Nutrient removal and biofuel potential of MaB-floc biomass from an integrated algal pond system treating domestic sewage

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Abstract

Integrated algal pond systems (IAPS) are a passive water treatment technology derived from the Oswald designed advanced integrated wastewater pond systems (AIWPS®) and effect wastewater treatment based on biological activity of microorganisms within the system, solar energy and gravity. The technology consists of an advanced facultative pond (AFP), a series of interconnected high rate algal oxidation ponds (HRAOP) and algal settling ponds. The symbiotic relationship between microalgae and bacteria facilitated by paddlewheel mixing of HRAOP results in the formation of biomass aggregates known as MaB-flocs. MaB-floc formation enhances nutrient abstraction, gravitational sedimentation and separation from water hence forming two product streams; recyclable water and biomass, both with valorisation potential. This work aimed to determine the suitability of MaB-floc biomass generated in the HRAOP of an IAPS treating domestic sewage as feedstock for biofuel production based on the content of carbohydrate and lipid. Nutrient removal efficiency, biomass productivity and bulk lipid and carbohydrate concentration were monitored for two consecutive three-month periods in the winter and summer seasons of 2018/19. Maximum removal efficiencies of nitrogen and phosphorus were determined as 71% and 75% respectively, demonstrating the efficiency of IAPS as a wastewater treatment technology. MaB-floc biomass productivity in winter and summer was 9.4 g/m^2/d and 16.5 g/m^2/d respectively indicating the heavy influence of seasonal temperature, possibly day length, and solar irradiation on biomass productivity in the HRAOP. Summer productivity was lower than the maximum theoretical productivity of 25 g/m^2/d possibly due to photoinhibition of photosynthesis as well as grazing pressures caused by the proliferation of rotifers mainly of the *Brachionus* genus. MaB-floc biomass consistently contained higher amounts of carbohydrate than lipid despite the changes in species dominance from *Scenedesmus* sp. and *Desmodesmus* sp. in winter to *Pediastrum* sp. in summer. Variations in MaB-floc biomass carbohydrate content were linked to changes in nitrogen concentration, mainly in the form of nitrates. Lower nitrogen concentration significantly increased the carbohydrate content of MaB-floc biomass from 17.5 ± 0.15% to 33.5 ± 0.3 % recorded in summer. In winter, biomass carbohydrate increased from 18.3 ± 1.2% to 35.8 ± 0.3%.To induce accumulation of carbohydrates through nitrogen starvation, isolated microalgal species native to the HRAOPs of the IAPS at Institute for Environmental Biotechnology Rhodes University(EBRU) were used. The outcome from the laboratory studies showed that carbon partitioning within isolated strains could be altered

from carbohydrate to lipid which is more energy-rich. Hence, exploring the biodiesel production option using HRAOP MaB-floc biomass, which had a lipid content ranging between 12.1 ± 0.64 % and 13.9 ± 0.5 %, would require a preconditioning step in the form of nitrogen starvation to enhance its lipid content. Overall, the outcome of outdoor monitoring studies on biomass biochemical composition indicated that HRAOPs operating under natural environmental conditions preferentially generated a biomass rich in carbohydrate. Therefore, anaerobic digestion may be a more viable option for HRAOP MaB-floc biomass because of the high carbohydrate levels ranging between 24.9 ± 0.6 % and 25.6 ± 1.3 % of the dry MaB-floc biomass weight. Despite the low biomass C/N ratio (7.1 to 7.8), the MaB-floc biomass can be anaerobically co-digested with a higher C/N ratio (24) substrate such as in-pond digester sludge, to improve methane yields calculated to be between 0.31 m^3 CH4/ kg MaB-floc biomass and 0.33 m^3 CH4/ kg MaB-floc biomass. Anaerobic digestion of biomass also produces CO2 which can be recovered and added to HRAOPs to enhance MaB-floc biomass productivity while lowering greenhouse gas emissions from a wastewater treatment plant. The digestate from the anaerobic process, which is rich in nitrogen and phosphorus can be used as a biofertiliser. Thus, a potential MaB-floc biomass biorefinery consisting of biogas and bio-fertiliser pathways can be established using IAPS treating sewage as the platform technology. IAPS is a system designed to operate in a way that is passive and without substantial environmental impact but technological innovations and a reduction in the size of the system are required to make the technology more acceptable.

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List of Abbreviations

AFP	**Anaerobic Facultative Pond**
AIWPS	**Advanced Integrated Wastewater Pond**
APHA	**American Public Health Association**
ASP	**Algal Settling Pond**
C	**Carbon**
DCW	**Dry Cell Weight**
DNA	**deoxyribonucleic acid**
dNTP	**deoxyribonucleotide triphosphate**
DWA	**Department of Water Affairs**
dwt	**dry weight**
EBRU	**Institute for Environmental Biotechnology Rhodes University**
EPS	**Extracellular Polymeric Substance**
FP	**Facultative Pond**
GC-MS	**Gas Chromatography-Mass Spectrometry**
HRAOP	**High Rate Algal Oxidation Pond**
HRT	**Hydraulic Retention Time**
H	**Hydrogen**
HPLC	**High-Performance Liquid Chromatography**
HTL	**Hydro-Thermal Liquefaction**
IAPS	**Integrated Algal Pond System**
IPD	**In-Pond Digester**
MaB-floc	**Microalgal Bacterial floc**
MLSS	**Mixed Liquor Suspended Solids**
N	**Nitrogen**
PCR	**Polymerase Chain Reaction**
S	**Sulphur**
SAIAB	**South African Institute for Aquatic Biodiversity**
SE	**Standard Error**
WRC	**Water Research Commission**

Chapter 1: General Introduction

1.1 Introduction

In a world where urbanisation and industrialisation are rapidly taking place, the biggest challenges that sub-Saharan African countries face today are water scarcity and water pollution (Maizatul et al. 2017). According to the 2018 national water and sanitation master plan report (National Water and Sanitation Master Plan Brief description of crucial revisions: Issue Date 2018), South Africa's water deficit is estimated will by 2030 have increased by 17% from the current water demand. Water scarcity in South Africa is attributed to low annual rainfall averaging 495 mm and a high evaporation rate of 1800 mm per annum on average (Adewumi et al. 2010). It is, therefore, imperative to adopt treatment technologies with high efficacy in order to reclaim and reuse treated water as one of the strategies to reduce the gap between supply and demand. In this regard, more appropriate, robust, easy to deploy and sustainable wastewater treatment technologies are required to treat wastewater to an acceptable level of nutrient discharge into the environment for reclamation purposes. For a technology to be termed sustainable it should possess the following aspects: high nutrient recovery, low operation and maintenance costs, biomass with valorization potential, and low land usage (Balkema et al. 2002). Based on the above characteristics, most waste water treatment technologies certainly meet one of more of these criteria.

South Africa's urban municipalities have adopted activated sludge systems (AS) attached to biological nutrient removal (BNR) systems due to high volumes of sewage produced from industrial and urban activities and stringent regulatory discharge limits. While AS treat sewage to levels that adhere to regulations for discharge into the environment in a short space of time, their limitations are associated with high energy consumption, need for highly skilled labour and use of sophisticated mechanical equipment (Mahmood et al. 2013). The cost of operating and maintaining these systems goes far beyond the affordability of most developing countries such as South Africa. This has left most sewage treatment works derelict and unable to treat sewage to a quality that meets regulatory discharge limits (Mambo et al. 2014). Inadequate treatment of sewage exposes receiving water bodies to heavy pollution by nitrogen and phosphorus, the causative agents of eutrophication, which is a condition of high nutrient load that results in toxic algal blooms and low oxygen waters that can kill aquatic life. Additionally drawing water from eutrophic areas for production of drinking water may pose a health risk to human populations through ingestion of algal bloom and bacterial toxins (Adewumi et al. 2010). Addressing the issue of water pollution in

South Africa requires the adoption of wastewater treatment technologies with high efficacy yet manageable.

On the other hand, considering the affordability aspect, Municipalities in rural areas of South Africa have adopted waste stabilisation pond (WSP) technology, which is a rather passive and less energy- consuming type of pond treatment system (Mahmood 2013; Mambo et al. 2014, Butler et al. 2017). Sewage treatment in such systems mainly relies on the symbiotic interactions between bacteria and microalgae utilising sunlight as a source of energy for the removal of nutrients and pollutants from sewage (Oswald et al. 1964; Buchanan et al. 2018). However, this type of pond technology has its shortcomings which include sludge accumulation over time, production of unpleasant odours, long hydraulic retention times spanning a month and tendency to eutrophy reducing their effectiveness with time. (Picot et al. 1992; Mambo et al. 2014; Buchanan et al. 2018). The reduction of nutrients from wastewater is therefore imperative to reduce eutrophication, improve water quality and reusability.

Integrated algal pond systems (IAPS) can be considered as a sustainable wastewater treatment technology. Integrated algal pond systems (IAPS) are a pond treatment technology derived from AIWPS and consists of an in-pond digester (IPD) and facultative pond (FP), together termed an advanced facultative pond (AFP), a series of interconnected high rate algal oxidation ponds (HRAOP) and algal settling ponds (Mambo et al. 2014; Cowan et al. 2016). This technology combines biological activity from microorganisms within the system, solar energy and gravity to effect wastewater treatment (Rose et al. 2007b). The symbiotic relationship between microalgae and bacteria is facilitated by paddlewheel mixing resulting in the formation of biomass aggregates known as MaB-flocs (Green et al. 1995; Jimoh and Cowan 2017). Formation of MaB- flocs facilitates gravitational sedimentation and separation from water (Jimoh and Cowan 2017), hence forming two utilisable products, namely water for recovery and reuse and biomass with beneficiation potential. These enhanced pond systems (i.e. AIWPS and IAPS) create a platform for the establishment of a biorefinery system as the biomass can be beneficiated into biofuel, fertiliser, fish and animal feed (Rawat et al. 2011; Craggs et al. 2014).

The growing interest in microalgal based biofuels is closely associated with declining fossil fuel reserves and global warming due to the associated increase in greenhouse gas emissions (Brennan and Owende 2010). Previous studies have strongly recommended amalgamating wastewater treatment and biofuel production, where costs associated with microalgal production and harvesting form part of the wastewater treatment cost, hence producing free feedstock for biofuel

production (Lundquist et al. 2010; Pittman et al. 2011). Thus, IAPS can be the technology to bridge the existing gap between wastewater treatment and energy production.

1.2 History of integrated algal pond systems

The research and development of IAPS as a sustainable sewage treatment technology began in the 1950s, led by Professor Oswald from California. Much of Professor Oswald's work was carried out at Lawrence Berkeley National laboratory. One of Professor Oswald's earliest works focused on the role of microalgae in sewage ponds (Oswald et al. 1957). This led to the inception of microalgae- containing high rate algal oxidation ponds (HRAOPs) in 1957, which served as aeration and nutrient removal ponds through photosynthetic oxygenation and microalgal assimilation respectively (Mambo et al. 2014). HRAOPs were then implemented at Concord and Richmond research sites in California (Oswald et al. 1994), where further studies on biogas analysis and sludge digestion were carried out. Based on extensive studies on HRAOPs and Parker's research on waste stabilisation ponds in Australia, Professor Oswald merged these two pond technologies to establish advanced integrated wastewater pond systems (AIWPS). This pond bioprocess system consisted of series of uniquely designed ponds that enabled removal of suspended solids, support growth of methanogenic bacteria, provide microalgal photosynthetic oxygenation of primary treated sewage and subsequent disinfection (Oswald et al. 1957).

Further research was conducted on AIWPS by Professor Oswald, Doctor Greene and Benemann, which included nutrient removal efficiency, methane production and microalgal biomass harvesting (Mambo et al. 2014). Over the years, similar systems were built and research was undertaken to optimise these systems to the regional conditions in which they were deployed. Notable works include those of Shelef from Kuwait, Banat from Israel and more recently Craggs from New Zealand and Sutherland from Australia.

In 1994, the water research commission (WRC) funded the construction of an IAPS similar to the AIWPS in design at the Institute for Environmental Biotechnology (EBRU), Makhanda (formerly Grahamstown) South Africa. The pilot plant served as a demonstration of a low cost and effective sewage treatment technology that can be implemented in small communities and rural municipality treatment works (Rose et al. 2007b). The primary focus areas on the demonstration pilot plant IAPS were the monitoring of its performance under South African conditions, optimisation of process performance and the applicability of this technology in treating other types of wastewater (Rose et al. 2007b).

Integrated algal ponding system (IAPS) is a passive bioprocess technology that makes use of gravity, solar energy and biological activity to abstract nutrients from wastewater. A typical IAPS consists of an IPD, AFP, HRAOPs, ASP and maturation pond (Green et al. 1995; Lundquist and Oswald 1995). Due to its passive nature of treating wastewater, this system requires no skilled labour for operation and maintenance nor does it require any chemical dosing that may otherwise result in residual sludge build-up (Whitton et al. 2015). In terms of faecal sludge handling, IAPS technology enables sludge accumulation and high residence time of approximately 10 years within an IPD before it can be removed and disposed of. Thus, IAPS overcomes one of the major problems encountered by WSPs systems (Craggs et al. 2011).

Although the large land requirement may pose a hindrance to the dissemination of IAPS, this technology offers more than just nutrient reduction. The technology provides a platform for resource recovery of two valuable products, namely reusable water and biomass (Park et al. 2011b; Sutherland et al. 2015). In its deployment in different countries globally, IAPS has been demonstrated to treat various types of wastewater including a tannery, piggery, brewery, acid mine drainage and abattoir wastewater (Rose 1996; Craggs 2003; Boshoff et al. 2004).

The IAPS ~~system~~ located at Belmont Valley, Makhanda (formerly Grahamstown), South Africa was initially designed without a tertiary treatment component in the form of maturation ponds, trickling filter or ultraviolet treatment (Cowan and Render 2012; Cowan et al. 2016). The design of this particular IAPS-was based on the first generation AIWPS design shown in Figure 2.1.

So far, consistently high chemical and biological oxygen demand and total suspended solids have been reported and this has been attributed to apoptosis and inefficient biomass removal thus suggesting the need for additional treatment (Cowan et al. 2016; Jimoh and Cowan 2017). Therefore, the performance expectations of the Belmont Valley IAPS are to produce an effluent of secondary water quality; hence it does not meet the department of water affairs (DWA) specifications (Table 1.1) for discharge into the environment (Mambo et al. 2014). However, IAPS technology may be implemented as an add on system to poorly performing wastewater treatment plants to enhance the effluent quality through nutrient removal and subsequent UV disinfection (Rose et al. 2007b; Cowan and Render 2012). Alternatively, due to its high performance in nutrient removal, the HRAOP component of an IAPS may independently be incorporated into waste treatment plants to enhance the effluent quality before reuse, with added benefits of biomass recovery for beneficiation (Craggs et al. 2014; Young et al. 2017).

Table 1.1 Water quality parameters for discharge into the environment as specified by the department of water affairs (Republic of South Africa, Water Act 1998)

Parameter	**General limit**
pH	5.5-9.5
Suspended Solids (mg/L)	25
Electrical Conductivity	70 mS/m above intake to a maximum of 150 mS/m
Chlorine as free Chlorine (mg/L)	0.25
Ortho-Phosphate as phosphorus (mg/L)	10
Nitrate/Nitrite as Nitrogen (mg /L)	15
Ammonia (ionised and un-ionised) as Nitrogen (mg/L)	6
Chemical Oxygen Demand (mg/L)	75

1.2.1 HRAOPs description and operational process

HRAOPs are the primary nutrient removal components that contribute to high-quality effluent discharged from an IAPS system (Young et al. 2017; Arashiro et al. 2019). Also, renewed global interest in these ponds is due to their minimal energy consumption when treating wastewater compared to AS systems, while they produce a valorisable biomass (Young et al. 2017).

HRAOPs are shallow (0.3 m - 0.5 m) paddlewheel-driven raceway ponds that promote microalgal and bacterial growth (Craggs et al. 2014; Mambo et al. 2014). The main control variables are mixing velocity, hydraulic retention time (HRT) and organic loading rate (Olguín 2003; Olguín 2012; Craggs et al. 2014; Mehrabadi et al. 2015).

Mixing velocities within HRAOPs range between 10 and 30 cm/s (Olguín 2003; Park et al. 2011a; Craggs et al. 2014; Sutherland et al. 2015). Typically, HRAOP paddle-wheels are driven by an electric motor utilising approximately 250 – 370 J/s (Mambo et al. 2014). However, power installed to drive the paddle-wheel also depends on raceway length and channel velocity (Moulick et al. 2002). Gentle mixing promotes the growth of large flocculating species such as *Micractinium, Pediastrum* and *Scenedesmus* while keeping them in suspension (Park et al. 2011b; Sutherland et al. 2015). The non-motile algal species tend to be outcompeted in facultative ponds as they settle faster compared to unicellular microalgae in stagnant water (Craggs et al. 2014; Mehrabadi et al. 2017).

Continuous gentle mixing also allows for microalgal periodic exposure to sunlight as well as enhancing temperature and oxygen dispersions, thus preventing thermal and oxygen stratification (Craggs et al. 2014; Mambo et al. 2014).

Hydraulic retention time (HRT) in HRAOPs varies between 4 and 10 days, depending on climatic conditions (Olguín 2003; Craggs et al. 2014 Sutherland et al. 2015). During winter the retention time is set to 7 - 10 days while in summer the retention is reduced to 3 – 4 days (Sutherland et al. 2015). HRT has been associated with altering the microalgal bacterial ratios within HRAOPs (Mehrabadi et al. 2015; Mehrabadi et al. 2017). Studies carried out by Park et al. (2011a) showed an increase of microalgal composition in HRAOP biomass from 56% when retention was set at 8 days to 80% when the HRT was 4 days.

Ultraviolet disinfection is another prominent occurrence within HRAOPs (Oswald et al. 1957). This is an essential aspect of HRAOPs technology in wastewater treatment as faecal coliform removal results in a pathogen-free effluent (Cowan et al. 2016; Green et al. 1995).

The symbiotic relationship between microalgae and heterotrophic bacteria results in nutrient assimilation and wastewater remediation (Mambo et al. 2014). The breakdown of organic matter by heterotrophic bacteria results in an effluent rich in inorganics such as NH_4^+, NO_3^{2-} and PO_4^{2-} (Jimoh et al. 2019). Removal of these nutrients is via assimilation into microalgal biomass (Picot et al. 1991). Inorganic nitrogen (N) removal is assimilated into microalgal biomass in the preference $NH_4^+ > NO_3^{2-} > NO^{2-}$ (Van Den Hende 2014). NO_3^{2-} is reduced to $NH_4^{+,}$ which is subsequently incorporated into amino acids for the formation of proteins (Whitton et al. 2015). Phosphorus (P) is actively transported into microalgal cells in the preferred forms of $H_3PO_4^-$ and HPO_4-, where it is assimilated into nucleotides during DNA synthesis (Whitton et al. 2015). Microalgae are also able to uptake excess P which is converted and stored as polyphosphates within microalgal cells (Markou et al. 2012). The stored polyphosphates may be utilised in the future by the microalgal cell when external phosphorus levels are low (Whitton et al. 2015).

Nitrogen and phosphorus may also be lost via volatilisation and precipitation. Substantially, microalgal photosynthesis during daylight elevates the concentration of dissolved oxygen (> 200 %) which consequently increases pH to above 10 in the medium (Craggs et al. 2014). In such high pH conditions, NH_4^+ is oxidised to ammonia (NH_3) while inorganic phosphorus precipitates out as calcium phosphate hence facilitating removal of both nutrients through volatilisation and precipitation respectively (Park et al. 2011b; Craggs et al. 2014; Mambo et al. 2014).

Elevated dissolved oxygen levels also prevent the growth of faecal coliforms and other pathogenic microbes, while ultraviolet light acts as a disinfectant to further reduce the pathogenic bacterial numbers (Kumar et al. 2010; Cowan et al. 2016). However, a biological counter mechanism enables excess oxygen liberated by microalgae to be utilised by aerobic heterotrophic bacteria in degrading the sewage organic matter into carbon dioxide (CO_2), ammonia and phosphates. While CO_2 availability in HRAOPs is dependent on oxidation of organic matter by heterotrophic bacteria and aeration (ingress from the atmosphere), domestic wastewater does not provide sufficient carbon for nitrogen assimilation by microalgae (C/N ratio of 3:1 in domestic wastewater compared to 6:1 in microalgae (Mambo et al. 2014). Additionally, carbon limitation is attributed to high light intensities which increase water temperature causing a decrease in the solubility of atmospheric CO_2 (Sutherland et al. 2015). However, carbon augmentation through CO_2 addition has been shown to increase microalgal productivity and reduce the loss of nitrogen through volatilisation so that the bulk of the nitrogen is incorporated into algal biomass (Craggs et al. 2014; Mambo et al. 2014; Sutherland et al. 2015).

1.2.2 HRAOPs and MaB-floc biomass production

HRAOPs were first developed in the 1950s by Oswald for combined wastewater treatment and nutrient recovery via biomass harvesting (Greenwell et al. 2010; Christenson and Sims 2011; Craggs et al. 2014; Sutherland et al. 2014b; Lynch et al. 2015). Previous works focused on the recovery of MaB-floc biomass as a source of feed and other chemical compounds (Johnson 2010; Olguín 2012). It was only later in the 1970s when the oil crisis first struck, that an urgent call for alternative and sustainable biofuel sources was made (Olguín 2012). Mass cultivation in HRAOPs coupled to wastewater treatment was perceived as a better option for microalgal production due to their low cost and ease of scaling up (Acién et al. 2016).

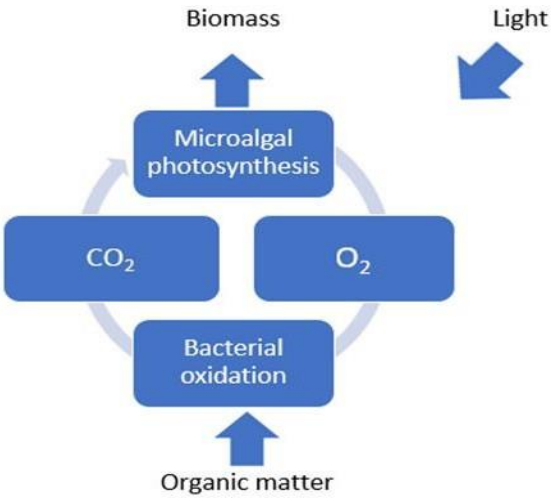

Figure 1.1: Schematic diagram illustrating the symbiotic relationship between microalgae and heterotrophic bacteria in HRAOPs (Rawat et al. 2011).

Biomass production in HRAOPs is driven by the symbiotic relationship between microalgae and bacteria (Rose et al. 2007a; Rawat et al. 2011). The generation of O_2 by microalgae supports the breakdown of organic matter by heterotrophic bacteria into CO_2, which in turn is assimilated by photosynthesising microalgae (Figure 1.1) (Rawat et al. 2011).

MaB-flocs consist of approximately 60 to 80% microalgae and 20 to 30% of bacteria (Jimoh et al. 2019). Other organisms such as fungi and ciliates also form part of the MaB-floc but are in the minority (Jimoh et al. 2019). MaB-floc formation is facilitated by extrapolymeric substance (EPS) production by microalgae and bacteria. EPS comprises of carbohydrate, protein, lipids, nucleic acids, other organic compounds secreted by microorganisms and insoluble material (Jimoh and Cowan 2017). The secreted EPS forms a gel matrix around the MaB-floc to give it structural and biochemical support (Jimoh et al. 2019). Therefore the generation of EPS together with paddlewheel mixing is what drives flocculation into MaB-flocs (Green et al. 1995).

1.2.3 Factors affecting MaB-floc biomass productivity

MaB-floc biomass productivities in HRAOPs treating sewage can reach an annual average of 25 $g/m^2/d$ if well operated (Park et al. 2011a). Mixotrophic growth mainly exhibited in wastewater HRAOPs due to the assimilation of both dissolved organic and inorganic compounds attributes to these high biomass productivities (Chisti 2016).

HRAOPs are susceptible to seasonal and diurnal variations in light and temperature (Waller et al. 2012). This, in turn, impacts on biomass production within HRAOPs (Waller et al. 2012, Sutherland et al.

2015). Higher productivities are achieved during summer while they are relatively low in winter (Whitton et al. 2015; Sutherland et al. 2018).

The quality and quantity of light determine the photosynthetic rate in microalgae (Markou and Georgakakis 2011; Benavente-Valdés et al. 2016). Biomass productivity increases with light intensity and reaches a maximum when the light intensity gets to a saturation point (Park et al. 2011a). Irradiance beyond the light saturation point results in photoinhibition where photons absorbed into the photosynthetic apparatus exceeds the required amount for the photosynthetic reaction (Mehrabadi et al. 2015). This results in damage to microalgal light receptors, thus decreasing biomass productivity (Mehrabadi et al. 2015). Moreover, an increase in algal biomass concentration, in turn, decreases light penetration, a phenomenon known as "self-shading" (Park et al. 2011a; Juneja et al. 2013; Mehrabadi et al. 2015). Algae within the top 15 cm of the pond will absorb most of the available light while the rest of the pond depth remains in a dark zone which reduces photosynthetic efficiency (Park et al. 2011a; Markou et al. 2012;).

Significant proportions of biomass are lost at night time during respiration which is estimated to be approximately 10% of the total productivity (Park et al. 2011b). Park et al. (2011b) measured biomass productivity of 24 $g/m^2/d$ from the Hamilton pilot-scale HRAOP they studied during summer. The productivity of 24 $g/m^2/d$ also factored in biomass losses.

With regards to temperature, biomass productivity doubles for each 10 ˚C increase in temperature (Mehrabadi et al. 2015). At the optimum level, maximum algal productivity is observed. However, the optimum temperature for maximum algal growth is species-dependent but is often in the range of 28 ℃ - 35℃ (Park et al. 2011b; González-Fernández and Ballesteros 2012; Markou et al. 2012; Mehrabadi et al. 2015). Any value above optimum reduces algae growth as oxidative damage occurs and rubisco's affinity to CO_2 is reduced (González-Fernández and Ballesteros 2012).

Temporary succession is a phenomenon that has been observed in HRAOPs. The success of a microalgal consortium in HRAOPs is influenced by the performance of each species under prevailing environmental conditions and changes in these conditions result in temporary successions (Sutherland et al. 2014a). For instance, for the Belmont Valley IAPS HRAOPs, *Pediastrum* species was succeeded by *Micractinium* species in October 2012, which in turn was succeeded by *Pediastrum* species once again in December 2012. *Cyclotella* species succeeded *Pediastrum* in March 2013 and was succeeded by *Pediastrum* species again in December 2013.

Finally, *Pediastrum* was supplanted by *Dictyosphaerium* species in January 2014 (Cowan et al. 2016).

Temporary successions thus pose a challenge in HRAOPs, that is, maintaining desirable algal species throughout the year for consistent biomass production. Several environmental factors come into play. Some of these factors include seasonal and diurnal changes (irradiance, temperature), zooplankton grazing, and nutrient availability. For example, Cowan et al. (2016) noted elevated concentrations of ammonium ions which was 2.5 times higher than the standard limit during the period that diatom species *Cyclotella* was dominant. The authors attributed this increase to a hypothesis that suggested ammonium replete diatoms released nitrite ions, ammonium ions or dissolved organic nitrogen after a quick increase in irradiance. Winter temperatures enhance the growth of benthic algal species such as *Scenedesmus* species while summer temperatures favour the growth of Chlorophytes such as *Chlorella* species (Mutanda et al. 2011). In tropical zones, water temperature ranges from 10 ℃ to 30 ℃ (Mutanda et al. 2011). Prior research points out that algal species shifts are caused by the presence of grazers in the form of zooplankton and protozoa (Rawat et al. 2013). It has been observed that significant periodic grazing of *Chlorella* species had resulted in anaerobic conditions in ponds (Hoffmann 1998).

Where nutrient availability is concerned, literature revealed that some microalgal species dominate in nutrient-limited environments while others not (Johnson 2010; Li et al. 2010). For instance, Cyanobacteria are well known for their ability to fix atmospheric nitrogen which enables them to survive in low dissolved nitrogen concentrations, hence out competing other algal species (Tilman et al. 1986). On the other hand, a study carried out by Sutherland et al. (2014a) revealed that none of the environmental variables had an impact on the dominant species but rather algal morphology. In their study, *Micractinium pusillum* was found to be the dominant species throughout the study period and the authors attributed this to the presence of spikes which made it difficult to be grazed on (Sutherland et al. 2014a).

Nevertheless, avenues have been explored to select for beneficial algal species and sustain their dominance. Focused on improving harvesting efficiency, Park et al. (2011a) successfully maintained dominance (76 % - 99 % dominance) of readily settleable algal species *Pediastrum* in a pilot-scale wastewater treatment HRAOP using the technique of recycling *Pediastrum* biomass. Another alternative method used is adjusting the hydraulic retention time (HRT), which selects species based on their growth rate (Hoffmann 1998; Park et al. 2011a).

Mab-floc formation relies on adequate mixing (Quijano et al. 2017). Mixing enhances nutrient and gas exchange between microalgae and bacteria (Anderson 2005). Adequate mixing also increases the number of light/dark cycles, thus increasing the growth of the MaB-flocs (Park et al. 2011b; Mambo et al. 2014; Sutherland 2015). Gentle mixing in HRAOPs is facilitated by paddlewheels at velocity range of 0.2 m/s to 0.3 m/s to overcome frictional losses (Anderson 2005). High mixing velocities have been attributed to shear stress in MaB-flocs hence resulting in the breakdown of the structure (Lumar 2015). Rawat et al. (2011) and Chisti (2007) have highlighted that biomass concentrations in wastewater HRAOPs still remain low because of poor mixing with less than 1 g/L being achieved (Juneja et al. 2013). Also, HRAOP design limits sufficient sunlight exposure to photosynthesising algal flocs within the deeper depths of the pond (Acién et al. 2016). Yet still, HRAOPs treating wastewater are rendered most suitable for biomass production because of their low energy requirements and operational costs compared to tubular and flat panel photobioreactors and other conventional wastewater treatment systems (Whitton et al. 2015). The generated wet biomass can be digested anaerobically to produce biomethane or used as a biofertiliser. Another alternative would be drying the biomass from which biochemical substrates such as lipid and carbohydrate preferable for biofuel production can be extracted (Acién et al. 2016). The remainder of the biomass still containing protein and pigments can then be used as an animal or aquatic feed (Johnson 2010). This forms the basic concept of biorefinery (Rawat et al. 2011; Markou and Nerantzis 2013).

1.3 Microalgae biochemical composition

MaB-floc biomass generated from an IAPS primarily consists of 70% to 90% algae, bacteria, fungi, viruses and invertebrates (Craggs et al. 2014). MaB-floc biomass is abundant in algal-based biochemical substrates that mainly include carbohydrates (15.7 kJ/g), lipids (37.6 kJ/g) and proteins (16.7 kJ/g), pigments and vitamins (Williams et al. 2010; Mehrabadi et al. 2015). Microalgae biochemical components are made up of the following elements: carbon, hydrogen, oxygen, nitrogen and phosphorus with an approximate biochemical composition formula of $C_{106}H_{18}O_{45}N_{16}P$ (Mehrabadi et al. 2015). The biochemical composition with respect to lipids, carbohydrates and proteins determines the overall value of biomass as well as the appropriate bioconversion method to utilise (Mata et al. 2010; Pittman et al. 2011; Yen et al. 2013; Kim et al. 2014; Drira et al. 2016; Mehrabadi et al. 2017). Microalgal biochemical composition and energy content varies with species and can be affected by several factors. Table 1.2 below shows the biochemical composition of various microalgal species.

Table 1.2: Biochemical composition in different algal species (Becker 2007)

Algal species	Protein % dry cell weight	Carbohydrate % dry cell weight	Lipid % dry cell weight
Chlamydomonas rheinhardii	48	17	21
Chlorella pyrenoidosa	57	26	2
Chlorella vulgaris	51-58	12-17	14-22
Scenedesmus obliquus	50-56	10-17	12-14
Spyrogyra sp.	6-20	33-64	11-21
Arthrospira maxima	60-71	13-16	6-7
Arthropira platensis	46-63	8-14	4-9
Dunaliella salina	63	15	11

1.3.1 Lipid and carbohydrate accumulation in MaB-floc biomass

Microalgae take up carbon and convert it to its major biochemical components namely carbohydrates, lipids and proteins. Some HRAOP operational parameters such as HRT and organic load may impact on carbon allocation into microalgal intracellular lipid or carbohydrate or protein (Arcila and Buitrón 2017; Peng et al. 2019). Apart from operational parameters, the significant influence in lipid and carbohydrate accumulation in microalgae is stress (Markou et al. 2012). When stress conditions are introduced, microalgae shift their metabolic pattern to counter the altered conditions with a subsequent change in biochemical composition (Hu et al. 2008a; Ho et al. 2013; Markou and Nerantzis 2013; Kim et al. 2014). Kim et al. (2014) highlight that while lipid and carbohydrate accumulation is favoured during the stress conditions, total proteins decrease significantly. Stress factors that enhance carbohydrate or lipid accumulation are divided into nutritional (carbon source, nitrogen, phosphorus) and environmental (light, temperature, pH). Numerous studies have demonstrated this phenomenon as shown in Table 1.3 (Markou et al. 2012; Chen et al. 2013; Benavente-Valdés et al. 2016).

According to Markou et al. (2012), nutrient limitation or deprivation is considered the most affordable approach for the production of microalgae rich in carbohydrate and lipid. Under nutrient limitation, algae cultures adapt to the insufficient supply of the limiting nutrient during nutrient deprivation, exogenous nutrient supply is exhausted and the algae are forced to utilise the endogenous reserves (Dragone et al. 2011; Markou et al. 2012; Markou and Nerantzis 2013). It has been observed that non-oleaginous species divert assimilated carbon into storage as carbohydrates while the oleaginous microalgae divert their biosynthetic pathways in favour of

neutral lipid accumulation which is mainly made up of triacylglycerides (TAGs) (Hu et al. 2008a; Rodolfi et al. 2009).

Table 1.3: Impact of stress factors on microalgal biomass productivity and biochemical composition (Cheng and He 2014).

Microalgae	**Biomass productivity mg/L/d**		**Biochemical content (% of dry cell weight)**		**Environmental stresses**
	Before stress	After stress	Before stress	After stress	
			Carbohydrate		
Arthrospira platensis	193	87	11	67	Phosphorus limitation
Nannochloropsis sp	633	437	8	11	High CO2
Scenedesmus obliquus CNW-N	841	732	38	52	Nitrogen limitation
			Lipid		
Chaetoceros muelleri	70	-	19	36	Silicon limitation
Chlorella vulgaris	138	133	6	15	Nitrogen limitation
Cyclotella cryptica	-	-	18	38	Silicon limitation
Scenedesmus obliquus CNW-N	841	742	12	22	Nitrogen limitation
Scenedesmus sp LX1	37-64	27	23-28	53	Phosphorus limitation

While lipid or carbohydrate accumulation is enhanced in microalgae due to stress conditions, biomass productivity is significantly reduced due to slow growth or halted growth (Dragone et al. 2011; Markou and Nerantzis 2013). Rodolfi et al. (2009) were able to demonstrate the effects of nitrogen deprivation which resulted in the reduction in biomass productivity of 15% and a 60% increase in lipid content of *Nanochloropsis* species.

However, two-stage cultivation alleviates the problem of low biomass productivity while the accumulation of desired metabolites is achieved. In the first stage of culturing, microalgae are exposed to optimum conditions aimed at maximising biomass productivity while the second stage focuses on enhancing the accumulation of metabolites of interest (Markou and Nerantzis 2013; Yen et al. 2013; Benavente-Valdés et al. 2016). Johnson (2010) suggested that biomass productivity and carbohydrate accumulation in an IAPS could be enhanced by running one HRAOP in continuous mode and the other HRAOP in batch mode where the accumulation of

carbohydrate can take place. The latter noted higher biomass productivities in continuous mode compared to batch mode and also that carbohydrate was the major component of the biomass when the HRAOP was operated in batch mode. Similar strategies have been used to accumulate lipid and high-value products in HRAOP operated systems (Huntley and Redalje 2007). In a study by Dragone et al. (2011) using *Chlorella vulgaris*, a two-stage culture strategy was used to investigate the effect of nitrogen and iron deprivation on carbohydrate accumulation. The starch content accumulated reached 41% (dry cell weight), which was 8 times more than the control.

1.3.2 Factors affecting lipid/carbohydrate ratios

Microalgae growth and biochemical composition depend on the following factors: hydraulic retention time (HRT), organic load and flow rate. Among the abiotic factors, light, temperature, CO_2, optimal pH, macronutrients and micronutrients (Mutanda et al. 2011). The above factors play a role in altering carbon fixation and allocation into different types of biochemical molecules (Cheng and He 2014; Benavente-Valdés et al. 2016).

Hydraulic retention time (HRT) affects species composition, which subsequently alters the biochemical composition of MaB-floc biomass (Johnson 2010). A study by Arcila and Buitrón (2016) on the effect of HRT on HRAOP MaB-floc biochemical composition showed an increase in biomass carbohydrate from 14% to 22.1% at an HRT of 10 days under *Scenedesmus* species dominance while no significant change was observed at HRT of 2 days. The lipid content of MaB-floc biomass remained unchanged (9%) on both HRTs that were investigated. The conclusion was that longer HRTs favoured the accumulation of intracellular carbohydrates compared to lipids (Arcila and Buitrón 2016).

Organic load

Sewage waste contains organic matter that is made up of carbohydrates, protein and volatile fatty acids (VFAs). Extensive research focusing on the impact of organic load on the biochemical composition of MaB-floc biomass in HRAOPs is still scarce. Thus far, Peng et al. (2019) demonstrated at laboratory-scale that sewage organic concentration of 302 TOC mg/L and high in carbohydrate induced lipid accumulation in *Chlorella vulgaris* grown under mixotrophic conditions. Findings showed an increase of intracellular lipid content from 27% to 31.4%. Furthermore, Peng et al. (2019) showed that *Chlorella vulgaris* preferentially accumulated carbohydrate up to 30% of the dry cell weight when grown in sewage with an organic concentration of 301 TOC mg/L and rich in protein. Therefore, high organic load-induced

biochemical accumulation, however intracellular carbon allocation was governed by the type of organic carbon.

Temperature

Temperature significantly influences carbohydrate content in microalgae (Cheng and He 2014). For instance, carbohydrate content in *Arthrospira* species increased from 14% to 21% when the temperature was raised to 40 ℃ from an initial of 25 ℃ (Markou et al. 2012; Cheng and He 2014). On the contrary, the diatom *Chaetoceros* species observed high carbohydrate accumulation at lower temperatures (Markou et al. 2012).

The lipid profile is majorly affected by changes in temperature. In various algal species, the ratio of polyunsaturated to saturated fatty acids decreases with an increase in temperature (Juneja et al. 2013). Previous studies had shown that saturated fatty acids of *Monoraphidium* species increased from 30 to 34% (DCW) when the temperature was elevated from 25 ℃ to 35 ℃ (Mehrabadi et al. 2015). It has also been noted that cell fluidity is reduced at low temperatures; hence cells counter this change by increasing the content of polyunsaturated fatty acids to increase fluidity (Hu et al. 2008a; Mehrabadi et al. 2015).

Light

Light is one of the significant factors that impact photosynthesis in microalgae (Markou and Georgakakis 2011; González-Fernández and Ballesteros 2012; Markou et al. 2012; Chen et al. 2013; Cheng and He 2014; Benavente-Valdés et al. 2016).

It has been reported that carbohydrate accumulation is enhanced with increased light intensity. A previous study showed a three-fold (10% to 34% dry cell weight) increase in *Arthrospira maxima* carbohydrate content when irradiance was increased (Markou et al. 2012). High light intensity favours the accumulation of saturated and mono-unsaturated fatty acids. Triacylglyceride (TAG) synthesis consumes twice the amount of reductant NADPH hence making them an effective electron sink (Hu et al. 2008a; González-Fernández and Ballesteros 2012; Mehrabadi et al. 2015).

Carbon

Microalgae can utilise carbon in its various forms that are CO_2, HCO_3^- (bicarbonate) and CO_3^{2-} (carbonate). Microalgae also utilise organic carbon such as acetate, glucose and ethanol by osmotrophy which requires the use of transporters and enzymes to enable uptake as dissolved organic carbon (Lee 2001). Their metabolism is divided into four processes namely

photoautotrophy, heterotrophy, photoheterotrophy and mixotrophy (Markou et al. 2012). Carbon makes up approximately 50% of the organic biomass and growth can become limiting if demand exceeds supply (Sutherland et al. 2015). In HRAOPs treating wastewater, heterotrophic metabolism of organic matter generates CO_2 which contributes 25% to 50% of the dissolved inorganic carbon (DIC) in addition to the inorganic carbon already present in wastewater (Mambo et al. 2014; Mehrabadi et al. 2015).

Microalgal productivity in HRAOPs is carbon limited due to low carbon to nitrogen (C/N) ratio in wastewater (3:1) compared to (6:1) microalgal biomass (Mehrabadi et al. 2015; Sutherland et al. 2015). This means that wastewater contains insufficient carbon for effective nitrogen removal by direct assimilation into algal biomass which in turn lowers algal productivity (Craggs et al. 2014). Carbon limitation is mainly due to pH elevation to above 8.5 (Park et al. 2011b; Craggs et al. 2014; Mehrabadi et al. 2015; Sutherland et al. 2015). pH elevation results in the equilibrium shifting towards the formation of more HCO_3^- and CO_3^{2-} carbon species with a reduction of CO_2. Microalgae preferentially uptake CO_2 over the energy-consuming dissolved inorganic carbon species that additionally require carbon concentrating mechanisms (Park et al. 2011b; Juneja et al. 2013; Sutherland et al. 2015).

CO_2 concentration affects microalgae carbohydrate accumulation (Craggs et al. 2014; Mehrabadi et al. 2015). Studies have shown that a decrease in CO_2 concentration increased the carbohydrate content in algal biomass (González-Fernández and Ballesteros 2012; Markou et al. 2012). The increase is attributed to carbon concentrating mechanisms that are induced at low CO_2 concentrations enabling microalgae to take up dissolved inorganic carbon species from the extracellular environment (Markou et al. 2012). For example, a 2.5 fold increase in carbohydrate content was observed when CO_2 concentration decreased from 3% to 0.04% (Markou et al. 2012).

On the other hand, reports by Mehrabadi et al. (2015) and Cheng and He (2014) concluded that elevated CO_2 concentrations improved fatty acid content due to enhanced de novo fatty acid synthesis. Another study revealed a 30% increase in fatty acid content when CO_2 concentration was raised from 2% to 12% (Mehrabadi et al. 2015).

Nutrients

Nitrogen. Nitrogen is an essential nutrient in the production of algal biomass. Typical wastewater total nitrogen content varies between 20 and 40mg/L (Olguín 2012). The nitrogen content of biomass can range from 1 – 10% depending on the availability and type of nitrogen source

(Markou and Georgakakis, 2011). Microalgae can utilise the following nitrogenous forms: nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) (Chen et al. 2009). Furthermore, some microalgae species (e.g. Cyanobacteria) are "diazotrophic", meaning they can reduce N_2 to NH_4^+ by the use of nitrogenase (Markou and Georgakakis 2011). Algae also preferentially assimilate NH_4^+ to synthesise compounds such as chlorophyll and protein (González-Fernández and Ballesteros 2012). On the other hand, NO_3^- is directly linked to the synthesis of storage compounds (carbohydrate and lipid) (Van Den Hende 2014). However, in the presence of NO_3^-, nitrate reductase reduces NO_3^- to NO_2^- and subsequently to NH_4^+ by nitrite reductase during the active growth phase (Van Den Hende 2014). High NH_4^+ concentration inhibits the uptake of NO_3^- because NH_4^+ represses NO_3^- reductase enzyme, whereas elevated NO_3^- concentrations inhibit NH_4^+ uptake (Markou and Georgakakis 2011).

The availability of various nitrogenous forms is dependent on pH. At pH above 9.5, free ammonia (NH_3) seems to dominate compared to NH_4^+ ion (Markou and Georgakakis 2011; Mambo et al. 2014). High temperatures also favour the formation of free NH_3. Under nitrogen replete conditions, nitrogen form also affects carbon allocation. Photosynthesis is higher with NH_4^+ ion than NO_3^- as a nitrogen source (Benavente-Valdés et al. 2016). Carbon tends to be allocated to proteins with NH_4^+ as a nitrogen source (González-Fernández and Ballesteros, 2012).

Nitrogen deprivation/limitation affects the photosynthetic systems and pigments (Markou et al. 2012; Chen et al. 2013). Photosynthetically fixed carbon is diverted from the protein synthesis pathway to lipid or carbohydrate synthetic pathways (Williams et al. 2010; Dragone et al. 2011). Response to nitrogen deprivation/limitation in microalgae is species dependent. The consensus among scientists is that nitrogen limitation in green algae results in lipid and carbohydrate accumulation (Hu et al. 2008a; Rodolfi et al. 2009; Williams et al. 2010; Markou et al. 2012). Studies related to carbohydrates reported carbohydrate accumulation of 38% and 40% DCW in nitrogen-starved cultures of *Chlorella vulgaris* (Dragone et al. 2011). Another study showed that nitrogen starvation resulted in the accumulation of neutral lipids from starch synthesis (González-Fernández and Ballesteros 2012). Mehrabadi et al. (2015) report that algal lipid content can double or triple under these nitrogen limiting/deficient conditions.

Phosphorus. Phosphorus is an essential macronutrient that takes part in microalgal growth. It is a limiting growth factor, especially in natural environments (Markou and Georgakakis 2011; Benavente-Valdés et al. 2016). In wastewater environments, phosphorus ranges between 1 and 10 mg/L (Olguín 2012). Phosphorus constitutes 1% dry cell weight of algae hence low concentrations

of it are required (Brennan and Owende, 2010; Juneja et al., 2013). However, microalgae tend to take up and store excess phosphorus as polyphosphates which are utilised during phosphate starvation (Markou et al. 2012). Polyphosphates regulate intracellular orthophosphate and enzyme activity (Markou et al. 2012; Benavente-Valdés et al. 2016).

It has been illustrated that phosphate limitation results in carbohydrate accumulation (Markou et al. 2012). The enzyme that is responsible for carbohydrate synthesis is induced by 3-phosphoglycerate and inhibited by inorganic phosphate (Markou and Georgakakis 2011; Markou et al. 2012; Cheng and He 2014). Thus, carbohydrate synthesis is determined by the ratio of 3-phosphoglycerate to intracellular inorganic phosphate. Markou et al. (2012) reported a carbohydrate increase of 63% in *Arthrospira platensis* at low phosphate concentrations.

The effect of phosphorus limitation is also significant in lipid accumulation (González-Fernández and Ballesteros 2012; Markou et al. 2012; Juneja et al. 2013). In some studies, it has been shown that *Scenedesmus* species accumulated lipid content of up to 53% under phosphate-limited conditions (Markou et al. 2012; Juneja et al. 2013; Cheng and He 2014). In terms of biomass, phosphorus concentration is inversely proportional to biomass yields. This is because phosphorus is essential for cellular processes such as synthesis of nucleic acids, phospholipid and energy transfer purposes (Becker 2008). Li et al 2010 reported a lower final biomass cell density of 0.26 x 10^6 cells in *Scenedesmus* sp grown in 0.1 mg/L phosphorus containing media while a higher final cell density of 0.98 x 10^6 cells was observed in *Scenedesmus* sp. grown in growth media containing 2.0 mg/L phosphorus .

1.3.3 Quantification of lipids and carbohydrates

Lipids and carbohydrates form part of the biomass primary energy reserve which can be extracted and valorised into multiple commodity products and various types of biofuel (Zhu et al. 2016). The possibility of finding suitable valorisation pathways lies in finding the most appropriate quantification method that accurately measures biomass lipid and carbohydrate concentrations to employ an efficient and economic valorisation pathway (Johnson 2010).

Various conventional quantification methods such as gravimetric and Nile Red assays for lipids have been reliably used for decades in research due to their simplicity and accuracy (Lu et al. 2008). Similarly, phenol-sulphuric acid and anthrone assays still prove to be popular techniques for carbohydrate analysis (Masuko et al. 2005). However, some of the reagents utilised in the analysis process pose a hazard both to human health and the environment due to their high levels

of toxicity (Mishra et al. 2014). Moreover, these conventional methods require large quantities of both the sample and reagents, have a small detection range, are time-consuming and tedious to carry out especially where a large sample size is being dealt with (Lu et al. 2008). This motivated the development of improved alternative approaches such as GC-MS, HPLC micro-SPV (lipids) and SUV (carbohydrates). While the first two former approaches require minute sample quantities and give accurate results, the labour intensive sample preparation becomes a disadvantage. Meanwhile, apart from requiring small quantities of samples, both the micro-SPV and SA-UV methods have been demonstrated to be rapid and straightforward, hence reducing turn around times and eliminating the handling of toxic reagents (Albalasmeh et al. 2013; Byreddy et al. 2016).

Sulfo-phospho-vanillin (SPV) method is a relatively old and established method that was first introduced in 1937 by Chabrol and Charronat for routine quantification of total lipids in human cerebrospinal fluid (Anschau et al. 2017). Applicability of this method extends to food, serum and aquatic samples (Cheng et al. 2011). A microquantity SPV approach was developed by Van Handel (1985) solely for measuring total lipids from mosquitoes. Approximately 0.05 mL of reconstituted biomass and less than 1mL of reagents are required for a reaction that takes a total of 30 minutes (Cheng et al. 2011). Moreover, this method is versatile enough to exclude the solvent extraction of lipids before spectrophotometric analysis (Mishra et al. 2014).

Concerning carbohydrates, the sulphuric acid-UV (SA-UV) approach is a significant improvement to the popular phenol sulphuric acid (PSA) approach by DuBois. This method is based mainly on the inherent absorption of dehydrated carbohydrates (furfurals) in the UV region (Itagaki 1994). In comparison to the phenol sulphuric acid approach, the sulphuric acid-UV technique omits the colour development step achieved through the addition of phenol, hence reducing the total reaction time and more importantly preventing the handling of phenol (Albalasmeh et al. 2013). Continuous exposure to phenol has been reported to cause skin dermatitis, lung oedema and severe damage to the central nervous system (Van Wychen et al. 2017).

1.3.4 The biofuel potential of wastewater generated MaB-floc biomass

Several studies have demonstrated the capability of microalgae to grow in different types of wastewater (Pittman et al. 2011; Drira et al. 2016). Additional research has also revealed that microalgae growing in wastewater environments also accumulates significant lipid and carbohydrate into their biomass (Bohutskyi et al. 2018a; van den Broek et al. 2018). This energy-rich biomass can potentially be used as a biofuel feedstock. The idea of biofuel from wastewater

algae was first proposed by Oswald & Golueke (1960). From the biomass lipid and carbohydrate extracts, two main types of biofuel can be generated, viz biodiesel and bioethanol (Laurens et al. 2015). Alternatively, biomass in its entirety can be converted through biochemical (anaerobic digestion) and thermochemical (liquefaction, pyrolysis, gasification) means to yield biomethane, biocrude oil and biohydrogen (Driver et al. 2014).

The use of algae biomass as a substrate for bioethanol generation has been viewed as an attractive option in comparison to food crops initially used for bioethanol production (Tijjani-Oshungboye 2011). In addition, algae biomass is rich in cellulose which, upon hydrolysis or saccharification yields fermentable hexose sugars (LewisOscar et al. 2015). Bioethanol production follows two crucial steps before fermentation that is: cell wall decrystallisation which releases stored carbohydrates, followed by saccharification to fermentable sugars. A suitable yeast (*Saccharomyces cerevisiae*) is added to commence the fermentation process which yields bioethanol with an approximate energy value of 31 MJ/kg. According to Balat and Balat (2009), 1 kg of glucose is converted to 0.51 kg of ethanol yield. Based on this equation, an experimental study by Laurens et al. (2015) showed that *Scenedesmus* sp. biomass harvested mid-phase and containing 46% fermentable carbohydrate was capable of yielding of 0.27 L bioethanol/kg biomass. Estimations of bioethanol from algal biomass have been pegged at 0.13 L bioethanol/kg algal biomass (Craggs et al. 2011). This corroborates findings from a study by Tijjani-Oshungboye (2011) which explored the potential of wastewater generated MaB-floc biomass for bioethanol production and revealed inordinately low yields (115 mg bioethanol/g biomass equivalent to 0.146 L bioethanol/kg biomass). This has been attributed to the low availability of fermentable sugars (Craggs et al. 2011). Thus, the selection of a robust biomass pretreatment method to efficiently hydrolyse and extract fermentable sugars becomes an essential aspect of bioethanol production (Laurens et al. 2015; Souza et al. 2017).

Given that algal biomass with lipid content of 30% can give oil yields ten times more than any terrestrial oil crop on an equal area basis, then algae may be the only potential source of biodiesel to displace fossil diesel entirely. Biodiesel is derived from transesterification of lipid triglycerides in the presence of an alcohol group and a strong base to form 3 moles of fatty acid methyl esters (FAME) with an energy content ranging between 39 and 41 kJ/g and a mole of glycerol (Brennan and Owende 2010; Greenwell et al. 2010). Biodiesel conversion efficiency is profoundly impacted by the lipid profile which mainly consists of triacylglycerides and polyunsaturated fatty acids (Hu et al. 2008b; Brennan and Owende 2010). Mehrabadi et al. (2015) reported overall

biodiesel yields ranging between 80% - 90% although the conversion efficiency of triacylglycerides could reach higher than 90%.

Algal biodiesel, just like all other biofuels, has a total net energy ratio (NER) of 0.93, where 0.43 represents feedstock input such as water and nutrients (Sturm and Lamer 2011). Coupling algae-based wastewater treatment and algal biodiesel production would offset the cost of nutrient and water inputs thus giving an overall positive net energy ratio (Lundquist and Oswald 1995). According to Rupiper (2016), giving an example of a 37.85 ML wastewater treatment plant where biomass productivity is approximately 25 $g/m^2/d$, lipid content of 30% and an oil density of 0.88 kg/L, the potential output of biodiesel for that plant would be 4125 L/day. Another case scenario, a wastewater treatment plant treating 45 ML per day, with biomass productivity of 12 $g/m^2/d$ and a biomass lipid content of 10% can potentially output 480 L biodiesel/day (Sturm and Lamer 2011).

Fluctuations in wastewater chemical composition result in wastewater generated biomass forming a unique biochemical composition which comprises of low carbohydrate and lipid while protein and ash tend to be high (Li et al. 2018). Therefore, thermochemical conversion pathways (pyrolysis and HTL) become more useful in ensuring full valorisation of biomass less than ideal. The biomass, either wet or dry for HTL and pyrolysis respectively is subjected to high temperatures of 270 ^oC to 600 ^oC and high pressure (10 to 25 MPa) in which biocrude oil is produced along with char, aqueous and gas co-products (Li et al. 2018). However, HTL is highly preferred over pyrolysis as it can be applied to wet biomass to produce an oil that contains twice the energy content of pyrolysis oil (Kligerman and Bouwer 2015). The past decade has seen the implementation of this technology to largescale demonstration projects from several startup companies. One good example is Aquaflow Bionomic Corporation (now NXT fuels), a startup company that was able to produce biocrude oil from wastewater grown algae through its patented processes (www.biomssmagazine.com; www.patents.justia.com).

Anaerobic digestion is a well-established simple technology that has been successfully implemented in wastewater treatment setups. The process entails bacterial conversion of organic material (in this case algal biomass) into biogas (biomethane, CO_2 and biohydrogen) at temperatures ranging between ambient to 55 ℃ in the absence of oxygen (Heaven et al. 2011; Mehrabadi et al. 2015). Apart from biogas, this technology also yields useful co-products that can be used as bio-fertiliser, thus offering greater sustainability benefits through the recycling of crucial nutrients. Biogas output may be improved by employing effective pretreatment strategies

(chemical and/or physical) to increase the biodegradability of the biomass (Tijjani-Oshungboye 2011; Passos et al. 2015). A theoretical estimation of methane yields based on algal biochemical ratios indicates that lipids give the highest yield, followed by protein and lastly carbohydrates (Heaven et al. 2011). Methane yields could be maximised byco-digesting high C/N ratio biomass with high carbon-containing material such as wastepaper (Yen and Brune 2007). Furthermore, co-digestion has been shown to lower the rate of ammonia release that could otherwise compromise anaerobic bacterial performance (Mehrabadi et al. 2015). Current methane yields from wastewater cultivated biomass range between 0.10 - 0.30 L methane/g VSS (Passos et al. 2015).

1.4 Rationale

IAPS has been demonstrated to be a robust wastewater treatment system achieving 99.99% disinfection with COD (400 to 600 mg/L), nitrogen (80 mg/L) and phosphate (50 mg/L) removals of 87%, 55% to 90% and 50% to 76% respectively (Banat et al. 1990). In addition to bioremediation, IAPS generates abundant MaB-floc biomass from which various biofuel product streams can be formed. The concept design of this system also indicates that native mixed algal species in HRAOPs offer a more resilient type of system that can be optimised to accumulate more of lipid and or carbohydrate through nutrient limitation/starvation strategies (Whitton et al. 2015). This study aimed to determine the impact of IAPS operation with the standard configuration on MaB-floc biomass carbohydrate and lipid accumulation in a HRAOP treating domestic wastewater and evaluate whether the biomass is a suitable feedstock for biofuel production.

Hypothesis

MaB-floc biomass generated in HRAOPs of IAPS used to treat wastewater can be induced to accumulate either lipid or carbohydrate to derive bespoke feedstock for biofuel production.

Specific objectives

1. Quantify MaB-floc biomass from HRAOPs treating domestic wastewater during winter and summer and determine productivities for each season. Isolate and identify native microalgal strains from HRAOPs with the potential to accumulate either lipid or carbohydrate.
2. Evaluate methods for estimating carbohydrate and lipid concentration of MaB-floc biomass and determine the effect of nitrate starvation on carbohydrate and lipid concentration using isolated native species.
3. Determine the theoretical biofuel potential of MaB-floc biomass based on biochemical composition for each season.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Apparatus

EC Testr 11 dual range conductivity meter, Beckman Coulter Avante-JE centrifuge (J-20 rotor), VIS-TIR Benchtop SLC benchtop freeze drier, Elementar Vario MICRO Cube CHNS analyser, filter discs (Whatman GF/C, pore size 0.45 µm), vacuum filtration apparatus, aluminium pans, Shimadzu UV mini 1240 spectrophotometer, Accublock dry heat bath thermo-block, Zeiss Axiostar plus light microscope, Labnet prism mini centrifuge, UVP BioDoc-It™ UV visualiser, Veriti 96 well thermocycler.

2.1.2 Reagents and chemicals

Proteinase K, TNES buffer, 99% ethanol, 70% ethanol, 5 M NaCl, Cleaver™ all-purpose agarose, PCR-grade water, vanadium pentoxide, concentrated sulphuric acid, phospho-vanillin reagent, chloroform, methanol, 0.5% calcium chloride, 5% phenol solution, 1.5% BBM media, 0.2 mg/mL glucose stock solution, 2mg/mL canola oil, Merck analysis kits (NO_3^--N, NH_4^+-N, PO_4^{2-}-P), HotStar taq® master mix kit, AccuPol™ DNA polymerase kit, 0.2 mM dNTPs, ammonium buffer, TBE buffer (1 X), 10 mg/mL ethidium bromide, FavorPrep™ gel purification mini kit.

2.2 Integrated algal pond system configuration and operation

The Belmont Valley IAPS pilot plant is located at the Institute for Environmental Biotechnology Rhodes University (EBRU), Makhanda (33° 19’ 07” South, 26° 33’ 25” East), South Africa. IAPS operates as a continuous system consisting of an 840 m^2 AFP which incorporates an AD (50 m^2), two 500 m^2 HRAOPs and two 12.5 m^2 ASPs. The effluent treatment capacity for IAPS is 75 m^3/day. Raw domestic effluent is introduced into the system via the AD where anaerobic digestion of biosolids takes place. The HRTs in the AD and AFP are 20 and 3 d respectively. Effluent (75 m^3) from the AFP flows to HRAOP A in which constant mixing occurs at a retention time of 2 d by a motor (0.25 kW) driven paddle wheel at a velocity of 0.3 m/s. HRAOP A effluent flows to ASP A where half of the effluent (37.5 m^3) is channelled to HRAOP B after settling for 0.5 d. Effluent from HRAOP B which has a retention time of 4 d, flows to ASP B for another 0.5 d of settling to recover biomass slurry concentrated to 2-3%. The slurry is pumped out to drying beds. Treated effluent is discharged back to the Belmont Valley Wastewater Treatment Works. The present study focuses on HRAOP B (Figure 2.2) from which sampling took place in winter and

summer.

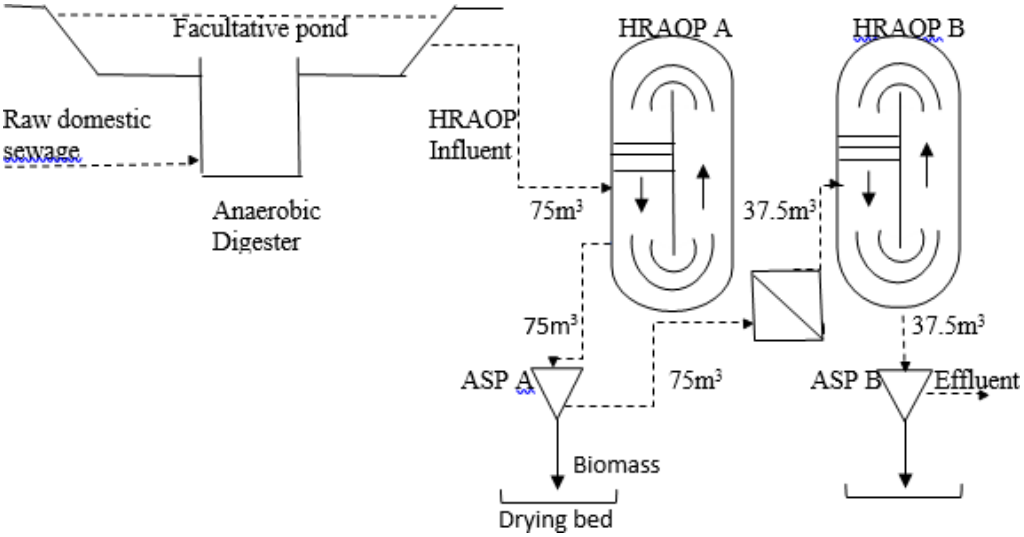

Figure 2.1 Configuration and flow diagram of the Belmont Valley pilot-scale IAPS. HRAOP= High rate algal oxidation pond; ASP= Algal settling pond (Cowan and Laubscher 2010).

2.3 Sampling routine and MaB-floc biomass collection

The sampling point was just upstream before the paddlewheel, as shown in Figure 2.2. The temperature of HRAOP water was measured in situ using an EC Testr 11 dual-range (Eutech, Singapore). Weather data was obtained from Weather Rhodes throughout sampling (weatherrhodes.ru.ac.za). Sample collection was carried out twice daily in the morning and afternoon or once every week in the morning. Biomass recovery was achieved by centrifugation of MLSS at 15000 x g for 15 min using a Beckman Coulter Avante-JE centrifuge (J-20 rotor). After centrifugation, the supernatant was discarded, and the biomass lyophilised using a VIS-TIR Benchtop SLC benchtop freeze drier (SP Industries, USA).

Figure 2. 2: HRAOP B at EBRU in Belmont Valley. The sampling point is labelled A.

2.4 Metagenomic analysis of MaB-floc biomass

2.4.1 DNA extraction

DNA from MaB floc biomass was extracted using the salting-out method according to Sunnucks and Hales (1996). MaB-floc biomass (5 mg) weighed out into a 1.5 mL Eppendorf tube was suspended in a solution of 25 µL Proteinase K and 600 µL of TNES buffer consisting of 50 mM Tris pH 7.5, 400 mM NaCl, 20 mM EDTA and 0.5% SDS. The suspension was incubated in a shaker incubator at 37 ^{o}C overnight to facilitate digestion. Duplicate sets of 1.5 mL tubes were set up and 700 µL of cold 99% (v/v) ethanol added into each of them before freezing. Upon digestion of MaB-floc biomass, 240 µL of 5 M NaCl was added and the mixture vortexed on a vortex mixer (Labnet International, Inc) for 15 s to precipitate out proteins. The MaB-floc biomass NaCl mixture was then centrifuged in a Labnet prism mini centrifuge (Labnet, International, Inc) at 21 200 x g for 10 min to pellet the proteins. After centrifugation, the supernatant was carefully pipetted out and transferred into a tube containing the cold ethanol (from the freezer). The supernatant ethanol mixture was centrifuged at 21 200 x g for 8 min to pellet the DNA and the ethanol discarded. The DNA pellet was rinsed using 500 µL of 70% ethanol and agitating gently, followed by centrifugation at 21 200 x g for 5 min. The ethanol was discarded and the DNA pellet dried using an AccuBlock digital dry bath heating block (Labnet International, USA) at 56 ℃ for 5 min. The dried DNA pellet was resuspended in 100 µL of DNA elution buffer and stored overnight at 4℃. To determine the quality of the DNA, gel electrophoresis was carried out on 1% (w/v) Cleaver™ all-purpose agarose gel using TBE buffer (1 X) containing 10 mg/mL ethidium bromide. The DNA samples were electrophoresed at 100 V and 5 A for 30 min and visualised under UV light with a UVP BioDoc-It™.

2.4.2 18S and 16S PCR

DNA extracted from MaB-floc biomass was PCR- amplified using 18S and 16S universal primers. Universal primer pairs 1391f (5’-GTACACACCGCCCGTC-3’) and EukBr (5′-TGATCCTTCTGCAGGTTCACCTAC-3′) were used to amplify the V9 region of the 18S rRNA gene. For 16S amplification, primer pairs 515f (5’-GTGYCAGCMGCCGCGGTAA-3’) and 926r (5’-CCGYCAATTYMTTTRAGTTT-3’) were used to amplify the V4 and V5 regions of the 16S rRNA gene (www.earthmicrobiome.org). The regions were amplified from 5 µL of whole genomic DNA extracted from MaB-floc biomass using either 13.5 µL HotStar taq® master mix (Qiagen, catalogue number 203446) or 0.8 µL AccuPol™ DNA polymerase (Ampliqon, catalogue number

A210002), PCR-grade water, 2.5 µL of 1 X ammonium buffer and 0.7 µL of 0.2 mM of each dNTP to make up a 25 µL PCR reaction. PCR was performed using a Veriti 96 well thermocycler (Applied Biosystems, USA) and conditions for amplification of the 18S rRNA V9 region were as follows: 90 ℃ for 15 min, 35 cycles of 94 ℃ for 1 min, 60 ℃ for 1 min, 72 ℃ for 1 min and a final step of 72 ℃ for 7 min. Amplification conditions for 16S rRNA V4 and V5 regions were as follows: 1 cycle of 98 ℃ for 5 min, 5 cycles of 98 ℃ for 45 s, 44 ℃ for 30 s, 72 ℃ for 1 min, 20 cycles of 98 ℃ for 45 s, 50 ºC for 30 s, 72 ºC for 45 s and 1 cycle of the final step at 72 ℃ for 5 min. To determine successful amplification, amplicons were visualised under UV light on 1% (w/v) Cleaver™ all-purpose agarose gel using a UVP BioDoc-It™. The sizes of the amplicons were estimated by comparison to a DNA marker (Kappa express ladder, 100 bp).

2.4.3 Gel purification of PCR products

Following PCR and visualisation, a portion of the agarose gel containing amplification products was cut out and purified using a FavorPrep™ gel purification mini kit (Favorgen, catalog number FAGPK 001-2). Briefly, 200 mg of the gel slices were transferred to a 1.5 mL Eppendorf tube. A volume of 600 µL of FAGP buffer was added to each sample and mixed by vortexing. The sample mixtures were incubated at 55℃ using an AccuBlock dry bath heating block for 5 min and mixing at intervals until the gel slice dissolved completely. Following incubation, the sample mixtures were cooled to room temperature. A volume of 750 µL of each sample mixture was transferred to an FAGP column attached to a collection tube and this was centrifuged at 11 000 x g for 30 s. The flow-through in the collection tube was discarded. Centrifugation was repeated at 18 000 x g for an additional 3 min to dry the column matrix. The FAGP columns containing the amplification products were then placed in elution tubes. This was followed by the addition of 40 µL of elution buffer and centrifugation at 18 000 x g for 1 min to elute the purified amplification products. Purified amplification products were visualised under ultraviolet light on 1% (w/v) Cleaver™ all-purpose agarose gel using a UVP BioDoc-It™. The sizes of the amplification products were estimated by comparison to a DNA marker (Kappa express ladder, 100 base pairs) (Appendix B1). The samples were sent to SAIAB Molecular laboratory for shot-gun metagenomic analysis.

2.4.4 Analysis of sequence reads using Mothur software

Analysis of sequence reads generated from shot-gun metagenomics was performed using Mothur software package version 1.41.3 (Schloss et al. 2009).

2.5 Elemental analysis of MaB-floc biomass

Harvested HRAOP biomass was analysed for elemental content (CHNS) using an Elementar Vario Micro Cube CHNS analyser (Elementar Americas, USA). The lyophilised biomass was ground into a fine powder using a pestle and mortar. A portion of the ground biomass (2 mg) was mixed with an oxidising agent vanadium pentoxide (V_2O_5) before combustion at 1000℃. The combustion gas products (N_2, NO_x, CO_2, SO_2 and H_2O) are carried by helium (carrier gas) through a copper filled column where NO_x is reduced to elemental nitrogen while H_2O is absorbed in a separate column. The gases are separated through a programmed temperature raise system within the temperature-programmed desorption (TPD) column and detected by a thermal detector that gives off an electrical signal proportional to concentrations of C, H, N and S expressed as a molar weight percentage.

2.6 MaB-floc biomass concentration and productivity measurements

Biomass concentration was analysed according to Standard methods for the examination of water and wastewater (APHA 1998). A 20 mL aliquot of mixed liquor suspended solids (MLSS) was filtered through pre-dried and pre-weighed filter discs (Whatman GF/C, pore size 0.45 µm, Merck, South Africa) using the vacuum filtration apparatus. The filters were oven-dried at 105 ℃ overnight while the filtrate was retained for chemical analyses. Dried filter discs containing biomass were cooled to room temperature in a desiccator before being reweighed. Biomass concentration expressed as mg MLSS/L was calculated as follows:

$$\frac{(MF-MI)}{V} \times 1000 \qquad \textbf{Equation 2.1}$$

Where MF denotes the weight (mg) of dried filter disc and biomass, MI is the weight (mg) of the pre-dried filter disc before filtering and V is the volume (mL) of MLSS filtered.

The areal productivity of HRAOP in $g/m^2/d$ was calculated from the MLSS concentrations using Equation 2.2 (Al-Shayji et al., 1994).

$$P = pd/t \times r \times MLSS \qquad \textbf{Equation 2.2}$$

where P is pond productivity ($g/m^2/d$), pd represents pond depth (m), t denotes hydraulic retention time of the pond in days (d), MLSS is total mixed liquor suspended solids (mg) and r is the algae ratio in the MLSS (0.9-1.0) as estimated by Al-Shayji et al. (1994).

2.7 Settling efficiency

Settling efficiency was carried by drawing out 5 mL of liquor from the halfway point of the Imhoff cone and optical density measured using a spectrophotometer. Efficiency was then calculated according to the equation described by (Guo et al. 2013).

$$settling\ efficiency\ \% = \frac{A-B}{A} \times 100\% \quad \textbf{Equation 2.3}$$

where A and B are the optical density (OD_{680}) of algal culture at initial time 0 and the time interval, respectively.

2.8 Chemical analyses

MLSS filtrate nutrients were measured using test kits: NO_3^- -N (catalog number 1.14773.0001), $NH4^+$-N (catalog number 1.14752.0001) and PO_4^{2-}-P (catalog number 1.14848.0001) according to the manufacturer's instructions (Merck, Chem. Co., Germany).

Calculation of percentage removals for nitrogen (NO_3^- -N and NH_4^+-N) was done according to equation 2.2 below (Spellman 2013).

$$\%\ \text{Nitrogen Removal} = \frac{[\text{Influent Concentration} - \text{Effluent Concentration}]}{\text{Influent Concentration}} \times 100 \quad \textbf{Equation 2.4}$$

2.9 Biochemical analyses

2.9.1 Determining the accuracy of micro- sulpho-phospho-vanillin and sulphuric acid ultraviolet methods using standards

Accuracy of the micro-sulpho-phospho-vanillin and sulphuric acid-ultraviolet methods was assessed and compared to conventional gravimetric and phenol sulphuric acid methods respectively. For this purpose, varying concentrations of standards were used. Results were reported as percentage mean recovery, which was calculated as follows:

$$\%\ \text{recovery} = 100 \times \frac{\mathbf{CM}}{\mathrm{CP}} \quad \textbf{Equation 2.5}$$

where CM denotes the concentration of the measured standard and CP represents the concentration of the prepared standard.

2.9.2 Lipid quantification from MaB-floc biomass

MaB-floc biomass for lipid quantification was prepared according to Mishra et al. (2014). Briefly, 5 mg of lyophilised biomass was suspended in 1 mL distilled water in a test tube, followed by

transferring an aliquot of 0.1 mL to a new test tube to give a final amount of 0.5 mg biomass. Biomass lipids were quantified using a modified micro-sulpho-phospho-vanillin method described by Cheng et al. (2011). A total volume of 0.5 mL of concentrated sulphuric acid (Sigma 95-97% Puriss ACS grade) was added to the 0.1 mL aliquot of suspended biomass. The mixture was incubated at 90 ℃ for 10 min and allowed to cool to room temperature. Upon cooling, 0.5 mL of phospho-vanillin reagent (0.2 mg vanillin/mL 17% phosphoric acid) was added to the mixture, gently mixed and left for 15 min to allow for colour development. The absorbance of the samples was measured at 540 nm (Shimadzu UV mini 1240 spectrophotometer) against a blank prepared with distilled water and phospho-vanillin reagent. Lipid yield in micrograms was determined by interpolation from a standard curve prepared using known amounts from canola oil dissolved in chloroform to make up 2 mg/ml stock solution (Appendix Figure A6). The lipid yield was expressed as a percentage of the biomass dry weight.

Lipid content measured using the micro-sulpho-phospho-vanillin method was compared with the gravimetric method. Lipid extraction and purification were done according to Folch et al. (1957) as described by Tijjani-Oshungboye (2011). Chloroform:methanol in the v/v ratio of 2:1 (1.5 mL) was added to a pre-weighed 5 mg sample of lyophilised biomass in a test-tube, followed by vortexing and centrifugation at 4000 x g for 5 min. The lipid-chloroform (lower) phase was collected. The extraction procedure was repeated three times and the collected lipid-chloroform phases combined followed by washing with 0.5% calcium chloride (0.2 volumes of chloroform phase) to purify the lipid phase. The mixture was centrifuged at 4000 x g for 5 min to separate the phases. After noting the volume of the lipid chloroform phase, 2 mL aliquots were transferred into pre-weighed aluminium pans. The aluminium pans were dried at 40 ºC overnight to evaporate the solvent and then reweighed. Total lipids were expressed as a percentage of dry biomass calculated as follows:

$$\% \text{ Total lipid} = \frac{(A1 - A_2)\, V_c}{V_s M_a} \times 100 \qquad \textbf{Equation 2.6}$$

where A_1 is the mass of lipid (mg) and pan after drying, A_2 is the mass of the empty pan (mg), V_C is the volume (mL) of the lipid-chloroform phase after calcium chloride washing and centrifugation, V_S is the volume (mL) of lipid-chloroform aliquot, M_a is the dry mass of biomass (mg)

2.9.3 Carbohydrate quantification from MaB-floc biomass

MaB floc biomass hydrolysis followed a method described by Silkina et al. (2015). Briefly, lyophilised biomass (5 mg) was first hydrolysed in 1M concentrated sulphuric acid (1 mL) at 100 ^{o}C for an hour using a thermo-block. Carbohydrate content in the biomass was measured using the sulphuric acid- ultraviolet method, according to Albalasmeh et al. (2013). Upon cooling, 3 mL of concentrated sulphuric acid was added to an aliquot of the hydrolysate (0.025 mL made up to 1 mL with distilled water). The mixture was gently mixed and cooled to room temperature. Absorbance was read at 315 nm using a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Japan) against a blank prepared with 1 mL distilled water and concentrated sulphuric acid. Carbohydrate content was determined through interpolation from a standard curve using a series of known concentrations of glucose (0.2 mg/mL) stock solution and expressed as a percentage of dry biomass (Appendix Figure A5).

Carbohydrate content assayed using the sulphuric acid- ultraviolet method was compared with the phenol-sulphuric acid method by DuBois (1956). An aliquot of the hydrolysate (0.05 mL diluted to 0.5 mL with distilled water) was added to 5% phenol solution (0.5 mL) followed by the addition of 2.5 mL concentrated sulphuric acid. The mixture was vortexed and left to cool to room temperature and allow for colour development. Absorbance was measured at 490 nm using a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Japan). A blank was prepared by adding phenol and concentrated sulphuric acid to distilled water. Carbohydrate concentration was determined through the interpolation from a standard curve prepared using 0.2 mg/mL glucose stock solution (Appendix Figure A4).

2.9.4 Protein analysis

Lyophilised biomass (5 mg) was first hydrolysed in 1M concentrated sulphuric acid (1 mL) at 100 ^{o}C for an hour using a thermo-block (Silkina et al. 2015). A procedure adapted from Bradford measured the protein fraction of MaB-floc biomass. Bradford reagent (1.5 mL) was added to an aliquot of the hydrolysate (0.025 mL made up to 0.25 mL with distilled water). The mixture was gently mixed and left for 5 minutes to allow colour development. Absorbance was measured at 595 nm using a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Japan). A blank was prepared by adding Bradford reagent to distilled water. Protein content was determined through interpolation from a standard curve using a series of known concentrations of bovine

serum albumin (BSA) (2 mg/mL) stock solution and expressed as a percentage of dry biomass (Appendix Figure A7).

2.10 Calculations for theoretical outputs of methane, bioethanol and biodiesel from HRAOP generated MaB-floc biomass

Theoretical methane yields for the biomass was calculated based on biomass biochemical composition using equation 2.7, according to Choudhary et al. (2016) as follows:

$$\text{Theoretical methane potential} = \frac{1}{100}(A \times C_L + B \times C_c + C \times C_P) \qquad \textbf{Equation 2.7}$$

where A, B and C are the % dry weight contents of lipids, carbohydrate and protein respectively while C_L, Cc and Cp represent the specific methane yields for lipids (1.014 L CH_4/g VS), carbohydrates (0.415 L CH_4/g VS) and proteins (0.851 L CH_4/g VS).

Ethanol theoretical yields were calculated (equation 2.8) assuming all the cellulosic and starch material were hydrolysed to glucose which is further converted through fermentation to give a 51% theoretical ethanol fermentation yield (Chin and Hng 2013).

$$\text{Theoretical ethanol yield (L/kg)} = \frac{X \times 0.51}{0.789} \qquad \textbf{Equation 2.8}$$

where X represents the mass of glucose in kg, 0.51 is the theoretical ethanol yield (kg) and 0.789 is the density of ethanol in kg/L.

Theoretical yields of biodiesel from algal biomass were calculated (equation 2.9) based on the assumption that there were no losses during lipid extraction and transesterification to yield 80% biodiesel (Chisti 2007).

$$\text{Theoretical biodiesel yields (L/kg)} = \frac{Y \times 0.78}{0.864} \qquad \textbf{Equation 2.9}$$

where Y denotes the mass of neutral lipids (kg),

0.8 = biodiesel theoretical yield in kg,

0.864 = density of algal biodiesel in kg/L (www.oilgae.com).

2.11 Experimental work: Effect of lipid and carbohydrate accumulation induced by nitrate starvation in *Scenedesmus* sp. and *Chlorella* sp.

2.11.1 Isolation of microalgae species from HRAOP

Microalgae were isolated from the HRAOP by spread-plating 0.2 mL of MLSS onto 1.5% BBM solid media prepared by dissolving 15 g Agar-agar in 1 L BBM. The culture plates were incubated under constant environment (25 ℃; 12:12 light: dark cycle; light intensity 70 - 90 µmol/m/s) until growth was observed. Following growth, single colonies were aseptically transferred into 100 mL conical flasks containing 50 mL sterilised BBM. The inoculated flasks were kept under constant environment on a shaker at 120 pm. Sub-culturing was carried out every 14 d with 10% (v/v) inoculum until unialgal cultures were obtained. The cultures were viewed under a Zeiss Axiostar plus light microscope (Carl Zeiss Jena, Germany), where they were identified to the genus level using identification keys for freshwater algae (Prescott 1970).

2.11.2 Effect of Nitrate starvation on growth, lipid and carbohydrate accumulation in *Scenedesmus* sp. and *Chlorella* sp.

A 10% (v/v) inoculum of two distinct unialgal cultures (*Chlorella* sp. and *Scenedesmus* sp.) were inoculated in nitrate-free BBM media and one containing $NaNO_3$ (7 mg/L) to determine the effects of nitrate starvation on growth, lipid and carbohydrate accumulation. The cultures were cultivated in a constant environment for 8 d. Growth measurements were carried out every 2 d by measuring absorbance at 760 nanometers and the result interpolated from standard curves of absorbance versus biomass concentration generated for each unialgal culture. Lipid and carbohydrate yields were measured every 2 d using the micro-sulpho-phospho-vanillin and sulphuric acid-ultraviolet methods, respectively, as described in section 2.9.

2.12 Statistical analysis

Mean values and standard error were computed using Microsoft Office Excel 2013. All statistical analyses were carried out using Sigma Plot software version 11. A t-test (alpha level 0.05) was used to determine the level of significance between the mean values of different data sets. Pearson's correlation test was used to test the relationships between the biochemical variables and the environmental factors.

Chapter 3: Generation of MaB-floc biomass in high rate algal oxidation ponds of an IAPS treating municipal sewage

3.1 Introduction

The primary function of wastewater treatment is the removal of nutrients to acceptable discharge limits which consequently reduces nutrient recycling into receiving water bodies (Banat et al. 1990). Due to more stringent effluent discharge limits, there has been a growing need for robust yet cost-effective wastewater treatment technologies (Mambo et al. 2014). IAPS being one of them is a wastewater treatment technology that integrates algal photosynthesis and bacterial aerobic processes to break down and extract nutrients from wastewater. The end results are reusable treated effluent and readily settleable biomass that can be valorised into several product streams (Cowan et al. 2016).

HRAOPs are a component of an IAPS and support the growth of microalgal, bacterial, ciliates fungal and zooplankton. HRAOPs are characterised by shallow depths ranging between 0.3 m to 0.6 metres and paddlewheel mixing at velocities of 0.1 metres/second to 0.3 metres/second (Green et al. 1995). Continuous gentle mixing serves to keep microalgae in suspension; enhance between microalgae and bacteria while enhancing their aggregation to form MaB-flocs or mixed liquor suspended solids (MLSS). MLSS is the biomass component typically produced in activated sludge systems. Similarly, HRAOPs serve as an aeration basin that enhances nutrient exchange and subsequent growth of microalgal and bacterial populations through symbiotic interactions. This interaction together with gentle paddlewheel mixing and EPS production stimulates the formation of readily settleable aggregates known as MaB-flocs or MLSS (van Den Hende 2014; Green et al. 1995; Cowan et al. 2016). A readily settleable MaB-floc biomass enhances harvestability and thus lowers harvesting costs which already contribute as much as 50% of biofuel production costs (Craggs et al. 2011). Additionally, presence of large-sized microalgal species such as *Pediastrum* sp. and *Micractinium* sp. are characteristic of good settling biomass (Park et al. 2011a; Craggs et al. 2014).

Since HRAOP technology heavily relies on natural processes governed by weather dynamics within the environment, performance, as well as MaB-floc biomass output, are bound to be inconsistent (Sutherland et al. 2014b; Sutherland et al. 2018). This may subsequently affect biofuel quality and quantity; hence it becomes imperative to establish an appropriate harvesting period during which biomass could be capitalised the most (Wendt et al. 2019).

This chapter gives a summary of the results on HRAOP nutrient removal efficiency, biomass concentration, biomass productivity, biomass settling efficiency and biomass species composition obtained during pond monitoring in winter and summer.

3.2 Total nitrogen and phosphorus removal in HRAOPs during winter and summer

Pond water influent and the pond water effluent were sampled weekly for ammonium, nitrate and phosphorus concentrations. Total nitrogen was calculated ammonium and nitrate, which were the primary nitrogen forms in the pond water. Nitrogen and phosphorus removal efficiencies in the HRAOP were then calculated from their respective total nitrogen and phosphorus concentration values and the results are summarised in Table 3.1.

Table 3.1 Seasonal averages of HRAOP nutrient removals during winter (June 2018 to August 2018) and summer (November 2018, January 2019 and February 2019). Data are presented as the mean ± SE of samples collected between June 2018 and February 2019. Numbers in the parenthesis represent the number of data used to generate the means.

Season	Total Nitrogen Removal (% influent pond water nitrogen)	P Removal (% influent pond Water phosphorus)
Winter	68 ± 2.3 (10)	70 ± 3.1 (10)
Summer	71 ± 2.9 (8)	75 ± 1.7 (8)

Throughout the study, on average, 68% of the sewage total nitrogen was removed in the HRAOP during winter while 71% total nitrogen removal was achieved in summer. There was no significant difference ($p > 0.05$) in total nitrogen removal in winter and summer. Average phosphorus removal efficiencies attained in HRAOPs in winter and summer were 70% and 75% respectively.

3.3 HRAOP conditions and MaB-floc production during winter and summer

Pond monitoring was carried out during winter months June, July and August of 2018 while summer monitoring was conducted in November 2018, January and February of 2019. Figure 3.1a shows water temperature and solar radiation measured during sampling. Water temperatures in winter varied between 8 ℃ (July 2018) and 20 ℃ (July 2018). On the other hand, summer water temperatures remained low during the beginning of the summer sampling season in November 2018 with the lowest reaching 8℃. Gradually, temperatures increased in January and February with a temperature of 27 ℃ being the maximum reached during the season. Daily Solar radiation during winter was between 0.25 W/m^2 to 250 W/m^2 while summer solar ration ranged between 0.34 W/m^2 and 500 W/m^2.

Figure 3.1b shows the dynamics in MaB-floc biomass concentration during the winter and summer expressed as dry weight MLSS. MaB-floc biomass concentration was influenced by changes in water temperature and solar radiation with the outcome being low Mab-floc biomass concentrations during winter ranging between 99 mg MLSS/L and 198 mg MLSS/L and respective productivities of 7.4 $g/m^2/d$ and 13.9 $g/m^2/d$. As water temperature increased during summer, higher MaB-floc biomass concentrations were observed with a range between 85 mg MLSS/L and 377 mg MLSS/L and corresponding productivities of 6.4 ± 0.9 $g/m^2/d$ and 28.3 ± 1.6 $g/m^2/d$ respectively. Average biomass productivities for summer and winter were 16.5 $g/m^2/d$ and 9.4 $g/m^2/d$ respectively. However, in January, the HRAOP used in this study experienced a mechanical failure which disrupted the regular inflow of influent. Increased evaporation rates due to high temperatures resulted in increased biomass concentrations of 492 mg MLSS/L and 457 mg MLSS/L.

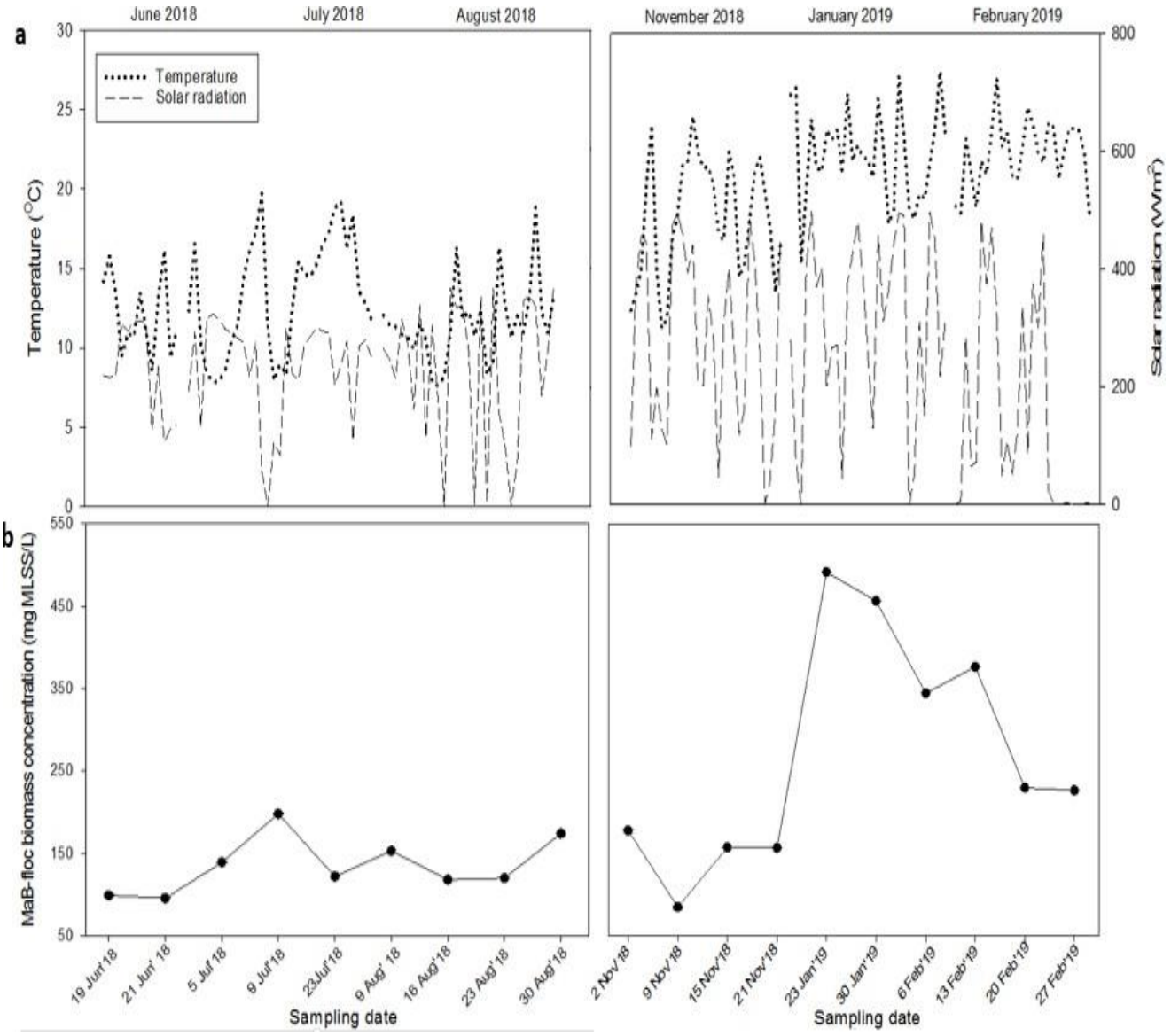

Figure 3. 1: a) Changes in water temperature and solar radiation and b) MaB-floc biomass concentration in HRAOPs of an IAPS treating municipal sewage. Data were collected from June 2018 till February 2019 and are presented as the mean ± SE.

3.4 Diurnal variations MaB-floc biomass concentrations in HRAOPs

MaB-floc biomass concentration was monitored in the morning and afternoon to observe any changes effected by diurnal water temperature variations. Average morning water temperatures in winter ranged between 9 ℃ and 17 ℃, while afternoon water temperatures were in the range of 12 ℃ and 19℃. Summer morning water temperatures from the end of January till the end of February ranged between 18 ℃ and 23 ℃. The respective afternoon water temperatures ranged between 25 ℃ and 28 ℃. Diurnal changes in MaB-floc biomass concentration and corresponding productivities were significant (t-test, $p < 0.05$).

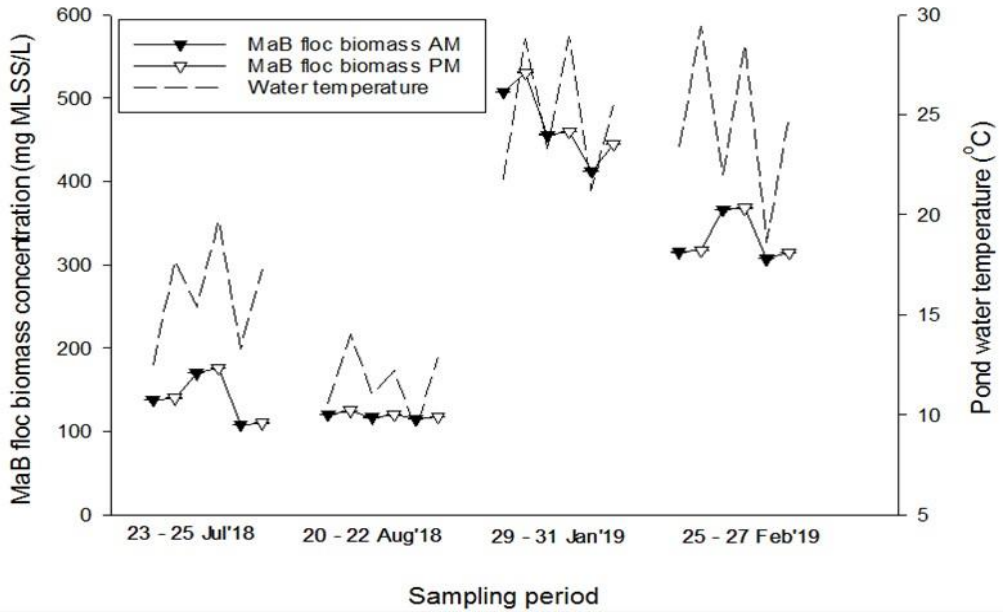

Figure 3.2: Diurnal changes in water temperature and MaB-floc biomass concentration in HRAOPs treating municipal sewage. Data were collected from July 2018 till February 2019 and are presented as the mean ± SE.

3.5 Settleability and microbial diversity of MaB-flocs generated in HRAOPs

To ensure that the biomass generated from a HRAOP of an IAPS is harvestable, settleability efficiencies were determined. A settleability test showed that the HRAOP generated biomass readily settled within an hour as illustrated in Figure 3.3a. Good settleability was attained during summer with a settling efficiency of 82 ± 0.9%. Light microscopy analysis showed that the predominating species within the flocs generated in summer included *Pediastrum sp.*, *Scenedesmus* sp. and *Chlorella* sp. (Figure 3.3c). A low average settling efficiency of 37 ± 1% was recorded in winter and microscopic analysis indicated a floc mainly consisting of *Scenedesmus* sp. as shown in Figure 3.3d. Small flocs were also observed during winter and this was coincidental with low nutrient concentrations. A metagenomic study of the biomass produced in both seasons gave further identification and ratios of predominating microorganisms. *Desmodesmus* sp. was the most abundant algal genus within MaB-flocs generated in winter (Figure 3.5). *Chlorella* sp. and *Scenedesmus* sp. also formed part of the biomass. MaB-flocs generated in summer had *Pediastrum* sp.and *Scenedesmus* sp. as the dominant genera within the Chlorophyta. Fungi belonging to the genus *Purpureocilium* were also abundant within the summer generated floc collected from HRAOPs (Figure 3.4). Rotifers namely *Brachionus* also dominated in HRAOP MLSS during summer constituting 34.5% of the biomass (Figure 3.4).

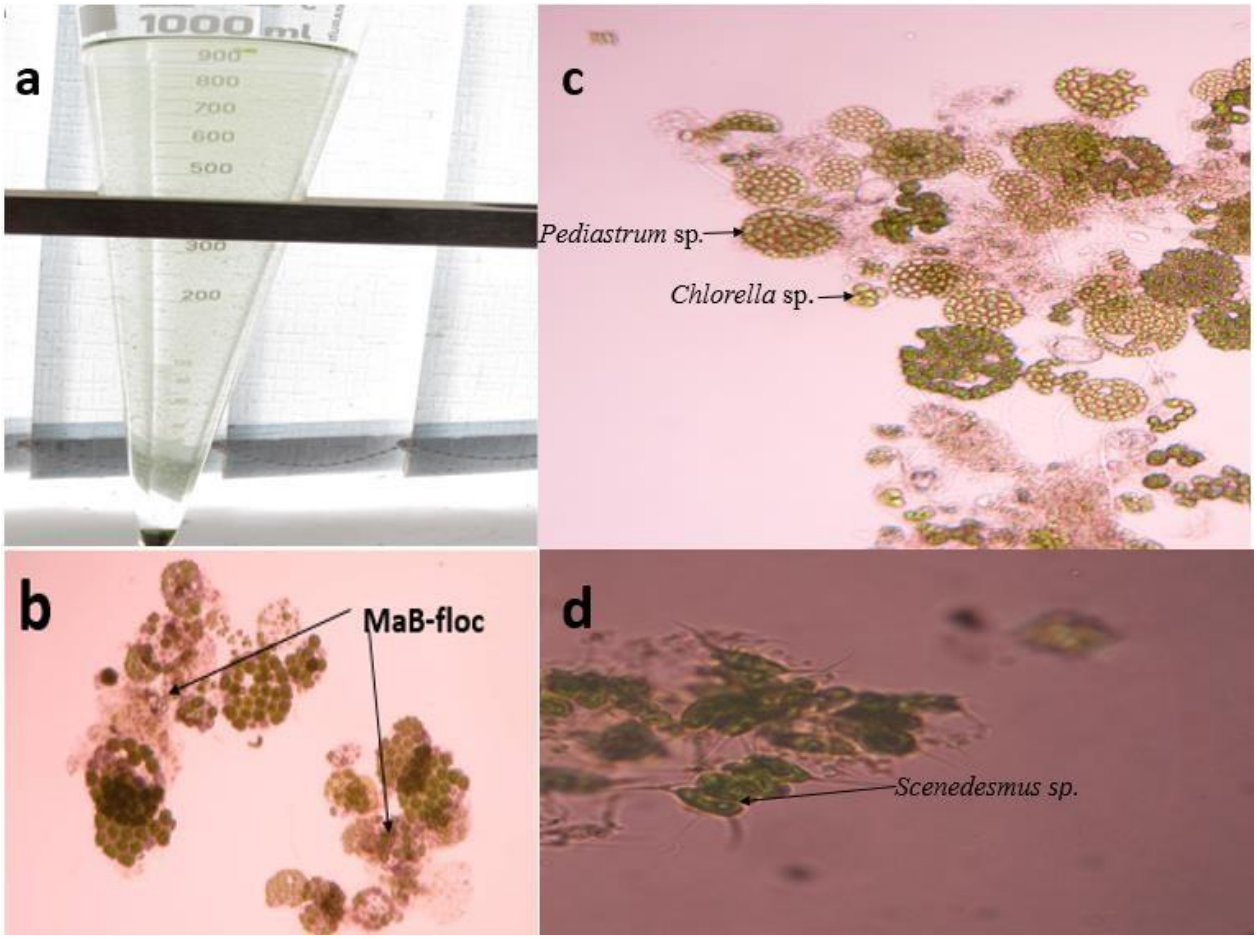

Figure 3.3: Settleability and light micrographs of MaB-flocs generated in high rate algal oxidation ponds of an integrated algae pond system treating municipal sewage. a) Settleability, b) low resolution of the MaB-flocs (10 ×), c) high resolution of summer generated MaB-floc (40 ×) and d) high resolution of winter generated MaB-floc (40 ×) as viewed under a light microscope.

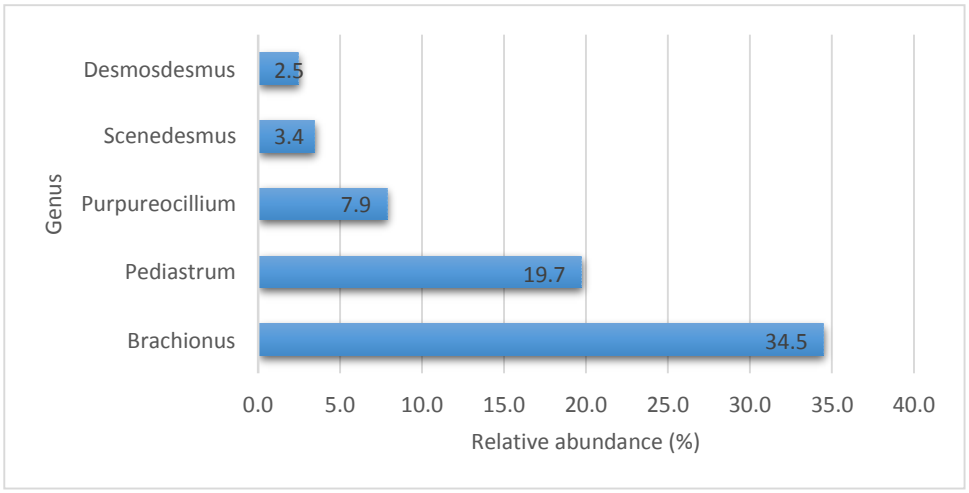

Figure 3.4: Graph shows relative abundance for the most abundant eukaryotes forming part of the summer MaB-floc biomass from high rate algal oxidation ponds treating primary treated domestic sewage. The results were generated through a metagenomics approach.

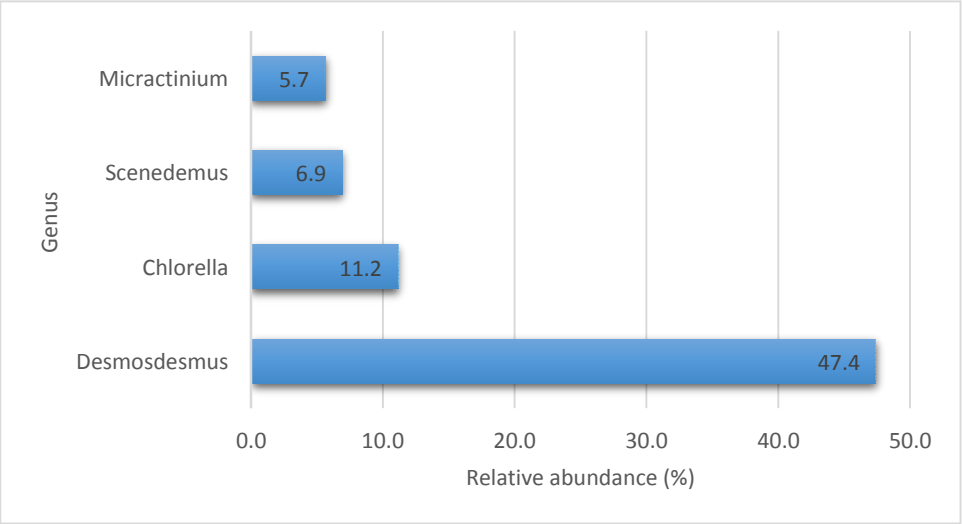

Figure 3.5: : Graph shows relative abundance for the most abundant eukaryotes forming part of the winter MaB-floc biomass from high rate algal oxidation ponds treating primary treated domestic sewage. The results were generated through a metagenomics approach.

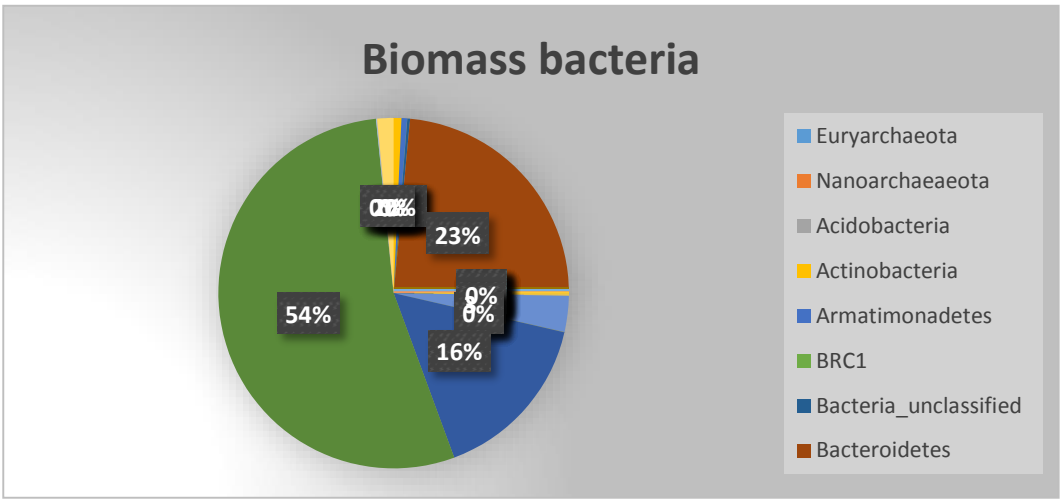

Figure 3. 6: Typical bacteria found in MaB-floc biomass generated in high rate algal oxidation ponds treating primary treated domestic sewage.

The metagenomic study on MaB-floc biomass bacterial communities showed that there were in total 8 phyla shared by 2 independent samples collected. *Proteobacteria* predominated the bacterial portion of the biomass constituting (54%), followed by *Bacteroidetes*, which made up 23% of the bacterial biomass (Figure 3.6).

3.6 Discussion

This chapter aimed to determine whether HRAOPs of an IAPS treating municipal sewage under prevailing winter and summer conditions, was able to efficiently remove nitrogen and phosphorus while producing significant quantities of MaB-floc biomass that could be used as a feedstock for

biofuel production. Additionally, MaB-floc composition and settleability were determined. The results revealed an insignificant change in nitrogen and phosphorus removal efficiencies between winter and summer within the HRAOP under study. These results are in contrast with findings by Sutherland et al. (2014a) who observed a higher nutrient removal in summer compared to winter. The reason for the insignificant difference in nutrient removal between the two seasons may likely have been increased light attenuation brought about by high biomass concentrations which subsequently decreased nutrient assimilation into microalgal biomass (Cromar et al. 1996). Indeed during this study, high biomass concentrations were measured in summer and this could have affected nutrient removal within the HRAOP. The phosphorus removal efficiencies of 70% and 75% from this study were in agreement with findings by Wells et al. (2003) during their study on nutrient removal efficiencies within the same HRAOP currently studied. However, were higher than the 50% obtained by Wells et al. (2003). The nitrogen removal efficiencies of 68% and 71% during winter and summer respectively were within the range of 60% to 75% previously reported by Sutherland et al. (2014a). Findings also showed that the HRAOP currentlystudied was able to reach acceptable productivities (16.5 $g/m^2/d$) of volarisable MaB-floc biomass in summer although the value is lower compared to biomass productivities attained under similar conditions in previous findings (Park et al. 2011b; Passos et al. 2015). The low productivity in summer corresponded to the abundance of rotifers of the genus *Brachionus* sp. and this could have exerted grazing pressure.

Besides grazing pressure, seasonality also plays a role in MaB-floc productivities in HRAOPs (Cromar et al. 1996; Sutherland et al. 2015). This is in agreement with the findings in this study. Summer average biomass productivity was 16.5 $g/m^2/d$ while an average of 9.8 $g/m^2/d$ was measured during winter. High solar radiation in summer increases water temperature (Cromar et al. 1996). According to Mehrabadi et al. (2015), biomass productivity doubles for each 10˚C increase in temperature. At the optimum temperature, maximum algal productivity is observed. The optimum temperature for maximum algal growth is species-dependent but is often in the range of 28 ℃ to 35 ℃ (Park et al. 2011b; Markou et al. 2012). However, proliferation of MaB-floc biomass under high temperature and solar irradiance may result in photoinhibition of photosynthesis which subsequently reduces biomass productivities (Cromar et al. 1996).

Significant diurnal variations in MaB-floc biomass concentrations were recorded during this study (figure 3.2). MaB-floc biomass was observed to be lower in the morning compared to biomass in the afternoon. The same observation was made by Park et al.

(2011b) and this reduction in productivity in the mornings was attributed to biomass losses at night time during respiration.

The highest measured biomass concentration in this study was during a mechanical breakdown of the HRAOP, which disrupted regular inflow of influent. Because no regular topping up of influent was occurring, most of the water in the HRAOPs was lost to evaporation, thus increasing MaB-floc biomass concentration. Whitton et al. (2015) state that high evaporation rates are typical in open pond raceways such as HRAOPs due to their large surface area.

Harvesting contributes 20% to 50% of the production costs in biofuel production; therefore it is imperative that good settling biomass is attained to enhance harvestability thus, lowering the cost of harvesting (Quijano et al. 2017; Jimoh et al. 2019). Microalgal species composition significantly contributes to MaB-floc biomass settleability (Van Den Hende 2014). Presence of larger microalgal species such as *Pediastrum* sp., *Micractinium* sp. and cyanobacteria is an indicator of a readily settleable biomass (Craggs et al. 2011; Jimoh et al. 2019). During the study, MaB-floc biomass with a high settling efficiency was observed during summer and its composition was predominantly *Pediastrum* sp. and Rotifera *Brachionus*. On the other hand, poor settling efficiencies of MaB-floc biomass dominated by *Scenedesmus* sp. or *Desmodesmus* sp. were observed during winter and this coincided with the absence of grazers. According to Lampert et al. (1994) and Lurling and Van Donk (2000) *Scenedesmus* sp. use colony formation as a defence mechanism to prevent being grazed on by rotifers. Biochemical substances released by rotifers induce colony formation as a defence mechanism by microalgae (Lürling and Van Donk 1997). Previous studies have reported the existence of grazer colony formation as a defence mechanism in *Scenedesmus* sp. (Hessen and Van Donk 1993; Lampert et al. 1994; Lürling and Van Donk 1997). This indicates the importance of the presence of rotifers in the flocculation of microalgae.

Bacterial populations in the MaB-floc biomass were predominated by phyla *Proteobacteria* and *Bacteroidetes* which was consistent with results obtained by Ye and Zhang (2013), Ju et al. (2014) and Zhang et al. (2019). The primary role of *Proteobacteria* in wastewater treatment is to remove organic pollutants and nitrogen and phosphorus (Zhang et al. 2019). This phylum is made up of *Gammaproteobacteria* which are subdivided into denitrifying bacteria or bacteria that accumulate phosphates. Among the *Proteobacteria* are the *Betaproteobacteria* which are involved in nitrification, and are potentially also involved in denitrification (Jabari et al. 2016). *Bacteroidetes* play an essential role in the degradation of complex polymers such as

polysaccharides, proteins and lipids into acetate, long-chain fatty acids, CO_2, formate and hydrogen (Jabari et al. 2016).

Seasonal variations play a significant role in microalgal species dominance in HRAOPs (Craggs et al. 2014; Sutherland et al. 2014a). Similar MaB-floc compositions obtained during this study in summer and winter were observed in the same HRAOPs under study by Potts (1998), Johnson (2010) and Jimoh (2017) where *Pediastrium* sp. dominated during summer months and *Scenedesmus* sp. predominated during winter months.

To conclude, the HRAOP under study was able to demonstrate its efficiency in nitrogen and phosphorus removal and its ability to form settleable biomass consisting of various microalgal species, bacteria and fungi. Biomass consisting of larger sized microalgal species such as *Pediastrum* sp. is much more favourable for biofuel production as it is easily harvestable thereby cutting the estimated 20% to 30% of biofuel production costs attributed to harvesting. However, the MaB-floc biomass productivity was lower than the value of expected productivities in HRAOPs treating wastewater due to photoinhibition of photosynthesis and grazing pressure exerted by rotifers.

Chapter 4: Biochemical characterisation of MaB-floc biomass from high rate algal oxidation ponds

4.1 Introduction

High rate algal oxidation ponds (HRAOPs) are an energy-efficient wastewater treatment approach and a production unit for affordable and sustainable MaB-floc biomass (Bohutskyi et al. 2018b). MaB-floc formation is resultant of symbiotic interactions between microalgae and heterotrophic bacteria through which bacteria use oxygen to breakdown organic matter into inorganic C, NH_4^+, NO_3^{2-} and P. In the process, photosynthetic microalgae assimilate and fix inorganic carbon into carbohydrate, protein and lipid via various biosynthetic pathways (Jimenez et al. 2013; Li et al. 2018; Ohemeng-Ntiamoah and Datta 2018). Thus far, MaB-floc biomass productivities in HRAOPs treating sewage have been reported to reach an annual average of 25 $g/m^2/d$ (Park et al. 2011b) with cellular protein being the most abundant (40-50%) fraction of the biomass (Bohutskyi et al. 2018a).

MaB-floc biomass is made up of approximately 80% microalgae, 20% bacteria and up to 10% fungi, detritus and invertebrates (Mehrabadi 2017). The availability of N and P in wastewater HRAOPs regulates carbon allocation in microalgal cells (Driver et al. 2014; Whitton et al. 2015). If wastewater N and P are limiting, carbon uptake continues and allocation is directed towards primary and secondary energy storage in the form of carbohydrates and lipids respectively (Whitton et al. 2015; Bohutskyi et al. 2018a). Accumulation of cellular carbohydrates in nutrient-limited HRAOPs treating sewage can reach 40% of the total biomass (Johnson 2010) while lipid has been reported to reach 26% (Mehrabadi 2017). Microalgae alter their biochemical composition to accumulate energy storage biomolecules that can subsequently be used as feedstock for production of liquid biofuels and methane through various valorisation pathways.

Biomass lipid and carbohydrate, the primary energy reserves for biofuel production, require appropriate estimation for selection of the most economical biomass valorisation route. While conventional methods have been heavily relied upon for their accuracy, these methods are tedious to carry out, time-consuming and require the use of reagents that are hazardous to human health (Albalasmeh et al. 2013).

The micro-sulpho-phospho-vanillin method, on the other hand, is a highly sensitive quantification technique based on the reaction of lipid and sulphuric acid derivative (carbonium ion) and sulpho-

phospho-vanillin (Byreddy et al. 2016). Several studies have measured algal lipid content regarding plant-based oils such as olive oil and canola oil due to their similar unsaturated fatty acid composition (Cheng et al. 2011; Mishra et al. 2014; Byreddy et al. 2016). With regards to carbohydrate estimation, the primary advantage of the sulphuric acid-ultraviolet method over the phenol sulphuric acid method is the elimination of the colour development step of carbohydrate furfural derivatives using phenol, well known for its detrimental effects on human health (Albalasmeh et al. 2013). This is because the sulphuric acid-ultraviolet method is based on the ultraviolet absorption of furfural derivatives (Zhang et al. 2015).

This result chapter gives an insight into the general biochemical composition of MaB-floc biomass with a particular focus on carbohydrate and lipid ratios in HRAOPs of an IAPS treating municipal sewage during winter and summer. Two methodologies for lipid and carbohydrate analyses were compared in terms of accuracy in measuring biomass lipid and carbohydrate proportions to establish suitability for use in biofuel production. Further laboratory work was conducted to investigate the possibility of increasing the stored energy of the biomass by accumulating more carbohydrates using nitrate starvation.

4.2 Assessing the accuracy of sulpho-phospho-vanillin and sulphuric acid –ultraviolet methods for lipid and carbohydrate quantification using standards

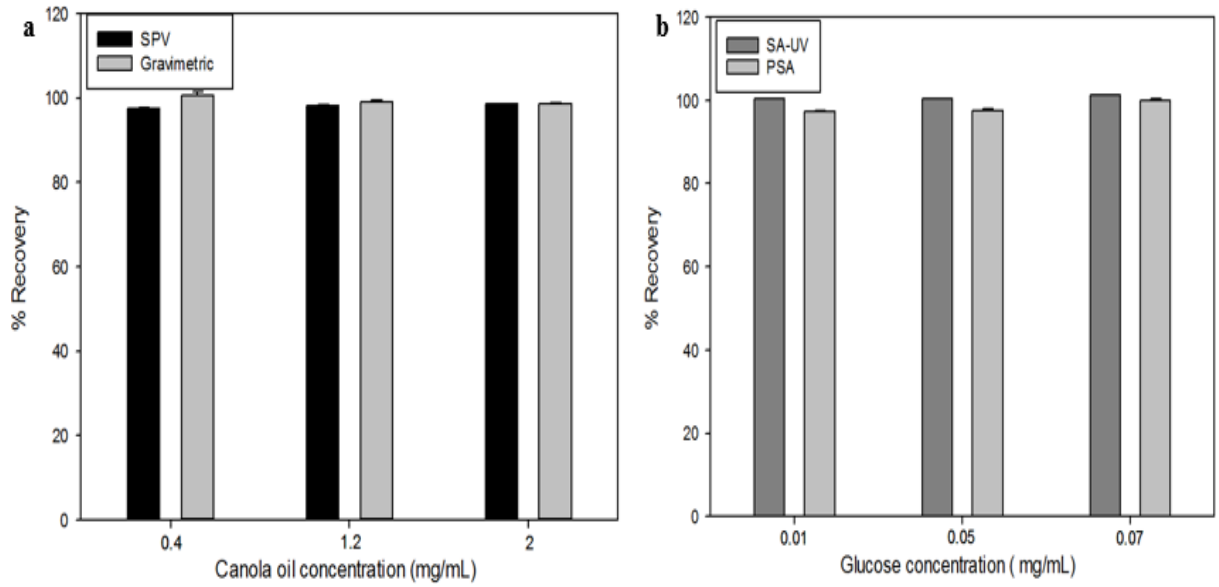

Figure 4.1: Comparison of **a)** sulpho-phospho-vanillin and gravimetric methods for lipid using canola oil and **b)** sulphuric acid-ultraviolet and phenol sulphuric acid for carbohydrates using glucose solution. Percentage recovery from each concentration was carried out in triplicate. Bars indicate standard error.

To determine the best and most reliable method, accuracy becomes an important vetting parameter and is expressed as a percentage mean recovery value. In this experiment, sulpho-phospho-vanillin and sulphuric acid-ultraviolet methods were assessed for accuracy using canola and glucose standards. The graphically illustrated (Figure 4.1) percentage mean recovery values were compared to the values obtained from the conventional gravimetric and phenol sulphuric acid methods.

Assessment of the sulpho-phospho-vanillin method for accuracy gave a percentage mean recovery of 98.0 ± 0.1% while the mean lipid recovery using the gravimetric method was 100.4 ± 0.7%, as shown in figure 4.1. Statistically, no significant difference was found between the two methods (t-test, $p > 0.05$).

With regards to the alternative carbohydrate method assessed, the outcome showed that Sulphuric acid–ultraviolet % mean recovery of 95.6 ± 0.8% was comparable (t-test, $p > 0.05$) to the conventional phenol sulphuric acid method with a % mean recovery of 97.3 ± 0.25%.

4.3 Application of sulpho-phosphovanillin and sulphuric acid-ultraviolet methods to quantify lipids and carbohydrates in MaB-floc biomass

Gravimetric and spectrophotometer based sulpho-phospho-vanillin methods for measuring lipid content were compared to determine if the two methods generated similar values with MaB-floc biomass samples. The gravimetric method generated a slightly higher value of 17.8 ± 0.23% compared to the sulpho-phospho-vanillin method, which generated a mean value of 16.3 ± 0.55% as shown in Figure 3.2. However, statistically, the difference was insignificant (t-test, $p > 0.05$).

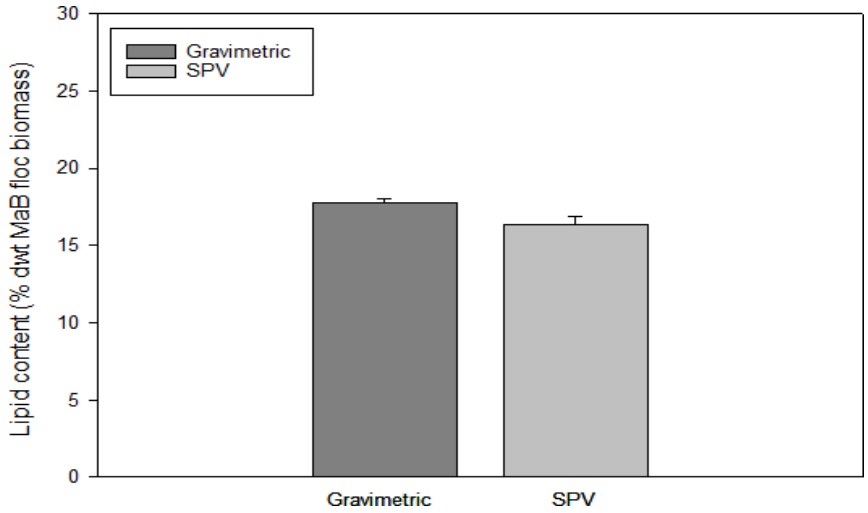

Figure 4.2: Comparison between gravimetric and sulpho-phospho-vanillin methods for lipid determination in MaB-floc biomass. Lipid content values are an average of triplicate samples. Bars indicate standard error.

Similarly, comparison between the phenol sulphuric acid and the sulphuric acid-ultraviolet (Figure 4.3) for MaB-floc biomass carbohydrate quantification showed that the phenol sulphuric acid method gave a higher estimate of carbohydrate (25.7 ± 2%) compared to the SA-UV method that gave an average estimate of 24.8 ± 1% (Figure 3.2). However, this difference was statistically not significant (t-test, $p > 0.05$).

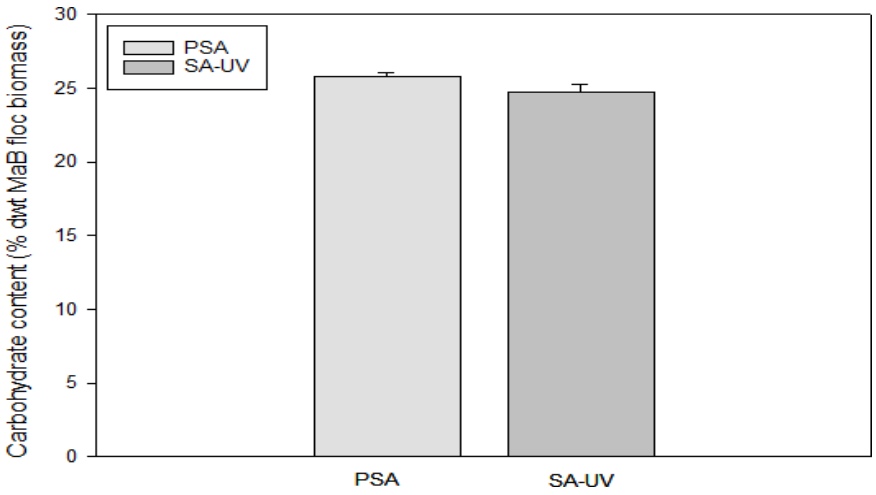

Figure 4.3: Comparison of phenol sulphuric acid and sulphuric acid- ultraviolet methods for carbohydrate determination in MaB-floc biomass. Carbohydrate content values are an average of triplicate samples. Bars indicate standard error.

4.4 Seasonal and diurnal changes in MaB-floc biomass carbohydrate and lipid

MaB-floc biomass carbohydrate and lipid contents were measured in the morning and the afternoon in July 2018, August 2018, January 2019 and February 2019. As shown in Figure 4.5, carbohydrate and lipid contents were lower in the morning and slightly higher in the afternoon which is directly proportional to an increase in water temperature. Statistically, this increase proved to be insignificant (t-test, $p > 0.05$). Nevertheless, the small increase in both carbohydrate and lipid suggested that water temperature changes diurnally do alter MaB-floc carbohydrate and lipid ratios. However, the minimal influence over carbohydrate and lipid variation also suggests the existence of other factors that significantly impact on MaB-floc biochemical composition within a HRAOP system.

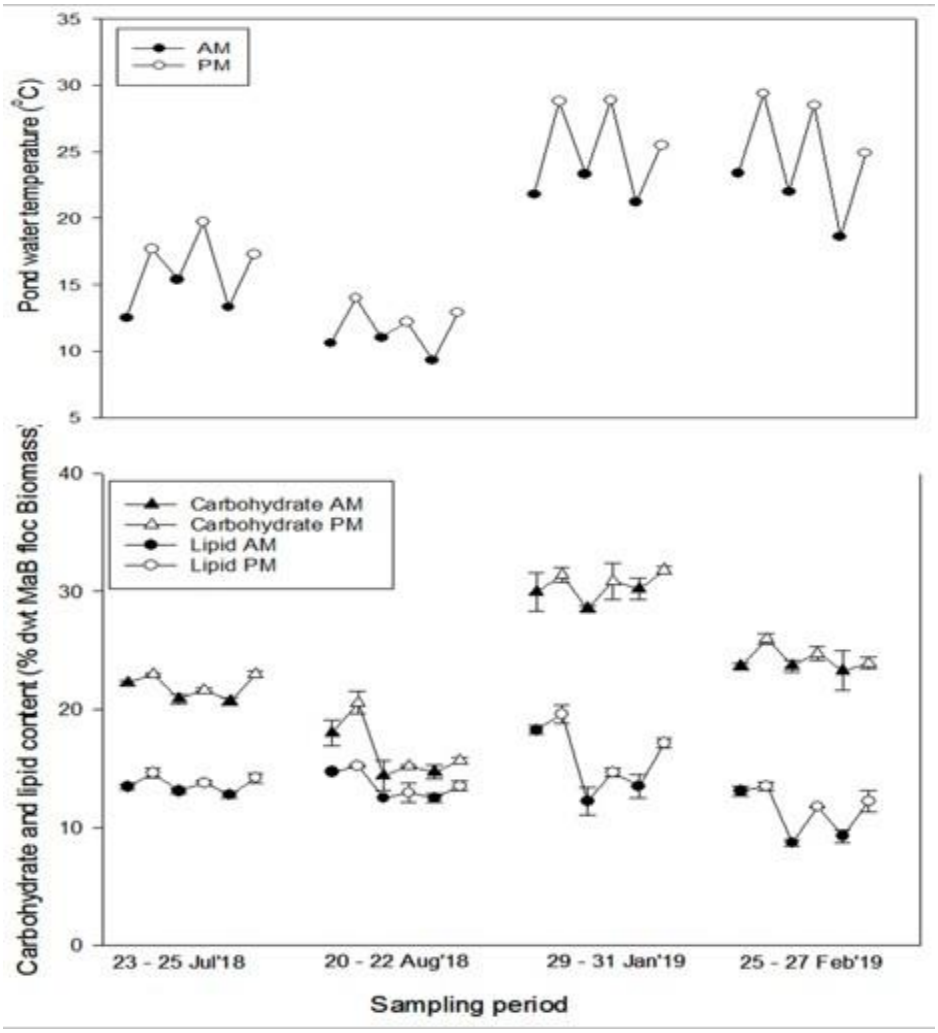

Figure 4.4: Diurnal variations of biomass carbohydrate and lipid in HRAOPs. Carbohydrate and lipid content samples were measured in triplicate. Bars indicate standard error.

4.5 Effects of wastewater nutrients on MaB-floc biochemical composition carbohydrate and lipid in HRAOPs

Since wastewater nutrients affect MaB-floc biochemical composition, the latter were monitored during winter and summer to determine their correlation and effects on MaB-floc biochemical composition, mainly carbohydrate and lipid. Sampling and analyses of the MLSS filtrate for P, NO_3^{2-} and NH_4^+ were carried out while the MLSS biomass fraction was subjected to analyses for carbohydrate, lipid and protein weekly. The relationships were determined using Pearson's correlation test, while t-tests were carried out to test for significance of the effects.

During winter, pond water ammonium and pond water phosphorus concentrations exhibited similar gradual increasing trends which were then followed by a sharp decline as shown in figure 4.5a and 4.5b. The sharp concentration decline in both nutrients was again observed during summer, followed by consistent low concentrations of almost zero for pond water phosphorus and

< 1.5 mg/L for pond water ammonium. This suggests the removal of both nutrients either via microalgal assimilation or ammonification for pond water ammonium and phosphate precipitation in the case of pond water phosphorus.

Fluctuations in pond water nitrate concentration were observed both in winter and summer (Figure 4.5). The increase in pond water nitrate ranging between 5 mg/L and 7 mg/L during the summer period may be due to the increase in nitrification by nitrifying bacterial populations. The lowest pond water nitrate and pond water ammonium concentrations of < 1 mg/L were recorded in June and beginning of July. The low values were as a result of Belmont valley IAPS operating in recycle mode whereby a fraction of the HRAOP treated (secondary) effluent was recycled as influent to reduce its high organic load.

Pond water ammonium concentration showed a weak positive linear relationship ($r = 0.48$) with carbohydrate content while pond water phosphorus, on the other hand, had little influence on carbohydrate content ($r = 0.15$). No significant correlation was found between lipid content variation and pond water ammonium concentration (t-test, $p > 0.05$). MaB-floc carbohydrate negatively correlated with pond water nitrate concentration ($r = -0.79$). The highest MaB-floc carbohydrate content of 35.8 ± 0.3% was recorded in winter (July 2018) and coincided with the lowest pond water nitrate concentration of 0.2 ± 0.01 mg/L while the lowest was carbohydrate content of 17.5 ± 0.15% was recorded in summer (February 2019) coinciding with the highest recorded nitrate concentration of 7.1 ± 0.04 mg/L. Similarly, MaB-floc lipid content had a positive correlation with pond water nitrate concentration ($r = -0.61$) with the highest recorded being 15.7 ± 0.9% recorded in July 2018 and 10.4 ± 0.6% being the lowest recorded in November 2018. Throughout the monitoring period, MaB-floc carbohydrate content was observed to be higher than the MaB-floc lipid. This could have been due to the presence of microalgal and bacterial species with similar carbohydrate and lipid content composition, which subsequently affects the overall carbohydrate and lipid content of the biomass.

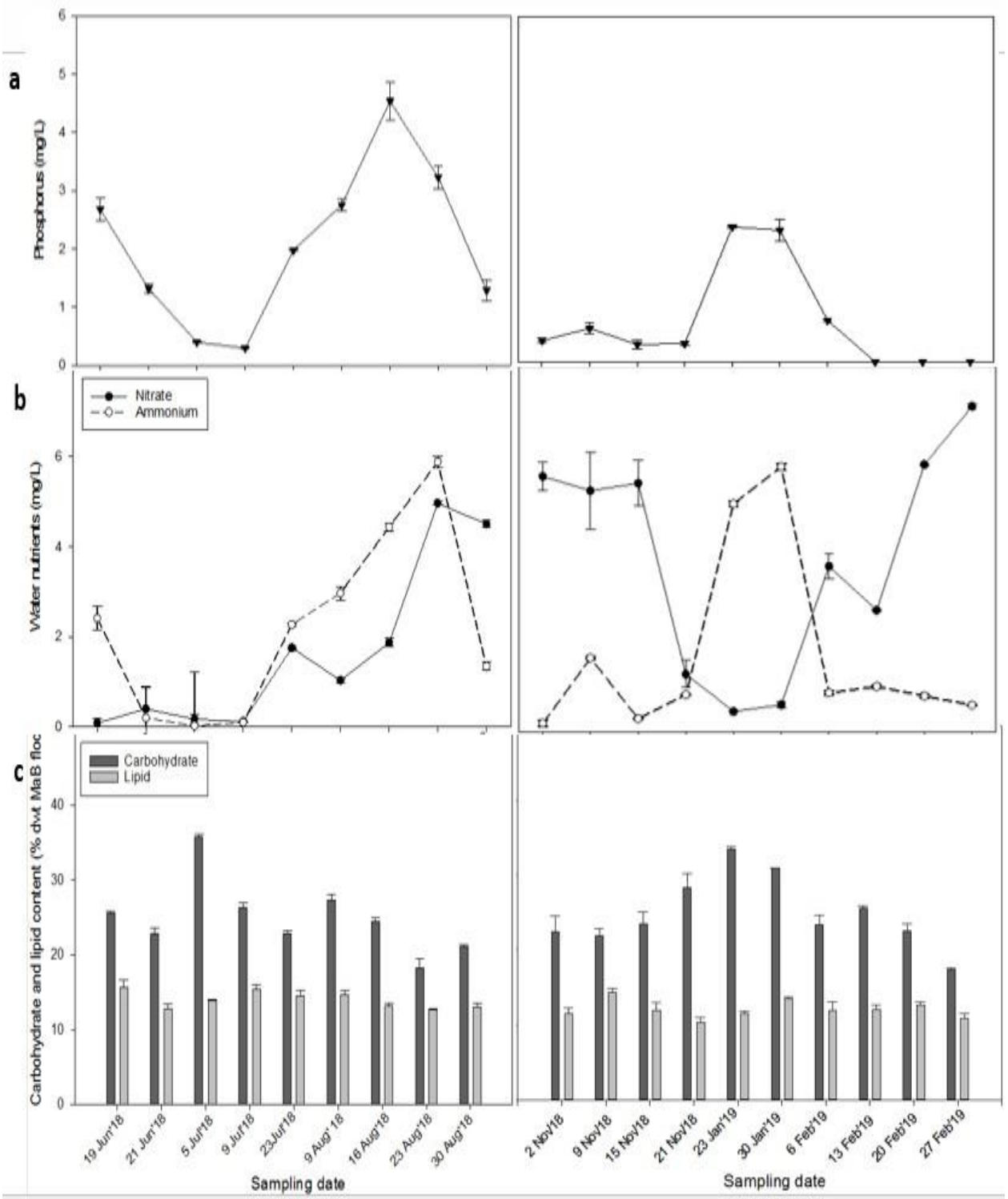

Figure 4.5: Seasonal variation of **a)** phosphorus, **b)** nitrate and ammonium and **c)** MaB-floc carbohydrate and lipid content. Data are presented as the average of two samples collected in the morning and afternoon. Bars indicate standard error.

4.6 Investigation of nitrate starvation on carbohydrate and lipid accumulation and growth using HRAOP native microalgal species

4.6.1 Effect of nitrate starvation on carbohydrate and lipid accumulation in *Scenedesmus* species and *Chlorella* species

Findings from outdoor monitoring showed that carbohydrate-rich microalgal species determined overall MaB-floc biomass carbohydrate. Also, nitrogen limitation in the form of low pond water nitrate concentration significantly increased the microalgal carbohydrate content. Further work was carried out at a laboratory scale to investigate the possibility of increasing carbohydrate content of biomass by inducing accumulation of carbohydrate using nitrate starvation. Isolated unialgal *Scenedesmus* sp. and *Chlorella* sp., commonly found in wastewater HRAOPs and well known to grow unaffected by extremely low nutrient concentrations were used. Moreover, both microalgal species have been associated with EPS production. In green microalga, EPS production is a defensive mechanism against stressful conditions.

On day 8, biomass was harvested and carbohydrate and lipid content measured. Nitrate starved *Scenedesmus* sp. had a higher proportion of lipid than carbohydrate, indicating a preference of carbon allocation towards lipid synthesis pathway during stress conditions (Table 4.1). The results also showed a three-fold accumulation of lipid from 8.1 ± 0.7% to 25.5 ± 0.9% in nitrate starved (nitrate 0 mg/L) *Scenedesmus* culture compared to the *Scenedesmu*s sp. control (nitrate 7 mg/L). The high lipid content in the *Scenedesmus* sp. culture starved of nitrate is indicative of nutrient stress in which the response is the accumulation of either lipid or carbohydrate for energy storage. However, carbohydrate content in nitrate starved *Scenedesmus* sp. was observed to be lower than in *Scenedesmus* sp. control. Accumulation of carbohydrate and lipid contents were insignificant ($p > 0.05$) in *Scenedesmus* sp. control culture.

Table 4.1: Change in carbohydrate and lipid content (± SE) of *Scenedesmus* sp. after cultivation for 0 and 8 d in the presence and absence of nitrate. Data are mean of 2 experiments**.**

	Nitrate 0 mg/L		**Nitrate 7 mg/L**	
	Carbohydrate (% dwt MaB-floc biomass)	Lipid (% dwt MaB-floc biomass)	Carbohydrate (% dwt MaB-floc biomass)	Lipid (% dwt MaB-floc biomass)
Day 0	10.5 ± 0.4	8.1 ± 0.7	10.5 ± 0.4	8.1 ± 0.7
Day 8	7.3 ± 0.2	25.5 ± 0.9	11.6 ± 0.1	11.7 ± 0.6

Table 4.2: Change in carbohydrate and lipid content (± SE) of *Chlorella* sp. after cultivation for 0 and 8 d in presence and absence of nitrate. Data are mean of 2 experiments..

	Nitrate 0 mg/L		**Nitrate 7 mg/L**	
	Carbohydrate (% dwt MaB-floc biomass)	Lipid (% dwt MaB-floc biomass)	Carbohydrate (% dwt MaB-floc biomass)	Lipid (% dwt MaB-floc biomass)
Day 0	9.7 ± 0.6	8.9 ± 0.9	9.7 ± 0.6	8.9 ± 0.9
Day 8	20.8 ± 1.8	24.9 ± 0.8	11.5 ± 0.8	6.3 ± 2.3

Nitrate starved *Chlorella* sp. culture yielded 24.9 ± 0.8 % lipid, which was four times more than the final lipid content recorded in *Chlorella* sp. control culture with 6.3 ± 2.3 % lipid content. Similarly, carbohydrate content in nitrate starved (nitrate 0 mg/L) *Chlorella* sp. culture was significantly higher ($p < 0.05$) than carbohydrate content in *Chlorella* sp. control culture (Table 4.1).

4.6.2 Effect of nitrate starvation on the growth of *Scenedesmus* species and *Chlorella* species

Growth of nitrate starved *Scenedesmus* sp. and *Chlorella* sp. were measured every 2 days up to the end of the experiment. The results are shown in Figure 4.2. After the first 2 days of nitrate starvation, *Scenedesmus* sp. biomass concentration increased two-fold from 0.03 to 0.06 mg MLSS/L while a three-fold increase in biomass concentration was observed in *Scenedesmus* sp. control. By day 6, nitrate starved *Scenedesmus* sp. had reached late growth / early stationary phase whereas *Scenedesmus* sp. control remained in the log phase till the end of the experiment on day 8. The final biomass concentrations for nitrate starved *Scenedesmus* sp. and the *Scenedesmus* control cultures were 0.25 ± 0.05 mg/L and 0.28 ± 0.02 mg/L respectively.

***Scenedesmus* species** ***Chlorella* species**

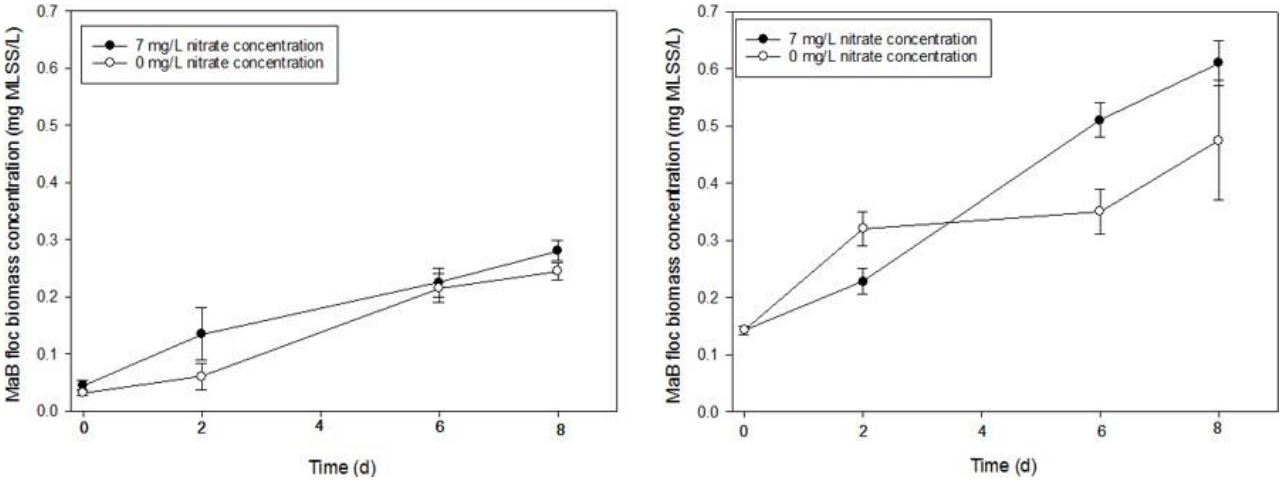

Figure 4.6: Growth curves for *Scenedesmus* sp and *Chlorella* sp. grown in nitrate replete and nitrate deplete conditions for 8 days. Error bars indicate ± SE of duplicate samples.

The biomass concentration of nitrate starved *Chlorella* sp. doubled from 0.14 ± 0.01 mg MLSS/L to 0.32 ± 0.03 mg MLSS/L within 2 days of initial culturing. However, from day 2 to day 6, growth of *Chlorella* sp. control was prolonged, which was reflected in the insignificant increase in biomass concentration. A gradual increase in biomass concentration from day 0 to day 8 was observed in *Chlorella* sp. control likely due to the metabolism of available storage products to support growth. The final biomass concentration of *Chlorella* sp. control measured was 0.61 ± 0.04 mg MLSS/L. Better biomass accumulation was generally observed in *Chlorella* sp. than in *Scenedesmus* sp. as shown in Figure 4.6.

4.7 Discussion

Characterisation of biomass is desirable as it ultimately dictates the most appropriate valorisation pathway. However, no methodologies have been standardised for biochemical characterisation of MaB-floc biomass. The first part of this result chapter reported on the comparison of quantitative methods in terms of accuracy using percentage recovery between phenol sulphuric acid and sulphuric acid-ultraviolet for carbohydrate as well as gravimetric and micro-sulpho-phospho-vanillin (SPV) for lipids to determine the best spectrophotometric methods to measure carbohydrate and lipid content from HRAOP generated MaB-floc biomass. The result chapter also gave an insight into the biochemical composition of MaB-floc biomass particularly the variations in carbohydrate and lipid in response to season and inorganic nutrients present in the pond water.

Further work was conducted in the laboratory in an attempt to induce either carbohydrate or lipid and enhance MaB-floc formation using nitrogen starvation.

For lipid quantification, the alternative micro-sulpho-phospho-vanillin method gave lipid estimates which were relatively comparable to the conventional gravimetric method. Likewise, the sulphuric acid- ultra violet method for carbohydrates proved to be as accurate when compared to the phenol sulphuric acid method. Results also indicated that the applicability of the alternative methods (micro-sulpho-phospho-vanillin and sulphuric acid-ultraviolet) could be extended to MaB-floc biomass samples. However, a slightly higher lipid content value from the gravimetric method in comparison to the micro-sulpho-phospho-vanillin method was observed. Similar observations have been made from previous studies and this has been attributed to the presence of nonlipid compounds within the lipid extract (Folch et al. 1957; Gardner et al. 1985; Lu et al. 2008). With regards to carbohydrate measurement, the difference in measurement values obtained from both phenol sulphuric acid and sulphuric acid ultraviolet methods was negligible. From the results in figures 4.1a and 4.2, the sulphuric acid –ultraviolet method proved to be a reliable method with an added advantage of non-usage of phenol known for its toxicity (Albalasmeh et al. 2013; Zhang et al. 2015).

Findings from the outdoor monitoring showed a slight increase in both lipid and carbohydrate in response to diurnal variations. This showed that temperature had an impact on carbohydrate and lipid accumulation in MaB-floc biomass, although the changes were negligible. A similar conclusion was drawn by Mehrabadi (2017) after observation of a slight increase in MaB-floc biomass lipid content due to diurnal temperature variations of different seasons.

Throughout the monitoring period, carbohydrate content was more than the lipid content in MaB-floc biomass. Results obtained from chapter 3 of this study showed biomass predominated by *Pediastrum* sp. in summer and *Desmodesmus* sp., *Scenedesmus* sp. and *Chlorella* sp. in winter. Microalgal species composition and bacterial compositions within the biomass were part of the contributory factor to the overall biochemical composition of the biomass. Microalgal populations constitute at most 80% of the biomass with carbohydrate, lipid and protein contents of 30 to 60 dwt %, 15 to 30 dwt %, 10 to 30 dwt % of intracellular biochemical composition respectively (Jimoh et al. 2019). Common microalgal species found in HRAOPs treating wastewater *Pediastrum* sp., *Micractinium* sp., *Chlorella* sp. and *Scenedesmus* sp. are considered carbohydrate-rich with dwt % of 54%, 59%, 55% and 49% respectively Johnson (2010). When preconditioned by being cultured in low nitrogen, green microalgae tend to accumulate more carbohydrates

than lipids (Cowan and Laubscher 2010). This explains the consistent high carbohydrate content of the MaB-floc biomass for both sampling seasons compared to lipid content even during periods of low nitrogen availability. On the other hand, bacterial populations in MaB-flocs make up approximately 20% and the respective intracellular carbohydrate, lipid and protein contents are 20-40 dwt %, <10 dwt % and 25-50 dwt % (Mehrabadi 2017). However, results obtained from this study contradict findings from previous studies on biochemical characterisation of biomass generated in HRAOPs operating in continuous mode. For instance, Johnson (2010) and Bohutskyi et al. (2018a) observed protein-rich MaB-floc biomass, while Mehrabadi (2017) reported lipid-rich biomass consistently throughout all four seasons.

Variability in biochemical content of MaB-floc biomass is a function of pond water nitrogen availability (Park et al. 2011a; Whitton et al. 2015; Michelon et al. 2016). The primary forms of nitrogen in the HRAOP under study were pond water ammonium and nitrate. Of the two forms of available nitrogen, a correlation was observed between pond water nitrate concentration and MaB-floc carbohydrate during winter and summer monitoring. MaB-floc biomass carbohydrate was observed to increase with a decrease in pond water nitrate concentration. These findings are in agreement with those of Johnson (2010) in which nitrogen limitation within HRAOPs operating in batch mode resulted in biomass constituting of carbohydrate as the significant biochemical component. Both nitrogen starvation and limitation can induce energy storage in microalgal cells (Rodolfi et al. 2009). However, carbon allocation between carbohydrate and lipid is species-specific (Hu et al. 2008a; Williams et al. 2010; Markou et al. 2012). It can be deduced that HRAOPs treating sewage undergo nitrogen limitation time and again due to several factors and depending on predominating microalgal species, this directly affects overall MaB-floc biomass accumulating either carbohydrate or lipid.

Laboratory work was done to establish the possibility of using nitrogen starvation as a potential strategy to further enrich the biomass biochemical composition in terms of carbohydrate. EPS producing microalgal species *Scenedesmus* sp. and *Chlorella* sp. were used (Jimoh and Cowan 2017). Three-fold lipid accumulation was observed in nitrogen starved *Scenedesmus* sp. unialgal culture while biomass concentration was significantly reduced. These results are in agreement with findings by (Rawat 2011). Nitrogen is an essential macronutrient that increases lipid or carbohydrate content but at the same time halt culture growth (Illman et al. 2000; Singh and Gua 2010). Under nitrogen starvation conditions, microalgal cells cease to divide (Rawat 2011). However, due to the availability of light and carbon dioxide, excess energy generated in the form

of electrons is directed towards lipid and carbohydrate biosynthesis in which the biomolecules become energy sinks thus preventing cellular damage by photooxidation (Rodolfi et al. 2009). The preference in accumulating lipids by *Scenedesmus* sp.was due to the higher energy storage capacity by lipids compared to carbohydrates (Packer 2009).

Nitrogen starvation triggered the accumulation of both lipid and carbohydrate in *Chlorella* sp. unialgal culture with reduced biomass concentration compared to the control. A similar pattern of accumulation was observed in *Chlorella zofingiensis* by Zhu et al. (2014a). According to Gao et al. (2019), induction of carbohydrate accumulation was usually a quick response to stress by microalgae; hence, carbohydrates act as a primary energy sink. On the other hand, prolonged stress to microalgae leads to long term or secondary energy storage in the form of lipids (Gao et al. 2019). Additionally, lipids store twice the amount of energy stored by carbohydrates hence this could be the reason most microalgal species preferentially accumulate lipid in response to prolonged nitrogen starvation (Hu et al. 2008b; Rawat 2011).

To conclude, the outcome from the experimental work demonstrated the possibility of enriching green microalgal cellular composition with energy-rich lipids making the biomass highly suitable for biodiesel production. However, lipid or carbohydrate accumulation is species-specific; hence, this may affect the overall biomass biochemical composition. Based on findings from outdoor monitoring, nitrogen limitation in the form of nitrates may be used to control the overall carbohydrate content of MaB-floc biomass. Nitrogen limitation can be implemented into the process operation of HRAOPs by increasing hydraulic retention time. Nitrogen concentrations should be low enough to induce accumulation without affecting biomass productivity.

Chapter 5: Theoretical biofuel potential of MaB-floc biomass generated in HRAOPs of an IAPS

5.1 Introduction

An estimated 88% of global energy consumption is mostly attributed to fossil fuel combustion, with oil and coal combined accounting for 64% of the usage (Brennan and Owende 2010). However, continued reliance on fossil fuels cannot sustain the increasing worldwide energy demand due to the non-renewability of these types of fuel (Khan et al. 2009). To conserve and manage what is left of the natural resources, sustainability is a crucial principle. Biofuels derived from energy crops are renewable and can provide energy that will sustain future generations (Demirbas et al. 2009). However, energy crop production dedicated to biofuel requires large quantities of land which would compete with food production and compromise food security (Pittman et al. 2011). Algal biomass, on the other hand, has a distinct advantage over other land-based energy crops as it can be cultivated on non-arable land utilising wastewater (Brennan and Owende 2010). Several reports have recommended coupling algal biomass production to wastewater treatment as a way of reducing the associated costs of mass production (Lundquist et al. 2010; Pittman et al. 2011).

Integrated algal pond systems (IAPS) have attracted increasing attention as inexpensive biotechnology that can treat wastewater and provide biomass from which bioproducts such as biofuel, fertiliser and fish and animal feed can be derived (Oswald et al. 1964). Of particular interest in an IAPS, are the mixed high rate algal oxidation ponds (HRAOPs) which are shallow raceway ponds with paddlewheels to facilitate mixing and aeration (Park et al. 2011b). These ponds support the growth of mixed algal and bacteria consortia in the form of microalgal bacterial (MaB) flocs which form the bulk of the biomass. These flocs effectively remove and recover nitrogen and phosphorus nutrients from wastewater, preventing eutrophication in receiving water bodies (Olguín 2012; Young et al. 2017). Flocs also facilitate settling and separation of biomass from water. The settled biomass is rich in energy stored in the form of lipids and carbohydrates, hence making the biomass a suitable substrate for biofuel production (Yuan et al. 2018). Depending on the proportions of lipid and carbohydrate energy reserves in microalgae, a variety of biofuels can be derived from MaB-floc biomass through thermochemical and biochemical conversion pathways. Lipid-rich biomass can be transesterified into biodiesel whereas jet fuel and green diesel can be obtained through other thermochemical conversions of the same biomass. Fermentation into bioethanol is typically employed on algal biomass with a higher proportion of

carbohydrates. With regards to anaerobic digestion to yield methane, the C/N ratio is an essential factor to consider. Ideally, a C/N ratio of higher than the ratio 20:1 gives better yields of methane (Yen and Brune 2007; Sialve et al. 2009). IAPS is a low-cost technology hence biomass valorisation options should be such that the process gives a positive net energy balance.

This result summarises the biochemical and elemental composition for MaB-floc biomass obtained from HRAOPs of an IAPS treating sewage during summer and winter. To establish the most suitable valorisation pathway for IAPS generated MaB-floc biomass, measured biochemical and elemental values during winter and summer were used to calculate theoretical biofuel potential values.

5.2 MaB-floc biomass biochemical, elemental compositions and theoretical biofuel potential

Table 5.1 Biofuel potential values, biochemical and elemental compositions of MaB-floc biomass for winter and summer. Biochemical and elemental composition data are presented as the mean of 10 independent samples for winter and summer (± SE).

Parameter	Winter	Summer
Carbohydrate (% dwt MaB-floc biomass)	24.9 ± 0.6	25.6 ± 1.3
Lipid (% dwt MaB-floc biomass)	13.9 ± 0.5	12.1 ± 0.64
Protein (% dwt MaB-floc biomass)	11.9 ± 0.5	9.6 ± 0.9
C (% dwt MaB-floc biomass)	43.2 ± 0.4	39.2 ± 0.5
H (% dwt MaB-floc biomass)	7.2 ± 0.2	7.1 ± 1.1
N (% dwt MaB-floc biomass)	6.9 ± 1.5	5.9 ± 0.9
S (% dwt MaB-floc biomass)	0.9 ± 0.05	0.9 ± 0.07
C/N ratio	7.3	7.8
TMP (m^3 CH_4/kg MaB-floc biomass)	0.34	0.31
Theoretical bioethanol yield (L/kg)	0.15	0.17
Theoretical biodiesel yield (L/kg)	0.13	0.11

Both winter and summer MaB-floc biomass composition was characterised by high carbohydrate content with averages of 24.9 ± 0.6% and 25.6 ± 1.3% respectively. On the other hand, protein content was the lowest during the two sampling seasons with an average of 11.9 ± 0.5% for winter and 9.6 ± 0.2% for summer. Lipid contents for winter and summer were comparable averaging 13.9 ± 0.5% and 12.1 ± 0.64% respectively. Average theoretical bioethanol yields for winter and

summer were 0.15 L bioethanol /kg MaB-floc biomass and 0.17 L bioethanol/kg MaB-floc biomass respectively, which implies a low probability of exploring this valorisation route. Similarly, theoretical biodiesel yields were low for both sampling seasons with averages being 0.13 L biodiesel/kg MaB-floc biomass and 0.11 L biodiesel/kg MaB-floc biomass. There was no significant difference (t-test, $p > 0.05$) in theoretical bioethanol and biodiesel yields obtained for winter and summer.

Theoretical methane potential values for HRAOPs generated MaB-floc biomass was relatively low for winter and summer averaging 0.33 m^3 CH_4/ kg MaB-floc biomass and 0.31 m^3 CH_4/ kg MaB-floc biomass respectively. The C/N ratio for both seasons ranged between 7:1 – 8:1, which was far from the ideal 20:1 - 30:1 required for good biogas and methane production.

There was no significant difference (t-test, $p > 0.05$) in MaB-floc biomass elemental composition measured in winter and summer. The intracellular carbon contents were reasonably high, averaging 43.2 ± 0.4% and 39.2 ± 0.9% for winter and summer respectively. Intracellular nitrogen content was well within the limits of total intracellular nitrogen found in algal-bacterial biomass harvested from nitrogen replete and deplete conditions which range from 6% - 12% (Laurens et al. 2018). However, with total biomass nitrogen being well within range, this did not tally with the low biomass protein content. It was also observed that IAPS generated MaB-floc biomass contained high levels of sulphur with the highest recorded being 0.9 ± 0.05%.

5.3 Discussion

MaB-floc biomass elemental composition in winter was similar to the one measured in summer. The sulphur content was observed to be high for both sampling seasons. Carbon content in MaB-floc biomass was reasonably high which translated to the acceptable energy value of the biomass. These measurements were in agreement with findings by Mehrabadi (2017) and Laurens et al. (2018). On the other hand, protein content remained low despite the high nitrogen content.

The characteristics of HRAOP generated MaB-floc biomass in this study summarised in Table 5.1 showed a high carbohydrate content and relatively low average lipid and protein contents for both winter and summer. The biochemical composition of this MaB-floc biomass was different from biomass composition obtained from HRAOPs treating sewage in previous studies. Instead, protein-rich biomass was observed while average lipid and carbohydrate were relatively low (Tijjani-Oshungboye 2011; Mehrabadi 2017; Bohutskyi et al. 2018a; Li et al. 2018). Due to the low lipid content and high extraction and purification costs, it would, therefore, be uneconomical

to utilise the HRAOP generated biomass as feedstock for biodiesel production. This is also depicted by the low theoretical biodiesel yields.

According to calculations, the bioethanol theoretical value was low (0.15 L/kg and 0.17 L/kg) implying the unsuitability of the MaB-floc biomass for this valorisation route. This corroborates findings from a study by Tijjani-Oshungboye (2011) which explored the potential of MaB floc biomass from the HRAOP under study for bioethanol production and revealed inordinately low yields (115 mg bioethanol/g biomass equivalent to 0.15 L bioethanol/kg biomass). Craggs et al. (2011) also estimated bioethanol from microalgal biomass to be 0.13 L bioethanol/kg algal biomass. The low yields have been attributed to the low availability of fermentable sugars (Craggs et al. 2011). Thus, the selection of a robust biomass pretreatment method to efficiently hydrolyse and extract fermentable sugars becomes an essential aspect of bioethanol production (Laurens et al. 2015; Souza et al. 2017).

Theoretical methane potential values for the MaB-floc biomass from the HRAOP under study were between 0.33 m^3 CH_4/ kg MaB-floc biomass and 0.31 m^3 CH_4/ kg MaB-floc biomass. These values were comparable with the maximum theoretical methane potential value of 0.30 m^3 CH_4/ kg MaB-floc biomass for sewage grown biomass (Passos et al. 2015). However, the theoretical value obtained was lower than the value of 0.70 m^3 CH_4/ kg MaB-floc biomass previously reported by (Tijjani-Oshungboye (2011) for the biomass obtained from the same HRAOP under study. The theoretical C/N ratios of 7.3 and 7.8 obtained in this study were well below the optimum range of between 20:1-30:1 reported in the literature by Parkin and Owen (1986). Taking this into consideration, the low C/N ratio, therefore, explained the low theoretical methane potential values obtained. Also, Heaven et al., (2011) highlighted that high N proportions result in low C/N ratios that release more ammonia in the digester which in turn compromises anaerobic bacterial performance.

To conclude, HRAOPs generated MaB-floc biomass could preferably be harvested in summer when biomass concentration and productivities are highest as shown in Chapter 3. To explore the biodiesel production pathway, the biomass may require to be preconditioned to improve lipid content through nitrogen starvation as demonstrated in Chapter 4. The outcome of outdoor monitoring of biomass biochemical composition indicated that HRAOPs operating under natural environmental conditions preferentially generated a biomass rich in carbohydrate, thus making the biomass more suitable for anaerobic digestion to produce methane and CO_2. Despite the low C/N ratio which reduces methane yields, this can easily be overcome by co-digesting the biomass with

a higher C/N ratio co-substrate such as in-pond digester sludge.

Chapter 6: General discussion and conclusion

6.1 Discussion

The growing concern over increasing water scarcity and poor water quality emanating from the discharge of high nutrients from poorly treated wastewater into the environment has continued to compromise water security (Adewumi et al. 2010; Brennan and Owende 2010). Water reclamation and reuse have been adopted as a strategy to alleviate water scarcity while more stringent water nutrient discharge regulations have been imposed regardless of the technology in use to ensure discharge of a water quality worthy of recycling (Sutherland et al. 2014a; Tram et al. 2014). This would require treatment technologies with high efficacy in nutrient removal and sustainable in the long run. For a technology to be termed sustainable, it should possess the following aspects: high nutrient recovery, low operation and maintenance costs, biomass with valorisation potential, and low land usage (Balkema et al. 2002). Based on the above characteristics, alternative technologies including integrated algal pond systems (IAPS) certainly meet most of the criteria.

IAPS is a derivation of AIWPS® developed by Oswald in 1956 as a low-cost wastewater treatment technology. This passive yet robust system combines the principles of fermentation, photosynthesis and microbial metabolism to remediate wastewater producing a reusable effluent and substantive biomass with a volarisation potential. Volarisation of microalgal biomass for biofuel production has been gaining momentum in the last couple of years. Suggestions for coupling microalgal biomass production to wastewater treatment as a cheaper route to producing biofuels at a larger scale have been made (Lundquist et al. 2010; Pittman et al. 2011). The WWT plant would offset the production and harvest costs of the biomass. While several studies have focused on biomass production and composition using small scale HRAOPs operating under controlled conditions, little information is available on HRAOPs being part of an integrated system treating sewage under natural environmental conditions, the generated biomass quality produced and its suitability for biofuel production. The work entailed in this thesis sought to demonstrate the nutrient removal efficiency of an integrated algal pond system and evaluating the biofuel potential of the resultant MaB-floc biomass.

In an IAPS, the bulk of nutrient removal takes place in the HRAOPs. The efficiency of HRAOPs in nutrient removal notably nitrogen has been reported to range between 60% and 75% while phosphorus removal ranges between 50% and 76% (Banat et al. 1990; Wells et al. 2003; Sutherland et al. 2014b). Seasonal nutrient removal of the HRAOPS currently studied had maximum average

nitrogen and phosphorus removal efficiencies of 71% and 75% respectively, both recorded in summer and within the range previously reported by Banat et al. (1990), Wells et al. (2003) and Sutherland et al. (2014a). While the performance expectations of a HRAOP is to produce an effluent of secondary water quality (Mambo et al. 2014), nitrogen and phosphorus concentrations from the treated effluent met nutrient discharge limits under South African regulations. Therefore, there is a possibility of utilising this component of IAPS as an additional final nutrient removal step to existing wastewater treatment plants that are unable to produce effluent that is within DWA discharge limits.

In addition to efficient nitrogen removal, an added benefit of a HRAOP of an IAPS is the production of a volarisable MaB-floc biomass. For pond monitoring, the mean winter and summer MaB-floc biomass productivities attained were 16.5 $g/m^2/d$ and 9.4 $g/m^2/d$ which were below the HRAOP theoretical productivity value of $25g/m^2/d$. MaB-floc biomass productivities were highly dependent on seasonal changes in temperature and solar radiation. Thus, operational parameters such as HRT can be altered according to seasonality. Longer hydraulic retention times (HRTs) can be implemented to cater for the low photosynthetic activity of microalgal cells and also allow enough time for nutrient assimilation into microalgal biomass while shorter HRTs may be implemented during summer where higher microalgal photosynthetic activity and increased nutrient assimilation rates influence higher MaB-floc productivities.

Settling efficiency of the MaB-floc biomass generated in HRAOPs was higher in summer compared to winter (Chapter 3). MaB-floc biomass in summer was dominated by the easily settleable *Pediastrum* sp. while winter biomass was predominated by the smaller size *Scenedesmus* sp. and *Chlorella* sp. and less settleable due to the absence of MaB-flocs. Since settleability is a function of species composition, maximum harvestability can, therefore, be achieved during summer when an easily settleable *Pediastrum* genus predominates the biomass. Alternatively, for biomass comprising of *Scenedesmus* sp., settleability can be facilitated by maintaining low nitrogen levels within HRAOPs with an added advantage of inducing either lipid or carbohydrate accumulation(Trainor and Egan 1988). Rotifers, through controlled grazing, can also be used to enhance the aggregation of microalgal cells for better settling biomass (Lurling and Van Donk 2000).

The success of a microalgal consortium in HRAOPs is influenced by the performance of each species under prevailing environmental conditions and changes in these conditions result in temporary successions (Sutherland et al. 2014a). Temporary successions thus pose a challenge in

HRAOPs, that is, maintaining desirable algal species throughout the year for consistent biomass production and biochemical composition. However, characterisation of MaB-floc biomass carbohydrate and lipid during winter and summer revealed a consistently high carbohydrate content compared to lipid (Chapter 4). This is because the genera native to HRAOPs, namely *Pediastrum, Scenedesmus, Chlorella* and *Micractinium* are naturally rich in carbohydrate (Cowan and Laubscher 2010; Johnson 2010).

Both nitrogen starvation and limitation can induce energy storage in microalgal cells (Rodolfi et al. 2009). Variations in carbohydrate content of the MaB-floc biomass during summer and winter outdoor monitoring were influenced by nitrogen levels, particularly pond water nitrate. An increase in carbohydrate was observed when pond water nitrate levels were low, while a decrease was observed with increasing pond water nitrate levels. This subsequently led to the laboratory studies carried out to investigate whether nitrogen starvation was capable of inducing carbohydrate accumulation to increase the carbohydrate content. Instead, lipid accumulation at the expense of biomass productivity was observed in both green alga genera used in the study. Thus, to generate biomass high either in carbohydrate or lipid in HRAOPs, nutrient levels in particular nitrogen levels should be considered. Nitrogen levels to either create nitrogen limiting or starvation conditions can be controlled through mode of operation. Recycling of effluent can significantly lower nitrogen levels to almost zero as observed between 21 June and 9 July 2018 and graphically shown in Figure 4.5 (Chapter 4). Alternatively, increasing the hydraulic retention time in HRAOPs can be used as a strategy to create nitrogen limiting or starvation conditions (Mehrabadi 2017).

Since the outcome from the experimental work demonstrated the possibility of enriching green microalgal cellular composition with lipids using nitrogen starvation, this strategy can be used to improve HRAOP MaB-floc biomass lipid content which from outdoor monitoring ranged between 12.1 ± 0.64% and 13.9 ± 0.5%. Lipid enriched biomass in terms of energy is ideal for biodiesel production. This is because lipids produce twice the amount of energy (37 kJ/g) produced by carbohydrates (17 kJ/g) on combustion as a biofuel (Mehrabadi et al. 2015). The outcome of outdoor monitoring of biomass biochemical composition indicated that HRAOPs operating under natural environmental conditions preferentially generated a biomass rich in carbohydrate, thus making the biomass more suitable for anaerobic digestion to produce methane and CO_2. Despite the low biomass C/N ratio (7.1 to 7.8), the high carbohydrate levels ranging between 24.9 ± 0.6% and 25.6 ± 1.3% means that the MaB-floc biomass can be anaerobically co-digested with in-pond digester sludge, which according to Tijjani-Oshungboye (2011) has a high C/N ratio of 24:1.

Anaerobic digestion of biomass also produces CO_2 which can be recovered and added to HRAOPs to enhance MaB-floc biomass productivity while lowering greenhouse gas emissions from a wastewater treatment plant. Another added advantage of methane production on-site means it can be used directly as an energy feedstock for clean electricity sufficient to drive HRAOP paddlewheels. Anaerobic digestate is rich in macronutrients such as nitrogen and phosphorus; hence, it can be used in the agricultural sector as bio-fertiliser. Thus, a potential pathway for a biorefinery can be established in an IAPS treating sewage.

The introduction of IAPS into South Africa was meant to demonstrate it as a low cost, efficient and environmentally friendly technology that could be deployed in rural communities. Indeed, the technology's simplicity in operation and maintenance, as well as added benefits of a volarisable biomass, makes it appealing. However, the lack of technological advancement, the requirement for large land area and construction costs have hindered the deployment of microalgal wastewater treatment at a commercial scale. For instance, as of 2019, the cost of building such a technology roughly ranges between R26 million and R13 million for sewage treatment capacities of 1 megalitre and 0.25 megalitres respectively. This defeats its purpose as a low-cost technology where no technological upgrades have been made from the time of its inception in 1956 and where land has become limited.

6.2 Conclusion

IAPS demonstrated to be an efficient nutrient removal system with maximum average nitrogen and phosphorus removal efficiencies of 71% and 75% respectively. The MaB-floc biomass generated from HRAOPs demonstrated good settling efficiency in summer where it was predominated by *Pediastrum* sp. Settleability of biomass predominated by *Scenedesmus* sp. or *Desmodesmus* sp. can be improved by nitrogen limitation within HRAOPs. This is because nitrogen limitation induces intracellular accumulation of storage compounds which eventually force the *Scenedesmus* or *Desmodesmus* cells to sink. MaB-floc biomass productivity is a function of environmental conditions, particularly solar irradiance and pond water temperature. Photoinhibition of photosynthesis due to higher temperatures and solar irradiance significantly reduce biomass productivities. Rotifers that generally thrive in summer because of warm temperatures also contribute to reducing MaB-floc biomass productivities through exerting grazing pressure. HRAOP biomass biochemical composition is significantly influenced by species dominance. Biomass can be preconditioned using nitrogen starvation to enhance lipid content in

HRAOP MaB-floc biomass which makes an ideal feedstock for biodiesel. The outcome of outdoor monitoring of biomass biochemical composition indicated that HRAOPs operating under natural environmental conditions preferentially generated a biomass rich in carbohydrate, thus making the biomass more suitable as a co-substrate for anaerobic digestion to produce methane and CO_2. The CO_2 produced can be used to enhance MaB-floc biomass productivities while the digestate can be applied as a biofertiliser. IAPS is a perfectly designed system that operates in the way it is supposed to, but technological innovations are essential for the technology to compete with other wastewater treatment technologies.

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Appendices

Appendix A

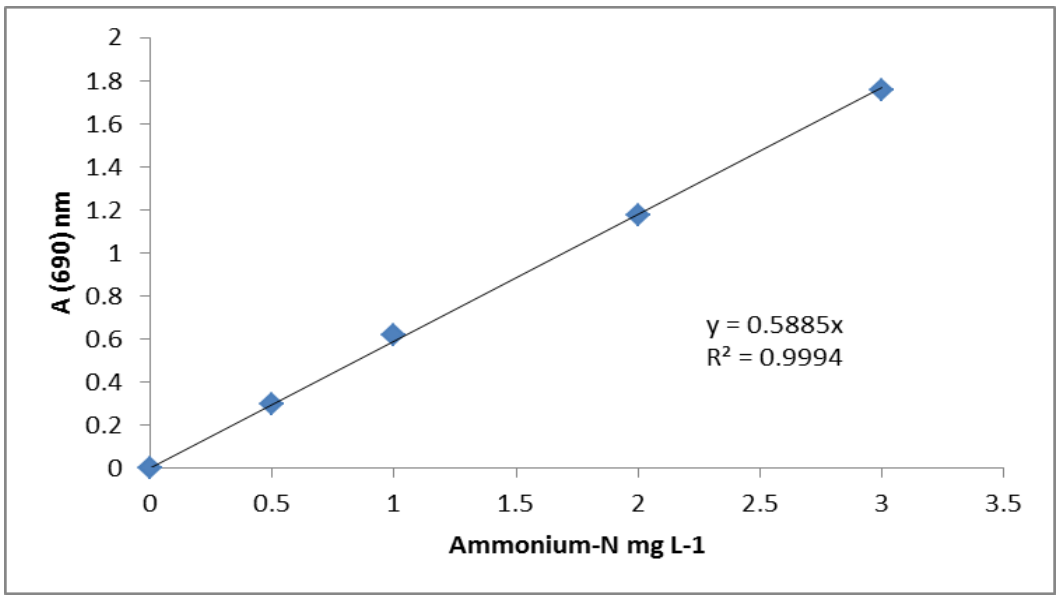

Figure A1: Graph showing the increasing concentrations of Ammonium-N at a wavelength of 690 nm. The curve was used to interpolate unknown Ammonium-N concentration in water samples.

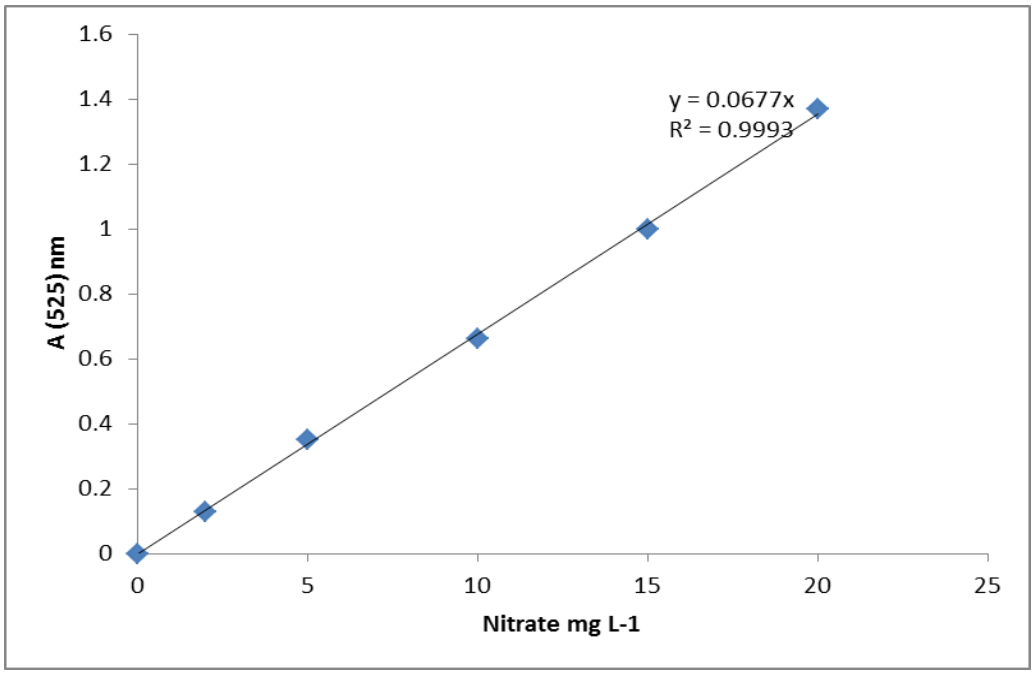

Figure A2: Graph showing the increasing concentrations of Nitrate-N at a wavelength of 525 nm. The curve was used to interpolate unknown Nitrate-N concentration in water samples.

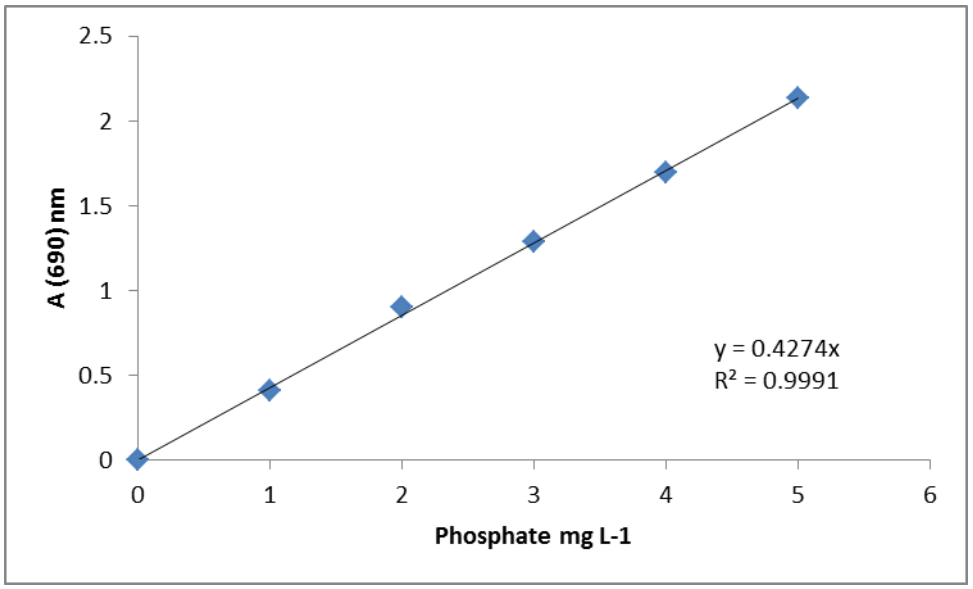

Figure A3: Graph showing the increasing concentrations of Phosphate-P at a wavelength of 690 nm. The curve was used to interpolate unknown Phosphate-P concentration in water samples.

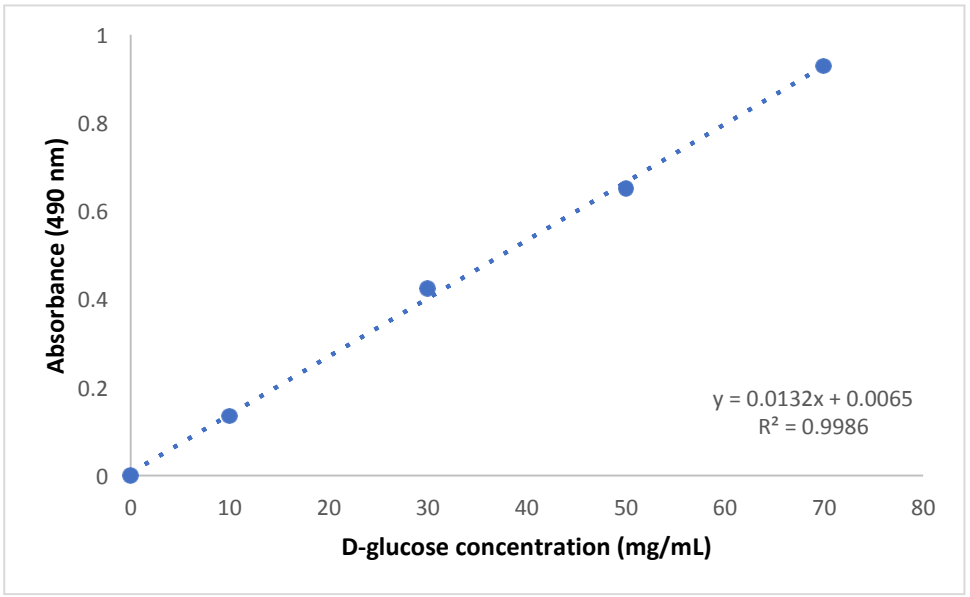

Figure A4: Graph showing the increasing concentrations of D-glucose at 490 nm using the Phenol-sulphuric acid assay. The curve was used to determine unknown carbohydrate concentrations in MaB-floc biomass.

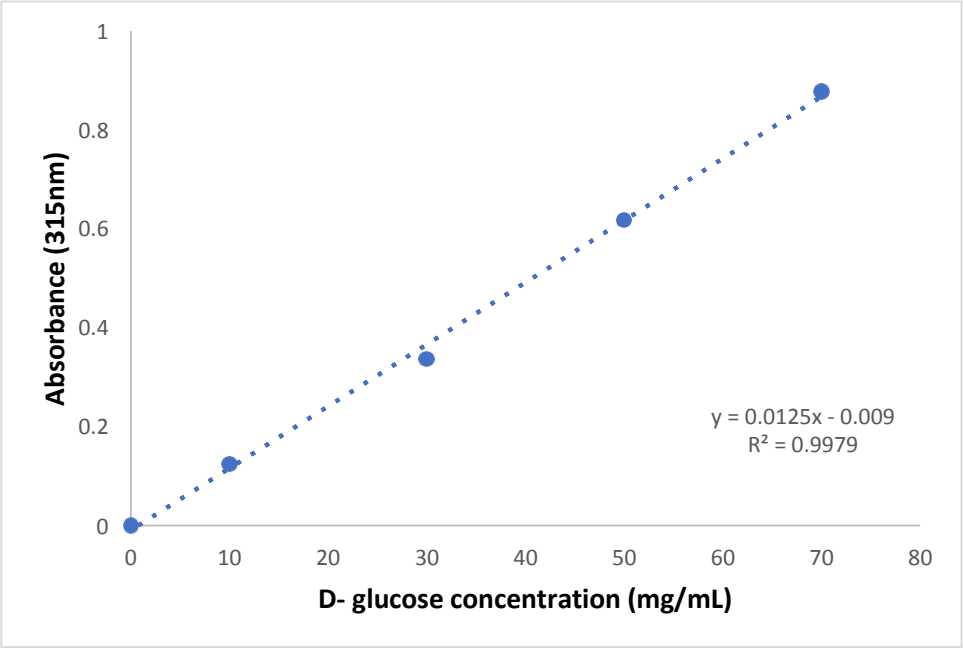

Figure A5: Graph showing the increasing concentrations of D-glucose at 315 nm using the Sulphuric acid- ultraviolet assay. The curve was used to determine unknown carbohydrate concentrations in MaB-floc biomass.

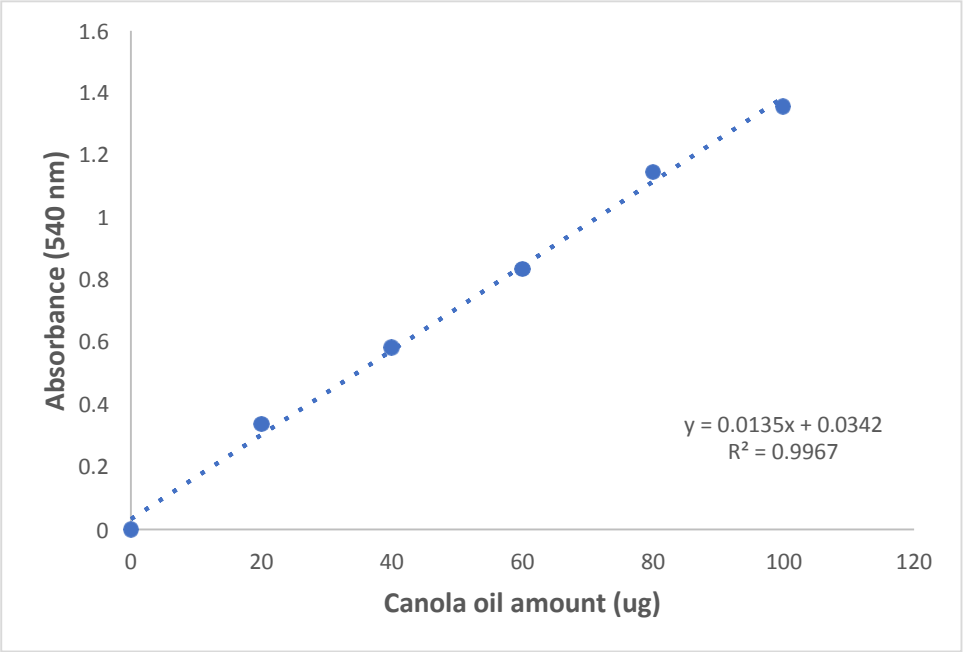

Figure A6: Graph showing the increasing amount of lipids in the form of canola oil at 540 nm using the Sulpho-phosphovanillin assay. The curve was used to determine unknown lipid concentrations in MaB-floc biomass.

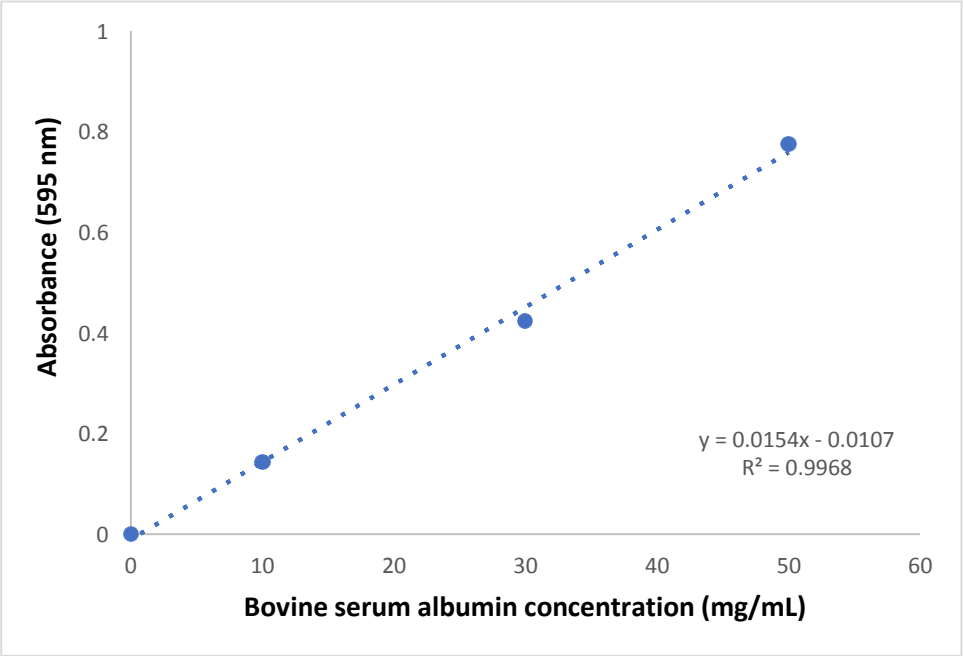

Figure A7: Graph showing the increasing amount of bovine serum albumin at 595 nm using the Bradford assay. The curve was used to determine unknown protein concentration in MaB-floc biomass.

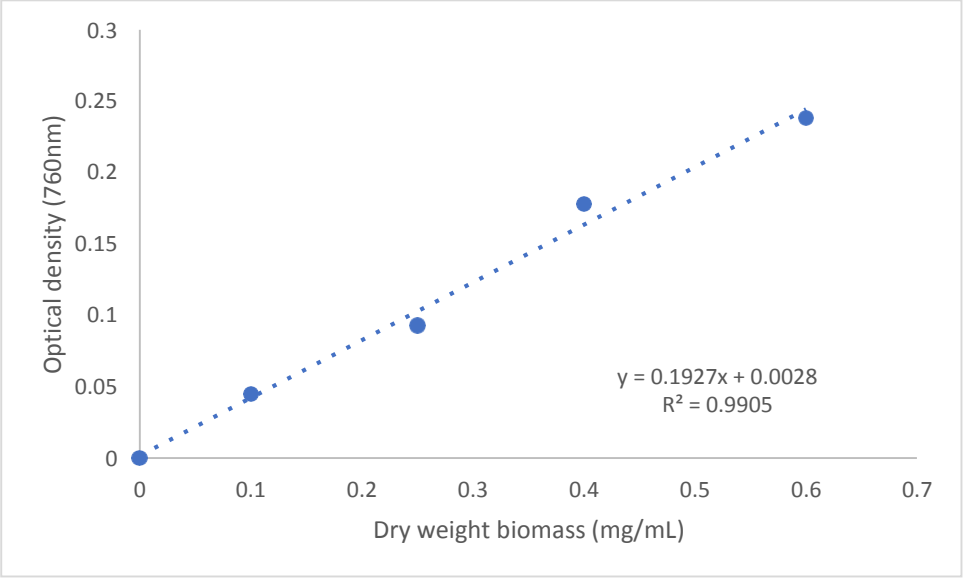

Figure A8: Graph showing increasing dry weight of *Scenesdesmus* culture biomass. The curve was used to determine unknown dry weight biomass in *Scenedesmus* culture.

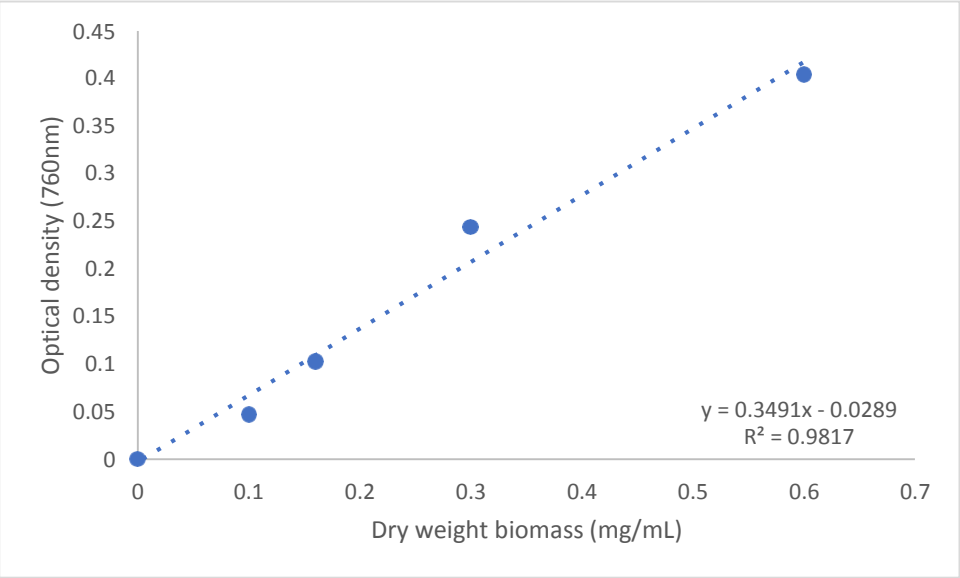

Figure A9: Graph showing increasing dry weight of *Chlorella* culture biomass. The curve was used to determine unknown dry weight biomass in *Chlorella* culture.

Appendix B

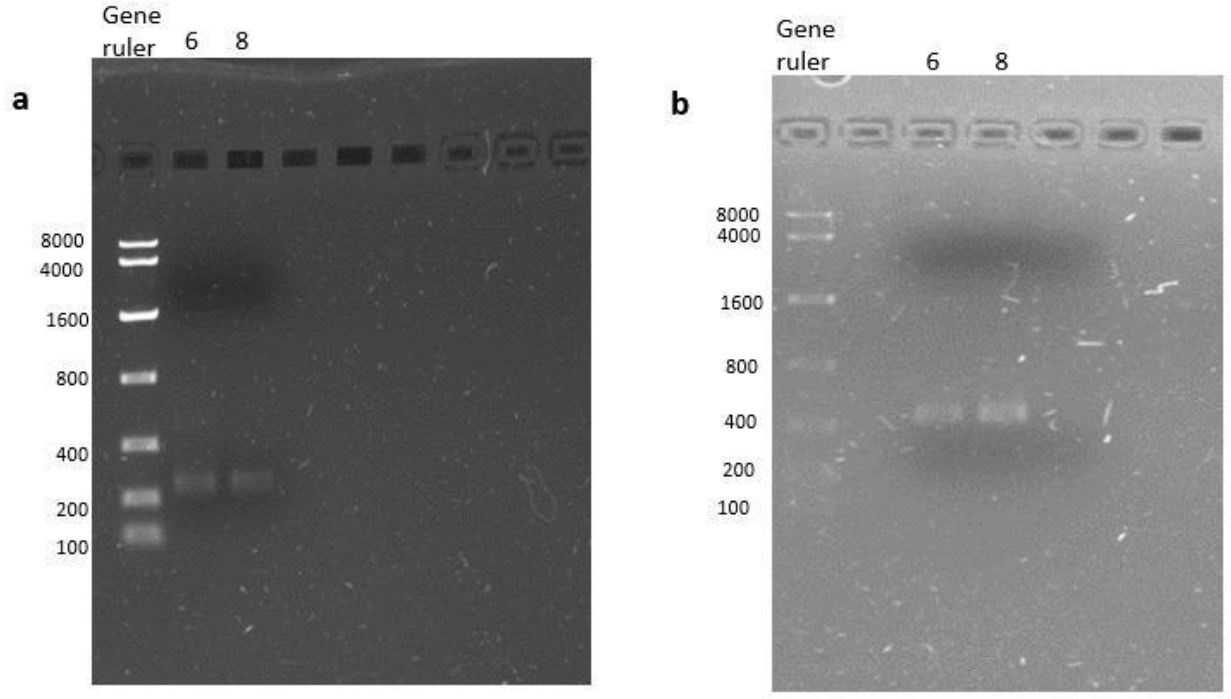

Figure B1: Illustration of **a)** 18S gel-purified 200 base pair PCR product using primers Miseq 1391f/EukBr (5uL loaded) **b)** 16S gel-purified 450 base pair PCR product(5uL loaded) using Primers: Miseq 16S 515f/9261r. The PCR product sizes were measured using a Kappa Express ladder.

Table B1: Metagenomic results of the most abundant genera making up the MaB-floc containing mixed liquor suspended solid collected **a)** in summer and **b)** in winter.

a	Genus	relative abundance	b	Genus	relative abundance
	Brachionus	14705		Desmosdesmus	9598
	Pediastrum	8406		Chlorella	2259
	Purpureocillium	3357		Scenedemus	1403
	Scenedesmus	1455		Micractinium	1144
	Desmosdesmus	1049			

Table B2: Metagenomic results of the most abundant bacterial phyla making up the MaB-floc containing mixed liquor suspended solids from high rate oxidation ponds.

Phyla	relative abundance
Euryarchaeota	8
Nanoarchaeaeota	3
Acidobacteria	359
Actinobacteria	358
Armatimonadetes	531
BRC1	9
Bacteria_unclassified	255
Bacteroidetes	23292
Chlamydiae	14
Chloroflexi	153
Cloacimonetes	2
Cyanobacteria	28
Deinococcus-Thermus	158
Dependentiae	15
Epsilonbacteraeota	1
Firmicutes	404
Gemmatimonadetes	3391
Hydrogenedentes	3
Kiritimatiellaeota	2
Nitrospirae	1
Omnitrophicaeota	1
Patescibacteria	13
Planctomycetes	16007
Proteobacteria	54762
Spirochaetes	80
Synergistetes	4
Tenericutes	29
Verrucomicrobia	1524

Table B3: Fatty acid composition of canola oil used as lipid standard

Fatty acid type	**% content of total lipid**
Saturated fatty acids	7.3
Polyunsaturated fatty acids	28.9
Omega-6 fatty acids	19.2
Omega-3 fatty acids	9.7
Alpha-linolenic acid	9.7
Monounsaturated fatty acid	63.7

Table B4: Bold Basal medium composition

Stock Solution	Volume (per litre distilled water)Concentration
$NaNO_3$ (25 g/ L)	10 mL
$CaCl_2$ (2.5 g/ L)	10 mL
$MgSO_4 .7H_2O$ (7.5 g/ L)	10 mL
K_2HPO_4 (7.5 g/ L)	10 mL
KH_2PO_4 (17.5 g/ L)	10 mL
NaCl (2.5 g/ L)	10 mL
P-IV metal solution	6 mL
Soil water	40 mL
Vitamin B_{12}	1 mL