Raceway-based production of microalgae for possible use in making biodiesel

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Abstract

Oils from microalgae are of interest as a potential feedstock for producing renewable transport fuels including gasoline, diesel, biodiesel and jet fuel. For producing feedstock oils, an alga must be capable of being grown easily in readily available seawater and have a high productivity of biomass and oil. This study explored the biomass and lipid production potential of the microalga *Chlorella vulgaris* in seawater media, as a potential producer of feedstock oils. The alga was grown photoautotrophically under various conditions in ~2 L Duran bottles and a pilot scale (~138 L) raceway system. Initially, eight species of microalgae of different classes were assessed under nutrient sufficient growth conditions for the production of biomass and lipids in ~2 L Duran bottles. Two of the promising species (*C. vulgaris* and *Nannochloropsis salina*) were then further evaluated extensively under various conditions (i.e. salinity stress, different levels of nitrogen in growth media, continuous light and light-dark cycling). Based on these assessments *C. vulgaris* stood out as the best alga for further detailed study. *C. vulgaris* was evaluated for biomass production and lipid production. The consumption rates of major nutrients (N and P) were quantified. Biomass was characterized for elemental composition and energy content at the end of the growth cycle. A maximum lipid productivity of ~31 mg L^{-1} d^{-1} was attained in Duran bottle batch culture under nitrogen starvation in continuous light with a lipid content in the biomass of ~66% (dry weight). This appears to be the highest lipid content reported for *C. vulgaris* grown in seawater and demonstrates an excellent ability of this alga to accumulate high levels of oil. Under a 12:12 h light-dark cycle, the lipid content and productivity in Duran bottle batch culture were decreased by 13% and 41%, respectively, relative to the case for continuous illumination. Energy content of the biomass produced in Duran bottle batch culture exceeded 30 kJ g^{-1} both in continuous light and the 12: 12 h light-dark cycle.

Batch and continuous culture kinetics of *C. vulgaris* in the raceway system were assessed. The alga was subjected to various light regimes and nitrogen starvation conditions. Although the N starvation enhanced the lipid accumulation by 42% relative to nutrient sufficient growth in batch culture, the highest biomass and oil productivities were attained under nutrient sufficient conditions in continuous mode of cultivation. Under nutrient sufficiency in continuous culture with a constant illumination of 91 $\mu mol \cdot m^{-2} s^{-1}$, the productivities of biomass and lipid in the raceway were >61 mg L^{-1} d^{-1} and >8 mg L^{-1} d^{-1}, respectively.

This work represents the first detailed study of *C. vulgaris* in a raceway pond in full strength seawater media. Previous studies of this alga were almost always carried out in freshwater media.

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In the name of Allah, the Entirely Merciful, the Especially Merciful

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Abbreviations

A	Surface area for light absorption
Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
A_{xxx}	Spectrophotometric absorbance at *xxx* nm
BG11	A medium formulation for growing cyanobacteria/microalgae
CCALA	Culture Collection of Autotrophic Organisms, Czech Republic
CCAP	Culture Collection of Algae and Protozoa, Argyll, United Kingdom
D	Dilution rate
DCW	Biomass dry cell weight
DF	Dilution factor
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EPA	Eicosapentaenoic acid
F	Feed flow rate
FAME	Fatty acid methyl esters
F/2-Si	A medium formulation for growing diatoms
GC	Gas chromatography
G3P	Glyceraldehyde-3-phosphate
HRAP	High Rate Algal Ponds
I	Irradiance
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
I_L	Local irradiance at any depth *L* from the surface

ISO	International Organization for Standardization
L	Depth from the surface
LED	Light emitting diode
MBL	A medium formulation for growing cyanobacteria/microalgae
NA	Not available
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
ND	Not determined
N_f	Final nitrate concentration
NCMA	Provasoli-Guillard National Center of Marine algae and Microbiota, Maine, USA
N_i	Initial nitrate concentration
NIWA	National Institute of Water and Atmospheric Research Ltd, New Zealand
N_1	Nitrate concentration at time t_1
N11	A medium formulation for growing cyanobacteria/microalgae
N_2	Nitrate concentration at time t_2
PAR	Photosynthetically active radiation (light in the wavelength range of 400-700 nm)
P_b	Biomass productivity
PBR	Photobioreactor
P_f	Final phosphate concentration
PFD	Photon-flux density
P_i	Initial phosphate concentration
P_l	Lipid productivity
ppt	Part per thousand
PUFA	Polyunsaturated fatty acid
P_1	Phosphate concentration at time t_1

P_2	Phosphate concentration at time t_2
q_N	Average biomass specific nitrate consumption rate
q_P	Average biomass specific phosphate consumption rate
RNA	Ribonucleic acid
rpm	Revolutions per minute
RTD	Resistance temperature device
RuBP	Ribulose-1,5-bisphosphate
S	Surface area
t	Duration time of a batch (i.e. the time required to attain the biomass concentration X_f
TAG	Triacylglycerols
UTEX	The Culture Collection of Algae of University of Texas, Austin, USA
V	Volume
V	Volume in the culture vessel
v/v	Volume by volume
w	Weight fraction of the lipids in the biomass
w/w	Weight by weight
w_s	Steady state weight fraction of the lipids in the biomass
x	x-Variable
X	Biomass concentration at any time t
X_i	Initial biomass concentration
X_f	Final biomass concentration
X_s	Steady state biomass concentration
X_1	Biomass concentration at time t_1
X_2	Biomass concentration at time t_2
y	y-Variable

Y_N, $Y_{X/N}$	Biomass yield coefficient on nitrate
Y_P, $Y_{X/P}$	Biomass yield coefficient on phosphate
$Y_{X/L}$	Biomass yield coefficient on light

Greek symbols

μ	Specific growth rate

Chapter 1

INTRODUCTION

Microalgae are sources of energy-rich oils and other valuable products (Whyte, 1987; Becker, 1994). The amount of a desired compound in an algal biomass determines its value (Williams and Laurens, 2009). Microalgae have been produced commercially for several decades to obtain high value compounds such as β-carotene, phycobilin, astaxanthin, and long chain polyunsaturated fatty acids (Becker, 1994; Dufossé *et al.*, 2005; Spolaore *et al.*, 2006; Williams and Laurens, 2009; Borowitzka, 2010; Borowitzka, 2013). Microalgae are also used as aquaculture feeds and in the future may provide fuels such as biohydrogen and bioethanol (Matsumoto *et al.*, 2003; Ho *et al.*, 2013); methane (Zamalloa *et al.*, 2011); diesel, gasoline, kerosene (jet fuel) (Lestari *et al.*, 2009); and oils obtained via thermochemical conversion of algal crude oil or biomass (Kröger and Müller-Langer, 2012).

Fuels from microalgae are of interest as they are potentially renewable and their production/use may reduce emissions of climate changing greenhouse gases (Farrell *et al.*, 2006; Huntley and Redalje, 2007; Lam and Lee, 2012). According to a recent review by British Petroleum (2013), fossil fuels provide nearly 87% of the total energy consumption of the world. Renewable energy sources currently provide less than 2% of the global energy consumption. Therefore there is a tremendous potential for expanding the supply of renewable energy. According to the International Energy Agency (2010), renewable energy derived from combustion of waste and other forms of renewable sources has a substantial scope for expansion.

Biofuels from microalgae have been receiving increasing attention (Banerjee *et al.*, 2002; Guschina and Harwood, 2006; Hu *et al.*, 2008) but there is also the recognition that commercializing them would require sustained long-term research. Although the technology of algal biomass production is available and has been practiced for decades (Williams and Laurens, 2009), it is expensive. Furthermore, the currently used relatively low-cost open pond production system for algae are suitable only for a few species that either grow rapidly or require extremophilic culture conditions in which other algae do not thrive. Examples of algae and cyanobacteria produced in open ponds currently include *Chlorella*, *Dunaliella salina*, *Haematococcus pluvialis* and *Spirulina* (Borowitzka, 1999a). These species have been cultivated for high value compounds and for use as dietary supplements. As far as cultivating microalgae for fuel oil is concerned, the cultivation needs to be extremely low cost as fuel oils are low-value products (Williams and Laurens, 2009). In 1978, an extensive study of algal fuel production concluded it to be economically nonviable (Sheehan *et al.*, 1998). Increasing price of petroleum oil has renewed the interest in algal fuels. Although commercial production of biomass is feasible (Borowitzka, 1997), it is economic only for high-value products. This and other limitations to commercialization of algal biofuels have been reviewed by Chisti (2013).

At present, the research on microalgae biofuels is focused on assessing the potential in terms of lipid productivity, cultivation methods, production system design and construction, and harvesting techniques (Chen *et al*., 2011; Lam and Lee, 2012). The use of genetically modified algae (Radakovits *et al.*, 2011; Yu *et al.*, 2011b) is in infancy. Genetic and metabolic engineering (Waltz, 2009; Lü *et al.*, 2011) for improving algal oil production and reduce costs have barely commenced. Much of the existing literature is focused on freshwater algae although a general global shortage of

freshwater requires that algae that can be grown in seawater should be focused on. Limited information is available on algal biomass and lipid production kinetics (Chen *et al.*, 2010) in seawater systems.

This study examined how various growth parameters (e.g. different light regimes, nutrients concentrations, levels of salinity) affect kinetics of algal growth and oil productivity in seawater media. The effect of these growth parameters on the energy content of the biomass and elemental composition of the biomass was studied. Effects of the nitrogen starvation on the lipid production in seawater were a focus of the study as nutrient starvation offers a relatively simple and inexpensive method of increasing the oil content (Sharma *et al.*, 2012). Although the response to nitrogen limitation is well documented in several microalgae (Shifrin and Chisholm, 1981; Piorreck *et al*., 1984; Illman *et al*., 2000; Scragg *et al*., 2002; Widjaja *et al*., 2009; Chen *et al*., 2010; Lv *et al*., 2010; Chen *et al*., 2011; Wahlen *et al*., 2011; Yeh and Chang, 2011) , this is almost exclusively in freshwater. Extensive work is reported in this study in a relatively large (~138 L) raceway pond as growth systems of this type are most likely to be used in actual commercial practice.

The relevant literature is reviewed in Chapter 2. Chapter 3 documents the experimental methods. Chapter 4 is concerned with the results and their discussion. Summary of the major findings and conclusions of the study are presented in Chapter 5.

Chapter 2

LITERATURE REVIEW

2.1 Microalgae

Microalgae are microscopic photosynthetic plants that do not have roots, stem and leaves (Lee, 1989). Microalgae occur in diverse environments (Tomaselli, 2004) including deserts and polar regions (Harwood and Guschina, 2009). More than 40,000 species of algae have been estimated to exist (Hu *et al.*, 2008). A few common microalgae are shown in Figure 2.1. The main groups of algae include Chlorophyta (green algae), Bacillariophyta (diatoms), Xanthophyta (yellow-green algae), Chrysophyta (golden algae), Rhodophta (red algae), Phaeophyta (brown algae), Dinophyta (dinoflagellates) and Eustigmatophyte (pico-plankton) (Hu *et al.*, 2008). The detailed anatomy, biochemistry and classification can be found in the literature (Van den Hoek *et al.*, 1995; Barsanti and Gualtieri, 2005).

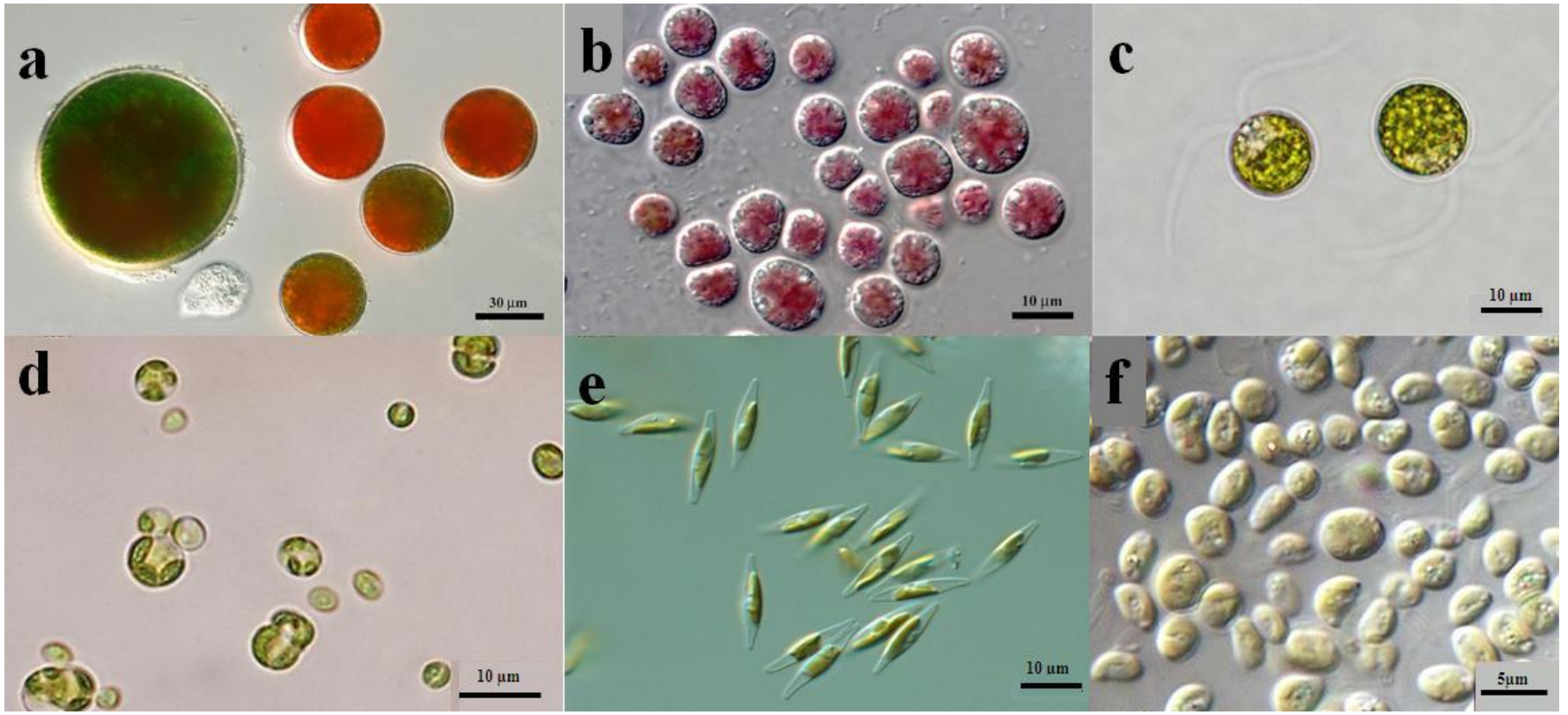


Figure 2.1 Some examples of microalgae belonging to different classes: a) *Haematococcus pluvialis*, Chlorophyta (CCALA – Culture Collection of Autotrophic Organisms, Czech Republic); b) *Porphyridium cruentum*, Rhodophyta (CCALA – Culture Collection of Autotrophic Organisms, Czech Republic); c) *Dunaliella salina*, Chlorophyta (UTEX – The Culture Collection of Algae of University of Texas, Austin); d) *Chlorella vulgaris*, Chlorophyta (UTEX – The Culture Collection of Algae of University of Texas, Austin); e) *Phaeodactylum tricornutum*, Bacillariophyta (NCMA – Provasoli-Guillard National Center of Marine algae and Microbiota) and f) *Nannochloropsis salina*, Eustigmatophyte (www.sb-roscoff.fr/Phyto/gallery/main.php).

Microalgae use energy from sunlight to combine water and carbon dioxide to produce cell mass as described further in this chapter. Typically, algae account for 50% of the photosynthesis occurring on Earth (Moroney and Ynalvez, 2001). As photosynthetic microorganisms, algae are referred to as a primary producers and form the base of the marine food web (Longhurst *et al.*, 1995; Morel and Antoine, 2002). The environmental and ecological significance of microalgae has long been known. Increasingly, microalgae are being examined as sources of high value compounds (Figure 2.2). Microalgae are being cultivated commercially for food, aquaculture feeds, nutraceuticals, pharmaceuticals, cosmetics, and environmental applications (Apt and Behrens, 1999; Muller-Feuga, 2000; Pulz and Gross, 2004; Dufossé *et al.*, 2005; Patil *et al.*, 2005; Carvalho *et al.*, 2006).

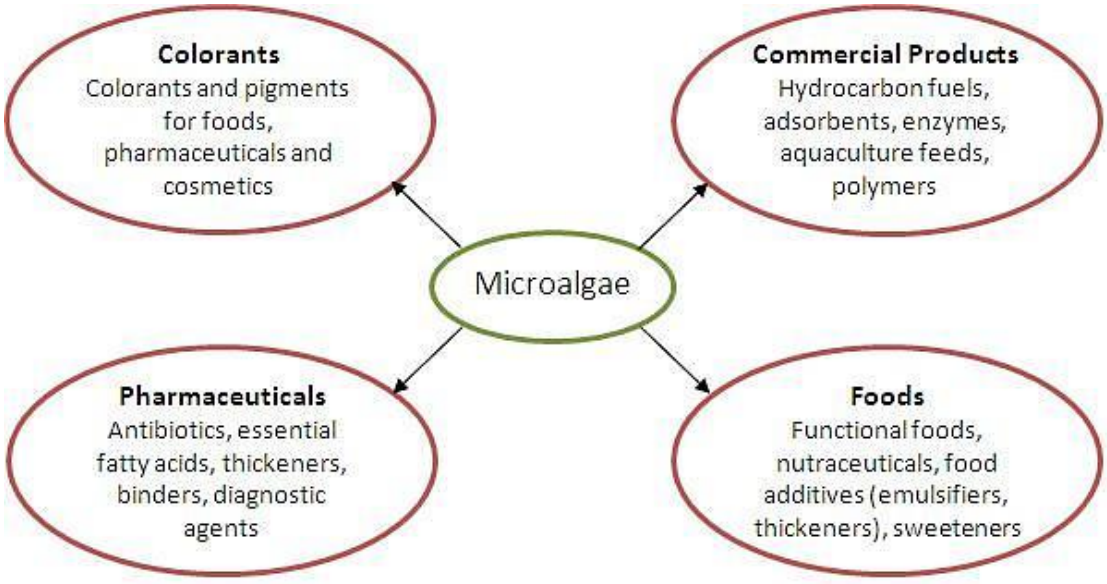


Figure 2.2 Microalgae applications in various fields. Modified from Dufossé *et al.* (2005).

2.2 Microalgae as a fuel source

The concept of obtaining liquid fuels from microalgae is not new (Meier, 1955; Chisti, 1980-81; Nagle and Lemke, 1990; Sawayama *et al.*, 1992). Fuels derived from algal oils are potentially renewable. Algal fuels can be potentially produced and used without a net increase in the concentration of carbon dioxide in the atmosphere (Antolin *et al.*, 2002; Vicente *et al.*, 2004). Algal fuels have not previously received significant attention because they are expensive to produce (Chisti, 2007).

Interest in algae for fuels is reemerging because of considerations of global warming and therefore a need to move away from fossil fuels (Kessel, 2000; Gavrilescu and Chisti, 2005; Pahl, 2005). Various other renewable biofuels have the same advantages (Cook and Beyea, 2000; Lang *et al.*, 2001; Antolin *et al.*, 2002; Vicente *et al.*, 2004) as potential fuels from algae. Unlike biofuels derived from higher plants, algae have much greater oil productivity (Shay, 1993) and appear to be able to provide far greater quantity of oil compared to what could be realistically produced from oil crops (Chisti, 2007, 2008).

Algae can be grown using carbon dioxide in the flue gases produced by fossil fuel burning power plants (Talec *et al.*, 2013; Kumar *et al.*, 2014). Compared to higher plants, microalgae have higher photosynthetic efficiency (Ginzburg, 1993; Shay, 1993), short growth cycles (Chisti, 2007; Meng *et al.*, 2009) and can be harvested daily (Chisti, 2007; da Silva *et al.*, 2009; Meng *et al.*, 2009). Depending on species, microalgae can produce more than 70% their dry weight in the form of lipids, or oils (Andersen, 1992; Sheehan *et al.*, 1998; Banerjee *et al.*, 2002; Tsukahara and Sawayama, 2005; Guschina and Harwood, 2006; Spolaore *et al.*, 2006; Chisti, 2007). Furthermore, algae can be grown on nonarable land (Chisti, 2007, 2008; Dismukes *et al.*, 2008) using seawater. Algae can be produced without compromising the supply of food, feed and freshwater (Chisti, 2007, 2008; Dismukes *et al.*, 2008; da Silva *et al.*, 2009). For all these different

reasons, microalgae are considered ideal candidates for producing biofuels. Microalgae have been viewed as suitable for producing biodiesel (Borowitzka, 2010). Therefore many studies have focused on triacylglyceride oils of microalgae as they are readily converted to biodiesel (Xu *et al.*, 2006; Schenk *et al.*, 2008). Microalgae contain many other kinds of oils and not just triglycerides. All these oils can potentially provide various types of biofuels, although commercial production of algal biofuel remains challenging due to technical (Greenwell *et al.*, 2010) as well as economic reasons (Benemann and Oswald, 1996; Borowitzka, 1999b; Stephenson *et al.*, 2010a; Chisti, 2013). There is, therefore, the need to better understand the various aspects of production of microalgal biomass and lipids (Chen *et al.*, 2010).

2.3 Algal cultivation

Depending upon the requirements and the type of strain, different types of algal cultivation approaches can be used. These include photoautotrophic, heterotrophic, photoheterotrophic and mixotrophic growth (Chojnacka, 2004; Mata *et al.*, 2010). In photoautotrophic growth light is the only source of energy. The heterotrophic growth utilizes only organic compounds as a source of carbon and energy. In photoheterotrophic growth, light and exogenous organic compounds are the sources of energy. In mixotrophic growth, energy source is light and organic carbon (Chojnacka, 2004), but inorganic carbon (CO_2) is also used.

Growth on organic carbon can greatly enhance production of biomass and lipids (Chen, 1996; Miao and Wu, 2004; Xu *et al.*, 2006; Li *et al.*, 2007) compared to photoautotrophic (light as energy source) growth (Liang et al., 2009). Some microalgae possess the features necessary for all these different types of metabolism. Examples of such algae are *Chlorella vulgaris*, *Dunaliella salina* (Scarsella *et al.*, 2009) *Haematococcus pluvialis* and the cyanobacterium *Arthrospira* (*Spirulina*) (Mata *et al.*,

2010). However using organic compounds as a source of energy is inefficient overall as all the organic carbon must be produced ultimately by photosynthesis (e.g. in plants) for feeding the heterotrophic algae (Chisti, 2007). This review therefore mostly focuses on photoautotrophic growth of algae, its requirements and challenges.

2.3.1 Photosynthesis and algal production

Photosynthesis is a unique process which is responsible for nearly all the life on Earth (Rubio *et al.*, 2003). The plants, algae and cyanobacteria are capable of photosynthesis. The algae generally perform C3 photosynthesis which is similar to the C3 photosynthesis of most terrestrial plants. In C3 photosynthesis the first product of assimilation of CO_2 is a 3-carbon sugar, glyceraldehyde-3-phosphate (G3P) (Lobban and Harrison, 1994; Ehleringer and Cerling, 2002). The photosynthetic process has been described in detail in the literature (Whittingham, 1952; Lobban and Harrison, 1994; Alberts *et al.*, 2002; Douglas *et al.*, 2003; Lv *et al.*, 2010). A summary follows.

In photosynthesis, light energy is captured by the antenna complex of the photosynthetic apparatus and transferred rapidly to the neighboring chlorophyll molecules present in the photochemical reaction centers in the chloroplast. This light energy (four photons) splits water molecules to remove four electrons from two water molecules. This produces H^+ ions and O_2 is released as a by-product. To complete a photosynthetic reaction a total of 8 photons are required. This process produces ATP (adenosine triphosphate) and NADPH. ATP (with NADPH) is used as an energy source for fixing the inorganic carbon (CO_2) to a three carbon organic compound, glyceraldehyde-3-phosphate (G3P). Most of this three-carbon sugar (G3P) in chloroplast is converted into starch and stored as large granules during rapid photosynthesis. The biochemical reactions of photosynthesis are controlled by numerous enzymes which are located in the chloroplast. From the chloroplast stroma,

glyceraldehyde-3-phosphate (G3P) is transported into the cytosol to serve as the basis for the biosynthesis of many organic molecules and metabolites. Some of G3P is used for carbohydrate biosynthesis. Some G3P molecules are converted into pyruvate for protein synthesis and some (G3P) is used for fatty acid synthesis for lipid accumulation. All of these organic products contain stored chemical energy, which is utilized by the cell at different stages of growth. The process of photosynthesis is illustrated in Figure 2.3.

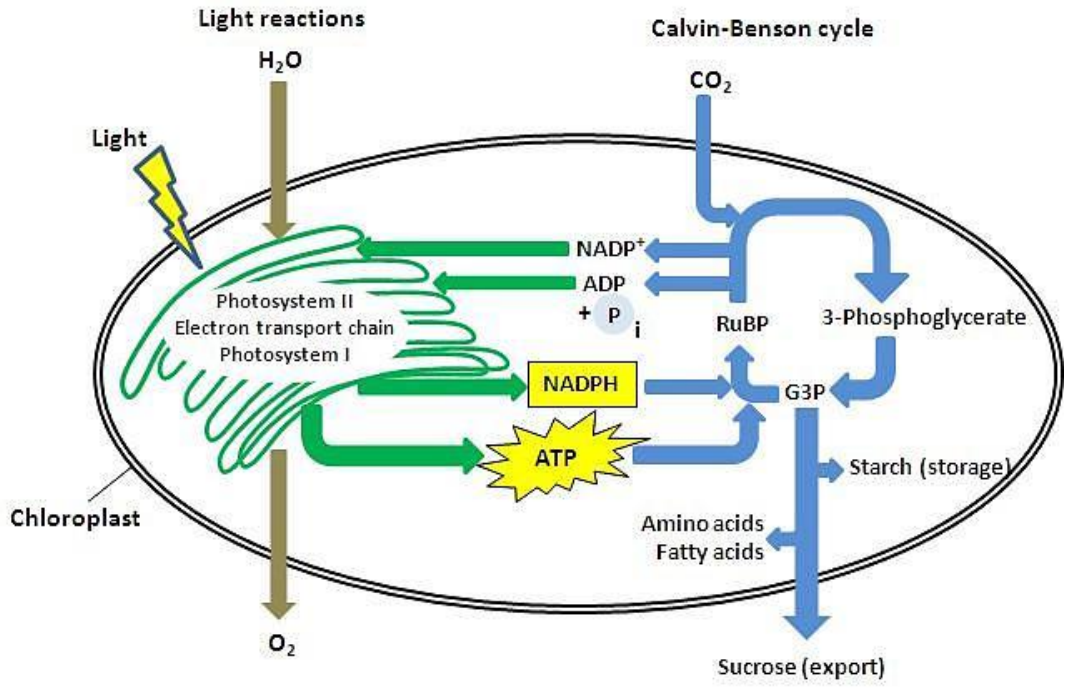


Figure 2.3 Conversion of solar energy into chemical energy by oxygenic photosynthesis in the chloroplast. Modified from Campbell and Reece (2005).

Many factors influence the rate of photosynthesis and any variations in these may affect the rate of biomass growth or change its composition (Hu, 2004). These factors include the concentration of CO_2, the quality and quantity of light, temperature (Vonshak and Torzillo, 2004), and the availability of micronutrients.

2.3.2 Growth parameters and limitation to biomass production

For biomass production in practice, growth is affected by:

1) Environmental factors such as light level, temperature, and salinity;
2) Chemical factors, i.e. the quality and quantity of nutrients (Borowitzka, 2005);
3) Operational factors (e.g. dilution rate, mixing) relating to the culture system; and
4) Biotic factors, i.e. contamination from unwanted species (Borowitzka, 1998).

Some of the major factors influencing biomass production are discussed in the following sections.

2.3.2.1 Light

Light is the major controlling factor influencing growth. Availability of light is directly related to the algal production regardless of the optimal level of nutrients and temperature in a cultivation system (Smith, 1983; Richmond, 1999). The effective light utilization by algae depends on cell concentration in the broth, the culture depth in a production system (Richmond, 1999) and the pigment composition of the cells (Carnicas *et al.*, 1999; Six *et al.*, 2009; Sforza *et al.*, 2012). The algal culture is affected by variations in light intensity and its cycles during diurnal and seasonal changes (outdoor) as well as by a very short light-dark cycle, i.e. a split-second cycle (flashing light effect) induced by mixing (Kok, 1953; Vonshak and Torzillo, 2004; Sato *et al.*, 2010).

In a production system with an increasing light intensity, the biomass concentration continues to increase as long as the other nutrients are not limiting. If the biomass concentration is kept low, a maximum value of the specific growth rate is achieved (Figure 2.4) at a certain light level known as the light saturation point (Bouterfas *et al.*, 2002; Macedo *et al.*, 2002; Torzillo *et al.*, 2003). Increasing light intensity from light saturation level may inhibit the growth (Figure 2.4), a phenomenon known as

photoinhibition. The spectrum of light radiation used by algae (i.e., the photosynthetically active radiation, PAR) is within the range of 400-700 nm. PAR is measured as a photon-flux density (PFD) incident on the surface of broth. (Photon flux density is the number of micromoles of photons of 400-700 nm wavelength received per square meter of a surface in 1 second.) The PAR value measured at peak solar irradiation in a tropical region is about 2,000 $\mu mol \cdot m^{-2}s^{-1}$ (Chisti, 2012). However, for most of the algal strains light saturation level occurs at a PAR value of less than 200 $\mu mol \cdot m^{-2}s^{-1}$ (Ogbonna and Tanaka, 2000; Torzillo *et al.*, 2003; Chisti, 2012). The effect of increasing light intensity on the growth rate is shown in Figure 2.4.

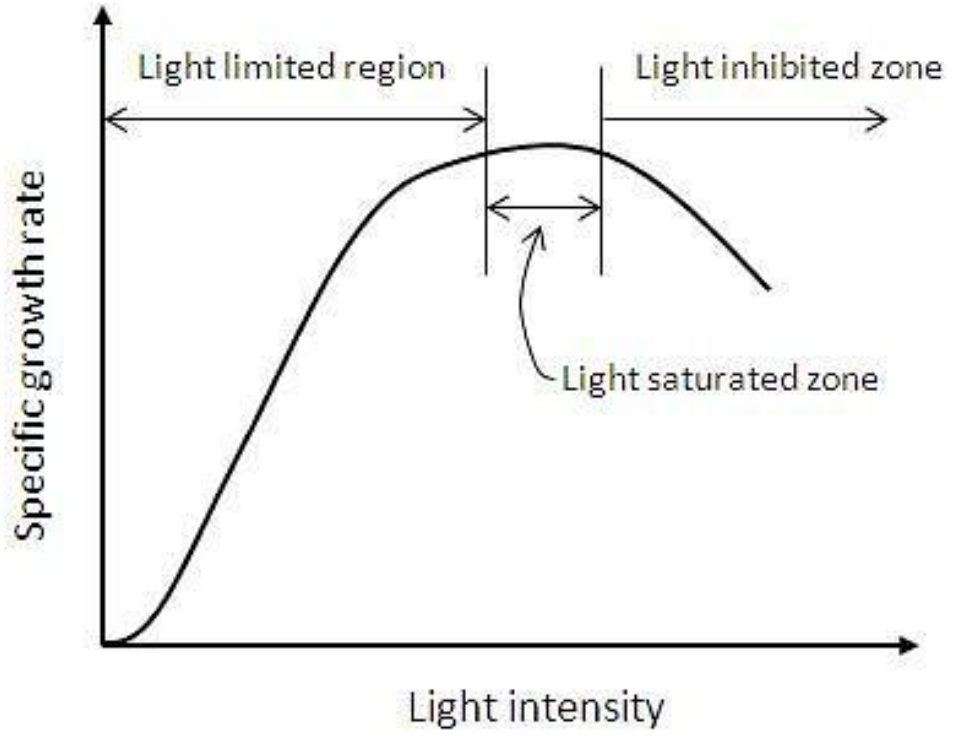


Figure 2.4 Light intensity levels and its effect on growth. Adapted from Ogbonna and Tanaka (2000).

The energy from light is converted into biomass in the form of chemical energy. The fraction of the total incident sunlight energy converted into biochemical energy is known as the photosynthetic efficiency. Theoretically, the photosynthetic efficiency of algae is estimated to be 13% of incident light (Bolton and Hall, 1991), but in practice in outdoor mass culture, the maximum efficiency is about 2.7% (Chisti, 2012). In some earlier studies, the flashing light effect was used to raise the photosynthetic efficiency to a value of 10% (Laws *et al.*, 1983; Sheehan *et al.*, 1998) by generating vortices using foil arrays.

The variation in light intensity also modifies the chemical composition of biomass. The content of carbohydrate, protein and lipids are affected by light-induced changes in intracellular pH. Mg^{2+} and NADPH levels in stroma regulate the key enzymes for fatty acid synthesis (Sukenik and Livne, 1991; Lv *et al.*, 2010). Enzyme activity may be reduced under light limitation and photoinhibition conditions. The effect of light is further discussed in the context of lipid accumulation later in this review (Section 2.4.1.2).

2.3.2.2 Temperature

Temperature is another important factor (Richmond, 1988; Richmond *et al.*, 1990; Torzillo *et al.*, 1991; Richmond, 1999) that regulates algal growth. Temperature affects the rates of all biochemical reactions and therefore the metabolic rates. Any variation in growth temperature can influence the composition of the algal biomass (Geider, 1987; James *et al.*, 1989; Davison, 1991). The optimal growth temperature of algal strains varies according to the climatic conditions of their natural habitat. For most algae of interest, the optimal growth temperature ranges between 24-40 ºC (Hanagata *et al.*, 1992). Algae that thrive at 24-40 ºC, often can tolerate temperatures as low as 15 ºC, but temperatures of 2-4 ºC higher than their optimal could cause a culture crash (Richmond,

1999). Irradiance affects culture temperature and effectively interacts with temperature in influencing productivity (Borowitzka, 1998).

2.3.2.3 Nutrients

Algal cells require nutrients to grow which are easily accessible to the cell from its surrounding environment (Chisti and Yan, 2011) (i.e. the aqueous medium). Composition of medium is an important factor in influencing growth via the supply of inorganic nutrients (Chen *et al.*, 2010). Main nutrients for algal culture are carbon, nitrogen and phosphorus (Richmond, 1988). Optimal nutrient requirements depend on species (Maddux and Jones, 1964; Rhee and Gothan, 1981; Smith, 1983; Cromar and Fallowfield, 1997). Kaplan *et al.* (1986) mentioned 30 elements as being essential for the autotrophic growth of algae. These nutrients are typically divided into macronutrients and micronutrients.

2.3.2.3a Macronutrients

These are the most important nutrients and their deficiency can arrest the growth of algae. They include carbon, nitrogen, phosphorus, hydrogen and oxygen, which are essential for all algae. Silicon is an important element for the growth of diatoms which use it to build the structural part of the cell wall (Healey, 1973). Water is the main source of hydrogen and oxygen (Knud-Hansen, 1998). The role of the other major elements and their sources are discussed here.

Carbon as a main nutrient constitutes approximately 50% by weight of the algal biomass (Becker, 1994). Usually for photosynthetic algal production, the source of carbon is CO_2, which is either supplied directly to the nutrient media as pure CO_2 or through an air/CO_2 mixture. Carbon is needed to form all essential metabolic compounds including DNA/RNA, carbohydrates, proteins and lipids. According to an

estimate, producing 1 kg of biomass requires 1.5 to 2 kg of CO_2 (Sobczuk *et al.*, 2000). Sodium bicarbonate ($NaHCO_3$) can also be used as an inorganic carbon source (Chen *et al.*, 2010; Yeh and Chang, 2010).

After carbon, nitrogen is the next key element needed in relatively large amounts for algal growth. Nitrogen constitutes about 7-10% of the cell dry mass (Hu, 2004). Nitrogen is generally provided to algal cultures in the form of nitrates, ammonium salts or urea. The nitrogen supply affects growth rate of algae, the lipid content (Chen *et al.*, 2010), the fatty acid composition and the general health of culture (Fidalgo *et al.*, 1998). Cells assimilate nitrogen to produce chlorophyll, proteins, nucleic acids and coenzymes (Liu, 2012). As a result of nitrate assimilation, the pH of the culture rises (Becker, 1994). pH is controlled in a production system usually by supplying CO_2. According to an estimate, 50-80 kg of N is required to produce 1000 kg of algal biomass (Borowitzka and Moheimani, 2013).

Oxygen is another major element found in most organic cellular materials. Nearly 20% of cell biomass is oxygen. Oxygen molecule is the final electron acceptor during aerobic respiration of cell (Liu, 2012). The main sources of oxygen are water (Knud-Hansen, 1998). Hydrogen is also required for algal nutrition and occurs in water and nearly all organic molecules. The cell biomass consists of approximately 8% hydrogen by weight (Liu, 2012).

Phosphorus is another essential element for algal metabolism. Although it constitutes only 1-3% by weight of algal biomass, its supply regulates many aspects of growth, composition of the biomass and the cellular metabolic processes. For example, energy transfer during photosynthesis, synthesis of nucleic acids and DNA are affected by the supply of phosphorus (Becker, 1994; Grobbelaar, 2004). Synthesis of phospholipids and certain coenzymes requires phosphorus (Liu, 2012). Phosphorus is provided to the cultures as inorganic salts (Kuhl, 1974), usually as orthophosphate

(PO_4^{2-}) and its use by the algal cell is an energy dependent process (Becker, 1994). The necessary energy is supplied through photosynthesis or respiration.

2.3.2.3b Micronutrients and trace elements

Micronutrients are required in smaller amounts compared to the macroelements but influence algal growth and participate in metabolic activity. Examples of microelements are sulfur, calcium, sodium, potassium, magnesium, iron, manganese, zinc, copper, and cobalt (Goldman and Horne, 1994). A sufficient supply of all these micronutrients is needed to ensure a high yield of the biomass; however, limiting amounts of some nutrients may be used to enhance production of specific metabolites (Grobbelaar, 2004).

2.3.2.4 Salinity

Salinity refers to the saltiness of saline water and is commonly defined as the amount of total dissolved salts (g) per kg of water. The preferred method of determining salinity is by measuring the electrical conductivity of water (Lewis and Perkin, 1978). The salinity scale (Bradshaw and Schleicher, 1980; Culkin and Smith, 1980; Dauphinee *et al.*, 1980; Lewis, 1980) is used for measuring seawater salinity. The average salinity of seawater is about 35 g/kg or 35 parts per thousand (ppt, ‰).

Freshwater algae often fail to grow in saline waters (Luangpipat, 2013). For marine algae, salinity is an important parameter that influences the growth and composition of the cell (Gomez *et al.*, 2003). The optimum salinity range is different for different algae (Brand, 1984), but many marine strains appear to tolerate a wide range (Borowitzka and Borowitzka, 1990; Tredici and Materassi, 1992; Becker, 1994; Fabregas *et al.*, 2000; García-González *et al.*, 2003) without necessarily growing. Increase in salinity beyond the optimal level induces osmotic stress, ionic stress and may modify the ionic ratio of

the cell by affecting cellular water activity (Kirst, 1989). Ion uptake and loss and membrane permeability for some ions may be affected by salinity (Kirst, 1989).

2.3.2.5 Mixing

Another important operational factor for attaining high productivity in all types of algal production systems is mixing (Thomas *et al.*, 1995; Borowitzka, 1996). The mixing requirement depends on the algal strain and the scale of culture operation. Mixing could be induced mechanically or by bubbling an air mixture in the culture broth. Mixing prevents cells from settling into relatively dark zones of a culture system (Barbosa, 2003) and increases light utilization efficiency. Mixing prevents thermal stratification and enhances the nutrients distribution. Mixing facilitates absorption of carbon dioxide in the culture medium and the removal of dissolved oxygen generated by photosynthesis (Terry and Raymond, 1985; Mata *et al.*, 2010). Mixing can also induce damage to algal cells (Thomas and Gibson, 1990; Eriksen, 2008) and to prevent a decline in the biomass yield, the limits to acceptable mixing need to be investigated (Barbosa, 2003).

2.3.2.6 Biotic factors (contamination)

Algal production in open cultivation system is often adversely impacted by unwanted algae, bacteria, yeasts, fungi (Becker, 1994; Borowitzka, 1998; Sheehan *et al.*, 1998), algae grazers (zooplankton) (Richmond, 1990) and sometimes viral infections (Wommack and Colwell, 2000). An algal broth with a low concentration of cells is more prone to contamination (Chisti, 2012). Closed photobioreactors are also not totally free from contamination from accumulation of debris and waste products on the inner surfaces (Richmond, 2004b). Control of contamination is not easy once it has prevailed. To minimize the risk of predators and other unwanted microorganisms getting in the culture, pretreatment of water by sterilization or filtration is recommended (Chisti,

2012). There are other strategies as well that could be applied to minimize contamination; for example the use of extremophilic algae and those capable of growing rapidly (Rodolfi *et al.*, 2009).

2.4 Lipids in microalgae

By definition, lipids are substances that are insoluble in water and soluble in nonpolar solvents such as petroleum ether, chloroform and hexane (Becker, 1994). Lipids are essential components of cells and functionally important as a part of cell membranes. Lipids also constitute energy storage compounds and are involved in the synthesis of some secondary metabolites (Becker, 1994; Qin, 2010).

Generally, lipids are classified based on their chemical characteristics as either neutral or polar (Christie, 2003). Neutral lipids are nonpolar and mainly storage lipids such as mono-, di- and triglyceride oils, waxes, (Greenwell *et al.*, 2010), prenyl derivatives (e.g. carotenoids, tocopherols, quinones, terpenes), pyrrole derivatives (e.g. chlorophyll) as well as hydrocarbons (Hu *et al.*, 2008; Sharma *et al.*, 2012). Polar lipids are usually structural lipids such as phospholipids, glycolipids, sphingolipids, and sterols that have ionizable or otherwise water soluble functional groups in the molecule (Becker, 1994).

Lipid synthesis in microalgae is similar to that of higher plants; however, eukaryotic algae synthesize a unique variety of fatty acids which are not produced elsewhere (Harwood and Guschina, 2009). Fatty acids are the basic components of the triglyceride lipids and some other lipids. Some marine algae produce very long chain polyunsaturated fatty acid (PUFA) (Becker, 1994) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). DHA and EPA are used as a nutraceuticals (Riekhof *et al.*, 2005; Sharma *et al.*, 2012). The composition of fatty acids may vary in different classes of lipids. Saturated fatty acids and monounsaturated fatty acids may occur in

storage lipid while polyunsaturated fatty acids (PUFA) typically occur in structural lipids (Hu *et al.*, 2008).

Under optimal growth conditions, algal cells predominately synthesize polar lipids. These may constitute approximately 5-20% of the dry cell mass (Aakanksha *et al.*, 2010). These polar lipids (i.e. membrane lipids such as phospholipids, glycolipids and sterols) have structural and maintenance roles; provide platforms for various metabolic processes; and are involved in bioactive signaling (e.g. sphingolipids) for the regulation of cell growth and differentiation (Bartke and Hannun, 2009; Sharma *et al.*, 2012). The polar lipids are located in the plasma membrane, thylakoid membrane and endoplasmic reticulum (Hu *et al.*, 2008). These membrane lipids tend to be rich in polyunsaturated fatty acids (PUFA). The main components of the storage lipids (i.e. non-polar lipids) are triglycerides (TAGs), which are stored in the cell cytosol in the form of lipid bodies. TAGs synthesis occurs when free fatty acids are transferred from the chloroplast to the endoplasmic reticulum and from there they are released into cytosol in the form of oil droplets (Scott *et al.*, 2010). However, in some green algae, the biosynthesis and accumulation of TAGs may takes place in inter-thylakoid space of the chloroplast (Ben-Amotz *et al.*, 1989). TAGs synthesis generally occurs during light period and its degradation happens in the dark, to provide energy for cell division (Thompson Jr, 1996; Greenwell *et al.*, 2010). TAGs accumulation commonly increases in the stationary phase of growth (Siron *et al.*, 1989; Sicko-Goad and Andresen, 1991; Lombardi and Wangersky, 1995; Alonso *et al.*, 1998; Alonso *et al.*, 2000) when cell division stops and the energy supply in the form of fixed carbon exceeds the metabolic requirements of the cells (Greenwell *et al.*, 2010). TAGs are then accumulated as an energy reserve. TAGs contain saturated, monounsaturated and polyunsaturated fatty acids (Alonso *et al.*, 1998; Bigogno *et al.*, 2002; Aakanksha *et al.*, 2010). Stress responses may lead to dismantling of membranes to storage lipids (Makewicz *et al.*,

1997; Bigogno *et al.*, 2002; Khozin-Goldberg and Cohen, 2006). A brief overview of the metabolic pathways that lead to lipid biosynthesis is shown in Figure 2.5.

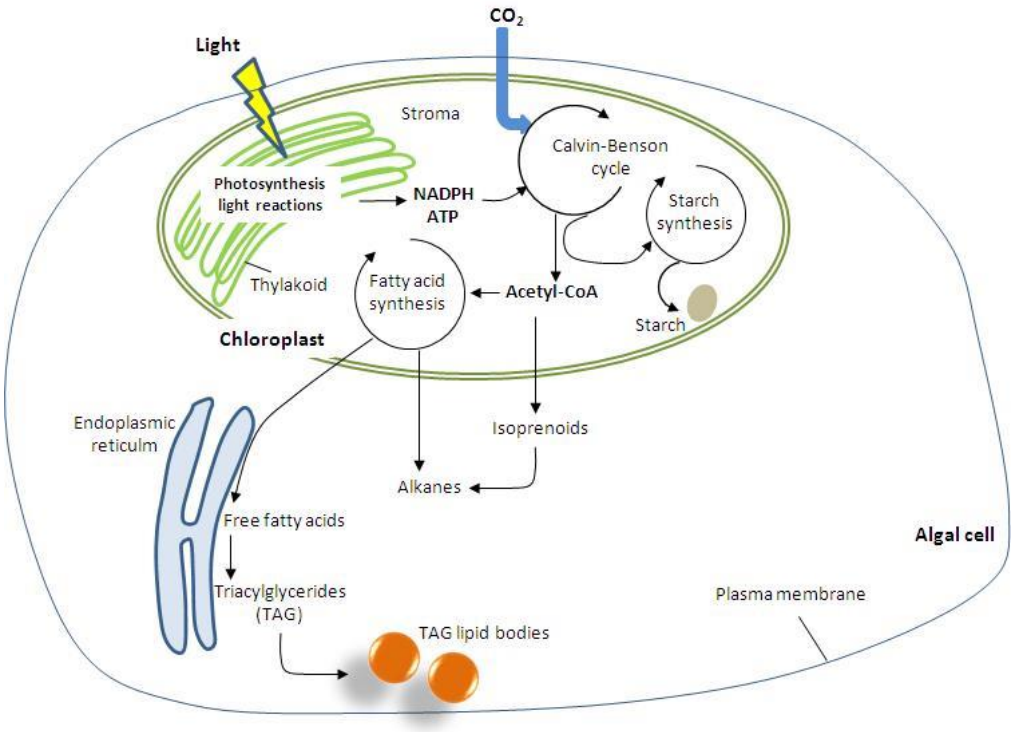


Figure 2.5 Microalgal metabolic pathways contributing to production of lipids. Based on Radakovits *et al*. (2010).

Proportion and composition of the different classes of lipids varies with the stage of growth, the nutrient stress, the diurnal cycle (Shifrin and Chisholm, 1981; Sukenik and Carmeli, 1990; Ekman *et al.*, 2007), cultivation conditions, and the harvesting time (Greenwell *et al.*, 2010). Only a few microalgae have been investigated extensively for lipid production (Harwood and Guschina, 2009).

Knowing the composition of algal lipids is important for assessing a strain's suitability for producing various biofuel (Greenwell *et al.*, 2010). The main focus of microalgal biofuel research has been the production of biodiesel (Chisti, 2013) and,

therefore, the tendency has been to try to maximize the production of TAGs and fatty acids (Courchesne *et al.*, 2009; Khozin-Goldberg and Cohen, 2011; Yu *et al.*, 2011a; Blatti *et al.*, 2012; Sharma *et al.*, 2012). However, the many other types of oils produced by algae can be converted to liquid fuels such as gasoline, diesel and kerosene (Chisti, 2012).

2.4.1 Lipid accumulation

A given alga exhibits different lipid metabolism under different growth conditions. For example, the level of nutrients in the growth medium, the irradiance, the temperature, CO_2 supplementation and salinity influence lipid accumulation and the types of lipids accumulated (Shifrin and Chisholm, 1981; Roessler, 1990). These culture conditions determine the quality and amount of lipid produced (Hu *et al.*, 2008; Rodolfi *et al.*, 2009; Pruvost *et al.*, 2011). Different algal species may respond differently to stressors (Shifrin and Chisholm, 1981). From the perspective of biofuels, modifications in the culture conditions have been used to enhance the lipid contents of the biomass (Illman *et al.*, 2000; Liu *et al.*, 2008; Mazzuca Sobczuk and Chisti, 2010). Lipid content and composition can also vary significantly in different phases of growth (Hu *et al.*, 2008); under favorable growth conditions, more polar lipids may be synthesized (Cagliari *et al.*, 2011) and less TAGs (Hu, 2004). Under stationary phase of growth or adverse growth conditions, production of neutral lipids in the form of TAGs is enhanced (Berge *et al.*, 1995; Tonon *et al.*, 2002; Hu *et al.*, 2008).

The various factors that trigger lipid accumulation and affect its composition have been reviewed for certain algae (Hu *et al.*, 2008; Leonardi *et al.*, 2011; Sharma *et al.*, 2012). Some of these factors are discussed briefly in the following sections.

2.4.1.1 Effect of nutrient starvation

Nutrient starvation can trigger lipid accumulation. Several nutrients including nitrogen, phosphorus, silicon and sulfur are known for inducing lipid accumulation (Sharma *et al.*, 2012). Nitrogen deprivation has often been found to greatly increase the lipid content of many microalgae (Hsieh and Wu, 2009; Yeh and Chang, 2011; Praveenkumar *et al.*, 2012). In addition to the concentration of nitrogen, the source of nitrogen also impacts the accumulation of lipids (Hsieh and Wu, 2009; Yeh and Chang, 2011). The first reported study on lipid accumulation under variable nitrate concentration was by Spoehr and Milner (1949) for *Chlorella pyrenoidosa*. An increase in lipid content of up to 85% was recorded. Extensive work has been reported on the effect of nitrogen deficient conditions on lipid accumulation in green microalgae (Largeau *et al.*, 1981; Piorreck *et al.*, 1984; Sawayama *et al.*, 1992; Illman *et al.*, 2000; Scragg *et al.*, 2002; Khozin-Goldberg and Cohen, 2006; Griffiths and Harrison, 2009; Hsieh and Wu, 2009; Widjaja *et al.*, 2009; Gardner *et al.*, 2009; Chen *et al.*, 2011; Praveenkumar *et al.*, 2012) . Diverse responses are sometimes seen within different species of a given genus. For example, most *Chlorella* species accumulate neutral lipids under N starvation, but some species accumulate starch (Hu, 2004). In *Tetraselmis suecica* and some *Dunaliella* species, no change in lipids levels has been seen under N starvation (Borowitzka, 1988). *Nannochloropsis* species have been extensively studied for lipid production (Boussiba *et al.*, 1987; Suen *et al.*, 1987; Sukenik *et al.*, 1989; Chini Zittelli *et al.*, 1999; Hu and Gao, 2006). Except for *Nannochloropsis salina* (Boussiba *et al.*, 1987), members of this genus have often been reported to accumulate lipids under a combination of nitrate depletion condition and high irradiance.

Diatoms (golden or brown algae) usually show multiple responses with regards to nitrate deprivation (Shifrin and Chisholm, 1981; Benemann and Oswald, 1996). Some

diatoms show an increased lipid content, specifically the TAGs, under N deprivation. Examples of such diatoms are *Cyclotella cryptica*, *Nitzschia palea*, *Navicula pelliculosa* (Shifrin and Chisholm, 1981), *Phaeodactylum tricornutum* (Parrish and Wangersky, 1987), and *Chaetoceros muelleri* (McGinnis *et al.*, 1997). Other diatoms may increase the lipid level only a little on N starvation. This happens in *Skeletonema costatum*, for example. Yet other diatoms, e.g. *Synedra ulna* and *Biddulphia aurita*, may show no increase on N starvation while in species such as *Thalassiosira weissflogii* nitrate starvation may actually decrease the lipid content (Shifrin and Chisholm, 1981). The silicate starvation has resulted in lipid content increase in diatoms (Shifrin and Chisholm, 1981). This happens, for example, in the diatoms *Cyclotella cryptica* (Werner, 1966; Shifrin and Chisholm, 1981; Roessler, 1990; Griffiths and Harrison, 2009), *Amphiprora hyalina, Chaetoceros muelleri, Nitzschia dissipata* and several species of *Navicula* (Griffiths and Harrison, 2009). In the case of *Cyclotella cryptica*, different silicon stress levels enhanced lipid accumulation significantly. Production of TAGs as well as saturated and monounsaturated fatty acids was also enhanced (Roessler, 1990).

A deficiency of other nutrients may also trigger lipid accumulation. For example, deficiency of phosphorus and sulfur may do this in some but not all algae. Phosphate deficiency induced lipid accumulation in *Scenedesmus obliquus* (Mandal and Mallick, 2009) and fatty acid production in *Dunaliella tertiolecta* (Siron et al., 1989), but decreased the lipid level in *Nannochloris atomus* and *Tetraselmis* sp, (Reitan *et al.*, 1994). Deficiency of sulfur caused an increase in lipid content of *Chlorella* sp. and *Chlamydomonas reinhardtii* (Matthew *et al.*, 2009). A deficiency or excess of micronutrients also influence lipid accumulation. For example, Fe^{3+} supplementation in certain stages of growth has increased the lipids content of *Chlorella vulgaris* (Liu *et al.*, 2008).

In diatoms, phosphorus limitation increased the lipid content in *Phaeodactylum tricornutum* (Siron *et al.*, 1989; Valenzuela *et al.*, 2013), *Chateoceros* sp. (Reitan *et al.*, 1994) and *Chaetoceros gracilis* (Lombardi and Wangersky, 1995).

2.4.1.2 Light stress

The level of light (both the extent of the photoperiod and the intensity of light) (Brenckmann *et al.*, 1985) also influence lipid production. A high irradiance induces neutral lipid production (Spoehr and Milner, 1949; Orcutt and Patterson, 1974; Sukenik *et al.*, 1989; Roessler, 1990; Napolitano, 1994; Brown *et al.*, 1996) as they tend to protect the cell from photooxidative stress. The latter is usually associated with the production of secondary carotenoids (Rabbani *et al.*, 1998; Zhekisheva *et al.*, 2002). A low irradiance level increases the synthesis of membrane lipids such as glycolipids and phospholipids (Hu *et al.*, 2008). In sunlight driven production processes the light level is not readily manipulated, unfortunately.

2.4.1.3 Temperature stress

Temperature is known to influence the lipid content and composition (Roessler, 1990; Hu, 2004; Guschina and Harwood, 2006; Hu *et al.*, 2008), but no generalized patterns have been identified with respect to the effects of temperature (Hu *et al.*, 2008). This is because of a relative lack of studies in this area possibly because temperature is not easily controlled in many outdoor large-scale commercial culture operations. Some algae have been reported to increase lipid content with increasing temperature. This occurs in the range of 15-30 °C in *Ochromonas danica* (Aaronson, 1973); in the range of 17-35 °C in *Nannochloropsis salina* (Boussiba *et al.*, 1987); in the range of 20-25 °C in *Nannochloropsis oculata*; and in the range of 25-30 °C in *Chlorella vulgaris*

(Converti *et al.*, 2009). Lipid content of *Chlorella sorokiniana* has not shown any obvious effect under variable temperatures of 14 °C, 22 °C and 38 °C.

Interactive effects of temperature and light intensity have been reported (Sorokin and Krauss, 1962; Collins and Boylen, 1982).

2.4.1.4 Salinity stress

The effect of salinity on lipid accumulation has been reported for some green algae (Sonnekus, 2010). In some cases, salinity changes had no significant effect on lipid accumulation but affected the lipid composition (Vazquez-Duhalt and Arredondo-Vega, 1990; 1991). *Dunaliella tertiolecta* has been found to increase its lipid content with increasing salinity (Takagi *et al.*, 2006). Lipid contents of *Isochrysis* sp. and *Nannochloropsis oculata* (Prymnesiophyceae and Eustigmatophyceae, respectively) were also elevated by increasing salinity (Renaud and Parry, 1994). Lipid contents of some diatoms declined at salinity of more than 35 ppt (Renaud and Parry, 1994; Sonnekus, 2010).

As outlined above, many factors can be used to influence lipid accumulation in microalgae. Some of these factors (e.g. temperature) are not easily manipulated in large scale outdoor operations, or are expensive to manipulate. Factors such as N starvation are broadly applicable, easy to control and may actually reduce the cost of producing the algal oils. What specific factors are used to influence oil productivity would depend very much on the specific alga (Pruvost *et al.*, 2011).

2.5 Strain selection

Over the years, thousands of strains of different algae classes have been examined for their lipid contents. Of these hundreds have been characterized as being oleaginous

strains (Hu *et al.*, 2008). An oleaginous microorganism is one that is able to produce and store a significant amount of TAGs under various stress conditions (Hu *et al.*, 2008; Damiani *et al.*, 2010; Gardner *et al.*, 2010).

Most of the oil-rich strains have belonged to the algal class Chlorophyceae (green algae) and Bacillariophyceae (diatoms) (Sheehan *et al.*, 1998), but some belong to other algal classes (Hu *et al.*, 2006). Typically, the oil-rich strains of different algal classes show an average lipid content of ~27% of biomass dry weight under optimal growth conditions and ~44% of dry weight, under unfavorable growth conditions (Hu *et al.*, 2008). However, the lipid content alone is an unsatisfactory criterion for selecting a strain for oil production (Griffiths and Harrison, 2009) as the oil content does not consider the time needed to achieve it. A better criterion is the lipid productivity (Griffiths and Harrison, 2009; Rodolfi *et al.*, 2009; Mata *et al.*, 2010; Pruvost *et al.*, 2011). Other considerations include the ease of growth, harvest and lipid extraction (Greenwell *et al.*, 2010). Once a strain is selected, the production process needs to be optimized for maximal oil productivity. Productivity is the quantity (mass) of oil produced per unit culture volume per unit time.

2.5.1 *Chlorella vulgaris*

Chlorella species have been generally the most favored for large scale production because they typically grow rapidly, are easy to cultivate (Lv *et al.*, 2010) and resistant to contamination (Huntley and Redalje, 2007). Among the various strains of *Chlorella* sp., the freshwater *Chlorella vulgaris* has been investigated most commonly (Görs *et al.*, 2010) but mainly for dietary purposes. This strain was first isolated and cultivated by Beijerinck (1890).

C. vulgaris has been cultivated on a mass scale since 1950 (Görs *et al.*, 2010) and is produced by more than 70 commercial companies (Spolaore *et al.*, 2006). However,

most of the industrial biomass production of *C. vulgaris* is carried out by mixotrophic cultivation (Iwamoto, 2004) for use in nutritional products. Due to difficulties in distinguishing different *Chlorella* species by morphology, most of the studies only state the genus. This poses problems as many metabolites are species specific (Görs *et al.*, 2010). Most of the investigations on *C. vulgaris* have been in freshwater media with a few exceptions reporting the use of marine media (Liu *et al.*, 2008; Lv *et al.*, 2010). Although, *C. vulgaris* is a robust alga, few studies have addressed the effect of pH on its growth for example (Azov, 1982; Goldman *et al.*, 1982; Powell *et al.*, 2009) and the effect of phosphorus level on growth (Grover, 1989).

Freshwater *C. vulgaris* has been suggested as the best algal strain for biodiesel production (Lee *et al.*, 2010; Yeh and Chang, 2010; Choi *et al.*, 2011; Yeh and Chang, 2012; Pignolet *et al.*, 2013) primarily because of its potential to accumulate a substantial amount of lipids under various conditions (Widjaja *et al.*, 2009; Amaro *et al.*, 2011). The other attractive characteristics of *C. vulgaris* for use on a large scale (Ugwu *et al.*, 2008) are its competitive nature under diverse growth conditions (Kessler, 1976; Maxwell *et al.*, 1994; Wilson and Huner, 2000) and tolerance to a pH range of between 5 and 10 (Widjaja *et al.*, 2009; Yeh and Chang, 2011).

Generally, the biomass of *C. vulgaris* comprises 50-58% protein, 12-17% carbohydrate and 14-22% lipids (Spolaore *et al.*, 2006). A similar lipid contents range of 14-25% has been reported for *C. vulgaris* in other studies under nutrient sufficient conditions (Illman *et al.*, 2000; Griffiths and Harrison, 2009; Rodolfi *et al.*, 2009). However, lipid levels of up to 58% of dry weight may be achieved (Mata *et al.*, 2010; Amaro *et al.*, 2011) by manipulating the culture conditions.

N starvation has been found to be particularly promising for enhancing lipid accumulation in *C. vulgaris*. The effect of N has been extensively studied in freshwater *C. vulgaris* (Illman *et al.*, 2000; Scragg *et al.*, 2002; Converti *et al.*, 2009; Liang *et al.*,

2009; Widjaja *et al.*, 2009; Lv *et al.*, 2010; Stephenson *et al.*, 2010a; Yeh and Chang, 2011; Mallick *et al.*, 2012). N starvation leads to a progressive increase in lipid content of the biomass but reduces growth. Some of the above studies also identified the effects of other conditions on lipid accumulation. Lipid content may be influenced by the concentration of CO_2 (Widjaja *et al.*, 2009; Lv *et al.*, 2010), the levels of irradiance (Lv *et al.*, 2010; Yeh and Chang, 2010), the temperature (Converti *et al.*, 2009) and supplementing with iron (Liu *et al.*, 2008). Recently a detailed study was carried out on biomass and lipid productivity of *C. vulgaris* in seawater media in photobioreactors (Luangpipat, 2013). The published studies for lipid accumulation by *C. vulgaris* under various growth conditions are summarized in Table 2.1. With a few exceptions, nearly all reported studies focused on freshwater media. The only extensive study in full strength seawater (Luangpipat, 2013) did not consider the open raceway pond culture systems as would be necessary for any large-scale outdoor culture.

Some studies in *C. vulgaris* have optimized some of the cultivation conditions for lipid production in a laboratory scale (~4 L working volume) photobioreactor (Chen *et al.*, 2010; Lv *et al.*, 2010) in freshwater. No work at all has been reported on *C. vulgaris* culture in open raceway types of systems in full strength marine media.

Table 2.1 Summary of *C. vulgaris* studies on lipid production

Salinity	Production system	Cultivation method	Nutrient medium	Growth variables	Lipid extraction method	Findings	References
Freshwater	8 L tank	Autotrophic	NA[1]	-different sources of N (NH_4Cl and KNO_3) -different concentration of N	Bligh and Dyer (1959)	-highest lipid content of 62.9% attained with 10 mg of KNO_3 but decreased lipid productivity of 1.2 mg L^{-1} d^{-1}	(Piorreck *et al.*, 1984)
Freshwater	200 mL flask	Autotrophic	WC medium[2]	-N starvation	Soxhlet extraction[3]	-highest lipid content was 40.6%	(Shifrin and Chisholm, 1981)
Freshwater	2-L bioreactor	Autotrophic	Watanabe	-low N, 5% CO_2	Bligh and Dyer (1959)	-lipid content increased from 18 to 40% by N-starvation -highest lipid productivity was 29 mg L^{-1} d^{-1} -calorific value increased from 18 to 23 kJ g^{-1} by N-starvation	(Illman *et al.*, 2000)
Freshwater	230-L bioreactor	Autotrophic	Watanabe	-low N	Bligh and Dyer (1959)	-lipid content increased from 28 to 58% by N-starvation -calorific value increased from 21 to 28 kJ g^{-1} by N-starvation	(Scragg *et al.*, 2002)
Seawater (natural)	250-500 mL flask	Autotrophic	F/2-Si	-iron supplementation at late exponential phase	Bligh and Dyer (1959)	-lipid content increased from 7.8 to 56.6% by low iron concentration	(Liu *et al.*, 2008)
Freshwater	5-L fermenter	Autotrophic	Modified Fitzgerald	-N depleting media -different CO_2 concentrations	Bligh and Dyer (1959)	-highest lipid content was ~42% and 13 folds high TAG content under N depletion condition at CO_2 concentration of 0.33%	(Widjaja *et al.*, 2009)
Freshwater	2-L Erlenmeyer flask	Autotrophic	Bold's Basal	-low N -temperature increase from 25-30°C	Ultrasound and Folch[4] (1957) method	-lipid content increased from 5.9 to 16.4% under N limitation -lipid content decreased from 14.7 to 5.9% with increasing temperature	(Converti *et al.*, 2009)
Freshwater	250 mL flask	Autotrophic	BG11	-N replete condition	Charring (1966) method[5]	-highest lipid content of 18% and lipid productivity of 36.9 mg L^{-1} d^{-1}	(Rodolfi *et al.*, 2009)

Table 2.1 Summary of *C. vulgaris* studies on lipid production (Cont.)

Salinity	Production system	Cultivation method	Nutrient medium	Growth variables	Lipid extraction method	Findings	References
Freshwater	1-L bottle	Autotrophic, heterotrophic, mixotrophic	Bristol medium+proteose peptone	-different cultivation methods with different C sources -N starvation	Bligh and Dyer (1959)	-highest lipid content of 38%, attained in autotrophic, N starvation condition -highest lipid productivity of 54 mg L^{-1} d^{-1} attained with mixotrophic condition	(Liang *et al.*, 2009)
Freshwater	1-L photobioreactor	Autotrophic	NA[1]	-different levels of N were tested -$NaHCO_3$ used as a source of C, various concentrations were tested	Bligh and Dyer (1959)	-highest lipid productivity of 114.3 mg L^{-1} d^{-1} (attained at KNO_3 concentration of 650 mg L^{-1} and $NaHCO_3$ of 1500 mg L^{-1})	(Chen *et al.*, 2010)
Seawater (~ 34 ppt)	5-L photobioreactor	Autotrophic	NA[1]	-different levels of N, CO_2 and irradiance were tested	FT-IR spectroscopy[6] (2009)	-highest lipid content and lipid productivity was 20% and 40 mg L^{-1} d^{-1}, respectively, attained at 1.0 mM of KNO_3, 1.0% CO_2 and 60 $\mu mol \cdot m^{-2}s^{-1}$	(Lv *et al.*, 2010)
Freshwater	1-L photobioreactor	Autotrophic	Basal	-effect of C source, i.e. $NaHCO_3$ and its concentration -effect of light intensity and light source	Bligh and Dyer (1959)	-lipid content of 30-40% attained at 1000 mg L^{-1} of $NaHCO_3$ of and 41 $\mu mol \cdot m^{-2}s^{-1}$ (fluorescent light)	(Yeh and Chang, 2010)
Freshwater	9-L jar	Autotrophic	BG11	-N replete condition	Bligh and Dyer (1959)	- highest lipid content of 7.8 and lipid productivity of 11 mg L^{-1} d^{-1} by N sufficient condition	(Lee *et al.*, 2010)
Freshwater	130-L airlift photobioreactor	Autotrophic	Bold's Basal	-N depletion conditions	Folch[4] (1957) method	-lipid content was 20-23% i.e. unchanged under N starvation, but increase in TAG content from 3 to 14%	(Pruvost *et al.*, 2011)

Table 2.1 Summary of *C. vulgaris* studies on lipid production (Cont.)

Salinity	Production system	Cultivation method	Nutrient medium	Growth variables	Lipid extraction method	Findings	References
Freshwater	1-L photobioreactor	Autotrophic	Basal	-2-stage method to induce lipid accumulation -low initial N in one-stage cultivation	Direct transesterification[7] (1984)	-two-stage approach did not increase lipid content -one-stage approach maximum lipid content of 55.9% and lipid productivity of 132.4 mg L^{-1} d^{-1} in low N concentration of 313 mg L^{-1}	(Yeh and Chang, 2011)
Freshwater	2-L glass cylinder photobioreactor	Autotrophic	BG11	-variable light intensity	Gravimetric method using chloroform/methanol (2:1, v/v)	-highest lipid productivity within a range of 80.5 to 88.5 mg L^{-1} d^{-1} by light intensity of 200 μmol·$m^{-2}s^{-1}$	(Hempel *et al.*, 2012)
Freshwater	1-L bubble column photobioreactor	Autotrophic	BG11	-2-stage method -effect of various N and P concentrations, irradiance and aeration rate was tested in stage-2	Bligh and Dyer (1959)	-highest lipid productivity of ~77 mg L^{-1} d^{-1} attained with N and P limitation[8], light intensity of 100 μmol·$m^{-2}s^{-1}$ and 2% of CO_2	(Mujtaba *et al.*, 2012)
Freshwater	150 mL Erlenmeyer flask	Autotrophic, Mixotrophic	N11, N11+glucose	-limitation of N, P and Fe	Bligh and Dyer (1959)	-highest lipid content of 55% and lipid productivity of with 171.4 mg L^{-1} d^{-1} with N11 medium -highest lipid content of 48.5% and lipid productivity of 1973.9 mg L^{-1} d^{-1} with N11+glucose medium	(Mallick *et al.*, 2012)
Freshwater	1-L photobioreactor	Autotrophic, heterotrophic, mixotrophic, photoheterotrophic	Bold Basal, modified Bristol's, MBL medium	-different nutrient media -different cultivation methods	Direct transesterification[7] (1984)	-highest lipid content of 53% in MBL medium - highest lipid productivity in phototrophic growth was ~56 mg L^{-1} d^{-1} (Basal medium) -highest lipid productivity in mixotrophic growth was 143.9 mg L^{-1} d^{-1} (Bristol's medium)	(Yeh and Chang, 2012)

Table 2.1 Summary of *C. vulgaris* studies on lipid production (Cont.)

Salinity	Production system	Cultivation method	Nutrient medium	Growth variables	Lipid extraction method	Findings	References
Seawater	1-L Duran bottle, 4-8 L stirred tank photobioreactor, tubular photobioreactor	Autotrophic	BG11 medium (seawater)	-nutrient sufficient medium, 25 to 27 °C - N depletion condition and temperature of 10 to 20°C	Bligh and Dyer (1959)	-highest lipid content of 33% and lipid productivity of >37 mg L^{-1} d^{-1} by N sufficient condition -highest lipid content of 59% and lipid productivity of >42 mg L^{-1} d^{-1} by N starvation and low to normal temperature condition	(Luangpipat, 2013)

[1]Medium composition is provided in the article, but the name is not mentioned; [2](Guillard, 1975); [3](Soxhlet, 1879) ; [4](Folch *et al.*, 1957); [5](Marsh and Weinstein, 1966); [6](Pistorius *et al.*, 2009);

[7](Lepage and Roy, 1984); [8]2% and 10% of initial N and P levels, respectively, in BG11 media.

2.6 Microalgal production systems

Mass production of microalgae is carried out in two types of systems (Figure 2.6) (Becker, 1994; Tredici, 2004; Borowitzka and Moheimani, 2012): either open ponds (i.e. natural ponds and lagoons, inclined circular ponds, oblong raceway ponds), or closed photobioreactors of different designs.

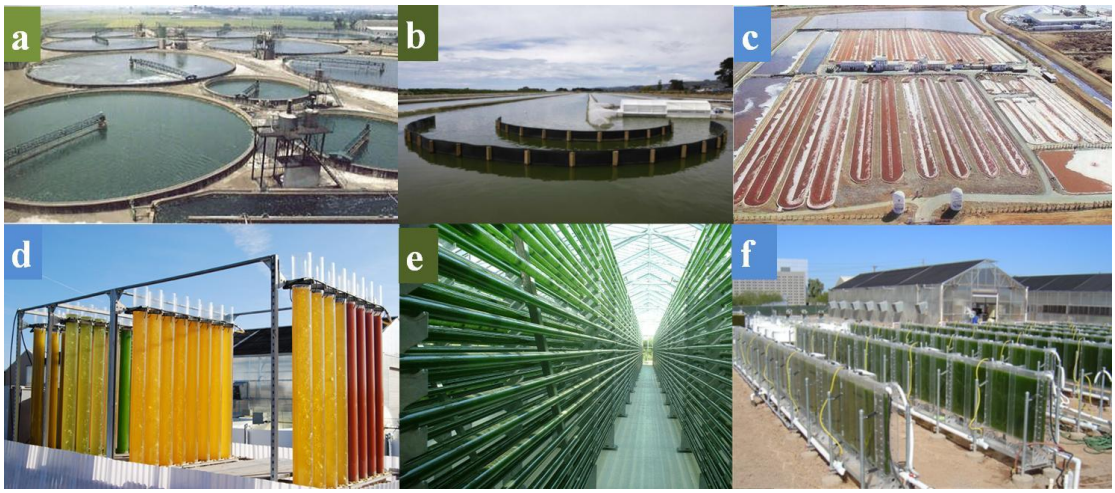


Figure 2.6 Some designs of open and closed microalgal production systems: a) Centre-pivot ponds used for the production of *Chlorella*, Taiwan (www.pureplanet.de); b) Algae wastewater treatment raceway pond at the NIWA's research site, Christchurch, New Zealand (National Institute of Water and Atmospheric Research Ltd); c) *Dunaliella bardawil* cultivation in raceway ponds (Nature Beta Technologies Ltd, Eilat, Israel, subsidiary of Nikken Sohonsha Co. Gifu, Japan); d) Bubble column photobioreactors growing algal strains for different pigments at Arizona State University's Polytechnic campus in Mesa, Arizona, USA; e) Tubular photobioreactor used for biomass production in Klötze, Saxony Anhalt, Germany (© Bioprodukte Prof. Steinberg GmbH); f) Plate-type photobioreactors, NanoVoltaicsk Inc. (www.nanovoltaics.com), Arizona, USA.

Both types of systems can be considered as reactors in view of their utility for the cultivation of microalgae (Tredici, 1999). The performance of closed reactors, i.e. photobioreactors (PBR), is far better than that of open reactors (open ponds). This is because closed systems allow a good control over growth parameters, have a higher surface-to-volume (S/V) ratio for light capture and reduce contamination (Becker, 1994; Tredici, 2004; Chisti, 2007). The scale of operation of photobioreactors is, however, smaller compared to that of conventional raceway systems and photobioreactors tend to be expensive (Lehr and Posten, 2009; Tredici *et al.*, 2010). The other major challenges associated with photobioreactors are: an accumulation of oxygen that inhibits photosynthesis at a high concentration; management of an even liquid flow in multiple long tubes; and hydrodynamic stress that can lead to cell damage (Gudin and Chaumont, 1991; Williams and Laurens, 2009). The design and characteristics of photobioreactors have been extensively reviewed in the literature (Lee, 1986; Prokop and Erickson, 1995; Torzillo, 1997; Tredici and Chini Zittelli, 1997; Pulz and Scheibenbogen, 1998; Tredici, 1999; Carvalho *et al.*, 2006; Eriksen, 2008; Lehr and Posten, 2009; Tredici *et al.*, 2010).

2.6.1 Raceway and its operation

A raceway is a shallow open pond that is shaped like a closed loop race track (Figure 2.7). Raceways are most commonly used for commercial microalgal biomass production (Borowitzka, 1999a; Olaizola, 2000). Depending on the type of ground, a pond is constructed either by digging into the ground or erecting walls of concrete blocks or bricks. The ponds are generally lined with a plastic liner (Tredici, 2004; Chisti, 2007). The principles of construction and design of paddlewheel raceway ponds have been extensively reviewed (Dodd, 1986; Oswald, 1988; Borowitzka, 2005; Chisti, 2012). Large scale raceway ponds were first introduced in the 1950s as High Rate Algal

Ponds (HRAP) for wastewater treatment (Oswald *et al.*, 1957). They were later used in commercial production of algal biomass mainly for food and nutraceuticals applications. Algae and cyanobacteria such as *Chorella* sp., *Spirulina platensis*, *Haematococcus* sp. and *Dunaliella salina* (Jiménez *et al.*, 2003a; Borowitzka, 2005) have been successfully cultivated in open ponds. The raceway ponds were first used in attempts to produce algal biofuels under the Aquatic Species Program (Sheehan *et al.*, 1998) in the United States. This extensive demonstration project started in 1976 and continued for several years. These studies were focused on optimization of operational conditions and examination of their effect on biomass and lipid productivities. As an outcome of this program, the inexpensive raceway technology was concluded to be potentially feasible for production of oil for fuels.

Figure 2.7 Commercial algal biomass production in raceways: a) and b) Cultivation of *Spirulina* (a blue-green cyanobacterium) and *Haematococcus pluvialis* (an orange pigment producing green alga) by Cyanotech Corporation, Kona, Hawaii (www.cyanotech.com); c) *Dunaliella bardawil* cultivation in raceway ponds (Nature Beta Technologies Ltd, Eilat, Israel, subsidiary of Nikken Sohonsha Co. Gifu, Japan).

A raceway pond is typically filled with algal culture to a depth of 0.2–0.3 m (James and Boriah, 2010; Chisti, 2012). The culture is circulated in the pond typically by means of a paddlewheel (Figure 2.7). Raceways driven by paddlewheels are one of the most commonly used systems for commercial production of algal biomass (Dodd, 1986; Richmond, 1992; Tredici, 2004). The agitation by paddlewheel is supposed to enhance light utilization for algal cell (Vonshak and Guy, 1992) and keep the cells suspended; however, intensive mixing increases the cost of energy consumption (Béchet *et al.*, 2010; Mata *et al.*, 2010; Stephenson *et al.*, 2010b). In practice, the velocity of flow in the channel is a little higher than the minimum needed to keep the cells suspended. The recommended velocities are 0.15 m s^{-1} (Park and Craggs, 2010), 0.18 m s^{-1} (Hase *et al.*, 2000) and 0.3 m s^{-1} (Lin and Lin, 2011).

Various other types of mixing systems have been used in raceways. These include airlift pumps, propellers, Archimedes screws and water jets (Borowitzka and Moheimani, 2012). Paddlewheels have proven most successful and are widely used because of their low cost and a relatively low energy consumption (Borowitzka and Moheimani, 2012).

Propellers require the same amount of energy as required by paddlewheels, but propellers have not been tested in large ponds (Borowitzka and Moheimani, 2012). The Archimedes screws are reported to have a higher energy consumption compared to paddlewheels (Laws and Berning, 1991). Water jets have also been used in small ponds and found to use more energy than paddlewheels (Becker, 1994). Nevertheless, currently the jet-type of circulation mechanism is being used for large-scale raceways by Aurora Algae (Australia). This patented (Paresh *et al.*, 2010) system is claimed to minimize energy inputs.

The airlift-based mixing systems have been investigated (Persoone *et al.*, 1980; Märkl and Mather, 1985), but require almost twice as much energy as the paddlewheels.

An airlift raceway (23 L) configured by Ketheeshan and Nirmalakhandan (2011, 2012) was assessed for algal cultivation. The biomass productivity of this system was close to the biomass productivity of photobioreactors and the energy efficiency was higher.

To improve the biomass production, CO_2 or air/CO_2 mixture is generally supplied to the culture broth (James and Boriah, 2010) by bubbling at the bottom of the raceway (Ketheesan and Nirmalakhandan, 2012). A drawback of open raceways is that some of the CO_2 supplied is released into the atmosphere (Weissman *et al.*, 1989; Carvalho *et al.*, 2006). This can be a significant economic loss. Different strategies have been proposed to overcome the technical challenges relating to CO_2 supply in a raceway. For example, the use of a carbonation column (Putt *et al.*, 2011) and supplementation with dissolved carbonates (Campbell *et al.*, 2011). The other major limitation of a raceway is water loss through evaporation (Carvalho *et al.*, 2006). This needs to be compensated for by adding freshwater. Although raceways are open to atmosphere, removal of oxygen produced by photosynthesis can be difficult (Richmond, 1990; Moheimani and Borowitzka, 2007). Another problem is contamination through the open surface (Sheehan *et al.*, 1998; Borowitzka, 2005; Mata *et al.*, 2010).

Under conditions of nutrient sufficiency and otherwise optimal physical factors, availability of light is the only factor that governs the photosynthetic biomass productivity in a raceway (Richmond, 2004a). As the culture density increases, the light penetration is reduced by mutual shading by cells (Tamiya, 1957). In such a dense culture, two light zones develop: the photic zone (i.e. the illuminated surface of the broth, where cells can harvest light energy for photosynthesis) and the dark zone (i.e. the volume of the broth where light cannot reach therefore no photosynthesis can occur) (Richmond, 2004a). In addition to cell density, the culture depth also plays a vital role in determining the biomass productivity in a raceway (Richmond, 2004a). Once the

culture reaches a concentration of about 0.5 g L^{-1} light cannot penetrate to its full depth in a raceway and more than 80% of the culture becomes unproductive (Chisti, 2012). The irradiance-depth profile is typically as shown in Figure 2.8.

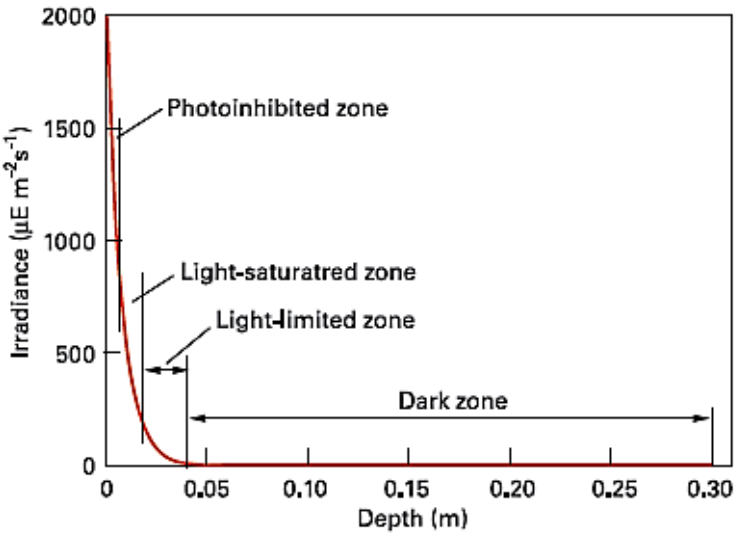


Figure 2.8 Light profile in a 0.3 m deep raceway at a dry biomass concentration of 0.5 g L^{-1}. The profile was calculated for a suspension of the marine diatom *Phaeodactylum tricornutum* at an incident irradiance level of 2,000 μmol·m^{-2} s^{-1} at the surface of the raceway. The zones of different metabolic activity are: the photoinhibited zone ($I_L \geq$ 800 μmol·m^{-2} s^{-1}); the light-saturated zone (170 $\leq I_L \leq$ 800 μmol·m^{-2} s^{-1}); the light-limited zone (4 $\leq I_L \leq$ 170 μmol·m^{-2} s^{-1}); and the dark zone ($I_L \leq$ 4 μmol·m^{-2} s^{-1}). I_L is the local irradiance at any depth L from the surface. Source: Chisti (2012).

Another factor that regulates the biomass productivity in a raceway is the rapid light-dark cycling as the cells at the illuminated surface periodically move deeper in the darker zones (Richmond, 2004a). Mixing affects this light-dark cycling. If light-dark cycling is sufficiently rapid, the photosynthetic apparatus can use the energy of the photon before the next photon arrives (Richmond, 2004a).

For a given culture volume, the productivity of a culture system generally increases with increasing surface area for light absorption (Pirt *et al.*, 1980; Richmond, 2004a), but large raceways (e.g. 0.5 ha in surface area) cannot be made shallower than about 0.2 m because of issues relating to evaporative losses and difficulties in ensuring flatness of construction so that the flow is not adversely influenced by differences in depth along the channel.

The algal production in a raceway may be by a batch operation, a continuous operation or semicontinuous operation. As summarized in Table 2.2, in a batch culture of raceway that lasts for a period of 4-6 weeks, the maximum biomass productivity is 0.05-0.1 g L^{-1} d^{-1} (Pulz, 2001). In a 13-month long semicontinuous culture, the averaged biomass productivity was 0.19 g L^{-1} d^{-1} (Moheimani and Borowitzka, 2006). In a continuous culture, a biomass productivity of 0.204 g L^{-1} d^{-1} (Boussiba *et al.*, 1987) and 0.19 g L^{-1} d^{-1} (Ketheesan and Nirmalakhandan, 2012) have been achieved. The biomass productivity in all these different modes of cultivation is generally similar. However, semicontinuous and continuous culture modes of operation are preferred for commercial algal production because culture operations can be run for long periods of time (Moheimani and Borowitzka, 2006). Based on available information (Table 2.2), the best areal productivities are about 20-22 g·m^{-2} d^{-1} with lipid contents of about 33% (Moheimani and Borowitzka, 2006). A productivity of 22 g·m^{-2} d^{-1} with a lipid contents of 20-40% has also been reported (Sheehan *et al.*, 1998). According to Richmond (2004a), in a well-managed raceway the algal productivity does not generally exceed 20–25 g·$m^{-2}$$d^{-1}$.

Table 2.2 Summary of raceway algal cultivation

Algal strain	Cultivation Mode	Culture volume (L)	Surface area (m^2)	Biomass concentration ($g\ L^{-1}$)	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Areal productivity ($g\ m^{-2}\ d^{-1}$)	Lipid content (%, w/w)	Location	References
Micractinium sp[1]	-	-	-	-	-	33	-	Israel	(Shelef, 1982)
Actinastrum sp[1]	-	-	-	-	-	35	-	Israel	(Shelef, 1982)
Phaeodactylum tricornutum	-	-	-	-	0.0028–0.13	2.4–11.3	-	Hawaii	(Laws *et al.*, 1983)
Nannochloropsis salina	Continuous	300	2.5	0.35-1.3	0.204	3.5-24	16.5-21	Israel	(Boussiba *et al.*, 1987)
Cyclotella cryptica	-	-	48	0.155	-	29.7	-	Hawaii	(Laws *et al.*, 1988)
Chlorella sp	-	-	100	-	-	20	-	USA	(Weissman *et al.*, 1988)
Porphyridium cruenetum	-	120	-	-	0.04-0.20	5.0-24	-	Israel	(Cohen *et al.*, 1988)
Mixed algal culture[1,2]			-	-	-	15	-	Kuwait	(Banat *et al.*, 1990)
Spirulina platensis	Semicontinuous	750	2.4	0.4-0.7	0.06–0.18	15-27	-	Israel	(Richmond *et al.*, 1990)
Tetraselmis suecica[3]	-	-	-	-	0.103	-	-	Hawaii	(Laws and Berning, 1991)
Spirulina platensis	Semicontinuous	-	2.5	-	-	11.5-20.8	-	Israel	(Vonshak and Guy, 1992)
Scenedesmus and *Coelastrum*[1]	-	-	45	-	-	20-40	-	Kuwait	(Al-Shayji *et al.*, 1994)
Tetraselmis sp	-	600	-	-	0.008-0.060	5-40	-	Japan	(Matsumoto *et al.*, 1995)

Table 2.2 Summary of raceway algal cultivation (Cont.)

Algal strain	Cultivation Mode	Culture volume (L)	Surface area (m^2)	Biomass concentration ($g\ L^{-1}$)	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Areal productivity ($g\ m^{-2}\ d^{-1}$)	Lipid content (%, w/w)	Location	References
Chlorella and *Ankistrodesmus* sp[1]	Continuous	-	-	-	-	18	-	Australia	(Cromar *et al.*, 1996)
Spirulina platensis	-	282	3.8	0.75	0.18 ± 0.02	14.47 ± 0.16	-	Italy	(Pushparaj *et al.*, 1997)
Spirulina platensis	-	-	-	-	-	8.2	-	USA	(Belay, 1997)
Platymonas sp	-	-	48	-	-	26	20-40[a]	Hawaii	(Sheehan *et al.*, 1998)
Tetraselmis suecica	2-day batch	-	-	-	-	37.5	-	Hawaii	(Sheehan *et al.*, 1998)
Chlorophyta sp	Semicontinuous	200	~0.1	0.3	0.050	8.2	-	Japan	(Hase *et al.*, 2000)
Chlorella sp	Semicontinuous	200	~0.1	0.5	0.081	13.2	-	Japan	(Hase *et al.*, 2000)
Unknown alga	Batch	500	75,000	0.3-0.5	0.05-0.1	-	-	Germany	(Pulz, 2001)
Anabaena sp	Semicontinuous	300	1.0	-	~0.031–0.078	9.4–23.5	-	Spain	(Moreno *et al.*, 2003)
Spirulina sp	Semicontinuous	135,000	450		0.006–0.07	2–15	-	Spain	(Jiménez *et al.*, 2003b)
Dunaliella salina	Semicontinuous	180-360	3	-	-	1.6–3.5	-	Spain	(García-González *et al.*, 2003)
Spirulina sp	Semicontinuous	-	6.03	-	-	9–13	-	Mexico	(Olguín *et al.*, 2003)
Pleurochrysis carterae	Semicontinuous	160-200	1	-	0.11–0.21	16–33.5	33	Australia	(Moheimani and Borowitzka, 2006)

Table 2.2 Summary of raceway algal cultivation (Cont.)

Algal strain	Cultivation Mode	Culture volume (L)	Surface area (m^2)	Biomass concentration ($g\ L^{-1}$)	Volumetric productivity ($g\ L^{-1}\ d^{-1}$)	Areal productivity ($g\ m^{-2}\ d^{-1}$)	Lipid content (%, w/w)	Location	References
Dunaliella salina	Semicontinuous	110	1	-	0.22–0.34	20–37	35	Australia	(Moheimani and Borowitzka, 2006)
Haematococcus pluvialis	Batch[6]	50,000	417	0.202	0.126	15.1	25	Hawaii	(Huntley and Redalje, 2007)
Muriellopsis sp	Semicontinuous	100	1.0	0.2-0.77	-	8-20	23.5±2.5	Italy	(Blanco *et al.*, 2007)
Cyclotella (wild type)	Semicontinuous	-	2.8	0.76-1.76	0.58-1.23	12	-	USA	(Huesemann *et al.*, 2009)
Cyclotella sp. CM1-1 (mutant)[4]	Semicontinuous	-	2.8	0.32-1.50	0.35-1.04	9	-	USA	(Huesemann *et al.*, 2009)
Mixed algal culture[1,5]	Continuous	8,000	31.8	0.27-0.31	-	9-24.7	-	NZ[b]	(Park and Craggs, 2010)
Scenedesmus sp	Continuous	23	-	-	0.16 ± 0.02	-	-	USA	(Ketheesan and Nirmalakhandan, 2011)
Scenedesmus rubescens	-	-	-	0.37 ± 0.03	0.023	4.6	-	USA	(Lin and Lin, 2011)
Botryococcus braunii Kutz.	Batch	1800	6	1.8 ± 0.13	0.114	-	19	India	(Ashokkumar and Rengasamy, 2012)
Scenedesmus sp	Continuous	23	-	1.39 ± 0.03	0.19 ± 0.003	-	20-22	USA	(Ketheesan and Nirmalakhandan, 2012)

Adapted from: (Moheimani and Borowitzka, 2006; Ketheesan and Nirmalakhandan, 2011; Park *et al.*, 2011; Borowitzka and Moheimani, 2012); all blank fields show a lack of data.

[1]High rate algal ponds (HRAP); [2]the dominant species were *Euglena, Chlamydomonas, Scenedesmus* and *Coelastrum*; [3]Archimedes screws used for circulating water in raceway pond; [4]small antenna size; [5]the dominant species were *Scenedesmus* sp, *Micractinium* sp, *Pediastrum* sp and *Ankistrodesmus* sp; [7]coupled system of continuous culture photobioreactors and batch culture operation. [a]Increased lipid content during N and P limitation, [b]Christchurch, New Zealand.

Irrespective of the reasons for a low biomass production, raceways remain preferred for large scale algal cultivation because they are relatively cheap to build and operate (Sheehan *et al.*, 1998; Hase *et al.*, 2000; James and Boriah, 2010; Mata *et al.*, 2010). Raceways seem to be the only sustainable system for algal biofuel production (Borowitzka and Moheimani, 2013). So far only a few algae/cyanobacteria are grown commercially on a large scale in raceways. These include the fast growing alga *Chlorella* and extremophilic organisms such as *Spirulina* sp (*Arthrospira*), *Dunaliella salina* and *Haematococcus pluvialis* (Sheehan *et al.*, 1998; Pulz and Gross, 2004). Most of the strains being evaluated for biofuel production have not been optimized for production in raceways (Rodolfi *et al.*, 2009). A promising strain needs to be monitored for biomass production, lipid production (for example, neutral lipids), the harvesting time, and optimized at normal growth conditions as well as unfavorable conditions for lipid accumulation. Competitiveness of selected strain over the other potential algal contaminants needs to be tested (James and Boriah, 2010). Raceway ponds may be effectively utilized in conjunction with photobioreactors in a hybrid system (Rodolfi *et al.*, 2009). A two-stage algal cultivation has been demonstrated (Pushparaj *et al.*, 1997; Huntley and Redalje, 2007). (A two-stage culture involves growing the inoculum in a closed photobioreactor which is then used to inoculate the open raceway.) This may minimize the possibility of culture crash and contamination.

2.2 Contributions of this study

Considering a global shortage of freshwater, any production of algal biofuels must make use of only the algae capable of reliably growing in full-strength seawater. This research focused on raceway-based production of algal biomass and lipids using algae capable of growing in seawater. The impact of culture conditions on production of biomass and lipids was studied under nutrient replete and deficient conditions. The characteristics of

the biomass such as the total lipid content, the energy content and the proportion of different elements in it were quantified under specified growth conditions. The research was driven by the following key hypotheses:

1. Stable long-term culture of seawater microalgae in open ponds as generally used for freshwater algae was hypothesized to be feasible given a judicious selection of the algal species; and
2. As demonstrated for certain freshwater algae, suitable seawater grown algae were hypothesized to able to produce heightened levels of lipids through deprivation of certain nutrients that were essential for biomass growth.

As discussed in the next chapter, the normally freshwater alga *Chlorella vulgaris* was identified as the focus of the study because it could grow robustly in full-strength seawater and had not been previously evaluated for lipid production in seawater media in raceways as revealed by the summary in Table 2.1. The specific objectives of the study were:

1. Identification of a suitable alga capable of growing easily and stably in full-strength seawater media in raceway pond type of culture systems;
2. Characterization of the selected alga in terms of growth and lipid production in batch and continuous culture under different light conditions to quantify production capabilities and stability in nutrient replete media;
3. Assessment of the selected alga in terms of its lipid production response to deprivation of nutrients necessary for growth;
4. A baseline characterization of the energy content of the algal biomass produced under various culture conditions; and
5. An assessment of the types of oils that may be produced by the chosen alga.

Chapter 3

MATERIALS AND METHODS

3.1 Introduction

As an overall plan, the study consisted of the following main steps:

1. A screening of several microalgae for ability to grow in seawater media and rapidly attain a high final biomass concentration (e.g. 2 g L^{-1}) in batch cultures while showing a relatively high (e.g. ≥20% by dry weight) lipid content in the dry biomass. This reduced the focus on 2–3 algae for further screening.
2. Further screening of algae from the previous step to see that they were capable of surviving salinity levels exceeding that of the normal seawater as such elevated salinity values would occur in any large-scale open outdoor culture system through evaporative loss of water. This narrowed the choice of the algae to two species for further assessment.
3. Further screening of the algae from the previous step to assess their ability to substantially enhance the cellular lipid content in response to nitrogen starvation post growth. This reduced the choice to one algal species for further indepth assessment.
4. A detailed characterization nutrient sufficient growth of the selected alga in batch raceway cultures with different illumination regimes, to establish growth potential, the baseline lipid production and other growth kinetic parameters.
5. An evaluation of the effect of post growth nutrient starvation on lipid production in raceway batch culture and an identification of the minimum level of nitrogen supply to allow the alga to attain the maximum possible biomass concentration for the available light prior to entering a nitrogen starvation phase.

6. A characterization study of continuous raceway culture of the selected algae at different dilution rates to quantify biomass production capability and the culture stability.

Eight species of microalgae were preliminary screened for biomass and lipid productivity. Based on preliminary screening, one algal species was selected for a detailed evaluation. Experiments were carried out in Duran bottles and a purpose-built raceway operated under various conditions. Both batch and continuous culture operations were evaluated in the raceway. The progress of the cultures was followed in terms of the biomass produced, the nitrate and phosphate consumed, and the total oil produced in the biomass. The total energy content of the selected biomass samples were characterized in terms of the calorific values. The objective was to identify how the culture operation might be used to improve the total oil production in the selected algae. The relevant experimental methods are explained in the following sections.

3.2 Microalgal strains, sources, maintenance and cultivation

For preliminary assessments, eight species of microalgae were used (Table 3.1).

All these microalgae were maintained by aseptic sub-culturing on agar plates and slants at 25±2 °C under continuous irradiance of 109 to 116 $\mu mol \cdot m^{-2} s^{-1}$ (at the surface of agar plates/slants). Illumination was provided by daylight fluorescent lights (Philips TLD 18W/840, cool white, Thailand). Agar media (Section 3.2.1.1) had the same composition as the BG11 media (Section 3.2.1; Table 3.2), but contained the vitamins specified in Table 3.3 and agar (1% Difco^{TM}, Agar Noble, Becton, Dickinson and Company, USA). Algal colonies started developing within 1-2 weeks after inoculation.

Afterwards the agar plate cultures were stored at 4 °C under an irradiance of 3 to 8 $\mu mol \cdot m^{-2} s^{-1}$. The agar plates were refreshed every two months.

Table 3.1 Microalgal strains

Alga	**Normal habitat**	**Class**
Chlorella vulgaris	Originally freshwater	Chlorophyceae
Nannochloropsis salina (CCAP 849/3)	Marine	Eustigmatophyceae
Tetraselmis subcordiformis (CCAP 161/1A) synonym *Platymonas subcordiformis*	Marine	Chlorophyceae
Cylindrotheca fusiformis (CCAP 1017/2)	Marine	Bacillariophyceae (diatom)
Cylindrotheca closterium (CCAP 1017/9) synonym *Nitzschia closterium*	Marine	Bacillariophyceae (diatom)
Ditylum brightwellii (CCAP 1022/1) synonym *Triceratium west*	Marine	Bacillariophyceae (diatom)
Hymenomonas elongata (CCAP 961/3)	Marine	Prymnesiophyceae
Chroomonas salina fo. oculus-bovis (CCAP 978/21A)	Marine	Cryptophyceae

C. vulgaris was purchased from Landcare Research, Lincoln, New Zealand. All the other algae were purchased from the *Culture Collection of Algae and Protozoa* (CCAP), Argyll, United Kingdom.

3.2.1 Growth media

For maintenance and growth of all microalgal species, BG11 (Stanier *et al.*, 1971) medium made with artificial seawater was used (Section 3.2.1.1). The components of artificial seawater are given in Section 3.2.1.4.

The BG11 is normally made in freshwater. It was originally developed for freshwater cyanobacteria, but has been widely used to grow other freshwater algae. This medium formulated in artificial seawater was used for the marine algae in this work. For growing the marine diatoms, the medium was additionally supplemented as described in Section 3.2.1.3.

3.2.1.1 Preparation of BG 11 medium

Stock solutions 1–4 were made (Table 3.2) separately, sterilized (121 °C, 15 min), cooled, and stored at 4 °C until further use. The storage period did not exceed two months. For preparation of BG11 working medium, the Stock solution 1 (10 mL), Stock solution 2 (10 mL), Stock solution 3 (10 mL), and Stock solution 4 (1 mL) were mixed; 1.5 g of sodium nitrate (LabServ Biolab Australia Ltd.) and 0.02 g of sodium carbonate (LabServ Biolab Australia Ltd.) were added and the volume was made up to 1 L with artificial seawater (Section 3.2.1.4). The final concentration of $NaNO_3$ and Na_2CO_3 in the culture medium was 1.76×10^{-2} M and 1.89×10^{-4} M, respectively.

Table 3.2 Components of BG11 medium

Component	**Source**	**Quantity in stock solution ($g \cdot L^{-1}$ deionized H_2O)**	**Concentration in medium (M)**
Stock 1			
$CaCl_2 \cdot 2H_2O$	LabServ Biolab Australia Ltd.	3.6	2.44×10^{-4}
Citric acid monohydrate ($C_6H_8O_7 \cdot H_2O$)	BDH VWR International Ltd, Poole, England	0.6	3.12×10^{-5}
Ferric III ammonium citrate ($C_6H_{11}FeNO_7 \cdot H_2O$)	BDH Chemicals Limited, Poole, England	0.6	2.12×10^{-5}
Na_2EDTA	BDH AnalaR VWR International Ltd, Poole, England	0.084	2.25×10^{-6}
Stock 2			
$MgSO_4 \cdot 7H_2O$	LabServ Biolab Australia Ltd.	7.5	3.04×10^{-4}
Stock 3			
K_2HPO_4	LabServ Biolab Australia Ltd.	3.05	1.75×10^{-4}
Stock 4			
H_3BO_3	LabServ Biolab Australia Ltd.	2.86	4.62×10^{-5}
$MnCl_2 \cdot 4H_2O$	AnalaR NORMAPUR, VWR International, France	1.81	9.14×10^{-6}
$ZnSO_4 \cdot 7H_2O$	BDH VWR International Ltd, Poole, England	0.222	7.71×10^{-7}

Table 3.2 Components of BG11 medium (Cont.)

Component	**Source**	**Quantity in stock solution ($g \cdot L^{-1}$ deionized H_2O)**	**Concentration in medium (M)**
$CuSO_4 \cdot 5H_2O$	BDH Laboratory Supplies, Poole, England	0.079	3.16×10^{-7}
$CoCl_2 \cdot 6H_2O$	BDH Chemicals Ltd, Poole, England	0.050	2.10×10^{-7}
$Na_2MoO_4 \cdot 2H_2O$	BDH Chemicals Ltd, Poole, England	0.390	1.61×10^{-6}

The pH of the final medium was adjusted to 7.5 with 1 M HCl.

For preparing the solid medium 10 g of agar (Difco™, Agar Noble, Becton, Dickinson and Company, USA) per liter of BG11 seawater medium was heated to boiling to dissolve the agar. This medium was autoclaved (121 °C, 15 min) cooled to 40-50 °C and supplemented with filter-sterilized vitamins solution (Table 3.3). This medium was then aseptically poured (15 mL) into a petri-dish and allowed to solidify. The fresh plates were kept at 4 °C and used within a few days of preparation.

3.2.1.2 Vitamins solution

Vitamins (Table 3.3) were added to BG11 media used for maintaining cultures on agar plates and slants. Vitamins were added also to all shake flask stages of inoculum preparation. The vitamin solution was filter-sterilized by using a 0.20 μm membrane filter (28 mm diameter Minisart® NML, syringe driven filter unit; Sartorius Stedim

Biotech GmbH, Goettingen, Germany). The final concentration of the vitamins and the type of vitamins added were as used normally in the f/2 medium (Guillard and Ryther, 1962; Guillard, 1975).

Table 3.3 Vitamins added to media

Vitamin	**Source**	**Final concentration (M)**
Thiamine – HCl (Vitamin B_1)	Sigma-Aldrich, USA	2.97×10^{-7}
Biotin (Vitamin H)	Sigma-Aldrich, USA	2.25×10^{-9}
Cyanocobalamin (Vitamin B_{12})	Sigma-Aldrich, USA	3.70×10^{-10}

3.2.1.3 Silicate solution (for diatom culture only)

Silicate stock was prepared as for the f/2 medium (Guillard and Ryther, 1962; Guillard, 1975). Thus, 30 g of $Na_2SiO_3 \cdot 9H_2O$ (Fisher Scientific, UK) was dissolved in 1 L of deionized water. One milliliter of this solution was added to 1 L of the BG11 seawater medium. The final concentration of $Na_2SiO_3 \cdot 9H_2O$ in the culture medium was 1.05×10^{-4} M.

3.2.1.4 Artificial seawater

Seawater was prepared by dissolving 40 g of seasalt (Pacific natural seasalt; Dominion Salt, Marlborough, South Island, New Zealand) in 1 L of deionized water, unless specified otherwise. The salinity of resulting seawater was 37.0 to 38.9 ppt (EcoSense® EC300 conductivity/salinity meter; YSI Inc., Yellow Springs, OH, USA; calibrated with

a 1.0 molal aqueous solution of KCl (conductivity at 25 °C = 108,621 μS/cm; (Pratt *et al.*, 2001; Shreiner and Pratt, 2004)).

Seawater for the raceway pond (138 L) was prepared by dissolving the required amount of the above specified sea salt in 30–40 L of tap water. This solution was passed through two cartridge filters in series. The filters were nominally rated at 50 μm and 10 μm (Filterpure, 10 micron, E10PP10-FG and 50 micron E50PP10-FG, 10" standard, polyester pleated filter; USA). The solution was then filter sterilized (0.45 μm membrane filter; Millipak-100, catalog number MPHL10CA3; Millipore Corporation, Billerica, MA, USA) to remove any marine microorganisms that might be present in the natural sea salt mix. Non-sterile prefiltered (1 μm nominal; E1PS10-FG, 10" standard, polypropylene spun melt filter; USA) tap water was then added to make up the volume to the required concentration.

3.2.2 Duran bottle batch culture

Duran bottles (borosilicate glass 3.3, LabServ, Biolab, Auckland, New Zealand) were used in preparation of inocula and in most small scale experiments (Figure 3.1). Prior to use the culture bottles, tubings and fittings that directly contacted the culture were sterilized by autoclaving (121 °C, 15 min).

Typically, 2 L Duran bottles which contained sterilized (121 °C, 15 min) BG11 seawater medium were used. The working volume was ~1.8 L. The broth in the bottles was sparged (2–5 L/min at NTP) with filtered humidified air mixed with 5% (v/v) carbon dioxide. The exhaust gases were also filter sterilized (0.2 μm Teflon membrane filter; Midisart® 2000; Sartorius AG, Goettingen, Germany). The other culture conditions were: an ambient temperature of 25±2 °C and a continuous irradiance at the surface of bottles of between 105 and 220 $\mu mol \cdot m^{-2} s^{-1}$ (for irradiance used in specific

experiments see Chapter 4) provided by daylight fluorescent tubes (Philips TLD 18W/840, cool white, Thailand).

The inocula for the bottles were grown aseptically in shake flasks for 7–14 days, depending upon the species cultured. These shake flasks were seeded from agar plates and slants. Volume of the inoculum was between 10 and 28% (v/v) of the total inoculated culture volume. The bottles were generally sampled daily from the time of inoculation for monitoring of growth and nutrient consumption.

Figure 3.1 Sparged Duran bottle cultures.

3.2.2.1 Harvesting of biomass from Duran bottles

The culture broth from the Duran bottles was harvested usually after the stationary phase had been reached. Harvesting was achieved by centrifugation (Hitachi high-speed refrigerated centrifuge CR22GII; Hitachi Koki Co., Ltd., Tokyo, Japan) at 8,370 × g, 4 °C, for 10 min. The supernatant was discarded and the recovered biomass was re-suspended in 1 L of distilled water and centrifuged to wash away the salts. Washing of

the biomass was repeated four times. The final biomass collected by centrifugation was stored at −80°C (Thermoscientific Forma 900 series freezer, Model 995; Ohio, USA) overnight before being freeze dried. In the case of the marine microalgae (i.e. *N. salina*, *C. fusiformis*, *T. subcordiformis*) ammonium formate (0.5 M; Sigma-Aldrich Co., USA) was used for washing (Zhu and Lee, 1997) instead of distilled water.

3.2.3 Raceway pond culture system

A raceway pond (~138 L working volume, 0.23 m depth, 0.61 m^2 surface area) was built specifically for this project (Figures 3.2–3.5) by Massey University technicians. The approximate dimensions of the raceway and the paddlewheel for circulating the broth are shown in Figure 3.2. The stainless steel raceway was equipped with a central baffle, two stainless steel frits (200 μm; GKN Filters GmbH, Radevormwald, Germany) for sparging with carbon dioxide, a water cooled heat exchanger for temperature control, a drain, and an overflow harvest tubing (Figure 3.3). The paddlewheel was partly covered with a protective Plexiglas® shroud (Figure 3.4). The speed of rotation of the paddlewheel could be controlled (Section 3.2.3.1). The raceway was equipped with sensors for pH (Cole-Parmer EW-27301-21 in-line double junction pH electrode with probe guard and a temperature sensor (100 ohm platinum RTD) for automatic temperature compensation) and temperature (Omega Engineering Inc., Stamford, CT, USA; platinum RTD Sensor PR-19-2-100-1/4-9-E).

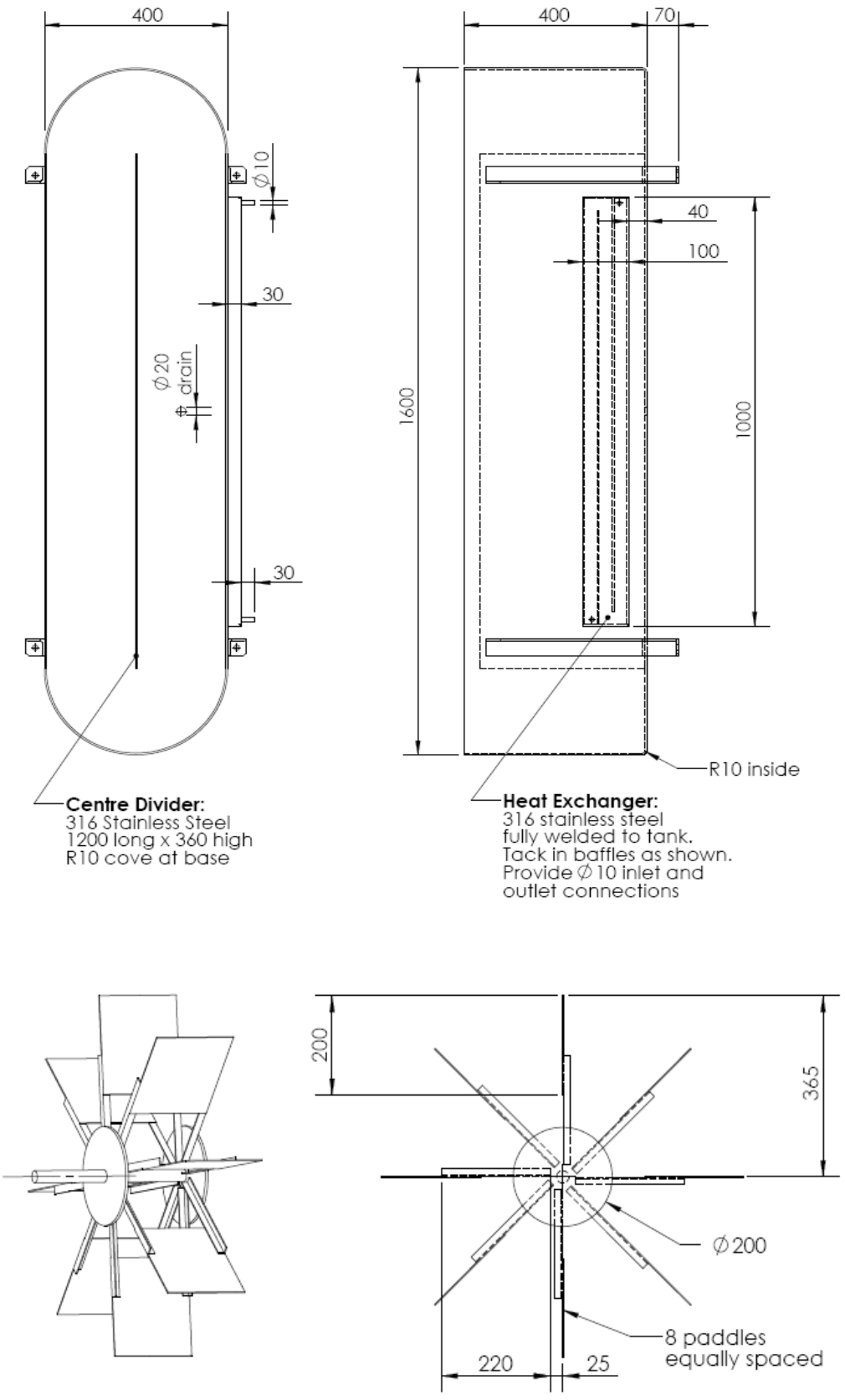


Figure 3.2 Raceway and paddlewheel. Dimensions in mm.

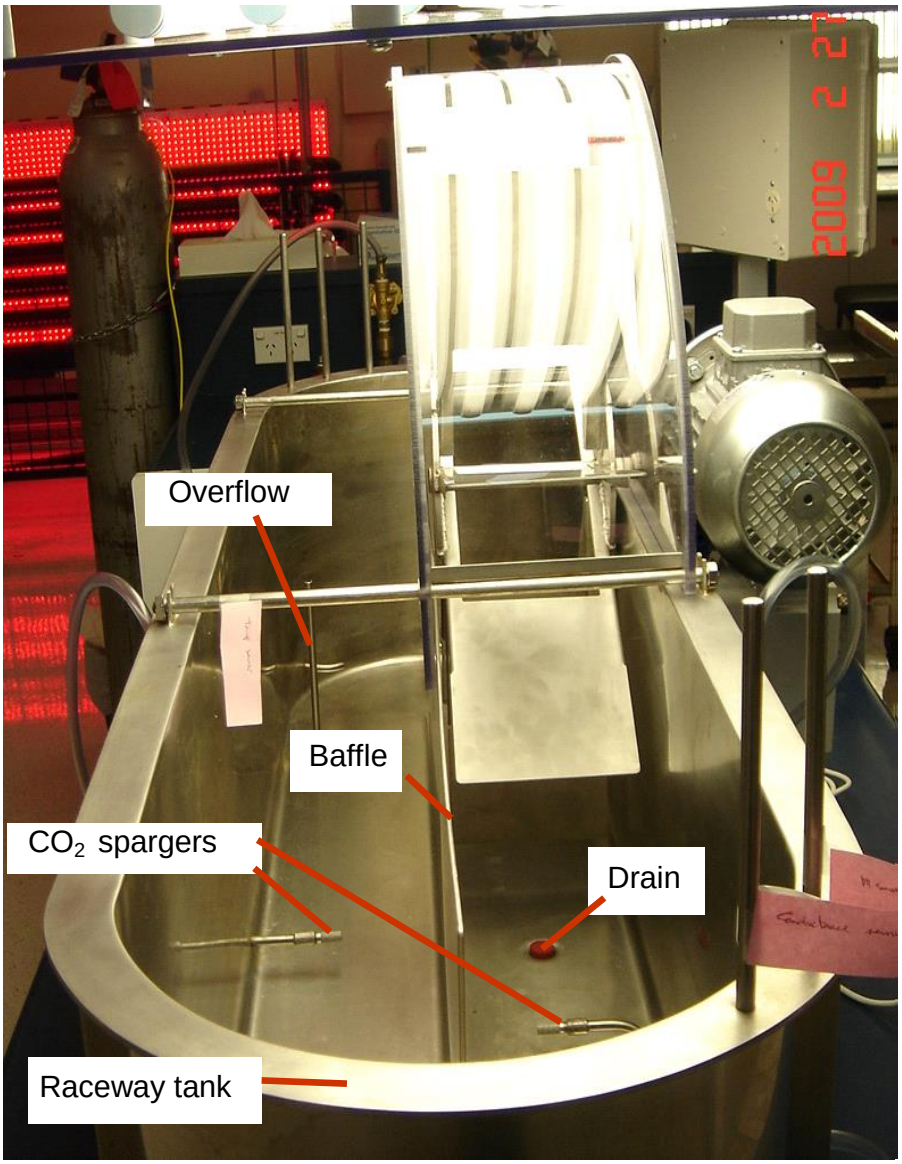


Figure 3.3 Raceway pond (empty).

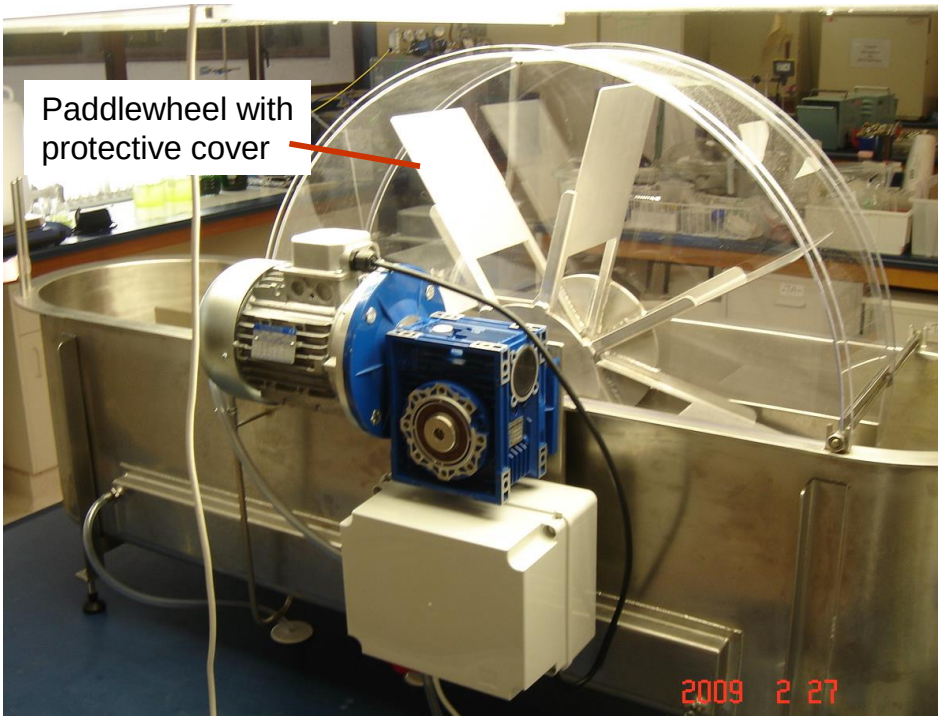


Figure 3.4 Raceway paddlewheel with transparent protective cover.

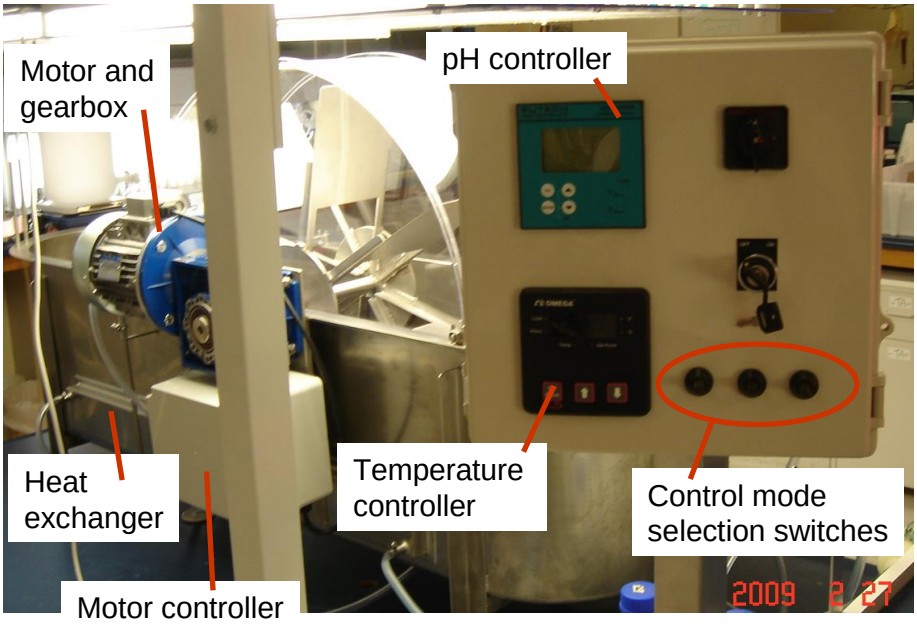


Figure 3.5 The raceway main control panel, the heat exchanger and the paddlewheel motor.

The temperature could be controlled automatically (Omega Engineering Inc., Stamford, CT, USA; 1/4 DIN Compact Temperature Controller model CN2110-R20) by on/off switching (solenoid valve) of the cooling water supply. The water flowed through the heat exchanger (Figure 3.4, Figure 3.5) at a preset value. The temperature measured by the aforementioned sensor provided the control data. The system provided only a cooling capability to remove the heat absorbed as a consequence of illumination. The raceway was located in an air-conditioned room.

The pH was controlled (Figure 3.5) by bubbling carbon dioxide in response to the output of the above mentioned pH sensor. Carbon dioxide was sparged at a preset flow rate (15 L/min) if the pH rose above a specified level. Sparging was discontinued once the set point pH was re-established. This ensured that carbon dioxide did not becoming a limiting factor to photosynthesis. An on/off controller was used (Eutech Instruments 1/4-DIN pH 800 on/off controller KH-56705-05; Cole-Parmer Corp., Vernon Hills, IL, USA) to manage the carbon dioxide flow. Prior to injection into the raceway the carbon dioxide was passed through a sterilizing filter (0.2 μm Acropak 800 PTFE membrane; Pall Corporation, Portsmouth, U.K.). The pH sensor was calibrated every few months in the usual way using standard buffers of pH 7.0 and 4.0.

Initially, raceway was illuminated by an array of daylight fluorescent lights (Sylvania, Premium*extra*, 58 W lamps, China) in which incident light level was controlled at two preset values with maximum output level of 91 $\mu mol \cdot m^{-2}s^{-1}$. Later on, illumination was switched to a custom built array (Agricultural Biological Engineering Ltd, Pukekohe, New Zealand) of 660 nm LEDs with a maximum power output of around 280 $\mu mol \cdot m^{-2}s^{-1}$at a distance of 0.3 m from the face of the array (Figure 3.6). The output of the LED array could be adjusted manually or automatically by an internal clock. The light output profiles at different peak settings of the LED array for a diurnal cycle are shown in Figure 3.7. The irradiance received at the surface of the raceway for

a given output of the LED array is shown in Figure 3.8. The irradiance at different depths of freshwater in the raceway at various output light levels on the surface is shown in Figure 3.9 for illumination with LED lights and fluorescent lights. The irradiance profile in a culture broth of *C. vulgaris* (0.5 g L^{-1} biomass concentration) illuminated using fluorescent light is shown in Figure 3.10. Ingress of light from other than the illumination source was prevented by hanging opaque plastic sheets around the raceway as in Figure 3.6.

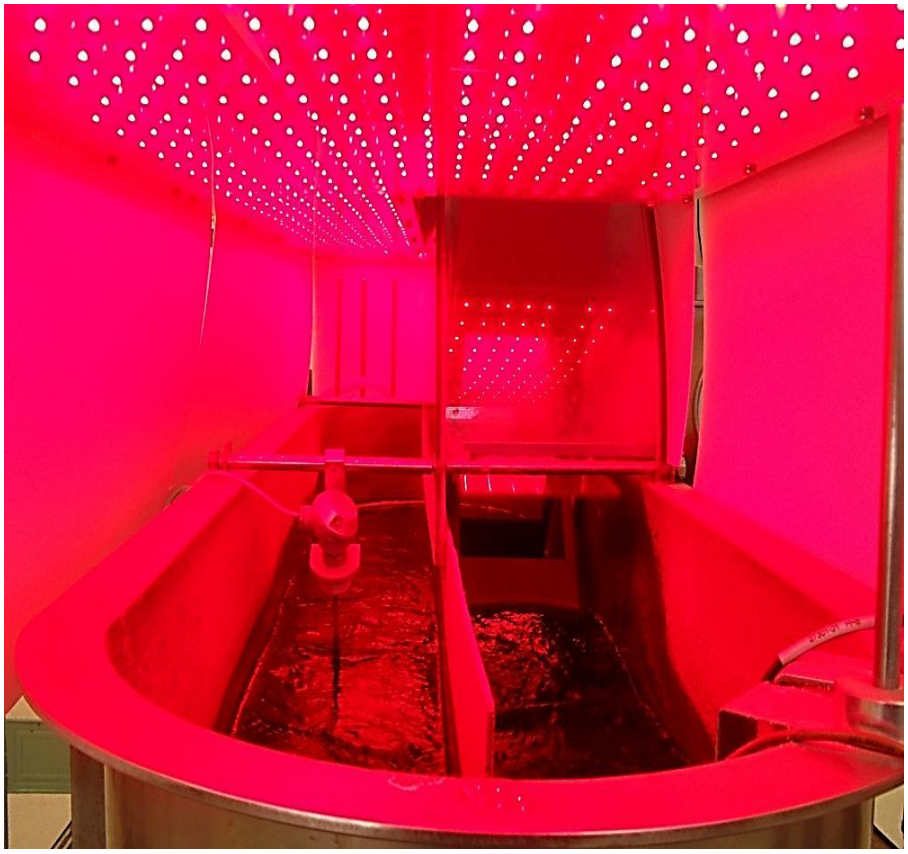


Figure 3.6 Raceway with LEDs in day mode of operation.

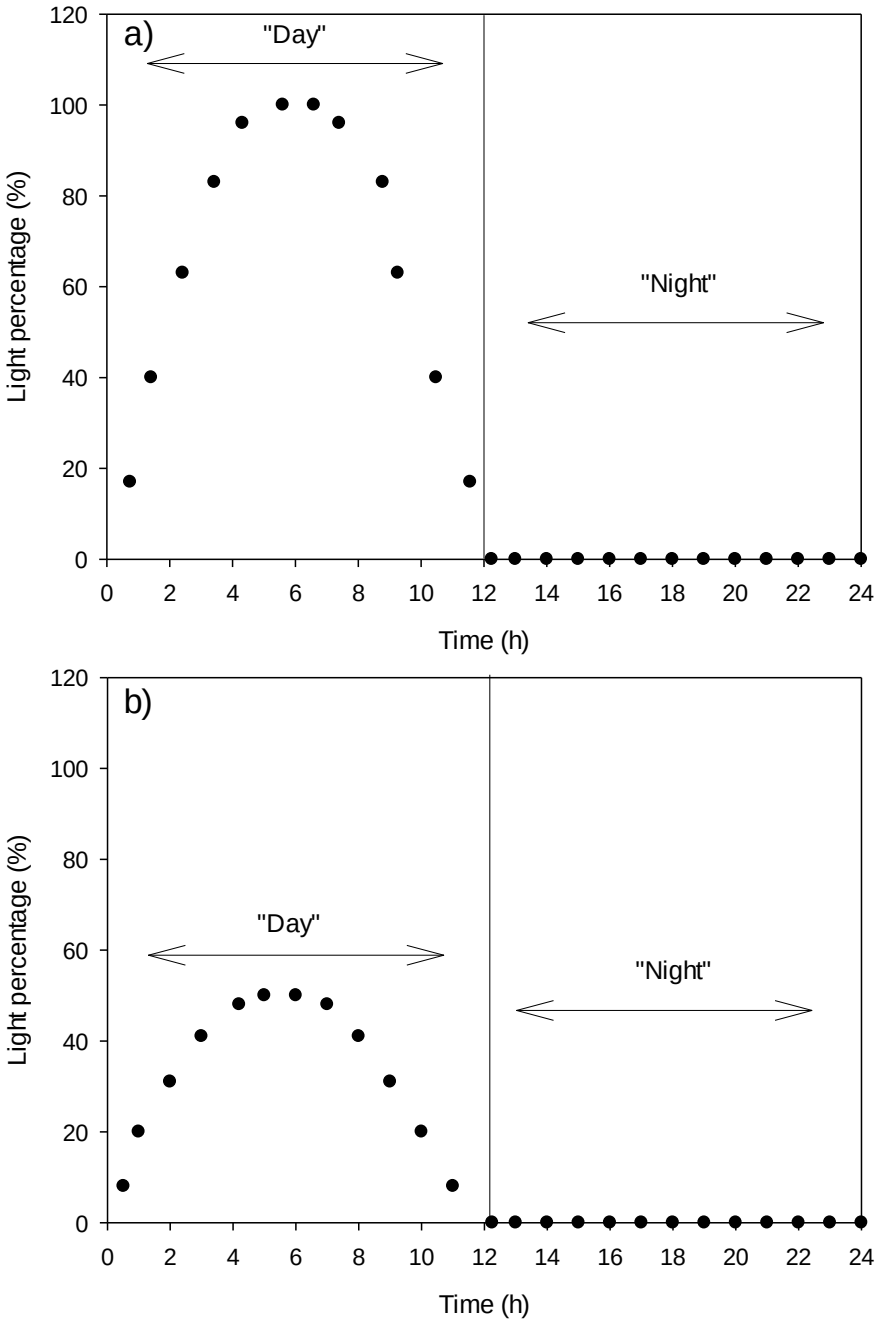


Figure 3.7 Diurnal light output profile of raceway LED array: a) at peak value of 100% light level, the day-night averaged irradiance was 280 μmol·m^{-2}s^{-1}; b) at the peak output set to 50% of full light level, the day-night averaged irradiance was 165 μmol·m^{-2}s^{-1}.

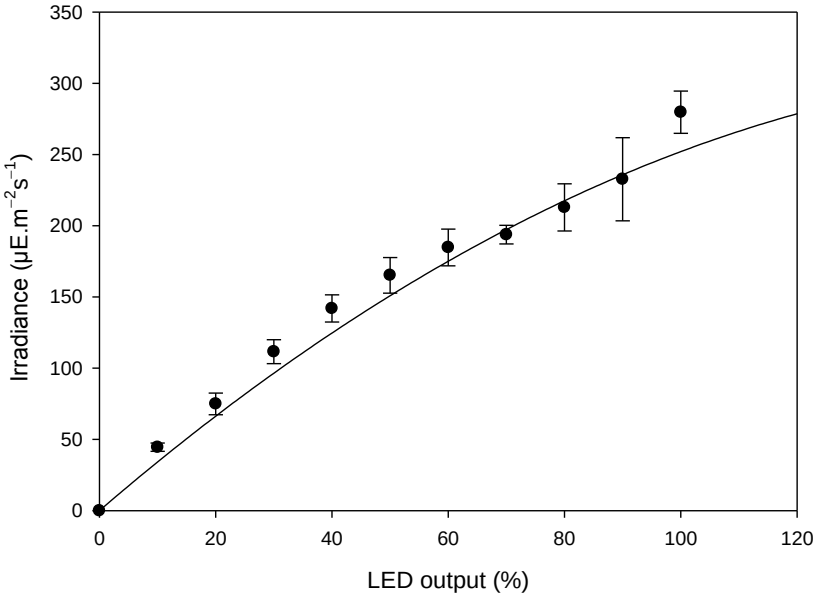


Figure 3.8 Irradiance on the surface of culture broth in the raceway at various output settings of the LED array. Standard deviation values are based on four replicates.

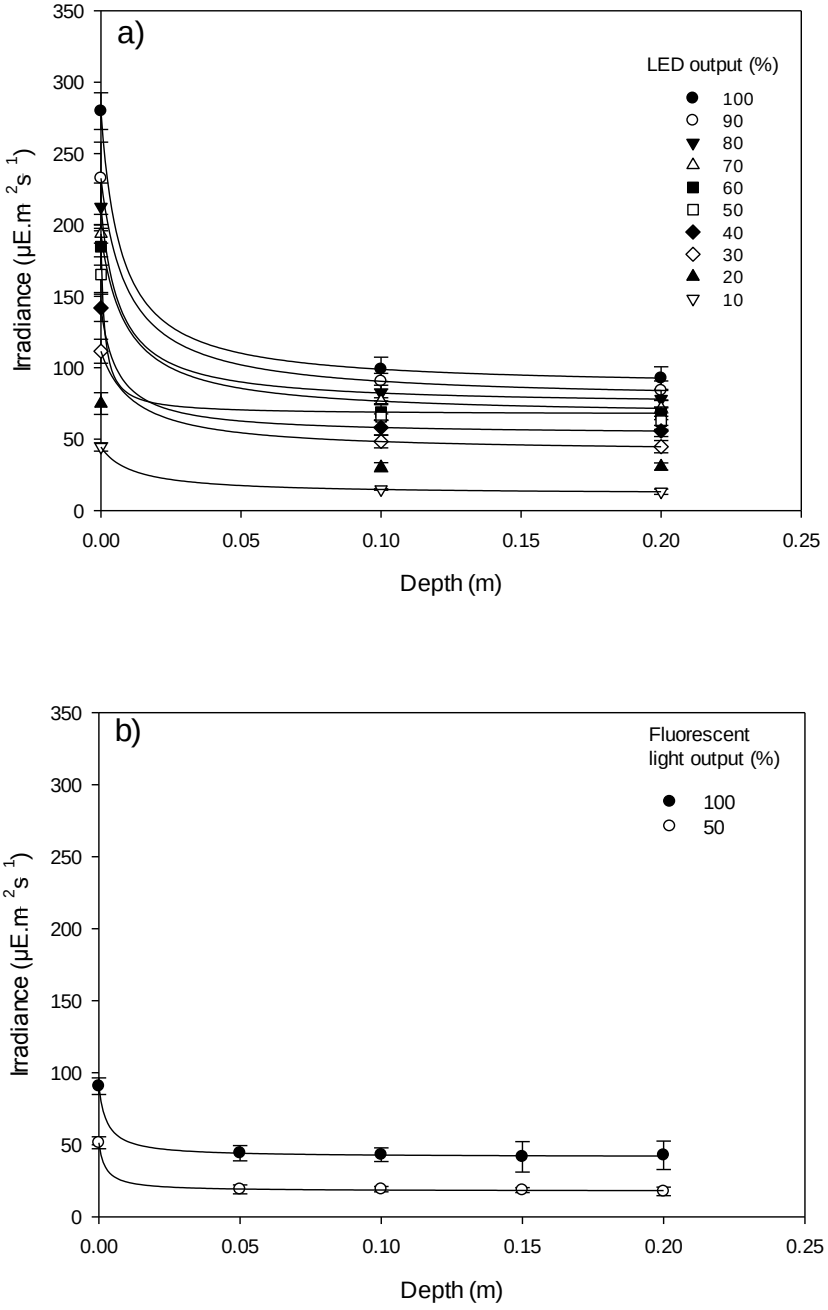


Figure 3.9 Variation of irradiance at different depths of freshwater in the raceway: a) at different LED light output levels; b) at different fluorescent light levels. The total depth was 0.23 m. Standard deviation values are based on four replicates.

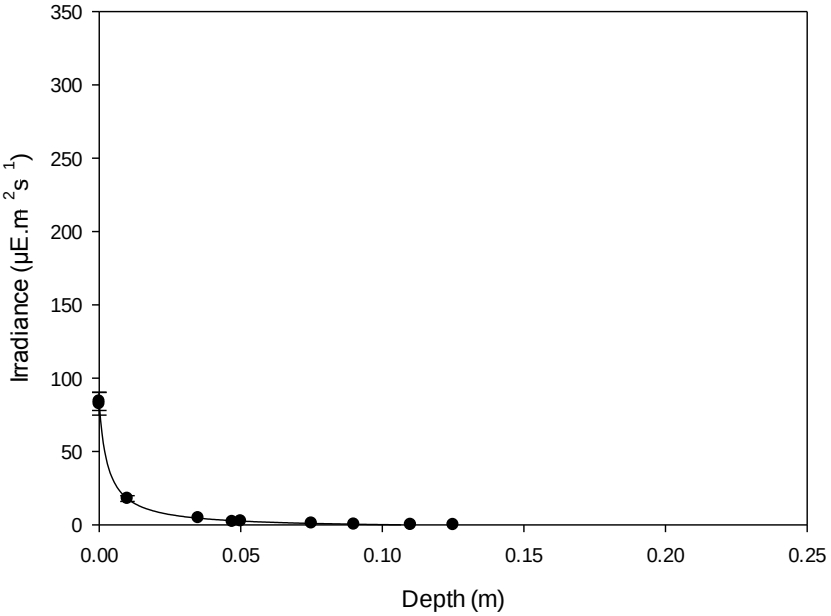


Figure 3.10 Irradiance profile of fluorescent light at different depths in the raceway culture broth of *C. vulgaris* with 0.5 g L^{-1} of biomass concentration. The total culture depth was 0.23 m. The fluorescent light output level was 100%. Standard deviation values are based on duplicates except the value at surface. The latter is based on nine replicates.

3.2.3.1 Calibration of the impeller speed

Agitation was provided by an eight-bladed paddle impeller installed in the raceway pond (Figures 3.2 and 3.4). The rotational speed of the impeller was regulated by a potentiometer, which was normally set at position 5. A calibration curve between the potentiometer setting and the actual measured impeller speed (rpm) is shown in Figure 3.11. The impeller speed was measured as the number of rotations per minute using an optical laser speed counter.

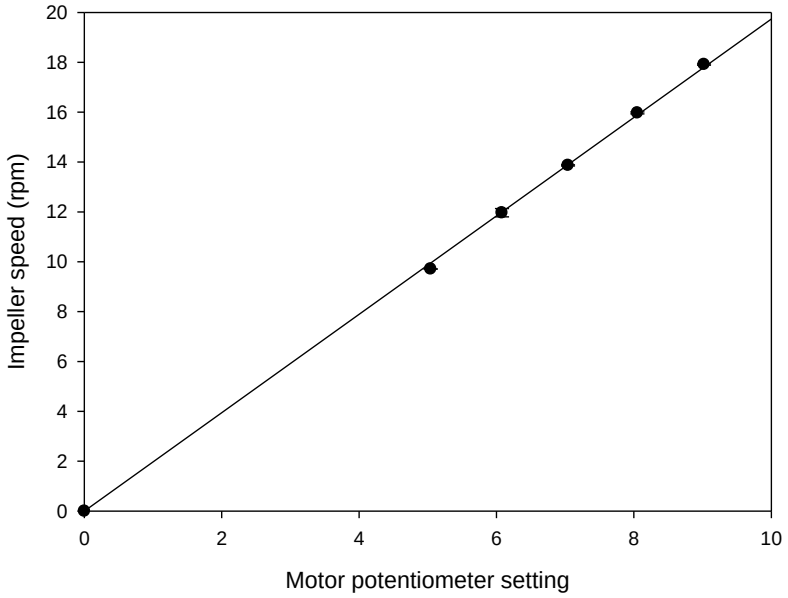


Figure 3.11 Impeller speed versus motor potentiometer settings. Standard deviation values are based on six replicates.

3.2.3.2 Relationship between the impeller speed and liquid flow velocity

Culture broth was circulated in the raceway by the impeller. The full circulation loop length was 2.6 m. At any setting of the impeller speed, the liquid flow velocity was measured by the time taken by a neutrally buoyant flow follower (a bright colored particle of ~1 cm diameter) to complete a circuit. Numerous measurements were made at any setting of the impeller speed and the values were averaged. The relationship

between the flow velocity and the impeller speed is shown in Figure 3.12. Tap water was used in the raceway for these measurements made at room temperature.

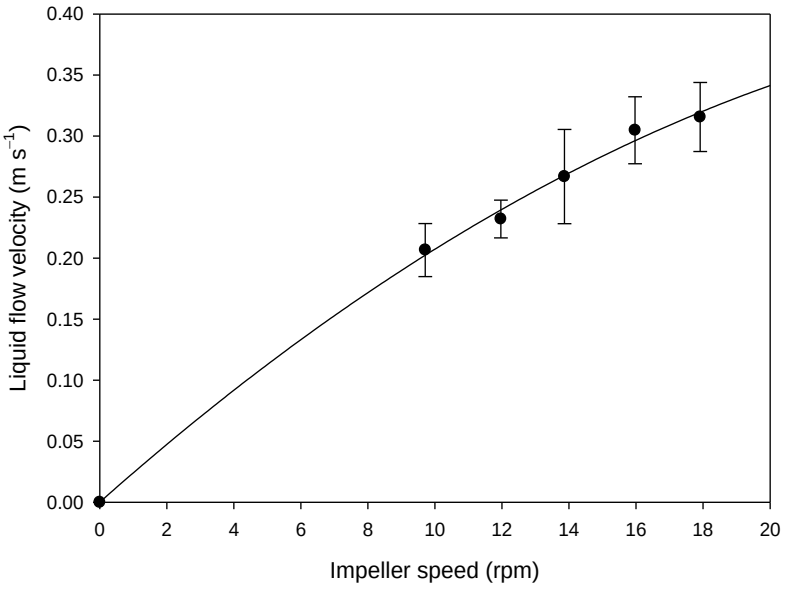


Figure 3.12 Relationship between impeller rpm and the liquid flow velocity. Standard deviation values are based on fifteen replicates.

3.2.3.3 Raceway batch culture

Prior to use, the raceway was thoroughly washed with tap water. The raceway was then filled to above the normal operating level with tap water containing ~5 L of bleach (2-4% v/v sodium hypochlorite; Jeyes Scrubbs Bleach, JohnsonDiversey, New Zealand) and left standing (~1 h) with the paddlewheel running (set at position 5 of the potentiometer unless specified otherwise). All internal surfaces were then scrubbed with a brush. The raceway was then drained and hosed repeatedly with tap water to remove all traces of bleach.

The raceway pond that had been filled with the medium was inoculated using cultures grown in Duran bottles (Section 3.2.2) to a density of around 2–3 g/L. The initial working volume was 138 L. The inoculum was generally 5% (v/v) of the initial

working volume of the raceway. The culture used the BG11 medium made with seawater, unless specified otherwise. The temperature and the pH set points were at 25 °C and 6.8, respectively. The mixing velocity of the culture broth was 0.21 ± 0.02 $m \cdot s^{-1}$ at a paddlewheel rotational speed setting of 5 on the potentiometer (paddlewheel speed of 9.71 rpm). The irradiance at the surface of the raceway culture was either constant (continuous illumination) at various specified values, or varied with time in accordance with a diurnal cycle (12:12 h day/night mode; with the light level peaking at "midday" during the daylight hours). The conductivity of the medium was monitored using a conductivity meter as explained in Section 3.2.1.4. Conductivity was maintained within the range of 52 to 54 mS/cm (equivalent to a salinity of 37 to 38 ppt). The culture was sampled daily for measuring the biomass concentration (Section 3.3.1) and the nutrients (Sections 3.3.3 and 3.3.4). The temperature, pH and salinity were measured daily. Evaporative losses were compensated by adding distilled water daily to the required level before taking the sample.

3.2.3.4 Raceway continuous culture

Prior to use the raceway was thoroughly washed and sanitized as explained in Section 3.2.3.3.

In continuous culture operations, *C.vulgaris* was grown at various dilution rates and irradiance levels. The continuous mode of operation was initiated by switching a raceway from a batch culture that had been running for 30 days. For continuous culture, fresh medium was pumped (peristaltic pump; Masterflex model no. 7554-60; Cole Parmer Instrument Co., Chicago, IL, USA) into the raceway at an specified flow rate to achieve the desired dilution rate, *D*. The dilution rate was calculated from the feed flow rate *F* and the constant broth volume *V* in the raceway; thus,

$$D = \frac{\text{Feed flow rate}}{\text{Total culture volume in raceway}} = \frac{F}{V} \quad (3.1)$$

The broth volume in the raceway was kept constant at ~138 L by means of an overflow mechanism. The overflow broth was collected into a reservoir held at 4 °C. The harvest reservoir was continuously mixed by a magnetic stirrer (IKA® C-MAG MS IKAMAG™, IKA® Werke GmbH & Co. KG, Germany) set at stirring position 4. The broth harvested over specified periods was centrifuged (Section 3.2.3.5) to recover the biomass. Periodic samples for analyses (biomass concentration, nitrate concentration, phosphate concentration) were taken directly from the raceway. The culture temperature and pH were controlled automatically as explained in Section 3.2.3. The salinity was measured daily.

3.2.3.5 Harvesting of biomass from the raceway broth

The algal biomass from the raceway culture broth was recovered by continuous flow centrifugation (CR22GII refrigerated centrifuge, Hitachi Koki Co., Ltd, Tokyo, Japan) at 11,800 × g, 4 °C. The culture broth (~117 L) was siphoned out into large buckets (50 L each), placed on a trolley and transported to the centrifuge. A peristaltic pump (Masterflex model no. 7554-60; Cole Parmer Instrument Co., Chicago, IL, USA) was used to pump the broth into the centrifuge at a flow rate of 1 L/min (Figure 3.11). The supernatant was continuously collected from the centrifuge outlet hose into a waste bucket. The waste was treated with bleach and discarded.

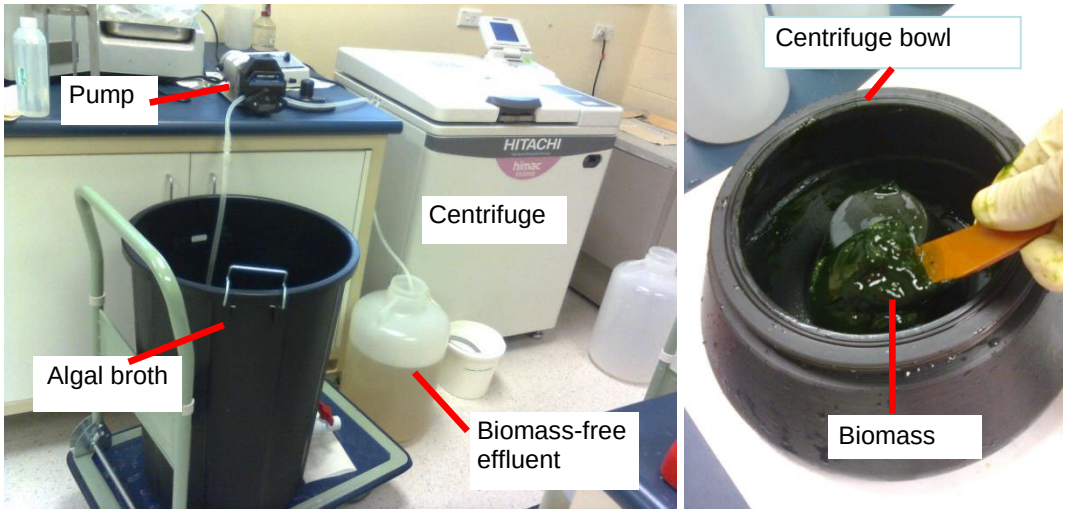


Figure 3.13 Continuous flow centrifugation – Algal broth from the black tank is pumped to the centrifuge where the biomass is retained in the bowl shown on the right. The biomass-free effluent leaves the centrifuge and is collected in the waste bottle.

The biomass collected as a paste was re-suspended in 1 L of distilled water for washing. This suspension was centrifuged in a batch centrifuge at 8,370 × g, 4 °C, for 10 min. The washing step was repeated four times, each time with 1 L of distilled water. The recovered biomass paste was stored at −80°C for freeze drying later on.

3.2.4 Freeze drying of microalgal biomass

The recovered biomass from Duran bottles and raceway pond (Sections 3.2.2.1 and 3.2.3.5) was freeze dried in a laboratory freeze dryer unit (CRYODOS −80; Telstar Industrial, S. L., Barcelona, Spain). For this, the biomass paste was spread as a thin layer (<20 mm thick) in a sample bottle and frozen overnight at −80°C (Thermoscientific Forma 900 series freezer, Model 995; Ohio, USA). Frozen biomass samples were immediately transferred to the vacuum desiccator connected to the condenser cylinder valve of the freeze dryer. The valve was opened slowly to begin the process of freeze drying. (The freeze dryer had been turned on at least 1 h prior to

attaching the sample and displayed a stable vacuum pressure of <1 mbar and a temperature of −80 °C). Depending on the sample size, the drying process typically completed within 24 h to 7 days.

3.3 Analytical methods

3.3.1 Biomass concentration

Dry cell weight in the culture broth was measured by vacuum filtration of a 20 mL sample of the broth through a preweighed Whatman GF-C (0.45 µm) 90 mm microfiber filter. The biomass was washed twice, each time with 20 mL of deionized water (for *C. vulgaris*) or 20 mL of 0.5 M ammonium formate for the other algae. The filter membrane was dried in an oven (Contherm Digital Series Five Oven, Lower Hutt, New Zealand) at 105 °C overnight. Filter membrane was then cooled to room temperature in a desiccator and weighed to calculate the dry biomass in 20 mL of the culture broth.

An identical sample of the broth as used for the dry weight measurements was serially diluted with the fresh culture medium and measured in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Model 80-2106-00) at 680 nm to obtain several data points in the absorbance range of 0 to 0.7. The blank was the fresh BG11 freshwater/seawater medium. The samples were diluted serially with fresh BG11 medium with a known dilution factor. The absorbance was plotted against dry weight to obtain a calibration curve. Subsequently, for determining the biomass concentration in an unknown sample, the sample was diluted with the fresh medium to bring the absorbance in the range of 0-0.7. The measured absorbance (A_{680}) and the dilution factor (DF) were used to calculate the biomass dry weight concentration (*DCW*, g L^{-1}) using equations (Equations 3.2-3.10) of the calibration curves.

Separate calibration curves were made for the different algae and also for the cases when an alga was grown under different physiological conditions. In cases of *C.*

vulgaris and *N. salina*, calibration curves also were prepared at different stages of the growth in media with different concentration of nitrate and different illumination regimes (continuous light; 12 h: 12 h light-dark cycle). The calibration curves obtained under different initial concentration of nitrate in BG11 media were essentially identical. However, the calibration curves obtained under continuous light and the 12 h: 12 h light-dark cycle conditions had different slopes for a given alga. For *C. vulgaris* raceway culture, separate calibration curves were prepared under continuous light as well as for 12 h:12 h diurnal light-dark cycle. For the experiments run under specific conditions, the relevant calibration curves were used for estimating the biomass concentration from spectrophotometric data. The calibration curves are shown in Figures 3.14-3.22. Samples (2 mL) for spectrophotometric measurements of the biomass were taken in duplicate or triplicate.

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{4.7327} \times (\mathrm{DF}) \text{ for } \textit{C. vulgaris} \text{ (a)} \tag{3.2}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{1.3758} \times (\mathrm{DF}) \text{ for } \textit{C. vulgaris} \text{ (b)} \tag{3.3}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{1.6231} \times (\mathrm{DF}) \text{ for } \textit{C. vulgaris} \text{ (c)} \tag{3.4}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{2.2310} \times (\mathrm{DF}) \text{ for } \textit{C. vulgaris} \text{ (d)} \tag{3.5}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{2.6253} \times (\mathrm{DF}) \text{ for } \textit{C. vulgaris} \text{ (e)} \tag{3.6}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{5.4389} \times (\mathrm{DF}) \text{ for } \textit{N . salina} \quad \text{(a)} \tag{3.7}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{5.9515} \times (\mathrm{DF}) \text{ for } \textit{N . salina} \quad \text{(b)} \tag{3.8}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{1.9416} \times (\mathrm{DF}) \text{ for } \textit{C. fusiformis} \tag{3.9}$$

$$\mathrm{DCW} = \frac{\mathrm{A}_{680}}{1.8203} \times (\mathrm{DF}) \text{ for } \textit{T. subcordiformis} \qquad (3.10)$$

The other algae mentioned in Table 3.1 (i.e. *Cylindrotheca closterium, Ditylum brightwellii, Hymenomonas elongate* and *Chroomonas salina fo. oculus-bovus*) could not be grown despite repeated efforts and therefore were not further examined.

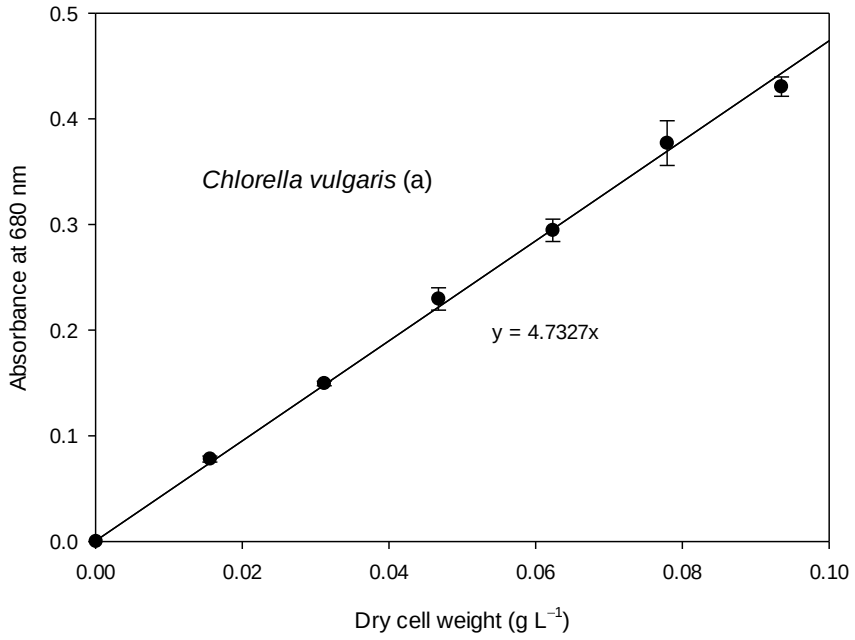


Figure 3.14 Spectrophotometric calibration curve for freshwater *C. vulgaris* obtained under continuous light in Duran bottle using the standard BG11 medium. Standard deviation values are based on duplicate samples.

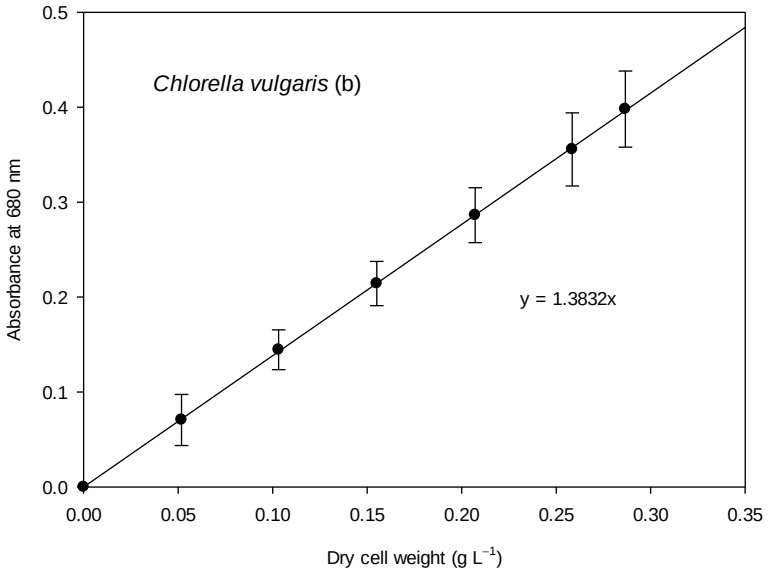


Figure 3.15 Spectrophotometric calibration curve (averaged) for *C. vulgaris* (b) obtained under continuous light in Duran bottles using BG11 seawater medium with different initial nitrate concentrations (0.1 to 1.1 g L^{-1}). Standard deviation values are based on 36 runs.

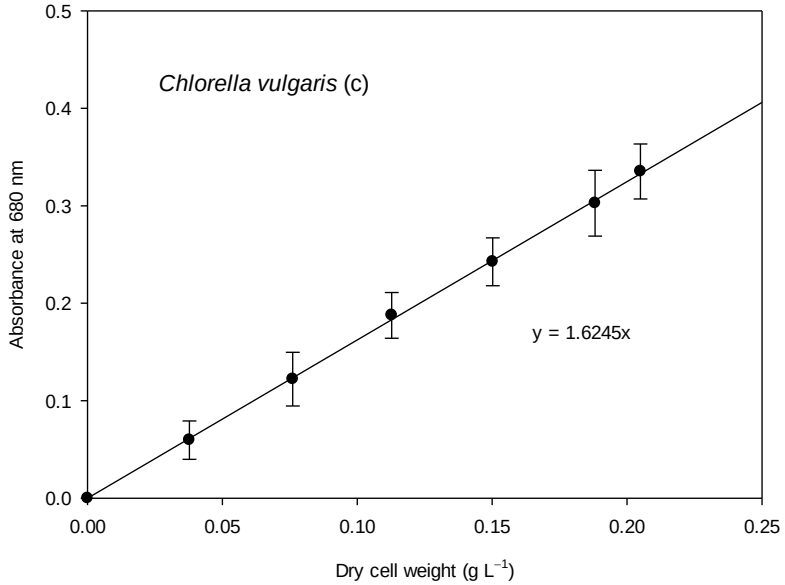


Figure 3.16 Spectrophotometric calibration curve (averaged) for *C. vulgaris* (c) obtained under 12 h:12 h light-dark cycle in Duran bottles using BG11 seawater medium with different nitrate concentrations (0.1 to 1.1 g L^{-1}). Standard deviation values are based on 32 runs.

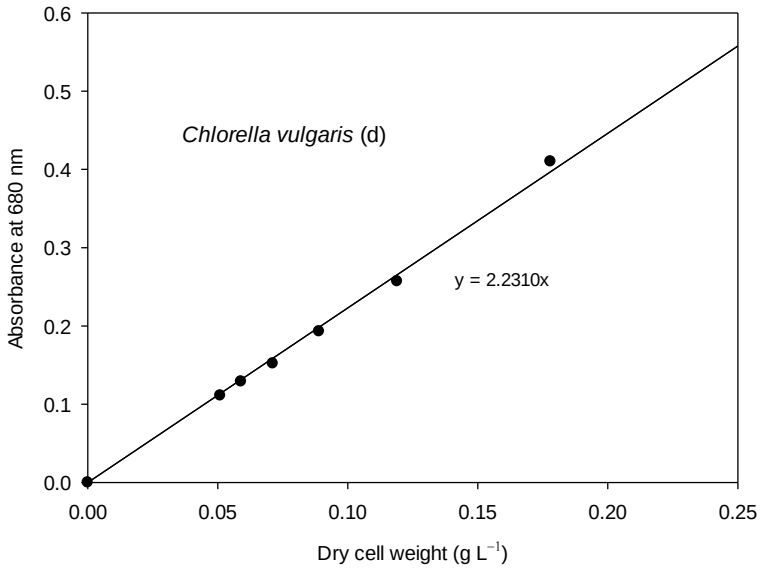


Figure 3.17 Spectrophotometric calibration curve for *C. vulgaris* (d) obtained under continuous light in raceway in BG11 seawater medium.

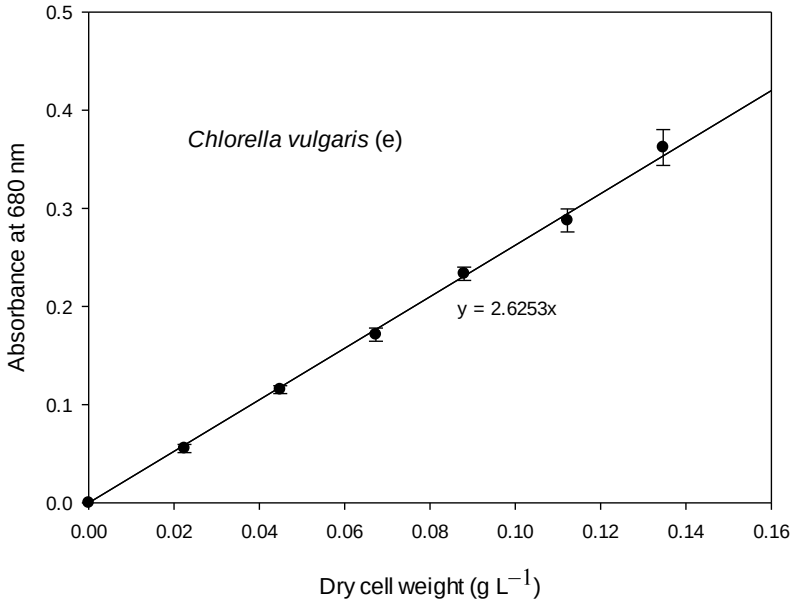


Figure 3.18 Spectrophotometric calibration curve for *C. vulgaris* (e) obtained under 12 h: 12 h diurnal light-dark cycle in raceway in BG11 seawater medium. Standard deviation values are based on triplicate samples.

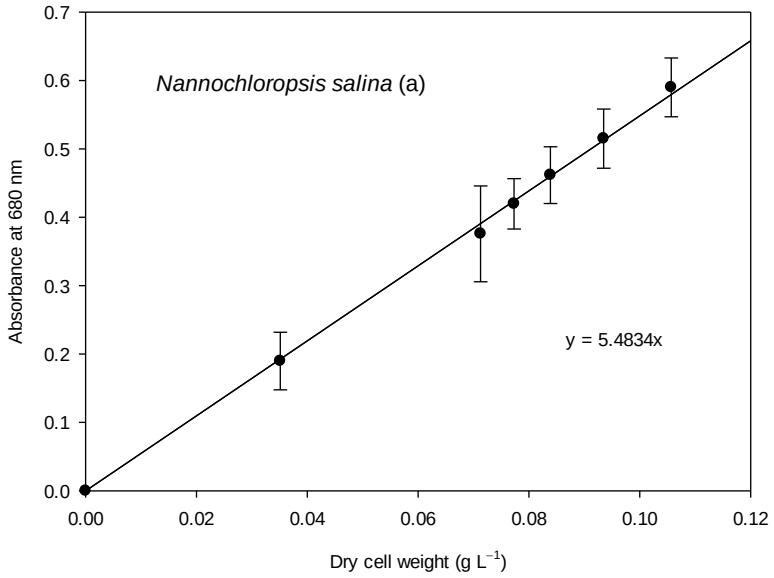


Figure 3.19 Spectrophotometric calibration curve (averaged) for *N. salina* (a) obtained under continuous light in Duran bottle using BG11 seawater medium with different initial nitrate concentrations (0.1 to 1.1 g L^{-1}). Standard deviation values are based on 35 runs.

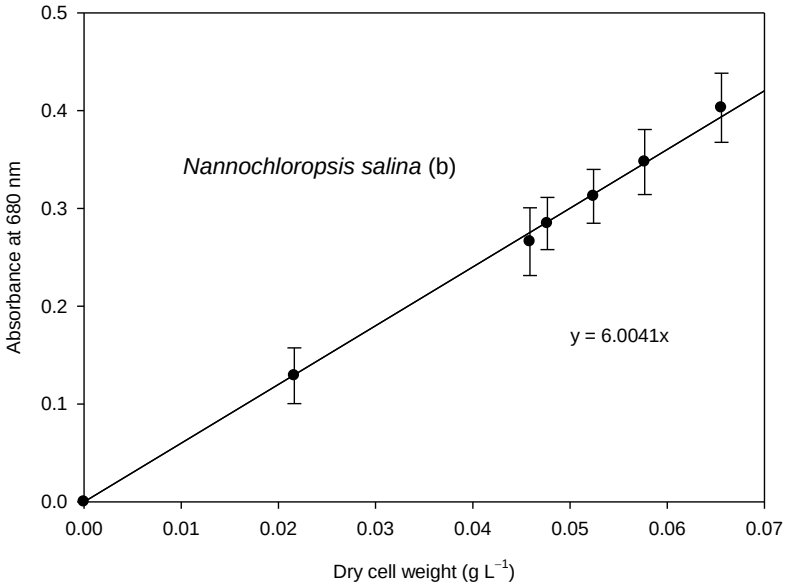


Figure 3.20 Spectrophotometric calibration curve (averaged) for *N. salina* (b) obtained under 12 h:12 h light-dark cycle in Duran bottles using BG11 seawater medium with different initial nitrate concentrations (0.1 to 1.1 g L^{-1}). Standard deviation values are based on 45 runs.

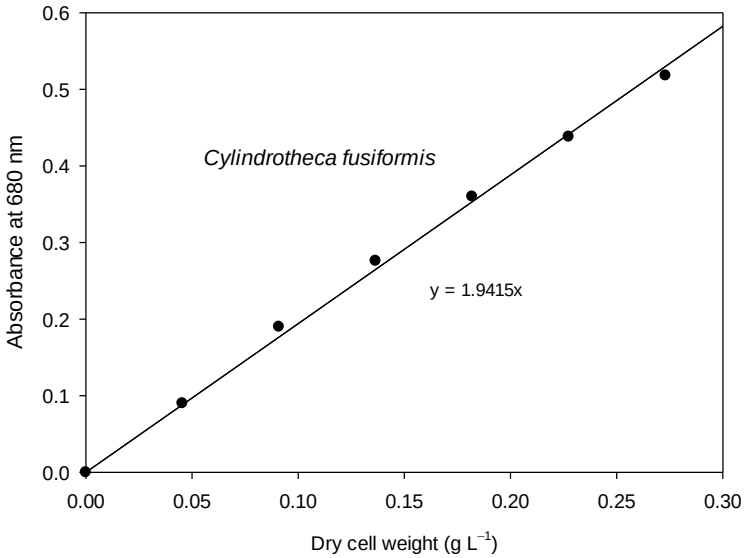


Figure 3.21 Spectrophotometric calibration curve for *C. fusiformis* obtained under continuous light in Duran bottle using BG11 seawater medium.

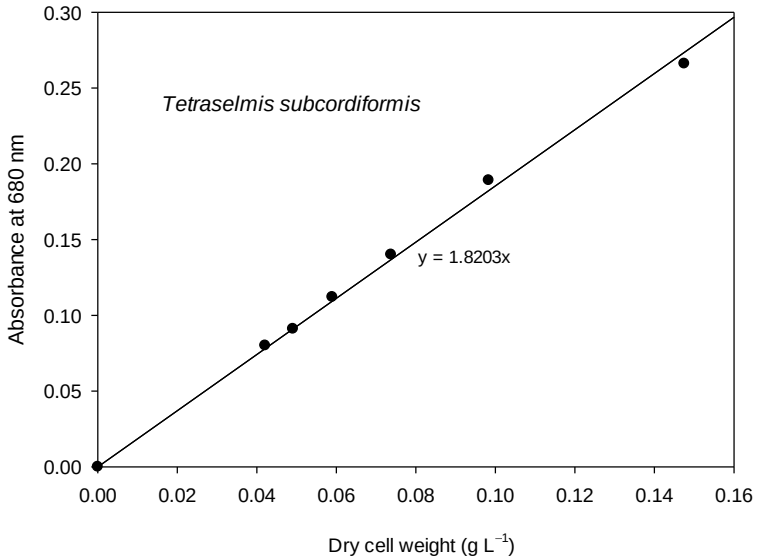


Figure 3.22 Spectrophotometric calibration curve for *T. subcordiformis* obtained under continuous light in Duran bottle using BG11 seawater medium.

3.3.2 Irradiance

Photosynthetically active radiation (PAR) incident at the surface of the culture bottles and the raceway pond, was measured using either a QSL-2101 quantum scalar irradiance sensor (Biospherical Instruments Inc, San Diego, CA, USA) or a Li-Cor LI-189 quantum irradiance meter (Li-Cor Inc., Lincoln, NE, USA). Readings of the two instruments were comparable. PAR values at various depths in the raceway were measured using the submersible QSL-2101 sensor.

3.3.3 Nitrate analysis

Nearly all the nitrogen (>99.9% by weight) in the media used was present as nitrate. Residual nitrate in filtered (0.45 μm membrane filter; 28 mm diameter Minisart® NML, syringe driven filter unit; Sartorius Stedim Biotech GmbH, Goettingen, Germany) algal broth was measured using the cadmium reduction method (Hach method 8039) as available from Hach Company (Loveland, CO, USA). Contents of a NitraVer® 5 nitrate reagent sachet, for a 10 mL sample (Hach Co., Loveland, Co, USA; catalog number 2106169) were added to 10 mL of the culture filtrate diluted with nitrate-free BG11 seawater medium such that the nitrate concentration in the diluted sample was <30 mg L^{-1}. The reaction mixture was vortexed for 1 min exactly and left standing at room temperature for a further 5 min. The amber color developed was measured at 525 nm without disturbing any sediments. The spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Model 80-2106-00) used in the measurements had been zeroed with a 10 mL blank of the nitrate-free BG11 seawater medium treated with the NitraVer® 5 reagent in exactly the same way as the samples.

The measured absorbance was converted to a nitrate concentration using a calibration curve and the dilution factor (DF). The calibration curve (Figure 3.23) had the following equation:

$$\text{Nitrate}\left(\text{mg L}^{-1}\right) = \frac{A_{525}}{0.0017} \times (\text{D F}) \qquad (3.11)$$

The calibration curve had been prepared by serially diluting the BG11 seawater medium with nitrate-free BG11 seawater medium to obtain samples with known nitrate concentrations in the range of 0-30 mg L^{-1}. These samples were then treated with NitraVer® 5 reagent and their absorbance was measured. A plot of absorbance (corrected for dilution) versus the nitrate concentration was made (Figure 3.23).

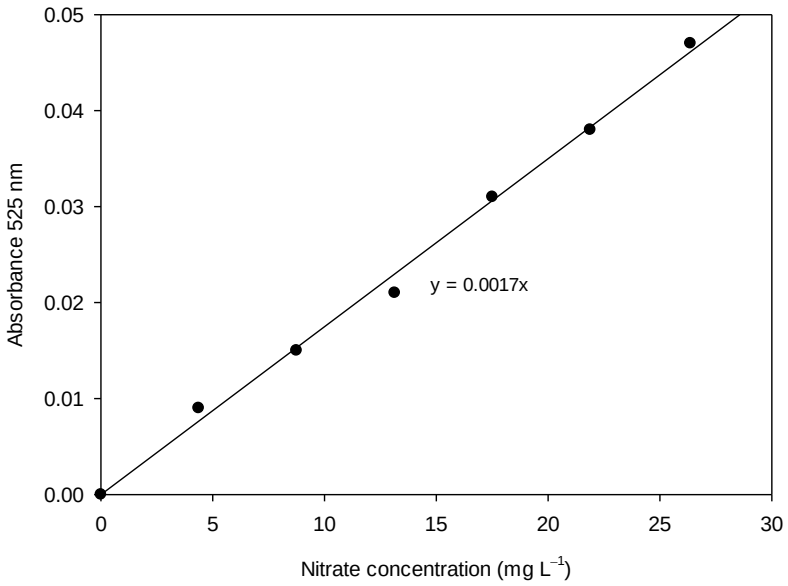


Figure 3.23 Nitrate standard curve prepared with dilutions of BG11 medium in seawater.

3.3.4 Phosphate analysis

Phosphate in the cell-free culture broth was measured using the ascorbic acid-molybdate method (Strickland and Parsons, 1968). The broth samples were prefiltered (0.45 μm membrane filter; 28 mm diameter Minisart® NML, syringe driven filter unit; Sartorius Stedim Biotech GmbH, Goettingen, Germany).

The sample filtrate was diluted with distilled water to bring the phosphate concentration within the range of 0–0.3 mg PO_4^{2-} per liter. All glassware used had been acid washed and rinsed with distilled water to prevent potential interference from phosphate contained in some soaps and detergents.

The mixed reagent for phosphate analysis was prepared just before use, by mixing the following:

1. Ammonium molybdate solution (50 mL)
2. Sulfuric acid (125 mL)
3. Ascorbic acid reagent (50 mL)
4. Potassium antimonyl-tartrate solution (25 mL)

The above solutions had been prepared as follows:

1. By dissolving 15 g of ammonium molybdate (VWR, BDH, Prolabo, Belgium) in 500 mL of distilled water. This solution was stored in the dark in a plastic bottle.
2. Adding 140 mL of concentrated sulfuric acid (~2.4 M; Sharlab S.L., Spain) to 900 mL of deionized water and storing in a glass bottle.
3. Dissolving 5.4 g of ascorbic acid (BDH, VWR International, Poole, England) in 100 mL of deionized water. This solution was made fresh every time the phosphate analysis was performed.
4. Dissolving 0.272 g of potassium antimonyl-tartrate (BDH Chemicals Ltd, Poole, England) in 200 mL of deionized water and storing in a plastic bottle.

To 5 mL of the diluted sample, 0.5 mL of a mixed reagent (see above) was added. The resulting solution was mixed and left to stand at room temperature for 20 min. Absorbance of this solution was measured at 885 nm using a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, Model 80-2106-00). The blank was distilled water.

The absorbance was converted to a phosphate concentration using a calibration curve (Figure 3.24). The equation of the calibration curve was:

$$\text{Phosphate}\left(\text{mg L}^{-1}\right) = \frac{A_{885}}{0.1203} \times (\text{DF}) \qquad (3.12)$$

where DF is the dilution factor.

The calibration curve (Figure 3.24) had been prepared by measuring the absorbance of six dilutions of a phosphate standard solution (8.0×10^{-6} M K_2HPO_4; LabServ Biolab Australia, Ltd) after treatment with the mixed reagent solution. The dilutions of the standard phosphate solution were such that the maximum phosphate concentration was <0.3 mg/L. The absorbance was plotted against the known phosphate concentrations.

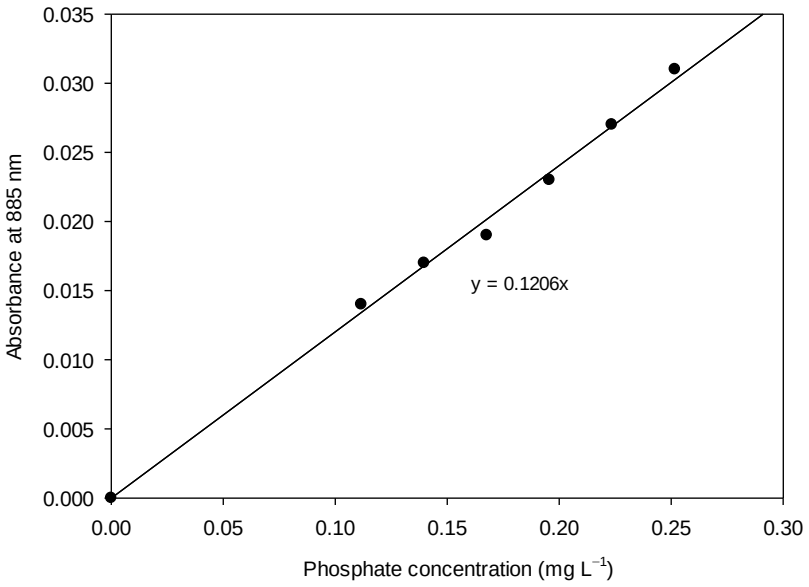


Figure 3.24 Phosphate standard curve.

3.3.5 Total lipids extraction

Freeze-dried biomass was used for quantitative extraction of the total lipids using a modified method of Bligh and Dyer (1959). The volume ratio of chloroform, methanol

and water was always 1:2:0.8 in the extraction step and 2:2:1.8 in the biphasic separation step.

Dry biomass (1 g) was homogenized with 5 mL of chloroform, 10 mL of methanol and 4 mL of distilled water, for 4 h in a 100 mL Duran bottle while being stirred by a magnetic stirrer at 700 rpm (IKA® KMO 2 Basic IKAMAG™, IKA® Werke GmbH & Co. KG, Germany) at room temperature. 5 mL of chloroform was then added and the mixture was vigorously stirred for 30 s. 5 mL of distilled water was added and vigorously mixed for 30 s. The resulting dispersion was centrifuged (Hitachi High-speed refrigerated centrifuge CR22GII, Hitachi Koki Co., Ltd., Tokyo, Japan) at 4,000 × g, 4°C, for 10 min. This resulted in separation into three layers. The upper phase (methanol/water) was discarded. The second layer from the top was the biomass residue. The lowest layer was the chloroform layer that contained the dissolved lipids. This layer was pumped out using a capillary glass pipette. The biomass residue (the second layer) was kept for further extraction. This biomass was extracted again using exactly the same protocol as described above. A third extraction of the residual biomass was carried out with 5 mL of chloroform, by mixing for 30 s (vortex mixer) and leaving for 1 h. The chloroform extracts collected from the three extractions were combined. The total lipids were determined gravimetrically by evaporating a known volume of the chloroform extract at room temperature (fume hood) in a preweighed aluminum pan (12 h) followed by further drying (12 h) at room temperature in a desiccator.

The percentage of total lipids in the biomass (Figure 3.25) was determined using the measured volume of the chloroform extract, the total lipids concentration in the chloroform extract and the amount of the dried biomass used in the extraction, as follows:

Total lipids (% w/w)

$$= \frac{\text{volume of chloroform extract (mL)} \times \text{total lipid concentration in extract (mg/mL)}}{\text{mass of biomass extracted (mg)}} \times 100 \qquad (3.13)$$

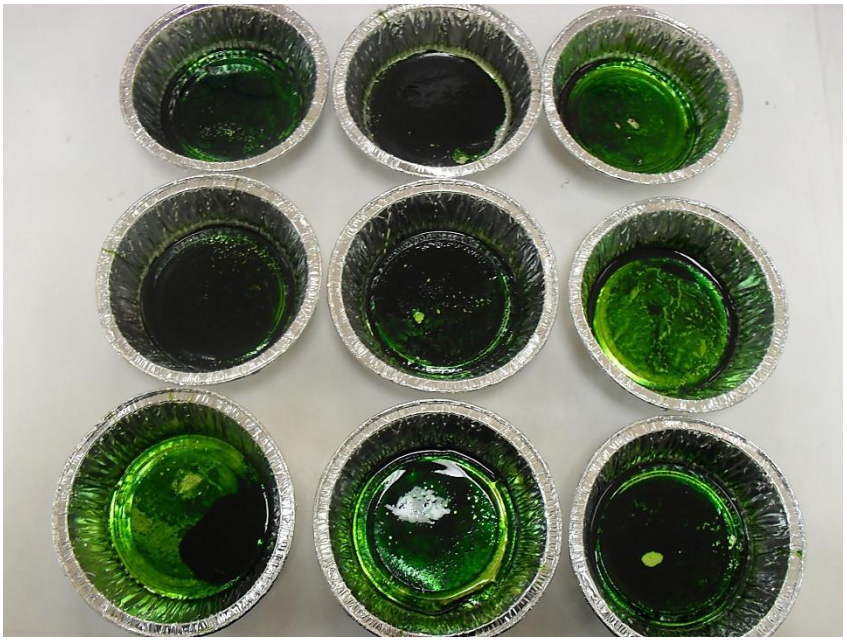


Figure 3.25 Total lipids (crude oil) after evaporation of chloroform.

3.3.6 Nile red staining

For visualization of lipids in microalgal cells, Nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one, $C_{20}H_{18}N_2O_2$) fluorescent dye (Sigma-Aldrich, USA) was used. The protocol was adapted from Elsey *et al.* (2007). The stained microalgal cells were imaged by a confocal microscope (Leica SP5 DM6000B) at Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North.

A sample of algal broth was diluted with BG11 medium to an spectrophotometric absorbance of 0.1 to 0.2 at 720 nm. A 3 mL sample of the diluted broth was vortexed (1 min) with 10 µL Nile Red (7.8×10^{-4} M in acetone) to give a final Nile Red concentration of 0.26 µM. The sample was kept at room temperature for 30-40 min. Then vortexed for 30 s and placed on a microscope slide, covered with a cover slip, and

photographed under a confocal microscope. Neutral lipids stained yellow and the other lipids stained red. Typical images are shown in Figure 3.26.

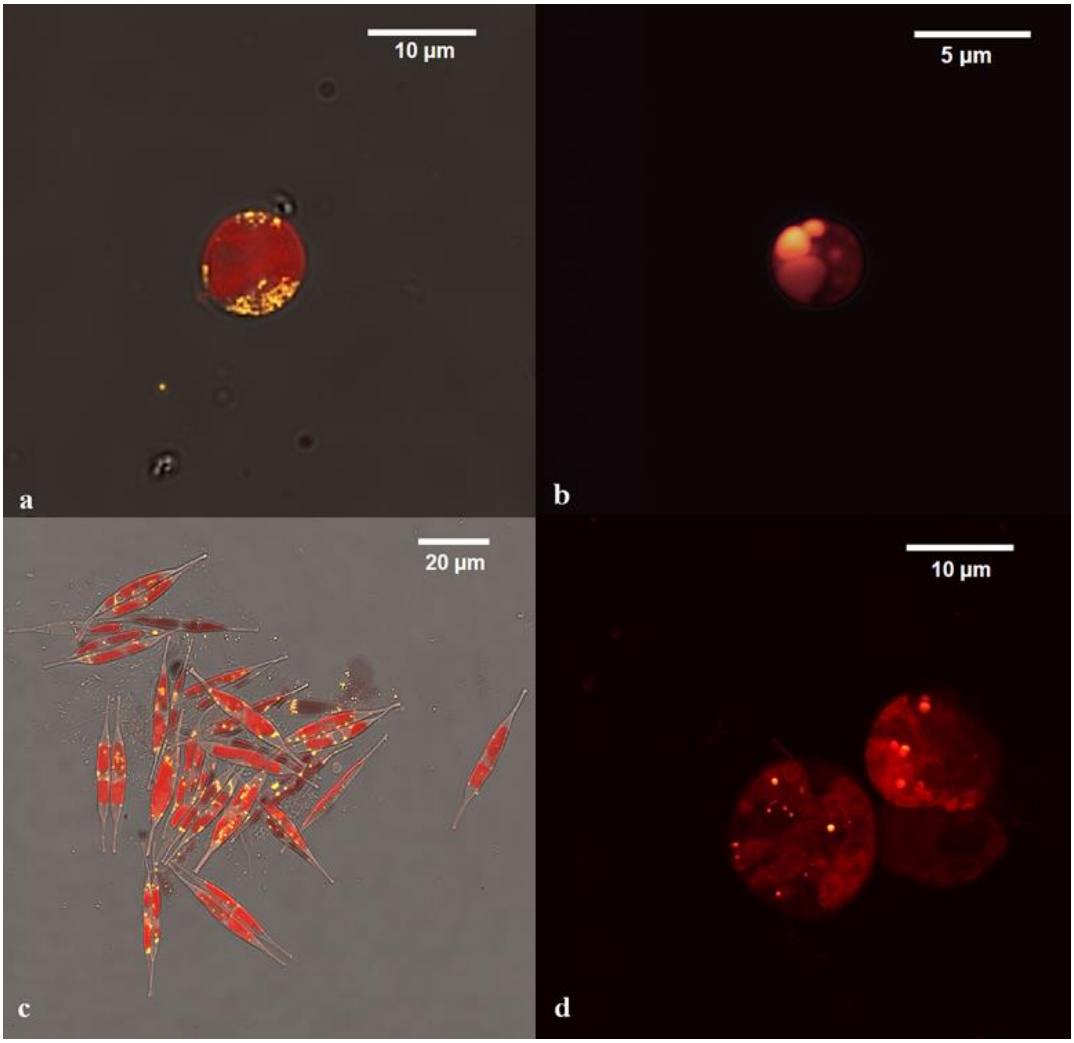


Figure 3.26 Fluorescence confocal microscope images of algae stained with Nile Red: (a) *C. vulgaris*; (b) *N. salina*; (c) *C. fusiformis*; (d) *T. subcordiformis*.

3.3.7 Fatty acid profile

Fatty acid methyl ester from algal crude oil was separated by gas chromatography (GC) according to ISO 15304 [International Standard ISO 15304]. Dried oil sample (~400 mg) was characterized for fatty acid profile at Nutritional Laboratory, Institute of Food, Nutrition and Human Health (Massey University, Palmerston North, New Zealand).

3.3.8 Calorific value

Total energy content of the algal biomass was established by measuring the heat of combustion, or the calorific value. The freeze-dried biomass samples were measured in a bomb calorimeter (Leco AC-350 calorimeter, Leco Corporation, St Joseph, MI, USA). For each measurement, ~1 g of the biomass sample was used. In some cases the calorific value of algal crude oil was measured if sufficient oil (~1 g per measurement) could be extracted. All measurements were made by the Nutritional Laboratory, Institute of Food, Nutrition and Human Health (Massey University, Palmerston North, New Zealand). Replicate measurements on a given sample were generally reproducible to within ±3% of the average measured value.

3.3.9 Elemental analyses

The content of carbon (C), nitrogen (N), sulfur (S) and phosphorus (P) in some freeze-dried samples of the algal biomass were measured. For this, the dried biomass samples were sent to the Nutritional Laboratory, Institute of Food, Nutrition and Human Health (Massey University, Palmerston North, New Zealand). C and N were measured by total combustion in a Leco elemental analyzer. The elemental analyzer used the Dumas combustion method for N (AOAC Official Method 968.06; AOAC International, www.aoac.org; *Official Methods of Analysis of AOAC (OMA)*, 18th edition, AOAC International, Gaithersburg, MD, USA, 2006). S and P were measured by inductively coupled plasma optical emission spectrometry (ICP-OES).

3.3.10 Microscopy

Algal cultures were periodically examined by light microscopy (Leica DMBRE Compound Light Microscope) to assess the cell morphology, general condition and

possible contamination. Images of the normal cells of the various algae are shown in Figure 3.27.

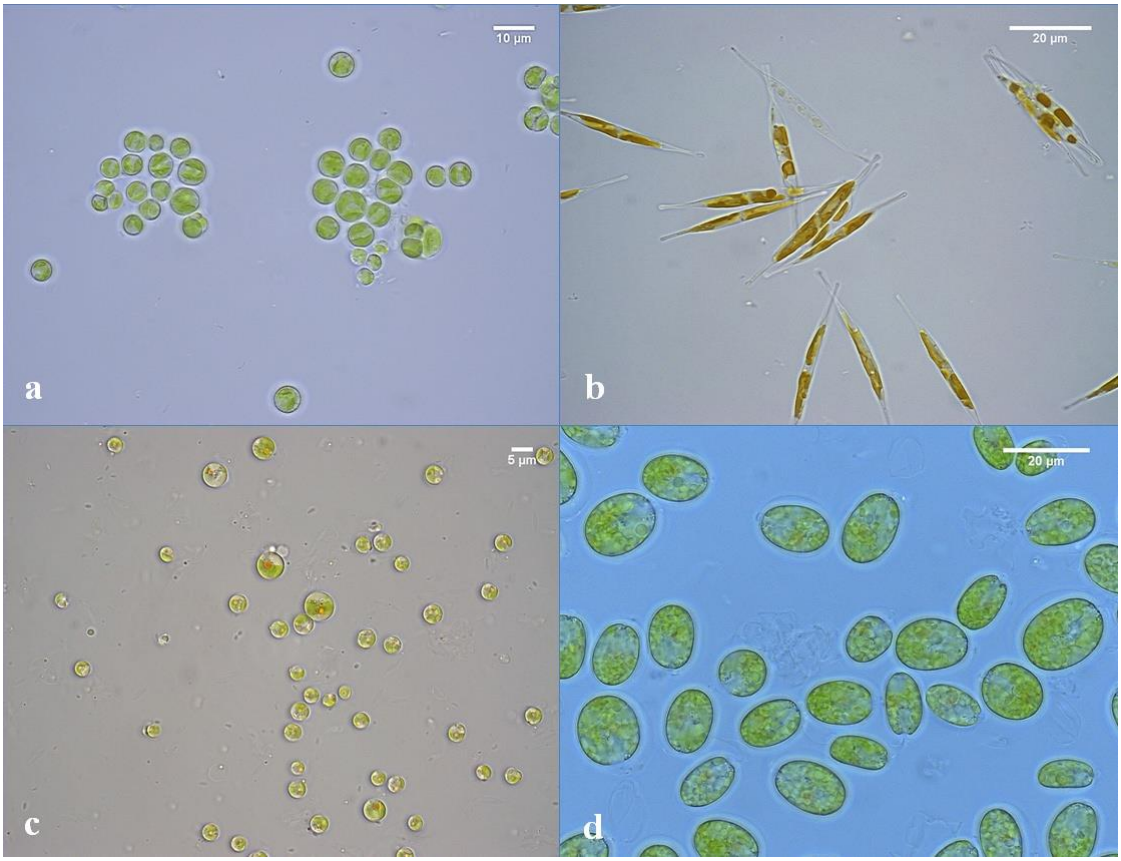


Figure 3.27 Light microscopic images of: (a) *C. vulgaris*; (b) *N. salina*; (c) *C. fusiformis*; (d) *T. subcordiformis*.

3.3.11 Isolation of bacteria from raceway

A few loopfuls of culture from the raceway were streaked on agar plates (1% Difco^{TM}, Agar Noble, Becton, Dickinson and Company, USA) prepared in BG11 seawater (Section 3.2.1) to check for contamination with bacteria. These agar plates were placed at 25±2 °C under continuous irradiance of 109 to 116 µmol·m^{-2}s^{-1}. Colonies of bacteria started developing within 2-3 days of inoculation. The algal colonies appeared later after 1-2 weeks of inoculation.

3.4 Calculations of culture kinetic parameters (Doran, 1995; Shuler and Kargi, 2002)

The various algae culture kinetic parameters were calculated as explained here.

3.4.1 Batch culture

Specific growth rate, μ (h^{-1})

$$\mu = \frac{1}{t_2 - t_1} \ln \frac{X_2}{X_1} \quad (3.14)$$

Where, X_1 is biomass concentration (g L^{-1}) at time t_1 (h) and X_2 is biomass concentration (g L^{-1}) at time t_2 (h). Equation (3.14) applies during exponential growth. Semilog plots of ln X/X_i versus time were made from the growth curve. (Here X_i is the initial biomass concentration and X is the biomass concentration at any time *t*.) The exponential growth phase was identified as the period in which the above plot had the maximum slope.

Biomass productivity, P_b (g L^{-1} d^{-1})

$$P_b = \frac{X_f - X_i}{t} \quad (3.15)$$

Lipid productivity, P_l (g L^{-1} d^{-1})

$$P_l = \frac{(X_f - X_i)\, w}{t} \quad (3.16)$$

Biomass yield coefficient on nitrate, Y_N (g mg^{-1})

$$Y_N = \frac{\mathrm{X_f} - \mathrm{X_i}}{N_i - N_f} \tag{3.17}$$

Biomass yield coefficient on phosphate, Y_P (g mg^{-1})

$$Y_P = \frac{\mathrm{X_f} - \mathrm{X_i}}{P_i - P_f} \tag{3.18}$$

In the above equations, X_f is final biomass concentration (g L^{-1}); X_i is initial biomass concentration (g L^{-1}); t is duration (d) of the batch (i.e. the time required to attain the biomass concentration X_f); w is weight fraction of the lipids in the biomass; N_i is initial nitrate concentration (mg L^{-1}); N_f is final nitrate concentration (mg L^{-1}); P_i is initial phosphate concentration (mg L^{-1}); and P_f is final phosphate concentration (mg L^{-1}).

Average specific nitrate consumption rate, q_N (mg g^{-1} h^{-1})

$$q_N = \frac{N_1 - N_2}{(X_2 - X_1)\,(t_2 - t_1)} \tag{3.19}$$

Average specific phosphate consumption rate, q_N (mg g^{-1} h^{-1})

$$q_P = \frac{P_1 - P_2}{(X_2 - X_1)\,(t_2 - t_1)} \tag{3.20}$$

In the above equations, X_1 is the biomass concentration (g L^{-1}) at time t_1 (h); X_2 is biomass concentration (g L^{-1}) at time t_2 (h); N_1 is nitrate concentration (mg L^{-1}) at time t_1 (h); N_2 is nitrate concentration (mg L^{-1}) at time t_2 (h); P_1 is phosphate concentration (mg L^{-1}) at time t_1 (h); and P_2 is phosphate concentration (mg L^{-1}) at time t_2 (h).

3.4.2 Continuous culture (at steady state)

Specific growth rate, μ (d^{-1})

$$\mu = D = \frac{F}{V} \tag{3.21}$$

Where D is dilution rate (d^{-1}), F is feed flow rate (mL d^{-1}) and V (mL) is the volume in the culture vessel. Equation (3.21) applies to steady state operation.

Biomass productivity, P_b (g L^{-1} d^{-1})

$$P_b = DX_s \tag{3.22}$$

Lipid productivity, P_b (g·L^{-1} d^{-1})

$$P_l = DX_s w_s \tag{3.23}$$

In the equations (3.21) – (3.23), D is dilution rate (d^{-1}), X_s is steady state biomass concentration (g L^{-1}) and w_s is the steady state weight fraction of the lipids in the biomass.

Biomass yield coefficient on nitrate, $Y_{X/N}$ (g mg^{-1})

$$Y_{X/N} = \frac{X_s}{N_f - N} \tag{3.24}$$

Biomass yield coefficient on phosphate, $Y_{X/P}$ (g mg^{-1})

$$Y_{X/P} = \frac{X_s}{P_f - P} \tag{3.25}$$

In the equations (3.24) and (3.25), $Y_{X/N}$ is biomass yield coefficient (g mg^{-1}) on nitrate; X_s is steady state biomass concentration (g L^{-1}) in the culture vessel; N_f is nitrate concentration in the feed (mg L^{-1}); N is steady state nitrate concentration (mg L^{-1}) in the

culture vessel; $Y_{X/P}$ is biomass yield coefficient (g mg^{-1}) on phosphate; P_f is phosphate concentration (mg L^{-1}) in the feed; and P is steady state phosphate concentration (mg L^{-1}) in the culture vessel.

Biomass yield coefficient on light, $Y_{X/L}$ (g µmol^{-1}) (continuous light only)

$$Y_{X/L} = \frac{FX_s}{IA} \tag{3.26}$$

Where F is feed flow rate (L s^{-1}); X_s is steady state biomass concentration (g L^{-1}); I is irradiance (µmol·$m^{-2}s^{-1}$); and A is surface area (m^2) for light absorption.

Average specific nitrate consumption rate, q_N (mg g^{-1} h^{-1})

$$q_N = \frac{D(N_f - N)}{X_s} \tag{3.27}$$

Average specific phosphate consumption rate, q_P (mg g^{-1} h^{-1})

$$q_P = \frac{D(P_f - P)}{X_s} \tag{3.28}$$

In the equations (3.26) – (3.28), X_s is steady state biomass concentration (g L^{-1}); D is dilution rate (h^{-1}); N_f is nitrate concentration (mg L^{-1}) in the feed; N is steady state nitrate concentration (mg L^{-1}) in culture vessel; P_f is phosphate concentration (mg L^{-1}) in the feed; and P is steady state phosphate concentration (mg L^{-1}) in the culture vessel.

Chapter 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents and discusses the key experimental results. Eight microalgae were initially preliminarily screened for their ability to produce biomass and lipids. The relatively promising algae were then further screened for their salinity tolerance with the aim of identifying one that was relatively highly productive in seawater and retained a reasonable productivity under somewhat hypersaline conditions as would occur in an outdoor commercial raceway because of evaporative loss of water. Based on this data, one alga (*C. vulgaris*) was identified as the focus of this study, but work on a second promising alga (*N. salina*) was continued for comparison until it was eliminated from the study as being unresponsive to nitrogen limitation as a means of enhancing oil productivity. Extensive studies are carried out in seawater with *C. vulgaris* in a large (~138 L) raceway in nutrient sufficient and nutrient limited conditions. Batch and continuous culture operations at various steady states were evaluated. Effects of different illumination regimes and turbulence levels on biomass/lipid productivities, lipid contents and calorific values of the biomass were assessed.

4.2 Screening of microalgae in Duran bottles

4.2.1 Batch cultures under normal growth conditions

Initial screening was based on biomass and lipid productivities. Growth profiles of the four screened microalgae were obtained in 2 L Duran bottles (Figure 4.1).

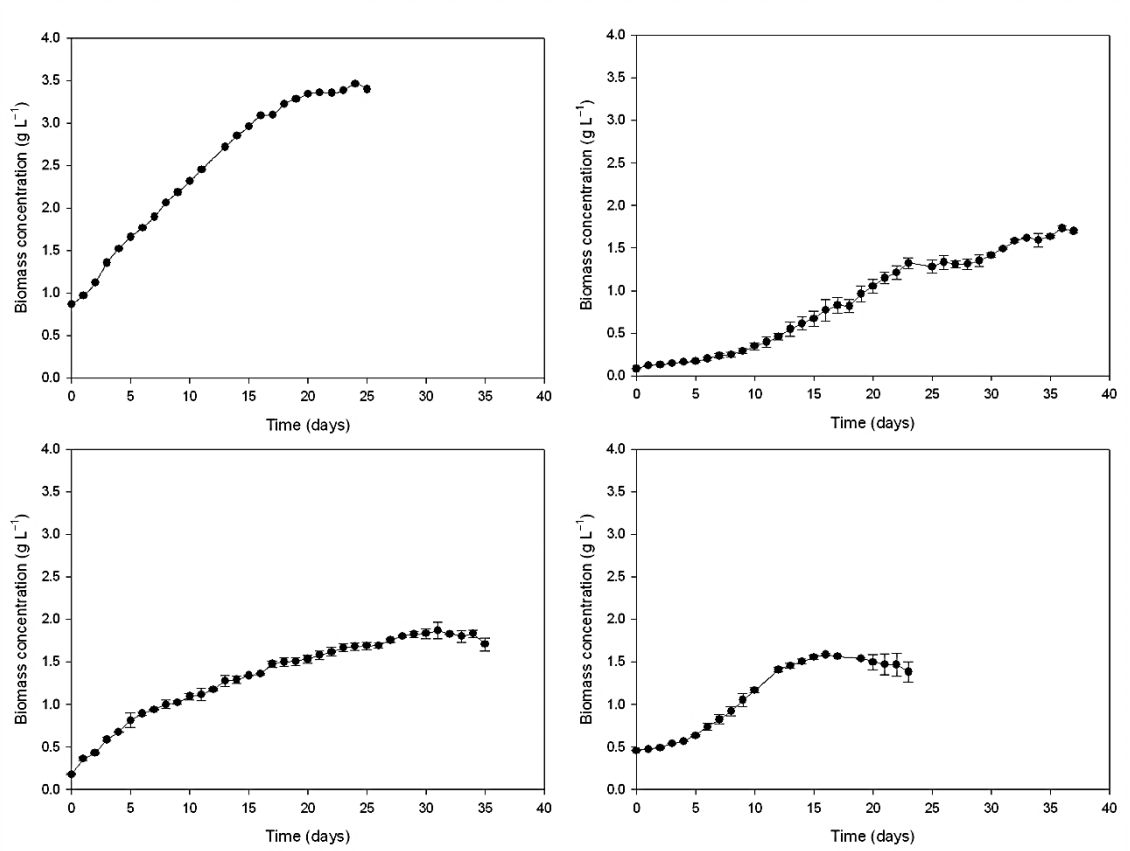


Figure 4.1 Growth profile of: a) *C. vulgaris*, irradiance of 105 μmol·m^{-2}s^{-1}; b) *N. salina*, irradiance of 124 μE·m^{-2}s^{-1}; c) *T. subcordiformis*, irradiance of 124 μmol·m^{-2}s^{-1}; d) *C. fusiformis*, irradiance of 105 μmol·m^{-2}s^{-1}. All algae were grown in BG11 seawater medium, 24 ± 2 °C, bubbled with 5% (v/v) CO_2 in air. Silicate was added to the medium for *C. fusiformis*. Standard deviations are based on duplicate runs.

The culture growth and lipid production parameters based on Figure 4.1 are summarized in Table 4.1. The diatom *C. fusiformis* was relatively slow growing (Table 4.1) although its final biomass productivity was comparable to the other algae (Table 4.1) except *C. vulgaris*. *C. fusiformis* cells tended to clump and settle. Also, the cultures tended to require a high initial inoculum. Formation of clumps and sedimentation of cells may have been due to the unique shape (pennate diatom) and relatively large cell size (~30 μm) of this strain (Figure 3.27 in Chapter 3).

The estimated lipid content of this strain was high at 27% (w/w) (Table 4.1) and consistent with an earlier report (Suman *et al.*, 2012). Other *Cylindrotheca* sp have been reported to have a high lipid content (Cooksey *et al.*, 1987; Priscu *et al.*, 1990; Elsey *et al.*, 2007). The lipid productivity was low compared to *C. vulgaris* and *N. salina* (Table 4.1).

Table 4.1 Summary of findings from preliminary screening of the microalgae

Alga	**Specific growth rate, μ (d^{-1})**	**Maximum biomass concentration[a] ($g\ L^{-1}$)**	**Biomass productivity, P_b($mg\ L^{-1}\ d^{-1}$)**	**Lipid content (%, w/w)**	**Lipid productivity, P_l ($mg\ L^{-1}\ d^{-1}$)**
C. vulgaris	0.142	3.46	108.0	22.8	24.6
N. salina	0.132 ± 0.020	1.73 ± 0.02	45.9 ± 0.7	41.8 ± 0.3	19.2 ± 0.1
C. fusiformis	0.125 ± 0.010	1.47 ± 0.12	48.2 ± 6.0	-	-
[b]*C. fusiformis*	-	1.48	39.9	27.4	10.9
T. subcordiformis	0.223 ± 0.014	1.87 ± 0.10	54.6 ± 3.0	11.4 ± 0.9	6.2 ± 0.5

[a]Attained on day 24 for *C. vulgaris*, on day 36 for *N. salina*, on day 21 for *C. fusiformis*, and on day 31 for *T. subcordiformis*. Standard deviations are based on duplicate runs.

[b]Different batch (measurements were made at harvest on day 25).

In view of the data in Table 4.1, *T. subcordiformis* did not appear to be a promising candidate for oil production. Therefore no further work was done on this alga. Further studies were carried out on the other three algae in Table 4.1, to select a single alga for a more detailed study in pilot-scale raceway production system.

4.2.2 Effect of salinity

Salinity of the culture medium strongly affects the growth and survival of many algae. An open culture system such as an outdoor raceway will inevitably loose water by evaporation and this will increase salinity. Therefore, the effects of salinity mainly on

growth and lipid content of the selected microalgae (*C. vulgaris*, *N. salina* and *C. fusiformis*) were examined.

Three different concentrations of seasalt (40, 50, 60 g L^{-1}) were made in the BG 11 medium (Section 3.2.1) for a batch operation in Duran bottles as described in Section 3.2.2. The irradiance was 135-145 $\mu mol \cdot m^{-2}s^{-1}$. Identical inocula (15% v/v, prepared in standard BG11 seawater medium) were used for different salt concentration cultures for each individual strain and final working volume was 2 L.

4.2.2.1 Salinity tolerance of *C. vulgaris*

For *C. vulgaris*, a parallel batch of freshwater BG11 was also run along with the different salinities to compare the findings, as originally *C. vulgaris* is a freshwater strain. The inoculum for freshwater *C. vulgaris* culture was prepared in freshwater BG11 medium. The measured salinities at different seasalt concentrations are shown in Table 4.2.

The growth profiles are shown in Figure 4.2. The final biomass concentration and the specific growth rate (Table 4.3) (calculated from the data in Figure 4.2) declined with increasing salinity.

Biomass production of *C. vulgaris* in freshwater BG11 was 50% higher than the culture grown in normal seawater salinity as shown in Table 4.2. The alga was quite sensitive to salinity exceeding the normal seawater (37 ppt) level. For cultures started identically, but at different salinities, differences in the biomass concentrations were reflected in remarkable differences in the intensity of green color as shown in Figure 4.3.

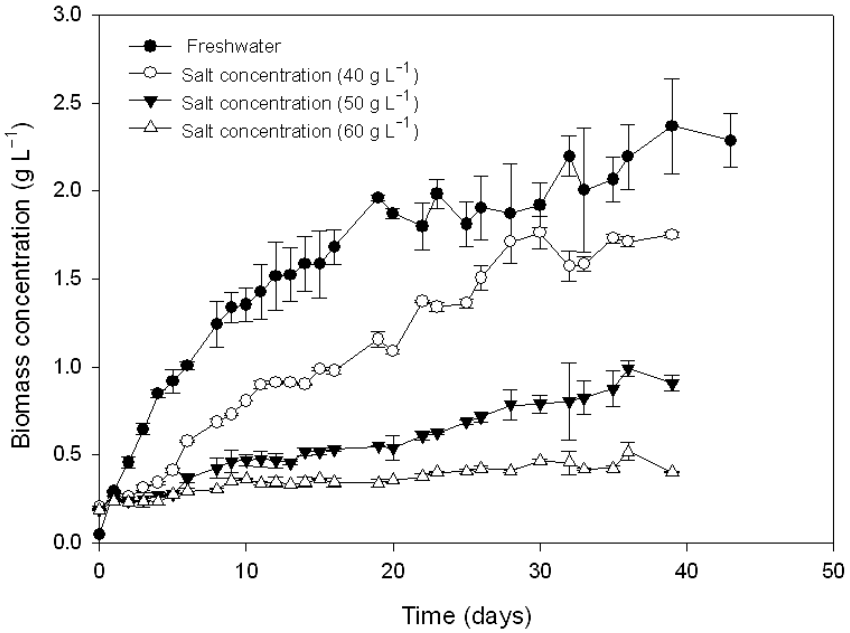


Figure 4.2 Growth profiles of *C. vulgaris* in freshwater and different seasalt concentrations. Standard deviations are based on duplicate runs except for the seasalt concentration of 40 g L^{-1}. The latter standard deviations are based on duplicate samples of a single run.

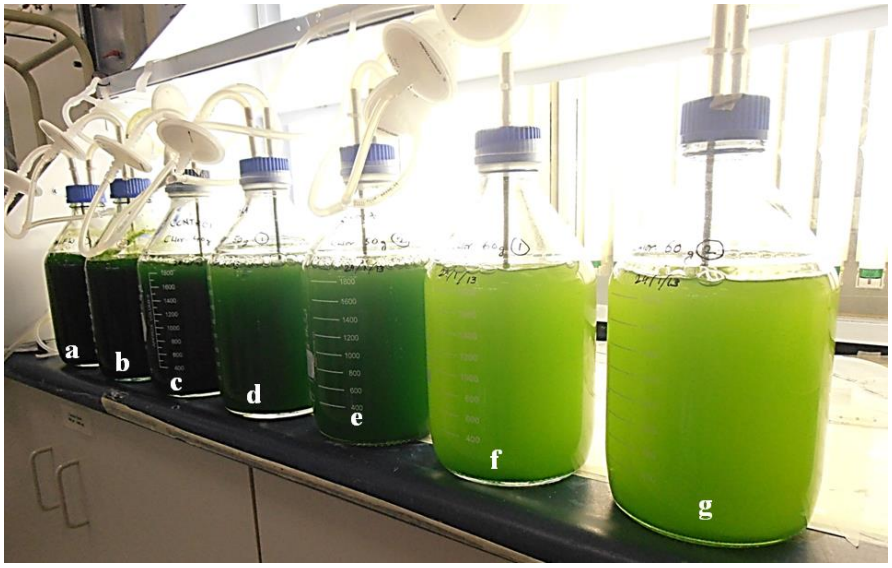


Figure 4.3 *C. vulgaris* cultures on day 23: (a, b) Freshwater culture in duplicate bottles; (c) culture with a salt concentration of 40 g L^{-1} (control, salinity 37 ppt); (d, e) cultures with salt concentrations of 50 g L^{-1} (salinity 46 ppt) in duplicates; (f, g) cultures with salt concentrations of 60 g L^{-1} (salinity 57 ppt) in duplicates. Low biomass concentration can be seen in cultures with a salt concentration of 60 g L^{-1}. The exact biomass concentrations are given in Figure 4.2.

Table 4.2 Biomass characteristics of *C. vulgaris* under different salinities

Seasalt concentration (g L^{-1})	**Salinity in the final medium (ppt)**	**Specific growth rate, μ (d^{-1})**	**Maximum biomass concentration**[a] **(g L^{-1})**	**Biomass productivity, P_b (mg L^{-1} d^{-1})**	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**	**Elements in biomass**			
								C (%)	**N (%)**	**S (%)**	**P (%)**
0 [b]	1≤	0.317 ± 0.02	2.37 ± 0.27	59.6 ± 6.9	22.2 ± 3.5	14.7 ± 0.0	24.9 ± 0.8	54.5 ± 1.2	6.4 ± 0.2	0.3 ± 0.0	0.2 ± 0.0
40 (Control)	37	0.175 ± 0.00	1.75 ± 0.02	39.7 ± 0.5	42.8 ± 0.5	17.0 ± 0.2	27.8	58.4	5.8	0.6	0.2
50	46	0.133 ± 0.05	0.91 ± 0.04	22.3 ± 1.1	41.4 ± 1.8	9.2 ± 0.4	27.4 ± 0.3	57.8 ± 0.6	6.0 ± 0.4	0.5 ± 0.0	0.3 ± 0.0
60	57	0.076 ± 0.03	0.40 ± 0.00	9.2 ± 1.3	19.6 ± 0.8	1.8 ± 0.1	ND	ND	ND	ND	ND

[a]Attained on day 39 for freshwater culture and control (40 g L^{-1}) culture and on day 36 for cultures grown in seasalt concentration of 50 g L^{-1} and 60 g $L^{-}1$;

all standard deviations are shown for duplicates runs, except for control (for the latter the standard deviations are based on duplicate samples of a single run).

[b]Freshwater culture; a salinity of ~1 ppt was measured.

ND = Not determined

Lipid contents of *C. vulgaris* grown in freshwater media and at different seasalt concentrations are shown in Table 4.2. The lipid contents of biomass from seawater cultures (i.e. within the salinity range of 37-46 ppt) were higher compared to the lipid content of the biomass of freshwater culture and the hypersaline culture (salinity of 57 ppt) (Table 4.2). Calorific value of the biomass of the seawater cultures was comparatively higher than the biomass produced in freshwater (Table 4.2). The C, N and S contents of the biomass samples produced at different salinities (salt concentration $\geq$40 g L^{-1}) were similar, but different relative to freshwater grown culture. The P content in the biomass was always 0.2-0.3% (Table 4.2). A positive correlation was consistently seen between the biomass calorific value, the lipid content and the C content. The correlation between these parameters is further discussed in Section 5.1.

Earlier studies on the effects of salinity on *C. vulgaris* (Abdel-Rahman *et al.*, 2005; Hiremath and Mathad, 2010) reported no effect on growth with increasing salt concentration of up to 14 g L^{-1}, but higher salt concentration decreased growth. The maximum salt concentration tested was 23 g L^{-1}, well below the seawater salinity level. The data in Figure 4.2 show that although the alga grew best in the freshwater, it could grow in full strength seawater. Salinities of greater than seawater adversely affected growth. The lipid accumulation in *C. vulgaris* grown in BG11 saline media (i.e., salt concentration of 40 g L^{-1} and 50 g L^{-1}) was ~1.9 times higher than in *C. vulgaris* grown in freshwater (Table 4.2) after 39 days of culture.

4.2.2.2 Salinity tolerance of *N. salina*

N. salina was grown at various salinities (37-57 ppt) in the BG11 nutrient media as described earlier in Section 4.2.2. The growth profiles of the alga and culture set up are shown in Figure 4.4 and Figure 4.5, respectively. The characteristics of the biomass produced at various salinities are shown in Table 4.3.

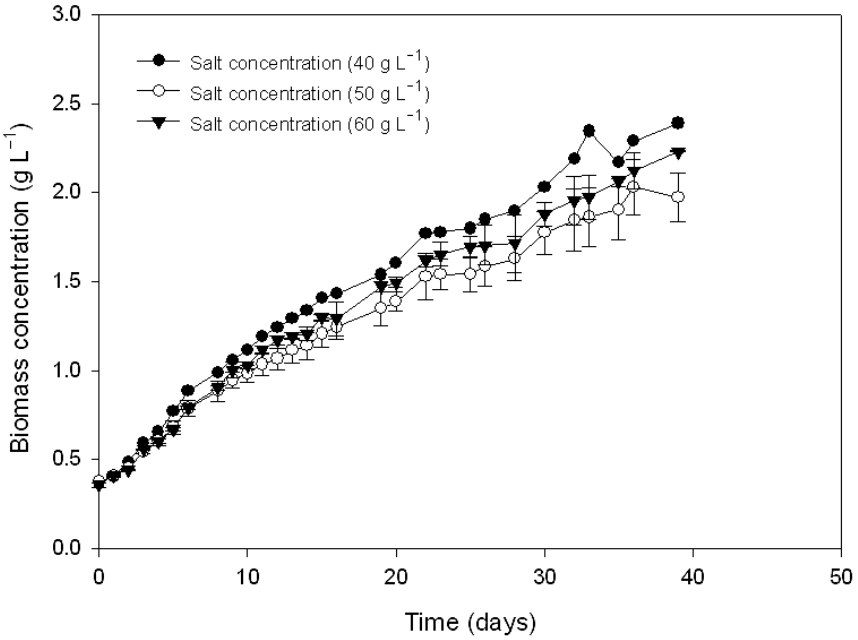


Figure 4.4 Growth profiles of *N. salina* under different seasalt concentrations. Standard deviations are based on duplicate runs.

Figure 4.5. *N. salina* cultures on day 23: (a, b) cultures with a salt concentration of 60 g L^{-1} (salinity 57 ppt) in duplicates; (c, d) cultures with salt concentrations of 50 g L^{-1} (salinity 47 ppt) in duplicates; (e) culture with a salt concentration of 40 g L^{-1} (control, salinity 37 ppt).

Table 4.3 Biomass characteristics of *N. salina* grown under different salinities

Seasalt concentration ($g\ L^{-1}$)	**Salinity in the final medium (ppt)**	**Specific growth rate, μ (d^{-1})**	**Maximum biomass concentration[a] ($g\ L^{-1}$)**	**Biomass productivity[a], P_b ($mg\ L^{-1}\ d^{-1}$)**	**Lipid content[a] (%, w/w)**	**Lipid productivity[a], P_l ($mg\ L^{-1}\ d^{-1}$)**	**Calorific value[a] ($kJ\ g^{-1}$)**	**Elements in biomass[a]**			
								C (%)	**N (%)**	**S (%)**	**p (%)**
40 (Control)	37	0.154	2.39	51.8	43.1 ± 0.5	22.3 ± 0.2	26.8	55.8	7.8	0.5	0.2
50	47	0.108± 0.02	1.97 ± 0.14	40.9 ± 3.5	46.6 ± 0.9	19.1 ± 0.4	27.5 ± 0.1	57.2 ± 0.1	7.7 ± 0.2	0.5 ± 0.0	0.2 ± 0.0
60	57	0.145 ± 0.01	2.23 ± 0.00	48.1 ± 0.5	46.0 ± 3.6	22.1 ± 1.7	27.9 ± 0.4	58.3 ± 0.4	7.1 ± 0.2	0.5 ± 0.0	0.2 ± 0.0

[a]On day 39; standard deviations are shown for duplicate runs, except for the control (for the latter the lipid estimation is based on duplicate measurements of a single run).

The growth of this marine alga was not substantially affected by increasing salinity levels of up to 57 ppt (Table 4.3). Within the salinity range of 37-57 ppt, alga appeared to be halotolerant and able to grow and produce lipids (Table 4.3). The other characteristics of algal biomass such as the calorific value and the elemental composition (C, N, S and P) were unaffected within the specified range of salinity (Table 4.3).

4.2.2.3 Salinity tolerance of *C. fusiformis*

C. fusiformis was also found to be quite halotolerant as shown in Figure 4.6. The maximum biomass concentrations attained in the salinity range of 37-57 ppt were quite comparable (Figure 4.6, Table 4.4). Visually, the cultures grown at different salinity levels were similar (Figure 4.7).

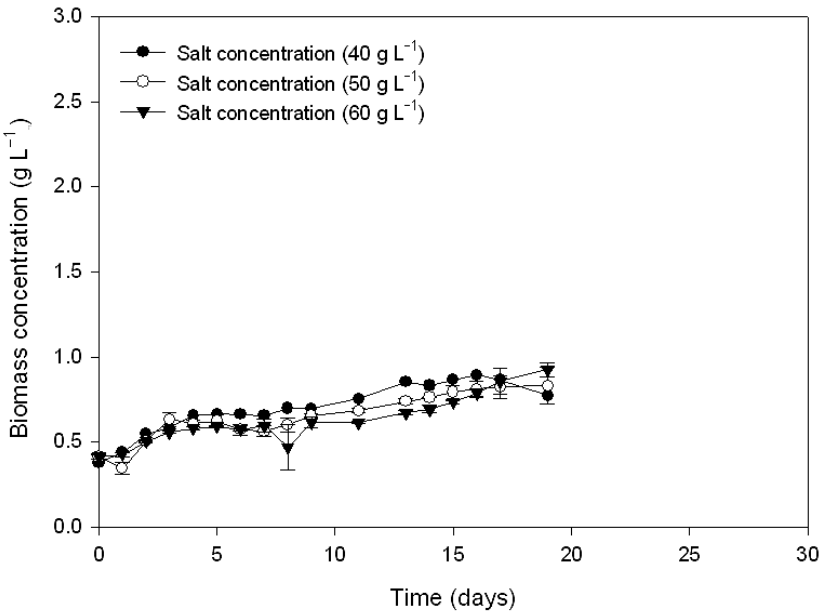


Figure 4.6 Growth profiles of *C. fusiformis* under different seasalt concentrations. Standard deviations are based on duplicate runs.

Figure 4.7 *C. fusiformis* cultures on day 4: (a) culture with a salt concentration of 40 g L^{-1} (control, salinity 37 ppt); (b, c) cultures with salt concentrations of 50 g L^{-1} in duplicates (salinity 47 ppt); (d, e) cultures with salt concentrations of 60 g L^{-1} (salinity 57 ppt).

Table 4.4 Biomass characteristics of *C. fusiformis* grown under different salinities[a]

Seasalt concentration (g L^{-1})	**Salinity in the final medium (ppt)**	**Specific growth rate, μ (d^{-1})**	**Final biomass concentration (g L^{-1})**	**Biomass productivity, P_b (mg L^{-1} d^{-1})**	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**
40 (Control)	37	0.134	0.89	32.3	12.3	4.0	ND
50	47	0.320± 0.08	0.83 ± 0.11	22.0 ± 4.8	16.4 ± 4.3	3.6 ± 0.9	19.9
60	57	0.142 ± 0.03	0.92 ± 0.04	26.8 ± 1.6	14.8 ± 2.4	4.0 ± 0.7	ND

[a]Attained on day 16 for control and day 19 for the other cultures; standard deviations are shown for duplicate runs except for the control sample.

The lipid contents of the *C. fusiformis* biomass (Table 4.4) were not too much affected by the different salt concentrations. This alga is known for its tolerance to a broad range of salinities in its natural environment (Paul, 1979). However, this alga was not studied further due to difficulty growing it to a high concentration as explained in Section 4.2.1.

In the approximate salinity range of 37-57 ppt, all microalgae tested here were quite salt tolerant. The highest productivity of biomass in *C. vulgaris* was achieved at the normal seawater salinity (~37 ppt) whereas the biomass productivities of *N. salina* and *C. fusiformis* remained high within the tested salinity range.

The adaptability to an elevated salt concentration varies in different species of microalgae (Hiremath and Mathad, 2010). Growth rate generally decreases with increasing salt concentration above the standard seawater level in marine microalgae. A decrease in growth rates with increasing salinity was seen in the case of *C. vulgaris* in this study, but growth rates of the other two halotolerant algae (i.e. *N. salina* and *C. fusiformis*) were not too much affected by increase in salinity. Reduction in growth rate at higher salinities has been associated with a reduced rate of photosynthesis (Vonshak and Richmond, 1981; Gilmour *et al.*, 1984; Ben-Amotz *et al.*, 1985; Kirst, 1989; Endo *et al.*, 1995). The algal cells respond to salinity induced changes in osmotic pressure, either by changing the internal ionic ratios or by degrading or accumulating organic solutes such as glycerol, mannitol, sucrose, glycosides and the amino acid proline (Hellebusi, 1976; Kauss, 1977; Ben-Amotz and Avron, 1983; Wegmann, 1986).

In the cyanobacterium *Spirulina platensis*, changes in salinity are known to affect the biosynthesis of low molecular weight carbohydrates (Warr *et al.*, 1985) and a high salinity induces lipid accumulation in microalgae such as *Dunaliella tertiolecta*, *Isochrysis* sp. and *Nannochloropsis oculata* (Renaud and Parry, 1994; Takagi *et al.*,

2006). In the present study, a salinity of ~57 ppt inhibited lipid accumulation in *C. vulgaris,* but lipid accumulation in *N. salina* and *C. fusiformis* was unaffected with changes in salinity in the range tested.

4.2.3 Effect of nutrients concentration

4.2.3.1 Effect of initial phosphate concentration

Phosphorus is an important macronutrient that is necessary for algal growth and metabolism. The aim of this study was to ascertain whether phosphate concentration of the normal BG 11 seawater medium was limiting. *C. vulgaris* and *N. salina* were used in this study. The algae were grown in parallel in the standard BG11 seawater medium and the same medium formulated to have twice the normal concentration of phosphate.

4.2.3.1a *C. vulgaris*

C. vulgaris batch cultures were grown in two 2 L Duran bottles. One of the bottles contained the standard BG11 seawater medium and the other bottle contained the same medium supplemented to have twice the normal initial phosphate concentration. All other growth conditions were identical (continuous fluorescent illumination at 125 $\mu mol \cdot m^{-2} s^{-1}$, 22.4 ± 2 °C, salinity of 37.5 ppt, 5% (v/v) CO_2 in air). Both bottles were inoculated (17% (v/v) inoculum) using the same inoculum that had been grown in the standard BG11 seawater medium. The culture volume was 1.8 L in both cases. Sampling was done once a day for measuring growth and consumption of phosphate and nitrate.

The culture profiles are shown in Figure 4.8. A quantitative comparison of the culture kinetic parameters is shown in Table 4.5. A doubling of the phosphate concentration did not affect the average growth rate of *C. vulgaris* and the final biomass concentration relative to control. The peak biomass concentrations attained at day 23

were essentially the same, i.e. 3.37 g L^{-1} in control and 3.40 g L^{-1}, in the phosphate supplemented medium. Similarly, the biomass productivity values were comparable: 128.6 mg L^{-1} d^{-1} (control) and 127.1 mg L^{-1} d^{-1} for the high phosphate culture (Table 4.5). Specific growth rate, however, was 35% higher in the high phosphate culture compared to the control bottle (Table 4.5).

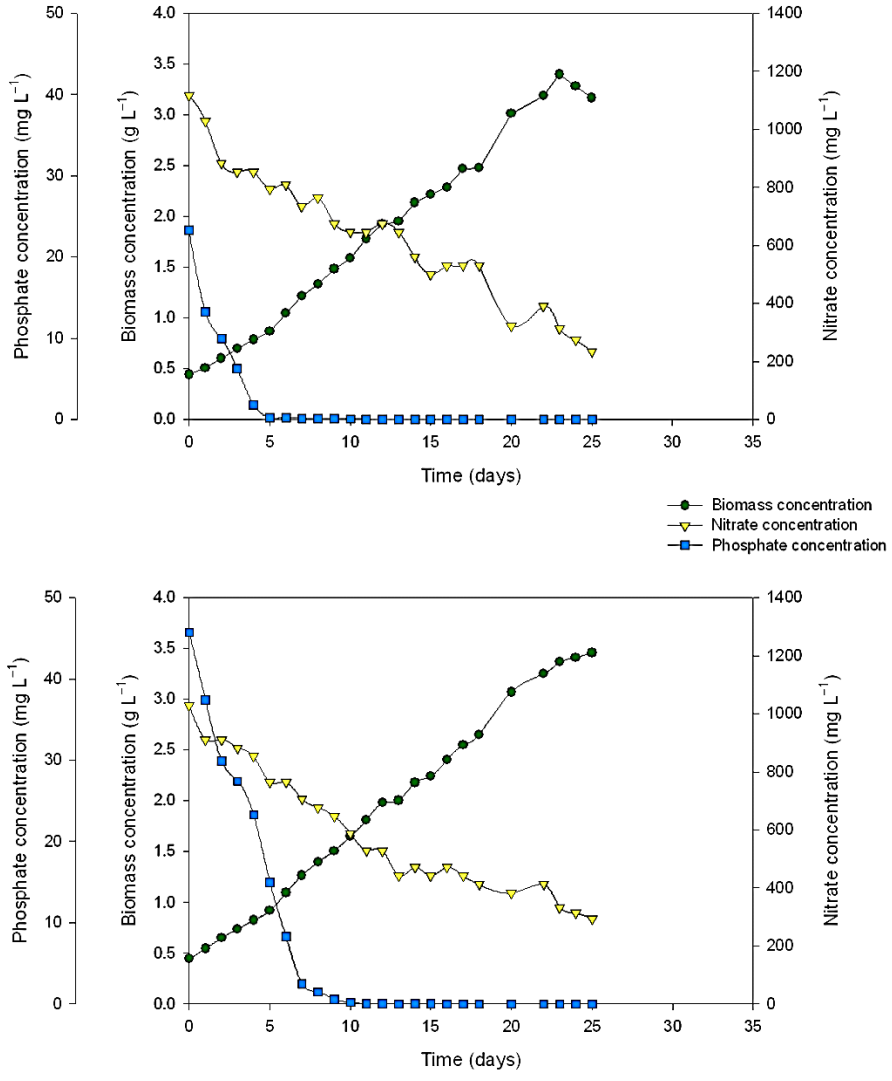


Figure 4.8 Growth and nutrient consumption profiles of *C. vulgaris* in Duran bottles: a) control, i.e. the standard phosphate concentration in BG11 seawater medium; b) twice the normal initial phosphate concentration in the BG11 seawater medium.

In the control culture, the phosphate was consumed by day 5 whereas in the high phosphate culture, phosphate remained in the medium until day 10 (Figure 4.8). In both cases, the alga continued to grow normally until the end of growth on day 24. Both cultures took up phosphate quantitatively, but had similar levels of the final biomass concentrations. Therefore, the biomass yield on P in phosphate supplemented culture was only ~50% of the yield in the control culture Table 4.5.

Nitrate consumption profiles for the two cases (Figure 4.8) were comparable and at harvest >24% of the nitrate remained in the culture broth.

Table 4.5 *C. vulgaris* Duran bottle batch culture kinetics (different phosphate concentrations)

Kinetic parameters[1]	**Duran bottle-1**[a]	**Duran bottle-2**[b]
Specific growth rate, μ (d^{-1})	0.113	0.161
Maximum biomass concentration (g L^{-1})	3.40	3.37
Biomass productivity, P_b (mg L^{-1} d^{-1})	128.6	127.1
Biomass yield coefficient on N, Y_N (g mg^{-1})	0.004	0.004
Biomass yield coefficient on P, Y_P (g mg^{-1})	0.127	0.064
Average N consumption rate, q_N (mg g^{-1} d^{-1})	11.8	10.4
Average P consumption rate, q_P (mg g^{-1} d^{-1})	0.3	0.7
Lipid content (%, w/w)	6.5	9.2
Lipid productivity, P_l (mg L^{-1} d^{-1})	8.4	11.7

[1]Kinetic parameters were calculated at the point of peak biomass concentration attained on day 23

[a]Duran bottle with standard phosphate concentration in BG11 seawater medium; [b]twice the normal initial phosphate level in BG11 seawater medium.

In view of the results, the normal BG11 seawater medium is phosphate sufficient for attaining biomass concentrations in excess of 3 g L^{-1}. The lipid content of the biomass and the lipid productivity (Table 4.5) were not substantially influenced by a doubling of the initial phosphate concentration in media that were phosphate sufficient.

4.2.3.1b *N. salina*

The culture profiles of *N. salina* in the control medium and the phosphate enriched medium are shown in Figure 4.9. The cultures were grown in otherwise identical conditions (continuous fluorescent illumination at 116 μmol·$m^{-2}s^{-1}$, 22.4 ± 2 °C, salinity of 37.5 ppt, 5% (v/v) CO_2 in air, inoculum level of 20% (v/v)). The culture volume was 2 L in each bottle. Culture bottles were set up in duplicates for each medium. A quantitative comparison of the culture kinetic parameters in the two media is provided in Table 4.6.

As was seen in the previous section for *C. vulgaris* (Section 4.2.3.1a), phosphate supplementation of the medium did not improve the final biomass concentration (Figure 4.9, Table 4.5), the biomass productivity and the specific growth rate of *N. salina*. Lipid content of the biomass and the lipid productivity were unaffected by phosphate supplementation (Table 4.6) as both media were phosphate sufficient. Phosphate supplementation reduced the biomass yield on phosphate by ~50% relative to control. In both cases, the phosphate had been fully consumed by the culture at day ≥10 (Figure 4.9). Both sets of cultures (Figure 4.9) were always nitrate sufficient.

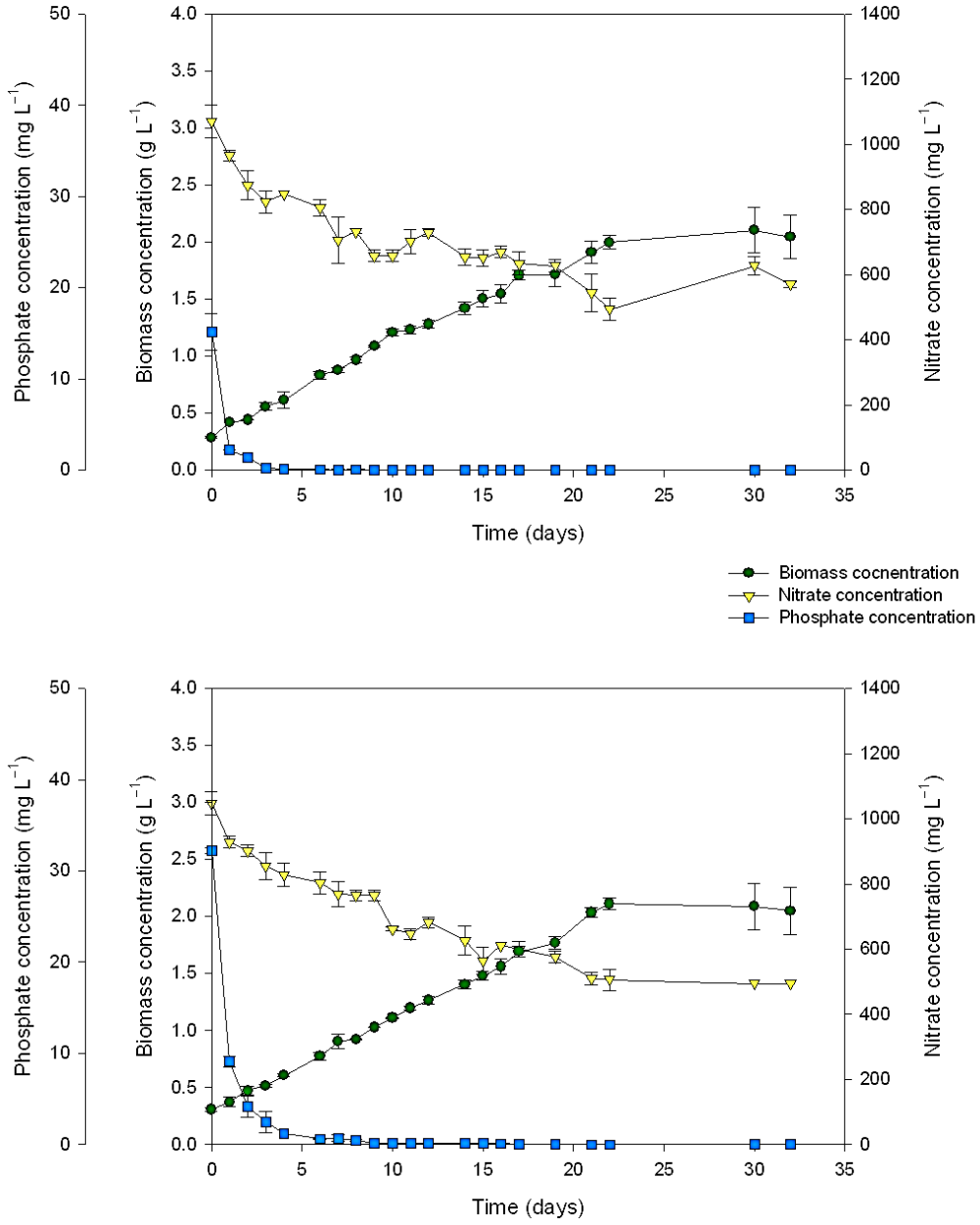


Figure 4.9 Growth and nutrient consumption profiles of *N. salina* in Duran bottles: a) control, i.e. standard phosphate concentration in BG11 seawater medium; b) twice the normal initial phosphate level in BG11 seawater medium. Standard deviation of biomass is based on duplicate runs. Standard deviations of nitrate and phosphate are based on triplicate and duplicate samples of a single run, respectively.

Table 4.6 *N. salina* Duran bottle batch culture kinetics (different phosphate concentrations)

Kinetic parameters[1]	**Duran bottle-1**[a]	**Duran bottle-2**[b]
Specific growth rate, μ (d^{-1})	0.181 ± 0.03	0.138 ± 0.01
Maximum biomass concentration (g L^{-1})	2.22 ± 0.21	2.20 ± 0.21
Biomass productivity, P_b (mg L^{-1} d^{-1})	81.2 ± 3.2	86.4 ± 3.0
Biomass yield coefficient on N, Y_N (g mg^{-1})	0.003	0.004
Biomass yield coefficient on P , Y_P (g mg^{-1})	0.119	0.059
Average N consumption rate, q_N (mg g^{-1} d^{-1})	14.5	12.9
Average P consumption rate, q_P (mg g^{-1} d^{-1})	0.4	0.8
Lipid content (%, w/w)	37.3 ± 0.2	40.4 ± 1.3
Lipid productivity, P_b (mg L^{-1} d^{-1})	30.3 ± 2.1	34.9 ± 2.3

[1]Kinetic parameters were calculated at the point of peak biomass concentration attained on day 30

[a]Duran bottle with standard phosphate concentration in BG11 seawater medium; [b]twice the normal initial phosphate level in BG11 seawater medium. Standard deviations are shown for duplicate runs.

The conclusion of the results of this section is that BG11 seawater medium is phosphate sufficient for attaining a biomass concentration of ≥3 g L^{-1} of *C. vulgaris* and at least 2.5 g L^{-1} for *N. salina*.

Earlier studies have shown that the excess phosphate in a culture medium is taken up and stored within the cell by microalgae as polyphosphate granules (Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi *et al.*, 1964; Cembella *et al.*, 1984; John and Flynn, 2000). Furthermore, it has been shown that the phosphate starved

microalgal cells transferred to a phosphate rich medium readily absorb and store phosphate (Hernandez *et al.*, 2006). The stored phosphate is used for growth after the phosphate in the medium has been depleted (Miyachi *et al.*, 1964; Kanai *et al.*, 1965; Elgavish and Elgavish, 1980). Cell division and growth continues for many generations on stored phosphate (Jansson, 1988; John and Flynn, 2000). Thus, the rapid decline in phosphate level upon inoculation (Figure 4.8, Figure 4.9) is attributed to rapid absorption of phosphate by cells transferred from the previous stage of culture that was approaching phosphate depletion.

4.2.3.2 Effect of initial nitrate concentration

Nitrogen is essential for algal growth. Nitrogen limitation in the medium affects the cell growth rate and the composition of the biomass. Nitrogen limitation frequently leads to accumulation of lipids (Piorreck *et al.*, 1984; Sawayama *et al.*, 1992; Illman *et al.*, 2000; Scragg *et al.*, 2002; Griffiths and Harrison, 2009). The need was to determine if a low nitrate concentration could induce lipid accumulation without significantly affecting the biomass production. For this, the cultures of *C. vulgaris* and *N. salina* were carried out at different low initial nitrate concentrations and compared with the controls (standard concentration of nitrate in BG11 seawater media). Effect of a reduced initial nitrate was tested under continuous light as well as in a 12 h:12 h light/dark cycle.

4.2.3.2a *C. vulgaris*

C. vulgaris batch cultures were grown in BG11 (seawater) media with 100%, 50%, 20% and 10% of the normal initial nitrate concentration. All cultures were grown under continuous light as well as under a 12 h:12 h day/night cycle at an incident irradiance of 138-142 $\mu mol \cdot m^{-2} s^{-1}$. All experiments were conducted in duplicate in Duran bottles. All other growth conditions were identical (22.4 ± 2 °C, salinity of 37.5 ppt, aeration with

5% (v/v) CO_2 in air). All Duran bottles were inoculated with a 13% (v/v) inoculum that had been produced in the normal BG11 seawater medium. Total volume of the culture broth was 2 L in all cases. Sampling was done once a day, or on alternate days, for measuring growth. Samples for measurements of nitrate and phosphate were taken every 5^{th} day. The cultures under light/dark cycle were not sampled for nitrate and phosphate, except for the control bottle culture (normal nitrate concentration in BG11 seawater medium).

As the pigment composition of microalgal cells can change with time under nitrate-limited conditions (Ben-Amotz *et al.*, 1982; Sheehan *et al.*, 1998) and this can potentially affect the spectrophotometric biomass measurements, separate calibration curves were prepared every fifth day of culture to rule out possible discrepancies in biomass measurements due to changed pigmentation (see Section 3.3.1). The calibration curves obtained at various initial nitrate concentrations were essentially identical and did not vary with time. However, the calibration curves obtained under continuous light differed from those obtained under the 12 h: 12 h light/dark cycle, by ~16%. Therefore, two separate sets of calibration curves (Figure 3.15, Figure 3.16) were prepared for the different illumination regimes by averaging the calibration curve data for a given regime at different initial nitrate levels.

The culture profiles of *C. vulgaris* are shown in Figure 4.10 for the two light regimes and various initial nitrate levels. There was no obvious effect on growth of the various initial nitrate concentrations so long as the concentration was ≥50% of the control level. For example, the peak biomass concentration of the control culture on day 46 was 2.45 ± 0.01 g L^{-1} (Table 4.7), or nearly the same as a biomass concentration of 2.33 ± 0.08 g L^{-1} for the culture with 50% of the normal initial nitrate level. The peak attainable biomass concentration progressively declined (Table 4.7) once the initial nitrate level was reduced to <50% of the normal level. In culture with 50% of the initial

nitrate, nitrate ran out on day 20 (Figure 4.10) but slow growth continued until day 40, presumably on internal nitrogen reserves. In cultures with ≤20% of the initial nitrate level, nitrogen ran out by day 10 and the growth slowed. For otherwise identical conditions, continuous illumination provided a higher final concentration of biomass and a higher biomass productivity compared to 12 h: 12 h light dark/cycle (Table 4.7).

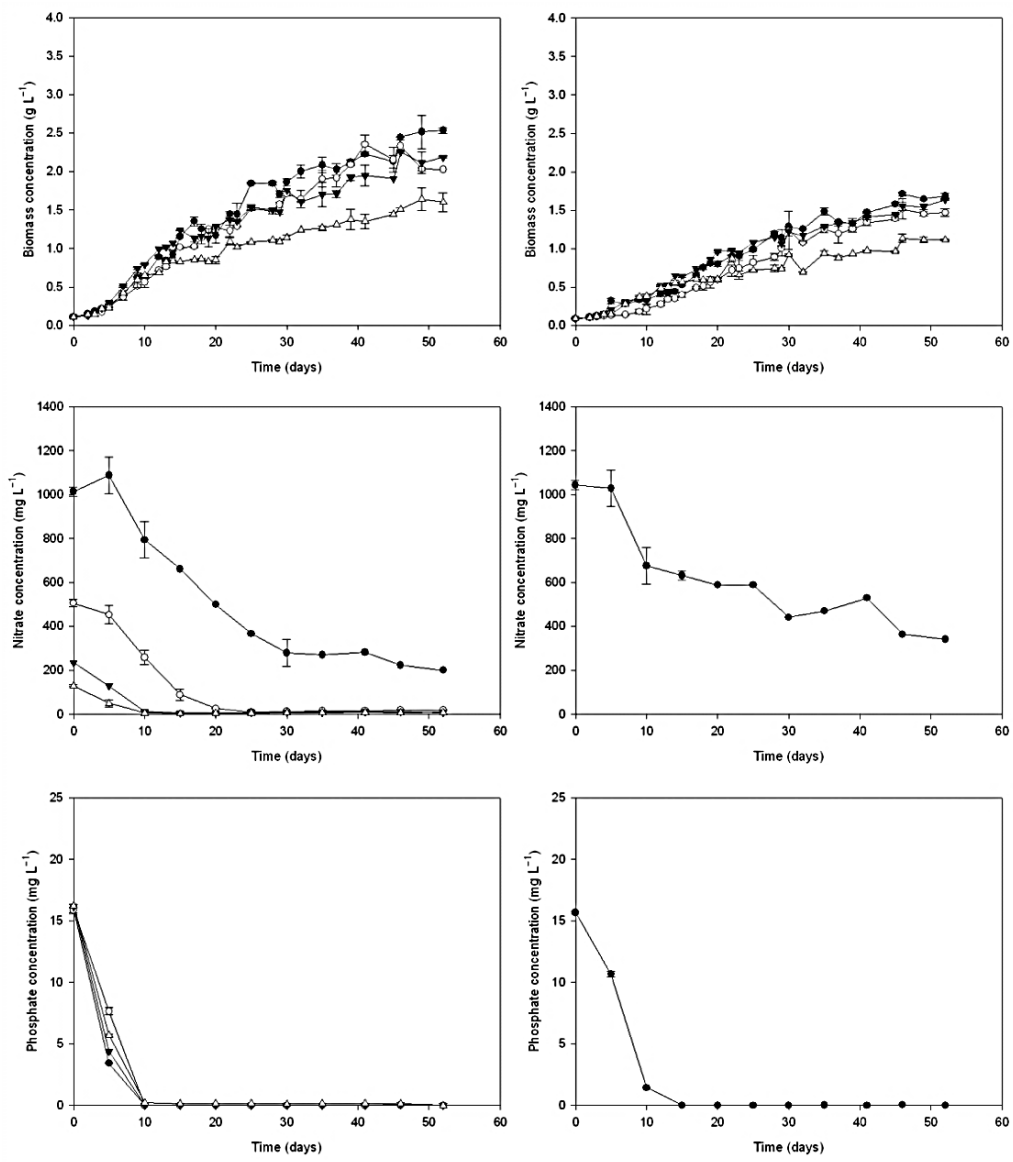


Figure 4.10 Growth and nutrient consumption profiles of *C. vulgaris* in Duran bottles with different initial nitrate concentrations of 100% (control), 50%, 20% and 10% in BG11 seawater medium: a-1) and b-1) biomass concentrations; a-2) and b-2) nitrate concentration; a-3) and b-3) phosphate concentration. All 'a' culture profiles were obtained under continuous light. All 'b' culture profiles were obtained under a 12 h:12 h light/dark cycle. In b-2 and b-3 the N and P consumption profiles were obtained only from the control bottles. Biomass standard deviations are shown for duplicate runs, except for the control. The standard deviation for the control are based on duplicate measurements of a single run. Standard deviations of nitrate and phosphate are based on duplicate samples of single run.

In control cultures, nitrate was not fully consumed by harvest (day 46; Figure 4.10), but complete consumption occurred by day 25 or earlier, if the initial nitrate level was ≤50% (Figure 4.10, a-2). More biomass was produced in continuous light (Table 4.7) because photosynthesis occurred for nearly twice as long as in the 12 h:12 h light/dark cycle condition. In nitrate-limited media with 20% and 50% of initial nitrate levels relative to control, although nitrate ran out from the media within 10-25 days, growth continued until day 46 (Figure 4.10, a-1 and b-1) without a significant loss in the final biomass concentration (Table 4.7). In the culture with 10% initial nitrate level relative to control, nitrate ran out on day 10, but growth continued at a somewhat reduced pace (Figure 4.10). The main reason for continued growth in the nitrate-limited medium was a continuous flow of carbon (from CO_2) to form starch (and lipids) via photosynthesis but reduced cell division as a consequence of a reduced protein synthesis and DNA synthesis (Hu, 2004). As confirmed later in this study, in nitrogen limitation in *C. vulgaris*, lipids accumulation was increased (Table 4.8) with a simultaneous increase in the C content of the algal biomass (Table 4.8) when compared to the nitrate-sufficient growth.

N uptake rates were directly correlated to the initial nitrate concentration in the media. A higher initial nitrate concentration, increased nitrate consumption rate (Table 4.7). These uptake rates (Table 4.7) were measured in the cultures grown under continuous light. Under light/dark cycled condition, the nitrate consumption profiles of only the control cultures (nitrate sufficient BG11 media) were obtained and the biomass specific uptake rate of nitrate was virtually the same as that of continuously illuminated culture (Table 4.7). This meant that although less biomass was produced under reduced illumination in the control medium, the nitrogen content of the biomass were essentially the same as for the biomass grown in the same medium under continuous light. The biomass yield on N changed with changes in initial concentration of nitrate in the

growth media. Biomass yield on N was higher (3.6 times) in the medium that was the most deficient in nitrate (10% nitrate relative to normal BG11) (Table 4.7).

The biomass specific phosphate uptake rate was independent of the nitrate concentration in the media and was generally similar in all cultures (Table 4.7). On day 10, all phosphate disappeared from culture broths run under continuous light and this occurred later (on day 15) in light/dark cycled cultures (Figure 4.10, a-3 and b-3). This was simply a consequence of slower biomass growth in light/dark cycled culture relative to otherwise identical continuously illuminated cultures.

Table 4.7 *C. vulgaris* Duran bottles culture kinetic parameters (different initial nitrate concentrations in BG11 seawater medium)[1]

Initial nitrate concentration (% of normal)	**Light period[2]**	**Specific growth rate, μ (d^{-1})**	**Maximum biomass concentration (g L^{-1})**	**Biomass Productivity, P_b (mg L^{-1} d^{-1})**	**Biomass yield coefficient on N, Y_N (g mg^{-1})**	**Biomass yield coefficient on P, Y_P (g mg^{-1})**	**Average specific N consumption rate, q_N (mg g^{-1} d^{-1})**	**Average specific P consumption rate, q_P (mg g^{-1} d^{-1})**
100 (control)	Continuous	0.180 ± 0.01	2.45 ± 0.01	50.9 ± 0.1	0.003	0.148	7.4	0.1
100 (control)	12 h:12 h	0.116 ± 0.00	1.71 ± 0.03	35.3 ± 0.6	0.002	0.107	8.8	0.2
50	Continuous	0.181 ± 0.03	2.33 ± 0.08	48.3 ± 1.9	0.005	0.140	4.8	0.2
50	12 h:12 h	0.157 ± 0.04	1.52 ± 0.13	31.0 ± 2.9	ND	ND	ND	ND
20	Continuous	0.298 ± 0.03	2.26 ± 0.02	46.7 ± 0.3	0.010	0.133	2.3	0.2
20	12 h:12 h	0.126 ± 0.02	1.55 ± 0.05	31.8 ± 1.3	ND	ND	ND	ND
10	Continuous	0.250 ± 0.06	1.51 ± 0.02	30.4 ± 0.4	0.011	0.087	1.9	0.2
10	12 h:12 h	0.172 ± 0.09	1.14 ± 0.05	22.7 ± 1.0	ND	ND	ND	ND

[1]Kinetic parameters were calculated at the point of peak biomass concentration attained on day 46; ND = not determined. Standard deviations are shown for duplicate runs, except for control. For the latter the standard deviations are based on duplicate samples of a single run.

[2] Irradiance ~138-142 µmol·$m^{-2}s^{-1}$ continuously for 24 h or 12 h of light period followed by 12 h of dark period.

Table 4.8 Lipid content, calorific value and elemental content of *C. vulgaris* biomass (different initial nitrate concentrations)

Initial nitrate concentration (% of normal)	**Light period[1]**	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**	**Elements in biomass**			
					C (%)	**N (%)**	**S (%)**	**P (%)**
100 (control)	Continuous	38.9 ± 0.9	19.8 ± 0.5	27.3	56.2	6.4	0.7	0.2
100 (control)	12 h:12 h	29.2 ± 3.3	10.3 ± 1.0	25.0	53.5	7.1	0.7	0.3
50	Continuous	56.0 ± 1.8	27.4 ± 0.3	30.1 ± 0.5	61.8 ± 0.3	4.7 ± 0.2	0.5	0.2
50	12 h:12 h	32.3 ± 2.4	10.3 ± 1.8	25.7 ± 0.9	54.3 ± 1.1	6.9 ± 0.5	0.7 ± 0.0	0.3 ± 0.0
20	Continuous	65.6 ± 1.4	30.6 ± 0.5	31.3 ± 0.1	63.6 ± 0.0	3.6 ± 0.2	0.4 ± 0.0	0.2 ± 0.0
20	12 h:12 h	56.9 ± 1.5	18.1 ± 1.2	30.2 ± 0.6	61.5 ± 0.2	3.8 ± 0.4	0.5 ± 0.0	0.3 ± 0.0
10	Continuous	64.6	19.6	31.7 ± 0.1	63.8 ± 0.3	2.9 ± 0.4	0.4	0.2
10	12 h:12 h	62.5 ± 1.5	14.2 ± 0.3	30.8 ± 0.1	62.5 ± 0.0	3.7 ± 0.1	0.4 ± 0.0	0.4 ± 0.0

[1]Irradiance ~138-142 µmol·$m^{-2}s^{-1}$ continuously for 24 h or 12 h of light period followed by 12 h of dark.

Standard deviations are shown for duplicate runs, except for control. For the latter duplicate samples of a single run were used to calculate the standard deviations.

N limited growth resulted in elevated lipid content in the biomass (Table 4.8). In continuously illuminated cultures the lipid content increased by 44-66% relative to the N-sufficient controls. In light/dark cycled cultures, the lipid content increased by 10-114% relative to N-sufficient cultures. However, due to less biomass production under light/dark cycling, the average lipid productivity was lower relative to the corresponding continuously illuminated cases (Table 4.8).

The calorific values of the biomass were positively correlated with the lipid content (Table 4.8). Nitrate starvation enhanced the lipid content as well as the calorific value (Table 4.8). The highest measured calorific value was ~31 kJ g^{-1}. The C, N, S and P contents of the biomass grown under various conditions are shown in Table 4.8. Low N and S contents were found in the biomass produced in low-nitrate media. Low nitrogen levels reduced protein-synthesis and this also resulted in low S levels even though the S content of the medium were not changed in any experiment. The relevance of the elemental composition of the biomass and its energy content is discussed in Section 4.3.1.1.

In view of the results in Table 4.8, a 20% initial nitrate concentration relative to the standard BG11 seawater medium is promising for producing a biomass concentration (2.26 ± 0.02 g L^{-1}) which is comparable to the control (2.45 ± 0.01 g L^{-1}). However, the lipid level was the highest under nitrate deficient condition (20% of normal initial nitrate level); i.e. ~66% lipid content in the biomass and a lipid productivity of ~31 mg L^{-1} d^{-1} compared to the control productivity ~20 mg L^{-1} d^{-1} in Duran bottles under continuous light. Production of the biomass and therefore the lipid production was reduced by light/dark cycling relative to the case for continuous illumination.

4.2.3.2b *N. salina*

The culture profiles of *N. salina* in media with different initial N-levels are shown in Figure 4.11 both for continuous light and 12 h:12h light/dark condition cycle. All cultures were grown at otherwise identical conditions of irradiance of 135-145 $\mu mol \cdot m^{-2} s^{-1}$, 22.4 ± 2 °C, salinity of 37.5 ppt and 5% (v/v) CO_2 in air. The initial culture volume was 2 L in each bottle and the inoculum level was ~13% (v/v). All inocula were grown in the standard BG11 seawater medium. All cultures were run in duplicates.

Under continuous light, the highest biomass concentration of 2.93 ± 0.00 g L^{-1} was attained on day 49 in nitrate sufficient medium. Lower initial nitrate concentrations of 50% and 20% of control were still capable of providing biomass concentrations of 2.72 ± 0.11 g L^{-1} and 2.42 ± 0.12 g L^{-1}, respectively (Table 4.9). However, the culture grown under 10% of the initial nitrate concentration relative to control had a 35% lower final biomass concentration relative to control (Table 4.9). At identical initial nitrate concentrations, the 12 h:12 h light/dark cycling decreased biomass concentrations by 31-40% compared to continuous light cultures. This was explained in Section 4.2.3.2a for *C. vulgaris*. The culture kinetic data for different initial nitrate levels and light conditions are compiled in Table 4.9.

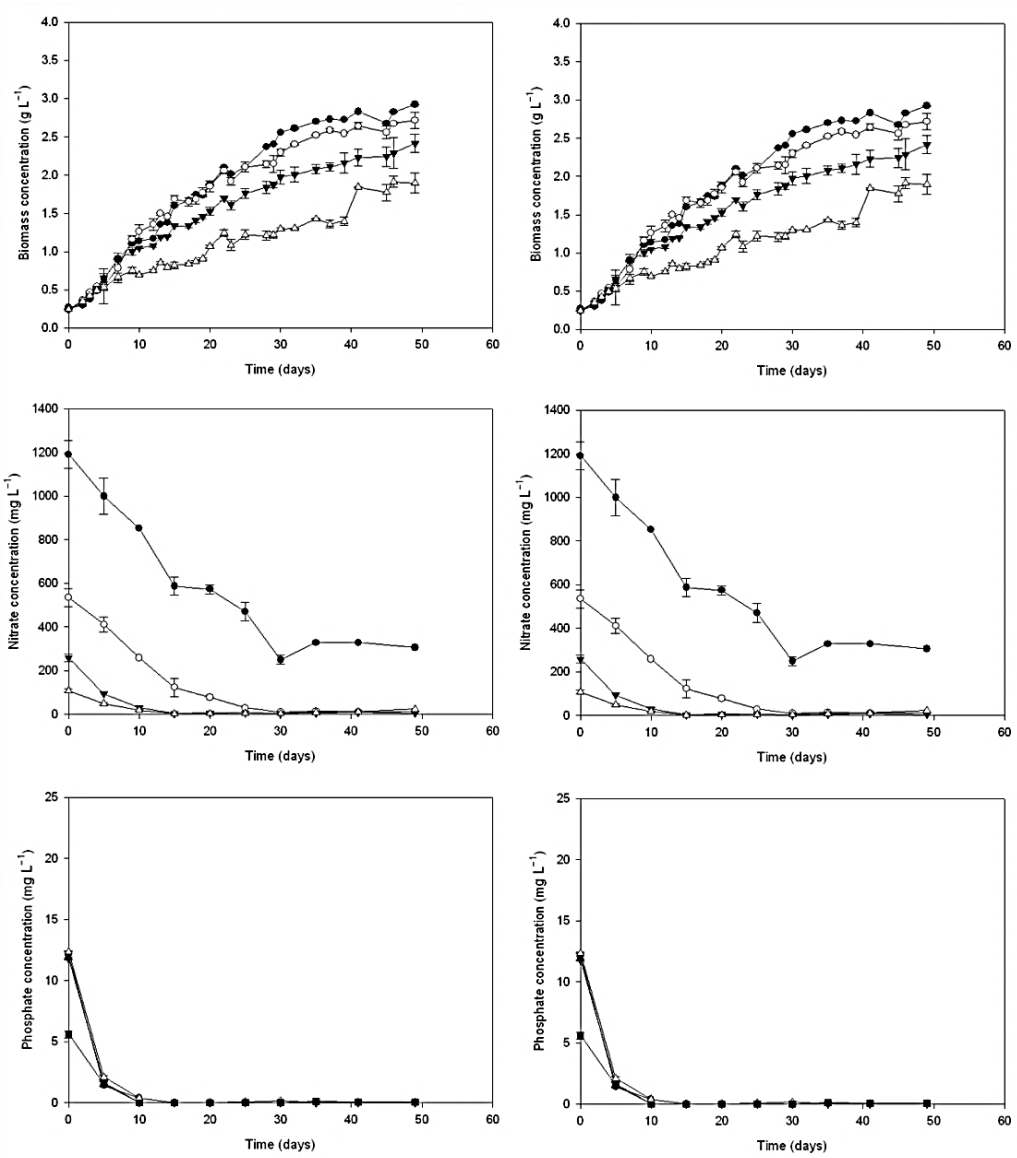


Figure 4.11 Growth and nutrient consumption profiles of *N. salina* in Duran bottles with different initial nitrate concentrations of 100% (control), 50%, 20% and 10% in BG11 seawater medium: a-1) and b-1) biomass concentrations; a-2) and b-2) nitrate concentration; a-3) and b-3) phosphate concentration. All 'a' culture profiles were obtained under continuous light. All 'b' culture profiles were obtained under a 12 h:12 h light/dark cycle. (In b-2 and b-3 the N and P consumption profiles were obtained only from the control bottles.) Biomass standard deviations are shown for duplicate runs, except for the control. For the latter duplicate measurements of a single run were used to calculate the standard deviations. Standard deviations of nitrate and phosphate are based on duplicate samples of single run.

Table 4.9 *N. salina* Duran bottles culture kinetic parameters (different initial nitrate concentrations in BG11 seawater medium)[1]

Initial nitrate concentration (% of normal)	**Light conditions[2]**	**Specific growth rate, μ (d^{-1})**	**Maximum biomass concentration ($g\ L^{-1}$)**	**Biomass productivity, P_b ($mg\ L^{-1}\ d^{-1}$)**	**Biomass yield coefficient on N, Y_N ($g\ mg^{-1}$)**	**Biomass yield coefficient on P, Y_P ($g\ mg^{-1}$)**	**Average specific N consumption rate, q_N ($mg\ g^{-1}\ d^{-1}$)**	**Average specific P consumption rate, q_P ($mg\ g^{-1}\ d^{-1}$)**
100 (control)	Continuous	0.227 ± 0.01	2.93 ± 0.00	54.2 ± 0.1	0.003	0.220	6.8	0.1
100 (control)	12 h:12 h	0.127 ± 0.00	1.84 ± 0.00	33.3 ± 0.0	0.003	0.137	7.1	0.1
50	Continuous	0.125 ± 0.01	2.72 ± 0.11	50.6 ± 2.1	0.005	0.211	4.3	0.1
50	12 h:12 h	0.151 ± 0.01	1.62 ± 0.12	28.5 ± 2.3	ND	ND	ND	ND
20	Continuous	0.257 ± 0.01	2.42 ± 0.12	44.5 ± 2.4	0.009	0.177	2.4	0.1
20	12 h:12 h	0.144 ± 0.00	1.61 ± 0.02	28.4 ± 0.4	ND	ND	ND	ND
10	Continuous	0.115 ± 0.01	1.90 ± 0.13	33.7 ± 2.6	0.019	0.298	1.1	0.1
10	12 h:12 h	0.146 ± 0.01	1.31 ± 0.07	22.5 ± 1.5	ND	ND	ND	ND

[1]Kinetic parameters were calculated at the point of peak biomass concentration attained on day 49; ND = not determined.

Standard deviations are shown for duplicate runs, except for the control (for the latter duplicate measurements of a single run were used to calculate the standard deviation values).

[2] Irradiance ~135-145 $\mu mol \cdot m^{-2} s^{-1}$ continuously for 24 h or 12 h of light period followed by 12 h of dark

Table 4.10 Lipid content, calorific value and elemental content of *N. salina* biomass (different initial nitrate concentrations)

Initial nitrate concentration (% of normal)	**Light period[1]**	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**	**Elements in biomass**			
					C (%)	**N (%)**	**S (%)**	**P (%)**
100 (control)	Continuous	40.9 ± 0.3	22.1 ± 0.1	26.1 ± 0.1	52.5 ± 0.0	9.8 ± 0.0	0.5 ± 0.0	0.1 ± 0.0
100 (control)	12 h:12 h	39.2 ± 0.0	13.1 ± 0.0	25.6 ± 0.3	53.3 ± 0.0	9.5 ± 0.2	0.5 ± 0.0	0.1 ± 0.0
50	Continuous	38.7 ± 4.6	19.6 ± 1.5	25.7 ± 1.5	52.9 ± 2.2	8.7 ± 0.9	0.5 ± 0.0	0.1 ± 0.0
50	12 h:12 h	44.4 ± 0.7	12.6 ± 0.8	26.6	55.0 ± 0.6	8.6 ± 0.4	0.5 ± 0.0	0.2 ± 0.0
20	Continuous	ND	ND	ND	ND	ND	ND	ND
20	12 h:12 h	44.3 ± 3.0	12.6 ± 0.7	26.9 ± 1.1	54.7 ± 2.1	7.8 ± 1.2	0.5 ± 0.0	0.2 ± 0.0
10	Continuous	ND	ND	ND	ND	ND	ND	ND
10	12 h:12 h	ND	ND	ND	ND	ND	ND	ND

[1]Irradiance ~138-142 µmol·$m^{-2}s^{-1}$ continuously for 24 h or 12 h of light period followed by 12 h of dark.

Standard deviations are shown for duplicate runs, except for the control (duplicate samples of a single run were used to calculate the standard deviations values for the control); ND = not determined

For *N. salina*, the lipid content in the biomass was unaffected by nitrate starvation (Table 4.10) and therefore the lipid productivity of the control culture was highest at 22.1 ± 0.1 mg L^{-1} d^{-1}. Consistent with the lipid levels, the calorific values were not affected by N starvation (Table 4.10). This behavior is peculiar for *N. salina* and has been reported before (Boussiba *et al.*, 1987). As for the elemental content of the biomass, the N content was low in the biomass produced under low nitrate conditions.

In summary, an initial nitrate level of 20% of control was found to not affect biomass productivity and because of a high lipid content of ~66% in the biomass, the lipid productivity was 1.5 fold higher than in *C. vulgaris* control. Therefore, *C. vulgaris* was assessed to be the most promising of the algal strains tested. A highest lipid productivity of ~31 mg L^{-1} d^{-1} under nitrate deficient conditions could be achieved for this alga. In the literature, the highest lipid productivity reported for *C. vulgaris* under N starvation has been ~132 mg L^{-1} d^{-1} (lipid content ~46%) in a freshwater medium in a 1 L photobioreactor (Yeh and Chang, 2011). The biomass concentration of the reported study and present work were comparable at ~2 g L^{-1}. Unfortunately, most freshwater strains of *C. vulgaris* have been found to not grow in full strength seawater.

In the present study, N starvation altered the composition of the biomass and caused a marked increase (~39-66%) in the lipid content of *C. vulgaris*. In *N. salina*, the lipid content was unaffected by N starvation but other changes in the composition of biomass may have occurred that were not considered in this study. Based on earlier studies, under nutrient sufficiency and absence of stress, *C. vulgaris* grown in freshwater media has been reported to have a lipid content of 14-22%, a carbohydrate content of 12-17% and a protein content of 51-58% (Spolaore *et al.*, 2006). In freshwater *C. vulgaris*, Yeh and Chang (2012) reported a lipid content of ~22%, a carbohydrate content of ~58% and a protein content of ~20% under N sufficient conditions. Under N deficiency, the

lipid content increased to ~53%, the carbohydrate level was reduced to ~40% and the protein content was 7%.

4.2.3.2a.a Biomass coloration

Algal biomass coloration appeared to be affected by nitrate limitation. Under nutrient sufficient conditions in continuous light and 12 h: 12 h light/dark cycling, the culture was dark green at peak biomass concentrations for both *C. vulgaris* and *N. salina*. This is expected for most green algae. The biomass is made green by a high content of chlorophyll. However, for both algae, grown under progressively nitrate-limited conditions, the intensity of green progressively reduced to pale green or yellow (Figure 4.12). This was because nitrate limitation induced changes in the composition of photosynthetic pigments. The chlorophyll content (green) was reduced and the content of secondary carotenoids (yellow, orange) may have increased (Ben-Amotz *et al.*, 1982). Nitrogen is essential for the synthesis of chlorophyll as each molecule of this pigment contains the equivalent of two molecules of nitrogen. The level of light is also known for altering the level of photosynthetic pigments. For example, under nitrogen sufficiency, chlorophyll content in the biomass is decreased at high light intensity (Hu, 2004) as less chlorophyll is needed to capture the readily available light. In this study irradiance of 138-142 $\mu mol \cdot m^{-2} s^{-1}$ was constant but the duration of light period induced changes in the culture color (Figure 4.13).

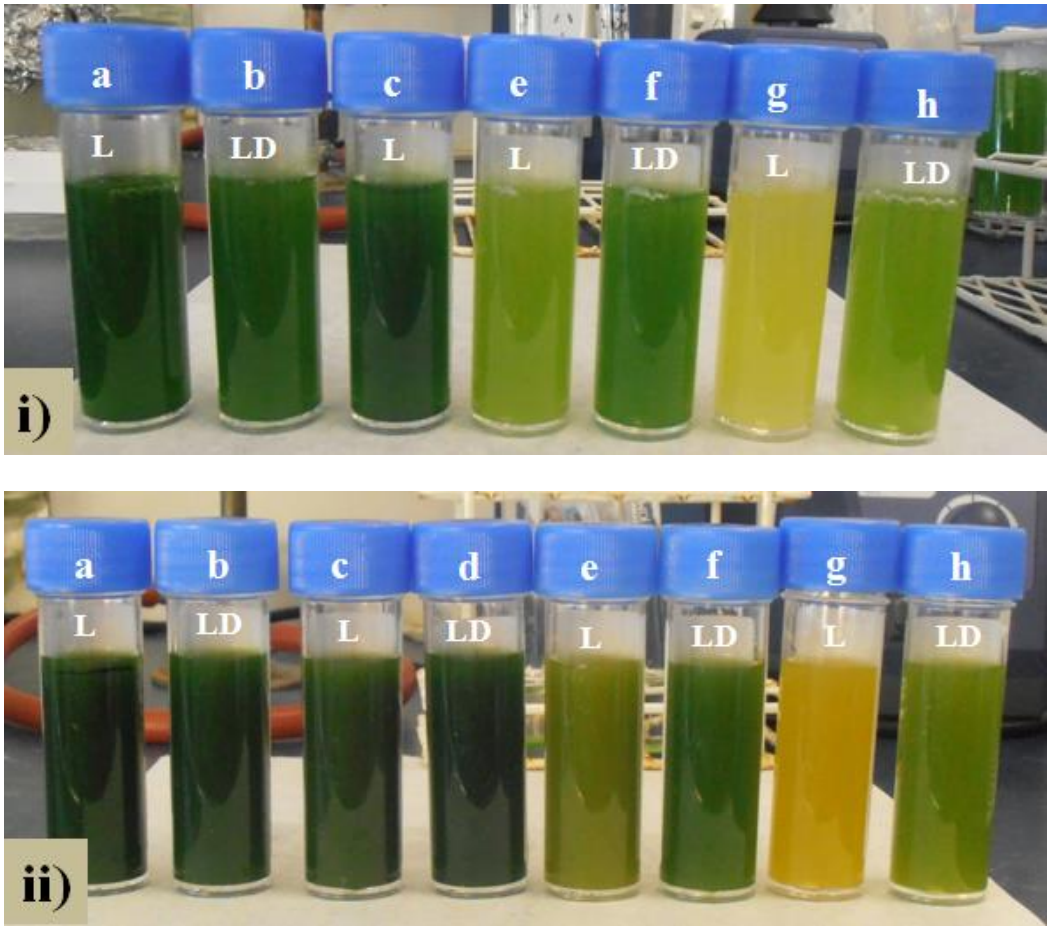


Figure 4.12 Different colors of algal broth as a consequence of different initial nitrate levels in the media: i) *C. vulgaris* (on day 52); ii) *N. salina* (on day 49). (a, b) 100% initial nitrate; (c, d) 50% initial nitrate; (e, f) 20% initial nitrate; (g, h) 10% initial nitrate. All samples labeled "L" had been continuously illuminated. All samples labeled "LD" had been illuminated by a 12 h:12 h light/dark cycle. For biomass concentrations in individual samples, see Table 4.7 and Table 4.9.

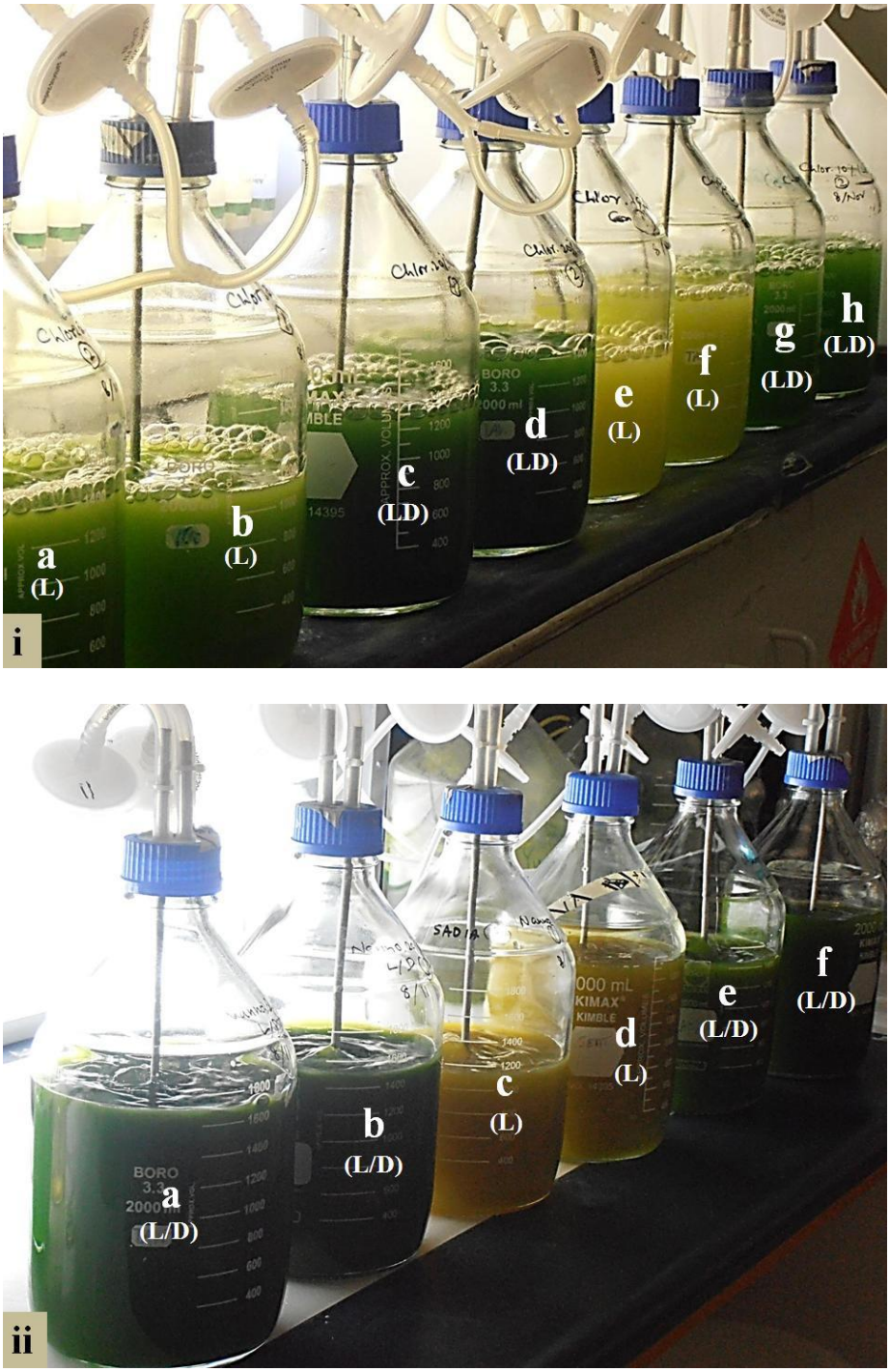


Figure 4.13 Duran bottles cultures of two microalgae on day 46: (i) *C. vulgaris* (a, b, c, d) 20% initial nitrate, (e, f, g, h) 10% initial nitrate; (ii) *N. salina* (a, b) 20% initial nitrate, (c, d, e, f) 10% initial nitrate. All bottles labeled "L" had been continuously illuminated. All bottles labeled "LD" had been illuminated by a 12 h:12 h light/dark cycle.

The microscopic images of the live cells of *C. vulgaris* and *N. salina* were taken (Figure 4.14 and Figure 4.15) at harvest on day 46 and day 49, respectively, to view possible morphological changes. It was noticed that the chlorophyll pigment content of cells were significantly reduced and barely visible in the culture grown at 10% initial nitrate concentration (Figure 4.14 and Figure 4.15). In the control culture, normal levels of chlorophyll pigment could be seen (Figure 4.14 and Figure 4.15). In *C. vulgaris*, rounded bodies could be seen in the cells (Figure 4.14). These occupied roughly 50% of the space in the cells and were more pronounced in cells grown under nitrate-deficient condition. The intracellular inclusions in *C. vulgaris* may have been lipid droplets, but Nile red staining could not be performed on these cultures. (The results in Table 4.8 did show an increased lipid level in this alga as a consequence of N starvation.)

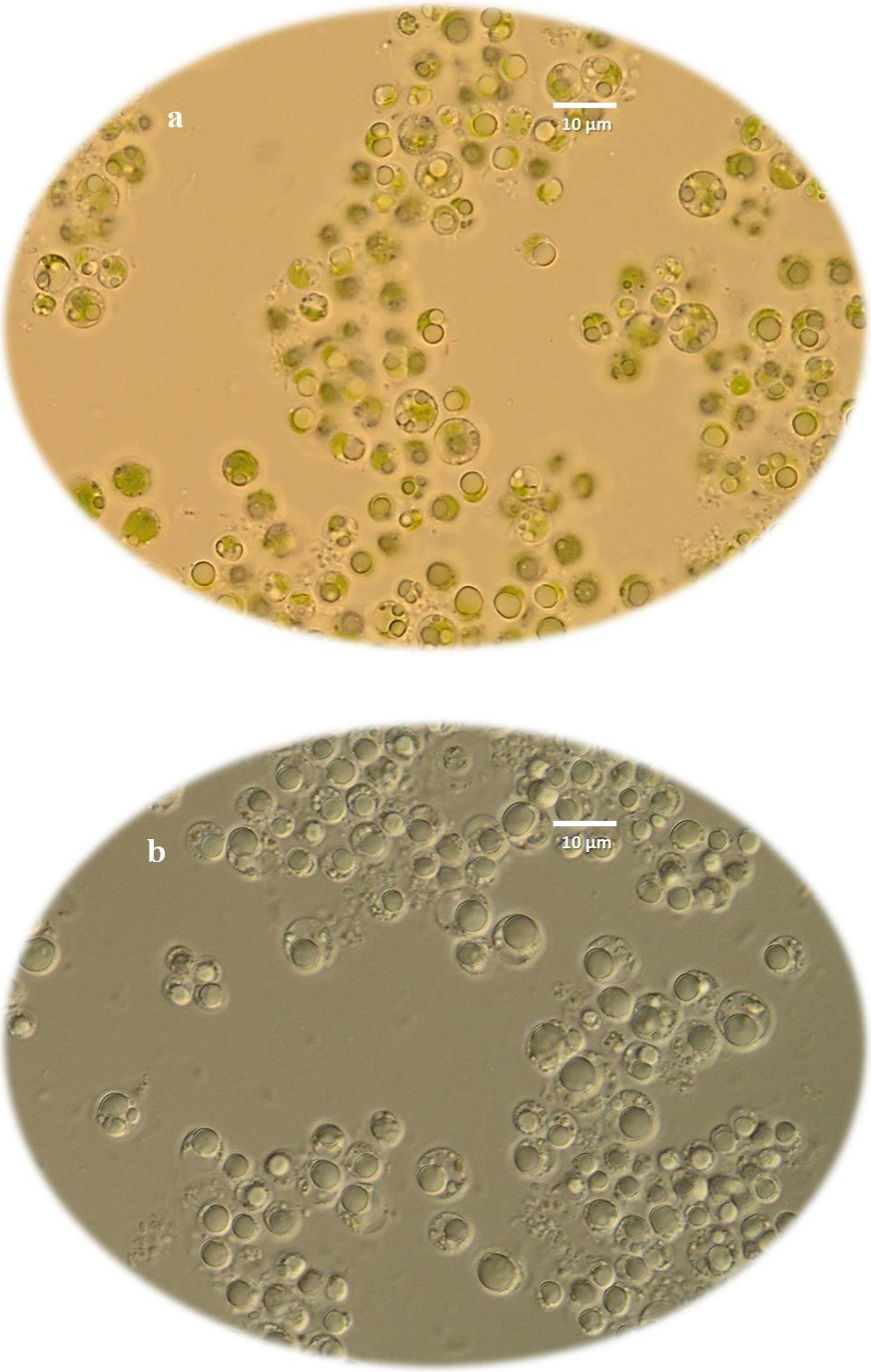


Figure 4.14 Light microscopic images (day 46) of *C. vulgaris* cells grown in continuous light with an initial nitrate concentration of: (a) 100% (control) and (b) 10%.

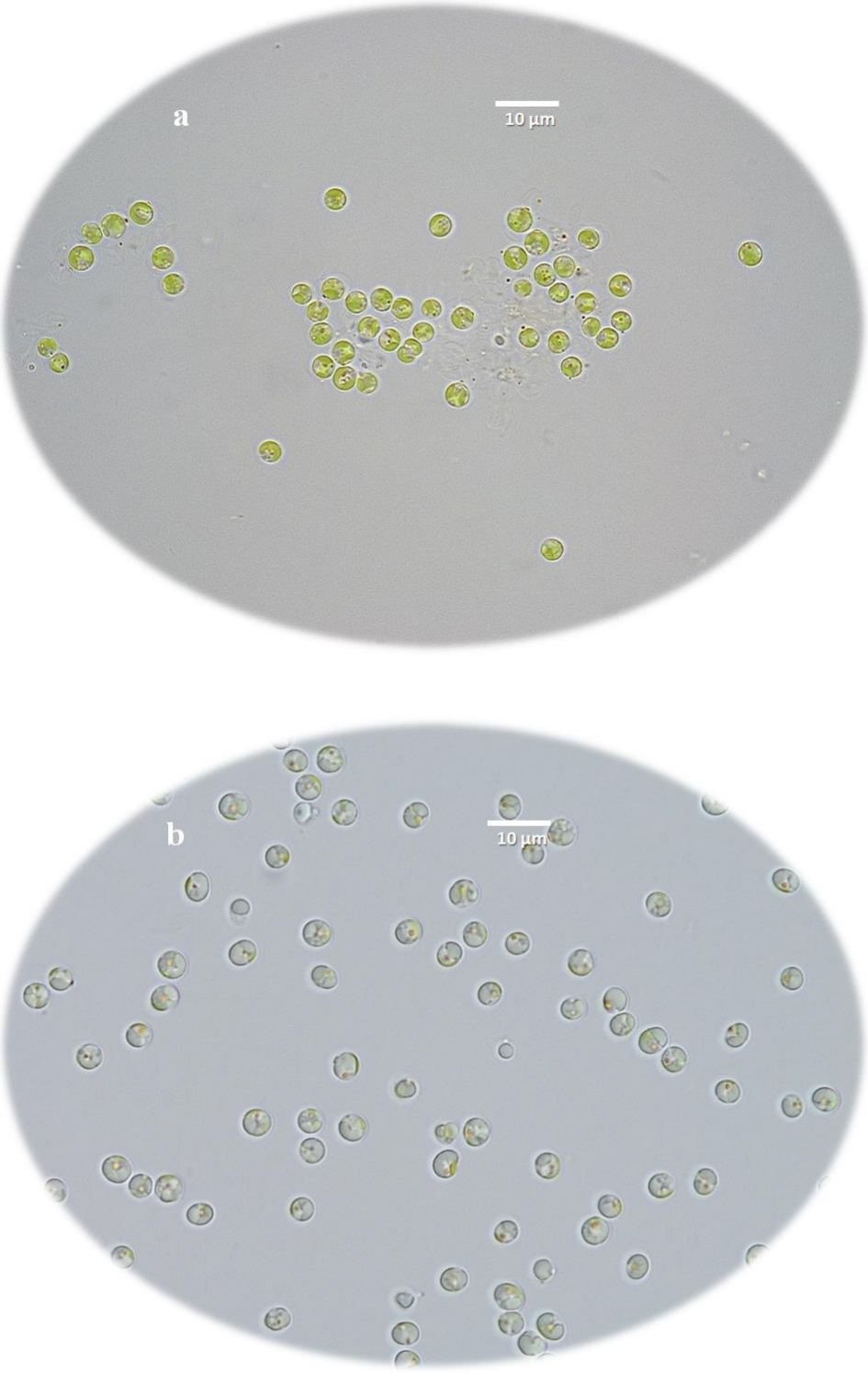


Figure 4.15 Light microscopic images (day 49) of *N. salina* cells grown in continuous light with an initial nitrate concentration of: (a) 100% (control) and (b) 10%.

4.3 Biomass production in the raceway

4.3.1 Raceway batch culture

Based on Duran bottle results, *C. vulgaris* was chosen for further evaluation in the raceway pond. Raceway batch experiments were intended to establish the maximum attainable biomass concentration and lipid contents in the biomass under normal growth conditions and other conditions (e.g. different initial nitrogen levels).

4.3.1.1 Standard raceway batch culture (normal operational conditions)

C. vulgaris was grown in the raceway to obtain baseline data using the standard BG11 seawater medium under continuous illumination and 12 h: 12 h diurnal cycle at various levels. Three batches were run as specified in Section 3.2.3. The batches ran for ~30-65 days each. In all cases the temperature was controlled at 20.5 ± 0.9 °C and the pH was controlled at 6.7 ± 0.1 by automatic feeding of CO_2. The rotational speed of the paddlewheel was 5 on the potentiometer to produce a flow velocity of 0.21 ± 0.02 m s^{-1} as shown in Figure 3.11 and Figure 3.12. The working volume of raceway was ~138 L, except batch-1 which was ~133 L. Salinity of the individual batches varied a little because of the evaporation and make up, but was generally within the range for seawater, i.e. ~37.2 ppt. The batches 1-3 had salinities of 43.1 ± 0.5 ppt, 38.0 ± 0.3 ppt and 31.9 ± 0.3 ppt, respectively.

The batches 1 and 2 were illuminated using continuous fluorescent light at a PAR level of 91 μmol·$m^{-2}s^{-1}$. Batch 3 was illuminated using LED lights (Section 3.2.3) under a 12 h:12 h diurnal cycle. The irradiance level varied from zero to the peak value (100% output) from the start of the day to the midday point. Then the irradiance level declined. The actual irradiance profile is shown in Figure 3.7 and Figure 3.8. The inoculum size for the batches was 10% (v/v) in batch 1 and 5-6% (v/v) for batches 2 and 3. The inoculum age was 49 days for batch-1 and 15 days for batches-2 and 3.

The culture profiles of the three batches are shown in Figure 4.16. The batches 1 and 2 that were run under continuous light attained peak biomass concentrations of 0.44 g L^{-1} and 0.66 g L^{-1}, respectively, in 30-35 days (Figure 4.16). The batch-3 with a day/night irradiance cycle, attained a maximum biomass concentration of 1.11 ± 0.03 g L^{-1} in 60 days. These biomass concentrations were within the range reported for 25 cm deep outdoor raceways located in tropical regions (Becker, 1994). Biomass productivities in the raceway batch-2 and batch-3 were similar (17.4 and 20 mg L^{-1} d^{-1}, respectively; Table 4.11). Although the time to reach the maximum concentration was longer in batch-3 due to day/night cycling, the peak biomass concentration was higher than in batch-2 because the day-night averaged irradiance was nearly 3-fold the level in batch-2.

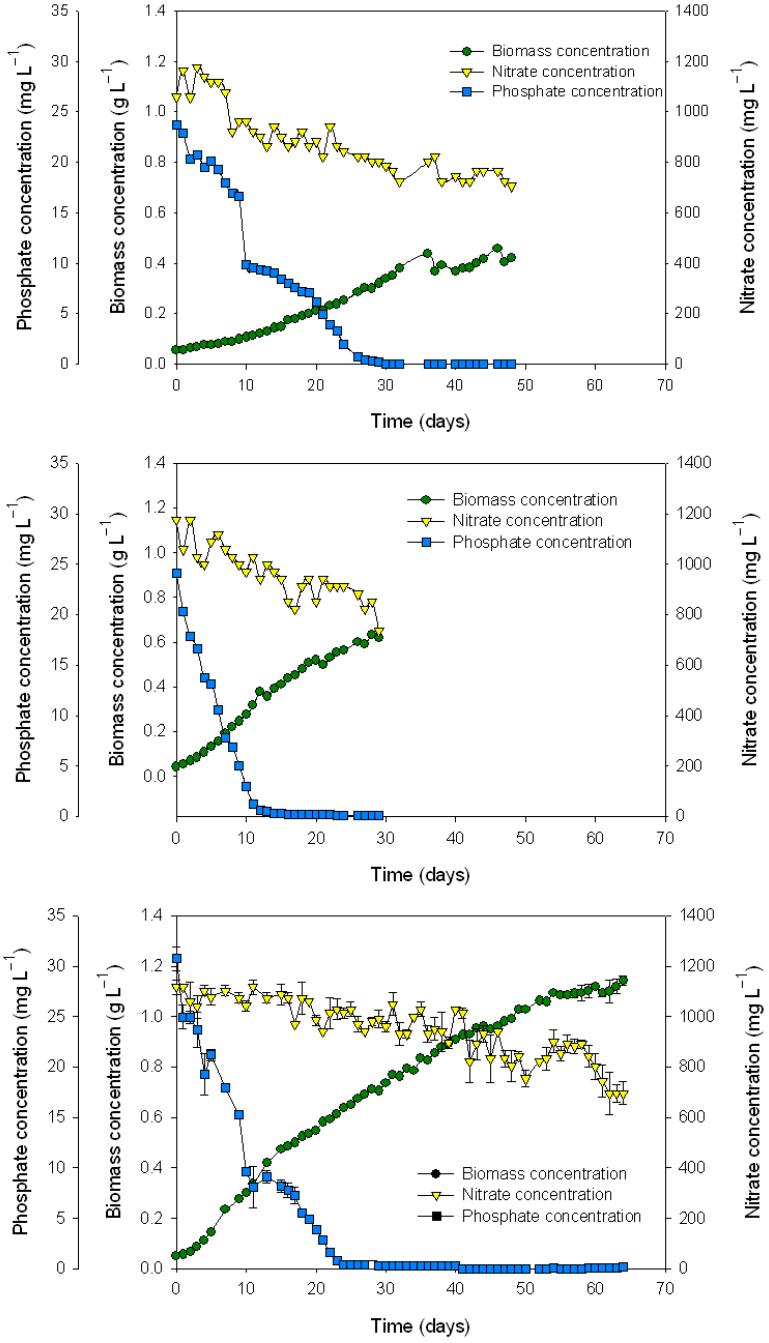


Figure 4.16 Growth and nutrient consumption profiles of *C. vulgaris* in raceway: a) Batch-1 with continuous irradiance of 91 μmol·m^{-2}s^{-1} from fluorescent light; b) Batch-2, same as batch-1; c) Batch-3 cultured under LED light (100% light output at midday (day-night averaged irradiance of 280 μmol·m^{-2}s^{-1}), 12 h:12 h light/dark cycle). All batches started with 5-6% (v/v) inoculum except batch-1 for which the inoculum size was 10.5% (v/v). Standard deviations for the raceway batch-3 are based on triplicate samples for biomass and nitrate and duplicate samples for phosphate.

The raceway batch-2 attained the highest specific growth rate at 0.185 d^{-1} (Table 4.11) demonstrating the capability of the culture system. However, as in all algae culture systems, the growth rate declined (Figure 4.16) as the cell concentration increased and self-shading limited the availability of light, the growth controlling factor.

Nitrate and phosphate concentrations declined with time because of consumption by the biomass. The cultures (Figure 4.16) were always nitrate sufficient and at harvest, >600 mg L^{-1} of nitrate remained in the broth. This nitrate concentration was enough to support further growth, but the culture did not grow further due to light limitation as explained later in this section.

In view of the extensive prior experience with reproducibility of the various measurements from the Duran bottle cultures, replicate measurements were made only for a selection of samples in the profiles shown in Figure 4.16. Within a sample, the biomass measurements were reproducible within ±3.5 % of the mean value. The fluctuations in the nitrate measurements (Figure 4.16) are a result of evaporation and daily addition of the make-up water.

Phosphate concentration declined to nil within 27 days (Figure 4.16). All cultures continued to grow even after phosphate had run out, a phenomenon that has been commonly seen in microalgae (Miyachi and Tamiya, 1961; Miyachi *et al.*, 1964; Cembella *et al.*, 1984; John and Flynn, 2000) as discussed in Section 4.2.3.1b. The rate of phosphate consumption in the raceway (Figure 4.16) was much slower than seen previously in Duran bottles (Section 4.2.3.1). This was because the biomass growth in the raceway was slower than in the Duran bottles because of light limitations.

The culture kinetics of the three raceway batches are given in Table 4.11.

Table 4.11 *C. vulgaris* raceway batch culture kinetics (standard BG11 medium)

Kinetic parameters[1]	**Batch-1**[a]	**Batch-2**[b]	**Batch-3**[c]
Specific growth rate, μ (d^{-1})	0.072	0.185	0.106
Maximum biomass concentration (g L^{-1})	0.44	0.66	1.11± 0.00
Biomass productivity, P_b (mg L^{-1} d^{-1})	10.5	20.5	17.6 ± 0.55
Biomass yield coefficient on N, Y_N (g mg^{-1})	0.001	0.002	0.003
Biomass yield coefficient on P, Y_P (g mg^{-1})	0.015	0.026	0.035
Average N consumption rate, q_N (mg g^{-1} d^{-1})	18.6	20.7	4.9
Average P consumption rate, q_P (mg g^{-1} d^{-1})	1.7	1.3	0.5

[1]Biomass kinetic parameters were calculated at the point of peak biomass concentration attained on day 36 (batch-1), day 30 (batch-2) and day 60 (batch-3).

[a]Batch-1 = fluorescent continuous light; [b]Batch-2 = fluorescent continuous light; [c]Batch-3 = LED light and 12:12 h day/night cycle.

Standard deviations are based on triplicate samples.

For otherwise identical conditions, the alga grew best under a 12:12 h light-dark cycle as would normally occur in nature (Table 4.11). In this production regimen, the day-night averaged irradiance level was 280 μmol·$m^{-2}s^{-1}$ (Figure 3.7) compared with an average level of 91 μmol·$m^{-2}s^{-1}$ for continuous illumination. Although the source of light can have an impact on the photosynthetic efficiency (Matthijs *et al.*, 1996) comparing light sources based on the actual measured PAR (photosynthetically active radiation), as in this work, is independent of the source. That is, in principle, red LEDs with a given PAR output should provide the same growth performance as fluorescent light with the same PAR output.

The main reason for the low final biomass concentration in the raceway compared with the Duran bottles was limitation of light. Figure 3.9 shows the measured irradiance profiles in freshwater at various incident irradiance values in the raceway. Even in clear

water, the local irradiance value declined rapidly with depth. The measured irradiance profile in *C. vulgaris* culture with 0.5 g L^{-1} of biomass concentration is shown in Figure 3.10. A sharp decline in irradiance was found just below the surface of the broth. At a depth of 0.02 m from the surface, barely any light remained. This demonstrates that at a concentration of 0.5 g L^{-1} algal cells receive light only at the surface of broth. In commercial raceway ponds, the depth ranges from 0.2 to 0.3 m (Borowitzka, 2005). In some experimental and commercial raceways that were 0.11 to 0.25 m deep, the reported maximum biomass concentrations has ranged between 0.2 and 1 g L^{-1} under conditions of outdoor natural illumination (Richmond, 1990; Hase *et al.*, 2000; Pulz, 2001; Tredici, 2004; Moheimani and Borowitzka, 2006) .

In 2 L Duran bottle experiments, the biomass concentration was considerably higher, e.g. ~3.3 g L^{-1} (Table 4.1) and was attained in a shorter period (20 days) with an irradiance of 100-150 μmol·$m^{-2}s^{-1}$ (fluorescent light). The culture depth in 2 L Duran bottles was ~0.11 m (Figure 3.1), which made light penetration easier than in the raceway.

Live cells of *C. vulgaris* were checked for the presence of stored neutral lipids using the Nile red dye method (Elsey *et al.*, 2007) explained in Section 3.3.6. A photomicrograph is shown in Figure 4.17. The lipid droplets appear as golden-yellow fluorescence while red auto-fluorescence was from chlorophyll (Figure 4.17). The neutral lipids were visible (clear yellow lipid bodies in Figure 4.17) but in a relatively small quantity, as expected, because under nutrient sufficient growth conditions, microalgae generally synthesize polar lipids (i.e. phospholipids and glycolipids) (Guckert and Cooksey, 1990) whereas neutral lipids accumulate (as triglycerides) under nutrient limiting conditions (Hu *et al.*, 2008).

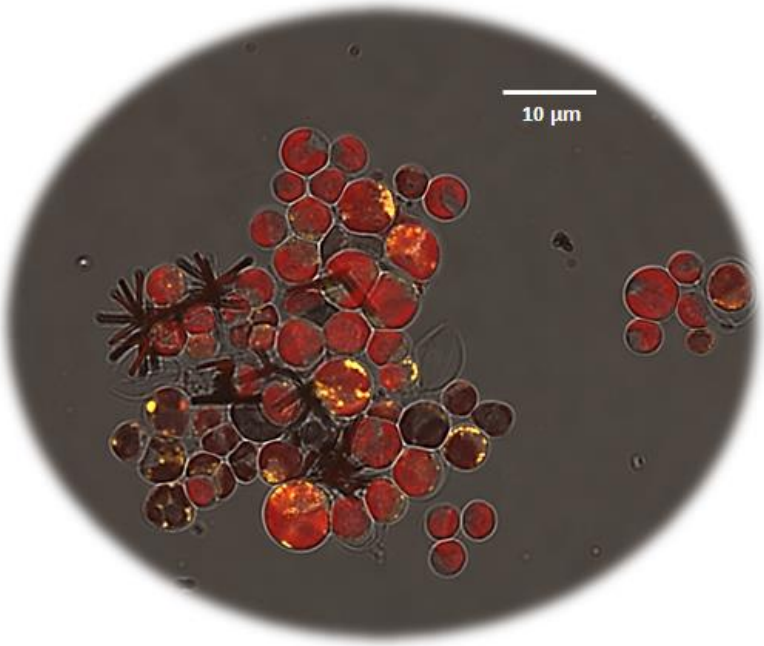


Figure 4.17 Fluorescent confocal microscopic image of *C. vulgaris* culture (stained with Nile Red) from Raceway batch-3 (image taken at day 34 of growth cycle, some debris can also be seen in the image).

The biomass recovered from the culture broth was analyzed for total lipids, the calorific values and the fraction of C, N and S in the biomass. The relevant data are shown in Table 4.12 and Table 4.13.

Table 4.12 Lipid contents and calorific values of biomass samples

Batch	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**
Batch-1	11.5 ± 0.7	1.2	20.9[1]
Batch-2	ND	ND	ND
Batch-3	25.1 ± 0.2	4.4 ± 0.0	21.6 ± 0.3

[1]The calorific value of the oil was 31.7 kJ g^{-1}; ND = not determined; standard deviations are based on duplicate samples.

Lipid contents of the biomass were in the range of 11.5-25.1% (w/w) (Table 4.12). Lipid content of the biomass of batch-2 could not be estimated because the batch was switched to a continuous mode of operation. The lipid contents of these batches that were run under nutrient sufficient conditions, were within the expected range of ~18-25% w/w.

The calorific values of the biomass samples (Table 4.12) were relatively low but consistent with the other studies. For example, Illman *et al.* (2000) and Scragg *et al.* (2002) reported calorific values of 18 kJ g^{-1} and 21 kJ g^{-1}, respectively, for *C. vulgaris* grown in nutrient sufficient media in freshwater.

Generally, lipids are the most energetic compounds in a cell and, therefore, the calorific value is expected to directly correlate with the lipid content of the biomass (Illman *et al.*, 2000). This notwithstanding, other compounds (carbohydrates, proteins, etc.) also contribute to the energy content of the cell. The composition of the biomass determines its energy content (Sudhakar *et al.*, 2012). The net calorific values can be estimated for a given microalgae by using the following calorific values for individual compounds: 38.3 kJ g^{-1} for lipid, 13 kJ g^{-1} for carbohydrate and 15.5 kJ g^{-1} for protein (Lardon *et al.*, 2009). Thus, for an alga with a composition of 40% lipid, 40% carbohydrate and 20% protein the estimated calorific value would be (0.4 × 38.3) + (0.4 × 13.0) + (0.2 × 15.5), or 23.6 kJ g^{-1}.

The C, N, and S contents of biomass (Table 4.13) were consistent with expectation for general algal biomass (Williams and Laurens, 2009). Carbon of course is an essential part of all organic molecules that make up the cell. N is found mainly in proteins, DNA, RNA and chlorophyll. S is a constituent of certain amino acids. The carbon content was around 47% (w/w) and similar to what has been reported for other

algae (Sánchez Mirón *et al.*, 2003). All the carbon in the biomass is acquired from carbon dioxide, an essential nutrient required by algae.

Calorific value of the oil was also measured in one case and was 31.7 kJ g^{-1} (Table 4.12). This value was lower than a calorific value of 36.2 kJ g^{-1} as estimated for algal lipids by Williams and Laurens (2009), or a value of 38.3 kJ g^{-1} as estimated by Lardon *et al*. (2009). The estimates published are reasonable for only triglyceride oils, but not all algal oils are triglycerides.

Table 4.13 Elements (%, w/w) in *C. vulgaris* biomass from various raceway batches

Raceway batch	**Carbon (%)**	**Nitrogen (%)**	**Sulfur (%)**
Batch-1	47.6	7.5	0.8
Batch-2	ND	ND	ND
Batch-3	48.2	6.8	0.8

ND = Not determined because the raceway batch-2 was switched to continuous operation

4.3.1.1a Analysis of *C. vulgaris* crude oil from raceway batch-1

C. vulgaris crude oil extracted from the biomass of the batch-1 was fractionated into different lipid classes (i.e., neutral lipids, glycolipids and phospholipids; Table 4.14). Fatty acid profile of the total lipids was determined (Table 4.15). All these analyses were intended to characterize the crude oil with regards to its constituents. These aspects are discussed in the following sections.

4.3.1.1b Fractionation of *C. vulgaris* lipids into different lipid classes

The crude oil consisted of neutral lipids (14.1%), phospholipids (19.4%) and glycolipids (66.2%) (Table 4.14). The neutral lipid content was low, and the neutral lipids were not fractioned further for estimating the triglycerides.

Table 4.14 Fractionation of lipids from raceway batch-1

Lipid class	**Lipids fraction in total lipids[1, 2] (%, w/w)**	**Lipids fraction in biomass (%, w/w)**
Neutral lipids	14.1	1.3
Phospholipids	19.4	1.8
Glycolipids	66.2	6.0

[1]Fractionation according to Kates (1986)

[2]Total lipid sample ~257 mg dissolved in chloroform was used for analysis

4.3.1.1c Fatty acid profile of *C. vulgaris* oil

Fatty acid profile of one algal crude oil sample (raceway batch-1) was characterized as explained in Section 3.3.5.2. This is potentially of interest if the intention is to produce biodiesel from algal oil as the characteristics of the biodiesel are influenced by fatty acids present in the triglyceride oil used for making the biodiesel (Williams and Laurens, 2009). The fatty acid profile is shown in Table 4.15. From the data in Table 4.15, fatty acids comprised only 2% (w/w) of the algal crude oil. Of the fatty acids present, 21.2% (w/w) were saturated, 17.2% were monounsaturated and 61.6% were polyunsaturated. This was generally consistent with expectations, as algal oils are known to be rich in polyunsaturated fatty acids (Jamieson and Reid, 1972; Belarbi *et al.*, 2000) . In view of a high concentration of polyunsaturated fatty acids, *C. vulgaris* oil is unlikely to be suitable for making biodiesel, but may be used to make diesel, gasoline and kerosene through catalytic treatment (Chisti, 2012).

Table 4.15 Fatty acid profile of crude oil of *C. vulgaris* (raceway batch-1)

Fatty acid profile[1]	**Concentration in oil**[2] **(g/100 g)**
C6:0 Caproic	ND
C8:0 Caprylic	ND
C10:0 Capric	ND
C11:0 Undecanoic	ND
C12:0 Lauric	0.03
C13:0 Tridecanoic	ND
C14:0 Myristic	ND
C14:1n5 - cis-9-Myristoleic	ND
C15:1n5 - cis-10-Pentadecenoic	ND
C16:0 Palmitic	0.38
C16:1n7 - cis-9-Palmitoleic	0.05
C17:0 Margaric	ND
C17:1n7 - cis-10-Heptadecenoic	ND
C18:0 Stearic	0.02
C18:1n9t Elaidic	ND
C18:1n7t Vaccenic	ND
C18:1n9c Oleic	0.19
C18:1n7c Vaccenic	0.11
C18:2n6t Linolelaidic	ND
C18:2n6c Linoleic	0.64
C20:0 Arachidic	ND
C18:3n6 - cis-6,9,12-Gamma linolenic	ND
C20:1n9 - cis-11-Eicosenoic	ND
C18:3n3 - cis-9,12,15-Alpha linolenic	0.61
C21:0 Heneicosanoic	ND
C20:2n6 - cis-11,14-Eicosadienoic	ND

Table 4.15 Fatty acid profile of crude oil of *C. vulgaris* (raceway batch-1) (Cont.)

Fatty acid profile[1]	**Concentration in oil**[2] **(g/100 g)**
C22:0 Behenic	ND
C20:3n6 - cis-8,11,14-Eicosatrienoic	ND
C22:1n9 - cis-13-Erucic	ND
C20:3n3 - cis-11,14,17-Eicosatrienoic	ND
C23:0 Tricosanoic	ND
C20:4n6 - cis-5,8,11,14-Arachidonic	ND
C22:2n6 - cis-13,16-Docosadienoic	ND
C24:0 Lignoceric	ND
C20:5n3 - cis-5,8,11,14,17-Epa	ND
C24:1n9 - cis-15- Nervonic	ND
C22:5n3 - cis-7,10,13,16,19-DPA	ND
C22:6n3 - cis-4,7,10,13,16,19-DHA	ND

[1]Method used: FAME, GC separation (Section 3.3.7).

[2]Dry oil sample ~400 mg sent to Nutritional Laboratory, Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand.

ND = Not detected.

4.3.1.1c Concentrations of certain elements in *C. vulgaris* oil

Chemical catalytic processes have been developed to convert crude oils to gasoline, diesel and kerosene (Chisti, 2012). These processes are quite different from the process used in making biodiesel from vegetable oils that are predominately triglyceride type of oils. Alternative methods of making conventional fuels from algal crude oil are necessary because triglycerides often constitute only a small portion of the total algal oil and therefore cannot become a viable basis for making biodiesel as a large proportion of the oil would not be used.

Unfortunately, some of the chemical catalysts used in transforming algal oils to useable fuels are susceptible to poisoning by elements such as Ca, Mg, P, Cu, Na, S, Fe and N. Therefore, there was some interest in determining the concentration of the relevant elements in some crude oil samples. As shown in Table 4.16, significant quantities of the undesired elements were present in *C. vulgaris* crude oil. In fact, for most of these elements, the levels present were higher than would be acceptable. Therefore, treatment processes will be required to reduce the concentration of the unwanted trace elements. Possible options may be to wash the crude oil repeatedly with dilute acid, or with dilute acid mixed with a chelating agent such as EDTA, to remove some of the metal ions.

Table 4.16 Elemental content of crude *C. vulgaris* oil from the raceway batch-1

Element	**Method used[1]**	**Measured concentration in oil[2] (mg/kg)**	**Detection limit (mg/kg)**
Calcium, Ca	ICP-OES	78	0.20
Magnesium, Mg	ICP-OES	1700	0.20
Phosphorus, P	ICP-OES	3800	1.0
Sodium, Na	ICP-OES	1400	5.0
Copper, Cu	ICP-MS	42	0.005
Sulfur, S	ICP-OES	3700	1.0
Iron, Fe	ICP-OES	40	0.10
Chloride, Cl	Potentiometric titration	50	<1.0
Nitrogen, N	Dumas combustion	13000	20

[1]ICP-OES (inductively coupled plasma optical emission spectrometry) and [1]ICP-MS (inductively coupled plasma mass spectrometry); [2]Dry oil sample ~500 mg sent to Hills Laboratories, Hamilton, New Zealand, for analysis.

4.3.1.2 Effect of low irradiance

The raceway batch-4 was setup with a relatively low irradiance to determine the maximum attainable biomass concentration and the lipid contents of the biomass. The peak LED light output level was 50% of the maximum and corresponded to a day-night averaged PAR value of 165 $\mu mol \cdot m^{-2}s^{-1}$. Understanding the effect of light is important in view of its seasonal variations. Other than irradiance, all growth conditions of batch-4 were the same as for raceway batch-3 (Section 4.3.1.1).

The *C. vulgaris* starter culture for raceway batch-4 had been grown in Duran bottles for 21 days under normal culture conditions (standard BG11 seawater medium; continuous fluorescent illumination at 153 $\mu mol \cdot m^{-2}s^{-1}$). The inoculum constituted 6% of the total volume of the raceway culture. The growth temperature (20.6 ± 1.6 °C), the pH (6.7 ± 0.1) and salinity (39.7 ± 1.2 ppt) were monitored regularly at samplings and were constant within the above limits.

The illumination was from LED lights (Section 3.2.3) at 12 h:12 h diurnal cycle. The light level varied from zero to the peak value (50% of full light output) in 12 h and then declined, as shown in Figure 3.7b. The day-night averaged PAR value was 165 $\mu E \cdot m^{-2}s^{-1}$.

The culture profiles of the batch-4 are shown in Figure 4.18. The specific growth rate was relatively low, i.e. 0.086 d^{-1}, in comparison with the batch-3 raceway experiment (Table 4.11). (Batch-3 had a specific growth rate of 0.106 d^{-1}.) In raceway batch-4, the peak biomass concentration was 0.39 ± 0.00 g L^{-1} attained in 56 days with a day/night cycle (Table 4.17). This was only about 35% compared to the biomass concentration of 1.11 ± 0.00 g L^{-1} (Table 4.11) under 100% light level with 12 h:12 h diurnal cycle. Biomass productivity was reduced to 5.8 ± 0.0 mg L^{-1} d^{-1} compared to the full light condition productivity of 17.6 mg L^{-1} d^{-1} (Table 4.11).

Nitrate consumption ceased (Figure 4.18) once the biomass ceased to grow. The phosphate ran out on day 34 but the culture continued to grow. Similar nutrient consumption patterns were discussed previously in Section 4.3.1.1.

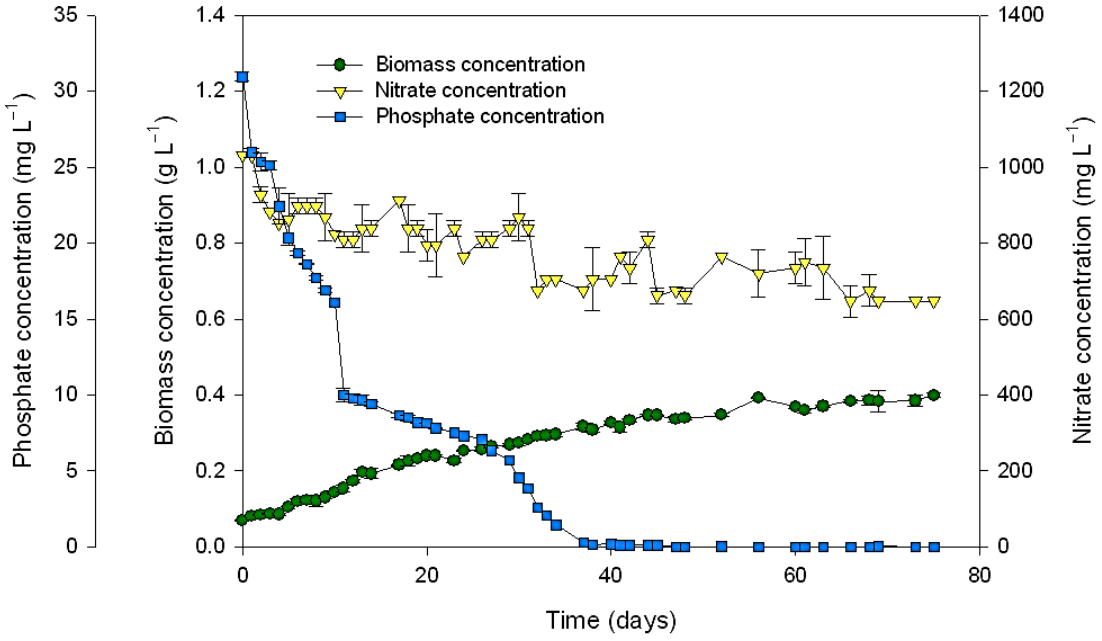


Figure 4.18. Growth and nutrient consumption profiles of *C. vulgaris* in raceway batch-4 with LED light (50% of full light output at midday (day-night averaged irradiance of 165 μmol·m^{-2}s^{-1}), 12 h:12 h light/dark cycle). Standard deviations are based on triplicate samples for the biomass measurements and duplicate samples for the nitrate and phosphate measurements.

During stationary phase of growth, a volume of 5-10 L of culture broth was harvested (at different times) to see if the lipid content of the biomass varied with age in the stationary phase (Table 4.18). No significant change in lipid contents was found (Table 4.18). The calorific values, and the contents of C, N, S, P in the biomass are shown in Table 4.18.

Table 4.17 *C. vulgaris* raceway batch culture kinetics (low irradiance condition)

Kinetic parameters[1]	**At 50% light output level**[2]
Specific growth rate, μ (d^{-1})	0.086
Maximum biomass concentration[a] (g L^{-1})	0.39 ± 0.00
Biomass productivity[a] , P_b (mg L^{-1} d^{-1})	5.8 ± 0.02
Biomass yield coefficient on N, Y_N (g mg^{-1})	0.001
Biomass yield coefficient on P, Y_P (g mg^{-1})	0.010
Average N consumption rate, q_N (mg g^{-1} d^{-1})	17.1
Average P consumption rate, q_P (mg g^{-1} d^{-1})	1.7
Lipid content[b] (%, w/w)	21.1 ± 0.1
Lipid productivity[b] , P_l (mg L^{-1} d^{-1})	1.2 ± 0.0

[1]Kinetic parameters were calculated at the point of peak biomass concentration attained on day 56.

[2] LED lights with a 12:12 h diurnal cycle with 50% of normal full irradiance at peak (Figure 3.9).

[a]Triplicate samples; [b]duplicate samples.

Table 4.18 Biomass characteristics of *C. vulgaris* at various harvesting times in the raceway batch-4

Batch harvest time[1]	**Biomass concentration (g L^{-1})**	**Biomass productivity, P_b (mg L^{-1} d^{-1})**	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**	**Elements in biomass**			
						C (%)	**N (%)**	**S (%)**	**P (%)**
Day 45	0.35 ± 0.20	6.2 ± 0.0	16.5 ± 0.1	0.6 ± 0.0	ND	ND	ND	ND	ND
Day 56	0.39 ± 0.00	5.8 ± 0.0	21.1 ± 0.1	1.2 ± 0.0	23.1	48.6	7.0	0.7	0.9
Day 68	0.39 ± 0.01	4.7 ± 0.0	22.9 ± 2.6	1.1 ± 0.0	23.3	ND	ND	ND	ND
Day 75	0.40 ± 0.01	4.2 ± 0.1	22.4 ± 0.2	1.0 ± 0.0	23.8 ± 0.1	51.9 ± 0.4	6.8 ± 0.0	0.8 ± 0.0	0.6 ± 0.0

[1]Harvesting was done after the culture had entered the stationary phase of growth.

ND = Not determined because the amount of the dried biomass was not sufficient for analyses.

Biomass standard deviation is based on triplicate samples; all other standard deviations are based on duplicate samples.

Lipid content measured at different harvest times was within the range of 16.5 to 22.4% (w/w) (Table 4.18). Also, the calorific values were essentially constant at ~23 kJ g^{-1} (Table 4.18). All these measurements were fairly stable throughout the stationary phase from Day 56 to 75. The calorific values as well as the C, N, S and P contents of biomass (Table 4.18) were comparable to the earlier measurements (Table 4.12 and Table 4.13) in the raceway carried out under nutrient sufficient conditions both with continuous light as well as with the day-night cycle (Section 4.3.1.1).

It is obvious from the results that a reduction in the day-night averaged light level to 165 $\mu mol \cdot m^{-2}s^{-1}$ reduced growth, but the lipid accumulation in the biomass was the same as was found under full light (i.e. 280 $\mu mol \cdot m^{-2}s^{-1}$ day-night averaged light level). Therefore the irradiance level affects the production of the biomass and lipids, but not the lipid content in the biomass at least in the irradiance range of 165-280 $\mu mol \cdot m^{-2}s^{-1}$. A low biomass productivity is expected under reduced irradiance as light always drives photosynthesis (Vonshak *et al.*, 1982; Richmond, 1986).

4.3.1.3 Effect of nitrate stress

The raceway batches were setup with low initial nitrate concentrations to determine the effect of this parameter on the maximum attainable biomass concentration and the lipid contents of the biomass. Based on the studies of nitrate stress in Duran bottles, a ~20% initial nitrate level relative to standard BG11 was used initially for raceway batch culture.

At first, two raceway batches (batch-5 and batch-6) of *C. vulgaris* were run with ≤21% nitrate in BG11 (i.e. ~21% of standard BG11 nitrate level in batch-5 and ~18% of standard BG11 nitrate level in batch-6; Section 4.3.1.3a). Furthermore, a concentration of ~10% of normal initial nitrate in BG11 was also tested for *C. vulgaris* in raceway batch-7 (Section 4.3.1.3b). Other than the nitrate level in BG11 (seawater), all other

growth conditions of the various batches were similar: a 12 h:12 h diurnal cycle with 100% peak light output via LEDs (Section 3.2.3 and Section 4.3.1.1.), an average temperature of 19.6 ± 1.5 °C and pH of 6.7 ± 0.1. The individual salinities of raceway batches 5-7 were 35.0 ± 0.5 ppt, 37.1 ± 0.7 ppt and 38.9 ± 0.4 ppt, respectively. The effect of increased rotational speed of the paddle wheel (i.e. from 5 to 8 on the potentiometer) was tested in raceway batch-6 only, during stationary growth phase (Section 4.3.1.3a). The inocula for all three batches were produced under nitrate sufficient condition in continuous fluorescent light (from 135 to 153 $\mu mol \cdot m^{-2}s^{-1}$). The inocula constituted 6% (v/v) of the initial batch volume.

Raceway batch-8 was run under nitrate stress using *N. salina*. During this batch the rotational speed of the paddlewheel was changed as described in Section 4.3.1.3c. The growth conditions were as for the other raceway batches.

4.3.1.3a Effect of ≤21% of normal initial nitrate level on *C. vulgaris*

The culture profiles of batch-5 and batch-6 are shown in Figure 4.19. Both batches attained the maximum biomass concentration around day 60. The averaged specific growth rate of batch-5 and batch-6 was 0.131±0.025 d^{-1} (Table 4.19), or comparable to a specific growth rate of 0.106 d^{-1} for the nutrient sufficient batch-3 (Table 4.11). The batch-5 and batch-6 attained comparable biomass concentrations of 0.91 g L^{-1} and 0.66 ± 0.00 g L^{-1} (Table 4.19), respectively. The nutrient sufficient batch-3 (Section 4.3.1.1) had a peak biomass concentration of 1.11 ± 0.03 g L^{-1} (Table 4.11).

In the growth profile of batch-5 the decline in the biomass concentration on day 76 (Figure 4.19a), was due to harvesting of a large volume (30 L) of culture broth and making it up with fresh BG11 (seawater) medium without nitrate and phosphate. Subsequently, periodic harvests occurred as identified in Table 4.20 and each time the

harvested volume was replaced with an equal volume of nitrate and phosphate-free BG11 seawater medium. The final harvest occurred on day 159. Due to periodic harvests, the biomass concentration progressively declined after about day 90 (Figure 4.19a). The biomass of each harvest was characterized for total lipids, calorific value and the elemental profile (Table 4.20).

In batch-6 with similar culture conditions as the batch-5, the speed setting of the paddlewheel was increased to 8 on the potentiometer to give a flow velocity of 0.30 ± 0.03 m s^{-1}. This was done on day 97. This caused vigorous turbulence in the culture broth and the very next biomass sample on day 98 showed a sudden increase (~16% increase) in the biomass concentration (Figure 4.19b). This was attributed to resuspension of biomass that had settled at the bottom of the raceway pond and not to actual growth. The culture continued to run at the new speed setting for about 70 days (i.e. from day 96 until day 167). During this period the biomass was harvested several times as noted in Table 4.21. On day 162, paddlewheel speed was returned to normal potentiometer setting of 5, consequently to a flow velocity of 0.21 ± 0.02 m s^{-1}.

The nutrient profiles of batch-5 and batch-6 (Figure 4.19) showed that by day 60 when the biomass stopped growing, the nitrate level in the media was low, but measurable. For both batches, the phosphate (Figure 4.19) had been consumed by around day 20.

Nitrate consumption rates in batch-5 and batch-6 were similar (Table 4.19) and comparable to the consumption rate in batch-3 (Table 4.11). Thus a low initial nitrate level of ~20% of normal, did not affect the specific nitrate consumption rate relative to the nitrate sufficient condition. The average specific phosphate uptake rate of batch-5 and batch-6 were comparable at 0.65 ± 0.15 mg g^{-1} d^{-1} (Table 4.19). The nutrient sufficient batch-3 had a similar phosphate uptake rate at 0.5 mg g^{-1} d^{-1} (Table 4.11).

The above results suggest that a reduced initial nitrate level of 18-20% of the normal level of BG11 seawater medium produces essentially identical growth results to the nutrient sufficient medium and allows average peak biomass concentration of about 0.8 g L^{-1} to be attained. At the end of the culture in the low nitrate medium, i.e. at the instance of peak biomass concentration, a detectable level of nitrate remained. Thus, in the raceway, where the light level limits the peak biomass concentration to an average of about 0.8 g L^{-1}, an initial nitrate level of ~20% of normal constitutes essentially a nutrient sufficient environment.

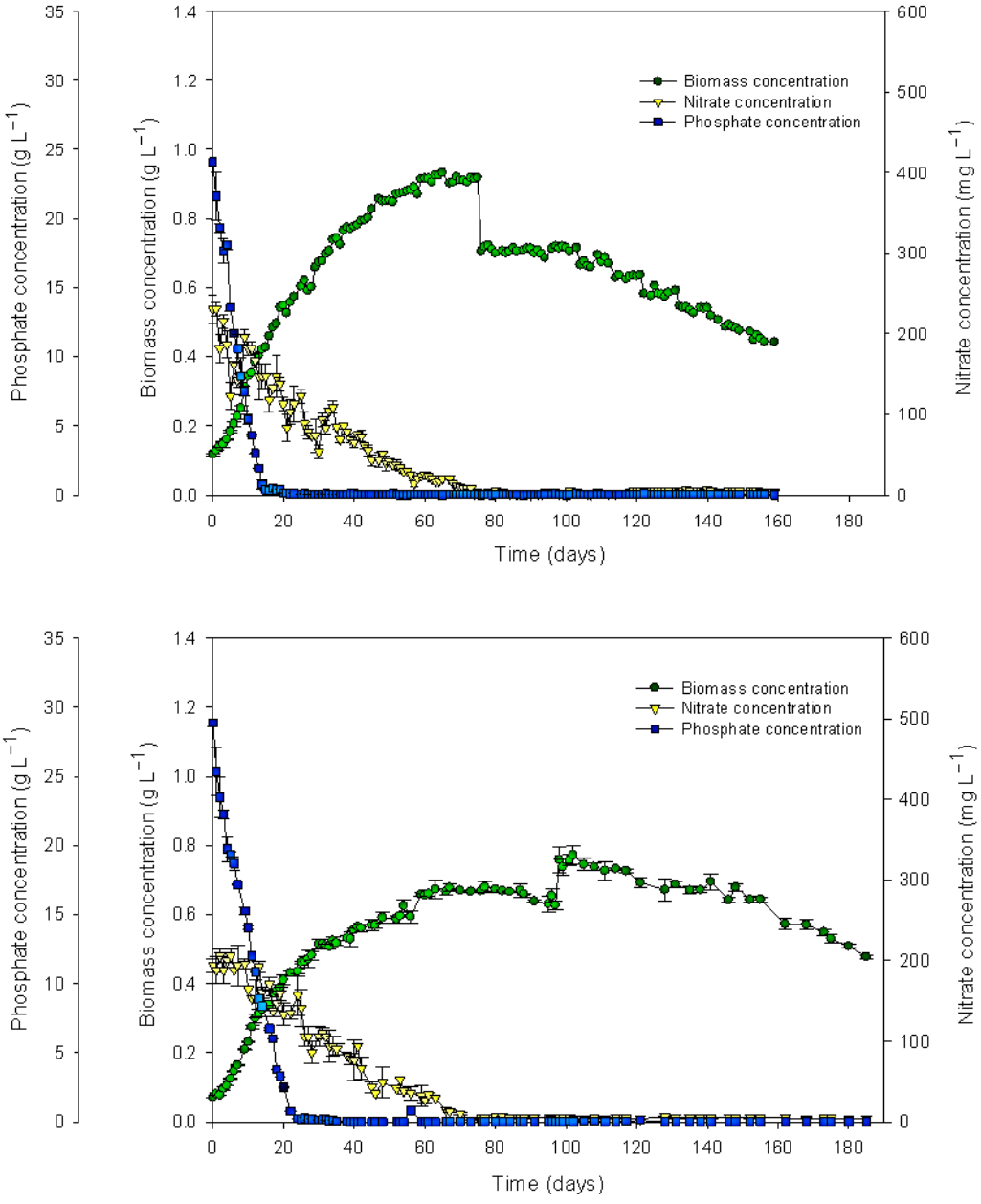


Figure 4.19 *C. vulgaris* growth and nutrient consumption profiles under nitrate stress: a) raceway batch-5 grown with ~20% of normal initial nitrate in BG11 (seawater); b) raceway batch-6 grown with ~18% of normal initial nitrate in BG11 (seawater). Biomass and nitrate standard deviations are based on triplicate samples; phosphate standard deviation is based on duplicate measurements.

Table 4.19 *C. vulgaris* raceway batch culture kinetics (low initial nitrate concentration)

Kinetic parameters[1]	**Batch-5**[a]	**Batch-6**[b]
Specific growth rate, μ (d^{-1})	0.106	0.156
Maximum biomass concentration (g L^{-1})	0.91	0.66 ± 0.00[c]
Biomass productivity[c], P_b (mg L^{-1} d^{-1})	13.5 ± 0.1	10.0 ± 0.1
Biomass yield coefficient on N, Y_N (g mg^{-1})	0.004	0.004
Biomass yield coefficient on P, Y_P (g mg^{-1})	0.033	0.020
Average N consumption rate, q_N (mg g^{-1} d^{-1})	4.4	4.7
Average P consumption rate, q_P (mg g^{-1} d^{-1})	0.5	0.8
Lipid content[d] (%, w/w)	23.7 ± 1.7	23.4 ± 0.2
Lipid productivity[d], P_l (mg L^{-1} d^{-1})	3.8 ± 0.3	2.5 ± 0.0

[1]Kinetic parameters were calculated at the point of peak biomass concentration attained on day 59.

[a]Raceway batch with ~20% of normal initial nitrate concentration; [b]raceway batch with ~18% of normal initial nitrate.

[c]Triplicate samples; [d]duplicate samples.

The lipid contents of the biomass from the nitrate-deficient and nitrate-sufficient raceway batches were similar (Table 4.19 and Table 4.12 batch-3) at the time of peak biomass concentration on day 59. This lends further credence to the argument that an initial nitrate level of ~20% of that found in normal BG11 (seawater) medium is in fact a nutrient sufficient level for attaining average peak biomass concentration of ~0.8 g L^{-1}. After day 59, the raceway cultures with ~20% of the normal initial nitrate level became progressively nitrogen deprived as time progressed. Nitrogen deprivation increased the lipid content of the biomass in batch-5 (Table 4.20, data from day 131 onwards). An increase in lipids to a lesser level occurred in batch-6 (Table 4.21).

Table 4.20 Biomass characteristics at various harvesting times in the raceway batch-5 (~20% of normal initial nitrate)

Batch harvest time[1]	**Biomass concentration (g L^{-1})**	**Biomass Productivity, P_b (mg L^{-1} d^{-1})**	**Lipid Content[2] (%, w/w)**	**Lipid Productivity[2], P_l (mg L^{-1} d^{-1})**	**Calorific value[3] (kJ g^{-1})**	**Elements in biomass[3]**			
						C (%)	**N (%)**	**S (%)**	**P (%)**
Day 48	0.85	15.3	24.1 ± 0.5	3.7 ± 0.1	22.5	ND	ND	ND	ND
Day 65	0.93	12.6	23.7 ± 1.7	3.0 ± 0.2	22.5	ND	ND	ND	ND
Day 75	0.92	10.7	22.5 ± 0.1	2.4 ± 0.0	22.8	ND	ND	ND	ND
Day 85	0.72	7.1	18.0 ± 0.0	1.3 ± 0.0	23.2	ND	ND	ND	ND
Day 90	0.71	6.6	16.2 ± 0.2	1.1 ± 0.0	23.5	ND	ND	ND	ND
Day 96	0.71	6.2	16.3 ± 0.1	1.0 ± 0.0	23.3	ND	ND	ND	ND
Day 103	0.72	5.8	18.0 ± 1.0	1.0 ± 0.1	23.8	52.0	5.4	0.8	ND
Day 112	0.67	4.9	22.5 ± 0.1	1.1 ± 0.0	24.1	49.4	5.0	0.8	0.3
Day 121	0.64	4.3	22.00	0.9	24.4	50.2	4.8	0.8	0.3
Day 131	0.59	3.6	29.0	1.1	25.2	54.7	4.5	0.8	0.3
Day 141	0.52	2.9	36.8	1.1	25.4	54.9	4.2	0.8	0.3
Day 152	0.47	2.3	41.0	1.0	26.2	56.4	4.0	0.8	0.2
Day 159	0.44	2.1	42.6 ± 1.3	0.9 ± 0.0	26.5 ± 0.2	59.2 ± 0.7	3.8 ± 0.0	0.8 ± 0.0	0.2 ± 0.0

[1]Harvesting was done after culture entered the stationary phase of growth. [2]Duplicate samples; [3]triplicate samples (on day 159 only); ND = not determined

This behavior is consistent with the previously reported (Illman *et al.*, 2000; Scragg *et al.*, 2002; Converti *et al.*, 2009) elevation in lipid levels as a consequence of nitrogen starvation in *C. vulgaris* grown in freshwater, but has not been reported in seawater grown *C. vulgaris* in raceways.

In batch-5, the calorific value of the biomass increased with increasing lipid content (Table 4.20), as expected. This occurred to a lesser level in batch-6 as the oil content in this batch did not increase a lot on nitrogen starvation. In both batches, the N content of the biomass declined with progressive nitrogen starvation (Table 4.20, Table 4.21).

Table 4.21 Biomass characteristics at various harvesting times in the raceway batch-6 (~18% of normal initial nitrate)

Batch harvest time[1]	**Biomass concentration[2] (g L^{-1})**	**Biomass productivity[2], P_b (mg L^{-1} d^{-1})**	**Lipid content[3] (%, w/w)**	**Lipid productivity[3], P_l (mg L^{-1} d^{-1})**	**Calorific value[3] (kJ g^{-1})**	**Elements in biomass[4]**			
						C (%)	**N (%)**	**S (%)**	**P (%)**
Day 80	0.67 ± 0.00	7.5 ± 0.1	23.4 ± 0.2	1.8 ± 0.0	22.7 ± 0.2	ND	ND	ND	ND
Day 88	0.66 ± 0.00	6.9 ± 0.4	23.5 ± 0.9	1.6 ± 0.1	22.7 ± 0.2	ND	ND	ND	ND
Day 96	0.65 ± 0.02	6.1 ± 0.2	21	1.3	22.4 ± 0.2	49.1	6.3	0.8	0.5
Day 102	0.77 ± 0.03	6.9 ± 0.2	22.4 ± 0.2	1.5 ± 0.0	23.1 ± 0.1	51.1	6.5	0.8	0.5
Day 111	0.73 ± 0.03	5.9 ± 0.2	21.3 ± 1.7	1.3 ± 0.1	23.0 ± 0.0	51.1	6.2	0.8	0.5
Day 117	0.73 ± 0.01	5.6 ± 0.0	23.9 ± 0.6	1.3 ± 0.0	23.0 ± 0.0	50.2 ± 0.4	5.7 ± 0.2	0.8 ± 0.2	0.5 ± 0.0
Day 131	0.69 ± 0.01	4.7 ± 0.1	25.7 ± 0.4	1.2 ± 0.0	23.3 ± 0.3	51.3	5.6	0.8	0.4
Day 141	0.70 ± 0.02	4.4 ± 0.2	26.3 ± 1.0	1.1 ± 0.0	23.3 ± 0.2	50.5	5.4	0.8	0.4
Day 148	0.68 ± 0.01	4.1 ± 0.1	27.1 ± 1.8	1.1 ± 0.1	23.3 ± 0.2	51.7	5.8	0.8	0.4
Day 155	0.64 ± 0.01	3.7 ± 0.0	23.1 ± 0.5	0.9 ± 0.0	22.8 ± 0.5	50.8	5.0	0.9	0.4
Day 162	0.57 ± 0.01	3.2 ± 0.1	26.6	0.8 ± 0.0	23.3 ± 0.2	48.9	5.2	0.9	0.4
Day 168	0.57 ± 0.01	3.0 ± 0.1	23.7 ± 1.8	0.7 ± 0.1	22.8 ± 0.2	49.8 ± 0.1	5.5 ± 0.1	0.9 ± 0.0	0.4 ± 0.0
Day 175	0.53 ± 0.01	2.7 ± 0.1	22.0 ± 1.8	0.6 ± 0.0	23.2 ± 0.0	51.6	5.5	0.9	0.3
Day 185	0.48 ± 0.00	2.2 ± 0.0	28.0 ± 0.1	0.6 ± 0.0	23.7 ± 0.0	52.6 ± 0.4	5.1 ± 0.0	0.9 ± 0.0	0.3 ± 0.0

[1]Harvesting was done after culture entered the stationary phase of growth. [2]Triplicate samples; [3]duplicate samples, [4]duplicate samples (on day 117, day 168 and day 185 only); ND = not determined

Lipid accumulation in microalgae may start within 1-2 days of nitrogen deprivation (Solomon *et al.*, 1986) but the rate of lipid accumulation may be different for different algae. The rate of lipid accumulation may also depend on the other conditions of a nitrogen deprived culture. Lipid accumulation at the cellular level was visualized by transmission electron microscopy of some samples as described in Section 4.3.1.3d.

4.3.1.3b Effect of 10% of normal initial nitrate on *C. vulgaris*

The aim of this run (raceway batch-7) was to determine if the lipid accumulation in the biomass could further be enhanced relative to the results of Section 4.3.1.3a. Other than the nitrate concentration in BG11 seawater medium, the culture conditions of the raceway batch-7 were similar to those of batch-5 and batch-6, discussed in the previous section. The growth profile of batch-7 is shown in Figure 4.20.

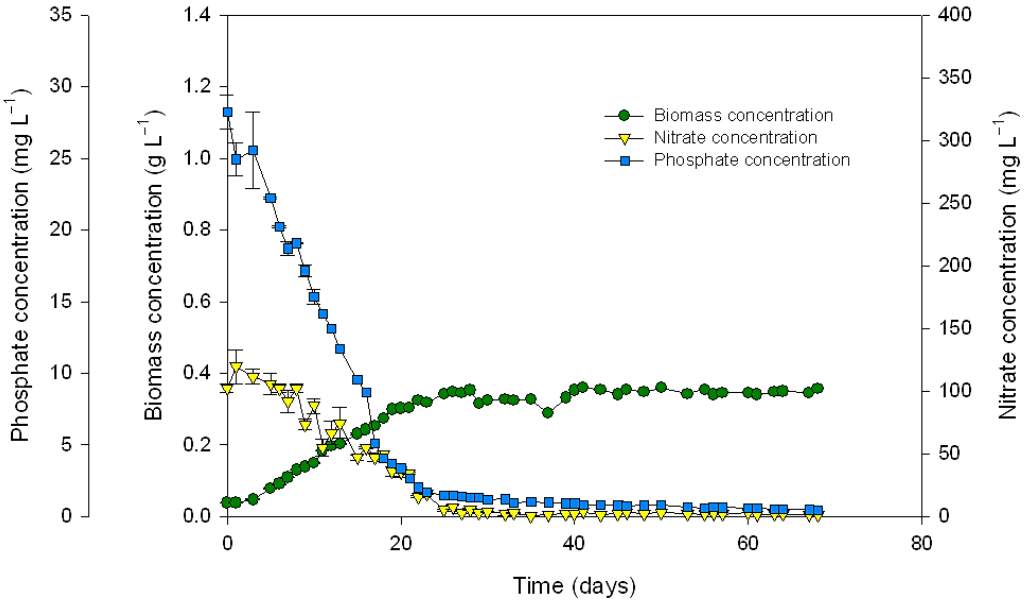


Figure 4.20 Growth and nutrient consumption profiles of *C. vulgaris* in raceway batch-7 with ~10% of normal initial nitrate in BG11 (seawater). Biomass and nitrate standard deviations are based on triplicate samples; phosphate standard deviation is based on duplicate measurements.

The maximum biomass concentration (0.34 g L^{-1}) attained on day 25 coincided with nitrate depletion from the culture broth (Figure 4.20). Clearly, the peak biomass concentration was limited by nitrate depletion. The growth was rapid with a specific growth rate of 0.214 d^{-1} (Table 4.22) until being arrested by nitrate depletion. Until nitrate depletion on day 25, the culture was essentially nutrient sufficient. Thus, on day 25, the biomass yield on N in batch-7 (Table 4.22) was comparable to the other raceway batches regardless of the initial nitrate concentration in the BG11 media (Table 4.11 and Table 4.19). However, the biomass yield on P was low in raceway batch-7 (Table 4.22) compared to the other batches (batches 3, 5 and 6; Table 4.11 and Table 4.19). This was simply because the biomass production was being limited by nitrate supply and not by phosphate.

Table 4.22 *C. vulgaris* raceway batch culture kinetics (~10% of normal initial nitrate in BG11)

Kinetic parameters[1]	**Batch-7**[a]
Specific growth rate, μ (d^{-1})	0.214
Maximum biomass concentration (g L^{-1})	0.34
Biomass productivity[b], P_b (mg L^{-1} d^{-1})	12.2 ± 0.0
Biomass yield coefficient on N, Y_N (g mg^{-1})	0.003
Biomass yield coefficient on P, Y_P (g mg^{-1})	0.011
Average N consumption rate, q_N (mg g^{-1} d^{-1})	12.7
Average P consumption rate, q_P (mg g^{-1} d^{-1})	3.5
Lipid content[c] (%, w/w)	18.8 ± 0.2
Lipid productivity[c], P_l (mg L^{-1} d^{-1})	2.1 ± 0.0

[1]Kinetic parameters were calculated at the point of peak biomass concentration attained on day 25

[a]Raceway batch with ~10% of normal initial nitrate concentration

[b]Triplicate samples; [c]duplicate samples

Once the culture had attained the maximum biomass concentration and all nutrients had exhausted, it was kept running to monitor the effects of nutrient starvation on lipid accumulation. During this period harvests (4 harvests) occurred as shown in Table 4.23. The lipid content progressively increased from ~19% to >41% on day 68 (Table 4.23). Clearly, therefore nitrate starvation leads to progressively increased lipid levels (Table 4.23). The calorific value of the biomass also increased with the increase in lipid level (Table 4.23).

Although the lipid content in the biomass increased with starvation period, the lipid productivity did not increase (Table 4.23). This was because for a fixed biomass concentration, the increased lipid level with time was countered by the negative impact of time on productivity.

Table 4.23 Biomass characteristics of *C. vulgaris* at various harvesting times in the raceway batch-7 (10% of normal initial nitrate)

Batch harvest time[1]	**Biomass concentration (g L^{-1})**	**Biomass productivity, P_b (mg L^{-1} d^{-1})**	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**	**Elements in biomass**			
						C (%)	**N (%)**	**S (%)**	**P (%)**
Day 28	0.35	7.9 ± 0.2	18.8 ± 0.2	1.5 ± 0.0	21.9	41.4	7.6	0.8	1.1
Day 35	0.33	5.6 ± 0.1	22.7 ± 1.5	1.3 ± 0.1	ND	ND	ND	ND	ND
Day 55	0.35	4.1 ± 0.1	29.4 ± 2.3	1.2 ± 0.1	ND	ND	ND	ND	ND
Day 68	0.36	3.3 ± 0.1	41.1 ± 0.2	1.4 ± 0.0	27.0 ± 0.0	55.2 ± 0.7	4.1 ± 0.1	0.6 ± 0.0	0.7 ± 0.0

[1]Harvesting was done after culture entered the stationary phase of growth.

ND = Not determined.

Biomass standard deviation is based on triplicate samples; all other standard deviations are based on duplicate measurements.

In view of the results, a maximum biomass concentration of 0.66-0.91 g L^{-1} can be attained with an initial nitrate level of ~20% of normal in BG11 seawater medium. This is the same as would occur in a full nitrate medium. Reducing the initial nitrate level to ~10% of normal, significantly reduces the peak attainable biomass concentration to 0.34 g L^{-1}, or <50% of the peak value of the normal full strength medium. Nitrate starvation subsequent to growth, builds up the lipid content in the biomass and the calorific value of the biomass. Earlier studies on raceways under N starvation reported a maximum biomass concentration of 0.3 g L^{-1} for *N. salina* (Boussiba *et al.*, 1987) and ~0.4 g L^{-1} for *Scenedesmus rubescens* (Lin and Lin, 2011) with lipid contents of 16% and 24%, respectively. For *N. salina*, the lipid content did not increase with N starvation as this species does not respond to N stress. However, *Scenedesmus rubescens* showed an increase in lipid content but this was accompanied by a decrease in the biomass growth.

4.3.1.3c Effect of 22% of normal initial nitrate level on *N. salina*

N. salina was studied in the raceway for nitrate stress effects. An initial nitrate level of ~22% of normal in BG11 seawater medium was used. The growth conditions were as described earlier in the Section 4.3.1.3. (This alga had been inoculated accidently, but the experiment was continued to gain information for comparison with *C. vulgaris*.) The paddlewheel potentiometer setting was at 5 to give a flow velocity of 0.21 m s^{-1}. After the onset of the stationary growth phase, on day 73, the rotational speed was increased gradually to the potentiometer setting of 9 (i.e. a flow velocity of 0.32 m s^{-1}). This was done to check for possible effects of agitation/mixing as they have been found to affect the biomass productivity in some cases (Gudin and Chaumont, 1991; Hu and Richmond, 1996).

The growth profile for this batch-8 is shown in Figure 4.21. After a prolonged lag phase a maximum biomass concentration of 0.44 g L^{-1} was attained on day 69. Unlike in *C. vulgaris* raceway culture at a similar initial nitrate level, the nitrate did not run out completely from the culture broth (Figure 4.21) and ~47 mg L^{-1} of nitrate remained until final harvest on day 125. Phosphate was fully consumed by around day 50 (Figure 4.21). Nutrients uptake rates were similar to the values seen for *C. vulgaris* at a similar initial nitrate level (Table 4.19 and Table 4.24). The biomass yield on N (Table 4.24) was comparable to the values for *C. vulgaris* (Table 4.19). However, the biomass yield on P was 2.2 times lower (Table 4.24) than in *C. vulgaris* (Table 4.19).

The slight jump in biomass concentration on day 103 was a consequence of increase in paddlewheel speed from a potentiometer setting of 5 to a setting of 7 (Figure 4.21). During the period when the paddlewheel setting was 7-9 on the potentiometer, the growth rate improved (Figure 4.21). This may have been a consequence of turbulence associated improvements in desorption of oxygen (an inhibitor of photosynthesis) or improved mixing resulting in more frequent exposure of the biomass from deeper darker zones of the raceway to the better illuminated surface. Once the paddlewheel speed was lowered from 9 on the potentiometer to 5, biomass concentration declined a little (Figure 4.21) possibly because of some sedimentation.

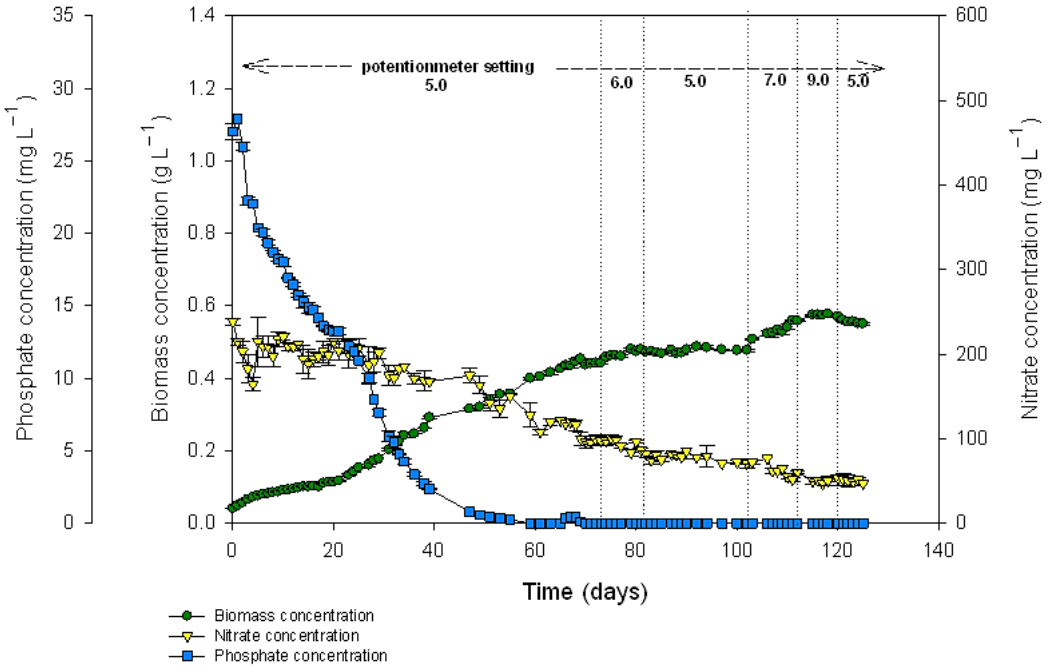


Figure 4.21 Growth and nutrient consumption profiles of *N. salina* in raceway batch-8 with ~22% of normal initial nitrate in BG11 (seawater). Vertical dotted lines demarcate periods of different settings of paddlewheel speed on the potentiometer: 5, 6, 7 and 9 (the corresponding velocities were 0.21± 0.02 m s^{-1}, 0.23 ± 0.02 m s^{-1}, 0.27 ± 0.04 m s^{-1} and 0.32 ± 0.03 m s^{-1}, respectively). Standard deviations of biomass and nitrate are based on triplicate samples; standard deviation of phosphate is based on duplicate samples.

Table 4.24 *N. salina* raceway batch-8[a] culture kinetics (at different rotational speeds[1])

Kinetic parameters[2]	**Paddlewheel potentiometer setting**					
	5	**6**	**5**	**7**	**9**	**5**
Specific growth rate, μ (d^{-1})	0.060	ND	ND	ND	ND	ND
Maximum biomass concentration[b] (g L^{-1})	0.44 ± 00	0.46 ± 00	0.47 ± 00	0.52 ± 01	0.56 ± 00	0.54 ± 00
Biomass productivity[b], P_b (mg L^{-1} d^{-1})	5.3 ± 0.1	4.8 ± 0.0	4.3 ± 0.0	4.1 ± 0.1	4.1 ± 0.0	3.8 ± 0.0
Biomass yield coefficient on N, Y_N (g mg^{-1})	0.003	0.003	0.003	0.003	0.003	0.003
Biomass yield coefficient on P, Y_P (g mg^{-1})	0.015	0.016	0.017	0.018	0.019	0.018
Average N consumption rate, q_N (mg g^{-1} d^{-1})	5.0	4.4	4.1	3.5	3.1	3.1
Average P consumption rate, q_P (mg g^{-1} d^{-1})	0.7	0.7	0.6	0.5	0.4	0.4
Lipid content[c] (%, w/w)	34.9	31.9	ND	ND	ND	31.8 ± 0.2
Lipid productivity[c], P_l (mg L^{-1} d^{-1})	1.9	1.5	ND	ND	ND	1.2 ± 0.0

[a]Raceway batch with ~22% of normal initial nitrate concentration in BG11 (seawater)

[1]Paddlewheel potentiometer setting corresponded to the following flow velocities; 5 = 0.21± 0.02 m s^{-1}; 6 = 0.23 ± 0.02 m s^{-1}; 7 = 0.27 ± 0.04 m s^{-1}; 9 = 0.32 ± 0.03 m s^{-1}

[2]Kinetic parameters were measured when the biomass concentration had stabilized at each rotational speed on day 69, day 81, day 92, day 110, day 118 and day 123, respectively, for potentiometer settings of 5, 6, 5, 7, 9 and 5

[b]Triplicate samples; [c]duplicate samples

Culture broth samples from raceway batch-8 were harvested on days 69, 81 and 125 (Table 4.25). The lipid level in the biomass remained stable at ~33% (Table 4.25) and the calorific value was always in the range of 23-26 kJ g^{-1} (Table 4.25).

The culture in Figure 4.21 was never nitrogen deprived, but earlier work with this alga (Section 4.2.3.2b), showed that nitrate-deprivation does not promote lipid accumulation. This behavior has been previously reported (Boussiba *et al.*, 1987) and is different to that of *C. vulgaris*.

Use of *N. salina* in the raceway was discontinued because of the low biomass and lipid productivities in low-nitrogen conditions and the failure of this alga to respond to nitrogen deprivation by accumulating lipids.

Table 4.25 Biomass characteristics of *N. salina* at various harvesting times in the raceway batch-8 (20% of normal initial nitrate)

Batch harvest time[1]	**Pontentio-meter setting**	**Biomass concentration (g L^{-1})**	**Biomass productivity, P_b (mg L^{-1} d^{-1})**	**Lipid content (%, w/w)**	**Lipid productivity, P_l (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**	**Elements in biomass**			
							C (%)	**N (%)**	**S (%)**	**P (%)**
Day 69	5	0.44 ± 00	5.3 ± 0.1	34.9	1.9	25.7	ND	ND	ND	ND
Day 81	6	0.46 ± 00	4.8 ± 0.0	31.9	1.5	24.8	ND	ND	ND	ND
Day 125	5	0.53 ± 00	3.7 ± 0.0	31.8 ± 0.2	1.2 ± 0.0	23.4 ± 0.2	49.3 ± 0.1	8.8 ± 0.0	0.6 ± 0.2	0.6 ± 0.2

[1]Between day 81-125, the paddlewheel potentiometer setting corresponded to the following flow velocities; 5 = 0.21± 0.02 m s^{-1}; 7 = 0.27 ± 0.04 m s^{-1}; 9 = 0.32 ± 0.03 m s^{-1} (Table 4.24).

ND = Not determined.

Biomass standard deviation is based on triplicate samples; all other standard deviations are based on duplicate measurements.

4.3.1.3d Lipid accumulation

Lipid bodies in individual algal cells can be observed via Nile red fluorescent microscopy (Elsey *et al.*, 2007) and the transmission electron microscopy (Bozzola and Russell, 1998). *C. vulgaris* and *N. salina* collected from batch-6 and batch-8, respectively), were observed using these methods.

In fluorescent images of *C. vulgaris*, the neutral lipid bodies (i.e. yellow fluorescence) appeared very few and smaller compared to the *N. salina* (Figure 4.22).

TEM (transmission electron microscope) showed a cross section of the algal cells (Figure 4.23). Multiple small lipid droplets were seen in the cytoplasm of *C. vulgaris* (Figure 4.23a) and one or two large lipid bodies occurred in *N. salina* cytoplasm (Figure 4.23b). The number of lipid droplets was not the same in all cells of a given sample of the two strains (Figure 4.23), suggesting that cells were at different stages of lipid accumulation in the same population. The lipid droplets were identified according to the electron micrographs of de-Bashan *et al*. (2002) and Přibyl *et al*. (2013).

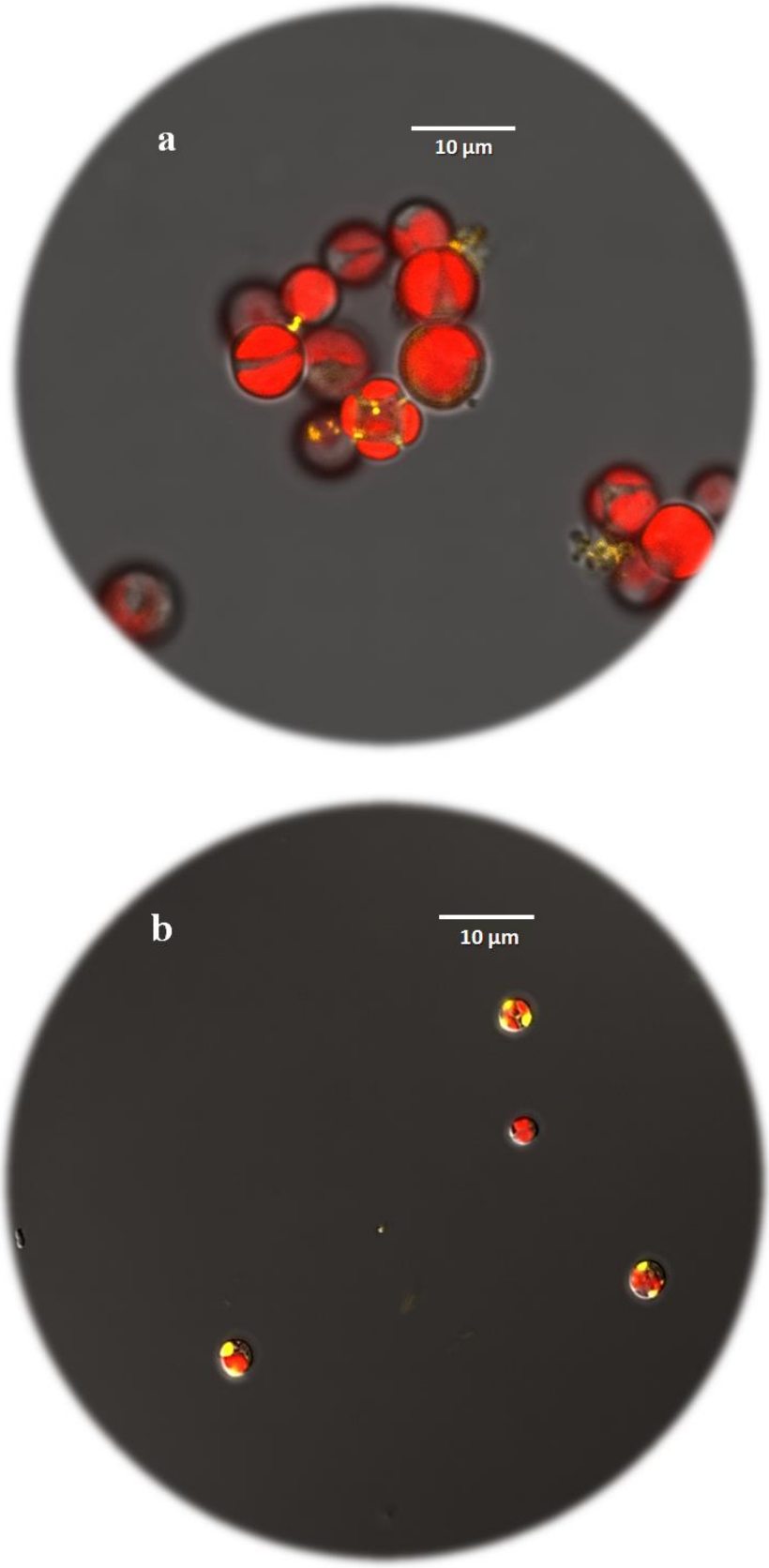


Figure 4.22 Fluorescent microscopy: (a) *C. vulgaris* at day 81; (b) *N. salina* at day 103.

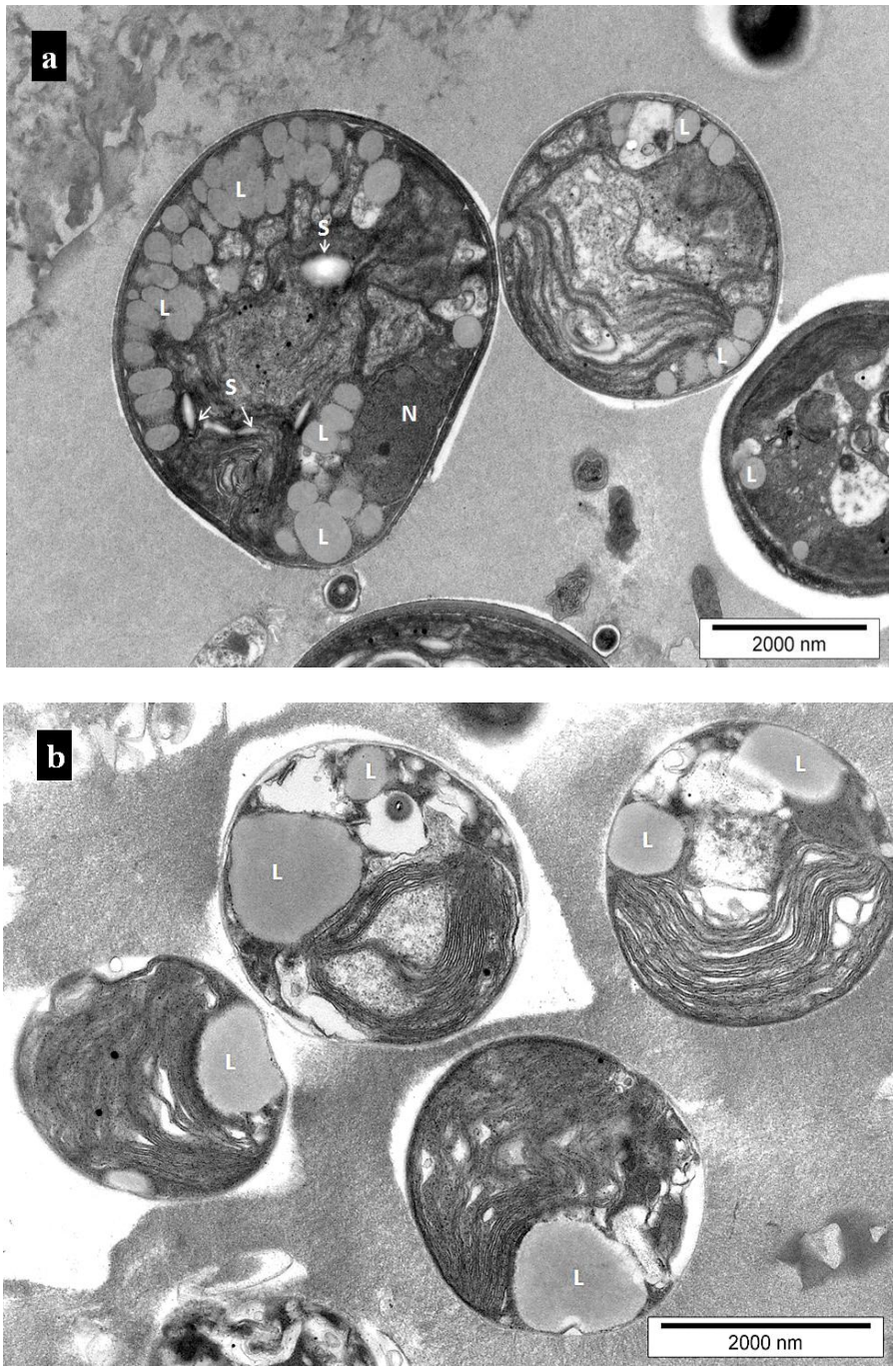


Figure 4.23 Transmission electron microscopy of algal cells from raceway: (a) *C. vulgaris* on day 111 (batch-6); (b) *N. salina* on day 108 (batch-8). L, represents lipid droplets in cells; S, represents starch; N, represents nucleus. Images taken at Manawatu Microscopy and Imaging Centre, Massey University (sample prepared by glutaraldehyde-osmium tetroxide standard fixation protocol).

4.3.2 Raceway continuous culture

C. vulgaris was grown in continuous mode of operation for baseline studies on growth and lipid production. The raceway batch culture shown in Figure 4.16b was switched to a continuous culture mode of operation on day 30. Continuous culture setup was explained in Section 3.2.3.4. At various times, the dilution rate (i.e. the ratio of the feed flow rate (L d^{-1}) to the total culture volume in the raceway (L)) values were 0.276 d^{-1} and 0.173 d^{-1}. Illumination was either continuous at 91 $\mu mol \cdot m^{-2}s^{-1}$, or at 46 $\mu mol \cdot m^{-2}s^{-1}$. In specific cases a 14 h: 10 h light-dark cycle was used. The culture medium was BG11 seawater (Section 3.2.1). The operational temperature and pH were constant throughout at 20 ± 1 °C and 6.5-6.8, respectively. The rotational speed of the paddlewheel was fixed at setting 5 (flow velocity of 0.21 ± 0.02 m s^{-1}) on the potentiometer.

The growth profile of the continuous culture is shown in Figure 4.24. At a dilution rate of 0.276 d^{-1} the steady states 1-5 (Figure 4.24) were attained at different illumination regimes (Table 4.26). By definition, at any steady state in a continuous culture, the dilution rate is equal to the specific growth rate (Doran, 1995; Shuler and Kargi, 2002). The biomass concentration at each steady state was different as recorded in Table 4.26. At any given steady state, different combination of biomass concentration and the light regimen were such as to produce the same average light level in the raceway and therefore the same specific growth rate.

A large volume of the broth was continuously harvested at each steady state in a refrigerated (5 °C) carboy and processed later for determining the lipid level, the calorific value and the elemental composition of the biomass (Table 4.27). Feeding and harvesting of the culture which operated under light-dark cycling (Figure 4.24) was stopped each night to prevent washout.

The biomass concentration profile in Figure 4.24 was generally consistent with expectations. Commencing at 0 h, the biomass concentration began to decline from the maximum value attained in the previous batch run (Figure 4.16b). This was because of dilution by the feed. The biomass concentration declined until a steady concentration (steady state 1, SS-1) was attained. At this steady state (and any other steady state in a continuous culture), the specific growth rate of the biomass was assumed equal to the dilution rate (Doran, 1995; Shuler and Kargi, 2002). The dilution rate remained fixed at the values shown in Figure 4.24. Steady state 1 (SS-1) was obtained at the full possible light level (Table 4.26). At the end of steady state 1, the light level was reduced (Table 4.26). This caused the biomass concentration to decline further to a lower steady state value (i.e. steady state 2, SS-2). At the end of steady state 2 (SS-2), the light level was raised again to the initial value of (Table 4.26) and, therefore, the biomass concentration rose to attain the new steady state 3 (SS-3). The light level now remained at full intensity (91 $\mu mol \cdot m^{-2} s^{-1}$ continuous illumination) but switched to light-dark cycling (Table 4.26). Consequently, the biomass concentration began to decline and oscillate – a high concentration during 'day' and a low concentration at 'night' (Figure 4.24, Table 4.26). Eventually, the oscillations settled around a pseudosteady state biomass concentration (steady state 4, Figure 4.24 inset a1). At the end of the steady state 4 (SS-4), the light was switched back to full intensity continuous operation and the biomass concentration rose to a higher steady state value (SS-5). The culture operation was continued beyond 1500 h at a new fixed dilution rate of 0.173 d^{-1} (Figure 4.24). The nutrients (nitrate and phosphate) profiles are shown in Figure 4.24b.

At 1500 h, the dilution rate was reduced to 0.173 d^{-1}, but all other operational conditions were same as described earlier. As specific growth rate at each of the steady states 6-8 was constant at 0.173 d^{-1}, the alga was growing ~62% slower than in the previously discussed steady states 1-5. The steady states 6-8 (Figure 4.24) were solely a

consequence of changes in the incident irradiance at a fixed dilution rate of 0.173 d^{-1}. The biomass concentrations at various light regimens for the steady states in Figure 4.24a are summarized in Table 4.26. The relevant lipids contents and productivities are provided in Table 4.27.

Table 4.26 *C. vulgaris* raceway continuous culture kinetics (standard BG11 seawater medium)

Steady state[1]	**Dilution rate[2], μ (d^{-1})**	**Maximum biomass concentration[3] (g L^{-1})**	**Biomass productivity[3], P_b (mg L^{-1} d^{-1})**	**Biomass yield coefficient on N, Y_N (g mg^{-1})**	**Biomass yield coefficient on P, Y_P (g mg^{-1})**	**Biomass yield coefficient on light, $Y_{X/L}$ (g μE^{-1})**	**Average N consumption rate q_N (mg g^{-1} d^{-1})**	**Average P consumption rate q_P (mg g^{-1} d^{-1})**
1	0.276	0.13 ± 0.01	34.9 ± 1.4	0.004	0.008	8.5×10^{-7}	66.0	35.1
2	0.276	0.08 ± 0.00	20.8 ± 0.7	0.000	0.004	1.2×10^{-6}	899.8	66.5
3	0.276	0.18 ± 0.00	50.5 ± 0.5	0.017	0.030	3.0×10^{-6}	16.1	9.4
4	0.276	0.14 ± 0.00[a]	38.4 ± 0.5[a]	0.001	0.014	ND	198.0	20.0
5	0.276	0.16 ± 0.00	43.5 ± 0.3	0.002	0.012	1.1×10^{-6}	164.4	22.0
6	0.173	0.36 ± 0.00	61.9 ± 0.5	-0.006	0.025	1.4×10^{-6}	-29.2	7.1
7	0.173	0.21 ± 0.01	36.4 ± 1.3	0.009	0.040	2.1×10^{-6}	18.8	1.6
8	0.173	0.26 ± 0.00	45.7 ± 0.5	0.003	0.027	1.1×10^{-6}	69.2	6.4

[1]Biomass kinetic parameters were calculated at steady states 1-8; where SS-1 = continuous light at 91 µmol·$m^{-2}s^{-1}$, SS-2 = continuous light at 46 µmol·$m^{-2}s^{-1}$, SS-3 = same conditions as SS-1, SS-4 = light/dark cycle (14 h:10 h) at illumination level of 91 µmol·$m^{-2}s^{-1}$, SS-5 = same conditions as SS-1, SS-6 = continuous light at 91 µmol·$m^{-2}s^{-1}$, SS-7 = continuous light at 46 µmol·$m^{-2}s^{-1}$, SS-8 = same conditions as SS-6.

[2]Specific growth rate; [3]averaged steady state biomass concentration; standard deviations of all steady state values are based on 4-12 samples (one sample per day) taken within the steady state.

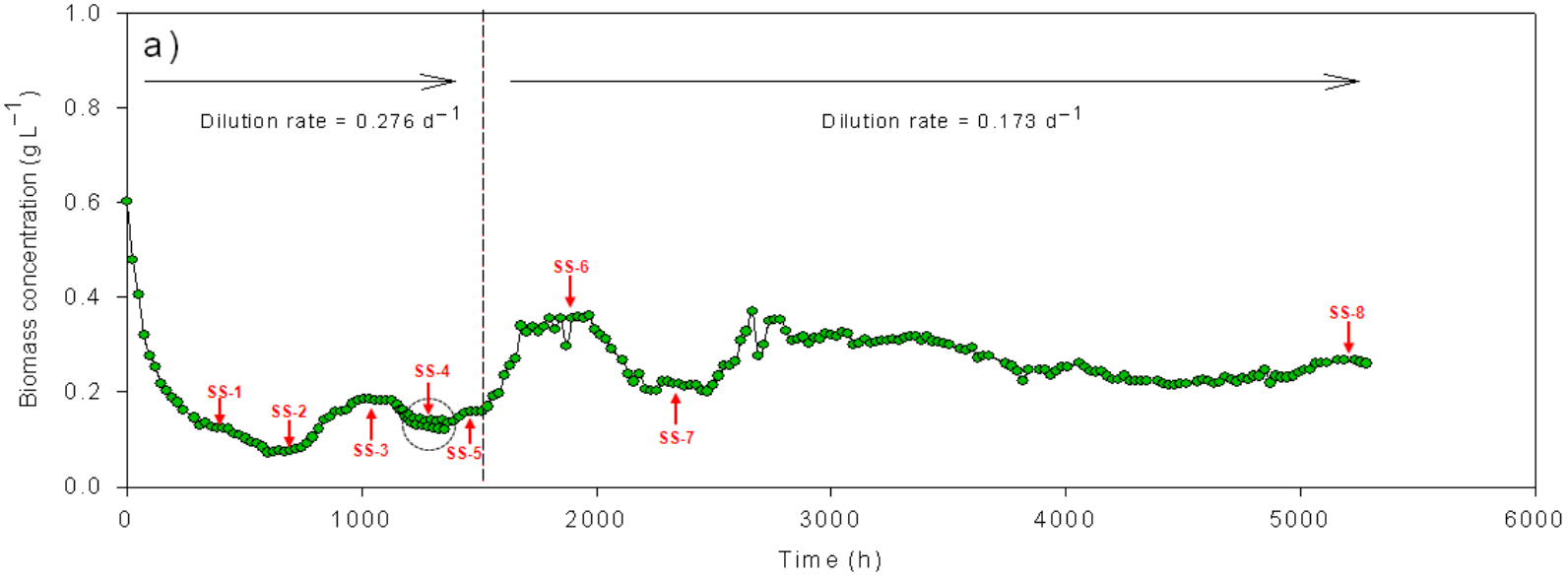
[a]Light period (14 h) biomass concentration and productivity; the dark period (10 h) biomass concentration and productivity were 0.12 ± 0.00 g L^{-1} and 33.9 ± 0.5 mg L^{-1} d^{-1}, respectively.

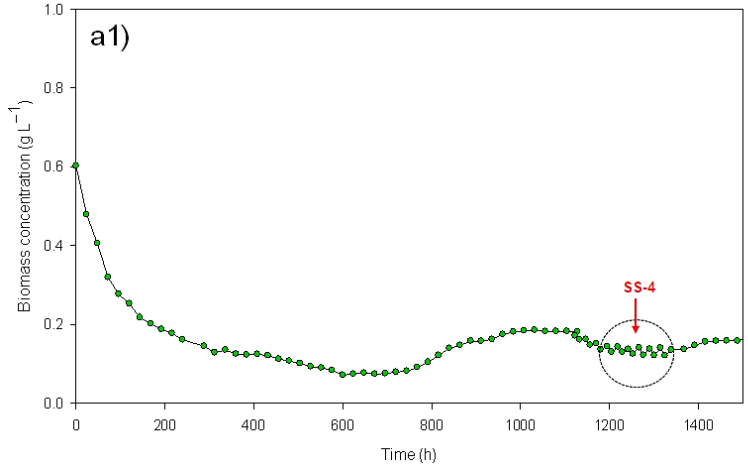
Table 4.27 Biomass characteristics at various steady states (Figure 4.24)

Steady states[1]	**Dilution rate, μ (d^{-1})**	**Lipid content (%, w/w)**	**Lipid productivity, P_b (mg L^{-1} d^{-1})**	**Calorific value (kJ g^{-1})**	**Elements in biomass**		
					C (%)	**N (%)**	**S (%)**
1	0.276	12.0	4.2 ± 0.2	20.6	46.2	7.8	0.7
2	0.276	13.9	2.9 ± 0.1	20.5	45.9	8.0	0.7
3	0.276	16.1	8.1 ± 0.1	21.5	47.9	8.4	0.7
4	0.276	13.9	5.0 ± 0.3	20.2	44.9	7.8	0.7
5	0.276	13.9	6.1 ± 0.1	19.6	43.9	7.8	0.6
6	0.173	13.6	8.4 ± 0.1	20.6	45.8	7.8	0.7
7	0.173	14.2	5.2 ± 0.2	21.1	46.8	7.8	0.8
8	0.173	15.1	6.9 ± 0.1	21.5	47.4	8.5	0.8

[1]Steady state conditions as described in Table 4.26.

Lipid productivity standard deviations are based on 4-12 samples (one sample per day) taken within a steady state.





Inset (a1) expands the 0-1400 h time scale and the biomass concentration scale of (a) to clearly show the day-night oscillations in biomass concentrations in steady state SS-4

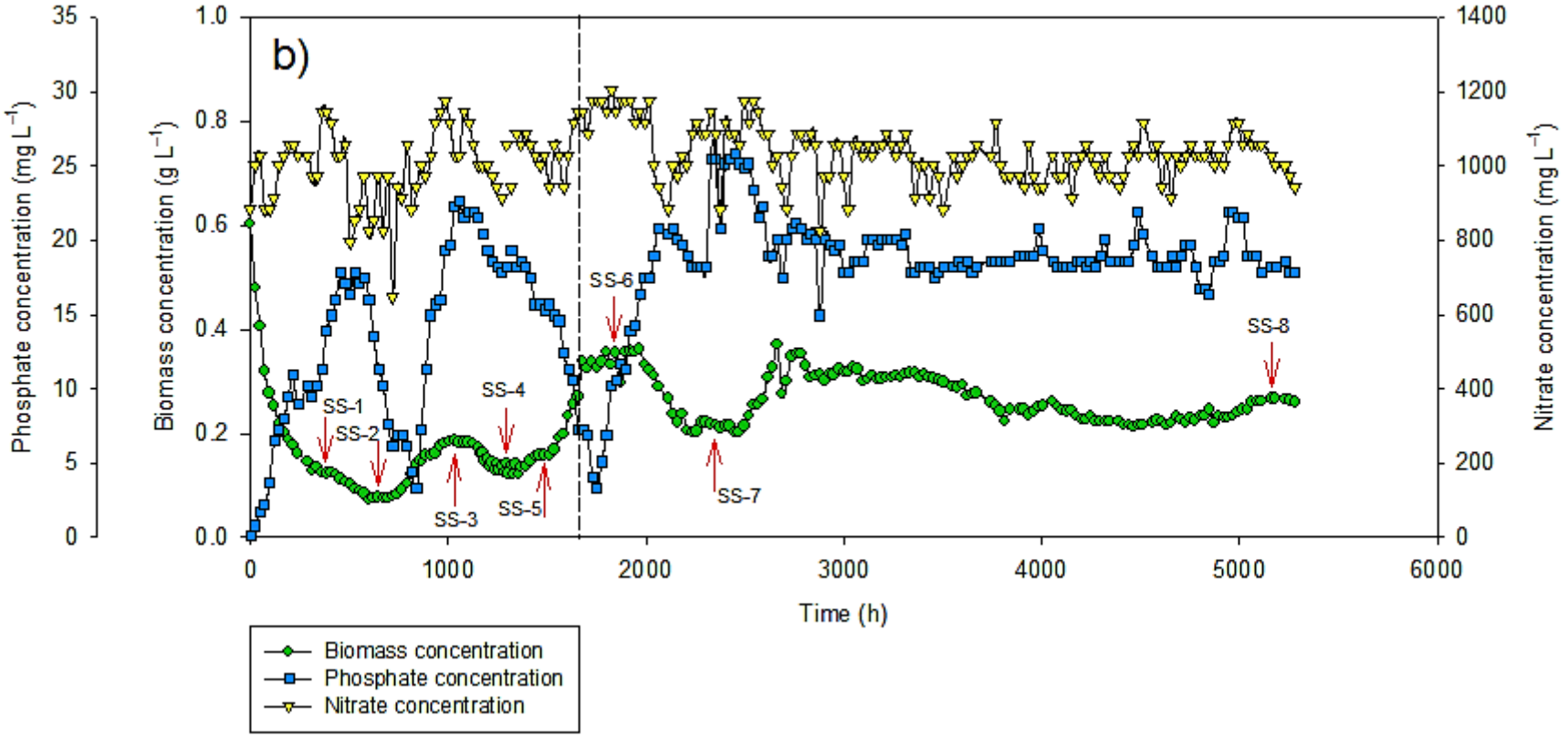


Figure: 4.24 *C. vulgaris* culture in continuous raceway operation (arrows indicate steady states): a) biomass concentration at steady states 1-8, where SS-1 = continuous light at 91 µmol·m^{-2}s^{-1}, SS-2 = continuous light at 46 µmol·m^{-2}s^{-1}, SS-3 = same conditions as SS-1, SS-4 = light/dark cycle (14 h:10 h) at illumination level of 91 µmol·m^{-2}s^{-1}, SS-5 = same conditions as SS-1, SS-6 = continuous light at 91 µmol·m^{-2}s^{-1}, SS-7 = continuous light at 46 µmol·m^{-2}s^{-1}, SS-8 = same conditions as SS-6; a1) oscillation in biomass concentration at SS-4 (due to 14 h: 10 h day/night cycle); b) growth and nutrient profile of C. *vulgaris* culture in continuous raceway.

The stable biomass concentrations for steady states 6-8 were higher than for the steady states 1-4 (Figure 4.24, Table 4.26). This was because the dilution rate was higher in the latter set of steady states (i.e. steady states 1-4). The biomass concentrations for steady states 6 (SS-6) and 8 (SS-8) were comparable because of the identical conditions of operation. At steady state 7, the biomass concentration was lower than in steady states 6 and 8 because the incident light level was lower (Table 4.26).

The productivity of the raceway cultures was clearly limited by light as at a culture density of ~0.6 g L^{-1}, the measured photosynthetically active irradiance level at full incident light was almost nil at a depth of 0.23 cm from the surface (Figure 3.10). Thus, nearly 87% of the volume of the raceway constituted an essentially non-productive dark zone. This of course is an important limitation of also the full-scale commercial raceways placed in full sunlight.

The lipid content and productivity data for the biomass samples from steady states 1-8 are shown in Table 4.27. The maximum biomass productivity was ~62 mg L^{-1} d^{-1} attained in steady state 6 and showed the highest lipid productivity of 8.4 mg L^{-1} d^{-1}. Lipid content of the biomass of all steady states were comparable indicating that the proportion of lipid in the biomass was independent of the specific growth rate and the irradiance level in nutrient sufficient growth in continuous culture. The lipid content of the biomass obtained from raceway continuous culture was not necessarily similar to the lipid content of the biomass of raceway batch cultures (i.e. both ran under nutrient sufficient growth conditions, Section 4.3.1.1 and Section 4.3.1.2). In a raceway continuous run, biomass was harvested continuously and the culture remained in exponential phase of growth and showed a lipid content range of 12-16% (Table 4.27). Whereas in raceway batch cultures, the biomass was usually harvested when the cultures had entered the stationary growth phase and showed more accumulation of lipids at levels of 21-25% in the dry biomass (Table 4.12 and Table 4.18).

Calorific value of the biomass at different steady states was comparable at 20.7 ± 0.7 kJ g^{-1} (Table 4.27) irrespective of the light regime and the dilution rate. Also, the calorific value of the biomass of raceway continuous culture (Table 4.27) was consistent with the calorific value of the biomass of raceway batch culture (Table 4.12) produced under nutrient sufficient condition. This suggested that certain variables, i.e. different modes of cultures, various levels of incident light and the specific growth rate, have little effect on energy content of the biomass (i.e. the calorific value) under nutrient sufficient conditions.

The fractions of C, N and S in the biomass obtained at different steady states were essentially independent of the operational conditions (Table 4.27).

In conclusion, among batch and continuous culture operation, the latter was found to be more efficient in terms of attaining highest biomass and lipid productivities of ~62 mg L^{-1} d^{-1} and 8 mg L^{-1} d^{-1}, respectively. This was achieved under nutrient sufficient conditions. Irrespective of the strain and the culture parameters used in other studies in raceways, the biomass and lipid productivities were much lower in the current study. Recently, Ketheesan and Nirmalakhandan (2012) reported biomass and lipid productivities of 190 mg L^{-1} d^{-1} and 40 mg L^{-1} d^{-1}, attained with *Scenedesmus* in continuous mode of cultivation in an airlift raceway system in freshwater. Overall, in the raceway pond, the best biomass productivities were 220-340 mg L^{-1} d^{-1}, achieved under semi-continuous mode of cultivation for *Dunaliella salina* (Moheimani and Borowitzka, 2006). As there is no published data on productivity of *C. vulgaris* in seawater in raceways (see Table 2.1 and Table 2.2), the data obtained here cannot be compared with the literature. For the same *C. vulgaris* as used here, lipid productivities in photobioreactors in seawater have been as high as 37 mg L^{-1} d^{-1} (nutrient sufficient

conditions) (Luangpipat, 2013). A lipid productivity of 8 mg L^{-1} d^{-1} in the nutrient sufficient raceway is ~22% of the nutrient sufficient lipid productivity of photobioreactors. However, algae production in photobioreactors tends to be much more expensive (e.g. 6 to 10-fold more expensive) than in raceways (Chisti, 2012; Chisti, 2013) and therefore, despite a lower productivity raceways can be a more cost-effective production system than photobioreactors.

Chapter 5

SUMMARY AND CONCLUSION

5.1 Summary

This research focused on production of lipids by microalgae in seawater media. Several microalgae were initially screened for lipid productivity, biomass productivity, salinity tolerance and response of lipid productivity to nutrient starvation postgrowth. Based on these studies, *Chlorella vulgaris* was identified as the most promising alga. This alga was examined in detail for biomass and lipid production in marine media in a pilot-scale (~ 140 L) purpose-built open raceway.

Studies revealed that *C. vulgaris,* normally a freshwater alga, could be effectively grown in full strength seawater media that would be necessary for any large-scale production as freshwater is in short supply globally. Previous studies of *C. vulgaris* under saline conditions either used media with salinity far below that of seawater (e.g. salt concentrations of $\leq$23 g L^{-1}; Abdel-Rahman *et al*. 2005, Hiremath and Mathad, 2010), or focused on photobioreactors as production systems (Luangpipat, 2013).

Although normal seawater salinity significantly slowed the growth of *C. vulgaris* compared with the case for freshwater medium, the lipid content of the biomass was 1.9-fold higher in seawater. Consequently, this alga had a higher lipid productivity in full strength seawater than in freshwater under nutrient replete conditions.

The effect of salinity on growth, biomass production, lipid production, and the elemental composition of the biomass were reported for the first time for *C. vulgaris* (Table 4.2) and *N. salina* (Table 4.3), another alga of interest. Although the latter

marine alga was more halotolerant than *C. vulgaris*, it had a lower oil productivity and the oil content in the biomass could not be positively influenced by nutrient limitations.

In all cases, the use of a 12 h: 12 h light-dark cycle reduced biomass and lipid productivity of *C. vulgaris* relative to continuous illumination.

Studies confirmed that the phosphate concentration of the standard BG11 seawater medium was nonlimiting to growth at peak biomass concentrations that could be sustained by available light. Although all phosphate was generally consumed within the first few days of the start of a batch culture, growth continued unabated on intracellular stored phosphate. A doubling of the initial phosphate concentration did not influence either the biomass productivity or the final attainable concentration. This was consistent with the behavior previously described for microalgae (Miyachi and Miyachi, 1961; Miyachi and Tamiya, 1961; Miyachi *et al.*, 1964; Cembella *et al.*, 1984; John and Flynn, 2000).

Extensive work on the effects of nitrogen limitation on oil productivity and biomass characteristics was reported for *C. vulgaris* in marine media and also for *N. salina*. Nitrogen starvation postgrowth of *C. vulgaris* generally caused an increase in the lipid content of the biomass and its calorific value. In *N. salina*, nitrogen starvation did not trigger lipid accumulation in keeping with earlier studies (Boussiba *et al.*, 1987).

In raceway cultures at operating depth of ~0.23 m as is typical of industrial operations, the available light can only support a peak biomass concentration of ~0.8 kg m^{-3} and, therefore, nitrogen starvation cannot be achieved if the initial nitrate level is the same as in a standard BG11 medium. Therefore, an initial nitrogen level that could support the peak biomass concentration determined by light availability was experimentally identified. In the raceway, an initial nitrate concentration of 20% of the normal BG11 was found to provide the same lipid content and productivity as the normal BG11 medium, thus representing a nonnitrogen limiting condition for the

attainable biomass level while just consuming all the initial nitrate. Thus, with a 20% initial nitrate relative to standard BG11, a nitrogen limiting regime could be attained postgrowth in the raceway. Prolonged nitrogen starvation in the raceway led to an increase in the lipid content of the biomass so that the biomass contained ~42% of lipid on a dry basis. However, the lipid productivity was adversely affected as the biomass did not grow. Effects of nitrogen limitation in raceway culture of *C. vulgaris* have not been previously reported either in marine media or in freshwater cultures. Unlike in *C. vulgaris*, the lipid content of *N. salina* did not respond to nitrogen starvation in the raceway. Effect of dilution rate in steady state continuous raceway culture of *C. vulgaris* were discussed for the first time in relation to biomass productivity, lipid content, lipid productivity, biomass composition and calorific values.

Growth conditions and nutrient limitation affected the relative amounts of lipids, proteins, carbohydrates and other biomolecules in the biomass and this was reflected in changes in the levels of N, P and C in the biomass. In this work on *C. vulgaris*, depending on the conditions, the C content of the biomass varied from 41% to 64%; the nitrogen content varied from 2.9% to 8.5%; and the P content varied from 0.2% to 1.1%. There was an inverse relationship between the N content of the biomass and the lipid content. The energy content (calorific value) generally positively correlated with the C content. The *C. vulgaris* biomass had up to ~66% lipids under nitrogen deprived condition and a calorific value of >31 kJ g^{-1}. Under these conditions the C content of the biomass exceeded 63%.

5.2 Conclusion

1. Based on biomass and lipid productivity in full strength seawater media, *C. vulgaris* proved to be the most promising alga compared to the other species tested in this study.

2. Originally a freshwater strain, *C. vulgaris* was successfully grown at normal seawater salinity; higher salinities adversely affected this alga.
3. *C. vulgaris* grown in ~2 L Duran bottle in seawater, attained a lipid content of ~66% of dry biomass and a lipid productivity of ~31 mg L^{-1} d^{-1} under nitrogen starvation and continuous irradiance. A 12:12 h light-dark cycle reduced lipid content by 13% and lipid productivity by 41% relative to the case for continuous illumination.
4. The energy content and the C content of the biomass generally increased with increasing lipid content. Under nitrogen deficient condition in Duran bottles, the energy content and C content of the biomass increased up to 31 kJ g^{-1} and >63%, respectively.
5. Lipid contents of the biomass produced in nitrogen sufficient raceway batches were 22-25%, or similar to the biomass grown in Duran bottles under nitrogen sufficient condition. Relative to 2 L Duran bottles, the peak biomass concentration in the raceway was 69% lower because of light limitations.
6. In raceway batch culture, the highest biomass and lipid productivities occurred under nutrient sufficient condition. These were >17 mg L^{-1} d^{-1} and >4 mg L^{-1} d^{-1}, respectively. Under N starvation condition, lipid accumulation rate was slow and the lipid productivity was reduced relative to the nutrient sufficient case.
7. The continuous mode of operation in the raceway was promising, allowing a comparatively high biomass productivity of >61 mg L^{-1} d^{-1} and a high lipid productivity of >8 mg L^{-1} d^{-1} at a dilution rate of 0.173 d^{-1} under nitrogen sufficient condition.

8. The highest energy content and C content of biomass produced in the raceway batch culture were ~27 kJ g^{-1} and 55-59%, respectively, obtained in low nitrogen media.
9. The proportions of the lipid classes in crude oil of *C. vulgaris* were ~86% of polar lipids and ~14% non-polar (i.e. neutral lipids) for the oil produced in raceway batch culture under nutrient sufficient conditions. The proportion of fatty acid in the algal oil was only 2% and most of the fatty acids were of the polyunsaturated type.

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APPENDIX

Experimental data

Data for diurnal light output profile for Figure 3.7

a) At peak value of 100% LED output

Time (h)	LED output (%)
0.7	17
1.4	40
2.4	63
3.4	83
4.3	96
5.6	100
6.6	100
7.4	96
8.8	83
9.3	63
10.5	40
11.6	17
12.3	0
13	0
14	0
15	0
16	0
17	0
18	0
19	0
20	0
21	0
22	0
23	0
24	0

b) At peak value of 50% LED output

Time (h)	LED output (%)
0.5	8
1	20
2.0	31
3.0	41
4.2	48
5.0	50
6.0	50
7.0	48
8.0	41
9.0	31
10.0	20
11.0	8
12.3	0
13	0
14	0
15	0
16	0
17	0
18	0
19	0
20	0
21	0
22	0
23	0
24	0

Data for irradiance on the surface of broth for Figure 3.8

LED output (%)	Irradiance ($\mu mol \cdot m^{-2} s^{-1}$)
0	0
10	44.5 ± 2.9
20	74.9 ± 7.6
30	111.6 ± 8.4
40	142.0 ± 9.5
50	165.2 ± 12.5
60	184.8 ± 12.9
70	193.8 ± 6.5
80	212.9 ± 16.5
90	232.7 ± 29.2
100	279.7 ± 14.9

Standard deviation values are based on four replicates

Data for irradiance at different depths for Figure 3.9

a) at different LED light output levels

Depth (cm)	Irradiance (μmol·m^{-2}s^{-1}) at different light levels									
	100%	90%	80%	70%	60%	50%	40%	30%	20%	10%
0	279.7 ± 12.9	232.7 ± 25.3	212.6 ± 16.7	193.8 ± 6.5	184.8 ± 12.9	165.2 ± 12.5	141.9 ± 9.5	111.6 ± 8.4	74.9 ± 7.6	44.5 ± 2.9
10	98.9 ± 8.5	90.6 ± 5.5	82.3 ± 5.5	76.6 ± 2.4	68.9 ± 5.9	65.5 ± 2.2	58.2 ± 5.2	48.3 ± 4.4	30.4 ± 3.2	14.8 ± 0.5
20	92.8 ± 7.9	84.1 ± 6.7	78.1 ± 6.0	71.6 ± 5.5	68.2 ± 4.5	62.6 ± 5.8	55.7 ± 3.9	44.7 ± 4.3	30.8 ± 2.5	13.2 ± 1.9

Standard deviation values are based on four replicates

b) at different fluorescent light levels

Depth (cm)	Irradiance (μmol·m^{-2}s^{-1}) at 100% LED output	Irradiance (μmol·m^{-2}s^{-1}) at 50% LED output
0	90.7 ± 5.8	51.4 ± 4.1
5	44.2 ± 5.3	19.1 ± 3.1
10	43.1 ± 4.7	19.2 ± 1.9
15	41.6 ± 10.6	18.6 ± 1.7
20	42.7 ± 9.9	17.7 ± 3.0

Standard deviation values are based on four replicates

Data for irradiance at different depths for Figure 3.10

Depth (cm)	Irradiance ($\mu mol \cdot m^{-2} s^{-1}$)
0.0	82.5 ± 7.7[a]
0.0	84.2 ± 6.2[b]
1.0	17.9 ± 2.0 [b]
3.5	4.7
4.7	2.2
5.0	2.5
7.5	1.0
9.0	0.4
11.0	0.1
12.5	0.1

Standard deviation values are based on nine[a] replicates and [b]duplicates

Data for impeller speed for Figure 3.11

Potentiometer dial setting	Impeller speed (rpm)
5.0	9.7 ± 0.0
6.1	11.9 ± 0.2
7.0	13.9 ± 0.0
8.1	15.9 ± 0.0
9.0	17.9 ± 0.0

Standard deviation values are based on six replicates

Data for relationship between impeller and liquid flow velocity for Figure 3.12

Impeller speed (rpm)	Liquid flow velocity ($m\ s^{-1}$)
0.0	0
9.7	0.21 ± 0.02
11.9	0.23 ± 0.02
13.9	0.27 ± 0.04
15.9	0.30 ± 0.03
17.9	0.32 ± 0.03

Standard deviation values are based on 15 replicates

Data for calibration curve for Figure 3.14

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.016	0.078 ± 0.003
0.031	0.150 ± 0.002
0.047	0.230 ± 0.011
0.062	0.295 ± 0.011
0.078	0.377 ± 0.021
0.094	0.431 ± 0.009

Standard deviation values are based on duplicate samples

Data for calibration curve averaged for Figure 3.15

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.052	0.071 ± 0.027
0.103	0.144 ± 0.021
0.155	0.214 ± 0.023
0.207	0.286 ± 0.029
0.258	0.356 ± 0.038
0.287	0.398 ± 0.040

Standard deviation values are based on 36 runs

Data for calibration curve averaged for Figure 3.16

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.038	0.060 ± 0.020
0.076	0.122 ± 0.027
0.113	0.188 ± 0.023
0.150	0.243 ± 0.025
0.188	0.303 ± 0.034
0.205	0.335 ± 0.028

Standard deviation values are based on 32 runs

Data for calibration curve for Figure 3.17

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.178	0.410
0.119	0.257
0.089	0.193
0.071	0.152
0.059	0.129
0.051	0.111

Data for calibration curve for Figure 3.18

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.022	0.060 ± 0.021
0.045	0.122 ± 0.043
0.067	0.187 ± 0.065
0.088	0.242 ± 0.085
0.112	0.303 ± 0.104
0.135	0.336 ± 0.105

Standard deviation values are based on triplicate samples

Data for calibration curve averaged for Figure 3.19

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.035	0.190 ± 0.042
0.071	0.376 ± 0.070
0.077	0.420 ± 0.037
0.084	0.462 ± 0.042
0.093	0.515 ± 0.043
0.106	0.590 ± 0.043

Standard deviation values are based on 35 runs

Data for calibration curve averaged for Figure 3.20

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.022	0.129 ± 0.028
0.046	0.266 ± 0.035
0.048	0.285 ± 0.027
0.052	0.312 ± 0.028
0.058	0.347 ± 0.033
0.066	0.403 ± 0.035

Standard deviation values are based on 45 runs

Data for calibration curve for Figure 3.21

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.045	0.090
0.091	0.190
0.136	0.276
0.182	0.360
0.227	0.438
0.273	0.518

Data for calibration curve for Figure 3.22

Dried biomass concentration (g L^{-1})	Absorbance at 680 nm
0.0	0.0
0.148	0.266
0.098	0.189
0.074	0.140
0.059	0.112
0.049	0.091
0.042	0.080

Data for nitrate calibration curve for Figure 3.23

Nitrate concentration (g L^{-1})	Absorbance at 525 nm
0.0	0.0
4.38	0.009
8.75	0.015
13.14	0.021
17.50	0.031
21.88	0.038
26.36	0.047

Data for phosphate calibration curve for Figure 3.24

Phosphate concentration (g L^{-1})	Absorbance at 885 nm
0.0	0.0
0.112	0.014
0.140	0.017
0.168	0.019
0.196	0.023
0.224	0.027
0.252	0.031

Data for biomass concentration (g L^{-1}) for Figure 4.1 (a, b, c and d)

Time (d)	*C. vulgaris*	*N. salina*	*T. subcordiformis*	*C. fusiformis*
	a	b	c	d
0	0.87	0.08 ± 0.00	0.18 ± 0.01	0.46 ± 0.00
1	0.97	0.12 ± 0.00	0.36 ± 0.02	0.48 ± 0.00
2	1.12	0.13 ± 0.00	0.43 ± 0.01	0.49 ± 0.00
3	1.35	0.15 ± 0.00	0.59 ± 0.02	0.54 ± 0.01
4	1.52	0.16 ± 0.01	0.68 ± 0.01	0.57 ± 0.01
5	1.66	0.17 ± 0.01	0.81 ± 0.08	0.63 ± 0.01
6	1.77	0.20 ± 0.02	0.89 ± 0.02	0.74 ± 0.04
7	1.90	0.23 ± 0.03	0.94 ± 0.00	0.83 ± 0.06
8	2.06	0.25 ± 0.03	1.00 ± 0.05	0.92 ± 0.06
9	2.18	0.29 ± 0.03	1.02 ± 0.02	1.05 ± 0.08
10	2.31	0.34 ± 0.04	1.09 ± 0.04	1.17 ± 0.03
11	2.45	0.39 ± 0.06	1.12 ± 0.07	1.09 ± 0.09
12	2.39	0.46 ± 0.04	1.18 ± 0.01	1.41 ± 0.02
13	2.72	0.55 ± 0.08	1.28 ± 0.06	1.46 ± 0.03
14	2.85	0.61 ± 0.07	1.29 ± 0.04	1.50 ± 0.03
15	2.96	0.67 ± 0.09	1.34 ± 0.00	1.56 ± 0.03
16	3.09	0.77 ± 0.13	1.36 ± 0.01	1.56 ± 0.02
17	3.09	0.83 ± 0.10	1.48 ± 0.04	1.57 ± 0.00
18	3.22	0.82 ± 0.08	1.50 ± 0.05	1.56 ± 0.00
19	3.28	0.96 ± 0.09	1.51 ± 0.05	1.54 ± 0.01
20	3.34	1.05 ± 0.08	1.54 ± 0.05	1.50 ± 0.09
21	3.36	1.15 ± 0.07	1.58 ± 0.05	1.47 ± 0.12
22	3.35	1.21 ± 0.08	1.62 ± 0.05	1.47 ± 0.14
23	3.38	1.32 ± 0.06	1.66 ± 0.05	1.38 ± 0.12
24	3.46	1.42 ± 0.02	1.68 ± 0.04	-
25	3.40	1.28 ± 0.07	1.69 ± 0.04	-
26	-	1.33 ± 0.08	1.69 ± 0.03	-
27	-	1.31± 0.04	1.76 ± 0.02	-
28	-	1.31 ± 0.06	1.80 ± 0.01	-
29	-	1.35 ± 0.07	1.83 ± 0.03	-
30	-	1.42 ± 0.02	1.84 ± 0.06	-
31	-	1.49 ± 0.01	1.87 ± 0.10	-
32	-	1.58 ± 0.02	1.83 ± 0.00	-
33	-	1.62 ± 0.00	1.80 ± 0.07	-
34	-	1.59 ± 0.08	1.83 ± 0.04	-
35	-	1.64 ± 0.01	1.71 ± 0.07	-
36	-	1.73 ± 0.02	-	-
37	-	1.70 ± 0.02	-	-

Standard deviation values are based on duplicate runs

Data for biomass concentration (g L^{-1}) for Figure 4.2

Time (d)	Freshwater	Salt concentration		
		40 g L^{-1} (control)	50 g L^{-1}	60 g L^{-1}
0	0.04 ± 0.00	0.20 ± 0.01	0.19 ± 0.01	0.19 ± 0.01
1	0.29 ± 0.00	0.25 ± 0.01	0.26 ± 0.02	0.23 ± 0.00
2	0.46 ± 0.03	0.26 ± 0.02	0.24 ± 0.01	0.23 ± 0.00
3	0.65 ± 0.03	0.31 ± 0.02	0.24 ± 0.04	0.24 ± 0.00
4	0.85 ± 0.02	0.34 ± 0.01	0.27 ± 0.01	0.24 ± 0.02
5	0.92 ± 0.06	0.41 ± 0.01	0.28 ± 0.01	0.28 ± 0.00
6	1.01 ± 0.02	0.58 ± 0.01	0.37 ± 0.02	0.29 ± 0.02
8	1.24 ± 0.13	0.69 ± 0.02	0.42 ± 0.06	0.30 ± 0.01
9	1.34 ± 0.09	0.73 ± 0.01	0.46 ± 0.06	0.35 ± 0.02
10	1.35 ± 0.10	0.81 ± 0.00	0.47 ± 0.03	0.36 ± 0.00
11	1.43 ± 0.15	0.90 ± 0.02	0.47 ± 0.05	0.34 ± 0.01
12	1.52 ± 0.19	0.91 ± 0.01	0.47 ± 0.04	0.35 ± 0.03
13	1.52 ± 0.15	0.91 ± 0.00	0.45 ± 0.01	0.33 ± 0.00
14	1.58 ± 0.15	0.90 ± 0.00	0.52 ± 0.02	0.34 ± 0.03
15	1.58 ± 0.19	0.99 ± 0.01	0.52 ± 0.02	0.36 ± 0.00
16	1.68 ± 0.10	0.98 ± 0.02	0.53 ± 0.01	0.34 ± 0.02
19	1.96 ± 0.02	1.16 ± 0.04	0.55 ± 0.00	0.34 ± 0.02
20	1.87 ± 0.03	1.09 ± 0.01	0.54 ± 0.07	0.35 ± 0.01
22	1.80 ± 0.13	1.37 ± 0.01	0.61 ± 0.02	0.37 ± 0.02
23	1.98 ± 0.08	1.34 ± 0.02	0.62 ± 0.01	0.40 ± 0.00
25	1.81 ± 0.13	1.36 ± 0.03	0.69 ± 0.01	0.40 ± 0.01
26	1.90 ± 0.18	1.50 ± 0.07	0.72 ± 0.03	0.42 ± 0.02
28	1.87 ± 0.28	1.71 ± 0.00	0.78 ± 0.09	0.41 ± 0.01
30	1.92 ± 0.13	1.76 ± 0.09	0.79 ± 0.05	0.46 ± 0.01
32	2.20 ± 0.12	1.57 ± 0.09	0.80 ± 0.22	0.46 ± 0.07
33	2.00 ± 0.35	1.58 ± 0.04	0.82 ± 0.09	0.42 ± 0.01
35	2.06 ± 0.13	1.73 ± 0.03	0.87 ± 0.10	0.42 ± 0.02
36	2.19 ± 0.18	1.71 ± 0.03	0.99 ± 0.05	0.52 ± 0.05
39	2.37 ± 0.27	1.75 ± 0.02	0.91 ± 0.04	0.40 ± 0.00
43	2.29 ± 0.15	-	-	-

Standard deviation values are based on duplicate runs, except for the control (for this duplicate measurements of a single run were used to calculate standard deviation)

Data for biomass concentration (g L^{-1}) for Figure 4.4

Time (d)	Salt concentration		
	40 g L^{-1} (control)	50 g L^{-1}	60 g L^{-1}
0	0.37	0.38 ± 0.00	0.36 ± 0.01
1	0.41	0.41 ± 0.01	0.41 ± 0.00
2	0.49	0.45 ± 0.01	0.44 ± 0.00
3	0.59	0.54 ± 0.01	0.55 ± 0.00
4	0.65	0.60 ± 0.03	0.60 ± 0.01
5	0.77	0.68 ± 0.04	0.67 ± 0.01
6	0.89	0.79 ± 0.04	0.79 ± 0.01
8	0.99	0.88 ± 0.06	0.90 ± 0.01
9	1.06	0.95 ± 0.05	1.00 ± 0.04
10	1.11	0.98 ± 0.05	1.03 ± 0.02
11	1.19	1.03 ± 0.06	1.12 ± 0.02
12	1.24	1.07 ± 0.06	1.17 ± 0.02
13	1.29	1.11 ± 0.07	1.19 ± 0.02
14	1.33	1.14 ± 0.08	1.21 ± 0.04
15	1.41	1.21 ± 0.07	1.30 ± 0.02
16	1.43	1.25 ± 0.07	1.29 ± 0.09
19	1.54	1.35 ± 0.10	1.47 ± 0.03
20	1.61	1.39 ± 0.05	1.49 ± 0.03
22	1.77	1.53 ± 0.13	1.62 ± 0.03
23	1.78	1.54 ± 0.09	1.65 ± 0.07
25	1.80	1.54 ± 0.10	1.69 ± 0.06
26	1.85	1.58 ± 0.11	1.70 ± 0.11
28	1.90	1.63 ± 0.12	1.71 ± 0.16
30	2.03	1.77 ± 0.12	1.88 ± 0.07
32	2.19	1.85 ± 0.18	1.96 ± 0.14
33	2.34	1.86 ± 0.17	1.97 ± 0.12
35	2.17	1.90 ± 0.17	2.06 ± 0.03
36	2.29	2.03 ± 0.16	2.12 ± 0.10
39	2.39	1.97 ± 0.14	2.23 ± 0.00

Standard deviation values are based on duplicate runs

Data for biomass concentration (g L^{-1}) for Figure 4.6

Time (d)	Salt concentration		
	40 g L^{-1} (control)	50 g L^{-1}	60 g L^{-1}
0	0.38	0.41 ± 0.02	0.41 ± 0.01
1	0.44	0.34 ± 0.03	0.43 ± 0.01
2	0.55	0.51 ± 0.00	0.50 ± 0.01
3	0.58	0.63 ± 0.04	0.56 ± 0.01
4	0.65	0.61 ± 0.02	0.58 ± 0.01
5	0.66	0.62 ± 0.04	0.59 ± 0.00
6	0.66	0.57 ± 0.03	0.57 ± 0.01
7	0.66	0.57 ± 0.03	0.59 ± 0.04
8	0.70	0.60 ± 0.04	0.47 ± 0.14
9	0.70	0.65 ± 0.01	0.61 ± 0.03
11	0.75	0.68 ± 0.01	0.61 ± 0.00
13	0.85	0.74 ± 0.02	0.67 ± 0.01
14	0.83	0.76 ± 0.00	0.69 ± 0.02
15	0.87	0.79 ± 0.04	0.73 ± 0.01
16	0.89	0.81 ± 0.05	0.79 ± 0.02
17	0.87	0.82 ± 0.07	0.86 ± 0.07
19	0.77	0.83 ± 0.11	0.92 ± 0.04

Standard deviation values are based on duplicate runs

Data for *C. vulgaris* culture profile for Figure 4.8: a) Control (standard phosphate concentration in BG11 seawater medium); b) Twice the normal initial phosphate level in BG11 seawater medium.

Time (d)	(a)			(b)		
	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.44	1117.6	23.28	0.44	1029.4	45.72
1	0.51	1029.4	13.30	0.55	911.8	37.41
2	0.60	882.4	9.98	0.65	911.8	29.93
3	0.70	852.9	6.23	0.74	882.4	27.43
4	0.79	852.9	1.87	0.82	852.9	23.28
5	0.87	794.1	0.25	0.92	764.7	14.96
6	1.05	808.8	0.21	1.09	764.7	8.31
7	1.22	735.3	0.18	1.26	705.9	2.49
8	1.33	764.7	0.16	1.40	676.5	1.46
9	1.48	676.5	0.09	1.51	647.1	0.55
10	1.59	647.1	0.08	1.65	588.2	0.21
11	1.78	647.1	0.06	1.81	529.4	0.04
12	1.93	676.5	0.01	1.98	529.4	0.02
13	1.95	647.1	0.02	2.00	441.2	0.01
14	2.14	558.8	0.01	2.18	470.6	0.03
15	2.22	500.0	0.01	2.24	441.2	0.02
16	2.29	529.4	0.01	2.40	470.6	0.01
17	2.47	529.4	0.01	2.55	441.2	0.00
18	2.48	529.4	0.00	2.65	411.8	0.00
20	3.01	323.5	0.00	3.07	382.4	0.00
22	3.19	392.1	0.00	3.25	411.7	0.00
23	3.40	313.7	0.00	3.37	333.3	0.00
24	3.28	274.5	0.00	3.41	313.7	0.00
25	3.17	235.3	0.00	3.46	294.1	0.00

Data for *N. salina* culture profile for Figure 4.9: a) control (standard phosphate concentration in BG11 seawater medium); b) twice the normal initial phosphate level in BG11 seawater medium

Time (d)	(a)			(b)		
	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.30 ± 0.01	1070.6 ± 49.9	15.17 ± 2.06	0.33 ± 0.01	1047.1 ± 35.3	33.21 ± 0.29
1	0.44 ± 0.00	964.7 ± 16.6	2.25 ± 0.05	0.39 ± 0.00	929.4 ± 16.6	9.14 ± 0.59
2	0.47 ± 0.00	874.5 ± 44.5	1.38 ± 0.00	0.50 ± 0.00	901.9 ± 17.9	4.16 ± 1.18
3	0.59 ± 0.04	823.5 ± 33.3	0.28 ± 0.00	0.55 ± 0.04	854.9 ± 41.3	2.49 ± 1.18
4	0.65 ± 0.07	847.1 ± 0.0	0.12 ± 0.02	0.64 ± 0.07	827.5 ± 35.9	1.25 ± 0.00
6	0.88 ± 0.04	805.9 ± 24.9	0.08 ± 0.08	0.82 ± 0.04	803.9 ± 33.9	0.62 ± 0.10
7	0.93 ± 0.02	705.9 ± 71.6	0.05 ± 0.05	0.95 ± 0.02	768.6 ± 37.8	0.71 ± 0.34
8	1.02 ± 0.02	733.3 ± 6.8	0.08 ± 0.08	0.97 ± 0.02	764.7 ± 16.6	0.49 ± 0.11
9	1.15 ± 0.01	658.8 ± 16.6	0.00	1.08 ± 0.01	764.7 ± 16.6	0.18
10	1.27 ± 0.03	658.8 ± 16.6	0.03	1.17 ± 0.03	662.7 ± 6.8	0.15
11	1.30 ± 0.04	701.9 ± 37.8	0.02	1.26 ± 0.04	647.1 ± 16.6	0.13
12	1.35 ± 0.04	729.4 ± 11.8	0.00	1.33 ± 0.04	682.4 ± 16.6	0.12
14	1.50 ± 0.06	654.9 ± 24.5	0.01	1.48 ± 0.06	627.5 ± 44.5	0.13
15	1.59 ± 0.07	650.9 ± 24.5	0.04	1.56 ± 0.07	564.7 ± 42.4	0.12
16	1.63 ± 0.08	670.6 ± 16.6	0.03	1.65 ± 0.08	611.8 ± 0.0	0.10
17	1.81 ± 0.04	635.3 ± 35.3	0.05	1.78 ± 0.04	600.0 ± 23.5	0.09
19	1.81 ± 0.11	627.5 ± 17.9	0.00	1.86 ± 0.11	576.5 ± 16.6	0.05
21	2.01 ± 0.10	545.1 ± 58.0	0.01	2.15 ± 0.10	509.8 ± 17.9	0.00
22	2.11 ± 0.06	494.1 ± 33.3	0.02	2.23 ± 0.06	505.9 ± 33.3	0.00
30	2.22 ± 0.21	627.5 ± 27.2	0.01	2.20 ± 0.21	494.1 ± 0.0	0.05
32	2.16 ± 0.20	570.6 ± 8.3	0.02	2.16 ± 0.20	494.1 ± 0.0	0.02

Biomass standard deviation is based on duplicate runs; standard deviations of nitrate and phosphate are based on triplicate and duplicate samples of a single run, respectively

Data for *C.vulgaris* for Figure 4.10:

a-1) Biomass concentration (g L^{-1}) at continuous light and different initial nitrate concentrations in BG11 seawater media

Time (d)	Initial nitrate concentration relative to normal BG11 media			
	100% (control)	50%	20%	10%
0	0.11 ± 0.00	0.11 ± 0.01	0.11 ± 0.00	0.11 ± 0.01
2	0.15 ± 0.01	0.13 ± 0.00	0.13 ± 0.01	0.14 ± 0.00
3	0.18 ± 0.00	0.16 ± 0.00	0.18 ± 0.00	0.14 ± 0.02
4	0.22 ± 0.01	0.17 ± 0.00	0.22 ± 0.01	0.22 ± 0.01
5	0.26 ± 0.02	0.25 ± 0.03	0.29 ± 0.02	0.23 ± 0.02
7	0.40 ± 0.02	0.37 ± 0.04	0.51 ± 0.01	0.43 ± 0.03
9	0.62 ± 0.02	0.51 ± 0.01	0.74 ± 0.02	0.59 ± 0.09
10	0.64 ± 0.02	0.56 ± 0.07	0.79 ± 0.01	0.65 ± 0.02
12	0.89 ± 0.02	0.72 ± 0.00	0.99 ± 0.00	0.68 ± 0.02
13	0.84 ± 0.01	0.76 ± 0.02	1.01 ± 0.03	0.82 ± 0.01
14	0.92 ± 0.04	1.01 ± 0.03	1.06 ± 0.02	0.83 ± 0.02
15	1.16 ± 0.02	1.01 ± 0.02	1.24 ± 0.01	0.83 ± 0.00
17	1.35 ± 0.06	1.02 ± 0.02	1.13 ± 0.04	0.86 ± 0.01
18	1.25 ± 0.06	1.13 ± 0.01	1.17 ± 0.10	0.86 ± 0.01
19	1.23 ± 0.02	1.25 ± 0.01	1.14 ± 0.12	0.83 ± 0.02
20	1.17 ± 0.10	1.27 ± 0.02	1.29 ± 0.00	0.85 ± 0.05
22	1.45 ± 0.03	1.23 ± 0.08	1.37 ± 0.02	1.09 ± 0.05
23	1.44 ± 0.15	1.29 ± 0.01	1.36 ± 0.01	1.02 ± 0.02
25	1.85 ± 0.01	1.52 ± 0.02	1.54 ± 0.03	1.08 ± 0.01
28	1.85 ± 0.02	1.48 ± 0.03	1.50 ± 0.02	1.11 ± 0.01
29	1.71 ± 0.04	1.57 ± 0.03	1.47 ± 0.03	1.09 ± 0.03
30	1.86 ± 0.05	1.71 ± 0.03	1.76 ± 0.02	1.14 ± 0.03
32	2.00 ± 0.08	1.64 ± 0.12	1.61 ± 0.01	1.24 ± 0.00
35	2.09 ± 0.10	1.91 ± 0.11	1.70 ± 0.15	1.26 ± 0.03
37	2.03 ± 0.08	1.92 ± 0.12	1.71 ± 0.05	1.31 ± 0.00
39	2.12 ± 0.04	2.09 ± 0.02	1.92 ± 0.04	1.38 ± 0.13
41	2.23 ± 0.02	2.35 ± 0.12	1.95 ± 0.14	1.35 ± 0.09
45	2.13 ± 0.08	2.15 ± 0.16	1.91 ± 0.00	1.44 ± 0.02
46	2.45 ± 0.01	2.33 ± 0.08	2.26 ± 0.02	1.51 ± 0.02
49	2.52 ± 0.22	2.03 ± 0.02	2.12 ± 0.14	1.64 ± 0.15
52	2.53 ± 0.04	2.03 ± 0.00	2.19 ± 0.01	1.60 ± 0.12

Standard deviations are based on duplicate runs except for the control (duplicate samples of a single run were used for the control)

b-1) Biomass concentration (g L^{-1}) at 12 h: 12 h light/dark cycle and different initial nitrate concentrations in BG11 seawater media

Time (d)	Initial nitrate concentration relative to normal BG11 media			
	100% (control)	50%	20%	10%
0	0.08 ± 0.00	0.09 ± 0.00	0.09 ± 0.01	0.09 ± 0.01
2	0.11 ± 0.00	0.10 ± 0.01	0.11 ± 0.02	0.10 ± 0.01
3	0.13 ± 0.00	0.11 ± 0.00	0.13 ± 0.00	0.12 ± 0.01
4	0.14 ± 0.00	0.12 ± 0.00	0.16 ± 0.02	0.14 ± 0.00
5	0.32 ± 0.00	0.13 ± 0.01	0.20 ± 0.01	0.14 ± 0.01
7	0.28 ± 0.00	0.14 ± 0.01	0.31 ± 0.01	0.28 ± 0.03
9	0.33 ± 0.00	0.18 ± 0.03	0.35 ± 0.03	0.37 ± 0.02
10	0.34 ± 0.00	0.22 ± 0.07	0.33 ± 0.07	0.38 ± 0.03
12	0.41 ± 0.00	0.28 ± 0.01	0.52 ± 0.01	0.48 ± 0.03
13	0.42 ± 0.00	0.34 ± 0.01	0.53 ± 0.02	0.50 ± 0.04
14	0.44 ± 0.00	0.35 ± 0.03	0.65 ± 0.00	0.56 ± 0.03
15	0.53 ± 0.00	0.40 ± 0.03	0.64 ± 0.01	0.57 ± 0.01
17	0.66 ± 0.00	0.49 ± 0.01	0.73 ± 0.04	0.60 ± 0.03
18	0.75 ± 0.00	0.52 ± 0.05	0.75 ± 0.03	0.59 ± 0.01
19	0.80 ± 0.00	0.56 ± 0.07	0.85 ± 0.02	0.59 ± 0.00
20	0.80 ± 0.00	0.60 ± 0.03	0.96 ± 0.01	0.60 ± 0.00
22	0.90 ± 0.00	0.72 ± 0.07	0.98 ± 0.01	0.87 ± 0.01
23	0.90 ± 0.00	0.74 ± 0.10	0.95 ± 0.01	0.66 ± 0.04
25	0.99 ± 0.00	0.82 ± 0.10	1.08 ± 0.00	0.72 ± 0.05
28	1.20 ± 0.00	0.89 ± 0.05	1.15 ± 0.01	0.74 ± 0.01
29	1.14 ± 0.00	1.00 ± 0.09	1.06 ± 0.02	0.74 ± 0.05
30	1.28 ± 0.00	1.24 ± 0.25	1.22 ± 0.05	0.92 ± 0.01
32	1.25 ± 0.00	1.08 ± 0.01	1.17 ± 0.07	0.70 ± 0.01
35	1.48 ± 0.00	1.25 ± 0.04	1.29 ± 0.01	0.94 ± 0.01
37	1.35 ± 0.00	1.20 ± 0.12	1.33 ± 0.03	0.88 ± 0.03
39	1.33 ± 0.00	1.25 ± 0.03	1.34 ± 0.02	0.93 ± 0.03
41	1.47 ± 0.00	1.34 ± 0.04	1.41 ± 0.01	0.97 ± 0.00
45	1.58 ± 0.00	1.39 ± 0.02	1.45 ± 0.01	0.97 ± 0.01
46	1.71 ± 0.00	1.52 ± 0.13	1.55 ± 0.05	1.14 ± 0.05
49	1.65 ± 0.00	1.45 ± 0.03	1.54 ± 0.04	1.11 ± 0.03
52	1.68 ± 0.00	1.47 ± 0.05	1.64 ± 0.01	1.11 ± 0.01

Standard deviations are based on duplicate runs except for the control (duplicate samples of a single run were used for the control)

a-2) Nitrate concentration (mg L^{-1}) at continuous light and different initial nitrate concentrations in BG11 seawater media: b-2) nitrate concentration (mg L^{-1}) at 12 h: 12 h light/dark cycle in control

Time (d)	Initial nitrate concentration relative to normal BG11 media				
	100% (control)		50%	20%	10%
	a-2	b-2	a-2	a-2	a-2
0	1014.7 ± 20.8	1044.1 ± 20.8	505.8 ± 16.6	235.2 ± 0.0	129.4 ± 4.2
5	1088.2 ± 83.2	1029.4 ± 83.2	452.9 ± 41.6	129.4 ± 0.0	50.0 ± 16.6
10	794.1 ± 83.2	676.5 ± 83.2	258.8 ± 33.3	11.8 ± 0.0	5.9 ± 8.3
15	661.8 ± 0.0	632.4 ± 20.8	88.2 ± 24.9	5.6	3.5
20	500.0 ± 0.0	588.2 ± 0.0	26.2 ± 0.4	6.5	4.7
25	367.6 ± 0.0	588.2 ± 0.0	10.6 ± 3.3	8.2	4.7
30	279.4	441.2 ± 0.0	13.5	7.7	6.5
35	270.6	470.6	15.3	8.8	7.1
41	282.4	529.4	16.5	11.2	8.8
46	223.5	364.7	19.4	11.2	7.1
52	200.0	341.2	19.4	8.2	8.8

Standard deviation of nitrate is based on duplicate samples of a single run

a-3) Phosphate concentration (mg L^{-1}) at continuous light and different initial nitrate concentrations in BG11 seawater media: b-3) nitrate concentration (mg L^{-1}) at 12 h: 12 h light/dark cycle in control

Time (d)	Initial nitrate concentration relative to normal BG11 media				
	100% (control)		50%	20%	10%
	a-3	b-3	a-3	a-3	a-3
0	15.86 ± 0.26	15.69 ± 0.09	15.88 ± 0.18	16.17 ± 0.00	16.21 ± 0.18
5	3.43 ± 0.03	10.68 ± 0.24	7.65 ± 0.29	4.41 ± 0.00	5.71 ± 0.09
10	0.00	1.43	0.00	0.00	0.22
15	0.00	0.00	0.00	0.00	0.14
20	0.00	0.00	0.00	0.00	0.14
25	0.00	0.00	0.00	0.00	0.14
30	0.00	0.00	0.00	0.00	0.11
35	0.00	0.00	0.00	0.00	0.12
41	0.00	0.00	0.00	0.00	0.12
46	0.00	0.00	0.00	0.00	0.12
52	0.00	0.00	0.00	0.00	0.00

Standard deviation of phosphate is based on duplicate samples of a single run

Data for *N.salina* for Figure 4.11:

a-1) Biomass concentration (g L^{-1}) at continuous light and different initial nitrate concentrations in BG11 seawater media

Time (d)	Initial nitrate concentration relative to normal BG11 media			
	100% (control)	50%	20%	10%
0	0.27 ± 0.00	0.24 ± 0.00	0.24 ± 0.00	0.25 ± 0.00
2	0.30 ± 0.00	0.36 ± 0.01	0.31 ± 0.02	0.34 ± 0.03
3	0.38 ± 0.00	0.47 ± 0.01	0.42 ± 0.01	0.42 ± 0.02
4	0.49 ± 0.00	0.54 ± 0.00	0.52 ± 0.00	0.49 ± 0.00
5	0.64 ± 0.00	0.65 ± 0.08	0.65 ± 0.06	0.53 ± 0.04
7	0.90 ± 0.00	0.78 ± 0.20	0.89 ± 0.01	0.67 ± 0.04
9	1.10 ± 0.00	1.16 ± 0.05	1.00 ± 0.04	0.75 ± 0.04
10	1.14 ± 0.00	1.26 ± 0.09	1.05 ± 0.01	0.69 ± 0.02
12	1.17 ± 0.00	1.35 ± 0.07	1.08 ± 0.02	0.75 ± 0.02
13	1.35 ± 0.00	1.50 ± 0.02	1.19 ± 0.02	0.85 ± 0.02
14	1.38 ± 0.00	1.46 ± 0.02	1.20 ± 0.01	0.80 ± 0.03
15	1.60 ± 0.01	1.69 ± 0.04	1.34 ± 0.01	0.82 ± 0.04
17	1.66 ± 0.00	1.65 ± 0.05	1.34 ± 0.03	0.84 ± 0.02
18	1.75 ± 0.00	1.68 ± 0.06	1.41 ± 0.03	0.87 ± 0.02
19	1.73 ± 0.00	1.77 ± 0.06	1.46 ± 0.03	0.91 ± 0.01
20	1.87 ± 0.00	1.86 ± 0.07	1.53 ± 0.05	1.07 ± 0.02
22	2.10 ± 0.00	2.06 ± 0.04	1.69 ± 0.03	1.24 ± 0.04
23	2.02 ± 0.00	1.92 ± 0.05	1.61 ± 0.06	1.08 ± 0.07
25	2.11 ± 0.00	2.11 ± 0.07	1.76 ± 0.07	1.22 ± 0.06
28	2.37 ± 0.00	2.14 ± 0.04	1.84 ± 0.08	1.21 ± 0.06
29	2.41 ± 0.00	2.15 ± 0.11	1.88 ± 0.04	1.22 ± 0.04
30	2.56 ± 0.00	2.30 ± 0.05	1.98 ± 0.09	1.29 ± 0.01
32	2.61 ± 0.00	2.40 ± 0.01	2.01 ± 0.09	1.30 ± 0.00
35	2.70 ± 0.00	2.52 ± 0.00	2.08 ± 0.06	1.43 ± 0.01
37	2.73 ± 0.00	2.59 ± 0.02	2.11 ± 0.06	1.36 ± 0.05
39	2.73 ± 0.00	2.55 ± 0.00	2.16 ± 0.13	1.40 ± 0.06
41	2.83 ± 0.01	2.64 ± 0.04	2.23 ± 0.11	1.84 ± 0.01
45	2.68 ± 0.00	2.56 ± 0.08	2.24 ± 0.13	1.77 ± 0.11
46	2.83 ± 0.00	2.68 ± 0.00	2.29 ± 0.21	1.91 ± 0.07
49	2.93 ± 0.00	2.72 ± 0.11	2.42 ± 0.12	1.90 ± 0.13

Standard deviations are based on duplicate runs, except for the control (duplicate samples of a single run were used for the control)

b-1) Biomass concentration (g L^{-1}) at 12 h: 12 h light/dark cycle and different initial nitrate concentrations in BG11 seawater media

Time (d)	Initial nitrate concentration relative to normal BG11 media			
	100% (control)	50%	20%	10%
0	0.20 ± 0.00	0.22 ± 0.00	0.21 ± 0.00	0.20 ± 0.00
2	0.24 ± 0.00	0.23 ± 0.00	0.27 ± 0.05	0.23 ± 0.00
3	0.29 ± 0.01	0.30 ± 0.01	0.31 ± 0.00	0.27 ± 0.00
4	0.32 ± 0.00	0.33 ± 0.00	0.36 ± 0.00	0.30 ± 0.00
5	0.42 ± 0.00	0.37 ± 0.03	0.43 ± 0.00	0.36 ± 0.00
7	0.49 ± 0.00	0.52 ± 0.00	0.55 ± 0.00	0.49 ± 0.00
9	0.58 ± 0.00	0.57 ± 0.01	0.61 ± 0.01	0.51 ± 0.00
10	0.58 ± 0.00	0.60 ± 0.02	0.63 ± 0.00	0.52 ± 0.00
12	0.59 ± 0.00	0.67 ± 0.01	0.66 ± 0.00	0.53 ± 0.00
13	0.69 ± 0.00	0.79 ± 0.02	0.76 ± 0.01	0.62 ± 0.00
14	0.66 ± 0.00	0.72 ± 0.02	0.71 ± 0.02	0.58 ± 0.00
15	0.81 ± 0.00	0.85 ± 0.01	0.82 ± 0.03	0.64 ± 0.00
17	0.76 ± 0.01	0.80 ± 0.01	0.81 ± 0.01	0.62 ± 0.00
18	0.89 ± 0.00	0.89 ± 0.02	0.90 ± 0.01	0.68 ± 0.00
19	0.86 ± 0.00	0.90 ± 0.00	0.89 ± 0.00	0.69 ± 0.00
20	0.97 ± 0.00	0.97 ± 0.01	0.94 ± 0.01	0.74 ± 0.00
22	1.13 ± 0.00	1.13 ± 0.02	1.12 ± 0.02	0.88 ± 0.00
23	1.02 ± 0.00	1.03 ± 0.01	0.99 ± 0.00	0.76 ± 0.00
25	1.20 ± 0.00	1.12 ± 0.01	1.10 ± 0.02	0.86 ± 0.00
28	1.32 ± 0.00	1.19 ± 0.04	1.13 ± 0.00	0.90 ± 0.00
29	1.32 ± 0.00	1.23 ± 0.04	1.13 ± 0.01	0.91 ± 0.00
30	1.40 ± 0.00	1.26 ± 0.03	1.22 ± 0.01	0.95 ± 0.00
32	1.46 ± 0.00	1.32 ± 0.02	1.26 ± 0.02	1.00 ± 0.00
35	1.61 ± 0.00	1.40 ± 0.08	1.33 ± 0.00	1.09 ± 0.00
37	1.63 ± 0.00	1.40 ± 0.06	1.33 ± 0.01	1.06 ± 0.00
39	1.71 ± 0.00	1.46 ± 0.03	1.43 ± 0.03	1.12 ± 0.00
41	1.76 ± 0.01	1.53 ± 0.10	1.49 ± 0.04	1.18 ± 0.00
45	1.73 ± 0.00	1.44 ± 0.16	1.49 ± 0.01	1.20 ± 0.00
46	1.85 ± 0.00	1.64 ± 0.12	1.58 ± 0.02	1.28 ± 0.00
49	1.84 ± 0.00	1.62 ± 0.12	1.61 ± 0.02	1.31 ± 0.00

Standard deviations are based on duplicate runs, except for the control (duplicate samples of a single run were used for the control)

a-2) Nitrate concentration (mg L^{-1}) at continuous light and different initial nitrate concentrations in BG11 seawater media: b-2) nitrate concentration (mg L^{-1}) at 12 h: 12 h light/dark cycle in control

Time (d)	Initial nitrate concentration relative to normal BG11 media				
	100% (control)		50%	20%	10%
	a-2	b-2	a-2	a-2	a-2
0	1191.2 ± 62.4	1132.4 ± 62.4	535.3 ± 41.6	258.8 ± 16.6	108.8 ± 8.3
5	1000.0 ± 83.2	1029.4 ± 41.6	411.8 ± 33.3	94.1 ± 0.0	48.5 ± 6.2
10	852.9 ± 0.0	882.4 ± 0.0	258.8 ± 0.0	29.4 ± 8.3	19.1 ± 2.1
15	588.2 ± 41.6	794.1 ± 41.6	123.5 ± 41.6	3.5	2.9
20	573.5 ± 20.8	764.7 ± 0.0	78.2	6.5	3.5
25	470.6 ± 41.6	691.2 ± 20.8	30.0	6.5	5.9
30	250.0 ± 20.8	602.9 ± 20.8	11.2	3.5	3.5
35	329.4	623.5	14.7	6.5	8.8
41	329.4	635.3	11.8	8.8	11.8
46	305.9	564.7	12.4	5.3	23.5

Standard deviation of nitrate is based on duplicate samples of a single run

a-3) Phosphate concentration (mg L^{-1}) at continuous light and different initial nitrate concentrations in BG11 seawater media: b-3) nitrate concentration (mg L^{-1}) at 12 h: 12 h light/dark cycle in control

Time (d)	Initial nitrate concentration relative to normal BG11 media				
	100% (control)		50%	20%	10%
	a-3	b-3	a-3	a-3	a-3
0	12.09 ± 0.29	11.99 ± 0.03	11.74 ± 0.03	12.36 ± 0.09	5.61 ± 0.29
5	1.41 ± 0.06	2.24 ± 0.06	1.66 ± 0.24	2.12 ± 0.12	1.56 ± 0.21
10	0.38	0.63	0.00	0.40	0.00
15	0.02	0.13	0.03	0.00	0.00
20	0.02	0.02	0.02	0.03	0.00
25	0.03	0.00	0.00	0.08	0.02
30	0.00	0.00	0.00	0.16	0.02
35	0.07	0.03	0.03	0.04	0.11
41	0.05	0.06	0.03	0.04	0.09
46	0.02	0.00	0.01	0.03	0.07

Standard deviation of phosphate is based on duplicate samples of a single run

Data for raceway culture profiles for Figure 4.16: (a, b and c) normal operational conditions

a) Raceway batch-1

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.06	1058.7	23.69
1	0.06	1162.6	22.86
2	0.06	1058.7	20.37
3	0.07	1176.4	20.78
4	0.08	1137.1	19.53
5	0.08	1117.5	20.08
6	0.08	1117.5	19.29
7	0.09	1078.3	17.98
8	0.09	921.5	16.93
9	0.10	960.7	16.63
10	0.11	960.7	9.81
11	0.11	921.5	9.59
12	0.12	901.9	9.38
13	0.13	862.7	9.24
14	0.14	941.1	9.04
15	0.15	901.9	8.45
16	0.17	862.7	8.03
17	0.18	882.3	7.63
18	0.19	921.5	7.20
19	0.20	862.7	7.03
20	0.21	882.3	6.15
21	0.21	823.4	4.89
22	0.23	941.1	3.92
23	0.24	862.7	3.24
24	0.25	843.1	2.00
26	0.29	823.4	0.73
27	0.30	823.4	0.43
28	0.30	803.8	0.33
29	0.32	803.8	0.25

a) Raceway batch-1 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
30	0.34	784.2	0.00
31	0.35	764.6	0.00
32	0.38	725.4	0.00
36	0.44	803.8	0.00
37	0.37	823.4	0.00
38	0.39	725.4	0.00
40	0.37	745.0	0.00
41	0.38	725.4	0.00
42	0.38	725.4	0.00
43	0.40	764.6	0.00
44	0.42	764.6	0.00
46	0.46	764.6	0.00
47	0.40	725.4	0.00
48	0.42	705.8	0.00

b) Raceway batch-2

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.04	1176.5	24.11
1	0.06	1058.8	20.37
2	0.07	1176.5	17.87
3	0.08	1029.4	16.63
4	0.11	1000.0	13.72
5	0.13	1088.2	13.16
6	0.15	1117.6	10.56
7	0.19	1058.8	7.84
8	0.22	1029.4	6.83
9	0.25	1000.0	5.07
10	0.28	970.6	3.01
11	0.32	1029.4	1.21
12	0.38	941.2	0.58
13	0.36	1000.0	0.47
14	0.39	970.6	0.28
15	0.41	941.2	0.28
16	0.44	852.9	0.25
17	0.45	823.5	0.22
18	0.48	911.8	0.22
19	0.51	941.2	0.21
20	0.52	852.9	0.21
21	0.50	941.2	0.20
22	0.53	911.8	0.19
23	0.55	911.8	0.16
24	0.56	911.8	0.12
26	0.60	882.4	0.12
27	0.59	823.5	0.14
28	0.63	852.9	0.10
29	0.62	735.3	0.08

c) Raceway batch-3

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.05	1117.6 ± 29.4	30.76 ± 1.18
1	0.06	1117.6 ± 0.0	24.94 ± 1.18
2	0.07	1058.8 ± 77.8	24.94 ± 0.59
3	0.09	1039.2 ± 44.9	23.69 ± 1.76
4	0.11	1102.9 ± 20.8	19.33 ± 2.06
5	0.15	1078.4 ± 33.9	21.19 ± 0.59
7	0.24	1102.9 ± 20.8	17.92 ± 0.22
9	0.28	1073.5 ± 20.8	15.34 ± 0.35
10	0.30	1044.1 ± 20.8	9.63 ± 0.18
11	0.34	1117.6 ± 29.4	8.09 ± 2.08
13	0.42	1073.5 ± 20.8	9.14 ± 0.60
15	0.48	1088.2 ± 41.6	8.19 ± 0.60
16	0.49	1073.5 ± 20.8	7.82 ± 0.72
17	0.50	970.6 ± 0.0	7.27 ± 0.82
18	0.53	1073.5 ± 62.4	5.55 ± 0.02
19	0.54	1058.8 ± 0.0	4.92 ± 0.05
20	0.55	985.3 ± 20.8	3.92 ± 0.00
21	0.58	941.2 ± 0.0	2.89 ± 0.09
22	0.59	1014.7 ± 62.4	1.63
23	0.61	1029.4 ± 41.6	0.85
24	0.64	1014.7 ± 20.8	0.43
25	0.65	1029.4 ± 29.4	0.45
26	0.67	970.6 ± 29.4	0.38
27	0.69	941.2 ± 0.0	0.40
28	0.71	980.4 ± 16.9	0.41
29	0.71	990.2 ± 33.9	0.32
30	0.74	960.8 ± 20.8	0.37
31	0.77	1049.0 ± 44.9	0.33
32	0.76	931.4 ± 44.9	0.37
33	0.79	931.4 ± 16.9	0.35
34	0.79	1000.0 ± 0.0	0.37
35	0.83	1029.4 ± 29.4	0.37
36	0.83	931.4 ± 33.9	0.31
37	0.85	951.0 ± 44.9	0.29

Standard deviations of nitrate and phosphate are based on triplicate and duplicate samples, respectively

c) Raceway batch-3 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration $mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
38	0.88	941.2 ± 77.8	0.29
39	0.89	897.1 ± 20.8	0.30
40	0.91	1029.4 ± 0.0	0.28
41	0.93	1014.7 ± 20.8	0.00
42	0.93	823.5 ± 83.2	0.02
43	0.95	892.2 ± 61.2	0.02
44	0.96	931.4 ± 33.9	0.02
45	0.95	833.3 ± 94.5	0.03
46	0.96	941.2 ± 0.0	0.02
47	0.98	833.3 ± 33.9	0.02
48	0.99	803.9 ± 61.2	0.00
49	1.03	843.1 ± 16.9	0.02
50	1.03	754.9 ± 33.9	0.00
52	1.06	823.5 ± 0.0	0.00
53	1.06	833.3 ± 44.9	0.02
54	1.09	902.0 ± 44.9	0.12
55	1.08 ± 0.00	852.9 ± 29.4	0.02
56	1.08 ± 0.01	892.2 ± 33.9	0.03
57	1.09 ± 0.02	882.4 ± 29.4	0.01
58	1.09 ± 0.03	892.2 ± 16.9	0.03
59	1.10 ± 0.03	843.1 ± 44.9	0.07
60	1.12 ± 0.00	801.5 ± 51.9	0.06
61	1.09 ± 0.01	745.1 ± 62.4	0.10
62	1.10 ± 0.05	696.1 ± 84.9	0.12
63	1.12 ± 0.03	696.1 ± 33.9	0.08
64	1.14 ± 0.02	696.1 ± 44.9	0.17

Standard deviations of biomass and nitrate are based on triplicate samples

Data for raceway culture profile for Figure 4.18: (effect of low irradiance)

Raceway batch-4

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.08 ± 0.00	1029.4 ± 0.0	30.96 ± 0.29
1	0.10 ± 0.00	1029.4 ± 0.0	25.98 ± 0.29
2	0.10 ± 0.00	926.5 ± 20.8	25.35 ± 0.59
3	0.10 ± 0.00	882.4 ± 0.0	25.15 ± 0.29
4	0.10 ± 0.00	852.9 ± 0.0	22.44 ± 1.18
5	0.12 ± 0.00	862.7 ± 67.9	20.37 ± 0.00
6	0.14 ± 0.01	897.1 ± 20.8	19.35 ± 0.15
7	0.14 ± 0.01	897.1 ± 20.8	18.61 ± 0.04
8	0.14 ± 0.02	897.1 ± 20.8	17.72 ± 0.15
9	0.15 ± 0.00	867.6 ± 62.4	16.91 ± 0.16
10	0.17 ± 0.00	823.5 ± 0.0	16.08 ± 0.00
11	0.18 ± 0.01	808.8 ± 20.8	10.02 ± 0.46
12	0.20 ± 0.01	808.8 ± 20.8	9.78 ± 0.27
13	0.22 ± 0.00	838.2 ± 62.4	9.66 ± 0.33
14	0.23 ± 0.01	838.2 ± 20.8	9.38 ± 0.13
17	0.25 ± 0.00	911.8 ± 0.0	8.65 ± 0.14
18	0.26 ± 0.00	838.2 ± 62.4	8.51 ± 0.19
19	0.27 ± 0.01	838.2 ± 20.8	8.19 ± 0.06
20	0.28 ± 0.01	794.1 ± 41.6	8.15 ± 0.02
21	0.28 ± 0.00	794.1 ± 83.2	7.82 ± 0.01
23	0.30 ± 0.00	838.2 ± 20.8	7.51
24	0.30 ± 0.01	764.7 ± 0.0	7.30
26	0.31 ± 0.00	808.8 ± 20.8	7.08
27	0.32 ± 0.01	808.8 ± 20.8	6.32
29	0.32 ± 0.01	838.2 ± 20.8	5.69
30	0.33 ± 0.01	867.6 ± 62.4	4.54
31	0.34 ± 0.01	838.2 ± 20.8	3.84
32	0.34 ± 0.00	676.5 ± 0.0	2.57
33	0.34 ± 0.00	705.9 ± 0.0	2.07
34	0.35 ± 0.01	705.9 ± 0.0	1.45
37	0.37 ± 0.01	676.5 ± 0.0	0.29

Standard deviation of biomass is based on triplicate samples; standard deviations of nitrate and phosphate are based on duplicate samples

Raceway batch-4 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
38	0.36 ± 0.01	705.9 ± 83.2	0.16
40	0.38 ± 0.01	705.9 ± 0.0	0.17
41	0.37 ± 0.01	764.7 ± 0.0	0.12
42	0.39 ± 0.01	735.3 ± 41.6	0.10
44	0.41 ± 0.00	808.8 ± 20.8	0.10
45	0.41 ± 0.00	661.8 ± 20.8	0.11
47	0.39 ± 0.01	676.5 ± 0.0	0.00
48	0.40 ± 0.00	661.8 ± 20.8	0.00
52	0.41 ± 0.01	764.7 ± 0.0	0.02
56	0.46 ± 0.00	720.6 ± 62.4	0.01
60	0.43 ± 0.01	735.3 ± 41.4	0.00
61	0.42 ± 0.01	750.0 ± 62.4	0.00
63	0.45 ± 0.01	735.3 ± 83.2	0.00
66	0.45 ± 0.00	647.1 ± 41.6	0.00
68	0.46 ± 0.00	676.5 ± 41.6	0.00
69	0.43 ± 0.00	647.1	0.03
73	0.47 ± 0.02	647.1	0.00
75	0.45 ± 0.01	647.1	0.00

Standard deviations of biomass and nitrate are based on triplicate and duplicate samples, respectively

Data for raceway culture profile for Figure 4.19: effect of nitrate stress (a) ~20% of normal initial nitrate and (b) ~18% of normal initial nitrate

a) Raceway batch-5

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.12 ± 0.00	230.4 ± 16.9	24.11 ± 0.00
1	0.13 ± 0.00	230.4 ± 8.5	21.61 ± 1.76
2	0.14 ± 0.00	181.4 ± 16.9	19.33 ± 0.29
3	0.15 ± 0.01	215.7 ± 8.5	17.66 ± 0.88
4	0.16 ± 0.00	186.3 ± 16.9	18.08 ± 0.29
5	0.18 ± 0.00	122.5 ± 16.9	13.55 ± 0.24
6	0.21 ± 0.01	161.8 ± 0.0	11.72 ± 0.00
7	0.23 ± 0.01	142.2 ± 8.5	10.60 ± 1.03
8	0.25 ± 0.01	142.2 ± 8.5	8.60 ± 1.32
9	0.31	196.1 ± 8.5	7.48 ± 0.35
10	0.34	181.4 ± 8.5	5.49 ± 0.35
11	0.35	181.4 ± 8.5	4.32 ± 0.06
12	0.38	166.7 ± 8.5	3.01 ± 0.03
13	0.40	147.1 ± 29.4	1.93 ± 0.03
14	0.42	147.1 ± 14.7	0.75 ± 0.36
15	0.43	147.1 ± 14.7	0.36 ± 0.09
16	0.46	117.6 ± 14.7	0.29 ± 0.00
17	0.48	132.4 ± 0.0	0.50 ± 0.00
18	0.50	147.1 ± 25.5	0.35 ± 0.03
19	0.54	137.3 ± 8.5	0.39 ± 0.03
20	0.55	112.7 ± 8.5	0.15 ± 0.03
21	0.53	83.3 ± 16.9	0.10 ± 0.03
22	0.56	102.9 ± 14.7	0.07 ± 0.00
23	0.57	112.7 ± 22.5	0.10 ± 0.01
25	0.60	122.5 ± 8.5	0.04 ± 0.01
26	0.62	88.2 ± 14.7	0.08 ± 0.01
27	0.59	78.4 ± 8.5	0.06 ± 0.02
28	0.60	73.5 ± 0.0	0.05 ± 0.00
29	0.66	73.5 ± 20.8	0.05 ± 0.01

Standard deviations of biomass and nitrate are based on triplicate samples; standard deviation of phosphate is based on duplicate samples

a) Raceway batch-5 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
30	0.67	53.9 ± 8.5	0.06 ± 0.00
31	0.68	93.1 ± 8.5	0.06 ± 0.01
32	0.70	83.3 ± 8.5	0.08 ± 0.01
33	0.71	103.9 ± 1.7	0.05 ± 0.01
34	0.74	108.8 ± 7.8	0.05 ± 0.01
35	0.74	84.3 ± 3.4	0.03 ± 0.01
36	0.72	68.6 ± 3.4	0.04 ± 0.02
37	0.77	85.3 ± 4.2	0.05 ± 0.03
38	0.77	78.4 ± 3.4	0.05
39	0.77	68.6 ± 3.4	0.08
40	0.78	64.7 ± 5.9	0.07
41	0.78	74.5 ± 3.4	0.03
42	0.79	72.5 ± 6.8	0.02
43	0.80	60.8 ± 3.4	0.06
44	0.80	54.9 ± 6.8	0.07
45	0.83	43.1 ± 6.8	0.05
47	0.86	43.1 ± 8.9	0.06
48	0.85	51.0 ± 3.4	0.04
49	0.85	39.2 ± 8.9	0.03
50	0.85	41.2 ± 3.4	0.02
51	0.85	37.3 ± 0.0	0.07
52	0.87	37.3 ± 6.8	0.03
53	0.87	33.3 ± 3.4	0.01
54	0.88	29.4 ± 3.4	0.02
55	0.88	29.4 ± 0.0	0.01
56	0.88	25.5 ± 0.0	0.05
57	0.89	13.7 ± 3.4	0.05
58	0.87	20.6 ± 3.4	0.02
59	0.91	23.5 ± 0.0	0.02
60	0.92	25.0 ± 2.9	0.04
61	0.92	22.5 ± 2.1	0.03
62	0.91	19.6 ± 1.7	0.04
63	0.93	16.3 ± 1.7	0.02
64	0.93	17.1 ± 0.3	0.07

Standard deviations of nitrate and phosphate are based on triplicate and duplicate samples, respectively

a) Raceway batch-5 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
65	0.93	20.0 ± 0.6	0.00
67	0.90	20.0 ± 0.3	0.03
68	0.91	13.7 ± 1.6	0.05
69	0.92	10.4 ± 0.3	0.02
70	0.91	9.4 ± 0.9	0.02
71	0.91	7.3 ± 1.6	0.02
72	0.91	7.6 ± 0.7	0.06
73	0.92	7.8 ± 1.0	0.03
74	0.91	2.2 ± 0.7	0.04
75	0.92	2.6 ± 0.6	0.04
76	0.71	1.6 ± 0.7	0.01
77	0.72	2.2 ± 0.3	0.02
78	0.72	2.9 ± 0.3	0.02
79	0.71	2.2 ± 0.0	0.01
80	0.70	4.5 ± 0.0	0.03
82	0.71	3.3 ± 0.0	0.02
83	0.70	2.5 ± 0.0	0.02
84	0.71	1.2 ± 0.0	0.05
85	0.72	1.8 ± 06	0.05
86	0.71	1.2 ± 0.6	0.03
88	0.71	1.8 ± 0.6	0.00
89	0.71	1.6 ± 0.0	0.02
90	0.71	0.6 ± 0.3	0.02
91	0.70	2.4 ± 0.0	0.04
92	0.71	1.6 ± 0.6	0.02
93	0.70	1.4 ± 0.3	0.02
94	0.69	3.7 ± 0.3	0.05
96	0.71	2.7 ± 0.3	0.04
97	0.72	2.5 ± 0.6	0.07
98	0.71	1.0 ± 0.3	0.02
99	0.72	2.2 ± 0.7	0.03
100	0.72	0.8 ± 0.3	0.00
101	0.71	5.3 ± 0.7	0.06

Standard deviation of nitrate is based on triplicate samples

a) Raceway batch-5 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
103	0.72	2.7 ± 0.0	0.09
104	0.67	2.4 ± 0.3	0.02
105	0.68	2.2 ± 0.0	0.06
106	0.66	2.0 ± 0.3	0.03
107	0.66	0.4 ± 0.3	0.03
109	0.69	3.3 ± 0.3	0.03
110	0.67	1.8 ± 0.3	0.05
111	0.69	2.2 ± 0.6	0.02
112	0.67	1.6 ± 0.9	0.03
114	0.63	1.4 ± 0.9	0.05
115	0.64	2.4 ± 0.3	0.05
117	0.62	2.2 ± 0.6	0.05
118	0.63	2.9 ± 0.9	0.03
119	0.64	5.1 ± 0.0	0.04
120	0.63	5.1 ± 0.3	0.02
121	0.64	5.6 ± 0.3	0.05
122	0.58	5.7 ± 0.3	0.07
124	0.58	4.9 ± 0.9	0.05
125	0.61	4.7 ± 0.7	0.05
126	0.58	4.7 ± 0.0	0.03
127	0.58	5.3 ± 0.0	0.04
128	0.57	4.7 ± 0.6	0.05
129	0.59	5.0 ± 0.0	0.06
131	0.59	5.5 ± 0.4	0.02
132	0.55	4.1 ± 0.6	0.04
133	0.54	5.0 ± 0.6	0.04
134	0.54	6.5 ± 0.4	0.05
135	0.53	5.5 ± 0.0	0.02
136	0.53	2.2 ± 0.7	0.05
138	0.54	5.7 ± 0.3	0.04
139	0.54	3.3 ± 0.9	0.05
140	0.54	5.9 ± 0.3	0.03
141	0.52	5.0 ± 0.4	0.06

Standard deviation of nitrate is based on triplicate samples

a) Raceway batch-5 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
143	0.51	3.8 ± 0.9	0.07
145	0.49	3.1 ± 0.4	0.03
146	0.49	4.4 ± 0.3	0.02
147	0.49	5.3 ± 0.4	0.04
148	0.48	5.0 ± 0.6	0.02
149	0.48	4.7 ± 0.4	0.02
152	0.47	5.6 ± 0.0	0.02
153	0.45	4.9 ± 0.3	0.05
154	0.46	4.5 ± 0.3	0.02
155	0.45	4.1 ± 0.3	0.03
156	0.44	3.9 ± 0.6	0.02
159	0.44	3.8 ± 0.3	0.00

Standard deviation of nitrate is based on triplicate samples

b) Raceway batch-6

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.07 ± 0.00	194.1 ± 8.3	28.89 ± 0.29
1	0.08 ± 0.00	188.2 ± 16.6	25.35 ± 1.76
2	0.08 ± 0.00	205.9 ± 8.3	23.48 ± 1.47
3	0.10 ± 0.01	188.2 ± 16.6	22.24 ± 0.29
4	0.10 ± 0.00	200.0 ± 0.0	19.74 ± 0.88
5	0.12 ± 0.00	205.9 ± 8.3	19.33 ± 0.18
6	0.15 ± 0.00	188.2 ± 0.0	18.70 ± 0.47
7	0.16 ± 0.00	194.1 ± 24.9	17.17 ± 0.06
9	0.21 ± 0.00	196.1 ± 6.8	15.25 ± 0.29
10	0.23 ± 0.00	164.7 ± 0.0	14.05 ± 0.12
11	0.27 ± 0.01	152.9 ± 0.0	12.01 ± 0.00
12	0.30 ± 0.01	152.9 ± 23.5	10.87 ± 0.03
13	0.31 ± 0.01	192.2 ± 6.8	8.92 ± 0.09
14	0.33 ± 0.00	160.8 ± 6.8	8.35 ± 0.12
16	0.34 ± 0.01	170.6 ± 8.3	6.75 ± 0.09
17	0.37 ± 0.01	137.3 ± 6.8	6.01 ± 0.03
18	0.38 ± 0.01	147.1 ± 8.3	3.79 ± 0.01
19	0.39 ± 0.01	158.8 ± 8.3	3.28 ± 0.01
20	0.41 ± 0.01	133.3 ± 13.6	2.50 ± 0.01
22	0.43 ± 0.01	135.3 ± 8.3	0.75 ± 0.02
24	0.44 ± 0.01	156.9 ± 24.5	0.22 ± 0.01
25	0.46 ± 0.00	141.2 ± 23.5	0.18 ± 0.00
26	0.46 ± 0.02	105.9 ± 11.8	0.29 ± 0.01
27	0.47 ± 0.02	105.9 ± 11.8	0.17 ± 0.01
28	0.48 ± 0.01	86.3 ± 13.6	0.20 ± 0.01
30	0.51 ± 0.01	105.9 ± 11.6	0.16 ± 0.01
31	0.51 ± 0.01	109.8 ± 6.8	0.18 ± 0.00
32	0.51 ± 0.00	105.9 ± 0.0	0.18 ± 0.01
33	0.51 ± 0.01	94.1 ± 20.4	0.10 ± 0.01
34	0.52 ± 0.01	94.1 ± 11.8	0.12 ± 0.01
35	0.52 ± 0.01	90.2 ± 6.8	0.07 ± 0.01
38	0.53 ± 0.01	82.4 ± 0.0	0.07 ± 0.00
39	0.53 ± 0.02	78.4 ± 6.8	0.00

Standard deviations of biomass and nitrate are based on triplicate samples; standard deviation of phosphate is based on duplicate samples

b) Raceway batch-6 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
40	0.56 ± 0.00	76.5 ± 24.9	0.00
41	0.56 ± 0.00	94.1 ± 0.0	0.00
42	0.56 ± 0.01	66.7 ± 13.6	0.00
45	0.57 ± 0.02	43.1 ± 6.8	0.00
46	0.57 ± 0.01	35.3 ± 0.0	0.00
48	0.59 ± 0.01	49.0 ± 18.9	0.00
52	0.59 ± 0.01	41.2 ± 0.0	0.00
53	0.60 ± 0.00	52.9 ± 0.0	0.00
54	0.63 ± 0.02	38.2 ± 4.2	0.00
56	0.59 ± 0.02	35.3 ± 8.3	0.82
59	0.66 ± 0.00	32.4 ± 12.5	0.00
60	0.66 ± 0.00	27.5 ± 3.4	0.00
61	0.66 ± 0.01	33.3 ± 3.4	0.00
63	0.67 ± 0.03	29.4 ± 0.0	0.00
66	0.67 ± 0.01	14.7 ± 0.0	0.00
67	0.68 ± 0.01	13.2 ± 2.1	0.00
70	0.67 ± 0.00	10.3 ± 2.1	0.02
73	0.67 ± 0.00	2.1 ± 0.4	0.00
76	0.67 ± 0.00	5.3 ± 0.3	0.02
77	0.68 ± 0.02	5.3 ± 0.4	0.02
80	0.67 ± 0.00	5.9 ± 0.0	0.00
82	0.67 ± 0.00	5.9 ± 0.6	0.00
84	0.67 ± 0.00	5.6 ± 0.4	0.00
87	0.67 ± 0.02	4.4 ± 0.4	0.00
88	0.66 ± 0.00	5.3 ± 0.8	0.00
91	0.64 ± 0.00	5.3 ± 0.0	0.00
95	0.63 ± 0.02	5.6 ± 0.4	0.00
96	0.65 ± 0.02	4.1 ± 0.8	0.00
97	0.63 ± 0.01	4.1 ± 0.8	0.00
98	0.76 ± 0.04	5.0 ± 1.3	0.00
99	0.74 ± 0.02	5.6 ± 0.4	0.00
101	0.76 ± 0.02	5.3 ± 0.8	0.04
102	0.77 ± 0.03	3.2 ± 1.3	0.00

Standard deviations of biomass and nitrate are based on triplicate samples

b) Raceway batch-6 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
105	0.75 ± 0.02	3.5 ± 0.0	0.07
108	0.74 ± 0.01	4.7 ± 0.8	0.03
111	0.73 ± 0.03	4.1 ± 0.0	0.00
114	0.73 ± 0.00	5.0 ± 0.4	0.00
117	0.73 ± 0.01	4.7 ± 0.0	0.05
121	0.69 ± 0.01	4.1 ± 0.0	0.12
128	0.67 ± 0.03	6.2 ± 0.4	0.00
131	0.69 ± 0.01	5.0 ± 0.4	0.00
135	0.67 ± 0.01	5.0 ± 1.3	0.00
138	0.67 ± 0.01	5.0 ± 0.4	0.00
141	0.70 ± 0.02	5.0 ± 0.4	0.00
146	0.64 ± 0.01	5.3 ± 0.0	0.00
148	0.68 ± 0.01	4.7 ± 0.0	0.00
152	0.64 ± 0.01	5.3 ± 0.8	0.00
155	0.64 ± 0.01	4.7 ± 0.0	0.00
162	0.57 ± 0.01	5.3 ± 0.0	0.00
168	0.57 ± 0.01	2.9 ± 0.0	0.00
173	0.55 ± 0.01	4.7 ± 0.0	0.00
175	0.53 ± 0.01	4.7 ± 0.0	0.00
180	0.51 ± 0.01	3.8 ± 0.0	0.00
185	0.48 ± 0.00	4.1 ± 0.0	0.00

Standard deviations of biomass and nitrate are based on triplicate samples

Data for raceway culture profile for Figure 4.20: effect of nitrate stress (~10% of normal initial nitrate)

Raceway batch-7

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.04 ± 0.00	102.0 ± 3.4	28.26 ± 1.18
1	0.04 ± 0.00	119.6 ± 13.6	24.94 ± 1.18
3	0.05 ± 0.00	111.8 ± 5.9	25.56 ± 2.65
5	0.08 ± 0.00	105.9 ± 8.3	22.24 ± 0.06
6	0.09 ± 0.00	102.0 ± 3.4	20.24 ± 0.06
7	0.11 ± 0.00	92.2 ± 8.9	18.70 ± 0.47
8	0.13 ± 0.00	102.0 ± 3.4	19.08 ± 0.06
9	0.14 ± 0.00	73.5 ± 4.2	17.17 ± 0.41
10	0.15 ± 0.00	88.2 ± 5.9	15.34 ± 0.53
11	0.18 ± 0.00	54.9 ± 6.8	14.13
12	0.20 ± 0.00	66.7 ± 8.9	13.09
13	0.20 ± 0.00	74.5 ± 12.3	11.72
15	0.23 ± 0.00	47.1 ± 2.0	9.56
16	0.24	54.7 ± 0.8	8.69
17	0.25	47.1 ± 3.3	5.08
18	0.27	49.4 ± 0.0	4.09
19	0.30	35.7 ± 3.8	3.68
20	0.30	35.3 ± 4.1	3.36
21	0.30	34.5 ± 0.7	2.64
22	0.32	16.1 ± 0.7	2.03
23	0.32	18.0 ± 0.7	1.67
25	0.34	5.1 ± 0.7	1.50
26	0.35	7.5 ± 1.7	1.50
27	0.35	3.4 ± 1.0	1.39
28	0.35	5.5 ± 0.9	1.31
29	0.32	2.9 ± 0.6	1.30
30	0.32	3.7 ± 0.3	1.18
32	0.33	2.1 ± 1.3	1.22
33	0.32	2.7 ± 0.3	0.96

Standard deviation of biomass is based on triplicate samples; standard deviations of nitrate and phosphate are based on duplicate samples

Raceway batch-7 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
35	0.33	0.6	1.06
37	0.29	1.2	0.99
39	0.33	2.4	0.91
40	0.35	1.8	0.92
41	0.36	3.5	0.82
43	0.35	1.2	0.80
45	0.34	2.4	0.77
46	0.35	2.9	0.71
48	0.35	2.4	0.76
50	0.36	2.9	0.82
53	0.34	2.4	0.67
55	0.35	1.8	0.61
56	0.34	2.4	0.67
57	0.35	1.8	0.62
60	0.35	2.9	0.57
61	0.34	1.2	0.56
63	0.35	1.8	0.51
64	0.35	1.8	0.52
67	0.35	1.2	0.48
68	0.36	1.5	0.41

Data for raceway culture profile for Figure 4.21: effect of nitrate stress (~22% of normal initial nitrate)

Raceway batch-8

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
0	0.04 ± 0.00	238.2 ± 4.2	27.02 ± 0.59
1	0.05 ± 0.00	214.7 ± 4.2	27.85 ± 0.00
2	0.06 ± 0.00	202.9 ± 12.5	25.98 ± 0.29
3	0.07 ± 0.00	182.4 ± 16.6	22.24 ± 0.29
4	0.07 ± 0.00	164.7 ± 8.3	22.03 ± 0.00
5	0.08 ± 0.00	214.7 ± 29.1	20.37 ± 0.00
6	0.08 ± 0.00	208.8 ± 12.5	20.03 ± 0.24
7	0.08 ± 0.00	208.8 ± 12.5	19.33 ± 0.18
8	0.08 ± 0.00	197.1 ± 12.5	18.70 ± 0.12
9	0.09 ± 0.00	217.6 ± 8.3	18.20 ± 0.12
10	0.09 ± 0.00	220.6 ± 4.2	18.00 ± 0.29
11	0.09 ± 0.00	208.8 ± 4.2	16.92 ± 0.06
12	0.09 ± 0.00	208.8 ± 4.2	16.46 ± 0.12
13	0.10 ± 0.00	211.8 ± 0.0	15.71 ± 0.12
14	0.10 ± 0.00	194.1 ± 16.6	15.25 ± 0.41
15	0.10 ± 0.00	188.2 ± 16.6	14.88 ± 0.35
16	0.10 ± 0.00	194.1 ± 8.3	14.67 ± 0.29
17	0.10 ± 0.00	197.1 ± 4.2	14.13 ± 0.00
18	0.11 ± 0.00	202.9 ± 12.5	13.59 ± 0.06
19	0.11 ± 0.00	199.0 ± 11.9	13.30 ± 0.12
20	0.11 ± 0.00	214.7 ± 2.9	13.26 ± 0.18
21	0.12 ± 0.00	203.9 ± 11.1	13.22 ± 0.24
23	0.13 ± 0.00	200.0 ± 16.6	12.22 ± 0.47
24	0.14 ± 0.00	202.0 ± 9.5	11.85 ± 0.06
25	0.15 ± 0.00	207.4 ± 10.4	11.22 ± 0.00
27	0.16 ± 0.00	187.3 ± 21.3	9.98 ± 0.35
28	0.17 ± 0.00	191.2 ± 11.8	8.56 ± 0.00
29	0.18 ± 0.00	201.5 ± 6.2	7.65 ± 0.24

Standard deviation of biomass is based on triplicate samples; standard deviations of nitrate and phosphate are based on duplicate samples

Raceway batch-8 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
31	0.20 ± 0.00	174.5 ± 12.3	5.98 ± 0.36
32	0.21 ± 0.00	171.6 ± 8.9	5.59 ± 0.38
33	0.23 ± 0.00	182.4 ± 0.0	4.75 ± 0.04
34	0.24 ± 0.01	185.3 ± 0.0	4.26 ± 0.15
36	0.25 ± 0.00	170.6 ± 5.9	3.36 ± 0.12
38	0.26 ± 0.00	168.6 ± 11.9	2.73 ± 0.14
39	0.29 ± 0.00	167.6 ± 0.0	2.36 ± 0.05
47	0.32 ± 0.00	174.5 ± 8.9	0.79
49	0.32 ± 0.00	163.2 ± 10.4	0.57
51	0.34 ± 0.00	142.2 ± 8.9	0.43
53	0.36 ± 0.00	135.3 ± 10.6	0.34
55	0.36 ± 0.00	150.0 ± 0.0	0.23
59	0.40 ± 0.00	127.9 ± 14.6	0.00
61	0.40 ± 0.00	107.8 ± 3.4	0.00
63	0.42 ± 0.01	119.6 ± 4.5	0.00
65	0.43 ± 0.00	121.6 ± 1.7	0.00
66	0.44 ± 0.00	117.6 ± 0.0	0.35
67	0.43 ± 0.01	119.6 ± 1.7	0.41
68	0.45 ± 0.01	116.2 ± 6.2	0.41
69	0.45 ± 0.00	100.0 ± 7.8	0.10
70	0.44 ± 0.00	96.1 ± 6.8	0.00
71	0.44 ± 0.00	95.1 ± 1.7	0.00
72	0.44 ± 0.00	98.5 ± 6.2	0.00
73	0.44 ± 0.00	97.1 ± 2.9	0.00
74	0.46 ± 0.00	97.1 ± 5.1	0.00
75	0.46 ± 0.00	101 ± 3.4	0.00
76	0.46 ± 0.00	98.0 ± 4.5	0.00
77	0.46 ± 0.00	91.2 ± 2.9	0.00
79	0.48 ± 0.00	83.3 ± 3.4	0.00
80	0.47 ± 0.01	96.1 ± 1.7	0.00
81	0.48 ± 0.00	86.8 ± 2.1	0.00
82	0.47 ± 0.00	81.4 ± 3.4	0.00
83	0.47 ± 0.00	74.5 ± 4.5	0.00

Standard deviation of biomass is based on triplicate samples; standard deviations nitrate and phosphate are based on duplicate samples

Raceway batch-8 (Cont.)

Time (d)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)
84	0.47 ± 0.00	80.4 ± 4.5	0.00
85	0.47 ± 0.00	75.0 ± 2.1	0.00
87	0.48 ± 0.00	81.4 ± 1.7	0.00
88	0.47 ± 0.01	80.4 ± 3.4	0.00
89	0.47 ± 0.00	78.4 ± 1.7	0.00
90	0.48 ± 0.00	85.3 ± 0.0	0.00
92	0.49 ± 0.00	77.9 ± 2.1	0.00
94	0.48 ± 0.00	79.4 ± 12.5	0.00
97	0.48 ± 0.00	70.6 ± 0.0	0.00
100	0.48 ± 0.00	72.1 ± 2.1	0.00
102	0.48 ± 0.00	70.6 ± 5.9	0.00
103	0.51 ± 0.00	72.1 ± 2.1	0.00
106	0.52 ± 0.01	76.5 ± 0.0	0.00
107	0.52 ± 0.01	61.8 ± 2.9	0.00
108	0.53 ± 0.00	61.8 ± 0.0	0.00
109	0.53 ± 0.01	64.7 ± 0.0	0.00
110	0.54 ± 0.01	54.9 ± 1.7	0.00
111	0.56 ± 0.00	51.5 ± 2.1	0.00
112	0.56 ± 0.00	60.3 ± 2.1	0.00
115	0.57 ± 0.00	50.0 ± 4.5	0.00
116	0.58 ± 0.00	51.0 ± 1.7	0.00
117	0.57 ± 0.00	47.1 ± 0.0	0.00
118	0.58 ± 0.00	50.0 ± 5.1	0.00
120	0.57 ± 0.00	52.9 ± 4.2	0.00
121	0.56 ± 0.00	52.0 ± 6.8	0.00
122	0.55 ± 0.00	50.0 ± 7.8	0.00
123	0.56 ± 0.00	49.0 ± 4.5	0.00
124	0.55 ± 0.00	51.5 ± 2.1	0.00
125	0.55 ± 0.01	47.1 ± 0.0	0.00

Standard deviations of biomass and nitrate are based on triplicate and duplicate samples, respectively

Data for continuous raceway culture profile for Figure 4.24:

Time (h)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Dilution rate (h^{-1})
0	0.60	882.4	0.15	
24	0.48	1000.0	0.80	D = 0.0115 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
48	0.41	1029.4	1.68	
72	0.32	882.4	2.19	
96	0.28	882.4	3.65	
120	0.25	911.8	6.56	
144	0.22	1000.0	7.29	
168	0.20	1029.4	8.02	
192	0.19	1058.8	9.48	
216	0.18	1058.8	10.94	
240	0.16	1029.4	8.95	
288	0.15	1029.4	10.21	
312	0.13	970.6	9.48	
336	0.14	970.6	10.21	
360	0.13	1147.1	11.31	
384	0.12	1147.1	13.86	
408	0.12	1117.6	14.95	
432	0.12	1029.4	16.05	D = 0.0115 (continuous illumination of 46 $\mu mol \cdot m^{-2}s^{-1}$)
456	0.11	1029.4	17.87	
480	0.11	1058.8	17.14	
504	0.10	794.1	16.41	
528	0.09	852.9	17.87	
552	0.09	882.4	17.14	
576	0.08	970.6	17.51	
600	0.07	823.5	16.05	
624	0.07	852.9	13.49	
648	0.08	970.6	11.31	
672	0.07	823.5	10.21	
696	0.08	970.6	7.66	
720	0.08	647.1	6.20	
744	0.08	941.2	6.93	
768	0.09	911.8	6.93	

Data for continuous raceway culture profile for Figure 4.24 (Cont.):

Time (h)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Dilution rate (h^{-1})
792	0.10	1058.8	6.20	D = 0.0115 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
816	0.12	882.4	4.38	
840	0.14	941.2	3.28	
864	0.15	1000.0	7.29	
888	0.16	970.6	11.31	
912	0.16	1029.4	14.95	
936	0.16	1117.6	15.68	
960	0.17	1147.1	16.05	
984	0.18	1176.5	19.33	
1008	0.18	1117.6	19.69	
1032	0.19	1029.4	22.25	
1056	0.18	1029.4	22.61	
1080	0.18	1147.1	21.52	
1104	0.18	1117.6	21.88	
1128	0.18	1058.8	21.88	
1152	0.17	1000.0	21.52	D = 0.0115 (14 h: 10 h Light/dark cycle)
1162	0.16	-	-	
1176	0.16	1000.0	20.42	
1186	0.15	-	-	
1200	0.15	1000.0	19.33	
1210	0.14	-	-	
1224	0.14	970.6	18.60	
1234	0.13	-	-	
1248	0.14	941.2	18.23	
1258	0.13	-	-	
1272	0.14	911.8	17.87	
1282	0.13	-	-	
1296	0.14	1058.8	18.23	
1306	0.12	-	-	
1320	0.14	941.2	19.33	
1330	0.12	-	-	
1344	0.14	1088.2	18.23	

Data for continuous raceway culture profile for Figure 4.24 (Cont.):

Time (h)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Dilution rate (h^{-1})
1354	0.12	-	-	
1368	0.14	1058.8	18.60	D = 0.0115 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
1392	0.14	1088.2	18.23	
1416	0.15	1058.8	17.51	
1440	0.16	1029.4	15.68	
1464	0.16	1000.0	15.68	
1488	0.16	1029.4	15.32	
1512	0.16	941.2	15.68	
1536	0.17	1058.8	14.95	D = 0.0072 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
1560	0.19	1029.4	14.59	
1584	0.20	941.2	12.40	
1608	0.23	1029.4	11.31	
1632	0.26	1117.6	10.58	
1656	0.27	1147.1	7.29	
1680	0.34	1147.1	7.29	
1704	0.33	1088.2	6.93	
1728	0.34	1176.5	4.01	
1752	0.33	1176.5	3.28	
1776	0.34	1176.5	5.11	
1800	0.36	1147.1	6.93	
1824	0.33	1205.9	10.21	
1848	0.35	1147.1	10.58	
1872	0.30	1176.5	11.67	
1896	0.36	1176.5	11.31	
1920	0.36	1176.5	13.86	
1944	0.36	1117.6	14.22	
1968	0.36	1147.1	16.41	D = 0.0072 (continuous illumination of 46 $\mu mol \cdot m^{-2}s^{-1}$)
1992	0.33	1117.6	17.51	
2016	0.32	1176.5	17.51	
2040	0.31	1000.0	18.96	
2064	0.29	941.2	20.79	
2112	0.27	882.4	20.42	

Data for continuous raceway culture profile for Figure 4.24 (Cont.):

Time (h)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Dilution rate (h^{-1})
2136	0.24	1000.0	20.79	D = 0.0072 (continuous illumination of 46 $\mu mol \cdot m^{-2}s^{-1}$)
2160	0.22	970.6	20.06	
2184	0.24	1029.4	19.69	
2208	0.21	1000.0	18.96	
2232	0.20	1088.2	18.23	
2256	0.20	1117.6	18.23	
2280	0.22	1088.2	18.23	
2304	0.22	1088.2	18.23	
2328	0.22	1147.1	25.53	
2352	0.22	1088.2	25.53	
2376	0.21	882.4	20.79	
2400	0.21	1117.6	25.16	
2424	0.21	1088.2	25.53	
2448	0.20	1088.2	25.89	
2472	0.20	1058.8	25.16	D = 0.0072 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
2496	0.21	1176.5	24.80	
2520	0.23	1117.6	25.16	
2544	0.25	1176.5	23.34	
2568	0.26	1147.1	21.52	
2592	0.27	1088.2	22.25	
2616	0.31	1088.2	18.96	
2640	0.33	1000.0	18.96	
2664	0.37	1029.4	20.06	
2688	0.28	941.2	17.51	
2712	0.30	882.4	20.06	
2736	0.35	1029.4	20.79	
2760	0.35	1088.2	21.15	
2784	0.35	1088.2	20.79	
2808	0.33	1058.8	20.06	
2832	0.31	1088.2	20.42	
2856	0.31	1058.8	20.06	
2880	0.32	823.5	14.95	

Data for continuous raceway culture profile for Figure 4.24 (Cont.):

Time (h)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Dilution rate (h^{-1})
2904	0.30	970.6	20.06	D = 0.0072 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
2928	0.31	970.6	19.69	
2952	0.31	1058.8	19.33	
2976	0.32	1058.8	19.69	
3000	0.32	970.6	17.87	
3024	0.32	882.4	17.87	
3048	0.33	1058.8	18.60	
3072	0.32	1058.8	18.60	
3096	0.30	1029.4	18.60	
3120	0.30	1058.8	20.06	
3144	0.31	1029.4	20.06	
3168	0.30	1058.8	19.69	
3192	0.31	1058.8	20.06	
3216	0.31	1088.2	20.06	
3240	0.31	1058.8	20.06	
3264	0.31	1029.4	20.06	
3288	0.31	1058.8	19.69	
3312	0.31	1088.2	20.42	
3336	0.32	1029.4	17.87	
3360	0.32	911.8	17.87	
3384	0.31	1000.0	18.23	
3408	0.32	1000.0	18.23	
3432	0.31	911.8	18.23	
3456	0.31	1000.0	17.51	
3480	0.30	970.6	17.87	
3504	0.30	882.4	18.23	
3552	0.29	1029.4	18.23	
3576	0.29	970.6	18.60	
3600	0.29	1000.0	18.23	
3624	0.27	1029.4	18.60	
3648	0.28	1029.4	17.87	
3672	0.28	1058.8	18.23	

Data for continuous raceway culture profile for Figure 4.24 (Cont.):

Time (h)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Dilution rate (h^{-1})
3744	0.26	1029.4	18.60	D = 0.0072 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
3768	0.25	1117.6	18.60	
3792	0.24	1000.0	18.60	
3816	0.22	970.6	18.60	
3840	0.25	970.6	18.60	
3888	0.25	970.6	18.96	
3912	0.25	941.2	18.96	
3936	0.23	1058.8	18.96	
3960	0.24	970.6	18.96	
3984	0.25	941.2	20.79	
4008	0.25	941.2	19.33	
4056	0.26	1029.4	18.60	
4080	0.25	970.6	18.23	
4104	0.24	970.6	18.23	
4128	0.24	1029.4	18.23	
4152	0.24	911.8	18.23	
4176	0.23	1000.0	18.60	
4200	0.23	1029.4	18.60	
4224	0.23	1058.8	18.23	
4248	0.23	1029.4	18.60	
4272	0.22	1000.0	18.23	
4296	0.22	970.6	18.96	
4320	0.22	1029.4	20.06	
4344	0.22	970.6	18.60	
4392	0.22	941.2	18.60	
4416	0.22	970.6	18.60	
4440	0.22	1029.4	18.60	
4464	0.21	1058.8	19.69	
4488	0.22	1029.4	21.88	
4512	0.22	1117.6	20.42	
4560	0.22	1029.4	18.96	
4584	0.23	1058.8	18.23	

Data for continuous raceway culture profile for Figure 4.24 (Cont.):

Time (h)	Biomass concentration ($g\ L^{-1}$)	Nitrate concentration ($mg\ L^{-1}$)	Phosphate concentration ($mg\ L^{-1}$)	Dilution rate (h^{-1})
4608	0.22	941.2	18.23	D = 0.0072 (continuous illumination of 91 $\mu mol \cdot m^{-2}s^{-1}$)
4632	0.22	1029.4	18.23	
4656	0.22	911.8	18.96	
4680	0.23	1029.4	18.23	
4704	0.23	1000.0	18.96	
4728	0.22	1029.4	19.69	
4752	0.23	1058.8	19.69	
4776	0.23	1029.4	18.23	
4800	0.23	1029.4	16.78	
4824	0.23	1029.4	16.78	
4848	0.25	1058.8	16.41	
4872	0.22	1000.0	18.60	
4896	0.23	1029.4	18.60	
4920	0.23	1000.0	18.96	
4944	0.23	1058.8	21.88	
4968	0.23	1117.6	21.88	
4992	0.24	1117.6	21.52	
5016	0.25	1058.8	21.52	
5040	0.25	1088.2	18.96	
5064	0.26	1058.8	18.96	
5088	0.26	1058.8	18.96	
5112	0.26	1058.8	17.87	
5160	0.27	1029.4	18.23	
5184	0.27	1000.0	18.23	
5232	0.27	1000.0	18.60	
5256	0.26	970.6	17.87	
5280	0.26	941.2	17.87	