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WITWATERSRAND,
JOHANNESBURG

Algae Biomass Cultivation in Fish Farm Effluent for Biodiesel Production

Uzochukwu Onyinyechukwu Enwereuzoh

1580825

A thesis submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Science in Engineering.

Johannesburg, 2021

Declaration

I declare that this thesis is my unaided work. It is being submitted for the degree Doctor of Philosophy in Engineering to the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination to any other University.

.....

Uzochukwu Onyinyechukwu Enwereuzoh

..... day of ..... year .....

Dedication

To my loving and supporting wife, Precious Adaku Enwereuzoh

To my kids, Chizaram Enwereuzoh and Ziora Enwereuzoh

To my parents Cyprain Enwereuzoh and Victoria Enwereuzoh

To the rest of my family, Chinenyeze, Nneka, Uche, Ijeoma, Ozioma, Kachi and kids

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I would like to express my profound gratitude to all with a mention of a few.

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Abstract

Microalgae research is driven by the need for alternative fuels for reasons such as climate change mitigation, depleting fossil fuel reserves, increased energy demand and economic growth. Some setbacks of producing commercial microalgae biodiesel include purchasing nutrients, the supply of large volumes of water, low biomass productivity, low lipid accumulation and desirable fatty acid composition. However, the increased cost of microalgae cultivation is influenced by the high cost of inorganic nutrients and large volumes of water required for propagation.

On the other hand, the use of fertiliser for the production of microalgae may be counterproductive, as the production of fertiliser through conventional technologies release carbon dioxide. These purchased synthetic nutrients can be replaced by nutrients in different agricultural wastes, for example. One type of suitable agricultural waste with suitable nutrient concentration for cultivating microalgae biomass is the aquaculture wastewater. Different studies have shown that nutrients required for microalgae cultivation such as ammonia, nitrate and phosphates are in a suitable range in aquaculture wastewater. These nutrients have supported the cultivation of microalgae in aquaculture wastewater for different purposes.

Previous studies in microalgae wastewater treatment have focused on cultivating microalgae in other sources of wastewater, with little studies on using aquaculture wastewater. The few studies using fish farm wastewater (FFW) have focused on growing microalgae for biomass production and nutrient removal with minimal research on the composition of the biomass obtained, biodiesel production and properties of biodiesel obtained, the economic and environmental implication of using FFW nutrients instead of purchased nutrients. The gap in using FFW for microalgae biodiesel production is the reason this research is aimed at reducing the cost of microalgae biomass cultivation for biodiesel production, by recycling nitrogen and phosphorus in nutrient-rich fish farm effluent which is released in a form that is

preferred by microalgae (that is NH_3 for nitrogen and PO_4^{-3} for phosphate) for microalgae growth. Therefore, instead of purchasing fertilizer, those nutrients from farm effluent were exploited in the cultivation of microalgae for biodiesel production. As a result, this supplied nutrients and large volumes of water required for microalgae cultivation treated the FFW by removing these nutrients and mitigate the environmental challenges of discharging nutrient-rich effluents.

Three high lipid accumulating algae species, according to other studies, were used in this research work (*Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii*). Each species was cultivated in a Tris-acetate phosphate (TAP) standard growth media and fish farm effluent at the same conditions. The specific growth rate, biomass yield, biomass productivity, lipid content, lipid productivity, carbohydrate content, carbohydrate productivity, protein content, protein productivity, rate of CO_2 uptake and carbon content were determined.

The biodiesel was produced from microalgae species that were cultivated in the standard growth medium in addition to modified and unmodified fish effluent. The same species were also grown in modified (low nutrient) fish farm effluent to compare fuel properties and level of accumulation of protein and carbohydrate with those obtained in unmodified fish farm effluent. The fatty acid methyl ester composition of the biodiesel aided in determining the properties of the produced biodiesel (cetane number, iodine value, cold filter plugging point, kinematic viscosity, density and degree of saturation). A theoretical cost estimation and a life cycle assessment was used to determine the environmental benefits of using fish farm effluent nutrients for the cultivation of microalgae feedstock for biodiesel production.

Higher growth, biomass yield, lipid content and lipid productivity was obtained in microalgae species cultivated in FFW. Microalgae grown in FFW had better uptake of CO_2 than the same species cultivated in the standard growth media. Comparable fuel property was obtained

across different growth media. Fuel properties had characteristics within specifications of both ASTM D6751-02 and EN 14214 standards. *Chlamydomonas reinhardtii* in all culture media had better CFPP property and is more suited for cold climates with freezing temperatures. FFW growth media had a lesser environmental and cost impact which came mostly from electricity used for supply of light and CO_2 gas supply.

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Nomenclature

1	*Tetradesmus obliquus*
2	*Heterochlorella luteoviridis*
3	*Chlamydomonas reinhardtii*
CFPP	Cold flow pour point
CN	Cetane number
DU	Degree of unsaturation
F	Unmodified fish farm wastewater
FA	Fatty acids
FAME	Fatty acid methyl esters
FFW	Fish farm wastewater
HHV	High heating value
IV	Iodine value
LCSF	Long chain saturated factor
LN	Modified fish farm wastewater
MSFA	Monosaturated fatty acid
PSFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid
T	Tris-Acetate-Phosphate standard

TAG	Triacylglycerol
TAP	Tris-Acetate-Phosphate
υ	Density (mm^2/s)
ρ	Kinematic viscocity (g/cm^3)

Chapter 1 Introduction

This chapter provides an overview of the work presented in this thesis.

1.1 Background

The need for a renewable biofuel replacement for fossil fuels has been spurred by different challenges facing the use of fossil fuel such as global warming effects from excessive release of carbon dioxide a major greenhouse gas into the atmosphere and air pollution (Sheehan et al., 1998), dwindling fossil fuel reserves like coal, oil and natural gas has been reported to run out in 122-, 42- and 66 years respectively (Islam et al., 2014). In addition to the need for energy security, foreign exchange savings and other socioeconomic factors relating to rural development (Demirbas, 2007).

The use of diesel vehicles in construction, commercial freight, and maintenance of infrastructure gives them unique importance as they play numerous roles in different sectors of the economy (Johnston and Holloway, 2007). There is a lot of focus on developing sustainable biofuels with the EU and other major developed countries having a similar ambitious goal of replacing 10% of all transport fuels with biofuels (Rösch and Skarka, 2009). A combination of waste cooking oil and fats plus biofuels obtained from traditional oil crops such as rapeseed, soybean oil, sunflower cannot meet up global needs for transport fuels (Chisti et al., 2007; Tyson et al., 2004). Therefore, biodiesel from microalga oil might be one of the main biofuel feedstock that can sustainably meet the growing needs of transport fuel (Chisti et al., 2007; Schenk et al., 2008).

Most existing large scale cultivation, harvesting, and processing of algae around the globe are used for high-value food additives instead of low-cost transport fuels (Passell et al., 2013). Large scale production of biodiesel requires enormous biomass through mass cultivation of microalgae (Gong and Jiang, 2011). The main parameters for efficient production of microalgae biodiesel production are using efficient strains, cultivation systems, and biomass production (Maity et al., 2014). It is unlikely for a particular algae species to possess the best

features for the production of biodiesel in all conditions (Simionato et al., 2013). The major parameters for consideration in selecting microalgae for commercial scale-up are biomass and lipid productivity, lipid composition including quality and quantity (Huerlimann et al., 2010). Currently, biodiesel is not economically feasible and requires more research and development to be commercially practicable (Atabani et al., 2012).

The cost of raw materials (oil and fats) constitute about 70% of biodiesel production cost (Behzadi et al., 2007). The main cost include the cost of nutrient supplements and large volumes of water, biomass harvest, oil extraction from biomass and cost of extra illumination to enhance photosynthesis and increase growth (Cho et al., 2013). A proposed method for cost reduction of biofuels from algal biomass is to combine production with wastewater treatment, animal feeds, and high-value products. The direct production cost approximation of some species of algae biomass dry cell weight is as follows: $10,000/t for Spirulina, $20,000/t for *Dunaliella*, $20,000/t for *Chlorella* and $200,000/t *Haematococus* (Benemann, 2013).

Studies have reported on the cultivation of different aquatic plants including algae using different farm effluents such as fish farm, poultry, and pig for different purposes like protein recovery, nutrient removal and municipal wastewater treatment. Previous studies have not reported the cultivation of algae specifically for biodiesel production, by optimizing algae growth conditions to maximise biodiesel production from algae cultivated in fish farm effluent coupled with CO_2 sequestration.

More specifically, in aquaculture production the fastest growing food production in the world there are numerous environmental issues associated with it. This includes the consumption of large volumes of water and large volumes of untreated discharge (Biao et al., 2004; Biao and Kaijin, 2007). The dependence of farmed fish, which supplies close to half of the fish directly

consumed by humans (Naylor et al., 2000), could increase if there is an increasing dependence on it (for example increasing world population) resulting in increased effluent. The large volumes of untreated discharge can be used in growing algae for biodiesel production, as growing algae in nutrient-rich wastewater may perform removal of nitrogen and phosphorus consequently reducing nutrient loads of the receiving water (Sturm and Lamer, 2011).

In this thesis, the focus is on using the best conditions of maximum lipid yield for biodiesel production from algae cultivated on fish farm waste with additional CO_2.

1.2 Research Questions

The work presented here will comprise of theoretical and experimental analysis, to answer the following research questions:

1. Will the supply of cheap and available nutrients in commercial fish farm effluent reduce the cost of algae cultivation for biodiesel production?
2. Are there sufficient nutrients (nitrogen and phosphorus) in commercial fish farm effluent for the cultivation of algae?
3. Can algae significantly deplete nitrogen and phosphorus in commercial fish farm effluent?
4. Will the cultivation of algae in nitrogen-rich effluent enhance the growth of algae?
5. Will the production of biodiesel from algae cultivated in commercial fish farm effluent have a lower environmental and lower economic impact on the overall process?

1.3 Aim, Objectives, and Contribution to Knowledge

This research aims to reduce the cost of cultivating algae, as a feedstock for biodiesel production, by reducing the cost of growth medium nutrients. This will be investigated by growing algae in nutrient-rich fish farm effluent. One of the major challenges facing large scale algae cultivation is a large amount of nitrogen and phosphorus fertilizer required for microalgae cultivation. This makes biodiesel from algae expensive and uncompetitive with fossil diesel despite the promises algae feedstock offers due to high lipid content and high productivity (Sialve et al., 2009). The objectives of this research are to:

- Exploit available nitrogen and phosphorus in commercial fish farm effluent as a cheap and available source of nutrients for algal biomass cultivation.
- Use three algae species to compare:
    - the rate of nutrient (nitrogen and phosphorus) removal.
    - algal growth and lipid accumulation under optimized lipid accumulation conditions such as low nitrogen concentrations, low temperature and high intensity of light.
- Determine the rate of CO_2 use by the three different algae species.
- Assess the economic and environmental effects of algae cultivation in fish farm effluent, including the biodiesel production system.
- Assess the quality of the biodiesel produced from algae cultivated in fish farm effluent medium.

The novelty of this research is the optimal cultivation of algae biomass in fish farm effluent, for maximum lipid accumulation for biodiesel production, coupled with wastewater treatment (ammonia, nitrogen and phosphorus removal) and CO_2 mitigation.

1.4 Thesis Layout

This thesis is arranged into six chapters including the introduction. The background and motivation, as well as the research goals of the thesis, were presented in the introduction chapter. Every chapter has a reference list of its own.

The reader will see that there is a summary of scientific literature relevant to the work of that particular chapter in each of the chapters three to five. The first and sixth chapters are written as regular chapters of the thesis. The explanation for this format is because the work is presented as a publication paper for each chapter. Chapters two to five can be read as an independent research paper. Therefore, chapters two four and five would have some relationship. However, each chapter will provide input to the overall goal of the thesis. A brief description of the content of each chapter is set out below:

Chapter 1: Introduction

This chapter introduces the research and contains the background, research questions, aim and objectives. It also contains a description of the thesis layout.

Chapter 2: Fish farm effluent as a nutrient source for algae biomass cultivation

In this chapter, a literature review of microalgae cultivation in fish farm wastewater (FFW) is provided.

Chapter 3: Microalgae cultivation using nutrients in fish farm effluent for biodiesel production

This chapter exploited nutrients in FFW for microalgae cultivation, modified FFW (lowered nutrient concentration in FFW) to improve lipid productivity. It also analysed the

biochemical compositions (lipid, carbohydrate, and protein) of the microalgae obtained. Furthermore, it determined the effects of CO_2 supply and uptake by cultivated microalgae.

Chapter 4: Biodiesel production from microalgae cultivated in fish farm wastewater

The growth media composition and environmental condition of algae cultivation affect the composition of the algae biomass feedstock. The composition of the feedstock determines the properties of fuel obtained from the feedstock. In this chapter, direct transesterification of microalgae biomass, analysis of FAME and determination of fuel properties from FAME was carried out to determine the effect of FFW nutrients on fuel properties obtained from the cultivated algae.

Chapter 5: Environmental and cost effects of algae cultivation in fish farm effluent

The environmental impact of growing algae in FFW was evaluated in this chapter with a life cycle assessment to determine the environmental benefits of using nutrients in FFW for microalgae biomass production and economic benefits also contained.

Chapter 6: Conclusion

This chapter contains an overall discussion of the major findings relating to the objectives of the research presented in chapters three to chapter five. This chapter also contains recommendations for future studies.

1.5 References

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Chapter 2 Fish Farm Effluent as a Nutrient Source for Algae Biomass Cultivation

This chapter has been accepted for publication by South African Journal of Sciences. An abbreviated form of this chapter was presented orally at the 8th cross-faculty postgraduate symposium at the University of the Witwatersrand, Johannesburg. 23 to 24th October,2017.

The contribution of the co-author's Professor Kevin Harding and Dr. Michelle Low towards the work is primarily that of supervision or collaboration, and the work and write-up were carried out by the author of this thesis.

This chapter aims to review the use of microalgae for biodiesel, identify the nutrients required for microalgae growth and their availability in FFW. It also gives a summary of current research that utilised FFW for microalgae cultivation for different applications.

Abstract

Microalgae biotechnology has gained significant importance in recent decades. However, one of the challenges of microalgae biotechnology is the cost of nutrients. The use of residual nutrient and nutrient recycling could overcome the high cost of algae biomass production, a major drawback in algae biotechnology in biodiesel production. Moreover, microalgae cultivation consumes enormous quantities of fertilisers; more than other oil crops. The cost of synthetic fertilizers traditionally used in mass cultivation of microalgae is associated with rising prices of crude oil and their usage for microalgae cultivation might also compete with fertilizers for traditional agriculture. The fact that fish farm wastewater (FFW) nutrients are released in the form preferred by microalgae (NH_3 for nitrogen and PO_4^{-3} for phosphate) and the ability of microalgae to use nitrogen from different sources, can be exploited by using fish farm effluent rich in nutrients (nitrogen and phosphorus) in the cultivation of microalgae biomass for production of biodiesel. The cultivation of algae biomass in FFW will serve the dual strategy of wastewater treatment and CO_2 sequestration. This chapter reviewed the benefits of fish effluent in algae cultivation, algae nutrient requirements, and cultivation methods. The ability of microalgae to utilise nutrients in FFW has been demonstrated in different applications and has added benefits of treating wastewater and reducing freshwater demand.

Keywords: Fish farm effluent; microalgae; biodiesel; nitrogen; phosphorus; carbon dioxide

Background

Algae are photosynthetic aquatic organisms. Algae has a similar photosynthetic mechanism as land-based plants, but have a simple cellular structure (Gouveia, 2011). According to Falkowski and Raven (1997) and Hu et al (2008), algae represent very diverse, yet a highly specialised group of micro-organisms that live in different ecological habitats like freshwater, marine, brackish and hypersaline, with unique nutrient availability, and a range of pH and temperature. In Hoek et al (1995), algae are classified in multiple major groupings as follows: green algae (*Chlorophyceae*), cyanobacteria (*Cyanophyceae*), yellow-green algae (*Xanthophyceae*), diatoms (*Bacillariophyceae*), golden algae (*Chrysophyceae*), brown algae (*Phaeophyceae*), red algae (*Rhodophyceae*), 'pico-plankton' (*Prasinophyceae* and *Eustigmatophyceae*) and dinoflagellates (*Dinophyceae*).

The composition and the amount of oil vary between microalgae species (Chisti, 2007; Griffiths et al., 2012, 2011; Purkayastha et al., 2017). The amount of oil is represented in Table 2.1. Some species of microalgae such as *Dunaliella Salina, Chlamydomonas reinhardtii, Chlorella* and *Botryococcus braunii* can contain more than 60% lipid of dry cell weight (Scott et al., 2010). For microalgae species with high lipid accumulation of 50 -70% of dry cell weight such as *Botryococcus braunii* generally have a slow growth rate due to gene composition and physiological conditions (Banerjee et al., 2002). *Nannochloropsis oculata* and *Chlorella vulgaris* are two examples of fast-growing and high lipid accumulation microalgae that have been used for biodiesel production (Converti et al., 2009).

Table 2.1 The oil content of some microalgae

Microalga	Oil content (% dry wt)	Source
Achnanthes sp.	42.8 – 46.2	(Doan et al., 2011)
Ankistrodes falcatus	21.78 – 59.6	(Singh et al., 2015)
Chorella Sorokiniana	26 – 39.1	(Guldhe et al., 2017)
Crypthecodinium cohnii	20	(Chisti, 2007)
Cryptomomas sp	28 – 29.2	(Doan et al., 2011)
Cylindrotheca sp.	16–37	(Chisti, 2007)
Dunaliella primolecta	23	(Chisti, 2007)
Isochrysis sp.	25–33	(Chisti, 2007)
Monallanthus salina N	20	(Chisti, 2007)
Nannochloris sp.	20–35	(Chisti, 2007)
Nannochloropsis sp.	37.6 -46.5	(Doan et al., 2011)
Neochloris oleoabundans	35–54	(Chisti, 2007)
Nitzschia sp.	45–47	(Chisti, 2007)
Phaeodactylum tricornutum	20–30	(Chisti, 2007)
Schizochytrium sp.	50–77	(Chisti, 2007)
Tetraselmis sueica	15–23	(Chisti, 2007)

The ability of microalgae to adapt in a diverse environment is reflected in exceptional different patterns of lipid as well as their ability to synthesise different unusual compounds (Guschina and Harwood, 2006). Kinetics of microalgae growth, lipid productivities and amount of biomass varies with the algal strain, culture and physiological conditions (Purkayastha et al., 2017). Cessation of cell division of microalgae occurs under environmental stress conditions switching the synthesis of CO_2 to lipid as energy storage thereby increasing lipid content (Xin et al., 2010b). Microalgae can be grown to accumulate more lipids in different culture conditions (Hsieh and Wu, 2009; Hu et al., 2008; Miao and Wu, 2004; Purkayastha et al., 2017) between 20–50% dry cell weight (DCW) of mostly triacylglycerol (TAG)(Hu et al., 2008). Some of the environmental stress conditions are;

- low nitrogen concentrations (Illman et al., 2000; Piorreck et al., 1984);
- at low temperature (Renaud et al., 2002);
- high intensity of light (Khotimchenko and Yakovleva, 2005); and
- high ion concentrations (Liu et al., 2008).

2.1 Benefits of Microalgae Biodiesel

There are several benefits of microalgae which includes high growth rate, less land requirement, lower water requirement and higher mitigation of carbon dioxide to name a few.

2.1.1 High Growth Rate

Microalgae has a higher growth rate when compared to land-based plants. This is as a result of their simple cell structure and existence in an aqueous environment, high substrate intake and photosynthesis efficiency due to availability of entire cell surface for mass transfer and capturing of light (Gao and McKinley, 1994; Gouveia, 2011; John Sheehan et al., 1998b). Furthermore, they can convert 3-8% of solar energy to biomass compared to only 0.5% solar energy conversion by terrestrial plants (Lewis and Nocera, 2006). Doubling one to three times per day in growth (Hu et al., 2008) and can be harvested in a few days unlike a land-based plant, which can take a longer time to harvest (Danielo, 2005). Besides, microalgae can be harvested in batches all year round which makes it a continuous and reliable supply of biofuel (Schenk et al., 2008).

Unlike land-based plants, where only seeds are harvested, every microalgae cell contains lipids which make biomass yield very high (Becker, 1994) and large desirable products obtained from biomass produced (Weissman et al., 1988). Also, aquaculture cultivation of

microalgae is favoured by the absence of non-photosynthetic supporting structures such as roots and stems (John et al., 2011). Again, microalgae have flexible metabolism which can be regulated to vary biochemical composition towards higher lipids, protein or carbohydrate accumulation (Tredici, 2010).

2.1.2 Less Land Use

Microalgae require less cultivation land than other oil crops due to high growth rate thereby, using less land to supply enough biofuel to meet up the massive biofuel demand without causing a deficit in biomass (Clarens et al., 2010; Gao and McKinley, 1994; Li et al., 2008). Microalgae do not compete with existing food commodities and can be grown on marginal lands not required by food cultivation, unlike other plant biodiesel feedstock such as soybean and corn (Mata et al., 2010; Sturm and Lamer, 2011).

Also, biofuels from microalgae will likely have a lower impact on the world's food supply and the environment when compared with conventional biofuel crops. Microalgae biomass has more suitable properties for biofuel than lignocellulosic materials such as low viscosity, high calorific value and low density comparable to fossil oil, and can be cultivated in different climates (Miao and Wu, 2004). Also, cultivation of microalgae does not require pesticides and herbicides and can be coupled with nutrient removal from wastewater streams and CO_2 uptake from industrial waste streams (An et al., 2003; Hodaifa et al., 2008; Rodolfi et al., 2009), minimizing the release of nitrous from use of fertilizers into the atmosphere and could likely be more cost-effective farming microalgae than usual land crops (Li et al., 2008). Microalgae feedstock has a high lipid production advantage of 15-300 times more of dry weight biomass over plant-based biomass (Lam and Lee, 2012; Lee et al., 2010) (Table 2.2).

Table 2.2 Average lipid productivities of some common oilseed crops compared to microalgae

Oil Source	Productivity ($L/m^2/yr$)	References
Algae	4.7 to 14	(J. Sheehan et al., 1998)
Palm Oil	0.54	(Mata et al., 2010)
Jatropha	0.19	(Sazdanoff, 2006)
Rapeseed	0.12	(Sazdanoff, 2006)
Sunflower	0.09	(Sazdanoff, 2006)
Soya	0.04	(Sazdanoff, 2006)

Source: (Griffiths et al., 2011)

2.1.3 Lower Water Requirement

Microalgae can make use of nutrients especially nitrogen and phosphorous from different sources of waste such as concentrated animal feed operations, industrial and municipal wastewater and agricultural run-off (Hu et al., 2008; Rodolfi et al., 2009). This offers cost-saving from the purchase of exogenous nutrients sodium nitrate and potassium phosphate (Wang et al., 2008), also reducing the use of freshwater greatly (Schenk et al., 2008; Wang et al., 2008) and providing additional bioremediation benefit of wastewater (Grobbelaar, 2010; Hu et al., 2008; Rodolfi et al., 2009). Again, there is a potential of combining treatment of wastewater using microalgae, like removal of nutrients, with biofuel production (Pittman et al., 2011). Furthermore, the hydraulic nature of microalgae cultivation systems suggests that

microalgae cultivation if operated properly, can eliminate the inherent challenges of conventional agriculture such as nutrient, CO_2 and water delivery (Weissman et al., 1988).

2.1.4 CO_2 Mitigation

Production of microalgae biomass offers real opportunities for solving issues of CO_2 sequestration (Grobbelaar, 2010) and at the same time, generates economic value by conversion of CO_2 into energy and chemical products (Klinthong et al., 2015), utilising about 1.83kg of CO_2 for production of 1kg microalgae biomass (Chisti, 2007). Microalgae like higher plants can biologically fix CO_2 but microalgae have a higher fixing capability than the higher plants and higher tolerance of microalgae to high CO_2 content in gas streams like flaring gas and flue gases (5% -15% CO_2 content) allows mitigation of CO_2 at high efficiency (Wang et al., 2008). A highly promising alternative to current CO_2 mitigation strategies can be provided by combining CO_2 fixation, wastewater treatment and biofuel production. This would also make microalgae CO_2 fixation more environmentally sustainable and economically cost-effective (Wang et al., 2008).

2.2 Microalgae Growth Requirements

The use of wastewater to efficiently grow microalgae is dependent on different variables. Like every other growth medium, main variables are the concentration of essential nutrients including nitrogen, phosphorus, organic carbon, temperature, pH of the medium, availability of light, CO_2 and oxygen (Pittman et al., 2011).

2.2.1 Nutrients

Microalgae require nutrients for growth, especially nitrogen and phosphorus, in addition to carbon dioxide, light energy and water (Chisti, 2007; Goldman, 1979). Nitrogen, Phosphorus, iron and in some instances silicon are organic compounds that must be present in an algae growth medium (Chisti, 2007). Table 2.3 contains a list of other nutrients required by algal cells and recipe of few algae culture medium in Table 2.4. The concentration of nitrogen and phosphorus in algae growth medium are considered fundamental factors affecting algae growth kinetics directly and is closely related to lipid accumulation and nutrient removal (Xin et al., 2010b). The main mechanism for nutrient removal by microalgae is by uptake into microalgae cells (Bich et al., 1999; Xin et al., 2010b), the rate of nutrient removal is directly affected by the microalgae population growth rate (Xin et al., 2010b).

The usual carbon source for microalgae photosynthetic culture is carbon dioxide. Carbon dioxide is usually supplied continuously or intermittently. Consumption of carbon during photolysis of water leads to the ultimate production of oxygen (Grima et al., 1999). The efficiency at which microalgae cell use carbon through photosynthesis is directly proportional to the microalgal biomass production rate (Williams and Laurens, 2010). Regular CO_2 sources for microalgae are CO_2 from industrial exhaust gases, atmospheric CO_2 and chemically fixed CO_2 in the form of soluble carbonates (e.g. $NaCO_3$ and $NaHCO_3$) (Kumar et al., 2010; Wang et al., 2008). The pH Changes in microalgae culture is majorly from the consumption of CO_2, pH changes due to degradation of metabolites excreted or from the uptake of other nutrients can be neglected (Rubio et al., 1999). Increasing concentration of CO_2 can result in higher production of biomass and decrease in pH which can cause harm to the physiology of microalgae (Oswald, 1988).

The next most important element required for nutrition of microalgae after CO_2 is nitrogen (Becker, 1994). Nitrogen is directly involved with primary metabolism as it constitutes protein and nucleic acid (Hu et al., 2012; Kumar et al., 2010). Nitrogen content of the biomass can vary from 1% to above 10% even within the same species and it depends on the type and availability of nitrogen source (Grobbelaar, 2004). Microalgae cultivation utilises a higher amount of chemical fertilizers (N-fertiliser), unlike other oil-bearing terrestrial plants like Jatropha, palm, rapeseed and sunflower (Lam and Lee, 2012) about 8 – 16 tons N/H (Markou and Georgakakis, 2011). Use of nutrient from wastewater especially of agro source rich in inorganic pollutants (nitrogen and phosphorus) can be one alternative to chemical fertilizers (Markou and Georgakakis, 2011).

Ammonia nitrogen is the preferred form of nitrogen for micro-organisms (Grobbelaar, 2004). Nutrients released from aquaculture are most suitable for cultivation of algae as they are released in a preferred and suitable form (NH_3 for nitrogen and PO_4^{-3} for phosphate) (Troell et al., 2003). On the other hand, microalgae species with fast growth rate prefer primary source of nitrogen in the form of ammonia to nitrate (Green and Durnford, 1996). Besides, microalgae can grow well in different sources of nitrogen (Alberto et al., 2001; Grobbelaar, 2004; Markou and Georgakakis, 2011).

Assimilation of either NH_4^+ or NO_3^- is related to the pH of the growth medium. The pH of growth medium could drop during active algal growth when ammonia is used as the only nitrogen source. This is due to the release of H^+ ions. On the other hand, pH increases when nitrate is used as the only nitrogen source in the growth medium. At high pH, nitrate could be lost due to volatilization. However, it is important to ensure an adequate supply of this important nutrient to achieve high growth rate in algae technology. Culture media are formulated to supply nutrients in excess to avoid nutrient becoming a limiting factor except in specific applications (Grobbelaar, 2004).

Another important nutrient for microalgae growth is phosphorus and is less than 1% in microalgae biomass (Grobbelaar, 2004). According to Kumar et al (2010), phosphorus is the third most important nutrient for microalgae growth and requires significantly excess supply because not all compounds of phosphorus are bioavailable, especially those combined with metals ions (Chisti, 2007; Grobbelaar, 2004; Martinez et al., 1999). Also, microalgae stores excess phosphorus in phosphate bodies which it can use when phosphorus becomes limiting. Again, the ratio of N: P in the growth medium is important both in determining the growth potential and maintaining the dominance of cultured species in the culture (Grobbelaar, 2004).

Algae use oxidative phosphorylation, photophosphorylation and phosphorylation at substrate level to convert phosphorus into high energy organic compound (Martinez et al., 1999). Uptake of phosphorus is affected by the presence of ions. Ca^{2+} increases the rate of phosphorus uptake significantly (Rigby et al., 1980). The energy-dependent accumulation of phosphorus within the cell is strictly dependent on the pH of the cytoplasm which varies during the light and dark stages of photosynthesis (Falkner et al., 1980).

Table 2.3 List of nutrients required by algal cells for growth

Elements	Compounds
Sodium	Several inorganic salts, NaCl, Na_2SO_4, Na_3, PO_4
Potassium	Several inorganic salts, KCl, K_3PO_4, K_2SO_4
Calcium	Several inorganic salts, $CaCO_3$, Ca^{2-} (as chloride)
Hydrogen	H_2O, organic molecules, H_2S
Oxygen	O_2, H_2O, organic molecules
Sulphur	Several inorganic salts, $MgSO_4.7H_2O$, amino acids
Magnesium	Several inorganic salts, CO_2^{3-}, SO_4^{2-}, or Cl^- salts
Chlorine	As Na^+, Ca^{2+}, K^+ or NH_4^+ salts
Iron	$Fe(NH_4)_2SO_4$, $FeCl_3$, ferric citrate
Zinc	SO_4^{2-} or Cl^- salts
Manganese	SO_4^{2-} or Cl^- salts
Bromine	As Na^+, Ca^{2+}, K^+ or NH_4^+ salts
Silicon	$Na_3SiO_3.9H_2O$
Boron	H_3BO_3
Molybdenum	Na^+ or NH_4^+ molybate salts
Vanadium	$Na_3VO_4.16H_2O$
Strontium	SO_4^{2-} or Cl^- salts
Aluminium	SO_4^{2-} or Cl^- salts
Rubidium	SO_4^{2-} or Cl^- salts
Lithium	SO_4^{2-} or Cl^- salts
Copper	SO_4^{2-} or Cl^- salts
Cobalt	Vitamin B12, SO_4^{2-} or Cl^- salts
Iodine	As Na^+, Ca^{2+}, K^+ or NH_4^+ salts
Selenium	Na_2SeO_3

Adapted from (Grobbelaar, 2004)

Table 2.4 Recipe of some selected growth medium for different algae.

Substrate*	BG11	Modified Allen's	Bold's Basal
$NaNO_3$	1.5	1.5	0.25
$K_2HPO_4.3H_2O$	0.04	0.039	0.075
KH_2PO_4			0.175
$MgSO_4.7\ H_2O$	0.075	0.075	0.075
$CaCl_2.2H_2O$	0.036	0.025	0.084
$Ca(NO_3)_2.4H_2O$		0.02	
$Na_2SiO_3.9H_2O$		0.058	
Citric acid	0.006	0.006	
Fe-Ammonium citrate	0.006		
$FeCl_3$		0.002	
$FeSO_4.7\ H_2O$			0.00498
EDTA, 2Na-Mg salt	0.001	0.001	0.005
Na_2CO_3	0.02	0.02	
NaCl			0.025
KOH	0.031		
H_3BO_4 (μgL^{-1})	2.86	2.86	11.42
$MnCl_2.4H_2O$ (μgL^{-1})	1.81	1.81	1.44
$ZnSO_4.7H_2O$ (μgL^{-1})	0.222	0.222	8.82
$Na_2MoO_4.2H_2O$ (μgL^{-1})	0.391	0.391	
$CuSO_4.5H_2O$ (μgL^{-1})	0.079	0.079	1.57
$Co(NO_3)_2.6H_2O$ (μgL^{-1})	0.0494	0.0494	0.049
MoO_3 (μgL^{-1})			0.71
Adjusted pH	7.4	7.8	

* All concentrations are in g/l and quantities are for 1 litre of culture solution.Adapted from (Grobbelaar, 2004)

Mostert and Grobbelaar (1987) reported nitrogen supply concentrations between 25 – 5000 mg/l algae mass culture have been reported, with suggested optimal concentration for maximum productivity between 2- and 619 mg/l and a variation on phosphorus between 0.98- and 179 mg/l. Studies with *Chlorella vulgaris* at different ammonia concentrations obtained algae growth in all concentration. Low algae growth were obtained in very high ammonia concentration above 750 mg/l and very low ammonia concentration below 10 mg/l while maximum cell density was obtained in nitrogen concentrations between 20- and 250 mg/l with no difference in specific growth rates. The growth rate in the different ammonia media studied were comparable to growth in commercial Bristol medium which had nitrate as the nitrogen source (Tam and Wong, 1996).

Under nitrogen-rich conditions, rapid cell division and chlorophyll accumulation occur while under depleted nitrogen condition, no cell division with high lipid biomass accumulation for several more days after with a rapid drop of chlorophyll (J. Sheehan et al., 1998). At 2.5 mg/l nitrogen limitation, *Scenedesmus sp.* LX1 accumulated up to 30% lipid of biomass and up to 53% at phosphorus limitation of 0.1 mg/l (Xin et al., 2010b). Different other studies have cultivated microalgae in different nitrogen and phosphorus concentrations. Some of the studies are Aslan and Kapdan (2006) with 13.2-410 mg/l ammonia concentration and 7.7-199 mg/l phosphorus 25-200 mg/l urea concentration (Hsieh and Wu, 2009).

2.2.2 Light

Light is an important requirement in microalgae growth, so there is a need to deliver optimal light to all microalgae cells within the culture. Highest photosynthetic efficiencies are realized at low light, as high light intensities not only cause inefficient use of absorbed light energy but also causes biochemical damage to photosynthetic machinery (photoinhibition)

and reduction in dry weight (Scott et al., 2010). Generally making light intensity requirement of microalgae cultivation lower than light intensity needed for higher plants (Kumar et al., 2010). Microalgae photosynthesis and productivity is equal to the efficiency of light conversion when the only limiting factor is light (Grima et al., 1999; Richmond et al., 2003). Generally, specific growth increases with an increase in irradiance to a maximum irradiance point beyond this point, inhibition may occur from a further increase in irradiance (Grima et al., 1999).

2.2.3 Temperature

One of the major factors controlling cellular, physiological and morphological responses of microalgae is temperature. Generally, an increase in temperature increases the rate of metabolism while a decrease in temperature decreases the growth of algae. Environmental parameter like light intensity affects optimal temperature with 20 – 25ºC reported as optimal temperature for some species, accumulating most cell density at 23°C (Kumar et al., 2010).

2.3 Fish Farm Wastewater

Globally, aquaculture has been one of the food production sectors with rapid development and production growth, significant investment and technical innovation (Muir, 2005). Growing at 5.8 % annually between 2004 to 2014 and produced 73.8 million tonnes of fish in 2014 (FAO, 2016). The main pollutants of concern in FFW are particulate and dissolved nutrients (nitrogen and phosphorus), specific inorganic and organic compounds (Piedrahita, 2003). The volume of waste discharged from aquaculture depends on feeding regime,

stocking density and feeding rate because these three factors combined determine the quantity of feed to be used (Islam, 2005).

2.3.1 Nitrogen

Transformations of nitrogen are key biochemical processes in aquaculture systems with protein as the major form of nitrogen in the fish feed (Hu et al., 2012). In every ton of fish produced, approximately 132.5 kg nitrogen and 25.0 kg phosphorus are released to the environment (Islam, 2005). Fish feed consumed is converted partially into fish biomass, egested as faeces or excreted through the gills as unionised ammonia, a major product of protein metabolism (Handy and Poxton, 1993; Hu et al., 2012; Krom et al., 1985)

Most ammonia produced in fish occurs in the liver and voided through the epithelia surface and renal routes while urea, is obtained through argininolysis or hepatic uricolysis and excreted through the gills, kidneys, skin or faeces. Production of ammonia also occurs in the kidney, intestine and muscle due to the presence of amino acid deamination enzyme in the tissues (Mommsen and Walsh, 1992). Ammonia in freshwater is excreted via passive NH_3 diffusion across the branchial epithelium. Next to the gill, this NH_3 subsequently gets trapped as NH_4^+ in an acidic boundary layer, which maintains the partial pressure gradient of blood-to-gill water NH_3 (Wilkie, 2002).

Therefore from the above text, the source of nitrogen loading in fish farms can be generally grouped into three (Handy and Poxton, 1993; Islam, 2005):

1. Feed wasted due to poor management and farm practice;
2. Poor feed quality leading to poor feed stability and rapid dissolution of fish feed in water; and

3. Low absorption and retention of food ingested that can be due to poor food digestibility of fish metabolism.

From the three groupings, large amounts of nitrogen in FFW is dissolved with only 7 to 30% in particulates (Cripps and Bergheim, 2000). Nitrogen in aquaculture is predominantly excreted as ammonia (NH_4) (Lazzari and Baldisserotto, 2008) and only about 20 to 40% of total nitrogen excreted as urea (CH_4N_2O) (Mommsen and Walsh, 1992). Ammonia nitrogen build-up is the second most limiting factor to an increased level of production in intensive aquaculture after dissolved oxygen (Ebeling et al., 2006). Even at very low concentrations, ammonia especially un-ionised (NH_3) are toxic to fishes with maximum concentrations usually not close to 0.0125 mg/l acceptable safe concentrations by fish culturist (Meade, 1985).

In flow-through aquaculture systems, most of the total nitrogen in the system is produced as ammonia while in recirculating systems with biofilters produces mostly nitrates (Bergheim and Asgard, 1996). As the most reactive nitrogenous species, the pelagic microbial community quickly take up ammonia and produce other nitrogenous species like nitrate (Black, 2008). The rate of ammonia reaction in water is rapid, having a half-life of fewer than 50 msec for interconversion of NH_4^+ to NH_3 (Stumm, W. and Morgan, 1981). However, temperature, pH and salinity of the water affects the relative proportion of the two forms of ammonia (Stumm, W. and Morgan, 1981). In natural water, ammonia exists as a component of pH and temperature-dependent equilibrium. Aqueous, an ionised form of ammonium (NH_4^+) is favoured within equilibrium pH (6.5 to 8.0) while high pH of more than nine favours unionised form of ammonia (NH_3) (Hargreaves, 1998).

2.3.2 Phosphorous

Phosphorus is a limiting nutrient in a freshwater ecosystem (Hudson et al., 1999; McDaniel et al., 2005) and is excreted through urine in fishes (Bureau and Cho, 1999). Excretion of phosphorus usually 60 – 86% of dietary phosphorus is related to sources of origin, which different species use in different ways (Lazzari and Baldisserotto, 2008). Water quality can be influenced by phosphorus from aquaculture (Reid and Moccia, 2007) as elevated levels of phosphorus cause premature eutrophication (McDaniel et al., 2005). Soluble phosphorus is not produced when feed with low phosphorus levels is consumed to a point after which soluble phosphorus increases with an increase in phosphorus in the feed (Coloso et al., 2003). The particulate total phosphorus (TP) and particulate total nitrogen (TN) fractions of effluent from a salmonid farm range from 30% to 84% and 7% to 32% respectively (Cripps and Bergheim, 2000; Schulz et al., 2003; Sindilariu, 2007).

Phosphorus is usually not lost in an aquatic environment but remains conserved in a series of fractions as a result of dissolution, adsorption and precipitation process (Paytan et al., 2003) and therefore, makes phosphorus a useful study parameter as an indicator of the environmental impact of fish effluent (Kus et al., 2008). Modern agriculture relies on non-renewable phosphate from rocks for phosphorus supply which is estimated to run out in 50 – 100 years with an estimated increase in phosphorus use (Cordell et al., 2009). This makes it very essential to recycle phosphorus in wastewater sources, manure and even with-in production process of biofuels to eliminate direct competition of phosphorus for algae cultivation with conventional agriculture (Borowitzka and Moheimani, 2013).

2.3.3 Impact of Aquaculture Discharge to the Environment

Aquaculture impact on the environment depends on feed type, stocking density, species, culture method and farm practices (Wu, 1995). The concentration or total amount of effluents released and the capacity of the environment to assimilate the particular constituent also affects the impact of aquaculture on the environment (Piedrahita, 2003). Nitrogen and phosphorus as major constituents of fish loading can affect the environment as a whole as well as the rearing of the fish (Lazzari and Baldisserotto, 2008). Introduction of organic and in-organ materials through food feed to fishes have significantly impacted the nutrient and organic matter loading in coastal waters.

Rapid growing intensive aquaculture system would lead to different adverse effects on the environment such as:

- Increased release of nutrients, which leads to eutrophication of coastal waters (Marinho-Soriano et al., 2009; Páez-Osuna et al., 1998; Paudel et al., 2015; Troell et al., 1999);
- Shortage of drinking water resources as a result of release toxic chemicals like ammonia (NH_3) and nitrite (NO_2) from aquaculture especially in intensive systems of fish culture (Heidari et al., 2011);
- Reduction of wild fish supplies which can affect the ecosystem through large input of wild fish feed, used in feeding carnivorous species and also, habitat modification for some aquaculture systems (Naylor et al., 2000);
- Competition for land and disturbance of wild ecosystem from escaped farmed fish (Bisson, 2006);
- Pollution from drugs residues used in the prevention and treatment of diseases in aquaculture can lead to a change in biodiversity (Tovar et al., 2000); and

- Environmental concerns from use of chemicals like (antifoulants, vitamins) and the introduction of new genetic strains and pathogens. Also, cleaning of fouled cages can add to the organic loading of the water (Wu, 1995).
- Microbial nitrification and denitrification are reactions common in aquaculture systems, which lead to the release of nitrous oxide (N_2O), a major greenhouse gas, with 310 times more global warming potential than CO_2 over a lifespan of hundred years (Figure 2.1). Nitrous oxide destroys the ozone and has a lifespan of 114 years. Aquaculture N_2O emission is estimated to contribute about 5.72% anthropogenic N_2O-N emission by 2030 if aquaculture maintains the same annual development rate of about 7.10% (Hu et al., 2012).

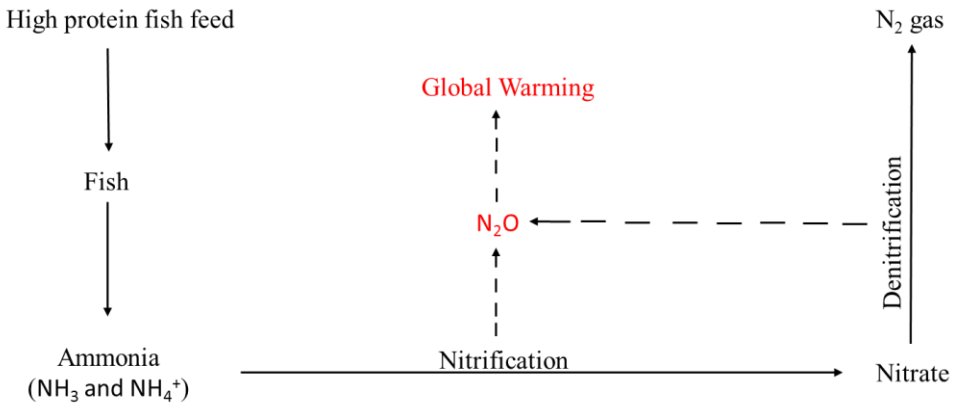

Figure 2.1 Nitrous oxide emissions from aquaculture. Source: (Hu et al., 2012).

2.3.4 Benefits of utilising Microalgae in Fish Farm Wastewater Nutrient Recovery

Use of algae for nutrient removal especially nitrogen and phosphorus has been demonstrated and has numerous advantages such as low operation costs (Aslan and Kapdan, 2006; Heidari et al., 2011; Wang et al., 2008):- saving money used for the purchase of exogenous nutrients like potassium phosphorus and sodium nitrate by minimising the use of exogenous nutrients and saving of freshwater resources (Heidari et al., 2011). Suitable growth and high tolerance

(Heidari et al., 2011), pollutant conversion to commercial crops like algae and loaded effluent to clean water (Neori et al., 2004) generates extra income when economic important species are used (Marinho-Soriano et al., 2009), increase in productivity by eliminating pollutant nutrients (Wu, 1995), recycling the nitrogen and phosphorus trapped in algae biomass as fertilizer thereby, avoiding the problems of sludge handling and oxygenated effluent discharge into receiving water body (Aslan and Kapdan, 2006). Use of algae for nutrient removal is not environmentally dangerous as it involves principles of natural ecosystem and also, does not lead to secondary pollutants as long as biomass produced is reused (Martínez, 2000).

Furthermore, the process is attractive for treatment of secondary sludge as it has no carbon requirement for nitrogen and phosphorus removal (Aslan and Kapdan, 2006). Moreover, the use of wastewater from agricultural, industrial and municipal activities can provide sustainable and cost-effective means of cultivating algae for biofuels (Pittman et al., 2011). Eliminating the cost of synthetic fertilizers traditionally used in mass cultivation of algae which is associated with rising prices of crude oil and as an alternative to synthetic fertilizer (Fenton and Ó hUallacháin, 2012). Additionally, the use of residual nutrient and nutrient recycling can overcome the high cost of algae biomass production, a major drawback in algae biotechnology for biodiesel production (Schlagermann et al., 2012). Cultivation of microalgae also benefits the fish farmer in several ways like saving cost associated with the treatment of aquaculture wastewater before discharge, reduction in demand for freshwater and supply of algae biomass fish feed for the cultivation of fishes (Malibari et al., 2018).

2.3.5 Microalgae Cultivation in Fish Farm Wastewater

Recently, studies using FFW has been carried out for different purposes (Ansari et al., 2017; Egloff et al., 2018; Gao et al., 2016; Guerrero-Cabrera et al., 2014; Guo et al., 2013; Halfhide et al., 2014; Malibari et al., 2018; M. H. A. Michels et al., 2014; Nasir et al., 2015). Most of the studies (Egloff et al., 2018; Gao et al., 2016; Guo et al., 2013; M. H. A. Michels et al., 2014; Nasir et al., 2015) focused on the growth rate of algae in aquaculture wastewater, rate of nutrient removal, the effect of aquaculture wastewater on algae composition, enhancing microalgae harvesting through bioflocculation by co-cultivation of microalgae with fungus and feed production to name a few. A few studies (Ansari et al., 2017; Guerrero-Cabrera et al., 2014; Halfhide et al., 2014; Malibari et al., 2018) determined the lipid content of the microalgae grown in FFW while fewer (Malibari et al., 2018) went further to determine the fatty acid composition of the lipid accumulated. However, all the studies reviewed characterised the FFW used, determined biomass yield and nutrient removal. The characteristics of the FFW are shown in Table 2.6 while specific growth rate, biomass yield, biomass productivity and Lipid content is shown in Table 2.5. All the studies utilising FFW for microalgae cultivation agree that FFW has sufficient nutrients to support microalgae cultivation. Also, microalgae utilised nutrients in FFW for growth and accumulation of biochemical compounds and biomass production. Also, the nutrient load of FFW reduced significantly after microalgae cultivation indicating the suitability of the use of microalgae in the removal of nutrients in FFW.

Table 2.5 Species, initial algae cultivation concentration, specific growth rate, biomass yield, biomass productivity and lipid content of some microalgae cultivated in aquaculture wastewater.

Species	Initial Concentration (g/l)	Specific growth rate (day^{-1})	Biomass yield (g/l)	Biomass Productivity (mg/l/d)	Lipid content (%)	References
Ankistrodesmus falcatus			2.96	211.64	25.2	(Ansari et al., 2017)
Chlorella sorokiniana			1.51	107.85	31.85	(Ansari et al., 2017)
Chlorella sp.		0.018	0.07	0.047	9.2	(Guerrero-Cabrera et al., 2014)
Chlorella sp.		0.18	0.058			(Malibari et al., 2018)
Chlorella vulgaris	0.025	0.17	0.044	7.3		(Gao et al., 2016)
C. vulgaris in MPBR	0.41			42.6		(Gao et al., 2016)
Monoraphidium sp.		0.013	0.162	19	10.4	(Guerrero-Cabrera et al., 2014)
Nannochloropsis sp		0.16	0.073			(Malibari et al., 2018)
Scenedesmus obliquus	0.025	0.15	0.037	6.2		(Gao et al., 2016)
Scenedesmus obliquus			1.25	89.61	30.85	(Ansari et al., 2017)
Scenedesmus sp.		0.14	0.344	26	10.3	(Guerrero-Cabrera et al., 2014)

Table 2.6 Characteristics of aquaculture wastewater used for cultivating microalgae

Reference	NH_4 (mg/l)	NO_3 (mg/l)	NO_2 (mg/l)	P (mg/l)	pH	COD (mg/l)
(Malibari et al., 2018)	443	125.5	28.7	5.8	-	-
(Gao et al., 2016)	4.2	0.13	2	0.42	7.78	
(Nasir et al., 2015)	0.91	-	-	2.6	-	-
(Egloff et al., 2018)	-	67 – 157	-	-	-	-
(Halfhide et al., 2014)	-	2	-	16.9	6.94	238
(Michels et al., 2014)	0.48	40.7	0.14	4.96	7	115
(Guerrero-Cabrera et al., 2014)	24	-	-	10	7.5	-
(Ansari et al., 2017)	5.32	40.67	5.52	8.82	7.25	96
(Guo et al., 2013)	-	47.8	-	8.87	-	-
Range*	0.48 – 433	0.13- 157	0.14 - 28	0.42 – 16.9	6.94 – 7.78	96 - 238

*Range refers to minimum to maximum values from papers reviewed

2.4 Conclusion

The increasing world population and increased dependence on aquaculture for fish supplies, waste fish farm effluents is expected to grow alongside to provide nutrients for microalgae cultivation. Several studies have shown that cultivation of microalgae in aquaculture wastewater is suitable for microalgae growth and biomass productivity coupled with efficient nutrient removal. The replacement of inorganic fertilizer with nutrient-rich fish farm effluent will eliminate the cost of fertilizer purchase. This should lead to cheaper cultivation of microalgae biomass production for biodiesel production. When cost as one of the major setbacks of algae biotechnology is eliminated, the potential of microalgae biodiesel to replace fossil diesel will be enhanced. Also, fish farm effluent nutrient recycling for microalgae cultivation for biodiesel production will at the same time, eliminated numerous negative environmental effects associated with nutrient-rich effluent discharge to the environment, while reducing the volume of water used for fish farming. Therefore, resulting in a sustainable process.

As we look to the future, there is scope for improving microalgal technology across the value chain from pre-production to post-production as reported by several authors (ADD References – two/three that speak on algae tech for production).

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Chapter 3 Cultivation of microalgae in fish farm effluent

Part of this chapter has been by South African Journal of Chemical Engineering. An abbreviated form of this chapter was presented as a poster at the 9th cross-faculty postgraduate symposium at the University of the Witwatersrand, Johannesburg. 29 – 30th October 2018

The contribution of the co-author's Professor Kevin Harding and Dr. Michelle Low towards the work is primarily that of supervision or collaboration, and the work and write-up were carried out by the author of this thesis.

This chapter aims to achieve various objectives such as exploiting nutrients in fish farm wastewater (FFW) for the cultivation of microalgae in conditions favourable for high lipid accumulation. It also evaluates the rate of CO_2 uptake and the rate of removal by algae cells.

Abstract

There has been a lot of research in cultivating microalgae in different sources of wastewater for growth rate determination and nutrient removal. However, there has been little research on cultivating and determining microalgae composition in fish farm wastewater (FFW). In this study, the following species were compared *Tetradesmus obliquus, Heterochlorella luteoviridis,* and *Chlamydomonas reinhardtii*. Their growth in unmodified FFW and modified FFW (which had nutrient concentration lowered) and Tris-Acetate-Phosphate (TAP) standard growth media were studied. The following parameters of the algae species were investigated: specific growth rate, biomass yield, biomass productivity, lipid content, carbohydrate content, protein content. Additionally, lipids, carbohydrate and protein productivities were calculated and the rate of CO_2 uptake was determined. *Chlamydomonas reinhardtii* in unmodified FFW had the highest lipid and carbohydrate composition of 44.08 % and 35.04% respectively and most suited for biodiesel production and nutrient removal. The modified FFW only had the extra benefit of almost complete nutrient removal but comparable yield and biochemical composition with samples in unmodified FFW. A similar effect of CO_2 concentration was observed in unmodified FFW and modified FFW. The pH increased in the FFW culture media but reduced in the TAP media during cultivation. Therefore, this study showed that nutrients in FFW are suitable for algae biomass cultivation and lipid yield.

Keywords: Biomass yield, lipid content, carbohydrate content, protein content, CO_2 removal

3.1 Introduction

One of the most important environmental issues is pollution from combustion for electrical or mechanical power generation (Russo et al., 2012). Since the beginning of the first industrial revolution in the late 18th and early 19th century, energy has become vital to humans for increased economic development and sustenance (Atabani et al., 2012). While research into renewable energy has focused on electricity generation (*e.g.* solar, wind, hydroelectricity, geothermal, tidal), global energy demand is made up of mostly non-renewable fuels (about 66%) (Gouveia, 2011; Schenk et al., 2008). The transportation sector is the second-largest energy consumer after the industrial sector (Singh and Singh, 2015) and current technology cannot replace the unique role of liquid fuels in transportation with electricity (Simionato et al., 2013).

The explosion in biotechnology and the debate on food versus fuel has resulted in a significant commitment to the expansion of algae biofuels by the governmental and industrial sectors. The potentials microalgae offer through high productivity of lipids using small land areas and can also use non-arable land justifies recent investments in microalgae for fuels (Richard, 2010). Also, microalgae can utilise nutrients and organic load for example from FFW for the production of valuable biomass. Biomass from microalgae is rich in lipids and carbohydrates (Guldhe et al., 2017a) that can be converted to biofuels. As aquatic species, a large amount of water is required for cell proliferation and survival. In addition to intracellular water, algae suspend in culture medium for growth and reproduction (Farooq et al., 2015). This makes use of water in algae cultivation a challenge of high significance (Guieysse et al., 2013).

The profound rise in aquaculture over the years has been driven by a rise in demand for seafood and the reduction of captured fish (Neori et al., 2004; Paudel et al., 2015). FFW requires treatment before it can be reused or discharged to avoid eutrophication. Water treatment adds to the cost of aquaculture produce. Cultivating microalgae in FFW can be of biorefinery importance for economic production of microalgae biomass feedstock and wastewater treatment. Integrating of wastewater treatment and microalgae biodiesel production is a promising option to mitigate the energy crisis of diminishing fossil fuel reserves, the biggest challenge of the 21st century (Xin et al., 2010). Production of 1L of biodiesel from microalgae consumes about 3000L of water (Farooq et al., 2015). The use of FFW could supply this large volume of water required for algae biodiesel feedstock. Using nutrients in wastewater for microalgae cultivation can provide sustainable biorefinery with the dual advantage of microalgae feedstock for biofuels production and treatment of wastewater (Guldhe et al., 2017a).

3.1.1 Nutrients

Nitrogen supply is an important driver of the sustainability and commercialization of microalgae biofuels. The production of 1kg triacylglycerol from microalgae with 20% triacylglycerol yield requires about 0.36% nitrogen (0.46kg NH_4^+ or 1.6 kgNO_3^-). Finding new ways to reduce external nitrogen requirements in microalgae production for improved energy balance, sustainability and economics of microalgae biofuels is required. External nitrogen requirements for microalgae cultivation can be reduced by two main ways; recycling nitrogen in wastewater and recovering nitrogen from a non-triacylglycerol component of microalgae biomass (Peccia et al., 2013).

In conventional wastewater treatment, nutrient removal is a two-stage process: precipitation of phosphorus with metals and nitrification-denitrification of nitrogen to nitrogen gas. However, microalgae can remove nutrients in wastewater in a single process, eliminating the two-stage process. Microalgae produce biomass by utilising nitrogen and phosphorus in wastewater. The flexibility of microalgae biomass nitrogen and phosphorus concentration enables microalgae to adjust their intracellular nitrogen and phosphorus concentration to nitrogen and phosphorus supplied by wastewater (Beuckels et al., 2015).

The carbon system is the most critical buffer in freshwaters and is the best way to control and maintain different pH levels that are suitable for mass-cultivated organisms. CO_2 fixation during photosynthesis leads to the release of OH^- which builds up in the growth solution and leads to a steady pH increase. A pH static control through direct CO_2 emission into the growth media is the best and most practical method of pH control while at the same time providing CO_2 for high yield algae biomass (Richmond, 2004).

Although both algae and terrestrial photosynthetic organisms tend to grow faster in the presence of slightly elevated CO_2 and nutrient levels, these nutrients are more easily delivered to algae than to terrestrial plants (Clarens et al., 2010). Autotrophic production requires high rates of supply of CO_2 and HCO_3^- , and unlike land plants, atmospheric CO_2 is not capable of meeting the supply requirements of high-yielding autotrophic algal production systems.

The two main sources of CO_2 for microalgae cultivation are from the atmosphere and flue gases (Cheah et al., 2015). However, there are other sources of CO_2 like crude glycerol, a by-product of biodiesel production which has been considered as an important alternative carbon source for microalgae cultivation (Bahadar and Bilal Khan, 2013).

Biomass determination and growth rate assessments are important methods for determining microalgae performance in CO_2 bio-sequestration. In different CO_2 concentrations and cultivation methods, microalgae species had different CO_2 fixation rates and removal percentages (Cheah et al., 2015). High CO_2 concentration enhances microalgae photosynthetic ability to replicate in a shorter time. Nevertheless, CO_2 levels above 5% (v / v) are known to be harmful to the growth of microalgae. Supply of about 10 – 20% (v/v) can reduce pH to up to 5.5 and this can be toxic to algae growth (Zhao and Su, 2014).

3.1.2 Cultivation systems

Microalgae are cultivated in either closed systems (photobioreactors) or open systems (Ugwu et al., 2008). Photobioreactors can be designed as either open (high rate algae ponds or stabilization unit) or closed (flat plate or tubular) systems (Muñoz and Guieysse, 2006). Open systems can be grouped into natural waters (ponds, lakes, lagoons) and artificial ponds or containers. The systems commonly used are raceway ponds, tanks, shallow big ponds and circular tanks (Ugwu et al., 2008). The closed system (flat plate and tubular systems) can be grouped according to the orientation of tubes or panels, culture recirculation mechanism, type of gas exchange system, method of light delivery, construction materials used and the arrangement of individual growth unit (Grima et al., 1999).

Microalgae culture in a raceway or open pond is very developed but only a few microalgae species can be cultivated without contamination in a traditional open system using saline or alkaline control environment (Molina et al., 2001), while closed photobioreactor can be used in the production of various microalgae species and has high production rate unlike the raceway ponds (Lee and Lee, 2003). Use of enclosed photobioreactor can allow for

monoculture cultivation and can potentially avoid contamination from the environment (Chisti, 2007; Grima et al., 1999; Molina et al., 2001; Ugwu et al., 2008).

Raceway ponds and tubular photobioreactors are two practical methods of microalgae large scale production. A tubular photobioreactor is made up of transparent straight tubes usually made of glass or plastic for solar collection. Flow is produced by gentle airlift flow or more vigorous mechanical pump flow to prevent biomass sedimentation in tubes. Mechanical pumps are easy to design, install and operate (Chisti, 2007) but can destroy the biomass (Camacho et al., 2001), a supply of air is required in airlift pumps and are less flexible.

In the design of photobioreactors for the cultivation of microalgae biomass and products, many factors must be considered (Weissman et al., 1988). Some of the factors to be considered are effective and efficient supply of light, carbon dioxide supply while minimizing losses, removal of inhibitory oxygen from photosynthesis (Grima et al., 1999). Other important factors to consider are biomass use, process safety and cost of land (Muñoz and Guieysse, 2006). In tubular photobioreactors, establishing mixtures of flow rate, tube length and level of irradiance to avoid inhibitory dissolved oxygen build-up in the reactor (Canon-Rubio et al., 2016).

Photobioreactors have higher yield almost up to 30 folds more biomass yield than the raceway. This affects biomass concentration during harvesting and makes the cost of harvesting in photobioreactors lesser than that of the raceway. This is due to the small volume of broth to be processed to obtain a certain quantity of biomass (Chisti, 2007). Easy construction and operation of the open pond system unlike the closed system are one of the main advantages of open ponds. However, main limitations in open systems include evaporative losses, poor light utilization by the cells, the requirement of a large area of land, contamination by predators and other heterotrophs with rapid growth, CO_2 diffusion to the

atmosphere and low mass transfer rates due to inefficient mixing methods (Ugwu et al., 2008). Summary of advantages and disadvantages of photobioreactors and open ponds is shown in Table 3.1.

Production of biomass in a photobioreactor depends on a close relationship with the algal culture environment and to the needs of the microalgae strain selected. The environmental growth factors like nutrients and temperature can be adequately controlled in a photobioreactor (Grima et al., 1999).

Table 3.1 Summary of advantages and disadvantages of photobioreactors and open ponds

Factors	Photobioreactor	Open ponds
Monoculture	High	Low
Control of culture conditions light, nutrients, temperature, pH	High	Medium
Commercialization	Low	High
Contamination from environment	Low	High
Management	High	Medium
Evaporation of Water	Low	High
Escape of carbon dioxide	Low	High
Dissolved Oxygen accumulation	High	Low
Productivity	High	Low
Easy construction and operation	Low	High
Harvesting cost	Low	High
Land requirement	Low	High

3.1.3 Aim and Objective

In this chapter, the aim is to use nutrients in FFW for the cultivation of microalgae for the production of biodiesel. Microalgae were grown in unmodified and modified FFW, to determine if nutrients in FFW are appropriate for growing microalgae for the development of biodiesel were compared to TAP media. The majority of the research done on this topic (Egloff et al., 2018; Gao et al., 2016; Guo et al., 2013; M. H. A. Michels et al., 2014; Nasir et al., 2015) has focused on cultivating microalgae in wastewater with limited research in using aquaculture wastewater for microalgae biodiesel production. Research in microalgae aquaculture wastewater has focused on phototrophic cultivation and nutrient removal with little focus on the composition of biomass obtained from microalgae cultivation in wastewater (Ansari et al., 2017; Guldhe et al., 2017a).

3.2 Methodology

Fish farm wastewater was collected, characterised and prepared as a growth medium. This was followed by the cultivation of microalgae (in the prepared fish farm wastewater). Biomass yield determinations and pH measurement were carried throughout the propagation. After harvesting the cultivated microalgae, biochemical compositions (lipids, proteins and carbohydrates) and carbon dioxide uptake were determined.

3.2.1 Preparation of fish farm wastewater

Fish farm wastewater was collected from David Fincham Aquaculture, Johannesburg, South Africa. Farming of the *Oreochromis Niloticus* fish species (tilapia) was in a recirculating

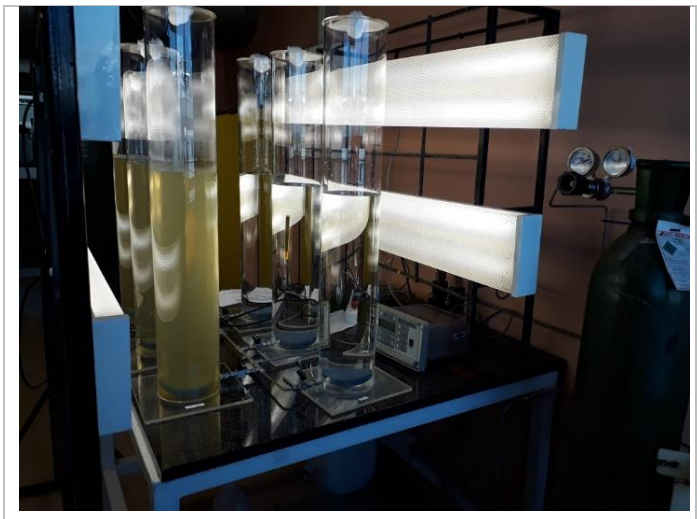
system using 10 000 L fish tanks, containing 200 kg of fish, fed 4% weight daily with fish feed containing 32% protein. Once a day, the system was back washed.

The collected farm wastewater was filtered using a vacuum pump and 509ws filter paper, followed by sterilisation in an autoclave at 120°C for 20 mins. A Hach ammonia nitrogen test kit model N1-8, nitrate test kit model N1-11 and orthophosphate test DOC326.98.00023 were used respectively to analyse for NH_4, NO_3 and PO_4. Distilled water was used to modify nutrient load in sterilized FFW while sterilised FFW was used in the original form in unmodified FFW cultivation.

3.2.2 Microalgae Cultivation

Three species of microalgae (*Tetradesmus obliquus*, *Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii*) were obtained from South African National Phycology Culture Collection, Evolution Studies Institute, University of the Witwatersrand, Johannesburg. They were selected because they were readily available and have been used in different studies as depicted in table 2.5 for high lipid accumulation when cultivated in ffw. Details on each of the species can be found in the reference; *Tetradesmus obliquus* (Di Caprio et al., 2018), *Heterochlorella luteoviridis* (Kim et al., 2017) and *Chlamydomonas reinhardtii* (Griesbeck et al., 2006). Tris-acetate phosphate (TAP) standard media was selected as the control growth media as preliminary results (data not shown) obtained better growth on TAP media. The three species were each cultivated (initial starting mass of 20 mg/l ±5 in batch using a 10L perspex (plexi glass) photobioreactor height of 100 cm and inside diameter of 14.5 cm, with a diffuser at the base of the reactor at 12/12 hours light and dark regime with the light intensity of 2388 lx (Figure 3.1.A and Figure 3.1.B). Air was used for the continuous supply of air at 7 ml/min and 1 hour daily supply of CO_2 at 2 ml/min. Brooks Instrument 0154 mass flow

meter was used to measure the flow rate of gases. The source of light for the bioreactor was supplied from the sides using eight fluorescence lamps (Osram L58 Watts/640 cool white) covered with a lamp cover. The light intensity was measured with MAJOR Tech MT940 light meter (Figure 3.1.C). After 15 days of cultivation, the microalgae were harvested. It was separated from the growth medium using a pressure filter (0.45 µm cellulose acetate filter paper) and washed with distilled water, followed by an oven drying process at 28°C for 48 hrs.



(A)



(B)



(C)

Figure 3. 1 Experimental setup: (A) Algae cultivation set-up showing reactors containing FFW to the left and control TAP media to the right before inoculation of microalgae species (B) Microalgae growing in photobioreactors in FFW (left) and in TAP (right) media (C). Light meter

3.2.3 Daily Growth and Biomass Analyses

Daily optical density reading of 680 nm using a Shimadzu UV-1800 spectrophotometer was used to determine the daily growth rate of microalgae species. The microalgae biomass yield was gravimetrically estimated every four days. To determine biomass yield (in dry cell weight) a 50 ml of culture samples were centrifuged at 4000 rpm for 5 mins, washed with distilled water followed by the transfer of microalgae pellets into filter papers of known weight (Figure 3.2 showing filter papers) dried in the oven at 50°C for 24 hours, and cooled in a desiccator. Biomass productivity (mg/l/d) was gravimetrically estimated as the dry biomass at the end of cultivation. Biomass productivity and specific growth rate were calculated using Equations 3.1 and 3.2 respectively as shown in Singh et al (2015). Calibration data and plots are shown in Appendix A.1

$$\text{Biomass productivity(mg/l)/d} = \text{amount of biomass (mg/l)/(number of days)} \quad (3.1)$$

$$\text{Specific growth rate } (\mu) = 1/t_m \ \text{In}(X_f/X_i) \quad (3.2)$$

Where X_f is biomass at last day of cultivation, X_i is biomass at initial cultivation and t_m is cultivation time in days.

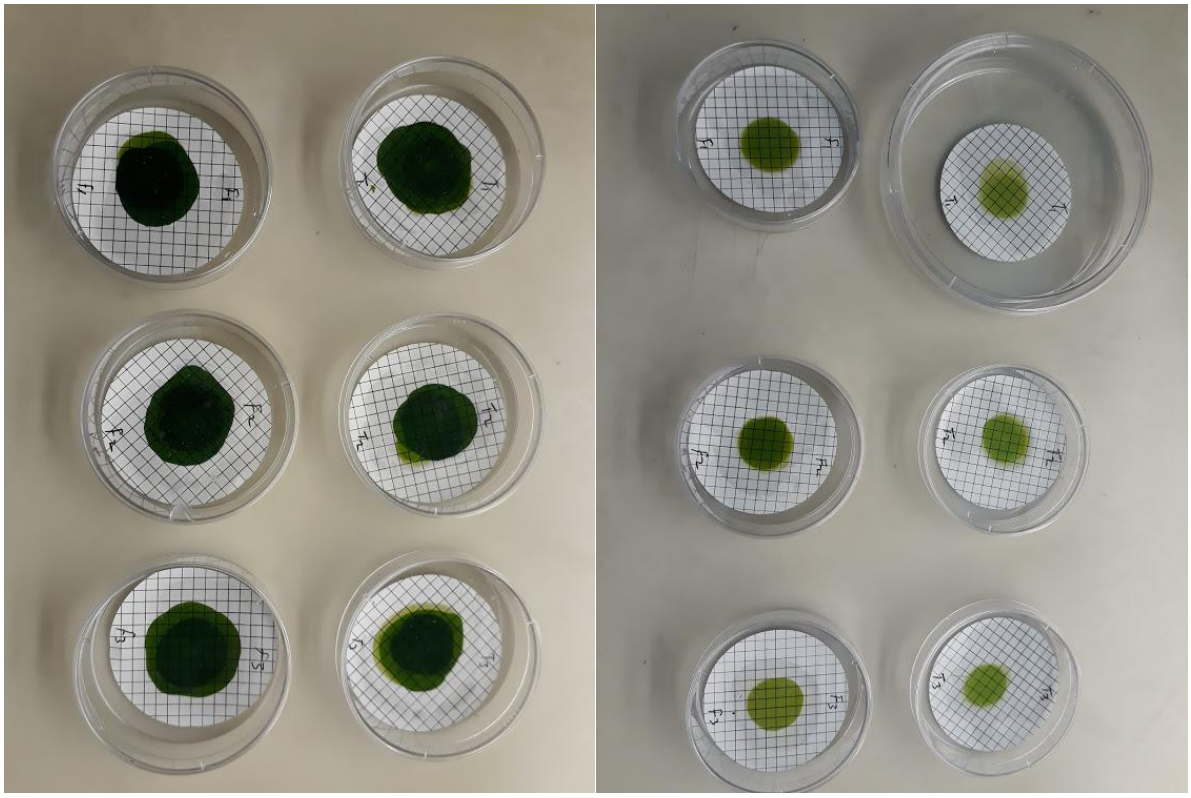


Figure 3. 2 Filter papers used to determine biomass dry cell weight in early and late stages of cultivation

3.2.4 Nutrient Removal

Nutrient removal was determined at the end of the microalgae propagation. A 50ml sample was collected for nutrient analysis and centrifuged at 4000 rpm. Using the Hach ammonia nitrogen test kit model N1-8, orthophosphate test DOC326.98.00023 and nitrate test kit model N1-11 respectively to measure ammonia, phosphate and nitrate. Percentage nutrient removal was calculated as in Guldhe et al (2017a) using Equation 3.3. Visual of colourimetric results obtained in *Tetradesmus obliquus* grown in unmodified FFW is shown in Figure 3.3, Figure 3.4 and Figure 3.5 respectively for ammonia, nitrate and phosphate.

$$\text{Percentage removal (\%)} = \frac{X_f - X_i}{X_f} \text{ x } 100 \tag{3.3}$$

Where X_f is initial concentration of nutrient, X_i is final concentration of nutrient

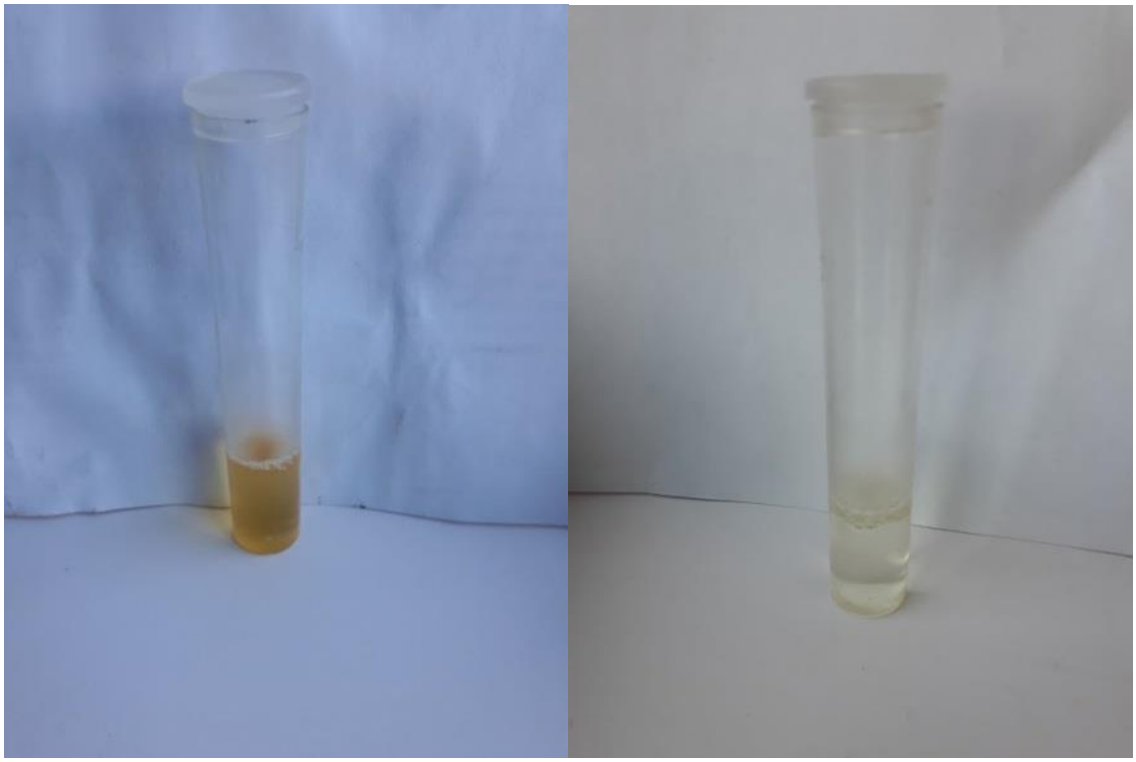


Figure 3. 3 Visual representation of ammonia concentration test in *Tetradesmus obliquus* grown in unmodified FFW before cultivation (left) and after cultivation (right)

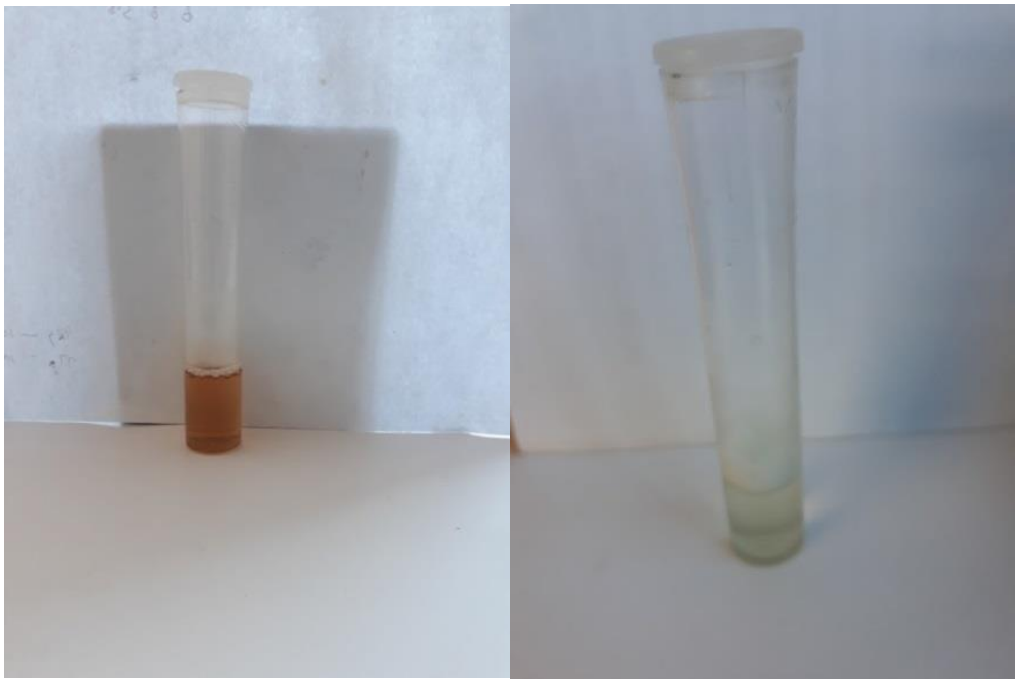


Figure 3. 4 Visual representation of nitrate concentration test in *Tetradesmus obliquus* grown in unmodified FFW before cultivation with higher nitrate (left) and after cultivation with low nitrate concentration (right)

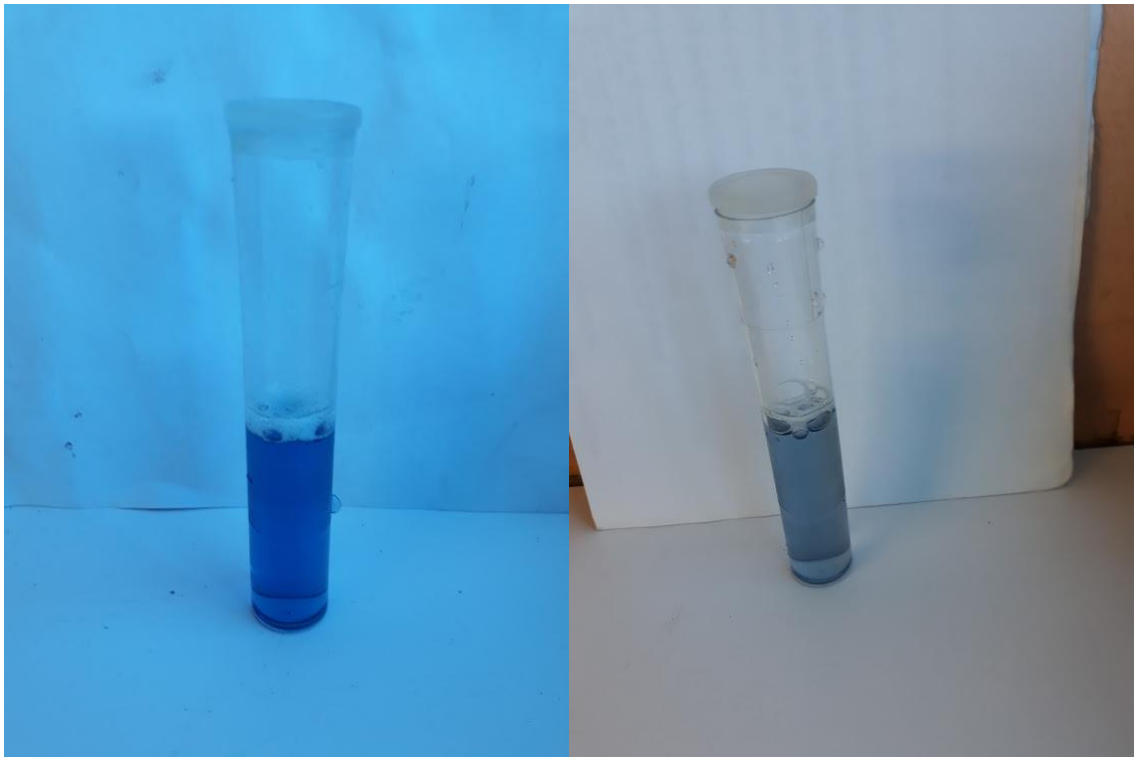


Figure 3. 5 Visual representation of phosphate concentration in *Tetradesmus obliquus* grown in unmodified FFW before cultivation with higher phosphate concentration (left) and after cultivation with lower phosphate (right)

3.2.5 Determining total lipid content

The total lipids were extracted using the modified Bligh and Dyer method as adapted from (Mujtaba et al., 2012). A final ratio of 1:1:0.8 (chloroform, methanol, water) was used in the total lipid extraction. An initial ratio of (1:2:0.8) of 1 ml of chloroform, 2 ml methanol and 0.8 ml of water was added to 20 mg of dried and crushed microalgae biomass. The mixture was sonicated for 2 mins at 150W and 50 kHz (Labotec Model 703, South Africa) and vortexed for about 30 Sec. The remaining ratio of chloroform and water were then added to make of the final ratio of 1:1:0.9 and the mixture vortexed again for 30 sec. The resulting solution was centrifuged at 4000 rpm for 5 mins. The lower part was transferred into a pre-weighed tube while the upper part was collected and the extraction process repeated with half the volume of reagents. The resulting chloroform mixtures were combined in the pre-weighed tube and dried in the oven at 80°C for 24 hours. The percentage lipid expressed as the dry cell

weight was used to represent the total lipids while lipid productivity was calculated using Equation 3.4 as in Singh et al (2015).

$$\text{Lipid productivity (mg/l/d)} = (\text{biomass productivity (mg/l/d)} \times \text{\% lipid content})/100 \quad (3.4)$$

3.2.6 Total Carbohydrate Test

The Phenol Sulfuric Acid Method with Spectrophotometer was used to determine the total carbohydrate content of the microalgae cultivated. This method was adopted from Dubois et al (1956). A small quantity of 14µl of phenol was added to 490µl of samples followed by rapid addition of 1.5ml of 95% H_2SO_4 in a fume hood. Sample readings were taken at 490nm using a Shimadzu UV-1800 spectrophotometer. D-glucose was used as a standard for making a calibration curve used to determine the carbohydrate concentration of the algae samples. 50mg of D-glucose was dissolved in 50ml of water to make a stock solution of 1mg/ml. 4ml of 0,5,10,15,20 and 25µg/ml concentration standards were prepared from the stock solution as presented in Appendix A.2. 80% phenol was prepared by dissolving 80g of phenol in 100ml of water. Microalgae samples of 5mg were dissolved in water and then, diluted to keep results within 0.3 to 1.5nm optical density range. The standard calibration curve was used to quantify carbohydrates and calibration data and graph is shown in Appendix A.3. Carbohydrate productivity was determined using Equation 3.5 as in Guldhe et al (2017a).

$$\text{Carbohydrate productivity (mg/l/d)} = (\text{biomass productivity (mg/l/d)} \times \text{(carbohydrate content))}/100 \quad (3.5)$$

3.2.7 Protein Analysis

Protein was extracted using 8M urea and was quantified using Bradford's method. Absorbance was measured at 595nm in a Shimadzu UV-1800 spectrophotometer. Bovine serum albumin (BSA) standard was used for the standard calibration curve. The calibration curve was used to quantify proteins and protein productivity was calculated with Equation 3.6 as in Guldhe et al(2017a).

$$\text{Protein productivity (mg/l/d)} = \frac{\text{biomass productivity (mg/l/d)} \times \text{(protein content)}}{100} \quad (3.6)$$

Bradford's reagent was prepared by dissolving 50mg of Coomassie brilliant blue in 50ml 95% ethanol, followed by addition of 100ml 85% phosphoric acid to the solution. The solution was filtered with a 0.45µm cellulose acetate filter paper and stored at 4 C in a brown bottle. $Na_2H_2PO_4$ buffer solution was prepared by adding 6g $Na_2H_2PO_4$ of to 800ml distilled water. The pH of the solution was brought to 7.4 using phosphoric acid and sodium hydroxide and the mixture made up to 1000ml with distilled water. To prepare 8M urea, 100ml of the buffer solution was gradually added to 54g urea in a beaker containing a stirrer and gradually heated to completely dissolve the urea. Dissolved urea solution was stored in a bottle at -4°C. Bovine serum albumin (BSA) standard was used for standard calibration. To make a BSA stock, 5ml of buffer was added in 5mg of BSA and homogenized in a vortex mixer for one minute.

Standard concentrations of 0, 10-, 20-, 30-, 40-, 50 µg/ml were prepared (Appendix A, Table A.4). 1 ml of 8 M urea was added to 1 mg of the sample in an Eppendorf tube and vortexed for 30 mins, then centrifuged at 12500 rpm for 15 mins. 200 µl of supernatant was diluted in 200 µg of 8 M urea to make up 400 µl, followed by the addition of 1600 µl of Bradford

reagent and mixed. The mixture was allowed to stand for 5 mins and absorbance was measured at 595 nm. The calibration data is shown in Appendix A.4 to Appendix A.5.

3.2.8 Effects of CO_2 Uptake

Rate of CO_2 uptake in the cultivation system was determined by monitoring daily, the pH of the culture media before the daily sparging of CO_2 was measured using an Ohaus Starter 3100 meter (Figure 3.6) and carbon content was determined with an organic elemental analyser Flash 2000 (Figure 3.7). About 1.75 mg to 1.85 mg of the sample was measured in a universal soft tin cup, the cup was then folded into a barrel shape using tweezers and put in the autosampler. The folded sample was pushed one at a time into a quartz reactor in the instrument Furnace at 950 °C for flash combustion with the help of oxygen using a Flash 2000 CHNS Elemental Analyzer from Thermofisher Scientific. The gaseous combustion compounds N_2, NOx, H_2O, SO_2, O_2 and CO_2 are transferred using helium as carrier gas through a column filled with copper oxide and from there to a Cu-column where nitrogen oxides are reduced to elemental nitrogen and O_2 to CuO. Water in another column can be absorbed. The remaining gasses are placed into a column of TPD (Temperature Programmed Desorption) where N_2 passes through it and the other gasses are added to the column. The gases are released separately with a scheduled temperature increase in the tube. The gasses pass through a detector of thermal conductivity (TCD) which generates an electrical signal proportional to the carbon concentration.

Using a mass balance, the quantity of CO_2 fixed (mg/L/d) by each cultivation was determined in from the relationship between the volumetric growth rate and carbon content of the microalgae cells as shown in Equation 3.7 as adapted from Ho et al (2012).

$$CO_2 \text{ fixation rate} = P * C_{Carbon} * (M_{CO2}/Mc) \quad (3.7)$$



Figure 3. 6 Ohaus Starter 3100 meter for measuring pH



Figure 3. 7 Organic Elemental Analyser for carbon content determination

3.3 Results and Discussion

All experiments were carried out in triplicates. Percentage nutrient removal, carbohydrate content, lipid content protein and CO_2 content were determined at the late log phase (15 days).

3.3.1 Characterisation of Wastewater

Wastewater was collected from the recirculatory system, used without modification (unmodified FFW) and modified (modified FFW) with distilled water to dilute the nutrient load to provide a more nutrient stress FFW growth media. FFW was analysed for nitrates, ammonia and phosphates required for the growth of microalgae. The characteristics (ammonia, nitrogen, phosphorus and pH) of both unmodified and modified FFW are shown

in Table 3.2. The nutrient removal efficiency was determined at the end of cultivation (Figure 3.8, Figure 3.9 and Figure 3.10). The nutrient composition of the FFW used in this study is in the range of some FFW previously used for microalgae cultivation for a different purpose (Table 2.6). This shows that the FFW has required nutrients to support microalgae growth and accumulation of biochemicals. The low nitrogen concentration in both FFW (ammonia 4.6 mg/l and nitrate 140 mg/l in unmodified FFW, ammonia 2.3 mg/l and nitrate 70 mg/l in modified FFW) is expected to create a nitrogen stress condition for higher lipid accumulation. High lipid production is beneficial for microalgae biodiesel production. Also, having mixed nitrogen sources in FFW (ammonia and nitrate) gives microalgae the option to utilise the nutrient preferred for the growth of the species. This could be the reason for the little lower lag phase observed in species grown in FFW (Appendix A.6).

The fast-growing aquaculture industry will continue to produce enormous volumes of wastewater. It is important to eliminate the nutrient loads of FFW before they are released to the environment to prevent eutrophication. These FFW can be utilised for the cultivation of algae to produce valuable biomass for biofuels production, reduce the cost of wastewater treatment (Guldhe et al., 2017a) and at the same time, reduce the cost of algae biodiesel feedstock production. In this study, three selected species have shown utilisation of nutrients in FFW for growth. Ammonia was almost completely removed from the culture media in both unmodified and modified FFW culture media (Figure 3.8). This removal can be attributed to uptake by microalgae for growth as preliminary analysis showed species used preferred Tris-acetate-phosphate (TAP) media to nitrate-based media (data not shown). Also, algae prefer ammonia to nitrate and therefore, will utilise ammonia first. Besides, the concentration of ammonia in FFW is lower than the concentration of nitrate and will be completely removed first even at similar rates of uptake of both nutrients. Although, the removal of nutrients in culture media especially ammonia and phosphates are not only

governed by algae uptake. Increasing temperature and pH can cause some quantity of ammonia to volatilize and phosphate to precipitate (Guerrero-Cabrera et al., 2014). This may not be the reason for near-complete removal of ammonia in this study even though temperature and pH were not regulated, pH rise in the media only occurred later during cultivation days after ammonia was completely removed from the media.

In the modified FFW, removal of all the nutrients was over 92% in all species cultivated. Ammonia (Figure 3.8) and nitrate (Figure 3.9) were almost completely removed and with less than 8 % phosphate (Figure 3.10) and 2% nitrate remaining in the media. The higher phosphate in the media maybe because not all compounds of phosphates are not bioavailable especially those bonded to metal ions (Grobbelaar, 2004). In the unmodified FFW, *Tetradesmus obliquus* had the best nutrient removal of 100%, 67.31% and 54.97% respectively for ammonia, nitrate and phosphate. The least nutrient removal efficiency was observed in *Chlamydomonas reinhardtii* with 98.12, 52.02 and 33.19% of ammonia, nitrate and phosphate respectively. The high removal efficiency observed in modified FFW can be attributed to low initial concentrations of nutrients. On the other hand, high nutrient absorption by algae observed in this study can also be attributed to the continuous supply of air and media sterilization as suggested by Guerrero-Cabrera et al (2014). The presence of ammonia and phosphates in water is toxic for aquatic organisms, algae utilise nutrients in FFW for the accumulation of biomass. This provides water with lower concentrations of ammonia and phosphate that can be reused in the cultivation of fish. Thereby, saves money for wastewater treatment and purchase of freshwater for fish farming. Furthermore, reduces eutrophication even when discharged. The suggestions of providing water that can be reused in fish farming and reducing cost associated with fish farming are in agreement with suggestions made in the studies using shrimp farm wastewater by Malibari et al (2018).

Table 3.2 Characteristics of unmodified and modified FFW utilised for microalgae cultivation

Characteristic	Value
Unmodified Fish WasteWater	
NH_4	4.6 ± 0.16 mg/ml
NO_3	140 ± 6.55 mg/ml
PO_4	15 ± 2.09 mg/ml
pH	7.3 ± 0.72
Modified Fish Farm Wastewater	
NH_4	2.3 ± 0.08 mg/ml
NO_3	70 ± 3.27 mg/ml
PO_4	7.5 ± 1.04 mg/ml
pH	7.3 ± 0.72

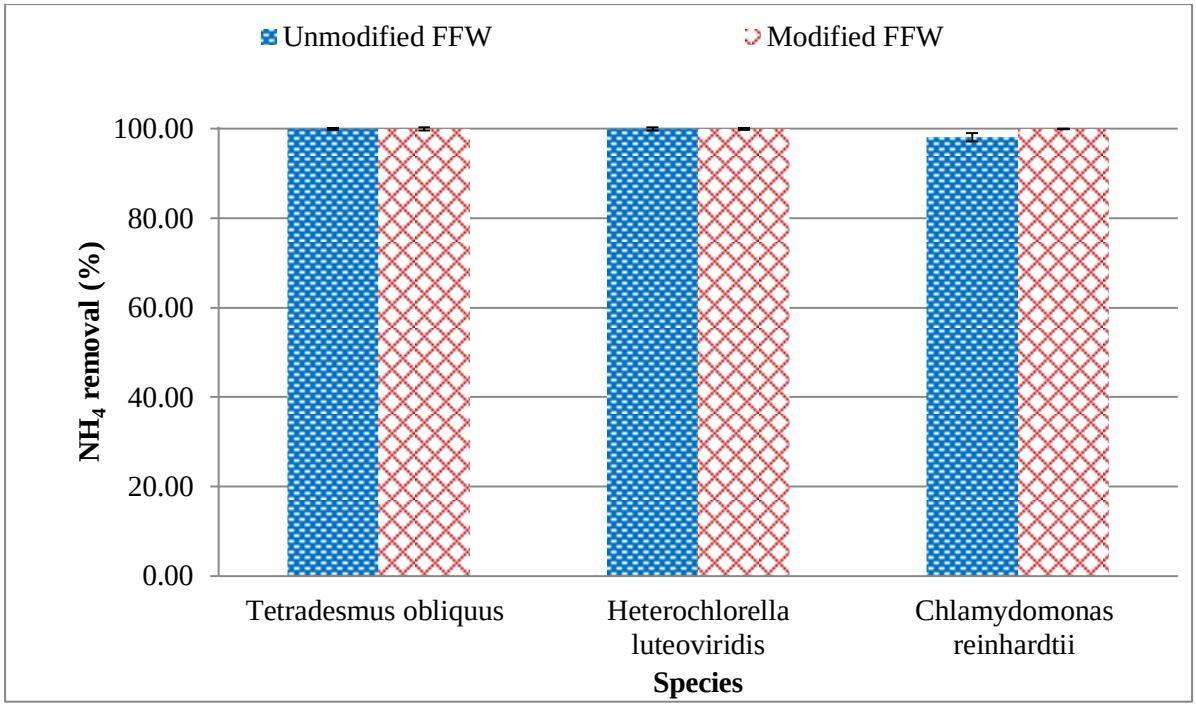


Figure 3. 8 Percentage NH_4 removal of *Tetradesmus obliquus*, *Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* in unmodified FFW and modified FFW

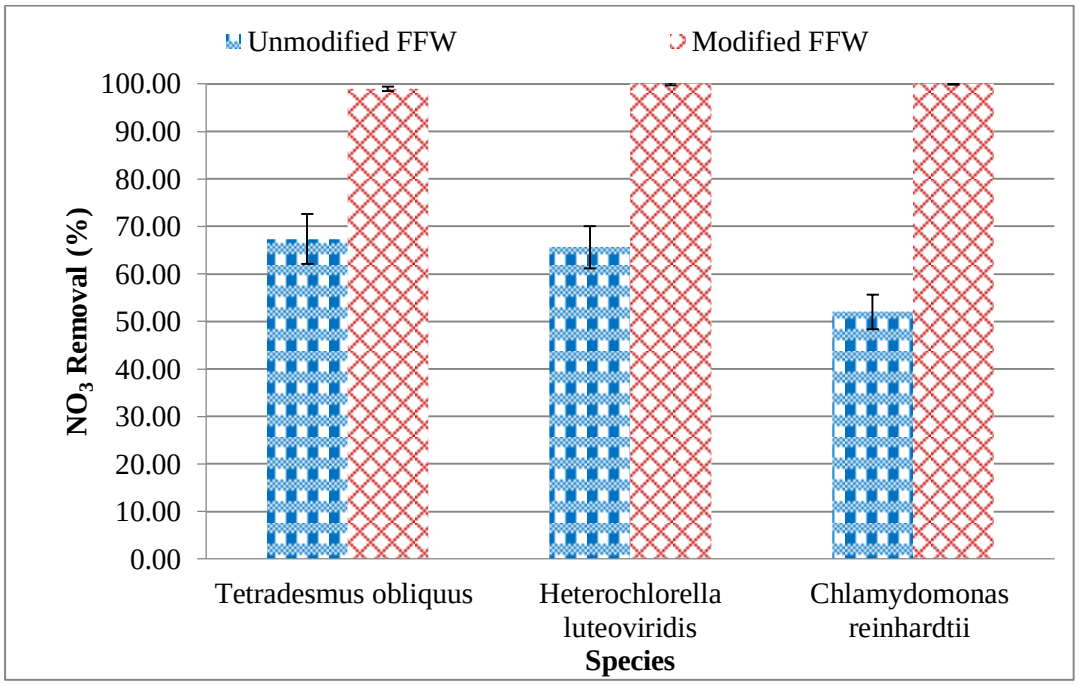


Figure 3. 9 Percentage NO_3 removal of *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* in unmodified fish farm wastewater and modified fish farm wastewater

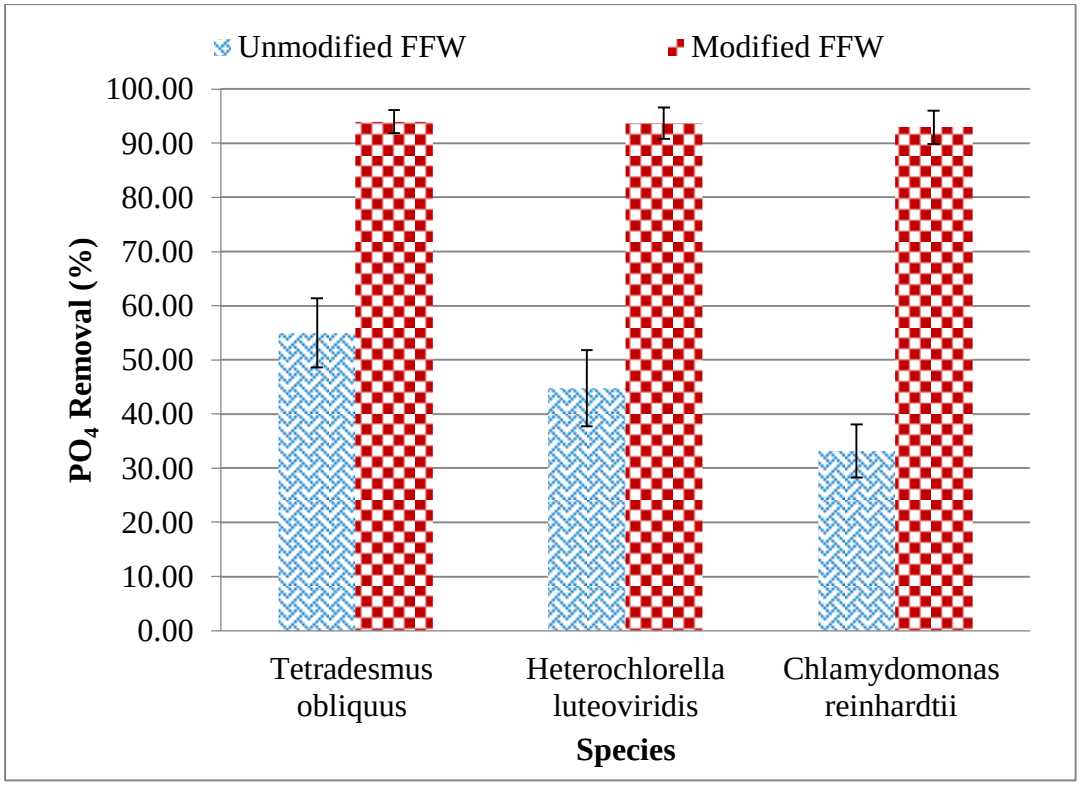


Figure 3. 10 Percentage PO_4 removal and of *Tetradesmus obliquus*, *Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* in unmodified FFW and modified FFW

3.3.2 Growth, Biomass Yield and Biochemical Content

Table 3.3 Specific growth rate, biomass yield, lipid, carbohydrate and protein content of the three algae species in standard growth media, unmodified FFW and modified FFW

Samples	Specific growth rate (day^{-1})	Biomass yield (mg/l)	Lipid content (%)	Carbohydrate content (%)	Protein content (%)
T1	0.15 ± 0.02	116.08 ± 3.75	31.25 ± 0.18	22.25 ± 0.14	10.11 ± 0.14
T2	0.19 ± 0.04	105.64 ± 6.15	33.06 ± 0.09	26.64 ± 0.14	7.74 ± 0.13
T3	0.15 ± 0.02	94.72 ±3.2	33.75 ± 0.08	22.70 ± 0.44	8.32 ± 0.24
F1	0.15 ± 0.017	158.75 ± 3.09	36.42 ± 0.05	23.10 ± 0.29	6.74 ± 0.23
F2	0.18 ± 0.04	173.71 ± 5.59	42.33 ± 0.32	27.00 ± 0.12	7.39 ± 0.10
F3	0.16 ± 0.05	97.29 ± 5.63	44.08 ± 0.35	35.04 ± 0.13	7.26 ± 0.20
LN F1	0.14 ± 0.01	182.52 ± 1.67	37.85 ± 0.25	21.99 ± 0.32	5.91 ± 0.25
LN F2	0.17 ± 0.01	185.62 ± 1.34	42.07 ± 0.07	21.21 ± 0.28	4.90 ± 0.21
LN F3	0.15 ± 0.02	118.71 ± 1.35	40.92 ± 0.10	18.90 ± 0.27	11.47 ± 0.27

T- Tris-acetate-phosphate, F- Unmodified Fish Farm Wastewater, LN F- Modified Fish Farm Wastewater, 1-*Tetradesmus obliquus,* 2- *Heterochlorella luteoviridis,* 3- *Chlamydomonas reinhardtii.*

Specific growth rate and biomass yield are depicted in Table 3.3 while biomass productivity is depicted in Figure 3.11. There was no relationship between the specific growth rate to both biomass yield and biomass productivity and therefore, presented as reference as was also presented in Guerrero-Cabrera et al (2014). But there was a correlation between biomass yield and biomass productivity. The biomass yield and productivity obtained in this study is similar to the biomass yield and productivity obtained in Guerrero-Cabrera et al (2014).

Lowest biomass yield of the same species was obtained in Tris-Acetate-Phosphate growth media and the highest biomass yield was obtained in modified FFW media. *Chlamydomonas reinhardtii* in both FFW had biomass yields within ranges obtained in Tris-Acetate-Phosphate growth media while *Tetradesmus obliquus* and *Heterochlorella luteoviridis* in both FFW had a wider biomass yield than the same species in Tris-Acetate-Phosphate. *Heterochlorella luteoviridis* had the highest biomass yields in all three cultivation media, with the highest yield of 185.62 mg/l in modified FFW, 173.71 mg/l in unmodified FFW and 105.64 mg/l in Tris-Acetate-Phosphate. *Tetradesmus obliquus* had the next highest yield which followed the same yield trend as *Heterochlorella luteoviridis* in all the different growth media.

Algae in nitrate as nitrogen source attains higher growth rate and biomass productivity than algae cultivated in ammonia-based media as a nitrogen source (Guerrero-Cabrera et al., 2014). FFW also contains nitrates in addition to ammonia, the nitrate content of FFW might be the reason why species cultivated in FFW obtained higher biomass yield and productivity than same species cultivated in the Tris-Acetate-Phosphate media which contains only ammonia even with a higher concentration of ammonia (400 mg/l).

However, lower biomass yield and productivity were obtained in this study when compared to biomass yield and productivity obtained in studies by Ansari et al (2017) and Gao et al (2016). Lower concentrations of nitrate in the FFW and the absence of nitrate in the standard media could be a reason for lower yield and productivity obtained in all samples (between 185.62 mg/l to 94.72 mg/l for biomass yield and 6.77 to 13.26 mg/l/d biomass productivity). The values are about 10 times lower than values for biomass yield and biomass productivity obtained in studies by Ansari et al (2017) using BG11, FFW and FFW supplemented with different concentrations of sodium nitrate. In their study, biomass yield and biomass productivity were higher in FFW supplemented with sodium nitrate and increased with

increasing concentration of the supplemented nutrient. Another reason why this study obtained lower biomass yield and productivity can be attributed to lower initial biomass concentration of about 20mg/l unlike about 200mg initial concentration in studies by Ansari et al (2017) as initial concentration affects biomass yield and nutrient removal Gao et al (2016). On the other hand, the biomass yield and productivity range of this study did not vary much from results obtained in some other studies Doan et al (2011) and Song et al (2013). Studies by Song et al (2013) cultivated different microalgae species in BG 11 and f2 growth while studies by Doan et al., (2011) cultivated microalgae in Walne and f2 growth media. The comparable results obtained from other studies justifies that nutrients in FFW are suitable for microalgae biomass production. The higher biomass yield and productivity obtained in this study in FFW suggest that FFW are more suitable for biomass yield and productivity. Again, this could be attributed to the presence of different nitrogen sources (ammonia and nitrate) in FFW. Biomass productivity of all species cultivated in the different growth media had the same trend with biomass yield as explained above.

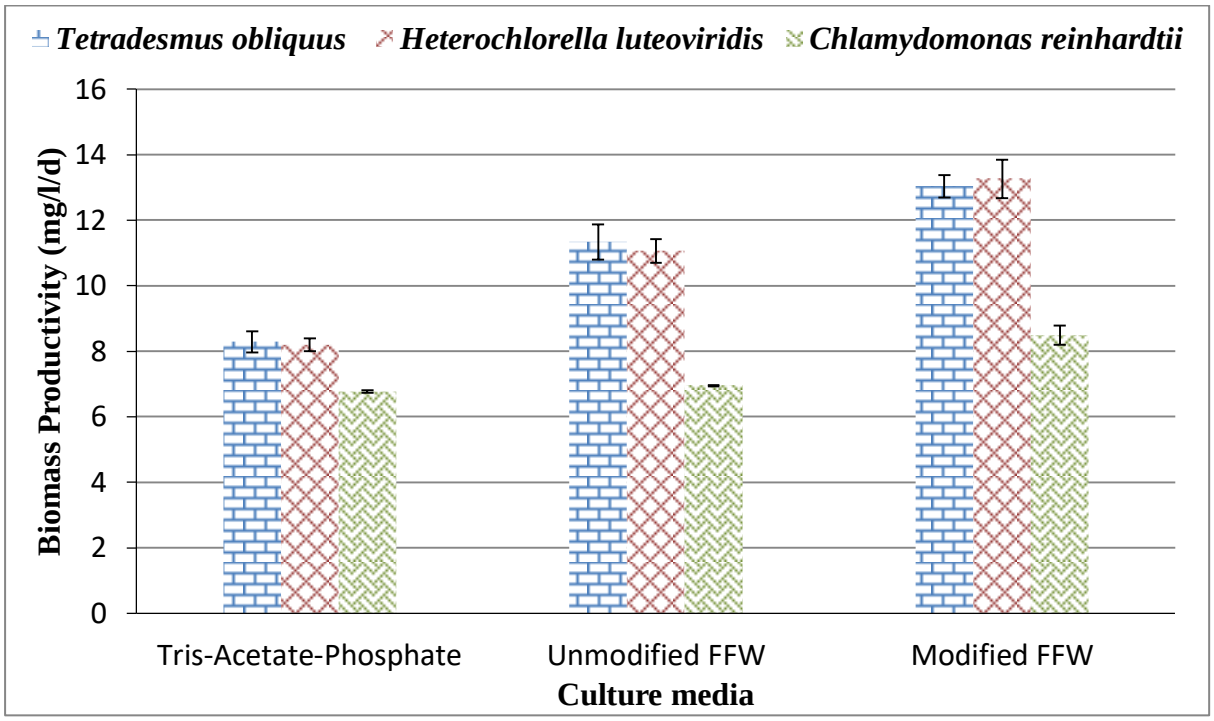


Figure 3. 11 Biomass productivity of the three algae species cultivated in standard Tris-Acetate-Phosphate standard growth media, unmodified FFW and modified FFW.

3.3.3 Biochemical Composition

Harvested algae biomass cultivated in FFW was analyzed for lipids, carbohydrates and proteins. The composition of the biochemical compositions obtained is shown in Table 3.3. The biochemical composition of algae (lipids, carbohydrate and proteins) depends on the nutrient makeup of the culture media and cultivation conditions (Guldhe et al., 2017b).

3.3.3.1 Lipid Content of Microalgae

Lipid content of *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* cultivated in standard Tris-Acetate-Phosphate (TAP) had lipid contents of 31.25-, 33.06- and 33.75% dry cell weight respectively. The lipid content of the same species cultivated in fish farm wastewater (FFW) growth media was higher than the lipid content of the Tris-Acetate-Phosphate media. *Tetradesmus obliquus*, *Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* in unmodified FFW had 36.42-, 42.33- and 44.08% respectively and 37.85-, 42.07- and 40.92% in the same order in modified FFW. In FFW, the highest lipid accumulation of 44.08% was obtained in *Chlamydomonas reinhardtii* in unmodified FFW while *Tetradesmus obliquus* in modified FFW had the lowest lipid content of 37.85%. The higher lipid accumulation of *Chlamydomonas reinhardtii* in unmodified FFW, when compared to the same species in modified FFW, can be due to lower biomass yield obtained in unmodified FFW. Even with lower biomass yields obtained from species cultivated in Tris-Acetate-Phosphate, higher lipid content obtained from same species cultivated in FFW can be attributed to lower nitrogen concentration. Microalgae accumulate more lipids in nutrient-deficient media (Ansari et al., 2017). Lipid productivity was higher in FFW. Highest lipid productivity was obtained in species cultivated in modified FFW, followed by species

cultivated unmodified FFW and then species cultivated in Tris-Acetate-Phosphate standard growth media. The higher mean lipid productivity of species in modified FFW even with slightly lesser lipid content can be attributed to higher biomass yield of species grown in modified FFW. However, the lipid productivities of species in FFW did not significantly vary from the other (Figure 3.12).

Studies by Ansari et al (2017) using *Scenedesmus obliquus*, *Chlorella sorokiniana* and *Ankistrodesmus falcatus* achieved a higher percentage lipid content in microalgae cultivated in FFW when compared to the lipid content of the same species cultivated in a synthetic BG-11 growth media. This is similar to the results obtained in this study with higher lipid contents obtained in species cultivated in FFW. This can be attributed to lower nitrogen concentrations in the FFW media causing a nitrogen stress condition, a condition that favours lipid accumulation.

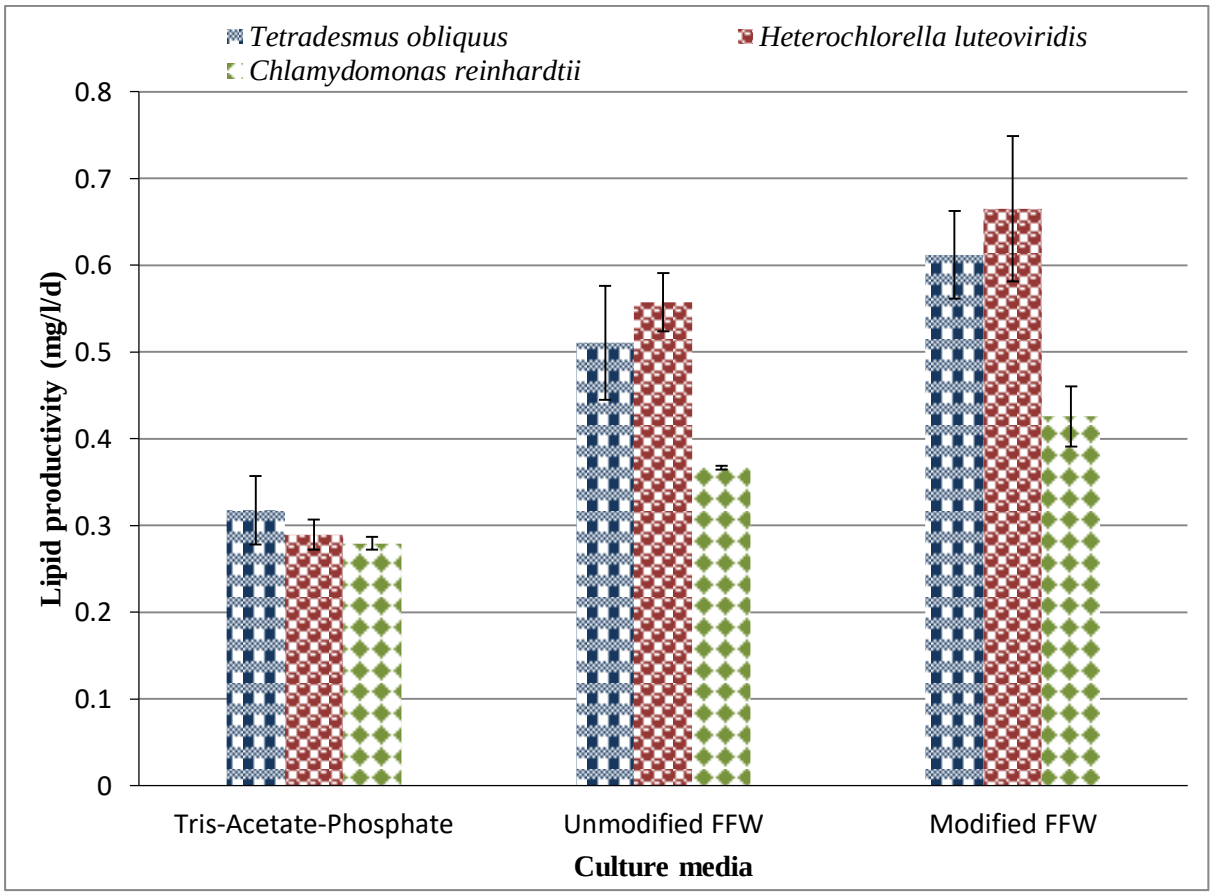


Figure 3. 12 Lipid productivity of the three algae species cultivated in standard Tris-Acetate-Phosphate (TAP) standard growth media, unmodified FFW (UFFW) and modified FFW (MFFW).

3.3.3.2 Carbohydrate Content of Microalgae

Carbohydrate content (Table 3.3) for *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* in Tris-Acetate-Phosphate media were 22.25, 26.64 and 22.70% dry cell weight respectively. There was increased carbohydrate composition in the unmodified FFW of 23.10, 27.00, 35.04% respectively for *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii.* This increase was similar to the trend observed by (Guldhe et al., 2017b) which he attributed to the lower nutrient content of the culture media as microalgae accumulate more lipids and carbohydrates under nutrient stress. The low nutrient concentration in unmodified FFW can be associated with higher carbohydrate content obtained in species cultivated in FFW. Going by the accumulation of more carbohydrates under stress level, the higher increase was expected of *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* in modified FFW with 21.99, 21.21 and 18.90% which were even lower than what was obtained in Tris-Acetate-Phosphate media with higher nutrient composition. The higher growth rate of species cultivated in modified FFW could be the reason for lower carbohydrate composition (species used nutrients for growth instead). In terms of carbohydrate productivity (Figure 3.13), productivity increased with reducing concentrations of nutrients per species with *Chlamydomonas reinhardtii* in modified FFW marginally more than same species in Tris-Acetate-Phosphate standard growth media and lower in unmodified FFW. *Tetradesmus obliquus, Heterochlorella luteoviridis* in both FFW had significantly higher carbohydrate productivity to sample in Tris-Acetate-Phosphate standard growth media.

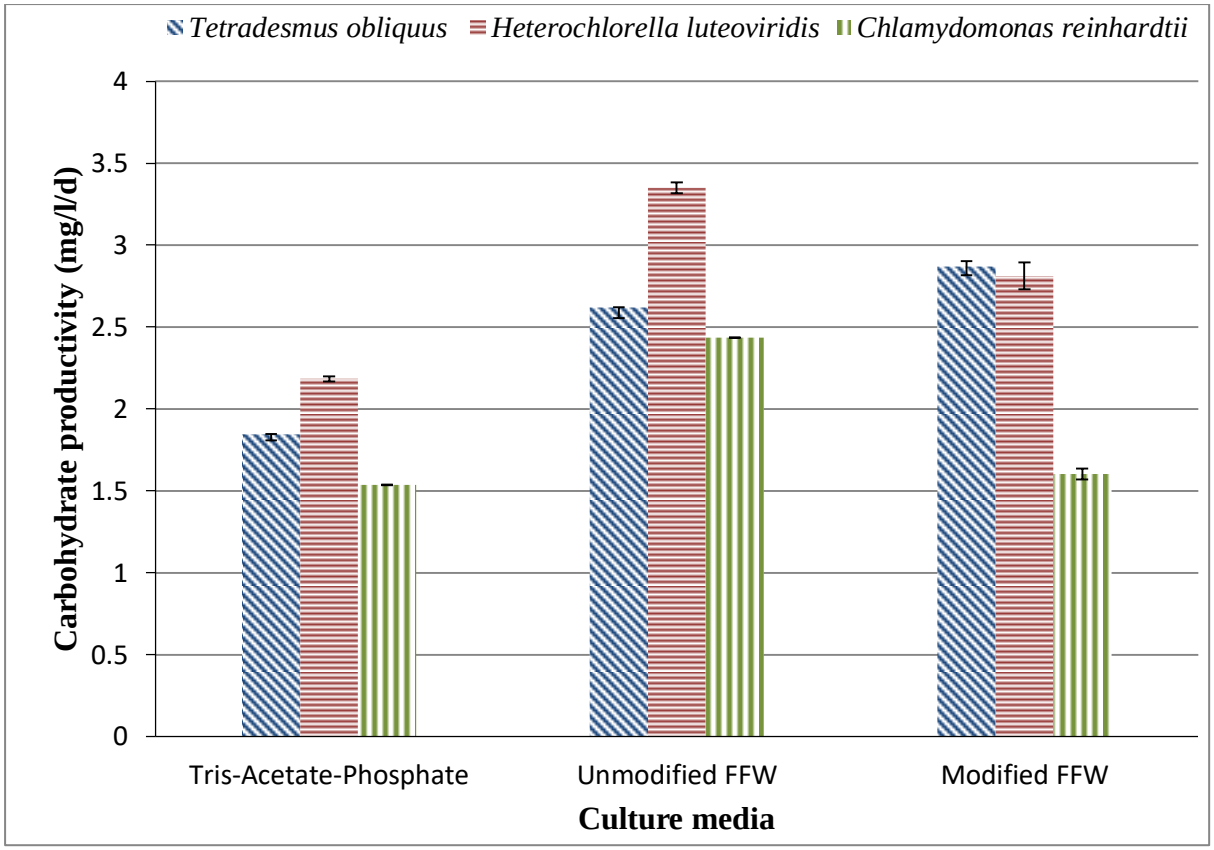


Figure 3. 13 Carbohydrate productivity of the three algae species cultivated in standard Tris-Acetate-Phosphate standard growth media, unmodified FFW and modified FFW.

3.3.3.3 Protein Content

Percentage protein contents by species were higher in species cultivated in Tris-Acetate-Phosphate growth media with 10.11, 7.74 and 8.32% in *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* respectively. Following the same species order for cultivation in unmodified FFW, protein contents were 6.74-, 7.39- and 7.26% and for unmodified FFW 5.91, 4.9, 11.47% accordingly. The protein composition of the different species in the different culture media is shown in table 3.3. The higher protein content of species cultivated in Tris-Acetate-Phosphate growth media can be attributed to higher concentrations of nitrogen in the standard media (Ansari et al., 2017). Protein productivity of *Tetradesmus obliquus* cultivated in the different culture media did not significantly vary from the other as shown in Figure 3.14. *Tetradesmus obliquus* had similar

protein content in all media while Heterochlorella and *Chlamydomonas* accumulated most protein in unmodified FFW and modified FFW respectively.

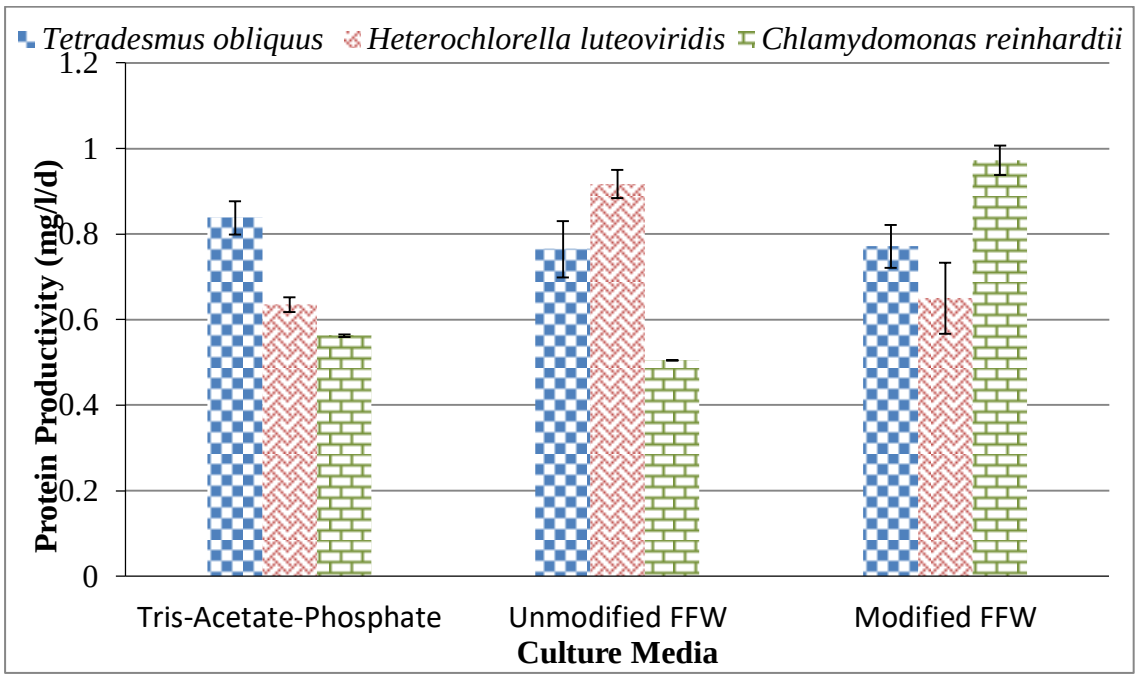


Figure 3. 14 Protein productivity of the three algae species *Tetradesmus obliquus*, *Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* cultivated in standard Tris-Acetate-Phosphate standard growth media, unmodified FFW and modified FFW.

3.3.4 Carbon content and CO_2 Fixation

The carbon content of the different microalgae and quantity of CO_2 fixed is shown in Table 3.4 while Figures 3.15, 3.16 and 3.17 compare pH measurement of the same species in the three different growth media. A similar pH trend was observed in different growth media. pH decreased in the standard growth media with time while pH in both FFW increased with time (Figures 3.15 to 3.17). pH rise in both FFW can be attributed to higher uptake of CO_2 which leads to the release of higher quantities HO^-. HO^- in the media is responsible for the rise in pH of growth media (Richmond, 2004). This could be the reason for higher biomass yield obtained in species cultivated in FFW as shown in Table 3.3. Using ammonia as the only nitrogen source leads to a decrease in pH due to the release of H^+ ion into the media. Also,

the use of only nitrate as the only nitrogen source leads to a rise in pH (Grobbelaar, 2004). This could be the reason the pH concentration in the TAP growth media containing ammonia as the only nitrogen source was decreasing. The rise in pH in both FFW can also be attributed to the fact that ammonia in the FFW media was used up first as observed leaving nitrate as the only source of nitrogen in the media.

CO_2 fixation was least in species cultivated in TAP growth media, higher in unmodified FFW and highest in modified FFW (Table 3.4). CO_2 had a similar fixation trend in all growth media with highest fixation rate in *Tetradesmus obliquus* followed by *Heterochlorella luteoviridis* and lowest in *Chlamydomonas reinhardti*. The CO_2 fixation correlates to carbon content, biomass yield and productivity. As can be seen in Figures 3.15 to 3.17, the highest pH was observed in modified FFW, followed by unmodified FFW and least in TAP media. This shows that species cultivated in both FFW had better CO_2 removal rates. This is in accordance with the pH effects on the culture media where higher pH in the media depicted higher usage of CO_2, biomass yield and productivity. This suggests that FFW are better CO_2 sequestration media than TAP media and can be a better media in mitigating the environmental effects of CO_2 to the atmosphere.

The higher pH in FFW media was an indication that microalgae in FFW utilise CO_2 quicker which in turn, leads to a rise in pH. The higher biomass yield and productivities obtained in FFW also adds that microalgae in FFW used more CO_2. The higher CO_2 utilization can be attributed to the properties of the FFW media which contain both ammonia and nitrate as different forms of nitrogen. The presence of both forms of nitrogen could have enhanced CO_2 uptake and growth by providing microalgae preferred form for growth, especially at the lag phase. The lower lag phase obtained in FFW (Appendix A.6) also suggest the better performance of different nitrogen sourced in the FFW media.

Table 3.4 Carbon content and CO_2 fixation of cultivated microalgae species in the different growth media

Sample	Media	Carbon content (%)	CO_2 fixation (mg/L/d)
Tetradesmus obliquus	Tris-Acetate-Phosphate	43.73 ± 0.24	1328.71
Heterochlorella luteoviridis	Tris-Acetate-Phosphate	42.46 ± 0.11	1274.8
Chlamydomonas reinhardtii	Tris-Acetate-Phosphate	41.13 ± 0.45	1019.69
Tetradesmus obliquus	Unmodified fish farm wastewater	45.905 ± 0.13	1907.45
Heterochlorella luteoviridis	Unmodified fish farm wastewater	44.36 ± 0.49	2016.9
Chlamydomonas reinhardtii	Unmodified fish farm wastewater	44.035 ± 0.15	1121.31
Tetradesmus obliquus	Modified fish farm wastewater	45.9 ± 0.13	2192.84
Heterochlorella luteoviridis	Modified fish farm wastewater	46.405 ± 0.26	2254.62
Chlamydomonas reinhardtii	Modified fish farm wastewater	45.54 ± 0.25	1415.05

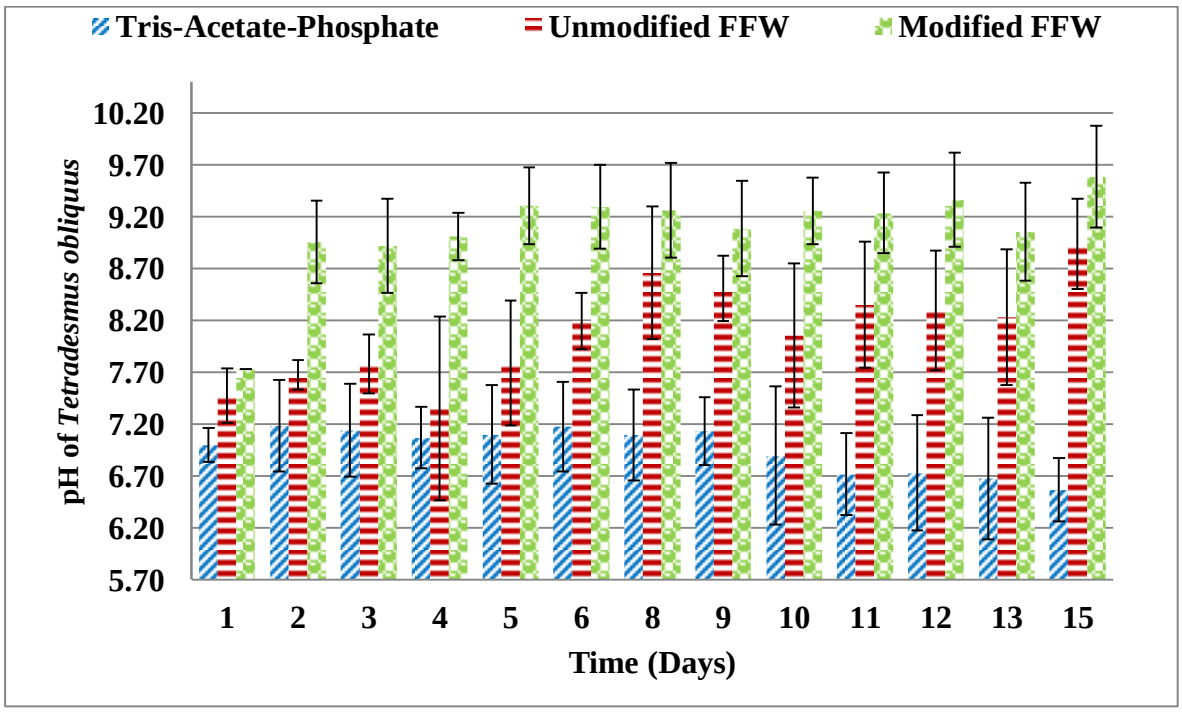


Figure 3. 15 pH of *Tetradesmus obliquus* in different culture media.

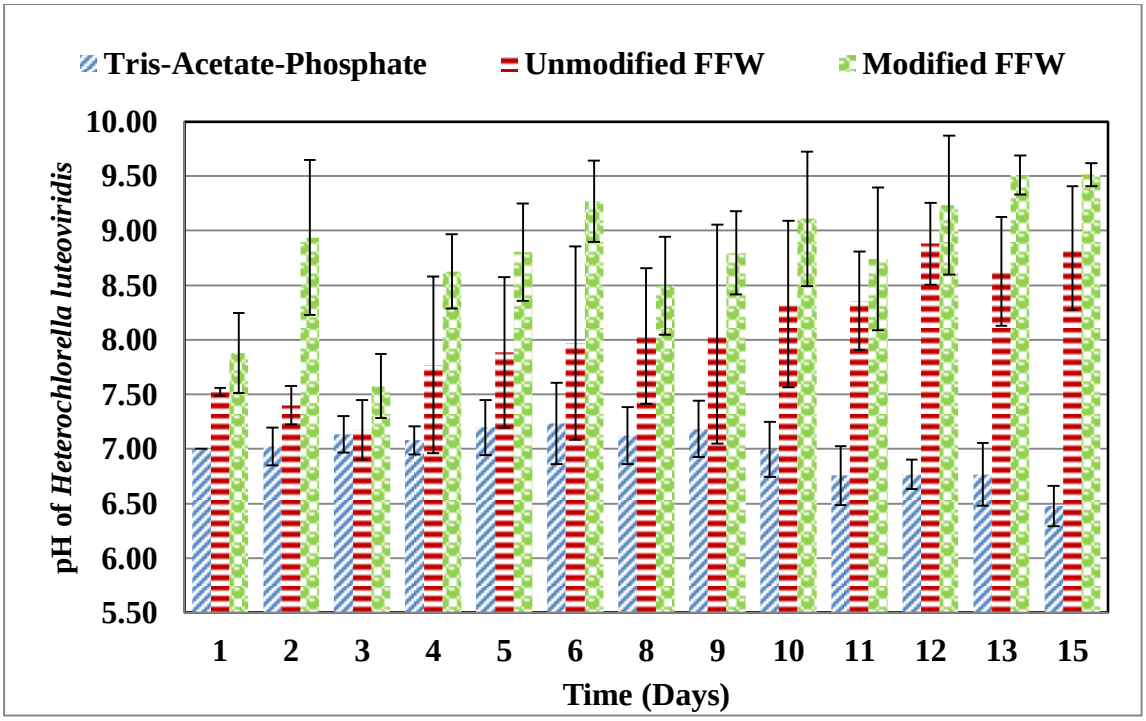


Figure 3. 16 pH of *Heterochlorella luteoviridis* in different culture media.

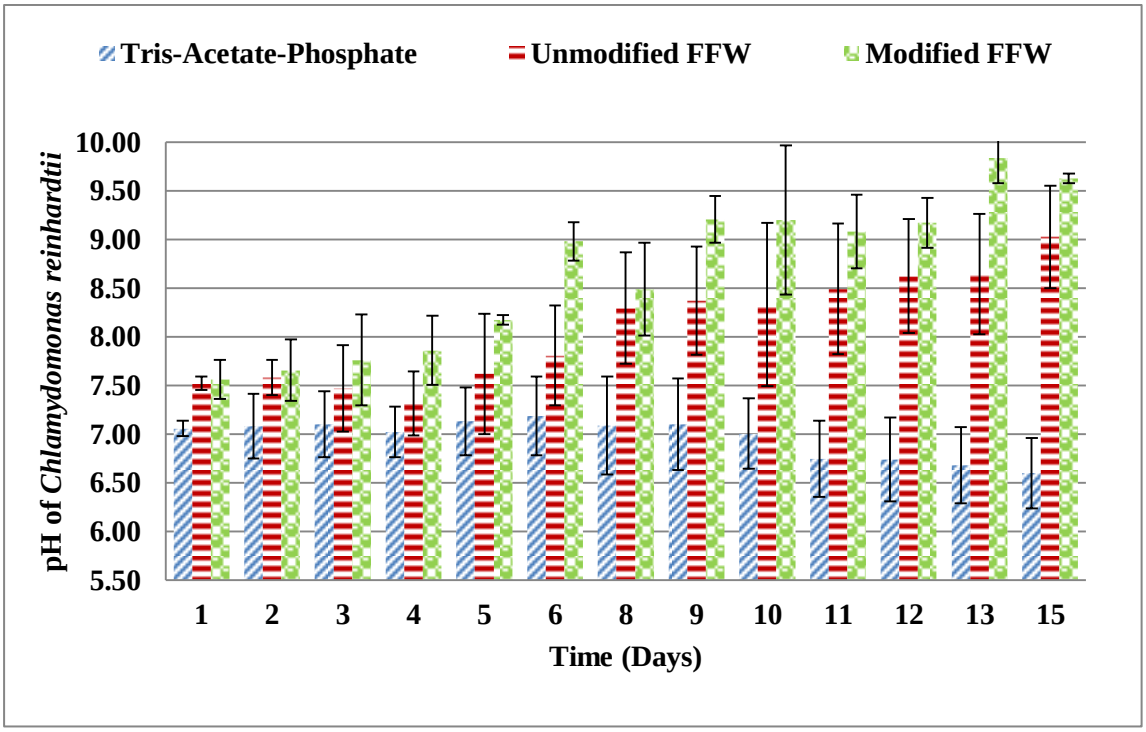


Figure 3. 17 pH of *Chlamydomonas reinhardtii* in different culture media.

3.4 Conclusion

Results obtained from this study shows that nutrients in fish farm wastewater (FFW) ammonia, nitrate and phosphorus- are suitable for cultivating microalgae. Additionally, the algae utilised these nutrients in FFW for growth, biomass productivity and accumulation of biochemical compounds (lipids, carbohydrates and proteins). When compared to Tris-Acetate-Phosphate standard growth media, microalgae cultivated in FFW had higher biomass yield and biomass productivity, higher lipid content and productivity, higher CO_2 uptake, CO_2 content and comparable carbohydrate composition. In this work, it was found that lower nutrient concentrations (ammonia, nitrate and phosphate) in FFW lead to accumulation of higher lipid and carbohydrate content in the species cultivated in FFW. Also, it found that the combination of ammonia and nitrogen in the FFW provided microalgae with a nitrogen preference which in turn, facilitated higher biomass yield and productivity in addition to higher CO_2 utilization. Higher lipid content and lipid productivity obtained in FFW shows that low ammonia and nitrate concentrations in FFW are suitable for growing microalgae for biodiesel production. The ability of FFW nutrients to support better growth of microalgae and higher lipid content shows that FFW can substitute purchased nutrients required for microalgae cultivation for biodiesel production and in turn, eliminated cost associated with the nutrient purchase. *Chlamydomonas reinhardtii* in unmodified FFW had the best biochemical composition (lipid and carbohydrates) for production of biofuels especially biodiesel. It also achieved a desirable nutrient removal of 98.12, 52.02 and 33.19% nutrient removal efficiency for ammonia, nitrates and phosphates respectively but had lower biomass yield. The higher lipid accumulation in *Chlamydomonas reinhardtii* in unmodified FFW can be attributed to not only low nutrient content of the FFW but also to slow growth rate. Thereby, accumulating more lipids instead of growing. Results also show that similar biomass yield and biochemical compositions were obtained in both FFW and the extra

benefit of modified FFW was in higher lipid productivity and nutrient removal efficiency. The extra benefits can be attributed to low nutrient concentrations in modified FFW. Higher nutrient removal by microalgae cultivated in both FFW for biodiesel production will not only benefit the environment by reducing the eutrophication effects of discharged high nutrient load FFW but will also, save freshwater resources and cost of water treatment for the fish farmer. Compared with other studies, this was the first time the rate of CO_2 uptake and content of *Tetradesmus obliquus, Heterochlorella luteoviridis,* and *Chlamydomonas reinhardtii* cultivated in FFW was determined in addition to lipid, carbohydrate and protein content. Higher CO_2 uptake and CO_2 content were achieved in species cultivated in both FFW.

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Chapter 4 Characterisation of Biodiesel from Microalgae Cultivated in Fish Farm Wastewater

A research paper on the work of this chapter has been accepted for publication by the journal 'SN Applied Sciences'. Enwereuzoh U, Harding K, Low M, (2020). Characterization of biodiesel produced from microalgae grown on fish farm wastewater. *SN Applied Sciences,* 2020;(2)5.

The contribution of the co-author's (Prof Kevin Harding and Dr. Michelle Low) towards the work is primarily that of supervision or collaboration, and the work and write-up were carried out by the author of this thesis.

This chapter aims to determine the fatty acid composition of biodiesel obtained from microalgae cultivated in FFW and calculate the fuel properties from the fatty acid methyl ester composition.

The composition of the growth media affects the composition of the microalgae cultivated. The composition of FAME in the biomass determines the properties of biodiesel obtained. In this chapter, we tried to determine the suitability of nutrients in FFW for growing algae for biodiesel production.

Abstract

Most research on microalgae biodiesel has been focused on improving growth rate and lipid productivity in different sources of wastewater with limited research on the quality of the potential biodiesel which can be produced. The properties of lipids, which determine the quality of biodiesel produced, vary between algal species and with nutrient conditions of the growth medium used to grow the alga. It is, therefore, important to determine the fuel properties of biodiesel produced from microalgae cultivated using nutrients in fish farm wastewater (FFW). To determine the quality of biodiesel obtained from microalgae cultivated in FFW, it is also important to establish the suitability of nutrients in FFW as cheap alternative growth media for growing algae biomass for biodiesel production. This project investigated the influence of fish wastewater nutrients on properties of fatty acid methyl esters (FAME) of three microalgae species (*Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii*) cultivated in unmodified and modified FFW. The fuel properties of the FAME obtained were then compared to the fuel properties obtained from FAME cultivated in a standard growth media of modified Tris-Acetate-Phosphate (TAP). Fuel properties of biodiesel produced from microalgae species cultivated in FFW were similar to fuel properties of biodiesel from the same species cultivated in the standard growth media. Overall, *Chlamydomonas reinhardtii* (in both FFW media) had the most suitable biodiesel property, especially when looking at the cold filter pour point (CFPP).

Keywords: Biodiesel; microalgae; fish farm wastewater, fuel properties, fatty acid methyl ester

4.1 Introduction

Biofuels are gaseous or liquid fuels for internal combustion engines usually made from biomass (Russo et al., 2012). Biofuels are classified into primary and secondary biofuels. The primary biofuels such as wood chips and fuelwood are used in their raw form without processing for heating, electricity and cooking. Secondary biofuels are processed from biomass such as biodiesel and bioethanol and can be utilised in vehicles and different industrial processes (Nigam and Singh, 2011).

1st generation biofuels are made with properly established conversion methods using edible and commonly available feedstock. Most biofuels currently in use fall under this category like biodiesel made by transesterification of animal fats and vegetable oil, ethanol from fermentation of sugar (sugarcane, sorghum, corn, etc). 2nd generation biofuels are made from advanced, non-food feedstock with advanced processing methods. Non-edible triglycerides and lignocellulose (like algae and jatropha) are examples of advanced feedstocks while examples of advanced conversion methods are thermal conversion (pyrolysis and gasification) of lignocellulose and triglycerides catalytic hydroprocessing to produce renewable diesel (Hoekman et al., 2012). Land requirement for biofuels production has reduced along with transitions from first, second to third generation biomass due to increased energy yield per hectare and to non-agricultural land utilization (Fenton and Ó hUallacháin, 2012).

Microalgae can be used in the production of different kinds of biofuels such as biodiesel, bio-hydrogen, bio-ethanol, bio-oil and bio-syngas, through different conversion processes Biodiesel is a clear amber-yellow liquid with similar viscosity to that of fossil diesel, is non-flammable, unlike petroleum diesel, non-explosive with 423K flash point compared to 337K flash point of petroleum diesel (Yusuf et al., 2011) and has higher heating value (HHV) 39-

41 MJ/kg, higher than coal 32-27 MJ/kg and slightly lower than petrol diesel 43 MJ/kg (Demirbas, 2009). Any diesel fuel substitute obtained from renewable biomass source can be generally referred to as biodiesel (Demirbas, 2009; Sheehan et al., 1998).

Biodiesel is the best replacement for fossil diesel and is also more environmental friendly than the petrol-diesel (Atadashi et al., 2010). Biodiesel has other advantages over petrol diesel as renewable, biodegradable, has low exhaust emissions, not toxic and essentially free from sulfur and aromatics (Demirbas, 2009; Ma and Hanna, 1999; Van Gerpen, 2005; Vasudevan and Briggs, 2008) and can be used in any engine without modification because it has similar properties with diesel fuel (Agarwal, 2007; Demirbas, 2007). Biodiesel most often is used in different ratio blends with petrol diesel 2% (B2), 5% (B5) or 20% (B20). Also, it can be stored and transported using diesel tanks and equipment. Furthermore, it has better lubricating properties than diesel fuel because biodiesel is oxygenated, is more completely combusted and increasing the life of engines. Also, it is safer to use, store and handle due to the high flash point of biodiesel. In sensitive environments like heavily polluted cities, biodiesel is an ideal fuel to use due to relatively low emissions (Vasudevan and Briggs, 2008).

Biodiesel can be produced from a variety of natural oil such as soybean, rapeseed and other feedstock including recycled cooking oil and animal fat (Agarwal, 2007; Sheehan et al., 1998). Biodiesel is primarily made in four ways; (1) transesterification, (2) thermal cracking (pyrolysis), (3) direct use and blending and (4) microemulsions. Transesterification of animal fats and vegetable oil is the most commonly used method (Ma and Hanna, 1999). Transesterification is achieved by reacting biologically derived oils (animal fats and vegetable oils) that are made of fatty acids (FA), with monohydric alcohols in the presence of a catalyst. During the transesterification reaction, the FAs bond with the methyl group to produce FAME and glycerol (L. C. Meher et al., 2006).

The quality of biodiesel obtained is, therefore, directly influenced by the composition of the FAs produced by the algal species (Talebi et al., 2013) this makes lipid composition an important parameter in species selection for commercial microalgae biodiesel production (Huerlimann et al., 2010). The structural properties of the fatty acids, such as level of saturation, chain length and branching, affect the chemical and physical properties of biodiesel (Islam et al., 2013; Knothe, 2005). These fatty acid properties vary with species and nutrient conditions of the algal culture (Williams and Laurens, 2010) and in turn, determine the main parameters of the biodiesel including cetane number, oxidative stability, kinematic viscosity, cold flow and lubricity (Knothe, 2005).

FAs are the building blocks of Triacylglycerol (TAG) and every other cellular lipid. Under environmental stress conditions, some microalgae can accumulate TAGs up to 20-50% dry cell weight. TAGs serve as energy and carbon storage and do not perform structural roles like other lipids (Hu et al., 2008). There is a more diverse chain length distribution in different microalgae species than in higher plants. This makes it possible for selection of certain species for selected fuel properties, like the selection of species with less than C_{16} chain length for jet fuels and selection of species with very-long-chain length C_{20} for lubricants (Williams and Laurens, 2010). A schematic of TAG is shown in Figure 4.1 while FA names, acronyms and abbreviation is shown in Table 4.1

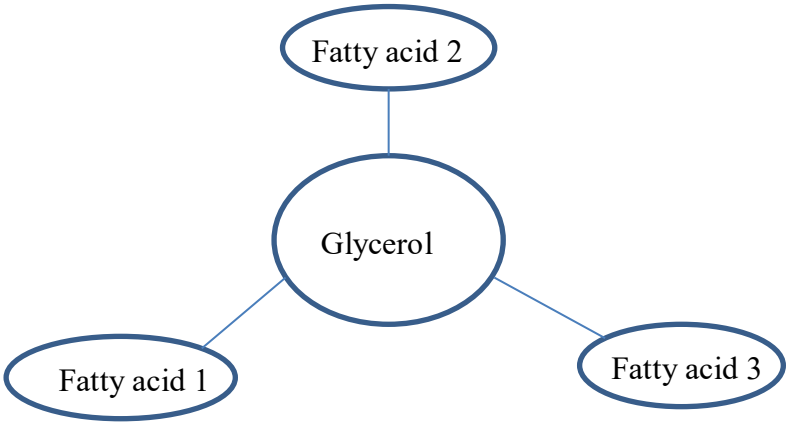

Figure 4. 1 Structure of triglyceride. Adapted from (Gopinath et al., 2009)

Table 4.1 Fatty acid names and acronyms. Adapted from (Su et al., 2011)

Fatty acid	Acronym
Butyric acid	C4:0
Caproic acid	C6:0
Caprylic acid	C8:0
Capric acid	C10:0
Lauric acid	C12:0
Myristic acid	C14:0
Palmitate	C16:0
Palmitoleic	C16:1
Margaric acid	C17:0
Stearic acid	C18:0
Oleic acid	C18:1
Linoleic acid	C18:2
Linolenic acid	C18:3
Arachidic acid	C20:0
Gadoleic acid	C20:1
Bechnic acid	C22:0
Erucic acid	C22:1
Gadolenic acid	C22:2
Lignoceric	C24:0

4.1.1 Transesterification

Transesterification (also known as Alcoholysis) is the formation of fatty acid esters and glycerol by oil or fat with an alcohol in the presence of a catalyst to improve yield. However, transesterification in supercritical methanol does not require a catalyst (Demirbas, 2007). Either a homogenous catalyst (acid or base) or a heterogeneous catalyst (acid, base, enzyme) can be used to catalyse a transesterification reaction (Atadashi et al., 2010). Transesterification reaction is reversible, so the addition of alcohol in excess shifts equilibrium to the products side (Ma and Hanna, 1999). Methanol, ethanol, butanol, propanol and amyl alcohol are among the alcohols that can be used for transesterification process (Ma and Hanna, 1999; Van Gerpen, 2005) with methanol and ethanol most commonly used especially methanol due to low cost and chemical and physical advantages (alcohol with the shortest chain and polar). NAOH dissolves in methanol easily and reacts with triglycerides quickly (Ma and Hanna, 1999).

Alkali-catalysed transesterification reaction with methanol is used in the production of most biodiesel today, due to relatively short reaction time. However, substantially anhydrous alcohol and vegetable oil with low free fatty acid content must be used to avoid saponification reaction, which is promoted by the presence of free fatty acids or water or both (Vasudevan and Briggs, 2008).

According to Demirbas (2007), transesterification process is required to reduce the viscosity of the oil and also, transesterification is ideally a potentially less expensive way of converting the large and branched structure of bio-oil into smaller and straight-chain molecules, like the type used in regular diesel combustion engines. Transesterification in supercritical methanol which does not require catalyst has advantages such as simpler purification of products, lesser reaction time, more environmental friendly and lesser energy use.

In the transesterification reaction, three moles of alcohol is required for each mole of triglyceride to give three moles of methyl esters and one mole of glycerol (Chisti, 2007) but generally, six to one mole ratio is used to accurately complete the reaction (Mata et al., 2010). Temperature of reaction and molar ratio of alcohol to oil are the most important variables affecting methyl ester yield during transesterification (Demirbas, 2007).

Different conventional methods used in the purification of biodiesel of contaminants to ensure quality and purified biodiesel is obtained are methods such as the use of adsorbent, evaporation, distillation, gravitational settling, washing with acid and water. While separative membrane and membrane reactors are the most improved methods used in separation of biodiesel from impurities. Purification of biodiesel is necessary to achieve stringent diesel standards and eliminate the problems of carbon deposits, coking, ring sticking and trumpet formation on injectors and gelling and thickening effects of lubricating oil and also to improve the performance of the combustion engine (Atadashi et al., 2010).

Most research on algae lipid as biodiesel feedstock has focused on improving growth rate and lipid productivity with limited research on biodiesel production and the quality of this potential microalgae biodiesel (Hoekman et al., 2012; Stansell et al., 2012). The main aim of this chapter was to produce biodiesel from microalgae feedstock cultivated using nutrients in FFW and to determine the quality of biodiesel thereafter. Also, compare the quality of biodiesel obtained from microalgae species cultivated in FFW to quality of biodiesel of same species cultivated in a microalgae standard growth media. This will, in turn, establish the suitability of nutrients in FFW as cheap alternative growth media for growing algae biomass feedstock for biodiesel production. Finding and a suitable cheap alternative growth microalgae growth media to purchased synthetic growth medium nutrients will eliminate the cost of purchased culture nutrients and thereby, reduce the cost biodiesel production by providing cheaper feedstock.

4.2 Methodology

In this section, FFW was collected and prepared for microalgae cultivation. The next step involved utilising prepared FFW for microalgae cultivation. After cultivation, harvested algal biomass was used for biodiesel production and produced biodiesel properties were calculated using Equation 4.1 to 4.12 from FAME properties obtained.

4.2.1 Preparation of Fish Farm Wastewater

Fish farm wastewater was collected from David Fincham Aquaculture, Johannesburg, South Africa. *Oreochromis Niloticus* (tilapia) was farmed in a recirculating system using 10,000L fish tanks, containing about 200 kg of fish, fed daily with a 32% protein fish feed at 4 wt%. The system ran a backwash to remove debris from the recirculating water once a day. Collected wastewater was filtered using a vacuum pump and a 509ws filter paper and sterilised in an autoclave at 120°C for 20 mins. pH was measured using a Ohaus Starter 3100 meter while nutrients (ammonia, nitrate and phosphate) were measured respectively using Hach ammonia nitrogen test kit model N1-8, nitrate test kit model N1-11 and orthophosphate test DOC326.98.00023. Distilled water was used to modify nutrient load in sterilized FFW while sterilised FFW was used in the original form in unmodified FFW cultivation.

4.2.2 Microalgae Cultivation

Cultivation of algae was done in batch using three species of microalgae (*Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii*) obtained from the South African National Phycology Culture Collection, Evolution Studies Institute, University

of the Witwatersrand, Johannesburg. Tris-acetate phosphate standard growth media was used as the control growth media. The microalgae species were cultivated in a 10L perspex (plexi glass) photobioreactor with a height of 100 cm and an inside diameter of 14.5 cm. The reactor included a diffuser at the base and a 12/12 hour light/dark regime, with an artificial light intensity of 2388 lx. An air flotation method was used for a continuous supply of air at 7 ml/min and CO_2 at 2 ml/min for one hour a day. A Brooks Instrument 0154 mass flow meter was used to measure the flow rate of gases. Lights were supplied from the sides of the reactors using eight fluorescence lamps (Osram L58 Watts/640 cool white) with protective lamp cover. Light intensity was measured with a MAJOR Tech MT940 light meter. Optical density at 680 nm was used to determine the daily growth rate of the algae species. Microalgae were harvested in the lag phase using a 0.45 µm cellulose acetate filter paper in a pressure a filter, washed with distilled water and oven-dried at 28°C for 48hrs.

4.2.3 Biodiesel Production

Microalgae biomass was directly transesterified to biodiesel because direct transesterification has potential large-scale production of biodiesel directly from microalgae cells without the need for oil extraction and is less expensive (Griffiths et al., 2010). Biodiesel was made by direct transesterification of algal biomass as adapted from (Johnson and Wen, 2009). 50 mg of dried and crushed algal biomass was weighed into a 15 ml glass bottle with a silicon screw cap. The sample was mixed with 2 mL of methanol and 600 µL 95% sulfuric acid and vortexed for roughly 30 s. The mixture was then heated for 60 min at 300 rpm at 90 C and allowed to cool to room temperature. After cooling, 2 mL of hexane was added to the reaction mixture and mixed, followed by the addition of 1 mL of distilled water to separate

the mixture into two phases. The tubes were centrifuged for 5 min at 4000 rpm, with the solvent layer containing the FAME separated and collected for characterization.



Figure 4. 2 Photo of the GC-MS used for the ananlysis of biodiesel

4.2.4 FAME Characterisation

A GC/MS (Figure 4.2) was used to measure the FAME composition. 1 µL of FAME extract was injected into a GC/MS with Rtx5 column (30 m x 320 mm x 1.0 µm film thickness) (Supelco, USA). Standard split injection was used with a split of 100. An injection temperature of 180°C and column oven temperature of 140°C was used, increased to 250°C at 4 °C /min, with the detector at 220°C with a carrier gas flow rate of 50 ml/min. A Supelco C14:0 to C22:0 FAME mixture was used as standard. Peak areas were used to quantify the FAME.

4.2.5 Calculation of Fuel Properties

FAME composition of biodiesel produced was used to calculate biodiesel properties: cetane number (CN), Iodine value (IV), cold filter pour point (CFPP, kinematic viscosity (υ), density (ρ), higher heating value (HHV), long-chain saturated factor (LCSF), degree of unsaturation (DU) and saponification value (SF). CN, CFPP and LCSF were calculated as in (Álvarez-Díaz et al., 2015; Stansell et al., 2012) while SV, IV, DU, υ, ρ and HHV were calculated as in (Islam et al., 2013). The equations are shown below.

CN of saturated fatty acid (SFA)

$$CN_{SFA} = -107.71 + 31.126n - 2.042n^2 + 0.0499n^3 \quad (4.1)$$

Where n is the number of carbon

CN of monosaturated fatty acid (MSFA)

$$CN_{MSFA} = 109 - 9.292n + 0.354n^2 \quad (4.2)$$

CN for polysaturated FA (PFA)

$$CN_{PFA} = -21.157 + (7.965 - 1.785db + 0.235db^2)n - 0.099n^2 \quad (4.3)$$

Where db is the number of double bonds

The CN of the biodiesel is given by the following equation:

$$CN = 1.068\sum(cn_i \times m_p) - 6.747 \quad (4.4)$$

Where cn_i is the CN of each FAME and m_p is the mass percentage of each FAME.

$$CFPP = (3.1417 \times LCSF) - 16.477 \quad (4.5)$$

Where LCSF is the long-chain saturated factor and is calculated by weighing up values of the longer chains (C16, C18, C20, C22, C24 and the m_p of each FA)

$$LCSF = (0.1 \times mp_{C16}) + (0.5 \times mp_{C18}) + (1 \times mp_{C20}) + (1.5 \times mp_{C22}) + (2 \times mp_{C24}) \quad (4.6)$$

$$SV = \sum (560 \times m_{pi}) / m_i \quad (4.7)$$

$$IV = \sum (254 \times db \times mp) \times m_i \quad (4.8)$$

Degree of unsaturation (DU) calculated based on the fraction of mono-unsaturated fatty acids and poly-unsaturated fatty acid

$$DU = \sum MUFA + (2 \times PUFA) \quad (4.9)$$

v, p and HHV calculated with Equations 4.10 – 4.12 respectively

$$\ln(v) = -12.503 + 2.496 \times \ln(M) - 0.178 \times db \quad (4.10)$$

Where M is the molecular weight of the FAME and ν is the kinematic viscosity at 40 C in mm^2/s.

$$\rho = 0.8463 + \frac{4.9}{M} + 0.0118 \times db \quad (4.11)$$

$$HHV = 46.19 - \frac{1794}{M} - 0.21 \times db \quad (4.12)$$

Where ρ is the density at 20 C in g/cm^3; and HHV is the higher heating value in MJ/kg of FAME.

4.3 Results and Discussion

The fuel properties of biodiesel (Table 4.2) were calculated from the composition of FAME (Figure 4.3 to Figure 4.4) with Equations 4.1 to 4.12.

4.3.1 Composition of FAME

As can be seen in Figure 4.3 to Figure 4.5, all species cultivated in the three different growth media accumulated less than 1% C12:0, C14:0, C16:1, C17:0, C20:2 and C22:0 and accumulated between 1 – 2% of C14:1 and C20:0 FAME except for all species cultivated in modified FFW that had values of C14:1 less than 1% and C20:0 in *Heterochlorella luteoviridis* in unmodified FFW above 2%. C18:0 was generally above 4% but less than 10% in all samples except in *Heterochlorella luteoviridis* in modified FFW with 13.09% and absent in *Chlamydomonas reinhardtii* in both FFW. C18:2 was above 20% in species cultivated in TAP standard growth media and unmodified FFW but were lower (11.20 to 13.59%) in all species cultivated in modified FFW. C16:0 was the second most abundant FA in all samples cultivated. Generally, most samples contained above 24% of C16:0, having the highest accumulation of 34.54% in *Tetradesmus obliquus* in modified FFW and lowest of 19.41% in *Chlamydomonas reinhardtii* in unmodified FFW. The most abundant FA in all samples is C18:1(x) with about 40% in all samples cultivated. *Chlamydomonas reinhardtii* in unmodified had 22.27% of SFA, the lowest concentration of SFA in all samples, about twice lesser than *Tetradesmus obliquus* in modified FFW with the highest SFA concentration of 46.60%. The concentration of MSFA was above 41% in all the samples with 59.01% as the highest concentration in *Chlamydomonas reinhardtii* in modified FFW and 41.36 as the lowest concentration in *Heterochlorella luteoviridis* in TAP. The concentration of PUFA was

generally lower in modified FFW samples nearly twice lower in concentration than in species cultivated in both TAP and unmodified FFW.

The main fatty acid components of all species were C16 to C18 comprising over 95% of all fatty acids. Saturated fatty acids were above 25% in all the samples except in *Chlamydomonas reinhardtii* in both FFW with 22.27% while unsaturated fatty acids were above 60% in all cultivations. This is in agreement with studies by Malibari et al (2018) and Wu and Miao (2014) that obtained mostly C16 to C18 fatty acids, above 25% saturated fatty acid and above 60% unsaturated fatty acids. Wu and Miao (Wu and Miao, 2014) suggested that the effects of nitrogen starvation on fatty acid composition are dependent on the species. In this study, the lowest composition of saturated fatty acid in *Chlamydomonas reinhardtii* in each media can be attributed to this fact. Studies by Huerlimann et al (2010) and Malibari et al (2018) suggest that the composition of microalgae fatty acids is dependent on growth media and duration of cultivation. Since research by Wu and Miao (Wu and Miao, 2014) suggest that fatty acid composition are species-dependent, the lower percentage composition of saturated fatty acids observed in *Chlamydomonas reinhardtii* cultivated in all media can be attributed to this.. On the other hand, the lower polyunsaturated fatty acids observed in all species cultivated in modified FFW can be attributed to the impact of media on fatty acid composition as suggested by Huerlimann et all and Malibari et al (Huerlimann et al., 2010; Malibari et al., 2018).

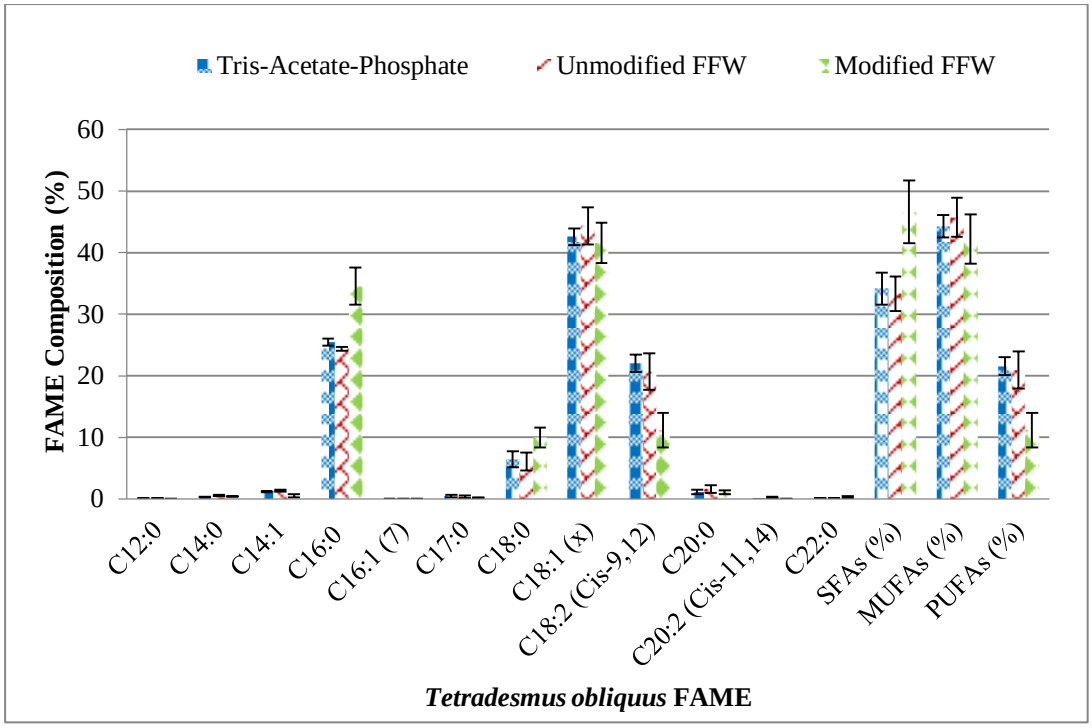

Figure 4. 3 FAMEs composition of *Tetradesmus obliquus* in the different growth media

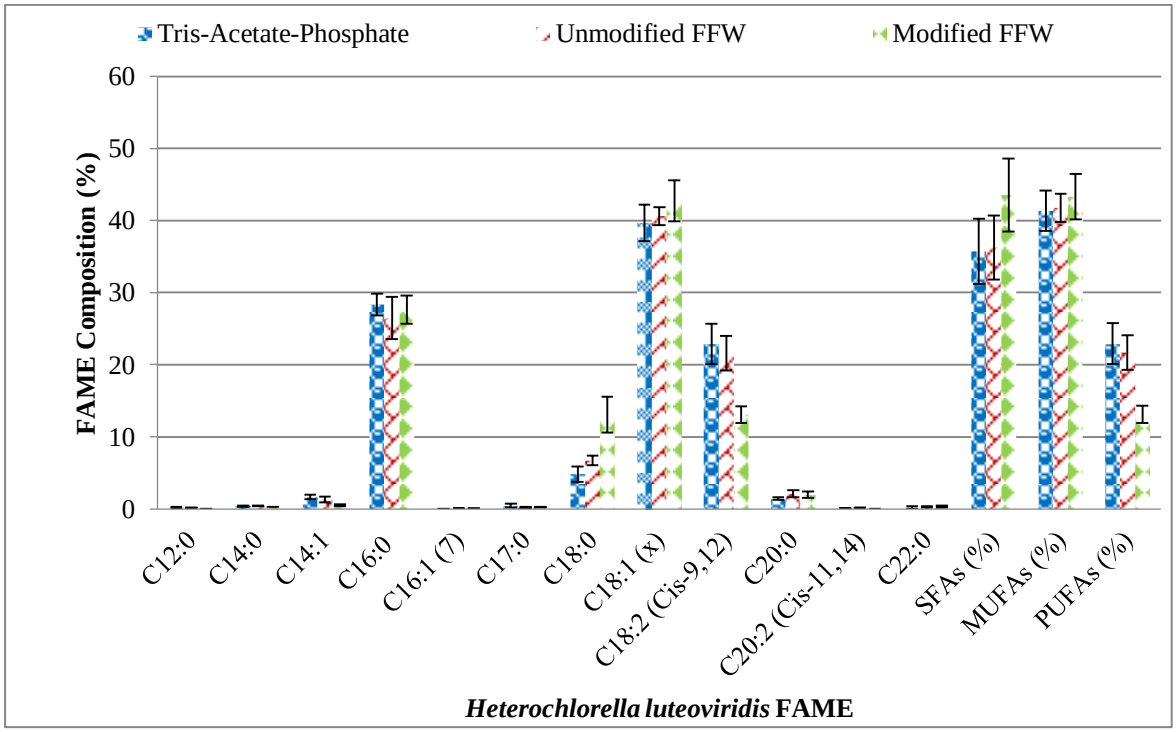

Figure 4. 4 FAMEs composition of *Heterochlorella luteoviridis* in the different growth media

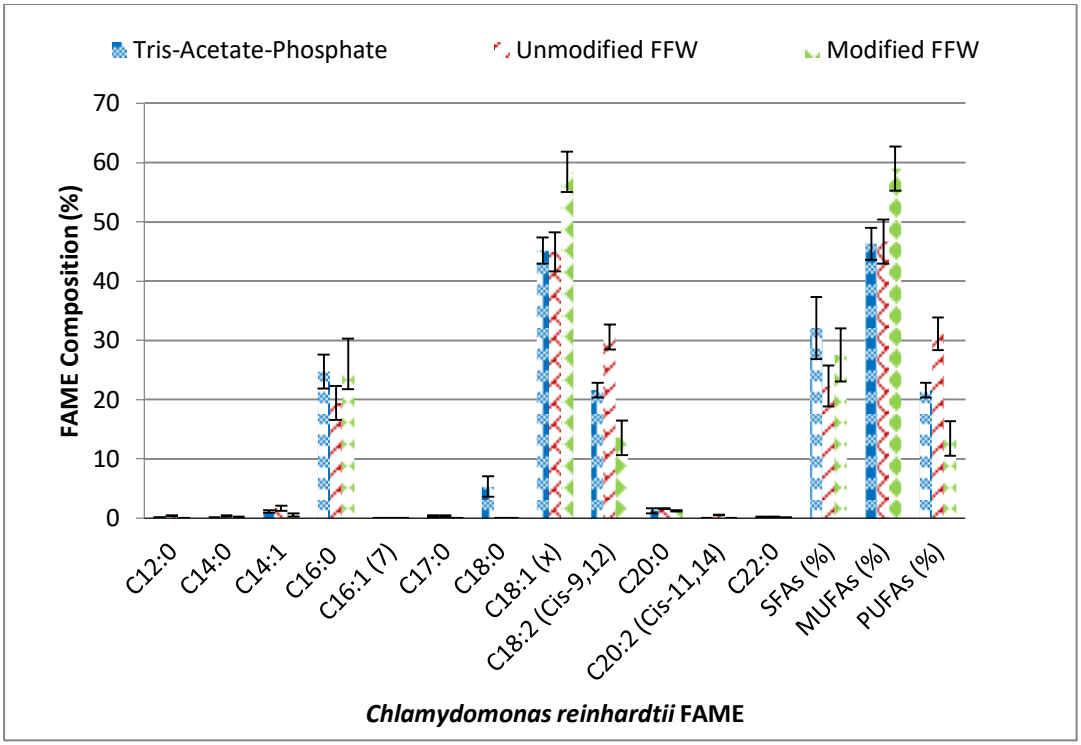

Figure 4. 5 FAMEs Composition of *Chlamydomonas reinhardtii* in the different growth media

4.3.2 Fuel Properties

Experimental procedures used to determine the fuel properties of biodiesel are difficult, long, expensive (Islam et al., 2013a; Talebi et al., 2015) and usually have insufficient microalgae to supply the amount of oil required (Talebi et al., 2015). Simple and reliable calculation methods are used instead of experimental procedures to estimate biodiesel fuel properties (Islam et al., 2013a; Talebi et al., 2015). As a theoretical work established reliable calculation (Equations 4.1 to 4.12) for estimating fuel properties of biodiesel from the composition of FAME were used to estimate biodiesel properties (Table 4.2) in this study.

4.3.2.1 Cetane Number

The CN deals with the ignition properties of fuels. It is associated with ignition delay time, combustion smoothness, knock time, stability, the efficiency of combustion and release of

carbon monoxide, nitrous oxides and hydrocarbons (Ramírez-Verduzco et al., 2012). Generally, ignition properties get better with increasing CN (L.C. Meher et al., 2006). The higher the CN, the lesser the ignition delay time (Knothe, 2006). CN increases with increase in molecular weight of the FAs (Ramírez-Verduzco et al., 2012). All the samples had CN above 59 which is higher than both the ASTM D6751-02 and EN 14214 standards limits of ≥47 and ≥51 respectively. The higher CN value can be attributed composition of longer chain fatty acids (C16 to C18) constituting upto 95% of FAME. The CN of this study is comparable to the CN in studies by Wu and Miao (2014) that obtained a CN above 58 in different concentrations of nitrate. *Heterochlorella luteoviridis* in unmodified FFW had the highest CN of 64.39 while *Chlamydomonas reinhardtii* in modified FFW had the least CN of 59.17. In as much as *Heterochlorella luteoviridis* in unmodified FFW biodiesel will have the least ignition delay time than others and highest ignition delay time with *Chlamydomonas reinhardtii* in unmodified FFW, all fuel samples have short ignition delay time which is good quality and will generally run smoothly with lesser emissions. This is as a result of the CN obtained having higher values than the required CN in both EN 14214 and the ASTM D6751 and the fact that CN properties get better with increasing CN values.

4.3.2.2 Kinematic viscosity

Kinematic viscosity directly affects the atomisation process during combustion. Viscosity increases with an increasing molecular weight of FAs and reduces with an increasing number of double bonds (Ramírez-Verduzco et al., 2012). High kinematic viscosity can lead to certain operation problems like engine deposits. Most issues associated with high viscosity are noticed during low ambient temperatures and cold-start engine conditions. Kinematic viscosity is also used to monitor the quality of stored biodiesel as it increases with reducing

biodiesel quality (Knothe, 2006). All calculated viscosity fall within the range of both ASTM D6751-02 and EN 14214 standard of 1.9 – 6.0 mm^2/s and 3.5 – 5.0 mm^2/s respectively with most of the calculated values falling within the upper limit of the EN 14214 standard but within the mean range of ASTM D6751-02. All samples of *Chlamydomonas reinhardtii* had lowest kinematic viscosity values even though the difference in value did not vary so much with other species. The biodiesel obtained from all samples will lead to a less operational problem to the engine due to proper atomization characteristic of the biodiesel produced. Biodiesel obtained from all samples of *Chlamydomonas reinhardtii* has the best viscosity because they had the lowest values when compared to values obtained from other species. Of all the samples, *Chlamydomonas reinhardtii* samples had the lowest composition of saturated FA and more unsaturated FA. This is in agreement with other research that kinematic viscosity reduces with the increasing number of double bonds (Ramírez-Verduzco et al., 2012). The values obtained were unvaried from each other because all samples accumulated similar FAs and had a similar molecular weight. The variations must have been from the level of unsaturation of each sample.

4.3.2.3 Density

Density also is known as specific gravity decreases with increasing molecular weight and increases with an increasing number of double bonds (Ramírez-Verduzco et al., 2012). It is a major parameter that affects engine performance. Fuel density determines the mass of fuel injected and also influences the energy content and air-fuel ratio within the combustion chamber (Hoekman et al., 2012). All densities fall within the EN 14214 standard of 0.86 – 0.90 g/cm^3. All densities had similar values for each sample cultivated in the different media with samples of all *Chlamydomonas reinhardtii* slightly higher. Similar density can be

associated with the accumulation of similar molecular weight FAs. The slight difference in fuel density obtained can be associated with higher levels of double bonds in all samples of *Chlamydomonas reinhardtii*. This is also in agreement with the fact that density increases with an increasing number of double bonds.

4.3.2.4 Higher Heating Value

HHV is the amount of heat produced when one gram of fuel is combusted to produce CO_2 and H_2O at its original temperature. HHV reduces with an increasing number of double bonds (Ramírez-Verduzco et al., 2012). Biodiesel has HHV of 39 to 41MJ/kg (Demirbas, 2009) about 10% lesser than petrodiesel that is ~46 MJ/kg (Refaat, 2009). There is no stipulated HHV limit for both ASTM D6751-02 and EN 14214 standards, but according to Demirbas (2009)s, the HHV for biodiesel is between 39 to 41MJ/kg. Biodiesel from all samples had HHV between 39.68 to 39.92 and fall within 39 to 41MJ/kg quoted by Demirbas (2009).. *Chlamydomonas reinhardtii* in unmodified FFW had the lowest HHV and can be related to a higher composition of unsaturated FAs.

4.3.2.5 Iodine value

Iodine value is the parameter used in measuring the rate of saturation of the fuel (Ramírez-Verduzco et al., 2012). All samples had IV with the ≤120 of EN 14214 with *Chlamydomonas reinhardtii* in unmodified FFW having the highest IV of 94 $I_2$100/g and *Tetradesmus obliquus* in modified FFW with the lowest IV of 54 $I_2$100/g. Generally, samples cultivated in modified FFW had lower IV while samples in TAP and unmodified FFW had similar value per species except for *Chlamydomonas reinhardtii* in unmodified FFW which had the highest

value overall. As a measure of saturation, the highest values obtained in *Chlamydomonas reinhardtii* in unmodified FFW show the least saturation while the lowest value obtained in *Tetradesmus obliquus* in modified FFW show high saturation. The iodine values as a measure of saturation also correspond to the highest values obtained from the calculated degree of unsaturation. Same reason, samples cultivated in modified FFW had lower iodine especially *Tetradesmus obliquus* in modifies FFW and *Heterochlorella luteoviridis* in modified FFW.

4.3.2.6 Cold Filter Plugging Point

The cold filter pour point (CFPP) of biodiesel determines its performance during the cold weather. Lower temperatures can cause the fuel to thicken and reduce the flow rate of the fuel thereby, impacting on the performance of the fuel pipes, pumps and injectors (L.C. Meher et al., 2006). The CFPP reduces with increasing unsaturation (Álvarez-Díaz et al., 2015). This is in agreement with the results of this study as can be seen in Table 3, Species of *Chlamydomonas reinhardtii* in both FFW had lowest CFPP values. Most of the samples exceeded the CFPP limits of EN 14214 standard of ≤5/≤-20 C with only all the samples of *Chlamydomonas reinhardtii* in the three different growth media falling within the limit with 4.45 C, -4.5 C and -4.02 C respectively. The remaining samples had CFPP values more than 5 C which exceeds the upper limit of the EN 14214 range. This means biodiesel from *Chlamydomonas reinhardtii* generally have good CFPP although with a better flow from samples cultivated in FFW which will not thicken during cold temperatures and affect fuel delivery. The wide variation of CFPP observed in *Tetradesmus obliquus* and *Heterochlorella luteoviridis* in different culture media can be attributed to the nutrient composition of the different media as it affects the properties of FAME accumulated (Williams and Laurens, 2010).

4.3.2.7 Saponification Value

Chlamydomonas reinhardtii in unmodified had the highest SV of 194 mg KOH/g while *Chlamydomonas reinhardtii* in modified FFW had the lowest SV of 188 mg KOH/g. The range of SV is not stated in both ASTM D6751-02 and EN 14214 but values obtained fall with the same range of samples measured in (Islam et al., 2013)

Table 4.2 Fuel properties of *Tetradesmus obliquus*, *Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* cultivated in Tris-Acetate-Phosphate standard growth medium, unmodified fish farm waste growth medium

Sample	Media	CN	IV ($gI_2/100g$ fat)	CFPP (°C)	SV (mg KOH/g)	DU	ρ (g/cm^3)	υ (mm^2/s)	HHV (MJ/kg)
Biodiesel Standard EN 14214		≥51	≤120	Summer ≤5 /Winter ≤-20	NA	NA	0.86-0.9	3.5-5.0	NA
Biodiesel Standard ASTM D6751-0002		≥47	NA	Summer ≤5/Winter ≤-20	NA	NA	NA	1.9-6.0	NA
Tetradesmus obliquus	TAP	63.59	74	5.83	190	87.40	0.871	4.71	39.71
Heterochlorella luteoviridis	TAP	63.60	73	5.52	191	87.19	0.871	4.71	39.92
Chlamydomonas reinhardtii	TAP	63.25	75	4.42	189	89.59	0.873	4.52	39.68
Tetradesmus obliquus	Unmodified FFW	63.68	73	6.64	189	87.58	0.871	4.71	39.71

Sample	Media	CN	IV (gI_2/100g fat)	CFPP (°C)	SV (mg KOH/g)	DU	ρ (g/cm^3)	υ (mm^2/s)	HHV (MJ/kg)
Heterochlorella luteoviridis	Unmodified FFW	64.31	72	10.18	191	85.15	0.871	4.67	39.68
Chlamydomonas reinhardtii	Unmodified FFW	59.70	94	-4.45	194	108.81	0.872	4.55	39.61
Tetradesmus obliquus	Modified FFW	63.49	54	14.93	191	64.62	0.871	4.71	39.71
Heterochlorella luteoviridis	Modified FFW	63.32	58	20.51	190	69.54	0.871	4.71	39.71
Chlamydomonas reinhardtii	Modified FFW	59.17	71	-4.02	188	85.91	0.872	4.64	39.68

4.4 Conclusion

All species accumulated FAMEs within C:12 to C:22 and had upto C16 to C18 FAMEs comprising upto 95% of FAMEs. Similar biodiesel properties were obtained from all microalgae species cultivated in both fish farm waste media when compared to the TAP standard growth media. *Chlamydomonas reinhardtii*, in both fish farm wastewater (FFW) media, had the most suitable biodiesel properties, especially the CFPP. This makes biodiesel obtained from *Chlamydomonas reinhardtii* in FFW most suitable for different weather in all regions, including cold temperature regions. Biodiesel from other algal species can only be suitable for use in hot regions or only during summer in temperate regions due to a poor CFPP. Most biodiesel parameters of all samples were within the range of both ASTM D6751-02 and EN 14214 biodiesel standards. This suggests that biodiesel obtained from FFW would generally have little ignition time as a result of cetane values obtained, less deposit and operational problems due to good kinematic viscosity. Biodiesel obtained would also have good engine performance according to the densities obtained. These properties indicate that nutrients in FFW are as suitable as the purchased TAP standard growth media for microalgae biodiesel feedstock production for *Tetradesmus obliquus, Heterochlorella luteoviridis* but more suitable for biodiesel production from *Chlamydomonas reinhardtii* species cultivated in FFW. This can be attributed to the inhibition of PUFA accumulation in *Chlamydomonas reinhardtii* cultivated in both FFW. This is the first time the biodiesel properties of *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* cultivated in FFW has been determined when compared to other published work. This has successfully shown that FFW contains nutrients to support the accumulation of desirable fatty acids in *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* and can be attributed to low nutrient concentrations and presence of mixed nitrogen sources. The desirable properties of biodiesel obtained from microalgae cultivated in FFW can be

attributed to the nutrient composition of FFW. This indicates that FFW nutrients are suitable for the cultivation of microalgae for biodiesel production fit for use as a renewable energy until diesel cars are replaced by other forms of energy such as electricity. The research carried out in this chapter suggests that FFW can replace purchased growth media for cultivation of microalgae for biodiesel production.

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Chapter 5 Environmental and economic impacts of using fish farm wastewater for microalgae cultivation

A research paper on this chapter has been submitted to Cleaner Environmental Systems and is under review for publication.

The contribution of the co-authors (Professor Kevin Harding and Dr. Michelle Low) towards the work is primarily that of supervision or collaboration, and the work and write-up were carried out by the author of this thesis.

This chapter aims to use a life cycle assessment to determine the effects of fertiliser use on the cultivation of and the mitigation of these effects using fish farm wastewater (FFW).

This chapter also aims to determine the cost benefits of replacing purchased nutrients with FFW.

Abstract

The ability of microalgae to utilise waste nutrients in fish farm wastewater (FFW) for biomass accumulation and removal of nutrients from the water, is promising for biodiesel production but the environmental benefits of recycling these nutrients for biodiesel production needs to be investigated. This chapter presents the environmental effects of utilizing nutrients in FFW for growing microalgae compared to purchase nutrients of Tris-acetate-phosphate standard growth media through life-cycle assessment (LCA). Extrapolated results conducted in an airlift photobioreactor were used. Cultivation in TAP media containing fertilisers had a higher environmental impact (3 times more on global warming (kg $CO_{2\ eq}$), 3.6 on marine ecotoxicity (kg 1,4-DCB), 2 on human carcinogenic toxicity (kg 1,4-DCB), 3.7 on freshwater ecotoxicity (kg 1,4-DCB), 3.9 on human non-carcinogenic toxicity (kg 1,4-DCB), 2 on freshwater eutrophication (kg P_{eq}), 8 on terrestrial ecotoxicity (kg 1,4-DCB) and 15 on water consumption (m^3)) more than the use of FFW. Use of fertilisers impacted the environment most in cultivation in TAP media while electricity consumption impacted the environment most in cultivation in FFW media. Utilising FFW for biodiesel microalgae cultivation also saved the cost of wastewater treatment, freshwater and nutrient up to R3386.78 for 1L biodiesel cultivatin especially for initial cost.

Keywords: Life cycle assessment, fish farm wastewater, biodiesel, cost, environment.

5.1 Background

Due to the environmental burden of commercial fertilizers on the cultivation of microalgae for the production of biofuels, this chapter used a life-cycle assessment (LCA) to assess the effect of fertilizer use on the cultivation of algae and how much environmental impact can be mitigated by replacing fertilizers with nutrients in fish farm wastewater (FFW).

The cost benefits and sustainability of replacing purchased nutrients with FFW was also considered in this chapter.

5.1.1 Life-cycle assessment

LCA is a methodology for evaluating the environmental aspects and potential impacts of a product by:

- Compiling an inventory of the applicable inputs and outputs of a product or process;
- Assessing the potential environmental impacts associated with those inputs and outputs; and
- Interpreting the findings of the inventory and evaluation.

Life cycle assessment studies the environmental aspects and potential impacts from raw material acquisition through processing, use and disposal throughout the life of a product (*i.e.* cradle-to-grave). The life cycle inventory (LCI) quantifies comprehensively, all the energy and environmental flows involved with a product from "cradle-to-grave". The LCI gives information on raw materials obtained from the environment, energy resources utilised and water, air and solid emissions produced.

Biofuel may reflect an improvement in fossil CO_2 emissions; however, such technology can also trigger negative environmental impacts, induced, for example, by fertilisers and pesticides, and may also create competition with food crops for land use (Lardon et al., 2009). Biodiesel production from microalgae is an emerging technology considered by many to be a promising source of energy, mainly due to its reduced competition for land (Lardon et al., 2009). Nevertheless, the environmental impact of this new technology must be assessed and guided by technological developments before large-scale production of microalgae (Collet et al., 2014). The impact assessment and the energy balance shows, however, that algal biodiesel suffers from several disadvantages at the current technological maturity level. High photosynthetic yields of microalgae significantly reduce land and pesticide use, but not fertilizer needs, in comparison with traditional energetic crops (Lardon et al., 2009).

The initial purpose of biodiesel was to solve the problem of energy security, but the recent focus on renewable diesel has moved to reduce carbon dioxide emissions to the environment. The use of more carbon dioxide from the environment by biodiesel feedstock plants than the carbon dioxide released when used as energy makes biodiesel carbon neutral (Atadashi et al., 2010). Besides, long-term reliance on fossil-based fertilizers or CO_2 from power plants makes diesel algae unsustainable (Clarens et al., 2010; Pfromm et al., 2011).

Nevertheless, growing biomass and turning it into a liquid fuel requires input, *e.g.* fertiliser and electrical power, each of which has a related environmental burden. Therefore, quantifying the potential greenhouse gas savings associated with the use of these biofuels is significant. Life-cycle assessment (LCA) can be used to evaluate and measure the overall environmental effects of a system or service, from raw material processing to waste disposal and goods at the “end of life”. Environmental impacts such as global warming potential (GWP), acidification potential, and water eutrophication potential can be quantified by

relevant emissions from the system to a chemical reference; *e.g.* GWP is quantified in terms of the equal amount of carbon dioxide emitted (Stephenson et al., 2010).

To achieve a significant reduction in GHG emissions, most literature studies focus on breakthrough technology (Collet et al., 2014). But recently, water consumption for biofuel production has been identified as a major limitation to the development of an expanded biofuel economy. Water consumption for most biofuels occurs almost entirely in the production of feedstock (Powers and Baliga, 2010). Using wastewater to grow microalgae is one solution to improve the sustainability of the production of microalgae biodiesel in practice, as it not only recovers energy in the form of biodiesel but also has environmental values in terms of wastewater purification (Zhu et al., 2016).

Algae systems have demonstrated mixed environmental impact quality (energy usage, water use and greenhouse gas emissions) on a 'per km ' basis to benchmark crops. This indicates that production and conversion methods need to be carefully considered to ensure the algae-to-energy processes is environmental viable (Clarens et al., 2011). A large proportion of environmental impacts, and in particular GHG emissions, do not occur directly at the production facility. They stem from the production of electricity required for the cultivation, harvesting and transformation of algae, the source of electricity and algae production technology appear to be relevant (Collet et al., 2014).

The use of wastewater as a culture media could significantly reduce the need for chemical fertilizers and their related life-cycle burdens, as well as the use of freshwater during algae cultivation (Clarens et al., 2010). Because of the environmental burden of fertilizers on the cultivation of microalgae for biofuels production, this study used an LCA to determine the impact of fertiliser use in algae cultivation and how much of the environmental impact can be mitigated by substituting fertilisers with nutrients in FFW.

5.1.2 Feasibility and Economics of Biodiesel from Algae

Biodiesel has been gaining great global significance for its sustainability, good quality and biodegradability (Atadashi et al., 2010). However, biodiesel has little commercial use due to high cost of production. Cost of feedstock (fats and oil) constitute between 70- to 80% of biodiesel production costs (Atabani et al., 2012; Behzadi and Fraid, 2007; Yusuf et al., 2011), making the selection of the best feedstock crucial for low production costs (Atabani et al., 2012). Other costs that impact the price of biodiesel are the cost of methanol, labour, and enzyme (Yusuf et al., 2011). Biodiesel needs to be produced at a lower price to be able to play active roles in energy demands (Behzadi and Fraid, 2007). The variation in the price of biodiesel depends on base feedstock, season to season variation of crop production, geographic area, price of crude and other factors (Carlos and Ba Khang, 2008).

Despite all the potential of algae, when compared to the production of land energy crops, algae production capacity is currently limited (Richard, 2010). The market size of algae biomass is about 5000 tonnes per year of dry matter, generates a turnover of about $1.25 billion per year without the addition of processed products. Successful algae biotechnology depends mainly on selecting the right algae with appropriate properties for particular culture conditions and products (Pulz and Gross, 2004).

The cost of algae oil production depends on numerous factors such as oil content, biomass yield, cost of oil extraction from algae biomass and scale of production systems (Kirrolia et al., 2013). A study by Huntley and Redalje (2007) reported that existing technology could generate $84/bbl oil (with no price attributed to the non-oil fraction), with possible technological advances reducing this cost to or below $50/bbl. One way of cost improvement of biodiesel from microalgae can be from co-products (Sun et al., 2011).

A meta-analysis study by Zhang et al (2015) suggests that under the base case (assumes moderate algal oil yield obtainable with present-day technology), algae biodiesel will sell for $5 to $10.3 1/gallon, more than the estimated benchmarks: $4.2 1/gallon for commercial biodiesel (B100) obtained from conventional animal fat and vegetable oil and $3.77/gallon petrol diesel. However, algae biodiesel selling price is expected to be competitive with anticipated development within estimated price of $2.76 – 4.92/gallon . Price of algae biodiesel is most sensitive to the cost of algae cultivation, algae lipid content and biomass productivity based on scenario-based sensitivity analysis. Other factors are oil recovery, biodiesel conversion efficiency (Zhang et al., 2015).

5.2 Methods

A life cycle assessment (LCA), to determine the environmental impacts of unmodified FFW for microalgae biodiesel production was carried out using SimaPro 9.0. while the cost-benefit was done theoretically. The method section here is associated with the LCA. Cost analysis was done theoretically from 2019 data obtained from online sources. Google rate was used to convert ZAR to USD as at the time of study in December 2019. Both LCA and cost analysis were on growth media (water and nutrients) because the objective was to determine the environmental and cost implication of replacing purchased media (freshwater and nutrients) with FFW. Cost estimation was limited to lab-scale trials and cost of lab-scale reagents assuming the cost of other factors such as labour and energy cost are constant.

5.2.1 Scope Definition

The scope of this study includes the processes needed for microalgae biomass cultivation for the production of 1L biodiesel. The system includes only cultivation of microalgae and not harvesting, drying, oil extraction or biodiesel production (Figure 5.1). The excluded sections were based on the assumption that the same processes will be used for harvesting, drying and biodiesel conversion regardless of the growth media used. To assess the impact of microalgae cultivation in FFW nutrients, Studies done in this research was considered for microalgae cultivated in FFW in an airlift photobioreactor (Chapter 3). Comparable growth and productivity were assumed for *Chlamydomonas reinhardtii* cultivated in FFW and Tris-acetate-phosphate growth media, as obtained in this study but with higher lipid content of 44.08% in FFW and 33.75% in TAP media. In the analysis, an attributional LCA is used that implies that other relationships like cost were not considered alongside physical relationships between each phase, contrary to consequential LCA where physical relationships and other factors are evaluated. SimaPro 9.0. with ReCiPe 2016 midpoint (H) impact was used for this study and the prominent impact categories chosen are Water consumption (m^3), Terrestrial ecotoxicity (kg 1,4-DCB), Marine ecotoxicity (kg 1,4-DCB), Global warming (kg $CO_{2\ eq}$), Freshwater eutrophication (kg P_{-eq}), and Freshwater ecotoxicity (kg 1,4-DCB). The system studied is limited to the nutrient composition of FFW, photobioreactor volume and electricity used in this system.

5.2.2 Microalgae Specification

Chlamydomonas reinhardtii was selected for this study because of the high lipid content obtained (Chapter 3). The analysed process chain is an assumption of similar growth in 3000 L of *Chlamydomonas reinhardtii* cultivation in FFW compared to Tris-acetate-

phosphate standard growth medium. The microalgae *Chlamydomonas reinhardtii* species were grown in a photobioreactor of 100 cm in 10L perspex (plexiglass) and 14.5 cm in diameter. 12/12 hours light and dark at ambient temperature with a light intensity of 2388 lx was used. The Air floatation system was used to supply air and CO_2 daily for 1 hour. During the test, the characteristics of growth are examined. Microalgae biomass was obtained after 15 days of cultivation using filtration as the method of processing, after which an oven was used as a dryer. *Chlamydomonas reinhardtii* in FFW had 44.08% lipid while *Chlamydomonas reinhardtii* in the standard media had 33.75% lipid. Assumptions of the volume of water and CO_2 required for the cultivation of microalgae biomass for 1 L biodiesel production were made as in Farooq et al (2015) and Chisti (2007). The fertiliser required for cultivating the microalgae were obtained from our study (Table 5.1). Local electricity was used (South Africa).

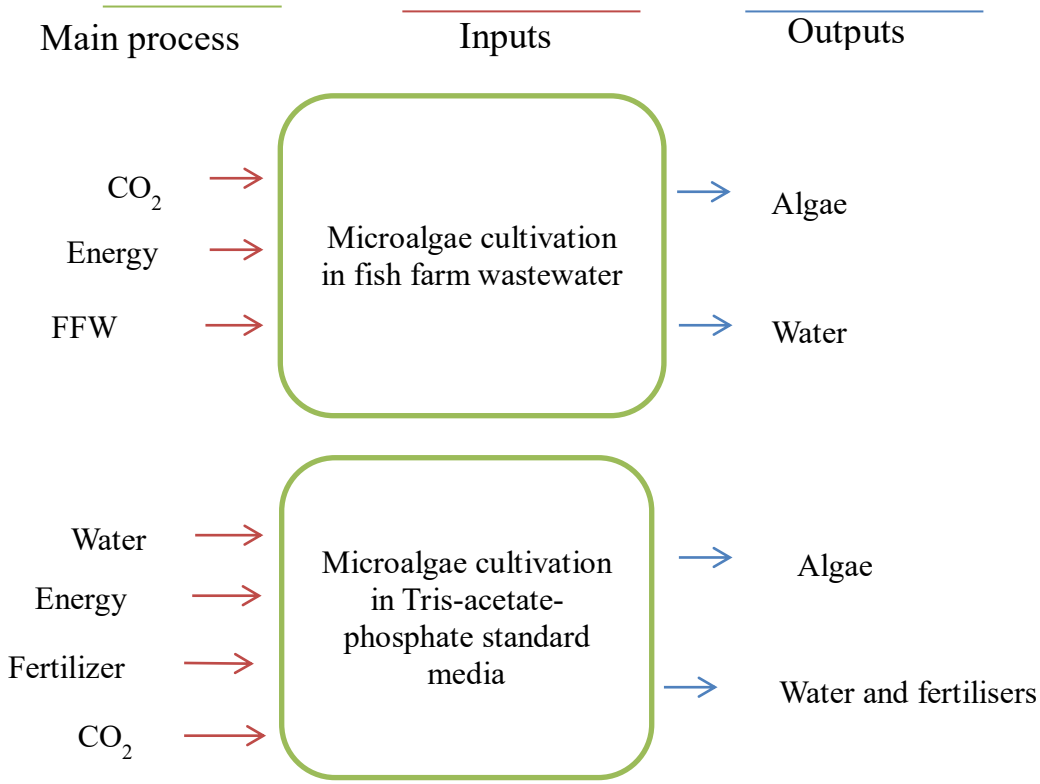

Figure 5. 1 Microalgae biodiesel production system boundaries of the two production processes

The input here (Table 5.1) refers to any input into the culture of microalgae. This includes water, fertilizers and chemicals and electricity.

Table 5.1 Energy and Material Requirement for the Cultivation of Microalgae in FFW and TAP Media

Utilities/Material	Units	FFW	TAP
Water	L	3000	3000
CO_2	kg	5.46	5.46
Electricity (light and aertation)	kW h	5.57	5.57
Ammonia	kg		1.20
Phosphate	kg		0.93
$CaCl_2.2H_20$	g		150
$MgSO_4.7H_2O$	g		300
Tris base	kg		7.26
$FeSO_4.7H_2O$	g		15
$ZnSO_4.7H_2O$	g		66
H_3BO_3	g		34.5
$MnCL_2.4H_2O$	g		15.3
$CuSO_4.5H_2O$	g		4.8
$Na_2MoO_4.2H_2O$	g		2.19
$CoCl_2.2H_2O$	g		4.8
EDTA	g		150
Acetic acid	L		3
Algal	Kg (DCW)	3	3

5.3 Results and Discussion

5.3.1 Life Cycle Assessment

Following the LCA method, once the functional unit, the study perimeter and the system are defined, each process included in the perimeter must be characterized in terms of technical inputs and outputs, energy and resource consumption and environmental emissions. Due to the lack of industrial information on microalgae cultivation, the inventory data compiled in the selected studies often rely either on extrapolation from the results of the laboratory, on adaptation from similar processes used under different conditions or with different feedstock (Collet et al., 2013).

Environmental burden of FFW and TAP media microalgae cultivation were analysed and compared for environmental impacts on marine ecotoxicity, human carcinogenic toxicity, freshwater ecotoxicity, human non-carcinogenic toxicity, freshwater eutrophication, terrestrial ecotoxicity were analysed (Table 5.2 and Figure 5.3). Cultivation in TAP media had a higher environmental impact than FFW media cultivation. Each environmental category had impacts on TAP and FFW respectively for global warming 0.735 and 0.228 kg $C0_2$ eq, marine ecotoxicity 0.027 and 0.00747 kg 1,4-DCB, human carcinogenic toxicity 0.0302 and 0.0151 kg 1,4-DCB, freshwater ecotoxicity 0.0198 and 0.00533 kg 1,4-DCB, human non-carcinogenic toxicity 0.539 and 0.14 kg 1,4-DCB, freshwater eutrophication 0.00033 and 0.000162 kg P eq, terrestrial ecotoxicity 1.59 and 0.201 kg 1,4-DCB and water consumption 1.42 and 0.0017 m3 Having 3 times more on global warming, 3.6 on marine ecotoxicity, 2 on human carcinogenic toxicity 3.7 on freshwater ecotoxicity, 3.9 on human non-carcinogenic toxicity, 2 on freshwater eutrophication, 8 on terrestrial ecotoxicity and 15 on water consumption.

The source of the environmental impact of all the impact categories studied is presented in the process contribution charts in Appendix B Figure B.5 to Figure B.22. Higher environmental impact in TAP originated mostly from the manufacturing process of the nutrients and electricity input with minimal contribution from the microalgae cultivation process. This is in agreement with Collet et al (2014) that most environmental burden of cultivating microalgae using fertilisers do not arise from the cultivation process facility. For instance, in marine ecotoxicity, microalgae cultivation contributed only 0.000000156 kg 1,4-DCB out of 0.02714 kg 1,4-DCB and 0.000000394 kg 1,4-DCB out of 1.5914 kg 1,4-DCB in terrestrial ecotoxicity. Other plots associated with the analysis of FFW and TAP media cultivation are presented in Appendix B.

In FFW, apart from water consumption, process contribution to other environmental impacts studied all originated from electricity generation (Figure 5.2) and also contributed 0.000701 out of 0.001m^3 in water consumption. In this study, electricity was used predominantly for the supply of light and gases (air and carbon dioxide) required for microalgae phototrophic growth. The gasses supplied through a diffuser were also used to mix the culture media and so, did not require extra energy for mixing the growth media. Electricity can make indoor phototrophic cultivation of microalgae unfavourable as required light for algae growth will have to be supplied through electricity. Both indoor and outdoor cultivation of algae requires mixing and mixing methods are powered by electricity. This makes reducing electricity consumption for mixing an important aspect of microalgae cultivation. Using electric efficient mixing methods can be a way to reduce the environmental burden of electricity to microalgae cultivation.

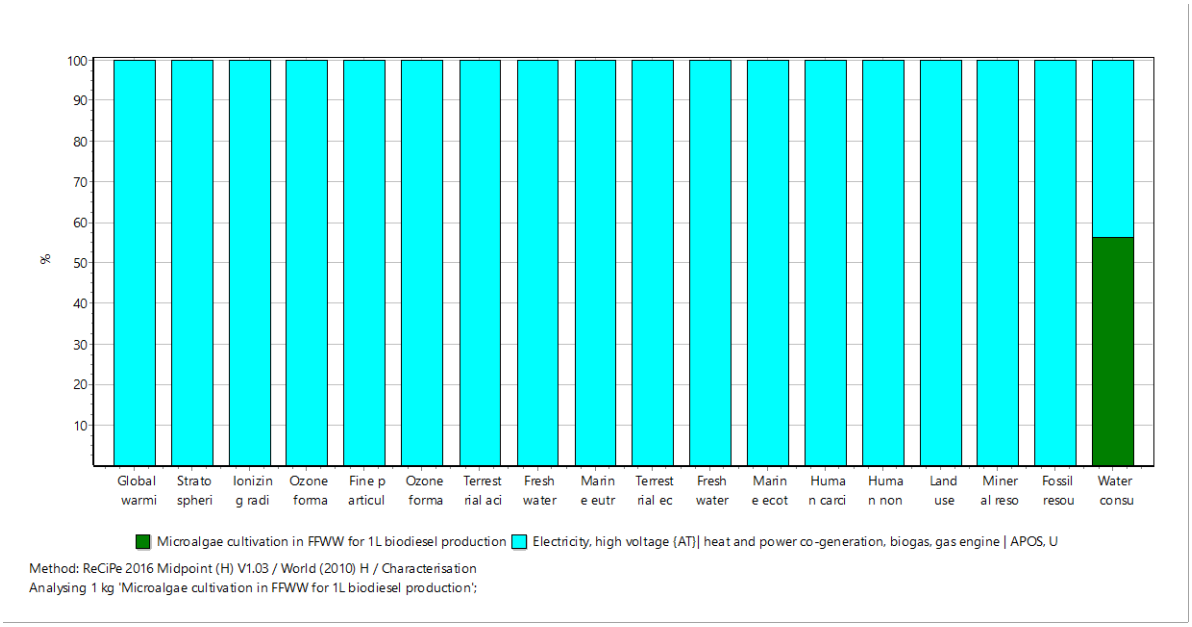

Figure 5. 2 Characterisation LCA plot of microalgae cultivation in FFW for the production of 1L biodiesel

Table 5.2 Environmental impacts of FFW and TAP microalgae cultivation

Impact category	Units	TAP	FFW
Human carcinogenic toxicity	kg 1,4-DCB	0.0302	0.0151
Global warming	kg CO_2 eq	0.735	0.228
Marine ecotoxicity	kg 1,4-DCB	0.027	0.00747
Human non-carcinogenic toxicity	kg 1,4-DCB	0.539	0.14
Freshwater ecotoxicity	kg 1,4-DCB	0.0198	0.00533
Terrestrial ecotoxicity	kg 1,4-DCB	1.59	0.201
Freshwater eutrophication	kg P eq	0.00033	0.000162
Water consumption	m3	1.42	0.0017

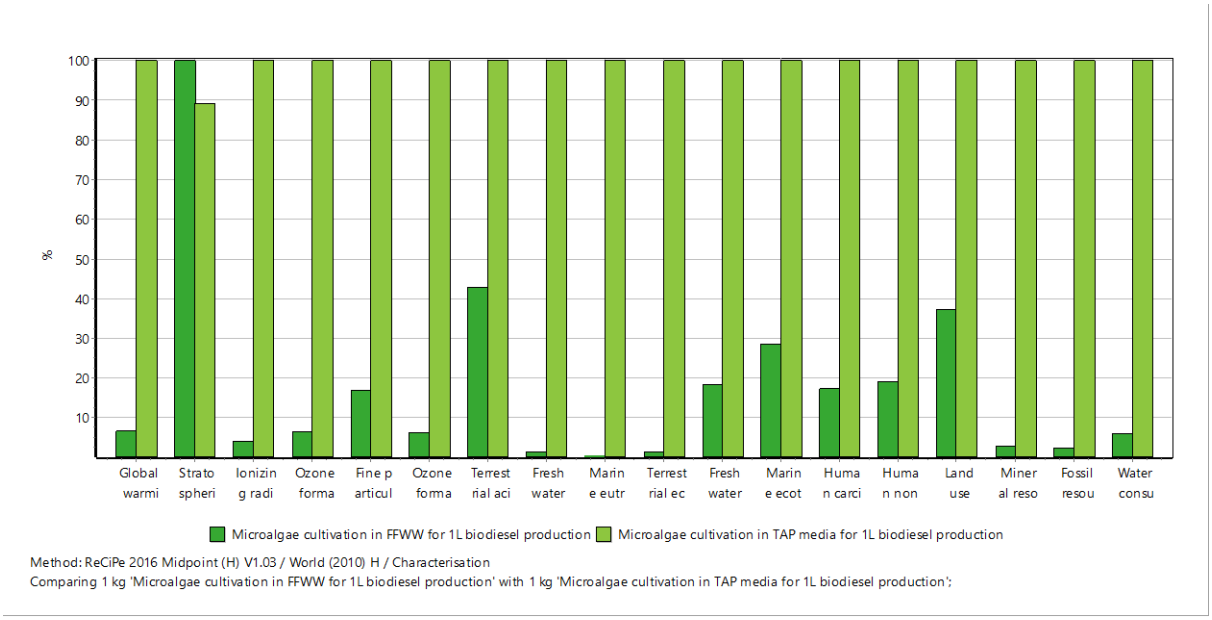

Figure 5. 3 Characterisation LCA plot comparing microalgae cultivation in FFW and TAP media for the production of 1L biodiesel

5.3.2 Sensitivity Test

The quantity of biodiesel that could be derived from a feedstock depends on that feedstock's lipid composition. Growth conditions and nutrient composition of the growth media influence microalgae lipid composition. A sensitivity analysis was conducted to assess the effect of the change in electricity consumption of FFW media and nutrient composition of the TAP media on environmental impacts. Similar to the base case, inventories for these cases were created and the impact assessment for the cultivation of microalgae required for the production of 1L biodiesel was analysed and compared to the reference case.

Sensitivity analysis is most apparent when all variables differ over similar confidence intervals. In this situation, the change in the objective function (here, nutrients in FFW compared to purchased nutrients) when each is varied equally likely, parameter by parameter, the parameters can be identified by the change in the objective function to determine sensitivities (Frank ED, Han J, Palou-Rivera I, Elgowainy A, 2011). A sensitivity analysis was carried out to determine the components of the life cycle of algae cultivation in FFW and standard growth media that contribute most directly to its burdensome footprint.

Electricity requirement for microalgae growth was varied higher and lower the base electricity used. Both scenarios showed that electricity consumption is sensitive to the cultivation of microalgae in FFW (Figure 5.4). Most impact parameters increased proportionally with increased electricity use except in water use and human non-carcinogenic toxicity. On electricity use, all parameters increased roughly by 50% with 50% increase in electricity use and decreased roughly by 50% with 50% lesser electricity use except for water consumption that increased and decreased by 20.6% in both scenarios.

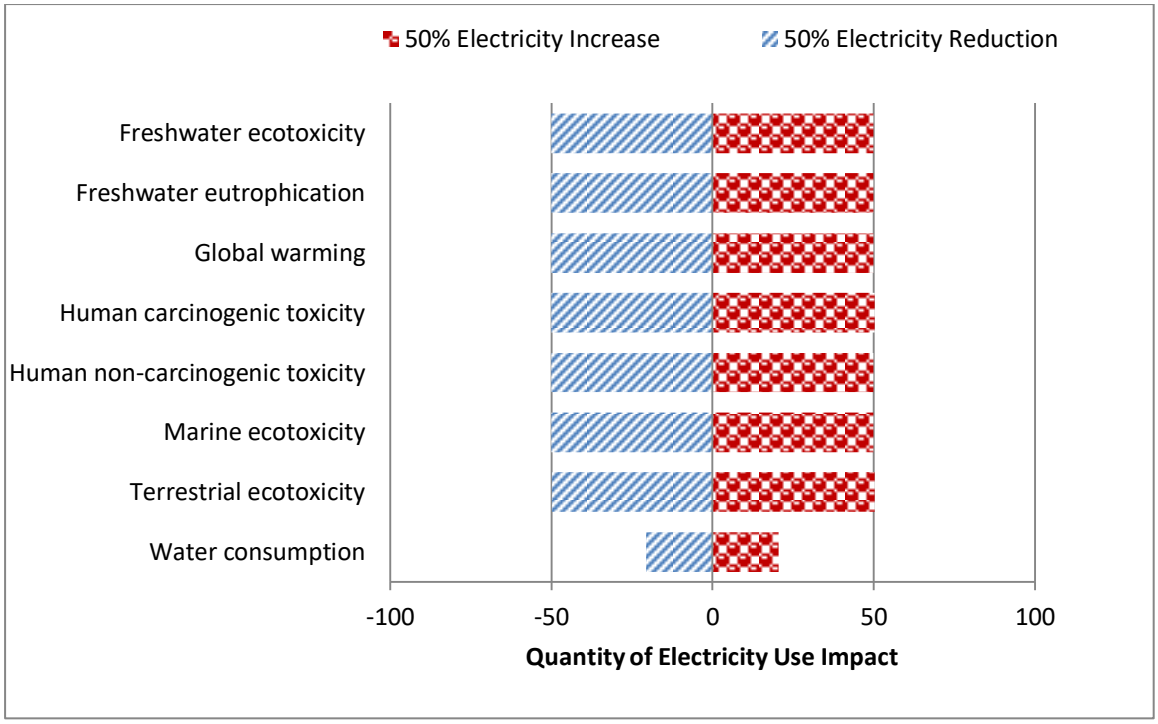

Figure 5. 4 Sensitivity plot of 50% electricity increase and decrease

In the TAP growth media, the concentration of fertilisers was sensitive to both higher and lower scenarios (Figure 5.). Increasing nutrient concentration in TAP media had the most impact on water use up to 86% and least impact on human carcinogenic toxicity of about 27% more. Impact of the remaining parameters was between about 35 and 49%. Reducing nutrients used in microalgae TAP cultivation had a similar impact reducing with reducing the concentration of nutrients) with increasing nutrient concentration except with water consumption. Water consumption did not reduce (about 47%) as much as it increased with increasing nutrients (about 86%). This makes fertiliser use in microalgae biodiesel cultivation a major environmental concern in microalgae biomass cultivation. If algae are to become a possible carbon-neutral replacement for fossil fuels, the areas that contribute to burdensome footprint should be areas of active research. Chemical fertilizer production is the main burden driver within the algae production process (Clarens et al., 2010).

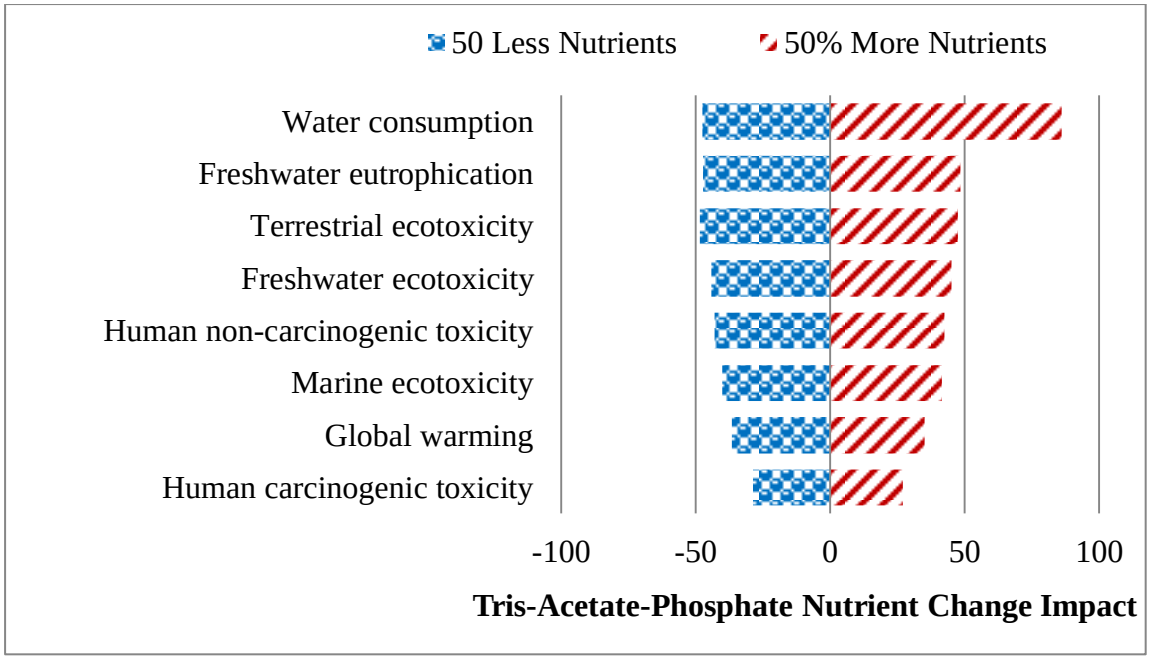

Figure 5. 5 Sensitivity plot of 50% Tris-Acetate-Phosphate nutrients increase and decrease

5.3.3 Cost

The cost contribution to microalgae cultivation media is studied in this section. Parameters that apply to both media during cultivation of microalgae such as labour and electricity were not considered. The difference in the cost of growing microalgae for biodiesel production here is limited to the microalgae cultivation media.

Cultivation of microalgae biodiesel feedstock requires large volumes of water. According to Farooq et al (2015), 3000 L of water is required to cultivate microalgae for the production of 1 L biodiesel. FFW supplies this large volume at no cost. According to Jones (2018), consumption of 10,500 to 25,000 L/M of water in Cape Town cost R 127/1000 L while above 35000 L/M cost R 768.64/1000L. In Cape Town, 3000 L would cost R 381 at R 127/1000 L. In as much as water can be reused, R 381 stands as the initial cost of water required for growing microalgae in TAP. Even with the recycling of water, this shows that water required for cultivating microalgae biomass for biodiesel production contributes to the cost of algae biodiesel feedstock production. Substituting purchased freshwater with free wastewater from

the fish farm will eliminate the freshwater cost contribution to microalgae biodiesel production and thereby, reduce the cost of microalgae biomass cultivation.

From a lab-scale cost calculation of TAP media made from analytical grade reagents, preparation of stock solution for 1 L TAP media cost almost R1. It would cost R 3000 in TAP media nutrients to cultivate microalgae in 3000L of water for 1 L of biodiesel without recycling the media. Even with about 10 times recycling of TAP media for microalgae production, price contribution of nutrient media will be up to R 300/ L of biodiesel produced. the cost of nutrients is the cost saved by replacing TAP media with FFW which contains free nutrients.

Treating wastewater costs $ 0.11/1000L ("Running costs of wastewater treatment plants," n.d.), which is approximately R 5.78 for 3000L of wastewater. Using FFW as microalgae cultivation media saves costs associated with treating wastewater in two ways. It saves the cost of treating FFW if FFW is not used for microalgae cultivation and saves the cost of that would have applied to TAP media treatment before discharge when TAP media is used for microalgae cultivation. Combining the cost of nutrients at R 3000/ L of biodiesel cost with R 381 for 3000 L of water and R5.78 wastewater treatment involved in the production of 1 L of biodiesel (Table 5.3) brings the total cost of growth media for 1 L biodiesel production to R 3386.94. Assuming water and nutrients in TAP media are recycled, using FFW instead of purchased water and nutrients for microalgae biodiesel cultivation would save the initial cost of R 3386.94 required for water and nutrients. Also, using FFW nutrients for cultivating biodiesel microalgae feedstock eliminates the cost of FFW treatment (Guldhe et al., 2017). This saves the cost of water treatment and in turn, reduces the cost of fish farming.

Table 5.3 Initial costs of TAP and FFW media for 1L biodiesel microalgae cultivation

Parameter	FFW	TAP
Water	Free	R 381
Nutrient	Free	R 3000
Wastewater Treatment	Free	R 5.78
Total	R 0.00	R 3386.78

5.4 Conclusion

In this work, the LCA findings have shown that Tilapia FFW with characteristics shown in table 3.2 is a better media for cultivating microalgae due to lesser environmental burden associated with FFW cultivation. FFW and TAP standard media cultivation of microalgae indicated that growing microalgae in both FFW nutrients and purchased TAP media fertilisers have different levels of environmental burden. FFW media burden came mostly from electricity used in supplying light and supply of air. Reducing the amount of electricity involved in growing microalgae by half would reduce the environmental burden associated with electricity by half as indicated by the sensitivity analysis. Growing microalgae in TAP media containing fertiliser had a greater impact on the environment in all parameters with (3 times more on global warming (kg $CO_{2\ eq}$), 3.6 on marine ecotoxicity (kg 1,4-DCB), 2 on human carcinogenic toxicity (kg 1,4-DCB), 3.7 on freshwater ecotoxicity (kg 1,4-DCB), 3.9 on human non-carcinogenic toxicity (kg 1,4-DCB), 2 on freshwater eutrophication (kg P_{-eq}), 8 on terrestrial ecotoxicity (kg 1,4-DCB) and 15 on water consumption (m^3)) when compared to using FFW nutrients. The use of fertilizers contributed mostly to the environmental burden of using TAP media for cultivation, while the use of electricity contributed to the most environmental burden of growing microalgae in FFW media. This suggests that replacing

TAP media with FFW would reduce the environmental impact associated with fertiliser use. Also, the supply of large volumes of water and nutrients required for the cultivation of microalgae feedstock with FFW will greatly reduce the cost of microalgae biodiesel feedstock production by R 3385 for the initial cost of 1L biodiesel before media recycling. There are savings when considering costs of energy and labour applies to the use of both media in microalgae cultivation. That is, the money saved from initial water and nutrient purchase (R 3385) for the production of 1 L biodiesel has shown to reduce. the initial cost of starting up even if both water and nutrients are recycled.

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Chapter 6 Conclusion and Recommendation

6.1 Conclusion

This research has found that FFW nutrients are suitable for cultivating microalgae. It also found that FFW supports the accumulation of desired fatty acids for biodiesel production and at the same time, saves cost and environmental impacts of microalgae fertiliser cultivation. Below are the conclusions to each of the objectives in the introduction.

Exploit available nitrogen and phosphorus in fish farm effluent as a cheap source of nutrients for algal biomass cultivation and compare the rate of nutrient removal, algal growth and lipid accumulation (Chapter 3)

It was shown that that fish farm wastewater (FFW) nutrients-ammonia, nitrate and phosphorus- are suitable for cultivating microalgae for biodiesel production. The characterization of FFW ascertained that FFW had ammonia, nitrate and phosphates in the range required to support cultivation (Chapter 3 Cultivation of microalgae in fish farm effluent). Secondly, the growth and biomass yield of algae cultivated in FFW also confirms that the nutrients in FFW are available and suitable to support algae growth and accumulation of compounds. Again, higher lipid content and lipid productivity of species cultivated in FFW also confirms the suitability of FFW nutrients for the production of biodiesel. This can be attributed to low nutrient concentration affecting more lipid accumulation and mixed nitrogen in FFW favouring higher biomass yield and biomass production. The uptake of nutrients in FFW by microalgae for growth and biomass accumulation was reflected in high FFW nutrient achieved in microalgae especially in species grown in modified FFW with almost complete nutrient removal.

Determine the rate of CO_2 use by the three different algae species (Chapter 3)

The higher CO_2 uptake rate in species of microalgae cultivated in FFW suggests that microalgae use CO_2 more efficiently in FFW. This could be further developed for mitigating greenhouse gas effects of CO_2 in the atmosphere. The better biomass yield, biomass productivity, better growth rate, higher lipid and carbohydrate content, higher CO_2 uptake and content of microalgae cultivated in both FFW can be attributed to the presence of mixed nitrogen sources (ammonia and nitrate) in the FFW media (Chapter 3 Cultivation of microalgae in fish farm effluent). The results obtained with lipid suggest that the low nutrient range stimulated the accumulation of more lipids especially *Chlamydomonas reinhardtii* with cultivated in FFW.

Quality of the biodiesel produced from algae cultivated in fish farm wastewater (Chapter 4)

The FAME composition and fuel properties which are affected by the culture media composition also show that FFW nutrients are suitable for growing algae biomass for biodiesel production. All species cultivated had values within both ASTM D6751-02 and EN 14214 standards specifications. This indicated that the biodiesel obtained had desirable biodiesel properties (Cetane number, kinematic viscosity, density, high heating, cold filter plugging point and iodine value). These desirable fuel properties of FFW were similar to that cultivated in the standard media. *Chlamydomonas reinhardtii* cultivated in both FFW had better cold filter plugging point properties and would be more suited in every climate. These desirable fuel properties of microalgae cultivated in FFW is an indication that FFW can substitute purchased nutrients in standard growth media for biodiesel microalgae cultivation. This would eliminate a fraction of cost associated with purchased nutrient microalgae cultivation and thereby, reduce the cost of microalgae biodiesel production.

Environmental and cost effects of algae cultivation in fish farm wastewater (Chapter 5)

The LCA comparison of cultivating microalgae in FFW and purchased fertiliser is another reason FFW should be used instead of purchased fertilisers in cultivating microalgae biomass feedstock for biodiesel production. It was shown that even though cultivating microalgae in FFW resulted in electricity usage from supplied light and gas input into the system, those burdens were lesser in comparison to the environmental burdens of TAP media (3 times more on global warming, 3.6 on marine ecotoxicity, 2 on human carcinogenic toxicity 3.7 on freshwater ecotoxicity, 3.9 on human non-carcinogenic toxicity, 2 on freshwater eutrophication, 8 on terrestrial ecotoxicity and 15 on water consumption) (Chapter 5 Environmental and economic impacts of using fish farm wastewater for microalgae cultivation). Suggestions to reduce the usage of electricity when cultivating microalgae in FFW include replacing electricity light supply with sunlight by outdoor photobioreactors, sourcing more energy-efficient methods to mix the media and deliver CO_2.

Nutrient composition of the growth media determines the composition of the algae biomass. The composition of the biomass is critical in selecting a feedstock for biodiesel production. Among the three species of algae selected for this study (*Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii*), *Chlamydomonas reinhardtii* had the least growth rate in each culture media but better lipid content of 44.08% in unmodified FFW and 42.07% in unmodified FFW and fuel properties (only species with most desirable CFPP). This makes selecting *Chlamydomonas reinhardtii* for cultivation in FFW for biodiesel production more favourable if growth rate can be improved.

In closing, this work has shown that microalgae, *Tetradesmus obliquus, Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii*, can be grown in fish farm wastewater. The production of microalgae can be carried out at a fraction of the cost, leading to affordable

biodiesel from microalgae cultivated in FFW. By using this method of cultivation shown in this thesis, eutrophication effects resulting from discharged high nutrient load FFW will be reduced. Additionally, the usage of freshwater resources and the cost of water treatment and purchase of fresh water for the fish farmer will be decreased. This work has shown that FFW can substitute purchased nutrients favourably for the cultivation of microalgae for biodiesel production. It has also shown that *Chlamydomonas reinhardtii* is a better algae species cultivation microalgae in FFW for biodiesel production with the best overall fuel properties and high lipid accumulation of 44.08% in unmodified FFW and 42.07% in unmodified FFW.

6.2 Recommendations

Based on the results obtained from this research, I would recommend FFW as a growth media to be used in cultivating microalgae species with higher biomass production and lipid accumulation. Cultivating microalgae in FFW will supply media that supports the accumulation of favourable fatty acids, high lipid accumulation, reduce the cost of purchased nutrients and supply large volumes of water required for microalgae cultivation.

A policy to regulate the amount of nutrients in FFW discharged is also recommended with incentives for using these large volumes of FFW are recycled for biodiesel microalgae feedstock cultivation to mitigate the environmental effects nutrient-loaded FFW. This would provide cheaper microalgae required for biodiesel production while saving the environment from the effects of discharging large volumes of nutrient-loaded wastewater. The cost of freshwater purchased for fish farming could also be reduced.

High light intensity was used in this study in an attempt to enhance lipid accumulation. High lipid accumulation was achieved but biomass yield and productivity was low. The high light intensity may need to be reduced in future studies due to low biomass yield obtained in all the culture media. Also, species with higher biomass yield and productivity can be added to the species used in future studies to improved biomass yield and productivity.

This research was batch cultivation, for future research, I recommend outdoor scale-up research on the fish farm, coupled with the fish farming process for continuous microalgae. The suggestion of outdoor cultivation is based on the need to reduce the environmental impact of electricity used to supply light for the indoor cultivation of microalgae. Sunlight may also supply a more suitable light intensity for higher biomass productivity.

Appendix
A. Calibrations Related to Chapter 3

This section contains calibration data for microalgae biomass determination, D-glucose standard calibration for phenol-sulfuric acid carbohydrate determination, Bovine serum albumin (BSA) standard calibration for protein analysis and microalgae growth rate.

Table A. 1 Growth Curve Calibration values

Sample	Media	Absorbance	Concentration
Tetradesmus obliquus	Tris-Acetate-Phosphate	(nm)	(g/l)
		0.071	0.016
		0.073	0.018
		0.315	0.05
		0.652	0.116
		0.95	0.158
Heterochlorella luteoviridis	Tris-Acetate-Phosphate		
		0.063	0.016
		0.081	0.018
		0.353	0.064
		0.598	0.098
		0.887	0.106
Chlamydomonas reinhardtii	Tris-Acetate-Phosphate	0.044	0.008
		0.051	0.012
		0.217	0.046

		0.27	0.082
		0.471	0.102
Tetradesmus obliquus	Unmodified FFW		
		0.093	0.012
		0.119	0.028
		0.438	0.06
		0.653	0.132
		1.187	0.196
Heterochlorella luteoviridis	Unmodified FFW		
		0.09	0.006
		0.153	0.036
		0.452	0.08
		0.653	0.126
		1.192	0.188
Chlamydomonas reinhardtii	Unmodified FFW		
		0.056	0.008
		0.063	0.018
		0.28	0.054
		0.441	0.106
		0.878	0.146
Tetradesmus obliquus	Modified FFW		

		0.15	0.03
		0.46	0.08
		0.83	0.13
		1.09	0.17
		1.20	0.22
Heterochlorella luteoviridis	Modified FFW		
		0.14	0.02
		0.55	0.07
		0.81	0.13
		1.14	0.19
		1.27	0.22
Chlamydomonas reinhardtii	Modified FFW		
		0.12	0.02
		0.24	0.04
		0.39	0.06
		0.64	0.10
		0.79	0.15

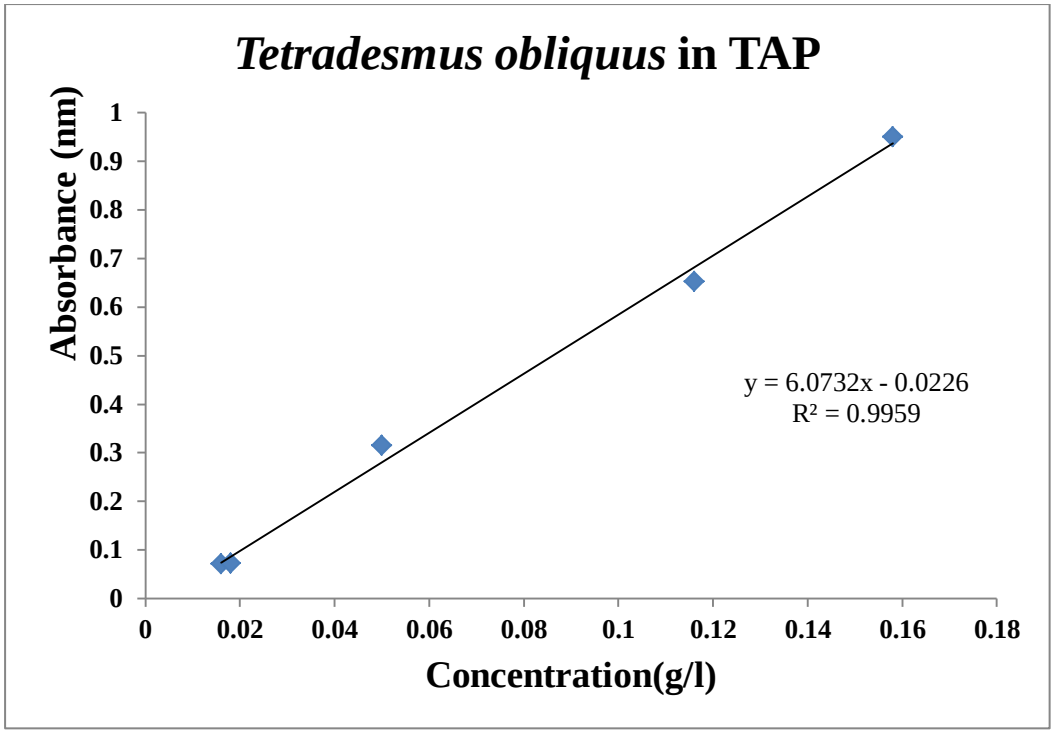


Figure A. 1 Biomass Yield Determination of *Tetradesmus obliquus* in TAP

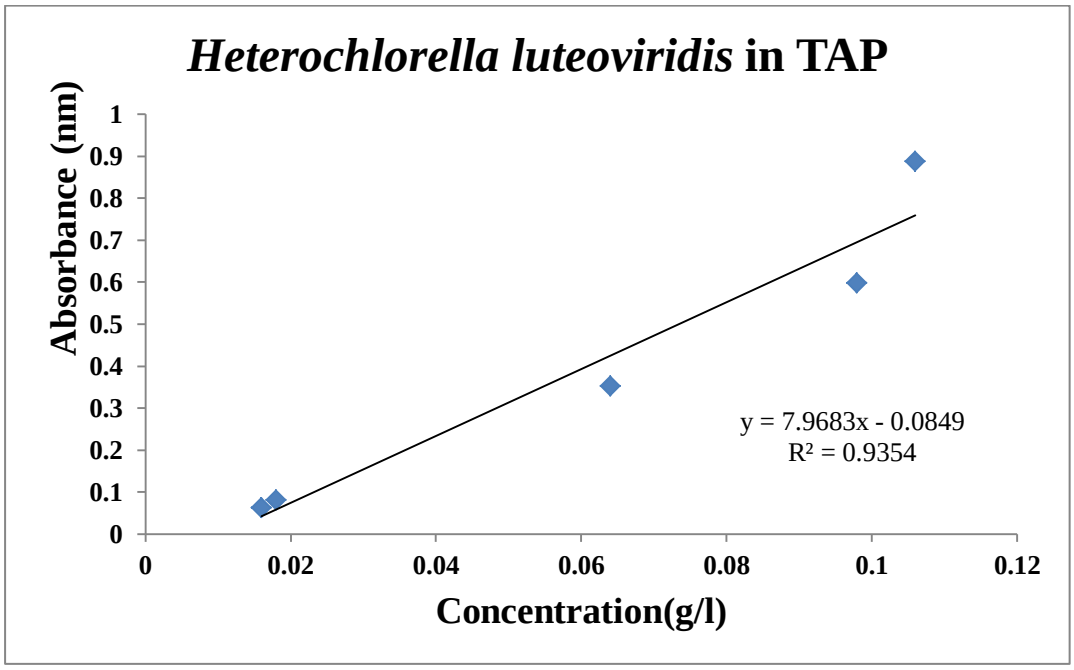


Figure A. 2 Biomass Yield Determination of *Heterochlorella luteoviridis* T2

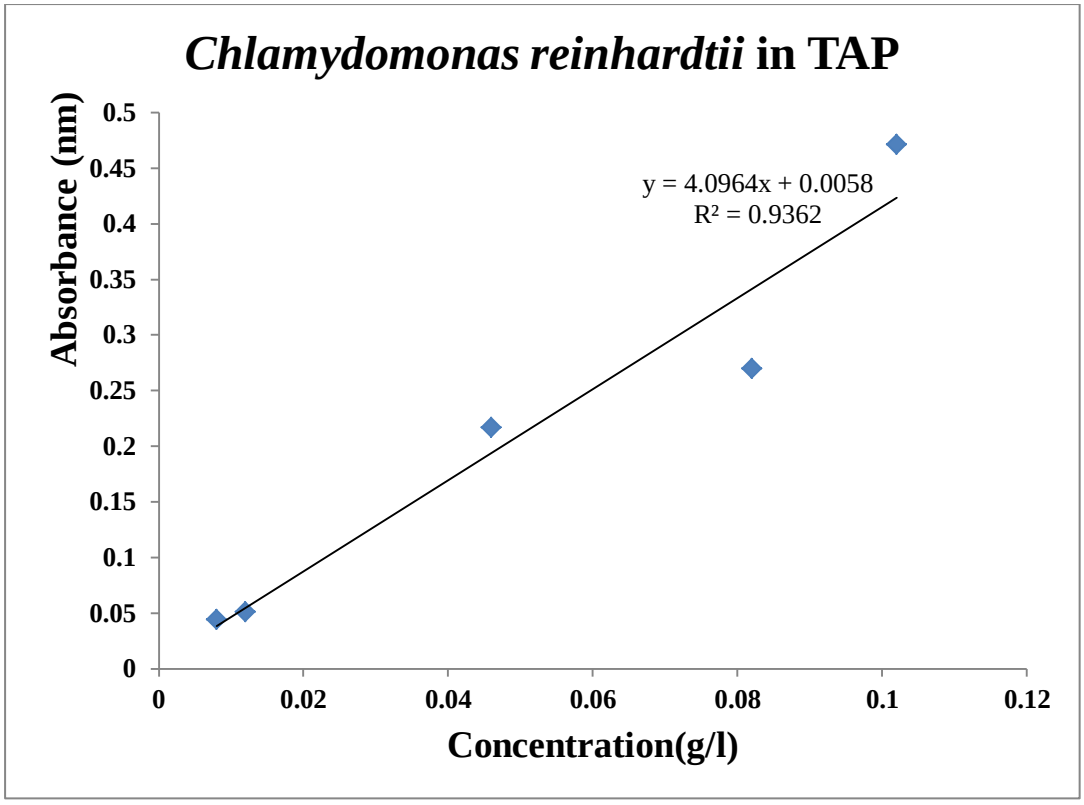


Figure A. 3 Biomass Yield Determination of *Chlamydomonas reinhardtii* in TAP

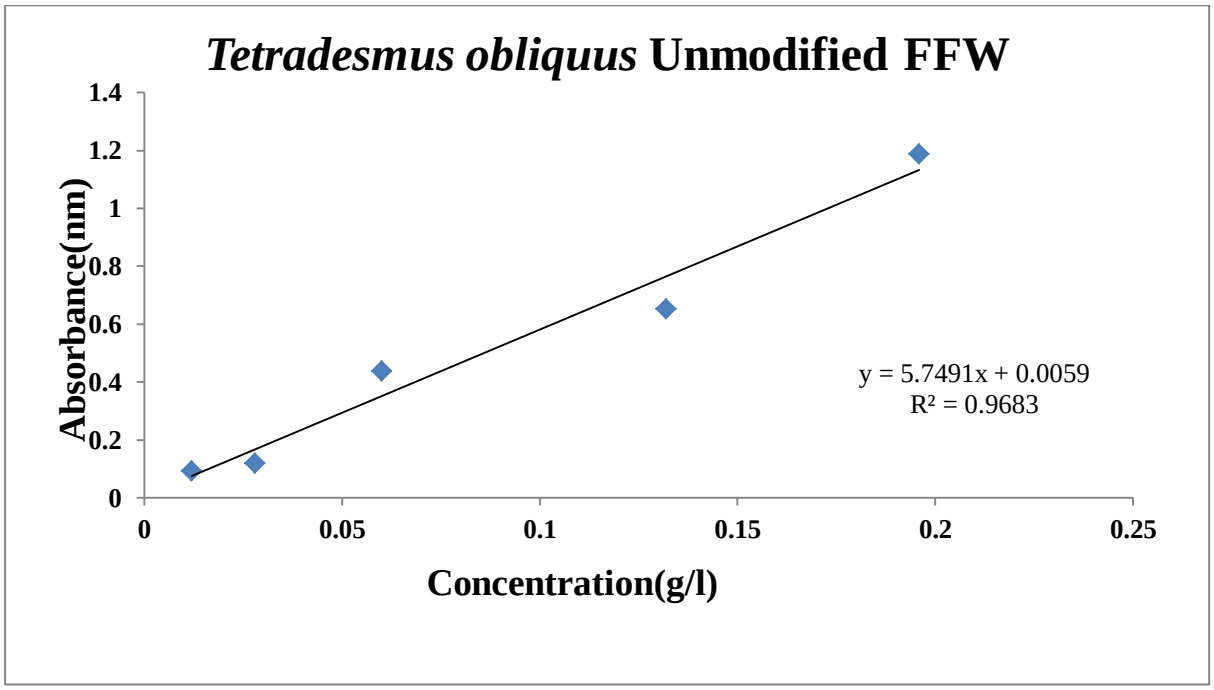


Figure A. 4 Biomass Yield Determination of *Tetradesmus obliquus* in unmodified FFW

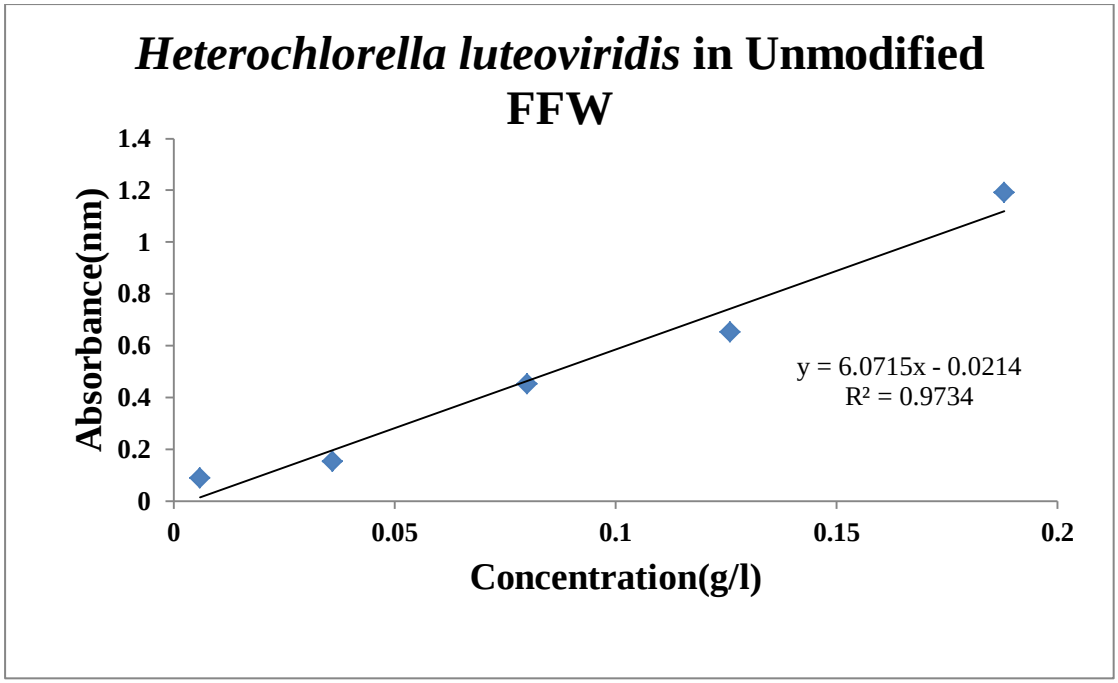


Figure A. 5 Biomass Yield Determination of *Heterochlorella luteoviridis* in Unmodified FFW

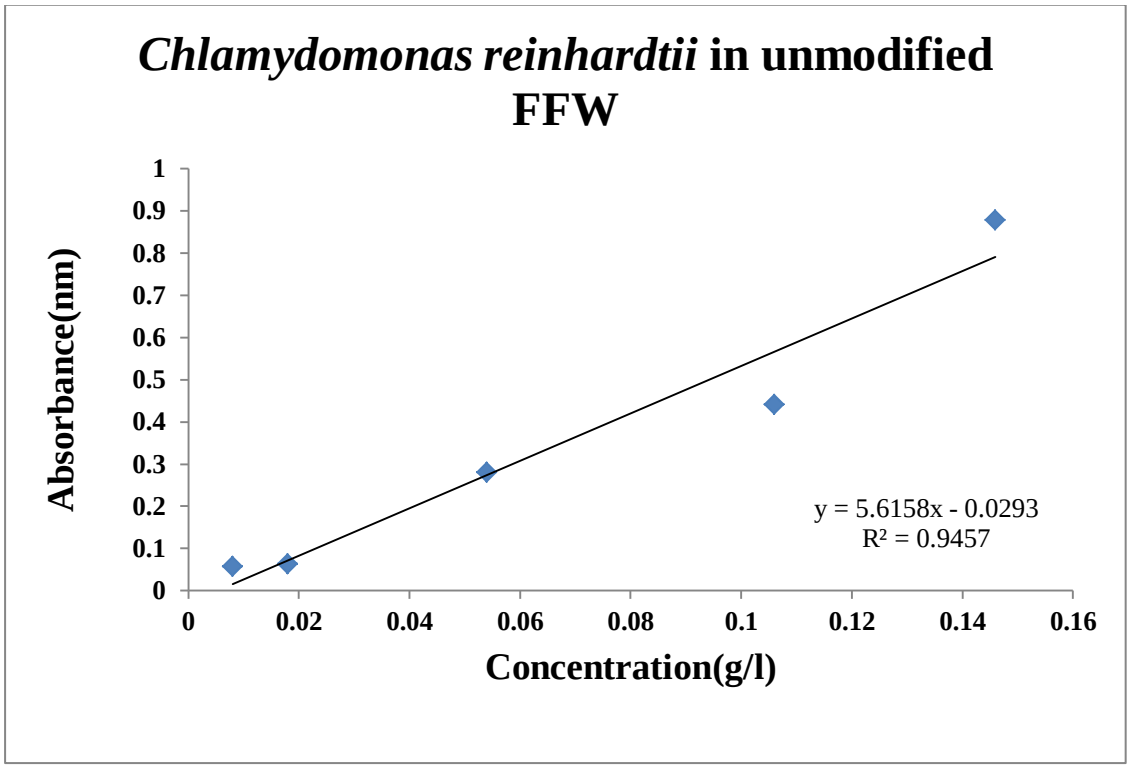


Figure A. 6 Biomass Yield Determination of *Chlamydomonas reinhardtii* in Unmodified FFW

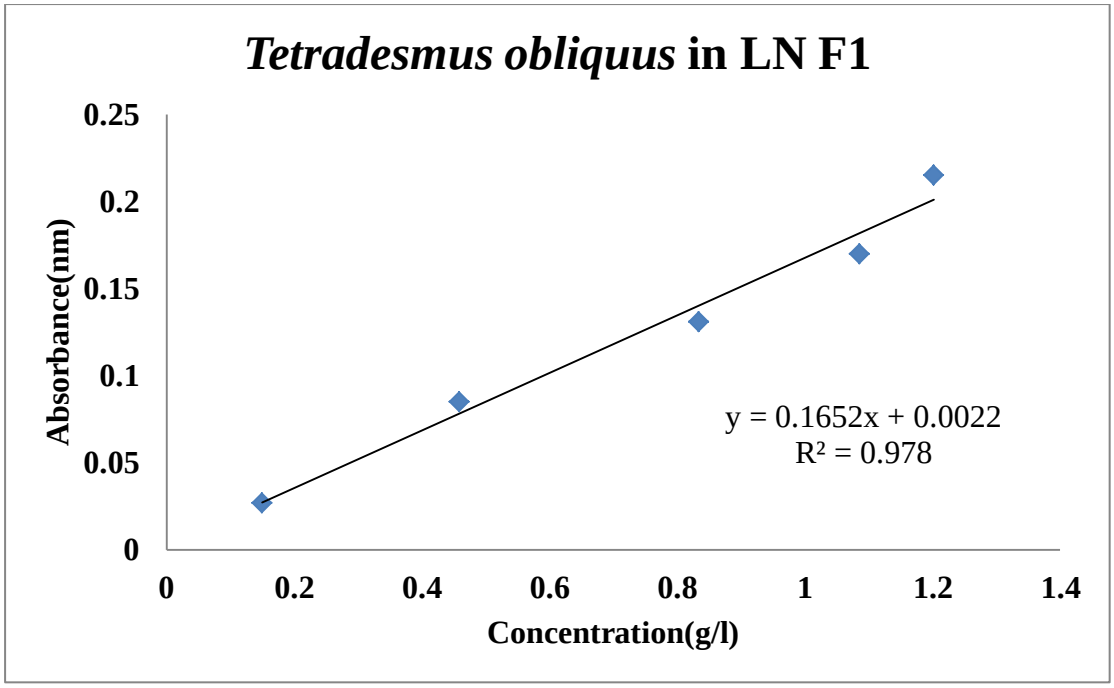


Figure A. 7 Biomass Yield Determination of *Tetradesmus obliquus* in Modified FFW

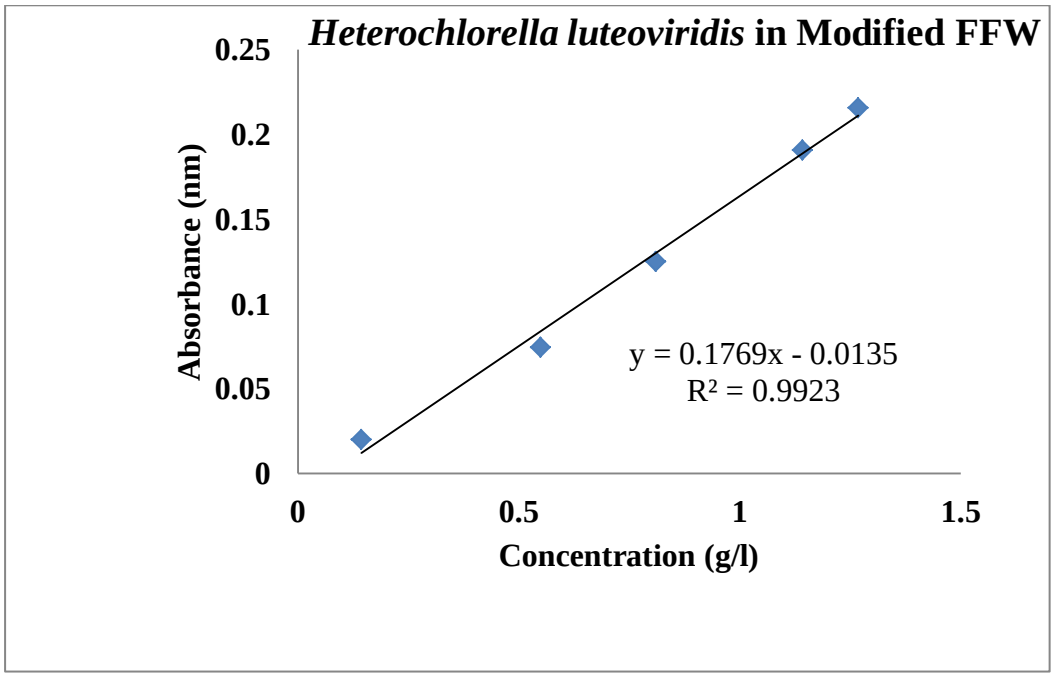


Figure A. 8 Biomass Yield Determination of *Heterochlorella luteoviridis* in Modified FFW

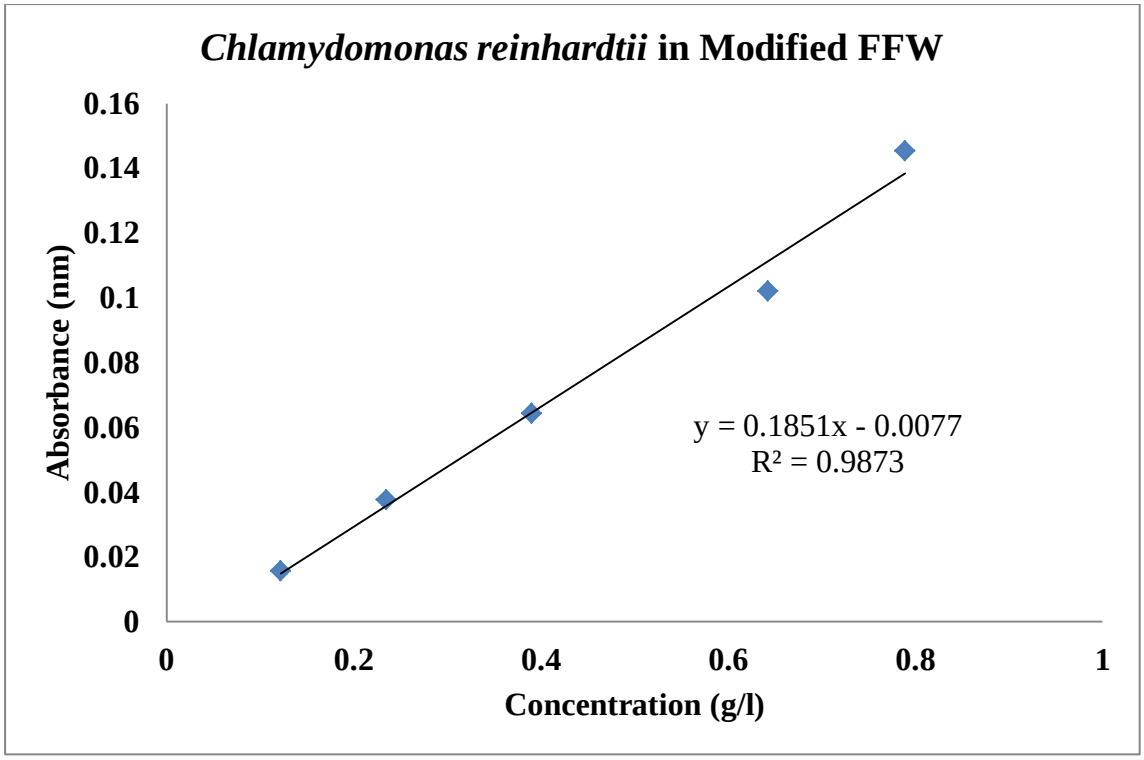


Figure A. 9 Biomass Yield Determination of *Chlamydomonas reinhardtii* in Modified FFW

Total Carbohydrate Using Phenol Sulfuric Acid Method with Spectrophotometer

Table A. 2 Preparation of Carbohydrate Standard Calibration Concentrations

Concentration of Stock mg/ml	Volume of Stock (µl)	Volume of Water (µg)	Final Concentration (µg/ml)
1	0	4000	0
1	20	3980	5
1	40	3960	10
1	60	3940	15
1	80	3920	20
1	100	3900	25

Table A. 3 Calibration Values for Carbohydrate Analysis

Absorbance (nm)	Concentration (µg/ml)
0.597	0
0.685	5
0.771	10
0.842	15
0.968	25

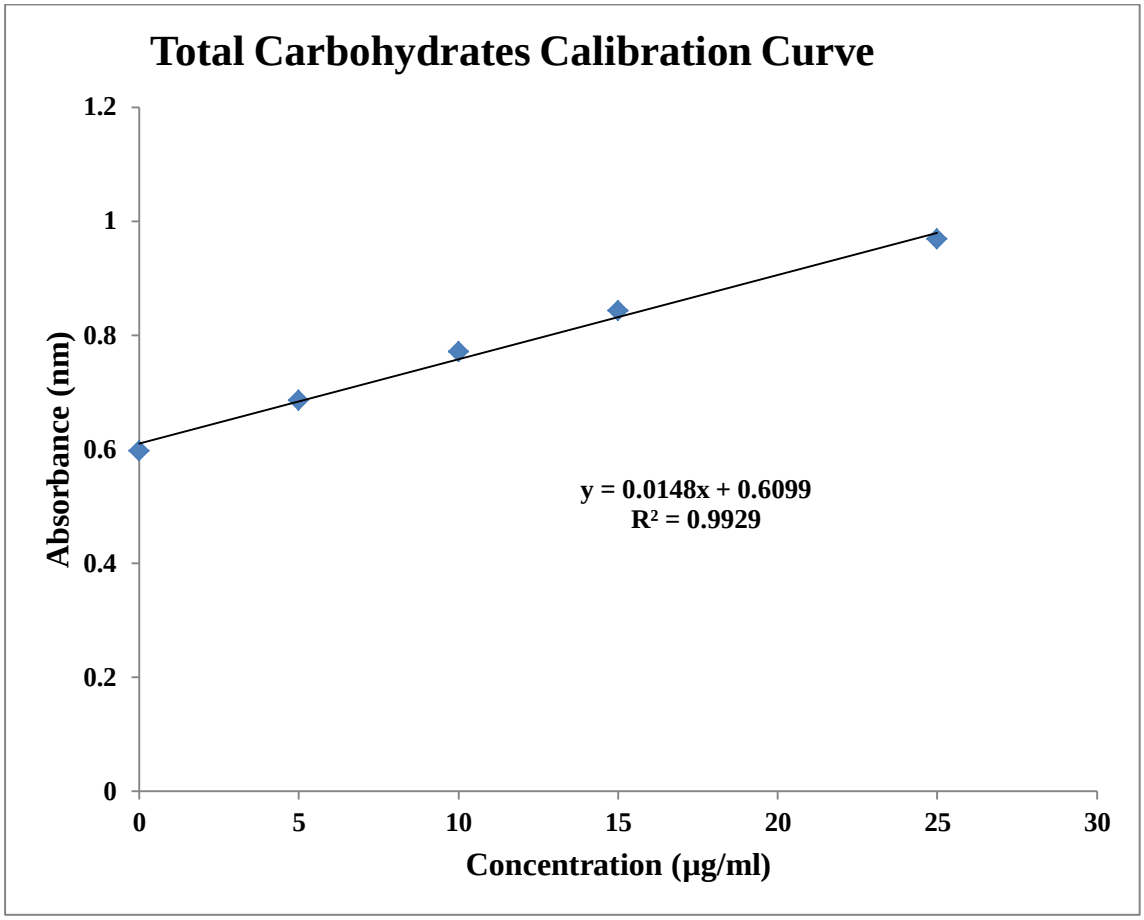


Figure A. 10 Total Carbohydrate Calibration Curve

Protein Analysis

Table A. 4 Preparation of Protein Standard Calibration Concentrations

Concentration of Stock mg/ml	Volume of Stock (µl)	Volume of Water (µl)	Final Concentration (µg/ml)
1	0	2000	0
1	20	1980	10
1	40	1960	20
1	60	1940	30
1	82	1920	40
1	100	1900	50

Table A. 5 Protein Analysis Calibration Data

Concentration (µl)	Absorbance
0	0.436
10	0.49
20	0.512
30	0.561
40	0.624
50	0.73

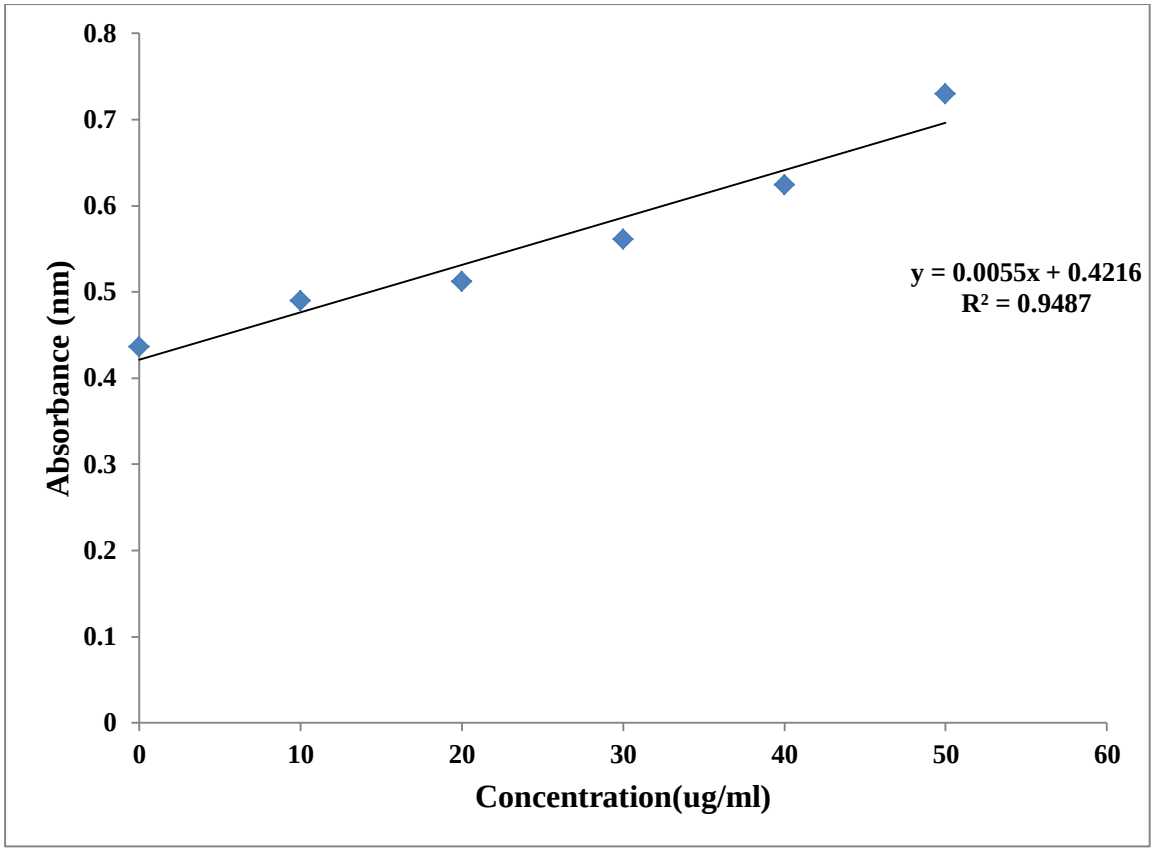


Figure A. 11 Protein Test Calibration

Microalgae Growth Curves

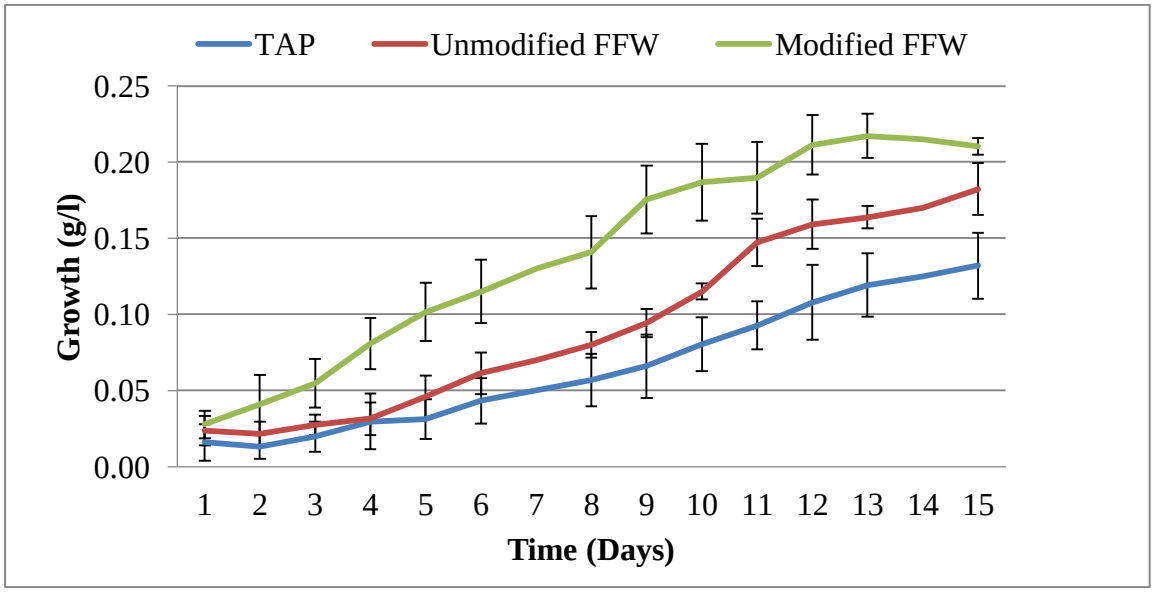


Figure A. 12Growth Curve of *Tetradesmus obliquus* cultivated in the three different growth media

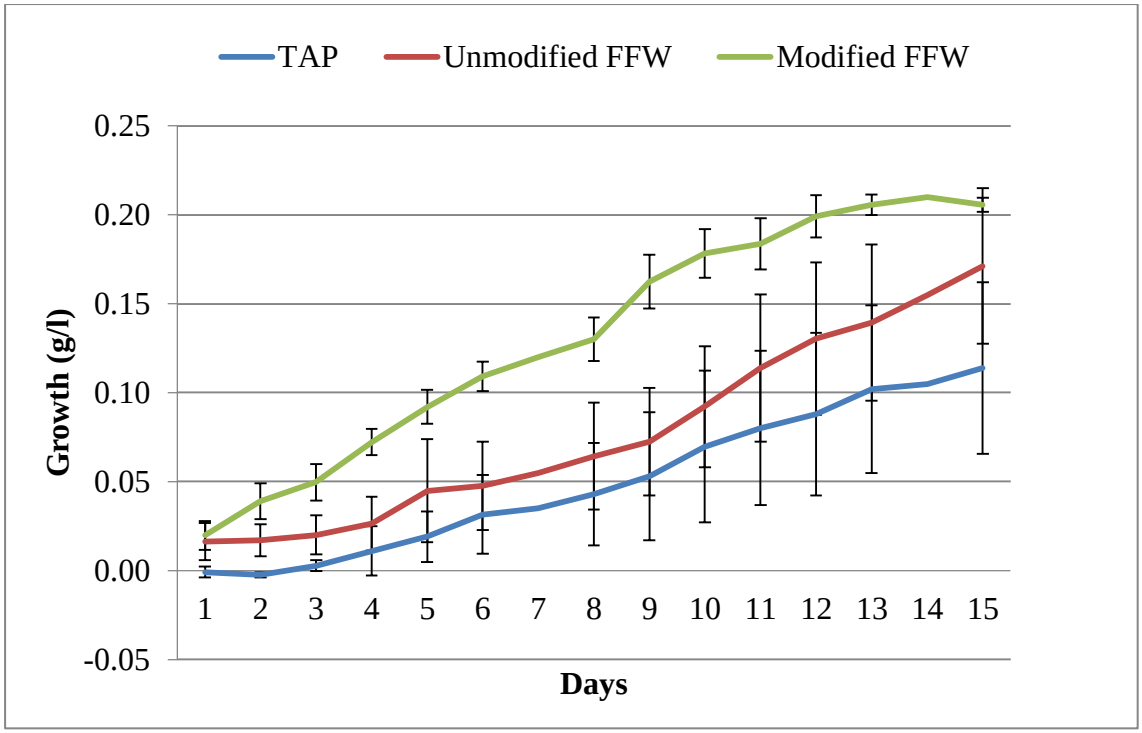


Figure A. 13 Growth Curve of *Heterochlorella luteoviridis* cultivated in the three different growth media

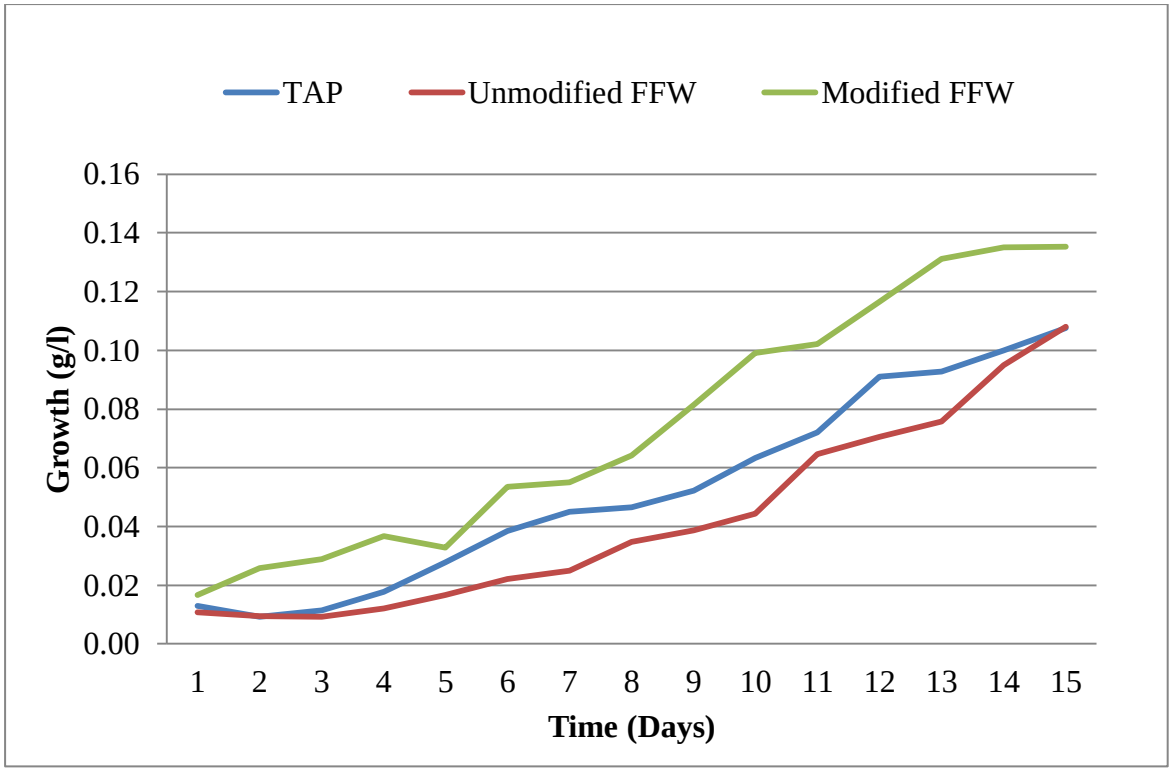


Figure A. 14 Growth Curve of *Chlamydomonas reinhardtii* cultivated in the three different growth media

B Related to Chapter 5

This section contains impact assessment and process contribution plots generated by SimaPro9.0 software cultivations in FFW and TAP media.

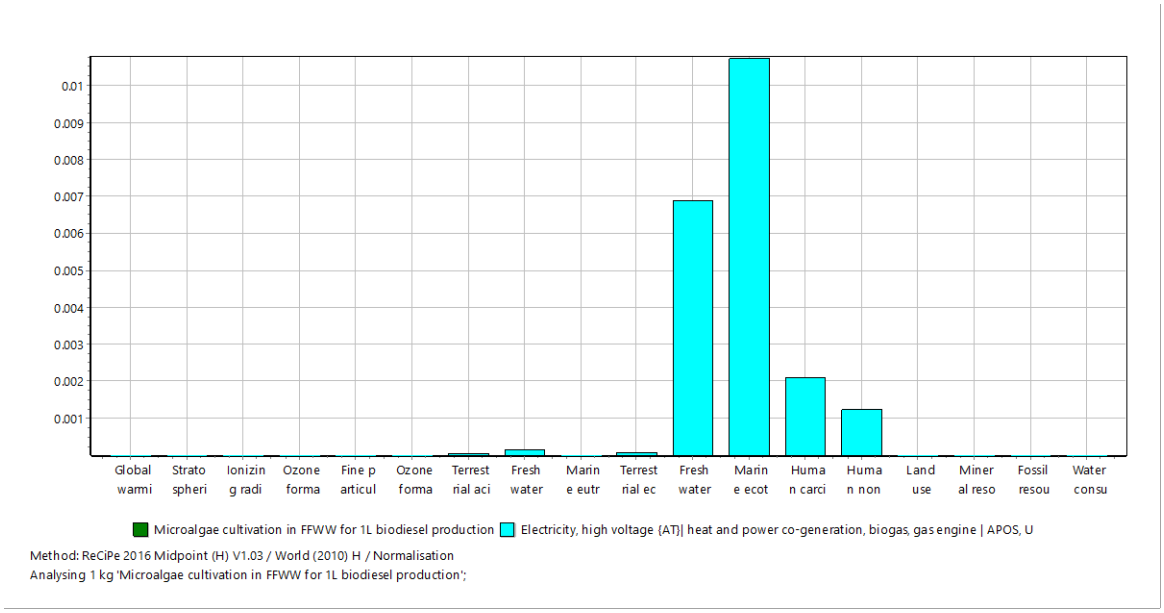


Figure B. 1 Normalisation LCA plot of microalgae cultivation in FFW for the production of 1L biodiesel

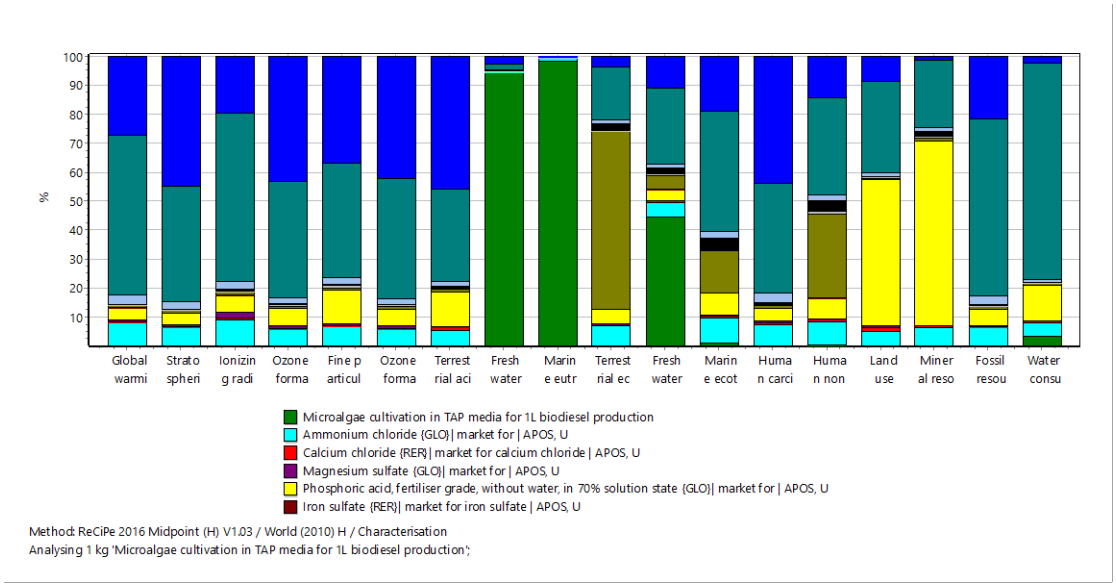


Figure B. 2 Characterisation LCA plot of microalgae cultivation in TAP media for the production of 1L biodiesel

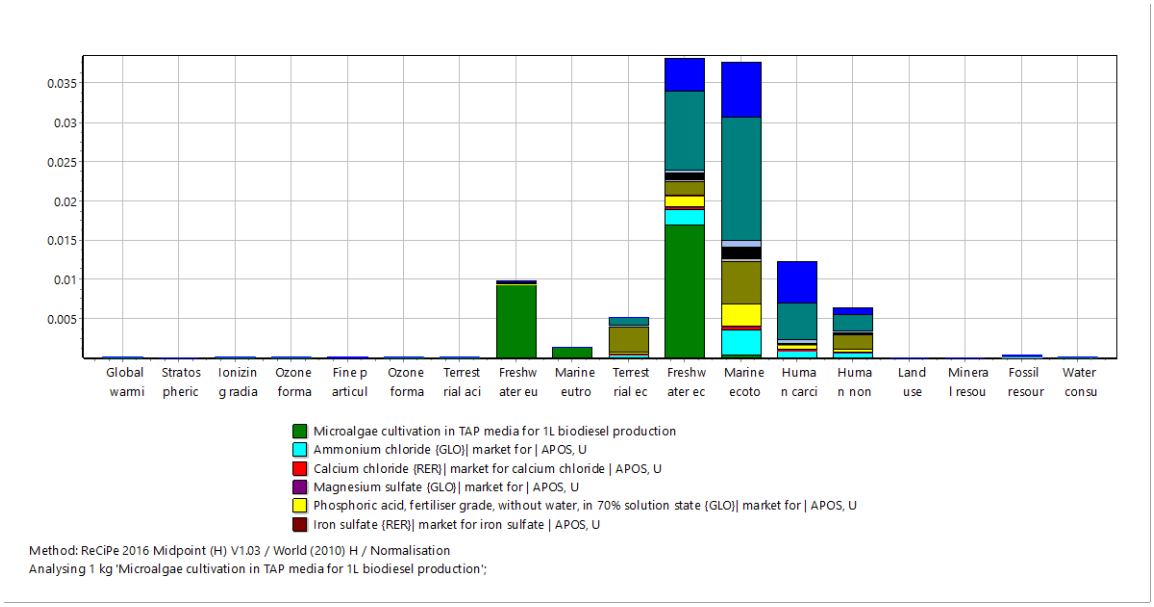


Figure B. 3Normalisation LCA plot of microalgae cultivation in TAP for the production of 1L biodiesel

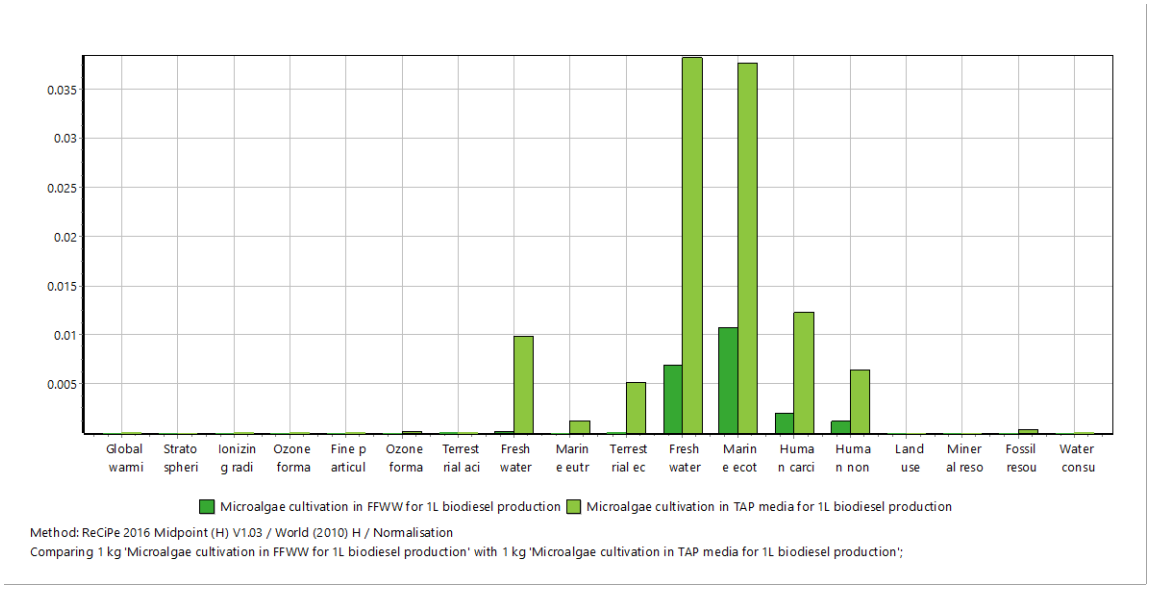


Figure B. 4 Normalisation LCA plot comparing microalgae cultivation in FFW and in TAP media for the production of 1L biodiesel

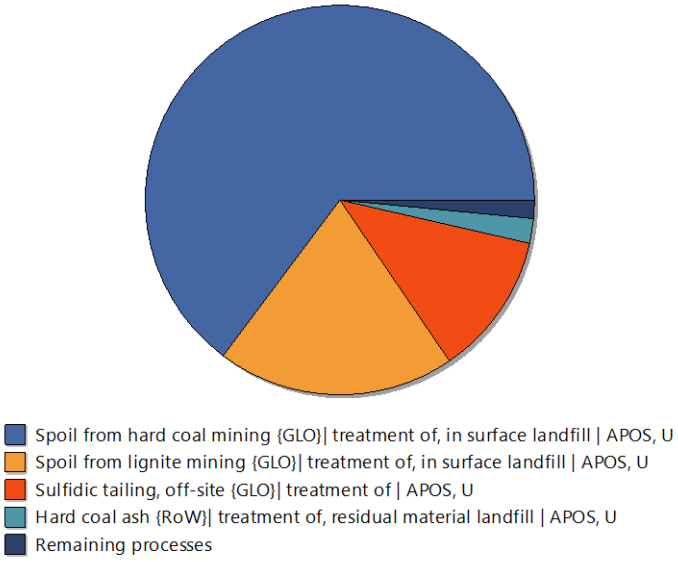


Figure B. 5 Process Contribution to Freshwater eutrophication in TAP

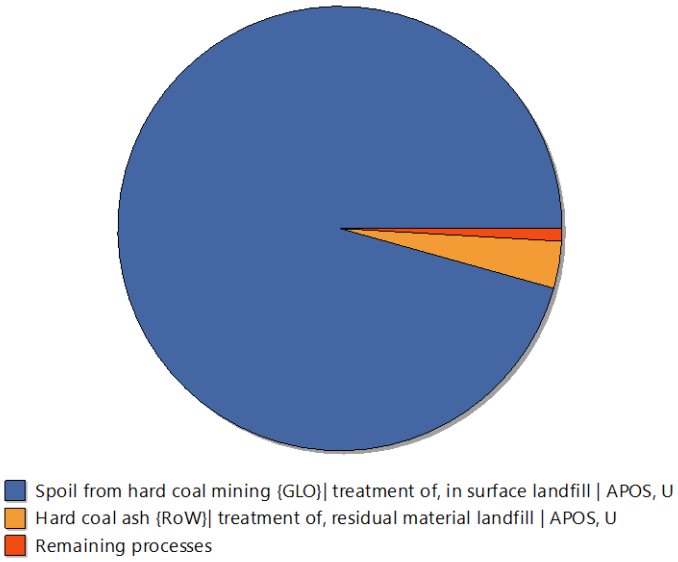


Figure B. 6 Process Contribution to Freshwater eutrophication in FFW

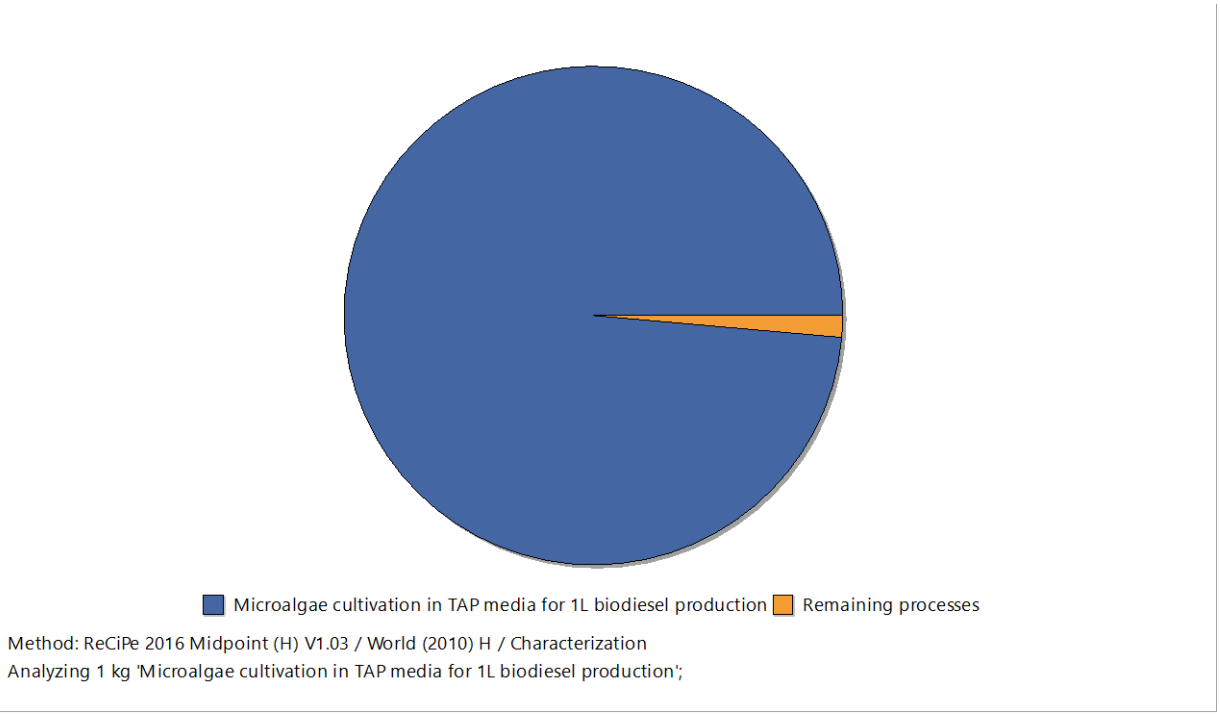


Figure B. 7 Process contribution to marine eutrophication in TAP

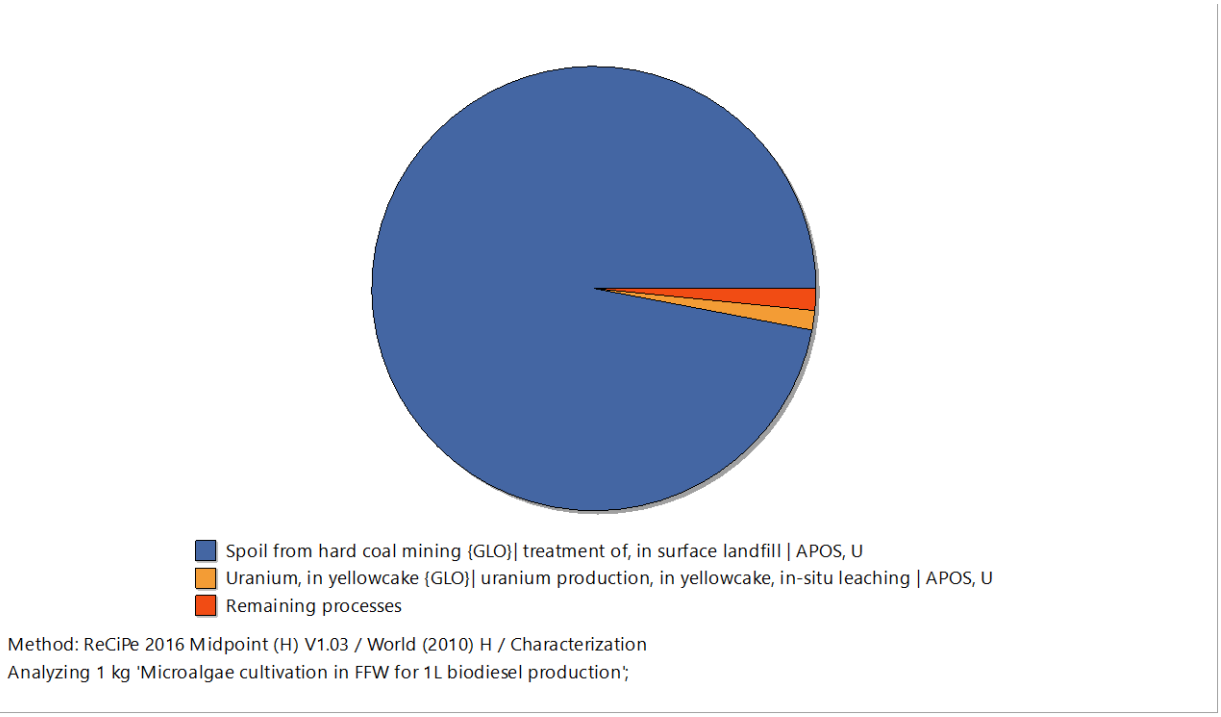


Figure B. 8 Process contribution to marine eutrophication in FFW

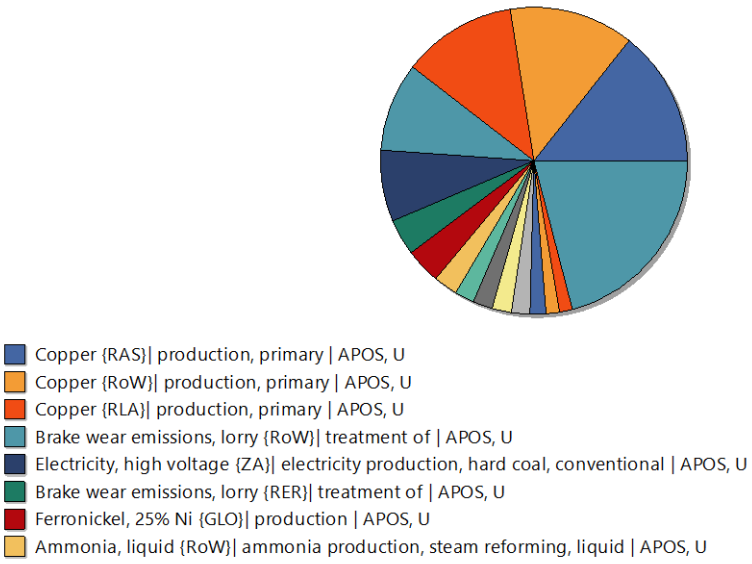


Figure B. 9 Process Contribution to Terrestrial Ecotoxicity in TAP

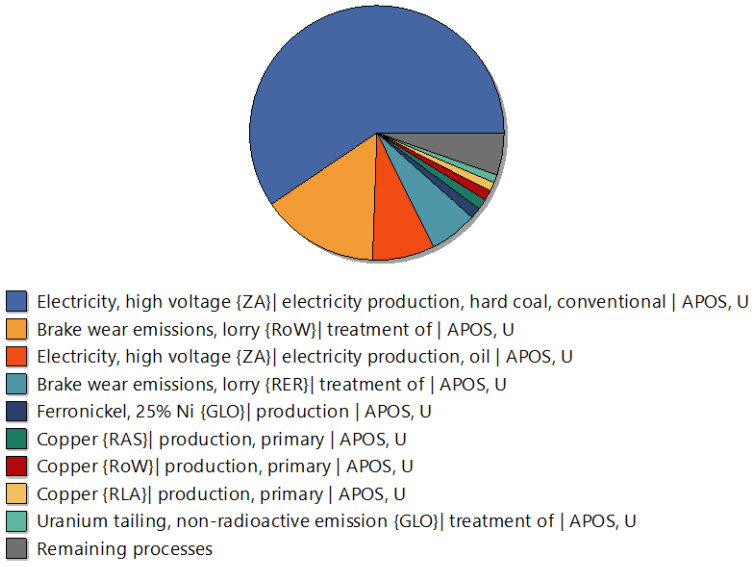


Figure B. 10 Process Contribution to Terrestrial Ecotoxicity in FFW

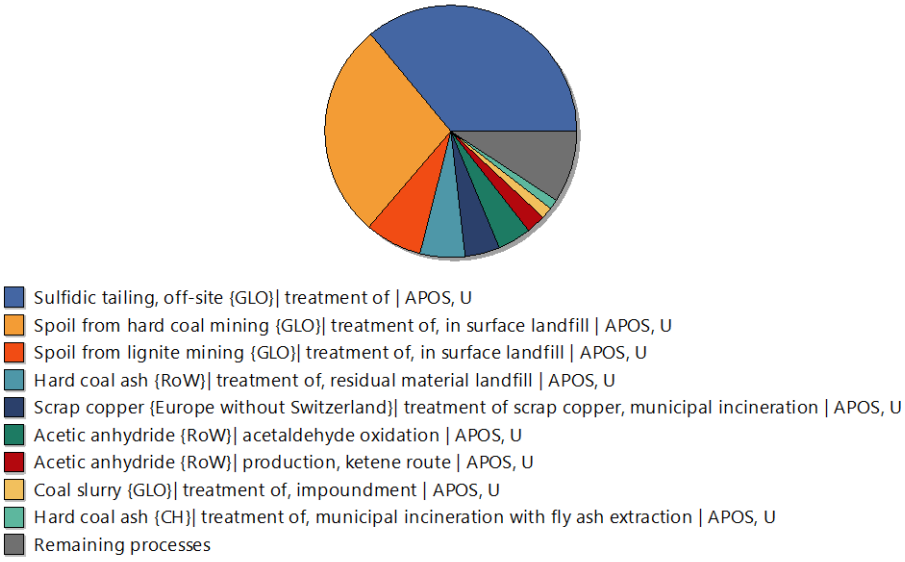


Figure B. 11 Process Contribution to Freshwater ecotoxicity in TAP

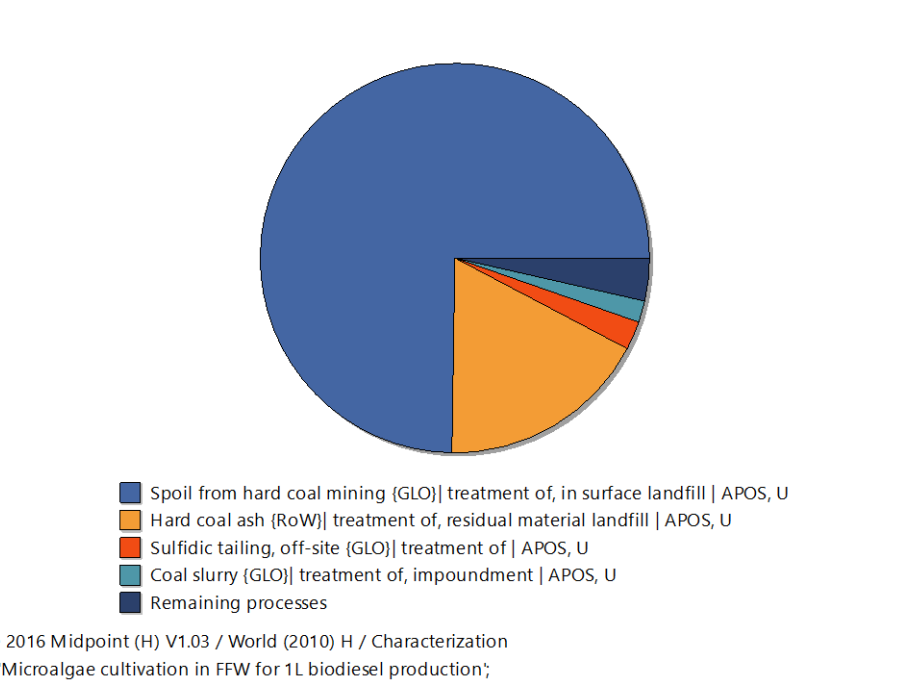


Figure B. 12 Process Contribution to Freshwater ecotoxicity in FFW

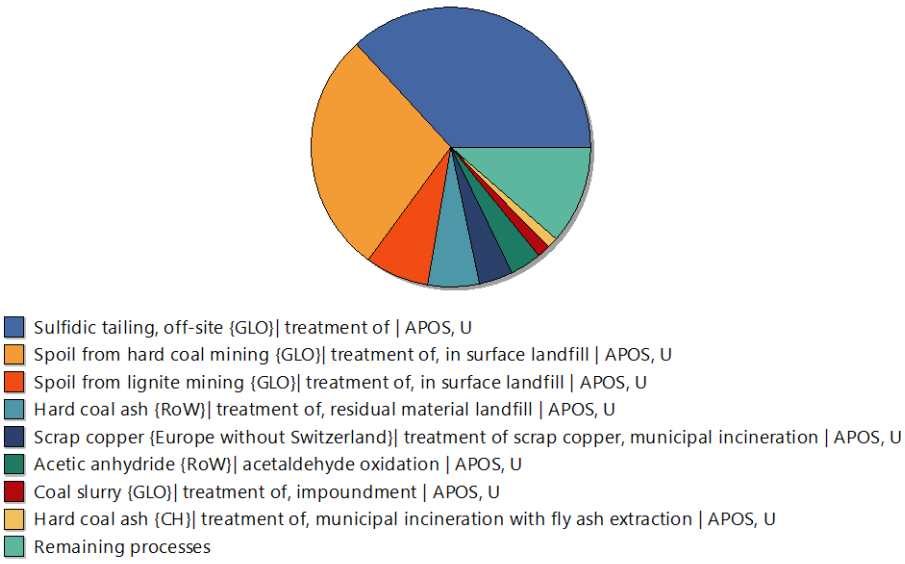


Figure B. 13 Process Contribution to Marine Ecotoxicity in TAP

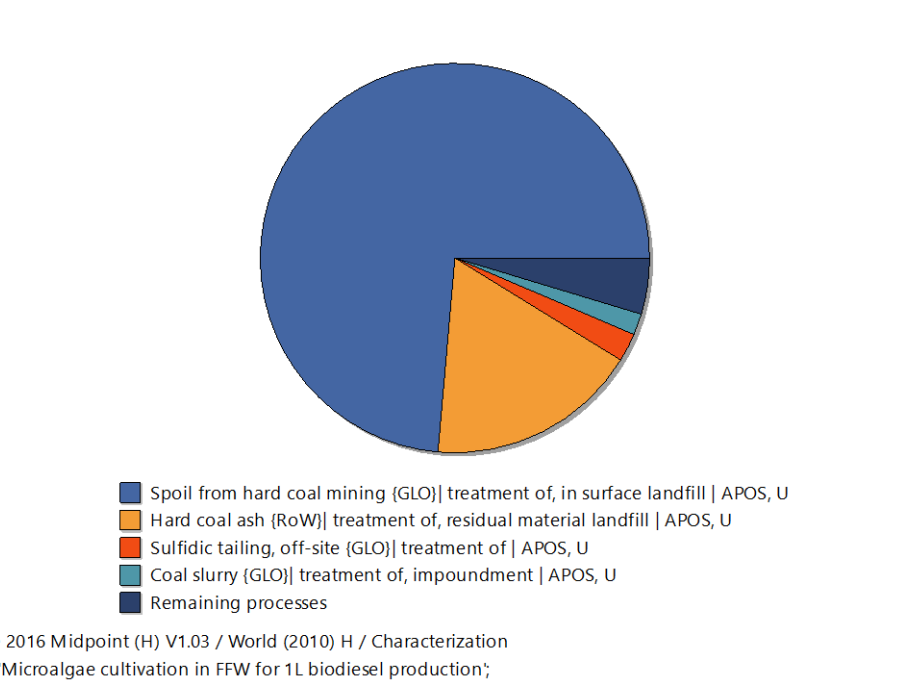


Figure B. 14 Process Contribution to Marine Ecotoxicity in FFW

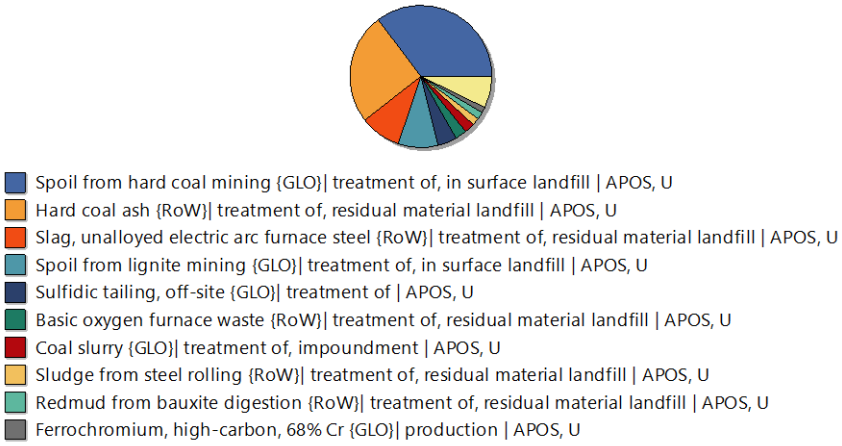


Figure B. 15 Process control to Human Carcinogenic Toxicity in TAP

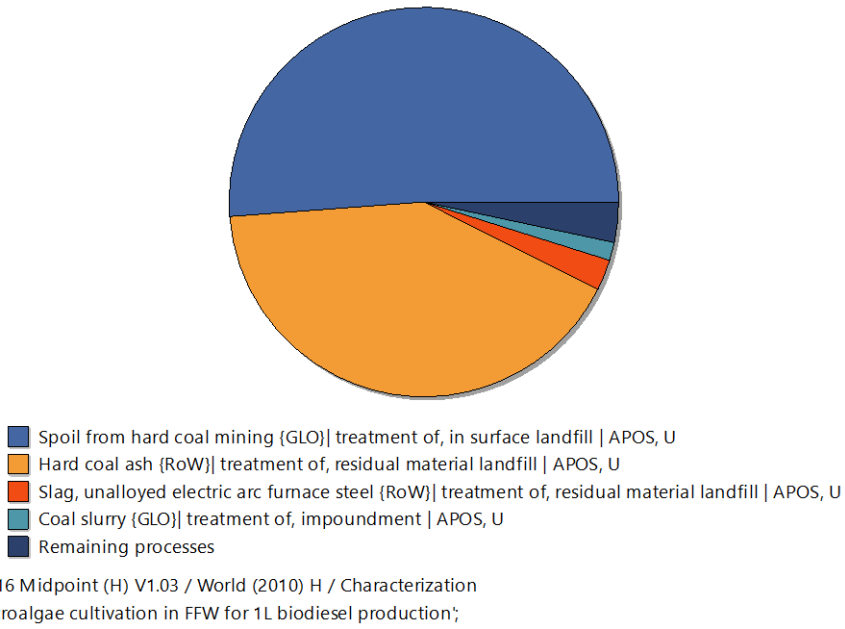


Figure B. 16 Process control to Human Carcinogenic Toxicity in FFW

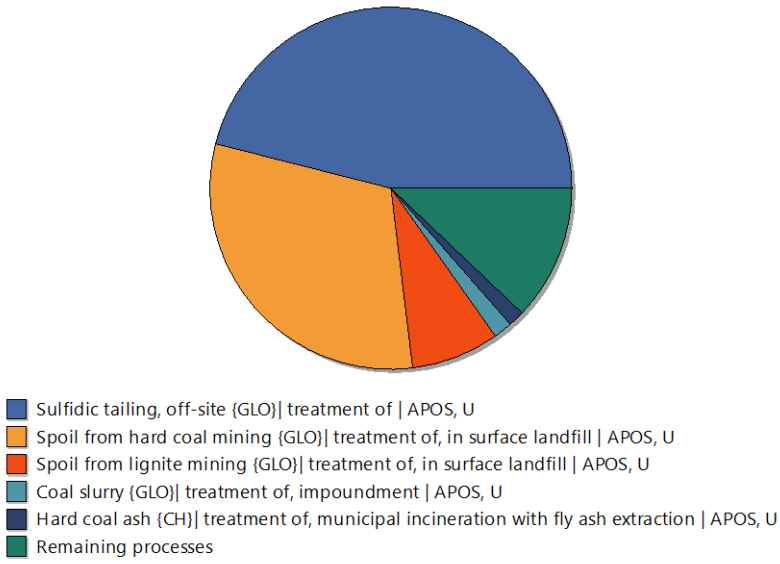


Figure B. 17 Process control to Human Non-carcinogenic Toxicity in TAP

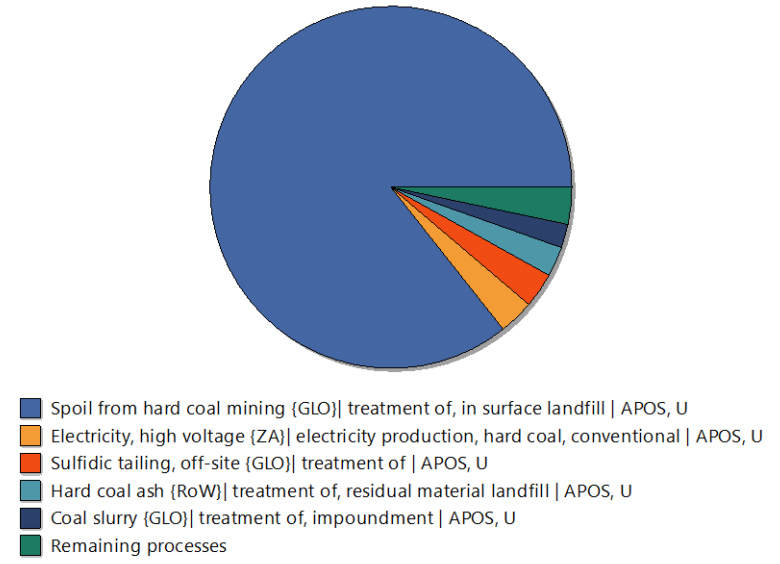


Figure B. 18 Process control to Human Non-carcinogenic Toxicity in FFW

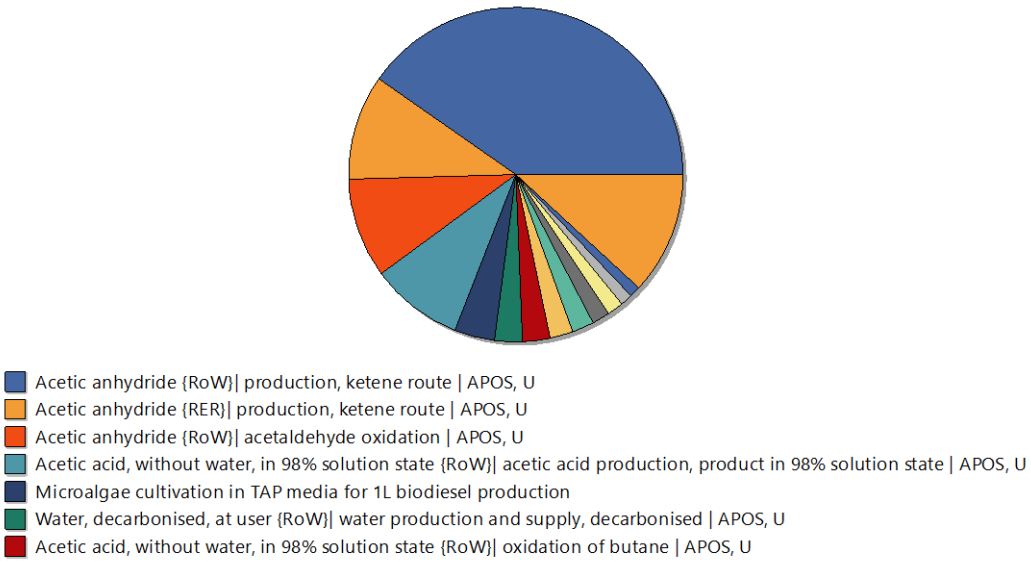


Figure B. 19 Process Contribution to Water Consumption in TAP

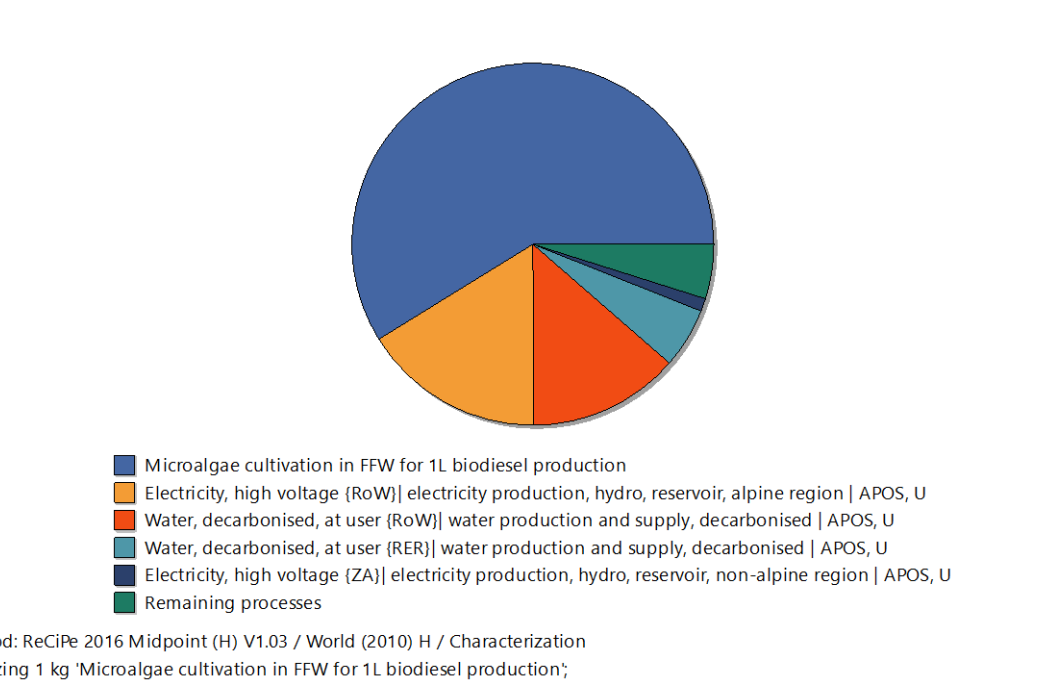
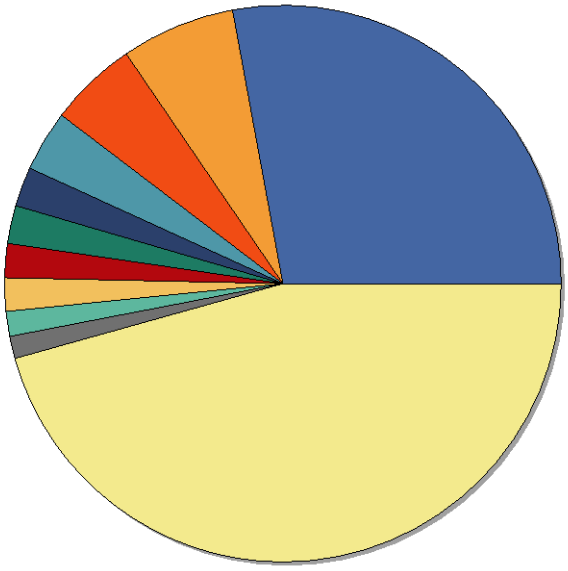


Figure B. 20 Process Contribution to Water Consumption in FFW



Method: ReCiPe 2016 Midpoint (H) V1.03 / World (2010) H / Characterization
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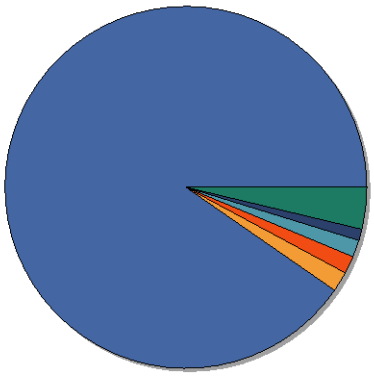
Figure B. 21 Process Contribution to Global Warming in TAP



Electricity, high voltage {ZA}| electricity production, hard coal, conventional | APOS, U
Electricity, high voltage {ZA}| electricity production, oil | APOS, U
Electricity, high voltage {ZA}| electricity production, hard coal, supercritical | APOS, U
Electricity, high voltage {ZA}| market for | APOS, U
Transport, freight, lorry 16-32 metric ton, EURO3 {RoW}| transport, freight, lorry 16-32 metric ton, EURO3 | APOS, U
Remaining processes

Method: ReCiPe 2016 Midpoint (H) V1.03 / World (2010) H / Characterization
Analyzing 1 kg 'Microalgae cultivation in FFW for 1L biodiesel production';

Figure B. 22 Process Contribution to Global Warming in FFW

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