Treatment of anaerobically digested brewery effluent in high rate algal ponds: An understanding of the microbial community structure in the ponds and the underlying mechanisms responsible for nutrient removal from the effluent

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Abstract

Tertiary treatment of wastewater using high rate algal ponds (HRAPs) is achieved through aerobic degradation of organic matter by bacteria and fungi. This aerobic degradation results in the release of inorganic substances such as carbon dioxide (CO_2), orthophosphate (PO_4^{3-}) and ammonia (NH_3). These inorganic substances are removed from the effluent through algal assimilation, NH_3 volatilization and PO_4^{3-} precipitation. When the HRAPs used in wastewater treatment are operating optimally, they can effectively remove nitrogen and phosphorus from the effluent.

High rate algal ponds were used to treat post-anaerobically digested (post-AD) brewery effluent at a pilot scale. The effects of seasonal variation on the microbial community structure and physicochemical parameters in HRAP A2 operating at a constant flow rate were investigated for 12 months. A flow rate experiment tested the effects of inflowing effluent flow rates on the microbial community structure and the removal of the effluent's nutrient load for a month in HRAP B2. An experiment in a controlled environment room tested the effects of temperature and pH on the microbial community structure and nutrient removal from the effluent.

The microbial community structure in HRAP A2 displayed seasonal trends. A light microscope identified the microalgae Chlorophyta, Cyanophyta, Euglenophyta and Bacillariophyta during summer, autumn, winter and spring, however, there was a seasonal shift in the species composition. Metagenomic techniques were used to identify microalgae and bacteria in summer, winter and spring. *Scenedesmus abundans* was the dominant species during summer, while in winter and spring the dominant species were *Mychonastes* sp. and *Nanofrustulum shiloi* respectively. In the bacterial community structure, Firmicutes, Proteobacteria and Planctomycetes were identified in all three seasons. Firmicutes were dominant in summer, while Actinobacteria and Proteobacteria were dominant during winter and spring respectively. Bacteroidetes, Verrucomicrobia, Acidobacteria, Cyanobacteria and Chloroflexi were present in some seasons yet absent in others. The algal biomass, chlorophyll (Chl) *a* and phaeophytin (Phaeo) *a* concentrations increased during summer, and then decreased in winter, and again increased in spring. Seasonal variation also had a marked effect on the abiotic parameters, with the maximum dissolved oxygen (DO), chemical oxygen demand (COD), pH and electrical conductivity (EC) recorded in summer. Changes in the algal biomass affected nutrient removal from the effluent. An increase in the removal efficiency of ammonium-nitrogen (NH_4^+-N) coincided with an increase in algal biomass. Whereas, there were no clear links between orthophosphate-phosphorus (PO_4^{3-}-P) removal and algal biomass.

The experiment testing the effects of different effluent flow rates resulted in a shift in the microbial community structure in HRAP B2. When using the microscope, the identified microalgae were Chlorophyta, Cyanophyta and Bacillariophyta, however, there were shifts in the species composition. When metagenomic techniques were used, at the start of the experiment, *Chlorella vulgaris*, *Nannochloris* sp., *Nanofrustulum shiloi*, *Scenedesmus abundans, Staurosira construens* and other microalgae with a low relative abundance were identified. As the flow rate was progressively increased by the end of the experiment, *Chlorella vulgaris* and *Scenedesmus subspicatus* were the only species identified. Firmicutes, Proteobacteria, Planctomycetes and Cyanobacteria were identified at the start and the end of the experiment, while Bacteroidetes, Chloroflexi, Actinobacteria and Euryarchaeota were identified occasionally. The algal biomass, Chl *a* and Phaeo *a* decreased as the flow rate was progressively increased. The DO, COD and pH remained constant, while the EC decreased with an increase in the effluent's flow rate. There was a relationship between the standing algal biomass and nutrient removal. The removal of NH_4^+-N remained constant, with a post-HRAP concentration of about 0.5 mg/L at flow rates that ranged from 1000 to 1800 L/d, but increased to about 70 mg/L at 2200 L/d. The removal of PO_4^{3-}-P decreased with a decrease in algal biomass.

When investigating the effects of temperature (20 and 30℃) and pH (7.0, 8.5 and 10.0) on nutrient removal, two pH adjustment methods: CO_2 and hydrochloric acid (HCl) were used. Similar microalgae and bacterial phyla identified in the seasonal variation and effluent flow rate experiments were identified at the start of the trial. When the temperature was increased from 20 to 30℃, the Chl *a* concentration also increased. The maximum Chl *a* concentration at both temperatures was obtained at pH 8.5. The removal of NH_4^+-N and PO_4^{3-}-P also increased with an increase in temperature. Ammonium-nitrogen removal from the effluent at pH 7.0, 8.5 and 10.0 was significantly different ($p < 0.001$) at both pH adjustment methods. Ammonium-nitrogen's removal was greater at pH 10.0 than at pH 7.0 and 8.5. At 20℃, the main mechanism of NH_4^+-N removal was through microbial activity (algal assimilation and nitrification) at all pH levels. However, at 30℃, a greater portion of NH_4^+-N was removed through microbial activity at pH 7.0 and 8.5, but at pH 10.0, volatilization was the main mechanism of NH_4^+-N removal. The PO_4^{3-}-P concentrations decreased significantly ($p = 0.02$) at all pH levels in the CO_2 method, however, in the HCl method, there was no significant difference ($p = 0.44$). Orthophosphate-phosphorus removal appeared to be due to algal assimilation. This could be supported by the increase in PO_4^{3-}-P removal which concurred with higher Chl *a* concentrations in the CO_2 method than in HCl method. Carbon dioxide addition in microalgae culture is known to increase algal biomass, thus the increase in algal assimilation.

Seasonal variation of temperature and pH affected the microbial community structure, which in turn affected the algal biomass and nutrient removal efficiency. An increase in temperature increases microalgal metabolism, thus the increased nutrient removal efficiency and algal biomass during summer and at 30℃. The preferred pH for most freshwater species is between eight and nine, this could be the reason the maximum growth was observed at pH 8.5. Besides temperature and pH, operational factors such as the hydraulic retention time (HRT) can also affect the microbial community structure, the algal biomass and nutrient removal efficiency. A HRAP with a depth of 11.5 cm and a surface area of 15.0 m^2 can retain algal biomass and treat effluent effectively up to a flow rate of 1800 L/d. The mechanisms which were possibly responsible for nutrient removal were NH_3-N volatilization, nitrification and algal assimilation. The laboratory experiment demonstrated that an increase in temperature and pH enhances NH_3-N volatilization, while an increase in pH from 7.0 to 10.0 decreases nitrification. It also demonstrated that an increase in temperature favours algal biomass and nutrient removal efficiency. The laboratory experiment provided an understanding of the mechanisms responsible for nutrient removal in the HRAPs. The removal of NH_4^+-N was through both microbial activity and volatilization of NH_3-N, whereas, PO_4^{3-}-P removal appeared to be through algal assimilation.

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Abbreviations

16S rRNA	16S subunit of ribosomal ribonucleic acid
18S rRNA	18S subunit of ribosomal ribonucleic acid
AD	Anaerobically digested
ATP	Adenosine triphosphate
AOB	Ammonium oxidizing bacteria
BLAST	Basic Local Alignment Search Tool
BMB	Department of Biochemistry, Microbiology and Biotechnology
BOD	Biological oxygen demand
CA	Carbonic anhydrase
CCM	Carbon concentration mechanism
CE	Controlled environment
Chl *a*	Chlorophyll *a*
Cl^-	Chloride
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DIFS	Department of Ichthyology and Fisheries Science
DO	Dissolved oxygen
DAFF	Department of Agriculture, Forestry and Fisheries
DWAF	Department of Water Affairs and Forestry
EBRU	Institute for Environmental Biotechnology, Rhodes University
HCl	Hydrochloric acid
HRAP	High rate algal pond
HRT	Hydraulic retention time
hL	Hectolitres
HPLC	High performance liquid chromatography
IAPS	Integrated algal ponding system
ISO	International Organization for Standardization
L/D	Light/darkness
MANOVA	Multivariant analysis of variance
$MgCO_3$	Magnesium carbonate
NaCl	Sodium chloride
$NADPH_2$	Nicotinamide adenine dinucleotide hydrogen phosphate

NaOH	Sodium hydroxide
NH_4^+-N	Ammonium-nitrogen
NGS	Next generation sequencing
nm	Nanometres
NO_2^--N	Nitrite-nitrogen
NO_3^--N	Nitrate-nitrogen
NOB	Nitrite oxidizing bacteria
OH^-	Hydroxyl ions
PCR	Polymerase chain reaction
PAR	Photosynthetically active radiation
PFD	Photon flux density
PFP	Primary facultative pond
PGA	Phosphoglyceric acid
Phaeo *a*	Phaeophytin *a*
PO_4^{3-}-P	Orthophosphate-phosphorus
PVC	Polyvinylchloride
RU	Rhodes University
SAB Ltd	South African Brewery Limited
SABWF	South African Breweries Water Footprint
SAIAB	South African Institute for Aquatic Biodiversity
SAWS	South African Weather Service
U.S. EPA	United States Environmental Protection Agency
WRC	Water Research Commission
WWF	World Wildlife Fund

Chapter one

General introduction

1. 1 Why treat wastewater?

Freshwater is widely used by industries and communities due to its easy accessibility, storage and the minimal costs associated with its treatment (Adewumi 2011). Globally, freshwater is scarce and this encourages the need to conserve it (Adewumi 2011). In South Africa, it has been predicted that in the next 25 years, if the country's water consumption patterns do not change, there will be an increasing demand for water supply that will present challenges in the equal distribution of water to citizens (Adewumi 2011). With the escalating usage of water by industries, there is an increase in wastewater production containing high levels of phosphorus and nitrogen (Abdel Hameed 2007, Choi & Lee 2012). This wastewater can be treated and reused for non-potable applications (Adewumi 2011). However, in South Africa, wastewater reuse is still at an infancy stage (Adewumi 2011). Several technologies in wastewater treatment have been considered and demonstrated through experimental research worldwide (Mata *et al*. 2012). Treatment systems such as aerobic and anaerobic reactors have proven to be effective, but are costly, difficult to operate, require high energy and generate large volumes of waste sludge (Mata *et al*. 2012). Therefore alternative wastewater treatment systems that are cheap and require less energy to operate have been explored such as the use of microalgae-bacteria assemblages (Abdel Hameed 2007).

1.2 Microalgae-bacteria assemblages

Algae are unicellular and multicellular eukaryotic organisms capable of photosynthesizing like higher plants, but lack leaves and roots (Richmond 2004). They are ubiquitous, preferring aqueous environments, but can also be found in soil (Richmond 2004). They can also form symbiotic relationships with other microorganisms *in situ* such as in the case of lichens (Richmond 2004). Algae have green chlorophyll pigments similar to that of plants while other photosynthetic pigments such carotenoids and phycobilins can also be present (Richmond 2004). Their sizes range from 0.5 µm (microalgae) to 50 m long (macroalgae) (Richmond 2004). Algae (microalgae) serve as food for some aquatic organisms, and in the case of macroalgae, they provide shelter (Madigan & Martinko 2006). They have an important role in geochemical cycling of nutrients, carbon fixation and oxygen release (Madigan & Martinko 2006; Karcher 2010). Algae can also be used in the production of nutraceuticals and aquaculture feeds (Spolaore *et al*. 2006; Hoff & Snell 2007). They can also be mass cultured to fill a variety of roles, such as in wastewater treatment (Spolaore *et al*. 2006; Hoff &

Snell 2007). Bacteria are unicellular prokaryotic microorganisms with cell walls, but lack organelles and a nucleus (Madigan & Martinko 2006). They are also ubiquitous and can be of a rod, sphere, spiral or cocci shape with a typical size of one to five micrometres long and a micrometre wide (Madigan & Martinko 2006). Bacteria are the primary decomposers of organic matter (Gurung & Urabe 2010).

1.3 Background of Ibhayi Brewery wastewater treatment system and the pilot scale effluent treatment study

The study site is situated at Ibhayi Brewery in Nelson Mandela Bay Metropolitan Municipality. The South African Brewery Limited (SAB Ltd) discharges its effluent to the municipal sewers. Only about 670 000 L of this effluent is treated on site, primarily to reduce the chemical oxygen demand (COD) load in the effluent sent to the municipal wastewater treatment works (Cilliers 2012). The brewery is billed by the municipality for treating this waste effluent, and there are considerable cost savings if there is a reduction in the COD content of this effluent (Cilliers 2012). The on-site treatment facility uses a drum filter as a primary treatment that separates solid items from the brewery effluent. Subsequently, this primary treated effluent stream is split, one portion is discharged directly to the municipal sewer and the remaining portion further undergoes secondary treatment in an anaerobic digester to biodegrade its organic matter content. The purpose of the anaerobic digestion process is to reduce the amount of COD reporting to the municipal wastewater treatment plant, for charges are levied according to the amount of COD in the effluent stream (Cilliers 2012). The post-anaerobically digested (post-AD) brewery effluent stream is further treated in an activated sludge digester, an aeration basin, a clarifier and then discharged into the municipal sewer after chlorination (Cilliers 2012). A portion of the effluent leaving the anaerobic digester is diverted to a small wastewater treatment pilot plant for further treatment, which is the focus of this study. Here, further treatment of the post-AD brewery effluent is the subject of an ongoing piloting study that uses both microalgae and bacteria biocatalysts to remove ammonium-nitrogen (NH_4^+-N) and orthophosphate-phosphorus (PO_4^{3-}-P) present in the final effluent stream. Microalgae-bacteria consortia are cultured in this effluent in shallow open ponds circulated by paddle wheels, which also provides the turbulent energy to ensure mixing of the biocatalysts with the effluent. These ponds are called high rate algal ponds (HRAPs) because of their high biological productivity (Figure 1.1).

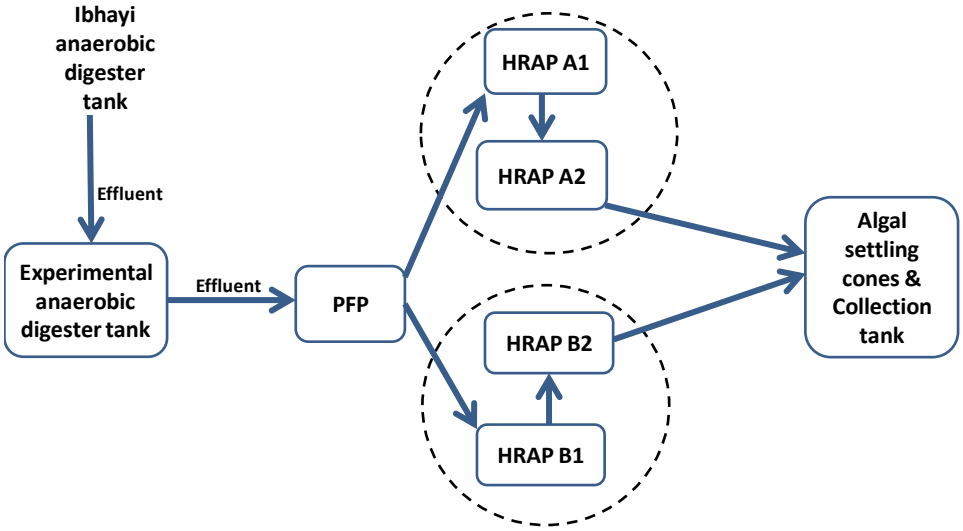

Figure 1.1 A schematic diagram of an integrated algal ponding system composed of an anaerobic digester, primary facultative pond (PFP), high rate algal ponds (HRAP A1, HRAP A2, HRAP B1, HRAP B2) and algal settling cones at the Ibhayi Brewery used to treat post-anaerobically digested (post-AD) brewery effluent at an experimental scale.

1.4 SABMiller water footprint

Beer is the world's fifth most widely consumed beverage amongst other drinks such as tea, milk, coffee and soft drinks (Fillaudeau *et al*. 2006). The average consumption of beer per person per year is estimated to be about 23 L (Fillaudeau *et al*. 2006). In 2002, the breweries sector produced over 1.34 billion hectolitres (hL) of beer globally and since brewing is a water intensive process, there is a need to conserve water in the brewery operations (Fillaudeau *et al*. 2006; Fakoya 2014). At the moment, the brewing industry uses an average of five litres of water to make a litre of beer (SABWF 2009). The SABMiller envisaged the need to conserve water and make it one of its priorities towards sustainable development. It aimed to reduce its water consumption in 2015 by reducing a substantial amount of water used to produce a litre of beer by 25% (SABWF 2009). The target was to reduce the water usage in the brewery operations to an average of 3.5 L of water per litre of beer produced (SABWF 2009). The SABMiller also emphasized the need for efficient and sustainable use of freshwater resources worldwide (Fillaudeau *et al*. 2006). The water footprint of beer in the value chain of South Africa and Czech Republic were the main focus of the SABMiller and World Wildlife Fund (WWF) in the Water Footprint Network (SABWF 2009). In South Africa, the SAB Limited (Ltd) total water footprint was 155 L of water per litre of beer while in the Czech Republic SABMiller, it was 45 L of water per litre of beer (SABWF 2009).

1.5 Legal vehicles driving water use and wastewater disposal in South Africa

The South African government has stipulated acts and standards that regulate the use of water and the disposal of effluent into a natural environment (DWAF 2004a). The relevant acts and standards include the National Water Act (Act 36 of 1998), Water Services Act (Act 108 of 1997), Water Research Act (Act 34 of 1971) and the International Organization for Standardization (ISO) such as the ISO 14001 and the ISO 14051:2011 system. The SAB Ltd in Ibhayi aims to meet the requirements of these acts and standards by further treating their brewing effluent (Hayim *et al*. 2001). Below is a brief description of the acts and standards:

- The National Water Act (Act 36 of 1998): provides a legal vehicle of effective and sustainable management of water resources. It stipulates how water resources are protected, used, developed, conserved, managed and controlled with integrated approaches (DWAF 2004b).
- The Water Service Act (Act 108 of 1997): provides rules for water services, such as provision of potable water and sanitation services by the municipalities to their communities, companies and other institutions. It stipulates how the municipalities should supply water to the community and companies for drinking, sanitation and other purposes (DWAF 2004b).
- The Water Research Act (Act 34 of 1971): This act encourages the promotion of research in connection with water affairs. It also encourages the establishment of the Water Research Commission (WRC) and Water Research Fund (DWAF 2007).
- The ISO 14001 system: this is an environmental management system that every food production organisation has to consider (Kitazawa & Sarkis 2000). The SAB Ltd has a sustainable development approach conceptualized in 2010, which includes ten priority areas, one of which is making more beer with less water and the mitigation of wastes generated from production processes (Fakoya 2014).
- The ISO 14051:2011 system: this is also termed the material flow costing account which focuses on encouraging organizations to be responsible for waste generated at their facility and also encourages the treatment and recycling of generated waste (Kitazawa & Sarkis 2000). It also takes into account the financial implications associated with waste treatment and recycling (Fakoya 2014).

The South African government has guidelines on effluent constituents permitted to be discharged into a natural resource (Table 1.1). South African citizens are encouraged to adhere to the guidelines.

Table 1.1 Wastewater limit values applicable for effluent discharge into a water resource (DWAF 2004a).

Parameter	General limit (mg/L) except pH	Special limit (mg/L) except pH
Ammonia as nitrogen	3.00	2.00
Nitrite as nitrogen	15.00	1.50
Nitrate as nitrogen	15.00	1.50
Orthophosphate as phosphorus	10.00	2.50
pH	5.50-9.50	5.50-7.50
Electrical conductivity (µS/cm)	70.00	50.00
Chemical oxygen demand	75.00	30.00
Chlorine as free chlorine	0.25	0.00

1.6 The brewing process and the brewery's effluent composition

The brewing process involves the handling of raw material, making of wort, alcoholic fermentation, filtration, bottling and packaging of beer (Figure 1.1; Driessen & Vereijken 2003). The quality and quantity of the brewery effluent fluctuates significantly as it depends on several processes taking place during the brewing of beer (Cronin 1996; Driessen & Vereijken 2003). The amount of wastewater produced from the brewing process is usually related to the water consumed and is expressed as hectolitres of water per hectolitre of beer brewed (Cronin 1996; Driessen & Vereijken 2003). Breweries consume water and produce wastewater that results in a water:beer:wastewater ratio of 4-11:1:2-8 m^3 for each cubic metre of beer produced (Connaughton *et al*. 2006).

A portion of the effluent produced during brewing is lost through evaporation while the remainder is discharged along with brewery solid waste (Cronin 1996; Driessen & Vereijken 2003). The brewery effluent typically consists of organic substances such as soluble starch, saccharides, fatty acids, spent grains and waste yeast (*Saccharomyces* sp.) (Cronin 1996; Driessen & Vereijken 2003). These organic substances are anaerobically digested in an anaerobic digester (Sayara 2010; Martin-Ryals 2012). They are further aerobically degraded by bacteria and fungi to produce ammonia (NH_3), PO_4^{3-} and carbon dioxide (CO_2) in a pond (Gurung & Urabe 2010). Zooplankton also contribute to organic matter degradation by breaking down organic particles with their mandibles, as well as releasing NH_3, PO_4^{3-} and CO_2 through egestion and excretion of undigested material and faeces in a water body (Korstad 1983). Cleaning chemicals containing NH_3 used at the brewery also contribute to the concentrations of NH_4^+-N in the brewery effluent (Heyneke, *pers. comm.*, water quality process specialist, SAB Ltd, Port Elizabeth, November 2014). All these factors influence the content of nitrogen and phosphorus in the brewery effluent (Table 1.2). Phosphorus levels in the effluent tend to escalate due to the use of phosphorus containing chemicals such as phosphoric acid in the clean in place process (Cronin 1996; Driessen & Vereijken 2003).

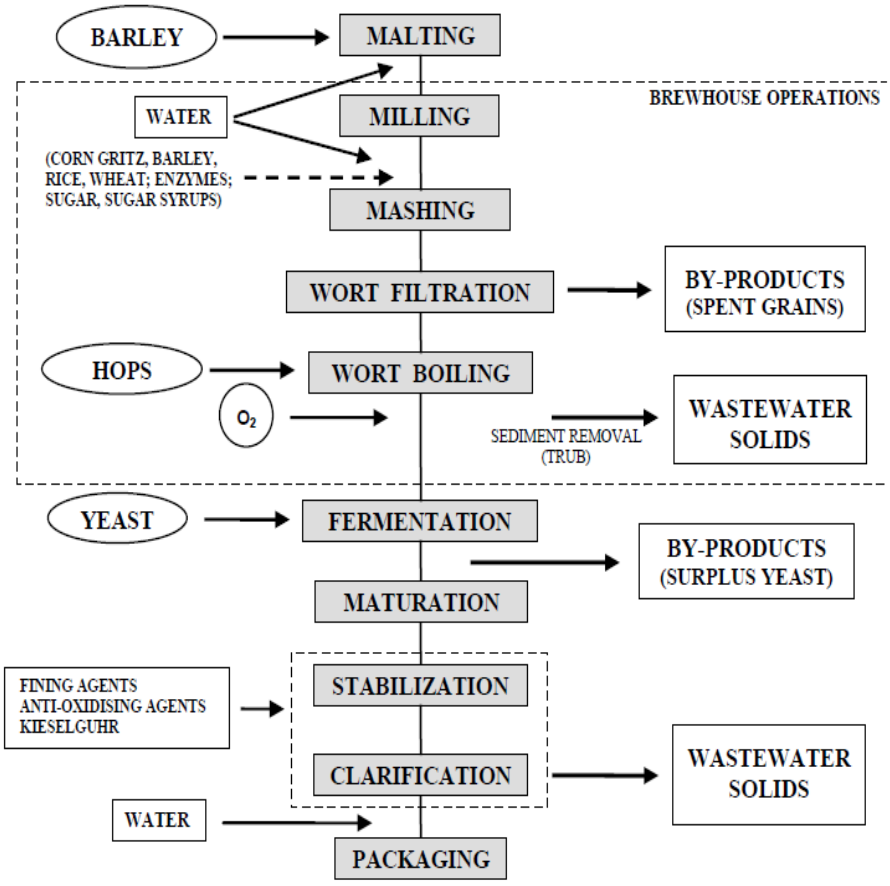

Figure 1.2 The brewing process (Brito *et al*. 2007).

Table 1.2 Ammonia, nitrate and total phosphate concentrations, the chemical oxygen demand and biological oxygen demand values of brewery effluent (Jones *et al*. 2014).

Parameter	Range of values (mg/L)
Ammonia	3.07-106.44
Nitrate	1.86-11.16
Total phosphates	56.98-325.75
Chemical oxygen demand	565.00-7837.00
Biological oxygen demand	560.00-4778.00

1.7 Anaerobic digestion of wastewater

Anaerobic digestion is a proven technology known to be effective in the treatment of industrial wastewater containing organic wastes (Connaughton *et al*. 2006). In anaerobic digestion, microorganisms, particularly bacteria, break down complex organic matter into simpler molecules in the absence of O_2, and biogases such as methane (CH_4), CO_2 and hydrogen sulphide (H_2S) are produced during this process (Cronin 1996). Anaerobic digestion is divided into four sequential stages, namely, hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1.3; Cronin 1996; Martin-Ryals 2012).

Detailed descriptions of the stages involved in an anaerobic digestion are as follows:

- Hydrolysis: this is the first stage of anaerobic digestion. Facultative and obligate anaerobic bacteria release enzymes such as hydrolases into the ambient environment which split covalent bonds within macromolecules such as carbohydrates, proteins and fats. These macromolecules are converted into sugars, amino acids and fatty acids respectively (Sayara 2010; Martin-Ryals 2012).
- Acidogenesis: this is the second stage of anaerobic digestion. Hydrolysis products are microbially degraded by a variety of facultative and obligate anaerobic acidogens such as *Bacteroids* spp*., Bifidobacterium* spp*., Clostridium* spp*., Lactobacillus* spp*.*and *Streptococcus* spp. into alcohol, H_2, CO_2 and volatile fatty acids (VFAs) such as butyric acid. The products of this stage vary extensively, depending on the active bacterial species and the environmental conditions (Sayara 2010; Martin-Ryals 2012).
- Acetogenesis: this is the third stage of anaerobic digestion. The products of acidogenesis are converted into acetate (CH_3CO_2) by obligate anaerobic acetogenic bacteria (Sayara 2010; Martin-Ryals 2012).
- Methanogenesis: this is the final stage of anaerobic digestion. Methane is generated in two pathways by various methanogens. In the first pathway, CH_3CO_2 is converted into CH_4 and CO_2 by acetoclastic methanogens such as *Methanosaeta* spp*.* (Navarro 2012). In the second pathway, hydrogenophilic methanogens such as *Methanosarcina* spp*.* use H_2 to reduce CO_2 to CH_4 (Cronin 1996; Madigan & Martinko 2006; Navarro 2012). Acetoclastic methanogenesis converts about 72% of volatile solids to CH_4, whilst 28% is converted by hydrogentropic methanogenesis (Figure 1.2; Sayara 2010; Martin-Ryals 2012; Navarro 2012).

The AD effluent can be treated further such as in an integrated algal ponding system (IAPS) (Wells 2005).

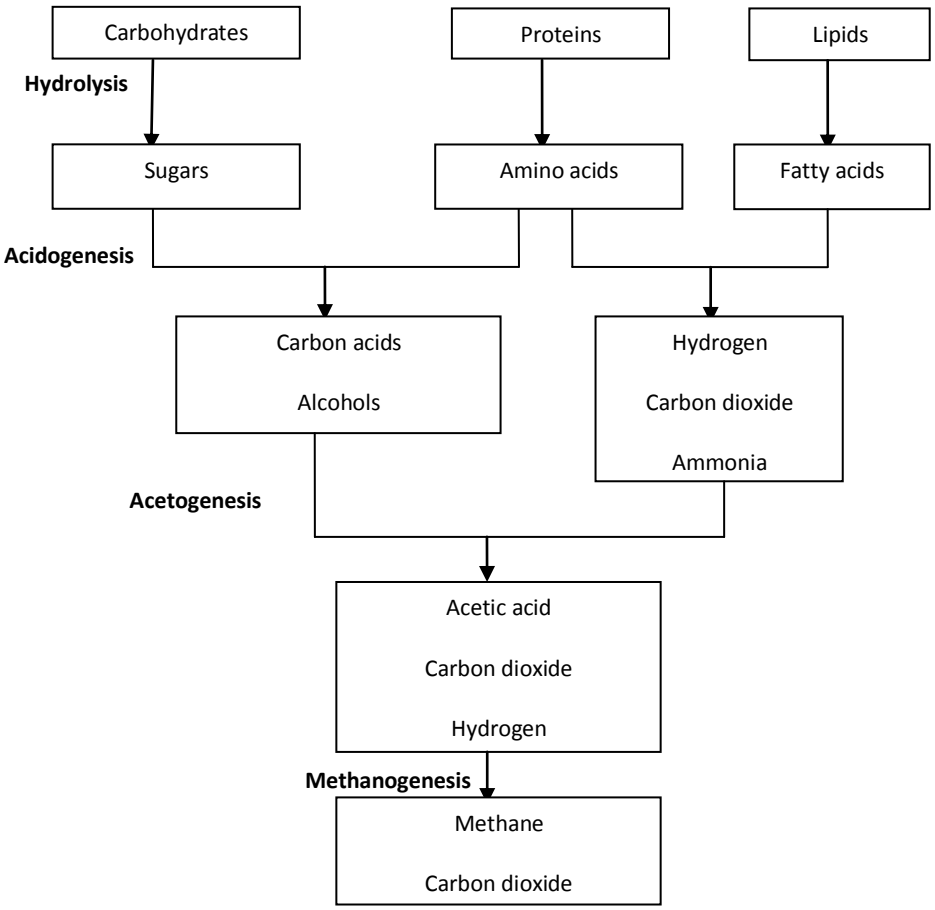

Figure 1.3 Anaerobic digestion pathways (Martin-Ryals 2012).

1.8 Components of an integrated algal ponding system

An IAPS for wastewater treatment refers to the integrated stages involved in the treatment of effluent with microalgae as a tertiary treatment system (Clark 2001; Wells 2005; Abdel Hameed 2007). Facultative ponds and HRAPs containing microalgae-bacteria consortia that decomposes organic matter, removes nitrogen and phosphorus in effluent form part of an IAPS for wastewater treatment (Clark 2001; Wells 2005). The HRAPs are typically followed by an algal settling pond (Wells 2005).

The ponds are operated optimally by adjusting their hydraulic retention time (HRT), which is the measure of the time liquids remain in a pond. The most commonly used HRT in HRAPs is between two to seven days, depending on the season the HRAPs are operated at and the volume of effluent that needs to be treated daily (Larsdotter 2006a; Muñoz & Guieysse 2006). A shorter HRT is preferably used in summer while a longer HRT is recommended in winter (Larsdotter 2006b).

1.9 Background of Project Eden

The Department of Ichthyology and Fisheries Science (DIFS) at Rhodes University (RU) is engaged in a wastewater treatment project, also known as Project Eden, situated in the SAB Ltd, Ibhayi Brewery. The objectives of Project Eden are to recycle effluent and potentially reduce the costs borne by SABMiller for water purchase and the costs involved in the brewery effluent treatment by the municipality. The municipality requires the COD in effluent to be less than 500 mg/L and have a pH greater than six. However, if it exceeds this limit, the brewery can incur financial penalties. If brewery effluent is recycled, it can be used for other water usage activities at the facility, i.e. the recycled effluent can be used for cleaning and irrigation purposes. By doing so, this will result in less water being purchased, since water for cleaning and irrigating will already be available at the site. The HRAPs and wetlands at the site are considered as a potential solution for brewery effluent treatment. Another objective of Project Eden was to test if HRAPs can produce effluent that meets the Department of Water Affairs (DWAF) general limits. Cilliers (2012) found that the HRAPs were efficient in the removal of NH_4^+-N and PO_4^{3-}-P from the post-AD brewery effluent. However, the microbial complexes and the underlying mechanisms responsible for the removal of NH_4^+-N and PO_4^{3-}-P from the post-AD brewery effluent were not fully understood.

A better understanding of which microalgae-bacteria consortia is present in the HRAPs and how NH_4^+-N and PO_4^{3-}-P are removed from the post-AD brewery effluent under different environmental conditions can give an idea of how the HRAPs operate. An understanding of how the HRAPs operate would provide wastewater treatment plants with the tools to optimise the treatment of effluent when using an algal ponding system.

1.10 Aims and objectives

The aim of this study was to better understand the biotic and abiotic changes occurring in the HRAPs at different times of the year and at different HRTs. It was also aimed at better understanding the underlying mechanisms responsible for the removal of NH_4^+-N and PO_4^{3-}-P from the post-AD brewery effluent. The specific objectives were to:

- establish the identity of microorganisms present in a HRAP using microscopy technique and metagenomics work, describe the microbial community structure (microalgae and bacteria) and nutrient removal from the post-AD brewery effluent at different times of the year (Chapter 3);

- establish the identity of microorganisms present in HRAPs using microscopy technique and metagenomics work, describe the microbial community structure (microalgae and bacteria) and nutrient removal from the post-AD brewery effluent at different HRTs (Chapter 4); and
- determine the effects of environmental parameters, such as temperature and pH on the microalgae community structure (microscopy technique), microalgae growth and nutrient removal from the post-AD brewery effluent (Chapter 5).

Chapter two

Literature review: microorganisms in high rate algal ponds, factors that affect their growth and roles in wastewater treatment

2.1 Microbial community structure and trophic levels in an aquatic environment

Microorganisms play a vital role in biogeochemical cycling of chemical elements, particularly carbon, sulphur, nitrogen, phosphorus and iron (Madigan & Martinko 2006; Matcher *et al*. 2011). This process enables the regeneration of elements essential for plant nutrition (Madigan & Martinko 2006). In an aquatic environment, the microbial community structure is composed of a variety of microorganisms such as microalgae, bacteria, fungi, protozoa and metazoa (Madigan & Martinko 2006). These microorganisms occupy specific trophic levels in a food web grouped into decomposers, producers and consumers (Gurung & Urabe 2010; Kang *et al*. 2013; Yu *et al*. 2014).

Bacteria and fungi are principal decomposers of organic matter releasing inorganic nutrients which become available for other trophic levels such as primary producers, for example microalgae (Gurung & Urabe 2010). Microalgae are autotrophs and occupy the first trophic level in a food web (Schroeder *et al*. 2012). Autotrophs are organisms capable of synthesizing organic carbon from inorganic nutrients using sunlight as an energy source (Richmond 2004; Markou & Georgakakis 2011; Schroeder *et al*. 2012; Medeiros *et al*. 2013). They serve as a food source for consumers such as zooplankton (Hoff & Snell 2007; Sellami *et al*. 2010; Yu *et al*. 2014). Zooplankton such as protozoa and metazoa are heterotrophic microorganisms (Medeiros *et al*. 2013). They feed on bacteria and algae, and in turn serve as a food source for fish and other large aquatic animals (Medeiros *et al*. 2013; Hisatugo *et al*. 2014; Yu *et al*. 2014). Protozoa provide the link between primary producers and higher trophic levels when they are consumed by other larger multicellular aquatic animals such as rotifers (Medeiros *et al*. 2013; Hisatugo *et al*. 2014). Metazoa such as rotifers are in turn consumed by fish and in this way, the carbon synthesized by primary producers is assimilated by other trophic levels (Wallace 2002).

2.2 Integrated wastewater treatment system

Facultative lagoons, also known as facultative ponds or waste stabilization ponds, have been used for over a century as a secondary treatment process in the management of wastewater (Griffiths 2010). They are a cost effective means of treating wastewater and range from small to large scale ponds (Griffiths 2010). Facultative lagoons are about 1.5-1.8 m deep with the water column vertically differentiated into an anoxic benthic zone and an aerobic upper zone (Mara *et al*. 2003). In

the benthic zone, anaerobic biodegradation of organic matter takes places with the release of carbon dioxide (CO_2) and methane (CH_4), while in the upper zone, dissolved organic matter is oxidised and CO_2 is also released (Cronin 1996; Wells 2005). The CO_2 in the aerobic zone is made available for assimilation by algae (Wells 2005). Effluent leaving the facultative ponds can receive further treatment in high rate algal ponds (HRAPs) where substantial removal of nutrients in the wastewater stream occurs (Wells 2005; Johnson 2010).

Tertiary treatment of wastewater using microalgae as a biocatalyst in HRAPs was conceptualised in the 1950s in California by William Oswald (Oswald 1991; Wells 2005; Larsdotter 2006a; Griffiths 2010). High rate algal ponds are shallow ponds with a depth of less than a metre, they have paddle wheels mixing the effluent continuously to keep algal cells in suspension (Wells 2005; Griffiths 2010). The design of HRAPs takes into consideration the organic loading of the waste stream and the available solar energy (Dunn 1997; Aguirre *et al*. 2011). The ponds are low-technology driven, require less energy inputs and are cost effective when compared to other conventional wastewater treatment systems (Al-Balushi *et al*. 2012; Al-Rajhia *et al*. 2012; Cilliers 2012).

In HRAPs, organic matter in the effluent is aerobically degraded by consortia of bacteria and fungi (Aguirre *et al*. 2011; Kshirsagar 2013). During the day, algae contribute to maintaining an aerobic environment by releasing O_2 into the effluent stream (Wells 2005; Larsdotter 2006a; Griffiths 2010). Aerobic degradation of organic matter by bacteria and fungi results in the release of CO_2 and ammonia (NH_3) which are in turn assimilated into biomass by the autotrophic algae population in the HRAPs (Chen *et al*. 2003; Aguirre *et al*. 2011; Kshirsagar 2013). The heterotrophic microorganisms secrete a range of enzymes such as amylase, lipase and protease for catabolising starch, lipids and proteins respectively (Madigan & Martinko 2006). High rate algal ponds efficiently remove nutrients from the effluent stream at a hydraulic retention time (HRT) of two to seven days, which is the most commonly used in wastewater treatment (Dun 1997; Wells 2005; Larsdotter 2006a; Muñoz & Guieysse 2006; Sen *et al*. 2013). When operating optimally, HRAPs are capable of removing about 80% of nitrogen and phosphorus from the effluent within a few days (Wells 2005; Sen *et al*. 2013). The treated effluent can be used for non-potable pursposes (Adewumi 2011). At the brewery, it can be used for cleaning and irrigation. Typically, the effluent from the HRAPs may decant into settling ponds where the algal community and particulate matter are allowed to sediment out (Dun 1997; Wells 2005). The overflow then decants into a maturation pond for final polishing of the HRAP's treated effluent stream (Sen *et al*. 2013).

In South Africa, at the Institute for Environmental Biotechnology, Rhodes University (EBRU) experimental field station, an integrated algal ponding system (IAPS) is used to treat sewage effluent

at a pilot scale (Figure 2.1). This system has been functional for fifteen years to date with modifications to the design being incorporated. The system is able to significantly reduce the organic loading of the wastewater stream and to reduce the nutrient load at a comparable rate with other conventional wastewater treatment systems (Wells 2005).

Figure 2.1 An integrated algal ponding system (IAPS) composed of primary facultative pond (PFP), high rate algal ponds (HRAPs), algal settling ponds and drying beds at the Institute for Environmental Biotechnology, Rhodes University (EBRU) for sewage treatment at an experimental scale (Wells 2005).

2.3 Factors affecting the performance of high rate algal ponds

An understanding of the microalgae community structure and its relationship with other biotic and abiotic factors in an aquatic system provide an insight into their roles in nutrient cycling (Madigan & Martinko 2006). Various factors affect the microalgae community structure in terms of their productivity and performance in nutrient removal (Madigan & Martinko 2006). These factors may be abiotic, biotic and operational. Abiotic factors include the absorption and scattering of light in water, the effects of the spectral quality of light on microalgae growth, effects of pH on nutrient solubility, the effects of temperature on microbial metabolism, the effect of CO_2 on pH levels and O_2 required by bacteria for organic matter degradation in a aquatic system (Johnson 2010). Operational factors include the effects of the depth of a pond on light penetration, effects of turbulence on nutrient availability and the HRT of a pond (Johnson 2010). Biotic variables include competition between microorganism for resources, zooplankton grazing on microalgae and the effect of infectious pathogens, such as parasites, on microalgae (Larsdotter 2006b; Johnson 2010;

Markou & Geogakakis 2011). These abiotic and biotic factors vary seasonally, particularly the microalgae community structure, a process known as algal succession (Addy & Green 1996).

2.3.1 Photosynthetic activity of algae

Algae, just like higher plants, utilize light as a source of energy through the process of photosynthesis (Richmond 2004). Phototrophic organisms utilize light within the wavelengths of 400 to 700 nanometres (nm) (Richmond 2004). This is the wavelenght range of photosynthetically active radiation (PAR) that overlaps with the visible light spectrum (Richmond 2004). The visible light is characterised by different colours of the light spectrum, each representing different wavelengths of light (Richmond 2004). The wavelengths in the PAR spectrum are referred to as the spectral quality of light (Richmond 2004). The light intensity in the PAR spectrum is quantified by photosynthetic photon flux density (PPFD), which is the measure of the number of photons in the PAR region falling on a metre square area in a second (Richmond 2004). The PPFD differs from that of the photon flux density (PFD) of the visible light spectrum because light wavelengths not utilized in photosynthesis are not included in PAR PFD, i.e. on a sunny day, the PFD can be about 4500 µmol m^{-2} s^{-1}, of which 40% (1800 µmol m^{-2} s^{-1}) is the PAR PFD obtained within 400 and 700 nm (Richmond 2004). When light energy is delivered in small pulses at a low PPFD, photoautotrophs can utilize light completely (Janssen *et al*. 2001). At higher PPFD, the photosynthetic apparatus becomes saturated and no additional photosynthesis takes place (Richmond 2004). Light intensity varies during the day and at different seasons (Muñoz & Guieysse 2006).

In water, light can be scattered or absorbed depending on the angle at which it penetrates a water body prior to reaching a specific depth (Curtis *et al*. 1994). If light penetrates a water body at an acute angle, there is less chance of it reaching a significant depth (Curtis *et al*. 1994). Light can also be reflected at the water surface depending on the sun's angle of incidence (Morris *et al*. 1995). The sun's angle of incidence is the angle at which the rays of the sun strike the surface of earth (Morris *et al*. 1995). The scattering and absorption depends also on water components such as gilvin and tripton particles (Curtis *et al*. 1994). Gilvin are humic substances absorbing light at a wavelength between 400 to 440 nm (Curtis *et al*. 1994). Tripton are particulate elements absorbing light of a similar spectrum to that of gilvin, however, the absorption is less (Curtis *et al*. 1994). Clear water has the ability to absorb light of wavelengths $<$ 550 nm moderately (Curtis *et al*. 1994). In turbid water, light is scattered forward with two percent of it scattered in the direction from which it came, which is known as back-scattered light (Curtis *et al*. 1994).

2.3.2 Effects of turbulence on light

In an aquatic environment, turbulence exposes phytoplankton to variable light by moving cells at random towards and away from the illuminated point in the water column (Fogg 1991; Richmond 2004). Turbulence creates fluctuating light regimes of flashing light or light and dark integration that affects the PPFD in the water column (Grobbelaar 1991; Grobbelaar 1994; Aydin 2007; Grobbelaar 2009). The flashing light occurs when short light/darkness (L/D) oscillations exist in a water body (Janssen *et al*. 2001). Benemann *et al*. (1987) stated that the flashing light effects created by turbulence in shallow microalgae culture reactors increased the productivity of the microalgae. Flashing light effects in *Chlorella* sp. culture also increased the species productivity (Janssen *et al*. 2001). In instances where there is algal shading, cells are exposed to L/D cycles that presumably take a few milliseconds to seconds (Richmond 2004). Exposure of a *Spirulina platensis* culture to L/D cycles of seconds to tenths of seconds resulted in a photosynthetic efficiency of 15% (Qiang & Richmond 1996).

2.3.3 Photosynthesis and light absorption pigments

Algae and plants contribute towards the availability of atmospheric gases through the production of O_2 and reduction of CO_2. Algae carry out photosynthesis and fix carbon through the Calvin-Benson cycle (Richmond 2004). The photosynthesis stoichiometric formula is illustrated below by Richmond (2004):

$$6CO_2 + 6H_2O + \text{light energy} \leftrightarrow C_6H_{12}O_6 + 6O_2$$

Photosynthesis is divided into two phases, the light and dark phases, which operate independently (Markou & Georgakakis 2011). During the light phase, light energy is absorbed by the photosystem pigments of the antennae complex or light harvesting complex in the chloroplast (Markou & Georgakakis 2011). In this process, light energy is converted into chemical energy through the production of reduced nicotinamide adenine dinucleotide hydrogen phosphate ($NADPH_2$) and adenosine triphosphate (ATP) (Markou & Georgakakis 2011). The $NADPH_2$ and ATP are utilized by algae to synthesis organic carbon compounds (Markou & Georgakakis 2011). The pigments responsible for absorption of light are chlorophyll (Chl), carotenoids and phycobilins (Markou & Georgakakis 2011). Chlorophyll absorbs red light between 650-700 nm and carotenoids absorb blue light ranging from 400 to 500 nm, while phycobilins absorbs orange to red light at 600-650 nm (Markou & Georgakakis 2011).

2.3.4 Photoinhibition of microalgae

Microalgae undergo alterations in their cell composition and physiological properties in response to sunlight (Richmond 2004). Light affects water variables such as water temperature and dissolved oxygen (DO) concentration (Rivkin 1989; Grobbelaar 1994; Lin & Beck 2001). With an increasing light intensity, microalgae growth rates tend to increase up to maximum at the optimal light intensity (Talbot *et al*. 1991). When light intensity is continuously increased, microalgae reach a saturation point, which can even result in photoinhibition that decreases growth rate (Talbot *et al*. 1991). At a low temperature and high light intensity, microalgae are more susceptible to photoinhibition (Larsdotter 2006a). The inhibition of microalgae is mainly due to photooxidation caused by visible light (Fogg 1991). In photooxidation, light is not absorbed directly by chlorophyll, but captured by a sensitizer molecule present in the algal cell and then transferred to O_2 (Benchokroun *et al*. 2003). The O_2 reacts with fatty acids which results in the formation of toxic lipid peroxides, hydrogen peroxides and hydroxyl radicals that kill microalgae (Benchokroun *et al*. 2003; Carvalho *et al*. 2011). At low temperatures, metabolic rates are slowed and this affects the ability of the algal cell to neutralise these free radicals (Richmond 2004). At higher temperatures, metabolic activity in the algae proceeds at a much higher rate and this allows a much quicker response to the build up of free radicals in the cell (Richmond 2004). Microalgae are able to recover from photoinhibition, which takes few hours and cells previously exposed to high irradiance become more tolerant to high irradiances (Fogg 1991). However, at optimal temperature ranges, microalgae can tolerate higher light intensities (Larsdotter 2006a).

Anabaena variabilis and *Chlorella sp*. were exposed to various combinations of light intensity and temperature (Richmond 2004). The lowest light intensity was tested 42 µmol m^{-2} s^{-1} and microalgae reached their maximum photosynthetic rate at 15°C (Richmond 2004). However, when the temperature was increased above 15°C combined with the low light intensity, the photosynthetic rate of microalgae decreased (Richmond 2004). When the temperature and light intensity were both increased, the photosynthetic rate also increased (Richmond 2004).

2.4 Algal–bacterial relationship

In algal–bacterial relationships, algae produce O_2 through photosynthesis, making it available for aerobic bacteria to degrade organic matter into inorganic matter (Muñoz & Guieysse 2006). In doing so, the aerobic bacteria produce CO_2 through respiration, which in turn is consumed by algae (Figure 2.2; Muñoz & Guieysse 2006). Microalgae can either enhance or inhibit the growth of bacterial cells (Bell & Lang 1974). They enhance bacterial growth by releasing extracellular compounds such as

carbohydrates, which are essential for bacterial growth (Bell & Lang 1974; Muñoz & Guieysse 2006). Cyanobacteria can also produce metabolites such as microcystins and antibiotics into a culture system, which have growth inhibitory effects on bacteria, eventually leading to bacterial cell lysis (Lopes & Vasconcelos 2011; Wang *et al*. 2014). Microalgae can even alter the environmental conditions of a culture system by elevating pH through photosynthesis, making the environment unfavourable for bacterial survival (Muñoz & Guieysse 2006).

Similarly, bacteria can either enhance or inhibit algal growth (Wang *et al*. 2014). Bacterial cells enhance algal growth by creating an environment favourable for algal growth through the reduction of O_2 and the release of CO_2 into a culture system through respiration (Muñoz & Guieysse 2006; Wang *et al*. 2014). Bacteria can produce algae growth promoting substances such as vitamins, inorganic nutrients, chelators and phytohormones (Wang *et al*. 2014). In some instances, bacterial cells can be detrimental to microalgae by releasing lytic substances into a culture system that lyses algal cells (Muñoz & Guieysse 2006; Wang *et al*. 2014).

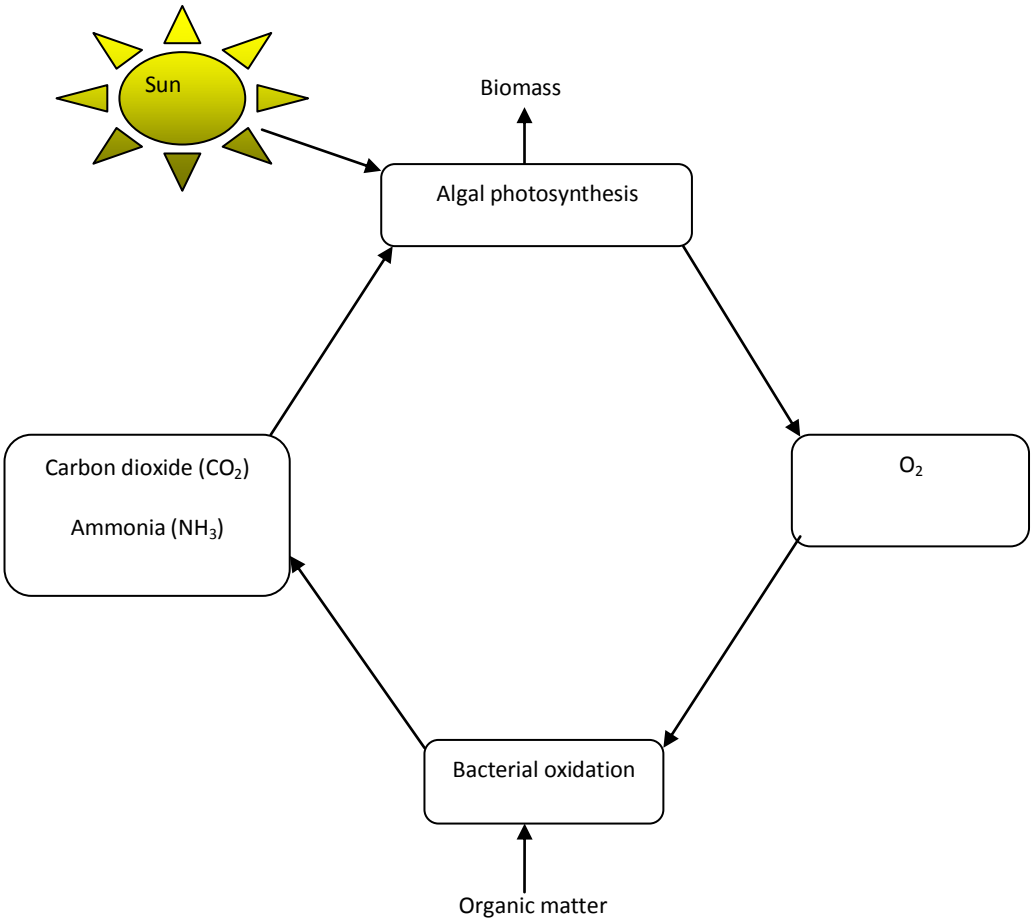

Figure 2.2 Algal-bacterial relationships in wastewater treatment using high rate algal ponds (Muñoz & Guieysse 2006).

2.5 The biological nitrogen cycle

In wastewater treatment plants, ammonification is one of the processes employed in organic matter decomposition (Park *et al*. 2006, You *et al*. 2009). This is the conversion of organic matter containing nitrogen such as proteins, into ammonium (NH_4^+) or NH_3 (Strauss 2000). Nitrification follows ammonification, whereby NH_4^+ is converted into inorganic nitrogen forms (Park *et al*. 2006, You *et al*. 2009). Nitrification is an aerobic process that converts NH_4^+ into nitrite (NO_2^-) and then nitrate (NO_3^-) within the temperature range of 20 to 30℃ (Mara *et al*. 2003). This process is facilitated by ammonium oxidizing bacteria (AOB) such as *Nitrosomonas* spp*., Nitrosospira* spp*., Nitrosovibrio* spp., *Nitrosococcus* spp*.* and nitrite oxidizing bacteria (NOB) such as *Nitrobacter* spp*., Nitrospira* spp. and *Nitrococcus* spp*.* (Mara *et al*. 2003; Park *et al*. 2006, You *et al*. 2009). In the first stage of nitrification, NH_4^+ is oxidized into NO_2^- with the aid of enzyme catalysts such as ammonium monooxygenase and hydroxylamine oxidoreductase (Figure 2.3; Mara *et al*. 2003). In the second stage of nitrification, the enzyme catalyst nitrite oxidoreductase oxidizes NO_2^- into NO_3^- (Figure 2.3; Mara *et al*. 2003). Park *et al*. (2006) found that bacteria from the *Nitrosomonas* and *Nitrosospira* genera were able to oxidize NH_4^+ into NO_3^- in activated sludge waste. You *et al*. (2009) found that archaea species also play a significant role similar to that of bacteria in nitrification. Archaea are prokaryotes consisting of species such as halophiles, thermophiles and methanogens (You *et al*. 2009). The archae *Candidatus* sp. and *Nitrosopumilus maritimus* have NH_4^+ monooxygenase and oxidized NH_4^+ into NO_2^- in activated sludge effluent (Park *et al*. 2006; You *et al*. 2009). The oxidation of NH_4^+ in effluent occurs within the pH range of 6.5 to eight (Khin & Annachhatre 2004). When the pH rises above this range, nitrification rate declines due to the formation of unionised NH_3 which is toxic to the nitrite oxidizers and hinders their growth (Khin & Annachhatre 2004).

Deammonification is another process employed in wastewater treatment systems (Wett *et al*. 2013). During this process, half of the NH_4^+ is oxidized into NO_2^- by aerobic AOB, while anammox bacteria, under anaerobic conditions, oxidize the residual NH_4^+ using NO_2^- to produce nitrogen gas (Wett *et al*. 2013). The partial nitritation process requires less O_2 as compared to complete nitrification and no source of carbon is required since the bacteria are autotrophs (Wett *et al*. 2013). Anammox is an anaerobic NH_4^+ oxidation process categorised under denitrification due to its release of nitrogen gas as its final product (Mara *et al*. 2003). Denitrification also produces free nitrogen gas as its final product (Mara *et al*. 2003). This process is microbially facilitated by anaerobic and facultative heterotrophs such as *Pseudomonas* spp., *Alcaligenes* spp. and *Paracoccus* spp. in reducing NO_3^- to nitrogen gas or other nitrogen-based gases (Mara *et al*. 2003; Bitton 2005). Denitrification has two

stages: firstly, NO_3^- is reduced to NO_2^- by nitrate reductase (Mara *et al*. 2003). Secondly, NO_2^- is reduced by nitrite reductase to nitrogen gas (Figure 2.3; Mara *et al*. 2003).

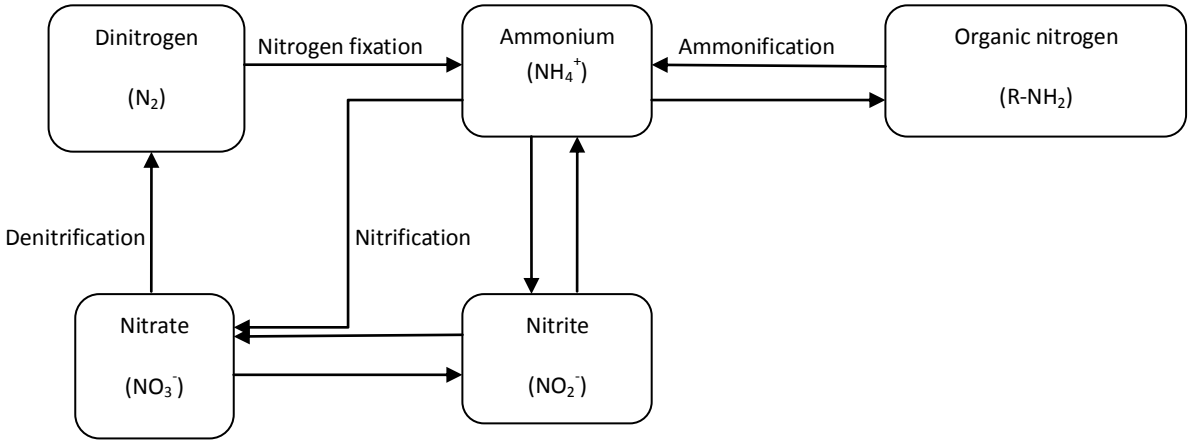

Figure 2.3 The biological nitrogen cycle. Redrawn from Mara *et al*. (2003).

2.6 Microalgae nutrition and various forms of nutrients

Algal cells grown in an aqueous environment might have more access to nutrients than plants (James & Boriah 2010). This contributes to the rapid growth rate of microalgae, with some strains capable of doubling their biomass within 24 h (James & Boriah 2010). Microalgae take up the inorganic nutrients that are made available through bacterial decomposition of organic matter (Mulholland *et al*. 1998; Anderson *et al*. 2002; Glibert *et al*. 2005). During bacterial hydrolysis, amylases, lipases and proteases catabolise organic matter into inorganic nutrients which can be assimilated by algae (Madigan & Martinko 2006).

Nitrogen and phosphorus are macro-nutrients essential in the nutrition of algae (Larsdotter 2006a). Sulphur, potassium, calcium, magnesium, manganese, molybdenum, copper, iron, zinc, boron, chloride and nickel are major micro-nutrients required by algae for growth (Larsdotter 2006a). Sodium, silicon, cobalt, iodine, vanadium and selenium are minor micro-nutrients required by some species of algae (Larsdotter 2006a). Nitrogen constitutes about 79% of this planet's atmosphere (Ssanyu 2003). It is chemically inert, making it inaccessible for plant consumption (Ssanyu 2003). However, species from the genera *Rhizobium*, *Clostridium* and *Azotobacter* can fix atmospheric nitrogen into a bioavailable inorganic form such as NH_4^+ (Mara *et al*. 2003). Nitrogen is the most limiting nutrient in algal growth (Ssanyu 2003). It is vital in the formation of proteins and nucleic acids and accounts for about one to 14% of microalgae dry weight (Mara *et al*. 2003; Zhou 2010). Phosphorus is required in the synthesis of phospholipids, ATP and nucleic acids. This accounts for about 0.05 to 3.3% of microalgae dry weight (Larsdotter 2006a; Zhou 2010).

In wastewater, nitrogen occurs in the form of amino acids, amines, urea, NH_4^+, NO_2^- and NO_3^- (Ssanyu 2003). Algae prefer NH_4^+ as a source of nitrogen and when it is present in effluent, no other source of nitrogen is assimilated (Larsdotter 2006a). Orthophosphate is utilized by microalgae as the preferred source of phosphorus (Ssanyu 2003; Larsdotter 2006a; Zhou 2010). The uptake of these nutrients by microalgae is dependent on environmental parameters such as water temperature, pH and turbulence in the water (Richmond 2004; Griffiths 2010).

2.6.1 The effects of temperature on microalgae growth and nutrient uptake

Temperature ranging from 15 to 25℃ supports the growth of most warm water microalgae (Griffiths 2010). Below 16°C, the growth of warm water microalgae slows down while temperature above 35°C is lethal (Richmond 2004). Antarctic microalgae tolerate temperature range from zero to 10°C, with the optimal temperature at five degrees celsius (Wiencke *et al*. 1994). In the northern hemisphere, Arctic species tolerate levels ranging from zero to 20°C, with an optimal temperature at 10°C (Wiencke *et al*. 1994).

The growth rate of microalgae increases with an increase in temperature until a maximum growth rate is reached (Robarts & Zohary 1987; James & Boriah 2010; Su 2012). Thereafter, it decreases with further increases in temperature (Robarts & Zohary 1987; James & Boriah 2010). Above the optimal temperature, a further increase in temperature might result in an increase in photorespiration which reduces algal productivity (Park *et al*. 2011).

In wastewater treatment plants where microalgae are part of the treatment process, nutrient removal efficiency decreases as temperature decreases in the medium (Muñoz & Guieysse 2006). Muñoz & Guieysse (2006) stated that when temperature was increased from 25 to 30°C in *Chlorella sorokiniana* and *Ralstonia basilensis* culture systems, the nutrient removal efficiency doubled. This could have been attributed to the temperature coefficient (Q_{10}) phenomenon, where every 10°C increase in water temperature doubles the uptake of nutrients by microalgae (Richmond 2004). Cold adapted strains of cyanobacteria can also remove nutrients from wastewater even at a temperature as low as 15°C (Muñoz & Guieysse 2006).

2.6.2 The uptake of carbon by microalgae

Carbon is an essential nutrient for microalgae, making up about half of its dry weight (Larsdotter 2006a; Lundquist *et al.* 2010). Inorganic CO_2 is an important source of carbon for photoautotrophic microalgae and the presence of CO_2 in water enhances microalgae growth (Yang *et al*. 2000; Moroney *et al*. 2001; Larsdotter 2006b; Markou & Georgakakis 2011). Enzymes such as carbonic anhydrase (CA) found in the mitochondria, cytoplasm and chloroplast of algal cells interconvert

HCO_3^- and CO_2 (Markou & Georgakakis 2011). In the carbon fixation phase during the photosynthesis, intracellular CO_2 is incorporated into ribulose-1,5-bisphosphate mediated by ribulose-1,5-bisphosphate carboxylase or rubisco (Jacob-Lopes & Franco 2010). Ribulose-1,5-bisphosphate is an organic molecule containing five carbon atoms. The product of this incorporation is catabolised into two molecules of phosphoglyceric acid (PGA), an organic molecule that contains three carbon atoms (Richmond 2004). The PGA is reduced by $NADPH_2$ into phosphorylated sugars and glucose. This metabolic transformation is known as the Calvin-Benson cycle (Jacob-Lopes & Franco 2010). The metabolism of carbon by microalgae can be categorised into photoautotrophy, heterotrophy, photoheterotrophy and mixotrophy (Yang *et al*. 2000; Markou & Georgakakis 2011). In photoautotrophy, light is the source of energy and inorganic compounds such as CO_2 are the source of carbon for microalgae (Markou & Georgakakis 2011). In heterotrophy, microalgae use organic compounds as a source of energy and carbon (Markou & Georgakakis 2011). In photoheterotropyhy, algal cells utilize energy from the sun and organic compounds as source of carbon (Markou & Georgakakis 2011). In mixotrophy, sunlight is still the source of energy, however, organic and inorganic compounds are used as carbon sources (Markou & Georgakakis 2011).

2.6.3 The effects of pH on ammonium and phosphate solubility and their uptake by microalgae

In HRAPs, an increase in pH due to the photosynthetic activity of microalgae occurs when carbonate and bicarbonate ions dissociate in the following manner:

$$2HCO_3^- = CO_3^{2-} + H_2O + CO_2$$

$$CO_3^{2-} + H_2O = 2OH^- + CO_2$$

The CO_2 is consumed by microalgae while the hydroxyl ions (OH^-) are distributed in the water, hence the rise in pH (Richmond 2004).

The optimal pH for most freshwater algal species lies between 7.5 and 8.5. However, marine species prefer a pH higher than this (Griffiths 2010). A marine phytoplankter such as *Phaeodactylum tricornutum* can tolerate pH as high as 10.3 (Chen & Durbin 1994). The pH affects the solubility of nutrients in water (Larsdotter 2006b; Aydin 2007).

When pH is below seven, NH_4^+ ions are dominant in the medium while at a pH above seven, NH_3 is the dominant form (Camargo Valero & Mara 2010). An increase in pH due to photosynthesis in algal ponds, especially when the algal culture is dense, results in the removal of nitrogen from effluent through volatilization rather than assimilation by microalgae (Camargo Valero & Mara 2010). Volatilization of NH_3 occurs at a pH greater than nine (Camargo Valero & Mara 2010). It is directly

proportional to the pH and temperature of effluent, as pH and temperature increases, NH_3 volatilization also increases (Sevrin-Reyssac 1998; Zimmo 2003).

At a pH of 11 and above, PO_4^{3-} in effluent can precipitate and form calcium phosphate. When cations such as magnesium and calcium are present in water they form metal phosphates of low solubility at a pH above 11 resulting in phosphate precipitation. The precipitated phosphate can redissolve if pH levels drops below 11. However, if carbonates are present in water, the phosphate precipitation can be inhibited even at a pH above 11. The precipitation of phosphate in wastewater can also occur at a neutral pH provided the concentration of phosphate is about 50 mg/L and the calcium concentration is about 100 mg/L (Larsdotter 2006a).

Phosphate can be removed from wastewater through microalgae assimilation (Larsdotter 2006a). Organic phosphate is converted into orthophosphates by phosphatase at the surface of bacterial cells (Larsdotter 2006a). Orthophosphates are soluble and easily assimilated by microalgae (Larsdotter 2006a). They are the most preferred forms of phosphorus for uptake by microalgae (Griffiths 2010). Phosphorus uptake by microalgae is inversely proportional to the phosphorus present in a cell (Griffiths 2010). When the phosphorus concentration in algal cells is insufficient to meet the cell's needs, more phosphorus is taken up from the medium (Griffiths 2010). However, when phosphorus in an algal cell is sufficient, less phosphorus is taken up from the medium (Griffiths 2010). This is due to intracellular phosphorus concentrations responsible for the kinetics of phosphorus uptake (Griffiths 2010). Previously phosphorus starved microalgae take up phosphorus in excess when exposed to it as compared to cells that were not deprived of phosphorus (Griffiths 2010). Some algal species such as *Chlorella vulgaris* and *Scenedesmus dimorphus* assimilate phosphorus as a luxury when it is in excess in effluent and store it as polyphosphate granules (Griffiths 2010). The excess phosphorus is reserved, and utilized at a later stage when phosphorus concentrations in wastewater are depleted (Larsdotter 2006a). The uptake of carbon, nitrogen, phosphorus (C:N:P) by microalgae during photosynthesis occurs in a ratio proportion approximately C:N:P-106:16:1 (Fogg 1991).

2.6.4 The effects of turbulence on nutrient availability and uptake by microalgae

In water, vertical mixing or turbulence exposes phytoplankton to a range of nutrient concentrations and light intensities (Fogg 1991; Winder & Sommer 2012). Turbulence ensures nutrient availability and increases the rates of nutrient transportation to an algal cell, in turn, stimulating phytoplankton growth (Fogg 1991; Winder & Sommer 2012). Vertical mixing also affects phytoplankton sinking velocities, giving small and buoyant phytoplankton species an opportunity to be resuspended (Fogg

1991; Grobbelaar 1994; Craggs *et al*. 2004; Winder & Sommer 2012). A study showed that the growth rate of an *Arthrospira* species increased significantly when the stirring speed in the culture system was doubled (Grobbelaar 1991). However, in some instances, turbulence can have damaging effects on phytoplankton (Fogg 1991). Minimal turbulence also reduces the exchange rates of nutrients between cells and their culture system (France 1995). This may lead to a thick boundary layer of nutrient depleted water developing between algal cells and the growth medium, resulting in a greater resistance to diffusion of nutrients into the cell (France 1995).

2.7 Competition for nutrients amongst microorganisms

In ponds, microorganisms such as such as bacteria, fungi and microalgae can compete for resources (Markou & Georgakakis 2011). Competition among microorganisms for available resources depends on several factors such as the microorganisms' growth rates, their metabolism and nutrient uptake (Markou & Georgakakis 2011). Different microorganisms in the same system depending on the same natural resources can result in interspecies competition when different species compete for the same resources (Markou & Georgakakis 2011). Intraspecies competition amongst microorganisms can also occur when the same species compete for the same resources in a medium (Madigan & Martinko 2006; Shank & Kolter 2009). In these competitions, the possibility of growth inhibition of the outcompeted microorganisms and antibiosis is likely to occur, particularly in cultures with high cell densities (Shank & Kolter 2009). Studies on algal antibiosis showed that some algal species are capable of excreting metabolites that hinder their own growth (autoinhibition) and that of other microorganisms (heteroinhibition) (Richmond 2004). In an autoinhibition situation, the growth of *C. vulgaris* was suppressed by the secretion of a growth inhibitory metabolite Chlorellin into the growth medium (Richmond 2004). Similarly, a culture of *Skeletonema costatum* also inhibited its own growth by releasing growth inhibitory metabolites into the culture system (Richmond 2004). In a heteroinhibition situation, a substance produced by *Chlamydomonas reinhardtii* was reported to be toxic to *Haematococcus pluvialis* (Richmond 2004).

In instances when nutrients are limited in a medium, the paradox of the plankton may occur (Hutchinson 1961). The paradox of the plankton is when nutrients are limited in a homogenous media with microbes competing for them, yet still accommodate a wide range of microorganisms (Hutchinson 1961). The Gause's law can also be observed during intraspecies and interspecies competition (Hutchinson 1961; Metzger *et al*. 2009). The law states that in a homogenous or heterogeneous environment, species competing for the same natural resources cannot coexist at similar population densities (Hutchinson 1961; Roy & Chattopadhyay 2007). Therefore one species shall outcompete the other species resulting in a population consisting of a single species

(Hutchinson 1961; Roy & Chattopadhyay 2007). A laboratory experiment showed that the number of species that can coexist in equilibrium cannot be greater than the number of limiting factors, unless additional factors are included (Roy & Chattopadhyay 2007). Closely related species compete more because they utilize the same resources (Wright *et al*. 1985). This competition may lead to a reduction of one of the other species productivity, which eventually results in that species' elimination (Wright *et al*. 1985). A study on the culture of filamentous microalgae and *S. platensis* in the same culture system showed that filamentous microalgae grew better than *S. platensis* (Markou & Georgakakis 2011). An investigation on competition amongst five marine phytoplankton species cultured in an outdoor system at various nutrient concentrations showed that some species were able to grow better than others (Ssanyu 2003).

When there is no competition amongst microorganisms, and instead nutrients happen to be in excess, microalgae can be intoxicated by the elevated nutrient loads (Chen *et al*. 2012). In a study on the effects of nutrient concentrations on cyanobacteria taxa and green algae showed that the densities of the cyanobacteria decreased to almost zero when cultured in anaerobically digested (AD) effluent with total nitrogen concentrations of 40, 100 and 200 mg/L for 15 days (Chen *et al*. 2012). Conversely, the green algae densities increased due to the excess NH_4^+ present in AD effluent (Chen *et al*. 2012). *Chlorella* sp. and *Scenedesmus* sp. grew optimally in high NO_3^- and NH_4^+ concentrations, while other microalgae were unable to grow (Chen *et al*. 2012). Excessive free NH_3 can inhibit photosynthesis and the uptake of NO_3^- (Chen *et al*. 2012). Excessive phosphorus in the medium can also be inhibitory (Chen *et al*. 2012). Concentrations of 50 mg/L and above were found to have an inhibitory effect on the growth of *Scenedesmus subspicatus* (Chen *et al*. 2012). Non-filamentous green algae seemed to have the ability to tolerate high nutrient concentrations (Chen *et al*. 2012).

2.8 Chlorophyll assays

Measurements of chlorophyll (Chl) *a, b* and *c* are used in the estimation of algal biomass (Wasmund *et al*. 2006). Chlorophyll *a* is the most measured photosynthetic pigment because it is universally present in all algae, unlike other Chl types (Richmond 2004). A Chl molecule consist of a chlorin ring, which is made up of four nitrogen atoms, with a magnesium ion in the centre, having attached side chains and a hydrocarbon tail (Richmond 2004). Methanol, ethanol and acetone are widely used as Chl extraction solvents, however, acetone is the most common one (Wasmund *et al*. 2006). Fluorometry, high performance liquid chromatography (HPLC) and spectrophotometry can be used in determining Chl *a, b* and *c* concentrations of phytoplankton (Ciotti *et al*. 2002). A fluorometer uses special filters at 436 and 680 nm to determine Chl *a* absorbance without any interference from

Chl *b* and *c* (Wasmund *et al*. 2006). However, this method has drawbacks: it also measures phaeopigments because their spectra overlap with Chl *a* and it also fails to distinguish between Chl *a* and chlorophyllide *a* (Wasmund *et al*. 2006). Chlorophyllides are degraded Chl molecules without a phytol tail (Wiltshire *et al*. 1998). Some other degraded molecules of Chl are molecules such as phaeophytin (Phaeo), which lacks a magnesium ion in its structure (Wiltshire *et al*. 1998). Measurement of Phaeo is an indirect method of measuring the grazing of zooplankton on microalgae (Sin *et al*. 2000). When using a fluorometer, acidification can be used to correct for phaeopigments (Wasmund *et al*. 2006). The HPLC method measures Chl *a*, *b* and *c* simultaneously, but it is impractical to use when frequent analysis of numerous samples is required. When using a spectrophotometer, Chl *a, b* and *c* absorbance is determined and the aliquot can be acidified to correct for Phaeo *a, b* and *c*, making this method more reliable than fluorometry and HPLC methods (Wasmund *et al*. 2006). Monochromatic and trichromatic equations are used to determine Chl *a*, *b* and *c* concentrations using the pigment absorbance values obtained from the spectrophotometer (Ciotti *et al*. 2002). A monochromatic equation only measures Chl *a* and corrects for Phaeo *a*, but does not rectify the interference of Chl *b* and *c* (Făgădar-Cosma *et al*. 2005). A monochromatic equation also underestimates Chl *a* concentrations in the presence of Chl *b* (Dos Santos *et al*. 2003). A trichromatic equation corrects for any interference of Chl *b* and *c* when measuring Chl *a* by using the maximum wavelengths of the particular Chl of interest (Ciotti *et al*. 2002). However, the drawbacks of the trichromatic equation is that it does not correct or even measure degraded molecules of Chl such as Phaeo (Făgădar-Cosma *et al*. 2005). It also overestimates the concentration of Chl *a* in the presence of Phaeo *a* (Dos Santos *et al*. 2003). So far, all Chl measuring methods have advantages and disadvantages. The type of method used can be influenced by the nature of the research and the available resources (Arar & Collins 1997).

2.9 Zooplankton grazing on microalgae

Zooplankton are filter feeders of phytoplankton in aquatic environments (Hoff & Snell 2007; Sellami *et al*. 2010). They reduce phytoplankton densities through grazing (Roche 1995; Park *et al*. 2011). The size of a phytoplankter grazed by a zooplankter depends highly on the size of the zooplankter (Bergquist *et al*. 1985). In a study conducted by Park *et al*. (2011) using HRAPs for wastewater treatment and biomass production, results showed that rotifers and cladocerans at densities > 105 counts/L reduced algal biomass by 90% in two days. In the same study, Chl *a* concentrations were reduced by 99% due to the zooplankton grazing. Goldman & Caron (1985) also found that a phagotrophic microflagellate such as *Paraphysomonas imperforate* could graze on a variety of both phytoplankton and bacterial species.

2.10 Value of microalgae

Microalgae have a significant nutritional value in human nutrition, aquaculture and livestock feeds (Becker 2007). The protein and carbohydrate content ranges from one to 63% and from eight to 64% respectively (Becker 2007). Their lipid content which is composed of glycerol and fatty acids also varies extensively ranging from one to 90% (Spolaore *et al*. 2006; Hoff & Snell 2007). Microalgae are also a source of vitamins such as vitamin A, B_1, B_2, B_3, B_5, B_6, B_9, B_{12}, C, E and H. As a result, they are used in aquaculture feeds, livestock feeds, human nutrition and also cosmetic products (Spolaore *et al*. 2006; Hoff & Snell 2007). In human nutrition, microalgae are incorporated in tablets, astaxanthins and liquid preparations (Spolaore *et al*. 2006). They are also added in food items such as pasta, snacks, beverages, candy bars or used as food colourants (Spolaore *et al*. 2006). The most used algal species are *Arthrospira* spp., *Chlorella* spp., *Dunaliella salina, Haematococcus pluvialis* and *Aphanizomenon flos-aquae* (Spolaore *et al*. 2006). In aquaculture and livestock feeds, about 30% of microalgae produced commercially in the world are used for animal feed (Spolaore *et al*. 2006). In cosmetic products, species such as *Arthrospira* spp. and *Chlorella* spp. are incorporated into skin care products such as anti-aging creams and sun blocks (Spolaore *et al*. 2006).

2.11 Value of wastewater

Treated effluent can be considered as wastewater with nutrients rather than just wastewater with no value (Zakria *et al*. 2011). The effluent can be used in agriculture, landscaping and aquaculture (Olguìn 2003; Fillaudeau *et al*. 2006; Zakria *et al*. 2011). Reusing of wastewater for these purposes minimizes the usage of clean water for irrigation, in turn conserving water (Zakria *et al*. 2011). In Turkey, at an experimental scale, brewery effluent was effective in the cultivation of sugar beets (Kütük *et al*. 2003). In India, also at an experimental scale, brewery waste (spent grains) was incorporated into a fish diet fed to carp (Kaur & Sxena 2004). The diet had more essential amino acids such as lysine, methionine and arginine when compared to a diet without the addition of brewery waste which had about three times less of the amino acids (Kaur & Sxena 2004). Fish fed the diet with brewery waste grew well, due to the protein content of the feed (Kaur & Sxena 2004). In South Africa, at an experimental scale, HRAPs' treated brewery effluent was used in the cultivation of tomatoes and results showed that the effluent was effective in providing nutrients required by tomatoes for growth (Jones *et al*. 2014).

2.12 Techniques used to identify microorganisms

Microscopy and metagenomic techniques are used in establishing the identity of microorganisms (Mosleh *et al*. 2012). Traditional microscopy methods focus on phenotypic characteristics of microorganisms while metagenomic techniques focus on the genotypic characteristics of organisms (Mosleh *et al*. 2012). Metagenomic studies provide genetic information of uncultured microorganisms, genomic linkages and profiles of community structures (Valenzuela *et al*. 2006; Kennedy *et al*. 2008; Kakirde *et al*. 2010; Thomas *et al*. 2012). It has been applied in various environmental samples, such as samples from the soil and aquatic environments (Kakirde *et al*. 2010). Firstly, samples are collected from the environment, then deoxyribonucleic acid (DNA) is extracted from them and the targeted genes are amplified by polymerase chain reaction (PCR) (Thomas *et al*. 2012; Gupta *et al*. 2013). Subsequently, the amplicons can be sequenced (Thomas *et al*. 2012; Gupta *et al*. 2013).

Polymerase chain reaction amplifies DNA templates recovered from a wide variety of organisms (Bott *et al.* 2010). In this reaction, about 20-30 cycles are run, with each cycle doubling the DNA (Gibbs 1991). The double helix DNA denatures at 95°C into single strands (Gibbs 1991). The temperature is then reduced to 50-65°C where two oligonucleotides of ribosomal ribonucleic acid (rRNA) primers anneal to the end of the targeted genes of the single strands (Gibbs 1991). The temperature is then increased to 70°C where Taq polymerase extends primers attached to the targeted genes of interest to produce more DNA strands (Gibbs 1991; Madigan & Martinko 2006). Once the reaction is complete, the amount of DNA is sufficient for use in other molecular applications such as in sequencing for establishing the identity of microorganisms (Bott *et al.* 2010; Thomas *et al*. 2012; Gupta *et al*. 2013).

Sequencing methods such as shotgun method can be used for sequencing meta samples (Albaggar 2014). Shotgun sequencing is the cloning of the whole genome of organisms (prokaryotes and eukaryotes) and sequencing the output clones (Madigan & Martinko 2006). The clones are then analyzed by some computer programme that tracks overlapping sequences of the clone (Madigan & Martinko 2006). The overlapping sequences are then assembled by the computer into the correct order (Madigan & Martinko 2006). Some of the shotgun sequencing fragments can be useless after the analysis (Madigan & Martinko 2006).

Next generation sequencing such as Illumina sequencing and 454 pyrosequencing can also be used in metagenomics (Albaggar 2014). The Illumina sequencing method has been used in metagenomics to understand bacterial community and their diversity in aquatic environments and soil samples

(Albaggar 2014). The Illumina sequencing (Illumina HiSeq 2000 and Illumina HiSeq 2500) is sequencing by synthesis method which yields about 200 to 600 Gb sequencing data per run which can take from five to 11 days to run to completion (Albaggar 2014). About a thousand samples can be sequenced simultaneously using the Illumina sequencer platform (Albaggar 2014). The 454 pyrosequencing produces about 0.7 Gb sequencing data per run within 24 hours (Albaggar 2014). Illumina sequencing is cheaper than 454 pyrosequencing, in the sense that its reagents are not expensive (Albaggar 2014). The Illumina sequencing technique produces sequencing data that can be obtained from 454 pyrosequencing (Albaggar 2014). Data yielded from Illumina sequencing and 454 pyrosequencing can be analysed using a Basic Local Alignment Search Tool (BLAST) programme (Bexfield & Kellam 2011). This programme compares the established DNA sequences to existing ones in the BLAST database (Bexfield & Kellam 2011). Metagenomic techniques have characterized microbial communities, i.e. the discovery of ammonium-oxidizing archaea and this technique has also developed a supposition of microbial functions (Thomas *et al*. 2012).

2.13 Conclusion

The purpose of this literature review is to outline the background of microorganisms present in HRAPs, their role and performance under different environmental conditions in wastewater treatment works and also the methods that are used in establishing their identity. Knowledge and an understanding of the topics discussed give insights on HRAPs dynamics in effluent treatment. The use of HRAPs in municipal and sewage effluent treatment through research has been successful, therefore the system has the potential to treat brewery effluent and provide SABMiller Ltd the tools to better manage nutrients in their effluent with fewer costs involved. Since HRAPs are cost effective and less technology driven than other conventional wastewater treatment systems (Al-Balushi *et al*. 2012; Al-Rajhia *et al*. 2012), adaptation of this system to the brewery needs could reduce the costs associated with effluent treatment in the brewery.

Chapter three

Seasonal variation of microorganisms and physicochemical parameters in a high rate algal pond

3.1 Introduction

Abiotic and biotic factors affect the performance of high rate algal ponds (HRAPs) in nutrient removal from wastewater (Wells 2005; Johnson 2010). The abiotic factors include the intensity of photosynthetically active radiation (PAR), temperature, the pH of the effluent and O_2 (Wells 2005, Johnson 2010) and photoperiod (Zucchi & Necchi 2001; Bouterfas *et al*. 2006). The intensity of PAR and temperature affects the metabolism of microalgae, which in turn affects microalgae growth and nutrient removal efficiency (Wells 2005, Johnson 2010; Markou & Geogakakis 2011). The pH of the effluent affects nutrient solubility and O_2 is required by bacterial species to decompose organic matter into inorganic substances (Wells 2005, Johnson 2010; Markou & Geogakakis 2011). Biotic variables include microbial community structure in HRAPs, competition between microorganisms for resources, zooplankton grazing on microalgae and the effect of infectious pathogens, such as parasites, on microalgae (Larsdotter 2006b, Johnson 2010; Markou & Geogakakis 2011). These abiotic and biotic factors vary seasonally and their seasonal variation results in shifts in microbial community composition and abundance (Affan *et al*. 2005). Shifts in microbial community have an important effect on nutrient removal efficiency (Winder & Sommer 2012). An understanding of the seasonal variation of these factors provides insights on the performance of HRAPs in nutrient removal at different times of the year (Affan *et al*. 2005).

Aim and objectives

The purpose of this study was to describe the seasonal changes in the microbial community structure in a HRAP and changes in nutrient removal from post-anaerobically digested (post-AD) brewery effluent at different times of the year. The specific objectives were to:

- establish the identity of microorganisms present in a HRAP at different times of the year using light microscopy and metagenomics;
- describe the microbial community structure in a HRAP at different times of the year;
- describe the removal of nutrients from post-AD brewery effluent at different times of the year; and
- correlate the algal biomass in the HRAP with environmental parameters.

3.2 Materials and methods

3.2.1 Study area and the integrated high rate algal ponding system design

The Ibhayi Brewery, South African Brewery Limited (SAB Ltd) is situated in Kohler Street, Perseverance, which is located 19.0 km northeast of Port Elizabeth in the Nelson Mandela Metropolitan Municipality, Eastern Cape Province (Figure 3.1; Public Process Consultants 2009). The site coordinates of the area are 33°50.305'S 25°32.426'E (Hayim *et al*. 2001). The average temperature and day length data from October 2013 to September 2014 were obtained from the Port Elizabeth South African Weather Services (SAWS 2015).

Figure 3.1 The location of the Ibhayi Brewery, South African Brewery Limited (SAB Ltd) situated in Kohler Street, Perseverance area, in the Nelson Mandela Metropolitan Municipality, Eastern Cape Province (Public Process Consultants 2009).

a. The integrated algal ponding system design

Brewery effluent also has items such as glass and plastics incorporated in it from the packaging of beer (Sharda *et al*. 2013). At the Ibhayi Brewery, brewery effluent with solid items is primarily treated in a drum filter (Autrex industrial screening R 015; serial no: A 140/02, size U-5/3.04) to separate the effluent from these solid items (Cilliers 2012). Subsequently, the effluent goes through secondary treatment in an anaerobic digester to biodegrade organic matter (Cronin 1996; Martin-

Ryals 2012). The effluent is then further treated in an activated sludge digester, an aeration basin, a clarifier and chlorinated (Cilliers 2012).

In this project, about 1000 L/d of effluent was drawn from the Ibhayi Brewery anaerobic digester tank and decanted into a 5000 L anaerobic buffer tank through a 25 mm diameter polyvinylchloride (PVC) pipe (Figure 3.2). The anaerobic buffer tank discharged effluent into a 1.08 m deep circular primary facultative pond (PFP) with a volume of 16730 L situated inside a greenhouse fabricated with clear polycarbonate sheeting; Rhino Plastics (PTY) Ltd (Figure 3.3). A submersible pump (Life Tech AP5800 Water pump, AC220-240 V 50 Hz 360 W, Hmax 5.6 m, Qmax 12000 L/h) was used to supply the PFP with effluent from the anaerobic buffer tank. The amount of effluent from the anaerobic buffer tank into the PFP was controlled by an inlet valve (Figure 3.4). Effluent from the PFP decanted into a splitter box that divided the effluent into two streams (Figure 3.5). Effluent from each side of the splitter box flowed by gravity into the first pond of each high rate algal pond (HRAP A and HRAP B) train (Figure 3.6). The HRAP A and HRAP B train were parallel to each other, with each train having two ponds in series, i.e. HRAP A1 and HRAP A2 (A-train), and HRAP B1 and HRAP B2 (B-train).

The HRAPs were made of green plastic PVC pond liner supported by a galvanised metal frame. The first pond of each train was approximately 25 cm deep, with a surface area of 14.8 m^2 and a volume of 3700 L/pond (Cilliers 2012). Effluent was gravity fed into the second pond of each train which had a depth of 11.5 cm, with a surface area of 15 m^2 and a volume of 1700 L/pond (Cilliers 2012). Each pond had a stainless steel paddle wheel driven by a 0.45 Kw electrical motor that continuously stirred the effluent and maintained the algal cells in suspension to avoid sedimentation. In HRAP A1 and HRAP B1, effluent flowed at an approximate velocity of 4.15 m/s while in HRAP A2 and HRAP B2, it flowed at 6.10 m/s (Cilliers 2012). Post-HRAP A2 and post-HRAP B2 effluent was decanted into a 500 L sump placed below the ground (Figure 3.6). Effluent from the collection tank was pumped using a submersible pump (Life Tech AP5800 Water pump, AC220-240 V 50 Hz 360 W, Hmax 5.6 m, Qmax 12000 L/h) into two algal settling cones connected in series to each other (Figure 3.6). Each settling cone had an opening valve at the bottom to enable the removal of settled algal cells. The valves were opened weekly to discharge algal cells and effluent for use in other experiments and for disposal into a municipal tank.

Figure 3.2 The 5000 L anaerobic buffer tank.

Figure 3.3 The post anaerobic digester settling tank, referred to as the primary facultative pond (PFP) in the text.

Figure 3.4 The inlet valve used to control the flow of anaerobically digested (AD) brewery effluent into the primary facultative pond (PFP).

Figure 3.5 The splitter box splitted primary facultative pond (PFP) effluent into two streams and directed the effluent into train A and train B of the high rate algal ponds (HRAPs).

Figure 3.6.a The high rate algal ponds (HRAP) consisted of two parallel trains, each train included two ponds run in series where the numbers 1 and 2 represented the first and second ponds in system A and B respectively. The two algal settling cones and the 500 L sump allowed the recovery of algal biomass.

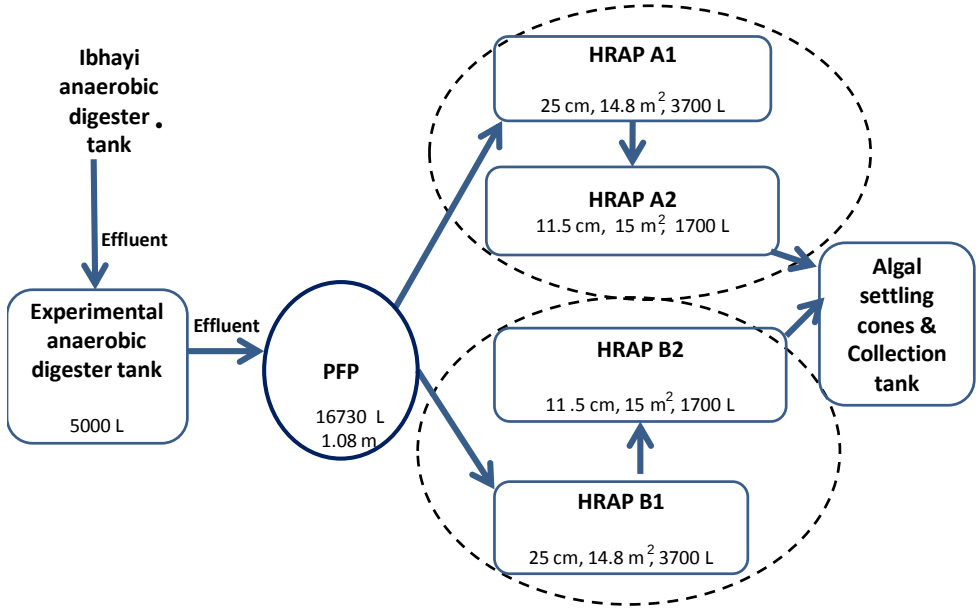

Figure 3.6. b A schematic diagram of the algal ponding system composed of an anaerobic digester, primary facultative pond (PFP), high rate algal ponds (HRAP A1, HRAP A2, HRAP B1, HRAP B2), algal settling cones and the depth, surface area and volume of each component.

b. Inoculation of algae into ponds

A new algal culture was prepared by mixing 850 L of PFP effluent with 150 L of algal cells obtained from the experimental HRAPs at the brewery. The culture was allowed to grow in a 1000 L inoculum tank. A stirrer fitted to the tank kept the growing algal culture in suspension. After seven days,

approximately 500 L of this algal culture was inoculated into HRAP A1 and the other 500 L was inoculated into HRAP B1. The HRAP A1 and HRAP B1 were operated as a batch culture for a further seven days. Thereafter, PFP effluent was diverted into each at a flow rate of 1000 L/pond/d using a splitter box for a further eleven days before experiments started. This allowed the algal culture in the HRAPs to adapt from a batch culture to a continuous culture.

c. Experimental design

Effluent from the PFP flowed into the first pond of train A (HRAP A1) and decanted into the second pond (HRAP A2). Overflow from the second pond decanted into the 500 L sump. The flow rate of the effluent from the PFP was set at 1000 L/d for 12 months. The system operated as a continuous flow system during daylight hours (08:00-16:00 h) and then ran as a batch culture for the remaining 16 h of the day, while paddle wheels remained operational throughout. The microbial community structure, chlorophyll (Chl) *a* and phaeophytin (Phaeo) *a* concentrations were analysed from water samples collected in the HRAP A2 during this period (Section 3.2.3). Furthermore, water quality parameters were also measured at intervals during the continuous flow operating period (Section 3.2.4). These data were recorded for 12 months.

3.2.2 Microbiological analysis

Water samples of 100 mL were collected from HRAP A2. These samples were stored in sterile containers, kept in a cooler box with ice and transported to Rhodes University (RU), Department of Ichthyology and Fisheries Science (DIFS) general laboratory for analysis.

a. Identification of microalgae using light microscopy

Eukaryotes recovered from the HRAP A2 were examined under a light microscope (Olympus, model: BX50, Japan) on the same day of collection or stored in a fridge at four degrees celsius and examined the following day. Between one to three drops of the sample was placed on a clear glass slide and covered with a coverslip. The contents of the slide were viewed using a light microscope at four, 10, 20 and 100 X (with oil immersion) objective lenses and the images were photographed using a camera (Olympus, model: SC30, Japan) attached to the microscope. The identity of microalgae was established visually with the aid of algae identification guides provided by Belcher & Swale (1976), Van Vuuren *et al*. (2006), Taylor *et al*. (2007) and Bellinger & Sigee (2010).

b. Identification of microalgae and bacteria using metagenomics

Molecular techniques were carried out at the South African Institute for Aquatic Biodiversity (SAIAB), Institute of Environmental Biotechnology, Rhodes University (EBRU) and The Department of

Biochemistry, Microbiology and Biotechnology (BMB). Water samples of 100 mL obtained from the HRAP A2 were filtered through a 0.2 µm membrane filter (Sterivex, Millipore Catalog # SVGPL10RC) with a vacuum pump at low pressure. Filter membranes with the retained biological material were then stored in a freezer at -20°C until deoxyribonucleic acid (DNA) was extracted.

Deoxyribonucleic acid extraction

Deoxyribonucleic acid recovered from the HRAP A2 samples was extracted using a commercially available DNA extraction kit (PowerWater ®Sterivex DNA isolation kit) from MO BIO Laboratories, Inc. The DNA isolation kit had eight different types of solutions labelled ST1A, ST1B, ST2, ST3, ST4, ST5, ST6 and ST7. The DNA extraction protocol had four major steps described below.

Sample preparation: Solution ST1A was added to solution ST1B, mixed thoroughly and stored in a fridge at four degrees celsius. A filter unit (Sterivex, microfunnel) with biological material was allowed to thaw for two to three minutes at room temperature. A 0.9 mL aliquot of solution ST1B was added to the microfunnel with a pipette. The microfunnel was vortexed for five minutes at a low speed.

Cell lysis: Solution ST2 was heated at 65℃ for 10 min to dissolve crystals and a 0.9 mL aliquot of it was added into the microfunnel. The microfunnel was heated at 90℃ for five minutes for further cell lysis, and then cooled for two minutes at room temperature. The microfunnel was vortexed at maximum speed for five minutes. Lysate from the microfunnel was drawn with a three millilitres syringe and transferred into a glass bead tube (PowerWater Sterivex). The glass bead tube was vortexed at maximum speed for five minutes for further lysis of cells and centrifuged at 4000 x G for a minute to separate the supernatant from the glass beads. Subsequently, contents of the glass beads tubes were transferred into a sterile 2.2 mL collection tube with a pipette.

Inhibitor removal technology: A 300 µL aliquot of solution ST3 was added into a 2.2 mL collection tube, vortexed and stored in a fridge at four degrees celsius for five minutes. The 2.2 mL collection tube was removed from the fridge and centrifuged at 13000 x G for a minute. The supernatant was removed from the 2.2 mL collection tube with a pipette and transferred into a five millilitres collection tube. The lysate in the supernatant was then transferred into a 20 mL syringe which was attached to a binding column. The lysate was expelled into the binding column. The binding column was removed from the syringe and placed into a clean 2.2 mL collection tube. The collection tube was centrifuged at 13000 x G for two minutes until the membrane was completely dry. The binding column membrane was washed with 0.7 mL of solution ST6 and then centrifuged for a minute at 13000 x G. The flow-through was discarded and binding column membrane was washed with 0.7 mL

of solution ST5, then centrifuged for a minute at 13000 x G. The binding column was placed into a clean 2.2 mL collection tube. The tube was centrifuged for two minutes at 13000 x G to dry completely.

Elute: The binding column was placed into a clean 2.2 mL collection tube and 100 µL aliquot of solution ST7 was added to it. The collection tube was centrifuged at 13000 x G for a minute to elute the DNA from the binding column (Instruction manual: Millipore catalog # SVGPL10RC, www.mobio.com). The eluted DNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Nanodrop 2000 spectrophotometer) and stored at -20℃ for polymerase chain reaction (PCR) application at a later stage.

Polymerase chain reaction and Illumina sequencing

The genomic material with a DNA concentration ranging from 36.6-80.7 ng/uL was amplified through PCR and sequenced using an Illumina sequencer by Inqaba Biotechnical Industries (Pty) Ltd, a next generation sequencing (NGS) service provider. The primer pair 566F 5'-CAG CAG CCG CGG TAA TTC C-3' and 1200R 5'-CCC GTG TTG AGT CAA ATT AAG C-3' was used to amplify the 18S rRNA region while the primer pair 27F 5'-AGA GTT TGA TCM TGG CTC AG-3' and 518R 5'-ATT ACC GCG GCT GCT GG-3' was used to amplify 16S rRNA region. The primers had Illumina adaptors added to them to enable amplification and sequencing of the libraries. Each sample was then labelled, indexed and barcoded at each end to enable demultiplexing of both forward and reverse reads. Reads are the produced nucleotide sequences. The sequencing kit used was Miseq 600 v3 kit and 2x300 base pair long paired end reads were produced. For every sample, data were trimmed and only q30 (i.e. high quality) reads were used, every read was analysed using a Basic Local Alignment Search Tool (BLAST) programme and the result file saved (E-value BLAST cut off of 0.005 was used). The top hit for every BLAST result was counted and a record was kept of how many times each species appeared as a hit. The reads percentage represented the relative abundance of the species.

3.2.3 Determination of microalgae biomass, chlorophyll *a* and phaeophytin *a* concentrations

Samples from the HRAP A2 were collected in sterile bottles and kept in a cooler box with ice packs. The samples were transported from the project sampling site to RU, DIFS general laboratory for analysis. Glass fibre filter paper (Whatman GF/F) with pore size of 0.7 µm and a diameter of 47 mm was weighed. Samples of 100 mL taken from the HRAP A2 were filtered at low vacuum pressure of less than 25 mm Hg to concentrate algal cells on the filter paper. The filter paper with algal cells was dried in an oven at 80℃ for 24 h. After 24 h, the filter paper with algae was weighed. The weight of

dry algal matter was obtained by subtracting the weight of the filter paper from that of filter paper with algae.

Samples were kept on ice in the darkness until they were analysed to avoid degradation of Chl *a.* Water samples of 100 mL from the HRAP A2 were filtered through a 0.7 µm glass fibre filter paper (Whatman GF/F) at low vacuum pressure of less than 25 mm Hg to concentrate algal cells. While filtering, two millilitres of one percent magnesium carbonate ($MgCO_3$) solution was added to the filter paper to prevent the loss of Mg^{2+} from the Chl *a* molecule. Filter papers with algal cells were stored in sterile 50 mL tubes with screw caps, labelled with their respective contents, wrapped in aluminium foil, placed in a cooler box and kept in a freezer at -20℃ until Chl *a* extraction. The filter papers with algae were kept in a freezer for no longer than two weeks, before Chl *a* extraction (Arar & Collins 1997).

Pigment extraction

The filter papers with algae were each transferred into a centrifuge tube containing 15 mL of 90% acetone. The centrifuge tubes were vortexed to completely mix and centrifuged at 2000 rpm for 10 min to separate cells and filter paper from the supernatant (Arar & Collins 1997). Ten millilitres of the supernatant was decanted into a glass cuvette. In situations when samples were too concentrated, they were diluted with a known amount of 90% acetone, and then the Chl *a* concentration was back calculated after the absorbance was recorded.

Absorbance measurements and pigments calculations

A spectrophotometer (VIS HACH, model: DR 2800, Germany) was used to measure absorbance of Chl *a* at 630, 647, 664, 665 and 750 nanometres (nm) wavelengths. Prior to any absorbance recording, the spectrophotometer was zeroed with 90% acetone (Arar & Collins 1997; Dos Santos *et al*. 2003). After recording the absorbance of Chl *a* at the aforementioned wavelengths, the supernatant was acidified with 0.1 mL of 0.1 M hydrochloric acid (HCl) to correct for Phaeo *a*. Phaeophytin *a* absorbance was recorded at 665 and 750 nm before and after acidification (Arar & Collins 1997; Dos Santos *et al*. 2003).

A trichromatic equation was used to determine Chl *a* in the extract uncorrected for Phaeo *a* (Făgădar-Cosma *et al*. 2005; Kim *et al*. 2014):

$$\text{Chl } a = 11.85(E_{664}-E_{750}) - 1.54(E_{647}-E_{750}) - 0.08(E_{630}-E_{750}) \quad (1)$$

where Chl *a* represents chlorophyll concentration in micrograms per litre (µg/L);

E_{630} = absorbance at wavelength 630 nm;

E_{647} = absorbance at wavelength 647 nm;

E_{664} = absorbance at wavelength 664 nm; and

E_{750} = absorbance at wavelength 750 nm.

Chlorophyll *a* in water samples was determined using the following equation (Parsons & Strickland 1963):

$$\text{Chl } a \text{ µg/L} = \text{Chl } a \times v/L \times V \quad (2)$$

where Chl *a* = Chl *a* concentration (µg/L);

Chl *a* = Chl *a* from the extract;

v = volume of extraction solvent in (mL);

L = path length of cuvette in (cm); and

V = volume of filtered water sample in (L).

Monochromatic equations were used to determine Chl *a* corrected for Phaeo *a* and Phaeo *a* in water samples (Sartory 1982; Wasmund *et al*. 2006):

$$\text{Chl } a \text{ µg/L} = [26.73 (E_{665}^{o} - E_{665}^{a}) v]/V \times L \quad (3)$$

$$\text{Phaeo } a \text{ µg/L} = [26.73 (1.7*E_{665}^{a} - E_{665}^{o}) v]/V \times L \quad (4)$$

where Chl *a* represents chlorophyll concentration (µg/L);

Phaeo *a* represents phaeophytin *a* concentration (µg/L);

E_{665}^{o} = absorbance at 665 nm before acidification;

E_{665}^{a} = absorbance at 665 nm after acidification;

v = volume of extraction solvent in (mL);

V = volume of filtered water sample in (L);

26.73 is a absorption coefficient correction of the pigment ratio with pure Chl; and

1.7 is the acid ratio.

3.2.4 Water quality parameter analysis

A 100 mL water sample of effluent was collected weekly from each of the post-AD, post-PFP and post-HRAP A2 sampling points. The sampling point for post-AD effluent was the inlet valve that drains AD effluent into the PFP. For post-PFP effluent, the sampling point was the splitter box. Samples of post-HRAP A2 effluent were collected from the pipe that drained effluent from the HRAP A2 into a sump tank placed below the ground. The effluent was filtered through eight micrometre filter disc with a diameter of 47 mm (Whatman, Ashless, Grade 42 circles, Cat. No. 1820-047) by vacuum at low pressure of less than 25 mm Hg. The filtrates were recovered instantly and used to measure ammonium-nitrogen (NH_4^+-N), nitrite-nitrogen (NO_2^--N), nitrate-nitrogen (NO_3^--N), orthophosphate-phosphorus (PO_4^{3-}-P), chemical oxygen demand (COD) and chloride (Cl^-) using the appropriate Merck test kits and a spectrophotometer (Merck Pharo 100 Spectroquant, product number 100706, Merck (Pty) Ltd, Darmstadt, Germany). Temperature, pH, electrical conductivity (EC) and dissolved oxygen (DO) were determined using an electronic probe and a meter (Hanna, HI 98129, United Kingdom).

Ammonium-nitrogen

Ammonium-nitrogen in water is partitioned between NH_4^+ ions and NH_3. A pH dependent equilibrium exists between the two forms. In an alkaline solution, the unionised NH_3 predominates. The concentration of NH_4^+ in water can be determined spectrophotometrically. Ammonium concentration of low range is determined by first reacting it with a chlorinating agent to form monochloramine. The monochloramine reacts with thymol to form a blue indophenol derivative which can be quantified spectrophotometrically. The method is analogous to EPA 350.1, US standard methods 4500 NH_3-D, ISO 7150/1. The measuring range of the reagent kit was 0.01-3.00 mg/L NH_4^+-N, (NH_4^+-N LR, Cat. No. 1.14752.0001, Merck (Pty) Ltd, Darmstadt, Germany). Five millilitres of effluent was added into a 10 mL test tube with a pipette followed by 0.6 mL of reagent NH_4^+-1. One level microspoon of reagent NH_4^+-2 was also added to the reaction tube. Contents of the reaction tube were then mixed thoroughly by swirling the tube to completely dissolve reagents. The tube and its contents were allowed to stand for five minutes to complete the reaction. After five minutes, four drops of reagent NH_4^+-3 were added and the tube was swirled thoroughly again. The contents of the tube were allowed to complete the reaction for another five minutes. Contents of the tube were then poured into a one centimetre length cuvette and the concentration of NH_4^+-N was measured spectrophotometrically.

In determining NH_4^+ concentration of high range, the sample reacts with hypochlorite ions to form monochloramine. The monochloramine reacts with phenol to form a blue indophenol derivative. The method used is analogous to EPA 350.1, APHA 4500-NH_3 D, ISO 7150/1, and DIN 38406 E5. The measuring range was four to 80 mg/L NH_4^+-N. Effluent of 0.1 mL was added into a reaction vial using a pipette and mixed thoroughly. Reagent NH_4^+-1 K was then added to the vial once, the vial was closed and mixed until contents were completely dissolved. The vial was allowed to stand for 15 min to complete the reaction. The vial was inserted into the spectrophotometer and NH_4^+-N concentration was measured.

Nitrite-nitrogen

In an acidic solution, NO_2^- ions react with sulfanilic acid to form diazonium salt. The diazonium salt then reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form a red-violet azo dye, which can be quantified spectrophotometrically. The measuring range of the method is 0.002 to one milligrams per litre NO_2^--N (NO_2^--N, Cat. No. 1.14776.0001, Merck (Pty) Ltd, Darmstadt, Germany). Five millilitres of the sample was added into a 10 mL tube with a pipette. One level microspoon of reagent NO_2^--N was added into the reaction tube. The contents of the tube were allowed to stand for 10 min to complete the reaction. The mixture was transferred into a one centimetre length cuvette and the concentration of NO_2^--N was measured spectrophotometrically.

Nitrate-nitrogen

The measurement of NO_3^--N in sulphuric and phosphoric solution, NO_3^- ions react with 2,6-dimethylphenol (DMP) forming 4-nitro-2,6-dimethylphenol which can be quantified using a spectrophotometer. The method was analogous to DIN 38405-9. The measurement range is 0.1-25 mg/L NO_3^--N (NO_3^--N, Cat. No. 1.09713.0002, Merck (Pty) Ltd, Darmstadt, Germany). Reagent NO_3^--1 of four millilitres was added into a test tube using a pipette and then followed by 0.5 mL of the sample added into the reaction tube. Reagent NO_3^--2 of 0.5 mL was also added into a reaction tube. The contents of the tube were allowed to stand for 10 min, and then poured into a one centimetre length cuvette and the NO_3^--N concentration was measured spectrophotometrically.

Orthophosphate-phosphorus

In a sulphuric acid solution, PO_4^{3-} ions react with ammonium vanadate and ammonium heptamolybdate, which results in the formation of an orange-yellow molybdovanadophosphoric acid. The amount of acid used was quantified with the spectrophotometer. The method was analogous to APHA 4500-PE. The measurement range of the method was 0.5-25 mg/L PO_4^{3-}-P. Five millilitres of the sample was added into a 10 mL clean reaction tube with a pipette. Reagent PO_4^{3-}-1

of 1.2 mL was added to the tube and mixed thoroughly until completely mixed. The mixed contents of the tube were then transferred into a one centimetre length cuvette and the PO_4^{3-}-P concentration was measured with the spectrophotometer.

Chemical oxygen demand

The water sample was oxidized in a hot sulphuric acid solution of potassium dichromate, with silver sulphate as a catalyst. Chloride was neutralised with mercury sulphate. The concentration of yellow $Cr_2O_7^{2-}$ ions that formed in the oxidation reaction was determined spectrophotometrically. The method corresponds to DIN ISO 15705 and is analogous to EPA 410.4, APHA 5220 D and ASTM D1252-06 B. The measurement range was 15-300 mg/L COD. A volume of two millilitres of effluent was added into a reaction tube containing the oxidising reagent. The reaction tube was screwed tightly and its contents were mixed thoroughly. The reaction cell was placed in a thermoreactor (Merck, model: TR300, Germany) and heated at 150℃ for 120 min. Once heating was complete, the reaction tube was allowed to cool for 30 min, then inserted into the spectrophotometer and COD was measured.

Chloride

In a solution, Cl^- ions react with mercury (II) thiocyanate, which forms partially dissociated mercury (II) chloride. In this process, thiocyanate is released, which reacts with iron (II) ions to form red iron (II) thiocyanate. The iron (II) thiocyanate is then quantified with a spectrophotometer. The method is analogous to EPA 325.1 and APHA 4500-Cl^-E. The measuring range of this method is from 2.5-250 mg/l Cl^- (Cl^-, Cat. No. 1.14897.0001, Merck (Pty) Ltd, chemicals, Darmstadt, Germany). Chloride was measured by adding 5.00 mL of filtrate into a clean 10 mL tube. Reagent Cl^--1 of 2.5 mL was then added into the reaction tube and the contents were mixed thoroughly. Fifty millilitres of reagent Cl^--2 of 0.5 mL was added to the tube and mixed thoroughly until the contents were completely mixed. The tube was left to stand for one minute for complete reaction, after which the contents were transferred into a one centimetre length cuvette and the Cl^- concentration was quantified with the spectrophotometer.

Temperature, pH, electrical conductivity and dissolved oxygen

Temperature was measured in degrees celsius (℃) together with pH values using an electronic probe (Hanna, model: HI 98129, United Kingdom). The raw pH values were anti-logged and then the average and error were calculated. Once the analysis was complete, the values were logged. Electrical conductivity was measured in µS/cm, also using an electronic probe (Hanna, model: HI

98130, United Kingdom). Dissolved oxygen concentration was measured in mg/L using a DO meter (Oxyguard, Handy polaris DO meter, Los Angeles, USA).

3.2.5 Statistical analysis

Data collected from the single algal pond (HRAP A2) were presented as a mean per month with a standard error. A linear regression model was used to describe the relationship between some of the water quality parameters and algal biomass or Chl *a* concentration, at $p \leq 0.05$. The R^2-value indicated the closeness of the data to the fitted regression line. The Pearson's R value (correlation coefficient) was also used in some water quality data to elucidated how closely related the data were.

3.3 Results

3.3.1 Climatic parameters of Port Elizabeth

The monthly average minimum and maximum temperature and day length data for Port Elizabeth from October 2013 to September 2014 were measured at 08:00 h and ranged from 9.2/20.7 to 18.7/26.6℃ and 09:55:46 to 14:22:22 h (Table 3.1).

Table 3.1 The average climate data for Port Elizabeth from October 2013 to September 2014 (Sampson, *pers. comm*., client liaison officer, South African Weather Services (SAWS), Port Elizabeth, February 2015).

Date	Minimum/maximum temperature (°C)	Day length (h)
Oct-13	13.0/21.9	12:56:52
Nov-13	15.0/23.1	13:53:08
Dec-13	16.1/24.0	14:22:22
Jan-14	18.6/26.8	14:06:47
Feb-14	18.7/26.6	13:18:16
Mar-14	15.3/25.3	12:16:59
Apr-14	12.5/23.3	11:13:58
May-14	11.9/22.3	10:21:47
Jun-14	7.4/20.5	09:55:46
Jul-14	9.2/20.7	10:08:20
Aug-14	10.4/21.1	10:53:20
Sep-14	11.8/21.2	11:52:59

3.3.2 Seasonal variation of the microbial community structure in the high rate algal pond

The microalgae community structure varied seasonally, with more taxa observed during summer and fewer taxa in winter (Table 3.2, Table 3.3, Figure 3.7). Some species such as *Chlorella* sp., *Scenedesmus* sp. and *Chlamydomonas* sp. were present in the pond throughout the year. There was also a shift in the bacterial community and its relative abundance at different seasons (Figure 3.8).

a. Identification of the microalgae community structure using light microscopy

Microalgae taxa in the HRAP A2 belonged to the Chlorophyta, Cyanophyta, Euglenophyta and Bacillariophyta phyla (Table 3.2, Figure 3.7). Chlorophyta species were *Scenedesmus* sp., *Chlamydomonas* sp., *Stigeoclonium* sp., *Pediastrum* sp., *Oocystis* sp., *Fritschiella* sp., *Chlorella* sp. *Chroococcus* sp., *Tribonema* sp., *Haematococcus* sp. and *Spirogyra* sp. Species belonging to Cyanophyta were *Oscillatoria* sp., *Synechococcus* sp., *Phormidium* sp., *Arthrospira* sp., and *Microcystis* sp. Euglenophyta consisted of *Euglena sp*. and Bacillariophyta was composed of *Diatoma* sp., *Navicula* sp. and *Nitzschia* sp. *Chlorella* sp., *Scenedesmus* sp., *Chlamydomonas* sp., *Diatoma* sp., *Microcystis* sp., *Synechococcus* sp. and *Phormidium* sp. were present in the pond all year round. *Pediastrum* sp. and *Stigeoclonium* sp. were observed in summer and spring while *Haematococcus sp*. was observed in summer, autumn, early winter and late spring. *Chroococcus* sp. and *Tribonema* sp. were observed in spring and early summer, while *Oocystis* sp. was only observed in spring. *Spirogyra* sp. was present in the HRAP in mid summer (January) and *Fritschiella* sp. was observed in mid spring (November). *Arthrospira* sp. was observed in summer and spring, whereas, *Oscillatoria* sp. and *Euglena* sp. were only observed in summer. Diatoms such as *Navicula* sp. and *Nitzschia* sp. were observed during summer and spring. Rotifers and nematodes were present in the pond during summer.

Table 3.2 Microorganisms, particularly microalgae in a high rate algal pond (HRAP A2) over 12 months identified using light microscopy. The tick (√) indicates the presence of a particular microorganism in the HRAP.

	Time (month-year)											
	2013			2014								
Microalgae species	**October**	**November**	**December**	**January**	**February**	**March**	**April**	**May**	**June**	**July**	**August**	**September**
Scenedesmus sp.	√	√	√	√	√	√	√	√	√	√	√	√
Chlorella sp.	√	√	√	√	√	√	√	√	√	√	√	√
Diatoma sp.	√	√	√	√	√	√	√	√	√	√	√	√
Chlamydomonas sp.	√	√	√	√	√	√	√	√	√	√	√	√
Microcystis sp.	√	√	√	√	√	√	√	√	√	√	√	√
Phormidium sp.	√	√	√	√	√	√	√	√	√	√	√	√
Synechococcus sp.	√	√	√	√	√	√	√	√	√	√	√	√
Arthrospira sp.	√	√	√	√	√						√	√
Pediastrum sp.	√	√	√	√	√						√	√
Navicula sp.		√	√	√							√	√
Rotifer		√	√	√	√							
Nitzschia sp.		√	√	√	√							√
Haematococcus sp.		√	√	√	√	√	√	√	√			
Euglena sp.		√	√	√								
Nematoda			√	√	√							
Macrothrix									√			
Oocystis sp.	√	√										
Ostracod	√											
Oscillatoria sp.			√	√	√							
Stigeoclonium sp.	√	√	√	√								√
Fritschiella sp.		√										
Chroococcus sp.	√	√	√									
Tribonema sp.	√	√	√									
Spirogyra sp.				√								

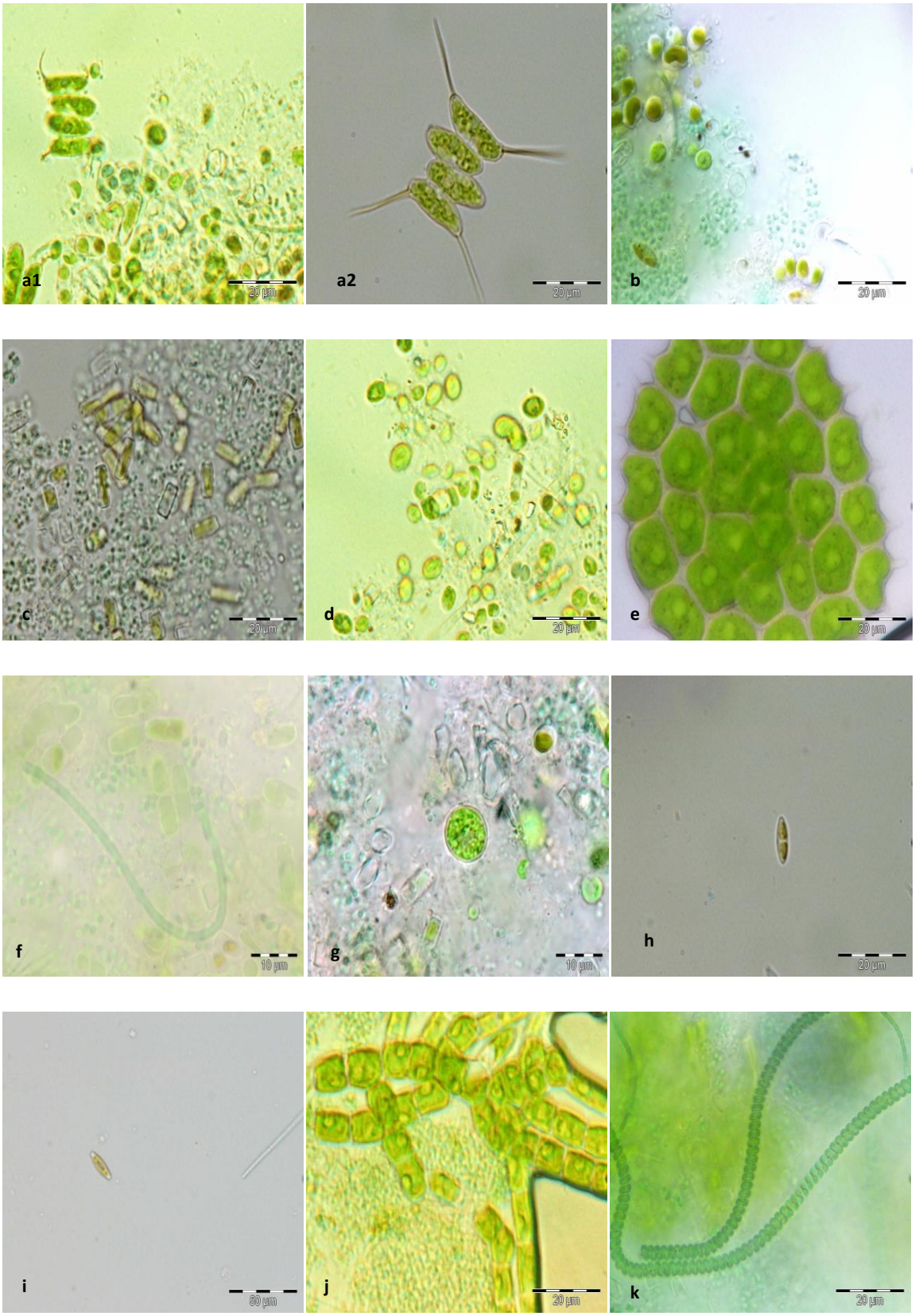

Figure 3.7 Photomicrographs of microorganisms in the high rate algal pond (HRAP A2) over 12 months: (a1-a2) *Scenedesmus* sp.; (b) *Chlorella* sp.*;* (c) *Diatoma* sp.*;* (d) *Microcystis* sp.*;* (e) *Pediastrum* sp.*;* (f) *Phormidium* sp.*;* (g) *Haematococcus* sp.*;* (h) *Nitzschia* sp.; (i) *Navicula* sp.*;* (j) *Fritschiella* sp. and (k) *Arthrospira* sp.

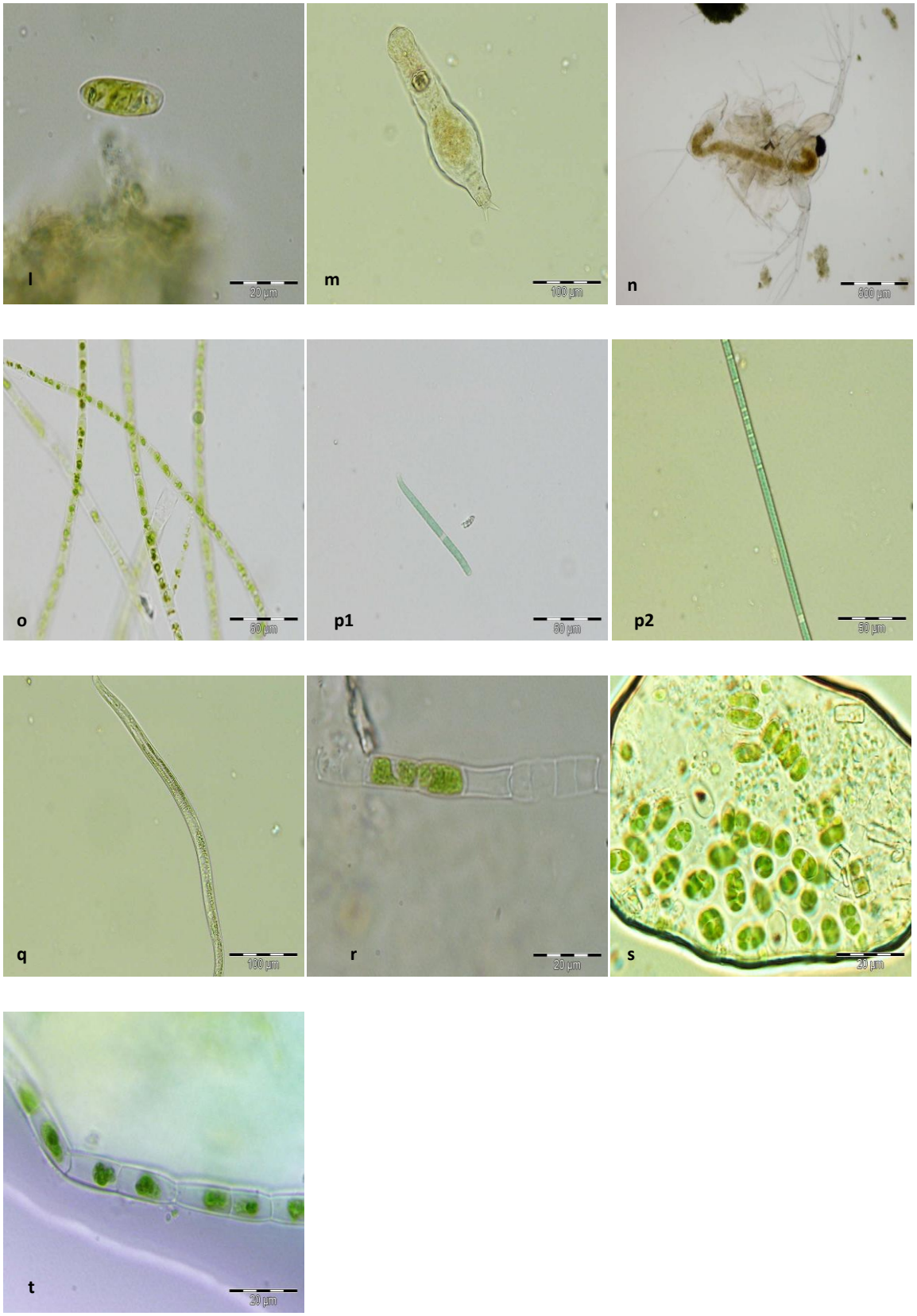

Figure 3.7 continued Photomicrographs of microorganisms in the high rate algal pond (HRAP A2) over 12 months: (l) *Euglena* sp.; (m) Rotifer*; (*n) *Macrothrix*; (o) *Stigeoclonium* sp.*;* (p1-p2) *Oscillatoria* sp *;* (q) Nematode; (r) *Tribonema* sp.; (s) *Chroococcus* sp*. and (t) Spirogyra* sp*.*

b. Identification of the microalgae community structure using metagenomics

Scenedesmus abundans was the dominant species during summer with a relative abundance of 66.41%, while *Mychonastes* sp. and *Nanofrustulum shiloi* were the dominant species in winter and spring with a relative abundance of 73.46% and 3.38% respectively (Figure 3.8). The relative abundance of *Scenedesmus abundans* decreased from 66.41% in summer to 2.82% in spring. *Mychonastes* sp. increased from 23.03% in summer to 73.46% in winter. *Nanofrustulum shiloi* was 4.46% in summer, and then declined to 3.38% in spring. The relative abundance of *Staurosira construens* was 5.38% in summer, which then decreased to 0.15% in spring (Figure 3.8, Table 3.3). Other algal species with a relative abundance of less than 0.25% were grouped under the identified microalgae category (Figure 3.8). The relative abundance of unknown eukaryotes was 0.30% in summer, which increased to 2.19% during winter, and then further increased to 69.49% in spring.

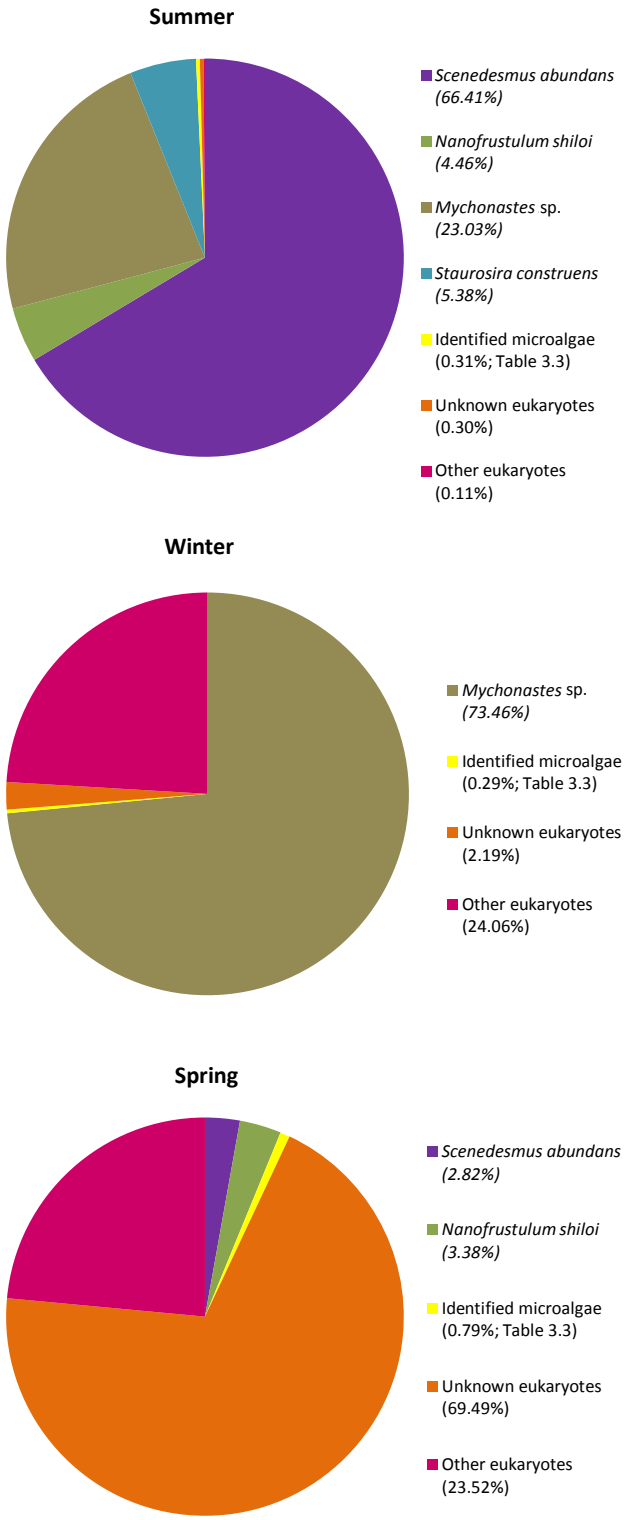

Figure 3.8 The microalgae community structure and the relative abundance as percentage (%) in a high rate algal pond (HRAP A2) with post-primary facultative pond (post-PFP) effluent flowing into HRAP A1, and then HRAP A2 at a rate of 1000 L/d during summer, winter and spring identified using metagenomics. Other eukaryotes refer to non-algal species that were also amplified with the universal primer pair 566F and 1200R.

Table 3.3 Microalgae species and the relative abundance as percentage (%) in a high rate algal pond (HRAP A2) grouped under the identified microalgae category in the pie chart (Figure 3.8).

Microalgae species	Summer (%)	Winter (%)	Spring (%)
Amphidinium carterae			0.01
Amphora montana	0.01		0.01
Bodomorpha minima		0.01	
Botryococcus sp.	0.01		
Chaetophora incrassata			0.01
Chaetosphaeridium globosum	0.01		0.01
Chlamydomonas sp.		0.01	0.02
Chlorella vulgaris	0.01		0.22
Choricystis sp.			0.01
Chrysocapsa vernalis	0.01		
Coccoid green	0.01		
Coccomyxa sp.		0.01	
Corethron inerme			0.02
Desmodesmus pirkollei	0.01		
Diatoma tenue	0.01		
Haematococcus zimbabwiensis		0.02	
Halosarcinochlamys cherokeensis	0.01		
Hydrodictyon reticulatum	0.01		
Lobochlamys segnis		0.02	
Microspora sp.		0.01	
Monodopsis subterranea	0.02		
Nannochloris sp.		0.01	0.11
Nannochloropsis salina			0.01
Nanofrustulum shiloi		0.01	
Navicula pelliculosa	0.01		
Nitzschia amphibia			0.01
Oocystis sp.	0.01		0.06
Pachycladella umbrina		0.01	
Paurantiaca	0.01		
Phaeocystis globosa	0.01		
Phaeodactylum tricornutum	0.01	0.01	
Pmalhamensis	0.03		
Pseudodictyosphaerium jurisii		0.01	
Pycnococcus sp.			0.01
Pyramimonas olivacea		0.07	
Pyrenomonas salina	0.01		
Scenedesmus deserticola	0.01		
Scenedesmus subspicatus	0.01	0.02	0.01
Scenedesnus abundans		0.05	
Scenedesmus costato-granulatus	0.01		0.03
Staurosira construens		0.01	0.15
Storeatula sp.	0.01		
Sphaeroeca	0.01		
Spinoclosterium cuspidatum			0.01
Symbiodinium sp.			0.01
Synechococcus pcc6307	0.01		
Thraustochytrium sp.	0.01		
Thalassiosiraceae			0.06
Trebouxia erici		0.01	
Uroglena americana	0.01		
Urospora neglecta	0.01		
Volvox sp.	0.01		0.01

c. Identification of the bacterial community structure using metagenomics

Bacteria belonging to the phyla Firmicutes, Proteobacteria, Planctomycetes, Bacteroidetes, Actinobacteria, Verrucomicrobia, Acidobacteria, Cyanobacteria and Chloroflexi were identified in the HRAP A2 (Figure 3.9). Firmicutes, Proteobacteria and Planctomycetes were observed during summer, winter and spring. Firmicutes were dominant in summer and accounted for 71.23% of the bacterial community, while Actinobacteria and Proteobacteria were dominant in winter and spring with a relative abundance of 16.22% and 22.93% respectively. Bacteroidetes decreased from 3.58% in winter to 2.19% in spring, while Actinobacteria increased from 3.47% in summer to 16.22% during winter. The relative abundance of Verrucomicrobia also increased from 0.23% during summer to 0.34% in winter. The phylum Acidobacteria was only observed in summer while Cyanobacteria and Chloroflexi were only observed in spring. During summer, 21.34% of the bacterial community was made up of unknown species. The relative abundance of the unknown species increased to 49.41% during winter, which then decreased to 37.65% in spring. Phyla representing less than 0.25% of the bacterial community were assigned to the category other.

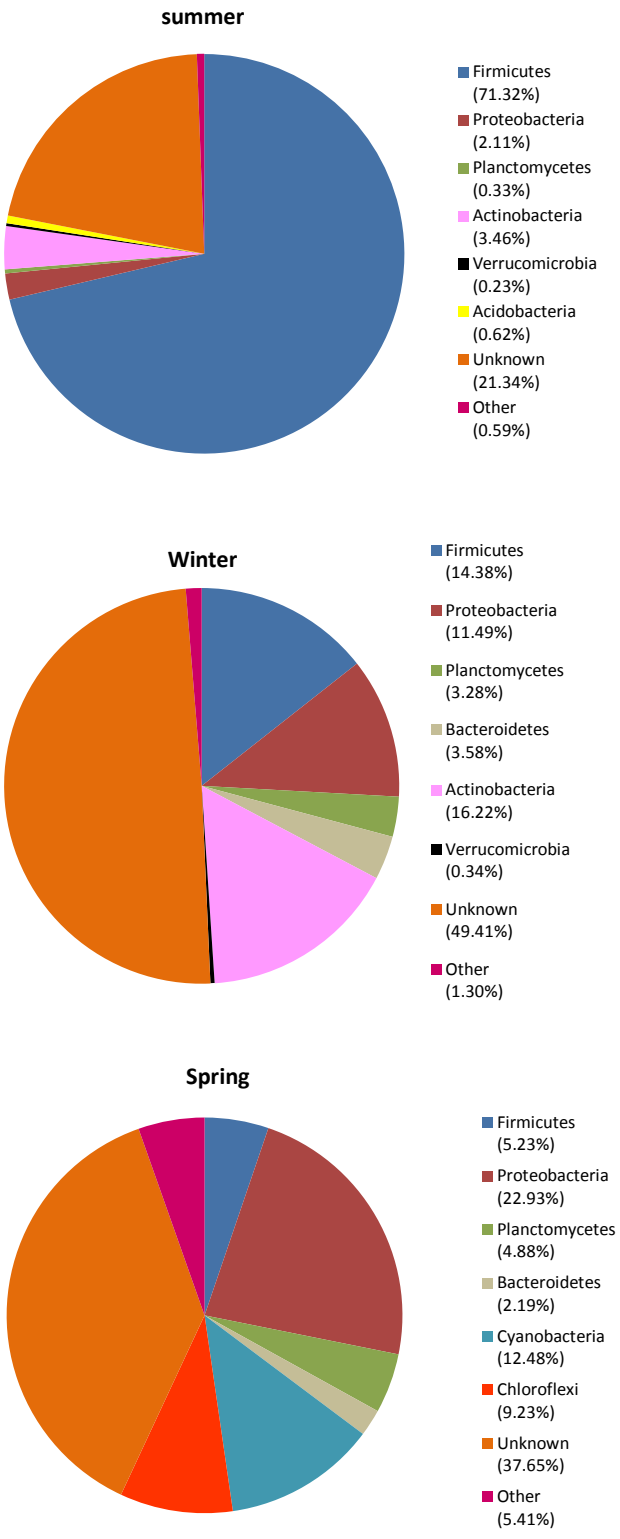

Figure 3.9 The bacterial community structure at a phylum level and the relative abundance as percentage (%) in a high rate algal pond (HRAP A2) with post-primary facultative pond (post-PFP) effluent flowing into HRAP A1, and then HRAP A2 at a rate of 1000 L/d during summer, winter and spring.

3.3.3 Microalgae biomass, chlorophyll *a* and phaeophytin *a* concentrations

Algal biomass in the HRAP increased from 0.10 g/L in October to 0.87 g/L in February, a period that covered the austral summer (Figure 3.10). The algal biomass then decreased during winter, with the lowest algal biomass 0.10 g/L recorded during winter (July). Algal biomass began to rise again in spring where 0.30 g/L was recorded in September. Chlorophyll *a* concentration, as expected, followed similar trends to algal biomass and when calculated using the trichromatic equation (Figure 3.11). The Chl *a* concentration increased from 877.81 µg/L in spring (October) to 1551.36 µg/L in autumn (March). During winter, Chl *a* concentration decreased to 583.24 µg/L in July. The Chl *a* concentration again increased to 989.33 µg/L in September. When using the monochromatic equation, similar trends were also observed (Figure 3.12). Chlorophyll *a* concentration increased from 371.78 µg/L in October to 604.63 µg/L in December. The Chl *a* concentration then decreased to 400.95 µg/L in January and then increased to 685.63 µg/L in February. After this, a gradual decrease in Chl *a* concentration was observed. In July, the Chl *a* concentration declined to 171.61 µg/L, the lowest measurement of the year, before increasing to 400.95 µg/L in September. Phaeophytin *a* concentration increased from 822.11 µg/L in October to 1945.41 µg/L in January (Figure 3.13). The Phaeo *a* concentration then decreased to its lowest point in the year, 678.81 µg/L, which was measured in July. Concentrations of this pigment increased to 991.15 µg/L in September. The algal biomass, Chl *a* and Phaeo *a* concentrations demonstrated seasonal trends.

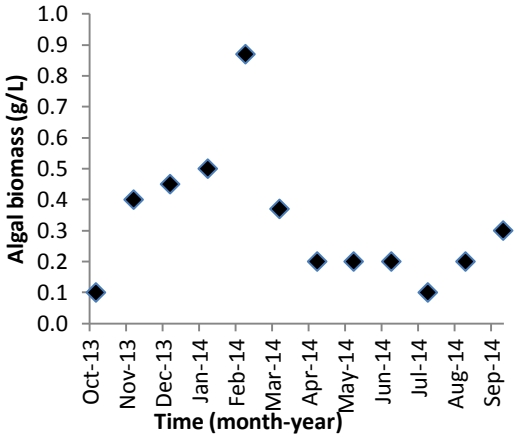

Figure 3.10 The algal biomass (dry matter) in the high rate algal pond (HRAP A2) over 12 months.

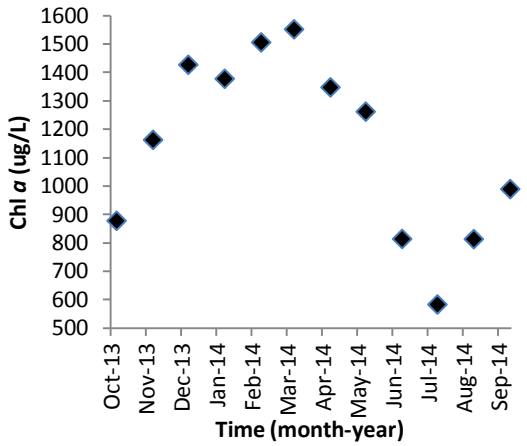

Figure 3.11 Chlorophyll (Chl) *a* concentration in high rate algal pond (HRAP A2) over 12 months using the trichromatic equation.

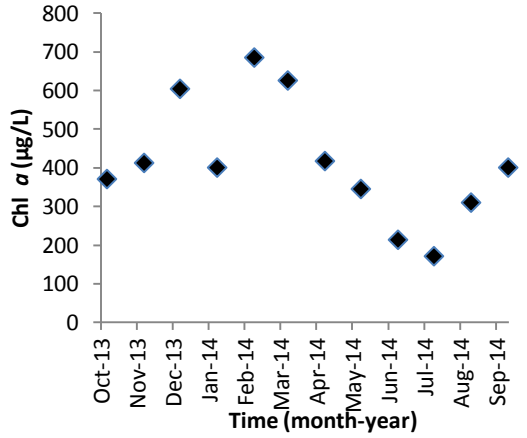

Figure 3.12 Chlorophyll (Chl) *a* concentration in high rate algal pond (HRAP A2) over 12 months using the monochromatic equation.

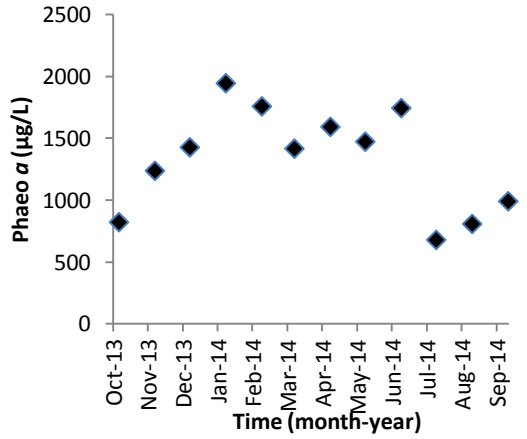

Figure 3.13 Phaeophytin (Phaeo) *a* concentration in high rate algal pond (HRAP A2) over 12 months using the monochromatic equation.

3.3.4 Water quality parameter analysis

Physical characteristics of the anaerobically digested brewery effluent

The monthly mean temperatures of effluent emerging from the anaerobic digester into the PFP and then the HRAP A2 showed a decrease along the effluent treatment system (Table 3.4, Figure 3.14). In the post-AD, PFP and HRAP A2 effluent, the lowest monthly mean temperatures were recorded during winter, while the highest mean temperatures were recorded in summer (Figure 3.14).

Table 3.4 The annual mean (± standard error) temperature in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent, the number of temperature samples taken over that period (n), and the change in temperature as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (°C)	n	Change (%)
post-AD	23.62 ± 0.92	30	0
PFP	23.02 ± 0.74	30	-3
HRAP A2	20.44 ± 0.73	31	-13

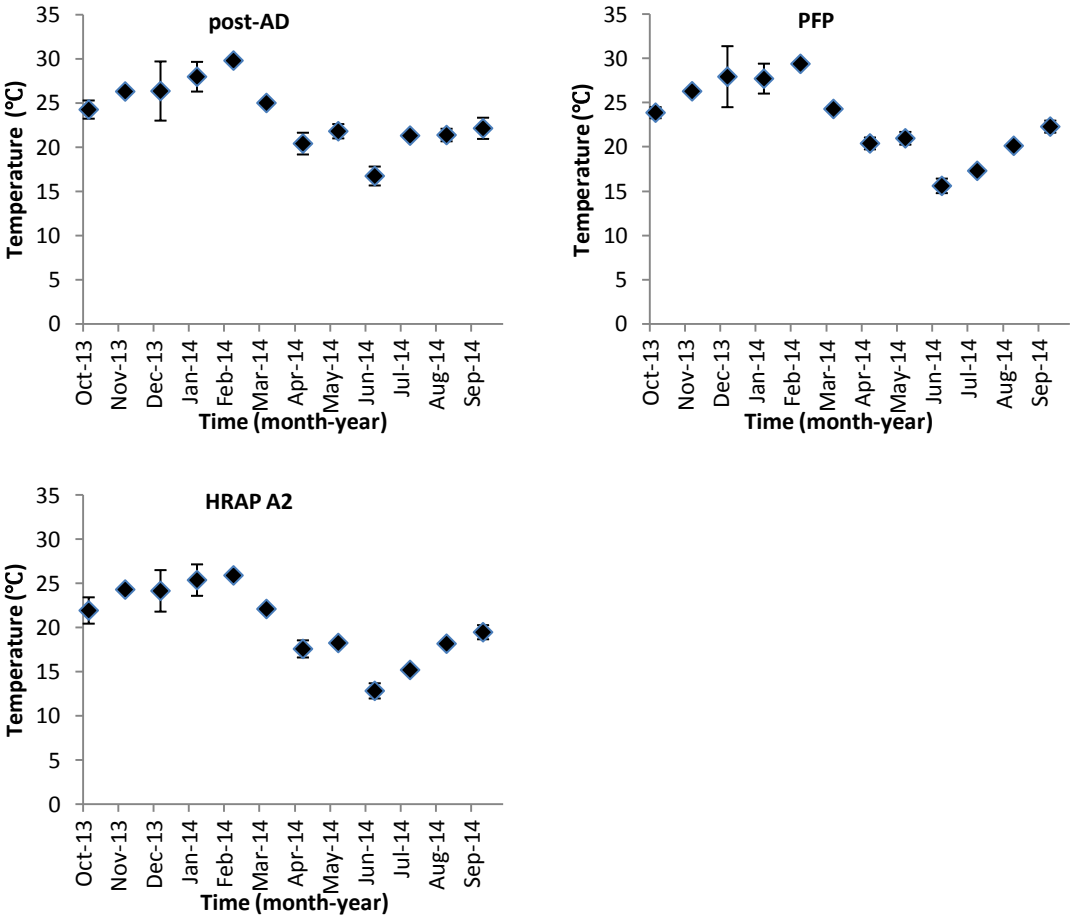

Figure 3.14 The monthly mean (± standard error) temperature in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent over 12 months.

Chemical characteristics of the anaerobically digested brewery effluent

The annual and monthly mean DO concentrations were generally higher in the HRAP A2, than in the PFP and post-AD effluent (Table 3.5, Figure 3.15). The monthly mean DO, however, increased from the lowest concentration of 0.04 mg/L, which was measured in the post-AD effluent, to higher mean concentrations measured in the PFP of 7.23±0.07 mg/L and in the HRAP A2 effluent of 18.64±0.01 mg/L (Figure 3.15). The COD was substantially higher in the post-HRAP A2, than in the post-PFP and post-AD effluent (Table 3.6, Figure 3.16). The highest monthly mean COD of 206.30±0.33 mg/L in the post-AD effluent increased to the highest COD of 303±42.01 mg/L in the post-HRAP A2 effluent (Figure 3.16). The COD in the post-PFP effluent was 225 mg/L, which was marginally higher than in the post-AD effluent. The pH of the effluent increased as effluent moved through the system from the anaerobic digester into the HRAP A2 (Table 3.7, Figure 3.16).

a. Dissolved oxygen

Dissolved oxygen concentrations in the post-AD and PFP effluent had no apparent seasonal trends (Table 3.5, Figure 3.15). In the HRAP A2 effluent, there were also no clear seasonal trends in DO concentrations, however, the highest mean concentrations were recorded in summer (January and February).

Table 3.5 The annual mean (± standard error) dissolved oxygen (DO) concentration in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent over 12 months, the number of DO concentration samples taken over that period (n), and the change in DO concentration as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (mg/L)	n	Change (%)
post-AD	2.3 ± 0.29	30	0
PFP	4.09 ± 0.52	30	78
HRAP A2	11.53 ± 0.47	20	401

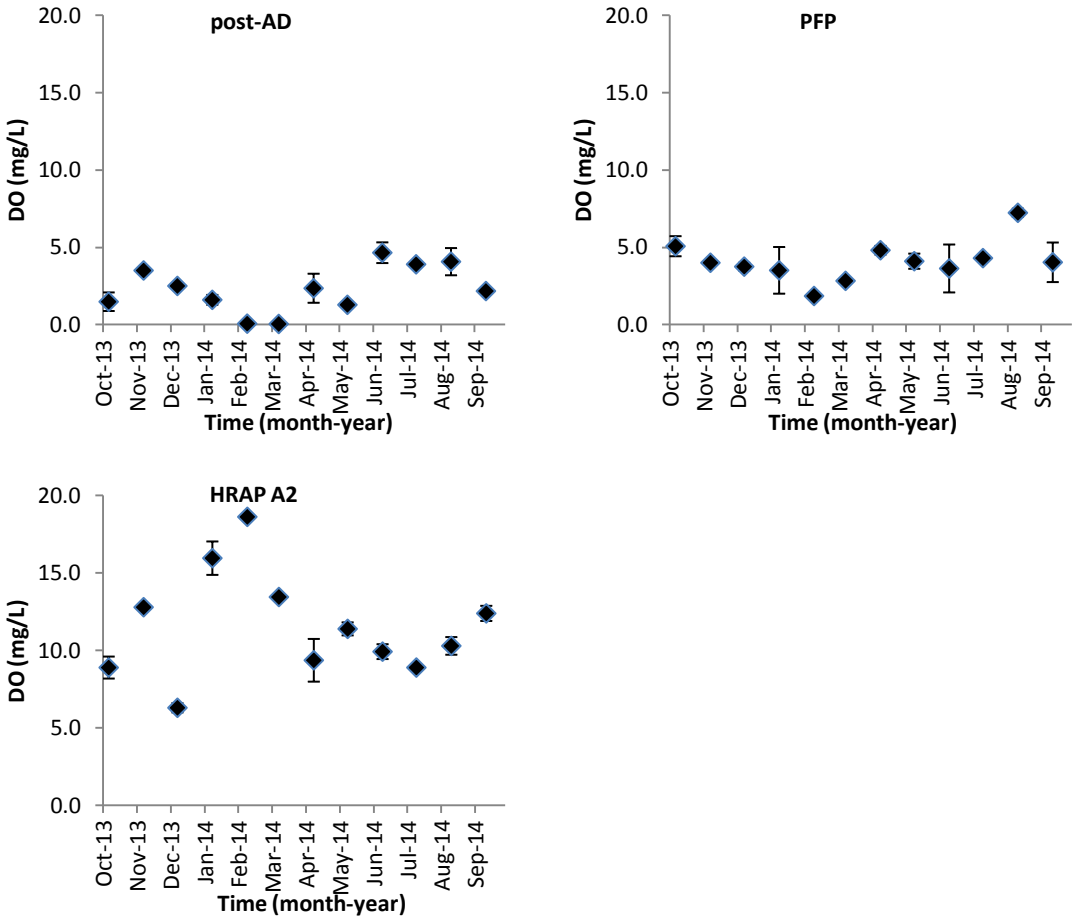

Figure 3.15 The monthly mean (± standard error) dissolved oxygen (DO) concentration in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent over 12 months.

b. Chemical oxygen demand

In the post-AD and post-PFP effluent, there was no apparent seasonal trend in the monthly mean COD (Table 3.6, Figure 3.16). In the post-HRAP A2 effluent, COD values also did not seem to have any seasonal trends, however, the highest mean value of COD was recorded in summer (January).

Table 3.6 The annual mean (± standard error) chemical oxygen demand (COD) in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent over 12 months, the number of COD samples taken over that period (n), and the change in COD as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (mg/L)	n	Change (%)
post-AD	192.38 ± 12.66	29	0
post-PFP	188.88 ± 13.89	29	-2
post-HRAP A2	236.67 ± 14.88	29	23

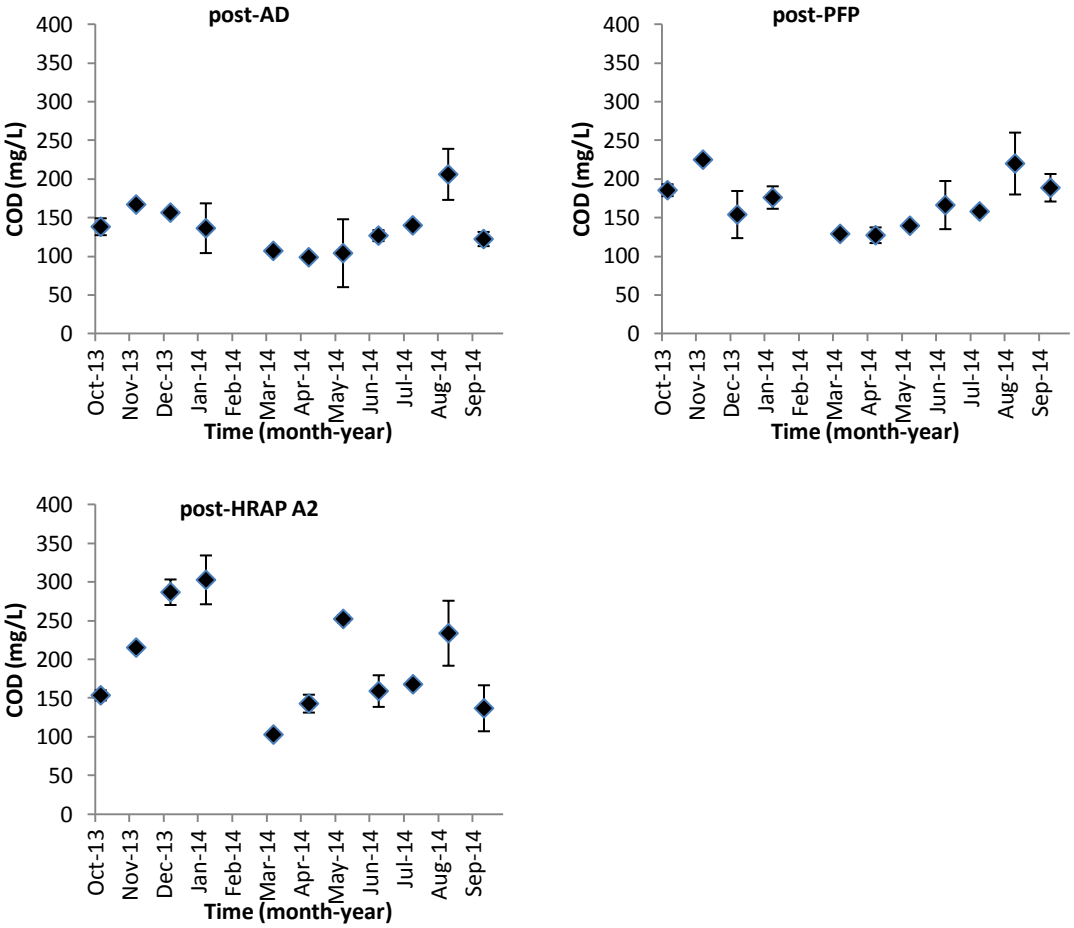

Figure 3.16 The monthly mean (± standard error) chemical oxygen demand (COD) in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent over 12 months.

c. pH

The average pH values in the post-AD and PFP effluent were lower than in the HRAP A2 effluent (Table 3.7, Figure 3.17). The values ranged from 6.39±0.61 to nine in post-AD effluent and PFP effluent (Figure 3.17). In the HRAP A2 treated effluent, the pH ranged from 7.25±0.75 to 10.23±0.21 with the highest average pH values recorded during spring and summer.

Table 3. 7 The annual average (± standard error) pH in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent, the number of pH samples taken over that period (n), and the change in pH as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean	n	Change (%)
post-AD	8.03 ± 0.17	30	0
PFP	8.17 ± 0.29	30	2
HRAP A2	9.44 ± 0.21	31	18

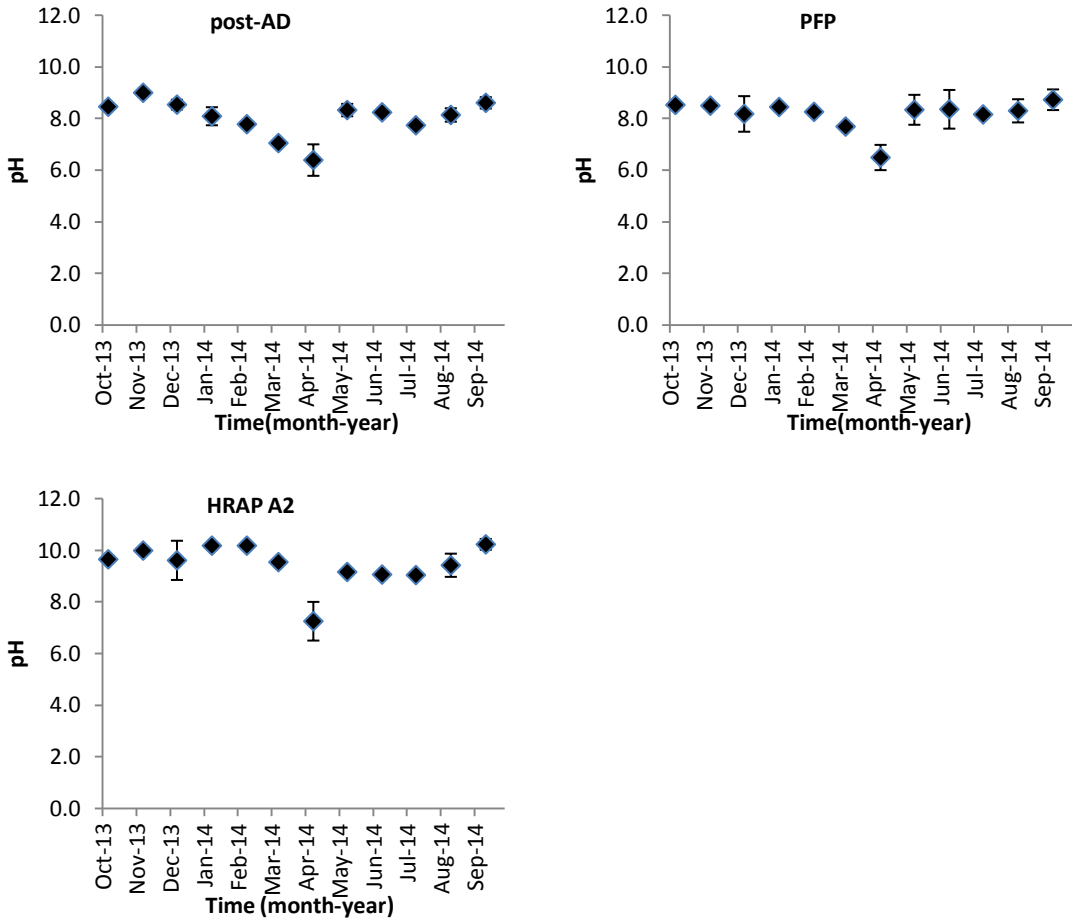

Figure 3.17 The monthly average (± standard error) pH in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent over 12 months.

The annual mean concentration of NH_4^+-N recorded in the post-AD effluent was 59.32±2.96 mg/L (Table 3.8), but it varied considerably throughout the year, ranging from a low concentration of 28.78±6.12 mg/L to a high concentration of 110 mg/L (Figure 3.18). The latter being the highest recorded values for NH_4^+-N across the process flow, a general trend in NH_4^+-N decrease was observed downstream of the post-AD effluent sample point. There was a noticeable drop in the monthly mean concentration of NH_4^+-N in post-PFP effluent. Monthly trends in the mean NH_4^+-N concentrations measured in the post-PFP effluent nominally tracked those measured in the post-AD effluent. In post-PFP effluent, the concentration ranged from 19.06±15.03 mg/L to 103 mg/L. However, the decline in NH_4^+-N concentration across the HRAP was substantial. The annual mean NH_4^+-N concentration measured in the post-HRAP A2 effluent was 1.04±0.14 mg/L (Table 3.8), ranging from a low monthly mean of 0.39±0.03 mg/L to 2.08±0.63 mg/L (Figure 3.18). A general decrease in NO_2^--N was observed across the process flow from the anaerobic digester into the PFP (Table 3.9, Figure 3.19). The annual and monthly mean concentrations of NO_2^--N decreased in the post-PFP effluent, and there was a further decline in NO_2^--N concentration in the post-HRAP A2 effluent. Nitrate-nitrogen concentrations increased slightly from the post-AD effluent to the post-

PFP effluent (Table 3.10, Figure 3.20). In post-HRAP A2 effluent, NO_3^--N concentration increased further to as high as 43.21±2.83 mg/L. The trends in the removal of PO_4^{3-}-P across the process flow showed a nominal decrease in the annual and monthly mean concentrations from the post-AD effluent to the post-PFP effluent (Table 3.11, Figure 3.21). There was a further notable decline in the monthly mean concentration of PO_4^{3-}-P in the post-HRAP A2 effluent (Figure 3.21). The PO_4^{3-}-P concentration decreased from the highest concentration recorded of 29.14±2.63 mg/L in post-AD effluent to as low as 8.49±0.22mg/L in post-HRAP A2 effluent.

a. Ammonium-nitrogen

The removal efficiencies of NH_4^+-N oscillated between 93 and 99% throughout the year, with the lowest measurement recorded in June with a removal efficiency of 93% (Table 3.8, Figure 3.18).

Table 3.8 The annual mean (± standard error) ammonium-nitrogen (NH_4^+-N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent, the number of NH_4^+-N samples taken over that period (n), and the change in NH_4^+-N concentration as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (mg/L)	n	Change (%)
post-AD	59.32 ± 2.96	30	0
post-PFP	46.6 ± 3.9	30	-21
post-HRAP A2	1.04 ± 0.14	31	-98

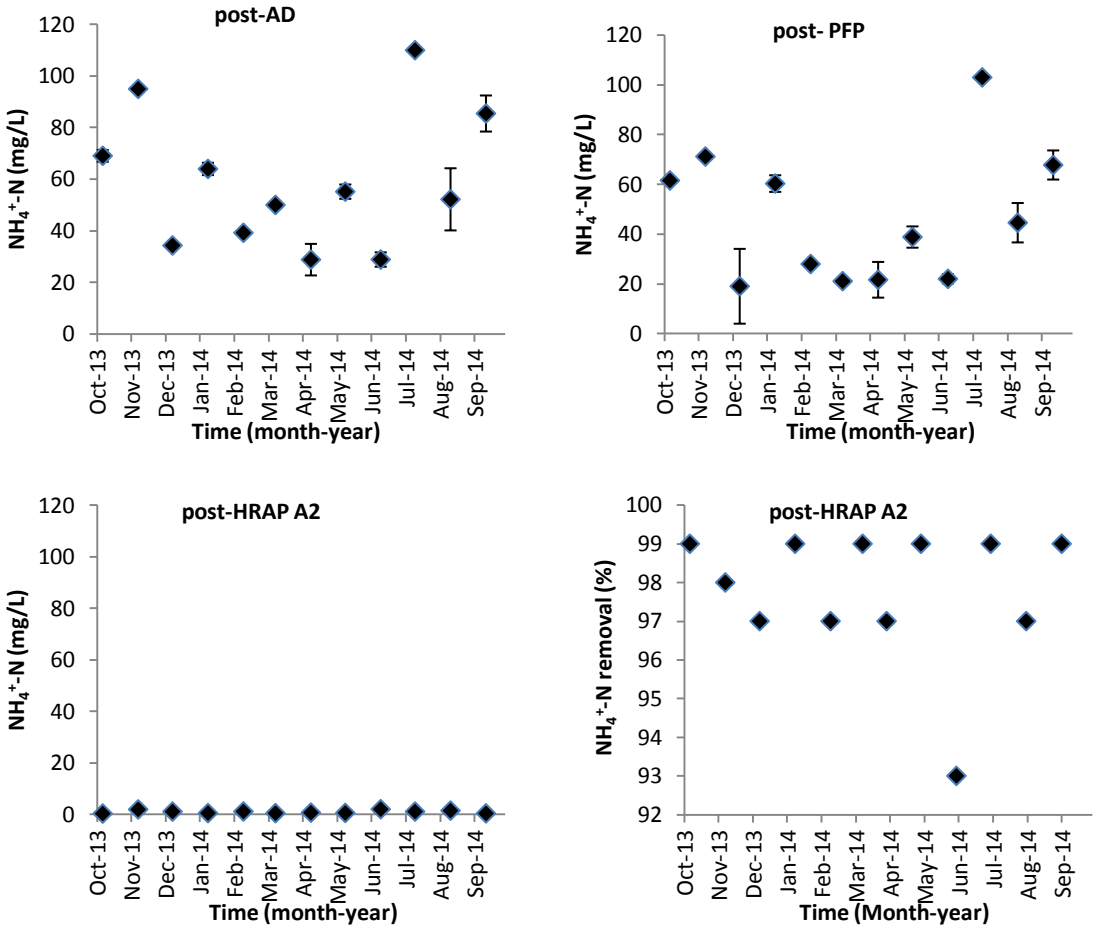

Figure 3.18 The monthly mean (± standard error) ammonium-nitrogen (NH_4^+-N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent over 12 months.

b. Nitrite-nitrogen

The lowest monthly mean measurement of NO_2^--N of 0.46±0.15 mg/L, was recorded in early summer (November) while the highest measurement, of 2.38±0.64 mg/L, was recorded in mid winter (June) (Figure 3.19). There appeared to be a seasonal signal in the removal efficiencies of NO_2^--N. Generally, higher efficiencies were recorded in the warmer months of the year, up to 84%, while the lower removal efficiencies coincided with the cooler months of the year down to 49% (Figure 13.19). However, the lowest and highest measurements recorded of 30% in September and 86% in October appeared as outliers and broke with the observed trend in seasonal associated removal efficiencies.

Table 3.9 The annual mean (± standard error) nitrite-nitrogen (NO_2^--N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent, the number of NO_2^--N samples taken over that period (n), and the change in NO_2^--N concentration as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (mg/L)	n	Change (%)
post-AD	1.05 ± 0.18	30	0
post-PFP	0.64 ± 0.11	30	-39
post-HRAP A2	0.42 ± 0.08	31	-60

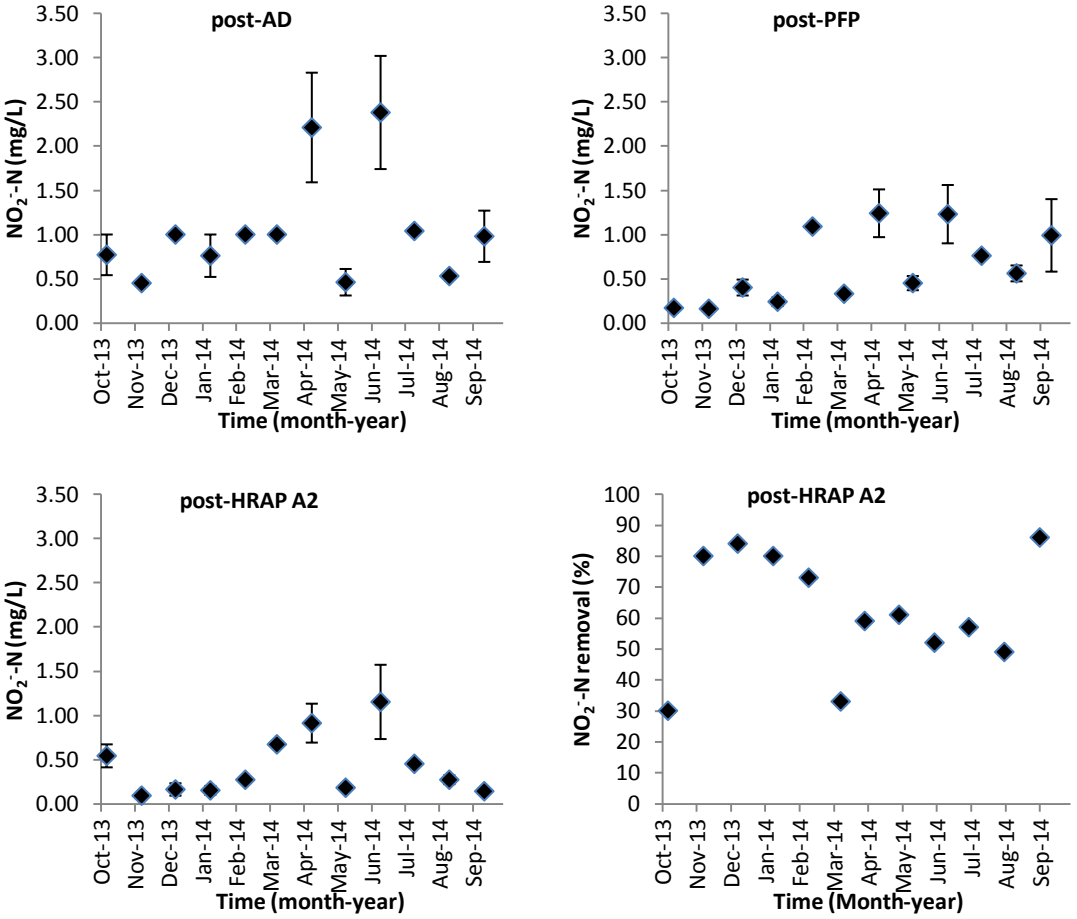

Figure 3.19 The monthly mean (± standard error) nitrite-nitrogen (NO_2^--N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent over 12 months.

c. Nitrate-nitrogen

The highest concentrations of NO_3^--N recorded in the post-AD effluent was 37.47 mg/L in winter (July) and the lowest concentrations measured in this effluent was 0.41 mg/L in late spring (November) (Figure 3.20). Similar seasonal trends were also observed in the post-PFP and post HRAP A2 effluents, in the former, 37.87 mg/L was measured in winter (July) and 0.59 mg/L was measured in late spring (November), while in the latter effluent, 43.21±2.83 mg/L was measured in

winter (August) and 4.15 mg/L was measured in late spring (November). The highest increase in NO_3^--N in the post-HRAP A2 effluent relative to the post-AD effluent occurred in winter.

Table 3.10 The annual mean (± standard error) nitrate-nitrogen (NO_3^--N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent, the number of NO_3^--N samples taken over that period (n), and the change in NO_3^--N concentration as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effuent	Mean (mg/L)	n	Change (%)
post-AD	16.35 ± 1.45	30	0
post-PFP	18.21 ± 1.79	30	11
post-HRAP A2	26.33 ± 2.16	31	61

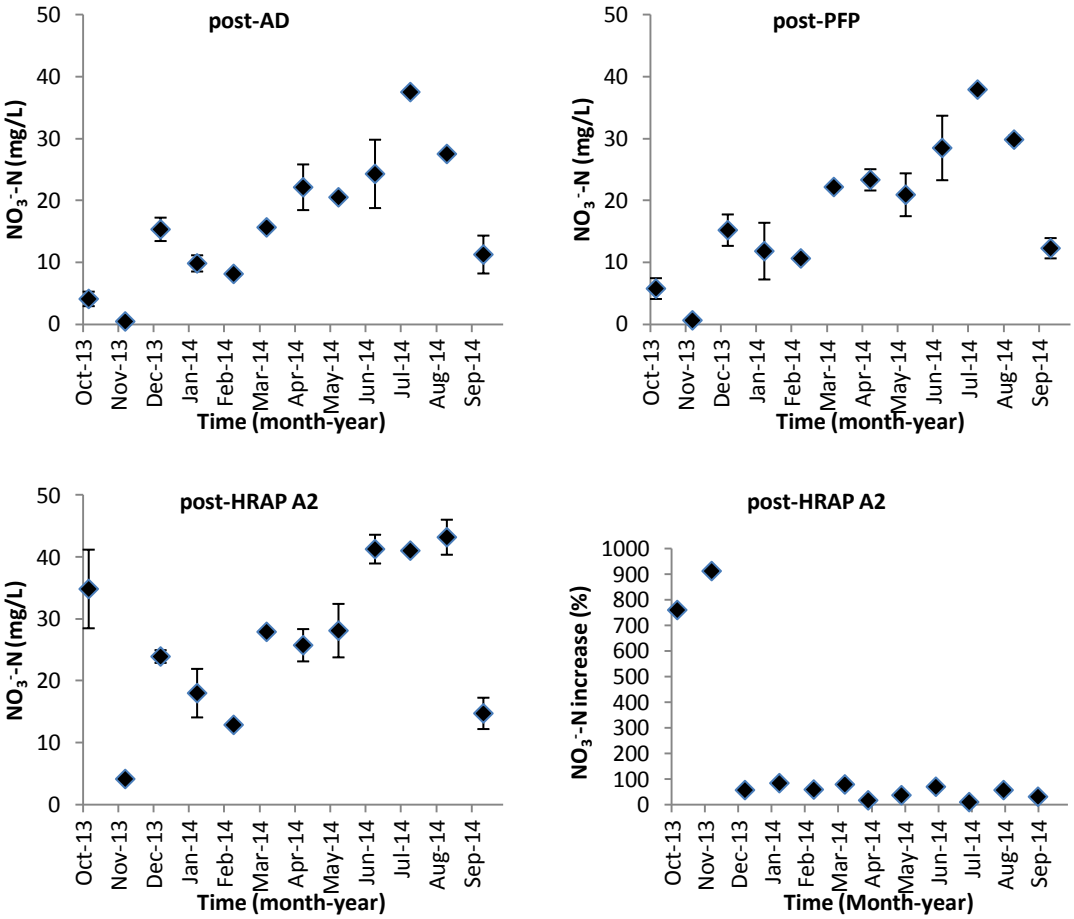

Figure 3.20 The monthly mean (± standard error) nitrate-nitrogen (NO_3^--N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent over 12 months.

d. Orthophosphate-phosphorus

The monthly mean concentration of PO_4^{3-}-P from the post-AD effluent to the post-PFP effluent decreased slightly (Table 3.11, Figure 3.21). In the post-HRAP A2 effluent, PO_4^{3-}-P removal efficiency was between 20 and 70%, however, the removal efficiencies had no clear seasonal trends throughout the year.

Table 3.11 The annual mean (± standard error) phosphate-phosphorus (PO_4^{3-}-P) concentration in post-anaerobically (post-AD) digested brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent, the number of PO_4^{3-}-P samples taken over that period (n), and the change in PO_4^{3-}-P concentration as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (mg/L)	n	Change (%)
post-AD	26.89 ± 1.74	30	0
post-PFP	23.14 ± 1.43	30	-14
post-HRAP A2	14.56 ± 2.16	31	-46

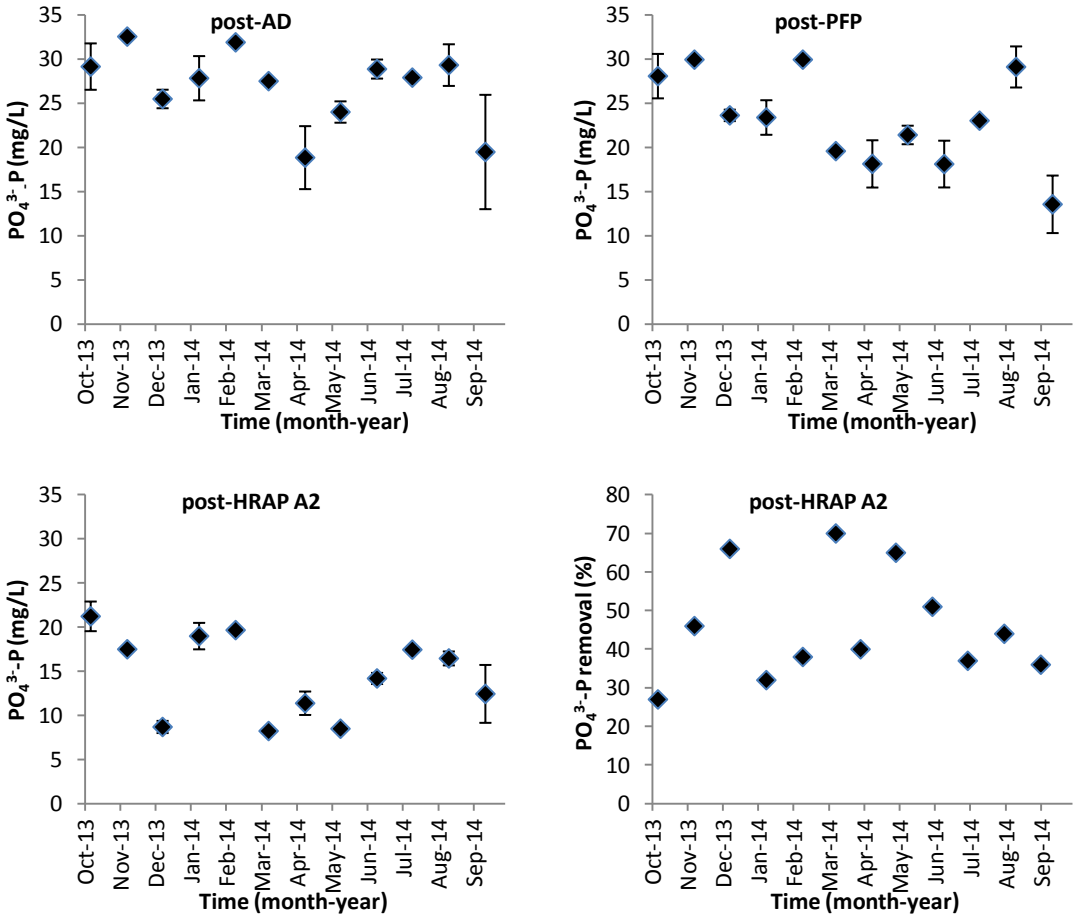

Figure 3.21 The monthly mean (± standard error) phosphate-phosphorus (PO_4^{3-}-P) concentration in post-anaerobically (post-AD) digested brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent over 12 months.

The monthly mean Cl^- concentration in post-AD and post-PFP effluent did not vary much, however, in post-HRAP A2 effluent, Cl^- concentration increased (Table 3.12, Figure 3.22). Similar trends were observed in the EC, which increased from the post-AD effluent into the post-HRAP A2 effluent (Table 3.13, Figure 3.23).

a. Chloride

There was little difference in mean Cl^- measurements between the post-AD and post-PFP effluents, but notably higher Cl^- measurements were recorded in the post-HRAP A2 effluent (Table 3.12, Figure 3.21). There were no clear trends in Cl^- concentration linked to season; although the highest recorded values in both post-AD and post-PFP effluents coincided with winter (June), where a maximum of 214.75±14.38 mg/L was recorded (Figure 3.21). The Cl^- minimum of 79 mg/L was recorded in post-AD effluent in late spring (November) and in post-PFP effluent, 36.50±23.89 mg/L was recorded in autumn (April). The maximum Cl^- concentration recorded in post-HRAP A2 effluent was 213.25±31.04 mg/L, which coincided with autumn (April), while a minimum of 95.75±4.91 mg/L was recorded in spring (October).

Table 3.12 The annual mean (± standard error) chloride (Cl^-) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent, the number of Cl^- samples taken over that period (n), and the change in Cl^- as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (mg/L)	n	Change (%)
post-AD	126.69 ± 19.99	30	0
post-PFP	127.03 ± 11.57	30	0
post-HRAP A2	154.31 ± 15.11	31	22

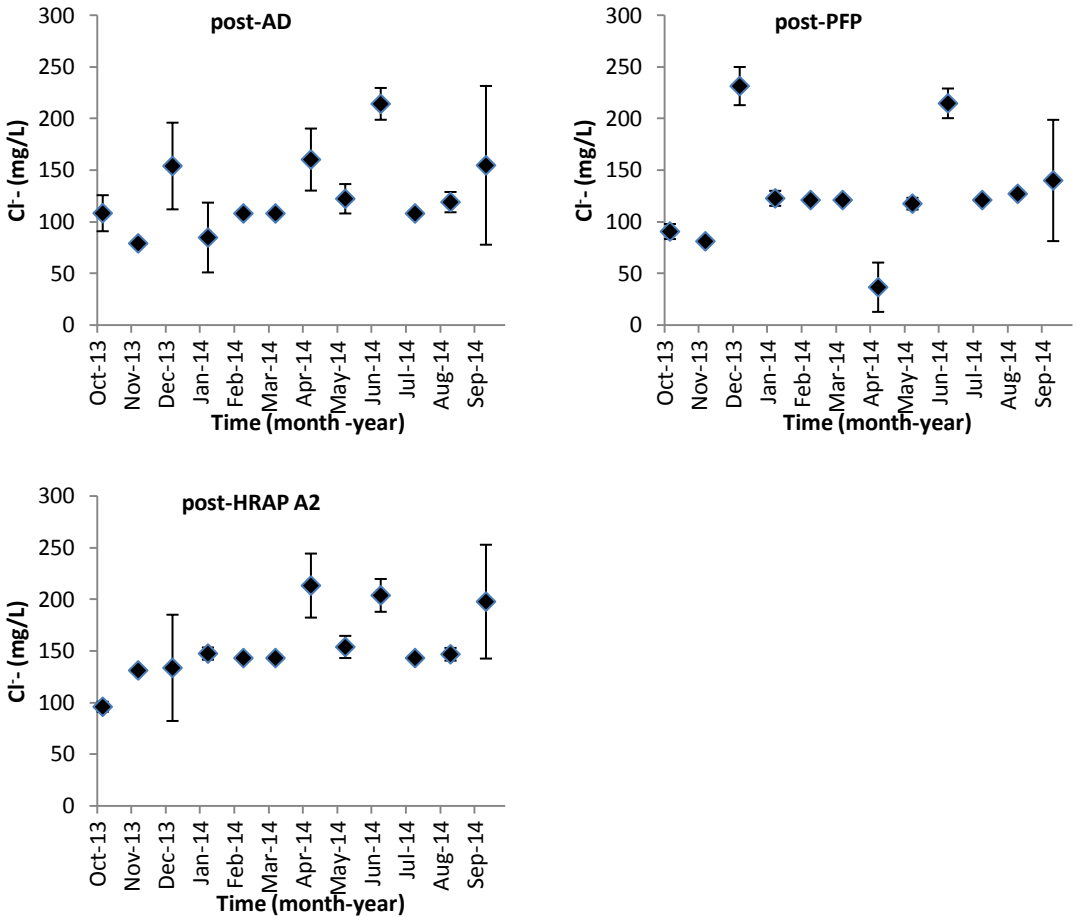

Figure 3.22 The monthly mean (± standard error) chloride (Cl^-) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal pond (post-HRAP A2) effluent over 12 months.

b. Electrical conductivity

The annual mean EC increased across the process flow, which ranged from 2502.48±171.21 µS/cm in the post-AD effluent, to 2839.51±40.88 µS/cm in the PFP effluent and 2998.14±60.97 µS/cm in the HRAP A2 effluent (Table 3.13). In effluents, EC generally trended higher from late summer to late winter (Figure 3.22). The monthly mean of EC in post-AD effluent ranged from 248.67±62.63 µS/cm in spring (September) to 3822 µS/cm in late summer (February). The lowest EC recorded in the PFP effluent was 2343 µS/cm, which was reported in spring (November), while the highest EC recorded in this effluent was 3410 µS/cm which was reported in late summer (February). In HRAP A2 effluent, the monthly mean EC ranged from 2425.75±152.43 µS/cm in spring (October) to 3495 µS/cm in late summer (February). In the HRAP A2 effluent, the highest EC was recorded during spring and summer.

Table 3.13 The annual mean (± standard error) electrical conductivity (EC) in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent, the number of EC samples taken over that period (n), and the change in EC as a percentage (%) of that entering the system in the post-AD effluent.

Integrated algal ponding system effluent	Mean (µS/cm)	n	Change (%)
post-AD	2502.5 ± 171.21	30	0
PFP	2839.5 ± 40.88	30	13
HRAP A2	2998.1 ± 60.97	30	20

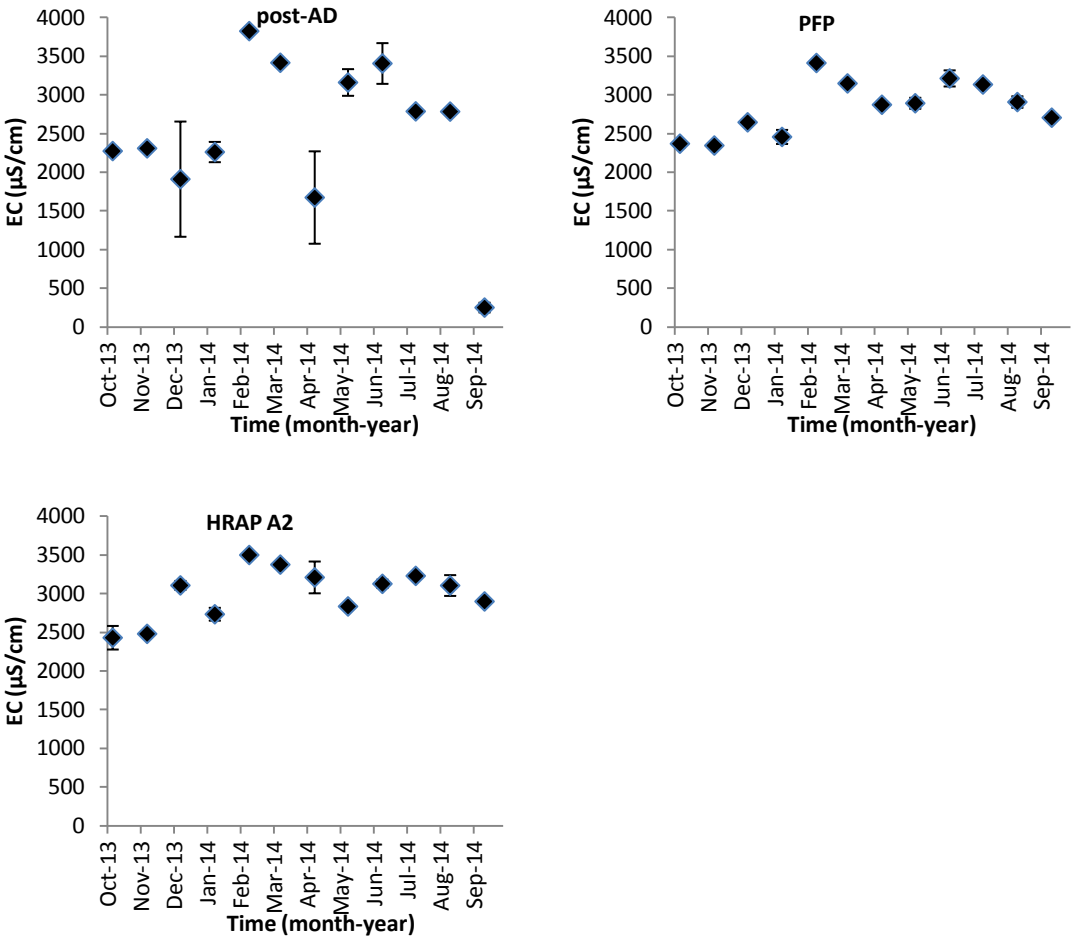

Figure 3.23 The monthly mean (± standard error) electrical conductivity (EC) in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal pond (HRAP A2) effluent over 12 months.

3.3.5 The relationship between microalgae biomass, chlorophyll *a* concentration and other abiotic parameters

There was a significant trend where algal biomass increased with an increase in temperature ($p < 0.001$, Figure 3.24). A similar significant trend was observed with the Chl *a* concentrations at different temperatures ($p < 0.001$, Figure 3.25; $p = 0.003$, Figure 3.26). There was a positive correlation between algal biomass and DO concentrations ($R = 0.76$, Figure 3.27).

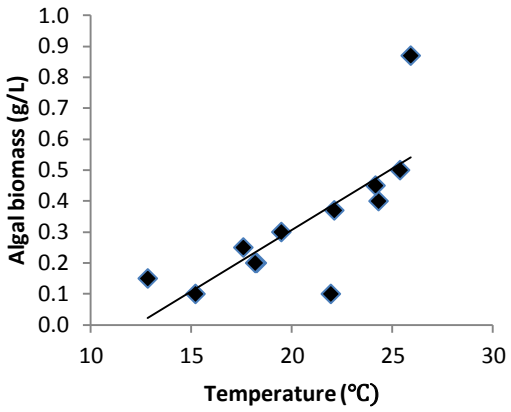

Figure 3.24 A relationship between the monthly mean temperature (℃) versus the monthly algal biomass (g/L) in high rate algal pond (HRAP A2) over 12 months (y = 0.0397x - 0.4881, R^2 = 0.58, $p < 0.001$).

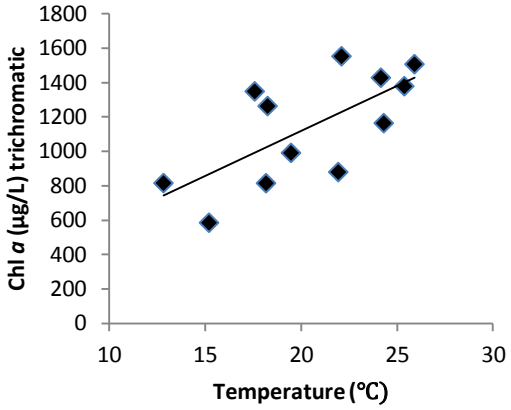

Figure 3.25 A relationship between the monthly mean temperature (℃) versus the trichromatic calculation for monthly chlorophyll (Chl) *a* concentration (µg/L) in high rate algal pond (HRAP A2) over 12 months (y = 52.217x + 75.271, R^2 = 0.47, $p < 0.001$).

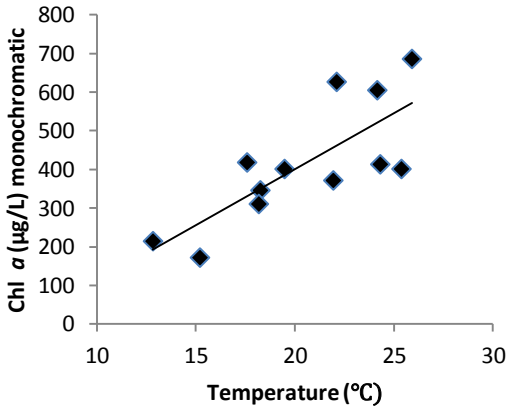

Figure 3.26 A relationship between the monthly mean temperature (℃) versus the monochromatic calculation for monthly chlorophyll (Chl) *a* concentration (µg/L) in high rate algal pond (HRAP A2) over 12 months (y =29.079x - 180.8, R^2 = 0.59, p = 0.003).

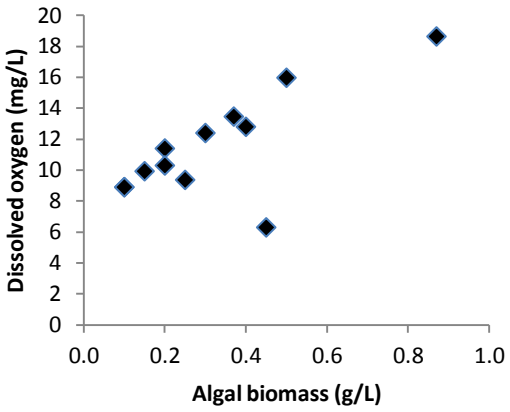

Figure 3.27 A relationship between the monthly algal biomass (g/L) versus monthly mean dissolved oxygen (DO) concentration (mg/L) in high rate algal pond (HRAP A2) over 12 months (R = 0.76).

3.4 Discussion

a. Seasonal variation of the microalgae community structure in a high rate algal pond

Seasonal variation resulted in changes in the algal species in the pond. In the austral spring and summer, more microalgae taxa were observed as compared to winter and autumn. Assemany *et al*. (2015) also found more phytoplankton taxa during spring and summer and fewer taxa in autumn when investigating the effects of solar radiance on phytoplankton community in HRAPs. In Port Elizabeth, the average minimum/maximum temperature ranged from 7.4/20.5℃ in winter to 18.7/26.6℃ in summer (Sampson, *pers. comm*., client liaison officer, SAWS, Port Elizabeth, February 2015). Solar PAR was also the highest during the warmer seasons when compared with the cooler seasons (Sampson, *pers. comm*., client liaison officer, SAWS, Port Elizabeth, February 2015). These environmental parameters varied seasonally, creating conditions that favour some algal species, while others were not favoured (Richmond 2004). This leads to species succession where new dominant algae taxa emerge (Addy & Green 1996). During the cooler seasons in Port Elizabeth, microalgae could have been exposed to sub-optimal temperature and solar PAR that probably limited the growth of some species (Robarts & Zohary 1987; James & Boriah 2010, Assemany *et al*. 2015). The effect of solar PAR on algal growth was evident in Assemany *et al*. (2015) investigation in which they compared algae growth in an 80% shaded and an unshaded HRAP. It was found that the microalgae in the unshaded pond had higher growth than those in the light limited shaded pond. Temperature and solar PAR are crucial in algal growth and should be within the tolerance range of algal species for the best growth (Fogg 1991). Above or below the tolerance range, the growth of the algae gets compromised (Fogg 1991; Talbot *et al*. 1991; Larsdotter 2006a).

Annual cycle affected the microalgae community structure in the HRAP. *Chlorella* sp*., Scenedesmus* sp*., Chlamydomonas* sp*.*, *Diatoma* sp*., Microcystis* sp. *Synechococcus* sp*. and Phormidium* sp*.* were present in the HRAP A2 throughout the year. However, some other algal species from the Chlorophyta, Cyanophyta, Euglenophyta and Bacillariophyta phyla were observed occasionally. Barthel *et al*. (2008) found that *Chlorella* sp*., Scenedesmus* sp*.* and *Chlamydomonas* sp. were present in the HRAPs throughout the entire study period when ponds were used to treat piggery waste. The relative abundance of these species probably varied seasonally, i.e. the relative abundance of *Scenedesmus abundans* was 66.41% in summer, which then decreased to 2.82% in spring. This decline was clearly visible under the light microscope. During summer, *Scenedesmus* sp. appeared to be the dominating species, whereas, during winter and spring, there were few *Scenedesmus* cells. In spring, there appeared to be no dominating species under the microscope and this was attested by the spring metagenomics data. Affan *et al*. (2005) also reported a high abundance of phytoplankton in summer which reduced gradually in winter. Some species such as *Chlorella* spp*., Scenedesmus* spp*., Chlamydomonas* spp*.*, *Diatoma* spp*., Microcystis* spp*., Synechococcus* spp*. and Phormidium* spp*.* have wide temperature tolerances ranging from 16-30℃, 16-48℃, 15-37℃, 20-36℃, 10-30℃ and 10-45℃ respectively (Tang & Vincent 1999; Richmond 2004; Vítová *et al*. 2011; Abdel-raouf *et al*. 2012; Suali & Sarbatly 2012; Wagley 2012). The temperature range at the study site was favourable for the growth of these species given in the previous sentence. Temperature affected the microalgae community structure and relative abundance, thus supporting the growth of some algal species, while other species were not favoured.

Besides seasonal variation, there are also biotic factors that may be seasonally linked and have an impact on the microalgae community structure and their relative abundance. Zooplankton grazing on microalgae also contribute to shifts of microalgae species composition and their relative abundance (Schlüter *et al*. 1987; Assemany *et al*. 2015). When using the light microscopy, rotifers were seen grazing on microalgae in summer, thus suggesting grazing pressure could be high during this season. Grazing on microalgae was also observed by Schlüter *et al*. (1987) when the rotifer *Brachionus rubens* selectively grazed on *Scenedesmus* sp*.* while rejecting other species such as *Micractinium pusillum* that have spines (setae) that impede ingestion by rotifers. Bergquist *et al*. (1985) reported grazing on *Euglena* sp. while *Scenedesmus quadricauda,* which has spines was not grazed, yet the cells were of the same size. This could imply that species such as *Chlamydomonas* sp*.,* cyanobacteria and *Chlorella* sp. which are very small cells can easily be grazed by zooplankton (Tiseer *et al*. 2008; Winder & Sommer 2012; Assemany *et al*. 2015) were present in the HRAP throughout the study period, possibly due to their fast growth rates that permit them to prevail despite being grazed (Assemany *et al*. 2015). However, algal species that were also grazed on, but

could not colonise the pond because the environment was not conducive for their rapid growth were absent in the pond at times (Shaari *et al*. 2011). Grazing could have contributed to the seasonal variation of the algal community in the HRAP A2.

Seasonal variations were seen to affect the Chlorophyta community structure. *Pediastrum* sp. and *Stigeoclonium* sp. were observed in summer and spring while *Spirogyra* sp. was only observed in summer (January). *Chroococcus* sp. and *Tribonema* sp. were also observed in spring and early summer while *Oocystis* sp. was only observed in spring (October). *Haematococcus* sp. was observed in late spring, summer, autumn and early winter. The relative abundance of these species probably differed seasonally, for example, the relative abundance of *Mychonastes* sp. was 23.03% in summer and then increased to 73.46% in winter. Simpson *et al*. (1987) reported *Mychonastes ruminatus* can grow at a temperature range of 5-30℃, this could be the reason the relative abundance increased in winter. Pieterse & Röhrbeck (1990) found *Pediastrum duplex* to be dominant during spring and summer when investigating the dominant phytoplankton species in Roodeplaat Dam, South Africa, for three years (1976, 1977 and 1978). Oosthuizen & Janse van Vuuren (2014) also found that *Pediastrum duplex* dominated during spring and summer at a Free State water purification plant, South Africa, when investigating the changes of the microalgae community. *Stigeoclonium* sp. was observed in October (spring) by Oosthuizen & Janse van Vuuren (2014). In the HRAP A2, *Pediastrum* sp., *Haematococcus* sp. and *Stigeoclonium* sp. were observed in spring and summer, which corresponded with their temperature tolerance range from 20-27℃ that favours their growth (Suali & Sarbatly 2012; Klochkova *et al*. 2013). *Spirogyra* sp. prefers a temperature ranging from 16-28℃ (Munir *et al*. 2015), which was the case in January, so this could explain their presence in the pond at this time of the year. *Oocystis* sp. prefer higher temperatures between 25-40℃ and a low PAR of 46-70 µmol m^{-2} s^{-1} (Jia-Qi *et al*. 2011). Temperature within this range was recorded in October in the HRAP. This phylum's wide range of temperature tolerance could have attributed to the wide range of microalgae species in the pond.

The Cyanophyta assemblage varied seasonally. *Arthrospira* sp. was observed in spring and summer, whereas, *Oscillatoria* sp. was only observed in summer. *Microcystis* sp. was found in the HRAP A2 throughout the year. Affan *et al*. (2005) also observed Cyanophyta species such as *Microcystis sp*., *Anabaena* sp., and *Planktolymbya* sp. in spring and autumn. The temperature in the HRAP during these seasons fell between the reported temperature tolerances of *Arthrospira* sp., *Microcystis* sp. and *Oscillatoria* sp., which ranges from 18-28℃ (Tiseer *et al*. 2008; Markou & Georgakakis 2011; Winder & Sommer 2012; Sipaúba-Tavares *et al*. 2014). Some *Arthrospira* species can even tolerate temperatures as high as 30-38℃ (Markou & Georgakakis 2011). De Pauw & van Vaerenbergh (1983)

observed an exponential growth rate increase in *Oscillatoria agardhii* with increasing temperatures of 20, 25 and 30°C. *Microcystis* sp. has gas vesicles that enable them to float to the water surface at any time of the year and be exposed to sufficient light (Robarts & Zohary 1987). This gives *Microcystis* sp. the ability to outcompete other species (Robarts & Zohary 1987), and this could have contributed to its presence throughout the year. Most cyanobacteria can easily adapt to environmental changes and are able to tolerate a wide range of temperature and PAR levels for prolonged periods (Fogg 1991).

Seasonal cycles affected the Euglenophyta and Bacillariophyta species composition. *Euglena* sp. was observed in summer only, while *Navicula* sp. and *Nitzschia* sp. were observed during spring and summer. *Nanofrustulum shiloi* was identified in summer with a relative abundance of 4.46%, while in spring, the relative abundance decreased to 3.38%. Affan *et al*. (2005) also observed *Euglena* sp. during summer and autumn in aquaculture ponds. Oosthuizen & Janse van Vuuren (2014) found *Nitzschia palea* prevalent from July to March (winter, spring, summer and autumn) in the Virginia water purification plant in the Free State. *Nitzschia* spp. grow optimally at a temperature range of 20-36°C (Wagley 2012). *Euglena* spp. grow well within a temperature range of 15-32°C (Cook 1966). *Euglena* spp. have motile abilities that enable them to manoeuvre to areas of sufficient illumination in the water column (Babu 2011). Diatoms such as *Navicula* spp. and *Nitzschia* spp. grow best at temperatures above 20°C (Lee & Kim 2002), this could have been the reason they were only observed in the warmer seasons.

The two methods used to identify microalgae gave different results in some instances. Some algal cells were not clearly visible under the light microscope and this could have made their identification a bit difficult. When using metagenomic techniques, the 18S rRNA gene of some microalgae was probably not amplified with the primer pair 566F and 1200R, thus the identification of some species was not established. These two reasons can account for the variation in species identified using the two methods. The lack of universal microalgae primers makes identification of microalgae community structure very difficult, but this work paved the way and gave a start point to an understanding of microalgae community structure in HRAPs.

b. Seasonal variation of the bacterial community structure in a high rate algal pond

Seasonal variation had a marked effect on the bacterial species composition and their relative abundance. Unidentified bacteria were the most abundant during summer, followed by winter and spring at a relative abundance of 31.23, 16.44 and 17.61% respectively. These unidentified species are bacterial species that have not yet been isolated, identified and cultured in the laboratory

(Moila, *pers. comm*., Next Generation Sequencing laboratory technician, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, September 2015). Tang *et al*. (2014) investigated the effects of temperature on the bacterial community structure and relative abundance in a freshwater aquaculture pond over a year. It was reported that there was a shift in the bacterial composition due to seasonal variation (Tang *et al*. 2014). The other bacterial species retrieved from the HRAP A2 were bacterial species whose role in wastewater treatment is unclear, i.e. literature states that they are not involved in nitrogen and phosphorus cycling.

Firmicutes composition and their relative abundance were affected by seasonality. Their relative abundance decreased from 71.32% in summer to 14.38 and 5.23% in winter and spring respectively. Tang *et al*. (2014) also observed the highest relative abundance of Firmicutes at 0.27% in the hot temperature seasons and a lower abundance of 0.05% at low temperatures. In the HRAP A2, the bacterial species belonging to this phylum were species such as *Bacillus* spp. Albaggar (2014) also reported that during summer, temperature affected the bacterial growth rate and their abundance positively, leading to an increase in the species relative abundance in temperate lakes, in the USA. This suggests that temperature in the HRAP A2 was one of the factors responsible for shifts in the Firmicutes composition and relative abundance (Fredriksson 2013; Albaggar 2014). These Firmicutes, such as *Bacillus* spp., are aerobic or facultative anaerobes that play a major role in the first stage of anaerobic digestion of organic matter, namely, hydrolysis (Mara 2004). These species play a vital role in nitrogen and phosphorus cycling in wastewater by degrading organic matter.

The Proteobacteria composition and their relative abundance were also affected by season. Proteobacteria increased from 2.11% in summer to 11.49 and 22.93% in winter and spring respectively. These results are comparable to Tang *et al*. (2014), where the lowest relative abundance of Proteobacteria of 18.77% was observed during the seasons with high water temperature and the highest relative abundance of 39.52% observed in the seasons with low water temperature. The phylum was represented by species of the genera *Eubacterium*, *Klebsiella, Rhizobium*, *Pseudomonas, Vibrio, Rhodopseudomonas, Rhodobacter, Methylobacteria and Paracoccus.* In spring, facultative anaerobes *Vibrio* sp. and *Vibrio mimicus* were 0.47% and 0.02% respectively. *Eubacteria* sp. was observed with a relative abundance of 3.37% in summer, while in winter, *Eubacteria* sp. abundance was 0.01%. The nitrogen fixing bacteria, *Rhizobium* sp. was observed in spring with a relative abundance of 0.17% while that of *Klebsiella* sp. were 0.01% in summer. The relative abundance of denitrifiers such as *Pseudomonas aeruginosa* was at 0.04% in spring, while *Methylobacteria* sp. was at 0.02% during spring. *Paracoccus sp*. was observed in summer at an abundance of 0.01%. Photosynthetic bacteria such as *Rhodopseudomonas palustrus*

accounted for about 0.03% of the bacterial composition during spring, while *Rhodobacter* sp. accounted for 1.25% and 0.78% during winter and spring respectively. This is supported by Tang *et al*. (2014) who reported shifts in the bacterial community structure and relative abundance due to seasonal variation. Proteobacteria such as *Vibrio* spp. are involved in the first stage of anaerobic digestion (hydrolysis) while *Eubacteria* spp. participate in the second stage of anaerobic digestion, acidogenesis (Mara 2004). *Vibrio* spp. are freshwater and marine aquatic bacteria that are able to biodegrade carbon, nitrogen and phosphorus substrates (Mansergh & Zehr 2014). They contain enzymes such as phosphatase which degrade phosphorus containing matter (Bitton 2005). *Rhizobium* spp. are aerobic bacteria that form nitrogen fixing nodules with legumes. The nodules form bacteroids which are swollen *Rhizobium* cells, capable of fixing nitrogen (Okafor 2007). *Klebsiella* spp., are facultative anaerobes also capable of fixing nitrogen (Bitton 2005). *Pseudomonas aeruginosa, Methylobacteria* spp. and *Paracoccus* spp. play a role in the denitrification of nitrate into free nitrogen gas (Bitton 2005; Andersson 2009). The role of Proteobacteria in wastewater treatment varies amongst their respective class (Albaggar 2014).

Actinobacteria composition and relative abundance also varied with seasonally. In summer, the relative abundance of Actinobacteria was 3.47%, which then increased to 16.22% in winter. Ju *et al*. (2014) also reported that the relative abundance of Actinobacteria was higher in winter (26.00±3.10%) than in summer (17.90±6.90%) in a wastewater treatment plant. An opposite pattern to this of the relative abundance of Actinobacteria was reported by Tang *et al*. (2014). In the warmer seasons, the relative abundance of Actinobacteria was 53.53%, whereas, during the cooler seasons, it's relative abundance was 41.81% (Tang *et al*. 2014). In the HRAP, this phylum was represented by species of the genera *Streptosporangium, Nesterenkonia* and *Actinobacteria.* During summer, the relative abundance of *Streptosporangium* sp. was 13.93%. In winter, some unidentified bacterial species belonging to the Actinobacteria phylum appeared to have a relative abundance of 0.23% while *Nesterenkonia sandarakina* was 2.39%. Actinobacteria are aerobic bacteria that are capable of mineralizing phosphorus containing compounds with the aid of phosphatase enzymes (Bitton 2005). This phylum also plays a significant role in nitrogen and phosphorus cycling in wastewater.

Season influenced the Planctomycetes, Chloroflexi and Verrucomicrobia phyla composition and their relative abundance. Planctomycetes relative abundance was the lowest at 0.33% in summer, and then increased to 3.58 and 4.88% in winter and spring respectively. *Chloroflexi* sp. was seen in spring only at 9.23%. The relative abundance of *Planctomycetes* sp. was 3.92% in spring, while that of *Chloroflexi* spp. were 8.50%. This is also supported by Tang *et al*. (2014) who showed that with

season, the bacterial community structure and its relative abundance shifts. Verrucomicrobia were more prevalent in winter than in summer at relative abundances of 0.34 and 0.23% respectively. Ju *et al*. (2014) also observed the abundance of Verrucomicrobia to be higher in winter than in summer. The anaerobic Planctomycetes, which could have originated from the anaerobic digester are known to play a role in the anaerobic oxidation of NH_4^+ (Bitton 2005). Some of the species belonging to the phyla Planctomycetes, Chloroflexi and Verrucomicrobia are of importance in nutrients transformation in wastewater treatment plants (Bitton 2005).

The annual cycle had a marked effect on Bacteroidetes, Cyanobacteria, Nitrospirae and the Acidobacteria phyla. Bacteroidetes were recorded at a higher relative abundance in winter (3.58%) than in spring (2.19%). This is supported by Tang *et al*. (2014) who found the relative abundance of Bacteroidetes (17.29%) to be greater during the cooler seasons than in the warmer seasons (4.32%). Cyanobacteria were seen in spring only, at a relative abundance of 12.48%. Nitrospirae and Acidobacteria had the lowest relative abundance amongst the phyla identified in the HRAP A2, with Acidobacteria only observed in summer at a relative abundance of 0.62%. Ju *et al*. (2014) reported that Acidobacteria abundance was one to 6.5 times higher in summer than was observed in winter. *Nitrospira* sp. was observed in winter and spring with a relative abundance of 0.05% and 0.04% respectively. *Nitrobacter winogradskyi* and *Nitrosomonas* sp. were present in the pond in spring at a relative abundance of 0.01%. Nitrospirae species play a significant role in nitrification and some members of the cyanobacteria are capable of fixing nitrogen (Okafor 2007).

Apart from the seasonal changes in temperature, pH is another factor that affects bacterial community structure indirectly by altering the nutrient composition in effluent (Albaggar 2014). During summer, the pH in the pond was usually higher than in winter. Sahni & Yadav (2012) also found a pond pH to be the highest in summer and the lowest in winter. These changes in pH can result in disturbances in the bioavailability of some nutrients, which can make some nutrients limiting for bacterial growth (Albaggar 2014). Albaggar (2014) reported that in 23 streams, in the USA, when the bacterial community structure was similar, at a low pH, Acidobacteria and Actinobacteria were most prevalent, while at high pH, Proteobacteria was the dominant phylum. This confirms that pH affects the bacterial composition and its relative abundance.

Protozoa grazing on bacterial cells and bacteriophage infections are other factors that can result in shifts in the bacterial community structure and its relative abundance. Bacteriophages such as Enterobacteria phage were found in some of the samples. Albaggar (2014) stated that some studies reported changes in the bacterial community structure during summer linked with changes in bacteriophage abundance in the water body. Bacteriophages account for about 40% of bacterial

mortality rates in freshwater and this rate reportedly is higher than that caused by protozoa (Albaggar 2014).

c. Seasonal changes in temperature, algal biomass, chlorophyll a, phaeophytin a, dissolved oxygen, chemical oxygen demand and pH in the high rate algal pond

Seasonal changes in temperature resulted in changes in the algal biomass. The maximum temperature of 25.9℃ was recorded in late summer (February) and the lowest of 12.83℃ was recorded during early winter (June). The highest algal biomass recorded was 0.87 g/L, which was measured in mid-summer and the lowest biomass of 0.10 g/L was observed in mid-winter. Artmann *et al*. (2003) also found the highest algal biomass of 2.60 g of dry matter (DM)/m^2 during summer and the lowest biomass of 1.00 g DM/m^2 in winter. In a HRAP, an increase in temperature enhances microalgae metabolism, which in turn increases algal biomass (Sevrin-Reyssac 1998; Zimmo 2003). Beside temperature, other factors affecting microalgae growth such as solar radiance, day length and zooplankton grazing pressure also vary seasonally (De Pauw & van Vaerenbergh 1983; Barthel *et al*. 2008; Tiseer *et al*. 2008; Chen 2010; Khondker & Abed 2013). During winter, when the temperature, solar PAR and day length are lower than in summer, the growth rate of microalgae decreases (De Pauw & van Vaerenbergh 1983; Assemany *et al*. 2015). A shorter day length deprives algal cells from receiving maximum solar PAR and temperature during the day, which in turn reduces microalgae growth (De Pauw & van Vaerenbergh 1983; Assemany *et al*. 2015). During summer, the longer day length exposes algal cells to sufficient light and temperature, thus a greater biomass is produced (Assemany *et al*. 2015). The gain in algal biomass that was lower than expected from November 2013 to January 2014 was probably due to zooplankton grazing. During this period, microscopy revealed that rotifers were feeding on microalgae. Davis *et al*. (2012) reported that the grazing rates of *Daphnia galeata* and *Daphnia retrocurva* were responsible for about 85% of the grazing rate and this resulted in a decline in algal biomass in the western Lake Erie. The seasonal changes in abiotic and biotic factors affected microalgae growth, with the warmer seasons favouring their growth.

Temperature also influenced the Chl *a* concentration in the algal pond. The highest Chl *a* concentration obtained using trichromatic equation of 1551.36 µg/L was recorded in March and the lowest concentration of 583.24 µg/L was observed in winter (July). Similar trends were observed when the monochromatic equation was used. During summer and winter, the Chl *a* concentrations were 685.63 and 171.61 µg/L respectively. The monochromatic equation underestimated Chl *a* in the presence of Chl *b* while the trichromatic equation overestimated Chl *a* in the presence of Phaeo *a,* hence the concentrations differ although samples were collected at the same time (Dos Santos *et*

al. 2003). Sutherland *et al*. (2014a) reported low Chl *a* concentrations during autumn and winter, and the highest concentrations were recorded during summer in an algal pond used to treat wastewater in New Zealand. Seasonal variation of the Chl *a* concentration in the HRAP was affected by the same factors that affected the algal biomass (Chen 2010; Khondker & Abed 2013). The solar radiance, day length and zooplankton grazing pressure also affected the Chl *a* concentration in the same way that it affected the algal biomass (Chen 2010; Khondker & Abed 2013).

The concentration of Phaeo *a* was influenced by seasonal changes. The highest Phaeo *a* concentration of 1945.41 µg/L was recorded during summer (January) and the lowest concentration of 678.81 µg/L was recorded in winter (July). These findings are comparable to Khondker & Abed (2013) whose investigation on the seasonal variation of phytoplankton in Turag River in Dhaka revealed that the highest Phaeo *a* concentration was also observed during summer and the lowest in winter. Phaeophytin *a* concentration is influenced by zooplankton grazing on microalgae as Chl *a* is converted into Phaeo *a* (Sin *et al*. 2000). Phaeophytin *a* is excreted into the water body in the faecal matter, and when the concentrations are high, this is an indication of high grazing pressure (Sin *et al*. 2000). High grazing pressure during summer coincided with the highest Phaeo *a* concentrations recorded during summer. The concentrations of Phaeo *a* were directly proportional to the concentrations of Chl *a*, when the Phaeo *a* concentration decreased, the Chl *a* concentration also decreased and vice versa. This can be supported by the positive correlation of R = 0.85 between Phaeo *a* and Chl *a* concentrations that was reported by Khondker & Abed (2013).

The standing algal biomass in the HRAP was correlated to the DO concentrations in the pond. There was a positive correlation of R = 0.76 between algal biomass and DO concentrations. Dissolved oxygen concentrations of 6.30$\pm$0.3 and 18.64 mg/L were recorded during winter and summer respectively. Tiseer *et al*. (2008) observed similar trends, where DO concentrations of 4.03$\pm$0.46 and 6.05$\pm$0.51 mg/L were measured during winter and summer respectively. During photosynthesis, microalgae release O_2 into the water body and this contributes to the DO concentrations (Kunlasak *et al*. 2003; Muñoz & Guieysse 2006). The elevated DO concentrations during summer were most likely when high photosynthetic rates due to high temperature and solar radiance PAR prevail (Richmond 2004). Chlorophyll content also influences O_2 production, since each molecule of Chl produces about 50-400 mol of O_2 per hour (Mandalam & Palsson 1998).

Aeration in the HRAP A2 provided by paddle wheels also raises the DO concentrations to the ambient atmospheric concentration (Mishra *et al*. 2011). Also agitation of the pond surface improves the diffusion of O_2 between the water and the air (Fogg 1991; Winder & Sommer 2012).

The COD in the HRAP A2 fluctuated throughout the year, with no clear seasonal trends observed. However, the highest COD was recorded in December and January, which overlaps with the period of elevated algal biomass and Chl *a* concentrations. Kawabe & Kawabe (1997) also observed the highest COD values during the warmer season. Biodegradation of organic material in wastewater results in a decrease in DO concentrations since aerobic bacteria and fungi utilize it (Mishra *et al*. 2011). Decomposition of dead algal cells in HRAPs contributes to the escalating levels of COD in HRAPs (Cilliers 2012). In algal ponds, microalgae use carbon dioxide (CO_2) as an inorganic source of carbon, in turn releasing photosynthetic organic products such as glycolic acid into the water body, which also contributes to escalating levels of COD in HRAPs and this makes it difficult to reduce COD in algal ponds (Wang *et al*. 2009). Total dissolved solids (TDS) may also contribute to the COD in a sense that when TDS in ponds increases, the COD may also rise (Akan *et al*. 2010).

Changes in season had a substantial effect on pH. The lowest pH was recorded in winter and the highest in summer. Sahni & Yadav (2012) also observed more modest differences in winter and summer in pH of 8.54 and 8.92 respectively. The seasonal fluctuations in pH were probably due to the photosynthetic activity of microalgae (Markou & Georgakakis 2011). During photosynthesis, when microalgae sequestrates CO_2 and in turn release hydroxyl (OH^-) ions into the water body, this increases the pH (Richmond 2004). The ability of microalgae to sequestrate CO_2 is influenced by the carbon concentration mechanism (CCM) (Zhang 2015). The CCM present in almost all microalgae overcome the obstacles encountered by microalgae in sequestrating carbon from the aquatic body by concentrating CO_2 and bringing it close to the carbon fixing enzyme RuBisCO (Zhang 2015). And the enzyme carbonic anhydrase (CA) breaks down CO_2 and HCO_3^-, thus making carbon available for algal assimilation (Moroney *et al*. 2001; Zhang 2015). However, a constant increase in CO_2 in the water, which decreases the pH may inhibit CA activity (Zhang 2015). Also the lack of a buffering system in the HRAP could have contributed to pH fluctuations.

d. The removal of nitrogen from the anaerobically digested brewery effluent

Seasonal variation affected the removal efficiency of NH_4^+-N from the post-AD brewery effluent. The removal efficiency of NH_4^+-N was 99% during summer, which then decreased to 93% in winter. Sevrin-Reyssac (1998) also reported a decrease in the NH_4^+-N removal efficiency during winter when algal ponds were used to treat swine effluent. This finding is also comparable to the study of Sutherland *et al*. (2014a) where the highest NH_4^+-N removal efficiency of 79% was in summer and then decreased to 77% during spring and further decreased to 53% in winter. Cleaning chemicals with NH_3 conent used at the brewery contributed to the fluctuation of NH_4^+-N concentrations in the effluent and the degradation of organic matter (Heyneke, *pers. comm.*, water quality process

specialist, SAB Ltd, Port Elizabeth, November 2014). Aerobic *Bacillus* spp. found in the pond could have contributed to the mineralization of the nitrogenous organic compounds in the effluent into NH_4^+-N. Previous studies by Mansergh & Zehr (2014) have suggested that these species are capable of degrading nitrogenous organic compounds into NH_4^+-N. The group of the unknown bacterial species could have also been composed of species capable of degrading nitrogenous organic matter in the effluent into NH_4^+-N. One of the mechanisms responsible for the removal of NH_4^+-N from the effluent is algal assimilation (Sevrin-Reyssac 1998; Zimmo 2003). When a greater portion of NH_4^+-N was removed from the effluent, there were more microalgae taxa with a greater algal biomass in the pond. When the NH_4^+-N removal efficiency decreased, the microalgae taxa and the associated algal biomass were minimal. This could imply that a greater algae biomass was responsible for assimilating the NH_4^+-N than the reduced biomass during winter. It is also known that during summer, microalgae metabolism is greater than in winter (Sevrin-Reyssac 1998; Zimmo 2003).

Another factor that contributed to the loss of NH_4^+-N was the volatilization of the unionised ammonia-nitrogen (NH_3-N) (Makarewicz & Lampman 1994; Sevrin-Reyssac 1998). When the pH is above nine in a HRAP, this results in the loss of NH_3-N mainly through volatilization rather than microalgae assimilation (Sevrin-Reyssac 1998; Zimmo 2003). Meisinger & Jokela (2000) reported that when the pH is greater than nine and the temperature is close to the freezing point at a laboratory scale, greater than 40% of the total NH_3-N can be stripped off into the atmosphere in two days. It was predicted that as the temperature increases, the loss of NH_3-N through volatilization also increases by a factor of three for every 10°C increase (Meisinger & Jokela 2000). The volatilization of NH_3-N is further enhanced by air currents above the water surface, which increases the transfer of air between the pond and the atmosphere (Meisinger & Jokela 2000). Mixing in HRAP provided by paddle wheels, also improves volatilization by increasing the surface area through elongation of the water surface (Fogg 1991; Meisinger & Jokela 2000).

Nitrification is also one of the mechanisms of NH_4^+-N removal from wastewater (Khin & Annachhatre 2004). During nitrification, NH_4^+-N is oxidized to NO_2^--N, and then to NO_3^--N, provided the temperature of the effluent is above 15°C and the pH is below eight to support the growth of nitrifiers (Khin & Annachhatre 2004). The *Nitrosomonas* sp. found in the pond at a relative abundance of 0.01% during spring could have oxidized NH_4^+-N into NO_2^--N. Some of the nitrifying bacteria could have been incorporated in the unknown bacterial species category. However, nitrification is limited in microalgae and bacteria consortia ponds due to the slow growth rate of nitrifiers (Babu 2011). The nitrifiers are usually washed out of the pond and this reduces their population biomass to low levels (Babu 2011). In some instances, when the intention is to improve

nitrification in ponds, attachment surfaces can be incorporated in the pond to allow nitrifiers to grow sufficiently (Babu 2011).

The concentrations of NO_2^--N and NO_3^--N in the post-AD brewery effluent were also affected by seasonal variation. The removal efficiency of NO_2^--N in mid-winter was less than 60% while in mid-summer, it was greater than 70%. Kumar *et al*. (2014) also observed a high removal efficiency of NO_2^- in Lahru pond, in India during summer, when the lowest concentrations of NO_2^- were recorded. The concentrations of NO_3^--N were greater in winter than in summer. Khondker & Abed (2013) reported the opposite of this, when the highest NO_3^--N concentrations in Turag River in Bangladesh were recorded during summer. Sevrin-Reyssac (1998) found that during winter, there was no removal of NO_3^--N from swine effluent treated in algal ponds. In the HRAP A2, nitrite oxidizing bacteria (NOB) such as *Nitrospira* sp. and *Nitrobacter winogradskyi* were observed during winter and spring. This could imply that the decrease in NO_2^--N was due to its oxidation into NO_3^--N, thus the increase in NO_3^--N concentrations. Some of the NO_3^--N in summer could have been reduced to free nitrogen gas by the denitrifiers *Pseudomonas aeruginosa* and *Paracoccus* sp. that were seen during spring and summer, thus the lower NO_3^--N concentrations that were measured in summer than in winter. Denitrification does not only occur in an anaerobic environment, it can also occur in aerobic conditions, where NO_3^--N is reduced by aerobic denitrifiers such as *Paracoccus denitrificans* (Babu 2011). *Paracoccus denitrificans* can reduce NO_3^--N even in an O_2 saturated environment (Babu 2011). *Pseudomonas* spp. are aerobic nitrifiers, also capable of reducing NO_3^--N into nitrogen gas (Babu 2011). However, some researchers argue that denitrification in HRAPs should be considered less significant because HRAPs are aerobic environments (Larsdotter 2006a).

Some of the significant bacterial species in the nitrogen cycle found in the pond were the nitrogen fixing bacteria such as the aerobic *Rhizobium* sp., facultative anaerobic *Klebsiella* sp., anaerobic *Clostridium* sp. and cyanobacteria, which are known to fix nitrogen from the atmosphere (Bitton 2005). The anaerobic and facultative anaerobic bacteria could have originated from the inflowing effluent from the anaerobic digester and the facultative pond.

e. The removal of phosphorus from the anaerobically digested brewery effluent

The removal efficiency of PO_4^{3-}-P was not affected by seasonal changes. The maximum concentrations of PO_4^{3-}-P of 21.23 and 19.67 mg/L were noted in spring (October) and late summer (February) respectively. Khondker & Abed (2013) reported the highest PO_4^{3-}-P concentration of 405.25 µg/L during summer, which also indicated no seasonal trends in PO_4^{3-}-P removal as warmer seasons are expected to have a higher PO_4^{3-}-P removal efficiency than cooler seasons. Aerobic

bacterial species from the Actinobacteria found in the HRAP are presumed to have mineralized phosphorus containing compounds. The studies of Bitton (2005) and Mansergh & Zehr (2014) reported that Actinobacteria species are known to biodegrade phosphorus containing compounds. Some of the bacterial species in the unknown category could have also degraded phosphorus containing organic matter in the effluent into PO_4^{3-}. The removal efficiency of PO_4^{3-}-P from the effluent varied extensively even when the pH ranged between 10 and 10.84, an indication that there was no clear relationship between pH and PO_4^{3-}-P removal in this system. This implies that PO_4^{3-}-P removal from the post-AD brewery effluent was not through precipitation, but rather attributed to algal assimilation. Kim *et al*. (2014) also observed inconsistent PO_4^{3-}-P removal efficiencies from wastewater and no precipitation, yet the pH was between 10 and 10.6. When there were more algal taxa with a greater biomass during summer and low algal taxa diversity with a lower biomass in winter, the removal efficiency of PO_4^{3-}-P did not differ. There was no link between the algal biomass and removal of PO_4^{3-}-P. Algal species such as *Chlorella vulgaris* and *Scenedesmus dimorphus* tend to assimilate phosphorus as a luxury when it is in excess in effluent and store it as polyphosphate granules (Griffiths 2010). The excess phosphorus gets reserved, and utilized at a later stage when there is a depletion of phosphorus in the effluent (Larsdotter 2006a). The removal of PO_4^{3-}-P from the post-AD brewery appeared to be solely through algal assimilation.

f. Ionic composition of the anaerobically digested brewery effluent

Chloride and pH affected the EC of the brewery effluent. The highest EC was recorded in summer while the lowest was recorded in winter. Sahni & Yadav (2012) also observed high EC values during summer and low EC values during winter in algal ponds treating diary effluent. The Cl^- concentrations in the effluent were influenced by chlorine containing chemicals used as pasteurizers, water softener and conveyor lubricants at the brewery. Hydrochloric acid used to correct the pH in the brewery's anaerobic digester also contributed to escalating Cl^- concentrations (Heyneke, *pers. comm*., water quality process specialist, SAB Ltd, Port Elizabeth, November 2014). The Cl^- and pH are known to directly affect the EC of the effluent, the EC would increase with an increase in Cl^- and pH, and vice versa (Sahni & Yadav 2012). In this study, Cl^- concentration and EC had no trends, which might imply that the Cl^- did not influence the EC. The increase in EC in summer and spring (September) was probably due to an increase in evaporation rates, since evaporation concentrates the effluent (Cilliers 2012).

3.5 Conclusion

Seasonal changes altered the biotic, abiotic and chemical parameters in the HRAP. There was a shift in the microalgae community composition and their relative abundance, with more algal species observed during summer, while fewer species were noticeable during winter. There was also a shift in the bacterial community structure and the relative abundance of the species and groups involved. Temperature influenced algal growth, leading to greater algal biomass and Chl *a* concentration when temperature was maximal in summer. During winter, microalgae growth was compromised. Similar trends were observed with the Phaeo *a* concentration. Other abiotic water variables such as the pH of the effluent and DO concentrations corresponded with the standing algal biomass, i.e. a positive correlation between algal biomass and DO.

Ammonium-nitrogen removal efficiency was greater in summer and lower in winter. This was linked to an increase in the algal biomass and Chl *a* concentration during summer, thus the greater nutrient removal efficiency. Even the NH_4^+-N oxidizing bacteria *Nitrosomonas* sp. documented in the pond during spring could have contributed to the removal of NH_4^+-N from the effluent. Nitrite-nitrogen and NO_3^--N concentrations were greater in the cooler season when compared to the warmer season. This can be associated with the presence of the NOB *Nitrospira* sp. that was observed in winter and spring, and *Nitrobacter winogradskyi* which was present in the pond during spring. The removal efficiency of PO_4^{3-}-P had no apparent seasonal trends. The HRAP was not efficient in the reduction of COD from the effluent to acceptable levels for effluent discharge into a natural environment. The increase in algal biomass coincided with the highest mean COD recorded in summer (January), however, there were no clear seasonal trend in the COD. The Cl^- concentration showed no seasonal trends, however, the EC of the effluent was higher during summer than in winter. Possibly this was due to an increase in evaporation which elevates TDS in water (Cilliers 2012). Although NH_4^+-N was lowered well within the discharge limits, other parameters such PO_4^{3-}-P, COD and EC were not always lowered to within the discharge limits. The HRAP system's efficiency was interrogated in detail by Jones *et al*. (2014) and perhaps an additional process step is required to improve the quality of the effluent discharged.

The outcomes of this study clearly demonstrated that summer favoured algal productivity and the performance of the HRAP in removing NH_4^+-N from the effluent. However, during winter, algal productivity and nutrient removal efficiency were substantially reduced. This might have drawbacks if the intention is to simultaneously produce algal biomass and treat effluent throughout the year. However, there are certain interventions that can be put in place to make up for the decrease in

algal biomass and nutrient removal capacity during winter (Larsdotter 2006a; Muñoz & Guieysse 2006).

Different effluent flow rates can be used to manipulate the hydraulic retention time (HRT) of the pond (Larsdotter 2006a). A shorter HRT of about three days can be used in summer while a longer HRT of about seven days can be used during winter (Larsdotter 2006a; Muñoz & Guieysse 2006). At these recommended HRTs, a HRAP can sustainably produce high yields of algal biomass throughout the year and efficiently remove nutrients from wastewater (Larsdotter 2006a; Muñoz & Guieysse 2006; Sutherland *et al.* 2014b).

Chapter four

The microbial community structure and physicochemical parameters in high rate algal ponds at different inflowing effluent flow rates

4.1 Introduction

Different effluent flow rates are used when operating a high rate algal pond (HRAP) in order to manipulate the hydraulic retention time (HRT) of the effluent in the pond (Larsdotter 2006b). A pond's HRT refers to the measure of the time liquids and their suspended solids remain in the pond (Larsdotter 2006a; Muñoz & Guieysse 2006). It is calculated as the volume of the pond over the flow rate of influent (Babu 2011; Cilliers 2012). The most commonly used HRT is between two to seven days, which is influenced by a number of factors such as the season the HRAP is operated in and the volume of effluent that needs to be treated (Larsdotter 2006b; Muñoz & Guieysse 2006). A shorter HRT is desirable in summer while a longer HRT is recommended in winter (Larsdotter 2006b; Muñoz & Guieysse 2006).

In a HRAP, the HRT is a vital operational factor that affects the microbial community structure and its standing biomass (Babu 2011; Sutherland *et al.* 2014b). A longer HRT provides microalgae and bacteria sufficient time to grow, reduces the chances of washout from the HRAP, and increases their biomass (Babu 2011; Sutherland *et al.* 2014b). The HRT also influences nutrient loading into the system, and thus affects the water quality of the post-HRAPs treated effluent (Babu 2011; Sutherland *et al.* 2014b). In instances where the HRT is prolonged, this provides microalgae with sufficient time to assimilate nutrients from the effluent (Sutherland *et al.* 2014b). Some HRTs are better suited to optimise algal biomass production, while some HRTs are suitable for the efficient treatment of large volumes of effluent (Sutherland *et al.* 2014b). The choice of which HRT to use will be based on the goal that needs to be achieved (Sutherland *et al.* 2014b). A short HRT, provided it is more than 24 h, favours the growth of microalgae in a sense that their cells are kept in a logarithmic growth phase (Medina & Neis 2007). However, when the HRT is prolonged, algal cells end up reaching a steady state, however, in some instances, they can continue to multiply, but at a very slow rate (Medina & Neis 2007). On the contrary, a long HRT enhances the removal of nutrients from effluent rather than a shorter HRT (Medina & Neis 2007). An understanding and knowledge of how different HRTs affect the HRAP system improves biomass production and nutrient removal efficiencies (Babu 2011).

This study maximized the volume of effluent treated daily, thus reducing the HRT since the brewery's goal is to treat more effluent daily with fewer costs involved. The focus was on the operational process of the HRAP system, i.e. the HRT and the effects HRT has on nutrient removal and the microbial community. This was a descriptive study that compared the microbial community structure and effluent's characteristics in two ponds. One pond was maintained at a constant flow rate while in the second pond, the flow rate was progressively increased. It was not possible to replicate these treatments so the conclusions that were drawn here are limited to the description.

Aim and objectives

The purpose of this study was to describe the effects of different effluent flow rates on the microbial community structure and the removal of nutrients from post-anaerobically digested (post-AD) brewery effluent in the HRAPs. The specific objectives were to:

- establish the identity of microorganisms present in the HRAPs at different effluent flow rates using light microscopy and metagenomics study;
- describe the effects of different flow rates on the microbial community structure in HRAPs; and
- describe the effects of different flow rates on nutrient removal from post-AD brewery effluent in the HRAPs.

4.2 Materials and methods

The flow rate of post-primary facultative pond (post-PFP) effluent into the HRAP A train was maintained at 1000 L/d, while that into HRAP B train was progressively increased over 21 days in spring (Figure 4.1). The HRAP A and HRAP B trains were parallel to each other, with each train of the pond consisting of two ponds in series, i.e. HRAP A (effluent flowed from HRAP A1 into HRAP A2 in series) and HRAP B (HRAP B1 effluent flowed into HRAP B2) (Chapter 3, Section 3.2.1.a). Samples were collected from the post-AD, post-PFP, post-HRAP A2 and post-HRAP B2 between 09:00 and 10:00 am at the same time intervals. Effluent flowed through HRAP B2 at 1000 L/d for seven days before data were collected for the first time on day zero. After the collection of samples on day zero, the effluent flow rate was subsequently increased to 1400 L/d and samples were collected on day seven. The flow rate was then increased to 1800 L/d from day seven, and samples were collected on day 14. From 1800 L/d, the effluent flow rate was increased to 2200 L/d and the samples were collected on day 21.

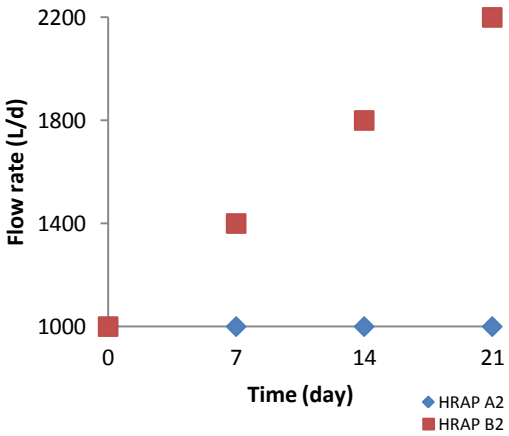

Figure 4.1 The flow rate of post-primary facultative pond (post-PFP) effluent into the parallel high rate algal ponds (HRAP A2 and HRAP B2).

4.2.1 Microbiological and water quality parameter analysis

Water samples (100 mL) were collected from the HRAP A2 and HRAP B2, stored in sterile containers, kept in a cooler box with ice and transported to Rhodes University (RU), Department of Ichthyology and Fisheries Science (DIFS) general laboratory for analysis. Deoxyribonucleic acid (DNA) samples of both the microalgae and bacteria were collected from samples collected at 1000 and 2200 L/d from HRAP B2 to describe the profile of the microbial community at the start of the experiment and when the system crashed (inflow rate was greater than the algae population growth rate resulting in the algae population being washed out of the HRAP). The identity of microalgae using a light microscope was established from samples collected in the HRAP A2 and HRAP B2 at seven days intervals. Water samples were collected for the analysis of ammonium-nitrogen (NH_4^+-N), nitrite-nitrogen (NO_2^--N), nitrate-nitrogen (NO_3^--N), orthophosphate-phosphorus (PO_4^{3-}-P), chemical oxygen demand (COD), chloride (Cl^-), temperature, pH, electrical conductivity (EC), dissolved oxygen (DO), chlorophyll (Chl) *a* and Phaeophytin (Phaeo) *a* concentrations at seven days intervals. The analysis of these parameters was carried out using the materials and methods described in (Chapter 3, Section 3.2.2, Section 3.2.3 and Section 3.2.4).

4.2.2 Statistical analysis

Some of the data collected from the HRAP A2 and HRAP B2 were described using a linear regression model (Statisica, version 12) and graphs were generated using Microsoft Excel (Version 2007). The slope of the linear regression model was determined at $p \leq 0.05$. The R^2-value indicated the closeness of the data to the fitted regression line.

4.3 Results

4.3.1 The microbial community structure in high rate algal ponds at different effluent flow rates

An increase in the flow rate of effluent from 1000 to 2200 L/d resulted in shifts in the microalgae species composition and abundance in the HRAP A2 and HRAP B2 (Table 4.1, Figure 4.2, Table 4.2). The bacterial community composition and abundance in the HRAP B2 also shifted as the flow rate of post-AD brewery effluent into it was progressively increased (Figure 4.3).

a. Identification of the microalgae community structure using light microscopy

When the flow rate of effluent into the HRAP A train and HRAP B train was 1000 L/d, the HRAP A2 and HRAP B2 had the same microalgae taxa, however, when the flow rate of effluent into the HRAP B was increased to 1400 L/d, *Pediastrum* sp. disappeared in the HRAP B2, whereas it remained in HRAP A2 with a flow rate of 1000 L/d (Table 4.1). When the flow rate of effluent into the HRAP B was further increased to 1800 L/d, even *Arthrospira* sp. disappeared in the HRAP B2, whereas it remained in HRAP A2 with a flow of 1000 L/d. At a flow rate of 2200 L/d, *Scenedesmus* sp. and *Chlorella* sp. were the only taxa observed in the HRAP B2, whereas all the taxa remained in HRAP A2 with a flow rate of 1000 L/d.

Table 4.1 Microalgae species in high rate algal ponds (HRAP A2 and HRAP B2) with post-primary facultative pond (post-PFP) effluent flowing into HRAP A at a rate of 1000 L/d while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d over 21 days. Algal cells were identified using light microscopy.

	HRAP A2	**HRAP B2**	**HRAP A2**	**HRAP B2**	**HRAP A2**	**HRAP B2**	**HRAP A2**	**HRAP B2**
	1000 L/d	1000 L/d	1000 L/d	1400 L/d	1000 L/d	1800 L/d	1000 L/d	2200 L/d
Scenedesmus sp.	√	√	√	√	√	√	√	√
Chlorella sp.	√	√	√	√	√	√	√	√
Diatoma sp.	√	√	√	√	√	√	√	
Chlamydomonas sp.	√	√	√	√	√	√	√	
Microcystis sp.	√	√	√	√	√	√	√	
Phormidium sp.	√	√	√	√	√	√	√	
Synechococcus sp.	√	√	√	√	√	√	√	
Pediastrum sp.	√	√	√		√		√	
Arthrospira sp.	√	√	√	√	√		√	

b. Identification of the microalgae community structure using metagenomics

At an effluent flow rate of 1000 L/d into HRAP B train, *Nanofrustulum shiloi* was the most common identifiable algal species with a relative abundance of 3.38%, followed by *Scenedesmus abundans* with a relative abundance of 2.82% in the HRAP B2 (Figure 4.2). Other microalgae species with a relative abundance of 0.01 to 0.15% were also present in the HRAP B2 (Figure 4.2, Table 4.2). At an

effluent flow rate of 2200 L/d, in the HRAP B2, *Scenedesmus subspicatus* and *Chlorella vulgaris* were the only microalgae identified with a relative abundance of less than 0.03%. The relative abundance of unknown eukaryotes was 69.49% at 1000 L/d, which decreased to 9.13% at 2200 L/d.

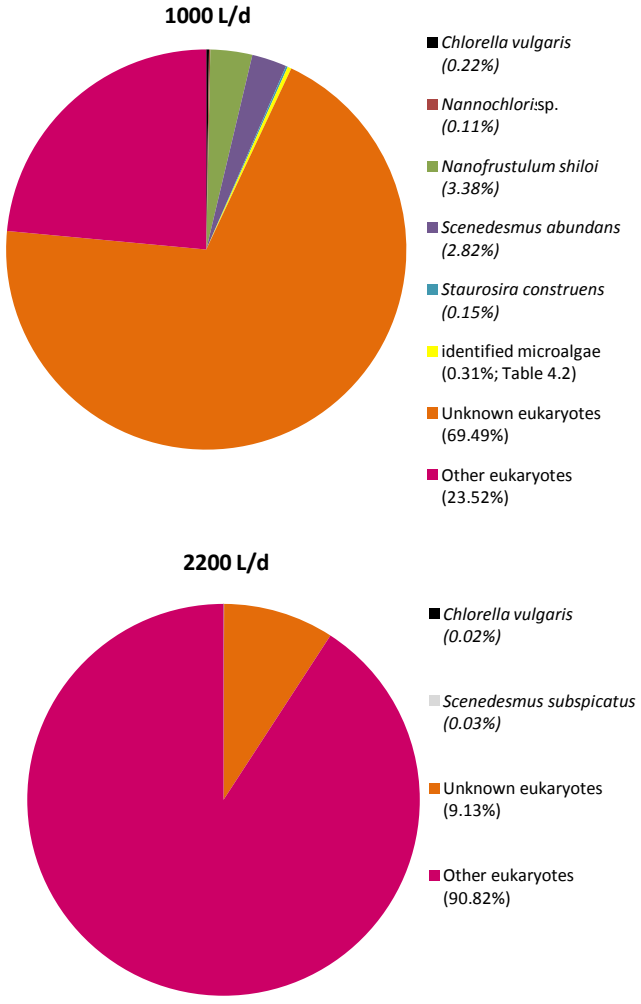

Figure 4.2 Microalgae species and the relative abundance as percentage (%) in a high rate algal pond (HRAP B2) with post-primary facultative pond (post-PFP) effluent flowing into the HRAP B train at a flow rate of 1000 L/d and 2200 L/d identified using metagenomics. Other eukaryotes refer to non-algal species that were also amplified with the universal primer pair 566F and 1200R.

Table 4.2 Microalgae species and the relative abundance as percentage (%) in a high rate algal pond (HRAP B2) grouped under the identified microalgae category at 1000 L/d in the pie chart (Figure 4.2).

Microalgae species	1000 L/d (%)
Amphidinium carterae	0.01
Amphora montana	0.01
Chaetophora incrassata	0.01
Chaetosphaeridium globosum	0.01
Chlamydomonas sp.	0.02
Choricystis sp.	0.01
Corethron inerme	0.02
Nannochloropsis salina	0.01
Nitzschia amphibia	0.01
Pycnococcus sp.	0.01
Oocystis sp.	0.06
Scenedesmus costato-granulatus	0.03
Scenedesmus subspicatus	0.01
Spinoclosterium cuspidatum	0.01
Symbiodinium sp.	0.01
Thalassiosiraceae	0.06
Volvox sp.	0.01

c. Identification of the bacterial community structure using metagenomics

The phyla Firmicutes, Proteobacteria, Planctomycetes and Cyanobacteria were observed in the HRAP B2 at a flow rate of 1000 and 2200 L/d (Figure 4.3). At a flow rate of 1000 L/d, the relative abundance of Firmicutes were 5.23%, which was lower than 43.13% that was observed at 2200 L/d. Proteobacteria relative abundance was also lower at 1000 L/d at a relative abundance of 22.93%, while at 2200 L/d, their relative abundance was 35.97%. The relative abundance of Planctomycetes at 1000 L/d was 4.88% which was higher than that of 0.82% that was observed at 2200 L/d. Cyanobacteria decreased from 12.84% at 1000 L/d to 0.89% at 2200 L/d. The phyla Bacteroidetes and Chloroflexi were observed at 1000 L/d while Actinobacteria and Euryarchaeota were only observed at 2200 L/d. At the flow rate of 1000 L/d, 37.65% of the bacterial community structure were unknown species, while at 2200 L/d, 18.05% of the bacterial species were not identified.

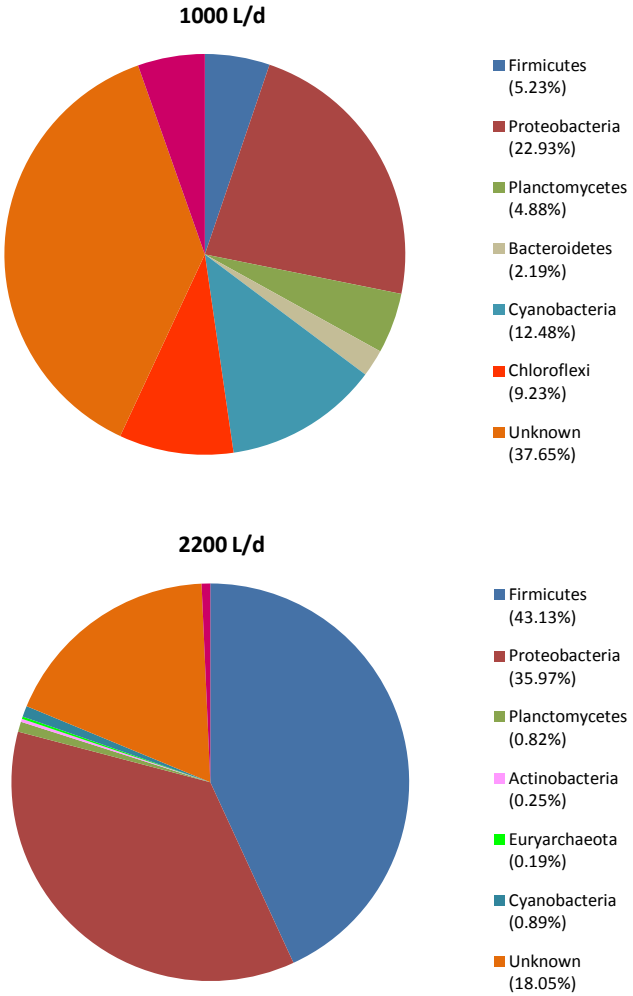

Figure 4.3 The bacterial community structure at a phylum level and the relative abundance as percentage (%) with post-primary facultative pond (post-PFP) effluent flowing into HRAP B2 at a rate of 1000 L/d and 2200 L/d.

4.3.2 Microalgae biomass, chlorophyll *a* and phaeophytin *a* concentrations

The algal biomass in HRAP A2 (1000 L/d) increased, while in HRAP B2 (1000-2200 L/d), the biomass decreased over the period of the experiment (Figure 4.4). Similar trends were observed with the Chl *a* concentration calculated using the trichromatic equation. The Chl *a* concentration increased from 989.33 to 1295.32 µg/L in HRAP A2 where flow rate remained constant while in HRAP B2, it decreased from 1088.91 µg/L to zero as the flow rate increased (Figure 4.5). When the monochromatic equation was used, the Chl *a* concentrations followed similar trends to that obtained when using the trichromatic equation (Figure 4.6). In the HRAP B2, the Chl *a* concentration decreased from 320.76 µg/L to zero. Phaeophytin *a* concentration remained constant, ranging from 1284.65 to 1457.05 µg/L in the HRAP A2 where the flow rate was 1000 L/d, whereas, in the HRAP B2, the concentration decreased from 1220.17 µg/L to zero when the flow rate was progressively increased (Figure 4.7).

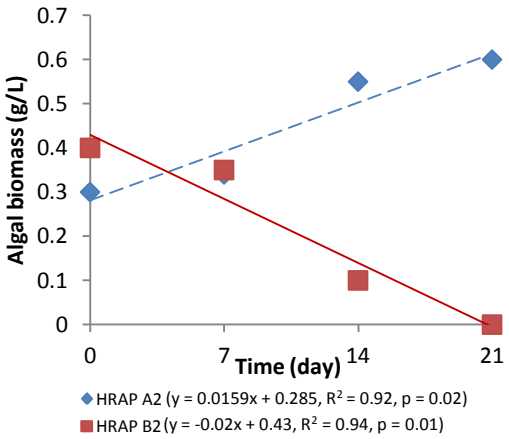

Figure 4.4 Algal biomass in high rate algal ponds (HRAP A2 and HRAP B2) with post-primary facultative pond (post-PFP) effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

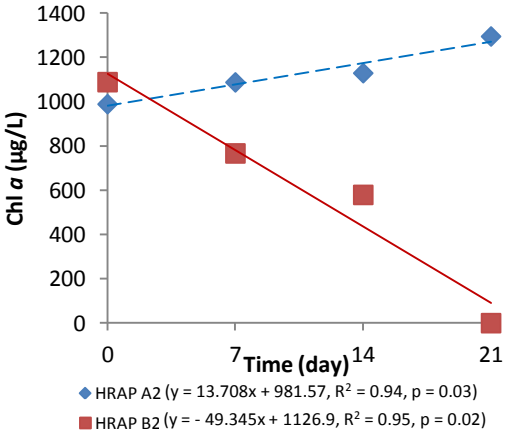

Figure 4.5 Chlorophyll (Chl) *a* concentration determined using a trichromatic equation in high rate algal ponds (HRAP A2 and HRAP B2) with post-primary facultative pond (post-PFP) effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

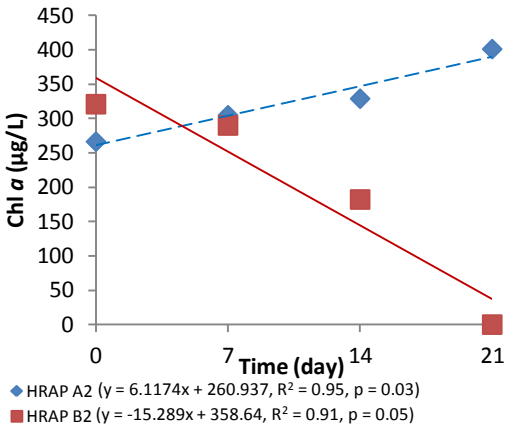

Figure 4.6 Chlorophyll (Chl) *a* concentration determined using a monochromatic equation in high rate algal ponds (HRAP A2 and HRAP B2) with post-primary facultative pond (post-PFP) effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

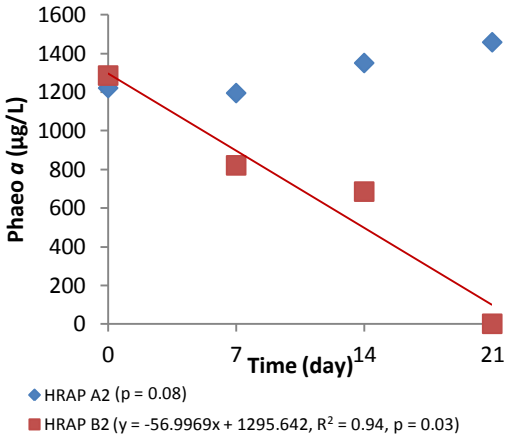

Figure 4.7 Phaeophytin (Phaeo) *a* concentration determined using a monochromatic equation in high rate algal ponds (HRAP A2 and HRAP B2) with post-primary facultative pond (post-PFP) effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

4.3.3 Water quality parameter analysis

Physical characteristics of the anaerobically digested brewery effluent

The temperature was on average about two degrees celsius lower in the HRAPs compared with the temperature in the post-AD effluent and PFP (Figure 4.8). The increase in flow rate did not appear to influence the temperature in the HRAPs.

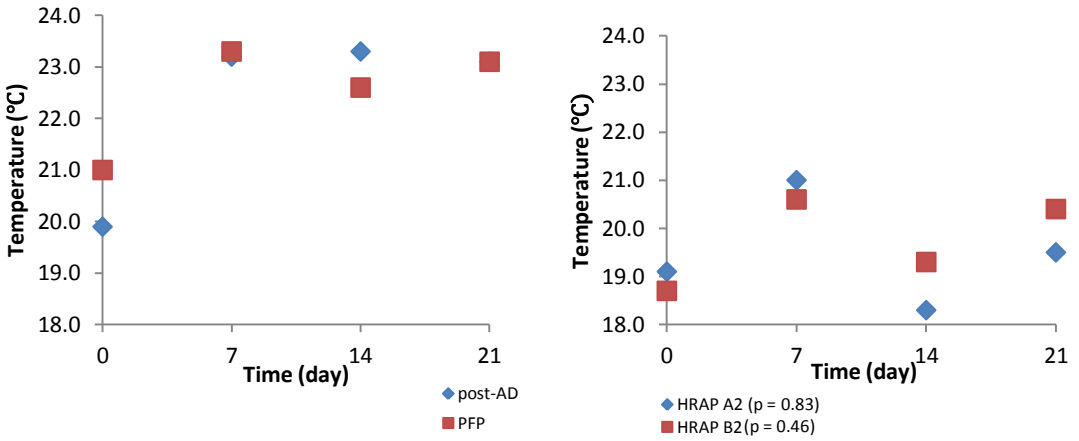

Figure 4.8 Temperature value in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal ponds (HRAP A2 and HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

Chemical characteristics of the anaerobically digested brewery effluent

The DO concentrations in the post-AD effluent and the PFP were lower than in the HRAPs (Figure 4.9). In both HRAPs, the DO concentrations were constant, even when the effluent flow rate in HRAP B2 was increased. The COD in the post-AD effluent was lower than in the post-PFP effluent on a number of occasions (Figure 4.10). In the post-HRAP A2 where effluent entered at a constant flow of 1000 L/d, the COD increased significantly over the 21 day trial ($p = 0.05$, Figure 4.10), while in post-HRAP B2 effluent, the COD remained constant over the same period as the flow rate increased from 1000 to 2200 L/d. The pH of the post-AD and PFP effluent was lower than that recorded in the HRAPs (Figure 4.11). In the HRAP A2 and HRAP B2, the pH did not differ.

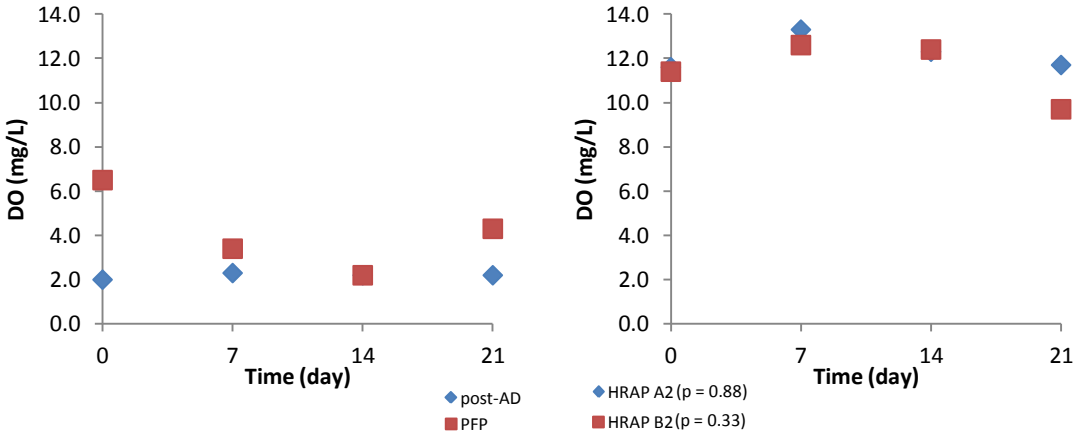

Figure 4.9 Dissolved oxygen (DO) concentration in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal ponds (HRAP A2 and HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

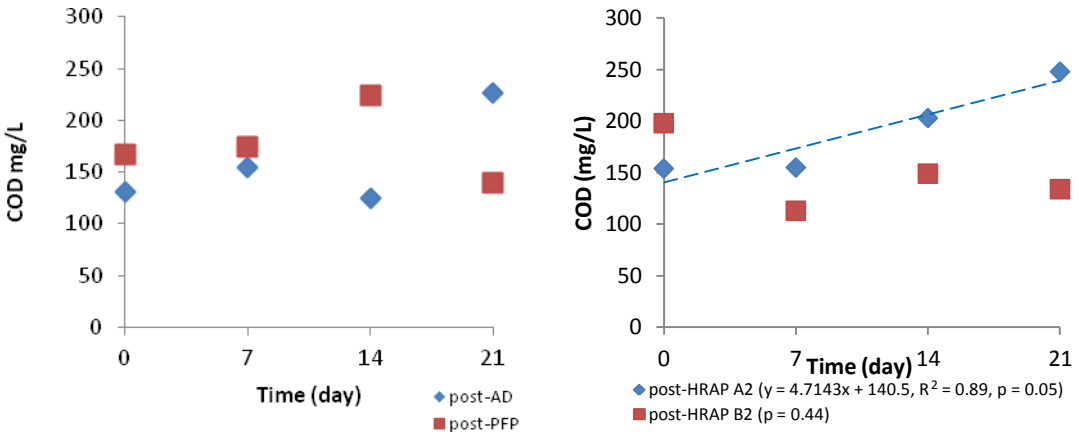

Figure 4.10 Chemical oxygen demand (COD) in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal ponds (post-HRAP A2 and post-HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

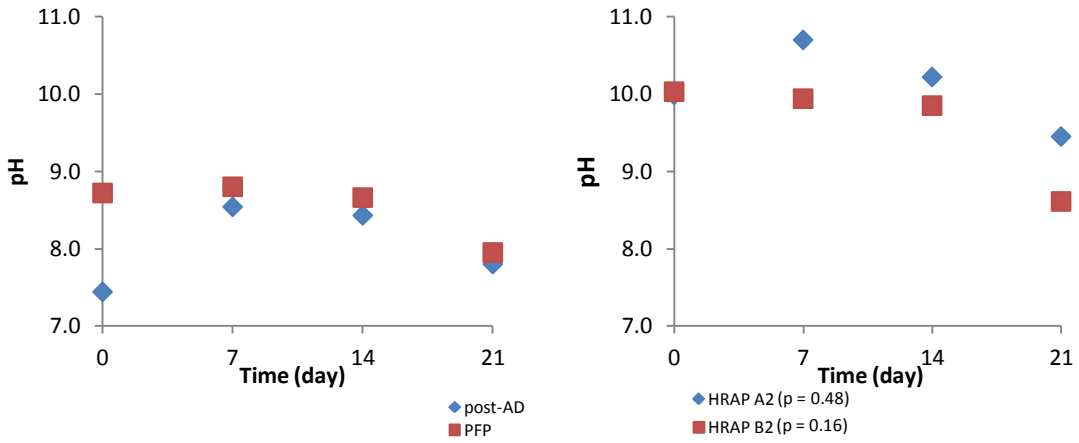

Figure 4.11 The pH values in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal ponds (HRAP A2 and HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

The concentration of NH_4^+-N decreased from the post-AD effluent into the post-PFP effluent (Figure 4.12). Ammonium-nitrogen concentrations were further decreased in the post-HRAPs effluent. The NH_4^+-N concentrations remained constant in the HRAP A2, which was operated at 1000 L/d. In the HRAP B2 where the effluent flow rate was increased, the NH_4^+-N concentrations were also constant at about 0.5 mg/L at a flow of 1000 to 1800 L/d, but increased to about 70 mg/L when effluent flow was increased to 2200 L/d (Figure 4.12). Nitrite-nitrogen concentrations decreased from the post-PFP effluent to the post-HRAPs effluent (Figure 4.13). The NO_2^--N concentration increased significantly when the flow rate was increased in HRAP B2 ($p = 0.02$, Figure 4.13), whereas there was no significant change in NO_2^--N concentration when the flow was maintained constant at 1000 L/d.

The NO_3^--N concentrations also decreased from the post-PFP effluent to the post-HRAP B2 effluent where the flow rate was increased from 1000 to 2200 L/d, however, there was no significant difference in NO_3^--N removal (Figure 4.14). In the HRAP A2 maintained at 1000 L/d, the concentrations of NO_3^--N increased significantly over time ($p = 0.04$, Figure 4.14). Concentrations of PO_4^{3-}-P decreased from the post-AD into the post-PFP effluent (Figure 4.15). The PO_4^{3-}-P concentrations further decreased in the HRAPs. In the post-HRAP B2, the concentrations of PO_4^{3-}-P appeared to follow the concentrations of PO_4^{3-}-P from the incoming post-PFP effluent. The removal of PO_4^{3-}-P in the HRAP B2 decreased significantly with an increase in the effluent flow rate ($p = 0.01$, Figure 4.15), while in the HRAP A2, the concentration of PO_4^{3-}-P remained constant.

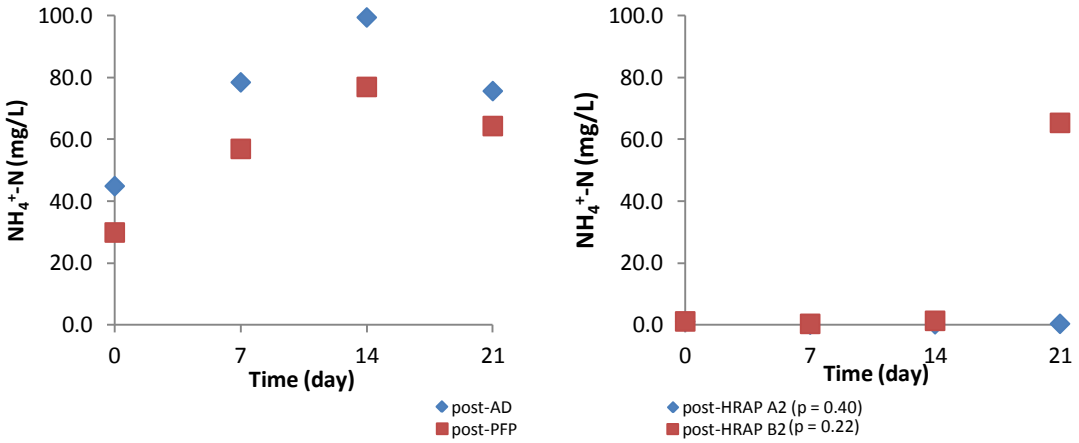

Figure 4.12 Ammonium-nitrogen (NH_4^+-N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal ponds (post-HRAP A2 and post-HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

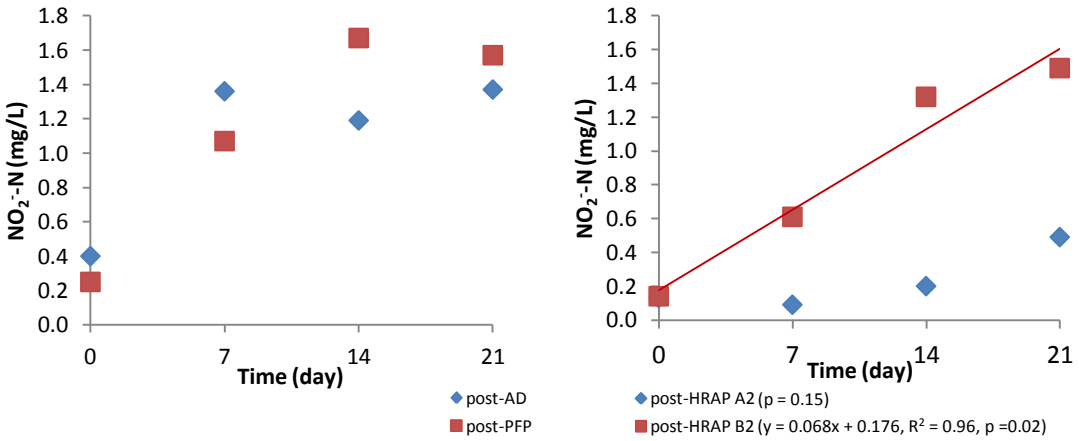

Figure 4.13 Nitrite-nitrogen (NO_2^--N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal ponds (post-HRAP A2 and post-HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

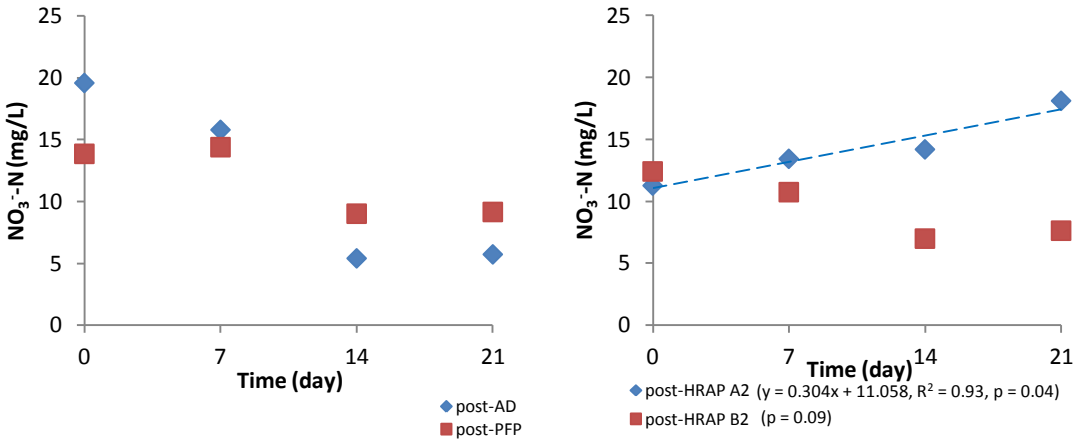

Figure 4.14 Nitrate-nitrogen (NO_3^--N) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal ponds (post-HRAP A2 and post-HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

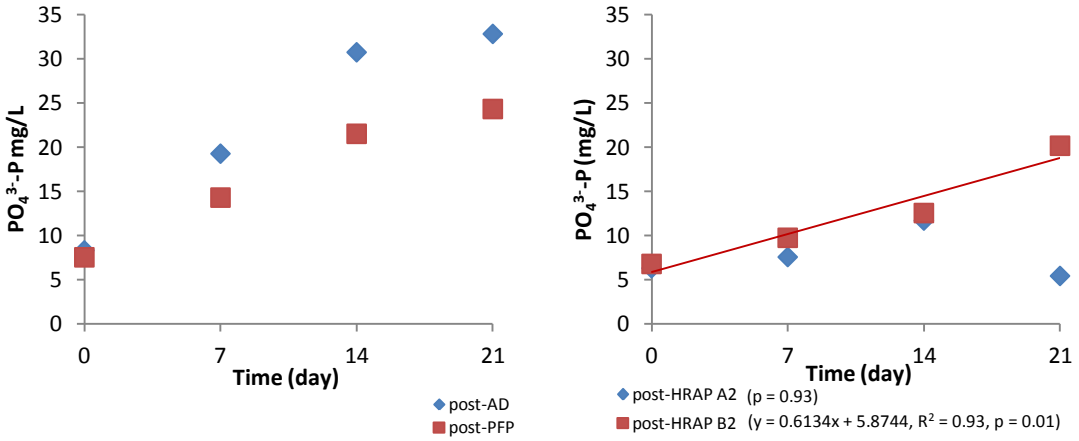

Figure 4.15 Phosphate-phosphorus (PO_4^{3-}-P) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal ponds (post-HRAP A2 and post-HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

The Cl^- concentrations were similar in the HRAP A2 effluent operated at 1000 L/d and in the HRAP B2 where flow rates progressively increased (Figure 4.16). In the post-PFP effluent, the EC was higher than in the post-AD effluent (Figure 4.17). The EC increased when effluent moved through the system into the HRAPs, but decreased significantly with an increase in flow rate in the HRAP B2 (p = 0.04, Figure 4.17), whereas this decrease was not apparent when flow was maintained at 1000 L/d (Figure 4.17).

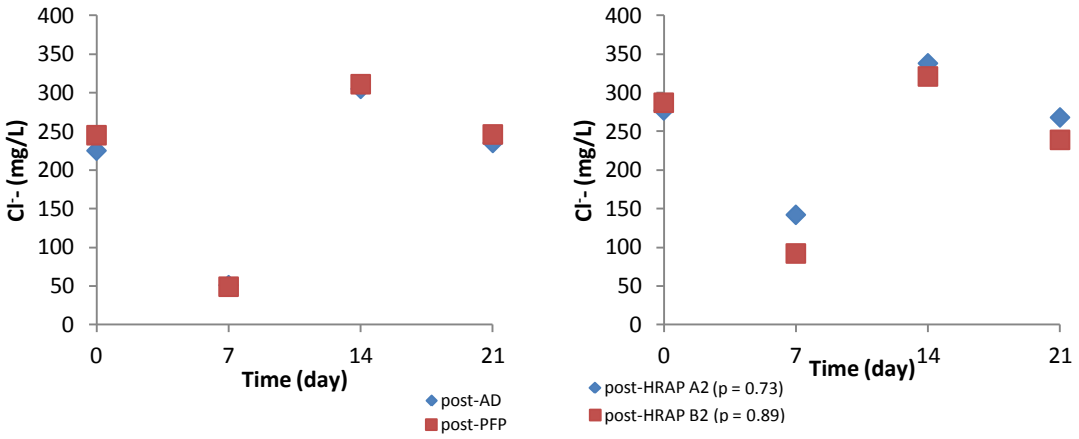

Figure 4.16 Chloride (Cl^-) concentration in post-anaerobically digested (post-AD) brewery effluent, post-primary facultative pond (post-PFP) effluent and post-high rate algal ponds (post-HRAP A2 and post-HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a rate of 1000 L/d, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

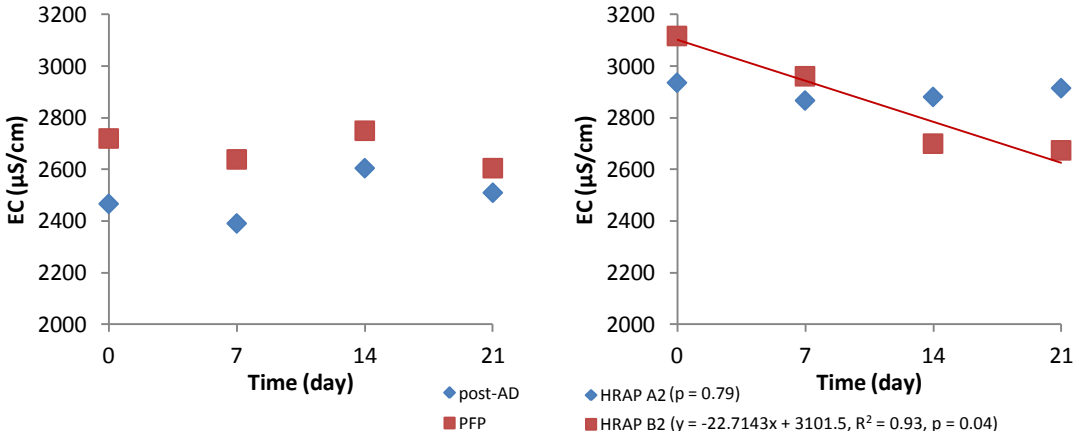

Figure 4.17 Electrical conductivity (EC) in post-anaerobically digested (post-AD) brewery effluent, primary facultative pond (PFP) effluent and high rate algal ponds (HRAP A2 and HRAP B2) effluent with post-PFP effluent flowing into HRAP A at a constant rate of 1000 L/d for 21 days, while HRAP B flow rates were progressively increased from 1000 L/d to 1400, 1800 and 2200 L/d at day 0, day 7, day 14 and day 21 respectively.

4.4 Discussion

a. The microalgae community structure in high rate algal ponds at different effluent flow rates

An increase in the flow rate of post-AD brewery effluent into the HRAP B train affected the microalgae community structure in the HRAP B2. When the flow rate of the effluent was progressively increased, some microalgae species were washed out in the HRAP B2, while in HRAP A2 at 1000 L/d, there was no apparent shift in the microalgae species composition. Metagenomics data also demonstrated shifts in the microalgae community structure and the relative abundance of the species involved. In the HRAP B2, at 1000 L/d, *Nanofrustulum shiloi, Chlorella vulgaris, Nannochloris* sp., *Scenedesmus abundans* and *Staurosira construens* had a relative abundance

between 0.11 and 3.38%. Whereas, at 2200 L/d, *Scenedesmus subspicatus* and *Chlorella vulgaris* were the microalgae identified with a relative abundance of less than 0.03%. El-Sayed *et al*. (2010) also observed a shift in microalgae taxa when water was frequently renewed as this shortened the HRT in aquaculture ponds. Shifts in the microalgae community structure and the relative abundance of the species in the HRAP B2 was due to a wash out of algal cells from the pond when HRT was shortened (El-Sayed *et al*. 2010; Sacasa Castellanos 2013). Shortening of the HRT reduced the microalgae community structure and changed its relative abundance in the HRAP B2.

Diatoma sp*., Chlamydomonas* sp*., Microcystis* sp*., Phormidium* sp*., Synechococcus* sp*., Pediastrum* sp*. and Arthrospira* sp*.* were washed out of the system at 2200 L/d. Johnson (2010) reported that at a shorter HRT of two days, algal species such as *Euglena* sp. can be washed out of a pond, whereas *Chlorella* sp. and *Scenedesmus* sp. were retained in the pond. Shortening of the retention time in the HRAP B2 did not provide some algal species sufficient time to grow, however, fast growing microalgae such as *Chlorella* sp. and *Scenedesmus* sp. were able to persist without being washed out (Johnson 2010; Sutherland *et al.* 2014b). This statement can be supported by Table 4.3 below which indicates that *Scenedesmus* sp. and *Chlorella* sp. have a greater biomass productivity than the other algal species identified in the HRAP B2.

Table 4.3 Microalgal biomass productivity.

Microalgae phylum	Microalgae species	Biomass productivity (g/L/day)
Chlorophyta	*Scenedesmus* sp.	0.26 (Mahapatra & Ramachandra 2013)
	Chlorella sp.	0.23 (Mata *et al*. 2010; Alhattab 2014)
	Nannochloris spp.	0.21 (Mata *et al*. 2010)
Cyanophyta	*Synechococcus* sp.	0.05 (Da Rós *et al*. 2013; Mahapatra & Ramachandra 2013)
	Arthrospira sp.	0.21 (Da Rós *et al*. 2013; Mahapatra & Ramachandra 2013)
	Microcystis sp.	0.06 (Mahapatra & Ramachandra 2013)
	Phormidium sp.	0.06 (Mahapatra & Ramachandra 2013)

b. The bacterial community structure in high rate algal ponds at different effluent flow rates

Changes in the flow rate of effluent into the HRAP B train resulted in shifts in the bacterial community structure and the relative abundance of the species in the HRAP B2. There was a substantial increase in Firmicutes and Proteobacteria when the flow rate was increased from 1000 to 2200 L/d. The relative abundance of Planctomycetes and Cyanobacteria decreased when the flow rate was increased from 1000 to 2200 L/d. Schneider *et al*. (2007) also reported shifts in the bacterial community structure at different HRTs in a recirculating aquaculture effluent system. At a two hour HRT, the dominant phylum was Acinetobacteria, while at seven hours HRT, Proteobacteria dominated (Schneider *et al*. 2007). In the HRAP B2, shifts in the bacterial community structure were probably due to an increase in the incoming post-AD effluent into the HRAP B2 (increasing dilution

rate) and this carried exogenous bacteria into the pond, thus contributing to changes in the community structure (Albaggar 2014).

Some bacterial phyla have a rapid growth rate and a high affinity for nutrients, especially Proteobacteria (Albaggar 2014). An increase in the flow rate of the effluent, thus increasing the organic load into the HRAP B2, could have attributed to the increase in the Firmicutes and Proteobacteria phyla. When decreasing the retention time, some bacterial species with a longer doubling period tend to grow slowly and this may result in a decline in the relative abundance (Albaggar 2014). Some bacterial speices such as nitrifiers have a slower growth rate than others (Table 4.4) and this could elucidate why some species were present at 1000 L/d yet absent at 2200 L/d. This could also be the reason why the relative abundance of Planctomycetes and Cyanobacteria decreased at 2200 L/d. In general, a shorter HRT is species selective, in a sense that it favours the growth of some bacteria, while others are washed out of a pond (Albaggar 2014).

Table 4.4 Bacterial species growth rate.

Bacteria nutritional modes	Phylum	Bacterial species	Growth rate (per day)
Chemolithotrophs	Proteobacteria	Nitrifiers	0.14-0.84 (Bitton 2005)
Heterotrophs	Proteobacteria Firmicutes Planctomycetes Bacteroidetes Cyanobacteria Chloroflexi Actinobacteria	Mixed culture	4.32-9.12 (Bitton 2005)

Zooplankton grazing on bacteria was probably also a factor affecting the bacterial community composition and abundance (Albaggar 2014). However, at the high flow rate of 2200 L/d, it is unlikely that bacterial shifts could have been attributed to protozoan grazing because it is known that when a flow rate is greater than the zooplankton reproduction rate, this limits their ability to develop (Albaggar 2014). Thus far, it appears that there has not been much research focused on the bacterial community structure in HRAPs at different HRTs.

c. Changes in temperature, algal biomass, chlorophyll a, phaeophytin a, dissolved oxygen, pH and chemical oxygen demand in the high rate algal ponds at different effluent flow rates

An increase in the flow rate of post-AD brewery effluent affected the algal biomass and Chl *a* concentration in HRAP B2. In the HRAP A2 (1000 L/d), the algal biomass increased over 21 days, while in the HRAP B2, the biomass decreased to zero when the effluent flow rate was increased. This is in agreement with Kim *et al*. (2014) who observed an increase in algal biomass was observed when the HRT was increased from two to four, six and eight days. The algal biomass in their study

was 0.99 $\pm$ 0.07, 1.26 $\pm$ 0.08, 1.45 $\pm$ 0.10 and 1.74 $\pm$ 0.16 g/L respectively (Kim *et al*. 2014). Sacasa Castellanos (2013) reported a decrease in *Chlorella vulgaris* biomass at a dilution rate of 0.75/d when investigating the effects of three dilution rates of 0.5, 0.6 and 0.75/d on the biomass of *Chlorella vulgaris* in a photobioreactor. Larsdotter (2006a) also observed a reduction in algal biomass at a shorter HRT. Chlorophyll *a* concentrations in the HRAP A2 increased over 21 days, while in the HRAP B2, there was a substantial decrease in Chl *a* concentrations when determined using the trichromatic and monochromatic equations. Similar trends were observed by Kim *et al*. (2014) where Chl *a* concentration was reported to be 3000 µg/L at a HRT of two days and 5000 µg/L at a HRT of eight days. A reduction in the standing algal biomass in HRAP B2 coincided with increasing dilution rates of the post-AD brewery effluent, which lead to a wash out of algal cells (Sacasa Castellanos 2013). At 2200 L/d, algal cells were not retained in the pond, however, in the HRAP A2, due to the prolonged HRT, the standing algal biomass increased (Larsdotter 2006a; Adesalu & Nwankwo 2012). The algal biomass in HRAP A2 doubled while the Chl *a* increased by about 30%. The type of solvent used for Chl *a* extraction and the cell wall of microalgae could have made it difficult to lyse some of the microalgae cell walls, hence the 30% increase (Sartory 1982). Also, the Chl *a* content in each microalgae species varies (Sartory 1982). All these could attribute for the less increase in Chl *a* concentration than expected. A reduction in the HRT of the pond reduced algal biomass and Chl *a* concentrations in the HRAP B2. There was no evidence of an accumulation of algal biomass at a shorter HRT (Medina & Neis 2007).

Phaeophytin *a* concentration in the HRAP B2 decreased as the flow rate of the post-AD brewery effluent was increased from 1000 L/d up to 2200 L/d in the HRAP B train. There was a significant difference in the Phaeo *a* concentration in HRAP B2, while in HRAP A2, there was no significant difference. A direct relationship between Phaeo *a* and Chl *a* concentrations was observed, where the Chl *a* increased with Phaeo *a* concentrations and vice versa. Khondker & Abed (2013) reported a positive correlation between Phaeo *a* and Chl *a*, i.e. both Phaeo *a* and Chl *a* concentrations increased, and vice versa. A decrease in the Phaeo *a* concentration was also probably influenced by the wash out of algal cells from the pond.

Dissolved oxygen concentrations and pH values in the HRAP B2 remained constant when the flow rate of post-AD brewery effluent was progressively increased. There were no significant differences in the DO concentrations and pH values in the HRAPs. Aeration provided by the paddle wheels may have contributed to the DO concentrations in the HRAPs (Mishra *et al*. 2011). Mixing in the pond probably improved the diffusion of O_2 between the water body and the air (Fogg 1991; Winder & Sommer 2012). In addition, microalgae contribute in the DO concentrations by releasing O_2 into the

water body during photosynthesis (Kunlasak *et al*. 2003; Muñoz & Guieysse 2006). The pH in HRAP A2 decreased following similar trends to the post-AD and PFP effluent.

A decrease in the algal biomass while the effluent flow rate was increased from 1000 to 2200 L/d in the HRAP B train kept the COD values constant in the HRAP B2. There was no difference in the COD values in the HRAP B2. However, in the HRAP A2, where the flow rate was maintained at 1000 L/d, the COD increased over 21 days. A COD removal efficiency of 27, 10 and 41% was observed at 1400, 1800 and 2200 L/d in HRAP B2. This is comparable to the COD removal efficiency of 85.44, 74.27, 68.41 and 63.59% at a HRT of two, four, six and eight days respectively as reported by Kim *et al*. (2014). The constant COD in the HRAP B2 could have been attributed to a decrease in the algal biomass. It is known that during photosynthesis, the standing algal biomass releases glycolic acid (organic carbon) into the water body, which contributes to escalating levels of dissolved COD (Wang *et al*. 2009; Kim *et al*. 2014), however, in the HRAP B2, this was probably not the case, thus the constant COD. An increase in COD in the HRAP A2 was concurrent with the accumulation of algal biomass, and this suggests that algae contributed to the increase in COD (Wang *et al*. 2009; Kim *et al*. 2014).

d. The removal of nitrogen from the anaerobically digested brewery effluent in the high rate algal ponds at different effluent flow rates

Ammonium-nitrogen concentrations in the post-HRAP A2 and post-HRAP B2 effluent were invariant, even when the post-AD brewery effluent flow rate was increased in the HRAP B train. The NH_4^+-N removal efficiency in the post-HRAP B2 effluent was between 98 and 100% up to 1800 L/d, however, at 2200 L/d the removal efficiency was only 14%. In the post-HRAP A2 effluent, the removal efficiency ranged from 98 to 100% when the effluent flow rate was maintained at 1000 L/d. Similarly, García *et al*. (2000) also found a higher NH_4^+-N removal efficiency when ponds were operated at a longer HRT as compared to those operated at a shorter HRT when treating urban wastewater in HRAPs. One of the mechanisms responsible for the removal of NH_4^+-N from wastewater is algae incorporating the NH_4^+-N into algal biomass (Sevrin-Reyssac 1998; Zimmo 2003). In the HRAP A2 and HRAP B2, the removal of NH_4^+-N from the effluent was associated with the standing algal biomass in the ponds. An increase in the algal biomass in HRAP A2 maintained a greater removal efficiency of NH_4^+-N. Even in the HRAP B2, a decline in the algal biomass and Chl *a* concentration up to 1800 L/d still maintained greater NH_4^+-N removal efficiency. This suggests that microalgae assimilated a portion of the NH_4^+-N.

Another mechanism known to be vital in the removal of NH_4^+-N from wastewater is volatilization of the unionised ammonia-nitrogen (NH_3-N). In HRAP A2, the pH was always above nine, making conditions suitable for NH_3-N volatilization (Sevrin-Reyssac 1998; Zimmo 2003). In the HRAP B2, with a flow rate up to 1800 L/d, pH rose above nine. However, at 2200 L/d, the pH was about 8.5. Below pH nine, the predominant form of ammonia is NH_4^+-N, which cannot be stripped into the atmosphere (Sevrin-Reyssac 1998; Zimmo 2003). Therefore, this implies that at 2200 L/d, where the pH was below nine, NH_3-N volatilization was not responsible for the removal of NH_4^+-N.

Nitrification is also responsible for the removal of NH_4^+-N from effluent (Khin & Annachhatre 2004). In the HRAP B2, at a flow rate of 1000 L/d, ammonium oxidizing bacteria (AOB), *Nitrosomonas* sp. was present at a very low relative abundance. At a flow rate of 2200 L/d, there was no trace of any AOB in the pond. This suggests that at 1000 L/d, NH_4^+-N was oxidized into NO_2^--N, while at 2200 L/d, there was no evidence of nitrification. At a shorter HRT, nitrifying bacteria are washed out of a pond (Babu 2011). These nitrifying bacteria have a slow growth rate and shortening the retention time does not favour their growth, but instead other faster growing bacteria (Albaggar 2014).

Nitrite-nitrogen in the HRAP B2 increased when the flow rate of post-AD brewery effluent was increased. In the HRAP A2, where the flow rate was maintained at 1000 L/d, NO_2^--N removal efficiency was constant. Similar results were reported by Cilliers (2012) when the NO_2^--N concentration was greater during summer when a shorter HRT was used as compared to other seasons when a longer HRT was used in HRAPs. An increase in NO_2^--N in the HRAP B2 can be associated with the oxidation of NH_4^+-N into NO_2^--N (Babu 2011). Ammonium oxidizing bacteria are known to grow faster than nitrite oxidizing bacteria (NOB) when the temperature is above 15°C (Khin & Annachhatre 2004). This suggests that when the flow rate was progressively increased, AOB and NOB were washed out of the HRAP B2, however, the rapid growth rate of AOB (Khin & Annachhatre 2004) probably enabled some species to remain in the pond. These AOB species probably oxidized NH_4^+-N into NO_2^--N, thus causing the increase in NO_2^--N. However, since NOB was presumed to be washed out, there was no oxidation of NO_2^--N into NO_3^--N. This is supported by the the NO_3^--N concentrations that remained constant in the HRAP B2.

In the post-HRAP B2 effluent with increased flow rates, there was no significant difference in the removal of NO_3^--N. While, in the post-HRAP A2 effluent (1000 L/d), NO_3^--N increased over 21 days. Buelna *et al*. (1990) also found an increase in the concentration of NO_3^--N at a longer HRT when treating piggery waste in a HRAP. An increase in NO_3^--N concentration in the HRAP A2 suggests that the longer HRT retained NOB and that NO_2^--N was oxidized into NO_3^--N.

e. The removal of phosphorus from the anaerobically digested brewery effluent in the high rate algal ponds at different effluent flow rates

The removal efficiency of PO_4^{3-}-P in the HRAP B2 increased up to a flow rate of 1800 L/d, and then decreased with a further increase of the post-AD brewery effluent flow rate. The PO_4^{3-}-P removal efficiency increased from 14 to 49%, and then decreased to 29% at 2200 L/d. In the HRAP A2, at 1000 L/d, the PO_4^{3-}-P removal efficiency was constant. Similar trends were observed by Kim *et al*. (2014) when the total phosphorus removal efficiency was 82.65±8.63% at a shorter HRT of two days and 95.49±13.81% at a longer HRT of eight days. The removal of PO_4^{3-}-P from HRAP A2 and HRAP B2's effluent could be through algal assimilation (Larsdotter 2006a; Kim *et al*. 2014). In the HRAP A2, the fixed removal of PO_4^{3-}-P from the effluent corresponded with an increase in the algal biomass and Chl *a* concentration. On the contrary, in the HRAP B2, a decrease in the removal efficiency also coincided with a substantial decrease in the algal biomass in the pond. At 2200 L/d, there were no algal cells in the pond, and thus no apparent removal of PO_4^{3-}-P. That microalgae were responsible for PO_4^{3-}-P removal could also be supported by the concentrations of PO_4^{3-}-P in the post-HRAP B2, which appeared to follow those from the incoming post-PFP effluent. An increase in effluent flow rate washed out algal cells in the HRAP B2, and thus it appeared there was no any other mechanism of PO_4^{3-}-P removal.

Precipitation did not contribute to the removal of PO_4^{3-}-P from the effluent. When the pH was above 10 in both ponds, there was no clear link between PO_4^{3-}-P removal and the pH. In the HRAP A2, with a longer HRT, it was expected that PO_4^{3-}-P would accumulate in the pond and this would increase PO_4^{3-}-P precipitation (Kim *et al*. 2014), however, this was not the case. Kim *et al*. (2014) also observed no relationship between PO_4^{3-}-P and pH when the pH was between 10 and 10.6. Up to a flow rate of 1800 L/d, the HRAP B2 was efficient in the removal of PO_4^{3-}-P from the post-AD brewery effluent, however, at 2200 L/d, the PO_4^{3-}-P removal capacity collapsed.

f. Ionic composition of the anaerobically digested brewery effluent in the high rate algal ponds at different effluent flow rates

The EC in the HRAP B2 was substantially affected by the increase of post-AD brewery effluent flowing into it. In the HRAP B2, EC gradually decreased from 3117 μS/cm at 1000 L/d to 2674 μS/cm at 2200 L/d, while in the HRAP A2 there was no noticeable change in the EC. El-Sayed *et al*. (2010) also reported a decrease in EC in aquaculture ponds when water was frequently renewed, thus increasing the dilution rate. Cilliers (2012) reported that in HRAPs, operated at a longer HRT, total dissolved solids (TDS) accumulate, making the effluent more concentrated, however, when the HRT

was shortened, the ponds were more diluted. This could have been the case in the HRAP B2 when it was operating at a flow rate of 2200 L/d: the effluent was flushed out of the system rapidly, which diluted the effluent and reduced the TDS. The EC and TDS in a pond are directly proportional to one another (Sahni & Yadav 2012). The HRT of the effluent in the HRAP B2 had a marked effect on the EC of the effluent.

4.5 Conclusion

This experiment demonstrated that a flow rate of up to 1800 L/d is sufficient for the removal of nutrients from the effluent. This flow rate can be recommended if the intention is to treat effluent, however, if the primary goal is to produce algal biomass, shortening of the HRT up to 1800 L/d is not recommended. The shorter the HRT, the more dilute the algal suspension and the higher the effort required to harvest microalgae (Richmond 2004). The main mechanism responsible for nutrient removal appeared to be algal assimilation. This was clearly seen at the flow rate of 2200 L/d, when the drop in algal biomass to zero concurred with failure to remove NH_4^+-N and PO_4^{3-}-P from the effluent. An increase in the effluent's flow rate kept the COD constant in HRAP B2. This probably implies that there was no substantial amount of glycolic acid (organic carbon) released by microalgae into the water body since the standing algal biomass declined gradually. Since the emphasis of this study was on effluent treatment rather than algal production, and because of this, the steady state of the HRAP B2 was determined using an analysis of nutrient composition. Future work can establish the steady state of the HRAPs using the microbial community as an indicator.

The HRT of a HRAP can be manipulated by changing a pond's depth, while considering the effects that seasonal variation has on HRAPs (De Pauw & van Vaerenbergh 1983). The depth of a pond can be increased in winter with a longer HRT, however, it is mostly recommended to increase the depth during summer (De Pauw & van Vaerenbergh 1983). In summer, maximum PAR is expected and algal cells are likely to receive sufficient PAR (Richmond 2004). Whereas, in winter, when PAR is minimal, algal cells can be deprived sufficient PAR (Richmond 2004).

Altering the HRT depends on the purpose of the HRAPs, whether to produce algal biomass or for treating effluent, or both (Medina & Neis 2007). To enhance biomass, CO_2 can be bubbled into the HRAPs (Park *et al*. 2011). However, prolonged bubbling of CO_2 into HRAPs decreases the pH of the culture medium if not controlled, which might negatively affect microalgae growth and nutrient removal efficiency (Park *et al*. 2011). Bubbling CO_2 into microalgae culture can be expensive, however, this can be economically beneficial if the produced biomass is used for other useful purposes (Kativu 2011).

Chapter five

The removal of nitrogen and phosphorus from anaerobically digested brewery effluent using microorganisms taken from the high rate algal ponds cultured under different temperatures and pH

5.1 Introduction

Abiotic factors such as temperature and pH in algal ponds are affected by seasonal cycles (Affan *et al*. 2005). These factors influence microalgae growth and their performance in nutrient removal from wastewater (Larsdotter 2006a, Johnson 2010). Khondker & Abed (2013) observed that the maximum microalgae growth estimated by Chl *a,* occurred during summer where the maximum temperature and pH when were also recorded. Larsdotter (2006a) reported changes in nutrient removal efficiency by algae grown in tanks related to seasonal variation of light and temperature. The pH of the effluent also affected the removal of nutrients (Larsdotter 2006a). Changes in microalgae growth and nutrient removal at different seasons were also noted by (Cilliers 2012). Chapter 3 data also present evidence that microalgae growth and nutrient removal efficiency in a high rate algal pond (HRAP) fed with post-anaerobically digested (post-AD) brewery effluent varies seasonally at the Ibhayi Brewery. However, the factors affecting microalgae growth and the mechanisms responsible for nutrient removal from the post-AD brewery effluent in HRAPs are not well understood. These mechanisms are likely to differ at different temperatures and pH levels (Assemany *et al*. 2015).

Temperature has an influence on the metabolism and growth of microalgae (Larsdotter 2006a, Johnson 2010; Markou & Geogakakis 2011). A temperature ranging from 15-25℃ supports the growth of most microalgae, especially warm water species (Griffiths 2010). Nutrient removal efficiency is also influenced by temperature, with increasing temperatures leading to increases in removal efficiencies (Muñoz & Guieysse 2006). The monthly mean temperature recorded in the HRAP at the Ibhayi Brewery ranged from 12.83±0.86 to 25.9℃ which was recorded between 09:00 and 10.00 am (Figure 3.14). This range motivated the selection of 20 and 30℃ for the following laboratory-based experimental work, which resembles the temperatures that would be recorded at midday.

The pH of the effluent affects the solubility of nutrients in HRAPs (Larsdotter 2006a, Johnson 2010; Markou & Geogakakis 2011). In the HRAP, at the Ibhayi Brewery, the pH of the post-AD brewery effluent ranged from 7.25±0.75 to 10.23±0.21 (Figure 3.17). This range determined the selection of

7.0, 8.5 and 10.0 as the pH range for the experimental variables. The pH of the effluent determines equilibrium shifts between ionised ammonium (NH_4^+) and unionised ammonia (NH_3) forms (Larsdotter 2006a, Johnson 2010). At a pH below seven, NH_4^+ is the dominant form, however, as the pH increases above seven, the form of ammonia shifts increasingly towards the unionised form (NH_3) (Larsdotter 2006a; Johnson 2010). In a typical algal pond, pH also influences orthophosphate (PO_4^{3-}) removal mechanisms from the effluent (Larsdotter 2006a). When the pH is above 11, with cations such as magnesium and calcium present in the effluent, the precipitation of PO_4^{3-} is one of the mechanisms responsible for phosphorus removal (Larsdotter 2006a). However, at a pH below 11, the removal of PO_4^{3-} from the effluent is mostly through algal assimilation (Larsdotter 2006a). Most freshwater algal species prefer a pH range of 7.5 to 8.5 for maximum growth (Richmond 2004).

In this experiment, the pH in the microalgae cultures was decreased by bubbling carbon dioxide (CO_2) into the medium whenever it rose above the set pH level during photosynthesis. A rise in pH is a result of the dissociation of carbonate and bicarbonate ions, in turn releasing OH^- into the medium (Abdel-Raouf *et al.* 2012). Carbon dioxide is a source of carbon, an essential nutrient for microalgae growth (Larsdotter 2006b; Lundquist *et al.* 2010), which in turn affects the removal of nutrients. This makes it unclear whether microalgae growth is solely responsible for nutrient removal, or whether temperature and pH variations also influence the removal of nutrients from the medium. To account for the masking effects of bubbled CO_2 on algae growth, a control using hydrochloric acid (HCl) to decrease the pH in the cultures was set up.

Thus far, no experimental research has confirmed the factors affecting microalgae growth and the mechanisms responsible for nutrient removal from the post-AD brewery effluent in the HRAP at the Ibhayi Brewery.

Aim and objectives

The purpose of this study was to investigate the effects of environmental parameters, such as temperature and pH, on microbial community structure and the removal of dissolved nutrients such as NH_4^+-N and PO_4^{3-}-P from the post-AD brewery effluent. This was undertaken to generate a better understanding of the mechanisms involved in nutrient removal from brewery effluent in HRAPs treatment systems. The specific objectives were to:

- compare the effects of temperature and pH on microalgae growth;
- compare the effects of temperature and pH on nutrient removal from the post-AD brewery effluent; and

- compare the effects of temperature and pH on microalgae community structure using light microscopy.

5.2 Materials and methods

5.2.1 Preparation of culture medium and microalgae

Anaerobically digested brewery effluent was collected from the Ibhayi Brewery experimental AD tank. The effluent was autoclaved at 121℃ for 20 min to sterilize it so that it could be used as a growth medium. The autoclaved effluent's pH was aseptically adjusted to between two to three and kept in a fridge at four degrees celsius to keep it sterile and avoid any change in its chemical properties until it was used (U.S. EPA 1983).

Effluent samples containing microalgae cultures and bacteria were collected from the experimental HRAPs at the Ibhayi Brewery. The culture was acclimated to the experimental conditions for two weeks prior to the initiation of the experiment and then centrifuged for five minutes at 2831 G using a centrifuge (Beckman Coulter, model: Avanti J-E) to separate algal cells from the effluent. The supernatant was decanted, and the pelleted material resuspended in 500 mL sterile sodium chloride (NaCl) isotonic solution and centrifuged for five minutes again at 2831 G. The supernatant was decanted and the algal cells were recovered and inoculated into the autoclaved growth medium.

5.2.2 Experimental system

The experiment was carried out in a controlled environment room at the Institute for Environmental Biotechnology, Rhodes University (EBRU). The effects of temperature (20 and 30℃) and pH (7.0, 8.5 and 10.0) at a photoperiod of 12 h darkness and 12 h light on the growth of microorganisms and the removal of nutrients from the post-AD brewery effluent were investigated over a period of six days. This duration was based on (a) a preliminary study that showed that nutrient removal took place within this period and (b) to simulate hydraulic retention times (HRT) commonly employed when HRAPs are used in effluent treatment.

Glass tanks were half filled with tap water and used as water baths. One hundred watt thermostatically controlled heaters were used to heat up and maintain the water at the selected temperature. The experiment was initially run at 20℃ and then subsequently repeated at 30℃. Due to constraints, the experiments were not run simultaneously, but everything was standardized, i.e. the effluent used had the same chemical properties, and other environmental conditions in the laboratory were the same. The temperature was monitored every hour during the light phase, to ensure it was within the desired range during the photosynthetic period.

Nine one litre glass round-bottom flasks were filled with 700 mL of microalgae culture in the CO_2 and in the HCl pH adjustment methods, i.e. where a flask represented the unit of replication (Figure 5.1).

Figure 5.1 Glass tanks (water baths) with flasks containing microalgae culture.

Fluorescent bulbs were used as a source of illumination providing an average photosynthetically active radiation (PAR) of 77.61 µmol m^{-2} s^{-1} which was measured using a light sensor (Skye Instrument PAR, model: SKP 210, United Kingdom) at the water level. The Skye Instrument PAR was linked to a data logger (Skye instrument data loggers, model: SDL 5000 series, United Kingdom) and data from the data loggers were processed using standard communications software (SkyeLynx Standard Communications Software, product: Software V 2.6, United Kingdom). A timer switch was used to maintain a photoperiod of 12 h darkness and 12 h light.

The pH in each flask was adjusted to either pH 7.0, 8.5 or 10.0. Parallel experiments were run simultaneously using two pH adjustment methods. The first method used CO_2 to decrease the pH. The CO_2 was supplied by a compressed CO_2 gas cylinder (Afrox, South Africa) which had a CO_2 regulator attached to it (Figure 5.2). If the CO_2 decreased the pH too much, 10 molar (M) sodium hydroxide (NaOH) was used to bring the pH to the required level. One molar HCl was used to

decrease the pH while 10 M NaOH was used to increase the pH in the cultures. Each level of pH was replicated three times in both pH adjustment methods, i.e. three flasks per treatment. The pH was monitored every 60 min for 12 h during the light period using a pH meter (Hanna, model: HI 8314, United Kingdom).

Figure 5.2 A compressed carbon dioxide (CO_2) gas cylinder with a CO_2 regulator.

Three flasks per treatment were continuously aerated to keep the algal cells in suspension. This was provided by air stones connected to a multiport manifold which was connected to an air pump. An in-line 0.2 µm filter (Whatman) was positioned between the air pump and the manifold to filter out air-borne contaminants. Valves on the manifold were used to control the air flow to each flask. Flasks were also regularly swirled to resuspend settled algal cells. The flasks were covered with a perforated transparent wrap to allow sufficient illumination while reducing influx of contaminants. The filtered air delivered into each flask provided positive air displacement.

To demonstrate the loss of NH_4^+-N from the effluent through mechanisms other than microbial activity, an additional control treatment was included for the NH_4^+-N data set. This included a flask of autoclaved growth medium that was not inoculated with microorganisms isolated from the HRAP. This control was also represented at the three pH levels, which were controlled using HCl or CO_2, and it was also represented in triplicate (i.e. three flasks per treatment).

5.2.3 Microbiological and water quality parameter analysis

Deoxyribonucleic acid (DNA) samples of both the microalgae and bacteria were collected at the start of the trial only, so that a profile of the microorganisms present in the microbial community could be described using metagenomics. Water samples of 20 mL were collected for the analysis of ammonium-nitrogen (NH_4^+-N), nitrite-nitrogen (NO_2^--N), nitrate-nitrogen (NO_3^--N) and orthophosphate-phosphorus (PO_4^{3-}-P) daily, while for the microscopic identification of microalgae and chlorophyll (Chl) *a* concentration, samples were collected at two days intervals. The analysis of the DNA and water parameters was carried out using the materials and methods described in Chapter 3, Section 3.2.2, Section 3.2.3 and Section 3.2.4.

5.2.4 Statistical analysis

Data were analysed with multivariate analysis of variance (MANOVA) (Statistica, version 12.0) and graphs were plotted using Microsoft Excel (Version 2007). A p-value of less than or equal to 0.05 ($p \leq 0.05$) indicated that there was a significant difference in the data while a p-value greater than 0.05 ($p > 0.05$) indicated no significant difference in the data. The collected data were fitted with a slope using a linear regression model (Statisica, version 12.0). The slope of the linear regression model was determined at $p \leq 0.05$. The R^2-value indicated the closeness of the data to the fitted regression line.

5.3 Results

Physical parameters

The temperature in the water baths fluctuated between 19.5 and 21.0℃ when the trial was run at 20℃, with an overall mean (± standard error) of 20.59±0.63℃. At 30℃, the temperature varied within a range of 29 to 32℃ with an overall mean of 31.06±0.97℃.

Biological parameters

Most of the microalgal species present in the algal community that was investigated belonged to the Chlorophyta, while a few species were from the Cyanophyta (Figure 5.3, Table 5.1, Table 5.2). The remaining algal species were distributed among the other phyla. In the bacterial community, Actinobacteria and Firmicutes were the dominant phyla with a relative abundance of 16.22 and 14.38% respectively, while Verrumicrobia had the lowest relative abundance of 0.34% (Figure 5.4). The concentrations of Chl *a* increased with an increase in temperature, with the maximum growth at pH 8.5 in both pH adjustment methods (Figure 5.5, Figure 5.6).

a. Identification of the microalgae community structure using metagenomics

The initial culture was composed of species belong to Chlorophyta and Bacillariophyta (Figure 5.3, Table 5.1). *Mychonastes* sp. was the dominant species of the Chlorophyta, while other species had a low relative abundance in the microalgae community.

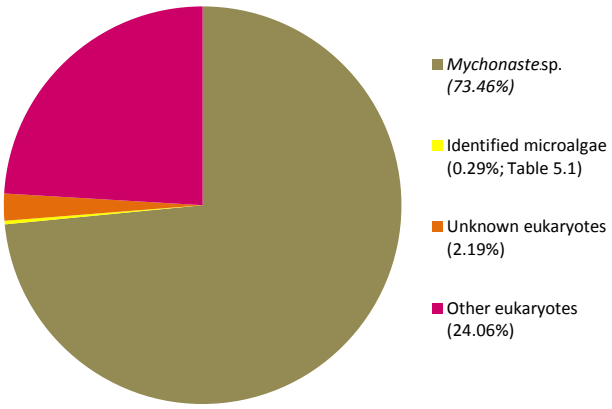

Figure 5.3 The microalgae community structure, other eukaryotes and the relative abundance as percentage (%) identified using metagenomics at the beginning of the experiment. Other eukaryotes refer to non-algal species that were also amplified with the universal primer pair 566F and 1200R.

Table 5.1 Microalgae species and the relative abundance as percentage (%) at the beginning of the experiment grouped under the identified microalgae category in the pie chart (Figure 5.3).

Microalgae species	(%)
Bodomorpha minima	0.01
Chlamydomonas sp.	0.01
Chlorella sp.	0.01
Coccomyxa sp.	0.01
Haematococcus zimbabwiensis	0.02
Lobochlamys segnis	0.01
Microspora sp.	0.01
Nannochloris sp.	0.01
Nanofrustulum shiloi	0.01
Pachycladella umbrina	0.01
Phaeodactylum tricornutum	0.01
Pseudodictyosphaerium jurisii	0.01
Pyramimonas olivacea	0.07
Scenedesmus subspicatus	0.01
Scenedesmus abundans	0.05
Staurosira construens	0.01
Trebouxia erici	0.02

b. Identification of the microalgae community structure using light microscopy

Microalgae belonging to the genera *Scenedesmus*, *Chlamydomonas, Chlorella*, *Diatoma, Microcystis, Phormidium, Arthrospira and Synechococcus* were identified throughout the entire experimental period from day zero to day six at all pH levels and temperatures (Table 5.2). Images of these algal species are presented in Chapter 3 (Section 3.3.3.a, Figure 3.7).

Table 5.2 Microalgae identified using light microscopy during the course of the experiment at 20 and 30°C. Number 0, 2, 4 and 6 on the columns represents the day on which samples were collected for microscopic analysis.

pH	7.0				8.5				10.0			
Time (day)	**0**	**2**	**4**	**6**	**0**	**2**	**4**	**6**	**0**	**2**	**4**	**6**
Scenedesmus sp.	√	√	√	√	√	√	√	√	√	√	√	√
Chlorella sp.	√	√	√	√	√	√	√	√	√	√	√	√
Diatoma sp.	√	√	√	√	√	√	√	√	√	√	√	√
Chlamydomonas sp.	√	√	√	√	√	√	√	√	√	√	√	√
Microcystis sp.	√	√	√	√	√	√	√	√	√	√	√	√
Phormidium sp.	√	√	√	√	√	√	√	√	√	√	√	√
Arthrospira sp.	√	√	√	√	√	√	√	√	√	√	√	√
Synechococcus sp.	√	√	√	√	√	√	√	√	√	√	√	√

c. Identification of the bacterial community structure using metagenomics techniques

Actinobacteria were the most abundant phylum with a relative abundance of 16.22%, followed by Firmicutes and Proteobacteria with a relative abundance of 14.38 and 11.49% respectively (Figure 5.4). Other phyla such as Planctomycetes, Bacteroidetes and Verrumicrobia had a relative abundance of between 0.34 and 3.58%. About 49.41% of the bacterial community structure were unknown species.

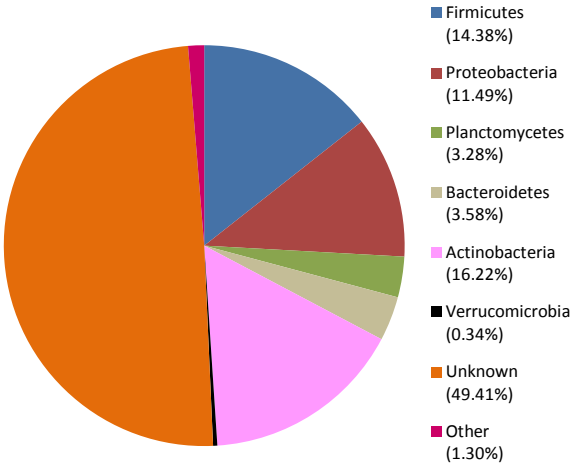

Figure 5.4 The bacterial community structure identified at the beginning of the trial and the relative abundance as percentage (%).

d. Chlorophyll a concentrations

The Chl *a* concentrations increased from day zero to day six (Figure 5.5, Figure 5.6). The Chl *a* concentrations showed a significant difference with an increase in temperature from 20 to 30℃ ($p < 0.001$) in both pH adjustment methods (Table 5.7). Also, with an increase in pH from 7.0 to 8.5 and 10.0, the Chl *a* concentrations were significantly different ($p < 0.001$) in both pH adjustment methods. When microalgae were cultured at 20℃, the concentration of Chl *a* was the greatest at pH 8.5, then followed by pH 10.0 and pH 7.0 (Figure 5.5). Similar trends were observed at 30℃. The Chl *a* concentration was also higher at pH 8.5, and followed by pH 10.0 and pH 7.0 (Figure 5.6).

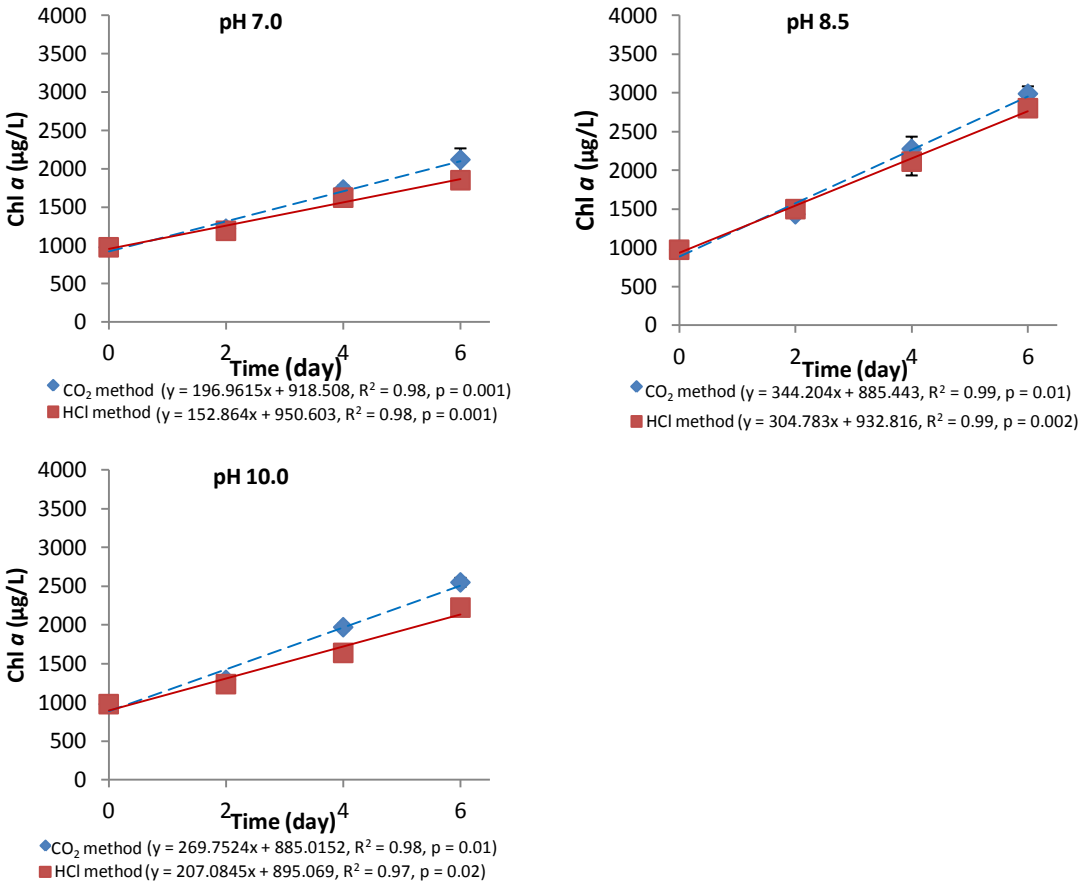

Figure 5.5 Changes in the mean (± standard error) chlorophyll (Chl) *a* concentration of microalgae cultured at 20℃ in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

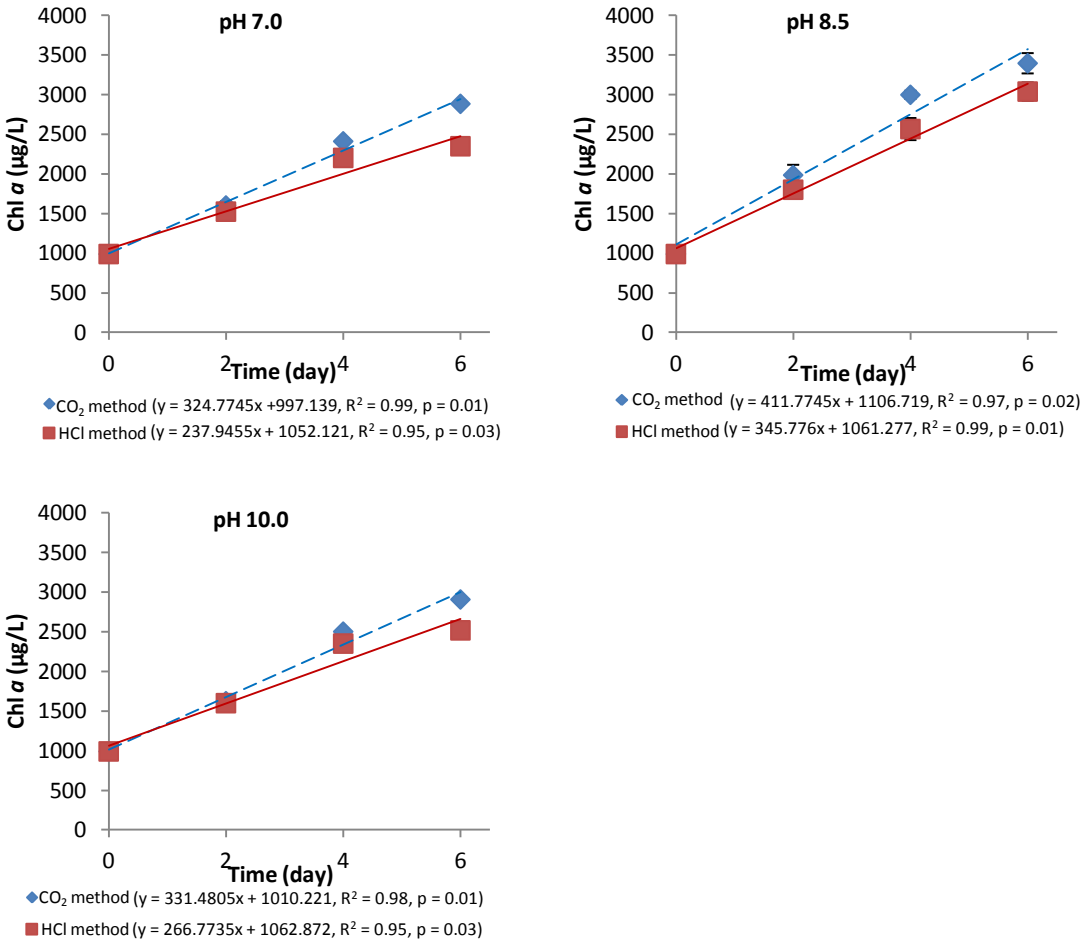

Figure 5.6 Changes in the mean (± standard error) chlorophyll (Chl) *a* concentration of microalgae cultured at 30°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

Chemical characteristics of the anaerobically digested brewery effluent

The mean concentrations of NH_4^+-N in the post-AD brewery effluent decreased at pH 7.0, 8.5 and 10.0 when microalgae and bacteria were cultured at 20 and 30°C using the CO_2 and HCl methods (Figure 5.7, Figure 5.8). In the autoclaved medium at 20°C, NH_4^+-N concentrations decreased at pH 7.0 and 10.0, while at pH 8.5, the concentrations remained constant (Figure 5.7). At 30°C, there was a decrease in NH_4^+-N at all pH levels (Figure 5.8). The concentrations of NO_2^--N at 20°C increased over time at pH 7.0 and 8.5, whereas, at pH 10.0, NO_2^--N concentrations were constant in the CO_2 and HCl methods (Figure 5.9). At 30°C, NO_2^--N concentration increased at all pH levels Figure (5.10). The mean concentrations of NO_3^--N at all temperatures and pH levels increased over six days (Figure 5.11, Figure 5.12). The concentrations of PO_4^{3-}-P in the effluent followed similar trends to NH_4^+-N and decreased at pH 7.0, 8.5 and 10.0 when microalgae were cultured at 20 and 30°C (Figure 5.13, Figure 5.14).

a. Ammonium-nitrogen

When microorganisms were cultured at 20℃, there was about a 65 to 85% decrease in NH_4^+-N concentration at pH 7.0, 8.5 and 10.0 in both the CO_2 and HCl methods (Figure 5.7). For both methods, there was a greater decrease of NH_4^+-N at pH 10.0 than at pH 7.0 and 8.5. Similar trends were observed in the autoclaved medium (with no microbial activity); also a greater portion of the NH_4^+-N was removed at pH 10.0 than at pH 7.0 and 8.5. For both pH adjustment methods, more NH_4^+-N was removed through microbial activity at pH 7.0, 8.5 and pH 10.0 rather than through volatilization (Table 5.3, Table 5.4).

At 30℃, in the microalgae cultures with bacteria, the concentration of NH_4^+-N decreased by 99% at pH 7.0, 8.5 and 10.0 for both pH adjustment methods (Figure 5.8). In the autoclaved medium, more of the NH_4^+-N was removed at pH 10.0 than at pH 7.0 and 8.5. A greater portion of about 82 to 84% of the NH_4^+-N in the microalgae cultures at pH 7.0 and 8.5 was removed through microbial activity in the CO_2 and HCl pH adjustment methods (Table 5.5, Table 5.6). At pH 10.0, a greater amount of NH_4^+-N was removed through volatilization for both methods of pH adjustment.

There was a significant difference ($p < 0.001$) in the removal of NH_4^+-N when temperature was increased from 20 to 30℃ for both pH adjustment methods (Table 5.7). There was also a significant difference ($p < 0.001$) in the removal of NH_4^+-N at different pH levels for both pH adjustment methods. Ammonium-nitrogen removal from the post-AD brewery effluent was influenced by an interaction between temperature and pH (MANOVA, $F_{(24,144)} = 7.84$, $p = 0.002$) in cultures where pH was adjusted using HCl.

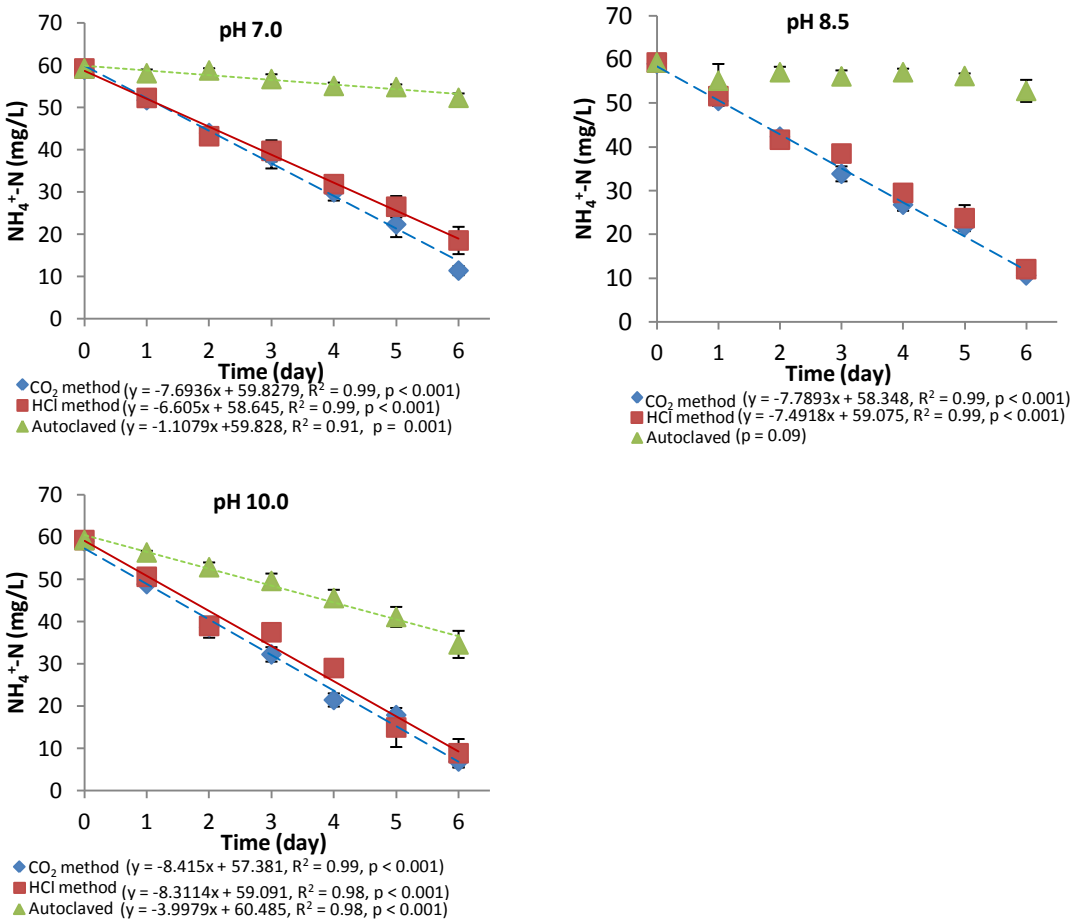

Figure 5.7 Changes in the mean (± standard error) ammonium-nitrogen (NH_4^+-N) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 20°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods and in the autoclaved medium at pH 7.0, 8.5 and 10.0.

Table 5.3 The estimated percentage (%) removal of ammonium-nitrogen (NH_4^+-N) from the post-anaerobically digested (post-AD) brewery effluent through microbial activity and volatilization at 20°C in the carbon dioxide (CO_2) treatment.

	Time (day)						
	0	**1**	**2**	**3**	**4**	**5**	**6**
pH 7.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	1.91	0.79	4.22	6.96	7.42	11.70
(b) With microbial activity	0.00	12.20	25.37	34.69	49.15	62.03	80.56
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	1.91	0.79	4.22	6.96	7.42	11.70
(d) Microbial activity (b-c) (%)	0.00	10.29	24.58	30.48	42.19	54.61	68.86
pH 8.5							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	7.03	3.76	5.36	3.59	5.23	10.96
(b) With microbial activity	0.00	14.41	28.25	42.66	54.63	63.00	82.03
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	7.03	3.76	5.36	3.59	5.23	10.96
(d) Microbial activity (b-c) (%)	0.00	7.37	24.49	37.29	51.04	57.77	71.07
pH 10.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	4.94	10.84	16.31	23.15	30.69	41.65
(b) With microbial activity	0.00	17.17	34.46	45.37	63.66	69.71	88.36
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	4.94	10.84	16.31	23.15	30.69	41.65
(d) Microbial activity (b-c) (%)	0.00	12.23	23.62	29.07	40.51	39.02	46.70

Table 5.4 The estimated percentage (%) removal of ammonium-nitrogen (NH_4^+-N) from the post-anaerobically digested (post-AD) brewery effluent through microbial activity and volatilization at 20°C in the hydrochloric acid (HCl) treatment.

	Time (day)						
	0	**1**	**2**	**3**	**4**	**5**	**6**
pH 7.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	1.91	0.79	4.21	6.96	7.42	11.70
(b) With microbial activity	0.00	11.75	27.03	32.88	46.16	55.18	68.63
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	1.91	0.79	4.21	6.96	7.42	11.70
(d) Microbial activity (b-c) (%)	0.00	9.84	26.24	28.67	39.19	47.76	56.93
pH 8.5							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	7.03	3.76	5.36	3.59	5.23	10.96
(b) With microbial activity	0.00	12.98	29.79	35.13	50.30	60.03	79.71
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	7.03	3.76	5.36	3.59	5.23	10.96
(d) Microbial activity (b-c) (%)	0.00	5.95	26.03	30.37	46.71	54.81	68.75
pH 10.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	4.94	10.84	16.31	23.15	30.69	41.65
(b) With microbial activity	0.00	14.67	34.28	36.81	51.09	74.82	85.11
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	4.94	10.84	16.31	23.15	30.69	41.65
(d) Microbial activity (b-c) (%)	0.00	9.73	23.44	20.51	27.94	44.13	43.46

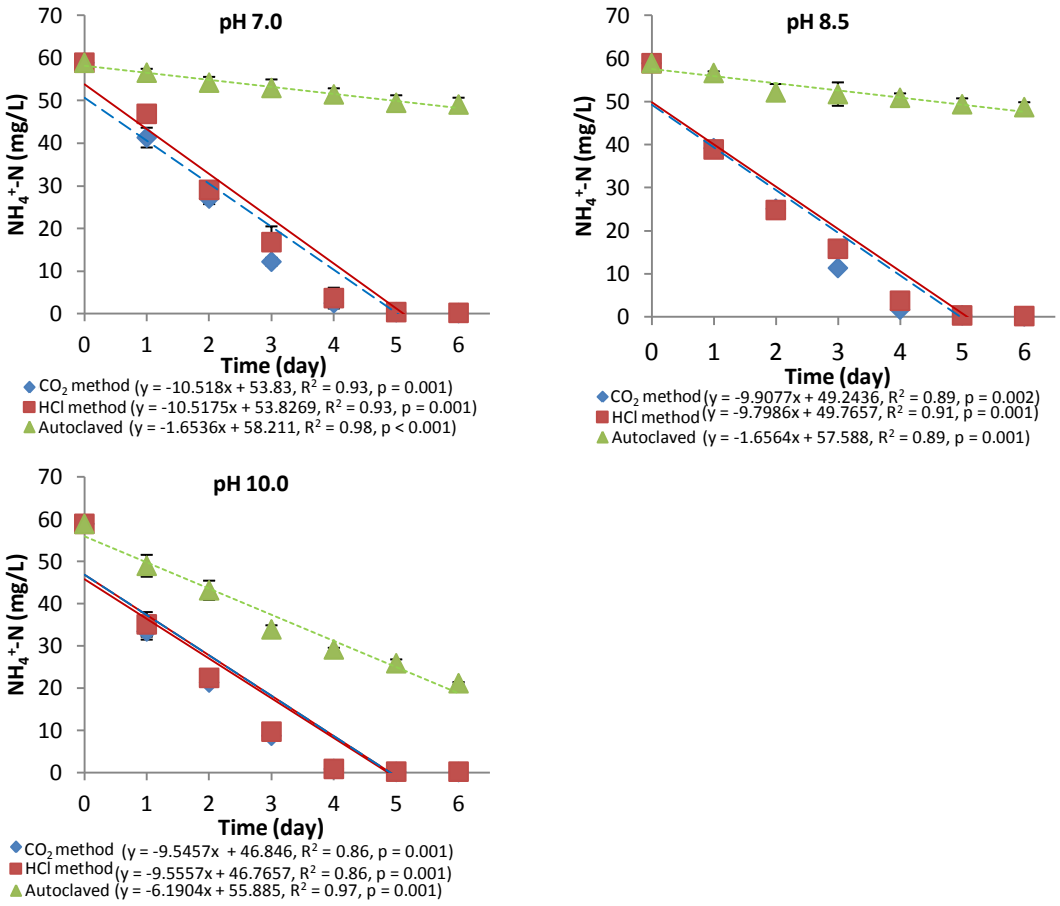

Figure 5.8 Changes in the mean (± standard error) ammonium-nitrogen (NH_4^+-N) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 30°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods and in the autoclaved medium at pH 7.0, 8.5 and 10.0.

Table 5.5 The estimated percentage (%) removal of ammonium-nitrogen (NH_4^+-N) from the post-anaerobically digested (post-AD) brewery effluent through microbial activity and volatilization at 30°C in the carbon dioxide (CO_2) treatment.

	Time (day)						
	0	**1**	**2**	**3**	**4**	**5**	**6**
pH 7.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	3.96	7.93	10.05	12.61	15.96	16.64
(b) With microbial activity	0.00	29.83	54.11	79.29	95.76	99.37	99.75
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	3.96	7.93	10.05	12.61	15.96	16.64
(d) Microbial activity (b-c) (%)	0.00	25.87	46.18	69.23	83.14	83.41	83.11
pH 8.5							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	3.85	11.49	12.17	13.63	16.18	17.32
(b) With microbial activity	0.00	33.49	57.39	80.76	97.16	99.39	99.81
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	3.85	11.49	12.17	13.63	16.18	17.32
(d) Microbial activity (b-c) (%)	0.00	29.64	45.89	68.59	83.53	83.21	82.49
pH 10.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	16.85	26.71	42.41	50.48	56.03	64.06
(b) With microbial activity	0.00	43.24	63.79	85.18	98.52	99.71	99.78
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	16.85	26.71	42.41	50.48	56.03	64.06
(d) Microbial activity (b-c) (%)	0.00	26.38	37.08	42.77	48.05	43.68	35.72

Table 5.6 The estimated percentage (%) removal of ammonium-nitrogen (NH_4^+-N) from the post-anaerobically digested (post-AD) brewery effluent through microbial activity and volatilization at 30°C in the hydrochloric acid (HCl) treatment.

	Time (day)						
	0	**1**	**2**	**3**	**4**	**5**	**6**
pH 7.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	3.96	7.93	10.05	12.61	15.96	16.63
(b) With microbial activity	0.00	20.37	50.64	71.48	93.77	99.34	99.64
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	3.96	7.93	10.05	12.61	15.96	16.63
(d) Microbial activity (b-c) (%)	0.00	16.42	42.72	61.43	81.16	83.38	83.01
pH 8.5							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	3.86	11.49	12.17	13.63	16.18	17.32
(b) With microbial activity	0.00	34.01	57.89	73.17	93.67	99.46	99.69
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	3.86	11.49	12.17	13.63	16.18	17.32
(d) Microbial activity (b-c) (%)	0.00	30.15	46.40	61.00	80.04	83.28	82.37
pH 10.0							
Mean drop in NH_4^+-N (% of starting conc.)							
(a) Autoclave (i.e. without microbial activity)	0.00	16.86	26.71	42.41	50.48	56.03	64.06
(b) With microbial activity	0.00	40.41	61.92	83.53	98.51	99.59	99.61
Estimated NH_4^+-N removal due to:							
(c) Stripping (=a) (%)	0.00	16.86	26.71	42.41	50.48	56.03	64.06
(d) Microbial activity (b-c) (%)	0.00	23.55	35.21	41.12	48.03	43.57	35.55

b. Nitrite-nitrogen

The NO_2^--N concentrations were significantly different ($p < 0.001$) at different pH levels in both pH adjustment methods (Table 5.7). Nitrite-nitrogen concentrations were greater at pH 7.0 and 8.5 than at pH 10.0 (Figure 5.9, Figure 5.10). An increase in temperature from 20 to 30°C elevated the NO_2^--N concentrations ($p < 0.001$) for both pH adjustment methods. The NO_2^--N concentrations were affected by an interaction between temperature and pH (MANOVA, $F_{(12,72)} = 28.28$, ($p < 0.001$) when microalgae cultures were treated with HCl. When CO_2 was used to adjust the pH, NO_2^--N concentrations were also influenced by an interaction of temperature and pH (MANOVA, $F_{(12,72)} = 8.22$, $p = 0.01$).

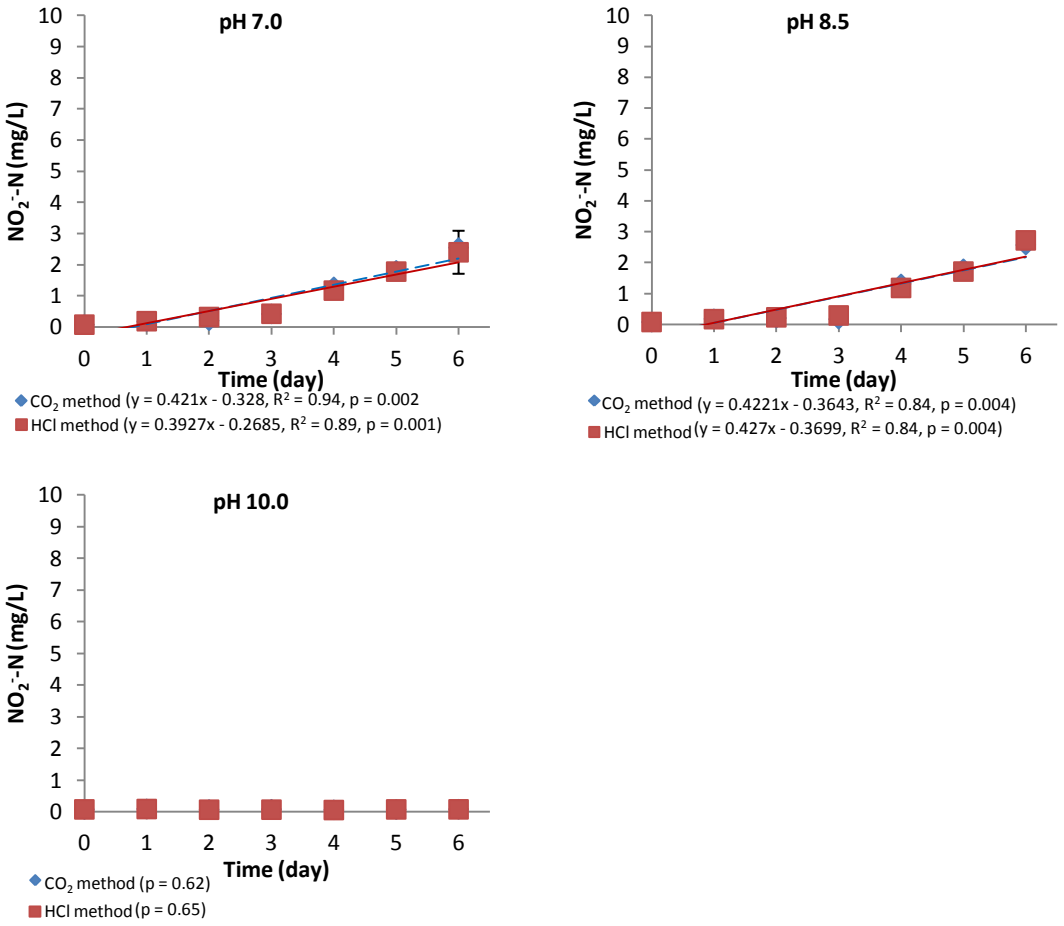

Figure 5.9 Changes in the mean (± standard error) nitrite-nitrogen (NO_2^--N) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 20°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

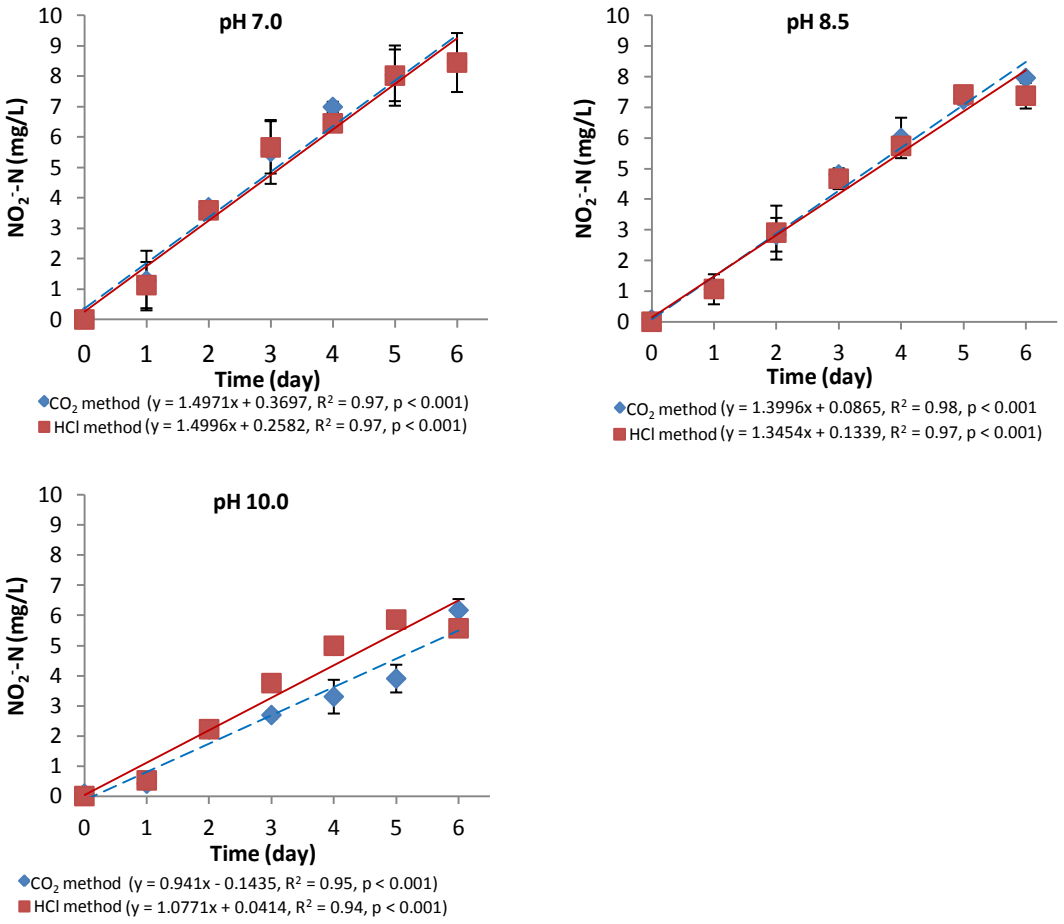

Figure 5.10 Changes in the mean (± standard error) nitrite-nitrogen (NO_2^--N) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 30°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

c. Nitrate-nitrogen

At 20 and 30°C, NO_3^--N concentrations were significantly different ($p < 0.001$) for both pH adjustment methods (Table 5.7). At 20°C, the concentration of NO_3^--N in the post-AD brewery effluent increased more at pH 7.0 and 8.5 in both pH adjustment methods (Figure 5.11). However, at pH 10.0, there was only a slight increase in NO_3^--N. Similar trends were observed when microalgae cultures were grown at 30°C. At pH 7.0 and 8.5, the concentrations of NO_3^--N also increased more than at pH 10.0 (Figure 5.12). These concentrations were affected by an interaction between temperature and pH (MANOVA, $F_{(12,72)} = 10.17$, $p < 0.001$) when microalgae cultures were treated with CO_2. In the HCl method, NO_3^--N concentrations were also influenced by an interaction of temperature and pH (MANOVA, $F_{(12,72)} = 5.82$, $p = 0.02$).

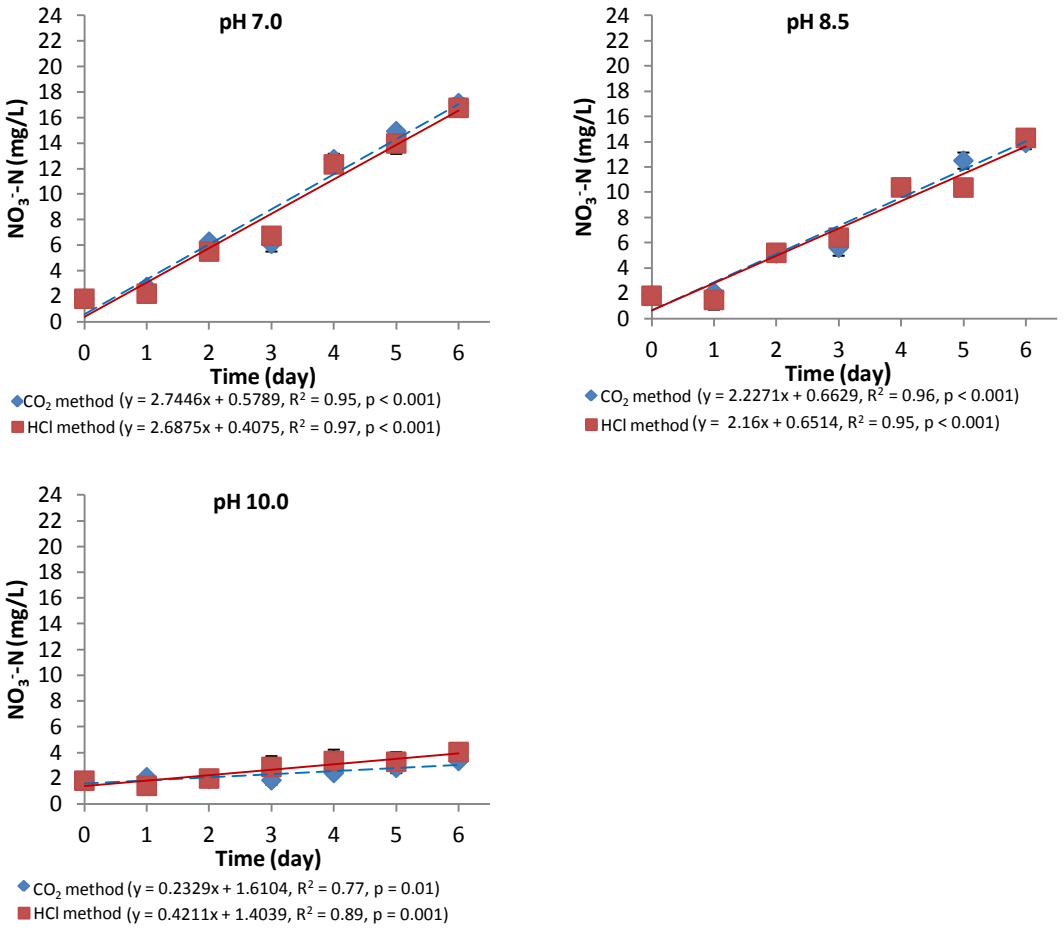

Figure 5.11 Changes in the mean (± standard error) nitrate-nitrogen (NO_3^--N) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 20°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

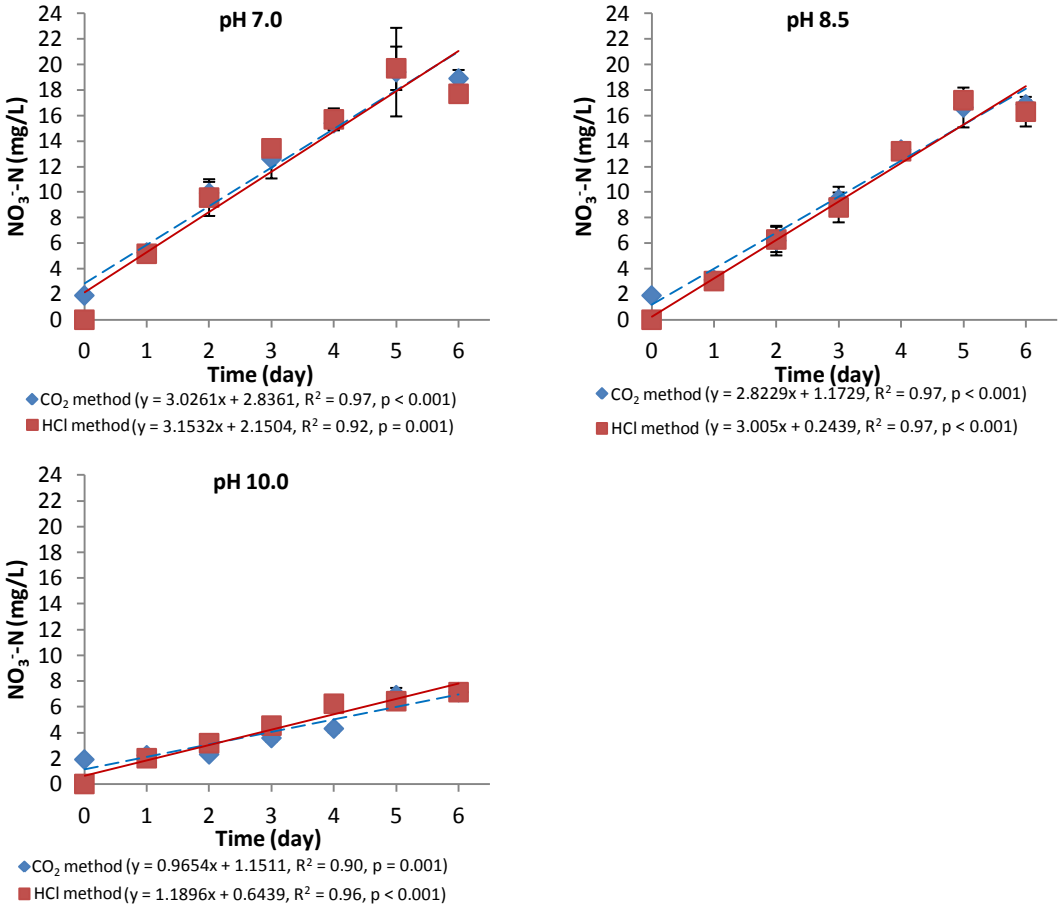

Figure 5.12 Changes in the mean (± standard error) nitrate-nitrogen (NO_3^--N) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 30°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

d. Orthophosphate-phosphorus

There was a significant difference ($p < 0.001$) in the removal of PO_4^{3-}-P with an increase in temperature (Table 5.7). About 72 to 76% of the PO_4^{3-}-P was removed when microalgae were cultured at 20°C for the CO_2 and HCl method of pH adjustment at pH 7.0, 8.5 and 10.0 (Figure 5.13). At 30°C, more than 90% of the PO_4^{3-}-P was removed at pH 7.0, 8.5 and 10.0 for both pH adjustment methods (Figure 5.14). There was also a significant difference ($p = 0.02$) at different pH levels in PO_4^{3-}-P removal when microalgae cultures were treated with CO_2, however, for the HCl method there was no difference ($p = 0.44$) in PO_4^{3-}-P removal at different pH levels.

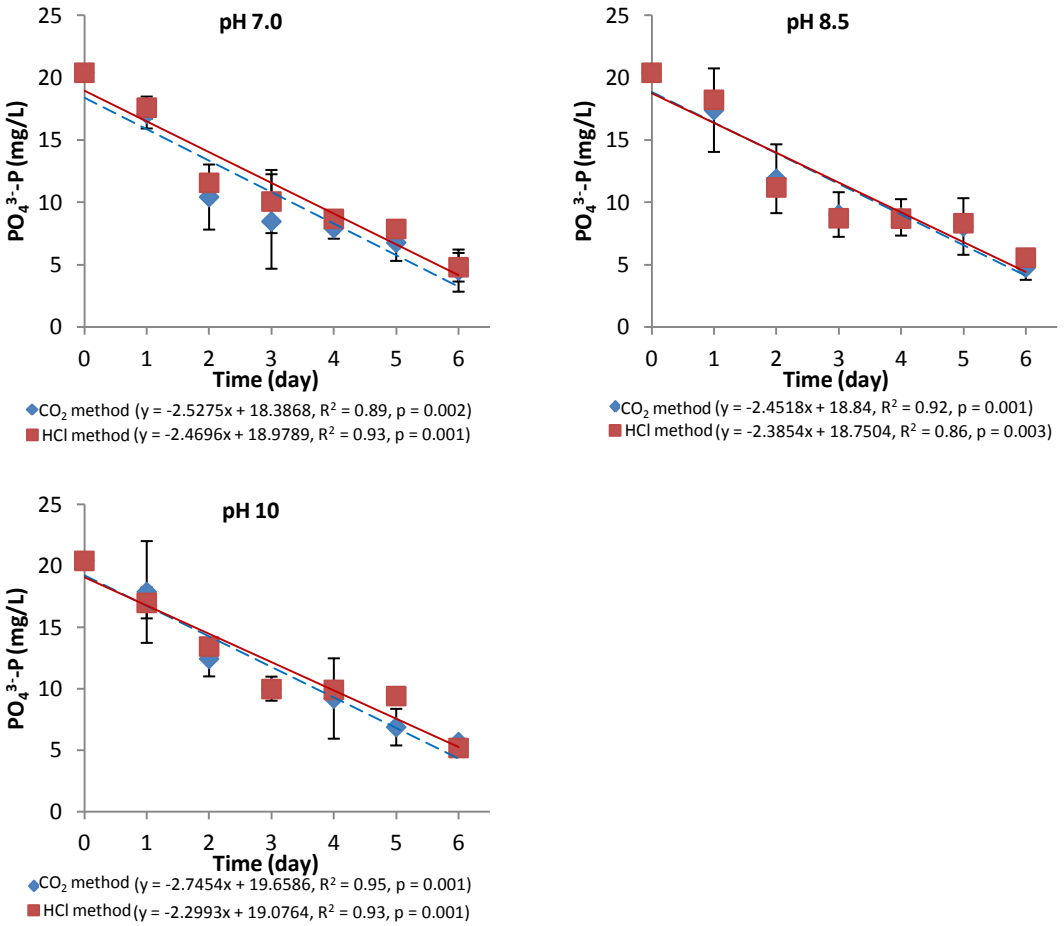

Figure 5.13 Changes in the mean (± standard error) orthophosphate-phosphorus (PO_4^{3-}-P) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 20°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

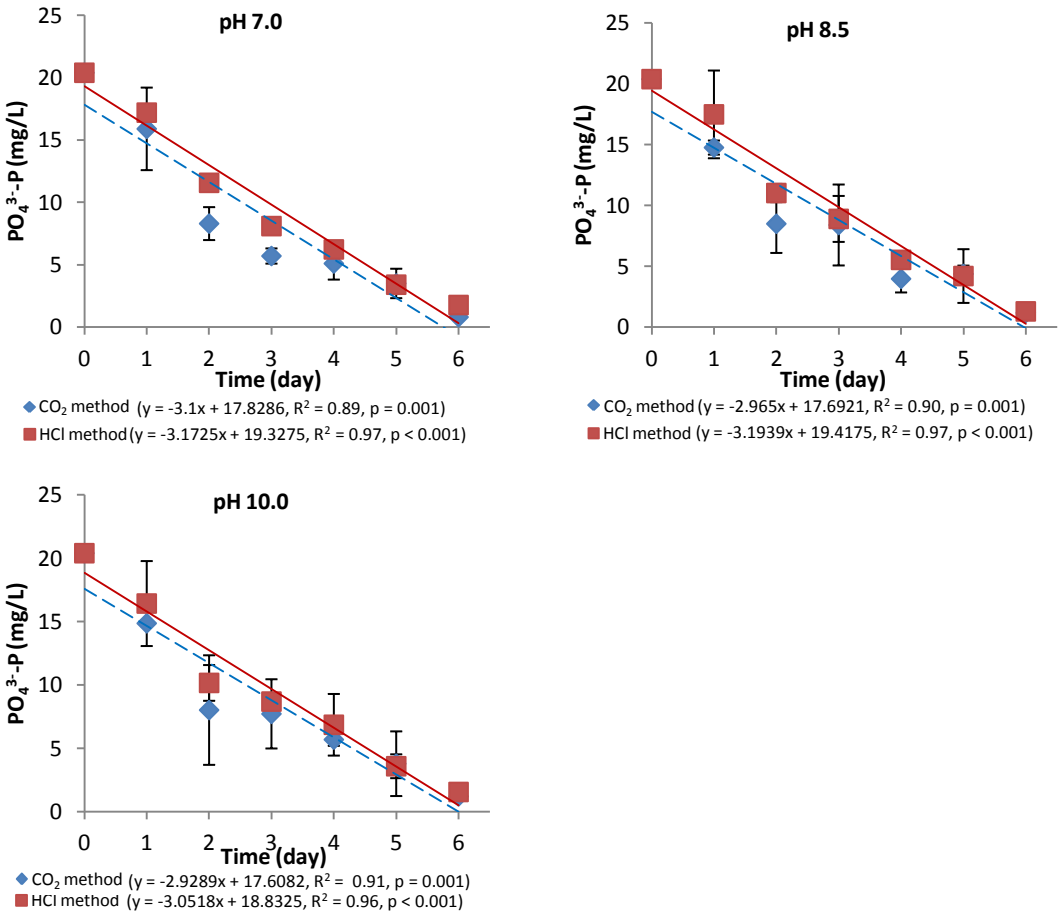

Figure 5.14 Changes in the mean (± standard error) orthophosphate-phosphorus (PO_4^{3-}-P) concentration in the post-anaerobically digested (post-AD) brewery effluent treated in microalgae cultures grown at 30°C in carbon dioxide (CO_2) and hydrochloric acid (HCl) pH adjustment methods at pH 7.0, 8.5 and 10.0.

Table 5.7 Multivariant analysis of variance (MANOVA) results of Chlorophyll (Chl) *a*, ammonium-nitrogen (NH_4^+-N), nitrite-nitrogen (NO_2^--N), nitrate-nitrogen (NO_3^--N) and orthophosphate-phosphorus (PO_4^{3-}-P) when microalgae cultures were treated with carbon dioxide (CO_2) and hydrochloric acid (HCl) as pH adjustment methods.

	CO_2 method			HCl method		
	DF	***F-value***	***P-value***	**DF**	***F-value***	***P-value***
Chl *a* (µg/L)						
pH	2	56.89	<0.001	2	72.36	<0.001
Temperature	1	202.45	<0.001	1	157.68	<0.001
pH*temperature	2	3.57	0.06	2	3.65	0.06
Residuals	12			12		
Total	36			36		
NH_4^+-N (mg/L)						
Method	6	560.15	<0.001	1	2576.20	<0.001
pH	12	10.92	<0.001	2	132.46	<0.001
Temperature	6		<0.001	1	640.90	<0.001
Method*pH	12	6.48	<0.001	2	41.28	<0.001
Method*temperature	6	42.91	<0.001	1	134.05	<0.001
pH*temperature	12	1.42	0.20	2	7.84	0.002
Method*pH*temperature	12	2.39	0.02	2	9.87	0.001
Residuals				24		
Total				144		
NO_2^--N (mg/L)						
pH	2	156.68	<0.001	2	323.97	<0.001
Temperature	1	4376.6	<0.001	1	7944.86	<0.001
pH*temperature	2	8.22	0.01	2	28.28	<0.001
Residuals	12			12		
Total	72			72		
NO_3^--N (mg/L)						
pH	2	1042.6	<0.001	2	415.81	<0.001
Temperature	1	300.78	<0.001	1	173.90	<0.001
pH*temperature	2	10.17	<0.001	2	5.82	0.02
Residuals	12			12		
Total	72			72		
PO_4^{3-}-P (mg/L)						
pH	2	6.12	0.02	2	0.88	0.44
Temperature	1	424.76	<0.001	1	162.19	<0.001
pH*temperature	2	3.16	0.08	2	2.51	0.12
Residuals	12			12		
Total	60			60		

5.4 Discussion

a. The effects of temperature and pH on microalgae growth

The maximum growth of microalgae estimated by changes in Chl *a* concentration over time was obtained at 30℃ and at a pH of 8.5 for both pH adjustment methods. The maximum Chl *a* concentrations obtained at pH 8.5 were subsequently followed by those recorded at pH 10.0. The

lowest concentrations were observed at pH 7.0. However, cultures bubbled with CO_2 grew better than those treated with the HCl method. This is comparable De Pauw & van Vaerenbergh (1983) study where the growth rate of *Oscillatoria agardhii* increased with an increase in temperature from 20℃, to 25 and 30℃. The response of Chl *a* concentrations to pH are supported by the observation of Chen & Durbin (1994) who found that the maximum growth rates of microalgae were obtained at a pH of 8.8 when the cultures were grown at a pH ranging from 7.0 to 10.0. The growth of microalgae at different pH levels is also in agreement with the findings of Fagiri *et al*. (2013) where the highest growth rate of *Arthrospira* sp. was at pH 8.0, followed by pH 7.0, and then pH 10.0. The increase in Chl *a* concentration could have been attributed to the Arrhenius effect of temperature, since temperature demonstrated to have affected microalgae growth (De Pauw & van Vaerenbergh 1983). The Arrhenius effect of temperature is a phenomenon in which an increase of 10℃ in a culture medium doubles the growth rate of microalgae (De Pauw & van Vaerenbergh 1983). However, the increase in Chl *a* concentration did not double, following the Arrhenius effect of temperature when the temperature was increased by 10℃.

The response of microalgae cultures to different pH levels was due to their pH preferences, with the optimum pH of most freshwater microalgae around eight (Park *et al*. 2011). Above and below this pH, microalgae growth declines, however, some species can grow slowly at a pH as high as 10 (Park *et al*. 2011).

Aeration could have also influenced the growth of microalgae because it keeps algal cells in suspension and distributes nutrients evenly throughout the medium, making them available for microalgae (Aydin 2007). It also exposes algal cells to favourable light conditions by allowing cells to move from highly illuminated areas to shaded areas and back (light and darkness fluctuations) (Janssen 2002). Temperature and pH had a marked effect on the growth of microalgae and this was observed for both methods of pH adjustment.

The addition of CO_2 to the culture medium also affected microalgae growth. A slight increase in Chl *a* concentrations from those treated with HCl was observed. Chen & Durbin (1994) also used HCl and bubbling of CO_2 in two parallel experiments to adjust pH in marine microalgae cultures. Results showed that the lack of bubbling CO_2 appeared to have limited the growth of microalgae where acid was used. Packer (2009) also observed different growth rates of *Chlamydomonas reinhardtii* when cultures were bubbled with and without CO_2 over 36 h. Microalgae bubbled with air containing one percent CO_2 had a Chl *a* concentration of 31 µg/mL, which was about double that of cultures bubbled with air containing 0.04% CO_2 (Packer 2009). Similar findings were also reported by Welch (2013) where *Chlorella* sp. bubbled with air had a Chl *a* concentration of 204.8 µg/L while those

bubbled with 10% CO_2 had a Chl *a* concentration of 436.6 µg/L over 11 days. Carbon dioxide serves as a source of inorganic carbon for microalgae (Karcher 2010; Markou & Georgakakis 2011). Carbon is essential in microalgae nutrition, making up about half of its dry weight (Larsdotter 2006a; Lundquist *et al.* 2010). The addition of CO_2 to microalgae culture can double algal productivity under controlled environmental conditions or even increase algal biomass by 30% in a HRAP during summer (Park *et al*. 2011). The effects of CO_2 on microalgae growth were evident in this study. However, bubbling large amounts of CO_2 for prolonged periods can have some growth inhibitory effects on microalgae as it might create an acidic environment if uncontrolled (Chen & Durbin 1994). This can negatively affect non-acidophilic species by impacting on microalgae physiology (Chen & Durbin 1994). In this study, addition of CO_2 to the culture medium enhanced the growth of microalgae, especially at pH 8.5.

b. The effects of temperature and pH on the removal of nitrogen

Temperature and pH affected the removal of NH_4^+-N from the post-AD brewery effluent for both pH adjustment methods. The removal of NH_4^+-N from the effluent was greater at 30℃ as compared to 20℃ in microalgae cultures and the autoclaved medium (no microbial activity). Ammonium-nitrogen's removal was greater at pH 10.0 than at pH 7.0 and 8.5. At 20℃, the main mechanism of NH_4^+-N removal was through microbial activity at all pH levels for the CO_2 and HCl pH adjustment methods. At 30℃, a greater portion of NH_4^+-N was removed through microbial activity at pH 7.0 and 8.5, however, at pH 10.0, the main mechanism of NH_4^+-N removal was through volatilization of unionised NH_3-N. Ammonium-nitrogen lost through NH_3-N volatilization was greater at pH 10.0, and then followed by pH 8.5 and pH 7.0 at 30℃. This is in agreement with Munezvenyu (2008) who reported that the rate of NH_3-N volatilization increased as the pH increased, with the maximum NH_3-N volatilization recorded at pH nine where there is microbial activity. Camargo Valero & Mara (2010) reported low volatilization rates of NH_3 from alkaline water with a pH of about 8.5 containing microalgae, which is comparable to the results of this study. In cultures with no microbial activity, NH_4^+-N was removed from the effluent through NH_3-N volatilization. This can be supported by the study of Camargo Valero & Mara (2010) where NH_4^+ was reported to be removed through volatilization from alkaline water with no biological activity at a pH greater than 8.5. An increase in temperature increased NH_3-N volatilization at pH 10.0 (Sevrin-Reyssac 1998; Meisinger & Jokela 2000; Zimmo 2003). The volatilization of the unionised NH_3-N could have also been enhanced by aeration that created air currents above the water surface which, increases the transfer of air between the flask and the atmosphere (Meisinger & Jokela 2000).

An increase in temperature also enhanced NH_4^+-N removal through an increase in microbial activity. In microbial activity, the removal of NH_4^+-N at pH 7.0 and 8.5 was attributed to both algal assimilation and nitrification (Sevrin-Reyssac 1998; Zimmo 2003). An increase in temperature enhances microalgae metabolism, in turn increasing nutrient uptake within the pH range of seven to 8.5, thus the observed NH_4^+-N removal, which coincided with the maximum algal growth rate at pH 8.5 (Sevrin-Reyssac 1998; Reay *et al*. 1999; Zimmo 2003; Richmond 2004*;* Park *et al*. 2011). Nitrification, which also occurs within the pH range of seven to 8.5 for nitrifying bacteria to oxidize NH_4^+-N into NO_2^--N and NO_3^--N could have been responsible for the removal of NH_4^+-N (Khin & Annachhatre 2004). This is supported by the metagenomics data, when nitrifying bacteria were observed. Nitrification is enhanced by aeration as it distributes O_2 throughout the entire culture medium, thus making it available for nitrifiers (Mata *et al*. 2012).

The concentrations of NO_2^--N and NO_3^--N in the post-AD brewery effluent were also affected by temperature and pH for both pH adjustment methods. Nitrite-nitrogen and NO_3^--N concentrations increased gradually over time, with higher concentrations found at 30 ℃. These concentrations were greater in cultures treated with CO_2. At 20℃, NO_2^--N mean concentrations were greater at pH 8.5 than at pH 7.0. However, at 30℃, NO_2^--N mean concentrations were greater at pH 7.0 as compared to pH 8.5. This is supported by Munezvenyu (2008) when higher NO_2^--N concentrations were observed at pH eight than at pH seven when investigating the effects of pH on nitrification in algae-bacterial biofilms exposed to a temperature range of 18-21℃. Nitrate-nitrogen concentrations were higher at pH 7.0 than at pH 8.5, while pH 10.0 had the lowest concentrations at both temperatures. Also at pH 7.0 and 8.5 when cultures were grown at 30℃, NO_3^--N concentrations decreased on day six. Munezvenyu (2008) observed similar trends, where there was more production of NO_3^--N at pH seven as compared to pH eight. As the pH was further increased, NO_3^--N production decreased. The elevated concentrations of NO_2^--N and NO_3^--N at pH 7.0 and 8.5 were probably due to nitrification because this is the preferred pH for nitrifying bacteria (Khin & Annachhatre 2004). The decrease in NO_3^--N concentrations on day six could imply that microalgae were utilizing NO_3^--N as source of nitrogen since NH_4^+-N was depleted (Richmond 2004). The high concentrations of NO_2^--N and NO_3^--N at pH 7.0 suggest that maximum nitrification rates occurred at pH 7.0 (Munezvenyu 2008).

c. The effects of temperature and pH on the removal of phosphorus

The removal of PO_4^{3-}-P from the post-AD brewery effluent was affected by an increase in temperature from 20℃ to 30℃ in both pH adjustment methods. However, in the CO_2 method, the PO_4^{3-}-P was reduced more rapidly than in the HCl method. At 20℃, on day six the concentrations of

pH was about 3.0 mg/L, while at 30℃ PO_4^{3-}-P was about 1.7 mg/L at all pH levels. Similar trends to this finding were observed by Powell *et al*. (2008) when microalgae were cultured at 15 to 25℃ in wastewater. There was more removal of phosphorus from the effluent at 25℃ which resulted in the accumulation of more phosphate in the algal cells compared to at the lower temperatures (Powell *et al*. 2008). It was reported that the removal of phosphate from wastewater was 15% in winter and 30% in summer in waste stabilization ponds with microalgae (Powell *et al*. 2008). The removal of PO_4^{3-}-P was probably due to algal assimilation rather than precipitation, because pH did not affect PO_4^{3-}-P removal. This was further supported by an increase in PO_4^{3-}-P removal in cultures bubbled with CO_2, which had higher Chl *a* concentrations than cultures treated with HCl. The addition of CO_2 to microalgae cultures is known to increase algal biomass, thus increasing the algal assimilation (Park *et al*. 2011). An increase in temperature enhances the luxury uptake of phosphorus by microalgae (Powell *et al*. 2008). Temperature as opposed to pH was the main role player in the reduction of PO_4^{3-}-P.

5.5 Conclusion

Microalgae growth and nutrient removal from the post-AD brewery effluent were influenced by temperature and pH. A greater portion of NH_4^+-N was removed from the effluent through microbial activity, however, with an increase of 10℃ in temperature, at pH 10.0, more of the NH_3-N was volatilized. Nitrite-nitrogen and NO_3^--N concentrations increases were more evident when the temperature was increased from 20 to 30℃ at pH 7.0 and 8.5. The removal of PO_4^{3-}-P by algal assimilation was also greatest at 30℃. The removal of nutrients from wastewater by microalgae and bacteria was influenced by temperature and pH which also influenced microalgae productivity.

Chapter six

General discussion

The microalgae and bacterial community structure and the relative abundance of the species in the high rate algal pond (HRAP) at the Ibhayi Brewery in Port Elizabeth, varied seasonally. In the pond, the algal biomass and chlorophyll (Chl) *a* concentrations increased during summer, and then decreased in winter. The algal biomass and Chl *a* concentrations began to rise again in spring. Phaeophytin (Phaeo) *a* concentrations followed similar trends to the Chl *a* concentrations. An increase in the algal biomass correlated positively with dissolved oxygen (DO) concentrations in the HRAP. The algal biomass also appeared to have increased with a rise in the pH of the effluent. The highest chemical oxygen demand (COD), recorded in summer (January) also concurred with an increase in algal biomass. When the algal biomass was maximal in summer, the removal efficiency of ammonium-nitrogen (NH_4^+-N) from the post-AD brewery effluent was greater than in the winter when there was a decline in algal biomass. Nitrite-nitrogen and NO_3^--N concentrations were higher in winter than in summer. This corresponded with the presence of the nitrifying bacterium *Nitrospira* sp. that was observed in winter and spring, and *Nitrobacter winogradskyi* and *Nitrosomonas* sp., which were found in the pond samples in spring. Orthophosphate-phosphorus (PO_4^{3-}-P) removal from the effluent had no clear seasonal trends. There was no apparent link between algal biomass and pH with PO_4^{3-}-P removal. The ionic composition of the effluent appeared to be influenced by seasonal variation, especially the electrical conductivity (EC), which escalated during summer, and then decreased in winter. The investigation of the influence of seasonal variation on microbial community structure and physicochemical parameters in the HRAP demonstrated that summer favoured microalgae growth and their performance in removing NH_4^+-N from the post-AD brewery effluent. However, in winter, microalgae growth and nutrient removal efficiency was substantially reduced. To optimise the performance of the HRAP, throughout the year, the hydraulic retention time (HRT) can be adjusted, with a shorter HRT in summer and longer HRT in winter (Larsdotter 2006a; Munoz & Guieysse 2006).

The flow rates experiment was run for a month where the HRT of the HRAP train B was progressively shortened. Shortening of the HRT resulted in a shift in the microbial community structure and their relative abundance in the HRAP B2. Algal cells were washed out of the pond when the HRT was further shortened, and this decreased the algal biomass, Chl *a* and Phaeo *a* concentrations. When the flow rate was progressively increased, NH_4^+-N was constantly removed from the effluent. There was a significant difference in the removal of NO_2^--N from the HRAP B2, however, there was no significant difference in the removal of NO_3^--N when the flow rate of post-AD brewery effluent was

increased to 2200 L/d. The removal of PO_4^{3-}-P decreased when the HRT was further shortened, which coincided with a decrease in the standing algal biomass. As the algal biomass decreased with an increase in flow rate, COD, DO and pH remained constant. Ionic constituents of the pond, as reflected by the measured EC, decreased when the HRT was gradually shortened.

Besides operational variables such as HRT, some of the important variables that affect the microbial community structure in a HRAP and nutrient removal efficiency from wastewater are temperature and pH that vary seasonally (Larsdotter 2006a, Johnson 2010; Markou & Geogakakis 2011). These abiotic factors also influenced microalgae growth and nutrient removal efficiency. The maximum growth of microalgae was obtained at pH 8.5 and at 30℃. The removal efficiency of NH_4^+-N was greater at 30℃ than at 20℃ and significantly different at pH 7.0, 8.5 and 10.0. The concentrations of NO_2^--N and NO_3^--N increased with an increase in temperature from 20 to 30℃. There was a significant difference ($p < 0.001$) in the concentrations of NO_2^--N and NO_3^--N at all pH levels. The removal efficiency of PO_4^{3-}-P increased with an increase in temperature. The removal of PO_4^{3-}-P at pH 7.0, 8.5 and 10.0 did not vary ($p = 0.44$) when hydrochloric acid was used to lower the pH of the effluent, while in the carbon dioxide pH adjustment method, PO_4^{3-}-P removal differed ($p = 0.02$) at all pH levels. This experiment clearly demonstrated that temperature and pH influences microalgae growth and nutrient removal efficiency. An increase in temperature which increased NH_4^+-N removal in Chapter 5 can explain the increase in NH_4^+-N removal efficiency during summer in Chapter 3.

The shifts observed in the microbial community structure, particularly microalgae were probably due to the seasonal variation of temperature and pH (Affan *et al*. 2005). The metagenomics study provided data that were helpful in better understanding the seasonal variation of the microalgae community structure and their relative abundance. However, there were some microalgae species that were not identified when the universal eukaryote primer set 566F and 1200R was used to amplify 18S rRNA from the deoxyribonucleic acid (DNA) template. To date, there is no universal primer set designed to cover a wide range of microalgae phyla (Arndt, *pers. comm*., Next Generation Sequencing manager, Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, August 2015). However, there are primers designed to target a specific phylum of microalgae currently available. Since the purpose of this study was to establish the identity of the entire microalgae community structure, this motivated the use of the universal primer set 566F and 1200R due to its wide coverage of the eukaryote community structure. Hadziavdic *et al*. (2014) used this universal primer pair in 454 pyrosequencing technique, and about 80% of the 18S rRNA eukaryote sequences yielded matched sequences in the SILVA database with no prokaryote sequences from marine sediment samples. The

18S rRNA of other marine sediment samples were also amplified using the universal primer set 566F and 1200R, and 71% of the sequences belonged to eukaryotes, with no prokaryote sequences generated (Hadziavdic *et al*. 2014). This primer set was the first to characterize a wide range of the 18S rRNA by generating sequences that matched all eukaryotes in the SILVA database (Hadziavdic *et al*. 2014). Thus far, it appears not much work has been done on investigating the microalgae and bacterial community structure in HRAPs. More research focused on the microbial community structure in HRAPs would contribute in the development of primer sets that would cover a wider range of the microbial community structure responsible for nutrient removal from wastewater.

Microbial activity, which includes algal assimilation and nitrification was one of the mechanisms responsible for the removal of NH_4^+-N from the post-AD brewery effluent (Sevrin-Reyssac 1998; Chen *et al*. 2003; Zimmo 2003). An increase in temperature enhances microalgae metabolism, which in turn increases nutrient uptake within the pH range of seven to 8.5, resulting in increased microalgae growth (Sevrin-Reyssac 1998; Zimmo 2003). This was demonstrated in Chapter 3, during summer, when the removal efficiency of NH_4^+-N was greater than in winter. This corresponded with an increase in algal biomass during summer and a decrease in winter. It was evident in Chapter 4 that the standing algal biomass in the HRAP influenced nutrient removal at 2200 L/d. When the algal biomass was zero, there was no apparent removal of PO_4^{3-}-P. Chapter 5 also clearly demonstrated that an increase in temperature from 20 to 30℃ enhanced nutrient removal efficiency and favoured the growth of microalgae. De Pauw & van Vaerenbergh (1983) also reported that the growth rate of *Oscillatoria agardhii* increased with an increase in temperature from 20℃, to 25 and 30℃. Maynard *et al*. (1999) and Camargo Valero & Mara (2007) reported that microalgae nutrient uptake can be the main mechanism of NH_4^+-N removal from wastewater during summer.

During nitrification, NH_4^+-N could have been oxidized into NO_2^--N and NO_3^--N. This can be supported by the nitrifying bacteria identified through metagenomics and elevated concentrations of NO_2^--N and NO_3^--N in the effluent when microalgae and bacteria consortia were cultured at pH 7.0 and 8.5 in Chapter 5. It is known that nitrification occurs within this pH range (Khin & Annachhatre 2004; Munezvenyu 2008). In Chapter 3 and 4, when the pH was between 7.0 and 8.5, nitrification could have been one of the mechanisms of NH_4^+-N removal. Microbial activity in wastewater is the dominant nitrogen removal mechanism in conditions that favour microalgae growth (Camargo Valero & Mara 2010).

Another underlying mechanism responsible for the removal NH_4^+-N from wastewater is the volatilization of the unionised ammonia (NH_3-N) into the atmosphere, especially at maximum temperatures during summer when the pH is above nine (Sevrin-Reyssac 1998; Zimmo 2003). This

was clearly demonstrated in Chapter 5, when the volatilization of NH_3-N was greater at 30℃ than at 20℃ at a pH of 10.0. However, the loss of nitrogen from wastewater through NH_3-N volatilization in algal ponds has not been considered significant when compared to the nitrogen removed through biological activity (Camargo Valero & Mara 2010). In Chapter 5, in the autoclaved medium, at pH 10.0, NH_4^+-N was removed from the effluent through NH_3-N volatilization. Camargo Valero & Mara (2010) also reported the volatilization of NH_3-N from effluent with no microbial activity when the pH was above 8.5.

The removal of PO_4^{3-}-P from wastewater could have been through algal assimilation. In Chapter 3, it was reported that there was no apparent link between PO_4^{3-}-P removal and algal biomass or pH. However, in Chapter 4 and 5, there was a link between PO_4^{3-}-P removal and algal biomass. In Chapter 5, the growth of microalgae was favoured by an increase in temperature and corresponded with an increase in the removal efficiency of PO_4^{3-}-P. This was also observed by Powell *et al*. (2008), when the removal of PO_4^{3-}-P from wastewater increased with an increase in temperature from 15 to 25℃ in microalgae cultures. This is an indication that PO_4^{3-}-P removal was through microalgal assimilation. An increase in temperature can also enhance the luxury uptake of phosphorus by microalgae (Powell *et al*. 2008).

Future work and recommendations

Future work that would add value to the understanding of the microbial community structure and nutrient removal mechanisms from post-AD brewery effluent when using HRAPs should focus on the following:

- Microalgae and bacteria must be isolated from the HRAPs and grown in nutrient media in order to obtain pure cultures so that they can be characterized using metagenomic techniques. This would allow for the development of primer sets that target a broader range of species in the microbial community in HRAPs.
- Characterize bacteria from the post-AD and post-PFP effluent as they would influence the bacterial community structure in the HRAPs.
- Controlled grazing experiments would provide further insight on the impact of zooplankton grazing on microalgae and bacteria. This would further enhance the understanding of the microbial shifts, i.e. to what extent were shifts in the microbial structure attributed to seasonal variation and also influenced by zooplankton grazing.
- The nitrification rates of NH_4^+ need to be measured since this would better elucidate the removal of NH_4^+-N through microbial activity.

- Measure the relative nitrogen/phosphorus ratios in algal biomass and link them with the removal of nitrogen and phosphorus from the effluent. Stable isotope analysis under controlled conditions could shed some light onto the fate of the different nutrients.
- Prolong timeframes between the changes in HRT and charactirize the microbial community structure at each HRT. This would provide an understanding of the changes in the microbial community structure at different HRT and the changes could be linked with nutrient removal.

It will also add value to future work if data could be collected from replicated HRAP systems. This would allow for more robust statistical analysis to be drawn between treatments, as the next phase of this research program.

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