

# *Synechococcus* PCC 7002 growth in anaerobic digestion effluent and off-gas

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## Abstract

Anaerobic digestion (AD) effluent is a low-cost and nutrient-rich medium produced at significant volumes, on which algae can be grown. The biogas that is produced by AD is commonly burnt, for heating or electricity generation. The off-gas from this process can be used to enrich the gas fed to an algal cultivation system, to enhance inorganic carbon supply to the growing photoautotrophic species. Therefore, a significant opportunity exists for the integration of the wastes from AD (the nutrient rich effluent, and the carbon dioxide (CO<sub>2</sub>) rich off-gas) with the production of microalgae.

The potential of using photosynthetic microalgae to produce valuable products is of interest currently. Not only does increasing dependence on non-renewable energy sources jeopardize sustainable supply but increasing CO<sub>2</sub> emissions also promote climate change. In addition, rising global energy demands of developing nations threaten the availability of sustainable energy for future generations. Since genetically modified strains on microalgae are available, integrating their growth with AD, which will produce nutrients and inorganic carbon, could support the cause for commercial sustainable chemical production.

In this study, AD effluent was treated with a cyanobacterial species, *Synechococcus* PCC 7002, to reduce turbidity and high concentrations of nutrients present. It was illustrated that by lowering the pH, increasing the total dissolved salts and adding a specific macro-nutrient necessary for growth to the AD effluent, the addition of valuable resources (such as water for effluent dilution and CO<sub>2</sub> enriched gas for enhanced growth) can be avoided. This ultimately increases the feasibility of AD-microalgae integrated systems. The study organism was grown in 150 mL batch reactors in modified AD effluent at moderate light conditions to biomass concentrations of up to 1550 mg/L within 310 hours – nearly 3 times faster than in synthetic growing media. By enriching the headspace gas with carbon dioxide to simulate conditions of burnt biogas generated from anaerobic digestion, this biomass concentration was increased to 2230 mg/L within 282 hours. The experiment was repeated, comparing the growth in synthetic media and the optimised AD media in an eight-litre air-lift reactor. Keeping conditions conservative by performing the experiments in low light conditions with air bubbling instead of carbon dioxide enriched air (at a gas hold up of 10% of the total volume of the reactor), the experiment illustrated what growth could be expected at large scale production with minimal costly enhancements. Although both experiments were nutrient limited, higher biomass yields were observed for cyanobacteria grown in AD media ( $\mu_{\max} = 0.018 \text{ hr}^{-1}$ ). For batch experiments in 85% AD effluent enriched with inorganic carbon, total nitrogen was reduced by 90% and in the airlift reactor with atmospheric air bubbling, by 60% within 10 days.

## Opsomming

Anaërobies verteerde afvalwater kan teen lae kostes in groot volumes vervaardig word en is ryk in voedingstowwe wat noodsaaklik is vir die groei van alge. Die biogas wat geproduseer word tydens anaërobiese vertering (AV) kan gebruik word om die gas wat in alge-kweek-sisteme ingevoer word te verryk met koolstofdiksied. Gevolglik is die absorpsie daarvan deur foto-outotrofiese spesies verhoog. Daar bestaan dus 'n merkwaardige geleentheid om the afval van AV ('n voedingstof-ryke vloeistof en 'n koolstof-ryke gas) te integreer met groot skaalse produksie van mikroalge. Die potensiële voordele om so 'n sisteem in plek te stel vir die produksie van waardevolle chemikalieë wat elders die vervaardig sou word is van kardinale belang. Toenemende afhanklikheid op nie-hernubare energiebronne plaas nie net druk op nasionale ekonomieë nie, maar die toenemende koolstof-uitlaatgasse bevorder wêreldwye klimaatsveranderinge. Die stygende aanvraag vir energie in ontwikkelende lande plaas ook druk op vermoëns vir die volhoubare produksie van energie vir toekomstige generasies. Siende dat 'n geneties gemodifiseerde spesies mikroalge ontwikkel is kan die groei daarvan met AV gekombineer word om die kommersiële produksie van biobrandstowwe te bevorder.

In hierdie studie was AV afvalwater behandel met 'n sianobakteriële spesie genaamd *Synechococcus* PCC 7002 om wolkerigheid en hoë konsentrasies van kontaminante te verlaag. Die byvoeging van waardevolle hulpbronne (soos water vir verwatering en koolstofdiksied-verrykte gas vir groei bevordering) kan tot 'n mate vermy word deur eksperiment bedryfde pH te verlaag, opeloste sout konsentrasies te verhoog en sekere makro-voedingstowwe noodsaaklik vir mikroalge groei by te voeg. Dit bevorder die lewensvatbaarheid van AV-mikroalg-integreerde sisteme. Die organisme was in 150 mL bondel reaktors in gemodifiseerde AV afvalwater teen middelmatige lig-blootstelling gegroei tot en met biomassa konsentrasies van 1550 mg/L in 310 ure. Die groeispoed is byne 3 keer vinniger as wat waargeneem was vir dieselfde eksperiment wat in sintetiese media uitgevoer is. Deur die hoofruimte-gas te verryk met koolstofdiksied (om verbrande biogas te simuleer wat deur AV geproduseer kan word), was die biomassa verhoog tot 2230 mg/L in 282 ure. Die eksperiment was herhaal in 'n 8 L lugbrug fotobioreaktor. Kondisies vir groei was in lae lig met atmosferiese lugborreling in plaas van koolstof-verrykte gas om die konserwativeit van resultate te bevorder. Dit is belangrik aangesien dit die geloofwaardigheid van grootskaalse produksie beter verteenwoordig, waar groeikondisies uitgelewer is aan natuurlike elemente en dikwels sub-optimaal is. Ten spyte van die feit dat albei eksperimente se groei beperk was, is hoër biomassa opbrengste waargeneem vir sianobakterië wat in AV afvalwater gegroei is ( $\mu_{\max} = 0.018 \text{ hr}^{-1}$ ). Vir bondel eksperimente in 85% AV afvalwater wat met 10 % (v/v) koolstof-verrykte gas gegroei is was totale stikstof met 90% verlaag en in die lugbrug fotobioreaktor met atmosferiese lug borreling tot 60%, beide binne 10 dae.

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## Nomenclature

Abbreviation	Description	Units
$\mu_g$	Gross specific growth rate	hr <sup>-1</sup>
$\mu_{max}$	Maximum specific growth rate	hr <sup>-1</sup>
$\mu_{net}$	Net specific growth rate	hr <sup>-1</sup>
$\mu_R$	Net specific replication rate	hr <sup>-1</sup>
$\lambda$	Wavelength	nm or 10 <sup>-9</sup> m
<b>A</b>	Surface area	m <sup>2</sup>
<b>AD</b>	Anaerobic digestion	-
<b>ATP</b>	Adenosine triphosphate	-
<b>c</b>	Speed of light	2.998 x 10 <sup>8</sup> m.s <sup>-1</sup>
<b>CUR</b>	CO <sub>2</sub> uptake rate	g.L <sup>-1</sup> .hr <sup>-1</sup>
<b>DMAPP</b>	Dimethylallyl diphosphate	-
<b>E</b>	Irradiance	W.m <sup>-2</sup>
<b>E<sub>p</sub></b>	Energy of one photon	J
<b>E<sub>QF</sub></b>	Photon flux at 1 m	μE.m <sup>-2</sup> .s <sup>-1</sup>
<b>h</b>	Planck's constant	6.63 x 10 <sup>-34</sup> J.s
<b>IPP</b>	Isopentyl diphosphate	-
<b>k<sub>d</sub></b>	Rate of cell mass loss	hr <sup>-1</sup>
<b>m</b>	Mass	mg
<b>A</b>	Electric current	C.s <sup>-1</sup>
<b>MEA</b>	Monoethanolamide	-
<b>MEP</b>	Methylerythritol Phosphate	-
<b>M<sub>w</sub></b>	Molecular weight	g.mol <sup>-1</sup>
<b>N<sub>A</sub></b>	Avogadro number	6.022 x 10 <sup>23</sup> mol <sup>-1</sup>
<b>NADPH</b>	Nicotinamide adenine dinucleotide phosphate	-
<b>N<sub>p</sub></b>	Number of photons	-
<b>P</b>	Power	W
<b>PBR</b>	Photobioreactor	-
<b>PCC</b>	Pasteur Culture Collection	-
<b>pK<sub>x</sub></b>	Logarithmic constant	-
<b>r</b>	Length	m
<b>t</b>	Time	hr
<b>T<sub>k</sub></b>	Temperature	K
<b>TN</b>	Total nitrogen	-
<b>TSS</b>	Total suspended solids	mg/L <sup>-1</sup>
<b>TP</b>	Total phosphorous	-
<b>X</b>	Cell mass concentration	g.L <sup>-1</sup>
<b>X<sub>max</sub></b>	Maximum biomass concentration	g.L <sup>-1</sup>
<b>Y</b>	Yield	Nm <sup>3</sup> .kg <sup>-1</sup>

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## 1. Introduction

Anthropogenic pollution is increasing proportionally with industrialisation and economic development. The wide applicability and increasingly strict enforcement of environmental and sustainability laws show that the fight against pollution and emissions has become a major issue on social, political and scientific agendas. Even if the rate of pollution decreases, it will not be enough to reverse the impact it has had on complex biotic systems. Biomimicry is an approach that has the potential to seek sustainable solutions to humanity's conventional processing challenges. Thus, the thorough understanding and optimisation of these equally elegant and efficient biological solutions is of vital importance and the best place to start is by understanding the problem.

### 1.1 Background

A major problem confronting our civilization is increased water scarcity and pollution. According to findings published by Carrington (2014), there is a 70% chance that the world population will rise continuously from 7 to 11 billion by 2100. This is a strong indicator that not only will sewage water, industrial wastewater, manure and other process wastewater increase proportionally, but water resources that are already depleted through climate change will have to be shared by greater numbers of people.

Liquid fossil fuels are the most widely used source of energy in the 21<sup>st</sup> century. This is mainly shaped by the transport sector, accounting for nearly a quarter of CO<sub>2</sub> emissions, one-third of global energy utilization and half of oil consumption (Alberts, et al., 2016). The fact that they are neither renewable nor sustainable marks a time when the production of valuable chemicals from petrol refineries will not be possible anymore. Additionally, when fossil fuels are burned, problems such as global warming (e.g. increased CO<sub>2</sub> levels in the atmosphere) and environmental pollution is accelerated. One of the major drivers for climate change is record levels of CO<sub>2</sub> in the atmosphere, which steadily increased from 391 ppm in 2012 to 403 ppm in February 2016 (Dlugokencky & Tans, 2016). 76% of the greenhouse gas (GHG) emissions in the atmosphere are CO<sub>2</sub> and 14.5% thereof comes from forestry and land use while the remaining 85.5% is a direct result of agricultural, fossil fuel and industrial processes (Edenhofer, et al., 2014).

It becomes easy to see that these issues will be harder to solve and mitigate as more time goes by and the need to design closed, sustainable systems to integrate and solve these problems in an economically viable way is not regarded as a luxury, but quickly becoming a necessity. The synergistic interaction of AD effluent and microalgae creates the opportunity to design systems where waste products can be used advantageously to not only circumvent sustainability issues mentioned in the previous two paragraphs, but also to possibly produce valuable chemicals. Implementing the process of photosynthesis for the generation of biofuels or high-value chemicals creates an opportunity in which a single host organism

can act both as a photo-catalyst (sunlight absorption) and processor (energy utilization), as well as a CO<sub>2</sub> assimilator.

## 1.2 Importance and benefit of research

Microalgae's large-scale production potential is constrained by the significant costs associated with cultivation, harvesting and product extraction (Alberts, *et al.* 2016). Slade and Bauen conducted a study in 2013 to determine the sustainability and economic viability of algae biofuel systems. The study considered the energy balance, environmental impacts and production costs for both raceway ponds and PBR's. For PBR's specifically, they found that the bulk of production costs are dominated by the capital costs of the PBR itself (26%) while the remainder of the process equipment sums to only 3.31%. They concluded that cost reductions in excess of 50% could be achieved if the main nutrients required for feasible microalgal growth (CO<sub>2</sub>, nutrients and water) can be supplied by valorised waste. One such waste-source which may prove a means to drastically enhance the techno-economic feasibility of algal bioprocesses is the effluent produced from anaerobic digestion.

Anaerobic digesters are used worldwide to produce bioenergy and sustainably treat organic wastes produced from municipal, industrial and agricultural operations. The technology is in many cases the optimal means of converting organic waste into useful products, capturing the energy content in the form of biogas. It has also been shown to reduce odours and pathogens, stabilize waste streams by reducing solids and organic content as well as mitigate GHG emissions compared to other waste management plans (Abbasi, *et al.* 2012). If applied, anaerobic digestion (AD) effluent can be an abundant low-cost source of nutrients. Feedstock for AD contain vast amounts of macro nutrients such as nitrogen and phosphorous, which are utilised by anaerobic bacteria to break down solid organic waste into dissolved organic carbon. Since most manure and other agricultural/industrial wastes are combined with water in a slurry for ease of transportation, it is ideal for growing microalgae. By burning the biogas produced from AD, the combustion process can serve as a source of energy, while microalgal growth will be enhanced by the enriched CO<sub>2</sub> flue-gas produced upon combustion, making it a cost-effective approach to meet a portion of the increasing demand for valuable chemicals, as well as reducing high nutrient content in wastewaters.

Although biofuels and bio-derived chemicals can be produced through a wide variety of methods, microalgae have a few unique advantages which sets them apart from terrestrial crops for this specific application:

- Their high lipid content makes for much larger oil yields per land area compared to terrestrial crops (Christi, 2007).
- They have the potential to add considerable value to non-arable land (Trentacoste, *et al.*, 2015).
- They are flexible with respect to the water sources they can be grown in, including waste and saline water (Dahiya, 2012).



- Some species are amenable to genetic manipulation, and so can be used to produce several valuable chemicals (Robertson, *et al.*, 2011)

### 1.3 Project aims

This project focused on the growth of *Synechococcus elongatus* PCC 7002 in shake flasks. The aim of this study was to investigate the growth of an organism in AD effluent with minimal addition of valuable resources such as water, nutrients and CO<sub>2</sub> and instead, supply these nutrients through AD effluent and other cheap, abundant resources. This can not only expand the application of the technology to water-stricken countries, but also strengthen the economic feasibility of bio-derived chemical processes. A more detailed breakdown of the project deliverables can be found in Chapter 3.

### 1.4 Approach

To compare the study organism's growth in synthetic media to that in AD effluent, it was important to establish growth conditions at which all experiments would be conducted, and to find accurate and trustworthy methods to control and measure them throughout the course of the project.

Once this was established, growing the study organism under ideal conditions found in literature, a base case was obtained to which the remainder of the experimental results could be compared with. This served not only as a source of data to compare the growth of the culture in AD effluent with, but also as a source of information regarding input conditions (such as nutrient speciation, pH, salinity, available nutrients, etc.) to identify which input factors with regards to the AD effluent could be beneficial or detrimental to algae growth.

Once experimentation in AD effluent commenced, the lack of growth under the same operating conditions led to two research paths, and ultimately, hypotheses to be tested:

- i. A nutrient or property necessary for algae growth was not present in the AD effluent.
- ii. A chemical in or property of the AD effluent was toxic to the algal cells, inhibiting growth.

This shaped the entire course of experiments conducted in this project. By designing experiments to test each hypothesis with respect to a specific property/nutrient, the properties of the AD effluent were adapted to host the algal cells and allow growth.

Another important aspect that shaped the research was to adapt the properties of the AD effluent in such a way as to minimise the addition of valuable and scarce resources, and in turn increase the chances of feasible scalability, should the research be applied in future projects. The focus was to reduce nutrient costs associated with producing high-value products through microalgae, and so some of these adaptations compromised the use of resources in other applications.

Growth was quantified through optical density measurements, dry biomass weights and nutrient depletion in the different media and growth conditions.

## **1.5 Scope**

The main aim was to evaluate the effect of replacing optimal synthetic media with AD effluent. Thus, only one synthetic media and AD effluent type was investigated. This enabled the identification of critical nutrients lacking from, and growth inhibiting compounds present in, the AD effluent. The other investigation was focused on the growth observed in a CO<sub>2</sub> enriched atmosphere. All other conditions (inputs) such as pH, temperature, light intensity, light effects, algae concentration and harvesting were to be kept constant, unless it was of specific interest in reaching an objective or testing a hypothesis. All experiments were performed in triplicates for biological repeats, and important variables were tested in triplicate as technical repeats.

## **1.6 Chapter overviews**

Chapter one of this thesis aimed at highlighting why this research is relevant and guides the reader from a broad perspective into the more detailed nuances of three major overlapping themes: valuable chemical production, microalgal growth and anaerobic digestion.

Chapter two contains the literature review and analysis. It expands on the three themes individually and exposes not only the areas where they supplement one another, but also identifies shortcomings and possible obstacles. First, high-value chemicals produced from microalgae are explored, followed by characteristics of microalgal growth. Anaerobic digestion is then placed in context as an alternative wastewater treatment process and thereafter, integration of these two technologies are discussed.

Chapter three focuses on the aims and deliverables of the study with literature input focusing the research.

Chapter four outlines all methodological aspects of the project relating to experimental design, performance and analysis.

In chapter five, the results of these experiments are presented and scrutinised to determine their contribution and effect on the project aims.

Conclusions and recommendations on these results follow in chapter six and seven respectively.

## 2. Literature review

### 2.1 Biofuels and high-value chemicals from microalgae

Concerns about global energy stability, climate change and petrochemical supply have made biomass-based fuels and chemicals a strategic focus in research with the aim of decarbonizing these sectors. Although the most widely applied for this purpose (Fortman, *et al.*, 2008), first generation biofuels (such as ethanol and biodiesel) are sub-optimal for current petroleum-centric transport infrastructures. Ethanol is produced naturally by some microbes during fermentation of sucrose or starch. Its high energy requirements for purification through distillation raises concern when comparing its energy value to that of petroleum ( $\pm 70\%$ ) (Stephanoloulos, 2007; Atsumi, *et al.*, 2008). Biodiesel is produced through the transesterification of vegetable or animal fats with methanol. It also has a lower energy content than gasoil (its petrobased counterpart), because of wax formation at lower temperatures (Peralta-Yahya, *et al.*, 2012). This causes transportation issues and places significant geographical limits on its application.

Besides these drawbacks, it has been argued that the link created between food and fuel prices by producing high volumes of biofuels from food crops are unethical, as it can result in the escalation of hunger worldwide. Although this is a morally compelling argument, adequate agricultural ethics for biofuels between the private and public sector developers should ensure that the positive attributes of first generation biofuels are realised (Thompson, 2012). Nevertheless, this “Food versus Fuel” argument drives the search towards using more abundant and underused non-food feedstocks such as lignocellulose, algal biomass and greenhouse gases for the production of high value chemicals..

Progress in metabolic and biological engineering has allowed the development of microbes that produce advanced “drop-in” biofuels with properties more like those of petroleum-based fuels. This is shown in Table 1 (extracted from Peralta-Yahya, *et al.*, 2012; Fortman, *et al.*, 2008; Nielsen & Keasling, 2011).

**Table 1: Transportation fuels and similar advanced biofuels**

<b>Fuel Type</b>	<b>Major components</b>	<b>Properties</b>	<b>Advanced biofuels</b>
<b>Gasoline</b>	C <sub>4</sub> -C <sub>12</sub> hydrocarbons	<ul style="list-style-type: none"> <li>Octane number: 87-91</li> </ul>	Butanol, isobutanol, short-chain alcohols, short branched-chain alkanes
<b>Diesel</b>	C <sub>9</sub> -C <sub>23</sub> hydrocarbons	<ul style="list-style-type: none"> <li>Cetane number: 40-60</li> <li>Good cold flow properties</li> </ul>	Fatty alcohols, alkanes, linear or cyclic isoprenoids
<b>Jet Fuel</b>	C <sub>8</sub> -C <sub>16</sub> hydrocarbons	<ul style="list-style-type: none"> <li>High energy density</li> <li>Low freezing temperature</li> </ul>	Branched alkanes, linear or cyclic isoprenoids

Microorganisms that have the ability to use non-sugar substrates are of interest, since a variety of feedstock can be used for the conversion to biofuels and high-value chemicals through the bacterium. Other applications of bio-engineered microalgae include lipids and carotenoids for the food and health industry, biohydrogen as biofuel, recombinant proteins for their pharmaceutical or medical values and other extracts for alcohol and cosmetic production (Jeon, et al., 2017).

Carotenoids produced by microalgae find wide application in the cosmetic (astaxanthin), food (beta-carotene as antioxidant and colouring agent) and therapeutic industries (Lutein) (Jeon, et al., 2017). These chemicals are mainly produced through the methylerythritol pathway (MEP) (Eisenreich, et al., 2004), an essential metabolic pathway present in eukaryotes, archaea and some bacteria. Also produced through this pathway are isoprenoids, one of the most diverse families of natural products (Cordoba, *et al.* 2009). As an example of genetic manipulation, through heterologous expression of an enzyme called isoprene synthase in the wild-type strain of *Synechococcus* PCC 7002, direct photosynthetic isoprene production could be achieved (Xu *et al.* 2011; Mayer and Kallas 2013 and Kallas, *et al.*, 2016).

Biohydrogen can be produced from microalgae. Being the most energy-dense fuel while not producing carbon dioxide upon combustion, it is considered as the most advanced green biofuel for the future (Barry, et al., 2016). Hydrogenases are the key enzymes responsible for its production and are extracted mainly through photobiological or fermentative pathways – the former being the eco-friendlier of the two (Gupta, *et al.*, 2013; Melis, *et al.*, 2000). Hydrogenases' fundamental sensitivity to oxygen requires additional research and understanding of how this problem may be overcome. Some avenues include creating conditional anaerobic conditions in the culture (Antal, *et al.*, 2011; Oncel, *et al.*, 2014; Song, *et al.*, 2011; Kumuraswamy, *et al.* 2013 and Ghirardi, *et al.* 2007).

Recombinant proteins find application in the medical industry in the form of antibodies, vaccines and hormones to name a few. Although mainly produced through bacteria, microalgae are superior hosts due to their ability to host large complex proteins (Demain & Vanishnav, 2009) and are less susceptible to immune responses and infectious agents such as pathogens (Rasala & Mayfield, 2015).

With the incentive to produce biofuels and valuable chemicals from microalgae firmly in place, one can proceed to explore the cultivation of algae itself and identify short-comings associated with commercial cultivation due to the high nutrient demand.

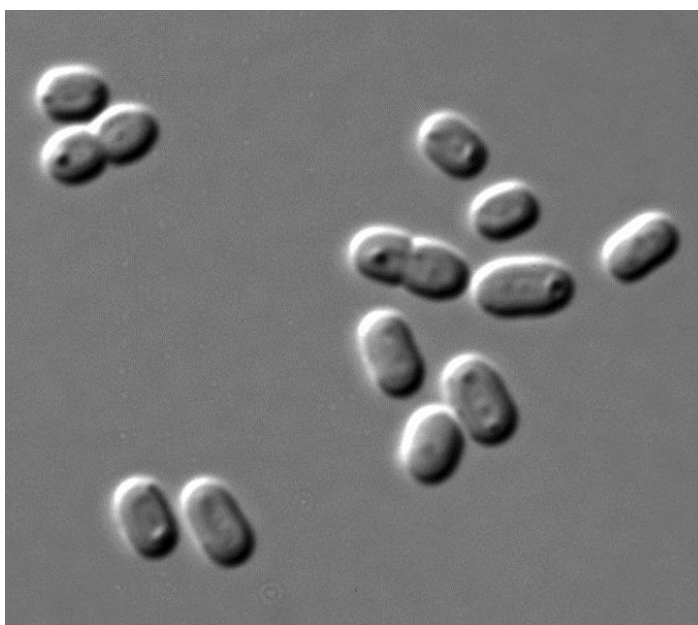
## **2.2 Characteristics and limitations of algae growth**

Algae is a collective term used to refer to highly diverse aquatic organisms that carry out photosynthesis and/or possess plastids (Clemens & Walter, 2012). For algae to grow, the four main requirements are: easy access to nutrients, water, inorganic carbon and sunlight. Moheimani and Borowitzka (2007) showed factors affecting algae growth can be subdivided into three major categories: physical factors

(nutrients, temperature, pH, and light), biotic factors (invasive species and predators) and operational factors (mixing, concentration, depth and harvesting frequency).

### 2.2.1 Study organism

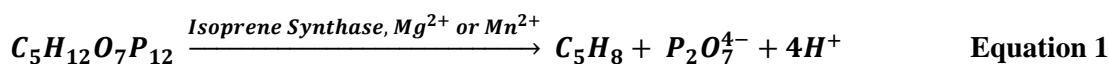
The microorganism chosen for this project is a cyanobacterium called *Synechococcus* PCC 7002. Cyanobacteria were initially disregarded by the research community for biofuel production due to their relatively low lipid densities compared to eukaryotic algae (Sheehan, et al., 1998). Interest in these organisms was renewed due to some species' ability to grow in extreme environments and their amenability towards genetic modification. These marine blue green algae are approximately one micron in size (Bryant & Kennedy, 2011). Furthermore, it is classified as a photoautotroph and can thus utilize CO<sub>2</sub> as a carbon source and light as an energy source (Shuler & Kargi, 2010). It reproduces cylindrical and ovoid shaped cells by binary fission and cells can exist either as single cells, pairs or short chains (Waterbury, et al., 1979) as can be seen in Figure 1.



**Figure 1: *Synechococcus* PCC 7002 in bright field microscopy. Imaging performed with the Olympus BX61 microscope and an UPlanSApo 100x NA 1.40 oil immersion objective. (Masur, 2010)**

Since *Synechococcus elongatus* utilises prokaryotic plasmids, which can be genetically engineered, isolated, changed and reintroduced into new cells, the organism is amendable to genetic manipulation (Shuler & Kargi, 2010). One example of this was the successful genetic modification of the wild-type *Synechococcus elongatus* strain by introducing an enzyme that converted dimethylallyl pyrophosphate (DMAPP) directly to isoprene and pyrophosphate by an elimination-rearrangement reaction (Silver & Fall, 1995). Enhancing cellular metabolic flux towards DMAPP predominantly contributed to the improved rates and yields of isoprenoid production by this cyanobacterium (Xian, 2015). It was

selectively synthesised from DMAPP rather than isopentenyl pyrophosphate (IPP) because of its longer half-life using the enzyme isoprene synthase according to eq. 1:



It was found to also be dependent on the presence of specific divalent cations and was optimal at a pH near 8. These conditions are similar to that found inside the chloroplast stroma during photosynthesis (Avron, 1981), suggesting that isoprene synthase located within the chloroplast might be activated by light-dependent changes in these parameters. Additionally, to grow the algae at higher cell concentrations the effect of overshadowing had to be limited. This was done by inactivating the gene *CpcB*, which codes for phycocyanin pigments (Zhao, et al., 2001). Thus, with less pigment being produced, light will penetrate deeper into the culture. Consequently, the genetically engineered form of *Synechococcus* PCC 7002 can grow at higher densities, which in turn correspond to higher isoprene yields. A fully sequenced genome is available for the strain. (Xu, et al., 2011)

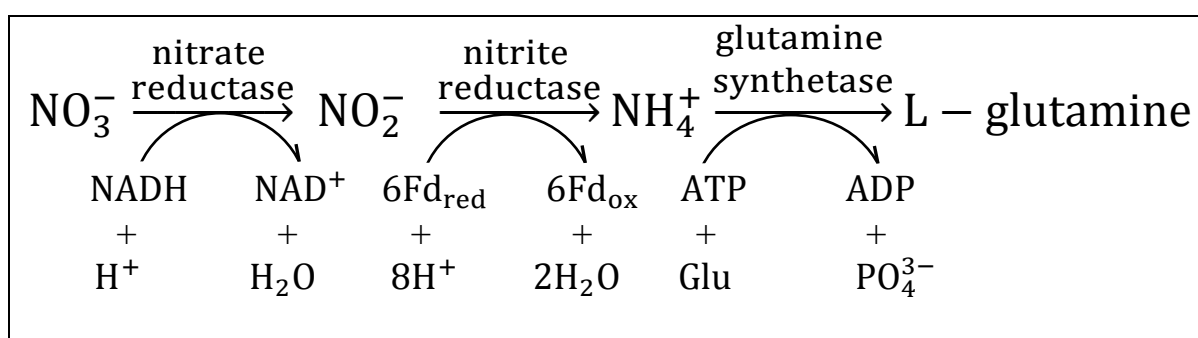
Although the isoprene producing engineered strain was not used during this project, its existence is still significant as it provides an opportunity to link waste valorization themes from this thesis to biofuel production. Various motivational factors for selecting *Synechococcus* PCC 7002 for the purpose of this research include:

- i. Its fast growth rate and accumulation of biomass with doubling times less than 3 hours in optimal growth conditions. (Van Baalen, *et al.* 1971)
- ii. Its ability to grow in a wide range of salinities ranging from nearly fresh water to more than 2 M of NaCl. This could also help reduce the invasion of cultures by other microorganisms. (Batterton & Van Baalen, 1971)
- iii. That it can fix nitrogen and utilize free ammonia/ammonium as nitrogen source.
- iv. That it can secrete energy dense exopolysaccharides. Exopolysaccharides are molecules released in response to the physiological stresses encountered in the natural environment and are structural components in which cells are embedded. Thus, the denser these structures, the better cells are protected from lethal environmental conditions and thus they are critical to cell survival (Davey & O'Toole, 2000)
- v. Its tolerance to high light intensities up to double that of sunlight ( $5000 \mu E.m^{-2}.s^{-1}$ ) (Sakamoto & Bryant, 1997) and the ability to still grow in low light (Van Baalen, *et al.* 1971).
- vi. Its natural transformability, meaning it can easily adapt to and accept new genes introduced into the genome (Mayer & Kallas, 2013).

## 2.2.2 Nutrients

### Nitrogen

Nitrogen is an essential element for microalgal growth and after carbon, it is the second most abundant element in microbial biomass ranging from 5 to 10% (dry weight) (Grobbelaar, 2004). The predominant path for nitrogen assimilation is through the glutamine synthetase enzyme system, by which glutamate reacts with ammonium to form the amino acid glutamine (Markou & Georgakakis, 2011). This is important for biochemical compounds such as amino acids, pigments and nucleic acids. It is illustrated schematically in Figure 2 (adapted from Markou and Georgakakis (2011)) and is useful to explain pH changes in the media depending on which nitrogen source is utilised. It can be taken up in inorganic, molecular or organic form.



**Figure 2: Simplified schematic pathway of nitrate conversion to glutamine within cyanobacteria including cofactors required for the conversion**

Nitrate ( $\text{NO}_3^-$ ) is the most frequently used nutrient form of nitrogen in synthetic media for microalgae and cyanobacteria cultivation (Grobbelaar, 2004). It is taken up actively<sup>1</sup> and does not display any toxic effects to cells and when the concentration is increased growth rates are slowed down. A possible explanation for this is that high nitrate concentrations result in enhanced activity of the enzyme nitrate reductase. This leads to high intracellular concentrations of nitrite and ammonium, which can be detrimental to cells (Markou, et al., 2014). According to Garbisu *et al.* (1992), nitrate uptake is inhibited in the presence of ammonia, which is toxic to cells with strong inhibitory effects at concentrations above 3000 mg/L (Ward, et al., 2014) and is dependent on temperature and pH of the media.

Nitrite ( $\text{NO}_2^-$ ) is commonly found in natural environments as an intracellular intermediate of metabolic processes in cells, where nitrate is reduced to nitrite by nitrate reductase, which in turn is reduced further to ammonium by nitrite reductase. It is mainly taken up through active transportation mechanisms, but

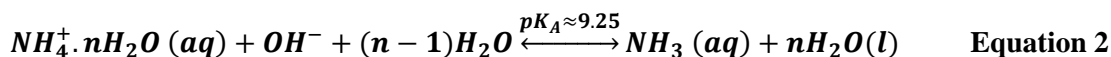
<sup>1</sup> Molecules can move across cell membranes through two major processes: passive or active transport. Some proteins can move molecules across cell membranes by utilising cell energy (in the form of ATP) and this is called active transport. It can move cells from high energies to low energies and *vice versa*. Passive transport on the other hand requires no energy from the cell and can be defined as the movement of molecules from a higher concentration to a lower concentration. In this work, transport refers to the process of solute passage through the plasma membrane while assimilation describes the incorporation of solute within the cell catabolism. Uptake refers to both since they cannot be separated.



diffusion mechanisms have also been reported for cyanobacteria (Flores, et al., 1987). Although it can be utilised as nitrogen source, Yang, *et al.* (2004) showed that at a concentration of 4 mM, the lag phase of *Botryococcus braunii* cultures was extended by 10 days, while a nitrite concentration of 8 mM completely inhibited growth. Also, work done by Flores *et al.* (1987) suggested that inorganic carbon is necessary for nitrite uptake and this was confirmed by Hu and Zhang in 2008 who succeeded in showing an increase in nitrite reductase activity for *Chaetoceros muelleri* cultures under elevated inorganic carbon concentrations.

An important consideration when using ammonia/ammonium as nitrogen source is the potential toxicity. Free ammonia is highly toxic to microalgae since it can diffuse passively into cells, resulting in little to no control over intracellular concentrations (Azov & Goldman, 1982). This phenomenon, occurring at concentrations as low as 2 mM at a pH of 8 (Azov & Goldman, 1982), has detrimental effects on photosynthetic systems by limiting the availability of inorganic carbon to the algae culture (Källqvist & Svenson, 2003). This degree of toxicity was investigated by Belkin and Boussiba (1991b) which concluded that it was related to the degree of deviation between extracellular and intracellular pH values. The smaller the difference, the higher the resistance to ammonia toxicity.

Ammonia (NH<sub>3</sub>), although it is a volatile molecule, has a high solubility (nearly 500 g per kg of water) at 25°C and atmospheric pressures, which enables it to exist as a liquid solution. When dissolved, a buffer system of ammonia/ammonium is formed (at 25°C) as shown in eq. 2:



Temperature also greatly influences the pK<sub>A</sub> value of this equilibrium system. When the temperature is higher, the pH value where free ammonia become dominant is lower and can be calculated by (valid for temperatures between 0 and 50 °C) eq. 3:

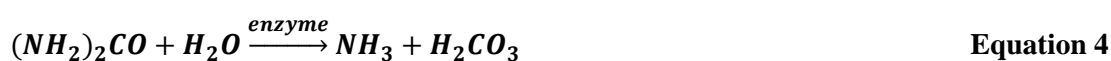
$$pK = 0.0901821 + \frac{2729.92}{T_K} \quad \text{Equation 3}$$

Where T<sub>K</sub> is the temperature in Kelvin (Ayre, 2013). Ammonium's (NH<sub>4</sub><sup>+</sup>) uptake and assimilation consumes less energy compared to other nitrogen sources, making it the preferred nitrogen source for microalgae and cyanobacteria (Boussiba & Gibson, 1991) and when it is available, cyanobacteria will not utilise other nitrogen sources until its levels are depleted. Slower growth rates have been associated with using NH<sub>4</sub><sup>+</sup> as opposed to nitrate as nitrogen source (Boussiba & Gibson, 1991). Lastly, ammonium is the only form of nitrogen requiring no enzymatic reduction that microalgae take up actively by transportation mechanism, enabling control of intracellular concentrations thereof. Thus, other forms of nitrogen such as nitrate and nitrite are reduced enzymatically to NH<sub>4</sub><sup>+</sup> so that it can be utilised in the well-known glutamine synthetase enzyme system (Boussiba & Gibson, 1991). The simultaneous presence of more than one nitrogen form in culture media affects not only the pH, but also the uptake of different nitrogen forms by the cells. This is discussed in further detail in Section 2.5.2.



Nitric oxide (NO) is a nitrogen form typically associated with flue gases. It has a very low aqueous solubility and if supplied as the sole nitrogen source to microalgae cultures, it has been found to always represent a rate-limiting factor (Markou, et al., 2014). It is a small non-polar molecule, which can diffuse directly into cells, where it is oxidised to nitrite or nitrate, which can both be utilised by microalgae. It is also a free radical and high intracellular concentrations will have detrimental effects on cells' health (Nagase, et al., 2001).

Some species of microalgae and cyanobacteria can utilize organic forms of nitrogen such as amino acids and urea, which are transported actively into cells (Flores & Herrero, 2005). The utilization of amino acids is species dependent and growth rates vary significantly depending on which microalgal species and amino acid is used (Flores & Herrero, 2005). Urea assimilation is far more significant since it can be hydrolysed through enzymes to ammonia and carbonic acid ( $H_2CO_3$ ), both of which are easily utilised by a wide range of microalgae and cyanobacteria. The reaction is shown in eq. 4:

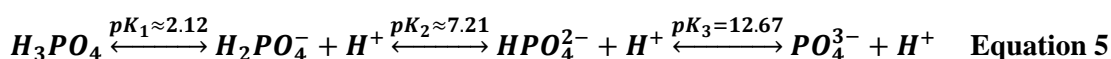


Although growth rates on urea have been reported to be even higher than other nitrogen sources, it has also been shown that urea triggered death and led to peroxidation of urease-producing cyanobacteria such as *Synechococcus* PCC 7002 (Sakomoto, et al., 1998). The primary event within the cell that triggers the phenomenon remains to be determined.

### Phosphorous

Although phosphorous contributes little to the biomass content of microalgae (0.05-3.3%) compared to carbon and nitrogen (Grobelaar, 2004), it is a significant contributor to microalgal growth. Low cell densities can be related to low phosphorous concentrations in growing media (Seale, et al., 1987) and unlike carbon and nitrogen, phosphorus originates from non-renewable mineral phosphate. Some cyanobacteria can store phosphorous as polyphosphate reserves when grown in phosphorous deficient media. When grown in these conditions, these cells take up phosphates at higher rates due to an increase in cellular production of phosphatase enzymes (Prieto, et al., 1997). This capability, known as 'luxury uptake', can be exploited for the removal of phosphorous in wastewaters since it enables them to outcompete nitrifying bacteria present in wastewater (Marchilhac, et al., 2014).

The molecular form of phosphorous present in media is also pH dependent and represented in eq. 5 (at 25°C):



Microalgae utilize only orthophosphate ( $PO_4^{3-}$ ) and all other available forms of organic phosphorous are hydrolysed intracellularly or extracellularly by phosphatase enzymes to  $PO_4^{3-}$  (Markou & Georgakakis, 2011), depending on the form of inorganic phosphorous molecules. Its utilization is

energy dependent and phosphate uptake is faster in illuminated and alkaline environments (Markou & Georgakakis, 2011). Lastly, it has been found that its uptake is increased in the presence of ions such as  $K^+$ ,  $Na^+$  and  $Mg^{2+}$  (Seale, et al., 1987). Provasoli (1969) reported that increased concentrations of monovalent and divalent cations favoured blue-green algal development over other green algae. The study also concluded that there is a lack of definitive information regarding the impact of these compounds in literature.

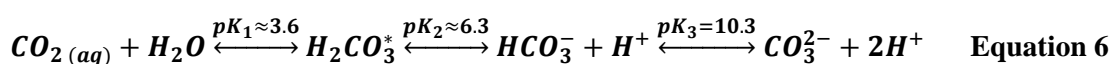
### Inorganic carbon

Inorganic carbon is one of the requirements of microalgae for photosynthesis and the optimal amount required is different for different algae strains (Posten and Walter, 2012). According to various literature sources, the optimal  $CO_2$  concentration is 8 % v/v  $CO_2$  in air and this was verified by Wu (2014) for *Synechococcus* PCC 7002. The highest known maximum reported where it could still grow is 60 % (v/v)  $CO_2$  enriched air. Miyairi's results are shown in Table 2.

**Table 2:  $CO_2$  assimilation of *Synechococcus Elongatus* at 52 °C in medium A with continuous  $CO_2$  enriched air bubbling under a tungsten lamp light source of 1400 lux (Miyairi, 1995)**

$CO_2$ % (v/v) enriched air	Doubling time [hr]
0.04	4.2
0.3	3.1
5	3.0
60	11.7

A decrease in doubling time was observed after some period when the algae was grown in 60% (v/v)  $CO_2$  enriched air, suggesting that the cells can adapt to high inorganic carbon concentrations. Growth was completely inhibited at 80% (v/v)  $CO_2$ . This effect is likely caused by the acidification of the medium through  $CO_2$  dissolution. As  $CO_2$  is dissolved, more complex speciation occurs in the aqueous medium containing the algae. The result is a weak acid-base buffer system called the bicarbonate-carbonate buffer system and it is represented in eq. 6:



where  $H_2CO_3^*$  refer to both  $CO_{2(aq)}$  and carbonic acid ( $H_2CO_3$ ). The fraction of a specific Dissolved Inorganic Carbon (DIC) species depends on the pH of the media (Markou, et.al. 2014). Thus, the predominant form of DIC present in pH ranges where microalgae thrive (between 6.5 and 10) is bicarbonate ( $HCO_3^-$ ). This is important, since the cyanobacteria employ many carbonic anhydrases and bicarbonate transport proteins that can transport both  $CO_2$  and  $HCO_3^-$  as an inorganic carbon source across the periplasmic membrane where it is converted to  $CO_2$  in the chloroplast (Markou and Georgakakis, 2011).

The buffer system provides the carbon necessary for photosynthesis as shown in eqns. 7-9.



The acidification caused by the formation of carbonic acid is countered by the accumulation of  $\text{OH}^-$  (Markou and Georgakakis, 2011) and a rise in pH is directly proportional to photosynthetic activity (Andrade & Costa, 1996).

### 2.2.3 Temperature

Photosynthetic capacity, specific respiration rate and growth rate of cyanobacteria are directly influenced by temperature and are optimal above 25 °C and the species' lethal upper limit (Robarts & Zohary, 1987). Additionally, they concluded that direct temperature effects on growth is secondary to indirect temperature effects such as mixing and nutrient availability and act synergistically with other factors in the growth process. According to Lavens and Sorgeloos (1996), media temperatures below 16 °C will significantly slow down algae growth, whereas temperatures higher than 35 °C can be lethal to most species. These limits are different for different microalgae strains and ultimately, each species' optimal growth temperature will be different. Sakamoto and Bryant (1997) showed that *Synechococcus* PCC 7002 grows optimally at 38 °C.

### 2.2.4 pH

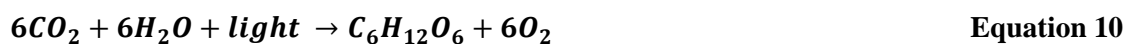
Most microalgae cultures can grow optimally in a pH range of 7 to 9 (Lavens & Sorgeloos, 1996). Failure to obtain the pH of a culture within acceptable bounds has detrimental effects on cellular processes and significantly hinders algae growth. The addition of a buffer to these batch scale experiments will circumvent this problem. To isolate the growth phenomena to the addition of specific nutrients, care should be taken that the addition of a buffer has no other effect on cell growth in the system, except regulating the pH. This will make monitoring and analysis of microalgal growth simpler. For example, if growth is carbon limited, the use of a carbonate buffer would compromise the growth results and should be avoided.

The addition of HCl has been shown to offer a short-term solution. This will also be expensive to add on a continuous basis and would lead to chlorine build up in the medium. The addition of  $\text{CO}_2$  has been shown to be much more effective for long-term cultivation and improves the stability of the culture (Moheimani, 2012). Since inorganic carbon can act as a pH regulator and growth stimulator for the algae, it promotes the argument of integrating AD flue gas in reasonable quantities with algae culturing.

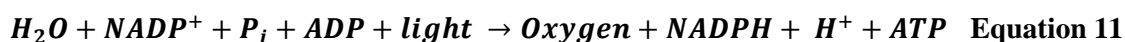
### 2.2.5 Light

At low light intensities, growth is limited by the rate of photosynthesis. As light intensities increase, growth rates increase until the point at which photo-inhibition (light-induced reduction in the photosynthetic capacity of an alga) occurs (Christi, 2007). This upper limit is different for different species. It is important to understand that as biomass concentration increases in the flasks, the effect of mutual shading (a phenomenon that occurs when tight spatial interrelationships between cells of a dense culture limits available light to other cells, depressing photosynthesis) becomes more important (Kunjapur & Eldridge, 2010). Some regions will receive less light than required due to light penetration paths being blocked by biomass. Light availability will also affect the biomass composition of the microalgae. For example, *Synechococcus* PCC 7002 prioritizes the production of amino acids under limited light conditions and sugars and starch under saturated light conditions (Fay, 1983). Most research associated with *Synechococcus* PCC 7002 was conducted at  $50 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for low light intensities and  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for high light intensities of which the latter is the optimal saturating irradiance. (Wu 2014; Sakamoto and Bryant 1997; Kuan 2010 and Ludwig and Bryant 2012)

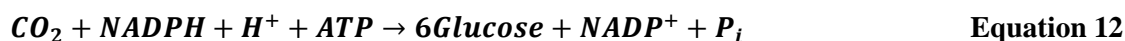
Since the strain of interest is a photoautotroph, it undergoes a physicochemical process in which light energy is used to drive the synthesis of organic compounds (Whitmarsh & Govindjee, 1999) through the photosynthetic electron transport chain. Water is oxidised to form dioxygen and atmospheric  $\text{CO}_2$  is fixed into organic carbon compounds for long-term energy conservation. The overall reaction can be summarised as shown in eq. 10:



This reaction takes place in two steps. The first, called the light-dependent reaction, involves light energy and is shown in eq. 11:



The light energy is captured and converted into ATP and NADPH, which are energy-storing molecules. As hydrogen containing water molecules are used to reduce  $\text{NADP}^+$ , hydrogen atoms are produced decreasing the pH as algae grows. (Masojidek, et al., 2004). During the second light-independent reaction, shown in eq. 12:



energy compounds produced previously (NADPH and ATP) are used to convert  $\text{CO}_2$  to glucose (Shuler & Kargi, 2010) leading to an overall increase in pH.

Microalgae have strong circadian rhythms of which the strongest external cue is daylight. This biorhythm will determine a wide variety of biological processes including reproduction, growth and

cell composition and thus this information is crucial to optimize algae culturing and harvesting (Van Alphen & Hellinger, 2015). The reason this timing mechanism exists in organisms is to cope with daily fluctuations in light and nutrient availability and temporally separate mutually incompatible processes (Tu & McKnight, 2006). Thus, the importance of light-dark cycles can influence the growth of microalgae significantly. Many experiments mimic the natural day light (12h-12h), but some reports have shown optimal growth under continuous lighting (Jacob-Lopes, *et al.* 2008; Sakamoto and Bryant 1997) and 16h-8h (Barsanti & Gualtieri, 2006) for *Synechococcus* PCC 7002. Since the study organism can maintain a free-running circadian rhythm in continuous light, it will significantly simplify experiments without compromising the validity of results if the algae are grown under continuous lighting.

Both sunlight and artificial light can provide the light energy used by microalgae. By using artificial lights, the conditions in which the algae grow can be controlled much more precisely. Lavens and Sorgeloos (1996) showed that lamps emitting either blue or red light (400 to 700 nm) are optimal for most algae species since these are the most active portions of the light spectrum for photosynthesis. The portion between these two frequencies are labelled the photosynthetic active radiation (PAR). Since most of light released by cool white fluorescent lights fall within this spectrum (with the highest photon absorbance at 670 nm for *Synechococcus* PCC 7002 (Alvey, *et al.*, 2011)), they are a suitable light source for the growth and study of cyanobacteria. For more information regarding the size and choice of the light sources used in this project, refer to Appendix B.1.

### 2.2.6 Growth patterns and kinetics in batch culture

Growth of microalgae results from replication and change in cell size and is an essential response to the physicochemical environment. Nutrients are extracted and utilised for energy production, biosynthesis and product formation. This leads to an increase in cell mass and the rate of microbial growth is characterised by the net specific growth rate, denoted  $\mu_{net}$  [ $hr^{-1}$ ], and defined in eq. 13 as

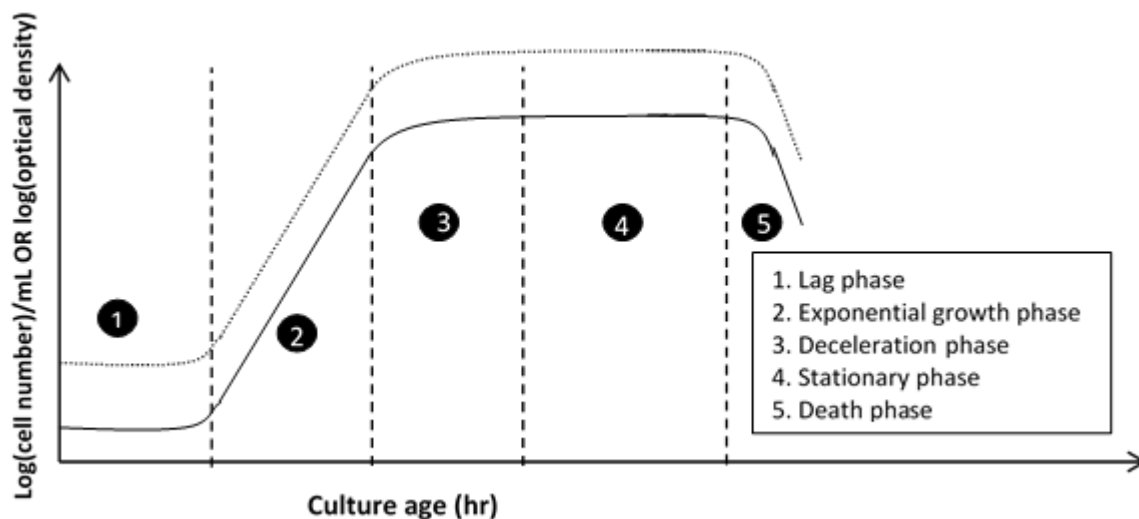
$$\mu_{net} = \frac{1}{X} \frac{dX}{dt} = \mu_g - k_d \quad \text{Equation 13}$$

where  $X$  [ $g \cdot L^{-1}$ ] is cell mass concentration,  $t$  [ $hr$ ] is time,  $\mu_g$  [ $hr^{-1}$ ] is the gross specific growth rate and  $k_d$  [ $hr^{-1}$ ] is the rate of cell mass loss due to endogenous metabolism (Shuler & Kargi, 2010). This is known as Monod growth kinetics. Alternatively, microbial growth can also be described in terms of cell number concentration, denoted  $N$  in eq. 14:

$$\mu_R = \frac{1}{N} \frac{dN}{dt} \quad \text{Equation 14}$$

where  $\mu_R$  [ $hr^{-1}$ ] is the net specific replication rate.

To simplify and modify Equation 14 and Equation 15, one needs to understand and examine the process by which cells extract nutrients and convert them to biomass. Batch grown microalgae can typically be characterised by five stages of growth and these are illustrated in Figure 3.



**Figure 3: Growth phases of microalgae**

The lag phase is characterised by little to no increase in biomass and no increase in cell number density due to the time required by cells to adapt to the pH, temperature, growing media composition, lighting, salinity and carbon source concentration associated with the growing environment (Shuler & Kargi, 2010). The more ideal these conditions are, the shorter the lag phase period will be. This phase is shorter for liquid-to-liquid inoculations compared to liquid cultures inoculated by plate colonies if the inoculum is already in the exponential growth phase when inoculated (Wu, 2014). It is beneficial to keep this phase as short as possible without compromising the culture's health. Rolfe *et al.* (2012) succeeded in illustrating that rushing this phase will have detrimental effects on DNA synthesis, transcription, translation and protein assembly during the exponential phase. Additionally, inoculum size should be large (5 to 10% by volume) and the nutrient media should be optimised (Shuler & Kargi, 2010).

After the adaptation period, cells multiply rapidly and cell mass and cell number density is observed to increase exponentially with time. Cells divide regularly by binary fission and balanced growth is achieved, meaning all components of the cell grow at the same rate (Shuler & Kargi, 2010). The implication of this is that the specific growth rate, denoted  $\mu$  [ $\text{hr}^{-1}$ ], would be the same regardless if it was determined from the cell mass or cell number. Also, since nutrient concentrations are high during this phase, the growth rate will be independent of nutrient concentration. This forms the basis for the first growth model employed, namely the exponential growth model. For initial condition  $X = X_0$  at  $t = 0$  and excluding the death rate, eq. 14 is transformed into eq. 15:

$$X = X_0 e^{\mu_{net} t}$$

**Equation 15**

Linearizing, eq. 15 is transformed into eq. 16:

$$\ln X = \mu_{net}t + \ln X_0$$

Equation 16

and thus, by plotting  $\ln X$  versus time, both the intercept and slope will yield values for the specific growth rate and the cell mass concentration at time zero. Assuming the specific growth rate is constant, eq. 15-16 are only valid for the exponential growth phase and when applied, care should be taken to exclude any other phases from the data used. Lastly, during the exponential growth phase cells produce both primary and secondary metabolites, which refer to growth-related and non-growth-related products, respectively.

Next, the deceleration phase describes a period where the growth rate approaches zero because of essential nutrient depletion or the accumulation of toxic by-products. Cell composition and size begin to change in an attempt to survive in the now hostile environment. The point where the growth rate equals zero (or the gross growth rate equals the death rate) marks the beginning of the stationary phase. In this phase, the cell mass concentration is dependent on the death rate ( $k_d$ ) and more importantly, only secondary metabolites are produced during this phase. This serves as the primary motivation for only considering the exponential growth phase for experimental runs and data collection, which would not only improve model accuracy but also significantly decrease the time required for one experimental run. Eventually, cells begin to disintegrate, and this process usually follows first-order kinetics.

Table 3 summarizes the growth conditions and lengths various sources achieved when growing *Synechococcus* PCC 7002 and serves as a guideline of what can be expected in the experiments performed for this project.

Table 3: Literary summary for growth phase lengths and conditions

Lag phase (days)	Exponential phase (days)	Deceleration phase (days)	Stationary phase (days)	Total dry biomass (g/L)	Conditions	Source
2	6	2	6	-	A+ medium, 38 °C, 250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 1 % (v/v) $\text{CO}_2$ in air	Xu (2010)
2	27	2	-	8.42	Medium A, 38 °C, 250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 1 % (v/v) $\text{CO}_2$ in air	Wu (2014)
7	-	-	-	0.20	Medium A, 30 °C, 600 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 0.04 % (v/v) $\text{CO}_2$ in air	Aikawa <i>et al.</i> (2014)
1	5	1	-	7.50	Medium A, 30 °C, 600 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 1 % (v/v) $\text{CO}_2$ in air	
1	4	2	-	9.20	Medium A, 30 °C, 600 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 2 % (v/v) $\text{CO}_2$ in air	
1	4	2	-	9.30	Medium A, 30 °C, 600 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 4 % (v/v) $\text{CO}_2$ in air	

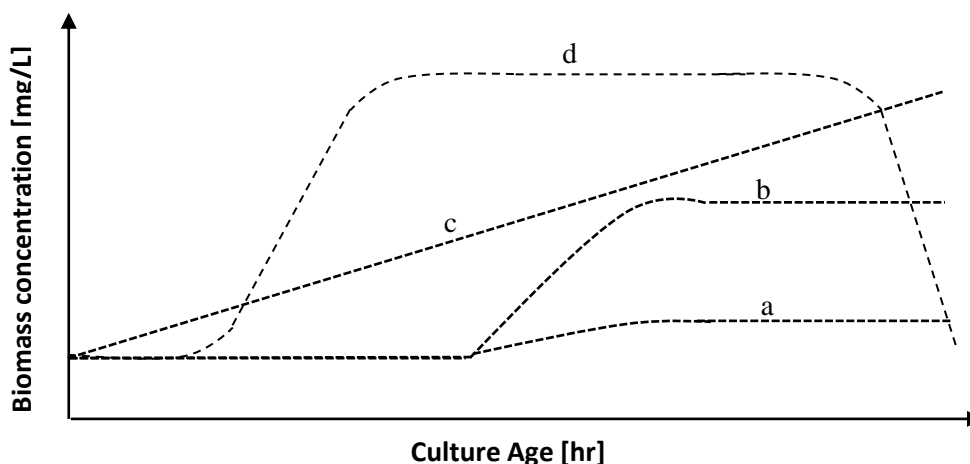
This data shows that carbon dioxide has the potential to speed up growth and shorten the time needed to reach the stationary phase. Based on this, it was expected that the growth would be severely limited

since the initial experimentation would be done with no carbon enrichment. This supports the cause to integrate AD flue-gas with microalgal growth.

For this project, all nutrients required for growth were assumed to be present in AD effluent from a theoretical point of view. Additionally, all external conditions (such as lighting, temperature, CO<sub>2</sub> concentration and mixing) would be held constant for all comparative runs. From this, it follows that if the organism grew sufficiently well in synthetic media under a certain set of growth conditions, one could presume that if it does not grow comparatively well in AD effluent under the same conditions, one of three things could be wrong:

- i. The presence/absence of a compound that is inhibitory/necessary for growth.
- ii. A nutrient is present in low concentrations and then exhausted by the study organism.
- iii. A nutrient is present in low concentrations and added continuously to the system.

These conditions form the basis of logical reasoning throughout the experimental process of this project and were justifiable by the shape and form of the growth curves obtained. It is discussed in more detail in the remainder of this Chapter.



**Figure 4: Growth curve associated with inhibiting compounds (a), growth curve associated with a limiting nutrient not continuously added to the system (b), growth curve associated with a limiting reagent added at a constant rate to the system (c) and growth curve associated with normal growth, when none of these limiting effects mentioned are present (d).**

#### Growth in the presence of a compound that is inhibitory to growth

In Figure 4, the growth curve associated with inhibitory- conditions or nutrient presence is illustrated by (a). It is normally characterised by little to no growth or an extensive lag phase if the cells can adapt to the condition over time. It can either be that the cells are killed by the toxic phenomenon or it could simply inhibit cell multiplication. A typical example of a toxic compound found in AD effluent is ammonia.



### Growth in the presence of low concentrations of a key nutrient not continuously added

Curve b in Figure 4 illustrates what growth curve could be expected when a limiting nutrient is depleted by the culture. If a compound necessary for growth is absent from the media, the cells will deplete whatever amount is still present in the inoculation media causing a slight rise in growth, but once depleted growth will stagnate. This would typically be a compound (such as nitrogen or phosphorous) in the media which is exhausted by the culture and characterised by a sudden stop in growth.

### Growth in the presence of low concentrations of a key nutrient continuously added

If a limiting reagent or nutrient is added at a constant, uniform rate to the system, one would expect to see an increase in growth at a steady (linear) rate as shown on curve c in Figure 4. Especially for liquid-to-liquid inoculations where the cells are already in the exponential growth phase, the growth curves would resemble the trend shown in curve c. The steeper the slope of the curve, the higher the ratio of constant nutrient addition to nutrient consumption, even though it's still not available in high enough quantities for the cells to multiply exponentially. A typical example of continuous limited growth is carbon limitations if the source of carbon is added at a constant rate.

## **2.2.7 Reducing nutrient costs associated with large scale algal growth**

Currently, the major barriers for deployment of biofuel and valuable chemical production from microalgae bioprocesses are the high costs associated with lighting, mixing, nutrient supply and product separation (Gao, et al., 2009). Generally, there are two major approaches to make this process more feasible: either reduce production costs or increase the value of products.

There are mainly two different ways of growing algae: open or closed PBRs. Open ponds are economical and simple but hard to monitor and control while closed PBRs are costlier but can provide a carefully controlled growth environment (Lee, 2001). If the high value product can be produced by microalgae in the gas phase, PBR's are the preferred method for growing the culture. Also, PBR's provide an easily controlled environment, limiting contamination and allowing accurate addition and recycling of nutrients such as CO<sub>2</sub>.

Progress has been made in terms of PBR designs (Singh & Sharma, 2012; Wang, *et al.*, 2012; Chen, *et al.*, 2011; (Pires, *et al.*, 2017); (Pham, *et al.*, 2017)) and for high-density culture cultivation, the optimization of environmental factors include light capturing, light utilisation, light distribution, CO<sub>2</sub>/O<sub>2</sub> gas exchange, temperature, pH, sterility and mixing. Through further advanced PBR engineering, commercialization of algal products was expected by 2013 (Suh & Lee, 2003). Since microalgae are typically very efficient at sequestering nutrients such as nitrogen and phosphorous when present in their environment, the nutrient requirements necessary for algae growth and the costs associated with it has often been ignored.

Besides sourcing macro nutrients for large scale algal growth, the Achilles heel of using microalgae for valuable product production on a global scale lies in the necessity of water addition. If algal growth is to be realised on non-arable land, water will be a central issue for large scale algae biofuel production. Not only will algae have to compete with the growing agricultural industry for water, but its growth also requires more water than most land crops, potentially triggering a future ‘water versus fuel’ debate (Hannon, *et al.*, 2010). Considering the high costs associated with large scale PBR design and construction, if the water and nutrient supply required for algae cultivation cannot be produced from existing waste or saline sources, the likelihood of industry expansion is increasingly slim.

### **2.3 Wastewater treatment potential**

Waterbodies can be polluted in varying strengths and volumes by different sources and the life styles and technologies practiced in communities are reflected in the wastewater’s composition (Gray, 1989). Complex mixtures of natural or fabricated organic and inorganic materials make up these waste streams. Up to 75% of organic carbon in biodegradable waste and sewage sludge manifests itself as carbohydrates, fats, proteins, amino acids and volatile fatty acids. Inorganic constituents include high concentrations of ammonium salts, bicarbonate, phosphate, chlorine, sulphur, magnesium, sodium, calcium, potassium and heavy metals (Horan 1990; Lim, *et al.*, 2010; Tebbutt, 1983). The presence of these constituents creates conditions in which a wide variety of microorganisms can thrive. The majority thereof is harmless, but can also contain pathogenic microorganisms, which can cause disease among humans and animals (Glynn, 1989). Anaerobic digestion can be applied to wastewater in order to reduce the solid organic carbon, recapturing this energy as biogas and producing an effluent rich in nutrients for growing microalgae. It is this ability to recapture energy from waste that makes AD a financially feasibly and scalable technology.

Water scarcity, energy and food requirements act as drivers for the exploration of feasible water recycling and resource recovery. Similarly, health concerns related to the unregulated release of toxic chemicals into the environment developed worldwide concern over environmental protection and a need to better understand adverse long term effects caused by wastewater discharge. An overview of current wastewater treatment processes followed by why anaerobic digestion and algae growth can replace some of these processing steps will follow in the remainder of this chapter.

#### **2.3.1 Conventional treatment methods**

The main goals of treating wastewater are the removal of suspended solids, biochemical oxygen demand (BOD – a key indicator of the carbon load present in wastewater), nutrients (such as ammonia, nitrate, nitrite, phosphorous and sulphur), pathogenic bacteria and toxins (Horan, 1990). These impurities are typically removed in three stages:

Preliminary treatment aims at removing large suspended solids such as clumps containing wood, faecal material and rags, which may obstruct or damage downstream processing units. The wastewater can be

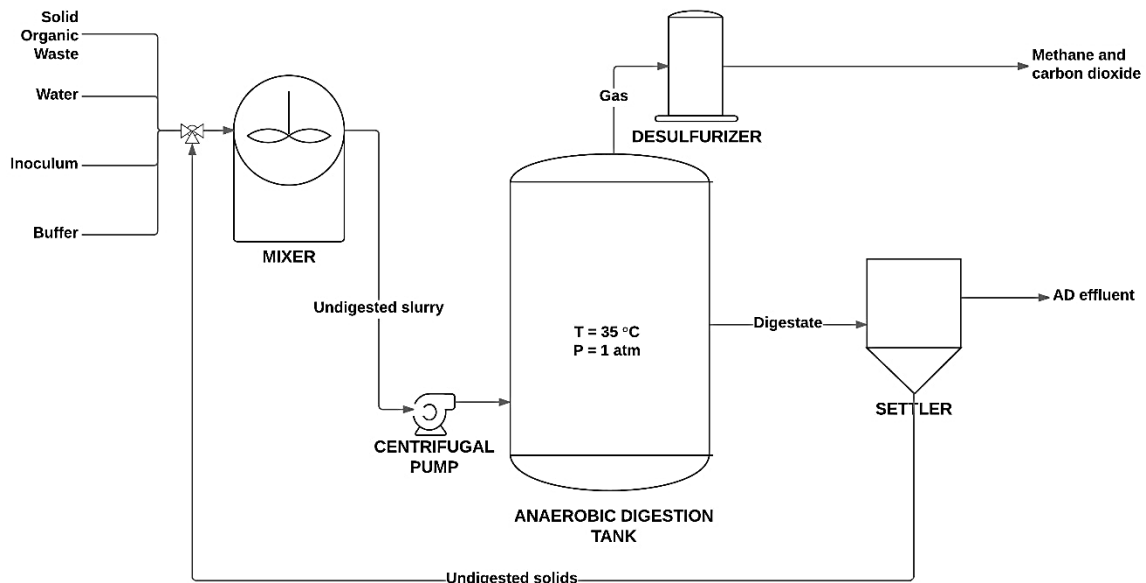
passed through bars or grids to remove most of these suspended solids (Tebbutt, 1983)). Grit or finer solid particles may be removed by reducing flow velocity, which will allow the particles to settle out (Gray, 1989).

Both BOD and pathogenic bacteria are removed to different extents in the primary and secondary treatment stages. (Horan, 1990). When considering the dependence of biological organisms to break down organic material in wastewater, it is crucial to remove as much BOD as possible in the primary stage (Abdel-Raouf, et al., 2012) to avoid the depletion of dissolved oxygen in receiving water bodies. Methods include the use of active sludge, where oxygen and a mixed population of heterotrophic bacteria are introduced into the wastewater. The microorganisms consume the organic material until a secondary sludge with a low energy generation potential is left behind. Pathogenic bacteria treatment in primary stages are either approached by removal processes or disinfection processes and methods for both are highly varied, producing different rates for different species (Gray, 1989; Bennett, 2008). In secondary treatment stages, the biological oxidation system caused by introducing activated sludge can remove over 90% of pathogenic bacteria present in suspended growth reactors (Gray, 1989).

The discharge of the remaining nutrient rich slurry into water bodies can stimulate the growth of unwanted aquatic plants and algae, which in turn can lead to eutrophication. More so, high levels of non-ionised ammonia are toxic to aquatic organisms and fish, can interfere with disinfection and cause methemoglobinemia (Lincoln & Earle, 1990). The removal of these ions can be done either chemically or biologically in a final tertiary processing step.

### **2.3.2 Anaerobic digestion as processing step**

Life cycle analysis of municipal solid waste management techniques revealed that anaerobic digestion is preferable with regards to GHG reduction and energy consumption, compared to incineration, aerobic composting, pyrolysis and landfilling (Environment Canada, 1995). Guiot & Frigon (2012) showed when considering combustion efficiencies, electricity prices, capital and operational costs, the energy revenue generated to make anaerobic digestion a profitable process on its own is unlikely. Integration with biofuel producing microalgae is one way to solve this problem. The process can be defined as the breakdown of organic waste by pureferic bacteria in the exclusion of air (Issah & Salifu, 2012) and is illustrated schematically in Figure 5.



**Figure 5: Schematic presentation of an anaerobic digestion process producing AD effluent and biogas under ideal conditions (35 °C and 1 atm).**

Organic waste, water, inoculum and a buffer is fed into a digestion tank. The organic matter contains complex proteins, lipids and sugars which are utilised through bacterial hydrolysis in order to break down insoluble organic polymers, making them available for other bacteria. These acidogenic bacteria will then convert the available sugars and amino acids into  $\text{CO}_2$ , hydrogen, ammonia and organic acids. Lastly, methanogens convert these products into methane and  $\text{CO}_2$  which can be collected and burned as biogas (Issah & Salifu, 2012). This method of treating wastewater can be applied to primary and secondary wastewater sludge (Guiot & Frigon, 2012) and not only lowers volatile organic compounds, but is also effective in mitigating pathogens and odor (Lusk, 1998).

In spite of this extensive breakdown of carbon into simpler compounds, the overall nutrient content with respect to nitrogen and phosphorus of the wastewater is not altered significantly and the high concentrations of these nutrients could cause environmental pollution if applied as fertilizer (Passero, et al., 2015). The bioavailability and uptake of nutrients in digested wastewater is dictated by their chemical forms (ionic, adsorbed, precipitated, and co-precipitated). These nutrient species also interact with other chemical compounds and this ultimately influences the composition of the effluent. Lastly, the extent of digestion and digester type also influences nutrient speciation (Mulkerrens, et al., 2004).

The fact that anaerobic digesters can in effect be considered net energy generators because of biogas production makes it an elegant replacement for conventional secondary treatment processes. Organic matter can be sourced from a wide variety of industries (ranging from agriculture, manure, sewage, etc.), while inoculum can be obtained from any process that contains the bacteria species required for AD. One example of a source of these bacteria species are beer breweries that implemented anaerobic digesters. The only valuable resource required for AD is water. Not only is it a necessity for bacterial hydrolysis, it also promotes transportation of the solid wastes. If the nutrient rich effluent produced

through AD can be exploited by microalgae in a profitable way while simultaneously reclaiming water, anaerobic digesters have the potential to turn “environmental liabilities into economic opportunities” (Guiot & Frigon, 2012). This requires a thorough understanding of the compounds present in the effluent and how they might affect downstream processes.

## 2.4 Characteristics of AD effluent and flue gas

### 2.4.1 Nutrients

The anaerobic digestion of organic wastes is associated with variability at this stage due to the diversity of substrates that can be used. Besides the different compositions of organic wastes, other factors such as carbon to nitrogen (C/N) ratio, temperature and pH contributes to the inherent variability of using AD effluent as a culturing media for microalgae. Various AD effluent’s nitrogen and phosphorous concentrations are shown in Table 4.

**Table 4: Total nitrogen (TN) and phosphorous (TP) content of AD effluents**

AD effluent type	TN $\left[\frac{mg}{L}\right]$	TP $\left[\frac{mg}{L}\right]$	Reference
Dairy manure	125-3456	18-250	(Wang, et al., 2010)
Poultry manure	1380-1580	370-32	(Yetilmezsoy and Sakar, 2008); (Yetilmezsoy and Sapci-Zengin, 2009)
Sewage sludge	427-467	134-321	(Montusiewicz, et al., 2010)
Food waste and dairy manure	1640-1885	296-302	(El-Mashad & Zhang, 2010)

Food waste and cattle manure are among some of the most abundant organic wastes produced both globally and in South Africa (Oelofse, 2013; National Agricultural Marketing Council (NAMC), 2015; Oelofse, 2014; Khan, *et al.*, 2015). Food waste is considered a very attractive feedstock for anaerobic digestion due to its exceptionally high methane production potential and biodegradability (Zhang, *et al.*, 2011; De Baere, 2006; Greben & Oelofse, 2009). It is food waste’s high lipid and fat content that favours methane production (Wan, et al., 2011), but its degradation leads to the formation of long-chain fatty acids (LCFAs). LCFAs are inhibitory at concentrations higher than 1 g/L and toxic to both syntrophic and methanogenic bacteria, which consequently limits the transport of nutrients to cells (Hanaki, *et al.*, 1981; Pereira, *et al.*, 2005). Additionally, anaerobic digestion of monosubstrates were found to be unstable due to low C/N ratios ranging from 5 to 8 (Li, et al., 2009). In 2010, Kumar *et al.* found that C/N ratios between 13.9 and 19.6 were acceptable for anaerobic digestion. The methane yield of livestock (including cow, pig and chicken) manure is unfeasibly low due to low solids and high fibre content, the latter being highly resistant to degradation by anaerobic digestion (Greben & Oelofse, 2009), which in turn leads to unfavorable economics (El-Mashad & Zhang, 2010).

An interesting approach to establish ideal C/N ratios for the anaerobic digestion of organic wastes is to combine multiple substrates as feedstock for the anaerobic digester, a process called co-digestion. Blending more than one organic waste type helps establish positive synergism in the digestion medium due to ideal C/N ratios and improved buffer capacity (originating mainly from the ammonia (Greben & Oelofse, 2009)). This has numerous benefits:

- An increase in the yield and stability of biogas production (El-Mashad & Zhang, 2010)
- Additional supply of missing nutrients required for microalgal growth (Mac & Llabr, 2000)
- Improved moisture content of digester feed (El-Mashad & Zhang, 2010)
- Limitation of inhibitory effects of high ammonia and sulfide concentrations (Hartmann, et al., 2003)

A study conducted by Wang, *et al.* in 2013 investigated the anaerobic co-digestion of *Chlorella sp.* and waste activated sludge. They found that not only did the microalgae secrete more extracellular polymeric substances in the activated sludge, but the biogas yield improved and the digestion period was shortened. Lastly, the dewaterability of these co-digested products were better combined compared to dewatering them individually.

#### **2.4.2 Turbidity**

Untreated anaerobic digestate is characterised by high turbidities due to dissolved and suspended materials. The effect of this on algae growth is discussed in Section 2.5.3. This cloudiness is produced as light reflects off the particles present in the water and is directly proportional to the concentration of suspended particles. As such, turbidity can be used to indicate changes in the total suspended solids (TSS) concentration of wastewater. In AD effluent, the main contributors of TSS are microorganisms, inorganic and undigested organic materials with poor settling characteristics such as silt and clay (United States Environmental Protection Agency, 2009). This, combined with low wastewater alkalinity due to nitrification increases floc formation and increases the turbidity of the digestate (Akpor, 2011).

A study conducted in 2006 by Ong *et al.* investigated the effect of phosphorus concentration on turbidity in swine AD effluent. Flasks containing 500 mL samples of water from a facultative lagoon were spiked with dicalcium phosphate resulting in total phosphate concentrations of 0 to 5550 mg/L (Ong, et al., 2006). The turbidity of all samples decreased steadily over a period of 10 days as a result of soluble phosphorus utilization by the algae present. Additionally, it was observed that the relationship between initial phosphorus concentration and turbidity was linear. They concluded that it could be possible that insoluble phosphorus precipitated out of the solution, appearing as suspended particles and resulting in increased turbidity. Eitherway, there is a significant amount of bacteria in AD effluent and in order to avoid contamination of experimental cultures and subsequent false data readings, proper treatment such as filtration and autoclave will be crucial to circumvent this problem. The majority of work in current literature report significant dilution of anaerobic digestates in order to reduce the ammonia

concentration and turbidity for algae to grow effectively (Sooknah & Wilkie (2004); Wang, *et al.* (2010); Cai, (2012); Paul, *et al.* (2016); Marchilhac, *et al.* (2014); Wahal (2010); Singh, *et al.* (2011); Kumar, *et al.* (2010); Ayre (2013); Park, *et al.* (2010); Cai, *et al.* (2013)). In light of increased water scarcity in South Africa, diluting AD effluent with water in order to circumvent these problems is neither economically feasible nor sustainable.

### 2.4.3 Carbon

Inorganic carbon is a primary nutrient required for sustainable algal cultivation. Although it can grow at atmospheric concentrations of CO<sub>2</sub> (400 ppm), biomass production can be increased significantly by supplementing the growing atmosphere with additional CO<sub>2</sub>. Its solubility however is dependent on temperature, pressure and concentration of total dissolved solids and the efficiency of CO<sub>2</sub> dissolution into aqueous solutions is dependent on the contact surface area, the contact time and the deviation from equilibrium of chemical conditions (Quinn, *et al.*, 2015). The effluent produced is rich in bicarbonate-carbonate alkalinity. The conversion of organic carbon to methane and CO<sub>2</sub> during anaerobic digestion allows the inorganic carbon to dissolve into the effluent, establishing a bicarbonate-carbonate buffer with elevated dissolved inorganic carbon concentrations, which is beneficial for algae growth (Markou & Georgakakis, 2011).

One of anaerobic digestion's intrinsic advantages lies in the fact that most of the gas produced will always be methane regardless of substrate chemistry, which translates to high theoretical yields for methane (Buckley & Wall, 2006). Typical composition of AD flue gas is shown in Table 5 (Braun, 2007; Cecchi, *et al.*, 2003).

**Table 5: Typical composition of biogas from anaerobic digestion**

Components	Symbol	Concentration (volume %)
<b>Methane</b>	CH <sub>4</sub>	50-75
<b>Carbon dioxide</b>	CO <sub>2</sub>	25-45
<b>Water</b>	H <sub>2</sub> O	6.5
<b>Hydrogen sulphide</b>	H <sub>2</sub> S	2
<b>Nitrogen</b>	N <sub>2</sub>	<2
<b>Oxygen</b>	O <sub>2</sub>	<2
<b>Hydrogen</b>	H <sub>2</sub>	<1

Eq. 17 can be used to calculate theoretical biogas composition and methane yield stoichiometrically from the net reaction given that the elemental composition of the substrate is known (assuming the substrate's composition consists of carbon, hydrogen, oxygen, nitrogen and sulphur only) (Braun, 2007)





where

$$y = \frac{c}{2} + \frac{h}{8} - \frac{o}{4} - \frac{3n}{8} - \frac{s}{4} \quad \text{Equation 18}$$

$$x = c - \frac{h}{4} - \frac{o}{2} + \frac{3n}{4} + \frac{s}{2} \quad \text{Equation 19}$$

Assuming ideal gas behaviour (molar volume of a gas =  $22.4 \frac{L_{STP}}{mol}$ ), the methane yield can be formulated as shown in eq. 20:

$$Y_{CH_4} \left[ \frac{Nm^3}{kg} \right] = \frac{22.4y}{cM_{W,C} + M_{W,H}h + oM_{W,O} + nM_{W,N} + sM_{W,S}} \quad \text{Equation 20}$$

where  $M_w \left[ \frac{g}{mol} \right]$  represents the molecular weights of carbon, hydrogen, oxygen, nitrogen and sulphur.

The fractions theoretical methane and  $CO_2$  of total carbon in the flue gas can then be calculated by eq. 21-22, respectively:

$$CH_4\% = \frac{y}{c} \cdot 100 \quad \text{Equation 21}$$

$$CO_2\% = \frac{c-y}{c} \cdot 100 \quad \text{Equation 22}$$

The expected fractions of methane and carbon dioxide is shown in Table 6.

**Table 6: Biochemical polymers' theoretical energy potential**

Substrate	Elemental formula	Reference	$Y_{CH_4}$ $\left[ \frac{Nm^3}{kg} \right]$	$CO_2\%$	$CH_4\%$
<b>Carbohydrates</b>	$C_6H_{10}O_5$	(Angelidaki & Sanders, 2004)	0.414	50.00	50.00
<b>Proteins</b>	$C_{106}H_{168}O_{34}N_{28}S$	(Hedges, et al., 2002)	0.517	48.35	51.65
<b>Lipids</b>	$C_{57}H_{104}O_6$	(Angelidaki & Sanders, 2004)	1.012	29.82	70.17
<b>Primary sludge</b>	$C_{10}H_{19}O_3N$	(Parkin & Owen, 1986)	0.696	37.50	62.50
<b>Microbial mass</b>	$C_5H_9O_{2.5}NS_{0.025}$	(Roels, 1983)	0.473	47.63	52.38
<b>Sewage sludge</b>	$C_{9.7}H_{17}O_{4.8}NS_{0.17}$	(Vassilev, et al., 2014)	0.522	44.77	55.23
<b>Grass</b>	$C_{63.7}H_{94}O_{42.5}NS_{0.063}$	(Vassilev, et al., 2014)	0.469	48.85	51.15
<b>Straw</b>	$C_{48}H_{70}O_{31.5}NS_{0.055}$	(Vassilev, et al., 2014)	0.470	48.99	51.01
<b>Microalgae</b>	$C_{2.11}H_{3.93}ON_{0.26}$	(Heaven, et al., 2011)	0.548	43.19	56.81
<b>Apples</b>	-	(Lane, 1984)	0.228	-	-

## 2.5 Valorisation of AD effluent and flue gas for algae cultivation

For microalgae to efficiently capture  $CO_2$  and extract nutrients from wastewater, the species should possess certain characteristics that makes it adaptable to grow in conditions found in AD effluent and flue gas. These include, but are not limited to:



- High CO<sub>2</sub> utilization rate to make anaerobic digestion flue gas recycling possible
- High growth rate for maximum biomass production
- Tolerance to high alkalinity to grow in AD effluent
- Tolerance to high temperatures and fluctuations for large scale operations
- Tolerance to high salinities if seawater is to be used to suspend wastes
- High potential for high value chemicals or biofuel production for process economic feasibility
- Easily collectable during harvesting process for low operational costs
- Ability to utilise free ammonia/ammonium as nitrogen source to grow in AD effluent

Various microalgae and cyanobacterial strains that can grow at high temperatures, alkalinity and inorganic carbon levels are shown in Table 7.

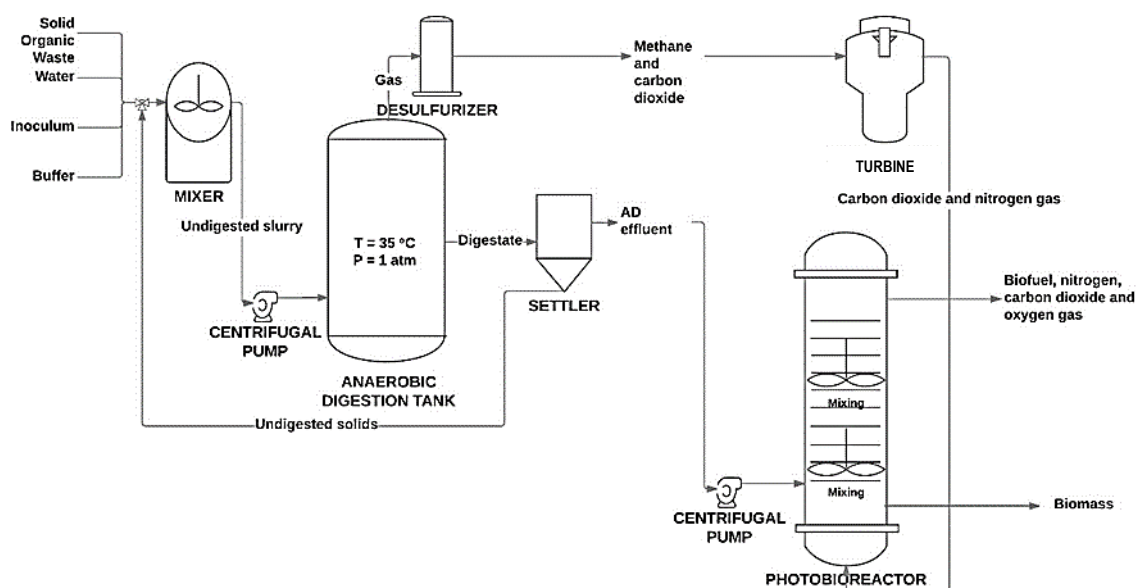
**Table 7: Growth parameters of various microalgae strains**

Species	pH	CO <sub>2</sub> % enrichment of air	Temperature (°C)	Doubling time (hr <sup>-1</sup> )	Source
<i>Chlorococcum</i> <i>species</i>	4-9	70	15-27	8	(Li, et al., 2006)
<i>Chlorella vulgaris</i>	3-7	60	15-45	2.5-8	(Li, et al., 2006)
<i>Chlamydomonas</i> <i>reinhardtii</i>	5.5-8.5	7	15-37	14	(Messerli, et al., 2005 and Vitova, et al., 2011)
<i>Galdieria sp.</i>	2-6	100	50	24	(Li, et al., 2006)
<i>Viridiella sp.</i>	2-6	100	15-42	13	(Li, et al., 2006)
<i>Synechococcus</i> <i>lividus</i>	8.2	5	40-55	2.9	(Li, et al., 2006)
<i>Synechococcus</i> <i>elongatus</i>	7-9	60	16-42	3 or less	(Xu, 2010)

Bogan *et al.* first suggested the intensive growth of algae in wastewater with the purpose of removing excess nutrients in 1960. The idea was investigated further in 1967 by Oswald *et al.*, who shifted the focus more towards treatment of wastewater with high-rate algal growth in ponds. This idea was put to the test in South Africa by Bosman and Hendricks in 1980. A large scale case study was conducted where industrial nitrogenous wastes was removed with multi-stage high-rate algal ponds. By combining AD with microalgal growth, the high costs of nutrient supply (inorganic carbon, nitrogen, phosphorous and water) could be significantly lowered through waste valorization. CO<sub>2</sub> enriched air can also be provided by the AD process. During anaerobic digestion, methane (CH<sub>4</sub>) and CO<sub>2</sub> produced can be burned in air to produce energy and a CO<sub>2</sub> enriched N<sub>2</sub> flue gas, which can serve as a carbon source for the algae. Lastly, due to the high ammonia content in AD effluent, dilution is usually required to lower its concentration below the toxic threshold.

The second high-cost barrier addressed by this project is the separation of valuable products from the algae. Rather than producing a high value liquid product, some cyanobacteria has been genetically modified to produce biofuels, which readily partitions itself to the gas phase. This circumvents the need for costly downstream liquid processing, and allows for the simpler collection of the valuable product from the gas phase, most likely through its condensation.

Over the last 30 years, a profound understanding of the biology and ecology of large scale algal cultures emerged which enabled genetic engineering of these simple organisms to produce high value products such as biofuels and pharmaceuticals. Furthermore, their ability to incorporate nutrients such as nitrogen and phosphorus with their photosynthetic ability enables them to convert solar energy into biomass which can be used for feedstocks, biofuels or high value products (Clemens & Walter, 2012). Their high tolerance to a wide variety of organic pollutants makes them effective in treating human sewage (Ibraheem, 1998), industrial wastes (Kaplan, et al., 1988), agro-industrial wastes (Zaid-Iso, October 1990), agricultural wastes (Phang & Ong, 1988) and livestock wastes (Lincoln & Earle, 1990). They also produce oxygen, remove heavy metals to some extent and create a disinfecting phenomena during growth because of an increase in pH (Abdel-Raouf, et al., 2012). A proposed process where anaerobic digestion and microalgal cultivation are combined is depicted in Figure 6.



**Figure 6: Process flow diagram for the integration of anaerobic digestion (35°C and 1 atm) with microalgal growth in a photobioreactor (38 °C and 1 atm), producing valuable chemicals/biofuel, carbon dioxide and oxygen.**

Cyanobacteria's robustness, simple growth needs and easy genetic modification makes them promising candidates for biofuel production. The final major tipping point in cyanobacteria's favour is the fact that their growth will not required the addition of freshwater. In fact, some species like *Nannochloropsis salina*, *Synechococcus* and *Synechocystis* are very robust strains that can live in brackish water or seawater and endure high salt stresses (Cai, 2012). This is important, since seawater can be used to

circumvent high turbidity, ammonia toxicity, contamination and low electrical conductivity issues associated with AD effluent. It is virtually cost free to obtain and is available in abundance. Lastly, the broad existance of *Synechococcus elongatus* in natural environments could provide large amount of feedstock for bioenergy production and so far, no research on growing this species in AD effluent has been reported.

### **2.5.1 Nitrogen and phosphorous removal**

With reference to the anaerobic digestion process described in Section 2.3.2, the digester effluent will contain valuable nutrients which algae can utilize for growth. It is not known from literature if AD effluent contains all the micro nutrients present in synthetic medias, but nitrogen, phosphate, potassium, calcium and magnesium should be in ample supply (Fricke, *et al.*, 2007 and Issah & Salifu, 2012). These compounds are key constituents of algal growth media. While the algae grow, they will absorb these nutrients from the AD effluent, effectively cleaning the water. It has been shown that over 99% of ammonium and phosphate can be removed from wastewater by algae under semi-continuous operation (Woertz, *et al.* 2009; Martínez, *et al.* 2000 and Ruiz-Marin, *et al.* 2010). The presence of dissolved organic carbon in the effluent will boost microalgal growth, but can also lead to contamination issues.

Table 8 provides a summary of literature publications where microalgae successfully lowered nitrogen and phosphorous levels in AD effluent, followed by a discussion of the data presented in it.

**Table 8: Algae growth on AD effluent studies in literature**

Species Name	Algae type	AD effluent source	NH <sub>3</sub> -N [mg.L <sup>-1</sup> ]	PO <sub>4</sub> -P [mg.L <sup>-1</sup> ]	pH range	Dilution	CO <sub>2</sub> enrichment (v/v)%	Light [ $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ]	Source
<i>Scenedesmus dimorphous</i>	Fresh water	Dairy manure	73	6.7	6.9-7	Deionised water	Sparged, unknown	485	(Wahal, 2010)
<i>Chlorella vulgaris</i>	Fresh water	Dairy manure	65	7.9	6.9-7	Deionised water	Sparged, unknown	485	(Wahal, 2010)
<i>Scenedesmus accuminatus</i>	Fresh water	Piggery manure	119.6	7.5	7.5	Deionised water	None	200	(Park, et al., 2010)
<i>Microspora willeana</i>	Fresh water	Dairy manure	178	24.4	7-7.5	Unknown	Unknown	140	(Wilkie, 2015)
<i>Chlorella vulgaris</i>	Fresh water	Piggery manure	1029.1 (undiluted)	23.9 (undiluted)	8.68-8.88	Deionised water	400 ppm	Unknown	(Kumar, et al. 2010)
<i>Chlorella minutissima</i>	Terrestrial	Poultry litter	76-152	6-12	Unknown	Deionised water	Unknown	75-80	(Singh, et al., 2011)
<i>Chlorella sorokiniana</i>	Fresh water/ terrestrial	Poultry litter	76-152	6-12	Unknown	Deionised water	Unknown	75-80	(Singh, et al., 2011)
<i>Scenedesmus bijuga</i>	Fresh water	Poultry litter	76-152	6-12	Unknown	Deionised water	Unknown	75-80	(Singh, et al., 2011)
<i>Nannochloropsis salina</i>	Marine	Municipal wastewater	68-546	11.43-91.44	7-10	Deionised water	400 ppm	200	(Cai, 2012)
<i>Synechocystis PCC6803</i>	Marine	Municipal wastewater	68-546	11.43-91.44	7-10	Deionised water	400 ppm	200	(Cai, 2012)
<i>Chlorella</i>	Fresh water	Dairy manure	89.3-223.2	9.98-24.97	Unknown	Deionised water	10	200	(Wang, et al., 2010)
<i>Eichhornia crassipes</i>	Fresh water	Dairy manure	69 and 130	16.5 and 32	7.81	Deionised water	None (bicarbonate)	Natural light	(Sooknah & Wilkie, 2004)
<i>Hydrocolyte umbellata</i>	Fresh water	Dairy manure	69	17.7	7.8	Deionised water	None (bicarbonate)	Natural light	(Sooknah & Wilkie, 2004)
<i>Pistia stratiotes</i>	Fresh water	Dairy manure	69	16.6	7.9	Deionised water	None (bicarbonate)	Natural light	(Sooknah & Wilkie, 2004)

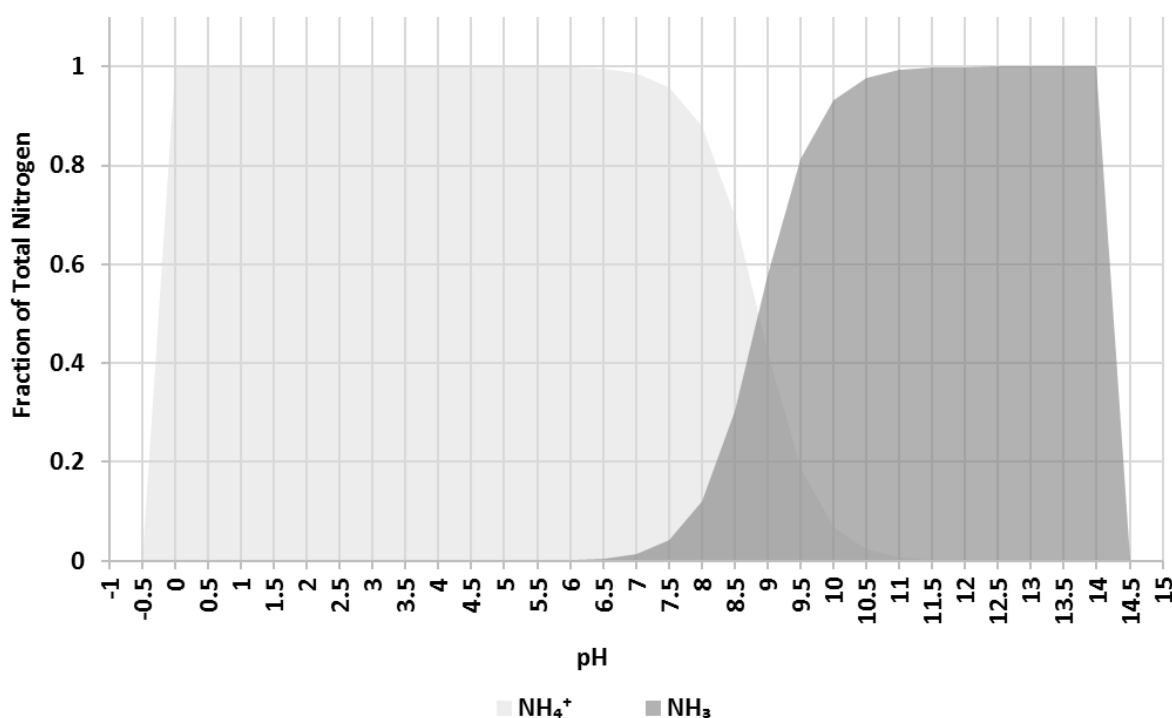
<i>Spirulina maxima</i>	Marine	Piggery manure	76	13.6	9.3	Seawater	None (bicarbonate)	60-70	(Olguin, et al., 1994)
<i>Botryococcus braunii</i>	Fresh water	Carpet industry waste/municipal sewage	17.58-25.85	20.31-35.10	6.54-8.04	None	400 ppm	75-80	(Chinnasamy, et al., 2010)
<i>Chlorella saccharophila</i>	Fresh water	Carpet industry waste/municipal sewage	17.58-25.85	20.31-35.10	6.54-8.04	None	400 ppm	75-80	(Chinnasamy, et al., 2010)
<i>Dunaliella tertiolecta</i>	Marine	Carpet industry waste/municipal sewage	17.58-25.85	20.31-35.10	6.54-8.04	None	400 ppm	75-80	(Chinnasamy, et al., 2010)
<i>Pleurochrysis carterae</i>	Marine	Carpet industry waste/municipal sewage	17.58-25.85	20.31-35.10	6.54-8.04	None	400 ppm	75-80	(Chinnasamy, et al., 2010)
<b>Consortium</b>	Fresh water	Dairy manure	18.5-73.8	24-96	Unknown	Deionised water	Unknown	67.5	(Ding, et al., 2015)

All the sources mentioned reported a nitrogen and phosphate reduction higher than 90%. It is clear that a variety of algae species can fulfill this niche and that the technology can be applied to various anaerobically digested wastewaters. There are however two major drawbacks in terms of scalability. Firstly, the studies do not attempt mixing substrates during anaerobic digestion in order to optimize C/N ratios for maximum biogas production. Secondly, nearly all studies dilute the AD effluent with deionised water in order to lower ammonia concentrations and turbidity in the AD effluent. Not only does this contradict the goal of growing algae in AD effluent, which is after all removing pollutants from wastewater, but clean water is also an extremely valuable resource. In short: it defeats the purpose of using microalgae as a sustainable and economical replacement for conventional wastewater treatment processes and should be avoided. Most of these experiments were conducted at ideal alkaline pH values, but this means that the AD effluent has to be diluted more in order to overcome ammonia toxicity issues. By operating as low as possible below the pK value of the ammonia ammonium buffer system, one can limit this toxic phenomenon while still using the maximum amount of AD effluent, essentially treating more of it per batch cycle.

### 2.5.2 pH dynamics

Many factors have an impact on algae growth medium pH and under some conditions, it has been reported that outdoor algae media can reach pH levels of up to 11 during the day (Moheimani, 2012).

According to the schematic representation of inorganic nitrogen assimilation in Figure 2,  $\text{NO}_3^-$  assimilation leads to an output of  $\text{OH}^-$  ions, causing an increase in the media pH. If  $\text{NH}_4^+$  is the dominant nitrogen source,  $\text{H}^+$  ions are produced leading to a decrease in overall pH of the media. Although  $\text{NH}_4^+$  requires less energy than other nitrogen sources to be utilised by algae cells, a drop in pH below the ammonia/ammonium  $\text{pK}_a$  value of 8.86 at 38 °C would be required to ensure the toxic effects of high concentrations of ammonia could be mitigated. Operating at a lower pH would result in an equilibrium shift towards ammonium instead of ammonia and this could possibly be achieved by the intentional reduction of the pH in the AD effluent. By using Eqns. 8 – 9 and aqueous ammonia equilibrium tables (Thurston, et al., 1979), Figure 7 illustrates this relationship:



**Figure 7: Ammonium/ammonia buffer system as a function of pH at 38 °C**

To buffer the system for a specific pH, it should be done with chemicals that are not limiting reagents in the media, but rather nutrients that are available in excess to ensure the growth observed is not affected in any way by the buffer agent.

### 2.5.3 Turbidity and light attenuation

The presence of dissolved and suspended material in anaerobic digestate can cause light attenuation and consequently limit photosynthetic activity in the effluent. In essence, microalgal growth is limited by the availability of photosynthetic active radiation (PAR). Any suspended or dissolved matter that

increases light absorbance between 400 and 700 nm can significantly reduce microalgal growth yield (Monlau, et al., 2015) as PAR is reduced. In 2010, Wang *et al.* succeeded in showing that the specific growth rate of *Chlorella sp.* is inversely proportional to the initial turbidity of digested dairy manure. In order to remove solid particles from the effluent chemical precipitation (Chen, et al., 2012), microfiltration (Bchir, et al., 2011), centrifugation (Marchilhac, et al., 2014) or decanting (Blier, et al., 1995) can be applied. The studies did not produce any data that established whether or not these clarification steps improved the optical characteristics of the digestate.

#### **2.5.4 Valorisation of AD flue gas through algae cultivation**

To avoid irreparable environmental damage, sequestration of industrially produced CO<sub>2</sub> from the atmosphere is essential. This calls for a transition into sustainable and carbon neutral systems, replacing present technologies that aims to mitigate and remediate CO<sub>2</sub> emissions. Most options proposed for capturing CO<sub>2</sub> has been classified as economically and environmentally short sighted. Commonly, immediate consequences of carbon emissions are set-off simply by “end-of-pipe” solutions such as injection into geological or oceanic sinks (Packer, 2009).

Before disposing of CO<sub>2</sub>, it must first be separated from flue gases. Physical methods employed for the removal of CO<sub>2</sub> on an industrial scale include scrubbing techniques, filtration and adsorption. The solvent monoethanolamide (MEA) can be used in scrubbers to adsorb CO<sub>2</sub> and this carbon rich solution is then reheated in a stripper where pure CO<sub>2</sub> is released. Despite recycling MEA, this technology typically requires large investments, equipment space and energy inputs, resulting in tremendous operation costs. Molecular sieves have demonstrated effective separation of CO<sub>2</sub> from methane, air and other gas mixtures. The method is based on separation according to molecule size and weight and they can easily be desorbed and released from the sieve by applying a low voltage across the sieve. Despite high investment costs, it is associated with low operational costs and minimal waste production. Lastly, the use of adsorbents such as zeolite proves to be effective in the removal of CO<sub>2</sub> at atmospheric pressures using a technique referred to as temperature swing. The presence of sulphur oxides in flue gases was found to impair this adsorbent (Stewart & Hessami, 2005).

After the effective separation of CO<sub>2</sub> from flue gases using the above-mentioned techniques (approximately 90% purity), popular disposal techniques include injection of the gas into the oceans, coal seams and oil reserves. Although the technology can serve as a potential interim solution while more sustainable energy sources are industrialised (Adams & Caldeira, 2008 and Tang, *et al.*, 2014), high associated risks (such as leakage and negative environmental impacts) combined with high investment costs, little to no economical return (Stewart & Hessami, 2005) and challenges with regards to legislation and public perception (Wilson, et al., 2008) calls the longevity of this treatment method into question.

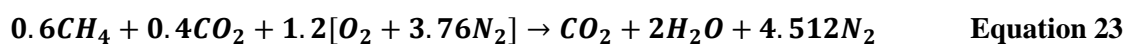
Turning CO<sub>2</sub> emissions into a fuel through photosynthesis is a way of recycling the carbon, reducing the demand for high-value resources. The impact on the global carbon cycle and environment would be reduced. True sequestration will not only require recycling, but reduction in amounts of CO<sub>2</sub> available globally. This would require sequestration into materials that do not release the gas back into the atmosphere. The concept of fixating the carbon into biomass through photosynthesis presents a way of sequestering CO<sub>2</sub> in a sustainable, profitable manner.

Algae's photosynthetic efficiency is higher than all other plant types – up to 12.5 kg.m<sup>-2</sup>.year<sup>-1</sup> (Shay, 1993). This equates to a lower land area demand for cultivation compared to terrestrial crops. In addition, they require neither arable land nor fresh water for cultivation. Apart from biofuels, the sequestration of CO<sub>2</sub> into algal biomass is deemed profitable through the production of high value products such as pigments and high-grade lipids (Stephens, et al., 2010). Comparisons between fossil fuels and biofuels profit fail to report some of the factors negatively affecting fossil products. Costs associated with initial exploration, pipeline installation and maintenance as well as the economic value of environmental impacts are rarely, if ever, mentioned (Campbell, et al., 2011). The economic viability of biofuels produced through CO<sub>2</sub> bio-fixation is an ongoing discussion in the waste valorisation field (Packer, 2009).

There is a flexibility on the type of energy that can be produced, ranging from heat, combined heat and electricity, purification (natural gas) or vehicle fuels (Guiot & Frigon, 2012). Thus, the flue gas from anaerobic digestion can be burnt for heat or energy generation while the CO<sub>2</sub> produced from this can be fixated into biomass through photosynthesis. CO<sub>2</sub> as a nutrient represents one of the costliest components in the cultivation of microalgae (Borowitzka, 1992). This way, CO<sub>2</sub> production from methane combustion can be sequestered in a sustainable manner. Carbon removal efficiency of biogas CO<sub>2</sub> to form biomass has been proved to reach up to 95% (Converti, et al., 2009).

#### Growth in burnt biogas

The combustion of methane in air follows the stoichiometry shown in eq. 23, assuming the biogas produced contains 40% CH<sub>4</sub> and 60% CO<sub>2</sub>:



The composition of burnt biogas will depend on the amount of excess air supplied to the combustion reaction.

#### Growth in pure biogas

Although biogas produced from anaerobic digestion is generally used as an energy source, the high volume of CO<sub>2</sub> in the gas reduces its heat content and some argue is not useful for the combustion of biogas to produce energy. Upgrading the biogas by removing the CO<sub>2</sub> will increase methane concentrations similar to levels associated with natural gas (95-98% CH<sub>4</sub>), typically referred to as



“biomethane”. Using microalgae to achieve this could result in a less costly method compared to conventional methods such as water washing, pressure swing adsorption and amine gas scrubbing.

## **2.6 Concluding thoughts**

To increase the feasibility of valuable chemical or biofuel production from microalgae, a significant opportunity exists if the nutrients required can be obtained through waste valorisation, with no requirement for potable water. AD effluent contains nutrients required for microalgal growth but has limitations in its application due to high ammonia concentrations, high turbidity and the lack of mineral salts which cyanobacteria require for growth. To understand how these variables influence the microalgal growth, mainly three phenomena are expected to arise from experiments in AD effluent:

- i. Properties and compounds that inhibit growth (ammonia and turbidity)
- ii. Absence of nutrients or conditions required for growth ( $\text{MgSO}_4$  and electrical conductivity)
- iii. Nutrient limited growth ( $\text{CO}_2$  and light)

### 3. Goals and deliverables

The specific goals and objectives of this study are:

#### Goal 1: Identify limitations or inhibitions of algae growth associated with AD effluent

1. Design an incubator (including the complete experimental setup required) in which the batch experiments are to be conducted.
2. Generate AD effluent using feedstock that is available.
3. Develop an accurate and sound methodology for growing the study organism in various altered media to establish inhibiting or limiting phenomena:
  - a. Under ideal light and temperature conditions in synthetic media with no CO<sub>2</sub> enrichment in the air.
  - b. Under the same ideal conditions with no CO<sub>2</sub> enrichment in the air as stipulated in point a, but using AD effluent as the primary growth media.
  - c. Under the same conditions as stipulated in points a and b, but adding CO<sub>2</sub> enrichment in air.

#### Goal 2: Assess dilution of AD effluent with seawater and its effect on algae growth

1. Develop an accurate and sound methodology for growing the study organism in AD effluent diluted with seawater:
  - a. Under ideal light and temperature conditions in synthetic media with no CO<sub>2</sub> enrichment in the air.
  - b. Under the same ideal conditions with no CO<sub>2</sub> enrichment in the air as stipulated in point a, but using AD effluent as the primary growth media.
  - c. Under the same conditions as stipulated in points a and b, but adding CO<sub>2</sub> enrichment in air.

#### Goal 3: Assessing scale-up in an airlift reactor

4. Scale up the process by growing the study organism in an 8 L airlift reactor:
  - a. Under lower light conditions and room temperature in synthetic media with air bubbling.
  - b. Under lower light conditions and room temperature in optimised AD effluent with air bubbling.

## 4. Materials and methods

### 4.1 Anaerobic digestion

#### 4.1.1 Generation

Apple fruit waste was obtained from Elgin Fruit Juices (Grabouw) and cow manure from Stellenbosch University's experimental farm, Welgevallen. These substrates were used throughout the entire project, aiming to achieve minimal variability of nutrient concentrations in the digestate. The raw media was made up according to the following procedure:

Equal parts of fruit waste and cow manure was added together in a Schott bottle to make up a total weight equal to 10% of the final working volume of the anaerobic digestion tank. Then, 10% of the final working volume of the anaerobic digestion tank was added as fresh, degassed inoculum.  $\text{CaCO}_3$  was added as buffering agent at a concentration of  $5 \text{ g}\cdot\text{L}^{-1}$ . The Schott bottle was topped up with deionised water to make up the final working volume and then mixed using a stirring plate and magnetic stirrer. While it was stirred, the pH was measured and adjusted with HCl or NaOH to be between 6 and 6.5. Thereafter, the bottle was sealed with a rubber stopper with two holes. While sparging with nitrogen through the one hole, the other was kept open to allow any oxygen present in the system to escape. Thereafter, both holes were sealed and once it was placed in the water bath allowing as much as possible water to cover the bottle surface (to limit temperature gradients in the reactor). A plastic pipe was inserted in to one hole, with its other end in the water to ensure no oxygen enters the system, but also to allow biogas to escape from the reactor and prevent pressure build-up. The temperature of the water bath was set to  $35 \text{ }^\circ\text{C}$  and the digestion process was allowed to continue for 21 days, or when all organic material was digested. The experimental setup is illustrated schematically in Figure 8.

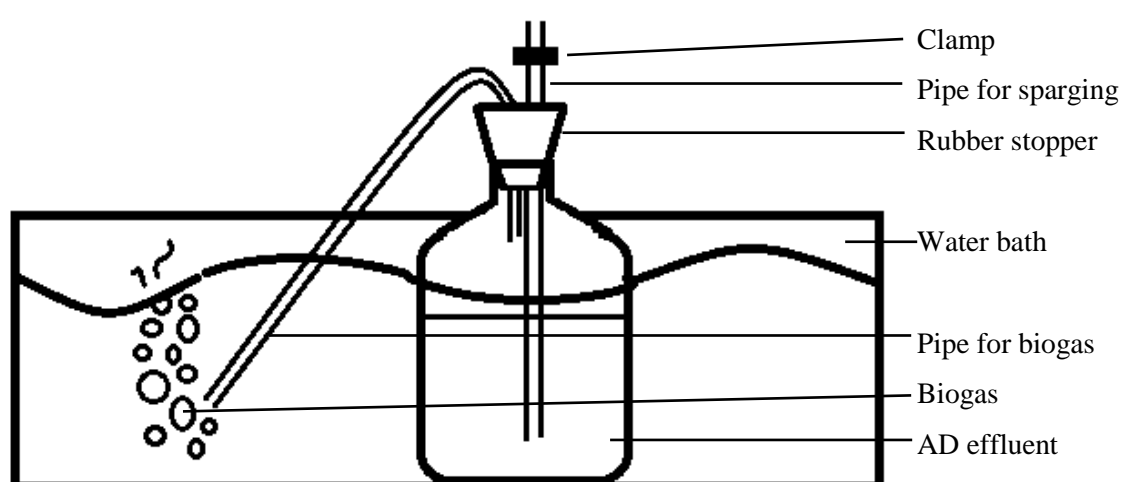


Figure 8: Schematic representation of anaerobic digestion experimental set-up

### 4.1.2 Processing

In order to remove any organic material and dead microorganisms, 120 mL batches of the digestate was first centrifuged using the Hettick Mikro 120 Benchtop centrifuge at room temperature and 1 atm for 20 minutes at 14400 rpm. Then, the centrate was vacuum filtered using glass fibre filters, 0.45 µm filters and lastly 0.22 µm filters in that specific order. The media was then sterilised according to procedures stipulated in Section 4.3.6.

## 4.2 Algae culturing

### 4.2.1 Synthetic media

Different synthetic growth media for *Synechococcus* PCC 7002 have been widely studied and the composition thereof adapted for optimal growth. Three growing media were established to provide optimal nutrient content: Medium A, medium A+ and medium A D7 (Wu 2014; Sakamoto and Bryant 1997; Stevens, *et al.* 1973; Ludwig and Bryant 2012; Wilhelm *et al.* 1996; Xu, *et al.* 2011; Christi 2007). Medium A D7 was used for this project and the composition of this growing media and procedures to produce it is explained in the remainder of this section.

The microalgae of interest were received from Prof Toivo Kallas of the University of Wisconsin (WISC). Medium A D7, a recipe developed at the University of Texas (UTEX) is the most widely used medium for the growth of this specific strain at a pH of 8.2. Research performed by the strain supplier at WISC illustrated that another set of nutrients termed D7 performs equally well. This was originally developed by Arnon *et al.* (1974).

Medium A D7 is a derivative of medium A and for clarity, its constituents can be classified into 4 sets: A base, AFM base, D7 micronutrients and complete medium A. The components and relative quantities of each of these stock solution batches is presented in Table 9 to

Table 12.

To approximately 390 mL of distilled water, the first 5 components were added in the order specified in Table 9 while stirring continuously. This was done by placing magnetic stirrers on a magnetic stirrer. The total volume was topped up to 500 mL with distilled water and in most cases, this recipe was doubled. For the micronutrients, the same was done according to Table 11.

#### All other compounds in Table 10 and

Table 12 was made up in the same way, except for the vitamin B12 mixture since autoclaving will cause the vitamins to disintegrate. Mixtures were filter sterilised instead and then placed in the fridge at 4 °C. After sterilisation, the respective quantities of Fe-EDTA, micronutrients, Tris-HCl, KH<sub>2</sub>PO<sub>4</sub> and vitamin B12 were added to A base recipe and stored in the fridge at 4 °C.

**Table 9: A base recipe**

Stock solution concentration [M]	Powder [g/L]		Equivalent volume of stock [mL/L]		Final (1x) concentration [M]
	1x	10x	1x	10x	
5 M NaCl (99.999%)	18	180	60	-	0.3
1 M KCl (99.0 - 100.5%)	0.6	6.0	8	80	0.008
1 M NaNO <sub>3</sub> (≥ 99.0%)	1.0	10	12	120	0.012
1 M MgSO <sub>4</sub> ·7H <sub>2</sub> O (≥ 99.5%)	5.0	50	20	200	0.2
1 M CaCl <sub>2</sub> (≥ 97%)	0.2775	2.78	2.5	25	0.0025

**Table 10: AFM base recipe (A base + Fe-EDTA + micronutrients)**

For each 1x A base add:	Volume added [mL]	Final (1x) concentration
15 mM Fe-EDTA* (≥ 95%)	1	15 μM
D7 micronutrients (1000x)**	1	1x

\*For 1L of 20mM Na<sub>2</sub>EDTA add 1mL of 5 mM FeSO<sub>4</sub> solution

**Table 11: Micronutrient stock solution recipe (diluted 1:1000 into the final medium)**

Compound	Amount [g/L]
H <sub>3</sub> BO <sub>3</sub> (≥ 99.5%)	2.86
MnCl <sub>2</sub> ·4H <sub>2</sub> O (99%)	1.81
ZnSO <sub>4</sub> ·7H <sub>2</sub> O (99%)	0.222
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O (≥ 99.5%)	1.26
CuSO <sub>4</sub> ·5H <sub>2</sub> O (≥ 98.0%)	0.079
NaVO <sub>3</sub> (≥ 98.0%)	0.239
CoCl <sub>2</sub> ·6H <sub>2</sub> O (98%)	0.0403

**Table 12: Complete medium A recipe**

Component solution concentration [M]	Volume [mL] added per 1L AFM base	Final concentration (1x) [mM]
1 M Tris-HCl (pH = 8.2)* (≥ 99%)	8.3	8.3
1 M KH <sub>2</sub> PO <sub>4</sub> ** (99.99%)	0.37	0.37
6μM vitamin B <sub>12</sub> *** (≥ 98.0%)	1	6

\*Add 24.22g per 200 mL; \*\*Add 13.6g per 100 mL; \*\*\*Add 8mg per 1L

### 4.2.2 Synthetic seawater media

Synthetic sea salt obtained from RED SEA was used to produce synthetic seawater to simulate the compounds present in natural seawater. It has the following properties according to the supplier when adding 38.2 g/L shown in Table 13:

**Table 13: Synthetic seawater properties**

Property	Value	Units
Concentration sea salt	38.2	g/L
Specific gravity	1.0255	Dimensionless (at 25 °C)
Salinity	35.5	ppt
pH	8.2 - 8.4	Dimensionless
Alkalinity	7.8 - 8.2	°dKH
Ca concentration	420 - 440	mg/L
Mg concentration	1250 - 1310	mg/L
K concentration	380 - 400	mg/L

The synthetic seawater was made up by adding 38.2 grams of the salt to 1 L of deionised water whilst continuously stirring at high speed on a thermostatic magnetic stirrer. It was left to dissolve at 50 °C for approximately 30 minutes and then sterilised according to procedures in Section 4.3.6.

## 4.3 Analytical methods

### 4.3.1 Media characterization

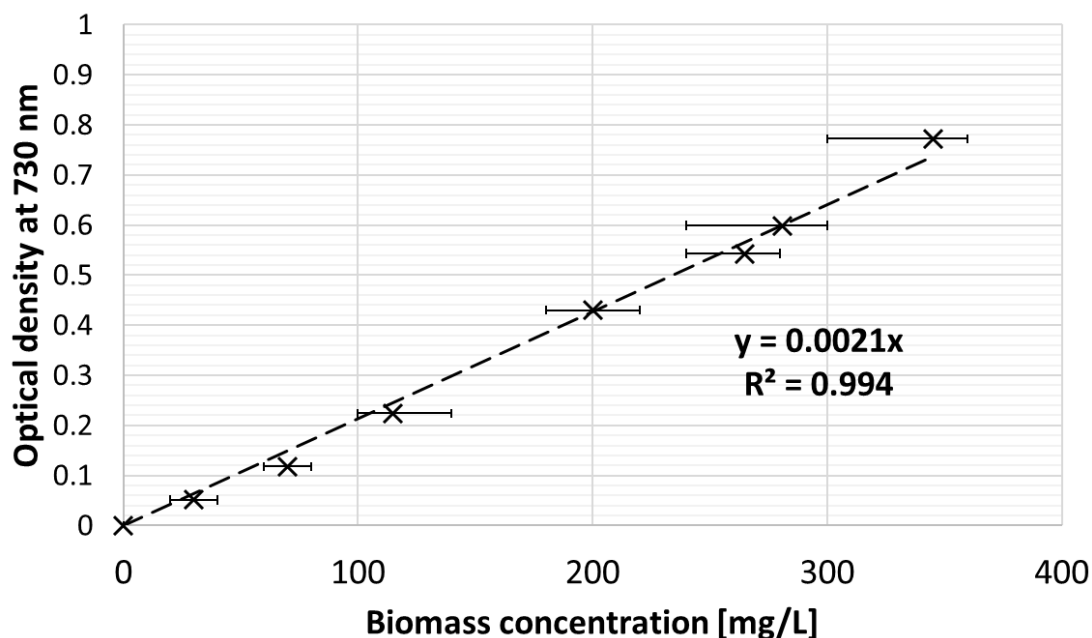
Elemental analysis of all medias (synthetic media, synthetic seawater and AD effluent) were performed at the CSIR, selectively choosing properties that would be relevant to the studies performed in this project. As the composition was available for media A D7 and synthetic seawater, these medias were analysed only once, while each batch of AD effluent made was analysed individually. This would not only ensure more accurate results when monitoring nutrient removal but would also serve as an example of the variability to be expected when employing AD technology in conjunction with other processes and what the effect of that variability in composition could be.

### 4.3.2 Biomass quantification

#### Spectrophotometry

To determine biomass concentration, optical density (OD) readings using a spectrophotometer is the most widely used method. The wavelength at which the spectrophotometer was set to quantify the OD for *Synechococcus* PCC 7002 is 730 nm and was kept constant. A calibration curve was generated by collecting a sample of known volume and OD and making serial dilutions that will have OD readings between 0 and 1. Distilled water was scanned by the spectrophotometer to represent an OD of 0. For

each of these samples, a small fixed volume was collected and weighed. The samples were vacuum filtered, and the remaining wet biomass was dried overnight in an oven at 80 °C. The dried sample biomass was re-weighed to determine the dry mass, and this was used to generate the calibration curve presented in Figure 9. This curve was obtained after repeating the process several times until the variation in results obtained were less than 10%.



**Figure 9: Calibration curve for biomass to optical density correlation**

#### Total Suspended Solids (TSS)

Since all medias are filtered before growing the culture, it can be assumed with reasonable certainty that the only suspended solids present in the medias will be algal cells or biomass. To generate calibration curves between optical density and biomass generated, total suspended solids had to be quantified.

Filter papers were first numbered to keep track of sampling and data and then rinsed with 20 – 30 mL of deionised water to remove any solids on the surface. From that point onward, they were only handled with tweezers to minimize any skin cells or oils that can attach to the surface. They were placed in aluminium weight pans and left overnight to dry at 80 °C. The next morning, the papers together with the pan was placed in a desiccator and then weighed individually. 20% of the filter papers were re-dried overnight, placed in the desiccator and weighed to ensure accuracy (within 0.5 mg) and consistency in the protocol followed. Once the weights were obtained, a fixed volume (recorded) of sample was collected from a stirred reactor and vacuum filtered through the pre-weighed filter paper. The biomass filtered wet papers were then placed onto the aluminium weight pans and dried at 80 °C for 48 hours. The papers, together with the aluminium pan was placed in a desiccator to cool down and then weighed. Again, 20% of the filter papers were re-dried overnight, placed in the desiccator and weighed to ensure

accuracy (within 0.5 mg) and consistency in the protocol followed. The total solids could then be determined by eq. 24.

$$TSS \left[ \frac{mg}{L} \right] = \frac{m_{Dried\ biomass\ containing\ filterpaper} - m_{Dried\ washed\ filterpaper} [mg]}{V_{sample} [L]} \quad \text{Equation 24}$$

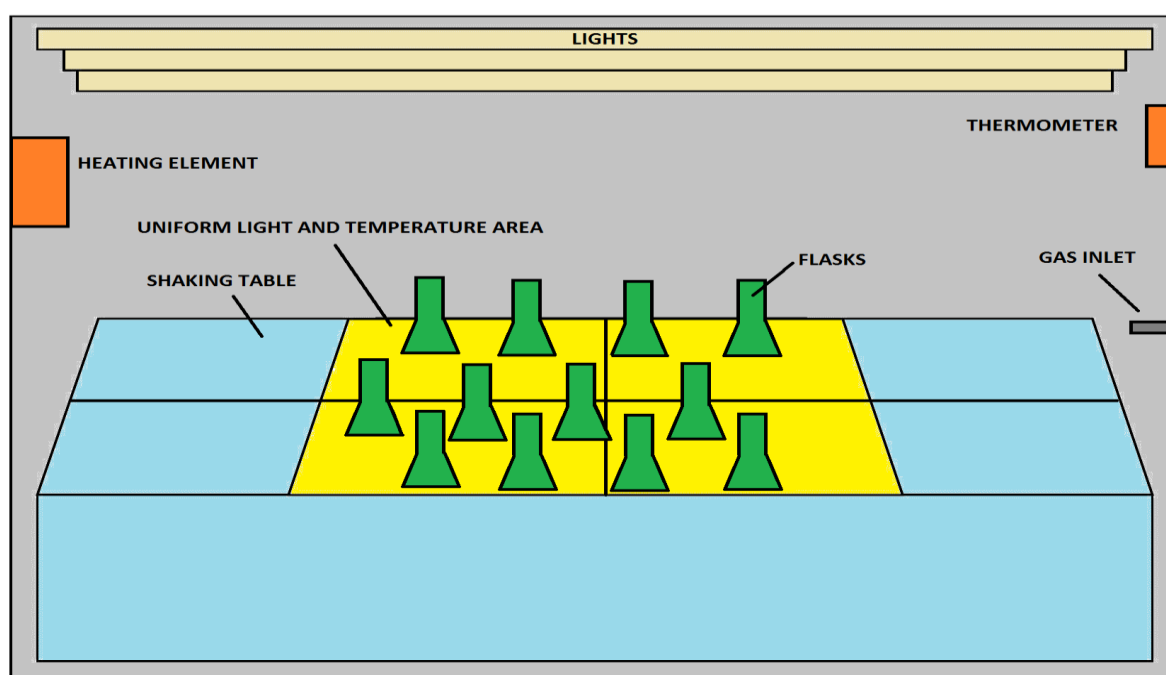
Due to the limited available working volume of each experiment, the samples could not be taken in triplicates. But the 20% of the papers that were re-checked aimed at reducing any systematic or human error and if any results were not within the reasonable bounds, the experiment was repeated.

### 4.3.3 Experimental condition regulation

Experiments were carried out in an incubator that allowed for:

- light adjustment by lifting or lowering the lights
- temperature adjustment by adjusting the thermostat setpoint. It was calibrated and continuously monitored with a thermometer

The experimental set-up is shown in Figure 10.



**Figure 10: Experimental set-up**

#### Temperature

A 60 W (260 mA) panel heater and thermostat provided heat and temperature regulation in the incubator. Three single fitted 30 W fluorescent tubes were installed which can be lowered to provide variable light intensities ranging from 160 to 1771  $\mu E \cdot m^{-2} \cdot s^{-1}$ . The operating temperature of 38°C was monitored by three thermostats placed in the incubator. Since the heat retention of the media is higher than air, the medias themselves were always measured to be 1 – 2 degrees higher than the surrounding air. To minimise chances of contamination, this was always measured at the end of each experimental



run. For the photobioreactor, no temperature control or adjustments were made. Experiments took place at room temperature, always varying between 24 and 26 °C. The media itself, absorbing light and having greater heat retention than air was always between 1 and 2 °C hotter than the surrounding air.

### pH

The pH of the cultures was monitored using a Hanna Bench meter with pH/temperature digital probe. The 1 mL that were extracted for OD analysis were also used for pH analysis to minimise volume loss during the growth period. Sterile NaOH and HCl solutions of known concentrations were added dropwise to adjust the pH to the set point of the specific experiment. These specific solutions were chosen as they do not contain any compounds that act as nutrients or inhibitors for the algae culture.

### Light

Light was measured monthly using a light meter from SPER SCIENTIFIC DIRECT. The light sensor was placed on the shake table surface at various points to determine any light gradients in the incubator. This surface area is shown in Table 14, where the yellow zones indicate the usable surface in the incubator. The light intensity was converted to photon flux using the methods described in Appendix B.1. The average of the central eight measurements were used to determine the photon flux and the distance of the lights from the surface to achieve a photon flux of  $225 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  was 23.8 cm.

1845	3588	3575	1945
1884	3510	3550	2018
1917	3585	3580	2043
1982	3584	3575	2021

**Table 14: Light distribution in incubator measured in lux for fluorescent lights (surface area of shaking table divided in 16 equal parts)**

As for the photobioreactor, two 36 W fluorescent lights provided lighting along the length of the airlift reactor. Placed 12 cm from the airlift reactor surface on either side, the average light intensity at the surface of the reactor was calculated to be  $180 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

## **4.3.4 pH adjustment procedures**

### Continuous pH adjustment

The magnetic stirring plate, stirring bars, pipettes and pH probe was wiped with 70% ethanol and then sterilised in the laminar flow cupboard under UV light for 10 minutes before the procedure was started. Once opened, the Bunsen burner was switched on and all procedures were performed near an open flame. The magnetic stirring bar was placed in the reactor flask and put on top of the magnetic stirring plate. The pH probe tip was once again washed with ethanol and then placed in the media. The pH was then adjusted dropwise with freshly autoclaved 1M solutions of NaOH or HCl.

### Phosphate buffers

A standard 50 mM phosphate buffer saline (Gunter & Gunter, 1953) was prepared using the Henderson Hassel Balch equation, depicted in eq. 25:

$$pH = pK_a + \log \left[ \frac{Na_2HPO_4}{NaH_2PO_4} \right] \quad \text{Equation 25}$$

From this, it was determined that 0.573 grams of  $Na_2HPO_4$  and 0.583 grams of  $NaH_2PO_4$  had to be added to each 500 mL batch volume, which would ultimately make up 3 repeat runs.

An important phenomenon to consider when using phosphate buffers in solutions containing magnesium is struvite precipitation in the form of crystals. The reaction proceeds according to eq. 26 (Moussa *et al.*, 2011):



This reaction is strongly dependent on the degree of supersaturation level and the crystal growth rate dependent on the mixing strength (Olinger *et al.*, 1999). Hao *et al.* (2009) reported that struvite formation was limited below a pH of 7 at 25 °C and thus operating at pH values below this will maximise phosphate and magnesium in dissolved form, making it available for nutrient utilisation. Additionally, higher temperatures impede the formation of crystals, and so operating at low pH values at high temperatures should minimise the potential for struvite precipitation.

### **4.3.5 Total nitrogen sample analysis and consumption**

For carbon enriched experiments (both batch and scale-up experiments), total nitrogen was measured to indicate nutrient depletion using Merck Nitrogen (total) Cell Tests. The cell tests provide accurate results in the range of 10-150 mg/L. 10 mL of sample is vacuum filtered using 0.22 µm filters to remove any cells and precipitate from the solution. For samples that are expected to have nitrogen levels out of range of the test, dilutions were made 1:10 using deionised water. One scoop of Reagent 1 is added to the cell, shaken well for one minute and placed in a Spectroquant® Thermoreactor TR320 at 120 °C for 60 minutes. Samples were allowed to cool down to room temperature without placing in a water bath. One scoop of Reagent 2 is added to the reaction cell and stirred vigorously for 1 minute. 1.5 mL of cooled sample is pipetted slowly into the reaction cell along the side of the cell as to avoid splashing. Add 6 drops of Reagent 3 and close the cell immediately, taking care to avoid the cell as it becomes extremely hot due to the exothermic reaction between the sample (mainly water) and the concentrated sulfuric acid in the reaction cell. All handling of reaction cells must be done wearing an acid-proof lab coat, inside the fume cupboard and wearing acid proof gloves. Once Reagent 3 has been added, allow the samples to react for 10 minutes. A signature reddish colour shows that the reaction has taken place successfully. Adjust the setting in the Spectroquant® Prove 600: Powerful UV/VIS Spectrophotometer for complex analyses to Total Nitrogen Test (either by placing in the auto selector cell into the

spectrophotometer or selecting the specific test form the drop-down menu). Place the reaction cell in the spectrophotometer, ensuring the mark on the cell lines up with the mark in the cavity. Cells are labelled for proper discarding in accordance with lab protocol and washed and sterilised for future use. Total nitrogen was measured at the start and end of each experiment, and during runs if necessary.

#### **4.3.6 General sterilisation techniques**

For all experiments (including AD effluent, synthetic media, seawater and mixtures thereof), glassware, stirring bars and any other equipment were autoclaved before use. After make-up the medias were autoclaved to ensure no viable microbes are present in the digestate. This was done at 121 °C and 100 kPa for 15 minutes, allowing the equipment to cool down inside the autoclave to room temperature. To minimise the loss of volatile fatty acids in anaerobic digestates or medias containing anaerobic digestate, the containers should be completely closed when autoclaved, leaving enough head space in the container to avoid pressure build-up.

When sterilizing flasks, gauze was stuffed into the opening of the flask and covered with foil to ensure no steam or dirty water enters the flask during sterilisation. Flasks were removed from the autoclave and wiped with 80% ethanol to minimise any bacterial growth on the surface of the flasks. Equipment, such as empty flasks (together with a sterilised volumetric flask, pipette and 5 mL pipette tips) are placed in the laminar and labelled before the UV lights are switched on for 10 minutes. UV light is switched off and the Bunsen burner is lit. Gloves are re-sterilised with 80% ethanol. The sterilised bottled medias are wiped down with 80% ethanol before placing in the laminar.

#### **4.3.7 Flask placement in incubator**

Flasks are then placed in the incubator under standard conditions, except they are positioned as far as possible from the one fluorescent light that is switched on. They are left like this for 24 hours under low light conditions to minimise the risk of photobleaching. All lights are switched on and the experiments left to proceed until the hypothesis of that experimental run has been proven true or false.

#### **4.3.8 Inoculation procedure**

Media is measured off (140 mL) into the volumetric flask and decanted into each flask, taking care to perform this close to the open flame and minimising flask exposure to the atmosphere when decanting. After that, each flask is inoculated with 10 mL of liquid culture. A new pipette tip for each sample is selected, taking care to close the container as soon as it's lodged onto the pipette. The gauze is briefly removed from the batch flask when inoculating, and then put back immediately.

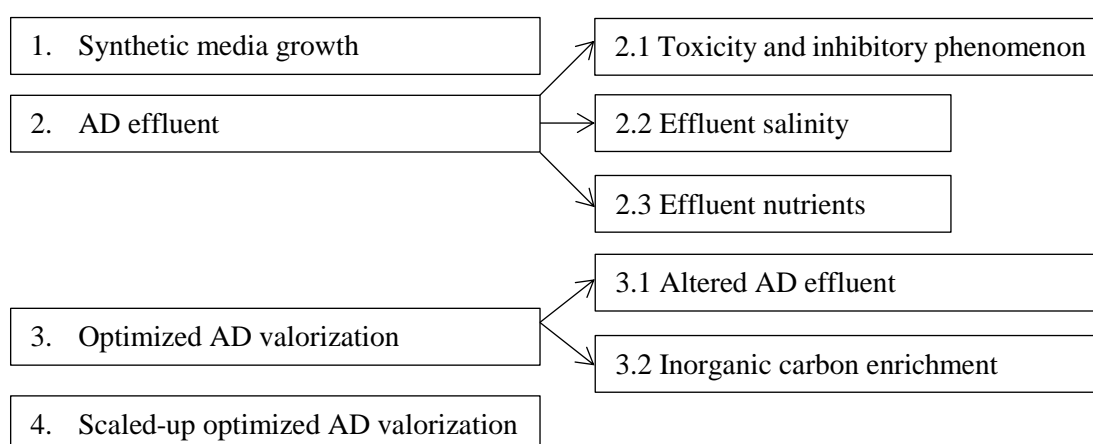
### **4.4 Laboratory scale experiments**

For any system, input variables, controllable variables and uncontrollable variables determined the output variables from it. The relevant variables in these experiments are described in Table 15.

**Table 15: Variable classification and description**

Variable classification	Variable name	Description
<b>Inputs</b>	Growing media	Synthetic growing media; AD effluent; seawater; mixtures
	CO <sub>2</sub> enrichment	400 ppm, 8 % (v/v) and 20 % (v/v)
<b>Controllable variables</b>	Temperature	38 °C
	pH	8.2
	Light intensity	250 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$
	Growing media volume	140 mL
	Mixing	150 rpm (orbital shaker)
	Algae inoculum volume	10 mL
	Light effects	Continuous light
<b>Uncontrollable variables</b>	Growth inhibitor in AD effluent	-
	Seasonal change in air composition	-
<b>Outputs</b>	Optical density	Dimensionless, but can be correlated to biomass concentration [ $\text{g}\cdot\text{L}^{-1}$ ]

The overall experimental investigation is illustrated in Figure 11. The bulk of experimental tests were aimed at decoupling and circumventing issues associated with the effluent as growing medium (2). Synthetic media growth experiments (1) form the benchmark to which results can be compared. All experiments in optimised AD effluent (3 and 4) serve as proof of concept for themes explored in (2) and illustrates the extent of growth improvement that can be achieved.

**Figure 11: Experimental framework**

The experimental conditions for each investigation shown in the framework is detailed in Table 16.

**Table 16: Experimental conditions**

<b>Nutrients</b>	<b>PH</b>	<b>Inorganic Carbon</b>
<b>1.</b> Media A D7	8.2	atmospheric
<b>2.</b> Undiluted AD effluent	8.2	atmospheric
Undiluted AD effluent with added micronutrients	8.2	atmospheric
AD effluent diluted at 74% and 90% with water	8.2	atmospheric
<b>2.1</b> 0.75*[(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ] + 0.25*[NaNO <sub>3</sub> ] = 0.012 M	8.2	atmospheric
0.25*[(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ] + 0.75*[NaNO <sub>3</sub> ] = 0.012 M	6 – 9	atmospheric
0.5*[(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ] + 0.5*[NaNO <sub>3</sub> ] = 0.012 M	6 – 9	atmospheric
[(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> ] = 0.1-1.5 M	6.5 - 7	atmospheric
<b>2.2</b> Synthetic seawater: [NaNO <sub>3</sub> ] = 0.012M; [KH <sub>2</sub> PO <sub>4</sub> ] = 0.37 mM	8.2	atmospheric
Media A D7, with synthetic seawater replacing NaCl	8.2	atmospheric
<b>2.3</b> Elemental analysis during processing of AD effluent		
<b>3.1</b> 60%, 75%, 85% seawater diluted AD effluent; [MgSO <sub>4</sub> ] = 0.2 M	6.8	atmospheric
<b>3.2</b> 60%, 75%, 85% seawater diluted AD effluent; [MgSO <sub>4</sub> ] = 0.2 M	6.8	10 v/v% CO <sub>2</sub>
<b>4.</b> 85% seawater diluted AD effluent; [MgSO <sub>4</sub> ] = 0.2 M	6.8	Air Bubbling

For investigation 1 to 3, batch mode experiments were performed in triplicates with a working volume of 150 mL. For investigation 4, the experiment was performed in an eight-litre airlift reactor.

#### 4.4.1 Standard sampling procedure

For the reactor flask tests, all sampling equipment and sterile HCl and NaOH were placed inside the laminar with the UV light switched on for 10 minutes. Sterilisation is done according to the standards stipulated in Section 4.3.6. Flasks were removed from the incubator and wiped with 80% ethanol to minimise any bacterial growth on the surface of the flasks. The UV light is switched off, the Bunsen burner is lit, and flasks are placed inside the laminar as far back as possible. Gloves are re-sterilised with 80% ethanol. A new pipette tip for each sample is selected, taking care to close the container as soon as it's lodged onto the pipette. The gauze is briefly removed from the batch flask when sampling, and then put back immediately.

For sampling from the airlift reactor, an autoclave sterilised falcon tube rinsed in 80% ethanol is immediately scooped at the top of the airlift reactor. Sterile gloves are used and the lid of the reactor is wiped with ethanol before replacing.

#### 4.4.2 Synthetic media growth tests

For growth experiments in media A D7, the growth media was made up in bulk according to the procedures in Section 4.2.1 and sterilized according to the procedures stipulated in Section 4.3.6. Inoculation is done according to procedures in Section 4.3.8. Samples are taken for analysis in accordance with Section 4.4.1. Flasks are placed in the incubator according to Section 4.3.7. Because the media A D7 is buffered, no pH adjustments were made during the experiments.

#### 4.4.3 AD effluent-based media tests

For growth experiments in AD effluent, the AD effluent was prepared in accordance with the procedures set out in Section 4.1. Sterilisation is done according to the standards stipulated in Section 4.3.6. Triplicate flasks were inoculated according to the following specifications:

- i. 140 mL of pure AD effluent, with pH adjustment to 8.2 using NaOH. (AD effluent)
- ii. 14 mL AD effluent diluted with 126 mL of media A D7, with pH adjustment to 8.2 using NaOH. (10% AD effluent)
- iii. 36.4 mL AD effluent diluted with 103.6 mL media A D7, with pH adjustment to 8.2 using NaOH. (26% AD effluent)
- iv. 0.14 mL of sterile micronutrient solution is added to 139.86 mL of AD effluent, with pH adjustment to 8.2 using NaOH. (AD effluent + micronutrients)

The bulk of the solution making up the growth media is measured off according to Section 4.3.8. Then, the remainder of solutions to be added are pipetted into each flask. Lastly, each flask is inoculated with 10 mL of liquid culture according to Section 4.3.8. Samples are taken for analysis in accordance with Section 4.4.1. Flasks are placed in the incubator according to Section 4.3.7. All pH adjustments are made in accordance with the procedure stipulated in Section 4.3.4.

#### 4.4.4 Nutrient utilization tests

For nutrient utilization experiments, media A D7 was prepared in accordance with the procedures set out in Section 4.2.1 and 4.4.2, except the nitrogen source ( $\text{NaNO}_3$ ) is replaced by ammonium ( $(\text{NH}_4)_2\text{SO}_4$ ). Thus, if a 1 M solution of  $(\text{NH}_4)_2\text{SO}_4$  is made up and sterilised, 6 mL of the bulk solution should be added per 1 L of media that is made up.

Sterilisation is done according to the standards stipulated in Section 4.3.6. Triplicate flasks were inoculated for different sets of conditions:

- i. 140 mL of ammonium media A D7. (Ammonium)
- ii. 140 mL of nitrate media A D7. (Nitrate)
- iii. 105 mL ammonium media A D7 diluted with 35 mL nitrate media A D7. (75% ammonium)
- iv. 135 mL ammonium media A D7 diluted with 105 mL nitrate media A D7. (25% ammonium)

The bulk of the solution making up the growth media is measured off according to Section 4.3.8. Then, the remainder of solutions to be added are pipetted into each flask. Lastly, each flask is inoculated with 10 mL of liquid culture according to Section 4.3.8. Samples are taken for analysis in accordance with Section 4.4.1. Flasks are placed in the incubator according to Section 4.3.7. All pH adjustments are made in accordance with the procedure stipulated in Section 4.3.4. No pH adjustments were made as the synthetic media is buffered for a pH of 8.2.

#### 4.4.5 Ammonia toxicity tests

For ammonia toxicity investigation, media A D7 was prepared in accordance with the procedures set out in Section 4.2.1 and 4.4.2, except the nitrogen source ( $\text{NaNO}_3$ ) is replaced by ammonium ( $(\text{NH}_4)_2\text{SO}_4$ ) in the same way as explained in Section 4.4.4.

Sterilisation is done according to the standards stipulated in Section 4.3.6. Triplicate flasks were inoculated for the following conditions:

- i. 140 mL of ammonium media A D7 with pH adjustment to 6 using HCl.
- ii. 140 mL of ammonium media A D7 with pH adjustment to 6.5 using HCl.
- iii. 140 mL of ammonium media A D7 with pH adjustment to 7 using HCl.
- iv. 140 mL of ammonium media A D7 with pH adjustment to 7.5 using HCl.
- v. 140 mL of ammonium media A D7 with pH adjustment to 8 using HCl.
- vi. 140 mL of ammonium media A D7 with pH adjustment to 8.5 using NaOH.
- vii. 140 mL of ammonium media A D7 with pH adjustment to 9 using NaOH.

Inoculation is done according to procedures in Section 4.3.8. Samples are taken for analysis in accordance with Section 4.4.1. Flasks are placed in the incubator according to Section 4.3.7. All pH adjustments at the start and duration of the experiment were done according to the procedure stipulated in Section 4.3.4.

After the results are obtained, the experiment is to be repeated but this time, the pH would remain constant at the value where optimum growth was observed in the previous experiment, while varying ammonium concentration. Thus, media A D7 was prepared in accordance with the procedures set out in Section 4.2.1 and 4.4.2, except the nitrogen source ( $\text{NaNO}_3$ ) is replaced by ammonium ( $(\text{NH}_4)_2\text{SO}_4$ ) in the same way as explained in Section 4.4.4. The solution was buffered for a pH of 6.8 according to the procedures stipulated in Section 4.3.4 using phosphate buffers.

Triplicate flasks were inoculated for the following sets of conditions:

- i. 0.1 M nitrogen concentration in ammonium media A D7.
- ii. 0.3 M nitrogen concentration in ammonium media A D7.
- iii. 0.9 M nitrogen concentration in ammonium media A D7.
- iv. 1.5 M nitrogen concentration in ammonium media A D7.

The same conversion as shown in eq. 25 can be applied to obtain the amount of  $(\text{NH}_4)_2\text{SO}_4$  stock solution to be added.

Inoculation is done according to procedures in Section 4.3.8. Samples are taken for analysis in accordance with Section 4.4.1. Flasks are placed in the incubator according to Section 4.3.7. All pH adjustments are made in accordance with the procedure stipulated in Section 4.3.4.

#### 4.4.6 Modified AD effluent tests

Combining all results generated from previous experiments, the AD effluent was prepared in accordance with the procedures set out in Section 4.1. Sterilisation is done according to the standards stipulated in Section 4.3.6. Triplicate flasks were inoculated for the following conditions:

- i. 119 mL of AD effluent diluted with 21 mL of synthetic seawater. (85% AD effluent)
- ii. 105 mL of AD effluent diluted with 35 mL of synthetic seawater. (75% AD effluent)
- iii. 91 mL of AD effluent diluted with 49 mL of synthetic seawater. (60% AD effluent)

The solutions were buffered for a pH of 6.8 according to the procedures stipulated in Section 4.3.4 and 2.8 mL of a 1 M  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  sterile solution was added to raise the magnesium and sulphate levels in the media.

The bulk of the solution making up the growth media is measured off according to Section 4.3.8. Then, the remainder of solutions to be added are pipetted into each flask. Lastly, each flask is inoculated with 10 mL of liquid culture according to Section 4.3.8. Samples are taken for analysis in accordance with Section 4.4.1. Flasks are placed in the incubator according to Section 4.3.7. All pH adjustments are made in accordance with the procedure stipulated in Section 4.3.4.

#### 4.4.7 Carbon enrichment tests

For the carbon enriched experiments, the experimental procedure stipulated in Section 4.4.6 was followed and the headspace gas in the incubator was enriched with carbon. The 10 %v/v carbon dioxide nitrogen gas cylinder was obtained from Afrox. The following details were supplied:

- Components:
  - Carbon dioxide: 10.2 %v/v
  - Balance: Nitrogen
- Cylinder content: 8.727 kg
- Pressure: 15000 kPa (g)
- Valve details: RH INT 5/3" BSP

The volume of the box to be filled with air was calculated to be 0.56 m<sup>3</sup>. Since both carbon dioxide and nitrogen gas are not ideal – especially at extreme pressures, the Van der Waal's equation can be used to calculate the volume of these gases in the cylinder according to eq. 27:



$$\left[ P + \frac{an^2}{V^2} \right] (V - nb) = nRT \quad \text{Equation 27}$$

For carbon dioxide and nitrogen, their volumes were calculated to be 2.22 L and 44.50 L, respectively. Now, assuming the ideal gas law between the cylinder and the incubator, if we want to displace all the cylinder contents into the incubator at a constant temperature, that means eq. 28 can be applied:

$$P_{cylinder}V_{cylinder} = P_{Incubator}V_{Incubator} \quad \text{Equation 28}$$

Applying the values available, we can conclude that

$$(15000 + 101.325) * V_{cylinder} = 101.325 * V_{Incubator}$$

$$V_{Incubator} = 149.04V_{cylinder}$$

This means that with that pressure difference; the volume available to fill the incubator at atmospheric pressure is 149 times the volume of the cylinder gas content at 15000 kPa. On a volume basis, this means there is almost 7 m<sup>3</sup> available to fill the incubator which means it can be filled with the gas 12 times. With a total cylinder pressure of 15000 kPa, that means 1250 kPa in pressure drop would result in filling up the incubator. Because we were essentially purging the incubator and wanted to ensure that carbon levels were high enough to ensure carbon diffusion remained the limiting step, and not the carbon concentration in the atmosphere, a volumetric equivalent of double the pressure drop calculated was allowed to flow through the incubator before it was sealed.

After samples were placed in the reactor, the doors were closed, and the two holes drilled on the opposite side of the gas inlet to the incubator was left open. The gas was allowed to pass through the incubator until the desired pressure drop of 2500 kPa and then rubber stoppers were placed in the holes, sealing the incubator and keeping the carbon rich gas inside. This limited the data to only six samples over the course of 13 days.

#### 4.4.8 Airlift reactor tests

The experiments were scaled-up to simulate a continuous process and to understand how the results would compare under conservative conditions typically associated with large scale algal growth. An airlift reactor designed at Stellenbosch University was available to perform the experiments. It is a pneumatic closed system which uses the velocity of sparged gas to circulate the fluid inside in a cyclic pattern through specific channels. It is made of a transparent material (acrylic) to allow photosynthesis. This specific design is based on an internal loop concentric tube lift reactor and more detail is available in Christi and Moo-Yong (1987). The main advantage of the design lies in the fact that there are no moving parts, which reduces maintenance needs and the occurrence of mechanical defects. Synthetic media was made up according to the procedures stipulated in Section 4.2.1. The reactor was sterilised using Perasafe and then washed with sterilised deionised water. Instructions regarding safety

information, dilution, procedures for applications and use of Perasafe can be found at [http://www.safmed.co.za/downloadable/download/sample/sample\\_id/111/](http://www.safmed.co.za/downloadable/download/sample/sample_id/111/).

6.9 L of sterile synthetic media was added to the column, followed by 800 mL of liquid inoculum. The media was sparged with air at a rate that achieved a gas to liquid volume ration of 2.5%. No temperature control was applied to the system. Again, no illumination was applied for the first 24 hours after which two fluorescent lights provided an average light intensity at the surface of the reactor of  $180 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Sampling was done in accordance with Section 4.4.1. The experiment was repeated in the exact same way, this time with 85% AD effluent, 15% seawater and  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  added to achieve the concentrations present in media A D7. Also, the media was buffered for a pH of 6.8 according to Section 4.3.4. Samples were analysed for Biomass concentration as well as total nitrogen.

#### 4.5 Statistical methods

For modelling purposes, the statistical technique used to illustrate if and how much the experimental data related to the model is correlation. The sum of error squared (SSE) was calculated between experimental data and model data and the closer the R-squared value is to one, the better the data fits the model. It is calculated by eq. 29 and is a simple way of quantifying if the data follows specific kinetics and serve as validation for results obtained.

$$SSE = \sum_{i=1}^n (x_i - \bar{x})^2 \quad \text{Equation 29}$$

All experiments performed in triplicate were evaluated for statistical soundness by calculating the standard deviation. The standard deviation is a quantity expressing by how much the members in a group (in this case, triplicates for each experimental run's sample) differ from the mean value of that group and it is calculated by eq. 30:

$$\sigma = \sqrt{\frac{\sum(x-\bar{x})^2}{n}} \quad \text{Equation 30}$$

It is associated with data where the average/mean is used to calculate central tendency. With small populations, one value can strongly affect the value of standard deviation and in that regard, it is a good indicator of outliers.

## 5. Results and discussion

### 5.1 Media Analysis

For medium A D7, elemental analysis was only performed on macronutrients to confirm accurate media make up. Other properties tested were electrical conductivity and turbidity. The remainder of the nutrients' concentrations were determined from mass balances based on the recipe for medium A D7. Raw data can be found in Appendix C. In total, three 20 L batches of AD effluent was produced during the project and elemental analysis of these are shown in Table 17. The data obtained confirmed the significant variability in the properties and composition associated with digesting organic waste anaerobically reported in literature. Major contributing factors include temperature, pH and the type of inoculum used. Both temperature and pH influence bacterial health in the digestate, while the consortium of bacteria present in the inoculum will vary depending on the source. When considering the concentrations of nitrogen and phosphorous sources present in the media, these far exceed the minimum requirement for algal growth.

**Table 17: AD effluent properties**

Property	AD 1 elemental analysis	AD 2 elemental analysis	AD 3 elemental analysis
Potassium [mmol.L <sup>-1</sup> ]	33.25	6.98	4.50
Calcium [mmol.L <sup>-1</sup> ]	1.33	1.67	5.76
Magnesium [mmol.L <sup>-1</sup> ]	1.65	1.81	1.07
Ammonia [mmol.L <sup>-1</sup> ]	21.42	15.35	9.78
Sulphate [mmol.L <sup>-1</sup> ]	0.14	0.27	0.15
Nitrate + nitrite [mmol.L <sup>-1</sup> ]	4.69	1.85 x10 <sup>-6</sup>	1.85 x10 <sup>-6</sup>
Nitrite [mmol.L <sup>-1</sup> ]	0.14	2.17 x10 <sup>-6</sup>	2.17 x10 <sup>-6</sup>
Ortho-phosphate [mmol.L <sup>-1</sup> ]	32.29	1.26	1.94
Dissolved Organic Carbon [mmol.L <sup>-1</sup> ]	10.36	1.70	10.16
Total Organic Carbon [mmol.L <sup>-1</sup> ]	10.36	1.89	10.32
Electrical Conductivity [mS.m <sup>-1</sup> at 25 °C]	84	500	290
Turbidity [NTU]	266	362	380

AD effluent 1 was produced from fresh inoculum, while the other two were formed using fridge stored inoculum. AD effluent 1 was used for experiments in Section 5.3, while AD effluent 2 was used for experiments reported in Section 5.4 to 5.7. AD effluent 3 was used for scaled-up experiments reported in Section 5.8.

By comparing the concentrations of nutrients in media A D7 to that found in AD effluent, it provided insight as to which properties associated with AD effluent could be responsible for the lack of growth. Assuming a 10% deviation in a property is within reasonable bounds to still ensure growth, the results are shown in Table 18 for pure AD effluent and Table 19 for diluted AD effluent, respectively.

**Table 18: Comparing properties of medium A D7 and AD effluent**

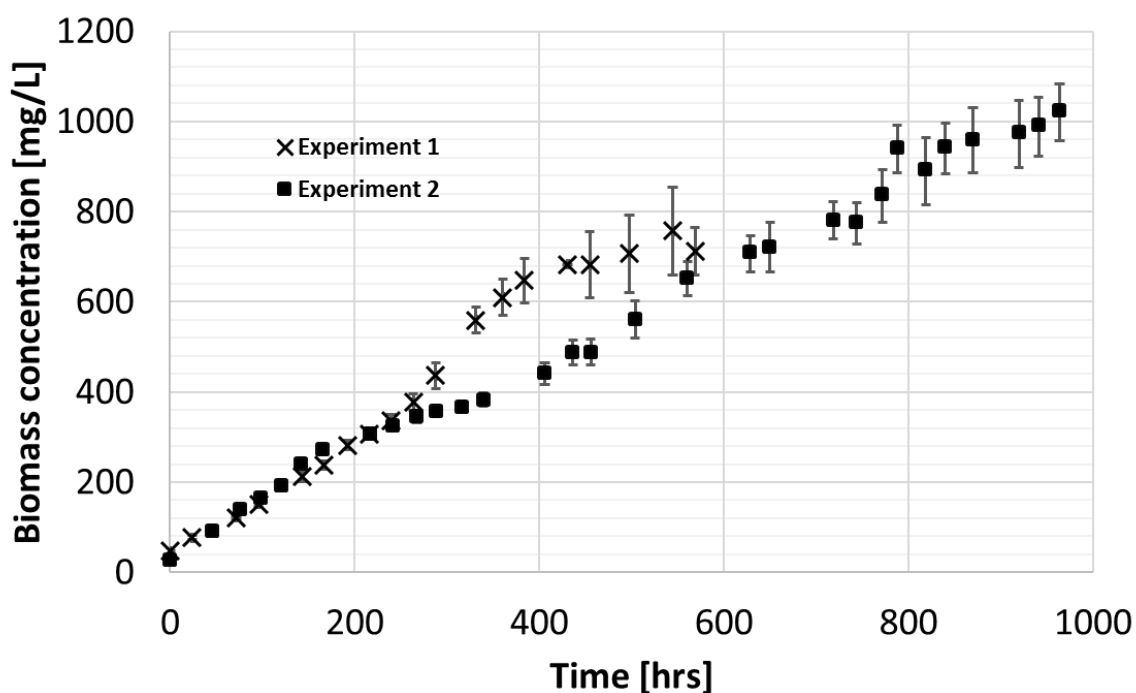
<b>Property</b>	<b>Average medium A D7</b>	<b>Average AD effluent</b>	<b>Conclusion</b>
<b>Potassium [mmol.L<sup>-1</sup>]</b>	11.70	4.91	Reasonable
<b>Calcium [mmol.L<sup>-1</sup>]</b>	2.50	2.92	Reasonable
<b>Magnesium [mmol.L<sup>-1</sup>]</b>	200.00	1.51	Possibly limiting
<b>Total Nitrogen [mmol.L<sup>-1</sup>]</b>	12.00	15.52	Reasonable
<b>Ammonia [mmol.L<sup>-1</sup>]</b>	0.00	15.52	Possibly Inhibitory
<b>Nitrate + nitrite [mmol.L<sup>-1</sup>]</b>	12.00	1.56	Reasonable (NH <sub>4</sub> present)
<b>Nitrite [mmol.L<sup>-1</sup>]</b>	0.04	0.05	Reasonable
<b>Sulphate [mmol.L<sup>-1</sup>]</b>	200.00	0.19	Possibly limiting
<b>Ortho-phosphate [mmol.L<sup>-1</sup>]</b>	0.36	11.83	Reasonable
<b>Dissolved Organic Carbon [mmol.L<sup>-1</sup>]</b>	0.00	7.41	Reasonable
<b>Total Organic Carbon [mmol.L<sup>-1</sup>]</b>	0.00	7.52	Reasonable
<b>Electrical Conductivity [mS.m<sup>-1</sup> at 25 °C]</b>	3500.00	291.00	Possibly limiting
<b>Turbidity [NTU]</b>	0.00	336.00	Possibly inhibitory

**Table 19: Comparing properties of medium A D7 and AD effluent diluted with medium A D7**

<b>Property</b>	<b>Average medium A D7</b>	<b>10% AD effluent</b>	<b>Conclusion</b>	<b>26% AD effluent</b>	<b>Conclusion</b>
<b>Potassium [mmol.L<sup>-1</sup>]</b>	11.70	12.02	Reasonable	12.53	Reasonable
<b>Calcium [mmol.L<sup>-1</sup>]</b>	2.50	2.54	Reasonable	2.61	Reasonable
<b>Magnesium [mmol.L<sup>-1</sup>]</b>	200.00	180.15	Reasonable	148.39	Too low
<b>Ammonia [mmol.L<sup>-1</sup>]</b>	0.00	1.55	Reasonable	4.03	Reasonable
<b>Sulphate [mmol.L<sup>-1</sup>]</b>	200.00	180.02	Reasonable	148.05	Too low
<b>Nitrate + nitrite [mmol.L<sup>-1</sup>]</b>	12.00	10.95	Reasonable	9.28	Reasonable
<b>Nitrite [mmol.L<sup>-1</sup>]</b>	0.04	0.04	Reasonable	0.04	Reasonable
<b>Ortho-phosphate [mmol.L<sup>-1</sup>]</b>	0.36	1.51	Reasonable	3.34	Reasonable
<b>Dissolved Organic Carbon [mmol.L<sup>-1</sup>]</b>	0.00	0.74	Reasonable	1.93	Reasonable
<b>Total Organic Carbon [mmol.L<sup>-1</sup>]</b>	0.00	0.75	Reasonable	1.96	Reasonable
<b>Electrical Conductivity [mS.m<sup>-1</sup> at 25 °C]</b>	3500.00	3179.00	Reasonable	2666	Reasonable
<b>Turbidity [NTU]</b>	0.00	34.00	Reasonable	87	Reasonable

## 5.2 Growth curve and biomass yield in synthetic media

The culture's growth characteristics were established during two independent batch-controlled experiments under the conditions established in literature. These results are depicted in Figure 12.



**Figure 12: Growth of *Synechococcus* PCC 7002 under standard conditions (medium A D7; 38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; pH = 8.2)**

Experiment 1 and Experiment 2 represent two repeats, each performed in triplicate and the error bars represent the standard deviation of these three triplicates. Error bars throughout the remainder of this chapter all represent the standard deviation of three triplicate runs. With reference to Experiment 1 in Figure 12, the linear nature of the curve suggests nutrient limited growth, and this was confirmed by repeating the experiment to determine if a stationary phase could be reached without carbon enrichment if it could be allowed to grow for a longer period. The results are depicted in Figure 12 as Experiment 2. Comparing the two experiments, a consistent biomass concentration yield of  $\pm 700$  mg/L at 560 hours is evident.

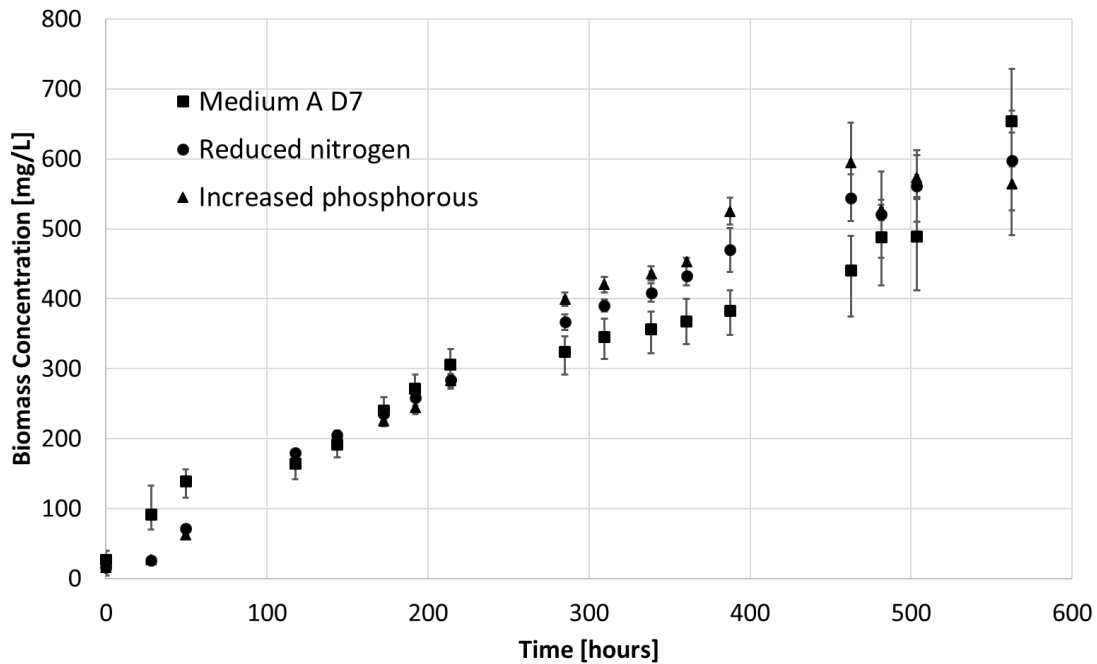
Compared to literature (Aikawa, et al. (2014), Miyairi (1995), Xu (2010), Wu (2014)) shown in Table 3, the growth for these cultures were severely limited when considering biomass yields over time. The similarity of the slopes of the two curves shown in Figure 12 suggests that the light irradiance is consistent throughout the incubator, if flasks are placed within the area specified in Section 4.3.3, where no light and temperature gradients exist in the incubator. The linearity of these growth curves was first believed to be due to light limitation only, but the culture depths ( $\pm 3$  cm) were too low to justify this assumption, as light limitation is usually associated with dense cultures as well as higher culture depths associated with large scale algal growth (Kim, et al., 2002). Instead, when growing high density

cultures, it has been proven that two factors become most important for biomass production: sufficient rate and concentration of inorganic carbon delivery to meet carbon uptake rates and sufficient light intensities to supply the energy demand of growing cells (Brune, 1980).

To ensure the concentration of CO<sub>2</sub> in the headspace of the incubator remained constant, air was allowed to purge the incubator at a constant rate. Thus, it can be argued that CO<sub>2</sub> was pushed through the incubator at a constant rate. Since no CO<sub>2</sub> enrichment was done at this stage, the concentration of CO<sub>2</sub> in the air was always 400 ppm. This would explain the limited growth of the culture, which didn't reach the stationary phase even after 1000 hours as seen in Figure 12. Since growth can only match the rate of nutrient addition if the nutrient is limiting, a constant growth rate was observed as opposed to an increasing (exponential) growth rate.

If this was true, all experiments conducted in the incubator without CO<sub>2</sub> enrichment would follow this linear growth pattern and even if the headspace gas were to be enriched with CO<sub>2</sub>, the lack of sparging and small surface area of the media inside the flasks exposed to the headspace would cause the growth to be limited by the diffusion of CO<sub>2</sub> into the media, rather than the concentration of CO<sub>2</sub> in the headspace gas. The decision to not sparge the media with air or CO<sub>2</sub> enriched air rests on two motivations: First, the high number of flasks (as high as 21 at a time) would call for apparatus to split gas streams to achieve uniform sparging in all the flasks simultaneously. This would be very challenging. Simultaneously, the aim was to keep experiments as simple as possible if scale-up were to be realised. The experimental philosophy was to keep results obtained in lab scale experiments conservative, therefore accounting for the inevitable challenges and inefficiency typically associated with scale-up, making it easier to understand and address.

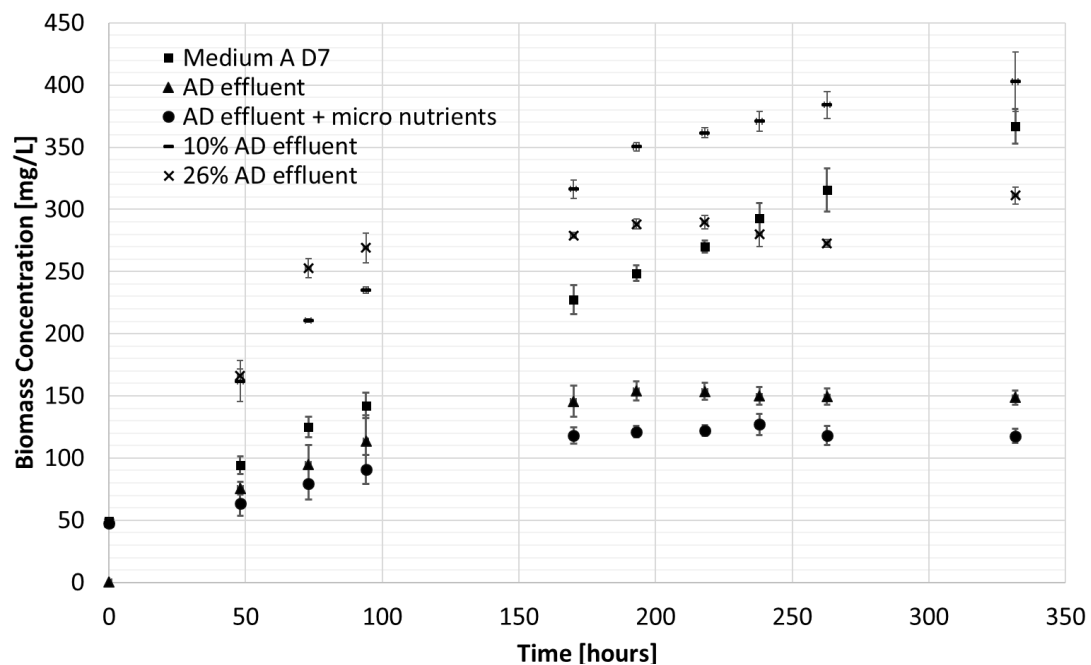
To ensure the growth was carbon limited, two experimental runs were conducted where the nitrogen source in medium A D7 was reduced by a factor of 8, and the concentration of the phosphorous source was increased by a factor of 4. The results are shown in Figure 13. The final deviation in biomass concentration for the decreased nitrogen and increased phosphorous flasks were 8.5% and 13.6%, respectively. Also, these final biomass concentrations fell within the maximum and minimum values of the final unchanged medium A D7 biomass concentrations. With such a small difference, it could be concluded with reasonable certainty that the limited growth observed was not due to nitrogen or phosphorous limitations, but solely carbon diffusion.



**Figure 13: Growth observed with reduced nitrate concentrations and increased phosphorous concentrations to investigate possible nutrient limitations (medium A D7; 38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; pH = 8.2)**

### 5.3 Growth in AD effluent

Cultures were grown in medium A D7, pure AD effluent and AD effluent with added micronutrients in accordance with that found in media A D7. The results are shown in Figure 14.



**Figure 14: Growth observed in pure AD effluent, investigating micro nutrient addition, diluted AD effluent at optimum dilutions as observed in literature compared with growth in media A D7 (38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; pH = 8.2)**



The growth observed in pure AD effluent was limited, but comparable to that in medium A D7 during the first 100 hours of the experiments. From approximately 150 hours, the growth in AD effluent stagnated. The addition of micronutrients to the AD effluent had no positive effect on the growth observed, establishing that none of the micronutrients could be limiting the growth. This seemingly instantaneous drop in cell division after 100 hours was probably caused by a phenomenon that shifted and changed as the experiment progressed. As all experimental conditions such as light intensity, temperature and culture mixing were fixed, it suggests that the inhibitory phenomenon is associated with a change in nutrient concentration within the AD effluent. Of all the compounds present in AD effluent, ammonia is the only component that has toxic effects on cell metabolism. And as the pH of the media is a function of ammonia assimilation, it could be that the pH was shifted beyond the culture's tolerance levels.

To support this argument and ensure that the AD effluent prepared for the experiments was adequate, experiments were designed to simulate those found in literature (refer to Table 8). The AD effluent was diluted with synthetic media to ensure all nutrients needed for growth are present, along with high salinities and raised pH values (according to Appendix C). The results are also shown in Figure 14.

The culture grown in 10% AD effluent grew at a similar rate than those in medium A D7, but still accumulated 10% more biomass at the end of the run. For the culture grown in 26% AD effluent, inhibition was observed at approximately 100 hours, like those grown in pure AD effluent (refer to Figure 14). Thus, whatever the cause for the lack of growth in AD effluent, it could be circumvented to some extent through dilution. This supports the argument that a shift in pH caused growth to stagnate, since dilution with media A D7 would not only buffer the growth media to some extent but would also dilute the ammonia present in growth media, limiting potential toxic effects. Alternatively, it could be argued that a key nutrient was not present in the effluent in high enough concentrations and brought growth to a halt.

The results showed strong correlation with findings in the experiments. For lack of growth in pure AD effluent, the phenomenon could be caused by too low concentrations of magnesium and sulphates, too low salinity or too high turbidity and ammonia concentrations. In the 26% diluted AD effluent, only magnesium and sulphate concentrations could be problematic. This can explain why the growth stops around 100 hours, but when the culture did grow, it grew better than in pure AD effluent. Lastly, in the 10% diluted AD effluent, all properties are within reasonable bounds, justifying why the culture did not only thrive, but also outgrew the culture in medium A D7. Simply put, more nutrients were available, resulting in slightly more biomass. The reason a significant increase in biomass accumulation is not evident could be due to carbon limitations.

From this, four possibilities arose which were explored to attempt successful algae growth in AD effluent through waste valorisation. These topics, along with the experiments performed are discussed in the next chapter.

## 5.4 Inhibitory phenomenon

### Ammonia toxicity

By replacing media A D7 with AD effluent, the nitrogen source changed from nitrate to ammonia. First, it was of value to see if the culture could utilise ammonium as nitrogen source, and if so, if it grew comparably well as opposed to nitrate as nitrogen source. Three sets of experiments were conducted simultaneously, and the results are presented in Figure 15.

All ammonium containing media showed better biomass yields than media containing nitrate as the sole nitrogen source, which confirms literature findings (as discussed in Section 2.2.2). Arguing that ammonium is in fact the preferred nitrogen source for this culture presents an opportunity to eliminate the toxicity issues of the high concentrations of ammonia present in AD effluent if the culture could withstand lower pH values (refer to Figure 7).

To establish a pH operating window, nitrate ( $\text{NaNO}_3$ ) was replaced with ammonium sulphate ( $(\text{NH}_4)_2\text{SO}_4$ ) in a ratio that results in the same total nitrogen concentration (0.012 M). Seven sets of experiments were performed, with pH values ranging from 6 to 9 in increments of 0.5. From these experiments, it was hoped to establish at which point the toxicity of ammonia is detrimental to cell growth as well as operational limits where lower pH does not affect growth. The pH was adjusted using 1 M solutions of NaOH and HCl every 24 hours. The results are shown in Figure 16.

The biomass concentrations of the two experiments were compared at  $\pm 210$  hours and this data of interest is marked by the dashed squares on the Figure 15 and Figure 16. The biomass concentration of the cultures grown in media with ammonium as nitrogen source at pH values between 6.5 and 7.5 are comparable to that of cultures grown with nitrates, ammonium or a mixture of the two as nitrogen source (at a pH of 8.2). In fact, when grown at lower pH's, cultures grown with ammonium shows a 6.7% improvement in biomass concentration.

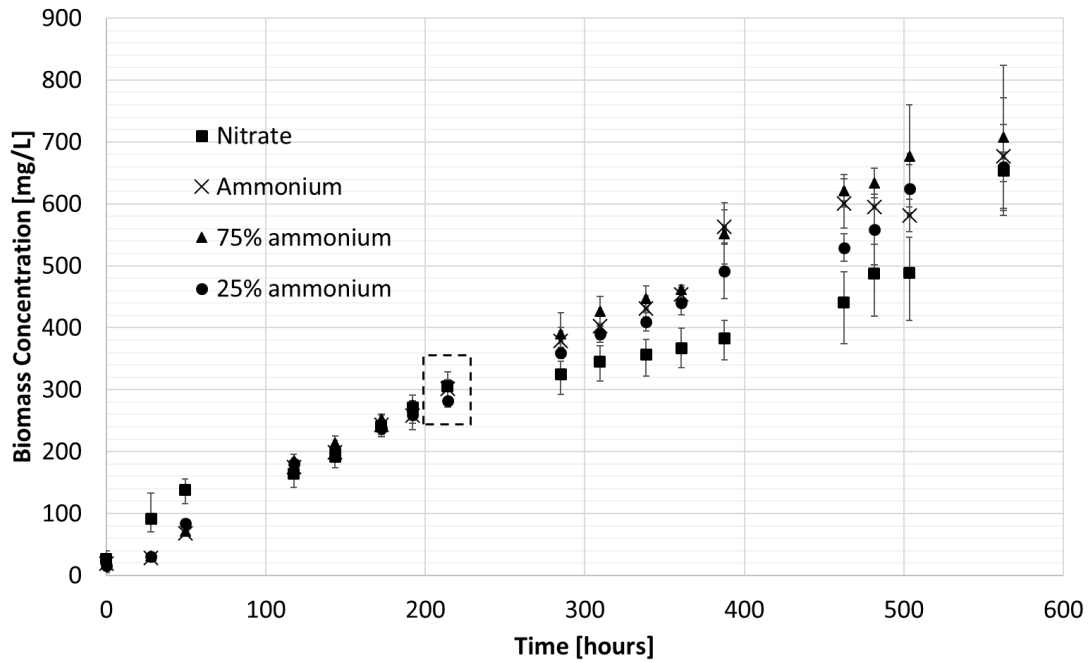


Figure 15: Growth observed when nitrate was replaced with ammonium as nitrogen source at the same concentration, the dashed square indicates time of comparison with Figure 16 (medium A D7; 38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ; pH = 8.2)

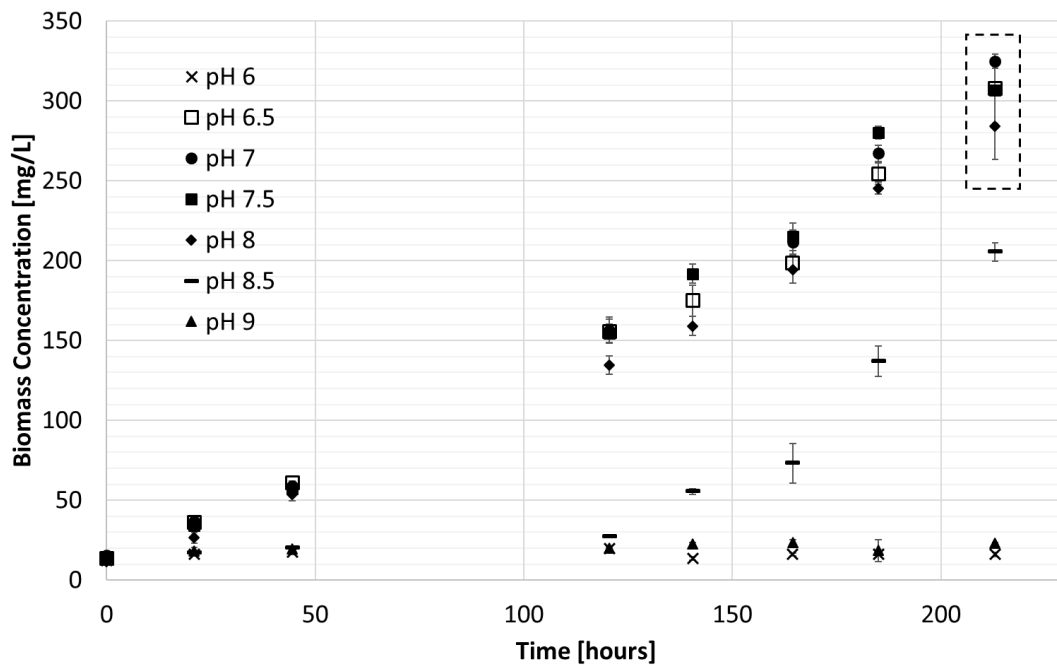
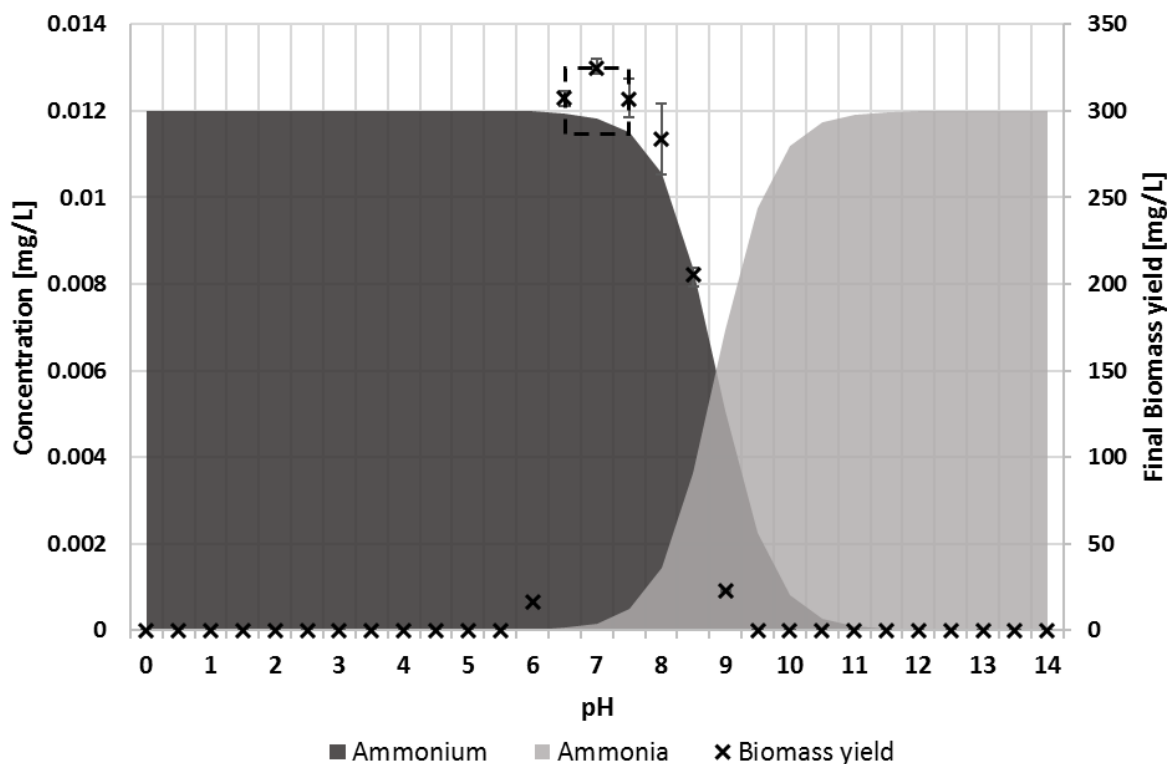


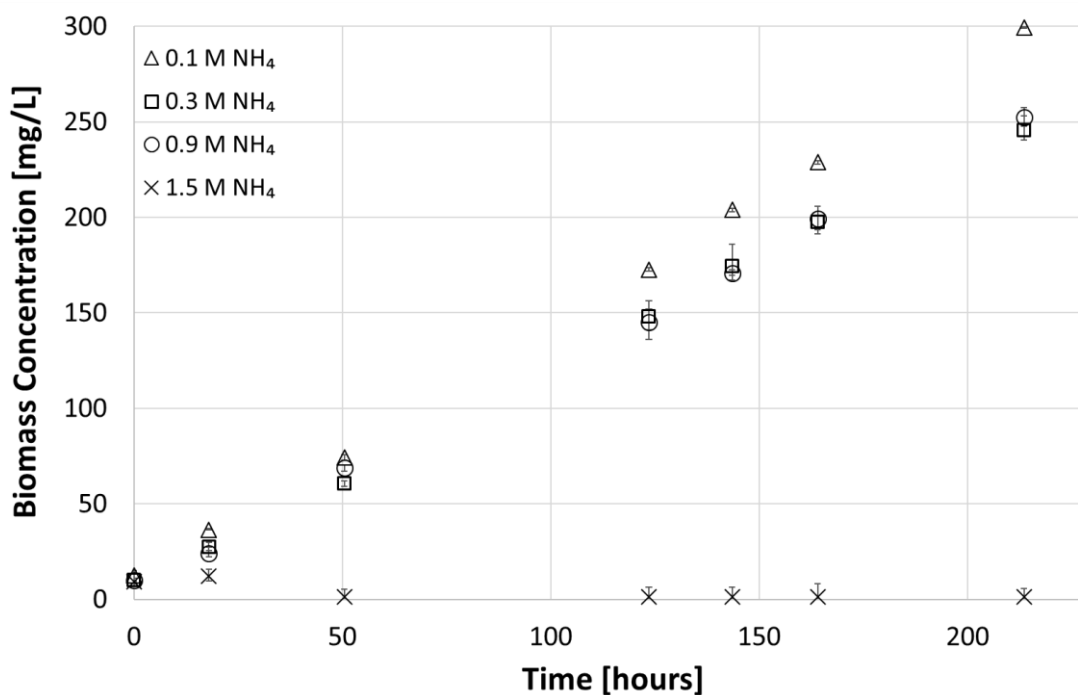
Figure 16: Growth observed in media with ammonium as nitrogen source at a constant initial concentration of 0.012 M with varying pH operation, the dashed square indicates time of comparison with Figure 15 (medium A D7; 38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )

With less than 10% variation in final biomass yield for cultures grown in media with a pH between 6.5 and 8, it was shown that the cyanobacteria could be grown in a more acidic environment, enough to minimise ammonia presence in the media. This is shown schematically in Figure 17. It is of interest to note that these battery limit pH values coincide with nutrient speciation. Referring to eq. 5 and eq. 6 in Section 2.2.2, phosphate speciation shifts from hydrogen phosphate to dihydrogen phosphate at a pH of 7.21 and carbonate speciation shifts from bicarbonate to carbonic acid at a pH of 6.3, as the pH is lowered. The optimal operational pH was chosen as 6.8, although this might not be the best choice for large scale cultivation systems where pH control is more difficult. Since the assimilation of ammonium causes  $H^+$  production (refer to Section 2.2.2), this could cause the pH to drop below 6.5, resulting in lowered productivity or cell death.



**Figure 17: Optimal pH range for growth in AD effluent, the dashed square indicates window optimal pH window (medium A D7; 38 °C; 225  $\mu E \cdot m^{-2} \cdot s^{-1}$ )**

From the three AD effluent batches, the highest concentration of ammonia present was 0.02 M. Depending on which substrate is used in the AD process, this value might be much higher. Since the toxic effect of ammonia on cells is a function of both pH and concentration, it was of value to get a rough estimate of where the concentration threshold of ammonia toxicity is at a pH of 6.7. Ammonia concentrations were varied from 0.1 M to 1.5 M at a constant light intensity, temperature and pH and the results are presented in Figure 18.



**Figure 18: Growth observed in media with varying ammonia concentration at a constant pH (medium A D7; 38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , pH = 6.8)**

From these experiments, it was clear that ammonia concentrations above 0.9 M in the media affected cell growth negatively. Although the experimental intervals were too high to establish exactly where between 0.9 M and 1.5 M the cells started being affected, it did provide insight for future experiments. For this project, the ammonia levels in the AD effluent used were far below these concentrations and it can be concluded that ammonia toxicity can be circumvented by pH reduction alone, without compromising biomass yields.

### Turbidity

One centrifugation step and three filtration steps were used to clarify the AD effluent to reduce its turbidity. Samples were taken of each processing step and tested for total phosphate concentration and turbidity, to confirm the published findings of Ong *et.al.* (The correlation between phosphate and turbidity), Bhcir *et.al.* (Microfiltration as clarifying technique) and Marchilhac *et.al.* (Centrifugation as clarifying technique). A turbidity chart for waste water (West, et al., 2011), along with the results obtained are shown in Table 20 and Table 21, respectively.

**Table 20: Turbidity classification chart**

1 - 10 NTU	10.1 - 40 NTU	40.1 – 150 NTU	> 150 NTU
EXCELLENT	GOOD	FAIR	POOR

**Table 21: Clarifying step effectiveness and phosphorous correlation**

Sample	Turbidity (NTU)	Total Phosphorous (%)					
		0%	20%	40%	60%	80%	100%
Raw AD	3525						
Centrate	362						
Filtrate (glass fibre)	253						
Filtrate (0.45 µm)	142						
Filtrate (0.22 µm)	6						

The high turbidities associated with AD effluent is evident in the results obtained. For this batch specifically, the most effective clarifying step was 0.22 µm filtration, lowering the turbidity of the previous step's filtrate by 95.8%. The second most efficient was centrifugation, in which the total turbidity was reduced by 89.7%. The least effective method was filtration of the centrate through glass fibre filters, lowering it by only 30.1%. The significant reduction in NTU's by 0.22 µm filtration was extremely resource intensive, requiring filter changes nearly every 5 mL, mounting up to 800 papers for one batch of AD effluent resulting. This would immensely increase the costs associated with the project. For this specific application, it was deemed unnecessary and the last filtration step was excluded from the remainder of the experiments.

### 5.5 Nutrient absence

Besides high turbidity and ammonia toxicity associated with AD effluent, the absence of important nutrients and characteristics in the media could also contribute to the poor growth observed in pure AD effluent.

#### Salinity

With reference to Table 17, the average electrical conductivity associated with AD effluent is one order of magnitude lower than that of medium A D7. *Synechococcus elongatus* is a non-heterocystous species (Lee, et al., 1999), meaning its growth would be supported by high salinity concentrations (> 4 dS.m<sup>-1</sup>) (Srivastava, et al., 2009). Furthermore, high salinity inhibits ammonia evaporation (El-Karim, et al., 2004), which means not only would a higher salinity be beneficial to the study organism's growth, but it would also help ensure that most of the ammonia present in the AD effluent would remain in solution where it can be utilised by the cyanobacteria for growth.

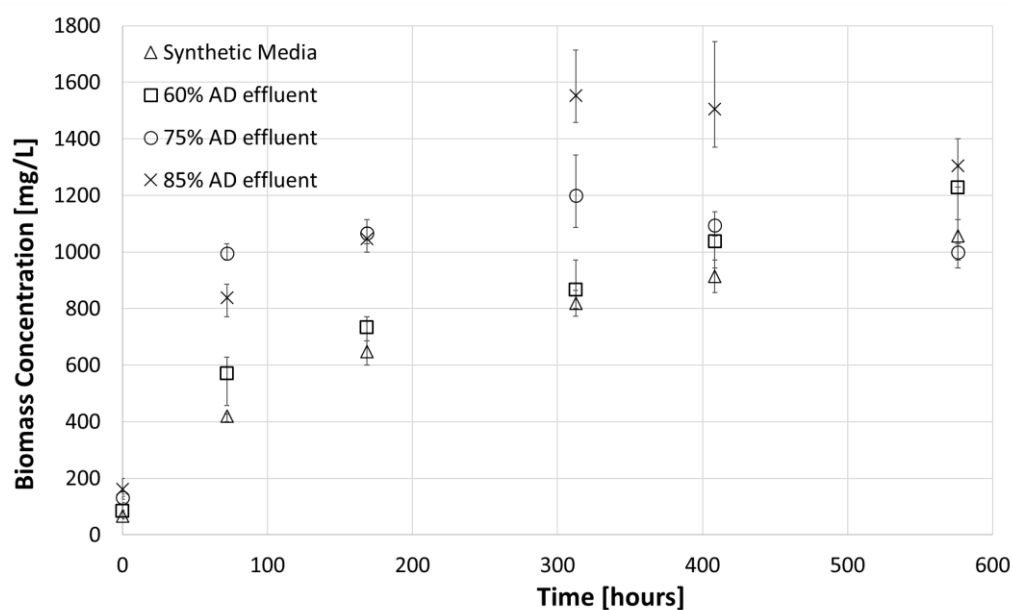
By raising the salinity of the AD effluent with seawater – a cheap and abundant resource – no extreme nutrient costs would be incurred. It also results in the dilution of the AD effluent, which means that growth inhibiting phenomenon associated with high ammonia concentrations and high turbidities could be overcome to some extent. To maximize waste valorisation from the AD effluent in various industries, seawater addition should be kept to a minimum.

### Magnesium and sulphates

Evident from Table 17 is the extremely low concentrations of magnesium and sulphate present in AD effluent – two orders of magnitude lower than which is found in medium A D7. Both magnesium and sulphate play an important role in cell size, RNA and protein synthesis (Utkilen, 1982). Under conditions of magnesium deficiency, cell division is inhibited and results in the formation of long filamentous cells (Webb, 1949). Thus, adding  $MgSO_4$  (Epsom salt) to the AD effluent is of vital importance for the cultivation of the study organism, and is a widely used and produced globally.

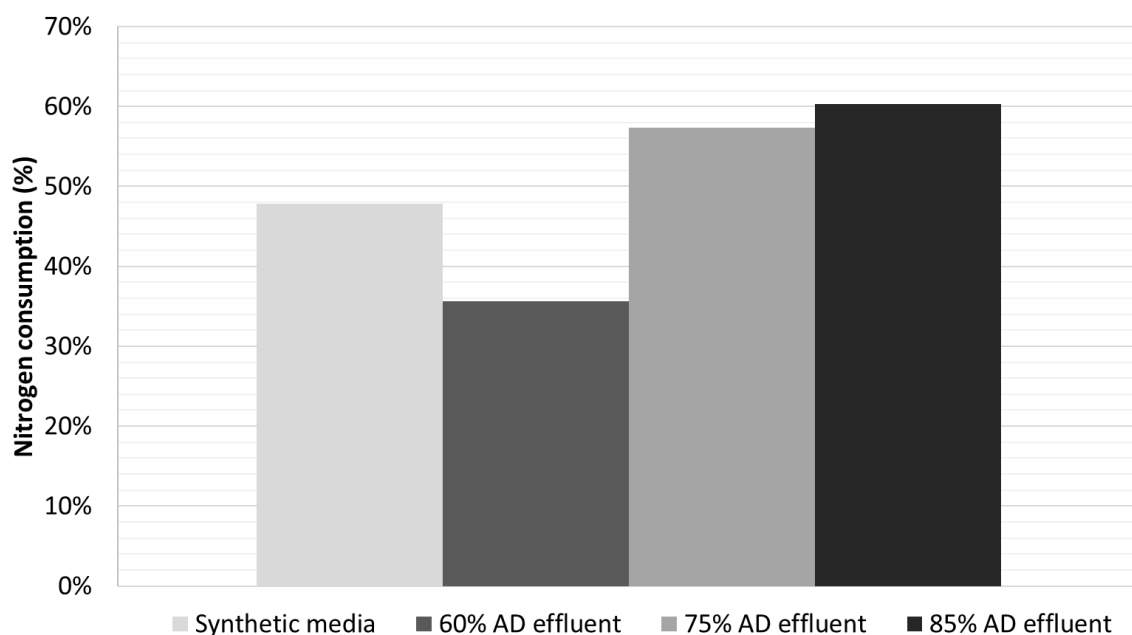
## 5.6 Growth and nutrient utilization in modified AD effluent

By adapting the AD effluent according to the methods stipulated in Section 4.4.6, the growth observed is shown in Figure 19.



**Figure 19: Growth observed in optimised AD media, adding  $MgSO_4$  to desired concentration and seawater in different levels ( $38\text{ }^\circ\text{C}$ ;  $225\text{ }\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ,  $\text{pH} = 6.8$ )**

The results indicated that the study organism could be grown successfully in up to 85% AD effluent with a maximum biomass concentration reached after 302 hours of 1550 mg/L. For both 75% and 85% AD effluent, total nitrogen concentration remained constant after 312 hours, and thus no more ammonium utilisation took place – which should have shown a stationary phase in the growth curve. Instead, a sudden drop in biomass is observed. The percentage of nitrogen utilised in 570 hours is shown in Figure 20. The method for calculation is explained in Section 4.3.5.



**Figure 20: Nitrogen consumption by cyanobacteria in synthetic media, 60% AD effluent, 75% AD effluent and 85% AD effluent (38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , pH = 6.8)**

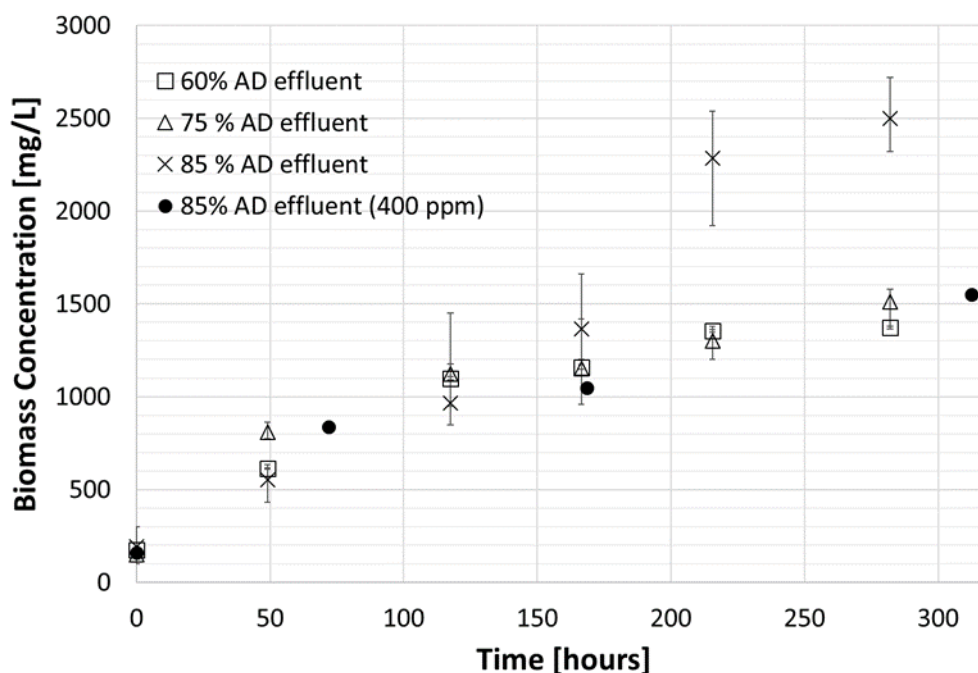
From this, it can be concluded that although higher biomass concentrations were observed for cultures grown in 85% AD effluent, the nitrogen available wasn't nearly depleted. Referring to Section 2.5.2, when ammonium is utilised as nitrogen source, this leads to a decrease in pH. As these experiments were operated at already low pH values to circumvent ammonia toxicity (pH = 6.8), it can be reasonably assumed that the cause of cell death was the pH dropping below 6.

### 5.7 Carbon enrichment in incubator

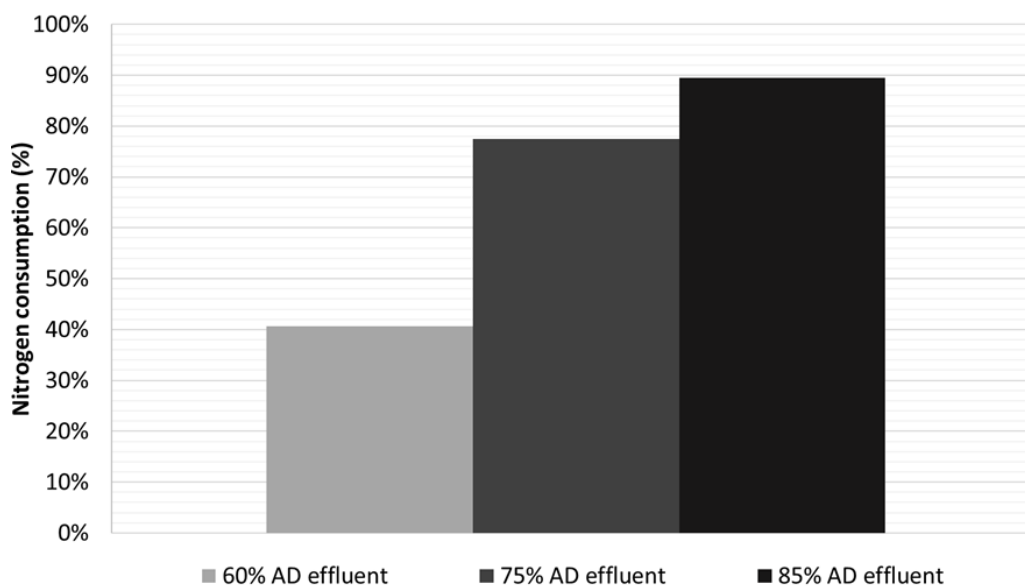
Experiments were progressed to include carbon enrichment to simulate biogas addition from anaerobic digestion, noting that the growth media had to be buffered for a pH of 6.8. This was done to prevent the media pH drifting below 6 where cell death occurs due to high ammonia concentrations. These procedures can be found in Section 4.3.4 and 4.4.7. By doing this, the full potential of the resources anaerobic digestion can offer cyanobacterial growth are valorised. Although the transfer of inorganic carbon into the media will still be growth limiting, it is expected that the cells will grow at a much faster rate, utilising more nutrients and ultimately, depleting the nitrogen and phosphorous levels in the effluent.

The results are presented in Figure 21 along with the nitrogen consumption for each AD effluent batch in Figure 22.





**Figure 21: Growth observed in buffered optimised AD effluent (60%, 75% and 85%) with carbon enrichment (10 %v/v) in the incubator head space gas (38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , pH = 6.8)**



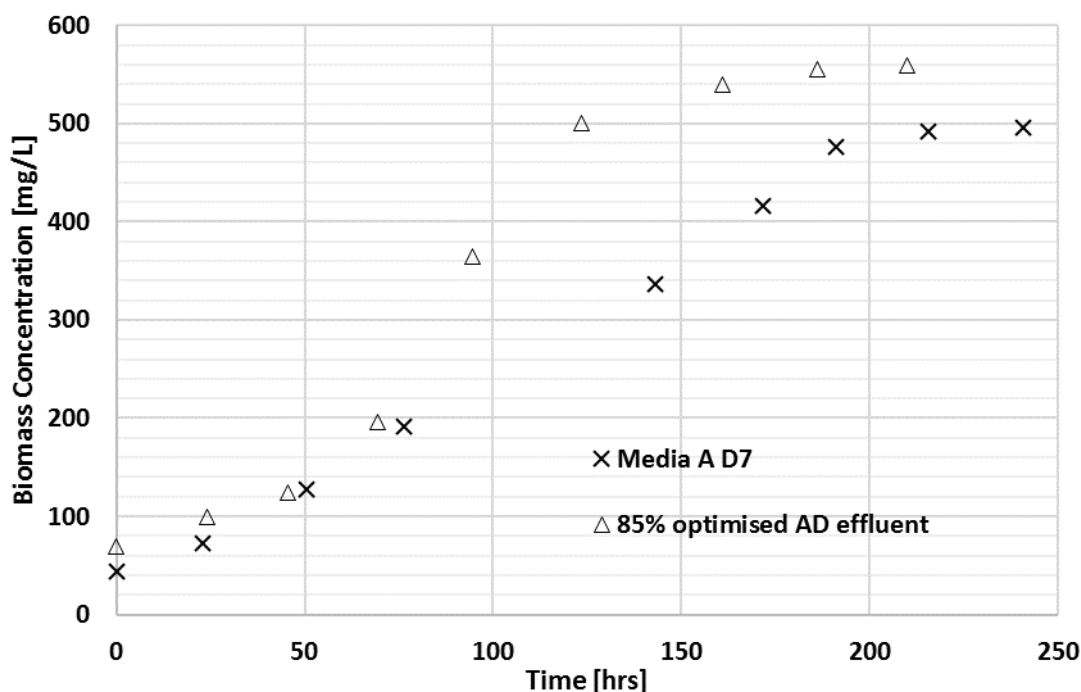
**Figure 22: Nitrogen consumption observed for cells grown in buffered optimised AD effluent (60%, 75% and 85%) with carbon enrichment (10 %v/v) in the incubator head space gas (38 °C; 225  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , pH = 6.8)**

With the highest quantity of nutrients available to be utilised in 85% AD effluent, the potential limiting effect of carbon on cell growth was the most pronounced for this experiment. In 300 hours, the addition of carbon in the headspace gas lead to a 38% increase in the biomass generated in 85% AD effluent. With regards to nutrient consumption, the microalgae reduced the nitrogen content in 85% AD effluent down to 10% of its original concentration. With biomass yields significantly higher in the case of carbon enrichment for 60 and 75% AD effluent media, the depletion in growth rate could be due to another

nutrient (such as phosphorous) being depleted which was not measured during the experiment. From these experiments, it was shown that the high concentration nutrients present in AD effluent can be reduced up to 30% further by microalgae when grown in a carbon enriched environment - supporting the hypothesis that the integration of AD effluent with microalgal growth is a means to lower the costs of biofuel production through waste valorisation.

## 5.8 Scale-up

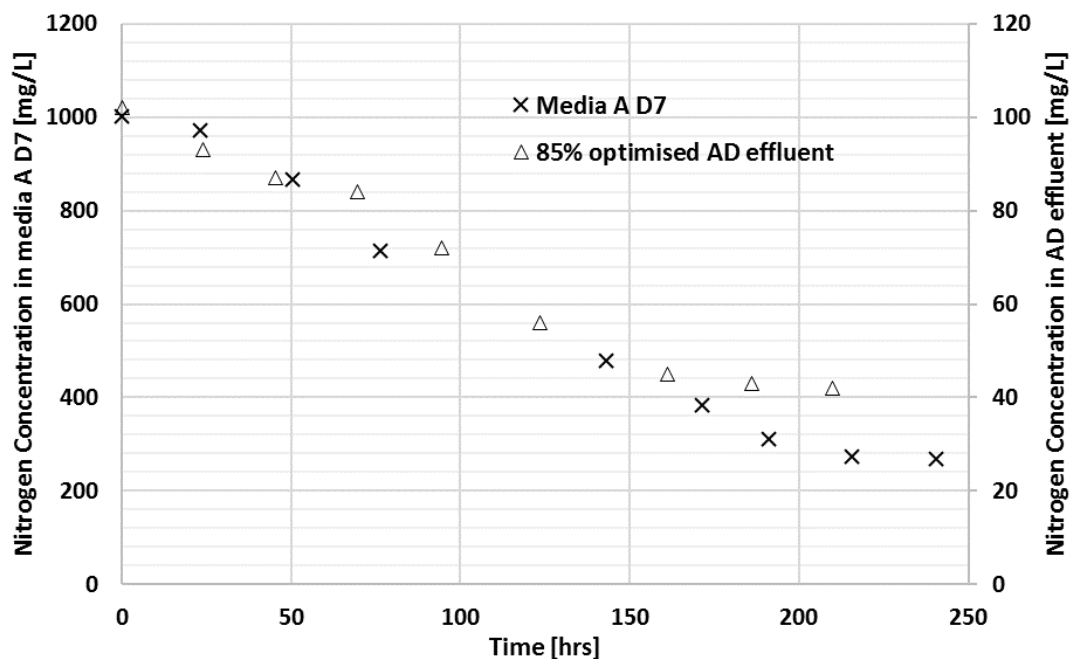
The culture was grown in an 8 L airlift reactor according to the methodology described in Section 4.4.8 in both synthetic media and AD effluent. Growth conditions were conservative, to give an indication of what growth can be expected if expensive and valuable resources are eliminated. Thus, the culture was exposed to low light conditions and lower temperatures compared to the batch experiments. It was grown at room temperatures and the temperature of the culture itself was measured daily using a thermostat. The culture media temperature (under a light intensity of  $180 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , which is about one order of magnitude smaller than natural sunlight) was measured daily during sampling. For the experiment in synthetic media, the average temperature was  $23.4 \pm 2.4 \text{ }^\circ\text{C}$  and for the AD effluent run it was  $23.1 \pm 3.2 \text{ }^\circ\text{C}$ . Although the mass transfer of carbon dioxide would be better in the airlift reactor, the gas used for sparging was atmospheric air. Thus, the results presented was grown at 400 ppm carbon dioxide, sparged. The biomass concentrations in the medias observed for these growth conditions and is shown in Figure 23:



**Figure 23: Biomass concentration observed for scaled-up growth in an 8L airlift reactor using optimised AD effluent and sparged with atmospheric air ( $23 \text{ }^\circ\text{C}$ ;  $180 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ,  $\text{pH} = 6.8$ )**

Although the weak light and low temperatures likely dampened the growth of the cells, the growth displayed in the airlift reactor resembled a typical growth curve with distinct lag, exponential, deceleration and stationary phases. The temperature, light conditions and level of air sparging were constant for both sets of experiments, and thus any difference in growth between the two experiments would be media nutrient related. For optimised AD effluent and synthetic media, the final biomass concentration achieved after 210 hours was 560 mg/L and 500 mg/L, respectively.

As for nitrogen removal, a similarly defined curve was generated and is shown in Figure 24.



**Figure 24: Nitrogen concentration observed for scaled-up growth in an 8 L airlift reactor using synthetic media and sparged with atmospheric air (23 °C; 180  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , pH = 6.8)**

For optimised AD effluent, nitrogen was reduced by 60% and the final biomass concentration achieved after 240 hours was 500 mg/L. For synthetic media, nitrogen was reduced by 70%.

The similarity between the final biomass concentrations of the two experiments suggests that the culture grew dense and as such, limited the available photosynthetic energy to reach dividing cells and promote growth. Using the modelling theory presented in Section 2.2.6, a comparison of the specific growth rate of each experiment could be calculated. Using Monod kinetics, the specific growth rate of the culture was calculated to be  $0.0185 \text{ hr}^{-1}$  ( $R^2=0.9766$ ) and  $0.0094 \text{ hr}^{-1}$  ( $R^2=0.983$ ) in optimised AD effluent and synthetic media, respectively. A comparison of the two cultures after 10 days is shown in Figure 25.



**Figure 25: Pictures of *Synechococcus* PCC 7002 grown in an airlift reactor after 10 days in optimised AD effluent (left) and synthetic media (right) (23 °C; 180  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , pH = 6.8)**

The experiment proved that *Synechococcus* PCC 7002 can be grown to equally concentrated cultures in optimised AD effluent compared to synthetic media. Although these culture densities achieved are far below that seen in the batch reactors, it is important to note that these experiments were conducted with the minimum operating condition optimisation to mimic the conditions under which the culture would be grown on an industrial scale – where the addition of heat and light would result in massive operational costs. The importance of the work lies in the fact that high concentrations of nutrients present in the effluent are significantly reduced whilst producing valuable by-products. For this, the only nutrient inputs required are  $\text{MgSO}_4$ ,  $\text{Na}_2\text{HPO}_4$  and  $\text{NaH}_2\text{PO}_4$ .

## 6. Conclusions

The aim of this study was to uncover and circumvent issues associated with growing microalgae in AD effluent using methods that would minimise the addition of costly resources.

Experiments were conducted in an incubator specifically designed for this investigation, and allowed for temperature control, light adjustment according to specifications, steady mixing of media and minimal operation gradients within the experimental space.

AD effluent was produced according to standard procedures (Section 4.1) and elemental analysis thereof revealed the inherent variability associated with the AD process. High ammonia concentrations, high turbidities, a lack of magnesium and sulphate and low dissolved salt concentrations were identified as key obstacles for integration with microalgae growth. Through the generation of AD effluent, centrifugation and filtration thereof revealed that a strong correlation exists between orthophosphate and suspended particles. Raw AD's orthophosphate concentration was reduced by 30% through centrifugation and filtration.

It was established that ammonium was the preferred source of nitrogen compared to nitrate, with nearly a 50% improvement in biomass concentration yield after 390 hours compared to cultures grown with nitrate as the sole nitrogen source. Growth in pure AD effluent and micro nutrient enriched AD effluent supported little microalgal growth. Dilutions with synthetic media as reported in literature resulted in a 10% increase in biomass concentration after 340 hours for AD effluent diluted by 90% as compared to growth in synthetic media. It was shown that the toxic effects of ammonia on cells could be circumvented if pH was maintained between 6.5 and 7. Operating above 6.5 was necessary for the health of this specific organism as the cells' metabolic functions are inhibited in more acidic environments. The upper limit of 7 is a function of ammonia concentration and temperature and is the maximum pH where ammonium is predominant in the media as opposed to ammonia in more alkaline environments. Carbonate and phosphate speciation could also be drivers for these pH limits. This strongly suggests that acidophilic microalgae might be better suited for growth applications in AD effluent.

At a maintained pH of 6.8, the maximum concentration of ammonium tested that tolerated cell growth was 0.9 M. No increase in biomass concentration was observed if the ammonium concentration was reduced from 0.9 M to 0.3 M with an 18% increase when the concentration was lowered further to 0.1 M. Growth was completely inhibited at ammonium concentrations of 1.5 M, suggesting the ammonia concentration limit lies between 0.9 M and 1.5 M.

Diluting AD effluent with synthetic seawater not only aided in lowering ammonia concentrations and turbidity, but it also increases the dissolved salts in the media that is required for the cyanobacterial study organism to grow. Adding as little as 15% of the total working volume in seawater was enough to meet the dissolved salt requirement for growth. Adding  $\text{MgSO}_4$  to the media in accordance with

concentrations suggested for media A D7 is also required, as this is a necessary nutrient for microalgal growth. Without any carbon enrichment, the microalgae showed comparable growth with cultures grown in media A D7 compared to 85% optimised AD effluent, with 10% more nitrogen assimilation in the optimised AD effluent. This illustrates that both dissolved salt and  $\text{MgSO}_4$  need to be present in high enough concentrations to support growth.

With carbon enrichment, the microalgal cells grew 3 times faster compared to growth in media A D7 with no carbon enrichment, with nitrogen consumption of up to 90%. The experiment was repeated, comparing the growth in synthetic media and the optimised AD media in an eight-litre air lift reactor. Keeping conditions conservative by performing the experiments in low light conditions with air bubbling instead of carbon dioxide enriched air (a gas hold up of 10% of the total volume of the reactor), the aim was to illustrate what growth could be expected at large scale production with minimal costly enhancements. Although both experiments were nutrient limited, higher biomass yields were observed for cyanobacteria grown in AD media ( $\mu_{\text{max}} = 0.018 \text{ hr}^{-1}$ ). Nitrogen was reduced by 60% and 70% within 10 days for growth in AD effluent and media A D7, respectively.

The results imply that high concentrations of nutrients associated with wastewater can significantly be reduced – up to 90% if temperature and light conditions are optimal and up to 70% in natural light and temperature conditions. This means that costs associated with nutrient supply can virtually be eliminated, lowering the overall operating costs of an industrial sized valuable-chemical producing microalgae plant. Most importantly, no clean water is required for the process and if incorporated with the flue-gas produced by AD, the process can not only utilise all wastes produced by AD but find benefit from incorporating other cheap waste sources such as seawater,  $\text{MgSO}_4$  rich effluents (such as mine wastewaters) and sunlight.

## 7. Recommendations for future work

This project yielded broadly defined results. Further suggested refinements include:

1. Investigating different sources of AD effluent, commercially produced, and investigating microalgae growth under the ideal conditions suggested by this project.
2. Repeating the process illustrated in this project for different types of microalgal strains, especially acidophiles - establishing lethal pH limits, ammonia concentrations and other possible growth limitations.
3. Zooming in on various conclusions by narrowing down ranges investigated,
  - a. Establish the exact optimum pH for *Synechococcus* PCC 7002 growth in AD effluent between 6.5 and 7 at the operating temperature.
  - b. Establish the exact maximum ammonia concentration that can be tolerated for feasible *Synechococcus* PCC 7002 growth, at the optimum pH (expected to be between 0.9 M and 1.5 M).
  - c. Establish the absolute maximum ratio of AD effluent to seawater that will allow *Synechococcus* PCC 7002 to grow, resulting in maximum AD effluent use.
  - d. Establish what the maximum turbidity is that can still allow for feasible *Synechococcus* PCC 7002 growth, and if successful, minimising AD effluent refinement through centrifugation and filtration – which was a tedious process.
  - e. Establish the minimum  $\text{MgSO}_4$  concentration requirement that supports microalgae growth in order to minimise nutrient costs associated with the resource on a large scale. Also, waste sources rich in  $\text{MgSO}_4$  (such as some mining effluents) can serve as a supplement to meet the demand required for microalgae growth.
  - f. Perform experiments with higher carbon dioxide concentrations in the gas phase, mimicking biogas produced from the anaerobic digestion process.
4. Consider repeating the experimental process with consortiums of microalgae and other bacteria, which could possibly improve nutrient sequestration and culture stability.
5. Perform experiments in a system that is set up outside where it will be exposed to natural elements in a less controlled environment. This will establish the robustness of species and provide insight into scalability and applicability of such systems in an economical way.
6. Perform experiments with genetically modified strains that produce biofuels in order to gauge possible differences in biofuel production when grown in synthetic media versus AD effluent. This can lead to valuable design considerations when integrating anaerobic digestion and algae growth in a closed system.
7. Performing more analysis on microalgal biomass generated in AD effluent with regards to pigments (chlorophyll).

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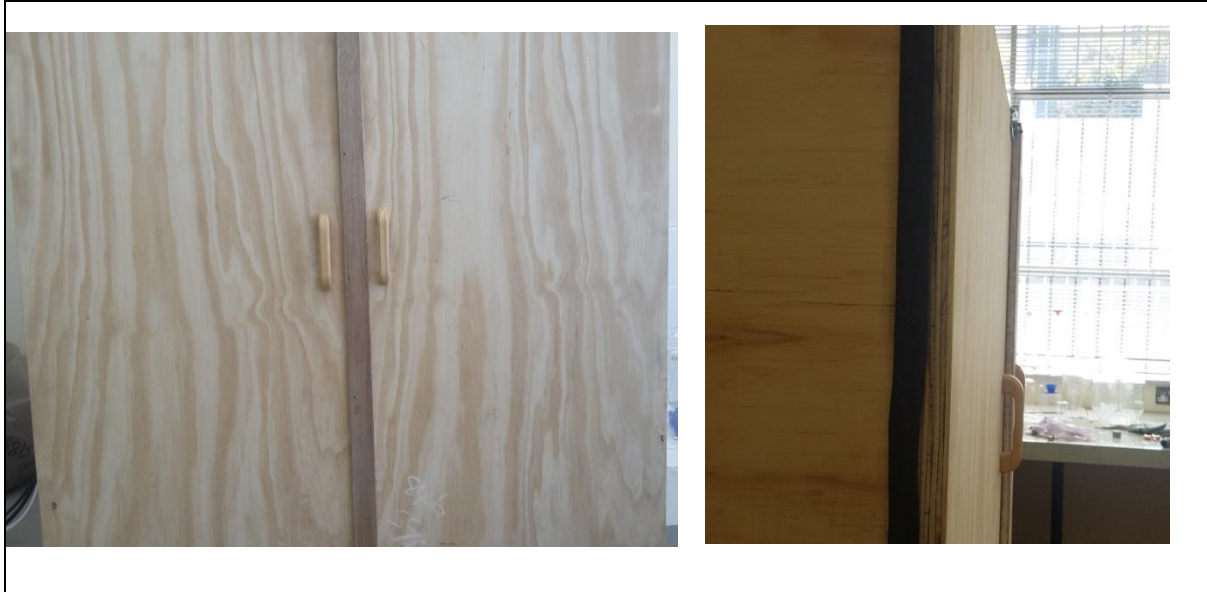
Zhu, Y., Graham, J.E., Ludwig, M., Xiong, W., Alvey, R.M., Shen, G., Bryant, D.A., 2010. Roles of xanthophyll carotenoids in protection against photoinhibition and oxidative stress in the cyanobacterium *Synechococcus sp.* strain PCC 7002. In: *Archives of Biochemistry and Biophysics*. Pennsylvania: The Pennsylvania State University, pp. 86-99.

## Appendix A: Incubator design

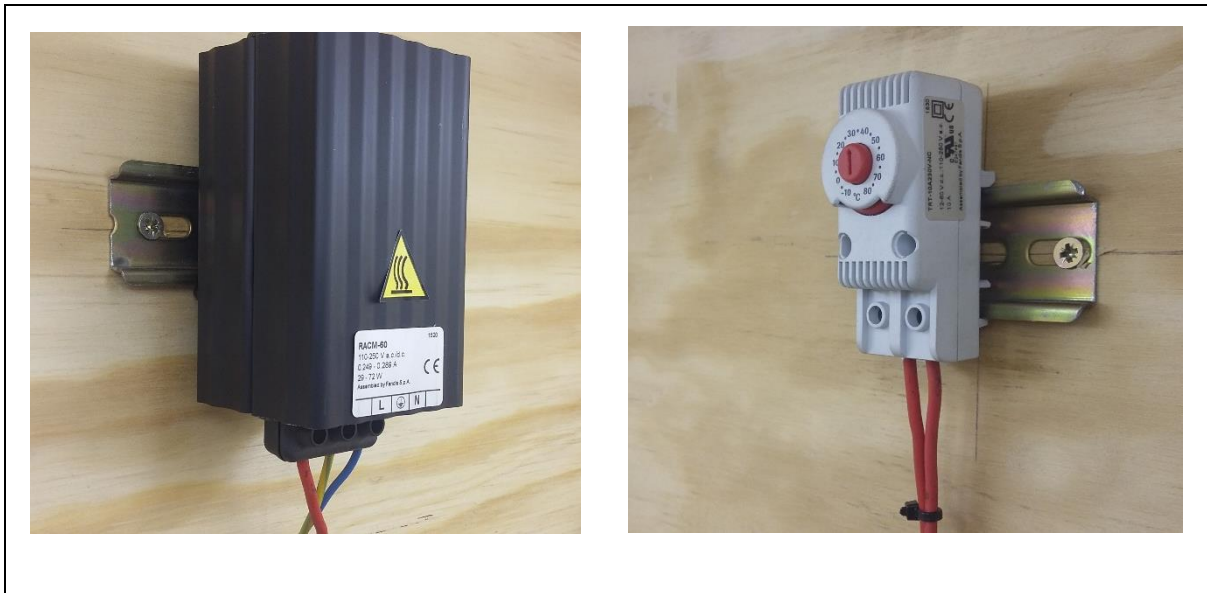
The lab-scale setup consists of an incubator, a shake table, an aeration system, a heating system and a lighting system. The batch PBRs will be individual 250 mL baffled flasks placed on top of the shake table and should provide sufficient mixing to ensure all cells get exposed to the light source. Following the literature review, adequate specifications for the parameters of all the experimental systems have been established and equipment was ordered accordingly. The flasks will be open to the atmosphere in the incubator and closed for 6 hours before sampling to allow sufficient accumulation of isoprene for GC-FID analysis. The incubator is constructed from wood and properly sealed to allow no gas to escape except at the outlet. The aeration system will be provided by a CO<sub>2</sub> cylinder. A 60 W (260 mA) panel heater and thermostat provides and regulates the temperature in the incubator. Three single fitted 30 W fluorescent tubes are installed which can be lowered to provide variable light intensities ranging from 160 to 1771  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . The incubator is shown in Figure 26, Figure 27 and Figure 28.



Figure 26: Incubator front view (open)



**Figure 27: Incubator front view closed (left), sealant (right)**



**Figure 28: Heating panel (left), thermostat (right)**

## Appendix B: Experimental solution preparations and calculations

### B.1 Light source quantification

The aim of this appendix is to justify the selection of three 30 W fluorescent tubes as light sources for growing the algae. Thus, 90 W in total will be available for the algae to absorb light from and, although the light will spread in all directions, the light of interest will be spread over a 0.652 m<sup>2</sup> surface (platform dimensions 632 mm by 1032 mm). Another important point is the fact that only 25% of the total energy input will be converted to visible radiation useful for the algae to grow (electrical4u, 2011-2016). Consequently, only 22.5 W is available to be converted to photon flux in following paragraphs. The irradiance [W.m<sup>-2</sup>] of the light source is defined by eq. 31:

$$E = \frac{P}{A} \quad \text{Equation 31}$$

Where P [Watts] is power and A [m<sup>2</sup>] is the surface area of the shake table that will be lit and has a value of 34.50 W.m<sup>-2</sup>. For biological processes the photon flux [ $\mu\text{E.m}^{-2}.\text{s}^{-1}$ ] of light with a distinct wavelength (in this case,  $\pm 553$  nm for fluorescent lights) has a higher relevance than the irradiance. Thus, a conversion between the two is required. A photon has some distinct energy quanta,  $E_p$ , defined in eq. 32:

$$E_p = \frac{h.c}{\lambda} \quad \text{Equation 32}$$

Where h is Planck's constant [ $6.63 \times 10^{-34}$  J.s], c is the speed of light [ $2.998 \times 10^8$  m.s<sup>-1</sup>] and  $\lambda$  is the wavelength of the light [nm]. Using these values, the energy amount of one photon is determined to be  $3.59 \times 10^{-19}$  J. Next, the number of photons denoted  $N_p$  can be calculated by eq. 33:

$$N_p = \frac{E}{E_p} \quad \text{Equation 33}$$

Using the values provided, the number of photons is calculated to be  $9.60 \times 10^{19}$  photons.m<sup>-2</sup>.s<sup>-1</sup>. Consequently, the photon flux,  $E_{QF}$ , can be determined from eq. 34:

$$E_{QF} = \frac{N_p}{N_A} \quad \text{Equation 34}$$

Where  $N_A$  is the Avogadro number [ $6.022 \times 10^{23}$  mol<sup>-1</sup>]. This results in a photon flux of  $1.59 \times 10^{-4}$  mol.m<sup>-2</sup>.s<sup>-1</sup> or  $159.38 \mu\text{E.m}^{-2}.\text{s}^{-1}$ . Important to note is that this is the photon flux 1 meter away from the light source. This number is inversely proportional to the distance between the light source and the surface area to be luminated. In effect, one can assume that the surface area was placed 1 meter away from the light source for the calculations above. As it is moved further and further away, the light intensity will decrease and this decrease is governed by the inverse-square law which states that brightness is inversely proportional to the square of the distance between the light source and the surface to be illuminated. This is illustrated in eq. 35:

$$\frac{E_{QF,1}}{E_{QF,2}} = \frac{r_2^2}{r_1^2}$$

**Equation 35**

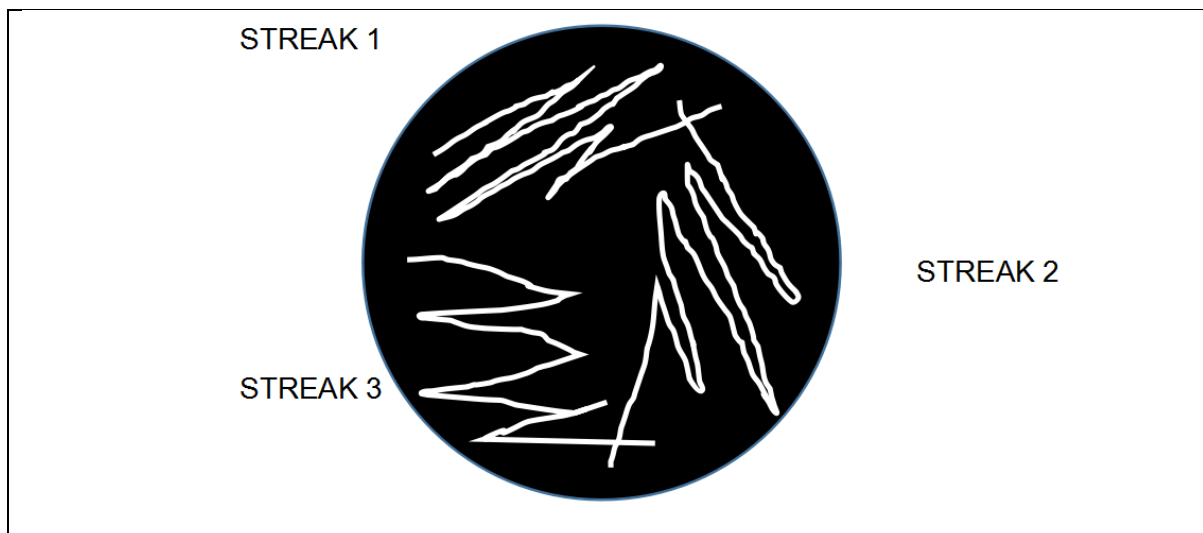
Where  $r$  is the new distance [m] between the light source and the surface area.

Since a head space 0.7 meters were given above the surface area of the shaker, the corresponding light intensities at the surface of the microalgae could be estimated. According to the algae supplier, it can successfully be grown at light intensities ranging from  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  to  $5000 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (which is double the intensity of full sunlight). Thus, for the purposes of this research, the highest photon flux achievable with the incubator dimensions and flask height constraining how close the lights could be placed to the surface was  $224.90 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  at a distance of 23.8 cm. This is 10% less than the recommended  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ .

## **B.2 Sub-culturing *Synechococcus* PCC 7002 from agar plates**

The cultures received from WISC will be in agar plates, from which more agar plates and liquid cultures will have to be prepared on a continuous basis to ensure healthy cultures are available for experiments. The former will be done by taking a single colony from the original plate and inoculating it on another agar plate. This will be done according to the following procedure and will be repeated for the wild-type strain of *Synechococcus* PCC 7002:

1. Obtain an LB agar plate with appropriate algae culture.
2. Label the bottom of the plate for future monitoring and reference with the plasmid name, batch number and date. The researcher's name and student number will also be added.
3. Ensure that a Bunsen burner or open flame is always active when working at the lab bench.
4. Using a sterile loop or pipette tip, pass it through the flame to sterilize it and be sure to allow enough time for it to cool before touching the bacteria. Touch the bacteria growing within the punctured area of the stab culture.
5. Gently spread the bacteria over various sections of the plate as indicated in Figure 29. The preparation of agar on which it should be streaked in petri dishes is explained in Appendix B.3.



**Figure 29: Streaking technique for a single colony**

6. Incubate the plate overnight (12-18 hours) at ambient level CO<sub>2</sub> under a light intensity of 150  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  and 30°C. (Xu 2010)
7. In the morning, single colonies should be visible. A single colony should look like a white dot growing on the solid medium. This dot is composed of millions of genetically identical algae that arose from a single bacterium. If the growth is too dense and no single colonies are visible, re-streak onto a new agar plate to obtain single colonies. Agar plates should be stored at 4°C.

For liquid-liquid inoculation, a single colony from the original agar plate is inoculated in 10 mL of medium A D7. A week later, this 10 mL culture is used as inoculum for the first 100 mL liquid culture. Tong (2014) prepared liquid cultures weekly. To prepare for a liquid-liquid subculture:

1. Add 90 mL of prepared medium A D7 and add 10 mL of previous liquid culture (as explained in previous paragraph) in the growth phase to complete the sub-culture.
2. Before using the sub-culture for inoculation during experiments, the OD was measured and if above 1, the sub-culture was diluted with sterilised media A D7. The final OD of the sub-culture has to be smaller than 1 before it is used to inoculate. This was to ensure consistency in the amount of cells introduced at the start of experiments, in order to compare growth results of different medias.
3. Again, be sure to sterilize all equipment when inoculating. The preparation of agar as stock solutions in which sub-cultures will be inoculated is explained in Appendix B.3.

### **B.3 Agar plate medium preparation**

For a 1L bulk solution of agar media:

1. Autoclave all glassware, stirring bars and any other equipment before use.



2. To approximately 390 mL of distilled water add the first 8 components (except  $\text{KH}_2\text{PO}_4$ ) in order specified in Table 9 and Table 10 while stirring continuously. This may be done by placing magnetic stirrers on a magnetic stirrer.
3. Bring total volume to 500 mL with distilled water.  
In a separate container add 12 g of agar to 500 mL of distilled water (final 1.5 % w/v). Also, include 2 g  $\text{Na}_2\text{S}_2\text{O}_3$  with the AFM base. Double the recipe.
4. Cover and autoclave both solutions at 121°C and 15 psi for 90 minutes. Although components do not have to be fully dissolved, ensure none is on the glass as this will cause caramelization.

**In a water bath mix the 1L AFM base and 1L agar and allow to cool down to 60 °C. Mix quick and well since agar solidifies quickly. Add trace elements, Tris-HCl, vitamin B12 and  $\text{KH}_2\text{PO}_4$  as it will precipitate when autoclaving. (Specified in Table 11 and**

5. Table 12)
6. Add filter sterilised solutions of antibiotics as necessary.
7. Allow to cool further to 45-50 °C.
8. Mix and pour plates (approximately 20mL per sterilised petri dish). Put petri dish covers on and place upside down in refrigerator for storage. Plates should be stored at 4 °C.
9. Repeat every month.

### B.5 Standard solution preparation

All standard solution preparations were done according to the general formula in eq. 36:

$$x \text{ M} \left[ \frac{\text{mol}}{\text{L}} \right] \text{ of compound} \mid M_w \left[ \frac{\text{g}}{\text{mol}} \right] = \text{amount added [g] per litre} \quad \text{Equation 36}$$

Where X is the molarity of the solution [ $\text{mol.L}^{-1}$ ] and  $M_w$  is the molecular weight of the compound added [ $\text{g.mol}^{-1}$ ]. Thus, by multiplying X with  $M_w$ , the amount to be added to 1 L in grams is calculated.

#### 5 M NaCl

$$X = 5 \text{ M}$$

$$M_w = 58.44 \text{ g.mol}^{-1}$$

Thus, add 292.2 g to 1 L.

#### 1 M KCl

$$X = 1 \text{ M}$$

$$M_w = 74.5513 \text{ g.mol}^{-1}$$

Thus, add 74.5513 g to 1 L.

#### 1 M $\text{NaNO}_3$



$$X = 1 \text{ M}$$

$$M_w = 84.9947 \text{ g.mol}^{-1}$$

Thus, add 84.9947 g to 1 L.

1 M MgSO<sub>4</sub>·7H<sub>2</sub>O

$$X = 1 \text{ M}$$

$$M_w = 246.5 \text{ g.mol}^{-1}$$

Thus, add 246.5 g to 1 L.

1 M CaCl<sub>2</sub>

$$X = 1 \text{ M}$$

$$M_w = 110.98 \text{ g.mol}^{-1}$$

Thus, add 110.98 g to 1 L.

1 M Tris HCl

$$X = 1 \text{ M}$$

$$M_w = 157.6 \text{ g.mol}^{-1}$$

Thus, add 157.6 g to 1 L.

Adjust the pH to 8.2 using NaOH.

1 M KH<sub>2</sub>PO<sub>4</sub>

$$X = 1 \text{ M}$$

$$M_w = 136.086 \text{ g.mol}^{-1}$$

Thus, add 136.086 g to 1 L.

6 μM vitamin B12

$$X = 6 \text{ μM}$$

$$M_w = 1355.37 \text{ g.mol}^{-1}$$

Thus, add 0.00813222 g to 1 L.

14 mM Fe-EDTA

For 1 L of 20 mM Na<sub>2</sub>EDTA add 1 mL of 5 mM FeSO<sub>4</sub> solution.

### 20 mM Na<sub>2</sub>EDTA

$$X = 20 \text{ mM}$$

$$M_w = 372.24 \text{ g.mol}^{-1}$$

Thus, add 7.444 g to 1 L.

### 5mM FeSO<sub>4</sub>

$$X = 5 \text{ mM}$$

$$M_w = 151.908 \text{ g.mol}^{-1}$$

Thus, add 0.75954 g to 1 L.

### 0.5 M EDTA

To prepare 1 mL of solution 186.1 g disodium EDTA dihydrate is dissolved in 700 mL of distilled water. A pH probe is calibrated and inserted into the solution. Add 10 M NaOH dropwise while stirring until the pH reaches 8.0. Then dissolve the EDTA powder into the solution and adjust the volume to 1L with distilled water.

### 1 M Tris-HCl

Add 121.4 g Tris Base to 500 mL of distilled water and by using a calibrated pH probe, add 10 M HCl solution dropwise until the pH reaches a value of 8.2. Adjust the final volume to 1 L.

## **B.6 Frozen stock cultures**

To preserve bacteria for prolonged periods, they must be stored at colder than normal temperatures. A major problem faced by bacteria stored in freezers is the formation of ice crystals. These crystals can damage cells by dehydration, which in turn is caused by increases in salt concentration within the cells. As water is converted to ice, solute accumulates in the residual free water, which denatures biomolecules. Additionally, it can also rupture cell membranes. To lessen this effect, glycerol is often used as a cryoprotectant. It depresses the freezing point of cells enhancing super cooling and thus protects them from the ice crystals. With bacteria, adding glycerol to a final concentration of 15% will keep cells viable. When stored at -20°C, the bacteria should be stored up to 1 year without any negative side effects. Dimethyl sulfoxide is also typically used for blue green algae.

To prepare frozen stocks, stock cultures with an optical density of between 1 and 2 were added to 2 mL culture volume to a final concentration of 15% (v/v) and cells may then be frozen at temperatures as low as -80°C.



**Appendix C: Raw data****Table 22: Medium A D7 properties**

<b>Property</b>	<b>Theoretical Medium A D7</b>	<b>Medium A D7 elemental analysis</b>
Potassium [mol/L]	0.01170	-
Calcium [mol/L]	0.00250	-
Magnesium [mol/L]	0.20000	-
Ammonia [mol/L]	-	-
Sulphate [mol/L]	0.20000	-
Nitrate+nitrite [mol/L]	0.01200	0.01199
Nitrite [mol/L]	0	0.00004
Ortho-phosphate [mol/L]	0.00037	0.00039
Dissolved Organic Carbon [mol/L]	0	-
Total Organic Carbon [mol/L]	0	-
Electrical Conductivity [mS/m at 25 °C]	-	3500
Turbidity [NTU]	-	19