Optimising Microalgal Lipid Productivity For Biodiesel Production

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Nobody makes a greater mistake than he who does nothing because he could only do a little.

- Edmund Burke

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Abstract

Microalgae are an alternative source of vegetable oil for biodiesel production, with several advantages over land-based crop plants. Algal biodiesel is technically but not yet economically feasible, largely due to costs of algal biomass production and harvesting. Enhancing algal lipid production would improve the economics of the process. Strategies to achieve this include careful choice of algal species as well as cultivation conditions to maximise lipid productivity. The lipid content of many algae can be increased through nitrogen (N) deprivation, but this comes at the expense of biomass productivity. The relative contribution of these determines the effect on overall lipid productivity (the product of biomass growth and lipid content).

The purpose of this work was to identify promising microalgal species and strategies of nitrogen deprivation that optimise lipid productivity for biodiesel production. This was carried out by I) developing the tools necessary to measure lipid productivity accurately, II) identifying and quantifying the key characteristics of promising microalgal strains, initially through a literature survey and then experimentally, and III) investigating the effect of different degrees of N limitation, and different N culture regimes, on cell physiology and lipid productivity in *Chlorella vulgaris*.

Accurate measurement of lipid productivity requires reliable assays for the quantification of biomass concentration and lipid content in microalgal cultures. The commonly employed methods were found to be unsatisfactory due to the impact of cell pigment content on the quantification of biomass by optical density, and the incomplete extraction of lipids from algal biomass respectively. In order to investigate the magnitude of error in dry weight estimation caused by variations in pigment content, *C. vulgaris* was grown in airlift photobioreactors under conditions designed to result in different pigment contents. Pigment content was measured daily, and biomass was quantified by dry weight and OD at wavelengths within the maximum (680 nm) and minimum (750 nm) range of pigment absorption. It was found that changes in pigment content over a 17 day growth cycle, particularly under N limited conditions, led to significant errors in quantification of biomass by optical density (up to 52% in *C. vulgaris* under N limited conditions). These errors could be minimized to between 4 and 8% through the use of a wavelength outside of the range of maximum absorption by the dominant pigments (for green algae, 750 nm) and generation of a standard curve either across the entire growth cycle, or at a point of average pigment content of the cells (during exponential or early stationary phase).

Assays for total lipid content in microalgae have, most commonly, been based on the Folch or the Bligh and Dyer methods of solvent extraction followed by quantification either gravimetrically or by chromatography. Direct transesterification (DT) is a method of converting saponifiable lipids *in situ* directly to fatty acid methyl esters that can be quantified by gas chromatography. This eliminates the extraction step and results in a rapid, one-step procedure applicable to small sample volumes. The effectiveness of the Folch, the Bligh and Dyer and the Smedes and Askland methods of lipid extraction

were compared to DT for the quantification of total fatty acids in *C. vulgaris*, *Scenedesmus* sp. and *Nannochloropsis* sp. The use of two catalysts in sequence and the effect of reaction water content on the efficiency of DT were investigated, as well as the effects of drying and storage of algae samples. Up to five times higher levels of fatty acid in the cells were determined by DT in comparison with the three extraction-transesterification methods. A combination of acidic and basic transesterification catalysts was found to be more effective than each catalyst individually when the sample contained water. The two-catalyst reaction was insensitive to water up to 10% of total reaction volume. Oven drying decreased measured lipid content and samples could be stored for up to 7 days with little effect on measured fatty acid content.

Lipid productivity was identified as a key characteristic influencing the choice of microalgal species for biodiesel production. Data on growth rate, biomass productivity and lipid content was gathered for 55 microalgal species across a range of literature sources. Using these data, with two assumptions in the conversion of areal to volumetric biomass productivities and specific growth rates to biomass productivities respectively, the lipid productivity (Q_P) of all species under N replete conditions was calculated. These data highlighted the following species for high lipid productivity (110 to 164 $mg.L^{-1}.day^{-1}$): *Neochloris oleoabundans*, *Chlorella sorokiniana, Navicula pelliculosa*, *Amphora* and *Cylindrotheca*. This ranking of species should be interpreted with caution as the data was collected under a wide range of growth conditions and comparison was restricted to N replete conditions. Nutrient limitation has been shown to enhance lipid content, but due to a lack in reporting of growth parameters under nutrient stress, lipid productivity could not be derived.

Eleven of the species from the literature study were chosen for further investigation. Each species was grown in airlift photobioreactors under two different conditions: N replete and nitrogen limited (starting nitrate concentrations of 1500 $mg.L^{-1}$ and 150 $mg.L^{-1}$ respectively). Biomass concentration and lipid content, as well as residual nitrate concentration in the media, were measured daily. After 14 days, settling tests were carried out. Data on the fatty acid profile of each species was collated from the GC profiles generated during lipid quantification. *Chlorella vulgaris*, *Scenedesmus* sp., *Cylindrotheca fusiformis* and *Nannochloropsis* sp. were identified as promising candidates for biodiesel production. All N limited cultures except for *S. platensis* were found to have a consistently higher average lipid productivity and volumetric lipid content than nitrogen replete cultures throughout the period of N limitation. A large variation in settling rates and biomass recovery was observed. Gravity sedimentation appears promising for some species, particularly *C. fusiformis*, *Tetraselmis suecica* and *Scenedesmus* sp. Estimation of the fuel properties from the fatty acid profile indicated that biodiesel from most of the species tested would require blending or additives to conform to biodiesel specifications in terms of cetane number, proportion of polyunsaturated fatty acids, cold flow properties and oxidative stability.

In order to test the effect of different degrees of N limitation on lipid productivity and cell physiology, *C. vulgaris* was grown in airlift reactors with nitrate concentrations of 0, 40, 70, 100, 170, 420, 570, 1200 and 2000 $mg.L^{-1}$. Media nitrate concentration, biomass and lipid content were measured daily for 20 days, and carbohydrate, pigment and protein content, along with elemental composition, were

measured every third day. The optimal tradeoff between biomass and lipid production was found at a starting nitrate concentration of 170 $mg.L^{-1}$. Through extrapolation, the highest lipid productivity and volumetric lipid content were predicted to occur at starting nitrate concentrations of 240 and 305 $mg.L^{-1}$ respectively, under the conditions tested. A strong correlation was found between the N content of the cells and the pigment, protein and lipid contents, allowing calculation of the optimal starting nitrate concentration under different conditions. In addition to enhancing overall lipid content, N limited culture has been reported to improve the triacylglycerol content and fatty acid profile of microalgae, improving biodiesel quality. The reduced use of N fertilizers could lead to savings in cost and energy, and lower environmental burden.

As the desirable characteristics of high lipid content and high biomass productivity were found to occur under different conditions of nitrogen supply, two-stage culture was investigated as a means of controlling nitrogen availability and hence further improving productivity. *C. vulgaris* was grown under various cultivation regimes: two-stage batch, fed-batch, two-stage continuous-batch and multi-stage continuous operation. Biomass concentration and lipid content were measured throughout the growth cycle, and key culture parameters compared to those achieved under nitrogen replete and limited batch culture. Although all of the N limitation culture regimes tested had a higher volumetric lipid yield and lipid productivity than the N replete batch culture, none improved upon N limited batch culture at the optimal starting nitrate concentration tested (170 $mg.L^{-1}$). The lower than expected lipid productivity in two-stage culture was due to a delay in lipid accumulation, postulated to be due to utilisation of intracellular nitrogen reserves, and decreased rate of lipid accumulation, due to light limitation in dense cultures. Careful process design and optimisation of light supply are required to further enhance productivity.

The key contributions of this study are, firstly, the investigation and development of rigorous methodology for the measurement of biomass productivity and lipid content in microalgae. Rapid and accurate quantification of these parameters using small sample volumes allows the analysis of lipid productivity throughout the time course of a growth cycle. Secondly, key assessment criteria, including lipid productivity, volumetric lipid content, ease of harvesting and fatty acid profile were used in the selection of algal species for biodiesel production. The use of lipid productivity as a criterion over lipid content or growth rate alone was shown to be important. Thirdly, an investigation of the use of nitrogen limitation to optimise lipid productivity and maximise yield of lipid per unit N was carried out. Both the level and timing of N limitation were shown to be critical in optimising the tradeoff between biomass and lipid production, avoiding the lag in lipid accumulation caused by intracellular nitrogen stores and minimising the effects of light limitation. The best results were found under N limited batch conditions with a starting nitrate concentration of 170 $mg.L^{-1}$. Further improvements in lipid productivity in these systems require the optimal provision of light to the cells.

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List of abbreviations

Af	*Ankistrodesmus falcatus*
ATP	Adenosine triphosphate
AU	Absorbance units
BBM	Bold's Basal Medium
BCA	Bicinchoninic acid
BF_3	Boron trifluoride
C	Carbon
Calc.	Calculated
CD	Continuous diluted
Cf	*Cylindrotheca fusiformis*
ChlA	Chlorophyll *a*
ChlB	Chlorophyll *b*
CR	Continuous raceway
CU	Continuous undiluted
Cv	*Chlorella vulgaris*
CvH	*Chlorella vulgaris* high nitrogen/pigment culture
CvL	*Chlorella vulgaris* low nitrogen/pigment culture
D	Depth (m) or dilution rate (day^{-1})
dH_2O	Distilled water
DHA	Docosahexaenoic acid
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DT	Direct transesterification
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
F	Freshwater or flow rate ($L.day^{-1}$)
FAME	Fatty acid methyl ester
GC	Gas chromatography
H	Nitrogen replete (starting concentration 1500 $mg.L^{-1}$ nitrate)
HPLC	High performance liquid chromatography
Iso	*Isochrysis*
L	Nitrogen limited (starting concentration 150 $mg.L^{-1}$ nitrate)
Lit.	Literature
LOD	Limit of detection
LOQ	Limit of quantification

M	Marine
MCM	Marine and Coastal Management
ME	Methyl ester
N	Nitrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
Nan	*Nannochloropsis*
Nol	*Neochloris oleoabundans*
NO_X	Nitrous oxide
OD	Optical density
P	Lipid content (% DW)
Pav	*Pavlova*
Pt	*Phaeodactylum tricornutum*
P_{VOL}	Volumetric lipid content ($mg.L^{-1}$)
Q_A	Areal biomass productivity ($g.m^{-2}.day^{-1}$)
Q_P	Lipid productivity ($mg.L^{-1}.day^{-1}$)
$Q_{P\ AVE}$	Average lipid productivity ($mg.L^{-1}.day^{-1}$)
$Q_{P\ INST}$	Instantaneous lipid productivity ($mg.L^{-1}.day^{-1}$)
Q_V	Volumetric biomass productivity ($g.L^{-1}.day^{-1}$)
$Q_{X\ AVE}$	Average biomass productivity ($g.L^{-1}.day^{-1}$)
$Q_{X\ INST}$	Instantaneous biomass productivity ($g.L^{-1}.day^{-1}$)
RAU	Relative absorbance units
S	Saline
Sc	*Scenedesmus*
SM	Sodium methoxide
SO_X	Sulphur oxide
Spir	*Spirulina platensis*
TAG	Triacylglycerol/triglyceride
TCA	Tricarboxylic acid
T_d	Doubling time (days)
τ	Residence time (days)
Ts	*Tetraselmis suecica*
UTEX	The culture collection of algae at the University of Texas at Austin
V	Volume (L)
WITS	University of the Witwatersrand
X	Biomass concentration ($g.L^{-1}$)
μ	Specific growth rate (day^{-1})

Outputs arising from this work

Published journal papers

Griffiths MJ and Harrison STL. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*. 21: 493-507

Griffiths MJ, van Hille RP and Harrison STL. 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. *Lipids*. 45: 1053-1060

Griffiths MJ, Garcin C, van Hille RP and Harrison STL. 2011. Interference by pigment in the estimation of microalgal biomass concentration by optical density. *Journal of Microbiological Methods*. 85: 119-123

Griffiths MJ, van Hille RP and Harrison STL. 2011. Lipid productivity, settling potential and fatty acid profile of eleven microalgal species grown under nitrogen replete and limited conditions. *Journal of Applied Phycology*. DOI: 10.1007/s10811-011-9723-y

Journal papers in progress

Griffiths MJ, van Hille RP and Harrison STL. 2011. Effect of nitrogen limitation on lipid productivity and cell composition in *Chlorella vulgaris*. Draft completed.

Griffiths MJ, van Hille RP and Harrison STL. 2011. Effect of culture regime on lipid productivity in *Chlorella vulgaris*. Draft in progress.

Book chapters

Harrison STL, Griffiths MJ, Langley N, Vengadajellum C and van Hille RP. 2009. Micro-algal culture as a feedstock for bio-energy, chemicals and nutrition. In *Manual of Industrial Microbiology and Biotechnology 3rd Edition* (Eds: RH Baltz, AL Demain and JE Davies), ASM Press, pp 577-590. ISBN: 978-1-55581-512-7

Griffiths MJ, Dicks R, Richardson C and Harrison STL. 2011. Advantages and challenges of microalgae as a source of oil for biodiesel. In *Biodiesel – Feedstocks and Processing Technologies* (Eds. Stoytcheva M and Montero G), Intech Open Access Publisher, pp 177-200. ISBN: 979-953-307-020-8

1 Introduction

1.1 Sustainable liquid fuels

Finding renewable, carbon neutral and economically viable sources of energy is one of the most pressing challenges facing mankind today (Mata *et al.* 2010). Climate change mitigation demands an immediate reduction in fossil fuel consumption. Fossil fuel use contributed 57% of global anthropogenic greenhouse gas emissions in 2004, with the transportation sector contributing just over 13% (IPCC 2007). Many applications of fossil energy could be substituted by alternative sources of heat or electricity, such as solar, hydroelectric, tidal, geothermal and wind. However, the internal combustion engine, and hence the majority of the world's transport system, runs on liquid fuels, which are more difficult to replace (Shay 1993). Ultimately, more efficient forms of transport utilising electricity or hydrogen gas need to be developed, however, in the short-term, sustainable liquid fuels are required urgently.

Liquid biofuels include bioethanol and biodiesel, as replacements for petrol and diesel respectively. Bioethanol is made by fermenting plant-derived sugars into ethanol. Common sources of sugar include sugarcane, sugar beet and starch from corn. Second generation technologies are under development to allow the use of more complex carbohydrates such as cellulose and thus expand the range of feedstocks to almost any plant material, including trees, grasses and agricultural waste (Antizar-Ladislao & Turrion-Gomez 2008).

Biodiesel is a mixture of fatty acid alkyl esters produced by transesterification of oils or fats. Although oil can be used unaltered, as a fuel, conversion to biodiesel decreases the viscosity and enhances other fuel properties (Bamgboye & Hansen 2008). Transesterification occurs on reaction of the fatty acids with an alcohol (usually methanol), in the presence of a catalyst and heat to form fatty acid methyl esters (FAME, the components of biodiesel) and the by-product glycerol (Figure 1.1). Biodiesel is biodegradable, non-toxic and can be used in most standard diesel engines without modification. It may be blended in any mixture with conventional diesel fuel. Biodiesel combustion results in significantly lower tailpipe emission of CO, SO_x, hydrocarbons and particulate matter than regular diesel, although emissions of NO_x can be higher (Ma & Hanna 1999; Meher *et al.* 2006; Shay 1993; Tong *et al.* 2010).

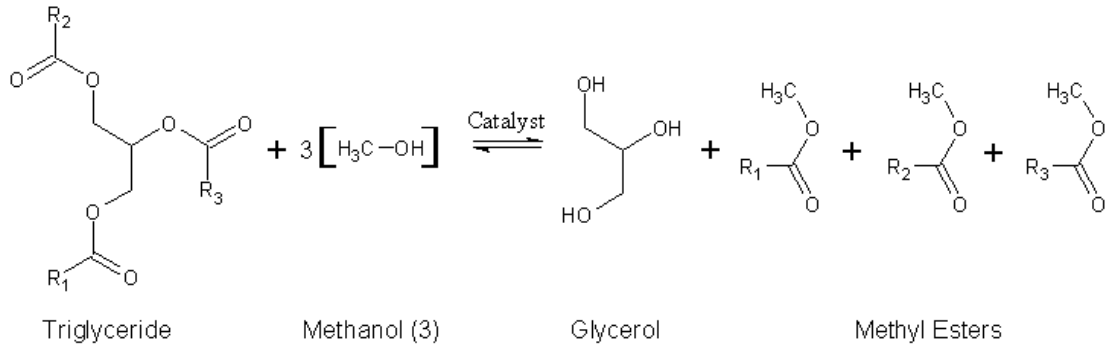


Figure 1.1 The transesterification reaction that converts triacylglycerides (TAG) to alkyl esters (biodiesel) by the addition of an alcohol such as methanol in the presence of a catalyst. Glycerol is a by-product

(Wikimedia Commons: http://en.wikipedia.org/wiki/File:Biodiesel_Reaction2.gif)

First generation biodiesel is primarily produced from oil seeds such as soybean, sunflower, rapeseed or oil palm (Ma & Hanna 1999; Miao & Wu 2006; Shay 1993). Several concerns have been raised about the large-scale production of biodiesel from these sources. The increased demand for oilseed crops will affect their price in the food market and place additional strain on the agricultural system, competing directly with food crops for land and water (Scott *et al.* 2010). The additional demand for arable land has led to the destruction of natural habitats (Ma & Hanna 1999) and release of additional CO_2 due to land tillage. Increased use of pesticides, herbicides and fertilizers could lead to toxicity and eutrophication, while increasing environmental burden. In addition, several life cycle assessment studies have questioned the net energy balance of crop-based biofuels (Stephenson *et al.* 2008; Von Blottnitz & Curran 2007). For a biofuel to be sustainable, the energy contained in the product must be greater than that demanded by the facilities, feedstocks and operations used in the production process.

Despite these issues, and although currently more expensive than fossil fuels, biofuel production and usage is being encouraged around the world. For example, the European Union has set the target of meeting 10% of road transport fuel needs from biofuel by 2020 (IEA 2007). In addition to reducing greenhouse gas emissions, biofuel production is expected to promote employment in rural areas, diversify income and fuel supply sources, and increase the security of energy supply (Mata *et al.* 2010). The potential market for biodiesel far surpasses the availability of feedstocks not designated for other uses. While oilseed crops already provide a small proportion of the liquid fuels used in several countries, second generation biodiesel from non-food sources such as microbes, and particularly microalgae, is currently receiving much attention (Chisti 2007).

1.2 Microalgae

The term 'algae' is used to describe a great variety of prokaryotic (strictly termed Cyanobacteria) and eukaryotic organisms with a range of morphologies and phylogenies. They represent a wide array of species, inhabiting environments from deserts to the Arctic Ocean, including both salt and fresh water. They vary in colour, shape and size, from picoplankton (0.2 to 2 μm) to giant kelp fronds up to 60 m in length (Barsanti & Gualtieri 2006). Microalgae are generally small (less that 2 mm in diameter) aquatic organisms, occurring unicellularly or in colonies. Some of the common taxonomic groups are described in Table 1.1. Macroalgae are larger (can be seen without the aid of a microscope), multicellular and often show some form of cellular specialisation.

Algae have been investigated as a source of energy in many different contexts, from direct combustion to the production of hydrogen gas (Benemann 2000; Miao *et al.* 2004) (Figure 1.2). Anaerobic digestion can be applied for the generation of methane (biogas) (Golueke *et al.* 1957). Liquid fuels can be produced through pyrolysis or liquefaction, as well as transesterification of the lipid fraction (Miao & Wu 2006). Bioethanol can be produced through fermentation of the carbohydrates in the whole algae, or the residue following oil extraction (John *et al.* 2011). Biodiesel is particularly promising as it has the potential to integrate directly with current petrodiesel infrastructure (Shay 1993).

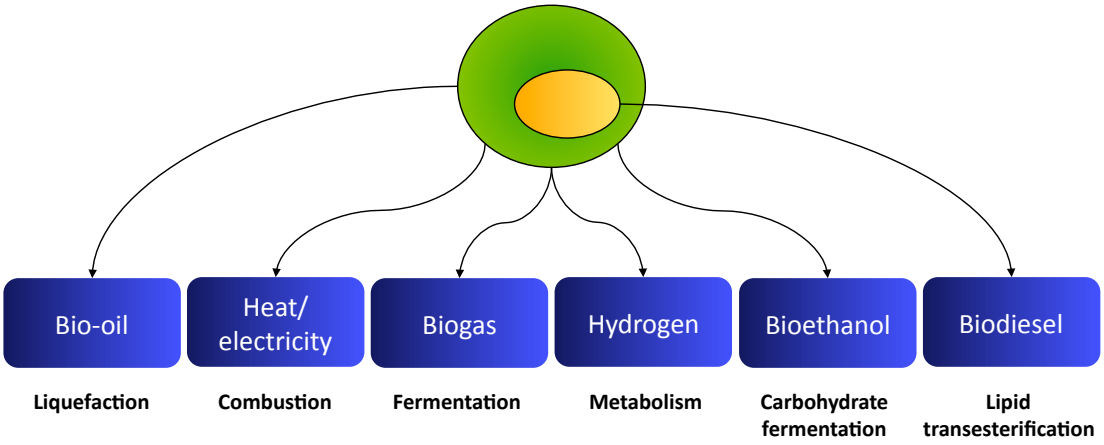


Figure 1.2 Some potential applications of microalgae as a source of energy

Research into algae for the mass-production of oil has focused on the microalgae, due to their high lipid, rather than macroalgae. Most algal species considered for biodiesel production are either green algae (chlorophyta) or diatoms (bacillariophyta) (Sheehan *et al.* 1998). They are generally photosynthetic, but several species are able to grow heterotrophically or mixotrophically (Barsanti & Gualtieri 2006).

Table 1.1 Characteristics of some microalgal groups with biotechnological applications (Apt & Behrens 1999; Pulz & Gross 2004; Spolaore *et al.* 2006)

Group	Description	Morphology	Well-known genera	Applications
Chlorophyta (Green algae)	Diverse group. Origin of higher plants. Usually photoautotrophic, but can be heterotrophic.	Large range of cellular structure – unicellular flagellates to complex multicellular arrangements. Cell walls contain cellulose.	*Chlamydomonas*, *Chlorella*, *Scenedesmus*, *Spirogyra*, *Volvox*	*Chlorella* commercially grown as nutraceutical and aquaculture feed. *Haematococcus pluvialis* used to produce astaxanthin. *Dunaliella salina* grown for β-carotene.
Cyanobacteria (Blue-green algae)	Non-motile, Gram-negative, prokaryotic eubacteria. Most widely distributed algal group. Dominate particularly in oceans. Important component of picoplankton.	Can be unicellular, filamentous or colonial.	*Spirulina, Anabaena, Oscillatoria*	*Spirulina* biomass sold commercially to health food and nutraceutical market.
Dinophyta (Dinoflagellates)	Important components of microplankton. Nutritionally diverse. Half the known species are obligate heterotrophs	Typically unicellular flagellates. Have armour-like cell-covering beneath cell membrane	*Gymnodinium*, *Crypthecodinium*	Known for blooms and toxin production, many exhibit bioluminescence. Responsible for red tides. Some species produce DHA.
Euglenophyta (Euglenas)	Occur in fresh, brackish and marine environments, mostly in soils and mud, especially in highly heterotrophic environments. Obligate mixotrophic as require B vitamins. Colourless species are phagotrophic.	Unicellular or colonial flagellates.	*Euglena gracilis*	Unique cellular and biochemical features, may have pharmaceutical applications
Haptophyta (Also known as Prymnesiophyta)	Generally marine. Mostly photosynthetic, but can be heterotrophic or phagotrophic.	Largely motile unicells. The best-known haptophytes are coccolithophores, which have an exoskeleton of calcareous plates called coccoliths.	*Pavlova, Isochrysis, Prymnesium*	Several species grown as food for fish and other aquaculture organisms such as bivalves and abalone
Heterokontophyta (Includes brown algae, golden algae and diatoms)	Large group containing the Chyrophyta, Xanthophyta, Eustigmatophyta and Bacillariophyta (diatoms). Largely marine, but some freshwater varieties.	Cells with two different flagella (as opposed to isokont – two the same). Bacillariophyceae are unicellular, brown cells with a silica cell wall	*Amphora, Nitzschia, Thalassiosira, Phaeodactylum, Nannochloropsis*	Several species grown as aquaculture feed, others known for EPA production
Rhodophyta (Red algae)	One of oldest and largest groups of algae. The accessory pigments phycobiliproteins give them their red colour	Free-living, unicellular	*Porphyridium*	Most economically important macroalgae are from this family, e.g. dulse (*Palmaria palmata*), nori (*Porphyra*) and species used to make agar, carrageenans and other food additives.

1.3 Microalgal culture

1.3.1 Reactor systems

Annual global microalgal production is currently estimated at about 10 000 metric tons, with the main algae cultivated being *Spirulina* (accounting for roughly half of the worldwide algal production), *Chlorella*, *Dunaliella* and *Haematococcus* (Benemann 2009). Microalgae have been cultivated for a variety of uses ranging from food and feed, pharmaceuticals and pigments, to high value chemical products (Apt & Behrens 1999; Pulz & Gross 2004; Spolaore *et al.* 2006). Technologically, microalgal production lies between agriculture, which requires large areas for sunlight capture, and fermentation, which involves liquid culture of microorganisms (Becker 1994). As light does not penetrate more than a few centimetres through a dense algal culture, scale-up is based on surface area rather than volume (as in heterotrophic fermentations) (Scott *et al.* 2010). There are two main types of cultivation system employed in algal culture: open and closed.

Open systems consist of natural water bodies such as lakes, ponds and lagoons, or artificial ponds or containers that are open to the air. Most commercial production to date has taken place in open ponds and raceways as these systems are easy and cheap to construct (Pulz 2001). The most common type of open system used is the raceway: an oblong looped pond mixed by a paddlewheel, with water depths of 15 to 20 cm (Becker 1994). Biomass concentrations of between 0.1 and 1 $g.L^{-1}$ and biomass productivities of between 0.05 and 0.1 $g.L^{-1}.day^{-1}$ are possible (Chisti 2007; Pulz 2001). The main advantages of open systems are their low cost, ease of construction and operation, low operating costs and energy requirements.

Disadvantages of open systems include contamination with unwanted species such as foreign algae, yeast, bacteria and predators, evaporation of water, diffusion of CO_2 to the atmosphere and poor control over environmental conditions, particularly temperature and solar irradiation (Becker 1994; Pulz 2001). In addition, the relatively low cell densities achieved can lead to higher cost of cell recovery (Chen 1996). Only a few microalgal species have been successfully cultivated in open ponds at a large scale. These tend to be either fast-growers, which naturally outcompete contaminating algae (e.g. *Chlorella* and *Scenedesmus*), or species that grow in a specialised environment such as high salt (e.g. *Dunaliella salina*) or high pH (*Spirulina platensis*), which limits growth of competitors and predators (Chen 1996). Due to the lack of control over cultivation conditions, resulting in low productivity, and the fact that many desirable species cannot be effectively maintained in open systems, attempts have been made to overcome some of these limitations through the use of enclosed reactor systems.

Closed systems, or photobioreactors, generally consist of clear plastic bags, tubes or vessels of various sizes, lengths and orientations (Pulz 2001). Commonly used designs include vertical flat plate reactors and tubular reactors, with the culture mixed either by mechanical pumping or by airlift (Scott *et al.* 2010). Closed reactors offer a much higher degree of control over process parameters, leading to improved heat and mass transfer, and thus higher yields. The risk of contamination and evaporation are reduced, CO_2 supply and release can be contained, production conditions can be reproduced and temperature can be controlled.

Productivity in closed systems can be much higher than open systems, with biomass concentrations of up to 8 $g.L^{-1}$ and productivities of between 0.8 and 1.3 $g.L^{-1}.day^{-1}$ (Pulz 2001). However, they are generally much more costly to build and more energy demanding to operate relative to open systems (Table 1.2). Closed systems can suffer fouling and oxygen build-up. Large systems can be difficult to clean and sterilize and long sections of enclosed tubing may require oxygen purging. High oxygen concentrations cause the key enzyme Rubisco to bind oxygen instead of carbon dioxide, leading to photorespiration instead of photosynthesis (Dennis *et al.* 1998). Although closed bioreactors offer a much higher degree of control over process parameters and can have higher yields, it is uncertain whether the increased productivity can offset the higher cost and energy requirements. For a commodity product such as vegetable oil for biodiesel, low cost, high volume production is demanded, while quality is less critical (Pulz 2001). In this case, the more favourable economics and energy requirements of open ponds may well outweigh the advantages of closed reactors.

Table 1.2 Comparison of open ponds and closed photobioreactors. Adapted from Pulz (2001).

Parameter	Open	Closed
Control over process	Low	High
Contamination risk	High	Low
Water loss due to evaporation	High	Low
CO_2 loss	High	Low
O_2 build-up	Low	High
Area required	High	Low
Productivity	Low	High
Consistency and reproducibility	Low	High
Weather dependence	High	Low
Cost	Low	High
Energy required	Low	High

1.3.2 Cultivation parameters

The genetic characteristics of an algal species determine the range of culture conditions within which they can grow and the type of compounds they produce. Within these ranges, there is large variation in the productivity achieved under different culture conditions. While optimal microalgal culturing systems vary greatly with application, the following characteristics should be considered for culture system design and operation: temperature, light provision, provision of CO_2 and removal of O_2, provision of nutrients such as nitrate, phosphate and trace metals, pH control, salinity and mixing (Figure 1.3) (Becker 1994; Grobbelaar 2000; Mata *et al.* 2010). Their optimal and tolerated ranges tend to be species specific.

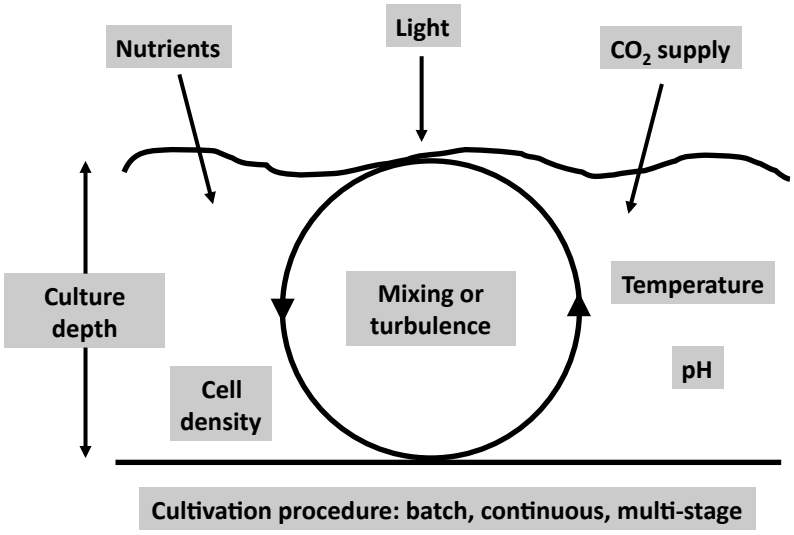


Figure 1.3 Major parameters that can be manipulated in microalgal culture (adapted from (Grobbelaar 2000))

Light and temperature are among the most difficult parameters to optimise in large-scale outdoor culture. Daily and annual fluctuations in temperature can lead to significant decreases in productivity. Optimal growth temperatures are generally between 20 and 30°C (Chisti 2008). Many algal species can tolerate temperatures of up to 15°C lower than their optimum, with reduced growth rates, but a temperature of only a few degrees higher than optimal can lead to cell death (Mata *et al.* 2010). Closed systems in particular often suffer from overheating during hot days, when temperatures inside the reactor can reach in excess of 50°C. Evaporative water cooling systems have been employed to counteract this (Mata *et al.* 2010). Low seasonal and evening temperatures can also lead to significant losses in productivity.

The efficient production of algal biomass relies on the optimal provision of light energy to all cells within the culture. Eight photons of photosynthetically active radiation are required to fix one molecule of CO_2 in photosynthesis. Most algal growth systems become light limited at high cell

densities, as light only penetrates a few centimetres into a dense algal culture due to absorption and shading by the cells (Richmond 2004). The average provision of light is linked to reactor depth or diameter, as well as culture density. Photosynthetic efficiency is highest at low light intensities. At high light levels, there is less efficient use of absorbed light energy, and damage to photosynthetic machinery can occur in a process known as photoinhibition (Scott *et al.* 2010).

In a dense unmixed culture exposed to direct sunlight, cells at the surface are likely to be photoinhibited, while those at the centre of the reactor are in the dark. Mixing is therefore important not only in preventing cell settling and improving mass transfer, but also exposing cells from within a dense culture to light at the surface. The frequency of light-dark cycling has been reported to affect algal productivity (Grobbelaar 1994; Grobbelaar 2000). Algae can be exposed to a higher light intensity when the light is supplied in short bursts. They are less likely to become photoinhibited because the photosystems have time to recover during the dark period of the flashes (Nedbal *et al.* 1996). The movement of cells between the light and dark sections of the reactor volume is governed by the hydrodynamics of the system. High rates of mixing facilitate transfer of gases, homogenise the distribution of heat and nutrients and ensure rapid circulation of cells between light and dark zones in the reactor, but the high liquid velocities generated can damage algal cells due to shear stress (Mata *et al.* 2010). High rates of mechanical mixing or gas sparging , while beneficial to mass transfer, also have large energy requirements, jeopardising the process energy balance and increasing cost (Richardson 2011).

In order to maintain a high photosynthetic rate, the influx of carbon and energy must be non-limiting. In photoautotrophic growth, energy is provided by light and carbon in the form of CO_2. In order to be taken up by cells, the CO_2 must dissolve in the water. The rate of dissolution is determined by the CO_2 concentration gradient as well as by the temperature, rate of gas sparging and surface area of contact between the liquid and gas (a function of agitation and bubble size). Reactor geometry, methods of gas introduction and reactor mixing can all influence the rate of CO_2 delivery (Bailey & Ollis 1977). Certain strains of microalgae can tolerate up to 12% CO_2. The 0.03% CO_2 content of ambient air is suboptimal for photosynthesis (Pulz 2001), hence for optimal microalgal growth, additional CO_2 must be provided. This is usually done by direct injection of a CO_2 enriched air stream.

The major nutrient requirements for microalgal growth are nitrogen and phosphorous, with certain diatoms, silicoflagellates and chrysophytes also requiring silicon (Anderson 2005). Requirements of nutrients, pH and osmolarity are species dependent. Deviation from optimal levels may cause a decrease in biomass productivity, but can have other advantages, for example, a high salinity may decrease contamination. Sufficient supply of all essential nutrients is a prerequisite for efficient photosynthesis and growth, but limitation of key nutrients (e.g. nitrate, phosphate or silica) may cause accumulation of desired products such as lipid.

Most microalgae are photoautotrophs (utilizing sunlight as their source of energy and CO_2 as a carbon source). This is the most common growth mode employed in algal cultivation (Chen 1996). However, several species (e.g. *Chlorella*, *Chlamydomonas, Phaeodactylum, Nitzschia, Tetraselmis* and *Crypthecodinium*) are also capable of heterotrophic growth (utilizing organic carbon such as glucose, acetate or glycerol as the sole source or carbon and energy) or mixotrophic growth (photoautotrophic growth supplemented by an organic carbon source).

The advantages of using an organic carbon substrate are that it decreases dependence on light provision, allowing growth in conventional fermenters in the dark. Higher cell concentrations and hence increased volumetric productivities can be reached (Chen 1996). Higher productivities of both biomass and lipid have been reported under heterotrophic growth compared to autotrophic (Ceron Garcia *et al.* 2000; Miao & Wu 2006). Disadvantages of feeding an organic carbon source include the fact that there are a limited number of algal species that can utilize organic carbon sources, the risk of bacterial contamination is greatly increased and the carbon substrate adds an additional cost, along with the environmental burden of its production. The use of a substrate such as glucose, commonly sourced from crop plants, adds a trophic level to the process, thereby removing the simplicity of the concept of microalgae as cellular factories producing liquid fuel from sunlight and CO_2.

The optimal cultivation strategy (e.g. batch, fed-batch or continuous cultivation mode) is determined by the kinetics of growth, product accumulation and substrate uptake (Shuler & Kargi 2005). For production of a primary product such as protein or biomass for food or feed, optimisation of biomass productivity is the main objective. In this case, batch or continuous systems are generally used. For production of a secondary product such as carotenoids or storage lipids, the use of two or more production stages to enhance yield has been proposed (Ben-Amotz 1995; Huntley & Redalje 2006; Richmond 2004). The first stage is designed to optimize growth, while the second stage provides conditions that retard growth and encourage product synthesis, usually by applying some form of stress, e.g. high light or salinity in the case of carotenoid production, or nutrient limitation in the case of lipid accumulation. Another potential two-stage strategy that could enhance lipid productivity is an initial photosynthetic stage, followed by a second heterotrophic phase, where feeding with an organic carbon source such as glucose may boost lipid content.

1.3.3 Harvesting and processing

The economic recovery of microalgal biomass remains a major challenge to the process of algal biodiesel production. Microalgae for biofuel are a low value product suspended in large volumes of water. Harvesting contributes 20 to 40% of the total cost of biomass production (Gudin & Therpenier 1986; Molina Grima *et al.* 2003). The difficulty in separation can be attributed to the small size of the cells (3 to 300 μm, (Henderson *et al.* 2008)), their neutral buoyancy and the fact

that photoautotrophic microalgal cultures are relatively dilute, achieving concentrations in the order of 1 to 8 $g.L^{-1}$ (Pulz 2001).

Harvesting requires one or more solid-liquid separation technique (Molina Grima *et al.* 2003). In order to achieve the levels of concentration required, various chemical, biological and physical separation steps may be necessary. Common methods of cell harvesting include flocculation, filtration, sedimentation, centrifugation and flotation (Mata *et al.* 2010). Several natural properties of microalgal cells affect the choice and efficiency of harvesting methods. Factors relevant to separation include density, surface charge, size, shape, hydrophobicity, salinity of the medium, adhesion and cohesion properties and settling or floating velocities (Jarvis *et al.* 2009; Petrusevski *et al.* 1995). Each algal species presents unique challenges due to the array of sizes, shapes, densities and cell surface properties encountered. A low-cost, energy efficient method with a high recovery efficiency and concentration is required, minimizing cell damage and allowing for water and nutrient recycle (Figure 1.4).

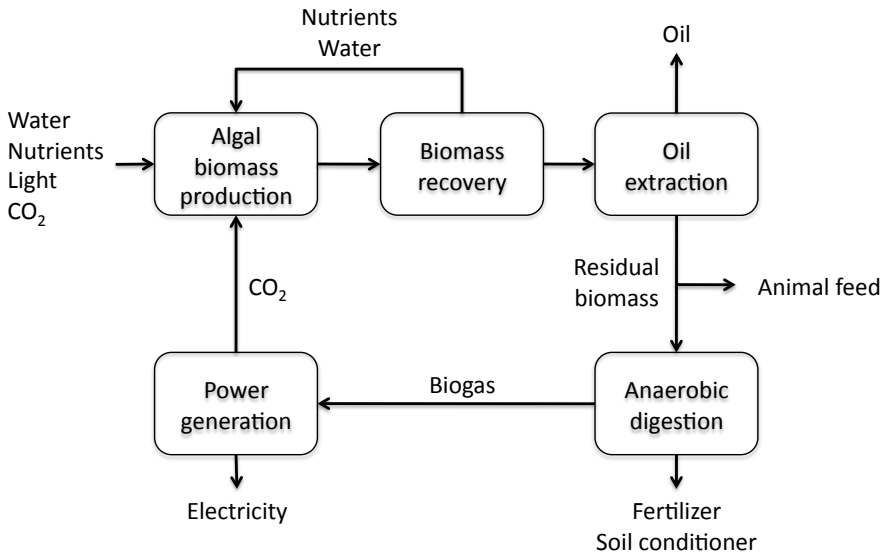


Figure 1.4 Conceptual overview of microalgae process options (adapted from Chisti, 2008)

After harvesting, the major challenge is in releasing the lipids from their intracellular location in the most energy efficient and economical way possible. Algal lipids must be separated from the rest of the biomass (carbohydrates, proteins, nucleic acids, pigments) and water. The residual biomass can be used for animal feed, fertilizer, anaerobic digestion or ethanol production (Chisti 2008; John *et al.* 2011; Mata *et al.* 2010).

Lipid extraction can be done in a number of ways. Solvent extraction techniques are popular, but the cost and toxicity of the solvent (e.g. hexane) is of concern and solvent recovery requires significant energy input. Other methods involve disruption of the cell wall, usually by enzymatic, chemical or physical means (e.g. homogenization, bead milling, sonication (Mata *et al.* 2010)),

allowing the released oil to float to the top of the solution. Ultrasound and microwave assisted extraction methods have been investigated (Cravotto *et al.* 2008). Supercritical CO_2 extraction is an efficient process, but is too expensive and energy intensive for anything but lab-scale production. Direct transesterification (production of biodiesel directly from algal biomass) is also possible. Drying of the algal biomass prior to extraction is often required.

Once the algal oil is extracted, it can be treated as conventional vegetable oil in biodiesel production. Biodiesel is most commonly produced by transesterification (Figure 1.1). One of the concerns for any biodiesel feedstock is the quality of the biodiesel produced. Biodiesel must meet certain international regulations (for example, the ASTM international standards or the EN 14214 in the EU) in terms of parameters such as viscosity, ignition properties, performance at low temperatures and susceptibility to oxidative degradation (Stansell *et al.* 2011).

1.4 Microalgal lipids

The main components of algae cells are proteins, carbohydrates and lipids (Becker 1994). Microalgae naturally produce lipids as part of the structure of the cell (e.g. in cell membranes and as signalling molecules), and as a dense form of carbon and energy storage, similar to fat stores in animals and humans (Tsukahara & Sawayama 2005). The term lipid encompasses a variety of compounds with different chemical structures (e.g. esters, waxes, cholesterol) (Palmquist & Jenkins 2003), but generally they are composed of a glycerol molecule bound to three fatty acids (triacylglycerol, TAG), or to two fatty acids with the third position taken up by a phosphate (phospholipids) or carbohydrate (glycolipids) group (Figure 1.5). Fatty acids are carboxylic acids with a long, unbranched hydrocarbon tail which can be saturated (no double bonds) or unsaturated (containing one or more double bonds). They are classified according to the number of carbon atoms in the chain and the number of double bonds. Microalgae contain fatty acids ranging from C8 to C28 (usually with an even number of carbon atoms), often with C16 and C18 unsaturates. Certain species contain significant amounts of polyunsaturated fatty acids (Stansell *et al.* 2011).

Storage lipids, generally in the form of triacylglycerols (TAG), accumulate in lipid vesicles called oil bodies in the cytoplasm. Most fast-growing species have a relatively low lipid content during normal growth, with these lipids mainly consisting of phospho- or glycolipids associated with cell membranes. Under certain conditions, generally triggered by stress or the cessation of growth, lipid content can increase to 60 to 80% of cell dry weight (DW), mostly composed of TAG (Shifrin & Chisholm 1981; Piorreck *et al.* 1984; Spoehr & Milner 1949; De la Peña 2007; Becker 1994).

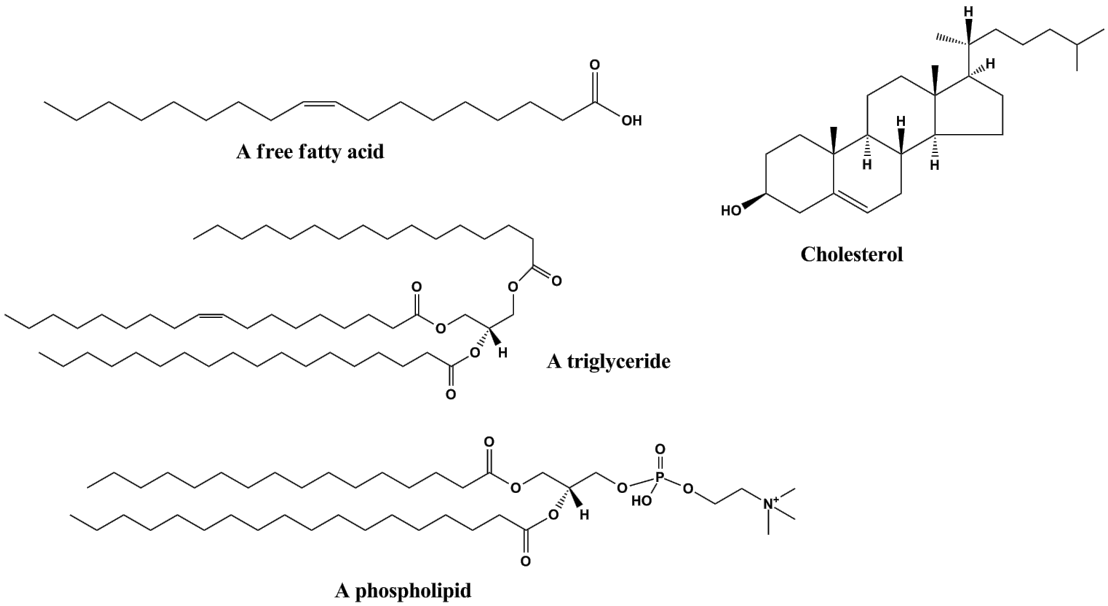


Figure 1.5 Some common lipids. Top: oleic acid and cholesterol. Middle: a triglyceride composed of palmitic (C16), oleic (C18:1) and stearic (C18) acid attached to a glycerol backbone. Bottom: a common phospholipid, phosphatidylcholine

(Wikimedia commons: http://en.wikipedia.org/wiki/File:Common_lipids_lmaps.png)

TAGs are the most suitable class of lipids for biodiesel production. They consist of a glycerol molecule attached by ester bonds to three fatty acids, while phospho- and glycolipids have only two fatty acids. Phospholipids are particularly undesirable for biodiesel production, as they increase consumption of catalyst and act as emulsifiers, impeding phase separation during transesterification (Mittelbach & Remschmidt 2004; Van Gerpen 2005). Phospholipids, and sulphur-containing glycolipids, also increase the phosphorous and sulphur content of the fuel respectively, which must both be below 10 $mg.L^{-1}$ to meet the European biodiesel standard EN 14214. In addition to the lipid profile, the type of fatty acids found in the oil can have a profound effect on the biodiesel quality. The fatty acid chain length and degree of saturation (determined by the number of double bonds) affect properties such as the viscosity, cold flow plug point, iodine number and cetane number of the fuel (Ramos *et al.* 2009). For biodiesel production, it is therefore important to maximize not only total lipid production, but also TAG content and appropriate fatty acid profile.

Lipid synthesis relies on carbon compounds generated from CO_2 by photosynthesis, as well as energy and reducing power (in the form of ATP and NAD(P)H respectively). The latter are produced during the light reactions of photosynthesis, while CO_2 uptake is mediated by the Calvin cycle during the dark reactions of photosynthesis (Figure 1.6). The output of the Calvin cycle is a three-carbon compound (glyceraldehyde 3-phosphate), which is converted through glycolysis into acetyl CoA. The conversion of acetyl CoA to malonyl CoA is the first committed step in lipid biosynthesis (Livne & Sukenik 1992). Throughout metabolism there are a number of

branch points at which metabolic intermediates are partitioned between the synthesis of lipids and other products such as carbohydrates and proteins (Lv *et al.* 2010). For example, acetyl CoA is a substrate for lipid synthesis as well as a feed molecule into the TCA cycle, which generates energy and biosynthetic precursors for proteins and nucleic acids. Both external and internal constraints, such as the availability of nutrients and the enzymatic reaction rates, limit the supply of metabolic intermediates. The production of storage lipids is particularly energy and resource intensive (Dennis *et al.* 1998; Roessler 1990) and therefore usually occurs under conditions of reduced growth.

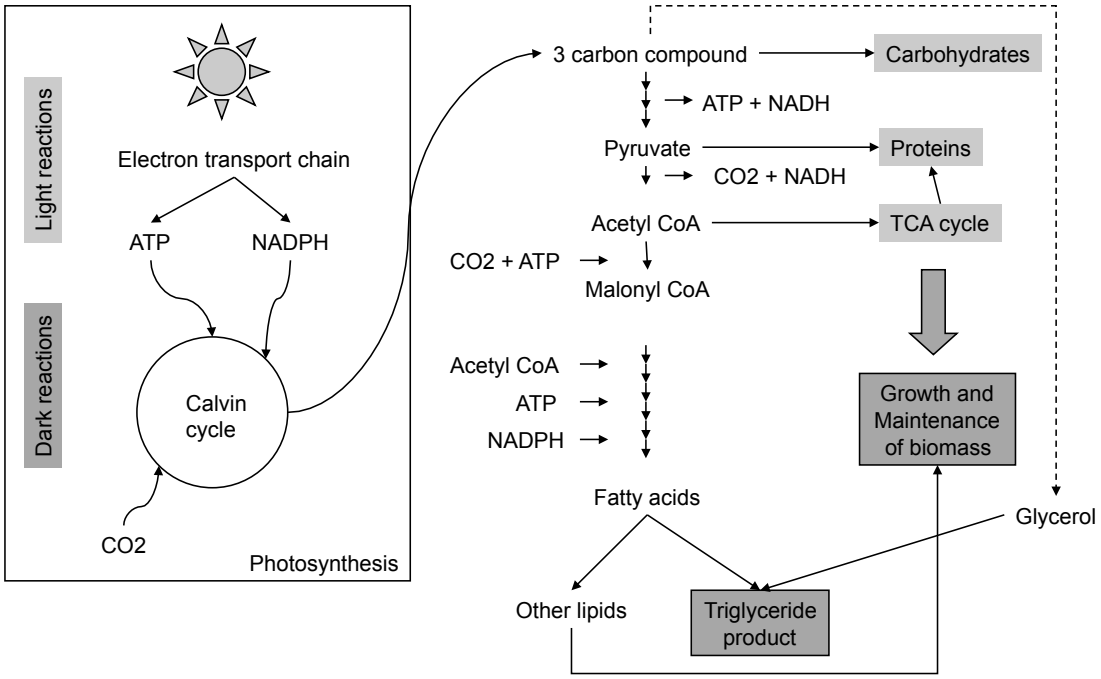


Figure 1.6 Simplified schematic diagram of plant lipid biosynthesis

1.5 Research into algal biofuel

The idea of using microalgae as a source of transportation fuel is not new. Research in this field has been conducted since the 1950s (Oswald & Golueke 1960). In the 1970s, several large, publicly funded research programs were set up in the USA, Australia and Japan (Regan & Gartside 1983; Usui & Ikenouchi 1997; Sheehan *et al.* 1998). The US Department of Energy invested more than US$ 25 million between 1978 and 1996 in the Aquatic Species Program to develop biodiesel production from algae (Sheehan *et al.* 1998). The main focus of the program was the production of biodiesel from high lipid-content algae grown in open ponds utilizing waste CO_2 from coal fired power plants.

Over 3000 species were collected and many of them screened for lipid content. Early in the program, it was observed that environmental stress, particularly nutrient limitation (nitrogen for

green algae and silicon for diatoms) led to an accumulation of lipids. Promising species were investigated to determine the mechanism of this 'lipid trigger'. Researchers in the program were the first to isolate the enzyme Acetyl CoA Carboxylase from a diatom. This enzyme catalyses the first committed step in the lipid synthesis pathway. Acetyl CoA Carboxylase was overexpressed successfully in algae, however, the anticipated increase in oil production was not demonstrated (Sheehan *et al.* 1998).

The program close out report (Sheehan *et al.* 1998) concluded that, although algae used significantly less land and water than traditional crops, and sufficient resources did exist for algal fuel to completely replace conventional diesel, the high cost of microalgae production remained an obstacle. Even with the most optimistic lipid yields, production would only have become cost effective if petro-diesel had risen to twice its 1998 price. In the last few years, an increase in oil prices, additional pressure to find alternatives to dwindling oil supplies and an urgent need to cut carbon emissions has led to a renewed interest in algae as a source of energy, particularly lipid producing algae as a source of biodiesel.

1.6 Algal biodiesel

The advantages of microalgae over land crops as a source of oil are numerous. Microalgae have higher growth rates than land-based plants. Due to their simple cellular structure and existence in an aqueous environment, the entire cell surface is available for light capture and mass transfer, leading to high rates of substrate uptake and photosynthetic efficiency (Miao & Wu 2006; Sheehan *et al.* 1998). In contrast to land-based oil crops, where only the seeds are harvested, each algal cell contains lipid and hence the yield of product from biomass is much higher (Becker 1994). Due to these differences, the oil yield per area of microalgal cultures potentially exceeds that of the best oilseed crops (Table 1.3).

Table 1.3 Average productivities of common oil crops compared to the theoretical productivity of microalgae

Oil source	Yield ($L.m^{-2}.yr^{-1}$)	Reference
Algae	4.7 to 14	Sheehan *et al.* 1998
Palm	0.54	Mata *et al.* 2010
Jatropha	0.19	Sazdanoff 2006
Rapeseed	0.12	Sazdanoff 2006
Sunflower	0.09	Sazdanoff 2006
Soya	0.04	Sazdanoff 2006

Other advantages of algae over oilseed crops include (Lardon *et al.* 2009; Mata *et al.* 2010; Rodolfi *et al.* 2009; Tsukahara & Sawayama 2005):

1) Algae potentially have very high photosynthetic yields (3 to 8% compared to 0.5% for land plants)
2) The only major inputs required are water, light, CO_2 and a source of nutrients such as nitrogen and phosphorous, all of which could potentially be obtained from free or waste sources
3) Cultivation can be on non-arable land, and use brackish, salt or waste water
4) Algae are not a food source, and do not compete directly for resources with conventional agriculture
5) Algal culture does not require herbicides or pesticides
6) Valuable co-products such as pigments, antioxidants and dietary supplements can be produced
7) The residual algal biomass after oil extraction is rich in protein and other nutrients and potentially valuable as feed or fertilizer
8) Cultivation can be coupled with the uptake of CO_2 from industrial waste streams and the removal of excess nutrients from waste water

The technical feasibility of algal biodiesel has been demonstrated, and fuel produced from *Chlorella* met most of the ASTM biodiesel standards (Miao & Wu 2006; Xiong *et al.* 2008). However, despite strong interest from the commercial and scientific sectors, there are currently no industrial facilities producing biodiesel from algae, largely due to its high cost of production (Chisti 2007; Miao & Wu 2006; Lardon *et al.* 2009). One of the major economic bottlenecks in the process is biomass and lipid production rates by the algae (Borowitzka 1992; Sheehan *et al.* 1998; Tsukahara & Sawayama 2005). The land area and size of culture vessels required, as well as the energy and water requirements for large-scale algal culture are strongly dependent on algal productivity (Sheehan *et al.* 1998). Enhanced productivity lowers the volume of liquid to be pumped, mixed, sparged, contained and harvested per unit product. More concentrated cell suspensions can also make downstream processing more efficient. All these factors lead to savings in terms of both cost and energy requirements.

The economics of algal biofuel production could also be improved through the production of co-products. For example, high value compounds such as pigments could be produced along with lipid. The residual biomass after lipid extraction could be sold as animal feed, fertilizer or soil conditioner, anaerobically digested to produce biogas, gasified or merely burned to provide some of the heat or electricity required in the process (Mata *et al.* 2010). Hence, for cultivation to be economically viable, productivities must be increased, costs lowered, or additional income streams developed.

In addition to economic feasibility, algal biodiesel must be environmentally desirable. It is critical that the energy embodied in the fuel produced is greater than the energy input required to produce it. Net energy analysis and life cycle analysis (LCA) are tools used to quantify the environmental burdens at every stage of production, from growth of the biomass to combustion of the fuel. Lardon *et al.* (2009) conducted a life-cycle analysis of a hypothetical algal biodiesel production facility. Two different culture conditions, fertilizer feeding and nitrogen starvation, as well as two different extraction options, dry or wet, were investigated. The study confirmed the potential of microalgae as an energy source, but highlighted the necessity of increasing productivity and decreasing energy and fertilizer consumption. Energy inputs, such as the energy required for mixing and pumping, the embodied energy in the materials used and the energy cost of harvesting and processing must be minimized. Recycling of material and energy from waste streams is also important wherever feasible (Scott *et al.* 2010). The use of nitrogen limitation, as well as the optimization of wet extraction were indicated as desirable options. The anaerobic digestion of residual biomass was also suggested as a way of reducing external energy usage and recycling of nutrients.

In order to compete economically with fossil diesel, and maintain a positive energy balance, further research into both the biological and engineering aspects of the process is required. Among the challenges that need to be addressed in microalgal culture are (Lardon *et al.* 2009; Rodolfi *et al.* 2009):

1) Increasing productivity in large-scale outdoor cultures
2) Minimizing contamination by other algal species and predation
3) Mitigating temperature changes and water loss due to evaporation
4) Optimizing supply of light and CO_2
5) Developing cheap and efficient reactor designs
6) Decreasing the overall energy and cost requirements, particularly for pumping, gas transfer, mixing, harvesting and dewatering
7) Producing valuable co-products
8) Recycling of water, energy and nutrients

1.7 Enhancing lipid productivity

The genetic characteristics of an algal species determine the range of its productivity. The levels reached in practice within this range are determined by the culture conditions. There are thus two main approaches to enhancing productivity:

1) Selection of highly productive algal species
2) Designing and maintaining optimal conditions for productivity

The choice of algal strain is a key consideration. The diversity of algal species is much greater than that of land plants (Scott *et al.* 2010), allowing selection of species best suited the local

environment and goals of the project. Although there have been several screening programs, building on the work of the Aquatic Species Program, the majority of strains remain untested. Few species have been studied in depth and the data reported in the literature is often not comparable due to the different experimental procedures used.

Lipid productivity is determined by both growth rate and lipid content. In addition to optimal temperature and pH, conditions that maximize autotrophic growth rate are optimal light, carbon and nutrient supply. Microalgal lipid accumulation is affected by a number of environmental factors (Guschina & Harwood 2006; Roessler 1990), and often enhanced by conditions that apply a 'stress' to the cells. Lipids appear to be synthesised in response to conditions when energy input (rate of photosynthesis) exceeds the capacity for energy use (cell growth and division) (Roessler 1990). Enhanced cell lipid content has been found under conditions of nutrient limitation (Hsieh & Wu 2009; Illman *et al.* 2000; Li, Y *et al.* 2008; Shifrin & Chisholm 1981; Takagi *et al.* 2000), high light intensity (Rodolfi *et al.* 2009), high temperature (Converti *et al.* 2009), high salt concentration (Takagi *et al.* 2000) and high iron concentration (Liu *et al.* 2008). Nitrogen (N) limitation is the most frequently reported method of enhancing lipid content, as it is cheap, easy to manipulate and reduction in nitrogen usage is a key parameter in reducing environmental burden (Harding *et al.* 2007; Richardson 2011). N limitation has a reliable and strong influence on lipid content in many species (Chelf 1990; Rodolfi *et al.* 2009; Shifrin & Chisholm 1981).

Stress conditions that enhance lipid content, such as nitrogen limitation, typically also decrease the growth rate, and thus the net effect on lipid productivity must be ascertained (Lardon *et al.* 2009). One of the main conclusions of the Aquatic Species Program was that oil accumulation through N limitation did not result in increased lipid productivity due to the lower biomass productivities achieved (Sheehan *et al.* 1998). More recent studies by Rodolfi *et al.* (2009), Stephenson *et al.* (2010), Hsieh and Wu (2009) and Takagi *et al.* (2000) have suggested that, by careful choice of 1) a species that can maintain a high productivity under nitrogen-limiting conditions, 2) intermediate levels of nitrogen limitation and 3) cultivation strategy, N limitation could be used successfully to enhance lipid productivity.

1.8 Gaps in current knowledge

Enhancing the lipid productivity of microalgae could assist in achieving economic algal biodiesel production. In order to improve lipid productivity (a factor of both biomass and lipid production by the algae), productive strains and culture conditions able to produce cells with a simultaneously high growth rate and lipid content are required (Rodolfi *et al.* 2009). Previous studies have highlighted the importance of species choice and suggested a range of important physiological characteristics (reviewed in Chapter 5). However, few studies have compared species on any quality other than lipid content.

Little consensus has been reported between research groups on the algal species most suitable for biodiesel production, and the number of strains used in algal biotechnology remains low (Grobbelaar 2000). Data has been collected under a range of culture conditions, and reported in a variety of units, making comparison difficult. Although N limitation is well known to enhance lipid content, there is a particular lack of information on biomass productivities achieved under conditions of N limitation, preventing calculation of lipid productivity.

Due to the tradeoff between growth rate and lipid accumulation, there is uncertainty in the literature as to whether N limitation improves lipid productivity. The Aquatic Species Program reported that overall lipid productivity was not enhanced under N limited conditions compared to N replete conditions (Sheehan *et al.* 1998). More recent studies have suggested that, with careful design of culture regime, N limitation could be used to increase lipid yield, although the optimal N feeding strategy has not been determined (Hsieh & Wu 2009; Rodolfi *et al.* 2009; Stephenson *et al.* 2010; Takagi *et al.* 2000).

The majority of studies have tested only the two extremes of N replete and N limited cultures. Few studies have investigated the effect of intermediate levels of N limitation. In addition, lipid content is often measured at only a few points in the growth cycle, e.g. before and after N limitation. A temporal profile of lipid accumulation and growth rate with time and degree of N limitation would allow a more accurate calculation of lipid productivity and elucidate the interaction between N limitation and cell physiology. There has been no comprehensive comparison of a range of different N limited cultivation strategies in improving lipid productivity.

Early on in this project, it became clear that common methods of biomass and lipid quantification were unsatisfactory and required rigorous inquiry and further development. Accurate measurement of lipid productivity depends on accurate quantification of biomass concentration and lipid content. It was noted during initial experiments that the pigment content of the cells influenced the estimation of DW by OD. Due to variations in the pigment content of microalgal cells with age and culture conditions, errors in DW estimation could be introduced. This has not been reported previously and the magnitude of the error associated with biomass quantification by OD is unknown.

Various methods have been used to measure the lipid content of cells, usually involving lipid extraction, followed by quantification by weight or chromatography (Bligh & Dyer 1959; Folch *et al.* 1957). In addition to being labour-intensive, traditional methods of lipid quantification are prone to both underestimation due to incomplete extraction, and overestimation of nutritive value or energy content due to extraction of non-fatty acid containing substances. Direct transesterification is a method of converting saponifiable lipids *in situ* directly to fatty acid methyl esters, which can be quantified by gas chromatography. This eliminates the extraction

step and results in a rapid, one-step procedure applicable to small sample volumes. This method has been applied previously, but infrequently, to microalgae. However, its effectiveness in comparison to extraction techniques is not known. In addition, there is concern about the effect of the water content of samples on the efficiency of the transesterification reaction.

1.9 Aims and objectives

The purpose of this work was to identify promising microalgal species and culture strategies that optimise lipid productivity for biodiesel production. Specific objectives were to:

1) Develop the tools necessary to measure lipid productivity accurately, specifically by investigating:
    a. the problem of interference by pigment in the quantification of microalgal biomass concentration by optical density
    b. direct transesterification as an alternative to lipid extraction methods in quantifying the lipid content of microalgal cells
2) Identify and quantify the key characteristics of promising microalgal strains, initially through a literature survey and then experimentally, to guide selection of the most appropriate species for large-scale biodiesel production
3) Investigate the effect of different degrees of nitrogen limitation on cell physiology and lipid productivity in one promising species
4) Explore the use of different cultivation strategies in improving lipid productivity

The context of this project is the economic production of algal oil for biodiesel. However, the study has been designed such that its primary outputs are relevant to algal biomass and oil production for other applications of general interest and to CeBER. These include production of specialty oils and polyunsaturated fatty acids, manufacture of algal oil for human or animal consumption, or as an ingredient in products such as lubricants and cosmetics. In addition, production of the carotenoids β-carotene, astaxanthin and lutein, amongst others, is associated with lipid accumulation. Increased oil content would also increase the nutritional content of algal cells for applications such as aquaculture feed (Palmquist & Jenkins 2003). Where algal biomass is produced for energy products other than biodiesel, enhanced oil content positively impacts its calorific value and hence the potential energy yield, regardless of the processing method.

1.10 Research hypotheses

1) Lipid productivity under both N replete and N limited conditions is a critical parameter for selecting species for biodiesel production
2) Nitrogen limited culture will produce greater overall lipid productivity than nitrogen replete culture
3) There is an intermediate degree of nitrogen limitation that optimizes lipid productivity in batch culture
4) Two stage culture leads to greater lipid productivity than nitrogen limited batch culture

1.11 Thesis overview

Materials and methods are presented in Chapter 2. The thesis thereafter falls into three main parts. Part I (Chapters 3 and 4) deals with method development. Chapter 3 investigates the sources of error in quantification of biomass concentration by optical density due to the pigment content of cells. Chapter 4 details the critical evaluation and optimization of the method of direct transesterification for quantification of lipid content.

Part II (Chapters 5 and 6) investigates species selection. Chapter 5 presents a comprehensive survey of the literature, in which 55 different algal species are compared according to their reported or calculated lipid productivity. This work is extended and validated in Chapter 6, where a study comparing the lipid productivity, settling rates and fatty acid profiles of 11 of the most promising species is presented.

Part III (Chapters 7 and 8) explores the effect of different degrees of nitrogen limitation and different culture regimes on lipid productivity in *Chlorella vulgaris*. Experiments on the effect of different starting concentrations of nitrate on cell growth, lipid content, lipid productivity and cell physiology are presented in Chapter 7. Chapter 8 introduces data on the productivities achieved in other culture modes such as two-stage, fed-batch and continuous culture. Final conclusions are presented in Chapter 9.

2 Materials and Methods

2.1 Introduction

This chapter presents the details of the microorganisms, media, reactor design, cultivation conditions and general analytical methods used in this study. Further methods specific to each section are presented in the relevant chapter. The methods detailed here for biomass quantification by absorbance (Section 2.4.2) and lipid measurement by direct transesterification (Section 2.4.3) underwent significant development and validation during the early phase of this project. The detailed investigations into these two methods are presented in Chapters 3 and 4 respectively.

2.2 Cultures and their maintenance

2.2.1 Microalgal cultures

The microalgal species cultivated, along with their taxonomy and origin are summarized in Table 2.1. These included four freshwater species (grown in 3N Bold's Basal Medium (BBM) (Bold 1949)), six marine species (grown in either f/2 (Guillard & Ryther 1962) or Walne's medium (Walne 1970) depending on their nutrient requirements) and one halophilic species (grown in Zarrouk's medium (Zarrouk 1966)).

Long-term stock cultures of freshwater species were maintained on agar plates. Liquid stock cultures of all species were maintained in 200 ml glass medicine bottles, bubbled with 2% CO_2, under constant illumination of 120 $\mu mol.m^{-2}.s^{-1}$ provided by two cool white 18W fluorescent light bulbs (Osram). Inoculum for the airlift reactors, in which experiments were carried out, was prepared by scaling up stock cultures into 500 ml glass bottles, or 2 L volumetric flasks where more than two columns were inoculated. These were cultivated under stock culture conditions for 7 to 10 days. All experiments were inoculated to an optical density of 0.1 at 750 nm (approx. 0.05 $g.L^{-1}$), except for the diatoms and haptophytes, which required a starting concentration of between 0.1 and 0.2 $g.L^{-1}$.

Table 2.1 Abbreviations, media, taxa and origins of the microalgal cultures used

Culture	Abbr.	Medium	Taxa	Origin
Chlorella vulgaris	Cv	3N BBM	Chlorophyte	UTEX 395
Scenedesmus sp.	Sc	3N BBM	Chlorophyte	own isolate, Upington, SA
Neochloris oleoabundans	Nol	3N BBM	Chlorophyte	UTEX 1185
Ankistrodesmus falcatus	Af	3N BBM	Chlorophyte	UTEX 242
Spirulina platensis	Spir	Zarrouk's	Cyanobacteria	own isolate, abandoned tannery treatment pond, Wellington, SA
Nannochloropsis sp.	Nan	f/2	Eustigmatophyte	University of Hawaii culture collection
Tetraselmis suecica	Ts	f/2	Chlorophyte	UTEX LB 2286
Cylindrotheca fusiformis	Cf	Walne's	Diatom	UTEX B2087
Phaeodactylum tricornutum	Pt	Walne's	Diatom	University of Hawaii culture collection
Pavlova sp.	Pav	Walne's	Haptophyte	MCM culture collection, Cape Town, SA
Isochrysis C4	Iso	Walne's	Haptophyte	WITS culture collection, Johannesburg, SA

2.2.2 Media

To facilitate comparison of the performance of different species, the media were chosen to be as simple and as similar as possible, while still meeting the nutritional and osmotic requirements of each alga. It was attempted to use a single media for each of the freshwater, halotolerant and marine groups of species, but not all of the marine species grew on the f/2 media, hence a second marine media (Walne's) was introduced.

Freshwater species were grown in 3N BBM medium, composed of 0.75 $g.L^{-1}$ $NaNO_3$; 0.025 $g.L^{-1}$ $CaCl_2.3H_2O$; 0.075 $g.L^{-1}$ $MgSO_4.7H_2O$; 0.075 $g.L^{-1}$ $K_2HPO_4.3H_2O$; 0.175 $g.L^{-1}$ KH_2PO_4; 0.025 $g.L^{-1}$ NaCl and 6 $ml.L^{-1}$ of PIV metal solution. The PIV metal solution was composed of 0.75 $g.L^{-1}$ Na_2EDTA; 0.097 $g.L^{-1}$ $FeCl_3.6H_2O$; 0.041 $g.L^{-1}$ $MnCl_2.4H_2O$; 0.005 $g.L^{-1}$ $ZnCl_2$; 0.002 $g.L^{-1}$ $CoCl_2.6H_2O$ and 0.004 $g.L^{-1}$ $Na_2MoO_4.2H_2O$.

S. platensis was cultivated in Zarrouk's medium, composed of 18 $g.L^{-1}$ $NaHCO_3$; 0.5 $g.L^{-1}$ K_2HPO_4; 0.75 $g.L^{-1}$ $NaNO_3$; 1 $g.L^{-1}$ K_2SO_4; 0.04 $g.L^{-1}$ $CaCl_2.2H_2O$; 0.2 $g.L^{-1}$ $MgSO_4.7H_2O$; 1 $g.L^{-1}$ NaCl; 0.08 $g.L^{-1}$ Na_2EDTA; 0.01 $g.L^{-1}$ $FeSO_4.7H_2O$ and 1 $ml.L^{-1}$ each of micronutrient solution and trace element

solution. Micronutrient solution was composed of 2.86 g.L^{-1} H_3BO_3; 1.81 g.L^{-1} $MnCl_2.4H_2O$; 0.22 g.L^{-1} $ZnSO_4.7H_2O$; 0.08 g.L^{-1} $CuSO_4.5H_2O$ and 0.014 g.L^{-1} $Na_2MoO_4.2H_2O$. Trace element solution comprised 46.6 mg.L^{-1} K_2CrO_7; 47.8 mg.L^{-1} $NiSO_4.7H_2O$ and 4.2 mg.L^{-1} $CoSO_4.7H_2O$.

Marine species were grown in either f/2 or Walne's medium. Filtered natural seawater was obtained from the Marine and Coastal Management facility in Seapoint, Cape Town. F/2 medium consisted of natural seawater, to which 0.75 g.L^{-1} $NaNO_3$ and 4.4 mg.L^{-1} NaH_2PO_4 were added, along with 0.5 ml.L^{-1} of Fe/EDTA solution and 0.5 ml.L^{-1} of trace metal solution. Fe/EDTA solution was made up of 5.71 g.L^{-1} $FeC_6H_5O_7$ and 17.35 g.L^{-1} $Na_2EDTA.2H_2O$. Trace metal solution consisted of 19.6 mg.L^{-1} $CuSO_4.5H_2O$; 44 mg.L^{-1} $ZnSO_4.7H_2O$; 20 mg.L^{-1} $CoCl_2.6H_2O$; 0.36 g.L^{-1} $MnCl_2.4H_2O$; 12.6 mg.L^{-1} $Na_2MoO_4.2H_2O$ and 1.64 g.L^{-1} $Na_2EDTA.2H_2O$.

Walne's medium consisted of natural seawater enriched with 0.75 g.L^{-1} $NaNO_3$; 20 mg.L^{-1} NaH_2PO_4; 0.42 mg.L^{-1} $MnCl_2.4H_2O$; 1.32 mg.L^{-1} $FeCl_3.6H_2O$; 33.4 mg.L^{-1} H_3BO_3; 45 mg.L^{-1} $Na_2EDTA.2H_2O$, 60 mg.L^{-1} $Na_2SiO_3.5H_2O$, 1 ml.L^{-1} of trace metal solution and 0.1 ml.L^{-1} vitamin solution. The trace metal solution consisted of 2.1 mg.L^{-1} $ZnCl_2$; 2 mg.L^{-1} $CoCl.6H_2O$; 0.9 mg.L^{-1} $(NH_4)_6Mo_7O_{24}.4H_2O$ and 2 mg.L^{-1} $CuSO_4.5H_2O$. The vitamin solution was made up of 0.1 g.L^{-1} cyanocobalamin (vitamin B12) and 0.1 g.L^{-1} thiamine hydrochloride (vitamin B1).

The $NaNO_3$ concentration was adjusted to 0.75 g.L^{-1} in all media for culture maintenance. Further adjustments to the nitrate concentration were made according to experimental requirements, described in each chapter. Addition of vitamins was not necessary for growth of the freshwater or f/2 medium species. Media recipes are provided in Appendix A.

2.3 Reactor design and operation

All experiments were conducted in tubular airlift photobioreactors, as illustrated in Figure 2.1. The glass and stainless steel reactors were 60 cm high with an external diameter of 10 cm, a draft tube of 5 cm diameter and a working volume of 3.2 L. The sparger at the bottom of the draught tube was a 0.22 µm stainless steel HPLC inlet filter. Air enriched with CO_2 (0.29%) was prepared by mixing air and 100% CO_2. The flow rate of each was controlled using a Brooks 5850S Thermal Mass Flow Controller. The two gas streams were sent through an inline mixer and fed at 2 L.min^{-1} to each column. The overall mass transfer coefficient, $k_La(CO_2)$, was found to be 0.0094 ± 0.00026 s^{-1} (Langley 2010). This rate of carbon supply has been shown to be non-limiting for growth of *C. vulgaris* in these reactors under the given conditions (Langley 2010).

Constant light (250 µmol.m^{-2}.s^{-1} at the reactor surface) was provided by three cool white 18 W fluorescent light bulbs (Osram). The total circulation time in the reactor was approximately 7 s, with a riser time of 1 s and a downcomer time of 6 s. Culture temperature was monitored daily and remained constant at 25 ± 1°C. All reactors and media were sterilised by autoclaving prior to

use. Approximately 100 ml of sterile, distilled water was added daily to replace that lost to evaporation. A 20 μl aliquot of antifoam was added to each reactor to reduce foaming.

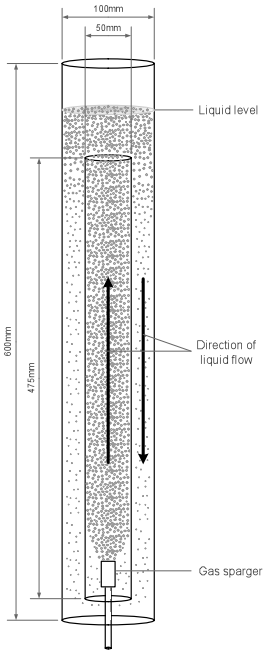


Figure 2.1 Diagram of airlift reactor (Langley 2010)

2.4 Analytical methods

2.4.1 Biomass quantification by dry weight

Between 5 and 20 ml culture suspension was filtered onto pre-weighed 0.45 μm cellulose nitrate filters (Sartorius stedim) and dried at 80°C overnight. Filters were cooled to room temperature in a desiccator and weighed. In order to account for the dissolved solutes in the media, equivalent amounts of sterile culture media were filtered, dried and the mass subtracted from the sample weight. All dry weight measurements were done in duplicate. The average relative error in duplicate dry weight measurements was 4%.

2.4.2 Biomass quantification by absorbance

Optical density (OD) at 750 nm was used as a convenient and rapid way to estimate dry weight. A discussion on the selection of the specific wavelength and the potential introduction of error by variation in cell pigment content can be found in Chapter 3. OD measurements were taken in duplicate using a Helios α spectrophotometer (Thermo Scientific). Cultures were diluted with medium to an OD below 1.0, to fall within the linear range of measurement. Dilution and OD were used to calculate dry weight using standard curves generated from OD and dry weight

measurements taken throughout the growth cycle. The average relative error between duplicate OD readings over the course of a growth cycle was 1%.

2.4.3 Quantification of cell lipid content

For the purposes of this work, cell lipid content was measured as total fatty acid content. This was considered the most relevant measurement for biodiesel production as biodiesel is produced from fatty acids. Fatty acid content was measured by direct transesterification of lipids *in situ*, using centrifuged algal cell pellets. Validation of this method and comparison to common extraction and quantification methods is presented in Chapter 4. A sequential combination of a basic catalyst followed by an acid catalyst in an excess of methanol was used to convert fatty acids in algal lipids to fatty acid methyl esters (FAME), which could be quantified by GC. Two internal standards were used: glyceryl triheptadecanoate (C17-TAG) was added prior to the reaction as a quantitative internal standard, and methyl nonadecanoate (C19-ME) was added in the final solvent extraction step to verify the completeness of the transesterification and efficiency of extraction into the hexane layer.

The algal cells were recovered from 15 ml samples of algal suspension by centrifugation at 1520 g for 10 min in glass test tubes with silicone-lined screw-cap lids (Kimax). The supernatant was removed and pellets frozen at -20°C to allow the assay to be performed in batches. Defrosted pellets were dissolved in 500 µl toluene containing 0.1 mg C17-TAG, in order to solubilise the lipids. One ml of 0.5 N NaOH (the basic catalyst) in methanol (the alkylating agent) was added and the samples mixed briefly by vortexing. Samples were incubated at 80°C for 20 min, with shaking at 300 rpm. After cooling for 5 min to room temperature, 1 ml 5% HCl (the acid catalyst) in methanol was added before repeating the incubation. Samples were returned to room temperature. In order to facilitate two-phase separation, 400 µl dH_2O and 400 µl hexane, containing 0.1 mg C19-ME, were added and mixed by vortexing. Tubes were centrifuged at 1520 g for 1 min to separate the upper hexane-toluene layer, containing the FAME extract, which was transferred to vials for GC.

The FAME extract (1 µl), containing internal standards, was injected into a Varian 3900 GC equipped with a flame ionisation detector and SupelcoWax 10 column (30 m x 320 mm x 1.0 µm film thickness) (Supelco, USA). Standard split/splitless injection was used with a split of 100 and injector temperature of 270°C. The column temperature was increased from 180°C to 260°C at $2°C.min^{-1}$. Nitrogen ($2 ml.min^{-1}$) was used as the carrier gas and the detector temperature was 260°C. Peaks were identified by retention time using Supelco 37 Component FAME and C14:0 to C22:0 FAME mixtures. Peak areas were used to quantify each FAME relative to the internal standards. The difference in the response factor of the detector to the range of FAMEs in the samples was negligible. The total fatty acid content was calculated by adding all the individual FAME peak areas.

2.4.4 Quantification of cell carbohydrate content

Total carbohydrate content of cells was measured according to the phenol-sulphuric acid method (Dubois *et al.* 1956). There are many slight variations on the basic protocol. The method used here is based on (François 2006). Samples of algal culture (2 ml) were centrifuged in 2 ml Eppendorf tubes at 16000 g for 3 min. The supernatant was discarded and the cell pellet resuspended in 2 ml distilled water. Between 50 and 450 nmol of monosaccharide are required for analysis (Masuko *et al.* 2005). Depending on the density of the culture, samples were diluted between 2 and 8 times to achieve this (generally a culture sample with an OD of less than one was in the right range). Phenol (200 µl of a 5% aqueous solution) was added to a 200 µl sample of diluted culture in a test tube and mixed by vortexing. Concentrated sulphuric acid (1 ml, 98%, Kimix) was carefully added in a direct stream onto the sample and immediately mixed by vortexing. Samples were incubated at room temperature for 10 min and then in a 30°C water bath for 20 min. Aliquots of each sample (200 µl) were transferred to a 96-well microtitre plate and the absorbance was read at 490 nm using a FLUOstar Omega microtiter plate reader (BMG Labtech). Total carbohydrate content was quantified relative a glucose standard curve (between 4 and 40 µg glucose per sample).

It was found that the addition of the sulphuric acid was a critical step in ensuring reproducibility of the assay. The acid must be added rapidly and directly onto the sample, not touching the sides of the test tube. This ensures rapid mixing and heat development. All carbohydrate assays were done in duplicate. The average relative error for each experiment was between 3 and 9%.

2.4.5 Quantification of cell protein content

Several different methods of quantifying the total protein content of algal cells were investigated (a discussion and comparison of these is presented in Appendix B). The bicinchoninic acid (BCA) method (Walker 1994) was chosen, due to its ease of use and higher tolerance to interfering substances, as the protein assay for this work, preceded by extraction into 0.5 N NaOH according to (Rausch 1981).

A sample of algal culture (2 ml) was centrifuged in a 2 ml Eppendorf tube at 16000 g for 3 min, and the supernatant discarded. The pellet was resuspended in 600 µl of 0.5 N NaOH and incubated in a water bath at 80°C for 10 min. After centrifugation at 16000 g for 3 min, the supernatant (protein extract) was collected in a fresh tube. The pellet was resuspended in another 600 µl of 0.5 N NaOH and the incubation repeated. Extraction was repeated a third time at 100°C for 10 min. All three supernatants were combined and the volume made up to 2 ml with 0.5 N NaOH.

To a 100 µl sample of extract or standard in a test tube, 2 ml reagent C (consisting of 50 ml reagent A: 1 $g.L^{-1}$ sodium bicinchoninate; 17.1 $g.L^{-1}$ Na_2CO_3; 1.6 $g.L^{-1}$ sodium tartrate dibasic

dihydrate; 4 $g.L^{-1}$ NaOH; 9.5 $g.L^{-1}$ $NaHCO_3$ (pH 11.25) and 1 ml reagent B: 40 $g.L^{-1}$ $CuSO_4.5H_2O$) was added and the mixture vortexed. Samples were incubated in a water bath at 60°C for 30 min. Aliquots of each sample (200 μl) were transferred to a 96-well microtitre plate and the absorbance was read at 562 nm using a FLUOstar Omega microtiter plate reader (BMG Labtech). Protein concentration was calculated using a bovine serum albumin standard curve. The average relative error in duplicate assays was less than 10%.

2.4.6 Quantification of cell pigment content

Pigments were extracted from algal cells using dimethylsulphoxide (DMSO), and their concentration measured by spectrophotometry (see appendix B for a discussion of choice of solvent and protocol). Culture samples (2 ml) were centrifuged in 2 ml Eppendorf tubes at 16000 g for 3 min and the supernatant discarded. Hot (60°C) DMSO (2 ml, 99%, Saarchem) was added and cells resuspended by vortexing. Samples were incubated at 60°C, with occasional shaking, for 10 min before centrifuging. The supernatant (pigment extract) was removed and diluted with DMSO to an OD of less than one. The OD at 649, 665 and 480 nm was determined and the pigment content calculated using Equations 2.1 to 2.4 (Wellburn 1994). The detection limit of this assay was approximately 0.1 $mg.L^{-1}$ pigment and the average relative error in duplicate assays was less than 6%.

Chlorophyll *a* (ChlA) ($mg.L^{-1}$) = $12.47(OD_{665}) - 3.62(OD_{649})$ Equation 2.1

Chlorophyll *b* (ChlB) ($mg.L^{-1}$) = $25.06(OD_{649}) - 6.5(OD_{665})$ Equation 2.2

Total carotenoids ($mg.L^{-1}$) = $[1000(OD_{480}) - 1.29(ChlA) - 53.78(ChlB)]/220$ Equation 2.3

Total pigment ($mg.L^{-1}$) = sum of the above Equation 2.4

2.4.7 Elemental analysis

The carbon (C), hydrogen (H) and nitrogen (N) composition of algal cells was determined by elemental analysis. Lyophilised samples were combusted in an excess of oxygen and the yield of the combustion products (e.g. CO_2, NO_2, H_2O) used to determine the CHN composition. Samples (50 ml) of culture were centrifuged in 50 ml Falcon tubes at 1520 g for 10 min. The supernatant was discarded and the pellets frozen at −60°C before freeze-drying for 24 h. Elemental analysis was carried out in the Department of Chemistry (UCT) using a Thermo Flash EA 1112 series elemental analyser. The reproducibility of this analysis was within 1%.

2.4.8 Quantification of media nitrate concentration

Nitrate concentration in the media was measured either by HPLC or spectrophotometry (Clesceri *et al.* 1998). In the HPLC method, culture samples (2 ml) were centrifuged at 16000 g for 3 min and the supernatant filtered through a 0.22 μm filter. Filtered samples, diluted to less than 200

$mg.L^{-1}$ nitrate in distilled water, were analysed for nitrate concentration using a Waters IC-Pak Anion High Resolution column. Sodium borate-gluconate mobile phase was used at a flowrate of 1 $ml.min^{-1}$. The injection volume was 50 µl. Detection was by conductivity with a gain of 0.01, range of 500 µS, positive polarity and temperature of 35°C. Nitrate eluted at between 6 and 7 min. Peak areas were used to quantify nitrate relative to $NaNO_3$ standards. The average relative error was within 2%. The detection limit was 5 $mg.L^{-1}$.

In the spectrophotometric method, the absorption of nitrate in the UV range is used to quantify its concentration relative to a nitrate standard curve. Note that dissolved organic matter, turbidity, chromium, nitrite and surfactants can interfere with this method. Filtered samples of supernatant were diluted to less than 12 $mg.L^{-1}$ nitrate, using nitrate-free media. OD at 220 nm was measured using a quartz cuvette and quantified using a standard curve of $NaNO_3$ (Clesceri *et al.* 1998). Average relative error in duplicate measurements was 1% and the detection limit was 1 $mg.L^{-1}$.

All early experiments were analysed by HPLC. The spectrophotometric method was introduced when it became clear that seawater samples could not be analysed by HPLC due to the large chloride peak eclipsing the nitrate peak. In order to verify the results obtained by spectrophotometry, samples of the culture media across the growth cycle of two cultures of *C. vulgaris* were analysed by both methods. The results obtained using the two different methods were very similar ($R^2 = 0.99$), validating the spectrophotometric assay. In later experiments, all nitrate determinations were done spectrophotometrically.

2.5 Research approach

To investigate the magnitude of error caused by variations in pigment content in biomass quantification by optical density, *C. vulgaris* was chosen as the model organism due to its fast growth rate and high pigment content. Results were verified using *Scenedesmus* sp., *Nannochloropsis* sp. and *S. platensis*. Cultures were grown in airlift reactors under conditions designed to result in different pigment contents: normal, nitrogen replete conditions; low pigment, nitrogen limited conditions; and high pigment, GRO-LUX illumination conditions. Biomass was quantified daily throughout a 17 day growth cycle, by both dry weight and OD at wavelengths within the maximum (680 nm) and minimum (750 nm) range of pigment absorption. Pigment content was also measured daily. Standard curves relating OD to DW were constructed at days 3, 7, 10 and 14 during the growth cycle.

To investigate, compare and validate the direct transesterification (DT) method of lipid quantification, *C. vulgaris*, *Scenedesmus* sp. and *Nannochloropsis* sp. were grown in airlift bioreactors under nitrogen replete conditions. Single batches of biomass of each species, harvested in stationary phase, were used for the determination of lipid content by three different extraction-transesterification methods, as well as by DT. DT was also carried out on the biomass

residue from each extraction to determine the efficiency of fatty acid recovery. The DT reaction was further investigated by comparing the results of using two catalysts in series to those obtained using an acidic or basic catalyst alone. The effect of reaction water content was examined by the addition of different volumes of water to samples of freeze dried algal biomass. Different methods of drying and storing the biomass were also investigated for their effect on fatty acid quantification by DT.

The question of species choice for biodiesel production was tackled in two ways. First a literature study was carried out. Data on growth rate, biomass productivity and lipid content was gathered from a range of literature sources, for 55 microalgal species. To allow comparison and calculation of lipid productivity, data were converted into comparable units. This necessitated assumptions in: 1) the conversion of areal to volumetric biomass productivities and 2) the conversion of specific growth rates to biomass productivities. Evaluation of average reported growth and lipid content, as well as literature and calculated values for lipid productivity, allowed an initial comparison of 55 species under nutrient replete conditions.

Eleven of these species were chosen for further investigation. Each species was grown in airlift photobioreactors under two different conditions: N replete (1500 $mg.L^{-1}$ nitrate) and N limited (150 $mg.L^{-1}$ nitrate). Biomass concentration and fatty acid content, as well as residual nitrate concentration in the media, were measured daily. After 14 days, settling tests were carried out to determine the rate and efficiency of gravity sedimentation. To predict the quality of the biodiesel produced, data on the fatty acid profile of each species was collated from the GC profiles generated during fatty acid quantification.

Due to its high lipid productivity, rapid response to N limitation and ease of cultivation, *C. vulgaris* was chosen to be used in further testing of the optimal degree of N limitation and the effect of culture regime on lipid productivity. To investigate the effect of intermediate degrees of N limitation, *C. vulgaris* was cultured in airlift reactors with starting nitrate concentrations of 0, 40, 70, 100, 170, 420, 570, 1200 and 2000 $mg.L^{-1}$. Media nitrate concentration, biomass and fatty acid content were measured daily for 20 days. In order to investigate the effect of nitrogen limitation on cell physiology, carbohydrate, pigment and protein content were measured every third day, and samples taken for elemental analysis.

To investigate the effect of N culture regime on lipid productivity, *C. vulgaris* was cultivated using two-stage batch, fed-batch, two-stage continuous-batch and multi-stage continuous regimes. Biomass concentration and lipid content were measured throughout the growth cycle, and key culture parameters compared to those achieved under N replete and limited batch culture.

Part I – Method development

3 Biomass quantification: Interference by pigment in the estimation of microalgal biomass concentration by optical density

3.1 Introduction

The accurate measurement of microalgal biomass concentration is a vital tool in the optimisation of lipid productivity, and important in most studies of algal physiology and biotechnology. Optical density (OD) is often used as a convenient indirect measurement of biomass in microbial cell suspensions. Absorbance of light at a particular wavelength by a suspension can be related directly to cell density. A range of wavelengths has been used to monitor microalgal cultures. In the initial stages of this project, 680 nm (an absorption maxima of chlorophyll *a*, the major pigment of green algae) was chosen due to the fact that microalgal suspensions absorb maximally at this wavelength.

It was noted during early experiments that inaccuracies in the estimation of dry weight (DW) from OD could be introduced when the pigment content of the cells changed. Pigment content was found to vary widely with age and culture conditions. For example, under the culture conditions used in nitrogen limited batch cultures, pigment content of *Chlorella vulgaris* varied from 0.5 to 5.4% DW. It was found that changes in pigment content could lead to significant errors in biomass quantification due to absorbance by the pigments, and that these errors could be reduced through selection of a wavelength that minimises absorbance by the pigment (e.g. 750 nm in cases where chlorophyll is the dominant pigment).

This chapter investigates the error in biomass quantification using optical density, relative to actual DW measurements, in cells with variable pigment content. *Chlorella vulgaris* was chosen as a representative microalgal species. It has small, round cells (2 to 4 μm in diameter) and a relatively high total pigment content (up to 6% DW) during exponential growth. The effect of using wavelengths within (680 nm) and outside (750 nm) the maximal absorbance range of the major pigments was investigated, as well as the use of standard curves generated under different culture conditions, or at different time points in the growth cycle.

3.2 Literature review

Optical density (OD), also known as absorbance or turbidity, is used frequently as a rapid and non-destructive measurement of biomass in cultures of bacteria and other unicellular microorganisms (Toennies & Gallant 1949; Shuler & Kargi 2005). The amount of light absorbed by a suspension of cells can be related to cell mass or cell number. The correlation is a complex function of particle size, shape and refractive index (Clesceri *et al.* 1998). Inaccuracies can be introduced when estimating biomass during growth, as cell morphology and composition, and hence optical properties, may change.

When a beam of light is shone through a suspension of particulate matter, such as a cell culture, light is both scattered and absorbed by the particles. The decrease in the amount of light transmitted, relative to a blank containing media and no particles, is called the absorbance (Clesceri *et al.* 1998). Absorbance of light at a particular wavelength can be related to cell concentration using a suitable standard curve. The wavelength of maximum absorbance is usually used, as this provides the greatest range of sensitivity.

Pigmented samples can bias absorbance, or optical density, as the colour absorbs in a particular region of the light spectrum (Clesceri *et al.* 1998). This can affect measurement of cell concentration by OD in pigmented cells. For example, a suspension of highly pigmented cells would absorb more light, making the culture appear more dense than a suspension of similar concentration of less pigmented cells (Figure 3.1). The increase in absorbance by pigmented cells can be accounted for by using an appropriate standard curve. However, this correlation is only accurate under growth conditions in which the pigment content of the cells is the same as that used to generate the standard curve.

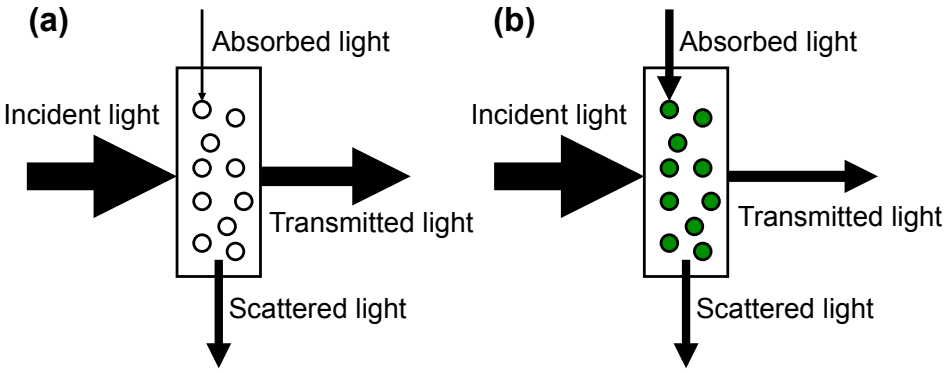


Figure 3.1 Diagram showing the absorption of light through a suspension of (a) non-pigmented and (b) pigmented cells at a wavelength of light absorbed by the pigment (e.g. 680 nm for chlorophyll).

Although the pigmented and non-pigmented cells are at the same concentration, more light is absorbed by the pigmented cells. This can lead to an overestimate of biomass concentration in the pigmented culture if a suitable standard curve is not used.

Microalgae have relatively high pigment contents, consisting of mainly chlorophylls and carotenoids (Becker 1994). Pigment content can vary from 0.1 to 9.7% of the wet biomass (Nicholls & Dillon 1978). Healey and co-workers (1975) reported that ratios of chlorophyll *a* (the major pigment in most microalgal cells) to DW varied more than 30-fold with changes in species composition, temperature and nutrient and light availability. No studies quantifying the error due to changes in pigment content when measuring biomass by OD have been reported.

Wavelengths that minimise absorbance by media components while maximising turbidity measurement (600 to 700 nm) are often used for the measurement of OD in bacterial cultures (Shuler & Kargi 2005). Algal culture media are usually clear and there is little consensus in the literature about which wavelength to use to measure OD in microalgal cultures (see Table 3.1). Absorbance by pigment is greater in certain parts of the light spectrum. Choosing a wavelength within the maximal absorbance range of the pigment is expected to give the largest signal, but also the largest error should the pigment content of the cells change. Despite this, wavelengths within the maximal absorbance range of chlorophyll *a* (400-460 nm and 650-680 nm) have been reported frequently (An *et al.* 2003; Bopp & Lettieri 2007; Chiu *et al.* 2008; De Morais & Costa 2007; Hsieh & Wu 2009; Piorreck *et al.* 1984; Sung *et al.* 1999; Takagi *et al.* 2000).

Table 3.1 Wavelengths used for biomass determination in microalgae

Reference	Species	λ (nm)
Chrismadha & Borowitzka 1994	*Phaeodactylum tricornutum*	350
Piorreck *et al.* 1984	multiple	440
Bopp & Lettieri 2007	*Thalassiosira pseudonana*	450
Scragg *et al.* 2003	*Chlorella vulgaris*	500
Gouveia *et al.* 2009	*Neochloris oleoabundans*	540
Gouveia & Oliveira 2009	multiple	540
Carvalho *et al.* 2006a	*Pavlova lutheri*	540
Vonshak & Richmond 1985	*Spirulina*	560
Hodaifa *et al.* 2008	*Scenedesmus obliquus*	600
Li *et al.* 2008b	*Neochloris oleoabundans*	600
Contreras *et al.* 1998	*Phaeodactylum tricornutum*	625
Converti *et al.* 2009	*Nannochloropsis oculata* and *Chlorella vulgaris*	625
Sung *et al.* 1999	*Chlorella sp.*	660
De Morais & Costa 2007	*Spirulina and Scenedesmus obliquus*	670
An *et al.* 2003	*Botryococcus braunii*	680
Chiu *et al.* 2008	*Chlorella sp.*	682
Hsieh & Wu 2009	*Chlorella sp.*	682
Patil *et al.* 2006	*Tribonema*	710
Sheehan *et al.* 1998	multiple	750
Rodolfi *et al.* 2009	multiple	750
McGinnis *et al.* 1997	*Chaetoceros muelleri*	750
Dempster & Sommerfeld 1998	*Nitzschia communis*	750

3.3 Methods

3.3.1 Cultures

C. vulgaris was grown in 3N BBM medium (NO_3 1500 mg.L^{-1}) in airlift reactors as described in Section 2.3. Batch cultures were inoculated to an OD of 0.1 at 750 nm and grown for 17 days. To manipulate the pigment content, the lighting or nitrogen concentration of the media was changed. Cultures with very high pigment content were obtained by constant illumination with three 18W GRO-LUX bulbs (Sylvania), which resulted in very dark green cultures. Cultures with low pigment content were obtained by growth in low nitrogen 3N BBM medium (NO_3 150 mg.L^{-1}). Cultures with 1500 mg.L^{-1} NO_3 were shown to still have sufficient nitrogen at the end of the growth period (as measured by HPLC, data not shown), producing normal, high pigment content cells, whereas cultures grown on 150 mg.L^{-1} nitrate became nitrate limited between day 3 and 5. Nitrogen limitation has been reported to result in low chlorophyll content (Piorreck *et al.* 1984; Porra & Grimme 1974). The results obtained with *C. vulgaris* were verified using three other species: *Scenedesmus* sp., *Spirulina platensis* and *Nannochloropsis* sp. These were all grown under the same conditions as *C. vulgaris*, except Zarrouk's medium was used for *S. platensis* and f/2 medium for *Nannochloropsis* sp.

3.3.2 Biomass quantification and standard curves

DW and OD were measured according to the methods in Sections 2.4.1 and 2.4.2. Standard curves of OD with respect to DW were generated at specified times in the growth cycle. A sample of the culture was diluted to an OD of less than one. Five serial dilutions were made from this stock and the OD at 680 and 750 nm measured in duplicate. The DW of each dilution was calculated from the DW of the stock. Cell size was estimated using an Olympus BX40 microscope equipped with a digital camera and AnalySIS software (manual function, approx. 50 cells per culture).

3.3.3 Pigment quantification

Pigment content was quantified by the method given in Section 2.4.6. Pigments were extracted from algal cells using DMSO and quantified spectrophotometrically. To compare the absorbance spectra of cells with and without pigment, the bleached, white algal cell pellet after DMSO extraction was resuspended in 2 ml dH_2O. Scans of absorbance across the range of visible wavelengths were carried out on an original culture sample, the pigment extract, and the bleached cells, each diluted to a maximum OD of less than 1.

3.3.4 Quantification of bacterial contamination

Algal cultures used in this work were monocultures, but not axenic. The bacterial load was monitored throughout the growth cycle by spotting 10 μl of serial dilutions of the algal culture onto LB-agar plates. The lowest dilution at which colonies were formed was used to estimate the

bacterial cell count. The bacterial DW was estimated by multiplying the cell count by the average weight of a bacterial cell (taken to be 2.9 x 10^{-13} g (Neidhardt *et al.* 1990)).

3.4 Results and discussion

3.4.1 Influence of absorbance by pigments on the absorbance spectrum of whole cells

To determine the influence of algal pigments on the absorbance of whole algal cells, absorbance across the range of visible wavelengths was determined for *C. vulgaris* cells, as well as cells from the same culture that had been bleached by the extraction of pigment (largely chlorophyll *a* and *b*). Bleached cells were examined by microscopy to confirm that they were intact and had similar morphologies to unbleached cells. The pigment extract was also scanned. Maxima in the absorbance of whole cells were found at wavelengths of 443, 487 and 684 nm (Figure 3.2). These correlated closely with those of the pigment extract (436, 461 and 667 nm). The scan of whole cells that had been bleached by pigment extraction did not exhibit any absorbance maxima. This indicated that the peaks in absorbance of *C. vulgaris* cells in the region of 440, 480 and 680 nm were due to absorbance by the pigments contained in the cells.

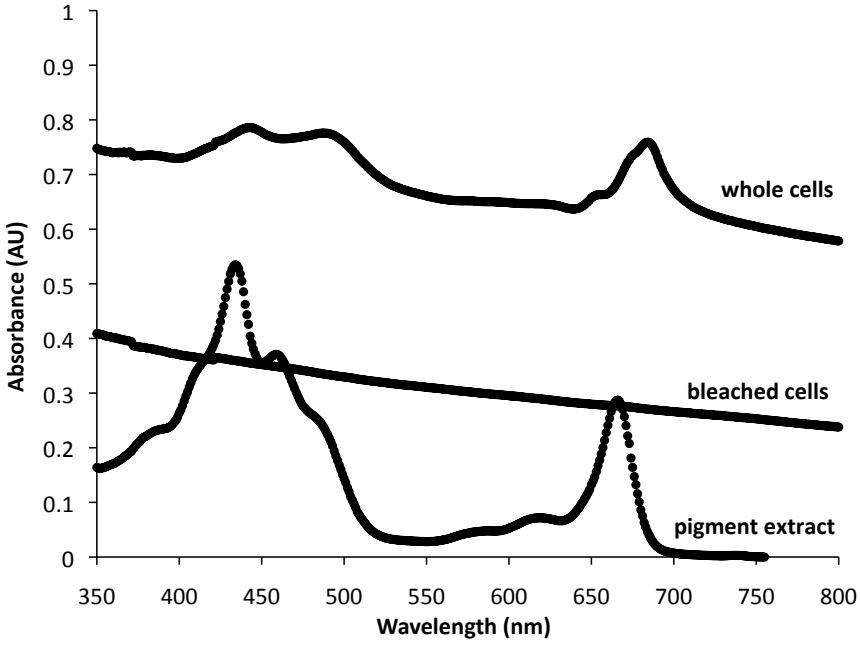


Figure 3.2 Visible wavelength scans of whole *C. vulgaris* cells, cells bleached following pigment extraction, and the pigment extract.

3.4.2 Effect of pigment content on the correlation between DW and absorbance at 680 nm and 750 nm

In order to test the effect of pigment content on the absorbance of whole *C. vulgaris* cells, the pigment content of *C. vulgaris* was manipulated by varying the nitrate concentration in the media. From Figure 3.2, the wavelengths chosen to represent maximum and minimum pigment absorbance were 680 and 750 nm respectively (550 nm has also been suggested as an absorbance minimum (Becker 1994)). Samples of *C. vulgaris* cultures grown under high and low pigment conditions for 14 days were used to generate standard curves of DW as a function of OD at 680 nm and 750 nm (Figure 3.3). Absorbance at 680 nm was higher (slope of the regression line 3.9) in the culture with a high pigment content (CvH) than in the culture with a low pigment content (CvL; slope 3.3). Absorbance at 750 nm was much less affected by pigment content (slope of the regression lines 3.3 and 3.2 for CvH and CvL respectively). In the low pigment content culture, the standard curves for both wavelengths were similar (slopes 3.3 and 3.2 for 680 nm and 750 nm respectively). This indicated that, for a given biomass concentration, absorbance at 680 nm was greater if the cells contained a high concentration of pigment, while absorbance at 750 nm was similar despite differences in pigment content.

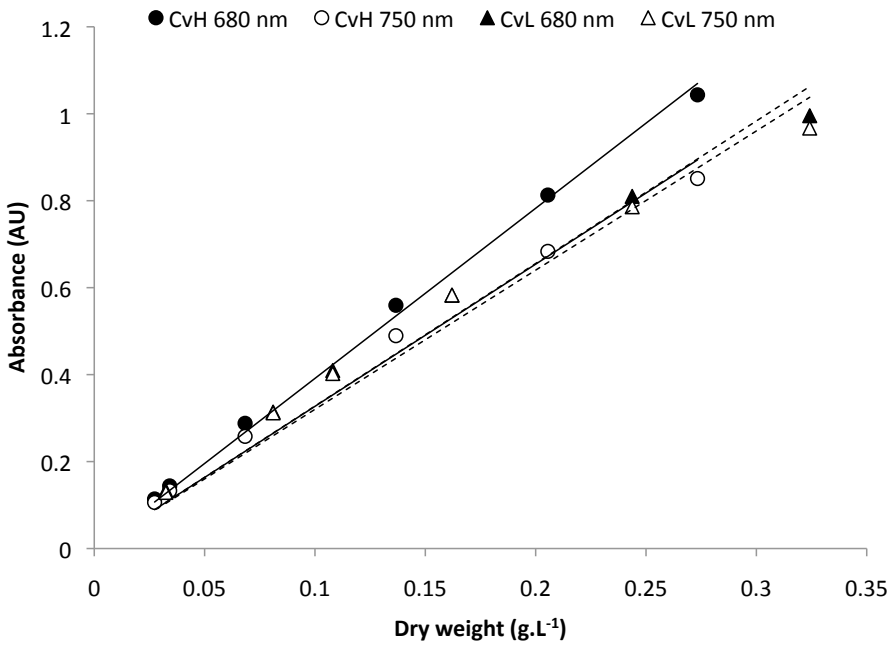


Figure 3.3 Standard curves of absorbance at 680 (solid symbols, solid lines) and 750 nm (empty symbols, dashed lines) as a function of dry weight for dilutions of high pigment (CvH, ●) and low pigment (CvL, ▲) containing *C. vulgaris* cultures. Error bars indicating standard deviation in the OD readings are too small to be seen.

3.4.3 Changes in pigment content and absorbance over the growth cycle

The greater absorbance at higher pigment contents could be taken into account by the use of an appropriate standard curve to convert OD to DW, if the pigment content of the cells were constant. However, the pigment content of microalgal cultures is known to vary with culture age and environmental conditions (Healey 1975; Nicholls & Dillon 1978). In order to investigate the magnitude of the change in pigment content and absorbance over the growth cycle of *C. vulgaris*, cultures were grown under high (CvH) and low (CvL) pigment conditions, and the DW and OD at 680 nm and 750 nm were recorded every day for 17 days. The absorbance data were normalised to 1 g.L^{-1} DW in order to elucidate any changes. Relative absorbance at 680 nm and 750 nm, along with the pigment content, is shown in Figure 3.4a and Figure3.4b for the CvH and CvL cultures respectively.

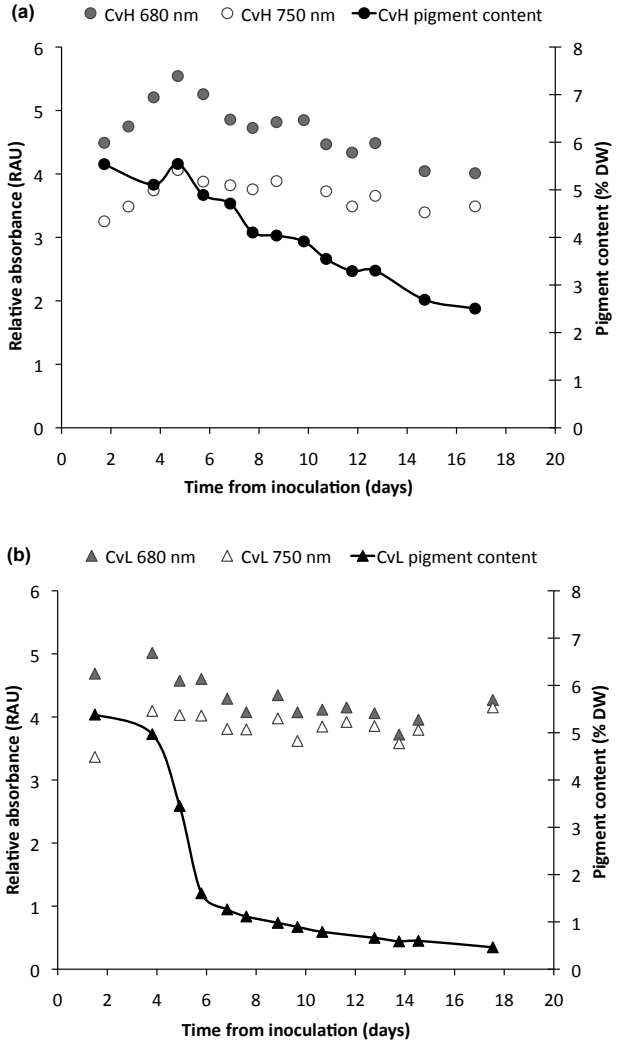


Figure 3.4 Pigment content and absorbance at 680 and 750 nm, normalised to 1 g.L^{-1} dry weight, during growth of *C. vulgaris* under (a) nitrogen replete (CvH) and (b) nitrogen limited (CvL) conditions.

The pigment content of *C. vulgaris* cells varied between 5.5 and 2.5% DW (decreasing with culture age) in the nitrogen replete culture. Pigment content varied even more widely (5.4 to 0.5%) in the nitrogen limited culture, decreasing dramatically with the onset of nitrogen limitation (day 3 to 5). The relative absorbance at 750 nm remained fairly constant (between 3.2 and 4.2 absorbance units per g dry biomass) over the growth cycle for both the CvH and CvL cultures, despite the variation in pigment content. The relative absorbance at 680 nm was higher (3.7 to 5.5 absorbance units per g dry biomass) and showed a peak (5.5 and 5 for the CvH and CvL cultures respectively) correlating with the maximum pigment content. The absorbance at 680 nm decreased with the decrease in pigment content over the growth cycle and was consistently lower for the CvL culture. When the pigment content of the cells was very low (less than 1% DW in CvL in the late growth phase), absorbance at 680 nm was similar to that at 750 nm.

3.4.4 Standard curves

The absorbance per unit biomass at 680 nm changed significantly over a normal microalgal growth cycle, and was related to the change in pigment content of the cells. The change in the correlation between OD and DW over the growth cycle was minimised, but not eliminated, by using a wavelength outside of the range of pigment absorption (e.g. 750 nm). Hence, in order to accurately estimate DW from OD, a standard curve corresponding to the culture age and conditions at which the measurements were taken becomes necessary. The use of a standard curve generated under high pigment conditions to convert OD measured under low pigment conditions to DW, would lead to an under-estimation of DW. Similarly, a standard curve generated under low pigment conditions would overestimate DW under high pigment conditions. The following experiments were conducted to investigate the magnitude of the error should standard curves generated under different conditions, or at different time points, be used:

Using standard curves generated under different growth conditions

Standard curves of absorbance as a function of DW in *C. vulgaris* cultures grown under very high pigment conditions (GRO-LUX lights) and low pigment conditions (low nitrogen) for 14 days were used to estimate the DW in a normal pigment content (CvH) culture (Figure 3.5). Using the standard curve from a very highly pigmented culture resulted in an underestimation of the actual DW (by up to 12% for 680 nm and 5% for 750 nm) between days 12 and 17. Using the standard curve from the low pigment culture, the actual DW was overestimated by an average of 52% over the growth cycle, with maximum error (up to 74%) between days 2 and 6, when absorbance was measured at 680 nm. Similar trends were seen at 750 nm, but the errors were less substantial (average of 25% and max 34%).

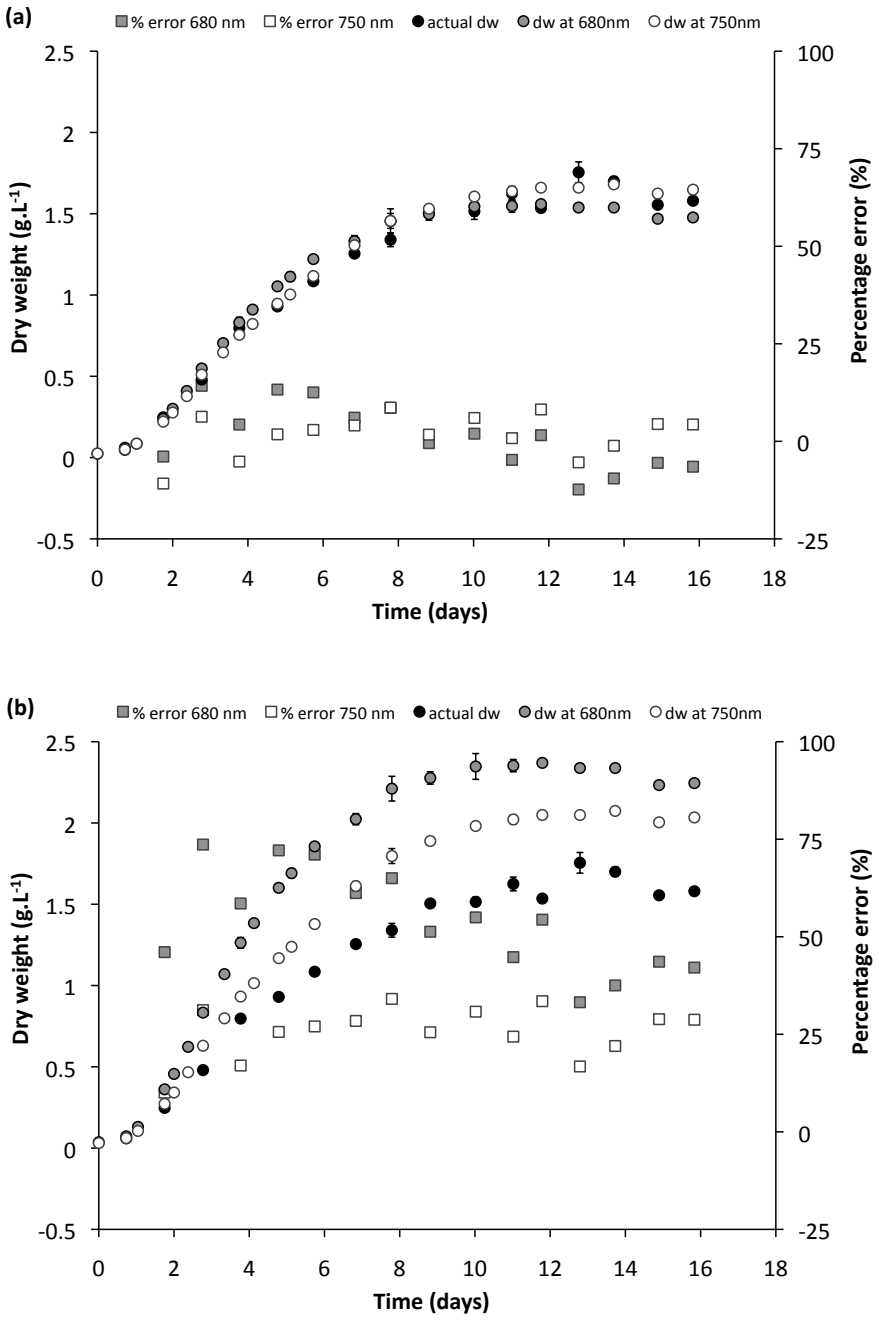


Figure 3.5 Actual dry weight and dry weight determined from absorbance at 680 and 750 nm, using standard curves generated after 14 days in (a) a very high pigment content culture (grown with GRO-LUX lights) and (b) a very low pigment content culture (low nitrogen). The percentage error in the estimations of dry weight from OD readings at 680 and 750 nm are shown by the filled and empty squares respectively.

Using standard curves generated at different time points in the growth cycle

Standard curves of OD as a function of DW were generated at 3, 7, 10 and 14 days for the CvH culture (Figure 3.6). The pigment content reached a maximum between days 2 and 6 and decreased steadily thereafter. There was a decrease in the absorbance at 680 nm over time, as the pigment content of the cells decreased, whereas the absorbance at 750 nm remained fairly constant over time.

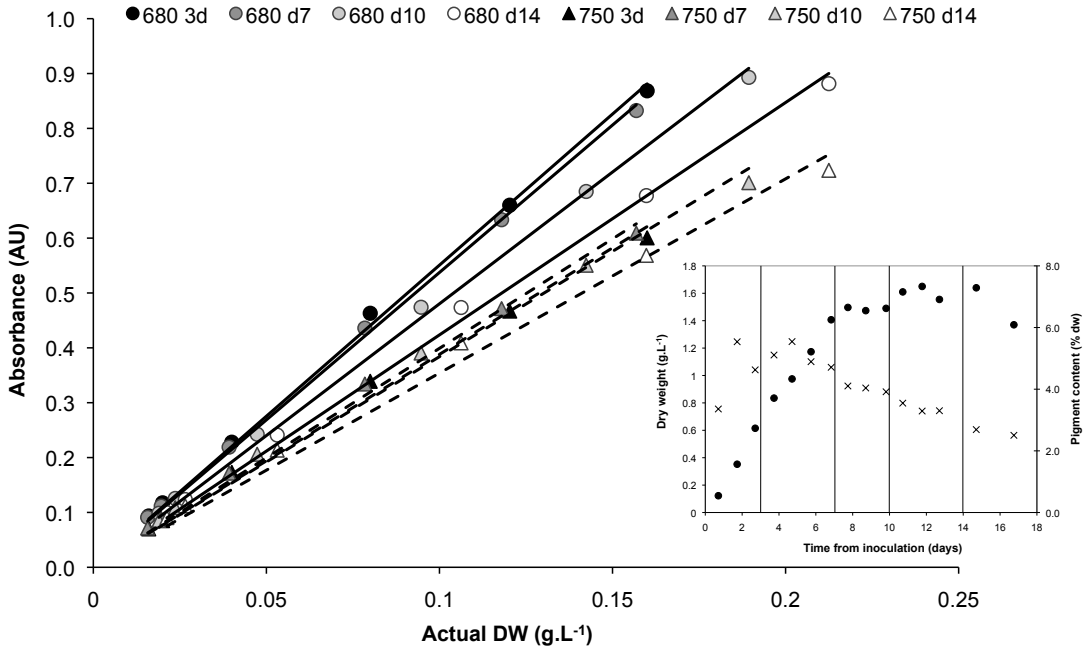


Figure 3.6 Standard curves of absorbance (680 (●, solid lines) and 750 nm (▲, dashed lines)) with respect to dry weight of a high pigment content *C. vulgaris* culture generated after 3, 7, 10 and 14 days growth. Inset shows dry weight (●) and pigment content (×). Vertical lines indicate when standard curves were generated.

The standard curves in Figure 3.6 were used to calculate the DW from the OD at 680 and 750 nm during growth of the CvH culture. A standard curve across the entire growth cycle, using OD and DW measurements taken every day, was also used. The average and maximum errors in calculated DW, relative to actual DW measurements, are shown in Table 3.2. Overall, calculated biomass concentrations using OD at 750 nm were more accurate than at 680 nm. Standard curves generated during exponential and early stationary phase were more accurate over the entire experimental period than those generated in late stationary phase (towards the end of batch culture). The standard curve generated under conditions of reduced nitrogen availability (known to decrease pigment content) was the least accurate, with maximum errors of 74 and 34% for 680 nm and 750 nm respectively. The standard curve generated across the entire growth cycle was the most accurate (average errors of 8 and 4% for 680 nm and 750 nm respectively).

Similar trends were shown with the green alga *Scenedesmus* sp., the marine Eustigmatophyte *Nannochloropsis* sp. and the cyanobacterium *S. platensis* (Table 3.3). The direction and magnitude of the error was related to the difference in pigment content between the conditions of standard curve generation and biomass quantification. Generally, measurements were more inaccurate the further the time point was from the time of the generation of the standard curve. Hence, generation of a standard curve towards the middle of the growth period should minimise error. Overall, the use of 750 nm instead of 680 nm reduced the average relative error in DW estimation by 42%.

Table 3.2 Errors in DW calculated from OD at 680 and 750 nm, relative to actual DW measurements, over a 17 day growth cycle of *C. vulgaris*. Average and maximum percent errors across the growth cycle are shown using standard curves generated at days 3, 7, 10 and 14 in the CvH growth cycle, day 14 of the CvL growth cycle, and using a standard curve across the entire CvH growth cycle. The time point at which the maximum error occurred is indicated in brackets.

Using standard curve generated at day	Ave. error 680 nm (%)	Max. error 680 nm (%)	Ave. error 750 nm (%)	Max. error 750 nm (%)
3	11	21 (day 13)	6	10 (day 12)
7	9	19 (day 13)	5	7 (day 12)
10	9	18 (day 3)	6	9 (day 3)
14	18	34 (day 3)	13	21 (day 8)
GRO-LUX	8	12 (day13)	5	8 (day 12)
CvL 14	52	74 (day 3)	25	34 (day 8)
Over growth cycle	8	15 (day 3)	4	6 (day 12)

Table 3.3 Average errors in DW calculated from OD at 680 and 750 nm, relative to actual DW measurements, across the growth cycle for *Scenedesmus* sp., *S. platensis* and *Nannochloropsis* sp. using standard curves generated at days 3, 7, 10 and 14 in the high nitrate (1500 mg.L^{-1}) growth cycle, day 14 of the low nitrate (150 mg.L^{-1}) growth cycle, and using a standard curve across the entire high nitrate growth cycle.

Using standard curve generated at day	*Scenedesmus* sp. ave. error (%)		*S. platensis* ave. error (%)		*Nannochloropsis* sp. ave. error (%)	
	680 nm	750 nm	680 nm	750 nm	680 nm	750 nm
3	15	5	13	14	19	7
7	14	7	16	16	10	5
10	11	7	13	7	10	8
14	15	8	13	8	10	7
Low pigment 14	48	22	38	21	24	8
Over growth cycle	11	5	14	8	10	5

3.4.5 Additional sources of error

The errors attributed to differences in cell physiology should be evaluated relative to sources of error in the actual DW and OD measurements themselves. The average relative error in duplicate

OD measurements was less than 2%. The average relative error in the duplicate DW measurements presented here was 4%. In all but one case, the error in DW estimation from OD in the four species examined here was greater than the error of the OD and DW assays combined.

Another potential source of error to be considered is that dry weight measurements do not exclude contributions to the mass by contaminants such as bacteria. In these cultures, bacteria contributed a maximum of 4% to the DW (estimated based on bacterial cell concentration and typical cell mass). This was within the standard error of the DW measurements. If, however, samples contain significant contamination by non-photosynthetic biomass (for example in environmental samples, or commercial open-air ponding systems, where the medium contains an organic carbon source), then a measurement related to chlorophyll may be helpful in differentiating the contribution of the algal biomass.

Changes in optical properties other than pigment content, such as cell size, may also influence absorbance. Cell size was monitored during growth of the *C. vulgaris* cultures. No significant difference in diameter over the course of a growth cycle was found, and no correlation with absorbance noted. Stephenson (2009) also reported little variation in the size of *C. vulgaris* cells before and after N limitation.

3.5 Conclusion

The pigment content of cells affects their optical properties, which alters the correlation between absorbance and dry weight. The average relative error across the growth cycle between DW measured directly and that estimated from OD was up to 52% using 680 nm and 25% using 750 nm. This effect could be mitigated by use of a suitable standard curve if the pigment content of the cells remained constant. However, the pigment content of microalgae has been shown to vary over the growth cycle and with different environmental conditions. From the data presented here, strategies to minimise error in estimates of biomass concentration from OD include:

1) Using a wavelength outside the range of absorbance by the pigments. For cells where chlorophyll is the major pigment, wavelengths of 750 nm or 550 nm can be used. Across the four species tested, the use of 750 nm instead of 680 nm reduced the average relative error in DW estimation by 42%.
2) Using standard curves generated during exponential or early stationary phase, preferably towards the middle rather than the end of the experiment. Using a standard curve in early stationary phase (day 10) reduced the average relative error by 12% relative the use of standard curves at days 3, 7 and 14.
3) Using a standard curve generated across the growth cycle (for example by taking dry weight measurements every three to four days). This reduced the average relative error by 29% compared to the use of standard curves generated at any single point in the growth cycle.

4 Lipid quantification: Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae

4.1 Introduction

Accurate measurement of the lipid content of cells is a critical tool in the study of lipid productivity. Lipid productivity is calculated from biomass concentration and lipid content and therefore the validity of any evaluations or predictions of lipid productivity are based on the accuracy of these two measurements. Issues in the measurement of biomass concentration were discussed in Chapter 3. This chapter investigates and compares methods of lipid quantification in algal cells, and presents direct transesterification (DT) as a convenient and accurate method. The results of DT on three algal species were compared to those obtained by extraction using the Folch, the Bligh and Dyer, and the Smedes and Askland methods. The effects of using a sequential combination of base and acid catalysts, as well as the influence of water, on transesterification efficiency were investigated. The effect of drying of the algal biomass and storage of samples under different conditions was also considered.

4.2 Literature review

Measuring the fatty acid content of microalgae is an important assay in many fields. The fatty acid content of cells reflects their energy content as a feed or fuel (Pulz & Gross 2004; Spolaore *et al.* 2006), as well as the yield of products such as long chain polyunsaturated fatty acids, lipid extracts for pharmaceuticals, cosmetics or nutraceuticals (Apt & Behrens 1999; Pulz & Gross 2004) and fatty acid methyl esters (FAME) for biodiesel. Currently there is little consensus on the best methods of total lipid or fatty acid determination, and with increasing interest in microalgal biotechnology, there is a need for the accurate quantification of lipids and fatty acids in microalgae.

Numerous methods are used to assay microalgal lipid content. Most studies have used solvent extraction to remove lipids from the cells, followed by quantification by weighing or chromatography (Ben-Amotz *et al.* 1985; Mansour *et al.* 2005; Patil *et al.* 2006; Pratoomyot *et al.* 2005; Reitan *et al.* 1994; Renaud *et al.* 1994; Shifrin & Chisholm 1981; Suen *et al.* 1987; Volkman *et al.* 1989; Widjaja *et al.* 2009). Solvent extractions from microalgae are typically based on the methods using chloroform and methanol published in the 1950's by Folch *et al.* (1956) and Bligh and Dyer (1959). It is well recognised that solvent extraction often results in incomplete lipid

extraction, particularly of free fatty acids, and can extract significant quantities of non-nutritive, non-saponifiable material such as pigments (Palmquist & Jenkins 2003; Pruvost *et al.* 2009; Ratledge 1987). Small modifications in the protocol can have a large effect on extraction efficiency (Iverson *et al.* 2001). Smedes and Askland (1999) tested various ratios of chloroform and methanol, described by the Bligh and Dyer method, and suggested that the yield of lipid could be increased by using a higher methanol content. This implied that the original Bligh and Dyer method resulted in incomplete extraction. Iverson *et al.* (2001) compared the Bligh and Dyer and the Folch methods for a range of tissues from marine organisms, and concluded that the Bligh and Dyer method significantly underestimated the lipid content of samples containing more than 2% lipid. The effect was aggravated by increasing lipid content, resulting in an underestimation of 50% compared to the Folch method in the samples with the highest lipid content.

Gas chromatography (GC) can be used to quantify individual fatty acids as well as the total fatty acid present in a lipid extract. GC of lipids requires derivatisation to volatilise the samples (Liu 1994). This is normally achieved by converting saponifiable lipids (in biological samples, mainly triacylglycerols (TAG) and phospholipids) to FAME by addition of an excess of methanol and a catalyst, in a reaction known as transesterification (Carrapiso & García 2000; Liu 1994). This eliminates the problem of extraction of non-fatty acid substances, such as pigments, as GC does not detect these, but the accuracy of quantification is still dependent on the completeness of the extraction.

Instead of improving extraction methods, some investigators have eliminated extraction completely by transesterifying lipids *in situ*. Direct transesterification (DT) was first successfully performed by Abel and co-workers (1963). Since then, it has been verified by numerous researchers in a variety of tissues, as a simple and rapid method of quantifying fatty acids by combining extraction and transesterification into one step (Figure 4.1).

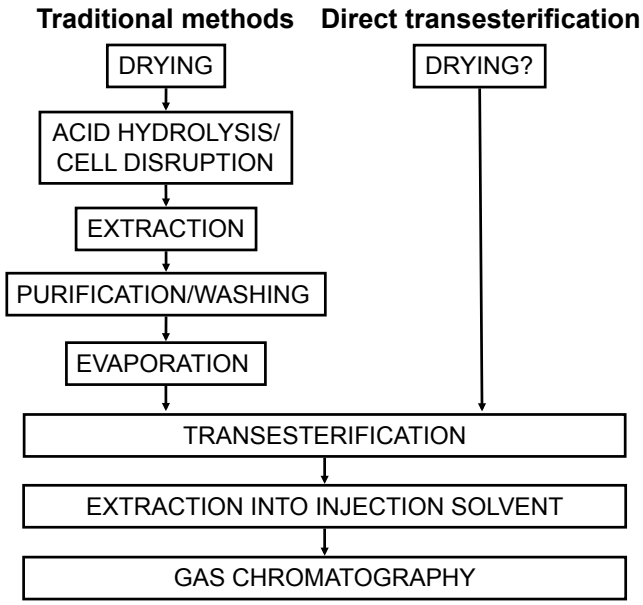


Figure 4.1 Classical techniques of lipid quantification by extraction and gas chromatography, compared to direct transesterification.

Diverse methods have been used for DT, but most involve the addition of an organic solvent, methanol, catalyst and heat to a small amount of dried sample (see Liu (1994) and Carrapiso and Garcia (2000) for comprehensive reviews of this). DT has previously, although infrequently, been applied to the quantification of fatty acids in microalgae (Table 4.1), however, no evaluation or comparison to alternative methods has been made.

Transesterification (reaction 1) involves cleaving of an ester bond by an alcohol (typically methanol). When FAMEs are formed by reaction of an alcohol with a free fatty acid, as opposed to a complex lipid, the reaction is simply esterification (reaction 2). Transesterification can be catalysed by either a base or an acid, while esterification by an acid only (Carrapiso & García 2000).

$$R-COO-R' + CH_3OH \xleftrightarrow{acid\,/\,base} R-COO-CH_3 + R'-OH \quad (1)$$

$$R-COOH + CH_3OH \xleftrightarrow{acid} R-COO-CH_3 + H_2O \quad (2)$$

Table 4.1 Direct transesterification protocols used for total fatty acid determination in microalgae and similar samples. The first entry shows the frequently referenced technique of Lepage and Roy (1984).

Reference	Sample	Drying	Catalyst	Solvent	Incubation
Lepage & Roy 1984	Human milk and adipose tissue	Dried under N_2 or freeze-dried	Acetyl chloride	Benzene	100°C 1 h
Montaini 1995	*Tetraselmis suecica*	Fresh or frozen	Acetyl chloride	Benzene	100°C 1 h
Rodríguez-Ruiz *et al.* 1998	Cod-liver oil, *Porphyridium cruentum* and *Phaeodactylum tricornutum*	Freeze-dried	Acetyl chloride	Hexane	100°C 10 min
Sönnichsen & Müller 1999	Fungus *Mucor circinelloides*	Freeze-dried	Hydrochloric acid	Hexane	100°C 1h
Lewis *et al.* 2000	Microheterotrophs	Freeze-dried	Hydrochloric acid	Chloroform	90°C 15 to 120 min
Wen & Chen 2001	*Nitzschia laevis*	Freeze-dried	Acetyl chloride		
Blokker *et al.* 2002	*Selanstrum chaetoceras* and *Tetraedron minimum*	Dried under N_2	BF_3	None	80°C 30 min
Carvalho *et al.* 2006b	*Pavlova lutheri*	Freeze-dried	Acetyl chloride		
Fajardo *et al.* 2007	*Phaeodactylum tricornutum*	Freeze-dried	Acetyl chloride	Benzene	100°C 1 h
Armenta *et al.* 2009	*Chlorella* and *Spirulina*	Freeze-dried	Hydrochloric acid	Chloroform	90°C 120 min
Hsieh & Wu 2009	*Chlorella*		BF_3		100°C 15 min
Johnson & Wen 2009	*Schizochytrium limacinum*	Freeze-dried and wet	Sulphuric acid	Chloroform	90°C 40 min

In order to shift the reaction equilibrium towards ester production, either an excess of alcohol, or removal of one of the products, is required. The presence of water in the reaction system can cause hydrolysis, the opposite of esterification (reverse of reaction 2 for acid catalysts or reaction 3 for base). Reaction 3 is irreversible because the carboxylate anion does not react with alcohol, but reacts readily with sodium or potassium to form soap (reaction 4), referred to as saponification (Liu 1994).

$$R-COO-R' + H_2O \xrightarrow{base} R-COO^- + R'-OH \quad (3)$$

$$R-COO^- + Na^+ \rightarrow R-COO-Na \quad (4)$$

The rate of the transesterification reaction depends not only on the catalyst and reaction conditions, but also the solubility of the lipids in the methanol reaction medium, which defines reactant concentration. Polar lipids such as phospholipids and free fatty acids dissolve well in

methanol, but nonpolar lipids such as TAG are poorly soluble, therefore it is necessary to add another solvent to the reaction system. Solvents used include benzene, toluene, diethyl ether, chloroform, dichloromethane, tetrahydrofuran and hexane (see Liu, 1994 and Carrapiso & Garcia, 2000) for more information on effects of solvent type and methanol ratio). Upon completion of transesterification, FAMEs are transferred to an organic solvent such as hexane to purify them for GC, and to minimize any further hydrolysis reactions (Liu 1994).

Usually a single catalyst is used and the choice is determined by the characteristics of the different catalysts. Alkaline catalysts, such as sodium methoxide, or sodium or potassium hydroxide in methanol, transesterify complex lipids quickly and at lower temperatures than required by acid catalysts, but they do not esterify free fatty acids (Liu 1994). Acid catalysts, such as hydrochloric or sulphuric acid in methanol or boron trifluoride (BF_3) methanol, require heating and longer reaction times than basic catalysts (Liu 1994), but can transesterify complex lipids as well as esterifying free fatty acids (Liu 1994). Considering the different capacities of acid and base catalysts, some investigators have used a combination of a basic catalyst or alkaline hydrolysis with NaOH or KOH in methanol, followed by an acid catalyst (Carrapiso & García 2000; Liu 1994). Metcalfe *et al.* (1966) first reported the use of alkaline hydrolysis followed by BF_3 esterification, resulting in a rapid and efficient procedure. This study investigated whether the sequential use of two catalysts would improve the efficiency of DT in microalgae, particularly in the presence of water.

Water is known to interfere with the transesterification reaction (Carrapiso & García 2000; Liu 1994) as it is a stronger electron donor than methanol. To avoid interference by water, it has been suggested that the sample be dried, or a water scavenger such as 2,2-dimethoxypropane added (Carrapiso & García 2000). However, there are reports which dispute the need for these practices below a critical water content. Lepage and Roy (1986), using the catalyst acetyl chloride, found recoveries (> 95%) to be unaffected by the presence of water up to 5% of the total reaction volume, while a water content of 10 to 15% significantly impaired the reaction. Most studies using DT for quantification of fatty acids in microalgae have used freeze-dried biomass, although there has been no investigation as to whether this is necessary.

4.3 Methods

4.3.1 Analytical reagents

All reagents used were of chromatography standard. Toluene (99.9%) and chloroform (99.8%) were from Sigma-Aldrich (USA) and hexane (98%) and methanol (99.9%) from Merck (Germany). Methanolic base (0.5N), also known as sodium methoxide (SM), and boron trifluoride (BF_3) methanol solution (14%) were obtained from Sigma-Aldrich (USA). Distilled water (dH_2O)

from a Millipore system was used for all analyses. Internal standards used were glyceryl triheptadecanoate (C17-TAG) and methyl nonadecanoate (C19-ME) from Sigma-Aldrich (USA).

4.3.2 Biomass

Two freshwater Chlorophyta (green algae): *Chlorella vulgaris* (Cv) and *Scenedesmus* (Sc), and a marine Eustigmatophyte: *Nannochloropsis* sp. (Nan) were cultured as described in Section 2.3. Cells were harvested in stationary phase. The culture was centrifuged at 1520 g for 10 min, rinsed in either distilled water (for freshwater cultures) or sterile seawater (marine cultures) and re-centrifuged. Biomass used in all the experiments was from a single batch of each species.

4.3.3 Determination of biomass water content

The wet pellet following centrifugation was weighed and resuspended in a known volume of distilled water or sterile seawater. A sample of this biomass concentrate corresponding to 0.1 g wet weight was filtered through a pre-weighed 0.45 μm Millipore filter and dried at 80°C overnight. After cooling to room temperature in a desiccator, filters were weighed to determine the relative dry biomass and water content. The 0.1 g wet weight samples of Cv, Sc and Nan corresponded to 19.6 ± 0.31, 19.8 ± 0.12, and 27.4 ± 0.85 mg dry weight respectively.

4.3.4 Extraction

All extractions and DTs were performed in triplicate, using samples of 0.1 g wet weight algal biomass. The control for each experiment was 80 μl dH_2O. Total lipid was extracted by the Bligh and Dyer method (Bligh & Dyer 1959), the Smedes and Askland method (Smedes & Askland 1999) and the Folch method (Folch *et al.* 1957). Each sample was extracted three times and the resulting solvent volumes combined. The combined extract was dried at room temperature overnight, transesterified and subjected to GC analysis for quantification of fatty acids. The residual biomass from each extraction was recovered and tested for any remaining fatty acids by DT.

Extraction protocols from the original references were followed as closely as possible, with the following two modifications:

- Sample size was scaled down by a factor of 1000, with extraction volumes scaled accordingly.
- Homogenisation was replaced by vortexing at maximum speed for 3 min.

According to Folch *et al.* (1956): "the procedure can be run on any scale that is otherwise technically feasible". In order to verify that the scale of the reaction did not affect the results, the three extraction techniques and DT were repeated in triplicate using 1 g of Sc biomass (i.e. volumes scaled down by a factor of 100). Results were found to be very similar to extractions from 0.1 g (data not shown).

Bligh and Dyer method

Samples were centrifuged (16000 g for 3 min) in Eppendorf tubes and the supernatant was discarded. Methanol (200 µl) and chloroform (100 µl) were added to the pellet, followed by vortexing at maximum speed for 3 min. A further 100 µl chloroform and then 100 µl dH_2O were added, with samples vortexed for 30 sec after the addition of each, and centrifuged for 3 min at 16000 g to separate the layers. The lower, organic layer was transferred to a glass test tube with silicone-lined screw-cap lid (Kimax, USA). Chloroform (200 µl) was added to the remaining cell pellet and aqueous phase in the Eppendorf tube, and the mixture vortexed for 3 min. Organic and aqueous layers were separated by centrifugation and the organic layer added to the first extract. This process was repeated three times for each sample. The combined extract was dried at room temperature overnight. The extract was then transesterified and subjected to GC analysis for quantification of fatty acids. The residual aqueous layer, containing the biomass, was resuspended in dH_2O, transferred to a glass tube and re-centrifuged at 1520 g for 10 min. Direct transesterification was then performed on this residual material to test for any remaining fatty acids.

Smedes and Askland method

This method is similar to the Bligh and Dyer method, except that 250 µl methanol, 200 µl chloroform and 50 µl water were added in a single step to the wet algal pellet and vortexed for 3 min. Layers were separated by centrifugation and the bottom layer transferred to a glass tube. Three chloroform washes and recovery of residual material were performed as above.

Folch method

Samples were centrifuged (1520 g for 10 min) in glass test tubes with silicone-lined screw-cap lids, and the supernatant was discarded. Twenty times the sample volume (2 ml) of chloroform/methanol (2:1) was added and the mixture vortexed at maximum speed for 3 min before centrifugation at 1520 g for 10 min. The supernatant was removed to a new glass tube and fresh solvent added to the pellet. Extraction was repeated three times and the resulting solvent volumes combined. The combined extract was washed with 0.2 v/v 0.05 N NaCl. The water/chloroform/methanol mixture was vortexed and centrifuged briefly to separate the layers. The top water/methanol layer was removed with a Pasteur pipette and discarded. The lower chloroform layer was dried at room temperature in a fume hood overnight and then at elevated temperature briefly to accelerate evaporation of the solvent. The residual biomass was used for direct transesterification.

4.3.5 Transesterification

The final transesterification method, used in the work presented in the following chapters (protocol given in Section 2.4.3), differs from the method used in the initial testing phase presented here in two ways: 1) the catalysts used and 2) the addition of water scavenger. The

catalysts used in the testing phase were sodium methoxide and BF_3 methanol. These are toxic and expensive, hence a variety of other catalysts were tested in subsequent work. It was found that sodium hydroxide in methanol (0.5 N) and hydrochloric acid in methanol (5%) could be substituted for sodium methoxide and BF_3 methanol respectively, with 99% of the transesterification efficiency of the purchased catalysts. The data in Figure 4.3 indicate that, with a combination of catalysts, it is not necessary to add a water scavenger. Hence this step was omitted in the final protocol.

For DT, reagents were added directly to either internal standards, olive oil, or algal biomass. For DT of wet algal biomass, negative controls of 80 µl water were included, as well as a control for each algal species containing biomass but no internal standards, to test for the presence of C17 or C19 in the algal cells. For extraction-transesterification, reagents were added to the dried extract or residual biomass from lipid extractions. Two internal standards were used: glyceryl triheptadecanoate (C17-TAG) was added prior to the reaction as a quantitative internal standard, and methyl nonadecanoate (C19-ME) was added in the final solvent extraction step to verify the completeness of the transesterification and efficiency of extraction into the hexane layer. A combination of base followed by acid catalysis was performed as follows: samples were dissolved in 500 µl toluene containing 0.1 mg C17-TAG in glass test tubes with silicone-lined screw-cap lids. A volume (100 µl) of the water scavenger 2,2-dimethoxypropane was added. Sodium methoxide (1 ml) was then added and the samples mixed briefly by vortexing before being placed in an 80°C incubator, with shaking at 300 rpm for 20 min. Samples were cooled for 5 min to room temperature and 1 ml BF_3 methanol was added before repeating the incubation. After cooling for 5 min to room temperature, 400 µl dH_2O and 400 µl hexane containing 0.1 mg C19-ME were added and tubes mixed by vortexing. Tubes were centrifuged at 1520 g for 1 min and the upper hexane-toluene layer, containing the FAME extract, was transferred to vials for GC. GC was carried out as detailed in Section 2.4.3.

4.3.6 Catalyst tests

To test the efficiency of transesterification using a single catalyst, as opposed to a combination, C17-TAG samples (0.1 mg) were transesterified with 1 ml SM, 1 ml BF_3 methanol, or a combination of the two catalysts. The tests were repeated with the addition of 80 µl water (equivalent to the amount of water in the algal pellets), with and without 100 µl of the water scavenger 2,2-dimethoxypropane. The efficiency of transesterification was calculated by dividing the peak area of the resulting C17-ME, by the peak area of the C19-ME added post-reaction. As equal quantities of these two standards were added (the mass of the three methyl esters formed is very similar to that of the TAG), their ratio gave an indication of how much of the C17-TAG was converted to fatty acid methyl esters.

4.3.7 Accuracy, precision, and limits of detection and quantification

To test the accuracy, precision, linearity, and limits of detection (LOD) and quantification (LOQ) of the assay, triplicate samples of 10, 5, 2.5, 1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 mg 100% extra virgin olive oil (Borges, Spain) were made up in toluene. Negative controls containing no olive oil were included. All samples were transesterified and quantified by GC. To estimate inter-experimental error, fresh 5 and 2.5 mg samples were analysed on three consecutive days. The LOD was defined as the minimum concentration at which distinct peaks could still be discerned above the baseline noise. The LOQ was defined as the lowest concentration of fatty acid that could be quantified with an accuracy and precision (calculated according to Wu *et al.* (2009)) within 15%.

4.3.8 Sensitivity to water

The effect of water on the reactions was tested using 0.1 mg of the C17-TAG internal standard as substrate, with dH_2O addition up to 50% of the final total reaction volume. Transesterification was carried out using C19-ME as the internal standard, with or without 100 µl of the water scavenger 2,2-dimethoxypropane. Reactions were done in duplicate.

4.3.9 Storage and drying of algal biomass

To investigate the effect of storage conditions on fatty acid content, centrifuged wet algal pellets were stored in glass test tubes sealed with silicone-lined caps, either on the bench at room temperature, at 4°C or at -20°C for two different time periods: short-term (overnight) and long-term (16 days). Samples were also subjected to DT immediately, and the hexane extract stored with the rest of the reaction mixture in the glass reaction tube at room temperature for 1, 7 and 16 days. To investigate the effect of drying of algal biomass, algae was either dried in an oven at 50°C or 80°C overnight, or lyophilised for 24 hrs. It was found that oven-dried samples became hard pellets, hence grinding of the oven dried samples prior to DT was performed in an attempt to increase the surface area available for solvent penetration. Tests were carried out in duplicate.

4.4 Results and discussion

4.4.1 Direct transesterification versus extraction-transesterification

Total fatty acid content determined by DT and by the three extraction-transesterification methods in Cv, Sc and Nan are shown in Figure 4.2. For all three species, DT yielded a higher estimation of fatty acid content than any of the extraction-transesterification methods. The commonly used extraction method of Bligh and Dyer was the least effective extraction method tested. It significantly underestimated fatty acid content (by up to 81% in the case of Cv), accounting for 19 to 63% of fatty acids quantified by DT.

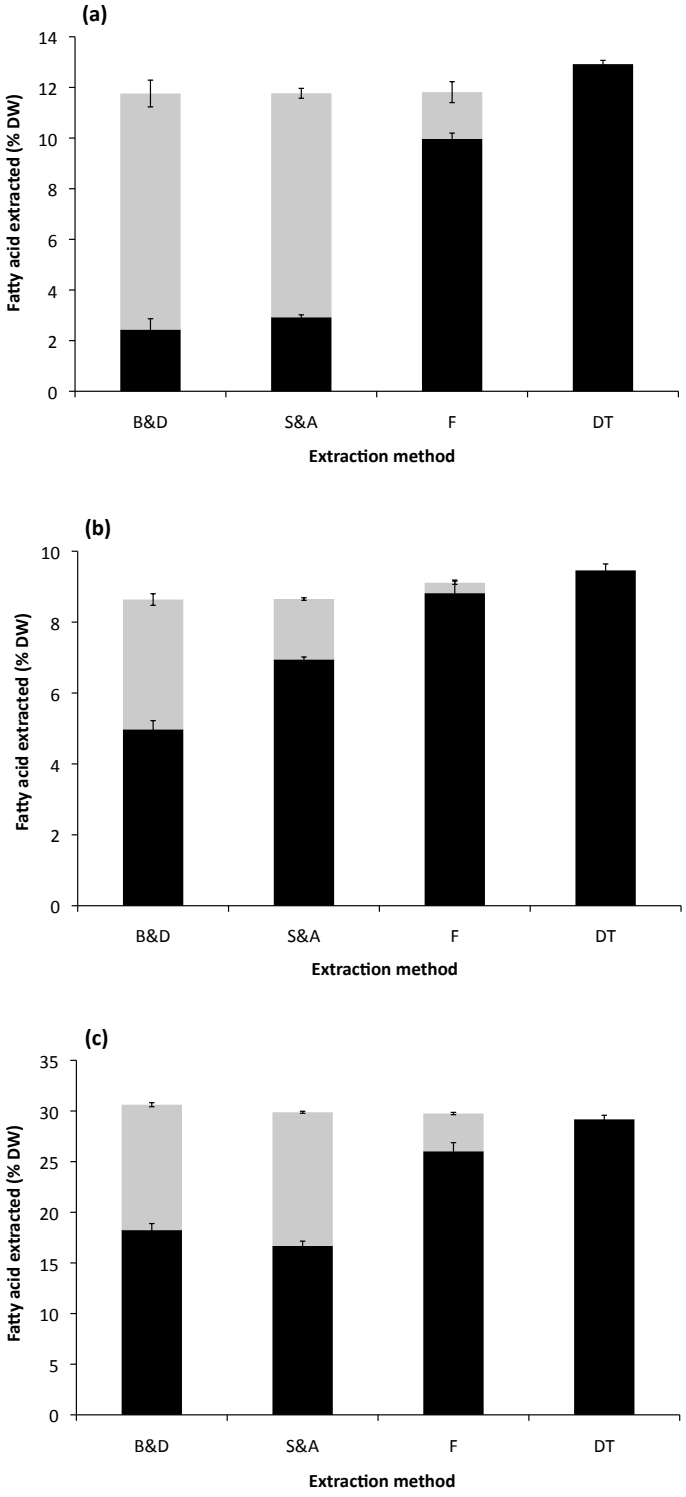


Figure 4.2 Fatty acid content (as percentage of the algal biomass dry weight) of (a) *Chlorella vulgaris*, (b) *Scenedesmus* sp. and (c) *Nannochloropsis* sp. measured by extraction with the Bligh and Dyer (B&D), Smedes and Askland (S&A) or Folch (F) methods, followed by transesterification and GC, or DT of the wet algal pellet. Black bars are the fatty acid content measured by the four different methods. Light grey bars are the fatty acids obtained by the DT carried out on the biomass residue from the extractions.

Smedes and Askland (1999) investigated the solvent ratios used by Bligh and Dyer and proposed that a greater proportion of methanol would result in a better recovery of lipids. This was true for the green algal species, as the Smedes and Askland method yielded 17 to 28% more fatty acids than the Bligh and Dyer method (although 6% less fatty acid than the Bligh and Dyer method for Nan). However, the Smedes and Askland method still underestimated lipid content, measuring 23 to 73% of the fatty acid content measured by DT. The Folch extraction method was the most effective, yielding 77 to 94% of the total extracted by DT. These findings are in agreement with Lepage and Roy (1984) who reported that fatty acid recovery from milk and adipose tissue was improved by DT due to the elimination of multiple extraction and purification steps. Fatty acid recoveries from samples of human milk and adipose tissue were 11 to 16% better than the Folch extraction. Iverson *et al.* (2001) also showed that the Bligh and Dyer method greatly underestimated lipid content compared to the Folch method in fish samples. DT on the residual biomass after the solvent extractions yielded additional fatty acids. The sum of fatty acids extracted plus fatty acids remaining in the residual biomass was similar to that obtained by DT, indicating that the extraction procedures did not extract all the fatty acids.

4.4.2 Single catalyst versus sequential combination of catalysts

The ratio of C17-ME to C19-ME following the sequential combination of a basic catalyst followed by an acid catalyst demonstrated the degree to which C17-TAG was transesterified (Figure 4.3). Values greater than 100% were obtained when the area of the C17-TAG peak was greater than that of the C19-ME peak. This could have been due to minor errors in dilution or pipetting of standard solutions.

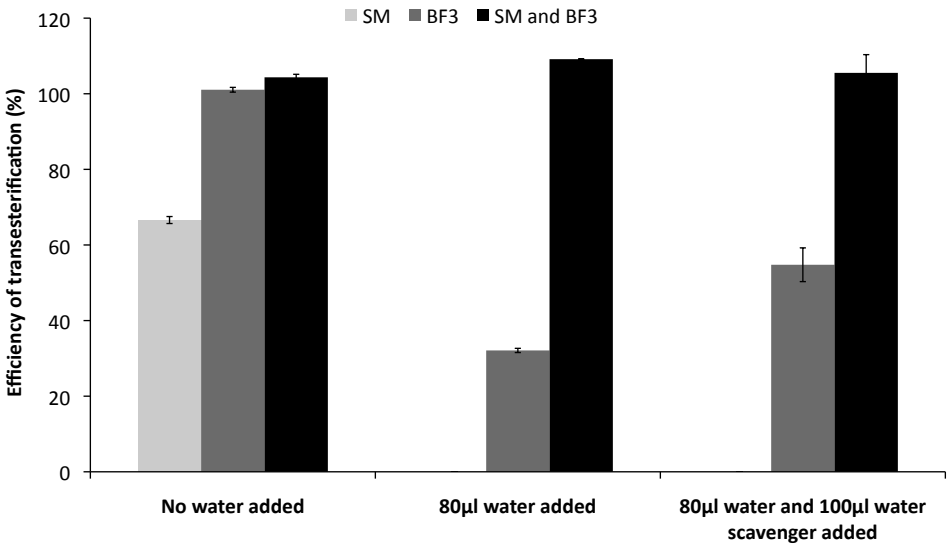


Figure 4.3 The efficiency of transesterification of glyceryl triheptadecanoate by the basic catalyst sodium methoxide (SM), the acid catalyst boron trifluoride-methanol (BF_3) and a combination of the two (SM and BF_3), compared under three different reaction conditions: anhydrous, with 80 µl water, and with 80 µl water and 100 µl water scavenger.

When the two reagents were used sequentially, 100% conversion of TAG to FAME was obtained in the presence or absence of water. In the absence of water, the acid BF_3 catalyst alone produced an equivalent degree of transesterification to both reagents together, but the basic SM catalyst resulted in only 67% conversion. In the presence of water, no transesterification occurred with SM, while with BF_3 32% conversion was obtained. The addition of a water scavenger to the reaction containing 80 µl of water improved the conversion with BF_3 to 55%, but did not improve SM conversion.

Thus, the sequential addition of a basic and acid catalyst improved the efficiency of DT, particularly when there was water present in the reaction. With the addition of an amount of water equivalent to that in the centrifuged microalgal samples (80 µl), the basic catalyst SM was completely inhibited and the activity of BF_3 was reduced, while the combination of catalysts yielded complete conversion. This might have been due to the basic catalyst, in the presence of water, resulting in saponification (or alkaline hydrolysis) and cleaving the ester bonds between fatty acids and glycerol. This removed the water and allowed the acid catalyst to esterify the free fatty acids at a faster rate (Liu 1994). If dry samples are used, BF_3 on its own is as effective as a combination of catalysts. However, under conditions where there is water present in the reaction, a combination of base and acid catalysts should be used, or alkaline hydrolysis should be performed before methylation.

4.4.3 Accuracy, precision and limits of detection and quantification

To test the accuracy, scalability and reproducibility of the DT assay, serial dilutions of olive oil in toluene were directly transesterified and quantified by GC. The slope of the linear regression line of fatty acid concentration determined from the assay to that added to the samples was 0.96 with a correlation coefficient of 0.9999. The LOD was 5 µg fatty acid, and the LOQ was 50 µg. For olive oil samples between 500 µg and 10 mg, the accuracy and precision of the assay were within 5% and 2.5% respectively. The inter-experimental accuracy and precision were both within 5%.

The assay was found to be accurate and reproducible to 5% above 0.5 mg and 15% (defined as the LOQ) above 0.05 mg. Assuming a relatively low fatty acid content of 10% dry weight and a biomass concentration of 1 $g.L^{-1}$, this LOQ equates to an algal sample of 0.5 mg dry weight or 2.5 mg wet weight.

4.4.4 Sensitivity to water

The addition of water up to 10% of the total reaction volume had little effect on the efficiency of the two-catalyst reaction (Figure 4.4). With the addition of larger volumes of water, the efficiency decreased dramatically and recovery was variable, as shown by the large error bars with

between 20 and 50% water. Results with and without the water scavenger below 10% water were similar. Above 30% water, reactions containing the water scavenger resulted in an increase in the efficiency of transesterification. However, this still represented a substantial under-estimation of the fatty acid content and there was a high degree of variation between samples.

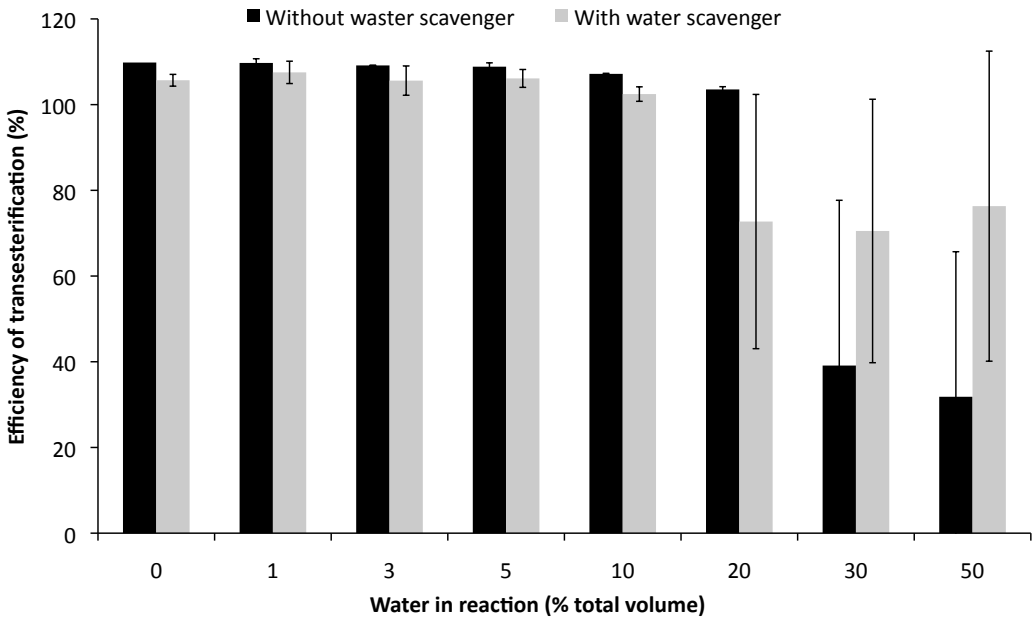


Figure 4.4 Efficiency of transesterification, measured by conversion of C17-TAG to C17-ME, as a function of reaction water content, with and without addition of a water scavenger.

The majority of previously reported DT protocols for microalgae used lyophilised biomass and a single acid catalyst (Armenta *et al.* 2009; Blokker *et al.* 2002; Carvalho *et al.* 2006b; Fajardo *et al.* 2007; Hsieh & Wu 2009; Rodríguez-Ruiz *et al.* 1998; Wen & Chen 2001). The two-catalyst transesterification reaction was unaffected by water up to 10% of the total reaction volume (290 μl). The water content of the algal samples contributed 80 μl of water (3% of total reaction volume) and was well within the acceptable range, thus centrifuged samples could be processed immediately without drying. It was not necessary to add a water scavenger at water contents less than 10%, although this may have been beneficial at high water contents (> 30%).

4.4.5 Effect of drying

Biomass is often dried prior to extraction or DT, therefore the effect on the fatty acid content of various drying methods was investigated. The fatty acid content of freeze-dried samples was on average 94% that of fresh samples, while samples dried in the oven at 50°C or 80°C and ground in a mortar and pestle were 86 and 87% of fresh samples, respectively (Figure 4.5). The fatty acid content of oven dried samples without grinding was 72% that of fresh samples.

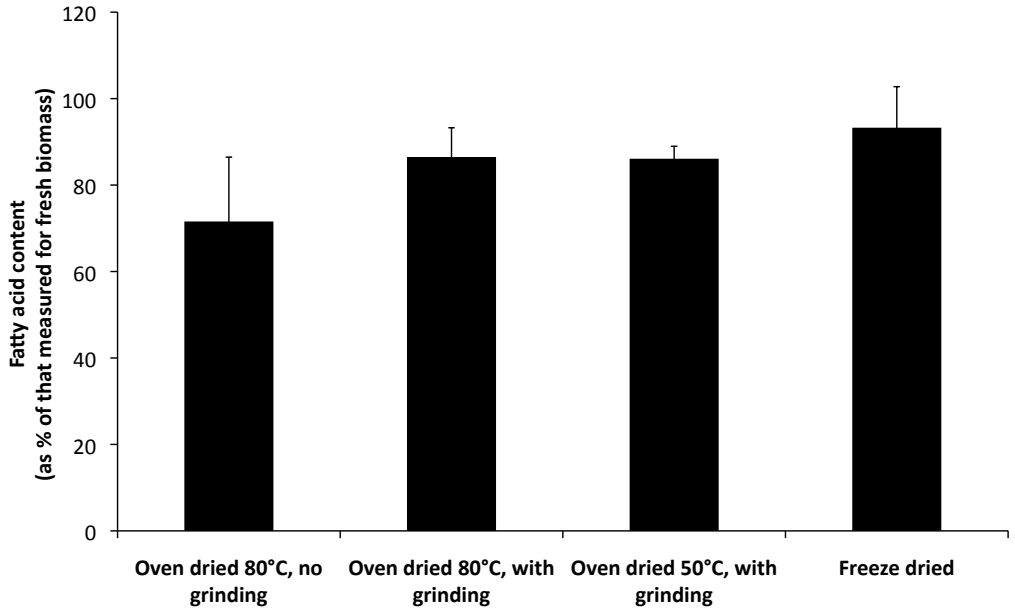


Figure 4.5 Fatty acid content of dried algal samples, normalised relative to the fatty acid content of fresh wet pellets from the same batch, as measured by DT. Results shown here are the average across three species (*Cv*, *Sc* and *Nan*), each tested in duplicate.

Drying of samples prior to analysis appeared to reduce the measured fatty acid content relative to fresh samples. Oven drying significantly affected the results obtained, resulting in a 14 to 28% underestimation of fatty acid content. This could be due to heat degradation (Carrapiso & García 2000), oxidation, polymerization and changes in unsaturation of fatty acids (Leszkiewicz & Kasperek 1988). In addition, oven-dried biomass became very hard, negatively influencing penetration of solvents into the algal cells. The above effects were avoided by freeze-drying, as this does not require heating and naturally forms a fine powder, maximising surface area for solvent contact. Widjaja *et al.* (2009) also reported a decrease in measured lipid content following the drying of biomass. The decrease was slight at a drying temperature of 60°C and significant at 80°C and 100°C, compared to drying at 0°C in a freeze-dryer. This appeared to be due to the oxidation of fatty acids upon exposure to high temperatures. As the DT reaction seems insensitive to water up to 10% reaction volume, it is recommended not to dry the biomass. If cells are to be dried, freeze-drying should be used.

4.4.6 Storage conditions

If samples cannot be processed immediately, it is important to quantify the effect of storage on the analysis. In order to test this, biomass or completed DT reactions were stored under various conditions. The results showed that storage under any of these conditions for 24 hours yielded 97 to 100% of the fatty acid content compared to immediate processing. After storage for 16 days, the results for the biomass stored at room temperature, in the fridge, and freezer were on

average 91%, 88% and 87% of immediate processing. The samples stored as DT reaction mixtures yielded 97% and 90%, relative to immediate quantification, after storage for 7 and 16 days respectively.

Samples can be stored either as DT extracts, or as biomass at room temperature, in the fridge, or freezer, for at least 24 hours with little effect on the results. Similarly, DT reactions stored for 7 days gave results within the experimental error. Samples stored for 16 days under any of the conditions tested showed a lower measured fatty acid content, possibly due to degradation of the sample. Cold storage does not appear beneficial for preserving fatty acids, however, it may prevent the growth of contaminants such as fungi or bacteria in the stored samples.

4.5 Conclusion

This chapter presents a rigorous comparison of DT to the most common alternative methods of lipid extraction and quantification used to analyse microalgae. It was shown, across three algal species, that DT was a simple, accurate and reliable method, capable of determining total fatty acid content more efficiently than traditional solvent extraction methods. DT completely eliminates the need for lipid extraction and purification and can be performed on small sample volumes. Classical lipid extraction techniques are lengthy and involve multiple steps, while DT is rapid and can be performed in a single tube, minimising sample losses. This provides the critical advantage of the collection of lipid accumulation data regularly throughout the time course of algal cultivation.

Drying of centrifuged algal biomass was not necessary if a sequential combination of basic and acid catalysts were used. Samples could be stored at room temperature, cold (4°C) or frozen (-20°C), for up to 7 days with little effect on results. If drying is required, freeze drying is recommended as oven drying (70°C) reduced the measured fatty acid content. Additional reactants, such as 2,2-dimethoxypropane, can cause extra peaks in the chromatogram, so it is recommended that water scavengers are not used at low reaction water contents (< 10%).

Part II – Species choice

5 Species choice, literature study: Lipid productivity as a key characteristic for choosing algal species for biodiesel production

5.1 Introduction

In any microalgal project, one of the most important decisions is the choice of species to use. Pulz and Gross (2004) observed that: "successful algal biotechnology mainly depends on choosing the right alga with relevant properties for specific culture conditions and products". Rigorous selection is challenging owing to the large number of microalgal species available, the limited characterisation of these algae, and their varying sets of traits. There are many factors to take into account and the final choice depends on the products, aims, location, environment, culture system and harvesting and processing techniques of the venture. It was hypothesized that there is useful information on the characteristics of different microalgal species available in the literature and that this could be collated and used to guide decision-making in choosing species for biodiesel production.

This chapter reviews the desirable characteristics of species for large-scale biodiesel production and presents the information available in the literature on growth rates, lipid contents and lipid productivities under nitrogen replete and limited conditions for 55 species of microalgae.

5.2 Literature review

There are a huge number of naturally occurring microalgal species (over 100 000 (Sheehan *et al.* 1998)), with a range of different characteristics. The qualities desirable in an algal species for large-scale culture are summarised in Table 5.1. A single algal species is unlikely to excel in all categories, hence, prioritisation is necessary. Production of the desired product, environmental conditions, available resources and choice of culture system influence the characteristics required.

Selection of fast-growing, productive strains is of fundamental importance to the success of any algal mass culture – particularly for low-value products such as biodiesel. Fast growth, high biomass density and high product content increase yield per culture volume and reduce cost of cultivation, extraction and purification per unit product (Borowitzka 1992).

Table 5.1 Desirable characteristics of algae for mass culture

Characteristic	Advantages	Reference
Rapid growth rate	Reduces culture area required. Competitive advantage over competing species	Borowitzka 1992
High product content	Higher value of biomass. Lower culture area required	Borowitzka 1992
Growth in extreme environment	Reduces contamination and predation	Grobbelaar 2000
Ease of biomass harvesting and processing	Reduces harvesting and downstream processing costs	Mata *et al.* 2010
Wide tolerance of environmental conditions	Less control of culture conditions required. Growth over range of seasons and ambient weather conditions	Borowitzka 1992 Grobbelaar 2000
CO_2 tolerance and uptake	Greater potential for CO_2 sequestration and use of waste CO_2	Grobbelaar 2000
Tolerance of shear force	Allows use of cheaper pumping and mixing methods	Borowitzka 1992
Tolerance of contaminants	Allows growth in polluted water and on flue-gases containing high CO_2, NO_x and SO_x	Sung *et al.* 1999
No excretion of autoinhibitors	Reduces autoinhibition of growth at high biomass densities	Grobbelaar 2000
Production of valuable co-products	Improves economics and sustainability	Mata *et al.* 2010

Resistance to contamination is critical to the successful maintenance of desired species in outdoor algal culture. Most commercial facilities to date have used raceway ponds open to the atmosphere, where maintaining a dominant monoculture is challenging (Rodolfi *et al.* 2009; Sheehan *et al.* 1998). The only species which have been successful so far have been those with a very high growth rate (e.g. *Chlorella*), which naturally out-compete slower-growing species, or those that grow in an extreme environment such as a high pH (e.g. *Spirulina*) or salt concentration (e.g. *Dunaliella*) (Sheehan *et al.* 1998). Closed photobioreactors can be used to reduce contamination risk, but on a large scale, even in closed systems, sterility is difficult to maintain (Lee 2001; Pulz 2001). Other strategies employed to concentrate the desired species include enriching reactors with stock culture and the use of size-selective recycle (Benemann *et al.* 1977). Although it is likely that dominance of specific oil-rich species is desirable, monoculture is not mandatory for biodiesel production and the possibility of using mixed, wild or volunteer cultures, for example species that grow naturally in sewage or waste water ponds, should be investigated (Oswald & Golueke 1960).

Tolerance of a range of environmental conditions is an obvious advantage in outdoor culture where season and time of day often dictate the temperature and light availability, while rainfall or evaporation lead to dilution or concentration of nutrients respectively. Freedom to adjust the culture conditions can also assist in limiting contamination. These factors are better controlled in closed reactors, but at higher cost (Pulz 2001).

An often-overlooked criterion during species selection is ease of harvesting. The absence of cost-effective methods of harvesting dilute microalgal biomass has contributed to the lack of success in commercialising low-value products. Algal biomass often consists of cells less than 20 μm in diameter – too small for low-cost straining or filtration (Benemann *et al.* 1977). Harvesting of such cells is a significant capital and operating cost, hence it is desirable to select an alga with properties that simplify harvesting. Examples include large cell size, colonial or filamentous morphology, high specific gravity compared to the medium and reliable autoflocculation (Borowitzka 1997).

A characteristic specific to biodiesel production is the suitability of the lipid profile of the species. The chain length and degree of saturation of the fatty acids, as well as the proportion of different lipid classes, e.g. triacylglycerides, influences the quality of the biodiesel produced (Ramos *et al.* 2009). The fatty acid profile of microalgae varies with species and environmental conditions (Grima *et al.* 1994; Rodolfi *et al.* 2009).

In selecting species for a particular application, particularly fuel production, the possibility of producing valuable co-products such as fine chemicals, nutraceuticals or a nutrient rich biomass should be borne in mind. Choosing a species well suited to the biorefinery approach (production of multiple products), could contribute to both economic success and environmental sustainability (Li, Q *et al.* 2008).

The use of genetic or metabolic engineering to develop microalgae with the correct combination of desirable traits has been considered. The single, non-differentiated cells of microalgae are simpler systems to modify than higher plants. Systems of genetic modification have been developed for some algal species, but progress has been slow. Promising advances in this area should be regarded with caution as regulations in many countries are likely to ban the large-scale release of transgenic organisms into outdoor cultivation systems (Grobbelaar 2000; Mata *et al.* 2010).

Many characteristics are critical to the success of large-scale microalgal production, however, for the purposes of this work, insufficient information is available in the literature to be able to compare more than a handful of the best-known species on all of the criteria given above. The only two characteristics relevant to biodiesel production that have been measured quantitatively for a wide variety of species are growth rate and lipid content. Lipid productivity, the combination of the two, was identified as the quality on which species for biodiesel production should be compared. This is seldom reported, but can be calculated as the product of biomass productivity and lipid content.

There is a large and diverse body of literature on microalgae, encompassing a variety of fields (from botany and ecology to biotechnology and bioengineering) and goals (from fundamental research into ecosystems and photosynthesis to applications in the production of aquaculture feed, nutraceuticals and fine chemicals). Data on characteristics relevant to biodiesel production is often embedded in these reports.

Two of the largest studies comparing species under similar growth conditions were undertaken by Shifrin and Chisholm (1981), who measured the lipid content of 30 species in response to nitrogen and silicon limitation, and Rodolfi *et al.* (2009) who screened 30 species for high lipid productivity and found the marine *Nannochloropsis* and *Tetraselmis* to be particularly promising. Due to the large number of strains used, the growth conditions (e.g. 250 ml flasks on an orbital shaker) were optimized for ease of use rather than microalgal productivity.

The Aquatic Species Program was a large project funded by the US Department of Energy from the 1970s to the 1990s, to develop renewable transportation fuels from microalgae (Sheehan *et al.* 1998). A part of the project focused on the isolation of high lipid content algae for growth in open ponds supplemented with CO_2 from coal-fired power stations. Over 3000 species were isolated and several of these screened for lipid production, but a final recommendation of species was not provided. Towards the end of the program, the focus shifted towards understanding oil production in algae, with the aim of modifying the performance of existing strains, rather than finding one that satisfied all the criteria.

Several other studies have compared smaller numbers of species, almost always with the focus on lipid content rather than productivity. Ben-Amotz *et al.* (1985) and Reitan et al (1994) each compared the effect of nutrient limitation on the lipid profile and content of 7 algal species, while Piorreck *et al.* (1984) investigated two green and four blue-green algae for biomass production and lipid content under different nitrogen regimes. Studies focusing on the energy content of microalgae for food or feed often provide data useful in comparing species for fuel production. For example, Mourente *et al.* (1990) and Patil *et al.* (2006) each compared the lipid content and fatty acid composition of 12 marine species used as aquaculture feed. The data presented in this review are collated from these studies, as well as a variety of other work, usually focused on goals other than biofuel production.

In summary, the issue of species choice for algal biodiesel production has received some attention in the literature. Several studies have highlighted the importance of species choice and suggested important physiological characteristics (Borowitzka 1992; Grobbelaar 2000; Mata *et al.* 2010). These are usually a range of qualities and a multicriteria decision strategy is recommended (Mata *et al.* 2010). However, few studies have compared species on any quality other than lipid content. Little consensus is reported between research groups on the algal species most suitable for biodiesel production, and the number of strains used in algal

biotechnology remains few (Grobbelaar 2000). This chapter aims to summarise the available information on microalgal lipid productivity in order to highlight species promising for biodiesel production.

5.3 Methods of data collation and analysis

5.3.1 Data collection

Growth rates, lipid contents and lipid productivities were gathered from a broad range of literature, spanning a variety of algal species and purposes, including fuel production, calorific value as a food or feed, and production of specialty oils and chemicals. A range of reactor configurations, designs and scales are reported under various conditions of nutrient supply, hence the data was sorted into the following subcategories:

- **Culture method:** Laboratory, outdoor pond or outdoor photobioreactor
- **Metabolic mode:** Photoautotrophic, heterotrophic or mixotrophic
- **Nutrient availability:** Nitrogen replete or nitrogen limited

The data for outdoor culture conditions, as well as hetero- and mixotrophic culture were sparse, using a very limited number of species, hence the information presented here is restricted to photoautotrophic growth under laboratory conditions. Nitrogen limitation is well known to enhance the lipid content of algae. Several studies have investigated this effect, but nutrient levels have been reduced by different amounts in different studies (Roessler 1990; Shifrin & Chisholm 1981). For the purpose of this study, the following definitions were used:

1) **Nitrogen replete (also referred to as nitrogen sufficient):** nitrogen supply in excess of stoichiometric requirements for growth. This was assumed where no evidence of nitrogen reduction or depletion in the medium was provided.
2) **Nitrogen limited (also referred to as nitrogen deficient or deprived):** nitrogen supply below stoichiometric requirements for growth. This was brought about either when nitrogen was removed by changing the medium, or insufficient nitrogen was supplied and nitrogen levels in the culture medium were shown to become depleted before the end of the culture period.

All microalgal species commonly cultivated, either commercially or in the laboratory, or considered in the context of lipid or biodiesel production were initially recorded. The resultant list was refined by excluding species where reliable data for biomass productivity and lipid content could not be found (e.g. *Biddulphia (Odontella) aurita, Chlorococcum*, *Emiliania huxleyi, Micractinium, Ochromonas danica, Ostreococcus tauri*, *Pseudokirchneriella subcapitata*, *Synechocystis aquatilis*), and species where the algal lipids produced were unsuitable for

biodiesel (e.g. hydrocarbons produced by *Botrycoccus braunii* have a chain length of greater than C30 (Banerjee *et al.* 2002; Li, Y *et al.* 2008) while vegetable oils currently used for biodiesel are mainly C16 and C18 (Harrington 1986)).

Data were collated at species level wherever possible. In a few cases, however, the references used described the organisms to genus level only, e.g. *Amphor*a (De la Peña 2007; Sheehan *et al.* 1998) and *Cylindrotheca* (Chisti 2007; Sheehan *et al.* 1998).

5.3.2 Units of quantification

In order to compare results between species and studies, all biomass and lipid parameters were converted into standard units. Units were chosen on the basis of how frequently they were reported in literature, as well as their independence of other parameters such as cell weight or reactor geometry.

Lipid content is typically reported as percentage dry weight (% DW). Data presented in pg lipid.cell^{-1} were discarded if no cell weight was available for conversion.

Growth rates are reported as doubling time (T_d) or specific growth rate (μ). These were inter-converted according to Equation 5.1.

$$T_d = \frac{\ln 2}{\mu} \qquad \text{Equation 5.1}$$

Standard units of g.L^{-1}.day^{-1} were chosen for biomass productivity. Specific growth rate (μ, in units of day^{-1}) can be converted to volumetric biomass productivity (Q_V, in g.L^{-1}.day^{-1}) if the biomass concentration (X, in g.L^{-1}) is known (Equation 5.2).

$$Q_V = \mu.X \qquad \text{Equation 5.2}$$

Biomass productivity is often reported on the basis of surface area (Q_A), in units of g.m^{-2}.day^{-1}. This can be converted to Q_V using Equation 5.3 if the depth (D, in m) of the culture vessel can be calculated from the reactor geometry.

$$Q_V = \frac{Q_A}{D.1000} \qquad \text{Equation 5.3}$$

Lipid productivity (Q_P) was infrequently reported in the literature, but was generally reported in g.L^{-1}.day^{-1} or mg.L^{-1}.day^{-1}. This parameter could be calculated from volumetric biomass

productivity (Q_V, in $g.L^{-1}.day^{-1}$) and lipid content (P, in% DW) where appropriate data were available (Equation 5.4).

$$Q_P = Q_V . P \qquad \text{Equation 5.4}$$

5.3.3 Assumptions

In order to allow direct comparison, and for calculation of lipid productivity, all biomass productivities in T_d, μ or $g.m^{-2}.day^{-1}$ were converted into $g.L^{-1}.day^{-1}$. Frequently, insufficient information was provided for conversion. The two most common problems were:

- data presented as a specific growth rate without a biomass concentration
- data given as areal productivity without geometry of the culture vessel

These limitations in the data necessitated two assumptions, which were applied to all unit conversions:

1) In order to calculate Q_V according to Equation 5.2, a biomass concentration of 0.15 $g.L^{-1}$ was assumed, based on experimental results of the average biomass concentration during the exponential phase of the growth curve (from data presented in Chapter 6)
2) In order to calculate Q_V according to Equation 5.3, an average depth of 0.1 m was assumed, based on best overall fit across species of converted Q_A with Q_V

The conversion of each reported growth parameter into biomass productivity as $g.L^{-1}.day^{-1}$ was carried out as shown in Figure 5.1. The average biomass productivity was calculated for each species, and multiplied by the average nitrogen replete lipid content to determine the calculated (calc.) lipid productivity. Lipid productivities, where directly reported in the literature, were also gathered and averaged separately as literature (lit.) lipid productivity.

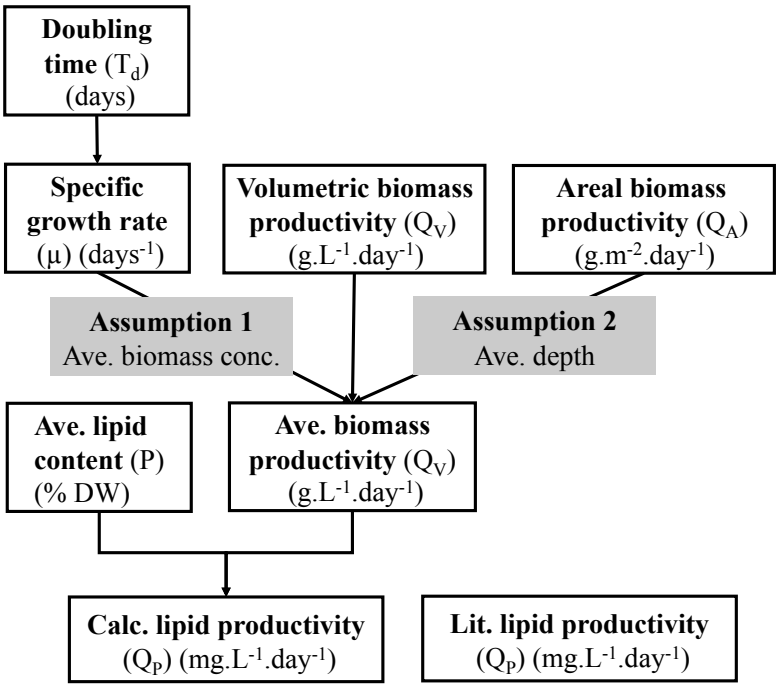


Figure 5.1 Assumptions used in lipid productivity calculations

5.3.4 Species names

Algal taxonomy is evolving as molecular methods improve. This has resulted in algal species being re-classified, making collation of information confusing. Species reported by a variety of names in the literature are summarised in Table 5.2, and their currently accepted classification specified.

Table 5.2 Current species names and their previous classification

Current name	Previous names
Dunaliella salina (Dunal) Teodoresco	*Dunaliella bardawil*
Neochloris oleoabundans (S. Chantanachat & H. C. Bold)	*Ettlia oleoabundans*
Monodopsis subterranea (J.B. Petersen) Hibberd	*Monodus subterraneus*
Monoraphidium minutum (Nägeli) Komárková-Legnerová	*Ankistrodesmus minutissimus* *Selenastrum minutum*
Porphyridium purpureum (Bory de Saint-Vincent) K. Drew & Ross	*Porphyridium cruentum*
Selenastrum gracile Reinsch	*Ankistrodesmus gracilis*

5.4 Results and discussion

Data collected for 55 different species under laboratory conditions are presented in Table 5.3 (Adam 1997; Ahmad & Hellebust 1990; Apt & Behrens 1999; Baker *et al.* 2007; Becker 1994; Ben-Amotz *et al.* 1985; Benider *et al.* 2001; Beudeker & Tabita 1983; Bhaud *et al.* 1991; Bopp & Lettieri 2007; Burlew 1953; Butterwick *et al.* 2004; Chelf 1990; Chisti 2007; Coleman *et al.* 1988; Collyer & Fogg 1955; Constantopoulos & Bloch 1967; Cook 1966; Coombs *et al.* 1967; De la Peña

2007; De Morais & Costa 2007; Dempster & Sommerfeld 1998; Exley *et al.* 1993; Ferguson *et al.* 1976; Fisher *et al.* 1996; Gatenby *et al.* 2003; Goldman & Peavey 1979; Göksan *et al.* 2007; Haury & Spiller 1981; Hu & Gao 2003; Illman *et al.* 2000; Ishida *et al.* 2000; Janssen *et al.* 2001; Johansen *et al.* 1987; Lee & Bazin 1991; Lee 2001; Li, Y *et al.* 2008; Liu *et al.* 2008; Maddux & Jones 1964; Mansour *et al.* 2005; Matsukawa *et al.* 2000; McGinnis *et al.* 1997; McKnight 1981; Miao & Wu 2006; Moheimani & Borowitzka 2006; Moore 1975; Mourente *et al.* 1990; Ostgaard & Jensen 1982; Parrish & Wangersky 1987; Patil *et al.* 2006; Piorreck *et al.* 1984; Price *et al.* 1998; Reitan *et al.* 1994; Renaud *et al.* 1994; Richardson *et al.* 1969; Rodolfi *et al.* 2009; Roessler 1990; Sheehan *et al.* 1998; Shehata & Kempner 1977; Shifrin & Chisholm 1981; Siron *et al.* 1989; Sorokin & Krauss 1962; Spoehr & Milner 1949; Stephenson 2009; Suen *et al.* 1987; Taguchi *et al.* 1987; Tomaselli *et al.* 1997; Ugwu *et al.* 2007; Vieira Costa *et al.* 2002). Each number is the average of all data collected for that species. The number of references used per value ranged from one to 20. Growth rates and biomass productivities under nitrogen limited conditions are rarely reported, hence all growth data is for nitrogen replete conditions. Lipid content is given for both nitrogen replete and limited conditions. Average literature values for growth and biomass productivity are reported as T_d, Q_A and Q_V, as well as the overall average biomass productivity calculated by converting T_d and Q_A into Q_V. The majority of growth data was reported as T_d or μ. The conversion of these to Q_V greatly expands the set of lipid productivities that can be calculated. Lipid productivity is divided into two columns: values calculated from the average biomass productivity and the nitrogen replete lipid content, and the average of productivities collated directly from literature. Overall averages for various groupings of algae, according to taxa and media, are given in the final rows of the table.

Table 5.3 Growth and lipid parameters of 55 species of microalgae, along with their taxonomy and media type. The average of literature values for lipid content under nitrogen (N) replete and limited growth conditions, doubling time (T_d), and areal (Q_A) and volumetric (Q_V) biomass productivities are shown in columns 4 to 8. Average biomass productivity calculated from T_d, μ, Q_A and Q_V is shown in column 9, and calculated and literature lipid productivity in columns 10 and 11 respectively. Blanks represent no data available.

[a] Key to taxa: B = Bacillariophyta, C = Chlorophyta, Cy = Cyanobacteria, D = Dinophyta, E = Eustigmatophyta, Eg = Euglenozoa, H = Haptophyta, O = Ochrophyta, Pr = Prasinophyta, R = Rhodophyta

[b] Key to media: F = Freshwater, M = Marine, S = Saline

			Lipid content			Biomass productivity			Lipid productivity	
			N replete	N defficient	T_d	Q_A	Q_V	Ave Q_V	Calculated	Literature
Species	**Taxa[a]**	**Media[b]**	**% dw**	**% dw**	**days**	**$g.m^{-2}.day^{-1}$**	**$g.L^{-1}.day^{-1}$**	**$g.L^{-1}.day^{-1}$**	**$mg.L^{-1}.day^{-1}$**	**$mg.L^{-1}.day^{-1}$**
Amphiprora hyalina	B	M	22	28	0.41			0.30	67	
Amphora	B	M	51		0.83	40.0		0.23	117	160
Anabaena cylindrica	Cy	F	5	5	1.00			0.10	5	
Ankistrodesmus falcatus	C	F	24	32	0.33	31.6	0.46	0.36	85	
Chaetoceros calcitrans	O	M	40				0.04	0.04	16	18
Chaetoceros muelleri	O	M	19	27	0.46		0.07	0.26	50	22
Chlamydomonas applanata	C	F	18	33						
Chlamydomonas reinhardtii	C	F	21		0.26			0.40	83	
Chlorella emersonii	C	F	29	63	0.80		0.03	0.08	23	
Chlorella minutissima	C	M	31	57	1.60		0.03	0.05	15	
Chlorella protothecoides	C	F	13	23	1.68			0.07	8	
Chlorella pyrenoidosa	C	F	16	57	0.31			0.34	55	
Chlorella sorokiniana	C	F	18	18	0.13		0.73	0.81	143	45
Chlorella vulgaris	C	F	19	42	0.70	10.7	0.11	0.16	31	30
Crypthecodinium cohnii	D	M	25		0.38			0.28	70	
Cyclotella cryptica	O	M	18	34	0.56			0.20	36	
Cylindrotheca	B	M	27	27	0.30			0.43	114	
Dunaliella primolecta	Pr	S	23	14		9.1		0.09	21	
Dunaliella salina	Pr	S	19	10	0.44			0.27	53	
Dunaliella tertiolecta	Pr	S	15	18	0.48			0.22	35	
Euglena gracilis	Eg	F	20	35	0.60			0.18	37	
Hymenomonas carterae	H	M	20	14	1.71			0.06	12	
Isochrysis galbana	H	M	25	29	0.89	11.5	0.16	0.15	37	38
Monodopsis subterranea	E	F	25	13			0.19	0.19	48	30
Monoraphidium minutum	C	F	22	52	0.35			0.30	65	
Nannochloris	C	M/F	28	30	0.49	31.9	0.23	0.27	74	77
Nannochloropsis	E	M	31	41	1.20		0.21	0.19	58	52
Nannochloropsis salina	E	M	27	46		13.9		0.14	38	
Navicula acceptata	B	F	33	35	0.42			0.29	96	
Navicula pelliculosa	B	F	27	45	0.23			0.46	124	
Navicula saprophila	B	F	24	51	0.38			0.28	68	
Neochloris oleoabundans	C	F	36	42			0.46	0.46	164	136
Nitzschia communis	B	M			0.96			0.18		
Nitzschia dissipata	B	M	28	46	0.39			0.27	73	
Nitzschia frustulum	B	M	26							
Nitzschia palea	B	M	47	40						48
Oscillatoria	Cy	F	7	13	0.28			0.37	27	
Ourococcus	C	F	27	50	3.01			0.03	9	
Pavlova lutheri	H	M	36				0.21	0.21	75	50
Pavlova salina	H	M	31				0.16	0.16	49	49
Phaeodactylum tricornutum	B	M	21	26	1.02	20.0	0.34	0.18	38	45
Porphyridium purpureum	R	M	11		0.45		0.23	0.23	24	35
Prymnesium parvum	H	M	30		0.74			0.14	42	
Scenedesmus dimorphus	C	F	26		0.46			0.23	57	
Scenedesmus obliquus	C	F	21	42	2.74		0.12	0.10	22	
Scenedesmus quadricauda	C	F	18				0.19	0.19	35	35
Selenastrum gracile	C	F	21	28						
Skeletonema costatum	O	M	16	25	0.66		0.08	0.15	24	17
Spirulina maxima	Cy	S	7		1.34			0.16	11	
Spirulina platensis	Cy	S	13	10	0.60	25.0		0.23	29	
Synechococcus	Cy	M	11		0.36			0.29	32	75
Tetraselmis suecica	C	M	17	26	1.51	28.1	0.59	0.39	65	32
Thalassiosira pseudonana	O	M	16	26	0.49		0.08	0.26	43	17
Thalassiosira weissflogii	O	M	22	24	0.58			0.18	41	
Tribonema	O	M	12	16	1.82		0.51	0.28	33	
			Average	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**	**Average**
Total			23	32	0.80	22.2	0.24	0.23	52	51
Freshwater			21	35	0.81	21.2	0.26	0.26	54	35
Marine			25	31	0.82	22.7	0.21	0.21	49	47
Chlorophyta			23	41	0.99	24.7	0.26	0.26	58	65
Other taxa			25	30	0.72	20.4	0.24	0.23	57	50
Cyanobacteria			8	9	0.72	25.0		0.23	21	75

5.4.1 Lipid content

Lipid content data were readily available and consistently reported in the literature. Figure 5.2 shows the average lipid content for green algae (Chlorophyta) and blue-green algae (Cyanobacteria) grown under nitrogen replete and limited conditions in the laboratory. The nitrogen replete lipid content for green algae ranged from 13 to 36% DW, with an average of 23%, while the Cyanobacteria range was markedly lower, between 5 and 13%, with an average of 8%. Nitrogen limitation is well recognised to influence lipid content (Sheehan *et al.* 1998; Shifrin & Chisholm 1981). All green algae were reported to have a higher lipid content under nitrogen limitation. The average lipid content for Chlorophyta under nitrogen limitation was 41%, almost double that under nitrogen replete conditions. In the Cyanobacteria, however, only *Oscillatoria* showed an increase in lipid content with nitrogen limitation.

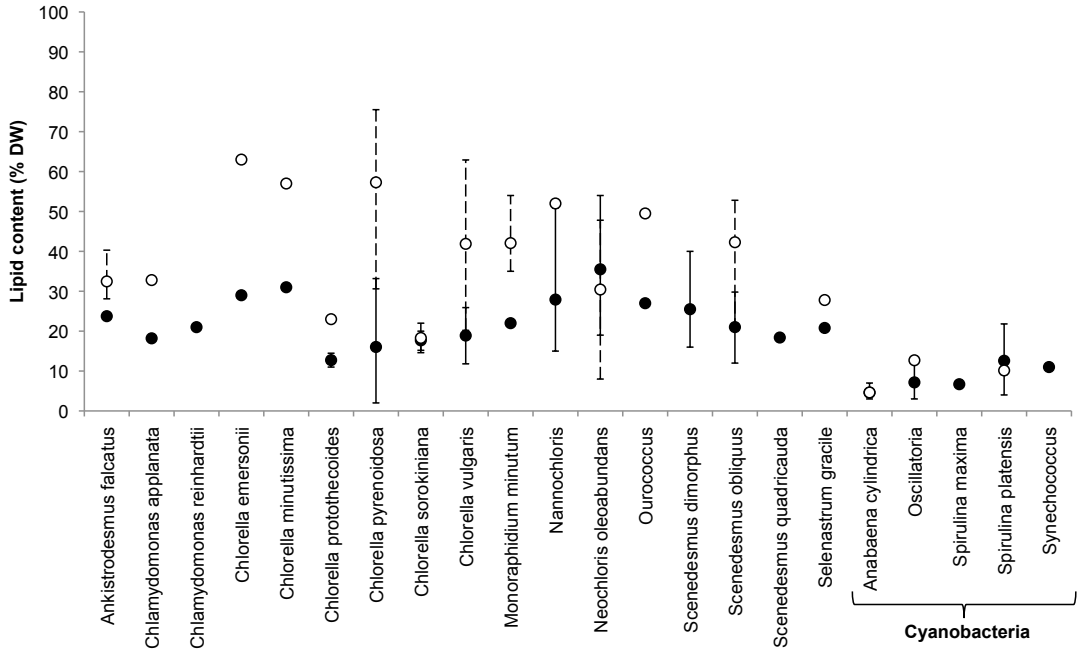


Figure 5.2 Average lipid content for laboratory cultures under nitrogen replete (filled circles) and nitrogen limited (empty circles) conditions for Chlorophyta and Cyanobacteria. Error bars show the minimum and maximum recorded values for each species (solid lines nitrogen replete, dashed lines nitrogen limited).

The average lipid content in laboratory cultures of other taxa (including diatoms, golden algae, Dinophyta and Eustimatophyta) under nutrient replete and nitrogen limited conditions are summarised in Figure 5.3. Nitrogen replete lipid content ranged from 11 to 51% DW, with an average of 25%, similar to that for green algae. The response to nitrogen limitation was varied. Eighteen of the 24 species for which information was available showed an increase in lipid content, with six showing a decrease, or no change. The average lipid content with nitrogen limitation was 30%.

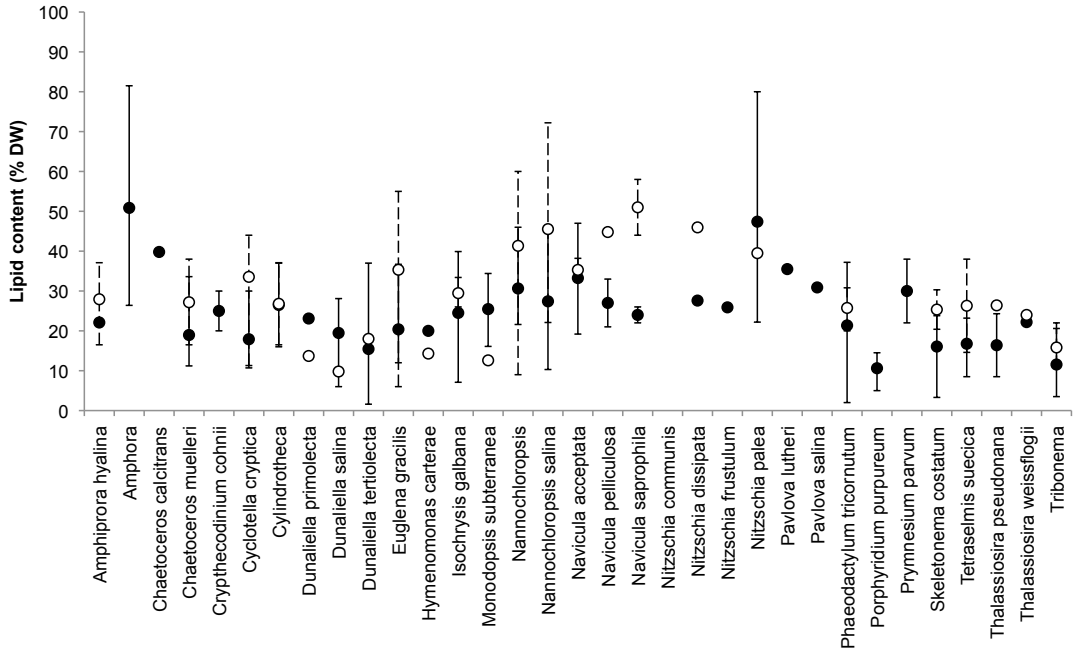


Figure 5.3 Average lipid content for laboratory cultures grown under nitrogen replete (filled circles) and nitrogen limited (empty circles) conditions for Dinophyta, Eustigmatophyta, Euglenozoa, Haptophyta, Ochrophyta and Prasinophyta. Error bars show the minimum and maximum recorded values for each species (solid lines nitrogen replete, dashed lines nitrogen limited).

The effect of nutrient depletion is further demonstrated in Figure 5.4, showing the shift in lipid contents with N limitation for Chlorophyta (Figure 5.4a) and diatoms and other taxa (Figure 5.4b). Most of the Chlorophyta had a lipid content between 20 and 30% DW under nitrogen replete conditions. Under nitrogen limitation, a shift in lipid content to the right is clearly seen with resultant contents from 18 to 64%. The diatoms and other taxa had a wider distribution under nitrogen replete conditions, although, similarly to the green algae, the majority fell between 20 and 30%. The varied response to nutrient limitation is demonstrated by the resultant bimodal distribution, with one species dropping below 10% and an increase in the number of species between 40 and 50% DW.

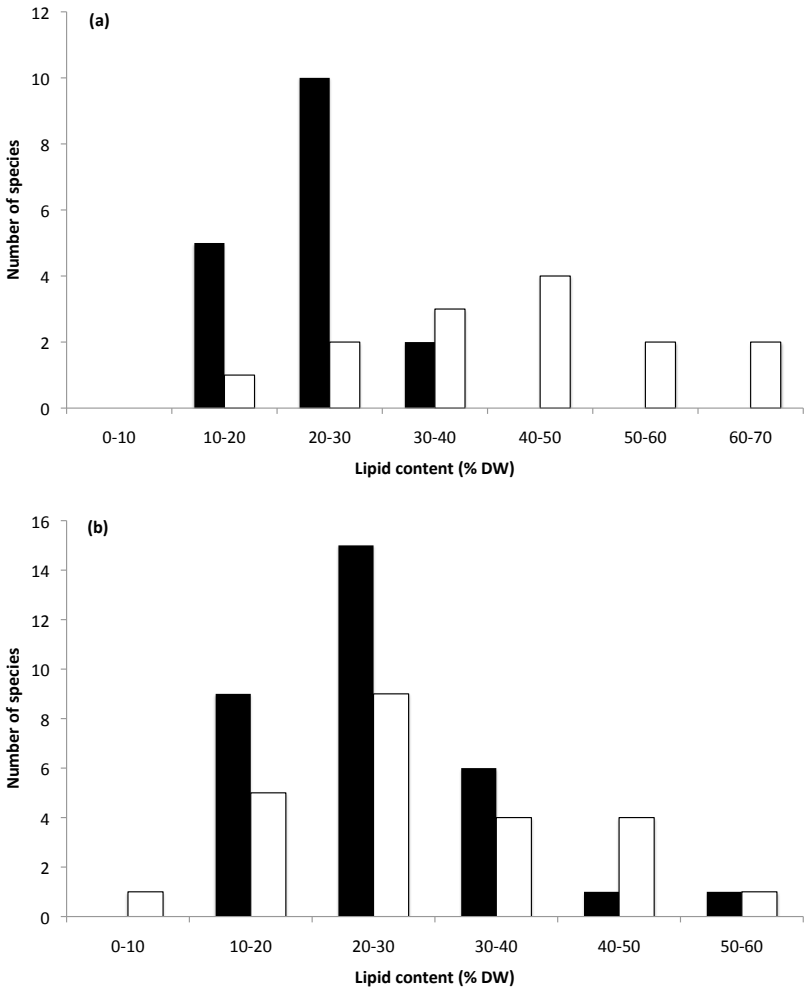


Figure 5.4 Number of algal species in each lipid content category under nitrogen replete (filled bars) and limited (empty bars) conditions. (a) = Chlorophyta, (b) = diatoms, golden algae and Eustigmatophyta.

When species were grouped according to culture environment rather than taxonomy, average lipid contents (for nitrogen replete and nitrogen limited conditions respectively) were 21% and 35% for freshwater species and 25% and 31% for marine species. The greater nitrogen limited lipid content for freshwater species is largely because most green algae (which show a greater response to nitrogen limitation) are freshwater species.

5.4.2 Biomass productivity

Average algal biomass productivities, the combination of values reported in $g.L^{-1}.day^{-1}$ and those converted from μ, T_d or $g.m^{-2}.day^{-1}$, are reported in Figure 5.5 and

Figure 5.6. The average biomass productivity was 0.26 $g.L^{-1}.day^{-1}$ for green algae, 0.23 $g.L^{-1}.day^{-1}$ for Cyanobacteria and 0.23 $g.L^{-1}.day^{-1}$ for all other taxa. Freshwater species had a slightly higher

average biomass productivity (0.26 $g.L^{-1}.day^{-1}$) than marine (0.21 $g.L^{-1}.day^{-1}$). The large range of biomass productivities reported for some species could be due to different studies reporting the biomass productivity, T_d or μ as either the maximum during the culture period, or the average over the entire culture period.

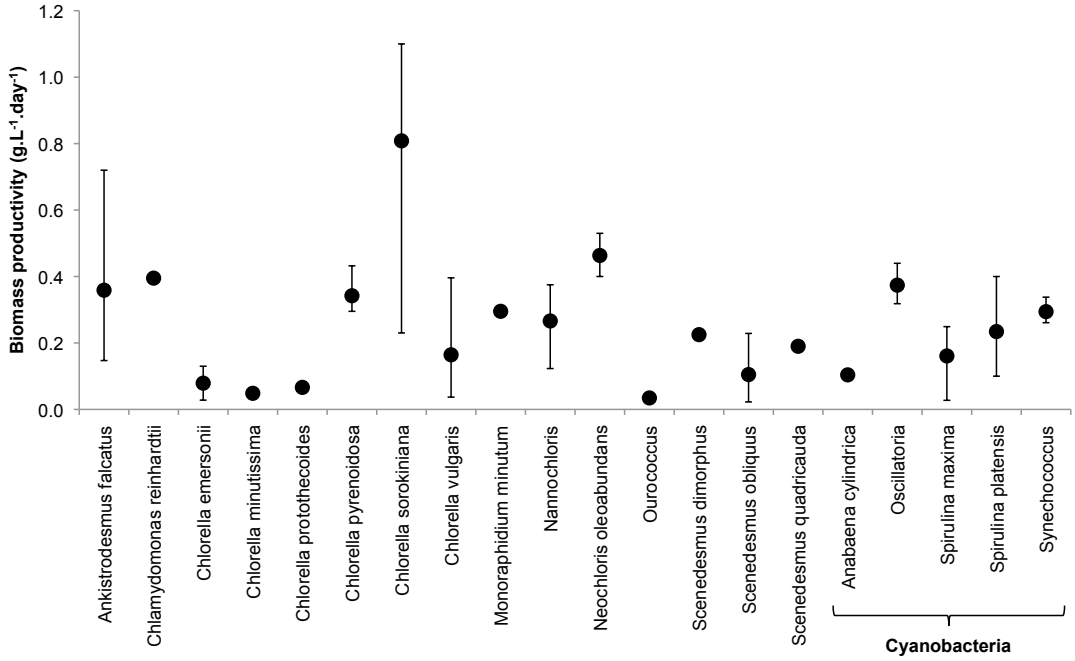


Figure 5.5 Average biomass productivity for Chlorophyta and Cyanobacteria. Error bars represent highest and lowest recorded values.

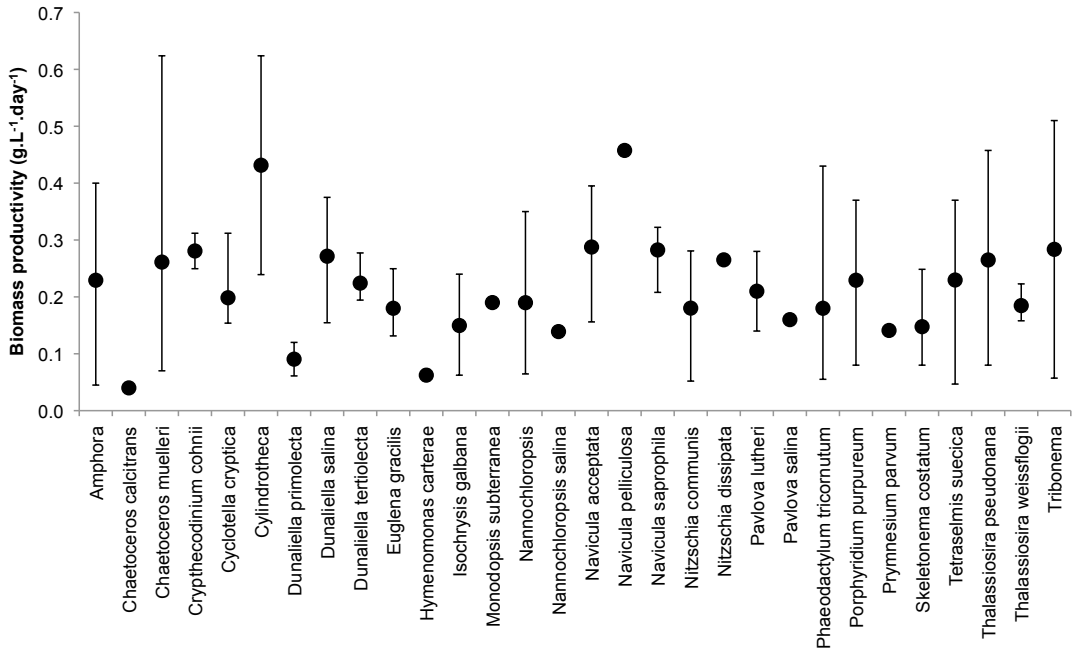


Figure 5.6 Average biomass productivity for other taxa. Error bars represent highest and lowest recorded values.

5.4.3 Lipid productivity

Average lipid productivities, either calculated or directly reported in literature, are summarised in Figure 5.7 and Figure 5.8. Lipid productivity was calculated as the product of average nutrient replete lipid content and biomass productivity in $g.L^{-1}.day^{-1}$. Productivities under nutrient limited conditions were not included due to a lack of reported growth rates under these conditions. The overall average lipid productivity for all species from literature-reported values (lit.) was 51 $mg.L^{-1}.day^{-1}$, compared with an average calculated (calc.) value of 52 $mg.L^{-1}.day^{-1}$. The similarity of the literature and calculated averages points to the validity of the two assumptions used in calculation. Green algae had a higher average lipid productivity, at 58 (calc.) and 65 (lit.) $mg.L^{-1}.day^{-1}$, than other taxa at 57 (calc.) and 50 (lit.) $mg.L^{-1}.day^{-1}$. There was a large discrepancy between the calculated (21 $mg.L^{-1}.day^{-1}$) and reported (75 $mg.L^{-1}.day^{-1}$) average lipid productivities for Cyanobacteria. This is due to two high lipid productivities for Synechococcus reported in Sheehan *et al.* (1998). Lipid productivities for Cyanobacteria are generally reported to be lower than the other taxa included here (Piorreck *et al.* 1984).

Minimum and maximum values for lipid productivity span a wide range for some species. This is due to the variation in biomass productivities and lipid contents reported in the literature, possibly due to the use of different strains, quantification methods, culture periods, reactor designs and culture environments.

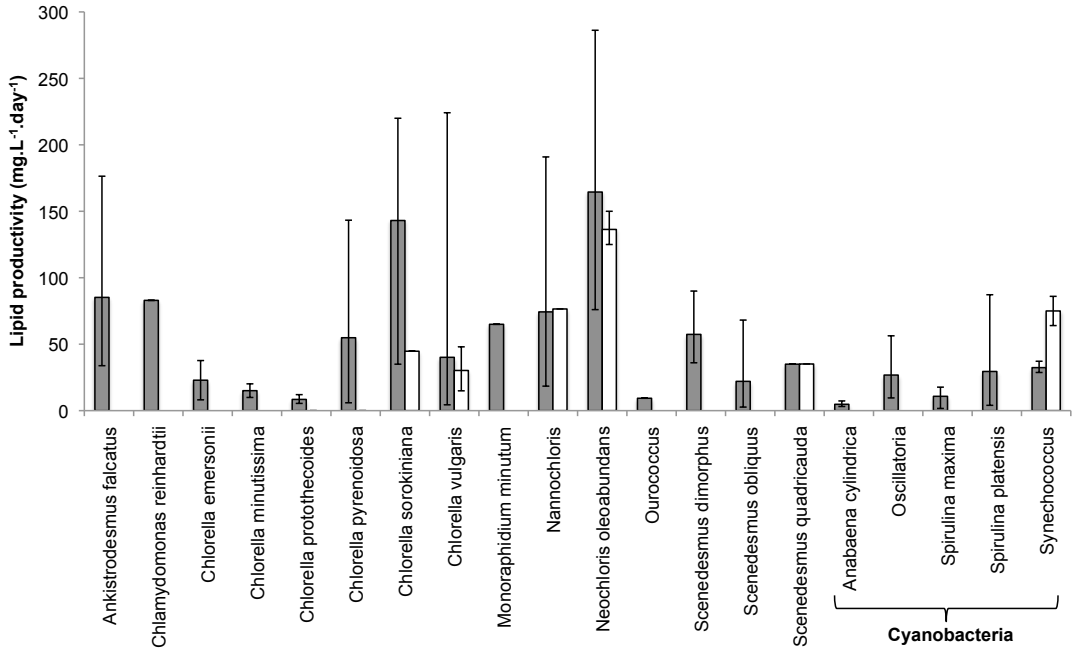


Figure 5.7 Average calculated (grey bars) and literature (empty bars) values for lipid productivity in green algae and Cyanobacteria. Error bars show the minimum and maximum calculated and recorded lipid productivities for calculated and literature values respectively.

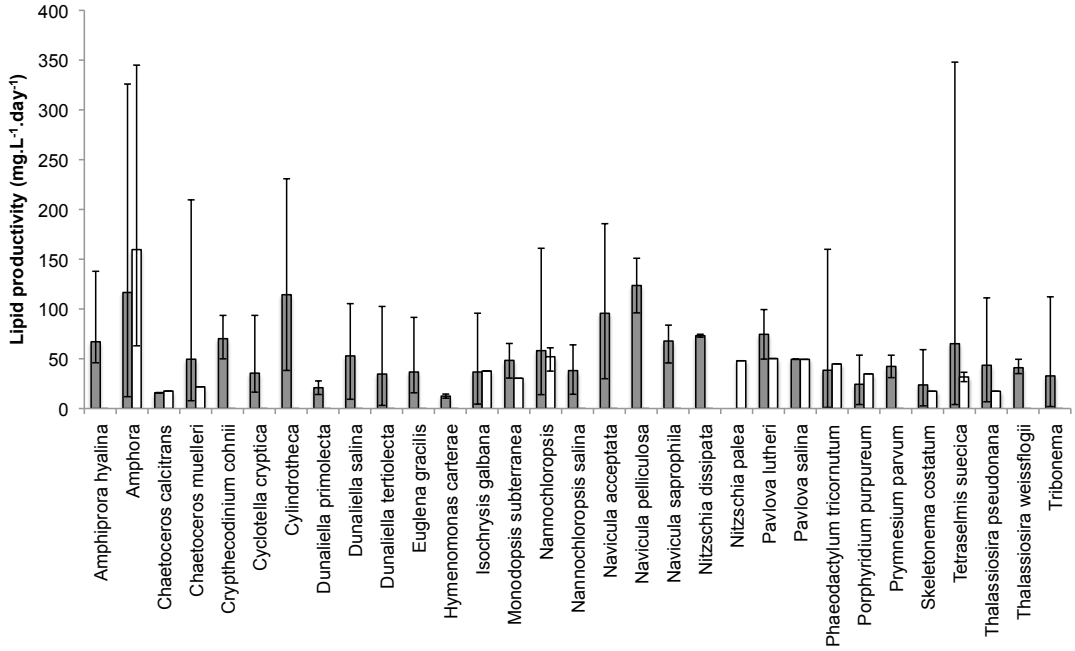


Figure 5.8 Average calculated (grey bars) and literature (empty bars) values for lipid productivity in other taxa. Error bars show the minimum and maximum calculated and recorded lipid productivities for calculated and literature values respectively.

The average calculated and literature-reported lipid productivities for the 20 most productive species are shown in Figure 5.9. Five species stand out as having very high lipid productivities, above 100 mg.L^{-1}.day^{-1}: *Neochloris oleoabundans* (164 calc. and 136 lit. mg.L^{-1}.day^{-1}), *Chlorella sorokiniana* (143 calc. and 45 lit.), *Navicula pelliculosa* (124 calc.), *Amphora* (117 calc. and 160 lit. mg.L^{-1}.day^{-1}), *Cylindrotheca* (114 calc.). Another promising species that almost reaches 100 mg.L^{-1}.day^{-1} is *Navicula acceptata* (96 calc.).

The lipid productivities of *Neochloris oleoabundans* and *Navicula pelliculosa* are a function of their high biomass productivities (both 0.46 g.L^{-1}.day^{-1}) and relatively high lipid contents (36 and 27% DW respectively). Both *Cylindrotheca* and *Chlorella sorokiniana* have very high biomass productivities (0.43 and 0.81 g.L^{-1}.day^{-1} respectively). In *Cylindrotheca* this is combined with an above average lipid content (27% DW), while in *Chlorella sorokiniana*, the high growth rate offsets a below average lipid content (18% DW).

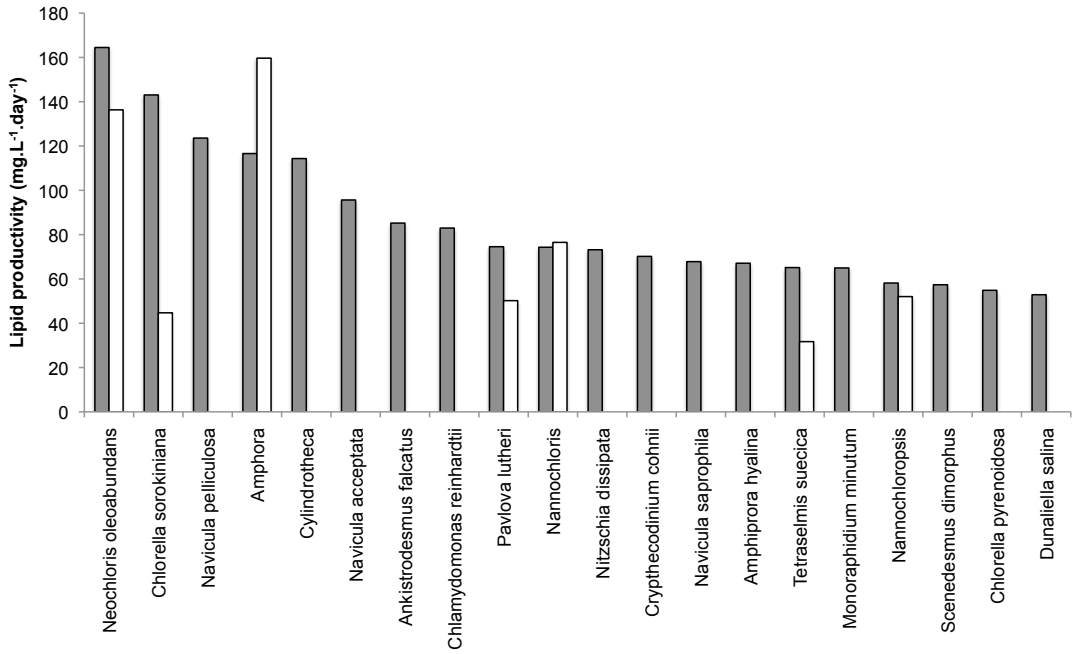


Figure 5.9 Average calculated (grey bars) and literature (empty bars) lipid productivity for the 20 most productive species investigated.

The high lipid productivity of *Amphora* is the product of a very high reported lipid content (average 51% DW) and an average growth rate of 0.23 $g.L^{-1}.day^{-1}$. These values should be regarded with caution due to the wide range of recorded lipid productivities for *Amphora* (63 to 345 $mg.L^{-1}.day^{-1}$, as shown by the error bars in Figure 5.8). The literature lipid productivity for *Amphora* is the average of values for three different strains, known as AMPHO27, AMPHO45 and AMPHO46, collected by M. Sommerfield, 1986-1987 (Sheehan *et al.* 1998). Inter-strain differences may account for the variation. In addition, these values, along with those for the Cyanobacteria *Synechococcus*, which, rather surprisingly given its low lipid content, has a relatively high reported lipid productivity (75 $mg.L^{-1}.day^{-1}$), were measured by Nile Red staining as triolein equivalents (Sheehan *et al.* 1998). According to Sheehan *et al.* (1998) a major problem with Nile Red is that species vary widely in their ability to take up this lipophilic dye. This limits the accuracy of these measurements. There has been no rigorous comparison of Nile Red staining and lipid quantitation across species.

This ranking of species should be interpreted with caution due to the nature of the data set. Lipid productivity was only available in the literature for 20 of the 55 species studied. The ranking of species was therefore largely based on calculated lipid productivities, which necessitated the use of two assumptions in the conversion of biomass productivities into units of $g.L^{-1}.day^{-1}$. In addition, the data collated here was collected under a wide range of growth conditions, usually not optimized for the species in question. Comparison of lipid productivities across algal species has also been restricted to photoautotrophic, laboratory, nitrogen replete conditions. Nutrient

limitation has been shown to enhance lipid content, but due to a lack in reporting of growth parameters under nutrient limitation, lipid productivity could not be derived. Hence, the ranking of species according to lipid productivity may change as more data becomes available.

From a practical perspective, it should be noted that *Amphora*, *Navicula*, *Cylindrotheca*, and *Nitzschia* are all benthic diatoms (Chen 2007), living in the lowest level of a body of water, often attached to the substrate bottom. Certain species may be able to be grown planktonically with sufficient agitation. If they require culture on solid substrates, this may render them unsuitable for large-scale production due to harvesting complexity.

5.4.4 Trends

In Figure 5.10, the impact of biomass productivity and lipid content on calculated lipid productivity is analysed through correlation. A relationship is demonstrated between lipid productivity and biomass productivity. All species with a high biomass productivity (above 0.4 $g.L^{-1}.day^{-1}$), and all but one above 0.3 $g.L^{-1}.day^{-1}$, have a high lipid productivity, greater than 60 $mg.\ L^{-1}.day^{-1}$. However, there are a few species with a high lipid productivity despite an average biomass productivity, indicating that lipid content is also a factor. Lipid content correlates poorly with lipid productivity, indicating that lipid content alone is not a good indicator of suitability for biodiesel production. There are several species with a low lipid productivity despite an above-average lipid content (> 22%). The species with high lipid productivities (> 60$mg.\ L^{-1}.day^{-1}$) range in lipid content from 16% DW to 51%. Further, species with a high lipid content (> 30%) vary in lipid productivity between 15 and 164 $mg.L^{-1}.day^{-1}$.

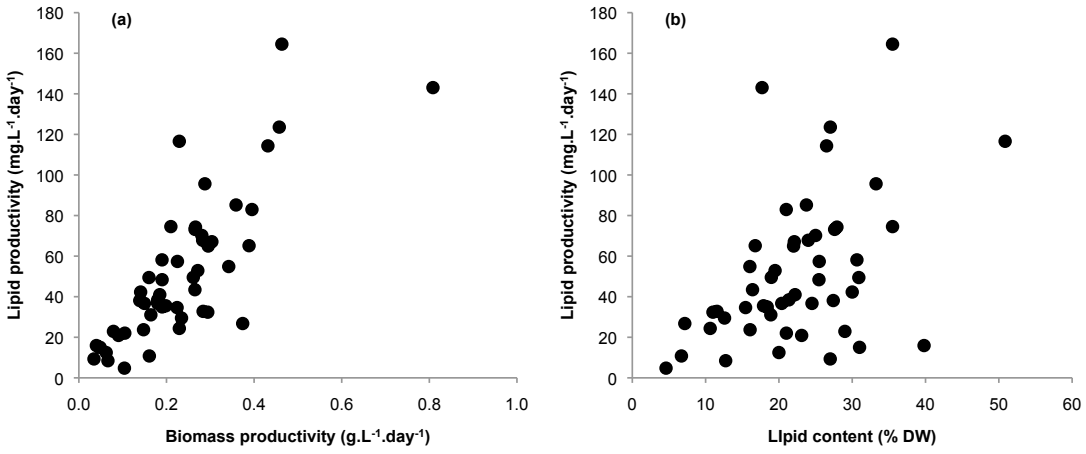


Figure 5.10 Correlation of calculated lipid productivity with (a) biomass productivity and (b) lipid content under nutrient replete conditions.

Figure 5.11 shows average biomass productivity as a function of lipid content. Contrary to the popular belief that the large metabolic demand of high lipid content necessitates slow growth rate, there appears to be no significant correlation between these. Of the nine species with a lipid content greater than 30%, three have an above average biomass productivity (> 0.23 $g.L^{-1}.day^{-1}$), while six are below average.

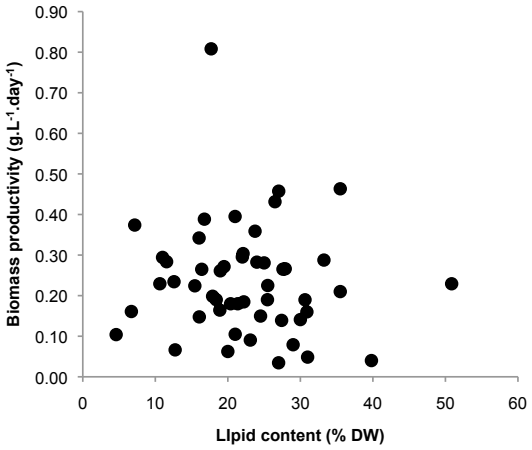


Figure 5.11 Relationship between biomass productivity and lipid content under nutrient replete conditions.

Lipid productivity is a critical variable for evaluating algal species for biodiesel production, as it gives an indicator of oil produced on a basis of both volume and time. Figure 5.10 illustrates that lipid content, reported in the absence of growth rate or biomass productivity, does not allow rational species selection for lipid production, as faster growing species may demonstrate lipid productivity greater than those with a very high lipid content. The data presented here were reported under nutrient replete conditions. Under conditions of nutrient limitation, known to enhance lipid content in several algae species, while frequently decreasing growth rate (Illman *et al.* 2000), it is particularly important to use the indicator of lipid productivity rather than lipid content.

5.5 Conclusion

This chapter demonstrates the role of information available in the literature in providing early stage guidance on culture selection. The data available is far from complete and rigorous comparison across experiments carried out under different culture conditions and using different analytical techniques has limited value. The presentation of data in a variety of different quantification units without the information necessary for interconvertion is an additional challenge. Lipid productivity was demonstrated to be a more important selection parameter than lipid content or growth rate individually. Lipid productivity is rarely reported in the literature, and in its absence, the data loses value in evaluating strains from an application perspective. In this review, two assumptions were introduced to allow the calculation of lipid productivity for a wide range of species.

Analysis of lipid content, biomass productivity and their combination to yield lipid productivity was conducted across literature data collected on 55 algal species, including 17 Chlorophyta, 5 Cyanobacteria and 11 Bacillariophyta as well as other taxa. The data clearly illustrates that lipid content can be enhanced by nitrogen limitation, although the effect varies across species. Lipid productivity calculated under nutrient replete conditions highlighted the following species for high lipid productivity (110 to 164 $mg.L^{-1}.day^{-1}$): *Neochloris oleoabundans*, *Chlorella sorokiniana, Navicula pelliculosa*, *Amphora* and *Cylindrotheca*. However, due to a lack of reported growth rates, lipid productivities under N limited conditions could not be calculated.

The collated information provides a framework for decision-making and a starting point for further investigation of species selection. In addition, this literature review highlights gaps in the knowledge and informs further experimental work that needs to be done, particularly with respect to comparing species under nutrient limited growth conditions. The following chapter addresses this by comparing the lipid content, biomass productivity and hence lipid productivity of 11 species selected from this literature review, under both nutrient replete and limited conditions.

6 Species choice, experimental study: Lipid productivity, settling potential and fatty acid profile of eleven microalgal species grown under nitrogen replete and limiting conditions

6.1 Introduction

Chapter 5 compared 55 microalgal species with potential for biodiesel production according to information available in the literature on their lipid content, biomass productivities and lipid productivities. One of the conclusions from this theoretical study was that there was a need for more experimental work, particularly: 1) testing of species under similar conditions to allow rigorous comparison of growth and lipid characteristics, 2) reporting of lipid productivities rather than lipid content 3) comparison of lipid productivities under nitrogen replete and limiting conditions and 4) investigation of key characteristics other than lipid productivity (such as ease of harvesting and tolerance of environmental conditions).

This chapter builds upon the literature review by comparing experimentally, under similar culture conditions in airlift photobioreactors, 1) the lipid productivity, 2) the potential for harvesting by gravity sedimentation and 3) the suitability of lipids for biodiesel production of eleven promising species, under both nitrogen replete (1500 $mg.L^{-1}$ nitrate) and limiting (150 $mg.L^{-1}$ nitrate) conditions. From previous work, it was clear that nitrogen limitation enhanced lipid content in most species. It was not clear whether this increase in lipid content would lead to increased lipid productivity, due to a simultaneous decrease in growth rate. The characterisation of growth and lipid accumulation under both nitrogen replete and limited conditions should assist in identifying the most promising species for lipid production, as well as testing whether nitrogen limitation leads to higher overall lipid productivities and yields.

6.2 Literature review

The desirable characteristics of microalgae for biodiesel production were reviewed in Chapter 5. Lipid productivity is one of the most easily quantifiable characteristics, and is a key parameter affecting the economic feasibility of oil production (Hsieh & Wu 2009). With greater productivity, lower culture volumes are required to yield the same amount of product. The lower reactor, pumping, mixing and harvesting volumes required would reduce the capital and energy requirements and hence the cost of the process. Biomass concentration and lipid content can also affect downstream processing cost (Li, Y *et al.* 2008; Stephenson 2009).

Comparison of lipid productivities is complicated by the fact that the metabolism of many species can vary greatly under different environmental conditions. Various forms of environmental stress such as limitation of nitrogen, silicate, phosphate, sulphur and iron, as well as certain conditions of temperature, light and salinity have been reported to increase lipid content in microalgae (Guschina & Harwood 2006; Hsieh & Wu 2009; Li, Y *et al.* 2008; Liu *et al.* 2008; Roessler 1990; Shifrin & Chisholm 1981). Nitrogen (N) limitation is the most commonly reported factor, often employed as it is cheap and easy to manipulate, and consistently induces high lipid content in a range of species (Illman *et al.* 2000; Rodolfi *et al.* 2009; Shifrin & Chisholm 1981). An additional advantage to using nitrogen limited cultures is the reduced use of the nitrogen source. In addition to reducing cost, lower use of nitrogen fertilizer could reduce the environmental burden and improve the overall energy balance of the process (Lardon *et al.* 2009).

While several studies have investigated the effect of nitrogen limitation on final lipid content (Ben-Amotz *et al.* 1985; Illman *et al.* 2000; Piorreck *et al.* 1984; Reitan *et al.* 1994; Rodolfi *et al.* 2009; Sheehan *et al.* 1998; Shifrin & Chisholm 1981), there is little information on the temporal profile of lipid accumulation, which is critical to evaluating productivity. The translation of an increase in lipid content into an increase in lipid productivity is dependent on the degree of growth retardation caused by the nitrogen limitation. Sheehan *et al.* (1998) reported that nitrogen limitation did not result in increased lipid productivity due to the high lipid content being offset by lower biomass productivities. The response of biomass productivity to nitrogen limitation, however, varies widely between species, and there have been cases where nitrogen limitation has been shown to improve lipid productivity in the short term, e.g. *Nannochloropsis* (Rodolfi *et al.* 2009). Currently, insufficient data on the impact of nutrient limitation on biomass productivity limits comparison between species of lipid productivity under nitrogen limitation.

Cost- and energy-effective harvesting is vital to the success of large-scale algae production. A large portion of the theoretical energy use of the algal biodiesel process was found to be in harvesting and oil extraction (Lardon *et al.* 2009). Microalgal cells are often less than 10 μm in diameter with densities just exceeding water. This requires harvesting techniques such as disc-stack centrifugation or micro-filtration, which are energy intensive and too expensive for large-scale fuel production (Lardon *et al.* 2009; Piorreck *et al.* 1984). For a low-cost, high-volume product such as biodiesel, flotation or gravity sedimentation (possibly assisted by flocculation) seem promising methods (Mata *et al.* 2010). Gravity sedimentation is particularly appealing, requiring few inputs other than transfer to a settling tank and sufficient time for the cells to settle. If the addition of chemical flocculants can be avoided (or selected appropriately), the culture medium could potentially be recycled directly back to the culture vessel.

An important characteristic for any biodiesel feedstock is the suitability of the fatty acid profile for biodiesel production. Few studies have investigated the quality of microalgal biodiesel (Francisco *et al.* 2010; Miao & Wu 2006; Xiong *et al.* 2008). Algal lipid composition is influenced by growth conditions such as temperature and nutrient availability (Stansell *et al.* 2011). The different carbon chain lengths, and number and position of unsaturated bonds found in fatty acids influence the cetane number (CN), iodine value, oxidative stability, cold flow properties and viscosity of the fuel (Ramos *et al.* 2009). Biodiesel quality is governed by different regulations in different countries, for example the ASTM D6751 in the United States, and the EN 14214 in Europe. Fuel that does not meet these specifications could still be used if blended with petroleum diesel, however, this would be undesirable for the goal of producing sustainable, carbon-neutral fuel.

Cetane number is a dimensionless parameter related to the tendency of the fuel to ignite in the engine. The higher the cetane number, the shorter the ignition delay time and the better the ignition properties. An adequate cetane number is required for good engine performance, cold start properties and minimisation of exhaust emissions (Ramos *et al.* 2009; Tong *et al.* 2010). The cetane number of methyl esters decreases with increasing unsaturation (more double bonds) and shorter chain length. A cetane number of above 51 is required by the European biodiesel standard (EN 14214).

The total unsaturation of a mixture of fatty acids is measured by the iodine value. It is expressed as grams of iodine required to react with 100 g of a sample. The upper limit in EN 14214 is 120 g I_2/100 g (Ramos *et al.* 2009). The total proportion of linolenic acid methyl ester (C18:3), and polyunsaturated fatty acids (methyl esters with more than 4 double bonds) in biodiesel are also regulated to below 12% and 1% respectively.

Oxidative stability is one of the major issues affecting biodiesel due to the degradation of polyunsaturated methyl esters. Biodiesel made from most common raw materials does not meet the European standard EN 14214 without addition of antioxidants. Oxidative stability decreases with an increase in the number of double bonds in the fatty acid methyl esters present, and the number and position of double bonds affect the rate of autoxidation. Oils rich in linoleic and linolenic acids tend to have poor oxidative stability (Ramos *et al.* 2009).

Another major problem with biodiesel is its relatively poor cold properties due to the presence of saturated fatty acids with relatively high melting points. Wax settling and plugging of filters and fuel lines can occur at overnight temperatures reached routinely in many parts of the world. Regulatory requirements for cold flow plug point differ by country (Ramos *et al.* 2009). For Spain, a country with a relatively mild climate, a cold filter plug point of below 0°C and -10°C are specified for summer and winter respectively (Ramos *et al.* 2009). CFPP is increased by the

presence of saturated fatty acids, particularly those with a long chain length, corresponding to a relatively high melting point (Ramos *et al.* 2009). The properties of fatty acids that produce a favourable CN (long, saturated carbon chain) also cause poor cold flow properties and *vice versa*, therefore a mixture of saturated and unsaturated fatty acids is required (Knothe 2009). For further discussion of the influence of microalgal lipid profiles on fuel properties, see Stansell *et al.* (2011).

6.3 Methods

6.3.1 Cultures

Batch cultures were inoculated and grown for 14 days in tubular airlift photobioreactors, according to the methods presented in Section 2.3. Four freshwater species, six marine species and one halophilic species were tested. The species used, along with their media requirements and the abbreviations used are presented in Table 6.1.

Table 6.1 Abbreviations, media, taxa and origins of the cultures used. Freshwater species are listed first (3N BBM medium), followed by marine (f/2 and Walne's) and finally one halophilic Cyanobacteria (Zarrouk's)

Culture	Abbr.	Medium	Taxa	Origin
Ankistrodesmus falcatus	Af	3N BBM	Chlorophyte	UTEX 242
Chlorella vulgaris	Cv	3N BBM	Chlorophyte	UTEX 395
Neochloris oleoabundans	Nol	3N BBM	Chlorophyte	UTEX 1185
Scenedesmus sp.	Sc	3N BBM	Chlorophyte	own isolate, Upington, South Africa
Cylindrotheca fusiformis	Cf	Walne's	Diatom	UTEX B2087
Isochrysis C4	Iso	Walne's	Haptophyte	WITS culture collection, Johannesburg, South Africa
Nannochloropsis sp.	Nan	f/2	Eustigmatophyte	University of Hawaii culture collection
Pavlova sp.	Pav	Walne's	Haptophyte	MCM culture collection, Cape Town, South Africa
Phaeodactylum tricornutum	Pt	Walne's	Diatom	University of Hawaii culture collection
Tetraselmis suecica	Ts	f/2	Chlorophyte	UTEX LB 2286
Spirulina platensis	Spir	Zarrouk's	Cyanobacteria	own isolate, abandoned tannery treatment pond, Wellington, South Africa

The nitrate concentration in the media was modified to either 1500 mg.L^{-1} (nitrogen replete condition, H) or 150 mg.L^{-1} (nitrogen limited condition, L). At 1500 mg.L^{-1}, all the cultures still had sufficient nitrate for unlimited growth at the end of 14 days, whereas at 150 mg.L^{-1}, the nitrate had been completely utilized by day 3 to 5 in all cultures (data not shown).

6.3.2 Biomass and lipid quantification

Biomass concentration was quantified by optical density at 750 nm. Dry weight measurements taken daily throughout the growth cycle (according to the protocol in Section 2.4.1) were used to construct a standard curve for each culture. Fatty acid content and profile were measured by direct transesterification and gas chromatography, according to the protocols in Section 2.4.3. For the purposes of this work, lipid content was measured as total fatty acid content.

6.3.3 Cell dimensions and settling tests

At the end of the culture period, cell shape was noted and cell size estimated using an Olympus BX40 microscope equipped with a digital camera and AnalySIS software (manual function, approx. 30 cells per culture). At the end of the 14 day growth period, 1 L of each culture, diluted in medium to 1 g.L^{-1} (except for *N. oleoabundans* which had a biomass concentration of less than 1 g.L^{-1} at the end of 14 days and was settled at its final concentration of 0.33 and 0.56 g.L^{-1} for the H and L cultures respectively), was settled in a conical settling funnel for 24 h. The supernatant and the settled fraction were sampled and the dry weight of each estimated by optical density at 750 nm. The settling rate was determined by monitoring the volume of the sedimented cell pellet over time, and multiplying by the concentration of the cell pellet to calculate the biomass settled out as a function of time. The settled biomass recovered after 24 h was calculated as a percentage of the original biomass present in the culture. The concentration factor was calculated as the concentration of cells in the settled fraction after 24 h, divided by the concentration of cells in the culture before settling.

6.3.4 Calculations

Instantaneous biomass productivity ($Q_{X\ INST}$) was calculated as the change in biomass concentration (X) per unit time between two consecutive sampling times (Equation 6.1), while average biomass productivity ($Q_{X\ AVE}$) was calculated by dividing the biomass concentration at each time point by the time from inoculation (Equation 6.2).

$$Q_{X\,INST} = \frac{X_2 - X_1}{t_2 - t_1} \qquad \text{Equation 6.1}$$

$$Q_{X\,AVE} = \frac{X_1}{t_1} \qquad \text{Equation 6.2}$$

Specific growth rate (μ) was determined from the slope of the natural logarithm of biomass concentration as a function of time across each pair of sample points (Equation 6.3). Doubling time (T_d) was calculated from μ using Equation 5.1.

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \qquad \text{Equation 6.3}$$

Volumetric lipid content (P_{VOL}) was calculated as the product of biomass concentration (X) and lipid content (P) (Equation 6.4). Instantaneous lipid productivity ($Q_{P\,INST}$) was calculated as the change in P_{VOL} as a function of time (Equation 6.5), and average lipid productivity ($Q_{P\,AVE}$) as P_{VOL} at each time point divided by the time from inoculation (Equation 6.6).

$$P_{VOL} = X.P \qquad \text{Equation 6.4}$$

$$Q_{P\,INST} = \frac{P_{VOL\,2} - P_{VOL1}}{t_2 - t_1} \qquad \text{Equation 6.5}$$

$$Q_{P\,AVE} = \frac{P_{VOL1}}{t_1} \qquad \text{Equation 6.6}$$

Maximum parameters were defined as the highest value reached within the total culture period. For the calculation of μ_{max}, $Q_{X\,max}$ and $Q_{P\,max}$, three consecutive instantaneous values of μ, Q_X and Q_P were averaged across the time-course to provide a rolling average, and the maximum of each determined.

6.3.5 Prediction of biodiesel properties from fatty acid content

The FAME profile was used to calculate the cetane number (CN) and cold filter plug point (CFPP) according to Stansell *et al.* (2011). The iodine value was calculated from the degree of unsaturation according to (Ramos *et al.* 2009).

6.4 Results and discussion

6.4.1 Growth and lipid parameters

The biomass concentration and lipid content as a function of time for each species, under nitrogen replete (H) and limited (L) conditions, are provided in Appendix C. The growth rates, lipid content and productivities for each species under H and L conditions, over a 14-day growth cycle, are summarised in Table 6.2. The error across experiments was investigated using two species. Five replicate cultures of *C. vulgaris* showed a relative error in biomass concentration throughout the growth cycle of less than 5%. Duplicate cultures of *C. vulgaris* and *Scenedesmus* under both H and L conditions were found to have average relative errors for the different growth and lipid characteristics as shown in Table 6.3. The average relative error varied from 3% to 15%, due to propagation of error in calculations involving more than one measurement.

Table 6.2 Minimum doubling time ($T_{d\ min}$), maximum specific growth rate (μ_{max}), biomass concentration (X_{max}), instantaneous biomass productivity ($Q_{X\ INST\ max}$), lipid content (P_{max}), instantaneous and average lipid productivity ($Q_{P\ INST\ max}$ and $Q_{P\ AVE\ max}$) and volumetric lipid content ($P_{VOL\ max}$) measured over a 14 day batch growth cycle for eleven species of microalgae under nutrient replete (H) and limited (L) conditions.

Species are listed alphabetically, with the freshwater species grouped first, followed by the marine species and finally the halotolerant *S. platensis*. The best recorded values are highlighted in grey

	μ_{max} (day^{-1})	$T_{d\ min}$ (days)	X_{max} ($g.L^{-1}$)	$Q_{X\ INST\ max}$ ($g.L^{-1}.day^{-1}$)	P_{max} (% DW)	$Q_{P\ INST\ max}$ ($mg.L^{-1}.day^{-1}$)	$Q_{P\ AVE\ max}$ ($mg.L^{-1}.day^{-1}$)	$P_{VOL\ max}$ ($mg.L^{-1}$)
AfH	0.99	0.70	1.12	0.25	12	13	16	96
AfL	0.95	0.73	1.48	0.23	30	55	31	397
CvH	1.34	0.52	1.65	0.28	14	27	22	231
CvL	1.30	0.53	1.05	0.24	57	67	47	597
NolH	1.56	0.45	1.13	0.29	13	29	24	140
NolL	1.69	0.41	0.98	0.29	44	50	41	428
ScH	1.15	0.60	2.61	0.30	9	29	21	242
ScL	1.20	0.58	1.73	0.36	43	106	60	649
CfH	0.72	0.96	1.52	0.35	27	55	45	357
CfL	0.69	1.00	1.39	0.29	32	52	43	366
IsoH	1.32	0.52	2.48	1.23	7	24	20	165
IsoL	0.79	0.88	1.72	0.34	15	42	27	235
NanH	0.76	0.91	1.74	0.24	24	42	31	413
NanL	0.73	0.95	1.32	0.21	35	63	43	471
PavH	0.60	1.15	1.00	0.17	11	13	14	105
PavL	0.58	1.19	0.94	0.18	14	12	15	122
PtH	0.68	1.02	1.74	0.34	18	30	27	308
PtL	0.69	1.00	1.51	0.32	28	46	37	406
TsH	1.20	0.58	2.32	0.49	9	37	31	177
TsL	1.32	0.53	2.10	0.49	13	48	38	259
SpirH	0.43	1.63	2.74	0.29	4	14	9	95
SpirL	0.68	1.02	1.48	0.34	2	5	8	23

Table 6.3 Average relative error (ARE) in the growth and lipid characteristics measured across duplicate experiments with *C. vulgaris* and *Scenedesmus* under both H and L conditions

	μ_{max} (day^{-1})	$T_{d\,min}$ (days)	X_{max} ($g.L^{-1}$)	$Q_{X\,max}$ ($g.L^{-1}.day^{-1}$)	P_{max} (% DW)	$P_{VOL\,max}$ ($mg.L^{-1}$)	$Q_{P\,max}$ ($mg.L^{-1}.day^{-1}$)
ARE (%)	3.0	3.0	4.6	8.1	3.6	8.7	14.6

Specific growth rate

Figure 6.1 shows the maximum μ and X reached during 14 days of batch growth for each species under N replete and limited conditions. The specific growth rate (μ) depends on the microorganism and growth conditions. As μ_{max} is the maximum increase in biomass per unit biomass per time, it occurs very early in the growth cycle, when the biomass concentration is very low and before any nutrients become limiting. It gives an indication of the potential doubling time of the species, independent of culture conditions. The μ_{max} for each species was very similar under nitrogen replete and limiting conditions, as it occurred early in the growth cycle when the N limited cultures had not yet run out of nitrate. *N. oleoabundans* had the highest μ_{max} (day^{-1}) (1.56 and 1.69 for nitrogen replete and limited respectively), followed by *C. vulgaris* (1.34 and 1.30), *Isochrysis* (1.32 and 0.79), *T. suecica* (1.20 and 1.32) and *Scenedesmus* (1.15 and 1.20). The species with the highest μ_{max} were all chlorophyta, except for *Isochrysis*.

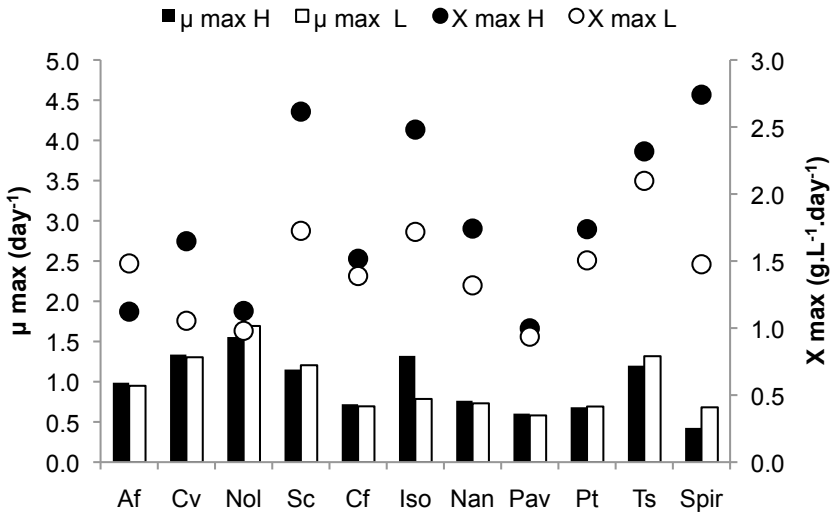


Figure 6.1 μ_{max} (bars) and X_{max} (circles) during a 14 day growth period for 11 species of microalgae under nitrogen replete (H, filled bars and circles) and limited (L, empty bars and circles) conditions

Biomass concentration

Maximum biomass obtained is important not only for calculating maximum product yield, but a high biomass concentration can also facilitate harvesting. In all species, except for *A. falcatus*, the

nitrogen replete culture reached a higher final biomass concentration than the nitrogen limited culture (Figure 6.1). The N limited cultures reached between 54% (*S. platensis*) and 94% (*Pavlova*) of the X_{max} of the N replete culture, with an average across all species of 84%. The cultures that reached the highest X_{max} ($g.L^{-1}$) were *S. platensis* H (2.74), *Scenedesmus* H (2.61), *Isochrysis* H (2.48) and *T. suecica* H (2.32). Interestingly, *T. suecica* L had the next highest X_{max} (2.10), indicating that the biomass concentration reached by this species was not significantly influenced by nitrate limitation. The lipid content of *T. suecica* was also not significantly affected by nitrate limitation. *N. oleoabundans* had a surprisingly low X_{max}, which peaked at day 6 and decreased from day 8. Growth of *N. oleoabundans* has been shown to be inhibited at a nitrate concentration above 900 $mg.L^{-1}$ (Li, Y *et al.* 2008).

Biomass productivity

The maximum $Q_{X\ INST}$ reached by each species was similar under N replete and limited conditions, except for *Isochrysis* H which was very much higher than any other culture (1.2 vs 0.2 to 0.35 $g.L.day^{-1}$, Figure 6.2). Similarly to μ_{max}, $Q_{X\ INST\ max}$ occurred early in the growth cycle, before the influence of nitrate limitation had become apparent. The highest $Q_{X\ INST\ max}$ was reached by *Isochrysis* H, followed by *T. suecica* H and L. The maximum instantaneous biomass productivity gives an indication of the productivity that could be maintained in continuous culture.

Lipid content

The highest lipid contents were found in *C. vulgaris* L, *N. oleoabundans* L and *Scenedesmus* L (all above 40% DW). The lipid content of *C. vulgaris* and *Scenedesmus* was still increasing at the end of 14 days and may have reached higher values if cultivation had been continued. Some of the N replete cultures began to increase in lipid content towards the end of 14 days. This can be seen in the difference between the average lipid content (grey bars, Figure 6.2), and the maximum lipid content (black bars, Figure 6.2) across the culture period. *Nannochloropsis*, *C. fusiformis* and *P. tricornutum* in particular showed an increase in lipid content with culture age, despite having sufficient nitrogen.

In all cases, except for *S. platensis*, nitrogen limited conditions resulted in a higher lipid content than nitrogen replete conditions. Lipid content was particularly enhanced (between 2.5 and 4.6 times larger under N limited conditions) in the freshwater green algae (*C. vulgaris*, *Scenedesmus*, *N. oleoabundans* and *A. falcatus*), while the marine species (*Nannochloropsis*, *T. suecica*, *C. fusiformis*, *P. tricornutum*, *Pavlova* and *Isochrysis*) showed less of an effect (lipid content in N limited cultures was 1.3 to 2.1 times that of N replete). The only blue-green algae tested (*S. platensis*) had a very low lipid content under both conditions. Previous studies have also shown that cyanobacteria have a relatively low lipid content and that N limitation has less impact on lipid accumulation than for freshwater algae (Griffiths & Harrison 2009). Piorreck *et al.* (1984)

reported that the lipid content of *S. platensis* was relatively unresponsive to the different nitrogen levels tested (between 0.0003% and 0.1% NH_4Cl or KNO_3).

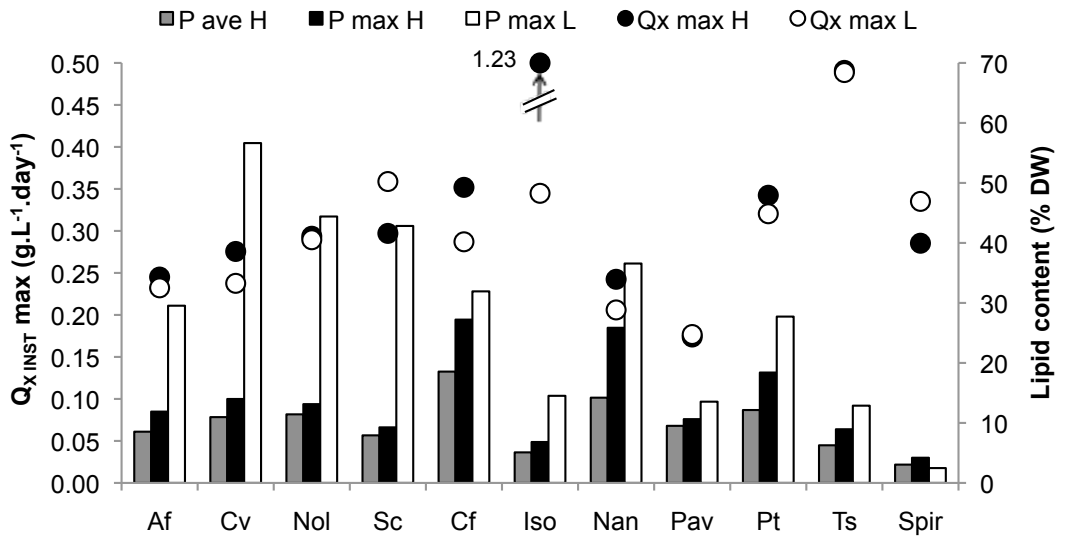


Figure 6.2 $Q_{X\,INST\,max}$ (filled and empty circles for H and L respectively), P_{ave} for H (grey bars) and P_{max} for H and L conditions (filled and empty bars respectively) during a 14 day growth period for 11 species of microalgae. In order for the lower points to be seen, $Q_{X\,max}$ for Iso H is shown on a different scale

Volumetric lipid content (P_{VOL}) is a measure of the lipid yield per volume of culture at a particular time point. Figure 6.3 shows that, in all cases other than *S. platensis*, the maximum P_{VOL} was higher for nitrogen limited than for nitrogen replete cultures. This was true throughout the period of N limitation and was caused by the higher lipid content of the N limited cultures, despite their lower biomass concentration. The highest P_{VOL} were achieved by *Scenedesmus* L (649 $mg.L^{-1}$) and *C. vulgaris* L (597 $mg.L^{-1}$). Freshwater chlorophyta showed higher levels of lipid accumulation with nitrogen limitation than the marine species.

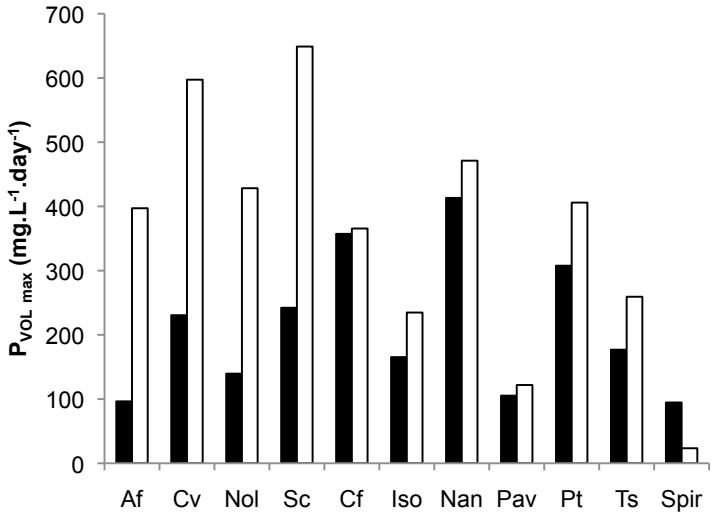


Figure 6.3 Maximum volumetric lipid content (P_{VOL}) during 14 days of culture for 11 species of microalgae under nitrogen replete (filled bars) and limited (empty bars) conditions

Lipid productivity

Lipid productivity is the product of biomass productivity and lipid content. It gives an indication of the highest lipid production rate that could be maintained in continuous culture ($Q_{P\ INST}$) or achieved after a certain length of time in batch culture ($Q_{P\ AVE}$). As with P_{VOL}, the $Q_{P\ INST}$ and $Q_{P\ AVE}$ was higher for nitrogen limited than nitrogen replete cultures in all cases except *C. fusiformis* (Cf) and *S. platensis* (Spir) (Figure 6.4). *Scenedesmus* L showed the highest instantaneous and average lipid productivity (106 and 60 mg.L^{-1}.day^{-1} respectively). This was followed by *C. vulgaris* L (67 and 47) and *Nannochloropsis* (63 and 43). The high productivity of *Scenedesmus* L was due to its high lipid content under nitrogen limited conditions, but also the fact that it could maintain a high biomass productivity (equivalent to *Scenedesmus* H) until day 7, when the lipid content of *Scenedesmus* L had accumulated to nearly 30% DW. *C. vulgaris* L was found to accumulate lipid rapidly under nitrogen limitation, to 39% DW by day 7 and 57% by day 14. The high lipid productivity of *Nannochloropsis* H and L was due to a relatively high lipid content. Other species worth mentioning are *N. oleoabundans* L. which had a relatively high lipid productivity due to a rapid accumulation of lipids, and *T. suecica* H and L which showed rapid growth and high biomass concentrations, although a relatively low lipid content. Nitrogen limitation did not significantly affect the growth rate or lipid content of *C. fusiformis*, however this species maintained a combination of reasonably high biomass productivity and above typical lipid content.

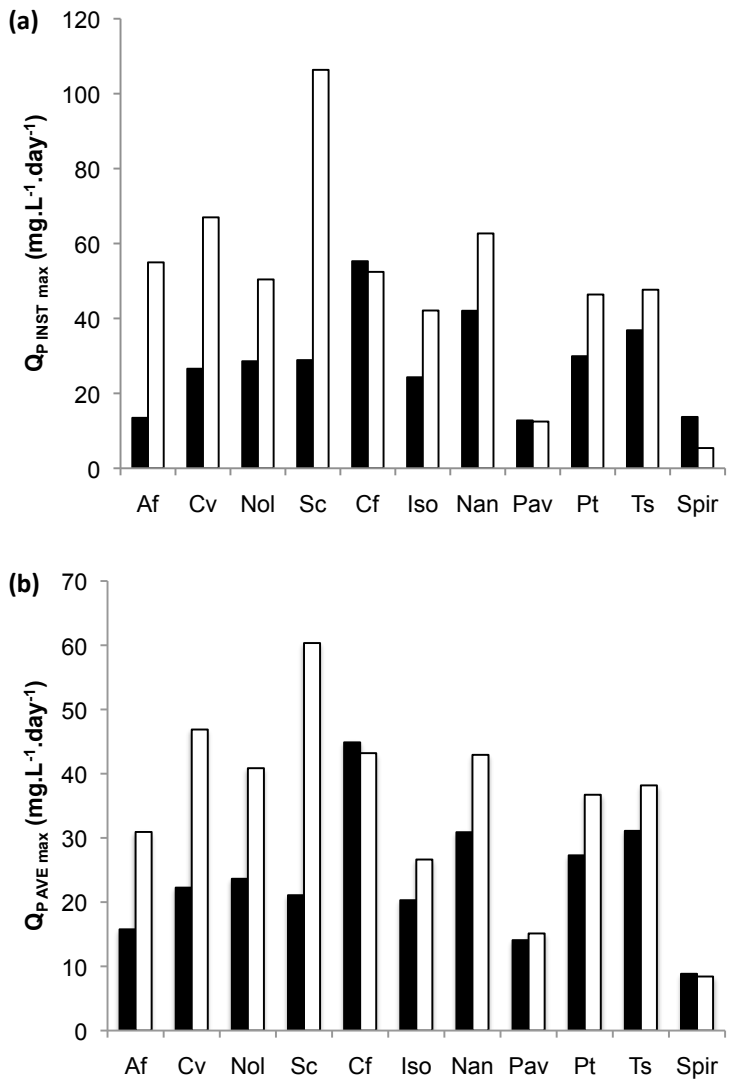


Figure 6.4 (a) $Q_{P\ INST\ max}$ and (b) and $Q_{P\ AVE\ max}$ during a 14 day growth period for 11 species of microalgae under nitrogen replete (filled bars) and limited (empty bars) conditions

6.4.2 Comparison between species

Comparison of maximum values reached during a set time period, for example maximum biomass concentration or product content reached during 14 days of growth, does not take into account the time at which the maximum value was reached. For example, *N. oleoabundans* H had reached its peak biomass concentration by day 6, while *Scenedesmus* H biomass concentration continued to increase up to day 14. In addition, maximum specific growth rate and instantaneous biomass productivity both occur very early in the growth cycle, before the 150 mg.L^{-1} nitrate cultures became nitrogen limited. A more accurate representation of the influence of nitrate depletion would be provided by comparing the average biomass productivity across the growth curve. However, the growth curve of different species spans different lengths of time. For these reasons, it would arguably be more appropriate to compare species at relative points in the growth curve, rather than after an absolute length of time.

An attempt to address these problems was made by choosing a point for comparison in the late exponential/early stationary phase, defined as the point at which the biomass concentration (X) reached 90% of the maximum obtained within 14 days. Table 6.4 compares some key parameters of the productivity of the 11 microalgal species at this point. $Q_{X\ AVE}$ is used for comparison, instead of $Q_{X\ INST\ max}$ and μ_{max} which were identical to the maximum values over the entire 14 days.

Table 6.4 Average biomass productivity ($Q_{X\ AVE}$), lipid content (P), average lipid productivity ($Q_{P\ AVE}$) and volumetric lipid content ($Q_{P\ VOL}$) of the cultures at the point where the biomass concentration had reached 90% of the maximum for each species. The corresponding time points are shown in column 2. Maximum values are highlighted in grey.

	Time (days)	90% X_{max} ($g.L^{-1}$)	$Q_{X\ AVE}$ ($g.L^{-1}.day^{-1}$)	P % DW	$Q_{P\ AVE}$ ($mg.L^{-1}.day^{-1}$)	$Q_{P\ VOL}$ ($mg.L^{-1}$)
AfH	10.8	1.08	0.10	8	8	87
AfL	9.8	1.33	0.13	22	30	290
CvH	7.8	1.48	0.18	11	20	157
CvL	11.8	1.02	0.08	52	44	515
NolH	4.7	1.01	0.20	12	24	111
NolL	7.6	0.88	0.11	34	38	285
ScH	11.0	2.47	0.22	8	19	208
ScL	6.8	1.55	0.22	25	55	374
CfH	5.1	1.37	0.26	16	42	212
CfL	5.7	1.25	0.22	19	41	235
IsoH	3.3	2.23	0.65	3	19	65
IsoL	7.8	1.55	0.20	9	17	133
NanH	9.7	1.70	0.16	17	27	260
NanL	9.7	1.28	0.13	27	34	334
PavH	6.7	0.90	0.12	10	12	84
PavL	6.7	0.84	0.12	10	12	84
PtH	6.9	1.56	0.21	12	26	176
PtL	6.9	1.35	0.20	18	35	241
TsH	7.7	2.20	0.28	8	22	173
TsL	3.8	1.89	0.39	9	34	128
SpirH	10.8	2.47	0.22	4	8	83
SpirL	4.8	1.33	0.21	2	4	20

The difference in biomass productivity between N replete and limited cultures is shown more clearly by $Q_{X\ AVE}$ at 90% X max (Figure 6.5). However, the difference in lipid content between replete and limited cultures is less as cultures have not yet had time to accumulate their maximum lipid content. In order to fully appreciate the effects of N limitation on lipid accumulation, cultures must be grown beyond the onset of stationary phase. For this reason, $Q_{P\ AVE}$ at 90% X_{max} is lower than the maximum recorded over a 14 day period. However, the comparison between species is similar (Figure 6.6).

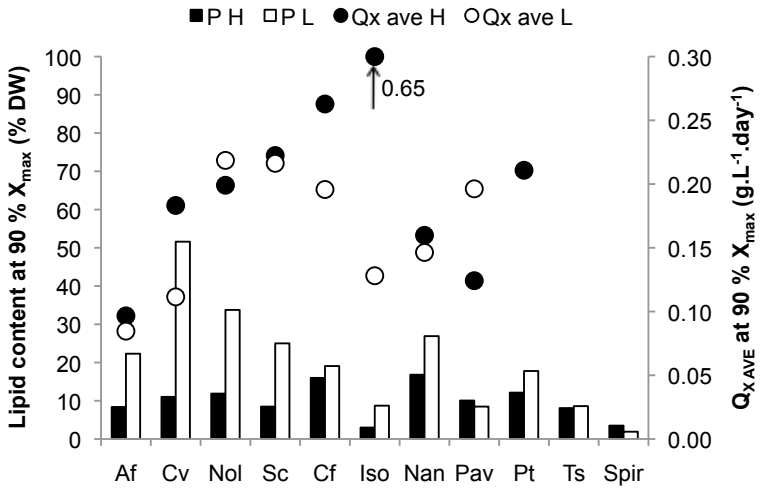


Figure 6.5 P (filled and empty bars for H and L respectively)and $Q_{X\ AVE}$ (filled and empty circles for H and L respectively) at the time point at which the biomass concentration reached 90% of X_{max} over 14 days

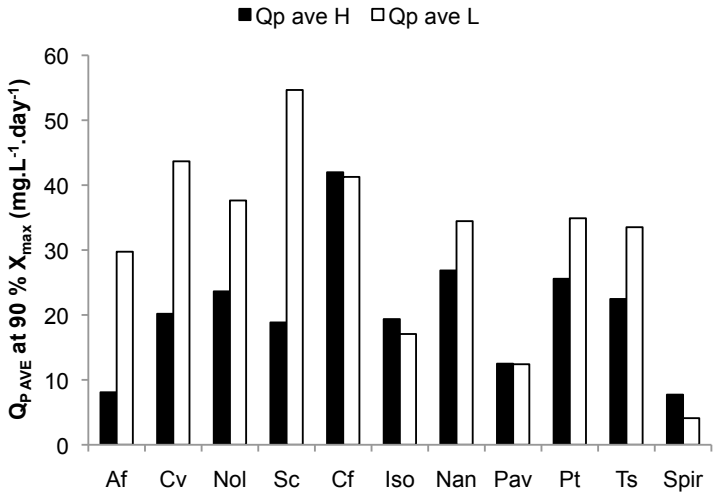


Figure 6.6 $Q_{P\ AVE}$ at 90% X max (filled and empty bars for H and L respectively)

6.4.3 Comparison to literature values

The values obtained experimentally for the eleven species over a 14 day growth period are compared in Figure 6.7 and Figure 6.8 to the average literature values determined in Chapter 5. Literature values for lipid content under both N replete and limited conditions were obtained, but values for biomass and lipid productivity were only available under N replete conditions. Where the strain was not identified to species level, average literature values for the genus were used.

Experimental and literature values for biomass productivity were comparable, other than *Isochrysis* H, which showed an extremely high biomass productivity in the lab (Figure 6.7a). This

was due to a consistently high growth rate between days 2 and 4, therefore it cannot be attributed to an error in a single measurement (see Appendix C). Measured lipid contents under N limited conditions compared well with literature values (Figure 6.7b), other than *S. platensis* L, *T. suecica* L and *Isochrysis* L, which had lower experimental values, and *C. vulgaris* L, which had a very high experimental lipid content. The measured lipid contents under N replete conditions were generally lower than those reported in literature.

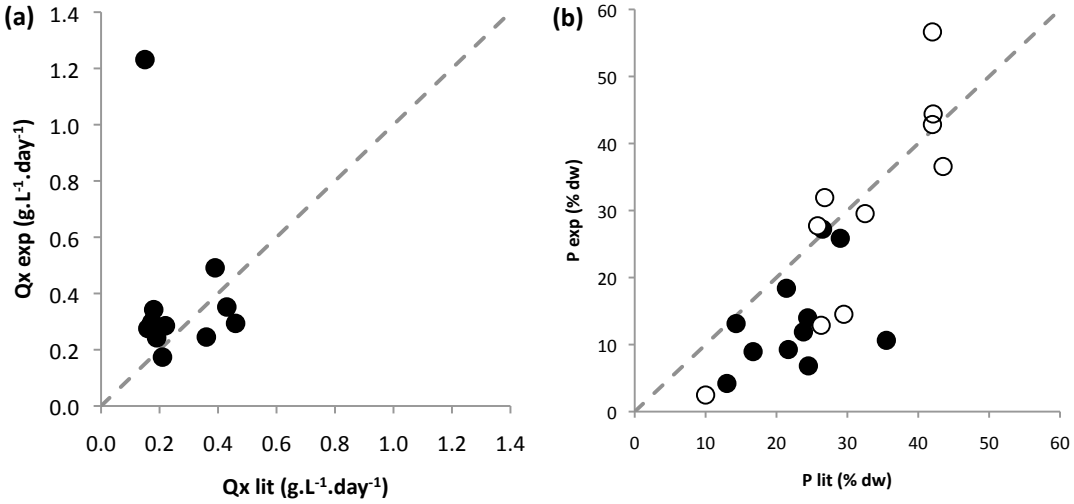


Figure 6.7 Parity plots of experimental (exp, y-axis) and literature (lit, x-axis) values for (a) biomass productivity (Q_X) under nitrogen replete conditions and (b) lipid content (P) under N replete (filled circles) and N limited (empty circles) conditions. The dashed line indicates y = x.

The experimental lipid productivities of *N. oleoabundans* H, *A. falcatus* H, *C. fusiformis* H and *Pavlova* H were significantly lower than those reported in literature, or calculated from biomass productivities and lipid contents reported in literature in Chapter 5 (Figure 6.8). The difference is due to the lower lipid contents found experimentally in the N replete cultures. This could be due the use of different strains or different methods of lipid quantification. Gravimetric quantification of lipid extracts includes the mass of non-saponifiable compounds (such as pigments, sterols and waxes) soluble in the extraction solvents (Palmquist & Jenkins 2003; Pruvost *et al.* 2009; Ratledge 1987)). This could lead to a higher reported lipid content. In this study, lipid content has been quantified as fatty acid content, because non-saponifiable compounds do not contain fatty acids and are therefore not relevant to biodiesel production. Another possibility is that studies assumed to have been conducted under nutrient replete conditions may not have provided sufficient N, Si or P to remain replete to the end of the cultivation period. For example, the original recipe for BBM media (Bold 1949) provides 250 $mg.L^{-1}$ $NaNO_3$ (182 $mg.L^{-1}$ nitrate). In this study, 150 $mg.L^{-1}$ nitrate was shown to be depleted in the medium between days 3 and 5, therefore cultures grown in the original recipe BBM media are likely to become N limited by day

6 or 7. Algae inadvertently placed under nutrient limitation would have been reported with a higher lipid content and hence higher lipid productivity.

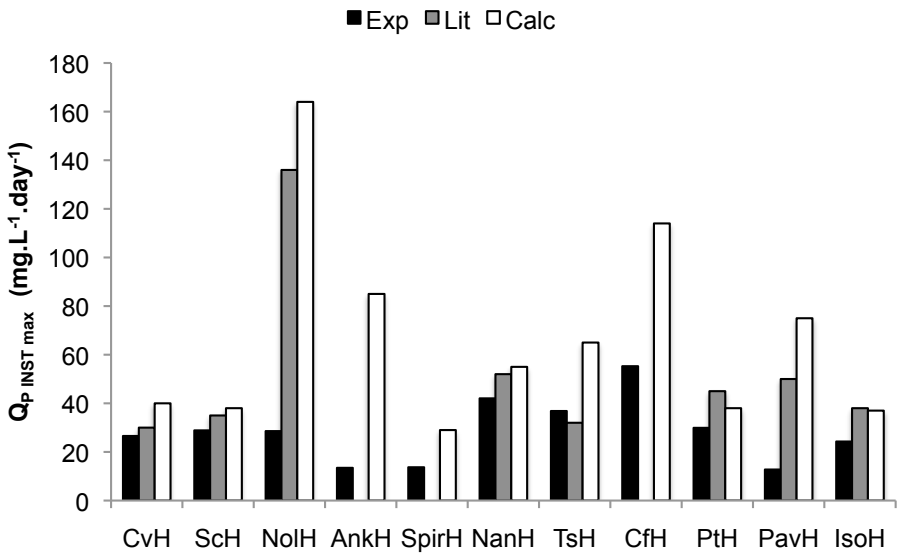


Figure 6.8 Comparison of lipid productivity achieved experimentally (exp), compared to average values collected (lit) and calculated (calc) from literature. Only values under N replete conditions are shown.

Direct comparison of the biomass and lipid productivity of different microalgal species is complicated by their requirement for and unique metabolic response to different growth conditions. Ideally experiments should mimic as closely as possible the final conditions under which the organisms will be grown, including the harvesting and biomass processing steps. However, for a general study such as this, these were not yet identified, hence standard conditions have been used. Preferably, culture conditions and levels of nitrogen limitation should be optimized for each species, in order to find the maximum productivity possible with that strain. This approach is, however, time consuming and often impractical. In this study, due to their different osmotic and nutrient requirements, it was necessary to use different culture medium for different groups of species. It is likely that some or all of the species tested here may perform better under different conditions, e.g. higher light intensity. Only two starting nitrate concentrations were tested here. It is possible that a lower or intermediate starting nitrate concentration could improve on the productivities achieved here.

6.4.4 Cell size/shape and settling parameters

Ease of harvesting was compared by measuring the rate and efficiency of gravity sedimentation of each culture after 14 days of growth. The rate of accumulation of the cell pellet, and the proportion of the suspended biomass that had settled into a recoverable pellet after 24 h were used as crude indicators of the potential of a species to be harvested by this method. Figure 6.9

shows the biomass recovery after 24 hours for each species under nitrogen replete and limited conditions. The species which settled best in a conical funnel at a dilution of 1 $g.L^{-1}$ (measured as% recovery in 24 h) were *S. platensis* (95%), *C. fusiformis* (94 to 96%), *T. suecica* (80 to 94%) and *Scenedesmus* (79 to 86%). *S. platensis* and *C. fusiformis* settled very quickly with *C. fusiformis* H and L cultures reaching maximum sedimented pellet volume within 5 min, while *S. platensis* H and L cultures took 3 h and 10 min respectively. *A. falcatus* H and *Nannochloropsis* H reached recoveries of over 50% within 24 h, while all other cultures had recoveries of less than 43%.

The size and shape of cells in the different cultures at the end of the growth period were also recorded (Table 6.5) as this could influence the choice of harvesting technique. There are several alternative harvesting methods, including centrifugation, flotation, flocculation and microfiltration, as well as techniques for enhancing settling, such as bioflocculation, addition of chemicals or changes in pH (Mata *et al.* 2010). In addition, the dimensions of the settling vessel, concentration of the culture and environmental conditions may significantly influence the settling rates recorded here.

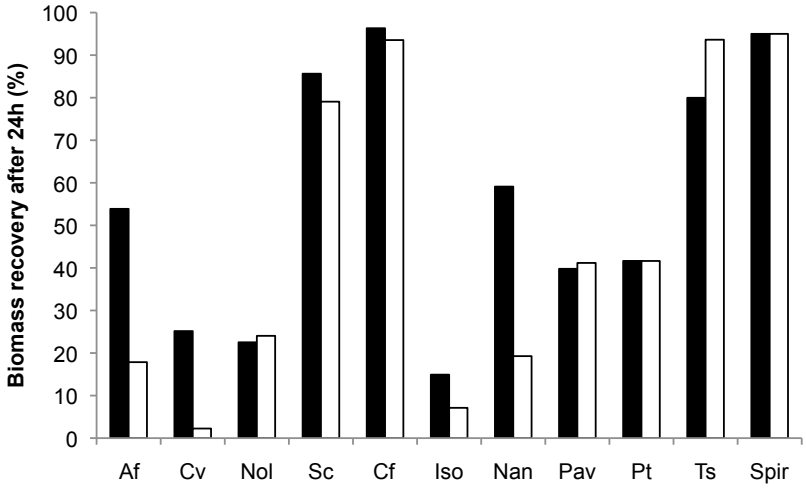


Figure 6.9 Biomass recovery after 24 h settling for 11 species grown under N replete (filled bars) and limited (empty bars) conditions

Table 6.5 Cell size, shape, maximum biomass settling rate, recovery and concentration factor after 24 h of gravity sedimentation for 11 species of microalgae grown for 14 days under nitrogen replete (H) and limited (L) conditions

	Cell shape	Average cell length (μm)	Average cell width (μm)	Maximum settling rate ($g.L^{-1}.h^{-1}$)	Biomass recovery after 24 h (%)	Biomass concentration factor at 24 h (x)
AfH	spindle	50	4.0	0.6	54	18
AfL	spindle	50	3.0	0.008	18	14
CvH	sphere	2.5	2.5	-	25	52
CvL	sphere	2.5	2.5	-	2	15
NolH	sphere	4.0	4.0	0.07	23	35
NolL	sphere	3.5	3.5	0.2	24	65
ScH	pointed oval	9.0	3.8	0.3	86	167
ScL	pointed oval	9.1	4.7	0.04	79	103
CfH	spindle	30	4.0	16	96	23
CfL	spindle	30	4.0	24	94	19
IsoH	sphere	5.2	5.2	-	15	12
IsoL	sphere	5.2	5.2	0.005	7	50
NanH	sphere	3.0	3.0	0.01	59	36
NanL	sphere	3.0	3.0	-	19	15
PavH	tapering sphere	5.0	5.0	0.005	40	19
PavL	tapering sphere	5.6	5.6	0.001	41	17
PtH	spindle	18.5	5.2	0.004	42	31
PtL	spindle	11.5	5.0	0.08	42	6
TsH	oval	12.5	8.3	0.4	80	102
TsL	oval	12.2	9.5	0.07	94	109
SpirH	filament	60 to 500	10	11	95	15
SpirL	filament	60 to 90	10	29	95	136

6.4.5 Suitability of lipids for biodiesel

The properties of biodiesel are heavily influenced by the fatty acid composition of the feedstock oil. In order to investigate the suitability of their oil for biodiesel production, the fatty acid profile of each species under nitrogen replete and limited growth conditions was analysed. FAME profiles at days 5, 10 and 14 were very similar, indicating that the relative proportions of fatty acids remained the same through the growth cycle. The FAME profiles after 14 days of growth are shown in Table 6.6. These correlate well with lipid profiles reported in literature (Ben-Amotz *et al.* 1985; Chen & Johns 1991; Colla *et al.* 2004; Harwood & Jones 1989; Patil *et al.* 2006; Reitan *et al.* 1994; Renaud *et al.* 1994; Suen *et al.* 1987; Tornabene *et al.* 1983; Volkman *et al.* 1989).

The most common fatty acid methyl esters found in the microalgae tested here were C16 and 18:1, with several species having high proportions of C16:1, C18:2 and C18:3 and some of the marine species C14:0 and C20:5. There were significant differences between species and between growth conditions. Similarly to Piorreck *et al.* (1984), all the freshwater Chlorophyta species showed a large increase in C18:1 with nitrogen limitation, along with a decrease in C16:0, C18:2 and C18:3. *S. platensis* showed an increase in C14:1 and C18:2 with nitrogen limitation. The fatty acid profile of the marine species was less influenced by nitrogen limitation, although *Nannochloropsis* showed an increase in C18:1 and a decrease in C14:0, C16:0 and C20:5. *C.*

fusiformis showed an increase in C16:1 and polyunsaturated C20 with a decrease in C16 and C18:1.

The biodiesel properties calculated from the fatty acid profile of each species under nitrogen replete and limited conditions are shown in Table 6.7. The cetane number of the oil from each culture was calculated from the fatty acid composition. None of the nitrate replete freshwater cultures (CN of 43 to 50) satisfied the EN 14214 criteria of a cetane number (CN) greater than 51, although *C. vulgaris* L, *Scenedesmus* L and *N. oleoabundans* L did. *A. falcatus* L, *Pavlova* H and *S. platensis* L also did not make the cut-off. The lower cetane numbers in algal oils are largely related to the proportion of unsaturated fatty acids such as C18:2 and C18:3. *C. vulgaris* H, *Scenedesmus* H, *N. oleoabundans* H and *A. falcatus* H have a high proportion of polyunsaturates, leading to a low cetane number. The decrease in C18:2 and C18:3, along with an increase in C18:1 was responsible for the N limited cultures reaching the specification. Oils rich in C16:0 and C18:0, such as those from *Nannochloropsis*, *T. suecica* and *C. fusiformis*, have a higher cetane number and theoretically better combustion. In agreement with Piorreck *et al.* (1984), N limitation resulted in an increase in C18:1 and a decrease in polyunsaturated fatty acid content, which lead to a higher CN and lower linolenic acid content.

The iodine values calculated for all cultures were within the EN 14214 requirement (Table 6.7). *C. vulgaris* H, *N. oleoabundans* H and *A. falcatus* H and L had a linolenic acid content above the upper limit of 12% and all the seawater species had a content of polyunsaturated fatty acids that was outside of the EN 14214 limit of 1%. All the freshwater species, as well as *S. platensis*, had high linoleic and linolenic contents, particularly under nitrogen replete conditions (Table 6.6). The cold flow plug point varied from -2°C (*Isochrysis* H) to 19°C (*S. platensis* H). Cold flow properties depend mostly on the saturated fatty acid content, as well as the chain length of the saturated esters. Longer chain lengths have higher melting points, hence those species with a lower content of saturated fatty acids, or those with mostly shorter chain length saturated fatty acids (e.g. C14:0), are predicted to have better low-temperature properties (e.g. *A. falcatus* L, *C. vulgaris* L, *Isochrysis* and *Pavlova*).

There are no models relating the oxidative stability of a fuel to the fatty acid composition. However, fuels with a high proportion of PUFA and linolenic acid (C18:3) are likely to have poor oxidative stability due to the large number of double bonds susceptible to autoxidation (Stansell *et al.* 2011). This includes *A. falcatus*, *C. vulgaris* H, *N. oleoabundans* H, *Pavlova*, *P. tricornutum* and *Nannochloropsis* H. Species with a low proportion of highly unsaturated fatty acids, and therefore likely to be more oxidatively stable, include *N. oleoabundans* L and *Scenedesmus* H and L. Oxidative stability is a significant problem in biodiesel fuels and most will require the addition of antioxidants.

Table 6.6 Fatty acid profile of 11 microalgal species after 14 days growth under nutrient replete (H) or limited (L, highlighted in grey) conditions.

The content of each fatty acid is given as a percentage of the total fatty acid content. The contents of minor fatty acids (each found to constitute less than 2% of the total in all species tested) are grouped in the last column under 'other'. Blanks indicate level below detection limit. Fatty acids are abbreviated with the number before the colon indicating the number of carbon atoms and the number after the colon the number of double bonds

	C14:0	C14:1	C15:0	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3	C18:4	C20:1	C20:3	C20:4	C20:5	Other
AfH	0.6	1.2		25.7	2.4	3.2	0.8		15.9	18.7	22.8						8.6
AfL	0.2			10.5	1.2	2.8	0.3	2.5	52.6	13.5	12.0		1.3				3.1
CvH	0.5	0.6	0.6	23.1	0.2	7.4	5.8	5.2	16.1	20.9	18.0						1.7
CvL	0.1	0.2		16.9	0.6	2.0	5.1	6.5	48.2	8.5	11.6						0.3
NolH				29.8	2.0	5.1	9.7	2.3	1.6	29.0	17.3						3.1
NolL	0.3	0.6		25.6	1.1	1.9	1.6	4.0	42.4	22.4							0.2
ScH	0.3	1.0		24.5	2.1	2.3	6.0	4.9	19.8	34.2	2.5			0.8			1.5
ScL	0.2	0.1		24.3	1.9	3.3	1.0	4.1	46.2	15.9	1.0						2.1
CfH	6.6	0.3	0.7	42.8	29.0		0.3	0.5	16.4	1.2				0.6	1.1	0.6	
CfL	5.7	0.3	0.6	34.9	35.3		1.0		9.2	2.8				2.4	4.9	2.8	
IsoH	25.5	1.0	0.8	10.8	4.7		0.8	0.6	35.2	5.1	4.9	6.9				1.7	2.1
IsoL	25.4		0.5	17.2	3.0		0.4	1.0	34.1	2.9	3.0	9.9				1.4	1.3
NanH	8.4	0.4	0.5	40.9	26.3			1.0	7.0	1.5					3.3	10.0	0.7
NanL	6.5	0.1	0.4	36.1	27.6		0.1	1.1	19.7	1.2					2.3	4.5	0.3
PavH	32.7			17.7	11.0				3.7	0.6						28.9	5.3
PavL	31.8			19.9	16.2				3.8	0.6						23.4	4.4
PtH	6.3			23.7	45.5	0.8	3.2		5.7	0.9						14.0	
PtL	5.8			26.3	48.8	0.7	1.8		7.1	0.5						9.0	
TsH	0.7	0.4		31.7	5.4	1.9	0.3	0.8	40.2	8.8	4.7		0.5	0.9	1.0		2.6
TsL	0.7	0.1		35.0	4.4	1.4	0.2	1.0	42.9	6.9	3.5		0.5		0.6	0.8	1.9
SpirH		1.2		51.8	3.9				7.4	23.7							11.9
SpirL	2.6	5.5		30.8	6.2				2.1	48.1							4.6

Table 6.7 Predictions of feedstock characteristics, calculated from the fatty acid profile.

S = saturated fatty acids, MU = monounsaturated fatty acids, PU = polyunsaturated fatty acids, CN = cetane number, IV = iodine value, Lin = proportion of linolenic acid (C18:3), PUFA = proportion of polyunsaturated fatty acids with more than 4 double bonds and CFPP = cold filter plug point. Dash indicates value below detection limit. EN 14214 specifications are shown in the final row. Values that fall outside the specification are highlighted in grey

	S (%)	MU (%)	PU (%)	CN	IV (g I_2/100g)	Lin (%)	PUFA (%)	CFPP (°C)
AfH	26	20	45	43	98	22.8	-	10
AfL	13	55	29	48	100	12.0	-	-1
CvH	29	17	52	47	107	18.0	-	8
CvL	23	49	27	52	92	11.6	-	4
NolH	32	4	61	45	111	17.3	-	11
NolL	30	44	26	55	86	0.0	-	9
ScH	30	23	46	50	102	2.5	-	8
ScL	28	48	21	54	82	1.0	-	8
CfH	51	46	4	59	50	-	1.7	15
CfL	41	45	14	55	66	-	7.7	12
IsoH	38	41	19	52	72	4.9	1.7	-2
IsoL	44	37	18	54	66	3.0	1.4	4
NanH	51	34	15	56	59	-	13.3	14
NanL	44	47	8	57	59	-	6.7	13
PavH	50	15	29	50	67	-	28.9	4
PavL	52	20	24	51	63	-	23.4	6
PtH	30	51	19	51	80	-	14.0	7
PtL	32	56	12	53	72	-	9.0	9
TsH	33	47	18	54	74	4.7	1.0	12
TsL	37	48	13	56	68	3.5	1.4	13
SpirH	52	13	24	51	56	-	-	19
Spir L	33	14	48	48	98	-	-	11
EN 14214				>51	<120	<12%	<1%	

None of the species were predicted to meet all the EN 14214 requirements for biodiesel quality, but this does not mean that they should be ruled out for biodiesel production. *C. vulgaris* L, *N. oleoabundans* L, *Scenedesmus* L, *T. suecica* H and *S. platensis* H were excluded only due to CFPP. Of these, *C. vulgaris* L had the lowest CFPP (4°C), which met the summer specification for South Africa. Additionally, *Isochrysis* was excluded only due to a PUFA content just above the cut-off point. If this limitation could be overcome, the lower C18:2 and C18:3 content of this species suggest a more favourable oxidative stability compared to the freshwater species. It should be noted that the biodiesel fuel specifications calculated here only approximate the actual measured qualities. As evidenced by the different conditions of N availability, the fatty acid profile of microalgae can be adjusted through modification of growth conditions. Several properties, particularly the oxidative stability and cold flow properties, may be readily overcome by the use of additives or blending.

6.5 Conclusion

This chapter investigated characteristics relevant to biodiesel production in eleven microalgal species under both nitrogen replete and limited conditions. The growth and lipid content of some of the marine species was minimally affected by nitrogen limitation (e.g. *T. suecica* and *C. fusiformis*). However, in all species tested except *A. falcatus* and *S. platensis*, nitrogen limitation led to lower final biomass concentration and higher lipid content. The species *Scenedesmus* L and *C. vulgaris* L obtained the highest lipid productivity and volumetric lipid content. These showed the greatest lipid accumulation (43 and 57% DW) and a rapid growth rate (μ of 1.2 and 1.3 day^{-1}), despite an intermediate final biomass concentration (1.7 and 1 $g.L^{-1}$).

All nitrogen limited cultures except for *S. platensis* were found to have a consistently higher average lipid productivity and volumetric lipid content than nitrogen replete cultures, throughout the period of N limitation (see graphs of $Q_{P\ AVE}$ and P_{VOL} in Appendix C). This was because the increase in lipid content (1.3 to 4.6 times that of the H culture) was greater than the decrease in biomass concentration (0.54 to 0.94 times that of the H culture). Harvesting the L culture at any point in the growth cycle gave a higher lipid yield than that from the H culture. Hence, for high lipid productivity in batch culture, nitrate limitation is recommended.

A large variation in settling rates and biomass recovery was observed between species. Gravity sedimentation appeared promising for some species, particularly *C. fusiformis*, *T. suecica* and *Scenedesmus*. However, it is not the optimal method of biomass concentration for other species such as *Chlorella* and *Isochrysis*, which showed poor settling.

All species examined had an iodine value that conformed to EN 14214 specifications (<120 g I_2.100 g^{-1}). Seven of the 22 cultures tested had cetane numbers too low to meet EN 14214 standards. The CFPP predicted from the fatty acid profile of the algal species investigated here were relatively poor. None of the species examined would meet the winter specification, even for a relatively warm country such as Spain (-10°C) or South Africa (-3°C). Only two would meet the Spanish summer standard (0°C) and five the South African summer standard (4°C). In addition, oxidative stability is likely to be a problem for most species examined here, due to the high proportion of polyunsaturated fatty acids. The fatty acid profile of many species was found to be improved by growth under nitrate limited conditions.

Among the most promising marine species were *Nannochloropsis*, *C. fusiformis* and *P. tricornutum*, due to their high lipid productivities and potential for settling. *C. fusiformis* was particularly easy to harvest by sedimentation, and had a relatively high lipid productivity, despite the fact that it did not respond to nitrogen limitation. *T. suecica* was also notable due to its high growth rate and biomass concentration, although it had a low lipid content and did not respond to nitrogen limitation.

The most promising freshwater species tested were *Scenedesmus* and *C. vulgaris*. *Scenedesmus* had the highest recorded lipid productivity under nitrogen limitation and a good biomass recovery after 24 h settling. *C. vulgaris* had the highest lipid content, and the second-highest volumetric lipid content and lipid productivity under nitrogen limitation. Both species are known to be resilient and easy to grow (as evidenced by the fact that they are common contaminants of other algal cultures, and *C. vulgaris* is one of few microalgal species that have already been cultivated on a commercial scale). Both species also met all of the EN 14214 requirements, except for CFPP, under nutrient limited conditions.

Part III – Nitrogen limitation

7 Optimising the degree of nitrogen limitation for lipid productivity in *Chlorella vulgaris* batch culture

7.1 Introduction

The work presented in Chapter 6 showed that nitrogen limited cultures, inoculated at a nitrate concentration of 150 $mg.L^{-1}$, accumulated a greater volumetric lipid content, and had a higher lipid productivity than nitrogen replete cultures, inoculated at 1500 $mg.L^{-1}$ nitrate. These nitrate concentrations were chosen in order to produce cultures that a) exhausted the nitrate in the media early in the growth cycle (day 3 to 5, 150 $mg.L^{-1}$), and b) did not run out of nitrate during the growth cycle (1500 $mg.L^{-1}$). Lower starting nitrate concentrations, resulting in earlier nitrate exhaustion, may have resulted in even greater lipid accumulation and higher lipid productivities. Alternatively, a starting nitrate concentration between 150 and 1500 $mg.L^{-1}$ may have enhanced lipid productivity by resulting in a higher biomass concentration and only slightly lower lipid content. The work presented in this chapter tests intermediate nitrate concentrations in order to explore the effect of degree of nitrogen limitation on lipid productivity and cell physiology.

Chlorella vulgaris was chosen due to its high lipid productivity and ease of cultivation. *Chlorella* is a resilient, fast-growing genus and one of few microalgae that have already been grown commercially for various purposes including food, feed and nutraceuticals (Apt & Behrens 1999; Spolaore *et al.* 2006).

To determine the optimal level of N for lipid productivity, batch cultures of *C. vulgaris* were grown at different nitrate concentrations. The growth rate, lipid content and lipid productivity were monitored for 20 days. The pigment, protein and carbohydrate content and elemental composition of the cells were also measured in order to investigate the interaction between cell N content and key growth and lipid parameters. An understanding of these correlations could allow prediction of optimal N limitation levels and hence feed concentrations.

7.2 Literature review

Nitrogen limitation is well known to enhance microalgal cell lipid content, but this is not often related to the change in growth rate and hence overall lipid productivity (Griffiths and Harrison, 2009). In determining the effects of N availability on microalgal cells, the majority of studies have tested only the two extremes of N replete and N limited. Few studies have investigated the application of intermediate levels of N limitation. In addition, lipid content is often measured at only a few points in the growth cycle, e.g. before and after N limitation. A temporal profile of lipid accumulation and growth rate with time would allow a more accurate calculation of lipid

productivity at specific time points in the growth cycle. This could assist in determining the optimal cultivation strategy and harvesting times.

Piorreck *et al.* (1984) investigated the growth of *Chlorella* and *Scenedesmus* and four species of Cyanobacteria at six different levels of nitrogen between 0.0003% and 0.1% NH_4Cl or KNO_3 (equivalent to between 2 and 1159 $mg.L^{-1}$ nitrate in terms of moles of N supplied). They reported that higher nitrogen concentrations led to greater biomass, while low N levels led to an increase in total lipid content, with up to 70% made up of neutral lipids such as triacylglycerol (TAG), as well as a lower protein and pigment content. The main focus of the work was the effect of different N levels on cell, lipid and fatty acid composition, and the authors do not calculate lipid productivity or propose an optimal nitrogen level for any purpose.

Stephenson *et al.* (2010) investigated starting nitrate concentrations of 10, 100, 200 and 550 $mg.L^{-1}$ in *C. vulgaris*, while Hsieh and Wu (2009) used urea in cultures of a marine *Chlorella* species, with starting concentrations of 25, 50, 100, 150 and 200 $mg.L^{-1}$ (equivalent to 52, 103, 207, 310 and 413 $mg.L^{-1}$ nitrate in terms of moles of N supplied). Both studies showed an inverse relationship between lipid content and biomass concentration at different N levels. The optimal nitrogen concentration for lipid productivity was 200 $mg.L^{-1}$ nitrate and 100 $mg.L^{-1}$ urea (equivalent to 207 $mg.L^{-1}$ nitrate) respectively. In neither study were cultures shown to be N replete at the maximum nitrogen concentration tested, therefore lipid productivity under N limited conditions could not be compared to that under N replete conditions.

The accumulation of lipid under N limitation is thought to result from two mechanisms: (1) breakdown and conversion of the carbon structures of existing cell components (e.g. proteins) into lipid, (2) channelling of newly fixed carbon from photosynthesis away from protein and into lipid biosynthesis (Rodolfi *et al.* 2009). Suen *et al.* (1987) cultured *Nannochloropsis* with ^{14}C labelled CO_2 and demonstrated that enhanced lipid synthesis under nitrogen limited conditions resulted principally from *de novo* CO_2 fixation.

Under nitrogen limiting conditions, cells are compromised in their ability to synthesize nitrogen-containing compounds, such as proteins, nucleic acids and chlorophyll. As these compounds are necessary for cell growth and division, the growth rate becomes dependent on the intracellular nitrogen concentration. Growth eventually ceases, but, given conducive conditions, photosynthesis continues, albeit at a reduced rate. Metabolic flux of carbon taken up during photosynthesis is diverted from protein synthesis to lipid or carbohydrate production (Li, Y *et al.* 2008). Storage lipids, primarily composed of triacylglycerol (TAG), are a compact and efficient cellular store of carbon and energy. They are relatively inert and can be packed into lipid vesicles easily. Lipids generate more energy than carbohydrates on oxidation, forming an excellent reserve for re-building the cell once nitrogen becomes available (Roessler 1990).

As well as enhancing lipid productivity, there are additional benefits to growth under N limitation. Under nitrogen limited conditions, both the total lipid content and the proportion of lipid made up of triacylglycerol (TAG, the storage form of lipid most suited to biodiesel production) increase. Stephenson *et al.* (2010) reported that, in *C. vulgaris* under N replete conditions, approximately 3% of the total lipid content was TAG, while after 12 days of N limitation, over 50% of the total lipid was TAG. Fatty acid composition also changes with N limitation, as demonstrated in Chapter 6. *C. vulgaris* metabolism shifted from the production of polyunsaturated fatty acids (C18:2 and C18:3) to the production of mainly saturated or monounsaturated fatty acids (C18:0 and C18:1) under N limitation. This shift in fatty acid composition resulted in the calculated qualities of biodiesel from *C. vulgaris* lipid meeting the European biodiesel standards (EN 14214) under N limited conditions. This was not the case under N replete conditions (see Chapter 6).

Piorreck *et al.* (1984) suggest an explanation for the changes in lipid composition with N limitation. The polar lipids and PUFA, which increase at higher N content, are mostly located in chloroplasts. With N limitation, levels of chlorophyll in cells drop, indicating a rapid reduction or breakdown of the chloroplast apparatus. During this process, chloroplast lipids and fatty acids appear to be catabolised. Hence there is a breakdown of structural lipids and synthesis of storage lipids. This shift in fatty acid composition may be due to the fact that saturated fatty acids provide more energy upon oxidation, and their more linear conformation may allow for more efficient packing within the cell (Roessler 1990). These changes are advantageous for the suitability of the lipid profile for biodiesel production.

From a life-cycle perspective, Lardon *et al.* (2009) highlight the necessity of decreasing fertilizer and energy use in achieving a positive energy balance in microalgal biodiesel production. Nitrogen fertilizer has a large embodied energy due to the extreme process conditions required to generate combined nitrogen in the manufacturing process. Growth of algal cultures under N limited conditions increases the lipid productivity per unit N (Figure 7.8), and hence decreases the process demand for nitrogen. This in turn has the potential to decrease the cost and environmental burden of the process.

7.3 Methods

7.3.1 Cultures

C. vulgaris was grown in batch culture in 3.2 L airlift reactors as described in Section 2.3. Nine reactors were inoculated to an OD of 0.1 at 750 nm, with starting media nitrate concentrations of 0, 40, 70, 100, 170, 420, 570, 1200 and 2000 $mg.L^{-1}$ respectively. Biomass concentration was measured by optical density (OD) every day, and by dry weight (DW) every third day (according to the protocols in Section 2.4.1 and 2.4.2). Individual calibration curves of OD as a function of DW were constructed for each culture, and used to calculate DW from OD.

7.3.2 Quantification of cell contents

Cell lipid content (measured as total fatty acid content) and medium nitrate concentration were measured daily according to the methods in Sections 2.4.3 and 2.4.8. Every third day, the carbohydrate, pigment and protein content of the biomass, as well as its elemental composition, were measured (protocols in Section 2.4.7). At the commencement of the experiment, the inoculum was also tested for nitrate concentration of the media and nitrogen (N) content of the cells. In addition to quantification by the BCA assay, protein content was calculated from the N content by multiplying by the conversion factor of 6.25.

7.3.3 Calculation of biomass and lipid productivity parameters

Maximum biomass concentration (X), specific growth rate (μ), biomass productivity (Q_X), lipid content (P), volumetric lipid content (P_{VOL}), and instantaneous and average lipid productivity ($Q_{P\ INST}$ and $Q_{P\ AVE}$) were calculated as detailed in Section 6.3.4.

7.4 Results and discussion

7.4.1 Nitrate uptake and growth

The average nitrate uptake rate in all cultures, calculated from the residual nitrate concentration in the medium (Figure 7.1a), was between 40 and 89 $mg.L^{-1}.day^{-1}$ with an average of 63 $mg.L^{-1}.day^{-1}$. The greater the nitrate concentration, the greater the final biomass concentration (Figure 7.1b). The time at which the nitrate became exhausted in the medium, along with some of the growth and lipid characteristics, are shown in Table 7.1. The age at which a culture stopped growing was estimated as the time from inoculation at which μ (averaged over 3 time points) became less than 0.1 (corresponding to a doubling time of one week). Cultures with a nitrate concentration less than 170 $mg.L^{-1}$ exhausted the nitrate in the medium during exponential growth (before day 2.2). Cultures with 420 and 570 $mg.L^{-1}$ nitrate depleted the nitrate in the media during linear growth (day 4 to 10). The 1200 $mg.L^{-1}$ nitrate culture ran out of nitrate after

20 days and the 2000 mg.L^{-1} culture still had a concentration of 660 mg.L^{-1} nitrate at the end of the experiment (Figure 7.1a).

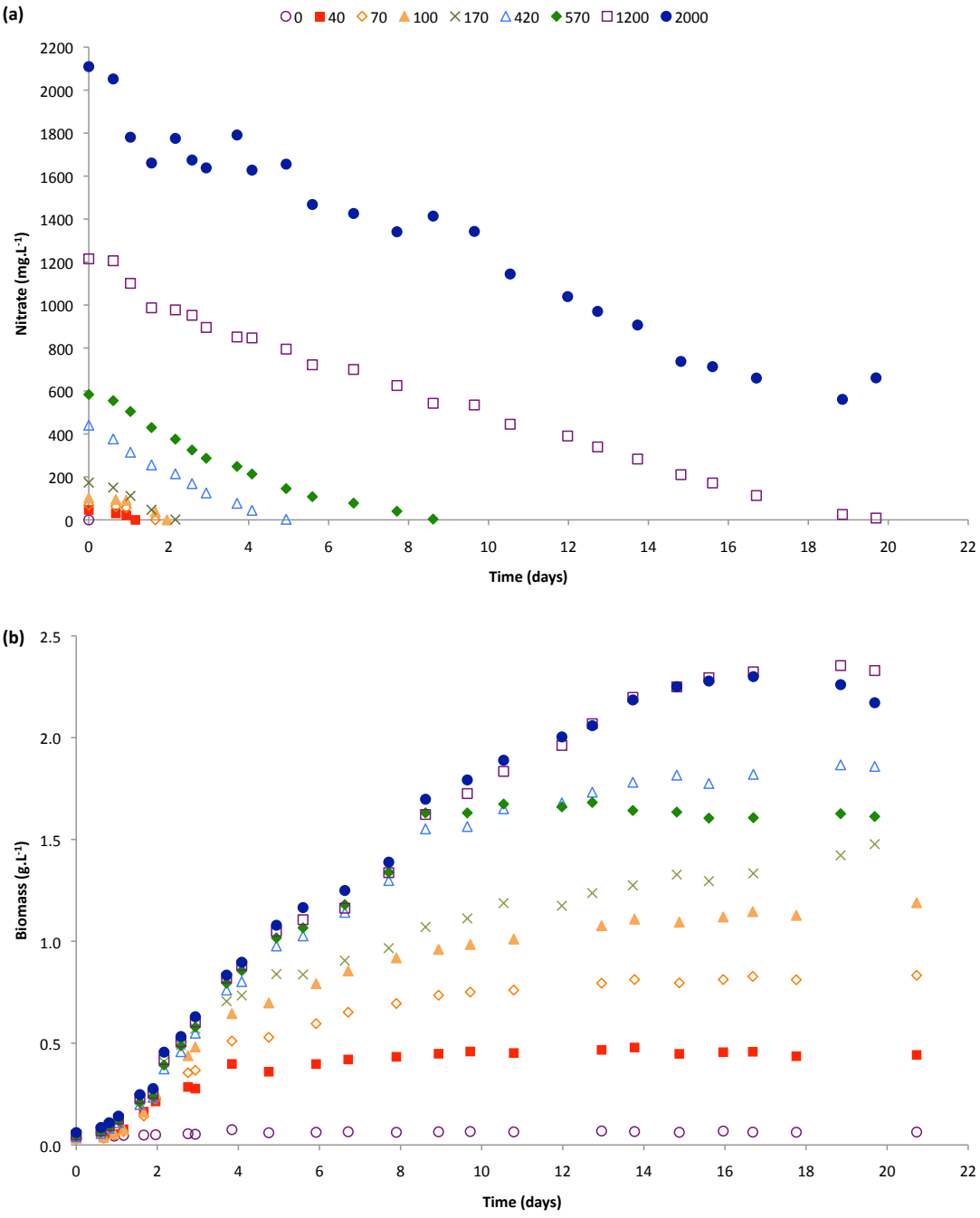


Figure 7.1 Nitrate concentration in the media (a) and biomass concentration (b) of *C. vulgaris* batch cultures over 20 days at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

Table 7.1 Growth parameters of *C. vulgaris* cultures with different starting concentrations of nitrate.

Initial nitrate concentration ($mg.L^{-1}$)	N exhaustion (days from inoculation)	μ_{max} (day^{-1})	X_{max} ($g.L^{-1}$)	$Q_{X\ max}$ ($g.L^{-1}.day^{-1}$)	Earliest time at which μ < 0.1 (days from inoculation)	Ratio of final biomass conc. to that at time of N exhaustion
0	0	0.4	0.08	0.016	2.0	2.2
40	1.2	1.5	0.48	0.149	3.8	6.2
70	1.7	1.5	0.83	0.200	5.9	5.9
100	2.0	1.5	1.19	0.265	6.7	4.7
170	2.2	1.4	1.48	0.326	4.9	3.6
420	4.9	1.4	1.87	0.325	9.6	1.9
570	8.6	1.4	1.68	0.352	9.6	1.0
1200	19.7	1.3	2.35	0.366	10.5	-
2000	-	1.1	2.30	0.373	9.6	-

The maximum specific growth rate (μ_{max}) of all cultures, except those with 0 $mg.L^{-1}$ and 2000 $mg.L^{-1}$ nitrate, was very similar (between 1.3 and 1.5 day^{-1}, Table 7.1) as μ_{max} occurred early in the growth curve (between day 0.8 and 1.2), when there was sufficient nitrate in the media. The culture containing 0 $mg.L^{-1}$ nitrate experienced immediate nitrogen limitation, resulting in a lower μ_{max} (0.4 day^{-1}). The lower μ_{max} of the 2000 $mg.L^{-1}$ culture (1.1 day^{-1}) may have been due to substrate inhibition. The maximum biomass concentration measured was up to 2.4 $g.L^{-1}$ in N replete cultures, between 1.7 and 1.9 $g.L^{-1}$ in cultures where N became exhausted during linear growth or early stationary phase, and less than 1.5 $g.L^{-1}$ in cultures that ran out of N during exponential growth. X_{max} in the 2000 $mg.L^{-1}$ nitrate culture was no higher than the 1200 $mg.L^{-1}$ culture, indicating that these cultures were N replete. The trends observed in these results agree with those reported by Hsieh and Wu (2009), using a marine species of *Chlorella*. An X_{max} of 2 $g.L^{-1}$ occurred under conditions of maximum urea supply (200 $mg.L^{-1}$, equivalent to 413 $mg.L^{-1}$ nitrate in terms of moles of N supplied). The μ_{max} was 1.4 day^{-1} at all urea concentrations above 100 $mg.L^{-1}$ (equivalent to 207 $mg.L^{-1}$ nitrate in terms of moles of N supplied), while below this critical urea concentration, μ_{max} decreased.

Another important issue is the influence of biomass concentration on ease of harvesting. Harvesting has been reported to contribute a significant proportion of the cost of algal biodiesel (Grima *et al.* 2003; Gudin and Therpenier 1986). Cultures with a higher starting nitrate concentration have a higher final biomass concentration (for example the 420 $mg.L^{-1}$ nitrate culture reached 1.9 $g.L^{-1}$ as opposed to 1.5 $g.L^{-1}$ and 1.2 $g.L^{-1}$ in 170 and 100$mg.L^{-1}$ nitrate respectively), which could facilitate downstream processing.

Growth became insignificant in all cultures, even those N replete, by day 14. This may have been due to light limitation. At day 14, N replete cultures had reached a biomass concentration of approximately 2.2 $g.L^{-1}$. At this biomass concentration, light becomes attenuated to below 10% of

the incident light within approximately 1 to 2 cm of the reactor surface, leading to an average light intensity of less than 20% of the incident light (>50 $\mu mol.m^{-2}.s^{-1}$) (Fraser 2011).

Nitrogen limited cultures continued to increase in DW for between 2 and 5 days after the exhaustion of nitrate in the medium. Li *et al.* (2008b) also observed that cell growth continued after exhaustion of N in the media of *Neochloris oleoabundans* cultures. As the cell content of N-containing compounds, such as chlorophyll and protein, also decreased with N limitation, they concluded that the additional growth must be supported by the consumption of these intracellular nitrogen pools.

In many cases, the increase in biomass after nitrate depletion was greater than that during N replete growth (Figure 7.2). The additional biomass, including intracellular storage compounds such as lipids, accumulated in the absence of any external N up to six times the DW at the time of nitrate exhaustion. By analyzing TEM photographs of *C. vulgaris* cells before and after 20 days of N limitation, Stephenson (2009) reported that the cell morphology changed dramatically, with large lipid storage vesicles taking up a large fraction of the cytoplasm after severe N limitation, but the spherical cells remained roughly the same size. If cell size remains constant, a 6 times increase in DW (found in cultures 40 and 70 $mg.L^{-1}$ nitrate) would correspond to between 2 and 3 cell doublings. The mass of the accumulated lipid contributed between 28 and 65% of the increase in DW (hatched areas, Figure 7.2).

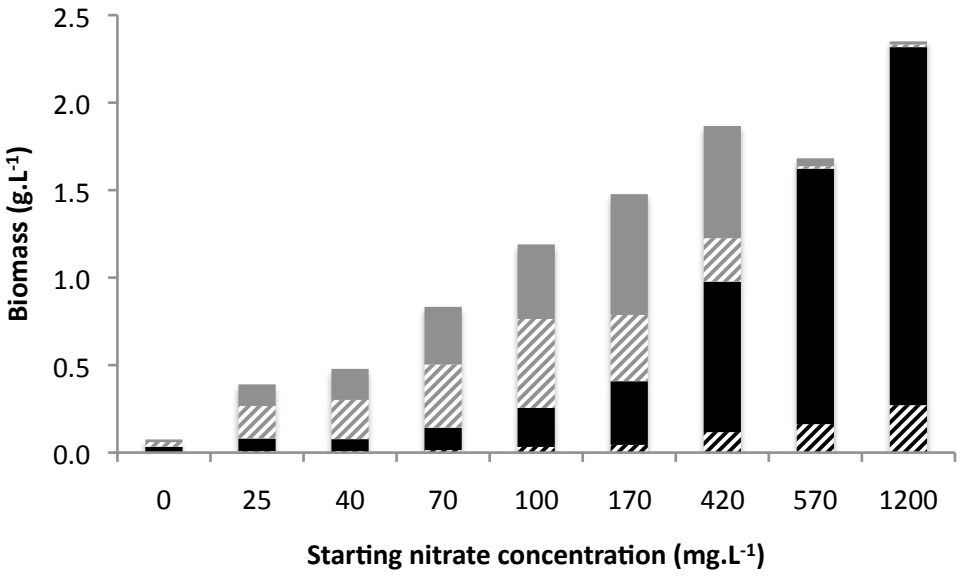


Figure 7.2 Final biomass concentration of *C. vulgaris* cultures grown at different nitrate concentrations, showing the biomass accumulated before (black bars) and after (gray bars) the exhaustion of nitrogen in the media. Hatched areas indicate the proportion of the accumulated biomass made up of lipid

7.4.2 Lipid content

N replete cultures (starting nitrate concentrations of 1200 and 2000 mg.L^{-1}) maintained a stable lipid content of between 10 and 12% throughout the growth period (Figure 7.3). Cultures with 420 and 570 mg.L^{-1} nitrate at inoculation showed a gradual increase in lipid content, beginning 4 to 6 days after N exhaustion in the medium. The lipid content of these cultures reached 35 and 28% DW respectively at 20 days and was still rising linearly with time, hence a higher content was expected if cultivation had continued. Cultures with an initial nitrate concentration of 100 and 170 mg.L^{-1} increased in lipid content steadily over 20 days, asymptoting to a maximum of between 50 and 55% DW. Cultures which became N limited during the first two days of growth (70 mg.L^{-1} and below) showed an immediate and rapid increase in lipid content, reaching a maximum of between 55 and 65% DW from day 16. The final lipid contents correlated with previous reports, where the lipid content of *C. vulgaris* varied between 12% and 30% under high N conditions, and 40% and 66% under low N conditions (Hsieh & Wu 2009; Illman *et al.* 2000; Piorreck *et al.* 1984; Widjaja *et al.* 2009).

There was a significant delay in lipid accumulation in those cultures (420 and 570 mg.L^{-1} media nitrate concentration) which ran out of nitrogen during the linear or early stationary phase, compared to those that ran out during exponential growth. This is shown clearly on analysing lipid content as a function of time from nitrogen exhaustion in the media (Figure 7.3b). The reason for this could be the greater cell density at the time of N exhaustion. Lipid synthesis requires significant metabolic resources in terms of carbon compounds as well as energy-carrying metabolites such as ATP and NADPH. The substrates for lipid synthesis are provided by both the light and carbon sources available to the culture, as well as the carbon and energy already contained within the cells as macromolecules at the end of the growth period. Cultures that reach nitrogen exhaustion at a higher cell density accumulate lipid at a slow and linear rate, indicative of nutrient supply rate controlling synthesis. This is presumably because, when N is exhausted at low cell density, cells have more light available to them, resulting in greater metabolic flux from photosynthesis available for lipid accumulation (Li, Y *et al.* 2008). Cultures that become N limited at greater cell densities experience greater mutual shading and hence lipid accumulation is limited by light availability.

N limitation also leads to reduced pigment content, which would decrease the rate of photosynthesis per cell even in the presence of more light. However, the low pigment content could allow a larger fraction of the culture to utilize light more efficiently due to decreased mutual shading. This could explain the faster lipid accumulation in dense cultures towards the very end of the experiment (Figure 7.3a and Figure 7.4), as they lose pigment content. An alternative explanation is that cultures that run out of N during exponential growth are still dividing rapidly, as opposed to cultures that run out as they are entering stationary phase. Cell division would dilute intracellular N rapidly, leading to a lower cell N content. If a low cell N

content triggers lipid synthesis, this could happen more quickly in cultures where the N runs out early in the growth cycle. Cultures that run out of N during exponential growth could also be more metabolically active than cultures entering stationary phase, and hence able to alter their cellular machinery and adapt to stress conditions more quickly.

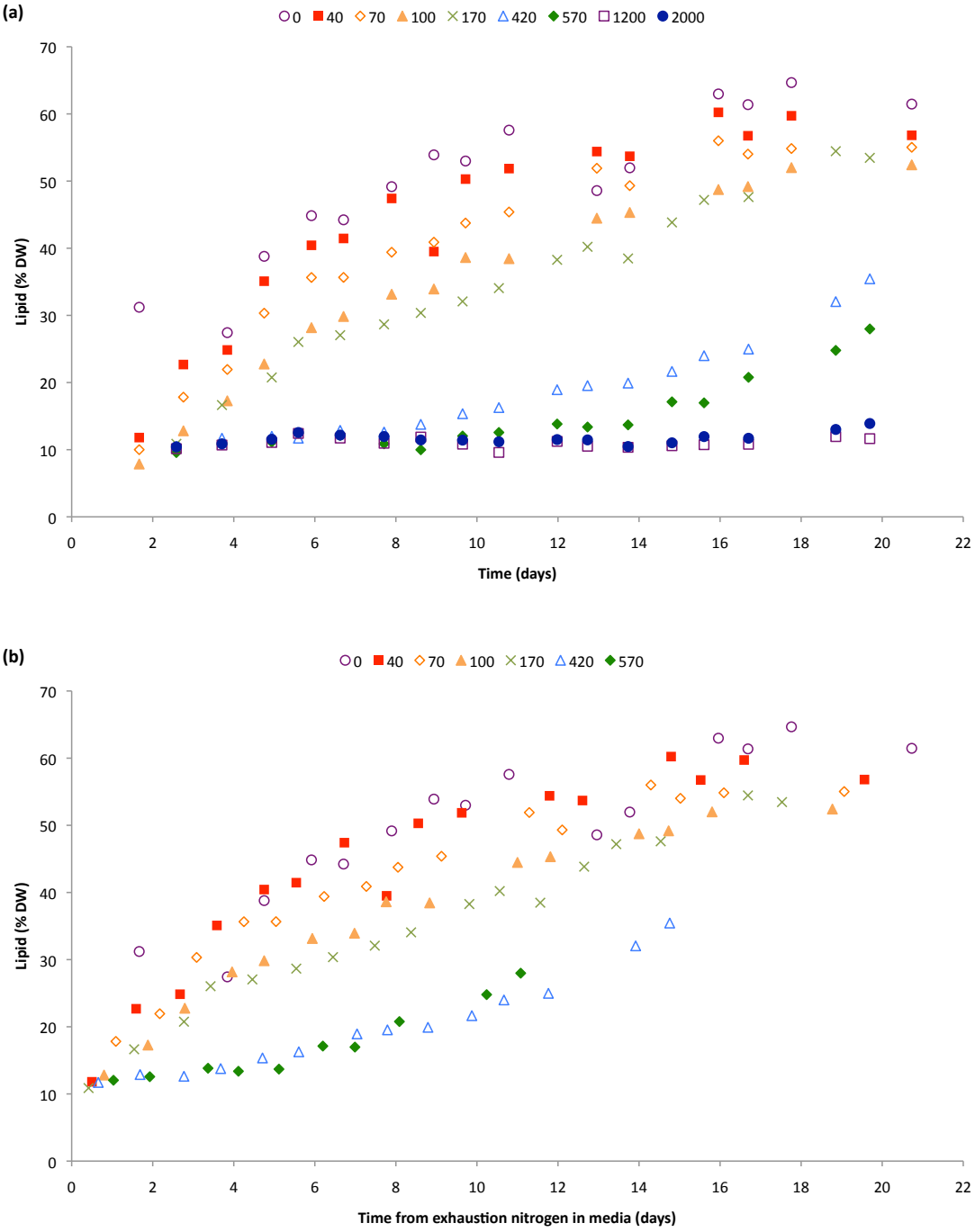


Figure 7.3 Lipid content (a) and lipid content from time of exhaustion of nitrate in the media (b) of *C. vulgaris* batch cultures over 20 days at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

Volumetric lipid content (P_{VOL}, the product of biomass concentration and lipid content) was greatest throughout the experiment in the culture grown on media containing 170 mg.L^{-1} nitrate (Figure 7.4). This culture maintained a constant rate of increase in P_{VOL} over 20 days and reached a maximum of 790 mg.L^{-1}. The cultures with nitrate concentrations of 70 and 100 mg.L^{-1} initially maintained an equivalent P_{VOL}, up to day 8 and 14 respectively. Lipid productivity declined thereafter due to a cessation of growth and the attainment of maximum lipid content. Cultures with nitrate concentrations of 40, 420, 570, 1200 and 2000 mg.L^{-1} all had very similar P_{VOL} up to day 8. This is because cultures above 420 mg.L^{-1} were nutrient replete to day 5 and had similar growth rates and lipid content. In the 40 mg.L^{-1} culture, the reduced biomass concentration was made up for by an increase in lipid content. From day 9, the 420 mg.L^{-1} culture began to increase in lipid content and by day 20 had nearly matched the P_{VOL} of 170 mg.L^{-1}. The culture with a starting nitrate concentration of 570 mg.L^{-1} followed a similar pattern, just 6 days later.

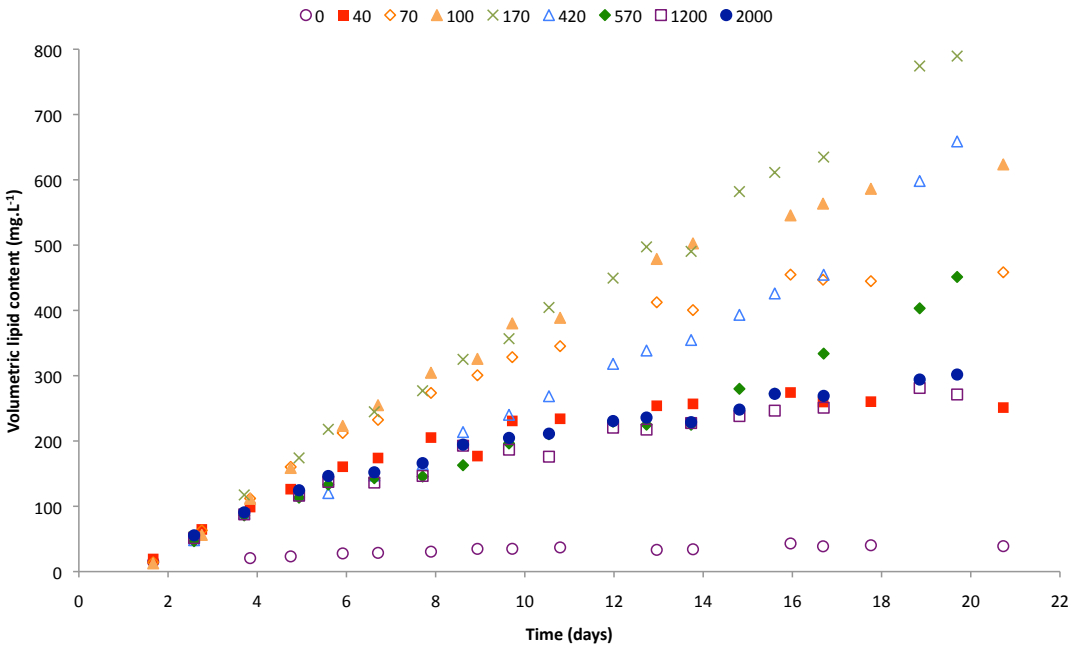


Figure 7.4 Volumetric lipid content of *C. vulgaris* batch cultures over 20 days at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

It is possible that the 420 and 570 mg.L^{-1} nitrate cultures would have reached higher volumetric lipid contents than the 170 mg.L^{-1} nitrate culture given a longer culture period. Assuming that these cultures would maintain a constant biomass and continue to accumulate lipid at the rate achieved over the last three days of cultivation, they can be predicted to achieve a P_{VOL} of greater than 800 mg.L^{-1} by day 22 and 30 respectively. As productivity is concerned not only with final yield, but also the time taken to reach it, this needs to be taken into account by comparing lipid productivity.

7.4.3 Lipid productivity

Maximum cellular and volumetric lipid content, as well as instantaneous and average lipid productivity for the different cultures are shown in Table 7.2. Instantaneous lipid productivity was calculated from the average slope of each four consecutive points for P_{VOL} as a function of time in Figure 7.4. An increase in $Q_{P\ INST}$ could be caused by an increase in either biomass concentration, lipid content or both. The 170 and 100 mg.L^{-1} nitrate cultures gave the most consistent $Q_{P\ INST}$ across 20 days due to a steady increase in both biomass and lipid (Figure 7.5). The 420 and 570 mg.L^{-1} nitrate cultures showed an intermediate lipid productivity for the first few days due to the low lipid content and high growth rate, but reached a higher productivity later due to the rapid increase in lipid content with a constant but high biomass. Assuming a constant biomass and continued lipid accumulation rate equivalent to that achieved in the last three days of cultivation, the 420 mg.L^{-1} nitrate culture can be predicted to reach a maximum $Q_{P\ INST}$ of 67 mg.L^{-1}.day^{-1} on day 21. This would exceed the highest $Q_{P\ INST\ max}$ recorded (55 mg.L^{-1}.day^{-1} in the 170 mg.L^{-1} nitrate culture). The 570 mg.L^{-1} nitrate culture was predicted not to exceed its maximum $Q_{P\ INST}$ of 48 mg.L^{-1}.day^{-1} during continued culture. The 40 and 70 mg.L^{-1} nitrate cultures both had high initial lipid productivities due to rapid lipid accumulation, but productivity decreased steadily to zero at day 18 as their lipid content reached a plateau at 55 to 60% and biomass no longer increased. The 2000 and 1200 mg.L^{-1} nitrate cultures maintained a constant lipid content and therefore the lipid productivity was dictated by the biomass productivity which decreased with time.

Table 7.2 Lipid parameters of *C. vulgaris* cultures with different starting concentrations of nitrate.

Nitrate concentration (mg.L^{-1})	P_{max} (% DW)	$P_{VOL\ max}$ (mg.L^{-1})	$Q_{P\ INST\ max}$ (mg,L^{-1}.day^{-1})	$Q_{P\ AVE\ max}$ (mg.L^{-1}.day^{-1})
0	65	43	3	9
40	60	274	30	27
70	56	458	48	35
100	52	624	53	39
170	54	790	57	41
420	35	659	55	33
570	28	451	48	24
1200	12	281	29	25
2000	14	302	31	26

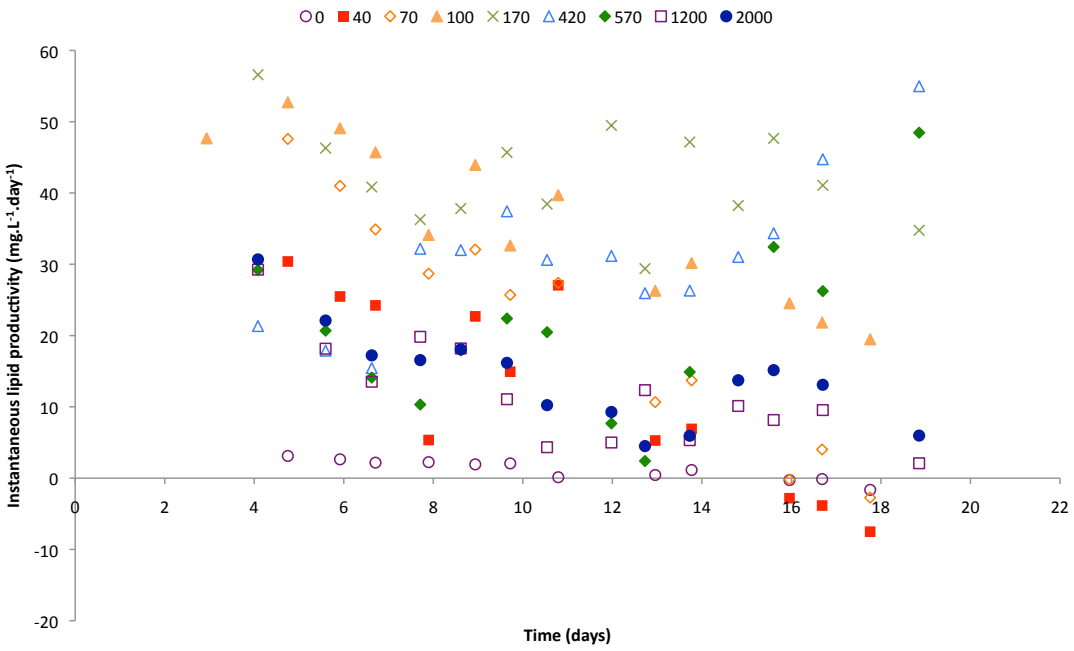


Figure 7.5 Instantaneous lipid productivity of *C. vulgaris* batch cultures over 20 days at initial nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

The overall average lipid productivity ($Q_{P\ AVE}$) shows the overall productivity achieved by each culture at a certain time point. Ideally cultures should reach maximum $Q_{P\ AVE}$ as quickly as possible and maintain it for as long as possible. As long as the $Q_{P\ AVE}$ remains constant, a greater lipid yield is obtained by maintaining the culture rather than starting a new one (due to the turnaround time and initial lag phase involved in batch culture). $Q_{P\ AVE}$ indicates which culture has been the most productive overall at a certain time point. For example, at day 3, most cultures had a similar $Q_{P\ AVE}$, except for the 40 and 70 mg.L^{-1} nitrate cultures which were slightly higher due to the onset of nitrogen limitation and thus a higher lipid content (Figure 7.6). By day 5, however, the cultures 70, 100 and 170 mg.L^{-1} nitrate had been more productive. The culture with 170 mg.L^{-1} nitrate maintained the highest $Q_{P\ AVE}$ of approximately 40 mg.L^{-1}.day^{-1} from day 5 to 20. It is uncertain how much longer this productivity could be maintained. Using the assumptions above, and assuming that they would eventually reach a P_{max} equivalent to that of the 170 mg.L^{-1} nitrate culture (54% DW), the cultures with 420 and 570 mg.L^{-1} nitrate can be predicted to reach a $Q_{P\ AVE}$ of 40 and 28 mg.L^{-1}.day^{-1} on day 25 and 31 respectively. The 420 mg.L^{-1} nitrate culture may therefore have approximately equalled the $Q_{P\ AVE}$ of the 170 mg.L^{-1} nitrate culture on day 25 if cultivation had been continued.

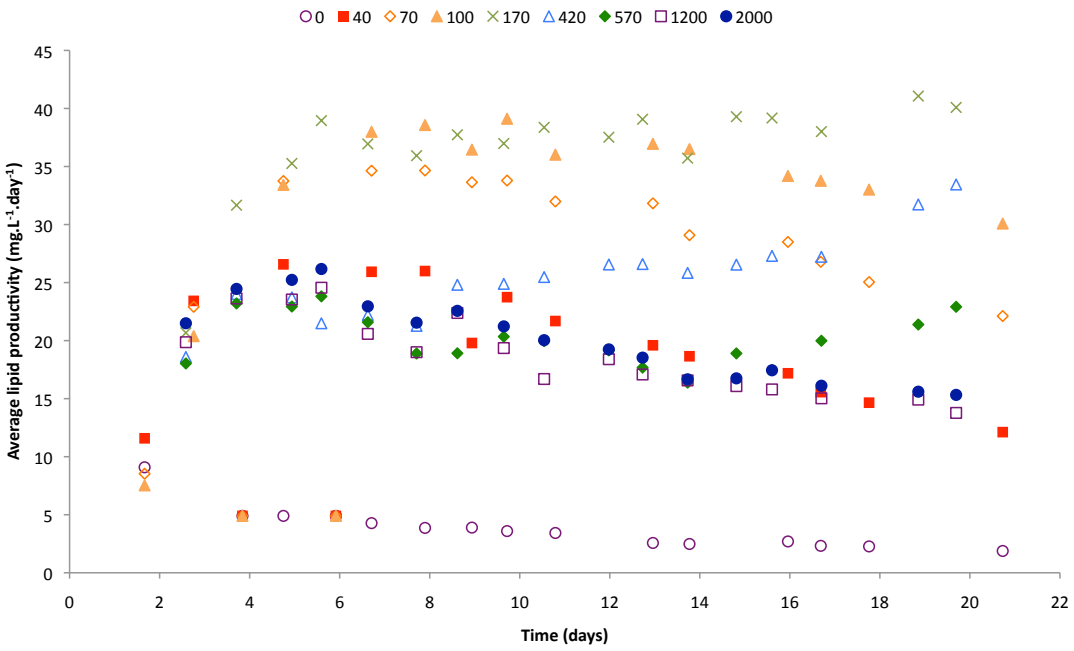


Figure 7.6 Overall average lipid productivity of *C. vulgaris* batch cultures over 20 days at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

7.4.4 Optimum starting nitrate concentration for lipid productivity

In Figure 7.7, key growth and lipid parameters are considered against starting nitrate concentration. Maximum specific growth rate was similar for cultures with 40 to 1200 mg.L^{-1} nitrate (Figure 7.7a), but lower for cultures with 0 mg.L^{-1} nitrate and 2000 mg.L^{-1} nitrate. Maximum biomass concentration (Figure 7.7b) and biomass productivity (Figure 7.7c) increased with increasing nitrate concentration. The maximum lipid content showed the inverse trend (Figure 7.7d), illustrating the tradeoff between biomass concentration and lipid content. The optimal starting nitrate concentration maximised volumetric lipid content and lipid productivity under the given culture conditions. Extrapolating from Figure 7.7e and f, by fitting a second order polynomial through the points from 70 to 570 mg.L^{-1} nitrate, the starting nitrate concentrations to achieve maximum volumetric lipid content and lipid productivity were estimated to be 305 mg.L^{-1} and 240 mg.L^{-1} respectively. Below this nitrate concentration, cells achieved a high lipid content, but the biomass concentration was too low for optimal productivity. Above this nitrate concentration, the higher biomass concentration of cultures was offset by a lower lipid content, and there was a lag phase between N exhaustion and lipid accumulation (Figure 7.3a). Note that these optimum starting nitrate concentrations are specific to this culture environment, particularly the light availability and CO_2 mass-transfer provided by the hydrodynamics and geometry of the airlift reactors.

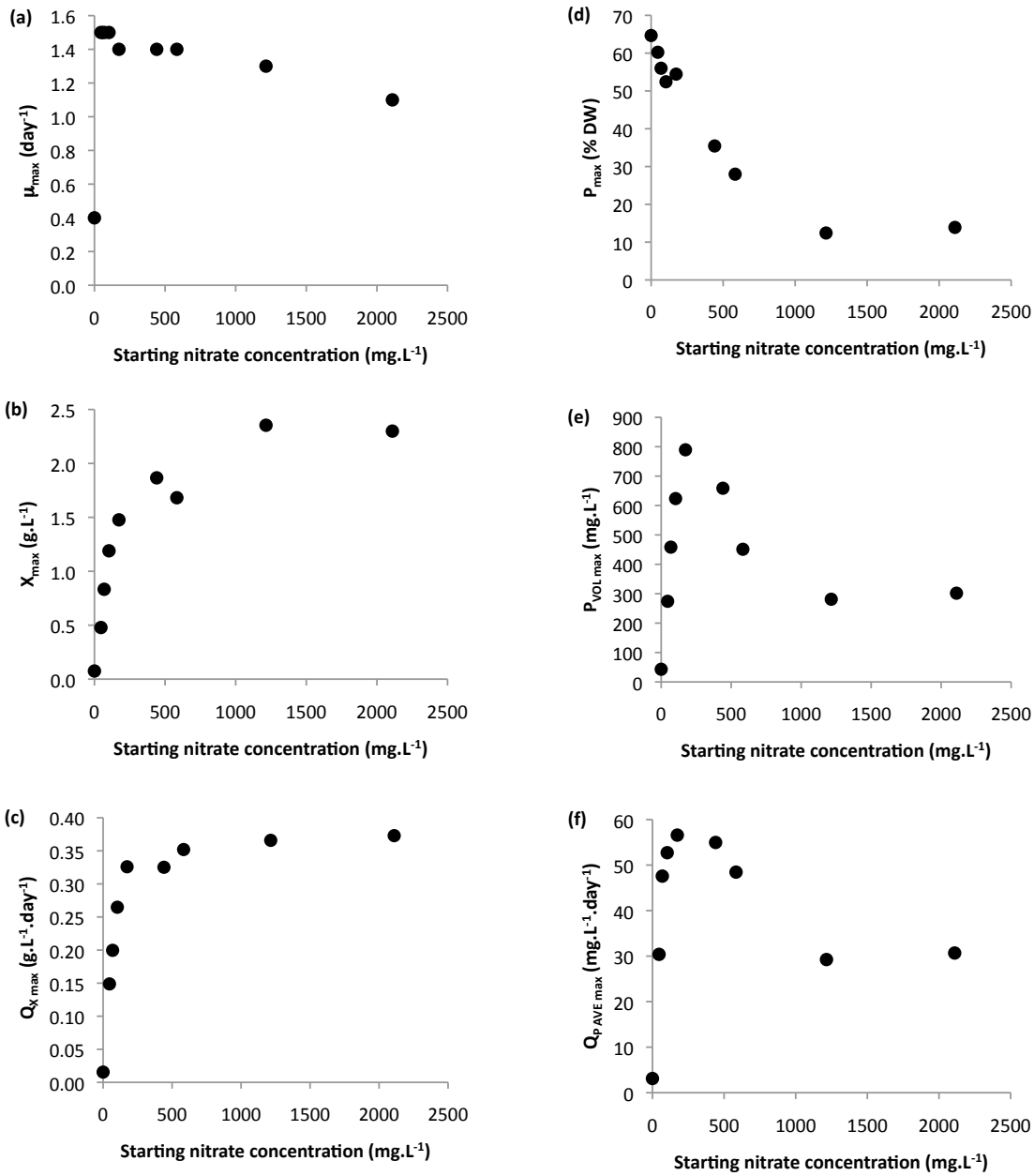


Figure 7.7 Maximum specific growth rate (a), maximum biomass concentration (b), maximum biomass productivity (c), maximum lipid content (d), maximum volumetric lipid content (e) and maximum average lipid productivity (f) achieved over 20 days of batch cultures of *C. vulgaris* containing 0, 40, 70, 100, 170, 420, 570, 1200 and 2000 mg.L^{-1} nitrate

These results agree closely with the optimal initial nitrate concentration of 200 mg.L^{-1} reported by Stephenson *et al.* (2010). Re-analysed results from Stephenson's study are summarized in Table 7.3. The experiment was continued to 40 days, but lipid content and productivity decreased after day 20. In contrast to the findings here, the maximum lipid content was reported at an intermediate starting nitrate concentration rather than the lowest starting nitrate concentration. Lower lipid contents were found overall, possibly due to the use of the Bligh and

Dyer extraction method for lipid quantification (known to incompletely extract lipids, see Chapter 4), along with significantly higher biomass concentrations, possibly due to different growth conditions, especially a greater light supply per cell, or a different strain of *C. vulgaris*. The higher biomass resulted in higher lipid productivities, but the trends in productivity were similar to this work.

Table 7.3 Summary of results from Stephenson *et al.* (2010). Growth and lipid parameters in *C. vulgaris* after 20 days of batch cultivation with starting nitrate concentrations of 10, 100, 200 and 550 mg.L^{-1}

Nitrate concentration (mg.L^{-1})	P_{max} (% DW)	X_{max} (g.L^{-1})	$P_{VOL\ max}$ (mg.L^{-1})	$Q_{P\ INST\ max}$ (mg.L^{-1}.day^{-1})	$Q_{P\ AVE\ max}$ (mg.L^{-1}.day^{-1})
10	15	0.84	128	3	7
100	39	2.17	846	65	45
200	46	2.9	1320	111	71
550	6	5.2	334	20	18

Hsieh and Wu (2009), found lipid productivity in a marine strain of *Chlorella* to be maximum (124 mg.L^{-1}.day^{-1}) at 100 mg.L^{-1} urea . This is equivalent, in terms of moles of N, to 207 mg.L^{-1} of nitrate.

Li *et al.* (2008b) investigated the effect of nitrogen concentrations between 3 and 20 mM $NaNO_3$ (equivalent to 186 to 1240 mg.L^{-1} nitrate) on lipid productivity in *Neochloris oleoabundans*. They reported that maximum overall lipid productivity (133 mg.L^{-1}.day^{-1}) occurred at an intermediate $NaNO_3$ concentration (5 mM, equivalent to 310 mg.L^{-1} nitrate). This starting concentration was higher than that found for *C. vulgaris*. This could be due to species used or the much higher light provision (16 light bulbs rather than 3).

If the environmental or economic cost of the provision of N to the cultures is significant, then the yield of lipid on N is an important optimisation parameter. Lardon *et al.* (2009) highlighted the importance of decreasing fertilizer and energy use in achieving a positive energy balance in microalgal biodiesel production. Nitrogen fertilizer has a large embodied energy due to the use of fossil fuel in the manufacturing process. Growth of algal cultures under N limited conditions decreases the process demand for nitrogen, which decreases cost and the environmental burden. Cultures with nitrate concentrations below 170 mg.L^{-1} yielded over 20 g lipid per g nitrogen taken up at the end of 20 days, while cultures above 170 mg.L^{-1} nitrate yielded below 6 g lipid per g nitrogen taken up (calculated as volumetric lipid content divided by nitrate removed from the medium, Figure 7.8). Therefore the yield of lipid on nitrogen can be improved by at least 300% under N limited conditions.

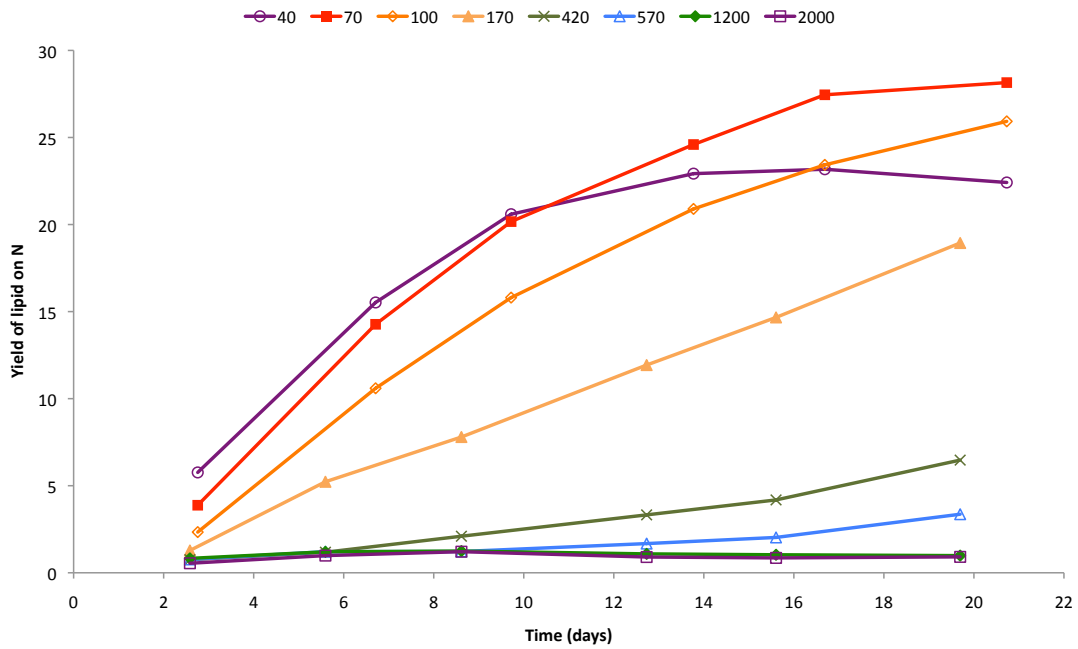


Figure 7.8 Yield of lipid on nitrogen in *C. vulgaris* batch cultures over 20 days at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

7.4.5 Changes in cell contents with N limitation

In addition to biomass concentration and lipid content, carbohydrate, protein and pigment content as well as elemental analysis for carbon (C), hydrogen (H) and nitrogen (N) were performed every three days over the growth cycle.

Elemental analysis

The carbon content of the cells remained fairly stable over the course of the growth cycle in all cultures except the 100 and 170 mg.L^{-1} nitrate cultures (Figure 7.9a). Those that became N limited early in exponential growth (cultures 0 to 70 mg.L^{-1}) had an average C content of 61%, while those that became N limited in stationary phase, or remained N replete, (cultures 420 to 2000 mg.L^{-1}) had an average C content of 48%. The C content of cultures 100 and 170, which became N limited in mid-exponential growth, changed from 46% to 64 and 60% respectively over the course of the growth cycle. The higher C content in N limited cultures was due to a relative increase in C-containing compounds such as lipids in the cells. A very similar pattern was seen with the H content (up to 9% DW in N limited cultures, and between 5 and 7% DW in N replete cultures), because C-containing compounds such as lipids and carbohydrates are H-rich compounds.

The N content of cells decreased with time and N limitation (Figure 7.9b). A slow decrease occurred in the N replete cultures (1200 and 2000). In the N limited cultures, a sharp decrease in N content was observed immediately following the exhaustion of N in the medium. Cells reached a minimum N content of between 0.5 and 1%.

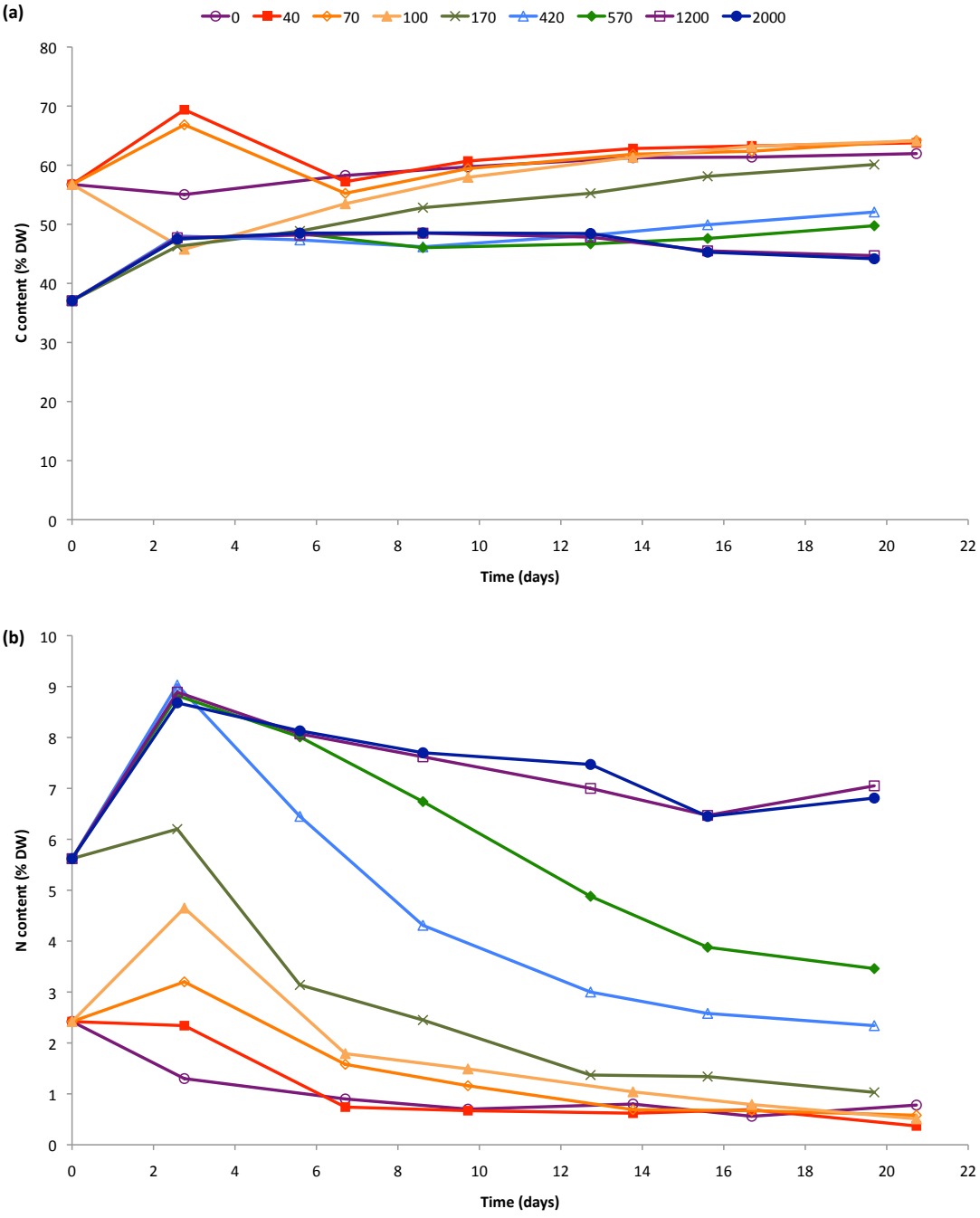


Figure 7.9 C content (a) and N content (b) of cells of *C. vulgaris* batch cultures over 20 days at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

Protein

Protein content was measured by NaOH extraction and the bicinchoninic acid assay (method in Section 2.4.5). An alternative method is to calculate protein content from N content using a multiplication factor. The conversion factor of 6.25 is frequently used, but it has been shown that microalgae can contain significant amounts of non-protein, nitrogen-containing substances such as pigments, nucleic acids, free amino acids and inorganic nitrogen (nitrate, nitrite and ammonia), which would cause this factor to overestimate the protein content (Barbarino & Lourenço 2005).

Lourenço *et al.* (2004) have suggested specific nitrogen to protein conversion factors for 12 species of marine microalgae, varying from 3.75 to 5.72, with an average of 4.78. In the N replete cultures measured in this work (570, 1200 and 2000 $mg.L^{-1}$), the average conversion factor was 6.5, while for N limited cultures (420 $mg.L^{-1}$ and below) it was 21.6 (Figure 7.10). Interference by lipids is known to cause erroneously high values for protein content with the BCA assay (Kessler & Fanestil 1986). Therefore, it is likely that protein content was overestimated by the BCA assay in N limited cultures with a high lipid content. As the conversion factor in cultures with a low lipid content was very close to the widely used conversion factor of 6.25, it was decided for comparison purposes to calculate protein content using this conversion factor.

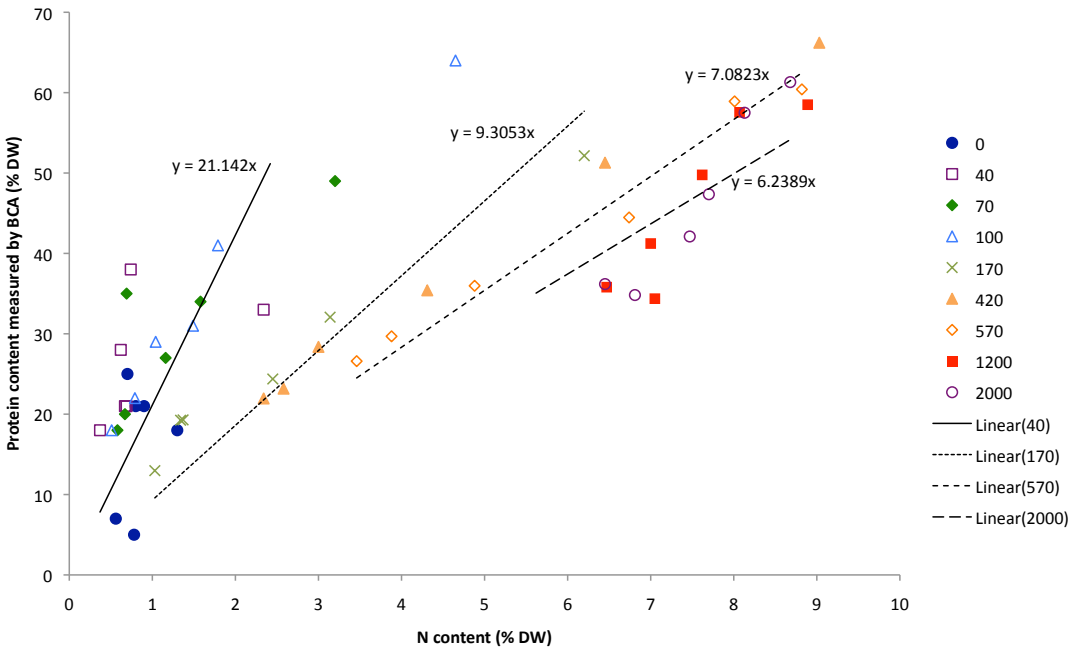


Figure 7.10 Nitrogen to protein conversion factors in cultures with varying levels of nitrogen limitation and hence different lipid content.

Due to the calculation method, the protein content estimated corresponded directly with the nitrogen content of the cells. Protein content declined steadily with culture age and N limitation (Figure 7.11). Minimum protein content reached was between 2 and 5%. Despite the higher values, similar trends were found in the measured protein results.

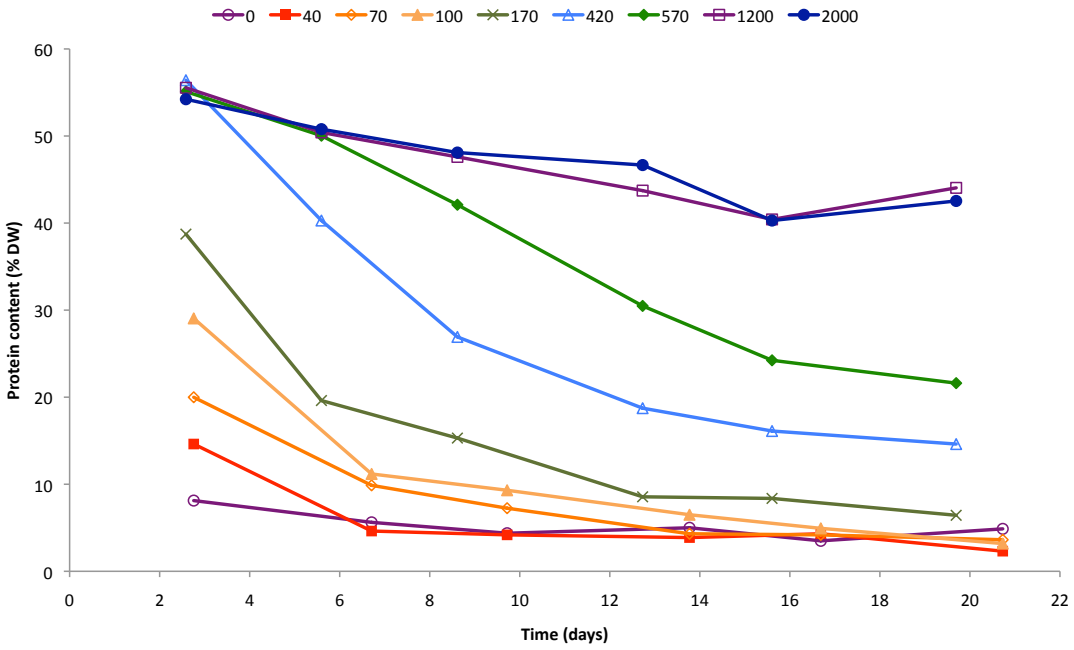


Figure 7.11 Protein content, calculated from N content, of cells of *C. vulgaris* batch cultures at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

Carbohydrate

Carbohydrate content decreased with culture age in N limited cultures (0 to 170 mg.L^{-1}) from an average of 26% at day 3, to 13% after 20 days. In the 420 and 570 mg.L^{-1} nitrate cultures, the carbohydrate content rose from 19% three days after inoculation to 38 and 35% respectively over the first 12 days, before stabilising at 33 and 34% respectively at day 20. N replete cultures (1200 and 2000 mg.L^{-1} nitrate) showed a gradual increase in carbohydrate content from 16 and 18% three days after inoculation to 27 and 28% after 20 days respectively (Figure 7.12).

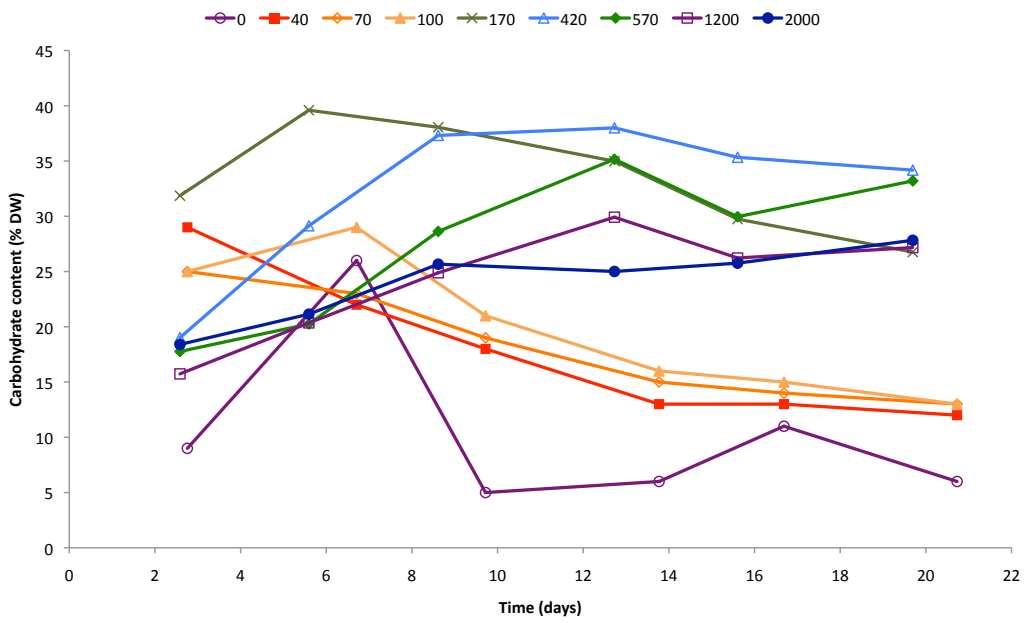


Figure 7.12 Carbohydrate content of cells of *C. vulgaris* batch cultures at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

Pigment

Pigment content changed very predictably with N limitation. It followed a very similar trend to the N content of cells, declining gradually with culture age and rapidly with the onset of N limitation (Figure 7.13).

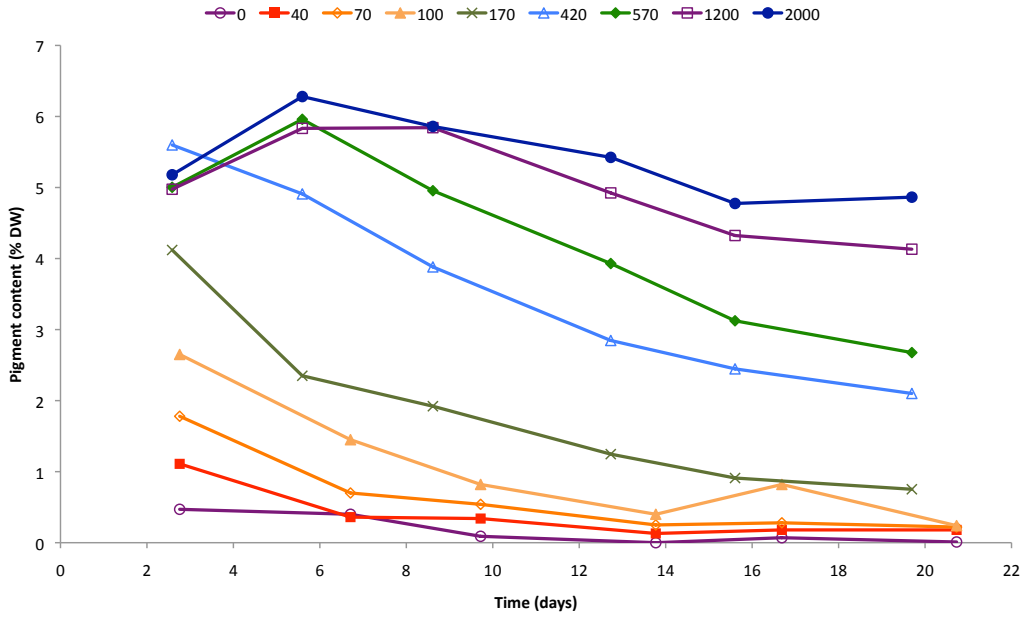


Figure 7.13 Pigment content of cells of *C. vulgaris* batch cultures at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

These findings are summarised in Figure 7.14. With increasing N limitation, lipid content increased, along with a decrease in all other major cell components. These changes in pigment, protein and carbohydrate content with N limitation are in agreement with a number of other studies (Illman *et al.* 2000; Li, Y *et al.* 2008; Piorreck *et al.* 1984; Stephenson 2009).

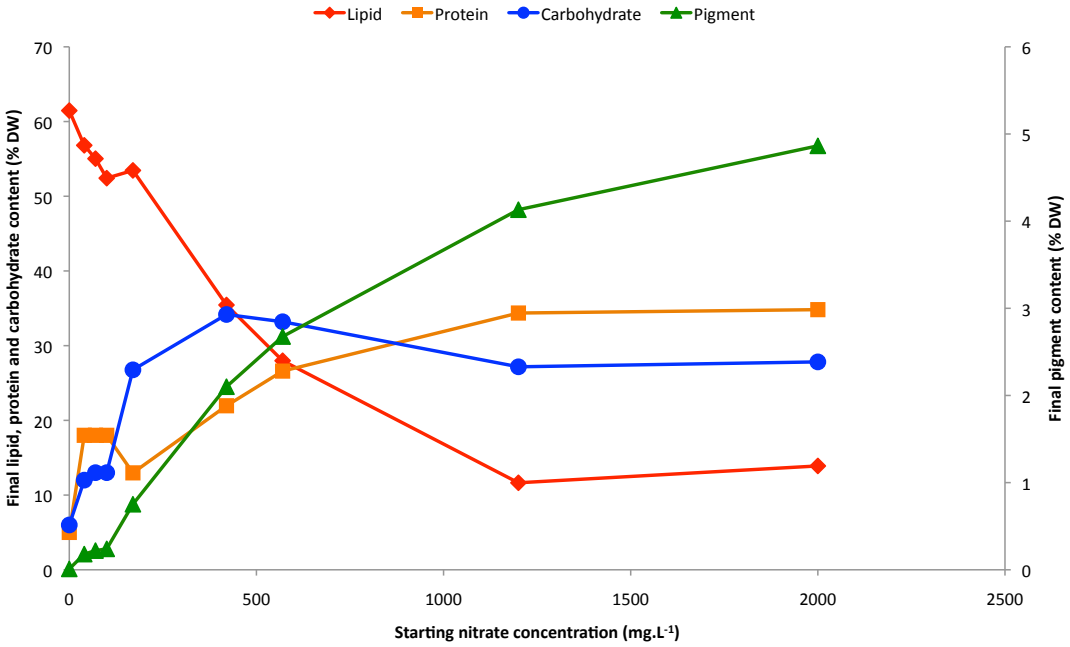


Figure 7.14 Final lipid (◆), protein (■), carbohydrate (●) and pigment (▲) content of *C. vulgaris* cultures containing 0, 40, 70, 100, 170, 420, 570, 1200 and 2000 mg.L^{-1} nitrate

These changes in composition have been calculated relative to the total biomass. As the lipid content of the biomass changed dramatically in some cultures (e.g. from 12% to 60% DW in the 40 mg.L^{-1} nitrate culture), it was interesting to determine whether the changes in composition were simply relative to the accumulation of lipid, or whether they represented changes in the composition of the non-lipid, catalytic portion of the biomass which controls the biology and metabolism of the cell. In order to investigate this, the N, protein, carbohydrate and pigment contents were recalculated as proportions of the non-lipid biomass fraction. The N, protein and pigment content, as a proportion of the non-lipid biomass, showed the same trends as for total biomass (Figures 7.9 to 7.14), although the proportions were larger, particularly for the cultures with higher lipid contents. For example, the protein content of the non-lipid biomass also decreased with time and N limitation (Figure 7.15a). Carbohydrate content, expressed as a proportion of the non-lipid biomass, could be seen to make up an increasing proportion, with age, of the non-lipid biomass in the cultures with 170, 420 and 570 mg.L^{-1} nitrate (Figure 7.15b).

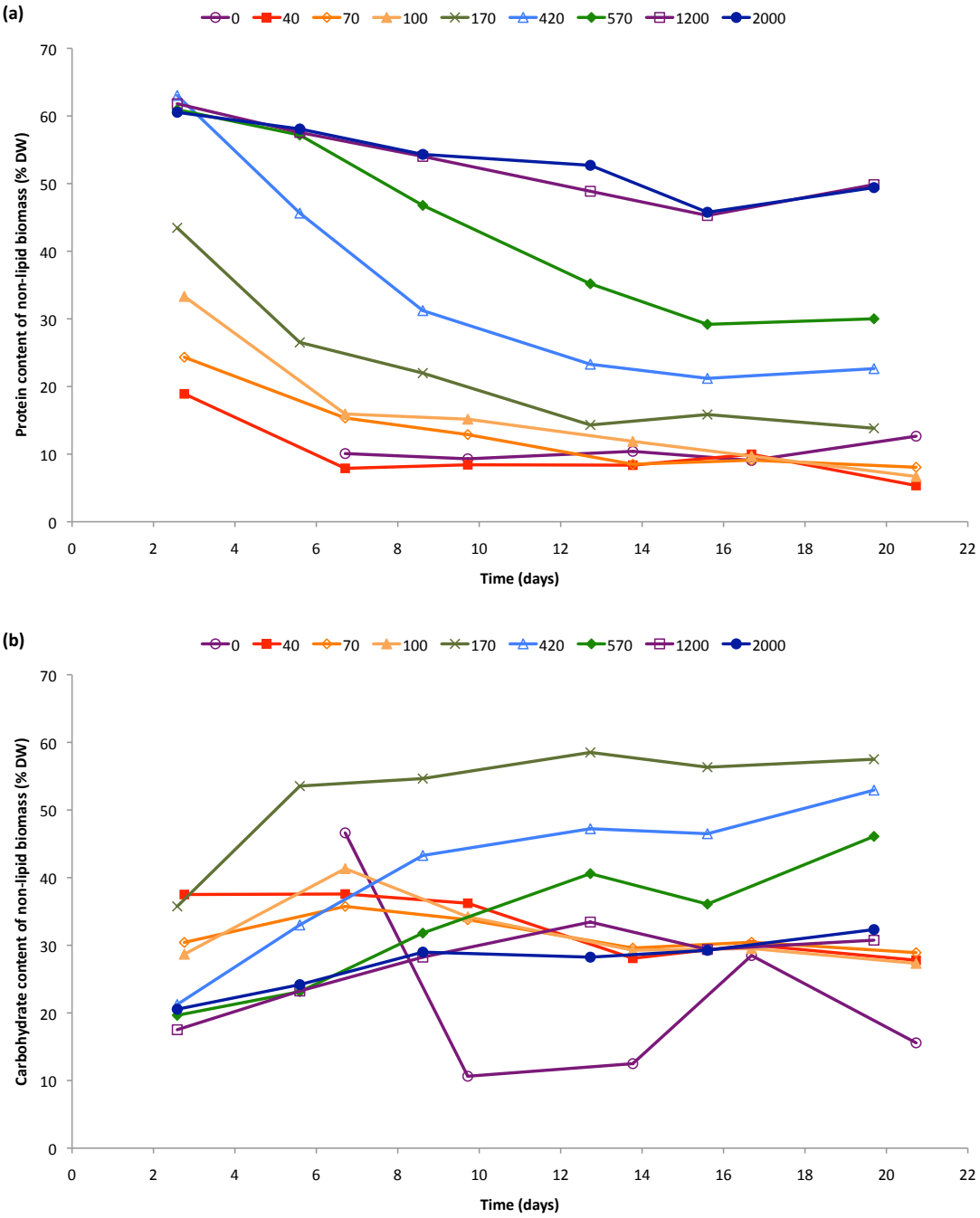


Figure 7.15 Protein (a) and carbohydrate (b) content of the non-lipid portion of cells of *C. vulgaris* batch cultures at nitrate concentrations of 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1}

7.4.6 Correlations in cell biochemistry with cellular nitrogen content

Pigment

The pigment content of cells was linearly correlated with their N content (Figure 7.16). The slope of the regression line was 0.69, with an R^2 of 0.95. Nitrogen limitation, as well as other stress conditions such as C limitation and high temperature are known to lead to blocked synthesis, and

breakdown, of pigment molecules in Cyanobacteria (Richaud *et al.* 2001). Li *et al.* (2008) suggested that chlorophyll was utilized as an intracellular N pool to support synthesis of cell material for further cell division. Pigments are one of the most readily accessible intracellular N pools (Li, Y *et al.* 2008), although they contain proportionally less N than protein. Chlorophyll *a* contains four N atoms per molecule, or 0.063 g $N.g^{-1}$ DW, while protein (calculated based on the relative abundance of amino acids in algal proteins) contains 0.16 g $N.g^{-1}$ DW (Geider & la Roche 2002).

It has also been suggested that chlorophyll degradation occurs to limit oxidative stress. The chloroplasts have a high protein content and therefore represent a store of proteinaceous N as well as pigment. In *C. vulgaris* cells grown at 0.03% KNO_3, chloroplasts were at least 40% protein, while whole cells averaged 27% protein (Piorreck *et al.* 1984). The thylakoid membranes (the site of the photosynthetic reaction centers in the chloroplasts) are rapidly degraded in Cyanobacteria at the onset of N limitation (Piorreck *et al.* 1984), possibly to mobilise N for other uses, or to downregulate photosynthesis. During extreme N limitation, the proteins holding chlorophyll molecules in place in the thylakoid membrane may be broken down. Free chlorophyll is dangerous to the cell as it emits free radicals, therefore chlorophyll must be actively broken down to prevent oxidative stress.

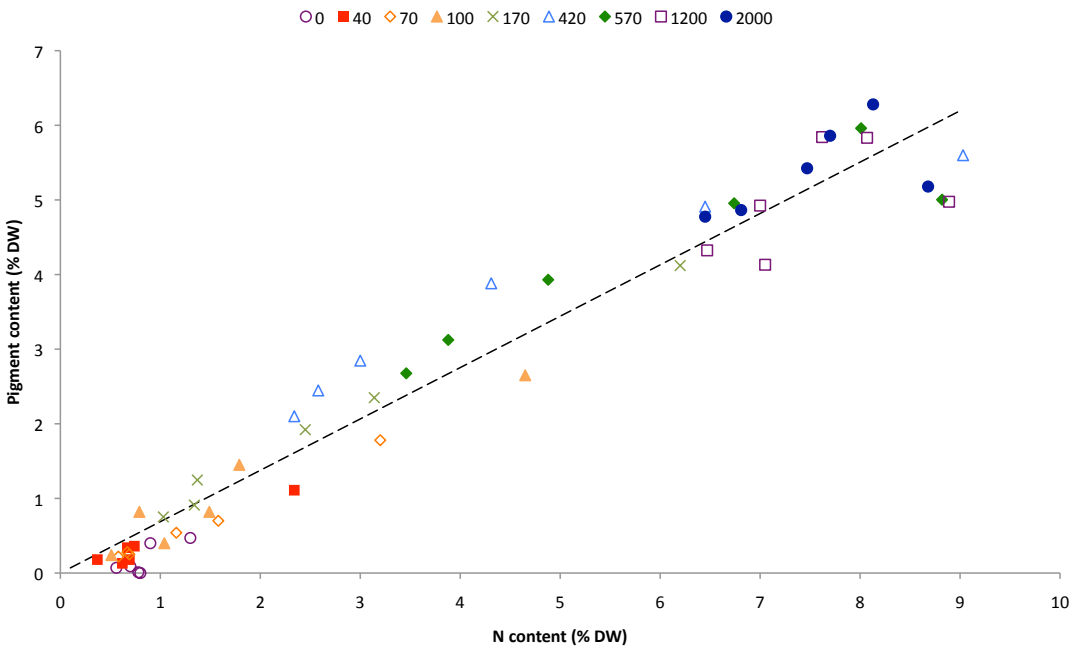


Figure 7.16 Pigment content plotted against nitrogen content of cells of *C. vulgaris* cultures inoculated at starting nitrate concentrations of 0, 40, 70, 100, 170, 420, 570, 1200 and 2000 $mg.L^{-1}$ respectively

Biomass productivity

Biomass productivity during exponential growth decreased with a decrease in the N content of the cells (Figure 7.17). There was a critical minimum N content required for growth (1% DW). Above this point, the productivity increased with increasing N content, showing a typical Monod growth response, asymptoting to a maximum Q_X of between 0.35 and 0.37 $g.L^{-1}.day^{-1}$. At high N availability, there was a maximum N content of the cells (9% DW).

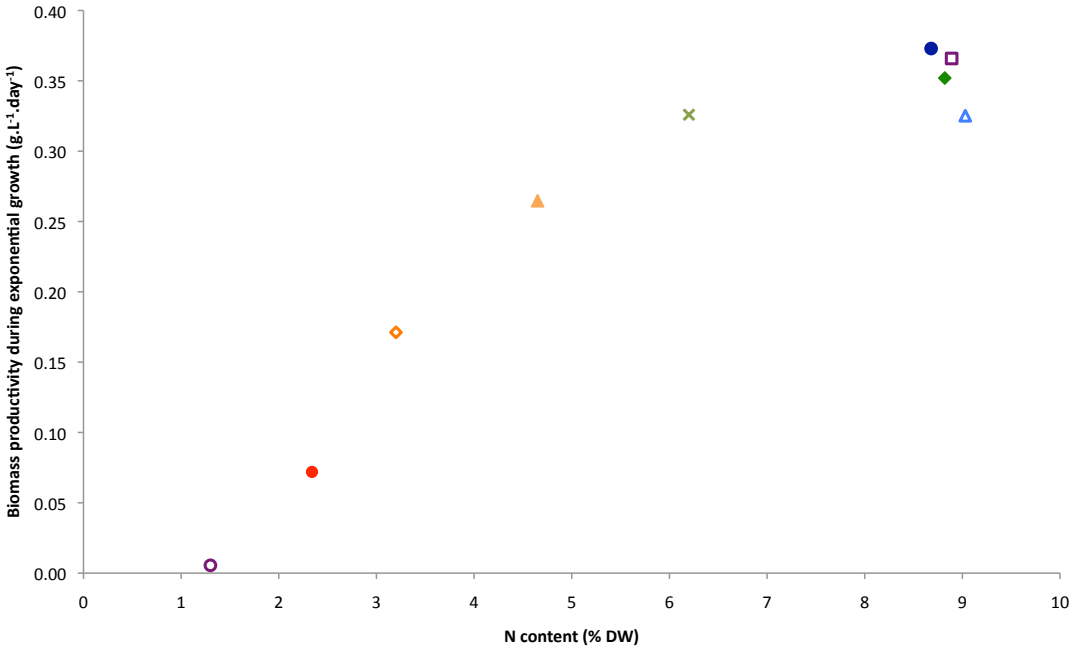


Figure 7.17 Biomass productivity during the exponential phase plotted against nitrogen content of *C. vulgaris* cultures on day three of cultivation in media containing 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) $mg.L^{-1}$ nitrate

Lipid content

Lipid content was inversely correlated with N content (Figure 7.18). There was a strong trend across cultures with different levels of N limitation. Above a cell N content of 5%, lipid content remained approximately 10% DW. There was almost no change in lipid content with change in cellular N content (slope of regression line -0.31). Once the N content of the cells dropped below 5%, lipid content became a function of the N content. Between a cell N content of 2 and 5%, lipid content increased with a decrease in N content (slope of regression line -6.5), up to intermediate levels (13 to 35% DW). Lipid contents of above 30% DW generally only occurred when the N content of cells was below 2%. In this region, lipid content increased as an even stronger function of cellular N content (slope of regression line -17.8).

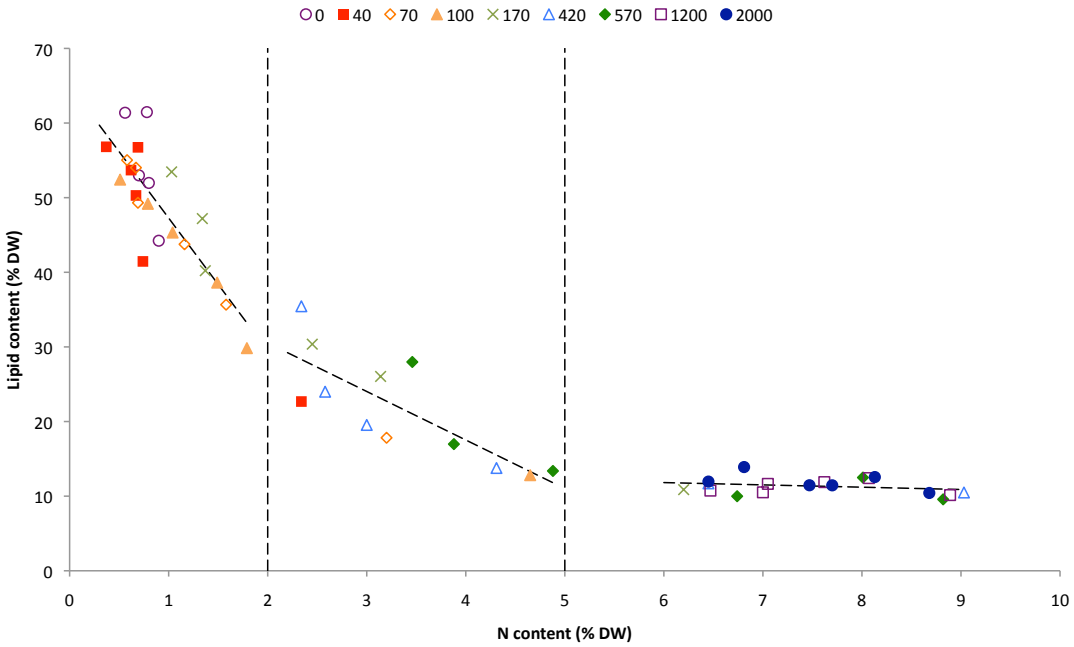


Figure 7.18 Lipid content plotted against nitrogen content of cells of *C. vulgaris* cultures containing 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1} nitrate

Lipid productivity

Overall average lipid productivity was at a maximum (34 to 40 mg.L^{-1}.day^{-1}) at a cell N content of between 1 and 2%. (Figure 7.19). This is the region where the tradeoff between lipid content and biomass productivity produced maximum lipid productivity. At cellular N contents greater than 3%, the lipid productivities were in the range of 14 to 26 mg.L^{-1}.day^{-1}, owing to a higher biomass productivity but lower lipid content. Below a cell N content of 1%, there was a large variation in lipid productivity, due to the range of biomass productivities in cultures that experienced N limitation before or during exponential growth (0, 40, 70 and 100 mg.L^{-1} nitrate).

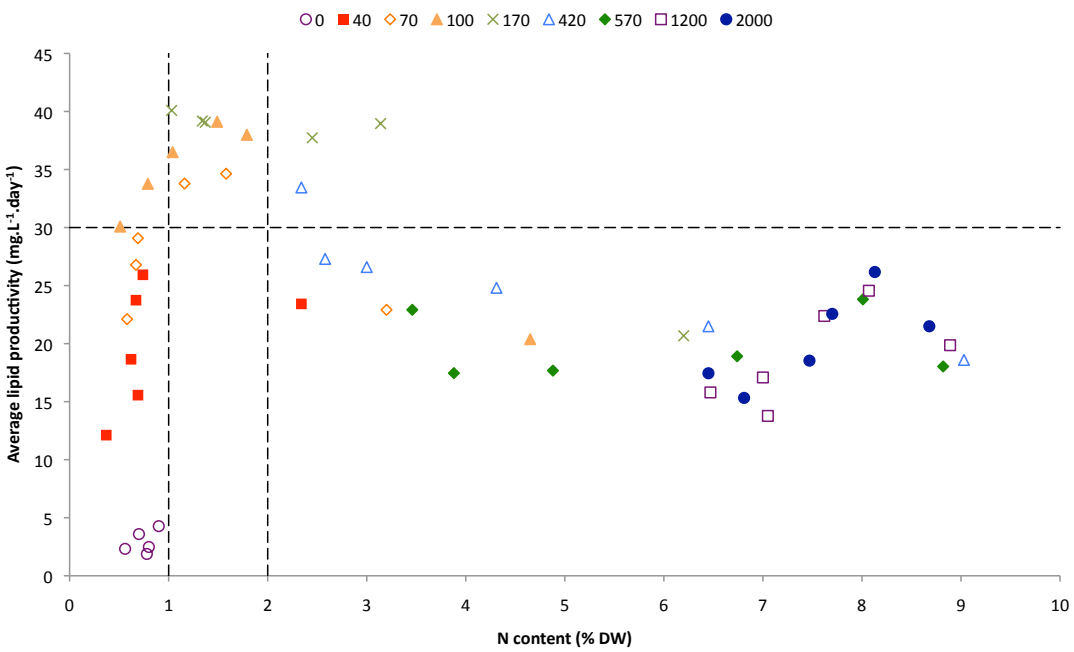


Figure 7.19 Average lipid productivity ($Q_{P\ AVE}$) plotted against nitrogen content of cells of *C. vulgaris* cultures containing 0 (○), 40 (■), 70 (◇), 100 (▲), 170 (×), 420 (△), 570 (◆), 1200 (□) and 2000 (●) mg.L^{-1} nitrate

7.5 Conclusion

In *C. vulgaris* batch culture, maximum biomass productivity and maximum lipid content occurred under opposite conditions in terms of N availability. There was an optimal level of nitrogen limitation that balanced the tradeoff between enhanced lipid content and decreased growth rate with associated decreased biomass productivity. Enforcing a level of N limitation that maintained a reasonable biomass productivity whilst accumulating maximum lipid content led to a lipid productivity and volumetric lipid content higher than either nitrogen replete (higher biomass but lower lipid content), or more N limited (lower biomass and only slightly higher lipid content) cultures. Under the growth conditions tested here, and over a 20 day growth period, the optimal degree of N limitation tested corresponded to a starting nitrate concentration of 170 mg.L^{-1}. This starting nitrate concentration allowed the cells a short initial period of N replete growth, but the culture medium became N depleted while the cells were still in the exponential phase. Cells responded to this treatment by maintaining a slow but steady increase in biomass, while lipid content increased dramatically, immediately following N depletion, leading to a high and sustained lipid productivity. The nitrate concentrations that were predicted to achieve the highest volumetric lipid content and average lipid productivity were 305 and 240 mg.L^{-1} respectively.

Cultures that became N exhausted later in the growth cycle reached higher biomass concentrations, with associated higher biomass productivities, but the onset of lipid

accumulation was delayed, and initially slower than cultures that ran out of N earlier. Assuming a continued increase in lipid content to 54% DW and no further growth, the 420 $mg.L^{-1}$ nitrate culture is predicted to have exceeded the volumetric lipid yield (1004 $mg.L^{-1}$) and equalled the overall lipid productivity (40 $mg.L^{-1}.day^{-1}$) of the 170 $mg.L^{-1}$ nitrate culture at approximately day 25. However, the 170 $mg.L^{-1}$ nitrate culture has the advantages that 1) it could be harvested earlier with the same average productivity, and 2) it required less nitrogen. The possibility of harvesting earlier with an equivalent overall productivity could be an advantage if long-term culture maintenance were difficult, for example due to contamination. If the financial or energy cost of the nitrogen fertilizer were significant, the 100 $mg.L^{-1}$ nitrate culture would be even more favourable than the 170 $mg.L^{-1}$ nitrate culture if harvested within the first two weeks. This is due to its minimal N requirements and comparable lipid productivity to the 170 $mg.L^{-1}$ nitrate culture for the first 14 days.

C. vulgaris cell physiology changed dramatically with N limitation. Increasing N limitation led to higher lipid content and lower protein, pigment and carbohydrate content. The correlations between the N content of cells and their pigment, protein and lipid content were so predictable that one of these measurements could be used as an indirect measure of the others. For example, once calibrated for a certain species, and in the absence of other causes of chlorosis, the pigment content could be used as an indicator of N limitation, and hence lipid content and productivity. As pigment content is rapid and easy to measure, this could facilitate monitoring of commercial cultures.

In addition to determining product content, N limitation affects other cell properties such as nutrient supply, ease of downstream processing, and product range and quality. The effect of these on the overall energy balance and cost of the process should be taken into account. N limitation was shown to improve the fatty acid profile for biodiesel production (Section 6.4.5). Growth of algal cultures under N limited conditions was also shown to decrease the nitrogen requirement per unit lipid produced (Figure 7.8), decreasing the process demand for nitrogen, along with the associated cost and environmental burden. N limitation decreased final biomass concentration and may alter cell properties (e.g. density), influencing the optimal harvesting method. One of the most attractive ways of improving the economics of an algal biodiesel process is the co-production of other valuable products in a biorefinery approach. In this case, the radical changes in cell composition under N limitation may have consequences for the suite of products that could be produced.

All experiments in this work were conducted in batch culture. The need for both high biomass concentration and high lipid content, which occur under different growth conditions, suggested that two-stage culture should be explored. Alternative culture regimes are investigated in Chapter 8.

8 Effect of culture regime on lipid productivity in *Chlorella vulgaris*

8.1 Introduction

The data presented in Chapter 7 showed that maximum biomass productivity and lipid content in *Chlorella vulgaris* occurred under N replete and N limited conditions respectively. In order to achieve maximum lipid productivity in batch culture, a compromise between these two situations can be found by applying an intermediate level of N limitation. Another approach to the problem is to apply the optimal conditions for growth and product accumulation sequentially, using a two-stage cultivation strategy. The N replete stage is designed to produce maximum biomass, while the N limited stage aims to produce maximum lipid content as quickly as possible.

This chapter investigates the potential of different culture regimes by comparing the overall lipid productivity to that achieved in batch culture. Culture regimes investigated were:

1) two-stage batch culture
2) fed-batch culture
3) two-stage continuous-batch culture
4) multi-stage continuous culture.

The effect of the initial nitrate concentration during N replete growth on lipid accumulation after removal to N-free culture medium was investigated, as well as the effect of light availability. It was hypothesized in Chapter 7 that the delay in lipid accumulation in more mature, concentrated cultures could be due to light limitation. This was tested by diluting cultures upon transfer to the second, lipid accumulation stage, to restrict light limitation.

8.2 Literature review

In bioprocesses, the kinetics of cell growth and product accumulation, and concomitant substrate uptake, yield of product on substrate, cost and requirements for downstream processing are among the factors that determine the optimum cultivation strategy. Products can be either primary (formed in association with growth, in which case their production is a function of the production of biomass), secondary (formed when growth has ceased) or mixed growth associated (formed during slow growth and stationary phase) (Shuler & Kargi 2005). For production of a growth-associated product, optimizing biomass productivity is the main objective. In the case of a non-growth associated, secondary product, two production stages are often required: first the culture is grown to a high cell density and then conditions are altered to

stop growth while maintaining culture viability and encouraging product synthesis. Growth and product accumulation in bioprocesses are usually controlled by adjusting the supply of nutrients and inducers. There are three major bioreactor configurations: batch, fed-batch and continuous. Due to their low solubility, gases are supplied continuously, so it is the supply of liquid feed that determines the classification (Doran 1997).

The merits of the different culture strategies depend on the kinetics of the reaction in question, as well as practical considerations. Comparison of culture regimes can be considered according to the product concentration obtained from vessels of the same size over a certain time period. In most processes, the rate of growth depends on the concentration of the limiting substrate. In a batch reactor, growth rate is high at the start (provided nutrient inhibition is not experienced) and falls as substrate is consumed. When growth enters the stationary phase, the production of secondary metabolites begins. In fed-batch culture, the period of biomass production can be extended by the addition of extra nutrients. In a continuous reactor, high rates of biomass accumulation can theoretically be maintained indefinitely. As cells are maintained in exponential growth, continuous reactors are typically used for primary products, or when the biomass is the product. However, where product formation is induced at low, but not zero, growth rates, a two-stage continuous system can be used with an initial biomass production stage operated at the optimal dilution rate to achieve μ_{max}, followed by a secondary stage of larger volume and thereby reduced dilution rate and specific growth rate to induce product formation (Doran 1997).

The joining of two or more continuous cultures in series produces a multi-stage process in which conditions such as pH, temperature and medium composition can be varied in each reactor (Shuler & Kargi 2005). This is advantageous if conditions required for growth differ from those required for product synthesis. One method of operation is for the product stream from the first reactor to be fed directly into the second. In the absence of a supplementary feed, the only supply of substrate to the second reactor is that in the product stream from the first (equal to the residual substrate concentration in the first reactor). The biomass concentration in the second reactor is the sum of the biomass concentration entering from the first reactor, plus any new growth in the second reactor. If more than two continuous reactors are joined in series, the conditions in the subsequent reactors mimic those in a plug flow reactor, or a batch reactor where each growth stage is confined to a different reactor (Shuler & Kargi 2005).

The choice of culture regime for the production of lipid by microalgae is complicated by the fact that lipid is a biomass-dependent but non growth-associated product. Structural lipids are a primary, growth-associated product, but storage lipids such as TAG (ideally suited to biodiesel production) are a secondary metabolite formed in large quantities during the stationary phase. Although ideas of extracellular production of lipid have been entertained, lipid storage vesicles are generally contained within cells, up to a maximum proportion of about 65% DW in *C. vulgaris*

(Figure 7.3), therefore the total product yield is constrained by the biomass concentration. Not only do biomass and product accumulation occur under different conditions, but it has also been shown in Chapter 7 that the rate of lipid accumulation is greater when the biomass concentration is lower, possibly due to greater light availability.

The culture strategies applied here relate to the supply of nitrogen. An additional complication in microalgal culture is that the two major nutrients required for autotrophic growth (light and CO_2), are not generally supplied in liquid form and therefore their availability is independent of the liquid flow rate. CO_2 supply in the experiments conducted here was calculated to be sufficient for balanced growth (Langley 2010). Supply of light, however, was limiting at high cell densities. Therefore, after an initial period of nutrient replete growth, cultures were co-limited by light and nitrogen.

Stephenson *et al.* (2010) found that maximal average lipid productivity in *C. vulgaris* (46 $mg.L^{-1}.day^{-1}$) was achieved by a two-stage method, where cultures were initially grown in N replete medium, and then transferred to medium with an intermediate nitrate concentration (200 $mg.L^{-1}$). They also investigated the effect of altering the cell concentration of cultures upon transfer to N limited medium, and concluded that there was an optimal cell concentration for lipid production. Cells transferred to N limited media at a lower cell density accumulated more lipid, however, due to lower cell concentration, the overall productivity of cultures inoculated at lower cell density was lower.

In order to further test the idea that light availability affected lipid accumulation, Stephenson *et al.* (2010) grew cultures with nitrate concentrations of 200 and 550 $mg.L^{-1}$ in two different volumes, resulting in two different culture depths. No difference was found between the two cultures. However, Rodolfi *et al.* (2009) found that increasing the illumination supplied to a flat panel reactor enhanced both the biomass productivity and the lipid content.

Hsieh and Wu (2009) investigated the feeding of different amounts of additional urea at different time points in fed-batch culture of a marine *Chlorella* species. Final biomass concentration increased with greater amounts of urea fed, but lipid content increased with decreasing amounts fed. Higher lipid contents were found in cultures fed in the stationary phase, rather than late log phase. A single feeding of additional urea did not improve lipid productivity in fed-batch (123 $mg.L^{-1}.day^{-1}$) over that in batch culture (124 $mg.L^{-1}.day^{-1}$). However, semi-continuous cultivation in which 25% of the culture was withdrawn and fresh medium with 25 $mg.L^{-1}$ urea (equivalent to 52 $mg.L^{-1}$ nitrate) supplied daily, resulted in an average biomass concentration of 1.1 $g.L^{-1}$ and lipid content of 44%. The lipid productivity was 139 $mg.L^{-1}.day^{-1}$, 26% higher than batch culture.

Takagi *et al.* (2000) showed that feeding of small amounts of nitrate (0.9mM, equivalent to 56 $mg.L^{-1}$) in *Nannochloris* culture could increase biomass concentration to twice that obtained

without feeding, while maintaining lipid content at a high level. In contrast to Hsieh and Wu (2009), the timing of a single feeding (log phase, constant growth phase or stationary phase) was found to have little effect on the lipid content. Intermittent feeding during log phase resulted in equivalent biomass concentrations and higher lipid contents than N replete batch culture. In Hsieh and Wu (2009), lipid content decreased when cultures were fed 100 $mg.L^{-1}$ urea (equivalent to 207 $mg.L^{-1}$ nitrate in terms of moles of nitrogen supplied) during stationary phase, but was maintained if a smaller amount (25 $mg.L^{-1}$ urea, equivalent to 52 $mg.L^{-1}$ nitrate) was fed. Jin *et al.* (2006) have also shown fed-batch cultivation to improve biomass productivity and the efficiency of CO_2 fixation.

Various microalgal species have been grown in continuous culture, usually at laboratory-scale for the purposes of producing uniform biomass or studying the effect of culture conditions on physiology (e.g. (Maddux & Jones 1964; Okay *et al.* 2003)). Although the first continuous culture apparatus for a microalgae (*Chlorella pyronoidosa*) was developed in 1944 (Myers & Clark 1944), much less is known about the growth of microalgae in a chemostat than bacteria (Okay *et al.* 2003). Continuous culture has also been investigated for the production of microalgal products, e.g. EPA from *Phaeodactylum tricornutum* (Reis *et al.* 1996). Multi-stage continuous culture has been investigated for the bioremediation of nitrate-contaminated groundwater, using *Scenedesmus* (Hu & Sommerfeld 2006).

Huntley and Redalje (2006) proposed a two-stage process to optimise both oil and astaxanthin production from *Haematococcus pluvialis*. In the first stage, conditions for optimal cell division and prevention of contamination were maintained in a photobioreactor. Biomass was transferred into a second stage where cells were exposed to nutrient limitation and other environmental stresses in an open pond. Upon transfer to the open pond, cell concentration increased initially for 1 to 2 days, before oil content and then astaxanthin content increased. The system was operated successfully on a large scale for several years with an average lipid productivity of 32 $mg.L^{-1}.day^{-1}$ (equivalent to 3.78 $g.m^{-2}.day^{-1}$ given a pond depth of 12 cm).

8.3 Methods

C. vulgaris was grown in airlift reactors as described in Section 2.3. Biomass concentration by OD and lipid content were measured daily, and protein, pigment, carbohydrate and DW quantification and elemental analysis were performed every 3 to 4 days according to the methods in Section 2.4. A selection of culture regimes were tested.

8.3.1 Two-stage batch culture

Cultures inoculated at starting nitrate concentrations of 1500, 750, 570 and 420 $mg.L^{-1}$ (all providing nitrogen replete growth for at least 5 days) were grown for between 5 and 6 days, at

which time a biomass concentration of approximately 1 $g.L^{-1}$ was reached. Biomass was harvested by centrifugation at 1520 g for 10 min, and resuspended in N free or N limited media, in the original reactor, before continued cultivation.

8.3.2 Fed-batch culture

Two different starting nitrate concentrations: 40 and 170 $mg.L^{-1}$, and four different feeding regimes were tested:

1) 40 $mg.L^{-1}$ every second day on days 4, 6, 8 and 10
2) 80 $mg.L^{-1}$ every second day on days 4, 6, 8 and 10
3) 40 $mg.L^{-1}$ every fourth day on days 4, 8, 12 and 16
4) 40 $mg.L^{-1}$ every eighth day on days 8 and 16.

Nitrate concentrate was added in a volume less than that added to the reactor daily to compensate for evaporation, hence, biomass was not removed from the reactors during the culture period and the volume of the reactors remained constant.

8.3.3 Two-stage continuous-batch culture

Continuous cultures were initiated by growing *C. vulgaris* in batch culture, with a nitrate concentration of 420 $mg.L^{-1}$, for 5 days. On day 5, continuous culture was initiated using a peristaltic pump to supply fresh feed consisting of 3N BBM medium with a nitrate concentration of 300 $mg.L^{-1}$. A dilution rate of 0.3 day^{-1} was maintained. In a 3.2 L airlift reactor, this equated to a feed rate of 0.97 $L.day^{-1}$ and residence time of 3.2 days. In order to compensate for evaporation from the reactor, an additional 100 $ml.L^{-1}$ of dH_2O was added to the feed and the feed rate adjusted to 1.1 $L.day^{-1}$. Culture was removed by another peristaltic pump and the outflow was level controlled by a tube suspended at the correct height to maintain a 3.2 L volume. This necessitated the frequent addition of antifoam to the cultures to prevent the flotation of biomass in the bubbles at the top of the reactor. If antifoam was not added, the biomass concentration in the reactor dropped due to the loss of too much biomass in the outflow.

Three identical continuous reactors were run in parallel. Once the reactors had reached steady-state (as indicated by a constant biomass and nitrate concentration, approximately 10 to 12 days from initiation of continuous culture and equivalent to 3.12 to 3.75 residence times), the biomass produced from the reactors was collected until sufficient volume to fill a fresh reactor was generated (2 days). This pooled culture was transferred to an airlift reactor and batch culture was initiated. No further nutrients were added. Due to the low residual N concentration in the continuous reactor, N in the media was exhausted within one day of batch culture.

8.3.4 Multi-stage continuous culture

Four continuous reactors were connected in series, as shown in Figure 8.1. The first reactor was run under the conditions described above. The outflow from each reactor provided the feed for the next reactor in series. The outflow of each reactor was level-controlled and no fresh media was added other than to the first reactor. Each was supplied with light and CO_2 as detailed in Section 2.3.

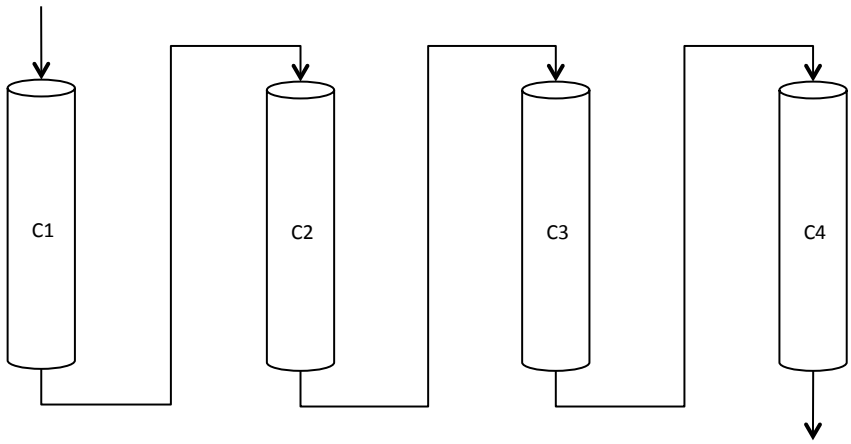


Figure 8.1 Setup of continuous reactors in series

8.3.5 Calculations

Calculations conducted using the batch and fed-batch cultivation data are described in Section 6.3.4. For steady state continuous culture, dilution rate (D) was calculated as the quotient of volume (V) and flow rate (F) of the feed. Residence time (τ) was calculated as the inverse of D. The specific growth rate is equal to the dilution rate, hence biomass productivity (Q_X) can be calculated from Equation 8.1.

$$Q_X = \mu.X = D.X \qquad \text{Equation 8.1}$$

In order to determine the overall productivity of culture regimes, the maximum biomass concentration, lipid content, lipid productivity and grams of nitrate required per gram of lipid produced were compared.

8.4 Results and discussion

8.4.1 Two-stage batch culture

In Chapter 7, results were presented illustrating that cultures inoculated with a limiting concentration of N accumulated lipid after N became exhausted in the medium. The final biomass concentration was determined by the N supply. Higher growth rate, biomass concentration and

hence biomass productivity were achieved under conditions of higher N supply, but overall lipid productivity was lower due to the length of time required for the N to become exhausted. From these, it was hypothesized that lipid productivity could be improved if the culture were grown initially under N replete conditions, until a high biomass concentration was reached, and then lipid accumulation triggered by removal of the N from the medium.

An initial test of the effect of sudden removal of N from a N replete culture was carried out. *C. vulgaris* was inoculated at a starting concentration of 1500 $mg.L^{-1}$ nitrate. Cultures in which the nitrate concentration had been suddenly reduced to zero or 170 $mg.L^{-1}$ at day 6 continued to accumulate biomass up to day 20, although at a slower rate than the equivalent nutrient replete culture (Figure 7.1). Maximum biomass concentrations of 1.9 and 2 $g.L^{-1}$ were reached in the 0 and 170 $mg.L^{-1}$ nitrate cultures respectively (Figure 8.2a). Up to day 6, the lipid content was on average 11%, typical of N replete *C. vulgaris* (Figure 7.3a). After transfer to N limited media, the lipid content increased slowly to 28 and 21% DW at day 20 in the cultures transferred to 0 $mg.L^{-1}$ and 170 $mg.L^{-1}$ nitrate respectively.

The volumetric lipid content (P_{VOL}) of both cultures increased linearly over the culture period to a maximum of 477 and 390 $mg.L^{-1}$ at day 19 for the 0 and 170 $mg.L^{-1}$ nitrate cultures respectively. Results are compared to those of single-stage batch culture with 170 $mg.L^{-1}$ nitrate (the N-limited batch culture with the highest P_{VOL} from Chapter 7), and 1200 $mg.L^{-1}$ nitrate (N replete for 20 days) (red symbols, Figure 8.2b). The P_{VOL} of two-stage cultivation with 0 $mg.L^{-1}$ nitrate was greater than N replete culture from day 10, but the maximum P_{VOL} reached was only 60% of that achieved in the 170 $mg.L^{-1}$ nitrate batch culture. Maximum average lipid productivities ($Q_{P\ AVE}$) of 25 and 21 $mg.L^{-1}.day^{-1}$ were reached on day 19 in the 0 and 170 $mg.L^{-1}$ cultures respectively.

Stephenson *et al.* (2010) found that gradual depletion of 200 $mg.L^{-1}$ nitrate after growth at 550 $mg.L^{-1}$ nitrate resulted in higher lipid productivity than complete removal of nitrate. In contrast, in this experiment, complete removal of N from the media resulted in higher lipid productivity due to a higher lipid content and almost equivalent biomass concentration to the culture resuspended in media with 170 $mg.L^{-1}$ nitrate.

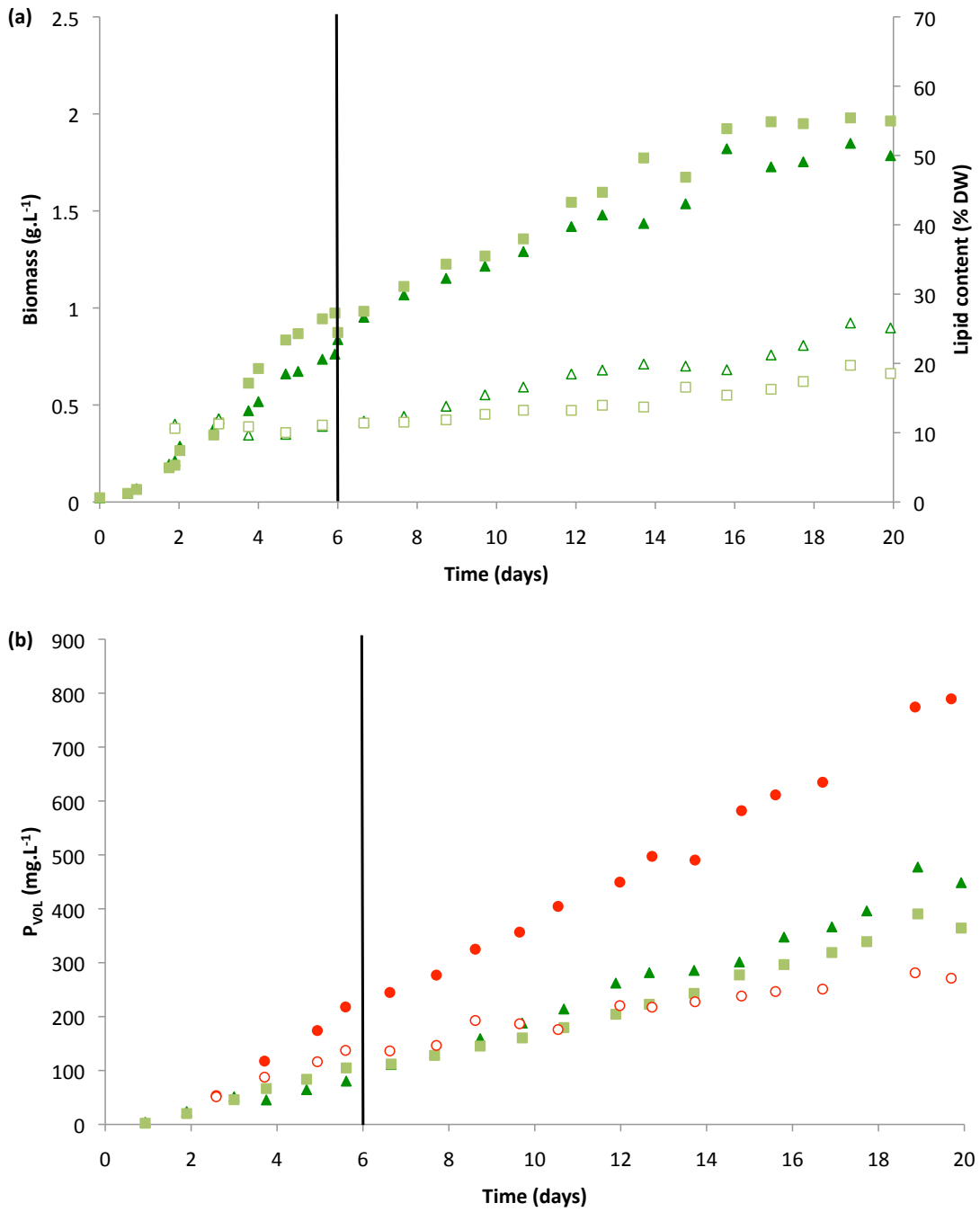


Figure 8.2 (a) biomass (filled symbols) and lipid content (empty symbols) and (b) volumetric lipid content (P_{VOL}) over 20 days in *C. vulgaris* grown initially under N replete conditions, harvested on day 6 (indicated by vertical line) and resuspended in fresh media with 0 mg.L^{-1} (▲, dark green) or 170 mg.L^{-1} (■, light green) nitrate before continued cultivation. The volumetric lipid contents of single-stage batch cultures with 170 mg.L^{-1} nitrate (●, red, highest yielding N-limited batch culture) and 1200 mg.L^{-1} nitrate (○, red, N replete batch culture) are shown for comparison

Upon sudden removal of an external N source, cells had sufficient intracellular nitrogen reserves to double in biomass. Lipid content trebled over 15 days without N, but the increase was linear rather than the exponential increase seen in cultures which became N limited very early in the

growth cycle (Figure 7.3). Microalgal cells are known to store N, not only in macromolecules such as protein, pigment and nucleic acid, but also as inorganic nitrate, nitrite and ammonia within the cell (Lourenço *et al.* 2004). Storage of extra N under conditions of N sufficiency could be responsible for the continued growth and slow lipid accumulation upon N removal, as N limitation would be delayed until cells had used up their N stores. This is a disadvantage for optimizing lipid productivity using N-limited two-stage culture, as it causes a delay in lipid accumulation after N removal.

It was hypothesized that the initial N concentration during the N replete phase could affect the amount of N stored and hence the physiology of the cells after N removal. In order to test this, identical *C. vulgaris* cultures were grown at starting nitrate concentrations of 750, 570 and 420 $mg.L^{-1}$ for 5 days and then transferred into N-free media. The biomass concentration and lipid content in all three cultures were within the average relative error for replicate cultures (less than 5%, see Section 6.4.1) over the entire culture period (Figure 8.3), and very similar to the performance of the cultures with a nitrate concentration of 1500 $mg.L^{-1}$ (Figure 8.2). In addition to identical growth rates and lipid contents, the protein, pigment and carbohydrate contents of the three two-stage cultures were also very similar (Figure 8.4). These results indicate that the initial nitrate concentration in the medium did not influence the physiology of the cells. Cells do not appear to accumulate greater N stores at higher medium N concentrations, as their response to N deprivation was identical. This is supported by the fact that the N uptake rate of cultures was similar regardless of the external nitrate concentration, up to 2000 $mg.L^{-1}$ (Figure 7.1). If cultures were responding to a higher medium N concentration by storing more N, the medium nitrate concentration would be expected to drop more rapidly, but evidence of this was not found.

The single-stage batch culture from Chapter 7, with a starting nitrate concentration of 420 $mg.L^{-1}$ (originally presented in Figure 7.1 and Figure 7.3) exhausted the N supply naturally at day 5. These data are included in Figure 8.3 for comparison. The culture allowed to exhaust 420 $mg.L^{-1}$ nitrate naturally, reached a lower biomass concentration and higher lipid content than the culture removed to fresh medium devoid of N. In the 420 $mg.L^{-1}$ culture, the nitrate in the media was exhausted at day 4.9, before transfer into fresh nutrient-free medium, therefore the only difference between the two cultures was the transfer step. The greater growth and lower lipid content could be due to the removal of an autoinhibitor, or the replacement of a nutrient other than N (e.g. phosphate or a micronutrient) that became limiting in the culture not transferred to fresh medium. It is possible that the exhaustion of an additional nutrient such as phosphate could have augmented the effects of N depletion. Phosphate limitation has been shown to result in lipid accumulation (Rodolfi *et al.* 2009).

The protein and pigment composition of the three two-stage cultures were similar to that of the 420 $mg.L^{-1}$ culture allowed to exhaust the nitrate naturally (Figure 8.4a and c), but the

carbohydrate content was lower, particularly at the end of cultivation (Figure 8.4b). This may have been an underestimation of the carbohydrate content on day 20 as the sum of the lipid, protein, pigment and carbohydrate contents at this time point in the three two-stage cutures was significantly less than 100%.

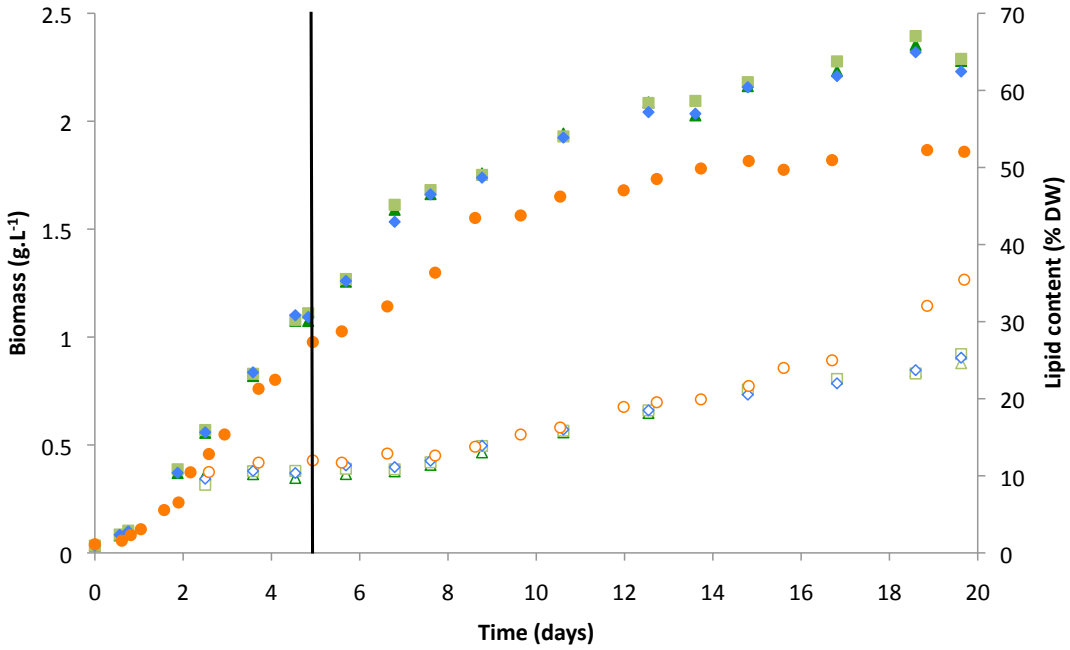


Figure 8.3 Biomass concentration (filled symbols) and lipid content (empty symbols) over 20 days in cultures of *C. vulgaris* inoculated with nitrate concentrations of 750 (▲, dark green), 570 (■, light green) and 420 (◆, blue) mg.L^{-1}, and transferred to N-free media at day 5 (indicated by vertical line). Results for a culture with a nitrate concentration of 420 mg.L^{-1}, allowed to exhaust the N naturally (●, orange), without centrifugation and transfer to new media are included for comparison.

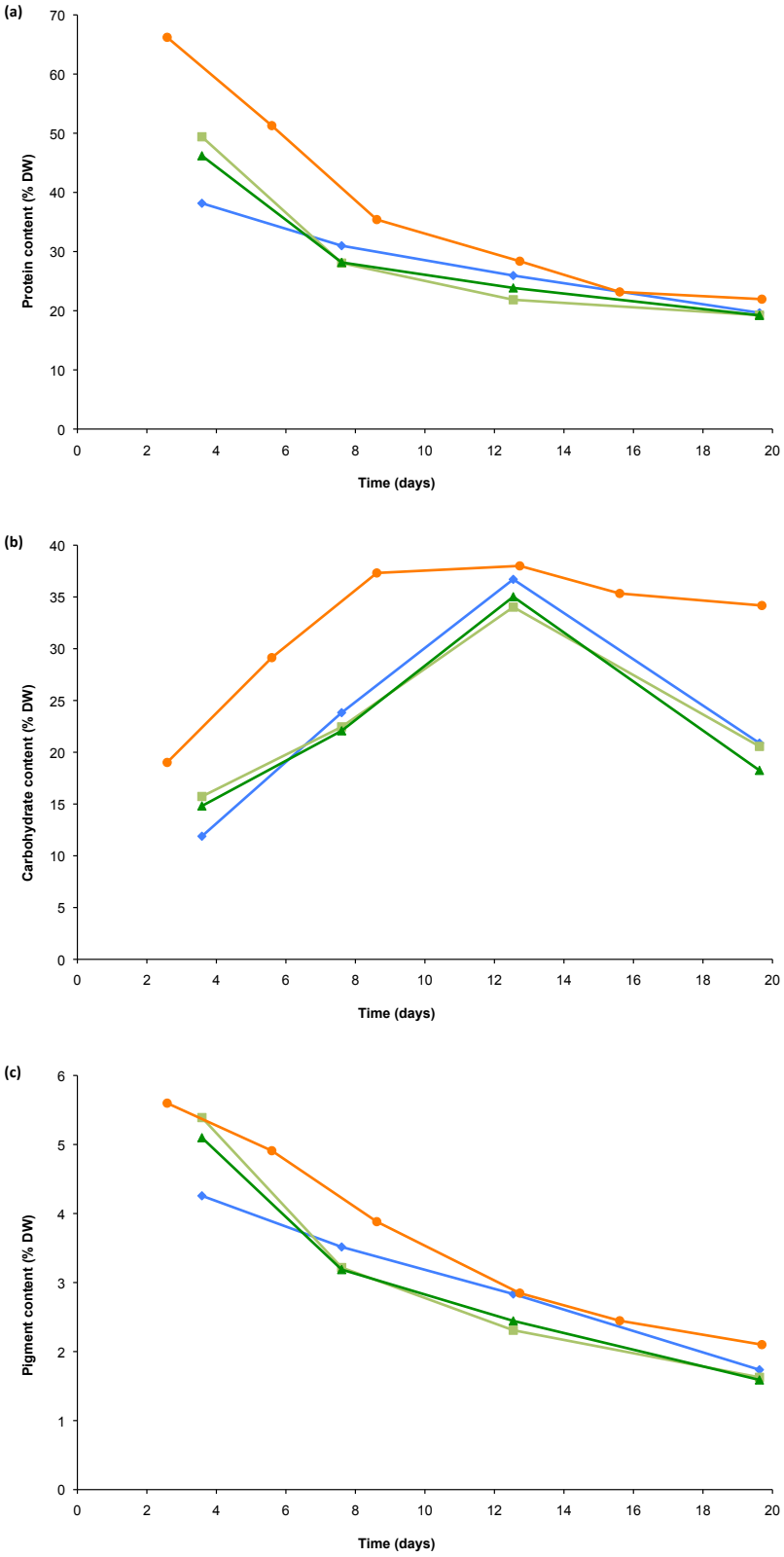


Figure 8.4 (a) protein, (b) carbohydrate and (c) pigment content over 20 days in cultures of *C. vulgaris* inoculated with nitrate concentrations of 750 (▲, dark green), 570 (■, light green) and 420 (◆, blue) mg.L^{-1}, and transferred to N-free media at day 5 (indicated by vertical line). Results for a culture with a nitrate concentration of 420 mg.L^{-1}, allowed to exhaust the N naturally (●, orange), without centrifugation and transfer to new media are included for comparison.

The two-stage strategy of batch culture under N sufficiency to a biomass concentration of 1 $g.L^{-1}$, followed by immediate N limitation yielded lower lipid productivities than those achieved in N limited batch culture (Figure 8.2b). A higher initial nitrate concentration did not promote N storage and had no effect on the subsequent growth, lipid accumulation or biochemistry of the cells. The single-stage batch culture was the most productive overall, due to a higher lipid content (Table 8.1). This may have been due to a concomitant limitation in another nutrient, possibly phosphate, towards the end of the culture period.

Table 8.1 Growth and lipid parameters of two-stage cultures of varying nitrate concentrations, compared to a batch culture in which the 420 $mg.L^{-1}$ nitrate was naturally exhausted after 5 days (420 nat ex) – the time point at which the other cultures were harvested and resuspended in N limited medium.

Initial $[NO_3]$ in stage 1 ($mg.L^{-1}$)	Initial $[NO_3]$ in stage 2 ($mg.L^{-1}$)	Max X ($g.L^{-1}$)	Max P (% DW)	Max P_{VOL} ($mg.L^{-1}$)	Max $Q_{P\ AVE}$ ($mg.L^{-1}.day^{-1}$)
1500	0	1.9	28	525	25
1500	170	2.0	22	434	21
750	0	2.4	25	561	31
570	0	2.4	26	590	31
420	0	2.3	25	565	30
420 nat ex	n/a	1.9	35	659	33

8.4.2 Effect of light availability

In Section 7.4.2, the difference in lipid accumulation profile between batch cultures that became N limited early in the growth cycle and those that became N limited later in growth was hypothesized to be due to the difference in cell concentration and hence light availability. Cultures that become N limited at lower cell density experience less competition for the light resource in the form of mutual shading and hence a greater flux of carbon and energy from photosynthesis. As growth is constrained by N availability, additional fixed carbon is stored as lipid.

This hypothesis was tested by diluting cultures to different degrees at the beginning of the N limitation stage in the two-stage batch culture described above. Cultures with a nitrate concentration of 420 $mg.L^{-1}$ nitrate were grown for 5 days, to a biomass concentration of between 1.1 and 1.2 $g.L^{-1}$. The biomass concentration after centrifugation and resuspension in N free medium was 1.1, 0.7 and 0.5 $g.L^{-1}$ for the cultures diluted 1:1, 1:2 and 1:3 respectively. The increase in biomass concentration per unit time was approximately the same in the diluted and undiluted cultures. However, the diluted cultures reached a lower final biomass concentration (2.3, 1.8 and 1.4 $g.L^{-1}$ in the 1:1, 1:2 and 1:3 dilutions respectively) due to a lower starting biomass concentration (Figure 8.5a).

Lipid accumulation was enhanced in the diluted cultures, and to a greater extent the more dilute the culture (Figure 8.5b). The culture diluted 1:3 reached the highest lipid content (39 % DW), followed by the 1:2 (33 % DW) and then the undiluted culture (1:1, 25 % DW). Although the diluted cultures achieved a higher lipid content, this was offset by the lower biomass concentration throughout the growth cycle. As a result, all three cultures yielded an equivalent volumetric lipid content and identical overall lipid productivity over 20 days, although the characteristics of the cultures were different (Table 8.2). On increasing the dilution factor, a lower biomass concentration and higher lipid content resulted. This could have consequences for the ease and cost of harvesting and lipid extraction. Maximum volumetric lipid content was 565, 555 and 545 $mg.L^{-1}$ in the cultures diluted 1:1, 1:2 and 1:3 respectively (Figure 8.5c), less than that of the best N limited batch culture.

Table 8.2 Characteristics of cultures grown under N sufficiency for 5 days and then transferred to N limited medium at different dilutions (equal, a half and a third of the culture concentration at day 5) for a further 15 days

Dilution	Max X ($g.L^{-1}$)	Max P (% DW)	Max P_{VOL} ($mg.L^{-1}$)	Max $Q_{P\ AVE}$ ($mg.L^{-1}.day^{-1}$)
1:1	2.3	25	565	30
1:2	1.8	33	555	30
1:3	1.4	39	545	29

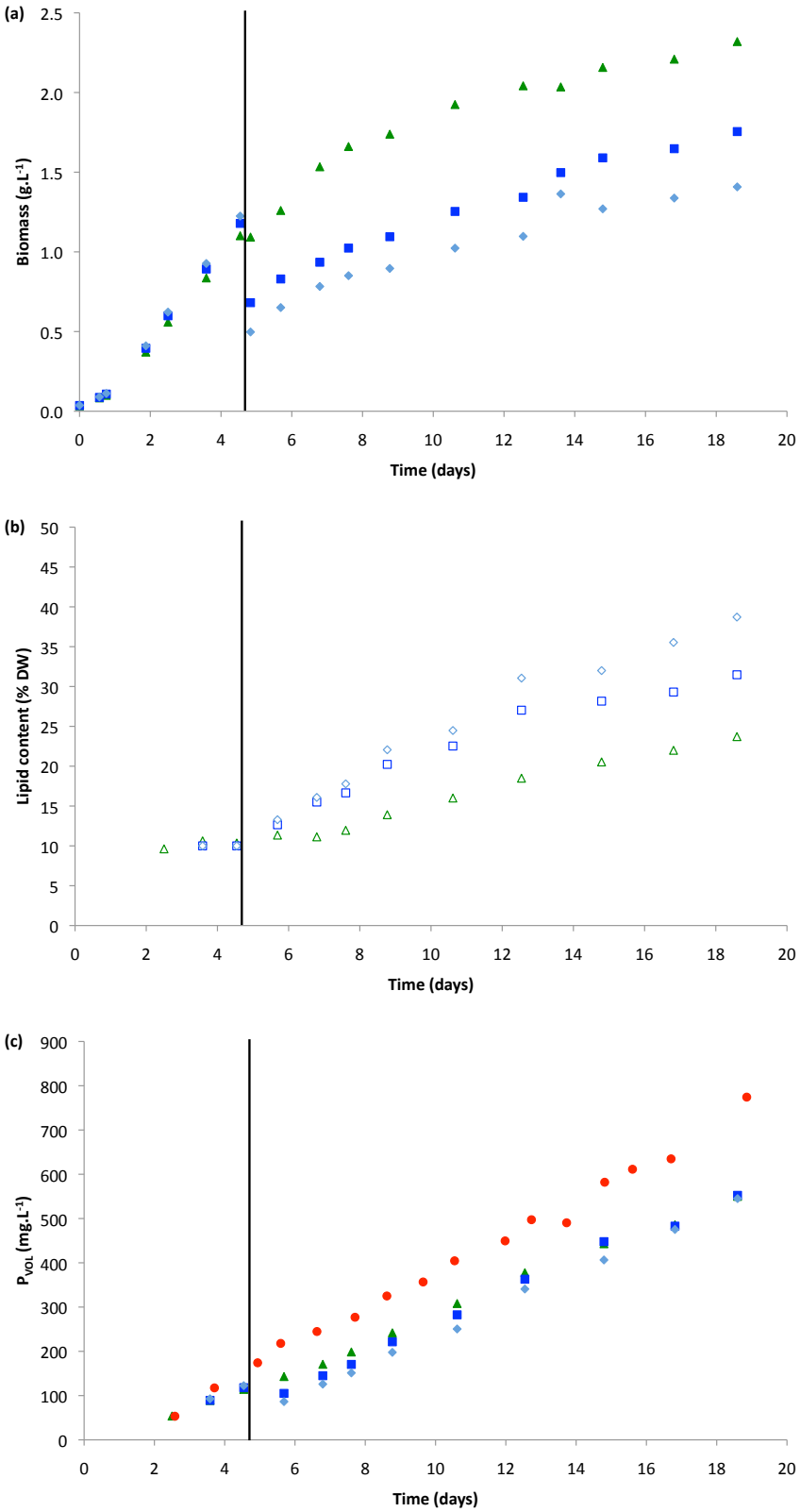


Figure 8.5 (a) biomass, (b) lipid content and (c) volumetric lipid content (P_{VOL}) over 20 days in *C. vulgaris* grown initially under N replete conditions, harvested on day 5 (indicated by vertical line) and resuspended in fresh media to dilutions of 1:1 (▲, dark green), 1:2 (■, dark blue) or 1:3 (◆, light blue) before continued cultivation. The volumetric lipid content of single-stage batch culture with 170 mg.L^{-1} nitrate (●, red) is shown for comparison

8.4.3 Fed-batch

In two-stage batch culture, the goal of the first stage was to optimise biomass concentration, followed by a second, nitrogen starvation stage in an attempt to increase lipid content. In contrast, fed-batch cultures were inoculated at a limiting nitrate concentration, in order to first achieve a high lipid content but low biomass concentration. Then, small quantities of nitrate were fed at various time points in an attempt to enhance biomass concentration while maintaining a high lipid content.

Starting nitrate concentrations of 40 $mg.L^{-1}$ and 170 $mg.L^{-1}$ were chosen, as these were the concentrations that led to the most rapid lipid accumulation and highest overall lipid productivity respectively in single-stage batch culture (Section 7.4.3). From data presented in Section 7.4.1, nitrate depletion in the media was expected by day 1.2 and 2.2 in the 40 $mg.L^{-1}$ and 170 $mg.L^{-1}$ reactors respectively.

In the first set of fed batch experiments, *C. vulgaris* cultures were grown in medium containing 40 $mg.L^{-1}$ nitrate and fed with an additional 40 or 80 $mg.L^{-1}$ nitrate every second day between day 4 and day 10. Results are compared to those of a single-stage batch culture with 40 $mg.L^{-1}$ nitrate (empty red circles, Figure 8.6) and 170 $mg.L^{-1}$ nitrate (solid red circles, Figure 8.6c) (from Figures 7.1, 7.3 and 7.4). Biomass concentration increased during and after N feeding and was greater in the culture fed with a higher concentration of nitrate (Figure 8.6a). The maximum biomass concentrations reached (1.6 and 1.3 $g.L^{-1}$ in the cultures fed 40 and 80 $mg.L^{-1}$ respectively) were over three times that in normal batch culture with 40 $mg.L^{-1}$ nitrate.

Lipid content was decreased by nitrate feeding. Fed-batch cultures initially accumulating lipid at the same rate as the batch culture, but lipid content decreased upon N feeding, remaining at an average of 24 and 14 % DW in the cultures fed 40 and 80 $mg.L^{-1}$ respectively, until feeding was stopped at day 10. Once N feeding ceased, lipid content increased slowly again (Figure 8.6b). As a result of the lower lipid content, the volumetric lipid content of the fed-batch cultures was initially lower than that of the batch culture. Once feeding ceased, the lipid yield of the fed-batch cultures was greater than that of the 40 $mg.L^{-1}$ nitrate batch culture, but lower than that of the 170 $mg.L^{-1}$ nitrate batch culture (Figure 8.6c).

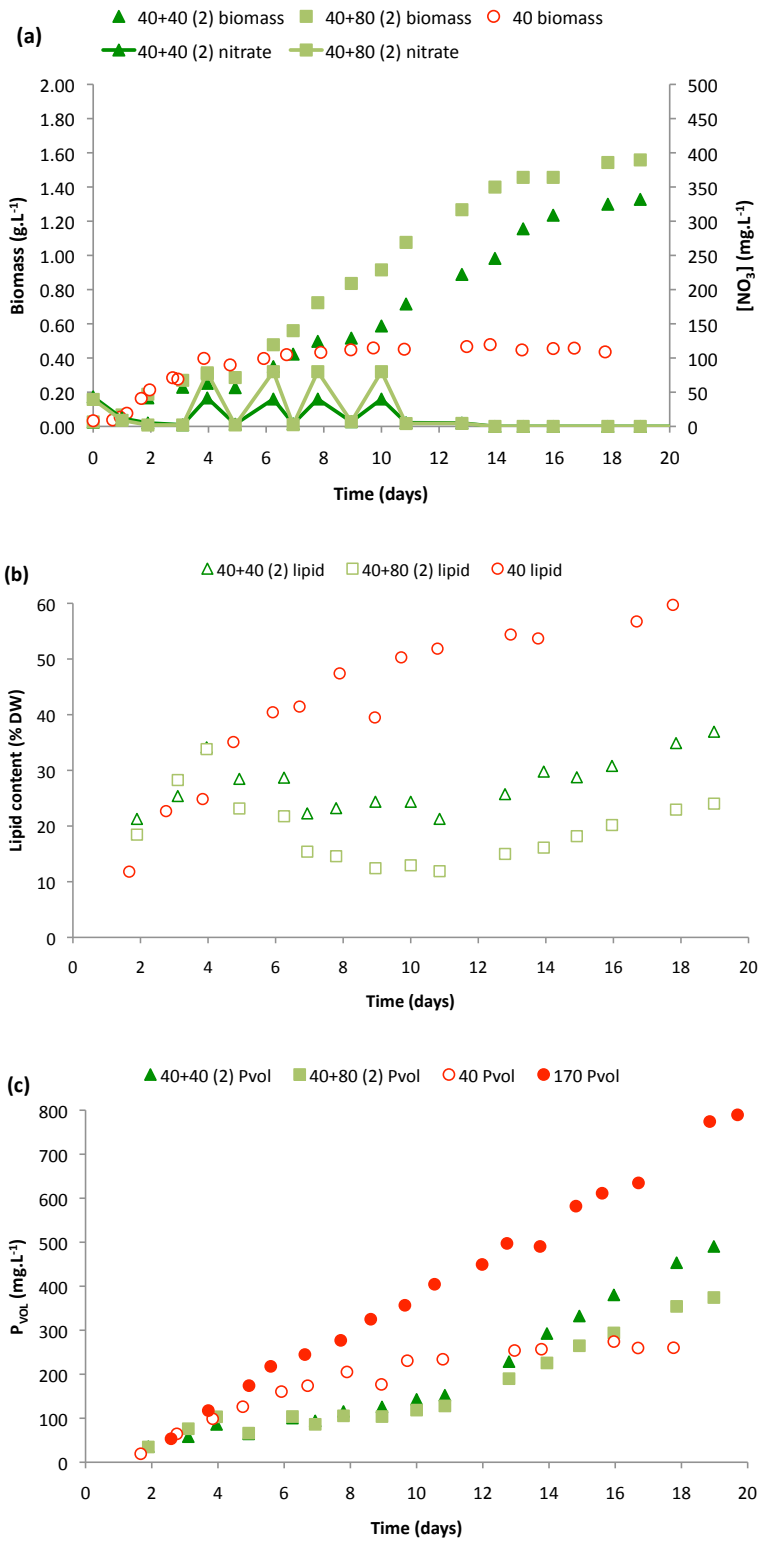
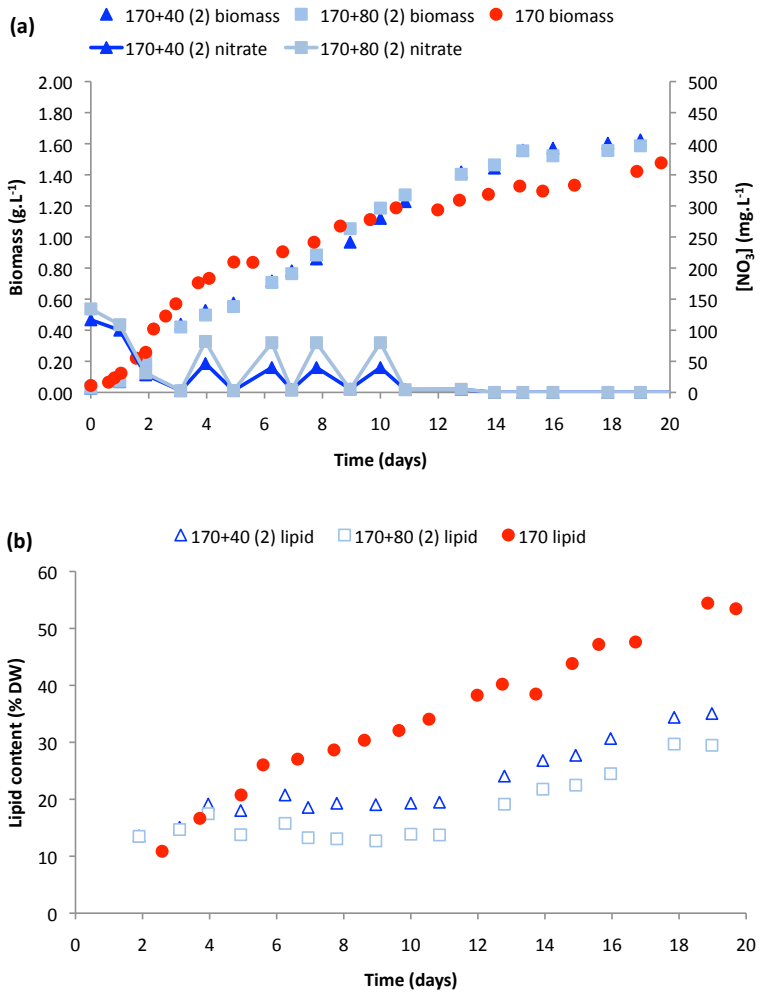


Figure 8.6 (a) biomass (scatter plot) and media nitrate concentration (line graph), (b) lipid content and (c) volumetric lipid content (P_{VOL}) over 20 days in *C. vulgaris* grown under fed batch conditions, with starting nitrate concentration of 40 mg.L^{-1}, fed either 40 (▲, dark green) or 80 mg.L^{-1} (■, light green) nitrate on days 4, 6, 8 and 10. The results of single-stage batch culture with 40 mg.L^{-1} nitrate (○, red) and 170 mg.L^{-1} nitrate (●, red) are shown for comparison

In the second set of experiments, the same feeding regimes were carried out in cultures with a starting nitrate concentration of 170 mg.L^{-1}. Results are compared to batch culture at 170 mg.L^{-1} (red circles in Figure 8.7). Again, the final biomass concentration was higher in fed-batch cultures, although the biomass concentration between days two and eight was lower in fed-batch than batch culture (Figure 8.7a). Feeding with 80 mg.L^{-1} showed no improvement over 40 mg.L^{-1}. Nitrate feeding retarded lipid accumulation, with 80 mg.L^{-1} having a greater effect than 40 mg.L^{-1} (Figure 8.7b). Volumetric lipid productivity in fed-batch cultures was lower than batch, due to the lower lipid content (Figure 8.7c).



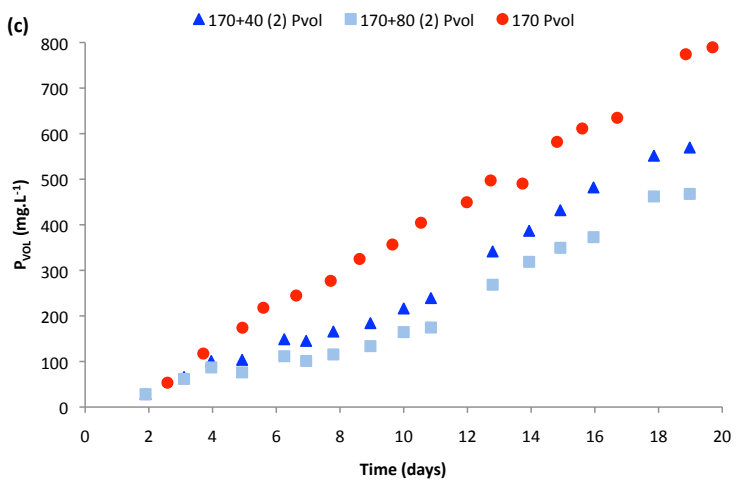


Figure 8.7 (a) biomass (scatter plot) and media nitrate concentration (line graph), (b) lipid content and (c) volumetric lipid content (P_{VOL}) over 20 days in *C. vulgaris* grown under fed batch conditions, with starting nitrate concentration of 170 mg.L^{-1}, fed either 40 (▲, dark blue) or 80 mg.L^{-1} (■, light blue) nitrate on days 4, 6, 8 and 10. The results of single-stage batch culture with 170 mg.L^{-1} nitrate (●, red) are shown for comparison

It was hypothesised that more infrequent feeding may mitigate the decrease in lipid content, while still enhancing biomass concentration. In order to test this, cultures with starting nitrate concentrations of 40 and 170 mg.L^{-1} were fed with 40 mg.L^{-1} nitrate either every four days, or every eight days over a 20-day period. With more infrequent feeding, biomass concentration increased more slowly (Figure 8.8a). The culture fed every four days reached the same final biomass concentration as that fed every two days. This is to be expected as both cultures received the same total amount of nitrogen (200 mg.L^{-1}). The culture fed every eight days received less nitrogen in total and had a lower biomass concentration throughout. An increase in growth rate can be seen after feeding (days 8 and 16) in the culture fed every 8 days.

Lipid accumulation was again retarded by nitrate feeding, but to a lesser degree the further apart the feedings (Figure 8.8b). In the culture fed every four days, lipid content remained constant (between 34 and 38% DW) throughout the period of nitrate feeding. In the culture fed every eight days, lipid content decreased for four days after feeding at day eight, but then increased again to the same level as the batch culture. Upon feeding at day 16, lipid content dropped again, as biomass concentration increased. Volumetric lipid productivity was greater in the culture fed every four days (621 mg.L^{-1}) than that fed every eight days (574 mg.L^{-1}) or two days (490 mg.L^{-1}), but still less than that achieved in batch culture at a starting nitrate concentration of 170 mg.L^{-1} (Figure 8.8c).

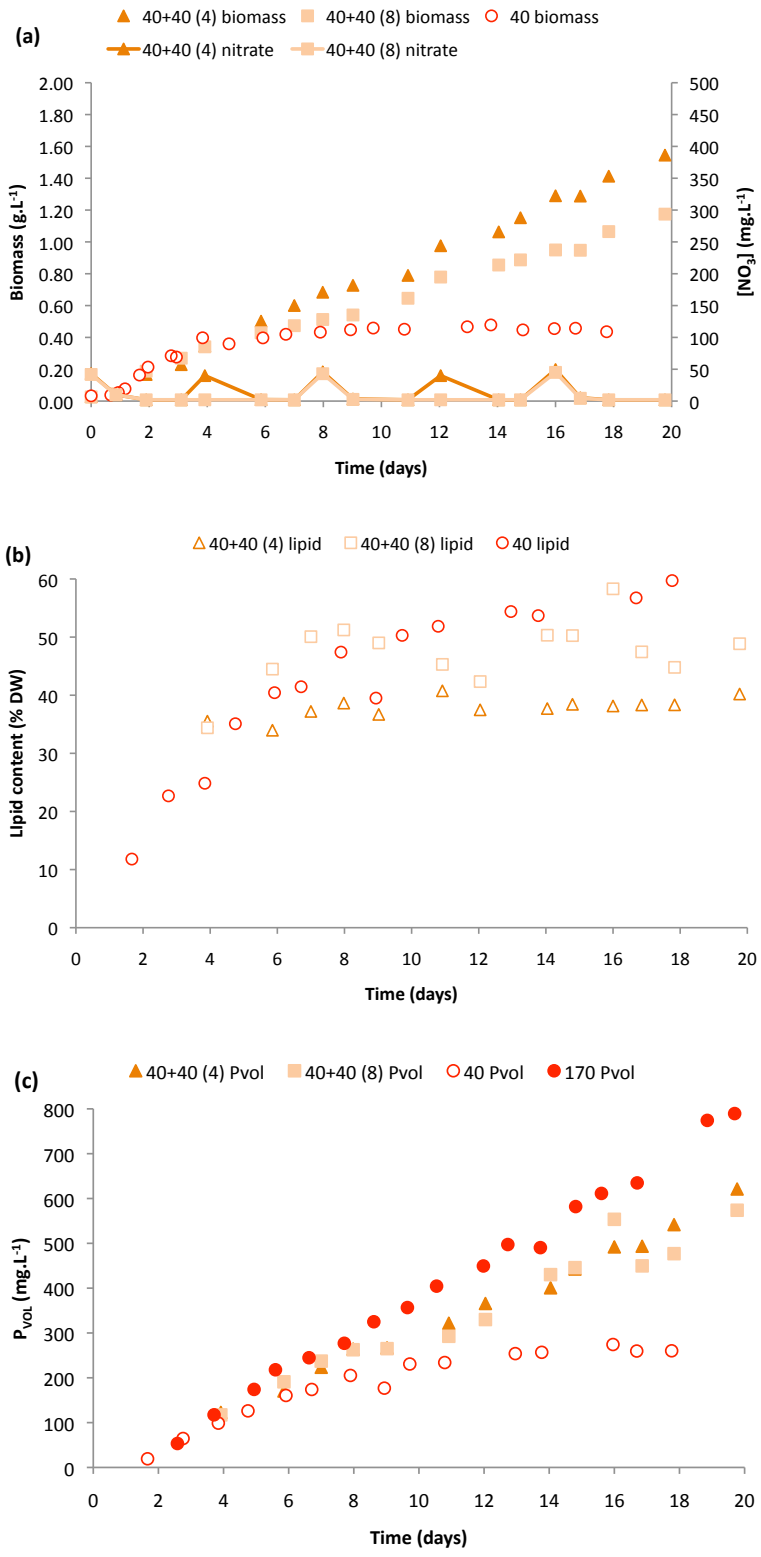
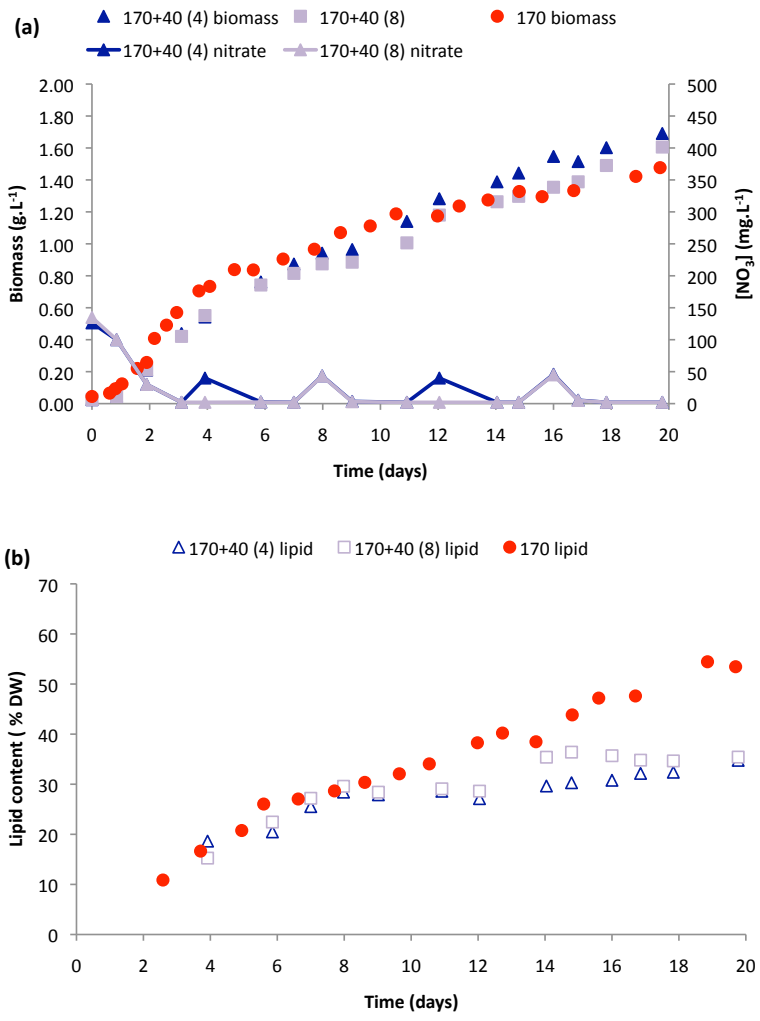


Figure 8.8 (a) biomass (scatter plot) and media nitrate concentration (line graph), (b) lipid content and (c) volumetric lipid content (P_{VOL}) over 20 days in *C. vulgaris* grown under fed batch conditions, with starting nitrate concentration of 40 mg.L^{-1}, fed 40 mg.L^{-1} nitrate every four (▲, dark orange) or eight (■, light orange) days. The results of single-stage batch culture with 40 mg.L^{-1} nitrate (○, red) and 170 mg.L^{-1} nitrate (●, red) are shown for comparison

The same feeding regimes were repeated with a starting nitrate concentration of 170 mg.L^{-1}. In these cultures, feeding with 40 mg.L^{-1} nitrate every four or eight days did not have a significant effect on biomass concentration (maximum biomass concentration was 1.7 and 1.6 g.L^{-1} in the cultures fed every four and eight days respectively, as opposed to 1.5 g.L^{-1} in the normal batch culture) (Figure 8.9a). Lipid accumulation, however, was retarded and remained relatively constant (between 19 and 31 % DW) over the period of feeding (Figure 8.9b). In the culture fed every eight days, lipid content increased between feeds, but to a lesser extent than in the 40 mg.L^{-1} starting nitrate culture (Figure 8.8b). As a result of the lower lipid content, the volumetric lipid content of the fed-batch cultures was lower than that of the batch culture (Figure 8.9c).



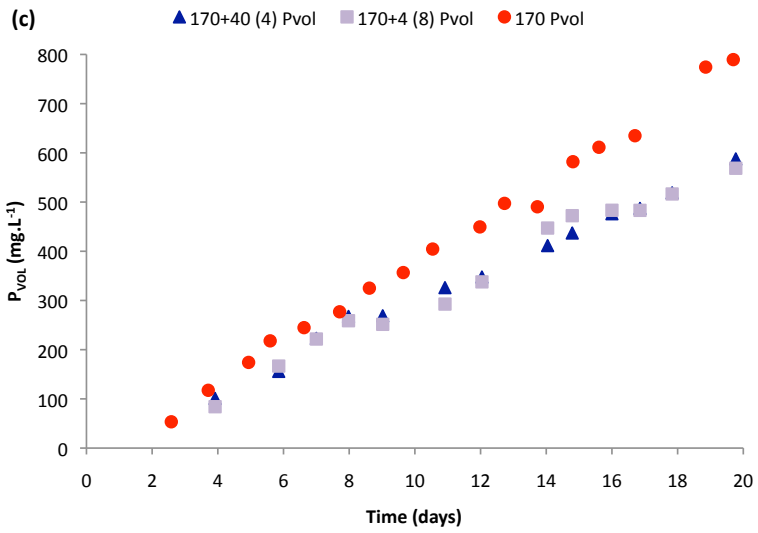


Figure 8.9 (a) biomass (scatter plot) and media nitrate concentration (line graph), (b) lipid content and (c) volumetric lipid content (P_{VOL}) over 20 days in *C. vulgaris* grown under fed batch conditions, with starting nitrate concentration of 170 mg.L^{-1}, fed 40 mg.L^{-1} nitrate every four (▲, dark purple) or eight (■, light purple) days. The results of single-stage batch culture with 170 mg.L^{-1} nitrate (●, red) are shown for comparison

The results of the fed-batch experiments are summarised in Table 8.3. Of the different feeding regimes tested, feeding of 40 mg.L^{-1} nitrate every 4 days produced the greatest volumetric lipid content and average lipid productivities with both starting nitrate concentrations. With a starting nitrate concentration of 40 mg.L^{-1}, nitrate feeding enhanced P_{VOL} and $Q_{P\ AVE}$ above that in batch culture, but not above that of the best batch culture (starting at 170 mg.L^{-1}). The mass of nitrate used per gram of lipid produced varied from 0.15 in the 40 mg.L^{-1} nitrate batch culture to 1 g.g^{-1} lipid in the 170 mg.L^{-1} fed-batch culture fed 80 mg.L^{-1} nitrate every two days. From the perspective of minimising the nitrate used in the process, the less frequent the feeding, the better.

At a starting nitrate concentration of 170 mg.L^{-1}, batch culture still outperformed any of the fed-batch strategies tested. This is in agreement with Hsieh and Wu (2009) who found that a single feeding of urea could enhance lipid productivity, but not above the maximum found in batch culture. They went on to show that a strategy of semi-continuous cultivation, where culture was removed and urea added every day from days 3 to 7, produced a higher lipid productivity than batch or fed-batch. The removal of culture may have facilitated light penetration, and hence lipid accumulation, by reducing cell density. It was shown in Section 8.4.2 that culture dilution led to higher rates of lipid accumulation, however, lipid productivity was not improved over the undiluted culture due to lower biomass concentrations. The combination of dilution by harvesting (to allow greater light penetration and encourage lipid accumulation) with feeding of small amounts of N (to allow cell growth) may be a promising strategy for further investigation.

Table 8.3 Summary of maximum biomass concentration (X), lipid content (P), volumetric lipid content (P_{VOL}), average lipid productivity ($Q_{P\ AVE}$) and mass of nitrate used per g lipid produced in fed-batch and batch experiments with starting nitrate concentrations of 40 and 170 mg.L^{-1}

Starting [NO_3] (mg.L^{-1})	Feeding regime (mg.L^{-1} NO_3)	Max X (g.L^{-1})	Max P (% DW)	Max P_{VOL} (mg.L^{-1})	Max $Q_{P\ AVE}$ (mg.L^{-1}.day^{-1})	NO_3 used (g.g^{-1} lipid)
40	none	0.5	60	274	27	0.15
40	40 every 2 days	1.3	37	490	26	0.41
40	80 every 2 days	1.6	34	374	26	0.96
40	40 every 4 days	1.5	41	621	33	0.32
40	40 every 8 days	1.2	58	574	29	0.21
170	none	1.5	54	790	41	0.22
170	40 every 2 days	1.6	35	570	31	0.58
170	80 every 2 days	1.6	30	468	26	1.05
170	40 every 4 days	1.7	35	588	34	0.56
170	40 every 8 days	1.6	36	569	32	0.44

8.4.4 Continuous culture

Instead of a batch reactor, a continuous reactor could be used as the first stage of two-stage culture. This would provide a continuous, uniform supply of N replete biomass and eliminate the labour and turnaround time associated with batch reactors. An initial study on the productivity achievable in a continuous reactor was conducted. Growth kinetics from N replete batch culture (starting nitrate concentration 1500 mg.L^{-1}) were used to estimate the dilution rate (D) for a continuous culture. The maximum instantaneous biomass productivity ($Q_{X\ INST}$, 0.27 g.L^{-1}.day^{-1}) occurred early in batch growth, between days two and three, when the biomass concentration (X) was relatively low (0.5 to 0.6 g.L^{-1}) (Figure 8.10). The corresponding specific growth rate (μ), and hence dilution rate, at this point was approximately 0.75 day^{-1}.

Although the initial production stage should be designed to produce biomass as fast as possible, the biomass concentration entering the second stage is also important as this will determine the final lipid yield, the volume of the culture vessel required and the cost of harvesting. Hence a dilution rate that would optimise the compromise between a high $Q_{X\ INST}$ and a high biomass concentration was sought. The biomass concentration and instantaneous and average biomass productivity of the same N replete batch reactor were plotted as a function of μ (equivalent to D) (Figure 8.11). A dilution rate of 0.3 day^{-1}, corresponding to a biomass concentration of 0.8 g.L^{-1} and a $Q_{X\ INST}$ of 0.2 g.L^{-1}.day^{-1}, was chosen.

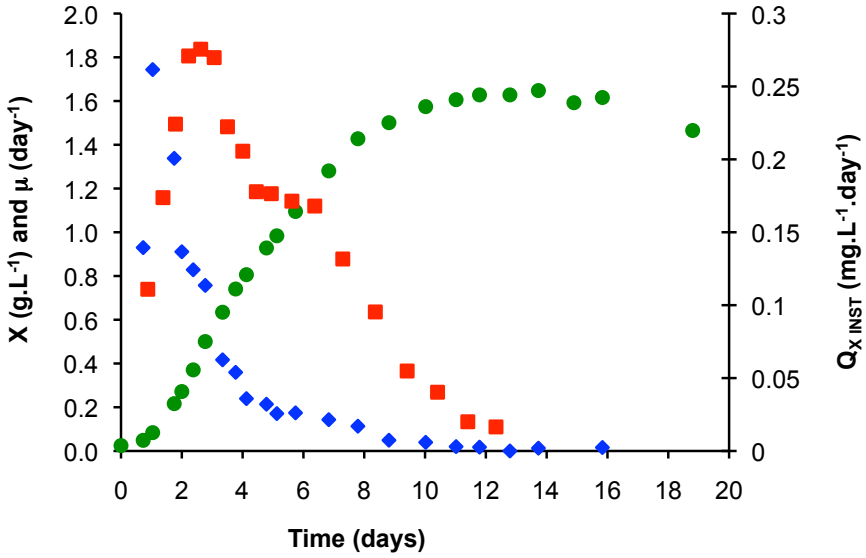


Figure 8.10 Biomass concentration (X, ●, green), specific growth rate (μ, ◆, blue) and biomass productivity ($Q_{X\ INST}$, ■, red) with time in a batch culture of *C. vulgaris* with a nitrate concentration of 1500 mg.L^{-1}

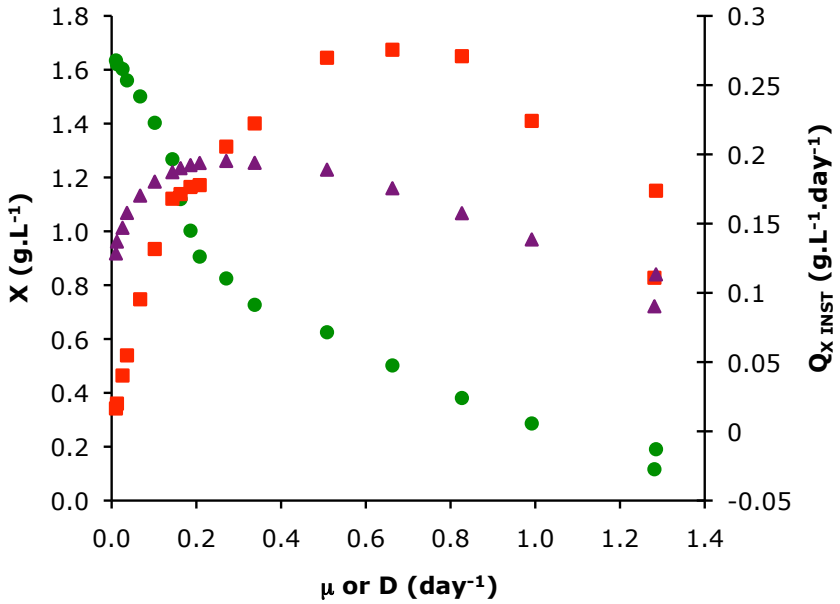


Figure 8.11 Biomass concentration (X, ●, green), instantaneous biomass productivity ($Q_{X\ INST}$, ■, red) and average biomass productivity ($Q_{X\ AVE}$, ▲, purple) as a function of specific growth rate (equivalent to the dilution rate in chemostat culture) in a batch culture of *C. vulgaris* with a nitrate concentration of 1500 mg.L^{-1}

Three identical continuous reactors were maintained at a dilution rate of 0.3 day^{-1} with a feed nitrate concentration of 300 mg.L^{-1}. An example of the biomass and residual nitrate concentration over time is shown in Figure 8.12. The biomass concentration reached a steady

state of between 0.3 and 0.5 $g.L^{-1}$ in the different reactors. The feed nitrate concentration was calculated to provide excess N, resulting in a residual nitrate concentration of between 50 and 70 $mg.L^{-1}$, indicating N sufficiency. In order to test for C limitation, the CO_2 concentration in the gas sparged to one of the reactors was doubled. There was no change in biomass concentration, indicating that cultures were C replete. The only limiting input in these cultures was therefore light. Cultures were maintained for up to 2 months before significant wall-growth occurred, blocking light to the cells and biomass concentration dropped. $Q_{X\ INST}$ was between 0.09 and 0.16 $g.L^{-1}.day^{-1}$ for a biomass concentration of 0.3 and 0.5 $g.L^{-1}$ respectively. The average lipid content in the steady-state, N replete continuous cultures was 12% DW. This translates to a $Q_{P\ INST}$ of between 11 and 18 $mg.L^{-1}.day^{-1}$.

The X and $Q_{X\ INST}$ measured in continuous culture were lower than that predicted from batch culture data. Predictions of steady-state X and Q_X from batch culture data may not be accurate owing to the dynamic conditions within the batch reactor. The X achieved in batch culture is a result not only of the growth rate at that point, but also the accumulated biomass from prior growth. Hence, the X at steady-state in continuous culture was lower than predicted. The lipid content was equivalent to that in N replete batch culture.

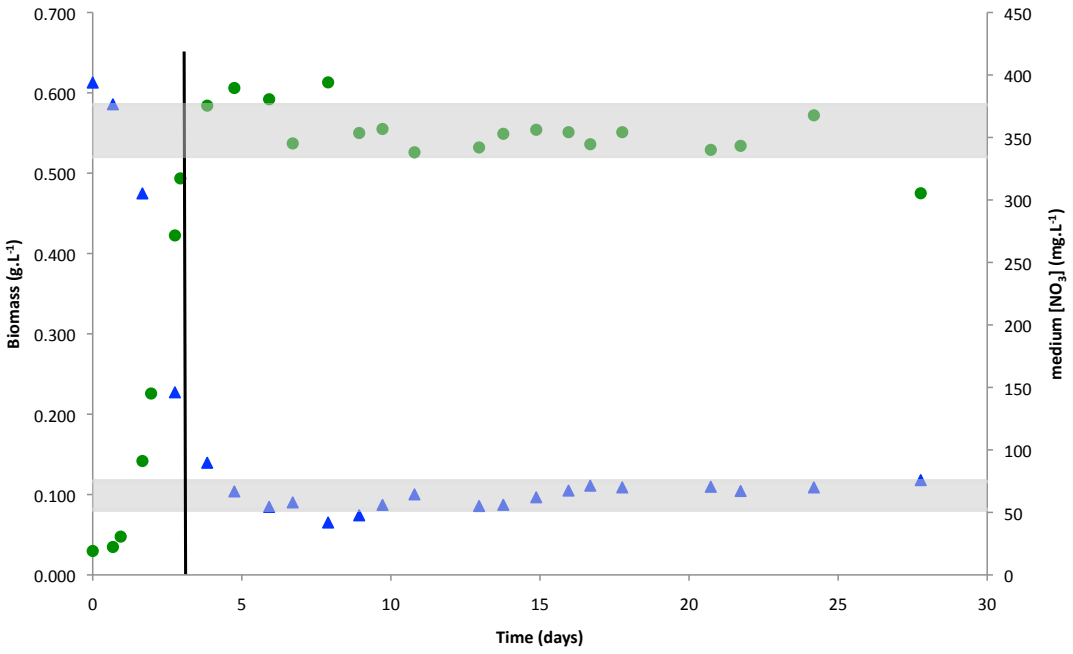


Figure 8.12 Biomass (●, green) and residual nitrate concentration (▲, blue) in a continuous culture of *C. vulgaris* maintained at a D of 0.3 day^{-1} and feed nitrate concentration of 300 $mg.L^{-1}$. Vertical line indicates start of continuous culture. Shaded areas indicate one standard deviation either side of the average biomass and nitrate concentration from the start of continuous culture.

8.4.5 Two-stage continuous-batch culture

Outflow from the steady-state continuous reactors described above was collected and used to fill batch airlift reactors. Culture and medium was transferred either undiluted (CU), or diluted 1:2 (CD) by adding an equal volume of fresh nitrate-free media. In order to mimic transfer from a continuous reactor to a raceway pond, a 2 L flask was also filled to a working volume of 1 L with undiluted continuous culture. The flask (CR) was continuously stirred using a magnetic stirrer, but unsparged, and illuminated with 2 fluorescent light bulbs at 160 $\mu mol.m^{-2}.s^{-1}$. The effluent from the continuous reactors had a residual nitrate concentration of between 50 and 70 $mg.L^{-1}$. This was consumed by the second stage batch cultures within 12 hours, resulting in N limitation.

The results of two-stage continuous-batch culture (Figure 8.13) were very similar to those of two-stage batch culture (Figure 8.5). The culture produced by the continuous reactors essentially mimicked that of a 3-day old N-replete batch culture (the time point with an equivalent biomass concentration of 0.8 $g.L^{-1}$). Lipid accumulation in CU and CD was initially more rapid than the diluted two-stage batch cultures. This could be because cells were transferred to N free medium at a lower biomass concentration (0.6 and 0.3 $g.L^{-1}$ as opposed to 1.1 and 0.7 $g.L^{-1}$ in the 1:1 and 1:2 dilutions of the continuous and batch cultures respectively). The diluted continuous culture accumulated lipid more rapidly and reached a higher lipid content than the undiluted culture, confirming the results from the diluted batch culture. Again, due to a lower biomass concentration, the volumetric lipid content (Figure 8.13c) and overall lipid productivity in the diluted culture was equivalent to that of the undiluted culture, and both were lower than the batch culture with 170 $mg.L^{-1}$ initial nitrate concentration.

The 2L flask culture (CR), designed to mimic transfer to a raceway pond, showed an initial increase in biomass concentration, whereafter the growth rate quickly declined to zero. This was probably due to carbon limitation in the non-sparged 2 L flask. Light conditions were also lower than in the airlift reactors. The lipid content of the CR culture remained below 10% DW until day 16. Due to the limited growth after transfer to N free medium, it was likely that cells retained intracellular nitrogen reserves and hence did not experience N limitation to the same degree as the airlift reactor cultures. Lipid content rose gradually to 16% DW at day 20 and may have reached similar levels to the airlift reactors given sufficient time. However, the productivity was much lower due to the response time and the low biomass concentration.

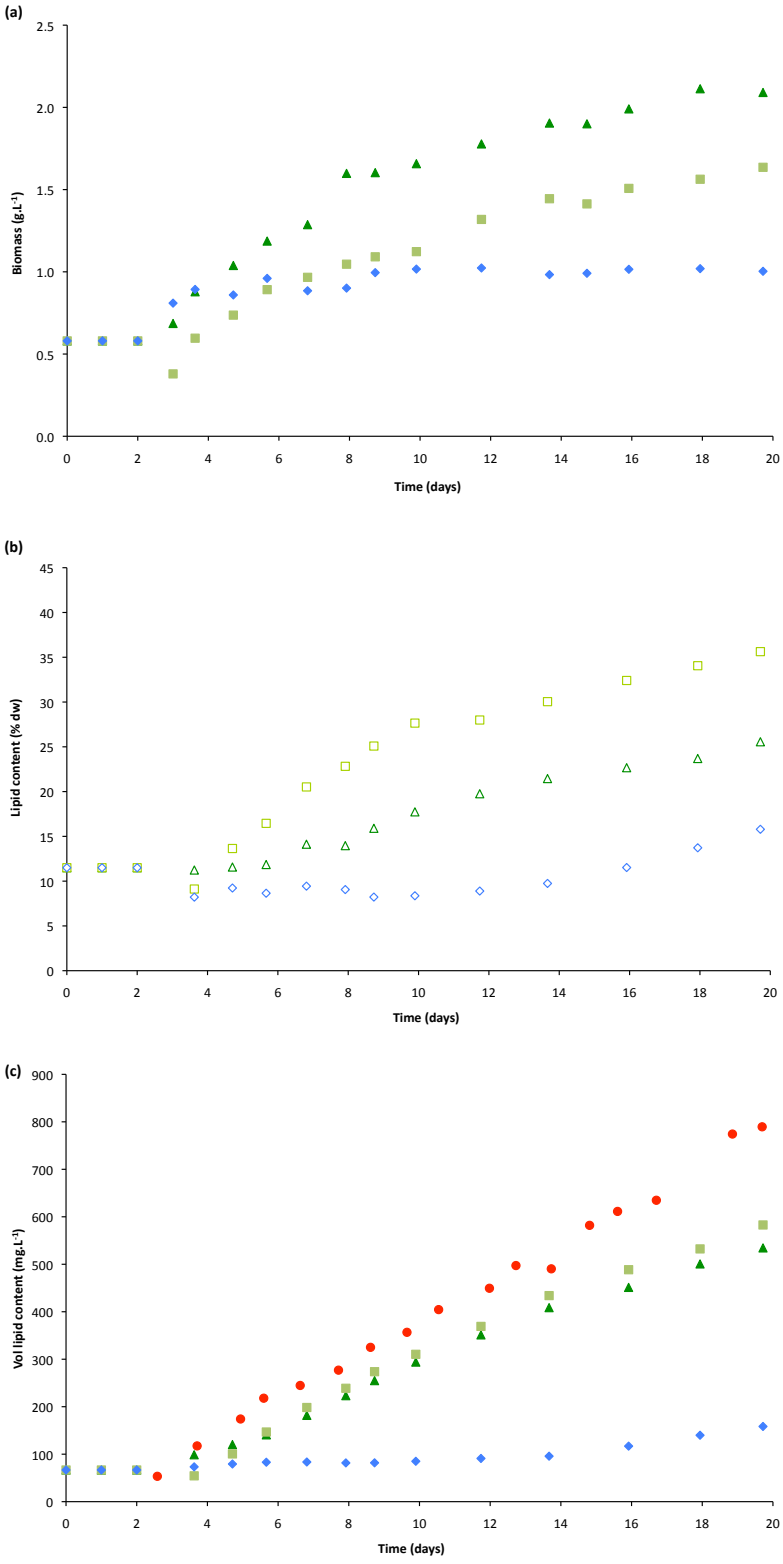


Figure 8.13 (a) biomass concentration, (b) lipid content and (c) volumetric lipid content with time, in cultures transferred from N replete continuous culture to N limited batch culture, either in airlift reactors, undiluted (CU, ▲, dark green) or diluted 1:2 (CD, ■, light green), or in a 2 L flask mimicking raceway conditions (CR, ◆, blue). Average values for the steady state continuous culture are shown for the first 3 days. The results of single-stage batch culture with 170 mg.L^{-1} nitrate (●, red) are shown for comparison.

8.4.6 Multi-stage continuous culture

A set of four continuous reactors in series was set up as shown in Figure 8.1. The first reactor (C1) was supplied with fresh, aseptic, N replete feed at a D of 0.3 day^{-1}, as described for the continuous reactors above. The subsequent three reactors (C2, C3 and C4) were set up in series after C1, each receiving, as feed, the outflow from the reactor before them. The first reactor was the N replete growth stage, while the subsequent three reactors formed the N limited lipid accumulation stage. The effluent from C1 had a residual nitrate concentration of between 50 and 70 $mg.L^{-1}$, and the effluent from C2 contained no nitrate.

The kinetic parameters of each of the reactors in series are shown in Table 8.4. Evaporation was compensated for in the feed rate to the first reactor. However, due to the level-control method of controlling effluent flow rate, this could not be done in the subsequent reactors and hence the dilution rate and residence time were different in each reactor. The effective flow rate takes into account the volume lost to evaporation (approximately 100 ml per day per reactor). Biomass concentration (X) and lipid content (P) were measured in each reactor once steady state was reached (approximately 14 days from the time of filling of the final reactor). Biomass productivity (Q_X) was calculated as the product of X formed in the reactor (i.e. for reactor C2, X_{C2} - X_{C1}) and D. Instantaneous lipid productivity ($Q_{P\ INST}$) was calculated as the product of Q_X and P, volumetric lipid productivity (P_{VOL}) as the product of X and P, and $Q_{P\ AVE}$ as P_{VOL} divided by the residence time (τ) of the reactor, plus the τ of any previous reactors.

Table 8.4 Parameters of each of the four continuous reactors in series

	C1	C2	C3	C4
Actual F ($L.day^{-1}$)	1.1	1	0.9	0.8
Effective F ($L.day^{-1}$)	1	0.9	0.8	0.7
D (day^{-1})	0.31	0.28	0.25	0.22
V (L)	3.2	3.2	3.2	3.2
τ (days)	3.2	3.6	4.0	4.6
X ($g.L^{-1}$)	0.31	1.10	1.52	1.83
Q_X ($g.L^{-1}.day^{-1}$)	0.10	0.22	0.11	0.07
P (% DW)	14	15	20	23
P_{VOL} ($mg.L^{-1}$)	44	165	297	411
$Q_{P\ INST}$ ($mg.L^{-1}.day^{-1}$)	14	33	21	15
$Q_{P\ AVE}$ ($mg.L^{-1}.day^{-1}$)	14	24	28	27

The total residence time of the four reactors in series was 15 days. Biomass concentration increased in subsequent reactors, with the largest increase occurring in the second reactor. The nitrate concentration in C2 was below the detection limit, therefore the increase in biomass was due to growth of the entering biomass on internal N stores. Lipid content also increased in the subsequent reactors, from 14% DW in C1 to 23% DW in C4. C4 had both the highest biomass concentration and lipid content and therefore the highest volumetric lipid content. Instantaneous lipid productivity ($Q_{P\ INST}$) was highest in the second reactor due to the high biomass productivity, but average lipid productivity was greatest in the third reactor, due to the higher volumetric lipid content.

The culture in subsequent reactors has the characteristics of subsequent portions of a batch growth cycle. The culture in C4 has similar characteristics to that of an undiluted two-stage batch reactor between days 11 and 15 (Figure 8.5), except the biomass concentration was slightly higher (1.9 to 2 $g.L^{-1}$) and the lipid content slightly lower (16 to 21% DW) in the two-stage batch culture. If a fifth reactor had been added, extending the total residence time to 20 days, the effluent may have reached the volumetric lipid content of two-stage batch or continuous-batch culture.

8.4.7 Comparison of culture regimes

In order to determine the best nitrogen culture regime for lipid production, the results obtained in the best of each of the different culture strategies were compared on the basis of maximum biomass concentration, lipid content, volumetric lipid content and average lipid productivity (Table 8.5). One of the advantages of using N limitation is that it decreases the process demand for nitrogen. If non-waste sources of N are used, their usage should be minimised in order to decrease the associated cost and environmental burden. As different culture regimes required different N inputs, this should be taken into account in their comparison. The mass of nitrate used per g lipid produced was therefore calculated for each culture regime (Table 8.5).

The most appropriate parameter to use in comparing different culture strategies is the rate of lipid produced per volume of culture (lipid productivity). The best culture strategy, in terms of lipid productivity, as well as lipid yield and N used, was N limited batch culture with a starting nitrate concentration of 170 $mg.L^{-1}$ ($Q_{P\ AVE}$ 41 $mg.L^{-1}.day^{-1}$). The second best strategy was fed-batch with feeding of 40 $mg.L^{-1}$ every four days ($Q_{P\ AVE}$ 33 and 34 $mg.L^{-1}.day^{-1}$ for starting nitrate concentrations of 40 and 170 $mg.L^{-1}$ respectively). The only parameter in which these cultures performed poorly was in the final biomass concentration. The final X reached (1.5 $g.L^{-1}$) was approximately two thirds of that achieved in the N replete or two-stage cultures (2.2 to 2.4 $g.L^{-1}$). If final biomass concentration is determined to be a significant factor in the cost of harvesting and therefore the cost of the overall process, and offsets the savings in terms of N used, then two-stage culture (using either batch or continuous for the first stage) could be the best option.

Table 8.5 Comparison of different culture regimes

	Max X ($g.L^{-1}$)	Max P (% DW)	Max P_{VOL} ($mg.L^{-1}$)	Max $Q_{P\ AVE}$ ($mg.L^{-1}.day^{-1}$)	NO_3 used ($g.g^{-1}$ lipid)
Batch – N replete (starting NO_3 1200 $mg.L^{-1}$)	2.4	12	281	25	4.27
Batch – N limited (starting NO_3 170 $mg.L^{-1}$)	1.5	54	790	41	0.22
Two-stage batch Undiluted	2.3	25	565	30	0.74
Two-stage batch Diluted 1:2	1.8	33	555	30	0.38
Fed-batch – fed 40 $mg.L^{-1}$ every 4 days (starting NO_3 40 $mg.L^{-1}$)	1.5	41	621	33	0.32
Fed-batch – fed 40 $mg.L^{-1}$ every 4 days (starting NO_3 170 $mg.L^{-1}$)	1.7	35	588	34	0.56
Two-stage continuous-batch	2.2	26	561	29	1.71
Multi-stage continuous	2.3	18	406	28	2.36

It is interesting to note that, although none of the two-stage cultivation strategies improved on the lipid productivity over the nitrogen limited batch culture, they were all better than N replete batch culture, which was the worst strategy in every parameter except biomass concentration.

8.5 Conclusion

This chapter compared the effect of various nitrogen culture regimes on lipid productivity in *C. vulgaris*. It was hypothesised that a N feeding strategy designed to optimise biomass and lipid production in different stages would improve lipid productivity over that of N limited batch culture. However, none of the culture regimes tested improved on the lipid productivity, lipid yield and efficiency of nitrogen use of N limited batch culture (Chapter 7).

Of the other cultivation strategies, fed-batch culture was the most promising. N feeding successfully increased the rate of biomass formation due to the removal of N limitation, but this was always accompanied by a decrease in lipid content as cells utilised the stored carbon and energy for growth. Feeding of small amounts of nitrogen (40 $mg.L^{-1}$ nitrate) relatively infrequently (every four to eight days) was found to be the best strategy for minimising loss of lipid content while increasing biomass concentration.

Two-stage batch culture did not lead to the expected increase in lipid productivity, due to the delay between the onset of nitrogen limitation and lipid accumulation. Upon transfer from N replete to N limited medium at day 5, cells continued to grow and accumulated minimal lipid for

up to a further eight days. Cultures achieved significant biomass growth in the absence of any external nitrogen supply. This is postulated to be due to cells maintaining growth on intracellular nitrogen reserves. The nitrate concentration during the N replete growth phase had no effect on the N uptake rate or subsequent performance of the culture and therefore did not affect the magnitude of intracellular N reserves.

In Chapter 7, it was demonstrated that once internal N reserves were exhausted, the rate of lipid accumulation was slower in cultures with a higher cell concentration. This was postulated to be due to light limitation in dense cultures. Diluting two-stage cultures at the point of transfer to N free medium showed that cultures at a lower cell density had higher rates of lipid accumulation. This confirms that lipid production is light limited in dense cultures. Although diluted cultures accumulated lipid faster per unit biomass, they achieved similar volumetric lipid concentration and lipid productivity as undiluted cultures due a lower biomass concentraton.

Multi-stage continuous culture of *C. vulgaris* was maintained successfully for several weeks. The results of both continuous-batch and multi-stage continuous culture were similar to those of two-stage batch culture, although greater amounts of N were required per unit lipid produced. When continuous culture was transferred to a 2 L volumetric flask (designed to mimic a raceway pond) for the second, N limited batch phase, biomass and lipid accumulation were both very low. This was likely due to carbon limitation in the unsparged flask. All strategies of N limitation enhanced lipid content over N replete culture. However, the results of the 2 L flask and the similarity of the best results across two-stage nitrogen culture regimes indicate that N limited lipid productivity is limited by the carbon and energy (light) supply to the reactor. In the flask, carbon and light were co-limiting. As the CO_2 supply to the airlift reactors was known to be non-limiting, lipid accumulation was limited by light provision.

N limited batch growth at a starting nitrate concentration of 170 $mg.L^{-1}$ achieved the highest lipid productivity as N became depleted early in the growth cycle, when the culture was still relatively dilute. This allowed high light penetration and hence rapid lipid accumulation. The fact that lipid accumulation began immediately upon N limitation indicates that internal N stores were minimal during this phase. Rapid cell division during exponential growth may have diluted internal N supplies between daughter cells. It is also postulated that metabolically active cells may have been able to modify their cellular machinery (e.g. changing the amount of key enzymes or manufacturing lipid storage vesicles) more rapidly than cells that became N limited in early stationary phase.

9 Conclusions

9.1 Method development

The first part of this thesis addressed the development of accurate quantification methods for biomass concentration and lipid content in microalgae – both critical in the measurement of lipid productivity. Chapter 3 concluded that, while optical density (OD) was a convenient indirect measure of biomass concentration, in pigmented cells such as microalgae, variation in pigment content due to culture age or growth conditions could lead to significant error in the estimation of dry weight (DW) from OD. Researchers using OD to measure biomass in pigmented cells should be aware of the potential magnitude and sources of inaccuracy. Errors were greater using a wavelength within the range of maximal absorbance by the major pigments, and using standard curves generated at a time point or culture condition of different pigment content. It is recommended that a wavelength outside the range of absorbance by the pigments (e.g. 750 nm) be used, and that the generation of standard curves be considered carefully. For batch cultures, it may be necessary to generate different standard curves, applicable at different points in the growth cycle, or to generate a standard curve across the entire growth cycle.

In Chapter 4, the method of direct transesterification (DT) was investigated rigorously as an alternative to lipid quantification by extraction and transesterification. DT proved convenient and more accurate than any of the extraction techniques for quantifying total fatty acid content in microalgae. Fatty acids in the cells were more completely quantified with DT in comparison with the three extraction-transesterification methods tested. The fact that DT on the residue from the extractions yielded further fatty acid confirmed that the extraction methods were incomplete.

A combination of acidic and basic transesterification catalysts was found to be more effective than each catalyst individually when the sample contained water. The two-catalyst reaction was insensitive to water up to 10% of total reaction volume. This meant that centrifuged algal samples could be assayed directly without the need for drying of the biomass. Drying of samples was shown to reduce measured lipid content. Storage of samples for up to seven days gave results within the experimental error of the assay, while longer storage, regardless of temperature, showed slightly lower values for lipid content.

9.2 Species selection

Part II of the thesis addressed the issue of choice of microalgal species for biodiesel production. An initial paper study (Chapter 5) was followed by further experimental work on some of the most promising species identified (Chapter 6). A review of the literature indicated that the only characteristics for which there were sufficient data to allow comparison across a broad range of

species were growth rate and lipid content. This information was collated from a range of studies that focused on many areas of algal research.

It was hypothesised that lipid productivity was a critical parameter for comparison across species, and this was shown to be correct. Neither biomass productivity nor lipid content alone is a good indicator of lipid productivity. For example, stress conditions such as nutrient limitation are known to enhance lipid content, but do not necessarily improve lipid productivity, either due to the lower biomass concentration achieved, or the increased time required for successive growth and lipid accumulation periods. Lipid productivity was infrequently reported in the literature, but could be calculated from biomass productivity or growth rate and lipid content, given two assumptions allowing unit conversion. These were 1) an assumed culture depth in the conversion of areal biomass productivities in $g.m^{-2}.day^{-1}$ to to volumetric biomass productivities in $g.L^{-1}.day^{-1}$, and 2) an assumed biomass concentration for the conversion of specific growth rate or doubling time to biomass productivity.

Lipid productivities were calculated for the 55 species under nutrient replete conditions. The following species were found to have relatively high lipid productivities (110 to 164 $mg.L^{-1}.day^{-1}$): *Neochloris oleoabundans*, *Chlorella sorokiniana, Navicula pelliculosa*, *Amphora* and *Cylindrotheca*. The ranking of these species according to their calculated lipid productivity should be regarded with caution. In addition to the two assumptions necessary for the calculations, the data from which they were calculated were collected under a variety of conditions and degrees of culture optimization. The literature clearly indicated that lipid content could be enhanced by nitrogen limitation, however, growth rates under these conditions were not often reported, hence lipid productivity under nutrient deprivation could not be calculated.

In order to validate these lipid productivities, and add data on the productivities achievable under nitrogen limitation as well as additional species characteristics relevant to biodiesel production, eleven promising species were tested experimentally. Cultures of the freshwater *Ankistrodesmus falcatus*, *Chlorella vulgaris*, *Neochloris oleoabundans* and *Scenedesmus*, the marine *Cylindrotheca fusiformis*, *Isochrysis*, *Nannochloropsis*, *Pavlova, Phaeodactylum tricornutum* and *Tetraselmis suecica*, and the halotolerant Cyanobacteria *Spirulina platensis* were grown under both N replete (1500 $mg.L^{-1}$ nitrate) and limited (150 $mg.L^{-1}$ nitrate) conditions. In all species tested, except *S. platensis*, overall lipid productivity was found to be enhanced by nitrogen limitation. The decrease in growth rate was offset by the increase in lipid content to give a higher lipid yield throughout the growth cycle. This validates the hypothesis that nitrogen limited culture would produce greater overall lipid productivity than nitrogen replete culture. The cultures with the highest lipid productivity were the freshwater *Scenedesmus*, followed by *C. vulgaris*, both under N limited conditions. The marine species with the highest lipid productivity were *Nannochloropsis* under N limited conditons and *C. fusiformis* in which productivity was equivalent under N replete and N limited conditions.

Additional characteristics investigated were the potential of the species for harvesting by gravity sedimentation, as well as the suitability of their fatty acid profile for biodiesel production. Species which settled readily in response to gravity included *S. platensis*, *C. fusiformis*, *T. suecica* and *Scenedesmus*. The fatty acid profile of each species was used to estimate the properties of the biodiesel fuel it would produce. The characteristics were evaluated according to the specifications of the European standard for biodiesel (EN 14214). Seven of the 22 cultures had a cetane number below specification, including all of the freshwater algae under N replete conditions. Several species were found to exceed the limit for the proportion of polyunsaturated fatty acids and linolenic acid content. The cold flow plug point (CFPP) and oxidative stability of fuel produced from the majority of species tested were also relatively poor (five of the cultures were predicted to meet the South African summer standard for CFPP). The fatty acid profile of species changed, and the calculated fuel properties of many of the species improved, when cultivated under N limited conditions. In addition to improving the fatty acid profile by modifying the culture conditions, blending or fuel additives could also be used to overcome these problems. Considering productivity, ease of harvesting by settling, and fatty acid profile, the most promising species overall were the freshwater green algae *Scenedesmus* and *C. vulgaris*. Among the most promising marine species were *C. fusiformis* and *Nannochloropsis*.

9.3 Investigation of nitrogen limitation

In the Part III of the thesis, *C. vulgaris* was used to test the effects of different starting nitrogen levels and different culture strategies on lipid productivity. Chapter 7 presented the results of experiments where identical cultures of *C. vulgaris* were inoculated at nine different starting nitrate concentrations between 0 and 2000 $mg.L^{-1}$. Maximum biomass concentration was reached on using a starting nitrate concentration of 1200 $mg.L^{-1}$, while maximum lipid content was reached with a starting nitrate concentrate of 0 $mg.L^{-1}$. It was hypothesised that a level of N limitation between these two extremes would optimise lipid productivity in batch culture. This was proved correct in that the tradeoff between growth and lipid production led to an optimal lipid productivity at an intermediate starting nitrate concentration of 170 $mg.L^{-1}$. Cultures with a low starting N concentration exhausted the N in the medium during the exponential growth phase and showed a high rate of lipid accumulation. Those with a higher starting N concentration, which became depleted during the growth cycle, showed a delayed and more linear lipid increase. The increased rate of lipid accumulation could have been due to the greater light availability in the more dilute cultures. Protein, pigment and lipid content were found to correlate very closely with N content of the cells. It was proposed that the measurement of any one of them (e.g. pigment content) could be used to predict the others. Optimum lipid productivity was found in cells with a total N content of between 1 and 2% DW.

In Chapter 8 the effect of culture regime on lipid productivity was investigated. The lipid productivity and lipid yield in two-stage batch, fed-batch, two-stage continuous-batch and multi-stage continuous culture were studied and compared to that achieved in batch culture (Chapter 7). It was hypothesised that two-stage culture would lead to greater lipid productivity than batch culture, however, this hypothesis was proved incorrect. N limited batch culture, at a starting nitrate concentration of 170 $mg.L^{-1}$, was found to be the optimal strategy for lipid production. Overall lipid productivity, lipid yield and minimisation of N usage were not improved by using fed-batch, two-stage or multi-stage procedures, although all of them improved upon that of N replete batch culture. The most promising two-stage strategy was fed-batch. Relatively infrequent feeding of low amounts of nitrate were necessary to increase the biomass concentration while preventing loss of lipid content. However, the tradeoff between biomass and lipid production ultimately limited productivity. The productivity of two-stage batch, continuous batch and multi-stage continuous culture were lower than expected due to the delay in cessation of growth and lipid accumulation after transfer to N free medium. This was postulated to be due to the utilisation of intracellular nitrogen and the limitation of lipid accumulation due to insufficient light availability in concentrated algal cultures.

The dilution of cultures to lower cell concentrations when transferred to N limited media led to increased lipid accumulation, confirming that the availability of light influences the rate of lipid accumulation. However, dilution did not lead to increased lipid productivity due to the reduction in biomass concentration caused by the dilution. For high lipid productivity, optimal light and carbon provision is necessary not only for growth, but also for lipid formation. This was demonstrated in the culture transferred to a volumetric flask for the second stage. Biomass and lipid accumulation were low due to carbon and light limitation. This implies that two-stage processes need to provide optimal light and CO_2 mass transfer conditions in both stages.

Overall, nitrogen limitation was shown to enhance lipid productivity up to a point at which the carbon and energy supply become limiting. This point is determined by the characteristics of the algal species and the culture environment. For a given algal species, further improvements in productivity rely on improving the supply of energy and carbon, either through reactor design or metabolic engineering of the algae. Examples include enhancing CO_2 uptake or minimising mutual shading.

9.4 Implications and recommendations

The assays for biomass and lipid quantification developed and evaluated in this thesis have application in a range of different areas. Accurate measurement of biomass concentration and hence growth rate is important in most commercial applications of microalgae. Monitoring of microalgal biomass in the natural environment is also of interest in the determination of primary productivity of the oceans and monitoring of toxic algal blooms.

The optimized two-catalyst DT reaction is applicable to small sample volumes and could be used to measure the lipid content of almost any sample with a water content contributing less than 10% of the total reaction volume. It has already been applied in monitoring the lipid content of concrete from the floor of a dairy factory. This method has facilitated the development of rigorous time profiles of lipid accumulation across the algal growth phase. The major limitation of this method is that, by converting all lipids directly into FAME, lipids can no longer be separated and evaluated as different classes, e.g. polar phospholipids and glycolipids vs. non-polar or neutral triacylglycerols. If a study requires the differentiation of lipid classes, solvent extraction of the tissue may be necessary.

As FAME are the components of biodiesel and transesterification is the major method of biodiesel production, DT has potential large-scale application in the production of biodiesel directly from microalgal cells, or other feedstocks, without the need for oil extraction. Johnson and Wen (2009) showed that a one-step method of DT resulted in a higher FAME yield from *Schizochytrium limacinum* (a *Thraustochytrid*, taxonomically aligned with heterokont microalgae (Lewis *et al.* 1999)) than oil extraction followed by transesterification, and could produce biodiesel where 8 out of 11 parameters met ASTM fuel standards. Eliminating the step of oil extraction could make the process cheaper, but does not eliminate the need for harvesting and dewatering of the biomass. DT would need to be further developed to use cheaper and less toxic transesterification reagents, and allow recycling of the solvents.

This work also highlighted certain microalgal species with promising characteristics for biodiesel production. *Scenedesmus* and *Chlorella* are recommended, particularly if N limitation is used, due to their high lipid content and lipid productivity under N limited conditions, as well as their ease of cultivation. They are both 'weed' algae in the sense that they are robust and resilient, often contaminating other algal cultures and have been grown successfully at large-scale. The marine species *Nannochloropsis* also showed favourable characteristics and has been used in several algal biodiesel pilot studies (Rodolfi *et al.* 2009; Sheehan *et al.* 1998; Sukenik *et al.* 2009). While the traits used to compare species in this study are important, it should be noted that there are several other characteristics likely to affect the success of commercial algal culture, including tolerance of a range of environmental conditions and resistance to predation. No known algal species satisfies all the desirable criteria. It is likely that many other promising species remain undiscovered, and that genetic and metabolic engineering of existing strains may play a role in improving their characteristics.

It is recommended that N limitation be applied to cultures grown for lipid production. There are several advantages to using N limited culture. Not only was N limitation shown to improve lipid productivity, but other advantages over N replete conditions include improved lipid profile for biodiesel production, both in terms of higher TAG content and improved fatty acid profile

(Piorreck *et al.* 1984; Stephenson *et al.* 2010). Nitrogen limited cultures also produce more lipid per mass of nitrogen used. Unless N can be sourced from a waste stream, the use of N fertilizer comes with associated financial costs and, from a lifecycle perspective, the energy used in its manufacture. In order to reduce both the environmental and cost burden, the use of N in the process should be minimized.

There are also potential disadvantages to N limitation. The reduced biomass concentrations reached may increase harvesting cost. Although the yield of lipid is higher, the concomitant decrease in the content of virtually every other cell component would decrease revenue from any co-products. It remains to be seen whether N limited cultures are sufficiently robust, resistant to contamination and resilient to changes in environmental conditions to succeed in large-scale outdoor culture. In the design of the reactor and culture regime, it should be borne in mind that both growth and lipid accumulation require sufficient energy and carbon supply.

Although this work has focused on the production of lipid for biodiesel, it is relevant to the production of lipids and associated products from microalgae for other applications. These include the production of oils as a food or feed or as an ingredient in lubricants, cosmetics or other products. Many algal species are rich in the essential polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These Omega-3 fatty acids are valuable as a food supplement, particularly in infant formula. Enhancing the lipid content of algae also increases their calorific content as a fuel in other energy application, or as feed for livestock or aquaculture (Apt & Behrens 1999; Converti *et al.* 2009).

9.5 Suggestions for future work

Lipid content, growth rate, biomass productivity and lipid productivity are key characteristics for biodiesel production and formed the focus of the species selection componenet of this work. A literature review showed that data on growth rates and lipid contents were available from a diverse array of literature sources. Although this data provided a framework for decision making and informed further experimental work, its utility was limited by the different experimental methods and reporting units used. There is a need for further data generated under comparable growth conditions, reporting of kinetic parameters in standard units, or with sufficient data for their interconversion, reporting of lipid productivities and recording of growth rates under conditions that cause increased lipid content.

The experimental study compared eleven species on growth rate, lipid content, biomass and lipid productivity, sedimentation potential and fatty acid profile. While these are important traits, it should be recognized that strains that performed best in the laboratory, in terms of these parameters, might not thrive in large-scale, outdoor culture due to factors such as contamination and failure to tolerate a wide range of environmental conditions. Traits such as ease of growth

and the cost of oil extraction should also be taken into account. However, insufficient published information currently exists to enable comparison across these aspects of a variety of species. Collection of a rigorous suite of data on promising algal species is thus required.

Determining the resilience of species by measuring the range of environmental conditions (e.g. pH, temperature, nutrient levels, CO_2 levels, light, etc.) within which the algae remains productive, and quantifying the ease of algal cell harvesting (e.g. by flocculation, filtration or sedimentation) of promising species would be valuable additions to the literature. Mata *et al.* (2010) suggest that experiments used to compare species in the laboratory should mimic real cultivation conditions and include the biomass processing stages. This would select for species ideal for that particular location and system. Alternatively, the culture conditions of selected promising species could be optimised individually, to provide a measure of the maximum biological productivity expected. The culture system would then be designed to mimic these conditions.

Lipid productivity can be achieved using nitrogen limitation, however, the overall productivity in this work is still constrained by the availability of light. In order to further improve yield, novel solutions to the optimal provision of light to the cultures are needed. In this work, light availability was increased by diluting the culture. An alternative strategy is to supply more light to the cultures. Rodolfi *et al.* (2009) reported that higher irradiance increased the lipid productivity of *Nannochloropsis*. Enhanced light supply could be achieved through a variety of biological or engineering means, for example increasing the light intensity (e.g. sunlight) and penetration, surface area to volume ratio of the reactor, or altering the antennae size of the algae to maximise photosynthetic efficiency and reduce mutual shading. Previous literature has indicated that a fed-batch (or semi-continuous) strategy where biomass is removed (reducing the density of the culture and enhancing energy provision for lipid production) along with nitrogen feeding (to allow increase in biomass) may improve productivity.

An alternative to photoautotrophy is mixotrophic or heterotrophic growth. Here carbon and energy are supplied in the form of an organic carbon source, decreasing the dependence on light. Work in this area has shown great potential for the enhancement of productivity (Chen & Chen 2006; Miao & Wu 2006; Xiong *et al.* 2008), particularly during the night time hours when the organic C source replaces photosynthesis entirely. However, the benefits need to be carefully considered in terms of the additional cost and energy input associated with the C source. Some of the best sources (e.g. glucose) are expensive and energy intensive to make. Others, such as crude glycerol, could be derived from waste streams (e.g. from biodiesel manufacture). Further investigation on the use of photoautotrophy with heterotrophy at key points in cultivation (e.g. during the high cell concentration, lipid accumulation phase) is warranted.

In order to better predict and manipulate the behaviour of microalgal cells, a deeper understanding of the signalling and metabolic processes governing lipid accumulation is required, particularly those processes governing the flux of carbon to lipid formation. The fact that growth and lipid accumulation occur under different conditions is a fundamental evolutionary design. Lipid accumulation is a carbon and energy storage mechanism. Under conditions favourable for growth, organisms naturally favour reproduction over energy storage. It is possible that genetic modification could override these systems, however, our understanding of the complex regulation of enzymes in the lipid biosynthesis pathway, and the interdependence of metabolic pathways, is limited (Scott *et al.* 2010). Further work in this area may lead to novel solutions to enhancing lipid productivity. For example, recent studies have indicated that blocking synthesis of starch in *Chlamydomonas* can lead to increased lipid formation (Li *et al.* 2010; Work *et al.* 2010). While genetic modification holds promise, such strategies should be approached with caution, as the release of a genetically modified organism on the scale required by a fuel production plant would be restricted in many countries. Much scope exists for the use of physiological control to manipulate carbon flux.

The work contained in this dissertation has contributed knowledge in the areas of biomass and lipid quantification, species choice and the effects of N limitation on lipid productivity in microalgae. However, the challenges to economic algal biodiesel production, in the areas of both biology and engineering, remain numerous. The lipid productivities achieved here are not yet economically viable as a single product based on current algal process costs. Along with the discovery or development of highly productive strains, cheap and energy efficient reactor designs, optimized light provision and low energy harvesting methods are required. Light provision is particularly challenging to optimize in outdoor culture. Direct sunlight can cause photoinhibition in dilute cultures, while dense cultures result in limited light penetration. Innovative reactor geometry and manipulation of culture depth, density and mixing regimes is necessary to optimize light provision.

Algal biodiesel continues to hold promise as a sustainable, carbon neutral source of transportation fuel, although the economics of production require substantial improvement. The necessary changes appear attainable through the enhancement of productivity, the reduction of cost and energy demand for key processes and the application of the biorefinery concept (co-production of valuable products or processes). Although the conversion of sunlight directly to electrical energy is likely to be more efficient than production of liquid fuel, the fact that the majority of global transport systems are liquid fuel based urgently demands alternatives to fossil petroleum and diesel, at least in the short term. Reducing worldwide carbon emissions will require global changes in thinking about economics, development, progress, value and quality of life. It is likely that a suite of technological solutions will need to accompany this. Algal biodiesel is unlikely to be the only answer, but it has the potential to be one of them.

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Appendix A – Culture media recipes

3N BBM medium

Recipe
3 ml of each macroelement stock
6 ml PIV metal solution
Make up to 1 L with distilled water and autoclave
Once cooled, add 1 ml of each of the two vitamin stocks

Note: do not autoclave the vitamins as they degrade when heated

Macroelement stocks
Make up each salt stock separately in distilled water to avoid precipitation. Autoclave. Adding 3 $ml.L^{-1}$ of each stock yields the final concentrations given below.

Component	**Final conc. in medium**	**Stock sol. conc.**	**Units**
$NaNO_3$	0.75	250	$g.L^{-1}$
$CaCl_2.3H_2O$	0.025	8.3	$g.L^{-1}$
$MgSO_4.7H_2O$	0.075	25	$g.L^{-1}$
$K_2HPO_4.3H_2O$	0.075	25	$g.L^{-1}$
KH_2PO_4	0.175	58.3	$g.L^{-1}$
NaCl	0.025	4.17	$g.L^{-1}$

PIV metal solution
Add the following, in order, to 1 L distilled water:

Component	**Amount**	**Units**
Na_2EDTA	0.75	$g.L^{-1}$
$FeCl_3.6H_2O$	0.097	$g.L^{-1}$
$MnCl_2.4H_2O$	0.041	$g.L^{-1}$
$ZnCl_2$	0.005	$g.L^{-1}$
$CoCl_2.6H_2O$	0.002	$g.L^{-1}$
$Na_2MoO_4.2H_2O$	0.004	$g.L^{-1}$

Solution should look yellow. Autoclave and store in the dark.

Vitamin stocks
Add 0.12g vitamin B1 (Thiamin HCl) to 100 ml distilled water and filter sterilise.
Add 0.1g vitamin B12 (cyanocobalamin) to 1 L dH_2O. Take 1 ml of this and add to 99 ml of distilled water. Filter sterilise.

Reference:
CCAP culture collection (http://www.ccap.ac.uk/media/pdfrecipes.htm)

f/2 medium

Recipe
To 1 L natural seawater, add:

Component	Conc. or vol.	Units
$NaNO_3$	0.75	$g.L^{-1}$
NaH_2PO_4	0.0044	$g.L^{-1}$
Fe/EDTA	0.5	ml
Trace metals	0.5	ml
Vitamins	0.5	ml

Sterilise by autoclaving before addition of vitamins.

Note: do not autoclave the vitamins as they degrade when heated

Fe/EDTA stock
Dissolve the following in 800 ml water:

Compound	Conc.	Units
$FeC_6H_5O_7$	5.71	$g.L^{-1}$
$Na_2EDTA.2H_2O$	17.35	$g.L^{-1}$

Neutralize to pH 7 with NaOH. Boil to dissolve, cool, and adjust volume to 1 L. Store in a dark bottle.

Trace metals stock

Compound	Amount	Units
$CuSO_4.5H_2O$	3.92	g per 200 ml
$ZnSO_4.7H_2O$	8.8	g per 200 ml
$CoCl_2.6H_2O$	4	g per 200 ml
$MnCl_2.4H_2O$	72	g per 200 ml
$Na_2MoO_4.2H_2O$	2.52	g per 200 ml
$Na_2EDTA.2H_2O$	See instructions	

Make individual 200 ml 1000x stocks of the first 5 components (store at 4°C).
To make the trace metal stock, add 250 µl of each of the 5 stocks to 200 ml water.
Add 0.41 g Na_2EDTA. Boil for 3 min. Cool, then adjust volume to 250 ml.
Store at room temperature in a dark cupboard, discard if precipitate forms.

Vitamin stock
To make primary stocks:
Dissolve 0.0114 g vitamin B12 (cyanocobalamin) in 10 ml distilled water. Dissolve 0.0104 g biotin in 100 ml distilled water (adjust pH to 10 to dissolve). Adjust stocks to pH 4.5 to 5 and store at -20°C.
To prepare vitamin solution, add the following to 100 ml distilled water:

B12 primary stock	0.1	ml
Biotin primary stock	1	ml
Thiamine HCl	0.02	g

Filter sterilize, dispense in small aliquots and store at -20°C.

Walne's medium

Recipe
To 1 L natural seawater, add:
0.75 g $NaNO_3$
1 ml enrichment solution
1 ml sodium metasilicate stock
100 µl vitamin solution

Enrichment solution
Heat 500 ml of water in a flask. As the flask heats up, dissolve in the following order:

Compound	**Amount**	**Units**
$MnCl_2.4H_2O$	0.42	g
$FeCl_3.6H_2O$	1.32	g
H_3BO_3 (boric acid)	33.4	g
Na_2EDTA	45	g
$NaH_2PO_4.H_2O$	20	g
Trace metal solution	1	ml

Before the liquid boils, make up to 1 L with distilled water.
Continue to stir and warm until salts dissolve. Store in fridge.

Trace metal solution
Dissolve in 100 ml warm, weak HCl (0.01N):

Compound	**Amount**	**Units**
$ZnCl_2$	2.1	g
$CoCl.6H_2O$	2	g
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.9	g
$CuSO_4.5H_2O$	2	g

Solution should be purple. Store frozen.

Sodium metasilicate stock
Dissolve 6 g of $Na_2SiO_3.5H_2O$ in 100 ml distilled water by warming slightly.

Vitamin solution
Dissolve in 100 ml distilled water:

Compound	**Amount**	**Units**
Vitamin B12 (Cyanocobalamin)	10	mg
Vitamin B1 (Thiamine HCl)	10	mg

Zarrouk's medium

Recipe

Component	Amount	Units
$NaHCO_3$	18	$g.L^{-1}$
K_2HPO_4	0.5	$g.L^{-1}$
$NaNO_3$	0.75	$g.L^{-1}$
K_2SO_4	1	$g.L^{-1}$
$MgSO_4 \cdot 7H_2O$	0.2	$g.L^{-1}$
NaCl	1	$g.L^{-1}$
$CaCl_2$	0.04	$g.L^{-1}$
$FeSO_4 \cdot 7H_2O$	0.01	$g.L^{-1}$
Na_2EDTA	0.08	$g.L^{-1}$
Micronutrient solution	1	$ml.L^{-1}$
Trace element solution	1	$ml.L^{-1}$

Micronutrient solution

Component	Amount	Units
H_3BO_3	2.86	$g.L^{-1}$
$MnCl_2 \cdot 4H_2O$	1.81	$g.L^{-1}$
$ZnSO_4 \cdot 7H_2O$	0.22	$g.L^{-1}$
$CuSO_4 \cdot 5H_2O$	0.08	$g.L^{-1}$
$Na_2MoO_4.2H_2O$	0.014	$g.L^{-1}$

Trace element solution

Component	Amount	Units
K_2CrO_7	46.6	$mg.L^{-1}$
$NiSO_4 \cdot 7H_2O$	47.8	$mg.L^{-1}$
$CoSO_4.7H_2O$	4.2	$mg.L^{-1}$

Appendix B – Notes on analytical method selection

Comparison of methods for quantification of cell protein content

It is commonly accepted that the most accurate method of measuring total protein content is acid digestion followed by quantification of individual amino acids by liquid chromatography (Lourenço *et al.* 2004). However, this technique is expensive, laborious and requires dedicated equipment. Protein content can be estimated from the amount of nitrogen in the cell (determined by elemental analysis). Nitrogen content is commonly multiplied by 6.25 to derive the protein content. This, however, depends on the assumption that most of the intracellular nitrogen is stored as protein, which does not hold true for microalgae. (Walker 1994) investigated the distribution of intracellular nitrogen in marine microalgae and established that conversion factors between N and protein content were lower (on average 4.78) than the traditionally used 6.25, and varied with species and growth conditions.

Colorimetric assays such as the Bradford, Lowry and bicinchoninic acid (BCA) methods are widely used (Lourenço *et al.* 2004; Rausch 1981). Protein must be extracted from the cells in order to be assayed by any of these methods, hence the accuracy of the method is influenced by the efficiency of extraction. The characteristics of the algae (particularly cell wall composition) and the extraction procedures used can significantly affect results (Walker 1994). The assays are also subject to interference by a number of substances. For example, the Bradford method is affected by detergents, the Lowry by sugars, buffers, EDTA, detergents and nucleic acids and the BCA assay by lipids and EDTA (for a full list of interfering substances, see Walker (1994)). In addition, due to the mechanisms of colour development, the amino acid composition of the protein can affect results. For example, in the Bradford method, colour development relies on the binding of the dye Coomassie blue G250 to protein. The dye binds most readily with arginyl residues (Lourenço *et al.* 2004). As a result, proteins rich in arginine or phenylalanine could result in overestimation of protein content. Similarly, the Lowry method is sensitive to tyrosine and tryptophan (Walker 1994). In all of these assays, absolute concentration cannot be determined, but is relative to the standard used (e.g. bovine serum albumin (BSA), casein or bovine γ-globulin) (Walker 1994).

Although sensitive to amino acid composition and extraction efficiency, the Bradford and Lowry methods have been so widely used that these estimations are an acceptable alternative to rigorous absolute determination where protein mixtures or crude extracts are involved. The question of which of these assays to use is not an easy one. The Bradford technique is simpler, faster and more sensitive than the Lowry method (Barbarino & Lourenço 2005), however, it has been shown to underestimate protein content in microalgae (Walker 1994). The Lowry assay requires several steps, but its sensitivity is more constant between different proteins. The BCA

assay is similar to the Lowry assay, but has the advantage that it can be carried out in a single step. Both are based on the Biuret reaction, where Cu^{2+} reacts with peptide bonds under alkaline conditions to produce Cu^{+}. This reacts either, in the case of the Lowry method, with Folin reagent to produce a blue colour, or, in the BCA method, with BCA to produce an intense purple colour. The BCA assay is more tolerant to interfering compounds than the Lowry, but it is known to be sensitive to reducing sugars and lipids (Rausch 1981).

Preliminary tests were done to compare extraction and assay methods. (Barbarino & Lourenço 2005) compared various methods for extracting protein from microalgal cells and suggested a standard method of extracting cells three times at elevated temperatures in 0.5 N NaOH. This method was compared to the more convenient method of extraction in 1 N NaOH overnight and to complete cell disruption using a Misonix XL-2000 sonication probe (30 min, setting 19). It was found that the Rausch extraction yielded higher protein results than the overnight extraction, and very similar results to those from sonicated samples (results not shown).

In agreement with (Bollag & Edelstein 1993), the Bradford assay (BioRad kit handbook) was found to detect less protein than the Lowry (Walker 1994) and BCA assays (Walker 1994) (Figure B1). The quantification by the Lowry and BCA methods were similar, with the results of the Lowry assay being slightly higher than the BCA method. This could be due to the different response to interfering substances present in the samples. Due to its ease of use and higher tolerance to interfering substances, the BCA method (Rausch 1981) was chosen as the protein assay for this work, preceded by extraction into 0.5 N NaOH according to (Rausch 1981).

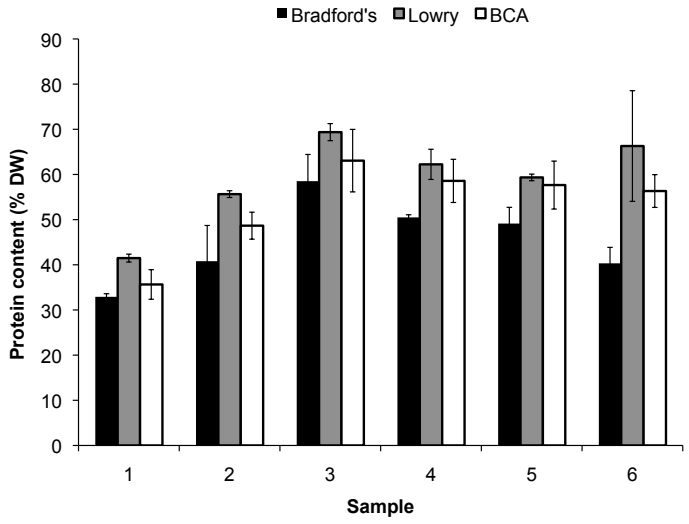


Figure B1 Comparison of protein content in six microalgal cultures grown under different nitrate conditions, as determined by the Bradford, Lowry and BCA assays. Protein was extracted into 0.5 N NaOH according to Rausch (1981) (Wellburn 1994).

Comparison of solvents for extraction of pigments

The pigment content of cells can be measured by HPLC, fluorometry or spectrophotometry. HPLC is costly, both in terms of time and materials, while spectrophotometry is rapid and easy and hence the method of choice. The accuracy of total pigment quantification is influenced by the efficiency of extraction of pigments from the cells. A variety of solvents can be used for pigment extraction, e.g. methanol, ethanol, acetone, dimethylformamide (DMF) and dimethylsulphoxide (DMSO) (Wellburn 1994). Different types of plant tissue are suited to different extractants. Some solvents require maceration or grinding, while others (DMF and DMSO) can extract pigments from cells with merely shaking. The absorbance of the extract is usually determined at multiple wavelengths and equations based on the solvent and the specific absorption coefficients of the various pigments (e.g. chlorophyll *a*, *b* and *c* and carotenoids) are used to quantify them (Wellburn 1994).

Preliminary tests were carried out to determine the best solvent to use. The amount of chlorophyll (the sum of chlorophylls *a* and *b*) extracted from identical samples of three algal species by various solvents is shown in Figure 2.3. Of the solvents tested, DMSO was found to extract the most pigments. DMSO is widely used as a solvent as it dissolves a broad range of compounds. It penetrates cells easily and is often used as an extractant in biochemistry and microbiology. In addition to being the most efficient extractant tested here, it is also less toxic than many other solvents. For these reasons, DMSO was chosen as the pigment extraction solvent to be used in this work.

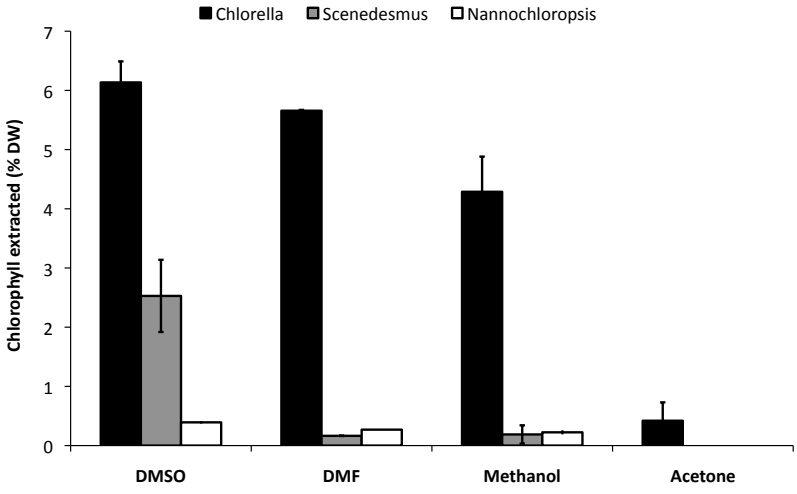


Figure B2 Amount of chlorophyll (sum of chlorophylls *a* and *b*, expressed as% DW) extracted by various solvents from samples of *Chlorella*, *Scenedesmus* and *Nannochloropsis*

The original pigment quantification protocol of Njodzi Zizhou (personal communication, 2007), on which the method presented in Section 2.4.6 is based, called for 5 ml of extraction solvent and

homogenisation with glass beads during the heating step. In order to streamline the assay and reduce the size of the culture sample required, it was desirable to adjust the volume to 2 ml, and omit the homogenisation step. In order to test whether this had a significant effect on results, the 2 ml extraction used in this work, without homogenisation, was compared to the original 5 ml extraction using homogenisation. The extraction using the modified method was almost identical to the original method in *Scenedesmus* and *Nannochloropsis* (99 and 101% respectively), while for *Chlorella* the modified method yielded 90% of extraction using the original method (Figure B3). It was felt that the extraction efficiency of the modified method was similar enough to the original to justify the savings in time and material by using the reduced volume and omitting homogenisation.

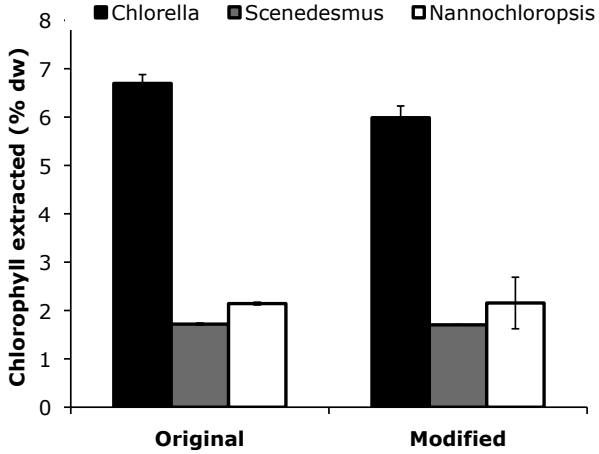
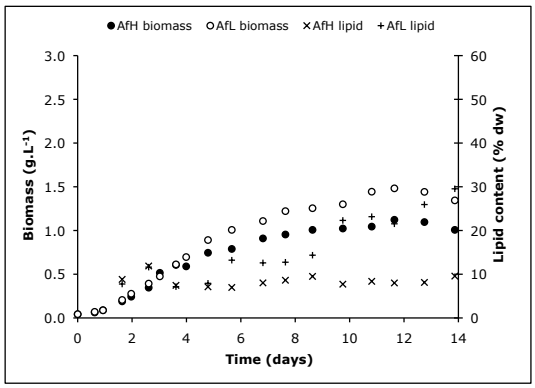


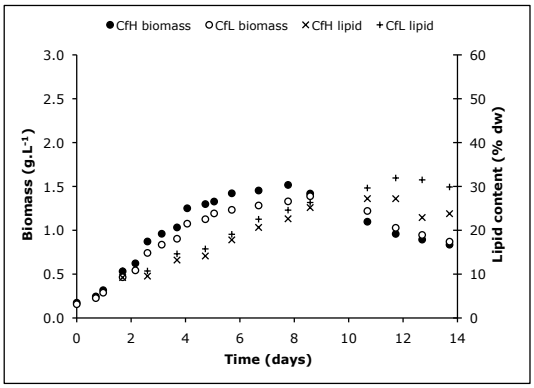
Figure B3 Extraction of chlorophyll *a* and *b* using the original (5 ml with homogenisation) and the modified (2 ml without homogenisation) method

Appendix C – Additional data from Chapter 6

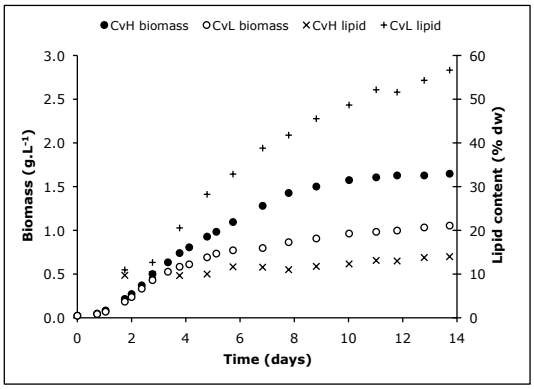
Biomass concentration and lipid content



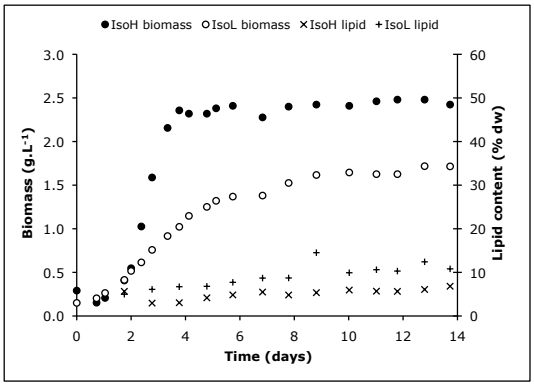
(a) ***Ankistrodesmus falcatus***



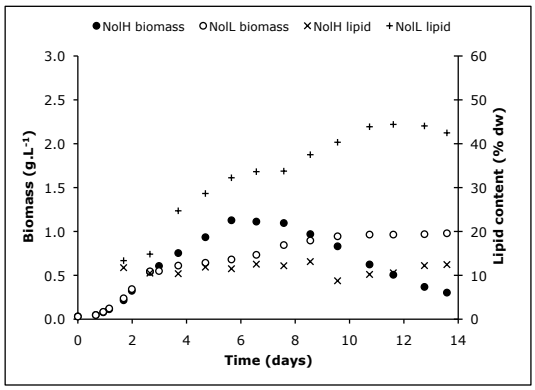
(e) ***Cylindrotheca fusiformis***



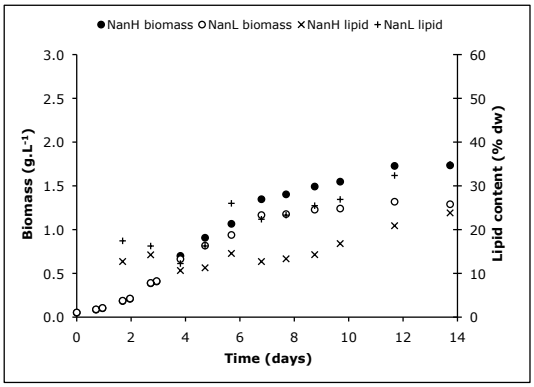
(b) ***Chlorella vulgaris***



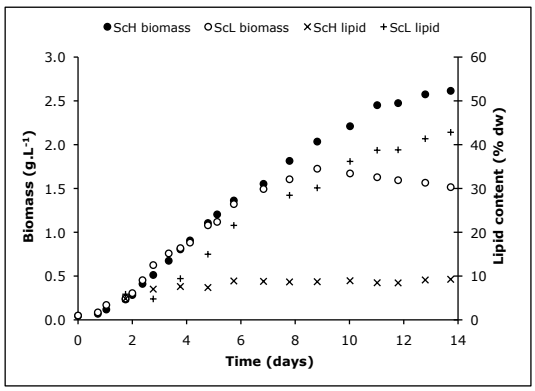
(f) ***Isochrysis***



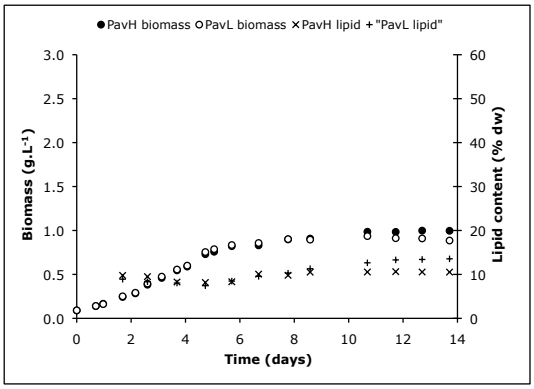
(c) ***Neochloris oleoabundans***



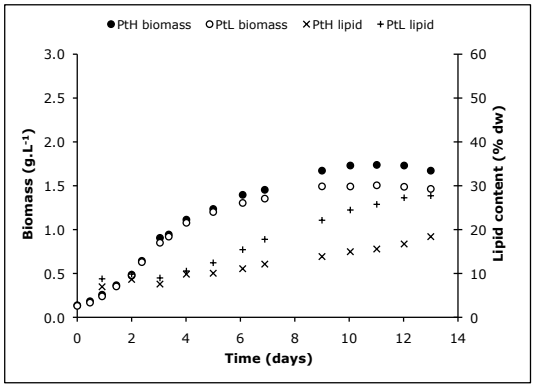
(g) ***Nannochloropsis***



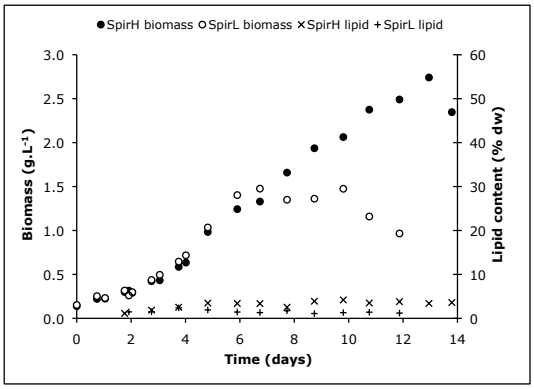
(d) ***Scenedesmus***



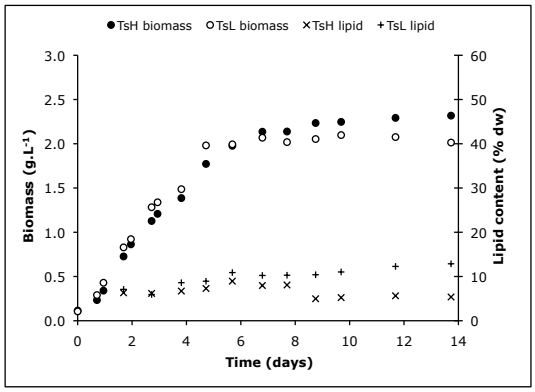
(h) ***Pavlova***



(i) ***Phaeodactylum tricornutum***



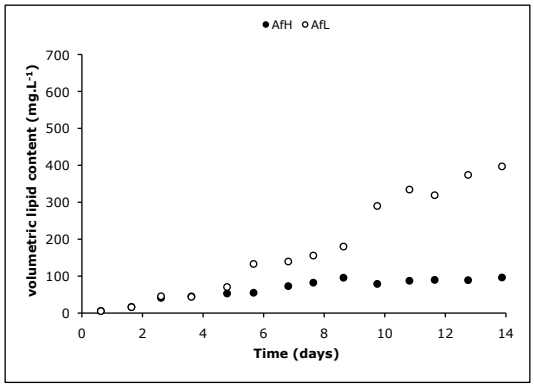
(k) ***Spirulina platensis***



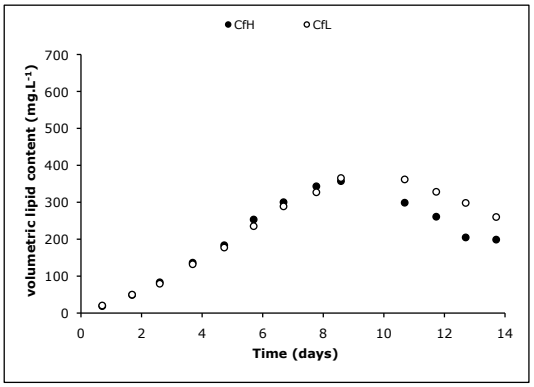
(j) ***Tetraselmis suecica***

Figure C1 Biomass concentration and lipid content during a 14 day growth period for 11 species of microalgae under nitrogen replete (H, ● and ×) and limited (L, ○ and +) conditions

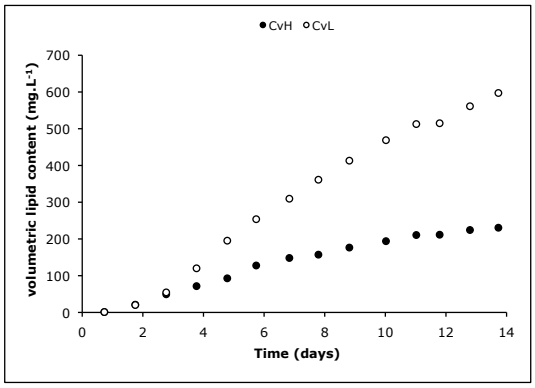
Volumetric lipid content



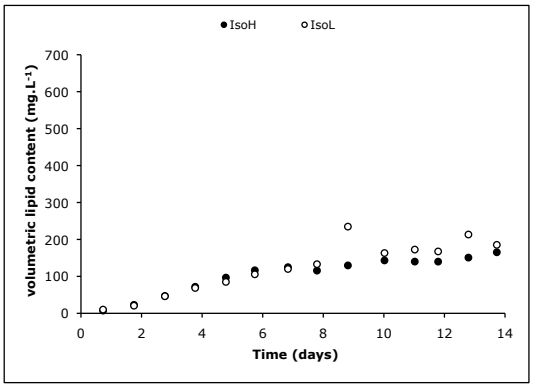
(a) ***Ankistrodesmus falcatus***



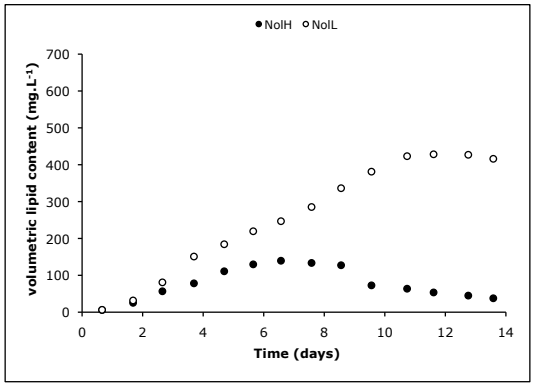
(e) ***Cylindrotheca fusiformis***



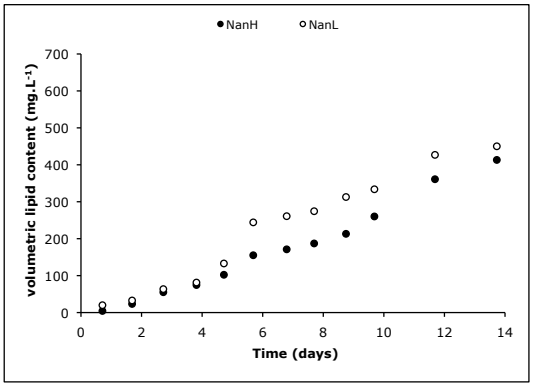
(b) ***Chlorella vulgaris***



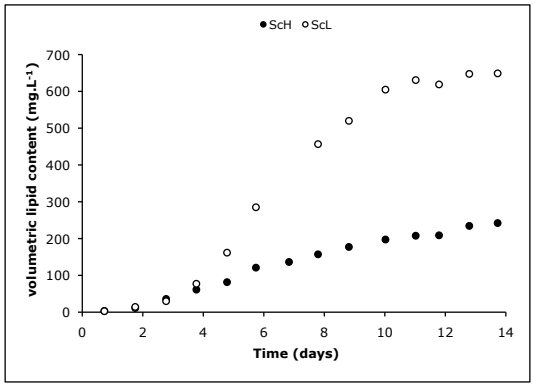
(f) ***Isochrysis***



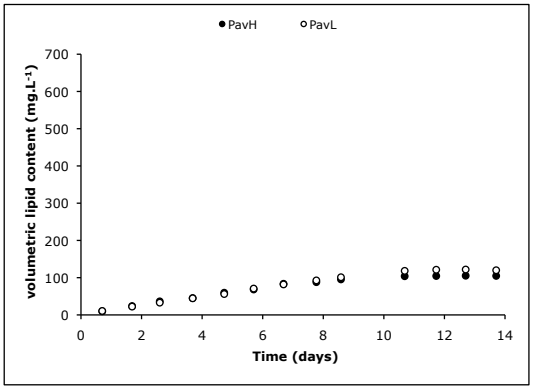
(c) ***Neochloris oleoabundans***



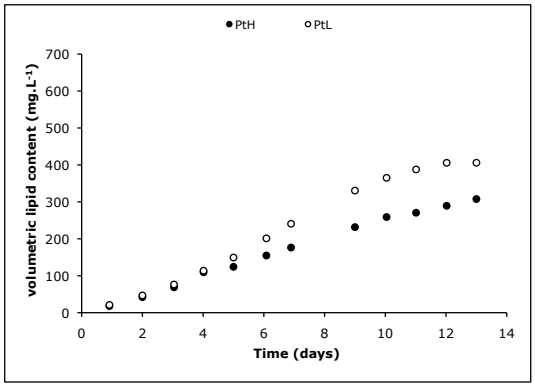
(g) ***Nannochloropsis***



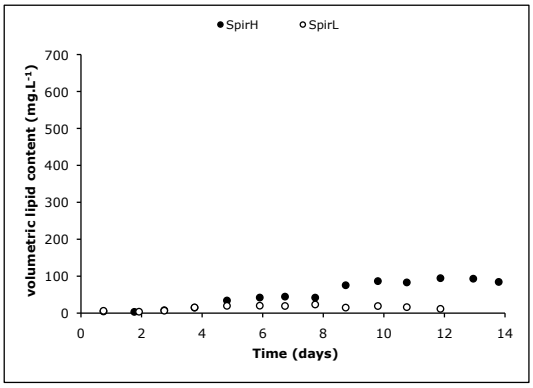
(d) ***Scenedesmus***



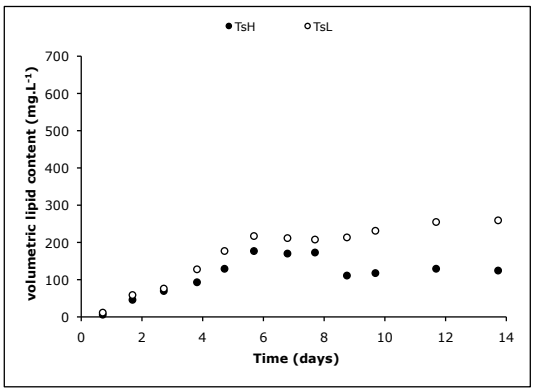
(h) ***Pavlova***



(i) ***Phaeodactylum tricornutum***



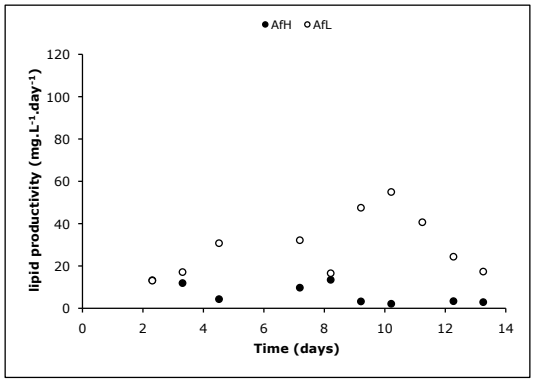
(k) ***Spirulina platensis***



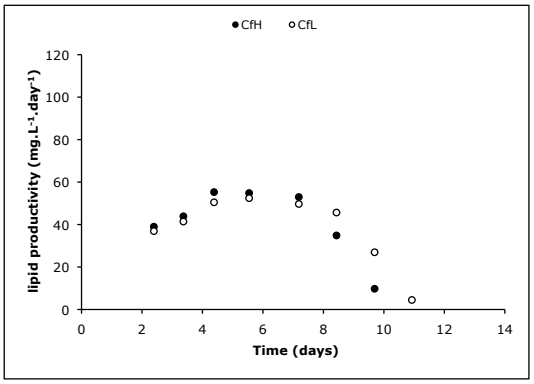
(j) ***Tetraselmis suecica***

Figure C2 Volumetric lipid content during a 14 day growth period for 11 species of microalgae under nitrogen replete (H, ●) and limited (L, ○) conditions

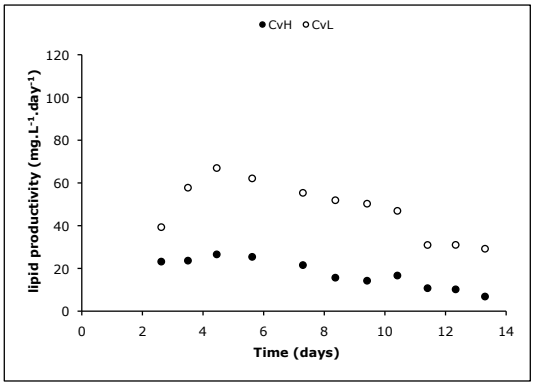
Instantaneous lipid productivity



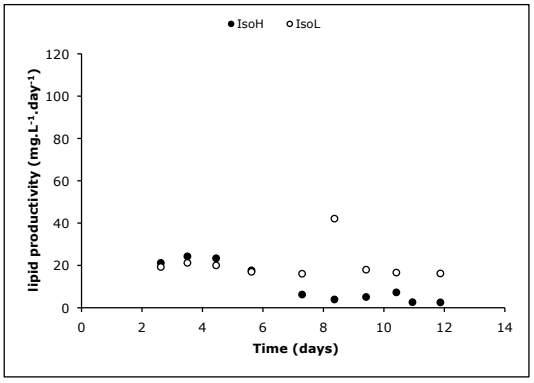
(a) ***Ankistrodesmus falcatus***



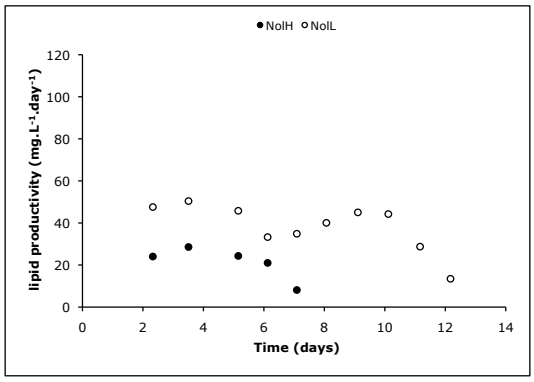
(e) ***Cylindrotheca fusiformis***



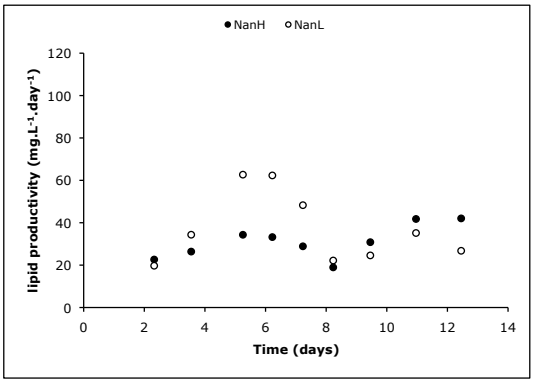
(b) ***Chlorella vulgaris***



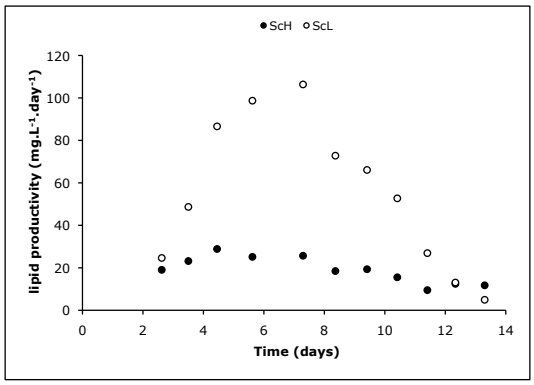
(f) ***Isochrysis***



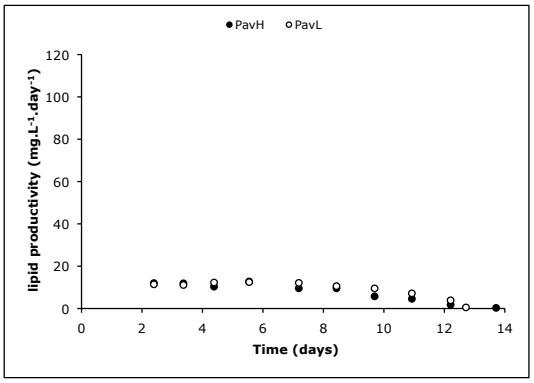
(c) ***Neochloris oleoabundans***



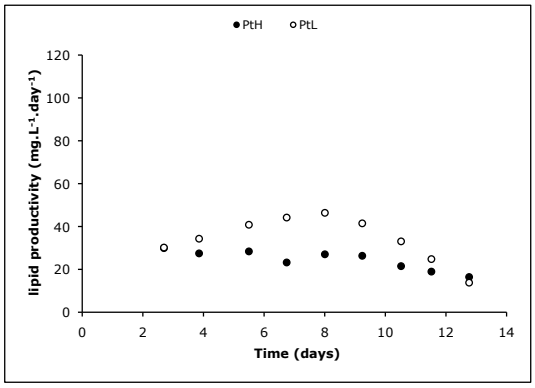
(g) ***Nannochloropsis***



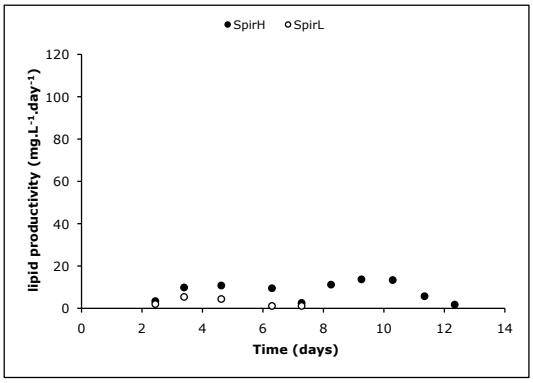
(d) ***Scenedesmus***



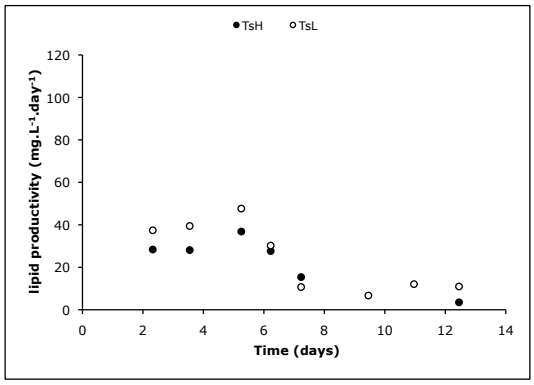
(h) ***Pavlova***



(i) ***Phaeodactylum tricornutum***



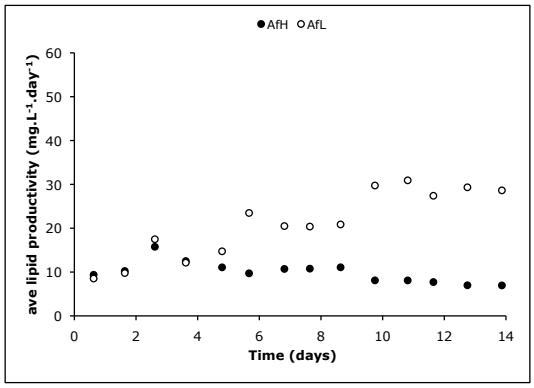
(k) ***Spirulina platensis***



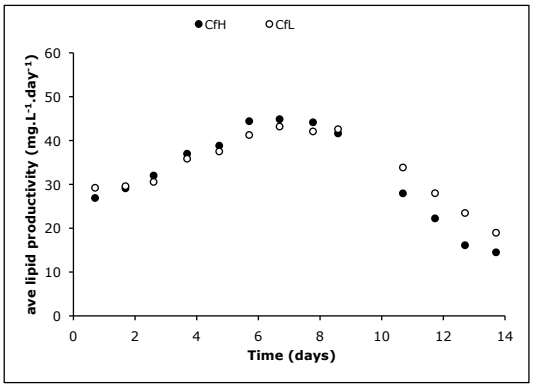
(j) ***Tetraselmis suecica***

Figure C3 Instantaneous lipid productivity during a 14 day growth period for 11 species of microalgae under nitrogen replete (H, ●) and limited (L, ○) conditions

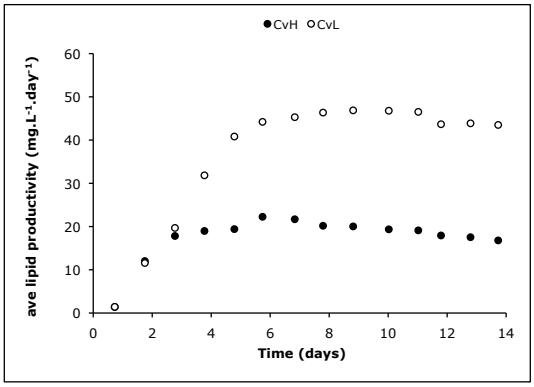
Overall average lipid productivity



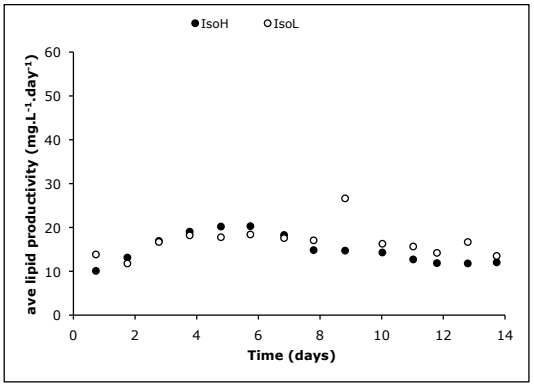
(a) ***Ankistrodesmus falcatus***



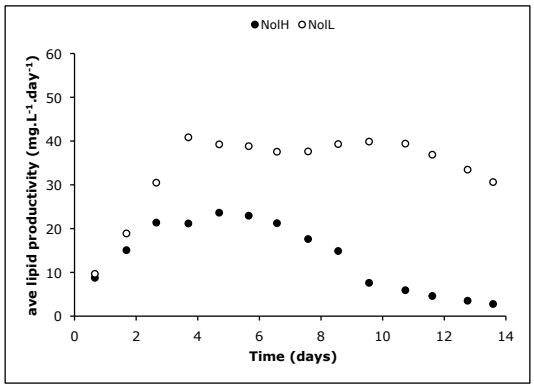
(e) ***Cylindrotheca fusiformis***



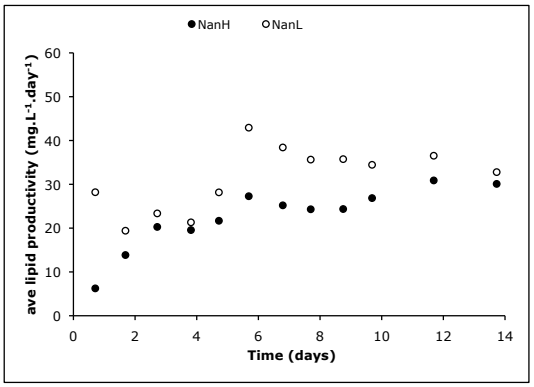
(b) ***Chlorella vulgaris***



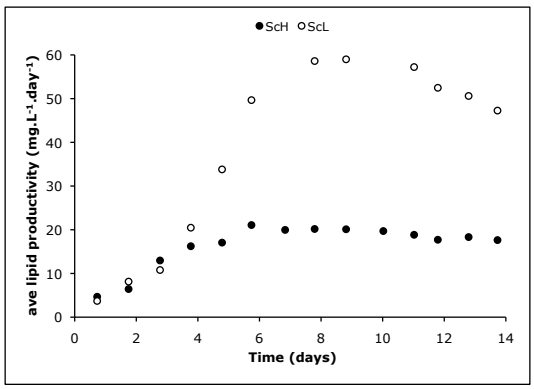
(f) ***Isochrysis***



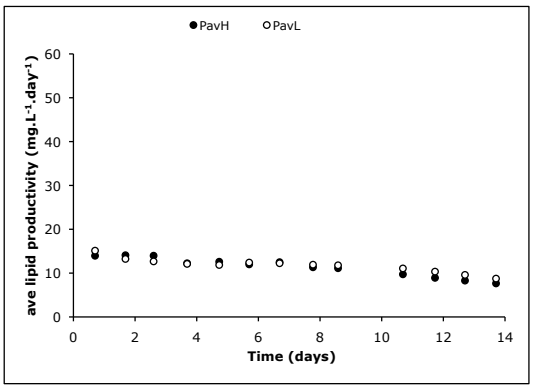
(c) ***Neochloris oleoabundans***



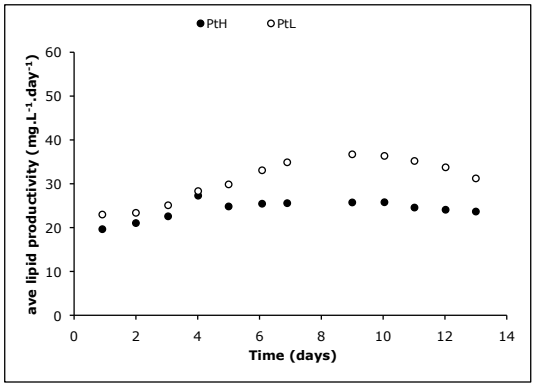
(g) ***Nannochloropsis***



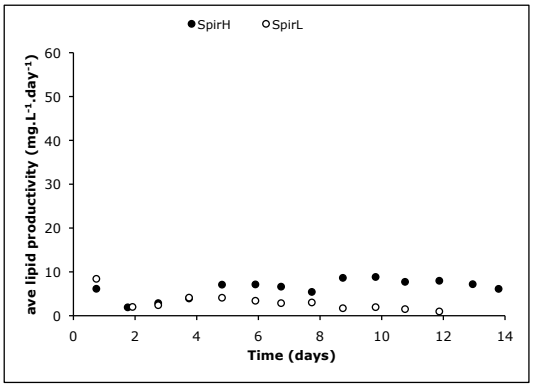
(d) ***Scenedesmus***



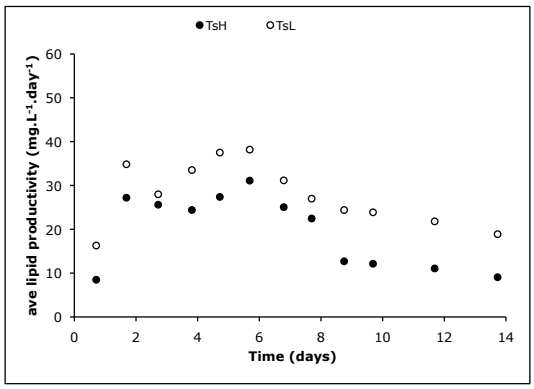
(h) ***Pavlova***



(i) ***Phaeodactylum tricornutum***



(k) ***Spirulina platensis***



(j) ***Tetraselmis suecica***

Figure C4 Overall average lipid productivity during a 14 day growth period for 11 species of microalgae under nitrogen replete (H, ●) and limited (L, ○) conditions