BIO-PROSPECTING MICROALGAE FROM CENTRAL QUEENSLAND FOR LIPID PRODUCTION BY ASSESSING BIOMASS AND LIPID CONCENTRATION USING NEAR INFRARED SPECTROSCOPY

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STATUTORY DECLARATION

I hereby certify that “Bio-prospecting microalgae from Central Queensland for lipid production by assessing biomass and lipid concentration using near infrared spectroscopy” is a presentation of my original research work and all the sources used were acknowledged by means of complete reference. The material has not been submitted, either in whole or in part, for a degree at this or any other universities.

I acknowledge and certify that I have complied with the rules, requirements, procedures and policy of the Central Queensland University, Australia relating to research higher degree award. I authorize Central Queensland University to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Name: Vineela Challagulla

Signature:

Date:

DEDICATION

This thesis is dedicated to my loving parents Sulochana Rani and Ramarao, who taught me that even the important task can be accomplished if it is done one step at a time. Without your encouragement and support this work would have been impossible.

Vineela Challagulla, 2012

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Abstract

For commercial biodiesel production, species bioprospecting and optimization of biomass and lipids detection techniques play a most important role. During the dry season of 2010, ninety water samples were collected from a range of water bodies across Central Queensland, Australia. The waters had a pH range of 5.8-8.8, a conductivity range of 184-2351 µS cm^{-1} and temperature range of 17-21 °C. Microalgal species containing neutral lipid reserves were identified using Nile Red and epi-fluorescence microscopy. One species of the genus *Chlorella*, two species of the genus *Navicula* and one species of the genus *Nitzschia* were identified. Uni-algal cultures of these species were grown in a standard nutrient solution as well as N, P and Fe deficient growth media. Cell counts and biomass measured using dry weight were well correlated to optical density read at 625 nm. *Chlorella* sp. recorded the highest mean biomass of 1.46±0.58 g L^{-1}, achieved with 22 d of growth in standard nutrient media and mean lipid content of 0.274±0.09 g L^{-1}, accounting for 69% of dry weight, under P deficient growth media.

The technique of Near Infrared Spectroscopy (NIRS) was assessed with respect to analysis of dry matter and lipid content. Microalgal culture samples were filtered through GF/C filter papers and spectral measurements of oven dried (60°C overnight) filter papers over the ranges of 300-1100 nm and 1100-2500 nm were recorded. Partial least square (PLS) models for combined species data were poor in terms of root mean square error of cross-validation (RMSECV), cross validation coefficient of determination (R_{CV}) and the ratio of standard deviation of reference

mean to RMSECV (SDR). A single species model for *Chlorella* sp., based on 1100-2500 nm spectra of dry filtrate, supported a model with RMSECV, R_{CV} and SDR values of 0.32 g L^{-1}, 0.955 and 3.38 for biomass and 0.089 g L^{-1}, 0.874 and 2.06 with lipid, respectively. Poor model results were obtained for spectra of wet filtrates and culture flasks. However, the best model performed poorly in the prediction of samples drawn from an independent series of *Chlorella* sp. cultured under N, P and Fe deficient conditions. A model based on all data available resulted in RMSECV, RP and SDR values of 0.54 g L^{-1}, 0.94 and 1.329 with biomass and 0.077 g L^{-1}, 0.85 and 0.279 with lipid respectively. The NIRS technique thus has the potential for dry matter and lipid determination of microalgal cultures. However, attention is required, to the issue of model robustness across species and growth conditions.

This study concludes that a *C. vulgaris* strain isolated from the Central Queensland region has lipid content equal to that reported in the literature and can be a potential candidate for biodiesel production if growth parameters are further optimized. Bioprospecting of microalgae may be extended to different seasons, locations and water habitats (especially marine, waste waters and hyper saline) in Queensland. Biomass and lipid content assessed using NIRS on dry filtrates was successful and can be further applied to assess lipid profile parameters such as fatty acid chain lengths and degree of un-saturation, which play a major role in biodiesel fuel quality.

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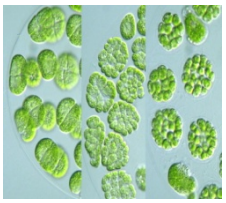
Chapter 1: Introduction



Increasing demand for energy has led to depletion of petroleum resources (Muradov & Veziroglu 2008). Transportation consumes almost two thirds of the primary energy use, and is particularly dependent on petroleum-based fuels (World Energy Outlook 2007). There is slow global move from a petro-economy to a bio-economy (Mathews 2008). Biomass derived energy sources include methane and thermal (from wood, agricultural, animal, sewage, organic and municipal solid wastes), ethanol (from crops such as corn, sugarcane, and sweet sorghum) and oil (from oil seed crops and oleaginous microorganisms) (Abraham & Srinivasan 1984 ; Muradov & Veziroglu 2008). Ethanol and biodiesel are particularly useful for transportation (Armbruster & Coyle 2006; Fulton *et al.* 2004).

1.1 Biofuels

First generation biofuels are derived from carbohydrates and lipids of edible seeds like corn, soybean, sunflower, safflower, canola, wheat (Demirbas 2008). Products include biodiesel, vegetable oil, biogas, bioalcohols, bioether, solid biofuels and syngas. The uses of these biofuels have raised moral and ethical issues due to the use of erstwhile food crops. To overcome these issues second generation biofuels have been considered derived from non edible crops (Demirbas 2008).

Second generation biofuels are derived from non edible crops such as *Pongamia*, *Jatropha*, stalks of wheat, corn, wood and waste biomass (Taylor 2008). Products include bio-ethanol, diesel, mixed alcohols, and wood diesels are still under consideration (Schumacher *et al.* 2004). Although non edible feed stocks are being

used, cultivation resources such as water, fertilizers and land first often continued to compete with those used for food crops

Third generation biofuels (from algae, fungi, yeast; also lignocellulosic ethanol) are claimed to offer advantages to the first (e.g. corn, sorghum, canola) and second generation (e.g. trees, grasses, non-edible oils), on the basis of having a better energy balance, and that they do not compete with food crops. One of the major sources of third generation biofuels are oleaginous microorganisms such as bacteria, fungi, algae and yeast which have lipid contents in excess of 20 % by dry weight (Meng *et al.* 2009). Among the oleaginous microorganisms, the use of microalgae as a fuel source has advantages such as absence of intractable biopolymers, metabolic and ecological diversity, biosynthetic control of chemical composition by nutrient and environmental stresses, low water usage (if in closed bioreactors) and valuable by-products (Dismukes *et al.* 2008).

1.2 Microalgae as feed stock for biofuels

Microalgal biomass production systems require light, carbon dioxide, water (fresh and brackish water from rivers, lakes, aquifers) and inorganic nutrients (nitrates, phosphates, iron, and a few trace elements). Large-scale production of biofuels from microalgae, however, requires refining of species selection and optimisation of growth conditions. Since the productive performance of species varies with climatic conditions and the properties of water used in the production, it is critical that these conditions are optimised for every location where microalgal production is undertaken.

Fuel production from microalgae is based on photo bioreactors (PBRs) and open pond systems. Although photo bioreactors have advantages like minimal contamination and low water use, construction cost is expensive. Open pond systems can provide more economical production, but suffer limitations such as contamination of cultures, and varied growth conditions are the major drawbacks of open cultivation systems (Huntley & Redalje 2007).

Microalgal biofuels as major source of bioenergy (Brennan & Owende 2010; Chisti 2006; Chisti 2007; Jones & Mayfield 2012), bio-prospecting microalgae for value added by-products (Mutanda *et al.* 2011; Sheehan *et al.* 1998), bio-processing for biofuels (Blanch 2012; Lam & Lee 2011; Sharma *et al.* 2008), and economics, challenges and opportunities of microalgal production systems (Chisti *et al.* 2003; Hoekman 2009; Molina Grima *et al.* 2003) are well established reviews. However, microalgal biodiesel is still not a commercial industry due to the limitations such as high energy requirements and low energy exchange percentage compared to oil seed crops.

1.3 Microalgal biomass and lipid estimations

Numerous authors have reported that the economics of microalgal biofuel production depend on the microalgal strain selection (Mutanda *et al.* 2011), biomass and lipid composition and detection methods (Laurens & Wolfrum 2011), and the efficiency of harvesting and conversion techniques (Brennan & Owende 2010).

Microalgal strains with the capacity to accumulate neutral lipids can be optimized for higher lipid production by modifying parameters such as temperature (Cho *et al.*

2007), light cycle and intensity (Kim *et al.* 2006; Perner-Nochta & Posten 2007) water quality, pH and salinity (Abu-Rezq *et al.* 1999; Cho *et al.* 2007; Ranga Rao *et al.* 2007a; Ranga Rao *et al.* 2007b). Lipid production can also be increased by limiting nutrients such as iron (Liu *et al.* 2008), nitrogen (Illman *et al.* 2000), silicon (Ghirardi *et al.* 2000) and phosphate (Reitan *et al.* 1994) in growth media.

Collection and identification of microalgal strains with high lipid content is always followed by an assessment of the lipid content and biomass productivity. Evaluation of lipid quality and quantity currently depends on conventional wet chemistry techniques such as chromatography for example High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC) (Yang *et al.* 2004; Zhila *et al.* 2005) and spectroscopy (Nile red fluorescence and mass spectroscopy) (Cooksey *et al.* 1987; Lee *et al.* 1998). Microalgal biomass is estimated by cell counts (cell counting chambers, flow cytometers, and Coulter counters) (Chiu *et al.* 2008; Fidalgo *et al.* 1998; Sukenik & Wahnon 1991), optical density (Lee *et al.* 2002; Takagi *et al.* 2006), centrifugation, freeze drying and dry weight measurement (Zhu *et al.* 2007), and chlorophyll estimation (Sukenik & Wahnon 1991; Zhang *et al.* 2011). Rapid cost effective, non-destructive and less time consuming measuring tools for assessing lipid and biomass are prerequisites for microalgal biofuel research.

1.4 Current microalgal research in Australia

Dunaliella salina grows well in saline conditions because of its high intracellular glycerol which provides protection against osmotic pressure (Borowitzka & Hallegraeff 2007). Since 1980's production of beta carotene (used as vitamin A

supplement) from *Dunaliella salina* is the major industry in Australia and Western Biotechnology Ltd. (Perth, Western Australia) and Betatene Ltd. (Melbourne, Victoria) are the world's largest producers. Large and shallow ponds (approximately 20 cm in deep) are constructed on a hypersaline coastal lagoon, or by artificially expanding a lagoon. For example, Western Biotechnology Ltd. each of the commercial ponds is 5 ha with total pond area of 50 ha constructed on a lagoon floor. The rate of harvesting and growth period is based on weather conditions across the year (Curtain *et al.* 1987).

During 2008-2009 a major project on "Development of a sustainable microalgal biofuel industry" was conducted in Flinders University with chief investigator Dr. Stephen Clarke (Flinders University) and Co-Investigators A/Prof Wei Zhang (FMC) and Dr. Sasi Nayar (South Australian Research and Development Institute). The work was focussed on developing microalgae as a second generation feedstock for sustainable biofuel production and value-adding to the biofuel production via a biorefinery approach. This project established optimum growth conditions of *Botryococcus braunii* which blooms to form large green mats at temperature (23° C), light (30–60 W/m^2 irradiance), salinity (8.8%) and photo period of 12 h light and 12 h dark in the laboratory.

During 2008-2009 a team led by Professor Michael Borowitzka of Murdoch University together with University of Adelaide, researchers received $1.9 million funding from the Federal Department of Environment, Water, Heritage and the Arts to research turning saline ponds of microalgae into clean, affordable fuel. This was the only biofuel project in Australia worked simultaneously on all steps in the process

of microalgal biofuels production, from microalgae culture, harvesting of the algae and extraction of oil suitable for biofuels production (Budge & Yovich 2008).

During 2010, a team led by Associate Professor Ben Hankamer (Institute for Molecular Bioscience, University of Queensland) received $ 1.48 million for research into high-efficiency microalgal biofuel systems that aim to produce a range of biofuels through “photo-bioreactors”. The consortium backing the project includes global engineering and construction company Kellogg Brown & Root Pty Ltd, Neste Oil Corp, Cement Australia Pty Ltd, North Queensland and Pacific Biodiesel Pty Ltd, the University of Karlsruhe, the University of Bielefeld and University of Queensland.

During 2010, a project in partnership with James Cook University (JCU) and Australian company MBD Biodiesel Ltd. was funded $166,000 from the Queensland Government to develop a sustainable biofuel for transport and industry (John 2010). Professor Rocky de Nys, Head of Aquaculture at JCU, will lead the first stage of the project in Townsville with Associate Professor Kirsten Heimann, Director of JCU’s North Queensland Algal Identification/Culturing Facility. The first stage of the project is to identify and develop suitable alga strains to achieve stable, continuous, high yield alga production.

1.5 Aim and objectives of the present study

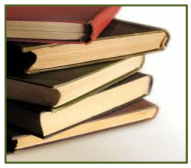
The present study aims to add to this effort through a bio-prospecting exercise of identification and isolation of oil accumulating microalgae from Central Queensland provenance, followed by an assessment of biomass and lipid content under nutrient sufficient and deficient cultivations. Additionally, method development on techniques

for assessing biomass and lipid content using near infrared spectroscopy were considered.

The objectives are:

- To collect, screen and isolate microalgal strains from various waters bodies in the Central Queensland region and select for high neutral lipid content.
- To characterize selected microalgae for increased biomass and lipid productivities under nutrient sufficient and deficient conditions.
- To apply Near Infrared Spectroscopy (NIR) to assess biomass and lipid content of microalgal samples.

Chapter 2: Literature review



This literature collection summarises knowledge related to experiences with bioprospecting for algae for oil production. It is followed by analysis of nutrient induced variations of biomass and lipids in *C. vulgaris*. Various techniques to assess biomass and lipid content and the associated disadvantages are also reviewed.

2.1 Bioprospecting of microalgae

Various advantages such as diversity, valuable co-products and low water usage have attracted the attention of researchers to develop viable renewable biofuels from microalgae (Dismukes *et al.* 2008). Microalgae are unicellular microscopic organisms ranging from 2–200 µm in diameter. They are autotrophic and have chlorophyll *a* as the primary photosynthetic pigment (Graham & Wilcox 2000). Major algal groups such as Cyanobacteria, Glaucophyta, Eustigmatophytes and Rhodophyta have chlorophyll a whereas groups such as Prochlorophyta, Euglenophyta, Cryptophyta, Haptophyta, Dinophyta, Ochrophyta and Chlorophyta are represented in microalgae (Graham & Wilcox 2000). Large scale production of biofuel from microalgae depends upon refining the process of species selection (bio-prospecting) (Mutanda *et al.* 2011). To date, much of the focus in the field of microalgal biofuel research has been on processing consideration (Mutanda *et al.* 2011) (harvesting, lipid extraction and lipid conversion techniques) with relatively less attention given to species and strain selection.

Chlorophyte genera such as *Botryococcus*, *Chlorella*, *Dunaliella*, *Nannochloris* and *Neochloris* are recognised as potential fresh water algae with potential production of

biofuel convertible lipids (20-75 % dry weight), whereas genera such as *Crypthecodinium*, *Isochrysis*, *Nannochloropsis*, *Phaeodactylum* and *Schizochytrium* are recognised as marine species with potential for production of lipids for biofuel (20-77 % dry weight) (Chisti 2007). To a large extent the microalgal biofuel research has been limited to selected species of microalgae, available from various culture collections. They have been imported by researchers (Table 1), (Liu *et al.* 2010; Liu *et al.* 2011; Widjaja *et al.* 2009; Xu *et al.* 2006). However, the same microalgal species, if grown in open pond production systems may not reasonably perform as well as those in controlled conditions (Chisti *et al.* 2003). For commercial scale production systems, it is important to focus on microalgal isolates from local habitats having a potential for lipid accumulation, as these are robust and have exceptional adaptation to the local environments (Sheehan *et al.* 1998).

Bioprospecting of microalgae involves various steps such as sampling of various habitats, isolation, purification and identification of species and screening for lipids (Mutanda *et al.* 2011). The success of bioprospecting depends upon efficient technologies for wide search of aquatic habitats for lipid producing microalgal species (Mutanda *et al.* 2011).

Table 1. Examples of microalgal species from various habitats isolated and deposited at culture collections, which were used for research in a different location.

Source	Microalgal Species/habitat	Isolated/deposited	Imported	Research conducted
Fidalgo *et al*. (1998)	*Isochrysis galbana* Marine	Culture Centre of Algae and Protozoa, England	Spain Israel	Effects of nitrogen source on lipid classes and fatty acid profile.
Rodolfi *et al*. (2003)	*Nannochloropsis* sp. Marine	Oceanographic and Limnological Research, Israel	Italy	Growth medium recycling in mass cultivation
Acien Fernandez *et al*. (2003)	*Phaeodactylum tricornutum* Fresh water	University of Texas, Austin	Spain	Biomass in helical reactor
Rangarao *et al*. (2007a)	*Botryococcus braunii* Fresh Water	University of Texas, USA	India	Effect of salinity on growth
Liu *et al*. (2010)	*Chlorella zofingiensis* Fresh water	American type culture collection (ATCC), U.S.A	China	Production potential for biodiesel and fatty acid profiles

The very first attempt at bioprospecting microalgae for high end products was conducted between 1978 to 1996, by The National Renewable Research Laboratories (NREL), United States of America (USA). Over 3000 species of algae from various locations of the west, the northwest and the south-eastern regions of the United States of America and Hawaii were collected, isolated and maintained (Sheehan *et al.* 1998). Furthermore, these numbers were reduced to 300 species based on screening, isolation and characterization and no single species recommended for large scale production (Sheehan *et al.* 1998).

In recent years, Yang *et al*. (2010) from Taiwan, collected microalgal strains from native environments. Their study aimed at screening heterotrophic microalgae for high docosahexaenoic acid (DHA, C22:6n-3) concentration. Moazami *et al*. (2011) collected 147 (marine) strains of microalgae from mangrove forests of Qeshm Island and the Persian Gulf (Iran) to study biomass and lipid productivities. Species belonging to the genera *Nanochloropsis*, *Chlorella* and *Nitzschia* and one unidentified diatom were selected from 147 species of microalgae for biomass and lipid productivity experiments. The biomass and lipid productivity ranged between 13.9 to 46.5 g L^{-1} and 13 to 46.5 mg L^{-1} d^{-1} across the microalgal species.

A similar study was conducted by Zhou *et al*. (2011), where microalgal samples were collected from fresh and waste water habitats of Minnesota and were tested for lipid productivity when grown in concentrated municipal waste (CMW). Sample collection was conducted between October, 2006 and January, 2010 and approximately 64 sites were sampled. Species belonging to the genus *Chlorella*, *Scenedesmus*, *Hindakia*, *Micractinium*, *Auxenochlorella*, *Heynigia* were isolated and tested for

biomass and lipid production capacities when grown in CMW. Biomass and lipid productivity ranged between 120 to 275 mg L^{-1} d^{-1} and 0.03 to 77 mg L^{-1} d^{-1} respectively across the microalgal species.

Project like the Aquatic Species Program has not been undertaken to date in Australia in spite of its rich ecological diversity of algae. However, efforts were made by the South Australian Research and Development Institute (SARDI) and Murdoch University, Australia in bioprospecting microalgae from local environments. Australia and Central Queensland (CQ) in particular, have a diversity of algal species from which a variety of products can be extracted. In Australia 12,000 species of marine, fresh water and terrestrial species of microalgae have been recorded (McCarthy *et al.* 2007) out of which 182 species of microalgae were recorded in CQ, and of these 2 % are endemic to the region (Fabbro 1999). Furthermore, the climate of the region is characterized by variable rainfall, leading to long periods when the water quality in a water body may be low in nutrients. Under conditions such as thermal and nutrient stress, microalgae have a tendency to modify their lipid metabolism to accumulate triacylglycerides (TAG's) (Singh *et al.* 2011). In these conditions, the growth of algae ceases leaving the synthesized triacylglycerides to accumulate in the cell (Meng *et al.* 2009). It is therefore postulated that a survey of CQ microalgae could lead to the discovery of strains with high lipid content.

2.2 Nutrient induced variation in biomass and lipid in *Chlorella* sp.

Accepted as a promising alternative source of biofuel feedstock, algal biomass is composed of carbohydrates, proteins and lipids. Biomass from microalgal production systems depends on CO_2 concentration (Chiu *et al.* 2008; Ge *et al.* 2011), nitrogen and phosphorus concentration (Chih-Hung & Wen-Teng 2009; Zhila *et al.* 2005), pH, salinity and culture media (Abu-Rezq *et al.* 1999; Cho *et al.* 2007; Ranga Rao *et al.* 2007b), culture media (Dayananda *et al.* 2007), light cycle and intensity (Kim *et al.* 2006; Perner-Nochta & Posten 2007), and temperature (Converti *et al.* 2009). The economic viability of microalgal biofuel research depends on high biomass, lipid yields and low production and processing costs (Liu *et al.* 2008). For the reason of low biomass and lipid content of *Navicula* and *Nitzschia* species compared to *Chlorella vulgaris* in this study, the literature review was focussed on biomass and lipid variations in *Chlorella* species.

Chlorella is a unicellular chlorophyte. It is spherical, non-motile, 2-10 µm in diameter and found in fresh and marine waters (Graham & Wilcox 2000). In recent years, *Chlorella* has gained popularity as a source of renewable bioenergy (Illman *et al.* 2000; Scragg *et al.* 2002; Xu *et al.* 2006). However, under normal cultivation conditions the lipid content varies between 14-30 % of dry weight (Spolaore *et al.* 2006) and for commercial production of biofuel the lipid content needs to be enhanced (Liu *et al.* 2008). Numerous studies were conducted on *Chlorella* species under nutrient limited and sufficient cultivation to increase the lipid content (Table 2).

Table 2. Summary of biomass and total lipid variations of *Chlorella* sp. to various nutrients reported in recent years towards biofuel research.

Source	*Chlorella* species	Biomass g L^{-1}	Cells mL^{-1}	Total lipid % dry weight	g L^{-1}	Cultivation conditions
Xu *et al*. (2006)	*Chlorella protothecoides*	3.4	-	15		Autotrophic growth
		3.74	-	55		Heterotrophic growth
Liu (2008)	*Chlorella vulgaris*	-	$13x10^6$ to $20x10^6$	57-8		Five levels of chelated iron as $FeCl_3.6H_2O$/EDTA
Chih-Hung & Wen-Teng (2009)	*Chlorella* sp.	0.849-2.02	-		0.33-0.66	Five urea concentrations (0.025-0.2 g L^{-1})
Widjaja *et al*. (2009)	*Chlorella vulgaris*	0.0086		45		Nitrogen limited growth medium on day 20
Ming (2010)	*Chlorella vulgaris*	0.68			0.65	KNO_3 variations (1.0 mM)
Phukan (2011)	*Chlorella* sp.	0.83-0.42		-		Various growth media (BG-11, BBM, MC-13 and Basal)

2.2.1 Role of pH and conductivity on the growth of algae

Measure of the hydrogen ions in a solution is defined as pH. Numerous studies have shown that pH plays a major role in terms of algal growth (Brock 1973; Celia & Edward 1994; Yoo *et al.* 2010; Yoo 1991) and also the species abundance in natural habitats (Leavitt *et al.* 1999; Pendersen & Hensen 2003).

In microalgal culture systems supplement with enhanced carbon sources such as CO_2 modify the pH of the growth media to enormous range (<4). In such case, pH is monitored and regulated throughout the experiment (Silva & Pirt 1984; Sung *et al.* 1998; Yue & Chen 2005). Other nutrients such as nitrogen sources affect the pH of

the media in either way. For example, if NH_4^+ is used by the algae the pH of the growth media will decrease and if NO_3^- is utilized the pH of the growth media will increase (Davis *et al.* 1953).

A recent study by Dayanada *et al* (2007) on the lipid yield of *Botryococcus braunii* grown under varies pH range of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 using chu 13 media. The culture was incubated for three weeks under 25 ± 1 °C and 1.2 ± 0.2 klux light intensity and 16:8 h photo period. It was reported that pH did not significantly affect the biomass and lipid yield however, at pH 7.5 the biomass (0.85 g L^{-1}) and lipid content (0.9 % w/w) were high. However, literature is limited regarding pH trends in normal and limited nutrient media.

Conductivity is a measure of the ability of water to conduct an electric current. They are numerous studies on conductivity in relation to algal ecology in the natural habitats (Baker & Humpage 1994; Bek & Robinson 1991; Grace *et al.* 1997). In a closed cultivation conditions temperature and evaporation rates influence conductivity measurements in a growth media (www. hydroponics.com). However, refilling with fresh media to compensate evaporation can help maintain conductivity at constant levels (Jiménez *et al.* 2003). Not much data is available on the influence of conductivity on algal biomass and lipid content and in most of the case it is considered as an indicator of evaporation rates.

2.3 Biomass estimation in microalgae

Microalgal biomass is defined as the total mass of cells in a given volume and is more often expressed as dry weight per unit volume. It is an important parameter for microalgal biofuel research. Numerous methods such as cell counting chambers

(manual and automated), optical density and dry weight and chlorophyll estimations are used to measure biomass concentration in microalgal cultures. The advantages and disadvantages of the methods are presented in this section. The economic and success of downstream (harvesting, extracting and conversion to biodiesel) process is dependent on the techniques used to assess biomass and lipid content of the microalgal species (Mutanda *et al.* 2011).

2.3.1 Microalgal biomass estimation using counting chambers

Conventional methods of enumerating microalgal cells employ a microscope with counting chambers such as Utermohl, Sedgwick-Rafter, Neubauer, Lund cell or haemocytometer (FAO 1996; Hotzel & Croome 1999). Choice of the chamber depends on the microscope, the species and the personnel experience (Hotzel & Croome 1999). Calibration of chambers is a prerequisite for each microalgal species used (FAO 1996). Time required to enumerate each sample depends on cell density. The sample needs to be fixed with solutions such as Lugol's iodine or formaldehyde (Wawrik & Harriman 2010) in order to prevent the movement of flagellates and diatoms which might lead to inaccurate counts. Microscope magnification needs to be selected depending on the size of the species (Hotzel & Croome 1999). The enumerated cell is expresses in cells mL^{-1} and is considered as a measure of biomass.

Counting algae using cell counting chambers is time consuming, laborious, expensive, and prone to inaccuracies (due to clumping of cells). To overcome this problem, electronic particle counters such as flow cytometers and coulter counters are widely used.

2.3.2 Microalgal biomass estimation using electronic particle counters

Conventional methods are recently being replaced by electronic particle counters such as flow cytometers, and coulter counters. With the development of these counters, enumeration of various cell types can turn out to be remarkably easy (Benson *et al.* 2005).

2.3.2.1 Flow cytometer

Operation of the flow cytometer is based on hydrodynamic focusing (Silva *et al.* 2012) The samples are injected into a flow cell (100 µm in diameter approximately) which has the capacity to transport fluids at the rate of 20 Km h^{-1}. (Veal *et al.* 2000). Light sources such as mercury vapour lamps or multiple laser beams are used with the photomultiplier tubes measuring the forward scatter (measures cell size), side scatter (provide information on cell internal features) and fluorescence of the sample particle passing through the light source (Veal *et al.* 2000). Cells can be differentiated and identified based on the scattering signals and to obtain cell counts microsphere standard is added to the samples(Silva *et al.* 2012).

Flow cytometry is a rapid and sensitive technique that can quantitatively measure cell counts (Maftah *et al.* 1993). Characterization and population dynamics (Fleck *et al.* 2006; Thyssen *et al.* 2011), toxicity studies (Franqueira *et al.* 2000; Hadjoudja *et al.* 2009; Prado *et al.* 2009; Yu *et al.* 2007) and metabolic activity (Xiao *et al.* 2011) of microalgae are some of the applications of flow cytometric techniques that are being applied. However, it is difficult to monitor low cell concentrations of microalgae using flow cytometry and this can result in insignificant counts (Vardon *et al.* 2011). To overcome this limitation the authors came up with a novel laser scanning

cytometry (LSC), where membrane filtration is combined with laser source to quantify microalgal cells. Vardon *et al*. (2011) constructed low cost LSC and its ability to count microalgal cells was tested against fluorescence microscopy (FLM) and flow cytometry.

2.3.2.2 Coulter counters

Invented in 1949 by W. H Coulter, coulter counters were initially used to count blood cells. Since their invention, coulter counters have been used in numerous applications such as bioengineering (Ebertz & McGann 2004), pharmaceuticals (Mosharraf & Nyström 1995), medicine (Cabrita *et al.* 1999) food and construction industries and various scientific studies of bacteria, viruses and microalgae.

The principle of the coulter counter is based on application of an electric field between two electrodes (one in the instrument aperture and the other immersed in the sample) and measuring the impedance between the electrodes. As the particles in the sample pass through the aperture or the light source, the scatter under the voltage curve is measured (Nieuwenhuis *et al.* 2004). Physical properties such as cell shape, size and refractive index influence the light's scattering properties. Any particulate material suspended in an electrolyte solution, ranging from 0.4 µm to 1600 µm can be measured using a coulter counter (Rodriguez-Trujillo *et al.* 2008).

Classic coulter counters have limitations such as small aperture (chances of clogging) (Washington 1992), and are expensive and use mercury to regulate pressure (Benson *et al.* 2005). However, these drawbacks were overcome with the development of new instruments, over a period of time.

Nieuwenhuis *et al*. (2004) developed a coulter counter based on a two dimensional liquid aperture control. The aperture is defined by a non-conductive sheath liquid that covers the conductive sample on three sides. The size of the aperture is controlled by changing the flow rates of sample and sheath liquid. This instrument increased the sensitivity and prevented clogging of the channel. However, the detection speed was low limited to 20 particles per second and the device classification was limited, (Rodriguez-Trujillo *et al.* 2008)

Benson *et al*. (2005) demonstrated mercury-free operation of the Multisizer II model coulter counter by inserting a pressure control to the sample stand. However this method has limitations such as aperture clogging. Also, as the flow rate increases, the measured volume decreases under increased pressures. For these reasons the flow rates (pressure) need to be monitored carefully at regular intervals of time (Benson *et al.* 2005).

A high speed coulter counter was developed with a two dimensional adjustable aperture was developed by Rodriguez-Trujillo *et al*. (2008), addressing the drawbacks of Nieuwenhuis *et al*. (2004). The particle detection rate is up to 1000/s with sample flow rate of 32 μm x 25 μm and 20 μm x 20 μm cross-sectional areas, respectively. The clogging problem was addressed by increasing the channel size. To reduce the cost, this device was built with transparent biocompatible materials as previously reported by Nieuwenhuis *et al*. (2004), who used silicon glass. However the aperture pump is operated manually and no effort was made to automate it. Despite the pros and cons of coulter counters, they are widely used to enumerate

microalgae (Fidalgo *et al.* 1998). A brief overview of various cell counting methods adapted in recent years is presented in Table 3.

In summary, microalgal biomass estimation using conventional counting chambers are laborious, inaccurate, and expensive and time consuming. Microalgal biomass estimation using electronic particle counters has draw backs such as small aperture size (requiring samples with high cell concentrations), aperture clogging (reason to monitor flow rates at regular intervals of time) and use mercury to regulate pressure.

Table 3. Summary of various cell counting chambers used to estimate microalgal cell counts.

Source	Microalgal species	Cell counting techniques
Sukenik and Carmeli (1991)	*Nannochloropsis* sp.	Coulter multisizer (Coulter Electronics Ltd., Luton, U.K).
Fidalgo *et al*. (1998)	*Isochrysis galbana*	Coulter counter
Elsey *et al*. (2007)	*Nannochloropsis* sp. *Tetraselmis* sp.	Z1 Coulter counter and Haemocytometer
Chiu *et al*. (2008)	*Chlorella* sp.	Haemocytometer and a Nikon Eclipse TS100 inverted metallurgical microscope
Liu *et al*. (2008)	*Chlorella vulgaris*	Haemocytometer
Chih-Hung & Wen-Teng (2009)	*Chlorella* sp.	Haemocytometer and a Nikon Eclipse TS100 inverted metallurgical microscope
Chiu *et al*. (2009)	*Nannochloropsis oculata*	Haemocytometer and a Nikon Eclipse TS100 inverted metallurgical microscope
Wawrik and Harriman (2010)	*Phaeodactylum tricornutum* *Chlorella vulgaris*	Epifluorescence microscopy under blue excitation using a micrometer grid on an Olympus BX-61 microscope.

2.3.3 Microalgal biomass estimation by optical density technique

Optical density (OD) or turbidity measurements are extensively used to measure the biomass of microalgae as such methods are relatively simple. The principle is based upon absorbance as a measure of light transmitted or scattered through a sample when exposed to a particular wavelength (nm) (Griffiths *et al.* 2011). Chlorophylls and carotenoids (Becker 1994) in microalgae range from 0.1 to 10 % of wet biomass and vary depending on the growth nutrients, temperature, light intensity and light cycle (Dolan *et al.* 1978). No particular wavelengths were specified in the literature to estimate microalgal biomass. However, a chlorophyll *a* absorbance range of 580 to 680nm was frequently reported in the literature (Table 4).

Error due to the impact of variation in growth conditions on pigment measurements using optical density was not often reported in the literature. One such effort was made by Griffiths *et al*. (2011) to investigate the error associated with OD values to dry biomass estimated in *Chlorella vulgaris* under various growth conditions across the growth cycle. They demonstrated that selecting wavelengths outside the range of pigment absorption (750nm or 550 nm) can minimise the error rate. Standard curves generated from entire growth cycle are recommended over curves from late exponential phase.

Table 4. Summary of publications on biomass quantification using optical density measurments.

Source	Microalgal species	Optical density methodology
Yue and Chen (2005)	*Chlorella* sp.	OD at 680 nm using a spectrophotometer.
Takagi (2006)	*Dunallelia tertiolecta*	OD at 680 nm and converted to ash-free dry cell weight employing the coefficient of OD_{680} to cell concentration of 0.01 ($g \cdot l^{-1} \cdot UOD^{-1}$).
Yen-Hui Lin (2007)	*Isochrysis galbana*	OD at 680 nm.
Chiu *et al*. (2008)	*Chlorella* sp.	Cell density (cells mL^{-1}) was measured by the OD at 682 nm (A_{682}) in an Ultrospec 3300 pro UV/Visible spectrophotometer (Amersham Biosciences, Cambridge, UK).
Liu *et al*. (2008)	*Chlorella vulgaris*	OD at 500 nm with a spectrophotometer (UV757CRT).
Chih-Hung and Wen-Teng (2009)	*Chlorella* sp.	Cell density (cells mL^{-1}) was measured by the OD at 682 nm (A_{682}) in an Ultrospec 3300 pro UV/Visible spectrophotometer (Amersham Biosciences, Cambridge, UK).
Chiu *et al*. (2009)	*Nannochloropsis oculata*	Cell density (cells mL^{-1}) was measured by the OD at 682 nm (A_{682}) in an Ultrospec 3300 pro UV/Visible spectrophotometer (Amersham Biosciences, Cambridge, UK).
Converti *et al*. (2009)	*Nannochloropsis oculata* *Chlorella vulgaris*	OD at 625 nm using UV-vis Spectrophotometer (Lamba 25, PerkinElmer, Italy).
Matsumoto *et al*. (2010)	*Navicula* sp. *Chlorella* sp.	Absorbance at 750 nm.
Xin (2010)	Scenedesmus sp.	OD at 650 nm.
Bhola *et al*. (2011)	*Chlorella vulgaris*	OD measurements at 686 nm using a Spectrophotometer (SpectroquantR Pharo 300, Merck, USA).
Moazami (2011)	*Nannochloropsis* sp *Nitzschia* sp (PTCC 6001)	Cell density measured with a spectrophotometer (Unicom UV–Vis Spectrometry) at 540 nm.
Abou-Shanab (2011)	*Scenedesmus obliquus*	OD at 680 nm using a spectrophotometer (HACH, DR/4000v, USA).

2.3.4 Microalgal biomass estimation by gravimetric technique

Gravimetric measurement of microalgal biomass is the most direct way of estimating biomass (FAO 1996). This procedure involves filtering a known volume of microalgal sample through glass fibre filter paper and drying the filter paper to constant (low) moisture (Table 5). The difference in weights of the filter papers before and after filtration is expressed as dry weight per unit volume. Dry weight estimations are simple and accurate but time consuming.

Alternatively, biomass can be determined by centrifuging a known volume of sample and measuring the weight of the pellet or concentrate. To obtain a good sized pellet and high density samples large sample volumes are required. Extracted samples are freeze dried for further estimation. Estimating biomass using centrifugation and freeze drying is widely used for microalgal research. Centrifugation speeds depend on the species of microalgae (Table 5). However, centrifugation is relatively expensive (capital costs of instrument).

2.3.5 Microalgal biomass estimation from chlorophyll measurements

Measuring biomass by estimating chlorophyll *a* involves sample filtration and extraction of the pigment with hot ethanol (Clark & Lidston 1993) or with cold acetone (Marker *et al.* 1980; Nusch 1980). Extracted chlorophyll *a* is quantified using various methods such as spectrophotometry, fluorometry or high-performance liquid chromatography (HPLC). All these extraction methods have their own advantages and disadvantages. A summary of literature on microalgal chlorophyll estimation is provided in Table 5.

HPLC has the potential to differentiate the type of pigments present in the microalgal culture. However it is relatively expensive and uses hazardous chemicals such as dimethyl sulfoxide, methyl alcohol and diethyl ether (Berkman & Canova 2007).

The fluorometric method is considered more economical compared to HPLC and requires less sample size than spectrometry, and requires use of fewer hazardous chemicals. However, it cannot distinguish between other pigments present in the sample leading to over or under estimations of chlorophyll *a* (Berkman & Canova 2007). The spectrophotometric method on the other hand is simple compared with the other two methods and has the capacity to distinguish between chlorophyll *a, b* and *c*. The disadvantage with this method is that it is less sensitive than fluorometric and HPLC methods (Berkman & Canova 2007).

Table 5. Summary of microalgal biomass estimations made using gravimetric and pigment assessment approaches in recent years.

Source	***Microalgal species***	**Methods**
		Microalgal biomass (dry weight) determination
Ranga Rao *et al*. (2007a)	*Botryococcus braunii*	Centrifugation at 5000 rpm, pellet freeze dried.
Zhu *et al*. (2007)	*Schizochytrium limacinum*	Centrifugation, pellet freeze dried.
Chiu *et al*. (2009)	*Nannochloropsis oculata*	Centrifugation, pellet dried at 105°C for 16 h.
Jacob Lopes *et al*. (2009)	*Aphanothece microscopica Nageli (RSMan92)*	Known amount of sample filtered through 0.4 µm filter, dried at 60°C for 24 h.
Yoo *et al*. (2010)	*Chlorella vulgaris* KCTC AG10032 *Scenedesmus* sp. KCTC AG20831 *Botryococcus braunii* UTEX 572	Cells filtered through glass fibre filter discs and dried at 105°C for 1 h.
Lee *et al*. (2010)	*Chlorella vulgaris* and *Scenedesmus* sp.	Centrifugation, cell mass frozen overnight at -70 °C and freeze dried at - 70°C under vacuum.
Matsumoto *et al*. (2010)	*Navicula* sp. Strain JPCC DA0580 *Chlorella* sp. NKG400014	Centrifugation, pellet freeze dried.
Abou-Shanab (2011)	*Scenedesmus obliquus* YSR01 *Nitzschia* cf*. pusilla* YSR02 *Chlorella ellipsoidea* YSR03	Centrifugation, pellet dried at 105°C over night.
Bhola *et al*. (2011)	*Chlorella vulgaris*	Centrifugation at 3000 rpm for 15 min at 4°C and pellet dried at 60°C for 24 h.
Ge *et al*. (2011)	*Botryococcus braunii* 765	Centrifugation at 1000 *g* for 10 min at 4°C, pellet freeze dried.
Liu (2011)	*Chlorella zofingiensis* (ATCC 30412)	Centrifugation at 3800 *g* for 5 min, pellet freeze dried at 70°C in a vacuum oven.

		Microalgal biomass determined by chlorophyll
Sukenik and Carmeli (1991)	*Nannochloropsis* sp.	Chlorophyll extracted using 90% methanol and spectra recorded on a Hewlett Packard diode array spectrophotometer.
Ranga Rao *et al*. (2007a)	*Botryococcus braunii*	Chlorophyll extracted using methanol and estimated spectrophotometrically at 652 and 665 nm
Zhang *et al*. (2011)	*Botryococcus braunii*	Chlorophyll extracted with 90% (v/v) acetone estimated spectrophotometrically at four wavelengths (630, 647, 664 and 730 nm)

2.4 Quantification of lipids in microalgae

2.4.1 Solvent extraction

Solvents can be used to separate lipids from cell constituents, by dissolving and extracting the lipids into a different phase (Smedes & Thomasen 1996). The quality and efficiency of the extraction process depends upon the solvent used and the concentration of solvents employed (Folch *et al.* 1957). For example, non polar solvents such as hexane and chloroform extract the neutral lipids from the cytoplasm by forming solvent-lipid complex. Whereas, polar solvent such asmethanol and isopropanol extract the lipid bound to protein forming hydrogen bonds (Halim *et al.* 2012). Selection of solvent proportions is important in extracting the lipids efficiently (Bligh & Dyer 1959). For example, chloroform dissolves almost all the non-polar lipids. (Smedes & Thomasen 1996). In pure methanol, triglycerides are almost insoluble.

Solvent extraction efficiency can be increased when used in conjugation with other applications such as bead beating, autoclaving, microwave, ultrasonication and osmotic shock (Table 6). The principle behind these applications is to induce pressure and temperature variation in cells forcing the contents into contact with the solvent the solvent phase. However, solvent extraction methods to extract lipids from microalgae are time consuming, use hazardous and carcinogenic chemicals and often requiring large sample size, and are destructive in nature.

Table 6. An overview of various solvents and processing methods used to extract lipids from microalgal biomass in recent years. Table adapted and modified from Lam and Lee (2011).

Source	Microalgal species	Solvent used	Processing method	Lipid (% dry weight)	Conclusions
Shen *et al*. (2009)	*Chlorella protothecoides* and *Scenedesmus dimorphus*	Ethanol /Hexane and Hexane only	Direct extraction, Sonication, French press, Bead-beater and Wet milling	26 and 30 using wet milling and Hexane respectively in *S. dimorphus*	Wet milling followed by Hexane was best for lipid yield and Bead beating followed by hexane was most effective for lipid recovery in *S. dimorphus*. These methods were not significant in case of *C. protothecoides*
Samorì *et al*. (2010)	*Botryococcus braunii*	n-hexane: chloroform: methanol =4:2.67:1.33 (v/v/v)	Freeze-dry	2.7	-
Lee *et al*. (2010)	*Botryococcus* sp.	Chloroform: methanol=1:1	Autoclave Bead-beater Microwave Ultrasonication Osmotic shock	11 28 28.5 8.5 10	Microwave cell disruption was the best among other methods. *Botryococcus* sp. had twice the lipid content of other microalgal species.
	Chlorella vulgaris	Chloroform: methanol=1:1	Autoclave Bead-beater Microwave Ultrasonication	10 8 10 6	

	Scenedesmus sp.	Chloroform: methanol=1:1	Autoclave Bead-beater Microwave Ultrasonication Osmotic shock	4 8 10 6 5.8	
Ranjan *et al*. (2010)	Scenedesmus sp.	n-hexane	Soxhlet Ultrasonication Chloroform:methanol =3:1 (v/v)	0.8 0.9 6	-
de Godos *et al*. (2011)	*Chlorella pyrenoidosa*	Chloroform: methanol=2:1	Magnetic strirring	19.7	Used as a lipid extraction method.
Halim *et al*. (2012)	*Chlorococcum* sp.	n-hexane		1.5	-

2.4.2 Supercritical fluid extraction

A supercritical fluid has physical properties intermediate to liquid and gas, therefore enhancing its diffusion into cellular materials (Halim *et al.* 2012). Application of supercritical fluid to extract microalgal lipids is a recent development. Solvents such as methanol, ethanol, benzene and toluene as well as gases such as CO_2 and ethylene have recently being explored as sources of supercritical fluids to extract lipids from microalgae (Couto *et al.* 2010; Halim *et al.* 2012).

Couto *et al*. (2010) conducted a comparative study of lipids extracted using supercritical carbon dioxide (SC-CO_2) and solvent extraction of *Crypthecodinium cohnii* (heterotrophically cultivated in a bioreactor). Lipid yields of 49.5% w/w were reported using solvent extraction which was double that of lipid yield achieved through SC-CO_2 extraction.

The advantage of SC-CO_2 extraction over solvent extraction is that it is non-toxic and non-oxidising and avoids thermal degradation of the lipid due to low critical temperature, easy separation of CO_2 and low surface tension (Halim *et al.* 2012; Lam & Lee 2011). However, high installation and operational cost (Lam & Lee 2011; Punín Crespo & Lage Yusty 2005) and safety issues are the major drawbacks of the SC-CO_2 extraction technique.

2.4.3 Chromatography

The basic principle of chromatography is the distribution of molecules between mobile and stationary phases. Separation of lipid classes is the initial step to lipid profile analysis. No single separation method is described in the literature that can separate all the lipid classes. Conventional methods involve the extraction of lipids followed by fatty acid conversion to methyl esters. These esters are then analysed and estimated using chromatographic techniques.

Chromatographic techniques like column, thin layer (TLC) and gas (GC) have been used to separate and estimate lipid classes from microalgal cells. Cziczo (2004) considered that thin layer chromatography was the preferred technique to separate neutral lipid classes from small samples. However, complex mixtures of lipids can only be separated using two dimensional thin layer chromatography (Hitchcock & Nichols 1971). A large number of non destructive reagents like 2, 7-dichlorofluorescein, Rhodamine 6G and Iodine are available to detect and characterize lipids on TLC (Hitchcock & Nichols 1971). Compared with column chromatography, TLC is quick and sensitive with good resolution. However, lipid class separation and estimation using TLC is being replaced by gas chromatography.

Gas chromatography (GC) is a powerful tool to analyse components of fatty acids in algal cells. Total lipids extracted from cells have to be converted to fatty acid methyl esters (FAME'S) using methods such as acid or direct trans-methylation (Christie 2003). The extracted, methyl esters are injected into the gas chromatograph equipped with a capillary column and flame ionization detectors (FID). The quantity

of lipid classes is estimated from the sample peaks in comparison with the internal standards such as heptadecanoic acid (Fidalgo *et al.* 1998; Liu *et al.* 2010; Liu *et al.* 2011). A summary of various chromatographic techniques applied to microalgal hydrocarbons is presented in Table 7.

However, slow loss of the stationary phase from the column makes it difficult to identify compounds in chromatography (Cziczo 2004). A combination of gas chromatography and mass spectrometry is therefore recommended, where the sample is split and exposed to the detector and mass spectrometer for analysis at the same time (Hitchcock & Nichols 1971). Limitations such as sample preparation time, destructive nature, complexity of instrument use, use of hazardous chemicals and long analysis time are the drawbacks of the present techniques.

2.4.4 Spectroscopy

2.4.4.1 Lipid soluble dyes

In vivo lipid detection within microalgae has been achieved using lipophilic fluorescent stains such as Nile red (Alonzo & Mayzaud 1999; Greenspan & Fowler 1985) and BODIPY (Cooper *et al.* 2010). These dyes provide for practical and rapid identification and isolation of microalgal species with lipid contents from a mixed culture of cells using microscopic techniques (Cooper *et al.* 2010).

Table 7. An overview of various chromatographic techniques applied to quantify microalgal lipids.

Source	Microalgal Species	Extraction Solvent	Separation	Analyser/Detector	capillary column
Dayananda *et al*. (2007)	*Botryococcus braunii*	n-hexane	Column chromatography on silica gel	GC/FID (Flame Ionization detector)	BP-5
Rangarao *et al*. (2007)	*Botryococcus braunii*	n-hexane	Column chromatography on silica gel	GC/FID	BP-5
Zhu *et al*. (2007)	*Schizochytrium limacinum*	n-hexane		GC/FID	DB-23
Vieler *et al*. (2007)	*Chlamydomonas reinhardtii* and *Cyclotella meneghiniana*	Chloroform/Acetone	Column chromatography on silica gel	MALDI-TOF mass spectrometry in combination with thin-layer chromatography	-
Petkov and Garcia (2007)	*Chlorella*	Chloroform/Methanol	TLC on silica gel	GC/FID	Supelcowax-10
Yihze *et al*. (2008)	Biodiesel sample	Methanol	-	Column chromatography	-
Chih-Hung and Wen-Teng (2009)	*Chlorella*	Methanol	-	GC/FID	-
Widjaja *et al*. (2009)	*Chlorella vulgaris*	Chloroform/Methanol	-	GC/FID	DB-5HT non-polar
Converti *et al*. (2009)	*Nannochloropsis oculata* and *Chlorella vulgaris*	Chloroform/Methanol	-	GC/FID	ZB Vax column
Lee *et al*. (2010)	*Botryococcus* sp. *Chlorella vulgaris* and *Scenedesmus* sp.	Chloroform/Methanol	-	GC/FID	-

Nile red is a benzophenoxazone dye produced from Nile blue by boiling it with diluted H_2SO_4 and extracting into xylene (Greenspan & Fowler 1985). It is a vital-dye with the capacity to penetrate living cells; is excited by wavelengths in the range 485-525 nm; and exhibits fluorescence in the visible range, with intensity and peak wavelength varying with the solvent used to dissolve the dye (peak emission wavelength shifting to shorter wavelengths with increasing hydrophobicity of the lipid molecule) (Greenspan & Fowler 1985). Fowler *et al*. (1979) also reported that Nile red has a low quantum yield in water. The fluorescence colours range from golden yellow to deep red depending on the lipid types. For golden yellow fluorescence, an emission filter of 450 to 500 nm is usually employed, while for red fluorescence, the range 515 to 560 nm is used (Stanley & Greenspan 1985).

Nile Red has been used for qualitative estimation of lipid content in various tissues for many decades. Given the reported change in emission wavelength with lipid type, the dye may also be useful for qualitative estimation of lipid type. Quantitative estimation of neutral lipids using Nile red was earlier reported by correlating the fluorescence data to gravimetric determination of lipids (Table 8).

In vivo application of this dye in microalgae studies has increased in recent years (Table 8). However, absorption of Nile red by microalgal cells is dependent on the thickness of cell wall and cytoplasm (Kimura *et al.* 2004). In order to overcome this issue, improved techniques such as microwave assisted cell membrane disruption with addition of dimethyl sulfoxide (Chen *et al.* 2011) and addition of phosphate buffer to minimize side reactions have been employed (Lee *et al.* 1998) (Table 8). Nile Red will generally not stain dead micro-algal cells as these cells lack neutral

lipids (the lipids are used by the microalgal cells during the decline stage) (Elsey *et al.* 2007). Other workers have reported on the species specific optimization of Nile red concentration, incubation temperature and time. However, variation in quantum yield by solvent composition and in emission wavelength by lipid type makes the use of Nile red application difficult to use in a quantitative sense in the context of cells grown under different conditions (Kimura *et al.* 2004)

BODIPY, chemically known as 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4adiaza-s-indacene (Haugland 1996), has been used to detect lipid droplets within animal and human tissue (Cooper *et al.* 1999; Listenberger & Brown 2007). Cooper *et al*. (2010), reported the use of green fluorescent dye BODIPY 505/515 to qualitatively visualise lipid in *Ophiocytium maius* Naegeli (Xanthophgycea), *Chrysochromulina* sp. (Haptophyceae) and *Mallomonas splendens* (Synurophyceae). BODIPY 505/515 has a narrow emission spectra compared to Nile red (Haugland 1996) and does not localize with cytoplasmic organelles other than lipid droplets (Cooper *et al.* 2010). In this work of Cooper (2010) BODIPY 505/515 was dissolved in anhydrous dimethyl sulfoxide (DMSO), and then directly added to the microalgal culture (of cultures grown using various growth media, light intensity, temperature and light cycles). Epifluorescence microscopy was employed using excitation wavelengths of 450-490 nm to visualise lipid particles. Lipid bodies appeared green, providing good contrast to the other major auto fluorescence source, chloroplasts, which appeared red.

Govender *et al*. (2012) used BODIPY 505/515 to stain intracellular lipid within *Chlorella vulgaris*, *Dunaliella primolecta*, and *Chaetoceros calcitrans*. A fluorescence spectrophotometer (Varian Australia Pty. Ltd.) was used to quantitatively measure

fluorescence, with the authors exploring optimisation of labelling conditions (concentrations of BODIPY ranging from 0.25 to 5 µL mL^{-1} and staining times from 0-30 min were trialled). A cell concentration of 1 x 10^6 cells mL^{-1} and a BODIPY 505/515 concentration of 0.067 µg mL^{-1}, incubated for 2 to 4 min were recommended as optimum for all the microalgae tested. The authors reported that BODIPY 505/515 was a potential dye for the determination of neutral lipids in microalgae.

2.4.4.2 Colorimetry (optical spectrophotometry)

The basic principle of colorimetry is that the amount of light absorbed by a substance is proportional to the concentration of the material and the optical path length. Colorimetric analysis is regularly applied to proteins and protein fractions (Fountoulakis & Lahm 1998; Gerald 1977; Hyman 1957; Spies & Chambers 1949), lipids and lipid fractions (Amenta 1970; Bragdon 1951; Duncombe 1963; Sperry & Brand 1955; Wawrik & Harriman 2010), carbohydrates and their fractions (Deriaz 1961; Dubois *et al.* 1956; Gilbert 1957, 1966).

Colorimetric analysis of lipids dates back to the 1900's, with application to microalgal lipids since the 2000's. Wawrik and Harriman (2010) described a colorimetric method to quantify lipids in *Phaeodactylum tricornutum* and *Chlorella vulgaris* in which microalgal lipids were hydrolysed to fatty acids, then HCl, copper reagents and chloroform were added, the mixture centrifuged, and the organic phase separated. Sodium diethyldithiocarbamate in 2-butanol was added to the organic phase, and a yellow colour developed. The resultant colour was measured by the authors at 440nm using an IMPLEN NanoPhotometer.

Table 8. An overview of literature on the use of Nile red to quantify neutral lipids in microalgae.

Source	Microalgal species	Coprocessing	Fluorescence measurement	Comments/conclusions
Lee *et al*. (1998)	*Botryococcus braunii*	Bead beating, sonication, high pressure (170 atm), lyophilisation, homogenization	Digital fluorimeter (Turner, Model 450) excitation 490 nm and emission 585 nm filters	R^2 of 0.998 between gravimetric measure and Nile red fluorescence
Elsey *et al.* (2007)	*Nannochloropsis* sp. *Tetraselmis* sp.	No processing Microalgal suspension of 3 mL was used	Spectrofluorimeter ISS excitation 486 nm and emission 570 nm	Stock solution of hexane was used for day to day calibration of the instrument *Nannochloropsis* sp. exhibited 6.5 times more fluorescence under nitrogen limited condition
Chen *et al*. (2009)	*Nannochloris* sp. *Nannochloropsis* sp. *Pavlova pinguis* *Chlorella zofingiensis* *Chlorella vulgaris*	Temperature 20-80 °C	Varian 96-well plate spectrofluorometer Excitation 530 emission 575 nm	No significant differences were detected in the lipids quantified using gravimetric methods and Nile red method
Huang *et al*. (2009)	*Chlorella pyrenoidosa* *Chlorella vulgaris*	Lyophilized culture	Spectrofluorometer (HITACHI F-4500, Japan) excitation 480 nm and emission 590nm filter	R^2 value of 0.99 between gravimetric quantification of lipids and Nile red method

Chen *et al*. (2011)	*Pseudochlorococcum* sp. *Chlorella zofingiensis* *Scenedesmus dimorphus*	Microwave	Varian 96-well plate spectrofluorometer Excitation 490 and emission 580 nm	Microwave assisted staining was optimized Quantification of neutral lipids using Nile red needs calibration curve of intracellular neutral lipids
Bertozzini *et al*. (2011)	*Chaetoceros socialis* *Alexandrium minutum* *Skeletonema marinoi*	Addition of lipid standard triolein to samples	Spectrofluorophotometer RF-5301PC (Shimadzu, Japan)	Detects low concentration of neutral lipids

However, it was noted that precaution was needed to avoid presence of water as a reaction with the dissolved copper salt resulted in a false signal. Further, the signal from co-extracts such as chloroform must be subtracted in order to avoid overestimation of lipid content.

Colorimetric analysis is relatively inexpensive and rapid. However limitations such as use of hazardous chemicals, the requirement for precise heating procedures and careful preparation of standard solutions are major factors limiting adoption of such methods.

2.4.4.3 Nuclear Magnetic Resonance (NMR) spectroscopy

The principle of NMR is based on the relaxation time of the hydrogen molecule in a compound of interest (Todt *et al.* 2001). Time-Domain Nuclear Magnetic Resonance (TD-NMR), also known as low resolution NMR, was introduced 35 years ago through the efforts of Unilever Research in the Netherlands (Todt *et al.* 2006). It was developed for analysis of fat composition in terms of solid to liquid ratio (Todt *et al.* 2006). This method, known as Solid Fat Content (SFC) determination, is now accepted as an international standard method by organization such as the American Oil Chemist Society and the International Standards Organization (ISO).

However, the technique cannot differentiate between molecules with the same transverse and longitudinal relaxation properties - for example fat and water molecules (Todt *et al.* 2006). Thus, for analysis of fat content in a biological sample, TD-NMR is a two-step procedure requiring drying of the sample (using oven, chemicals such as Cu_2SO_4, microwave or infrared drying) to remove water prior to TD-NMR analysis (Todt *et al.* 2006).

The first application of TD-NMR to estimation of microalgal lipids was in 2008. Gao *et al*. (2008) compared Nile Red staining and time-domain nuclear magnetic resonance (TD-NMR) against gravimetric determination of lipids in *Chlorella protothecoides*. Lipid content was analysed using a spin-echo NMR pulse sequence, which was calibrated using pure lipid from *C. protothecoides.* Cells were freeze dried before analysis. Cell lipid percentage ranged from 9 to 57 % dry weight and the correlation coefficient of determination against the gravimetrical estimates of extracted lipids was higher for TD-NMR (R^2=0.9973) than the Nile Red staining (R^2=0.9067). It was concluded that TD-NMR is equivalent to conventional solvent extraction for total lipid analysis.

In summary, TD-NMR estimation of lipids in microalgae is rapid and sample presentation involves only drying. However, the technique requires a pure component for calibration, limiting its application to biological materials (Hitchcock & Nichols 1971), and it involves access to an expensive piece of instrumentation.

2.4.4.4 Infrared Spectroscopy

The principle of Infrared spectroscopy (IR) is based on the absorption of infrared radiation (2500–16,000 nm) principally by fundamental and first overtones of stretching and bending of asymmetric bonds, such as C-H, O-H, S-H. These features create a spectral fingerprint allowing identification of the unknown sample. This technique can be used in quantitative analysis. Various optical geometries can be used, such as transmission, reflectance, attenuated total reflectance and diffuse reflectance, depending on the sample type. However, high absorption in IR (e.g. by water) limits the effective transmission depth.

Fourier transform infrared spectroscopy (FTIR) is probably the most common infrared spectroscopic method in use. This technique allows a wavelength resolution far superior to that achievable using a wavelength dispersive element (such as a grating). FTIR was once used only in the study of chemical structures, however in recent years has been applied in the fields of forensic science, the pharmaceutical industry and material sciences, and in animal and plant cell biology (Murdock & Wetzel 2009). Sampling accessories are selected based on the state of the sample, i.e. solid, liquid or gas (Hsu 1997). The technique has also been coupled to microscope optics. Sample preparation is generally minimal; however water is a very strong absorber of regions within the IR, so the water content of the sample should be removed if possible.

The Environmental Protection Agency (EPA) (USA) has established semi-quantitative methods using FTIR to determine the presence of petroleum hydrocarbons, oils and grease present in surface, saline, domestic and industrial waters (Hsu 1997). Petroleum hydrocarbons, oils and grease have carbon-hydrogen bonds which give rise to C-H stretching absorption in the 3100-2700 cm^{-1} region of the IR spectra. Semi-quantitative analysis is estimated by comparing the infrared spectra of the samples with the spectra of the standards.

Much work has been done using FTIR spectroscopy to study the biochemical variations of microalgal species and of cultures grown under various growth conditions (Table 9). Sample processing is minimal as the cell walls and cell size of microalgae are thin enough to allow acquisition of clear spectra (Murdock & Wetzel 2009). For example, Murdock *et al*. (2008) studied the feasibility of FTNIR micro-

spectroscopic imaging to assess the cellular and sub-cellular variability to nutrient availability of *Cladophora glomerata*. This study described a method where filamentous benthic algae (*Cladophora glomerata*) were placed on an IR reflective slide (Low-e, Kevley Technology, Chesterfield, OH, USA) and sufficient deionised water was added to allow even spreading of the sample. Excess water was removed and the slides were dried in a desiccator. Only cells without any obvious physical changes from drying were used for spectra acquisition.

FTIR microscopy is a promising technique for selection of potential oil accumulating species, and for estimation of lipid accumulation under different growth conditions. However, as noted above, the technique is relatively non-quantitative, and requires the removal of water from the sample. Near Infrared spectroscopy is an alternative technique, as absorption by water is much lower in this region.

Table 9. An overview of infrared spectroscopy using FTIR application to assess biochemical composition of microalgae in recent years.

Source	Microalgal species	Experimental condition	Wave range (cm^{-1})	Spectral resolution (cm^{-1})
Giordano *et al*. (2001)	*Chaetoceros muellerii*	N starvation/FTIR spectromicroscopy	3650-700	8
Beardall *et al*. (2001)	*Scenedesmus quadricauda* *Nitzchia* sp	Algae under P limitation/FTIR microspectrometer	7000-600	8
Hirschmugl *et al*. (2006)	*Euglena gracilis*	Cells in fresh media/FTIR absorbance coupled to IR microscope	4000-700	8
Sigee *et al*. (2007)	*Scenedesmus subspicatus*	P limited (0.05, 0.5 and 5 mg L^{-1}) /Synchrotron-based Fourier transform infrared (FTIR) microspectroscopy	4000-900	4
Dean *et al*. (2008)	*Chlamydomonas reinhardtii*	Carbohydrates, lipids and protein changes to P availability/FTIR microspectroscopy	4000-900	4
Dean *et al*. (2010)	*Chlamydomonas reinhardtii* *Scenedesmus subspicatus*	Lipid and carbohydrate N-limited conditions/FTIR spectroscopy	4000-600	-
Laurens and Wolfrum (2011)	*Nannochloropsis* sp. *Chlorococcum* sp. *Spirulina* sp.	Exogenous lipid estimated. Lyophilized biomass was mixed with lipid spikes/FTIR attenuated total reflectance	4000-500	4
Odlare *et al*. (2011)	Algal species in lake Malaren	Carbohydrate and lipids and species classification/FTIR absorbance	4000-600	6

2.4.4.5 Near Infrared Spectroscopy

On impacting a sample, light may be reflected, transmitted or absorbed. Visible light from 450-700 nm generally cannot penetrate more than 1 cm into biological tissues due to strong absorption and scattering (Owen-Reece *et al.* 1999). Near infrared radiation between approximately 1100-2500 nm is strongly absorbed by water, and thus has an effective path length of around 1 mm within biological tissue. However, the short wavelength NIR region (700-1100 nm) has the capacity to penetrate relatively deep into biological tissue, yielding information on composition and structure.

The history of NIR dates back to 1800's (Barton 2002). However, its utility as an analytical technique was recognised and developed by Karl Norris of the United States Department of Agriculture (USDA) in the 1960/70s (Mark & Campbell 2008). The near infrared region of electromagnetic spectrum is now utilised for analytical purposes in the fields of agriculture, medicine, pharmaceuticals, forensic sciences, material sciences, remote monitoring, particle measurement and industrial use. NIR measurements are relatively rapid and accurate, non-destructive, avoid use and disposal of hazardous and expensive chemicals, have the capability for analysis of large and inhomogeneous samples and various components from a single spectral measurement (Mark & Campbell 2008). The use of the NIR region is justified given the lower absorptivity of water in this region, compared to the IR, allowing assessment of whole cells and tissues. However, absorbance features are broader and more overlapped in the NIR than the IR region, thus a statistical (chemometric) approach is required (Mark & Workman 2003; Martens & Naes 1992). This involves

the collection of spectra and reference analyses of a large number of samples. Due attention is also required to an appropriate optical geometry for the attribute and sample type under consideration (i.e. reflectance, interactance, transmittance).

NIR spectroscopy (NIRS) is considered a secondary method as it needs another method (primary) to validate and calibrate the data. A detection limit using NIR spectroscopy of around 1 % on a weight basis is typically reported (Mark & Campbell 2008). Near infrared methods to assess physical and chemical properties of materials requires the application of calibration and statistical analysis to validate a spectral model (Mark & Workman 2003; Martens & Naes 1992). The scanning of a large number of samples and acquisition of valid reference values is thus the basis of quantitative analysis of unknown samples. Sample selection and spectra acquisition conditions (geometry, signal to noise etc) are also important steps in NIR application.

NIRS is widely used in assessment of hydrocarbon content of both natural products and petrochemicals (Table 10). For example, in the petrochemical industry, NIR based technology used to estimate quality based on hydrocarbon composition, octane number, cetane number and Reid vapour pressure (Hidajat & Chong 2000). NIR cannot directly measure the olefins or naphthenes present in hydrocarbons but it can measure the functional groups of methyl, methylene and methane, which can be related to the hydrocarbon composition of the samples (Mark & Campbell 2008).

Table 10. An overview of NIR applications to estimate lipids and their components in various materials in recent years.

Source	Sample analysed	Analysed for	Instrument	Resolu-tion nm	Spectral range nm	Spectral processing	Regre-ssion	RMSEP (%)	Reference method
Hidajat and Chong (2000)	Crude oil	Density and total boiling point	Perkin-Elmer Spectrum 2000 spectrometer	-	1600-2600	MSC	PLS	1.20 (diesel fraction)	Density (ASTM D1298) Total boiling point (ASTM D2892)
Almendingen *et al*. (2000)	Homogenized diets	Fat content	Technicon 500	4	1100-2500	MSC	PLS	0.49 (fat content varied from 6.50-17.11)	Folch procedure
Solberg *et al*. (2001)	Minced Capelin	Fat content	Infratec 1255 Food and Feed analyser	2	850-1050	-	PLS, PCA	>0.25 (fat content varied from 0-12 5)	Ethyl acetate extraction
Tajuddin *et al*. (2002)	Soybean seeds	Lipid content	NIRSystems 6500	-	700-1100	Second derivative spectra	PLS	-0.14 (≥6 mm group) -0.09 (<6 mm group)	Solvent extraction
Gonzalez-Martine *et al*. (2003)	Iberian breed swine	Fatty acids	Foss NIR Systems NR-5000 system	2	1100-2498	SNV DT	MPLSR	0.02-1.06 (across the fatty acids)	Solvent extraction and GC
Blazquez *et al*. (2004)	Processed cheese	Fat content	NIRSystems 6500 scanning monochromator	2	400-2498	SNV, DT	MPLSR	0.48 (1100-2500 nm)	IDF 5B:1986 solvent extraction
Golebiowski	Canola seed	Oil content	Foss NIRSystem	2	1100-	SNV, DT	-	-	Soxhlet

(2004)					2500				method
Pizarro *et al*. (2004)	Roasted coffee	Lipid content	NIRSystems 50	2	1100-2500	SNV	PCA, PLS	8.3	-
Yang *et al*. (2005)	Edible oils and fats		Nicolet 870 spectrometer	16	5500-6000		PCA, PLS	-	Iodine number
Suehara *et al*. (2007)	Waste water from biodiesel fuel production plant	Oil content	NIRS6500SPL spectrophotometer	2	800-2500	Second derivative spectra	MLR	-	Hexane extraction
Müller and Scheeder (2008)	Raw pig fat	Fatty acid composition	TA-XT2 Stabel Micro Systems	-	2400-9600	-	PLS	0.9-SFA 1.6-MUFA 4.7 -PUFA	GC
Garrido-Varo *et al*. (2008)	Animal origin fats in fat blends	Fat content	Foss NIRSystems 6500 SY-I scanning monochromator	-	400-2500	SNV, DT	MPLS	1.3	-
Sohn *et al*. (2009)	Processed cereal products	Fatty acid composition	NIRSystems 6500 spectrometer	2	400-2498	SNV	MPLS	3.5-4.2 (across fatty acids)	GC
Fernández-Cabanás *et al*. (2011)	Iberian pork dry-cured sausages	Fatty acid profile	Foss-NIRSystems 6500 SY-II monochromator	2	400-2498	SNV DT	MPLS	0.98 (SFA), 1.47 (MUFA) and 0.88 (PUFA)	GC

MSC- multiplicative scatter correction, SNV-standard normal variant, DT-Detrend, PLSR-Partial Least Square Regression, PCA-Principle Component Analysis, MPLSR-Multi way Partial Least Square Regression, MLR-Multi linear Regression, GC-Gas Chromatography, SFA-Saturated Fatty Acids, MUFA- Monounsaturated Fatty Acids, PUFA-Polyunsaturated Fatty Acids

NIRS has been used for the determination of fat content of biological materials such as meats and products (Folkestad *et al.* 2008; Lanza 1983; Norris *et al.* 1976; Viljoen *et al.* 2007), oils seeds (Golebiowski 2004; Hymowitz *et al.* 1974; Krishnan *et al.* 1994; Tajuddin *et al.* 2002) and milk (Coppa *et al.* 2010). NIRS has been effectively used to determine the lipid composition of edible oils (Safar *et al.* 1994), milk (Coppa *et al.* 2010; Soyeurt *et al.* 2006) and meat products (González-Martín *et al.* 2005; Pla *et al.* 2007). However, despite widespread application for lipid analysis, there are few reports of the use of this technology in the context of microalgae.

NIRS has also been applied to process control, with measurement occurring via fibre optic probes to the reactor vessel or within piping to control the quality of the final product (Brudzewski *et al.* 2006). For example, it is used in the pharmaceutical industry to monitor blend and content uniformity and thickness measurements of tablets (Blanco *et al.* 2010; Moes *et al.* 2008; Scotter 1990), in the chemical industry (Zachariassen *et al.* 2005), in the petroleum industry to monitor the quality of gasoline (Balabin *et al.* 2010; Balabin & Smirnov 2011), to monitor the adulteration of diesel fuel (Fernanda Pimentel *et al.* 2006; Oliveira *et al.* 2007), motor oil classification (Balabin *et al.* 2011) and in the food industry to monitor quality and undesirable substances in food products (Collell *et al.* 2012; Fernández Pierna *et al.* 2012; Sinelli *et al.* 2011). If using the 1100 – 2500 nm wavelength range, these applications usually employ a reflectance or an Attenuated Total Reflection (ATR) probe designed for measuring highly absorbent samples, to avoid the problem of strong absorbance by water. A practical issue is then that of mixing, such that the sample in contact with the probe is representative of the whole vessel. Alternatively,

if the Vis-SWNIR region (550 to 1100 nm) is used, a transmission or transflectance optical geometry is often used.

Biomass of microalgal cultures is often measured from culture samples through cell counts, dry weight, chlorophyll estimation or optical density measurements within the wavelength range 550-700 nm (Chapter 2, Table 4). However, a measure of algal biomass *in situ* within culture vessels would be very useful in cell culture management (Sandnes *et al.* 2006). Sandnes (2006) reported growth of *Nannochloropsis oceanic* using f/2 medium in a photo bioreactor, with cell density monitored using optical density sensors consisting of NIR light emitting diodes (LEDs) of 880 nm emission. The optical sensors were calibrated to dry weight (obtained by centrifugation and drying at 100 °C overnight) and cell counts (measured using a Coulter Multisizer particle counter) of *Nannochloropsis oceanic*. The authors concluded that the sensor developed could be used to estimate the cell density during the mass cultivation.

2.5 Conclusion

Bio-prospecting microalgae for value added product from local environments is of particular importance for large scale production. Numerous works suggest that microalgae imported from culture collections may not perform similarly to those in laboratory conditions when grown outdoors. On the other hand, importing microalgae from culture collections has limitations such as the requirement to clear the biosecurity policies and procedures of that particular country. For example, to import microalgal cultures into Australia from other parts of the world for commercial purposes, one has to clear the Australian Quarantine and Inspection Services

(AQIS) requirements. In a few cases this needs justification and can be expensive. Once imported, there is no assurance that the strain will perform in a manner similar to that in the previous environments. Such reasons illustrate the importance of bio-prospecting microalgae for value added products.

Biomass estimation of microalgae using manual counting chambers or electronic particle counters is time consuming, laborious and expensive. Biomass estimation using optical density measurements is relatively simple however; the technique requires calibration, optimised for each individual species of microalgae and growth conditions, which is time consuming. Biomass estimation using a gravimetric method and chlorophyll content in the microalgal cells is time consuming, expensive and uses hazardous chemicals.

Lipid estimation methods in microalgae are generally based on a solvent extraction which use hazardous chemicals, are time consuming, require a large sample size, destructive in nature, expensive and require technical expertise in most of the methods.

In practice, the adoption of a technology relies on a number of factors that include price, ease of use, user support and consumer acceptance. NIRS equipment such as the FOSS NIRSystems 6500 and the Thermo Nicolet FTNIR are well established in the research community and in process control applications; however the cost approaching $100,000 AU per unit limits its use to high throughput/high value applications. SWNIR equipment is generally cheaper, e.g. the handheld Nirvana unit is marketed at around $15,000. This wavelength range and geometry should be suited to the assessment of liquid cultures of microalgae.

It may be possible to apply VIS-SWNIRS to assessment of algal biomass and lipid content *in situ*, i.e. of the microalgal culture directly. Alternatively, it may be possible to assess these parameters of dried samples using NIRS (1100-2500 nm). In any application, attention to factors such as sample presentation, optical geometry, reference method and data pre-processing methods is required. The robustness of any model also needs to be demonstrated in terms of the accuracy of prediction across multiple populations not included in the calibration set.

In this study, the Central Queensland region was bio-prospected for microalgal species containing neutral lipid and the isolated species were assesses for biomass and lipid content using Near Infrared Spectroscopy.

Chapter 3. Bioprospecting of native microalgal species with lipid content from Central Queensland

3.1 Introduction

The first objective of this thesis is “To collect, screen and isolate microalgal strains from various waters bodies in the Central Queensland region and select for neutral lipid content”.

To a large extent microalgal biofuel research has been limited to selected species of microalgae, sourced from various culture collections. These species/strains have been demonstrated to accumulate lipids under certain growth conditions (Liu *et al.* 2010; Liu *et al.* 2011; Widjaja *et al.* 2009). However, the same microalgal species/strains, if grown in open pond production systems may not perform as well as in controlled conditions (Chisti *et al.* 2003). Selection of microalgal species with high growth rates and lipid contents from local environments has also the major advantage of avoiding bio-security issues.

Most nations including Australia, have acted policies and procedure that restrict importation and use of imported microalgae (http://www.daff.gov.au/ba). For example, AQIS (Australian Quarantine and Inspection Services) has become conscious of the potential for contamination of imported cultures of microalgae with various disease agents. Given successful importation, onerous use restrictions are also likely (around restricting release to environment). Such considerations drive a need to the identification of endemic strains for oil production.

Microalgal species of green algae and diatoms have received most consideration as candidates for biofuel production. Numerous authors have reported isolation of microalgae for production of value-added products such as carbohydrates, lipids and proteins (Mutanda *et al.* 2011; Sheehan *et al.* 1998; Yang *et al.* 2010; Zhou *et al.* 2011). Commercial scale culturing of microalgae currently occurs for use in proteins from *Spirulina* and *Chlorella* (Lee 1997), Beta Carotene from *Dunaliella* sp, whole cell aquaculture feed from *Tetraselmis, Nannochloropsis, Isochrysis, Nitzschia*, Polyunsaturated fatty acids from *Crypthecodinium*, *Schizochytrium*, biofuels from *Botryococcus, Chlamydomonas, Chlorella, Dunaliella, Neochloris* (Rosenberg *et al.* 2008).

Australia and Central Queensland (CQ) in particular, have a diversity of microalgal species from which a variety of products could be extracted. However, microalgae strain selection projects like the Aquatic Species Program undertaken in the U.S.A have not been undertaken to date in Australia. Comparatively modest efforts have been made by South Australian Research and Development Institute (SARDI) and Murdoch and James Cook Universities in bioprospecting of microalgae from local environments.

Approximately 100,000 species of diatoms (Bacillariophycea), 8000 species of green algae (Chlorophyceae), 2000 species of blue-green algae (Cyanophyceae) and 1000 species of golden algae (Chysophyceae) exist globally (Khan *et al.* 2009). In Australia approximately 12,000 species of marine, fresh water and terrestrial species of microalgae are documented to occur, however, it is recognised that many species are not documented (www.environment.gov.au/biodiversity/). Within Australia, 182

species of microalgae have been documented in Central Queensland region of which 2 % are endemic to the region (Fabbro 1999). These identified endemic species belong to the Cyanobacteria reflecting a focus in the work of Fabbro (1999) on this group. Thus in terms of documented species diversity, it cannot be said that the CQ region is especially rich in microalgal diversity. However, the climate of the region is characterized by variable rainfall, leading to long periods when the water quality in a water body may be poor. Under conditions such as thermal and nutrient stress, microalgae have a tendency to modify their lipid metabolism to accumulate triacylglycerides (TAG's) (Singh *et al.* 2011). In these conditions, the growth of algae ceases, leaving the synthesized triacylglycerides to accumulate in the cell (Meng *et al.* 2009). It is therefore postulated that a survey of CQ microalgae could lead to identification and isolation of microalgal strains with high lipid content.

Previous studies considering 'bio-prospecting' of microalgae have utilized a range of growth media. For example Bolds Basal Medium is suitable for fresh water Chlorophycea (although unsuitable for microalgae with vitamin requirements), while Guillard f/2 media is used to grow diatoms and ASM is used to grow Cyanobacteria. However, growth media can be modified according to the species requirements (Mutanda *et al.* 2011).

3.2 Materials and Methods

3.2.1 Microalgal species Collection

Field trips for sample collection were conducted from May to September, 2010 and were focused on water habitats such as creeks, rivers, lakes, manmade ponds and

small water reservoirs of fresh, saline and brackish waters in Central Queensland. Approximately 90 sites were covered in seven localities (Figure 1) (Table 11). Out of 90 sites approximately 15-25 were from the Bundaberg region (collected by other persons) for which the site locations were not available.

Sampling was undertaken using a phytoplankton net (25 µm Silk Screens mesh, 30 cm diameter, 50 cm long). The samples collected were transferred to a 500 mL labelled transparent plastic bottles (BTL 28/410 STD RND, PB packing, Australia). Water samples were collected randomly from 3 to 5 locations at each water source and covered the length and width of the water body. Physiochemical parameters such as temperature, conductivity, and pH were monitored of each sample site using a Horiba 131 meter (Horiba, Japan). Samples collected were transported (in < 3 hours after collection) to the laboratory for screening, isolation and purification of microalgal species containing neutral lipids.

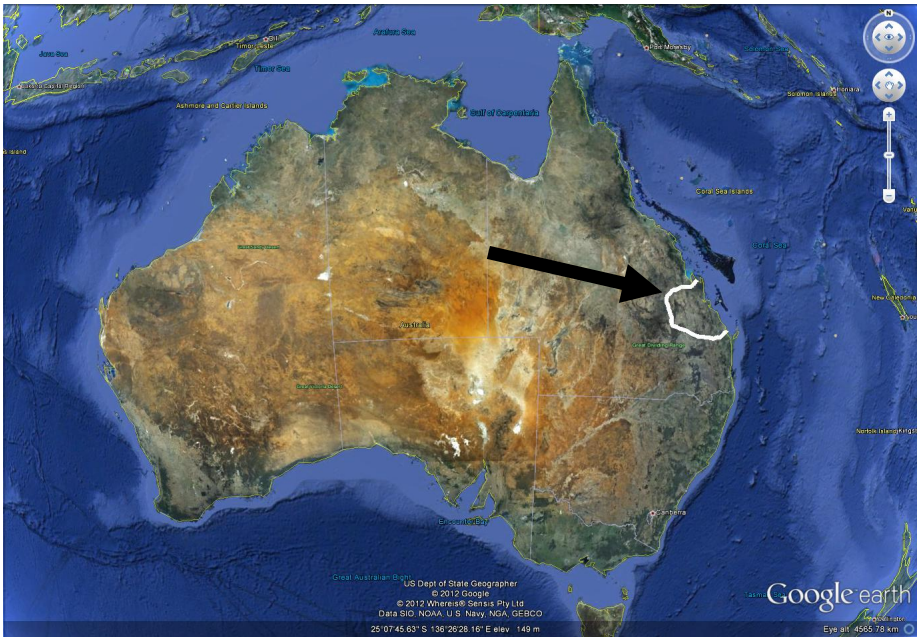


Figure 1. Region of Central Queensland from which water samples for microalgal species having lipids were collected. Sample collection area is arrowed.

Table 11. Water collection sites and GPS coordinates and pH and EC monitored of each sample site

	Water sample collection site	GPS co-ordinates		pH	EC (µS cm^{-1})
		Latitude	Longitude		
1	Moores creek, German street, Rockhampton	23°20'11.71"S	150°32'33.91"E	8.1	456
2	Moores creek, Dean street, Rockhampton	23°20'25.68"S	150°32'17.37"E	7.5	403
3	Moores creek, Musgrave street, Rockhampton	23°21'17.04"S	150°31'28.67"E	7.5	457
4	Near united 24 petrol station, Bruce highway, Rockhampton	23°24'18.47"S	150°30'10.90"E	8.4	756
5	Breakspear street, Gracemere	23°27'1.70"S	150°28'11.58"E	7.8	456
6	Four mile creek, Gracemere	23°28'8.91"S	150°29'45.76"E	8.6	832
7	Gracemere Lagoon	23°26'8.65"S	150°26'37.71"E	7.6	511
8	Fairy bower road, Gracemere	23°24'43.23"S	150°26'28.95"E	7.7	523
9	Nine Mile road, Pink Lily	23°22'13.66"S	150°27'30.94"E	7.7	516
10	Paradise Lagoon, Gracemere	23°23'3.54"S	150°24'23.92"E	8.3	787
11	Fig farm, Alton Down	23°18'3.16"S	150°23'51.76"E	8.2	659
12	400 m away from Fig farm, Alton Down	23°17'44.77"S	150°23'37.64"E	8.1	577
13	800 m from Fig Farm, Alton Down	23°17'13.20"S	150°23'40.81"E	8.1	605
14	1200 m away from Fig farm, Alton Down	23°16'59.26"S	150°24'18.02"E	7.9	453
15	1600 m from Fig farm, Alton Down	23°16'36.95"S	150°24'9.74"E	7.7	414
16	Yeppeen lagoon, Fairy Bower, Rockhampton	23°24'37.40"S	150°29'56.66"E	7.8	523
17	Botanical gardens, Rockhampton	23°24'0.54"S	150°29'17.88"E	7.6	429
18	Orams farm, pond one, Pink Lily	23°20'29.48"S	150°28'40.17"E	7.6	499
19	Orams farm, pond two, Pink Lily	23°20'40.90"S	150°28'44.68"E	7.5	528
20	Fitzroy river, Under the bridge, Rockhampton	23°22'15.64"S	150°30'30.70"E	7.2	452
21	Kershaw gardens, Rockhampton	23°21'37.73"S	150°31'1.18"E	6.5	468
22	Turf grass, Rockhampton	23°24'44.07"S	150°36'37.57"E	7.0	469
23	800 m away from Turf grass, Rockhampton	23°24'13.82"S	150°36'53.02"E	7.2	456

24	Splitters creak, Rockhampton	23°21'13.13"S	150°30'2.58"E	7.8	623
25	Gigg road, way to Yeppoon	23°17'5.91"S	150°33'17.89"E	7.1	452
26	500 m from car racing, on the way to Yeppoon	23°17'4.13"S	150°33'32.68"E	7.9	469
27	Mount Jim Crow National Park, 29 Km North-East of Rockhampton	23°12'53.39"S	150°37'47.51"E	7.0	593
28	Corduroy Creek, Queensland	23° 7'32.80"S	150°41'33.64"E	6.5	782
29	200 m up to Corduroy Creek, Queensland	23° 7'24.96"S	150°41'31.29"E	7.2	645
30	Property 168, way to Byfield	-	-	7.7	593
31	Prestone road, way to Byfield	-	-	6.6	586
32	Bungundarra road, Bungundarra	23° 3'43.67"S	150°39'31.98"E	6.8	792
33	Stoney creek, Byfield	22°55'26.31"S	150°39'44.85"E	7.0	592
34	Upper stony creek, Byfield	22°55'23.60"S	150°39'49.71"E	6.6	483
35	Upper stony creek, Byfield	22°55'23.22"S	150°39'36.88"E	6.4	563
36	Stony creek, Byfield	22°55'23.50"S	150°39'34.09"E	7.6	452
37	Water Park Creek, Byfield,	22°52'28.60"S	150°42'8.71"E	7.6	623
38	Water Park Creek, Byfield,	22°52'15.28"S	150°42'10.36"E	7.9	596
39	Water source near Water Park Creek, Byfield	22°51'0.24"S	150°39'26.24"E	7.2	488
40	CQUniversity cane field, Rockhampton	23°19'25.25"S	150°31'25.11"E	7.6	465
41	Curragh mines, Blackwater	23°30'13.83"S	148°51'24.76"E	7.2	453
42	Opposite to the creek inside the Curragh mine, Blackwater	23°29'47.97"S	148°54'3.40"E	7.6	526
43	Small water source, Bluff	23°34'49.54"S	149° 3'58.72"E	7.2	469
44	Bridge water creek, way to Blackwater	-	-	7.6	362
45	Wawoon creek, way to Rockhampton	-	-	6.9	492
46	Estuarine Crocodile Farm, 65 Savages Road, Coowonga	23°18'41.20"S	150°45'39.91"E	5.8	263
47	Blackwater creek, inside Curragh mines	23°30'13.83"S	148°51'24.76"E	7.6	526
48	Elphinstone street, Rockhampton	23°21'53.95"S	150°31'53.54"E	6.8	485
49	Under the bridge near Callagen park, Rockhampton	23°22'27.42"S	150°30'56.96"E	7.9	456

50	Moore creek, Kershaw gardens, Rockhampton	23°21'40.28"S	150°31'2.50"E	8.2	1394
51	Blackwater creek, inside the Curragh mines	23°30'13.83"S	148°51'24.76"E	7.6	389
52	Opposite to Moores creek, Kershaw garden, Rockhampton	23°21'38.11"S	150°30'59.94"E	8.2	452
53	Aligator creek, Rockhampton	-	-	7.6	526
54	Belmont Creek, Rockhampton	-	-	7.2	526
55	Midegee creek, 26 km from Rockhampton towards Gladstone	23°25'50.27"S	150°30'31.33"E	6.6	1589
56	Station creek, 35.5 km from Rockhampton towards Gladstone	23°25'55.77"S	150°30'37.81"E	7.7	2351
57	Okay creek, 36 km from Rockhampton towards Gladstone	23°27'13.51"S	150°31'46.47"E	8.1	964
58	Opp to Bajool plant 42 km from Rockhampton towards Gladstone	23°39'10.54"S	150°39'7.39"E	8.2	319
59	Six mile creek 45 km from Rockhampton towards Gladstone	23°38'53.43"S	150°39'35.59"E	7.7	1569
60	Central Queensland salt Ind water supply pond 2 Km inside the Port Alma	23°35'7.35"S	150°51'44.18"E	7.3	716
61	Eight mile road 48 km from Rockhampton towards Gladstone	23°39'59.99"S	150°39'24.81"E	8.2	408
62	St. Arnauds creek, Gladstone	23°41'17.73"S	150°43'58.45"E	8.1	1508
63	Twelve mile creek 57 km Rockhampton towards Gladstone	23°42'28.49"S	150°48'29.42"E	8.0	2054
64	Vallis creek, Gladstone	23°45'15.73"S	150°51'57.05"E	8.2	313
65	Calliope river	23°50'42.48"S	151°12'36.59"E	7.9	-
66	Lake Awoonga recreation park one, Benaraby, Gladstone	24° 3'55.71"S	151°17'31.89"E	8.8	310
67	Lake Awoonga recreation park two, Benaraby, Gladstone	24° 3'51.57"S	151°17'28.65"E	8.6	600
68	On the way to Awoonga park, Benaraby Gladstone	24° 2'23.50"S	151°19'14.38"E	8.5	1705
69	Farmers creek	23°53'24.53"S	151°11'46.33"E	7.3	1544
70	Laidley Creek, Glenore Grove, Queensland	27°31'23.56"S	152°23'55.57"E	8.3	184
71	Laidley tree pond Glenore Grove, Queensland	27°31'48.39"S	152°23'51.51"E	7.9	202
72	Pamora creek, Glenore Grove, Queensland	-	-	7.5	588

3.2.2 Nutrient media composition and preparation

Guillard f/2 (Guillard 1983) media was prepared using standard composition (Table I in Appendix A) and the pH of the media was adjusted to 7.5. ASM media (Gorham *et al*. 1996) was prepared according to the composition (Table II in Appendix A) and the pH of the medium was adjusted to 7.6–7.8. Bolds Basal medium (BBM) (Nichols & Bold 1965) was prepared according to the composition (Table III in Appendix A) and the pH of the medium was adjusted to 6.6.

All media stock solutions were stored in the refrigerator at 4°C until further use. The pH of the working media of Guillard f/2, BBM and ASM were measured using pH meter (Lab Chem pH/ conductivity/ temperature bench top unit, TPS, Australia) and were set to the required pH using HCl or NaOH. Once the pH was adjusted, media were sterilized at 121°C and 15 psi for 20 min in an autoclave (HS 5510 EC-1, Getinge, Sweden).

3.2.3 Screening microalgal for neutral lipids

Microalgae in water samples collected from various sources were screened for neutral lipid accumulation immediately after collection and at the end of two weeks of inoculation into various growth media (ASM, BBM and f/2).

Immediately upon collection approximately 50 mL of well mixed water sample was taken from each sampling bottle and centrifuged at 2000 rpm for about 5 min to concentrate the cells. In the case of sample screened after 2 weeks, 10 mL of sample from culture flasks was samples and centrifuged at 2000 rpm for about 5 min. Screening for neutral lipid content was carried out using Nile Red (Sigma-

Aldrich N3013-1G), a lipophilic dye. A few drops of Nile Red (0.1 mg/ml acetone) were added to the pellet and incubated at 37 °C for 10 min. The sample was transferred onto a microscope slide and observed for fluorescence under an epi-fluorescence Nikon Eclipse E600 microscope using Excitation filter B-2A, Barrier 520 nm and emission 450-490 nm, with a Nikon pressure mercury lamp model HB-10101 AF as the light source. The cells with golden yellow fluorescence were photographed using a Nikon E995 camera. Unstained samples were used as a control.

3.2.4 Microalgal species isolation and identification

A single cell isolation method was used to isolate microalgal species positive for Nile red. In this method, the microalgal cells were selected under a stereo microscope using a straight glass capillary tube (internal 1.1-1.2 mm, external 1.5-1.6 mm, length 75 mm) (Livingstone International Pty Ltd, NSW, Australia) and the microalgal cells were transferred to fresh sterile liquid growth media (ASM, BBM and Guillard f/2). As this did not yield uni-algal cultures, samples were plated on solid media plates. Water samples known to contain microalgae positive for neutral lipids were centrifuged at 2000 rpm for 10 min. The resultant pellet was washed with sterile distilled water. The pellet was inoculated to Petri plates (using the spread plate method) containing solid media (1.5-2 % agar) of ASM, BBM and Guillard f/2. After 7 to 10 days of incubation at 25°C, 200–250 µmol m^{-2} s^{-1} light intensity and a 16:8 light: dark cycle, the colonies were observed under microscope (Nikon Eclipse E600) at various magnifications. Microalgal species that tested positive for Nile red colonies were identified and were transferred to fresh media plates (ASM, BBM and Guillard f/2) aseptically. The streak plate technique was adapted to obtain uni-algal strains of

the target species. The uni-algal cultures were then transferred to liquid media and were maintained at 25°C, 200-250 µmol m^{-2} s^{-1} light intensity and a 16:8 light: dark cycle.

The stock cultures were routinely sub-cultured every three weeks under aseptic conditions. Each time prior to sub-culturing the culture flasks were checked for the presence of contaminating species.

Uni-algal cultures of microalgal species were maintained in Iwaki tissue culture flasks (75 cm^2 code 3123-075) containing 150 mL of the culture medium. The microalgal cultures were maintained under 150-200 µmol m^{-2} s^{-1} light intensity using cool white fluorescent light, and 16:8 day/night light cycle at 25±1°C.

Microalgal species positive for Nile red screening was identified using microscopic techniques (light and Scanning Electron microscope) and morphological taxonomy (Prescott 1978). The scanning electron microscopic (SEM) pictures of diatoms were obtained using SEM model JSM 6360 LA operated at 15 kV. Sample preparation for the SEM was under taken according to Hildebrand *et al*. (2009) and Massé *et al*. (2001). Briefly, diatom samples were transferred to 10 mL centrifugation tubes and were centrifuged at 1000 *g* for 10 min. In order to maintain cell integrity, cells were fixed for 30 min at room temperature in a 3% glutaraldehyde solution prepared with the culture medium. Fixed cells were then centrifuged (1000*g,* 10 min), the supernatant removed and the fixation continued for an additional 2 h in a 3% glutaraldehyde solution buffered with Phosphate buffered saline (PBS) (PBS was prepared by adding 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, was added to 1 L distilled water and pH was adjusted to 7.4 with HCl. The buffer was

sterilized in an autoclave and was stored at room temperature) (Sambrook *et al.* 1989). Cells were re-centrifuged; the pellet rinsed with buffer (PBS) and centrifuged one last time. The pellet was left to dry in a sterile environment overnight. The processed cells were mounted to carbon tape and coated with gold.

Prior to identification the microalgal species were assigned sample codes for easy identification of the microalgal species (Table 13) The SEM pictures and the samples of diatoms (Figure 4) were sent to Prof Jacob John (International Diatom Herbarium, Dept of Environment and Agriculture, Curtin University) and Lindsay Hunt (senior scientist, Clinical and State wide Services Division, Queensland Health) in Australia for confirmation of the identifications.

3.3 Results

3.3.1 Microalgal species collection

Water samples collected from 90 sites, of seven regions, had a pH range of 5.8-8.8, conductivity range of 184-2351 µS cm^{-1} and temperature range of 17-21°C (Table 12). The lowest conductivity of 184 µS cm^{-1} was recorded from Laidley Creek (Glenore Grove, Queensland) while value of 2351 µS cm^{-1} was recorded from Station Creek (Bajool, Queensland). The lowest pH of 5.8 was recorded from the estuarine Crocodile Farm (65 Savages Road, Coowonga, Queensland) while the highest value of 8.8 was recorded in a water sample from Lake Awoonga (Benaraby, Queensland).

Table 12. Range of pH, EC and temperature recorded in water samples collected from seven locations and surrounding areas to the seven locations of Central Queensland region.

Location	pH	EC (µS Cm^{-1})	Temperature (°C)
Rockhampton	6.5-8.6	403 - 832	17 - 21
Yeppoon	6.5-7.9	452 - 792	19 - 21
Blackwater	6.9-7.6	362 - 526	19 - 20
Byfield	6.4-7.8	452 - 792	19 - 21
Gracemere	6.5-8.6	403 - 832	17 - 21
Bundaberg	5.8-7.9	463 - 599	19 - 20
Gladstone	6.6-8.8	310 - 2351	20 - 21

3.3.2 Screening microalgal for neutral lipids

Five strains of microalgae tested positive under the Nile red screening procedure (Figure 2; Table 13).

Table 13. Sample collection location, pH, EC and temperature of microalgal species which tested positive for Nile red staining.

No. from Table 11	Location	Sample code	pH	EC (µS cm^{-1})	Water source	°C
50	Moore creek, Kershaw gardens, Rockhampton	Species 65	8.2	1394	Brackish	21.3
66	Lake Awoonga, Benaraby	Species 80	8.8	310	Fresh	21.2
43	Small water source, Bluff	Species 52	7.2	469	Fresh	21.1
20	Fitzroy river, River st, Rockhampton	Species 15	7.2	452	Fresh	20.2
25	Gigg road, way to Yeppoon	Species 25	7.1	452	Fresh	21.2

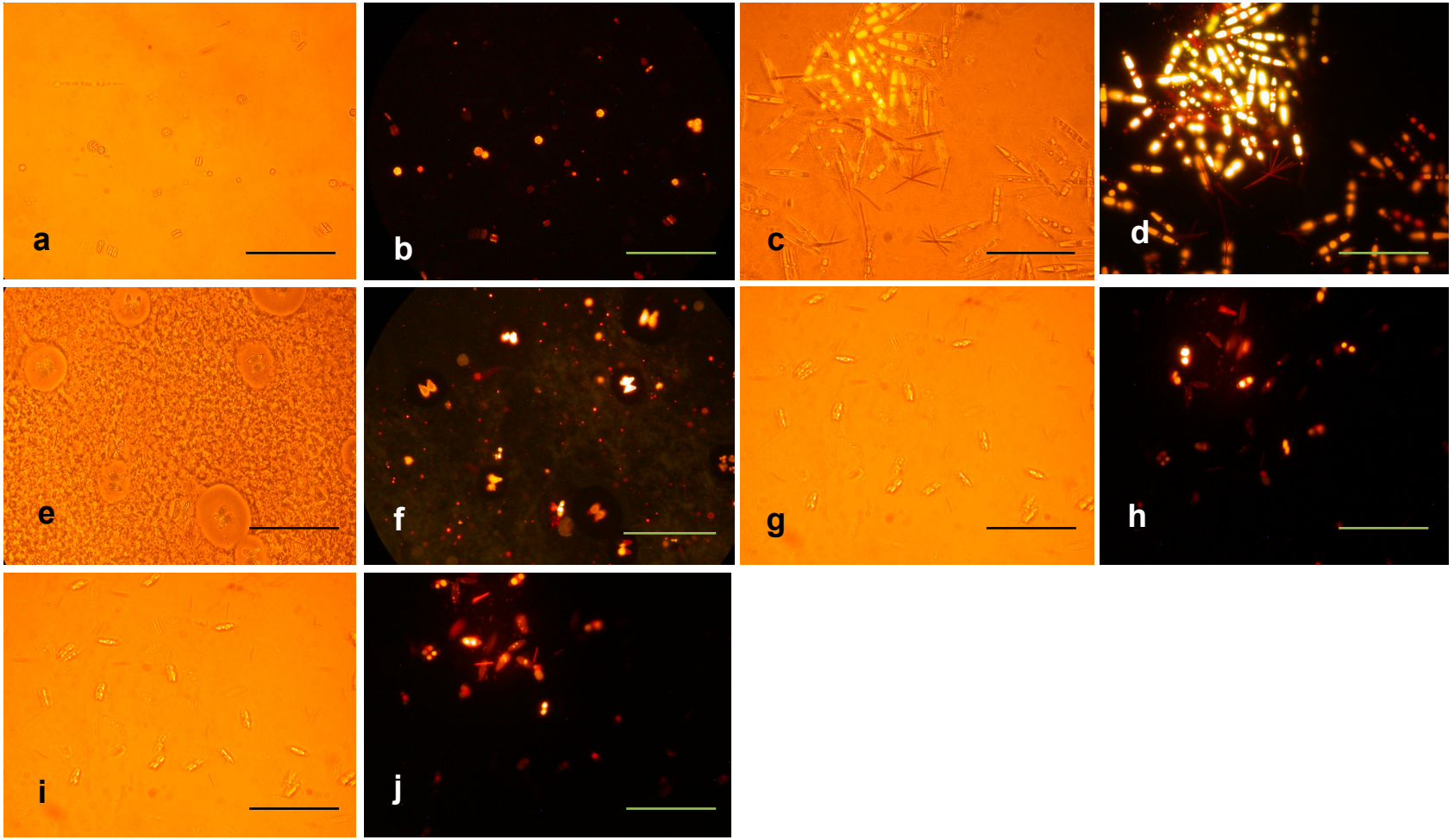


Figure 2. Photographs of microalgae showing control (a, c, e, g and i) and epi-fluorescence (b, d, f, h and j) (images of Nile Red stained samples) of Species 15, Species 65, Species 52, Species 25 and Species80 respectively at 400 x magnification. Scale bar indicates 30 µm. Excitation filter B-2A, Barrier 520 nm and emission 450-490 nm.

3.3.4 Microalgal species identification

Microalgal species with the sample codes species 15, species 25, species 65 and species 80 were identified as *Chlorella vulgaris*, *Navicula* sp. 1, *Nitzschia pusilla* Grunow and *Navicula* sp. 2 respectively using morphological keys (Gell *et al.* 1999; Prescott 1962, 1978; Thomas 1983) (Figure 3).

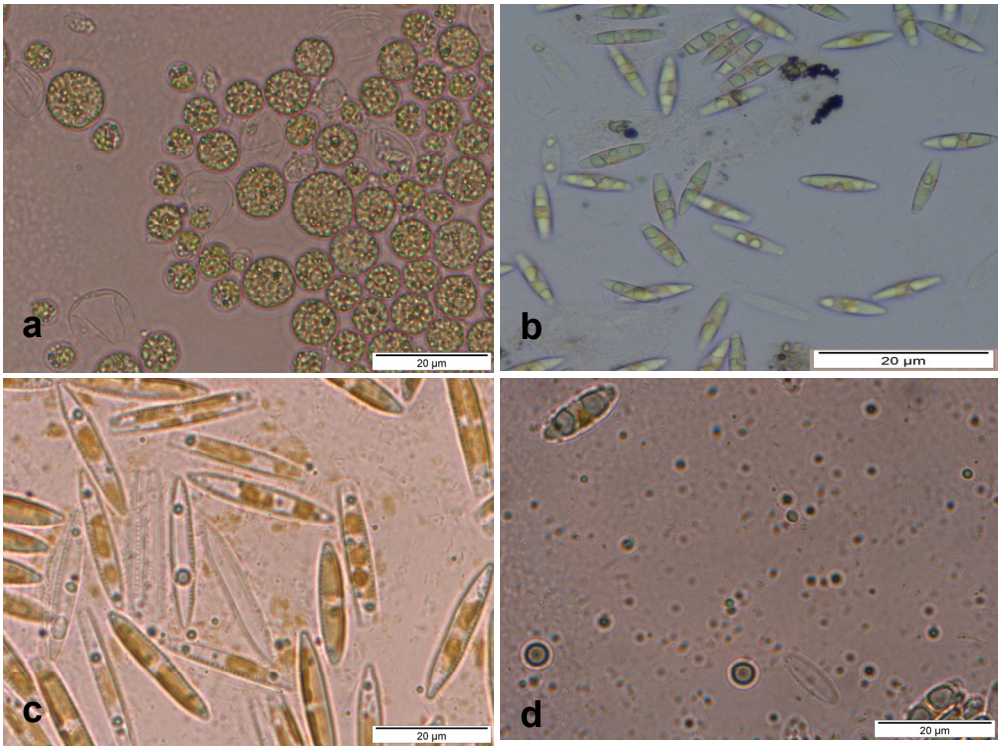


Figure 3. Photomicrographs (bright field) of (a) *Chlorella* (b) *Navicula* sp.1 (c) *Nitzschia pusilla* and (d) *Navicula* sp. 2 at 1000X and scale 20 µm.

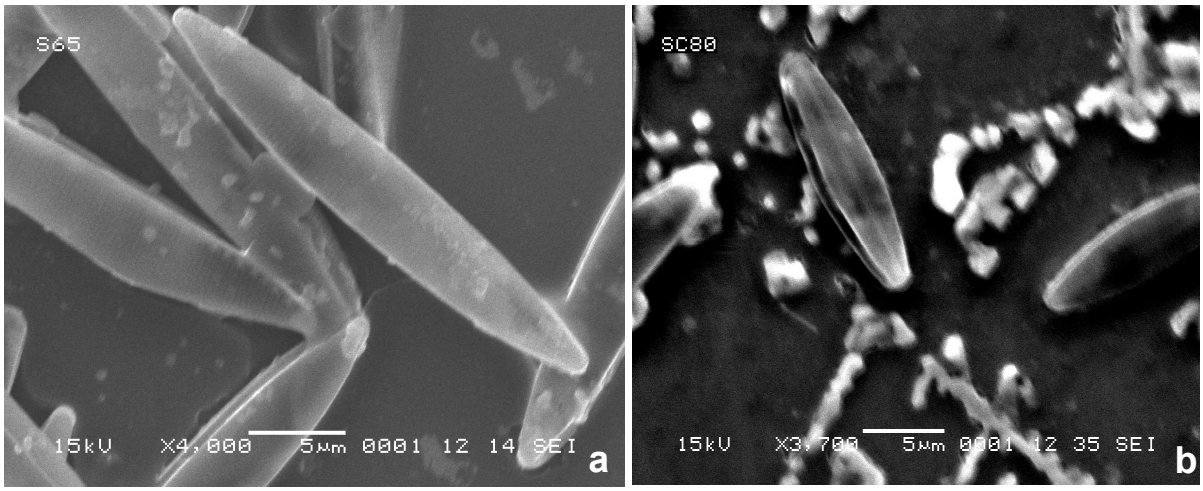


Figure 4. SEM images of (a) *Nitzschia pusilla* and (b) *Navicula* sp. 1

Microalgae with sample code "species 52" was identified as *Staurastrum* sp, however, the culture collapsed after a few weeks in all media (ASM, BBM and Guillard f/2). Microalgal species of *Scenedesmus*, *Tetraspora*, *Oscillatoria*, *Dolichospermum* were frequently observed in the collection (Figure 5), however these species did not test positive for neutral lipids.

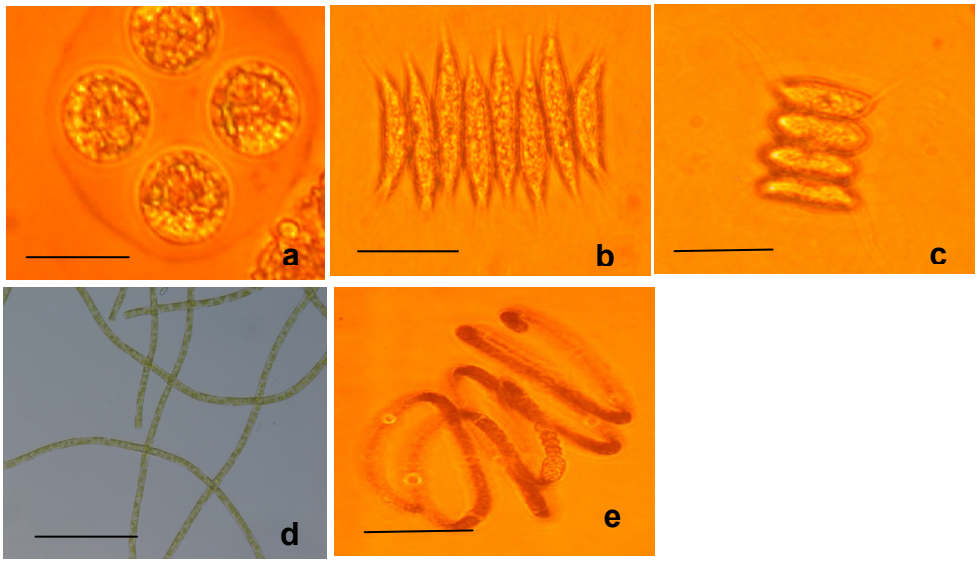


Figure 5. Various microalgal species frequently observed in the water samples collected from various locations in Central Queensland (**a**) *Tetraspora*, (**b**) *Scenedesmus dimorphus*, (**c**) *Scenedesmus quadricauda*, (**d**) *Oscillatoria*, (**e**) *Dolichospermum*.

3.4 Discussion

3.4.1 Bio-prospecting

To the knowledge of the author this work was the first attempt of bioprospecting native microalgal species with neutral lipid content within the Central Queensland region. Ninety sites in seven locations in Central Queensland region were screened for neutral lipid accumulating microalgae. One species of *Chlorella*, two species of *Navicula*, one species of *Staurastrum* and one species of *Nitzschia* were identified having neutral lipid content. There are no previous reports on the neutral lipid content of *Staurastrum*.

Numerous species belonging to Chlorophyta and Bacillariophyta have been reported as potential candidates for biofuel production (Chih-Hung & Wen-Teng 2009; Liu *et al.* 2010; Meng *et al.* 2009; Phukan *et al.* 2011; Widjaja *et al.* 2009; Xu *et al.* 2006). Previous studies on *Chlorella*, *Nitzschia* and *Navicula* species have reported lipid accumulation of 25-63, 16-47, 25-50 % dry weight respectively (Chisti 2007; Griffiths & Harrison 2009; Mata *et al.* 2010) under varied nutrient conditions. However, literature is limited on *Nitzschia* and *Navicula* as a potential source of biofuel.

Earlier studies on *Staurastrum* were mostly limited to taxonomic descriptions (Ngupula *et al.* 2011; Souza & Melo 2011). Unfortunately in the present study *Staurastrum* would not multiply in any of the media and gradually after a few weeks the culture was lost. The cells survived for longer periods in the original sample, but failed to multiply in any of the growth media used. The reason for the loss of the culture might be bacterial association in the original sample (turbid to visual observation at time of sample collection) which might have facilitated the survival of

Staurastrum. Earlier studies have noted a positive relationship between bacteria and algae (Caldwell & Caldwell 1978; Kochert & Olson 1970). It was reported that bacteria stimulate growth of algae by nitrogen mineralization (Axler *et al.* 1981), carbon generation (Marshall 1989), producing vitamins and organic growth factors (Haines & Guillard 1974; Ukeles & Bishop 1975), and sometimes modifying the pH of the media (Caldwell 1977).

The oil containing microalgae were isolated from water bodies ranging in pH from 7.1-8.8 and EC between 310-1394 μS cm^{-1}. *Nitzschia pusilla* was isolated from brackish water and species of *Chlorella*, *Staurastrum* and *Navicula* were from fresh water habitats. Earlier studies have reported isolation of microalgal species able to accumulate lipids from a broad range of environmental conditions (Mutanda *et al.* 2011; Sheehan *et al.* 1998; Yang *et al.* 2010; Zhou *et al.* 2011). It is concluded the oil accumulating microalgae occur in a variety of ecological habitats.

3.4.2 Screening techniques

Water samples containing microalgae were screened using Nile Red. Earlier research has recommended Nile Red as a potential dye to quantify neutral lipid content in microalgal cells (Chen *et al.* 2011; Elsey *et al.* 2007; Huang *et al.* 2009; Lee *et al.* 1998) because of it photo-stable properties (Cooksey *et al.* 1987). In previous reports, Nile red screening was always a second step followed after isolation and purification of microalgal species and requires data from gravimetric method to quantify lipids (Abou-Shanab *et al.* 2011; Moazami *et al.* 2011; Zhou *et al.* 2011). In the present study microalgal strains were isolated following the Nile Red screening of mixed population. The microalgal strains positive for neutral lipid

content were then quantified for lipid content using gravimetric methods. This approach decreased the time involved in screening and avoided isolation of unwanted microalgal strains.

Initially after Nile red screening, a single cell isolation technique was attempted, targeting uni-algal cultures of neutral lipid positive microalgal species. However this method failed to yield uni-algal cultures even after repeated transfers and was laborious and time consuming. The solid plate technique was then used with uni-algal cultures produced easily. Numerous isolation and purification procedures such as single cell isolation, using solid plates, dilution and gravimetric techniques (Andersen 2005) and advanced methods such as flow cytometry (Maftah *et al.* 1993) are being used, with each method having its own advantages and disadvantages (chapter 2, section 2.3.2.1). Ultimately, the choice of the isolation technique depends on the instrumentation and expertise availability (Mutanda *et al.* 2011).

Identification is an important step of bioprospecting (Godhe *et al.* 2002). The conventional microscope based procedures which requires significant taxonomic experience are slowly being replaced by advanced molecular techniques (Godhe *et al.* 2002; Mutanda *et al.* 2011). However, identification of microalgal strains to species level using molecular approaches, before lipid quantification, will add to the expense of a bio-prospecting exercise. For example, a recent study by Zhou *et al*. (2011) on bio-prospecting microalgae for lipid producing strains from concentrated municipal waste waters, adapted Polymerase chain reaction (PCR) amplification procedures were used to identify 17 strains of microalgae base on the growth rates.

However, after identification the lipid productivity estimated ranged from 0.037 to 94.8 mg L^{-1} d^{-1}. If the identification step was followed after the lipid quantification, the time and cost involved in identifying the no potential microalgae species would have been saved. These approaches might add to the life cycle analysis of the biofuel production procedures if based on bioprospecting native microalgal strains.

In the present study microalgal strains positive for neutral lipids were identified based on microscopic observation of morphological features and identification keys. The growth media to quantify biomass and lipid was selected based on the genus identified. Out of the four microalgal strains quantified for lipid content the strain with the highest biomass and lipid content was *Chlorella* followed by *Nitzschia* (data presented in chapter 4). The strains were then identified to species level using taxonomic expertise. This study recommends that the initial identification be restricted to morphological features using identification keys (identification to genus level) and molecular based techniques can be adapted (in case where taxonomic expertise is not available) after lipid quantification of microalgal species.

3.4.3 Non oil containing microalgae

The microalgal species of *Scenedesmus*, *Tetraspora*, *Oscillatoria*, *Dolichospermum* were negative for neutral lipid accumulation. Among the species, *Scenedesmus* was predominant in most of the water samples collected probably due to the low temperatures (Mutanda *et al.* 2011). *Scenedesmus* has been reported to be a potential candidate for lipid production (Balasubramanian *et al.* 2011; Rodolfi *et al.* 2009; Xin *et al.* 2010; Xin *et al.* 2011) in earlier reports. However in the current study,

no strains of *Scenedesmus* were tested positive for neutral lipid screening from the field collection or at different stages of growth under cultivation.

3.5 Conclusions and recommendations

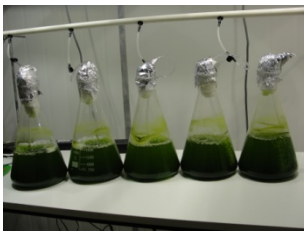
This study is the first initiation of bioprospecting microalgae for lipid containing species from the Central Queensland region.

Microalgal species of *Chlorella*, *Navicula*, *Nitzschia and Staurastrum* were identified as oil containing microalgal species. These genera belong to the division Chlorophyta, Heterokontophyta and Charophyta, in the order Chlorellales, Naviculales, Bacillariales, Desmidiales respectively. Thus there is no particular phylogenic distribution to this trait. As these species were present in a broad range of water bodies, there is also no particular ecological distribution associated with this trait. Bio-prospecting for these traits therefore remains a hit and miss affair.

This study recommends a protocol of screening of collected water samples using staining. This method is quite time efficient, however it may miss potential species for which dye penetration of the cell wall and membrane is poor, or that were present in a sample of carbon limited condition (i.e. lacking oil bodies at the time of screening). Several suggestions can be made to guide future work (a) water samples could be amended with a carbon source (for example glucose) and cultured for several days before screening to increase oil deposits in the microalgal species having the capacity to accumulate oil; (b) samples could be pre-processed with microwave and chemical treatment (dimethyl sulfoxide) to improve dye penetration; (c) DNA could be extracted from microalgae having oil content in the water samples

and probed for the presence of key genes in the oil accumulation pathway, followed by efforts to identify and isolate the responsible species.

Chapter 4. Biomass and lipid productivity of microalgal species isolated from Central Queensland, region



4.1 Introduction

The second objective of this thesis is “To characterize selected microalgae for increased biomass and lipid productivities under nutrient sufficient and deficient conditions”. In this chapter, the isolated microalgal species of *Chlorella*, *Navicula* and *Nitzschia* will be assessed for biomass and lipid content under nutrient sufficient growth trial. The species with highest biomass and lipid content will then be grown under nutrient limited growth trials and the best condition to yield highest biomass and lipid will be recorded.

Selection of microalgal species with a high growth rate, a high biomass (per unit volume of solution) and a high lipid content is the first step in establishing a commercially viable algal mass culture (Griffiths & Harrison 2009; Pulz & Gross 2004). However, production and accumulation of lipids in microalgae is species specific and dependent on the growth environments provided (Shifrin & Chisholm 1980). Any local microalgae selection growth environments should be benchmarked against international reports in terms of biomass and lipid accumulation. Numerous species of microalgae have been reported to accumulate between 1-70 % lipid by weight of biomass (Chisti 2006; Chisti 2007). Microalgae can be grown using batch or continues mode of cultivation systems. Continuous mode of cultivation has numerous advantages compared to batch mode of operation. However, laboratory

small scale experiments are mostly operated under batch mode of cultivation. Continuous modes of cultivations are preferred in mass cultivation of microalgae in closed culture systems (photo-bioreactors) (Mata *et al.* 2010).

Chlorella and *Nitzschia* are the most common candidate's species, with lipid yields of between 20-50 % (and up to 66 %) dry weights reported. Among microalgae of fresh waters, various species of *Chlorella* have been listed as potential candidates for biofuel production (Chisti 2007). Biomass content of between 0.04-51.2 g L^{-1} and biomass accumulation rates of 0.03-7.3 g L^{-1} d^{-1} respectively, have been reported, with associated lipid yields of 25-66 % dry weight and a lipid accumulation of 0.008-1.7 g L^{-1} d^{-1} respectively (Chisti 2007; Griffiths & Harrison 2009; Lam & Lee 2011; Mata *et al.* 2010) (Table 14). However, large variation in biomass and lipid content has been reported with varied cultivation condition and strains. There was no particular growth medium or cultivation condition reported in the literature which was optimal to grow *Chlorella*, *Nitzschia* and *Navicula* sp. This indicates that, for mass cultivation of microalgae each strain has to be optimized individually to suite the local environmental conditions.

Any factor (such as variation in nutrient, light availability and temperature) which disturbs the balance between growth rates and photosynthetic rates will impact on the accumulation of storage reserves (Chisti 2006; Chisti *et al.* 2003; Widjaja *et al.* 2009). The link between nutrition and carbon reserves levels is well established, with a N limitation associated with an increased C:N ratio and an increased accumulation of carbon rich storage products such as starch or lipids (Mata *et al.* 2010). Numerous authors have reported the *C. vulgaris* biomass to increase and lipid concentration (%

dry weight) to decrease as the supply of N is increased (Table 14). The literature related to P and Fe nutrition of *C. vulgaris* in relation to lipid accumulation is limited to relatively fewer studies.

Table 14. Brief overview of *Chlorella vulgaris* biomass and lipid yield and productivity grown under various nutrient conditions.

Reference	Culture condition	Nutrient	concentration (mg L^{-1})	Specific growth rate (day^{-1})	Biomass productivity (g L^{-1} d^{-1})	Biomass yield (g L^{-1})	Lipid productivity (g L^{-1} d^{-1})	Total lipid (% of biomass)	Comment
Illman *et al*. (2000)	Watanbe medium	$NaNO_3$	75	0.99	0.029	0.41	-	18	High lipid content was achieved through heterotrophic cultivation
	Nitrate sufficient and deficient cultivation		37.5	0.77	0.037	0.52	-	40	
Liu *et al*. (2008)	Gulliard f/2-Si medium	$FeCl_3$	0.67	-	-	-	-	57	Highest lipid content was achieved at 0.67 mg L^{-1} Fe^{3+}
Hsieh and Wu (2009)	Walne medium Urea as N source. Batch culture mode for 6 d	$CO(NH_2)_2$	25	0.86	-	0.464	0.051	66	Low cost nitrogen source and high yields of lipids were achieved.
			200	1.42	-	2.027	0.11	32	
Widjaja *et al*. (2009)	Modified Fitzgerald medium.	$NaNO_3$	70	-	0.043	0.86	0.0128	29	Increased lipid content was achieved

	Normal nutrient for 20 d, then 17 d N limited		0.02	-	-	-	-	44	compared to normal nutrition
Converti *et al*. (2009)	Guillard f/2 medium.	$NaNO_3$	1500	0.14	-	-	0.008	6	N limitation increased lipid yield
	Temperature and nitrate as limiting factor.		375	0.13	-	-	0.002	15	
Ordog *et al*. (2012)	Tamiya nutrient medium N limited cultivation for 14 d	KNO_3	70	-	-	3.03	0.044	-	Lipid yield was comparable with other N stressed experiments in earlier studies
Average				0.718	0.036	1.21	0.04	34	
SD				0.5	0.01	1.1	0.04	20	

Nitzschia and Navicula belong to the phylum Heterokontophyta (diatoms) with relatively limited information available on these diatom genera as potential candidates for biofuel production. In an earlier study, Coombs *et al*. (1967) reported *Navicula pelliculosa* grown under silicon starvation (8 μg mL^{-1}) on Tryptone medium, at 20 °C and 17,000 lux light intensity, achieved a lipid yield of 25-34 % dry weight over the exponential growth phase (54 h from exponential stage). *Nitzschia and Navicula* were identified as oil accumulating genera during the period 1978 to 1996, as part of the Aquatic Species Program conducted by National Renewable Research Laboratories (NREL). Working within this programme, Tadros and Johansen (1988) reported *Navicula* sp. biomass and lipid yield of 1.5 to 2.3 doublings d^{-1} and 26-47 % dry weight respectively, under varied temperature (10-35 °C), nutrient medium composition (SERL I and II and under varied NaCl concentrations with a specific conductance range of 10-70 μS cm^{-1}). Dempster and Sommerfeld (1998) reported *N. communis* growth rate and lipid yield of 0.5 to 2.5 doublings d^{-1} and 10-75 % dry weight respectively, under similar growth conditions to Tadros and Johansen (1998). Sheehan *et al*. (1998) reported biomass and lipid yield of 2.5-8.8 g dry weight m^{-2} d^{-1} and 25-47 % dry weight across numerous species of *Nitzschia* grown under varied growth conditions. There was one report of *Nitzschia pusilla* isolated from fresh water bodies and the biomass and lipid content was assessed as 1.37 and 0.66 g L^{-1} respectively. However, the species was limited for further research as the biomass and lipid content were low compared to *Chlorella* and *Scenedesmus* species used in the same study (Abou-Shanab *et al.* 2011).

Matsumoto *et al*. (2010) isolated *Navicula* sp. from a marine source near Kagoshima, Japan, reported biomass and lipid productivity of 346 mg/L and 185 mg L^{-1} $week^{-1}$ grown under f/2 medium with artificial sea water. Moazami *et al*. (2011), isolated *Nitzschia* sp. (PTCC 6001) from a marine source in the Persian Gulf region and reported an average biomass and lipid yield of 12.6 g L^{-1} and 32 % dry weight for cultures grown using sea salt and RM media at 180 µE m^{-2} S^{-1} light intensity and at 25 °C and batch mode of cultivation.

In summary *Chlorella* appears to be a better candidate for oil production than *Navicula* or *Nitzschia* sp. given higher reported growth rates and lipid content. The average lipid productivity of *C. vulgaris* across the reports is 0.04 g L^{-1} $d^{-1}$with a lipid content of 34 % biomass (dry weight basis) (Table 14). If these values were maintained under mass cultivation to produce biofuel then a pond 25 cm deep would yield 0.04 x 250 L x 10,000 m^2 biomass ha^{-1} d^{-1} i.e. 100 kg biomass, which contains 34 kg oil at the rate of 34 %. If these rates were maintained over 365 days in a year, then 36500 kg of biomass containing 12410 kg oil would be produced ha^{-1} $year^{-1}$ (approximately 10979 kg biodiesel ha^{-1} $year^{-1}$).

Australia's diesel consumption for the year 2010 was 19,000 ML and expected to reach 27,000 ML $year^{-1}$ by 2020 (IEA 2010). According to the above calculation, to produce the required diesel needs of 2020 land area of 2.5 million ha is required. Alternatively microalgal strain with high lipid productivity should be selected to reduce the land and resource requirement.

4.2 Materials and Methods

4.2.1 Estimation of cell densities and biomass content

Microalgal cells were counted using a Sedgwick Rafter Counting Cell under a Nikon Eclipse E600 microscope at 100X magnification. Results were expressed in cells/mL.

Dry weight was estimated using Whatman GF/C (2.5cm) filters. The filters were rinsed with deionised water and were dried at 70°C for 24 hrs. Once removed from the oven, they were stored in a vacuum desiccator over silica gel until further use. The initial weight of the filter discs was noted to 4 decimal points using an analytical balance (Mettler Toledo, classic AB 204-s, Australia). Aliquots (10 mL) of microalgal culture were filtered under vacuum and filtrates were dried at 60°C for 12 h. then cooled to room temperature in a vacuum desiccator. The weight of the filter discs was noted to 4 decimal points. Dry weight was expressed as g L^{-1}.

4.2.2 Estimation of total lipid content

Total lipid was estimated adopting the technique of Kates and Volcanic (1966) as modified by Moheimani (2005). Aliquots (10 mL) of microalgal culture were filtered using Whatman 2.5 cm GF/C filters as for biomass estimation. The filter discs were homogenised using a mortar and a glass pestle with 5 mL of methanol: chloroform: deionised water (2:1:0.8 v:v:v). The extracts were transferred to 10 mL graduated centrifuge tubes and centrifuged (Megafuse 2.0, Heraeus instruments) at 1008 *g* for 10 min at room temperature. The supernatant was carefully transferred to 15 mL graduated glass centrifuge tubes. The extract was made up to 5.7 mL with extraction solvent. To this solution 1.5 mL of chloroform was added followed by 1.5 mL of

deionised water and stirred until partial phase separation occurred. The sample was centrifuged at 1008 g for 5 min. The green phase was transferred to a pre-weighed 4 mL glass vial. A few drops of toluene were added to remove any water and the vials were dried under a nitrogen blanket. Further the vials were stored over KOH pellets in a vacuum desiccator overnight to remove any moisture remaining in the vials. The vials were then weighed and the total lipid content expressed as g L^{-1}.

4.2.3 Optical density, pH, EC and light intensity measurements

Optical density (OD) of the microalgal cultures was measured at 625 nm using a Varian Cary 50 Bio UV/VIS spectrophotometer (Converti *et al.* 2009). Culture media pH and electrical conductivity (EC) (µS cm^{-1}) was measured using a Lab Chem pH/ conductivity/ temperature bench top unit (TPS, Australia). Light intensity (Photosynthetically Active Radiation) of the experimental set up was measured using Ceptometer (LP-80 Accu PAR, Decagon Device, Inc).

4.2.4 Experimental design and growth trials

4.2.4.1 Nutrient sufficient growth trial (unmodified standard nutrient solutions)

The aim of the experiment was to select the microalgal species with the highest biomass and lipid productivity among *Chlorella vulgaris*, *Navicula* sp. 1, *Nitzschia pusilla* and *Navicula* sp. 2 grown under nutrient sufficient growth trial (no modification in nutrients from the standard composition of the growth media).

The growth trial employed a Randomized Complete Block Design (RCBD) with four species with five replications each. The microalgal species were grown using a

standard ASM media (Table II in Appendix A) in case of *C. vulgaris* and Guillard f/2 media (Table I in Appendix A) in case of *Navicula* and *Nitzschia*. Experiments were conducted at 25 °C and a light intensity of 350- 400 µmol m^{-2} s^{-1} (400 Watt metal halide lamp). Each replication consisted of a 2.0 L Erlenmeyer conical flask containing 1850 mL of respective media. To start the experiment, 550 mL of was transferred into each replicate flask (t=0)

Each flask was continuously aerated with filtered atmospheric air. The air was pumped using a Sakuragawa air pump SPP-40GJ-L having normal pressure of 13 k.Pa and flow rate of 43 L min^{-1} or 23.8 L^{-1} L^{-1} min^{-1}. The air pump was connected to a 13 mm low density Polyvinyl chloride (PVC) hose which was in turn connected to a high density PVC 15 mm pipe having outlets connected to a 4 mm clear flexible plastic tube with a PVC connecter and an air line regulator valve. The end of the clear tube was attached to an air stone aerator (15 x 25 mm). The air flow rate was controlled using the air line regulator valve and the air flow rate was fixed at 4 L min^{-1} for each flask.

A culture media sample of 25 mL was removed from each replication flask every 48 h to estimate the cell counts, dry weight, total lipid, pH and electrical conductivity (EC) of the microalgal culture.

4.2.4.2 Nutrient limited growth trial (modified N P and Fe concentration in the standard media)

A second growth trial was conducted to assess *C. vulgaris* biomass and lipid productivity under reduced concentrations of N, P and Fe, compared to the control

(standard composition of ASM media). The experimental design was a RCBD with three replications of each of three treatments.

Standard ASM media contains $NaNO_3$ at 85 g L^{-1} (N source), K_2HPO_4 at 17.4 g L^{-1} and Na_2HPO_4 at 14.2 g L^{-1} (P source) and $FeCl_3.6H_2O$ at 1.0835 g L^{-1} (Fe source). These sources were reduced to 75 % of standard levels. Other growth conditions were similar to nutrient sufficient growth trial.

4.2.5 Data analysis

The results were expressed as mean ± standard error (SE) of means of five replications in case of nutrient sufficient cultivation and three replications in case of nutrients reduced cultivation. Biomass and total lipid content of microalgal species were analysed by Analysis of Variance (ANOVA) using GenStat statistical package, Version 11.1 (VSNI Ltd, UK) after testing the data for outliers and normality, and homogeneity of error variances. A value of $p \leq 0.001$ was considered as significant.

4.3 Results

4.3.1 Nutrient sufficient growth trial

4.3.1.1 Cell numbers and relationship with OD_{625} nm

After 22 d of cultivation on normal nutrient media the microalgal cultures of *C. vulgaris*, *Navicula* sp. 1, *N. pusilla* and *Navicula* sp. 2 recorded the maximum cell concentration of 3 x 10^{-8}, 4 x 10^{-5}, 2 x 10^{-5} and 1 x 10^{-7} cells mL^{-1} respectively (Figure 6). Cell densities were significantly different ($p<0.001$) between the days and the species (Table I in Appendix H), with highest maximum cell density recorded in *C. vulgaris* (Figure 6).

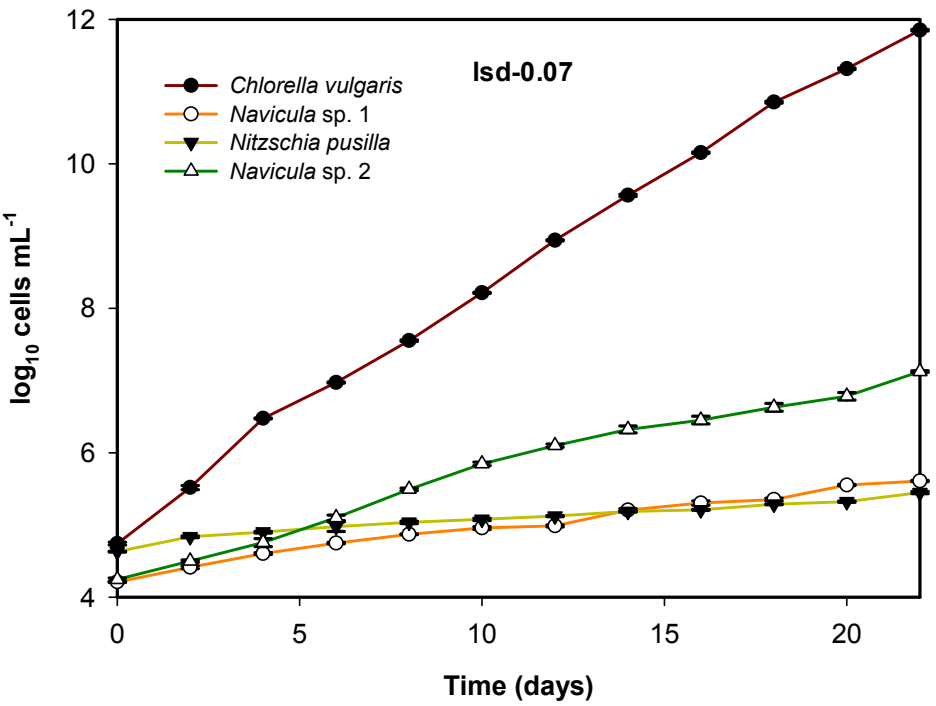


Figure 6. The number of cells (mean± SE, n=5) recorded every 48 h for 22 d for *C. vulgaris*, *Navicula* sp. 1, *Nitzschia pusilla* and *Navicula* sp. 2 grown in standard nutrient media (nutrient sufficient growth trial).

OD_{625} nm was linearly correlated with the cell counts as determined using the Sedgwick Rafter. Regression equations of y = 0.6423x + 4.3381, R^2 = 0.996 in case of *C. vulgaris*, y = 0.1212x + 4.1969, R^2 = 0.983 in case of *Navicula* sp. 1, y = 0.0619x + 4.6837, R^2 = 0.959 in case of *N. pusilla* and y = 0.2616x + 4.0788, R^2 = 0.981 in case of *Navicula* sp. 2 were obtained from the data analysis, where y is the cell number (log_{10} cells mL^{-1}) and x is the optical density of microalgal cultures at 625 nm.

4.3.1.2 Biomass content

The four microalgal species were tested for their biomass content by assessing the dry weight ever 48 h under nutrient sufficient growth trial. *C. vulgaris* showed the highest biomass content of 3.03±0.04 g L^{-1} while *Navicula* sp. 2 showed the lowest

content of 0.06±0.001 g L^{-1} compared among the species (Figure 7). A general ANOVA revealed significant differences in biomass content between days and species ($p < 0.001$) (Table II in Appendix H). On an average the mean biomass content of 1.46±0.58, 0.066±0.016, 0.10±0.014 and 0.04±0.008 g L^{-1} were recorded in *C. vulgaris*, *Navicula* sp. 1, *N. pusilla* and *Navicula* sp. 2 respectively after 22 d of nutrient sufficient growth trial.

The biomass content (g L^{-1}) compared with OD values at 625 nm indicated that the OD was linearly correlated with the biomass content determined using dry weight. Regression equations of $y = 0.2782x + 0.3515$, $R^2 = 0.997$ in the case of *C. vulgaris*, $y = 0.0079x + 0.0141$, $R^2 = 0.997$ in case of *Navicula* sp. 1, $y = 0.0068x + 0.0574$, $R^2 = 0.992$ in the case of *Nitzschia pusilla* and $y = 0.0037x + 0.0182$, $R^2 = 0.996$ in the case of *Navicula* sp. 2 were obtained from the data analysis, where y is the biomass content (g L^{-1}) and x is the optical density of microalgal cultures at 625 nm.

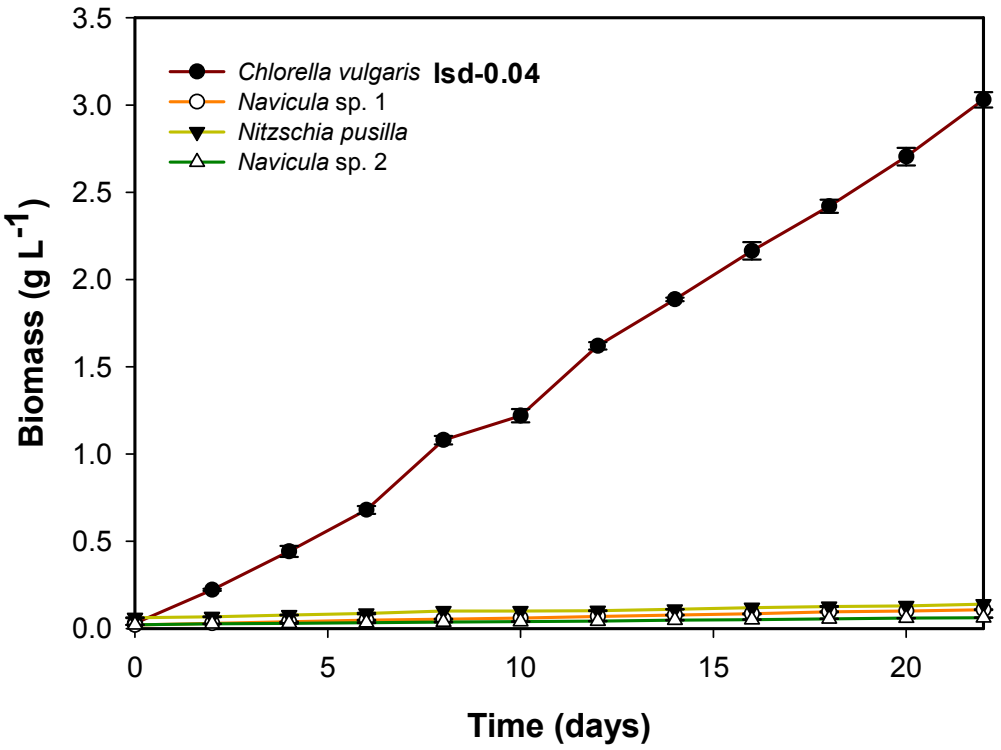


Figure 7. Biomass content (g L^{-1}) (dry weight) every 48 h for 22 d of five replicates in *Chlorella vulgaris*, *Navicula* sp. 1, *Nitzschia pusilla* and *Navicula* sp. 2 of standard nutrient cultivation (nutrient sufficient growth trial). The data points represent mean of five replicates and associated SE.

4.3.1.3 Lipid content

On average the lipid content of four microalgal species cultured under nutrient sufficient growth trial ranged from 17±4.68 to 50±5.40 % of the dry weight. The average lipid content of 0.20±0.05, 0.03±0.007, 0.04±0.008 and 0.04±0.001 g L^{-1} were recorded in *C. vulgaris*, *Navicula* sp. 1, *N. pusilla* and *Navicula* sp. 2 respectively after 22 days of nutrient sufficient growth trial (Figure 8). A general ANOVA revealed significant differences in lipid content between days and species ($p < 0.001$) (Table III in Appendix H) with average highest lipid content recorded in *C. vulgaris*.

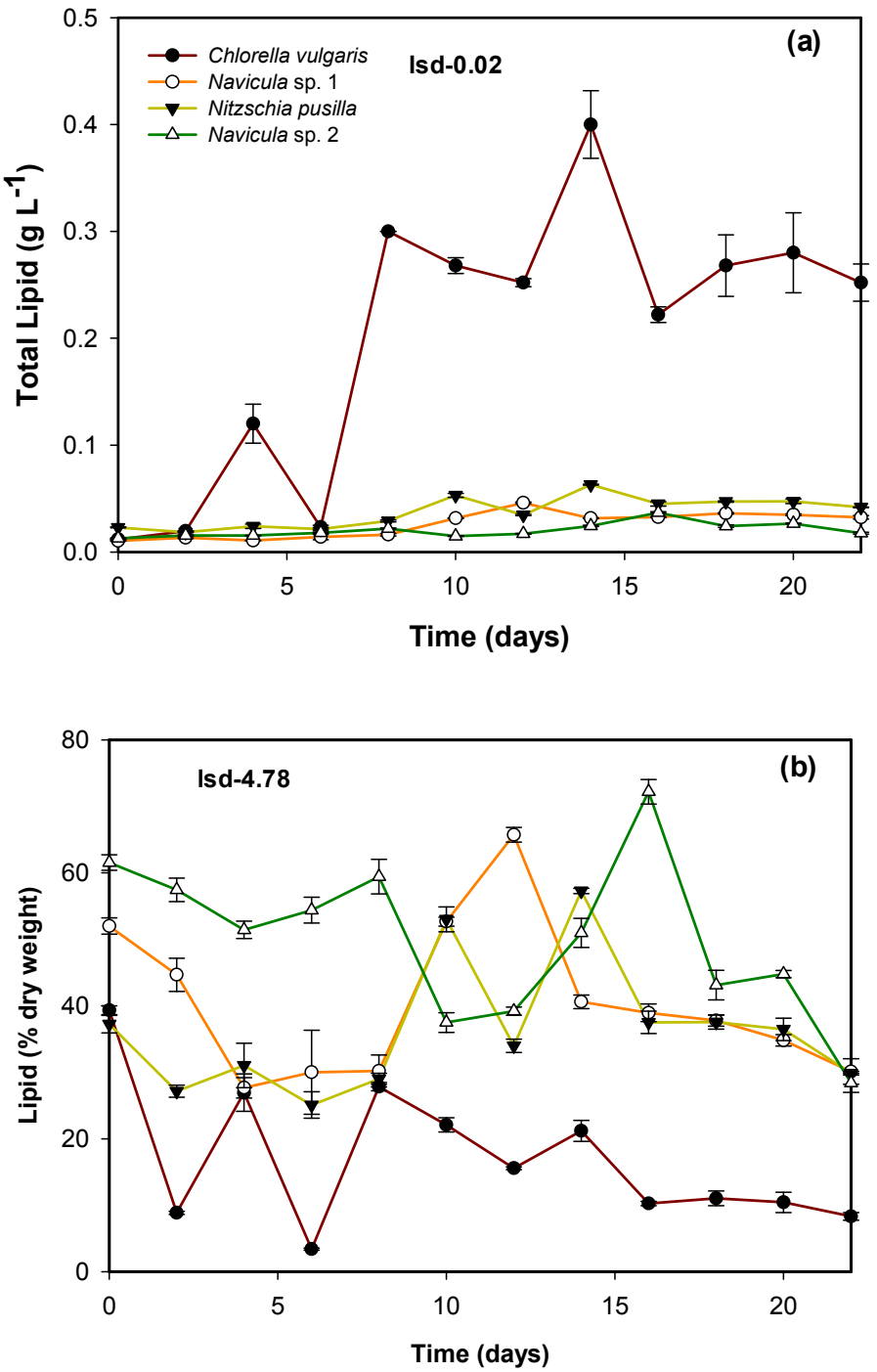


Figure 8. (a) Lipid content (g L^{-1}) and (b) lipid % dry weight, every 48 h for 22 d of *Chlorella vulgaris*, *Navicula* sp. 1, *Nitzschia pusilla* and *Navicula* sp. 2 on nutrient sufficient growth trial. The data points represent mean of five replicates and associated SE.

C. vulgaris accumulated maximum lipid content of 0.40±0.03 g L^{-1} on day 14 and the lipid content accounted to 21 % of dry weight on day 14. *Navicula* sp. 1 accumulated maximum lipid content of 0.05±0.001 g L^{-1} on day 12 and lipid content accounted to 66 % dry weight on day 12. *Nitzschia pusilla* accumulated maximum lipid content of 0.06 g L^{-1} on day 14 and lipid content accounted to 57 % dry weight on day 14. *Navicula* sp. 2 accumulated maximum lipid content of 0.04±0.001 g L^{-1} on day 16 and the lipid content accounted to 72 % dry weight on day 16.

4.3.1.4 pH and EC variations

A pH range of 6.84-8.07±0.02 was recorded in *C. vulgaris*, *Navicula* sp. 1, *N. Pusilla* and *Navicula* sp. 2 cultivated under nutrient sufficient growth trial (Figure 9a). Initially the pH of Guillard f/2 and ASM media were set at 7.5±0.2 and 7.6-7.8±0.2 respectively. A maximum and minimum pH of 8.07±0.02 and 6.84±0.02 were recorded in *C. vulgaris* on day 20 and *N. pusilla* on day four respectively. A significant correlation between pH with and biomass (R^2>0.98) and EC (R^2=0.99) was recorded after 22 days of nutrient sufficient growth trial. However, correlation of pH was poor to lipid content (R^2<0.64)

An EC range of 198-425 µS cm^{-1} was recorded in *C. vulgaris*, *Navicula* sp. 1, *N. pusilla* and *Navicula* sp. 2 cultivated under nutrient sufficient growth trial (Figure 9b). There was significant correlation of EC with biomass (R^2>0.98) and pH (R^2=0.99) recorded after 22 days of nutrient sufficient growth trial. A constant increase in EC was observed in nutrient sufficient growth trial.

Sampling 25 mL every 48 h up to 22 d should reduce the media to 1550 mL at the end of the experiment. However, towards the end of the experiment the remaining media in each replication flask measured was approximately 1200±100 mL across the species and replication flasks.

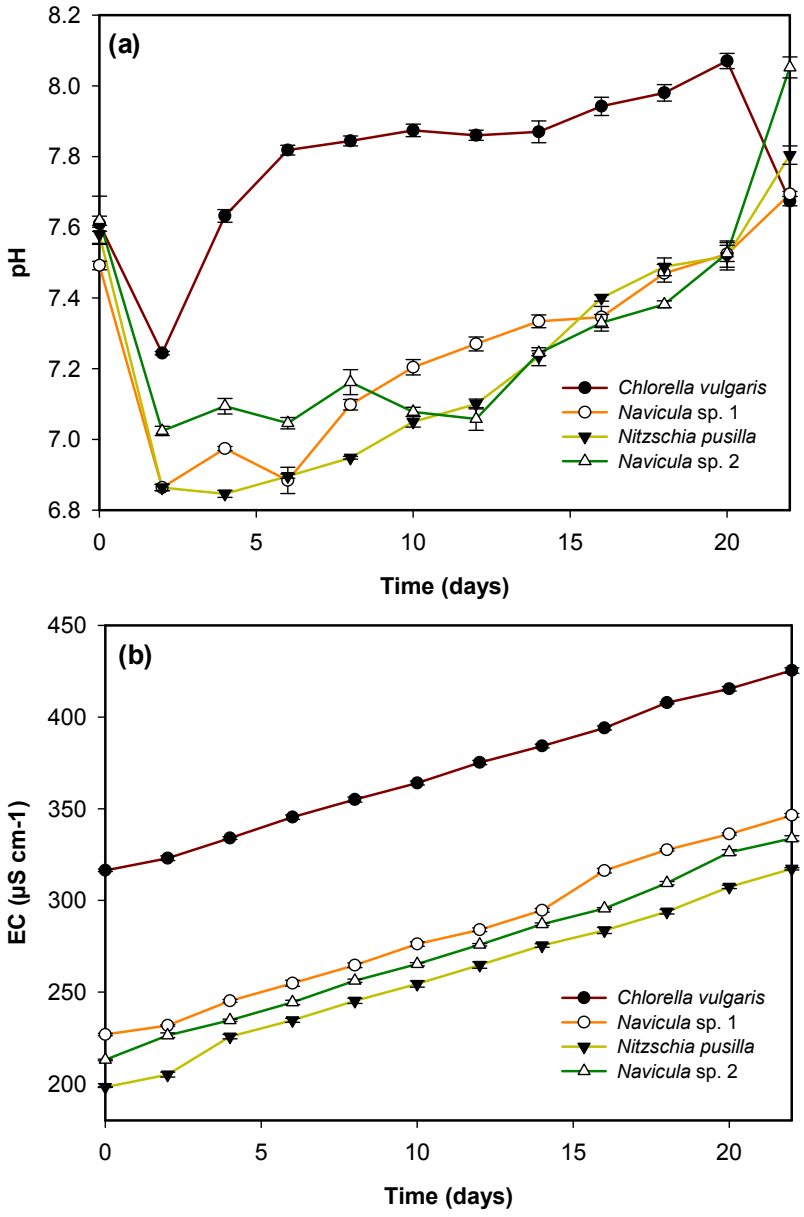


Figure 9. pH (a) and EC (b) recorded every 48 h for 22 days in *Chlorella vulgaris*, *Navicula* sp. 1, *Nitzschia pusilla* and *Navicula* sp. 2 under nutrient sufficient growth trial. The data points represent mean of five replicates and associated SE.

4.3.2 Nutrient limited growth trial

4.3.2.1 Biomass content and relation to OD_{625} nm

A mean biomass content of 1.301±0.49, 0.31±0.07, 0.38±0.11 and 0.40±0.11 g L^{-1} was recorded of control, N, P and Fe limited treatments respectively, after 20 days of cultivation (Figure 10). On average the biomass content of the control treatment was higher by 4.06, 3.41 and 3.25 times that of N, P and Fe limited cultures. A general ANOVA revealed significant differences in biomass content between days and treatments ($p<0.001$) (Table IV in Appendix H)

OD at 625 nm was linearly correlated with the biomass content determined using dry weight. Regression equations of $y = 0.2635x + 0.0279$, $R^2 = 0.992$ in case of control, $y = 0.0082x + 0.0111$, $R^2 = 0.981$ in case N limited cultivation, $y = 0.0597x + 0.0371$, $R^2 = 0.983$ in case of P limited cultivation and $y = 0.0061x + 0.0059$, $R^2 = 0.986$ in case of Fe limited cultivation were obtained from the data analysis, where y is the biomass content (g L^{-1}) and x is the optical density of microalgal cultures at 625 nm.

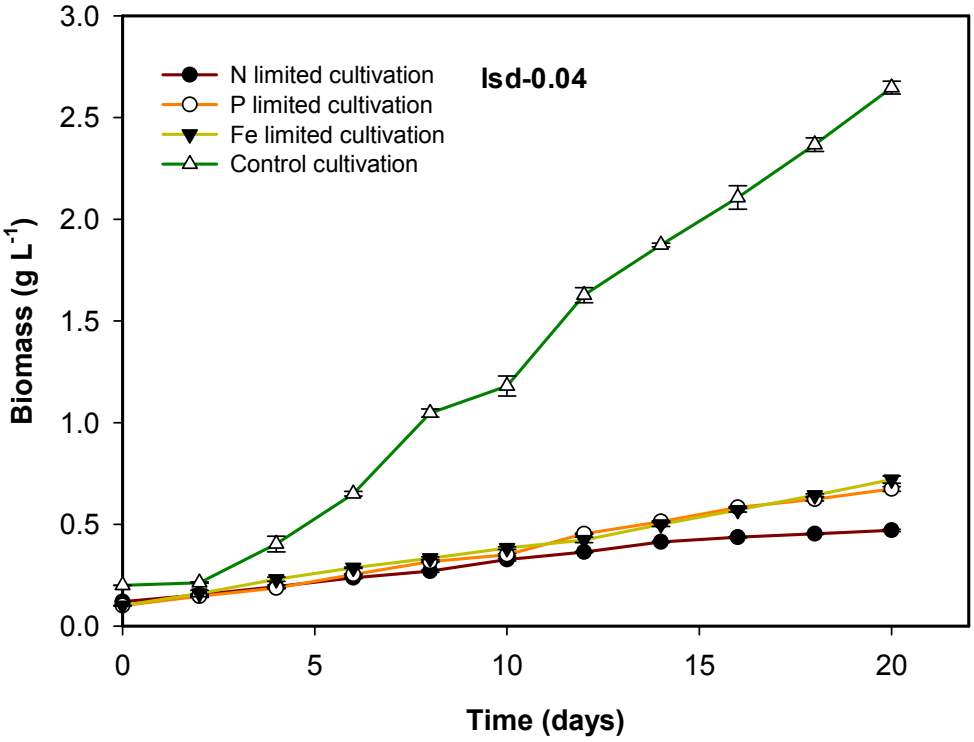


Figure 10. Biomass content (g L^{-1}) (dry weight) every 48 h for 20 days of *C. vulgaris* cultivated under nutrient limited growth trial of N ($NaNO_3$-21.25 g L^{-1}), P (K_2HPO_4-4.35 g L^{-1} and Na_2HPO_4-3.35 g L^{-1}) and Fe ($FeCl_3.6H_2O$-0.2708 g L^{-1}). Control was standard nutrient ASM media. The data points represent mean of three replicates and associated SE.

4.3.2.2 Lipid content

Mean lipid contents of 0.212±0.05, 0.23±0.07, 0.274±0.09 and 0.22±0.06 g L^{-1} were recorded after 20 days of cultivation in control, N, P and Fe limited conditions respectively (Figure 11). A general ANOVA revealed significant differences in lipid content between days and treatments ($p<0.001$) (Table IV in Appendix H). Under the control condition, the maximum lipid content of 0.33±0.01 g L^{-1} occurred on day 18, at which time the lipid content accounted for 14 % of dry weight. Under N limited cultivation the maximum lipid content of 0.38 g L^{-1} occurred on day 18, associated with lipid content of 84 % of dry weight. Under P limited cultivation, a maximum lipid content of 0.53±0.01 g L^{-1} occurred on day 18, associated with a lipid content of 86

% of dry weight. Under Fe limited cultivation the maximum lipid content of 0.38±0.01 g L^{-1} occurred on day 16, with a lipid content of 68 % of dry weight. Compared to the control the lipid % of dry biomass increased by 38-49 % under the nutrient limited growth trial of N, P and Fe.

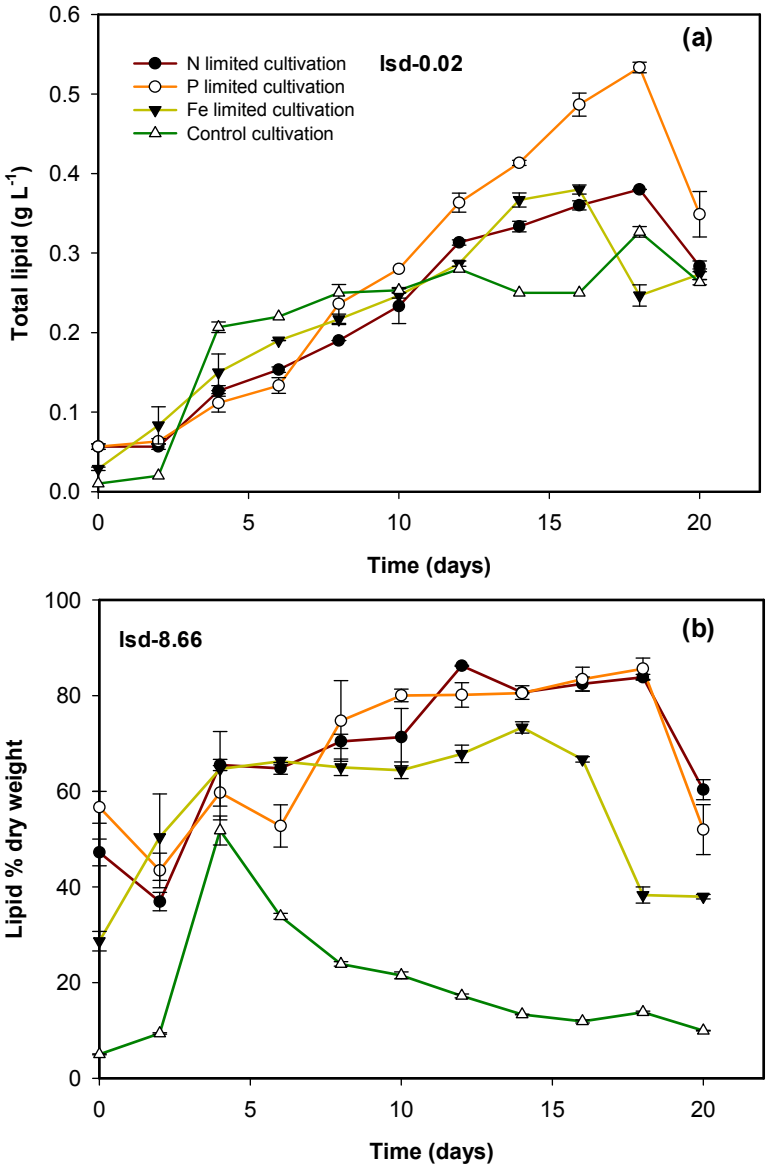


Figure 11. (a) Lipid content (g L^{-1}) (b) lipid % dry weight, ever 48 h for 20 days of *C. vulgaris* cultivated under N ($NaNO_3$-21.25 g L^{-1}), P (K_2HPO_4-4.35 g L^{-1} and Na_2HPO_4-3.35 g L^{-1}) and Fe ($FeCl_3.6H_2O$-0.2708 g L^{-1}) reduced growth trial. Control was standard nutrient media of ASM. The data points represent mean of three replicates and associated SE.

4.3.2.3 pH and EC variations

A pH range of 7.65-8.23±0.02 was recorded for *C. vulgaris* cultivated under control, N, P and Fe limited conditions (Figure 11a). Initially the pH of ASM media was set at 7.6-7.8±0.2. There was no correlation of pH to lipid content after 20 days in *C. vulgaris* cultivated under nutrient limited growth trial.

A constant increase in EC was observed in nutrient limited growth trial, with an EC range of 326-438 μS cm^{-1} (Figure 12b). There was no correlation of EC with biomass and lipid content and pH, recorded after 20 days of nutrient limited growth trial.

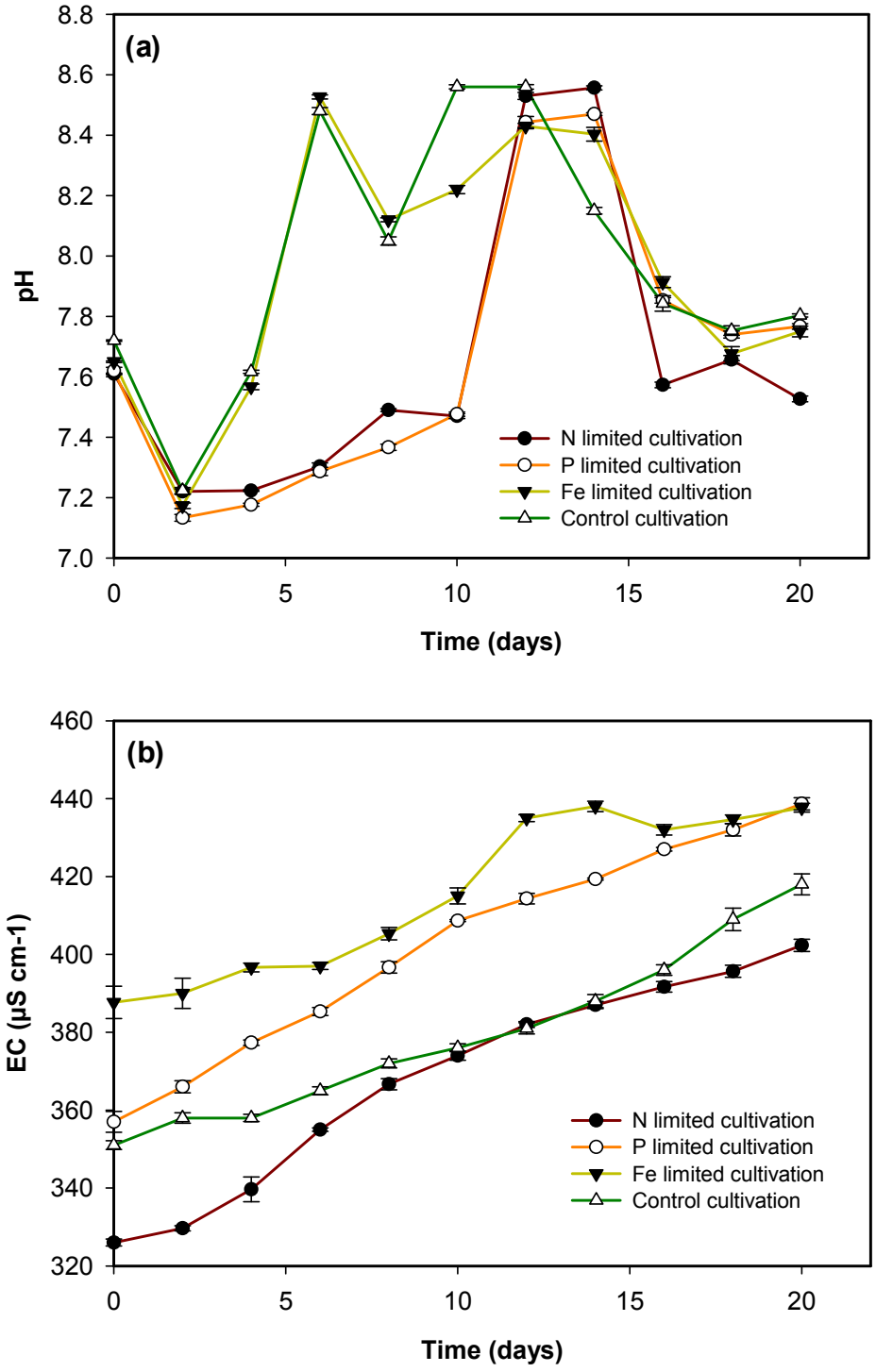


Figure 12. pH (a) and EC (b) recorded every 48 h for 20 days in *Chlorella vulgaris*, under reduced nutrient growth trial. The data points represent mean of three replicates and associated SE.

4.4 Discussion

The highest biomass and lipid content was recorded in *C. vulgaris* among *Navicula* and *Nitzschia* sp. cultivated for 22 days in nutrient sufficient growth trial. The biomass and lipid content were similar to that reported in the earlier studies (Table 14). *C. vulgaris* grown under N, P and Fe limited media resulted in 20-30 % less biomass and 38-49 % more lipid content compared to control (standard nutrient media). Among the nutrients highest lipid content was achieved in P reduced culture followed by N and Fe cultures.

4.4.1 Correlation between cell counts and dry weight to optical density

Microalgal culture growth was measured in terms of cell counts, optical density and dry weight. OD at 625 is a measure of scattering within a solution (e.g. by particles, or cells) as well as absorbance at that wavelength (e.g. by photosynthetic pigments in microalgae). The wavelength range of 580-680 nm has been used by numerous authors to measure cell densities and biomass in microalgae, with correlation coefficient values of 0.97-0.98 between cell numbers and OD reported (Chih-Hung & Wen-Teng 2009; Chiu *et al.* 2009; Chiu *et al.* 2008; Converti *et al.* 2009; Lin *et al.* 2007; Takagi *et al.* 2006; Xin *et al.* 2010; Yue & Chen 2005).

In the present study, the highest correlation coefficient determined between cell counts and optical density was recorded in *C. vulgaris*, followed by *Navicula* sp. and the lowest correlation value was in *N. pusilla*. The variation in correlation coefficient values across microalgal species was likely associated with the spread of data (SD) of each data set. The cell size of *Chlorella* and *Navicula* species were < 10 µm where as the size of *Nitzschia* sp. was > 20 µm. Particle size and number will

influence scattering, and thus a different slope would be expected for the relationship of OD to cell number between species.

The present study also demonstrated significant correlation between biomass estimated using dry weight and OD_{625} under nutrient sufficient and limited growth trials of microalgal species. Unlike the correlation values obtained between cells counts and optical density, the R^2 values obtained between dry weight and optical density were uniform across the microalgal species cultivated under nutrient sufficient growth trial.

Previous studies on various species of *Chlorella* under various growth conditions obtained a linear relation range of 0.97-0.99 between dry weight (g L^{-1}) and optical density measured at 540, 625 and 658 nm (Chih-Hung & Wen-Teng 2009; Converti *et al.* 2009; Feng *et al.* 2011; Xu *et al.* 2006). The R^2 values obtained in the present study were in agreement with the earlier results.

However, no particular wavelengths were specified in the literature to estimate microalgal biomass using optical density and the measurement are variable depending on the microalgal species and cultivation conditions (Nam 2009). The present study demonstrated that, to minimise the error occurring due to pigment variations among the microalgal species and varied growth conditions, wavelength range beyond the pigment absorptions (550 or 750 nm) are better options to develop precise independent calibration curves (Dempster & Sommerfeld 1998; Griffiths *et al.* 2011; Liu *et al.* 2008).

4.4.2 Biomass and lipid content of microalgae (nutrient sufficient growth trial)

Biomass and lipid content of C*. vulgaris* was 93-98 and 80-90 % respectively higher than that of *Navicula* and *Nitzschia* species. Lipid content in *C. vulgaris* was maximum (21 % dry weight) on day 14, then decreased, such that after 22 days of cultivation it was 17 % dry weight of biomass. The lipid content double on day 14 compared to the average lipid content after 22 days. The reason for the sudden increase in lipid content of *C. vulgaris* on day 14 in nutrient sufficient growth trial could be due to some unknown factors or might be an unnoticed sampling error, however, similar increase in lipid content on day 14 was not observed in control culture in nutrient limited growth trial of *C. vulgaris*, which had similar growth conditions as of nutrient sufficient growth trial.

In the present study, biomass and lipid content of *C. vulgaris* grown using ASM media for 22 days recorded biomass and lipid content of 1.46 g L^{-1} and 17 % dry weight respectively. ASM media is a common media used for blue green algal species. The present study demonstrated ASM media, as a potential media to grow *C. vulgaris* to achieve high biomass and lipid contents as equal to other media used to grown green algae. The reason for choosing ASM media to grown *C. vulgaris* in the present study was that the stock culture maintained using ASM media resulted in higher growth rates compared to BBM media. The N source ($NaNO_3$) is 29 % and P source (K_2HPO_4) is 43 % more in ASM media compared with that of BBM media. The low growth in the stock cultures maintained in BBM media might be due to the low concentrations of N and P. In the present study when the N and P

concentrations were reduced to 75 % less than the required media in nutrient limited growth trial, the biomass content decreased by 20-30 % less than the control media.

However, no standard growth media was listed in the literature and the choice of media to grown *Chlorella* is mostly restricted to green algae media (Chih-Hung & Wen-Teng 2009; Chisti 2006; Chisti 2007; Converti *et al.* 2009; Illman *et al.* 2000; Molina Grima *et al.* 2003; Widjaja *et al.* 2009).

In the present study, the strain which was a native isolate of the Central Queensland region performed equally good compared to the earlier reports under nutrient sufficient cultivation (Table 14). *C. vulgaris* has been reported as a potential candidate for biofuel production having lipid content range from 14-30 % of dry weight under normal cultivation conditions (Spolaore *et al.* 2006).

Limited information is available on *Nitzschia and Navicula* species as potential candidates for biofuel production. The lipid yield of various species of *Nitzschia* and *Navicula* range between 10-75 % (Dempster & Sommerfeld 1998; Moazami *et al.* 2011; Sheehan *et al.* 1998) and 25-47 % dry weight respectively (Coombs *et al.* 1967; Matsumoto *et al.* 2010; Tadros & Johansen 1988) under varied growth conditions.

In the present study, significant variation of biomass and lipid content was observed among the microalgal species of *Chlorella*, *Navicula* and *Nitzschia*. Biomass and lipid content of *Nitzschia pusilla* and *Navicula* sp. isolated from Central Queensland region were low compared to the *C. vulgaris*. These species also exhibited the characteristic of sticking to the culture vessels, character expected of a benthic algae

Such type of species needs sufficient agitation if considered for mass cultivation (Griffiths & Harrison 2009). Based on the reasons of low biomass and lipid content and sticky nature of *Nitzschia* and *Navicula* sp. These species were excluded from further growth trial.

4.4.3 Biomass and lipid content of microalgal species (nutrient limited growth trial)

Numerous authors have reported an increase lipid content associated with a decreased supply of N, P and Fe nutrients (Chih-Hung & Wen-Teng 2009; Converti *et al.* 2009; Illman *et al.* 2000; Liu *et al.* 2008; Ördög *et al.* 2012; Widjaja *et al.* 2009). Based on this information an additional study was conducted using *C. vulgaris* to assess the biomass and lipid content of *Chlorella* grown on reduced concentrations of N, P and Fe from the standard ASM media.

Reduced concentrations of N, P and Fe in the growth media demonstrated that *C. vulgaris* was able to accumulate 38-49 % dry weight of lipid content more than that of control culture, which was in agreement with the reported literature. Whereas, the average biomass content of the N, P and Fe limited cultures was 20-30 % less that the control culture. Similar results were observed in *C. zofingienis* grown under N and P deficient conditions (Feng *et al.* 2012). The nutrient deficient medium might have limited the cell growth in *C. vulgaris* the possible reason for the low biomass content compared to the control culture (Feng *et al.* 2012).

The maximum biomass in *C. vulgaris* was stimulated by Fe limited culture followed by P limited culture and the lowest biomass content was in N limited culture ($p<0.05$).

Although Fe limited culture stimulated maximum growth in *C. vulgaris* the lipid content was highest in P limited culture accounting to 69 % of dry weight ($p<0.05$).

The present study demonstrated that limiting iron in the standard media reduced the biomass by 30 % where as lipid content was similar to control culture in *C. vulgaris*. The result is in agreement with Liu *et al*. (2008), who suggested various level of iron in the media might have affected the lipid metabolic pathways. The function of iron in microalgae cell was well established in earlier studies (Behrenfeld *et al.* 2006) however, iron as limiting factor on biomass and lipid content in microalgal species is limited (Liu *et al.* 2008).

Phosphate which is considered as a major nutrient source to grow microalgae was proved to enhance the biomass concentration of microalgal if the growth conditions are optimised (Banerjee *et al.* 2002; Bhola *et al.* 2011; Chisti 2006; Chisti 2007). In the current study, the biomass content was 29 % lower and lipid content was 28 % higher than the control culture. These results were similar to that of Bhola *et al.* (2011) who demonstrated that in reduced P limited growth; *C. vulgaris* cell division was significantly slow leading to lipid accumulation in the cells.

Numerous studies reported that N limitation and various sources of N would trigger maximum accumulation of lipids (Converti *et al.* 2009; Hsieh & Wu 2009; Illman *et al.* 2000; Ördög *et al.* 2012; Widjaja *et al.* 2009). In the present study, *C. vulgaris* grown under reduced N concentration decrease the biomass content by 23 % and increase lipid content by 9 %. Although the % increase in lipid content was low compared to P limited cultured, the results obtained under N limited culture were in agreement with

the previous reports (Converti *et al.* 2009; Hsieh & Wu 2009; Illman *et al.* 2000; Ördög *et al.* 2012; Widjaja *et al.* 2009).

In the present study, biomass content in C*. vulgaris* under P and Fe limited cultivation was linear where as the biomass concentration of N limited culture was constant after day 14. The maximum lipid content across N and P limited culture was on day 18, where as in P limited culture it was on day 16. After which there was a steady decline in lipid content across the treatments.

This study demonstrated that reduced P concentration was a suitable condition for high lipid content in *C. vulgaris* strain isolated from Central Queensland region.

4.4.4 pH and EC variation under nutrient sufficient and deficient cultivations

The culture media used with the three species varied in pH but there were species variations in the change in pH during culturing. In the nutrient limited growth trial N and P limited cultures showed similar trend of pH where as control and Fe limited cultures showed a similar trend. The differences were because of the nutrient concentration in the growth media. The present study, showed no correlation between pH to lipid contents. pH is considered as an important parameter and varies widely depending on the CO_2 concentration of the experiments and lower pH inhibits the growth of microalgae (Yue & Chen 2005). A study by Dayanada et al (2007) on *Botryococcus braunii* reported no significant effect of pH on the biomass and lipid content over the pH range of 6.0 to 8.5 using Chu 13 media. Similar results were observed in the present study.

In the present study, a constant increase in EC was observed across the growth trials which could be due to the evaporation rates. The volume of the media measured at the end of each growth trial (nutrient sufficient and limited) was 300±100 mL less than the total sampled volume during the experiment. This accounts to 13±2 mL d^{-1} of evaporation rates in each replication flask the reason for constant increase in EC. However, there was no correlation of EC to biomass and lipid contents in the growth trials.

The present study demonstrated that *C. vulgaris* strain isolated from Central Queensland region has potential biomass and lipid content equal to numerous *C. vulgaris* reported in the literature. However, other parameters such as temperature, light intensity and other nutrient requirements needs to be optimized in the further, to consider this strain for outdoor cultivation.

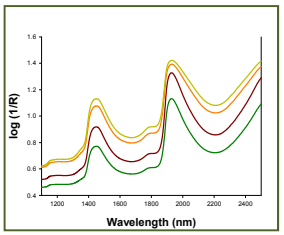
4.5 Conclusions and recommendations

Biomass and lipid contents of *Chlorella*, *Navicula* species isolated from fresh water and *Nitzschia* isolated from brackish water were tested under standard nutrients media and reduced concentrations of N, P and Fe in the media. The cell counts and dry weight values of biomass were significantly correlated to optical density of microalgal cultures measured at 625 nm. The growth curve was linear in all the four microalgal species. Biomass and lipid content of 1.46±0.58 and 0.20±0.05 g L^{-1} was highest in *Chlorella vulgaris* among the other microalgal species grown under standard nutrient media (nutrient sufficient growth trial). *C. vulgaris* grown under N, P and Fe reduced concentrations biomass content decreased by 20-30 % and lipid

content increased by 38-49 % dry weight compared to the control culture. Among the reduced nutrients particular interest was the P reduced culture of *C. vulgaris* with highest lipid content of 0.274±0.09 g L^{-1}. No correlations were recorded between pH and EC to that of biomass and lipid content of microalgal species. *C. vulgaris* isolated from Central Queensland region has potential lipid content equal to that of earlier studies.

This study recommends further efforts be focussed on assessing the lipid profile of *C. vulgaris* for parameter such as fatty acid chain lengths and degree of un-saturation, which play a major role in biodiesel fuel quality and optimization of varying light intensities and CO_2 requirements needs to be established as a primary step before the strain is cultivated outdoors.

Chapter 5. Assessing biomass and total lipid content of microalgal species with near infrared spectroscopy



5.1 Introduction

The third objective of this thesis is "To apply Near Infrared Spectroscopy to assess biomass and lipid content of microalgal samples". Relative to other analytical techniques, NIRS offers the advantage of rapid and *in situ* assessment of certain parameters. NIRS is suited to analysis of compounds rich in O-H, C-H, N-H or S-H bonds, which demonstrate absorption related to the stretching of these bonds (Murray & Williams 1987; Thyholt & Isaksson 1997). The C-H, OH_2 and CH_3 absorption bonds in NIR spectra are generally associated with fats, oils and carbohydrates in biological materials (Dull and Giangiacomo 1984). As such, the technique is relevant to the assessment of lipid production in microalgal culture. This potential was recently reported by Laurens and Wolfram (2011) who reported on the feasibility of use of NIR reflectance spectroscopy to assess exogenous lipids added to ground, lyophilized microalgal biomass.

Absorbance associated with OH and CH bond vibrations feature strongly in the NIR spectra of biological material. This is reflected in the use of NIRS to assess dry matter content (the inverse of moisture content) in a wide array of materials and to assess total lipids and lipid classes. For example, a Scopus search on the keywords NIR and DM yields a report of 79 publications over the years 2005-2012, while a

Scopus search on the keywords NIRS and lipid yields a report of 66 publications. However, little research has been reported in terms of the estimation of microalgal biomass and lipid content. For example, a Scopus search on the keywords NIRS and algae biomass or lipids yields a report of one publication over the same period.

Laurens and Wolfrum (2011) were the first to report the application of NIRS to the measurement of microalgal lipids. In their study, lyophilized biomass of *Nannochloropsis*, *Chlorococcum*, *Spirulina* and an unknown diatom were ground, and the lipids trilaurin (a triglyceride) and phosphatidylcholine (a phospholipid) were added at nine levels (0-3% w/w) resulting in 36 samples (9 addition levels and 4 microalgal species). Reflectance spectra were collected with a Foss NIR Systems model 6500 Forage Analyzer over the range of 400-2500 nm (at 2 nm steps). Partial Least Squares (PLS) regression models were based on the 1100-2500 nm region of spectra pre-treated using second derivative (d_2) and multiple scatter correction (MSC) treatments. A first derivative treatment significantly reduced the quality of the model relative to raw reflectance data. The PLSR model developed for the triglycerides and the phospholipids was reported to have an R^2 value of 0.97 and 0.95, respectively. The model was used to predict test spectra obtained from spiked biomass not included in the calibration sets. Single species and combined species models were used for the predictions. It was reported that the individual species models accurately predicted the levels of spiked lipids, compared to the combined species models. Models developed to assess the triglycerides added to *Spirulina* sp. biomass was used to predict the triglycerides added to *Chlorococcum* sp. biomass which resulted in root mean square error of prediction (RMSECP) values of

9.95±4.15. Similarly models developed to assess the phospholipids added to *Nannochloropsis* sp. biomass which resulted in an RMSECP value of 41.08±14.52.

This report is apparently encouraging; however the experimental design involves spiking of a single matrix (ground, lyophilized algae) with different levels of two lipids. This approach will result in a marked overestimation of the ability of the technique to detect lipids in a variable matrix (i.e. algae grown under different conditions, or at different growth stage, and thus of different composition). It is important for application development that the model be tested over a number of test populations that are independent of the calibration set. If the prediction is poor, then work is needed to understand if the model can be made robust to the new condition, or if a separate model should be created that is specific to the new condition, or indeed if the initial attempt was over-fitted, such that it will always fail in prediction of independent sets. For example, chlorophyll content may be correlated with biomass under one set of culture conditions but the relationship may not hold under another set of culture conditions. For example, if a biomass model weighted a spectral feature related to chlorophyll, it would perform poorly in prediction of biomass in cultures grown under a range of nutrient deficiencies.

Another critical issue in NIRS is sample presentation relative to light source and detector. To assess attributes of algal cultures, an *in-situ* (in flask) technique would be highly desirable for process control reasons. However the water content of the culture flask allows the water absorption bands is likely to dominate other components of the sample (Dull and Giangiacomo 1984). An obvious analytical solution to this issue is to remove water from the sample before analysis. A dried

filter paper approach using glass fibre filter papers was first reported by Asher *et al*. (1982) to assess fungal spore load on Barley and wheat. In this work, spore suspensions were diluted to, approximately 60 x 10^3 spores/mL. Using automated pipette the aliquots were transferred to GF/A glass micro-fibre discs (55 mm diameter) and the discs were dried at 40 °C for 12 h and cooled in a silica gel before scanning using the Neotec 6350 Research Composition Analyser over the range of 1100-2500 nm. Glass fibre discs have also been used as a substrate on which to concentrate a compound of interest for measurement using reflectance infrared or NIRS in a technique termed Dry Extract Sample Infrared Spectroscopy. For example this technique was used to detect the chemical contamination on fruit surfaces (Saranwong & Kawano 2005, 2007). The use of glass fibre filter papers to concentrate microalgae was first reported by Mitchell (1990), and the technique has been widely used subsequently, including for the study of carotenoids and fatty acid changes (Solovchenko *et al.* 2011; Solovchenko *et al.* 2010; Solovchenko *et al.* 2009). Such filtrates require relatively minor sample preparation, but should be able to be used directly with in a NIRs reflectance instrument.

In the present study, an attempt was made to estimate biomass and total lipid content of growing microalgal species using NIRS. In the previous chapter titled "Study of biomass and lipid content of microalgal species isolated from Central Queensland, Australia" four microalgal species were cultured and biomass and total lipid contents assessed, under nutrient sufficient and limited growth trials. These values of biomass and total lipid were used as reference values to PLSR models based on spectra of either culture flasks or filtrates.

5.2 Materials and Methods

5.2.1 Experimental design

In a first growth trial, four species of microalgae (*Chlorella vulgaris*, *Navicula* sp. 1, *Nitzschia pusilla* and *Navicula* sp. 2) were each grown in 2 L culture vessels (five replicates each), employing a complete nutrient solution (ASM in the case of *C. vulgaris* and f/2 in the case of *Navicula* and *Nitzschia* species), maintained at 25°C and at a light intensity of 350-400 µmol m^{-2} s^{-1} (400 W metal halide lamp). The experimental conditions were described in Chapter 4, section 4.2.4. Aliquots (10 mL) of the (well mixed) culture solution were collected every 48 h for 22 days for each culture flask, resulting in five samples/species/day, or 60 samples/species over the entire experiment (0-22 days).

In a second growth trial, *C. vulgaris* was grown under conditions of the nutrient sufficient growth trial, except that the nutrient solution was altered (complete, N, P and Fe reduced concentration), with three replications per treatment. The standard nutrient sources of N (21.5 g L^{-1} of $NaNO_3$), P (4.35 g L^{-1} of K_2HPO_4 and 3.55 g L^{-1} of Na_2HPO_4) and Fe (0.27 g L^{-1} of $FeCl_3.6H_2O$) were reduced by 75 % in the respective treatments. Aliquots (10 mL) were collected every 48 h for 20 days resulting a total number of samples collected during the experiment of 33 samples/nutrient treatment (0 to 20 days).

5.2.2 Sample presentation, reference measurements and spectral acquisition

Two different sample presentations and spectrophotometers were used:

In-situ scanning of the culture vessels was undertaken using a Nirvana (Integrated Spectronics) spectrometer that operated over the 300-1100 nm range and employed interactance geometry (Figure 13). This equipment incorporates a Zeiss MMS1 (Jena, Germany) spectrometer module fitted with an 8 mm diameter lens, matched to the numerical aperture of the fibre optics. The detector assembly is mounted in front of the tungsten halogen lamp reflector assembly, such that a shadow is cast into the sample. Light scattered by the sample can emerge from the sample within the shadowed zone, and within the angle of acceptance of the detector assembly. Thus interactance spectra or partial-transmittance spectra are obtained. The integration time on the shutter was varied with each sample. The culture vessels were mixed thoroughly and the spectra were acquired through the base of the culture vessel, with the visible light of the tungsten halogen bulb illuminating approx. 10 cm into the solution. An internal gold plated shutter was used as a 'white' reference for the entire sample. A dark reading was obtained with shutter in place and lamp turned off.

Other than the aeration provided in the flask, each flask was mixed thoroughly by shaking the flask before *in situ* scanning and sample collection. Each flask/sample was scanned twice, resulting in collection of 10 spectra/day/species, or 120 spectra/species over 22 days for the nutrient sufficient growth trial, and 6

spectra/day/treatment, or 66 spectra/treatment over 20 days in case of the nutrient limited growth trial.

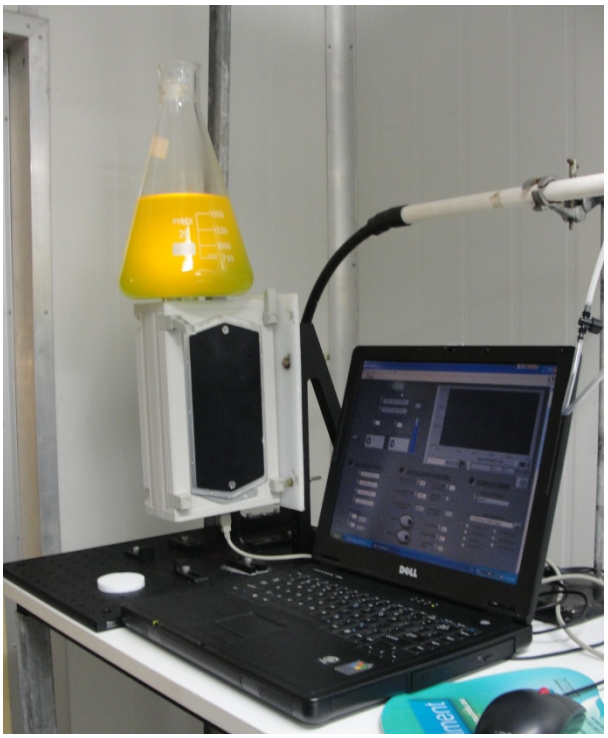


Figure 13. Spectral (300-1100 nm) acquisition of a culture flask, using the Nirvana unit to acquire interactance spectra through the base of the culture flask.

Aliquots (10 mL) of culture solution were filtered under vacuum through 2.5 cm diameter GF/C (Whatman, UK) filter paper, held within a filter holder (Sartorius GmbH, Gottingen, Germany). The filter discs were dried at 60 °C for 12 h and held in a vacuum desiccator for 12 h. Spectra of the wet and dry filter paper discs were collected using a Fourier Transform Near Infrared (FTNIR) (Figure 14) model Nicolet Aantaris Near IR Analyzer, USA over the range 1100-2500 nm at a resolution of 8.0 cm^{-1} with 64 scans per sample. Reflectance spectra were acquired using a rotating cup holder in conjunction with an integrating sphere.

Microalgal biomass was estimated by dry weight and total lipid content was estimated using the method of Kates and Volcanic (1966) described in chapter 4 (section 4.2.1 and 4.2.2 respectively).

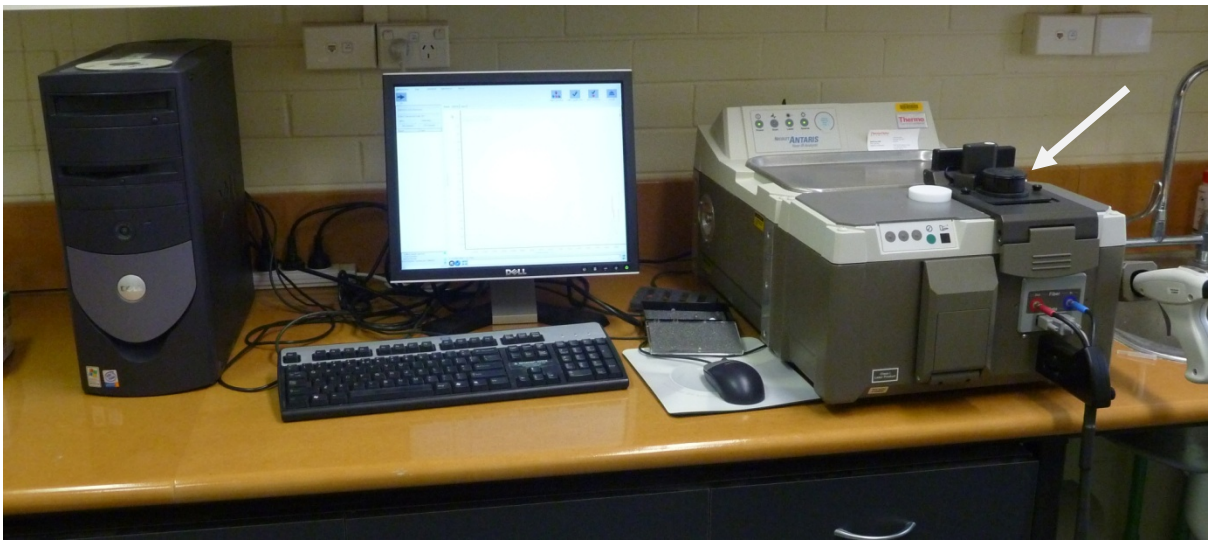


Figure 14. Spectroscopic instrument used for the spectral acquisition over 1100-2500 nm, FTNIR (Nicolet Aantaris Near IR Analyzer, USA). The spinning cup/reflectance accessory is arrowed.

5.2.3 Multivariate calibration and statistics

The Unscrambler software 10.1v (CAMO. Oslo, Norway) was used for data pre-processing and to develop Partial Least Squares regression models. Data pre-processing algorithms such as a second order polynomial Savitzky-Golay second derivative (left and right windows of around 13 nm for both FTNIR and SWNIR instruments), standard normal variant (SNV) and multiplicative scatter correction (MSC) were used with the spectral data sets. The impact of the spectral pre-treatments all commonly used to remove the effect of baseline shifts, and to correct for particle scatter effects in reflectance spectra, were trialled using 'culture flasks' as cross validation groups, over the range of 300-1100 nm and 1100-2500 nm.

Partial least square multivariate regressions were built using the PLS 1 routine using mean centred data. For PLSR cross validation, the maximum number of principle components was fixed at 20. Segmented cross validation was used, in which one group is kept out of calibration and prediction is made on other groups, continuing the process till all the groups are covered. Samples with high residuals from the other spectra and reference values were removed as outliers based on the visual inspection of the scatter plots. The percentage removal was kept below 2% of the total number of samples.

The PLSR models were compared in terms of elements (n), population mean ($\overline{X}$) and standard deviation (SD), the number of principle components (PC's), cross validation coefficient of determination (R_{CV}) and root mean square error of cross-validation (RMSECV). Models were also tested on sample sets not included in the calibration population, with model performance reported in the terms outlined above, prediction coefficient of determination (R_P), root mean square error of prediction (RMSEP) the ratio of standard deviation of reference mean to RMSECV (SDR), bias and slope. An R_{CV}=0.85 and SDR=2 was considered as the lowest acceptable result for a workable model.

Wavelength optimization was carried out based on visual observations of the spectral peaks. An alternative approach was also used to optimise the wavelength window, involving comparison of the Rcv of various combination of start and stop wavelengths. This approach employed a Matlab program using PLS Toolbox (Eigenvector Research, Inc., 3905 West Eaglerock Drive, Wenatchee, WA 98801), as described by Guthrie et al. (2005).

5.3 Results

5.3.1 Spectral features

Herschel region (300-1100 nm) Nirvana interactance spectra of the culture flask and NIRSystems 6500 reflectance spectra of wet and dried filtrates of the four microalgal species were dominated by features in the visible region (Figure. 15). The Herschel region contained obvious features around 840 and 960 nm for spectra of culture vessels, while these features were minor in the spectra of wet filter papers (Figure. 15c) and the region was featureless in spectra of dry filter paper (Figure. 15e).

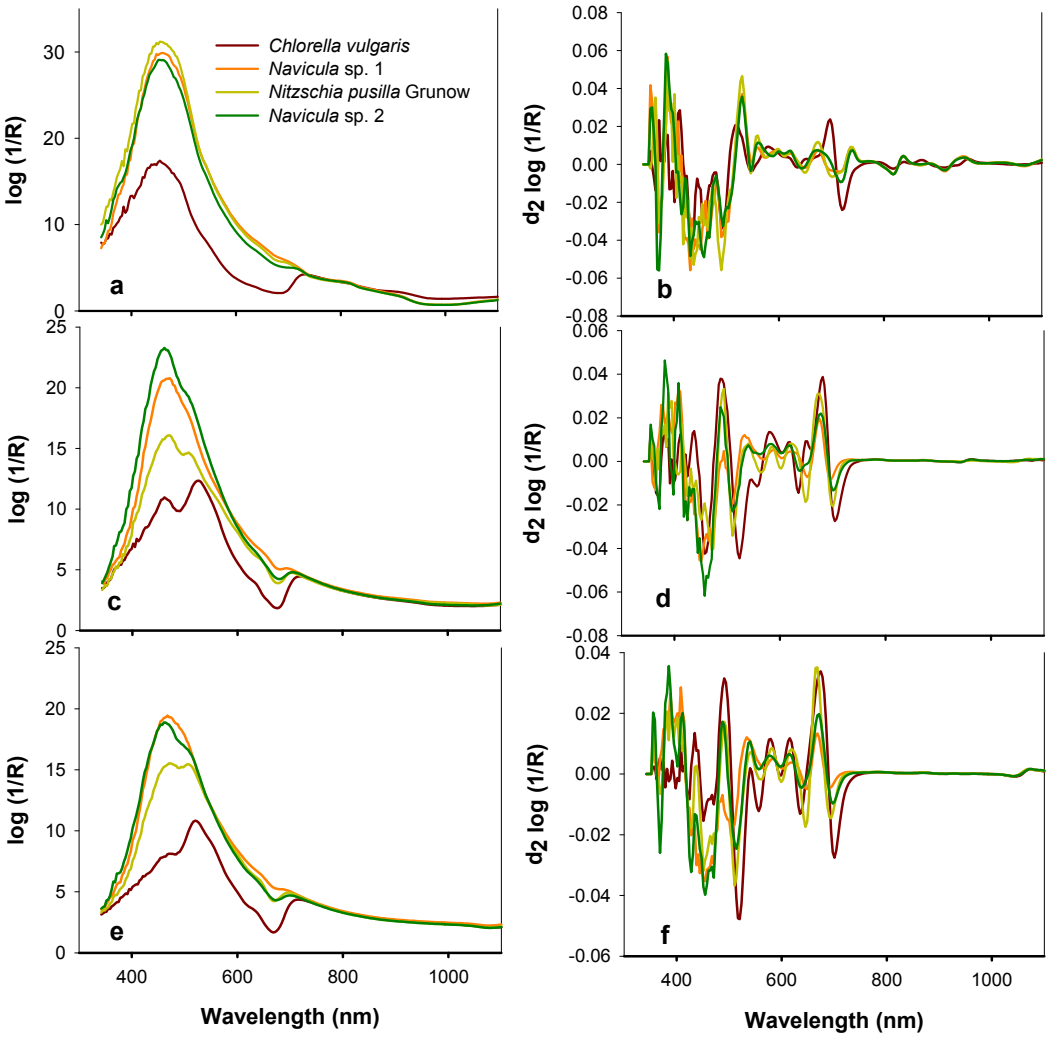


Figure 15. Spectra (Herschel region; 300-1100 nm) of four microalgal species: Left side panel (a,c,e) are of log (1/R) spectra while right side panel (b,d,f) are d^2 log (1/R) spectra of (a,b) culture vessels; (c,d) wet filtrates and (e,f) dry filtrates.

Reflectance spectra of NIR (1100 – 2500 nm) of wet filter paper of the four microalgal species were dominated by absorption features at around 1400 and 1900 nm, with a small peak evident at 1780 nm (Figure. 16b). For dry filtrates, the water peaks at 1400 and 1900 nm were greatly reduced, and spectra were characterised by absorption bands at 1350-1500, 1850-2000 and 2250-2400nm (Figure. 16a).

The absorption peaks recorded of nutrient deprived *C. vulgaris* over the range 1100-2500 nm (Figure I in Appendix B) and 300-1100 nm (Figure II in Appendix B) were similar to that described here, although with differences between treatments apparent in the visible region.

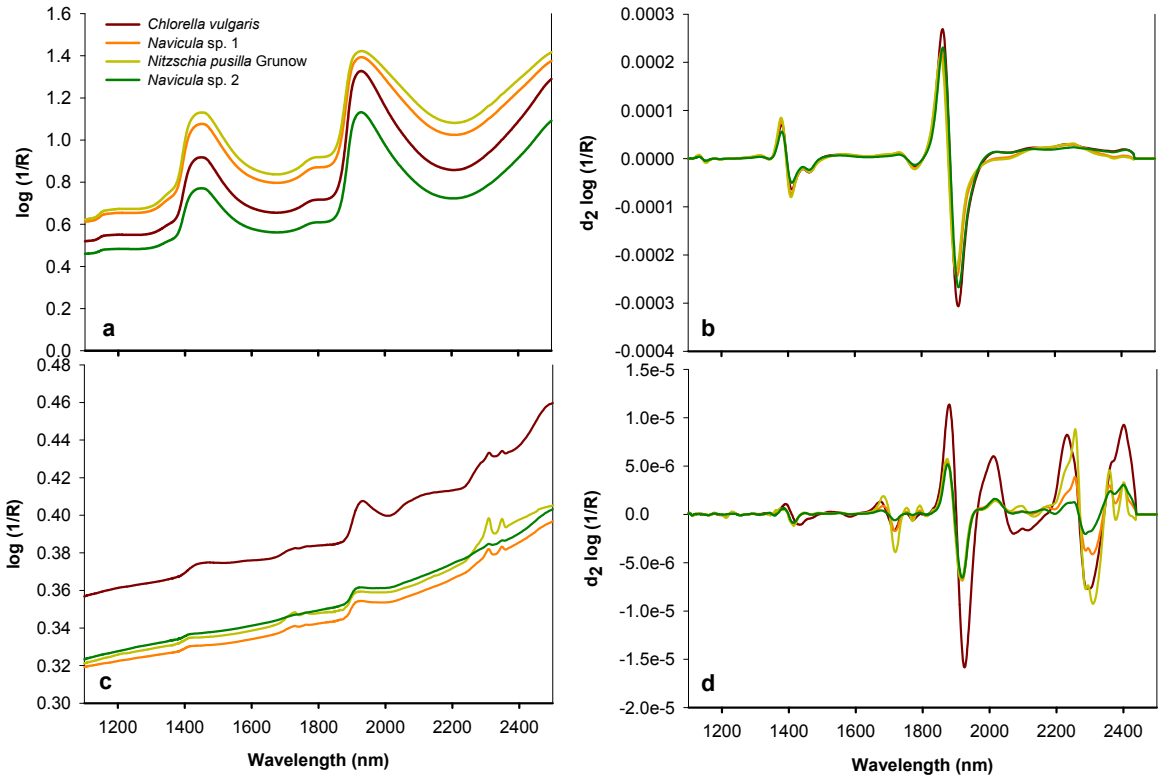


Figure 16. Spectra (1100-2500 nm; NIR region) of four microalgal species: Log (1/R) spectra of (a) wet and (b) dry filtrates; and d_2 log (1/R) spectra of (c) wet and (d) dry filtrates.

5.3.2 Reference analysis

The correlation coefficient of determination for biomass estimated using dry weight and optical density read at 625 nm was 0.98-0.99 across the microalgal species.

Total lipid was not correlated (R^2 < 0.64) with biomass (estimated using dry weight, optical density or cell count) across the microalgal species.

5.3.3 Spectral pre-processing

For completeness, all data is reported in here. For presentation flow, however, supporting Tables of 'less promising' results have been separated to an Appendix.

There was negligible impact from pre-processing (d2, MSC, SNV) of NIR or Herschel spectra in terms of the statistics of biomass models for *Chlorella* based on dry or wet filtrates, however MSC or SNV pre-treatments of Herschel region spectra of culture flasks improved model performance relative to that based on raw log (1/R) data (Table 15). Likewise, PLS models developed of total lipids were not improved by spectral pre-treatments (Table I in Appendix C).

Similarly, PLSR models of biomass and total lipids in *Navicula* sp. 1 and sp. 2 or in *N. pusilla* were not improved through use of pre-processed (d2, MSC, SNV) spectra (Table I-VII in Appendix D). An exception was noted for PLSR models of *N. pusilla* biomass based on spectra of dry filtrates using the range 1100-2500 nm with the pre-processing method of d2, which resulted in a R_{CV} value of 0.89, relative to the no pre-treatment result of 0.85 (Table IV in Appendix D)..

Based on these results, further analysis was based on 'raw' log (1/R) data.

5.3.4 Partial Least Squares regression model statistics for biomass

The Herschel region interactance spectra of cultures *in situ* supported PLS regression models on biomass with low values of R_{CV} and SDR in most cases (i.e. R_{cv} <0.85, RPD < 1; Table I in Appendix C). The exception was for models developed of *C. vulgaris* (using 'days' or 'flasks' as cross validation segments), *Navicula* sp. 2 (using 'days' as the cross validation segment) and *N. pusilla*, for which the R_{CV} values were >0.85 and <0.90. These models were associated with the highest population standard deviations.

Table 15. Summary statistics of biomass PLS models developed using various spectral pre-processing methods applied to *Chlorella vulgaris* log (1/R) spectra over two wavelength regions. Treatments included multiplicative scatter correction (MSC) and standard normal variance (SNV), imposed either on raw log (1/R) data, or following a second derivative treatment. Population n=120, $\overline{X}$ =1.36 g L^{-1} and σX=1.08 g L^{-1}. Rcv values above 0.85 are shown bolded.

Wave Length (nm)	Filter Paper	Pre-processing method	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log (1/R)	4	**0.95**	0.32	3.38	0.00	0.93
		d2 log (1/R)	2	**0.96**	0.31	3.48	0.01	0.92
		MSC	4	**0.96**	0.31	3.48	0.00	0.93
		SNV	4	**0.96**	0.31	3.48	0.00	0.93
		d2 + MSC	4	**0.96**	0.32	3.42	0.00	0.93
		d2 + SNV	6	**0.97**	0.28	3.88	0.00	0.95
	Wet	log (1/R)	11	**0.95**	0.34	3.17	0.02	0.92
		d2 log (1/R)	6	**0.94**	0.36	3.02	0.00	0.90
		MSC	9	**0.95**	0.35	3.08	0.01	0.92
		SNV	9	**0.95**	0.35	3.12	0.00	0.92
		d2 + MSC	5	**0.94**	0.36	2.96	0.00	0.89
		d2 + SNV	5	**0.94**	0.38	2.85	0.00	0.89
300-1100	Dry	log (1/R)	3	**0.96**	0.28	3.83	0.00	0.93
		d2 log (1/R)	9	**0.94**	0.36	2.97	-0.01	0.91
		MSC	3	**0.91**	0.36	3.01	-0.01	0.90
		SNV	3	**0.95**	0.33	3.27	-0.01	0.93
		d2 t+ MSC	11	**0.87**	0.59	1.8	-0.05	0.96
		d2 + SNV	7	**0.92**	0.41	2.64	0.00	0.86
	Wet	log (1/R)	5	**0.88**	0.51	2.10	-0.01	0.84
		d2 log (1/R)	20	**0.88**	0.54	1.99	0.00	0.90
		MSC	2	**0.87**	0.52	2.07	0.00	0.75
		SNV	2	**0.88**	0.50	2.15	0.00	0.76
		d2A + MSC	1	0.05	1.09	0.99	0.01	0.01
		d2A + SNV	12	**0.87**	0.53	2.03	0.00	0.84
	Flask	log (1/R)	1	**0.87**	0.53	2.03	0.00	0.77
		d2 log (1/R)	7	0.82	0.62	1.75	0.03	0.76
		MSC	4	**0.92**	0.43	2.49	0.01	0.89
		SNV	4	**0.95**	0.32	3.37	-0.01	0.91
		d2A + MSC	1	0.09	2.42	0.45	-0.19	0.20
		d2A + SNV	10	**0.92**	0.41	2.63	0.00	0.89

The Herschel region spectra of wet filtrates supported PLS regression models that were also generally poor, except for the case of *C. vulgaris* (single species model). This model supported an RMSECV, R_{CV} and SDR values of 0.51 g L^{-1}, 0.88 and 2.10 respectively (with' day of sampling' as the cross validation group) and 0.52 g L^{-1}, 0.88 and 2.1 respectively (with 'culture vessel' as the cross validation group) (Table II in Appendix C). These models were associated with the highest population standard deviation.

The Herschel region spectra of dry filter paper supported PLS regression models with RMSECV, R_{CV} and SDR values of 0.40 g L^{-1}, 0.87 and 2.01 respectively in the case of a combined species model (using flasks as cross validation groups) (Table III in Appendix C). In the case of single species models, the best RMSECV, R_{CV} and SDR values of 0.28 g L^{-1}, 0.96 and 3.83 respectively was achieved with the *C. vulgaris* data set, followed by *Navicula* sp. 1 (Table III in Appendix C). The R_{CV} values of *N. pusilla* and *Navicula* sp. 2 models were less than 0.85 (Table III in Appendix C).

The NIR region reflectance spectra of wet filtrates supported PLS regression models on biomass with RMSECV, R_{CV} and RPD values of 0.21 g L^{-1}, 0.96 and 2.85 respectively in the case of a combined species model (using flasks as cross validation groups) (Table 16). In case of single species models, the best RMSECV, R_{CV} and SDR values of 0.34 g L^{-1}, 0.95 and 3.18 respectively were associated with *C.vulgaris,* followed in order by *Navicula* sp. 1, N*. pusilla* and *Navicula* sp. 2*.* (Table 16). The number of PC's ranged from 6-17 across the models, and were lower than that used for models over the Herschel region.

Table 16. Wet filtrates: Summary statistics of PLS calibration models of biomass developed on NIR region (1100-2500 nm) log (1/R) spectra of wet filtrates. Cross validation results are reported for validation groups based on species (n=4), on replicate flasks (n=5) and on day of sampling (n=12). The highest Rcv value for models of a given species is shown bolded.

Species Model	Cross Validation Segment	Sample mean (g L^{-1})	Sample SD (g L^{-1})	Elements	PC	R_{cv}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Combined	Species	0.39	0.781	475*	14	0.94	0.274	2.85	0.02	0.85
	Flask				13	**0.96**	0.207	3.77	0.00	0.95
Chlorella vulgaris	Days	1.36	1.082	120	11	0.94	0.379	2.86	0.00	0.94
	Flask				11	**0.95**	0.401	2.71	0.02	0.92
Navicula sp. 1	Days	0.07	0.028	120	7	0.82	0.016	1.75	0.00	0.74
	Flask				6	**0.86**	0.014	1.97	0.00	0.75
Nitzschia pusilla	Days	0.10	0.024	120	11	0.86	0.013	1.89	0.00	0.88
	Flask				15	**0.91**	0.011	2.44	0.00	0.87
Navicula sp. 2	Days	0.04	0.012	117**	17	0.85	0.007	1.85	0.00	0.75
	Flask				15	**0.92**	0.005	2.47	-0.40	0.88

*Five spectra removed as outliers, ** Three spectra removed as outliers

The NIR region spectra of dry filter paper supported PLS regression models with RMSECV, R_{CV} and RPD values of 0.19 g L^{-1}, 0.97 and 4.11 respectively in case of a combined species model (using flasks as cross validation groups) (Table 17). In the case of single species models, the best RMSECV, R_{CV} and RPD values of 0.34 g L^{-1}, 0.94 and 3.38 respectively (using flask as cross validation segments) was achieved with the *Chlorella vulgaris* data set, followed by that for *Navicula* sp. 1., *N. pusilla* and *Navicula* sp. 2 (Table 17). The number of PC's ranged from 2-11 across the models, and was lower compared to the models of wet filter paper.

5.3.5 Partial Least Square regression model statistics for total lipid

The Herschel region interactance spectra of *in situ* cultures supported PLS regression models on total lipid with low values of R_{CV} and RPD for either single species or combined species data (Table IV in Appendix C).

The Herschel region reflectance spectra of wet filtrates supported PLS regression models on total lipid that were poor, with low values of R_{CV} and RPD across the combined and single species models. The R_{CV} values ranged from 0.09-0.62 across the models (Table V in Appendix C). Similarly, the Herschel region spectra of dry filter paper supported PLS regression models that were also poor, with low values of R_{CV} and RPD across the combined and single species models. The R_{CV} values ranged from -0.18-0.75 across the models (Table VII in Appendix C).

Table 17. Dry filtrates: Summary statistics of the PLS calibration models of biomass developed on NIR region (1100-2500 nm) log (1/R) spectra of dry filtrates. Cross validation results are reported for validation groups based on species (n=4), on replicate flasks (n=5) and on days (n=12). The highest Rcv value for models of a given species is shown bolded.

Species Model	Cross Validation Segment	Sample mean (g L^{-1})	Sample SD (g L^{-1})	Elements	PC	R_{cv}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Combined	Species	0.39	0.775	477*	7	0.22	0.878	0.88	-0.27	0.15
	Flask				6	**0.97**	0.188	4.11	0.00	0.96
Chlorella. vulgaris	Days	1.36	1.082	120	4	0.93	0.388	2.79	0.00	0.91
	Flask				4	**0.95**	0.320	3.38	0.00	0.93
Navicula sp. 1.	Days	0.07	0.028	120	10	0.91	0.012	2.35	0.00	0.81
	Flask				11	**0.95**	0.009	3.10	0.00	0.92
Nitzschia pusilla	Days	0.10	0.024	120	9	0.87	0.012	2.01	0.00	0.77
	Flask				8	**0.90**	0.011	2.26	-4.41	0.88
Navicula sp. 2.	Days	0.04	0.012	117*	2	0.78	0.008	1.59	0.00	0.65
	Flask				8	**0.87**	0.006	2.02	-1.09	0.81

*Three spectra removed as outliers.

Models of total lipid based on the NIR region reflectance spectra of wet filtrates were also not acceptable (Table VI in Appendix C). The model with the R_{CV} value (0.84) closest to the acceptability limit was the combined species model (using flasks for cross validation groups).

The NIR region reflectance spectra of dry filtrates supported PLS regression models of total lipid with RMSECV, R_{CV} and SDR values of 0.06 g L^{-1}, 0.89 and 2.09 respectively in case of a combined species models (using flask as cross validation segments) (Table 18). In case of single species total lipid models, the best RMSECV, R_{CV} and SDR values of 0.09 g L^{-1}, 0.87 and 1.46 respectively (using flask as cross validation segments) was achieved for *C. vulgaris,* followed by *N. pusilla* (Table 18). The poorest calibration results were achieved with *Navicula* sp. 2

5.3.6 Sample volume

To test the influence of sample size, another set of filtrates of *Navicula* sp. 2 culture were prepared using twice the sample aliquots (20 mL) as previous use (10 mL).The PLS calibration models developed of biomass over 1100-2500 nm resulted in R_{CV} value of 0.96, with a sample mean of 0.06 g L^{-1} which was significantly higher compared to the R_{CV} value of 0.87 having a sample mean of 0.04 of previous experiment. In case of total lipid the R_{CV} value was 0.95, with a sample mean of 0.027 g L^{-1}. Compared to the previous experiment the R_{CV} value were significantly higher (Table 16). However, the biomass and total lipid content of the microalgal species were not predicted accurately over 300-1100 nm even after increasing the sample load on to the filter paper.

Table 18. Dry filtrates: Summary statistics of PLS calibration models developed of total lipid using NIR region (1100-2500 nm) log (1/R) spectra of dry filtrates. Cross validation results are reported for validation groups based on species (n=4), on replicate flasks (n=5) and on day of sampling (n=12). The highest Rcv value for models of a given species is shown bolded.

Species Model	Cross Validation Segment	Sample mean (g L^{-1})	Sample SD (g L^{-1})	Elements	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Combined	Species	0.09	0.130	477*	14	0.66	0.097	1.34	0.00	0.44
	Flask				8	**0.89**	0.060	2.09	0.00	0.80
Chlorella vulgaris	Days	0.20	0.183	120	9	0.82	0.104	1.25	0.00	0.70
	Flask				8	**0.87**	0.089	1.46	0.00	0.80
Navicula sp. 1	Days	0.03	0.017	120	11	0.78	0.011	1.58	0.00	0.67
	Flask				10	0.79	0.017	1.64	0.00	0.71
Nitzschia pusilla	Days	0.04	0.023	120	6	0.70	0.023	1.36	0.00	0.63
	Flask				10	**0.85**	0.013	1.83	0.00	0.86
Navicula sp. 2	Days	0.03	0.014	120	1	-0.38	0.015	0.95	0.00	-0.05
	Flask				11	0.63	0.011	1.28	0.00	0.46

*Three spectra removed as outlier values.

5.3.7 Wavelength optimization

Of the various 'manual' selections of wavelength ranges, for spectra of cultures in flask (*in situ*) higher R_{CV} values and lower number of principle components were achieved for biomass PLSR models developed using a window that included the chlorophyll absorption peak (within the region 641 to 885 nm), relative to models developed over the full visible and Herschel region of 300-1100 nm (Table 19). For the wet filtrate data sets, no improvement of biomass model statistics for the use of manually chosen restricted wavelength ranges than the full wavelength regions (Table 19). However, a narrow wavelength encompassing the chlorophyll absorption was notable for supporting models of comparable statistics to that of full window models (Table 19). For dry filtrate data sets, biomass model statistics were also, in general, little improved for the use of manually chosen restricted wavelength ranges than the full wavelength regions (Table 19). For *Chlorella*, the highest Rcv was achieved using the window 2200-2400 nm (0.97, compared to 0.95 for a full NIR region model).

Table 19. Cross validation statistics for microalgal biomass PLSR models based on manually selected wavelength ranges of log (1/R) spectra over NIR (1100-2500 nm) and Herschel regions (300-1100 nm). Cross validation groups were based on flasks (n=5). The wavelength region supporting the highest absolute R_{cv} for a given species and sample type are displayed in bold.

Species	Sample	Wave length (nm)	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Chlorella vulgaris	Dry	1100 to 2500	4	0.95	0.32	3.38	0.00	0.93
		1350 to 1450	3	0.94	0.36	2.97	0.00	0.90
		1881 to 1949	6	0.95	0.34	3.19	0.00	0.93
		2200 to 2400	5	**0.97**	0.27	3.93	0.00	0.94
		300 to 1100	3	0.96	0.28	3.83	0.00	0.93
		500 to 1027	3	**0.97**	0.27	3.98	0.00	0.95
		570 to 746	9	0.96	0.31	3.52	0.00	0.93
		726 to 975	6	0.90	0.48	2.26	0.00	0.83
	Wet	1100 to 2500	11	**0.95**	0.34	3.17	0.02	0.92
		1700 to 1900	7	**0.95**	0.34	3.15	0.00	0.90
		300 to 1100	5	0.88	0.51	2.10	-0.01	0.84
		645 to 723	4	0.94	0.36	2.98	0.00	0.90
	Flask	300 to 1100	10	0.87	0.53	2.03	0.00	0.77
		684 to 846	8	**0.97**	0.27	4.05	0.00	0.95
Navicula sp. 1	Dry	1100 to 2500	11	**0.95**	0.01	3.10	0.00	0.92
		1350 to 1450	6	0.90	0.01	2.25	0.00	0.83
		1881 to 1949	11	**0.95**	0.01	3.26	0.00	0.94
		2200 to 2400	4	0.87	0.01	2.02	0.00	0.79
		300 to 1100	2	0.81	0.01	1.71	0.00	0.67
		641 to 694	3	0.82	0.01	1.76	0.00	0.70

	Wet	1100 to 2500	6	0.86	0.01	1.97	0.00	0.75
		1700 to 1900	6	**0.87**	0.01	2.07	0.00	0.78
		300 to 1100	10	0.85	0.01	1.90	0.00	0.79
		564 to 707	4	0.84	0.02	1.84	0.00	0.73
	Flask	300 to 1100	3	0.88	0.01	2.15	0.00	0.79
		710 to 878	3	**0.94**	0.01	2.99	7.47	0.90
Nitzschia pusilla	Dry	1100 to 2500	8	**0.90**	0.01	2.26	-4.41	0.88
		1350 to 1450	3	0.82	0.01	1.73	0.00	0.71
		1881 to 1949	7	0.83	0.01	1.80	0.00	0.75
		2200 to 2400	4	0.86	0.01	1.97	0.00	0.78
		300 to 1100	4	0.74	0.01	1.50	0.00	0.59
		648 to 700	4	0.84	0.01	1.86	0.00	0.74
	Wet	1100 to 2500	15	**0.91**	0.01	2.44	0.00	0.87
		1700 to 1900	8	0.85	0.01	1.86	0.00	0.77
		300 to 1100	1	-0.05	0.02	0.98	0.00	-0.01
		648 to 713	1	0.16	0.01	1.01	0.00	0.05
	Flask	300 to 1100	6	0.86	0.01	1.95	9.63	0.80
		651 to 885	4	**0.93**	0.01	2.81	7.86	0.88
Navicula sp. 2	Dry	1100 to 2500	8	0.87	0.01	2.02	-1.09	0.81
		1350 to 1450	2	0.82	0.01	1.73	0.00	0.70
		1881 to 1949	2	0.79	0.01	1.63	0.00	0.66
		2200 to 2400	6	**0.90**	0.00	2.35	0.00	0.84
		300 to 1100	3	0.72	0.01	1.43	0.00	0.59
		641 to 700	7	0.84	0.01	1.83	3.07	0.74
	Wet	1100 to 2500	17	0.85	0.01	1.85	0.00	0.75

	1700 to 1900	8	0.76	0.01	1.53	0.00	0.64
	300 to 1100	10	0.78	0.01	1.56	0.00	0.69
	651 to 703	6	**0.91**	0.00	2.47	0.00	0.85
Flask	300 to 1100	15	0.84	0.01	1.81	-5.46	0.79
	609 to 836	9	**0.93**	0.00	2.79	-4.42	0.89

Similar results to that for biomass PLSR models were obtained for total lipid models, although model statistics were much lower overall. For culture flask spectra, a restricted range encompassing the chlorophyll peak supported better results than use of the full visible and Herschel region (Table 20). For spectral sets of both wet and dry filtrates, full NIR region data sets supported models with better calibration statistics than those based on restricted wavelength regions (Table 20).

The alternative approach for optimisation of the wavelength window involved comparison of the RMSECV of all combinations of start and stop wavelengths within the region 1400-2400 and 550-1000 nm of the dry filtrate spectral data of *C. vulgaris.* The lowest RMSECV on biomass models within the NIR region was achieved with a start wavelength of 2000 to 2300 nm, and an end wavelength of 2400 nm, however the improvement over a full window was minimal (Figure 17a). The lowest RMSECV on biomass models within the visible-Herschel region was achieved with a start wavelength of around 650 nm, and an end wavelength of 725 to 1050 nm, although again the improvement over a full window was small (Figure 17b).

The lowest RMSECV on total lipid models within the NIR region was achieved with a start wavelength of around 1900 nm, and an end wavelength of around 2100 nm, however the improvement over a full window was minimum (Figure 18a). The lowest RMSECV on lipid models within the visible-Herschel region was achieved with a start wavelength of around 500 to 640 nm, and an end wavelength of 675 to 1050 nm, although again the improvement over a full window was minimum (Figure 18b).

Table 20. Cross validation statistics for microalgal lipid PLSR models based on manually selected wavelength ranges of log (1/R) spectra over NIR (1100-2500 nm) and Herschel regions (300-1100 nm). Cross validation groups were based on flasks (n=5). The wavelength region supporting the highest absolute Rcv.for a given species and sample type are displayed in bold.

Species	Sample	Wave length (nm)	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Chlorella vulgaris	Dry	1100 to 2500	8	**0.87**	0.09	1.46	0.00	0.80
		1350 to 1450	6	0.77	0.12	1.55	0.00	0.66
		1881 to 1949	3	0.79	0.11	1.63	0.00	0.66
		2200 to 2400	8	0.83	0.10	1.80	0.00	0.75
		300 to 1100	3	0.73	0.12	1.04	0.00	0.66
		500 to 1027	6	0.79	0.11	1.64	0.00	0.66
		570 to 746	6	0.79	0.12	1.58	0.00	0.66
		726 to 975	7	0.63	0.14	1.28	0.00	0.48
	Wet	1100 to 2500	10	**0.74**	0.12	1.05	0.00	0.61
		1700-1900	6	0.61	0.14	1.27	0.00	0.42
		300 to 1100	2	0.51	0.16	0.83	0.00	0.29
		645 to 723	7	**0.74**	0.12	1.48	0.00	0.62
	Flask	300 to 1100	2	**0.77**	0.12	0.08	-0.00	0.61
		684 to 846	4	**0.77**	0.12	1.56	0.00	0.62
Navicula sp01.	Dry	1100 to 2500	10	0.79	0.01	1.64	0.00	0.71
		1350-1450	6	0.77	0.01	1.56	0.00	0.68
		1881 to 1949	15	**0.80**	0.01	1.65	0.00	0.75
		2200 to 2400	6	0.43	0.02	1.07	0.00	0.28
		300 to 1100	3	0.57	0.01	1.20	0.00	0.41
		641 to 694	5	0.53	0.01	1.17	0.00	0.33

	Wet	1100 to 2500	17	**0.63**	0.01	2.01	0.00	0.47
		1700-1900	6	0.58	0.01	1.21	0.00	0.39
		300 to 1100	2	0.32	0.02	1.05	0.00	0.15
		564 to 707	4	0.40	0.02	1.08	0.00	0.21
	Flask	300 to 1100	2	0.30	0.02	1.02	0.00	0.16
		710 to 878	2	**0.46**	0.02	1.13	0.00	0.24
Nitzschia pusilla	Dry	1100 to 2500	10	**0.85**	0.01	1.83	0.00	0.86
		1350-1450	6	0.77	0.01	1.53	0.00	0.68
		1881 to 1949	8	0.69	0.02	1.37	0.00	0.57
		2200 to 2400	4	0.77	0.01	1.58	0.00	0.65
		300 to 1100	4	0.76	0.02	1.52	0.00	0.64
		648 to 700	4	0.74	0.02	1.50	0.00	0.60
	Wet	1100 to 2500	15	**0.70**	0.02	1.38	0.00	0.58
		1700-1900	3	0.60	0.02	1.27	0.00	0.37
		300 to 1100	1	0.17	0.01	1.01	0.00	0.05
		648 to 713	1	0.16	0.01	1.80	0.00	0.05
	Flask	300 to 1100	3	0.56	0.02	1.21	0.00	0.35
		651 to 885	6	**0.79**	0.01	1.63	9.41	0.66
Navicula sp02.	Dry	1100 to 2500	11	**0.63**	0.01	1.28	0.00	0.46
		1350-1450	5	0.55	0.01	1.05	0.00	0.37
		1881 to 1949	2	0.33	0.01	1.05	1.79	0.14
		2200 to 2400	1	-0.15	0.01	1.05	0.00	-0.02
		300 to 1100	3	0.36	0.01	1.04	0.00	0.22
		641 to 700	7	0.54	0.01	1.18	8.10	0.36
	Wet	1100 to 2500	1	-0.27	0.01	0.96	0.00	-0.03

	1700-1900	1	-0.34	0.01	1.05	0.00	-0.03
	300 to 1100	1	-0.09	0.01	0.99	0.00	-0.01
	651 to 703	1	-0.02	0.01	0.99	0.00	-0.00
Flask	300 to 1100	3	**0.34**	0.01	1.04	0.00	0.18
	609 to 836	1	0.06	0.01	0.99	0.00	0.01

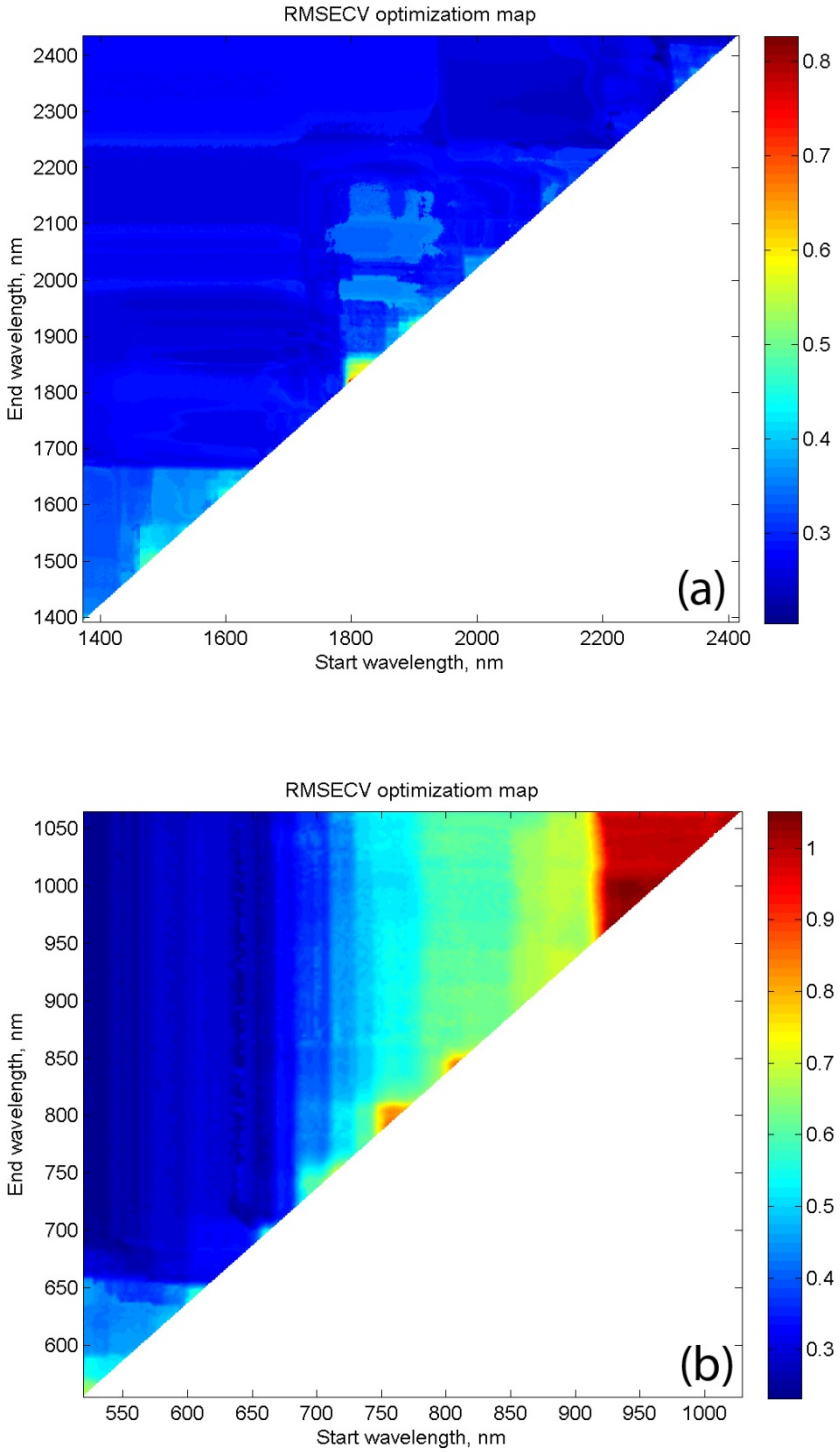


Figure 17. Optimization of wavelength region used in PLSR modelling of *Chlorella vulgaris* dry filtrate estimation, with presentation of RMSECV values on a colour scale, index shown on right side, for two regions : (a) 1400-2400, and (b) 550-1000 nm. The number of PLS factors for each model was varied up to 9 (Table I in Appendix E).

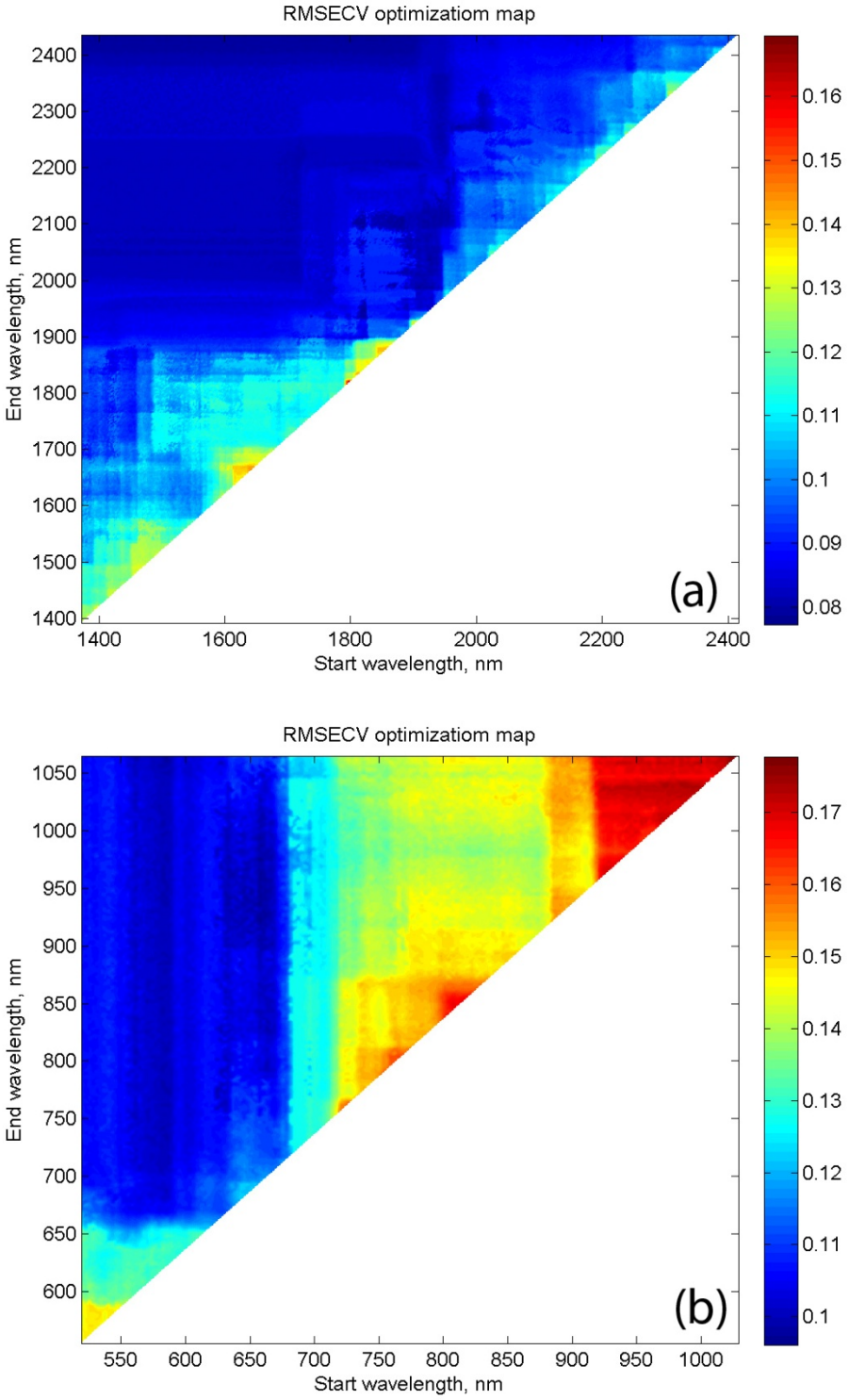


Figure 18. Optimization of wavelength region used in PLSR modelling of *Chlorella vulgaris* total lipid estimation, with presentation of RMSECV values on a colour scale, index shown on right side, for two regions : (a) 1400-2400 and (b) 550-1000 nm. The number of PLS factors for each model was varied up to 11 (Table II in Appendix E).

5.3.8 Calibration for biomass and total lipid of nutrient limited cultures

An independent data set to those used in the PLS modelling exercises described above was created through an growth trial involving *Chlorella* culture under a range of nutrient limitations. PLSR biomass models developed on log (1/R) NIR region spectra of dry filtrates from each nutrient supply condition were comparable in cross validation statistics to that of the first experiment, except in the case of P limited growth (Table 21). Similarly, models based on the visible-Herschel region gave similar results for all nutrient limited growth trial (Table 21); however the results of the control treatment (R_{CV} of 0.84) were low than that obtained with the nutrient sufficient growth trial (Rcv of 0.96).

PLSR total lipid models developed on log (1/R) NIR region spectra of dry filtrates from each nutrient supply condition were also comparable in cross validation statistics to that of the nutrient sufficient growth trial (Rcv> 0.90; Table 21). Models based on the visible-Herschel region gave similar results for control and N limited treatments (Rcv >0.94); however low-grade results were obtained with the P and Fe limited treatments (Rcv<0.89).

No spectral pre-processing methods consistently improved model performance, relative to the use of 'raw' log (1/R) data (Table 1 and II in Appendix F) even when spectral pre-processing methods of d2, MSC and SNV were also trialled with these data sets (i.e. for both biomass and total lipid models), parallel to the activity reported in Table 21. This result is consistent with that of the nutrient sufficient growth trial.

Spectra were also recorded of culture flasks over the range of 300-1100 nm and wet filtrates over the range of 300-1100 nm and 1100-2500 nm of the nutrient limited growth trial of *C. vulgaris* (Table III and IV, Appendix E). Overall model performance was inferior to that achieved with use of dry filtrates, consistent with the outcome of the nutrient sufficient growth trial. The spectral pre-processing methods applied did not increase the model statistics and considerably decreased the spectral quality compare to no treatment spectra.

5.3.9 Prediction performance of the PLS models

The test of a model is its ability to predict populations not directly represented in the calibration set. In this case the PLS models based on spectra 300-1100 and 1100-2500 nm absorbance of dry filtrates of *C. vulgaris* of nutrient sufficient growth trial were used in prediction of datasets from nutrient limited growth trial. Both single and combined species models were assessed.

A PLSR biomass model based on NIR spectra of dry filtrates of *Chlorella* grown in a complete nutrient solution worked well in prediction of *Chlorella* cultures growing under similar conditions, and in prediction of biomass of *Chlorella* grown under N and Fe limited conditions, but not of cultures grown under P limited conditions, or of the combined data set (Table 22). A PLSR biomass model based on NIR spectra of dry filtrates of a number of microalgal species, including *Chlorella*, grown in complete nutrient solution, performed similarly to the single species model in prediction of the test populations (Table 22).

Table 21. Summary statistics of biomass PLSR models developed using log (1/R) spectra of dry filtrates of a time course of growth of cultures varying in nutrient composition. Cross validation groups were based on culture flasks (n=5).

Growth Condition	Sample mean (g L^{-1})	Sample SD (g L^{-1})	Elements	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias g L^{-1}	Slope
NIR region 1100-2500:									
Control	1.30	0.85	66	4	0.96	0.23	3.62	0.00	0.94
N limited	0.31	0.12	66	4	0.94	0.04	2.98	-0.01	0.92
P limited	0.38	0.19	66	9	0.86	0.10	1.84	0.00	0.92
Fe limited	0.39	0.19	66	4	0.96	0.05	3.75	-0.01	0.92
Herschel region 300-1100 nm									
Control	1.26	0.82	64	2	0.84	0.45	1.84	0.02	0.74
N limited	0.31	0.12	64*	3	0.94	0.04	2.86	0.01	0.89
P limited	0.39	0.19	64*	2	0.90	0.08	2.30	0.00	0.84
Fe limited	0.39	0.19	64*	2	0.87	0.09	2.15	0.00	0.85

*Two spectra removed as outliers.

In contrast, the combined species biomass model based on the visible-Herschel region failed in prediction of biomass of the nutrient limited independent sets (maximum Rp of only 0.6; Table 22). The single species model fared better, except in prediction of the Fe-limited culture data set (Table 22).

A PLSR total lipid model based on NIR spectra of dry filtrates of *Chlorella* growing in a complete nutrient solution predicted the lipid content of *Chlorella* cultures growing under similar conditions with a maximum R_{CV} of 0.90 (Table I in Appendix G). A combined species model failed in prediction of the nutrient limited independent sets Table I in Appendix G). Both models performed poorly in prediction of nutrient limited cultures (Table I in Appendix G).

Table 22. Prediction of nutrient limited growth trial data using models for biomass created using nutrient sufficient growth trial data (based on *Chlorella* data only, and on all species considered). Models were based on log (1/R) spectra of dry filtrates. Prediction set mean and SD was 1.30 and 0.85; 0.31 and 0.12; 0.38 and 0.19; 0.39 and 0.19; and 0.60 and 0.60 g L^{-1} for the data sets of control, N limited, P limited, Fe limited and combined data sets, respectively. R_p values <0.85 are shown in bold.

Model	Predicted Spectra	Region (nm)	R_p	RMSECP (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Single species	Control	1100-2500	**0.90**	0.69	1.23	-0.58	0.72
	N Limited		**0.87**	0.50	0.24	0.26	3.82
	P Limited		0.72	0.32	0.60	-0.24	1.13
	Fe Limited		**0.87**	0.64	0.30	0.36	3.16
	Combined		0.58	0.55	0.23	-0.04	0.59
Combined species	Control	1100-2500	**0.90**	0.65	1.30	-0.52	0.95
	N Limited		**0.86**	0.61	0.20	0.22	4.79
	P Limited		0.70	0.35	0.55	-0.27	1.15
	Fe Limited		**0.89**	0.97	0.20	0.50	4.72
	Combined		0.58	0.68	0.88	-0.02	0.78
Single species	Control	300-1100	0.81	0.64	1.32	-0.29	0.96
	N Limited		**0.91**	0.96	0.12	-0.03	8.14
	P Limited		**0.91**	0.80	0.24	-0.59	3.32
	Fe Limited		0.78	1.40	0.14	0.96	4.86
	Combined		0.57	0.49	1.22	0.00	0.36
Combined species	Control	300-1100	0.16	1.07	0.79	-0.63	0.08
	N Limited		0.12	0.28	0.43	-0.03	0.26
	P Limited		0.53	0.40	0.48	0.01	1.31
	Fe Limited		0.60	0.43	0.44	0.27	1.39
	Combined		0.31	0.63	0.96	-0.09	0.22

5.4 Discussion

5.4.1 Technique recommendation

Relative to other analytical techniques, near infrared spectroscopy (NIRS) offers the potential advantage of rapid and *in situ* assessment of certain parameters. NIRS is suited to analysis of compounds rich in O-H, C-H, N-H or S-H bonds, which demonstrate absorption related to the stretching of these bonds (Murray & Williams 1987; Thyholt & Isaksson 1997). The C-H absorption bonds in NIR spectra are associated with fats, oils and carbohydrates in the biological materials (Dull and Giangiacomo 1984). As such, the technique is relevant to lipid production in microalgal culture. This potential was recently reported by Laurens and Wolfram (2011) who reported on the feasibility of use of NIR reflectance spectroscopy to assess exogenous lipid spikes added to ground, lyophilized microalgal biomass.

In the current study the work of Laurens and Wolfrum (2011) has been extended in several ways: (a) work is not based on 'spiked' samples with a common matrix (which is likely to support 'over optimistic' PLSR models); (b) a reduced sample preparation methodology (eliminating the lyophilisation and grinding steps used in the study of Laurens and Wolfram); (c) trialling of low-cost silicon diode array technology operating in the Herschel region, and (d) *in situ* assessments of liquid cultures, rather than dried extracts. The latter goal was established by Laurens and Wolfrum (2011).

Use of the dry filtrate technique for reflectance spectroscopy using a NIR window was demonstrated to have the potential to assess and predict biomass and total lipid content of samples of microalgal cultures grown under various nutrient growth trials.

In prediction of independent test sets, a maximum RMSEP of approximately 0.90 g L^{-1} each was achieved for biomass and total lipids. Prediction of biomass using a visible-Herschel region window was also reasonable, indicating that lower cost silicon diode instrumentation could be used for this attribute.

Use of the wet filtrate procedure is not recommended, as the loss of model performance is disproportional to the effort required to dry the filtrates. The *in situ* assessment of culture flasks was demonstrated to hold promise for the assessment of total biomass of cultures, but not for the assessment of total lipid content, at least with the interactance optical arrangement used.

5.4.2 Optical geometry and sample presentation

A limitation of any measurement technique is representiveness, that is, how well the measured sample represents the whole sample. For example, if microalgae adhere to culture vessel walls, or clump and settle, the assessed sample may not represent the whole culture.

In the current exercise, reference measurements were made of the filtrates, after acquisition of spectra, and thus the issue of measurement sample representativeness was not addressed. The *in situ* flask spectra measurements were referenced to the filtrate reference measurements, with the assumption made that this sample represented the assessed optical volume. The flask was aerated to effect mixing, nonetheless *C. vulgaris* formed sediments at the bottom of the culture flask (increasing with time of cultivation), while *Navicula* strains tended to stick to the walls of the culture flasks.

A mechanism of flask surface cleaning should be considered in future studies. A transmission geometry might also be considered, ideally with estimation of both reflected and transmitted light, towards an estimation of both scattering (related to cell size and number) and absorption (related to chemical composition) components.

5.4.3. Spectral pre-processing

The calculation of derivatives of spectral data is commonly undertaken to remove baseline shifts between spectra, while the MSC and SNV algorithms are typically employed to reduce the effects of light scattering on the spectrum (e.g. in relation to particle size in reflectance spectra). Numerous studies using NIR to predict milk fats (Coppa *et al.* 2010; Šašić & Ozaki 2001), lipid added to lyophilized microalgae (Laurens & Wolfrum 2011) and lipid content of edible oil seeds (Golebiowski 2004; Tajuddin *et al.* 2002; Yang *et al.* 2005) have reported a significant improvement in the spectral quality on using various spectral pre-processing treatments. However, there are no 'hard rules', and the general advise in relation to development of a NIRS method is one of trial and error, i.e. empirical trials of the benefit of such methods for the particular application. In the current study the application of pre-treatments did not consistently improve calibration statistics. This result is attributed to a consistent presentation methodology.

5.4.4. Wavelength region optimization

The *C. vulgaris* PLSR models were developed of various wavelength ranges within 300-1100 nm and 1100-2500 nm. For a biomass PLSR model based on *in situ* spectra of culture vessels, the optimum wavelength range included chlorophyll

absorption feature at 680 nm. Chlorophylls are measured over 550-700 nm and carotenoids over 450-470 nm (Lichtenthaler & Claus 2001). *C. vulgaris* has chlorophyll *a* and *b* as major photosynthetic pigments (Becker 1994). This result is attributed to a correlation between chlorophyll content and biomass however, it is likely that conditions that change the chlorophyll content of the microalgae, e.g. nutrient limitations, will impact on model performance.

For a biomass model based on spectra of dried filtrates, the highest R_{CV} value was obtained using a wavelength range of 2200-2400 nm. This can be interpreted as due to the composition of biomass which includes carbohydrate, proteins and lipids (Williams & Sobering 1993).

5.4.5. Population structure and model performance

Population mean and SD for biomass and total lipids was much higher for *Chlorella* than the other three genotypes – indeed the mean biomass was 3 to 7 times higher than that of the other genotypes (mean biomass for *C. vulgaris, N. pusilla, Navicula* sp. 1 and *Navicula* sp. 2 was 1.46, 0.10,. 0.06 and 0.04 g L^{-1}), and mean total lipid content was 13 to 20 times higher (mean lipid content for *C. vulgaris, N. pusilla, Navicula* sp. 1 and *Navicula* sp. 2 of 0.20, 0.04, 0.03 and 0.027 g L^{-1}). For a given RMSECV, R will be proportional to SD of the population, through the relationship:

$R^2 = 1 – (RMSEC/SD)^2$

Thus an SDR (SD/RMSEC) value of 2 represents a R^2 of 0.75, and an RPD of 1 indicates that RMSEC was equal to SD.

Thus only the *Chlorella* data set was suited to PLSR model development, given the achieved values of RMSECV for biomass and total lipid and the SD of the populations.

A simple solution to this issue is to increase the amount of sample loaded on to the filter paper. To test this assertion, twice as much sample (20 mL) of *Navicula* sp. 2 (species with lowest biomass) was filtered. The PLSR model developed on biomass using a wavelength range of 1100-2500 nm resulted in a R_{CV} value of 0.96, compared to the R_{CV} value of 0.87 of the previous experiment. In the case of total lipid, the R_{CV} value was increased to 0.95, compared to 0.63 in the previous experiment. However, the biomass and total lipid content of the microalgal species were not predicted accurately over 300-1100 nm even after increasing the sample load on to the filter paper.

5.4.6. Cross validation procedure

As for any multivariate regression modelling technique, model over-fitting, through use of too many PLS factors and too wide a wavelength range, is a risk in PLSR model development, particularly for data sets in which the number of Y variables (wavelengths) greatly exceeds the number of samples. The standard method to test for over-fitting is cross validation, in which PLS models are built on a series of subsets of the data set and tested on the remaining data, with the number of factors in the model optimised to achieve the lowest average RMSECV across the test sets. The model cross validation result (R_{cv}^2, RMSECV) is often reported in the literature. However, the choice of sets for the cross validation process can strongly influence the result. For example, Laurens and Wolfrum (2011) reported use of a 'full' cross

validation procedure. This is likely a leave-one-out procedure, in which one sample at a time is dropped out of the calibration set, a model developed and used to predict the single sample (Efron 1983). As sample numbers become large, this procedure will typically yield optimistic results, relative to the prediction of a larger test set.

In this study, the performance of PLSR models were assessed on cross validation segments based on time of culture, replicate flasks and algal species (the latter for combined species models). In general, poor cross validation results were obtained when using species as a cross-validation group criterion, indicating that the created models were not robust across new species. This issue is problematic for species survey work. Whether a calibration set could be extended to create a model that could cope with prediction of species not included in the calibration set is a question for future consideration. It may be that multivariate regression methods other than the straightforward PLSR approach could be employed. For example, Foss (www.foss.com) report the use of a neural network based calibration based on a very large calibration set to predict grain parameters across a worldwide network of NIR instruments. Shenk and Westerhaus (1997) report on the utility of a 'local'-PLS procedure, in which an unknown sample is matched to spectra within a large database, and the unknown sample, is predicted using a PLSR model developed on these 'like' spectra.

The use of day of culture as the cross validation group represents a relatively harsh test of a model, as the first and last day of the culture is likely to contain extremes of attribute level (ie. low and high values, respectively), and thus will involve development of a model developed over a different attribute range to that of the

validation group. Indeed model statistics were always poorer when cross validation was based on 'day of culture', relative to that for 'culture flask'.

The use of "culture flask" as the basis for cross validation group represents a reasonable approach, with each culture vessel being independent of other flasks. As the culture vessels were all similar in biomass and lipid content at a given time of culture, the removal of data associated with any specific culture vessel would have little effect on the mean and SD of the remaining calibration group. The use of cross validation segments based on culture flasks is recommended.

5.4.7 Model robustness

As a further test of model robustness, a second batch of *Chlorella* cultures were produced, with cultures grown under a range of limited nutritional conditions. The different nutritional conditions resulted in a change in the visible wavelength range spectra, as was expected due to changes in cell pigmentation. In consequence, it is not surprising that models based on combined species in the Herschel region failed in prediction of biomass obtained in nutrient limited independent sets (maximum R_p of only 0.6; Table 22).

The PLSR biomass models of biomass and lipid, developed using NIR spectra of dry filtrates of *Chlorella* grown on complete nutrient solution, worked well in prediction of *Chlorella* cultures growing under similar conditions, although a notable bias existed. The prediction of biomass and lipid in *Chlorella* grown under N, P and Fe limited conditions was compromised in terms of the low mean and SD of these attributes in these populations, nonetheless the R_p results for N and Fe limited cultures were

encouraging (>0.87). The poor result in prediction of the combined data set (Table 22) is indicative of a need for a broader calibration set. The data collected in this trial (across two trials and four nutrient conditions) forms a useful foundation for further trials on model robustness.

A PLSR biomass model based on NIR spectra of dry filtrates of a number of microalgal species, including *Chlorella*, grown in nutrient sufficient growth trial, performed similarly to the single species (*Chlorella*) model in prediction of the test populations (Table 16 and 17). This result is encouraging for the development of multi-species models, as recommended by Laurens and Wolfrum (2011).

In case of total lipid, the combined species PLSR calibration models developed using NIR spectra of dry filtrates performed equally well as of single species (*Chlorella*) model in prediction of the test sets.

5.5 Conclusions and recommendations

In this exercise, a low effort sample preparation method (dry filtrates) was successfully utilised for NIR reflectance spectroscopy. Nonetheless it remains to explicitly compare this method against that used by Laurens and Wolfram (2011), which involved lyophilisation and grinding of algal biomass. For direct filtrate method, the minor modification of varying culture sample volume to ensure filtrate sample biomass of at least 5-20 mg is suggested.

Further work is required to verify the robustness of the PLSR models, across growth conditions. This is a quality control exercise that requires multiple cultures. The calibration set can be expanded using these samples, and standard techniques (e.g.

Mahalanobois distance) used to determine whether a given test sample 'belongs' to the set described by the calibration samples.

Another challenge lies in testing the capacity of NIRS to resolve between classes of algal lipids, e.g. between the storage lipid triglycerides and the membrane phospholipids). Earlier research reports the application of NIRS to determine the fatty acid compositions (C_4 to C_{24}) of oils (Safar *et al.* 1994), milk (Coppa *et al.* 2010; Soyeurt *et al.* 2006), various meat products (González-Martín *et al.* 2005; González-Martín *et al.* 2003; Pla *et al.* 2007) and peanuts (Fox & Cruickshank 2005).

Consideration should also be given to different optical geometries for *in-situ* monitoring of parameters (biomass, total lipids, lipid classes, pigmentation, and microalgal species identification). Transmission or transflectance probes for online monitoring of biomass, lipid and lipid classes should be trialled.

Summary and future directions

Summary

Advantages such as diversity, high growth rates and less usage of water have attracted algae as a source of biofuels. Bio-prospecting and biomass and lipid detection techniques play an important role in microalgal biofuels research. Evaluation of biomass and lipids depend on conventional methods which are time consuming, expensive, and use hazardous chemicals. A cost effective, non destructive and rapid measuring tool to assess biomass and lipid content of microalgal cultures is the current requirement. The aim of this study is to isolate neutral lipid containing microalgae species from the Central Queensland region and to assess the feasibility of NIRS to measure biomass and lipid content of microalgae

This work is the first initiation of bio-prospecting microalgae for neutral lipid content in the Central Queensland Region. Ninety water samples from Rockhampton, Byfield, Yeppoon, Gracemere, Blackwater, Gladstone and Bundaberg were collected from May to September, 2010. Nile red was used to screen the presence of neutral lipid in the microalgal samples. This study recommends species screening (for presence of lipid) as a primary step before isolation. This approach decreased the time involved in isolation of unwanted microalgal strains. Species of *Chlorella*, *Navicula* (two species), *Nitzschia and Staurastrum* were initially identified as neutral lipid containing microalgal species. However, *Staurastrum* sp could not survive in the growth media for longer time and the reason was tentatively confirmed to the bacterial association of *Staurastrum* in the original sample. *Nitzschia pusilla* was isolated from brackish water and species of *Chlorella*, *Staurastrum* and *Navicula*

were isolated from fresh water habitats. The pH range from 7.1-8.8 and EC between 310-1394 μS cm^{-1} in the water bodies from where the neutral lipid containing microalgal specie were isolated. Isolations of uni-algal cultures were initially based on single cell isolation techniques. However, this method failed to yield uni-algal cultures even after repeated transfers and was laborious and time consuming. The solid plate technique was then used with uni-algal cultures produced easily. Identification of microalgal species was restricted to microscopic observation of morphological features and identification keys. Microalgal species of Scenedesmus, *Tetraspora*, *Oscillatoria*, *Dolicnospermum* were reported negative for Nile red screening.

Collection, screening, isolation and identification procedures were followed by characterization of biomass and lipid productivities. Species of *Chlorella*, *Navicula* (two species) and *Nitzschia* were grown under nutrient sufficient growth trials and the species with high biomass and lipid content was selected for further experiments under nutrient limited (N, P and Fe) growth conditions. Growth was measured in terms of cell counts (Sedgwick Rafter Counting Cell), optical density (625 nm) and dry weight. Total lipid was estimated adopting the technique of Kates and Volcanic (1966) as modified by Moheimani (2005). Among the four microalgal species grown under nutrient sufficient cultivation, biomass and lipid content of *Chlorella* was 93-98 and 80-90 % respectively higher than that of *Navicula* and *Nitzschia* species. OD_{625} nm was linearly correlated with the cell counts and dry weight measurement of biomass. However, the R^2 values ranged from 0.95-0.99 (OD to cell counts) and 0.99 (OD to dry weight). Reduced concentrations of N, P and Fe in the growth media

demonstrated that *C. vulgaris* was able to accumulate 38-49 % dry weight of lipid content more than that of control culture, which was in agreement with the reported literature. Whereas, the average biomass content of the N, P and Fe limited cultures was 20-30 % less that the control culture. The maximum biomass in *C. vulgaris* was stimulated by Fe limited cultivation whereas; maximum lipid content was demonstrated under P limited cultivation. The present study demonstrated that reduced P concentration was a suitable condition to grow *C. vulgaris* strain isolated from Central Queensland region. pH recorded during the experiment showed no correlation with lipid content. Constant rise in EC values was due to the evaporation rates in the culture flaks. This study recommends fatty acid profiling of *Chlorella vulgaris* and optimization of carbon and nutrient concentrations as a primary step before the strain is adapted for outdoor cultivation.

Biomass and lipid content was assessed using Near Infrared Spectroscopy. Spectra of culture vessels (*in-situ*) and microalgal sample filter through filter papers (oven dried and wet) were collected using FTNIR (1100-2500 nm) (Nicolet Aantaris Near IR Analyzer) and SWNIR (300-1100 nm) (Nirvana unit). Unscrambler software 10.1v (CAMO. Oslo, Norway) was used for data pre-processing and to develop Partial Least Squares regression models. The PLSR models were compared in terms of elements (n), population mean and standard deviation (SD), the number of principle components (PC's), cross validation coefficient of determination (RCV) and root mean square error of cross-validation (RMSECV). A single species model for *Chlorella vulgaris* based on 1100-2500 nm spectra of dry filtrate, supported a model with RMSECV, R_{CV} and SDR values of 0.32 g L^{-1}, 0.955 and 3.38 for biomass and

0.089 g L^{-1}, 0.874 and 2.06 with lipid, respectively. Model statistics were low for spectra obtained using wet and culture flasks. However, the best model performed poorly in the prediction of samples drawn from an independent series of *Chlorella vulgaris* cultured under N, P and Fe deficient conditions. A model based on all data available resulted in RMSECV, RP and SDR values of 0.54 g L^{-1}, 0.94 and 1.329 with biomass and 0.077 g L^{-1}, 0.85 and 0.279 with lipid respectively. The NIRS technique thus has the potential for dry matter and lipid determination of microalgal cultures. However, attention is required, to the issue of model robustness across species and growth conditions and fatty acid profile assessment as future recommendations.

Future directions

It was estimated that 2.5 million ha of microalgal pond production (at 0.04 g L^{-1} d^{-1}) and 34 % lipid dry weight) would be required to supply Australia's diesel needs for 2020. This is obviously impractical, given the limitations on fresh water availability in Australia. Two paths forward are suggested. The first involves use of brackish and saline water, both coastal and inland. This strategy would involve bio-prospecting for species of high growth rate and lipid content. The second path involves continued use of *Chlorella*, but under intensive cultivation. For example, light distribution through a culture is currently a likely limit to production. The use of 'light pipes' delivering light from reflectors adjacent to the cultivation areas to depth within cultures could allow an order of magnitude higher growth rate per unit area (e.g. by allowing cultivation of greater depths of solution) as could selection of species with high growth rates. 'Agronomic' protocols could be developed, likely involving media

and conditions that favour a high cell division at first, followed by conditions (example N and P deficiency) that favour oil accumulation.

If lipid yield per unit area of land could be increased by two orders of magnitude (great challenge!), the diesel required to support Australia's transport needs could be produced from half the required resources. No single best technology is likely to come up to produce biofuel from microalgae, since production depends upon optimizing regional microalgal strains using the existing resources. It might take few more years for the biofuel extracted from microalgae to be reasonably commercial. Earlier than it is worth to invest in isolating microalgae with high lipid contents and optimize the growth parameters.

Challenge of NIRS lies in the development of robust models across varies growth conditions and species and to resolve between lipid classes useful for biofuel production (to assess percentage of triglycerides). Consideration should also be given to different optical geometries for *in-situ* monitoring of parameters and transmission or trans-reflectance probes for online monitoring of biomass, lipid and lipid classes should be trialled. Continued development of NIRS applications has the ability to make major contributions to online monitoring of biomass and lipid composition in microalgal cultures.

Appendix A

Table I. Media composition of Guillard f/2 stock solutions. Each stock is made to 1 L. To prepare working solutions, 1 mL of stock solutions from 1-7 were added to 993 mL distilled water and mixed thoroughly.

S:No	Reagents	Composition g L^{-1}
1	$NaNO_3$	150
2	**Trace metals**	
	$CuSO_4.5H_2O$	0.0196
	$ZnSO_4.7H_2O$	0.044
	$CoCl_2.6H_2O$	0.022
	$MnCl_2.4H_2O$	0.36
	$Na_2MoO_4.2H_2O$	0.0126
3	$Na_2SiO_3.5H_2O$	0.0227
4	**Fe citrate**	
	Ferric citrate	9.0
	Citric acid	9.0
5	$NaH_2PO_4.2H_2O$	11.3
6	$Na_2EDTA.2H_2O$	30
7	**Vitamins**	
	Vitamin B12	0.001
	Biotin	0.001
	Thiamine HCl	0.02

Table II. Media composition of ASM stock solutions. Each stock is made to 1L. To prepare working solutions, one mL of each of the stock solutions was added to 10 L distilled water and mixed thoroughly.

S:No	Reagents	Composition g L^{-1}
1	$NaNO_3$	85
2	K_2HPO_4	17.4
3	Na_2HPO_4	14.2
4	$MgCl_2.6H_2O$	40.62
5	$MgSo_4.7H_2O$	49.33
6	$CaCl_2.2H_2O$	29.4
7	$FeCl_3.6H_2O$	1.0835
8	H_3BO_3	2.47
9	$MnCl_2.4H_2O$	1.3683
10	$ZnCl_2$	0.44
11	Na_2 E.D.T.A	6.64
12	$CoS0_4.7H_2O$	0.216
13	$CuCl_2.2H_2O$	0.013

Table III. Media composition of Bolds Basal stock solutions. Each stock is made to 1 L. To prepare working solutions, 10 mL each solution from 1-6 was added to 940 ml distilled water and mixed thoroughly. Then 1 mL of stock solutions from 7-10 were added.

S:No	Reagents	Composition g L^{-1}
1	$NaNo_3$	25
2	$CaCl_2.2H_2O$	2.5
3	$MgSo_4.7H_2O$	7.5
4	K_2HPO_4	7.5
5	KH_2PO_4	17.5
6	NaCL	2.5
7	EDTA	50
	KOH	31
8	$FeSO_4.7H_2O$	4.98
	H_2SO_4	1 mL
9	H_3BO_3	11.42
10	**Micronutrients**	
	$ZnSO_4.7H_20$	8.82
	$MnCl_2.4H_2O$	1.44
	MoO_3	0.71
	$CuSO_4.5H_20$	1.57
	$Co(NO_3)_2.6H_2O$	0.49

Appendix B

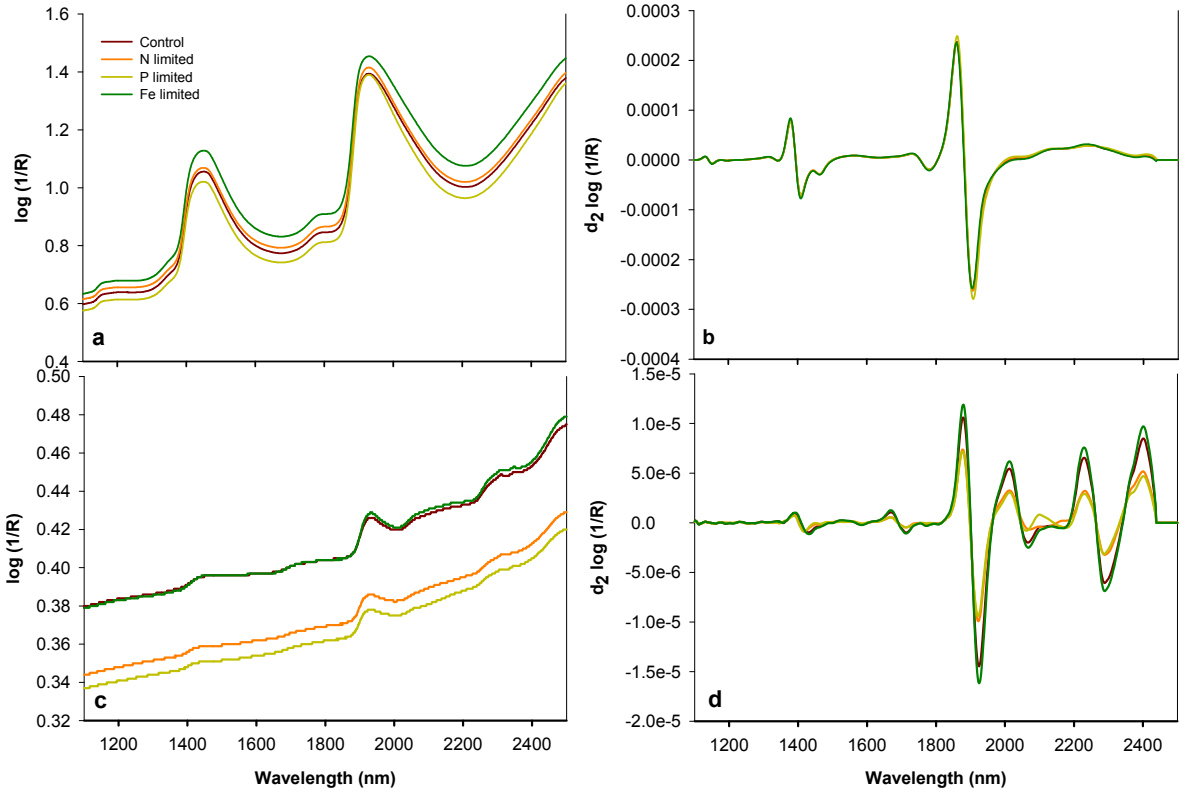


Figure I. Spectra (NIR region; 1100-2500 nm) of *Chlorella vulgaris* under nutrient limited growth trial: log (1/R) spectra of (a) wet and (c) dry filtrates; and d_2 log (1/R) spectra of (b) wet and (d) dry filtrates.

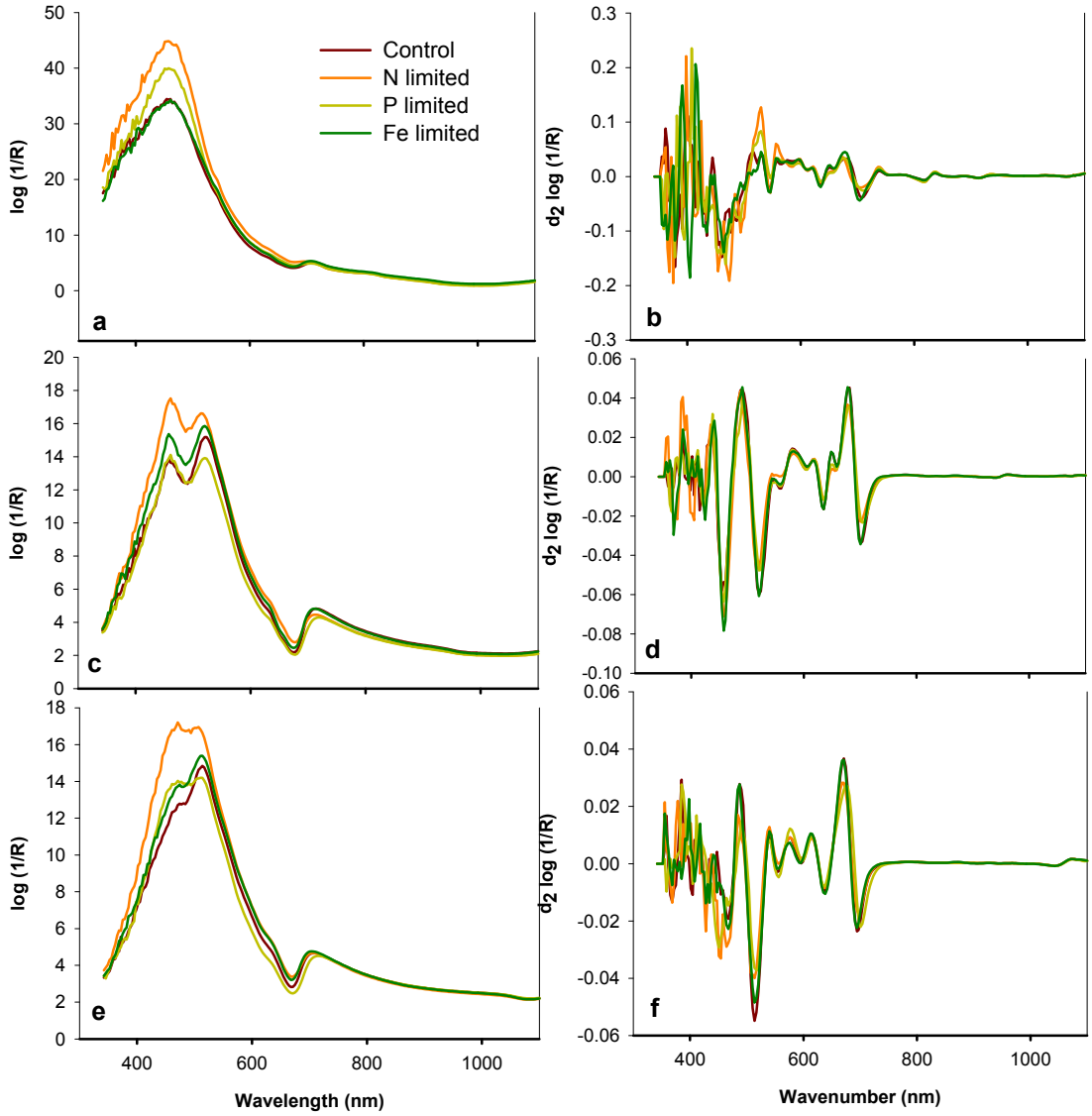


Figure II. Spectral (Herschel region; 300-1100nm) of *Chlorella vulgaris* under nutrient limited growth trial (a) log (1/R) and (b) d2 log (1/R) of interactance spectra of culture vessels; and (c) log (1/R) wet filtrates (d) d_2 log (1/R) of reflectance spectra of wet filtrates and of dry filtrates (e, f, respectively).

Appendix C

Table I. Culture flask: Summary statistics of PLSR calibration models of biomass developed on Herschel region (300-1100 nm) log (1/R) spectra of replication flasks. Cross validation results are reported for validation groups based on species (n=4), on replication flask (n=5) and on days (n=12).

Species Model	**Cross Validation Segment**	**Sample mean (g L^{-1})**	**Sample SD (g L^{-1})**	**Elements**	**PC**	**R_{CV}**	**RMSECV (g L^{-1})**	**SDR**	**Bias (g L^{-1})**	**Slope**
Combined	Species	0.39	0.781	478*	1	-0.17	0.951	1.86	-0.20	-0.09
	Flask				3	0.81	0.454	1.86	0.00	0.67
Chlorella vulgaris	Days	1.36	1.082	120	1	0.83	0.597	1.81	-0.01	0.74
	Flask				1	**0.87**	0.534	2.03	0.00	0.77
Navicula sp. 1	Days	0.06	0.028	120	3	**0.89**	0.012	2.23	0.00	0.75
	Flask				3	**0.88**	0.013	2.15	0.00	0.79
Nitzschia pusilla	Days	0.11	0.023	120	9	0.84	0.013	1.82	0.00	0.77
	Flask				6	**0.86**	0.012	1.95	9.63	0.80
Navicula sp. 2	Days	0.04	0.013	120	14	0.77	0.008	1.55	0.00	0.67
	Flask				15	0.84	0.007	1.81	-5.46	0.79

*Two spectra removed as outliers.

Bold - values showing higher R_{CV} among species, days and flasks.

Table II. Wet filtrates: Summary statistics of PLSR calibration models developed of biomass on Herschel region (300-1100 nm) log (1/R) spectra of wet filtrates. Cross validation results are reported for validation groups based on species (n=4), on replication flask (n=5) and on days (n=12).

Species Model	**Cross Validation Segment**	**Sample mean (g L^{-1})**	**Sample SD (g L^{-1})**	**Elements**	**PC**	**R_{CV}**	**RMSECV (g L^{-1})**	**SDR**	**Bias (g L^{-1})**	**Slope**
Combined	Species	0.43	0.813	450**	14	0.70	0.593	1.37	-0.02	0.59
	Flask				15	**0.84**	0.398	1.86	-0.01	0.75
Chlorella vulgaris	Days	1.36	1.082	120	19	0.87	0.558	1.94	0.02	0.88
	Flask				5	**0.88**	0.515	2.10	-0.01	0.84
Navicula sp. 1	Days	0.07	0.028	120	12	0.81	0.017	1.67	0.00	0.77
	Flask				10	**0.85**	0.015	1.90	0.00	0.79
Nitzschia pusilla	Days	0.11	0.017	90*	4	0.40	0.017	1.01	0.00	0.31
	Flask				1	-0.05	0.021	0.98	0.00	-0.01
Navicula sp. 2	Days	0.04	0.013	120	10	0.69	0.009	1.35	0.00	0.54
	Flask				10	0.78	0.008	1.56	0.00	0.69

* Spectra were not recorded for day 0,2,4,6 due to technical issues.

**includes missing values of *

Bold - values showing higher R_{CV} among species, days and flasks.

Table III. Dry filtrates: Summary statistics of the PLSR calibration models developed of biomass on Herschel region (300-1100 nm) log (1/R) spectra of dry filtrates. Cross validation results are reported for validation groups based on species (n=4), on replication flask (n=5) and on days (n=12).

Species Model	**Cross Validation Segment**	**Sample mean (g L^{-1})**	**Sample SD (g L^{-1})**	**Elements**	**PC**	**R_{CV}**	**RMSECV (g L^{-1})**	**SDR**	**Bias (g L^{-1})**	**Slope**
Combined	Species	0.40	0.787	446***	20	0.76	0.547	1.44	-0.18	0.61
	Flask				13	**0.87**	0.404	2.01	0.02	0.84
Chlorella vulgaris	Days	1.36	1.082	120	3	0.95	0.326	3.31	0.01	0.87
	Flask				3	**0.96**	0.282	3.83	0.00	0.93
Navicula sp. 1	Days	0.06	0.025	118**	3	0.75	0.017	1.52	0.00	0.58
	Flask				2	**0.81**	0.015	1.71	0.00	0.67
Nitzschia pusilla	Days	0.11	0.017	90*	4	0.68	0.013	1.35	0.00	0.56
	Flask				4	0.74	0.011	1.50	0.00	0.59
Navicula sp. 2	Days	0.04	0.013	120	2	0.63	0.010	1.28	0.00	0.44
	Flask				3	0.72	0.009	1.43	0.00	0.59

* Spectra were not recorded for day 0,2,4,6 due to technical issues.
**Two spectra removed as outliers.
***Includes * and **
Bold - values showing higher R_{CV} among species, days and flasks.

Table IV. Culture flask: Summary statistics of PLS calibration models developed of total lipid on Herschel region (300-1100 nm) log (1/R) spectra of culture flasks. Cross validation results are reported for validation groups based on species (n=4), on replication flask (n=5) and on days (n=12).

Species Model	**Cross Validation Segment**	**Sample mean (g L^{-1})**	**Sample SD (g L^{-1})**	**Elements**	**PC**	**R_{CV}**	**RMSECV (g L^{-1})**	**SDR**	**Bias (g L^{-1})**	**Slope**
Combined	Species	0.09	0.130	478*	1	-0.20	0.158	1.86	-0.03	-0.09
	Flask				3	0.76	0.084	1.86	0.00	0.59
Chlorella vulgaris	Days	0.20	0.183	120	2	0.75	0.121	1.07	0.00	0.62
	Flask				2	0.77	0.116	0.08	0.00	0.61
Navicula sp. 1	Days	0.03	0.017	120	1	0.07	0.019	0.93	4.22	0.02
	Flask				2	0.30	0.018	1.02	0.00	0.16
Nitzschia pusilla	Days	0.04	0.023	120	3	0.37	0.022	1.04	0.00	0.24
	Flask				3	0.56	0.019	1.21	0.00	0.35
Navicula sp. 2	Days	0.03	0.014	120	1	-0.57	0.020	0.70	0.00	-0.35
	Flask				3	0.34	0.013	1.04	0.00	0.18

*Two spectra removed as outliers.

Table V. Wet filtrates: Summary statistics of PLSR calibration models developed of total lipid on Herschel region (300-1100 nm) log (1/R) spectra of wet filtrates. Cross validation results are reported for validation groups based on species (n=4), and on replication flask (n=5) and on days (n=12).

Species Model	**Cross Validation Segment**	**Sample mean (g L^{-1})**	**Sample SD (g L^{-1})**	**Elements**	**PC**	**R_{CV}**	**RMSECV (g L^{-1})**	**SDR**	**Bias (g L^{-1})**	**Slope**
Combined	Species	0.08	0.136	450**	2	0.10	0.146	0.93	-0.02	0.05
	Flask				3	0.58	0.106	1.23	-3.70	0.36
Chlorella vulgaris	Days	0.20	0.183	120	2	0.42	0.168	0.77	0.00	0.24
	Flask				2	0.51	0.157	0.83	0.00	0.29
Navicula sp. 1	Days	0.03	0.017	120	1	-0.14	0.019	0.93	0.00	-0.04
	Flask				2	0.32	0.017	1.05	0.00	0.15
Nitzschia pusilla	Days	0.04	0.017	90*	3	0.62	0.018	1.28	0.00	0.40
	Flask				1	0.17	0.013	1.01	0.00	0.05
Navicula sp. 2	Days	0.03	0.014	120	1	-0.69	0.018	0.75	0.00	-0.29
	Flask				1	-0.09	0.014	0.99	0.00	-0.01

* Spectra were not recorded for day 0,2,4,6 due to technical issues.

**Includes *

Table VI. Wet filtrates: Summary statistics of PLSR calibration models developed of total lipid on NIR region (1100-2500 nm) log (1/R) spectra of wet filtrates. Cross validation results are reported for validation groups based on species (n=4), on replication flask (n=5) and on days (n=12).

Species Model	**Cross Validation Segment**	**Sample mean (g L^{-1})**	**Sample SD (g L^{-1})**	**Elements**	**PC**	**R_{CV}**	**RMSECV (g L^{-1})**	**SDR**	**Bias (g L^{-1})**	**Slope**
Combined	Species	0.09	0.131	477**	17	0.39	0.128	1.02	-0.02	0.26
	Flask				12	0.84	0.071	1.84	0.00	0.72
Chlorella vulgaris	Days	0.20	0.183	120	9	0.56	0.156	0.83	0.00	0.44
	Flask				10	0.74	0.124	1.05	0.00	0.61
Navicula sp. 1	Days	0.03	0.017	120	13	0.56	0.015	1.15	0.00	0.47
	Flask				17	0.63	0.014	2.01	0.00	0.47
Nitzschia pusilla	Days	0.04	0.017	90*	2	0.35	0.022	1.05	0.00	0.20
	Flask				15	0.70	0.016	1.38	0.00	0.58
Navicula sp. 2	Days	0.03	0.014	120	1	-0.73	0.016	0.86	0.00	-0.16
	Flask				1	-0.27	0.015	0.96	0.00	-0.03

* Spectra were not recorded for day 0,2,4,6 due to technical issues.

**Includes *

Table V11. Dry filtrates: Summary statistics of PLSR calibration models developed on total lipid on Herschel region (300-1100 nm) log (1/R) spectra of dry filtrates. Cross validation results are reported for validation groups based on species (n=4), on replication flask (n=5) and on days (n=12).

Species Model	**Cross Validation Segment**	**Sample mean (g L^{-1})**	**Sample SD (g L^{-1})**	**Elements**	**PC**	**R_{CV}**	**RMSECV (g L^{-1})**	**SDR**	**Bias (g L^{-1})**	**Slope**
Combined	Species	0.09	0.132	446**	1	-0.18	0.192	0.68	0.01	-0.16
	Flask				12	0.75	0.090	1.50	0.00	0.62
Chlorella vulgaris	Days	0.20	0.183	120	6	0.56	0.160	0.81	0.00	0.48
	Flask				3	0.73	0.125	1.04	0.00	0.66
Navicula sp. 1	Days	0.03	0.017	120	3	0.41	0.017	1.04	0.00	0.29
	Flask				3	0.57	0.015	1.20	0.00	0.41
Nitzschia pusilla	Days	0.04	0.017	90*	4	0.68	0.018	1.35	0.00	0.53
	Flask				4	0.76	0.016	1.52	0.00	0.64
Navicula sp. 2	Days	0.03	0.014	120	1	-0.53	0.017	0.81	0.00	-0.19
	Flask				3	0.36	0.013	1.04	0.00	0.22

* Spectra were not recorded for day 0,2,4,6 due to technical issues.

**includes * and four spectra removed as outliers.

Appendix D

Table I. Summary statistics of various spectral pre-processing methods applied on *Chlorella vulgaris* log (1/R) spectra of total lipid over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet and dry filtrates and replication flasks. Where n=120, $\overline{X}$ =0.20 g L^{-1} and σX=1.30 g L^{-1}. Cross validation groups based on replication flask (n=5).

Wave Length (nm)	Filter Paper	Pre-processing method	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log 1/R	9	0.82	0.10	1.25	0.00	0.70
		log 1/R to d2A	5	**0.86**	0.09	1.40	0.00	0.78
		log 1/R to MSC	8	0.85	0.10	1.34	0.00	0.78
		log 1/R to SNV	8	0.85	0.10	1.35	0.00	0.78
		d2A log 1/R to MSC	4	0.83	0.10	1.27	0.00	0.69
		d2A log 1/R to SNV	4	0.82	0.10	1.23	0.00	0.69
	Wet	log 1/R	2	0.42	0.17	0.77	0.00	0.24
		log 1/R to d2A	7	**0.77**	0.12	1.12	0.00	0.64
		log 1/R to MSC	8	0.70	0.13	1.00	0.00	0.55
		log 1/R to SNV	9	0.72	0.13	1.01	0.00	0.58
		d2A log 1/R to MSC	6	0.73	0.12	1.05	0.00	0.59
		d2A log 1/R to SNV	7	0.74	0.12	1.05	0.00	0.60
300-1100	Dry	log 1/R	6	0.56	0.16	0.81	0.00	0.48
		log 1/R to d2A	5	0.67	0.14	0.92	0.00	0.59
		log 1/R to MSC	4	0.69	0.13	0.98	0.00	0.57
		log 1/R to SNV	4	**0.71**	0.13	1.00	0.00	0.58
		d2A log 1/R to MSC	1	0.30	0.17	0.75	0.01	0.09
		d2A log 1/R to SNV	3	0.66	0.14	0.94	0.00	0.52
	Wet	log 1/R	10	0.74	0.12	1.05	0.00	0.61
		log 1/R to d2A	8	0.53	0.16	0.80	0.00	0.46
		log 1/R to MSC	2	0.51	0.16	0.83	0.00	0.30

	log 1/R to SNV	2	0.56	0.15	0.86	0.00	0.35
	d2A log 1/R to MSC	2	0.25	0.18	0.72	0.00	0.11
	d2A log 1/R to SNV	6	0.54	0.16	0.82	0.00	0.42
Flask	log 1/R	2	0.77	0.12	0.08	0.00	0.61
	log 1/R to d2A	6	0.70	0.13	0.99	0.01	0.56
	log 1/R to MSC	3	0.68	0.14	0.95	0.01	0.54
	log 1/R to SNV	2	0.75	0.12	1.07	0.00	0.59
	d2A log 1/R to MSC	1	0.07	0.24	0.54	-0.01	0.06
	d2A log 1/R to SNV	2	0.69	0.13	0.99	0.00	0.51

Bold- the spectral pre-treatment supporting the highest Rcv.

Table II. Summary statistics of various spectral pre-processing methods applied on *Navicula* sp. 1 log (1/R) spectra of biomass over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet and dry filtrates and replication flasks. Where n=120, $\overline{X}$ =0.07 g L^{-1} and σX=0.03 g L^{-1}. Cross validation groups based on replication flask (n=5).

Wave Length (nm)	Filter Paper	Pre-processing method	PC	R_{cv}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log 1/R	10	0.91	0.01	2.35	0.00	0.81
		log 1/R to d2A	5	0.93	0.01	2.68	0.00	0.89
		log 1/R to MSC	7	0.94	0.01	2.94	0.00	0.92
		log 1/R to SNV	10	**0.95**	0.01	3.22	0.00	0.91
		d2A log 1/R to MSC	7	**0.95**	0.01	3.24	0.00	0.91
		d2A log 1/R to SNV	7	**0.95**	0.01	3.30	0.00	0.91
	Wet	log 1/R	6	0.86	0.01	1.97	0.00	0.75
		log 1/R to d2A	8	0.85	0.01	1.92	0.00	0.79
		log 1/R to MSC	7	0.85	0.01	1.90	0.00	0.75
		log 1/R to SNV	6	0.85	0.01	1.90	-0.60	0.74
		d2A log 1/R to MSC	7	0.85	0.01	1.90	0.00	0.79
		d2A log 1/R to SNV	8	**0.88**	0.01	2.13	0.00	0.82
300-1100	Dry	log 1/R	2	0.81	0.02	1.71	0.00	0.67
		log 1/R to d2A	11	0.76	0.02	1.52	0.00	0.70
		log 1/R to MSC	2	0.79	0.01	1.66	0.00	0.63
		log 1/R to SNV	2	0.80	0.01	1.66	0.00	0.63
		d2A log 1/R to MSC	10	0.54	0.02	1.09	0.00	0.49
		d2A log 1/R to SNV	6	0.61	0.02	1.22	0.00	0.51
	Wet	log 1/R	10	0.85	0.02	1.90	0.00	0.79
		log 1/R to d2A	12	0.64	0.02	1.24	0.00	0.57
		log 1/R to MSC	19	0.85	0.02	1.81	0.00	0.86
		log 1/R to SNV	7	0.77	0.10	1.55	0.00	0.70

	d2A log 1/R to MSC	1	0.01	0.04	0.76	0.00	0.01
	d2A log 1/R to SNV	4	0.51	0.02	1.13	0.00	0.36
Flask	log 1/R	3	0.88	0.01	2.15	0.00	0.79
	log 1/R to d2A	18	0.86	0.01	1.94	-6.90	0.81
	log 1/R to MSC	2	0.86	0.01	2.00	0.00	0.75
	log 1/R to SNV	2	0.87	0.01	2.02	0.00	0.75
	d2A log 1/R to MSC	1	-0.02	0.03	0.99	0.00	0.00
	d2A log 1/R to SNV	17	0.79	0.02	1.58	0.00	0.76

Bold- the spectral pre-treatment supporting the highest Rcv.

Table III. Summary statistics of various spectral pre-processing methods applied on *Navicula* sp. 1 log (1/R) spectra of total lipids over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet and dry filtrates and replication flasks. Where n=120, $\overline{X}$ =0.03 g L^{-1} and σX=0.017 g L^{-1}. Cross validation groups based on replication flask (n=5).

Wave Length (nm)	Filter Paper	Pre-processing method	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log 1/R	10	0.79	0.02	1.64	0.00	0.71
		log 1/R to d2A	7	0.75	0.01	1.51	0.00	0.65
		log 1/R to MSC	10	**0.79**	0.01	1.62	0.00	0.68
		log 1/R to SNV	10	**0.79**	0.01	1.62	0.00	0.69
		d2A log 1/R to MSC	6	0.72	0.01	1.43	0.00	0.61
		d2A log 1/R to SNV	8	0.75	0.01	1.48	0.00	0.68
	Wet	log 1/R	17	0.63	0.01	2.01	0.00	0.47
		log 1/R to d2A	6	0.49	0.01	1.79	0.00	0.32
		log 1/R to MSC	6	0.48	0.01	1.13	0.00	0.30
		log 1/R to SNV	6	0.54	0.01	1.18	0.00	0.34
		d2A log 1/R to MSC	6	0.51	0.01	1.14	0.00	0.34
		d2A log 1/R to SNV	4	0.55	0.01	1.20	0.06	0.33
300-1100	Dry	log 1/R	3	0.57	0.02	1.20	0.00	0.41
		log 1/R to d2A	3	0.38	0.02	1.05	0.00	0.25
		log 1/R to MSC	2	0.52	0.01	1.17	0.00	0.32
		log 1/R to SNV	2	0.52	0.01	1.17	0.00	0.32
		d2A log 1/R to MSC	3	0.27	0.02	1.01	0.00	0.14
		d2A log 1/R to SNV	3	0.43	0.02	1.08	0.00	0.29
	Wet	log 1/R	2	0.32	0.02	1.05	0.00	0.15
		log 1/R to d2A	1	0.04	0.02	0.96	0.00	0.01
		log 1/R to MSC	1	0.04	0.02	0.97	0.00	0.03

	log 1/R to SNV	1	0.09	0.02	0.97	0.00	0.03
	d2A log 1/R to MSC	1	0.05	0.02	0.96	0.00	0.02
	d2A log 1/R to SNV	1	0.08	0.02	0.97	0.00	0.03
Flask	log 1/R	2	0.30	0.02	1.02	0.00	0.16
	log 1/R to d2A	1	0.04	0.02	0.98	-2.53	0.01
	log 1/R to MSC	2	**0.40**	0.02	1.02	0.00	0.22
	log 1/R to SNV	2	**0.40**	0.02	1.02	0.00	0.22
	d2A log 1/R to MSC	1	-0.15	0.02	0.86	0.00	-0.05
	d2A log 1/R to SNV	1	0.07	0.02	0.91	0.00	0.02

Bold- the spectral pre-treatment supporting the highest Rcv.

Table IV. Summary statistics of various spectral pre-processing methods applied on *Nitzschia pusilla* log (1/R) spectra of biomass over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet and dry filtrates and replication flasks. Cross validation groups based on replication flask (n=5).

Wave Length (nm)	Filter Paper	Pre-processing method	Sample mean g L^{-1}	Sample SD g L^{-1}	Elements	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log 1/R				8	0.90	0.01	2.26	-4.41	0.88
		log 1/R to d2A	0.11	0.024	120	7	**0.91**	0.01	2.45	0.00	0.88
		log 1/R to MSC				2	0.88	0.01	2.13	0.00	0.80
		log 1/R to SNV				5	**0.91**	0.01	2.48	0.00	0.84
		d2A log 1/R to MSC				2	**0.93**	0.01	2.73	0.00	0.87
		d2A log 1/R to SNV				3	**0.94**	0.01	2.86	0.00	0.88
	Wet	log 1/R	0.11	0.024	120	15	**0.91**	0.01	2.44	0.00	0.87
		log 1/R to d2A				8	0.88	0.01	2.11	0.00	0.81
		log 1/R to MSC				13	0.90	0.01	2.26	0.00	0.86
		log 1/R to SNV				14	**0.91**	0.01	2.47	0.00	0.86
		d2A log 1/R to MSC				9	0.90	0.01	2.21	0.00	0.84
		d2A log 1/R to SNV				9	0.88	0.01	2.09	0.00	0.83
300-1100	Dry	log 1/R	0.11	0.017	90*	4	0.74	0.01	1.50	0.00	0.59
		log 1/R to d2A				14	**0.82**	0.01	1.71	0.00	0.77
		log 1/R to MSC				3	0.71	0.01	1.42	0.00	0.55
		log 1/R to SNV				3	0.75	0.01	1.51	0.00	0.56
		d2A log 1/R to MSC				2	0.38	0.02	1.08	0.00	0.19
		d2A log 1/R to SNV				8	0.68	0.01	1.32	0.000	0.62
	Wet	log 1/R	0.11	0.017	90*	1	-0.05	0.02	0.98	0.00	-0.01
		log 1/R to d2A				1	-0.06	0.02	0.94	0.00	-0.10
		log 1/R to MSC				1	-0.09	0.10	0.16	-0.01	-0.44

	log 1/R to SNV				1	**0.02**	0.03	0.67	0.00	0.01
	d2A log 1/R to MSC				1	-0.03	0.02	0.83	0.00	0.00
	d2A log 1/R to SNV				1	-0.02	0.02	0.78	0.00	-0.01
Flask	log 1/R	0.11	0.024	120	6	0.86	0.01	1.95	9.63	0.80
	log 1/R to d2A				20	0.81	0.01	1.67	-7.26	0.76
	log 1/R to MSC				3	0.77	0.01	1.58	0.00	0.61
	log 1/R to SNV				3	0.78	0.01	1.60	0.00	0.62
	d2A log 1/R to MSC				1	-0.28	0.03	0.9	0.00	-0.05
	d2A log 1/R to SNV				16	0.75	0.02	1.465	0.00	0.70

* Spectra were not recorded of day 0,2,4,6 due to technical issues.

Bold- the spectral pre-treatment supporting the highest Rcv.

Table V. Summary statistics of various spectral pre-processing methods applied on *Nitzschia pusilla* log (1/R) spectra of total lipid over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet and dry filtrates and replication flasks. Cross validation groups based on replication flask (n=5).

Wave Length (nm)	Filter Paper	Pre-processing method	Sample mean (g L^{-1})	Sample SD (g L^{-1})	Elements	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log 1/R	0.04	0.023	120	10	0.85	0.01	1.83	0.00	0.86
		log 1/R to d2A				16	**0.89**	0.01	2.06	3.71	0.93
		log 1/R to MSC				10	0.87	0.01	1.98	0.00	0.83
		log 1/R to SNV				12	0.87	0.01	1.99	0.00	0.84
		d2A log 1/R to MSC				4	0.80	0.01	1.69	0.00	0.67
		d2A log 1/R to SNV				4	0.83	0.01	1.82	0.00	0.71
	Wet	log 1/R	0.04	0.023	120	15	0.70	0.02	1.38	0.00	0.58
		log 1/R to d2A				3	0.51	0.02	1.17	0.00	0.30
		log 1/R to MSC				2	0.47	0.02	1.13	0.00	0.26
		log 1/R to SNV				14	0.66	0.02	1.30	0.00	0.54
		d2A log 1/R to MSC				4	0.53	0.02	1.18	0.00	0.33
		d2A log 1/R to SNV				2	0.49	0.02	1.15	0.00	0.27
300-1100	Dry	log 1/R	0.04	0.017	90*	4	0.76	0.02	1.52	0.00	0.64
		log 1/R to d2A				8	0.68	0.02	1.30	0.00	0.62
		log 1/R to MSC				3	0.72	0.02	1.43	0.00	0.58
		log 1/R to SNV				3	0.74	0.02	1.50	0.00	0.56
		d2A log 1/R to MSC				6	0.57	0.02	1.18	0.00	0.47
		d2A log 1/R to SNV				7	0.69	0.02	1.32	0.00	0.62
	Wet	log 1/R	0.04	0.017	90*	1	0.17	0.01	1.01	0.00	0.05
		log 1/R to d2A				1	0.06	0.01	0.97	0.00	0.02
		log 1/R to MSC				1	-0.07	0.02	1.18	0.00	-0.08

	log 1/R to SNV				1	0.11	0.01	1.79	0.00	0.03
	d2A log 1/R to MSC				1	0.03	0.01	1.75	0.00	0.01
	d2A log 1/R to SNV				1	0.04	0.01	1.71	0.00	0.02
Flask	log 1/R	0.04	0.023	120	3	0.56	0.02	1.21	0.00	0.35
	log 1/R to d2A				1	0.06	0.02	0.96	0.00	0.02
	log 1/R to MSC				3	0.47	0.02	1.13	0.00	0.28
	log 1/R to SNV				3	0.52	0.02	1.17	0.00	0.31
	d2A log 1/R to MSC				1	-0.09	0.02	0.97	0.00	-0.02
	d2A log 1/R to SNV				1	0.08	0.02	0.96	0.00	0.03

* Spectra were not recorded of day 0,2,4,6 due to technical issues.

Bold- the spectral pre-treatment supporting the highest Rcv.

Table VI. Summary statistics of various spectral pre-processing methods applied on *Navicula* sp. 2 log (1/R) spectra of biomass over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet and dry filtrates and replication flasks. Where n=120, $\overline{X}$ =0.04 g L^{-1} and σX=0.013 g L^{-1}. Cross validation groups based on replication flask (n=5).

Wave Length (nm)	Filter Paper	Pre-processing method	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log 1/R	8	0.87	0.01	2.02	-1.09	0.81
		log 1/R to d2A	9	0.90	0.01	2.34	-4.88	0.86
		log 1/R to MSC	11	0.91	0.01	2.43	0.00	0.82
		log 1/R to SNV	11	0.92	0.01	2.56	0.00	0.84
		d2A log 1/R to MSC	4	0.91	0.01	2.37	0.00	0.83
		d2A log 1/R to SNV	4	**0.93**	0.01	2.66	0.00	0.87
	Wet	log 1/R	15	0.92	0.01	2.47	-0.40	0.88
		log 1/R to d2A	10	0.91	0.01	2.41	0.00	0.85
		log 1/R to MSC	11	0.87	0.01	1.99	0.00	0.80
		log 1/R to SNV	11	0.90	0.01	2.33	0.00	0.85
		d2A log 1/R to MSC	11	0.91	0.01	2.47	0.00	0.86
		d2A log 1/R to SNV	11	**0.92**	0.01	2.50	0.00	0.87
300-1100	Dry	log 1/R	3	0.72	0.01	1.43	0.00	0.59
		log 1/R to d2A	11	0.71	0.01	1.40	0.00	0.62
		log 1/R to MSC	2	0.63	0.01	1.29	0.00	0.44
		log 1/R to SNV	2	0.66	0.01	1.33	0.00	0.47
		d2A log 1/R to MSC	1	-0.03	0.01	0.99	0.00	0.00
		d2A log 1/R to SNV	8	0.58	0.01	1.18	0.00	0.48
	Wet	log 1/R	10	0.78	0.01	1.56	0.00	0.69
		log 1/R to d2A	14	0.75	0.01	1.48	0.00	0.65
		log 1/R to MSC	2	0.56	0.01	1.21	0.00	0.34

	log 1/R to SNV	4	0.65	0.01	1.30	0.00	0.50
	d2A log 1/R to MSC	1	-0.29	0.01	0.98	0.00	-0.02
	d2A log 1/R to SNV	10	0.64	0.01	1.25	0.00	0.55
Flask	log 1/R	15	0.84	0.01	1.81	-5.46	0.79
	log 1/R to d2A	18	0.73	0.01	1.42	-5.93	0.69
	log 1/R to MSC	3	0.54	0.01	1.18	0.00	0.36
	log 1/R to SNV	2	0.53	0.01	1.18	0.00	0.32
	d2A log 1/R to MSC	1	-0.19	0.01	0.85	0.00	-0.09
	d2A log 1/R to SNV	1	0.03	0.01	0.95	0.00	0.01

Bold- the spectral pre-treatment supporting the highest Rcv.

Table VII. Summary statistics of various spectral pre-processing methods applied on *Navicula* sp. 2 log (1/R) spectra of total lipid over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet and dry filtrates and replication flasks. Where n=120, $\overline{X}$ =0.03 g L^{-1} and σX=0.014 g L^{-1}. Cross validation groups based on replication flask (n=5).

Wave Length (nm)	Filter Paper	Pre-processing method	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
1100-2500	Dry	log 1/R	11	0.63	0.01	1.28	0.00	0.46
		log 1/R to d2A	2	0.22	0.01	1.01	-8.01	0.09
		log 1/R to MSC	11	0.66	0.01	1.34	0.00	0.49
		log 1/R to SNV	11	0.66	0.01	1.33	0.00	0.48
		d2A log 1/R to MSC	6	0.47	0.01	1.08	0.00	0.35
		d2A log 1/R to SNV	2	0.30	0.01	1.04	0.00	0.13
	Wet	log 1/R	1	-0.27	0.02	0.96	0.00	-0.03
		log 1/R to d2A	1	0.06	0.01	0.99	0.00	0.01
		log 1/R to MSC	1	-0.12	0.01	0.98	0.00	-0.01
		log 1/R to SNV	1	-0.11	0.01	0.98	0.00	-0.01
		d2A log 1/R to MSC	1	-0.12	0.01	0.99	0.00	-0.01
		d2A log 1/R to SNV	1	-0.13	0.01	0.99	0.00	-0.01
300-1100	Dry	log 1/R	3	0.36	0.01	1.04	0.00	0.22
		log 1/R to d2A	1	0.13	0.01	0.99	-1.74	0.04
		log 1/R to MSC	3	**0.37**	0.01	1.06	0.00	0.21
		log 1/R to SNV	2	**0.66**	0.01	1.47	0.00	0.47
		d2A log 1/R to MSC	1	-0.08	0.01	0.98	0.00	-0.01
		d2A log 1/R to SNV	1	0.13	0.01	0.99	0.00	0.04
	Wet	log 1/R	1	-0.09	0.02	0.99	0.00	-0.01
		log 1/R to d2A	1	-0.09	0.01	0.91	0.00	-0.03
		log 1/R to MSC	1	**0.06**	0.01	0.99	0.00	0.01

	log 1/R to SNV	1	**0.05**	0.01	0.99	0.00	0.01
	d2A log 1/R to MSC	1	**0.06**	0.01	1.00	0.00	0.01
	d2A log 1/R to SNV	1	**0.03**	0.01	0.95	0.00	0.01
Flask	log 1/R	3	0.34	0.01	1.04	0.00	0.18
	log 1/R to d2A	1	0.07	0.01	0.99	-1.97	0.02
	log 1/R to MSC	1	-0.11	0.01	0.93	0.00	-0.03
	log 1/R to SNV	2	0.23	0.01	1.02	0.00	0.09
	d2A log 1/R to MSC	1	-0.14	0.01	0.98	0.00	-0.01
	d2A log 1/R to SNV	1	0.00	0.01	0.94	0.00	0.00

Bold- the spectral pre-treatment supporting the highest Rcv.

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Appendix E

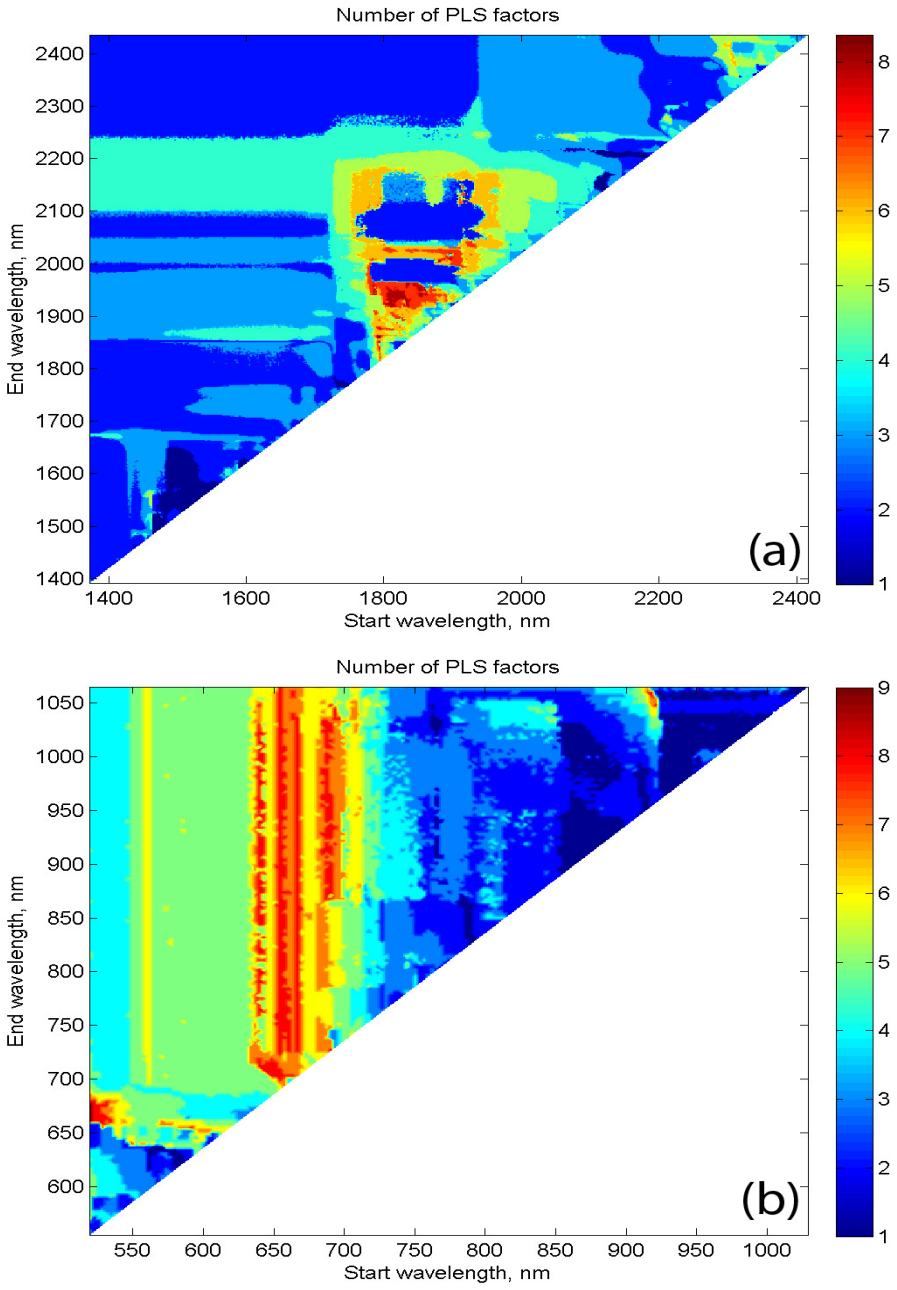

Figure I. Wavelength optimization of *Chlorella vulgaris* dry filtrate biomass spectral data, considering number of PLS factors (a) 1400-2400nm and (b) 550-1000nm.

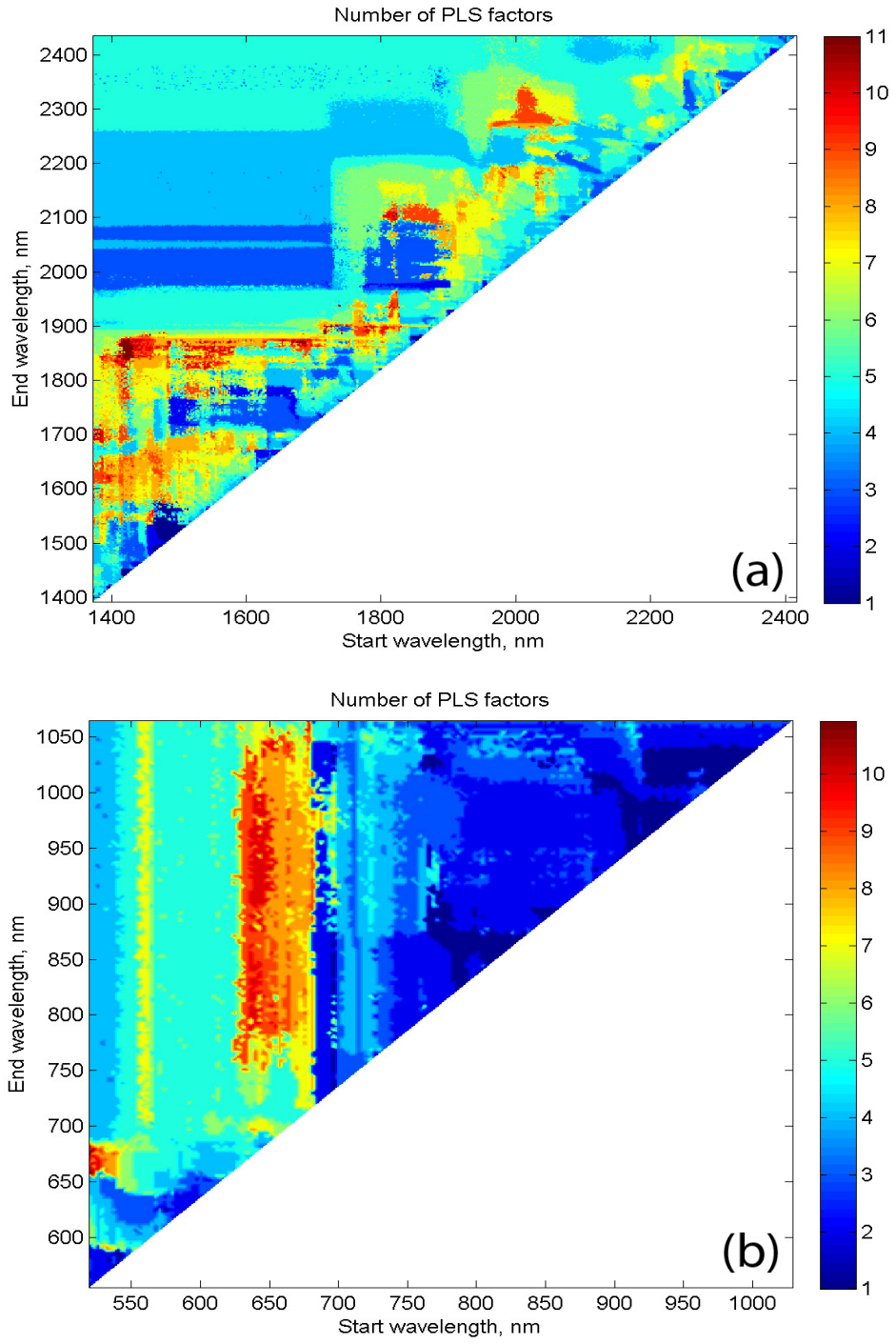

Figure II. Wavelength optimization of *Chlorella vulgaris* dry filtrate biomass spectral data, considering number of PLS factors (a) 1400-2400nm and (b) 550-1000nm.

Appendix F

Table I. NIR region (1100-2500): Summary statistics of PLSR calibration models developed of biomass on log (1/R) spectra and various spectral pre-processing methods of dry filtrates under nutrients limited growth conditions. Cross validation groups were based on replication flasks (n=5).

Growth Condition	Pre-processing method	Sample mean (g L^{-1})	Sample SD (g L^{-1})	Elements	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Control	ABS	1.30	0.85	66	4	**0.96**	0.23	3.62	0.00	0.94
	ABS to d2A				5	0.95	0.26	3.24	0.01	0.99
	ABS to MSC				3	**0.96**	0.23	3.74	0.01	0.95
	ABS to SNV				3	**0.96**	0.22	3.76	0.01	0.95
N limited	ABS	0.31	0.12	66	4	0.94	0.04	2.98	-0.01	0.92
	ABS to d2A				3	0.95	0.04	3.14	0.00	0.92
	ABS to MSC				4	**0.96**	0.03	3.78	0.00	0.95
	ABS to SNV				4	**0.96**	0.03	3.80	0.00	0.96
P limited	ABS	0.38	0.19	66	9	0.86	0.10	1.84	0.00	0.92
	ABS to d2A				6	**0.94**	0.06	2.97	0.00	0.95
	ABS to MSC				8	0.89	0.09	2.12	0.00	0.93
	ABS to SNV				8	0.90	0.09	2.18	0.00	0.94
Fe limited	ABS	0.39	0.19	66	4	0.96	0.05	3.75	-0.01	0.92
	ABS to d2A				4	0.94	0.05	2.92	0.00	0.90
	ABS to MSC				4	**0.97**	0.05	4.14	0.00	0.94
	ABS to SNV				4	**0.97**	0.05	4.15	0.00	0.94

Bold- the spectral preprocessing treatments supporting the highest Rcv.

Table II. Herschel region (300-1100): Summary statistics of PLSR calibration models developed of biomass on log (1/R) spectra and various spectral pre-processing methods of dry filtrates under nutrient limited growth trial. Cross validation groups were based on flaks (n=5).

Growth Condition	Pre-processing method	Sample mean (g L^{-1})	Sample SD (g L^{-1})	Elements	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
Control	ABS	1.26	0.82	64*	2	0.84	0.45	1.84	0.02	0.74
	ABS to d2A				2	0.71	0.58	1.45	0.05	0.53
	ABS to MSC				2	**0.86**	0.41	2.03	-0.01	0.76
	ABS to SNV				2	**0.86**	0.42	2.00	-0.01	0.75
N limited	ABS	0.31	0.12	64*	3	**0.94**	0.04	2.86	0.01	0.89
	ABS to d2A				3	0.79	0.07	1.64	0.00	0.69
	ABS to MSC				2	0.91	0.05	2.40	0.00	0.81
	ABS to SNV				2	0.91	0.05	2.46	0.00	0.82
P limited	ABS	0.39	0.19	64*	2	0.90	0.08	2.30	0.00	0.84
	ABS to d2A				2	0.85	0.10	1.96	0.00	0.76
	ABS to MSC				2	**0.97**	0.05	4.04	0.00	0.93
	ABS to SNV				2	**0.97**	0.05	4.25	0.00	0.93
Fe limited	ABS	0.39	0.19	64*	2	0.87	0.09	2.15	0.00	0.85
	ABS to d2A				3	0.81	0.11	1.72	-0.01	0.68
	ABS to MSC				1	**0.93**	0.07	2.71	0.00	0.85
	ABS to SNV				1	0.91	0.07	2.71	0.00	0.85

*Two spectra removed as outliers.
Bold- the spectral preprocessing treatments supporting the highest Rcv.

Table III. Summary statistics of PLSR calibration models of C. vulgaris biomass developed on log (1/R) spectra and various spectral pre-processing methods over the range of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet filtrates and replication flasks under nutrients limited growth trial. Cross validation groups based on replication flask (n=3).

Growth Condition	Wave Length (nm)	Sample	Pre-processing method	Sample mean (g L^{-1})	SD (g L^{-1})	Elements (n)	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
N limited	1100-2500	Wet	log 1/R (ABS)	0.31	0.12	65*	12	**0.96**	0.03	3.52	0.00	0.96
			ABS to d2A				6	0.94	0.04	3.03	0.00	0.92
			ABS to MSC				7	0.90	0.05	2.32	0.00	0.88
			ABS to SNV				5	0.87	0.06	2.07	-0.01	0.79
		Replications	log 1/R (ABS)	0.31	0.12	64**	4	**0.90**	0.05	2.35	0.00	0.82
Fe limited	300-1100	Replications	log 1/R (ABS)	0.39	0.19	64**	3	**0.91**	0.08	2.31	0.00	0.90
			ABS to d2A	0.39	0.19	65*	5	0.69	0.14	1.34	0.02	0.58
			ABS to MSC				2	0.79	0.12	1.65	0.00	0.66
			ABS to SNV				3	0.86	0.10	1.96	0.12	0.81

*One spectra removed as outliers.

**Two spectra removed as outliers.

Bold- the spectra supporting the highest Rcv.

Table IV. Summary statistics of PLSR calibration models of *Chlorella vulgaris* total lipid developed on log (1/R) spectra and various spectral pre-processing methods of NIR (1100-2500 nm) and Herschel regions (300-1100 nm) of wet filtrates and replication flasks under nutrients limited growth trial. Cross validation groups based on replication flask (n=3).

Growth Condition	Wave Length (nm)	Sample	Pre-processing method	Sample mean (g L^{-1})	SD (g L^{-1})	Elements (n)	PC	R_{CV}	RMSECV (g L^{-1})	SDR	Bias (g L^{-1})	Slope
N limited	1100-2500	Wet	log 1/R (ABS)	0.23	0.12	65*	12	**0.96**	0.03	3.73	0.00	0.95
			ABS to d2A				7	0.95	0.04	3.10	0.00	0.93
			ABS to MSC				7	0.89	0.05	2.21	0.00	0.88
			ABS to SNV				8	0.92	0.04	2.52	0.00	0.92
Fe limited	300-1100	Replications	log 1/R (ABS)	0.23	0.10	65*	1	**0.86**	0.05	1.92	0.00	0.76
			ABS to d2A				4	0.57	0.09	1.15	0.00	0.47
			ABS to MSC				3	0.79	0.07	1.54	0.00	0.76
			ABS to SNV				3	0.81	0.06	1.66	0.01	0.75

*One spectra removed as outliers.
Bold- the spectra supporting the highest Rcv.

Appendix G

Table I. Prediction of nutrient limited growth trial data using models for total lipid developed using nutrient sufficient growth trial data (based on *Chlorella vulgaris* data only). Models were based on log 1/R dry filtrates of NIR (1100-2500 nm) and Herschel regions (300-1100 nm).

Model	Predicted Spectra	Wave Length (nm)	Sample mean (g L^{-1})	Sample SD (g L^{-1})	RP	RMSECP (g L^{-1})	SDR	Bias (g L^{-1})	Slop
Single species	Control	1100-2500	0.25	0.09	0.57	0.23	0.42	0.14	1.25
	N Limited		0.23	0.11	0.82	0.56	0.20	0.35	3.79
	P Limited		0.27	0.16	0.58	0.28	0.59	-0.14	1.08
	Fe Limited		0.23	0.10	0.67	0.82	0.13	0.53	4.52
	Combined		0.23	0.12	0.48	0.19	0.66	-0.15	0.37
Combined species	Control	1100-2500	0.21	0.10	0.59	0.14	0.72	-0.11	0.56
	N Limited		0.23	0.11	0.77	0.13	0.86	-0.10	0.86
	P Limited		0.27	0.16	0.47	0.26	0.64	-0.21	0.14
	Fe Limited		0.22	0.10	0.49	0.20	0.53	-0.18	0.35
	Combined		0.23	0.12	0.36	0.19	0.64	0.03	0.56
Single species	Control	300-1100	0.21	0.10	**0.90**	0.15	0.65	-0.13	0.22
	N Limited		0.23	0.11	0.74	0.19	0.61	-0.16	0.18
	P Limited		0.27	0.16	0.83	0.24	0.68	-0.20	0.17
	Fe Limited		0.22	0.10	0.83	0.17	0.62	-0.14	0.19
	Combined		0.23	0.12	0.78	0.08	1.60	0.00	0.6
Combined species	Control	300-1100	0.22	0.09	0.75	0.17	0.57	0.03	1.72
	N Limited		0.23	0.11	0.82	0.19	0.58	-0.12	1.71
	P Limited		0.27	0.16	**0.85**	0.23	0.73	-0.20	1.12

Fe Limited	0.23	0.10	0.72	0.16	0.63	0.02	1.55
Combined	0.23	0.12	0.79	0.19	0.66	-0.16	0.18

Bold- values showing R_{CV}=>0.85.

Appendix H

Table I. Analysis of variance for Cell counts of *C. vulgaris*, *Navicula* sp. 1, *N. Pusilla* and *Navicula* sp. 2.

Variate: log_10_cell_mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
DAYS	11	215.323318	19.574847	7033.71	<.001
SPECIES	3	493.778520	164.592840	59142.10	<.001
DAYS.SPECIES	33	145.551062	4.410638	1584.85	<.001
Residual	192	0.534337	0.002783		
Total	239	855.187237			

Table II. Analysis of variance for Biomass (g L^{-1}) content of *C. vulgaris*, *Navicula* sp. 1, *N. Pusilla* and *Navicula* sp. 2 grown on standard nutrient media.

Variate: BIOMASS_content_g L^{-1}

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REPLICATION stratum	4	0.042403	0.010601	9.58	
REPLICATION.*Units* stratum					
DAYS	11	15.770290	1.433663	1296.10	<.001
SPECIES	3	86.693802	28.897934	26125.13	<.001
DAYS.SPECIES	33	39.823933	1.206786	1090.99	<.001
Residual	188	0.207953	0.001106		
Total	239	142.538382			

Table III. Analysis of variance for lipid content (g L^{-1}) of *C. vulgaris*, *Navicula* sp. 1, *N. Pusilla* and *Navicula* sp. 2 grown on standard nutrient media.

Variate: LIPID_content_g L^{-1}

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REPLICATION stratum	4	0.0023959	0.0005990	1.31	
REPLICATION.*Units* stratum					
DAYS	11	0.3213186	0.0292108	63.82	<.001
SPECIES	3	1.3904222	0.4634741	1012.60	<.001
DAYS.SPECIES	33	0.6077656	0.0184171	40.24	<.001
Residual	188	0.0860489	0.0004577		
Total	239	2.4079512			

Table IV. Analysis of variance for biomass content (g L^{-1}) of *C. vulgaris*, *Navicula* sp. 1, *N. Pusilla* and *Navicula* sp. 2 grown on reduced concentrations of N, P and Fe and standard ASM media (control).

Variate: BIOMASS_content_g L^{-1}

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REPLICATIONS stratum	2	0.0188364	0.0094182	13.42	
REPLICATIONS.*Units* stratum					
DAYS	10	14.7034015	1.4703402	2094.79	<.001
TREATMENT	3	21.9060515	7.3020172	10403.18	<.001
DAYS.TREATMENT	30	11.3554652	0.3785155	539.27	<.001
Residual	86	0.0603636	0.0007019		
Total	131	48.0441182			

Table V. Analysis of variance for lipid content (g L^{-1}) of *C. vulgaris*, *Navicula* sp. 1, *N. Pusilla* and *Navicula* sp. 2 grown on reduced concentrations of N, P and Fe and standard ASM media (control).

Variate: LIPID_content_g L^{-1}

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
REPLICATIONS stratum	2	0.0030022	0.0015011	5.56	
REPLICATIONS.*Units* stratum					
DAYS	10	1.6730716	0.1673072	619.73	<.001
TREATMENT	3	0.0771812	0.0257271	95.30	<.001
DAYS.TREATMENT	30	0.2575654	0.0085855	31.80	<.001
Residual	86	0.0232173	0.0002700		
Total	131	2.0340376			

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