Optimization of biomass and lipids production from microalgae using wastewater in a pilot scale raceway pond

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy: Biotechnology in the Faculty of Applied Sciences at the Durban University of Technology

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DECLARATION

Optimization of biomass and lipids production from microalgae using wastewater in a pilot scale raceway pond

Ismail Rawat

I hereby declare that the thesis represents my own work. It has not been submitted before for any diploma/degree or examination at any other Technikon/University.

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APPROVAL

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This 19 day of April, 2021, at the Durban University of Technology.

*** SUBMISSION APPROVED FOR EXAMINATION**

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ABSTRACT

Microalgae provide a sustainable renewable solution for the production of commodity products such as liquid biofuels. There are numerous benefits to using algae for the production of biofuels, however, the cost of production is a major hurdle to commercial-scale development. Major factors influencing the production of algae are the cost of nutrients, availability of water, contamination, and grazers. Research into algal biomass for biofuels production at laboratory scale does not translate directly to cultivation at large scale due to the change in cultivation conditions and the constant flux of environmental factors. This study focuses on the upstream processes of cultivation of biomass in a ~ 1146 m^2 raceway pond. It demonstrates biomass productivity under different climatic conditions and utilisation of post-chlorinated wastewater as a water and nutrient source. The study further elucidates the population dynamics of the system and provides insight into the challenges faced during the cultivation of algae at large scale.

An indigenous *Scenedesmus* sp. gave biomass productivity of 31.23 g/m^2/d with lipid production of 29.6 % lipid/g DCW in a 10 m^2 raceway pond in a greenhouse using BG11. Biomass productivity was reduced to 13.09 g/m^2/d with a lipid content of 22.9 % lipid/g DCW under 3-fold higher irradiance. Biomass productivity of circular 3000L ponds at the large scale site resulted in the highest biomass and acceptable lipid content using 250mg/L $NaNO_3$ although significantly lower than the 10 m^2 raceway ponds. Wastewater has shown potential to replace conventional media. Post-chlorinated wastewater was found to have low levels of nitrogen and phosphorus but contained metals that act as micronutrients for algae. Supplemented wastewater proved to be an effective growth.

Six individual runs of a covered 1146 m^2 raceway pond driven by paddlewheel were conducted over 15 months. The average water temperature ranged from 20.61±0.68°C during mid-winter to 31.03±2.22°C in late summer. Daylight ranges from 10.25 to 14 hours in winter and summer respectively. The highest average light intensity was 359.00±212.71 µmol/m^2/s from Mid-winter to early spring and 645.44±330.58 µmol/m^2/s in late summer. Biomass productivities were low ranging from 2.7 to 7.34 g/m^2/d for most runs of the raceway pond, mainly due to the long periods of cultivation. Average productivity at day 7 for all raceway runs was 7.25 g/m^2/d. Adaptive Neuro-Fuzzy Inference System (ANFIS) modelling of the system elicited that the major factors affecting biomass productivity in the raceway pond were light intensity, pH, and depth for the raceway pond. The model showed that maximum biomass productivity is possible at a depth between 20 and 22 cm at light intensities between 200 and 400 µmol/m^2/s. pH in the range of 9 to 9.5 correlated positively with light intensity ranging from 200 to 1000 µmol/m^2/s with maximum biomass expected in the region of 400 to 500 µmol/m^2/s.

The main algal constituents for the raceway ponds were *Scenedesmus obliquus*, *Scenedesmus dimorphus*, *Chlorella*, *Keratococcus,* and species of unidentified cyanobacteria. Either *Scenedesmus* or *Chlorella* was dominant for extended periods. Bacteria in open systems can have a positive or negative effect on the growth of microalgae but is dependent on the strains of microalgae and bacteria as well as prevailing conditions making these systems highly complex. *Rhodobacteraceae, Plactomycetaceae*, *Xanthomonadaceae, Flavobacteriaceae, Phycisphaeraceae, Comamonadaceae, and Cyclobacteriaceae* were found to be the major families of bacteria that proliferate at different levels during the cultivation period in the circular ponds and the raceway pond. These families of

bacteria have several beneficial traits to algae cultivation however further investigation is required.

Modelling the system revealed that pH, depth, and light intensity were factors having a substantial effect on biomass productivity. As the system was carbon limited addition of CO_2 (preferably a waste stream) could significantly enhance the overall biomass productivity. A major factor negatively affecting biomass productivity was the size of the pond. Inadequate mixing impacts biomass productivity in terms of access to nutrients and gaseous exchange. Shorter periods of cultivation resulted in higher productivities. For the scale of the system, semi-continuous harvesting would be required to achieve shorter residence time. This must be balanced against the energy utilization and cost of harvesting potentially lower culture densities.

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PREFACE

Aspects of the work covered in the following thesis can be found in the following publications and conference presentations:

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Singh, B., Guldhe, A., Rawat, I. and Bux, F. (2014). An overview of sustainable approaches in the development of biodiesel from plant and microalgae derived feedstocks. Renewable and Sustainable Energy Reviews. 26:216-245

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Book Chapters

Rawat, I., Ranjith Kumar, R., and Bux, F. (2013) Phycoremediation by High Rate Algal Ponds in: Potential Biotechnological Applications of Microalgae: Biodiesel and Value Added Products. F Bux (eds) Taylor and Francis, New York, USA ISBN: 978-1-46-651529-1

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Mutanda, T., Rawat, I., and Bux, F. (2012) Application of Municipal Domestic Wastewater for Microalgal Biomass Propagation for Biofuels Production. 20th EU Biomass conference and exhibition. Milano Convention Centre, MiCo, Italy. 18-22 June 2012

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CHAPTER 1: INTRODUCTION

1.1 The Need for Biofuels

Currently, one-fifth of the CO2 emissions globally are produced by the transport sector. The outlook for reduction of emissions from this sector does not look promising as the number of light motor vehicles on the roads globally is estimated to increase to over 2 billion vehicles by 2050 (Balat & Balat, 2010). Current global crude oil consumption is estimated to be 11.6 million tons per day and peak oil production is expected to be reached between 2020 and 2030 (Ashokkumar et al., 2019). Due to the diminishing supply of crude oil, it will continue to rise in cost thereby making the production of fuels from alternate sources more feasible (Anto et al., 2020; Zhang et al., 2020a). The ever-increasing effects of climate change will be accentuated by the increase in greenhouse gas emissions brought about by the continued use of fossil fuels. Biodiesel produced from renewable sources has the potential to offset a portion of the demand for crude oil in the transportation sector (Brennan & Owende, 2010).

Biodiesel is a monoalkyl ester on renewable feedstocks, produced by the transesterification of triglycerides or free fatty acids with short-chain alcohols and can be used in conventional diesel engines with little or no modification (Ahmad et al., 2011; Du et al., 2008). Biodiesel has been experimentally shown to be less eco-toxic than petro-diesel. Biodiesel is non-toxic at total soil saturation. Biodiesel contributes no net carbon dioxide or sulphur and overall less gaseous pollutants to the atmosphere than petro-diesel (Hulatt & Thomas, 2011; Williams & Laurens, 2010). With growing concern for the environment, these factors play an important role in the acceptability of biodiesel.

1.1.1 Crops based biodiesel feedstocks

Renewable sources of commercial biodiesel include canola oil, palm oil, corn oil, waste cooking oil, animal fat, and jatropha oil (Chisti, 2007; Yew et al., 2019). First-generation biofuels, or biofuels produced from edible crops, have been around for over a century (Campbell et al., 2011). The use of plant oils for fuel production is however highly controversial due to the requirement for arable land and other resources which could greatly affect food security (Javed et al., 2019; Lee, 2011). In 2010, 1% of the arable land available globally, is used for crop-based (1st and 2nd generation) biofuels. This fuel is sufficient only to meet 1% of the global requirement (Brennan & Owende, 2010). The use of crop-based biofuels may also not provide any reduction in GHGs when inputs into their productions are taken into account (Campbell et al., 2011).

More than 95% of biodiesel sources are first-generation agricultural edible crop oils. First-generation biofuels have a great impact on food security and have the potential to increase the cost of food crops. This has a knock-on effect which also increases the cost of biodiesel production (Campbell et al., 2011; Javed et al., 2019; Yew et al., 2019). Second-generation biofuels such as jatropha oil, waste cooking oil, and animal fats do not affect food security. However, the sustainability of second-generation biofuels is not favourable due to limited supply. Moreover, the production of crop derived biofuels gives rise to challenges such as poor cold flow properties and saturated fatty acids contained in animal fats give rise to production difficulties and may constitute a bio-safety hazard due to their solid nature at room temperature (Ahmad et al., 2011; Javed et al., 2019).

1.1.2 Limitations of crop-based biodiesel

First-generation crop-based biodiesel, such as rapeseed, soybean, palm, and sunflower oils directly compete with food supply which can result in increased cost of edible oil as well as biodiesel (Ahmad et al., 2011; Anto et al., 2020). Palm oil, due to its high productivity, represents one of the major raw materials used for the production of biodiesel and is ranked as the third-largest biodiesel feedstock providing the source for 10% of the biodiesel produced worldwide (Ahmad et al., 2011). Deforestation has become a concern in certain countries as more forest land is re-designated for the cultivation of commercial crops for biofuel production (Brennan & Owende, 2010). Production of palm oil in Malaysia has resulted in the clearing of vast areas of natural forest. This has indirectly increased the area's vulnerability to devastating fires and causes irreparable damage to biodiversity and ecosystems. Further, the overproduction of palm oil has resulted in the production of millions of tons of solid waste and palm mill effluent that has been shown to negatively impact terrestrial as well as aquatic environments (Ahmad et al., 2011).

Second-generation biofuels, such as jatropha, mahua, jojoba oil, tobacco seed, salmon oil, and sea mango are thought to be a suitable replacement for first-generation biofuels in that they are non-edible and can be grown in wastelands that are not suitable for crop cultivation and can sustain reasonably high yields without the management required for the production of food crops (Chia et al., 2018). Second-generation biofuels however contain high amounts of free fatty acids which increase the energy demand of process and production costs as they require additional production steps and may reduce the overall quality of the biodiesel produced (Demirbas, 2008)

The major limitation associated with the use of second-generation biofuels is the issue of sustainability. For the production of biodiesel from jatropha to completely satisfy the diesel

requirements of the UK (estimated 25 billion litres in 2008), half the land area of the UK (17.5 Million hectares) would be required. There is also some debate as to whether a positive energy balance is attained by the use of crop-based biodiesel. The total energy input for the life cycle of rapeseed and soybean is estimated to be half the total energy of the fuel (Javed et al., 2019; Scott et al., 2010).

A further criticism levelled against the use of first and second-generation biofuels is that the greenhouse gas balances may be affected due to indirect land use not being taken into account (Anto et al., 2020; Yin et al., 2020). This may result in zero net greenhouse gas benefit to the use of these fuels as compared to petro-diesel. Production of biodiesel from palm oil grown on dried peat marsh could be responsible for an increase in greenhouse gas emissions (Campbell et al., 2011).

1.1.3 Algal based biofuels

Microalgal lipids are regarded as third-generation biofuels source in terms of social and economic acceptability and greater energy security. Research into the production of biofuels from alternate sources has resulted in biodiesel from microalgae being deemed the most appropriate petro-diesel alternative (Anto et al., 2020; Chisti, 2007). Algal productivities can be anywhere from 9 to 251 twenty times that of oilseed crops per hectare and is thus a viable alternative (Ahmad et al., 2011; Anto et al., 2020; Chisti, 2007; Yew et al., 2019)

Microalgae have faster growth rates than plants and are capable of growth in saline waters which are unsuitable for agriculture. Microalgae have a greater quantum yield of PSII and require very few simple nutrients for growth. They utilise a larger fraction of solar energy making them more effective solar to chemical energy converters (Huber et al., 2006; Javed et al., 2019). The lipid

content of microalgae generally varies between 20 and 50% on a dry cellular weight basis (Anto et al., 2020; Ma & Hanna, 1999). Microalgae have the potential to produce 25 to 220 times higher triglycerides than terrestrial plants (Ahmad et al., 2011; Huber et al., 2006) and can be readily converted to biodiesel by the transesterification process (Ma & Hanna, 1999). As compared to biomass from trees and crops, microalgal oil is more economical in that transportation costs are relatively low. The quality of oil derived from microalgae is more suitable for biofuels than that of bio-oil derived from pyrolysis of lignocellulosic material (Ahmad et al., 2011).

1.1.4 Challenges to the commercial production of algal biofuels

Despite having many advantages over first and second-generation biofuels, algal biofuels production has a number of challenges to commercialisation. The major challenge to commercial production is biodiesel from algae is the cost, which is significantly higher than the cost of fossil diesel. Major factors influencing the production of algae are the cost of nutrients (Ashokkumar et al., 2019), availability of water, contamination, and grazers. Crucial steps in the production of lipids from biomass include 1. Selection of an appropriate species, 2. Culture conditions including light, temperature, and CO2, 3. Nutrient concentration (Ahmad et al., 2011; Ashokkumar et al., 2019).

Much research into algal biomass for biofuels production has been conducted at laboratory scale (Yin et al., 2020). These results, however, do not translate directly to cultivation at large scale due to the change in cultivation conditions and the constant flux of environmental factors during outdoor cultivation using pond systems (Fulbright et al., 2018; Sutherland et al., 2020). A large proportion of available literature on large-scale cultivation of algae using wastewater is for wastewater treatment rather than the cultivation of algae for designated product/purpose using

wastewater. Furthermore, literature on the challenges faced at large scale is very limited. This study focuses on the upstream processes of cultivation of biomass in a 1146 m^2 (~300 000L) raceway pond. It expounds on the productivity under different climatic conditions and utilisation of post-chlorinated wastewater as a water and nutrient source. The study will further elucidate the population dynamics of the system and provide insight into the challenges faced during the cultivation of algae at large scale.

1.2 Aim and Objectives

Aim

Determination of the effects of factors on microalgae cultivated using wastewater in a large-scale raceway pond

Objectives

- Optimisation of nitrogen concentration for increased biomass yield using pilot-scale outdoor systems
- Characterisation and adaptation of domestic wastewater for microalgal growth
- Elucidation of factors affecting microalgae cultivation in a large-scale raceway under outdoor conditions over different seasons for biomass production
- Determination of algal and bacterial population dynamics within the raceway pond

CHAPTER 2: LITERATURE REVIEW

2.1 Microalgae

Microalgae are the largest group of autotrophic microorganisms' in the world. They have simple structures that are primarily for energy conversion for cell division. This allows them to readily adapt to prevailing conditions (Brennan & Owende, 2010). They have the largest diversity with reports ranging from 10 000 to 150 000 million algal species that have been identified (Yew et al., 2019). These species, especially microalgae occur in diverse aquatic environments including freshwater, lacustrine, brackish, marine, maturation ponds, and hyper-saline. They have the potential to rapidly accumulate substantial amounts of lipids as compared to terrestrial plants due to their higher growth rates (Ahmad et al., 2011; Ashokkumar et al., 2019). Microalgal lipids offer great potential as biodiesel feedstocks. They contain twice the stored energy (per carbon atom) than carbohydrates, which translates into a two-fold increase in fuel energy content thereby outcompeting terrestrial plants for biofuel production. Biomass and lipid productivity vary significantly among different species (Chisti, 2007). Accumulation of neutral lipids by alteration of cultivation conditions is an established method of enhancing lipids accumulation (Chia et al., 2018). Microalgae require less freshwater for cultivation than terrestrial plants and can be cultivated on non-arable land, in brackish water thus reducing strain on resources required to produce food crops whilst reducing other environmental effects. They require significantly less land, an estimated 2% of the land required to produce the same amount of biodiesel from oil-bearing crops, and do not require the use of herbicides or pesticides (Ahmad et al., 2011).

Microalgal biodiesel has properties similar to those of petro-diesel including density, viscosity, flash point, cold flow, and heating value. In addition, they are carbon-neutral due to the fixation

of CO_2 biologically binding 1.83 kg of CO_2 for every kg of biomass produced. Residual biomass after extraction may offer a number of methods for improving economics. This includes use as fertilizer or for producing other energy products (Ahmad et al., 2011)

2.2 Abiotic factors influencing microalgae growth

Cyanobacteria and microalgae capture light energy through photosynthesis and convert inorganic substances into simple sugars using captured energy. Several factors influence algal growth which includes but are not limited to: light (intensity, photoperiod), temperature, nutrient concentration, O_2 saturation, carbon availability, pH, salinity, undesirable chemicals; biotic factors such as pathogens (bacteria, fungi, viruses) and competition by other weedy microalgae species; operational factors such as mixing, depth, and frequency of harvest.

2.2.1 Light

Light as the energy source for photoautotrophic life is the principal limiting factor in photo-biotechnology (Brennan & Owende, 2010; Zhang & Ogden, 2019). The supply of light greatly affects photosynthesis, cell composition, and metabolic pathways. The rate of photosynthesis is directly proportional to the illumination until high light intensities are reached. Photoinhibition occurs at high light intensities by damage to the photosynthetic receptor system (Raeisossadati et al., 2020).

The light within algal culturing systems decreases exponentially with the depth of the culture from the surface to the culture to the bottom of the reactor and is a function of light attenuation and shading from other cells (Jebali et al., 2018a). In dense algal cultures, light attenuation occurs on a steep gradient with a decrease in magnitudes over cm scale (Sutherland et al., 2014). Only the

top 10 cm of a raceway pond is likely to receive light whilst the rest of the culture remains in darkness thus limiting biomass productivity. An increase in light intensity will need to have a high amount of turbulence in order to illuminate more of the culture without causing photoinhibition. This is usually impractical in terms of cost at large scale (Raeisossadati et al., 2020). The absence of light reduces photosynthesis and thus the conversion of solar to chemical energy compounds which support cell growth (Yew et al., 2019).

2.2.2 **Carbon**

Other than light, carbon is the most important nutrient, making up about half the dry weight of microalgae. Generally, carbon is supplied as CO_2, but the residence time of dissolved CO_2 in the growth medium is limited and dependent on alkalinity and temperature (Sheehan et al., 1998). Sparging CO_2 gas has a profound effect on cell growth and lipid accumulation. It acts to give a measure of control of pH as well as provides a carbon source for growth (Anto et al., 2020).

If CO_2 is provided in excess, it will be released back to the atmosphere from the pond surface. In recent years, carbon dioxide has become the focus of attention because of its position as the primary greenhouse gas. Carbon dioxide is a critical nutrient for all photosynthetic plant species, but all conventional higher plant production systems can obtain it from the air, algae production is the exception in that it requires an enriched source, as atmospheric CO_2 is not sufficient. For a high photosynthetic rate, the CO_2/O_2-balance must be adjusted in a way that the prime carboxylating enzyme, Rubisco, furnishes CO_2 for the Calvin cycle but does not use O_2 for photorespiration. Hence, in algal cultures of high cell densities, sufficient CO_2 must be available, while evolved O_2 has to be removed before reaching inhibitory concentrations.

2.2.3 Temperature

Temperature is the most important limiting factor, after light and CO_2, for culturing microalgae in both closed and open systems (Zhang & Ogden, 2019). It affects cellular processes, nutrient uptake, CO_2 adsorption capacity, and the ability to capture light (Ahmad et al., 2011; Yew et al., 2019). The optimal temperature regimes for microalgae strains currently used in mass cultures show steep productivity declines below ~20°C and, on the high end, above ~35°C. Many microalgae can easily tolerate temperatures up to 15°C lower than their optimal but exceeding this range by only 2–4°C may result in total culture loss (Mata et al., 2010). Temperature exerts a greater influence on respiration and photorespiration than photosynthesis. When CO_2 or light is limiting for photosynthesis, the influence of temperature is greatly reduced. Another way to overcome temperature limitations is to find microalgae strains that maintain high productivity at lower temperatures, and over a greater temperature range. Strains that adapt more quickly to dual temperature regimes would also be beneficial. *Scenedesmus* sp. is known to tolerate a wide range of temperatures from 10°C to 40°C (Yew et al., 2019). Some microalgae can grow rapidly under both low and high temperatures in natural environments (Lundquist et al., 2010).

2.3 Biotic factors influencing algal growth

An appropriate growth medium providing essential nutrients in adequate amounts is necessary for the cultivation of microalgae. Microalgal cells screen their environment for suitable nutrients and energy which they store. By doing this they are able to optimize the efficiency of resource consumption (Amaro et al., 2011). Carbon (C), N, P, and sulphur (S) are major elements required by microalgal cells; whilst inorganic salts such as iron (Fe) and magnesium (Mg) as well as trace elements are required in lesser amounts. Silicon is required in the case of marine and a few other

strains. These elements are integral microalgal cell constituents. For optimum growth and CO_2 fixation, a balanced medium must be developed. Different media formulations are required depending on the product of interest (Zeng et al., 2011). Algae use organic and inorganic N for the synthesis of amino acids whilst P, preferentially inorganic, is used for cellular processes related to energy transfer and synthesis of nucleic acid (Martinez et al., 1999).

Nitrogen constitutes about 7 to 10% of microalgae cell dry weight (Richmond, 2004) and is the main constituent of proteins. Ammonia, nitrite, nitrate, and urea are used as nitrogen sources in microalgae cultivation. Nitrate is used as a nitrogen source in many green microalgae. Ammonium ($NH4^+$) is more readily utilised by algae as a nitrogen source as there is no requirement for a redox reaction which means a lower energy requirement (Jebali et al., 2018a). Nitrates and nitrites are reduced to ammonium enzymatically before uptake into the cell. In the presence of ammonium, nitrate, and nitrite uptake is controlled by the repression of the uptake proteins and enzymes (Nagarajan et al., 2019). However, High levels of free ammonia brought about by high concentrations of ammonium at high pH limit photosynthesis (Iasimone et al., 2018; Nagarajan et al., 2019).

Nitrogen is a critical factor in the lipid content regulation of microalgae. Lipid levels above 40% g^{-1} Dry cell weight (DCW) generally signifies that nitrogen has become the growth limiting factor (Park et al., 2011). The general principle is that when there is insufficient nitrogen for the protein synthesis required by growth, excess carbon from photosynthesis is channelled into such storage molecules as triacylglycerols or starch (Anto et al., 2020; Scott et al., 2010). This is accompanied by autophagy of proteins and some organelles to avail nitrogen for critical functions (Zhang & Ogden, 2019).

Phosphorus is a macronutrient that plays an important role in the growth and metabolism of algae. It is required for most cellular processes, that involving energy transfer and nucleic acid synthesis (Sánchez Zurano et al., 2020). Microalgae stores phosphorus mainly in the form of polyphosphates and metaphosphates. Phosphorus, although required in smaller amounts, must be supplied in excess as it complexes with metal ions and is thus not fully bio-available for cell uptake (Chisti, 2007). The phosphorus consumption rate in microalgae depends on phosphorus concentration in both the environment and the cells, and pH and temperature (Nagarajan et al., 2019).

Iron greatly affects the growth and biochemical composition of microalgae because of its redox properties. It is the nutrient involved in various processes such as photosynthesis, respiration, nitrogen fixation, and DNA synthesis (Richmond, 2004). Chlorophyll may be degraded when iron becomes limiting. Iron is also responsible for enhancing phytoplankton biomass in oceanic waters (Liu et al., 2008). Liu et al., (2008) showed that high iron concentration could also induce considerable lipid accumulation in marine strain *C. vulgaris*. This suggests that some metabolic pathways related to the lipid accumulation in *C. vulgaris* are probably modified by a high level of iron concentration in the initial medium (Singh et al., 2016).

The composition and amount of nutrients supplied depend on the species of microalgae to be cultivated. Large scale cultivation of microalgae requires substantial amounts of nitrogen and phosphorus. These are generally supplied as nitrates and ortho-phosphates (Lam & Lee, 2012; Levine et al., 2011). Nitrates and phosphates are generally supplied in the form of chemical or organic fertilizer to obtain the large quantities of biomass required. Chemical fertilizer is preferred as it allows for water recycling (Lam & Lee, 2012). Nutrients are generally supplied in excess for large-scale microalgal cultivation. An appropriate carbon source, light intensity, and

photoperiod are critical to the large-scale cultivation of microalgae. (Amaro et al., 2011). Microalgae of typical composition ($C_{1.06}H_{1.81}O_{0.45}N_{0.16}P_{0.01}$) undergoing phototrophic growth requires the addition of fertilizer with an N:P ratio of 16:1 (7.3 g N:1 g P). This is highly variable depending on algal species and nutrient availability. Ratios can vary from about to 4:1 to almost 40:1 (Putt et al., 2011). The production of chemical fertilizer may account for half the cost and greenhouse gas emissions related to the microalgal feedstock production (Lam & Lee, 2012; Levine et al., 2011; Yin et al., 2020). The price of mined P is ever increasing due to diminishing supply. At the current rate of agricultural use, the world's mineral P reserves are expected to last only 50 to 100 years (Chowdhury et al., 2012).

The costs associated with the supply of chemical media are estimated to be approximately $3000 ton^{-1} of biomass produced based on the production of 100 tons per annum (Chisti, 2007). The production of low-value products such as biofuels is thus not economically feasible using conventional media (Ashokkumar et al., 2019). Production of microalgae using fresh water and chemical fertilizer has higher environmental impacts in terms of energy use, GHG emissions, and water consumption as compared to the biofuels feedstocks such as corn, canola, and switchgrass. These environmental impacts are driven by upstream processes. An additional drawback to large-scale microalgal cultivation using chemical fertilizer is the consumption of up to 10 times the N requirement of palm cultivation (Olguín, 2012). Fossil diesel production uses 2.5 times less energy than current algal biodiesel production. This is due to direct and indirect energy inputs required for the production of fertilizer, ponds, harvesting facilities, and transport (Olguín, 2012). Nutrient recycling must be carried out in order to minimize the cost and give a better net energy balance to the system (Stephens et al., 2010).

2.4 Modes of Algal Cultivation

Microalgae naturally utilize suitable nutrients and energy sources from their environment, optimizing the efficiency of utilization for growth and survival. They are resilient organisms in that a single species may be able to undergo various types of metabolism dependant on the available nutrients for growth as well as other environmental factors (Amaro et al., 2011). *Chlorella vulgaris, Chlorella sorokiniana, Haematococcus pluvialis, Arthrospira (Spirulina) platensis, Selenastrum capricornutum,* and *Scenedesmus acutus* amongst others, are examples of species that can grow under phototrophic, heterotrophic, and mixotrophic conditions (Amaro et al., 2011; Yin et al., 2020). The mode of cultivations can be distinguished according to pH changes. Organic carbon (such as sugars, proteins, and fats), vitamins, salts, and other nutrients (nitrogen and phosphorous) are vital for algal growth, however, equilibrium between operational parameters (oxygen, carbon dioxide, pH, temperature, light intensity, and product and byproduct removal) is key to the successful cultivation of algae (Mohd Udaiyappan et al., 2020).

Phototrophic cultivation uses sunlight and CO_2 as an inorganic carbon source for energy production and growth (Mata et al., 2010; Yin et al., 2020). Freely available CO_2 from flue gas makes this particularly attractive. Phototrophic cultivation is less prone to contamination than other types of cultivation due to the lack of organic carbon. Heterotrophic growth occurs in the absence of light using organic carbon sources such as glucose, acetate, glycerol, fructose, sucrose, lactose, galactose, and mannose (Amaro et al., 2011; Nagarajan et al., 2020). Heterotrophic cultivation gives rise to higher lipid productivities than phototrophic growth (Chen et al., 2011). Higher cell densities and lipid productivity and cell size can be achieved thus translating to better economic viability (Borowitzka, 1999; Javed et al., 2019). Setup costs of reactors are minimal and the process is very well understood (Brennan & Owende, 2010; Javed et al., 2019). This is

however offset by the high cost of the carbon source. Heterotrophic cultures are more prone to contamination (Amaro et al., 2011), and the chemical composition of the algae change under these conditions. Furthermore, the numbers of species that undergo heterotrophic growth are limited (Borowitzka, 1999). Organisms that are able to undergo mixotrophic growth can photosynthesize or use organic substrates as a carbon source. Mixotrophic cultivations result in higher yields than autotrophic cultivation but lower than heterotrophic yields. Mixotrophic growth is preferred for high-strength opaque wastewater treatment as organic carbon compensates for light limitation (Nagarajan et al., 2019). Mixotrophic production reduces photoinhibition and decreases the loss of biomass due to dark phase respiration (Brennan & Owende, 2010; Javed et al., 2019; Yin et al., 2020).

2.5 Wastewater as a resource

It is desirable to use cheap and readily available media such as wastewater streams for sustainable biofuel production. The use of artificial media such as BG-11 for most freshwater and marine microalgae is economically undesirable due to the costs involved. The cost of conventional media for microalgae production is not feasible for low-value products such as biofuels (Park et al., 2011; Viswanath et al., 2010). Various authors have suggested the utilization of waste substrates such as wastewater for nutrient supply (Brennan & Owende, 2010; Javed et al., 2019; Lam & Lee, 2012). Microalgae have been previously used for the treatment of wastewater. Findings have shown that wastewater may be economically viable and sustainable for the production of microalgal biofuels (Boelee et al., 2011; Rawat et al., 2011). Coupling wastewater treatment with biofuels production is a very attractive option for energy, freshwater, and fertilizer reduction (Li et al., 2019). Wastewater is rich in organic and inorganic chemical nutrients. Treatment is essential before discharge in order to protect receiving waters from eutrophication and accumulation of other

nutrients. N and P and COD are the primary constituents that require removal before wastewater can be discharged. Wastewater treatment for N removal is most commonly carried out in the form of biological treatment mainly by bacterial action in order to reduce the organic load to within discharge standards (Rawat et al., 2011). Phosphorus is generally mitigated by Physico-chemical dephosphatization (Martinez et al., 1999). Both processes have high energy consumption and operational costs. The growth of microalgae can effectively remove phosphates and nitrates from wastewater, thus making it an ideal substrate for the cultivation of microalgae for biofuels production whilst acting as a treatment process for wastewater (Pagnussat et al., 2020). Martinez et al., (1999) showed that wastewater serves as a complete medium equivalent to chemical media from a kinetics standpoint. Wastewater treatment with the production of microalgae for biofuels significantly improves the economics of biomass production as secondary and tertiary wastewaters contain nitrates and phosphates. This reduces the cost of treatment that would normally be incurred for nutrient removal by conventional methods (Javed et al., 2019).

High N and P content promote microalgal growth whilst removing nutrients before discharge. Ammonia N and phosphates in secondary-treated wastewater are generally in the range of 20-40 mg/L and 10 mg/L respectively. These are deemed sufficient to support the highly productive growth of most freshwater microalgae (Olguín, 2012).

The coupling of wastewater treatment with the production of microalgae for biofuels significantly improves the economics of biomass production as secondary and tertiary wastewaters contain nitrates and phosphates. This reduces the cost of treatment that would normally be incurred for nutrient removal by conventional methods (Lam & Lee, 2012; Zhou et al., 2011). Wastewater

utilisation can reduce nitrogen by 94% and eliminate the need for the addition of elements such as potassium, magnesium, and sulphur (Jebali et al., 2018a; Yang et al., 2011b).

CO_2 rich wastewater promotes the growth rates of microalgae as it balances the ratio of carbon: nitrogen: phosphorus. This decreases harvesting costs due to higher biomass concentrations and overall costs by increased lipid production (Brennan & Owende, 2010). The usage of wastewater reduces the need for enormous amounts of freshwater in microalgae cultivation. Cultivation of microalgae using wastewater has the potential to use 90% less freshwater. This further improves the economic viability whilst being and is an eco-friendly means to the production of renewable microalgal biomass (Brennan & Owende, 2010; Zhou et al., 2011). The use of post-chlorinated municipal domestic wastewater compares favourably with conventional artificial media. However, to be highly effective as a growth medium, the wastewater requires supplementation with trace metals, nitrates, and phosphates. The cost-benefit is that while using the wastewater as a source of nutrients for microalgal growth, the procedure has a phyco-remediation role. The beneficiation of the water is environmentally significant.

The growth of microalgae on wastewater provides a means of removal of organic contaminants, heavy metals, and pathogens, thus saving on the costs of chemical remediation (Brennan & Owende, 2010). The cost of conventional removal of nitrogen (N) and phosphorus (P) is reported to be $4.4 /kg N and $3.05 /kg P removed. Utilizing wastewater as a nutrient source for large scale can thus reduce the cost of wastewater treatment significantly. Zamalloa et al., (2011) showed that a 70-110 ton/ha/annum facility using wastewater can result in a saving of $48 400 - $ 74 800 /ha/annum for nitrogen removal and $ 4575 - $ 7625 /ha/annum for phosphorus removal. The combination of saving from wastewater treatment and reduction of microalgae

production costs is thus a win-win strategy when used for the production of energy or liquid fuels (Lam & Lee, 2012).

Medium strength domestic wastewater in the U.S. contains sufficient N and P in each litre to produce 0.6 g of microalgae for a total of 77.6 million/kg/day. Theoretically, 90% removal of the limiting nutrient, coupled with a 10% biodiesel yield with a density of 0.801 kg/l, and 9 months per year operation could produce roughly 1.7 million gallons/day of biodiesel. Even though this represents a small fraction of the 378 million gallons of transportation fuel required by the U.S. per day, wastewater plants remain the best option for the development of commercial-scale algae biofuels facilities in the future (Christenson & Sims, 2011).

Pittman et al., (2011) stated that the potential of microalgae as a source of renewable energy and based on current technologies was able to conclude that without the utilization of wastewater, microalgal cultivation for biofuels production is unlikely to be economically viable or provide a positive energy return. Christenson & Sims, (2011) considered several approaches towards microalgae-based biofuels production coupled with wastewater treatment and suggested that only those studies that gave emphasis to wastewater treatment were able to yield cost-competitive biofuels. From these findings, they were able to conclude that large-scale algae biofuels production would not be feasible in the near future without wastewater treatment as a primary goal (Ho et al., 2011).

Despite the favourable outlook for the use of wastewater-mediated biomass production, the real potential must be explored practically at large scale. A potential challenge associated with wastewater utilization is viral and bacterial contamination that may or may not negatively affect the production process. These factors as well as the presence of inhibitory substances could

impede the growth of microalgae (Lam & Lee, 2012). High nutrient concentrations are also known to be inhibitory to certain microalgal strains. Excess ammonia is inhibitory to microalgae resulting in decreased photosynthesis and thus growth rate. These factors cannot be controlled and close monitoring and adjustment of nutrient levels by augmentation or dilution may be required depending on the type and pre-treatment of wastewater used. Utilization of wastewater will further necessitate frequent cleaning of the culturing system (Lam & Lee, 2011).

2.6 Water Footprint

Water footprint in terms of biorefineries is defined as the water used for microalgal cultivation and biomass processing into products and co-products. One of the conditions of technical and economical biofuels is that they should require minimal water (Brennan & Owende, 2010). Water is directly related to biomass and lipid productivity in that higher productivity requires more water to achieve target production (Lam & Lee, 2012). The impact of large-scale biofuel production on water utilization has generated great debate. The water footprint of large-scale microalgal cultivation utilizing seawater or wastewater is significantly smaller than that of crop-based biofuels production (Amaro et al., 2011). Microalgal cultivation requires relatively large amounts of water for growth and various processes. Water utilisation during the cultivation of algal is reported to be 700 L/kg biodiesel with the recycling of harvest water and 3700 L per/kg without recycling (Olguín, 2012; Khawam et al., 2019). Freshwater use can be reduced by up to 90% if seawater, brackish water, or wastewater is used for microalgae culturing (Rawat et al., 2011).

In open pond systems replenishment of water is required due to losses incurred by evaporation and harvesting. In the absence of a water recycle, 84.1% of the water is discharged post-harvest.

Evaporative losses from open ponds can be as high as 10 L/m^2/day and consequently, losses of up to 410 kg water /kg biomass produced can occur (Sheehan et al., 1998). Recycling of water has the potential to reduce nutrient addition by up to 55% (Yang et al., 2011a). The drawback to recycling however is the accumulation of inhibitory metabolites produced by certain microalgae and cyanobacteria (Stephens et al., 2010). Recycling of water concentrates contaminants and inhibitory substances and should thus be carried out after taking the relevant precautions.

Wastewater utilization reduces the requirement for freshwater whilst providing a source of nutrients. This improves the economic viability and offers an eco-friendly means to the production of renewable microalgal biomass (Brennan & Owende, 2010). The requirement for freshwater cannot be totally negated as some amount of water is required for the prevention of excessive changes to osmoregularity and to compensate for evaporative losses (Yang et al., 2011a). This can further be reduced by the utilization of treated wastewater. Factors such as the microalgal species choice and cultivation system further impact the water footprint. Photobioreactors require less than 33% of the amount of water required for raceway pond cultivation (Davis et al., 2011). *Chlorella vulgaris* utilizes 17% of the amount of water required for the growth of *Chaetoceros gracilis, Cyclotella cryptic,* and *Nannochloropsis* (Yang et al., 2011a).

2.7 Culturing systems

Large scale culturing systems fall within two broad categories: - i) open ponds and ii) photobioreactors. Open ponds are designed in a variety of configurations from large open ponds, circular ponds with a rotating arm for culture mixing, and raceway ponds (Borowitzka, 1999; Brennan & Owende, 2010). Photobioreactors typically are configured as vertical reactors, tubular

reactors, annular reactors, and plastic bags. They are generally mixed by pumping or airlifting (Amaro et al., 2011; Zeng et al., 2011).

2.7.1 Open Pond systems

Raceway ponds are the most commonly used cultivation systems (Anto et al., 2020; Brennan & Owende, 2010). They are generally cheaper to build and easier to operate than photobioreactors (Ashokkumar et al., 2019). They are generally constructed from concrete but can be plastic lined and earth-lined ponds are not uncommon (Brennan & Owende, 2010). Most paddlewheel driven raceway ponds are limited to 20cm to 30cm in depth. The paddle wheel prevents settling of the culture and reduces the shading effect (Brennan & Owende, 2010; Norsker et al., 2011). Shallow ponds are required to allow adequate penetration of sunlight (Borowitzka, 1999). (Sutherland et al., 2014) showed that shallower ponds (200mm) were more constrained than deeper ponds (400mm) which produced higher areal biomass productivity despite the lesser light attenuation and better mixing. This may be more applicable to systems with higher nutrient loading when using wastewater for cultivation as it tends to be a function of carbon limitation. Open ponds are generally regarded as the more cost-effective method of microalgal biomass production (Ashokkumar & Rengasamy, 2011; Javed et al., 2019; Schenk et al., 2008).

Open ponds however have various advantages and limitations. Amongst the major limitations are low productivity as compared to photobioreactors and environmental factors which to a large extent cannot be controlled (Borowitzka, 1999; Chisti, 2007; Zeng et al., 2011). Low productivities in open ponds occur as a result of a number of factors. Evaporative losses result in changes to the ionic composition of the media with potentially detrimental effects on culture growth. Changes in temperature, photoperiod, and seasonal variation are beyond control in open systems and directly affect productivity (Chisti, 2007; Li et al., 2019). Major contributors to low

productivity are CO_2 transfer rate and light limitation due to increasing culture densities (Yin et al., 2020). Atmospheric carbon dioxide is usually used to satisfy the carbon requirement. Techniques to enhance CO_2 absorption into the culture media such as aerators or bubbling may improve the overall biomass productivity but with additional cost. Improved mixing can minimize the impacts of both CO_2 and light limitation thus improving biomass productivity (Brennan & Owende, 2010).

Due to low productivities, large areas of land may be required to meet the desired output of cultivation (Amaro et al., 2011; Li et al., 2019). Open pond systems tend to become contaminated with undesirable species including undesirable algae, grazers, fungi, and bacteria fairly quickly (Anto et al., 2020; Ashokkumar et al., 2019) Contamination by protozoa and other microalgae may be reduced by utilization of highly selective culture conditions (Brennan & Owende, 2010). This limits the number of suitable species for open pond cultivation. A few examples include i) *Chlorella* species which require nutrient-rich media, ii) *Dunaliella salina* is adaptable to high salinity and iii) *Spirulina* species which grow well under high alkalinity (Borowitzka, 1999).

The selection of a culturing system must also take into account the intrinsic properties of the microalgal species to be cultivated. Natural climatic conditions and the cost of land and water availability also play a role in the determination of the culturing system (Borowitzka, 1999). Usage of marginal and non-arable land is a major advantage. Maintenance and cleaning of open systems are easier and less energy-intensive than photobioreactors (Brennan & Owende, 2010). The overall energy input for raceway pond operation is lower than for photobioreactors and therefore has the potential for a larger net energy production (Rodolfi et al., 2009).

Commercially produced raceway culture of *Dunaliella salina* cost approximately $2.55 /kg of dry biomass in 2008. This cost is considered to be too high to justify production for biofuels (Brennan

& Owende, 2010). It must be noted that the culture was grown on media for the production of high-value products.

2.7.2 Closed systems

The limitations of open pond culture have led to much research into photobioreactors, as a method of primarily overcoming contamination and low productivity (Amaro et al., 2011; Brennan & Owende, 2010). They are generally used for culturing microalgae for high-value products such as pharmaceuticals, nutraceuticals, and cosmetics as they cannot be grown as a monoculture in open systems. The increased utilisation of photobioreactors may be a result of a higher degree of process control and higher biomass productivities (Brennan & Owende, 2010). The most popular photobioreactor configurations are tubular, vertical or column, flat plate, and annular reactors (Amaro et al., 2011; Anto et al., 2020; Brennan & Owende, 2010; Zhang et al., 2020a). The fundamental principle behind most photobioreactors is the reduction of the light path thereby increasing the amount of light received by each cell. They are generally mixed by airlift or mechanically stirring/pumping. Mixing provides a benefit in phototrophic systems in that mixing as well as aeration can be accomplished simultaneously (Amaro et al., 2011). Mixing is essential for gaseous exchange within the system (Brennan & Owende, 2010).

Photobioreactors are more versatile than open ponds in that they can use sunlight, artificial light, and various combinations of light sources thus allowing the potential to increase photoperiod and enhance low light intensities given by sunlight variation. The stability of light intensity and photoperiod provided by artificial light has the potential to enhance yearly total oil yields from microalgae by 25% to 42% (Amaro et al., 2011).

Big bag reactors were used commonly for the cultivation of cultures up to 1000L. They consist of a number of large sterile plastic bags of 0.5 m in diameter. Mixing is provided by aeration and

the system is operated in batch or semi-continuous culture (Borowitzka, 1999). Column photobioreactors are constructed from transparent columns that are aerated from the bottom. These reactors give the best controllable growth conditions and the highest volumetric mass transfer rate when compared to other systems (Brennan & Owende, 2010).

Tubular photobioreactors are regarded as one of the most suitable types for large scale outdoor culturing. The solar collector is generally made of glass or transparent plastic with a diameter of 0.1m or less to allow penetration of light through dense culture (Chisti, 2007). The orientation of the solar collector may be horizontal, vertical, inclined, or as a helical coil around a supporting frame (Amin, 2009; Brennan & Owende, 2010). Cultures are generally reticulated by a pump passing through a degasser at regular intervals in order to remove excess oxygen. High levels of oxygen lead to lower productivities due to photooxidative stress. Mixing within the reactor is achieved by creating turbulence. This is costly in terms of energy utilization and increases wear on the pumps (Norsker et al., 2011). Tubular photobioreactors have large surface areas that are exposed to light and are thus regarded as suitable for outdoor cultivation (Brennan & Owende, 2010). Tubular reactors are currently used for the cultivation of pure cultures producing high-value products such as astaxanthin (Norsker et al., 2011).

Flat plate photobioreactors are capable of achieving high cell densities due to the large surface area available for solar capture. To allow maximum sunlight capture, the reactors are constructed from transparent materials with a layer of dense culture flowing over the flat plate (Brennan & Owende, 2010). Mixing is achieved by sparging with air at the rate of 1L air per litre of reactor volume per min (Norsker et al., 2011). Due to the high quantum yield of PSII and low accumulation of dissolved oxygen, flat plate reactors are more suitable for large scale culture than

tubular reactors. However, this incurs higher mixing and capital costs than tubular photobioreactors (Brennan & Owende, 2010; Norsker et al., 2011; Zhang et al., 2020a).

2.7.3 Benefits and limitations

Photobioreactors allow a larger range of species to be cultivated than open ponds (Davis et al., 2011). The main benefits of photobioreactors over open ponds are their higher productivities and the level of control in terms of environmental conditions. Photobioreactors offer the opportunity to optimize light path length and thus improve biomass productivity (Amaro et al., 2011). They are able to achieve and operate at high biomass concentrations due to their larger surface area to volume ratio (Amin, 2009; Davis et al., 2011). Since atmospheric contamination can be avoided, monocultures are possible for extended periods if the photobioreactors are operated in a sterile manner (Amaro et al., 2011; Brennan & Owende, 2010). Tubular photobioreactors are highly adaptable and therefore can be erected in any open space (Amin, 2009). Greater control of culture conditions results in the final product of microalgal propagation being of more consistent quality and composition (Borowitzka, 1999).

Table 2.1 Generalized comparison of two different cultivation methods of algae production (Modified from (Anto et al., 2020) Pulz (2001); Harun et al. (2010); Mutanda et al 2011).

Factors	**Open Ponds**	**Photobioreactors**
Cultivation		
Cultivation Species	No Monoculture	Monoculture
Contamination	High	Low to none
Cleaning of cultivation system	easy	intensive due to build up on walls

Control of cultivation conditions	Very difficult	Easy
Temperature	Highly variable	Requires cooling
Biomass Production		
Biomass quality	Variable	Reproducible
Biomass productivity	Low	High
Lipid productivity	Low	High
Light utilization efficiency	Low	High
Operational mode		
Shear Stress	Low	High
CO_2 transfer rate	Poor	Excellent
Mixing efficiency	Poor	Excellent
Water loss	Very high	Low
Evaporation	High	No evaporation
O_2 concentration	Low due to out gassing	Build up occurs and requires gas exchange device
CO_2 loss	High depending on pond Depth	Low
Economics		
Space required	High	Low
Periodical maintenance	Less	More
Capital investment	Low	High
Operating costs	Lower	Higher
Scale-up technology for commercial level	Easy to scale up	Most photobioreactors are difficult to scale up due to limitations

Table 2.1 provides a generalised list of positive and negative attributes of open ponds and photobioreactors. Scale-up of photobioreactors may present engineering and design challenges. Tubular reactors are limited on the length of the tubes due to inefficient gaseous exchange. Extensive tube lengths give the potential for excess oxygen accumulation, CO_2 depletion, and pH variation. All of which negatively affect biomass productivity. The photo-array is also prone to culture attachment on the walls of the tubes. This leads to fouling due to culture decay (Brennan & Owende, 2010). Despite their advantages and superior productivity of flat-bed reactors as compared to tubular reactors, they are difficult to scale up and maintain temperatures at desirable levels (Brennan & Owende, 2010). The major limitations to utilizing photobioreactors for large-scale cultivation of microalgae are the high capital and operational costs (Amaro et al., 2011). Estimates of capital and production costs may vary greatly. High power consumption is one of the major shortcomings of many photobioreactors. The use of artificial light adds to power consumption and increases capital costs (Amaro et al., 2011).

2.7.4 Hybrid production systems

Considering the advantages and drawbacks of raceways and photobioreactors, the logical step in cost-effective microalgal biomass production would be a combination of the technologies. Hybrid systems combine photobioreactors and raceway ponds at different stages of production (Javed et al., 2019). Hybrid systems have been used historically in aquaculture for the growth of inoculum. This allows the production of inoculum free of contamination and provides a large enough volume to give the culture of choice a competitive advantage in the open system (Schenk et al., 2008).

The use of hybrid systems for microalgal biofuels production utilises large scale photobioreactors and open pond cultivation sequentially. The first stage of growth is undertaken

within a bioreactor to maintain culture purity and achieve high biomass concentrations due to increased productivity (Amaro et al., 2011). The second stage is undertaken in a raceway pond as this is ideal for nutrient stress (Brennan & Owende, 2010; Yun et al., 2018). Hybrid systems can produce as much as 20-30 ton/ha of lipid annually but are climate dependant (Javed et al., 2019; Rodolfi et al., 2009).

2.8 Biomass production

Microalgal biomass production rates vary greatly depending on the levels of inputs and can even vary between strains of the same species (Campbell et al., 2011). Biomass yields of 0.5 to 1 g/L are accepted as a standard for raceway ponds. Photobioreactors are generally limited to 4 g/L before the shading effect greatly limits further growth (Davis et al., 2011). The theoretical maximum biomass productivity is estimated to be within the range of 77 to 96 g DCW/m^2/day. This translates to 280 to 350-ton DCW/ha/ammun (Zamalloa et al., 2011). This however is generally not achievable and productivities in the order of 27 to 62 g DCW/m^2/day (100 -227 ton DCW/ha/ammun) are regarded as reasonable targets (Stephens et al., 2010). Based on the potential oil yield of 30 to 50 % oil yield, the theoretical yield of 47000 to 308000 L/ha/ammun (Demirbas, 2010). The cost of biomass production is the only relevant factor for comparison between raceway ponds and photobioreactors as the cost of downstream processing remains constant irrespective of the cultivation system used (Chisti, 2007).

Microalgal productivities in open pond systems range from 5 to 50 g DCW/m^2/day. This is species, climate, and operation dependant (Demirbas & Demirbas, 2011; Park et al., 2011; Sheehan et al., 1998; Zamalloa et al., 2011). Raceway ponds with water depths of 15 to 20 cm allow for the productivity of 10 to 25 g DCW/m^2/day (Schenk et al., 2008). In practice, these are generally lower than the projected theoretical maximum (Zamalloa et al., 2011). Demirbas & Demirbas,

(2011) reported an average of close to 10 g DCW/m^2/day with a maximum productivity of 50 g DCW/m^2/day for biomass achieved by a pilot-scale raceway pond in Roswell, New Mexico. Researchers in Spain were able to achieve 8.2 g DCW/m^2/day (Brennan & Owende, 2010). Richmond, (2004) reported that an average of 19 to 25 g DCW/m^2/day may be achieved in well-managed ponds with peak productivities ranging from 12 to 40 g DCW/m^2/day. Tubular photobioreactors are able to achieve cell densities ranging from 2 g/L to 6 g/L. Higher surface-to-volume ratios give superior productivities (Davis et al., 2011). Cell densities of up to 10 g/L are possible in well-designed photobioreactors (Stephens et al., 2010). Reported productivities for photobioreactors range from 20 to 40 g DCW/m^2/day (Christenson & Sims, 2011).

2.9 Key limitations to biomass productivity in culturing systems

Large scale microalgal production has a number of challenges that need to be overcome in order to make it commercially viable. These include strain selection, maintaining culture integrity, nutrient supply, photosynthetic activity, energy requirements, and gaseous exchange (Javed et al., 2019; Mantovani et al., 2019). Open ponds are able to culture only certain species of microalgae (Richmond, 2004). Microalgae that grow under extreme conditions (high pH, nutrient level, etc.) provide a competitive advantage thus limiting contamination by other microalgae. Contamination is however inevitable and requires constant propagation of seed culture in order to maintain the culture of choice as the dominant culture (Christenson & Sims, 2011; Day et al., 2011). Contamination by non-target microalgae is only regarded as problematic should the contaminating species have a trait that is not desired, have a negative impact on the culture, or be capable of outgrowing the culture of choice. Increased control of the growth environment can effectively reduce contamination but increases the cost. Biofouling becomes a possibility if the microalgae adhere to the walls of the bioreactor. This effectively increases shading thereby

reducing productivity. Biofouling can also impede culture flow, requiring more energy and thus increasing the cost of production (Day et al., 2011).

The supply of photosynthetically active radiation (PAR) becomes a limiting factor in dense cultures above specific concentrations in both open and closed systems thus reducing productivity (Christenson & Sims, 2011). The supply of carbon dioxide is essential for the prevention of carbon limitation. Despite ambient air containing CO_2 that supports microalgae growth, CO_2 needs to be dissolved in the culture medium for uptake. Less than 10 % of the CO_2 resources in nature are available to the microalgae for uptake. Bubbling of air is not an effective delivery system for open ponds due to short residence time (Mata et al., 2010). Optimization of bubbling technology for the dissolving of CO_2 remains an engineering challenge. Removal of oxygen is imperative for the prevention of photo-oxidative stress in photobioreactors. Oxygen above atmospheric concentration inhibits photosynthesis. This problem is usually remedied by sparging of air through the reactor or a section of the reactor in order to strip away excess oxygen. This increases energy consumption and thus cost (Christenson & Sims, 2011).

Commercial operations have produced monocultures of *Spirulina* and *Dunaliella*, with little or no seed culture production being required, due to the use of selective growth media (alkaline or saline). Techniques need to be developed for the stable cultivation of microalgae at large scale. Mass cultivation of microalgae requires the use of inoculum stocks to rapidly restart the culture in the event of a culture failure (Grobbelaar 2009). In order for microalgal biofuels to be cost-effective, the culture will need to be able to grow for sustained periods without the need for frequent re-inoculation.

Although open ponds are currently used for mass microalgal culture for high-value products (e.g., nutritional supplements: Spirulina) and other uses (e.g., wastewater treatment), a cost-effective

system for producing microalgal oil for transportation fuel has the potential to be quite different. The larger scale will affect the liner type, mixing methods, the supply of flue gas, water, and nutrients, harvesting technique, processing system, waste treatment, mixing, etc. Therefore, it is necessary to understand at an early stage how these cost factors can constrain the design and engineering of a microalgae oil production plant and its component systems, and the implications they may have for the type of microalgae that can be grown in such systems (Yin et al., 2020). Besides cost, it is also important to understand the energy inputs into the process (both the embodied energy in the system and the operational energy requirement), and the overall greenhouse gas emissions associated with algal fuel production.

Additional factors that should be considered include:

• Site-specific factors, in particular local climate, water resources, the potential for integration with wastewater treatment (a source of nutrients and water), the potential for integration with CO2 emitters, and sustainability issues associated with land use and biodiversity.

• The potential for valuable co-products (e.g., animal feeds and animal feed supplements, etc) to create an algal biofuel production system that delivers commercially competitive transport oil.

The approach should also involve detailed engineering design and economic modelling and will likely also require the development of and/or analysis/testing/experimentation with component technologies, such as oil extraction. The possibility for integration with other industries should be taken into consideration, and valuable co-products that can be obtained from the process should be investigated in order to help improve the overall economics of the plant. In addition to cost, two other key factors should be considered in the engineering design: energy balance and GHG emissions.

2.10 Contamination of open systems

Microalgae grown in open pond systems are prone to contamination by bacteria, other undesirable species of algae, and zooplankton (Fulbright et al., 2018; Yin et al., 2020; Yun et al., 2018). Bacterial contamination can actively compete for nutrients and oxidise organic matter that could lead to putrefaction of the culture. The majority of contaminants to algal systems are yet to be identified, thus the development of control measures is still in their infancy (Fulbright et al., 2018). Control of heterotrophic bacteria may be achieved by an increase in pH. Aerobic bacteria generally found in algal ponding systems have an optimum pH of 8.3. An increase in pH beyond this level gives effective inhibition thus preventing competition by influencing nitrogen efficiency (Craggs, 2005; Park et al., 2011). Open systems are also susceptible to grazers in the form of protozoa and zooplankton. These organisms actively consume microalgae and can devastate algal concentration in relatively short periods (2 to 3 days). Zooplankton can reduce microalgal concentration by up to 90% of the original density in 48 hours (Oswald, 1980) and *Daphnia* can lower microalgal density by a massive 99% over a few days (Park et al., 2011). Several methods to control these organisms are available including filtration, centrifugation, low dissolved oxygen (DO), application of hormones, and increase in free ammonia. These methods however have drawbacks in that filtration is difficult due to the size of microalgal species such as *Chlorella* sp. making separation technically difficult. Centrifugation is prohibitively expensive at large scale requiring high capital and energy inputs. Photosynthetic microalgae produce oxygen thus actively increasing the DO as a function of growth.

An increase in free ammonia as a control method may be achieved by pH elevation by volatilisation of ammonia. It has been eluded that the toxicity of high pH may be due to increased free ammonia levels that are brought about by the volatilisation of ammonia at high pH (Oswald,

1988). Thus the most appropriate method of controlling zooplankton and bacterial populations is to increase pH to 11 for a short time, usually 3 to 4 hours (Park et al., 2011). The range of optimal pH for algae varies with species. The optimal level of growth for many freshwater microalgae is close to 8 and deviation from this level subsequently leads to a reduction in biomass (Kong et al., 2010). Most microalgae will grow at pH between 7 and 9, however *Amphora* sp. and *Ankistrodesmus* sp. have been shown to grow uninhibited at pH 9 and 10 respectively (Park et al., 2011; Yadav et al., 2020). pH exceeding 11 is reported to occur in high-rate algal pond systems due to the consumption of carbon dioxide and carbonic acid by the process of photosynthesis (Craggs, 2005).

Weedy species of algal compete for nutrients and influence the dynamics of the system often affecting the quality of the biomass for downstream processing (Yun et al., 2018). Control of undesirable algae is usually accomplished by the selection of species with fast growth rates, species grow under stress, such as temperature, pH, salinity, or maintaining higher levels of nutrients. Key to the control of undesirable algal species is higher inoculum levels and reseeding of the cultivation system as required. Hybrid cultivation systems are a method of continual reseeding of open cultivation systems due to the PBRs' resistance to contamination (Yun et al., 2018).

2.11 Upstream Processes of algal cultivation

2.11.1 Strain Selection

The selection of the appropriate species of microalgae is crucial to the success of large-scale cultivation especially for the production of biofuels (Chew et al., 2018). These organisms can be specific to certain environments and prevailing climatic conditions (Brennan & Owende, 2010; Yin et al., 2020). The species selected must meet the requirements for large-scale microalgal

cultivation. The selection of high lipid producers is paramount to success; however, this is just one of the considerations to be taken into account. The species selected should have some competitive advantage such as growth in a selective environment (such as a high nutrient or alkaline environment) to enable successful culturing at large scales (Brennan & Owende, 2010; Chew et al., 2018). The ability to adapt to various changes in conditions needs to be considered as a factor in the selection as temperature fluctuations and diurnal cycles are very difficult to control in open systems and are prohibitively expensive (Brennan & Owende, 2010; Yin et al., 2020). The strain of choice should preferentially be isolated from an area close to the site of production. This allows for a reduction in the time required for acclimatisation to climatic conditions.

Ideally, the species of choice should balance requirements for biofuels and the production of value-added co-products (Chew et al., 2018). This is generally a difficult task in that bioprospecting of a large number of species is required. Production systems need to be tailored towards attaining high photosynthetic activities. Contamination by undesirable algae, bacteria, and grazers is often difficult or costly to control at large-scale. There is a requirement for the development of techniques to prevent CO_2 losses due to diffusion and high rates of evaporation.

Strains may be selected from locally available culture collections, bioprospecting or selection of a suitable lipid producing starter microalgal cultures from reputable and specialized culture collections such as UTEX (USA), ANACC (Australia), CCAP (UK), NIES (Japan), SAG (Germany), CPCC (Canada), etc. However, the rule of thumb is that researchers should cultivate microalgal strains that adapt to their local environmental conditions. Therefore, the use of robust endemic indigenous microalgal strains is highly recommended since these strains are already adapted to the local environmental conditions (Mutanda et al., 2011; Xin et al., 2010; Yun et al., 2018).

2.11.2 Potential of indigenous strains

Culture collections contain strains from a variety of aquatic environments. These strains are acclimatised to the environment of isolation at the time of isolation. As cultures age, due to continual sub-culturing to ensure viability, they become adapted to the environment of cultivation and may lose characteristics such as competitive advantages that are of major benefit to open pond culture systems. These microalgae will generally have longer acclimatisation periods to climatic conditions. Cultures may also lose lipid productivity if stored continually under non-stressed conditions. The adaptability of culture collection strains may also be impeded. The benefit of the use of strains from culture collections is that much of the characterisation has been done with regard to growth requirements. Indigenous strains have inherent adaptability that may be the competitive edge required for open pond systems. They generally can adapt quickly to changes in environment and climate (Nagarajan et al., 2020). Indigenous strains however require isolation, the establishment of optimal culturing conditions, and determination of the feasibility of production by experimental techniques. This is time-consuming, labour intensive, and is more costly than strain selection from culture collections. The benefit of adaptability however may outweigh the drawbacks in the longer term (Munoz & Guieysse, 2006).

2.11.3 Selection of aquatic environments

Selection of environments similar in climatic conditions to an area in which cultivation, as proposed, is beneficial in that the strain finally selected for cultivation becomes easily acclimatised to cultural conditions. This will aid in the ease of culturing at large scale due to a shorter acclimatisation period. Understanding of environmental conditions allows mimicking of those environments to encourage growth. The simplest way of accomplishing this is the measurement

of abiotic factors such as pH, temperature, etc., nutrient levels, and other physic-chemical properties at the sampling point. It is prudent to collect the water from the microalgal sampling point and filter sterilise it and use this for media formulation in the laboratory. The use of culture enrichment for microalgal growth before purification is a particularly useful tool for isolation. Some knowledge of the taxonomic group being targeted is also useful to encourage *in vitro* growth. The addition of essential nutrients should occur as soon as possible after sample collection. This will help prevent culture death that may occur due to a change in growth conditions. The collection of samples during warmer months has been shown to be more adaptable to change in temperature (Andersen & Kawachi, 2005). Contaminants in the form of bacteria, algal grazers, and undesirable cultures may be eliminated by a combination of one or more techniques. Filtering of samples at the collection with a 100µm sieve is useful for the removal of debris and some zooplankton (Mutanda et al., 2011). Serial dilution, streaking on agar plates and the use of single-cell isolation techniques are extremely useful for the elimination of contaminants. Continued sub-culturing allows the determination of the supply of essential nutrients. Culture death after a number of sub-cultures indicates the lack of essential nutrients or the accumulation of metabolic wastes in the culturing environment (Andersen & Kawachi, 2005).

2.11.4 Screening of microalgae for lipid production

Screening for oil-producing microalgae among the isolates is a vital part of the optimisation of biodiesel production. As lipid is formed as a storage product there is a general inverse relationship between lipid production and biomass yield. Certain strains of microalgae such as *Botryococcus braunii* have high lipid storage potential (up to 75% lipid/g dry cell weight) but this is accompanied by low productivity. Cultures with moderate lipid accumulation levels (20-50%) but higher lipid

productivities are preferred for mass cultivation (Amaro et al., 2011; Mata et al., 2010). Factors other than lipid productivity such as lipid profile need to be taken into account such as the ability to grow under specific environmental conditions. Lipid profiles determine the suitability of lipid for the production of biodiesel. Lipid profiles are affected by nutritional, processing, and cultivation conditions. The selection criterion should be based on a number of factors including growth rate, lipid quantity, and quality, strong adaptability to changes in the environment, and determination of preferred nutrients and nutrient uptake rates (Mata et al., 2010). In order to gain high oil yields, it is necessary to ascertain the amount of oil, if any, produced under normal conditions. This is likely to allow overproduction under stress.

The viability of a species of microalgae for use in the production of biodiesel is often dependent on the yield. Healthy, actively dividing cells usually have a low percentage of TAG. TAG may be found in elevated proportions, in some algal species during the stationary phase. The stationary phase may be induced by limiting one or more variables that control growth, e.g. nitrogen, phosphorus, or silicon limitation (Mansour et al., 2005). Adaptation of microalgae to environmental change is generally a result of the change in lipid patterns and synthesis of various unusual compounds (Guschina & Harwood, 2006). This is likely the reason that microalgae overproduce fatty acids when subjected to stressful conditions. It is thus possible to induce or enhance lipid content by nitrogen starvation or other stress factors. Nutrient deficiencies such as silicon depletion may lead to an increase in cellular lipid content. A lipid content increase of 60% is possible by silicon starvation of the diatom *Navioua pelliculosa* (Miyamoto, 1997). Lipid fractions as high as 70 to 85% on a dry weight basis have been reported. Such high lipid contents exceed that of most terrestrial plants (Miyamoto, 1997).

Characterisation of lipids is required as different species of microalgae produce different types of fatty acids. Some fatty acids are more suitable for transesterification to biodiesel than others. Most microalgal oils are rich in polyunsaturated fatty acids with four or more double bonds. The problem associated with this degree of polyunsaturation is, these fatty acids and fatty acid methyl esters (FAME) are susceptible to oxidation during storage, thus reducing their acceptability for use in biodiesel (Chisti, 2007). The strain selected for large scale cultivation must produce the appropriate lipid under the envisaged culture conditions at large-scale. This is however difficult to ascertain as mimicking of open systems is dependent on a number of factors that cannot be accurately replicated in the laboratory. The cost of culturing at large scale must also be considered when selecting the strain for large-scale cultivation.

2.11.5 Are we selecting the correct strains? Natural vs. stressed conditions

Chlorophyceae have been reported to contain high levels of neutral lipids therefore they may represent a large pool of producers that may be useful for lipid production (Chen et al., 2011). Determination of lipid production capacity of microalgae growing in the natural environment is near impossible. Furthermore, different strains of the same species of microalgae are known to react differently under the same growth conditions. The only effective method of determining stressed conditions is the growth of the microalgae *in vitro* and experimentation to evaluate the effects of stress conditions on biomass and lipid productivity. Under nutrient-depleted conditions, cells do not proliferate abundantly. A lower amount of light intensity is required for biomass maintenance and excess energy in the form of free electrons is directed to lipid production as an energy sink to prevent photooxidation (Packer et al., 2011). Under normal cultural growth conditions, i.e., with no nutrient stress, photosynthesis increases with an increase in light intensity until light saturation sets in, at which point the maximum growth rate will be attained (Richmond,

2004). Photoinhibition and consequently decrease in microalgal growth rate is as a result of irradiance of the culture above the level of light saturation (Packer et al., 2011).

2.11.6 Selection of media

The production of microalgae requires important inorganic nutrients in the forms of nitrogen and phosphorus (Brennan & Owende, 2010). Microalgae are known to have different nutrient requirements not only by composition but also by the concentration of the nutrients supplied. Microalgal growth media are therefore composed of macronutrients generally consisting of a nitrogen source, phosphate, and a metal chelator. Iron is generally supplied as a micronutrient. Some microalgal species have inherent adaptability to cultural conditions due to the environment of isolation being in constant flux. *Chlorella* sp. are known to grow fairly well in nutrient-rich media (Illman et al., 2000). Illman et al., (2000) showed growth of various species of *Chlorella* in Watanabe media containing 1.25g/l KNO_3. Nutrient-rich media may cause culture shock and result in death brought on by nutrient toxicity (Watanabe et al., 2000). Media for screening should ideally range from low to high nutrient concentration so as not to exclude potential cultures by the lack or excessive nutrient supply. Care should be taken to avoid excessive bacterial growth by the addition of components that may support bacterial growth. Overgrowth of bacteria can cause the death of the microalgae by inducing anoxic conditions or causing culture toxicity (Andersen & Kawachi, 2005).

2.11.7 Evaluating freshwater and marine microalgae

Successful cultivation of marine microalgae is seen as favourable for improving the economics of biomass production of microalgae (Uduman et al., 2010). The selective environment will serve to reduce extensive contamination. Seawater can be used directly instead of freshwater sources

(Amaro et al., 2011). Choice of microalgal species for cultivation should depend on lipid and biomass productivities as well as the location of the cultivation plant. Marine and freshwater species have shown similar biomass and lipid productivities thus making strain selection dependant on other factors (Ahmad et al., 2011).

A major factor to be considered is water availability (Mata et al., 2010). Despite the cultural benefit of lower contamination, the feasibility of the production is hinged on the cost of production, thus marine production plants are limited to coastal regions due to the availability of seawater. This limits the sites available to set up a commercial plant and the chosen sites come at a high capital cost. Furthermore, seawater generally contains marine flora that consumes microalgae and large amounts of water need to be filtered or marine flora is removed which will potentially negatively impact the economics of production. High evaporative rates could increase salinity thus adjusting cultural conditions. Salinity needs to be monitored on a regular basis and corrected by the addition of freshwater (an additional cost). An increase in salinity results in osmotic shock and may result in the rupturing of cells under conditions that may not be suitable for lipid recovery (Mata et al., 2010). Cultivation of freshwater microalgae with similar productivities may be more appropriate in inland areas. Furthermore, the cultivation of freshwater species allows a more diverse consortium of species to be propagated.

Marine microalgal species have been shown to produce higher levels of phospholipids than triacylglycerols (TAGs) (Singh et al., 2011). These types of lipid are unsuitable for the production of biodiesel via transesterification. Freshwater strains have been shown to produce large quantities of saturated neutral lipids making them ideal candidates for biodiesel production. *Scenedesmus obliquus* contains predominantly saturated fatty acids and monounsaturated fatty acids. This imparts the property of oxidative stability (Ahmad et al., 2011). The fatty acid

compositions vary between species of freshwater microalgae as well as between species of marine microalgae. Further, it is possible to manipulate the type of lipid produced by adjustment of cultural conditions (Griffiths & Harrison, 2009). Each strain selected must be investigated to find the best fit of characteristics for the climatic conditions and location selected. The cost and feasibility of adjusting the cultural conditions must be considered. Adjustment of cultivation temperature is generally used at a laboratory scale to induce changes in lipid characteristics. Large scale cultivation in the form of raceway ponds generally does not incorporate temperature control. This negates the adjustment of temperature as a viable option. Moreover, the incorporation of such systems into raceway ponds increases the energy requirement for cultivation thus affecting the overall energy balance and economic feasibility.

2.11.8 Synergistic interactions in the environment

Microalgae occur under various nutrient conditions in nature. Much of the nutrients supplied are via the nutrient cycling brought about by bacterial degradation of organic matter. Interactions within the natural environment may dictate the ability of microalgae to colonise that environment and proliferate abundantly. This in turn will determine the ability of such a strain of microalgae to be successfully isolated in the laboratory. Isolation of microalgae from the natural environment may result in non-proliferation of cultures due to the lack of some essential metabolites required for growth that are not supplied in artificial media. These metabolites may be produced by various organisms within the natural environment (Ramanan et al., 2016). A common bacterium, *Azospirillum brasilense* was found to promote the growth of *Chlorella vulgaris* as well as induce changes in the lipid profile and pigment production by the production of Indole acetic acid (IAA) (Pagnussat et al., 2020). IAA has been shown to increase growth in algal species by increasing carotenoid biosynthesis and increased expression of antioxidant genes (Liu et al., 2020).

Production of auxins has also been shown to promote the growth of *Scenedesmus obliquus* under nitrogen stressed conditions, giving a 3-fold increase in biomass by a proposed mechanism of alleviation of oxidative stress (Pagnussat et al., 2020). The area of research on the mechanisms of algal-bacterial interactions is still emerging and warrants further investigations (Mohd Udaiyappan et al., 2020).

CHAPTER 3: OPTIMIZATION OF BIOMASS YIELD IN MEDIA AND WASTEWATER AT PILOT SCALE

3.1 Introduction

Production of commodity products such as biofuels requires the technology used to be inexpensive thereby reducing the cost of the commodity. This limits the propagation of algal biomass for lipids production to pond systems utilizing natural light and waste products to enhance the economic viability. Much of the current research on algal cultivation and media and wastewater is at laboratory scale (Yin et al., 2020). Scale-up from laboratory scale to pilot and large-scale presents several challenges that may be unique to each system. It is thus necessary to undertake research trials in environments as close to large scale cultivation as possible in order to achieve reliable results as several parameters are in constant flux in outdoor systems (Sutherland et al., 2020).

The supply of macronutrients is critical to algal cultivation, however, the use of conventional media is prohibitively expensive (Ashokkumar et al., 2019). The amount and composition of the nutrients will however depend on the species of algal cultivated and prevailing conditions. The role of nitrogen limitation in algal lipid accumulation has been well established but at the expense of biomass productivity (Park et al., 2011). It is thus imperative to obtain an appropriate balance. The use of algal biomass for the treatment of wastewater has been conducted extensively, however using wastewater as a resource for algal propagation for the production of products is limited at pilot and large scale and presents an attractive opportunity (Li et al., 2019).

This chapter focuses on the cultivation of biomass in pilot-scale raceway and circular ponds. It seeks to reduce the inclusion of media and assess the suitability of final effluent from a domestic

WWTP for the cultivation of algal biomass. The chapter furthers tracks the quality of final effluent from Kingsburgh WWTP in order to determine the stability of the effluent over a period of 9 months.

3.2 Materials and Methods

3.2.1 Microalgae cultivation and biomass production

Scenedesmus sp. used in this study was isolated from Kingsburgh wastewater treatment plant, KwaZulu Natal, South Africa. Raceway ponds of working volume 3000 L (5 x 2 x 0.6 m^3) at the Durban University of Technology, South Africa operated with modified BG11 medium for cultivation algal cultivation under natural sunlight (100-1200 µmol/m^2/s) and temperature of 18 - 27°C. Biomass was harvested using gravitational settling and the supernatant was discarded followed by centrifugation using a centrifuge (Thermo electron Corporation Multifuge 4KR) of concentrate biomass to obtained thick algal slurry.

3.2.2 Analysis of microalgal quantum yield (Algal Physiological health)

Microalgal culture samples were withdrawn at regular intervals. A Dual-Pulse amplitude modulation-100 (PAM) (Heinz Walz Gmbh, Effeltich, Germany) was used for non-invasive fluorescence analysis. Prior to analysis, the algal samples were adapted in dark (20-30 mins) to close Photosystem II (PSII) reaction centres. The maximum quantum efficiency of PS II charge separation (Fv/Fm) was calculated in accordance with the Equation 1 (Ramanna et al 2014).

$$F_v/F_m = (F_m - F_o)/F_m \qquad \text{Equation 1}$$

Where F_v is the variable fluorescence resulting due to maximum fluorescence F_m and minimum fluorescence F_o dark-adapted sample.

3.2.3 Microalgae growth analysis

The optical density of the microalgae was done routinely in which 1 ml of the microalgae culture was collected and the absorbance values were measured at 680 nm using a Jenway 7205 UV/Visible Spectrophotometer (Lasec, South Africa). Biomass yield was determined by taking 10 ml of the well-mixed culture and centrifuged (Hermle Z326K, Laboterchnik GmbH, Germany) at 1509 g at 4 °C for 10 min. The supernatant was discarded, and the pellet was washed with distilled water. The culture was further centrifuged to remove the water, and the pellet was transferred to dried pre-weighed watch glasses and kept in the oven at 70 °C overnight. The dry cell weight was determined gravimetrically using an analytical balance. The biomass productivity was determined according to Equation 2.

$$Biomass\ productivity\ mg/L/d = \frac{(biomass)\ mg/L}{cultivation\ time} \qquad \text{Equation 2}$$

Where biomass is the Dry Cell Weight in mg/L and cultivation time in days.

3.2.4 Lipid extraction

Total lipids were extracted using a 2:1 (v/v) mixture of chloroform and methanol as per the method of Folch et al. (1957) with microwave-assisted cell disruption. A total of 20 ml of solvent was added to 1g of dried biomass and digested in a microwave digester (Milestone S.R.L., Italy; 1200 W of output power) at 1000 W and 100 °C for 10 min. Solvent containing extracted lipids was centrifuged and vacuum filtered. The solvent was evaporated to dryness in an oven at 60 °C. The crude microalgal lipid was measured gravimetrically and the lipid yield was calculated according to Equation 3 (Talukder et al., 2012).

$$Total\ lipid\ yield\ (\%) = \frac{Extracted\ lipid}{Total\ lipid\ in\ biomass} \times 100 \qquad \text{Equation 3}$$

3.2.5 Proteins analysis in whole algae and residual biomass

Protein estimation from algal biomass was carried as per (Lowry et al., 1951). Reagents were prepared as per (López et al., 2010). A 25 ml lysis buffer solution per 100 mg of dried biomass was added and ground with mortar and pestle for five mins, and then vortexed for two mins. The supernatant was collected after centrifugation at 3000 *g* for 10 min. The pellet was resuspended in the same amount of lysis buffer solution, then ground and vortexed. Finally, the supernatant was collected by centrifugation and pooled from both runs. 0.5 ml of SDS solution was mixed with 0.5 ml of pooled supernatant and vortexed. This mixture was then added to 5 ml of reagent-C and vortexed. After 10 min, 0.5 ml of Folin reagent was added and left to rest for 30 min. A spectrophotometer (Spectroquant Pharo300, Merck) was used to measure the absorbance of this mixture at 750 nm. Standards for calibration were prepared by dissolving bovine serum albumin in lysis buffer, and the calibration curve was used for protein quantification and yield was quantified according to Equation 4 (López et al., 2010)

$$\text{Protein yield } (\%) = \frac{CVD}{m} \times 100 \qquad \text{Equation 4}$$

Where C is the protein concentration (mg L^{-1}) obtained from the calibration curve, V is the volume (L) of the lysis buffer used to resuspend the biomass, D is the dilution factor and m is the amount of biomass (mg).

3.2.6 Carbohydrates analysis in whole algae and residual biomass

Total carbohydrates in algae biomass were determined by the sulphuric acid method (DuBois et al., 1956). An aliquot (100 mg) of dry cell biomass was mixed with diluted sulphuric acid (2% v/v) and hydrolysis was carried out by autoclaved for 30 min at 121°C. Afterward, the mixture was neutralized by adding 1M H_2SO_4 until the effervescence ceased. To obtain supernatant, the

mixture was subsequently centrifuged at 1509 g for 10 min. 0.1 ml of supernatant transfer into the test tube and diluted to 1 ml. Consequently, 1 ml of phenol solution (5% w/v) and 5 ml of 96% H_2SO_4 were added. The mixture was kept at 30 C in a water bath for 30 min, the absorbance of the solution measured at 490 nm using a spectrophotometer (Spectroquant Pharo300, Merck). Glucose was used as a standard for the analysis (Kassim et al., 2014). The % carbohydrate yield was calculated according to Equation 5

$$Carbohydrate\ yield\ (\%) = \frac{C}{V} \times \mathrm{M} \qquad \text{Equation 5}$$

Where C is the carbohydrate content (mg/ml) obtained from the calibration curve, V is the volume (ml) of the supernatant used for the analysis and M is the total volume (ml) of the microalgal sample solution.

3.2.7 Nutrient Analysis

Nutrients (NO_3-N; NO_2-N; NH_3-N and PO_4) were analysed by the Gallery™ Discrete Analyser (Thermo Scientific, Germany). Tests were conducted as single measurements and were validated against a known standard. Analysis within 5% error was accepted.

3.2.8 Wastewater Characterisation

Physical parameters such as pH, electrical conductivity (EC), temperature, and dissolved oxygen (DO) were measured using a YSI MPS 556 multiparameter system (Yellow Spring Instrument Co.; USA). The suspended solids, alkalinity, and chemical oxygen demand (COD), following the American Public Health Association (APHA, 1998).

Chemical oxygen demand (COD) was conducted using the closed reflux titrimetric method. 2.5 ml sample was digested with 1.5 ml $K_2Cr_2O_7$ and 3.5 mL sulphuric acid reagent for 2 hours at a

temperature of 150 °C. The samples were left disturbed and allowed to cool to ambient temperature. Tubes were shaken and allowed to settle for a further 30 min to allow precipitation. The digested sample was titrated against ferrous ammonium sulphate until the end achieved (wine red colour). Two blanks solutions were prepared using distilled water instead of a sample and digested. The COD was calculated by Equation 6

$$COD = \frac{(A-B)*8000*(0.1M\ FAS)}{Sample\ Volume} \quad \text{Equation 6}$$

Where, A is the titrant used for blank and B is the titrant used for sample

3.2.9 Experimental setup

3.2.9.1 Pilot-scale raceway ponds

Media optimization for nitrate concentration is performed in 4 pilot-scale raceway ponds. Ponds were 5 m x 2 m x 0.6 m with a working volume of 0.3 m. The pilot-scale raceway ponds were situated in a greenhouse on the roof of the Institute for Water and Wastewater Technology Building at the Durban University of Technology (Figure 3.1). The culture was mixed by paddlewheel on a variable speed controller. Cultures were mixed at 0.48 m/s.

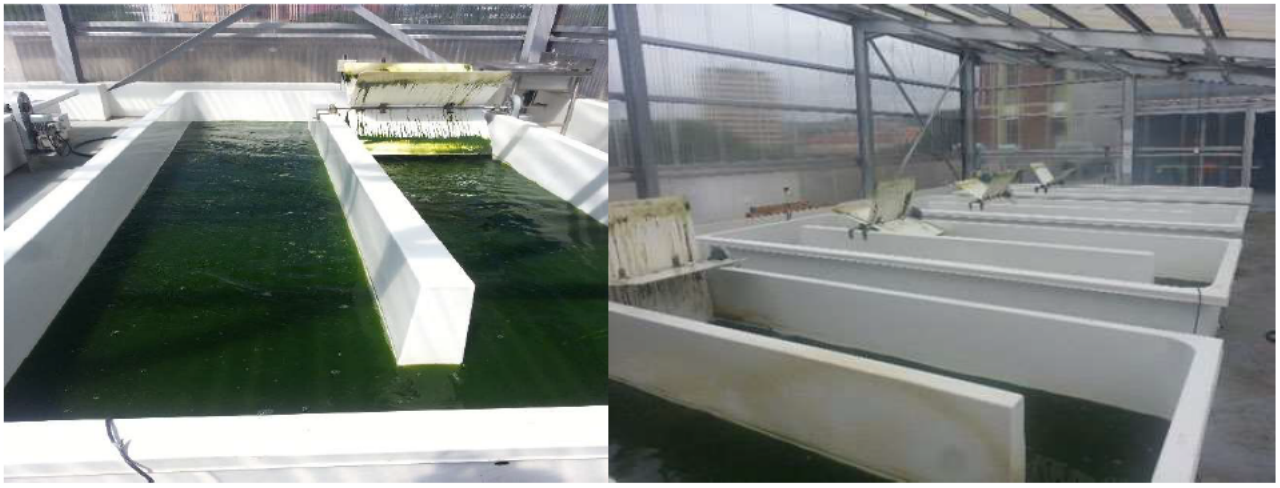

Figure 3.1. Raceway Ponds situated in a greenhouse on the roof of the Institute for Water and Wastewater Technology at Durban University of Technology

3.2.9.2 Circular Ponds at Kingsburgh Wastewater Treatment Plant

Cultivation of seed culture and experimentation under Kingsburgh climatic conditions were carried out in open circular ponds of diameter 3.55 m at a working depth of 0.3 m (Figure 3.2). the circular ponds were mixed by HQB 5500 fountain pumps at a rate of 6000 L/h. Ponds were opened and placed on the island of the raceway pond at Kingsburgh WWTP.

Figure 3.2. Algal culturing in 3000L circular ponds mixed by 6000 L/h submersible fountain pumps.

3.2.9.3 Media optimization

Sodium nitrate levels from BG11 were reduced to 750, 500, and 250 mg/L with full BG11 (1500 mg/L) as the control in pilot scale raceway ponds at Durban University of Technology. The experiment was conducted for 20 days under operating conditions as per section 3.2.9.1. Biomass, Biomass productivity, metabolites, and Fv/Fm were determined as per sections 3.2.1-3.2.7 above. Further experimentation utilized the same setup with a further reduction from the optimal levels of the preceding experiment at increments of 50mg/L. Effective concentrations of $NaNO_3$ were 300, 250, 200 and 150mg/L.

Sodium nitrate levels from BG11 were reduced to 300, 250, and 150 mg/L in opened circular ponds as per section 3.2.9.2. The experiment was conducted for 42 days under operating conditions as per section 3.2.9.2. Biomass, Biomass productivity, metabolites, and Fv/Fm were determined as per sections 3.2.1-3.2.7 above.

3.2.9.4 Replacement of $NaNO_3$ with Urea

Urea levels of 71, 83, and 106 mg/L equivalent in N to 200, 250, and 300mg/L $NaNO_3$ mBG11 (250 mg/L $NaNO_3$) as the control in pilot scale raceway ponds at Durban University of Technology. The experiment was conducted for 10 days under operating conditions as per section 3.2.9.1. Biomass, Biomass productivity, metabolites, and Fv/Fm were determined as per sections 3.2.1-3.2.7 above.

3.2.9.5 Cultivation of algae using post-chlorinated wastewater

Algal biomass cultivated on media variations using post-chlorinated wastewater as a water source where BG11 is BG11 media components freshwater as a water source; MBG11 is modified BG11 with $NaNO_3$ reduced to 250mg/L and PCW waster source; WW+N+P is PCW with the addition of 250mg/L $NaNO_3$ and 40mg/L KH_2PO_4; WW+N is PCW with the addition of 250mg/L $NaNO_3$.in opened circular ponds as per section 3.2.9.2. The experiment was conducted for 33 days under operating conditions as per section 3.2.9.2. Biomass, Biomass productivity, metabolites, and Fv/Fm were determined as per sections 3.2.1-3.2.7 above.

3.3 Results

3.3.1 Strain selection

Scenedesmus obliquus was selected for cultivation due to its robustness to change in climatic conditions and production of acceptable amounts of lipids under nutrient stressed conditions. The strain was isolated from the maturation pond at Kingsburgh WWTP located on the east coast of South Africa. This serves as the site for the project housing the large-scale pond. The strain occurred as a contaminant which outcompeted the strain of choice grown under outdoor conditions in a circular pond as described in 3.2.8 in one of the preliminary trials and was isolated and characterised. Under laboratory conditions, *S obliquus* peaked at maximum biomass of 0.311 g/L in 14 days using BG11 medium at an illumination of 80 µmol/m^2/s with a photoperiod of 16:8 light: dark cycle.

3.3.2 Optimisation of nitrate in BG11 media at pilot scale

Standard BG11 medium contains 1500 mg/L $NaNO_3$ which is not feasible for large-scale algal cultivation. The main aim of the experiment was to determine the based return in investment in terms of biomass productivity as a result of reduced $NaNO_3$ concentrations. As seen in Figure 3.3 and Figure 3.4, biomass reached a maximum of 1.34 g/L in 7 days with maximum biomass productivity of 32.78 $g/m^2/d$ for the 1500mg/L $NaNO_3$ trial. This was closely followed by 250 mg/L $NaNO_3$ with a biomass level of 1.25 g/L at biomass productivity of 31.23 $g/m^2/d$. over the full cultivation period, however, the highest productivity was observed for 250 mg/L $NaNO_3$ at 18.09$g/m^2/d$.

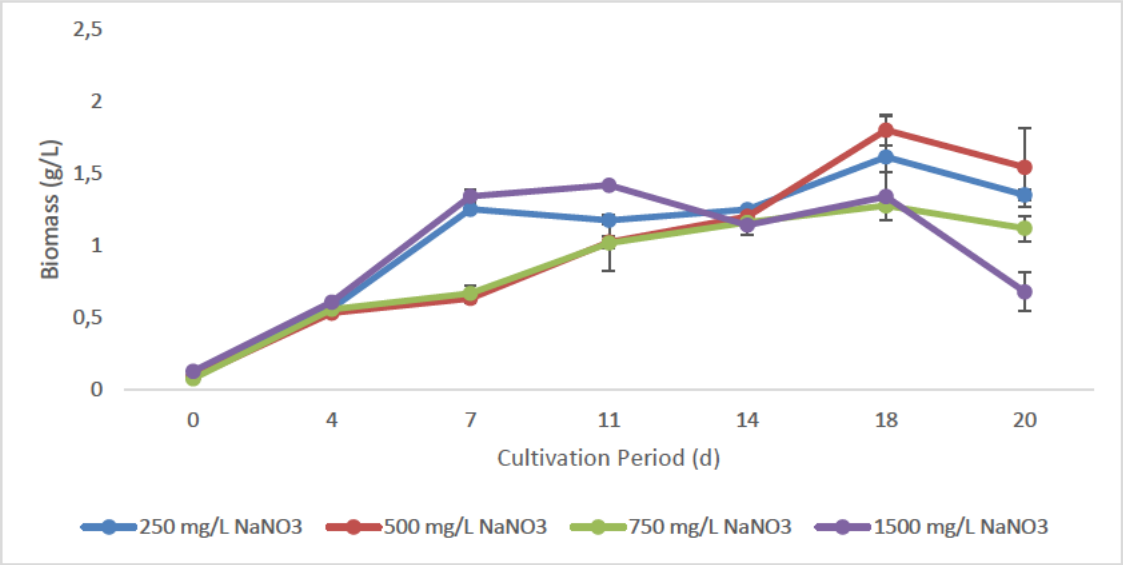

Figure 3.3. Growth curves of algal biomass cultivated in BG11 media with reduced nitrate in the form of $NaNO_3$ in a 10 m^3 pilot-scale raceway pond housed in a greenhouse

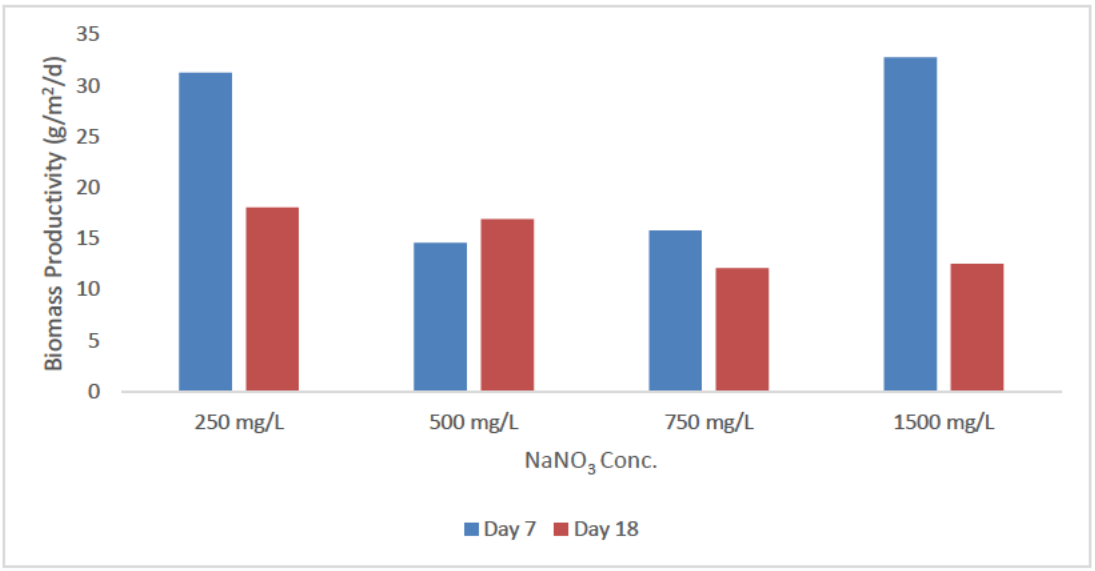

Figure 3.4. Biomass productivity of algal biomass cultivated in BG11 media with reduced nitrate in the form of $NaNO_3$ in a 10 m^3 pilot-scale raceway pond housed in a greenhouse on day 7 and day 18.

Higher overall biomass was achieved for 500 mg/L $NaNO_3$ however overall productivity was still higher 250 mg/L $NaNO_3$ which was comparable to standard BG11 medium. The biomass yields and productivity achieved for the trial is comparatively high. This is due to the favourable conditions provided by the greenhouse. The average temperature was maintained slightly below 30°C due to automated exhaust fans to removed hot air. Water temperatures in the region of 27.18±5.1°C with the average light intensity of 370±7.2 µmol/m^2/s over the first 4 to 7 days. It should be noted that the seed inoculum for the raceway ponds was cultivated under laboratory conditions and was therefore acclimatised to light intensities between 80 and 120 µmol/m^2/s at ±25°C. The increase in light intensity was high enough to stimulate the fast growth of the algae without reaching photoinhibition levels. The combination of optimal temperature and elevated light intensity with the supply of enough nutrients allowed the culture to attain high biomass

productivity. The decrease in productivity from day 7 to day 18 was an expectation due to an increase in culture density which limits light penetration.

Nitrogen concentration on the addition of media on day 0 demonstrated that incomplete dissolution of $NaNO_3$ took place in that, the concentrations of NO_3-N were lower than expected. Expected concentrations for 250, 500, 750 and 1500 mg/L $NaNO_3$ were 41.17, 82.35, 123.52 and 247.05 mg/L of N, respectively (Figure 3.5). Measured results however were 48, 64, 59 and 53 mg/L for the 250, 500, 750 and 1500 mg/L $NaNO_3$ trials, respectively. Due to the concentration of other salts from the BG11 medium and inadequate mixing, it is possible that dissolution took place at a slower rate. Nitrogen utilisation gave highly variable results for nitrate-N showing slight decreases from days 1 to 4 for 250 and 500 mg/L $NaNO_3$ and an increase in the concentrations for 750 and 1500 mg/L $NaNO_3$. The increase is likely due to the action of bacteria in the system, either by decomposition of dead organic matter or nitrification. Nitrite-N concentrations increase from 3 mg/L to 34 mg/L on day 20. NH_3-N concentrations remain below 0.5 mg/L. As no NO_2 or NH_3 were added to the media, the N species are likely to be a product of enzymatic reduction of nitrate which is the known mechanism for algal utilisation of N.

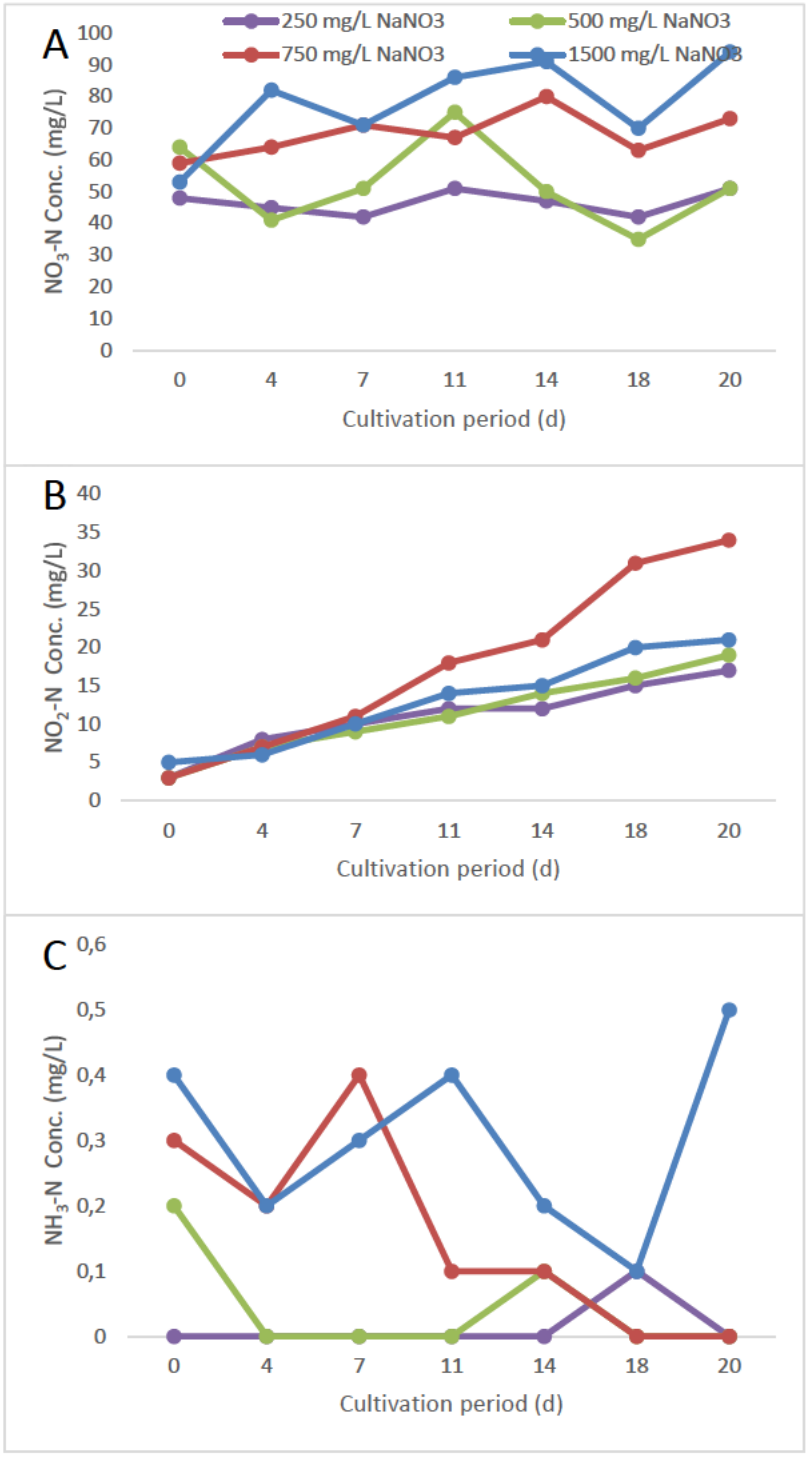

Figure 3.5. Nitrogen levels in the form of (A) NO_3-N; (B) NO_2-N and (C) NH_3- N of the 10 m^3raceway ponds over the full period of cultivation for optimisation of nitrogen concentration. No error bars shown as the results were validated using quality controls.

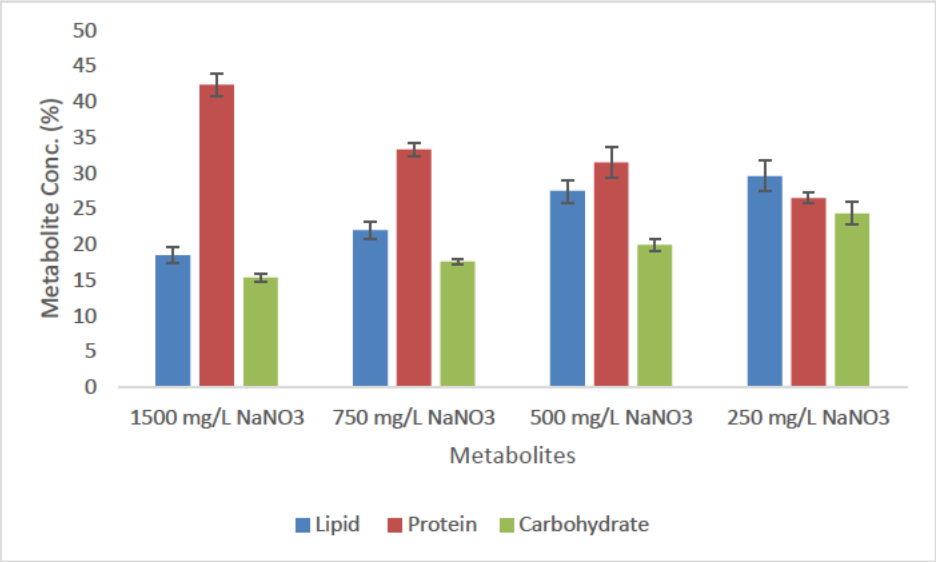

Figure 3.6. Metabolite production of algal biomass cultivated in BG11 media with reduced nitrate concentrations in a 10 m^3 pilot scale raceway pond

Nutrient concentrations as shown in Figure 3.5A, with the exception of 250 and 500 mg/L $NaNO_3$ displayed increased concentration over the first 4 days of cultivation. This is likely due to the high concentration of $NaNO_3$ which resulted in a slower dissolution rate. $NaNO_3$ was supplied in a granular form directly into the culture medium. The lower concentrations (250 and 500 mg/L) showed utilisation of NO_3-N over the initial cultivation period. This was followed by an increase in NO_3- N concentrations for all concentrations. A steady increase in nitrate concentration over the cultivation period to reach a maximum of 34mg/L NO_2-N Figure 3.3 B. Ammonia nitrate remained at levels below 0.5 mg/L throughout the cultivation period Figure 3.3C. The effects of nitrogen limitation on lipid, carbohydrate, and proteins are demonstrated in Figure 3.6. An increase in lipids is noted for each of the concentrations tested with BG11 (1500mg/L); 750mg/L; 500mg/L producing 18.5±1.17; 22.19±1.12 and 27.5±1.61 % Lipid/g DCW. The highest lipid content of 29.6±2.12 % Lipid/g DCW was achieved with 250mg/L $NaNO_3$. A similar trend is

noted for carbohydrates which increased with the decrease in NO_3-N. Protein concentration decreased as was expected. Under nitrogen stressed conditions lipids and carbohydrate accumulation take place as an energy sink in order to prevent damage to photosynthetic machinery. The lack of nitrogen availability shifts metabolism from growth to survival which results in a reduction of proteins.

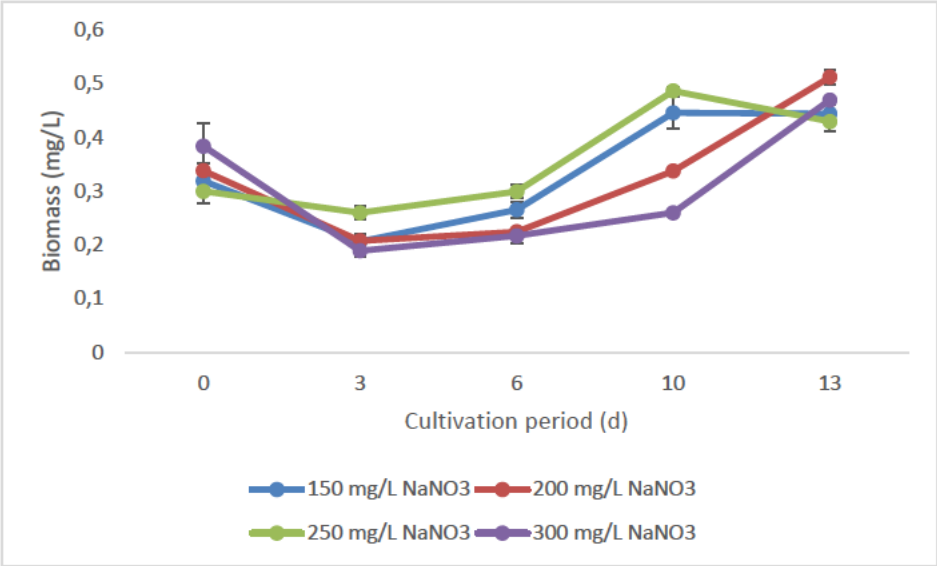

Figure 3.7. Growth curves of algal biomass cultivated in BG11 media with reduced nitrate (150 to 300 mg/L) as optimised from the previous experiment in the form of $NaNO_3$ in a pilot-scale raceway pond

Further optimisation of NO3-N concentration showed similar growth patterns for the range of 150 mg/L to 300 mg/L as per Figure 3.7. There is however a large disparity between biomass levels obtained in Figure 3.7 and Figure 3.3 where the biomass levels exceeded 1g/L by day 11 as compared to maximum biomass of 0.48 g/L (13.09 g/m^2/day) in the former. Lipid content ranged

from 22.9±1.21 to 35.12±0.44 % lipids. This is likely due to high irradiance during the validation of the NO_3-N level. The average light intensity observed in Figure 3.3 was 248±168 µmol/m^2/s whereas the average light intensity for Figure 3.7 was observed to be 942±459 µmol/m^2/s. This much higher light intensity is like to have led to photoinhibition which explains the decrease in biomass levels from days 1-3. The higher lipid content and low biomass productivity of the culture are also indicative of stress conditions being experienced by the algae.

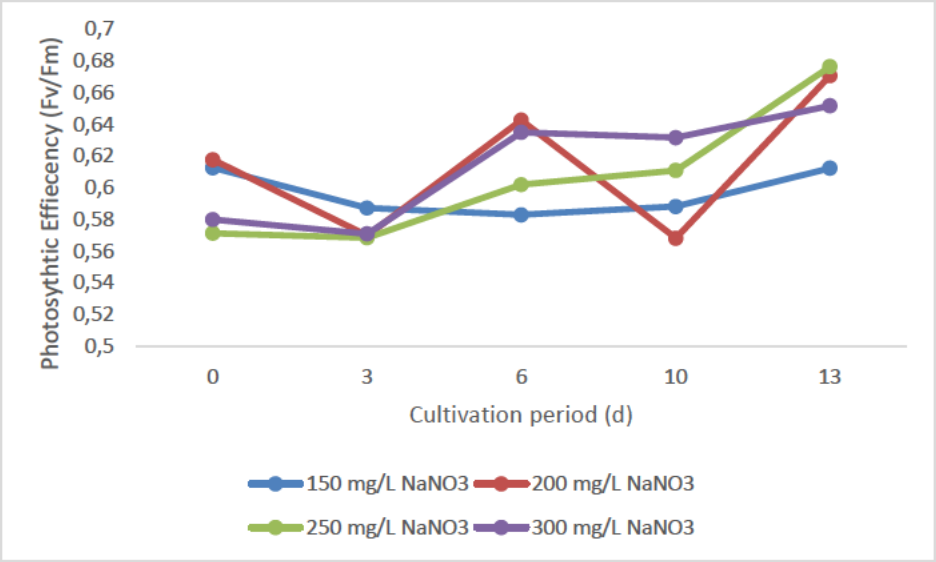

Figure 3.8. Quantum yield of PSII of algal biomass cultivated in a pilot scale raceway pond under reduced nitrogen conditions.

From Figure 3.8 it is observed that the culture undergoes stress from days 0 to 3 indicated by a lower Fv/Fm ratio during this period. The physiological health of the culture improves drastically for 250 mg/L $NaNO_3$ over the cultivation period. A decrease in Fv/Fm is usually associated with adaptation to change in light conditions such as the huge increase in light intensity as seen in the previous experiment. This occurs as a method to prevent damage to the photosystem. Lipids accumulation, being an energy sink is triggered to remove excess light energy results in an increase of lipids to a max of 35%.

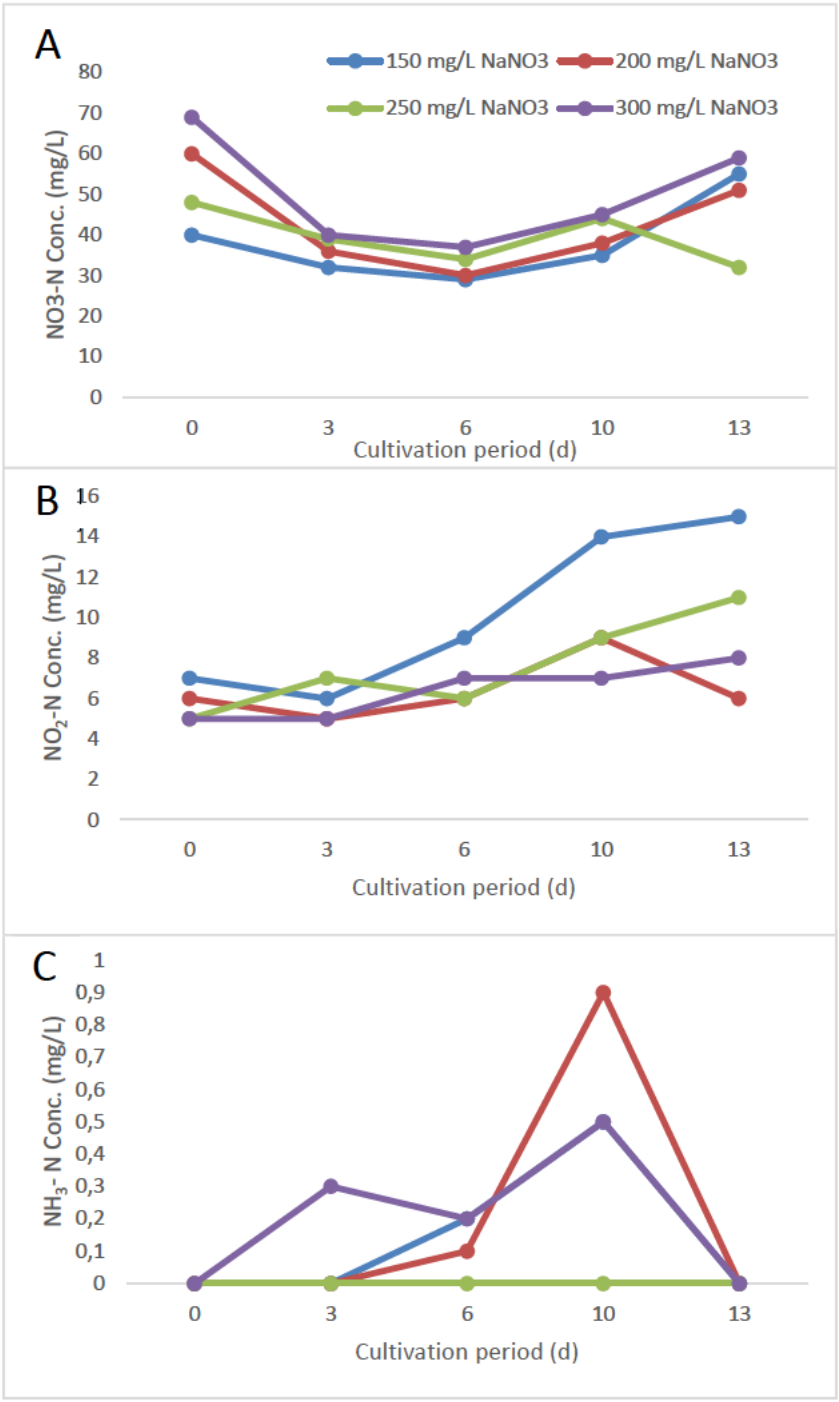

Figure 3.9. Nitrogen levels in the form of (A) NO_3-N; (B) NO_2-N and (C) NH_3- N of pilot scale raceway ponds over the full period of cultivation for optimisation of nitrogen concentration from 150- 300 mg/L $NaNO_3$. No error bars shown as the results were validated using quality controls.

Initial NO_3-N concentrations were measured to be 40, 48, 60, and 69 mg/L NO_3-N for the trials with 150, 200, 250 and, 300 mg/L $NaNO_3$ as per Figure 3.9. A similar trend is to Figure 3.5 is noted as the expected nitrate levels were lower than expected on day 0. A decrease in concentration is evident from days 0 to 6 followed by an increase in concentration for all trials beyond day 6. NO_2-N increased from day 0 to a maximum of 15 mg/L on day 13 for 300 mg/L $NaNO_3$. NH3-N concentrations peaked at 0.9 mg/L for the 200 mg/L $NaNO_3$ trials and remained at very low concentrations (below 0.9 mg/L) throughout the cultivation period. The results obtained display similar trends to the previous experiment with a higher concentration of $NaNO_3$ (Figure 3.5)

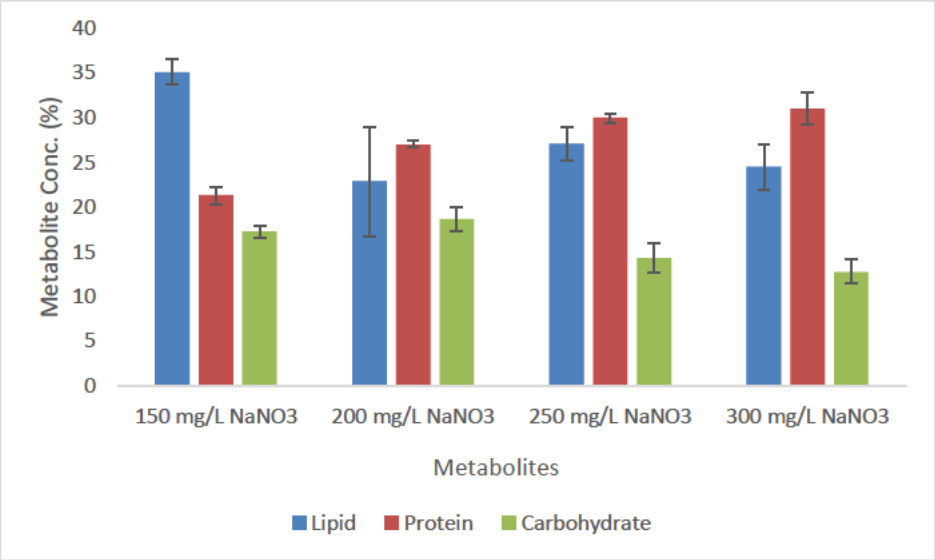

Figure 3.10. Metabolite production by algal biomass cultivated in BG11 media with reduced nitrate (150-300 mg/L) concentrations in pilot-scale raceway pond

The highest lipid accumulation was achieved for 150 mg/L $NaNO_3$ at 35.12% per g DCW (Figure 3.10). The was followed by 27.13, 24.54, 22.89 % lipid/g DCW for 250 mg/L, 300 mg/L and 200 mg/L $NaNO_3$ respectively. The results were similar to that of earlier trials using 250 mg/L $NaNO_3$ (Figure 3.6) yield of 29.6% lipid/g DCW. Although 150 mg/L $NaNO_3$ showed maximum lipid production, 250 mg/L $NaNO_3$ showed slightly higher quantum yield and biomass production. From earlier experimentation, we also noted an increase in cyanobacteria over the cultivation period for lower nitrogen levels.

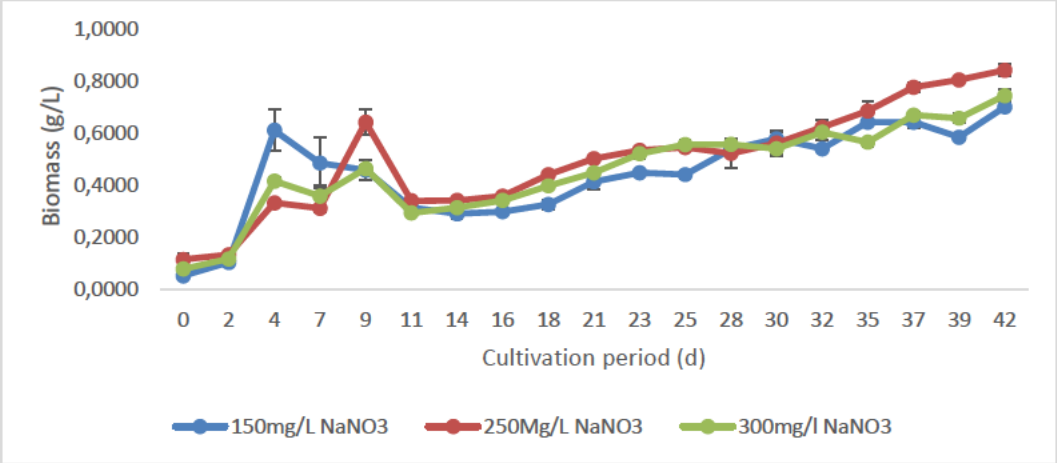

Figure 3.11. Growth curve of algae grown on BG11 medium with reduced nitrogen at 150, 250 and 300 mg/L $NaNO_3$ in a 3000 L open circular pond at Kingsburgh WWTP

The main objective of the experiment (Figure 3.11) was to determine growth rates in the open system as compared to the raceway ponds at the university as Kingsburgh is the site of the large-scale raceway pond as described in section 4.2.2. All nitrate concentrations showed initial rapid growth with maximum biomass of 0.61, 0.64, and 0.46 g/L for 150, 250, and 300 mg/L $NaNO_3$ on days 4, 9, and 9 respectively. On day 11, biomass yield fell sharply to just under 350mg/L on day

11. This was chiefly due to lower light intensities experienced from days 9 to 11 with an average intensity of 278.4 µmol/m^2/s as compared to an average intensity of 685.32 µmol/m^2/s for the first 7 days of cultivation. The culture showed steady biomass accumulation from day 11 to day 42. pH shock was implemented on day 32 due to the presence of grazers. Due to early intervention, the culture density was not significantly reduced. Overall productivities were 3.29, 3.51, and 3.22 g/m^2/day for150, 250, and 300 mg/L $NaNO_3$ trials respectively.

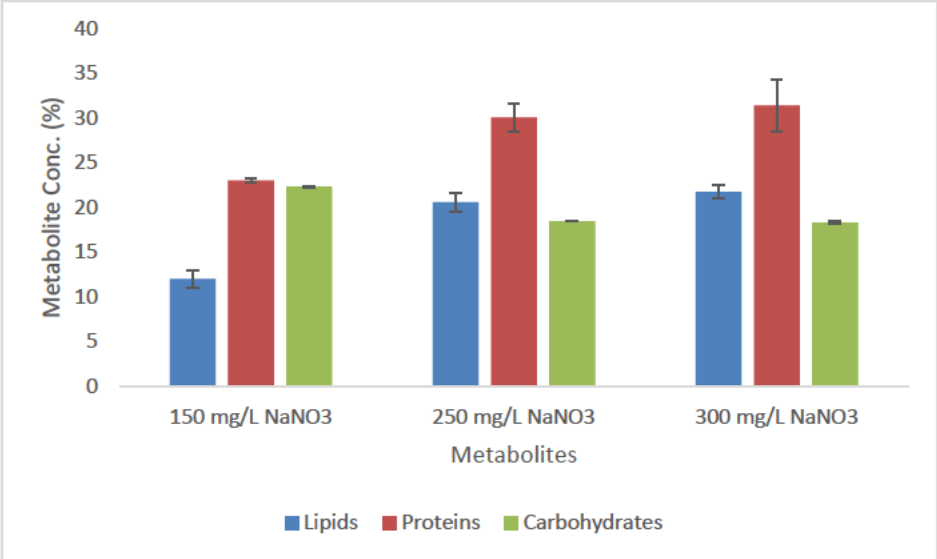

Figure 3.12. Metabolite production algal biomass cultivated in BG11 media with reduced nitrogen at 150, 250 and 300 mg/L $NaNO_3$ in a 3000 L open circular pond at Kingsburgh WWTP.

The highest lipid content obtained was for 300 mg/L $NaNO_3$ at 21.76 ±0.76 % followed by 250 mg/L $NaNO_3$ at 20.55±1.02 %. The lowest yield obtained was 12.14±0.98 % lipid at a concentration of 150mg/L $NaNO_3$ (Figure 3.12). This was lower than the yield obtained for the greenhouse trials as per Figure 3.4 and is likely due to lower temperatures averaging 19.86±2.3°C

as compared to 27.18±5.1°C. Light intensity for this trial was also lower than that recorded for the greenhouse trial at 638.10±236.15 µmol/m^2/s. The trial as per Figure 3.8 gave a more accurate representation of what could occur at large scale due to the location being at the same site and was therefore used as a reference point for further experimentation.

3.3.3 Replacement of $NaNO_3$ with Urea as a cost-effective N source

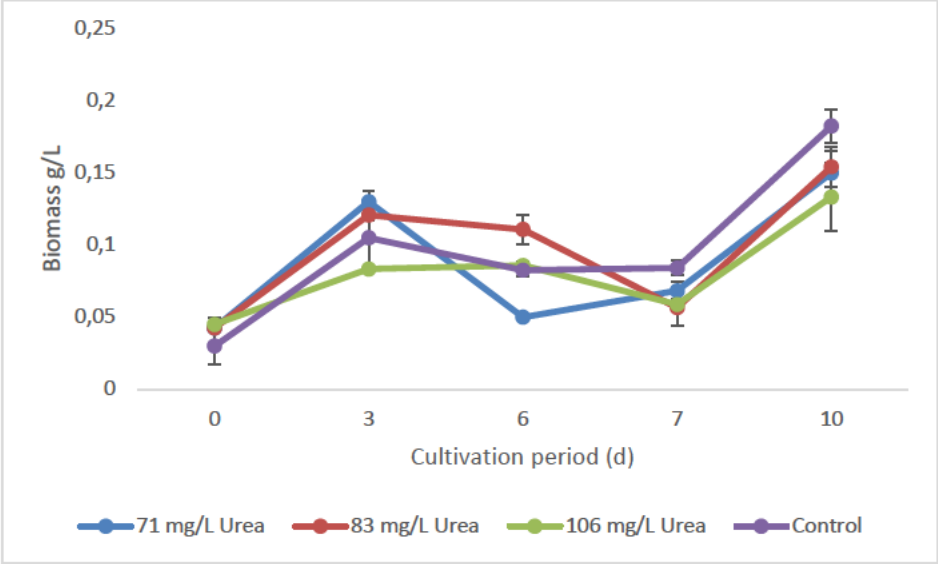

Figure 3.13. Growth curves of algal biomass cultivated in BG11 media with Urea as an N source in a pilot-scale raceway pond

Sodium nitrate constitutes the highest concentration of all the nutrients in the media utilised for algal cultivation. Urea contains a much higher stoichiometric ratio of N than sodium nitrate and costs ~50% less. The dissociation of urea to CO_2 and ammonium is beneficial in that it provides a carbon as well as a nitrogen source. (Ramanna et al., 2014). The higher ratio of N in Urea

effectively means that 71, 83, and 106 mg/L Urea is equivalent in N to 200, 250, and 300mg/L $NaNO_3$. The control used for the experiment was 250mg/L $NaNO_3$.

Growth of the culture increased rapidly from day 0 to 3 with 71 mg/L Urea reaching a maximum of 0.13 g/L for all urea concentrations in Figure 3.13. This was followed by a decline in biomass for the next 4 days and subsequent recovery of the culture. The control (250 mg/L $NaNO_3$) showed a marginal decline for 0.105 to 0.084 g/L during the same period. The reduction in biomass is likely due to overcast conditions and resultant the low light intensity of 182.5 µmol/m^2/s. The pH (Figure 3.14) of the culture is an important factor due to the volatilisation of ammonia which is toxic to the algae. The pH of the cultures using urea increased to 9.46 on day 3 which is likely to have volatilised ammonium in the media to ammonia resulting in inhibition of photosynthesis.

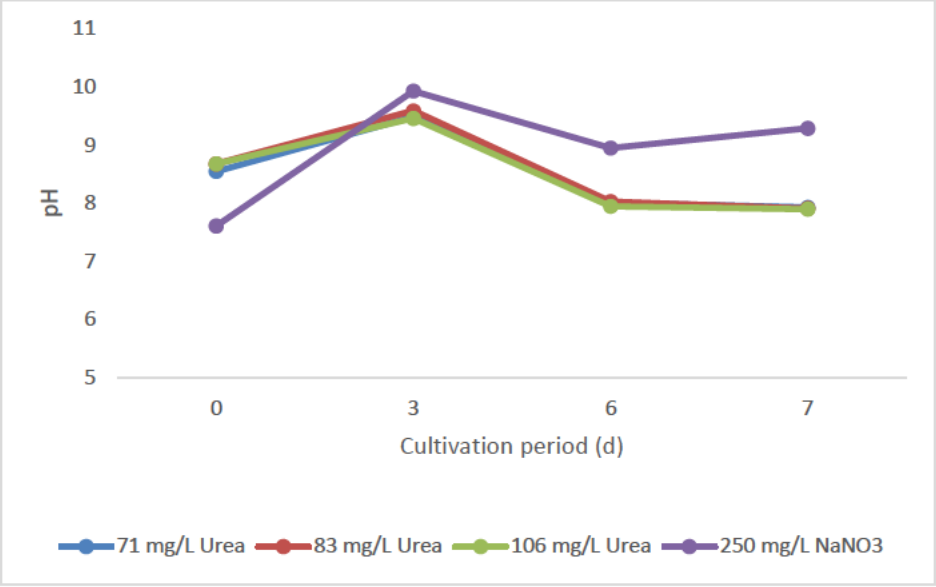

Figure 3.14. Change in pH during cultivation of algal biomass using urea as a source of nitrogen.

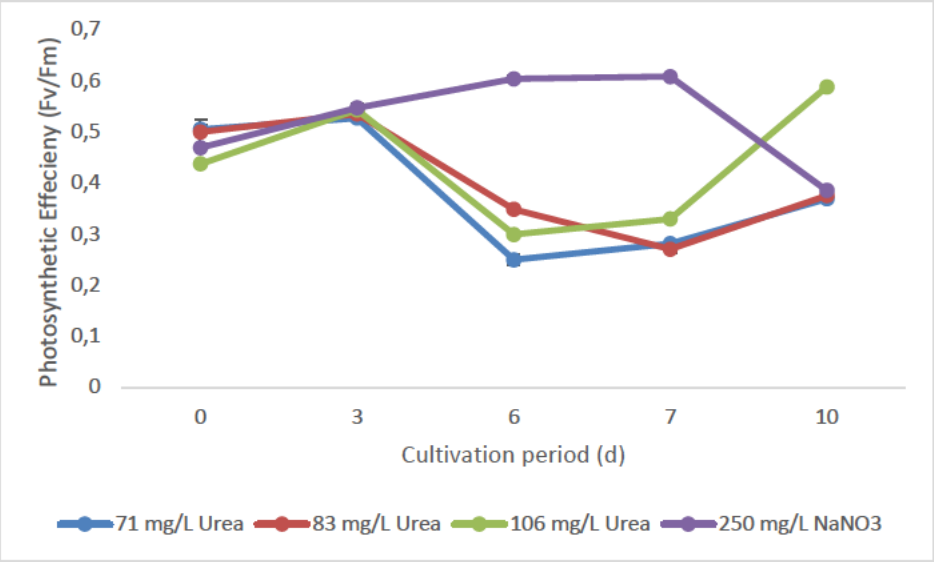

Figure 3.15. Quantum Yield (Fv/Fm) of algal biomass cultivated in BG11 using Urea as a source of nitrogen.

A marked decrease in the quantum yield of PSII is observed from days 4 to 7 (Figure 3.15) indicating that the culture undergoing physiological stress (Fv/Fm ≥ 0.5). This was not evident for the culture using $NaNO_3$ as a nitrogen source until day 7 thus reinforcing the postulation that ammonia inhibition leads to a decrease in quantum yield of PSII and thus culture decline. The decline in Fv/Fm for the NO_3-N trial is likely to be a function of adaptation to increasing light intensity from 182.5 µmol/m^2/s on day 6 to 603.3 µmol/m^2/s on day 7 (Table 3.1). Recovery of the culture was noted from days 7 to 10 which correlates with the highest amount of ammonium being present in the media. Ammonium is preferentially utilised by many algae as a primary N source due to its reduced nature. Breakdown of $NaNO_3$ is an enzymatic process that requires more energy.

Table 3.1. Light Intensity and N concentrations of the media for the duration of the urea trials

		71 mg/L Urea			83 mg/L Urea			106 mg/L Urea			250 mg/L $NaNO_3$		
Days	Light Intensity ($umol/m^2/s$)	NH_3-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	NH_3-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	NH_3-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)	NH_3-N (mg/L)	NO_3-N (mg/L)	NO_2-N (mg/L)
0	101.8	1.8	4	1	1.76	4	1	1.93	7	1	0.2	23	1
3	253.3	5.2	4	1	6.2	9	2	5.4	10	1	1.0	29	2
6	182.5	4.8	1	0	3.5	1	1	7.7	0	2	0.1	24	2
7	603.3	4.6	3	3	4.9	0	1	8.3	1	1	0.6	19	3
10	585.3	0.2	6	1	0.8	8	1	4.1	8	3	0.0	19	4

Nutrient levels for the trial were very low as per Table 3.1. despite the N levels in urea being equivalent to that of 250mg/L $NaNO_3$, N concentrations were considerably lower than expected. NH_3- N peaked at a maximum of 8.3 on day 7 for the 106 mg/L Urea trial. The reason for the low N levels could be due to the high pH which was observed in all the ponds on day 3 due to carbonic acid utilisation as per Figure 3.14. A further possibility for low nitrogen is the low dissociation of urea in water. The dissociation of urea to ammonium and CO_2 is usually enzymatic by the production of urease by the algae.

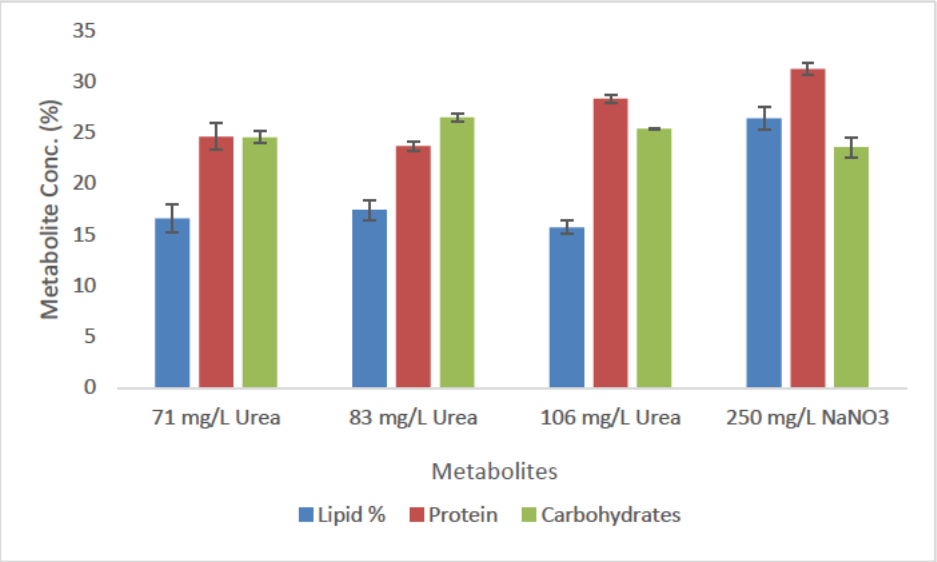

Figure 3.16. Metabolite production of algal biomass cultivated in BG11 media using Urea as an N source in a pilot-scale raceway pond

The highest lipid yield achieved was 26.4% for the control (Figure 3.16). The corresponding N equivalent in urea produced only 17.4% Lipid. Protein yield was similar for all the urea concentrations ranging from 24.6 to 28.3% but lower than $NaNO_3$ control at 31.2%. A maximum carbohydrate yield of 26.5% was observed for 83 mg/L urea. Carbohydrate yields for all the urea trials amongst the highest produced for the optimisation experiments conducted (Figure 3.6; Figure 3.12 and Figure 3.16). A trend of higher carbohydrate production has been noted for cultures grown under severe nutrient stress. Similar results were achieved by another study whereby a significant increase in total lipid and sugar were observed for *Scenedesmus obliquus* culture after nutritional stress. There was an increase in lipid content from 8% up to 25% (w/w). This alga has proved to be a good source of single-cell oil for biodiesel production (Batista et al., 2015)

3.3.4 Characterisation of post-chlorinated wastewater

The wastewater treatment system at works is divided into the east and west plants. The east plant is an extended aeration system and the west plant is a modified UCT process. Final effluent for data was recorded for the east plant as it is in the vicinity of the raceway pond thus reducing the distance required to pump the effluent. Wastewater characteristics were measured for 9 months to ascertain typical levels. Average data is represented in Figure 3.17 and Figure 3.18 where n=19, thereby giving rise to the large standard deviations. pH data was stable as expected with average readings between 6.8 and 7.4. The lower pH levels were noted for the winter period as was expected due to lower temperatures. Levels of total suspended solids (TSS) ranged from 1.2. to 6.1 mg/l with the highest TSS being noted for May.

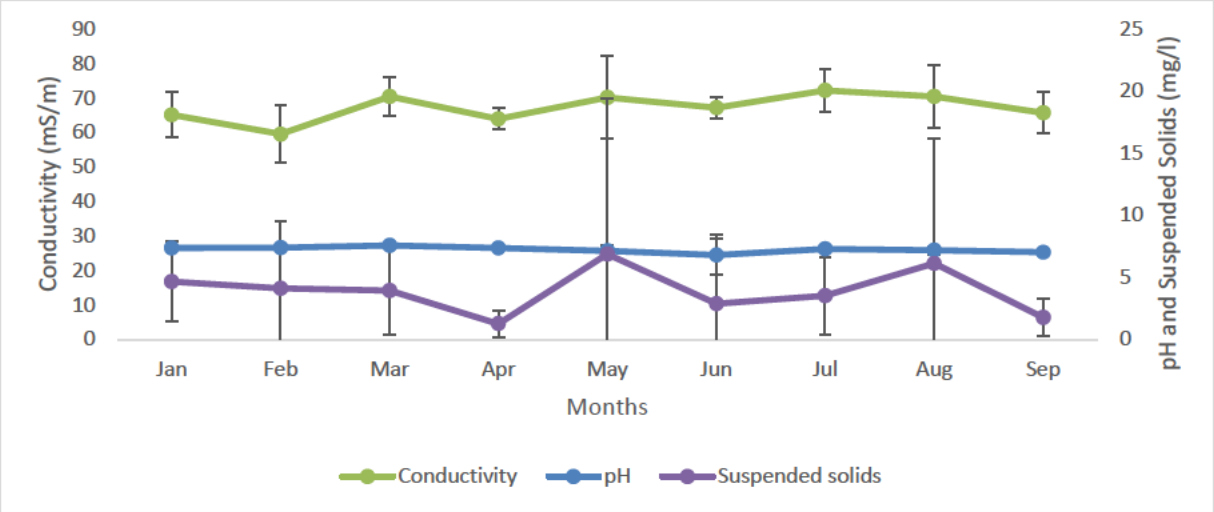

Figure 3.17. Average physical characteristics of post-chlorinated wastewater for 9 months from mid-summer to early spring (stdev n=19)

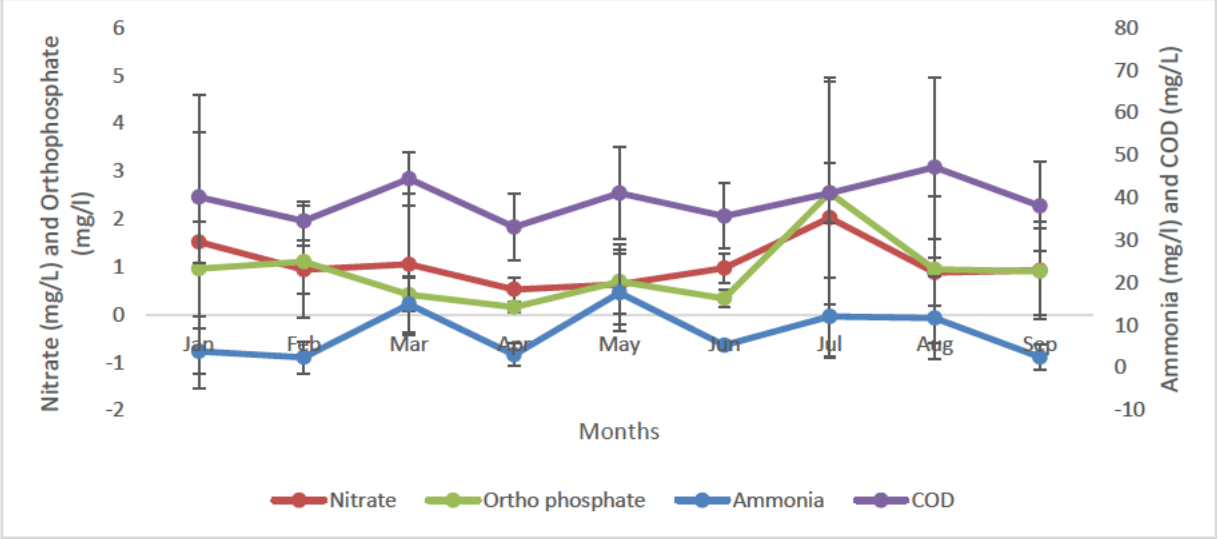

Figure 3.18. Average chemical characteristics of post-chlorinated wastewater for 9 months from mid-summer to early spring (stdev n=19)

The consistently low levels of suspended solids mean the effluent was very clear making it a good water source for algal cultivation as it does not impede penetration of light. Conductivity ranged from 59.4 to 72.1 mS/m for the full period.

Nitrate and ortho-Phosphate levels remained below 1mg/l consistently for 6 of the 9 months monitored. Maximum concentrations of 2.0 and 2.55 mg/l for observed for Nitrogen and Phosphorus respectively. These small ranges indicate that the treatment plant was performing consistently well. Ammonia was the most variable with concentrations ranging from 2.49 to 17.7 mg/L. COD concentrations varied between 33 and 47 mg/l. These levels are expected in domestic wastewater effluent and are usually recalcitrant materials that are non-biodegradable (Pizzera et al., 2019). Biodegradable COD if present adds the potential of mixotrophic growth, which could improve biomass and lipids production.

Metals concentrations of the wastewater were determined for a period of 6 weeks from April to June. All the metals are within acceptable limits for domestic wastewater as per Table 3.2. Iron

and Magnesium concentrations averaged 3.6±0.46 mg/L and 11.3±2.15 respectively for the period tested. All the metals detected are micronutrients or are required in trace concentration for the production of algal biomass. Magnesium, iron, manganese, and copper are particularly important as they play roles in photosynthesis, energy transfer, cell division, respiration, protein, and lipid synthesis (Singh et al., 2016).

Table 3.2. Concentrations of selected metals in post-chlorinated wastewater from Kingsburgh WWTP for 6 weeks from April to June 2018.

Month	Week	Co	Mg	Mo	Al	Fe	K	Zn	Mn	Cu
April	1	3,35	8,65	0,15	1,35	2,65	17,00	0,30	0,20	0,85
	2	0,00	9,15	0,10	1,50	2,60	15,65	0,55	0,20	0,75
	3	1,30	15,65	0,05	1,20	2,85	35,50	0,39	0,25	0,08
	4	0,00	9,50	0,05	2,35	4,05	16,35	0,30	0,20	1,15
May	1	2,30	13,45	0,05	1,70	3,65	30,00	0,40	0,20	0,90
	2	4,85	11,35	0,00	2,10	2,90	19,65	0,25	0,30	0,95
	3	2,10	12,60	0,00	1,75	3,05	7,40	0,35	0,10	1,00
	4	0,45	11,40	0,00	1,75	2,80	15,65	0,15	0,10	1,15
June	1	2,25	11,20	0,00	1,35	2,80	14,50	0,25	0,15	1,20
	2	0,90	10,05	0,00	2,35	3,25	16,95	0,20	0,15	1,20
Average		1,75±1,55	11,3±2,15	0,04±0.05	1,74±0,41	3,06±0,47	18,87±8,07	0,31±0,11	0,19±0,06	0,92±0,33

3.3.5 Cultivation of algae using post-chlorinated wastewater

Post chlorinated wastewater was chosen as a water source due to the reduced bacterial load which is known to negatively impact microalgae growth. The concentration of macronutrients N and P in the wastewater was too low to support sustained algal growth (Figure 3.18) thus necessitating supplementation of the wastewater. Modified BG11 (mBG11) using PCW as a water source was compared to standard BG11 using freshwater and PCW supplemented with N and P

as per mBG11 and PCW supplemented with 250 mg/L $NaNO_3$ optimised as per section 3.3.2 was compared.

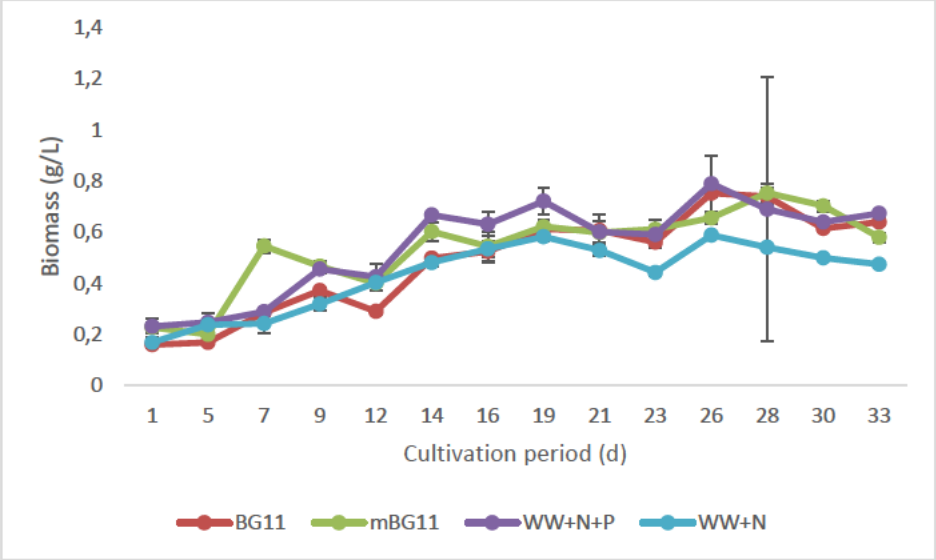

Figure 3.19. Algal biomass cultivated on media variations using post-chlorinated wastewater as a water source where BG11 is BG11 media components freshwater as a water source; MBG11 is modified BG11 with $NaNO_3$ reduced to 250 mg/L and PCW waster source; WW+N+P is PCW with the addition of 250 mg/L $NaNO_3$ and 40 mg/L KH_2PO_4; WW+N is PCW with the addition of 250 mg/L $NaNO_3$.

Similar growth patterns were observed for all the treatments. WW+N+P gave the highest biomass productivity of 7.89 $g/m^2/d$ on day 14 at a biomass concentration of 0.66 g/L (Figure 3.19). Biomass of 0.565 g/L was observed for mBG11 on day 7 before a decline and subsequent peak at day 14 with a maximum productivity of 6.41 $g/m^2/d$. Biomass productivities of BG11 and WW+N were 6.17 and 5.80 $g/m^2/d$ on respectively on day 14. WW+N+P is likely to have performed better than the BG11 due to the presence of micronutrients in the WW. BG11, mBG11, and WW+N+P showed very similar growth trends for the period of cultivation with growth on WW+N dropping slightly from day 19.

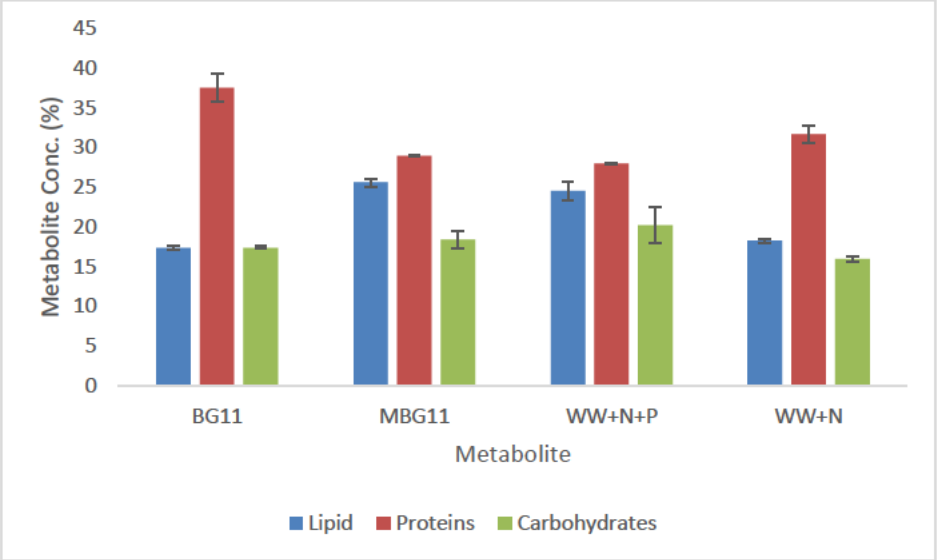

Figure 3.20. Metabolite production of algal biomass cultivated on media variations using post-chlorinated wastewater as a water source where BG11 is BG11 media components freshwater as a water source; MBG11 is modified BG11 with $NaNO_3$ reduced to 250 mg/L and PCW waster source; WW+N+P is PCW with the addition of 250 mg/L $NaNO_3$ and 40 mg/L KH_2PO_4; WW+N is PCW with the addition of 250 mg/L $NaNO_3$.

The quantum yield of PSII of all the treatments was above 0.55 indicating that the cultures were not highly stressed. There was however a change in metabolite concentration from BG11 containing high nitrogen as compared to the lower nitrogen-containing media. Lipid content for mBG11 and WW+N+P was observed to be 25.6 and 24.5% Lipid/g DCW respectively (Figure 3.20). There was no significant difference ($p>0.05$) between the results obtained. BG11 and WW+N achieved lipid yields of 17.40 and 18.29 % Lipid/g/DCW respectively. The lipid content achieved by WW+N was similar to that of BG11 but lower than WW+N+P and mBG11. Protein content was observed to be lower for all treatments containing lower N however none of these

showed a significant decrease. Based on the similar growth rates and production of similar amounts of lipid and carbohydrates, WW+N+P is a viable medium for algal cultivation.

3.4 Discussion

3.4.1 Strain selection

The selection of an appropriate strain is paramount to the successful cultivation of algae at a large-scale. The selected strains need to be robust with the ability to adapt to uncontrollable conditions such as ever-changing temperature and diurnal cycles and invading organisms (Jebali et al., 2018b). *Scenedesmus obliquus* has in recent times become one of the most promising microalgae for biofuels production (Pagnussat et al., 2020). *Scenedesmus* sp. is considered a high protein algal species typically containing 50-56% protein, 10-17% carbohydrate, and 12-14% lipid with an amino acid profile in accordance with human protein requirements (Raeisossadati et al., 2020). The species is a versatile organism that finds application in wastewater treatment, feed applications, energy production and can be grown in raceway ponds (Raeisossadati et al., 2020). The estimated cost of biomass production is US$7.52 /kg. Sánchez Zurano et al., (2020) determined Scenedesmus sp. to be highly resilient to variation in light and temperature making it a good candidate for outdoor cultivation.

Chlorella sp. has attracted much interest in large-scale cultivation for feed purposes as it is a fast grower, relatively easy to grow, and produces high amounts of bioactive compounds (Belanger-Lepine et al., 2018; Chew et al., 2018). Despite *Chlorella* sp. entering the system during the course of cultivation, it is not considered a contaminant due to having the desired attributes of being robust and producing appreciable amounts of lipids.

Chlorella and *Scenedesmus* spp. are commonplace in the remediation of wastewater. This is in part due to their robustness to variable conditions and ability to grow in environments with high nutrient concentrations. In a study by Jebali et al., (2018a), they concluded that the native Scenedesmus culture chosen for cultivation in their open pond system was a good candidate for outdoor cultivation.

3.4.2 Nitrogen utilisation

Algae require nitrogen equivalent to about 1-10% of the dry cell weight. The phosphorus requirement is usually much smaller at less than 1% of the cell (Eze et al., 2018). Ammonium is the preferred substrate for algal growth due to its reduced nature that allows assimilation with lower energy requirements. Challenges associated with N supplied in the form of ammonium are the toxicity of free ammonia at high concentration and loss of N due to volatilisation (Eze et al., 2018). High concentrations of ammoniacal nitrogen, typically above 100 mg/L, are usually toxic to algae. *Chlorella* sp., however, are resistant to high nutrient toxicity and are able to grow well above this level (Jebali et al., 2018b).

Zhang & Ogden, (2019) observed that nitrogen supplied in the medium when culturing *Chlorella Sorokiniana* was taken up rapidly within the first few days of cultivation as postulated that this was a luxury update for future use. They found that maximum biomass production was obtained at 455 mg/L $NaNO_3$ with no significant increase in biomass above this level under controlled conditions at laboratory scale. *Chlorella* is known to be a higher nutrient requiring species therefore higher levels of nitrate are expected to be optimal for biomass production. Similar results were achieved as denoted by Figure 3.5 (A) and Figure 3.9 where initial uptake occurred at a rapid rate before levelling off or decreasing.

Eze et al., (2018) found an increase in nitrate from 1.8 mg/L at the start of cultivation to 40.5 mg/L on day 16 when *Desmodesmus* sp. was cultivated on wastewater and attributed the increase in nitrate to the active oxidation of ammonium by nitrifying bacteria. Nitrification is strongly affected by dissolved oxygen concentration in that higher DO has been shown to enhance nitrification (Jia & Yuan, 2018).

Similar to the results in our study, Jebali et al., (2018a) noted an increase in the final nitrate concentration as compared to the initial. This is suggested due to nitrification taking place. The presence of nitrifiers in raceway ponds is common. Ammonia Oxidising Bacteria (AOBs) have been shown to be more tolerant of higher levels of free ammonia than Nitrate Oxidising Bacteria (NOBs) (Pizzera et al., 2019). Ramirez-Lopez et al., (2019) observed an increase in NO_3 concentration in a raceway pond cultivating *Chlorella vulgaris* UTEX 26 in the latter stages of semi-continuous cultivation due to the presence of *Nitrosomonas* and *Nitrobacter* sp. in the system which competed for the ammonia supplied as an N source. An increase in *Nitrosomonas* sp. was also noted by Galès et al., (2019) in high-rate algal ponds who asserted that proliferation is likely due to high oxygen concentrations due to microalgal growth. In their study, an increase in NO_2 and NO_3 concentrations correlated with the increase in *Nitrosomonas.* Proteobacteria include bacteria such as ammonia oxidising bacteria, nitrite oxidising bacteria, and denitrifiers (Jia & Yuan, 2018). Environmental conditions including temperature and rainfall were not shown to significantly affect nitrifiers in the raceway pond in a study conducted by Pizzera et al. However, Mohsenpour et al., (2021) stated that nitrifying populations showed increased activity at very low light intensity resulting in the increased concentration of NO_2 and NO_3. The fluctuation in ambient light, as well as high culture density, could have lead to increased nitrification as increases in NO_2 and NO_3 are noted throughout our study (Figure 3.5; Figure 3.9; Figure 5.4).

Optimal nutrient content uptake of culture can differ considerably using the same medium in indoor and outdoor conditions. Jebali et al., (2018a) showed that the optimal nutrient concentration under outdoor conditions for *Scenedesmus* sp. cultivation was half that of the optimal concentration under indoor conditions. An increase in N concentrations over time could also result in hydrolysis of dead biomass (Pizzera et al., 2019).

3.4.3 Mechanism of lipids accumulation

Optimisation of growth and lipid yield is essential to the economic viability of the production of biodiesel from microalgae. The role of lipids in the growth of microalgae is as energy reserves and part of the structural components of the cell. Phospholipids and glycolipids are the primary components of cell wall structures and determine the fluidity of membranes under various conditions. This is achieved by being able to adapt quickly to changes in the environment by recycling lipids and *de novo* synthesis. A large proportion of phosphate is present in the cell wall (Williams & Laurens, 2010). Lipid accumulation under standard growth conditions is usually very low. Lipid accumulation occurs naturally as a mechanism for energy storage during unfavourable conditions (Li et al., 2011). Under stressed conditions, many microalgae alter their biosynthetic pathways to produce neutral lipids (Li et al., 2011). Nitrogen limitation or depletion is one of the most effective methods used for the induction or increase of lipid content in microalgae (Brennan & Owende, 2010; Singh et al., 2016). Upon reaching nutrient-limited status, carbon is assimilated into cells but cell proliferation does not occur (Meng et al., 2009). This carbon is converted to TAGs or carbohydrates within existing cells depending on species (Brennan & Owende, 2010). Triacylglycerols (TAGs) are the primary storage components as energy reserves (Mairet et al., 2011; Singh & Gu, 2010). The greater proportion of the lipids produced is TAGs which are produced as metabolic rates of microalgae slow (Williams & Laurens, 2010). This was evident

from our pilot-scale trials where 150 mg/L $NaNO_3$ under suitable conditions produced the highest lipid content

Changes in cultural conditions may be used as a mechanism for the manipulation of metabolic pathways resulting in the redirection of cellular function to the production of desired products such as neutral lipid (Singh et al., 2016). This method of metabolic manipulation is preferred over mutagenesis and the production of transgenic strains due to problems with the stability of transformants and the potential impact on environmental security, especially for large scale commercial applications. It is important to determine the trade-off between neutral lipid production and algal growth as part of the optimisation for biodiesel production (Pagnussat et al., 2020). Nitrogen limitation has variable effects on different types of microalgae in terms of growth and cellular content (Illman et al., 2000). Amounts of lipid accumulation may be variable depending on the amount of nitrogen available (Ge et al., 2011). Achieving this trade-off under large scale conditions may become increasingly difficult as the culture reaches stability and synergistic interactions in the form of bacterial nutrient cycling occur must be taken into account. Selection of microalgae that can tolerate high nutrient concentrations may be a more viable option for cultivation as it can afford the advantage of limiting contamination by weedy species.

3.4.4 Cultivation of algae using wastewater as a water source

Over the past decade, there is a multitude of literature based on the utilisation of algal systems for the treatment and remediation of wastewater. All types of wastewater from domestic to high strength agricultural wastewater, including industrial wastewater, anaerobic digestate, and centrate have in some form or the other been investigated (Belanger-Lepine et al., 2018; Bohutskyi et al., 2018; Eze et al., 2018; Iasimone et al., 2018; Sutherland et al., 2017). Many of

these studies use favour the use of native algae for wastewater treatment (Hong et al., 2017; Sutherland et al., 2017). It is however to determine the main aim of the research in order to select the correct stream of wastewater to suit the specific requirement. Treatment of wastewater will provide algal biomass as a byproduct from which benefit can be gained. Should the objective be the cultivation of algal biomass, treated wastewater such as used in this study is a more viable alternative as it has reduced bacterial load, is usually clear thus not presenting light limitation, and is usually not nutrient-rich, so nutrient levels can be controlled by supplementation to appropriate levels. Our study demonstrated that treated wastewater was able to sustain algal biomass and required N supplementation to achieve similar results to nutrient media (Figure 3.19; Figure 3.20) productivity levels equivalent to other studies using raceway ponds in excess of 500 m^2 (Table 4.3) P concentrations in the wastewater were found to be sufficient for the growth of the algae, however, biomass and metabolite production was lower. Algae have the ability to regulate their internal N/P ratio to compensate for environmental conditions and therefore grow at a wider range of N/P ratio as compared to the Redfield ratio (Pizzera et al., 2019).

3.5 Conclusions

The propagation of a specific type or types of algae towards the production of a product such as lipids requires the determination of the condition to obtain optimal biomass productivity during scale-up. *Scenedesmus obliquus* was utilized as the strain of choice on the basis of its robustness and production of suitable quantities of lipids. Optimisation of nitrate, being the largest cost factor, was carried out in 3000 L ponds giving the highest biomass productivity and producing lipids in excess of 29% lipid/g DCW at 250 mg/L $NaNO_3$. Further reduction in nitrate resulted in higher lipid but lower biomass productivity. Optimized results were applied and tested in circular ponds at the test site in circular ponds producing similar results. Replacement of nitrate with urea

as a more cost-effective N source resulted in similar lipid levels but proved toxic to the algae and greatly reduced biomass production. Post chlorinated wastewater was found to support the growth of the algae but supplementation due to consistently low levels. Supplementation of post chlorinated wastewater with N and P provided similar results to conventional media.

CHAPTER 4: LARGE SCALE CULTIVATION OF MICROALGAE USING A RACEWAY POND

4.1 Introduction

In order to test the feasibility of algal cultivation, it is essential to learn all the factors which affect such a system. Laboratory and small pilot-scale systems give an indication of the growth rates of the lipid production of a species of algae but are do not experience many of the challenges of larger systems. These include prevailing climatic conditions (light intensity, photoperiod, temperature, nutrient concentration, O_2 saturation, carbon availability, pH, salinity, undesirable chemicals; operational factors such as mixing, depth and frequency of harvest., issues of contamination, mechanical failures, and other events. Biomass productivities are highly dependent on the inputs, several of which cannot be controlled and fluctuate constantly (Campbell et al., 2011). The water depth of raceway ponds with water depths also affects biomass productivity (Schenk et al., 2008). Microalgal productivities range from 5 to 50 g DCW/m^2/day in open pond systems as reported by several researchers (Demirbas & Demirbas, 2011; Park et al., 2011; Sheehan et al., 1998; Zamalloa et al., 2011). This chapter focuses on the cultivation of algae at large scale and elucidates the cultivation of algae as well as some of the challenges.

4.2 Materials and Methods

4.2.1 Analysis and data generation

Analytical data and measurements were performed as per sections 3.2.1-3.2.7 of the previous chapter.

4.2.2 The Raceway Pond

The large-scale raceway pond is situated at Kingsburgh Wastewater Treatment plant (Figure 4.2) was an existing raceway shaped oxidation ditch that was retrofitted for our purposes. The depth was reduced to 0.6m using a G5 sand backfill and a 1500 micron white plastic liner was fitted to provide the pond surface. A paddlewheel driven by a 2 x 1.5 kW was constructed to provide mixing. The paddlewheel which was constructed from stainless steel (Figure 4.4) was later divided into 2 and a second motor was fitted. Raceway pond dimensions as per Figure 4.1 below. The pond has an area of ~ 1146 m^2 and a working volume of 343 m^3 at 30 cm depth. The raceway pond sits between 2 sets of aerators on the wastewater treatment plant and was thus covered using corrugated polycarbonate sheeting (Figure 4.3) to reduce the level of contamination by grazers such as amoeba and protozoa which are known to decimate algal cultures within short periods of time.

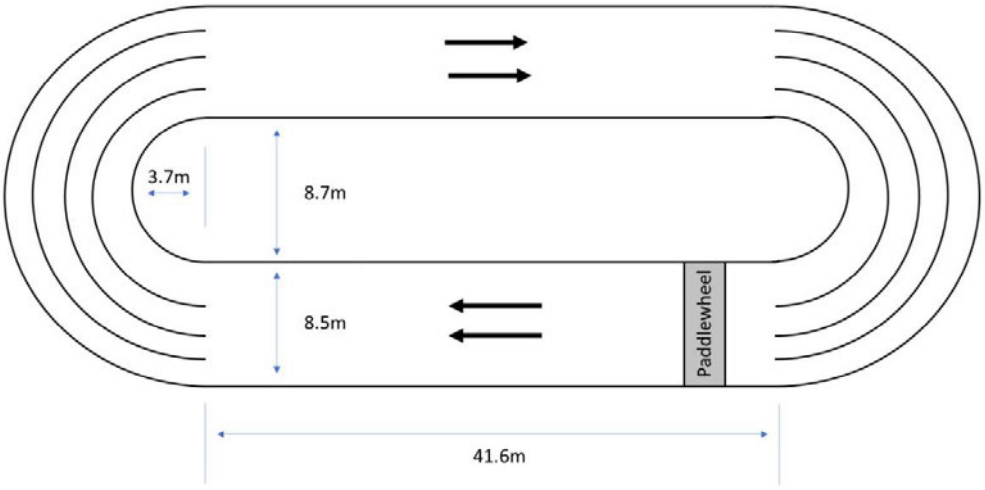

Figure 4.1. Schematic representation of the raceway pond at Kingsburgh Wastewater Treatment plant.

Figure 4.2. Picture of the raceway pond at Kingsburgh wastewater treatment plant

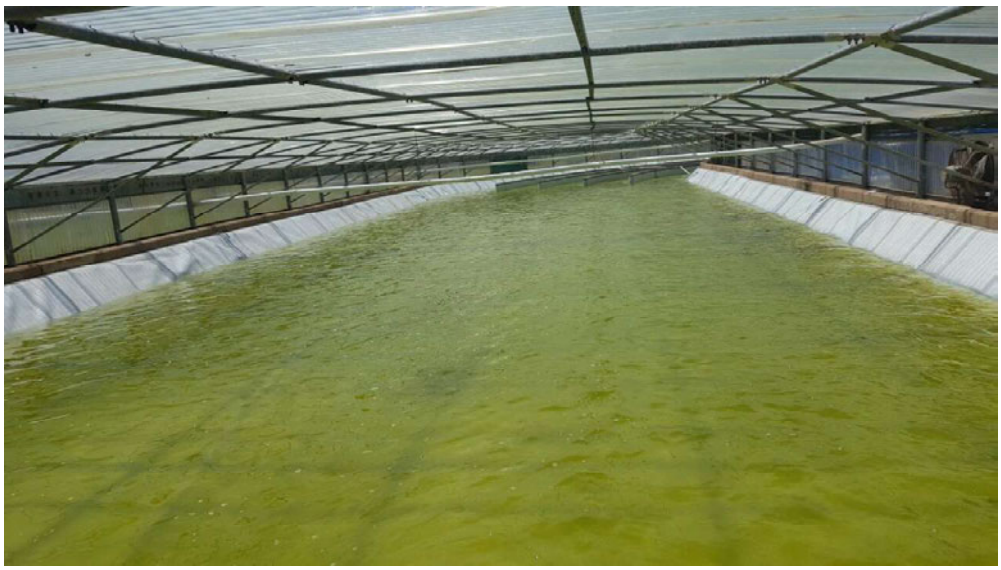

Figure 4.3. Internal section of the raceway pond showing polycarbonate cover

Figure 4.4. Paddlewheel in the raceway pond for mixing of the algal culture and baffles in the pond to alleviate dead spots around the circular sections of the pond.

4.2.3 Seed culture development

In order for cultivation at 300 m^3 scale to be effective, it is imperative to utilize an inoculum of ~10% of the total working volume of the pond. This limits contamination by weedy species of algae to some degree and provides a large enough inoculum for the desired culture to proliferate. Seed culture was developed stepwise from 500L culture from pilot-scale raceway ponds at DUT. Seed culture was generated in 7 x 3000L circular ponds, as per section 3.2.8.2, placed on the island of the raceway (Figure 4.2). The raceway pond was seeded with the culture at ~ $1x10^6$ cells/mL.

4.2.4 **Raceway pond cultivation**

A number of trials were conducted in the raceway pond over a period of 3 Years. For the purposes of this study, only data from 2018 and 2019 are being considered due to the challenges experienced with prior runs which will also be elucidated in this chapter. Due to the size of the pond and uneven mixing (4.4.10.3), composite samples were taken from 7 sampling sites with three samples per site and were mixed to give a more accurate reflection of the biomass yield. A circular pond, designated with pond # to match raceway #, at a depth of 0.3 m was run in parallel to each of the raceway runs. The culture for these ponds was pumped from the raceway after nutrients were dissolved to ensure equivalent inoculum and levels of nutrients

4.2.4.1 Raceway Runs

The raceway runs were initiated with nutrient supplemented PCW to determine growth rates and patterns. For ease of reference, each of the raceway results were designated with the respective number of each run as listed below (e.g. RW4 corresponds to Run4). The runs were done in reasonable successive taking into account time for cleaning of the system, repairs, and vacation periods. Differences in terms of season and operation changes were as follows:

Run 4: The raceway pond was run from mid-winter to very early spring (25 July- 3 September 2018).

Run 5: The raceway pond was run during mid-spring (1 to 31 October 2018). Evaporative losses were not made up during the cultivation period.

Run 6: The raceway pond was run mid to late summer (25 January to 25 February 2019). During the cultivation period, rolling blackouts were instituted in South Africa.

Run 7: The raceway pond run was started in autumn (15 April 2019).

Run 8: The raceway pond was run in early winter (6-20 May 2019). Evaporative losses were not made up after day 7 to provide a dense culture for harvesting.

Run 9: The raceway pond was run in mid-winter (24 June-24 July 2019).

Run 10: The raceway pond was run in Spring (8 August- 9 September 2019). Evaporative losses were not made up to determine potential for higher productivity.

4.2.5 Modelling

Inputs optimization and models development

The Adaptive Neuro-Fuzzy Inference System (ANFIS) model is a type of ANN, based on implementing the Takagi–Sugeno (TS) fuzzy approach, as shown in Figure 4.5. ANFIS implements fuzzy logic (FL) in the framework of ANN (Abunama et al., 2018). The development process of ANFIS modelling involves identifying the most relevant inputs that correlated to a targeted output. The defining the optimum rules, types, and numbers of the associated membership functions (MFs), aiming at selecting the optimum ANFIS model structure with the lowest modelling errors. As an example, two TS fuzzy sets of "if-then" rules in a typical ANFIS structure as following:

- Rule 1: If x_1 is A_1 and x_2 is B_1; then $f_1 = p_1x_1 + q_1x_2 + r_1$
- Rule 2: If x_1 is A_2 and x_2 is B_2; then $f_2 = p_2x_1 + q_2x_2 + r_2$

Where, p_1; q_1; p_2, *and* q_2 are ANFIS parameters, while *Ai* and *Bj* are the linguistic labels or grade (Ying, 1998). According to (Ying, 1998), ANFIS architecture consists of five layers (Figure 4.5), and a brief description of the role of these layers are described as follows:

- Layer 1 or fuzzification layer, receives the input values and identifies the associated MFs.
- Layer 2 or rule layer, generates the firing strengths for the rules.
- Layer 3 or normalization layer, normalizes the computed strengths.
- Layer 4, receives the normalized values and the consequence parameter sets.
- Layer 5 or defuzzification layer, returns the values to the final output.

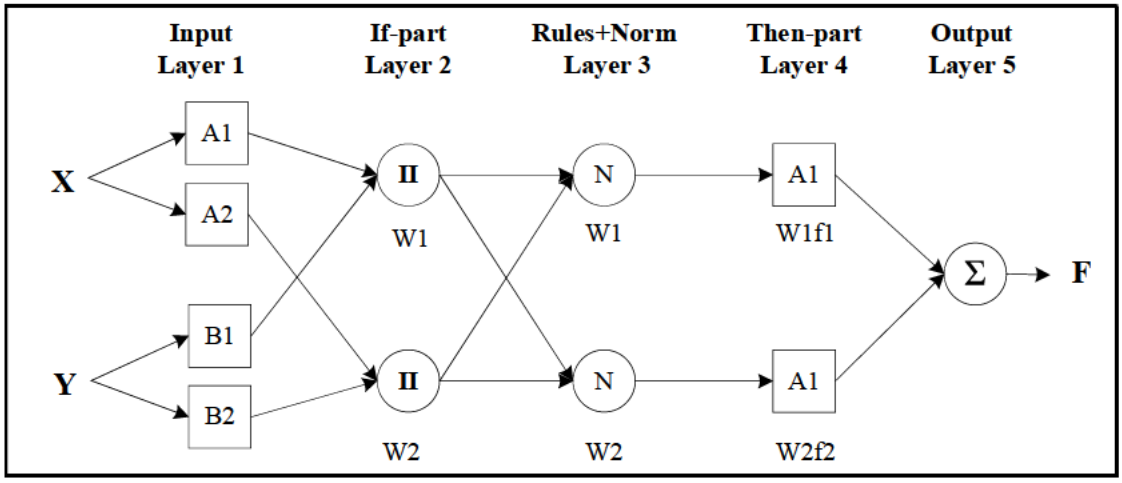

Figure 4.5. A typical ANFIS architecture, adopted from (Tang, 1993).

In this study, ANFIS edit toolbox and coding in MATLAB 2019b environment was used to train and develop the proposed models. As mentioned earlier, seven input variables and two output were used in the inputs selection and modelling process. All selected inputs parameters were related to targeted biomass productivity values. The training phase was conducted using the odd records, while the even records were used in the checking or testing phase. ANFIS learning process is repeated many epochs aiming at minimizing the yielded errors between the observed actual values and the output of the ANFIS model.

Models validation

The obtained results from the model were evaluated using numerous statistical checks. R squared (R^2) is used to evaluate the relationship between observed values and predicted values. The equation for calculating R^2 is denoted as Equation 7 as follows.

$$R^2 = \left(\frac{\sum_{i=1}^{n} (t_i - \bar{t})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(t_i - \bar{t})^2 \sum_{i=1}^{n}(y_i - \bar{y})^2}}\right)^2 \quad \text{Equation 7}$$

where n, t, and y are the number of observed data, observed values, and predicted values, respectively. Whereas, $\bar{t}$ and $\bar{y}$ are the average of observed and predicted values.

The range of R^2 values lies between zero and 1, with 1 as the highest accurate relationship possible. However, the values of R^2 greater than 0.7 are considered highly reliable in engineering models. In addition to R^2, percentage relative error (RE%) was used to assess the accuracy of the developed models. Equation 8 shows the mathematical expressions for the calculations of RE%.

$$RE\% = \left(\frac{(t_i - y_i)}{t_i}\right).100 \qquad \text{Equation 8}$$

The flowchart of data pre-processing, inputs optimization, and ANFIS structure development is presented in Figure 4.6:

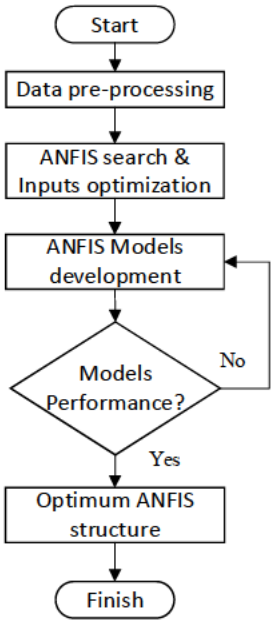

Figure 4.6. Flowchart of the modelling methodology.

4.3 **Results**

Growth patterns of the raceway ponds and circular ponds were very similar for the first 9 days of cultivation (Figure 4.7). Biomass productivities however differed with rates of 3.82; 5.87 and 4.38 g/m^2/d being achieved in the RW4, circular P4, and Control 4 respectively (Table 4.1). The biomass production in the raceway trended downwards from days 12 to 16 before increasing from day 16 to 28.

4.3.1 Raceway Run 4

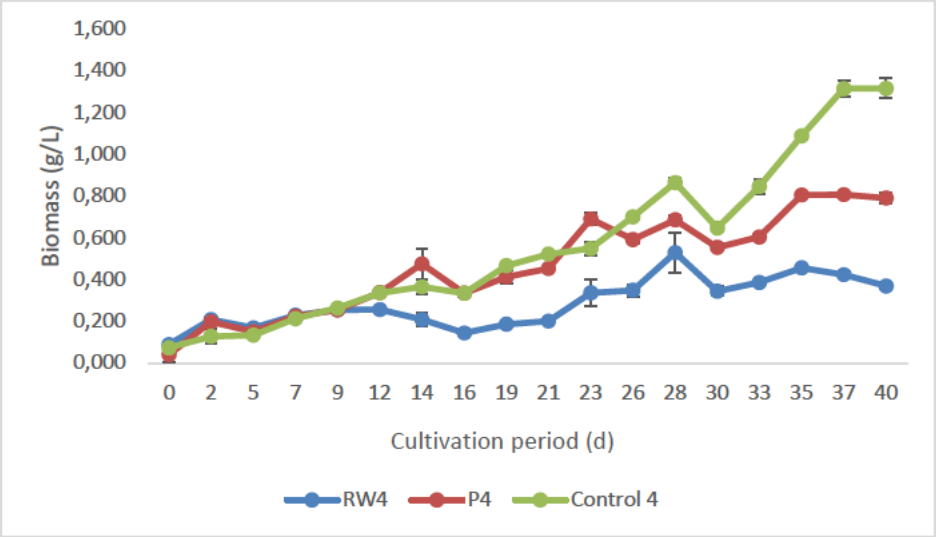

Figure 4.7. Cultivation of algal biomass in the raceway pond RW4 and P4 using mBG11 media and Control 4 from mid to late winter.

P4 had a similar growth pattern to the raceway albeit with higher biomass production. Control 4 showed a steady increase in biomass production until day 28 followed by a further increase in growth rate from days 30 to 37. Biomass levels achieved were relatively low due to lower temperatures during winter. Although the winters in Durban are mild, an average water temperature of 22.05±1.58°C was recorded for the cultivation period. *Scenedesmus* sp. prefer temperatures closer to 30°C for their optimum growth. It must be noted that the culture in the raceway was a polyculture with *Scenedesmus* being the dominant strain at more than 96.81% of the total algal community.

Table 4.1. Biomass productivity of algal biomass cultivated in the raceway pond (run 4) and outdoor circular ponds at Kingsburgh WWTP

	Biomass Productivity g/L/d	Biomass Productivity $g/m^2/d$	Average Water Temperature (◦C)	Average Ambient Temperature (◦C)	Average Light Intensity
RW4	0.00961	2.883	22.0525±1.58	21.77±2.10	359±212.70
P4	0.020494	6.148125	18.50±2.07	21.77±2.10	528.93±274.75
Control 4	0.036959	11.08781	18.23±1.94	21.77±2.10	528.93±274.75

Light is one of the major limiting factors to algal cultivation. Figure 4.8 shows the relationship between light intensity and biomass production. The photoperiod during the cultivation period was approximately 10:14 light to dark at the start of the cultivation period and shifted to ~11.5:12:5 light to dark cycle by the end of the run. This shorter photoperiod could potentially be a further reason for low biomass productivity. Light intensity was on average 32.12% lower in the raceway pond as compared to the ambient due to the polycarbonate sheeting cover. When the polycarbonate cover was installed, it impeded the light intensity by only 10%, however, due to weathering of the sheeting and attachment of particles light intensity was further impeded to 32%. The biomass concentration follows the pattern of the light intensity in Figure 4.6 A and B whereby a decrease in light is followed by a decrease in biomass for the period post decrease. The converse of this also holds where an increase in biomass is observed following an increase in light intensity. The pattern is noted for both the raceway pond and circular ponds (P4 and Control 4). RW4 was found to be under stress conditions from days 12 to 21 (Figure S 1) corresponding to the lower temperatures and light intensity.

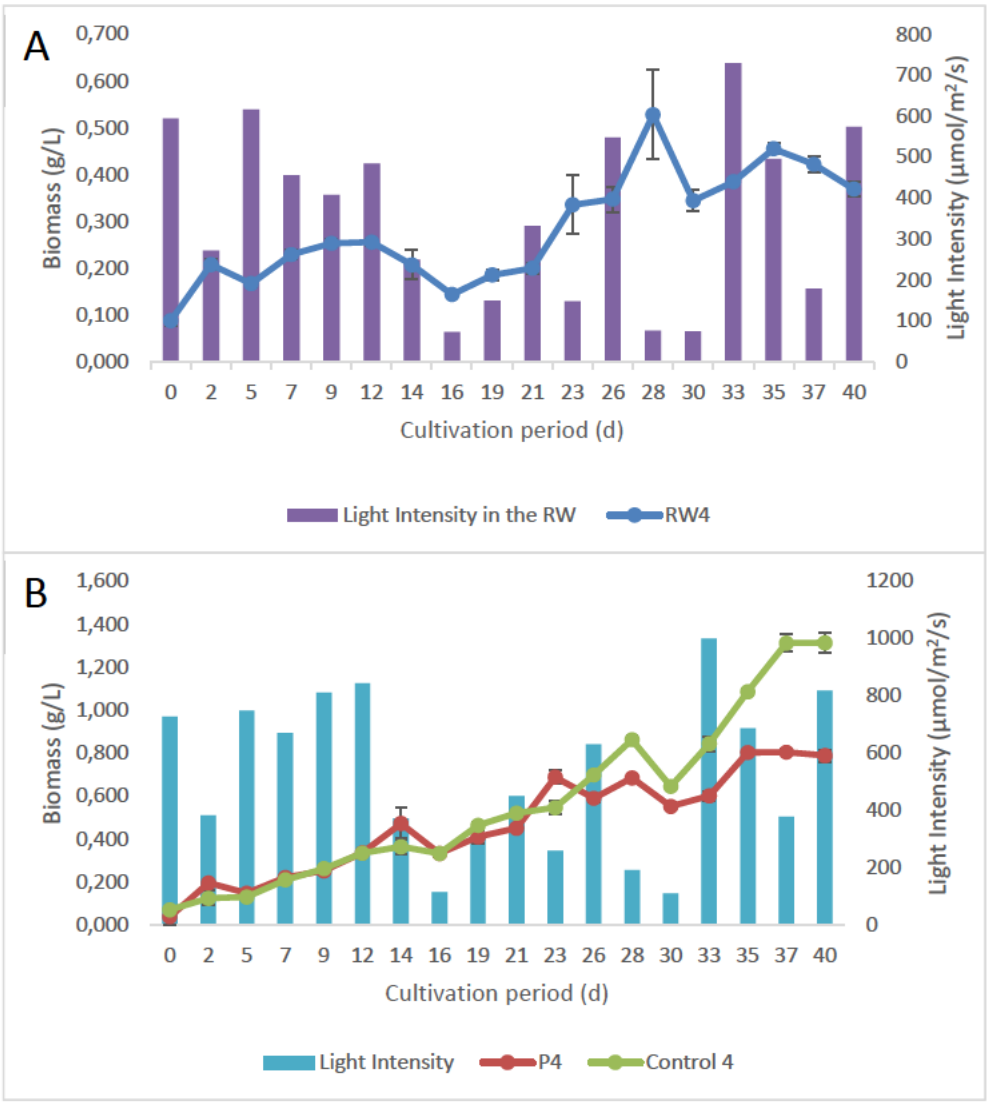

Figure 4.8. Relationship between biomass production and light intensity in the raceway pond, (A) light intensity and biomass concentration in the raceway pond, and (B) ambient light intensity and biomass concentration of Pond 1 and the control.

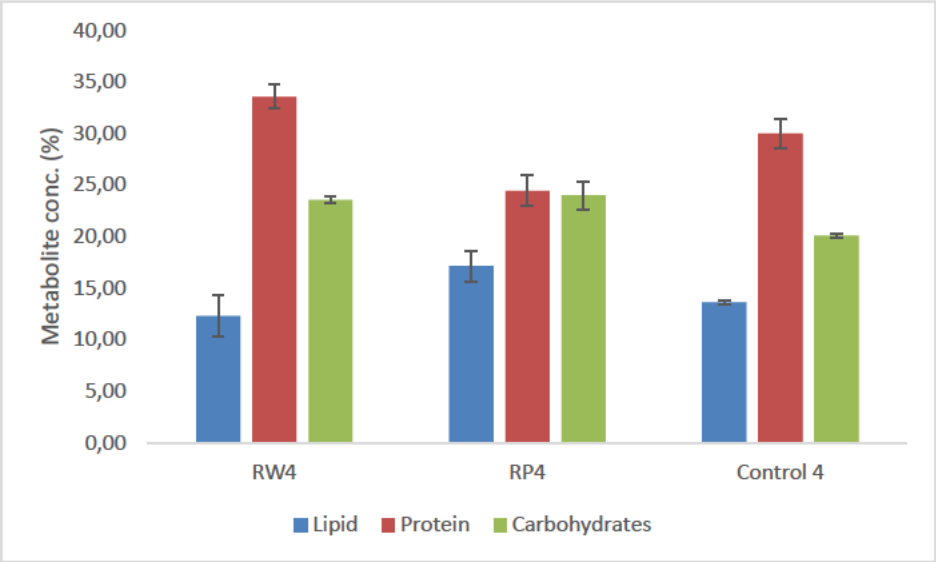

Figure 4.9. Concentration of major metabolites for RW4, P4, and control4

Lipid concentrations were observed to be 12.32±2.00; 17.14±1.04 and 13.61±0.25 % Lipid/g DCW for the raceway, circular pond, and BG 11 control respectively. Protein yield for the raceway pond at 33.56% was slightly higher than the control at 29.96% which was at the lower end of the expected range. Carbohydrate yields for RW4 and P4 trials were expected due to lower nitrogen content. Lipid yields for the raceway were significantly lower ($p<0.05$) than P4. Lower growth and metabolite production were most likely linked to the lower water temperatures (22.00 °C and 21.77 °C for the raceway and ponds respectively) experienced during the cultivation period.

4.3.2 Raceway Run 5

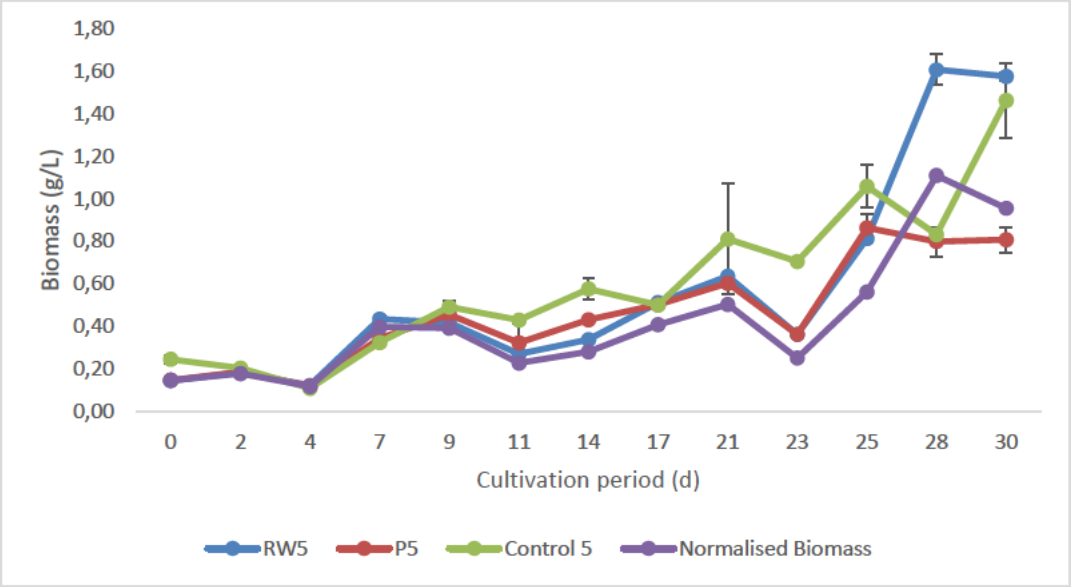

Figure 4.10. Cultivation of algal biomass in RW5 and P5 using mBG11 media Control5 using full BG11 from mid to late winter

Raceway productivities seldom follow a steady upward trend. This is due to a number of factors with varying degrees of influence. Raceway run 5 (Figure 4.10) is a clear example of this phenomenon whereby the overall biomass productivity for the period was 6.08 g/m^2/d whereas the maximum productivity noted was from days 23 to 28 of cultivation giving productivity of 24.92 g/m^2/d for the normalised biomass result calculated as a function of the reduced depth due to evaporation. The sharp increase in productivity is attributed to higher light intensities (Figure 4.11) averaging 963±31 µmol/m^2/s, higher water temperature averaging 31.37±1.03°C, and shallow depth of the pond at 14.5 cm (Figure 4.9). Days 9-21 gave slightly higher productivity than the full cultivation period of 4.60 g/m^2/d average light intensities (663±404 µmol/m^2/s) and moderate water temperatures (26.6±1.89°C) and an average depth of 17 cm.

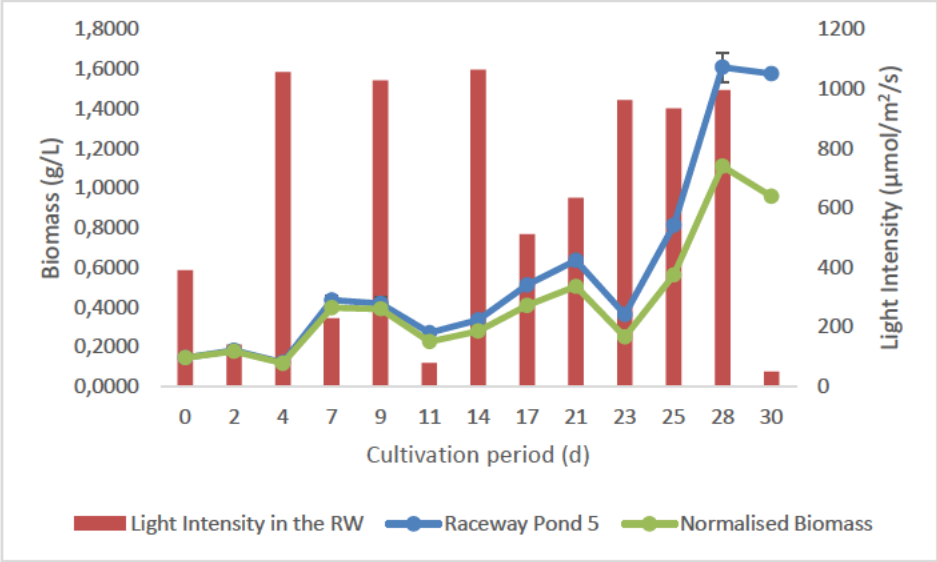

Figure 4.11. Algal biomass, normalised biomass in relation to light intensity for Run 5 of the raceway pond

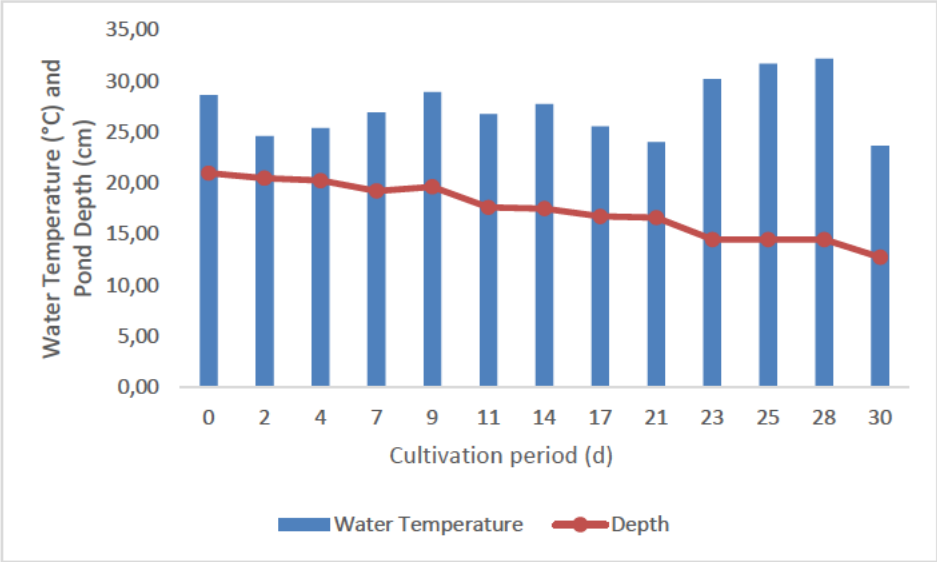

Figure 4.12. Water temperature and pond depth for Run 5 of the raceway pond for the full period of cultivation.

Light intensities in the raceway pond were highly variable over the cultivation period. The effects of the variability are observed in the biomass growth pattern. The spike in light intensity on day 4 (Figure 4.11) showed lower biomass and a corresponding quantum yield of PSII of 0.47 down from 0.51 on day 2 indication the culture undergoing stress which is most likely to be due to photoinhibition. A similar phenomenon is noted for day 11 however without the corresponding decrease in Fv/Fm (0.59). The variability in the light intensity from the beginning of the trial is likely to have allowed the culture to adapt better to the changing light conditions in the middle of the study. Evaporative losses averaged 0.275 cm/day with a total loss of 8.25 cm or 39% of the total volume over the cultivation period (Figure 4.12).

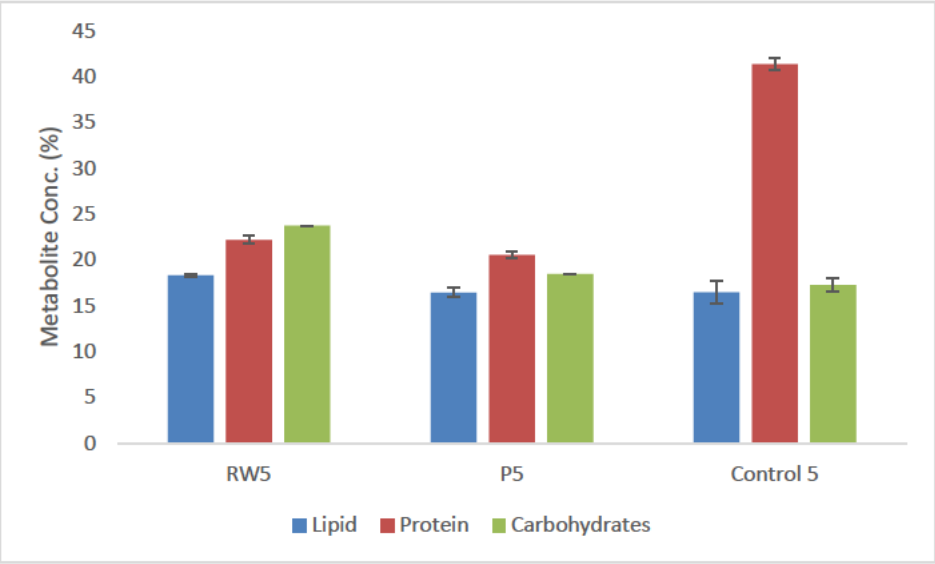

Figure 4.13. Concentration of lipid, protein and carbohydrates for Run 5 of the raceway, pond 5, and control pond

Lipid yield the raceway, Pond 5 and the control were 18.31, 16.46, and 16.49 % Lipid/g DCW respectively. Lipid yield for the raceway was found to be significantly higher ($p<0.05$) than Pond 5 and the control. Similarly, carbohydrate yields were significantly higher for the raceway than

pond 5 and the control at 23.77, 18.50, and 17.28 % respectively. The protein yield of the control at 41.40% was found to be significantly higher ($p<0.05$) than the raceway at 22.18% and pond 5 at 20.56%. The cultures were under limited stress conditions from inoculation of ponds until the day where they showed Fv/Fm> 0.5 throughout the cultivation period (Figure S 2). Lower protein production by RW5 and P5 is likely to be a result of lower N concentration than the control. This further agrees with the results of higher carbohydrate and lipid production in RW5 as compared to Control 5.

4.3.3 **Raceway Run 6**

As a deviation from the norm seen in runs 4 and 5, growth profiles of the 3 trials did not follow a similar trend over the 7 to 10 days of cultivation (Figure 4.12.). The control pond showed higher initial growth rates with a sharp decrease in biomass from day 5 to 7. This period encountered high rainfall which diluted P6 and Control 6.

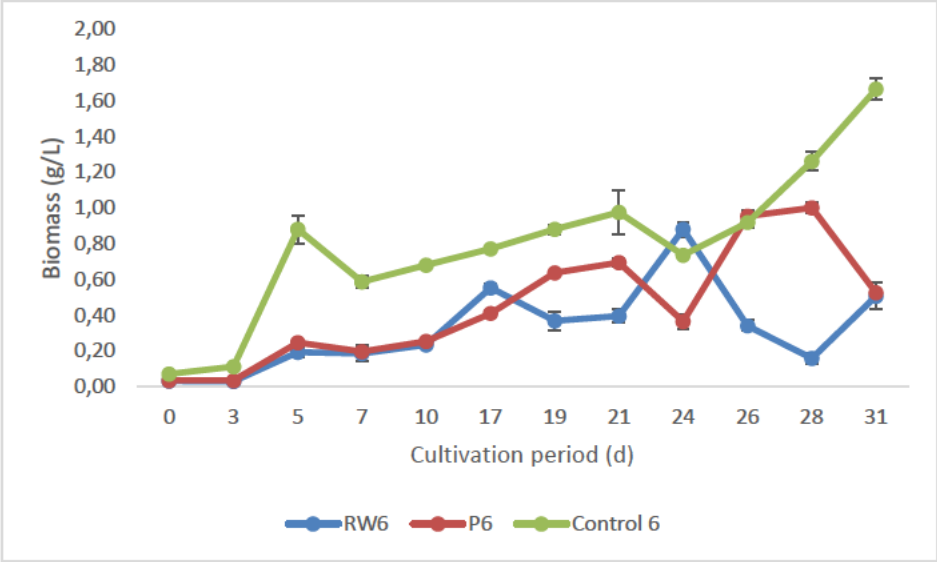

Figure 4.14. Cultivation of algal biomass in RW6 and P6 using mBG11 media Control 6 using full BG11 from mid to late summer

The added stress of lower light intensity of 579 µmol/m^2/s as compare to the average of 1019±277 µmol/m^2/s and dilution are most likely to be the main contributors to the decrease in biomass yield. The lower biomass is in agreement with the low quantum yield of PSII of RW6 and P6 (Figure 4.13) from inoculation until day 5 for P5 and day 7 for RW6. In contrast to the previous runs, the culture at the time of inoculation was predominantly *Chlorella* sp. Biomass productivity of 6.5 g/m^2/day was calculated from inoculation until day 17 where *Chlorella* sp. showed 80.38 % dominance in RW6. P6 and Control 6 gave productivities of 4.08 and 8.51 g/m^2/day for the same time period.

The run was undertaken during a period of rolling blackouts in South Africa whereby power was cut for up to 4 hours per day. This impacted directly on the mixing of the system as the paddlewheels have to be restarted manually after a power failure. Cyanobacteria were found to increase in abundance from day 17 and became the dominant culture around day 24 and showed 91.52% dominance by day 31. Due to the filamentous nature of the cyanobacteria species which overtook the pond, the culture was found to float on the surface after day 26 effectively impeding light penetration and reducing mixing in RW6. Whilst also increase potential on photoinhibition in the floating biomass due to constant exposure of light. P6 and Control six were mixed by submersible pumps which restart automatically once power is restored. *Chlorella* sp. maintained dominance in these ponds which leads us to postulate that the lack of mixing resulted in the settling of the unicellular algal species and allowed filamentous cyanobacteria to establish in the raceway pond.

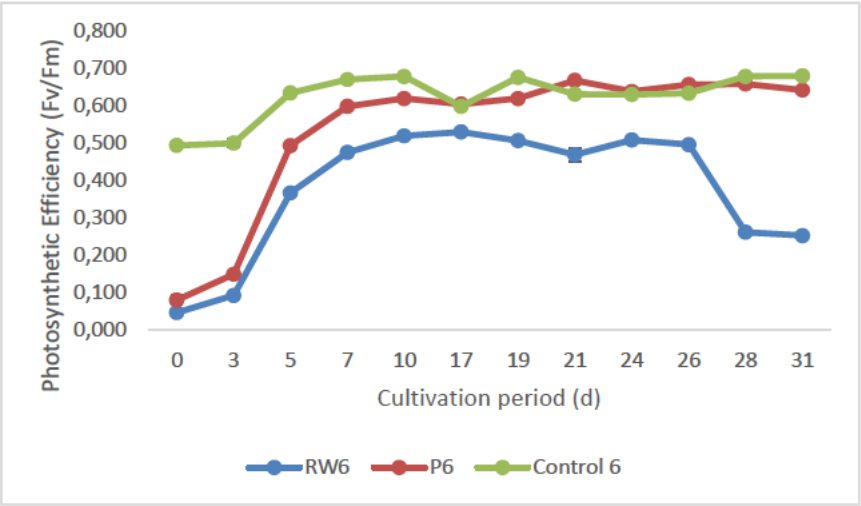

Figure 4.15. Quantum yield of PSII of algal cultivated in RW6, P6 using mBG11 and Control 6 from mid to late summer

Quantum yield of PSII of Control 6 indicated limited or no stress on the culture for the period of cultivation. P6 showed an increased quantum yield of PSII until day 7 after which no physiological stress was evident. RW6 however showed stress from inoculation until day 10, and further mild stress from days 19 to 24 before the quantum yield of PSII decreased drastically from day 26 (Figure 4.15). The decline from day 26 is attributed to the dominance of cyanobacteria. Cyanobacteria contain lower amounts of chlorophyll and utilize other pigments such as phycobilin proteins in light capture which cannot be measured by the PAM instrument utilized in the study. No significant difference ($p<0.05$) was observed in the lipid content with 21.44, 24.14, and 20.29% Lipid/g DCW being recorded for RW6, P6, and Control 6 respectively (Figure 4.16).

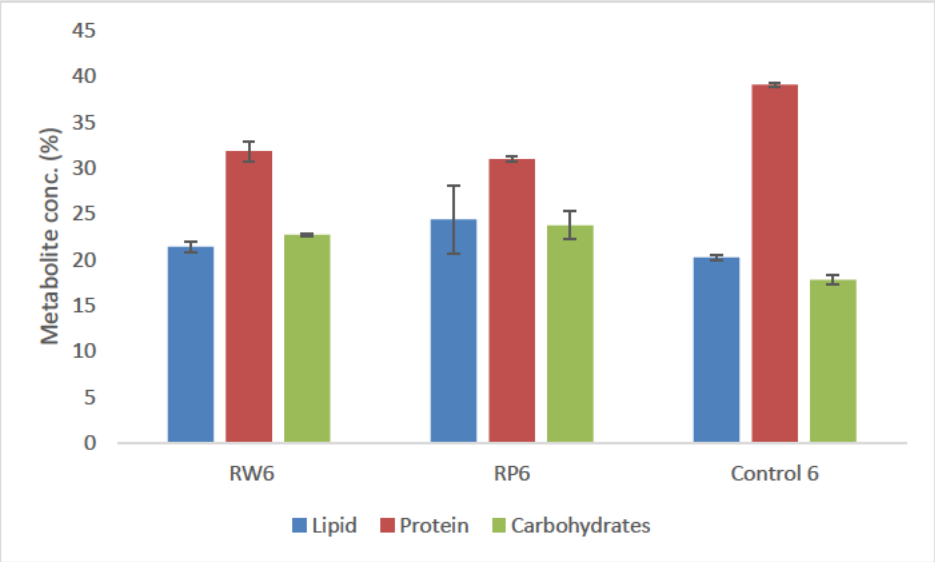

Figure 4.16. Concentration of lipid, protein and carbohydrates for Run 6 of the raceway, pond 6, and control 6 from mid to late summer

Carbohydrate concentration of 22.74% for RW6 was observed to significantly higher ($p<0.05$) than Control 6 at 17.83%. RP6 yielded 23.78% carbohydrate. The protein yield of Control 6 at 39.12% was found to be significantly higher ($p<0.05$) than the RW and P6 at 31.87 and 31.00% protein, respectively.

4.3.4 **Raceway Run 7**

The raceway pond was run was interrupted due to flooding of the wastewater treatment plant Figure 4.17. The pond sustained minor damage which was limited to damage of a few polycarbonate sheeting wall panels. Large amounts of soil were deposited into the raceway which gave the requirement of additional cleaning time. The raceway was repaired cleaned and readied for the next run. It was fortunate that the seed culture which is continually grown in circular ponds were diluted however were still viable. With the addition of nutrients, sufficient seed was cultivated to inoculate the raceway for the next run by the completion of the repairs and cleaning.

Figure 4.17. Run 7 of the raceway pond after flooding due to heavy rains.

4.3.5 **Raceway Run 8**

Growth patterns of RW8 and P8 were found to be similar for the cultivation period until day 7 (Figure 4.18). The biomass concentration of the raceway was normalised due to the reduction in pond depth from days 7 to 14. The normalised concentration was found to be much lower than the recorded results after day 7. This may be attributed to respiration losses and lower light penetration due to culture depth and reduced mixing. The paddlewheel is 5 cm above the surface of the raceway pond to prevent snagging of the plastic liner. The low water level of 10.75 cm means that the velocity of the water is likely to have been lower than required for efficient mixing throughout the length of the raceway. Overall biomass productivity was calculated to be of 2.99 g/m^2/day with a maximum productivity of being achieved 9.48 g/m^2/day being achieved on day 7.

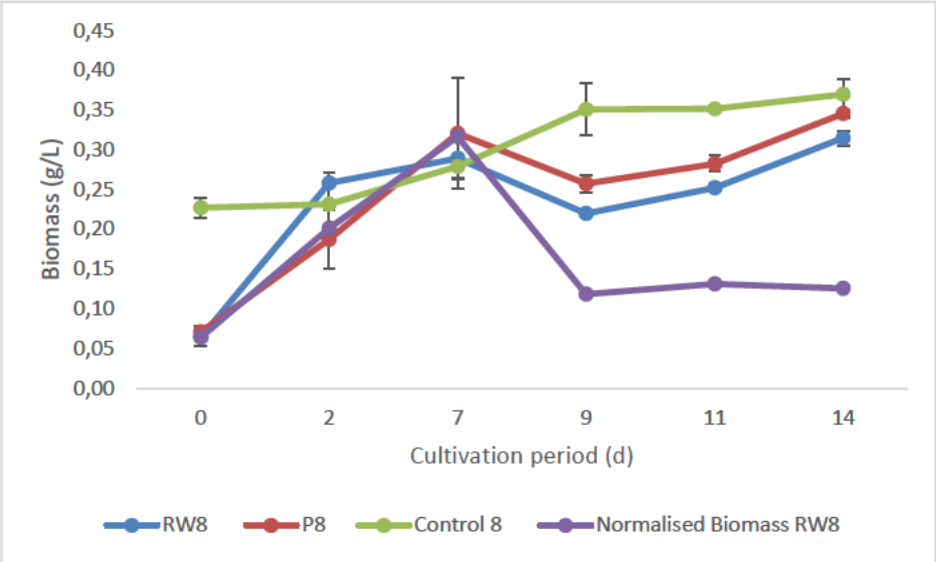

Figure 4.18. Cultivation of algal biomass in RW8 and P8 using mBG11 media Control 8 using full BG11 during autumn.

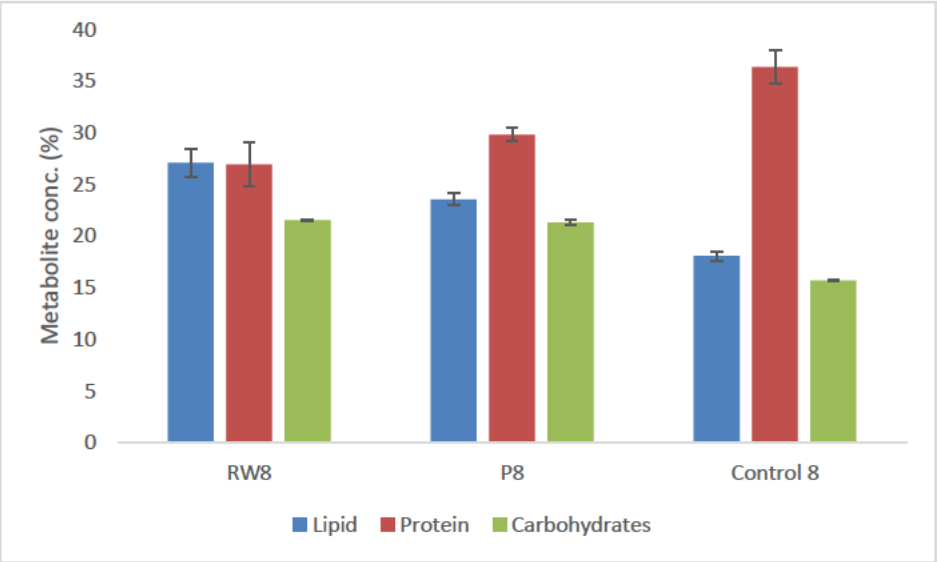

Figure 4.19. Concentration of lipids proteins and carbohydrates for Run 8 of the raceway, pond 8, and control 8 during autumn

Overall lipid concentration of 27.13 % Lipid/g DCW was achieved for the raceway pond (Figure 4.19). No significant difference ($p>0.5$) was observed between the RW8 and RP8 at 23.58 % Lipid/g DCW. There was however a significant increase ($p>0.5$) between control 8 at 18.08 % Lipid/g DCW and RP 8. The same trend is noted for carbohydrate levels. Proteins levels were observed to show the inverse relationship to lipids and carbohydrates which were higher for RP8 and significantly higher for Control 8 at 36.43%. Quantum yield of PSII for all trials increased from low levels of 0.233, 0.287, and 0.425 for RW8, RP8, and Control 8 respectively to above 0.5 on day 7 of cultivation after which it remained fairly constant (Figure S 3)

4.3.6 Raceway Run 9

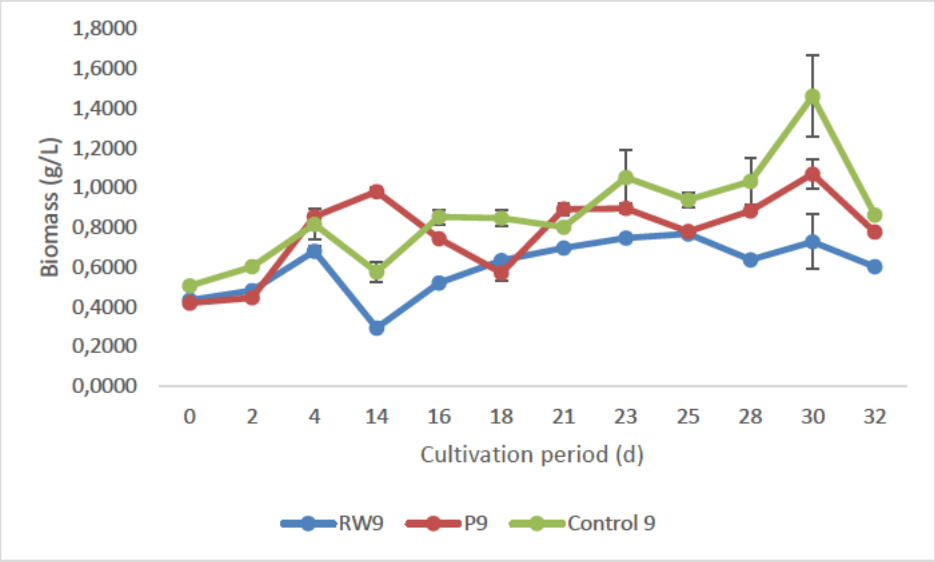

Figure 4.20. Cultivation of algal biomass in RW9 and P9 using mBG11 media Control 9 using full BG11 during winter.

Maximum biomass productivity was achieved for the first 4 days of cultivation at 13.3 g/m^2/day. This was likely due to the higher light intensities (<1000 µmol/m^2/s) and warmer water temperatures averaging 17±1.7 °C. Productivities of P9 and control 9 were noted to be 30.5 g/m^2/day and 21.7 g/m^2/day respectively. This was followed by lower water temperatures averaging 12.43±1.2 °C. This period was characterized by a decrease in biomass below the initial inoculum level followed by a gradual increase as seen in Figure 4.20. overall biomass productivities were 1.91, 4.94, and 7.20 g/m^2/day for RW9, P9, and Control 9 respectively. These low productivities are expected due to the lower temperatures experienced over the period of cultivation. Maximum biomass of 0.78 g/L was achieved on day 25 in the raceway pond.

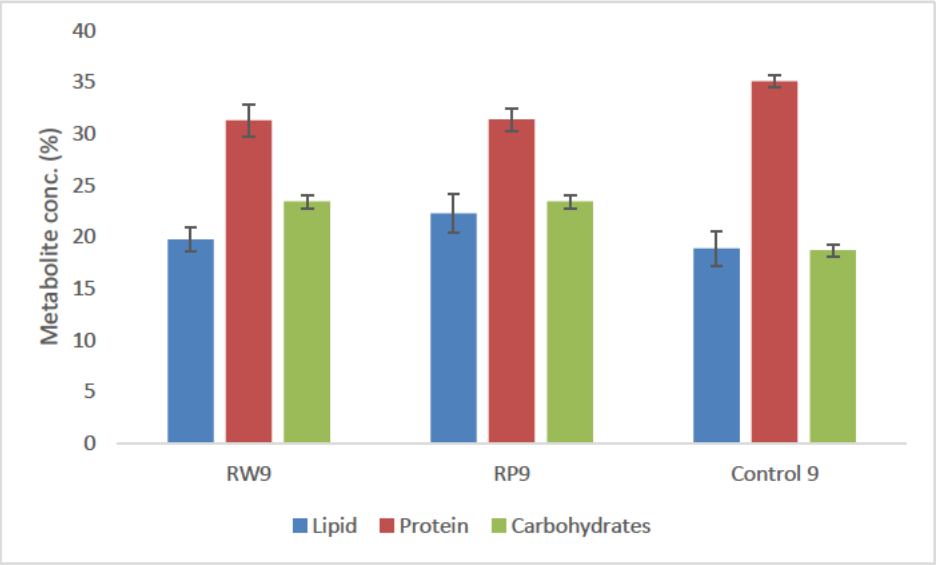

Figure 4.21. Concentration of lipid, proteins and carbohydrates for Run 9 of the raceway, pond 9, and control 9 during winter

Similar results were achieved for the raceway pond, with no significant difference ($p>0.5$) observed between the RW9 and P9 and RW9 and Control 9 for all the metabolites. Maximum lipid was achieved in RP9 at 22.3 % Lipid/g DCW followed by RW9 at 19.8 % Lipid/g DCW and Control 9 at 18.91 % Lipid/g DCW (Figure 4.21). Protein concentration ranged from 31-35 % and carbohydrates were 18-24% for the run. These results were expected as the culture did not undergo stress conditions for prolonged periods as observed from the quantum yield of PSII (Figure S 4). The lowest recorded efficiency was for the raceway pond on day 2 at 4.963 which subsequently increased and remained above 0.5 for the entire cultivation period.

4.3.7 Raceway Run 10

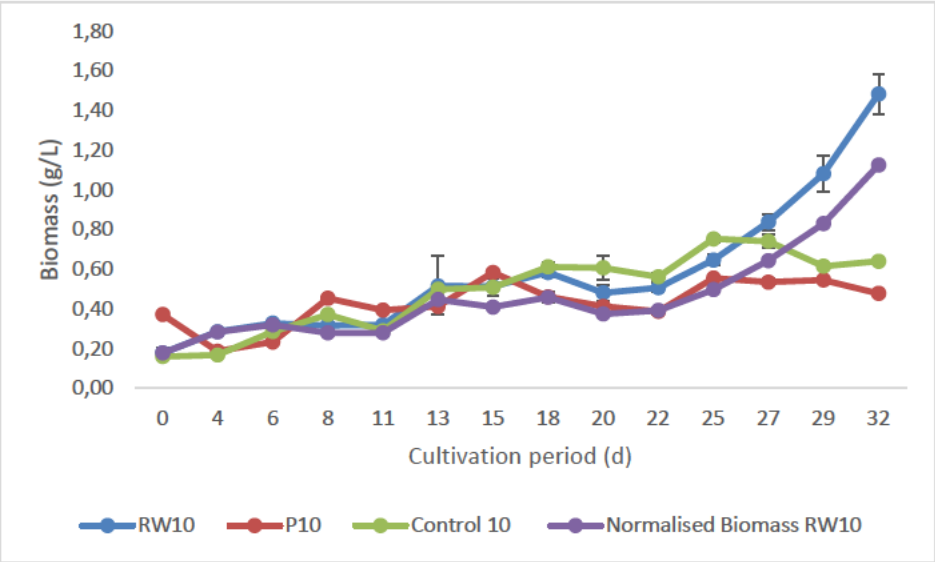

Figure 4.22. Cultivation of algal biomass in RW10 and P10 using mBG11 media Control 10 using full BG11 during spring

RW10, P10, and Control 10 displayed similar growth patterns until day 15 when a slight divergence is noted. RW10 biomass concentration increase significantly from days 22 to 32, reaching a maximum of 1.48 g/L. As evaporative losses were not compensated, the normalized maximum biomass achieved was 1.12 g/L. Overall biomass productivity was 5.83, 2.93, and 3.95 g/m^2/day for RW10 (normalized), P10 and control 10 respectively. This may have been due to slightly warmer water temperatures of the raceway averaging 23.05±1.03 °C as compared to P10 and control 10 averaging 19.53 ±1.40 °C and 19.64 ±1.65 °C respectively. It is prudent to note that *Chlorella* sp. was the dominant culture in RW10 from days 18 to 32 were as *Scenedesmus* dominated P10. RW10 was observed to achieve the optimal temperature for *Chlorella* at ~22°C

is several degrees lower than that of *Scenedesmus* thus allowing the culture to proliferate and achieve much higher biomass than P10 for the same cultivation period.

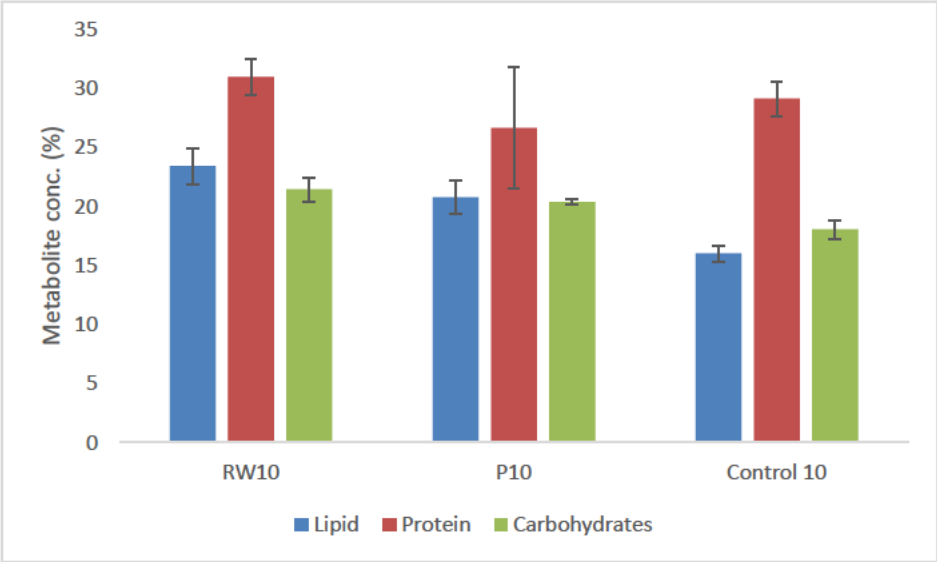

Figure 4.23. Concentration of lipids, proteins and carbohydrates for Run 10 of the raceway, pond 10, and control 10 during spring

A trend of higher lipid production is noted for raceway pond runs where evaporative losses are not compensated during the trial. This is seen for runs 5, 8, and 10 where lipid content in the raceway reached higher concentrations than that of the corresponding circular pond and control culture. Lipid yield for RW10, P10, and control 10 (Figure 4.23) were 23.47, 20.80, and 16.04% Lipid/g DCW respectively. Protein and carbohydrate content ranged from 26.71- 31.03% and 18.08- 21.48% respectively with no significant difference ($P<0.05$) observed. These results are likely to be a result of the culture not undergoing stress conditions as is evidenced by the quantum yield of PSII remaining above 0.5 (Figure S 5) for most of the cultivation period.

4.3.8 Changes in biomass productivity over different seasons.

Changes in biomass productivity are expected due to the prevailing climatic conditions and depending on the culture of algae dominating the raceway pond at the given time. Table 4.2 shows average water temperatures and light intensities recorded over each of the cultivation periods of the raceway pond and corresponding season in South Africa. It is noteworthy that the cultivation site is based on the east coast of South Africa which experiences fairly moderate climatic conditions where minimum temperatures reach 12.8°C with the average minimum for 2019 recorded as 16.7°C with the lowest daily temperature being reported at 5.2°C. Maximum average temperatures were recorded to be 27.4°C with the highest daily temperature of 38.3°C in 2019 (South_African_Weather_Service, 2020). As seen in Table 4.2, the average water temperature ranged from 20.61±0.68°C during mid-winter to 31.03±2.22°C in late summer. Daylight ranges from 10.25 to 14 hours in winter and summer respectively. The highest average light intensity was 359.00±212.71 µmol/m^2/s from Mid-winter to early spring and 645.44±330.58 µmol/m^2/s in late summer.

Table 4.2. Period, Season, average water temperature, and light intensity corresponding to each of the runs of the raceway pond

Run	Period	Season	Average Water Temp. (°C)	Average Light Intensity (µmol/m^2/s)
4	25 July-3 September	Mid-Winter to Early Spring	22.05±1.58	359.00±212.71
5	1 -31 October	Mid Spring	22.89±2.99	621.38±405.19
6	25 January -25 February	Late Summer	31.03±2.22	645.44±330.58
8	6-20 May	Early Winter	23.99±2.56	607.43±253.45
9	24 June - 24 July	Mid-Winter	20.61±0.68	588.95±211.73
10	8 August- 9 September	Late winter to Early Spring	23.05±1.30	446.12±300.28

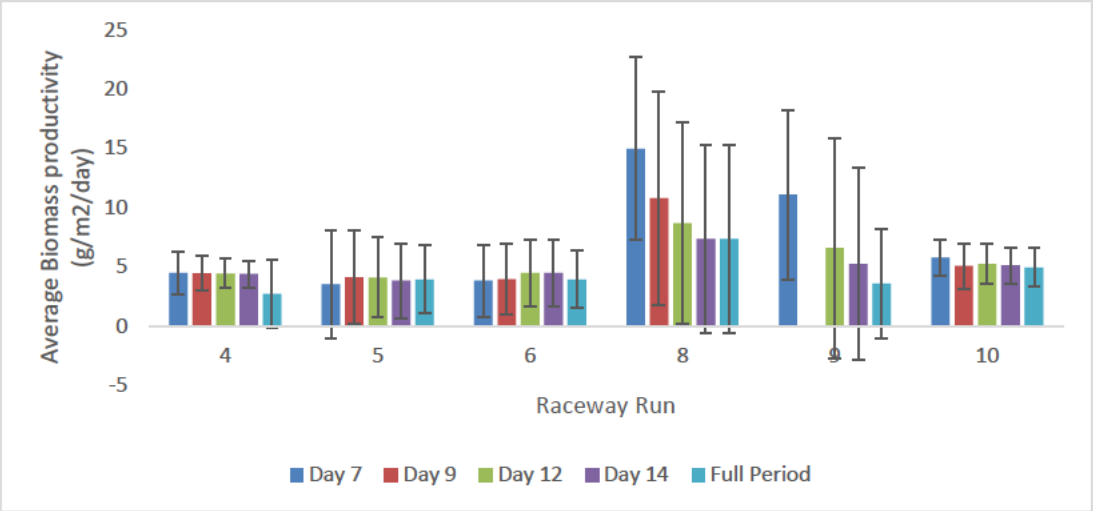

Figure 4.24. Changes in biomass productivity over the first 14 days of the cultivation for all runs of the raceway pond

For all 6 complete runs of the raceway, it was noted that higher biomass productivity was achieved in the first 7 to 14 days as illustrated in Figure S 6 to Figure S 11 in the supplementary data. This is an expectation due to lower biomass concertation during the initial stage of growth. Figure 4.24 shows biomass productivity as calculated for periods of cultivation of each run for days 7, 9,12, and 14. The biomass productivity achieved is dependent on multiple factors including the dominant species during the time of cultivation. The winter months which would have been expected to show lower productivities gave the converse result with the highest productivities being achieved. These productivities however also showed the highest amount of variability.

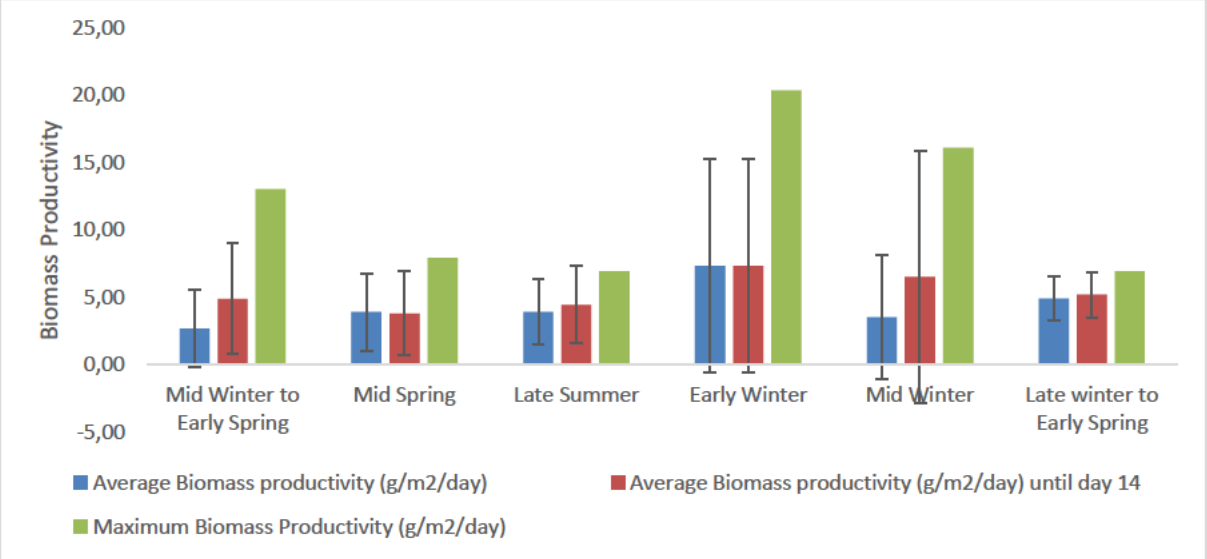

Figure 4.25. Comparison of biomass productivity for cultivation of algal biomass over different seasons of the raceway pond.

Changes in abiotic factors play a significant role in the biomass productivity of the raceway pond in the short term but appear to have a lesser effect for prolonged periods. Despite observing the highest average biomass productivity and highest overall productivity cultivation during early winter and late winter also showed the highest variability (Figure 4.25.) These periods correspond with Runs 8 and 9 of the raceway. Average biomass productivities for the full period of cultivation were low and shown to be more favourable at shorter cultivation periods. As the site is based in a temperate climatic region, early winter temperatures and moderate light intensities are still in the range beneficial to algal cultivation as shown by Figure 4.26 below. These observations concur with the results of ANFIS modelling in section 4.3.9

4.3.9 Modelling

It is important to gain an understanding of the relationship between biomass productivity and various factors that affect productivity. ANFIS modelling of the raceway data was carried out to elucidate these relationships and effects.

Data pre-processing

The data collected were statistically analysed to check consistency and reliability. The final dataset included six input variables i.e. LI: Light Intensity; DP: Depth (cm); pH; WT: Water Temperature; NT: NO_3-N (mg/L); PS: PO_4-P (mg/L). The targeted variables were DW: Dry Cell Weight Average; BP: Biomass Productivity (g/m^2/day).

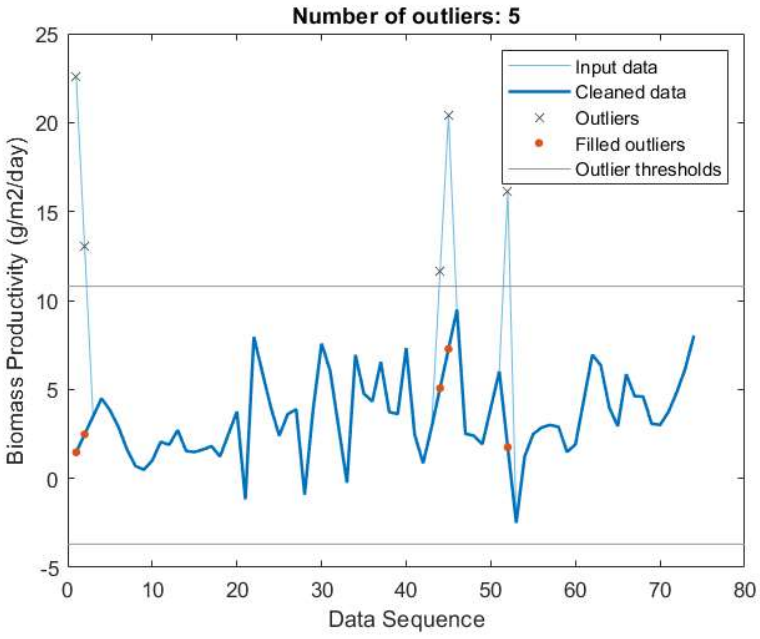

Figure 4.26. Data pre-processing for the target **BP** data.

Table 4.3. Summary statistics of all parameters

	LI	*DP*	*pH*	*WT*	*NT*	*PS*	*DW*	*BP*
Mean	568.4	20.4	9.8	24.6	28.3	1.2	0.4	3.4
Minimum	51.1	14.0	8.1	19.2	0.0	0.0	0.0	-2.5
Maximum	1323.8	26.9	10.9	33.5	70.0	4.6	1.2	9.5
Standard Deviation	341.1	2.9	0.7	4.0	14.6	1.2	0.2	2.3
Range	1272.8	12.9	2.8	14.3	70.0	4.6	1.2	12.0
Count	74	74	74	74	74	74	74	74

Figure 4.26 shows the data pre-processing and outliers' elimination of the targeted biomass productivity data. Similarly, the data cleaning procedure was applied to the other parameters. A detailed description of the summary statistics of all parameters is shown in Table 4.3.

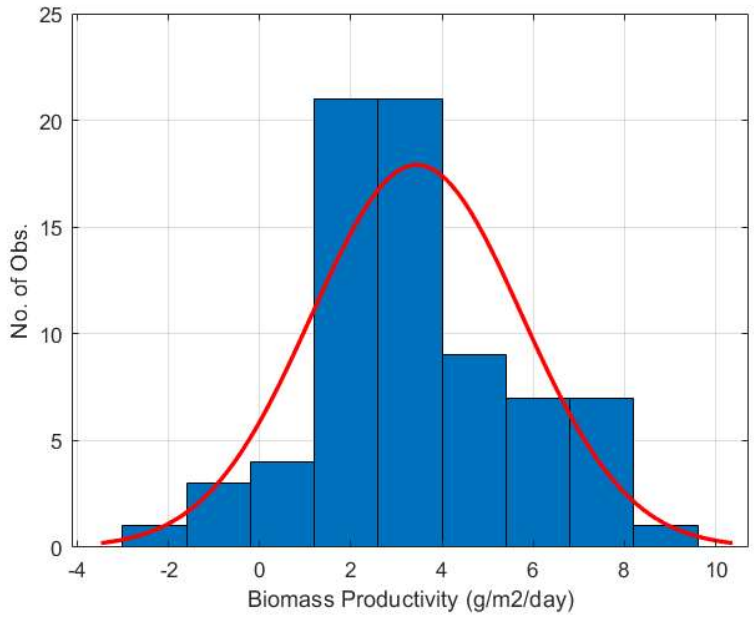

Figure 4.27. Normal probability curves of BP data.

Further, the fit goodness test was conducted using the normal probability curve of BP data as presented in Figure 4.27. A distribution is said to be normal when the probability curve is symmetrical and positioned around the mean of a data series (Abunama, 2019; Ramzan, 2013)

Inputs optimization

In this study, the datasets were separated into two, odd values for the training phase and even values for the testing phase. Subsequently, ANFIS exhaustive search function for inputs optimization was applied to identify the relevant inputs. Figure 4.28 shows the exhaustive errors results represented by RMSE values for training and checking datasets. The exhaustive search targeted selecting the optimum three inputs to represent the inputs-output relation.

Figure 4.29 presents the modelling results of the input optimization process of selecting 20 different combinations. The first input variables (from left) are the most correlated and relevant variables to the targeted output. This combination was selected because it showed the lowest RMSEtrn errors in the training data. It can be noticed that some of the other combinations have lower errors in checking data, however, they showed high training errors. Therefore, based on the results of inputs optimization using ANFIS executive search, in modelling BP levels, the three most relevant inputs were LI, DP, and pH.

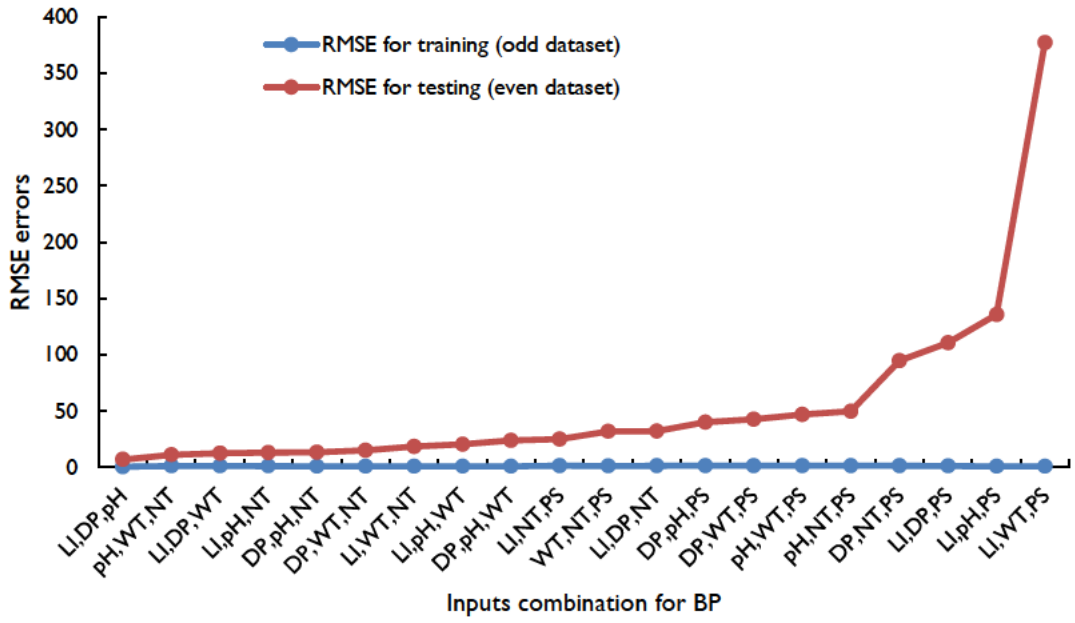

Figure 4.28. RMSE modelling results of the possible inputs combinations to model BP levels.

ANFIS models development

Upon defining the best input combination, the development of the best ANFIS structure was conducted by applying various types and number of membership functions (MFs), and different rules and epochs' numbers. This was performed aiming at testing all possibilities of ANFIS parameters, and compare their ability in modelling the BP. In the modelling, 70% of the data was used in the training phase, while the rest was used to check or test the model's performances. The following (Figure 4.29) shows the structure of ANFIS modelling with three inputs, 30 fuzzy rules, and one output, as well as the assigned rules.

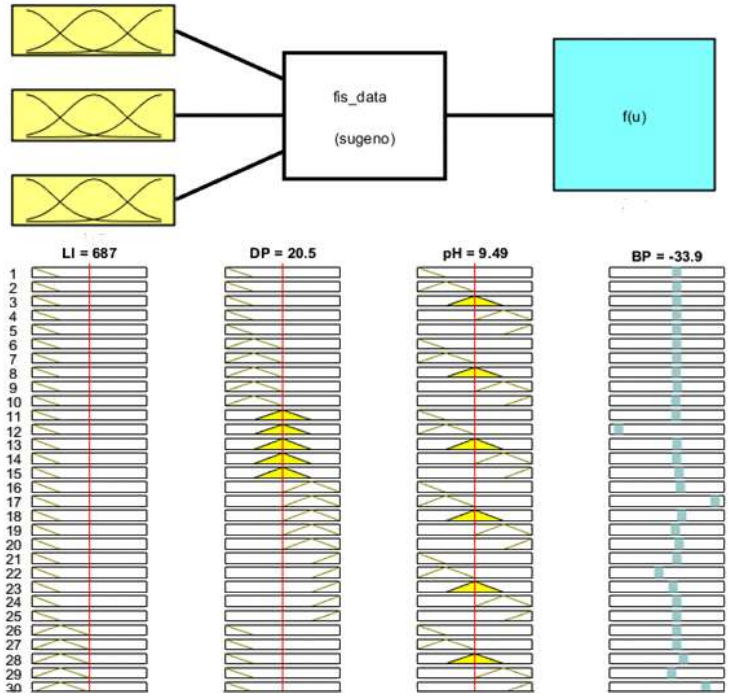

Figure 4.29.Comparison between the observed and modelled values for BP data by the selected structure of ANFIS models.

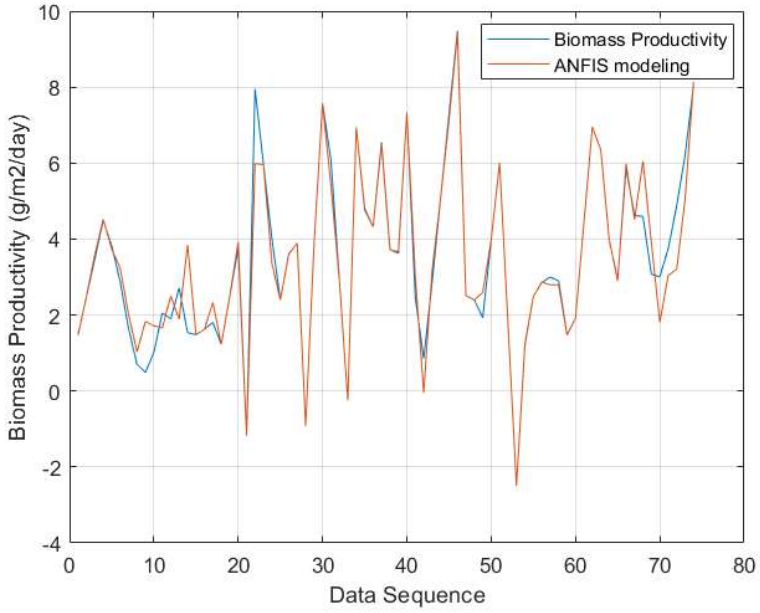

Figure 4.30. Comparison between the observed and modelled values for BP data by the selected structure of ANFIS models.

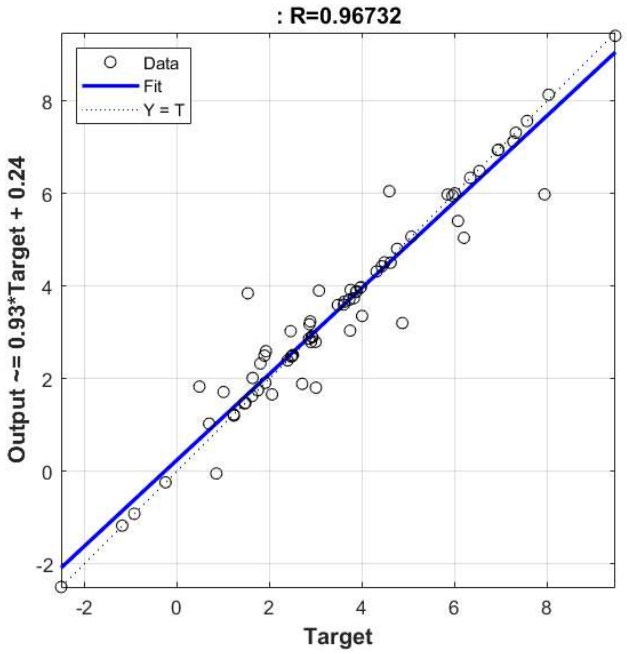

Figure 4.31. Regression curve between the observed and modelled datasets of BP data.

Figure 4.30 presents a comparison between the observed and modelled values of BP data by the selected structure of ANFIS models. Subsequently, Figure 4.31 presents the regression plots for both water salinity parameters between the targeted and predicted concentrations. A strong correlation was found with an R of 0.97632 and R^2 of 0.936, which verify the model's accuracy.

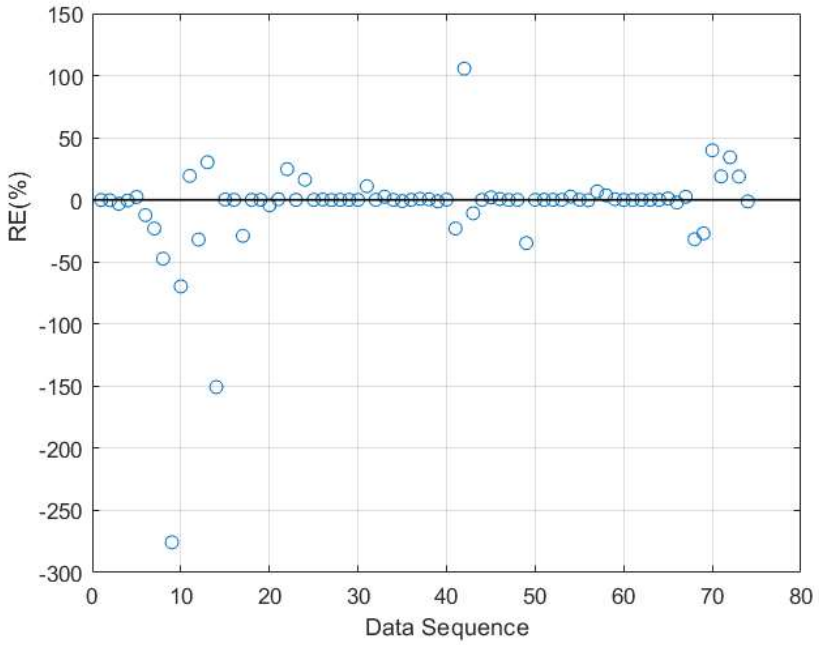

Figure 4.32. RE% results by the final ANFIS model for BP data. (6)

RE% test was used to demonstrate the accuracy of the proposed models. RE% plots are shown in Figure 4.32 for the optimum ANFIS model. Only one value had RE% above 50%, while all other values were less than RE% of 50%. The negative RE% results mean that the models overestimated the targeted BP levels. As shown in the figure, only three values were overestimated, while the rest were with RE% larger than -50%.

Finally, Figure 4.33, Figure 4.34, and Figure 4.35 present the 3D surface views of the selected inputs and the target output. DP, LI, and pH were the most correlated and optimum to model BP levels. A fluctuating trend in the figures is observed for BP data with the variation in input variables.

The ANFIS model was based on biomass productivity in relation to depth, pH, and light intensity.

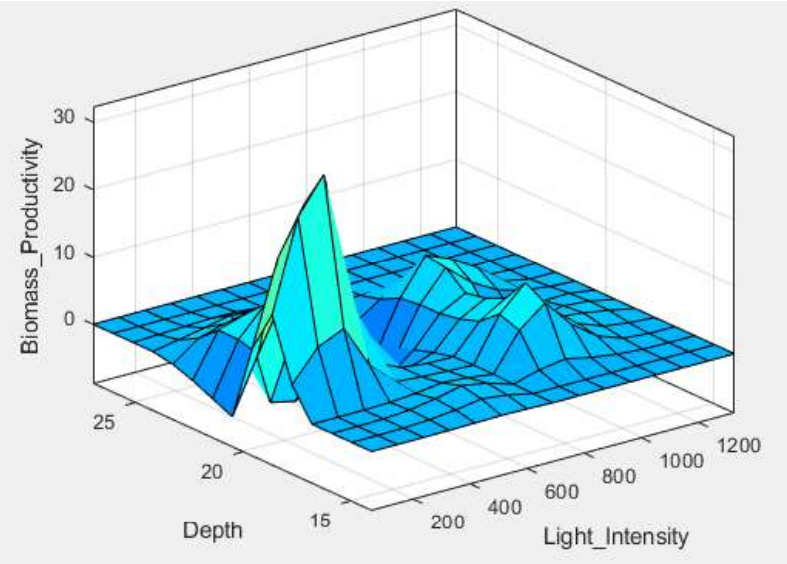

Figure 4.33. ANFIS model of the relationship between biomass productivity, depth, and light intensity based on runs 4 to 10 of the raceway pond.

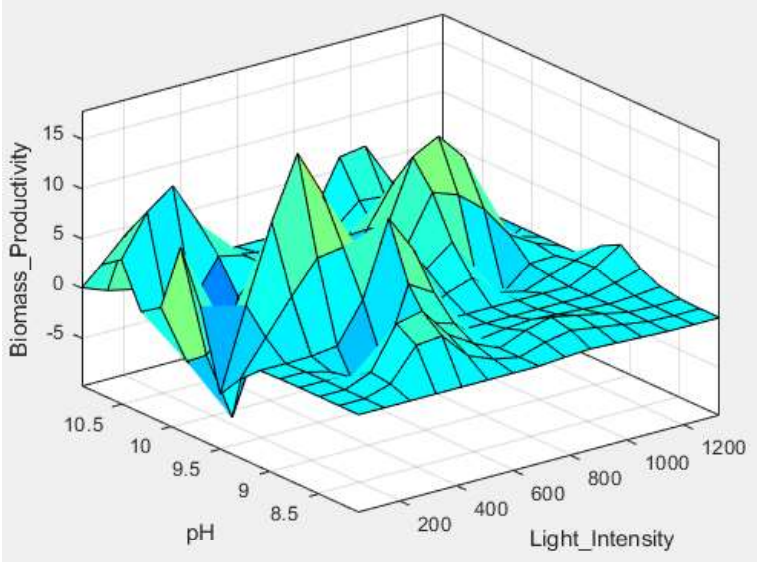

Figure 4.34. ANFIS model of the relationship between biomass productivity, pH, and light intensity based on runs 4 to 10 of the raceway pond.

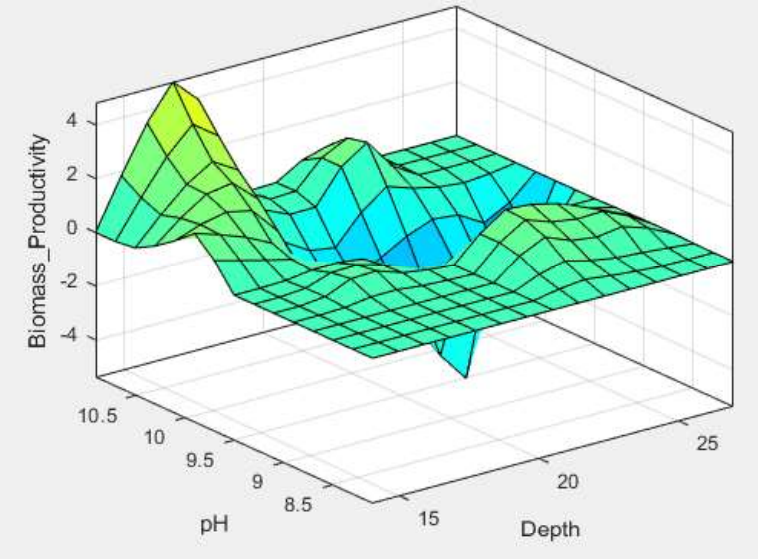

Figure 4.35. ANFIS model of the relationship between biomass productivity, pH, and depth based on runs 4 to 10 of the raceway pond.

Modelling of the system elicited that the major factors affecting biomass productivity in the raceway pond were light intensity, pH, and depth for the raceway pond. The model of the relationship between biomass productivity, depth, and light (Figure 4.33) showed maximum biomass productivity at a depth between 20 and 22 cm at light intensities between 200 and 400 µmol/m^2/s with lower productivities being evident from 800 to 1000 µmol/m^2/s. Higher light intensities at the same depth are likely to produce reduced biomass productivity due to the potential photoinhibition of the culture. Lower intensities would produce a negative effect on biomass productivity at depths above 22 cm due to the lack of light reaching cells.

The relationship between biomass productivity, pH, and light intensity (Figure 4.34) is more complex than the previous model (Figure 4.33). pH in the range of 9 to 9.5 correlated positively

with light intensity ranging from 200 to 1000 µmol/m^2/s with maximum biomass expected in the region of 400 to 500 µmol/m^2/s. a pH of 10 however gave a negative correlation which could be a result of nutrient limitation due to precipitation of phosphate and potential volatilization of ammonia. pH above 10 however correlated positively with biomass productivity at low light (200 µmol/m^2/s) and high light intensity (1000 µmol/m^2/s). High pH is commonly used for short periods of time to reduce the load of zooplankton which feeds on the culture. The reduction in grazers would result in higher biomass productivity. Autoflocculation of the culture, which contained an appreciable amount of Scenedesmus like species, would potentially negate some of the photo-inhibitory effect of higher light. This is however a postulation and would need to be verified. Culture depth above 20cm at pH 9.5 to 10 has a negative correlation with biomass productivity however at lower depth a positive correlation is seen for higher pH (Figure 4.28). This is likely due to the reduction of grazers at pH above 10.5, which would ordinarily reduce the algal density.

4.4 Discussion

4.4.1 Biomass Productivity

Biomass productivity of full-scale systems has been reported to be typically in the range of 3.7 to 11.5 g/m^2/day for full-scale algal cultivation systems in similar climatic conditions despite the season and dominant algal strain (Sutherland et al., 2020). Sutherland et al., (2020) found biomass productivity of 3 raceway ponds of scale 5 m^2, 330 $m^{2,}$ and 1 ha to be well below the theoretical maximum and on the lower end of reported literature values from other temperate climates. They suggested that the addition of CO_2 could have resulted in biomass levels 50 to 150% higher than that achieved. (Nagarajan et al., 2020) Asserted that supply if carbon has multifold benefits including control of pH which in turn prevents non-biological removal of N and P and increase in

biomass productivity (66 to 100% increase) by the potential negation of negative effects such as ammonia toxicity. Jebali et al., (2018a) growing *Scenedesmus* sp. in raceway pond at different depths, concluded that shallow ponds may provide better volumetric productivity and nutrient removal due to enhanced light penetration, however, deeper ponds are more commercially viable as they provide higher areal productivity and nutrient uptake capacity.

Several researchers have advocated for the supply of CO_2 in order to improve biomass productivity not only be the supply of additional carbon but also for pH control in order to limit competition for carbon by nitrifying and prevent loss of ammonia by stripping (Bagchi et al., 2019; Mantovani et al., 2019; Nagarajan et al., 2020; Yadav et al., 2020). Our study did not have supplementation of CO_2 due to ineffective delivery in shallow ponds and no waste CO_2 streams being available in close proximity to the experimental site. The supply of CO_2 at large-scale is prohibitively costly (Yun et al., 2018).

Ramirez-Lopez et al., (2019) cultivation of *Chlorella vulgaris* UTEX 26 in a 3.67 m^2 raceway pond housed in a greenhouse. Their initial productivity was calculated to be 5.85 $g/m^2/d$ for the first eight days of cultivation. Biomass productivity increased to 20.1 $g/m^2/d$ for cycles 2 and 3 however, the maximum volumetric biomass achieved was 0.62 g/L after 34 days of cultivation in a semi-continuous system in which half the volume of the pond was harvested every 8 days (third cultivation cycle). They found that both areal and volumetric productivity decreased substantially after day 3 of cultivation for each cycle. A similar trend is noted for our study as per Figure 4.22 when the culture was dominated by *Chlorella* sp. High productivity was noted for the first 5 to 7 days followed by a decline in productivity. Semi-continuous harvest has been shown to increase biomass productivity (Ashokkumar et al., 2019). This will form part of future experimentation.

Biomass productivity can be strongly affected by changes in the algal population. This will be discussed in Chapter 5.

Table 4.4 is a comparison of different scales of raceway ponds and the biomass productivity achieved by each of the studies. It is evident from the studies that biomass productivity was lower in larger-scale raceway ponds irrespective of the climatic conditions or the algal species being cultivated in agreement with the assertion made by Sutherland et al., (2020). Biomass productivity achieved in this study is within the range achieved by all other studies in ponds larger than 330 m^2. Pilot-scale raceway ponds at 10 m^2, in this study (Figure 3.4) achieved productivity of between 12 and 31 $g/m^2/day$ in agreement with smaller-scale systems.

Table 4.4. Recent studies on algal productivity in raceway ponds of varying scale

Scale of Pond (m^2)	**Working depth (m)**	**Algal Species**	**Medium**	**Nutrient Supplement**	**Biomass productivity ($g/m^2/d$)**	**Lipid Content (%)**	**Reference**
5,04	0,2	*Synechocystis*	Municipal WW Digestor	0.6 g/L NaHCO3)	24.4		(Ashokkumar et al., 2019)
7,2	0,15	*Scenedesmus*	Centrate (30%)	CO_2 on demand pH controlled	22.9	10,1	(Jebali et al., 2018a)
950L			Mixed carpet milling and municipal	6 % CO_2 supplied by bubbling	2.64-4.9		(Chinnasamy et al., 2010)
2,23		*M. pulchellum (85% dominant)*	Settled Domestic WW	1% CO_2 bubbled into pond			(Sutherland et al., 2014)
0,1	0,21	*Scenedesmus*	Bolds Basal Medium		7.89 ± 0.73	15 ± 0.28	(Raeisossadati et al., 2020)
0,403	0,16	*Chlorella vulgaris*	Tap water supplemented with $NaNO_3$, $MgSO_4$, K_2HPO_4, KH_2PO_4, NaCl, $ZnSO_4$,	Batch Culture	0.973 ± 0.02		(Yadav et al., 2020)

			$CuSO_4$, $CaCl_2$ and $FeSO_4$	Semi Continuous culture	1.29 ± 0.015		
				Batch Culture with Flue gas (10% CO_2 v/v)	3.27 ± 0.12		
				Semi Continuous culture with Flue gas (10% CO_2 v/v)	11.48 ± 0.005		
5	0,3	*Ankistrodesmus falcatus* (winter and Spring 85%) *Micractinium pusillum* Fresenius(Summer)	Digested Municipal effluent		4.2 - 7*		
330	0,3	*Ankistrodesmus falcatus* (winter and Spring 85%) *Micractinium pusillum* Fresenius(Summer)	Digested Municipal effluent		4.2-8.2*		
10000	0,3	*Ankistrodesmus falcatus* (winter and Spring 85%) *Micractinium pusillum* Fresenius(Summer)	Digested Municipal effluent		3.7-5.6*		(Sutherland et al., 2020)
1,2		*C. vulgaris* UTEX 26	M Medium				
6,37	0,18	*C. vulgaris* UTEX 26	M Medium		5,85	4,49	(Ramirez-Lopez et al., 2019)
52	0,3	*Scenedesmus accuminatus*	N11 Medium using nutrient analogues from fertiliser	Greenhouse during summer	10.15 ± 1.08		(Koley et al., 2019)

				Greenhouse during rainy season	7.83 ± 0.88		
				Greenhouse during winter	10.38 ± 1.18		
52	0,3	*Scenedesmus obliquus* GA45	N11 Medium using nutrient analogues from fertiliser	Greenhouse during summer-winter	12.44 – 13.12		(Bagchi et al., 2019)
675	0.2	Native consortium	commercial fertiliser		8.9	12.8	(Hong et al., 2017)
1146	0.2-0.3	*Scenedesmus* and *Chlorella*	Treated wastewater	250 mg/L $NaNO_3$	3.82-7.34	12.32-27.13	This study

* approximated values read off the graph.

There is a large gap between laboratory-scale optimization and large-scale cultivation of algae particularly due to the constant state of flux of physical factors such as temperature and irradiance (De-Luca et al., 2018). The use of models becomes necessary to start to understand the dynamics of an outdoor cultivation system. Supriyanto et al., (2019) found that, when using ANN to develop a predictive model on an open raceway pond for biomass production, light intensity and water temperature were key factors when medium, depth, HRT, and CO_2 were not limiting factors. It is noted that these are the 2 variables that would be prohibitively expensive to control, thus making it impractical. Our study found that depth, pH, and light were inextricably linked to biomass productivity. Factors that play a key role in biomass productivity are discussed below.

4.4.2 Light

Light intensity has a substantial effect on biomass accumulation. Insufficient or excessive light is known to reduce biomass productivity. Algae can grow rapidly under low light however increase

in cell density in cultivation systems often result in light limitation as to the depth increases and excessive light being supplied to cells at the surface of the system. The rate of photosynthesis increases linearly as a function of the irradiance until light saturation is reached (Chia et al., 2018; Jebali et al., 2018b; Mohsenpour et al., 2021). Excess energy resulting from the absorption of light beyond cellular requirements is dissipated as heat by non-photochemical quenching (NPQ) (Kim et al., 2019). Photoinhibition results due to the formation of reactive oxygen species (ROS) formed as a result of excess energy which is not utilized via the NPQ dissipation capacity of the cell. ROS reduces the quantum yield of PSII by reversibly damaging photosystems (Sánchez Zurano et al., 2020). Light availability to the cell and degree of light attenuation is a function of the incident light, biomass concentration, culture depth, and turbulence due to mixing (Jebali et al., 2018a).

From Figure 4.6 it is observed that biomass concentration tended to increase or decrease following periods of high or low light intensity respectively. A similar trend was noted by Mantovani et al., (2019) who cultivated a consortium of green algae in a 5.78 m^2 raceway pond on urban municipal centrate. Mohsenpour et al., (2021) suggested that the generally accepted illumination saturation point for freshwater algae is between 200 and 400 $\mu mol/m^2/s$. Results obtained from the ANFIS model in this study (Figure 4.33 and Figure 4.34) gave the same optimal light intensity for increased biomass productivity in our system.

Fv/Fm is commonly used as a measure of culture health. Fv/Fm represents the quantum yield of PSII and is an accurate measure of the stress of PSII due to photoinhibition, nutrient starvation, and other factors. Stress conditions and poor culture health are denoted by Fv/Fm below 0.5 (Kim et al., 2019). The quantum yield showed that most of the runs (Figure S 1-Figure S 5) underwent different levels of stress (Fv/Fm > 0.5) during the cultivation period. Run 4 (Figure S

1) showed an increase in quantum yield of PSII from inoculation to day 2 before the Fv/Fm levelled above 0.6 for the cultivation period in the circular ponds. The raceway ponds showed stress-induced from days 14 to 21 corresponding to a decrease in biomass density for this period. As the light intensity also declined sharply from day 14, it is likely to have been due to insufficient light rather than photoinhibition. Runs 5 (Figure S 2), Run 8 (Figure S 3), Run 9 (Figure S 4), and Run 10 (Figure S 5) did not demonstrate prolonged periods of stress with slight decreases occurring in the first few days of growth. Kim et al., (2019) cultivating *Chlorella* sp. HS2 in a 2L flat panel photobioreactor demonstrated a linear increase in biomass from light intensities of 50 to 450 μmol/m^2/s and achieved maximum productivity at 750 μmol/m^2/s whilst still maintaining a high growth rate up to 900 μmol/m^2/s. In their study, Fv/Fm decreased slightly with increasing light intensity up to 550 μmol/m^2/s and declined sharply from 550 to 900 μmol/m^2/s. Average light intensity in the raceway pond (this study) ranged from 359±212 μmol/m^2/s in mid-winter to 645±330 μmol/m^2/s in mid-summer with the maximum intensity of 1323 μmol/m^2/s.

The relationship between light intensity and biomass productivity cannot be oversimplified as it is affected by temperature, culture depth, culture density, and mixing (Sutherland et al., 2020) found that the quantum yield of PSII increased with light attenuation in open pilot-scale and full-scale raceway ponds. They determined that at low light, the quantum yield of PSII was proportional to light intensity irrespective of the size of the pond during summer and winter. The increased quantum yield of PSII in summer was a function of the light/dark cycles experienced by the cells due to higher mixing. Adequate dark periods allow relaxation of the photosynthetic quenching process and result in a higher relative electron transfer rate as opposed to extended exposure to irradiance which causes photoinhibition and thereby lower biomass productivity.

4.4.3 Temperature

The cultivation of microalgae in outdoor conditions is considerably influenced by temperature. The growth rate of microalgae increases exponentially with an increase in temperature until the optimal is reached after which growth rates see a steep decline. Temperate climates are beneficial to the cultivation of algae in order to enhance the potential of year-round cultivation. Photosynthetic carbon assimilation is enzyme-mediated and thereby dependant on temperature (Mohsenpour et al., 2021). Average water temperatures recorded for all periods of cultivation ranged between 20.61±0.68 in winter and 31.03±2.22 in summer (Table 4.2). Temperatures below 30°C enhance CO_2 solubility (Koley et al., 2019) and can affect the biochemical composition of the algae (Chia et al., 2018). Deeper ponds usually experience lesser temperature fluctuations ponds which may result in a lower average specific growth rate during the day and may result in a higher loss of biomass due to respiration at night (Jebali et al., 2018a; Khawam et al., 2019).

4.4.4 Mixing

The efficacy of mixing has a direct effect on biomass productivity. This affects the light intensity to which cells are exposed by keeping the culture in suspension and the level of gaseous exchange, especially in systems that do not supply an external source of CO_2 (Anto et al., 2020). Vertical mixing through the water column allows for more effective movement of cells from light to dark areas and the lack thereof will often light to cells in the upper portions of the column to become light-saturated and possible undergo photoinhibition whilst cells in the lower portion receive little to no light (Sutherland et al., 2020). Koley et al., (2019) cultivating *Scenedesmus accuminatus* in raceway ponds found that culture density more than doubled (0.49 to 1.11 g/L) in 30 days when

impellor speed was increased from 25 to 65 rpm giving a change in water velocity from 0.25 m/s to 0.35 m/s. The power consumption was found to be 66.3 W/m^2/d and 90 W/m^2/d for 0.25 m/s and 0.35 m/s respectively. The average water velocity achieved in our pond is between 0.15 and 0.21 m/s for different sections of the pond. The lower velocity achieved occurs as a function of the size of the pond being ~1145 m/s with paddlewheel driver by 2 x 1500 motors at a power consumption of 62.88 W/m^2/d. The biomass content achieved by our study ranged from 0.38 g/L to 1.11g/L which is in the range achieved by Koley et al., (2019) but at lower power consumption. As the paddlewheel rpm was kept constant for all our runs, increased mixing velocity would potentially be achieved by the lower depth of the pond. The paddlewheel has 50 mm clearance from the bottom of the pond which allows us to have the impellor blades at least 66.66% immersed at lower depths of 150 mm. An increase in pond width can result in poor mixing due to a decrease in water velocity (Javed et al., 2019)

4.4.5 **pH**

pH is an important factor for a number of reasons i) it affects nutrient availability; ii) it affects CO_2 uptake, iii) it affects cell wall-associated enzymes mainly affecting photosynthesis (Chia et al., 2018). Optimal pH is strain-specific however most algae grow at a pH of 6 to 10 (Chia et al., 2018). *Chlorella* sp. tends to accumulate higher amounts of lipid at elevated pH, but this comes with reduced biomass production (Belanger-Lepine et al., 2018; Chia et al., 2018). An increase in pH is commonly in algal cultivation systems and is usually indicative of CO_2 limitation at high levels. An increase in pH to ~10 has been noted by researchers during peak periods of photosynthesis and is usually aligned to high dissolved oxygen (Bagchi et al., 2019). ANFIS Modelling showed by pH shows up as an important factor to algal cultivation. Many algae species can tolerate a pH range of 7 to 9.5. Change in pH may negatively affect photosynthesis and enzyme

activity whilst also affecting nutrient availability (Yew et al., 2019). pH significantly affects the availability of carbon in the system. pH above 10 reduces the availability of carbon due to a shift in equilibrium from carbonic acid to inorganic carbonate which cannot be used by the algae (Eze et al., 2018). In a system that was already carbon limited, the effects can be more pronounced as seen in the model (Figure 4.35). pH shock (10.5-11) is commonly used to reduce the load of grazers in opened ponds. The phenomenon also takes place at lower depth, which would imply an increase in light penetration could have been enhanced. Culture depth affects photosynthetic activity, thereby affecting nutrient uptake.

4.4.6 HRT

Hydraulic retention time has a significant effect on biomass productivity in raceway systems. (Mantovani et al., 2019) suggested the use of HRT less than 10 days as a potential method of improving biomass productivity. We concur with the suggestion that higher productivities are achieved under shorter HRT's as we have observed a much lower overall productivity for each of the runs beyond day 14 for the majority of the trials (Figure 4.22). The semi-continuous operation of raceway ponds has resulted in higher biomass productivities with several researchers harvesting between 3 and 8 days (Yun et al., 2018). Bohutskyi et al., (2019) showed that HRT of 3 days gave a polyculture biomass productivity of 30-36 g/m^2/day using primary wastewater. Amini et al., (2018) cultivating *Chlorella vulgaris* showed that biomass productivity decreased with an increase in HRT from 7 to 14 to 21 days of cultivation. They further showed that periodic harvesting on biomass to low densities (0.1 g/L) had a significant effect on the overall productivity of the system irrespective of the cultivation depth.

4.4.7 Pond scale

Khawam et al., (2019) found that elevated raceway ponds were not representative of temperature and biomass productivity of raceway ponds set in the ground due to the damping effect of temperature fluctuations provided by the soil around the pond. Larger raceway ponds are more prone to loss of biomass as a result of sedimentation (cells under respiration in the dark) due to lower frequency of mixing due development of laminar flow and dead zones in long channels (Sutherland et al., 2020). Smaller ponds benefit from increased vertical mixing and therefore higher productivity by increased frequency light/dark cycles, CO_2 (if not supplemented), and reduction in respiration losses due to sedimentation. Although not directly comparably due to the configuration of the ponds, higher biomass productivity was achieved for the circular ponds in this study.

4.4.8 Biochemical composition

Light serves as a source of energy as well as a signalling mechanism in algae cultivation. Under nutrient replete conditions with sufficient light, algal will acquire sufficient energy to reach critical cell size and thus direct carbon to cell division. Under nutrient stress conditions, the lipid biosynthesis pathway is upregulated whilst protein degradation is active, and amino acid synthesis pathways are downregulated (Kim et al., 2019). The effect of high light intensity varies between algal species. Lipid productivity is usually a result of low biomass productivity due to an insufficient supply of light to the cells (Kim et al., 2019). In *Scenedesmus* sp., high light intensity triggers lipid accumulation under nitrogen-limited conditions (Jebali et al., 2018b; Kim et al., 2019). Lipid and carbohydrate biosynthesis pathways are competitive processes (Kim et al., 2019), thus an increase

in one metabolite would lead to a decrease in the other. Carbohydrates can be converted to lipid under stress conditions. (Jebali et al., 2018a)

4.4.9 Challenges experienced at large scale.

During the study, we had encountered several challenges. Although many of them are not unique to our system, we found it to be a learning experience at all stages.

4.4.9.1 Power failures

Power failures in South Africa tend to be fewer failures and more structured shutdowns of power to specific areas for specified periods of time in order to keep reduce pressure on the country's electricity grid. This is often due to breakdowns in generation especially during periods of peak electrical consumption. This is termed load shedding and is a common occurrence for several weeks at a time. Load shedding is usually for periods of 2 hours at a time. The frequency depends on supply and demand and can be as little as 1 session every 2nd day or up to 3 sessions per day. Load shedding occurring at the test site has an impact on the biomass productivity on the system as the paddlewheel shuts down, which in term results in the culture settling. The impact varies depending on the time of the shutdown and prevailing conditions. Frequent load shedding during daylight hours reduces cell irradiance thus stalling biomass production. Another dimension to this challenge is the paddle wheels needed to be started manually and the planted is only manned during working hours. This resulted in delays in starting the paddlewheel if load shedding occurred during the evening and the loss of daylight hours due to lack of mixing. Mitigation strategies that have been implemented are the use of a generator for backup power supply and more recently installation of an automated system for starting the paddlewheel once power has

been restored. We have also had a timer system installed to be able to switch off paddlewheels at night to try to reduce losses due to respiration and conserve energy input into the system.

4.4.9.2 Mechanical breakdown

Although not unique to our system, mechanical breakdowns of the paddlewheel, unfortunately, cost us years of downtime. At the outset, it is prudent to mention that the construction/ retrofitting of the raceway pond was started in 2009 when much less was known regarding large scale pond systems. The paddlewheel was manufactured from stainless steel (for longevity purposes) however this also meant that it was considerably heavy. Several of the breakdowns of the paddlewheel occurred due to the loss of structural integrity of the main shaft. This was likely due to the size and weight of the paddlewheel which spanned the full width of the pond and had a single shaft run by a single motor. The issue was resolved by splitting the paddlewheel into 2 sections and adding a second motor so that each of the sections is able to function as an individual unit should an issue arise rendering either of the paddlewheel sections inoperable. This has occurred on occasion and having one paddlewheel section running has allowed for partial mixing which allowed time for mitigation strategies to be implemented effectively saving the culture.

4.4.9.3 Movement of the Plastic Liner

As mentioned in section 4.2.2, the raceway pond was an existing structure on a wastewater treatment works that was retrofitted for our purposes. The original structure was that of an oxidation ditch which was backfilled with G5 sand and lined with a plastic liner. Breakdown of the paddlewheel thus often resulted in damage to the liner and loss of the culture into the backfill. Further challenges encountered included shifting of the backfill which raised the surface of the liner. When this occurred under the paddlewheel, it resulted in the paddlewheel blade snagging

the liner and ripping it. This challenge was mitigated by the construction of a concrete plinth under the paddlewheel and pinning the liner to the plinth.

4.4.9.4 Ingression of water

The main cause of this challenge was a high water table. As the system was an oxidation ditch that was retrofitted for our purposes, the original one-way values into the ditch were not sealed to prevent the structure from being lifted out of the ground by changes in the level of the water table. This unfortunately coupled with the choice of G5 backfill meant that water collected under the liner during periods of high rainfall which have increased in recent years. This results in the liner lifting, impeding the flow of the culture and drastically reducing effective mixing. Samples for analysis were taken as composite samples in order to minimize the effects of dead spots on the data analysis. The problem was mitigated by the construction of sumps with automatic pumps to continually drain the backfill.

4.4.9.5 Flooding

In recent years, we have had increased incidents of heavy rains over short periods of time. This has resulted in 2 instances of flooding which not only caused the loss of the culture but also damage to the raceway pond (Figure 4.17). There is no workable mitigation strategy for flooding, we do however ensure that stocks of culture are maintained in a pond in the university greenhouse to allow ease of scale-up for reinoculation of the raceway pond.

4.5 Conclusions

Seed culture for the raceway pond was cultivated in circular ponds at the project site on a continual basis. Several runs of the raceway pond were conducted using supplemented PCW over a period of 15 months from mid-winter to spring of the following year. Biomass productivity is

highly variable usually linked to prevailing climatic conditions. Moderate light intensity has been beneficial to biomass productivity until the point of saturation when lower productivities become more prevalent. Shorter cultivation periods have proved to be beneficial to higher biomass productivities. The scale of the cultivation system has also been noted to have an effect on biomass productivity in that smaller systems (raceway ponds and circular ponds) have been more productive than the large-scale raceway pond. This is partially due to more effective mixing in the smaller systems. The lack of mixing has a detrimental effect on biomass production and can allow the proliferation of undesirable species. ANFIS modelling of the raceway pond based on biomass productivity, rendered pH, depth, and light intensity as key factors affecting biomass productivity. Depths of 20-22 cm with light intensity between 200 and 400 μmol/m^2/s correlated positively with increased biomass productivity. Supplementation of CO_2 could be highly beneficial to the system in supply of carbon with is limited and control of pH which could improve nutrient availability to the algae.

CHAPTER 5: ELUCIDATION OF POPULATION DYNAMICS WITHIN THE RACEWAY POND

5.1 Introduction

Open pond systems for the cultivation of algae are preferred to photobioreactors for commodity products due to lower capital and operational costs despite having lower productivities. Contamination on open pond systems by algal and bacteria is common but can become a serious issue should the relevant checks and balances not be in place. This includes restriction in the type of species cultivated which usually will have some competitive advantage to allow use in open ponds. In this chapter, we seek to gain a basic understanding of the types of contamination that occur and the potential roles of the contaminants in the system.

5.2 Materials and Methods

5.2.1 Cell counts

Cell counts (cells/ml) were conducted using a haemocytometer with light microscopy (400× magnification, Zeiss, Germany) as per the method described by Andersen and Kawachi, (2005). Algae were tentatively identified microscopically based on morphological characteristics such as cell shape, size, colouration, branching, and presence of appendages.

5.2.2 DNA Extraction

Samples from the initial stage, the middle stage of cultivation, and the end of the cultivation period were chosen for sequencing to determine the change in bacterial populations Samples from the

experimental runs were collected as grab samples. DNA was extracted using the Qiagen Powersoil DNA Isolation Kit as per the manufacturer's instructions.

5.2.3 Next-Generation Sequencing

5.2.3.1 16S Metagenomic Library Construction

The amplicon library was constructed according to the Illumina MiSeq 16S Metagenomic Sequencing Library protocol (Illumina Inc., 2013) using the primer combination S-D-73-Bact-0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') targeting the V3-V4 region of the 16S gene directly (Klindworth et al., 2013). The amplicons were sequenced on an Illumina MiSeq platform at Inqaba Biotec (Pretoria, South Africa).

5.2.3.2 Data Cleanup using Mothur

The raw Illumina NGS sequencing output data were trimmed, screened, and filtered using the Mothur v1.4.3 software pipeline (Schloss et al., 2009) and chimeras were removed using the Uchime algorithm (Edgar, 2016). The forward and reverse reads were merged to yield one high-quality representative. Parameters were set at Mismatch cost = 1; Minimum score = 40; Gap = 4; and Maximum unaligned end mismatches = 5. Thereafter, cleaned sequences were aligned against the Silva v128 SEED database, according to the MiSEQ workflow (Kozich et al., 2013; Quast et al., 2013). The generated reads were clustered to representative sequences found in the SILVA 16S v128 reference database at 95 % similarity. The script used was modified to match the 16S rRNA V3-V4 region and can be found in Appendix B.

5.2.3.3 Data analysis using R

Illumina MiSeq NGS data were further analysed using the R open-source statistical analysis software (R Core Team, 2019), and the ampvis2 package. Alpha diversity estimates describe the number of species in a single sample, while beta diversity estimates differences in species diversity between samples. To measure alpha diversity, a phylogenetic tree was reconstructed using a Maximum Likelihood approach based on Multiple Sequence Alignment (MSA) of the OTU sequences as generated by Unipro Ugene (Okonechnikov et al., 2012).

5.3 Results

In order to gain an understanding of population shifts in algal ponds, we cultivated algal biomass in a circular pond using BG11 medium which was run 122 days when the pond crashed. A circular pond (Figure 5.1), designated S3, was inoculated with algae biomass with Scenedesmus being the most dominant species at 92.24% relative abundance (Figure 5.2). The pond was fed with BG11 medium at the start of the experiment and run until the system crashed, with only evaporative losses being made up. Biomass peaked initially on day 10 at 0.55 g/L before a steep decline until day 14. This trend was noted throughout the cultivation period of 122 days with peaks on day 28 at 0.528 g/L, day 59 at 0.667 g/L, day 77 at 1.07, day 94 at 1.12 g/L, and day 119 at 1.25 g/L. The Fv/Fm of the culture rose quickly to levels above 0.5 and remained fairly stable above 0.6 for most of the cultivation period except for a decrease to 0.42 on day 35.

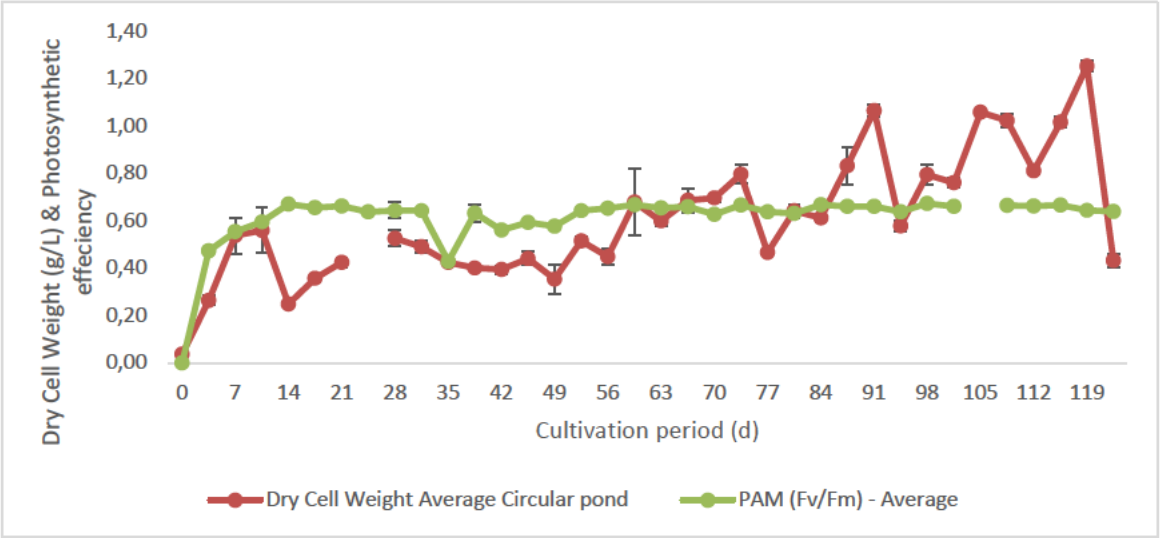

Figure 5.1. Growth profile and quantum yield of PSII of algal biomass grown in BG11 medium without addition in a circular pond under outdoor conditions at Kingsburgh WWTP during winter.

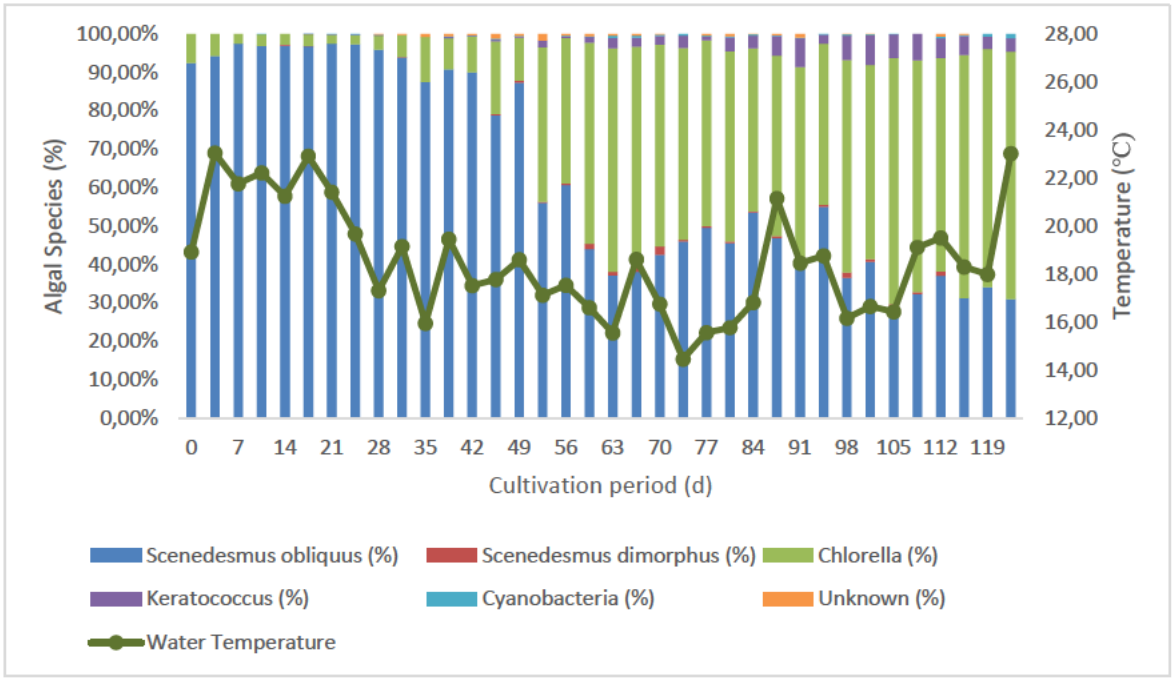

Figure 5.2. Relative abundance of algal species and water temperature over 122 days spanning winter (4 May to 3 September)

Scenedesmus obliquus was the dominant strain at the time of inoculation of the pond and remained more than 80 % dominant until day 42 of cultivation (Figure 5.2). *Chlorella* sp. became and remained dominant from day 59 until the end of the cultivation period with *Scenedesmus* dropping to below 40% after day 98. *Keratococcus* reached a maximum of 7.53%. The dominance of Scenedesmus species followed a trend of decreasing and increasing with a corresponding temperature change. Although *Scenedesmus* has a wide temperature range, it is known to prefer warmer temperatures whilst *Chlorella* sp. grows well in the range of 18°C to 20°C. The period in which *Chlorella* exhibited better growth was from winter to early spring when temperatures were in the preferred range of *Chlorella* sp.

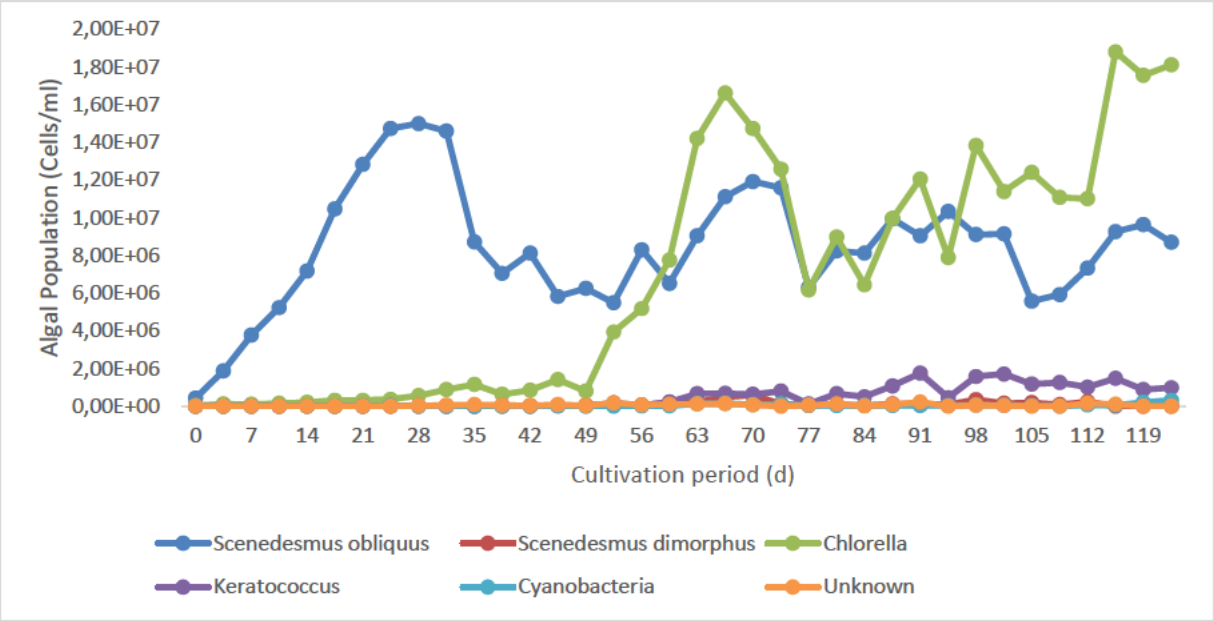

Figure 5.3. Algal population as a function of cell number per ml of culture grown in a circular outdoor pond at Kingsburgh WWTP for a period of 122 days during winter.

Figure 5.3 illustrates the actual number of cells in the culture and demonstrates that although *Scenedesmus* sp. became less dominant from day ~59, the species exhibited fairly constant numbers of cells from its initial decline around day 35. The dominance of *Chlorella* was a result of an increase in the number of *Chlorella* cells rather than a decrease in *Scenedesmus*. *Scenedesmus* showed exponential growth from day 0 to day 24 before the initial decline and stabilisation of cell numbers. *Chlorella* exhibited a similar trend from day 49 to day 66, once the species had established itself.

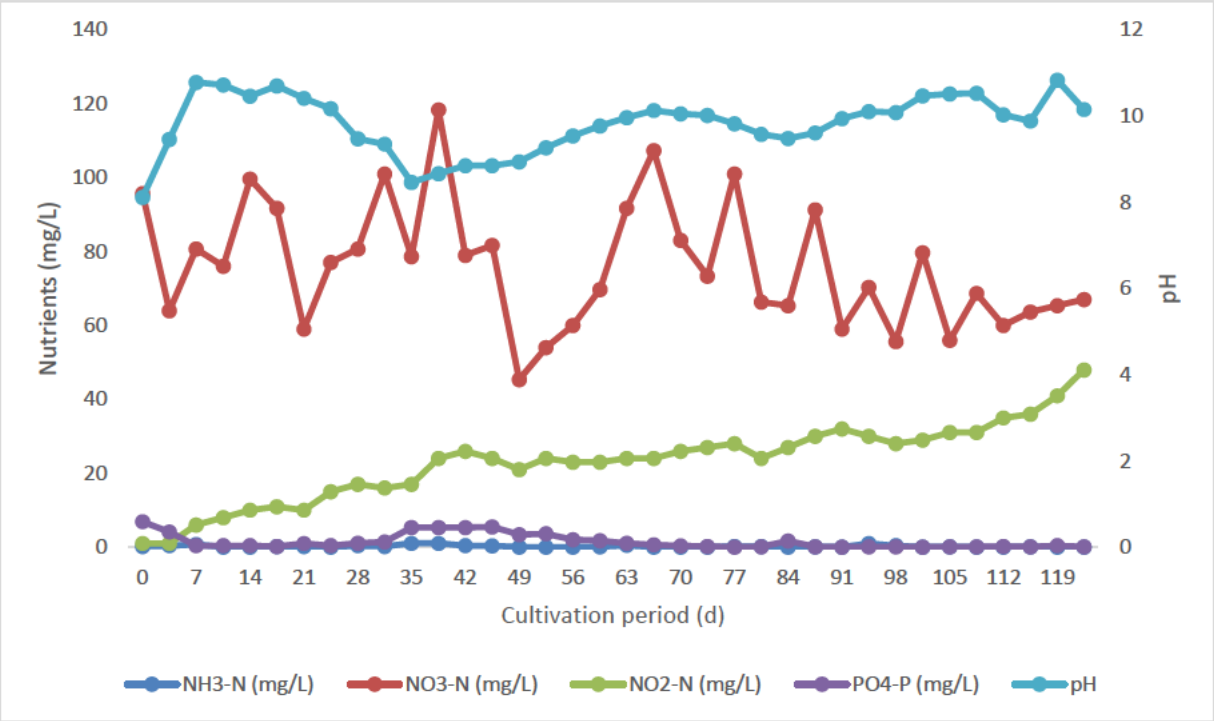

Figure 5.4. Nutrient concentration and pH for culture grown in a circular outdoor pond S3 at Kingsburgh WWTP for 122 days during winter. No error bars shown as the results were validated using quality controls.

Ammonium and phosphate levels were very low throughout the cultivation period (Figure 5.4). Nitrite showed a steady increase from day 3 and continued to increase gradually over the full period of cultivation. Nitrate was highly variable throughout the cultivation period indicating periods of higher utilisation which precede peaks of biomass on days 28, 59, 77, 94, and119 as per Figure 5.1. Changes in nitrate level are likely to be a function of bacterial action resulting in the generation of nitrate at a higher rate than utilisation occurs by the algae.

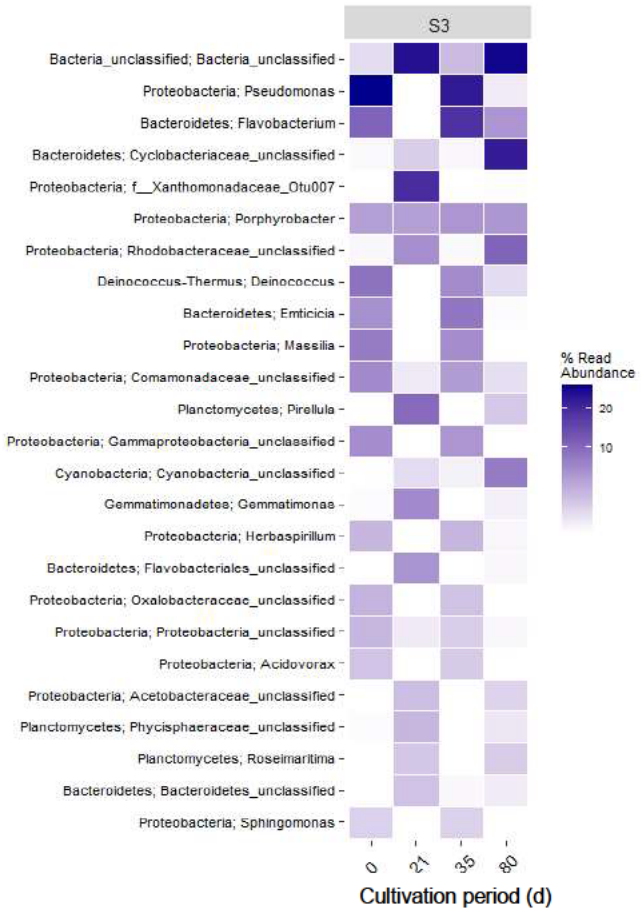

Figure 5.5. Heatmap of bacterial diversity at family level for pond S3 over 80 days of cultivation.

Bacterial populations in S3 also showed a shift for cultivation Figure 5.5. Unclassified bacteria were the most prevalent throughout the cultivation. *Pseudomonas* sp. were the most abundant on days 0 and 35 but were detected in much smaller quantities on days 21 and 80. *Flavobacterium* sp. although slightly less abundant followed a similar trend to *Pseudomonas* sp. Phophyrobacter sp. abundance remained fairly stable throughout cultivation. *Cyclobacteriacea*e and *Rhodobacteraceae* became more abundant towards the latter stage of cultivation.

Algal population dynamics of circular ponds optimizing $NaNO_3$ are shown in Figure 5.6 where *Scenedesmus obliquus* was more than 94% dominant for the entire cultivation period. *Scenedesmus dimorphus, Chlorella, Keratococcus,* and unknown species were prevalent only in small quantities over the cultivation period. The lack of algal diversity is across the three ponds is expected as inoculum was from the same source and the experimental condition was changed in nitrate within a fairly small range.

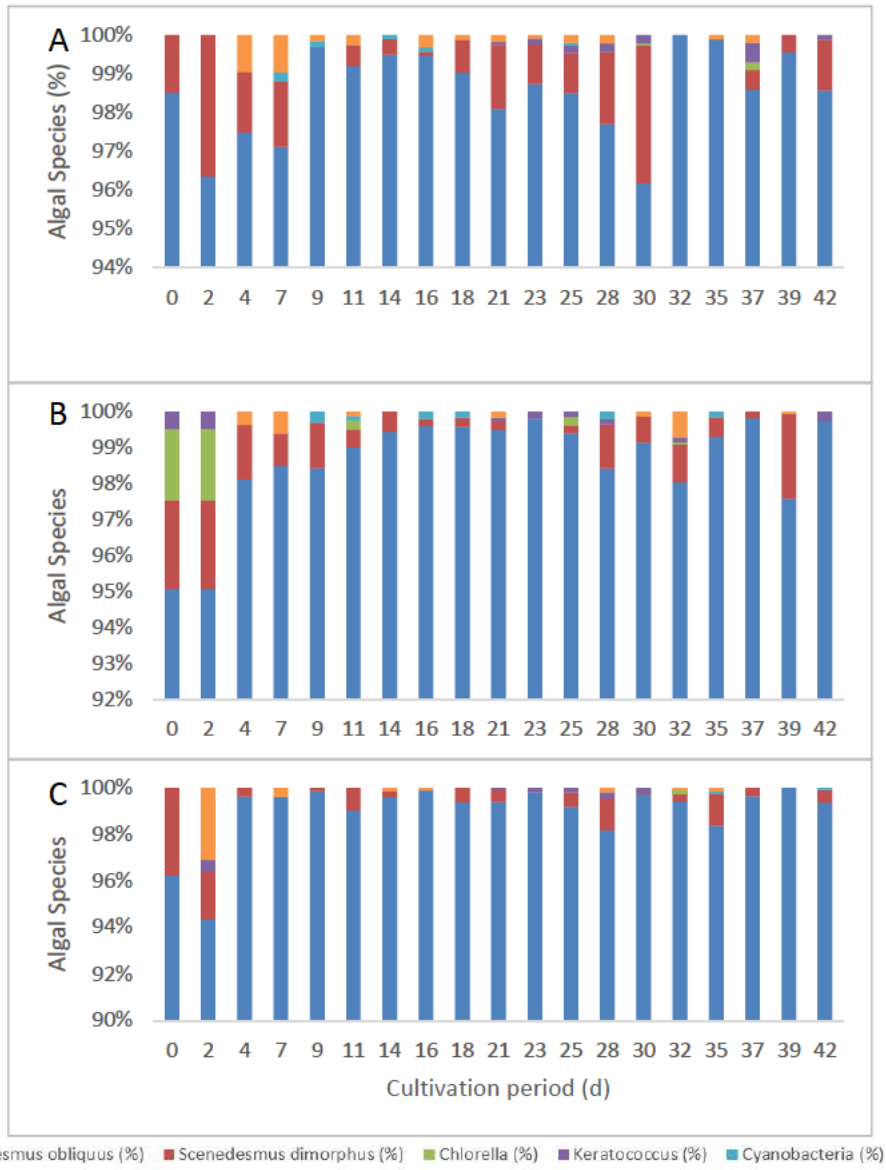

Figure 5.6. Population shifts of dominant species for circular ponds optimizing $NaNO_3$ concentrations with (A) 150 mg/L $NaNO_3$ (KSN 6); (B) 250 mg/L $NaNO_3$ (KSN 7); (C) 300mg/L $NaNO_3$ (KSN8)The most abundant bacteria present across all 3 ponds were *Comamonadaceae* which are denitrifying bacteria. As all the ponds were had a relatively high nitrate concentration at the start of cultivation, this is not unexpected and is further evidenced by the reduction of *Comamonadaceae* to become less abundant throughout the cultivation. This may have more to do with carbon limitation in the latter part of the cultivation period (Figure 5.7).

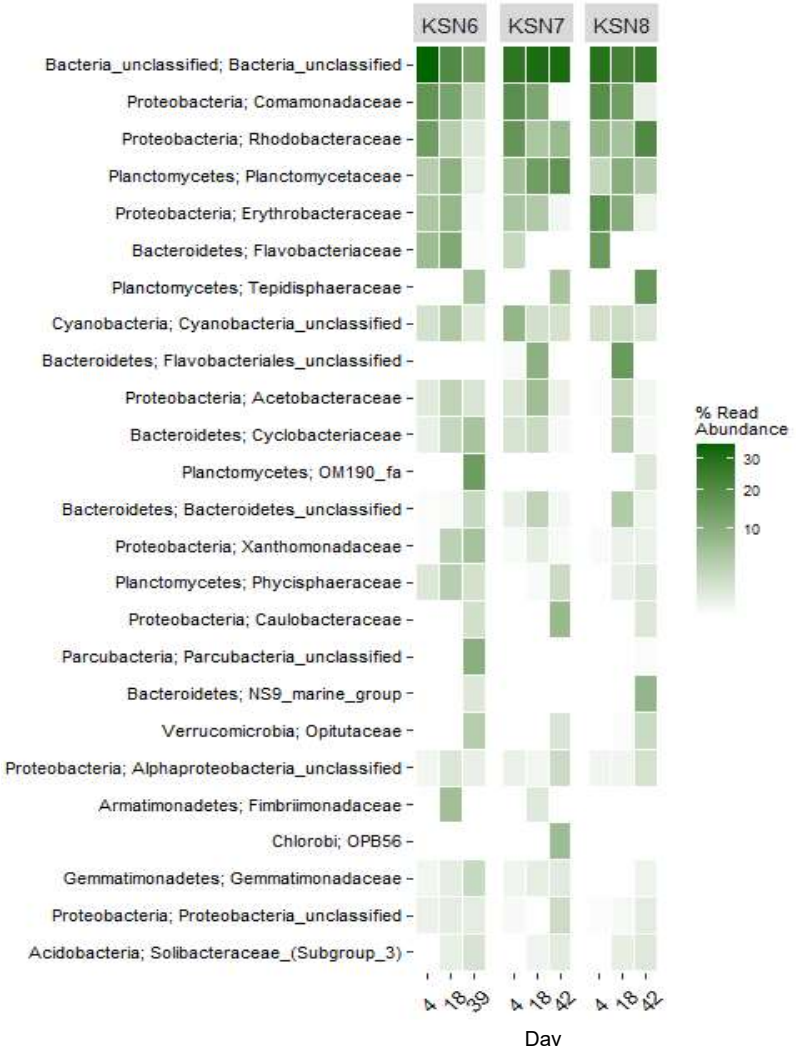

Figure 5.7. Heatmap of bacterial diversity at family level for nitrate optimisation ponds (150 mg/L NaNO3 (KSN 6); (B) 250 mg/L NaNO3 (KSN 7); (C) 300 mg/L NaNO3 (KSN8) over 42 days of cultivation

Erythrobacteriaceae, although less abundant *Comamonadaceae*, exhibited a similar trend. *Rhodobacteraceae* were amongst the most abundant species following *Comamonadaceae* but exhibited a different trend in that they were more prevalent at the start of cultivation in ponds KSN6 and KSN7 than the latter part of cultivation. KSN8 however showed a higher abundance

towards the latter part of cultivation. Plactomycetaceae did not display trends similar to the other bacterial groupings.

Figure 5.8 shows the relative abundance of the most prevalent algal species for all runs of the raceway pond. Although the relative abundances of the species which proliferated in the raceway pond, changed over time and with each run of the pond. The main constituents remained *Scenedesmus obliquus*, *Scenedesmus Dimorphus*, *Chlorella*, *Keratococcus,* and cyanobacteria which have not been further identified. Run 4 of the raceway pond (A) was dominated by *Scenedesmus obliquus* for the first 28 days of cultivation before diminishing to less than 20% from day 33. *Chlorella* sp. increase in abundance from day 28 to become the dominant species by day 40 at 60% of the population. In Run 5 the inoculum contained all strains with *Chlorella* accounting for 49% of the population and *Scenedesmus obliquus* accounting for 38%. By day 9, Chlorella has become 75% dominant and was 97% dominant by the end of the cultivation period on day 30. The major difference between raceway runs was an increase in the average light intensity to 621 μmol/m^2/s for run 5, up from an average of 359 μmol/m^2/s for run 4. Average temperatures for the period were 22.89±2.99 ° C and 22.05±1.58 °C (Table 4.2). Evaporative losses were not made up for Run 5 which could have resulted in higher nutrient conditions.

Run 6 (C) showed the proliferation of cyanobacteria sp. from inoculation of the system and contained 30% cyanobacteria. This decreased to 1.8% on day 7 and *Chlorella* became dominant at 80 %. From day 17 cyanobacteria increased 64% on day 24 and 91% at the end of cultivation on day 21. During this run, we experienced load shedding as detailed in section 4.4.10.1 which result in no mixing for periods from 2 hours upwards, depending on the time of day and when the paddlewheels were restarted. Our previous experience in preliminary trials also showed cyanobacteria proliferate during periods of poor mixing.

Run 8 was started with almost equal amounts of *Scenedesmus obliquus* and *Chlorella* and a small percentage of cyanobacteria. *Chlorella* sp. grew more rapidly than *Scenedesmus obliquus* to 77% abundance at day 2 and 84% for the rest of the cultivation period. We have suggested previously that semicontinuous harvesting could improve biomass productivity. Run 8 is a clear example whereby it was the shortest of the runs and the one with the highest initial and overall biomass productivity (Figure 4.24). Moreover, the results indicate that the dominant culture at the beginning of the run can go on to remain dominant for a period of up to 14 days. Despite their presence, cyanobacteria reached a maximum of 7% on day 2 and remained at lower levels for the rest of the cultivation period. The shorter cultivation period could become a viable strategy for maintaining a culture of choice at large scale.

Run 9 (E) was started with the relative abundance of *Chlorella* at 87% and *Scenedesmus* at only 10%. *Scenedesmus* however, became the dominant culture from day 14 to day 28. *Chlorella* thereafter maintained dominance at 60% abundance.

Run 10 (F) was similar to Run 9 in that shifts between *Chlorella* and *Scenedesmus* spp. are noted throughout the cultivation period. Cyanobacteria reached a maximum of 15% on day 6 and remained between 7 and 12 % of the population from day 13 until the end of the run. *Scenedesmus obliquus* was the dominant strain from day 8 to 18. *Chlorella* sp. was dominant for the first 8 days and from day 18 till the end of the cultivation period. It is important not to try to oversimplify these interactions and changes in population with time as the factors which may affect species dominance can change on a daily basis.

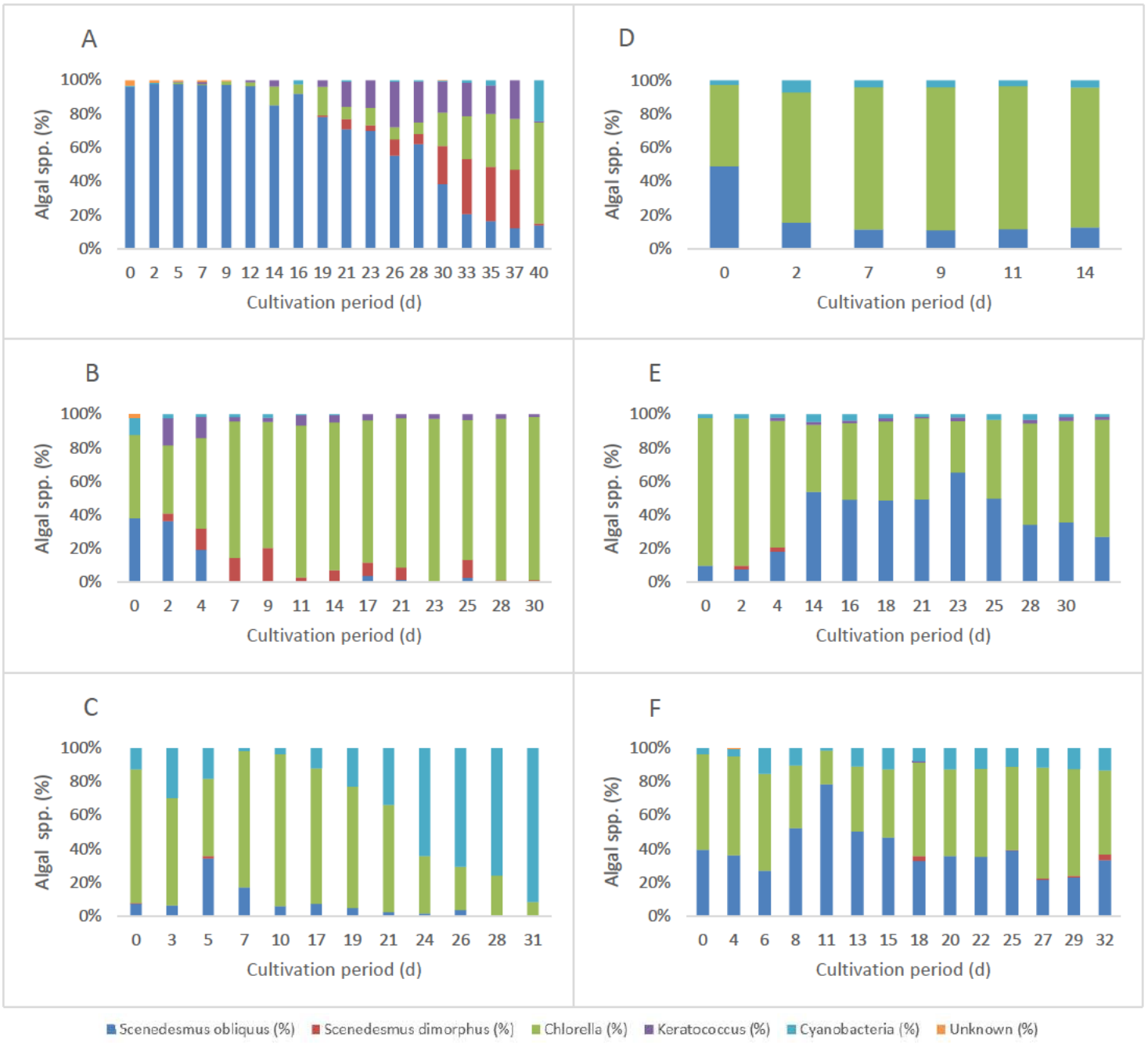

Figure 5.8. Relative abundance of algal species in the raceway pond for (A) Run 4; (B) Run 5; (C) Run 6; (D) Run 8; (E) Run 9 and (F) Run 10

Rhodobacteraceae, Planctomycetaceae, Xanthomonadaceae, Flavobacteriaceae, Phycisphaeraceae, and Comamonadaceae were common to both runs 4 and 5 of the raceway pond (Figure 5.9) despite the differences in running and climatic conditions. *Rhodobacteraceae,* Planctomycetaceae, *Xanthomonadaceae* were more prevalent midday through the cultivation period than at the start and end of Run 4. *Flavobacteriaceae* decreased in abundance over the cultivation period and *Phycisphaeraceae* increased in the same period. Despite the prevalence of the same families of bacteria, Run 4 and 5 did not necessarily exhibit similar abundance or changes in abundance. The abundance of *Rhodobacteraceae* remained stable in Run 5 as opposed to the increase in Run 4. A decrease in abundance was noted for *Planctomycetaceae, Phycisphaeraceae,* and *Comamonadaceae* whilst there was an increase in the abundance of *Xanthomonadaceae, Flavobacteriaceae.*

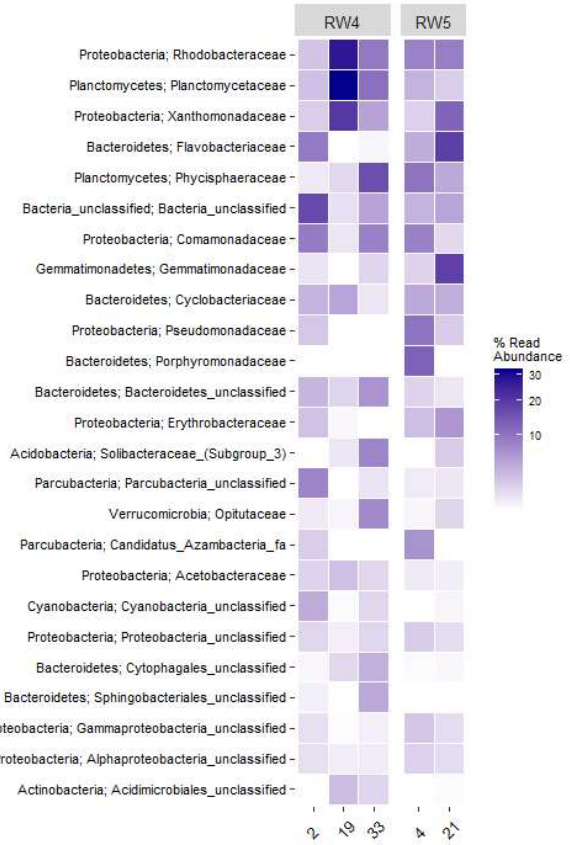

Figure 5.9. Heatmap of bacterial diversity at family level for raceway Run 4 and Run 5

5.4 Discussion

5.4.1 Algal population dynamics

The cultivation of a consortium of algae has shown several advantages over monoculture. Algae grown in consortia are more tolerant to free ammonia than single-species cultures (Pizzera et al., 2019). Cultivation of algae in a consortium has added benefits of imparting production stability, resistance to predation and contamination, and improved adaptability to changes in environmental conditions (Galès et al., 2019; Ramanan et al., 2016; Supriyanto et al., 2019).

Scenedesmus, Chlorella, and some cyanobacteria are common colonists of open systems reported in literature (Yun et al., 2018). Algal species dominance in open systems is dependent on a number of environmental and operational factors including light intensity, temperature, nutrient concentration, hydraulic retention time, and acclimatisation of cultures, selective predations (Sutherland et al., 2017). The mechanisms of maintaining the dominance of a specific culture in ponds using wastewater are still poorly understood and not clearly defined in literature for ponds of a similar scale. This affects the predictability of the harvest and can strongly affect the production of biofuels and bioproducts (Yun et al., 2018).

Chlorella sorokiniana and *Scenedesmus obliquus* are able to grow in 100% domestic wastewater (Galès et al., 2019). Shifts in algal populations are common in raceway ponds and are only regarded as a problem if the culture outcomes the culture of choice for nutrients and is not a producer of the desired product. Population shifts between *Chlorella* sp. and *Scenedesmus* sp. usually occur after prolonged growth periods. This shift can also occur as a result of lower temperatures. Optimal temperatures for *Chlorella* and *Scenedesmus* spp are 22°C and 29°C respectively (Pizzera et al., 2019). *Chlorella* sp. produces maximum lipids between 20 to 25°C with

a sharp decline at 30°C. *Scenedesmus* sp. has a wider growth range being able to survive between 10 and 40°C (Chia et al., 2018; Yew et al., 2019). The optimal temperature for the cultivation of *Chlorella sorokiniana* is between 30-40°C (Khawam et al., 2019). The optimal temperature range for *Scenedesmus obliquus* is between 22-37°C (Khawam et al., 2019). Pizzera et al., 2019) found that during raceway cultivation dominated by *Chlorella* and *Scenedesmus* spp, cultures of *Chlorella* dominated in the spring and autumn correlating with temperatures close to 20°C whilst *Scenedesmus* sp. dominated in the summer months due to their affinity to higher temperatures and irradiance. Ramirez-Lopez et al., (2019) observed *Scenedesmus* and *Chlorococcum* spp. predominated over *Chlorella vulgaris* UTEX 26 after 50 days in a semi-continuous raceway system.

Yun et al., (2018) observed shifts from *Parachlorella* sp dominance in the OPR of a hybrid PBR-ORP system using wastewater to colonizing species was more pronounced in the absence of CO_2 supplementation. OPRs with CO_2 supplementation maintained dominance of *Parachlorella* sp. at 72% and 52% based on biovolume over a 60 day cultivation period whilst the pond without supplementation lost dominance of *Parachlorella* sp. between day 20 and 25 of the cultivation period. Colonizing species were found to be *Scenedesmus, Chlorella*, and *Melosira* spp. which accounted for 90% of the biovolume of the pond by day 29. Supplementation of CO_2 was found to enhance the growth rate of *Parachlorella* sp. thus allowing a competitive advantage to the desired strain. Yun et al., (2018) concluded that operational parameters of the hybrid PBR ORP system were critical to maintaining the dominance of the culture of choice. We concern with the conclusion although control of parameters in the raceway pond is limited significantly more limited than possible in a PBR. Yun et al., (2018) found that algal species, grown simultaneously

using the same inoculum under the same climatic conditions varied greatly depending on the nutrient and CO_2 concentrations.

Chlorella spp. have a greater affinity to lower light concentrations than *Scenedesmus* spp. that have a more effective quenching mechanism. *Chlorella* sp. also has a higher affinity for light than cyanobacteria sp (Liu et al., 2017). As the light intensity during the cultivation period was moderate to high, it would likely favour the growth of the *Scenedesmus* and *Chlorella* sp. This is further evident from the lack of cyanobacteria detected by illumina sequencing (Figure 5.10).

Sutherland et al., (2017) whilst evaluating environmental factors that influence microalgal species in HRAPs found that it was a common occurrence for one species to be more than 60% dominant in the system, however, change in dominance occurred rapidly. A similar trend was noted for our trials (Figure 5.8) whereby either *Scenedesmus* or *Chlorella* was dominant for extended periods of time. Our study differs from Sutherland et al., (2017) in that it has established a natural selection of algae in the ponds rather than inoculation of desired algae as was our case. They further elaborated that low species diversity is common in systems with high nutrient concentrations. As our purpose was to maintain cultures that produce lipids as the dominant strain, maintain a relatively high nutrient concentration is like to have played a significant role in *Scenedesmus* and *Chlorella* remaining dominant.

Sutherland et al., (2017) further found that in their open pond system, the factors which affected algal communities were mainly changes in ammonium influent concentration and the presence of zooplankton grazers. Although grazers were found during the cultivation of the raceway in our study, they did not proliferate, potentially due to the high pH. Further control was instituted

where necessary in the form of pH shock whereby the pH was raised to 11 for 3 to 4 hours. This allowed effective control of grazing populations without major impact on the algal community.

5.4.2 Bacteria Population dynamics

Algae-bacterial consortia have been recently touted for the production of biofuels at large scale. They have potential for improved tolerance to unfavourable conditions (Yong et al., 2020) and usually outperform pure cultures of algae or bacterial due to metabolic compartmentalisation which allows the consortium to perform more complex tasks (Pagnussat et al., 2020). The type of bacteria present can have a positive or negative effect on the growth of microalgae but is dependent on the strains of microalgae and bacteria as well as prevailing conditions making these systems highly complex (Gonzalez-Camejo et al., 2020). This usually entails the exchange of nutrients and/or growth-promoting substances which is usually the basis of mutualistic relationships (Sánchez Zurano et al., 2020; Zhang et al., 2020a). The basics of this relation are usually algae produce oxygen which is used by bacteria who in turn use the oxygen for respiration and produce carbon dioxide which is available to the algae as a carbon source (Li et al., 2019; Mantovani et al., 2019; Yong et al., 2020). Algae release up to one-quarter of the photosynthetically fixed carbon into the system which supports bacterial growth (Fulbright et al., 2018). Bacteria can further fix nitrogen, produce vitamins, and siderophores which can enhance algal growth (Liu et al., 2017; Robles et al., 2020; Supriyanto et al., 2019). Apart from the provision of micronutrients and growth promoters, some bacteria provide macronutrients to algae which can enhance growth, this includes nitrogen by co-culture of nitrogen-fixing bacteria such as *Rhizobium* sp. and inorganic phosphate by a wide range of bacteria including *Escherichia Coli*, *Pseudomonas* sp. and *Bacillus* sp. (Zhang et al., 2020a). These interdependences often improve growth rates and can assist in preventing invasion from other species (Zhang et al., 2020a). Some

bacteria produce a range of antibiotics that prevent the invasion of the system by other bacteria and microorganisms (Liu et al., 2020). Although the interactions are not further elucidated in the scope of this study. Evidence of similar families of bacteria is noted for the different experimental trials as seen in Figure 5.5, Figure 5.7, and Figure 5.9.

The converse relationship whereby algae and bacteria can actively or passively inhibit each other usually occurs by the production of inhibitory substances such as allelochemicals or lytic enzymes produced by bacteria and exotoxins produced by algae (Mohsenpour et al., 2021; Ramanan et al., 2016; Zhang et al., 2020a). Some metabolites produced by *Alteromonas* and *Psuedoalteromonas* sp. are known to inhibit respiration and protein synthesis resulting in cell lysis in algae. Certain species of algae from *Prasinophyceae* and *Bacillariophyceae* are known to produce antibacterial substances in the form of certain fatty acids, chlorellin, terpenes, and chlorophyll-a derivatives. Certain algae metabolites have shown bactericidal activity against *Pseudomonas aeruginosa* (Mohsenpour et al., 2021) and could explain the reduction of *Pseudomonas* as seen in Figure 5.5 where the *Pseudomonas* goes from being amongst the most prevalent to not detected and the cycle repeats during the extended cultivation period. *Pseudomonas* is known to negatively affect algal growth (Mark Ibekwe et al., 2017). Depending on the species, *Pseudomonas* is capable of heterotrophic nitrification, aerobic denitrification, and aerobic conversion of ammonium to nitrogen (Luo et al., 2019; Shi et al., 2020). Pseudomonas has also been linked to polysaccharide degradation in algal blooms (Ye et al., 2020). This will require further investigation as bacteria were only determined to the family level.

Despite the rich nature of the algal phycosphere, such as the availability of organic carbon, not all bacteria are able to survive in such environments (Ramanan et al., 2016). Factors affecting microalgae bacteria interactions are most commonly nutrient availability, however abiotic

conditions such as pH, light intensity, temperature, mixing, and hydraulic retention time weigh in heavily. The relationship between algae and bacteria can shift from mutualism to parasitism depending on the prevailing conditions, including cultivation time, N:P ratio, carbon, and light intensity (Liu et al., 2020; Zhang et al., 2020a). Carbon limitation can improve the symbiotic relationship between algae and bacteria (Liu et al., 2017). Le Chevanton et al., (2013) demonstrated the growth of several bacteria species from *Flavobacteriaceae, Cyclobacteriaceae,* and *Rhodobacteraceae* amongst others which were grown in combination with algae with the addition of an organic carbon source, indicating that organic carbon was obtained from the algae. Mohsenpour et al., (2021) noted that photoperiod and light intensity has a significant effect on algae bacterial interactions. Fulbright et al., (2018) analysed various scales of photobioreactor systems and found that bacterial populations at different scales of cultivation were distinct in composition.

Our study showed that the type of bacteria most abundant in the cultivation systems were from similar families although the abundance changed over time and likely as a result of a combination of nutrients and prevailing climatic conditions. Proteobacteria and bacteriodetes are likely to be associated with green algae and perform similar to identical functions to plant growth-promoting rhizobacteria (Liu et al., 2017; Ramanan et al., 2016). This trend was observed in this study whereby proteobacteria and bacteriodetes were amongst the most dominant bacteria found in all the trials. *Rhodobacteraceae, Planctomycetaceae, Xanthomonadaceae, Flavobacteriaceae, Phycisphaeraceae, and Comamonadaceae* were found to be the dominant bacterial families throughout our investigation.

Rhodobacteraceae are generally accepted as algal growth promotors and play an important role in algal microbiomes. They produce indole acetic acid and phenylacetic acid which promote growth

and tropidithietic acid which stave of algicidal bacteria such as *Alteromonadaceae* (Chen et al., 2020). *Rhodobacteraceae* are known to be suppliers of vitamins such as B_{12} and siderophores to algae (Dogs et al., 2017; Shao et al., 2020). Some species within the family secrete siderophores which solubilize iron making is available to algae for enhanced growth (Dogs et al., 2017).

Members of *Planctomycetaceae* are thought to be specialized degraders of algal sulfated polysaccharides (Tait et al., 2015). Some *Planctomycetaceae* are anammox bacteria that convert ammonium to nitrate (Cheng et al., 2021). These potentially contribute to the increase in nitrate over the cultivation period for both the circular pond and raceway experiments.

Xanthomonadaceae typically contains species responsible for the degradation of organic matter (Hassan et al., 2019). They have the ability to improve phosphorus availability as they possess extracellular phosphatase (Mark Ibekwe et al., 2017).

Comamonadaceae species are common denitrifying bacteria (Cheng et al., 2021). Carbon is a key growth substrate for denitrifying bacteria. Reduction in *Comamonadaceae* at the middle and later stages of ponds KSN6-8 could also have been as a result of lower carbon availability which became the limiting substrate for both algae and bacteria (Liu et al., 2017). Some species of *Comamonadaceae* have the ability to accumulate phosphorus. They absorb phosphorus which is converted to polyphosphate for energy production (Cheng et al., 2021).

Flavobacteriaceae act as denitrifiers and degraders of organic matter including high molecular mass organic matter and several biopolymers (Shi et al., 2020). They can produce carotenoids that have antioxidant activity and are known to produce enzymes that degrade certain metabolites produced by microalgae. Some species within the *Flavobacteriaceae* play a role in the mineralisation of organic matter either directly or indirectly (Zhang et al., 2020b).

Although limited information is available on *Phycisphaeraceae* with regards to algal cultivation, the family is associated with nitrification and good nitrification performance in activated sludge (Schveitzer et al., 2020). As the project site is on a wastewater treatment plant, it is conceivable that the family is present in the wastewater treatment system.

5.5 Conclusions

Shifts in algal and bacterial species are expected of open systems during the cultivation period. Cultivation of algae over an extended period showed that although algal species shifted, strains of interest, *Chlorella* sp. and *Scenedesmus obliquus*, remained dominant. *Scenedesmus obliquus* dominance did not drop below 94%. This was run using a similar nitrate concentration under the same climatic conditions. The runs of the raceway ponds, however, showed changes in dominance using the same media but under different environmental or running conditions. We, therefore, conclude that environmental and running conditions have a great effect on algal succession. Bacterial population dynamics of the experiments found that *Rhodobacteraceae, Planctomycetaceae, Xanthomonadaceae, Flavobacteriaceae, Phycisphaeraceae, Comamonadaceae, Pseudomonas* were the most bacterial families present for all the experiments. The elucidation of bacterial families gave an indication as to the potential roles, however further in-depth studies are required in order to gain obtain any firm conclusions.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The propagation of a specific type or types of algae towards the production of a product such as lipids requires the determination of the conditions to obtain optimal biomass productivity. Open systems have many advantages in terms of the business case for biofuels from algae, however, they are complex ecosystems that have a number of factors that are cannot be feasibly controlled. Multi-stage optimization is required for biomass optimization as laboratory and pilot-scale results do not translate well to large scale.

Treated wastewater required nitrogen supplementation in order to be a viable medium for algal cultivation and nitrate was determined to be the most suitable nitrogen species for the system. Supplemented wastewater achieved similar results to conventional BG11 medium with increase lipid production which is beneficial to biofuels production due to lower cost.

Reasonably high biomass productivities and lipid content were achieved in 10 m^2 pilot-scale raceway ponds in the greenhouse. Algal productivities of the large scale raceway pond have been shown experimentally and through recent literature to be well below the theoretical maximums in open pond systems. Biomass productivities, although on the lower end of the spectrum, were still in line with published literature for cultivation in raceway ponds at a similar scale. Modelling the system is a valuable tool in determining the optimal conditions for biomass productivity. pH, depth, and light intensity were shown to be factors having a substantial effect on biomass productivity. Moderate light intensities in the region of 200 to 400 µmol/m^2/s at a depth of 20 to 22 cm correlated positively with increased biomass productivity. control of pH can only feasibly be carried out by the supply of CO_2. As the system was carbon limited addition of CO_2 could

significantly enhance the overall biomass productivity. This supply of CO_2 however would depend strongly on the availability of a waste CO_2 stream in close proximity to the culture ponds which is not always readily achievable as in our case.

One of the major factors negatively affecting biomass productivity was the size of the pond. Inadequate mixing impacts biomass productivity in terms of access to nutrients and gaseous exchange. Our raceway pond was excessively wide which was a major contributor to inefficient mixing. This coupled with the challenges encountered could have resulted in significantly lower productivity. Smaller ponds in this study and from literature tended to have better biomass productivities. The large-scale pond did however compare favourably with ponds of similar or larger size. The length of the cultivation is another factor that could have significantly affected biomass productivity. Shorter periods of cultivation resulted in higher productivities. For the scale of the system, semi-continuous harvesting would be required to achieve shorter residence time. This must be balanced against the energy utilization and cost of harvesting potentially lower culture densities.

Population dynamics of the algal cultivation systems are critical in culturing algae for a specific product such as lipid. Shifts in the algal population, although expected can be controlled to some degree in favour of cultures producing the desired product. This can be partially achieved by shorter cultivation periods. A large percentage of the bacterial population detected in the ponds were predominantly beneficial families involved in mutualistic relationships such as the supply of vitamins and siderophores or were involved in the degradation of organic matter and mineralization of compounds making them more available to algae.

The base requirement for the production of a fuel product is a positive energy balance. Consideration of the business case is essential for the uptake of any technology. The reality is that algal biofuels will only become viable once the price of fossil crude increases significantly. There is still great potential for algal biofuels technology in the long term, however technological advancements in terms of harvesting and conversion of not only the oil but the whole biomass to energy products are essential.

6.2 Recommendations

The addition of carbon is key to the improvement of biomass productivity as in temperate climates with adequate light as is the case in our study, carbon becomes the major limiting factor to biomass productivity. Proximity to a waste carbon source is greatly beneficial to achieving higher productivity. Future research should entail the design and application of an effective system for the supply of CO_2 to the raceway pond.

Shorter cultivation periods have been shown to produce higher biomass productivities. This can be accomplished by semicontinuous harvesting. We are in the process of installing a 25 m^3/h separator which will allow us to undertake semicontinuous harvesting and assess its effects in future research.

Bacterial interactions in algal cultivation systems have not been well detailed in literature. In the study, we determined bacterial populations to the family level. Investigation to genus and species level could give a wealth of knowledge towards understanding algal bacterial interactions and warrants significant investigation.

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APPENDIX A: SUPPLEMENTARY DATA

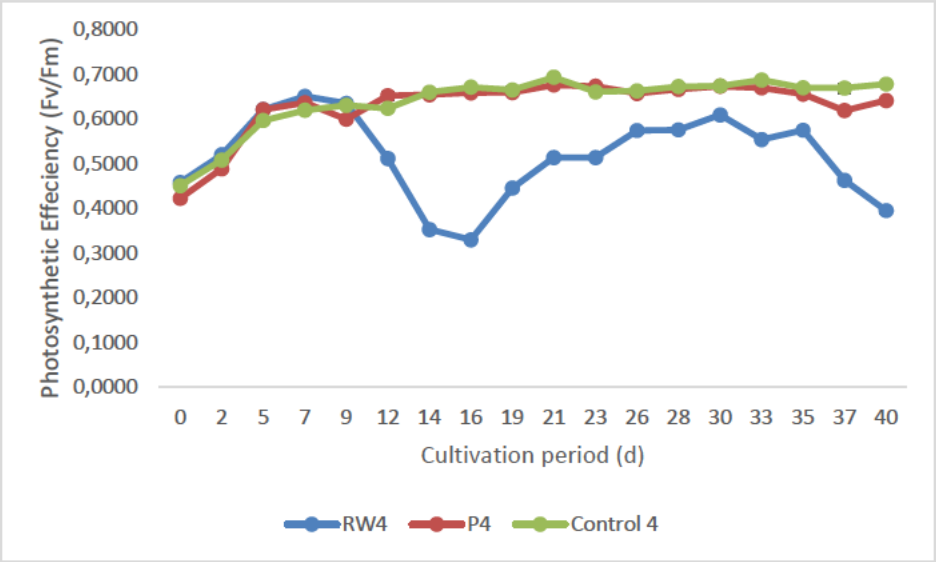

Figure S 1. Quantum yield of PSII of algae cultivated in a raceway pond, circular pond using mBG11+ PWC and BG11 control as per Run 4.

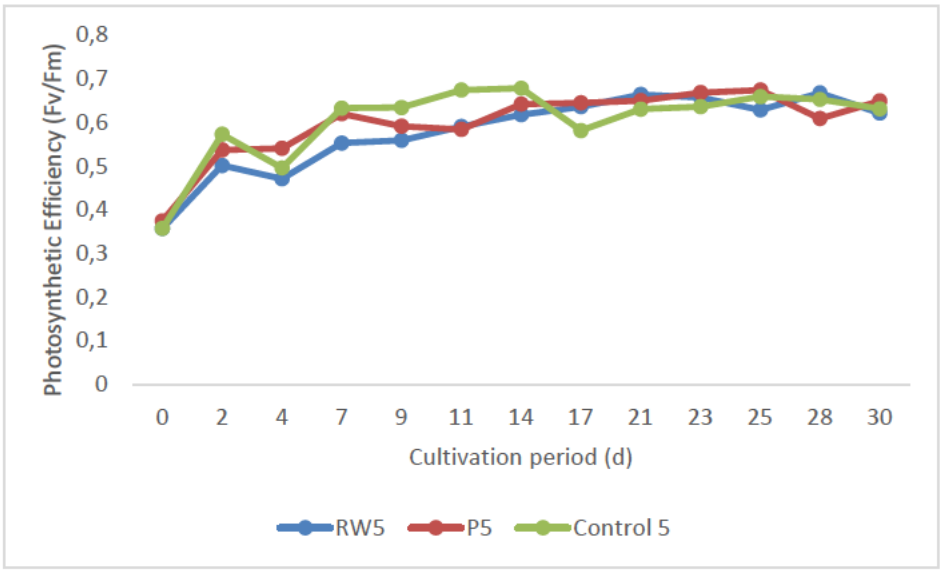

Figure S 2. Quantum yield of PSII of algae cultivated in a raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 5.

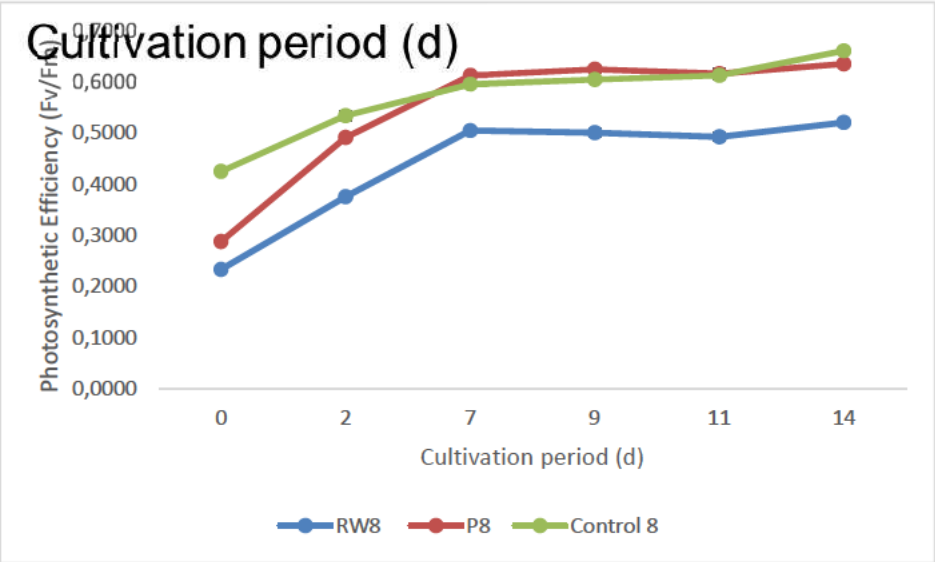

Figure S 3. Quantum yield of PSII of algae cultivated in a raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 8.

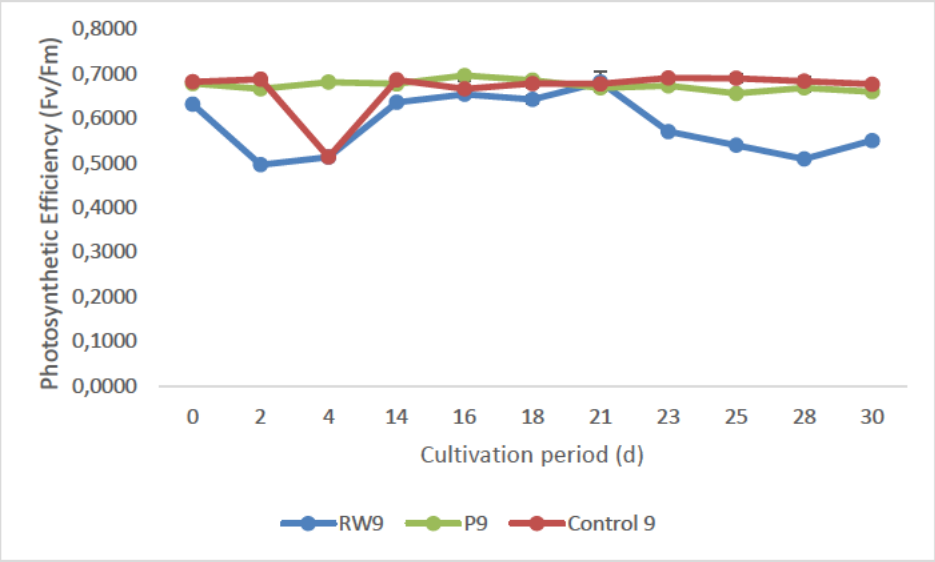

Figure S 4. Quantum yield of PSII of algae cultivated in a raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 9.

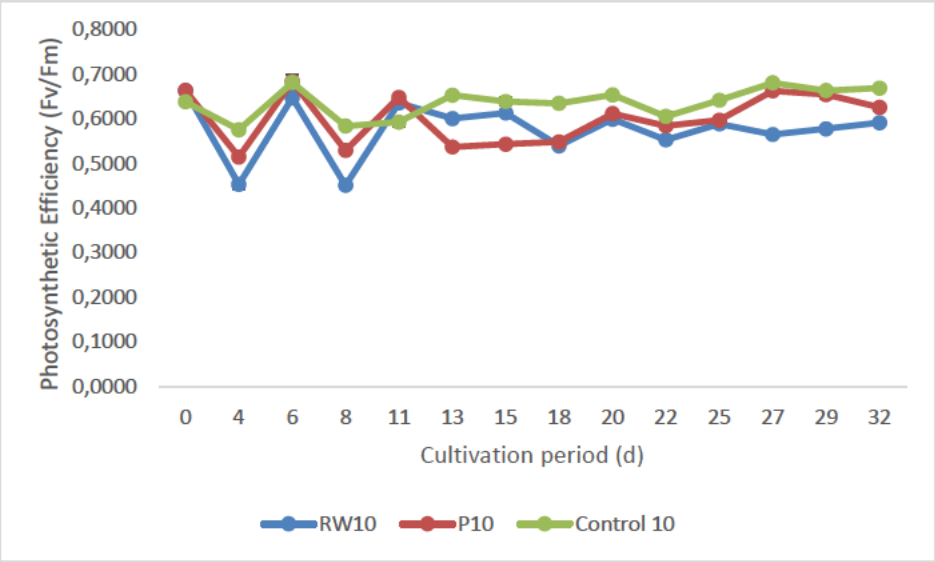

Figure S 5. Quantum yield of PSII of algae cultivated in a raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 10.

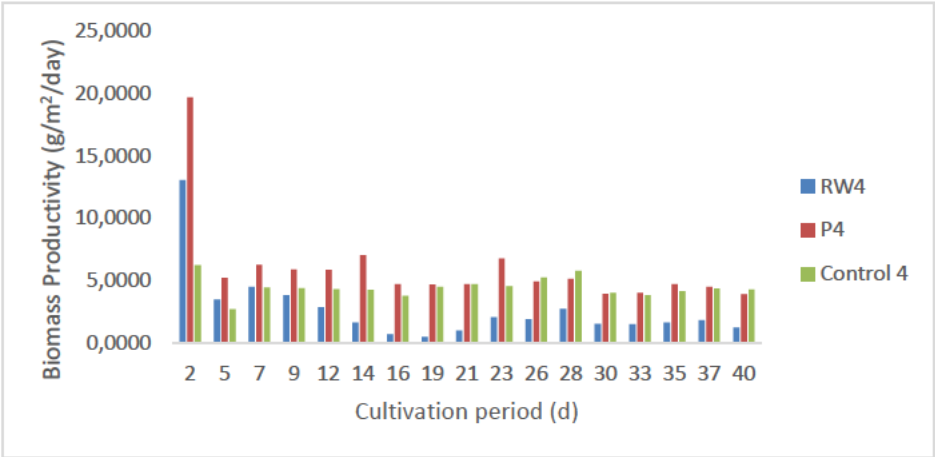

Figure S 6. Biomass productivities for the period of cultivation of the raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 4.

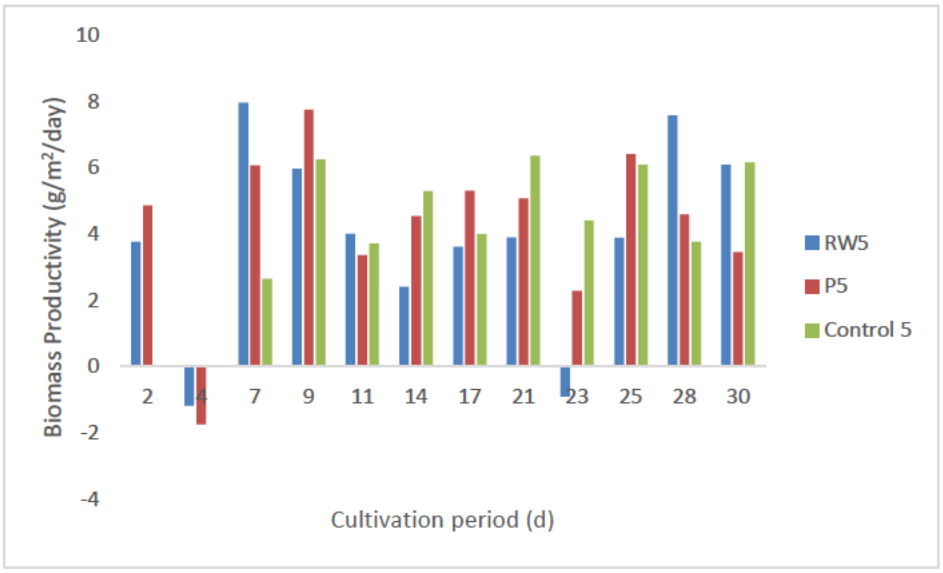

Figure S 7. Biomass productivities for the period of cultivation of the raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 5

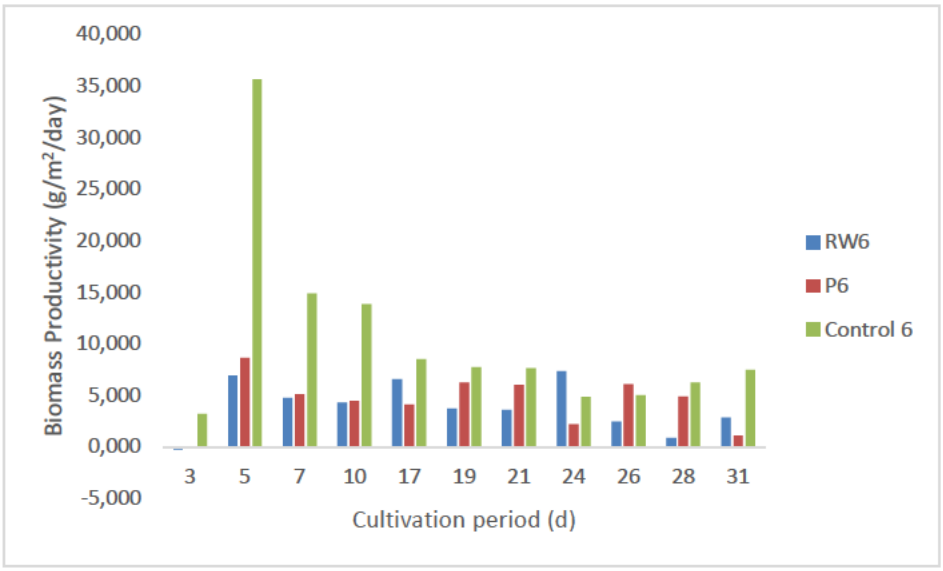

Figure S 8. Biomass productivities for the period of cultivation of the raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 6

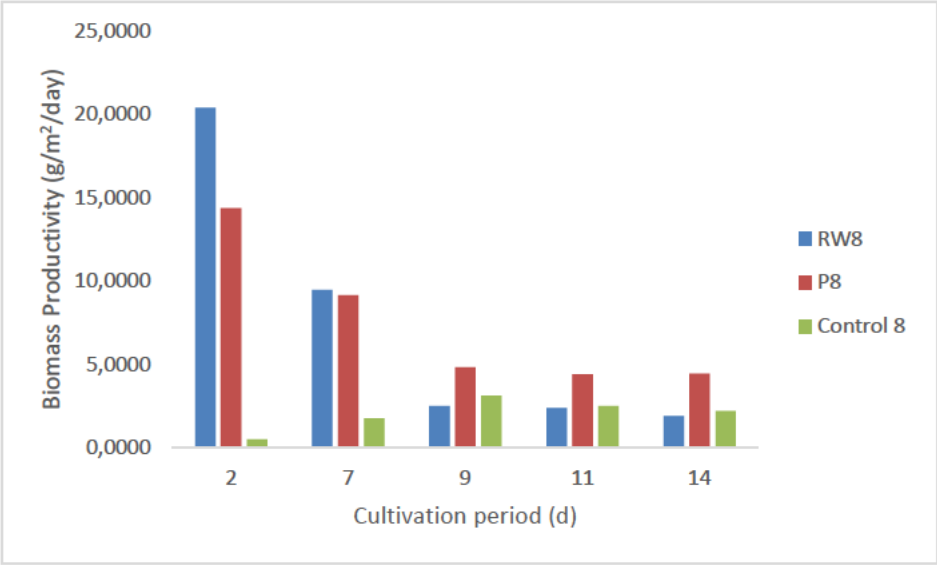

Figure S 9. Biomass productivities for the period of cultivation of the raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 8

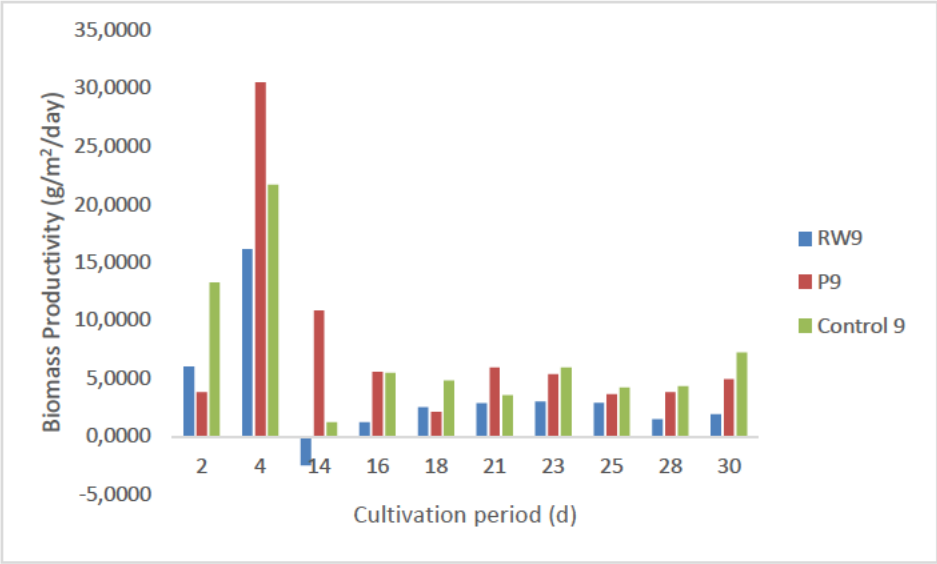

Figure S 10. Biomass productivities for the period of cultivation of the raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 9

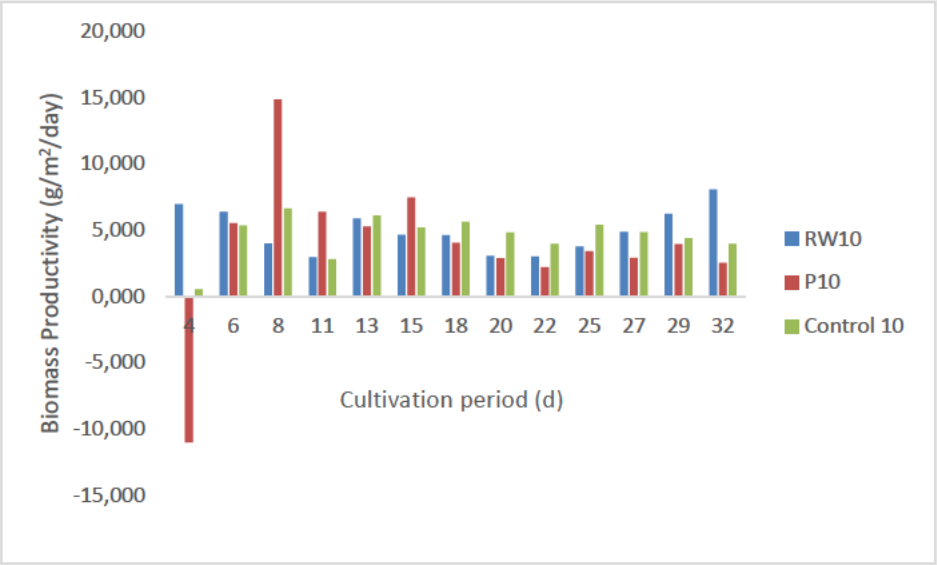

Figure S 11. Biomass productivities for the period of cultivation of the raceway pond, circular pond using mBG11+PWC and BG11 control as per Run 10

APPENDIX B: MOTHUR CODE

set.dir(output=D:\Documents\#Current_Work\Ismail\16S_Data\Mothur_output)

make.contigs(file=D:\Documents\#Current_Work\Ismail\16S_Data\Fastq_Files\Ismail.files,
inputdir=D:\Documents\#Current_Work\Ismail\16S_Data\Fastq_Files, processors=3)

summary.seqs(fasta=Ismail.trim.contigs.fasta)

trim.seqs(fasta=current, oligos=Primers2.oligos, pdiffs=2)

screen.seqs(fasta=current, group=current, maxambig=0, minlength=440, maxlength=480)

unique.seqs()

count.seqs(name=current, group=current)

summary.seqs(count=current)

align.seqs(fasta=current, reference=silvaPCR_DB_V3V4.fasta)

summary.seqs(fasta=Ismail.trim.contigs.trim.good.unique.align,
count=Ismail.trim.contigs.trim.good.count_table)

##change the start and end values based on the numbers in the above summary

screen.seqs(fasta=current, count=current, start=6388, end=25316, maxhomop=8)

filter.seqs(fasta=current, vertical=T, trump=.)

unique.seqs(fasta=current, count=current)

pre.cluster(fasta=current, count=current, diffs=2)

chimera.uchime(fasta=current, count=current, dereplicate=t)

remove.seqs(fasta=current, accnos=current)

summary.seqs(fasta=current, count=current)

classify.seqs(fasta=current, count=current, reference=silva.seed_v128.align, taxonomy=silva.seed_v128.tax, cutoff=70)

remove.lineage(fasta=current, count=current, taxonomy=current, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)

cluster.split(fasta=current, count=current, taxonomy=current, splitmethod=classify, taxlevel=5, cutoff=0.2)

make.shared(list=current, count=current, label=0.03)

classify.otu(list=current, count=current, taxonomy=current, label=0.03)

phylotype(taxonomy=current)

Genus level

make.shared(list=current, count=current, label=1)

classify.otu(list=current, count=current, taxonomy=current, label=1)

system(rename Ismail.trim.contigs.good.unique.good.filter.unique.precluster.pick.seed_v128.wang.pick.tx.shared Ismail.genus.wang.pick.tx.1.shared)

Phylum level

make.shared(list=Ismail.trim.contigs.good.unique.good.filter.unique.precluster.pick.seed_v128.wang.pick.tx.list,
count=Ismail.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.pick.count_table, label=5)

classify.otu(list=current, count=current, taxonomy=Ismail.trim.contigs.good.unique.good.filter.unique.precluster.pick.seed_v128.wang.pick.taxonomy, label=5)

system(rename Ismail.trim.contigs.good.unique.good.filter.unique.precluster.pick.seed_v128.wang.pick.tx.shared Ismail.phylum.wang.pick.tx.5.shared)

Rarefaction

rarefaction.single(shared=Ismail.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared, calc=sobs, freq=100)

Diversity indices

summary.single(shared=Ismail.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared)

##make biom file for Ampvis analysis

make.biom(shared=Ismail.genus.wang.pick.tx.1.shared,
constaxonomy=Ismail.trim.contigs.good.unique.good.filter.unique.precluster.pick.seed_v128.wang.pick.tx.1.cons.taxonomy)