

**CO-UTILISATION OF MICROALGAE
FOR WASTEWATER TREATMENT AND THE
PRODUCTION OF ANIMAL FEED SUPPLEMENTS**

A thesis submitted in fulfilment of the
requirements for the degree of

MASTER OF SCIENCE

of

RHODES UNIVERSITY

by

HAILEY E. JOHNSON

December 2010

Abstract

Microalgae have a variety of commercial applications, the oldest of which include utilisation as a food source and for use in wastewater treatment. These applications, however, are seldom combined due to toxicity concerns, for ethical reasons, and generally the requirement for cultivation of a single algae species for use as a feed supplement. These problems might be negated if a “safer” wastewater such as that from agricultural and/or commercial food production facilities were to be utilised and if a stable algae population can be maintained. In this investigation preliminary studies were carried out using an Integrated Algae Pond System (IAPS) for domestic wastewater treatment to determine the species composition in the associated High Rate Algae Ponds (HRAPs). The effect of different modes of operation, continuous versus batch, on nutrient removal, productivity and species composition was also investigated. Furthermore, indigenous species in the HRAP were isolated and molecularly identified as, *Chlorella*, *Micractinium*, *Scenedesmus* and *Pediastrum*. Additionally, the effect of the nor amino acid, 2-hydroxy-4-(methylthio)-butanoic acid (HMTBA) and its Cu-chelated derivative, on the growth and biochemical composition of *Chlorella*, *Micractinium*, *Scenedesmus*, *Pediastrum* and *Spirulina* was investigated.

Species composition in the HRAP was stable under continuous operation with *Micractinium* dominating > 90% of the algae population. Under batch operation the population dynamic shifted; *Chlorella* outcompeted *Micractinium* possibly due to nutrient depletion and selective grazing pressures caused by proliferation of *Daphnia*. Higher species diversity was observed during batch mode as slower growing algae were able to establish in the HRAP. Nutrient removal efficiency and biomass productivity was higher in continuous mode, however lower nutrient levels were obtained in batch operation.

HMTBA did not significantly affect growth rate, however treatment with 10 mg.L⁻¹ resulted in slightly increased growth rate in *Micractinium* and increased final biomass concentrations in *Chlorella*, *Micractinium* and *Spirulina* (although this was not statistically significant for *Micractinium* and *Spirulina*), which are known mixotrophic species. Algae treated with Cu-HMTBA, showed reduced final biomass concentration with 10 mg.L⁻¹, caused by Cu toxicity. Biochemical composition of the algae was species-specific and differed through the growth cycle, with high protein observed during early growth and high carbohydrate during late growth/early stationary phase. Additionally, 0.1 mg.L⁻¹ HMTBA and Cu-HMTBA

significantly reduced protein content in *Chlorella*, *Micractinium*, *Scenedesmus* and *Pediastrum*.

In conclusion, operation of the HRAP in continuous culture provided suitable wastewater treatment with high productivity of an ideal species, *Micractinium*, for use in animal feed supplementation. This species had 40% protein content during growth (higher than the other species tested) and dominated the HRAP at > 90% of the algae population during continuous mode. Addition of HMTBA (> 1 mg.L⁻¹) to algae cultivation systems and those treating wastewater, has the potential to improve productivity and the value of the biomass by enhancing protein content. Overall, the co-utilisation of microalgae for wastewater treatment and the generation of a biomass rich in protein, for incorporation into formulated animal feed supplements, represents a closed ecosystem which conserves nutrients and regenerates a most valuable resource, water.

Acknowledgements

First and foremost, I wish to express my gratitude to my supervisor, Prof. Keith Cowan, Director of EBRU, for his guidance and support throughout this project. I would like to thank all the staff at EBRU, and in particular Richard Laubscher for invaluable advice and input. Special thanks to all the students, Kubura, Prudence and Lerato for your support and for making my Masters experience that much more enjoyable ☺ Thank you also to Julie Coetzee, Nicodemus Mautsa and Michelle Isaacs for statistical and laboratory assistance; and thank you to Prof. Abdel-Hamid of the University of Mansoura, Egypt, for providing the *Spirulina* culture.

Special thanks to my digsmates, Hayley, Shan, Leanne, Xanthe, Lara, Sean, Matthys and Jessie, other friends and especially the GimpFace, for a really enjoyable couple of years – thanks for all the great times we had ☺ Also a very special thank you to my parents for your unfailing support throughout my University career.

Finally, I would like to acknowledge the Mellon Foundation and EBRU for providing financial support, without which I would not have been able to undertake this project.

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List of Abbreviations

AD	Anaerobic digester
ANOVA	Analysis of variance
ARA	Arachidonic acid
bp	Base pairs
BLAST	Basic local alignment search tool
BOD	Biological oxygen demand
BSA	Bovine serum albumin
CE	Constant environment
Cu	Copper
Cu-HMTBA	Copper-chelated 2-hydroxy-4-(methylthio)-butanoic acid
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
DWAF	Department of Water Affairs and Forestry
EBRU	Institute for Environmental Biotechnology, Rhodes University
EDC	Endocrine disrupting compound
EPA	Eicosapentaenoic acid
GLA	γ -linolenic acid
HMTBA	2-hydroxy-4-(methylthio)-butanoic acid
HPLC	High performance liquid chromatography
HRAP	High rate algae pond
HRT	Hydraulic retention time
IAPS	Integrated algae pond system
PCR	Polymerase chain reaction
PFP	Primary facultative pond
PUFA	Polyunsaturated fatty acid
SD	Standard deviation
TAE	Tris acetate ethylenediaminetetraacetic acid
WRC	Water Research Commission
WWTP	Wastewater treatment plant

Chapter 1. General Introduction

1.1 Mass culture of algae in high rate ponds and the generation of biomass

1.1.1 What are algae?

The term “algae” encompasses a diverse group of simple organisms, ranging from large multicellular seaweeds to microscopic single-celled organisms and includes both prokaryotic and eukaryotic phyla (Mata et al., 2010). Algae are typically photoautotrophic and generate organic compounds for growth and reproduction through photosynthesis. Some algae are however, mixotrophic, and hence are also able to obtain energy heterotrophically. Algae play a very important role in ecosystems as primary producers and contribute 40 to 50% of the oxygen in the atmosphere (Anderson, 2005).

Algae are difficult to define in terms of phylogeny as they do not descend from a common ancestor and are categorized in a variety of classes. Molecular sequencing, however, proposes eight or nine major phyla. These are the Eubacteria (cyanobacteria), Glaucophyta (sometimes classified as red algae), Euglenophyta, Cryptophyta, Haptophyta, Dinophyta (dinoflagellates), Orchophyta (flagellates, diatoms, brown algae), Rhodophyta (red algae) and Chlorophyta (green algae) (Graham and Wilcox, 2000). Microalgae, as the term implies, are microscopic algae found freely as single cells or in colonial form. The four most important classes of microalgae, in terms of abundance, are the diatoms (Bacillariophyceae), the green algae (Chlorophyceae), the blue-green algae (Cyanophyceae) and the golden algae (Chrysophyceae). Although predominantly aquatic, microalgae are found in terrestrial ecosystems ranging from cold Arctic regions to arid soils and are also found in extreme environments such as hot springs and alkaline and saline conditions (Masojidek and Torzillo, 2008).

1.1.2 Mass culturing of microalgae

Research in mass culturing of algae has been carried out in many parts of the world since the early 1950s. Initial interest was for the use of algae as a food source. This was sparked by early studies which indicated that the biochemical composition of algae, mainly protein and lipid content, could be manipulated by altering the growth conditions (Spoehr and Milner, 1949). It was believed therefore that algae could serve as a rich source of protein at

potentially a low cost. Further research at the time however, was restricted to short term studies which showed yields of less than $5 \text{ g.m}^{-2}.\text{day}^{-1}$ (Goldman, 1979). This changed in the late 1950s when Oswald and colleagues revolutionised large-scale algae culture by demonstrating simultaneous wastewater treatment and biomass production. In the process microalgae were grown in large outdoor cultures to remove nitrogen and phosphorous from wastewater streams and provide O_2 for the aerobic breakdown of organic compounds by bacteria. The bacteria in return provided CO_2 for photosynthesis, thereby facilitating the symbiotic relationship between these microorganisms (Figure 1-1).

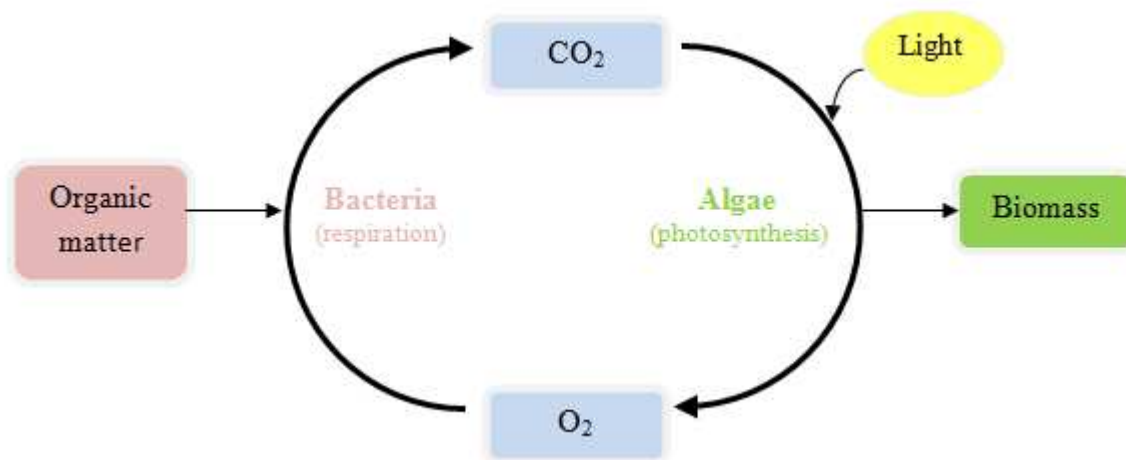


Figure 1-1: Inter-relationship between oxygen producing algae and oxygen consuming bacteria found in algae ponds treating wastewater (Adapted from Muñoz and Guieysse, 2006).

Algae biotechnology for wastewater treatment is still used today in the form of facultative ponds and high rate algae ponds (HRAPs). Facultative ponds are stagnant, deeper than one meter and contain algae growing near the surface in aerobic zones, creating a quiescent layer. Below this layer, wastewater is injected and suspended solids are allowed to settle into the anoxic zone where fermentation takes place. In this way suspended solids in the wastewater are digested, and the release of methane (CH_4) and CO_2 results (Oswald, 1988). In an Integrated Algae Pond System (IAPS), the wastewater stream flows from a facultative pond into a HRAP where nutrient removal and the breakdown of organic compounds take place. HRAPs are designed to optimise algae growth by maximising light penetration and by keeping the algae in suspension. HRAPs are shallow raceways (approximately 30 to 50 cm deep) which are mixed using a paddle wheel. Use of HRAPs is particularly prominent in wastewater treatment because photosynthetic oxygenation greatly reduces operational costs

and prevents the volatilisation of organic pollutants which can occur with mechanical aeration (Muñoz and Guieysse, 2006). Algae also facilitate wastewater treatment by enhancing the removal of heavy metals and pathogens (Aziz and Ng, 1992; Muñoz et al., 2006; García et al., 2008), and have also been shown to directly remove pollutants such as endocrine disrupting compounds (EDCs) through heterotrophic or mixotrophic feeding (Tarlan et al., 2002).

The first commercial-scale culturing of algae took place in the early 1960s in Japan with the production of *Chlorella* (Tsukuda et al., 1977). Since then a large amount of research has been carried out on the mass culture of algae for the generation of valuable products. Currently algae biomass can be generated in closed or open photobioreactors, either indoors or outdoors, or in closed fermenter systems heterotrophically (Borowitzka, 1999). Generally indoor cultures are limited to laboratory scale and require artificial illumination, whereas commercial culturing of algae is commonly carried out in rather unsophisticated outdoor systems as this is more cost-effective (Borowitzka, 1999; Ugwu et al., 2008). Whilst a large amount of work has focused on closed photobioreactors, as they prevent CO₂ and evaporative losses, and enable the maintenance of pure cultures, the majority of large scale production takes place in open systems due to the high cost and maintenance requirements of closed systems (Ugwu et al., 2008). Frequently used culture methods are large open ponds, circular ponds with a rotating arm to mix, raceway ponds with a paddle wheel, and large clear bags (Borowitzka, 1999).

In developing countries, such as South Africa, the benefits of open systems out-weigh those of closed systems due to ease of operation, minimal energy requirements, and the availability of large areas of non-arable land for the construction of ponds in remote areas. The utilisation of HRAPs for wastewater treatment has been established on a pilot scale in South Africa in the form of the IAPS. This system is based on the Algal Integrated Wastewater Ponding Systems (AIWPS) developed by William Oswald in the 1980s for low cost sewage treatment and utilises both anaerobic and aerobic biological processes for the treatment of wastewater. In 1990 the Environmental Biotechnology Research Unit (EBRU, now the Institute for Environmental Biotechnology) at Rhodes University undertook a program to study the application of IAPS for the treatment of different wastewaters. This program was initiated by the Water Research Commission (WRC) and over a period of 9 years set out to determine the value of IAPS for the treatment of domestic sewage, as well as effluents from abattoirs,

tanneries, wineries, distilleries and mines (acid mine drainage) (Horan et al., 2007). The results from the 9 year study showed that when the two HRAPs were operated in series, the effluent discharged was within the South African standard for nitrogen and phosphorous removal and for disinfection. Additionally, it was shown that a HRAP can be operated as a free-standing tertiary treatment and can be used as an add-on installation in under-performing treatment plants. The IAPS therefore demonstrated a low-cost, low-maintenance means for treating wastewater from small communities by utilising algae biotechnologies (Wells, 2005). This is particularly important for South Africa as water scarcity is an evident problem and the implementation of such technologies is vital for sustainability.

1.1.3 Commercial applications of microalgae

In order to add value to the wastewater treatment process, a means of further utilising the biomass is needed. To date, algae biomass has found a variety of uses, one of the foremost being as a source of feed for fish, crustacea, shell-fish, poultry, cattle, rabbits, pigs and humans (Aaronson et al., 1980). Although the predominant reason for utilising algae as a food source is due to its protein content, a variety of nutrients, namely carbohydrates, lipids, vitamins, pigments, minerals and trace elements, are also present (Becker, 2007). In most algae species the nutritional quality of protein (determined by the amino acid profile) is comparable to conventional protein sources (Becker, 2004). After processing of the biomass (usually to disrupt the cellulosic cell wall), the digestibility and protein efficiency ratio (weight gain per unit of protein consumed) are also similar to that of more conventional plant proteins (Becker, 2007). Although algae have been incorporated into a variety of products such as pastas, snack foods, and beverages, they are mainly marketed as health foods and supplements in the form of tablets, capsules and liquids. Despite the nutritional advantages, microalgae have not gained favour as a food substitute for human consumption due to its powdery consistency, dark green colour and slightly fishy smell (Becker, 2007). As health foods, generally it is claimed that algae act as immunostimulators, and *Spirulina* has been found to help maintain healthy intestinal bacteria (Pulz and Gross, 2004; Spolaore et al., 2006). Whilst it may not be feasible to utilise algae grown on domestic waste streams for human consumption due to sanitary and toxicity concerns, the use of this biomass for animal feed is still plausible.

Nutritional and toxicological assessment of algae biomass has shown that it is a suitable source of feed for animals (Becker, 2007). In small doses (5-10% feed supplement) algae are reported to positively affect the immune system, fertility, weight control, and condition of the skin and coat (Pulz and Gross, 2004; Spolaore et al., 2006). Currently the predominant use of algae as a feed supplement is in poultry production to increase the fatty acid composition and carotenoid content of egg yolks, and the docosahexaenoic acid (DHA) content of the flesh (Pulz and Gross, 2004; Fredriksson et al., 2006; Kalogeropoulos et al., 2010). In aquaculture microalgae are used as a food source for molluscs at all growth stages, crustaceans and some fish species in the larval stage, and for culturing of zooplankton which feed late-larvae and juveniles of some crustacean and fish species (Brown et al., 1997; Mitra et al., 2007; Ferreira et al., 2008). Of the total world production of algae it is estimated that 30% is sold for animal feed applications (Spolaore et al., 2006; Becker, 2007).

Other applications of algae include use as a fertilizer and soil amendment, as well as for the production of pigments, vitamins, polysaccharides, sugars, pharmaceuticals, amino acids, phytol, essential oils, enzymes and bioflocculants (Aaronson et al., 1980; Borowitzka, 1988). As a soil amendment, macroalgae have been used in coastal regions all over the world to increase water binding capacity and mineral composition (Pulz and Gross, 2004). Microalgae in soil ecosystems contribute to the soil fertility by producing polymers which assist with particle adherence and water storage. Some soil algae are also able to fix nitrogen and produce bioactive compounds which influence higher plants by promoting germination, leaf or stem growth, and flowering (Pulz and Gross, 2004). As a soil additive dry algae biomass can be used as a slow release fertilizer and shows comparable results to synthetic fertilizer with respect to dry weight production and nutrient composition (Mulbry et al., 2005).

Microalgae produce pigments such as chlorophylls, phycobiliproteins and carotenoids. In some species such as *Dunaliella salina* and *Haematococcus pluvialis*, secondary pigments, β -carotene and astaxanthin respectively, may be produced in larger quantities than the primary chlorophyll pigments (Borowitzka, 1988). The structures of various carotenoids found in algae are shown in Figure 1-2. Carotenoids have a variety of uses as “natural” food colourings and feed additives. As a feed additive, it is used to enhance, for example, the colour of salmonid flesh and the colour of egg yolks, and also to improve the health and fertility of lot-fed cattle (Borowitzka, 1988; Kerby and Stewart, 1988; Margalith, 1999). Whilst naturally produced astaxanthin cannot compete commercially with the synthetic form,

there are a few applications where the naturally produced product is preferred due to enhanced deposition in tissues (Spolaore et al., 2006). Biliproteins occur in Rhodophyceae, Cryptophyceae and Cyanophyceae and are sold as a natural blue pigment to be used in health foods and cosmetic products (Borowitzka, 1988). Additionally, they are used in industry and research immunology laboratories as powerful and highly sensitive fluorescent reagents (Spolaore et al., 2006).

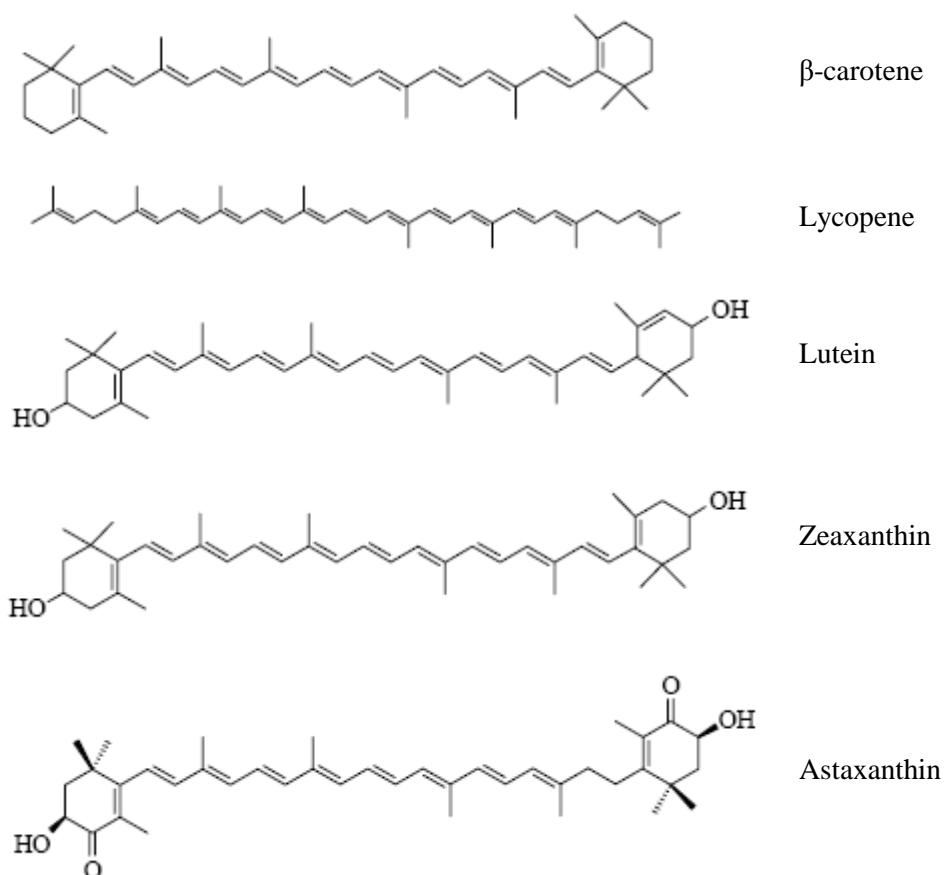


Figure 1-2: Structures of some commercially important carotenoid pigments obtained from algae.

Carotenoids are natural antioxidants produced to counteract the cell damaging effects of reactive oxygen species generated as by-products of photosynthetic activity (Pulz and Gross, 2004). In humans the free-radical scavenging capacity of antioxidants protects against numerous life-threatening diseases including various forms of cancer, coronary heart disease, diabetes, premature aging and arthritis. Humans are unable to synthesise the full complement of required antioxidants and therefore are dependent on obtaining these from the diet (Demmig-Adams and Adams, 2002; Dufosse et al., 2005). For this reason microalgal

carotenoids appear in the nutraceutical market and even though synthetic carotenoids are cheaper to manufacture, carotenoids from microalgae have the advantage of supplying natural isomers in their natural ratio (Spolaore et al., 2006). In addition to its antioxidant activity, β -carotene is a provitamin A. Vitamin A is important in the body as it acts as an immunostimulator, it protects against eye diseases and helps to maintain healthy skin (Dufosse et al., 2005; Spolaore et al., 2006).

Other carotenoids produced by microalgae which have good antioxidant properties include lycopene, zeaxanthin, and lutein (Demmig-Adams and Adams, 2002; Granado et al., 2003). Zeaxanthin and lutein are xanthophylls (oxygenated carotenoids) with no provitamin A activity. However, their consumption is directly associated with a reduction in the risk of arteriosclerosis, cataracts, age-related macular degeneration, multiple-sclerosis and cancers (Bhosale and Bernstein, 2005).

Another important group of compounds derived from algae are polyunsaturated fatty acids (PUFAs) which are essential components of cell membranes in higher eukaryotes as they confer flexibility, fluidity and selective permeability (Pulz and Gross, 2004; Ward and Singh, 2005). Animals lack the enzymes required for the synthesis of PUFAs of more than 18 carbons and therefore these need to be obtained through diet (Spolaore et al., 2006). The most common source of PUFAs in the human diet is fish and fish oils. The primary source of these PUFAs however is microalgae, either through direct consumption or through consumption of zooplankton which feed on microalgae (Jiang et al., 1999; Spolaore et al., 2006). Due to some of the disadvantages associated with fish oils such as fishy smell, unpleasant taste, presence of environmental pollutants, instability and presence of mixed fatty acids, microalgae have been considered for the direct production of PUFAs (Jiang et al., 1999; Ratledge, 2004; Ward and Singh, 2005; Spolaore et al., 2006). The major targets of production are γ -linolenic acid (GLA), arachidonic acid (ARA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) (Ward and Singh, 2005). The structures of these PUFAs are illustrated in Figure 1-3.

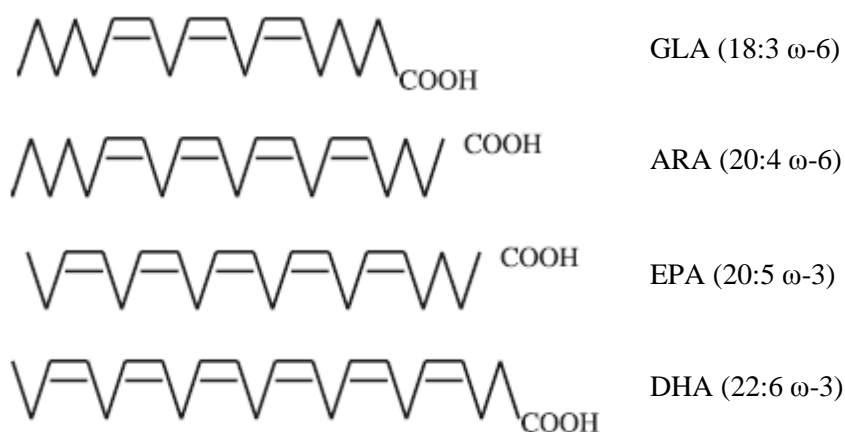


Figure 1-3: Structures of microalgae derived PUFAs with high pharmacological and nutritional value.

Currently DHA is the only commercially available algae-derived PUFA and is used in the preparation of infant milk formulas (Spolaore et al., 2006). DHA is a major structural component of grey matter in the brain and in infants it is necessary for the development of normal neural and retinal functions, and supports good cardiovascular health (Ratledge, 2004; Ward and Singh, 2005). This PUFA is commercially produced by Martek in the USA through the heterotrophic culturing of *Cryptocodinium cohnii* which can accumulate up to 40-50% DHA with no other PUFAs in excess of 1% (Jiang et al., 1999; Spolaore et al., 2006). EPA and ARA production have economic potential due to their pharmacological properties. These PUFAs are precursors of eicosanoids which are hormone-like compounds such as prostaglandins, thromboxanes and leukotrienes. A balanced uptake of EPA and ARA can prevent a number of eicosanoid dysfunctions such as rheumatoid arthritis, atherosclerosis, arrhythmias and thrombosis (Wen and Chen, 2003). To date however, algae productivities of EPA (by *Phaeodactylum triconutum* and *Nitzschia laevis*) are limited to 0.1-0.3 g.L⁻¹.day⁻¹ compared to DHA productivities of 0.4-1.2 g.L⁻¹.day⁻¹ (Veloso et al., 1991; Ward and Singh, 2005).

Carbohydrates accumulate in algae cells as storage materials, are present as osmoregulators and make up a significant proportion of biomass in the cell walls (Aaronson et al., 1980; Borowitzka, 1988). Polysaccharides have been harvested from macroalgae and are used as viscosifiers (thickening agents), flocculants and lubricants (Borowitzka, 1988). Some algal polysaccharides have shown pharmacological importance in having anti-cancer properties and the ability to stimulate the immune system (Borowitzka, 1988; Masojidek and Torzillo,

2008; Courtois, 2009). The microalga with the most potential for mass cultivation for polysaccharides is the red alga *Porphyridium* which produces large amounts of extracellular polysaccharides under certain conditions (Borowitzka, 1988).

Antibiotics produced by algae are largely unidentified but are thought to include fatty acids, phenolic substances, polysaccharides, alcohols, tannins and terpenoids, and are toxic to other algae, bacteria, fungi, viruses and protozoans (Kerby and Stewart, 1988). Some useful clinical compounds have been isolated from macroalgae. For example, an antihelminthic (for the treatment of parasitic worms) has been isolated from *Digenea simplex* (Aaronson et al., 1980). Whilst a wealth of novel pharmacologically useful compounds has been isolated from algae, often these compounds do not enter the market due to the economics of pharmacological development. Also, due to the difficulties of culture and collection as well as the fact that these compounds are usually only present in very small amounts, it is likely that an identified compound would be turned over to synthetic chemists to produce (Chapman and Gellenbeck, 1989).

Algae are autotrophic and therefore are ideally suited to incorporate stable (non-radioactive) isotopes ($^{13}\text{CO}_2$, $^{15}\text{NO}_3^-$, $^2\text{H}_2\text{O}$) into their biomass, generating labelled highly valued organic compounds such as amino acids, carbohydrates, lipids and nucleic acids (Behrens et al., 1994; Spolaore et al., 2006). Labelling of these compounds enables the determination of their structure at an atomic level, as well as the interaction of proteins with ligands. Isotopically labelled nutrients can also be used in clinical metabolic studies such as breath diagnosis tests to determine the metabolic activity of certain organs. Tests of this type are advantageous in that they are non-invasive and relatively simple to perform (Behrens et al., 1994).

Whilst there are many potential uses of microalgae, to date only a few algae species are commercially produced. The major ones are *Spirulina*, *Chlorella* and *Aphanizomenon flos-aquae* for health food and cosmetics (3 000, 2 000 and 500 t dry weight per year respectively), *Dunaliella* for β -carotene (1 200 t/year), *Haematococcus pluviialis* for astaxanthin (300 t/year), and *Cryptocodinium cohnii* for docosahexanoic acid (DHA, 240 t oil/year). On a smaller scale other species are cultured for aquaculture and the production of stable isotope biochemicals (Spolaore et al., 2006).

1.1.4 Factors affecting productivity

In the generation of algae biomass, optimisation of the system is important for improved productivity. In the case of combined biomass generation and wastewater treatment, removal of contaminants and nutrients must also be of the correct standard. Thus, wastewater treatment efficiency is closely linked to productivity of the biocatalyst (Azov and Shelef, 1982). Biomass generation in HRAPs is influenced by a number of factors which are vital in the development of large scale algae processes and these can be divided into biotic, abiotic, and operational (Table 1-1). Under conditions where a certain parameter limits photosynthesis, modification of that parameter will affect productivity whereas modification of other parameters will have little or no effect (Harlin and Darley, 1988).

Table 1-1: Factors influencing algae growth.

Abiotic	Biotic	Operational
Light	Algae competition	Turbulence
Temperature	Grazers	Depth (affects light intensity)
Nutrient concentration	Pathogens	Retention time
CO ₂ and pH		Dilution rate
O ₂		

Optimal operation for HRAPs is dependent mainly on location and prevailing climate as the major factors influencing productivity are solar irradiation and culture temperature (Azov and Shelef, 1982). Retention time in a HRAP, therefore, is usually regulated depending on temperature. In wastewater treatment there is a fixed flow rate and independent variables, area and depth, are manipulated to produce an optimal retention time. By increasing the depth during colder periods a longer retention time can be achieved without compromising light penetration as there will be a lower culture density. At the same time greater thermal stability is also provided (Azov and Shelef, 1982). Optimal retention time is the time required to achieve maximal productivity and is usually between 2-6 days. In practice, retention time is usually slightly longer than optimal in order to prevent washout of the culture (Muñoz and Guieysse, 2006).

Mixing of mass cultures is essential to promote efficient gas and nutrient exchange as well as better temperature and pH control (Anderson, 2005). In outdoor mass culture, paddle wheels

are often used to provide efficient but gentle mixing (Muñoz and Guieysse, 2006). This is important because increased turbulence increases the growth rate of an algae population due to decreased length of light/dark cycles and erosion of the boundary layer around cells that contains depleted nutrients; however, too much turbulence results in increased shear which damages sensitive algae (Richmond et al., 1980; Fogg, 1991; Camacho et al., 2000).

Algae and cyanobacterial diseases can occur in mass cultures and when they do it is important to prevent the spread of infection (Shilo, 1980). Infection can occur in the form of lytic bacteria, phycoviruses, fungi and cyanophages (Abeliovich, 1980; Shilo, 1980). Whilst there can be detrimental effects on the culture, it is possible to prevent such occurrences by knowing the biology of these organisms and their growth requirements. For example, in a study by Abeliovich (1980), infection by the fungus *Chytridium* was controlled by promoting an O₂-low period during the night which ensured that the fungus was not able to complete its lifecycle.

Infestation by zooplankton can have devastating effects on algae populations in mass culture. Rotifers and cladocerans are commonly found in HRAPs and are able to reduce algae populations by up to 90% in as little as two days (Lincoln et al., 1983). Some measures which have been implemented to control grazing include the addition of chemicals such as ammonia and quinine sulphate, and the use of screens (to remove larger grazers), and the reduction of dissolved oxygen levels in which aerobic zooplankton are unable to survive (Loosanoff et al., 1957; Lincoln et al., 1983; Moreno-Garrido and Cañavate, 2001). However, sometimes these measures are ineffective as they are usually species specific, and often eggs, juveniles or adults are left behind which can quickly re-introduce (Loosanoff et al., 1957). Rotifer grazing can also impact the population dynamics of algae in HRAPs. The presence of rotifers often triggers a population shift to predominantly *Micractinium*. This is believed to occur as *Micractinium* has spines which protect from the feeding activity (Schlüter et al., 1987). Fortunately, zooplankton do not always thrive in HRAPs as their growth is compromised by short retention times (Benemann et al., 1977).

1.1.5 Control of algae species composition in mass culture

The monoculture of algae species for specialised applications seems to have only limited success (Goldman, 1980). In large outdoor ponds, maintenance of a monoculture is usually

achieved using organisms that grow under extreme conditions thereby preventing competition from other organisms. For example, the culture of *Dunaliella salina* in hyper-saline conditions provides a selective advantage and therefore the generation of a relatively pure culture (Moreno-Garrido and Cañavate, 2001). *Spirulina* cultivation is another example as this species grows comfortably in highly alkaline solutions (Goldman, 1980).

Controlling and/or predicting algae species in a HRAP is of vital importance as certain genera have more desirable properties which make algae culture simpler and more economical (Azov et al., 1980; Tseng et al., 1991). For example, some algae are able to settle more quickly making harvesting easier; and certain species may be suitable for the production of commodity products (Azov et al., 1980).

A HRAP is a flexible system with a relatively low species diversity in which rapid succession of the predominant species can occur in response to various changes. Understanding algae population dynamics in a HRAP is therefore not an easy task as there are many factors which affect species composition, including environmental, biological, operational and historical factors, and the way in which these factors interrelate (Benemann et al., 1977). Although many algae species co-exist in a HRAP there are usually only one or two species that dominate at any particular time. The dominant species may change under certain circumstances and usually occurs during a transition period in which both the algae concentration and growth rate are quite low and there is no particular dominant species (Azov et al., 1980). This can have a major impact not only on the efficiency of wastewater treatment, but also on the biomass produced and the products of value being sought.

Factors which have been found to affect species dominance include season (irradiance and day length), temperature, pH, organic load, retention time, pond depth, agitation, method of charging the pond water (batch mode or continuous culture) and the concentration of nutrients (Azov et al., 1980; Tseng et al., 1991). In HRAPs, organic load varies greatly depending on the waste that is being treated and is one of the main factors influencing the population composition. Avoz et al. (1980) found that a biological oxygen demand (BOD) of 250 mg.L⁻¹ resulted in a dominance of *Scenedesmus*, whereas a BOD of 60 mg.L⁻¹ resulted in a dominance of *Micractinium*. Generally, in the most concentrated wastes, only anaerobic fermentative bacteria grow, and as the waste is diluted, photosynthetic bacteria, flagellated euglenoids, blue-green algae, green algae, diatoms and nitrogen-fixing bacteria appear in

succession (Benemann et al., 1977). A comprehensive compilation of data showed that the eight most pollutant-tolerant algae genera are *Euglena*, *Oscillatoria*, *Chlamydomonas*, *Scenedesmus*, *Chlorella*, *Nitzschia*, *Navicula*, and *Stigeoclonium*, and more specifically the five most tolerant species are *Euglena viridis*, *Nitzschia palea*, *Oscillatoria limosa*, *Scenedesmus quadricauda*, and *Oscillatoria tennis* (Palmer, 1969). In general, species diversity tends to be higher in more lightly loaded tertiary ponds as these ponds are more stable. In more heavily loaded ponds, the biomass is not allowed to fully stabilise and generally only one or two species predominate (Benemann et al., 1977). Contrary to these findings, de Godos et al. (2009) found that high organic loading supported a higher diversity; however this could also have been attributed to the moderate temperatures and irradiances of the summer period during which the study took place.

Food webs are complex systems which additionally affect species diversity, not only in terms of algae grazing but also by consumer-driven nutrient recycling (Schlüter et al., 1987; Kato et al., 2007). Different algae species utilize nutrients at different rates and ratios and, on a short timescale, nutrients such as N and P in the form of ammonium and phosphate, released by herbivores, are the main nutrient supplies for algae (Kato et al., 2007). This may be more applicable to batch cultures because in continuous cultures the population of herbivores may not be allowed to stabilise sufficiently to have a significant effect, and in wastewater treatment the nutrient supply is provided predominantly by the influent waste being treated (Benemann et al., 1977).

Some mechanisms have been described to control algae genera in HRAPs. Two such techniques are size-selective recycling and nutrient limitation (Benemann et al., 1977). In size selective-recycling a portion of harvestable algae (harvested by a microstrainer) is returned to the HRAP where it has an advantage over non-harvestable faster growing algae because washout is prevented. In this way and with time, most of the algae in the pond will be the desired species and a reduced amount of recycling is required. An example of species control by nutrient limitation is the growth of nitrogen-fixing algae in ponds enriched with phosphate and CO₂ but lacking N (Benemann et al., 1977). The success of these two methods, however, is debatable as the desirable species can be maintained for only a short period, after which other species that do not require recycling begin to dominate. This technique may therefore be useful only in establishing a harvestable culture more rapidly or maintaining it for longer (Benemann et al., 1980).

Operational features of HRAPs such as agitation method, pond loading, pond depth and hydraulic retention time can be altered to influence the population size and dynamic; however, environmental factors which cannot be controlled will also have an impact (Benemann et al., 1977). Hydraulic retention time affects population dynamics due to differences in specific growth rates. Algae with low growth rates such as *Euglena* may wash out at very short retention times (of 2 days), whereas with longer retention times, algae with rapid growth rates (such as *Chlorella*), may lose advantage. In deeper ponds motile algae such as *Euglena* have an advantage over *Chlorella* and *Scenedesmus*, whereas agitation of a pond is disadvantageous. Similarly, high shear forces, such as those generated by jet aerators, is unfavourable for sensitive algae such as *Micractinium* as it breaks their setae (Azov et al., 1980).

From the literature it appears that algae most commonly occurring in HRAPs are species such as *Chlorella*, *Scenedesmus* and *Micractinium*. Whilst dominance can be controlled to a certain extent, the fact that there are many aspects influencing their success makes the task quite daunting, especially as certain species may persist even under unfavourable conditions before being replaced by those more suited (Azov et al., 1980). Additionally, although distinct changes in populations occur seasonally, there appears to be very little comparable trend observed between studies done at different sites (Viviers and Briers, 1982; Canovas et al., 1996). This is probably due to the many other confounding elements that determine species dominance. In order to determine a means of valorising the biomass generated in a HRAP treating wastewater, a site specific study of the population dynamics and the influencing factors would need to be carried out over an extended period of time.

1.2 Problem statement

Utilisation of algae for wastewater treatment is a well established technology with a simple, efficient process that produces two benefits: sustainability of water value and the generation of valuable biomass. In wastewater treatment the biomass generated in HRAPs generally comprises a mixed population of algae and bacteria with changing population dynamics. The value of the biomass depends on the species present and the biochemical composition thereof. Biochemical characterisation is therefore necessary in order to determine the appropriate means of biomass valorisation. It is also important to establish factors which influence any changes in biomass content and composition to facilitate management of the system for maximum productivity. Although IAPS are known to be effective for wastewater treatment, in-depth characterisation of the resultant algae biomass has not always been carried out. This however is an absolute requirement if the system is to be effectively and sustainably managed for both wastewater treatment and biomass valorisation.

1.3 Hypothesis

Algae biomass generated in a HRAP treating wastewater can be biochemically altered to provide a suitable source of animal feed.

1.4 Aims and objectives

The aim of this project was to determine factors affecting the nutritional value of algae biomass generated in HRAPs treating domestic wastewater and determine whether biomass composition can be manipulated by altering the culture conditions.

The specific objectives were:

1. Characterise and compare the biomass generated in a HRAP operating in continuous and batch mode.
2. Determine the effects of 2-hydroxy-4-(methylthio)-butanoic acid (a methionine precursor) on the biochemical composition of algae species isolated from a HRAP.

Chapter 2. Species Isolation, Identification and Composition in a High Rate Algae Pond

2.1 INTRODUCTION

Pure algae cultures are necessary for fundamental research and are obtained by isolating species found in environmental samples and growing these in pure culture. Many of the techniques and media used today for culturing of algae were developed in the late 1800s and early 1900s, and enable the determination of species diversity and species specific characteristics (Preisig and Anderson, 2005). The first attempt to culture algae in a solution containing a few organic salts was by the Russian plant physiologist Famintzin, in 1871, and the first pure cultures were reported in 1890 by the Dutch microbiologist Beijerinck. These were obtained by mixing a water sample with gelatine; however, this proved unsatisfactory due to gelatine digestion by bacteria, and in 1896 Klebs in Switzerland was the first to use agar in Petri dishes. Other methods that were subsequently developed included the use of a micropipette to isolate algae cells and the dilution isolation method, developed by Miquel in the late 1800s (Preisig and Anderson, 2005).

By the early 1900s the successful isolation of many different species of algae had been documented and an understanding of nutrient and physiological requirements for growth began to emerge (Preisig and Anderson, 2005). Since the discovery that the biochemical composition of algae is species related and can be altered depending on growth conditions, many different species have been cultured and studied for the production of valuable products (Borowitzka, 1999; Ugwu et al., 2008). To date a number of methods have been described for isolating species of algae. One which is commonly used is to spread, streak or spray a field sample onto solid enriched agar medium and, after visible colony formation is observed, transfer single algae colonies into liquid medium for further cultivation. This method is simple and effective for isolating species for further studies under controlled laboratory conditions.

HRAPs are the major biocatalytic compartment in IAPS and are responsible for nutrient removal and disinfection of the water to be treated; processes of nutrient removal and disinfection occur as a result of the growing biomass. In well managed systems, this biomass comprises mostly bacteria and algae. However, rotifers, fungi, insects and protozoa also

occur (Hosetti and Frost, 1995). While the species composition of various HRAPs has been studied (Azov et al., 1980; Schlüter et al., 1987; Canovas et al., 1996; Tarlan et al., 2002; de Godos et al., 2009; Chinnasamy et al., 2010), it is assumed that the predominant species will be representative of geographical region and local conditions. In an effort to gain insight into the species composition and population dynamics of the HRAP in the pilot IAPS in Grahamstown, South Africa, samples containing microalgae (and bacteria and zooplankton) were taken at regular intervals for analysis. Algae species were identified phenotypically using light microscopy and four of the major species present were isolated and their identification confirmed by DNA sequencing.

2.2 MATERIALS AND METHODS

2.2.1 High rate algae pond configuration

The HRAP used in the present study (Figure 2-1) is part of the pilot IAPS for wastewater treatment located at the municipal wastewater treatment plant (WWTP) in Grahamstown (33° 19' 07" South, 26° 33' 25" East), South Africa.



Figure 2-1: The second HRAP in series in the IAPS at the municipal WWTP in Grahamstown, from which sampling took place.

Under continuous operation (Figure 2-2), the system comprises a primary facultative pond (PFP) incorporating an anaerobic digester (AD). Hydraulic retention time (HRT) in the AD is 3.5 d while in the PFP the HRT is 20 d. A transfer point is located 0.5 m below the water surface of the PFP and 80 m³ of water decants under gravity to the first HRAP per day (equivalent to influent). Two HRAPs (60 × 8 m) are connected in series via settling pond A

and are maintained at a water depth of 0.3 m to give a total volume of 150 m³ and surface area of ±500 m² per raceway. HRT is 2 d in HRAP A and 4 d in HRAP B with an influent rate of 80 and 40 m³.d⁻¹ respectively. Mixing, or turbulent flow, is essential to maintain optimum conditions for maximum algae productivity and in the current pilot system is set at 0.3 m.s⁻¹. Typically linear velocity is required to prevent stratification and is achieved using a paddle wheel powered with a small electrical motor (0.35 kWh). The biomass produced in the HRAPs is removed using inclined algae settling tanks. Outflow biomass from the HRAPs, due to paddlewheel mixing, auto/biofloculates and settles rapidly and thereafter is removed to drying beds or to a thickener tank (inverted cone). A HRT of 0.5 d is sufficient for adequate settling and yields a slurry of 2.5-4%.

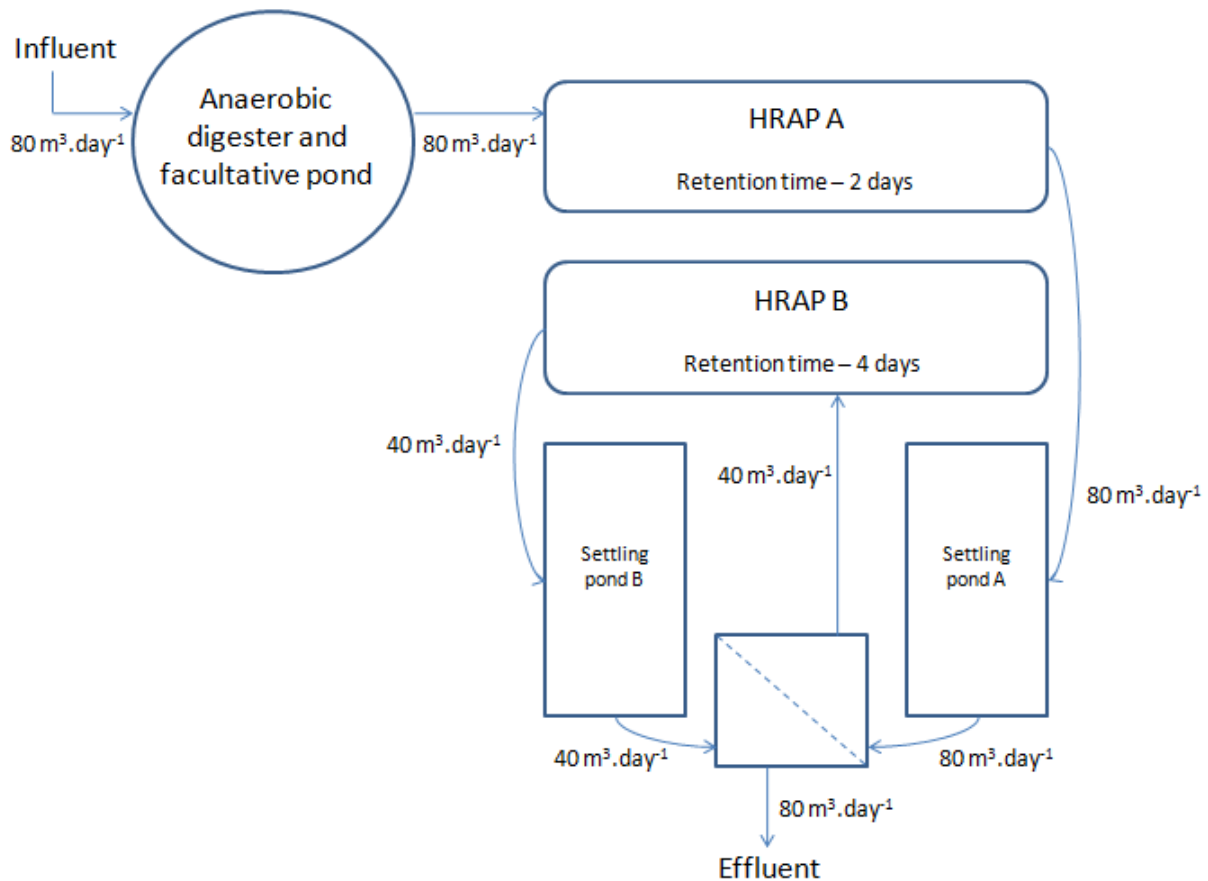


Figure 2-2: Configuration of the IAPS (continuous mode) at the time of sampling.

Under batch operation of the HRAPs there is no influent or effluent flow and the HRAPs are isolated from the IAPS. However, in order to offset water losses due to evaporation the HRAPs are intermittently topped up with tap water. In the current investigation, the

wastewater treated under batch operation was that which was in the HRAP following operation in continuous mode. No drainage of the HRAP took place in between treatments.

2.2.2 Sampling and sub-culturing procedures

Water samples for the isolation of algae species were collected from the HRAP (B) (at a position immediately before the paddlewheel, near the exit point of the effluent). Aliquots were diluted 1:10 and 1:100 and 200 μL from each inoculated onto enriched solid agar medium (1.5%) by spreading evenly using a sterile glass rod. The basal medium used for culturing was Bold 3N containing NaNO_3 (8.82×10^{-3} M), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.70×10^{-4} M), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3.04×10^{-4} M), K_2HPO_4 (4.31×10^{-4} M), KH_2PO_4 (1.29×10^{-3} M), NaCl (4.28×10^{-4} M), $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ (1.2×10^{-5} M), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2.16×10^{-6} M), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.26×10^{-6} M), ZnCl_2 (2.22×10^{-7} M), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (5.04×10^{-8} M), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (1.02×10^{-7} M), soil water ($40 \text{ mL} \cdot \text{L}^{-1}$) and vitamin B_{12} ($1.35 \times 10^{-4} \text{ g} \cdot \text{L}^{-1}$) (Internet reference 1). Media was autoclaved at 121°C for 15 min at 118 kPa.

Following development of colonies, single colonies were removed from the agar using sterile $10 \mu\text{L}$ plastic autopipette tips and inoculated into sterilised test tubes containing 5 mL liquid Bold 3N media. Test tubes were sealed and placed in a constant environment (CE) room (at 25°C , 12 h photoperiod, $50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) on a shaker at 100 rpm. After sufficient growth had occurred species were identified (Internet references 2-5) from light microscope analysis performed using a Zeiss Axiostar plus microscope (Carl Zeiss, Jena, Germany) and transferred to 100 mL fresh media. Light micrographs were captured using a Canon Powershot G6 digital camera. Pure cultures of algae were maintained in 100 mL volumes of Bold 3N media at 15°C with a 12 h photoperiod ($50 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). Sub-culturing was carried out regularly with 10% inoculum.

For quantification of algae composition in the HRAP, cell counts were carried out (using an improved Neubauer Haemocytometer) on samples collected 30 July (when the IAPS was in continuous mode), and 19 October 2009 (when the HRAP was in batch operation).

2.2.3 Molecular identification of isolated algae species

Freeze dried algae culture samples were extracted for DNA using a Qiagen DNeasy Plant extraction kit. Dried samples (± 10 mg) were homogenised with glass beads ($200 \mu\text{m}$) for 10×30 s (with 30 s intervals on ice) to rupture the cells. Extraction was then carried out

according to the manufacturer's instructions. In order to determine purity and quality of the DNA, samples (10 μL) were electrophoresed in a 1.2% (w/v) agarose gel using TAE ($\times 0.5$) electrophoresis buffer containing 1 $\text{mg}\cdot\text{mL}^{-1}$ ethidium bromide and visualised under UV light with a UVP BioDoc-It™ System transilluminator (Upland, California). The molecular weight of the DNA was estimated by comparison to a DNA marker (O'GeneRuler™ 100 bp DNA Ladder Plus). DNA was then PCR-amplified for sequencing. The two oligonucleotide primers used were universal eukaryotic primers which amplify the 18S rRNA gene: 5'-GTCAGAGGTGAAATTCTTGGATTTA-3' forward primer and 5'-AGGGCAGGGACGTAATCAACG-3' reverse primer (Rasoul-Amini et al., 2009). These primers amplify a ~700 bp region and were obtained from Whitehead Scientific (Pty) Ltd, South Africa. The PCR reaction volume was 50 μL and contained 25 μL of Qiagen HotStarTaq Master Mix Kit, 10 pmol of each primer and 500 ng DNA. The amplification was performed in a GeneAmp® PCR System 9700 thermocycler (Applied Biosystems, Carlsbad, California) and consisted of an initial denaturation step of 15 min at 95 °C followed by 35 cycles of 1 min each at 94 °C, 60 °C and 72 °C. There was a final extension step of 7 min at 72 °C.

Following PCR, samples were cleaned using a Qiagen PCR cleanup kit and DNA was quantified using a Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA). DNA sequencing was carried out by cycle sequencing using Big Dye sequencing kit. The reaction volume was 20 μL and contained 5 μL Big Dye Sequencing reagent, 2 μL Big Dye Sequencing Buffer, 10 pmol primer and 350 ng DNA. The PCR thermal cycle for sequencing consisted of an initial denaturation step of 2 min at 94 °C followed by 30 cycles of 30 s each at 94 °C, 55 °C and 72 °C, with a final extension step of 7 min at 72 °C. Samples were cleaned using the PCR cleanup kit and then air dried. Sequencing was carried out at the Rhodes University DNA Sequencing Unit. Edited sequences were used as queries in BLASTN searches (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), to determine the nearest identifiable match present in the complete GenBank nucleotide data base.

2.3 RESULTS

2.3.1 Determination of species diversity in a HRAP

HRAPs in WWTPs are typically populated by local species, the dynamics of which differ depending on the climatic conditions of the region, the waste stream being treated and the

operational features of the system. Water samples taken from the HRAP revealed the microflora and fauna diversity illustrated in Figure 2-3. Species diversity was affected by the mode of operation of the pond, with more species observed under batch operation in comparison to continuous. Microalgae dominated the HRAP numerically, however zooplankton were also observed. *Pediastrum*, *Scenedesmus*, *Micractinium*, *Chlorella*, *Pyrobotrys*, Diatoms, *Chlamydomonas*, *Euglena* and rotifers were observed in samples taken from the HRAP under continuous operation. Additional species *Actinastrum*, *Dictyosphaerium*, Blue greens, *Closterium* and *Daphnia* were observed under batch operation.

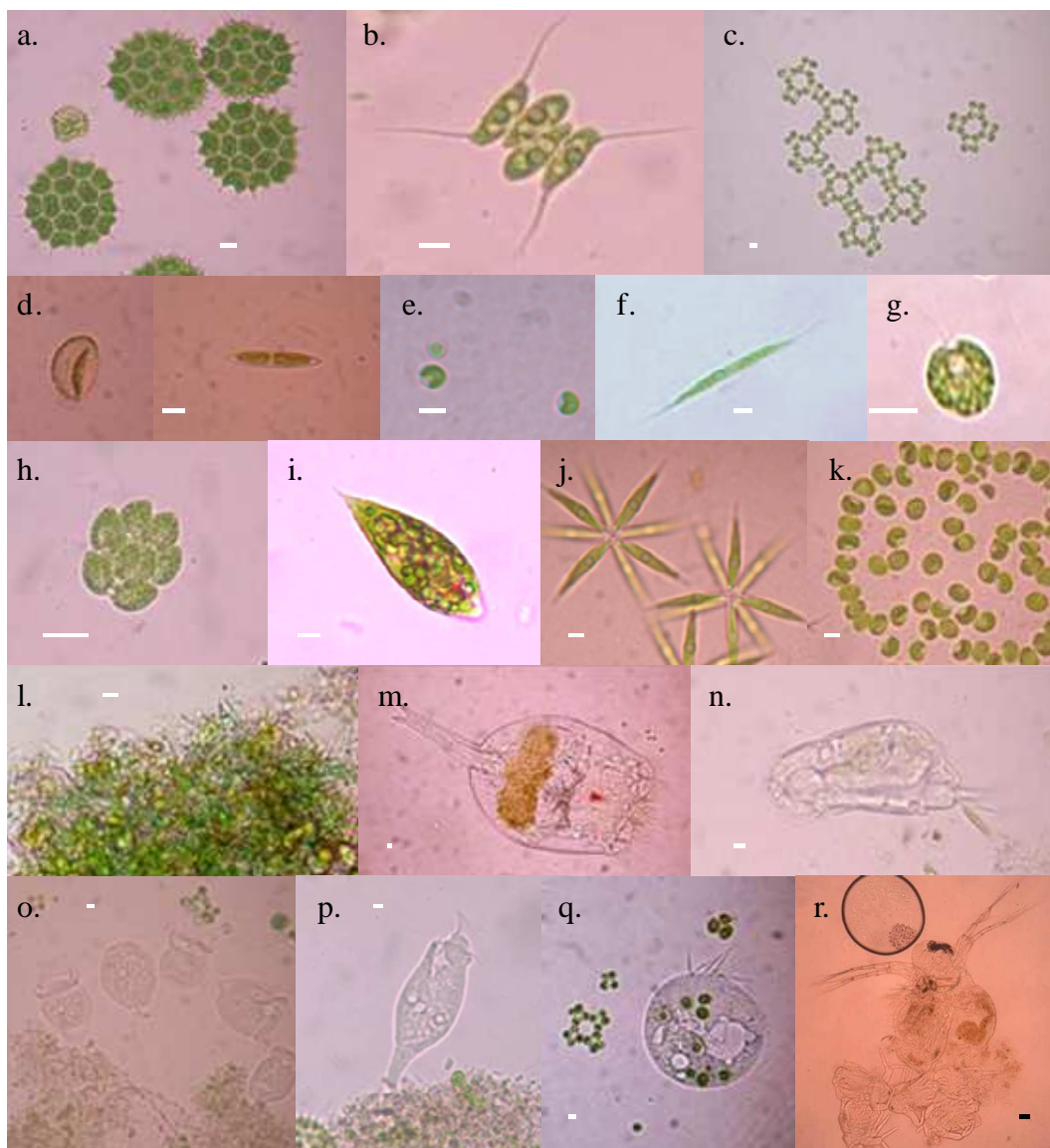


Figure 2-3: Microflora and fauna observed in water samples from the HRAP. Microalgae: a. *Pediastrum*, b. *Scenedesmus*, c. *Micractinium*, d. Diatoms, e. *Chlorella*, f. *Closterium*, g. *Chlamydomonas*, h. *Pyrobotrys*, i. *Euglena*, j. *Actinastrum*, k. *Dictyosphaerium*, l. Blue greens.

Zooplankton: m. *Brachionus*, n. *Lecane*, o. *Conochilus*, p. *Philodina*, q. *Cyclidium*, r. *Daphnia*. White scale bar is 5 μm (for microalgae and rotifers) and black scale bar is 50 μm (for *Daphnia*).

2.3.2 Isolation and identification of algae species

Solid agar medium inoculated with water samples from the HRAP had sufficient colony formation after four weeks of growth. The water sample diluted 1:100 was found to have best spacing of colonies ensuring that single colonies could be picked from the agar without cross contamination from surrounding colonies. Single colonies were inoculated into test tubes containing 5 mL of liquid Bold 3N medium. After 4 weeks, significant growth was observed and microscopic analysis revealed that pure cultures had been produced. Tentative identification, using databases (Internet references 1-5) and comparing morphology, indicated isolation of species with characteristics similar to *Chlorella*, *Pediastrum*, *Scenedesmus* and *Micractinium* (Figure 2-4).

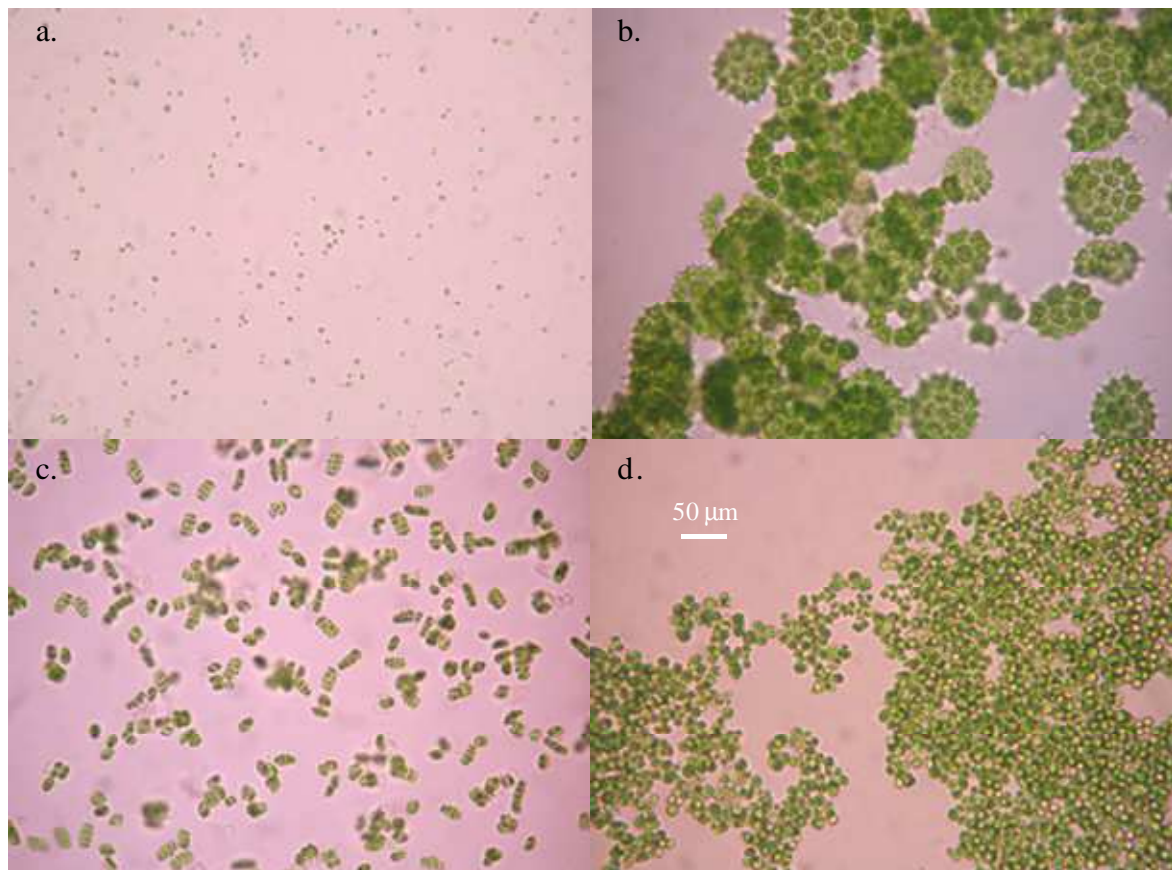


Figure 2-4: Pure culture of a. *Chlorella*, b. *Pediastrum*, c. *Scenedesmus*, and d. *Micractinium* viewed under the microscope at $\times 400$.

To obtain unequivocal information on the identity of these strains, cultures were grown in Bold 3N media and harvested in mid log-phase by centrifugation and subsequent freeze-

drying. DNA extraction and PCR amplification were carried out and a single PCR product of ~700 bp was recorded for each species (Appendix 1). Sequencing and BLAST analysis confirmed the species identity (Figure 2-5). The sequence of the EBRU *Chlorella* isolate was 100% matched to two *Chlorella* species, *Chlorella sorokiniana* (Accession number GQ122327.1) and *Chlorella vulgaris* (AB080308.1). The *Micractinium* isolate was 100% matched to *Micractinium pusillum* (AM231740.1), *Pediastrum* was 98% matched to *Pediastrum duplex* (AY780662.1) and *Scenedesmus* was 99% matched to *Scenedesmus communis* (X73994.1).

[gb|GQ122327.1|](#) *Chlorella sorokiniana* isolate BE1 18S ribosomal RNA gene, partial sequence
Length=1740

Score = 1170 bits (633), Expect = 0.0
Identities = 633/633 (100%), Gaps = 0/633 (0%)
Strand=Plus/Plus

```

Query 1      ATGTTTTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCCTAG 60
Sbjct 925     ATGTTTTTCATTAATCAAGAACGAAAGTTGGGGGCTCGAAGACGATTAGATACCGTCCTAG 984

Query 61     TCTCAACCATAAACGATGCCGACTAGGGATCGGCGGATGTTTCTTCGATGACTCCGCCGG 120
Sbjct 985     TCTCAACCATAAACGATGCCGACTAGGGATCGGCGGATGTTTCTTCGATGACTCCGCCGG 1044

Query 121    CACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAAC 180
Sbjct 1045    CACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAAC 1104

Query 181    TTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAA 240
Sbjct 1105    TTAAAGGAATTGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAA 1164

Query 241    CACGGGAAAACCTTACCAGGTCCAGACATAGTGAGGATTGACAGATTGAGAGCTCTTTCTT 300
Sbjct 1165    CACGGGAAAACCTTACCAGGTCCAGACATAGTGAGGATTGACAGATTGAGAGCTCTTTCTT 1224

Query 301    GATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTTCAGGTTGAT 360
Sbjct 1225    GATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTTCAGGTTGAT 1284

Query 361    TCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTCACGGTTGGTTCGCCAGCCGGCGG 420
Sbjct 1285    TCCGGTAACGAACGAGACCTCAGCCTGCTAAATAGTCACGGTTGGTTCGCCAGCCGGCGG 1344

Query 421    ACTTCTTAGAGGGACTATTGGCGACTAGCCAATGGAAGCATGAGGCAATAACAGGTCTGT 480
Sbjct 1345    ACTTCTTAGAGGGACTATTGGCGACTAGCCAATGGAAGCATGAGGCAATAACAGGTCTGT 1404

Query 481    GATGCCCTTAGATGTTCTGGGCCGCACGCGCCTACACTGATGCATTCAACGAGCCTAGC 540
Sbjct 1405    GATGCCCTTAGATGTTCTGGGCCGCACGCGCCTACACTGATGCATTCAACGAGCCTAGC 1464

Query 541    CTTGGCCGAGAGGCCCGGGTAATCTTTGAAACTGCATCGTGATGGGGATAGATTATTGCA 600
Sbjct 1465    CTTGGCCGAGAGGCCCGGGTAATCTTTGAAACTGCATCGTGATGGGGATAGATTATTGCA 1524

Query 601    ATTATTAATCTTCAACGAGGAATGCCTAGTAAG 633
Sbjct 1525    ATTATTAATCTTCAACGAGGAATGCCTAGTAAG 1557

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[emb|AM231740.1|](#) *Micractinium pusillum* partial 18S rRNA gene, strain CCAP 248/7
Length=1757

Score = 1203 bits (651), Expect = 0.0

Identities = 651/651 (100%), Gaps = 0/651 (0%)
Strand=Plus/Minus

Query	1	TAGGCATTCCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCATCACGATGCAGTTT	60
Sbjct	1563	TAGGCATTCCCTCGTTGAAGATTAATAATTGCAATAATCTATCCCCATCACGATGCAGTTT	1504
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Sbjct	1503	CAAAGATTACCCGGACCTCTCGGTCAAGGCTAGGCTCGTTGAATGCATCAGTGTAGCGCG	1444
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Sbjct	1383	CTAGTCGCCAATAGTCCCTCTAAGAAGTCCGCGGGCTGGCGAACCAACCGTGACTATTTA	1324
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Sbjct	1323	GCAGGCTGAGGTCTCGTTCGTTACCGGAATCAACCTGACAAGGCAACCCACCAACTAAGA	1264
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Sbjct	1263	ACGGCCATGCACCACCACCCATAGAATCAAGAAAGAGCTCTCAATCTGTCAATCCTCACT	1204
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Sbjct	1203	ATGCTGGACCTGGTAAGTTTTCCCGTGTGAGTCAAATTAAGCCGAGGCTCCACGCCT	1144
Query	421	GGTGGTGCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCAGCATACTCCCCCGGAA	480
Sbjct	1143	GGTGGTGCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCAGCATACTCCCCCGGAA	1084
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Sbjct	1083	CCCCAAAACCTTTGATTTCTCATAAGGTGCCGGCGGAGTCATCGAAGAAACATCCGCCGAT	1024
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sequence
Length=1668

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Strand=Plus/Plus

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Strand=Plus/Plus

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Sbjct 1553 |GCAATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCAATTCATCAGATTGCGTTG| 1610

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Figure 2-5: BLAST analysis of *Chlorella*, *Micractinium*, *Pediastrum*, and *Scenedesmus*.

2.3.3 Quantification of algae species in the HRAP

The algae species successfully isolated from the HRAP were the predominant species in the pond; however the population dynamics varied depending on the mode of operation (Figure 2-6). During continuous operation *Micractinium* dominated > 90% of the algae population, however this species was absent during batch operation. Conversely, *Chlorella* was absent during continuous mode and under batch it mode became dominant, at ~80% of the algae population. During both operation modes *Scenedesmus* and *Pediastrum* were present, however *Pediastrum* numbers were higher under batch operation (11% compared to 2%), when Diatoms also proliferated (5%). *Scenedesmus* remained at ~3% during batch and continuous operation.

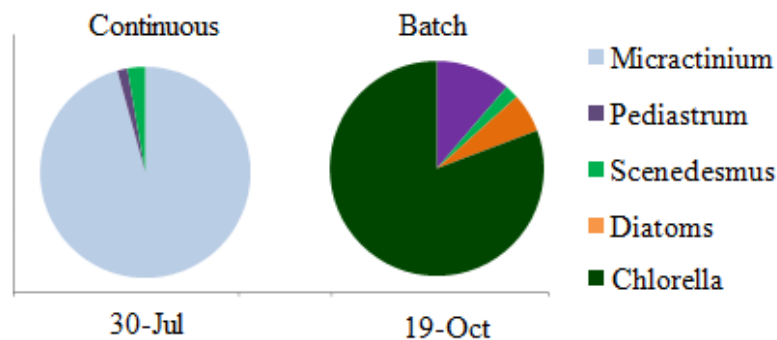


Figure 2-6: Algae species composition in the HRAP under batch and continuous operation.

2.4 DISCUSSION

Of the microorganisms found in the HRAP, the subject of interest in this study was algae as they play a fundamental role in its ecology and operation, and have the potential to generate valuable bioproducts. The species composition of the algae community in the HRAP investigated was variable, with relatively low species diversity and usually one numerically dominant species making up more than 80% of the algae counted. Low species diversity is generally found in HRAPs that are not allowed to fully stabilise due to heavy organic loading or fast dilution rate (Benemann et al., 1977; Canovas et al., 1996). The low species diversity observed under continuous operation may have been due to washout of slower growing algae caused by the short retention time coupled with low light intensities and temperatures (as sampling took place during late winter). Therefore, species recorded under conditions of continuous operation were able to sustain their growth rate above that of the dilution rate through the pond, and under batch conditions slower growing algae were able to establish in the HRAP, giving higher species diversity. Overall, only ± 10 algae species were observed,

which is commonly the case when using microscopic techniques. An alternative approach in determining species diversity is to use molecular techniques, which generally report higher species range (Ghosh and Love, 2010).

Under continuous operation, the presence of zooplankton in the pond may have accounted for the dominance of *Micractinium*, as this species can develop spines which prevent grazing by rotifers (Schlüter et al., 1987). Selective grazing pressure may have also removed smaller algae, thereby enabling colonial algae species (*Micractinium*, *Pediastrum* and *Scenedesmus*) to dominate (Van Donk, 1997). Under batch operation the dominant species changed and *Chlorella* became more prominent. *Daphnia* were also more prominent at this time and would have imposed a different grazing pressure as they have a broader food spectrum due to larger gape size (Burns, 1968; Cyr and Pace, 1992). Reduction in *Micractinium* numbers, therefore, may have been caused by *Daphnia* grazing, which would have allowed the faster growing *Chlorella* to outcompete this species. Further studies under controlled conditions should be carried out to determine the grazing effects of rotifers and *Daphnia* on the population dynamics because under conditions in the current investigation other factors (as discussed in Chapter 3) could have also influenced species composition.

In conclusion, preliminary results suggested that continuous operation of a HRAP was more advantageous as lower species diversity could be maintained with easily harvestable colonial species dominating (Azov et al., 1980). This is desirable from an operational and economic perspective as a more homogenous biomass is easier to characterise and determine a means of valorisation. Additionally, *Daphnia* are unable to flourish under continuous operation with a short retention time and therefore a reduced effect of grazing on productivity is obtained (Benemann et al., 1977).

Isolation of native algae species from the HRAP was successful and four pure cultures of the numerically dominant species, *Chlorella*, *Micractinium*, *Pediastrum* and *Scenedesmus*, were produced and maintained. The identity of these isolates was confirmed by DNA sequencing which also enabled classification to species level. Whilst the molecular approach used was not applicable to determining in depth species diversity, the pure cultures could be used for further analysis such as determination of biochemical composition and effects of changing growth conditions.

Chapter 3. Operation of a High Rate Algae Pond in Batch and Continuous Modes and Effect on Productivity and Biomass Composition

3.1 INTRODUCTION

High rate algae ponds (HRAPs) have been successfully used to cultivate a variety of microalgae for products of value and to treat a range of effluent streams (El Hamouri et al., 1994; Tarlan et al., 2002; Mulbry et al., 2005; Horan et al., 2007; de Godos et al., 2009; Chinnasamy et al., 2010). Typically, HRAPs are shallow, open photobioreactors and are usually configured as a series of interconnected raceways. Biomass produced and harvested from HRAPs can be converted via various pathways to useful commodity and high value end products. Some examples include the production of β -carotene in *Dunaliella*, astaxanthin production in *Haematococcus*, and the bulk production of *Spirulina* and *Chlorella* (Spolaore et al., 2006). Large scale production of algae biomass using wastewater treatment in HRAPs was pioneered by Oswald and Golueke (1960) and the biomass thus produced can be further utilised with minimal processing as feedstock for biogas, bioethanol, and high temperature conversion to bio-crude oil (Craggs et al., in press).

Wastewater HRAPs are components of IAPS, which comprise advanced facultative ponds and anaerobic digesters, and are designed to promote the symbiosis between microalgae and aerobic bacteria, each utilising the major metabolic products of the other. Thus, organic compounds are oxidised by aerobic bacteria utilising photosynthetic O_2 produced by microalgae which in turn utilise CO_2 and other nutrients released through bacterial oxidation. The combined action of aerobic bacteria and microalgae tends to increase pH of the water to above 9, providing a high rate of disinfection.

A consequence of continuous nutrient removal is not only a decline in BOD of the treated water but a substantial increase in biomass. Studies carried out recently have confirmed that the resultant biomass is suitable for use in agriculture and horticulture as a fertiliser and soil amendment (Mulbry et al., 2005). In addition, HRAPs have been suggested as good instruments for the production of algae biomass for high quality animal feed (Kuhad et al., 1997). Further support for the idea has been obtained from the treatment of dairy effluent

(Wilkie and Mulbry, 2002), and success achieved in with *Spirulina* cultivated using effluent from anaerobically treated swine wastewater (Cheunbarn and Peerapornpisal, 2010)

The present work attempted to investigate the characteristics of microalgae growth (population dynamic and nutrient content of the resultant biomass), along with the removal of nitrate, phosphate and ammonium from AD effluent, in a HRAP. The pond was operated first in continuous mode and the results compared and contrasted with batch mode to examine the effect of nutrient limitation.

3.2 MATERIALS AND METHODS

3.2.1 Pond operation

A detailed description of the IAPS and its operational characteristics is reported in Chapter 2. Two modes of operation of the HRAP were investigated, continuous and batch. Data collection during continuous mode took place from June to August 2009, and during batch operation from September to November 2009.

3.2.2 Pond monitoring

Sampling from the HRAP took place once or twice weekly (at 09.00 am) at a point upstream of the paddle wheel near the effluent discharge, where sufficient mixing took place. Three 1 L samples were collected for recovery of biomass and the subsequent determination of protein, carbohydrate and lipid composition. Three 250 mL samples were collected for determination of dry biomass and nitrate, phosphate and ammonium concentration. The pH of the wastewater in the HRAP was determined using a WTW pH 330 portable pH meter. Weather data (average ambient temperature and daily total solar irradiance) was obtained from logged data of the Rhodes University weather station (located approximately 5 km from the study site).

3.2.3 Biomass and ash determination

Cell counts were carried out weekly using an improved Neubauer haemocytometer at $\times 400$ magnification. The numbers of species present in the sample were expressed as a percentage of the total number of cells counted. Dry matter concentration was determined by filtering, under vacuum, a known volume of pond sample (between 50 and 250 mL) through a pre-

dried, weighed (using a Mettler Toledo PB 303-S balance) Whatman GF/C filter of 1.2 μm . Filters were dried in an incubator overnight at 80 $^{\circ}\text{C}$, and the filtrate was retained for determination of nitrate, phosphate and ammonium concentration. Dried filter papers with algae biomass were reweighed to determine the biomass, which was expressed per L of culture. Filtered dry samples were ashed (400 $^{\circ}\text{C}$ for 4 h) in a Carbolite muffle furnace. After cooling, the filters were reweighed and the ash content of the biomass calculated. Biomass productivity was calculated using the pond size, and average biomass concentration and flow rate during continuous operation, and maximum growth rate during batch operation.

3.2.4 Chemical analysis

Nitrate, phosphate and ammonium concentrations were measured using Merck analysis kits and a Merck Spectroquant SQ118 (according to the manufacturer's instructions).

3.2.5 Biochemical analysis

Biomass was recovered by centrifuging 3 \times 800 mL of culture at 2 325 \times g for half an hour in a Beckman Coulter Avante J-E centrifuge (J-10 rotor). Most of the supernatant was decanted, the biomass transferred to 3 \times 15 mL Falcon tubes and centrifuged at 2 325 \times g for 1 min (Eppendorf benchtop centrifuge, 5810 R). The supernatant was decanted and the biomass freeze-dried (Vir-Tis Benchtop SLC) and stored in a desiccator until required for further analysis.

For carbohydrate and protein determination, 10 mg of dry microalgae biomass was placed in a microcentrifuge tube and hydrolysed in 500 μL NaOH (0.5 M) at 30 $^{\circ}\text{C}$ for 24 h. Carbohydrate was determined using the phenol-sulphuric-acid assay, as described by Dubois et al. (1956). Total carbohydrate was assayed as follows; to 500 μL of hydrolysed sample (diluted 1:100), 500 μL phenol solution (5% in distilled H_2O) was added, followed by 2.5 mL conc. sulphuric acid. After mixing, samples were allowed to stand for 30 min to cool. Absorbance was measured at 490 nm using a Thermo Spectronic Aquamate spectrophotometer (ThermoFisher Scientific, Waltham, MA), and the mass of carbohydrate was determined by interpolation from a standard curve for D-glucose. Values in μg were expressed as a percentage of the biomass.

Protein was quantified using the protein dye-binding method described by Bradford (1976) with bovine serum albumin (BSA) as a standard; 1 mL Bradford reagent (Sigma-Aldrich, St Louis, MO) was added to 20 μ L of the hydrolysed sample (diluted 1:10) and allowed to stand for 5 min. The absorbance was measured at 595 nm using a Thermo Spectronic Aquamate spectrophotometer and the mass of protein determined by interpolation from a standard curve for BSA (also hydrolysed with 0.5 M NaOH at 30 °C for 24 h). Values in μ g were expressed as a percentage of the biomass.

Lipid was extracted using a modification of the method described by Folch et al. (1957); 500 μ L of distilled H₂O was added to a pre-weighed dry biomass sample (~100 mg) and mixed vigorously. After 15 min, 10 mL chloroform/methanol (2:1, v/v) was added and the sample was vortexed for 30 s, followed by centrifugation ($2\ 325 \times g$ for 1 min). The supernatant was removed, washed with 3.6 mL 0.09% NaCl, centrifuged ($2\ 325 \times g$ for 1 min) and the lower organic phase harvested. The extraction was repeated three times and the lower phases combined and evaporated in an incubator at 60 °C overnight. The amount of lipid extracted was determined gravimetrically and expressed as a percentage of dry biomass.

3.2.6 Statistical analysis

Measurements of biomass, carbohydrate, protein and lipid, nutrient concentrations and cell counts were carried out in triplicate. Mean values and standard deviations were determined. In order to smooth the data for temperature and irradiance readings, the running mean of 5 readings was calculated. Multiple regression analyses determined interactions between variables and T-tests were carried out to determine significant differences. Software used was STATISTICA 9.0 (StatSoft, 2009), with significance at $P < 0.05$.

3.3 RESULTS

3.3.1 Nutrient removal and productivity in a HRAP during continuous and batch modes

In continuous operation the HRAP was fed with anaerobic digestate high in nitrogen and phosphorous. A primary objective in operating wastewater treatment HRAPs is to remove dissolved inorganic nutrients by intracellular assimilation or chemical precipitation. In order

to examine nutrient removal and effect of pH, two modes of pond operation (continuous and batch) were investigated and the resultant profiles generated are illustrated in Figure 3-1.

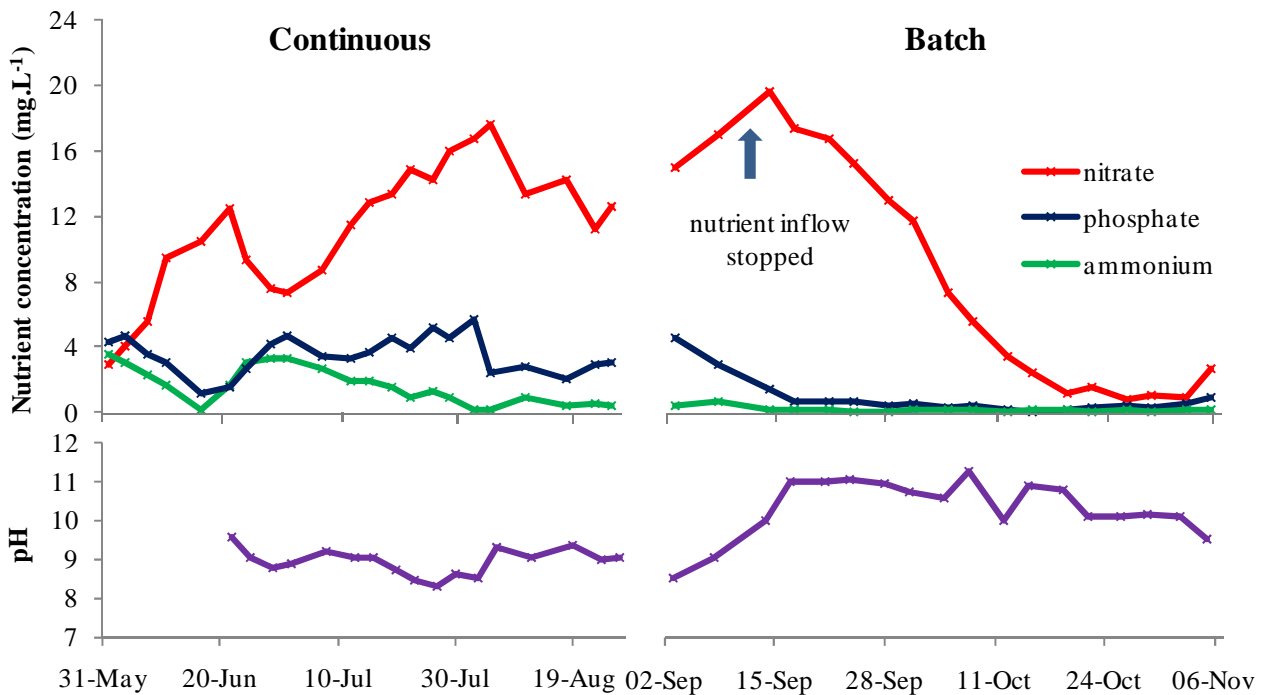


Figure 3-1: Nitrate, phosphate and ammonium concentrations, and pH of the HRAP water during batch and continuous operation.

Under continuous operation, chemical analysis of the pond water revealed a fluctuating nitrate concentration with an average of $11.2 (\pm 4.0) \text{ mg.L}^{-1}$, the result of a trade-off between influent nitrate and nitrate removal. Under batch operation, nitrate removal was steady and declined from 19.8 mg.L^{-1} to $\sim 1 \text{ mg.L}^{-1}$ in 2 months, demonstrating 98% removal efficiency. Removal of nitrate in batch operation was correlated with biomass ($P < 0.05$), as would be expected if nutrient assimilation by algae occurred.

Phosphate concentration in the pond water during continuous mode was $3.5 (\pm 1.2) \text{ mg.L}^{-1}$, and was negatively correlated with biomass concentration ($P < 0.05$), indicating assimilation during algae growth. During batch operation, phosphate concentration dropped from 2.9 mg.L^{-1} to $< 0.6 \text{ mg.L}^{-1}$ (80% removal efficiency) in 9 days, and negatively correlated with pH ($P < 0.05$), indicating removal by precipitation. During this time biomass concentration was increasing, therefore phosphate removal could also have been via intracellular fixation.

In continuous mode, ammonium concentration was $1.6 (\pm 1.1) \text{ mg.L}^{-1}$ and negatively correlated with nitrate concentration ($P < 0.05$), demonstrating preferential uptake of one form of nitrogen at a time. During batch operation, ammonium concentration was consistently low at $< 1 \text{ mg.L}^{-1}$, with an initial decline from 0.6 to 0.1 mg.L^{-1} , which coincided with an increase in pH and biomass. Ammonium removal during batch operation, therefore, could have been via intracellular immobilisation or volatilisation of ammonia or both.

Productivity in HRAPs is mainly influenced by the prevailing climatic conditions of the region. Operational features, however, can be adjusted in order to optimise the system and for improved biomass generation and subsequent nutrient removal. During continuous operation of the HRAP, biomass concentration fluctuated with changes in temperature and irradiance, an outcome of reduced growth rate during colder periods, which results in washout of the culture (Figure 3-2). Fluctuations were, however, small in comparison to biomass accumulated during batch operation, which showed little influence by temperature and irradiance fluctuations during exponential growth (Figure 3-2).

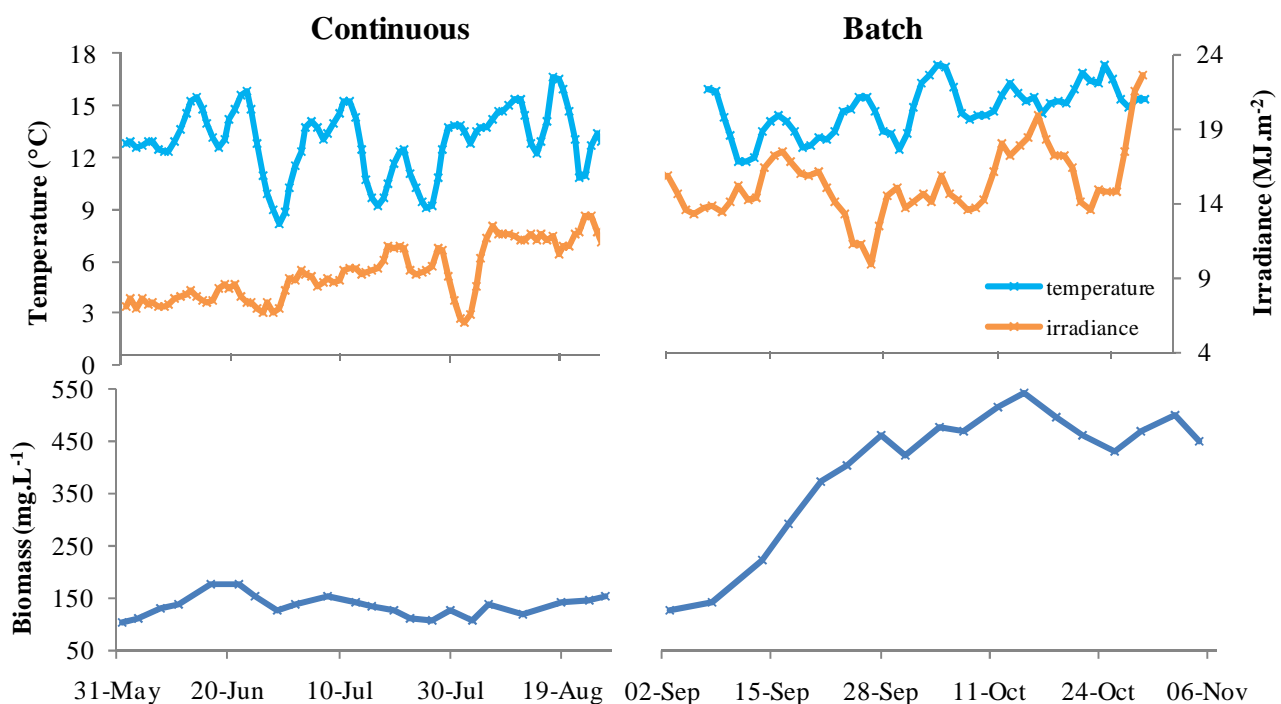


Figure 3-2: Climatic conditions - average ambient temperature and total solar irradiance, and HRAP biomass concentration, under batch and continuous operation.

Concentration of biomass in the pond does not reflect productivity because during continuous mode biomass is constantly being harvested, whereas in batch mode harvesting occurs only at the end of the run. Productivity of the HRAP was doubled during continuous operation in comparison to batch, at 10.8 and 5.4 g.m⁻².day⁻¹ respectively.

3.3.2 Species composition and nutritional quality of the biomass

Species composition of the HRAP showed considerable variation between the two modes of operation of the HRAP. *Micractinium* was the dominant species throughout continuous mode (Figure 3-3) with an initial increase from ~50% to ~90% of the population from 4 June to 6 July, and concurrent decrease in *Pediastrum* from 40 % to ~5%. The proportion of *Scenedesmus* remained relatively consistent at approximately 5-10% of the total algae population while Diatoms were only present during the two week periods of early June and late August, at ~3% of the total population.

In batch mode (Figure 3-3), the percentages of Diatoms and *Pediastrum* were higher than observed in continuous operation at 15 (± 5)% and 29 (± 11)% respectively; and when the biomass concentration reached ~470 mg.L⁻¹ there was a rapid decline in the *Micractinium* numbers. Coincident with nutrient depletion (reduction of nitrate to < 10 mg.L⁻¹), *Chlorella* became dominant and *Scenedesmus*, *Pediastrum* and Diatom numbers declined.

During continuous operation, an increase in protein content of the algae biomass (from 26% to 46%) was observed which coincided with the increase in proportion of *Micractinium* (Figure 3-3, 27 June – 8 July). Carbohydrate, lipid and ash content throughout continuous mode were 19 (± 2)%, 16 (± 4)% and 16 (± 3)% respectively. In batch operation, during the initial growth phase (Figure 3-3, 8 – 17 September) protein content of the biomass was at its highest, contributing 20 (± 0.8)% of the total biomass, whilst the carbohydrate content was 17.9 (± 2.6)%. As the population entered the stationary phase of growth (28 September), carbohydrate became the major cell constituent at 40.3 (± 4.9)% of the total biomass, while protein content was reduced to 13.7 (± 2.0)%. This change in biochemical composition also coincided with a change in the population dynamic with a decline in *Micractinium* numbers. Lipid content remained approximately constant during batch mode at 9.5 (± 1.5)%, and ash content throughout the sampling period was 20.5 (± 5.2)%.

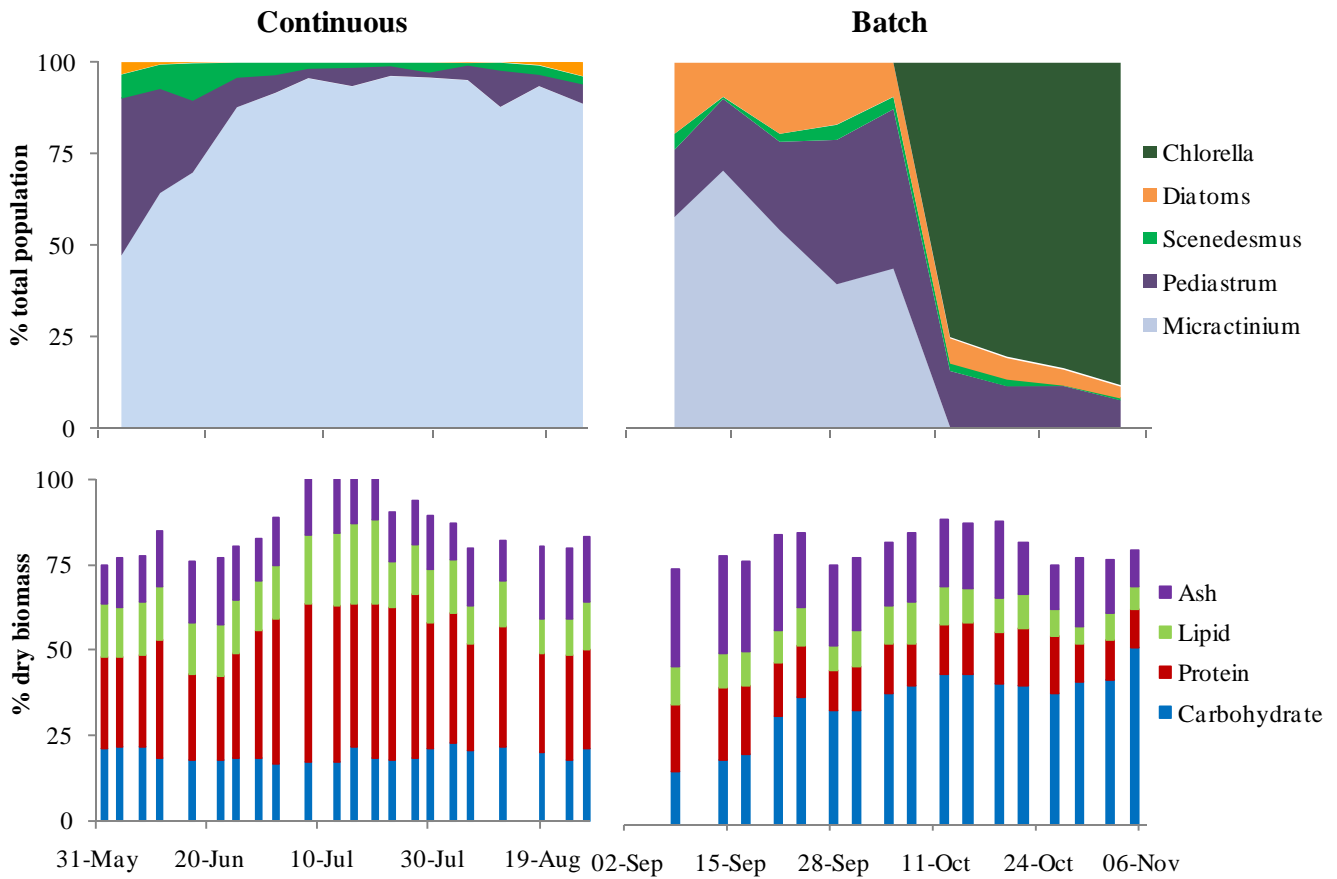


Figure 3-3: Species composition and nutritional value of algae in the HRAP in batch and continuous operation.

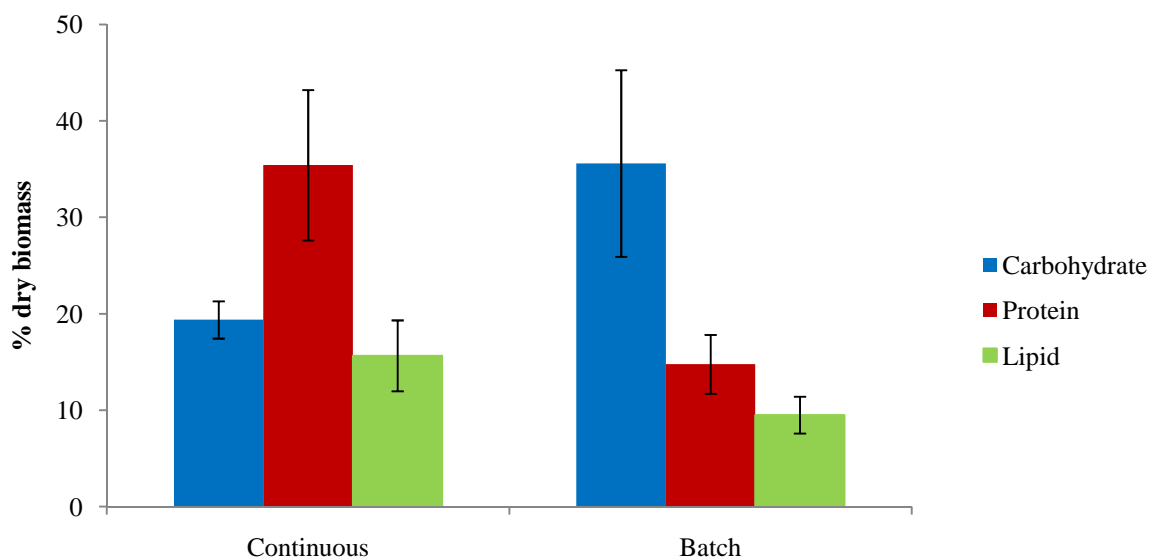


Figure 3-4: Mean carbohydrate, protein and lipid content of algae biomass generated during batch and continuous operation of the HRAP.

Comparing biochemical composition of algae biomass generated during continuous and batch operation of the HRAP, there were significant differences between protein, carbohydrate and lipid contents (Figure 3-4). During continuous operation the algae biomass generated high protein content, whereas under batch mode, the main cell constituent was carbohydrate. Lipid was the lowest biochemical component during both continuous and batch operation, however, it was higher during continuous mode.

3.4 DISCUSSION

High rate algae ponds (HRAPs) rely on interactions between natural populations of algae and bacteria for the removal of organic and inorganic nutrients found in waste streams. Inorganic nutrient removal by the algae population in the HRAP under investigation was effective in both continuous and batch operation, although levels of depletion differed. In continuous operation biomass productivity was higher in comparison to batch mode, and hence nutrient removal by assimilation into biomass was more efficient. The mean values of nitrate, phosphate and ammonium concentration in the pond water, and hence subsequent effluent, were below the South African discharge standard (DWAF, 2002), indicating that the HRAP provided adequate nutrient removal under continuous operation. In batch mode lower final concentrations of nutrients were achieved, and phosphate and ammonium removal was relatively rapid due to chemical precipitation and volatilisation respectively caused by raised pH in response to increase in biomass and depletion of dissolved CO₂ (Fallowfield et al., 1999; de Godos et al., 2009). Percentage removal rates of nutrients in batch mode were of similar order of magnitude to previous findings (Fallowfield et al., 1999; Martinez et al., 2000; Olguín et al., 2003; Aslan and Kapdan, 2006).

Although no direct relationship between nitrogen concentration and biomass was found in continuous mode there was a correlation under batch mode. Conversely, the opposite was evident with phosphate: under continuous mode phosphate concentration was strongly dependent on biomass whereas under batch mode removal was likely to be caused by precipitation. Similar nutrient removal and biomass correlations have been reported in both continuous and batch-fed HRAPS (Megharaj et al., 1992; Cromar et al., 1996; Fallowfield et al., 1999).

Biomass concentration during continuous mode was affected by temperature and solar irradiance, however the overall effects were minimal and productivity in the HRAP was higher during continuous operation, at double the productivity obtained during batch operation (in exponential growth). No direct correlations were found between biomass and temperature and solar irradiation, however a trend was observed during continuous operation. Similar results are reported in previous studies which found strong correlations of biomass with daily irradiance and temperature during continuous culture, but only weak correlations during batch culture (Cromar et al., 1996; Fallowfield et al., 1999). Low productivity during batch operation was likely to be caused by the accumulation of biomass, which results in increased self shading and lower light penetration (de La Noue and de Pauw, 1988), as well as concurrent CO₂ limitation induced by the high pH. The average concentration and productivity of biomass in the HRAP under continuous operation was low in comparison to previous findings (Goldman, 1979; García et al., 2006), however performance of a HRAP (in the absence of nutrient limitation) is dependent on environmental conditions.

A more stable population dynamic was observed during continuous mode, with *Micractinium* dominating. Other major species observed during both continuous and batch mode were *Scenedesmus*, *Pediastrum*, *Chlorella* and some unidentified Diatoms, all of which are commonly found in HRAPs treating wastewater and are known to be highly pollutant-tolerant (Palmer, 1969; Schlüter et al., 1987; Fallowfield et al., 1999; Chinnasamy et al., 2010). Under batch operation *Chlorella* dominated the HRAP as nutrient concentrations declined. This species has a smaller cell and therefore is able to out-compete larger colonial species under nutrient limitation (Fogg, 1991). Nutrient recycling by zooplankton during batch operation would have also had an effect on the algae population dynamic because different grazers release different N:P ratios which can favour the development of certain algae species (Attayde and Hansson, 1999).

Biochemical composition of algae biomass was dependent on both species specific characteristics and the growth state of the population. Under continuous operation, protein was the major component of the algae biomass. This coincided with both high numbers of *Micractinium* and the presence of an actively growing population. Although some variability in biochemical composition was observed under continuous culture, this mode of operation proved to be more consistent in producing biomass high in protein, as has been observed previously (Taub, 1980).

In batch mode, carbohydrate was the highest biochemical constituent of the algae biomass and was likely due to a combination of changing species composition, with reduced *Micractinium* and increased *Chlorella* numbers, and the presence of a less actively growing population (Valenzuela-Espinoza et al., 2002). Productivity during batch mode was lower in comparison to continuous culture, however in order to efficiently generate a biomass high in carbohydrate, a two-stage culturing process could be adopted. Firstly the biomass is generated in a HRAP running in continuous mode, with the effluent collected in a second HRAP where batch operation, and accumulation of carbohydrate, can take place. Similar processes have been adopted to improve the productivity of carotenoids and lipids (Borowitzka, 1999; Huntley and Redalje, 2007), and application in this instance would increase the productivity of a biomass rich in carbohydrate and lipid which could find further use in biofuel production (Craggs et al., in press).

Overall however, it was determined that operation of a HRAP in continuous mode is substantially more advantageous as it provides efficient nutrient removal to within standard levels for discharge, with higher biomass productivity. Furthermore, a more stable algae population is obtained with high protein content, suggesting that the biomass generated has the potential for further exploitation. Thus, the co-utilisation of HRAPs for wastewater treatment and biomass generation provides an ideal opportunity to investigate the efficient and economical use of the system for the production of algae biomass as an animal feed or feed supplement.

Chapter 4. Effect of 2-Hydroxy-4-(methylthio)-butanoic Acid and its Cu-chelate on Growth and Biochemical Composition of Selected Microalgae

4.1 INTRODUCTION

Results presented in Chapters 2 and 3 indicated that HRAPs integrated in WWTPs contain a plethora of microorganisms and in particular microalgae. Furthermore, a comparison of growth characteristics of microalgae from HRAPs operated in continuous and batch modes showed that these algae had good potential for use in animal nutrition due to accumulation of protein (in continuous) and carbohydrate (in batch).

Research and production activities are undergoing a paradigm shift and are moving more and more to the attainment of sustainable development. This change in attitude arises from the threat to an already fragile environment. However, utilisation of land for the production of food staples will continue as a high priority in areas with elevated population density. In this situation, for sustainable growth in animal production, new approaches are needed to identify alternative feed resources that are environmentally friendly and efficient utilisers of already dwindling resources, and at minimal cost.

Traditional efforts to increase ruminant productivity have centred on the quality of fibrous crops and by-products of grain harvesting (Preston and Murgueitio, 1992) and the addition of supplements to increase protein content. A feed additive is an ingredient (generally a chemical compound) or mixture of ingredients which are added to feed in small amounts to satisfy a particular need (Anon, 2005). One such supplement is 2-hydroxy-4-(methylthio)-butanoic acid (HMTBA, Figure 4-1b), a methionine hydroxy-analogue used to supplement methionine deficiency and enhance protein content of beef cattle, poultry and sheep (Barnes et al., 1995; Dibner et al., 2004; Lobley et al., 2006; Liu et al., 2007). Once inside animal tissues HMTBA is rapidly converted to L-methionine and shows identical metabolism to other L-methionine sources (Dibner, 2003).

In addition to protein supplements, animal feed formulations include trace minerals as these are required for many physiological processes (Mitchell, 1947). Organic trace elements are minerals which are chelated to an organic ligand such as an amino acid, polysaccharide or

organic acid. These are used in animal feed instead of inorganic trace minerals because of increased mineral bioavailability and absorption and to provide additional nutritional supplements (Predieri et al., 2005). The use of metal chelated HMTBA in animal feed formulations (particularly for broiler chicks) has been investigated in some detail and it was determined that HMTBA is fully available as a methionine source (Yi et al., 2007).

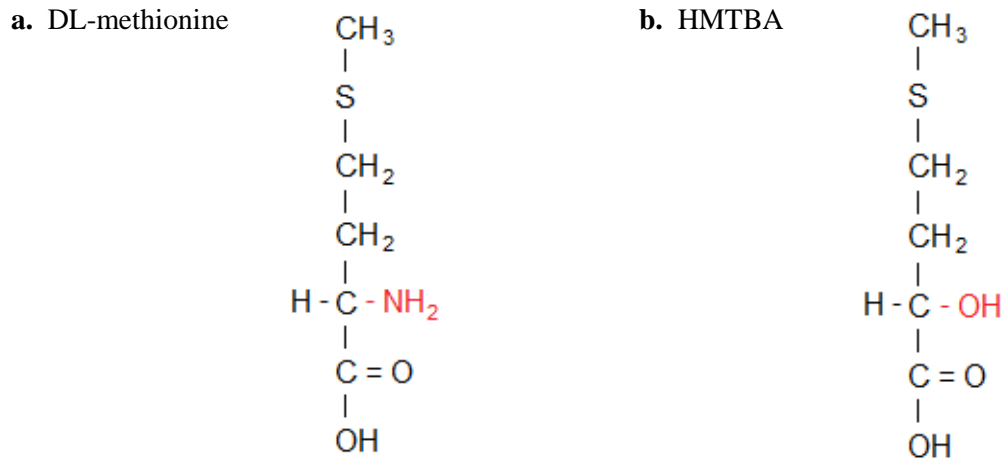


Figure 4-1: Structure of methionine and its hydroxy-analogue, HMTBA.

Algae are also used as feed additives in poultry production to increase the fatty acid composition and carotenoid content of egg yolks, and the DHA content of flesh (Pulz and Gross, 2004; Fredriksson et al., 2006; Kalogeropoulos et al., 2010). As algae are able to absorb and utilise various forms of carbon and nitrogen, including organic acids and amino acids (Neilson and Lewin, 1974; Bollman and Robinson, 1977; Linares, 2006), it is possible to stimulate uptake of HMTBA and HMTBA-chelates to enhance productivity of algae mass culture and the nutritional quality of biomass for use and supplementation as a feed source. This would provide an alternative means of administering HMTBA, and a more sustainable supply of animal feed supplements.

In the following investigation, the effect of HMTBA and its Cu-chelate derivative on growth and biochemical composition of five algae species was determined. Four of the strains used were isolated from a HRAP treating domestic wastewater as described in Chapter 2 and 3, and selected for study to determine the potential of the WWTP to provide a sustainable biomass of improved nutritive quality for use as a feed supplement. Addition of a Cu-HMTBA at the correct concentration could find further management applications in algae mass culture as Cu is a known bactericide, fungicide, herbicide and algicide (Roussel et al.,

2007). A fifth species of algae tested, the cyanobacterium *Spirulina platensis*, is currently commercially produced as a nutraceutical and animal feed supplement (Spolaore et al., 2006).

4.2 MATERIALS AND METHODS

4.2.1 Algae cultures

Four freshwater green algae, *Chlorella*, *Scenedesmus*, *Pediastrum* and *Micractinium*, isolated from a HRAP, as previously described (Chapter 2 and 3) were cultured in modified Bold medium. Stock cultures were grown in media containing NaNO_3 (2.94×10^{-3} M), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.70×10^{-4} M), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3.04×10^{-4} M), K_2HPO_4 (4.31×10^{-4} M), KH_2PO_4 (1.29×10^{-3} M), NaCl (4.28×10^{-4} M), EDTA (1.71×10^{-4}), KOH (5.53×10^{-4} M), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1.79×10^{-5} M), H_3BO_3 (1.85×10^{-4} M), $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ (3.07×10^{-5} M), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (7.28×10^{-6} M), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (6.29×10^{-6}), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (4.93×10^{-6} M), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (1.68×10^{-6} M) (Bold, 1949). Media was autoclaved at 121 °C for 15 min at 118 kPa.

A pure culture of the cyanobacterium *Spirulina platensis* was a gift from Professor Abdel-Hamid, Botany Department, University of Mansoura, Egypt. This species was cultured in the same media as described above with added NaNO_3 (final concentration 2.94×10^{-2} M), Na_2CO_3 (1.62×10^{-1} M) and NaHCO_3 (3.80×10^{-2} M). Cultures were grown in a CE room at 27 ± 1 °C under continuous illumination at $103 \pm 11 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ with gentle shaking (Labcon Shaker and Gerhardt Laboshake R0500) and bubbling of filtered ambient air. Airflow to each flask was controlled using a manifold. Stock cultures were inoculated into fresh medium every 5 or 6 d at 15% final volume to maintain cells in linear growth.

4.2.2 Experimental setup

HMTBA and its Cu^{2+} chelate (Cu-HMTBA, Figure 4-2) were assessed to determine their effects on the growth and biochemical composition of *Chlorella*, *Micractinium*, *Scenedesmus*, *Pediastrum* and *Spirulina*. Medium used was the same as that used for maintaining stock cultures except for addition of test compounds (with ~80% active component) at 0.1, 1 and 10 $\text{mg} \cdot \text{L}^{-1}$ for *Chlorella*, *Micractinium*, *Scenedesmus* and *Pediastrum*, and 10 $\text{mg} \cdot \text{L}^{-1}$ for *Spirulina*. The response was referenced against untreated

cultures (containing no test compounds). All treatments and controls were carried out in triplicate and a random block design was used to place the flasks.

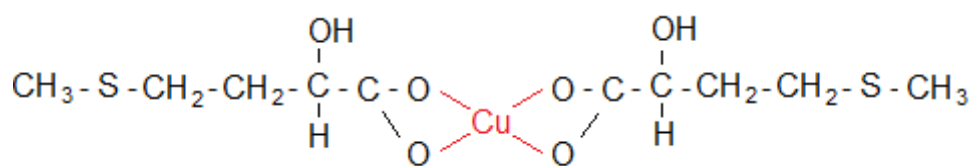


Figure 4-2: Structure of Cu-HMTBA.

Experiments were carried out in 250 mL Erlenmeyer flasks containing 150 mL culture. Medium was inoculated with 10% culture in log phase. Culturing took place in a CE room under the same conditions as described above.

4.2.3 Sampling

Cultures were sampled at intervals specified in the results by removing a 200 μL aliquot for determination of biomass by measuring the absorbance at 660 nm and interpolating from a standard curve for cell density vs. dry weight as described below. Samples were also taken for determination of carbohydrate, protein, lipid and total carotenoids.

4.2.4 Biomass determination

Standard curves of absorbance vs. biomass and absorbance vs. cell number were generated for each species. Biomass (algae culture density in mg.L^{-1}) and cell number were obtained by measuring the absorbance of 200 μL culture sample at 660 nm in a microplate reader (UV/Vis. Spectrum Finstruments™ Multiskan, ThermoFisher Scientific, Waltham, MA) and interpolation from the standard curves. Samples were diluted accordingly if the absorbance was greater than 1. Biomass for the standard curves was determined by filtering and drying 10 mL of culture using the method described in Chapter 3, and expressed per L of culture. Cell counts were carried out under a microscope using an improved Neubauer haemocytometer at $\times 400$ magnification.

4.2.5 Carbohydrate and protein determination

Carbohydrate and protein were determined using the same 500 μL or 1 mL sample, prepared by centrifuging for 5 min at $13\,000 \times g$ (Eppendorf benchtop centrifuge, 5810 R). Most of the

supernatant was removed (either 950 μL or 450 μL , leaving 50 μL of supernatant and the algae pellet). To this 50 μL NaOH (1 M) was added and the samples vortexed and incubated at 80 $^{\circ}\text{C}$ for 3 h with intermittent mixing (2-3 times).

Carbohydrate was determined using the phenol-sulphuric acid method adapted to a microtitre plate format (Masuko et al., 2005), and using D-glucose as a standard: 5 μL of the hydrolysed sample was diluted 1:10 with distilled H_2O . Concentrated sulphuric acid (150 μL) was added, followed by addition of 5% phenol in distilled H_2O (30 μL). Samples were mixed and then incubated for 10 min at 90 $^{\circ}\text{C}$. Samples were allowed to cool for 5 min at room temperature, mixed, and the absorbance then measured at 410 nm using a microplate reader.

Protein was determined using the Bradford assay (Bradford, 1976) with BSA (also hydrolysed with 0.5 M NaOH for 3 h at 80 $^{\circ}\text{C}$) as a standard. For protein analysis 250 μL of Bradford reagent was added to 5 μL hydrolysed sample and mixed. The absorbance was determined at 660 nm using a microplate reader.

4.2.6 Lipid determination

A 200 μL culture sample was added to 20 μL Nile red solution (50 $\mu\text{g}\cdot\text{mL}^{-1}$ in 50% dimethyl sulphoxide, DMSO) in a 96-well fluorescence plate, and mixed by pipetting. The relative fluorescence was determined at Ex 500 nm and Em 520 nm using a Fluoroskan® Ascent microplate fluorometer (ThermoFisher Scientific, Waltham, MA). Standard curves for each species were generated in which lipid was determined by extraction using a method modified from that described by Folch et al. (1956), and the dichromate assay for quantification (Pande et al., 1963) using peanut oil as a standard. For extraction, 1 mg of dry algae biomass was placed into a microcentrifuge tube, 50 μL of distilled H_2O was added and the sample was allowed to stand for 15 min prior to addition of 1 mL chloroform/methanol (2:1, v/v) and vortexing for 30 s. 210 μL CaCl_2 (0.5%) was added to wash the extract, and centrifuged at $4\,000 \times g$ for 1 min. The lower phase was collected and the extraction was repeated. The lower phases were combined and dried at 90 $^{\circ}\text{C}$. For lipid quantification, 2 mL potassium dichromate (2% in conc. sulphuric acid) was added to the dried sample and heated for 15 min at 100 $^{\circ}\text{C}$. The test tubes were then cooled in a water bath for 5 min and 4.5 mL of distilled H_2O was added cautiously, followed by gentle vortexing. After cooling absorbance at 590 nm was measured and referenced against dichromate.

4.2.7 Carotenoid determination

Cells in a 1 mL culture sample were pelleted by centrifugation for 5 min at $13\,000 \times g$ and 950 μL of the supernatant removed. DMSO was heated to $80\text{ }^\circ\text{C}$ and 950 μL was added to the algae pellet. The samples were vortexed, incubated at $80\text{ }^\circ\text{C}$ for 10 min, vortexed again and centrifuged for 5 min at $13\,000 \times g$. The absorbance of the supernatant was determined at 480 nm, 649 nm, 665 nm using a spectrophotometer (Thermo Spectronic Aquamate, ThermoFisher Scientific, Waltham, MA), with DMSO as a blank.

Carotenoid concentration ($\mu\text{g}\cdot\text{mL}^{-1}$) was determined using the following equations described by Wellburn (1994), and was then expressed per mg of biomass:

$$\text{Chl}_a = 12.47A_{665} - 3.62A_{649}$$

$$\text{Chl}_b = 25.06A_{649} - 6.5A_{665}$$

$$\text{Carotenoids} = (1000A_{480} - 1.29\text{Chl}_a - 53.78\text{Chl}_b)/220$$

4.2.8 Statistical analysis

For all statistical analyses, STATISTICA 9.0 software was used (StatSoft Inc., 2009). Significant differences between treatments and controls were measured with the Tukey test ($P < 0.05$).

4.3 RESULTS

4.3.1 Effect of HMTBA and Cu-HMTBA on growth and biomass accumulation

In order to determine the effect of HMTBA and Cu-HMTBA on growth and biomass accumulation of the algae species, specific growth rates were calculated (Table 4-1) based on changes in cell number, and the final biomass was recorded (Table 4-2). *Micractinium* showed a dose dependent response to HMTBA, with the $10\text{ mg}\cdot\text{L}^{-1}$ treatment having significantly higher growth rate than the $0.1\text{ mg}\cdot\text{L}^{-1}$ treatment, although individually these results were not significantly different in comparison to the control. While increased final biomass concentrations were observed with treatments of HMTBA at concentrations of 1 and $10\text{ mg}\cdot\text{L}^{-1}$ with *Micractinium*, and $10\text{ mg}\cdot\text{L}^{-1}$ with *Spirulina*, these results were not statistically significant in comparison to the controls. Treatment of *Chlorella* with $10\text{ mg}\cdot\text{L}^{-1}$ HMTBA resulted in significantly increased final biomass concentration.

Conversely, treatment of *Chlorella* and *Scenedesmus* with Cu-HMTBA at 10 mg.L⁻¹ showed significantly reduced growth rate; this reflected in the final biomass which was also lower than the controls. Similarly, Cu-HMTBA was lethal to *Spirulina* at 10 mg.L⁻¹. The *Scenedesmus* treatment with Cu-HMTBA 0.1 mg.L⁻¹ had reduced growth rate, however this did not affect the final biomass concentration. Other differences in growth rate and final biomass between treatments and controls were not significant.

Table 4-1: Growth rates of treated and untreated cultures of *Chlorella*, *Micractinium*, *Scenedesmus*, *Pediastrum* and *Spirulina* during exponential growth (* significantly different to control).

Treatment	Concentration (mg.L ⁻¹)	Growth rate (d ⁻¹)				
		<i>Chlorella</i>	<i>Micractinium</i>	<i>Scenedesmus</i>	<i>Pediastrum</i>	<i>Spirulina</i>
Control	0	0.28 (±0.02)	0.25 (±0.02)	0.38 (±0.02)	0.22 (±0.01)	0.52 (±0.03)
HMTBA	0.1	0.23 (±0.02)	0.21 (±0.02)	0.39 (±0.01)	0.22 (±0.01)	-
	1	0.23 (±0.03)	0.25 (±0.02)	0.36 (±0.01)	0.23 (±0.01)	-
	10	0.26 (±0.00)	0.27 (±0.02)	0.37 (±0.04)	0.21 (±0.01)	0.45 (±0.03)
Cu-HMTBA	0.1	0.21 (±0.02)*	0.22 (±0.00)	0.39 (±0.02)	0.23 (±0.01)	-
	1	0.24 (±0.01)	0.22 (±0.01)	0.38 (±0.03)	0.21 (±0.00)	-
	10	0.22 (±0.01)*	0.24 (±0.01)	0.34 (±0.00)*	0.22 (±0.01)	No growth*

4.3.2 Effect of HMTBA and Cu-HMTBA on biochemical composition

The effect of HMTBA and Cu-HMTBA on biochemical composition of algae species was determined by analysing biomass harvested during early stationary phase (Table 4-3). Protein content in *Chlorella*, *Micractinium*, *Scenedesmus* and *Pediastrum* treatments with HMTBA and Cu-HMTBA at 0.1 mg.L⁻¹ was significantly lower than the controls. Carotenoid content in *Scenedesmus* was lower in the treatment with 10 mg.L⁻¹ Cu-HMTBA, caused by a change in culture colour, which also occurred in *Pediastrum* with the same treatment. Other variations in biochemical composition were not significant to infer change caused by the treatments.

Table 4-2: Final biomass of treated and untreated cultures of *Chlorella*, *Micractinium*, *Scenedesmus*, *Pediastrum* and *Spirulina* (* significantly different to control).

Treatment	Concentration (mg.L ⁻¹)	Final Biomass (mg.L ⁻¹)				
		<i>Chlorella</i>	<i>Micractinium</i>	<i>Scenedesmus</i>	<i>Pediastrum</i>	<i>Spirulina</i>
Control	0	605 (±27)	709 (±12)	1287 (±87)	1316 (±60)	1080 (±36)
HMTBA	0.1	678 (±28)	682 (±98)	1290 (±21)	1241 (±63)	-
	1	584 (±59)	727 (±18)	1262 (±102)	1208 (±140)	-
	10	660 (±53)*	775 (±58)	1244 (±169)	1185 (±68)	1116 (±83)
Cu-HMTBA	0.1	624 (±32)	691 (±65)	1303 (±135)	1261 (±68)	-
	1	585 (±12)	674 (±77)	1262 (±97)	1236 (±55)	-
	10	526 (±14)*	674 (±65)	1034 (±43)*	1302 (±60)	No growth*

4.3.3 Biochemical profile of wastewater algae strains during one growth cycle

Changes in biochemical composition of each species during one growth cycle were determined in order to ascertain the best time for harvesting for the recovery of certain bio-products. Biochemical composition of harvested biomass differed between species and at different time points during the growth cycle (Figure 4-3). In all species carbohydrate was the highest reported cellular component and lipid was the lowest (excluding carotenoid). In *Chlorella*, protein, carbohydrate and lipid appeared to increase throughout the growth cycle, whereas in *Micractinium*, *Scenedesmus* and *Pediastrum* protein peaked during early exponential growth and carbohydrates and/or lipids accumulated in the late growth/early stationary phase.

The highest protein content reported, ~40%, was found in *Chlorella* during the stationary phase, and in *Micractinium* during early exponential growth. The highest carbohydrate and lipid contents were reported in *Micractinium* in the stationary phase.

Table 4-3: Biochemical composition of treated and untreated cultures of *Chlorella*, *Micractinium*, *Scenedesmus*, *Pediastrum* and *Spirulina* harvested at early stationary phase (* significantly different to control).

Species	Treatment	Concentration (mg.L ⁻¹)	Carbohydrate (%)	Protein (%)	Lipid (%)	Carotenoid (µg.mg ⁻¹)	
Chlorella	Control	0	40 (±5)	42 (±3)	14 (±4)	3.9 (±0.2)	
	HMTBA	0.1	37 (±1)	26 (±4) *	13 (±0)	3.1 (±0.8)	
		1	39 (±2)	40 (±1)	12 (±1)	3.0 (±0.6)	
		10	45 (±2)	39 (±3)	14 (±2)	3.4 (±0.1)	
	Cu-HMTBA	0.1	43 (±2)	28 (±1) *	10 (±1)	3.7 (±0.2)	
		1	40 (±2)	35 (±1)	11 (±3)	3.8 (±0.5)	
		10	40 (±5)	37 (±3)	14 (±4)	4.1 (±0.3)	
	Micractinium	Control	0	59 (±6)	32 (±1)	20 (±0)	3.9 (±0.3)
		HMTBA	0.1	55 (±6)	25 (±3) *	19 (±4)	4.3 (±0.4)
1			59 (±1)	30 (±2)	19 (±2)	4.3 (±0.6)	
10			61 (±7)	29 (±1)	15 (±3)	4.4 (±0.5)	
Cu-HMTBA		0.1	54 (±5)	24 (±2) *	19 (±1)	4.4 (±0.5)	
		1	56 (±1)	31 (±4)	18 (±3)	4.4 (±0.5)	
		10	52 (±4)	30 (±3)	17 (±2)	4.3 (±0.5)	

Table 4-3 cont.

Species	Treatment	Concentration (mg.L ⁻¹)	Carbohydrate (%)	Protein (%)	Lipid (%)	Carotenoid (µg.mg ⁻¹)	
Scenedesmus	Control	0	55 (±5)	14 (±1)	14 (±1)	2.8 (±0.1)	
	HMTBA	0.1	51 (±2)	10 (±0) *	15 (±1)	2.7 (±0.2)	
		1	51 (±4)	13 (±1)	16 (±3)	2.6 (±0.2)	
		10	50 (±3)	13 (±1)	13 (±2)	2.7 (±0.3)	
	Cu-HMTBA	0.1	53 (±6)	10 (±0) *	13 (±1)	2.8 (±0.2)	
		1	52 (±4)	14 (±1)	13 (±1)	2.7 (±0.2)	
		10	56 (±3)	15 (±2)	13 (±1)	2.3 (±0.1) *	
	Pediastrum	Control	0	54 (±3)	21 (±2)	16 (±4)	3.0 (±0.4)
		HMTBA	0.1	51 (±4)	17 (±1) *	16 (±2)	3.0 (±0.5)
1			50 (±2)	21 (±2)	15 (±5)	3.1 (±0.3)	
10			51 (±2)	22 (±1)	14 (±2)	3.0 (±0.1)	
Cu-HMTBA		0.1	47 (±4)	16 (±1) *	19 (±7)	3.0 (±0.3)	
		1	51 (±2)	21 (±1)	11 (±3)	3.0 (±0.1)	
		10	52 (±2)	20 (±2)	12 (±2)	2.8 (±0.3)	
Spirulina		Control	0	50 (±3)	27 (±2)	10 (±3)	3.4 (±0.6)
		HMTBA	10	42 (±8)	25 (±6)	9 (±5)	3.3 (±0.6)

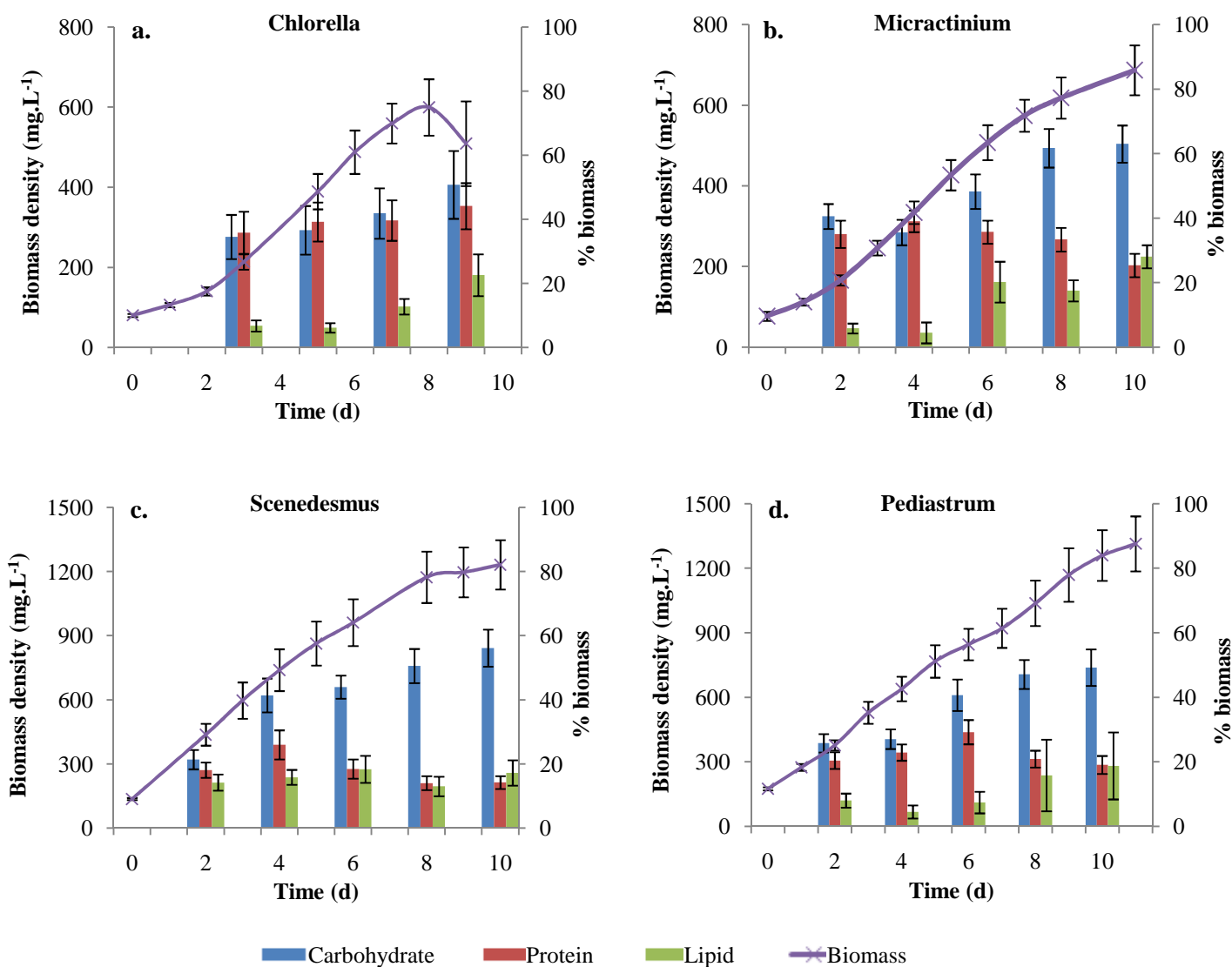


Figure 4-3: Culture density and biochemical composition of a. *Chlorella*, b. *Micractinium*, c. *Scenedesmus* and d. *Pediastrum* during one growth cycle.

4.4 DISCUSSION

With increasing population numbers, the efficient production of sustainable food sources is a major concern. Animal feed supplements provide a means of improving meat production and quality, however administration of chemically synthesised supplements is not an environmentally friendly solution and the demand for more “organic” food products is increasing. The ability to incorporate chemically synthesised supplements into algae provides an alternative, “natural” means of administering these supplements to animals.

Certain green algae and cyanobacteria are able to grow heterotrophically or mixotrophically. Heterotrophy is the ability to utilise various carbon sources, under dark conditions, for growth; whereas mixotrophic growth implies that carbon sources are assimilated in the presence of light (Bouarab et al., 2004). In the current investigation, continuous light was used for algae culture and hence utilisation of HMTBA and Cu-HMTBA would have been in a mixotrophic capacity. This may account for the increased (although this was not necessarily significant) biomass accumulation with 10 mg.L⁻¹ HMTBA, observed in *Chlorella*, *Micractinium* and *Spirulina*, which are species that have previously been found to have mixotrophic ability (Yang et al., 2000; Bouarab et al., 2004; Chojnacka and Noworyta, 2004). HMTBA is a simple organic acid and therefore it is not surprising that mixotrophic species could utilise this compound resulting in increased biomass accumulation.

Other Cu-chelated organometallic compounds, like Cu-HMTBA, have been studied before and have shown increased bioavailability due to lipid solubility which enables direct diffusion across the lipid cell membrane (Stauber and Florence, 1987). Once inside the cell, the ligand and copper may exert their own effects. In experiments with Cu-HMTBA, however, increased biomass accumulation was not observed in *Chlorella*, *Micractinium* and *Spirulina*, and 10 mg.L⁻¹ significantly reduced the growth rate and final biomass concentration of *Chlorella* and *Scenedesmus*. At this concentration Cu toxicity began to take effect, and was lethal in the case of *Spirulina*. Whilst copper is an essential mineral in trace amounts as an enzyme cofactor and electron transport carrier, in excess it reduces growth and photosynthetic and respiratory processes (Stauber and Florence, 1987; Perales-Vela et al., 2007). Cu toxicity is both species and dose dependent and therefore addition of Cu-HMTBA to HRAPs could additionally provide control of species diversity and infestations by grazers and fungi in the pond (Roussel et al., 2007). Further studies would however need to be carried out to determine the level of Cu-HMTBA tolerance by desired algae species.

Apparent decreased protein content of *Chlorella*, *Micractinium*, *Scenedesmus* and *Pediastrum* caused by 0.1 mg.L⁻¹ HMTBA and Cu-HMTBA may have been due to the method of protein quantification used. Percentage protein could have been comparable to the control, however with increased methionine content. The Bradford assay quantifies protein by the affinity of Coomassie brilliant blue for basic amino acids, particularly arginine. If the compounds had been incorporated into biomass protein as methionine residues, this would not be picked up in the Bradford assay. At higher concentrations (1 and 10 mg.L⁻¹) there may

have been higher protein content in the biomass; however with no reported difference due to methionine being the main contributor. For a more definitive answer, more accurate means of determining biochemical composition would need to be carried out. For example protein could be quantified by measuring total nitrogen, and changes in the amino acid profile by protein hydrolysis and high performance liquid chromatography (HPLC). Uptake and partitioning into the biomass could also be determined using isotopically labelled HMTBA (Mailhot, 1987; Collos et al., 2007).

Most species exhibited similar biochemical profiles through the growth cycles, with protein content at its highest during the early growth phase and increased lipid and carbohydrate content as the cultures entered the stationary phase. Such profiles have been reported previously, due to high metabolic activity during growth, and the accumulation of storage compounds during the stationary phase (Fernández-Reiriz et al., 1989). Carotenoid content was roughly the same in all the species tested throughout the growth cycles ($\sim 3\text{-}5 \mu\text{g}\cdot\text{mg}^{-1}$), however, late stationary phase was not monitored and this is generally the time that carotenoids accumulate (Guil-Guerrero and Reboloso-Fuentes, 2008). Similar carotenoid levels in green algae and *Spirulina* have been reported previously (Jaya Prakash Goud et al., 2007; Liao et al., 1993), yet this is much lower than the $290 \mu\text{g}\cdot\text{mg}^{-1}$ carotenoid that can accumulate in *Dunaliella* (Hu et al., 2008). As a feed supplement, however, it has been found that these lower levels and at only 3% supplemented diet, are sufficient for pigmentation of, for example, black tiger prawns (Liao et al., 1993).

The high carbohydrate content observed in *Chlorella* and *Micractinium* in the stationary phase, and *Spirulina* in the early growth phase (equating total biochemical composition to more than 100%) could have been due to the production of extracellular polysaccharides which have been reported in both green algae and cyanobacteria, particularly during the early and late stages of growth (Moore and Tischer, 1964; Marker, 1965; Maksimova et al., 2004; Trabelsi et al., 2009). As biomass was determined by absorbance, these soluble extracellular carbohydrates would not have been reported in the biomass determination; however they would have been part of the quantified carbohydrate. One can assume therefore that intracellular carbohydrate, determined by the methods described, is overestimated.

Of the four species isolated from the HRAP and tested, *Micractinium* showed the most promise for utilisation as an animal feed supplement due to its high protein content of ~40% during the growth phase. This species also accumulated more lipid and carbohydrate in the stationary phase, and therefore could find additional applications in biofuel production (Craggs et al., in press).

Further studies on the effect of HMTBA and Cu-HMTBA on the amino acid profile would provide valuable insight as to their incorporation into algae biomass as protein, and more specifically as the amino acid, methionine. Additionally, studies on the uptake and localisation of these organic compounds in the cell would need to be carried out as it is possible that Cu-HMTBA could be incorporated into the algae biomass via storage in neutral lipids in the cell, as previous studies on organometallic compounds have found (Wong et al., 1997). If this were the case, the algae biomass would still provide a more sustainable means of introducing HMTBA into animal diets.

Chapter 5. General Discussion and Conclusion

Possibly the most precious resource on planet Earth is potable water and there is substantial evidence to suggest that demand already exceeds availability. It is generally accepted that an individual uses between 150-200 L of water per day (UNEP, 2002). Thus, an average size town or city of 100 000 inhabitants generates a domestic wastewater stream of 15-20 ML every day. Clearly, the continued excessive use of an already scarce resource will place heavy burdens on those responsible for managing water supply and treatment. This is particularly true for arid regions (e.g. southern Africa, Australia and western USA) and in areas of rapid economic growth and urbanisation (e.g. India, China and South East Asia). It is therefore not surprising that many are advocating water recycle and reuse, a philosophy being championed by His Royal Highness the Prince of Orange Willem-Alexander, Chairman of the United Nations Secretary-General's Advisory Board on Water and Sanitation, and evidenced in recent publications such as "Charting Our Water Future - Economic frameworks to inform decision-making" (2030 Water Resources Group, 2009). Furthermore, the recent 'food versus fuel' debate has highlighted the potential risks of using arable land and fresh water to produce low value products such as plant oils for biodiesel production (Tilman et al., 2006; Chisti, 2007a, 2007b; Chisti, 2008; Reijnders, 2008; Posten and Schaub, 2009). Similarly, animal feed is produced on arable land and requires potable water for its production. Since microalgae biomass produced during conventional wastewater treatment has been demonstrated as a more than adequate fertilizer in row crop agriculture and high value horticulture it seemed pertinent to investigate the potential of wastewater treatment HRAPs to support a biomass that may lend itself for use in the formulation of animal feed. It is hoped that the outcome of the current investigation and future studies will facilitate a paradigm shift on how water is used and reused.

The present study has indeed successfully demonstrated the co-utilisation of HRAPs for wastewater treatment and the production of algae biomass for use as an animal feed supplement. A previous study indicated that HRAPs, as part of an IAPS, provide efficient treatment of a variety of wastewater streams (Horan et al., 2007), however in depth characterisation of the biomass has until now not been described. Furthermore, the current study presents information on the operation of HRAPs in both batch and continuous modes and describes the efficiency of each for generation of a quality biomass while maintaining nutrient removal capacity. Finally, using pure cultures of *Chlorella*, *Micractinium*,

Scenedesmus and *Pediastrum*, isolated from the HRAPs, and the cyanobacterium *Spirulina* (as a commercial control), the effect of HMTBA on nutrient content and composition of these microalgae was elucidated.

The four main species observed within the HRAP were, under continuous mode, *Micractinium*, *Scenedesmus* and *Pediastrum*, and under batch operation, *Chlorella*, *Pediastrum* and *Scenedesmus*. These species were successfully isolated from the HRAP and were unequivocally characterised by PCR of 18S rRNA gene, and DNA sequencing. BLAST analysis resulted in the identification of these microalgae as *Chlorella sorokiniana*, *Micractinium pusillum*, *Pediastrum duplex* and *Scenedesmus communis*. Cell counts of samples taken during continuous and batch operation revealed that species composition in the HRAP was considerably influenced by the mode of operation, particularly due to different grazing pressures exerted by the zooplankton present. Under continuous operation lower species diversity was observed, as slower growing algae species were unable to establish. Large colonial species dominated, and in particular, in the presence of rotifers, *Micractinium* flourished as this species develops spines which prevent grazing (Schlüter et al., 1987). *Daphnia* were unable to proliferate due to the short retention time which caused washout of these slower growing grazers (Benemann et al., 1977; Rothhaupt and Lampert, 1992), however in batch mode they were able to establish and exert a significant influence on the algae population dynamic. *Micractinium* numbers declined, allowing the faster growing *Chlorella* to dominate in nutrient depleted water.

Mixed populations are typical of open systems, and species diversity is influenced by a number of factors (Azov et al., 1980). Although it is difficult to determine a suitable application of a changing and mixed biomass, allowing natural populations of algae to establish provides an adaptable system which is less susceptible to losses in productivity. For this reason characterising the biomass over prolonged periods, under different conditions is essential so that some degree of predictability or control over the population can be established.

Nutrient removal was effective in both modes of operation to within South African standard for discharge. During continuous mode nutrient removal was more efficient due to higher biomass productivity with average concentrations of 11.2 mg.L⁻¹ nitrate, 3.5 mg.L⁻¹ phosphate and 1.6 mg.L⁻¹ ammonium obtained; however lower final nutrient concentrations

were achieved during batch operation with 1 mg.L⁻¹ nitrate, 0.5 mg.L⁻¹ phosphate and 0.1 mg.L⁻¹ ammonium. Biomass generation in the HRAP was influenced by changes in temperature and irradiance, but significantly more so by the mode of operation, with higher productivity obtained in continuous mode, due to reduced self shading.

Biochemical composition of the algae biomass was of similar order of magnitude to previous findings (Becker, 2007) and appeared to be mostly influenced by the species composition, with higher protein content observed during periods of *Micractinium* dominance. However, mode of operation also had an effect on biochemical composition as there was significantly higher carbohydrate accumulated by microalgae when HRAPs were operated in batch mode. Therefore, in order to generate biomass high either in protein or carbohydrate in open HRAP systems, mode of operation as well as species dominance should be considered.

As productivity was higher under continuous mode, which is usually operated for wastewater treatment, further studies were carried out to determine whether protein content could be further enhanced to improve nutritional quality of the resultant microalgae as an animal feed supplement. Of the four species isolated from the HRAP, it was determined that *Micractinium* was the best candidate for co-wastewater treatment and generation of an animal feed supplement, for the following reasons: Growth and biochemical analysis revealed that *Micractinium* has high protein content during exponential growth and demonstrated mixotrophic capacity resulting in increased growth rate and biomass accumulation when exposed to HMTBA at 10 mg.L⁻¹, and possibly as a result of incorporation of HMTBA into biomass to give a higher protein yield. Generation of *Micractinium* biomass with incorporated HMTBA as a by-product of wastewater treatment would provide a sustainable means of administering this feed supplement to animals. This process would also be economically feasible due to the ease of harvesting the readily settling colonial species (García et al., 2000). Additionally, this species was maintained in the HRAP under continuous operation and comprised > 90% of the total population, demonstrating an easily cultured species in outdoor photobioreactors.

Overall, work done in this study confirmed that operation of a HRAP in continuous mode is more productive for biomass generation and wastewater treatment than batch mode due to higher metabolic activity within the pond. Operation under these optimum conditions, subsequently results in the generation of a biomass high in protein with potential for further

use as an animal feed supplement. Furthermore, it was determined that addition of HMTBA to a HRAP treating a waste stream has the potential to improve the biomass productivity and protein yield, provided the resident microalgae species have mixotrophic growth ability.

Application of biomass for feed generated in such a system, however, encounters problems due to concerns of toxicity from the waste streams. Whilst this system may not become a reality in the avenue of production of microalgae for human consumption, due to unpalatability caused by its green colour, powdery texture and slightly fishy taste and smell (Becker, 2007), it can become a reality in the avenue of animal feed supplements. Secondary toxicology studies have been carried out to evaluate the safety of the meat (for human consumption) of broilers and carps raised on microalgae (grown on wastewater). The results of the study indicated no effect on the growth and well being of rats which were fed the chicken or fish meat, and although toxic metals were high in microalgae this was not reflected in the consumed flesh; there was however accumulation of arsenic in the chicken livers (Yannai et al., 1980).

Co-utilisation of microalgae for wastewater treatment and the generation of a biomass for feed applications creates an ideal nutritional ecosystem, in which waste nutrients are utilised and conserved by a variety of organisms which can then be utilised by man, other animals and plants (Figure 5-1). In this way waste generated is no longer a pollutant but rather it is a valuable resource, as recycling provides nutrients (energy, protein, vitamins and minerals), non-polluted water and air, and overall a more habitable environment for man (Kromann, 1980).

In the above mentioned nutritional ecosystem, simple-stomached animals such as swine and poultry conserve nutrients of non-fibrous products, and ruminant animals conserve those of fibrous products (Kromann, 1980). Although a variety of nutrients are available to these animals (particularly ruminant animals) through the consumption of microalgae, methionine is known to be a limiting amino acid (Williams and Burris, 1952). For this reason, HMTBA is added to animal feed formulas to supplement methionine and promote animal growth, as its absence results in incomplete utilisation of other important amino acids (Anon, 2005).

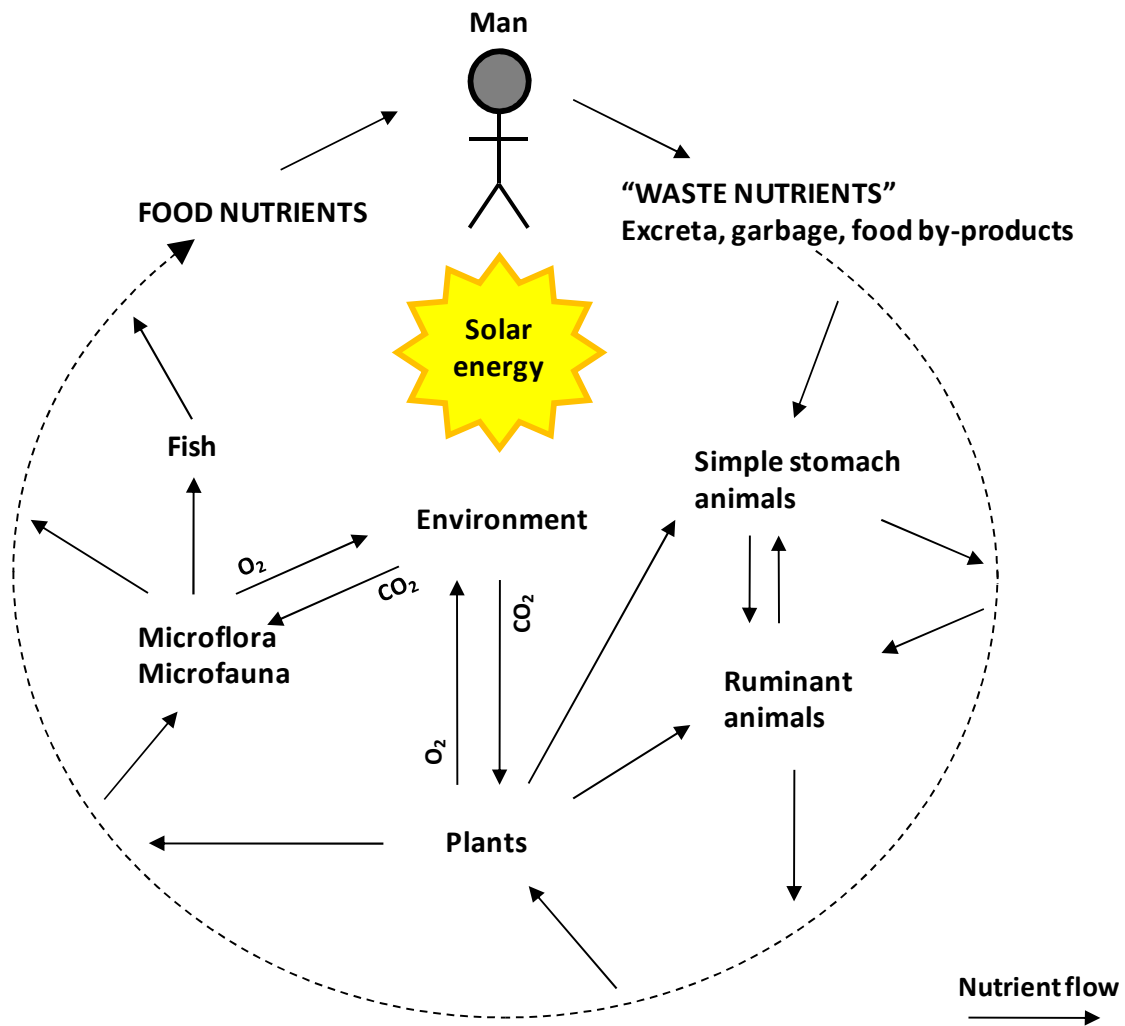


Figure 5-1: Proposed nutrient ecosystem (adapted from Kromann, 1980).

HMTBA is of particular importance as a feed supplement as animals lack the ability to assimilate sulphur which must therefore be obtained through diet. Methionine is generally the principal sulphur-containing contributor of proteins and is therefore the most important sulphur compound in any diet (Pirie, 1932). Sulphur is a vital constituent of a number of organic compounds essential for cellular processes such as protein biosynthesis and transfer of electrons or acyl groups (Hell, 1997). Therefore, addition of HMTBA to the nutritional ecosystem at the level of microalgae would enhance food production, which is vital for sustainability to nourish our growing population.

Conclusions and future work

Successful isolation of key species, in a HRAP treating wastewater, enabled confirmation of species identity, species quantification and the determination of factors influencing population dynamic and nutritional quality of biomass generated. Furthermore, studies on the effect of HMTBA on the biochemical composition of these native species could be carried out to determine the ability to improve nutritional quality of biomass generated in wastewater treatment systems for further application as an animal feed additive. Results indicated that microalgae biomass generated as a by-product of wastewater treatment can be utilised for animal feed supplementation, provided that the waste stream treated passes safety regulations. Addition of HMTBA to a HRAP treating a waste stream has the potential to improve the biomass productivity and protein yield, if the resident microalgae species have mixotrophic growth ability.

Future work should involve prolonged monitoring of the population dynamics of the HRAP in order to determine the effect of seasonal variation on productivity, species composition and nutritional quality of the biomass. Controlled grazing experiments would also provide further insight into the major effectors of species dominance. Additionally, in depth controlled laboratory studies on the uptake and incorporation of HMTBA and Cu-HMTBA into algae biomass, as well as their influence on amino acid profile, should be carried out. Toxicity studies on the effect of Cu-HMTBA on the growth of different algae species, grazers and fungi would determine whether this compound could find additional applications in pond management strategies. Finally, in order to add favour to the proposed co-utilisation process further investigation into the treatment of different wastewaters should be undertaken with additional toxicology studies to determine food safety.

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Appendices

Appendix 1: DNA extraction and PCR of *Chlorella*, *Micractinium*, *Pediastrum* and *Scenedesmus*

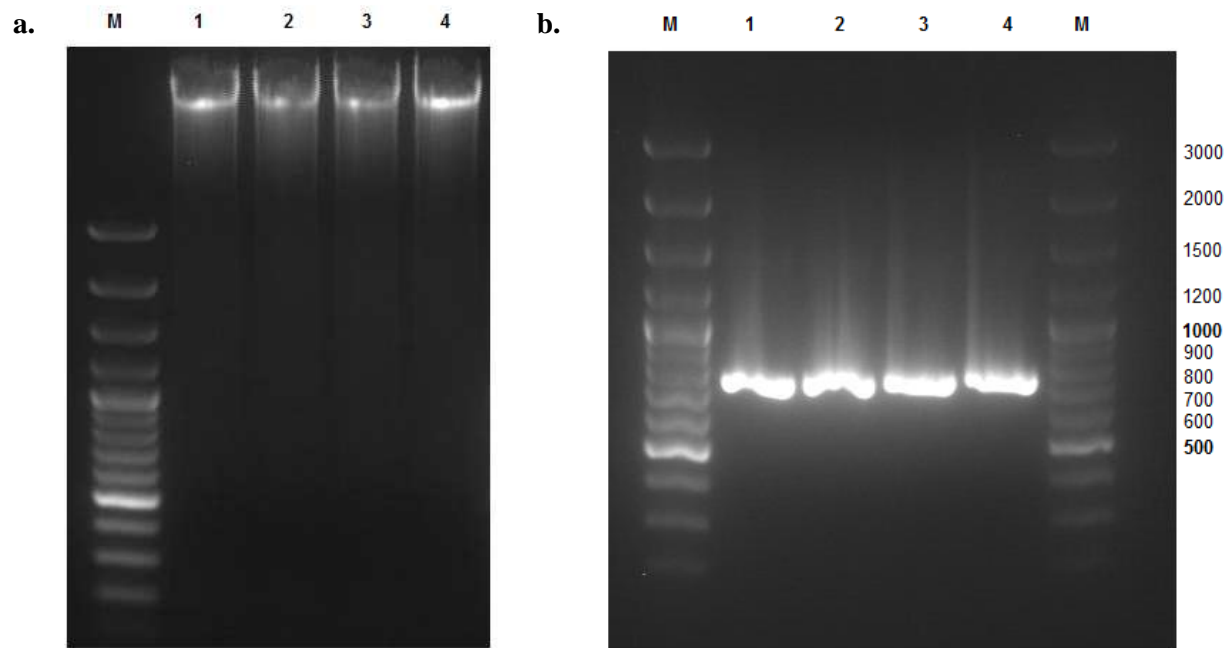


Figure 6-1: a. DNA extraction of pure cultures. b. PCR products for DNA sequencing to verify algae species identification. M – DNA marker (bp), 1 – *Chlorella*, 2 – *Micractinium*, 3 – *Pediastrum*, 4 – *Scenedesmus*.