and then the nitrogen concentration was further reduced to 0.2 mM (theoretical value) (a total reduction of $1/32^{nd}$ the original concentration, shift 2 (2^{nd} change in nutrient

concentration in influent media)) again by reducing ammonia and nitrate concentration by an equal amount. The flow-rate was also reduced again to 25% of the original flow-rate. Thus, the reactors were running under N-limited conditions (also called as N-limited reactors). Finally after reaching to a new steady-state condition, all the N-limited reactors were harvested.

3.5.2 Phosphorus Limitation

After the first steady-state condition was reached, the phosphorus concentration in influent media was reduced from 1.13 mM to 0.113 mM (theoretical value) (reduced to $1/10^{th}$, shift 1) by reducing phosphate concentration in media. The flow rate of influent media was not adjusted because no significant decrease in fluorescence level or mass density was seen. The system was again allowed to run for a few days until new steady-state condition was reached where phosphorus concentration was further reduced to 0.0113 mM (theoretical value) (reduced to $1/100^{th}$ of original concentration, shift 2) again by reducing phosphate concentration in media. The flow-rate was also reduced to 70% of original flow-rate this time to avoid wash out. Thus, the reactors were running under P-limited conditions (also called as P-limited reactors). Finally, after reaching to a new steady-state condition, all the P-limited reactors were harvested.

3.5.3 Sulfur Limitation

For this set of experiments, only four reactors were switched to continuous mode from batch mode due to peristaltic pump failure and thus no control reactors were harvested. All four reactors were switched to sulfur-limiting conditions. After the first steady-state condition was reached, the sulfur concentration in influent media was reduced from 0.43 mM to 0.03 mM (theoretical value) (reduced to $1/14^{th}$, shift 1) by

reducing the sulfate concentration in the media. The flow rate of influent media was not adjusted because no significant difference in fluorescence level or mass density was seen. The system was again allowed to run for few days until a new steady-state condition was reached, when the sulfur concentration was further reduced to 0.006 mM (theoretical value) (reduced $1/68^{th}$ of original concentration, shift 2) again by reducing sulfate concentration in media. The flow-rate was also reduced to 89% of original flow-rate this time to avoid wash out. Thus, reactors were running under S-limited conditions (also referred to in this work as S-limited reactors). Finally after reaching to a new steady-state condition, all the S-limited reactors were harvested.

3.6 Analytical Methods

In every set of experiments, there were two phases of harvesting (except for S-limitation), harvest 1 and harvest 2. Harvest 1 was for control reactors for which various parameters were measured to investigate the characteristics of cells under nutrient-replete conditions (control conditions), as well as to check the consistency of the performance of the reactors under control conditions on different days. Harvest 2 was for nutrient-limited reactors after the limitation was observed in the culture to analyze the characteristics of nutrient-depleted cells. In addition to from fluorescence and pH, the following parameters were measured:

3.6.1 Mass Density

Dry mass density was determined by taking 50 mL of algal cultures directly from the reactor and by vacuum filtering through a pre-dried and pre-weighed glass-fiber filter (Whatman GF/F). The filters were dried 24 hrs at 105^{o}C, cooled in a desiccators and then weighed again.

3.6.2 Carbon-nitrogen Analysis

For C N analysis, 250 mL of algal culture directly taken from the reactors at the time of harvesting was pelleted out by centrifugation (12 mins at 12000 g, Thermo scientific, Sorvall Legend RT+ Centrifuge) and were dried for 24hrs at 105^{o}C, cooled in desiccators to remove any moisture from pellets. These pellets were then analyzed in a C H O N analyzer (CE-440 Elemental Analyzer, Exeter Analytical Inc.) which determines cellular carbon and nitrogen content.

3.6.3 Dissolved Nutrient Analysis

Supernatant remaining from the previous analysis (CN analysis) after centrifugation process was used for measuring the ammonia, nitrate, sulfate and phosphate concentration in the effluent.

The ammonia concentration was measured using standard Ammonium-Phenate method as described in Standard Methods for the Examination for Water and Wastewater (Greenberg et al. 1992).

The nitrate and sulfate concentration in the effluent were determined by ion chromatography using a Dionex ICS-2000 Ion Chromatograph (Dionex Corporation, Sunnyvale, CA, USA) with anion-exchange column – the dionex IonPac® AS18 (4 x 250 mm), and is published in US-EPA (method 300.1) (Hautman et al. 1997).

The dissolved orthophosphate (soluble reactive phosphorus) concentration in the effluent was measured using an automated ascorbic acid Flow Solution® 3000 (FS3000) Analysis (FIA), and is a US-EPA approved method (method 365.1) for analyzing orthophosphate (USEPA 1983).

3.6.4 Cellular Phosphorus Analysis

Algal culture (25 mL) was taken directly from the reactor was filtered through a 1 micron PTFE membrane filter (Whatman filters) by vacuum filtration. The filtered sample was then placed in 10 mL borosilicate glass tubes with 1mL of Milli-Q water and 1mL of concentrated HNO_3 for acid digestion. It was then heated for 15 minutes at 90 ± 1.5^{o}C. The sample was allowed to sit for at least 4 hrs and allowed to cool to room temperature. H_2O_2 (0.3 mL) was then added to the sample and it was heated again for 15 minutes at 90 ± 1.5^{o}C. After cooling to room temperature the final volume was adjusted to 50 mL with Milli-Q water and was analyzed for cellular phosphorus content using ICP (Thermo Jarrell Ash).

3.6.5 Lipid Analysis

To determine the relative neutral lipid content of the algal cultures, the Nile Red Assay was used (Cooksey et al. 1987). The lipid readings were normalized with a known standard using a plate reader to measure the fluorescence of incorporated Nile Red at a specific wavelength.

Nile Red stock solution was prepared by adding 1 mL of 100% HPLC-grade acetone to 1 mg of Nile Red powder (Sigma-Aldrich) in a glass vial and wrapping it in foil. This stock solution (3 µL) was added to 1mL of culture taken from reactors in a 1.7 mL Eppendorf tube and was vortexed. 300 µL of the stained culture was then transferred to a black opaque 96-well plate which was then analyzed in a Biotek Synergy plate reader at an excitation wavelength of 540 nm and an emission wavelength of 590 nm. The fluorescence measured was then normalized by dividing the fluorescence measured for a 2% lipid solution (mixture of 1 mL of 2% lipid solution (Gibco) and 3µL of Nile Red stock solution) to account variability between plate reads. The normalized fluorescence was again divided by the cellular mass

concentration of the culture to obtain the fluorescence per unit mass of cells. Controls with no stain added showed negligible fluorescence.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Batch Growth

A typical growth profile of *C. reinhardtii* biomass (natural logarithm of fluorescence) and pH variation with time for batch reactors in NAPK media without PIPES buffer is shown in Figure 4.1. Fluorescence increased with time during exponential growth phase until it reached a peak (1398 FUs in this case) and began to drop after that. Initially, the pH of the media was 7.0 and decreased slowly till it reached 6.0 where the buffering capacity of the phosphate buffer was apparently exceeded and the pH rapidly dropped to 4. At peak fluorescence, the pH was 3.9.

A drop in fluorescence after the peak (net decay) can be because of exhaustion of an essential nutrient or lower pH. Only atmospheric CO_2 (~0.03% CO_2) was supplied carbon source for cell growth.

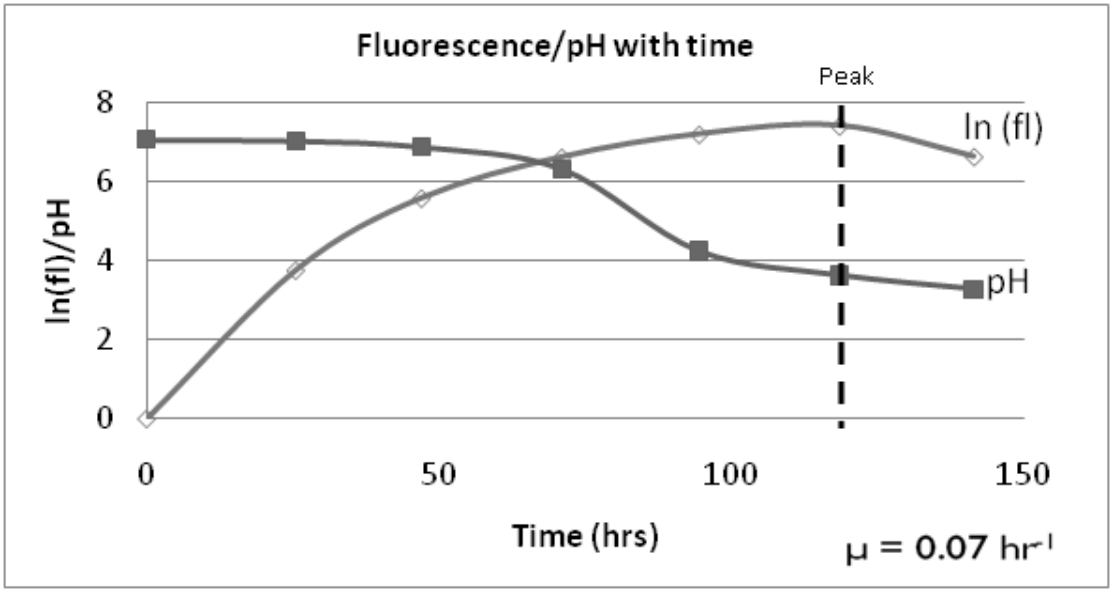


Figure 4.1 Growth of C. reinhardtii and variation in pH with time as measured in batch reactor. NAPK media without PIPES buffer was used.

The maximum specific growth rate was calculated as 0.07 hr^{-1} for media without PIPES buffer, but in regular experiments media containing PIPES was used, which maintained the pH level and a maximum specific growth rate observed for that media was 0.09 hr^{-1}.

4.2 Continuous Flow

In general, the growth profile for *C. reinhardtii* in NAPK media (with PIPES) is presented in Figure 4.2. Initially PBRs were allowed to run in batch mode and about 2 days after the inoculation, the fluorescence level of algae reached its previously determined peak value (around 1500 FUs, ln FU = 7) at which time the peristaltic pump was turned on to switch the reactors from batch mode to continuous mode. In about 2 days, reactors reached a steady-state condition in which fluorescence readings appeared to be constant (Figures 4.2, 4.3, 4.4, 4.5). For the example of a P-limiting experiment (Figure 4.2) control reactors were harvested 5 days later (harvesting 1). The rest of the reactors were shifted to nutrient limiting conditions (shift 1). After additional 7 days approximately, nutrient levels were further reduced (shift 2). Final harvesting of all reactors was done after these reactors reached a new steady-state condition (harvesting 2). During shift 1 and shift 2, the flow rate of influent media was also reduced to avoid wash-out. Table 4.1 presents growth rates, flow-rates, fluorescence, pH and nutrient values for all sets of experiments at harvest 1 and harvest 2. In each case, the pH varied between 6.01 and 7.15, which was favorable for algae. Growth curves for the other experiments are presented in Figure 4.3, 4.4 and 4.5.

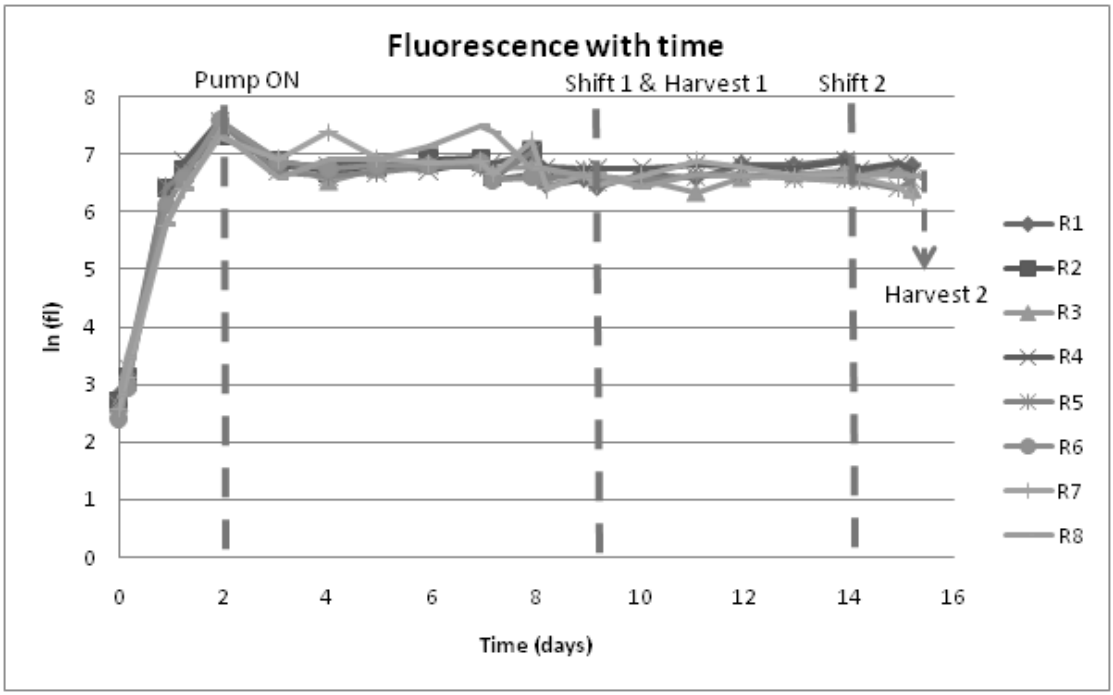


Figure 4.2 Growth curve of *C. reinhardtii* in continuous-flow reactors. This particular graph was from P-limitation experiment. Media used was NAPK with PIPES as an external buffer and pH values at steady-state were 6.52 (for control reactors) and 6.01 (under limiting condition, after shift 2)

It can be seen that fluorescence and pH value for all sets of experiments for control and limiting conditions remained nearly constant except for the N-limitation. As per Richmond (2004), nitrogen limitation led to a discoloration of the cells due to decrease in chlorophyll-a content and increase in carotenoids, which possibly explains the decrease in fluorescence. Thus, for N-limitation, the reduced flow-rate under N-limited conditions was chosen to maintain constant mass density, which is discussed in the next section.

Table 4.1 Variation in flow-rate, growth-rate, fluorescence and pH value with changing in nutrient concentration at steady-state condition.

	Experiment	N-Concentration (mM)	P-Concentration (mM)	S-Concentration (mM)	Flow-rate (ml/min)	Estimated Growth-rate (hr^{-1})	Fluorescence	pH
Harvest 1	N-Limited	7.50	1.13	0.43	0.825	0.09	787	6.34
	P-Limited	7.50	1.13	0.43	0.825	0.09	737	6.47
	S-Limited	7.50	1.13	0.43	0.825	0.086	NA	NA
Harvest 2	N-Limited	0.23	1.13	0.43	0.206	0.023	276	7.15
	P-Limited	7.50	0.011	0.43	0.578	0.063	708	6.22
	S-Limited	7.50	1.13	0.006	0.734	0.077	658	6.74

NA→Not available because of no harvesting of control reactors

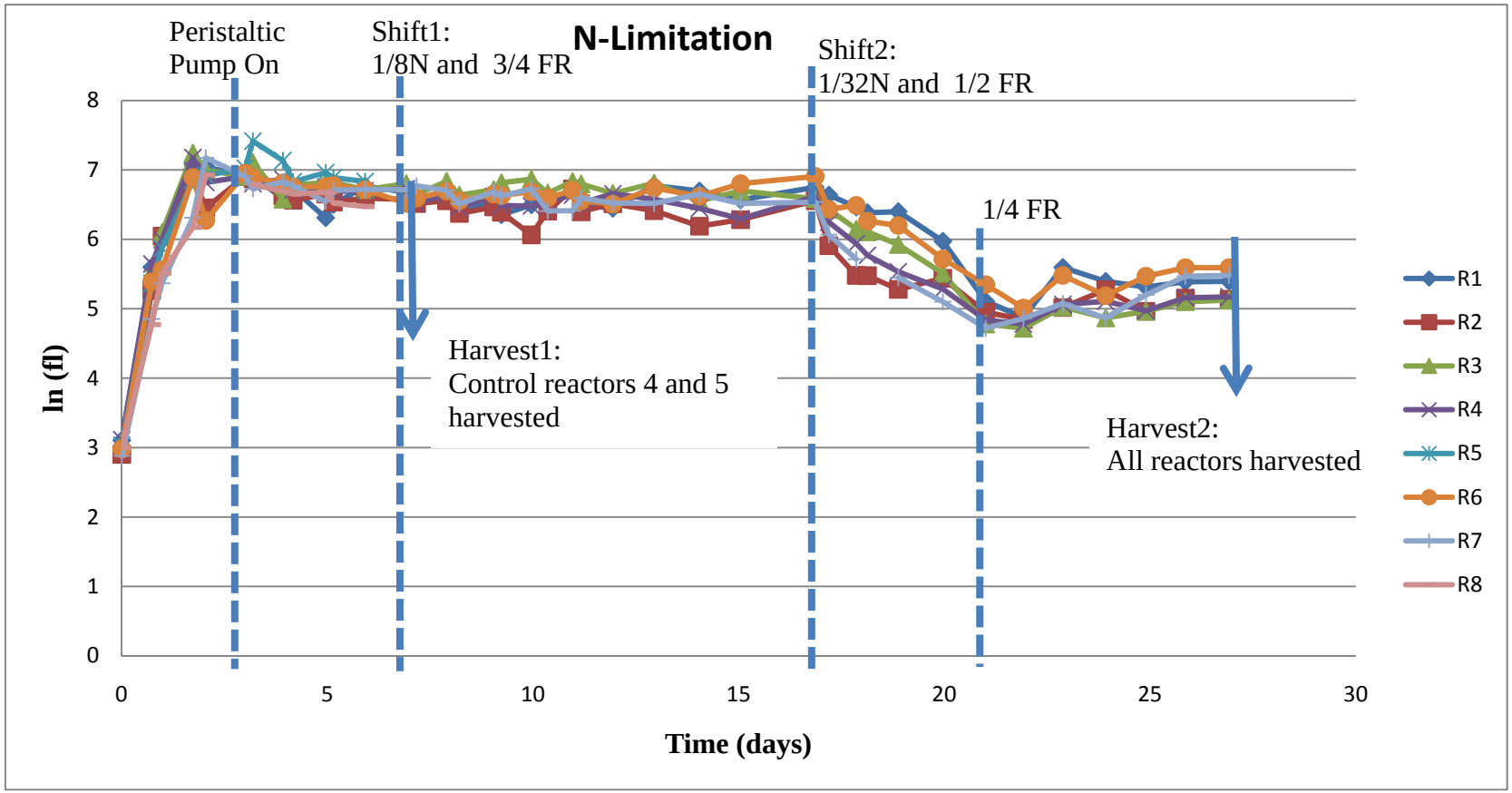


Figure 4.3 Growth curve of *C. reinhardtii* in continuous flow reactor for N-limiting experiment. N is the original nitrogen concentration in media which was 7.5 mM (theoretical value) and FR is initial flow-rate when peristaltic pump was turned on which was 0.825 ml/min (calculated on the basis of growth rate which was 0.09 hr^{-1}).

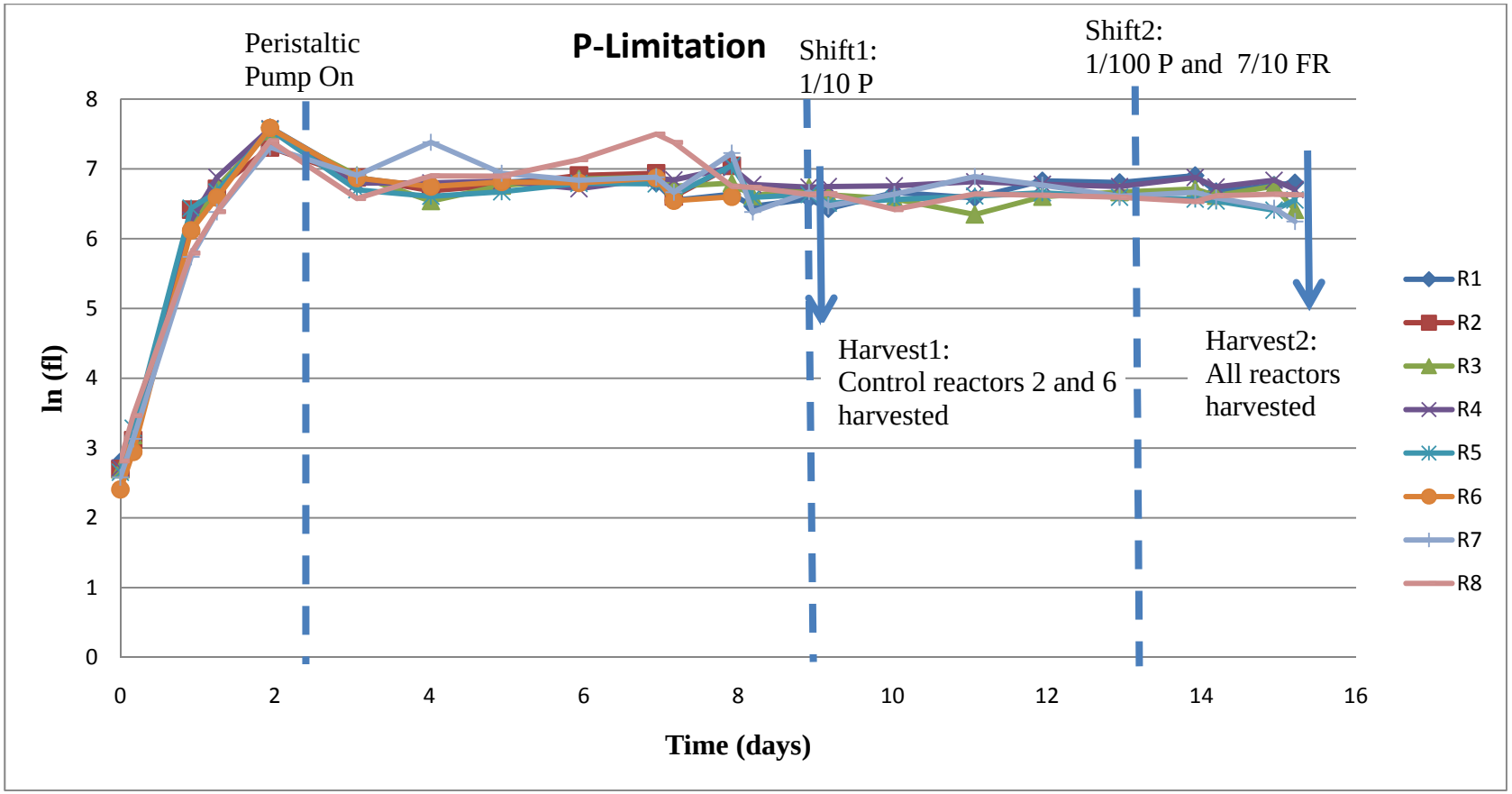


Figure 4.4 Growth curve of *C. reinhardtii* in continuous flow reactor for P-limiting experiment. P is the original phosphorous concentration in media which was 1.13 mM (theoretical value) and FR is initial flow-rate when peristaltic pump was turned on which was 0.825 ml/min (calculated on the basis of growth rate which was 0.09 hr^{-1}).

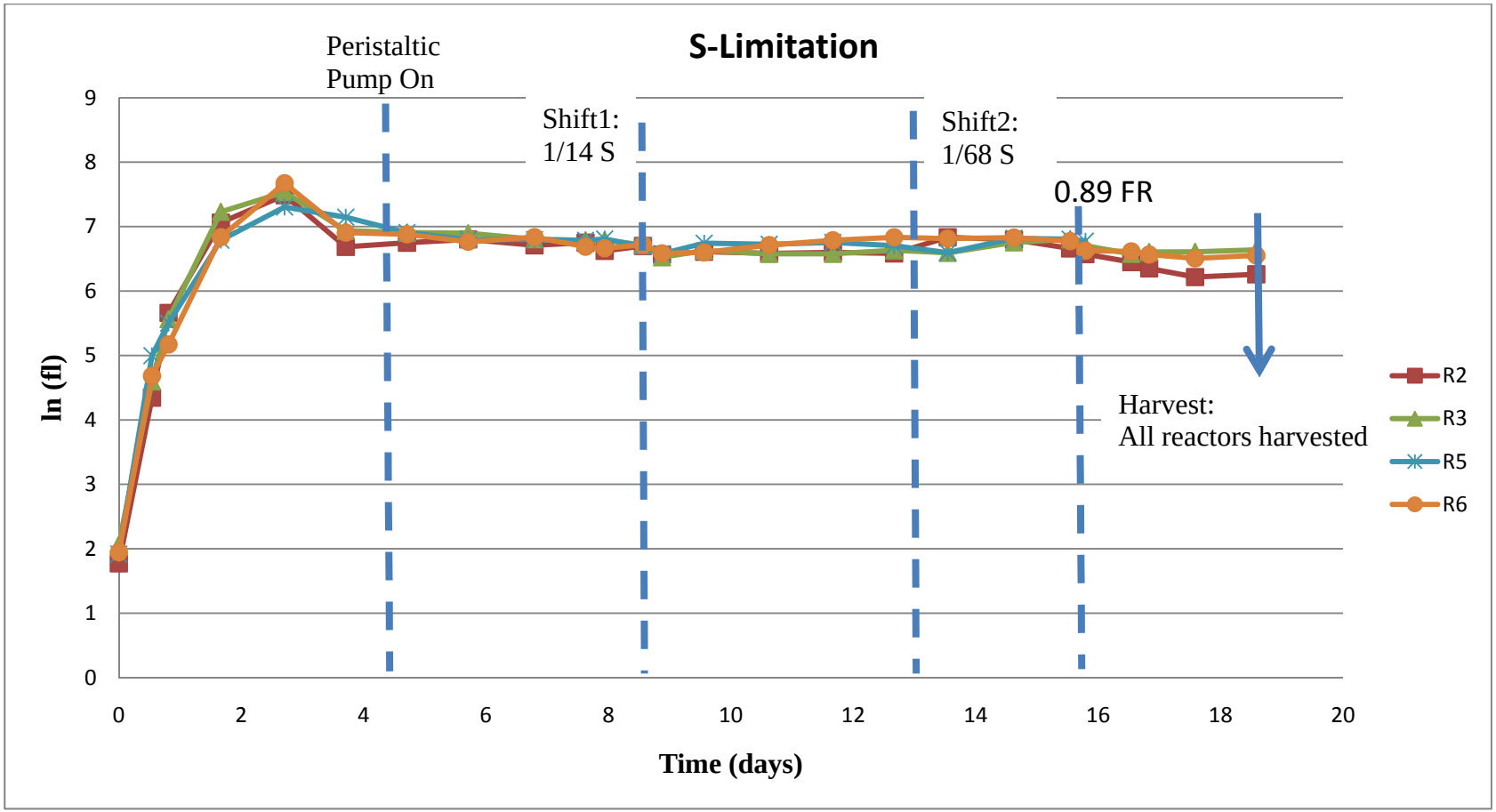


Figure 4.5 Growth curve of *C. reinhardtii* in continuous flow reactor for S-limiting experiment. S is the original sulfur concentration in media which was 0.43 mM (theoretical value) and FR is initial flow-rate when peristaltic pump was turned on which was 0.825 ml/min (calculated on the basis of growth rate which was 0.09 hr^{-1}).

The growth rate of cells depends on the growing method, nutrient concentrations, growth media, environmental conditions and many other factors. For this experiment, the growth rate of cells for batch-phase growth was calculated to be around 0.09 hr^{-1} and was consistent for all sets of experiments (Table 4.1). This value is similar to those determined in previous research, reported between 0.058 hr^{-1} to 0.084 hr^{-1} for *Chlamydomonas*, considering certain differences in experimental methods (Yang and Gao 2003; Fischer et al. 2006; Zhang et al. 1999; Chen and Johns 1996a). Growth rate can be enhanced further by increasing the supply CO_2 concentration (Yang and Gao 2003).

Growth rates under N-limiting conditions were reduced by a larger extent (25%) compared to P and S-limiting conditions (70% and 89% respectively), thus N-limited cultures in these experiments grew slowest, which is same as result obtained by (Kruskopf and Du Plessis 2004) who compared growth rate for *Chlamydomonas* and *Chlorella* species in batch mode under N limitation (0.01 hr^{-1})and P limitation (0.02 hr^{-1}) by changing the N: P ratio. This result also reflects on the importance of nitrogen for the growth of the culture and for biomass production. A decrease in growth rate implies a decrease in the uptake rate of nutrients under limiting conditions which can be responsible for changing cell compositions.

4.3 Mass Density

Figure 4.6 depicts the mass density (g/L) for biomass harvested from control reactors (harvest 1) and from different nutrient-limited reactors (harvest 2). Tabular forms of these results are presented in Appendix 2. The density for control reactors is presented as the mean (± standard deviation) mass density of all the control reactors from all sets of experiments.

As targeted, mass densities under different conditions were almost the same (around 0.075 g/L), except for S-limitation which was higher (0.089 g/L). Reducing the flow-rate more than required in this case can be the reason for getting higher biomass but as there were no control reactors for this set of experiment it cannot be said with certainty. Consistency in readings for the control reactors reflects the replicable performance of the bioreactors.

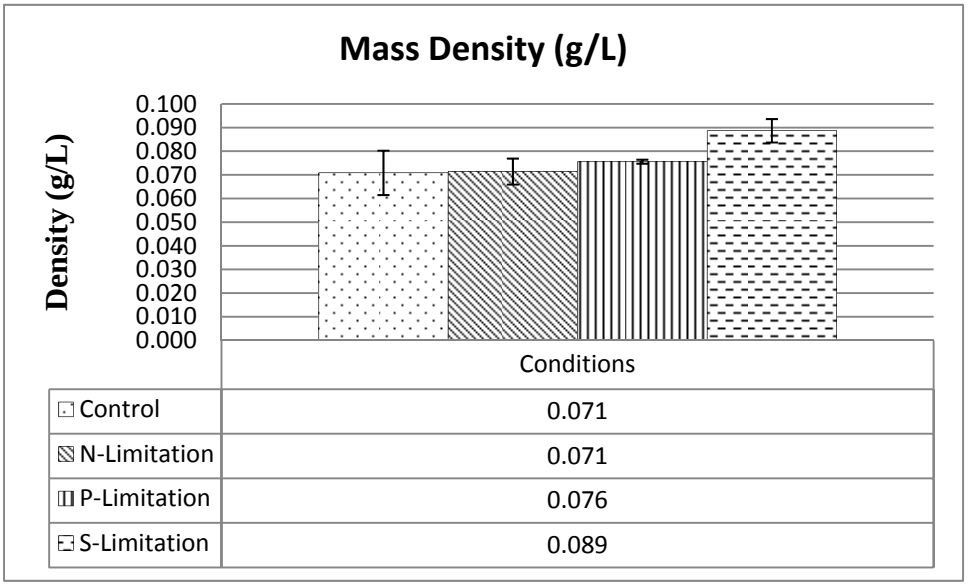


Figure 4.6 Mean mass density in g/L of dry algal biomass obtained after harvesting 1 (for control reactors) and harvesting 2 (for nutrient-limiting conditions). For control reactors n=7, for N-limitation n=3, for P-limitation n=6, for S-limitation n=3, where n is number of the samples.

Similar to growth rate, mass density depends on many factors, mainly reactor type, operational mode (batch vs continuous), media and nutrient concentrations. The mass densities obtained for this experiment were lower than the density researchers usually achieve (between 0.84 g/L to 1.5 g/L) (Chen and Johns 1996b), because for these sets of experiments reactors were running in continuous flow mode with a high flow-rate (with an aim to grow the culture at the maximum steady-state growth rate)

compared to others who used either batch reactors or continuous reactors with a very low flow-rate to maximize biomass levels. Also, a supply of organic carbon source like acetate is an additional factor which can be responsible for higher biomass density compared to only atmospheric CO_2 as a carbon source as in this experiment.

4.4 Ammonium and Nitrate

Figure 4.7 and 4.8 present the ammonium and nitrate levels in the influent media and in effluent under different conditions.

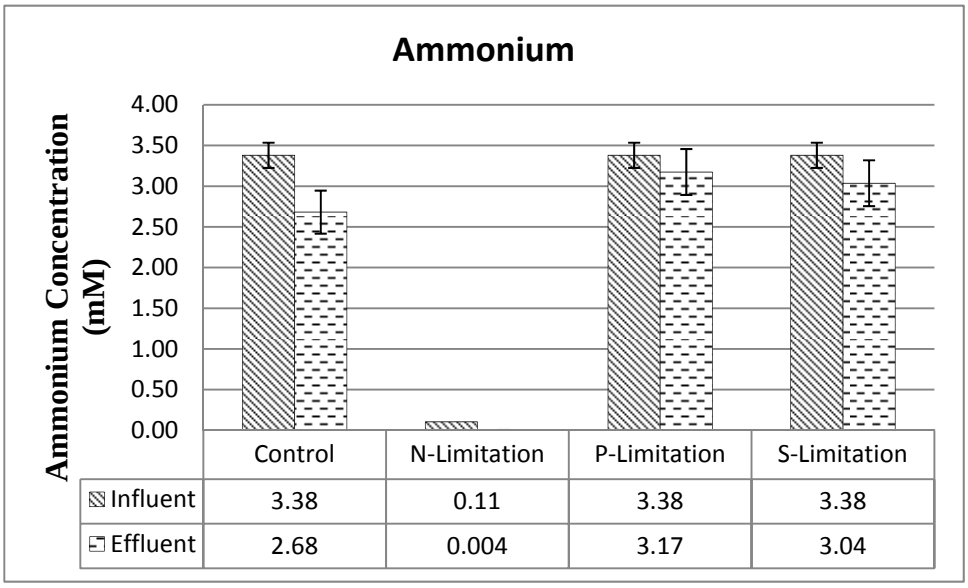


Figure 4.7 Mean ammonia concentration in mM in influent and effluent for control reactors and nutrient-limiting reactors.
Note: Ammonium concentration in influent was measured from P-limitation and S-limitation experiment

Observing the difference between influent concentration of ammonia in media and effluent ammonia concentration, there is consumption of about 8.84 mmoles of ammonia per gram dry weight of biomass for the control reactors, 2.70 mmoles/g dw for P-limited reactors and 3.85 mmoles/g dw for S-limited reactors (Appendix 3). Thus, consumption of ammonia was reduced under P-limiting condition which was

also tested statistically different (p=0.02 from t-test). However for S-limitation, consumption under limiting conditions was not that significantly different (p=0.2 from t-test). For N-limitation, the ammonia concentration in influent was finally reduced to 1/32 of the original concentration (in control reactors) and the reduction in effluent ammonia concentration obtained was almost 96%.

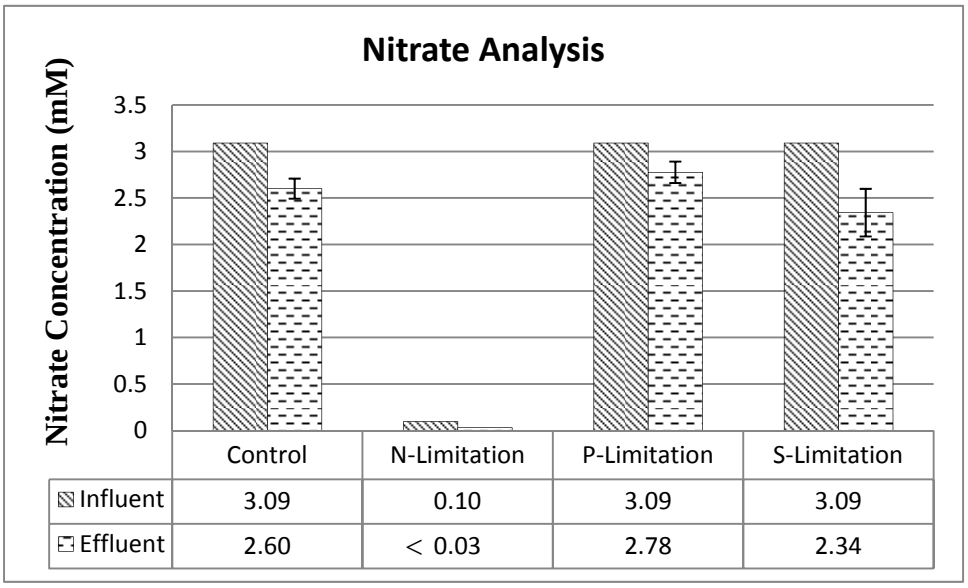


Figure 4.8 Mean nitrate concentration in mM in influent and effluent for control reactors and nutrient-limited reactors.

Observing the difference between the influent and effluent nitrate concentration, there is consumption of about 6.90 mmoles of nitrate per gram dry weight of biomass for the control reactors, 4.13 mmoles/g dw for P-limited reactors and 8.40 mmoles/g dw for S-limited reactors (Appendix 3). But again statistically consumption of nitrate didn't seemed to be that significantly different under limiting conditions (p=0.5 for P-limiting and p=0.3 for S-limiting). For N-limitation, the nitrate concentration in the influent was also finally reduced to 1/32 of the original concentration and because of the limitation on the lower detection limit of the

instrument (0.03 mM), the exact reduction of nitrate in could not be analytically determined but it was greater than 76%.

Comparing consumption of ammonium and nitrate, it was observed that consumption of nitrate was less than the consumption of ammonia for non-limiting condition which is also supported by literature mentioning that the ammonia is preferred over nitrate by *C. reinhardtii* (Hu et al. 2008). But strangely under P-limiting and S-limiting conditions, the consumption of nitrate was preferred over ammonia and higher consumption of nitrate was observed. The reason for this unexpected response is not known but it seems that under limiting conditions with higher concentrations of nitrate and ammonia, algal cells prefer nitrate over ammonia. Because nitrate consumption result for N-limiting conditions could not be determined accurately nothing certain could be said for preference of ammonia and nitrate for this case. Comparing total nitrogen concentration in an effluent to the half-saturation constant K_{SN} value (0.06 mM) for *C. reinhardtii* (Cunningham 1984) for nitrite in stirred chemostat, it can be said that cells were growing under N-limited conditions (K_{SN} is half-saturation constant for nitrogen and as no value was reported for *Chlamydomonas* for ammonia and nitrate, nitrite value is used to compare).

4.5 Phosphate

Figure 4.9 shows the phosphate concentration in the influent media and in effluent under different conditions.

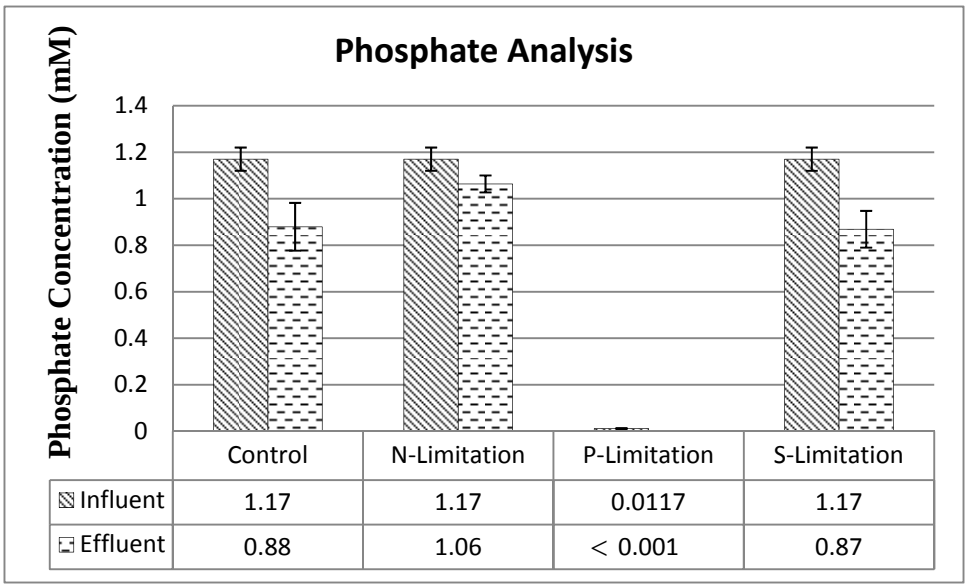


Figure 4.9 Mean phosphate concentrations in mM in influent and effluent for control reactors and nutrient-limited reactors.

For control reactors, phosphate consumption was about 4.09 mmoles/g dw which is close to consumption calculated for S-limitation (3.38 mmoles/g dw) (Appendix 3) (p=0.9 from t-test). As in the case of ammonia and nitrate, where limiting phosphate affected nitrogen consumption, in this case limiting nitrogen affected phosphate consumption. For N-limitation, consumption of phosphate was 1.5 mmoles/g dw which is lower than that in control (p=0.05 from t-test) and S-limitation conditions. The possible reason for this could be for maintaining the N to P ratio in the cell. For P-limitation, the concentration of phosphate under limiting conditions was finally reduced to 1/100 of the original concentration in media. The exact amount of consumption cannot be calculated because of the lower detection limit of an instrument (0.001 mM) but it was greater than 91%. Comparing effluent concentration of phosphate (<0.001 mM) for P-limited condition to the K_{SP} value (0.001 mM) for

PO_4^{3-}-P for *Chlamydomonas*, it can be said that cells were growing under P-limited condition (K_{SP} is half-saturation constant for phosphorus).

4.6 Sulfate

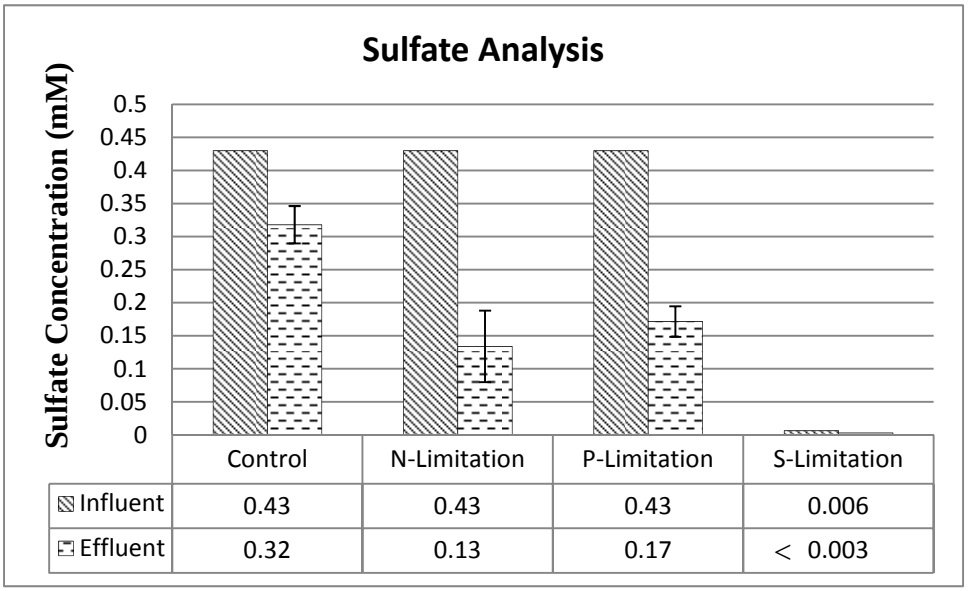
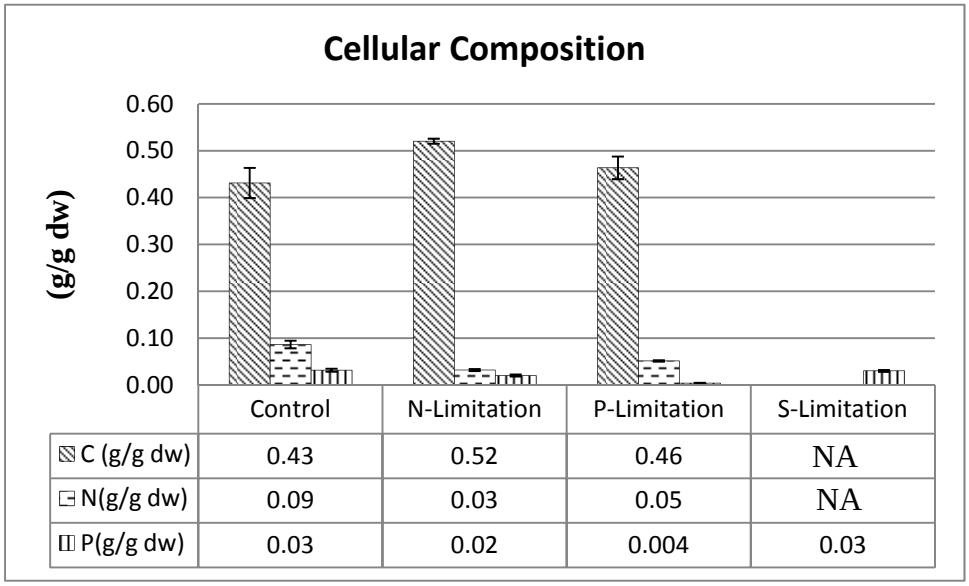


Figure 4.10 Mean sulfate concentrations in mM in influent and effluent for control reactors and nutrient-limited reactors.

Figure 4.10 presents the sulfate level in influent media and in the effluent under different conditions. In contrast to ammonia, nitrate and phosphate consumption, sulfate consumption increased during N and P-limitation. For control reactors, the calculated consumption was about 1.58 mmoles/g dw whereas for N-limitation and P-limitation, consumption was about 4.17 mmoles/g dw and 3.40 mmoles/g dw respectively (Appendix 3). This result shows that under nutrient-limiting conditions, for some reason algal cells start taking up more sulfur in form of sulfate. For the S-limiting condition, the exact amount of consumption cannot be calculated because of the lower detection limit of an instrument (0.003 mM) but it was greater than 53%.

4.7 Cellular Composition and C:N Ratio

C, N and P cellular contents were measured for all sets of experiments and are presented in Figure 4.11. Another important parameter most researchers used for determining the variation in cellular composition of algal cells is the carbon to nitrogen ratio (C:N ratio) which is depicted in Figure 4.12.



	Control	N-Limitation	P-Limitation	S-Limitation
C (g/g dw)	0.43	0.52	0.46	NA
N(g/g dw)	0.09	0.03	0.05	NA
P(g/g dw)	0.03	0.02	0.004	0.03

Figure 4.11 Cellular C, N and P content in g/g dw for control and nutrient-limited reactors. (NA→Not available)

Under normal condition as per the literature, carbon content is between 45 – 50%, nitrogen content is 7 – 10%, and phosphorus content is 1 – 2% (Richmond 2004; Chisti 2007) which is what we obtained in our experiments. For control reactors C, N and P content was 43%, 9% and 3% respectively of cell dry weight which changed under different limiting conditions. Also, in terms of C:N ratio, the result was similar to previous studies which mentioned the average C:N ratios of nutrient replete phytoplankton cultures to be around 6 (Geider and Roche 2002).

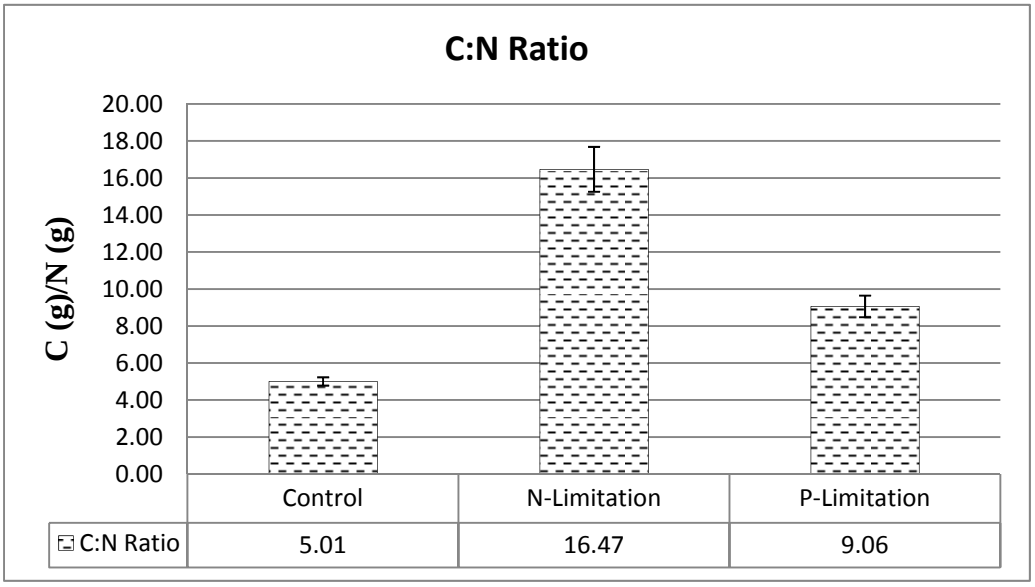


Figure 4.12 C:N ratio for control reactors and nutrient-limited reactors.

For N-limitation, as expected C content increased to 52% and N content was decreased to 3%. P content also decreased from 3% to 2%. The increase in carbon content can be due to accumulation of carbon rich carbohydrates or lipids (Dean et al. 2010; Becker 1994). Also, as reported earlier, N-limitation affected phosphate levels in effluent; it is also reflected in decreased P-content in cells. Increase in carbon content can also be shown through change in C: N ratio which increased from 5 to 16 under N-limitation conditions.

For P-limiting condition, there was slight increase in carbon content which went up to 46% and, as reported earlier, P-limitation affected nitrate consumption; nitrogen content in the cell went down to 5%. Cellular phosphorus content decreased by large extent, to 0.4% under the limiting condition from 3% in control reactors. We also saw an increase in C:N ratio under this condition which can be again because of accumulation of carbohydrates or lipids under phosphorus-limiting conditions.

Consistent with other studies, C:N ratio for N-limited reactors was found to be higher than P-limited and non-limited reactors (Tezuka 1989) which reported the C:N ratio as10 for N-deficient compared to ratio of around 6 for P-deficient and N-P moderate ones.

4.8 Relative Lipid

Table 4.2 Relative lipid content in algal samples per gram for non-limiting and limiting conditions

	Normalized Nile Red fluorescence/g cells
Control	7.0 ± 1.8
N-Limited	28.8 ± 6.7
P-Limited	8.1 ± 0.2
S-Limited	9.5 ± 2.7

*The relative lipid content was measured for control reactors only from the P-limitation experiment. For the discussion below, control reactors assumed to be similar for all experiments.

Table 4.2 presents the relative lipid content in control, N-limited and P-limited reactors. There was not a significant difference in the lipid concentration between non-limiting, P-limiting and S-limiting conditions. However, in comparing non-limiting conditions to N-limiting conditions, the relative lipid content increased by four times, suggesting possible accumulation of neutral lipids in nitrogen-limited cells.

CHAPTER 5

CONCLUSION AND FUTURE WORK

5.1 Summary and Conclusion

The bulk cell composition and growth of *C. reinhardtii* were investigated under nutrient-limited conditions in replicable chemostat photo-bioreactors.

1. The reactors were operated in a replicable way, supported by consistent readings obtained for non-limiting control reactors as well as nutrient-limited reactors.
2. Under N-limiting conditions, as expected, there was a reduction in phosphate consumption, which was also reflected in the reduction of the cellular P content. But, on the contrary, sulfate consumption was found to be higher in this condition for which the reason is not clear.

   N-deficient cells showed a higher level of cellular carbon in comparison to N-replete cells which is also supported by the higher level of relative lipid contents observed in cells (4 times) which shows the accumulation of carbon in the form of lipids as suggested by (Dean et al. 2010). The C:N ratio increased by 3 times under this condition. Also, of all the nutrients evaluated, nitrogen limitation was the most critical nutrient affecting cellular composition.
3. Under P-limiting conditions, similar to N-limiting condition, there was a reduction in nitrogen consumption (ammonia and nitrate) and also a reduction in cellular nitrogen content. But again, the reason for higher sulfate consumption in this condition too was not known.

   P-deficient cells showed slightly higher level of cellular carbon than P-replete cells which can be due to accumulation of carbon rich compounds as

observed in small increase in relative lipid content. C:N ratio also increased two fold under this condition.

4. Under S-limiting conditions, there was a reduction in ammonia consumption by a significant amount but phosphate consumption was almost unchanged. Moreover, for reasons not known yet, there was increase in consumption of nitrate under this condition. This should increase or maintain cellular nitrogen content in cells. Unfortunately C:N ratios for this biomass are not yet available.

This work confirms that the nutrient supply affects the growth rate of microalgae *C. reinhardtii* in addition to affecting its final biomass yield (g/L-day). We observed a decline in growth rates as well as changes in cell composition under nutrient-limited conditions. As reported earlier, responses to nutrient limitations were observed with the decrease in cell quota of that nutrient as well as other non-limiting nutrients and thus cell composition can be consider as a useful indicator of nutrient limitation. Moreover this work also confirms that growing algae under nutrient-limiting condition with reduced growth rate accumulates carbon, which is likely stored in useful carbon-rich compounds. Among the tested conditions, there was highest accumulation of carbon observed in the N-limiting condition.

5.2 Future Work

To further assess the value of accumulated carbon in the nutrient-deplete cells, lipid and carbohydrate analyses can be performed on the cells. Knowing the lipid and carbohydrate content in the cells can help to optimize for growing condition. Also, because of the replicable nature of the reactors, cells can be analyzed under various nutrients levels inducing one or more nutrients limiting conditions at a time with different dilution rates to optimize for biomass and lipid production.

Work can be also done on using recycled media i.e. recycling reactor effluent and using it in combination of fresh media in appropriate quantity as an influent for growing fresh microalgae as effluent contains some of the consumable nutrients which were present in excess and would be wasted and discarded. The common challenges associated with recycling media are increasing the possibility of contamination and lower pH value of effluent. Lower pH value can be controlled by using external buffer and for sterilizing effluent to remove contamination; research is required on analyzing the consumable nutrient concentrations in sterilized effluent.

Apart from reducing C, N, P and S level, the system can be analyzed with supply of varying CO_2 concentration as well as varying source for CO_2 like using flue gas which can make biomass production more economical. It has been reported that increasing CO_2 supply concentration enhances growth rate to a certain extent. Moreover, growth of *C. reinhardtii* can also be affected by varying intensity of light. Lots of work has already been done using different intensity of light and varying light/dark cycles but little information is available for growing microalgae with different light intensity along with limiting nutrient conditions.

Cells harvested under different nutrient-limiting conditions can be used to study the proteins that are indicative of overall culture health like cell division protein, stress response proteins, light harvesting proteins as well as to study an enzyme involved in lipid or carbohydrate accumulation. This can help to analyze the conditions which results in up-regulation of biomolecular pathways for oil production.

APPENDIX 1: NAPK MEDIA

Final composition of all major and minor nutrients in NAPK media is presented below in Table A1.1and Table A1.2

Table A1.1 Final composition of major nutrients in NAPK media

Major components	**Molarity (mM)**
NH_4^+	3.74
K^+	5.57
Na^+	0.05
Ca^{2+}	0.34
Mg^{2+}	0.41
NO_3^-	3.75
Cl^-	4.43
SO_4^{2-}	0.43
PO_4^{3-}	1.13

Table A1.2 Final composition of minor nutrients in NAPK media

Trace components	**Molarity (uM)**
Fe^{2+}	25.00
Zn^{2+}	1.00
Cu^{2+}	0.01
Co^{2+}	6.76
Mn^{2+}	1.00
Mo^{2+}	0.07
BO_3^{3-}	0.99
EDTA	24.98

APPENDIX 2: MASS DENSITY

Table A2.1 Mass density under N-limiting condition

Description	**Density (g/l)**
Full N (7.5 mM)	0.082 ± 0.009
1/32 N (0.2 mM)	0.071 ± 0.009

Table A2.2 Mass density under P-limiting condition

Description	**Density (g/l)**
Full P (1.13 mM)	0.067 ± 0.001
1/100 P (0.0113 mM)	0.076 ± 0.001

Table A2.3 Mass density under S-limiting condition

Description	**Density (g/l)**
Full S (0.43 mM)	NA
1/68 S (0.03 mM)	0 .089 ± 0.005

NA→Not available because of no harvesting of control reactors

Note: Concentration mentioned in each of these cases is theoretical concentration, not measured. Measured concentrations are mentioned in Appendix 4.

APPENDIX 3: NUTRIENT CONSUMPTION

Table A3.1 Nutrient consumption under non-limiting and limiting condition

Condition	Nutrients	Influent Concentration (mM)	Effluent Concentration (mM)	Consumption (mmoles/g dw)
Control (Replete condition)	Ammonia	3.38 ± 0.28	2.75 ± 0.38	8.87
	Nitrate	3.09 ± 0.11	2.60 ± 0.11	6.90
	Phosphate	1.170	0.88 ± 0.10	4.10
	Sulfate	0.430	0.32 ± 0.03	1.58
N-limited condition	Ammonia	0.106	0.004 ± 0.001	1.43
	Nitrate	0.097	<0.030	>0.94
	Phosphate	1.170	1.06 ± 0.04	1.49
	Sulfate	0.430	0.13 ± 0.05	4.17
P-limited condition	Ammonia	3.38 ± 0.28	3.17 ± 0.28	2.70
	Nitrate	3.09 ± 0.11	2.77 ± 0.12	4.13
	Phosphate	0.012	<0.001	>0.14
	Sulfate	0.430	0.17 ± 0.02	3.41
S-limited condition	Ammonia	3.38 ± 0.28	3.03 ± 0.28	3.85
	Nitrate	3.09 ± 0.11	2.34 ± 0.26	8.40
	Phosphate	1.170	0.87 ± 0.08	3.38
	Sulfate	0.006	<0.003	>0.04

APPENDIX 4: MASS BALANCE OF LIMITING NUTRIENTS

Table A4.1 Mass Balance on Nitrogen

		Influent (N_{in}) (mg N/l)		Effluent (N_{out1}) (mg N/l)		N_{out2} (mg N/l)	Total N_{in} (mg N/l)	Total N_{out} (mg N/l)	Difference (mgN/l)	Difference (%)
		Ammonia	Nitrate	Ammonia	Nitrate	Biomass N				
N-limited condition	Control	47.32	43.26	33.32	37.94	7.79	90.58	79.05	11.53	12.73
	Limited	1.48	1.35	0.06	0.42	2.27	2.83	2.75	0.08	2.82
P-limited condition	Control	47.32	43.26	43.40	36.12	5.59	90.58	85.11	5.47	6.04
	Limited	47.32	43.26	45.78	40.04	3.83	90.58	89.65	0.94	1.03

Table A4.2 Mass Balance on Phosphorus

		Influent (P_{in}) (mgP/l)	Effluent (P_{out1}) (mgP/l)	Biomass P (mg P/l)	Total Pin (mg P/l)	Total Pout (mg P/l)	Difference (mg P/l)	Difference (%)
N-limited condition	Control	36.27	30.38	2.79	36.27	33.17	3.10	8.55
	Limited	36.27	32.24	1.42	36.27	33.66	2.61	7.20
P-limited condition	Control	36.27	27.28	2.03	36.27	29.31	6.96	19.19
	Limited	0.36	<0.03	0.28	0.36	<0.31	>0.05	>13.92
S-limited condition	Control	NA	NA	NA	NA	NA	NA	NA
	Limited	36.27	26.97	2.937	36.27	29.907	6.363	17.54342

NA→Not available because of no harvesting of control reactors

Note: Cellular sulfur content was not determined, thus were not able to perform mass balance on sulfur.

APPENDIX 5: GROWTH CURVES OF TRIAL EXPERIMENTS

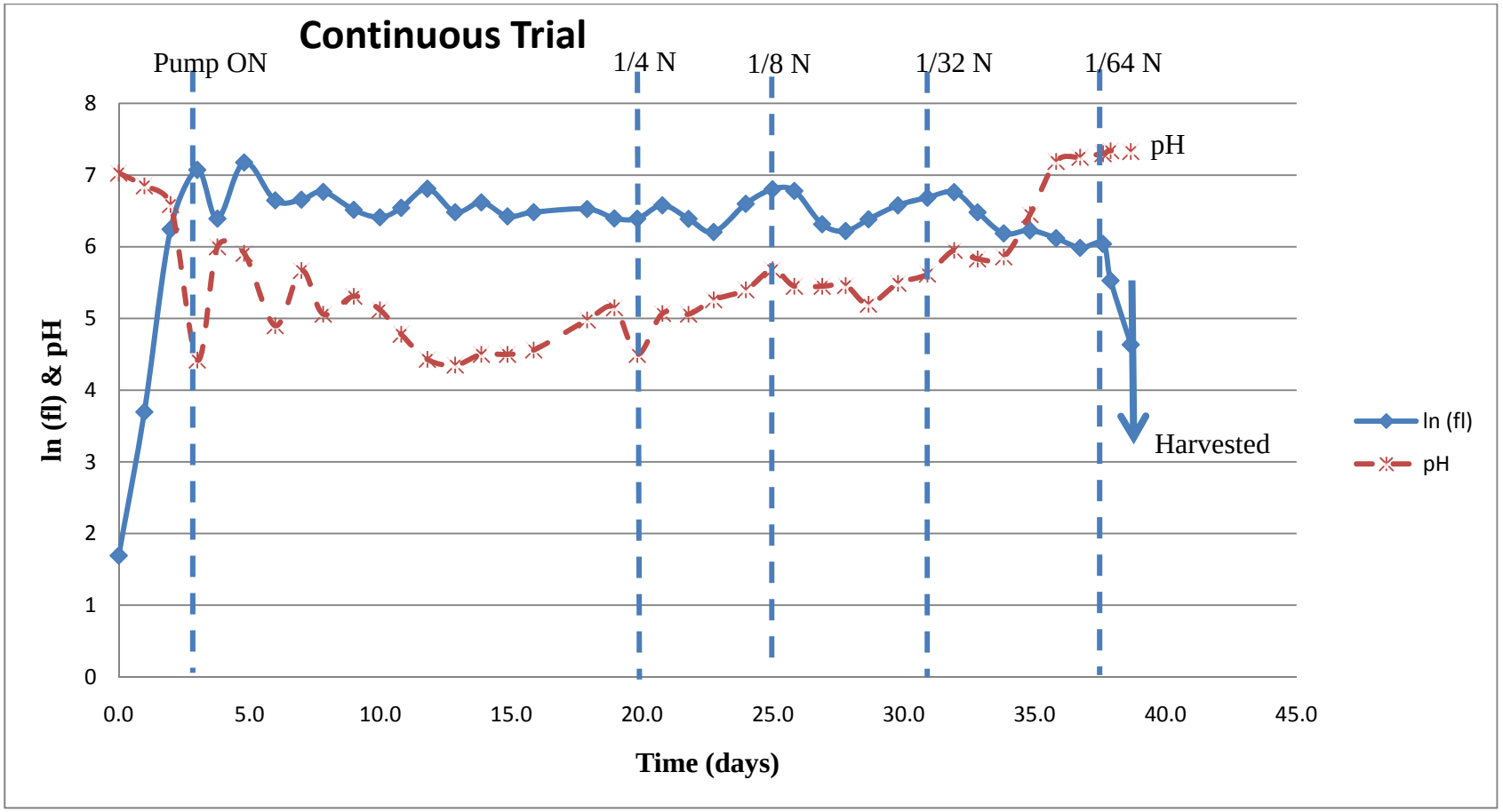


Figure A5.1 First growth curve of *C. reinhardtii* in continuous flow reactor in NAPK media (without PIPES) which was followed by N-limitation trial experiment. N is the original nitrogen concentration in media which was 7.5 mM (theoretical value) and FR is initial flow-rate when peristaltic pump is turned on which was 0.7 mL/min (calculated on the basis of growth rate which was 0.076 hr^{-1}).

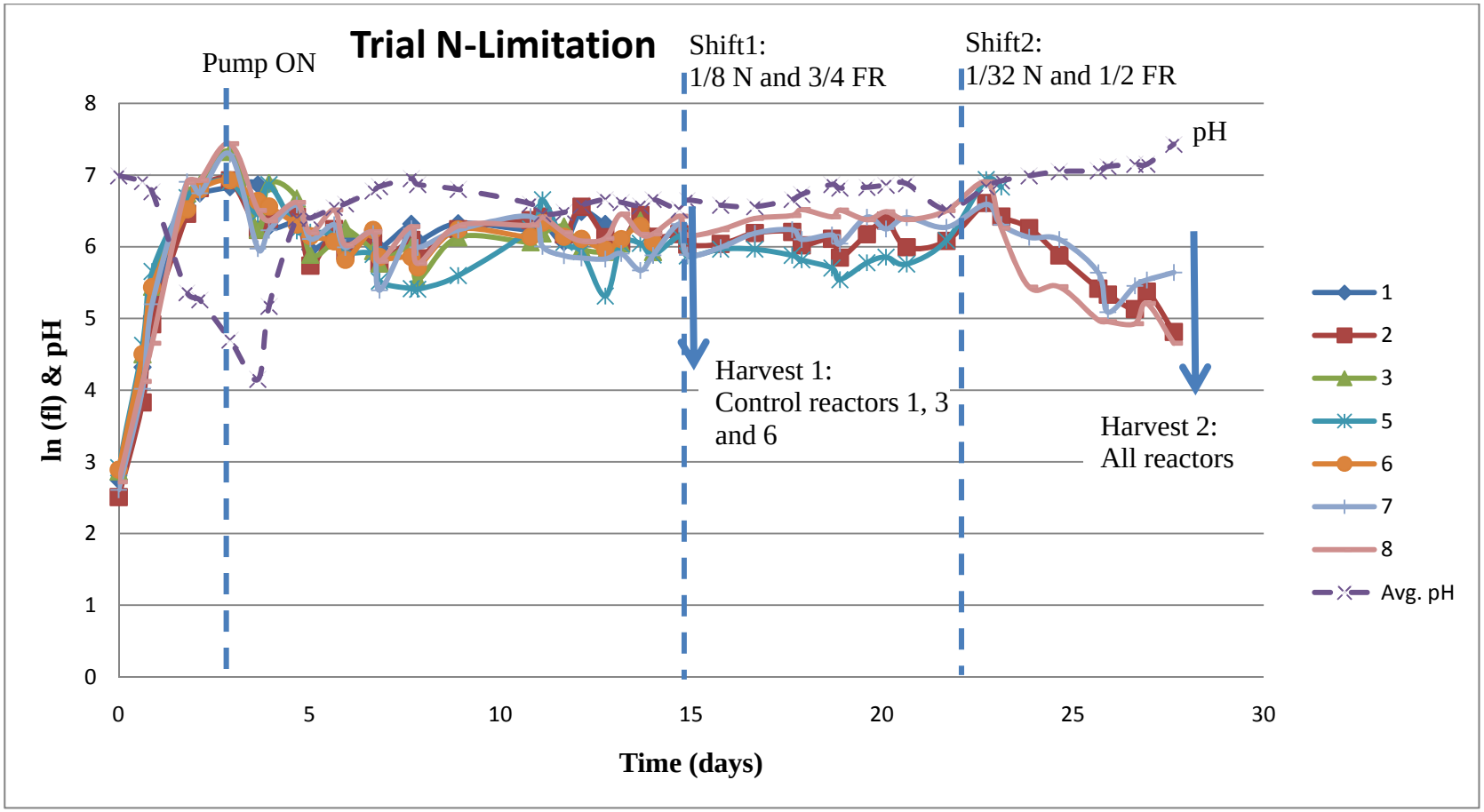


Figure A5.2 Growth curve of *C. reinhardtii* in continuous flow reactor for N-limiting trial experiment with NAPK media. N is the original nitrogen concentration in media which was 7.5 mM (theoretical value) and FR is initial flow-rate when peristaltic pump is turned on which was 0.825 ml/min (calculated on the basis of growth rate which was 0.09 hr^{-1}). Final nitrogen limiting concentration was decided on the basis of 1st continuous trial experiment in which at 1/32 N, cells starts to wash out pretty rapidly.

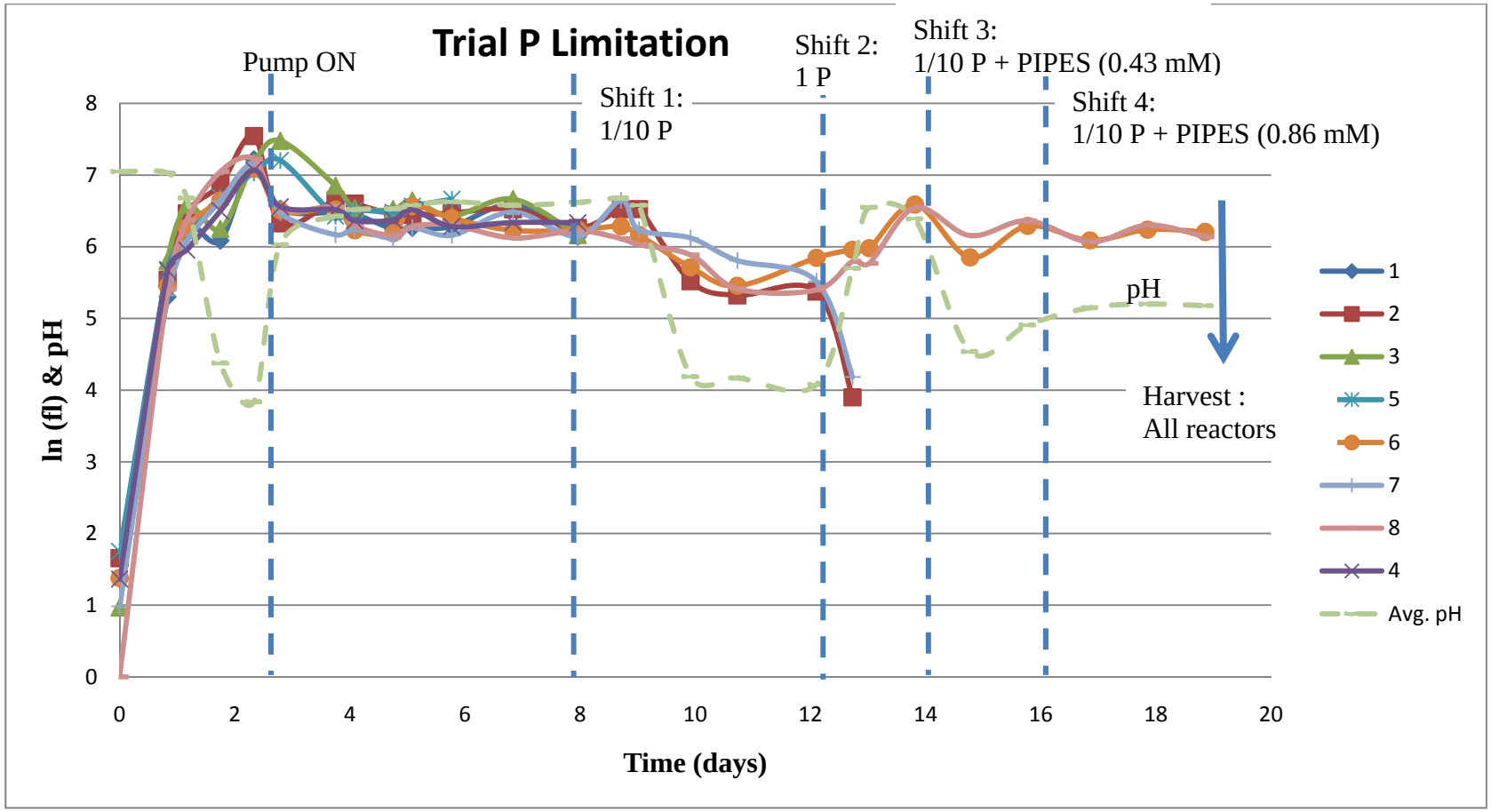


Figure A5.3 Growth curve of *C. reinhardtii* in continuous flow reactor for P-limiting trial experiment with NAPK media (no PIPES). P is the original phosphorus concentration in media which was 1.13 mM (theoretical value) and FR is initial flow-rate when peristaltic pump is turned on which was 0.825 ml/min (calculated on the basis of growth rate which was 0.09 hr^{-1}). Because of low pH, cells started to wash off and thus PIPES was introduced. Different concentrations were tried to bring pH back up to 6.

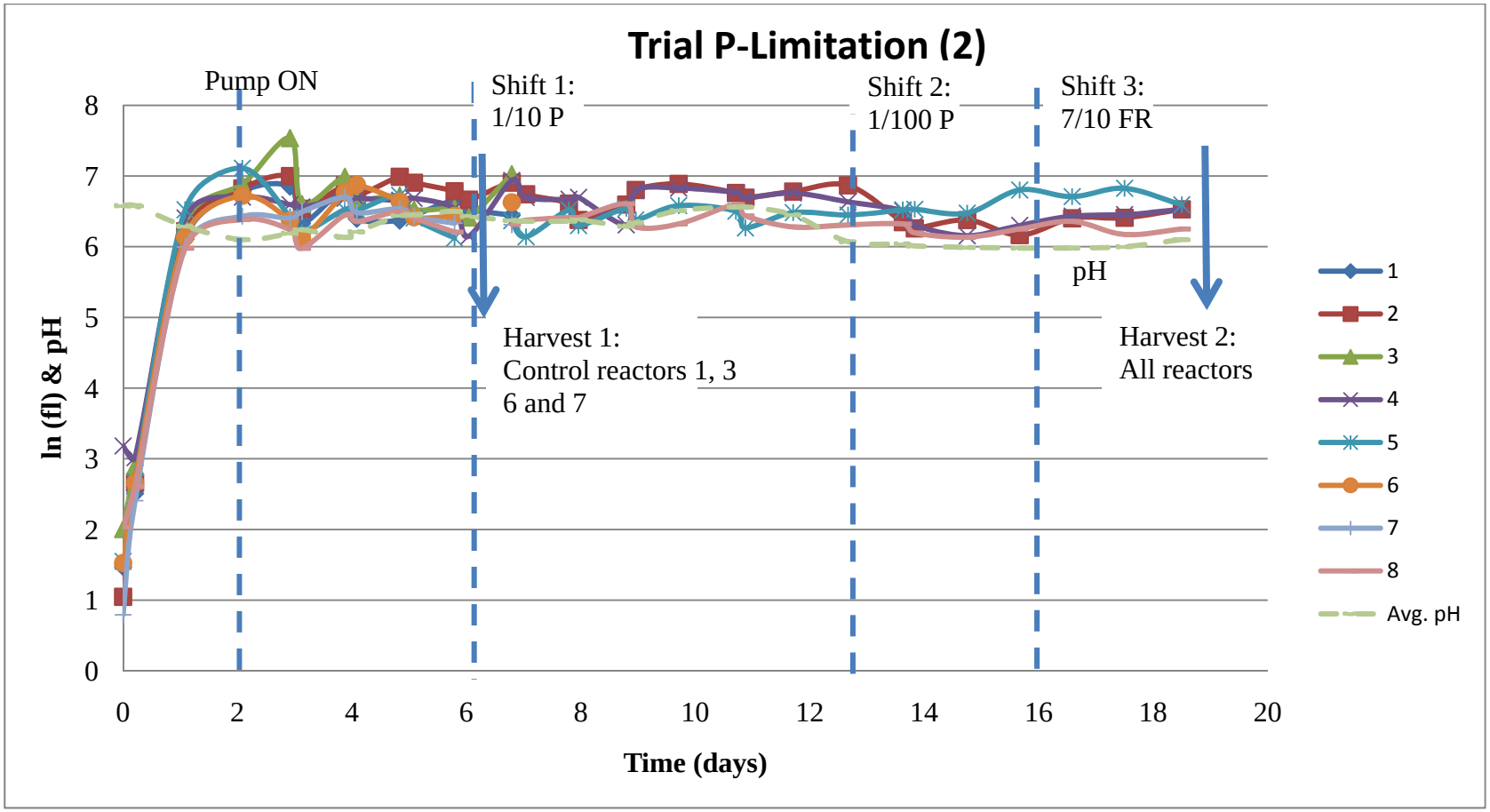


Figure A5.4 Growth curve of *C. reinhardtii* in continuous flow reactor for P-limiting second trial experiment with NAPK media (and 1.23 mM PIPES) as 0.86 mM of PIPES was not sufficient to maintain pH between 6 and 7. P is the original phosphorus concentration in media which was 1.13 mM (theoretical value) and FR is initial flow-rate when peristaltic pump is turned on which was 0.825 ml/min (calculated on the basis of growth rate which was 0.09 hr^{-1}).

APPENDIX 6: FLUOROSCENCE – MASS DENSITY RELATION

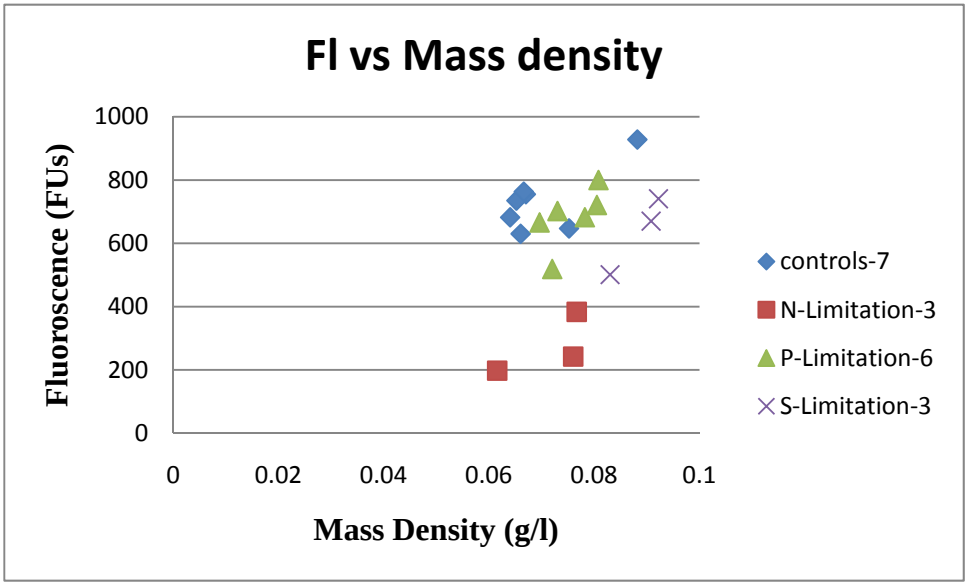


Figure A6.1 Fluoroscence vs Mass density for C. reinhardtii under different nutrient limiting conditions. As expected, fluorescence/g cell is lower for the N-limiting condition because of discoloration of the cells. Also for S-limitation because of reduction in photosynthesis activity, fluorescence/g cell is little off the cluster.

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