Integrating heterotrophic microalgae as a feedstock into the Brazilian biodiesel industry: A whole systems analysis

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The candidate confirms that the work submitted is her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The following jointly authored publications are part of the thesis:

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Details of contributions from the candidate and co-authors are listed below:

1. The candidate conducted the fieldwork and wrote the journal article, with comments and guidance from her supervisors Dr. Lea-Langton, Dr. Ross, Dr. Camargo Valero and Prof. Gale.
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Abstract

Biodiesel is a renewable transport fuel produced largely from terrestrial oil seed crops which, if used as an alternative to fossil diesel, can reduce our dependency on fossil fuels. Brazil is one of the largest biodiesel producers in the world, yet cultivation of the predominant feedstock, soybean, puts pressure on highly biodiverse ecosystems as well as threatening land ownership and access. In order to improve the environmental, social and economic sustainability of biodiesel production in Brazil, new feedstocks are being investigated.

The successes and weaknesses of the Brazilian “Programme for Biodiesel Production and Use” were analysed and the opportunity to introduce a new, potentially more sustainable feedstock was identified. Heterotrophic microalgae were investigated as an alternative feedstock, due to suggested benefits over other feedstocks such as high growth rates and lipid yields, potentially reducing production costs and energy inputs. To investigate the feasibility of supplying nutrients from different waste streams, the microalga *Chlorella vulgaris* was cultivated in a synthetic wastewater medium with addition of an organic carbon feedstock, either pure glucose, molasses from the sugar industry or crude glycerol from the biodiesel industry. The harvested biomass was converted to biodiesel by transesterification of hexane extracted lipids or by in situ transesterification to investigate the difference in yields. The properties of the biodiesel were then analysed to assess its quality. The life cycle energy use and greenhouse gas emissions were calculated and compared with autotrophic microalgae, followed by a whole systems analysis to identify risks and challenges to integrating heterotrophic microalgae into the biodiesel industry in Brazil.

The analysis found that the biodiesel programme in Brazil has made compromises to allow family farmers to contribute to the feedstock matrix, and the programme would face sustainability challenges if it were scaled up. Therefore a sustainable alternative feedstock would be required to provide for an increase in feedstock demand. Heterotrophic microalgae were selected as they may be capable of introducing additional social benefits, particularly associated with improving sanitation and waste management.

Heterotrophic cultivation growth trials demonstrated that biomass densities of up to 3 g l^{-1} d^{-1}, with a lipid content of 48% could be achieved where crude glycerol was the organic carbon source. The fatty acid methyl ester composition of the transesterified lipids and other fuel characteristics were determined using correlations based on the FAME composition, including a new technique for predicting cetane number. The results suggest that in situ transesterification can lead to higher biodiesel yields than extraction and transesterification, and that the algal biodiesel quality from either technique was comparable with soybean biodiesel. The rate and quality of the oil produced is significant as there is potential to integrate this oil into the existing blend as an economical product.

The energy ratio calculated for heterotrophic microalgae showed a potentially positive balance could be achieved when waste nutrients were utilised. This was compared to autotrophic microalgae feedstock, and found advantages for the heterotrophic systems due to lower energy and water requirements during cultivation. The opportunities and risks of integrating microalgae into the existing system for biodiesel production in Brazil, identified by the whole system analysis, determined that the existing infrastructure could be utilised, but highlighted the role of policy decisions and investor confidence in stimulating further development and potential deployment of microalgal feedstocks for biodiesel. However, the barriers to future development are significant and the gap between research and commercialisation must be bridged by working at the interface of different disciplines, in order to produce a truly sustainable biodiesel feedstock.

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Nomenclature

ANP	National Petroleum, Natural Gas and Biofuel Agency (Brazil)
BNDES	National Bank for Economic and Social Development (Brazil)
BOD	Biological oxygen demand
CAPEX	Capital expenditure
CCS	Carbon capture and storage
CFPP	Cold filter plugging point
CH_4	Methane
CN	Cetane number
CNPE	National Council for Energy Policy (Brazil)
CO_2	Carbon dioxide
CO_2eq	Carbon dioxide equivalent
COD	Chemical oxygen demand
CP	Cloud point
CV	Calorific value
DMSP	Dimethylsulfoniopropionate
EIA	Energy Information Administration (United States)
ETS	Emissions trading scheme (Europe)
EU	European Union
FAME	Fatty acid methyl ester
FAPSEP	Foundation for Support and Research in the State of São Paulo (Brazil)
GCMS	Gas chromatography - mass spectrometry
GDP	Gross domestic product
GHG	Greenhouse gas
GWP	Global warming potential
HBM	Heterotrophic basal media
HBMC	Heterotrophic basal media with crude glycerol
HBMG	Heterotrophic basal media with glucose
HBMM	Heterotrophic basal media with molasses
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ID	Indirect transesterification
IEA	International Energy Agency
IS	In situ transesterification
ISO	International Standardisation Organisation
LCA	Lifecycle assessment
LCI	Lifecycle inventory
LEA	Lipid extracted algae

LTFT	Low temperature filterability
MDA	Ministry of Agrarian Development (Brazil)
MTBE	Methyl tert-butyl ether
N_2O	Nitrous oxide
NDIR	Non dispersive infrared gas adapter
OECD	Organisation for Economic Co-operation and Development
OPEX	Operations expenditure
PBR	Photobioreactor
PNPB	National Program for Production and Use of Biodiesel (Brazil)
PESTEL	Political, economic, social, technological, environmental and legal
PP	Pour point
PRONAF	National Program to Strengthen Family-run Agriculture (Brazil)
PTFE	Polytetrafluoroethylene
RED	Renewable Energy Directive (EU)
RI	Refractive index
SEC	Size exclusion chromatography
SFS	Social Fuel Seal
SoI	System of Interest
SOA	Secondary organic aerosols
SWW	Synthetic wastewater
SWWC	Synthetic wastewater with crude glycerol
SWWG	Synthetic wastewater with glucose
SWWM	Synthetic wastewater with molasses
TE	Transesterification
TGA	Thermogravimetric analysis
TOC	Total organic carbon
TSS	Total suspended solids
US DOE	United States Department for Energy
USA	United States of America
USD	United States dollars
UV-VI	Ultraviolet visible
VOC	Volatile organic compound
WSoI	Wider System of Interest

Chapter 1 Research Motivation and Aims

1.1 Introduction

Access to affordable energy is considered a necessity in modern day life for billions of people across the globe. Fossil fuels include liquid fuels such as oil, gas fuels and solid fuels such as coal which are ideal for producing energy due to a high energy density and a range of products that can be produced via fractionation and refining. However, the continuation of provision is facing a challenge in terms of delivering sustainable, secure and affordable energy, described by the diagram in Figure 1.1. These challenges for future energy supply include:

- Technological provision of fuels, as wells are driven deeper and in more hostile environments.
- Geopolitical stability as supplies become more scarce, with protectionist policies covering the short, medium and long term
- Financial uncertainties as prices rise along with diminishing supply.
- Environmental concerns as oil exploration delves deeper into untouched lands, and emissions produced from the burning of fossil fuels cause changes to the climate systems.

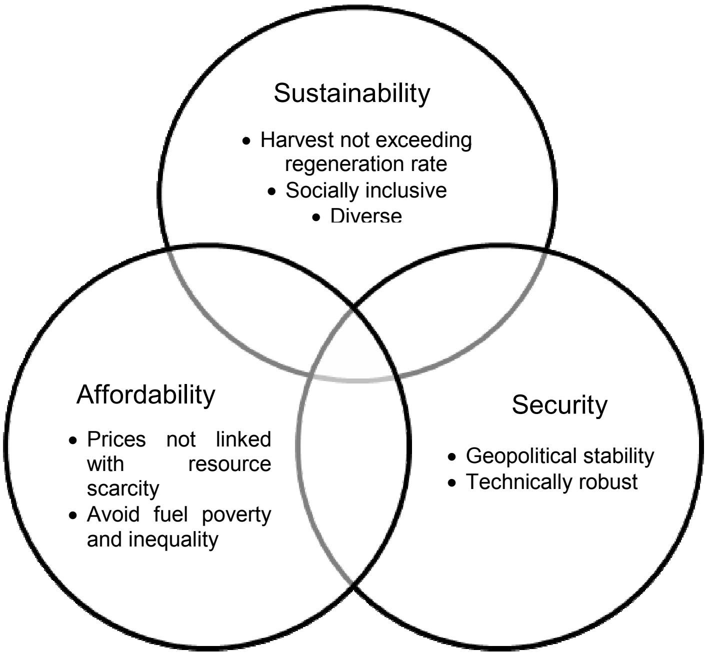


Figure 1.1 The "trilemma" facing the future of energy provision

The challenge for finding an alternative to petroleum is not limited to the production of fuel. A replacement is required for the “whole barrel” provided by oil. This means replacing not only fuel products, but also the raw materials for production of oil dependant products including solvents and plastics. Biomass is the only renewable energy source that will be able to address this in the near term due to compatibility with existing infrastructure [1]. As such, research into biomass for energy and bioproducts is key on the agenda of any government looking to address the future energy provision.

In 2010, 29.6 petawatt hours of liquid fuel were used for transportation internationally and of this, 32% of was diesel [2]. The demand for transportation is expected to continue to rise particularly in non-“Organisation for Economic Co-operation and Development” (OECD) countries. Diesel consumption is also expected to rise until 2050, despite rises in fuel economy and introduction of alternative fuel vehicles described by the roadmap produced by the International Energy Agency in 2011. Over the past 100 years, biodiesel has been introduced to begin to tackle some of the issues mentioned above relating to fossil fuel supply. The first diesel engine was patented by Rudolf Diesel in 1894, and although designed to run using coal dust, it could also be run using peanut (arachide) oil [3]. The supply of cheap fossil oil however, led to the development of an industry based on fossil diesel. Until today, biodiesel still only comprises 2% of the total transport energy mix [4] and in 2011, 44% was produced in Europe, 16% in the USA and 11% in Brazil [5].

The demand for biodiesel in Brazil reached 2.72 billion litres in 2012, although production capacity exceeds this significantly with only 35% of capacity currently in use [6]. Feedstocks consist of predominantly soybean (approximately 73%), although palm, cotton, castor, babassu, sunflower and animal fat are also used. The area of land harvested for soybeans was 27.2 million hectares in 2012/13, 8% (by weight) of which went towards oil production [7].

This scale of production inevitably impacts on the environment. Biofuels are thought to reduce some of the environmental impacts associated with fossil fuels for example lower greenhouse gas (GHG) emissions due to cleaner combustion because of oxygenated compounds in biofuels that improve combustion efficiency [8]. However, the cultivation of crops for biofuels have been criticised for being as energy intensive as fossil fuel extraction in some cases. For example, 180m tonnes of fertilisers are estimated to be needed between 2012-2016 [9] to sustain large scale production of biofuels, and the raw materials for the fertilisers require mining, processing and transportation. Water and energy are also required for the cultivation of crops, and the energy requirement is often met by using fossil energy. Pesticides used to maintain pest-free crops are found in waterways causing secondary effects to wildlife and are present in the atmosphere due to either aerial application or volatilisation from the soil. Soil erosion and emissions from soil are also of concern when cultivation is carried out on a large scale [10]. Soil erosion leads to the loss of nutrients from the soil, leading to higher requirements for fertilisers and often a lower yield. Emissions from soil can include N_2O, a potent GHG and nitrous oxides (NO_x) which can lead to health problems [11].

Alternative feedstocks that are high yielding, but do not pose threats to sustainability are needed. Options for alternative feedstocks have included high oil yielding, inedible crops such as castor and jatropha [12,13]. However, the yields still do not reach the proportions needed for large scale fuel production. Microalgae have been suggested as an alternative biodiesel feedstock that could overcome some of these issues [14]. This is due to the benefits it could deliver in terms of potential for low cost and low-tech production through high yields and the fact that the fuel quality is similar to that of other vegetable oils and therefore there is the ability to use existing infrastructure. Algae can be grown in environments previously rendered naturally unsuitable for crop growth, such as saline environments, which will reduce the demand for freshwater or marginal land with poor quality soil [1,15]. There is also potential for high value materials to be extracted from the algae [16], making it more cost effective. (e.g. [17,18,19,20,21]). However there are still many technological and financial barriers to commercialisation including strain selection, resource provision for cultivation and harvest and processing techniques.

Brazil has become the focus of this thesis due to the experience and scale of the biodiesel industry, the existing diversity of feedstock for production and the social programme for biodiesel production. There are studies that have suggested microalgae could be scaled up to produce significant quantities of biodiesel oil [21,22,23,24,25]. The Brazilian programme for biodiesel production and use offers a unique opportunity for small scale producers to supply an industry, which under normal market conditions would not occur, and as a consequence has the potential to deliver social benefits to many families, from which lessons can be learnt and applied in other countries. Brazil is also in a good geographical position to develop microalgae as a fuel source because of its location meaning it has a suitable temperature year round, water is readily available in many parts of the country, and it has a large land area suitable for cultivation including a long coast line that would potentially be useful for cultivation of saltwater microalgae strains.

In recent years there has been a surge in research activity for microalgal feedstocks, with these tending to focus on autotrophic cultivation of microalgae that is photosynthetic growth requiring sunlight and a source of CO_2. This approach has met a number of barriers including adequate penetration of light through cultures to ensure growth and in general low yields that are potentially uneconomical to harvest. An alternative cultivation system is to grow microalgae in the absence of light by providing an organic carbon feedstock as the energy source, also known as heterotrophic cultivation (discussed in detail in Chapter 5). Further advantages of heterotrophic systems over autotrophic systems are discussed in Chapters 2 and 7. The production of an energy balance, focussing on the heterotrophic system will increase the scope of the sustainability assessment and comparison. Assessment of this cultivation technique is novel, and will contribute a new insight into the benefits this system has in terms of energy requirements and GHG emissions.

1.2 Research scope, aims and objectives

The research presented in this thesis approaches one area of this complex problem, the provision of sustainable biodiesel. Biodiesel is a liquid fuel, produced from organic materials. It differs in structure from fossil diesel due to the presence of an ester functional group. This leads to slightly different properties, for example biodiesel can have a higher boiling point and cetane number but lower calorific value than fossil diesel. However, it is suitable for use in a conventional diesel engine without need for engine modification, thus providing a technically feasible alternative without the need for new infrastructure. Microalgae cultivated heterotrophically (that is in the absence of light with an organic carbon feedstock) has been selected as the feedstock under investigation as it is a promising technique for growing algae at high yields with good oil content, without the requirement to provide light and therefore allowing dense cultivation [20,26,27].

The aim of this thesis is to assess the feasibility for the addition of microalgal biodiesel as a sustainable feedstock for biodiesel production industry in Brazil, with regards to the social programme for biodiesel production and the environment. Despite an increasing range of literature on the subject of microalgal biomass, heterotrophic microalgae have seen relatively little interest in comparison with autotrophic microalgae (i.e. photosynthetic algal species) to date and there remains a need to assess the opportunities and barriers to integrating microalgae into an existing system. This requires investigation of governmental legislation, frameworks and political will, capacity for production and technological feasibility. The environmental, social and economic impacts of changing the current system also need reviewing in order to make a whole system assessment of the sustainability of microalgae as a biodiesel feedstock.

In order to achieve this aim this thesis will determine what already exists in terms of frameworks and legislation for biodiesel production, and assess the pros and cons of the existing programme for biodiesel production and use. This will allow us to identify the opportunities for including heterotrophic microalgae as a feedstock, by considering the technical aspect of biomass production such as nutrient provision, the quality of fuel that is expected by Brazilian standards, the environmental costs of proceeding and the stakeholders who will be impacted by a change.

The objectives have been defined below to allow the above aims to be achieved.

- The Brazilian programme for biodiesel production and use has been implemented for the past 7 years. The success of the existing programme for biodiesel production and use will be analysed using existing academic and commercial literature by considering how fit for purpose each stage of the production process is, using a systematic scoring model. This will allow a broad comparison of impacts on people, technological suitability of the fuel, economic viability of the programme, political credibility and environmental sustainability. The analysis will then go on to consider the introduction of microalgae as an alternative feedstock for biodiesel, using the same model to allow for comparison between the different systems.
- The productivity of cultivating microalgae heterotrophically using different carbon feedstocks and waste water will be investigated using experimental cultivation trials. In order for microalgae to be a sustainable feedstock, it should be able to utilise waste resources, so as not to require use of fertilisers, which require high energy input for production. A good quality, high oil yielding crop is also required, therefore this will be the metric used to determine the suitability of this stage for biodiesel production.

Considering the best way to convert the biomass to biodiesel is crucial to ensure a positive energy balance is achieved. The positive energy balance is indicated by a higher output when combusting the fuel (measured using its calorific value) compared with the energy that has been used in its production. An in situ transesterification method will be investigated in order to reduce requirements for solvent extraction, a step which would increase financial and energetic costs and environmental toxicity. The characteristics of the fuel will also be compared with other biodiesel from terrestrial crops to indicate the suitability of microalgal biodiesel for inclusion in the fuel matrix.

- The energy balance will be quantified to ensure the above steps have provided an efficient production process. The opportunity for microalgae to be incorporated in the fuel matrix is in part dependant on a positive energy balance, and as such requires all the energy inputs onto the production process to be quantified. The GHG emissions during the process will also be quantified to provide a metric for one of the environmental impacts of the production process. This will be done using GHG emission factors for existing processes found in the literature and from industry.
- In order to provide an assessment of how well microalgae could fit into the existing system for biodiesel production in Brazil, a whole system perspective is sought. This will be achieved through using the information gathered in this thesis as well as a systematic search of the literature to provide an evidence based evaluation of the suitability of microalgae as a sustainable biodiesel feedstock. This will include areas for future work, to improve upon this assessment and provide lessons for other locations.

1.3 Thesis structure

The introductory chapters 2 and 3 will present a review of the existing literature, and an introduction of the experimental techniques employed. An analysis of the Brazilian biodiesel industry will be made initially in Chapter 4, considering the stakeholders within the industry and looking at the drivers, followed by hypothesising the inclusion of microalgae as an additional biodiesel feedstock.

Following on from this work, the environmental and technical suitability of microalgae as a biodiesel feedstock will be investigated further using lab scale experimental work. The growth dynamics of heterotrophic microalgae will be monitored where different carbon feedstocks are added to a basal media and a synthetic wastewater medium in Chapter 5. This work explores the use of wastewater for heterotrophic cultivation of microalgae, which is an emerging area of research interest [26-33]. The resulting biomass will be converted to biodiesel using the transesterification method, and the fuel characteristics tested, the results of which are presented in Chapter 6. Using data obtained from this work, in Chapter 7 a life cycle assessment of the energy, mass and GHGs from the whole process will be calculated, and different scenarios will be tested to define energy and GHG hotspots. A critical and in depth review of the literature about the potential environmental impacts of large scale microalgae cultivation will explore other concerns that also will need to be faced before commercialisation of heterotrophic microalgal fuel. A discussion of finding and opportunities for further work are included in Chapter 8. Where applicable, references to work published by the author are included in the introductory comments and a list of references used in this thesis is provided at the end, followed by Appendices containing supplementary information.

Chapter 2 Introduction to Biodiesel

Governments around the world are already legislating for the inclusion of biofuels within the transport fuel sector. By 2020, EU policy requires 10% of road transport fuel to be from renewable sources. Brazil also has mandates in place for biofuel inclusion in its fuel mix and has become one of the largest producers of bioethanol and biodiesel. There has been a requirement for 25% ethanol in gasoline blends since 1st May 2013 [28] and 5% biodiesel in diesel blends since the beginning of 2013, 8 years after the social programme for biodiesel production was brought into law [29] (this programme is discussed in detail in Chapter 4). The USA included 34bn litres of renewable fuel, equivalent to 5.5%, in their gasoline blend in 2012, and have targets to increase this to 164bn litres by 2022 [30]. By 2050, the International Energy Agency (IEA) estimates 20% of liquid fuels will come from biofuels globally [31]. This level of demand for biofuels places enormous stress on biofuel producers in terms of land availability and resources for cultivation of feedstock crops. A source of biofuel is sought, which may relieve some of these pressures.

2.1 Drivers for biodiesel production and use

2.1.1 Energy security

There is a global increase in the demand for energy, yet supplies of energy from gas and oil resources is becoming increasingly restricted by physical, economic and political factors [32]. Access to energy relies on a complex system of global markets, cross border infrastructure networks and a small group of energy suppliers leading to vulnerability of nations that do not have their own supplies [33]. Concerns about energy security are fuelled by volatile prices, increasing demand, terrorism and the threat of natural disasters [34].

For global energy security to be achieved, the system of energy supply needs to overcome the following factors [35]:

- Volume of demand for fossil fuel energy needs to be reduced via improved efficiency of production, transportation and utilisation of energy.

- Management of external shocks to supply, including political unrest, terrorism and price fluctuations. Emergency preparedness for these shocks needs to be developed.
- Diversification of the energy mix and supply sources, leading to eventual self-sufficiency of supply and flexibility in the market to allow some shocks to be absorbed.

2.1.2 Economic drivers

Increasing the portion of biodiesel in the energy supply will lead to economic sustainability, represented in a number of ways for example, security of supply through diversification of sources, job creation in rural areas, technical jobs in manufacturing and creation of office based work in marketing, sales etc.. This will lead to an increase in income taxes, and depending on the tax framework, could lead to income on fuel taxes. However, biofuels tend to be exempt from these levies to encourage uptake, therefore the impact could be limited in this case.

Direct effects from biodiesel production include expenditure on feedstock oils (in particular soybean oil), utilities and labour. The effect of increasing biodiesel demand will circulate throughout the economy, creating jobs in sectors such as oil refining, utilities etc., supporting job creation therefore increasing household income and tax revenue. In 2007 $1.4 billion was spent by the global biodiesel industry on goods and services. Of this, feedstocks accounted for 83% of production costs. Indirect impacts from biofuel production were both local and national, and were estimated to add $4.1billion to gross domestic product (GDP) globally [36]. Biofuels are certainly being used as an opportunity for economic development, and between 2005 and 2010 the biofuel market has increased by 40%. Other benefits include agricultural development. With the development of second generation biofuels (described in section 2.3.1), previously degraded land can be used leading to an increased output from land.

2.1.3 Environmental drivers

Biodiesel is expected to provide an environmentally friendly fuel in the place of fossil diesel. Benefits of biodiesel include GHG reductions, air pollution reduction, biodegradability, carbon sequestration during growth and improved land and water use compared with fossil fuel extraction and processing [8]. Using biodiesel can lead to improvement in air quality due to a reduction in many emissions such as unburnt hydrocarbons because of the combustion efficiency and also lower sulphates, carbon monoxide, aromatics, nitrated compounds and particulate matter. However, NO_x emissions rise as the concentration of biodiesel in any biodiesel blend increases [37].

GHG emissions from biodiesel are the subject of much research and quantification largely through lifecycle assessment studies. The gases of main interest are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). CO_2 emissions receive the most attention and are released throughout the production chain of biodiesel, but are also sequestered during the growth phase of a photosynthetic feedstock with the aim of sequestering the same amount of CO_2 as is released during biodiesel production to provide a carbon neutral resource [38]. In addition, CH_4 can be a result of the decomposition of organic matter and N_2O is released in farming activities depending on the soil management and fertilisers used [39].

2.1.4 Oil supply and demand

Liquid fuels are a preferred fuel for transport because of the high energy content per volume. This allows the fuel source to be carried and consumed without the vehicle having to stop to refuel too often.

The global production of oil in 2013 was 86.8 million litres per day, compared with a consumption rate of 91.3 million litres per day. The developing world accounted for 80% of energy demand growth in 2013 and 51% of oil consumption now occurs outside of the OECD [40]. The area of highest production globally is in the Middle East, followed by Europe and Eurasia, although south and central America have seen the highest rise in oil production and have the highest production to consumption ratio of any region. Asia Pacific and Europe are all net importers of oil, consuming more than they produce [41]. This has economic and political consequences for countries supplying and depending on oil.

The price of oil is intrinsic in determining how economically competitive alternative fuel sources are [42]. Price fluctuations lead to changes in prices of diesel and biodiesel on a daily basis. The prices are also determined by internal taxing and subsidy policies. This is obvious in the comparison in Table 2.1. In the USA, biodiesel is more expensive than fossil diesel, regardless of the concentration of the blend and even when the energetic content of the biodiesel is taken into account (i.e. the energy content of biodiesel is lower, leading to a price of US$1.04 per litre of biodiesel B100 for the same energetic output in April 2014). In Brazil and the UK, this cost is absorbed into the price of the blended fuel, Brazil having a significantly lower price for diesel or biodiesel than in the USA, but the UK having a much higher cost. This demonstrates the influence of political intervention on pricing, as diesel in Brazil is heavily subsidised for its use in freight in order to keep inflation low, whereas in the UK diesel is heavily taxed.

Table 2.1 Fuel costs in the USA, UK and Brazil, comparing changes in prices since the beginning of 2014. Diesel in UK and Brazil has a mandated 5% biodiesel content, incorporated in the price [43,44,45]

	Fuel price (US$/ litre)
USA	**April 2014**
Diesel	$0.87
Biodiesel (B99-B100)	$0.93
UK	
Diesel (B5)	$2.31
Brazil	
Diesel (B5)	$0.66

2.1.5 Future of the transport fleet

As transport has become more affordable, demand has risen across most forms of transport. A shift to new and improved technology which results in lower carbon consumption will be key in meeting the future targets for emission reduction, which are set on a national and international basis, for example the Kyoto protocol. Both private and government sectors will play a major role in research and development, and close collaboration between these sectors will stimulate the development of low carbon technologies and reduce costs. The Stern report [46] suggests incentives for low carbon technologies should increase by up to $150 billion globally, up from $33 billion currently. According to the report, $20 billion of this should be invested in R&D for low carbon energy supply. However, a lack of certainty over the future pricing of the carbon externality will reduce the incentive to innovate.

Brazil already has a relatively low carbon intensity transport sector compared with other countries owing to the extent of ethanol use in cars. In 2009, Dilma Rouseff proposed to reduce carbon emissions by 38-42% by 2020 compared with 2005 levels, although this pledge is voluntary. The expanding biodiesel sector will also have a role given that the transport sector accounts for 43% of carbon emissions from fossil fuel use in Brazil [47] and 12% of total emissions [48]. Brazil is exemplary when it comes to using ethanol in its transport fuel, where 22% of the vehicular fuel is ethanol. However, when looking at the Brazilian vehicular fuel matrix, diesel outweighs all other vehicular fuel sources. Over 50% of the fuel is diesel as the majority of freight within the country is executed by road transport. Trucks cover vast distances in order to move produce and goods.

2.2 Legislative framework for biofuels

2.2.1 Current legislative framework for biofuels (EU and USA)

Legislation for production of biofuels varies geographically, and has implications for international trade. For example, the EU has developed an environmental sustainability criterion in article 17 of the Renewable Energy Directive. Countries outside of the EU wishing to trade with EU member states must comply. The criteria are fivefold, as stated in Figure 2.1. The lack of environmental sustainability criteria in the Brazilian National Program for Production and Use of Biodiesel (PNPB in Portuguese), which is analysed further in Chapter 4, may lead to repercussions if Brazil wishes to export to European markets.

The USA has a complex system for biofuel use, which varies from state to state. In 2005, the Energy Policy Act included the first Renewable Fuels Standard, which required 34 billion litres of biofuel by 2012. This was met by 2008 due to the replacement of the petrol additive MTBE with ethanol. The second Renewable Fuel Standard raised the required volume to 164 billion litres [49]. Large subsidies under the Volumetric Excise Tax Credits scheme, which are linked to consumption other than oil prices, has helped make biofuels economical in the market price.

1. *GHG savings must be 35%, this target will rise to 50% in 2017*
2. *Crops for biofuel shall not be from land that is "highly biodiverse", that is primary forest, designated nature protection areas or highly biodiverse grassland.*
3. *Crops for biofuel shall not be from area considered to have a high carbon stock, which is not continuously forested area, with 10-30% canopy cover or wetlands.*
4. *Crops for biofuels cannot be grown on land that was peat land, unless there is evidence that the land was previously undrained.*
5. *The materials cultivated and used for production must be done so in a way that meets standards and provisions in the common rules for direct support for farmers and under minimum requirements for good agricultural and environmental conditions under the common agricultural policy.*

Figure 2.1 Sustainability criteria set out under the European Renewable Energy Directive [50,51]

2.2.2 History of biofuel in Brazil

The Brazilian biofuels programme, PROALCOOL, was launched in 1975 following the global oil crisis. The scheme provided public sector subsidies and tax breaks which help farmers to plant more sugar cane and promoted the construction of more distilleries. The automobile industry was also part of the plan, designing flexi-fuel cars that could run on ethanol blends, and today they can run on both pure ethanol and ethanol blended with petrol. Fuel distributors such as Petrobras were involved in the policy as well as the Ministry for Science and Technology, the Ministry of Mines and Energy, the Ministry of Agriculture, the Ministry of Industry and commerce, the Ministry of Finance and Planning and the Ministry of the Environment [52]. It is still the most successful biofuels programme in the world, and has ensured all gasoline contains 20-25% bioethanol, compared with the 10% target in the EU for 2020.

The Brazilian government has taken a novel approach in relation to its biodiesel industry by using biodiesel as a tool for social development and environmental protection as well as an opportunity for fuel security, technology development and economic growth. This opportunity was incorporated into policy by President Lula, through the PNPB and a subsequent range of supporting laws and institutes [53]. Since then, the production of biodiesel has risen to 2.7bn litres per year in 2013.

2.3 Biodiesel sources and technologies

Biodiesel offers many advantages over diesel oils as a fuel. It has a higher combustion efficiency than diesel-oil due to it being more oxygenated [8], for example as mentioned above in section 2.1.3 reduction in many emissions.

Biodiesel can be used in a conventional engine without any major modifications, and can easily be blended with fossil diesel. It also has liquid nature portability, meaning it can be used within the existing infrastructure. Biodiesel can also have a good energy balance due to simplicity in the manufacturing process.

2.3.1 Feedstocks

In order for a feedstock to be considered, it must consist of triglycerides. The feedstocks that can be used for biodiesel are diverse, and consequently the GHG savings depend on the feedstocks chosen. Most feedstocks have relative advantages and disadvantages. They can be divided into the following categories: virgin oil feedstocks (edible and non-edible), waste vegetable oil, animal fats, algae and other halophytes.

First generation biofuels are those that have been derived from sources such as sugar, starch, animal fats or vegetable oil [54]. Biodiesel production via transesterification uses first generation feedstock (i.e. vegetable or animal oils and fats). The processes used are discussed in more detail below in sections 2.3.2 and 3.2.

Second generation technologies for biodiesel production can be produced via thermochemical reactions, gasification (normally to produce a syngas for further processing), pyrolysis, torrefaction or biochemical routes using a pre-treatment to separate out the lignin, cellulose and hemicelluloses found in biomass. For example, bio-dimethylester (bio-DME) can be produced from synthesis gas, a process still under development, or from catalytic dehydration where water is separated from methanol using chemicals. Bio-DME can replace diesel in conventional engines with minor modifications, but cannot be blended and has a lower energy content per volume than diesel. Fischer–Tropsh also uses a syngas to produce a diesel that can be mixed directly with fossil diesel. Synthetic kerosene can also be produced. The benefit of using a second generation biofuel is that the feedstock is generally not in competition with food crops as it can be produced from lignocellulosic or woody material such as stalks, agricultural residues or waste [54]. However, none of these processes are reproducible at an economic scale yet.

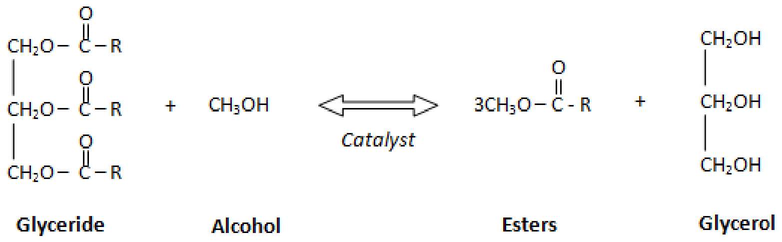
Third generation biofuels consider the use of micro-algae. This is not a process available at an economic scale yet as discussed below in section 2.3.3, but there is potential for the yield of oil from the micro-algae to be 15-300 times higher than first or second generation biofuels.

Feedstocks tend to be the largest cost involved in biodiesel production [55]. If a method that would reduce the feedstock cost could be developed, this would lead to the production of a fuel that would compete with oil prices. Whilst producing biodiesel from different feedstocks is an important aspect of environmental protection, fuel security and social involvement, this does result in technical issues with quality control.

2.3.2 Biodiesel production

The most common process to produce biodiesel, operated commercially worldwide, is transesterification using a base catalyst. This requires low temperatures and pressures and can give a 99% conversion yield under optimised conditions [56]. The transesterification process involves the reaction between a triglyceride (which is a fat or oil) and an alcohol (such as methanol or ethanol) in the presence of a catalyst to form esters and glycerol, shown in Equation 2.1. Prior to the transesterification process, the catalyst is dissolved into the alcohol using an agitator. Once dissolved the crude oil is added to the catalyst/methanol slurry in a closed system vessel in order to prevent evaporation of the alcohol. The reaction takes place at the alcohol's boiling point for an efficient reaction speed, and must use excess alcohol to ensure complete conversion as the reaction is reversible.

Equation 2.1 Transesterification reaction



The oil is composed of triglyceride molecules, which are made up of a glycerine molecule attached to three fatty acids. Catalysts are typically strong alkalis such as sodium or potassium hydroxide, sodium methoxide and sodium ethoxide. The type of catalyst depends on the manufacturer, because there are relative advantages of using either. Base catalysts result in a faster reaction than acid catalysts. However, they are also more selective with regards to the types of lipids to be transesterified, in particular with regards to free fatty acids IFFA) [57]. Base catalysts can cause a number of issues where microalgae are used as a feedstock for biodiesel production due to a generally high content of free fatty acids. FFA's can saponify in the presence of an alkaline catalyst, leading to difficulties in biodiesel purification [58]. Therefore, the majority of techniques cited in the literature use acidic catalysts for transesterification of oils with high FFA (e.g. [58,59,60]). Inorganic acids such as H_2SO_4 can also be less expensive, so can bring down the costs of production. A further reason for using acid catalysts for transesterification of microalgae derived lipids is that higher yields have been observed due to the role an acidic environment plays in extraction of the lipids. The vesicles that store triacylglycerol in the form of oil (known as spherosomes or oleosomes) are more labile in acidic conditions [61] and therefore the lipid is easier and quicker to extract.

Water and FFA levels are monitored in the feedstock during industrial transesterification processes as they lead to soap formation, and make separation of the glycerol from the ester more difficult downstream. Following the reaction, the mixture is allowed to settle, and then the denser glycerol layer is drawn off from the bottom. The oil is then washed with warm water to remove un-reacted alcohol, catalyst and residual glycerol. The excess alcohol can be removed from each material and recycled back to be mixed with the catalyst [62,63]. Un-reacted alcohol is removed from the biodiesel as it reduces the flash-point of biodiesel, and therefore it is a requirement that only trace levels are present, for example a requirement of the ASTM standards is for the methanol content to be below 0.2%.

Crude glycerol is a by-product of transesterification and is heavier than the biodiesel, therefore it can be separated by leaving the mixture to form layers. Once it has settled out, it can be extracted and used as a product for other industries. The crude glycerol can contain unused catalyst and soaps that must be neutralised with an acid. Salts such as sodium or potassium phosphate can form during this phase, which can then be used as a fertiliser. Pure glycerol is used in pharmaceuticals and cosmetics, but it does not have a very high market value due to a large amount of glycerol present in the market because of large scale biodiesel production. There is also a high cost associated with purifying the crude glycerol, which is not financially viable given the market conditions.

The conventional method to produce FAME from oil-crops consists of two steps; extraction of lipids followed by transesterification using alcohol in the presence of a catalyst. However, an alternative route in which the lipids are transesterified with the same solvent that is used for extraction is known as 'in situ transesterification'. The solvent is an alcohol, typically methanol or ethanol, and the reaction is also performed in the presence of a catalyst. An example of the two processing routes is shown by the schematic in Figure 2.2.The in situ method offers a number of advantages: reduction of solvent requirement which reduces the cost and negative environmental impacts associated with solvent production and recycling and can reduce processing time also reducing processing costs [64]. It has also been found to increase yields compared to the conventional route for microalgal feedstocks [58].

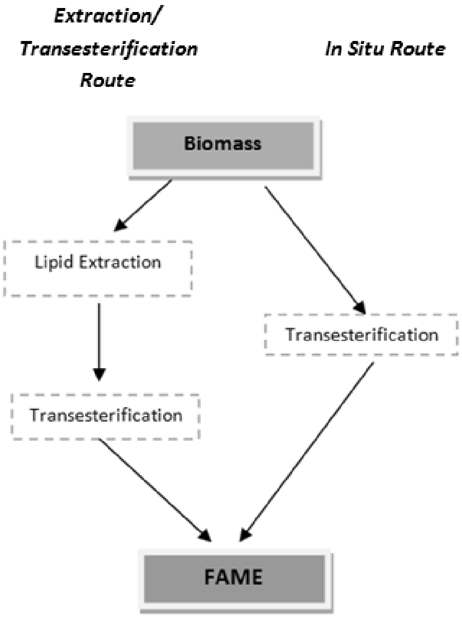


Figure 2.2 Processing routes for biodiesel production

2.3.3 Current situation for algae as a biodiesel feedstock

The area of microalgae biotechnology is rapidly developing, attracting funding and investment worldwide. Examples shown in the table in Appendix A indicate the range of products and the scale of production being reached currently. Large scale facilities for cultivation exist for nutritional supplements as these plants are economically feasible due to the high value end product (e.g. pigments and nutrients). Over 80% of the world's green algae producers are currently located in Taiwan, with Inner Mongolia in China and Israel being the top three producers of *Dunaliella* worldwide [65]. There is funding from governments in the US, EU, Brazil, China, India, Canada and other countries worldwide in both universities and commercial facilities.

Many petro-based companies including Exxon, Shell, BP, Statoil, ENAP, Chevron and Petrobras are investing in biofuel research and development for production of methanol, ethanol, bio-butanol, biodiesel, and biocrude as well as bio-based chemicals [66].

Table 2.2 Energy content of fuels from microalgae compared with existing biofuels

Fuel type [a]	Energy Content (MJ/kg)	Technologies	References
Biodiesel from algae	35-41	Transesterification	[60,67]
Bioethanol from algae	23.4	Fermentation	[67]
Biogas from algae	37.2	Anaerobic digestion, hydrothermal treatment	[67]
Bio-oil from algae	33-39	Hydrothermal liquefaction	[68]
Hydrogen from algae	144	Biological production, hydrothermal processing	[69]
Biodiesel from soybean	37.2	Transesterification	[70]
Gasoline	45	Distillation of crude oil	[71]
Diesel	48	Distillation of crude oil	[71]

[a] *The final energy density of the refined fuels is dependent on the composition of lipids and the biochemical composition of the starting microalgae*

Various components of the microalgae structure can be used to produce different fuel types, using similar technology to that which is used for other bioenergy crops. Microalgae have cultivation benefits compared with other bioenergy crops because of their high growth rates and the ability to grow them on marginal land. A report produced for the US DOE in 1984 looked at the chemical composition of eight strains of microalgae and calculated fuel production options based on their carbohydrate/protein/lipid content, demonstrating a combination of fuels which can be feasibly produced from an algal crop [67]. It is possible to produce biodiesel, bioethanol, biogas, bio-oil and even bio-hydrogen, as shown in Table 2.2 [72]. The energy content of biofuels from microalgae is comparable to those from other bio-crops and also fossil fuels. A summary of the energy contents are given in Table 2, based on an assumption of the following energy values for each characteristic: 38.93MJ/kg for lipids, 23.86 MJ/kg for proteins and 15.92 MJ/kg for carbohydrates [67].

Table 2.3 Comparison of autotrophic and heterotrophic microalgae as feedstocks for biodiesel production (species used for comparison was *C. vulgaris* unless otherwise stated)

	Heterotrophic	Autotrophic
Growth period	5-7 days [73]	<7 days
Biomass yield [22]	4 – 20 g l^{-1} d^{-1}	19 – 30 mg l^{-1} d^{-1} (open) 360 mg l^{-1} d^{-1} (PBR**)
Lipid yield	Up to 43% [74]	Up to 30% [75]
Land (area) required*	2.0 - 9.3 m^3/kg/d biodiesel	627m^3/kg/d biodiesel
Open v closed	Closed system reduces contamination, losses via evaporation and reduces risk of contamination to outside sources Can mitigate odours from wastewater treatment Climate independent (i.e. not reliant on solar radiation)	Open systems require large land area for solar penetration, are susceptible to contamination and have high evaporation losses increasing the WF***. Closed systems are energy intensive and light provision is often required.
GHG Emissions	No sequestration potential Lower energy use for refining due to lower impurity levels	CO_2 sequestration [38] Refining stage potentially more energy consuming
Conversion technologies	Potentially easier oil extraction due to thinner cell walls (observed for species T. suecica [76]). Extraction efficiency in this work as high as 95%.	Thick cell walls make oil extraction problematic (e.g. efficiency of 70% reported by [77])
Fuel quality	Low pigments, good FAME profile. Refining required to reduce ash content.	High level of contaminants, e.g. pigment chlorophyll-a, ash to be removed during refining stage. Good FAME profile

*based on the LCA used in Chapter 7; where heterotrophic *C. vulgaris* is assumed to have a growth rate of 0.34 -1.01 and a lipid content of 22 - 47% (scenario A) and autotrophic *C. vulgaris* is assumed to have a growth rate of 0.24 and a lipid content of 20%.

***Photobioreactor (PBR), ***water footprint (WF)*

To date, autotrophic microalgae have typically seen more research, but reducing land requirement remains restricted by the requirement for light by the organisms. Heterotrophic systems have also been investigated and show promising yields, but require a cheap carbon source or will remain uneconomical. There are a number of options, in particular in Brazil where there are industries with waste products rich in organic carbon from livestock farming and sugar cane processing to biodiesel production and oil and gas extraction. It potentially has several advantages over an autotrophic as a feedstock for biodiesel, as shown in Table 2.3. The advantages need to be made clear to help stimulate further research and development.

Use of heterotrophically cultivated microalgae is thought to lead to a number of advantages over autotrophic microalgae in terms of yields and fuel quality. For example, heterotrophic *C. vulgaris* contains fewer polar lipids [78] making processing easier and potentially reducing ash content (i.e. phosphorus). There is no chlorophyll present in heterotrophically cultivated microalgae which removes an additional processing step. The characteristics of biodiesel fuel produced in situ from heterotrophic *S. limacinum* cultivated using a crude glycerol feedstock showed properties that meet ASTM standards [79]. The FAME profile of heterotrophically cultivated microalgae does not appear to vary significantly from autotrophic counterparts (see Table 6.1), although it is difficult to quantify exact differences in the literature as different conditions are used for cultivation and processing by different authors.

2.4 Summary

An alternative biofuel feedstock is required that will not compete with food crops for land and nutrients, that can meet sustainability criteria set out by various governments, that has a good energy content and physical characteristics and is economically competitive to produce. Microalgae could potentially fit these criteria, but will require more work in order to develop a strategy that allows identification and development of an environmentally sensitive and reliable fuel at a reasonable price. Heterotrophic microalgae may provide several advantages over autotrophic microalgae and therefore will be investigated over the next chapters as a potentially environmentally friendly feedstock for biodiesel production in Brazil.

Chapter 3 Equipment and Experimental Techniques

This chapter will describe various experimental techniques used throughout this thesis and discussed in the following chapters. Some of the methods are well known and have been described in the literature, whilst some have been developed specifically for this thesis. The methods are described below, and are arranged into four sections to cover the cultivation methods, biodiesel production techniques, analytical techniques and reagents used.

3.1 Cultivation methods

3.1.1 Heterotrophic cultivation trials

A heterotroph is an organism that cannot assimilate inorganic carbon, such as CO_2, and uses an organic carbon source instead for growth. Heterotrophs benefit from being able to use all of the energy obtained from feeding for growth and reproduction, compared with autotrophs which must use some energy for carbon fixation. However, as they cannot produce energy through the assimilation of sunlight, as their autotrophic counterparts do, they obtain all energy through metabolism of nutrients in the water source.

All heterotrophic cultivation trials were carried out in the Public Health Laboratory, School of Civil Engineering at the University of Leeds. Heterotrophic microalgae were obtained from an autotrophic growth culture. The original culture of *Chlorella vulgaris* was obtained from the Scottish Association for Marine Science and cultures were maintained at the University of Leeds. The microalgae were transferred to opaque containers in order to provide dark conditions and stop the photosynthetic process. Heterotrophic algae utilise oxygen for respiration therefore the water was agitated using air stones in order to keep the conditions in the water aerobic. Two growth media were used, a heterotrophic basal media designed to be nitrogen limiting and an adapted synthetic wastewater formula, described in detail in Figure 3.1below.

Figure 3.1 Heterotrophic microalgae cultivation set-up

The cultivation of heterotrophic microalgae took place in 1-litre Duran bottles containing 500ml of culture media, with an air stone connected to an air pump. The air was first pumped through distilled water in order to be saturated with moisture and reduce water loses due to evaporation. The set-up is shown by the schematic in Figure 3.2. The media were autoclaved at 121°C for 1 hour to ensure any bacteria present were destroyed. The temperature remained between 24-27°C in the media throughout the trials.

Two growth media were used, described below. Once the inoculant had adapted to the new conditions, it was then used to inoculate further cultures. Each time a different carbon feedstock was used the microalgae were allowed to adapt to the environment before inoculating a new media, after which point measurements were taken. The *C. vulgaris* was considered well adapted to the new environment once the pH was steady. The trials were carried out in triplicate following an adjustment phase where a new feedstock was trialled.

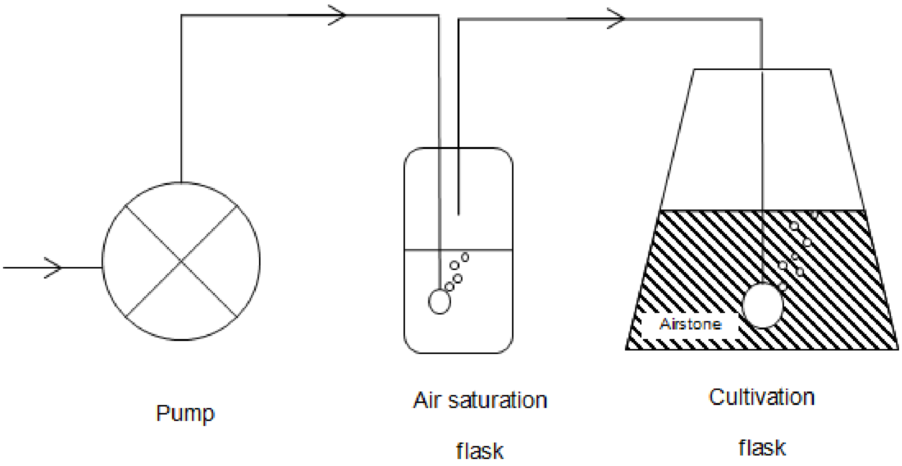


Figure 3.2 Cultivation trial equipment set up

Table 3.1 Composition of media used for cultivation trials

Media	Composition
Bolds Basal Media (BBM)	25g $NaNO_3$ 2.5g$CaCl_2.2H_2O$ 7.5g $MgSO_4.7H_2O$ 7.5g K_2HPO_4 17.5g KH_2PO_4 2.5g NaCl 1ml alkaline EDTA Stock solution 1ml acidified iron stock solution 1ml boron stock solution 1ml trace metal stock solution (8.82g $ZnSO_4.H_2O$, 1.44g $MnCl_2.4H_2O$, 0.71g MoO_3, 1.57g $CuSO_4.5H_2O$, 0.49g $Co(NO_3)_2.6H_2O$)
Heterotrophic Basal Media (HBM)	0.7g KH_2PO_4 0.3g K_2HPO_4 0.3g $MgSO_4.7H_2O$ 25mg CaCl2.H_2O 25mg NaCl 3mg $FeSO_4.7H_2O$ 1ml trace metal stock solution (as above) 4g yeast extract Organic Carbon
Adapted Synthetic Wastewater (SWW)	0.08g Peptone 0.055g Meat extract 0.015g Urea 0.0035 NaCl 0.002g $CaCl_2.H_2O$ 0.001 $MgSO_4$ 0.14g K_2HPO_4 1.5g yeast extract Organic Carbon

Culture media were prepared using the formulas described in Table 3.1. Two different cultivation media were used for heterotrophic cultivation. The first was a medium designed for heterotrophic cultivation by Wu et al. (1992) shown in Table 3.1, which was carbon limiting with respect to the carbon-to-nitrogen (C:N) ratio [80], and nitrogen limiting with respect to the nitrogen-to-phosphorus (N:P) ratio [81,82] and is henceforth known as the heterotrophic basal medium (HBM) [83]. The nitrogen limited design was to promote the accumulation of lipids, as observed by [80,84].

The heterotrophic *C. vulgaris* were also cultivated using a synthetic wastewater media (SWW). Data was provided by Professor Andre Calado from the University of Rio Grande do Norte in Brazil, shown in Table 3.2 as to the nutrient content of a series of waste stabilisation ponds in Brazil, discussed further in Chapter 5. The medium was based on the OECD synthetic wastewater media [85], but adjusted so as to match the nitrogen and phosphorus levels found within the ponds shown in Table 3.3. The C:N ratio was provided in excess of the stoichiometric ratio of $C_{73.5}N_{12.7}P_1$ defined by Sansawa & Endo, (2004) for heterotrophic *C. vulgaris.* The final ratios for C:N:P are shown in Table 3.3. Due to the nature of the waste organic carbon feedstocks, the carbon content of the crude glycerol and molasses could change by up to 3% between batches. The molar content of carbon for the HBM was 0.38M, which was equivalent to 9.8g glucose, 10g molasses and 20g crude glycerol. The characterisation of the crude glycerol is explained in Chapter 5. The carbon content of the media was measured using TOC, described below in section 3.3.4.

Table 3.2 Nutrient content of wastewater treatment facility in Ponte Negra in Natal, Brazil

	Raw centrate	Primary facultative pond	Maturation pond 1	Maturation pond 2
Phosphorus (mg/l)	5.70	3.90	4.40	4.30
Organic N (mg/l)	25.6	10.4	15.1	6.1
Ammonia (mg/l)	34.7	17.2	17.3	14.5

Table 3.3 Molar composition of different media (where organic carbon content per litre was 9.8g glucose, 10g molasses and 20g crude glycerol)

		SWW Media			**HBM Media**		
	WSP Natal	**Glucose**	**Crude**	**Molasses**	**Glucose**	**Crude**	**Molasses**
C (mol)	***0.1***	**0.39**	**0.38**	**0.38**	**0.39**	**0.39**	**0.36**
N (mol)	***0.014***	**0.018**	**0.018**	**0.018**	**0.019**	**0.019**	**0.019**
P (mol)	***0.0015***	**0.002**	**0.002**	**0.002**	**0.018**	**0.018**	**0.018**
C:N:P ratio	***78:11:1***	**208:9:1**	**198:9:1**	**199:9:1**	**22:1:1**	**22:1:1**	**20:1:1**

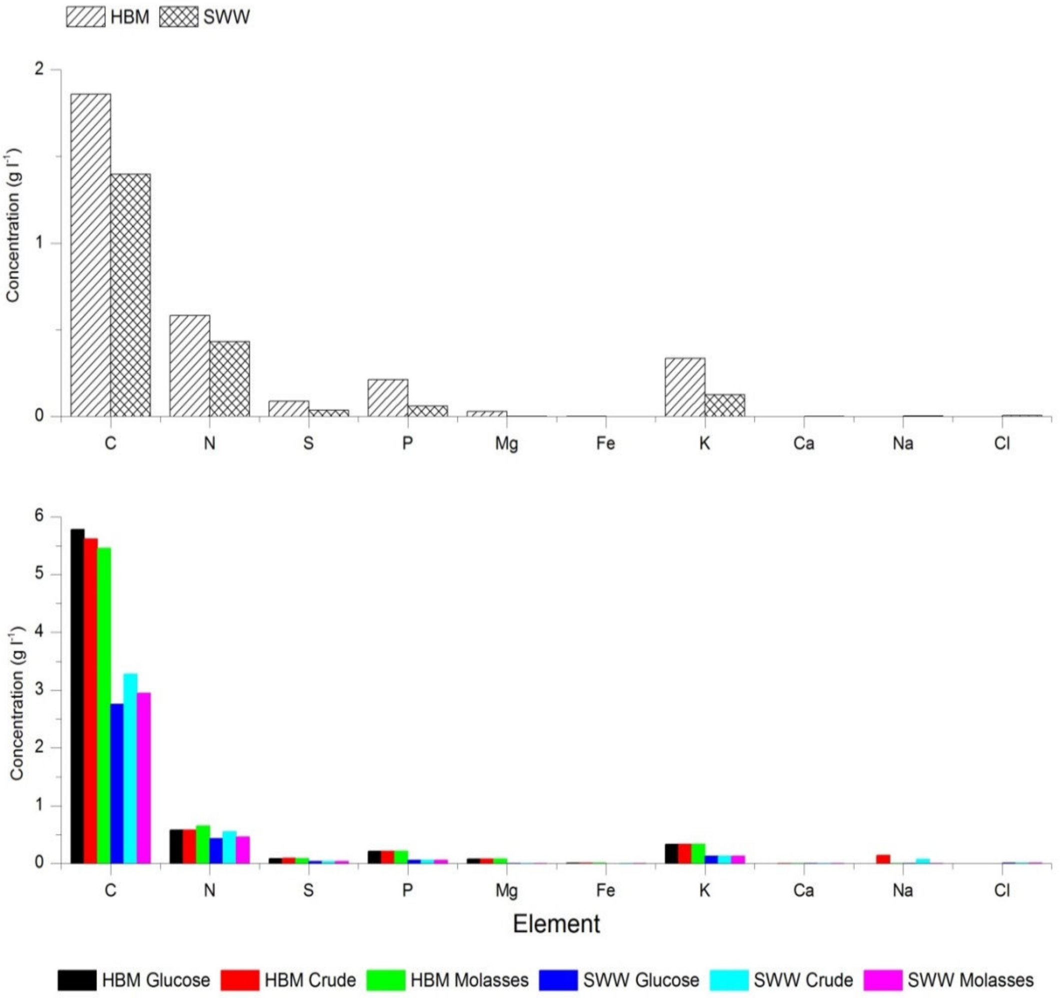


Figure 3.3 Cultivation media composition, (top) showing the two media before the addition of an organic carbon feedstock, and (bottom) the total elemental composition.

3.1.2 Measuring growth rates of algal biomass

The growth rate was calculated by using cell counting methods described by [86] and dry cell weight, as turbidity proved an unreliable proxy due to sedimentation of components of the media and presence of an emulsion when crude glycerol was neutralised with H_2SO_4 in the media. The algal population size was calculated via counting of cells using a Neubauer chamber (also known as a haemocytometer). The Neubauer chamber is a thick crystal slide with a cover glass slide. The central part of the chamber is slightly lowered, so as to create a depth of 0.1mm. It has a counting grid set on the glass shown in Figure 3.4. The grid is 3 x 3mm in size, with 9 subdivisions of width 1mm. A central square is used for algae cell counting due to their small size. This central square is split into 25 squares of width 0.2mm. Each square is subdivided into 16 small squares. The volume of the chamber for each small square is 0.004μl, and therefore the concentration of cells can be calculated using Equation 3.1. Where any dilution takes place, the dilution calculated by dividing the concentration by the dilution applied. This was a destructive technique (i.e. the culture was not returned to the cultivation flask) but only used a small volume (i.e., 1ml), therefore this allowed daily measurements without a great loss of culture medium.

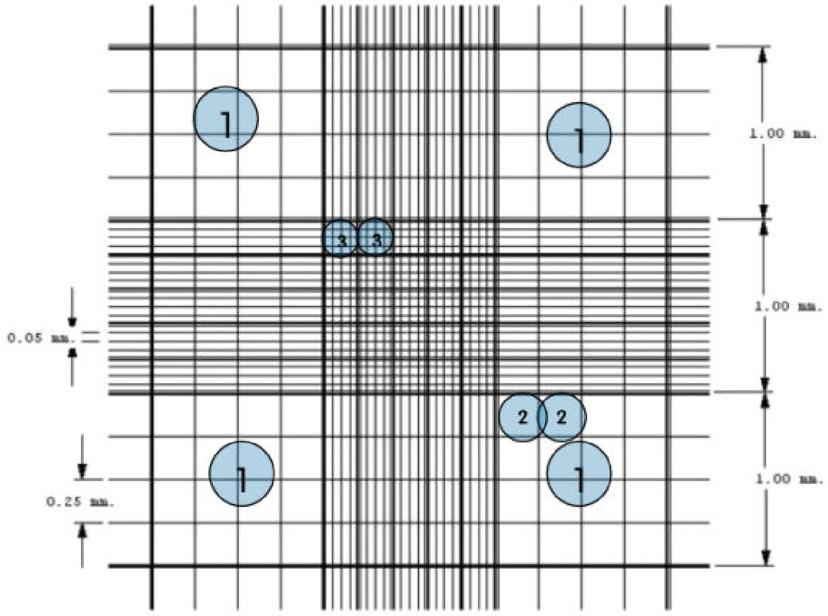


Figure 3.4 Neubauer chamber grid layout [87]

The total suspended solids (TSS) was calculated by filtering a known volume of the culture media through a pre-weighed *No. 1 Whatman Filter*, then drying in an oven at 105°C for 24 hours. The filter was then reweighed. A sample of the media, before addition of any biomass, was also weighed in order to discount the weight of any solids in suspension in the media. This was also a destructive technique, and used up to 10ml per measurement. Therefore it was carried out at a three day interval so as not to deplete the culture medium too quickly.

To calculate the exponential growth rate (r), the natural log of the population size at the beginning of the exponential growth phase (N_0) and end of the exponential growth phase (N_t) were calculated and divided by the time period (t) to calculate growth rate (mass of algae per day)(shown in Equation 3.2) [86].

$$Concentration\ (cell/ml) = \frac{\#\ cells}{volume\ (ml)}$$ **Equation 3.1**

$$r = \frac{ln(N_t/N_0)}{\Delta t}$$ **Equation 3.2**

Cell count was used as the preferred method to monitor algal growth as it proved more accurate than turbidity measurements, as absorbance (measured using a colorimeter) can change as metabolism of the culture changes (i.e. cell flocculation or emulsion formation).

Measurements of pH were taken daily, and observations on the appearance of the microalgae were made using an *Olympus BH-2* microscope with a 40x magnification (also used for cell counting).

3.1.3 Algal biomass harvesting and drying techniques

Once a culture had reached a stationary growth phase, it was harvested. The media containing the algae was placed in 500ml bottles at equal weights, and centrifuged for 10 minutes at 2000 x g, as speeds higher than this can lead to cell rupture [88]. The supernatant was removed and the algae slurry collected into 50ml sterile centrifuge tubes. The tubes were frozen and stored at -12°C in a freezer.

The biomass was dried in a *Christ Alpha 1-2 LD* freeze dryer (lyophilisation). The freeze dryer reduced the pressure within the chamber to less than 6 millibars, controlled through application of a vacuum, created through a pump attached to the chamber. At room temperature there is enough heat in this environment to allow the frozen water in the biomass to sublimate directly from a solid to a gas phase. A cold condenser plate allows the water vapour to re-solidify, preventing water from entering the pump. This process removes approximately 95% of the total moisture. The time for drying depends on the water content and mass of sample, but is a relatively slow process and can range from 1-4 days for a sample size 1-2g containing 30ml water.

3.2 Transesterification of oil

Fatty acid methyl ester (FAME) was produced using two methods: transesterification of extracted lipids and in situ transesterification of biomass (in situ route shown in Figure 3.5). The production of FAME from the oil harvested from the algal biomass was conducted via acidic transesterification using H_2SO_4 as catalyst in an excess of methanol. The catalyst quantity was based on the weight of the oil, with 1% of the oil weight being used [89]. The molar ratio used was 56:1 of methanol to oil (v/v wt%), calculated using molecular weight obtained from size exclusion chromatography SEC data (see section 3.3.10).

The in situ transesterification was developed and tested at different temperatures and for different time periods, explained in detail in Chapter 6. In a typical in situ reaction, shown in Figure 3.5, 200mg dry algal biomass was placed in a glass container, and 2ml methanolic acid was added. A lid was put on and the sample was heated to 70°C for 90 minutes. Once cooled, the mixture was washed with hexane and water to stop the reaction and allow phase separation. The top layer was removed and filtered using a syringe attached to a 0.2µm PTFE filter into a pre-weighed glass vial. The hexane was left to evaporate, the rate of which was increased by gentle heating at 30°C**.** Biodiesel yield relative to the weight of algae biomass and algae lipid fraction was estimated gravimetrically. The transesterification of lipids also used this method but with methanolic acid volumes adjusted to the weight of the lipid.

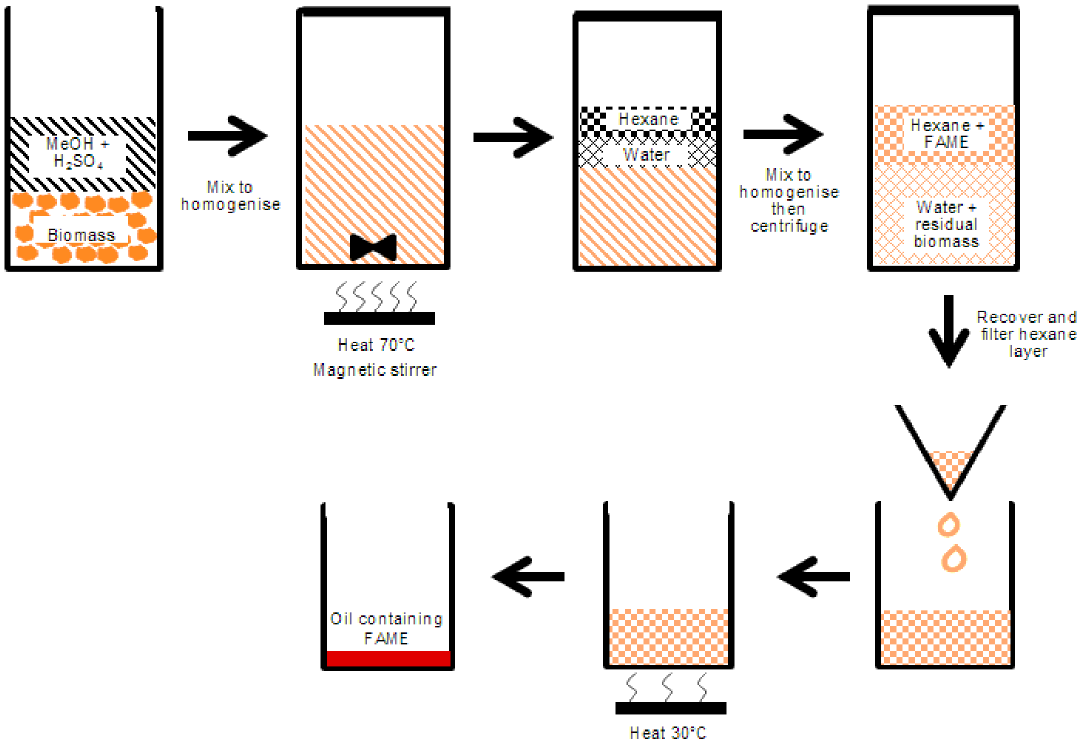


Figure 3.5 Schematic showing in situ transesterification method

3.3 Analytical techniques

3.3.1 Carbohydrates quantification

Carbohydrates are heterogeneous compounds, differing in primary and macromolecular structure, linkage type, degree of polymerisation and charge. The degree of polymerisation can be classified into mono-, oligo- or poly- saccharides. Monosaccharides are aliphatic aldehydes or ketones containing one carbonyl group and one or more hydroxyl groups – e.g., hexoses glucose, fructose, galactose and pentoses arabinose and xylose. Oligosaccharides are low molecular weight polymers of monosaccharides, bonded by glycosidic linkages. Polysaccharides are the most common form in which carbohydrates are found in nature and are high molecular weight monosaccharides including starch, cellulose, pectin, hemicellulose and gums. Carbohydrates can be analysed by spectrophotometry, using a strong acid to breakdown the carbohydrates into furan derivatives. These then condense to produce dark coloured complexes which can be measured using UV-VI light [90].

The method used here for analysis, began by hydrolysing the biomass with a concentrated sulphuric acid (H_2SO_4) catalyst, followed by addition of phenol. In brief, 3ml of 72% H_2SO_4 was added to 0.05g of the sample, mixed and heated to 40°C for 1 hour, after which 1ml 5% phenol solution was added. Samples were diluted to 100ml with distilled water, then centrifuged. A sample blank was also prepared for each sample, where 1ml distilled water was added instead of the 5% phenol solution. A glucose standard was also prepared using a 100mg/ml glucose stock solution. 5ml of concentrated H_2SO_4 was added to 1ml of the sample supernatant, and also to 1ml glucose standard and to 1ml distilled water for preparation of a reagent blank. All samples, standards and blanks were left for 1 hour [91].

The carbohydrate concentration is determined by refractive index. The refractive index (RI) of the solution increases with increasing carbohydrate content. The RI is temperature and wavelength specific and therefore all measurement were made at 485nm at 20°C. After 1 hour, a spectrophotometer was adjusted to 485nm and was zeroed using the reagent blank. The light absorbance of the samples, sample blanks and the standard were measured in a 1cm quartz cell and the absorbencies were recorded.

The total carbohydrate content of the microalgae biomass was calculated as a percentage of total biomass using Equation 3.3. Where A_1 and A_2 were the sample and standard absorbance respectively, B was the absorbance of the sample blank, W was the sample weight in grams, C was the concentration of standard in µg/ml and V was the initial volume in ml.

$$\%\ total\ carbohydrates = \frac{(A_1 - B)\ x\ V\ x\ C}{A_2\ x\ W\ x\ 10000}$$ **Equation 3.3**

Whilst there can be inaccuracies in this method caused by interference of amino acids, inorganics or ashes, or because not all derivatives exhibit the same colorimetric responses, this is still considered a good method for carbohydrate determination [92].

3.3.2 Protein quantification

Protein analysis was carried out using the Dumas method [92], whereby the sample was combusted at 900°C in the presence of oxygen leading to the release of carbon dioxide, nitrogen oxides and water vapour. The level of nitrogen was determined using a thermal conductivity detector, described in further detail in section 3.3.6. A conversion factor of 4.78 was then used to convert this value to the protein content [92]. The conversion factor depends on the amino acid sequence in the protein and can therefore be variable. There are also differences in the microalgae protein accumulation throughout the growth phase; for example, harvests taking place into the stationary phase can have a higher conversion of 5.06. However, the advantages of the Dumas method include the rapid procedure time of only a few minutes, there are no toxic chemicals used and a very small sample can be analysed to give approximate results. A number of authors have also used this method and found it to give a good indication of protein content within biomass [93] [94]. This method was used as opposed to a spectrophotometric method using Folin reagent following work by [92], who found that inaccuracies can be between a 17-53% overestimation of protein compared with up to 10% overestimation using the Dumas method.

3.3.3 Lipids quantification

Lipids are an essential part of the biomass structure in microalgae serving as an energy reserve and also as an aid in floating [95]. Lipids classification can be simply divided into two categories: polar and non-polar lipids, explained further in Chapter 5. Non-polar lipids are a potential feedstock required for biodiesel production, and as such, quantification of lipids within the biomass is an important metric to decide whether the microalgae are a suitable feedstock for biodiesel.

Solvent extraction is commonly used for extraction of oils from biofuel feedstock crops, such as soybeans. The solvent selected must have a high solubility for lipids [96]. Chloroform is also non-polar, therefore it should not bond with polar lipids such as phospholipids in the cell membrane. Several articles report a method using chloroform/methanol as the most reliable method for total lipid determination [96,97,98]. Hexane is used industrially as the most common extraction technique for oil-seed crops, as it is a non-polar solvent and therefore only extracts non-polar lipids. It was therefore decided to be the most suitable method for determining the lipid content as it should only extract the non-polar lipids which can be used for biodiesel production and it is also more likely to be scalable for use in industry, as hexane extraction is currently used for extraction of oil from other oil seed crops (e.g. soybean).

This lipid extraction method used the following steps: 5ml hexane was added to 200mg dry algal biomass in a glass container and placed on a magnetic stirrer for 10 minutes. Following this, 5ml of distilled water was added to wash any non-polar compounds from the extract and left for 2 hours to form 2 layers, the top layer containing hexane and lipid and the bottom layer containing biomass and any polar extracts. The hexane layer was recovered and passed through a 0.2μm PTFE filter using a syringe into a pre-weighed glass vial. The syringe was then rinsed with hexane. The hexane was allowed to evaporate over a gentle heat and the lipid extracted was determined gravimetrically. It was assumed that all of the lipid extracted using hexane was non-polar as hexane is a very non-polar solvent. Another 5ml hexane was added to the lipid extracted algae (LEA) to extract any remaining lipids, and the method was repeated as above. All extracts were carried out in duplicate.

3.3.4 Carbon content (total, organic and inorganic)

Total carbon measurements were required to investigate the level of carbon uptake from the media, investigated in Chapter 5. To prepare the sample, a known volume of the sample was filtered through a qualitative cellulose filter paper (Whatman No. 1) and the filtrate was collected. The filtrate was then frozen until all samples were collected, ensuring all analysis could be done together to allow consistency. Samples were taken after 0, 3, 6 and 9 days in the HBM and after 0, 3 and 5 days in the SWW to observe the rate of uptake of organic carbon from the media and to investigate any corresponding drops in growth rate. The reason for a shorter sample time was due to the fact the SWW culture had a shorter exponential growth period.

The total organic carbon was determined using a differential method. Both the total carbon and the inorganic carbon were determined separately, then used to calculated the organic carbon content by difference. A combustion method was used by the *Hach-Lange IL550 analyser*. 100µl of sample was injected into a heated combustion tube packed with an oxidation catalyst. The water was vaporised and the inorganic and organic carbon converted to carbon dioxide. The CO_2 was removed from the chamber via a carrier gas to a non-dispersive infrared gas analyser (NDIR), obtaining a concentration for CO_2. The inorganic fraction was measured by a subsequent injection of sample into a separate reaction chamber which was filled with phosphoric acid solution. This converted the inorganic carbon to carbon dioxide, and allowed quantification again by NDIR.

Figure 3.6 Total Organic Carbon (TOC) and Total Inorganic Carbon (TIC) Analyser (Hach-Lange IL550)

3.3.5 High performance liquid chromatography (HPLC)

HPLC is a chromatographic technique to separate, identify and quantify organic components in a mixture. To analyse the sugar content of the crude glycerol, the *Supelcogel C-610H* ion exchange HPLC column with refractive index detection was used. The liquid sample was pumped through a column packed with a solid phase sorbent at high pressure (above 5 x 10^6Pa). The column was packed with sulphonated polystyrene and divinyl benzene. The mobile phase was 0.1% H_3PO_4, with a flow rate of 0.5ml/min, run at a temperature of 30°C for 38 minutes.

An external calibration was set up in order to identify and quantify the concentration of sugars, alcohols and organic acids that may have been present. The sugars were glucose, sucrose, xylose, ribose and arabinose, the alcohols were glycerol, methanol and ethanol and the organic acids calibrated were oxalic, citric and acetic acid. Sucrose and maltose have similar retention times and therefore it was not possible to make a clear distinction between them.

3.3.6 Elemental analysis

Analysis of the carbon, hydrogen, nitrogen, sulphur and oxygen within the biomass and oil were determined using an elemental analyser (*CE Instruments Flash EA 1112 Series*). The sample was weighed into a tin capsule and sealed via crimping. 2.5mg of each of the following standards were used: atropine, BBot, dl-methionine, L-cystine and sulphanilamide. These were loaded via an auto sampling device, followed by 2.5mg of each sample in duplicate. The auto-sampler was used to deliver the sample at a preset time. Once loaded into the oxidation/reduction reactor, which is kept at 900-1000°C, oxygen was added for optimum combustion of the sample. The oxygen reacts with the tin capsule and creates an exothermic reaction causing temperature to rise to <1800°C for a few seconds. The sample was converted to CO_2, H_2, N_2 and SO_2 at these temperatures. They were then separated in a chromatographic column and detected by a thermal conductivity detector. Results allowed calculation of carbon, hydrogen, nitrogen and sulphur content. Oxygen content was determined by difference.

3.3.7 Thermogravimetric analysis (TGA)

The physical and chemical properties of the biomass and oil were determined using thermo-gravimetric analysis. This technique measures the changes in mass of the sample as a function of temperature, using a constant heating rate, and is useful for determining the moisture and ash content of a biological sample. It can also provide information on the structure of the biomass such as lipids, proteins and carbohydrates [99]. As the furnace is heated, heat flows across the systems boundary and the mass is reduced either by evaporation or decomposition, which is recorded by the balance.

A number of errors can arise leading to inaccuracies in the measurement. The instrumental errors can arise from sample container air buoyancy, furnace convection currents, turbulence and induction effects, random fluctuations in the balance or recording mechanisms or thermal expansion of the balance beam. Sample errors may arise from characteristics within the sample such as condensation of volatile products on the sample suspension, reaction of the sample with the container, sample packing or solubility of evolved gases. The following method explains how some of these issues were overcome.

The same technique was used for both solids (algal biomass) and liquids (extracted oils). 10mg of sample was placed into a crucible suspended from an arm of the TGA microbalance. The furnace, which was purged with N_2 to create an inert atmosphere, was brought up around the crucible. The temperature was increased at a rate of 10°C/min up to 700°C, which was sufficient to ensure all material was combusted. The chamber was then purged with O_2 to ensure complete combustion of any remaining material. The weight loss was recorded simultaneously. The proximate analysis of the sample allowed identification of moisture content, volatile material, ash and fixed carbon [100].

Figure 3.7 Stanton TGA used for thermogravimetric analysis of biomass and oil samples

3.3.8 Gas chromatography mass spectrometry (GCMS)

Oils produced from microalgal biomass were identified and quantified using a GCMS. The GCMS partitioned the components as they were passed along a capillary column at an elevated temperature. Lighter components were eluted first. The separated components were then characterised by the mass spectrometer (MS). The MS ionised each component by a high energy beam of electrons. The charged particle was deflected along a circular path with a radius that was proportional to the mass to charge ratio, m/e. The mass fragment spectrum produced allowed the component to be identified.

The specific method used to characterise the components within the oil after transesterification was a Restek RTX 1701 30m column with a diameter of 320 μm and film thickness of 25μm. The pressure used was 28 psi, with an initial flow of 4.8 ml/min and average velocity of 80cm/sec. Temperature in the oven began at 60°C. The temperature was ramped to 150°C over 6 minutes. The temperature was then ramped again to a maximum temperature of 280°C over 4 minutes. Helium was used as a carrier gas. The total run time was 64.5 minutes. A split ratio of 10:1 was used for calibration and for processing all samples.

An external calibration was carried out in order to quantify the concentration of FAME. A 100mg FAME Mix C_8-C_{24} obtained from Sigma Aldrich, *Supelco-18919-1AMP*, was used. In order to produce a calibration range, the FAME Mix was made up to 10ml with DCM, to produce a solution of 1mg/l FAME Mix concentration (1ppm). A further two dilutions were made of 0.25mg/l and 0.5mg/l. The three samples were injected into the GCMS using the conditions described above. The response factor was calculated from the calibration. The response is plotted in the chromatograph in Figure 3.8, and the carbon number is overlaid to indicate the compounds present.

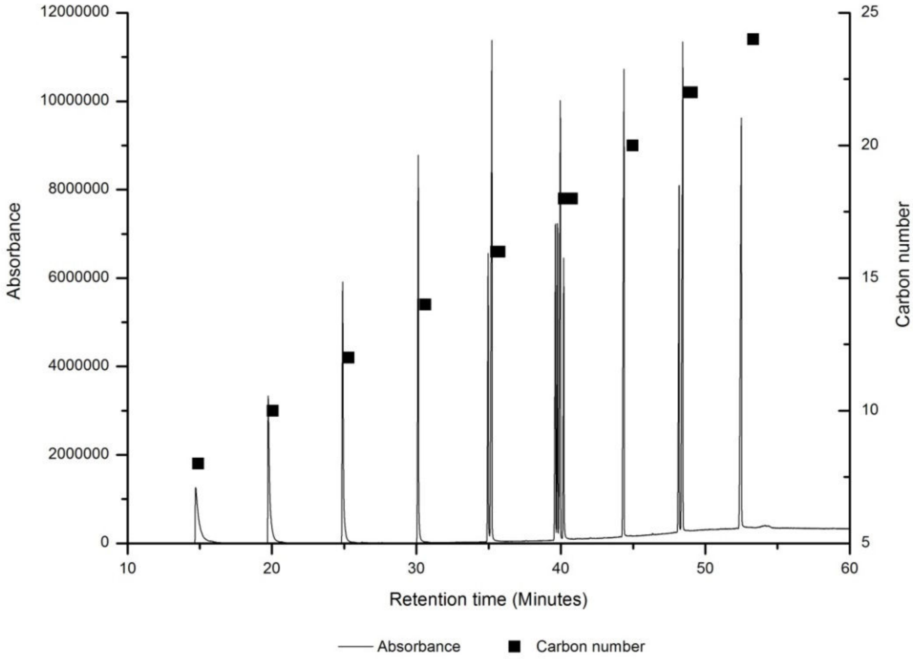


Figure 3.8 The number of carbon atoms per FAME, identified from mass spectroscopy, is shown overlaid upon the gas chromatography absorbance data

Figure 3.9 Agilent GC-MS used for analysis of FAME content in algae oil

3.3.9 Inductively coupled plasma mass spectroscopy (ICP-MS)

ICP-MS is capable of major and trace metals and other elements at ppb-ppt by ionising the sample using inductively coupled plasma. The plasma is ionised by inductive heating of the carrier gas (in this case argon) using an electromagnetic coil. The plasma contains ions and electrons which make the gas electrically conductive and electrically neutral as there are equal quantities of positively charged ions and free electrons. When the sample is introduced to the chamber, the high temperatures in the plasma cause the atoms within the sample to be ionised [101]. The ions are then quantified using a mass spectrometer (the same method as described in section 3.3.8.

To prepare the sample for the ICP-MS analysis, 0.2g of the sample was digested in 10ml nitric acid. Once digested the sample was serial diluted to 5000 times with deionised water. A *Perkin Elmer Elan DRCe ICP/MS* was used for the analysis, operated by laboratory technicians in the Energy Research Institute, University of Leeds.

Figure 3.10 Perkin Elmer Elan DRCe ICP/MS

3.3.10 Size exclusion chromatography (SEC)

The purity of the extracted oil and the conversion efficiency in the in situ transesterification was identified using SEC. Whilst this was not a fully quantitative method, it gave a range of molecular weights found within the oil. The benefit of using SEC was that heavy components which would not be detected on more sensitive gas chromatography methods could be seen. The molecules were separated by size, with the heaviest components eluting first. A *Perkin Elmer Series 200* liquid chromatography system, with a Varian PL Gel 3um 100A Column was used. The programme was set at 30°C for 12 minutes. A polystyrene standard was used for calibration, allowing approximate molecular weights to be identified.

There were errors associated with using this techniques, for example the analyte could interact with the stationary phase, leading to alter elution times and therefore underestimation of the analyte size. This is a particular problem with polar compounds in the analyte. However, the analysis gave a useful indication as to the molecular weight of compounds being extracted by lipid extraction and the efficiency of the transesterification reaction by identifying compounds with similar molecular weights to those of FAMEs.

3.4 Reagents

A list of the reagents used in this work, along with its grade and manufacturer are listed below. The chemicals used for the cultivation media were sourced from *ThermoScientific* in solid form unless otherwise stated. The carbon sources were obtained from various sources, listed below. The carbon sources not of scientific grade (i.e., molasses and crude glycerol) were analysed to determine elemental composition prior to use.

- Glucose, Thermo Scientific
- Unrefined molasses, International Food Store, Leeds
- Crude glycerol, East Yorkshire Biofuels, Hull

All reagents for the production of FAMEs and determination of carbohydrate, protein and lipid composition were sourced from *Sigma Aldrich* unless otherwise stated. The PTFE filters, syringes and Pasteur pipettes were obtained from *VWR international*. The purity is shown below:

- Chloroform; containing ethanol as stabilizer, ACS reagent, ≥99.8%
- Methanol; ACS reagent, ≥99.8%
- Hexane: anhydrous 95%
- Dichloromethane anhydrous; ≥99.8%, containing 50-150 ppm amylene as stabilizer
- Sulphuric acid; ACS reagent, 95.0-98.0%
- FAME Mix C_8-C_{24} standard; Sigma Aldrich, *Supelco-18919-1AMP*

Chapter 4 Identifying the opportunities for microalgal feedstock in Brazil’s biodiesel industry

4.1 Introduction

A new biodiesel feedstock needs to be compatible with existing infrastructure for it to be technically and economically feasible. This chapter investigates the possibility of introducing a new feedstock in Brazil, where currently the dominant feedstock is soybean, but at a cost to the environment and social development. By understanding the existing techno-socio system, opportunities for introducing heterotrophic microalgae as a new feedstock can be identified and developed.

Brazil’s “National Programme for Production and Use of Biodiesel” (PNPB in Portuguese) is a novel programme, aimed at improving the sustainability of biodiesel by promoting social inclusion, whilst simultaneously increasing food security, diversifying feedstocks and producing a lower carbon fuel. The achievements of the programme have been evaluated by conducting a qualitative analysis of the system. The existing system is then compared with 4 scenarios. The first considers the effect of increasing feedstock production via further technological and financial intervention, followed by a second scenario to estimate the impacts of a mandated increase in biodiesel volume within diesel blends. A third scenario looks into the impact of removing tax incentives to the programme in order to hypothesise the economic stability of the system. The final scenario utilises the findings from scenarios 1-3 and investigates the potential impacts of the introduction of heterotrophic microalgae as an alternative feedstock for biodiesel production and uses it as a basis for further work into exploring the feasibility of incorporating microalgae into the biodiesel supply chain.

4.1.1 Development of the biodiesel programme in Brazil

The development of biodiesel in Brazil started in the early 20th century, with support for research finally been recognised in 1960’s under the military government. Support was given due to national security considerations and logistical reasons for isolated communities where fuel needed to be produced locally. The Prodiesel Programme was set up and Professor Parente for the Universidade Federal do Ceará (UFC) developed the transesterification process for biodiesel production [102]. However, the cost of biodiesel was too high to compete with fossildiesel and therefore the programme did not continue [103]. Petrobras was also established under the military government in 1954, and although it was established as a petroleum company and a state-owned enterprise it is now a semi-independent enterprise with a biofuels subsidiary [104].

The strong political drive for the development and use of renewable fuels in Brazil was initiated by the dictatorship government, and continued to be part of government policy throughout subsequent governments but carried through because of its importance as a component of economic development and fuel security. The PNPB was established in 2004 and came into force in 2005 under President Lulas' government. The aims of PNPB when it was set up with regards to sustainability were social inclusion of farmers, food security, promotion of sustainable agriculture, regional development, feedstock diversity and a positive carbon and energy balance from biodiesel [105]. Figure 4.1 shows the design of the PNPB, from how it is driven, to obstacles it faces and the expected outcomes.

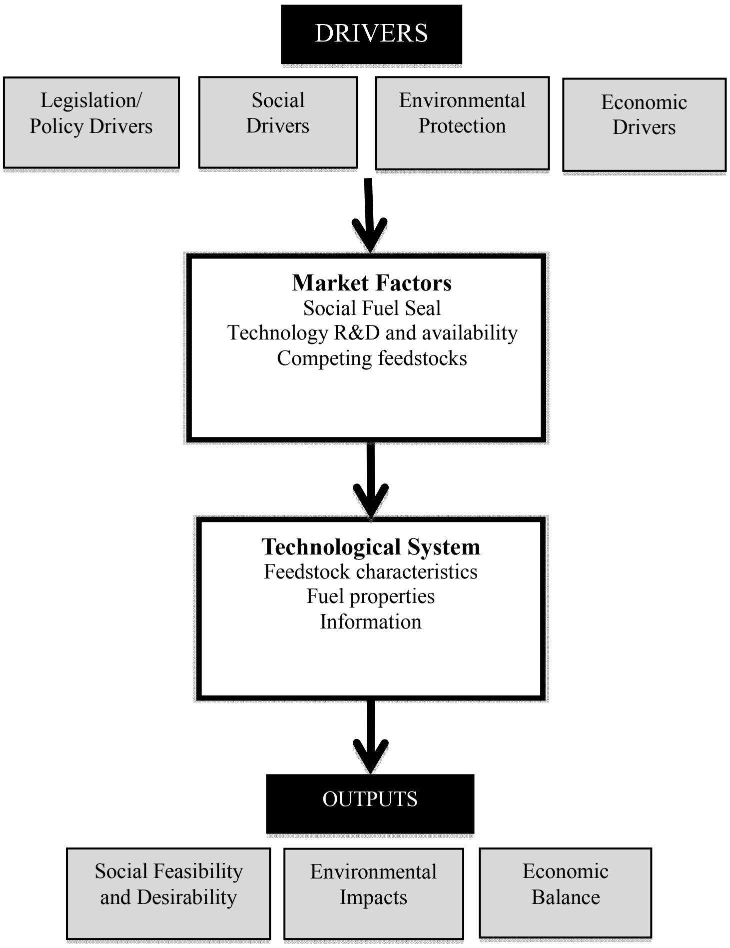


Figure 4.1 Brazilian biodiesel programme design

The PNPB is supported by two core aims, raising competitiveness of corporate farming and strengthening family farming. There were two federal laws introduced to support the programme [106,107,108]. Initially these were set up as two temporary measures, No. 214 and 227, in 2004 to guide the biodiesel industry as to which feedstocks to use and voluntary levels of biodiesel in the fuel mix. Temporary measure 214 was transformed to a conversion bill (PLV no. 60-2004), which added new components including a minimum of 5% biodiesel in diesel by 2012, definitions of biofuel and biodiesel, assignment of responsibility to the National Council for Energy Policy (CNPE in Portuguese) for deciding the mix of primary materials, industrial production, technology and participation of family farms, and a proposal to change the “National Petroleum Agency” to the “National Petroleum, Natural Gas and Biofuel Agency” (ANP in Portuguese). This became Federal Law No.11.097-05 in 2005. The law led to the amendment of rules, dictating where royalties from petroleum could be spent. Previously these royalties could only be used for financing projects relating to oil or gas. However, the amendment also allowed projects relating to biofuels to be included [105]. Law 11.116/05, created to incentivise biodiesel production and use set out the tax structure of the biodiesel programme. The role of the Executive branch was defined in the regulation of two taxes, the ‘PIS/PASEP’, a tax for the social integration programme and ‘COFINS’, the federal social security contribution. The level of these taxes for biodiesel varies depending on raw materials, producers, and the region of production. As a result of this structure, the taxes paid for biodiesel production and use can be zero [109]. In the north and northeast regions these optimal tax incentives are only applicable to palm and castor (see Table 4.1). The rules also establish a minimum amount of capital required for a biodiesel producer, set at R$0.5million for producers and R$0.1milion for importers. This is controversial as it excludes small producers, such as family co-ops, a problem seen with the PROALCOOL programme for bioethanol form sugar cane [105].

The price of biodiesel is kept competitive through biodiesel auctions, administered by the ANP [102,105,110]. [105]. The price of biodiesel can be significantly higher than the cost of fossil diesel, but the pricing ensures biodiesel is economically viable in the market place [102]. This model of biodiesel acquisition requires producers and importers to purchase biodiesel according to their market share. Petrobras has a 93% market share, and this market position is used by the state to control the purchase price and minimum price for family farmers. This combination of policies has led to the Brazilian government being able to control biodiesel production without expenditure of public funds.

The government has set up other policies and ministries to stimulate the production and use of biodiesel, the main tool being the Social Fuel Seal (SFS) discussed below. The aim of the SFS is to promote social inclusion through job creation and technical assistance to family farmers, by providing federal tax relief [111]. By providing federal tax relief, the Sectoral Chamber of Feedstock and Biodiesel Production Chain aims to improve the efficiency of feedstocks and procurement channels, and drives research and development through the Business Technological Development Programme and Brazilian Biodiesel Technology Network [107].

Table 4.1 Examples of tax breaks under the Social Fuel Seal scheme

Tax reduction	Any feedstock, any region	Castor or palm feedstock North/North-east region
With Social Fuel Seal	80%	100%
Without Social Fuel Seal	67%	77.5%

4.1.2 Technical considerations

There are advantages to using biodiesel in a standard engine, particularly in Brazil where there is low turnover of vehicles, the average age of a truck being 18 years [112]. Biodiesel has a high flash point, a good lubricity, a high cetane number and potentially lower toxicity than fossil fuels which can reduce engine wear [113]. But there are issues too, depending on the type of oil feedstock, such as viscosity, cold filter plugging point (CFPP) and oxidative stability [114]. Soybean is the most common feedstock for biodiesel in Brazil. It is more stable than castor oil, a feedstock promoted by the PNPB for family farmers to grow. However, castor oil has good properties for operation in cold climates because of its low CFPP, due to the majority of the oil being monounsaturated. This would be important if Brazil was to decide in the future to focus on fuel export rather than their internal market production [12,115]. It also has a high cetane number for this reason. However, there are problems with the kinematic viscosity and cetane number of castor oil being too high for it to be used without blending. Palm oil, a growing feedstock in Brazil, has over 85% saturated and monounsaturated fatty acids, making it a stable fuel. However, a high CFPP makes it unsuitable without additives or blending. Based on these features, a biodiesel blend from all of these feedstocks appears a good technical choice for end application in Brazil's warm climate.

Whilst producing biodiesel from a variety of feedstocks is an important aspect of environmental protection, fuel security and social involvement, this does result in technical issues with quality control. Whilst biodiesel can be incorporated into any blend with fossil diesel (i.e. up to 100%) each type of oil has different physiochemical characteristics, leading to different quality biodiesels being produced. While some types of biodiesel (e.g soybean and palm) have national and international quality standards already available, such as ASTM D-6751 in the US or EN14213 in the EU, biodiesel from other feedstocks such as castor oil still require the development of standard test methods for quality assurance and control [103,116]. The Brazilian National Petroleum Agency is responsible for setting quality standards (i.e. Resolution ANP 42/2004), but in their case they focus on performance based parameters in the specification, classifying properties relating to the "quality of the process" instead of "nature of raw materials" as is measured in the EU and US. The aim of that approach is to comply with end market use requirements, mainly as a blend component and so as not to limit the diversity of biodiesel sources [117]. This works to complement the PNPB which aims to increase diversification of feedstocks in order to promote regional development and fuel security.

4.1.3 Social inclusion goals

Social participation rules were designed with corporate social responsibility ideals in mind, to provide income generation for those living on the poverty line, to diversify feedstock in order to promote stability and ecological integrity of the regions and to see integration of the cultivation of food and fuel. As such it is a key aim of the PNPB.

The social fuel seal (SFS) is part of Law 11.097/05, and is a set of incentives granted by the Ministry of Agrarian Development (MDA in Portuguese) to industrial biodiesel producers to purchase raw materials from family farmers. Family farmers are defined in Brazil as those who hold a piece of land smaller than 100ha, have the majority of their labour from family members, derive an income from activities on their own establishments and run their establishment with their family (Act 11326, 24/7/2006). The farmers are registered within a framework known as the "National Programme to Strengthen Family-run Agriculture" (PRONAF in Portuguese). A record is kept of each farmer, contracts entered into, social position and training needs.

Producers who are awarded the SFS must purchase a minimum of 30% (in the northeast, south and southeast) or 10% (in the north and mid-west) of raw materials from family farmers. In order to ensure companies participate with the SFS, the CNPE restricts participation in the biodiesel auctions to producers who hold a social fuel certificate, or those recognised by the MDA of being worthy of participation. The producers must also close contracts with the family farmers and ensure technical assistance and training is provided. Incentives include differentiated tax reductions depending on type of farm, region and crop, and access to financial facilities provided by the National Bank for Economic and Social Development (BNDES in Portuguese) [118]. The SFS was developed as a way of ensuring small primary material suppliers (i.e. family farms) could participate in the supply chain, thus redistributing income to poverty stricken areas. It also offers the opportunity for the creation of cooperatives that provide the farmers with support, and has the advantage of allowing democratic decision making and stronger presence during decision making. Many farmers have benefited from the SFS as they have received training and inputs from biodiesel producers, which has improved food security and increased biodiesel feedstock volume. Family farmers often only own 1-4 hectares, but the scheme encourages them to acquire more land because they receive minimum prices and advantageous supply contracts. This allows them to plan over a longer time period and invest more into the land than they might without the security the SFS offers [111].

Figure 4.2 Petrobras Biodiesel facility in Quixadá

Criticisms of the PNPB system exist. For example, social inclusion is threatened because rules restrict family farmers to the role of primary material production and supply and makes them reliant on industrial producers. It is the industrial producers who benefit from the high added value of the biodiesel production, but family farmers are excluded from this. There is also a rule included in the legislation that limits farmers to being suppliers of primary materials. Under the Law No.11.116-05, as mentioned above, biodiesel producers need a minimum amount of capital to partake in the auctions. The majority of family farmers cannot raise this amount, and hence are excluded from a part of the market which is often where most value is added to the product [105]. There has also not been a noticeable effort through the scheme to resolve land conflicts. The conflicts tend to lie between family farms and industrial farms, and often rural dwellers do not have legal documents to support their claims for land ownership [119].

An additional problem is the lack of technical support for family farmers due to insufficient resources having been invested in the scheme and poor management has led to projects being abandoned. It has also indicated that family farms do not have favourable conditions for negotiating contracts to sell their biofuel crops, despite it being a prerequisite for family farming [105]. This is a particular problem in the northeast where the market is dominated by a few industrial producers, namely Brasil Ecodiesel and Petrobras [105].

A particular problem observed in the state of Ceará, (and this may be applicable elsewhere), is that the family farmers have been unwilling to combine resources and therefore they remain as low-production, separate entities, not learning from each other's experiences and not combining knowledge or capital lending to an uneconomical and inefficient production system [120]. Research in the state of Rio Grande de Norte has found problems with cooperatives, based on cultural behaviours determined by mistrust and corruption [121]. On a national scale, there are many problems associated with the transaction costs and management due to the large number of participants. In 2013, 12.3 million people were employed as family farmers, and it was expected that 250,000 people benefited under the terms of the PNPB, and this accounted for 31% of the oilseed acquisitions in Brazil [122]. These numbers show progress towards the PNPB aims, as previously the family farmer's market share of biofuel crops was negligible.

There have also been problems with the timely delivery of finance. Although R$450million in loans were promised from the BNDES, there have been delays. A scheme was set up to provide funds to renewable projects which covers up to 70% of capital costs excluding land acquisition and imported goods or services [123]. The Bank of Brazil can also finance biodiesel projects through other mechanisms. The northeast has been particularly hindered by lack of finance and this has led to a reduction in investment in palm and castor oil production.

Concerns over food prices are an argument against the expansion of biofuel crop production. Food prices are affected by a number of factors. For example the agricultural production chain is dependent on weather conditions, speculation in the financial world, and final demand. In Brazil, results so far show that no soybean has been diverted from food for consumption in Brazil to oil feedstock, and instead there have been fewer exports [124]. Castor beans are grown in the same space as food crops, owing to castor plants being tall allowing food crops to being grown beneath them. This means there is no displacement of food crops [120]. Palm plantations, however, may see a displacement of crops or other land use. This will depend on development in the coming years.

The town of Quixadá in the northeast state of Ceará saw a Petrobras owned biodiesel plant open in 2008. The plant has a capacity of 96,000t/yr and uses predominantly soybean feedstock, with palm and cotton oil in addition. 37% of the fuel purchased by Petrobras qualified for social fuel label status in 2010.

As a result of the project, local residents have seen a rise in income for both farmers and those in the wider local economy. In 2010 63,034 families and 15 co-operatives were under contract with Petrobras covering an area of 165,430 hectares. Acquisition from the family farms reached R\$78million (US\$38million) in 2010. 650 technicians were employed to provide technical assistance to farms, with an investment of R\$24.2million (US\$12million) from Petrobras. Employment was generated during construction for 1,200 people directly and a further 400 indirectly. Currently there are 104 people employed as operational staff although these roles generally require specialist knowledge that does not exist at present in the local labour force.

Petrobras is also involved with education schemes that see farmers visiting local education centres to talk to students about their experience and encourage young people to become involved in agriculture. This is, in part, an attempt to stem the flow of young people to cities such as Fortaleza in Ceará.

Figure 4.3 Case study of the Petrobras biodiesel facility in Quixadá [120]

4.1.4 Environmental impacts

The positive environmental aspects of the PNPB policy are that it promotes ecological integrity within regions by providing tax incentives for different crops, depending on region. In the northeast for example, 100% tax relief is offered for castor and palm oil produced by family farmers. Growing food and fuel together is advocated where possible. There is also a National Sustainable Oil Palm Production Programme, which is an international initiative aiming to avoid problems made with regards to deforestation and habitat loss due to cultivation of oil palm. Further legislation supports the programme such as the forest code (4771/65), the Environmental Pollution Code (997/76), the Soil Conservation in agriculture code (6171/88) and the Environmental Crime Law (decree 6680/08) which came into effect 2011/12. However, there are no zoning laws controlling biodiesel feedstocks, as there are for sugar cane cultivation [123]. However, it might be considered a missed opportunity that no environmental criteria were added to the requirements for primary materials from family farms, such as which agricultural techniques are acceptable, which agro-chemicals are approved of or which tillage methods should be used [105].

Family farming has a much lower negative effect on the environment than large scale farms on land quality, biodiversity and deforestation. Traditionally, soybean was farmed on small farms that produced several crops for subsistence and the domestic market. However, the expansion of the global market and trade liberalisation have led to small scale farming decreasing and an increase in large single crop plantations, controlled by foreign trade organisations. Manual labour has been replaced by mechanised farming systems which are more economical for farming on a large scale [125][111]. The crops that have been chosen for biodiesel feedstocks in the northeast are ones that thrive in a semi-arid climate, and are also suitable for use in an integrated system. Intercropping schemes such as growing cover crops to protect from erosion have been shown to improve soil quality and increase land productivity without the need for further fertiliser use. Cover crops combined with no-till systems can also increase the content of soil organic carbon, which is a form of carbon sequestration[126]. A case study in Rio Grande de Norte showed how cultivation of sunflower seeds for biodiesel feedstock was linked with honey production and fish farming in order to increase the value of the product. Cultivation of sunflower was also deemed preferential to castor beans because the product is non-toxic and therefore can be used for cattle feed should there be a lack of demand for the crop for biodiesel production, compared with castor beans which contain the toxic compound ricin, and therefore cannot be consumed [121].

Table 4.2 Feedstocks acquired from family farms in 2013

Oilseed	Peanut	Colza	Palm	Sunflower	Castor beans
Acquisitions from family farms (million USD)	0.66	0.36	3.54	2.43	0.87

Whether biodiesel is carbon neutral, and whether its energy balance is preferential depends on the feedstocks, the cultivation methods, harvesting, processing and transportation between each stage from production to distribution. A number of lifecycle assessments have been done on various feedstocks to establish the energy balance. For example, a study on palms in Brazil found the greatest energy user is fertilisers, followed by irrigation and fuel [127] [128]. However, this study fails to include the impact of changing the land use, and different agricultural practices such as no till methods which reduce erosion and the release of nitrogen oxide emissions.

Although Brazil has good conditions for agricultural production across a vast area, it has critical logistical issues. Other than a few rail links in the south of the country, freight is carried by, in many cases, poor quality roads. As an example, the Petrobras factory in Quixadá sources its soybean feedstock from up to 650km away in a neighbouring state. The final product must also travel a significant distance to filling stations across the northeast. Whilst this provides jobs in the logistics industry, there is a high environmental impact of moving large quantities of goods over this distance for example emissions from diesel engines, construction and maintenance of fleet and roads [63].

A concern relating to biofuel production across the world is how expanding fuel crop cultivation will affect the land use, and consequently how this will affect emissions. Deforestation is Brazil's largest source of GHG emissions. In 2009, President Dilma Rouseff proposed to reduce carbon emissions by 38-42% by 2020 compared with 2005 levels, although this pledge is voluntary. Targets for reduction in deforestation rates are 80% in the Amazon and 40% in the Cerrado (area of wooded grassland in Brazil). Emissions from direct land use change are the emissions when one type of land use (i.e. fallow land, forest, grassland, agriculture of a particular crop) is changed to another. Different land uses provide a carbon sink of varying quantity, for example the rainforest provides a large store of carbon, both from trees and in the soil, quantified to 112.5MgCO_2/ha [129][130][131]. They also estimated the Cerrado stores 45MgCO_2/ha, although the Cerrado has a high capacity to sequester CO_2 due to environmental factors such as humidity and rainfall. In northeast Brazil it is the highly biodiverse grasslands of the Cerrado that are being transformed to soybean plantation. 47% of these lands have already been replanted, an area of over 96 million ha

[125]. It has been estimated that between 1-3% of the forest clearance in Mato Grosso du Sul, including Amazon and Cerrado, is due to expansion of soybean plantations specifically for biodiesel feedstock [132]. Changes in soil organic carbon were evaluated between native and agricultural land, and findings also showed that agriculture led to a reduction in the soil organic carbon [133]. With direct environmental impacts from added fertilisers, loss of biodiversity and soil erosions aside, this change in emissions may be the balance between whether biodiesel is carbon neutral or not. The expanding biodiesel sector is expected to play a part in reducing GHG emissions, therefore some irony exists in the level of sequestered CO_2 lost through deforestation to provide a sustainable fuel.

4.2 Methodology

The aim of the research was to identify where the PNPB has had positive impacts, identify the limitations in the existing system and create an opportunity to compare the existing programme with a new microalgae feedstock scenario. Initially, the current and historical situation for biodiesel production in Brazil was assessed to understand the background information. A systematic analysis was then performed in order to characterise the PNPB as an integrated technological, social, political and economic system. The intent was to identify in which respects the PNPB is “fit for purpose” and to evaluate how plausible modifications to the PNBP are. The purpose was to develop a way to identify existing positive and negative impacts of the system, and use them to explore the potential impact of introducing a new feedstock into the biodiesel supply chain. The existing literature has been assessed and information has been obtained from interviews with farmers to make an informed judgement of the system, based on the criteria set out as follows. A look at both the technological robustness of the system, the social fuel programme, the political and economic landscape and the environmental impacts has been taken. Next, the extent to which the PNPB has addressed each of them with regards to the goals set in the policy has been assessed.

Each design feature has been rated in terms of the PNPB’s design as having a positive impact, negative impact, split/uncertain impact or there being insufficient information to permit analysis. The measurement of overall “success” as perceived by participants in the PNBP, is subjective depending on where a participant is in the system. Four scenarios were evaluated, as follows:

1) Increasing feedstock productivity via further intervention from the government.
2) Mandating an increase in demand for biodiesel by increasing statutory blend volume.
3) Removing tax incentives for biodiesel feedstock production.
4) Introducing microalgae as an additional biodiesel feedstock.

This study is qualitative and is intended to provide a sense of the various impacts of the programme, both in its present form and where a new feedstock, i.e. microalgae is introduced. The outcome provides an indication of fitness for purpose of different design features within the Brazilian biodiesel programme, and how changing aspects of the programmes will affect the impact to each stakeholder.

4.3 Results

4.3.1 Assessment of the current process

The current situation was the first to be evaluated, in order to create an assessment of impacts observed by other authors and via interviews with farmers and people working in the biodiesel sector to create a base case of the existing experience against which to compare new scenarios. Interviews comprised a conversation between family farmer owners and students from the University of Ceará, where the interviewees were asked their opinion on the PNPB, observations they had made on the changes to their livelihood and local communities where relevant, and issues observed from technical and financial operation of plant processing the feedstocks. The responses from interviews were amalgamated with comments found in a range of literature to form a definitive comment on the operation of the PNPB.

The study presented in this section depicts the compromise that is made between the social and environmental impact and the technical and economic viability. A summary of the results of this analysis are given in Table 4.4, and have been rated according to the key in Table 4.3.

Table 4.3 Key to assessment study

Positive Impact	Negative Impact	Split/Uncertain Impact	Insufficient Data

The programme is making progress towards social inclusion and reducing negative environmental impacts, but this analysis reveals agricultural producers cannot comply with the technical demand of biodiesel feedstock needed and the programme is economically viable only with financial incentives that are provided through the legislation described above. The literature explains that social goals are being met to some extent, but the impact varies from region to region [105,111,119,123,134,135]. Some areas studied saw incomes and provision of access to education rise, plus multiplier effects into local communities where incomes had also risen (see case study in Figure 4.3). However, problems with the existing PNPB system include insufficient resources in terms of technical assistance and a lack of government intervention where this is the case, and restriction of access to parts of the supply chain where value is added to the crop.

The farmers providing biodiesel feedstocks were formerly engaged in subsistence farming and the importance remains of not impairing their ability to continue to grow food for their own use. Farmers frequently have more land than they have the manpower and tools to farm for food crops and so energy crops can be grown on land not used hitherto for food production. This is applicable for small farmers, as per the rules of the programme, where to be eligible for the programme the land cannot exceed 100ha but with no limit on productivity within this area. The current scale of the PNPB programme does not lead to competition of land between production of biodiesel crops and production of food, although this could be an issue if scaled-up production causes a shortage of cultivatable land. Also, certain plants can be grown together such as tall plants for biodiesel production (e.g. sunflowers or castor) combined with low growing food plants (e.g. beans). This may have further benefits such as reduction of soil erosion which is particularly relevant to castor bean cultivation where soil loss can be high [136]. However some crops are not suitable for this technique, such as palm which casts too much shade after reaching maturity [137]. Therefore, Table 4.4 shows the compatibility of growing a biodiesel feedstock with the social goals as having a positive impact.

The greatest environmental benefits are realised by the small farmers because they use low levels of agrichemicals and intercropping (which can be at the expense of higher yields). There are also benefits from diversifying crops as opposed to mono-culture in terms of biodiversity, maintaining soil quality and increasing resilience to crop failure, hence the positive rating given to environmental impacts in Table 4.4.

Once the feedstock reaches the production process, production of GHG emissions increases threatening cleaner production of biofuels. However, the use phase sees positive environmental impacts through the reduction of GHGs. Land use change is an important component regarding the environmental impact of biodiesel feedstock production. The type of land use change will affect the overall carbon balance of biodiesel production, as well as other emissions (e.g. N_2O) and biodiversity changes. The inclusion of land use change is beyond the scope of this project at this point, except to point out that increasing the volume of feedstock produced will certainly have an impact on the area of land under cultivation.

A major problem with the system is the distribution of materials. Due to a poor road infrastructure and an ageing fleet (as discussed in Chapter 2) the emissions associated with transporting of feedstock and the end product remains high. In the study, the capacity to supply feedstock and the distribution are linked and improvements in the distribution network would lead to a higher number of participants in the family farming scheme.

4.3.2 Increase feedstock productivity via more intervention

The current situation, analysed above in section 4.3.1 demonstrates the compromises within the PNPB, and the limitations in terms of technological and economic feasibility, as well as environmental and social change. The alternative considers how further intervention would change the environmental and social impacts of the programme. The intervention could take the form of more rural assistance, including education, introduction of machinery and chemicals or tighter controls on which chemicals could be used and agricultural methods employed.

Benefits of the system from a social point of view would be to increase the number of families that might be willing to participate in the scheme and to improve productivity. Time for farmers to adapt to the new measures needs to be allowed in order to see the benefits.

The impact of this scenario on the environment at the agricultural sources level depends on the scale of change. The average yield of soybean is currently 2.6 tonnes per hectare. The environmental impact of the agricultural source is a split impact under the assumption that the quantity of feedstock would increase, therefore the intensity of the farming would have to increase. Not all intensive farming is irresponsible, and if, for example, the flow of nutrients can be managed well, intensive farming can have positive effects. However, from experience of intensive farming methods in Brazil and other tropical regions, farming practices have not managed the land well leading to problems with excessive fertiliser use and soil erosion [126,138,139].

There would continue to be a problem with road and fleet infrastructure producing high levels of exhaust emissions. The environmental impact of the distribution phase is not easy to quantify. If the output from family farmers increases within the vicinity of the plant, this will reduce the quantity of feedstocks that need to be brought in from further afield. However, collecting feedstocks from many locations could potentially offset this benefit.

Costs would inevitably rise as a result of providing more assistance which would lead to a strain further down the supply chain, resulting in either higher fuel prices or cuts in changes to fiscal policy elsewhere. Greater acceptance by farmers of receiving help is also required, as cultural issues already compromise productivity where farmers do not share resources. The viability of this scenario depends on the government’s willingness to invest in the programme. Before investments are increased, it must be ensured that the investment is going to family farmers and is not being lost within the system for example for use by large farms where family farms cannot fulfil their quotas.

Table 4.4 Assessment of impacts from the current process (see key in Table 4.3)

Criteria for "Success"	Design features				
	Biodiesel Feedstock	**Agricultural Producers (Family farms)**	**Production Processes**	**Distribution**	**End Use**
Technical fitness for purpose	Able to produce biodiesel from feedstock oil	Capacity and yield low	Technically mature method for biodiesel production.	Logistical issues	Fuel properties meet standards for diesel fuel
Compatible with social goals	Cultivation of fuel crops does not compromise cultivation of food crops.	Income increasing to family farmers. Lack of cooperation and resources to provide for all participants.	Some job creation Restrictions on participation for small businesses	Data required on distributors and their participation with family farmers	Positive health impacts locally and globally from lower CO_2 and PM emissions
Economic viability	Only with financial incentive	Only with financial incentive	Technique depends on feedstock. Capacity exceeds supply.	Can use existing infrastructure	Subsidises distort cost comparison but remain competitive
Environmental Impact	Multi-cropping allows biodiversity and resilience	Fewer agrochemicals, lower carbon intensity	High chemical use, energy input and water consumption	Emissions associated with distribution by road	Lower CO_2 and particulates. Higher NO_x

Table 4.5 Increase supply by improved feedstock productivity via more intervention (see key in Table 4.3)

Criteria for "Success"	**Design features**				
	Biodiesel Feedstock	**Agricultural Producers (i.e. Family farms)**	**Production Processes**	**Distribution**	**End Use**
Technical fitness for purpose	Suitable charateristics	Capacity exists to produce more feedstock	Feedstocks can be readily incorporated into the system.	Logistical issues	Fuel properties could change if biodiesel blend rises
Compatible with social goals	Higher productivity from feedstock	Increase control reduces use of indigenous farming techniques. More resources improve standards.	Provide few more jobs in production facilities	Provide few more jobs in distribution	Positive health impacts locally and globally from lower emissions of CO_2 and PM
Economic viability	Economies of scale	Higher input cost for resources (e.g. technical assistance)	High volumes lead to economies of scale if well managed	High volumes lead to economies of scale if well managed	Biodiesel subsidy can be reduced but has to equal diesel price
Environmental Impact	Feedstocks suitable for region. Multi-cropping allows biodiversity and resilience	Likely to increase intensity of farming. More land needed for agriculture (land use change impacts)	Higher volumes of processing chemicals for biofuels but reduces fossil diesel processing	Increase in road transport leads to higher exhaust emissions	Lower CO_2 and particulates, higher NO_x from transport emissions.

4.3.3 Mandating an increase in biodiesel demand by increasing statutory blending volume

The current legislation requires 5% biodiesel content in all diesel fuel blends. This has created a market for biodiesel in Brazil and increasing the volume will lead to increased demand for biodiesel production. Whilst an increase of up to 20% is technically feasible [124], this scenario considers what sort of effect the higher blend volume would have on family farmers and the environment. Table 4.6 on p.58 shows there are many mixed impacts. An increase in the proportion of biodiesel in the blend volume is unlikely to have a positive effect for family farmers at present, due to a lack of resources such as technical assistance and machinery. A higher blend could also lead to pressure being put on the growing system, which in turn could lead to a reduction in space for food production. This is a problem particularly for subsistence farmers.

The benefits of increasing the blends are more likely to be felt by industrial scale farmers of fuel crops than family farmers, and also at the production, refining, distribution and end use stages. These later stages are where more value is added to the chain, but are also the part of the chain that small companies are restricted from accessing, as discussed above in section 4.1.3. It was found that after being awarded the SFS, biodiesel producers are not necessarily monitored any further, therefore there is no guarantee they will continue to use family farmers for their supplies. Biodiesel would remain competitive as producers would still be required to use the ANP auctions to sell their biodiesel to distributors [140]. An increase in the mandatory blend is unlikely to change this structure, except by potentially lowering the price due to economies of scale enabling savings to be made throughout the production chain.

Distributing higher volumes of biodiesel using existing infrastructure would create a technical challenge due to the quality and capacity of existing infrastructure. It is also a problem environmentally as more road freight by an ageing fleet will lead to high emissions from exhaust fumes causing a local pollution problem and contributing to global CO_2 levels. The environmental impact from the processing step is split into the negative and positive impacts. The negative impact is that of higher chemical (e.g. solvent and alcohol use) and energy use for the biodiesel production process. However the positive impact is that of reducing production of fossil diesel for each unit of biodiesel produced, as fossil diesel also has high energy and chemical demands. The by-products of biodiesel production, mainly glycerol, also need to be dealt with. Although glycerol can be used in other industries, it needs a high purity to be of value. Therefore there may need to be a comprehensive management strategy including technology development and incentives to incorporate glycerol into other supply chains.

Therefore increasing the statutory blending volume will not necessarily have any impact on the demand of crops from the family farmers unless further incentives are provided to biodiesel producers to buy feedstocks from family farmers. Time would also be required for family farmers to adapt to the increased demand. This time lag has already been observed with the current scheme. An alternative step that would improve the position for family farmers would be to allow their involvement in the processing of the oil seeds in order to allow them to add value to their product.

4.3.4 Remove tax incentives

This scenario examines whether the programme could be expected to stand alone without the tax incentives scheme, the summary of the analysis is shown in Table 4.7 on p.59. Removal of tax incentives will raise the price of feedstocks from family farmers. This scenario demonstrates how the technical aspect of the programme is compromised to allow for the social inclusion programme to work. Reducing the diversity of suppliers and obtaining feedstock from industrial farms will lead to a more uniform biofuel product which will help with standardisation of the biofuel quality.

Removal of tax incentives is likely to reduce the demand for feedstocks from family farmers, as there will be no financial gain in buying these crops, and there is no mandatory requirement to do so. There will also be a reduction in uptake of the SFS leading to reduced technical assistance to farmers. The result will be yields falling and it is likely a collapse of the whole system will occur as this is one of the key building blocks for the programme. Therefore, the Brazilian biodiesel programme needs to use either tax incentives or legislation to make the SFS mandatory in order to succeed. Removing the tax incentives alone would make the programme uneconomical due to high costs involved with buying feedstocks from small farmers and the cost of providing them with technical assistance.

There would also be an environmental burden as a result of this change in parts of the country, particularly with regards to loss of biodiversity and reduction in drought resilience from intercropping if land is changed from small hold farms to large scale mono-crop cultivation. On the other hand, if the land is changed to wholly subsistence farming this may result in the land quality remaining the same.

Table 4.6 Mandating an increase in demand by increasing statutory blending volume (see key in Table 4.3)

Criteria for "Success"	**Design features**				
	Biodiesel Feedstock	**Agricultural Producers (Family farms)**	**Production Processes**	**Distribution**	**End Use**
Technical fitness for purpose	High oil content crops required	Yield not high enough from family farms.	Capacity exists	Requires expansion	Fuel quality will change depending on feedstock
Compatible with social goals	Pressure to produce fuel could compromise food production	Increase income. Insufficient supply of resources to provide for all participants.	Provide few more jobs in production facilities	Provide few more jobs in distribution	Positive health impacts locally and globally from lower emissions of CO_2 and PM. NO_x may increase.
Economic viability	Greater tax incentive needed	Greater tax incentive needed	High volumes lead to economies of scale if well managed	High volumes lead to economies of scale if well managed	Biodiesel price may fall due to economies of scale benefiting distributors and consumers
Environmental Impact	Depends on crop cultivation techniques	Depends on crop cultivation techniques. Increase in land use change to agriculture	Higher volumes of processing chemicals for biofuels but reduces fossil diesel processing	Increase in road transport leads to higher exhaust emissions	Lower CO_2 and particulates. Higher NO_x

Table 4.7 Remove tax incentives (see key in Table 4.3)

Criteria for "Success"	Design features				
	Biodiesel Feedstock	**Agricultural Producers (Family farms)**	**Production Processes**	**Distribution**	**End Use**
Technical fitness for purpose	Market changes will make soybean the most economic crop standardising production	No incentive to provide technical assistance, likely to result in even lower yields	Less diversity in oil stock due to less input from family farms leads to more standard processing method	Logistical issues remain	More standard fuel
Compatible with social goals	Some feedstocks used as food or animal fodder where unsold for biodiesel feedstock	No guarantee of funding will put farmers at risk.	Data needed about potential loss of jobs at production facility	Data needed about how distribution impacts society	Positive health impacts from lower emissions such as lower PM and CO_2 will be reduced
Economic viability	Uneconomical to continue producing biodiesel this way	Uneconomical to continue producing biodiesel this way	Costs will increase because of changes to pricing (beneficial pricing from SFS)	Lower demand will reduce efficiency of transportation	Cost of biodiesel will be higher, therefore loss in demand
Environmental Impact	Loss of biodiversity	All supply will be from industrial farming.	No change from initial scenario assuming overall volume remains constant	More feedstock will need to be brought from large farms, potentially in other states	No change from initial scenario assuming overall volume remains constant

4.3.5 Microalgae as a biodiesel feedstock and as part of the PNPB

Developing heterotrophic microalgae as an alternative, more sustainable feedstock for biodiesel presents many opportunities to improve biodiesel sustainability, summarised in Table 4.8 on p.63. The reasons for selecting heterotrophic cultivation methods (in particular over autotrophic) were explained in Chapter 2. Briefly, the advantages are their ability to produce higher yields of biomass with a lower water demand than existing feedstock crops. Brazil in particular could benefit owing to its suitable climate (i.e. warm year round promoting faster growth), water availability and available land area. There is also potential for lower environmental impact than scaling up existing processes using terrestrial crops. Whilst there is no commercial scale production of heterotrophic microalgae biodiesel at time of writing, the potential oil yield is confirmed as being much higher than terrestrial crops by a number of sources. Large scale production of autotrophic microalgae can produce over 1000 times more oil per year than soybean for example [141,142] due to the fact it has a fast growth rate and can be harvested many times throughout the year, and heterotrophic microalgae could potentially exceed this [143].

To produce biodiesel from microalgae, the cell wall is ruptured and the lipid fraction is extracted, typically using solvent extraction. In a similar fashion to vegetable oil derived biodiesel, the microalgal oil can be trans-esterified to FAME, the primary constituents of biodiesel, using methanol and either an acidic or alkaline catalyst [144], discussed in detail in Chapter 6.

If microalgae are to be considered as eligible for the SFS it needs to be cultivated by family farmers. While it is possible to cultivate algae at any scale, growing microalgae at small scale for a large scale production process will be a technical challenge, and could be subject to the same or worse economies of scale than apply to small farms of terrestrial crops. The biggest challenge is the set up costs and infrastructure. Therefore it would be necessary to create an incentive, potentially in the form of an addition to the SFS which allows tax breaks for algae cultivated at larger scale sites.

The feedstock cultivation can still meet a social development goal - although not necessarily the one the PNPB had in mind as it is unlikely microalgae could feasibly be grown by small holder farmers. This is due to technical capability and resources including capital, construction and maintenance including fertilisers. The social development goal would be in using microalgae as a wastewater treatment technique. If microalgae are cultivated in wastewater the microalgae can absorb nutrients from the water leading to a cleaner water product and a free source of nutrients for the microalgae, discussed in detail in chapters 5 and 7. This will lead to health benefits from cleaner water and also environmental benefits, as this method will reduce impact of water discharge into open waterways by reducing nutrient content, therefore reducing toxic/uncontrolled algal blooms. Microalgae also lowers costs and GHG emissions associated with manmade fertilisers, making the overall production process cleaner [145,146,147,148]. If ponds are constructed on marginal lands, this could reduce the pressure for land and thus be beneficial in easing land conflicts in sensitive regions. Job creation would occur at the algae farms through jobs in cultivation, harvesting, and drying plus further work in engineering, consultancy or contingency for example. However, the algae would not be grown together with other crops, and therefore it would take labour resources away from the land which may be detrimental to subsidence farming practices. There will be health risks associated with cultivating algae in wastewater, as there will be pathogens present where the water comes from domestic or dairy sources for example, which is why this item is given a split impact.

The environmental impacts of microalgae biodiesel could be far reaching. Briefly, the demand for land use will be reduced; therefore more natural habitat could be retained. Heterotrophic cultivation of microalgae could utilise waste streams such as sugar cane waste or waste glycerol from the biodiesel process as a carbon source [26,74,149]. This is still difficult on a small scale, but this process has the potential to significantly increase lipid yields [150].

The production step still requires chemical use but there is scope for cleaner production and integration with existing industry. The energy use for harvest and drying is also high at the moment, compromising the overall energy balance of the energy contained in the final product compared with the energy put in to produce the final product [17,19,148]. This will be investigated by calculating an energy ratio in chapter 7.

Distribution of the product can use existing infrastructure which means exhaust emissions will remain high. However, if co-location of wastewater treatment, microalgae cultivation and refineries was included into the planning phase of a microalgae biodiesel project, this has the potential to reduce transportation and provide jobs locally.

The fuel quality and emission from microalgae need to meet or improve upon other biodiesel feedstock, So far, research has shown this is possible

[89]. The technological challenge of producing microalgae biodiesel still exists as the characteristics of microalgae oil differ to that of terrestrial crops and varies by strain, and much research is still needed into the combustion characteristics to ensure investment is really leading to development of a sustainable feedstock.

The economic feasibility of microalgae as a feedstock is a major hurdle. Using data from autotrophic algae farms in the USA, estimates have shown algae biomass is the most expensive component of the biodiesel production process (no data is currently available for heterotrophic cultivation systems, but there are similarities in the infrastructure requirements). There are optimistic projections for autotrophic microalgae production of as low as \$1.44/litre [151]. This could reduce further if nutrients could be recycled instead of buying virgin fertilisers. This is a promising finding, and particularly if algae can be processed in the existing facilities (i.e. there is no CAPEX for new infrastructure).

Table 4.8 Microalgae as a feedstock for biodiesel as part of the PNPB (see key in Table 4.3)

Criteria for "Success"	Design Features				
	Biodiesel Feedstock	**Agricultural Producers (family farms)**	**Production Processes**	**Distribution**	**End Use**
Technical fitness for purpose	Promising, although still problems to overcome.	Can be grown at any scale.	Extraction technology needs developing. Potential use of existing infrastructure.	Use existing infrastructure.	Research needed on combustion characteristics and comparison with standards.
Compatible with social goals	Growth in wastewater provides water treatment.	Infrastructure and knowledge needs make small scale cultivation unlikely. Jobs created elsewhere.	May be more jobs created in harvesting and drying.	Data required on distributors and their participation with family farmers	Lower combustion emissions. Cleaner water has health benefits Lower land demand eases land disputes
Economic viability	Depends on cultivation method and nutrient/water sources.	High costs for small scale cultivation.	Requires investment in infrastructure. Use of some existing facilities.	Use existing infrastructure.	Current estimate are higher than diesel although potential for costs to be reduced.
Environmental Impact	Water treatment.	Reduce loss of vegetation/ biodiversity due to lower land area demand.	High energy input into harvesting. Research into alternative methods.	Logistical issues leading to high levels of emissions during distribution	Uncertain of emission composition at this point.

4.4 Discussion

The PNPB has provided a unique opportunity for family farmers to access the biodiesel feedstock market by creating a market that is accessible to the smallest of producers. However, more needs to be done to make sure this contribution can continue and grow, and allowances must be made for these producers to access more of the production value chain. The contributions of different feedstocks contribute to both a more environmentally sustainable fuel and in some cases, a technically superior fuel, making it fit for its purpose. However, it is unlikely the volume of existing feedstocks can be expanded without causing detrimental effects on the environment or on local communities who may either be displaced themselves or see their food crops displaced by crops for fuel.

Whilst increasing the level of government intervention seems like an ideal solution to increase social inclusion, financially it is not a real world option. There are refinery managers who show the cost of providing technical assistance to farmers exceeds the tax rebates gained under the current system [140]. The cost of including either higher levels of terrestrial biodiesel or microalgae in the programme are a key part of whether the program can continue to be successful as an increase in costs will have an impact on both the biodiesel producer and the customers. The majority of freight transporters are diesel vehicles, therefore a rise in the cost of fuel will lead to inflation of food and other consumable goods. Mandating an increase in biodiesel volume through the PNPB would not help small farmers who do not currently have the capacity to produce more. The increase would also place more strain on the environment in terms of land use, land quality and biodiversity as the production would lead to further large scale cultivation.

The optimal solution is a combination of increased efficiency of resource distribution and use, and expanding the fuel matrix to include other feedstocks, such as microalgae, either as a blended product with soybean or as an alternative given that it is technologically feasible. These analyses show microalgae could be a suitable supplement to the biodiesel industry, assuming it is fit for use technically (as is analysed in Chapter 6), and delivering social and environmental benefits. Inclusion could also meet a political agenda for development of sanitation, and expansion of biodiesel production without compromising land use. As a result, the Brazilian government will have allowed development of a biodiesel blend which is more sustainable, maximises clean production of biodiesel fuel and is economically sound and environmentally friendly.

4.5 Summary

The relationship between government, business and society (i.e. small scale farmers) in Brazil is novel, and other countries could stand to learn how investing in society without the motivation of monetary gain can be effective in bringing people out of poverty. However, no net monetary gain either for the state or private business does not mean the scheme should be without broader benefit beyond financial gain. Brazil has addressed this by producing a useful product utilising the inherent skill set of the people involved. However, should the government pull out of the scheme; it would collapse as the product is not economically viable without government support, as shown by the scenario in section 4.3.3. This creates a sense of vulnerability and suggests further technological advances are needed to bring the cost of biodiesel production down so that it can compete with fossil diesel without government support. This implies looking to second or third generation biofuels such as microalgae, and developing the PNPB to include training and development for farmers on how to grow other feedstocks. The social inclusion policy still needs significant investment of money and resources from those running the scheme and trust from those participating.

A real question remains as to what the Brazilian government’s priorities for social development and environmental protection are. If social inclusion is the priority, this scheme has shown strength, illustrated through case studies referred to in this paper, yet proves problematic in many others. Focussing on environmental priorities would inevitably lead to more investment in research of alternative feedstocks, as expanding the existing feedstocks will extend loss to highly biodiverse areas and fail to reach a larger production quota.

The following chapters explore the feasibility of incorporating microalgae into the biodiesel supply chain. Some of the benefits have been suggested here, but the practicality is as yet relatively unknown. Therefore, the next chapters will investigate some of the areas of uncertainty, in particular cultivation of heterotrophic microalgae in wastewater, quality of the fuel produced and the energy ratio of this system. Results will determine whether this strategy will deliver the benefits suggested in here in this chapter and begins to give a clearer view of what the real technological feasibility of such as system would be.

Chapter 5 Heterotrophic microalgal cultivation in wastewater for a biodiesel feedstock

5.1 Introduction

The case for developing an alternative biodiesel feedstock was developed in Chapter 4, which was to find an alternative feedstock that is more environmentally, socially and economically sustainable than terrestrial oil seed crops and can also meet the technical specifications required for a blended biodiesel fuel. Heterotrophic microalgae have been suggested as an alternative in Chapter 2 and 4. The challenge is to produce oil from microalgae at a low cost, requiring innovative thinking on the process design.

The following chapters discuss the technical and environmental impacts of producing and using heterotrophically cultivated microalgae as a biodiesel feedstock. The cultivation of microalgae on the scale required for biofuel production faces a number of issues including growth rates, access to light and cell composition. Heterotrophic growth of microalgae could overcome some of these issues leading to significant economic advantages [20] and potentially many environmental benefits too. To date, the majority of research has focussed on autotrophic microalgae for biodiesel production. If it can be demonstrated that microalgae can be cultivated heterotrophically to produce high yields of good quality oil, then heterotrophic microalgae could become a contender for biodiesel feedstock on a large scale.

The literature referred to in the sections below introduces the requirements for heterotrophic microalgal cultivation and the opportunities for reducing the monetary and environmental costs of production using waste resources. The experimental work, presented below, tests the feasibility of cultivating microalgae on waste resources in a heterotrophic environment.

5.1.1 Heterotrophic cultivation

Heterotrophy is defined as the utilisation of organic compounds for growth. For the purposes of this thesis, heterotrophic alga is that which uses oxidative assimilation of an organic carbon source for the production of energy, in the absence of light and with oxygen as the final electron acceptor [152]. In a heterotrophic environment, microalgae are in competition with bacteria and can be disadvantaged by size, metabolic speed and versatility in changing environments. However, the ability to grow in the absence of light or a CO_2 supply puts these algae at an advantage over other phytoplankton species when these resources may be in limited supply.

Benefits of heterotrophic growth over autotrophic growth in terms of commercial application for biofuel production include higher growth rates leading to high cell mass, protein and lipid accumulation [153], as described in Table 2.3 . Higher cell densities can be achieved as there is no restriction on light penetration caused by shading by biomass, and bioreactor operation can be simple and remain axenic as a closed system [20,154]. However, a drawback is the cost of the organic substrate that will be required, and the system must be properly maintained to ensure it does not become anoxic, as heterotrophic species require oxygen for their metabolism [152]. Another point worthy of mention is the fact that heterotrophic microalgae do not absorb CO_2. Whilst photoautotrophic microalgae cultivation has been using the idea of CO_2 uptake to its advantage to market a low carbon fuel, the same may not be said for heterotrophic as it is a CO_2 source rather than a temporary sink [143]. That said, heterotrophic microalgae as a feedstock may still provide environmental benefits that other biofuels do not, such as not being in competition for land, ability to recycle waste for nutrients and potentially wastewater treatment, and low energy inputs. This is explored further in Chapter 7. There may be also lower energy requirements for refining, although this is beyond the scope of this thesis.

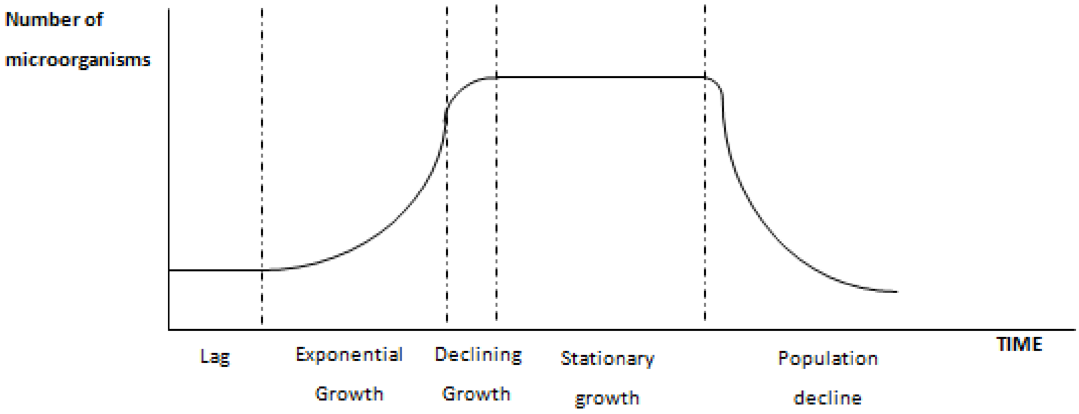


Figure 5.1 Typical growth profile of algae

There are five well-defined stages to algae growth, shown in Figure 5.1. The first stage is a lag phase, whilst the microalgae become accustomed to their environment. This is followed by an exponential growth phase. The duration of the growth phase depends on the strain of algae, the medium on which it is grown and the growth rate. Declining growth rate is the next stage, and it becomes apparent when there is a limiting factor which inhibits reproduction. This could be nutrient limitation, light limitation for autotrophic microalgae due to high density of biomass preventing penetration of light deep into the water or accumulation of inhibitors. The microalgae will reach a stationary growth phase where the net accumulation of biomass is zero. The cells undergo changes in this period, depending on what it was that limited the growth. If there is a nitrogen limitation, reduction in the protein content, and lipid and carbohydrate composition can change [155].

Cultivation of heterotrophic microalgae will typically take place in tanks or other enclosed containers. A number of factors will influence the growth, as discussed below. The key requirement for a heterotrophic species is the ability for diffusion of a carbon source across the cell membrane, and the presence of an enzymatic process which will incorporate the carbon into the central carbon metabolism [143].

5.1.2 Nutrients

Microalgae are highly adaptive to their environment and thrive by utilising nutrients available in the water body. A high surface area to volume ratio gives algae the potential to absorb large amounts of nutrients across their surface enhancing metabolic processes. The demand and rate of uptake of a nutrient depends on the strain and environmental conditions (e.g. temperature, light, limiting nutrients etc.) [156].

The essential macronutrients needed for algal growth are carbon (C), nitrogen (N) and phosphorus (P). A deficiency in one of these will limit the growth. In the ocean these nutrients are required by marine microalgae according to the Redfield ratio: 106:16:1 for C:N:P, yet specific research on heterotrophic *C. vulgaris* found under dark conditions a ratio of 73.5:12.7:1 was required [82]. However, other micronutrients are also essential for growth, including silica (S) and iron (Fe), trace metals and vitamins. The nutrients must be in a bio-available form for the microalgae to use.

Carbon is an essential nutrient required for biomass formation. It can be acquired by photosynthetic microalgae in an inorganic form from carbon dioxide via carboanhydrase activity. Heterotrophic microalgae cannot assimilate carbon in the same way and require an organic carbon source. In wastewater streams this would generally be by-products from the degradation of complex organic molecules, including acetate and other sugars from domestic and industrial liquid wastes e.g., wastewater from food or drink industries. It is assumed an additional carbon source would be required to ensure adequate levels of organic carbon were available for high growth rates.

Nitrogen can make up to between 1-10% of an algal cell (dry weight), and as such is one of the most important elements after carbon, hydrogen and oxygen. Metabolic routes for carbon and nitrogen are in fact linked in heterotrophic microalgae because they share the carbon assimilated from organic carbon and they also share the energy created in the oxidation of the carbon source for production of CO_2, ATP, and amino acid precursors (the citric acid cycle) [20]. The levels of nitrogen in a growth media have been shown to affect rates of growth and lipid accumulation, with high levels of nitrogen generally leading to high growth rate and low lipid accumulation [72,75,84]. The availability of the nitrogen source depends on the form in which it is present in a medium. The two most common inorganic sources of nitrogen are ammonium and nitrate ions. Ammonium ions are generally preferred by algae, although there are certain species which will prefer nitrate [157,158]. This has been shown to depend on growth stage and pH [159]. However, ammonium is generally preferred as it requires the least energy for uptake.

Organic nitrogen sources can also be used by some strains of algae. A number of studies have looked into the use of amino acids as the sole nitrogen source for microalgae e.g., *C. vulgaris* was grown in a medium containing 1% glucose and 56ppm organic nitrogen under light conditions [160]. The author identified a number of organic nitrogen sources that *Chlorella* would utilise, which included a few but not all species tested. The reason a certain amino acid could not be utilised include the inability of the *C. vulgaris* to metabolise it, or production of inhibitory metabolites. Another study discovered amino acids with an odd number of carbons served as the best substitute for nitrate, and higher growth rates were observed where no glucose was added to the solution, indicating that an organic carbon source, in this case glucose, may have an effect on the growth rate [161]. The ratio of carbon to nitrogen has been shown to be an important variable in lipid accumulation in some algae. A high carbon-to-nitrogen (C:N) ratio under heterotrophic conditions can increase the lipid content significantly [143]. This is particularly the case during the stationary growth phase, as synthesis of protein and other nitrogen containing compounds is reduced. Lipids are a preferred store of energy for cells as they are a more energy dense form of storage compared with carbohydrates [162,163]

Phosphorus is a key component to most cellular processes. In the form of phosphate, it is present in DNA, RNA, ATP and phospholipids in cell membranes. In photosynthesising plants, phosphorylated compounds are key in the conversion of light energy to biological energy [164]. Phosphorus is also essential for heterotrophs as it is key in the biological assimilation of oxygen. The optimum levels for phosphorus have been investigated by a number of authors, who have failed to reach a consensus other than that it is highly species dependant (e.g. [84,146]). However, even where phosphorus is present at very low concentrations, it can still be utilised and stored. High concentrations are potentially toxic to many strains with the tolerance for most species between 50µg l^{-1} – 20mg l^{-1} [165].

The use of microalgae as a method of phosphate recovery from wastewater is the topic of much current research due to worries of peak phosphate production for fertilisers since phosphate is a non-renewable resource [166]. Whilst a number of studies have looked at phosphorus removal from wastewater under autotrophic conditions [167,168], few have looked into heterotrophic conditions to date. Prathima Devi et al. (2012) found between 32-65% phosphorus recovery, depending on the ratio of C,N,P and K [169].

There must also be oxygen present in the liquid medium, as insufficient energy is available under anaerobic conditions for microalgae growth. Microalgae in general have been found to be obligate aerobes, that is, they are dependent on aerobic pathways. This is thought to be due to a lack of dehydrogenase in cells and consequent inability to re-oxidise $NADH_2$ anaerobically [152].

5.1.3 Microalgal biochemistry

The composition of the cell will change during the different growth phases, shown in Figure 5.1. Knowledge of when the cell may have optimum characteristics for harvest (i.e. the highest lipid content for biodiesel production) is therefore crucial.

5.1.3.1 Protein and inorganic nitrogen

Proteins are a variety of peptide-bonded amino acids. They perform a wide range of functions in a cell from catalysing metabolic processes to cell signalling and ligand bonding and are also used in cellular structures such as fibrous protein in higher plants and animals. Proteins are present in all cells, and are formed of nitrogen, carbon and oxygen. Microalgae will store nitrogen in case of shortage of supply in the environment, and this can be either as an inorganic form or organic form such as amino acids or functional proteins [170].

Not all the nitrogen present in the cell is protein; there is a certain level of inorganic nitrogen in the form of nitrate, nitrite or ammonium. The ratio of organic to inorganic nitrogen varied throughout the growth phase. Studies have found there are higher levels of inorganic nitrogen during the exponential growth phase, but that when nitrogen becomes a limiting nutrient in the media, intracellular inorganic nitrogen is consumed, demonstrating inorganic nitrogen is a nitrogen reserve in microalgae. The ratio of protein-nitrogen to total nitrogen will therefore increase during nutrient limited growth periods. The C:N ratio is an indicator for nitrogen limitation. Algae typically have a C:N ratio of between 1-20. Assimilation of nitrogen into protein is related to carbon availability, and the C:N ratio plays an important role in cell development. A study found the C:N ratio tends to be low during the exponential growth phase, with the highest ratio occurring during stationary growth [171]. Nitrogen limitation has been found to trigger lipid accumulation, therefore the nitrogen content of growth media and of algae is significant for biodiesel production.

Table 5.1 Optimum C:N ratio measured in algal biomass

Cultivation	Species	Ratio	Source
Heterotrophic	*C. sorokiniana*	23.5	[172]
Heterotrophic	*C. regularis*	5.7	[82]
Heterotrophic	*N. oleoabundans*	17	[143]
Autotrophic	Marine phytoplankton	6.6	[81]

5.1.3.2 Carbohydrates

Carbohydrates take the form of sugars, starches and cellulose. *C. vulgaris* have a rigid cell wall constructed from cellulose, whereas polysaccharides such as starch are used as energy storage. Because microalgae have a mainly-cellulosic cell wall, they are also an attractive feedstock for bioethanol production, as they would require little pre-treatment [15]. Bioethanol and biodiesel processes could utilise the same biomass, for example the lipids could be extracted for biodiesel production then the remaining lipid-extracted biomass for bioethanol, which would increase the fuel yield from microalgal biomass.

Carbohydrate has been observed to change by up to 10% depending on the harvesting point, and is also linked to nitrogen starvation, showing the highest carbohydrate content before the onset of lipid accumulation [92]. Studies have found that by adapting conditions and using engineering to adapt species, carbohydrate content of autotrophic *C. vulgaris* can be increased to achieve over 50% carbohydrate composition [173]. Factors that cause stress to the microalgae such as irradiance, nitrogen starvation, temperature variation, pH change or increased concentration of CO_2 can all cause changes in carbohydrate content too [15,173]. However, stressful conditions can also affect the lipid content (as discussed below) and therefore the optimum conditions of either carbohydrate or lipid production need to be balanced, depending on the product desired (i.e. lipids for biodiesel or carbohydrates for bioethanol).

5.1.3.3 Lipid accumulation

During growth, microalgae accumulate lipids within the cell. Lipids can cover a number of biochemical compounds. However they are grouped collectively as “non-water soluble” compounds that are soluble in organic solvents [57]. The way in which lipids are accumulated differs from higher plants due to the fact the fatty acid oil composition changes depending on the environmental conditions and stresses [165]. Lipids function as membrane components, storage products, metabolites, and energy sources in all cells. Lipids can be separated into two groups, “simple” and “complex” which refer to the number of breakdown products [174]. In turn these can loosely be defined as “neutral” and “polar”. The most common lipid class is fatty acids, linked by either an ester bond to glycerol (e.g. mono/di/tri-acylglycerol) or to other alcohols such as cholesterol, or by an amide bond to other amines. These lipids comprise the energy store in cells. More complex lipids are found in the cell membrane such as phospholipids and sterol lipids. The polarity of the lipid is of importance when selecting a solvent for extraction, as only the simplest lipids are desired for fuel production [175]. The neutral lipids can be extracted from the cells and can be transesterified into fatty acid methyl esters (FAME), creating biodiesel.

Studies to date have shown increasing lipid accumulation comes at the cost of reduced growth rates, even in genetically engineered strains, for example during a trial of a genetically engineered diatom *T. pseudonana,* aimed to reduce the catabolism of lipids during times of stress [176]. The breakdown of lipids is thought to occur after stress periods in order to provide quick release of energy in the form of free fatty acids and formation of polar lipids.

One author predicted maximum lipid accumulation could be achieved by firstly having fast growth under optimum conditions followed by changing conditions to nitrogen starvation for example, or stressing other inputs [165]. The composition of fatty acids depends on the species as well as the conditions.

5.1.4 Temperature and pH

To cultivate microalgae in Brazil, the ambient conditions need to be taken into account. Figure 5.2 shows that monthly average air temperatures across Brazil exceeds 20°C throughout the year, with the exception of the most southerly cities of Porte Alegre, Curitiba and Belo Horizonte. Although Rio de Janeiro is further south than Belo Horizonte, it is located on the coast and therefore temperature is moderated by the ocean, leading to higher temperatures particularly in June and July. These high temperatures could present problems for cultivation of microalgae in fermenters, as the internal temperature could be much higher due to metabolic activity. However, *C. vulgaris* has been shown to survive at temperatures up to 35°C, and there is potential for their growth to be adapted to new conditions.

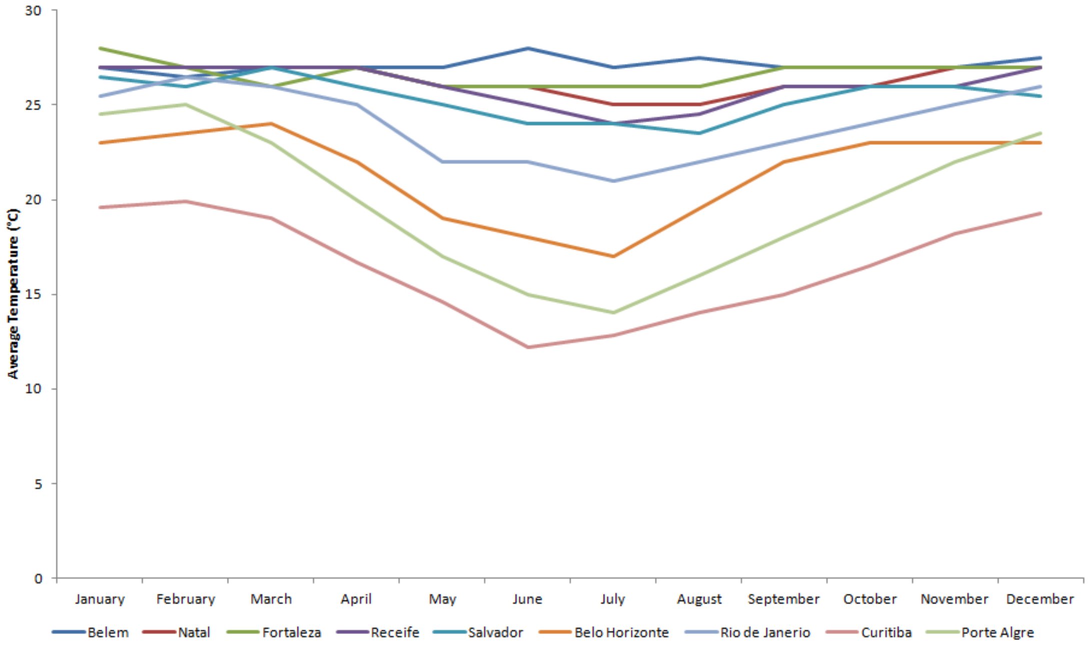


Figure 5.2 Average monthly air temperature in 9 cities in Brazil

Temperature affects the growth rate of algae, and the optimum temperature varies depending on species and strain. In general, optimum conditions exist between 20 – 30°C. Temperatures below 16°C will slow growth rates and temperatures above 35°C will kill many species, although *C. regularis* can be grown at 36°C [82], and some benthic microalgae have been found to survive temperatures in excess of 60°C [177]. Numerous studies emphasise that the relationship between autotrophic microalgae growth and temperature depends on the species. Some studies have found higher temperatures will benefit lipid accumulation. For example a study on *N. oculata* found lipid content doubled between 20-25°C [178], but a previous study using *N. laevis* found temperature of cultivation had little effect on lipid accumulation but temperature can affect TAG content, with it decreasing with decreasing temperature [179]. Work with *C. vulgaris* found temperatures between 25 – 30°C had little effect on lipid accumulation rates. The opposite pattern was found however, when growing species *Scenedesmus* sp. LX1, which displayed characteristics of storing lipids at lower temperatures [180]. To date, there have not been studies made on the effect of temperature on heterotrophic cultivation of these strains.

The tolerance of microalgae to pH is thought to be limited by either the chemical impact on the media or on the metabolic effect on the cells. The pH of the medium affects the availability of the nutrients, both due to the effect on the nutrient itself and because the pH has an effect on the electrical charge of the cell wall surface. For example, if the pH of the medium is lower than that of the cell, the cell will accumulate a weak acid, determined by the diffusion coefficient [181] . Cell walls tend to have a negative charge, giving an affinity to trace metals. This is of interest where microalgae may be used in wastewater treatment, as it may affect the accumulation of trace metals within the biomass. The range of pH at which microalgae can survive is wide, and it effects how the microalgae respond to nutrient availability. One particular study looked at how high pH affected inorganic carbon uptake in autotrophic *C. vulgaris*, discovering carbon uptake was severely limited above pH 8.9 [182]. It is unknown whether the same effect occurs with heterotrophic *C. vulgaris.*

5.1.5 Light and heterotrophic growth

Whilst the focus of this study is the cultivation of microalgae in the absence of light, it is worth noting that growth rates in some cases can be augmented where light and an organic carbon source is provided. The intensity of the light can have an impact, and there are certain strains of *Chlorella* that will not grow heterotrophically in dark conditions, but where a light source is provided they will utilise organic carbon below the photosynthesis threshold [183].

5.1.6 Heterotrophic cultivation using wastewater

Treatment of the required volumes of wastewater in Brazil is a problem because of rapid growth both domestically and industrially. Whilst Brazil has a large freshwater resource, care is needed to ensure resources do not become contaminated, and that equitable distribution of resources can be made, particularly in rapidly growing urban areas. Up until now, there are several studies where microalgae has been cultivated under heterotrophic conditions using a waste carbon source (e.g. [26,74,149,184,185]). The majority of these use either a basal media for heterotrophic growth or an artificial wastewater media, with only a few using real domestic wastewater source [27,169]. However, none of these studies have looked into combining wastewater with waste carbon sources.

Using reclaimed wastewater from industry, municipal wastewater and agricultural wastewater for microalgae growth is potentially a large resource for nutrient provision. The most common industrial sources are evaporative cooling water, especially from power stations, boiler feed water, process water and irrigation of grounds surrounding industrial plant [186]. The composition of the wastewater will affect the growth of the microalgae, therefore water analysis is needed prior to plans for a joined up system. High nutrients or contaminants in wastewater and the high costs involved in their removal make it an attractive nitrogen and phosphorus source [187]. Cultivation cost can be reduced because nutrients are provided in the wastewater, rather than them having to be added to the cultivation process. Therefore both the financial and environmental cost of their production is reduced, because fertilisers have to be neither produced nor bought. There could be challenges however with the quality of the water as it may vary and contain inhibitory substances.

A further benefit of using microalgae for wastewater treatment is that the microalgae will concentrate nutrients, as following harvesting and drying the algae will be in solid form and could be transported at low cost for use as a fertiliser for example. A complication to using microalgae as a water treatment process is the fact that water composition from industrial sources can be complex. Domestic wastewater tends to have lower biological oxygen demand (BOD) and chemical oxygen demand (COD) than industrial wastewater, but high inorganic content, therefore the correct microalgae strain should be identified first. A study investigating microalgae as a treatment option for municipal wastewater found microalgae reduced COD, nitrogen and phosphorus in the water, although the levels at which these nutrients were removed were dependant on environmental factors including CO_2 concentration [147]. Another study of microalgae cultivated in wastewater from carpet manufacturers effluent found a 96% rate of nutrient removal from the water [188]. Other industrial process waters such as soy sauce production, beer and brewing effluent, paper mill and pulp effluent have also been investigated as suitable media for cultivation of microalgae for biofuels and the use of algae for bioremediation [189,190,191]. This wide range of applications demonstrates the potential for microalgae to be used in this way, and justifies the need for further research into this area.

Previous work has also looked at heterotrophic microalgal growth patterns in sewage lagoons. *Chlamydomonas* was identified as the main species in an almost anoxic sewage oxidation pond in the Mojave Desert, California. Growth trials with the algae proved an organic carbon source was essential for its survival and growth [192]. More recently, a study has looked at cultivation of mixed cultures in domestic wastewater with the addition of major nutrients in combination of carbon, phosphorus or nitrogen supplement, or a combination of all three. The results showed phosphorus was the growth rate limiting nutrient, although when starved of carbon lipid accumulation was higher [169]. Another study isolated three strains (2 strains of *Scenedesmus* species and one of *Chlorella*) and cultivated these separately on a domestic wastewater which had been centrifuged to remove suspended solids and autoclaved, but no supplementary nutrients were added. They found the wastewater could support growth for these species for 4 days before it started to decline, and demonstrate no additional nutrients were needed for the cultivation. However, there are yet to be studies to demonstrate how productive microalgae cultivation grown on untreated water are, the range of growth rates that may be expected and how the toxicity of the environment might affect growth patterns [150].

Methanol can potentially be used by heterotrophic microalgae as an organic carbon source but it can be toxic at certain doses. The exact reason for the toxicity is not fully understood, but it may be due to formation of an intermediary product, formaline, which is toxic to algae. Experiments on dosage by one study found that microalgae *Chlorella minutissima* would withstand concentrations of up to 0.5-1% methanol, but beyond this methanol was considered toxic to the cells [193].

Although microalgae can thrive in chemical conditions that would normally kill other aquatic life, they are vulnerable to certain toxins. Herbicides and fungicides, such as sulfonylurea and imazlil sulphate, are toxic to microalgae, even at nannomolar concentrations [194,195]. These chemicals can enter the water ways via surface run-off from agricultural land. Sulphides can be found in detergents, which can enter the aquatic environment via wastewater. However, microalgae can also be used for bioremediation due to their capacity to synthesis certain heavy metals and other toxicants that may enter the environment. For example, *Spirulina* has been identified as an option for bioremediation of low concentration of lead in wastewater [196], whilst three species of *Chlorella* were found to remove Cadmium [197].

5.1.7 Organic carbon sources

Use of different carbon sources for heterotrophic microalgae cultivation has become the focus for many researchers investigating a low cost cultivation method, and is the topic of this research chapter. The issue for biofuel production is that the use of organic carbon by microalgae involves the conversion of one energy source to another. However, the energy content of organic carbon such as glucose or glycerol tend to be low and are not suitable for application in existing infrastructure as fuel, therefore a transformation is required. Heterotrophic microalgae can provide this service. Recent work includes looking at industrial waste products such as thin corn silage, soybean flakes, rice hydrolysate, sweet sorghum, Jerusalem artichoke tuber and crude glycerol [74,149,184,198,199]. The productivity of the microalgae will vary from species to species, and depending on different environmental conditions as well as the organic carbon source. Table 5.2 compares the biomass and lipid yield from various species and carbon sources found in the literature.

The type of organic carbon utilised by heterotrophs depends on the species. There are two main distinctions to be made between feedstocks, that they are either sugar feedstocks or acetate feedstocks. There are a number of algae species which will survive only on one feedstock or the other; however some species can use either. Within the range of substrates available in each category, certain species may prefer a particular substrate, for example acetate-utilising algae tend to prefer alcohols with an even number of carbons. Sugar-utilising algae largely prefer monosaccharides such as glucose, fructose or galactose, although some di-saccharides and polyhydric alcohols, in particular glycerol, are also found to be assimilated by sugar-utilising algae [152]. There are also a few species known that will utilise amino acids such as glycine as a sole source of carbon.

Glucose is one of the most widely used organic substrates assimilated by microbial species. This is probably due to its high free energy content (~13.8

kJ/mol for Glucose-6-phosphate) compared with other substrates (e.g. ~9.2 for glycerol-6-phosphate) [200]. Glucose is the simplest molecule to be broken down, and is done so via the process of glycolysis for energy production. The Embden-Meyerhof pathway is the most common type of glycolysis. This involves the glucose being broken down to pyruvate, releasing energy for ATP production. In order for this reaction to continue, organisms must be able to oxidize NADH back to NAD^+. Whilst bacteria can continue this reaction in anaerobic conditions, aerobic organisms such as microalgae require oxygen to continue the process. There are also other pathways that will lead to different products being metabolised from glucose, for example glucose assimilation by *C. vulgaris* leads to changes in cell size, starch, protein and lipid content, RNA and vitamin content [20].

Whilst CO_2 is an inorganic form of carbon that cannot be used to sustain heterotrophic growth, it can have an impact on the fatty acid composition within the accumulation of lipids. In a culture of *C. fusca*, increasing the CO_2 concentration from 1 – 30% saw a significant increase in not only the lipid content but also the composition, with lower amount of long poly-unsaturated fatty acids [201].

However, the utilisation of pure organic carbon feedstocks such as glucose or glycerol will prove expensive and energy intensive. Therefore alternatives must be found. There are benefits of using waste sources other than the lower costs because many wastes will contain additional nutrients that could increase productivity. “Crude” glycerol is a co-product of triglyceride transesterification, the process used for biodiesel production. During biodiesel production, triglycerides are mixed with alcohol (generally methanol) and catalysts to produce fatty acids. Up to 10% of the final product can be crude glycerol [149]. Crude glycerol can be up to 80% pure, the main impurity being water, although there are often low levels of methanol present from the transesterification reaction. There is a suitably sized market for glycerol, and pharmaceutical quality glycerol (99.5% purity) is priced at around £880 per ton in 2014. However, clean-up of glycerol is uneconomical and prices for crude glycerol remain low due to oversupply to the market from the biodiesel industry [202].

Molasses are a viscous by-product of sugarcane refinery. As such they are high in sugar content, but low cost. In Brazil, the expansion of the alcohol programme plus the large sugar market means a large volume of sugar cane is processed every year. Where molasses are further refined this leads to production of a silage which is still rich in nutrients. Brazilian sugar mills can release an average of 156l of silage per 1000kg cane processed, causing a significant amount of water pollution [203]. Waste molasses are strongly acidic, have potentially toxic levels of potassium and have a high COD and BOD [204]. The release of any waste from sugar mills can cause severe environmental problems if released into waterways such as rivers or estuaries [205]. Waste molasses can provide a good feedstock for heterotrophic microalgae cultivation. It contains around 50% sugars, plus other nutrients, proteins, fats and water [206]. The possibility of replacing an artificial medium completely using molasses was investigated, and found good biomass and lipid yields where they did so [205]. However, there may be additional benefits in terms of energy saving by using waste water as the medium due to the clean-up costs associated with treating sewage effluent.

Studies using other agricultural waste feedstocks include a study investigating the growth rates when *C. vulgaris* was cultivated using two industrial waste products; corn thin stillage (an acetate feedstock containing acetic acid, lactic acid and glycerol) and soybean flakes (a sugar feedstock containing sucrose, stachyose, galactose and glucose). The *C. vulgaris* exhibited higher cell mass and oil accumulation rates when grown using the corn thin silage feedstock than with soy flakes, and a diauxic growth pattern was observed in both cases. This is a result of the simplest sugars being consumed first, followed by a lag phase where the cell develops the ability to metabolise the second sugar source [74]. Sweet sorghum juice was found to increase lipid contents compared with a pure glucose feedstock [184]. Rice straw hydrolysate was investigated as a feedstock in China where there are large amounts of residue, and lead to maximum growth in only 2 days with 56% lipids [198]. However a pre-treatment of the rice straw was required adding time and energy to the process.

Table 5.2 Productivity of heterotrophic microalgae intended for biodiesel production on different carbon sources

Species	Carbon source	($g L^{-1}$) Biomass	(%) Lipid yield	Quantity Carbon (g/l)
C. zofingiensis	Glucose [185]	9.7	42.1	30
	Molasses [185]	12.9	50	30
C. kessleri	Glucose[207]	17.6	47.7	18
C. vulgaris	Glucose [208]	12.1	23	10
	Glycerol [208]	7.2	22	10
	Acetate [208]	9.87	31	10
	Ethanol thin silage [74]	9.8	43	4
	Soy whey [74]	6.3	11	4
	Modified bolds media [74]	8	27	4
C. protothecoides	Sweet sorghum[184]	5.1	52.5	10
	Crude glycerol [149]	23.5*	62	30
	Glucose [149]	15.3*	50	30
	Pure glycerol [149]	19 .2*	51	30
	Pure glycerol [26]	8.7	4.3	10
	Corn powder hydrolysate [89]	15.5	55.2	10
	Glucose [73]	15.5	46	10
	Molasses [205]	57.6	70.9	30
C. pyrenoidosa	Rice straw hydrolysate [198]	2.8	56.3	10
S. limacinum	Crude glycerol & corn steep [79]	-	51	100

*Batch cultivation

5.2 Methodology

The aim of this research is to investigate whether wastewater and waste carbon can be used to cultivate microalgae heterotrophically under conditions found in Brazil for biodiesel production. To achieve this synthetic wastewater was designed to imitate the nutrient levels found in raw centrate treatment ponds in Ponte Negra in Natal, northeast Brazil (shown in Figure 5.3). Due to restriction on lab equipment and space, the experiments were scaled down to 500ml, and for practical reasons of logistics and infection control, synthetic wastewater was used to allow for repeat experiments to take place in the University of Leeds under a controlled environment. This latter point is of importance as the composition of wastewater is liable to change from batch to batch. Therefore to gain an understanding of some of the mechanisms controlling growth a simplified experiment was required.

A control experiment was set up using a heterotrophic basal medium (HBM) optimised for heterotrophic growth and lipid accumulation and a synthetic wastewater (SWW) medium imitating the nutrients found in Ponte Negra, using the data supplied as described in Chapter 3. Pure glucose was used in the control experiments as one of the simplest sugars, free of any impurities. The variable introduced was the organic carbon source; either crude glycerol from biodiesel production or crude molasses. Each organic carbon source was added to both HBM and SWW media. All growth trials were monitored daily and were continued for 6 days after the beginning of the exponential growth phase. The procedures used, including equipment set-up and monitoring techniques and cultivation media composition are explained in detail in Chapter 3 (p. 22-26).

An additional experiment was run where a higher level of crude glycerol was added to the HBM and SWW media, to investigate the effect of increasing this waste feedstock on growth rates and lipid accumulation. In this experiment, 450g l^{-1} crude glycerol was added to the HBM and 100g l^{-1} to the SWW. Growth rates were measured using the techniques also described in Chapter 3. There were additional nutrients in the crude glycerol and molasses that were not present in the glucose feedstock. In particular, a higher concentration of sodium was found in the crude glycerol feedstocks compared with glucose or molasses, shown in Figure 3.3. The pH of the media became as high as 9 when the crude glycerol was added and was therefore neutralised using H_2SO_4, which also helped reduce foaming.

5.2.1 Crude glycerol characterisation

Before the crude glycerol was used as a feedstock, it was characterised using a number of techniques to determine physical, chemical and nutritional properties. The crude glycerol was obtained from East Yorkshire Biofuels (EYB) in Hull, UK. It was a by-product of biodiesel production from waste cooking oil feedstock. The biodiesel was produced by EYB by heating the oil to 40-50°C, followed by removal of any water. The oil was then passed through a 50 micron filter. Oil was next pumped into the reactor where a methoxide catalyst was added at a rate of 20% of the volume of oil. The mixture was heated to 80°C for the transesterification reaction to take place. Once the reaction was complete, the mixture was pumped into a settling tank where the crude glycerol settled to the bottom and was drained off and collected.

Table 5.3 Composition of crude glycerol from EY Biofuels (average of 3 repeats)

(detection limit 1ng l^{-1})

Composition	
Glycerol	13%
Methanol	12%
Water	54.7%
Ash	5.27%

Element	mg l^{-1}	Element	mg l^{-1}
Aluminium	16.8	Manganese	0.0
Boron	11.3	Phosphorus	53.0
Bromine	49.4	Potassium	61.5
Calcium	230.1	Silicon	26.5
Iron	10.2	Sodium	7001.8
Lithium	7.6	Sulphur	123.4
Magnesium	22.9	Zinc	13.9

The crude glycerol was dark brown in colour and received no further processing. A number of techniques were used to characterise the crude glycerol including high performance liquid chromatography (HPLC) to find the glycerol and methanol content, ICP elemental analysis to get a better understanding of the metal content, and the ash and moisture content were found via drying in an oven at 105°C for 48 hours, followed by a muffle furnace at 500°C for 2 hours. The composition is shown in Table 5.3, and is an average of 3 samples, all of which varied by up to 10%. The crude glycerol was found to have a high level of sodium, due to the sodium hydroxide catalyst used in transesterification. The presence of other metals such as calcium, iron, magnesium, potassium, silicon, zincs and non- metals such as bromine, phosphorus and sulphur were detected. Several of these elements could potentially have an effect on microalgae cultivation, in particular iron and phosphorus. The effect of iron on growth has been investigated previously and showed higher levels of lipid accumulation in media supplemented with iron [209].

5.2.2 Wastewater characterisation

The Ponte Negra treatment plant is a series of three ponds used to treat raw sewage effluent. Firstly the sewage enters the pond system as raw centrate into the primary facultative pond, and then it flows out to two maturation ponds in series. The projected volume of water that could be treated by the ponds is 8500m^3 per day by 2017. The ponds have been monitored since 2001 and nutrient levels and impact of the ponds on the underlying aquifer have been recorded since this time as 70% of the water for the city of Natal is supplied from the aquifer. A high rate of wild-type autotrophic algae grows on the maturation ponds.

In order to formulate a synthetic wastewater medium that represented the conditions found in Ponte Negra, Natal, but is suitable for heterotrophic cultivation, data from these ponds was used, as shown in Chapter 3. The concentration of nitrogen and phosphorus of the raw centrate were used as the basis for adaptation of the OECD synthetic wastewater medium, shown in Table 3.2 and the organic carbon was then added to reach the ratio stated in Table 3.3. The media was autoclaved to remove any organisms before the cultivation trials began. Although there would be many organisms present in the synthetic wastewater, this strategy was used in order to observe the behaviour of the *C. vulgaris* and identify patterns of growth before further parameters were introduced (such as competing organisms or parasites for example).

5.2.3 Determining nutrient limiting conditions

In order to investigate whether the level of carbon could have been limiting in the media, two techniques were used. The first was to measure the C:N ratio of the biomass using elemental analysis. The elemental analysis provided data on the total carbon and nitrogen content of the biomass, and therefore a ratio could be calculated to estimate which nutrient was limiting growth. Observed C:N ratio for heterotrophic and autotrophic microalgae are shown in Table 5.1. The range of values shown in the table made an estimation of a balanced C:N ratio for *C. vulgaris* difficult. Therefore, the organic carbon remaining in the media during cultivation could also be measured using the method described in Chapter 3. The results were then compared with the growth rates to see how carbon concentration corresponds with the growth dynamics.

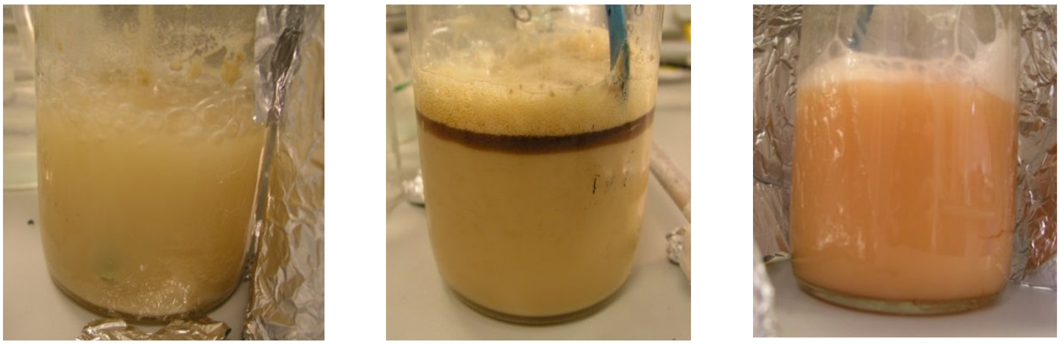
Figure 5.3 Location of the Ponte Negra facility in Natal, northeast Brazil (maps from Google©)

5.3 Results

Microalgae *C. vulgaris* was cultivated in two different media, a heterotrophic basal medium (HBM) and synthetic sewage medium (SWW). The media were supplemented with an organic carbon source; pure glucose, crude glycerol or unrefined molasses. The trial was started once the microalgae entered the exponential growth phase and harvested 6 days later, after which time it was dried for further analysis.

Initially, autotrophic *C. vulgaris* was cultivated under dark conditions with glucose to allow it to acclimatise to the new conditions. During the first 3 days of cultivation the pH was constantly adjusted to remain neutral. After 3 days a noticeable shift in the metabolism occurred where pH rose above 7 and dissolved oxygen (DO) dropped to below 1%, indicating respiration had become the principal operation.

Once acclimatised to the heterotrophic conditions, which was identified by a steady pH value, the microalgae was cultivated for 6 days using the medium on which the growth rates would be measured with the respective carbon source. The results of these growth trials are shown in section 5.3.1 for HBM and 5.3.2 for SWW. The composition of the microalgae is analysed to identify whether the carbon sources caused any significant changes in biomass composition in terms of lipids, proteins and carbohydrate content, the results of which are presented in section 5.3.3. This is followed by an analysis of the organic carbon uptake rates from the cultivation media in order to identify what may be affecting the growth rate and results from further growth trials with a higher crude glycerol content, in section 5.3.3.



Pure glucose **Crude glycerol** **Molasses**

Figure 5.4 Cultivation flasks showing *C. vulgaris* cultivated using three different organic carbon feedstocks. The photographs show the microalgae cultivated using HBM with (left to right) pure glucose, crude glycerol and molasses.

5.3.1 Cultivation using a medium optimised for high lipids

The first series of experiments were conducted by cultivating *C. vulgaris* using HBM, supplemented with pure glucose, molasses or crude glycerol, using the media shown in Chapter 3. The growth curves for these experiments are shown in Figure 5.5. The growth has been plotted from the beginning of the exponential phase. All trials show microalgae cultivation can be supported with the three different feedstocks in both HBM and SWW. However growth rate and exponential phase varies substantially. There is a good degree of variability in the growth pattern between samples using the same variables, which is to be expected when monitoring biological systems.

The length of the exponential growth phase varied for the different feedstocks. The length of the exponential phase was determined from the graphs in Figure 5.5, and was shown to last 6 or 7 days for crude glycerol and glucose feedstocks respectively, but only 4 days for the molasses feedstock. The fastest growth rate, measured by cell numbers, was observed in the molasses media, at 2.5g l^{-1} d^{-1} compared with 1.01g l^{-1} d^{-1} for glucose and 1.59g l^{-1} d^{-1} for crude glycerol. This indicates the cells in the HBM molasses were growing more quickly and hence consuming the molasses feedstock, thereby reducing the length of the exponential period. The greatest biomass weight was also observed where the molasses feedstock was used, and was measured at 9.99gTSS l^{-1}, closely followed by the crude glycerol media which measured 9.94gTSS l^{-1}. This was 30% higher than the glucose medium where the biomass weight measured 7.08gTSS l^{-1}.

The relationship between cell number and biomass weight was investigated by using a regression analysis, shown in Figure 5.7, to determine the accuracy of cell counting as a proxy for cell weight. A reliable relationship was observed between the cell numbers and the TSS for *C. vulgaris* cultivated with all three organic carbon feedstocks, with R^2 values of 0.88, 0.99 and 0.88 for glucose, crude glycerol and molasses respectively, and an R^2 of 0.95 where a higher crude glycerol concentration was used (discussed further in section 5.3.5). The profiles clearly show a difference in the cell development. Although a higher number of cells are present in cultures with glucose and crude glycerol, the weight of the biomass is lower compared with that for the culture containing molasses. This indicates the molasses cells are doubling more slowly but are gaining more mass; therefore the biomass at the end of the average exponential growth phase is still equal to that of the crude glycerol, despite the crude glycerol having a longer average exponential growth phase.

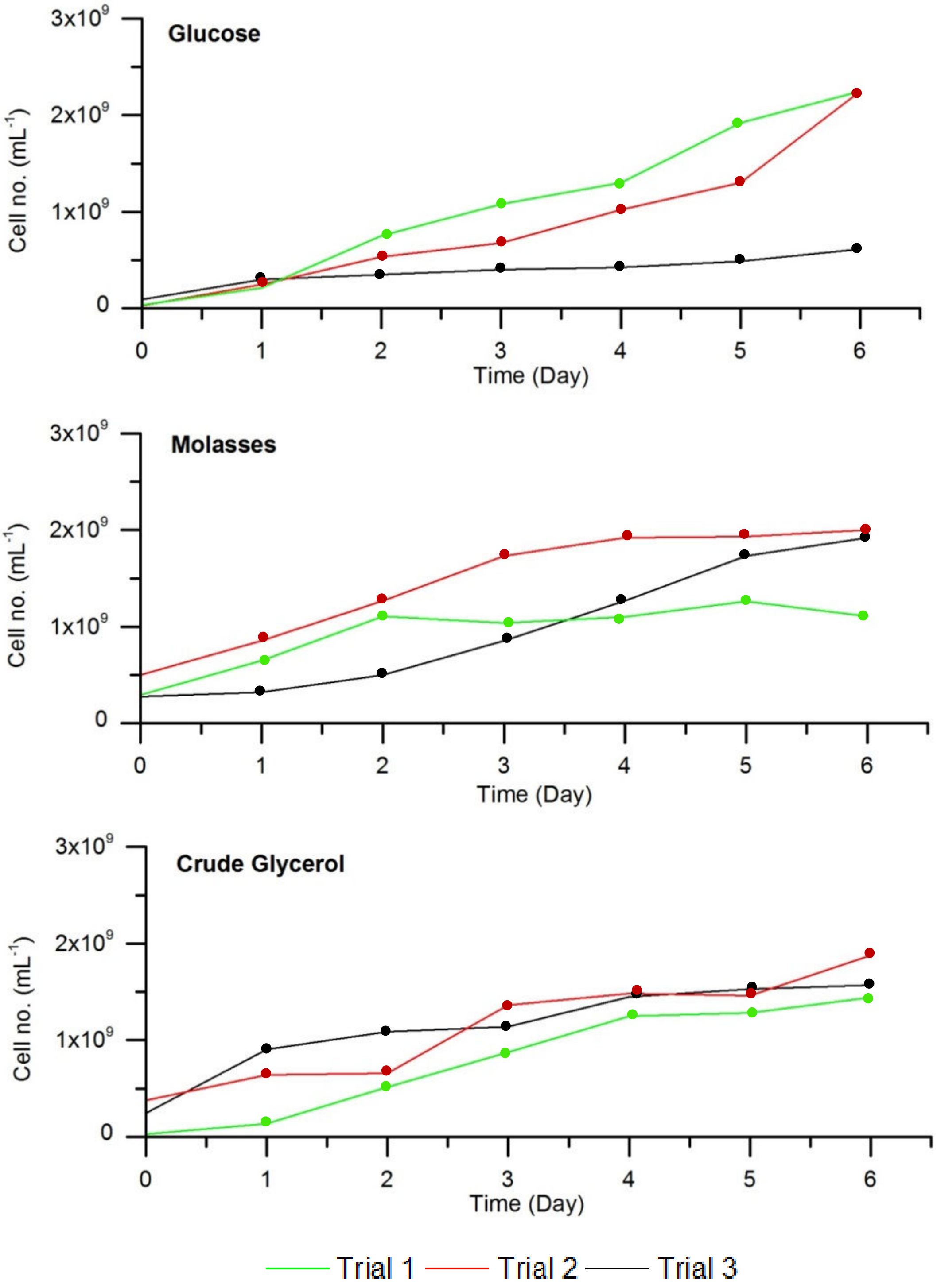
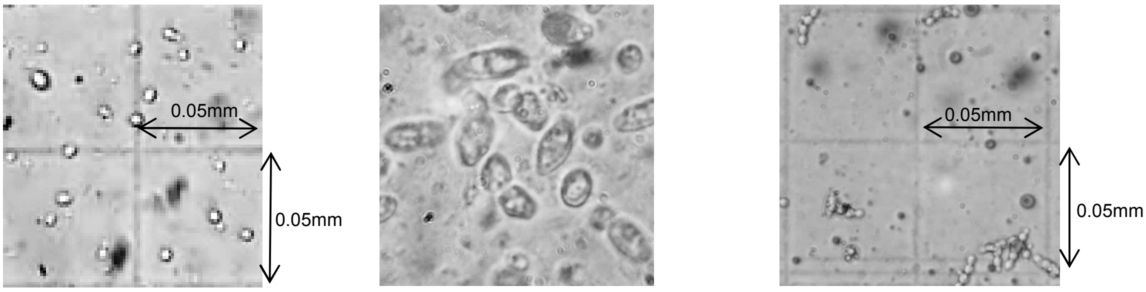


Figure 5.5 Growth curves for C. vulgaris cultivated in HBM with different carbon sources (top, pure glucose; middle, molasses; bottom, crude glycerol). The growth was measured by counting cells on a daily basis, and the graph shows an average of triplicate growth trials for each carbon source.

Table 5.4 Average growth rates and biomass accumulation in HBM

Organic carbon source	Media	Growth rate g l^{-1} d^{-1}	Exp. growth phase (days)	Maximum biomass (gTSS l^{-1})	Lipid content (%)
Glucose	HBM	1.01	7	7.08	22
Molasses	HBM	2.50	4	9.99	18
Crude Glycerol	HBM	1.59	6	9.54	38

*Measured at the end of the exponential growth phase



HBM + Glucose **HBM + Molasses** **HBM + Crude glycerol**

Figure 5.6 Appearance of cells cultivated in the feedstock (labelled below the photograph). Photographs of HBM + Glucose and HBM + Crude glycerol were diluted by a factor of 10 and photos were taken using an camera attached to a *Olympus BH-2* microscope with 40x magnification. Photograph of HBM + Molasses was taken at 100x magnification at a factor of 10 dilutions to give a clearer picture of the cell shape.

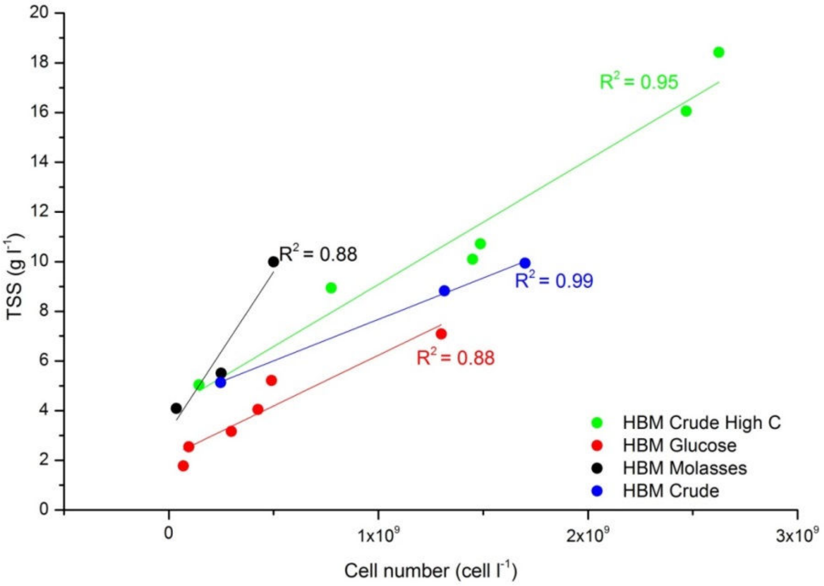


Figure 5.7 Relationship between biomass weight and cell number cultivated using HBM with different carbon feedstocks.

It is clear from the growth trials that the type of feedstock has an impact on the growth rate of the microalgae, and it also has an effect on the physiology of the cells. The cells were photographed using a camera attached to an *Olympus BH-2* microscope. The images in Figure 5.6 show there are differences in the cell sizes and shapes, and also patterns of growth. For example, the cells grown with molasses feedstock are more oblate compared with the glucose and crude cells which tended to be spherical. The cells grown in HBM + crude glycerol had a tendency to agglomerate in small chains, although this was not a consistent pattern. No relationship was found between pH and flocculation tendencies either.

5.3.2 Synthetic wastewater medium for heterotrophic cultivation

The synthetic wastewater was based on the OECD recipe for synthetic wastewater [85], and the design was adapted to imitate conditions found at Ponte Negra sewage treatment facility in Natal, Brazil. This led to a medium that differed considerably from the HBM, in particular in terms of the N:P ratio which was in excess of the 11:1 ratio observed in heterotrophic biomass [82] and was closer to the ratio of 16:1 observed for marine life [81] (i.e. 16:1 in SWW compared with 3:1 in HBM, shown in Chapter 3, Table 3.3), meaning the medium was less nitrogen limiting.

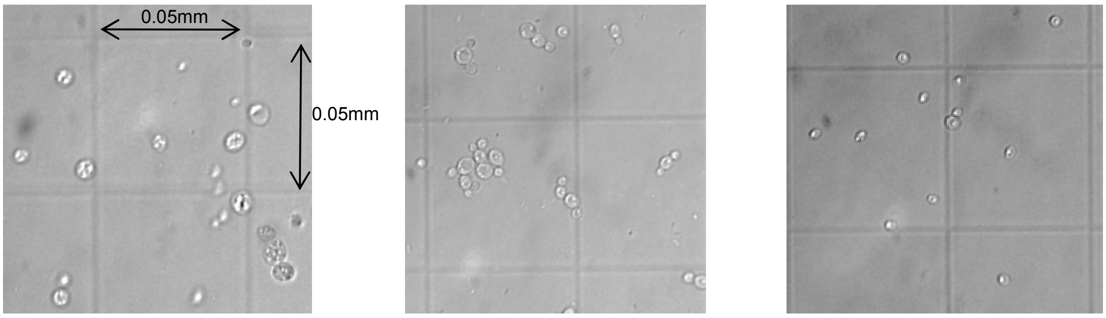
The most noticeable difference where the *C. vulgaris* was cultivated using SWW was the length of the exponential phase, evident from the growth curves in Figure 5.9. The exponential growth period was less than half that of growth rates observed where HBM medium was used, shown in Table 5.5. This resulted in lower levels of biomass in terms of TSS for all trials using SWW, with a maximum TSS gained from the molasses SWW of 1.44g TSS l^{-1}, compared with 1.24g TSS l^{-1} for crude glycerol SWW and 1.04g TSS l^{-1} for glucose SWW. Growth rates were also lower for glucose and crude glycerol feedstocks. In particular, the growth rate where glucose was used as the organic carbon feedstock was only 0.23$gl^{-1}d^{-1}$. This resulted in biomass accumulation levels reaching only 76%, 48% or 55% of the biomass accumulated in HBM for glucose, molasses and crude glycerol respectively.

The appearance of the cells was different for the different feedstocks. The cells grown in the glucose SWW appeared larger in general, whilst the cells grown in the molasses SWW were more spherical than ovoid, shown in Figure 5.8. The molasses SWW cells were more prone to flocculation, but this was not an issue with the cells in the glucose SWW or crude glycerol SWW. The reasoning for this is unknown but could be caused by a secretion by the cell, cell charge or consistency of the media. Counting of cells was problematic towards the end of the growth trials for molasses due to flocculation. This was overcome by ensuring the culture was homogeneously distributed by shaking of the flask prior to a sample being taken and taking repeated measurements using the haemocytometer. Duplicate measurements of TSS were also made, and this reduced the error margin. The cells grown in crude glycerol SWW remained smaller than with the other two feedstocks. Whilst comparison of cell size could be made from the images; actual measurements were not made due to the resolution of the microscope used.

Table 5.5 Average growth rates and biomass accumulation in SWW

Organic carbon source	Media	Growth rate ($g\ l^{-1}\ d^{-1}$)	Exp. growth phase (days)	Maximum biomass* (g TSS/l)	Lipid content (%)
Glucose	SWW	0.35	3	1.04	12
Molasses	SWW	0.46	3	1.40	15
Crude Glycerol	SWW	0.41	3	1.24	47

*Measured at the end of the exponential growth phase



SWW + Glucose **SWW + Molasses** **SWW + Crude glycerol**

Figure 5.8 Appearance of cells cultivated using SWW feedstock (labelled below the photograph). Photographs of were taken of culture after it had been diluted by a factor of 10 and photos were taken using an camera attached to a *Olympus BH-2* microscope with 40x magnification.

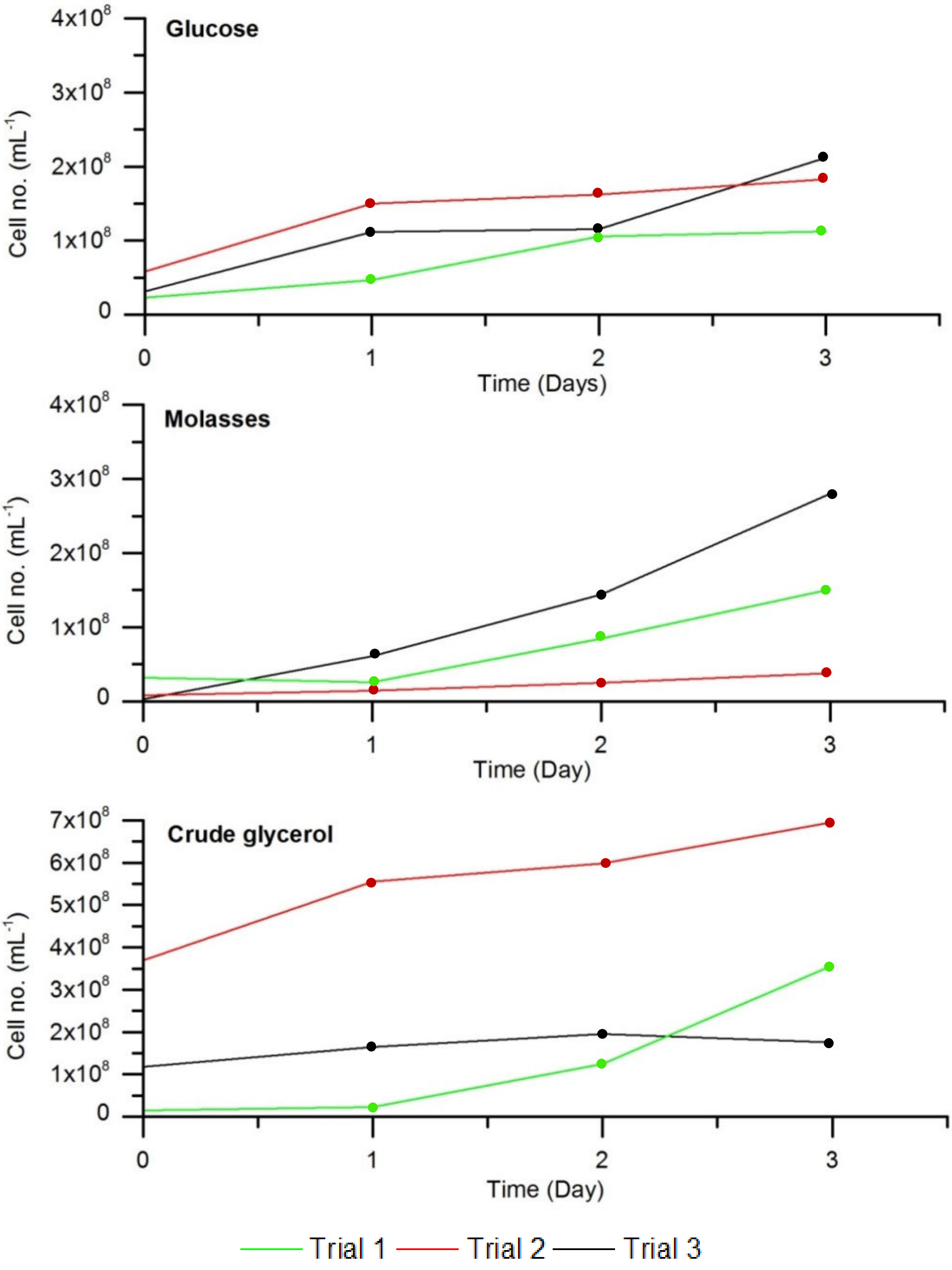


Figure 5.9 Growth curves for *C. vulgaris* cultivated in SWW with different carbon sources (top, pure glucose; middle, molasses; bottom, crude glycerol). The growth was measured by counting cells on a daily basis, and the graph shows an average of triplicate growth

5.3.3 Algae composition

The *C. vulgaris* cells were free of any chlorophyll compounds and had a yellow to brown appearance, shown in Figure 5.10. The medium with crude glycerol separated to leave an oily layer on top of the medium, however this was mixed once aeration was added. Once aeration was added to this medium, it caused foaming to occur. However, the foaming was prevented by acidifying the medium using H_2SO_4 (pH 6.5 or below).

Further analysis was carried out on the harvested biomass to investigate the impact the media had on the composition of the biomass. The biomass was harvested and dried, then analysed for carbohydrates, protein, lipids and ash content using the methods described in Chapter 3, the results of which are shown in Figure 5.11.

The component of greatest importance in organic feedstocks for biodiesel is the lipid content. From the results in Figure 5.11 it is clear the lipid content was higher where the crude glycerol feedstock was used, in both HBM and SWW. However, the largest error is associated with determining the lipid content, shown in Appendix B. The error could range between 2-14% based on the standard deviation of the lipid content measured by three repeats of lipid extraction on samples from the same cultivation trial. The trend that emerged was a higher lipid content where crude glycerol was used as a feedstock, reaching 40% for HBM and 47% for SWW. The lowest lipid contents were measured from the SWW glucose and molasses of 12 and 16% respectively where the lowest N:P ratio was also observed.

The protein content observed in the heterotrophically cultivated *C. vulgaris* was 37% for glucose HBM and 35% for molasses HBM, whereas the glucose and molasses SWW had lower protein contents of 20% and 19% respectively. The media with crude glycerol saw the opposite trend, with higher protein content from SWW (20%) than from HBM (6%). The protein content of the microalgae cultivated in SWW was similar for all three feedstocks.

Where *C. vulgaris* was cultivated using HBM, the carbohydrate content was higher than where SWW was used, although the difference in carbohydrate content was 12.5-24.1% overall. The lowest carbohydrate content was found in the SWW Crude glycerol, where the highest lipid content was also observed.

Figure 5.10 Heterotrophic microalgae cultivated on (left to right) HBM Glucose, HBM Crude, HBM Molasses, SWW Glucose, SWW Crude, SWW Molasses after harvesting and freeze-drying

A mass balance of the measured biochemical components was constructed in Figure 5.11. This included the lipid, protein, carbohydrate and ash content, measured on a moisture free basis. The errors associated with measurements of the lipid, protein and carbohydrates are given as a total (as a standard deviation from the mean). The error for the carbohydrate measurement was between 0.56 (HBM Crude and SWW Glucose) and 4.97 (SWW Molasses), and the error associated with protein measurements was between 0.72 (HBM Glucose) and 1.22 (HBM Crude). A full list of the components and errors is given in Appendix A.

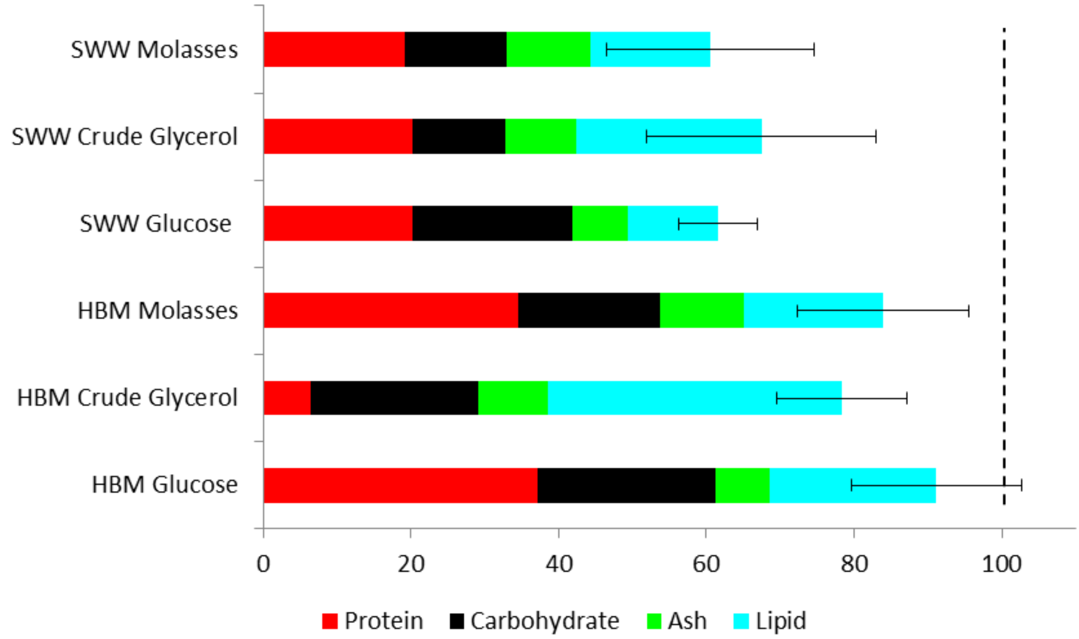


Figure 5.11 Composition of algal biomass on a dry basis, when cultivated on different media and different organic feedstocks. Errors associated with lipid content are shown.

5.3.4 Carbon as a limiting nutrient

In order to investigate whether the level of carbon could have been limiting in the media, two analysis techniques were used; the C:N ratio of the biomass and the organic carbon content of each medium during cultivation were detected.

The C:N ratio was calculated using elemental analysis, using the method described in Chapter 3. The C:N ratio could be used to identify where there may have been limitation in the nutrient supply. The results in Table 5.6 show different media had very different impacts on the C:N ratio. The HBM glucose and molasses saw a low C:N ratio of 4.9 and 5.4 respectively, whereas the HBM Crude had a much higher ratio of 26.9. The SWW feedstocks all showed a similar ratio of between 13 and 15.

Table 5.6 C:N ratio in heterotrophic algal biomass

Biomass	C:N ratio
HBM glucose	5.4
HBM crude	26.9
HBM molasses	4.9
SWW glucose	13.0
SWW crude	14.3
SWW molasses	15.1

The total organic carbon content was measured using a TOC elemental analyser with samples being taken every three days. This was compared with the growth curve, shown in Figure 5.13**.** All feedstock show a rapid drop in organic carbon in the first three days of cultivation. This coincides with the highest growth rates. Where a higher organic carbon (crude glycerol) concentration was used (see section 5.3.5), the same pattern existed (i.e. a fall in the first three days). However, there was still a significant amount of carbon left in the medium after 3 days (i.e. over 1g/l) and this did not change later on. This suggests another nutrient is limiting growth after 3 days, leading to a reduction in carbon uptake from the medium.

The sample was further analysed using HPLC. The results for SWW Glucose show the majority of the organic carbon is present as glucose, whereas the SWW molasses has the majority of the organic carbon as sucrose/maltose, with lower levels of glucose, xylose and glycerol. After 3 days there were only trace levels of sugars. A peak was observed near the beginning of the spectrum, but has so far been unidentified. Due to the retention time of the peak being less than that of any of the sugars and alcohols it may be possible that the peak is a light organic acid such as glycolic acid. A study found 0.8% of the organic acids produced by phototrophic marine *Chlorella* were in the form of glycolic acid [210], therefore it is possible that such a mechanism for its formation exists in heterotrophic algae. Small amounts of oxalic, citric and acetic acid were identified after 3 and 7 days of cultivation.

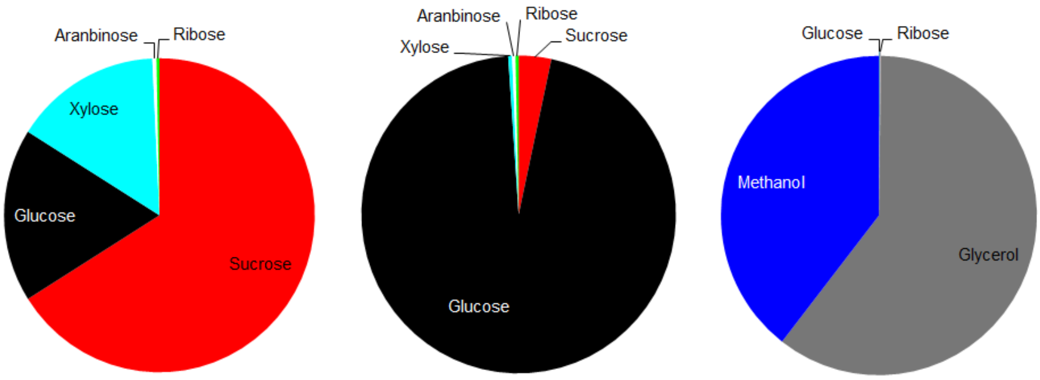


Figure 5.12 Carbohydrate compositions of SWW media (left to right, SWW with molasses, SWW with glucose and SWW with crude)

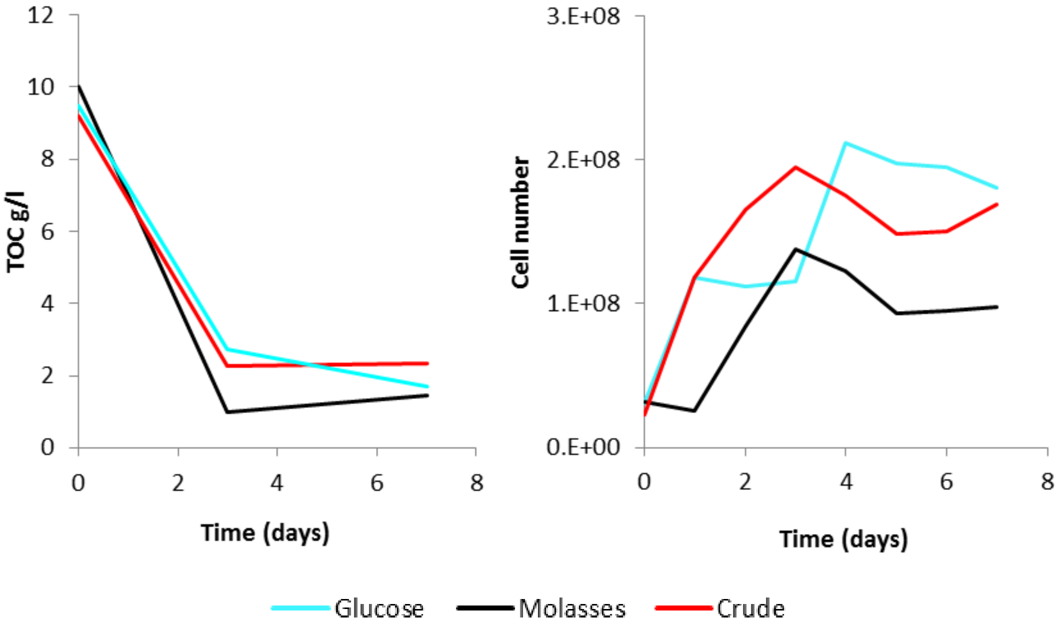


Figure 5.13 The uptake of organic carbon is shown in the left graph over the cultivation period, and is plotted (right graph) against the cell growth rate from the beginning of the cultivation period (i.e. before the exponential phase begins)

5.3.5 Increasing the organic carbon concentration to increase the biomass yield

The levels of biomass obtained from the media with only 10g l^{-1} organic carbon were not high enough to produce a feasible biodiesel feedstock. Therefore an additional set of cultivation trials was carried out to investigate whether increasing the organic carbon concentration would lead to higher productivity of heterotrophic algal biomass. These cultivation trials were carried out using crude glycerol as the organic carbon feedstock and increasing the level to 450g l^{-1} in the HBM and 100g l^{-1} in the SWW. The growth rates and total biomass was measured at the end of the exponential phase.

Increasing the crude glycerol concentration led to significant increases in the biomass yield (TSS g l^{-1}), shown in Table 5.7. Both the exponential growth phase and the maximum biomass accumulation increased, leading to a three-fold increase in biomass in the HBM and over ten-fold production in biomass for the SWW media. The lipid content also increased for the HBM from 38 to 52%. The lipid content of the SWW biomass remained at a similar level rising slightly from 47 to 48%.

Table 5.7 Growth parameters and algal characteristics where *C. vulgaris* was cultivated using higher concentrations of crude glycerol

Organic carbon source	Media	Growth rate ($g\ l^{-1}\ d^{-1}$)	Exp. growth phase (days)	Maximum biomass* (g TSS/l)	Lipid content (%)
Crude Glycerol (high C: 450g l^{-1})	HBM	2.18	13	28.4	52
Crude Glycerol (high C: 100 g l^{-1})	SWW	3.06	6	18.4	48

*Measured at the end of the exponential growth phase

There were observed differences in the C:N ratio where a higher concentration of carbon was added, shown in Table 5.8. The C:N ratios observed for the HBM and SWW Crude High C were similar despite more crude glycerol being added to the HBM media than the SWW. The main difference between the compositions of the two types of biomass is the lipid content, with much higher lipid content in the HBM Crude High C than the SWW Crude High C (52% compared with 24%) shown in Figure 5.14. The error on the lipid content was 8.72 for the HBM and 2.11 for the SWW, and therefore this does not account for the difference, indicating an alternative mechanism has promoted lipid accumulation.

Table 5.8 C:N ratio in heterotrophic algal biomass

Biomass	C:N ratio
HBM Crude High C	45.9
SWW Crude High C	48.5

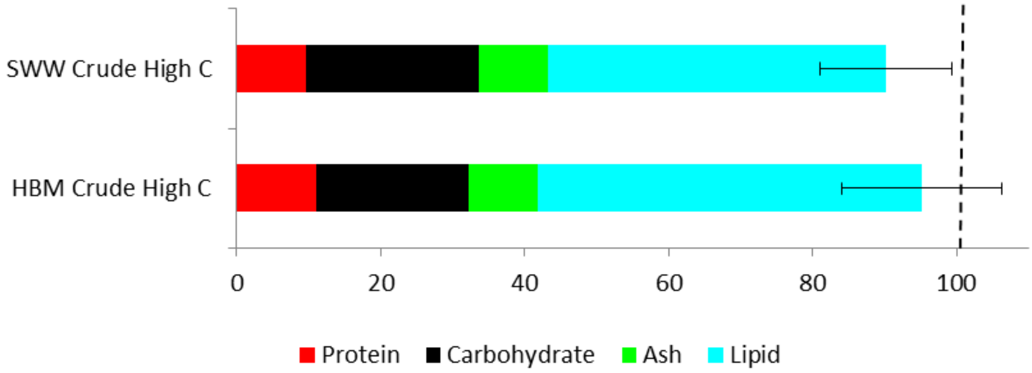


Figure 5.14 Biochemical composition of heterotrophic *C. vulgaris* cultivated using excess organic carbon.

The total organic carbon content of the media was measured for the media, and showed the organic carbon levels were not depleted as quickly as in the low carbon media. Carbohydrate composition was determined using HPLC and the profile obtained for the HBM Crude High C found the feedstock was mainly composed of glycerol and methanol. These levels were monitored over the next 11 days, and it can be seen in Figure 5.15 that the glycerol was taken up most quickly by the microalgae leaving only trace levels after 3 days, but that the methanol was consumed more slowly giving rise to a slower TOC reduction overall. The growth pattern for the crude glycerol was also different, as it exhibited two exponential phases, the first between days 4-6, and the next between days 7-9.

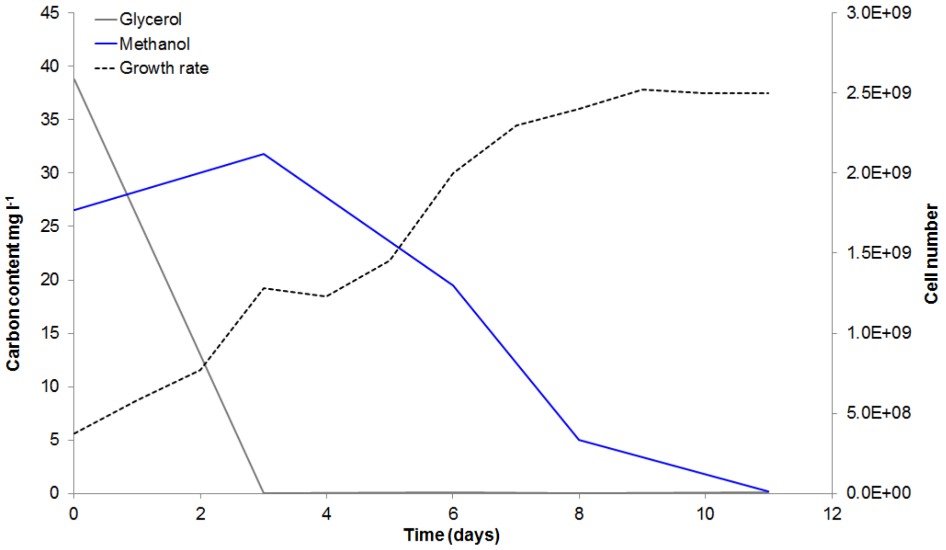


Figure 5.15 Rate of glycerol and methanol consumption in crude glycerol SWW

5.4 Discussion

5.4.1 Growth rates

The growth rates were measured as an average during the exponential phase. However, variations over the exponential phase could also be seen. The shorter exponential phase in the SWW media can be attributed to overall lower nutrient levels. It is not possible from this analysis alone to determine whether organic carbon is the limiting nutrient in this environment. However, since the same organic carbon level was present in both the HBM and SWW, the HBM supported a longer exponential phase leading to higher biomass production; therefore it is assumed there is another nutrient limiting growth in SWW.

The growth pattern shown by the HBM and SWW Crude High C media shows two separate exponential phases. This diauxic growth pattern occurs due to the microalgae assimilating the simplest form of organic carbon (in this case, glycerol). A period of stable growth follows, as the microalgae develop an enzyme to assimilate another form of organic carbon, in this case methanol. This is demonstrated by the results in Figure 5.15 where glycerol concentrations are reduced to below 0.1g l^{-1} in 3 days, but the methanol concentrations remain above 5g l^{-1} until day 11 when they are reduced to 0.2g l^{-1}.

5.4.2 Composition of algae

The composition of the *C. vulgaris* varied significantly depending on the media, and it is thought these differences are due to the way in which the cells develop and metabolise different nutrients in the medium. There are also expected to be other structural differences in the microalgae which were not measured here, such as changes in the cell wall and pigment production for example. Observations made where *T. suecica* were cultivated heterotrophically, following a period of autotrophic cultivation saw thinning of cell walls and decrease in chlorophyll content. If the same pattern occurs in other microalgae this could promote the ease of lipid extraction, hence providing a further benefit from heterotrophic microalgae in terms of ease of processing compared with autotrophic microalgae [211].

The loss of cholorphyll was clearly observed in the *C. vulgaris,* which changed from a dark green colour during autotrophic cultivation to a yellow to brown colour during heterotrophic growth, (heterotrophic biomass is shown in Figure 5.10 on p.94). As a result of lower chlorophyll levels it is expected that overall pigment levels might be lower and hence processing to biodiesel maybe easier. However, the pigments found in the microalgae are potentially useful and could add value to the biodiesel feedstock. Potential components include beta-carotene, astaxanthin and other pigments which could be used for nutraceutical supplements. The density of the microalgae varied, depending on the cultivation media. The images in Figure 5.10 all have the same mass of microalgae, but the HBM Crude and SWW Crude are denser.

5.4.2.1 Protein content

There was a noticeable reduction in the protein content of the biomass cultivated in the SWW medium compared with the HBM (30-40% reduction). The reasons for the observed differences in protein content between the HBM and SWW could be due to nitrogen limitation. Nitrogen was limited with respect to both phosphorus and carbon in the medium. The HBM had a low N:P ratio of 3:1. This would have caused stress to the microalgae and caused them to accumulate storage products such as lipids or carbohydrates. The N:P ratio in the SWW on the other hand was 16-18:1, which is the optimum ratio defined for marine microorganisms [81] and in excess of the ratio found previously for heterotrophic *C. vulgaris* [82], and therefore the microalgae may not have been so stressed in their environment.

Further stress may have been caused to the *C. vulgaris* where the crude glycerol was used as a feedstock due to the high levels of methanol in the feedstock, which has been found to be toxic and to affect growth rates [193]. The growth rates do not appear to have been affected, as the growth observed for the culture with the crude glycerol does not differ from the other two feedstocks (i.e. glucose or molasses). However, it has caused a very large reduction (up to 80%) in the protein content of the HBM Crude, HBM Crude High C and SWW Crude High C, indicating there is a component in the crude glycerol that is affecting the protein production mechanisms in the cell. The methanolic content of the media could lead to conformational changes in algal enzymes, as the methanol would disrupt the hydrogen bonding between water and the polar residues of the protein complex. This denaturing of the enzyme could therefore reduce protein production [212].

5.4.2.2 Lipid content

The accumulation of lipids is associated with stressful conditions within the culture, and these could have been cause by low N:P ratio in the HBM medium, different sugars in the crude glycerol and molasses feedstocks causing the microalgae to develop ways to assimilate them and high methanol concentration in the crude glycerol feedstock. N-limitation has been observed as a key factor in lipid accumulation in various strains and growth conditions of microalgae. It also potentially stressed the *C. vulgaris* and thus affected the characteristics of the biomass. However, what has been observed from the results is that although the exponential growth period was shorter, the lipid content was also lower. This could have been due to the fact that the C:N:P ratio was more preferable for the microalgae, and therefore all nutrients became depleted at a similar point thereby stopping growth.

There may also be other reasons for higher lipid content in the media with crude glycerol. For example it was observed that the accumulation lipids could be attributed to a higher rate of sugar accumulation than cell generation [80]. This would promote the conversion of the excess sugar to lipids via production of glucose-6-phosphate by glycosis, which is a precursor for triacyglycerol (TAG) synthesis. The results from the SWW Crude demonstrate this theory may be occurring because the rate of carbon uptake continues past day 3, although the growth enters the stationary phase indicating the sugar could be in excess (shown in Figure 5.13). The variability of the lipid content is of concern for biodiesel production and could affect the estimated biodiesel yield considerably. This experiment demonstrates the need for future work to determine the optimum level of crude glycerol required for feasible biomass production using the heterotrophic cultivation method.

5.4.2.3 Carbohydrate content

The metabolism for lipid and carbohydrate synthesis are in competition with each other because, as mentioned above, the precursor for TAG synthesis is glucose-3-phosphate, which is produced via glycosis [15]. Therefore it would be expected that the microalgae with higher lipid content would tend to have lower carbohydrate content. This was observed in a few cases in these experiments. For example, the comparison between *C. vulgaris* cultivated with a crude glycerol in HBM and SWW, the HBM saw a lipid content of 40% compared with 47% in the SWW, and the carbohydrate content was higher in the HBM where the lipid content was lower (i.e. carbohydrate content of 23% and 12.5% respectively for HBM and SWW). However, where a higher crude glycerol content was used in HBM, this pattern was not the case, as the lipid content was high (53%) but the carbohydrate content was also high (25%). Carbohydrates are an alternative storage product; therefore the mechanism causing energy storage by the cell needs to be understood. Having a high carbohydrate content may be useful in terms of creating a by-product for further biofuel production as bioethanol could then be produced from the lipid extracted microalgae [15].

5.4.2.4 Closure of mass balance

Once the protein, lipid, carbohydrate and ash content have been taken into account, there is a certain mass of unaccounted for material. This could include pigments, for example astaxanthin and beta carotene, minerals, or unidentified carbohydrates such as uronic acids and amino sugars [93]. There could also be a fraction of polar lipids that were not extracted using hexane. The content of phospholipids may be identifiable from the in situ transesterification analysis in Chapter 6, as acidic transesterification can promote extraction of lipids due to oleosomes being more soluble in an acidic environment.

5.4.3 Type of carbon affects growth rate and biomass accumulation

The results clearly show higher organic carbon content leads to the accumulation of more biomass. However, there are a some barriers to increasing the carbon content, including the financial cost of the organic carbon feedstock and the availability of the feedstock which may be limited in terms of capacity. Use of molasses as a feedstock would potentially be too expensive for the cultivation of microalgae as it is rich in nutrients which could be used for animal feed, and it can be processed to a food grade without too much extra processing. Crude glycerol, on the other hand, is a low value product which would require a good deal of purification before it could gain any added value. Due to the boom in the biodiesel industry since 2007, there has been an oversupply of glycerol to the market, and therefore even purified glycerol is not an economical product. The results above show that crude glycerol is a suitable feedstock for *C. vulgaris,* and deals with the issue of disposing of the crude glycerol. It is high in nutrients and therefore could not be discharged into a waterway due to risk of causing eutrophication and damaging the ecology of the receiving water body. The high levels of methanol in the crude glycerol would also be toxic to some life forms in the water.

The biomass accumulation was higher where an optimised media for lipid accumulation was used compared with synthetic wastewater because nutrient levels were higher. The lipid content of the cells was also higher. However, the cost to produce this feedstock would be substantially higher. The degree to which this would impact the overall energy balance of the system will be investigated using an energy balance model in Chapter 7.

Whilst observing the growth trials using the microscope, it was apparent that several of the cultures were susceptible to contamination, in particular the molasses and glucose trials. The crude glycerol became contaminated the fewest number of times, and when it did become contaminated, the bacteria were found to have disappeared after 5-6 days of cultivation. This suggests there may be something present in the crude glycerol that makes it more difficult for a bacteria culture to survive, or that it was outcompeted by the microalgae. More work would be needed to find out whether this pattern continued at larger cultivation sizes. There were also some problems with counting cells using the microscope, particularly in the media with crude glycerol as micro bubbles formed, making it difficult to distinguish between bubbles and cells. However, increasing the dilution helped with this, as did increasing the number of readings to improve the reliability of the cell counts. There would certainly be contamination of the culture if SWW was used as a medium, including bacteria and pathogens. Therefore a more detailed analysis of how these affect the survival and growth of microalgae under heterotrophic conditions would be required.

Cultivation using crude glycerol was problematic due to foaming of the medium when an air stone was added. In order to stop the foaming, the pH of the medium was reduced below 7 (in the range 6-7). The pH of the culture changed throughout the cultivation, and was not consistent for each trial, although most trials had a pH that remained between 6.5 and 7.5. The *C. vulgaris* proved tolerant to pH as low as 4 whilst still exhibiting a positive growth rate. The drop in pH suggests the *C. vulgaris* is producing organic acids that are affecting the pH of the solution. However, there seems to be some degree of buffering that allows the culture to change the pH to suit its requirements. The crude glycerol also appears to have a high buffering capacity, which was also observed by [149]. Neutralising the medium may not be practical should this scheme be scaled up, therefore an alternative anti-foaming agent would need to be investigated. There have been several studies to investigate whether antifoaming agents affect growth rates, such as [213]. High pH conditions were rarely observed, although trials using molasses feedstock remained above 7 and saw the pH reach 7.97 on day 6 from 7.09 at the beginning of the trial. Again the growth rate remained positive throughout cultivation using molasses feedstock.

5.5 Summary

Microalgae *C. vulgaris* was cultivated heterotrophically using a high nutrient medium and synthetic wastewater, utilising a waste carbon source either from crude glycerol or unrefined molasses and comparing it with a control medium using pure glucose. The growth was observed to be lower in the SWW, achieving a maximum of 0.5g l^{-1} d^{-1} where only 10g organic carbon was added due to lower nutrient levels. However, increasing the organic carbon content led to a longer exponential growth phase, higher biomass content of higher yields of up to 3g l^{-1} d^{-1} and higher lipid content of up to 52% in both media. The high growth rate that was observed from the SWW medium with high organic carbon content has potential benefit for the energy balance as virgin fertilisers would not be required.

Chapter 6 Producing biodiesel from heterotrophic microalgal feedstock

6.1 Introduction

Biodiesel is a commercial product, and is blended with fossil derived diesel fuel in over 30 countries worldwide. Feedstocks typically include soybean, rapeseed, corn and animal tallow. However, more diverse crops are also being investigated, and in some countries, such as Brazil, are being incorporated into the fuel mix. This offers an opportunity for new feedstocks such as microalgae to be developed if the quality required by the market can be achieved.

Biodiesel offers many advantages over fossil diesel as a fuel. Biodiesel can be added to conventional engines without any major modifications, and can easily be blended with diesel-oil. It also has ‘liquid nature portability’, meaning it can be used within the existing infrastructure. The ability to combine biodiesel into a blend with fossil diesel provides further benefits such as better lubricity which reduces engine wear, low sulphur emissions and high flash point. Biodiesel is also more biodegradable than fossil diesel which is particularly relevant when assessing the environmental impacts of spillages [214]. However, there are still some problems with biodiesel in that it can act as a solvent, degrading rubbers and plastics for example in vehicle seals [215]. Biodiesel is also hydrophilic, causing a number of issues such as reducing the heat of combustion of the fuel, corrosion of fuel system components, increased gelling of the fuel and potential for microbe colonies to establish in fuel tanks and lines [216]. The production of biodiesel via transesterification was described in Chapter 2.

A significant increase in the volume of biodiesel in diesel fuel mix means a consistently good quality biodiesel is required. The characteristics of both biodiesel and fossil diesel can vary significantly depending on feedstocks and type of oil. In order to maintain a good quality biodiesel standard, there are guidelines which differ from country to country. The Brazilian standard for biodiesel is regulated by the Agência Nacional do Petrôleo (ANP) under the Brazilian Biodiesel standard specifications; ANP 42. These specifications cover both fatty acid methyl esters (FAME) and fatty acid ethyl esters (FAEE) and describe the product for use as a blending component rather than as a stand-alone fuel [116]. Properties that are regulated in Brazil include oxidative stability, flash point, water content, viscosity, sulphur content, cetane number, cold flow properties and metal contaminants. However, the Brazilian market recognises biodiesel as a blend component and therefore does not require each individual feedstock to attain the standard but rather anticipate the blending of biodiesel from various feedstocks with fossil diesel will allow the standard to be achieved. This will produce a fuel that will attain the overall specifications, as discussed in Chapter 4. This is the same way in which fossil diesel is blended from a range of characterised diesel blend components [117].

6.1.1 Fuel properties

Fuel characteristics can be influenced by a number of factors, for example the fatty acid composition of the feedstock oil and the production process or its handling and storage [114,116]. The standards designed for biodiesel quality (e.g. international standard ASTM D6751-08 for biodiesel fuel blend stock, European standard for biodiesel EN 14214, and Brazilian ANP Resolution 7/2008) are aimed at providing a fuel which will ignite in a way that does not damage the engine, and does not cause undue wear to engine parts, has low levels of deposits and can be stored and transported to its point of use. There has been research to determine which composition of biodiesel would give the best combustion characteristics, with the decision being that fewer components is preferable but a mixture of components with advantageous properties could also be acceptable [214]. A number of the attributes discussed above are influenced by the FAME profile of the biodiesel, and could be used to engineer a more desirable fuel type. The FAME content of a vegetable oil is influenced by composition of the lipids in the oil seed crop, the climate and nutrients with which it is grown and the processing techniques used.

6.1.1.1 Cetane number

The cetane number (CN) is a measure of fuel ignition, in particular the ignition delay [217]. A higher CN means a shorter delay between fuel injection and ignition, and also indicates improved cold start properties in an engine [218]. A higher cetane number (between 55 and 60) also lessens tailpipe emissions through more complete combustion. CN increases with chain length of the hydrocarbons and decreases with the level of unsaturation. Biodiesel tends to have a cetane number of between 48 and 65, whilst fossil diesel has a lower cetane number between 40 and 55 [215,219]

6.1.1.2 Energy content

Current fossil fuels are extremely successful fuel sources for transport and currently surpass all alternatives in terms of energy density, allowing long journeys to be made before refuelling is required. Biodiesel has a lower energy density than biodiesel due to the oxygen content of the fuel. The energy content of different fuels is as shown by way of comparison to fossil fuels in Table 2.2.

The gross calorific value (CV) of a fuel is a measure of the fuel energy density and is measured by the number of heat units evolved when a unit of fuel is completely burned and the combustion products cooled to 288K. [100]. To determine the CV of a fuel experimentally, calorimetry is the most common method. For this method, electrical ignition of the fuel occurs in a stainless steel bomb containing a known mass of fuel in oxygen. The CV can also be calculated by knowing the elemental composition of the fuel. To determine the CV based on a dry, ash-free basis, the C, H, O and S content can be used. The Dulong formula (shown in Equation 6.1) is the relationship used in this work [220].

Equation 6.1: Dulong formula

$$CV(MJ/kg) = 0.3383\,C + 1.443\,(H - \frac{1}{8}\,O) + 0.0942\,S$$

Diesel has both a higher CV than biodiesel from any known feedstock [10], with biodiesel generally containing 8-9% less energy than fossil diesel per litre. The energy content affects the torque, power and fuel economy [215]. Fuel economy is proportional to the volumetric energy density and therefore the volumetric energy density of the fuel will be lower for biodiesel and biodiesel blends [217]. This is relevant from the point of view of consumers including freight and passenger vehicles which will not be able to travel such long distances on the same quantity of fuel or for the same cost.

6.1.1.3 Cold flow properties

Cold flow properties are dependent on the oil and alcohols used for biodiesel production and include cloud point (CP), pour point (PP), cold filter plugging point (CFPP) and low temperature filterability (LTFT). Cloud point is the temperature at which wax starts to form in biodiesel, giving a cloudy appearance. This has implications for operation of the engine as the presence of solidified wax will lead to filters and injectors becoming blocked. The CFPP is the lowest temperature at which a given volume of biodiesel will pass through a standardised filter in a specified time when cooled under certain conditions. Of the cold flow properties listed above, CP is the only property that can be determined thermodynamically. However, CFPP, PP and LTFT are linear functions of CP and can be calculated from its value [221].

The cold flow properties are dependent on the number of saturated FAMES's, and are not affected by unsaturated components. The PP of oil states the lowest temperature at which the oil remains liquid and is therefore able to flow through pipes or into an engine without causing blockage. It is related to how many long chain paraffins are present in the oil, as long chain molecules are the first to solidify. Chains of over 16 carbon molecules in a chain cause near ambient temperature precipitation, and less than 1% can be sufficient to cause solidification of the fuel [222]. The point at which crystallisation occurs is termed CP [223]. Presence of crystals will affect the viscosity, volatility, flowability and filterability of the biodiesel. Contaminants, impurities and unsaponifiable matter such as sterols or other hydrocarbons also impact the cold flow properties [217]

The geographical region in which the fuel will be used needs to be considered before an appropriate feedstock can be selected, for example a fuel to be used in the tropics can have a fuel with a higher pour point temperature. Blending of biodiesel with fossil diesel can lower the cloud point, extending its geographical range [215]. For example, coconut oil becomes a solid at 14°C, rendering it unsuitable for use outside of the tropics [224]. Additives can be used to help the cold flow properties, but this will consequently lead to higher prices and poor performance in an environmental life cycle analysis [202].

6.1.1.4 Oxidative stability

Oxidation of fuel occurs largely due to exposure to air and auto-oxidation is promoted by the presence of air, heat, trace metals and peroxides as well as the FAME structure. Poor oxidative stability makes storage more difficult and causes problems for fuel delivery systems and engines including blocking of filters and injectors, corrosion, hardening of rubbers, fusion of moving parts and deposits in the engine. This is due to an increase in viscosity, acidity, peroxide value and formation of gums after oxidation [225]. When biodiesel is in a pure form, it is less stable than petrol-diesel. Biodiesel can also act as a solvent, leading to removal of deposits elsewhere in the engine. However, this can lead to an accumulation of the sediments on the fuel filter and vehicles using diesel with high levels of biodiesel will often need an additional filter to remove sediment [202].

Biodiesel is prone to oxidation if it contains high levels of unsaturated hydrocarbons. Oxidative stability decreases with the unsaturation of the FAMEs in the biodiesel and depends on both the number and position of double bonds [117]. It is possible to correlate the oxaditive stability with the degree of unsaturation of fatty acid esters [218,226,227] .

6.1.1.5 Impurities

There are certain materials present in oil that are non-combustible, and following combustion will leave a residue, known as ash. Total ash content can be derived from TGA data. The ash can contain a variety of components, and depends on the feedstock. Fossil diesel can include organic-metals or inorganic metal salts, metal and silicon oxides, water soluble inorganics such as calcium or sodium chlorides and impurities arising from materials used in refining or foreign contaminants from storage [228]. Biodiesel will have similar issues with storage, but the composition of the ash will tend to be different and can contain higher levels of phoshorous, sodium and potassium and lower levels of calcium, magnesium and zinc. High levels of some elements such as Na, Zn and potentially K are associated with formation of injector deposits leading to power loss and potentially fuel delivery failure [229].

Sulphur is a contaminant that causes emission issues from diesel engines. Low sulphur levels are desirable because sulphur is very corrosive, can poison catalysts and also are a precursor to acid rain and other air pollution. Biodiesel has the benefit of having zero sulphur content [10]. Crude oil with high sulphur levels is expensive to treat and requires high energy inputs to extract. Different sulphur compounds require different extraction technology and can significantly change the economic efficiency of processing.

6.1.1.6 Tailpipe emissions

The levels of tailpipe emissions are regulated in Europe in order to protect the general public from poor air quality levels that can lead to a range of illnesses including respiratory problems and skin irritations. In vehicles, technologies such as catalytic convertors and particulate filters reduce tailpipe emissions in situ. As yet, Brazil has no vehicle fuel efficiency standards, and attempts to reduce air pollution is hindered by a low turnover rate in the heavy duty vehicle sector meaning new technologies are slow to be introduced.

Measurements can be made using a range of instrumentation at road side locations. Meteorological conditions and street design are also important to note in observations of pollution levels. Chemical reactions between species such as NO_x and ozone in different temperatures cause concentrations of these particular species to change.

Biodiesel has a higher combustion efficiency than fossil diesel-oil due to it being more oxygenated. This leads to a reduction in many emissions such as unburnt hydrocarbons and due to the nature of biodiesel will also reduce levels of sulphates, carbon monoxide, aromatics, nitrated compounds and particulate matter. However, NO_x emissions rise as the concentration of biodiesel in any biodiesel blend increases. This is partly due to the different cetane number of biodiesel and can be resolved to some degree by changing the injection timing.

6.1.1.7 Flash point and distillation temperature

The temperature at which the mixture of air and vapour ignite is the flash point. Diesel has an average flash point of between 60-80°C, and biodiesel has a flash point between 100-170°C. A minimum flash point of 93°C for biodiesel is required for fire safety standards, measured using the ASTM D93 closed cup method [223].

The distillation can demonstrate the FAME content of biodiesel. Because pure biodiesel contains only a small number of components (i.e. different FAMEs) which all boil at a similar temperature (between 325 and 360°C), any volume left after this upper temperature will indicate the presence of contaminants. Likewise, any components evaporating at low temperatures will indicate the presence of water, methanol or glycerol [217]. The boiling range is also directly influenced by the viscosity, calorific value, average molecular weight of components, contaminants and vapour pressure.

6.1.1.8 Viscosity

Viscosity is a measure of the resistance of a material to deformation. For a liquid, it is used as a measure of “thickness”. It affects how fuel is atomised upon injection into the ignition chamber and affects the level of deposits [218]. Viscosity increases with the FAME chain length and decreases with level of unsaturation, and is also affected by the level of contaminants including glycerol (increased viscosity) and methanol (decreased viscosity) [230]. It affects the fuel quality and is of importance for industrial biodiesel as in order to optimise costs of biodiesel production it is necessary to balance the formulation of biodiesel blends whilst still allowing the fuel to meet the required standard [231]. FAME generally reduces the viscosity of a blended fuel (i.e. fossil diesel with biodiesel) and therefore can deliver benefits to diesel engines for performance and maintenance.

6.1.2 Properties of algal biodiesel

Microalgae can accumulate high levels of lipids depending on species, strain and environmental conditions. Defining the properties of the extracted oils has been pursued by a range of industries because of the interesting properties algal oils contain. The use as a fuel is one of these, and many microalgal species have been identified as suitable to produce FAMEs for biodiesel production. The specifications for the FAME involve chain lengths between 12 to 24 carbon molecules, with low levels of poly-unsaturated FAMEs. Table 6.1 lists some examples of FAME profiles from heterotrophically cultivated microalgal species considered for use as a biodiesel feedstock.

The link between environmental conditions and FAME profile has been investigated by various authors, looking at links between different factors, particularly in autotrophic species of microalgae. Nutrient starvation and cell density also have an impact. For example nitrogen starvation can cause more neutral lipids to accumulate, and high light intensity in autotrophic microalgae was shown to decrease the number of saturated fatty acids in microalgae *N. closterium* and *E. gracilis*. Temperature has been proven to have an effect on the saturation of FAMEs, with lower temperatures leading to more unsaturated FAMEs in order to compensate for a decrease in membrane fluidity [232]. There is much work to be done on the impacts of nutrients and temperature on heterotrophic microalgae, although it is assumed some similarities may occur (e.g. lipid accumulation in nutrient stressed conditions).

Table 6.1 Comparison in the cultivation methods, transesterification catalyst and the FAME profiles of 5 strains of heterotrophically cultivated microalgae

Species	*C. Kessleri*[1]	*C. zofingiensi*[2]		*C. protothecoides*[3]	*S. limacinum*[4]	*C. vulgaris*[5]
Carbon source	Glucose	Molasses	Glucose	Glucose	Crude/corn steep	Unknown
Catalyst	H_2SO_4	H_2SO_4	H_2SO_4	lipase candida	H_2SO_4	H_2SO_4
C14:0				0-1.31	5.3	1.1
C16:0	21-30	22.8	21.8	10-13	56.7	17.1
C16:1	2-4	2.5	1.6			3.6
C16.2	1-2	7.5	8.2			2.8
C16:3	0.2-0.4	1.8	0.3			
C18:0	6-13	2.7	0.2	3		4.7
C18:1	14-21	34.2	38.2	61-67		10.8
C18:2	21-27	19.7	18.6	17-19		54.0
C18:3	12-18	7.3	7.9			6.5
C18:4		0.9	0.4			
C20				0.4-0.6		
C20:1				0.4-0.6		
C22:5					5.1	
C22:6					29.7	
Total FA (%dw)	21-48	41.9			31.5	Unknown

(1) Wang, Chen, & Qin, 2012 (2) Liu et al., 2011 (3) Li, Xu, & Wu, 2007 (4) Johnson & Wen, 2009 (5) Nichols, 1965

6.1.3 Biodiesel purification

Crude biodiesel can contain a number of impurities which will reduce the quality of the biodiesel product so that it may not comply with regulatory standard for biodiesel and purification will affect the final yield. Contaminants in the biodiesel can include methanol, water, catalyst, soap, free fatty acids, glycerides and glycerol. The extent to which contaminants are present will depend in part upon the catalyst used. For example an alkaline catalyst will lead to higher levels of soap (as discussed above) and hence there will be higher loss of yield as purification is more challenging.

In general, contaminants will lead to deposit formation which will damage injectors, cause corrosion and affect the durability of the engine. Residual glycerol for example is a problem because it causes storage problems due to deposits leading to injector fouling, aldehyde and acrolein emissions, and engine durability problems [117][217]. FFA's will affect the oxidative stability and therefore how long the fuel can be stored for is a problem. Methanol in the fuel poses a safety issue due to a low flash point and as a solvent it can cause rubber seals to deteriorate. Water on the other hand can reduce the heat of combustion, lowering energy output. Formation of ice crystals would be a particular issue in colder climates, and makes biodiesel unsuitable for consideration as a jet fuel in most cases. It can also lead to microbial growth causing further issues with blockages [233]. Contamination from inorganic metals is also an issue, and research has already been carried out extensively for fossil fuels and many terrestrial crops into this. Inorganic elements that have been observed in algal species include Si, Fe, Ca, Mg, P, Na, K, S, and Cl, and depend on growth environments and availability of nutrients [234]. These would potentially accumulate in the ash fraction during combustion but may also lead to formation of secondary species and contribute to gaseous emissions to the air.

Techniques for purification include wet or dry washing, or use of a membrane (organic or ceramic). Wet washing is typically the most utilised for removing soaps, catalysts, glycerol and residual alcohol. Techniques for wet washing include using deionised water, acid and deionised water or organic solvents. However, these add considerable time, cost and energy input into the biodiesel production process. Large amounts of wastewater are also produced from the washing phase of up to 10 litres per litre of biodiesel. Dry washing technologies include using silicates, ion exchange resins or activated carbon or clay. Membrane technologies are relatively new to the market but can offer considerable advantages in terms of lower water, energy, and cost of purification which can typically be in the range of 60-80% of the cost of the biodiesel production process (excluding feedstock production) [233].

6.1.4 Specifications for fuel quality

The standards used to specify the properties of biodiesel in Brazil were developed by the ANP. The original definition of biodiesel was *“a fuel consisting of alkyl esters of long chain fatty acids derived from vegetable oils or animal fats*” (ANP Resolution 37/2005). This was updated in 2012 to include the technique to be used, thereby making the definition, “*a fuel consisting of alkyl esters of long chain carboxylic acids produced from the transesterification and/or esterification of raw greases or fats of vegetable or animal origin and that meets the specifications in the Technical Regulation No. 4/2012 found in the Annex of ANP Resolution 14/2012”*. The requirements differ from other international standards in that many of the parameters are required for reporting, but no limit is set. For example, cetane number, ester composition and sulphur content are all required for reporting without definition of an upper or lower limit. There are also variations in the requirements depending on location, specifically related to the CFPP specifications. Southern states have a stricter control on CFPP owing to lower temperatures during winter months which could lead to problems with fuels crystallising or gelling. The structure of the ANP Resolution has allowed various feedstocks to be incorporated into the biodiesel feedstock matrix.

Currently, lab scale microalgae cultivation has low yields of biomass, and therefore the sample size for testing of oil properties is very low [57]. Therefore, innovative techniques are required to test the oil in order to warrant its scale up for biodiesel production. These techniques include estimating properties such as cetane number, cold flow properties, oxidative stability and viscosity from the FAME content and structure [214,218,235].

6.2 Methodology

6.2.1 FAME production and analysis

C. vulgaris was cultivated using the techniques described in Chapter 5 to produce six feedstocks for biodiesel production. The lipids were converted to FAME using the two methods described in Chapter 3. Briefly these methods were either:

1. Transesterification of the extracted lipids using methanolic acid at 60°C for 90 minutes, followed by recovery using hexane
2. In situ transesterification where methanolic acid was added directly to the dry biomass, followed by recovery using hexane

All experiments were carried out in duplicate, and all analysis on the subsequent oil was performed in duplicate. The FAME content of the extracted oil was analysed using GC-MS, as described in Chapter 3. Subsequent calculations were made using this data.

6.2.2 Calorific value

The gross calorific value of the oils produced was determined by analysing the C, H, S and O content of the oil, using Equation 6.1 to calculate the net CV on a dry ash free basis. The ash and moisture content of the fuel was determined by TGA using a simulated distillation to 700°C, as described in Chapter 3.

6.2.3 Cetane number

The cetane number is a measure of the ignition quality of the fuel. Longer fatty acid chains which are saturated will have a higher CN number compared with shorter or more branched chains [236]. The test to determine CN is complex and there can be considerable experimental error during measurement. For this reason, ways of calculating CN number are sought to increase the range of fuels that can be analysed. Since CN is based on the FAME profile of the oil, it is possible to use FAME as a way of estimating CN. A method based on the work by [237] was developed to calculate the CN based on the properties of the constituent FAME’s. The equation developed by these authors only calculated the CN based on 7 pure FAMEs, therefore the matrix was expanded to include 11 FAMEs and factors were recalculated based on figures for CN number found in the literature [217,238,239]. Where more than one value existed, an average value was calculated. These were then inserted into a matrix, shown in Table 6.2. A factor was calculated by assuming a 100% content of each FAME, shown in Table 6.3.

An equation was then developed, shown in Equation 6.2. In the equation, K is a constant, calculated previously [237], x is the factor calculated in Table 6.3, the methyl ester (e.g. C16:0 etc.) is represented by n, and y indicates the concentration of each FAME (%). The cetane number is a result of the addition of all FAMEs present after their percentage of composition has been multiplied against the relevant factor and added to the constant (K) of 61.1.

Table 6.2 Matrix developed to calculate the factors for estimating CN number

Pure FAME	% present in Biodiesel											
	C14	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1	C22:0	C22:1	CN
C14	100											69.9
C16:0		100										74.4
C16:1			100									51.0
C18:0				100								81.3
C18:1					100							56.1
C18:2						100						31.8
C18:3							100					22.7
C20:0								100				74.9
C20:1									100			73.2
C22:0										100		77.7
C22:1											100	74.2

Table 6.3 CN number of FAMEs found in literature from experimental work, and averaged where more than one value is available

n	Structure	Common name	Factor (X)
1	14:0	Myristic	0.0875
2	16:0	Palmitic	0.133
3	16:1	Palmitoleic	-0.101
4	18:0	Stearic	0.2015
5	18:1	Oleic	-0.05
6	18:2	Linoleic	-0.2935
7	18:3	Linolenic	-0.384
8	C20:0	Arachidic	0.138
9	C20:1	Gondoic	0.121
10	C22:0	Behenic	0.166
11	C22:1	Erucic	0.131

Equation 6.2 Cetane number [237]

$$CN = K + \sum_{n=1}^{n=11} (X_n * Y_n)$$

6.2.4 Cold flow properties

The cold flow properties of biodiesel are correlated with the degree of saturation of the FAMEs within the biodiesel. The unsaturated compounds are thought to have little effect on the low temperature properties overall [240,241].

Equation 6.3: Cloud point (CP) [240]

$$CP = 1.44\ (\Sigma Sats) - 24.8$$

Equation 6.4: Cold flow plugging point (CFPP) [240]

$$CFPP = 1\ (CP) - 4.5$$

Equation 6.5: Pour point (PP) [240]

$$PP = 0.98\ (CP) - 5.1$$

Equation 6.6: Low temperature filterability (LTFT) [240]

$$LTFT = 1\ (CP) + 5$$

The CP was calculated using the data collected on FAME composition from each feedstock and calculating the level of saturation, which was then inputted into Equation 6.3. ΣSats represents the total saturation as a percentage of the total FAME. The subsequent equations (Equation 6.4 - Equation 6.6) used the figure calculated for CP to calculate CFPP, PP and LTFT, as these all have linear relationships with the CP. The accuracy of the calculations depends on the level of saturation within the FAME, with low levels (<12%) leading to lower accuracy.

6.2.5 Density, viscosity and oxidative stability

The physical properties of density, kinematic viscosity and oxidative stability depend upon the molecular weight of the oil and the number of double bonds in the oil. Therefore several empirical equations have been developed to calculate these properties, shown in Equation 6.7 - Equation 6.9.

Density of biodiesel has been shown to decrease with an increase in molecular weight, but increases as the degree of unsaturation increases. The density of biodiesel at 20°C was quantitated experimentally and used to construct Equation 6.7, which proved to have 0.11% deviation from the experimental results, and was used to calculate the density of the microalgal FAMEs [242]. In the following equations, ρ represents density, v_i is the kinematic viscosity, M_i is the molecular weight of the ith FAME and N is the number of double bonds present.

Equation 6.7: Density [242]

$$\rho_i = 0.8463 + \frac{4.9}{M_i} + 0.0118 * N$$

The kinematic viscosity was calculated using a correlation between saturated and unsaturated FAMEs [242]. The calculations were compared with reported values and were found to have an absolute deviation of 1.65%. Equation 6.8 was used to calculate the kinematic viscosity of the microalgal FAMEs.

Equation 6.8: Kinematic viscosity [242]

$$\ln(v_i) = -12.503 + 2.496 * \ln(M_i) - 0.178 * N$$

Oxidation of fuel occurs due to free radical behaviour in unsaturated molecules. Oxidative stability is a measure of an oil's resistance to oxidation, and is mainly affected by temperature and exposure to air. A higher content of the saturated FAMEs palmitic and oleic acid increase oxidative stability. However, polyunsaturated fatty acids were found to be the most important for determining oxidative stability. When testing the influence of FAME mixtures in 3 types of oil (soybean, rapeseed and palm) the strongest relationship was found between C18:1 and C18:2 leading to the formulation of Equation 6.9 to estimate oxidative stability of a FAME mixture, where Y is the oxidative stability and X is the wt% of C18:1 and C18:2 [226]. This equation was used to calculate the oxidative stability of the microalgal FAME, the result was given in hours.

Equation 6.9: Oxidative Stability [226]

$$Y = \frac{117.9295}{X} + 2.5905 \; (0 < 100)$$

The following notation is used throughout the results section too identify the different samples:

- **HBMG**: HBM with glucose
- **HBMC**:HBM with crude glycerol
- **HBMM**: HBM with molasses
- **IS**: In situ transesterification
- **TE**: Transesterification
- **SWWG**: SWW with glucose
- **SWWC**: SWW with crude glycerol
- **SWWM**: SWW with molasses
- **ID**: Indirect (i.e. lipid extraction followed by transesterification)

6.3 Results

Microalgae were investigated for use as a feedstock for biodiesel production. Six samples were tested for a range of technical characteristics, all having been cultivated on different media and different carbon feedstocks. The technical characteristics investigated were those required for reporting under ANP Biodiesel specifications, plus further characteristics to allow comparison with diesel no. 2 fuel. Overall, the microalgae feedstocks show promising characteristics, with similarities to existing biodiesel feedstocks. Further refining and purification would inevitably produce even better results.

6.3.1 FAME yield from two methods for FAME production

The oil yield from both reactions was determined gravimetrically, and the FAME content of the oil was confirmed using SEC. The oil was found to contain between 94-100% FAME (dry, ash free basis), with up to 6% heavier components thought to be unreacted triglycerides or other heavier compounds.

The FAME yields were measured as a percentage of the total biomass, and are presented in Figure 6.1 with the error shown as 1 standard deviation of the mean. The repeatability of the experiment was generally good, with standard deviations remaining below 1.5% of the mean. The results show the in situ transesterification has a higher conversion rate for all the feedstocks, even once errors are taken into consideration. The highest yields were obtained from the HBMC-IS and SWWC-IS feedstocks, achieving 38 and 48% yield respectively. The highest yield from ID were also achieved from these feedstocks at 28 and 39% respectively.

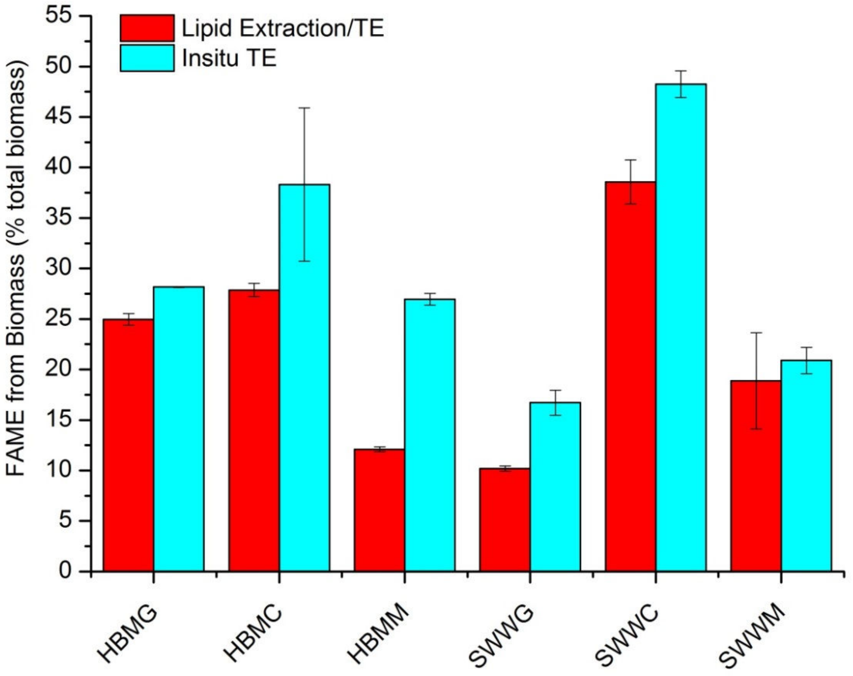


Figure 6.1 A comparison of FAME yields from 6 feedstocks using two techniques; indirect transesterification of lipids (ID) and in-situ transesterification (IS) of algal biomass. Errors are one standard deviation of the mean from triplicate experiments.

6.3.2 FAME profiles

The FAME profiles were analysed using GC-MS, and are of importance as they have a direct effect on the fuel characteristics. The FAME compositions are shown as an average of duplicate runs in Table 6.4, and a comparison of the conversion method (either transesterification of lipids (ID) or in situ transesterification (IS)) and the FAME profile from different feedstocks is given. The errors are not shown in the table but were found to be lower than 5%. shows the signal from four different oils when analysed using gas chromatography.

The data in Table 6.4 shows the saturation of FAMEs was not greatly affected by the conversion method, with less than 5% difference in the level of saturation. For 5 of the 6 feedstocks, the largest constituent of between 38.6-63.2% was mono-unsaturated FAME (the majority of which was C18:1), with between 21.3-35.5% poly-unsaturated (poly-unsaturated FAME refers to FAME with between 2-3 double bonds, as no unsaturation of 4 double bonds or more was observed). The exceptions to this pattern were the SWWM-IS which had a much higher level of saturation at 40.5% saturates compared with <28% saturates from other feedstocks. The crude feedstocks all include around 7% of 18:3, which is shown by the peak at 39.7 minutes in the chromatographs. The peak at 40.5 minutes was identified by MS as C18:0 and accounted for as much as 11% of the total. Cyclopropanoic acid methyl ester was identified in 6 of the oils from trace levels up to 8%.

Table 6.4 Average % composition of fatty acids of different feedstocks (Totals are average of duplicate analysis and therefore total is not 100%). (*tr indicates trace, i.e. <0.4% detected)(Transesterified (TE))*

	HBMG ID	HBMG IS	SWWG ID	SWWG IS	HBMC ID	HBMC IS	SWWC ID	SWWC IS	HBMM ID	HBMM IS	SWWM ID	SWWC IS
C14					tr				3.6		0.6	
C16:0	20.8	7.6	7.3	9	13.9	9.8	12.6	10.3	14.2	17.1	19.4	10.9
C16:1	5.5		0.3	6.8			0.6	0.5	4.9	16.0	1.3	0.7
C18:0			9.6	11.1	3.9	4.9					19.1	9.2
C18:1	41.3	38.6	39.5	35.7	54.6	51.4	59.2	58.9	47.1	37.5	44.1	45.1
C18:2	24.4	26.1	24.3	20.0	13.6	23.3	13.9	13.9	26.6	29.5	12.9	25.3
C18:3		5.2	4.8	1.7	7.1	8.0	7.8	7.4				2.8
C20:0			1.8	2.6	1.2	0.5	1.0	1.2			0.5	1.5
C20:1			3.4	3.3	1.9	1.1	2.0	2.6				1.8
C22:0			2.0	3.6	1.0	Tr	0.8	1.1				1.0
C22:1			3.1	3.7	0.8	tr	0.8					0.9
C24:0			0.7	1.3			tr	tr			0.6	
C24:1			0.9	1.1			tr	tr				
Cyclo-propane octanoic ME	8.0				tr		0.3	tr	3.6		0.5	
Other		22.5	2.3	tr	2.0	1.0	1.0	4.1			1.0	0.8
Saturated	20.8	7.6	21.4	27.9	20	15.2	14.4	12.6	17.8	17.1	40.2	22.6
Mono-unsaturated	46.8	38.6	47.2	50.6	57.3	52.1	62.6	62.0	52.0	53.5	45.4	48.5
Poly-unsaturated	32.4	31.3	29.1	21.7	20.7	31.3	22.0	21.3	30.2	29.5	13.4	28.1

6.3.3 Fuel properties

6.3.3.1 Calorific value and nitrogen content

Only small quantities of oil were produced (<1g), therefore testing for calorific value using calorimetry was not possible, and there was insufficient sample from HBMG-IS to be tested. Therefore the composition of the remaining oils was determined by elemental analysis, and was used to calculate the gross CV. The Dulong formula (see Equation 6.1) has been shown to give good results for CV compared with experimental testing up to carbon contents of 86%. The oils tested had an average carbon content of between 58-80% carbon, and therefore this method is appropriate. The CV was calculated on a dry, ash free basis.

The CV of HBMG-ID is significantly lower than any other feedstock. This is due to low carbon content in the oil, and high oxygen content. Figure 6.2 shows the CV plotted against the elemental composition of the oil. The CV of the majority of the oils was between 38 and 41 MJ/kg, with SWWG-ID being slightly higher at 44 MJ/kg. The nitrogen content of the oils was between 0.42 and 0.94%.

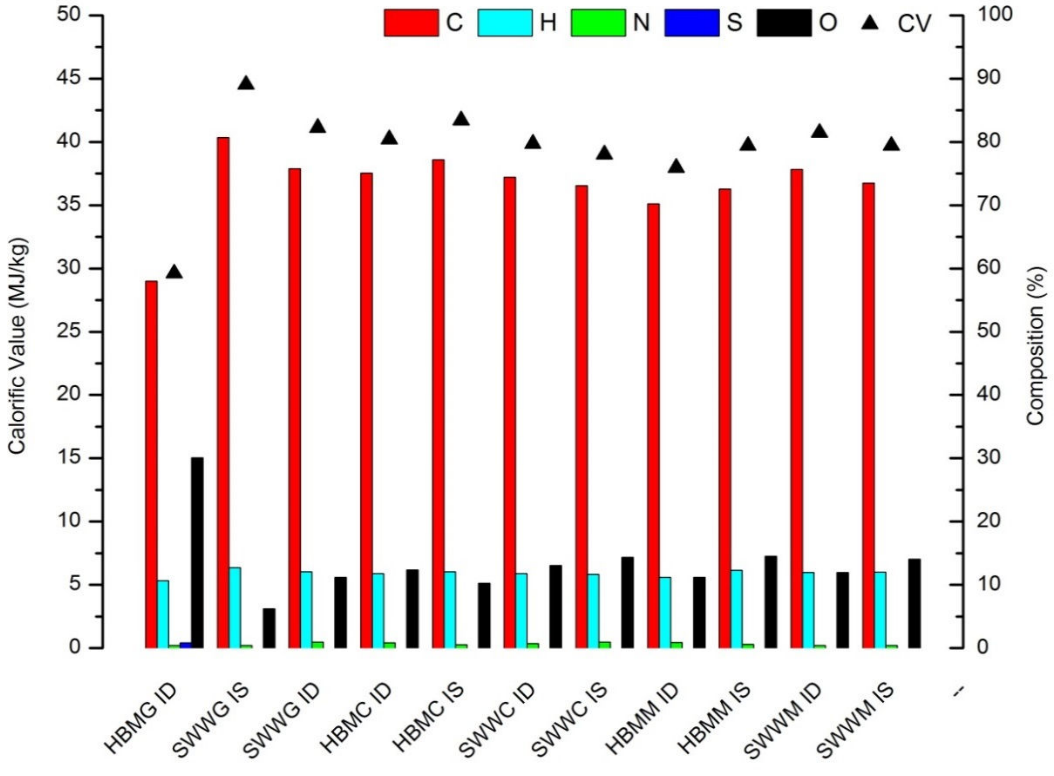


Figure 6.2 Calorific value of oil from 11 different feedstocks (no data available for HBMG-IS due to insufficient sample for analysis)

6.3.3.2 Cetane number (CN)

The CN was calculated using Equation 6.2, and the data obtained from the FAME analysis. It ranges between 49 from HBMC-IS to 61.5 for SWWM-ID. This difference is due to the levels of saturation, which also affects the cold flow properties of the biodiesel. SWWM-ID had the highest proportion of saturated FAME's, and subsequently has a higher CN number. HBMC-IS on the other hand had the highest level of polyunsaturates, and therefore the cetane number was lower. As discussed in section 6.1.1.1, cetane number is difficult to calculate experimentally and due to the small volumes of oil produced it was not possible to test the CN experimentally. However, the test is based on figures that have been tested in the literature, and therefore the outputs from the calculations are reliable enough to provide guidance as to this property of the biodiesel. However, the CN was calculated from the FAME composition on a dry ash free basis, and did not take into account other characteristics of the oil including nitrogen content (which was between 0.4 and 0.8%).

Table 6.5 CN calculated using Equation 6.1

	Processing method	
Feedstock	**ID**	**IS**
HBM Glucose	53.1	50.5
HBM Molasses	52.7	51.2
HBM Crude	54.9	49.0
SWW Glucose	54.4	57.4
SWW Molasses	61.5	54.6
SWW Crude	53.3	53.2

6.3.3.3 Cold flow properties

The cold flow properties were calculated using Equation 6.3 – 6.8, and are shown in Figure 6.3, plotted against the saturate content of the oil (as a % of total mass). A clear relationship exists between the level of saturation in the FAME and the estimated cold flow properties, with the higher saturation in SWWM-ID showing poorer cold flow properties, estimating that the fuel would begin to crystallise at 32°C. HBMG-ID has no saturated compounds and therefore the CP is -28°C. There is no indication of any relationship between similar feedstocks (i.e. same carbon type or same processing method). It is difficult to draw a conclusion as to what determines the saturation of the feedstock, and therefore how it can be managed.

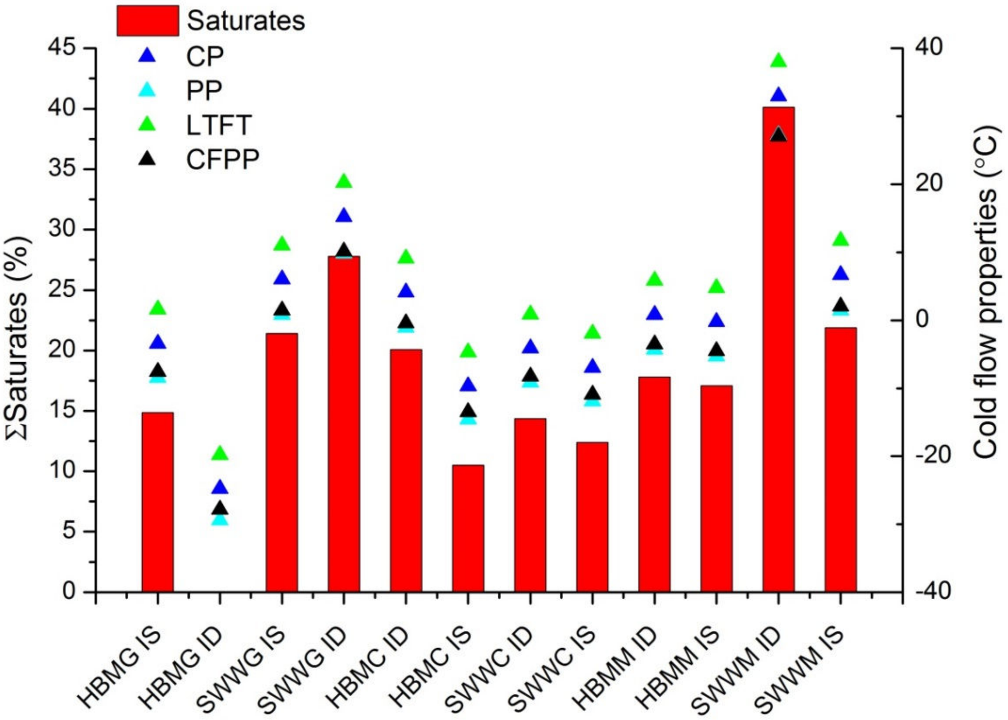


Figure 6.3 Cold flow properties are related to the content of saturated FAMEs, shown by the bar graph. The CFPP, PP and LTFT are linear functions of CP, calculated using Equation 6.3 - Equation 6.6.

6.3.3.4 Density, kinematic viscosity and oxidative stability

The density of the oils was calculated empirically using Equation 6.7, and the kinematic viscosity using Equation 6.8, and was based on the molecular weight of the oil and the number of double bonds. The density ranged between 0.87 and 0.88 kg/m^3, and the kinematic viscosity was calculated to range between 2.34 and 4.29, shown in Table 6.6. These values are comparable with soy biodiesel and fossil diesel values.

The oxidative stability was calculated using Equation 6.9, to obtain a value indicating the period of time (hours) for which the oil is stable, based on the C18:1 and C18:2 wt% content. As the oils all had similar C18:1 and C18:2 content, the oxidative stability was also similar, calculated at between 4.17 and 4.71 hours, shown in Table 6.6. The shortest oxidative stability period was calculated for HBMC-IS, which also had the highest C18:1 and C18:2 content at 74.7%. All the values fall below the specified limit set by the ANP of 6 hours.

Table 6.6 Oxidative stability, density and kinematic viscosity of oil produced from 12 feedstocks using 2 processing methods (ID indicates indirect transesterification, IS indicates in situ transesterification)

Feedstock	C18:1 + C18:2 (wt%)	Oxidative stability at 110°C (hours)	Density@20°C (g/cm^3)	Kinematic Viscosity @40°C (mm^2/s)
HBM G IS	65.7	4.39	0.8746	4.31
HBMG ID	64.7	4.41	0.8804	2.34
SWWG IS	63.8	4.44	0.8766	4.04
SWWG ID	55.7	4.71	0.8746	4.33
HBMC ID	68.2	4.32	0.8760	4.11
HBMC IS	74.7	4.17	0.8780	4.03
SWWC ID	73.1	4.20	0.8766	4.29
SWWC IS	72.8	4.21	0.8769	4.00
HBMM ID	73.7	4.19	0.8762	3.94
HBMM IS	67	4.35	0.8766	4.16
SWWM ID	57	4.66	0.8730	3.76
SWWM IS	70.4	4.27	0.8763	4.06

6.3.3.5 Distillation temperature

The FAME was heated at a constant gradient of 10°C/min up to 700°C in an inert N_2 environment. Once the temperature reached 700°C, O_2 was introduced. Only SWWC-IS was tested as there was insufficient sample size to test the other samples.

The boiling curve shows 80% of the mass was volatilised after 23 minutes where the temperature reached 260°C. A heavy fraction was then vaporised more slowly between 23 and 45 minutes. The volume was reduced by 90% after 32 minutes with a final temperature of 349°C. At the end of the heating time 3.9% weight remained in the crucible, indicating the final ash content. This profile indicates there are several different components within the biodiesel including light volatiles, heavy volatiles and ash. However, there was insufficient sample for further analysis of the ash.

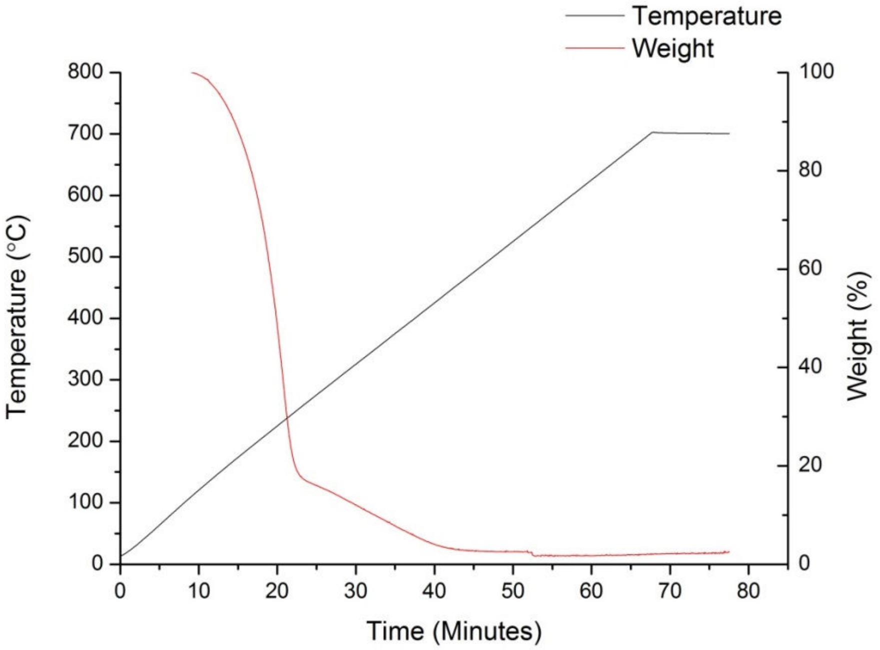


Figure 6.4 TGA simulated distillation of SWWC-IS oil used to determine the ash and moisture content, carbon residue and temperature at which 90% of the volume was recovered.

6.3.3.6 Comparison with biodiesel specifications in Brazil

The properties of the biodiesel have been both tested experimentally and calculated using known properties of the oil where not enough sample exists. There are some properties of the fuel that require a compromise, for example the CN and CFPs.

The ANP Resolution (14/2012) updated the definition of biodiesel and produced a set of technical specifications (Technical Regulation No. 4/2012). The properties of SWW-IS investigated in this chapter have been compared against the properties of No. 2 diesel fuel in Table 6.7 to provide a comparison between algal biodiesel, soybean biodiesel and fossil diesel.

Properties that were not measured but are important indicators of fuel property include viscosity, flash point, elemental content of calcium, potassium, sodium, magnesium and phosphorus, copper corrosion, acid number and iodine value. The methanol content was not measured, but is assumed to be very low as it would have appeared in the TGA analysis as a weight loss at ~65°C.

Table 6.7 Specifications for Biodiesel B100 compared with measured and observed values from heterotrophic algae oil produced from C. vulgaris cultivated using a crude glycerol feedstock with SWW (specifications from ANP Resolution 42/2004)

Property	Unit	Limit (Biodiesel)	Diesel (no. 2) [1,6,7]	Soybean biodiesel[4,5,6,7]	Algae biodiesel (unrefined)	Test method for Algae Biodiesel
Regulated						
Appearance	-	Clear & bright	-	Clear, pale yellow	Clear, yellow/brown	Observation
Cetane number	-	Report	40-55	47-56	53	Calculated
Density @ 20°C	kg/m^3	Report	0.8455	0.8848	0.8769	Calculated
Kinematic viscosity @ 40°C	mm^2/s	Report	2.60	4.01	3.99	Calculated
Oxidative stability @110°C	hours	6 (min)	-	4.6	4.2	Calculated
Water/sediment content	%vol	0.05	*Negliable*	*Negliable*	3.95	TGA
Distillation 90% vol. recovered	°C	360	315	340	349	TGA
Ester content/composition	%mass	Report	75% saturates, 25% aromatic HC	15-17% saturates, 25% mono-unsaturates	13-15% saturates, 63% mono-unsaturates	GC-MS
Sulphur content	% mass	Report	0.2 (max)	0.0	0.0	Elemental analysis
CFPP	°C	Variable	-18	-5	-11	Calculated
Not regulated						
Calorific value	MJ/kg		46.2[3]	37.4	36-40	Calculated
Carbon	%weight		85-88	77.2	77-80	Elemental analysis
Hydrogen	%weight		12-15	11.9	12.7	Elemental analysis
Oxygen	%weight		0	10.8	2-6	Elemental analysis

[1][243] [2][114] [3][127] [4][244] [5][245] [6][217] [7][10]

*Includes ash (3.96%) and moisture (0.36%) content

6.4 Discussion

The aim of producing biodiesel from microalgae was to produce a fuel that could meet technical specifications for a good and safe fuel, and is an efficient process, not requiring large amounts of energy or other resources. The oil produced from media with crude glycerol feedstock was of particular interest as this method utilised a waste resource for cultivation, therefore good results would prove economically attractive and environmentally promising as it would deal with the issue of disposal of a high nutrient waste stream into water ways.

6.4.1 Yields

The conversion efficiency of transesterification of extracted lipids and in situ transesterification were discussed in section 6.3.1. It was clear that the in situ transesterification led to higher yields from the microalgae biomass. A higher yield from in situ transesterification may be explained by several factors. The first is that by subjecting the whole cell to the esterification process, other parts of the cell may contribute to the overall ester yield, for example phospholipids in the cell membrane. This has been observed by other authors who investigated in situ reactions using oil seeds such as sunflower [222] or in other biological tissues [246]. However, this would not have a large effect on the yield from microalgae via in situ transesterification, particularly given the measurement accuracies when using very small samples.

Where lipids were extracted before transesterification, yields were lower, and this could be due to incomplete lipid recovery from the cell when using hexane extraction. There are a number of theories that could explain this. For example lipid vesicles are acid labile, and therefore the acid environment can also enhance lipid recovery. However, the acidic environment in the in situ environment may also have caused other materials to become soluble in the methanol solvent and therefore increase the yield. In particular, phospholipids may have become incorporated into the extracted material. The TGA shows the presence of less volatile components when the temperature exceeded 240°C which indicates the presence of other material aside from pure FAME. A study using acidified hexane found up to 35% more phosphorus was extracted than with hexane alone and the source of the phosphorus was from phospholipids [61]. If this is the case, this could prove problematic for fuel production due to ash formation. This is important for future developments of in-situ technology, and requires further work to quantify the phosphorus present in the ash and identify its source. The higher yield from in situ transesterification does indicate that potentially not all of the lipid was extracted using the method described in Chapter 5, and therefore one of the reasons for the mass balance not reaching closure was due to this (see section 5.4.2.4).

A higher rate of conversion from either method may be achieved by optimising the reaction parameters in both the lipid extraction and in situ transesterification reactions. Reasons for lower than optimum yields in these experiments may be due to insufficient time to complete the reaction or insufficient catalyst quantity. It is not thought the methanol volume would limit the conversion as it was provided in excess.

6.4.2 FAME Profile

The FAME profile was similar for both lipid transesterification and in situ transesterification. This had been observed previously [246]. Whilst the oil produced from the medium with molasses and glucose feedstocks tended to vary in composition depending on medium and processing method, the crude glycerol oils had a more constant composition, with mainly oleic acid (over 50%) and the remainder being palmitic (9.6-13.9%), linoleic (13.6-13.9% with the exception of HBM processed in situ)) and linolenic (7.1-7.8%). This is despite the crude glycerol being the most variable feedstock, with the properties in the crude glycerol varying from batch to batch by up to 3% (carbon content).

The property of the oil derived from the SWWC-IS microalgae complies with the ANP specifications, and also closely aligns with other biodiesel FAME profiles, in particular that of rapeseed biodiesel, shown in Figure 6.5. The most abundant FAME present in all oils was C18:1, exceeding 50% in all the crude glycerol oils. Oleic acid is considered a stable molecule due to the presence of only one double bond. The cetane number of oleic acid is also acceptable for fuel quality as a pure FAME at 56.1.

There was up to 8% of the natural alicyclic compound, cyclopropane octanoic acid methyl ester. Cyclopropane was present in all of the FAME oils processed via transesterification of extracted lipids. It has been found previously in other studies in phospholipids. This finding is unexpected, as it was theorised earlier that more phospholipids would be extracted using in situ transesterification, therefore it would be expected that the opposite trend would be observed. The presence of alicyclic compounds in the extracted oil may indicate the hexane is extracting phospholipids as well as neutral lipids. Another possibility is that there is bacteria present in the biomass, indicating the culture was not anoxic. Cyclopropane is often found in bacteria, composing up to 35% of membrane lipids [247].

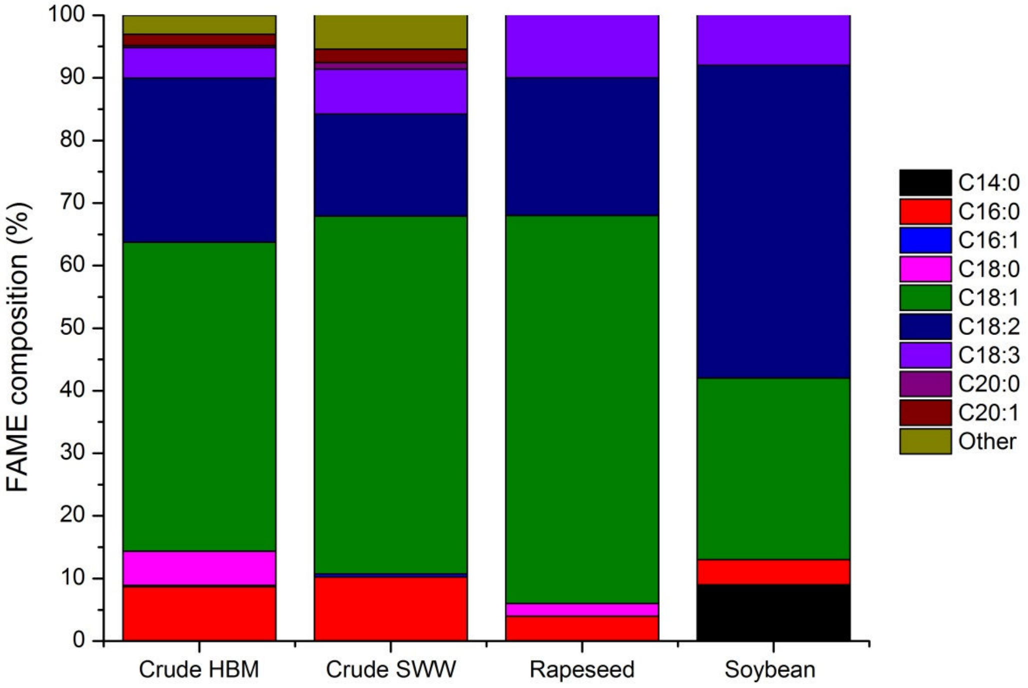


Figure 6.5 FAME composition of heterotrophic HBM and SWW Crude *C. vulgaris* in comparison with typical biodiesel feedstocks, rapeseed and soybean oil.

There could be a number of factors that affected the FAME profile in these experiments. However, no significant differences in the profiles were noticeable. All media were nitrogen limited, which may have resulted in a higher proportion of unsaturated C18 at the expense of saturated C18:0, which is the same pattern as was observed elsewhere in *Porphyridium cruentum* [248]. However, further research would be needed to ascertain the relevance of this. Whilst temperature is known to alter the FAME profile, all experiments were carried out at the same temperature (26-28°C). It is possible that cultures would reach higher temperatures in Brazil, up to 35°C would be well within the expected range. This could lead to a higher proportion of saturated methyl esters, as observed by [232], and hence affect the fuel properties, in particular cold flow properties.

6.4.3 Fuel properties

The CN and cold flow properties were both calculated using the FAME profile of the oil. The CN has an impact on the level of emissions from burning the fuel in an engine, in particular nitrogen oxides (NO_x) which tend to be higher from biodiesel than from fossil diesel (e.g. a study found 76.96g NO_x per litre of ultra-low sulphur diesel, compared with 102.6g NO_x per litre of a 20% biodiesel blend with diesel [249]). NO_x production is strongly correlated with temperature, with higher production rates at higher temperatures. It is estimated that where the CN is higher the NO_x emissions would be lower [13]. This is due to the effect of CN on the ignition delay. Higher CN has a shorter ignition time and therefore less pre-mixed burning and lower peak temperatures in the engine. However, it was not possible to measure the NO_x emissions in this work. The CN is in the range found for other biodiesels, (e.g. see Table 6.7), which indicates the possibility for including algal biodiesel into existing blends without need for an ignition promoter as is sometimes found in diesel fuels.

The cold flow property calculations showed a wide range of temperatures could be obtained from the algal biodiesel (CFPP between -27°C and 27°C), and that it was dependant on the saturation of the FAME, which in turn appeared to depend upon the organic carbon feedstock. The crude glycerol feedstocks gave the most consistently low CFPP (between -0.4 and -13.5°C). The results were calculated from the FAME profiles, and as good repeatability was obtained from the FAME quantification the results are thought to be reliable. From the results of the calculation, it could be determined that the FAMEs would be suitable for use in Brazil as part of a diesel blend. However, should the algal FAME be exported to lower latitudes, additives may be required to stop crystals forming.

The density, kinematic viscosity and oxidation stability of the oils were also calculated based on the structure of the molecules, found by GC-MS and SEC analysis. Whilst only the density and kinematic viscosity are required to be reported on by the ANP specifications, the oxidative stability has a minimum requirement of 6 hours. The oxidative stability of the oil does not meet this criterion therefore additives may be required to ameliorate this issue. Blending the fuel with diesel, as it would be expected for commercial sale in Brazil, would also reduce the problem [215]. The density and kinematic viscosity were compared with soy biodiesel in Table 6.7 and are similar. Therefore there is a possibility that the microalgal biodiesel would be suitable as a replacement or addition to soy biodiesel in a fossil diesel blend. Further testing is required to determine the impact blending microalgal biodiesel with fossil diesel would have on the overall physical and chemical properties.

The sulphur content of the fuel is below trace level. Fossil diesel contains sulphur which when emitted leads to air pollution and can contribute to acid rain. Desulphurisation can be used to lower sulphur content but is a costly and energy intensive process. Blending biodiesel with fossil diesel also helps reduce the level of sulphur, therefore low sulphur level in the microalgae biodiesel is a promising quality.

The oxygen content of the microalgal FAME was between 10-18%. Whilst oxygen contained in the FAME molecules can reduce pollution emissions through more complete combustion, it can also cause a reduction in peak engine power.

6.4.4 Refining and blending

The simulated distillation plotted in Figure 6.4 shows that there is nearly 4% of incombustible materials (ash). This may be composed of metals (Ca, Mg, P etc.) These components would present issues for use in an engine such as problems with deposit formation. Therefore a further refining of the fuel would be required. This could be via wet or dry filtering, as discussed in section 6.1.3.

The presence of residual methanol can be an issue with biodiesel production, and hence it is in the specifications that it must be below 0.2%. However, the simulated distillation indicates negligible methanol content, as there is no mass drop at 65°C, the boiling point of methanol.

It is more realistic to assume the microalgal FAME would be incorporated into a diesel blend, rather than be used as a pure biodiesel in vehicles. Current blend ratios in Brazil are 5%. Additives to fossil diesel are included at the refinery stage and can include cetane improvers, pour point reducers, stability additives, lubricity improvers and antifoaming agents, depending on the intended market. Adding biodiesel to fossil diesel will have an effect on the properties of the diesel, for example better ignition and combustion characteristics due to an increased cetane number, and therefore reduced exhaust emissions and improved engine lubricity, even at blends as low as 1%, and may therefore lead to a reduction in use of other chemicals. These factors however are currently beyond the scope of this project.

6.5 Summary

The results obtained from the experimental work using heterotrophic *C. vulgaris* to produce biodiesel are promising in terms of developing microalgae as a feedstock for industrial microalgae production. The maximum yield achieved was 48% of biomass from SWWC feedstock using the in situ method. There is a high FAME concentration in all the biomass analysed, and further analysis of the oils found promising characteristics for a good quality biodiesel fuel. Cultivation using SWW instead of nitrogen limited HBM does not appear to affect the FAME profile and therefore could provide a suitable alternative as a nutrient resource, but it does so at the expense of the yield under these conditions.

The impact of the yield from cultivation and of the yield from processing the biomass to biodiesel on the energy ratio will be crucial in deciding its viability as a biodiesel feedstock. In Chapter 7 these factors will be used to calculate a mass and energy balance and also to estimate the GHG contribution from these processes. The impact of using IS over ID on the energy ratio will also be investigated.

Chapter 7 Environmental impacts of heterotrophic microalgae feedstock for biodiesel

7.1 Introduction

Biodiesel is an alternative to petro-diesel with the potential to reduce reliance on fossil fuels and reduce GHG emissions from the transport sector. The environmental sustainability of biofuels depends on a number of factors from the energy requirements to produce the fuels, to emissions caused by the production from cradle to grave, and also includes chemical and biological changes in aquatic, terrestrial and atmospheric biospheres.

Whilst much of the research currently cited in the literature studies the production of autotrophic microalgae for biofuel feedstocks, this study has chosen to focus on heterotrophic microalgae, for reasons given in Chapters 1 and 2. Following this, the technical ability to produce a good quality biodiesel fuel from heterotrophically cultivated microalgae was demonstrated in Chapters 5 and 6. However, this system could only be a feasible alternative to fossil diesel if the energy gained from the biodiesel produced exceeded the energy required for production and if the environmental impacts can be shown to be lower than from autotrophic or terrestrial biodiesel feedstocks.

This chapter will discuss the potential environmental impacts of large scale cultivation of both autotrophic and heterotrophic microalgae, and then present a new methodology to calculate the energy ratio and GHG emissions associated with biodiesel production from heterotrophic microalgae. This can then be used as a comparison with autotrophic cultivation systems as well as against other terrestrial feedstock. This approach will be used to analyse four different cultivation and processing scenarios in order to assess where the energy and emission hotspots lie, define the ratio of energy input to output and propose mitigation strategies.

7.1.1 Potential environmental impacts from cultivating microalgae

Cultivation of microalgae for biofuels would require a large scale operation that would inevitably cause changes in the local environment and potentially further afield. Whilst microalgae promises to deliver many environmental benefits compared with existing biofuel technology, there are also issues to overcome in relation to wastewater management, emissions control, land use change and responsible development of genetically modified organisms. Some of the potential impacts that must be considered when planning autotrophic or heterotrophic cultivation plant are discussed below.

7.1.1.1 Impacts to aquatic system

7.1.1.1.1 Water footprint (WF)

A water footprint is the total amount of fresh water embedded in the production of goods and services and includes both surface and groundwater (blue water footprint) and rainwater (green water footprint). Calculation of WF is highly sensitive to evaporation rates, hydraulic retention time and also the design of the plant and processes. For example, the evaporation rate from an open system will vary depending on the local climate from 0.48m^3 m^{-2} yr^{-1} to 2.28 m^3 m^{-2} yr^{-1} in arid regions, where annual rainfall is less than 3 x 10^6 m^3 yr^{-1} [250]. One study found an open algal farm could lose up to 3.7 litres m^3 d^{-1} in Louisiana USA [251].

The WF of a closed photobioreactor (PBR) for biofuel production was found to be lower for microalgae biofuels than for other biofuels such as soybean or palm biodiesel, or bioethanol from sugarcane, shown in Table 7.1 [252]. A closed heterotrophic system will also experience evaporation, particularly with aeration to ensure adequate oxygen in the media. Saturation of the air prior to pumping it through the media will reduce evaporation rates, but not eliminate the issue. However, it is expected that the biomass density would be greater in a heterotrophic system, as described in Table 2.3, and therefore the water requirement per GJ energy output (i.e. in the form of biodiesel) would in fact be lower. Calculations of the volume of water required for cultivation of heterotrophic microalgae for biodiesel have been made using the methodology outlined below in section 7.2 to provide an estimate as no reference has yet been made in the literature.

Table 7.1 Water footprint of different transport fuels

Fuel type	Average annual water footprint (m^3/GJ)	Source
Natural gas	0.11	[253]
Petroleum diesel	0.04-0.08	[187]
Soybean biodiesel	287	[252]
Sugarcane ethanol	85-139	[252]
Microalgae biodiesel (autotrophic, open raceway)	14-87	[187][252]
Microalgae biodiesel (autotrophic, closed bioreactor)	1-2	[252]
Microalgae (heterotrophic, 20% lipid)*	*0.2*	*This study*

*Calculated assuming 6.6m^3/kg biomass/day, where the energy content of the biodiesel produced form the biomass is 39MJ/kg

7.1.1.1.2 Wastewater treatment

For microalgae cultivation, water quality requirements vary depending on alga strains. It is possible to use low-grade wastes as a water source, in order to reduce pressure on natural water resources (i.e., industrial and/or domestic wastewater) [72,145,146,147,175,254,255,256,257]. Sewage is abundant in most countries, although collection methods vary. In Brazil 47 million inhabitants have a wastewater collection system, and 66% of the collected wastewater is treated [258]. Using domestic and industrial wastewater sources could be economically and environmentally beneficial.

Autotrophic microalgae cultivation is a feasible wastewater treatment process for various wastewaters, as algae are able to cope with particular pollutants, and has commercial application in Brazil (e.g. autotrophic cultivation at the Ponte Negra wastewater treatment facility in Natal by CAERN and pilot scale heterotrophic cultivation by Petrobras) and elsewhere (e.g. Aquaflow Bionomic Corporation in New Zealand [259]). A summary of the potential pollutants found in wastewater, and their impact on humans, animals and microalgae is given in Table 7.2.

The use of microalgae as a wastewater treatment method reduces the need for energy intensive cleaning processes and chemical use as is standard in wastewater treatment across the world [260]. The mechanisms for nutrient removal depend on species but are generalised here to give a sense of the extent to which microalgae can be used for wastewater clean-up, and the problems faced. Waste stabilisation pond systems are one of the most popular and well established technologies for wastewater treatment using microalgae. These tend to be open air ponds with a variety of autotrophic microalgal species. They are used to reduce the nutrient loading (in particular N and P) in the wastewater. A recent study conducted in Taiwan showed complete nitrogen removal and 33% removal of phosphorus was achieved by *Chlamydomonas* sp. [145] and *Chlorella* sp. removed high levels of ammonia, total nitrogen, total phosphorus, and chemical oxygen demand (COD) in 14 days [261]. Heterotrophic activity can also occur in these ponds, demonstrated in the same study, where it was proven that strains could remove organic carbon from the water under mixotrophic conditions, leading to higher growth rates and lipid yields making it suitable for biodiesel [147]. Heterotrophic growth trials have also found promising results with regards to reducing dissolved organic carbon, N and P in water bodies, particularly where mixed cultures (autotrophs, mixotrophs and heterotrophs) were used [169] but also where axenic cultures of *C. vulgaris, C. sorokiniana, R. sphaeroides* or *Scendesmus* were used [27] and in fact, there could be potential for higher nutrient removal by heterotrophic species [262].

Heavy metals, phenols, endocrine disruptors, antibiotics, polychlorinated biphenyls, viruses, antibiotics, pesticides, oils and greases have all been detected in either industrial or domestic wastewater sources [257,263,264,265]. Microalgae respond to these in different ways, from bioaccumulation to biodegradation [265]. Compound uptake is highly species-specific, with toxic concentrations varying for different applications. Heavy metals can severely inhibit photosynthesis by blocking or replacing prosthetic metal atoms in enzyme active sites [266]. On the other hand, it has long been known that microalgae can be used to remove pesticides from water sources [267]. Bioengineering of microalgae and cyanobacteria could lead to further pollutant removal from water bodies [265]. A number of companies are investigating the use of microalgae for cleaning of process water from the oil and gas industry (e.g. Petrobras project in Extremos, Rio Grande do Norte in Brazil). The nutrient profile of the water is very low in nutrients such as N and P, but high in organic carbon, suggesting mixotrophic (photoheterotrophic) growth occurs. However, use of microalgae in further applications (e.g. fuel, food, pharmaceuticals etc.) could be compromised if toxic compounds were found to bioaccumulate leading to their release either through emission from combustion or ingestion [268]. Examples of studies showing accumulation of heavy metals by autotrophic *Chlorella* sp. [197] and *Scendesmus* and uptake and biodegrading of organic pollutants by *C. reinhardtii* [265]. However, no trials show the performance of heterotrophic species to date.

7.1.1.1.3 Viruses, pathogens and parasites

Viruses affecting microalgae are thought to be ubiquitous in aquatic environments and function as an ecological mechanism for controlling microalgae populations [269,270,271]. Two impacts for large scale autotrophic microalgae cultivation could result from this. On the one hand it may lead to a population collapse, thus resulting in loss of the algae and knock on effect on the supply chain for which it was intended. On the other hand, viruses could be used to control algal blooms. Further work is required into the potential for this and whether the same virus may affect heterotrophic microalgae.

Parasites may also threaten the health of the microalgae culture. A specific example is that of *A. protococcarum* which was identified as being a risk to microalgal cultures. Research found the parasite is diverse and requires further research to understand its behaviour in order to protect microalgal cultures [272].

Pathogens that could affect humans or animals will co-exist with microalgae. Where water is sourced from waste streams, particularly municipal or animal waste, there is a high chance that pathogens will be present. This will affect the end use of the microalgal product, or at least the post-treatment it must receive before it can be used in any product where it could present a potential health risk. There are also occupational health hazards for those managing the algal farms [268], although this risk may be minimised with heterotrophic microalgae where closed systems can be utilised.

Table 7.2 Compounds found in wastewater that could be assimilated by microalgae

	Nutrient recovery (C, N and P)	Endocrine disruptors	Heavy metals	Oils/grease	PAH's*/ PCB's**
Cultivation technique	Autotrophs/ heterotrophs	Autotrophs/ Cyanobateria	Autotrophs	Autotrophs/ heterotrophs	Autotrophs
Source	Municipal, industrial or animal wastewaters, fertilisers, anaerobic digestion effluent, industrial exhaust gas.	Pharmaceuticals, plasticisers, hormones, pesticides, polyaromatic hydrocarbons etc. [273].	Industrial wastewater, mining, municipal wastewater.	Spills, mining activity.	Oil/coal industry, diesel/gas engines, incinerators, asphalt production, coke stoves [274].
Potential effects of excess in humans/ animals	Nitrates can cause methemoglobinemia [275]. Excess phosphorus can lead to kidney damage in animals [276].	Neurological effects, birth defects, reproductive health problems [277].	Bio-accumulates in food chain. Range of health impacts.	Variable toxicity. Potentially lethal to aquatic wildlife. Bioaccumulation issues [278].	Carcinogenic, mutagenic, and teratogenic [279].
Effects in microalgae	Enhanced biomass accumulation, changes in biomass composition depending on water composition Eutrophication or population collapse.	Enhanced growth in cyanobacteria <100mg has no affect in marine microalgae >1mg/l photosynthesis completely inhibited in marine microalgae [273].	Sulphur accumulation Metal recovered by microalgae could limit application of microalgae Metals detected include: Cd^{2+}, Ag^{2+}, Bi^{3+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} [257,280].	Prolonged growth phase, higher biomass production [265].	Bio-accumulation and bio-transformation of PAH's (highly species specific). PCB's accumulate in lipids [265].

*PAH's: Polyaromatic hydrocarbons

**PCB's: Polychlorinated biphenyls

7.1.1.2 Impacts to terrestrial systems

Biofuel production has met with controversy regarding displacement of food crops for production of fuel. A key advantage of using microalgal biofuels is the reduction of land needed to grow the same quantity of fuel given faster growth rates and higher yields per unit area than terrestrial crops [141].

Many of the initial claims made for the amount of biofuels that could be produced from algae used prediction based on small scale cultivation, which could potentially be much lower in a scaled up operation. Estimates based on autotrophic systems suggest an oil production rate from algae of 5775 L ha^{-1} yr^{-1} (4620 L ha^{-1} $yr^{-1}$of biofuel considering the 80% conversion efficiency) [281]. There is potential for heterotrophic microalgae to reduce this figure further due to the ability to grow it at a higher density and also in deeper vessels, therefore increasing volume per area as light penetration is not an issue [27].

7.1.1.2.1 Land use change

The criteria for site selection for microalgae cultivation will be determined by the cultivation method. For autotrophic microalgae the criteria were defined as a water supply with appropriate salinity and chemistry, suitable land topography, geology and ownership, good climatic conditions and easy access to nutrients and carbon supply [144]. The same criteria would apply to heterotrophic microalgae although potentially with less emphasis on land topography because, as discussed above, heterotrophs could be grown in deeper vessels. A map was developed to identify suitable locations for autotrophic microalgae cultivation, illustrating where the criteria above could be met [282]. All areas identified for autotrophic microalgae as suitable are within the tropics, where the temperature is high enough to support growth throughout the year, there is a critical mass of population to provide the nutrients required through wastewater, and varied between inland and coastal locations. In Brazil the most suitable locations in terms of nutrients were located towards the coast, in particular in the northeast, central and southern regions. Availability of flat land and suitable infrastructure also highlighted the northeast and southern areas. The infrastructure would still be of importance for heterotrophic microalgae as locating near nutrient sources would be crucial, but an alternative requirement of being near a carbon-rich effluent would be required instead of near a CO_2 source. However, an additional benefit in terms of location exists for heterotrophic microalgae as small areas of land could be sought increasing the possibility of locating near nutrient and energy sources at lower cost (i.e. cost to buy land).

Whilst cultivation could take place on marginal land as described above, there would inevitably be changes to existing land use including pasture and forested areas. Direct land use change measures the direct GHG emissions caused from changing from one land use to another, for example how building cultivation tanks on arable land leads to changes in gas fluxes. Indirect land use change occurs where land previously used to cultivate food is used to grow fuel crops, thus displacing food production to another area of land. The indirect change is the change in emissions as a result of changes made to the land that will now grow food. In 2012 EU member states agreed to report indirect land use change (ILUC) by fuel suppliers into GHG figures [283].

7.1.1.2.2 Contamination and leaks

There are many designs for cultivation reactors, depending on whether autotrophic or heterotrophic cultivation is taking place, and designs are still being optimised for biomass growth.

Open ponds allow large scale autotrophic cultivation at lower cost than PBR’s. However, the open design makes them vulnerable to contamination. This risk can be minimised by altering culture conditions, making them unfavourable to native species. Ponds that are not correctly designed or constructed could pose a threat to the direct environment from leaching into the ground. Examples include salinisation in situations where marine algae are cultivated on land, or loss of toxicants where microalgae are also being used as a wastewater treatment facility [268]. Whilst the content of the ponds would not necessarily be toxic, it may lead to contamination of ground water.

Closed reactors are more likely to be used for cultivation of heterotrophic microalgae, and are less susceptible to contamination to and from the local environment. However, bioreactors that are not correctly engineered could still pose a threat to the direct environment from leaching of the contents into the ground. Leaks from either system would lead to problems including salinisation in situations where marine algae are cultivated on land, or loss of toxicants where microalgae are also being used as a wastewater treatment facility [268]. Depending on the volume, a leak from these containers could also have a significant environmental impact, for example if located near a natural source of water.

There is a high potential of contamination in media by bacteria, in particular where wastewater is used. This could also affect the emissions from the cultivation, discussed in more detail below. Species that may be affected in particular could be H_2S, N_2O which are the result of bacterial activity.

7.1.1.2.3 Impacts to terrestrial diversity

The construction of ponds could also lead to the displacement of local fauna through destruction of habitat. Environmental Impact Assessment surveys can be used to assess the level of impact the construction of large scale ponds would have. The US NRC identified effects on terrestrial biodiversity from changing the landscape pattern as a result of infrastructure development for algal biofuels. They suggested land required for the cultivation of algal biofuels is unlikely to compete with high quality land as non-fertile soil can be used [268]. However, the development of a large scale facility would also face challenges, in particular damage to local habitats, including pollution, and disturbance by presence of human activity. Whilst development of facilities would be relatively localised, there would also be need for development of further infrastructure such as roads and power and pipe lines.

Due to the fact that heterotrophic microalgae would require less land area because of the ability to grow microalgae heterotrophically in deeper ponds, these impacts could be reduced. Also, the nature of the ponds being closed reduces the risk to wildlife as they would not be able to drink from them and also reduces the risk of animals breeding in the ponds, in particular mosquitoes and other animals that use standing water to breed.

7.1.1.3 Atmospheric impacts

Whilst the direct impacts of microalgae cultivation are most apparent to water and land systems, large scale microalgae cultivation also has a range of potential impacts on the atmosphere. The scale of the impact will depend largely on the type of cultivation system, for example there may be a higher risk from large scale open systems than closed systems where some gases could be captured within the reactor. This section looks at potential gaseous and aerosol emissions from autotrophic microalgae cultivation in particular. It also looks at direct impacts and further atmospheric reactions that can take place as a result of the pollutant species emitted. A summary of the main species that could potentially be emitted is given in Table 7.3.

7.1.1.3.1 Nitrogen dioxide (N_2O)

N_2O emissions from microalgae are of concern if they can be proved to be significant. N_2O is 264 times more powerful than CO_2 as a GHG over a 20 year period [284], and therefore of concern, should the emissions prove to be significant during cultivation. Traditionally, two main routes have been proposed for N_2O production during microalgal biomass cultivation under non-axenic conditions; either from autotrophic bacteria which can use either hydrogen or sulphur compounds as the electron donor, or from heterotrophic denitrifiers, which can use organic compounds instead [285,286,287].

Generation of N_2O by bacterial denitrification occurs through a series of reduction reactions, shown in Figure 7.1. However, there have been few studies into the production of N_2O from microalgae cultivation. In open ponds of *N. salina*, N_2O levels were found to be negligible under oxic conditions, but they were increased where anoxic conditions develop [288]. The suggested route for N_2O production was from denitrifying bacteria in the culture. Another study from raceway ponds in Hawaii found that when NO_3^- was depleted in a raceway pond cultivating *Staurosira* sp., the water body would become a sink of N_2O rather than a source [289]. However, the same study concluded that the net N_2O mass transfer from the atmosphere represented an insignificant fraction of the overall CO_2 equivalent uptake by the microalgae culture. Whilst others suggest it may be possible to use antibiotic treatment to reduce N_2O fluxes to the atmosphere due to bacterial denitrification, this would inevitably lead to water quality concerns in relation to antibiotic immunity [288]. This is relevant to a heterotrophic cultivation system, which would not necessarily be axenic; it would be expected there would be presence of both various algae species and bacterial communities. On the other hand it may be easier to mitigate this from a heterotrophic system through some form of capture or abatement or by optimising culture conditions.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O$$

Figure 7.1 Denitrification pathway leading to GHG N_2O production [288]

A further source of N_2O exists where microalgal biomass (either lipid extracted or digestate from biogas production) is used as a fertiliser for nutrient recycle [290]. A study, following methods suggested in the IPCC AR4 report, calculated the use of microalgae digestate as a fertiliser can cancel any GHG saving benefits gained from displacing fossil fertilisers[291].

7.1.1.3.2 Ammonia

Ammonia (NH_3) is a reactive gas in the atmosphere as well as in water bodies. Emissions of ammonia can contribute to the formation of ammonium salts and nitrate aerosols within the atmosphere and thus to the formation of PM2.5 (particulate matter which passes through a size-selective inlet with a 50 % efficiency cut-off at 2.5 μm aerodynamic diameter) [85]. Via deposition processes, atmospheric ammonia can lead to water pollution through surface run off in the form of nitrites (NO_2^-), nitrates (NO_3^-), and ammonium (NH_4^+) and dissolved organic nitrogen potentially contributing to soil acidification, the leaching of soil nutrients, eutrophication and ground water pollution. In aqueous solution, ammonia gas (NH_3) remains in equilibrium with its ionised form ammonium (NH_4^+) and the relative concentration of NH_3 increases over the concentration of NH_4^+ when pH increases. Ammonia volatilisation has generally been reported as a main concern in open algal ponds, as it is assumed that ammonia nitrogen is lost to the atmosphere as a consequence of high in-pond pH values (>9) [292,293]. Such assumption considers the role of nitrogen algal uptake and algae-mediated denitrification (N_2O emissions). The question is whether this would remain an issue in a closed heterotrophic system, where either the emissions may be different from emissions from autotrophic cultivation systems, and it may be possible to scrub emissions from cultivation reactors should they be a cause for concern.

7.1.1.3.3 Hydrogen sulphide

In a water body that is depleted of oxygen, there is a risk of hydrogen sulphide being produced via sulphate reduction, as bacteria look for sources of energy to sustain growth [294]. This is a potential issue in large scale heterotrophic systems which are not axenic, where oxygen is depleted from the water for respiration by the heterotrophs. In order to manage this, good aeration of the culture is required.

7.1.1.3.4 Methane (CH_4)

There are only a few studies of CH_4 emissions from large scale microalgae facilities. Basic measurements from wastewater treatment plants, lakes or oceanic emissions could give an indication of potential levels of emissions [291,295,296]. However, due to the limited research in this area it is not possible to give a reasonable estimate. CH_4 is another potent GHG with a global warming potential (GWP) of 84 over a 20 year period and therefore large-scale emissions are of concern in the context of climate change. It also contributes to the formation of background ozone which has both air quality and climate implications [284].

It is widely acknowledged that CH_4 is produced via anaerobic decomposition by methanogenic bacteria. In a well-managed microalgae system, it would not be expected that any anaerobic conditions would exist due to constant aeration of the water. Therefore the production of aerobic CH_4 is of particular interest when calculating the potential GHG emissions from microalgae cultivation. Aerobic production of CH_4 was discovered in 2006, and is not a microbial process but rather an in situ process in living plants [297]. Studies have found that CH_4 is usually supersaturated above the surface water across the planet with respect to atmospheric levels, and have demonstrated that it is produced by the water under oxic conditions [289][296]. Therefore, any scale and type of microalgal cultivation facility is likely to make some contribution to CH_4 emissions to the atmosphere.

7.1.1.3.5 Biogenic halogenated emissions

Organohalogens are derived from CH_4 emissions, and therefore the level of CH_4 emitted by a cultivation site may have a direct impact on the level of halogenated species. Whilst the majority of halogenated compounds are thought to be produced by macroalgae on coastlines, microalgae have also been shown to emit a range of brominated and iodinated species [298,299]. The mechanism by which organohalogens are formed is biomethylation with a halogen ion, where sulphonium compounds are considered to be the main CH_3^+ donor [300]. Emissions could include dihalo- and trihalomethanes and further brominated and iodinated compounds [301].

Reactive halogen compounds can then be formed via the breakdown of organohalogens and impact on the oxidising capacity of the troposphere, as well as contributing to ozone depletion in the stratosphere [302][303]. Studies have also suggested that biogenic iodocarbon emissions may play a role in new particle formation in the atmosphere thus contributing to secondary aerosol production [304]. The size of the flux of halogenated compounds has been reported from only a few sources and requires further investigation, but these studies prove that large scale cultivation of microalgae, particularly on saline water, would have a certain degree of influence on the total halogenated species emission budget globally [299,305].

7.1.1.3.6 Isoprenes

The production of isoprene by microalgae has been observed from microalgae cultivated in seawater [299][306]. Isoprene is formed via enzymatic catalysis by isoprene synthase [307]. Isoprene is highly reactive due to the presence of a double bond and its effects on the global climate have been modelled with increasing interest over the past decade [308,309,310,311,312,313]. For example high concentrations of isoprene consume hydroxyl radicals, thus reducing their capacity to oxidize volatile organic compounds. This can lengthen the atmospheric lifetime (and hence climate change effects) of key global warming gases such as CH_4 [314]. The presence of sunlight and NO_x links VOC's to the production of tropospheric ozone (O_3) which has a positive radiative forcing potential [284].

Isoprene oxidation products have also been suggested to contribute to the formation and particle growth of secondary organic aerosols (SOA) which potentially have both air quality and climate impacts [305] . The amount of SOA formed is dependant of the level of oxidation, NO_x levels and organic aerosol loading. This could have an impact on the location of cultivation sites. If located near a source of NO_x, for example road links or industry, the levels of SOA could be higher [310]. However, this cannot currently be estimated and further work on the link between NO_x and cultivation is required.

7.1.1.3.7 Carbon dioxide (CO_2)

A number of studies have quantified the scale at which autotrophic microalgae can contribute to CO_2 uptake from the atmosphere via photosynthesis and have found the uptake rate varies between organisms. A surface response methodology developed by [315] quantified the contribution autotrophic microalgae could have for CO_2 uptake, if grown at optimum conditions. Using these figures and updating to 2013 levels of global CO_2 emissions; to remove 2.5% of emissions from the atmosphere (that is 900m tCO_2) requires 65,800km^2 land, equivalent to 0.43% global arable land (as defined at 15.3 million km^2 by the UN/FAO in 2009). Heterotrophic microalgae cultivation on the other hand would be a net contributor to CO_2 as it takes up oxygen and releases CO_2 during respiration [143]. However, there are currently no studies estimating the contribution heterotrophic microalgae cultivation for biofuels would have on a net CO_2 balance.

7.1.1.3.8 Emissions from application of chemical pest controls

In order to maintain a healthy microalgae crop, particularly where an axenic culture is required, the use of herbicides, insecticides or fumigants may be employed. Pesticides contain organochlorine compounds which, as mentioned above, lead to ozone destruction in the stratosphere [316]. However, it would be expected that the use of pest control would be lower than compared with terrestrial agricultural crops [317] as some species produce metabolites that act as natural pest control mechanisms [318].

7.1.1.3.9 Impacts of emissions to biodiversity

Particulate emissions can lead to impacts on human health by affecting the air quality as well as impacts to crops, trees and fragile micro-ecosystems. For example, tropospheric O_3, a by-product of VOC's (see section 7.1.1.3.5) has adverse effects for humans and wildlife for example damaging effects for crops, adverse health impacts such as respiratory problems etc. [319]. Ammonia is another problematic species for health and can pose a real threat to biodiversity. In particular the dry deposition of ammonia is suggested to be detrimental to sensitive ecosystems such as lichens and bryophytes.

7.1.1.4 Genetic modification

Genetic modification of microalgae is becoming appealing to some groups of scientists, especially due to the relative simplicity of the microalgae cell compared to higher plants which have cell differentiation. So far much attention has been paid to photosynthetic and metabolic pathways, particularly for antibody production and soil bioremediation. These species have been grown under controlled and concealed autotrophic and heterotrophic conditions [124]. However, concerns about biological contamination have been sensationalised by the media and are a hot topic for environmental campaigns so have restricted development in this area. On the other hand, with microalgae being one of the most fundamental parts of the ecosystem, a change in the natural ecosystem could have devastating effects for the whole food chain and beyond [126].

Table 7.3 Summary of emissions from microalgae and their potential impacts

Species	CH_4	N_2O	DMS/DMSP	VOC's	Halogenated Compounds	H_2O	NH_3	H_2S
Cultivation method of concern	Autotrophs/ heterotrophs	Autotrophs/ heterotrophs	Marine autotrophs	Autotrophs/ heterotrophs	Autotrophs	Autotrophs/ heterotrophs	Autotrophs/ heterotrophs	Heterotrophs
Potential Source	Anaerobic decomposition Aerobic bacterial production [289]	Bacteria	Biological interactions	Enzymatic (e.g. MVA or DOXP pathways [306]) Land use change [311]	Biogenic emissions Fumigants, herbicides[316]	Evaporation	Urea fertiliser	Anoxic water
Formation mechanism	$CH_2O \rightarrow ½CH_4 + ½CO_2$	Denitrification	Methionine → DMSP[320]	Dependant on sunlight and temperature Potential defensive mechanism to stress	e.g. $Cl + O_3 \rightarrow ClO + O_2$	Heating of water	Change in pH by photosynthesis activity [293]	Bacterial breakdown of organic material
Type of flux from microalgae*	Positive [289]	Range from negative to positive [289]	Negligible -positive [321]	e.g. isoprene: positive [299]	Negligible - positive [299]	(evaporation rate) 0 ± 2 $kg/m^2/day$	Unknown	Result of mixed cultures containing bacteria [294]
Direct Impacts	GWP: 84*	GWP: 264**	Sulphate aerosol production	Precursor for tropospheric O_3 production[311]	Stratospheric O_3 destruction [299]	Increase in OH• [311]	Formation of acid rain ammonium nitrate, salts, aerosols [322]	Odorous, toxic in high concentrations
Further Reactions	Decomposition to CO_2 Precursor for organohalogens [323]	Source of NO radicals leading to stratospheric O_3 destruction	Cloud condensation nuclei affect cloud albedo and hence global radiation budget	Sequesters NO_x as isoprene nitrate [311] Tropospheric ozone formation and secondary aerosol formation [310]	Secondary aerosol formation [324]	Reduce CH_4 lifetime [311]	Atmospheric oxidation of sulphur compounds, aerosol formation resulting in effects on global radiation budget [322]	Redox reactions leading to acid rain formation

*Fluxes vary depending on species, aquatic environment composition and environmental conditions therefore numbers presented here are an indication from the literature **GWP over 20 years.

7.1.2 Quantifying environmental impacts

A number of techniques can be used to quantify environmental impacts of plans, policies or projects. These include “Environmental Impact Assessments”; a comprehensive assessment of the biophysical, social and other relevant impacts of a proposed development, techno-economic evaluations which attempt to determine the externalities of a project in monetary terms (e.g. cost benefit analysis), and life cycle assessments (LCAs), as discussed below. The extents to which environmental assessments are employed vary between different regions and states, and hence the methodology and implementation will vary too.

7.1.2.1 Lifecycle assessment

An LCA is a tool used to evaluate the environmental impacts and resource consumption of a product. It takes into account all parts of a product’s lifecycle from the extraction of materials used to make the product, to its end of life management. It allows identification of highly polluting or energy intensive processes and can be used as a cost management and regulatory compliance tool within a wide array of industries. It also allows comparisons to be made between two or more products or services. This type of analysis can be used to take into account environmental impacts including carbon emissions, total energy, chemical use and water consumption.

An LCA follows a systematic approach, following four phases set out by the ISO standard 14044/2006 [325].

- Initially the goal and scope is defined, where the purpose, target audience and functional unit are defined. As part of the scope, boundaries to the system have to be set. System boundaries to consider include natural systems, geographical boundaries, time, production capital, labour force, other products LCA. Types of environmental impact and level of detail for use in the study also are defined at this early point in the project.
- Secondly a compilation of a lifecycle inventory (LCI) analysis is required. The LCI analysis builds a systems model of the technical components in the life cycle, generally using a flow model to illustrate the process. Data is collected for the activities identified, and the volumes of resources used are calculated in terms of the product of interest [326].

- The lifecycle impact assessment is carried out by assigning the LCI data to the relevant impact and resource categories. In a full LCA this will often include global warming contribution, acidification, eutrophication, photochemical oxidation, aquatic and terrestrial ecotoxicity, abiotic and biotic resource use and ozone depletion, alongside energy use.
- Finally, there is an interpretation stage where the results are reviewed and retested using sensitivity analysis. Conclusions are drawn from these, and compared with the goal and scope so that recommendations can be made.

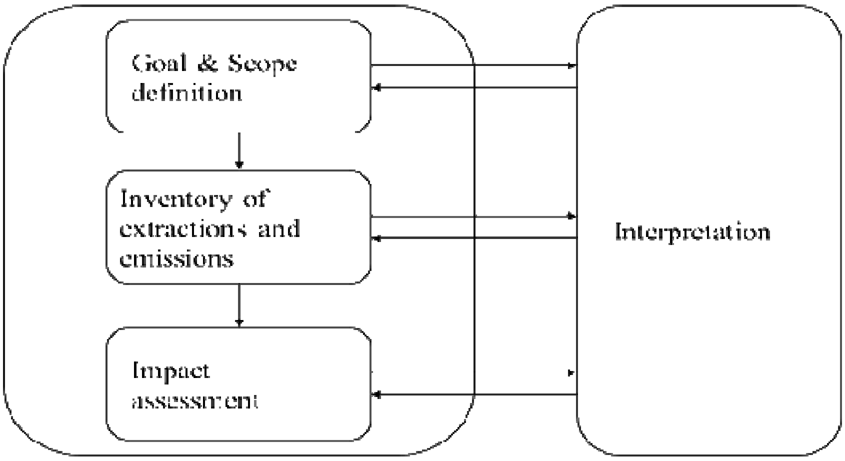


Figure 7.2 Lifecycle assessment methodology [325]

There are many applications for LCA, from product design, purchasing and development of policy instruments, to exploring possibilities for changes in production systems and communication methods such as eco-labelling and benchmarking [326]. However, because various techniques exist, there can be a lack of consistency [327]. This can cause problems for policy and design. The differences tend to be due to different system boundaries and lifetimes. Various software packages can be used to aid the process, and there is a standard LCA technique outlined by International Organization for Standardization (ISO) [325]. There are several well used LCA formats. The main difference between the techniques is the system boundary, which can be restricted to process data (as in process LCA) or expanded to include international economies data.

7.1.2.1.1 Allocation

Allocation is "*the act of assigning the environmental impacts of a system to the function of that system in proportional shares*" [328]. Allocation becomes a problem where a LCA includes multifunctional processes, such as recycled products being reallocated to various products or processes. The ISO guidelines state that allocation should be avoided where possible, and where there is no option, allocation should be first based on changes in inputs and outputs caused by changes in products, and based on economic relationships [325].

7.1.2.2 Biodiesel LCA’s

In order to investigate the environmental impacts of biodiesel production, and to be able to compare it with fossil diesel, many authors have worked on producing LCA’s for biodiesel from a range of feedstocks (e.g. [329,330,331,332]). The production of biodiesel requires many inputs, and each one of these has embedded energy which can be quantified. The placement of the systems boundaries is a key difference in many studies, with some authors focussing on production steps and the resources need to complete these steps, whilst others highlight the impacts involved with certain technologies, processing or use phases.

7.1.2.2.1 Edible crop feedstocks

Biodiesel from soybean can deliver many benefits including a high production capacity of 55,000 tonne/year and an input/output ratio of around 3.4 [127], plus a reduction in air pollution and reduced dependency on diesel imports. However, there are also many problems with soybean production including large scale monoculture, price volatility, low yields and land use change impacts [125]. A study comparing soy biodiesel with fossil diesel found the efficiency in converting raw energy (i.e. petroleum or soybean oil) to fuel is almost the same; the difference being biodiesel uses a renewable source. They calculated soy biodiesel produces 3.2 units of energy for every unit of fossil energy consumed in its production, compared with 0.83 units of energy produced from fossil fuels, per unit of fossil energy consumed [10].

Palm oil is a suitable feedstock for biodiesel as it exhibits similar properties to those of fossil diesel. However, palm oil has received a lot of negative press due to concerns about deforestation. The impacts on direct and indirect land use change have importance in this matter. An LCA that included inputs from agriculture, milling and transesterification within the system boundaries, with the outputs being biodiesel, glycerol, palm kernel, fibre and shells, empty fruit bunches and palm oil mill effluent found the energy ratio was 3.53, much higher than the energy ratio from rapeseed biodiesel in the EU, which was calculated to be 1.44 [332]. The same study also calculated that the emissions from oil palm were also 38% lower than fossil diesel, meaning palm oil feedstock complies with the sustainability criteria set by the EU for biofuels, which require at least 35% GHG savings [51]. Even after the land use change including transformation of peat land to plantation (common in many tropical countries, including Brazil) which causes a flux of 15-70 tons of CO_2 over 25 years, the net GHG balance was still found to be negative (i.e. CO_2 assimilation by palms). However, N_2O emissions were not accounted for even though they are a result of drainage and development of peat land.

Another study looking specifically at Brazilian and Columbian oil palm found an energy ratio on average of 4.8 for oil palm. However, state intervention has cut the use of fertilisers in Bahia, Brazil; meaning productivity from palm has fallen and therefore so has the energy ratio. On the other hand, in the north of Brazil fertilisers are still used and consequently production is higher [127]. The authors suggested sustainability could be improved by controlling or managing fertiliser use, co-generation, using ethanol (e.g. bioethanol) instead of methanol in the transesterification process and making oil extraction mills more efficient. Using bioethanol could lead to an increase in energy ratio to 8-9, as methanol accounts for 43% of fossil energy from the lifecycle energy in this study [127]. Co-generation of biodiesel and electricity production using a condensing steam turbine from the palm residues was investigated, and resulted in an energy ratio of 5.08, higher than any other terrestrial feedstock [333].

7.1.2.2.2 Non-food feedstocks

There are benefits of using non-food crops, such as castor or jatropha. For example they can often be cultivated on marginal land and therefore don't compete with food crops for fertile soil. Castor oil as a feedstock for biodiesel in Brazil has been shown to have a number of advantages, for example reduced air pollution during combustion, and it can be produced with an energy ratio of 2.0-2.9. However, there is a high opportunity cost involved with growing castor plants as they cannot be used for other applications, and the by-products are toxic therefore they cannot be used as an animal feed, although by-products can be used as a fertiliser. Long term agriculture involving castor beans can also lead to reduced soil quality if there is not proper agricultural management.

In China, a LCA investigating the impacts from soy, jatropha and microalgal biodiesel found improvements in the GWP, abiotic depletion and ozone depletion categories where biodiesel was used instead of fossil diesel. They suggested the best way to improve in other categories was linked to management of traffic and infrastructure rather than fuel source [334].

7.1.2.2.3 Microalgae

With microalgae being considered for use as a biofuel source, it is essential it has a beneficial energy ratio. The contribution it will make to GHG emissions are of utmost interest in terms of environmental impact, and are linked to the energy required in production, especially where this comes from fossil sources. The source and quantity of energy needed for cultivation is key to making it a sustainable and low emission product.

LCAs that consider energy use fail to agree on an absolute figure for the amount of energy required to produce a certain quantity of biomass. For example, one study compared eight LCAs from a range of authors (including [148,330,335,336,337,338,339]) in terms of MJ/kg dry biomass from raceway ponds [340]. Each study used different conditions, and hence the energy requirement varied considerably from 0.42 – 47MJ/kg algal biomass. Figure 7.3 shows the relative energy requirements as their energy ratio for biodiesel produced from different feedstocks. GHG emissions also depend on cultivation methods, and can range from lower to considerably higher than other feedstocks, between 0.4-4.4 kgCO_2eq/kg feedstock compared with 0.4-0.5 kgCO_2eq/kg feedstock for soybean for example.

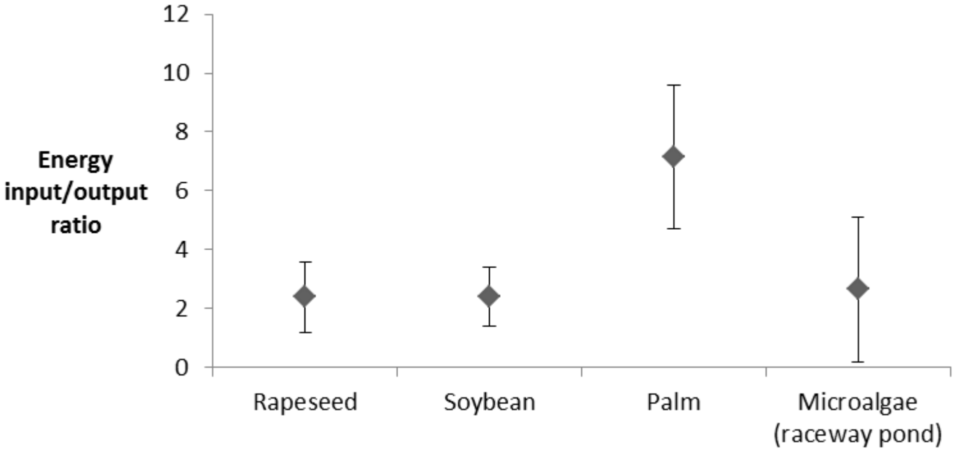


Figure 7.3 Energy ratio for production of biodiesel from different feedstocks [127,148,251,336,339,341,342]

In terms of energy demand for microalgal growth there is not a clear difference between the use of saline or freshwater sources [336,337,338]. However, there are significant energy input implications, associated with water use. The water-energy nexus is a relationship between the energy required to supply water and water required to produce energy. The amount of energy required to clean water to drinking standard is in the range of 5.4 - 25.55kWh/m^3. The energy required just for supply of surface water was measured to be 0.035kWh/m^3 in California [260]. A study on the water requirement for biodiesel production from autotrophic *C. vulgaris* in open ponds estimates between 1-11 billion m^3 would be needed to achieve the target of 1 million m^3 biodiesel [187]. This would lead to an energy demand of up to 281 TWh if clean water was to be used, equal to 88% the UK's electricity consumption for 2012. Where untreated water or seawater can be used there will be energy savings. In terms of availability for large scale cultivation, the use of freshwater cultivation will be limited due to the competing markets such as domestic and agricultural use.

The choice of cultivator will affect the energy usage, affecting the overall GHG emissions associated with microalgae cultivation. Heterotrophic microalgae will need to be cultivated in closed reactors, but will not require a light source, hence saving energy over a typical PBR used for autotrophic cultivation. With lower evaporation in a closed unit and higher density of biomass, it could be conceivable that heterotrophic cultivation could use significantly less water for cultivating the same quantity of biomass for biodiesel as autotrophic cultivation. The energy ratios in Figure 7.3 show the range that have been modelled from autotrophic cultivation in open raceway ponds and PBRs. PBRs generally have a lower energy ratio due to higher energy intensities during cultivation and despite generally having higher productivities. However, the comparison is difficult as all studies have different system boundaries and assumptions on growth rates and lipid content.

Co-location of microalgae cultivation with other industries could lead to a range of energy savings in production of microalgal biodiesel. For example, previous work looked at co-locating microalgae with sugar mills in Brazil to utilise CO_2 [251]. This is not a requirement for heterotrophic microalgae, however the effluent from the sugar mills is high in organic matter which could be suitable for cultivation [204]. This can also be supplemented with crude glycerol from the biodiesel production process, which is otherwise costly to refine and has a low market value. Heat produced from other industries could also be used in the transesterification steps.

7.2 Methodology

The energy ratio and GHG emissions from a microalgal biorefinery have been modelled in the present work in order to calculate the potential energy requirements to produce biodiesel from heterotrophic microalgae, and the potential contribution to GHG's. To the author's knowledge, this is the first study to focus on the use of heterotrophic microalgae instead of the more commonly studied autotrophic microalgae.

This study is based on a hypothetical system in order to simplify the process and identify trends; therefore the values are based on a range of estimates from existing studies, and new values are derived from the experimental work presented in Chapters 5 and 6. This is because there is no available information on large scale production of biodiesel from heterotrophic microalgae. The results are intended to identify high intensity energy and GHG emissions, referred to as "hotspots", in the production process.

Scenarios were built using Microsoft Excel. This allows a full transparency when building the model and avoidance of black boxes which can occur in some LCAs. The production system is shown in Figure 7.4, and is based on existing systems for biodiesel production. Data was taken from a variety of sources as the system being modelled does not exist a whole, nor does it exist on an industrial scale. Consistency within a process stage has been maintained by using data from the same source throughout this stage.

Where data does not exist in the literature, data from the author's experimental work or estimates have been used. GHG emissions are also calculated at each process stage based on emission factors from a range of sources [343,344,345]. There are many emission factors missing, particularly with regards to N_2O emissions from cultivation which have seen only a small amount of research [288,346].

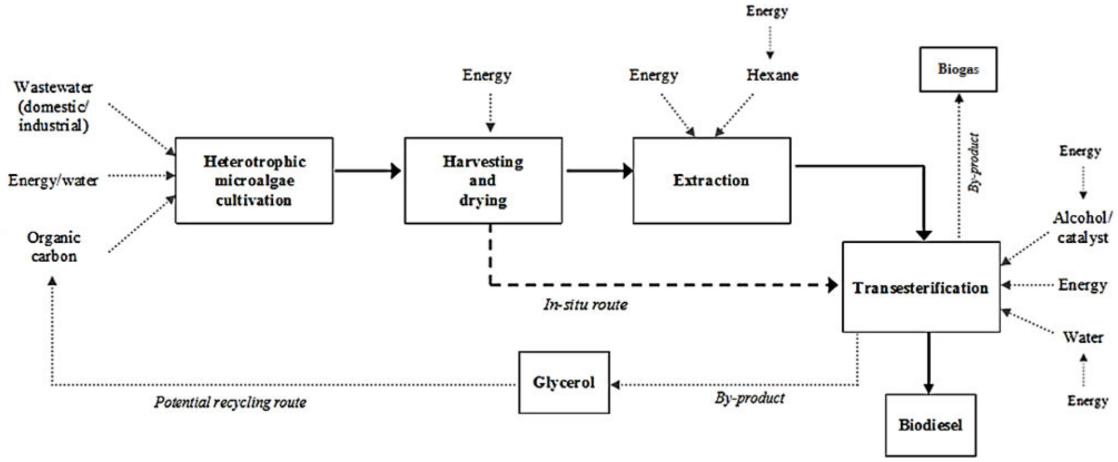


Figure 7.4 Systems boundary diagram of biodiesel production from heterotrophically grown microalgae

7.2.1 Scope

The aim of the model is to quantify the energy requirements for biodiesel production from heterotrophically cultivated microalgae. As a result, energy hotspots will be able to be identified, and comparisons will be possible with alternative biodiesel production methods.

The scope of the model starts with the production of the feedstock and ends with the output from the transesterification reaction. The system boundaries, shown in Figure 7.4, include the energy penalties of production of electricity and heat, clean water production [260] and production of virgin resources (e.g. nutrients, hexane, alcohol for transesterification etc.). Transport was not included, although this is considered a limitation of the study. The reason for excluding transport is that it is dependent on where the plant would be located. In order to maximise benefits of a microalgae biorefinery, it should be co-located with the nutrient source, the alcohol production unit and the processing plant. This would allow savings in both transport energy and heating energy. Infrastructure is also excluded.

A functional unit of 1kg biodiesel from heterotrophic microalgae, assuming energetic content of 39MJ/kg from the biodiesel was used. This functional unit was used as the basis for all calculations in the mass and energy ratio calculations.

The assumptions used within the model are discussed below. There were a number of limitations with regards to the data collection, due to the fact that this process does not exist at industrial scale. Therefore, data has been collected from literature assuming scaled up operation. Where possible, every effort was made to use data sources from infrastructure in Brazil. Where there was no existing data in the literature, results from experimental work in Chapters 5 and 6 was used. References to the sources of the data are made throughout this chapter.

The LCI is given in its entirety in Appendix C, with supporting references showing the data sources. The impact categories that have been reported on are the energy consumption of the process, and the total GHG emissions. The GHG emissions include emissions of CO_2, CH_4 and N_2O, and have been reported as kg CO_2 eq. based on their GWP [284].

7.2.2 Structure of model

The model is modular, with five units used to calculate the overall energy ratio. This structure is used as each process in the biodiesel production route is expected to be separate both in terms of location and operator. It also allows flexibility in using the model, for example to test different cultivation or processing techniques without needing to change other aspects of the model simultaneously. A mass balance was constructed based on the process steps outlined in Figure 7.4, then an input data sheet was created, based on the equations in Equation 7.2. The input sheet allowed calculation of the energy requirements for each resource used, in MJ, per unit of consumption (e.g. kg, kWh, m^3 etc.). It also allowed for calculation of the GHG emissions per unit. The requirement of each resource was then calculated per functional unit, to give a final energy demand and GHG emissions factor.

Equation 7.1 Calculation of energy ratio

$$Energy\ balance = \frac{energy\ out\ (MJ)}{energy\ in\ (MJ)}$$

Equation 7.2 Energy demand calculations, where E is the energy demand in MJ/unit required

$$E_{cultivation} = (E_{nutrients}) + (Pump) + (Water)$$

$$E_{nutrients} = (Organic\ carbon) + (Nitrogen) + (Potassium) + (Phosphorus) + (other)$$

$$E_{harvesting} = (Pump)$$

$$E_{transesterification} = (Alcohol) + (Catalyst) + (Heat) + (Water)$$

$$E_{biogas} = (Biogas\ yield) - (Production + Purification)$$

7.2.2.1 Cultivation

The data regarding the microalgae are based on the results gained from the cultivation of *C. vulgaris* in Chapter 5. Where parameters are missing, it is assumed a freshwater species such as *C. protothecoides* could obtain similar yield and growth rate [60]. The lipid content of microalgae varies between 12.2 and 53.4% in lab scale studies, shown in Chapter 6. For this reason the impact of lipid content on the energy ratio was investigated using sensitivity analysis. In scenarios S1-S6, the lipid content ranged from 10% to 70%, as described below in section 7.2.3.

The growth media for the base case was calculated from the use of virgin resources, including energy production requirements of fertilisers using industry data for fertiliser production (shown in Table 7.4), based on the recipe for HBM used in Chapter 6 (nutrient levels shown in Chapter 3, Table 3.1. Growth media for the recycled scenario (Scenario B, C and D; see below in section 7.2.3) are obtained from municipal wastewater. The nutrient levels are based on data from Ponta Negra waste stabilisation ponds in Natal, northeast Brazil. In the wastewater scenarios, the organic carbon source is assumed to be from industrial co-products and therefore no energy cost has been allocated. In addition, no energy discount has been applied despite the fact it may have led to savings in waste treatment energy and materials elsewhere. The type of carbon source affects the growth rate and lipid accumulation of the microalgae. Hence, the scenarios were tested with different lipid yields and growth rates based on experimental results. It is assumed that there is sufficient crude glycerol within the existing biodiesel industry to supply the organic carbon needs for microalgae cultivation. It was assumed that yeast extract could also be obtained as a waste product from other industries, for example breweries. No allocation has taken place in this study.

The energy cost of producing clean water has been included in the study as a comparison against using wastewater. A water energy nexus exists where water is required for power generation and power is required to clean water [260]. Therefore a figure of 0.036MJ/m^3 water treated is included in the base case scenario which is a low estimate based on water cleaning energy requirements from a range of countries.

The electricity source is assumed to be the Brazilian national grid, where the emissions factor is 0.0097kgCO$_2$/kWh. Where biogas production is included the energy savings are assumed after all electricity use has been accounted for. Therefore the emissions factor could potentially be lower where biogas is used onsite for electricity production. Electricity production from biogas is not included in the energy ratio.

Table 7.4 LCI for cultivation of heterotrophic microalgae

Step	Units	Value	Source
Nutrients			
Organic Carbon (Glucose)	MJ/kg	6.4	[347]
Nitrogen	MJ/kg	65.0	[348]
Potassium	MJ/kg	17.3	[348]
Phosphorus	MJ/kg	13.6	[348]
Pump			
Electricity	kWh/kg algae	0.2	[348]
Water			
Cleaning	MJ/m^3	0.036	[260]

7.2.2.2 Harvesting and drying

Harvesting was assumed to take place at the end of the stationary phase, and drying was achieved via air drying. This assumption is based on the layout of the wastewater treatment plant in Natal, Brazil, where by algae is air dried in the sun, and hence takes no further energy other than to pump the algae to the beds. A limitation of this stage is that no energy use was quantified for recovery of the dried algae from the drying beds. There were also no GHG emissions included in this stage.

Data was collected for electricity consumption for pumps from an existing LCA where natural settling was used as the harvesting method, assuming 95% bioflocculation rate after 6 hours. Whilst air flotation will be used as a harvesting method following completion of the waste treatment works in Pium, there is a lack of data relating to the energy requirement and therefore this method was not quantified in this study.

The harvesting by natural settling was followed by air drying to 80% moisture removal [119]. Flash drying was not considered as a feasible option due to the energy requirements of 3.5MJ/kg water removed [349].

Figure 7.5 Construction of drying beds for microalgae cultivated in wastewater in Pium, RN, Brazil. Beds have a capacity for 4 tonnes algae to be dried per day, the algae currently retails for £25 per tonne

7.2.2.3 Processing to biodiesel

In addition to two different cultivation techniques (virgin resources and wastewater), two scenarios for processing to biodiesel are calculated (lipid extraction and transesterification and in situ transesterification), as described in Chapter 6, Figure 6.1. The base case considers a lipid extraction using hexane followed by transesterification with methanol, as is conventional in industry for first generation crops. Data is not available for large scale extraction of oil from microalgae. However, it is stated in the literature that it has similar properties to soybeans, therefore data from EcoInvent covering soy mills has been used [77]. Yields obtained from experimental work in Chapter 6 are used for the mass balance. It is assumed the algae has been dried to 90% and is then subject to extraction via hexane solvent extraction. This also means the algae may be able to use existing infrastructure, although the construction of new infrastructure has not been included in this study therefore it is not possible to draw a comparison between utilising existing capacity and building new. The production of hexane was included in the energy ratio, the energy required to produce hexane is shown in Table 7.5. The products of the extraction are microalgae oil and lipid extracted biomass which can be used for either bioethanol or biogas production in alternative scenarios. This is compared to in situ transesterification with methanol (i.e. no extraction phase).

In the direct transesterification scenarios, lipids are not removed before transesterification, instead the biomass is directly subjected to transesterification conditions. This removes the demand for energy and chemicals involved with lipid extraction. The molar ratio of methanol to biomass is 6:1, using methanol production data from [343]. The rate of H_2SO_4 catalyst use is 2% per volume of oil. Water used for washing and biodiesel recovery is included.

Table 7.5 LCI for materials used in extraction and transesterification

Step	Units	Value	Source
Hexane	MJ/kg	0.52	[17]
Ethanol	MJ/kg	2.14	[350]
Methanol	MJ/kg	30.28	[343]
Sulphuric Acid (93% concentrated)	MJ/kg	2.4	[351]

7.2.2.4 Biogas production and purification

The biogas production step is based on anaerobic digestion of lipid extracted algae. The two products from anaerobic digestion are biogas and a solid residue known as digestate. During the digestion, organic materials in the LEA are converted to CH_4 and CO_2 via anaerobic microbial metabolism, at a yield of up to 70% CH_4, depending on nutrient ratio (C:N) in the digestate

[17]. In this study, values were based on those proposed which are based on LEA from phototrophic C*hlorella* [17]. The CH_4 production was calculated to have a mid-range of 0.3m^3/kg total solids in the digestate. The process is mesophyllic, in a completely stirred tank reactor. Thermal and electrical energy are required for operation of the reactor, accounting for 0.68kWh of thermal energy and 0.11kWh of electrical energy per kg of digestate. This energy consumption also depends upon the retention time, which was assumed to be 46 days (as in [348]).

Biogas production requires energy input in the form of energy for mixing of digesters and heating. Data for this study was taken from [348]. The efficiency of a turbine for electricity production is assumed to be 30%. A biogas upgrading step is also included in the energy requirements. The biogas can contain a number of impurities. A method for cleaning the gas includes bubbling through pressurised water. The CH_4 can be recaptured as it is not soluble in water, whereas CO_2 is. The process will also remove other trace gases and particulates that may be present such as H_2S, halogenated organics, siloxanes, particulates which will cause corrosion is combusted together with the gas in an engine or turbine [352]. The energy consumption for the biogas purification is 0.301 kWh/m^3 gas upgraded, resulting in a biogas product with 96% CH_4, based on the work by [348]. The use of solid waste as fertilisers is investigated by several authors [17,348]. However, this is considered outside the system boundaries set out in this study.

Anaerobic digestion will contribute to GHG emissions due to the use of electricity in the process, fugitive emissions from the reaction and combustion of the biogas and application of resultant digestate for fertilisers, either on land or for further microalgae cultivation. GHG emissions from normal operations were calculated to be 1-2% of the CH_4 yield but could increase up to 19% where flaring is used [353]. In this study, normal operations were assumed throughout the study. The complete LCI can be found in Appendix C.

7.2.3 Scenarios

The scenarios were based on variables in two areas: cultivation and processing. The nutrients and water for the cultivation were assumed to be either all derived from virgin sources, therefore the production of the nutrients and cleaning of water is included within the systems boundary, or all from waste sources, in which case no production energy is required. The processing from biomass to biodiesel was by one of two methods, either extraction of oil via hexane extraction, followed by acidic transesterification or direct transesterification where no pre-treatment was used. Each scenario reported the energy ratio for biodiesel production based on the assumptions listed below. The GHG emissions were calculated for scenarios A-D, and were based on kg CO_2eq per MJ for electricity consumption (based on the Brazilian electricity grid), or kg CO_2eq per kg of material input, which varied depending on the material in question. A full LCI including GHG emissions is given in Appendix C.

7.2.3.1 Scenario A: Virgin resources

This scenario investigates the production of biodiesel using microalgae cultivated using virgin nutrient sources, including a glucose organic carbon feedstock. The growth rate was 1.01g l^{-1} day^{-1}, and the lipid content of the algae was 22%. The biodiesel is dried via air drying, and then follows the process of oil extraction through to transesterification using a methanolic catalyst.

7.2.3.2 Scenario B: Wastewater media

The use of wastewater instead of media from virgin resources was used in Scenario B. The growth rate was lower than in Scenario A at 0.34g l^{-1} day^{-1}, but the lipid content was higher at 47%. The same drying, extraction and transesterification process was used as in scenario A.

7.2.3.3 Scenario C: In situ transesterification

The growth medium for the heterotrophic microalgae used in Scenario C was also wastewater (growth rate 0.34g l^{-1} day^{-1}, lipid content 47%), but a different processing method was used. In situ transesterification was used as the processing method; therefore no extraction stage is included.

7.2.3.4 Scenario D: High carbon

A scenario with optimised conditions refers to the growth conditions. The organic carbon content was increased to 100g l^{-1} in a wastewater media (as in the experiment described in Chapter 5, section 5.3.4). The growth rate was 3.06 g l^{-1} day^{-1}, and the lipid content was 24%.

7.2.3.5 Scenario “Autotrophic”

A further scenario was constructed to investigate a comparison between the energy required for heterotrophic microalgae compared with autotrophic microalgae, by modelling a system for autotrophic cultivation of microalgae in wastewater in open raceway ponds. This comparison was not possible from the literature due to different assumptions and system boundaries made in each study.

The scenario was based on growth characteristics taken from an open raceway pond, as this has lower energy consumption than PBRs in general (e.g. [17,339,354]) and therefore is proposed to be heterotrophic microalgae cultivation's closest competitor. The data for growth rate and lipid content used a range from between at 17.5 – 38.5% lipids and a growth rate of 0.19g l^{-1} d^{-1} based on data from [148]. It was assumed the microalgae were cultivated in a media composed of waste nutrients, as this has been shown to be possible by a number of authors (e.g. [145,168,175,261,355,356]) and at industrial scale as described in section 7.1.1.1.2. The oil extraction efficiency was assumed to be 70%, taken from [148] and the transesterification yield was assumed to be 98% taken from [251].

7.2.3.6 Sensitivity analysis

Local sensitivity scenarios were simulated to investigate the impact lipid content has on the energy content as it was assumed, along with growth rate, it could be one of the most important factors for a positive energy ratio. Scenarios S1-S3 are based on scenario A (i.e. virgin resources), and scenarios S4-S6 are based on scenario B (i.e. waste resources), with the lipid contents of 10, 40 and 70% generated respectively for S1-S6.

7.3 Results

The results from all scenarios were calculated using the model developed using Microsoft Excel. A mass balance was constructed initially, shown in Figure 7.6. This was then used to calculate the energy ratios from scenarios A-D, which are presented first, followed by results from the sensitivity analysis in scenarios S1-S6. The Autotrophic scenario is presented next and an analysis of the GHG emissions are then reported for scenarios A-D.

7.3.1 Mass balance

The mass balance was constructed from the functional unit. The original quantity of algal biomass required was calculated based on the efficiency of the process, taking into account any losses. Algae with a lower lipid content required more biomass for production of one functional unit. However, this resulted in more LEA available for biogas production. A refining step was included into the mass balance, in order to improve the accuracy in terms of the total biomass that needed to be produced. However, the energy requirements for the refining step have not been included in the energy ratio of GHG emissions.

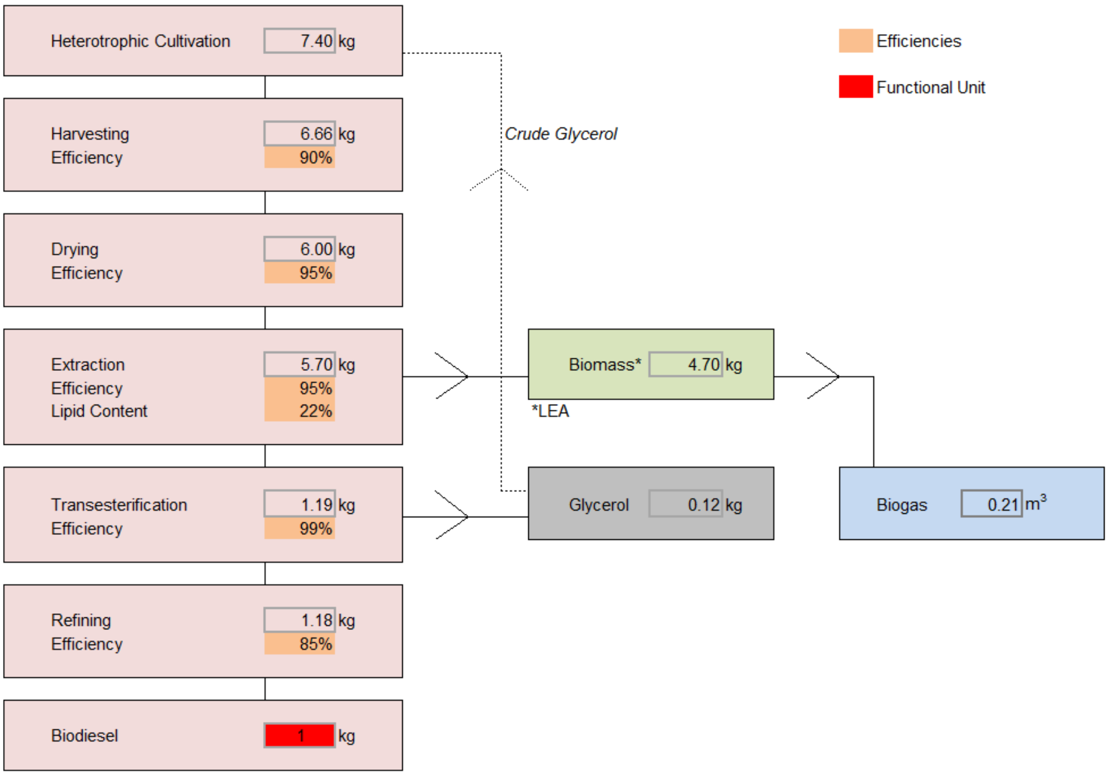


Figure 7.6 Mass balance for scenario B where the functional unit is 1kg biodiesel produced from heterotrophically cultivated microalgae

7.3.2 Energy ratio

Each scenario was constructed using different parameters, and these are reflected in the output energy requirements. The highest energy demand is in the cultivation with virgin resources in scenario A. The graph in Figure 7.7 represents the energy demand for cultivation in scenario A as scaled down by 1 x 10^{-2}, the actual demand being 449MJ/kg biodiesel. The contribution to the energy balance from biogas is shown as a negative energy consumption in Scenarios A and D due to the energy content of the biogas being higher than the energy required in the anaerobic digestion process. The reason for the energy yield being higher from scenarios A and D is due to the fact that lower lipid content was assumed. The effect lipid content has on the energy yield from biogas production is explored further in section 7.4.1.

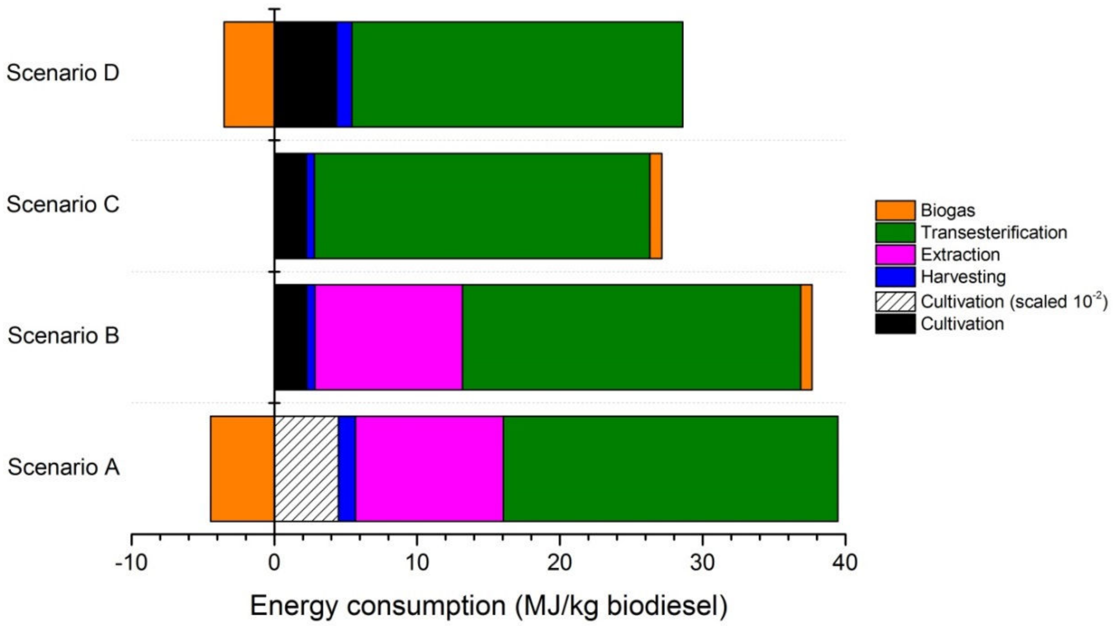


Figure 7.7 Energy requirement to produce 1kg biodiesel, assuming 39 MJ/kg, from heterotrophic microalgae under 4 different scenarios(Scenario A scaled down by a factor of 100)

The lipid content was also the major factor influencing the energy requirement for cultivation. Where the algae contained higher lipid content, cultivation of less biomass was required, and therefore the energy costs associated with 1kg biodiesel were lower, e.g. comparing scenarios B and C with D, where the lipid contents were 47% and 22% respectively.

Where the extraction step was avoided through in-situ transesterification in scenarios C and D, the overall energy demand was much lower, and gave an energy ratio of between 1.6-1.8. The energy demand is shown for each scenario in Figure 7.7. The transesterification stage created a large demand for energy use in biodiesel production, and represents a hotspot area for energy use that could be targeted. The main demand for energy was from methanol production.

The production of biomass from the LEA led to a net production of energy in scenarios A and D, but a net energy loss in scenarios C and D where a higher lipid content was assumed. The energy demand associated with producing biomass were 3.78MJ per kg LEA, including the electricity consumption of the digester mixing, centrifugation of digestate, and purification of the biogas (e.g. electricity and water requirements). This energy demand was subtracted from the total energy gained from the methane produced, which ranged between 2.9MJ/kg for scenario C and

8.3MJ/kg for scenario A to give a net energy requirement of between -4.5MJ/kg for scenario A to 0.8MJ/kg for scenario C.

The energy ratio is a measure of the energy input for production of biodiesel to the energy output which was assumed to be the energy content of the fuel, 39MJ/kg, shown in Table 7.6. A value greater than 1 indicates the process has a positive energy ratio. Two calculations of energy ratio were made, firstly without production of biogas from LEA, and secondly including the biogas production as part of an integrated system with biodiesel production. The energy ratio in scenario A where the energy requirements are very high resulted in a very low energy ratio. All other scenarios delivered a neutral (scenario B) or positive energy ratio (scenario C and D), the maximum being 1.6 in scenario D where the lipid content of the algae was 22%, and an in situ transesterification process was used, leading to lower energy requirements during production and allowing for more biogas to be produced.

Table 7.6 Energy ratio for production of one functional unit from a range of scenarios

	Scenario A	Scenario B	Scenario C	Scenario D
Energy ratio (no biogas)	0.08	1.00	1.41	1.40
Energy ratio (with biogas)	0.08	1.02	1.37	1.60

7.3.2.1 Sensitivity analysis

The sensitivity analysis investigated the effect of different lipid contents on the overall energy ratio. The lipid contents were assumed to be 10% in S1 and S4, 40% in S2 and S5, and 70% in S3 and S6. The lipid content of microalgae grown using virgin resources has a large impact on the energy ratio, with the total energy required for cultivation reduced from 988MJ/kg biodiesel to 141MJ/kg biodiesel where the lipid content is changed from 10% to 70%. Where waste resources are used in scenarios S4-S6, the energy requirements for cultivation are much lower in the range 10MJ/kg biodiesel where there the algae contains 10% lipids, to 1.5MJ/kg biodiesel where the algae contains 70% lipids. The harvesting energy is also lower where the algae was assumed to have a high lipid content due to the fact less biomass is required to produce the same quantity of biodiesel.

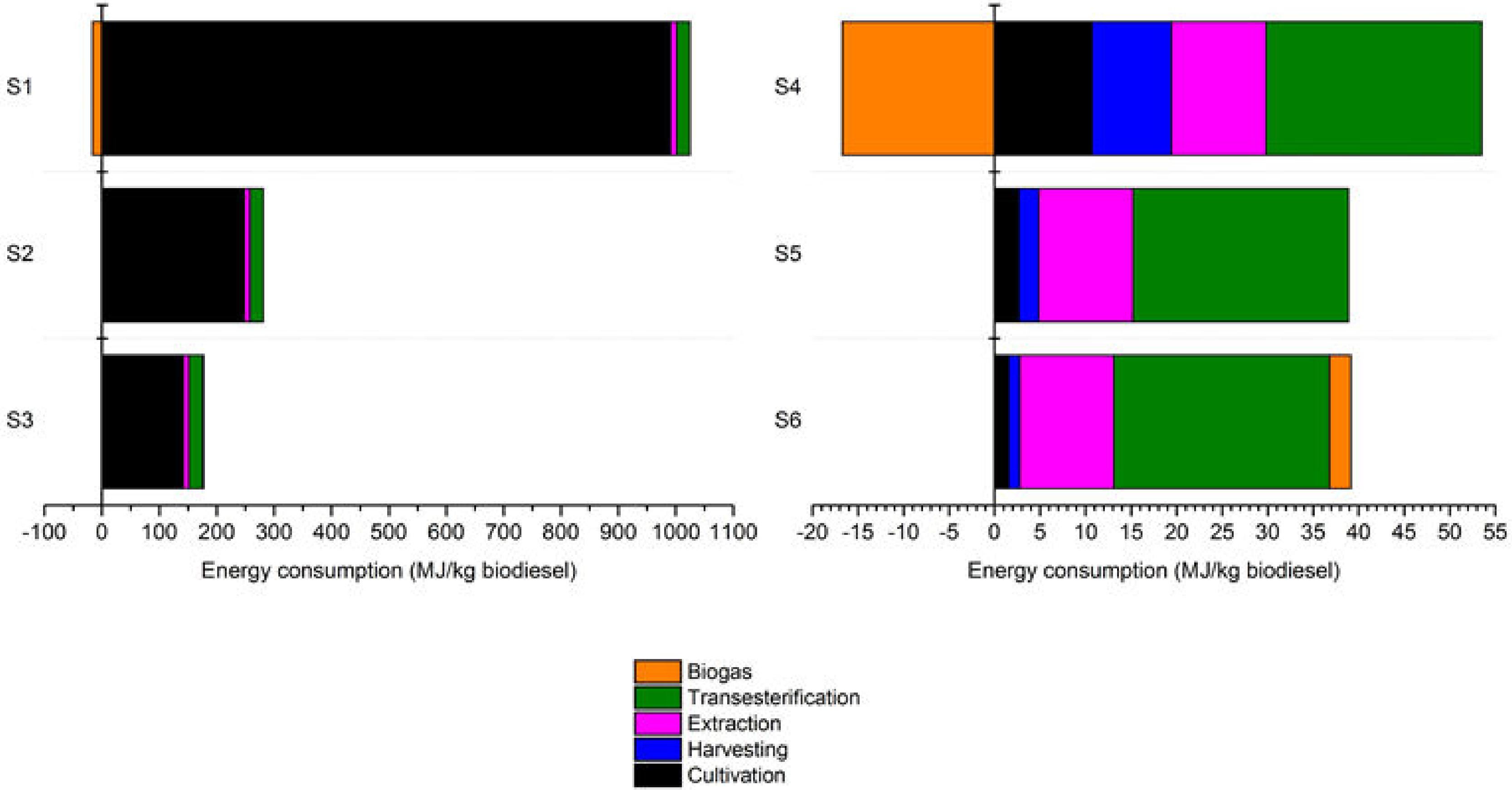


Figure 7.8 Sensitivity analysis based on the lipid content of microalgae where virgin materials (S1-S3) and waste materials (S4-S6) supplied the nutrients for cultivation.

7.3.3 Autotrophic microalgae

The cultivation of microalgae autotrophically using waste resources was modelled to allow for a comparison between heterotrophic and autotrophic microalgae to be made. Three simulations were run, the first with low lipid content (17.5%) the second with a high lipid content (38.5%) and high transesterification yield, the results of which are shown in Figure 7.9. The energy ratio for the low lipid content was 0.86 and for the high lipid content was 0.97, not accounting for any energy produced from biogas, shown in Table 7.7. Where biogas was included, the energy balance rose to 1.15 and 1.03 respectively for the low and high lipid content, giving a positive ratio overall.

Table 7.7 Energy balance from autotrophic microalgae containing depending on lipid content

	Autotrophic 17.5% lipid	Autotrophic 38.5% lipid
Energy ratio (no biogas)	0.86	0.97
Energy ratio (with biogas)	1.10	1.02

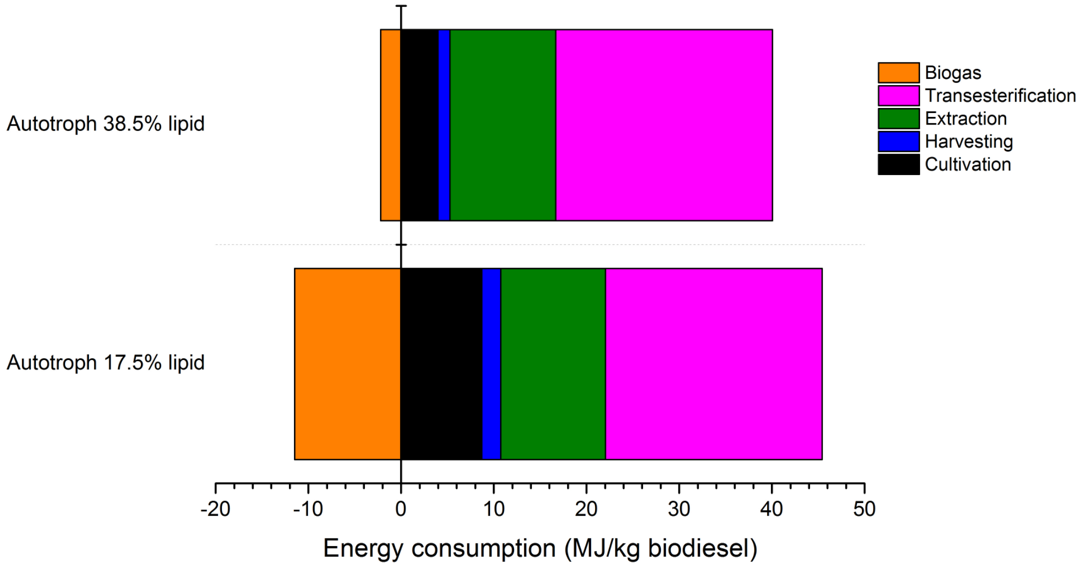


Figure 7.9 Energy consumption during production of 1kg biodiesel from autotrophic microalgae

7.3.4 GHG Emissions

The GHG emissions from the scenarios are closely linked with the energy ratio, with the scenarios having the highest energy input, also having a higher GHG output. This is clear in Figure 7.10, where the emissions of CO_2 from scenario A have been scaled down by a factor of 100. The majority of the emissions in scenario A are from the production of nutrient sources (i.e. fertilisers) for cultivation.

Cultivation of heterotrophic microalgae on waste resources (scenarios B-D) led to a relatively low level of emissions in comparison to the other process stages. There are several limitations in these calculations however as discussed below. The emissions are also linked to the quantity of feedstock required to make one functional unit, that is where the lipid content was lower, more feedstock was required and hence emissions were higher (i.e. in scenarios A and D). Scenarios B and C require less energy during the harvesting stage due to higher lipid content, and require less biomass to be pumped from the cultivation tanks for each kg biodiesel.

The extraction stage was the largest contributor to emissions after cultivation in scenario A, shown in Figure 7.11, therefore scenarios A and B which model the use of oil extraction before transesterification have a higher total of GHG emissions. The majority of these emissions are from CO_2 which was due to emissions caused during the production of hexane.

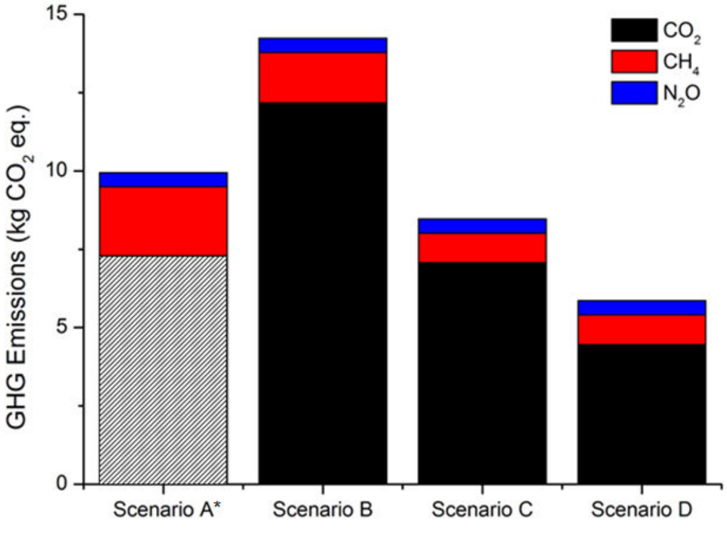


Figure 7.10 GHG emissions from scenarios A-D, shown per gas (kg CO_2eq) ***CO_2 emissions from Scenario A are scaled down by a factor of 100***

Production of methanol for transesterification was the largest contributor to emissions during the transesterification stage. This is due to the fact that it is produced from fossil fuel and refining is required, making it an energy intensive material to produce at 0.52MJ per kg. Emissions from the transesterification stage were similar for all scenarios as the volume of oil to be transesterified depended on the efficiency of the process which ranged from 97.6% for extraction and transesterification in scenario B to 100% for in situ transesterification in scenario D. Total emissions from scenarios S1-S6 are included in Appendix C.4. GHG emissions from autotrophic microalgae were not modelled and require further data for calculations to be made.

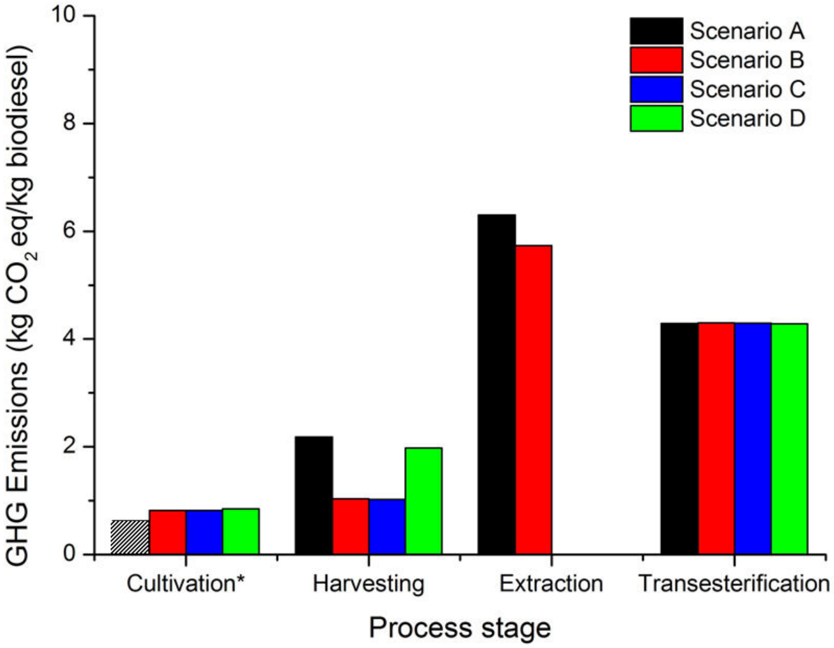


Figure 7.11 GHG by process step
CO_2eq. emissions from Scenario A have been scaled down by a factor of 100 (true value 62kg CO_2 eq/kg biodiesel)

7.4 Discussion

In order to assess the environmental impacts of producing biodiesel from heterotrophic microalgae, the literature concerning the challenges and potential issues involved with large scale cultivation of microalgae was reviewed. In order to gain new insight into what the energy and GHG emission hotspots might be in a new system using heterotrophic microalgae for biodiesel production, energy requirements and GHG emissions involved with the production process were quantified from cultivation through to a finished biodiesel product (excluding refining).

7.4.1 Energy requirements for biodiesel production

The use of virgin resources clearly indicates a higher energy requirement and makes the energy ratio unfeasible for algal biodiesel. The main reason for this is due to the energy intensity of fertiliser production, in particular nitrogen fertilisers which are largely produced using the Haber-Bosch method. The transport of fertilisers was not included within the systems boundary, but is of concern as fertilisers must be distributed from the place of production. In Brazil, the majority of fertiliser production takes place in the south, requiring long distances to be covered for distribution, particularly to the northeast region.

The comparison between scenario B and C highlights the difference between extracting oil before transesterification, and in situ transesterification. The result is evident in the energy ratio, which shows the in situ method leads to a more positive energy ratio due to lower energy requirements involved in oil extraction and higher yields due to greater efficiency. This is also clear in the sensitivity analysis where all scenarios tested (S1-S6) included oil extraction before transesterification, and all had poor energy ratio, the highest being 1.1 in scenario 6. This shows that whilst the lipid content does affect the energy ratio, it is not enough to make algal biodiesel energetically feasible. However, a challenge exists for the in situ method in that the infrastructure already exists for processing of oil, but alterations would have to be made to make plant suitable for in situ reactions. For example, removal of biomass following the in situ reaction would be required.

Scenario D represented an optimistic scenario in terms of growth rate, but the high growth rate was compromised by a lower oil yield, and this led to a higher overall energy ratio compared with scenario C. However, where a biogas route was included, a higher energy ratio was calculated, as there was more LEA for conversion to biogas.

Production of biodiesel from autotrophic microalgae showed a negative energy balance resulted from this process where no biogas production was included. There are several reasons that autotrophic microalgae was found to have a lower energy ratio than heterotrophic. For example, the biomass density is lower for autotrophic microalgae; therefore more water is required for cultivation, resulting in more water being pumped hence higher energy consumption. The lipid yield tends to be lower for autotrophic microalgae, although this is strain dependant hence two scenarios being modelled. The literature was used to obtain an oil extraction efficiency yield of 70% [148] which is lower than that found in experimentation with heterotrophic microalgae and therefore led to a higher energy consumption due to more biomass needing to be produced. However, once biogas production was included, the energy balance became more favourable than for heterotrophic microalgae under similar circumstances (e.g. in comparison with scenario B) because there was more LEA for the anaerobic digestion, therefore it would be possible to obtain a higher biogas yield.

Production of biogas was included to investigate the potential benefit of using LEA to produce a further energy source. The results showed that where the lipid content of the microalgae was low, a net energy gain could be made from the biogas. However, where the lipid content of the microalgae was high, this was no longer the case as there was not enough LEA to be digested in order to produce the biogas required to sustain the electricity demand of operating the biogas plant. The graph in Figure 7.12 shows that at about 40% lipids, the LEA will lead to a net expenditure on energy based on energy input into the biogas production plant. Potentially the LEA could be supplemented with sludge recovered from the waste stabilisation ponds; however this is beyond the scope of this work. The algae composition was also assumed to be consistent throughout each scenario. However, this may not be what experience in a real world scenario would be. Hence, the sensitivity analysis was used to investigate the effect of different lipid contents. The composition of protein and carbohydrates would also affect the biogas yield.

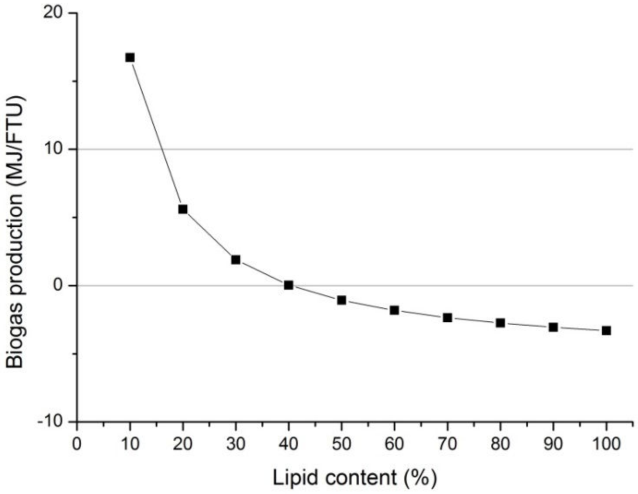


Figure 7.12 Biogas could lead to a net energy gain where the microalgae contain less than 41% lipids

The use of biogas may not be a realistic assumption under current circumstances in Brazil due to the economic value of biogas as an alternative fuel source. An example of where this is the case is at the Dom Nivaldo Monte urban wastewater treatment facility in Natal. The site treats wastewater from 450,000 people within the city, and produces 150kg biogas per hour from activated sludge. However, the gas is flared and not utilised for energy as it is not economical to capture and use it.

7.4.2 GHG emissions from biodiesel production

The GHG hotspots identified in the biodiesel production process were from the cultivation, where virgin nutrients were used, and from the extraction and transesterification stages. The production of fertilisers, as described above, are energy intensive and therefore lead to the production of large amounts of GHG, in particular CO_2 in the production of electricity and thermal energy. There were some GHG emissions from the extraction and transesterification stages associated with the production of materials too, for example hexane production for oil extraction (1.1kg CO_2 eq/kg biodiesel) and methanol production for transesterification (0.68 kg CO_2 eq/kg biodiesel). However, the majority of emissions were derived from the electricity requirement for the process, for example the energy required for electricity during oil extraction was 4.23kg CO_2 eq/kg biodiesel and for transesterification in terms of heat for the reaction accounted for 4.3kg CO_2 eq/kg biodiesel.

A limitation with regards to the system boundary was the exclusion of fugitive emissions from cultivation tanks and from biogas production.

Fugitive emissions include leaks of gases or vapours, and could include any of the species described in section 7.1.1.3 during cultivation. Fugitive emissions from biogas production could include methane in particular, a strong GHG. For this reason, the process needs to be monitored to ensure good practice is followed and maintenance of equipment is upheld. Calculation of GHG from autotrophic cultivation was beyond the scope of this study and should be considered for further development to provided further comparison between the two cultivation techniques.

7.4.3 Comparing heterotrophic cultivation with other biodiesel feedstocks

The energy ratio from autotrophic microalgae was found to be lower than that from heterotrophic microalgae, even based on an assumption of lipids being 38.5%. One of the reasons for this is that autotrophic microalgae generally have lower growth rates, therefore the same biomass yield takes longer to produce (i.e. growth rate of 0.02 – 0.03kg m^3 d^{-1} in autotrophic growth [77,335,336,339]) compared to 0.15-0.77kg m^3 d^{-1} in heterotrophic in this study and in [184,185,208,357]) and therefore the energy requirements are higher for powering pumps and other electrical infrastructure, or the volume of water required is larger again leading to a higher energy demand.

The energy ratios calculated for autotrophic microalgae biodiesel are within the range cited elsewhere in the literature, which has seen values of between 0.1 [77] and 6.8 [339] in open raceway ponds and between 0.2 [339]and 1.6 [336] in PBRs. The results found in this study resemble those found in the PBR studies. However, there are major differences in all studies which make this sort of comparison difficult. The major difference between studies tends to be the system boundary, and therefore which processes are included in the study. For example, in the study where the highest energy ratio of 6.8 was achieved, allocation of by-products was included, lowering the total energy consumption and therefore increasing the energy ratio [340].

This study has attempted to illustrate the impact of both the biological output (i.e. growth rates and lipid content) as well as the embedded energy in resources and energy input and as a result has led to lower energy ratio in comparison to those sometimes found in the literature.

7.4.4 Limitations and challenges

Whilst the aim of a lifecycle assessment is to provide a holistic view of a process, there are inevitably limitations and challenges specific to the method used. There may be limitations in the model due to its structure. Whilst the aim of using a model designed in Excel was to eliminate any "black boxes", that is unknown calculations taking place by predetermined calculations in the software, this extended the manual work time required for data collection and therefore restricted the achievable volume of data output. It also limited the number of impact categories that could be investigated for the purpose of this project.

Challenges arose during data collection. Due to the fact that a hypothetical situation was being modelled, there was not real time data to be modelled. Therefore data was collected from a number of sources including experimental work academic literature and industrial data. This allows the introduction of many errors, including reporting style and technique from different institutions, data quality and transparency and inconsistent assumptions across existing LCA's. In order to make this study as transparent as possible the LCI includes all references in Appendix C.

No allocation technique was used in this analysis as per the recommendations of the ISO framework for LCA and in the interest of avoiding double counting. The time frame for the GHG emissions was determined by using the unit of kg CO_2 equivalent. This unit is designed to take into account the effect any GHG will have during its residence time in the atmosphere over a 100 year period, based on the GWP (a metric that combines the radiative forcing effect of a particular gas over a set time horizon, in relation to that of CO_2 [284]).

The system boundaries were designed to include the production of materials used in the biodiesel production process. Inclusion of these components was considered a key element in identifying where the energy and emissions hotspots were. For example, production of fertilisers are an energy intensive process, therefore if alternative ways to deliver nutrients to crops for biofuel production can be found this will relieve pressure on production and thus reduce energy demand, reduce GHG emissions and also reduce pressure on supply for food crops. The choice to include energy required for cleaning water in the system boundary was made as it is often an overlooked resource. The energy water nexus has received some attention, as reviewed in section 7.1.1.1.1. The energy and chemicals required to clean water have an impact on the environment and therefore utilisation of untreated water for biofuel production reduces the energy demand, and in a more detailed LCA the impact of this could be investigated in other impact categories.

A major limitation in the system boundaries is the exclusion of transport. In Brazil the majority of freight transport is carried out by road, due to lack of rail, canal or pipelines nationally. This adds an energy and emissions burden to any manufactured good which must be transported, worsened by the quality of roads and the age of the freight fleet which is on average 18 years old. It could be assumed that a microalgae biodiesel production system could have advantages over soy biodiesel in Brazil, due to the fact that microalgae cultivation could take place closer to biodiesel refineries, should the land be available, as the quality of the land is not an issue. There could be symbiotic advantages of this co-location, for example the microalgae could be delivered directly to the biodiesel refinery after harvesting therefore management of supply might be improved, crude glycerol from the biodiesel production process could be delivered directly to the cultivation tanks and energy production from biogas could be utilised on site.

Energy requirements for refining of the oil were excluded from the system boundaries, although it was included in the mass balance. This is due to the fact that there is limited information on the exact refining the oil would require at a large scale. However, an approximation of efficiency was included in the mass balance to get a more accurate calculation of the amount of biomass that might realistically be required. Therefore the actual energy demand would be slightly higher on a cradle-to-grave analysis including refining energy.

7.5 Summary

An assessment of the environmental impacts of heterotrophic microalgae as a feedstock for biodiesel production was carried out through a literature review and construction of an energy ratio and quantification of GHG emissions associated with the process. The literature is optimistic on the use of microalgae biomass as biodiesel feedstock, and describes a number of potential environmental benefits if it is well managed, in particular with relation to wastewater treatment. However, there are still many unknown factors including real emissions of large scale cultivation, efficiency of bioaccumulation depending on algae species and pollutant and impact on land use change.

The results of the scenarios modelled here showed that whilst a positive energy ratio is achievable, it is dependent on reusing nutrients. The results also highlighted the use of electricity in the production process as a major barrier to a more positive energy ratio. It also showed that it is necessary that the system boundary continues to include processes, products, materials and energy use outside of the core mass balance as all energy use is interrelated.

A comparison between autotrophic microalgae and heterotrophic microalgae was made in terms of energy required to produce these two different feedstocks, following on from the comparison made in Table 2.3 in Chapter 2. This provides evidence towards expending further research efforts on developing heterotrophic microalgae as a feedstock for biodiesel.

There is a need to look beyond the physical aspects of energy ratio and GHG emissions in order to understand how well this system might fit into the existing biodiesel industry in Brazil. Therefore, Chapter 8 will take into account a perspective of the whole system in which biodiesel production is set, in order to identify potential barriers to the introduction of a new feedstock.

Chapter 8 Whole systems analysis for integrating microalgae feedstock into the Brazilian biodiesel industry

8.1 Introduction

> *"The whole is greater than the sum of its parts because things that will emerge from a whole system would not happen in isolation"* [358,359].

Biofuel production is a major industry in Brazil, with Brazil being the second largest producer of biodiesel in the world in 2013. The demand for biodiesel, which has been created through legislation in Brazil, has created social and economic opportunities and also social objectives that need to be preserved if biodiesel is to be adopted, as discussed in Chapter 4. A gap was also identified in Chapter 4, in terms of continuing to expand biodiesel feedstock production sustainably. The technological requirements for this were subsequently investigated in Chapters 5 and 6. Algal biodiesel will only become a reality if it can be embedded into an existing system in an economic and technologically feasible way. The impacts of introducing a new feedstock however, as shown in Figure 8.1, will also reach across the social, political and environmental spheres.

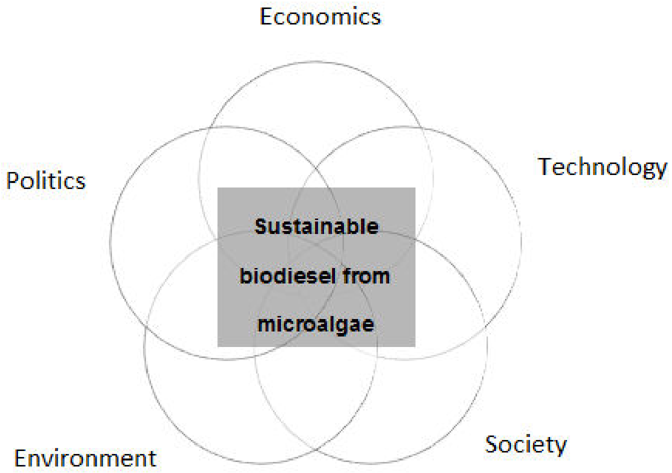


Figure 8.1 The sectors affecting the sustainability of algal biodiesel and its successful introduction into the supply chain

This chapter will work towards identifying how heterotrophic microalgal biodiesel can be developed and integrated into the existing industry. This will be approached by trying to understand the obstacles and potential risks to successful and sustainable production of algal biofuels and how these risks can be mitigated and obstacles overcome. A series of expanding system boundaries will be used to look at cause and effect within immediate systems and the wider environment and will identify the relative importance of political, economic, social, technological, environmental and legal inputs (commonly known as PESTEL analysis; environmental referring to the natural environment) in the context of producing a biodiesel product that delivers the three pillars of sustainability (i.e. environmental, social and economic). As a result, progress will be made towards the development of a roadmap to enable governments, industry and academia to identify steps needed to integrate microalgal feedstock into the existing system in a competitive, sustainable and integrative way.

In previous chapters, an approach was adopted in order to produce a quantitative analysis. This approach was mechanistic in that it analysed and reduced a system to form conclusions based on either experimentation, in this case the experimental work carried out in Chapters 5, 6 and 7; or analysis of parts which have been enacted, such as the PNPB which was analysed in Chapter 4. This chapter takes a holistic view to produce a more integrative picture of the biodiesel industry in order to identify barriers beyond the obvious that may hinder the introduction of microalgae as a biodiesel feedstock in Brazil. Barriers may be overcome through approaching obstacles such as a legal or policy driven framework supporting development, a strategy for managing the natural environment, stakeholder engagement at an early stage of the project to build on trust with those involved from investors to the general public, and stable funding and research efforts that address the PESTEL topics.

8.1.1 System design and whole system thinking

“Systems thinking” is an approach used to analyse networks in a diverse yet interrelated world. Complications arise due to ever increasing presence of technology in day to day life and gaps in scientific consensus combined with the irrationality and unpredictability of human behaviour. In general, the definition of a system is not well defined in the literature. Therefore the following definition has been used here to develop a way of looking at microalgal biodiesel in context:

> *“A system is an open set of complimentary, interacting parts with properties, capabilities and behaviours emerging from the parts and from their interactions”* [360].

The widest implications of systems are considered within a whole system analysis, including the environment in which it exists. Connections between systems and sub-systems and impacts they cause on each other are part of analysing a method.

Systems thinking has been employed in many disciplines to help develop more efficient systems, in particular the interface between human and technological operations. Examples demonstrating the range of topics that employ a systems thinking approach include technical product design methodologies [361], military and defence organisation [359] and organisation of the education system [362]. There are many methodologies, and hence the outcomes vary from quantitative assessments with the production of tools such as roadmaps and project plans, to qualitative dialogue with propositions for workshops and ethical discussions. Engineers in particular have an opportunity to utilise their technical knowledge and team it with other professional tools to manage, lead and understand complex interdisciplinary challenges [363]. A systems thinking approach also relies on an accumulation of experience and bringing together experts from traditionally separate disciplines to reflect on actions and modify behaviours, beliefs and interventions in order to improve operations and outcomes [364].

8.1.2 Shortfalls of thinking in isolation

In general, the approach to an engineering project is process driven, focussing on systematically dealing with problem situations in order to achieve an end product [365]. While this approach is successful in its intents, it can lead to provision of an unsustainable scenario (be this unsustainability economic, social or in the natural environment) whereby either the product is not fit for purpose, or there are externalities which could have been avoided through use of a more holistic analysis. For example, the use of electric vehicles is now a real prospect for consumers, but will not be practical without considering several dimensions. Technological considerations will include adaptations that need to be made to the grid by considering charging patterns and managing the electrical load [366], policy development will need to include ways to make electric cars affordable and the political and economic costs of competition with conventional motor vehicles need to be evaluated. Consumer safety issues are also part of the multi-disciplinary approach, for example with quieter vehicles that make it more difficult for drivers to perceive their speed or that other road users may not be able to hear, plus implications for journey planning of limited range [367,368].

Two approaches can be taken towards systems analysis, shown in Figure 8.2. Hard systems analysis assumes problems are relatively well-defined, may have a single and optimum solution and are dominated by technical factors. A soft systems approach, on the other hand, tries to analyse problems that are not necessarily well-defined and are therefore are more difficult or impossible to quantify. The issue with treating these two approaches as separate systems is that there is a risk of over-simplifying impacts or missing important drivers and therefore in the long term, the system is likely to fail and does not represent a sustainable analysis. Failures may be of an analytical nature, for example using incorrect or incomplete data or methods to prove a hypothesis, or occur in the real world, where observations of performance are not achieving pre-defined goals.

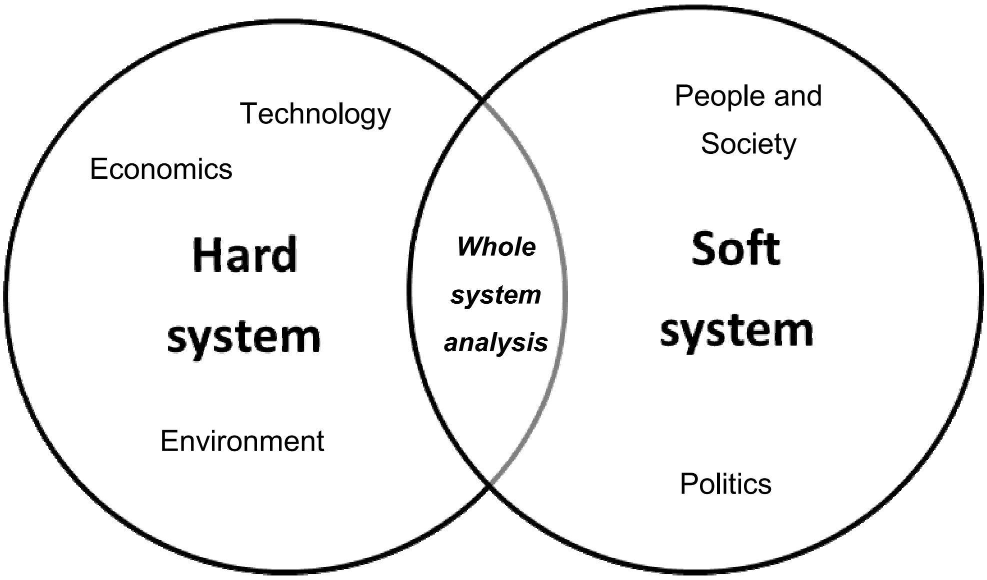


Figure 8.2 How systems can work together to provide a holistic view

There are also a number of obstacles that are commonplace in most organisations. These include pressure for uniformity in services (even where this favours an incumbent process or product, over an otherwise superior alternative), lack of evaluation, continuity or connection of previous policies, tight deadlines and inefficient project management, secrecy that stops knowledge transfer (despite protecting intellectual property at development stage), competition between industries in particular where political power is yielded, and use of command and control policies that have a high risk of failure within complex systems by alienating people of different cultures and goals by treating them instrumentally [364]. Identification of factors such as these can help them to be managed and the negative impacts minimised.

8.1.3 The need for measurement

Whilst it is important to appreciate the diversity of people and policies for any system, quantification is essential to assess feasibility and desirability of projects. Assigning value to a project can take a number of forms, for example assigning values during the projects life cycle by using cost benefit analysis must address tangible and intangible goods and services.

In trying to quantify the life cycle costs (including both accounting and economic costs) and risks, uncertainty and assumptions associated with hard to measure variables are introduced into a model. Drawbacks exist when modelling systems, in particular a downward spiral of lack of good data in modelling which leads to lack of confidence followed by lack of credibility for the results delivered through insufficient time or resources. However, identifying the gaps leads to opportunities for strengthening analysis and is a pre-requisite of building a successful model.

8.1.4 Uptake of new technologies

The uptake of technologies has been studied to identify why certain technologies are successfully introduced and consumed in the market place. Diffusion of innovation is a theory that tries to explain why certain technologies are more successful than others at spreading through consumer cultures, with the key elements influencing the spread being innovation, communication channels, time and the social system [369].

The diffusion process refers to how innovation spreads across a group to reach consumers, encompassing the adoption process over time. Adoption of new technologies is generally a result of competitive pressures and often a necessity of survival. The first companies to go ahead with the implementation of a new technology can often reap the benefits of higher returns, gaining of intellectual property and developing a new client base. However they will encounter major uncertainties which later adopters can learn from spillovers of knowledge and expertise. Spillovers can be differentiated in to the technology space where the spillover from R&D can lead to positive effects for other firms, and the product market space where spillovers can damage the value of companies due to business stealing [370]. An example of this would be the electronics market, where Samsung have benefited greatly from the technological and market expertise of rival company Apple [371]. The success of uptake of a new technology will also depend on the integrative capability of a firm to align the new technology into their business model [372].

8.1.5 Technological roadmaps

A technological roadmap matches specific technological solutions with short and long term plans. It considers the alignment of markets, products and technologies over time. Roadmaps are often compiled as a result of collaborative discussion between stakeholders including industries, political parties and individual interest groups, and can be internal technology roadmaps, for instance industry sector specific or overall technology (i.e. at a national or even international level e.g. IEA, as described below). At an industrial level, they are a way of dealing with competitive pressures, and nationally they can be used to ensure national security for trade and knowledge [373].

There are a number of biofuel roadmaps that have been developed over the last 10 years, giving the vision of individual countries and partnerships for biofuel development and deployment. An international perspective is provided by the IEA’s Technology Roadmap: Biofuels for Transport. The key challenges identified were creating policy frameworks for biofuels, finding funding and support, continuing to develop international sustainability criteria, linking financial support to sustainable performance, continuing research and development activities and adopting sustainable agricultural, forestry and land use management practices. The use of algal biofuel is also considered, with the verdict from the International Energy Agency being that the commercial viability of algal biofuels coming from “*effective strategies to generate high-volume, low- value biofuel along with high-value co-products*” [4].

National roadmaps for the production and use of biofuels have been produced by individual countries. For example the French Agency for Environment and Energy Management (ADEME in French) has developed a road map for Second-Generation Biofuels [374]. The REFUEL project (A European Road Map for Biofuels) developed in the Netherlands explores the expanding biofuel industry more generally within Europe [375]. However, neither of these explore the possibility of algal biofuels in any detail, The USA on the other hand, has developed a specific roadmap for algal biofuels, showing their commitment to development of these fuels, in the “National

Algal Biofuel Technology Roadmap”. This roadmap recognises algal biofuels are still in their infancy with considerable requirements for R&D, but also expects algal biofuels to have potential to contribute significantly to the renewable fuel production in the USA. It has identified the key resource requirements for heterotrophic microalgae as being “*the sourcing of suitable organic carbon feedstock, water, energy plus infrastructure required for siting and operating industrial bioreactor-based algae production and post-processing to fuels and other co-products”* [376]. The Natural Environment Research Council (NERC) in the UK has also released a technological roadmap exploring the sue of microalgae and macroalgae across a range of industries [399].

In Brazil, the Foundation for Support and Research in the State of São Paulo (FAPSEP in Portuguese) has developed a bioenergy programme called BIOEN to develop a roadmap for biofuels in Brazil, specifically for ethanol from sugarcane, but also for other plants that can be used for biofuel. The plan incorporates technological research for fuel development and engines, and also environmental assessment and policy design. They currently have a number of projects and workshops looking at the use of microalgae for CO_2 sequestration, production of biofuels and treatment of wastewater [377].

8.2 Methodology

It is necessary to understand the interrelated factors that would support the introduction and development of a new feedstock for biodiesel, whilst ensuring environmental sustainability measures, social inclusion levels and economic prosperity are adopted and upheld. In order to develop a way in which to analyse the system holding together biodiesel production in Brazil, and identify how microalgae could be integrated as a feedstock for biodiesel production it was necessary to develop system boundaries. The boundaries were divided into four sub-systems, which are nested inside each other. The context of this analysis is represented graphically in Figure 8.3 and is explained below.

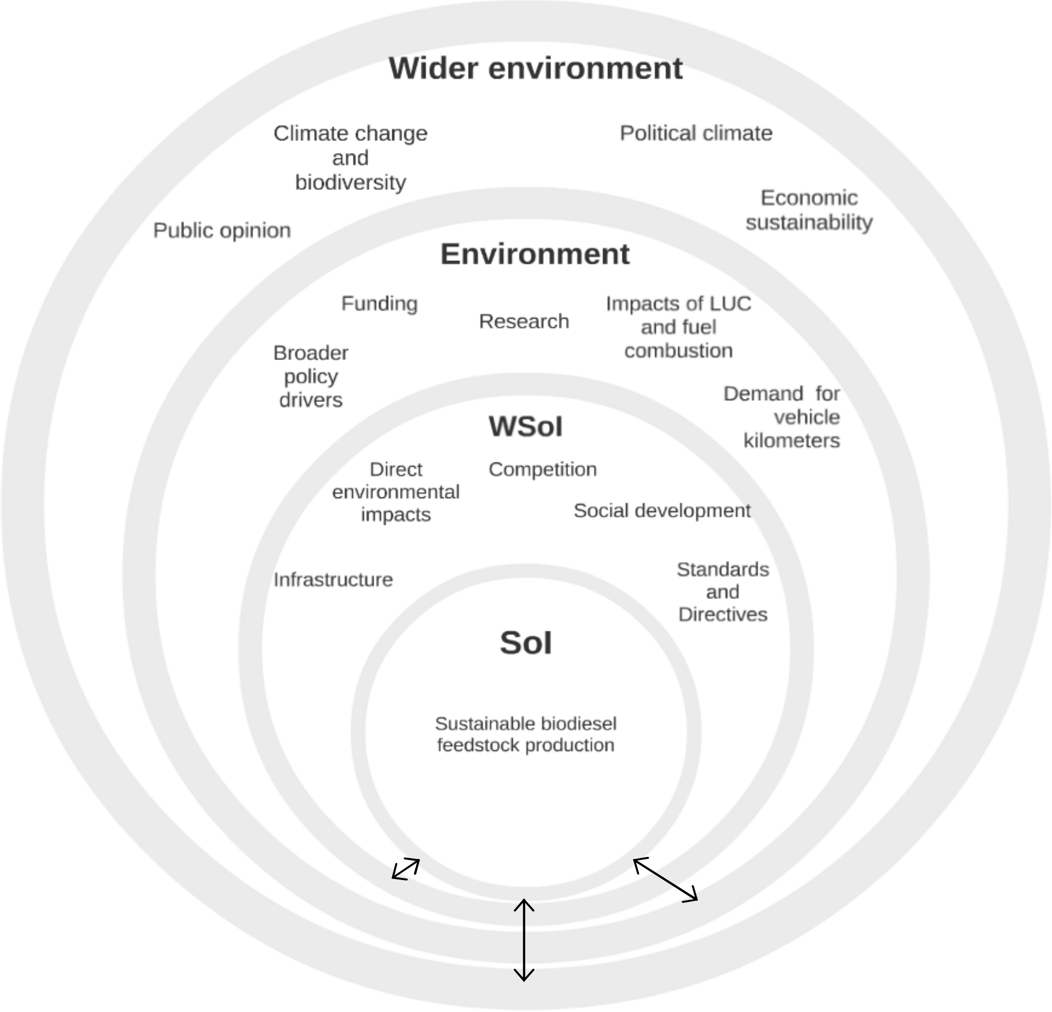


Figure 8.3 Context diagram showing the system boundaries (SoI = system of interest, WSoI = Wider system of interest)

An initial system of interest (SoI) and a wider system of interest (WSoI) were defined from the thesis objectives. The objectives were to analyse the success of the current biodiesel system by identifying and comparing the design features, followed by development of an alternative feedstock which would prove to be more environmentally sustainable than existing feedstocks. The SoI was defined as a "*renewable biodiesel feedstock produced from heterotrophic microalgae cultivated on wastewater and waste carbon"*. Analysis of the SoI will look at how the production could be impacted by external factors in the WSoI and beyond in the environment and the wider environment. The WSoI includes factors that will have a direct bearing on the introduction of microalgae into the feedstock market either technically, for example infrastructure for production or financing, or politically such as standards for fuel quality. The WSoI also investigates systems upon which microalgae would have an impact, in particular social development.

The environment and wider environment potentially fall beyond the control of the SoI and WSoI, but will still have cause and effect relationships inside and outside of these boundaries. The wider environment in which the SoI is set includes economic sustainability, which includes a subset of economic activities including competition and substitutes for biodiesel products, and also availability of funds for research and development. In the wider environment this interplays with the political climate and geopolitical stability. Climate change and biodiversity are included due to their wide temporal and spatial impacts, and public opinion which can include media and political viewpoints.

The following analysis discusses specifics within each system and attempts to define challenges in order to reduce the risks they might pose.

8.3 Analysis

Using the boundaries defined in Figure 8.3, the component parts were analysed, and where possible traced back to the SoI to examine the obstacles and risks. Where necessary, more detailed analysis of a particular component was constructed, or alternatively other examples were sought to draw on experiences of other systems. Each boundary defined in Figure 8.3 is explored in more detail below from the more specific SoI to the more holistic wider environment.

8.3.1 System of interest

The immediate aims of the project were to find a renewable feedstock for biodiesel production that would be technically feasible and sustainable in terms of environmental, social and economic impacts. In order to do this the product had to be defined. Through analysis of an existing system in Chapter 4, it was recognised that expanding production of existing terrestrial feedstocks further in Brazil would lead to environmental degradation through loss of biodiversity, reduction in land quality and therefore productivity, and more intense use of machinery and fertilisers. This in turn would put pressure on land availability for crop production and cattle grazing, and conflicts over land ownership. Therefore the opportunity for microalgae as a feedstock for biodiesel production was defined. This was further refined to looking into the use of heterotrophic microalgae to overcome the issues associated with providing light to cultures and hence increasing the density in which it could be grown. Nutrients were provided to the microalgae from sources that would not put pressure on provision of nutrients for food crops, and could deliver further benefits from treatment of wastewater from domestic sewage and waste carbon from biodiesel production at a low cost. The biomass produced was converted to biodiesel and the characteristics of the biodiesel were assessed against existing feedstocks to investigate its suitability as a feedstock.

8.3.2 Wider system of interest

The SoI is positioned within a wider set of objectives that will determine its outcomes. This wider system of interest (WSoI) has direct links with the SoI and cause and effect will be more obvious. The sub-components were identified as infrastructure, direct environmental impacts, social development and standards and directives and their relationship to the SoI was investigated in more detail.

8.3.2.1 Infrastructure

The technical system affecting the SoI is largely the provision of infrastructure. The requirements for infrastructure are wide ranging, and while some could be reallocated for microalgal biodiesel production, others may need to be developed leading the capital expenditure (CAPEX) requirements.

The logistics infrastructure is a particular issue. This is an existing problem in Brazil, where the majority of freight transport is carried out by road. Ideally the microalgae cultivation site could be located close to an existing biodiesel production facility. This would reduce the logistical requirement for both the delivery of waste carbon and the movement of the biomass to the biodiesel production plant, assuming an in situ transesterification process could be carried out, as discussed in Chapter 6, due to lower energy intensity, as calculated in Chapter 7. This would also move wastewater treatment away from urban areas, thus reducing the potential for health issues arising from pathogens in wastewater, and also social acceptance with regards to odours, noise and unsightliness of the plant.

Table 8.1 identifies the process stages for the SoI and assesses the existing infrastructure that could be utilised, and the new infrastructure that would need investment and development. A summary of the new requirements includes construction of a new site for cultivation which would require new administrative facilities as well as specific equipment for cultivation at a large scale, plus infrastructure for delivery of utilities, movement of wastewater to be treated and other resource inputs such as the waste carbon supply.

Table 8.1 Identifying existing capital infrastructure and requirements for new development

Process stage	Input	Output	Existing infrastructure	New infrastructure required
Bioreactors	Water/ nutrients	Biomass		Bioreactor construction Construction of on-site facilities, piping and electricity Development of control systems and instrumentation
Transport of biomass	Biomass	Biomass	Potential to use existing road fleet	Roads to cultivation site
Oil extraction	Biomass	Algal oil	Solvent extraction plant	Modify conditions to suit microalgae feedstock Retrain personnel
Transport of oil	Algal oil	Algal oil	Potential to use existing road fleet (tankers)	
Biodiesel production	Algal oil	Biodiesel	Use same plant as for other feedstocks	Modify conditions to suit algal feedstock Retrain personnel
Transport of biodiesel	Biodiesel	Biodiesel	Potential to use existing road fleet (tankers)	
Refining and distribution	Biodiesel	Blended diesel	Business as usual (Check capacity and storage time)	

8.3.2.2 Social development

Intellectual infrastructure is also required and includes training of personnel including operational staff and health and safety workers. There would be potential for job provision, during construction and for operations. These jobs would potentially be for skilled labour. However, a certain number of unskilled jobs may also be created within the facility. Opportunities for employment would gain a new facility public and political support, and could have a multiplier effect in the region with regards to the local economy and education, as was noticed in Quixadá in Brazil, when a new biodiesel facility started operations (see Figure 4.3).

Treatment of wastewater remains a challenge for the expanding population and economy of Brazil. Rapid urbanisation is leading to rising pollution levels in urban waterways due to the disposal of untreated domestic and industrial waste into rivers. Only 40-45% of homes are connected to a sewage network, although drinking water distribution now reaches 93% of the population. Of the wastewater collected, only 32% is treated, presenting major pollution and sanitation challenges, particularly in poor areas and slums in the cities [378]. The northeast of Brazil has the lowest number of people who have access to the sewage network, with 28% of people (15.3 million) not connected to any form of sewage collection compared to <1% (1.2 million) in the south of Brazil [379]. This presents an economic opportunity for wastewater treatment companies, and could also create a new market for the use of heterotrophic microalgae for wastewater treatment. As the cultivation of heterotrophic microalgae can potentially take place using a smaller land area than autotrophic microalgae [76], there is the opportunity to take the wastewater treatment method into more urban environments. An example of a wastewater treatment facility that makes use of heterotrophic organisms is the Dom Nivaldo Monte (ETE do Baldo) wastewater treatment facility in Natal, Brazil, run by the Water and Sewage Company of Rio Grande do Norte (CAERN in Portuguese), where sewage from 400,000 households is treated using activated sludge within the city boundaries. The plant is designed to treat 12 million m^3 of sewage per year, without releasing odorous emissions that would affect the densely populated neighbourhood surrounding the site. There could be potential to adapt this system to cultivating microalgae instead of heterotrophic bacteria in order to add value to the water treatment by producing fuel feedstocks.

Figure 8.4 Location of the Dom Nivaldo Monte (ETE do Baldo) treatment facility in a densely populated area of Natal.

The cultivation of microalgae will cause impacts the immediate surroundings, so the location for cultivation needs to be carefully selected. The location of plant is subject to conflicts of interest in terms of land use, existing infrastructure and land ownership as well as impacting on biodiversity. This could have knock-on effects for international trade and public perception as discussed below in sections 8.3.4.2 and 8.3.4.4 respectively, and would impact the potential for social development from the SoI.

8.3.2.3 Direct environmental impacts

The environmental impacts of large scale microalgae cultivation were discussed in detail in Chapter 7, and remain a concern for development of biofuels. Assessment of direct impacts can occur through EIA, LCA and CBA, also discussed in Chapter 7. Diversifying feedstock remains important for biodiesel production, and therefore whilst introducing heterotrophic microalgae as a new feedstock, it will serve best to add to a matrix of feedstocks alongside crops mentioned in Chapters 2 and 4, improving resilience and biodiversity locally and nationally.

The effect the local environment has on the microalgal cultivation should also be taken into consideration, in particular with regards to local climate. Extreme temperatures will affect growth rates and other extreme events such as drought will lead to shortage in cultivation media, or storms or flooding could lead to damaged equipment, spills and loss of harvest, as discussed in Chapter 7.

As mentioned above, the land area required for heterotrophic cultivation of microalgae can be much smaller than that of autotrophic microalgae and this is due to the fact that during heterotrophic cultivation the microalgae does not require light, therefore cultivation is not restricted to shallow depths. This makes it more suitable for urban locations and reduces the pressure on land use. Locating the plant however would be key to ensuring easy access to an additional organic carbon feedstock (e.g. crude glycerol form biodiesel production), and removal of the biomass for drying and processing.

8.3.2.4 Standards and directives

The policy tools directly affecting the SoI are the mandating of biodiesel inclusion in the fossil diesel blend (conversion bill PLV no. 60-2004) and the PNPB (part of Law 11.097/05) as discussed in Chapter 4. Microalgae technology is currently not an object in any specific legislation, or part of a technological roadmap in Brazil to date. However, there are a number of institutions developing technologies and therefore adding to existing directives, such as inclusion of a specific percentage of fuel derived from microalgal feedstocks would help grow the market and show commitment from the government. The inclusion of microalgal biofuels into the technological roadmap for biofuels is instrumental in generating support in terms of investor interest, industrial involvement and research and development.

8.3.2.5 Competition

Due to the fact there are a number of different feedstocks available for biodiesel feedstocks, this leads to a situation where there is also competition for investment and resources. These include terrestrial crops, as described in Chapter 2, autotrophic microalgae. There is also a potential that these crops will come into competition for resources with food crops which is a politically sensitive issue as well as being technically challenging. The diversity of feedstocks is important in providing a diverse and stable biodiesel market. However, the extent of the competition may become a threat to the development of heterotrophic microalgae biodiesel, and therefore it is important to establish what the key benefits of heterotrophic microalgal biodiesel would be. The comparison shown in Table 2.3 identifies the advantages the heterotrophic microalgal feedstock would have over autotrophic microalgae. This comparison was felt to be important due to the nature of the product being similar, and due to the fact that there has been only a small amount of research on heterotrophic biomass as compared with autotrophic; therefore the advantages need to be made clear to help stimulate further research and development. In summary the main advantages are less land for cultivation, higher yields in a shorter period of time and potentially easier processing due to lower levels of pigments.

There are a number of projects now either at pilot scale or under construction for commercial scale cultivation, which will lead to the production of algal biomass either as the desired product (e.g. joint Solazyme and Bunge project in Brazil) or a by-product (e.g. wastewater treatment). In order to maximise the benefits of using microalgae for wastewater treatment in terms of nutrient recycling and improving sanitation conditions, the opportunity to combine these systems is now. Producing a useful by-product that can be converted to biodiesel and potentially other bio-products is an additional benefit that could be integrated into plans.

8.3.3 Environment

8.3.3.1 Market and policy factors

Due to the fact microalgae is a new feedstock, there are many new firms that are investing in cultivation technology. New firms can develop their business model around a purpose built infrastructure and workforce with specialist skills and knowledge. The obstacle arises where this technology meets the existing market, in this instance joining of the new feedstock supply with existing biodiesel industry, in particular processing and blending facilities. There are 55 biodiesel producers in Brazil, the most predominant of which is Petrobras with a 25% market share [380], and another 25% is controlled by 3 other large firms; Ecodiesel Brazil, Archer Daniels Midland Co. and Granol. Petrobras also remains the major distributor of oil products in Brazil and owns a large part of the associated infrastructure including oil refineries and oil tankers [381]. If these companies invested in adapting their facilities, the economies of scale that could be achieved for microalgal biodiesel would be significant.

With this in mind, the diffusion of microalgal biodiesel into the Brazilian biodiesel market could take a number of forms. On one hand, the power of the market system is harnessed and companies invest in the SoI in the interest of profitability. If the production of microalgae oil can prove to be technically and economically attractive this would give biodiesel suppliers and producers a competitive advantage. There is currently a relatively elastic supply of biodiesel feedstocks, and therefore a cheaper substitute could lead to higher profits. Price elasticity of supply is an economic measure used to show the responsiveness (i.e. the elasticity) of the quantity supplied of a good or service to a change in its price, and due to the fact there are substitutes for biodiesel feedstock that would be available at a similar price, a price for algal feedstocks that is above that of substitutes could mean the quantity supplied remains low. Therefore it is imperative to ensure the low cost of production, with the biggest potential for cost reduction coming from the use of waste resources.

The other route leading to use of microalgae as a biodiesel feedstock would be political intervention in favour of more aggressive development of algal biodiesel (a term coined “authority innovation-decision” [369]) could be asserted by raising the mandate for biodiesel inclusion in the diesel blend, as discussed in Chapter 4.The capacity for production already exists in Brazil, and therefore this seems political intervention in terms of both legislating a rise in biodiesel quantity required in the diesel blend and subsidies for biodiesel production could be a requirement for the system to expand, as fossil diesel prices continue to be lower than biodiesel prices.

8.3.3.2 Demand for vehicle kilometres

In Brazil, the demand for diesel is largely driven by the demand for goods seeing as diesel is in large part only used for freight (rather than for passenger cars as is seen in Europe). The road network is relied upon for the mass transit of goods in the majority of the country in Brazil, the only substitute for overland transport being the railway network predominantly in the south of the country. The demand for diesel and therefore biodiesel is stable and is expected to stay that way, given that the fuel efficiency of Brazil’s truck fleet is unlikely to rise significantly in the near future, fluctuating with the supply and demand for goods and the price of oil, which is moderated by government subsidies. This also stems inflation which would be a result of rising oil process, as the price of freight is incorporated into the sale price of the goods, hence higher diesel prices could lead to higher retail prices.

8.3.3.3 Funding and Research

Funding for research and development of projects is available at a government level and also commercially, with different emphasis on wastewater treatment from domestic and agricultural sources, and for biofuel production.

Organisations initiating research projects include the Brazilian Enterprise for Agricultural Research (EMBRAPA in Portuguese), who have a range of projects including using stillage and CO_2 from ethanol production for microalgae cultivation, and the National Institute of Metrology, Standardization and Industrial Quality (Inmetro in Portuguese) who are investigating microalgae for biodiesel feedstocks. There are also a number of research groups within universities in Brazil that are considering microalgae for wastewater treatment, biofuel production via fermentation for ethanol, biogas production or biodiesel production (e.g. projects at federal universities in the states of Rio de Janeiro, Sao Paulo, Rio Grande do Norte, Espírito Santo and Rio Grande do Sul [382][383]). Commercial activity is also underway, including a project by Petrobras for the use of microalgae in the treatment of process water from the oil and gas industry (co-project with UFRN), and a project by Solazyme using heterotrophic microalgae for producing ethanol. Due to commercial sensitivity however, no more information on these projects is currently available.

8.3.4 Wider environment

8.3.4.1 Political climate

Biofuels have been fundamental in Brazil’s strategic plan for fuel security since the oil crisis in 1975 which led to the introduction of the Proalcool programme for ethanol production. One of the outcomes of this programme is that Brazil has become one of the world leaders on biofuel research, development and production, particularly with regards to ethanol. Biodiesel is also a growing sector and receives direct governmental support via the PNPB scheme for family farming. However, Brazil is a democratic republic, and hence there are elections held every 4 years, and potentially a change in government. This will lead to policy changes which could potentially change the emphasis for biofuel production. Therefore it is crucial that “microalgae products” are embedded into the value chain, as discussed below in section 8.3.4.2 to ensure stability.

On a global scale, there are international policies that already try to promote a reduction in GHG emissions, for example the Kyoto protocol of 2005 which committed OECD countries to reducing their GHG emissions [384]. Considering 51% of the growth in energy consumption in 2013 was among developing countries [40], there is an increasing need to include these countries in a new commitment to climate change, which will have impacts on biofuel policy and therefore on technology for provision of sustainable biofuels.

Currently, Brazil does not export biodiesel, consuming almost all of the biodiesel domestically due locations of production often being inland and therefore high costs involved in transportation to ports. There is also the issue of varying technical standards as a result of the feedstock matrix. If Brazil were to commence exporting biodiesel, this could have wider consequences for the whole scheme because the properties would have to meet with the specifications of the importing country. Producers in Brazil are also protected by a 14% import tax on biodiesel imports [49]. Recent developments in trade agreements between the EU and Mercosur (or “Common market of the south”, founded by Brazil, Argentina, Paraguay and Uruguay) have seen the agreement of an 87% reduction in trade tariffs, and this could lead to increased opportunities for trade of goods such as biodiesel [385]. However, this deal is still to be finalised.

If Brazil were to consider exporting to other nations, in particular Europe, the source of the biodiesel would be of particular interest. Historically, there have been a number of European nations that have taken particular interest in guardianship of the Amazon rainforest, and this is demonstrated by the number of joint projects the EU delegation to Brazil has (see [386] for full list). Acquiring biofuels whose production may have been linked to destruction of the rainforest through direct or indirect land use change will be of concern and as a reflection has been incorporated into the EU Renewable Energy Directive, as described in detail in Chapter 2 (also see Article 17, [50][51]). While the EU attempts to increase the mandated blend of biodiesel, it is restricted by land area and therefore will potentially look to other continents for its supply. This being the case, and assuming Brazil opened to international trade of biodiesel, heterotrophic microalgae could represent an interesting feedstock from a European point of view, due to the benefits described throughout this work, in particular the use of marginal land for cultivation. There is the possibility that Europe could use the same technology to produce feedstocks for themselves. Developing the technology in Brazil could lead to market spillovers, as discussed in section 8.1.4. This would have a positive impact in terms of developing a more robust technology more quickly. However, it may affect the demand for trade, although this may only be minor as the demand for biodiesel in Europe is high in light of the RED quotas for inclusion of biofuels in transport fuels, plus European pledges to reduce GHG emissions (e.g. the Kyoto Protocol of 2005)[51]).

8.3.4.2 Economic sustainability

In order for algal biofuels to become a stable fuel supply there is a need to deepen algal products into supply chains. In this way, value is added to the algal feedstocks and resilience to changes in market forces, examples of which are shown in Figure 8.5. There is also an interest in replacing oil based products, which has been termed “replacing the whole barrel”, indicating transport fuels are not the only products to be produced from oil and reducing dependence on oil will also require finding new sources for materials including plastics, solvents and lubricants as well as energy fuels [1]. Oil prices continue to play a major role in the pricing of other commodities. When the price of oil drops, the production costs for biofuels can also be reduced, as biodiesel production requires the use of fossil fuel products, in particular methanol for transesterification.

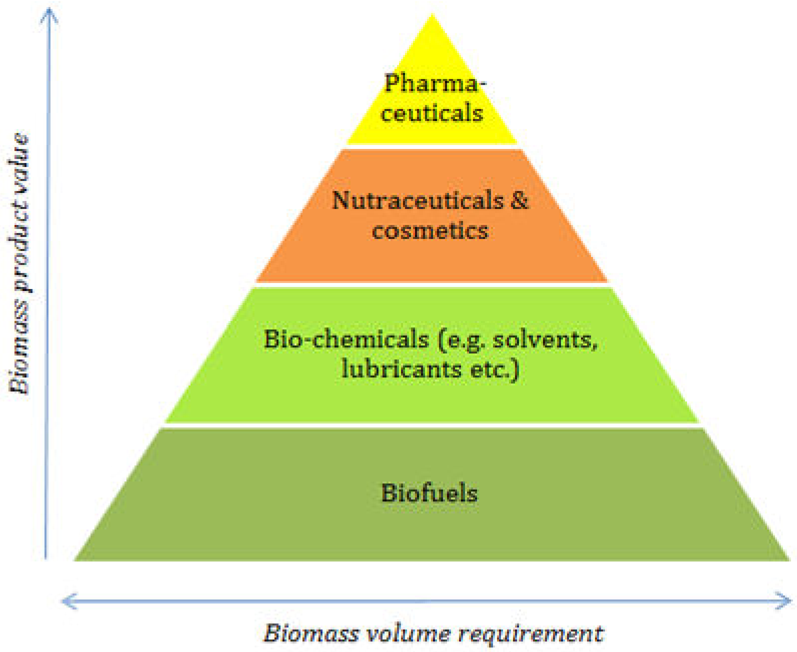


Figure 8.5 Adding value to algal biofuels will involve deepening algal products into other supply chains where more value can be added

Production of algal biodiesel will also be competing against the price of substitute feedstocks, in Brazil this is mainly soy beans, palm and tallow. Productivity of any of these crops can vary year upon year, and therefore the prices continue to fluctuate making economic predictions difficult. Techno-economic modelling is a useful tool in ensuring market driven pricing can be achieved when developing a new technology. By using this technique research and development teams can work within the financial constraints leading to innovation of solutions that are economical to implement. A complete techno-economic model is an extensive undertaking and was beyond the scope of this project. However, the constraints shown in Table 8.2 explore the financial constraints that would need to be quantified in order to produce a useful model for microalgal biodiesel.

Brazilian markets are protected by high import and export tax rates, for example the tax on biodiesel imports as mentioned above in section 8.3.4.1. This creates an environment for development of new products to be used within the country which is protected from external trading activities, but may hamper investment from overseas and also limit the flow of knowledge stunting growth.

8.3.4.3 Climate change and biodiversity

Climate change is included within the wider environment as it is a long term process that could affect technical operation, but the causality is loose and ill-defined. Particular climatic conditions that are worthy of mention are extreme temperatures, rainfall (or drought) and other extreme weather events. While the cultivation of heterotrophic microalgae would take place in a closed system, there would remain a requirement to keep temperatures at a level that will maximise growth (i.e. 25-32°C for *C. vulgaris*). In Brazil, a potential problem could be temperatures becoming too high, risking the death of the algal population. More regionally, for example in the northeast which is semi-arid, drought could become a concern, limiting the availability of water for both food and fuel crops and creating competition for scarce water resources.

In some markets, ownership of emissions has been used to promote environmentally responsible actions. In particular the “cap and trade” carbon trading scheme in Europe has been introduced to try to internalise the costs of releasing carbon dioxide into the atmosphere via the European Union Emissions Trading System (EU ETS) using the market system to decide the most economical way to reduce carbon emissions. The scheme so far has been fraught with difficulties including over-allocation of permits, windfall profits, price volatility and a carbon price that is too low to provide a strong incentive for decarbonisation, but it is only in the first phase which has been coined a “learning phase” by the programme’s advocates [387]. The Amazon Fund in Brazil is a scheme that has aimed to transform emissions reductions into a system that will fund conservation without the use of carbon credits, whilst still contributing to REDD+ (a UN initiative to reduce deforestation and forest degradation) [388]. Should a more integrative scheme be possible, that would link emitters of GHG financially with their emissions, this may lead to more pressure on finding alternative technologies with lower GHG footprints, hence stemming the contribution of these to manmade climate change [284].

8.3.4.4 Public opinion

Public opinion is also included within the wider environment as it can often be tied to political activity and hence is an important driver of cultural and technological development based on other components of the wider environment. Bias can come from the research community as well. For example if a researcher has a particular environmental worldview, their processing of knowledge and information sharing may be influenced [389].

Technology development has always been a part of human existence, and has always had side effects that were not identified beforehand, from the advent of agriculture that has led to mono-cultures which are less resistant to drought and disease than diverse ecosystems, to coolants for refrigeration and propellants in aerosols which cause depletion of ozone in the stratosphere. Now, technological assessment means there is more information available about the pros and cons of a new technology, but this extra information can also become “dis-information” when a full account of the details is not presented. Public support for technology varies across the globe as well as within countries, and opinions about technology affect policies and politics. For example, some members of the general public may be interested in the safety of a product, with the direct impact on health more relevant than that of long term climate change impacts, whereas others may display pro-environmental behaviours, looking at impacts that occur further from home. An example of an environmental issue which has divided opinion is that of carbon capture and storage (CCS). A study found that, based on interviews with the general public in Scotland, further policy would be required to address social acceptability of CCS, and also to help with the technical selection for a CCS site [390].

In order to increase the public support for biofuel development, an informed unbiased coverage is required that allows the public to engage with research and planning processes, encourage them to ask meaningful questions and express hopes and concerns to those in both political and science and technology fields.

8.4 Towards a technological roadmap for algal biodiesel in Brazil

A roadmap should identify technology goals and define the key actions that stakeholders must undertake to make algal biodiesel a reality and to ensure its sustainability in production and use [4]. The vision for this roadmap is to identify the key risks and challenges to integrating a microalgal feedstock into the existing biodiesel production industry, and to suggest a strategy to help overcome these. It will enable governments, industry and academia to identify steps needed to integrate microalgal feedstock into the market place in a competitive and sustainable way. The roadmap represents an amalgamation of the work in this thesis alongside a growing area of literature concerning microalgal technology, biofuels and long term environmental stewardship. Identification of dependencies is a key component of a technological roadmap and as such, key factors that could compromise successful deployment of heterotrophic microalgae are included below.

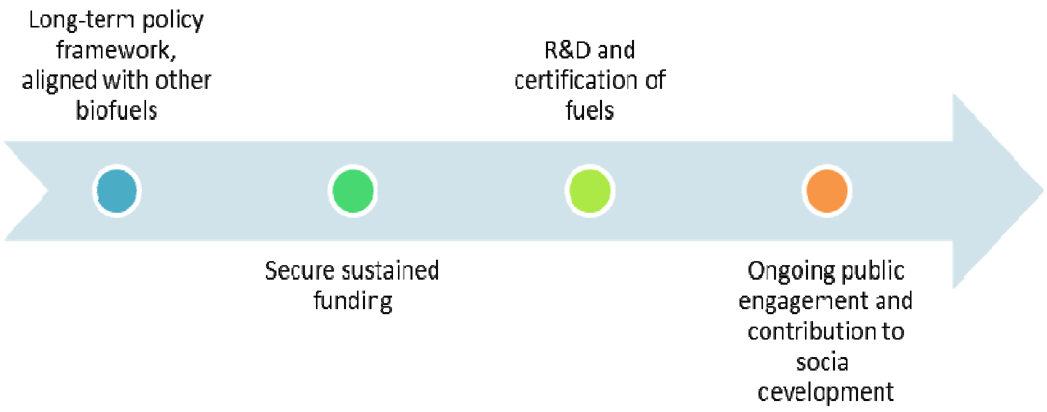


Figure 8.6 Points for inclusion in a roadmap for the successful integration of heterotrophic microalgae into the market for biodiesel feedstocks

8.4.1 Creating a timeline

In order to develop a robust roadmap, a timeline is required to steer the project and ensure deadlines are being met. The time line should include short and long term objectives, project phases, time phased activities, project structure and project criteria including any barriers to be cleared before the project can begin, document risks and issues, come up with a communication plan for example to business leaders, the public and the media, and a financial plan to fund the project.

The short term objectives with regards to heterotrophic microalgae would be proving the technological feasibility of up-scaling to commercial production and development of risk assessments and risk aversion strategies, and integrating this into the existing system for biodiesel production. In the longer term, the objectives would include an on-going financial case for the production and distribution of biodiesel from heterotrophic microalgae, a method of monitoring and quantifying the impacts from heterotrophic microalgae and a sound communication plan, and a framework for inclusion of algal fuels into policy in Brazil.

The starting point for heterotrophic microalgae is to ensure it is technically robust as a fuel source, and following this it needs to be scaled up and ensure the technical feasibility is maintained at large scale product (e.g. thousands of litres per day). The planning phase will include planning, design, testing, training of staff and production verification. Before the project can begin barriers should be identified for example political barriers with regards to the funding route. For example, in the case of heterotrophic biodiesel, can this feedstock be included in the existing SFS or will a new scheme be required to incentivise production (assuming under normal market conditions, heterotrophic microalgae will not be economically feasible)? Barriers can also include dependencies from other projects, which in this case will likely be issues with scale up technologies and provision of feedstocks. Other dependencies are discussed in more detail below. As a result of understanding the phases involved and potential barriers, a project structure can be developed to manage and execute the implementation of the roadmap. Time phased activities must be detailed in order to develop a consistent plan and ensure any dependent activities will be ready in order to execute the next phase. Documenting risks and other issues will be required before starting the project but must be continuously reviewed and will be developed as the project progresses.

The communication strategy is crucial as good communication can help avoid the spread of misinformation, doubt and rumours that may compromise the success of the project. This is true between all stakeholders from researchers and construction workers through to business leaders and the general public. A financial plan is also required, both before and then in tandem with development of the project plan. An initial financial case will need to be presented to stakeholders before scaling up (bearing in mind finances would already have had to have been gained for research purposes), and then a full costing of the project through to full scale operations.

Quantifying these times goes beyond the scope of this thesis, but identification of the points above, plus the dependencies below is a step towards formulating this plan.

8.4.2 Identifying dependencies

In order to identify the dependencies of the SoI, they were categorised into three sections; financial, technological and biological, shown in Table 8.2. The financial dependencies are the stability of the market which can be created through policy design, highlighted by the first objective in Figure 8.6. Long term policies will inspire investor confidence and should align biofuel development with agricultural planning, rural development and work within the oil industry. In order to strengthen the position of biofuels, finance should be linked to sustainability criteria for example benefits for utilising waste products or for reducing air or water pollution. Subsidies for biofuels already exist within Brazil and would have to be extended to algal biodiesel in order to make it economically feasible.

Table 8.2 Examples of dependencies for the successful development and deployment of heterotrophic microalgae as a biodiesel feedstock

	Dependencies	
Financial	**Technological**	**Biological**
Potential market size	Resource availability (e.g. nutrients)	Strain selection
Gross profit margin	Circulation hydraulics (pumping rates and pump sizes)	Growth rate
Interest rates on loans	Evaporation rates	Lipid content
Minimum order size	Harvest rates	Maximum density
CAPEX and OPEX (labour, materials, utilities)	Low temperature behaviour and long term storage stability	

Any development of microalgal biofuels will require avoidance of the use of fossil fuels as they are too expensive. The idea of a bio-refinery, where algal biofuels are produced alongside higher value products, as discussed in section 8.3.4.2. Any realistic research on bio-refineries must consider the whole systems and supply chains is discussed in this chapter and requires trustworthy links and stability between industries in order to make them productive and competitive both nationally and internationally. Collaborative capacity building and transfer of technologies may prove essential for a biorefinery to work.

Scientists are attempting to overcome some of the biological constraints of the system, through strain selection and genetic modification. Calculations of the theoretical maximum oil production from algae have been carried out and include the perfect conditions for photosynthetic algae including the photon transmission and utilisation efficiency should light be perfectly absorbed by the biomass, biomass accumulation efficiency accounting for energy used in cellular functions, and potential maxima for oil content and density. Estimates from one study using physical laws to investigate the limits for algal oil production found, under the assumptions that cultivation is in an equatorial location, achieving 50% oil content in cells, 354,000L ha^{-1} year^{-1} is the maximum possible yield (compared with 446L ha^{-1} as an annual average for soybean) [391]. Therefore, the key risks to the SoI identified in section 8.3.1, are long term planning and informed policy decision making from a supportive government, technological success of up-scaling with coordinated research projects across research institutions, investor confidence which will be influenced by technological outputs, economic activity including stability of the oil industry and media reporting and public opinion.

8.5 Summary

A whole systems analysis of introducing a new biodiesel feedstock, in this case heterotrophic microalgae, brings new insight to the challenges ahead. The analysis covered the particular to the general by considering 4 sub-systems nested inside each other. The analysis demonstrated the wide number of factors that could affect the viability of heterotrophic microalgae as a biodiesel feedstock and the importance of inspiring confidence in those with the potential to promote microalgal fuels either through policies or through the marketplace. This needs to be done by using robust scientific procedures to create a safe and clean fuel that will be economically profitable, politically popular and technologically stable in its supply and performance.

Chapter 9 Conclusion and recommendations

The global demand for cheap energy is increasing as the economic wealth of nations rises. Infrastructure throughout the developed and developing world has locked us in to a system reliant on fossil fuels. This has brought an array of environmental and social problems, as well as political tension and economic instability. Whilst it remains unlikely that there will be a step change away from fossil fuels, biofuels are being incorporated into the market place, which is leading to a gradual change in the energy supply. There is an opportunity to learn from gains made in fossil fuel and combustion sciences, due to the similarities in nature of fossil and bio-based fuels, as well as a chance to learn from environmental problems they have caused in order to predict consequences associated with biofuels. There may also be a chance to address social problems associated with conflicts over land ownership, rights to access and irresponsible corporate activity that has been observed with regards to fossil fuel extraction.

Brazil has developed a programme for biodiesel production which has been in place since 2005, where biodiesel is being used to create social development opportunities. Chapter 4 analysed how social and technical elements work together to provide feedstocks for biodiesel production in Brazil. In was found that whilst there has been success in the PNPB, apparent through the increase in income for family farmers and growing number of participants since the programme began, the prospect of family farmers producing a majority of the feedstock for biodiesel is unrealistic given the scale of production small scale farming can achieve, with the given resources, in comparison with industrial scale farming and the associated economies of scale. At the same time, cultivation of soybeans as a feedstock for biodiesel continues to expand into highly biodiverse regions causing negative impacts on the natural environment.

As a result, development of an alternative feedstock for biodiesel was considered; one which could produce a high yield of good quality oil, with the lowest possible negative environmental impacts whilst still creating social development prospects. Heterotrophic microalgae was investigated in this capacity, although it was recognised early on that the way in which this particular alternative could contribute to social development would have to be different from existing feedstocks as small farmers could not be expected to afford the infrastructure for large scale microalgae cultivation. There would potentially be social benefits instead through the treatment of domestic wastewater and thus improve sanitation.

The technical feasibility of heterotrophic microalgae to meet this additional demand for biodiesel feedstock in Brazil was investigated in Chapter 5 and 6. In Chapter 5 it was demonstrated that, at a lab scale, the cultivation of microalgae using waste resources, from domestic wastewater nutrients supplemented with waste carbon, led to growth rates which exceeded autotrophic microalgae and other terrestrial crops. Organic carbon was found to be the main factor limiting growth, and other nutrient stress may have been responsible for changes in the biochemical composition in the biomass. While it was not possible to optimise the nutrient ratios for lipid production within the scope of this work, the work demonstrated using an alcohol based feedstock, i.e. crude glycerol which contained methanol, led to a lipid yield which exceeded that from sugar feedstocks such as glucose or molasses, and both the growth rate and lipid content exceeded that observed in the literature from autotrophic *C. vulgaris*. The benefits associated with using crude glycerol as a feedstock include an opportunity for treating low value waste products which would be uneconomical to upgrade into a purer product, and could cause harm if disposed of in aquatic environments without treatment.

The oil extracted from the algae and transesterified produced biodiesel that contained over 95% FAME, with yields that exceeded autotrophic microalgal feedstocks, shown in Chapter 6. In situ transesterification was carried out as an alternative to transesterification of extracted lipids in order to investigate the efficiency, given that it could potentially reduce energy demand and costs of biodiesel production by omitting a process stage and the energy and solvents involved. The yields obtained from in situ transesterification exceeded those from the extracted transesterified lipids, with no loss in oil quality. The properties of the oil were analysed based on the FAME profile and other physical characteristics. Tests to calculate the properties of the biodiesel were based on existing techniques to determine properties such as cold flow properties, CV, density and oxidative stability, and a new technique was developed for calculation of CN from a more extensive range of FAMEs. These techniques allowed properties to be estimated where only small volumes of sample were available. It was suggested that blending the heterotrophic microalgal oil with other biodiesel feedstocks or with fossil diesel may be feasible, given its properties aligned with that of soybean biodiesel, shown in Table 6.7.

The energy required to produce biodiesel, using the methods described in Chapters 5 and 6, was calculated using a mass and energy balance in Chapter 7. The results indicated that heterotrophic microalgae could have a more favourable energy ratio than autotrophic microalgae. The largest energy penalty in each process was the transesterification stage, due to the methanol requirements. The main benefit in terms of lower energy consumption was during the cultivation stage, because heterotrophic microalgae had higher biomass densities, therefore a lower water demand per kg of biodiesel produced than autotrophic microalgae. This led to a lower energy requirement for pumping water. A smaller area would be required for heterotrophic cultivation than autotrophic cultivation, shown in Table 7.1, as it can be cultivated at greater depths given that light is not required to penetrate the medium, leading to a further benefit of this system. The differences in the results however, were subtle and could change as the technology develops. A number of challenges in applying LCA techniques were identified, including the labour intensive nature of data collection which would make this expensive as a commercial project, incomplete data regarding processes, the number of assumptions made in order to complete the study and difficulty in benchmarking against other biofuel production processes. This is a result of there not being a prescribed format for calculation of energy requirements for a process, for example with regards to system boundaries or the use of a standardised LCI.

Environmental impacts that may be associated with the large scale cultivation of heterotrophic microalgae were assessed, beyond the impacts from energy and GHG emissions. A number of areas of concern were found including the impact microalgae cultivation may have on emissions to the atmosphere and the impacts that would be caused by leaks. However, it was also suggested that a well-managed system should not be at risk of these problems, although much further work is required.

Chapter 8 assessed how heterotrophic microalgae could be integrated into the existing biodiesel industry, by identifying the key dependencies for development and deployment. It was determined that heterotrophic microalgae may be able to use some of the existing infrastructure, aiding its integration without large requirement for CAPEX. However, the work identified potential risks to integration as a result of lack of consumer and investor confidence; therefore government support would be essential for success. Furthermore, it identified limitations to the development of heterotrophic microalgae feedstocks for financial, technological and biological reasons. The whole system study emphasised the need for joined up thinking across academic, commercial and industrial sectors to ensure development of a biodiesel feedstock that would be technically robust, economical to produce and could limit negative external impacts on the natural environment and society, taking advantage of opportunities to promote social development.

The author concludes that the most valuable aspect of this thesis has been the holistic approach which was taken to identify the different inputs that must be considered when developing a new technology. There are a number of findings that are new to this field of research including the cultivation of microalgae heterotrophically in a wastewater medium with waste organic carbon, development of a new technique for estimating CN and the quantification of the energy requirement for a heterotrophic microalgae biodiesel system. Brazil is unique in its approach to integrating social development with biodiesel production. The findings of this thesis have application elsewhere in the world and there is an opportunity, particularly in developing countries, to learn from Brazil's experience. This would include ensuring feedstock production is integrated into society, taking the opportunity to embed the feedstock into additional supply so as to increase resilience against economic and political changes, and using biofuel feedstocks to reduce negative pressures on the natural environment.

9.1 Suggestions for future work

This thesis represents a scoping study for the use of heterotrophic microalgae as a sustainable biodiesel feedstock. However, it has not been able to investigate the potential for scale-up of the process. System optimisation for scale-up will require compromises of technical specifications, with interdependencies varying with the scale used. The further work suggested has been divided into topics, and a comprehensive programme would ideally coordinate these research activities to deliver a holistic review of progress.

There is a need to understand the scale up impacts of this project for both the cultivation and processing stages. Small problems identified during experimental work could lead to large problems once the scale is increased. A compromise between organic carbon concentration, cost and availability of feedstock would be needed in a scaled up operation. For example, the cultivation period may compromise the yield, as a longer exponential growth period would give rise to higher yields, but at the cost of operating time. Scaling up the size of cultivation is also essential as part of further feasibility studies. Controlling contamination and infection where SWW is used as a growth medium is essential, for both the health of the microalgae and the safety of employees working in the vicinity of the water treatment plant. Future trials would look towards using SWW collected from real world treatment ponds so that potential hazards could be identified, observed and controlled. There is also a requirement for further work into reactor design to ensure good mass transfer of oxygen through the media for example. The extent to which microalgae improves the quality of the water is also important in order to see the feasibility of this approach for wastewater treatment.

The work in Chapter 6 focussed on biodiesel production, but the impacts for the end use stage were only investigated in a preliminary fashion. The scope for further work involves ensuring the quality of the fuel is not only suitable for use in an engine but that the emissions are within the specified limits, depending on the country of intended use. Therefore, larger volumes of oil need to be produced to allow for parameters such as CV, viscosity, density, cold flow properties, CN and emissions including HC, CO_2, PM and NO_x to be tested experimentally. This could then lead to tests using blended and unblended biodiesel in the engines of light and heavy duty vehicles. There is potential to engineer culture conditions to allow microalgae to produce desirable FAME mixtures, and further work is needed to identify what triggers changes in the FAME composition and how these triggers can be managed to ensure consistent and optimised FAME profiles. There is also further work to do in such as ensuring separation of contaminants from the FAMEs such as metals, gums, polar lipids and other pigments which may complicate the biofuel processing steps.

The profile of the ash needs investigating to understand how metals are mobilised using different processing methods. Other conversion technologies also need to be investigated to find the most energy efficient method of converting feedstocks into biodiesel. An understanding of the way in which new infrastructure could integrate into the market in an economically feasible way is needed. Benchmarking of the production of biodiesel from heterotrophic microalgae by transesterification compared with other technologies is required under comparable conditions, and will change as the technologies develop.

The energy balance presented in Chapter 7 can be used to help guide technological development of heterotrophic microalgae biodiesel. Potential routes for further development include the investigation of ethanol as an alternative to methanol for transesterification in order to reduce the energy intensity of biodiesel production. Brazil has one of the largest and most developed ethanol industries in the world, and therefore this may be economically feasible. Energy recovery could also help reduce thermal energy demand through the process, thus improving the energy balance and reducing GHG emissions from the production of electricity for heat. This model could be improved by strengthening the LCI through further experimental work and by increasing the number of impact categories. However, the most accuracy would be gained come from scaling up the system in order to get more accurate data compared with lab scale work. The assessment of environmental impacts identified the need for further work into potential for fugitive emissions from cultivation, the aquatic environments including impact on other life, and how locating the plant in new areas might affect the terrestrial environment.

A key part of further work will include a techno-economic study, building on the dependencies identified in Chapter 8, and quantification of the time required for the development and deployment of this technology in order to strengthen the roadmap for microalgal feedstocks for biodiesel. A time scale for development is also crucial to ensure the heterotrophic microalgae are competitive with other emerging technologies for biofuel feedstocks. This will include a comprehensive assessment of environmental impacts, ideally with quantification of some of the issues outlined in this thesis.

The multidisciplinary nature of this project across the Schools for Chemical and Process Engineering and Civil Engineering at the University of Leeds, and the Centre for Research on Sanitation at the Federal University of Rio Grande do Norte in Brazil has opened up the possibilities of this project and has greatly enriched the discussion in this thesis. There remains scope for further research at the interface of the disciplines presented here in order to develop technologically robust and sustainable fuels for the future.

List of References

1 US EPA (2013) *Replacing the Whole Barrel to Reduce U.S. Dependance on Oil*, Washington D.C.

2 Zaretskaya, V. V., Chase, N. and Morris, M. (2013) “Transportation sector energy consumption,” in *International Energy Outlook 2013*, Washington DC, US Energy Information Administration, pp. 141–147. [online] Available from: www.eia.gov/ieo

3 Chalkley, Alfred Philip. (1922) *Diesel engines for land and marine work* 5th ed., London, Constable & company, limited.

4 International Energy Agency (2011) *Technology Roadmap: Biofuels for Transport*, France, OECD Publishing. [online] Available from: http://www.oecd-ilibrary.org/energy/technology-roadmap-biofuels-for-transport_9789264118461-en

5 US Energy Information Administration (2011) *International Energy Statistics* [online] Available from: http://www.eia.gov/cfapps/ipdbproject/

6 USDA Foreign Agricultural Service (2013) *Biofuels Annual Report 2013: Brazil*, Sao Paulo.

7 USDA Foreign Agricultural Service (2012) “Record Soybean and Corn Area for Brazil.” *Commodity Intelligence Report*. [online] Available from: http://www.pecad.fas.usda.gov/highlights/2012/08/Sinop_Brazil/ (Accessed 22 July 2014)

8 Demirbas, Ayhan (2009) “Political, economic and environmental impacts of biofuels: A review.” *Applied Energy*, 86, pp. S108–S117.

9 FAO (2012) *Current world fertilizer trends and outlook to 2016*, Rome, Food and Agricultural Organization of the United Nations.

10 Sheehan, J., Camoreco, V., Duffield, J., Graboski, M. and Shapouri, H. (1998) *Life Cycle Inventory of Biodiesel and Petroleum Diesel for Use in an Urban Bus*, Colorado, NREL.

11 European Commission (2013) *Nitrogen Pollution and the European Environment: Implications for Air Quality Policy*, Bristol. [online] Available from: http://ec.europa.eu/environment/integration/research/newsalert/pdf/IR6.pdf

12 Okullo, Aldo, Temu, A K, Ogwok, P and Ntalikwa, J W (2012) “Physico-Chemical Properties of Biodiesel from Jatropha and Castor Oils.” *Renewable Energy Research*, 2(1).

13 Jain, Siddharth and Sharma, M.P. (2010) “Biodiesel production from Jatropha curcas oil.” *Renewable and Sustainable Energy Reviews*, 14(9), pp. 3140–3147.

14 Chisti, Yusuf (2008) “Biodiesel from microalgae beats bioethanol.” *Trends in biotechnology*, 26(3), pp. 126–31.

15 Chen, Chun Yen, Zhao, Xin Qing, Yen, Hong Wei, Ho, Shih Hsin, et al. (2013) “Microalgae-based carbohydrates for biofuel production.” *Biochemical Engineering Journal*, 78, pp. 1–10.

16 Dragone, Giuliano, Fernandes, Bruno, Vicente, António A and Teixeira, José A (2010) “Third generation biofuels from microalgae,” in Medndez-Vilas, A. (ed.), *Current research, technology and eduction topics in applied microbiology and microbial biotechnology*, Portugal, Formatex, pp. 1355–1366.

17 Frank, E. D., Han, J., Palou-Rivera, I., Elgowainy, A.. and Wang, M. Q. (2011) *Life-Cycle Analysis of Algal Lipid Fuels with the GREET Model*, Energy System Division.

18 Pate, Ron (2011) *Assessment of Algae Biofuels Resource Demand and Scale-Up Implications for the U.S.*. Alberquerque, Sandia National Laboratories.

19 Lundquist, T. J., Woertz, I. C., Quinn, N. W. T. and Benemann, J. R. (2010) *A Realistic Technology and Engineering Assessment of Algae Biofuel Production*, Berkeley.

20 Perez-Garcia, Octavio, Escalante, Froylan M E, de-Bashan, Luz E and Bashan, Yoav (2011) “Heterotrophic cultures of microalgae: metabolism and potential products.” *Water research*, 45(1), pp. 11–36. [online] Available from: http://www.ncbi.nlm.nih.gov/pubmed/20970155 (Accessed 4 March 2013)

21 Rawat, I., Ranjith Kumar, R., Mutanda, T. and Bux, F. (2013) “Biodiesel from microalgae: A critical evaluation from laboratory to large scale production.” *Applied Energy*, 103, pp. 444–467

22 Tabernero, Antonio, Martín del Valle, Eva M. and Galán, Miguel a. (2012) “Evaluating the industrial potential of biodiesel from a microalgae heterotrophic culture: Scale-up and economics.” *Biochemical Engineering Journal*, 63, pp. 104–115.

23 Delrue, F, Li-Beisson, Y, Setier, P-a, Sahut, C, et al. (2013) “Comparison of various microalgae liquid biofuel production pathways based on energetic, economic and environmental criteria.” *Bioresource technology*, 136, pp. 205–12.

24 Zamalloa, Carlos, Vulsteke, Elien, Albrecht, Johan and Verstraete, Willy (2011) “The techno-economic potential of renewable energy through the anaerobic digestion of microalgae.” *Bioresource technology*, 102(2), pp. 1149–58.

25 Gendy, Tahani S. and El-Temtamy, Seham a. (2013) “Commercialization potential aspects of microalgae for biofuel production: An overview.” *Egyptian Journal of Petroleum*, 22(1), pp. 43–51.

26 Cerón-García, M.C., Macías-Sánchez, M.D., Sánchez-Mirón, A., García-Camacho, F. and Molina-Grima, E. (2013) “A process for biodiesel production involving the heterotrophic fermentation of Chlorella protothecoides with glycerol as the carbon source.” *Applied Energy*, 103, pp. 341–349.

27 Zhang, Tian-Yuan, Wu, Yin-Hu, Zhu, Shu-feng, Li, Feng-min and Hu, Hong-Ying (2013) “Isolation and heterotrophic cultivation of mixotrophic microalgae strains for domestic wastewater treatment and lipid production under dark condition.” *Bioresource technology*, 149, pp. 586–9.

28 Lane, Isobel (2013) “Brazil’s government announces rise in ethanol blending to 25%.” *Biofuels Digest*. [online] Available from: http://www.biofuelsdigest.com/bdigest/2013/03/04/brazils-government-announces-rise-in-ethanol-blending-to-25/

29 Ministerio de Minas e Energia (n.d.) “Objectivos e Diretrizes.” *Biodiesel - Programa Nacional de Producao e Uso de Biodiesel*. [online] Available from: http://www.mme.gov.br/programas/biodiesel/menu/programa/objetivos_diretrizes.html (Accessed 9 October 2013)

30 US EPA (2011) “Renewable Fuel Standard (RFS).” *Fuels and Fuel Additives*. [online] Available from: http://www.epa.gov/otaq/fuels/renewablefuels/index.htm (Accessed 3 June 2013)

31 IEA (2010) *Energy Technology Perspectives: Scenarios and Stratergies to 2050*, France: Paris.

32 Huntington, H (2004) “Energy security and global climate change mitigation.” *Energy Policy*, 32(6), pp. 715–718.

33 Umbach, Frank (2010) “Global energy security and the implications for the EU.” *Energy Policy*, 38(3), pp. 1229–1240.

34 Yergin, Daniel (2006) “Ensuring Energy Security.” *Foreign Affairs*, 85(2), p. 69.

35 Chester, Lynne (2010) “Conceptualising energy security and making explicit its polysemic nature.” *Energy Policy*, 38(2), pp. 887–895.

36 Urbanchuk, John M. (2007) *Economic Contribution Of The Biodiesel Industry*, Wayne, PA. [online] Available from: http://www.biodiesel.org/resources/reportsdatabase/reports/gen/20071119_GEN383.pdf

37 Petrobras (2011) “Processamento Industrial De Biodiesel.” *Presentation for Petrobras*.

38 Sudhakar, K, Suresh, S and Premalatha, M (2011) “An Overview Of CO_2 Mitigation Using Algae Cultivation Technology.” *International Journal of Chemical Research*, 3(3), pp. 110–117.

39 Metaya, Aurélie, Oliverc, Robert, Scopeld, Eric, Douzete, Jean-Marie, et al. (2007) “N_2O and CH_4 emissions from soils under conventional and no-till management practices in Goiânia (Cerrados, Brazil).” *Geoderma*, 141(1-2), pp. 78–88.

40 BP (2014) *Statistical Review of World Energy* 63rd Ed., London, BP plc

41 BP (2011) *Statistical Review of World Energy* 60th Ed., London, BP plc.

42 R. Zah, C. Binder, S. Bringezu, J. Reinhard, A. Schmid and H. Schutz (2010) *Future Perspectives of 2nd Generation Biofuels*, Zurich, vdf Hochschulverlag.

43 US DOE (2014) *Clean Cities Alternative Fuel Price Report*, Washington D. C.

44 DECC (2014) "Weekly road fuel prices." *Energy price statistics*. [online] Available from: https://www.gov.uk/government/statistical-data-sets/oil-and-petroleum-products-weekly-statistics (Accessed 30 June 2014)

45 IndexMundi (2014) "Diesel Monthly Price - Brazilian Real per Gallon." *Commodities Price Indicies*. [online] Available from: http://www.indexmundi.com/commodities (Accessed 30 June 2014)

46 Stern, Nicholas (2006) *Stern Review on the Economics of Climate Change*, Cambridge, Cambridge University Press.

47 Alvim, Carlos Feu (2008) "Transport Sector in Brazil," in *New Energy Indicators for Transport: The Way Forward*, Paris, International Transport Forum.

48 United Nations (2012) *Brazil Low Carbon Country Case Study*, [online] Available from: http://www.ncbi.nlm.nih.gov/pubmed/22878278

49 Lamers, Patrick, Hamelinck, Carlo, Junginger, Martin and Faaij, André (2011) "International bioenergy trade—A review of past developments in the liquid biofuel market." *Renewable and Sustainable Energy Reviews*, 15(6), pp. 2655–2676.

50 European Union (2010) "Communication from the Commission on the practical implementation of the EU biofuels and bioliquids sustainability scheme and on counting rules for biofuels." *Renewable Energy*, 17(443).

51 Council of the European Parliment (2009) "Directive 2009/28/EC on the promotion of the use of energy from renewable sources." , pp. 16–62.

52 World Resources Institute (2011) "National Alcohol Program (PROALCOOL)." *Special Report*. [online] Available from: http://projects.wri.org/sd-pams-database/brazil/national-alcohol-program-proalcool (Accessed 10 November 2011)

53 Harvey, M. and McMeekin, A. (2010) *Political Shaping of Transitions to Biofuels in Europe, Brazil and the USA*, University of Essex: Colchester.

54 Escobar, José C., Lora, Electo S., Venturini, Osvaldo J., Yáñez, Edgar E., et al. (2009) "Biofuels: Environment, technology and food security." *Renewable and Sustainable Energy Reviews*, 13(6-7), pp. 1275–1287.

55 Deng, Xiaodong, Li, Yajun and Fei, Xiaowen (2009) "Microalgae : A promising feedstock for biodiesel." *Journal of Microbiology*, 3(13), pp. 1008–1014.

56 Singh, A, He, B, Thompson, J and Gerpen, J Van (2006) "Process optimization of biodiesel production using alkaline catalysts." *Applied Engineering in Agriculture*, 22(4), pp. 597–600.

57 Laurens, Lieve M L, Quinn, Matthew, Van Wychen, Stefanie, Templeton, David W and Wolfrum, Edward J (2012) "Accurate and reliable quantification of total microalgal fuel potential as fatty acid methyl esters by in situ transesterification." *Analytical and bioanalytical chemistry*, 403(1), pp. 167–78. [online]

58 Ehimen, E.a., Sun, Z.F. and Carrington, C.G. (2010) "Variables affecting the in situ transesterification of microalgae lipids." *Fuel*, 89(3), pp. 677–684.

59 Mendow, G, Veizaga, N S, Sánchez, B S and Querini, C a (2012) "Biodiesel production by two-stage transesterification with ethanol by washing with neutral water and water saturated with carbon dioxide." *Bioresource technology*, 118, pp. 598–602. [online]

60 Miao, Xiaoling and Wu, Qingyu (2006) "Biodiesel production from heterotrophic microalgal oil." *Bioresource technology*, 97(6), pp. 841–6.

61 Hensarling, T. P and Jacks, T. J. (1983) "Solvent Extraction of Lipids from Soybeans with Acidic Hexane." *JAOCS1*, 60(4), pp. 783–784.

62 Ferreira, Isaac Quintino (2010) *Produção de Biodiesel: External Presentation*. Petrobras Biofuel: Brazil

63 Almeida, Antonio Carlos (2012) *Usina De Biodiesel De Quixadá: Operação e Produção*, Petrobras: Brazil.

64 Lam, Man Kee and Lee, Keat Teong (2012) "Microalgae biofuels: A critical review of issues, problems and the way forward." *Biotechnology advances*, 30(3), pp. 673–90.

65 CNA (2012) “Taiwan becomes top producer of algae medicine.” *Want China Times*. [online] Available from: http://www.wantchinatimes.com/news-subclass-cnt.aspx?id=20121228000001&cid=1104 (Accessed 3 December 2013)

66 Thurmond, Will (2011) “Global Biofuels, Drop-In Fuels, Biochems Markets and Forecasts,” in *Algae 2020 Volume 2*, Houston, Emerging Markets Online, pp. 1–4.

67 Feinberg, Daniel A. (1984) *Fuel Options from Microalgae with Representative Chemical Compositions*, Colorado. [online] Available from: http://www.nrel.gov/docs/legosti/old/2427.pdf

68 Biller, P and Ross, A B (2011) “Potential yields and properties of oil from the hydrothermal liquefaction of microalgae with different biochemical content.” *Bioresource technology*, 102(1), pp. 215–25.

69 Thomas, George (2000) “Overview of Storage Development,” in *US DOE Hydrogen Programme 2000 Annual Review*, California.

70 Pradhan, A, Shrestha, D S, Mcaloon, A, Yee, W, et al. (2011) “Energy Life-Cycle Assessment Of Soybean Biodiesel Revisited.” *American Society of Agricultural and Biological Engineers*, 54(3), pp. 1031–1039.

71 Lyons, P.E. and Plisga, BS (2004) *Standard Handbook of Petroleum and Natural Gas Engineering* 2nd ed., Burlington, Gulf Professional Publishing.

72 Olguín, Eugenia J (2012) “Dual purpose microalgae-bacteria-based systems that treat wastewater and produce biodiesel and chemical products within a Biorefinery.” *Biotechnology advances*, 30(5), pp. 1031–46. [online]

73 Li, Xiufeng, Xu, Han and Wu, Qingyu (2007) “Large-Scale Biodiesel Production From Microalga Chlorella protothecoides Through Heterotrophic Cultivation in Bioreactors.” , 98(4), pp. 764–771.

74 Mitra, Debjani, van Leeuwen, J. (Hans) and Lamsal, Buddhi (2012) “Heterotrophic/mixotrophic cultivation of oleaginous Chlorella vulgaris on industrial co-products.” *Algal Research*, 1(1), pp. 40–48.

75 Widjaja, Arief, Chien, Chao-Chang and Ju, Yi-Hsu (2009) “Study of increasing lipid production from fresh water microalgae Chlorella vulgaris.” *Journal of the Taiwan Institute of Chemical Engineers*, 40(1), pp. 13–20. [online]

76 Mohamed, Mohd Shamzi, Wei, Lai Zee and Ariff, Arbakariya B (2011) “Heterotrophic cultivation of microalgae for production of biodiesel.” *Recent patents on biotechnology*, 5(2), pp. 95–107.

77 Lardon, Laurent, Hélias, Arnaud, Sialve, Bruno, Steyer, Jean-Philippe and Bernard, Olivier (2009) “Life-cycle assessment of biodiesel production from microalgae.” *Environmental science & technology*, 43(17), pp. 6475–81.

78 Nichols, B. W (1965) “Light induced changes in the lipids of C. Vulgaris.” *Biochem. Biophys. Acta*, 106, pp. 274–279.

79 Johnson, Michael B. and Wen, Zhiyou (2009) “Production of Biodiesel Fuel from the Microalga Schizochytrium limacinum by Direct Transesterification of Algal Biomass.” *Energy & Fuels*, 23(10), pp. 5179–5183.

80 Chen, Feng and Johns, Michael R (1991) “Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic Chlorella sorokiniana.” *Journal of Applied Phycology*, 3, pp. 203–209.

81 Redfield, A. C. (1934) “On the proportions of organic derivations in sea water and their relation to the composition of plankton.” , pp. 176–192.

82 Sansawa, Hiroshi and Endo, Hiroshi (2004) “Production of intracellular phytochemicals in Chlorella under heterotrophic conditions.” *Journal of bioscience and bioengineering*, 98(6), pp. 437–44. [online]

83 Wu, Q.Y., Yin, S., G.Y., Sheng and Fu, J.M. (1992) “A comparative study of gases generated from stimulant thermal degradation of auto- trophic and heterotrophic Chlorella.” *Prog. Nat. Sci. (in Chinese)*, 3, pp. 435–440.

84 Xin, Li, Hu, Hong-ying, Ke, Gan and Sun, Ying-xue (2010) “Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga Scenedesmus sp.” *Bioresource technology*, 101(14), pp. 5494–500. [online]

85 OECD (2010) *Guidelines for the Testing of Chemicals* 12th Adden., OECD Publishing. [online] Available from: http://www.oecd.org/chemicalsafety/testing/43735667.pdf

86 Wood, A. M., Everroad, R. C. and Wingard, L. M. (2005) “Measuring Growth Rates in Microalgal Cultures,” in Andersen, R. A. (ed.), *Algal Culturing Techniques*, Burlington, Elsevier, pp. 269–286.

87 Bastidas, Oscar (2012) “Cell Counting with Neubauer Chamber - Basic Hemocytometer Usage.” Celeromics, Technical Note pp. 1–6.

88 Munir, Neelma, Sharif, Nadia, Naz, Shagufta and Saleem, Faiza (2013) “Harvesting and Processing of Microalgae Biomass Fractions for Biodiesel Production (A Review).” *Science, Technology and Development*, 32(3), pp. 235–243.

89 Xu, Han, Miao, Xiaoling and Wu, Qingyu (2006) “High quality biodiesel production from a microalga Chlorella protothecoides by heterotrophic growth in fermenters.” *Journal of Biotechnology*, 126, pp. 499–507.

90 Brummer, Yolanda and Cui, Steve W. (2005) “Understanding carbohydrate Analysis,” in Cui, S. W. (ed.), *Food Carbohydrates: Chemistry, Physical Properties, and Applications*, London, CRC Press.

91 Gerchakov, S.M. and Hatcher, P.G. (1972) “Improved technique for analysis of carbohydrates in sediments.” *Limnology and Oceanography,*, 17(6), pp. 938–943.

92 Laurens, Lieve M.L., Wychen, Stefanie Van, McAllister, Jordan P., Arrowsmith, Sarah, et al. (2014) “Strain, Biochemistry and Cultivation-Dependent Measurement Variability of Algal Biomass Composition.” *Analytical Biochemistry*, 452.

93 Laurens, Lieve M. L., Dempster, Thomas A., Jones, Howland D. T., Wolfrum, Edward J., et al. (2012) “Algal Biomass Constituent Analysis: Method Uncertainties and Investigation of the Underlying Measuring Chemistries.” *Analytical Chemistry*, 84, pp. 1879–1887.

94 Thompson, Michael, Owen, Linda, Wilkinson, Kate, Wood, Roger and Damant, Andrew (2002) “A comparison of the Kjeldahl and Dumas methods for the determination of protein in foods, using data from a proficiency testing scheme.” *Analyst*, 127, pp. 1666–1668.

95 Araujo, G. S., Matos, L. J., Gonçalves., L. R., Fernandes, F. A. and Farias, W. R. (2011) “Bioprospecting for oil producing microalgal strains: evaluation of oil and biomass production for ten microalgal strains.” *Bioresource Technology*, 102, pp. 5248–5250.

96 Smedes, Foppe and Thomasen, Torsten K (1996) “Evaluation of the Bligh & Dyer Lipid Determination Method.” *Science*, 32(1), pp. 681–688.

97 Iverson, S J, Lang, S L and Cooper, M H (2001) “Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue.” *Lipids*, 36(11), pp. 1283–7.

98 Mulbry, Walter, Kondrad, Shannon, Buyer, Jeffrey and Luthria, Devanand L. (2009) “Optimization of an Oil Extraction Process for Algae from the Treatment of Manure Effluent.” *Journal of the American Oil Chemists’ Society*, 86(9), pp. 909–915. [online]

99 Retief, Liezel, Faculty, Natural Science and Science, Polymer (2011) “Analysis of vegetable oils, seeds and beans by TGA and NMR spectroscopy.” , (March).

100 Harker, J. H. and Backhurst, J. R. (1981) *Fuel and Energy*, London, Acedemic Press.

101 Garbarino, J.R. and Taylor, H.E. (1996) *Inductively coupled plasma-mass spectrometric method for the determination of dissolved trace elements in natural water*, Colorado.

102 De Almeida, Edmar Fagundes, Bomtempo, Jose Vitor and De Souza E Silva, Carla Maria (2007) *The Performance of Brazilian Biofuels : An Economic, Environmental and Social Analysis*, Joint Transport Research Centre: Rio de Janeiro.

103 De Sousa, Paulo Teixeira, Dall’Oglio, Evandro L, Sato, Michele, Marta, Jose M., et al. (2008) “The Ethanol and Biodiesel Programmes in Brazil,” in Krieger, L. M. and Boyle, G. (eds.), *Making Choices About Hydrogen: Transport Issues for Developing Countries*, USA, United Nations University Press, pp. 118 – 140.

104 De Sousa and Dall’Oglio (2008) “The ethanol and biodiesel programmes in Brazil,” in *Making Choices about Hydrogen: transport issues for developing countries*, Canada, UNU Press/IDRC, pp. 118–140. [online] Available from: http://web.idrc.ca/en/ev-132144-201-1-DO_TOPIC.html

105 Vianna, De Souza, Aliana, Catherine and Garcez, Gucciardi (2011) “Brazilian Biodiesel Policy : Social and environmental considerations of sustainability.” *Energy Policy*, 34(2009), pp. 645–654.

106 Schaffel, Silvia Blajberg and La Rovere, Emilio Lèbre (2010) “The quest for eco-social efficiency in biofuels production in Brazil.” *Journal of Cleaner Production*, 18(16-17), pp. 1663–1670.

107 Padula, Antonio Domingos, Santos, Manoela Silveira, Ferreira, Luciano and Borenstein, Denis (2012) “The emergence of the biodiesel industry in Brazil: Current figures and future prospects.” *Energy Policy*, 44, pp. 395–405.

108 Hall, Jeremy, Matos, Stelvia and Severino, Liv (2009) “Brazilian biofuels and social exclusion : established and concentrated ethanol versus emerging and dispersed biodiesel.” *Journal of Cleaner Production*, 17, pp. S77–S85.

109 Ministry of Finance (2002) *Tax System and Administration in Brazil*, Federal Revenue Service: Brasilia.

110 Munir Y., Soares, Pavan, Margareth O., Barufi, Clara, Bermann, Célio and Parente, Virgínia (2007) “The Brazilian Biodiesel Program,” in *Amsterdam Conference on the Human Dimensions of Global Environmental Change*, Amsterdam, Vrije Universiteit Institute for Environmental Studies.

111 Van Den Berg, Jonas and Rademakers, Laurens (2007) “An in-depth look at Brazil’s ‘Social Fuel Seal.’” *Biopact*. [online] Available from: http://news.mongabay.com/bioenergy/2007/03/in-depth-look-at-brazils-social-fuel.html (Accessed 18 April 2012)

112 America Latina Logistica (2012) “Railroad and Trucking Industries.” [online] Available from: http://ir.all-logistica.com/all/web/conteudo_en.asp?idioma=1&tipo=323&conta=44#2 (Accessed 30 August 2012)

113 Kinast, J A (2003) *Production of Biodiesels from Multiple Feedstocks and Properties of Biodiesels and Biodiesel*, NREL: Illinois.

114 Lang, X, Dalai, a K, Bakhshi, N N, Reaney, M J and Hertz, P B (2001) “Preparation and characterization of bio-diesels from various bio-oils.” *Bioresource technology*, 80(1), pp. 53–62.

115 Ogunniyi, D S (2006) “Castor oil: a vital industrial raw material.” *Bioresource technology*, 97(9), pp. 1086–91.

116 Barabás, István and Todoruţ, Ioan-adrian (2011) “Biodiesel Quality, Standards and Properties,” in Montero, G. and Stoytcheva, M. (eds.), *Biodiesel- Quality, Emissions and By-Products*, Online, CC BY.

117 Goosen, Raoul, Vora, Kuntal and Vona, Candace (2007) *Establishment of the Guidelines for the Development of Biodiesel Standards in the APEC Region Development Initiative*, Texas.

118 Ministry of Mines and Energy (2004) “Decree N°5.297.” *Fuel*.

119 Marson, Renata, Andrade, Teixeira De and Miccolis, Andrew (2011) “Policies, institutional and legal framework in the expansion of Brazilian biofuels.” *Working paper 71*, CIFOR, pp. 1–36.

120 Almeida A. C. (2012) *Conversation with Philippa Usher*. 20 January

121 Magalhães, Cláudia, Drouvot, Hubert and Perluss, Martin (2010) *A coherent agri-energy policy to foster social inclusion for peasant families : the role of Petrobras on the João Câmara and Ceará-Mirim sites*, France.

122 Machado, Andre (2014) “The experience of social seal for biodiesel in Brazil,” in *Global Bioenergy Partnership*, Maputo, Mozampbique, MDA.

123 Talamini, E and Dewes, H (2012) “The macro-environment for liquid Biofuels in Brazilian science and public policies.” *Science and Public Policy*, 39(January), pp. 13–29.

124 Petrobras (2011) *Processamento Industrial De Biodiesel: Powerpoint Presentation*, Petrobras, Quixada, Brazil

125 Vitali, Isabella (2011) *Soya and the Cerrado: Brazil’s forgotten jewel*, WWF, Surrey

126 Metay, Aurélie, Moreira, José Aloisio Alves, Bernoux, Martial, Boyer, Thomas, et al. (2007) “Storage and forms of organic carbon in a no-tillage under cover crops system on clayey Oxisol in dryland rice production (Cerrados, Brazil).” *Soil and Tillage Research*, 94(1), pp. 122–132.

127 Yáñez Angarita, Edgar Eduardo, Silva Lora, Electo Eduardo, da Costa, Rosélis Ester and Torres, Ednildo Andrade (2009) “The energy balance in the Palm Oil-Derived Methyl Ester (PME) life cycle for the cases in Brazil and Colombia.” *Renewable Energy*, 34(12), pp. 2905–2913.

128 Ester da Costa, Rosélis and Lora, Electo Eduardo Silva (2006) “The Energy Balance In The Production Of Palm Oil Biodiesel - Two Case Studies: Brazil And Colombia.,” in *Proceding of the World Bioenergy*, Jonkoping, Sweden, pp. 4–8.

129 Finco, Marcus Vinicius Alves and Doppler, Werner (2010) “Bioenergy and sustainable development: The dilemma of food security and climate change in the Brazilian savannah.” *Energy for Sustainable Development*, 14(3), pp. 194–199.

130 Fearnside, Philip M and Imbrozio Barbosa, Reinaldo (1998) “Soil carbon changes from conversion of forest to pasture in Brazilian Amazonia.” *Forest Ecology and Management*, 108(1-2), pp. 147–166.

131 Fransen, Taryn (2009) “Brazil Pledges Ambitious Emissions Reductions.” *World Resources Institute*. [online] Available from: http://www.wri.org/stories/2009/11/brazil-pledges-ambitious-emissions-reductions#fn:1 (Accessed 1 August 2012)

132 Lima, Mendelson, Skutsch, Margaret and Costa, Gerlane De Medeiros (2011) “Deforestation and the Social Impacts of Soy for Biodiesel: Perspectives of Farmers in the South Brazilian Amazon.” *Ecology and Society*, 16(4).

133 Zinn, Yuri L., Lal, Rattan and Resck, Dimas V.S. (2005) “Changes in soil organic carbon stocks under agriculture in Brazil.” *Soil and Tillage Research*, 84(1), pp. 28–40.

134 Magalhães Drouvot, Cláudia, Drouvot, Hubert and Perluss, Preston Martin (2010) “A coherent agri-energy policy to foster social inclusion for peasant families : the role of Petrobras on the João Câmara and Ceará-Mirim sites (state of Rio Grande do Norte),” in *Family farming under pressure. Reassessing options for liveability and permanence*, Vienna, 9th European IFSA Symposium, pp. 1025–1035.

135 Ceasar, A. S. and Batalha, M.O. (2010) “Biodiesel production from castor oil in Brazil : A difficult reality.” *Energy Policy*, 38, pp. 4031–4039.

136 Comar, Vito, Tilley, David, Felix, Erika, Turdera, Mirko and Matias Chagas Neto (2004) “Comparative emergy evaluation of castorbean (ricinus,” in Ortega, E. and Ulgiati, S. (eds.), *IV Biennial International Workshop “Advances in Energy Studies,”* Campinas, pp. 227–237.

137 Kilham, S, Camargo, C and Willetts, J (2010) *Biodiesel: Farmer’s Perspectives*, Sydney.

138 Office of Solid Waste (1999) *Estimating Risk from Contaminants Contained in Agricultural Fertilizers Draft Report Estimating Risk from the Use of Agricultural Fertilizers Draft Report*, Washington.

139 Singh, Bijay, Singh, Yadvinder and Sekhon, G.S. (1995) “Fertilizer-N use efficiency and nitrate pollution of groundwater in developing countries.” *Journal of Contaminant Hydrology*, 20(3-4), pp. 167–184.

140 Matos, Stelvia and Silvestre, Bruno S. (2012) “Managing stakeholder relations when developing sustainable business models: the case of the Brazilian energy sector.” *Journal of Cleaner Production*, pp. 1–13.

141 Chisti, Yusuf (2007) “Biodiesel from microalgae.” *Biotechnology advances*, 25(3), pp. 294–306.

142 Groom, Martha J, Gray, Elizabeth M and Townsend, Patricia a (2008) “Biofuels and biodiversity: principles for creating better policies for biofuel production.” *Conservation biology : the journal of the Society for Conservation Biology*, 22(3), pp. 602–9.

143 Morales-Sánchez, Daniela, Tinoco-Valencia, Raunel, Kyndt, John and Martinez, Alfredo (2013) “Heterotrophic growth of Neochloris oleoabundans using glucose as a carbon source.” *Biotechnology for biofuels*, 6(100), p.1-12

144 Mata, Teresa M., Martins, António A. and Caetano, Nidia S. (2010) “Microalgae for biodiesel production and other applications: A review.” *Renewable and Sustainable Energy Reviews*, 14(1), pp. 217–232.

145 Wu, Li Fen, Chen, Pei Chung, Huang, Ai Ping and Lee, Chi Mei (2012) “The feasibility of biodiesel production by microalgae using industrial wastewater.” *Bioresource technology*, 113, pp. 14–18.

146 Christenson, Logan and Sims, Ronald (2011) “Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts.” *Biotechnology advances*, 29(6), pp. 686–702.

147 Li, Yecong, Zhou, Wenguang, Hu, Bing, Min, Min, et al. (2011) “Integration of algae cultivation as biodiesel production feedstock with municipal wastewater treatment: strains screening and significance evaluation of environmental factors.” *Bioresource technology*, 102(23), pp. 10861–7.

148 Lardon, Laurent, Hélias, Arnaud, Sialve, Bruno, Steyer, Jean-Philippe and Bernard, Olivier (2009) “Life-Cycle Assessment of Biodiesel Production from Microalgae.” *Environmental Science & Technology*, 43(17), pp. 6475–6481.

149 Chen, Y. H. and Walker, T. H. (2011) “Biomass and lipid production of heterotrophic microalgae Chlorella protothecoides by using biodiesel-derived crude glycerol.” *Biotechnology Letters*, 33(10), pp. 1973–83.

150 Liang, Yanna (2013) “Producing liquid transportation fuels from heterotrophic microalgae.” *Applied Energy*, 104, pp. 860–868.

151 Gao, Yihe, Gregor, Chapin, Liang, Yuanjie, Tang, Dawei and Tweed, Caitlin (2012) “Algae biodiesel - a feasibility report.” *Chemistry Central journal*, 6 Suppl 1(S1), pp. 1–16.

152 Droop, M. R. (1974) “Heterotrophy of Carbon,” in Stewart, W. D. P. (ed.), *Algal Physiology and Biochemistry*, Oxford, Blackwell, pp. 530–559.

153 O’Grady, J. and Morgan, J. A. (2011) “Heterotrophic growth and lipid production of Chlorella protothecoides on glycerol.” *Bioprocess and Biosystems Engineering*, 34(1), pp. 121–125.

154 Chen, Feng (1996) “High cell density culture of microalgae in heterotrophic growth.” *Trends in Biotechnology*, 1996(November), pp. 421–426.

155 Rodolfi, Liliana, Chini Zittelli, Graziella, Bassi, Niccolò, Padovani, Giulia, et al. (2009) “Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor.” *Biotechnology and bioengineering*, 102(1), pp. 100–12.

156 Usher, Philippa K, Ross, Andrew B, Camargo-Valero, Miller Alonso, Tomlin, Alison S and Gale, William F. (2014) “An Overview of the Potential Environmental Impacts of Large Scale Microalgae Cultivation.” *Biofuels*.

157 Cain, J. (1965) “Nitrogen Utilisation in 38 Freshwater Chlamydomonad Algae.” *Canadian Journal of Botany*, 43(11), pp. 1367–1378.

158 Morris, I. (1974) “Nitrogen Assimilation and Protein Synthesis,” in Stewart, W. D. P. (ed.), *Algal Physiology and Biochemistry*, Bristol, Blackwell Scientific Publications, pp. 583–609.

159 Stross, R. G. (1963) “Nitrate Preference In Haematococcus As Controlled By Strain, Age Of Inoculum, And pH Of The Medium.” *Canadian Journal of Botany1*, 9(1), pp. 33–40.

160 Bollard, E. G. (1966) “A comparative study of the ability of organic nitrogenous compounds to serve as sole sources of nitrogen for the growth of plants.” *Plant and Soil*, 25(2), pp. 153–66.

161 Dvořáková, Jiřina (1979) “The capability of the algae Chlorella vulgaris and Scenedesmus obliquus of utilizing amino acids as the sole nitrogen source.” *Biologia Plantarum*, 21(5), pp. 372–5.

162 Courchesne, Noémie Manuelle Dorval, Parisien, Albert, Wang, Bei and Lan, Christopher Q (2009) “Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches.” *Journal of biotechnology*, 141(1-2), pp. 31–41.

163 Rodolfi, Liliana, Chini Zittelli, Graziella, Bassi, Niccolò, Padovani, Giulia, et al. (2009) “Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor.” *Biotechnology and bioengineering*, 102(1), pp. 100–12.

164 Kuhl, A. (1974) “Phosphorus,” in Stewart, W. D. P. (ed.), *Algal Physiology and Biochemistry*, Bristol, Blackwell Scientific Publications, pp. 636–54.

165 Becker, E. W. (1994) *Microalgae: Biotechnology and Microbiology*, Cambridge, Cambridge University Press.

166 Cordell, Dana, Drangert, Jan-Olof and White, Stuart (2009) “The story of phosphorus: Global food security and food for thought.” *Global Environmental Change*, 19(2), pp. 292–305.

167 Aslan, Sebnem and Kapdan, Ilgi Karapinar (2006) “Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae.” *Ecological Engineering*, 28(1), pp. 64–70.

168 Boonchai, Rawiwan, Seo, Gyu Tae, Park, Da Rang and Seong, Chung Yeol (2012) “Microalgae Photobioreactor for Nitrogen and Phosphorus Removal from Wastewater of Sewage Treatment Plant.” *International Journal of Bioscience, Biochemistry and Bioinformatics*, 2(6), pp. 407–410.

169 Prathima Devi, M., Venkata Subhash, G. and Venkata Mohan, S. (2012) “Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during sequential growth and starvation phases: Effect of nutrient supplementation.” *Renewable Energy*, 43, pp. 276–283.

170 Pueschel, Curt M and Korb, Rebecca E (2001) “Storage of nitrogen in the form of protein bodies in the kelp Laminaria solidungula.” , 218(Pueschel 1994), pp. 107–114.

171 Lourenço, Sergio O, Barbarino, Elisabete, Lavín, Paris L, Lanfer Marquez, Ursula M and Aidar, Elizabeth (2004) “Distribution of intracellular nitrogen in marine microalgae: Calculation of new nitrogen-to-protein conversion factors.” *European Journal of Phycology*, 39(1), pp. 17–32.

172 Zapata-Vélez, Ana M. and Trujillo-Roldán, Mauricio a. (2010) “The lack of a nitrogen source and/or the C/N ratio affects the molecular weight of alginate and its productivity in submerged cultures of Azotobacter vinelandii.” *Annals of Microbiology*, 60(4), pp. 661–668.

173 Ho, Shih-Hsin, Huang, Shu-Wen, Chen, Chun-Yen, Hasunuma, Tomohisa, et al. (2013) “Characterization and optimization of carbohydrate production from an indigenous microalga Chlorella vulgaris FSP-E.” *Bioresource technology*, 135, pp. 157–65.

174 Fahy, Eoin, Subramaniam, Shankar, Brown, H Alex, Glass, Christopher K, et al. (2005) “A comprehensive classification system for lipids.” *Journal of lipid research*, 46(5), pp. 839–61.

175 Dufreche, Stephen, Hernandez, R., French, T., Sparks, D., et al. (2007) “Extraction of Lipids from Municipal Wastewater Plant Microorganisms for Production of Biodiesel.” *Journal of the American Oil Chemists’ Society*, 84(2), pp. 181–187.

176 Trentacoste, Emily M, Shrestha, Roshan P, Smith, Sarah R, Glé, Corine, et al. (2013) “Metabolic engineering of lipid catabolism increases microalgal lipid accumulation without compromising growth.” *Proceedings of the National Academy of Sciences of the United States of America*, 110(49), pp. 19748–53.

177 Tansey, M R and Brock, T D (1972) “The upper temperature limit for eukaryotic organisms.” *Proceedings of the National Academy of Sciences of the United States of America*, 69(9), pp. 2426–8.

178 Converti, Attilio, Casazza, Alessandro a., Ortiz, Erika Y., Perego, Patrizia and Del Borghi, Marco (2009) “Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production.” *Chemical Engineering and Processing: Process Intensification*, 48(6), pp. 1146–1151.

179 Chiu, Sheng-Yi, Kao, Chien-Ya, Tsai, Ming-Ta, Ong, Seow-Chin, et al. (2009) “Lipid accumulation and CO2 utilization of Nannochloropsis oculata in response to CO2 aeration.” *Bioresource technology*, 100(2), pp. 833–8.

180 Li, Xin, Hu, Hong-ying and Zhang, Yu-ping (2011) “Growth and lipid accumulation properties of a freshwater microalga Scenedesmus sp. under different cultivation temperature.” *Bioresource technology*, 102(3), pp. 3098–102.

181 Rubery, P. H. and Sheldrake, A. R. (1973) “Effect of pH and Surface Charge on Cell Uptake of Auxin.” *Nature*, 244(139), pp. 285–88.

182 Azov, Y (1982) “Effect of pH on Inorganic Carbon Uptake in Algal Cultures.” *Applied and environmental microbiology*, 43(6), pp. 1300–6.

183 Soeder, C. and Stengel, E. (1974) “Physio-Chemical Factors Affecting Metabolism and Growth Rate,” in Stewart, W. D. P. (ed.), *Algal Physiology and Biochemistry1*, Bristol, Blackwell Scientific Publications, pp. 714–740.

184 Gao, Chunfang, Zhai, Yan, Ding, Yi and Wu, Qingyu (2010) “Application of sweet sorghum for biodiesel production by heterotrophic microalga Chlorella protothecoides.” *Applied Energy*, 87(3), pp. 756–761.

185 Liu, Jin, Huang, Junchao, Sun, Zheng, Zhong, Yujuan, et al. (2011) “Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic Chlorella zofingiensis: assessment of algal oils for biodiesel production.” *Bioresource technology*, 102(1), pp. 106–10.

186 Hespanhol, I (1997) “Wastewater as a Resource,” in Helmer, R. and Hespanhol, I. (eds.), *Water Pollution Control - A Guide to the Use of Water Quality Management Principles*, WHO/UNEP.

187 Yang, Jia, Xu, Ming, Zhang, Xuezhi, Hu, Qiang, et al. (2011) “Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance.” *Bioresource technology*, 102(1), pp. 159–65.

188 Chinnasamy, Senthil, Bhatnagar, Ashish, Hunt, Ryan W and Das, K C (2010) “Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications.” *Bioresource technology*, 101(9), pp. 3097–105.

189 Shirai, F., Kunii, K., Sato, C., Teramoto, Y., et al. (1998) “Cultivation of microalgae in the solution from the desalting process of soy sauce waste treatment and utilization of the algal biomass for ethanol fermentation.” *World Journal of Microbiology and Biotechnology*, 14(6), pp. 839–842.

190 Simate, Geoffrey S., Cluett, John, Iyuke, Sunny E., Musapatika, Evans T., et al. (2011) “The treatment of brewery wastewater for reuse: State of the art.” *Desalination*, 273(2-3), pp. 235–247.

191 Ferdowshi, Z. (2013) “Screening of fresh water microalgae and Swedish pulp and paper mill waste waters with the focus on high algal biomass production.”

192 Eppley, R. W. and Macias, M. F. (1962) “Rapid Growth of Sewage Lagoon Chlamydomonas with Acetate.” *Physiologia Plantarum*, 15.

193 Kotzabasis, K, Hatziathanasiou, A, Bengoa-Ruigomez, M.V, Kentouri, M and Divanach, P (1999) “Methanol as alternative carbon source for quicker efficient production of the microalgae Chlorella minutissima: Role of the concentration and frequence of administration.” *Journal of Biotechnology*, 70(1-3), pp. 357–362.

194 Nystrom, Bo, Bjornsater, Bo and Blanck, Hans (1999) “Effects of sulfonylurea herbicides on non-target aquatic Growth inhibition of micro-algae and short-term inhibition of adenine and thymidine incorporation in periphyton communities.” *Aquatic Toxicology*, 47, pp. 9–22.

195 Ipatova, V. I., Prokhotskaya, V. Yu. and Dmitrieva, A. G. (2011) “The Structure Of Algal Population In The Presence Of Toxicants,” in *Unifying Themes in Complex Systems*, Berlin, Springer, pp. 182–189.

196 Chen, Hong and Pan, Shan-Shan (2005) “Bioremediation potential of spirulina: toxicity and biosorption studies of lead.” *Journal of Zhejiang University. Science. B*, 6(3), pp. 171–4.

197 Khoshmanesh, A, Lawson, F and Prince, I G (1997) “Cell surface area as a major parameter in the uptake of cadmium by unicellular green microalgae.” *Chemical Engineering Journal*, 65, pp. 13–19.

198 Li, Penglin, Miao, Xiaoling, Li, Rongxiu and Zhong, Jianjiang (2011) “In situ biodiesel production from fast-growing and high oil content Chlorella pyrenoidosa in rice straw hydrolysate.” *Journal of biomedicine & biotechnology*, 141207, pp. 1–8.

199 Cheng, Y., Zhou, W., Gao, C., Lan, K., et al. (2009) “Biodiesel production from Jerusalem artichoke (Helianthus tuberosus L.) tuber by heterotrophic microalgae Chlorella protothecoides.” *Soc Chem Ind*, 84, pp. 777–781.

200 D. Voet and J. Voet (1995) *Biochemistry* 2nd ed., New York, J. Wiley & Sons.

201 Dickson, Leal G, Galloway, Raymond A and Patterson, Glenn W (1969) “Environmentally-Induced Changes in the Fatty Acids.” , pp. 1413–1416.

202 Foreign Agricultural Service (2003) “EU: Biodiesel Industry Expanding Use of Oilseeds.” *Production Estimates and Crop Assessment Division*. [online] Available from: http://www.fas.usda.gov/pecad2/highlights/2003/09/biodiesel3/ (Accessed 14 November 2011)

203 Gunkel, Günter, Kosmol, Jan, Sobral, Maria, Rohn, Hendryk, et al. (2006) “Sugar Cane Industry as a Source of Water Pollution – Case Study on the Situation in Ipojuca River, Pernambuco, Brazil.” *Water, Air, and Soil Pollution*, 180(1-4), pp. 261–269.

204 Prabhu, T., Ramalingam, S., Manimaran, K., Narayanan, B. and Nagarajan, S. M. (2009) “A study on the physio-chemical parameters of sugar mills effluents.” *Journal of Current Sciences*, 14(1), pp. 123–127.

205 Yan, Dong, Lu, Yue, Chen, Yi-Feng and Wu, Qingyu (2011) “Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production.” *Bioresource technology*, 102(11), pp. 6487–93.

206 Liu, Jin, Huang, Junchao, Jiang, Yue and Chen, Feng (2012) “Molasses-based growth and production of oil and astaxanthin by Chlorella zofingiensis.” *Bioresource technology*, 107, pp. 393–8.

207 Wang, Yan, Chen, Tao and Qin, Song (2012) “Heterotrophic cultivation of Chlorella kessleri for fatty acids production by carbon and nitrogen supplements.” *Biomass and Bioenergy*, 47, pp. 402–409.

208 Liang, Yanna, Sarkany, Nicolas and Cui, Yi (2009) “Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions.” *Biotechnology letters*, 31(7), pp. 1043–9.

209 Liu, Zhi-Yuan, Wang, Guang-Ce and Zhou, Bai-Cheng (2008) “Effect of iron on growth and lipid accumulation in Chlorella vulgaris.” *Bioresource technology*, 99(11), pp. 4717–22.

210 Hellebust, J A (1965) “Excretion of some organic compunds by marine phytoplankton.” *Association for the Sciences of Limnology and Oceanography*, 10(2), pp. 192–206.

211 Azma, Mojtaba, Mohamad, Rosfarizan, Rahim, Raha Abdul and Ariff, Arbakariya B (2010) “Improved Protocol for the Preparation of Tetraselmis suecica Axenic Culture and Adaptation to Heterotrophic Cultivation.” *The Open Biotechnology Journal*, 4, pp. 36–46.

212 Alberts, Bruce, Johnson, Alex, Lewis, Julian, Raff, Martin, et al. (2008) *Molecular Biology of the Cell* 5th ed., New York, Garland Science.

213 Siegler, Hector De la Hoz (2012) “Optimization of Biomass and Lipid Production in Heterotrophic Microalgal Cultures.”

214 Knothe, Gerhard (2008) “‘Designer’ Biodiesel: Optimizing Fatty Ester Composition to Improve Fuel Properties.” *Energy & Fuels2*, 22, pp. 1358–1364.

215 NREL (2009) *Biodiesel Handling and Use Guide*, Springfield.

216 Bampi, Marlene, Scheer, Agnes De P. and de Castilhos, Fernanda (2013) “Application of near infrared spectroscopy to predict the average droplet size and water content in biodiesel emulsions.” *Fuel*, 113, pp. 546–552.

217 Graboski, Michael S. and McCormick, Robert L. (1998) “Combustion of fat and vegetable oil derived fuels in diesel engines.” *Progress in Energy and Combustion Science*, 24(2), pp. 125–164.

218 Igbum, O G, Leke, L, Okoronkwo, M U and Eboka, A (2013) “Evaluation of Fuel Properties from Free Fatty Acid Compositions of Methyl Esters Obtained from Four Tropical Virgin Oils.” *Internation Journal of Applied Chemistry*, 9(1), pp. 37–49.

219 Philips Petroleum Company (2002) “MSDS Summary Sheet: No. 2 Diesel Fuel.”

220 Corbitt, R.A. (1998) *Standard Handbook of Environmental Engineering*, New York, McGraw-Hill Inc.

221 Lopes, J C A, Boros, L, Meirelles, A J A, Daridon, J L, et al. (2008) “Prediction of Cloud Points of Biodiesel.” *Energy & Fuels*, 22, pp. 747–752.

222 Harrington, K. J. and D’Arcy-Evans, C. (1985) “Transesterification in Situ of Sunflower Seed Oil.” *Ind. Eng. Chem. Prod. Res. Dev.*, 24, pp. 314–318.

223 R. Nadkarni and A. Kishore (2007) *Guide to ASTM Test Methods for the Analysis of Petroleum Products and Lubricants* 2nd ed., West Conshohocken, ASTM International.

224 Karmakar, Aninidita, Karmakar, Subrata and Mukherjee, Souti (2010) “Properties of various plants and animals feedstocks for biodiesel production.” *Bioresource technology*, 101(19), pp. 7201–10.

225 Knothe, G (2007) “Some aspects of biodiesel oxidative stability.” *Fuel Processing Technology*, 88(7), pp. 669–677.

226 Park, Ji-Yeon, Kim, Deog-Keun, Lee, Joon-Pyo, Park, Soon-Chul, et al. (2008) “Blending effects of biodiesels on oxidation stability and low temperature flow properties.” *Bioresource technology*, 99(5), pp. 1196–203.

227 Islam, Muhammad, Magnusson, Marie, Brown, Richard, Ayoko, Godwin, et al. (2013) “Microalgal Species Selection for Biodiesel Production Based on Fuel Properties Derived from Fatty Acid Profiles.” *Energies*, 6(11), pp. 5676–5702.

228 Schmidt, Paul Frank (1985) *Fuel and Oil Manual* 4th ed., New York, Industrial Press Inc.

229 Barker, Jim, Cook, Stephen and Richards, Paul (2013) “Sodium Contamination of Diesel Fuel, its Interaction with Fuel Additives and the Resultant Effects on Filter Plugging and Injector Fouling.” *SAE Paper number 2013-2687*.

230 Pratas, Maria Jorge, Freitas, Samuel, Oliveira, Mariana B., Monteiro, Sílvia C., et al. (2010) “Densities and Viscosities of Fatty Acid Methyl and Ethyl Esters.” *Journal of Chemical & Engineering Data*, 55(9), pp. 3983–3990.

231 Pratas, Maria Jorge (2012) *“Biodiesel Fuel Formulation.”* PhD Thesis, Universidade de Averio

232 Cohen, Zvi, Vonshak, Avigard and Richmond, Amos (1988) “Effect of environmental conditions on fatty acid composition of the red alga porphyridium cruentum: correlation to growth rate.” *Phycology*, 332, pp. 328–332.

233 Atadashi, I.M., Aroua, M.K., Aziz, a.R. Abdul and Sulaiman, N.M.N. (2011) “Refining technologies for the purification of crude biodiesel.” *Applied Energy*, 88(12), pp. 4239–4251.

234 Lane, Daniel J, Zevenhoven, Maria, Ashman, Peter J, Eyk, Philip J Van, et al. (2014) “Algal Biomass : Occurrence of the Main Inorganic Elements and Simulation of Ash Interactions with Bed Material.” *Energy and Fuels,* 28(7), pp 4622–4632

235 Yuan, W., Hansen, A.C. and Zhang, Q. (2009) “Predicting the temperature dependent viscosity of biodiesel fuels.” *Fuel*, 88(6), pp. 1120–1126.

236 Pinzi, S, Garcia, I L, Castro, M D Luque De, Dorado, G and Dorado, M P (2009) “The Ideal Vegetable Oil-based Biodiesel Composition : A Review of Social , Economical and Technical Implications.” *Energy*, (3), pp. 2325–2341.

237 Bamgboye, A.I. and Hansen, A.C. (2008) “Prediction of cetane number of biodiesel fuel from the fatty acid methyl ester (FAME) composition.” *International Agrophysics*, (1), pp. 21–29.

238 Klopfenstein, W E (1982) “Estimation of Cetane Index for Esters of Fatty Acids Cetane Indexes of Fatty Acid Esters.” *JAOCS*, 59(12), pp. 531–533.

239 Knothe, Gerhard (2014) “A comprehensive evaluation of the cetane numbers of fatty acid methyl esters.” *Fuel*, 119, pp. 6–13.

240 Dunn, Robert O (2008) “Improving the Cold Flow Properties of Biodiesel by Fractionation,” in Ng, T.-B. (ed.), *Soybean - Applications and Technology*, Illinois. [online] Available from: http://www.intechopen.com/books/soybean-applications-and-technology/improving-the-cold-flow-properties-of-biodiesel-by-fractionation

241 Moser, Bryan R (2008) “Influence of Blending Canola, Palm, Soybean and Sunflower Oil Methyl Esters on Fuel Properties of Biodiesel.” , 22, pp. 4301–4306.

242 Ramírez-Verduzco, Luis Felipe, Rodríguez-Rodríguez, Javier Esteban and Jaramillo-Jacob, Alicia Del Rayo (2012) “Predicting cetane number, kinematic viscosity, density and higher heating value of biodiesel from its fatty acid methyl ester composition.” *Fuel*, 91(1), pp. 102–111.

243 Methanol Institute (2014) “Properties of Fuels.” [online] Available from: http://www.methanol.org/energy/resources/alternative-fuel/alt-fuel-properties.aspx (Accessed 20 March 2014)

244 Wang, P S, Thompson, J, Clemente, T E and Gerpen, J H Van (2010) “Improving the Fuel Properties of Soy Biodiesel.” *American Society of Agricultural and Biological Engineers*, 53(6), pp. 1853–1858.

245 Candeia, R.a., Silva, M.C.D., Carvalho Filho, J.R., Brasilino, M.G.a., et al. (2009) “Influence of soybean biodiesel content on basic properties of biodiesel–diesel blends.” *Fuel*, 88(4), pp. 738–743.

246 Dugan, L R, McGinnis, G W and Vadehra, D V (1966) “Low temperature direct methylation of lipids in biological materials.” *Lipids*, 1(5), pp. 305–8.

247 Harwood, J. L. and Scrimgeour, C. M. (2007) “Fatty Acid and Lipid Structure,” in Harwood, J. L. ., Gunstone, F. D. ., and Dijkstra, A. J. . (eds.), *The Lipid Handbook*, London, CRC Press.

248 Köst, H.-P., Senser, M. and Wanner, G. (1984) “Effect of Nitrate and Sulphate Starvation on Porphyridium cruentum Cells.” *Zeitschrift für Pflanzenphysiologie*, 113(3), pp. 231–249.

249 Tatur, M, Nanjundaswamy, H, Tomazic, D and Thornton, M (2008) “Effects of Biodiesel Operation on Light-Duty Tier 2 Engine and Emission Control Systems Preprint.”

250 Guieysse, Benoit, Béchet, Quentin and Shilton, Andy (2013) “Variability and uncertainty in water demand and water footprint assessments of fresh algae cultivation based on case studies from five climatic regions.” *Bioresource technology*, 128, pp. 317–23.

251 Lohrey, Christian and Kochergin, Vadim (2012) “Biodiesel production from microalgae: co-location with sugar mills.” *Bioresource technology*, 108, pp. 76–82.

252 Batan, Liaw, Quinn, Jason C. and Bradley, Thomas H. (2013) “Analysis of water footprint of a photobioreactor microalgae biofuel production system from blue, green and lifecycle perspectives.” *Algal Research*, 2(3), pp. 196–203.

253 Water Footprint Network (2013) “Water footprint and virtual water.” [online] Available from: http://www.waterfootprint.org/?page=files/Water-energy (Accessed 19 August 2013)

254 May, Peter I (2010) “Use of an Algal Turf Scrubber to Reduce Nutrient Loadings and Produce Biofuel at a Wastewater Treatment Plant on Jamaica Bay, New York City,” in *5th National Conference on Coastal and Estuarine Habitat Restoration*, Texas, Restore America’s Estuaries.

255 Woo, Sung-Geun, Yoo, Keunje, Lee, Juyoun, Bang, Sara, et al. (2012) “Comparison of Fatty Acid Analysis Methods for Assessing Biorefinery Applicability of Wastewater Cultivated Microalgae.” *Talanta*, 15(97), pp. 103–110.

256 Ramachandra, T.V., Durga Madhab, Mahapatra, Shilpi, Samantray and Joshi, N.V. (2013) “Algal biofuel from urban wastewater in India: Scope and challenges.” *Renewable and Sustainable Energy Reviews*, 21, pp. 767–777.

257 Abdel-Raouf, N., Al-Homaidan, A. A. and Ibraheem, I. B. M. (2012) “Microalgae and wastewater treatment.” *Saudi Journal of Biological Sciences*, 19(3), pp. 257–275.

258 Andreoli, C V, Garbossa, L H P, Lupatini, G and Pegorini, E S (n.d.) *Wastewater Sludge Management: A Brazilian Approach*, Curitiba, Brazil.

259 Wagner, Leonard (2007) *Biodiesel from Algae oil*,

260 Plappally, a.K. and Lienhard V, J.H. (2012) “Energy requirements for water production, treatment, end use, reclamation, and disposal.” *Renewable and Sustainable Energy Reviews*, 16(7), pp. 4818–4848.

261 Li, Yecong, Chen, Yi-Feng, Chen, Paul, Min, Min, et al. (2011) “Characterization of a microalga Chlorella sp. well adapted to highly concentrated municipal wastewater for nutrient removal and biodiesel production.” *Bioresource technology*, 102(8), pp. 5138–44.

262 Subashchandrabose, Suresh R, Ramakrishnan, Balasubramanian, Megharaj, Mallavarapu, Venkateswarlu, Kadiyala and Naidu, Ravi (2013) “Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation.” *Environment international*, 51, pp. 59–72.

263 Kathy E. Lee, Larry B. Barber, Edward T. Furlong, Jeffery D. Cahill, et al. (2004) *Antibiotics Field Data - Scientific Investigations Report 2004-5138*, Minnesota. [online] Available from: http://pubs.usgs.gov/sir/2004/5138/antibiotics.html (Accessed 19 August 2013)

264 Martín, J, Camacho-Muñoz, D, Santos, J L, Aparicio, I and Alonso, E (2012) “Occurrence of pharmaceutical compounds in wastewater and sludge from wastewater treatment plants: removal and ecotoxicological impact of wastewater discharges and sludge disposal.” *Journal of hazardous materials*, 239-240, pp. 40–7.

265 Subashchandrabose, Suresh R, Ramakrishnan, Balasubramanian, Megharaj, Mallavarapu, Venkateswarlu, Kadiyala and Naidu, Ravi (2013) “Mixotrophic cyanobacteria and microalgae as distinctive biological agents for organic pollutant degradation.” *Environment international*, 51, pp. 59–72.

266 Kumar, Amit, Ergas, Sarina, Yuan, Xin, Sahu, Ashish, et al. (2010) “Enhanced CO(2) fixation and biofuel production via microalgae: recent developments and future directions.” *Trends in biotechnology*, 28(7), pp. 371–80.

267 Butler, G L, Deason, T R and O’Kelley, J C (1975) “Loss of five pesticides from cultures of twenty-one planktonic algae.” *Bulletin of environmental contamination and toxicology*, 13(2), pp. 149–52.

268 National Research Council (2012) “Environmental Effects,” in *Sustainable Development of Algal Biofuels in the United States*, Washington D. C., The National Academies Press, pp. 139–190.

269 Fuhrman, J. A. and Suttle, C. A. (1993) “Viruses in Marine Planktonic Systems.” *Oceanography*, 6(2).

270 Beltrami, Edward and Carroll, T O (1994) “Modeling the role of viral disease in recurrent phytoplankton blooms.” *Journal of Mathematical Biology*, 32, pp. 857–863.

271 Bergh, O., Borsheim, K., Bratbak, G. and Heldal, M. (1989) “High abundance of viruses found in aquatic environments.” *Nature*, 340, pp. 467–468.

272 Letcher, Peter M, Lopez, Salvador, Schmieder, Robert, Lee, Philip a, et al. (2013) “Characterization of Amoeboaphelidium protococcarum, an algal parasite new to the cryptomycota isolated from an outdoor algal pond used for the production of biofuel.” *PloS one*, 8(2), p. e56232.

273 Perron, Marie-Claude and Juneau, Philippe (2011) “Effect of endocrine disrupters on photosystem II energy fluxes of green algae and cyanobacteria.” *Environmental Research*, 111, pp. 520–529.

274 EPA (2013) “Drinking Water Contaminants.” *National Primary Drinking Water Regulations*. [online] Available from: http://water.epa.gov/drink/contaminants/ (Accessed 19 August 2013)

275 US EPA (2013) “Nutrient Pollution.” [online] Available from: http://www2.epa.gov/nutrientpollution/problem (Accessed 8 October 2013)

276 Expert Group on Vitamins and Minerals (2003) *Risk assessment: Phosphorus*, Food Standards Agency. [online] Available from: http://multimedia.food.gov.uk/multimedia/pdfs/evm_phosphorous.pdf

277 National Institute of Environmental Health (2013) “Endocrine Disruptors.” (May). [online] Available from: http://www.niehs.nih.gov/health/materials/endocrine_disruptors_508.pdf (Accessed 19 August 2013)

278 US EPA (2013) “National Recommended Water Quality Criteria.” [online] Available from: http://water.epa.gov/scitech/swguidance/standards/criteria/current/index.cfm#U (Accessed 8 October 2013)

279 WHO Guidelines (1998) “Polynuclear aromatic hydrocarbons in Drinking-water,” in *Guidelines for drinking-water quality: Health criteria and other supporting information*, Geneva.

280 Howe, G and Merchant, S (1992) “Heavy Metal-Activated Synthesis of Peptides in Chlamydomonas reinhardtii.” *Plant physiology*, 98(1), pp. 127–36.

281 Wigmosta, MS, Coleman, AM, Skaggs, RJ, Huesemann, MH and Lane, LJ (2011) “National microalgae biofuel production potential and resource demand.” *Water Resources Research.*, 47(3), pp. 1–13.

282 Harmelen, Toon van and Oonk, Hans (2006) *Microalgae Biofixation Processes : Applications and Potential Contributions to Greenhouse Gas Mitigation Options*, TNO Built Environment and Geosciences, Apeldoorn.

283 European Commission (2012) “*New Commission proposal to minimise the climate impacts of biofuel production.*” [Press Release] 17 October.

284 IPCC (2013) *Working group I contribution to the IPCC fifth assessment report, Climate change 2013: the physical science basis*. [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J.

Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge UK and New York USA, Cambridge University Press

285 Poth, M and Focht, D D (1985) “N Kinetic Analysis of N(2)O Production by Nitrosomonas europaea: an Examination of Nitrifier Denitrification.” *Applied and environmental microbiology*, 49(5), pp. 1134–41.

286 Mampaey, K. E., Beuckels, B., Kampschreur, M. J., Kleerebezem, R., et al. (2013) “Modelling nitrous and nitric oxide emissions by autotrophic ammonia-oxidizing bacteria.” *Environmental Technology*, 34(12), pp. 1555–1566.

287 Sengupta, Sukalyan and Ergas, Sarina J. (2006) *Autotrophic Biological Denitrification with Elemental Sulfur or Hydrogen for Complete Removal of Nitrate-Nitrogen from a Septic System Wastewater*, Massachusetts.

288 Fagerstone, Kelly D, Quinn, Jason C, Bradley, Thomas H, De Long, Susan K and Marchese, Anthony J (2011) “Quantitative measurement of direct nitrous oxide emissions from microalgae cultivation.” *Environmental science & technology*, 45(21), pp. 9449–56.

289 Ferrón, Sara, Ho, David T, Johnson, Zackary I and Huntley, Mark E (2012) “Air-water fluxes of N_2O and CH_4 during microalgae (Staurosira sp.) cultivation in an open raceway pond.” *Environmental science & technology*, 46(19), pp. 10842–8.

290 Mulbry, Walter, Westhead, Elizabeth Kebede, Pizarro, Carolina and Sikora, Lawrence (2005) “Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer.” *Bioresource technology*, 96(4), pp. 451–8.

291 Frank, E. D., Han, J., Palou-Rivera, I., Elgowainy, A.. and Wang, M. Q. (2012) “Methane and nitrous oxide emissions affect the life-cycle analysis of algal biofuels.” *Environmental Research Letters*, 7(1), p. 014030.

292 Young, Ashton M. (2011) “Zeolite- Based Algae Biofilm Rotating Photobioreactor for Algae and Biomass Production.” *All Graduate Theses and Dissertations*, 986. [online] Available from: http://digitalcommons.usu.edu/etd/986

293 Bowmer, K H (1987) “Inhibition of algal photosynthesis to control pH and reduce ammonia volatilization from rice floodwater.” , 29(April 1986), pp. 13–29.

294 Yao, Wensheng and Millero, Frank J. (1995) “The chemistry of the anoxic waters in the Framvaren Fjord, Norway.” *Aquatic Geochemistry*, 1(1), pp. 53–88.

295 Ferrón, Sara, Ho, David T, Johnson, Zackary I and Huntley, Mark E (2012) “Air-water fluxes of N_2O and CH_4 during microalgae (Staurosira sp.) cultivation in an open raceway pond.” *Environmental science & technology*, 46(19), pp. 10842–8.

296 Karl, David M., Beversdorf, Lucas, Björkman, Karin M., Church, Matthew J., et al. (2008) “Aerobic production of methane in the sea.” *Nature Geoscience*, 1(7), pp. 473–478.

297 Keppler, Frank, Hamilton, John T G, Brass, Marc and Röckmann, Thomas (2006) “Methane emissions from terrestrial plants under aerobic conditions.” *Nature*, 439(7073), pp. 187–91.

298 Thorenz, U R, Kundel, M, Huang, R and Hoffmann, T (2012) “Trace analysis of short-lived iodine-containing volatiles emitted by different types of algae,” in *European Geosciences Union General Assembly*, Vienna, NASA, p. 202527.

299 Colomb, Aurélie, Yassaa, Noureddine, Williams, Jonathan, Peeken, Ilka and Lochte, Karin (2008) “Screening volatile organic compounds (VOCs) emissions from five marine phytoplankton species by head space gas chromatography/mass spectrometry (HS-GC/MS).” *Journal of environmental monitoring : JEM*, 10(3), pp. 325–30.

300 Carpenter, L J, Malin, G and Liss, P S (2000) “Novel biogenic iodine-containing trihalomethanes and other short-lived halocarbons in the coastal east Atlantic.” *Global Biogeochemical cycles*, 14(4), pp. 1191–1204.

301 Ballschmiter, Karlheinz (2003) “Pattern and sources of naturally produced organohalogens in the marine environment: biogenic formation of organohalogens.” *Chemosphere*, 52(2), pp. 313–24.

302 Ko, M.K.W., Poulet, G., Blake, D.R., Boucher, O., et al. (2003) “Very short-lived halogen and sulfur substances – Report No. 47,” in *Scientific Assessment of Ozone Depletion: 2002 Global Ozone Research and Monitoring Project*, Geneva.

303 Law, K.S., Sturges, W.T., Blake, D.R., Blake, N.J., et al. (2006) “Halogenated very short-lived substances – Report No. 50,” in *Scientific Assessment of Ozone Depletion: 2006 Global Ozone Research and Monitoring Project*, Geneva.

304 McFiggans, G., Coe, H., Burgess, R., Allan, J., et al. (2004) “Direct evidence for coastal iodine particles from Laminaria macroalgae - linkage to emissions of molecular iodine.” *Atmos. Chem. Phys. Discuss*, 4, pp. 939–967.

305 O’Dowd, Colin D., Jimenez, Jose L., Baherini, Roya, Flagan, Richard C., et al. (2002) “Marine aerosol formation from biogenic iodine emissions.” *Nature*, 417(June).

306 Matos, Cristina T, Gouveia, Luisa, Morais, Ana Rita C., Reis, Alberto and Bogel-Lukasik, Rafal Marcin (2013) “Green metrics evaluation of isoprene production by microalgae and bacteria.” *Green Chemistry*. [online] Available from: http://pubs.rsc.org/en/Content/ArticleLanding/2013/GC/c3gc40997j (Accessed 15 August 2013)

307 Kesselmeier, J and Staudt, M (1999) “Biogenic Volatile Organic Compounds (VOC): An Overview on Emission , Physiology and Ecology.” *Journal of Atmospheric Chemistry*, pp. 23–88.

308 Stone, D., Evans, M. J., Edwards, P. M., Commane, R., et al. (2011) “Isoprene oxidation mechanisms: measurements and modelling of OH and HO2 over a South-East Asian tropical rainforest during the OP3 field campaign.” *Atmospheric Chemistry and Physics*, 11(13), pp. 6749–6771.

309 Sanderson, M. G. (2003) “Effect of Climate Change on Isoprene Emissions and Surface Ozone Levels.” *Geophysical Research Letters*, 30(18), p. 1936.

310 Carlton, A. G., Wiedinmyer, C. and Kroll, J. H. (2009) “A review of Secondary Organic Aerosol (SOA) formation from isoprene.” *Atmospheric Chemistry and Physics Discussions*, 9(2), pp. 8261–8305.

311 Tai, Amos P. K., Mickley, Loretta J., Heald, Colette L. and Wu, Shiliang (2013) “Effect of CO2 inhibition on biogenic isoprene emission: Implications for air quality under 2000 to 2050 changes in climate, vegetation, and land use.” *Geophysical Research Letters*, 40(13), pp. 3479–3483.

312 Ferreira, J., Reeves, C. E., Murphy, J. G., Garcia-Carreras, L., et al. (2010) “Isoprene emissions modelling for West Africa: MEGAN model evaluation and sensitivity analysis.” *Atmospheric Chemistry and Physics*, 10(17), pp. 8453–8467.

313 Potter, Christopher S., Alexander, Susan E., Coughlan, Joseph C. and Klooster, Steven a. (2001) “Modeling biogenic emissions of isoprene: exploration of model drivers, climate control algorithms, and use of global satellite observations.” *Atmospheric Environment*, 35(35), pp. 6151–6165.

314 Monson, R.K. and Holland, E.A. (2001) “Biospheric trace gas fluxes and their control over tropospheric chemistry.” *Annual Review Ecology Systems*, 32, pp. 547–576.

315 Bilanovic, Dragoljub, Andargatchew, Arsema, Kroeger, Tim and Shelef, Gedaliahu (2009) “Freshwater and marine microalgae sequestering of CO2 at different C and N concentrations – Response surface methodology analysis.” *Energy Conversion and Management*, 50(2), pp. 262–267.

316 Ashworth, Daniel J, Luo, L. and Yates, Scott R. (2013) “Pesticide Emissions from Soil - Fate and Predictability.” *Outlook on Pest Managment*, 4(1), pp. 4–7.

317 Smith, Val H, Sturm, Belinda S M, Denoyelles, Frank J and Billings, Sharon a (2010) “The ecology of algal biodiesel production.” *Trends in ecology & evolution*, 25(5), pp. 301–9.

318 Beatriz Hernández-Carlos, M. Marcela Gamboa-Angulo (2011) “Metabolites from freshwater aquatic microalgae and fungi as potential natural pesticides.” *Phytochemistry Reviews*, 10(2), pp. 261–286.

319 EEA (2004) “Environmental impacts: Ozone and Health.” [online] Available from: http://www.eea.europa.eu/maps/ozone/impacts/bad-for-agriculture (Accessed 16 March 2011)

320 Yoch, Duane C (2002) “Dimethylsulfoniopropionate: It’s sources, role in the marine food web and biological degradation to dimethylsulfide.” *Applied and environmental microbiology*, 68(12), pp. 5804–5815.

321 Chen, G, Davis, D, Kasibhatla, P, Bandy, A, et al. (1999) “A mass-balance/photochemical assessment of DMS sea-to-air flux as inferred from NASA GTE PEW-West A and B observations.” *Journal of Geophysical Research*, 104(D5), pp. 5471–5482.

322 Renard, Jean J, Calidonna, Sheryl E and Henley, Michael V (2004) “Fate of ammonia in the atmosphere--a review for applicability to hazardous releases.” *Journal of hazardous materials*, 108(1-2), pp. 29–60.

323 Ballschmiter, Karlheinz (2003) “Pattern and sources of naturally produced organohalogens in the marine environment: biogenic formation of organohalogens.” *Chemosphere*, 52(2), pp. 313–24.

324 Kamilli, Katharina, Ofner, Johannes, Zetzsch, Cornelius and Held, Andreas (2013) “Formation of halogen-induced secondary organic aerosol (XOA).” , 15, p. 14214.

325 International Standard Organisation (2006) “Environmental managment - Lifecycle assessment - Principles and framework.”

326 Baumann H. and A. Tillman (2004) *A Hitchikers Guide to LCA*, Lund, Studentlitteratur.

327 Andrae, Anders S. G. and Andersen, Otto (2010) “Life cycle assessments of consumer electronics — are they consistent?” *The International Journal of Life Cycle Assessment*, 15(8), pp. 827–836.

328 Ekvall, Tomas and Tillman, Anne-Marie (1997) “Open-loop recycling: Criteria for allocation procedures.” *The International Journal of Life Cycle Assessment*, 2(3), pp. 155–162.

329 Acquaye, Adolf, Wiedmann, Thomas, Feng, Kuishang, Crawford, Robert H, et al. (2011) “Identification of ‘carbon hot-spots’ and quantification of GHG intensities in the biodiesel supply chain using hybrid LCA and structural path analysis.” *Environmental science & technology*, 45(6), pp. 2471–8.

330 Clarens, Andres F, Resurreccion, Eleazer P, White, Mark a and Colosi, Lisa M (2010) “Environmental life cycle comparison of algae to other bioenergy feedstocks.” *Environmental science & technology*, 44(5), pp. 1813–9.

331 Seabra, Joaquim E. A., Macedo, Isaias C, Paulo, São, Faroni, Carlos E and Sarto, Celso A (2011) “Life cycle assessment of Brazilian sugarcane products: GHG emissions and energy use.” *Biofuels, Bioproducts and Biorefining*, 5, pp. 519–532.

332 Yee, Kian Fei, Tan, Kok Tat, Abdullah, Ahmad Zuhairi and Lee, Keat Teong (2009) “Life cycle assessment of palm biodiesel: Revealing facts and benefits for sustainability.” *Applied Energy*, 86, pp. S189–S196.

333 Silva Lora, Electo E., Escobar Palacio, José C., Rocha, Mateus H., Grillo Renó, Maria L., et al. (2011) “Issues to consider, existing tools and constraints in biofuels sustainability assessments.” *Energy*, 36(4), pp. 2097–2110.

334 Hou, Jian, Zhang, Peidong, Yuan, Xianzheng and Zheng, Yonghong (2011) “Life cycle assessment of biodiesel from soybean, jatropha and microalgae in China conditions.” *Renewable and Sustainable Energy Reviews*, 15(9), pp. 5081–5091.

335 Campbell, Peter K, Beer, Tom and Batten, David (2011) “Life cycle assessment of biodiesel production from microalgae in ponds.” *Bioresource technology*, 102(1), pp. 50–6.

336 Jorquera, Orlando, Kiperstok, Asher, Sales, Emerson a, Embiruçu, Marcelo and Ghirardi, Maria L (2010) “Comparative energy life-cycle analyses of microalgal biomass production in open ponds and photobioreactors.” *Bioresource technology*, 101(4), pp. 1406–13.

337 Razon, Luis F. and Tan, Raymond R. (2011) “Net energy analysis of the production of biodiesel and biogas from the microalgae: Haematococcus pluvialis and Nannochloropsis.” *Applied Energy*, 88(10), pp. 3507–3514.

338 Khoo, H H, Sharratt, P N, Das, P, Balasubramanian, R K, et al. (2011) “Life cycle energy and CO2 analysis of microalgae-to-biodiesel: preliminary results and comparisons.” *Bioresource technology*, 102(10), pp. 5800–7.

339 Stephenson, Anna L., Kazamia, Elena, Dennis, John S., Howe, Christopher J., et al. (2010) “Life-Cycle Assessment of Potential Algal Biodiesel Production in the United Kingdom: A Comparison of Raceways and Air-Lift Tubular Bioreactors.” *Energy & Fuels*, 24(7), pp. 4062–4077.

340 Handler, Robert M., Canter, Christina E., Kalnes, Tom N., Lupton, F. Stephen, et al. (2012) “Evaluation of environmental impacts from microalgae cultivation in open-air raceway ponds:

Analysis of the prior literature and investigation of wide variance in predicted impacts.” *Algal Research*, 1(1), pp. 83–92.

341 Yanfen, Liao, Zehao, Huang and Xiaoqian, Ma (2012) “Energy analysis and environmental impacts of microalgal biodiesel in China.” *Energy Policy*, 45, pp. 142–151.

342 Xu, Lixian, Wim Brilman, Derk W F, Withag, Jan a M, Brem, Gerrit and Kersten, Sascha (2011) “Assessment of a dry and a wet route for the production of biofuels from microalgae: energy balance analysis.” *Bioresource technology*, 102(8), pp. 5113–22.

343 Pleanjai, Somporn and Gheewala, Shabbir H. (2009) “Full chain energy analysis of biodiesel production from palm oil in Thailand.” *Applied Energy*, 86(2009), pp. S209–S214.

344 Swiss Centre for Lifecycle Inventories (2014) “Ecoinvent Data v2.0.”

345 CEC Knowledge Network (2012) “Methane Emissions.” *North American Power Plant Air Emissions*. [online] Available from: http://www2.cec.org/site/PPE/ch4emissions (Accessed 23 July 2013)

346 Jenneman, G E, Montgomery, a D and McInerney, M J (1986) “Method for detection of microorganisms that produce gaseous nitrogen oxides.” *Applied and environmental microbiology*, 51(4), pp. 776–80.

347 Tsiropoulos, I. (2010) “Energy and emissions assessment on dextrose production from corn starch: Life cycle principles and multiple allocations.”

348 Collet, Pierre, Hélias, Arnaud, Lardon, Laurent, Ras, Monique, et al. (2011) “Life-cycle assessment of microalgae culture coupled to biogas production.” *Bioresource technology*, 102(1), pp. 207–14.

349 Borde, I. and Levy, A. (2007) “Pnematic and Flash Drying,” in Mujumdar, A. S. (ed.), *Handbook of Industrial Drying*, USA, CRC Press, pp. 397–410.

350 Seabra, Joaquim E. A., Macedo, Isaias C, Chum, Helena L., Faroni, Carlos E and Sarto, Celso A (2011) “Life cycle assessment of Brazilian sugarcane products : GHG emissions and energy use.” *Biofuels, Bioproducts and Biorefining*, 5, pp. 519–532.

351 Mu, Dongyan, Seager, Thomas, Rao, P Suresh and Zhao, Fu (2010) “Comparative life cycle assessment of lignocellulosic ethanol production: biochemical versus thermochemical conversion.” *Environmental management*, 46(4), pp. 565–78.

352 Mintz, M., Han, J., Wang, M. and Saricks, C. (2010) *Well-to-Wheels Analysis of Landfill Gas-Based Pathways and Their Addition to the GREET Model*, Illinos.

353 Flesch, Thomas K., Desjardins, Raymond L. and Worth, Devon (2011) “Fugitive methane emissions from an agricultural biodigester.” *Biomass and Bioenergy*, 35(9), pp. 3927–3935.

354 Benemann, John, Woertz, Ian and Lundquist, Tryg (2012) “Life Cycle Assessment for Microalgae Oil Production.” *Disruptive Science and Technology*, 1(2), pp. 68–78.

355 Feng, Yujie, Li, Chao and Zhang, Dawei (2011) “Lipid production of Chlorella vulgaris cultured in artificial wastewater medium.” *Bioresource technology*, 102(1), pp. 101–5.

356 Pittman, Jon K, Dean, Andrew P and Osundeko, Olumayowa (2011) “The potential of sustainable algal biofuel production using wastewater resources.” *Bioresource technology*, 102(1), pp. 17–25.

357 Kim, Sunjin, Park, Jeong-eun, Cho, Yong-Beom and Hwang, Sun-Jin (2013) “Growth rate, organic carbon and nutrient removal rates of Chlorella sorokiniana in autotrophic, heterotrophic and mixotrophic conditions.” *Bioresource technology*, 144, pp. 8–13.

358 Smuts, J. (1973) *Holism and Evolution*, Connecticut, Greenwood Press.

359 Sparks, Emma S (2006) “From capability to concept: Fusion of systmes analysis techniques for derivation of future solider systems.”

360 Hitchins, Derek K (1992) *Putting systems to work*, Chichester, John Wiley & Sons.

361 Luthe, Tobias, Kägi, Thomas and Reger, Jan (2013) “A Systems Approach to Sustainable Technical Product Design.” *Journal of Industrial Ecology*, 17(4), pp. 605–617.

362 Sterling, Stephen (2003) “Whole systems thinking as a basis for paradigm change in eduction: explorations in the context of sustainability.”

363 Grasso, Domenico and Burkins, Melody Brown (2010) “Beyond Technology: The Holitic Advantage,” in Grasso, D. and Burkins, M. B. (eds.), *Holistic Engineering Education*, New York, NY, Springer New York, pp. 1–11.

364 Chapman, Jake (2004) *System failure - Why govenrments must learn to think differently* 2nd ed., London, DEMOS.

365 Buede, D. M. (2009) *The engineering design of systems: models and methods*, New Jersey, Wiley.

366 IET (2013) *“What is a Smart Grid?”* [Breifing note] Institute for Engineering and Technology, Stevenage

367 Horswill, M. S. and Plooy, A. M. (2008) “Auditory feedback influences perceived driving speeds.” *Perception*, 37(7), pp. 1037–43.

368 Evans, Leonard (1970) “Speed Estimation from a Moving Automobile.” *Ergonomics*, 13(2), pp. 219–230.

369 Rogers, E. M. (1962) *Diffusion of innovations*, New York, Free Press of Glencoe.

370 Bloom, Nicholas, Schankerman, Mark and Reenen, John Van (2010) *Identifying technology spillovers and product market*, Paris.

371 Qiu, Jiaping and Wan, Chi (n.d.) *Technology Spillovers and Corporate Cash Holdings*, Boston.

372 Woiceshyn, J. (2005) “Integrative capability and technology adoption: evidence from oil firms.” *Industrial and Corporate Change*, 14(2), pp. 307–342.

373 Garcia, Marie L and Bray, Olin H (1997) *Fundamentals of Technology Roadmapping*, Alberquerque.

374 ADEME (2009) *Road Map for Second-Generation Biofuels*, France.

375 Londo, M., Deurwaader, E. and Lensink, S. (n.d.) “REFUEL: An EU road map for biofuels.”

376 US Department for Energy (2010) *National Algal Biofuels Technology Roadmap*, Maryland. [online] Available from: http://biomass.energy.gov

377 FAPSEP (2014) “BIOEN Program.” [online] Available from: http://bioenfapesp.org/index.php (Accessed 2 July 2014)

378 TechSci Research (2014) *Brazil Waste Water Treatment Plants Market & Forecast Opportunities, 2018*, Peterborough, UK. [online] Available from: http://www.slideshare.net/TechSci_Research/brazil-waste-water-treatment-plants-market-forecast-opportunities-2018

379 IBGE (2010) *Pesquisa nacional de saneamento basico*, Institute Brasileiro de Geografia e Estaistica, Rio de Janeiro.

380 Lane, Jim (2012) “Petrobras: Biofuels Digest’s 5-Minute Guide.” *BiofuelsDigest*. [online] Available from: www.biofuelsdigest.com/bdigest/2012/11/08/petrobras-biofuels-digests-5-minute-guide/ (Accessed 30 June 2014)

381 Sreeharsha, Vinod (2014) “Brazilian biodiesel bimonthly auction moves 463.8 million liters.” *Platts, McGraw Hill Financial*. [online] Available from: http://www.platts.com/latest-news/agriculture/saopaulo/update-brazilian-biodiesel-bimonthly-auction-21470262 (Accessed 30 June 2014)

382 UFES (2014) “Microalgae growth with wastewater effluent and different CO2 sources,” in *4th International Conference on Algal Biomass, Biofuels and Bioproducts*, Santa Fe NM, Elsevier.

383 Mezzari, M.P., Pirolli, M., Prandini, J.M., Nunes, E.O., et al. (2014) “Biomass harvest and bioethanol production from Chlorella vulgaris treating swine wastewater,” in *4th International Conference on Algal Biomass, Biofuels and Bioproducts2*, Santa Fe NM, Elsevier.

384 United Nations (2005) *Report of the Conference of the Parties serving as the meeting of the Parties to the Kyoto Protocol*, Montreal. [online] Available from: http://unfccc.int/resource/docs/2005/cmp1/eng/08a03.pdf

385 Meacham, Carl (2014) “Surprised? We May Finally See an EU-Mercosur Trade Agreement.” *Centre for Strategic and International Studies*, [online]. Available from: http://csis.org/publication/surprised-we-may-finally-see-eu-mercosur-trade-agreement (Accessed 25th April 2014).

386 Delegation of the European Union to Brazil (2014) “List of Projects.” *EEAS*. [online] Available from: http://eeas.europa.eu/delegations/brazil/projects/list_of_projects (Accessed 7 July 2014)

387 Gilbertson, Tamra and Reyes, Oscar (2009) *Carbon Trading - How it worksand why it fails*, Critical currents no. 7, Dag Hammarskjold Foundation, Uppsala.

388 BNDES (2013) *Amazon Fund Acitivity Report 2013*, Brazil.

389 Noblet, CL, Lindenfeld, LA and Anderson, MW (2013) “Environmental Worldviews: A Point of Common Contact, or Barrier?” *Sustainability*, 5(11), pp. 4825–4842.

390 Mabon, Leslie, Shackley, Simon and Bower-Bir, Nathan (2014) “Perceptions of sub-seabed carbon dioxide storage in Scotland and implications for policy: A qualitative study.” *Marine Policy*, 45, pp. 9–15.

391 Weyer, Kristina M., Bush, Daniel R., Darzins, Al and Willson, Bryan D. (2009) “Theoretical Maximum Algal Oil Production.” *BioEnergy Research*, 3(2), pp. 204–213.

392 Borowitzka, Michael A. (1990) “A Mass Culture of Dunaliella salina.” *Technical resource papers*, p. 180. [online] Available from: http://www.fao.org/docrep/field/003/ab728e/ab728e06.htm

393 He, H Y, Guo, X and Zhu, S L (2006) “Comparison of Membrane Extraction with Traditional Extraction Methods for Biodiesel Production.” *JAOCS*, 83(5).

394 BioGrace (2011) “Biofuel GHG calculation Tool (Version 4b, Public).”

395 Parlimentary Office of Science and Technology (2011) “Carbon Footprint of Electricity Generation.”

396 CEC Knowledge Network (2011) *North American Power Plant Air Emissions*, Quebec. [online] Available from: http://www2.cec.org/site/PPE/ch4emissions?page=51&order=field_plant_total_emissions_value&sort=asc

397 EPA (2014) *“Emission Factors for Greenhouse Gas Inventories.”* [online] Available from: http://www.epa.gov/climateleadership/documents/emission-factors.pdf (Accessed 8 July 2014)

398 Biomass Energy Centre (2011) “Carbon emissions of different fuels.” [online] Available from: http://www.biomassenergycentre.org.uk/portal/page?_pageid=75,163182&_dad=portal&_schema=PORTAL (Accessed 6 August 2013)

399 NERC (2013) “*A UK Roadmap for Algal Technologies”* NERC-TSB, London.

Appendix A A selection of current microalgae development projects (commercial)

Algae	**Pond type**	**Product**	**Location**	**Source**
Dunalialla	Closed (PBR)	β-carotene	Israel	*nikken-miho.com*
Dunaliella	Open (Raceway)	β-carotene	Australia	*[392]*
Haematococcus	Open (Raceway)	Astaxanthin	Israel	*algatech.com*
Haematococcus/ Spirulina	Open (Raceway)	Astaxanthin/ Dietary supplement	Hawaii	*cyanotech.com*
Haematococcus	Closed (PBR)	Astaxanthin	Sweden	*bioreal.se*
Spirulina	Open (Raceway)	Dietary supplement	California	*earthrise.com*
Spirulina/Chlorella	Open (Centre Pivot Ponds)	Dietary supplement	Taiwan	*wilson-groups.com*
Chlorella	Closed (PBR)	Dietary supplement	Klötze, Germany	*algomed.de*
Cyanobacteria	Closed (PBR)	Ethanol, diesel, jet fuel (0.46m litres/yr)	USA (Florida)	*algenolbiofuels.com*
Unknown	Closed (Cultivation Bags)	Jet fuel (4.5m litres/yr)	USA (New Mexico)	*sapphireenergy.com*
Unknown	Closed (Heterotrophic)	Biodiesel (>0.68m litres/yr)	Brazil	*solazyme.com*

Appendix B Microalgae Composition

Microalgal biochemical composition on a dry basis, with the error given as a standard deviation from the mean, calculated from duplicate experiments.

	Lipid	Protein	Carbs	Ash	Total
HBM Glucose	22.54	37.22	24.11	7.27	91.13
HBM Crude Glycerol	39.69	6.42	22.64	9.58	78.32
HBM Molasses	18.84	34.55	19.21	11.31	83.91
SWW Glucose	12.24	20.27	21.55	7.52	61.58
SWW Crude Glycerol	46.96	20.24	12.52	9.58	89.30
SWW Molasses	16.15	19.25	13.64	11.45	60.49
HBM Crude High C	53.42	10.96	21.25	9.58	95.21
SWW Crude High C	25.10	9.69	23.94	9.58	68.32
Error (Standard Deviation)					***Total + error***
HBM Glucose	8.88	0.72	1.96		*102.68*
HBM Crude Glycerol	7.50		0.56		*87.10*
HBM Molasses	8.59		2.31		*95.52*
SWW Glucose	4.09		0.56		*66.94*
SWW Crude Glycerol	13.73		1.11		*104.86*
SWW Molasses	8.39		4.97		*74.58*
HBM Crude High C	8.72	1.22	1.14		*106.29*
SWW Crude High C	2.11		5.82		*77.48*

Appendix C Lifecycle Inventory

C.1 Life cycle Inventory 1: Inputs

Stage	Parameter	Data entry	Choice/units	Variable Input	Notes
Heterotrophic Cultivation					
Algae	Water	Value	l	6.60	*Experimental work*
Algae	Growth Rate	Value	g/l/day	1.01	*Experimental work*
Algae	Lipid Content (DW)	Value	%	0.22	*Experimental work*
Algae	Cultivation period	Value	hours	84	[149]
Nutrients	Aeration - pumping energy	Value	MJ/kg DW	1.46	
Nutrients	N requirement	Value	g/kg biomass	46.0	[148]
Nutrients	P Requirement	Value	g/kg biomass	9.9	[148]
Nutrients	K requirement	Value	g/kg biomass	8.2	[148]
Nutrients	Mg Re	Value	g/kg biomass	3.8	[148]
Nutrients	S Requirement	Value	g/kg biomass	2.2	[149]
Nutrients	Yeast	Value	g/l	1.5	[348]
Nutrients	Carbon Source	Choice	glucose, crude, glycerol	Glucose	*Experimental work*
Nutrients	Carbon requirement	Value	g/l	10.0	*Experimental work*
Nutrients	Media Type	Choice	HBM, SWW	HBM	*Experimental work*
Harvesting and Drying					
De-watering	Flow out of settlers	Value	m^3	0.1	[348]
De-watering	Pumping out of settlers	Value	kwh	0.153	[348]
Drying	Pump to settlers	Value	MJ/kg	0.2	[348]
Flash drying	Flash Drying	Value	MJ/kg water evaporated	3.5	[349]
Lipid Extraction					
Extraction	Volume required	Value	kg/kg oil	0.015	[148]
Extraction	Homogenisation	Value	MJ/kg biodiesel	8	[339]
Extraction	Extraction efficiency	Value	%	95%	[17,148,339]
Extraction	Solvent	Choice	Hexane, Folch, SME	Hexane	[17,148,339]
Extraction	Solvent lost	Value	g/kg biodiesel	50%	[339]
Extraction	Energy Input	Value	kwh/kg oil	1.7	[17,148,339]
Extraction	Electricity Input	Value	kwh/kg oil	0.54	[17,148,339]
Refining	Heat Input	Value	MJ/kg biodiesel	0.6	[339]
Refining	Electricity Input	Value	MJ/kg biodiesel	0.1	[339]
Transesterification					
Conversion	TE method	Choice	Indirect (ID), In situ (IS)	ID	*Experimental work*
Conversion	Methanol requirement	Value	kg/kg biodiesel	0.79	*Experimental work*
Conversion	Catalyst	Choice	H_2SO_4, HCl	H_2SO_4	*Experimental work*
Conversion	Catalyst requirement	Value	% MeOH volume	1%	*Experimental work*
Conversion	Washing	Value	litres/ litres biodiesel	30%	*Experimental work*
Energy	Oil energy content	Value	MJ/kg	35	*Experimental work*
Losses	Transesterification yield	Value	%	98.8	*Experimental work*
Losses	Refinery losses	Value	%	15	[393]
Biogas					
Energy	HHV CH_4	Value	MJ/m^3	39	[19]
Energy	Electricity	Value	kwh/mm^3 raw biogas	0.25	[14]
Energy	CH_4 content of biogas	Value	%	90%	[14]
Energy	Yield	Value	m^3/kg dry biomass	0.15	[14]

C.2 Lifecycle Inventory 2: Primary energy and GHG factors

Input	Unit	Value	Source	CO_2 Factor kg CO_2/unit input	Source	CH_4 Factor kg CH_4/unit input	Source	N_2O Factor kg N_2O/unit input	Source
Cultivation									
Nutrients									
- Carbon substrate (glucose)	MJ/kg	6.4	[347]	0.965	[348]				
- Nitrogen	MJ/kg	65	[348]	2.827	[394]	0.01	[348]	0.010	[348]
- Potassium	MJ/kg	17.3	[348]	0.536	[348]	0.00	[348]		
- Phosphorus	MJ/kg	13.6	[348]	0.965	[348]	0.00	[348]		
Aeration Pump									
- Electricity	kwh	0.2	[348]	0.0097	[394]	0.0002	[394]		
Water									
- Pump	kwh/ha	2.4	[348]	0.0097	[394]	0.0002	[394]		
- Water cleaning	MJ/m^3	0.036	[148]	0.0097	[394]	0.0002	[394]		
Pump to settlers									
- Electricity consumption	MJ/kg	0.2	[348]	1.800	[394]	0.0002	[394]	0.0000108	[394]
Extraction									
- **Homogenisation**	MJ/kg biodiesel	0.1	[339]	1.800	[395]	0.0002	[345]	0.0000145	[396]
- **Natural Gas**	kwh/kg oil	6.12	[17]	0.075	[395]	0.0002	[345]	0.0000108	[396]
- **Electricity**	kwh/kg oil	1.944	[397]	1.800	[395]	0.0002	[345]		
- **Hexane**	MJ/kg hexane	0.52	[17]	0.543	[395]	0.2824		0.0000145	[396]
Transesterification									
Raw materials									
- Methanol	MJ/kg	30.28	[398]	0.7141	[397]				
Utilities									
Water	MJ/l	0.036	[148]	0.0097	[394]				
Electricity (heat)	MJ/kg	0.9	[339]	1.800	[395]	0.0097		0.0000108	[396]

Input	**Unit**	**Value**	**Source**	**CO_2 Factor** kg CO_2/unit input	**Source**	**CH_4 Factor** kg CH_4/unit input	**Source**	**N_2O Factor** kg N_2O/unit input	**Source**
Biogas Production									
Biogas yield	m^3/kg TS	0.3	[17]			0.0006	[17]		
Methane energy yield	MJ/m^3	39	[17]						
Utilities									
Electricity (digester mixing)	MJ/kg algae	0.3888	[339]	1.8	[395]	0.0002	[345]	0.0000108	[396]
Electricity (centrifugation of digestates)	MJ/kg algae	0.0907	[348]	1.8	[395]	0.0002	[345]	0.0000108	[396]
- **Purification**									
- Electricity consumption	MJ/m^3 upgraded	1.0836	[348]	1.8	[395]	0.0002	[345]	0.0000108	[396]
Water consumption	m^3	0.067	[348]						

C.3 Global warming potential (GWP) factors

Factors taken from the IPCC Fifth Assessment Report [284] and were determined by calculating the amount of CO_2 that would cause the same integrated radiative forcing over the given time horizon.

Gas	Unit	GWP
Methane	kg eq CO_2/kg CH_4	84
Nitrous Oxide	kg eq CO_2/kg N_2O	264

C.4 GHG emissions from scenarios S1-S6

Emissions are cumulative of the biodiesel production process as described by the methodology in section 7.2, and include CO_2, N_2O and CH_4, and converted to CO_2eq using the GWP factors given in Appendix C.3.

Scenario	Total GHG emissions (kg CO_2 eq)
S1	19.62
S2	13.89
S3	13.07
S4	19.72
S5	13.92
S6	13.09