WATER QUALITY, BIOMASS AND EXTRACELLULAR POLYMERIC SUBSTANCES IN AN INTEGRATED ALGAE POND SYSTEM

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Abstract

Integrated algae pond systems (IAPS) combine the use of anaerobic and aerobic bioprocesses to effect wastewater treatment. Although, IAPS as a technology process offers many advantages including efficient and simultaneous N and P removal, no requirement for additional chemicals, O_2 generation, CO_2 mitigation, and a biomass with potential for valorization, a lack of technological advancement and the need for large land area, has limited the reach of this technology at industrial scale. In mitigation, peroxonation was introduced as a tertiary treatment unit and its effect on COD and TSS of IAPS treated water investigated. An effort was made to characterize the soluble but persistent COD in IAPS treated water and, productivity of the HRAOP mixed liquor was investigated to gain insight into the potential use of this biomass. Results show that peroxone treatment effectively reduced COD, TSS, and nutrient load of IAPS water without any significant impact on land area requirement. Indeed, summary data describing the effect of peroxone on quality of IAPS-treated water confirmed that it complies with the general limit values for either irrigation or discharge into a water resource that is not a listed water resource for volumes up to 2 ML of treated wastewater on any given day. Extraction followed by FT-IR spectroscopy was used to confirm albeit tentatively, the identity of the soluble but persistent COD in IAPS treated water as MaB-floc EPS. Results show that MaB-flocs from HRAOPs are assemblages of microorganisms produced as discrete aggregates as a result of microbial EPS production. A relationship between photosynthesis and EPS production was established by quantification of the EPS following exposure of MaB-flocs to either continuous light or darkness. Several novel strains of bacteria were isolated from HRAOP mixed liquor and 16S ribosomal genomic sequence analysis resulted in the molecular characterization of *Planococcus maitriensis* strain ECCN 45b. This is the first report of *Planococcus maitriensis* from a wastewater treatment process. Productivity and change in MaB-flocs concentration, measured as mixed liquor suspended solids (MLSS) between morning and evening were monitored and revealed that MLSS is composed of microalgae and bacteria but not fungi. Concentration varied from 77 mg L^{-1} in September (winter) to 285 mg L^{-1} in November (spring); pond productivity increased from 5.8 g m^{-2} d^{-1} (winter) to 21.5 g m^{-2} d^{-1} (spring); and, irrespective of MLSS concentration in late afternoon, approximately 39% was lost overnight, which presumably occurred due to passive removal by the algae settling pond. The outcomes of this research are discussed in terms of the quality of treated water, and the further development of IAPS as a platform technology for establishing a biorefinery within the wastewater treatment sector.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

AFP	Advanced Facultative Pond
AIWPS	Advanced Integrated Wastewater Pond System
AOP	Advanced Oxidation Process
ASP	Algal Settling Pond
BOD	Biochemical Oxygen Demand
CFU	Colony forming Unit
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
CRF	Controlled Rock Filter
CTAB	Cetyltrimethylammonium Bromide
C/N	Carbon/Nitrogen ratio
DB	Drying Bed
DO	Dissolved Oxygen
DWA	Department of Water Affairs
DWS	Department of Water and Sanitation
EBRU	Institute for Environmental Biotechnology, Rhodes University
EC	Electrical Conductivity
ECCN	EBRU Culture Collection Number
EDTA	Ethylinediaminetetraacetic Acid
EPS	Extracellular Polymeric Substances
FC	Faecal Coliforms
FP	Fermentation Pit
FT-IR	Fourier Transformed Infrared Spectroscopy
HPLC	High Performance Liquid Chromatography
HRAOP	High Rate Algal Oxidation Pond
HRT	Hydraulic Retention Time

H_2O_2	Hydrogen Peroxide
IAPS	Integrated Algae Pond Systems
LB-EPS	Loosely Bound Extracellular Polymeric Substances
LHC	Light Harvesting Antenna Complexes
MaB-floc	Microalgal-Bacterial floc
MLSS	Mixed Liquor Suspended Solids
MPS	Maturation Pond Series
NMR	Nuclear Magnetic Resonance Spectroscopy
N/P	Nitrogen/Phosphorus ratio
$OH^{\cdot}$	Hydroxyl Radical
O_3	Ozone
PAR	Photosynthetically Active Radiation
PBS	Phosphate Buffer Saline
PCD	Programmed Cell Death
SE	Standard Error
SSF	Slow Sand Filtration
TB-EPS	Tightly Bound Extracellular Polymeric Substances
TE	Tris Ethylinediaminetetraacetic Acid
TSS	Total Suspended Solids
v/v	Volume/Volume
v/v/v	Volume/Volume/Volume
WWT	Wastewater Treatment
WWTW	Wastewater Treatment Works

Chapter 1: Literature Review

1.1 Introduction

In recent years, microalgae have emerged as alternative sources to first and second-generation biofuels (Delgado and Kafarov, 2012). This is due to the numerous benefits that can be provided such as smaller cultivation area, higher growth rate, more continuous biomass production, non-competition with food production and use of wastewater as a source of nutrients (Schenk *et al.*, 2008; Hernandez *et al.*, 2013). Despite these advantages, the economics of the processes make most algal-based technologies unviable in the long run as the cost of algae production and processing is comparatively high (Chisti, 2007).

However, studies have shown that the cost of algal biomass production can be reduced if the nutrient requirement is met through a more efficient use of the nutrients present in wastewater (Prajapati *et al.,* 2013; Hernandez *et al.*, 2013), which can be achieved using the integrated algae pond systems (IAPS). These systems are built upon effective and eco-friendly wastewater treatment with the added benefit of generating biomass for valorization. The systems were developed to maintain the advantages and mitigate the disadvantages of waste stabilization ponds to improve effluent quality (Mambo *et al.*, 2014a). High rate algal oxidation ponds (HRAOPs), as components of IAPS, provide efficient wastewater treatment and simultaneous biomass productivity of about 10 g m^2 d^{-1} (Sutherland *et al.*, 2013). Biomass generated from the system can be converted to various products such as biofuels, fertilizers as well as pharmaceuticals and nutraceuticals.

In terms of water treatment however, IAPS has performed inconsistently in the removal of chemical oxygen demand (COD) and total suspended solids (TSS) from wastewater in the past, suggesting the need for a tertiary treatment unit to give a final polish to the effluent before its eventual discharge (Mambo *et al.*, 2014b). Three tertiary treatment units including a maturation pond series (MPS), slow sand filtration (SSF) and controlled rock filter (CRF) have been studied (Mambo *et al.*, 2014b). All of these tertiary treatment processes are well accepted but contribute substantially to the footprint of the wastewater treatment process. In addition, the increased cost associated with implementation of any of these tertiary treatment systems results in very small adjustments to COD and TSS of the treated water. Thus, attention has turned to advanced oxidation processes, which have found application in the treatment and purification of potable water. These systems are reported as capable of

oxidation of organic and toxic pollutants in wastewater to products that are innocuous (Poyatos *et al.*, 2010; Swaminathan *et al.*, 2013). In the final analysis, this could be a cost effective method of reducing the footprint of IAPS and at the same time, allow for further water treatment particularly with respect to COD and TSS. In view of these potential benefits, an advanced oxidation treatment system, supplied by Puricare® International, was included in the process flow and evaluated accordingly.

To further reduce costs and improve efficiencies, there is a need to fashion out an economically feasible biorefinery concept to derive as many possible products from IAPS. A biorefinery concept implies converting biomass materials into bio-based products through a combination of both biotechnology and physico-chemical technology without damaging any product fraction (Vanthoor-Koopmans *et al.*, 2013; Safi *et al.*, 2014). This is a concept that will make IAPS a technology that, in addition to producing quality treated water for discharge, would allow for recovery of products of value for environmental, pharmaceutical, agricultural and industrial application. In this chapter, aspects of microalgae biotechnology in relation to wastewater treatment are reviewed and evaluated with a view to the recovery of biomass and high value products.

1.2 High Rate Algal Oxidation Ponds (HRAOPs)

1.2.1 Description of HRAOPs

HRAOPs (Figure 1.1) are shallow recirculating raceway-like oxygenated ponds with semicircular ends and depth between 0.2-1 m, configured as closed loop recirculation channels with flat bottom and vertical walls (Chisti, 2007; Park *et al.*, 2011; Chisti, 2016). The largest HRAOP for biomass production occupies an area of 440,000 m^2 (Chisti, 2007). Mixing and circulation are achieved by paddlewheels that operate continuously at a velocity between 0.15 m/s and 0.3 m/s (Park *et al.*, 2011). HRAOPs are used in the treatment of municipal, industrial and agricultural wastewaters. Rose *et al.* (1998) also reported the use of HRAOPs for treatment of acid- and metal- containing wastewaters. They have also been used in commercial production of microalgae (Chisti, 2016). HRAOPs are sustainable on industrial scale because of the organic matter assimilation from wastewater and concomitant biomass generation, making them the most cost-effective reactors for wastewater treatment (Rawat *et al.*, 2011; Vargas e Silva and Monteggia, 2015).

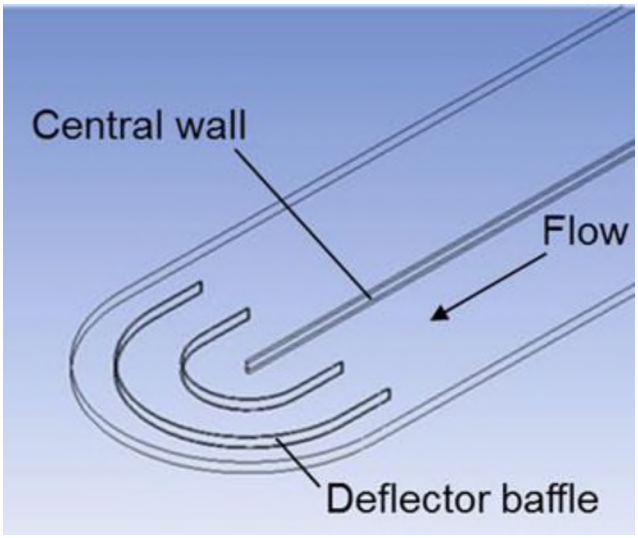

Figure 1.1: A typical high rate algal oxidation pond (Chisti, 2016).

1.2.2 Role of HRAOPs in wastewater treatment

HRAOPs are components of advanced integrated wastewater pond system (AIWPS^{TM}) proposed in 1950s by W. J. Oswald and C. G. Golueke for BOD, suspended solids and pathogen removal (Rawat *et al.*, 2011). The process was developed to maintain the advantages (simplicity and cost effectiveness) and mitigate the disadvantages (poor effluent quality, potential for odour and limited nutrient and pathogen removal) of conventional wastewater treatment systems (Mambo *et al.*, 2014a). A typical IAPS consists of:

(i) A deep advanced facultative pond (AFP) that incorporates an anaerobic digester that partially reduces the organic load through fermentation process,

(ii) HRAOPs for further reduction of organic load, disinfection and algal biomass production for beneficial use and,

(iii) Algal settling ponds (ASPs) for separating the algal cells from the treated water (Banat *et al.*, 1990).

HRAOPs promote the symbiotic relationship between algae and aerobic bacteria, each utilizing the major metabolic products of the other (Oswald *et al.*, 1955). The continuous mixing of the pond provides profuse algal growth, resulting in a high rate of photosynthesis, elevated pH (>11), and increased dissolved oxygen (3 times saturation) for the aerobic bacteria (Cowan and Render, 2012). The gentle paddlewheel mixing also maintains the surface velocity required to keep the algae and algal flocs in suspension near the surface for maximum light penetration, prevent sedimentation of biomass and better diffusion of nutrients for cell growth (Rawat *et al.*, 2011; Sutherland *et al.*, 2015a). Most nutrients in this

pond are assimilated into the algal biomass as indicated in the flow chart (Figure 1.2). At elevated pH, nutrient load is reduced, phosphate is precipitated and ammonia in the gas or volatile form is stripped off (Cowan and Render, 2012). Another unique contribution of HRAOP is the daily elevation of pH in the pond that provides 100% kill of *E. coli* and presumably most pathogenic anaerobic bacteria and as a result, provides a high disinfection rate (Ertas and Ponce, 2005). The combined activity of photosynthetic oxygenation by algae and oxidation by bacteria in HRAOPs provide remediation of wastewater and added benefit of biomass generation that can be removed periodically and used for its high fixed nutrient value.

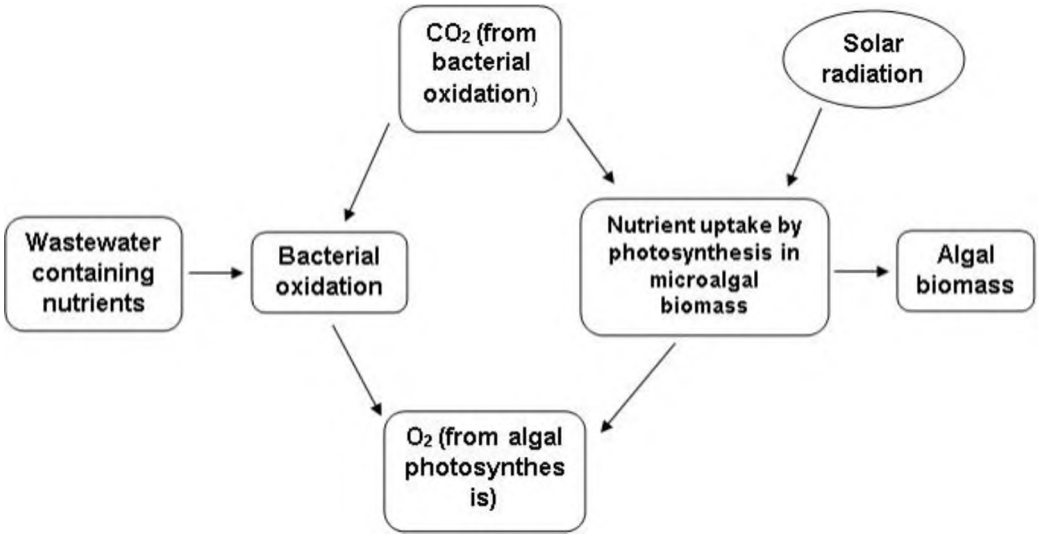

Figure 1.2: Photosynthetic carbon and nutrient flow in HRAOP (Andersson *et al.*, 2011).

Performance of HRAOPs in wastewater treatment is adequate for reduction of organic load, but data on nutrients, total suspended solids (TSS) and coliform removal is inconsistent (Park *et al.*, 2011). Mambo *et al.* (2014b) conducted extended research on performance of IAPS in municipal wastewater treatment. Their results confirmed that the system performed well in terms of reducing COD and nutrient concentration, but TSS and coliforms were above the South African discharge limits. However, it was noted that only the average value of COD data collected was in compliance and that individual values did not consistently comply with discharge standards. The high levels of COD and TSS were attributed to programmed cell death (PCD) in HRAOPs and ASPs resulting in elevation of these values (Mambo *et al.*, 2014b). COD remains an important indicator of water treatment quality and it is pertinent to note that discharge of treated water with inconsistent COD concentration may have a negative impact on receiving water bodies.

1.3 Microalgae and Biomass Production in HRAOPs

1.3.1 Biomass production in HRAOPs

Biomass produced in HRAOPs is mainly algae. Since HRAOPs are open, contamination is inevitable as they are exposed to various particles and debris (Chisti, 2016). Algae generally grow photoautotrophically using solar energy although some heterotrophic growth occurs in the dark. However, in HRAOPs, algae combine the two modes of nutrition for a mixotrophic growth. The mixotrophic growth mode is typical of wastewater treatment HRAOPs, believed to give higher productivity than pure photoautrophic growth due to the presence of dissolved organic compounds contributing to cell growth (Chisti, 2016). The perceived advantages of microalgae as mentioned earlier make them to be a target for scientific studies on biomass energy production and industrial applications (Al Darzins *et al.*, 2010).

Use of HRAOPs for commercial production of algae for industrial purposes dates back to 1960s (Chisti, 2016). Such ponds usually make use of freshwater and fertilizer for cell growth. However, algal production coupled with wastewater treatment has more advantages over the former. Taking biofuel production from microalgae as an example, operating HRAOP with potable water creates a water footprint, consumes more energy and results in higher greenhouse gas emissions (Park *et al.*, 2011). Therefore, algal production using wastewater is cost effective as the nutrients and medium are readily available; after all, it is just wastewater, which needs to be discharged.

Biomass productivity in HRAOPs varies greatly due to the vagaries of weather. Highest production is achieved in summer while it is relatively low in winter. Productivity of 25 g m^{-2} d^{-1} biomass or higher is achievable in HRAOPs especially those treating wastewater (Park *et al.*, 2011; Chisti, 2016). However, when weather condition is unfavorable as is the case in winter, productivity can decline to as low as 10 g m^{-2} d^{-1} (Chisti, 2012). On the other hand, when climatic conditions change, algae species composition and pond operation may vary, and productivity may range from 12-40 g m^{-2} d^{-1} (Park *et al.*, 2011). Because of contamination by unwanted microorganisms, poor mixing, inefficient use of carbon dioxide and high evaporation rate, biomass productivity remains low in HRAOPs (Chisti, 2007; Christenson and sims, 2011). Despite these drawbacks, HRAOPs remain the preferred algal production system given their low capital investment (Chisti, 2016).

1.3.2 Biochemical composition of microalgae

Microalgae are regarded as a promising sustainable energy resource due to their capacity to accumulate large quantities of lipids, proteins, carbohydrates, pigments, vitamins and minerals (Mata *et al.*, 2010; Gonzalez-Fernandez *et al.*, 2012). The major elements in microalgae are carbon, hydrogen, oxygen, nitrogen and phosphorus. The approximate composition of algal biomass is $C_{106}H_{181}O_{45}N_{15}P$ (Andersson *et al.*, 2011; Park *et al.*, 2011). The protein and carbohydrate contents in various strains of microalgae are up to 50% with lipid contents around 40% on dry weight basis (Singh and Gu, 2010). Table 1.1 shows the biochemical composition of some microalgae.

Table 1.1: Composition of some microalgae (% dry matter).

Algae	Protein	Carbohydrate	Lipid
Chlorella vulgaris	51-58	12-17	14-22
Chlamydomonas reinhardtii	48	17	21
Chlorella pyrenoidosa	57	26	2
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Scenedesmus obliquus	50-56	10-17	12-14
Spirogyra sp.	6-20	33-64	11-21
Arthrospira maxima	60-71	13-16	6-7
Spirulina platensis	46-63	8-14	4-9
Synechococcus sp.	63	15	11
Porphyridium cruentum	28-39	40-57	9-14
Anabaena cylindrica	43-56	25-30	4-7

Source: Becker (2007).

Proteins are important in the biochemistry of microalgae. They are involved in growth, repair, and maintenance of the algal cell. Almost 20% of total protein is bound to the cell wall, 50% is internal while 30% migrates in and out of the cell (Safi *et al.*, 2014). Lipids are compounds soluble in non-polar solvents but insoluble in water. Microalgae are mainly composed of glycolipids, phospholipids, triacylglycerol, hydrocarbons, and free fatty acids during optimal growth conditions for nutritional purposes. However, under stress or unfavorable growth conditions such as nutrient starvation, the lipid content of microalgae are exceptionally high (Safi *et al.*, 2014). Thus, microalgae cultured under such conditions are suitable for

commercial biodiesel production. Carbohydrates are one of the most important sources of energy for microalgae, comprising of starch, glucose, cellulose and various polysaccharides (Yen *et al.*, 2013; Safi *et al.*, 2014). The starch and glucose can be used in biofuel production such as bioethanol while the polysaccharides have many downstream applications in food, textiles, cosmetics, thickening agents and clinical drugs (Yen *et al.*, 2013). Microalgae contain a number of pigments for photosynthetic reactions, which gives them their colorful appearance. The major classes of pigments in algae are chlorophylls, carotenoids and phycobilins (Yen *et al.*, 2013). The chlorophylls are greenish pigments that absorb energy from sunlight while carotenoids are yellow, orange or red accessory pigments that serve as photo protectors against photo-oxidative damage (Yen *et al.*, 2013). These pigments possess therapeutic properties due to high antioxidant activity that fortifies the immune system, prevents chronic disease such as cancer and regulates blood cholesterol (Yen *et al.*, 2013; Safi *et al.*, 2014).

1.3.3 Factors affecting biomass productivity

Maintaining the microalgal/bacterial community in HRAOPs is important in order to achieve efficient wastewater treatment and high biomass production. However, biomass productivity in HRAOPs is limited by environmental, operational and biological conditions such as pH, temperature, retention time, nutrient and light availability among others (Sutherland *et al.*, 2015a; Mehrabadi *et al.*, 2016). These factors may differ depending on the type of wastewater and location of the treatment facility. In addition, algal growth depends on the type of species because different algal species tolerate different conditions (Pittman *et al.*, 2011). The major factors are as follows:

- **Light availability**

The two main factors that affect biomass productivity are irradiance and temperature (Picot *et al.*, 1993; Chisti, 2016). Nutrients can be stored and recycled by cells, but photons can only be absorbed once and have to be transformed immediately to chemical energy or dissipated non-photochemically (Sutherland *et al.*, 2015a). Photosynthetically active radiation (PAR) that saturates in HRAOPs for photosynthesis does not exceed 10-20% (200 $\mu E\ m^{-2}\ s^{-1}$) of the maximum PAR of 2000 $\mu E\ m^{-2}\ s^{-1}$ (Park *et al.*, 2011; Chisti, 2012). Therefore, biomass production can be inhibited at higher or lower light saturations than mentioned above.

The amount of light available for photosynthesis depends on the degree of attenuation in the pond, self-shading within the cell, size of the cell and pigment concentration in the cell. Microalgae use light harvesting antenna complexes (LHC) for capturing light (Sutherland *et al.*, 2015a). When attenuation is high, cells near the surface of the pond experience supersaturation, leading to excess photons, dissipated as heat or fluorescence to prevent photo-damage. However, cells near the bottom receive little or no light; they increase their LHC to capture the available light for photosynthesis. This leads to self-shading where absorption efficiency decreases with increasing chlorophyll content.

Light availability also depends on the biomass concentration in the pond, as high concentration also affects the amount of light reaching the bottom of the pond, which leads to self-shading (Sutherland *et al.*, 2015a). On the other hand, the cells near the surface can experience high amounts of radiation which can overpower the LHC leading to photoinhibition, decreased photosynthetic rate and cell damage (Christenson and Sims, 2011; Park *et al.*, 2011; Chisti, 2016). Paddlewheel mixing and turbulent flow provide vertical mixing to ensure that cells in the deeper zones of the pond are exposed to light (Park *et al.*, 2011). Studies have been focused on the utilization of light in microalgae using genetic approach. Mussgnug (2007) reported that reducing the size of antenna (light harvesting proteins) enhances photosynthetic efficiency, leading to higher light utilization. However, the cell's ability to dissipate excess photons is reduced leading to vulnerability of cells with reduced antenna size to photo-damage (Sutherland *et al.*, 2015a).

- **Temperature**

Temperature can affect biomass production in HRAOPs and sometimes even the biochemical composition of the generated biomass (Chisti, 2016). Temperature varies in HRAOPs seasonally and diurnally and its control is not practicable. Optimum temperature regime for most microalgal species is between 15°C and 25°C (Sutherland *et al.*, 2015a). When temperature is below this range, photosynthesis saturates at lower light intensities while temperature above this range increases respiration and photorespiration, which reduces algal productivity (Chisti, 2016; Park *et al.*, 2011). Optimal temperature can vary when there is limitation of nutrient and light conditions, which can also affect pH, O_2 and CO_2 solubility in the pond water (Park *et al.*, 2011; Sutherland *et al.*, 2015a).

- **Nutrient availability**

Microalgal biomass is composed of about 50% carbon, which is essential for growth (Sutherland *et al.*, 2015a). Light energy absorbed during photosynthesis is converted to chemical energy for CO_2 assimilation for the formation of carbohydrate molecules. Carbon limitation in wastewater treatment HRAOPs is due to the low C/N ratio (4-7:1) as compared to 15:1 required by algal biomass (Park *et al.*, 2011). During daytime when CO_2 concentration is reduced due to elevated pH (and temperature), it affects the inorganic carbon equilibrium because of increased carbonates and bicarbonates which also contributes to carbon limitation. This manner of carbon limitation affects photosynthesis, nitrogen removal efficiency from wastewater and hence, biomass production (Sutherland *et al.*, 2015a). CO_2 availability in HRAOPs depends on organic matter oxidation by heterotrophic bacteria (Park *et al.*, 2011). Addition of CO_2 gas has been demonstrated to improve wastewater treatment and biomass production in HRAOPs (Park and Craggs, 2010). Such addition brings about increased carbon concentration in wastewater for algal growth, and controls the pH of the water when less than 8.0 (Park *et al.*, 2011). It also improves light absorption, rate of photosynthesis and biomass yield (Sutherland *et al.*, 2015b).

Apart from carbon, other nutrients such as nitrogen and phosphorus are also essential for microalgal growth. Nitrogen and phosphorus have been shown to decrease photosynthesis and the ability of the cells to dissipate excess photons (Palmer *et al.*, 2013). Phosphorus rarely limits algal growth in wastewater when compared to nitrogen. However, nitrogen can limit algal growth even when carbon and light are not limiting (Sutherland *et al.*, 2015a). An N/P ratio of 16:1 is required for algal growth, which is attainable in wastewater treatment HRAOPs (Park *et al.*, 2011). Nutrient load into the HRAOPs can also affect removal efficiency as well as discharged water quality. When low, all the nutrients will be assimilated into biomass (Sutherland *et al.*, 2015a). However, lower nutrient load for improved water quality is at the expense of biomass yield, therefore nutrient load alteration will depend on the desired outcome, either wastewater treatment or biomass production. However, high biomass production and quality wastewater treatment is achievable when there is more than one HRAOP operating in series.

- **pH**

pH of HRAOPs affects the metabolic activity of the microbial community such as respiration, ionic composition of the water, and hence biomass productivity (Park *et al.*, 2011). HRAOP

pH increases to about 11 during the day due to photosynthetic activity, which indicates carbon is limitation in the culture medium, and decreases in the night due to respiration. Optimal pH for algal growth is 8 depending on the species, but some algae such as *Ankistrodesmus* sp. can grow at pH 10 (Park *et al.*, 2011). When pH is high in HRAOPs, there is volatilisation of ammonium to free ammonia and, phosphate precipitation, though this enhances nutrient removal from wastewater, it inhibits photosynthesis and reduces the growth of microalgae in the pond (Park *et al.*, 2011; Sutherland *et al.*, 2015a). Aerobic bacteria are also inhibited at high pH resulting in less organic matter oxidation to CO_2 (Sutherland *et al.*, 2015a). Addition of CO_2 controls pH and as well serve as source of carbon in HRAOPs. Its absorption by algae is reduced when pH is below 8 (Chisti, 2016).

- **Grazers and pathogens**

Culture contamination in HRAOPs is inevitable. Grazing by protozoa, zooplankton and viral infections can be a major problem and reduce algae concentration and production within a few days (Park *et al.*, 2011). Contamination with heterotrophic bacteria is also inevitable especially in wastewater treatment HRAOPs since they are open ponds. They are not necessarily harmful to microalgae since they cohabit in the environment (Chisti, 2016). The pathogenic microorganisms also compete with microalgae for essential nutrients present in wastewater (Pittman *et al.*, 2011). The only pragmatic method that inhibits growth of grazers in HRAOPs is adjusting pH to 11 (Park *et al.*, 2011). Alternatively, microfiltration can be used which is rather expensive bearing in mind sustainability and cost effectiveness. Therefore, Proper management is recommended as a possible way of reducing contamination in HRAOPs (Chisti, 2012).

1.3.4 Floc formation in HRAOPs

Harvesting accounts for 20-60% of total production cost of microalgae (Van Den Hende *et al.*, 2014; Barros *et al.*, 2015). Therefore, harvesting still remains a major setback for industrial production and applications. However, because of microalgae bacteria floc (MaB-floc) formation, biomass in HRAOPs settles rapidly in ASPs under gravity, giving high quality effluent for discharge. One method of harvesting microalgae is by flocculation using chemical flocculants, auto or bio flocculation (Safi *et al.*, 2014). Chemical flocculants are known to increase cost of production and contribute to contamination of the microalgae hindering its downstream application (Barros *et al.*, 2015). Auto and bio flocculation on the other hand are more or less natural methods of harvesting biomass. Auto-flocculation is the

spontaneous increase in pH of a culture medium leading to changes in microalgae cell surface properties, thereby accelerating settling (Vandamme *et al.*, 2013; Safi *et al.*, 2014; Barros *et al.*, 2015). Bio-flocculation is also a spontaneous process due to secretion of biopolymers (i.e. extracellular polymeric substances) by microorganisms, which cause aggregation of cells into flocs and, hence all biomass in the form of flocs can be more easily harvested and recovered. The latter method is cost effective in wastewater treatment and in biomass recovery systems because it does not result in further pollution of either the treated water or the resultant biomass. Formation of MaB-flocs in HRAOPs is therefore due to an association between microalgae and bacteria due to production of extracellular polymeric substances (EPSs) which is enhanced by elevated pH.

1.4 Extracellular Polymeric Substances

1.4.1 What are EPSs?

Extracellular polymeric substances (EPSs), also regarded sometimes as exopolysaccharides due to the high polysaccharide content, are polymers secreted by microorganisms into the surrounding medium as a result of cell lysis, cell degradation, metabolism and reaction to adverse environmental conditions (Sheng *et al.*, 2010; More *et al.*, 2014). In wastewater treatment systems, EPS matrices are regarded solely to be responsible for aggregation of cells to form flocs that facilitate settleability (Sheng *et al.*, 2010). The components of an EPS matrix are diverse depending on the source and method of EPS extraction, with carbohydrates and proteins being the major components (75-90%). Other components include humic substances, lipids, uronic acid, and nucleic acids (Sheng *et al.*, 2010).

EPSs are produced by both prokaryotic (archaea, bacteria) and eukaryotic (phytoplankton, algae, fungi) microorganisms. However, the most studied have been bacteria. There is limited research on EPS production by microalgae most especially green algae, as cyanobacteria have been the most studied so far. The few microalgae species reported in the literature for EPS production include *Chlorella, Dunaliella, Scenedesmus, Micractinum, Oscillatoria* and the diatoms (Staats *et al.*, 1999; Parikh and Madamwar, 2006; Mishra and Jha, 2009; Wang *et al.*, 2014). Due to the chemical and physical nature of these biopolymers, they are materials of biotechnological importance with application in bioremediation, and the food and pharmaceutical industries. This has therefore increased the rate of demand and interest for these natural polymers (Singha, 2012).

1.4.2 Characteristics of EPS

EPS is composed of high molecular weight compounds secreted by microorganisms, products resulting from cell lysis and hydrolysis of macromolecules (Sheng *et al.*, 2010). Some organic matter from the influent wastewater may also form part of the EPS matrix (Wang *et al.*, 2014). Molecular weight of EPS ranges from <0.5 kDa - >300 kDa (Kunacheva and Stuckey, 2014) with the largest reported reaching 2 MDa (Kehr and Dittmann, 2015).

EPS is distributed both inside (bound) and outside (soluble) microbial aggregates (Sheng *et al.*, 2010). As shown in Figure 1.3, soluble EPS is dissolved in the medium while bound EPS can either be loosely or tightly bound to the cell (Sheng *et al.*, 2010; Ahmed *et al.*, 2014) and, separated by centrifugation. Soluble EPS are unstructured, not associated with the cells but rather dispersed in the culture medium. Loosely bound EPS (LB-EPS) is less structured, less concentrated, less dense and not so tightly attached to the cells. Tightly bound EPS (TB-EPS) on the other hand is highly structured, very dense, concentrated and tightly attached to cells (Ahmed *et al.*, 2014; Ding *et al.*, 2015).

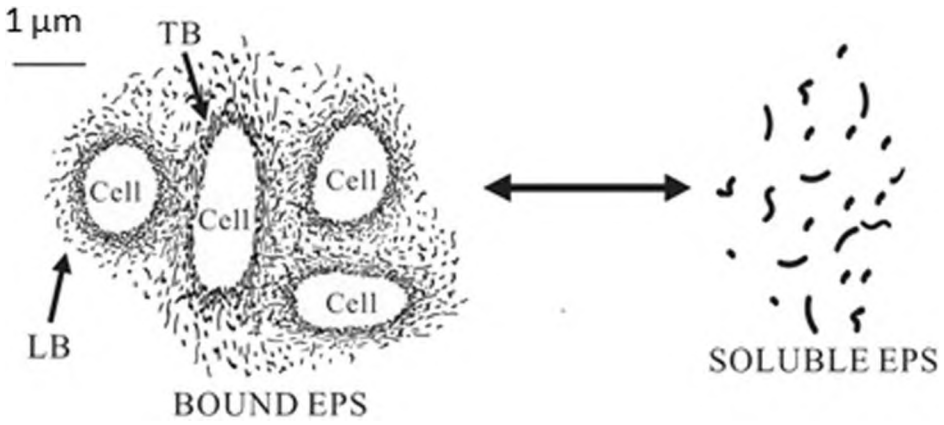

Figure 1.3: Structure of EPS distribution (Nielsen and Jahn, 1999).

EPS in wastewater treatment is an important component in biofilm formation (Sheng *et al.*, 2010). It strengthens the interaction between microorganisms, enhances settleability and sludge retention, facilitates attachment of cells to solid surfaces, and serves as a protective barrier for cells from biotic and abiotic stresses such as heavy metals, toxins, antibiotics, grazers and pathogens (Sheng *et al.*, 2010; Ahmed *et al.*, 2014; More *et al.*, 2014; Kehr and Dittmann, 2015). EPS (especially TB-EPS) binds to cells to form a very strong net-like matrix that protects against desiccation, shear intensity, and also serves as a source of carbon in cases of nutrient shortage (Sheng *et al.*, 2010; Yuan *et al.*, 2014; Ding *et al.*, 2015).

EPS have some important properties such as biodegradation, adsorption and hydrophobicity/hydrophilicity (More *et al.*, 2014). They have a strong binding capacity with heavy metals (lead, copper, nickel, zinc etc.), proteins, carbohydrates and organic pollutants (Pereira *et al.*, 2011; More *et al.*, 2014). This is due to the presence of many negatively charged functional groups like carboxyl, hydroxyl, phenolic, and phosphoric as binding sites (Sheng *et al.*, 2010; Pereira *et al.*, 2011). Soluble EPS is known to have higher binding capacity for metals than bound EPS because it contains a higher fraction of protein than bound EPS (Sheng *et al.*, 2010; More *et al.*, 2014). Research conducted by Micheletti *et al.* (2008) also revealed the presence of carboxyl and amide groups as the major band shifts upon DRIFT spectrometry analysis of soluble EPS after contact with Cu^{2+}, suggesting these functional groups as the most important binding sites for metals. Because of the hydrophobic/hydrophilic characteristic they possess, EPS can adsorb organic pollutants such as humic substances, phenanthrene, dyes, pesticides and benzene by electrostatic interaction (Sheng *et al.*, 2010; More *et al.*, 2014).

Another important characteristic of EPS in wastewater treatment systems is biodegradation. Degrading enzymes are abundant in these systems. They digest the large organic polymers of EPS into smaller molecules that are readily available to cells in case of nutrient limitation. (Park and Novak, 2007; Sheng *et al.*, 2010; More *et al.*, 2014). However, there are some fractions of EPS that are not degradable by microorganisms due to chemical structure or hydraulic retention time of the treatment system, which may not be long enough for total degradation (More *et al.*, 2014; Kunacheva and Stuckey, 2014). Biodegradation of EPS may lead to deflocculation of microbial aggregate, and the non-degradable parts may contribute to poor quality effluent in wastewater treatment systems (Sheng *et al.*, 2010; Kunacheva and Stuckey, 2014).

Some EPS molecules e.g. hydrophobic carbohydrates, ester-linked acetyl-groups, and aromatics and aliphatics in proteins are hydrophobic in nature because they are unable to form hydrogen bonds with water (More *et al.*, 2014). This helps in adsorption of organic pollutants and causes the whole EPS or parts to aggregate and/or separate from the culture medium (Sheng *et al.*, 2010; More *et al.*, 2014). On the other hand, some parts of microbial EPS such as carboxyl, hydroxyl, phenolic and phosphoric groups are hydrophilic. As such, EPSs are amphoteric in nature (Sheng *et al.*, 2010; More *et al.*, 2014).

1.4.3 EPS in biofilm formation

EPS producing organisms are abundant in environments with high amounts of organic substance (Singha, 2012), wastewater being a good example. Therefore, microbes can grow profusely and form aggregates due to the release of EPS, which aids settleability and dewatering of biomass. EPS is known as the major cause of well-structured aggregates and adhesion of organisms to solid surfaces, hence their stability (Sheng *et al.*, 2010). EPS molecules i.e. exopolysaccharides, exogenous proteins and nucleic acid but particularly exopolysaccharides, are said to be responsible for the morphology of biofilm, due to their high content (about five times more than proteins) (Czaczyk and Myszka, 2007). However, accumulation of EPS in these treatment systems results in fouling due to pore clogging and floc adhesion. This affects performance and effluent quality of many systems (Kunacheva and Stuckey, 2014; More *et al.*, 2014).

The microbial interactions resulting from EPS production have been explained by various mechanisms including the divalent cation bridging theory and the alginate theory. In the divalent theory, modelled by Higgins and Novak (1997), negatively charged sides of EPS macromolecules (especially proteins) bind with divalent cation e.g. Ca^{2+} and Mg^{2+}. This forms a very strong EPS matrix; hence, a well-structured biofilm (Ding *et al.*, 2015). Alginate-like EPS plays an important role in the gelation of EPS because of the gelling property and hydrophobicity. Microbes form alginate-like EPS that cross-link with divalent cations and thus bind cells together to form biofilm (Ding *et al.*, 2015). Other mechanisms of biofilm formation include lectin binding; EPS contain lectin-like proteins that play an important role in interaction and binding of cells (Ding *et al.*, 2015; Kehr and Dittmann, 2015).

1.4.4 EPS as an indicator of high COD in wastewater treatment systems

Efficiency of a wastewater treatment system can be evaluated by its ability to remove organic matter and pollutants, measured as COD. This test is usually reported as the amount of oxygen demanded for chemical oxidation of susceptible organic pollutants in a known volume of sample (Cowan *et al.*, 2016). Therefore, COD indirectly measures the amount of organic compounds present in the sample. COD in effluent of wastewater treatment systems are mostly soluble microbial products of which about 20% are EPS. This affects the performance and quality of final effluent from these systems (Kunacheva and Stuckey, 2014). As EPS contains some organic components, this indicates that high COD content may be due

to EPS in the sample. Persistence of COD in wastewater could therefore be attributed to EPS release into the medium by the indigenous microorganisms.

According to Jang *et al.* (2007), during wastewater treatment about 91% of COD in a submerged membrane bioreactor originates from carbohydrate and protein of soluble microbial products i.e. EPS. Aquino *et al.* (2009) also pointed out that about 45-63% of effluent soluble COD is produced by biomass as soluble microbial product (SMP) in demonstration-scale upflow anaerobic sludge blanket reactors (UASB) for treatment of raw wastewater. Organic loading rate and hydraulic retention time seem to have a direct relationship with EPS in biological treatment systems. When a treatment system is overloaded, microorganisms are not able to degrade all organic matter such that when the organic concentration is low, they decompose and contribute to EPS concentration in the system. Therefore controlling the organic load in these systems could help achieve minimum EPS and as such, a considerable decrease in COD levels in order to achieve quality and reusable water (Barker and Stuckey, 1999; Kunacheva and Stuckey, 2014).

1.4.5 Algal EPS production

EPS is not unique to bacteria and a number of algae ranging from chlorophytes, diatoms and blue-greens have been reported as abundant EPS producers. Mishra and Jha (2009) extracted and characterized EPS from *Dunaliella salina* under salt stress. Monosaccharide analysis by high performance liquid chromatography (HPLC) confirmed the presence of glucose, fructose, galactose and xylose. Fourier transformed infrared spectroscopy (FT-IR) analysis of the EPS also revealed the presence of carbohydrates. In another report on the same extracted EPS, nuclear magnetic resonance spectroscopy (NMR) analysis also confirmed the presence of amine and aromatic compounds, uronic acids, halides and sulphides (Mishra *et al.*, 2011). Different fractions of EPS produced by *Arthrospira platensis* strain MMG-9 were extracted and quantified by Ahmed *et al.* (2014). The eight monosaccharides detected included glucose, galactose, xylose, rhamnose, and fucose and these were common to all fractions. Other cyanobacteria studied for EPS production include, *Cynothece* sp., *Oscillatoria* sp., *Nostoc* sp. and *Nostoc carneum* (Parikh and Madamwar, 2006) and in these, the monosaccharide composition was similar to that mentioned above.

EPS production by diatoms was investigated by Staats *et al.* (1999) who reported that *Navicula salinarum* and *Cylindrotheca closterium* produced the highest EPS during transition

from exponential to stationary growth phase. Glucose and xylose were the main monosaccharide constituents while others such as galactose, mannose, and rhamnose were detected in smaller quantities. In addition, two Chlorophyte species, *Chlorella vulgaris* and *Micractinium* sp. were studied for their ability to produce EPS in wastewater with different nutrient concentrations. Both species were able to produce higher amounts of protein EPS than polysaccharide EPS in high-strength wastewater probably due to higher nitrogen content of the medium (Wang *et al.*, 2014). EPS released by microalgae have applications as antiviral agents, health foods, antioxidants, bioflocullants and anti-inflammatory agents (Raposo *et al.*, 2013).

Table 1.2: EPS production, extraction, and yield from some algae.

Algae	Medium	Yield	Extraction method	Reference
Dunaliella salina	De Walne's medium	944 mg L^{-1}	Ethanol extraction	Mishra and Jha (2009)
Cyanothece sp.		870 mg g^{-1}		
Oscillatoria sp.	BG 11/ASN III	700 mg g^{-1}	Acetone extraction	Parikh and Madamwar (2006)
Nostoc sp.		685 mg g^{-1}		
Nostoc carneum		560 mg g^{-1}		
Arthrospira platensis	Spirulina medium	561 mg g chl^{-1}	Centrifugation and EDTA extraction	Ahmed *et al.* (2014)
Chroomonas sp.	Seawater enriched with nutrients	7.22 mg L^{-1} day^{-1}	Phenol-sulphuric acid after centrifugation	Bermudez *et al.* (2004)
Cylindrotheca closterium	Kester medium	0.14 µg 10^6 cells day^{-1}	Ethanol extraction	Staats *et al.* (2000)
Oscillatoria augustissima		1,630 µg mL^{-1}		El-Sheekh *et al.*

Anabaena PCC 7120		476 μg mL^{-1}		(2012)
Scenedesmus obliquus	Allen's and Stainer's medium	378 μg mL^{-1}	Ethanol extraction	
Chlorella vulgaris		232 μg mL^{-1}		
Cyanothece sp.	F/2 medium	22.34 g L^{-1}	Ethanol extraction	Chi *et al.* (2007)

Microalgae also produce EPS as a defensive mechanism against stressful conditions. In a study conducted by El-Sheekh *et al.* (2012), four species of microalgae/cyanobacteria; *Anabaena* sp., *Oscillatoria augustissima*, *Scenedesmus obliquus,* and *Chlorella vulgaris* were tested for ability to sustain toxins produced by *Mycrocystis aeruginosa.* All the organisms were able to produce EPS as a response to cyanobacterial toxins. In addition, cyanobacteria and microalgae due to their ability to produce EPS can adsorb metals into their cell wall. An experiment by Van Hille *et al.* (1999) reported that EPS production has a great influence on the ability of *Spirulina* (*Arthrospira platensis*) cultured in HRAOP to adsorb metal ions from acid mine water. In their study, EPS was produced under nutrient stress conditions and was able to chelate metal ions particularly copper (94% removal) at low EPS concentration. The copper ions were soluble and remained in the overflow of treated water (Van Hille *et al.*, 1999). Rose *et al.* (1998) also reported about 40% metal removal by EPS fraction of *Spirulina* sp. (*Arthrospira platensis*) in acid mine drainage treatment under stress condition.

EPS production in wastewater treatment systems can also improve settleability. Park *et al.* (2013) demonstrated that recycling the liquid fraction of gravity harvested algal biomass of HRAOP treating domestic wastewater promoted algal-bacterial aggregation (>500 μm) and improved settleability of algae (>80%) due to release of EPS. This indicates that EPS production in settling pond of IAPS due to stressful conditions can increase the harvestability and recovery of algae from the system.

1.4.6 EPS composition

EPS is composed of repeating units of monosaccharides. Molecular composition pattern may vary depending on the culture medium, growth phase of organisms and extraction method adopted (Sheng *et al.*, 2010). However, in wastewater treatment systems, EPS composition

cannot be species dependent since it is a mixed culture environment (Ding *et al.*, 2015). EPS matrices are usually between 0.2-1.0 μm thick or less in some bacterial EPS (Czaczyk and Myszka, 2007). Carbohydrates, proteins and their derivatives are the major components of EPS. Other components such as lipids (phospholipids), uronic acid, humic-like substances and nucleic acids have also been reported (Ding *et al.*, 2015). The proportion of each component depends on the method of extraction, analytical tool used, type of wastewater, growth phase, and process parameter (Sheng *et al.*, 2010). Distribution and composition of EPS is heterogeneous, depending on the type of medium, operational conditions, microbial aggregate type, structures, and origin (Sheng *et al.*, 2010; Ding *et al.*, 2015).

Though microbial extracellular carbohydrates (exopolysaccharides) contain hexoses, pentoses, deoxyhexoses and sugar acids, they are mainly hexoses and pentoses with other substituents such as formates, phosphates, pyruvates, acetate esters, and succinates (Czaczyk and Myszka, 2007; Poli *et al.*, 2011; More *et al.*, 2014). Microbial exopolysaccharides consist of either homopolysaccharides or heteroplolysaccharides. The homopolysaccharides are neutral and made up of only one monosaccharide type either D-glucose or L-fructose. They are divided into three groups: α-D-glucans, β-D-glucans and fructans. Some of the microbial homopolysaccharides include dextran (glucose that contain consecutive α-(1, 6)- links in the chain), curdlan (glucose with β-(1,3)- linkage) and cellulose (a repetitive unit of D-glucose with β-(1,4)- linkage). Repeated units of monosaccharide, typically about 5-8 monosaccharides with exceptions in some cyanobacteria that reach about 15 monosaccharides as building blocks form heteropolysaccharides such as alginates, xanthan, gellan and hyaluronic acid (Kehr and Dittmann, 2015). EPS proteins contain 40-60% hydrophobic amino acids (Czaczyk and Myszka, 2007) with considerable amounts of protein as enzymes that degrade EPS components during starvation by acting on the EPS of the same organism or other species present in the substrate (More *et al.*, 2014). EPSs also contain proteins that are non-enzymatic (structural proteins) and some examples include lectins and polyamides (More *et al.*, 2014). Lectins are found in the matrix of activated sludge flocs, which help in bacterial aggregation.

EPS contain some extracellular DNA especially in wastewater (More *et al.*, 2014), and its secretion depends on the type of organism. Secretion of extracellular DNA is attributed to competent-signalling peptides, which support horizontal gene transfer in biofilm structure (Czaczyk and Myszka, 2007; More *et al.*, 2014). EPSs also contain lipids mainly as

phospholipids and lipid derivatives such as lipopolysaccharides (More *et al.*, 2014). There are also biosurfactants like surfactin and vicosin in EPSs component, which help in dispersal of hydrophobic substances in the medium (More *et al.*, 2014). Humic substances are an essential part of EPS especially in biological wastewater treatment systems but are not typically secreted by microorganisms. Rather, these are adsorbed by the biofilm matrix and influence some important properties of EPS such as adsorption and biodegradability (More *et al.*, 2014).

1.4.7 Factors that trigger EPS production

EPS production depends on different parameters and its application in biotechnology relies on identifying parameters that influence the synthesis and ways of optimizing production. C/N ratio is identified generally as the primary factor affecting EPS production (Pereira *et al.*, 2009; Czaczyk and Myszka, 2007). However, some of the parameters that are also important include other nutrients in the medium, temperature, pH, growth phase of the organism and aeration (Pereira *et al.*, 2009; Singha, 2012).

The nature of substrate and medium composition has been reported by many to influence EPS production and composition. Although some researchers still argue that, there is no effect (see More *et al.*, 2014). Microorganisms utilize carbohydrates as sources of carbon, and ammonium salts and amino acids as a source of nitrogen to synthesize exopolysaccharides. High glucose content (up to 70%) in the culture medium is the most efficient for EPS production, but other forms of carbohydrate such as fructose, lactose, xylose, and maltose can also influence EPS production, depending on the type of microorganism. Some microorganisms find one source of carbon more favourable for cell growth and EPS production than another (Czaczyk and Myszka, 2007; More *et al.*, 2014). In a study reported by Yuksekdag and Aslim (2008), *Lactobacillus* and *Streptococcus* species produced highest EPS with glucose as compared to other carbon sources investigated. In the same study, different concentrations of glucose were investigated and the highest glucose concentration stimulated EPS production. Similarly, Cerning *et al.* (1994) also reported a remarkable EPS yield from *Lactobacillus casei* CG11 with glucose rather than other carbon sources.

Differing C/N ratio has been reported in the literature to be causative due to the variation in the carbon-nitrogen source and type of microorganism involved (More *et al.*, 2014). Liu *et al.* (2010) reported C/N ratio of 0.5 to be the optimum while Ye *et al.* (2011) reported 20 to be

the most favourable C/N ratio for EPS production. Low C/N ratio increases the protein content of EPS while high C/N ratio favours carbohydrates. However, some researchers found that wastewater with low C/N ratio gave EPS with a high proteins/carbohydrates ratio (Sheng *et al.*, 2010). Low nitrogen concentration is considered to have a positive impact on EPS synthesis because it increases C/N ratio thereby providing enough carbon needed for EPS production (Pereira *et al.*, 2009). Blue green algae can use combined nitrogen sources or atmospheric nitrogen fixation in some cases. A combined nitrogen source is the best because of less energy needed for assimilation compared to atmospheric nitrogen (Pereira *et al.*, 2009). However, EPS production depends on the nitrogen source used for preparation of the medium and to some extent, the species or strain of microorganism (Pereira *et al.*, 2009). In addition, high nitrogen content in the culture medium brings about high exogenous protein content in the EPS produced.

Phosphate is a key growth parameter in aquatic environments. Phosphate concentration generally does not really affect EPS production, but in some cases, PO_4^{2-} starvation just like nitrogen can increase EPS production (Pereira *et al.*, 2009). Trace amounts (<1 mg/L) of other elements such as manganese, iron, copper, nickel, iodine, zinc and boron and vitamins such as B1, B2, B6, B12, K, biotin and niacin are also required to stimulate microbial growth and EPS production (More *et al.*, 2014).

Aeration is an important factor when it comes to algae production. Continuous mixing of the medium provides aeration and turbulence. This enhances release of EPS onto cell surfaces for new ones to be synthesized. It also improves light penetration hence photosynthetic activity of the cells (Pereira *et al.*, 2009). Aeration is also very important as oxygen limitation or depletion can suppress EPS production. High dissolved oxygen increases carbohydrate content of EPS with no change in protein content, whereas, low dissolved oxygen keeps both carbohydrates and protein at the same concentration (Shin *et al.*, 2001). Both aeration and turbulence are attributes of paddlewheel driven HRAOPs, therefore confirming this pond as a potential source of EPSs.

Temperature and pH are also important parameters to consider for EPS production. The estimated optimal temperature for EPS production is between 26 and 31°C (Czaczyk and Myszka, 2007). Reducing temperature by 10°C below optimum inhibits EPS production by microorganisms. However, optimum temperature required for microbial growth and EPS production could be different. Therefore, it is important to know the best temperature for both

with respect to species or strain (More *et al.*, 2014). Effect of temperature on EPS production could also be strain dependent; higher temperature (between 30 and 40^0C) tends to increase EPS production in some strains while it has no effect or decreases EPS production in other strains (Pereira *et al.*, 2009). The effect of pH depends on the type of microorganism, medium composition, and operational conditions (More *et al.*, 2014). Between pH 2.0-3.0 and >10, EPS synthesis is inhibited and pH 5.0-7.0 is therefore considered the optimum for maximum EPS production (Czaczyk and Myszka, 2007; More *et al.*, 2014).

Microbial community can also influence EPS production. Most EPS production is reported with single culture systems. However, the interaction between microbes is also important and therefore needs consideration. Co-cultivation of microorganisms or mixed culture can stimulate EPS production, improve biofilm growth, and flocculating efficiency of the produced EPS (Okaiyeto *et al.*, 2013; More *et al.*, 2014; Kehr and Dittmann, 2015). Viscosity, concentration and molecular weight of EPS in mixed culture is said to be higher than that of single culture (More *et al.*, 2014). Okaiyeto *et al.* (2013) recovered more EPS in consortium of two bacteria than from the individual strains. In addition, in a wastewater treatment system such as HRAOP, certain organisms might produce certain nutrients required by other microbes, thereby improving EPS production. Mixed culture condition can also increase the substrate utilization rate, hence increase EPS yield (More *et al.*, 2014). However, EPS concentration of mixed culture can also be very low especially in a natural environment.

Other conditions that can affect EPS production are retention time, light intensity, metal concentration, growth phase, and toxic substances. Some researchers conclude that solid retention time has a positive correlation with EPS production and its components i.e. EPS increases with increasing retention time (Pereira *et al.*, 2009). However, some find EPS production independent of retention time (Sheng *et al.*, 2010). Continuous light or light-dark cycles do not seem to affect quality or composition of EPS (Pereira *et al.*, 2009). However, EPS production is enhanced under continuous light and at a high intensity, depending on the culture volume and geometry of the bioreactor/flask (Pereira *et al.*, 2009). The relationship between EPS production and growth cycle depends on the type of organism. Production can occur during the endogenous stage, stationery phase or exponential phase (More *et al.*, 2014). EPS content also increases or decreases with cultivation time depending on the growth phase, type and strain of microorganism (Sheng *et al.*, 2010). High concentration of Ca^{2+} and Mg^{2+} increase protein content in EPS but decreases at high Na concentration (Higgins and Novak,

1997), while iron concentration also alters the components of EPS (Sheng *et al.*, 2010). Toxic substances stimulate microbial aggregates into producing more EPS, which serve as a form of protection (Sheng *et al.*, 2010).

1.4.8 Biotechnological applications of EPS

While EPS have some detrimental effects on wastewater treatment systems, these products still hold much beneficial potential in biotechnology. Because of absorption and adsorption properties, EPS has found application in environmental biotechnology for degradation of organic substances, denitrification of wastes, treatment of industrial and municipal wastewater and water purification (Czaczyk and Myszka, 2007; More *et al.*, 2014). EPS has been suggested as an efficient bioflocculant, serving as an alternative to conventional chemical polymers for flocculation, dewatering and settling of sludge and wastewater. EPS compounds are gaining interest in food industries to improve viscosity of food, as gelling, suspending and emulsifying agents, to produce low calorie food, hydration of food products, to inhibit crystal formation i.e. in ice cream, to improve water retention in confectionaries and as edible coatings to protect food from spoilage (Czaczyk and Myszka, 2007; Singha, 2012). Some EPS such as that from lactic acid bacteria are generally recognised as safe (GRAS) due to their anti-tumour and cholesterol lowering ability. They are therefore used in the dairy industry to improve texture of dairy products (Poli *et al.*, 2011).

Algal EPS can be considered suitable for biotechnological applications due to their reproducible physicochemical properties, stable cost and supply (Chug and Mathur, 2013). Because these organisms can be grown easily and can thrive in various environmental conditions, industrial scale production for various applications is possible (Chug and Mathur, 2013). Nutrient requirement for EPS production accounts for about 30% of the cost (Poli *et al.*, 2011). The required nutrients are abundant in wastewater and make algal-based wastewater treatment (WWT) systems like IAPS ideal technologies with which to maximize the cost-effectiveness of production.

1.5 Aims and Objectives

IAPS is a technology that was developed for passive wastewater treatment and which has subsequently assumed significance due to its ability to produce novel by-product streams that are being (re)investigated and evaluated at the water-energy-food nexus. Although, there are reports on performance of HRAOPs for water treatment and algae production (Park *et al.*,

2011; Kim *et al.*, 2014; Surtherland *et al.*, 2015a), successful utilization of this technology is dependent on environmental conditions and geographical location. In addition, a recent appraisal of IAPS as a wastewater treatment technology for implementation is South Africa revealed that COD and TSS concentrations were above the discharge standard and that reduction of these required tertiary treatment (Cowan *et al.*, 2016). Thus, a cost effective, small footprint alternative tertiary treatment process is required to reduce both COD and TSS in IAPS-treated water. Even so, the substance of the residual COD/TSS might constitute a product(s) with potential for beneficiation and this too, requires investigation.

The aims of this project were therefore to:

1. Study the potential of using an advanced oxidation process as a tertiary treatment unit for IAPS to reduce COD and TSS;
2. Investigate productivity in HRAOPs of an IAPS treating domestic sewage to give insight into the potential of the biomass for use in a biorefinery;
3. Quantify, extract and characterize EPS generated in HRAOP as a potential high value product.

Chapter 2: Advanced Oxidation as a Tertiary Treatment unit for IAPS

2.1 Introduction

Potable water is a scarce commodity. Water scarcity is on the increase in part due to water pollution. Unfortunately, South Africa is a water scarce country presently and therefore, there is continued need to avoid eutrophication of water bodies and to cultivate an attitude of water recycle and reuse. To achieve this, wastewater treatment systems must comply with the General Standard (Table 2.1) for effluent discharge as prescribed by the National Water Act (1998) and the recommendations set by the Department of Water and Sanitation (DWA 2013).

As discussed in Chapter 1, IAPS is a modification of AIWPS^{TM}, which relies on the combined activity of anaerobic fermentation in the AFP coupled with photosynthetic oxygenation by algae and biological oxidation by bacteria in HRAOPs to remediate domestic wastewater. Together, these processes provide the basis for primary and secondary wastewater treatment (Green *et al.*, 1995; Downing *et al.*, 2002; Mambo *et al.*, 2014a). The Belmont Valley IAPS in Grahamstown, Eastern Cape is a pilot scale system that was installed in 1996 to demonstrate the technology. Since then, the system has been subjected to myriad of research activities geared towards its improvement for wastewater treatment (Rose *et al.*, 2007; Mambo *et al.*, 2014a & b; Cowan *et al.*, 2016).

A report by Rose *et al.* (2007), following the commission of the system concluded that the water quality from the system does not meet requirement specified by the Department of Water and Sanitation (DWS) for COD and nutrient concentration. To overcome this issue therefore, Mambo *et al.* (2014a) proposed the incorporation of a tertiary treatment system to ensure that the final treated water would comply with the General Authorization for discharge (Table 2.1). Published results showed that nutrient concentration in IAPS effluent did indeed comply with standard, but TSS and COD concentration remained inconsistent (Mambo *et al.*, 2014b).

A tertiary treatment unit is typically part of all WWT systems including IAPS and is required to provide improved water quality prior to discharge and reuse (Green *et al.*, 1995). Maturation ponds series are one such conventional tertiary treatment unit for IAPS (Oswald,

1990; Green *et al.*, 1996; Craggs *et al.*, 2012). Others include sand filtration, rock filtration, constructed wetlands and more recently, peroxone treatment (Xu and Goddard, 2002).

Table 2.1: General Authorisation limits for discharge to the environment as specified by the Department of Water Affairs (DWA, 2013).

Variables and substances	General limit	Special limit
Faecal coliforms (CFU 100 mL^{-1})	1000	0
COD (mg L^{-1})	75	30
pH	5.5-9-5	5.5-7.5
Ammonia (ionised and unionised) as Nitrogen (mg L^{-1})	6	2
Nitrate/ Nitrite as Nitrogen (mg L^{-1})	15	1.5
Chlorine as free chlorine (mg L^{-1})	0.25	0
Suspended solids (mg L^{-1})	25	10
Electrical conductivity (mS m^{-1})	70 mS/m above intake to a maximum of 150 mS/m	50 mS/m above background receiving water, to a maximum of 100 mS/m
Ortho-phosphate (mg L^{-1})	10	1(median) and 2.5 (maximum)
Fluoride (mg L^{-1})	1	1
Soap, oil or grease (mg L^{-1})	2.5	0
Dissolved arsenic (mg L^{-1})	0.02	0.01
Dissolved cadmium (mg L^{-1})	0.005	0.001
Dissolved chromium (VI) (mg L^{-1})	0.05	0.02
Dissolved copper (mg L^{-1})	0.01	0.002
Dissolved cyanide (mg L^{-1})	0.02	0.01
Dissolved iron (mg L^{-1})	0.3	0.3
Dissolved lead (mg L^{-1})	0.01	0.006
Dissolved manganese (mg L^{-1})	0.1	0.1
Mercury and its compounds (mg L^{-1})	0.005	0.001
Dissolved selenium (mg L^{-1})	0.02	0.02
Dissolved zinc (mg L^{-1})	0.1	0.04
Boron (mg L^{-1})	1	0.5

Peroxone, a combination of ozone and hydrogen peroxide, is a strong oxidizing agent usually used for water and wastewater treatment. Ozone is produced from oxygen exposed to high voltage current, generating hydroxyl radicals that oxidize organic compounds into simpler

and less harmful or lower chain compounds otherwise impossible with the conventional systems (Zhou and Smith, 2002; Gogate and Pandit, 2004b; Cesaro *et al.*, 2013;). It is also very effective at destroying viruses and bacteria and decomposes back to oxygen rapidly without leaving harmful by products. This is why it is gaining much attention in utilization than the conventional chlorination, which is toxic to aquatic life even at low concentration (Abdel-Raouf *et al.*, 2012). Although not commonly used in municipal wastewater treatment due to high demand, ozone also removes colour and odour in addition to removal of coliforms and pathogens that are more resistant. It also partakes in coagulation via reaction with humic substances in municipal wastewater (Zhou and Smith, 2002). Hydrogen peroxide is equally effective in pollutant degradation, but is a slow oxidant. It has been used to reduce BOD, COD, and odour from domestic wastewater in the past (Ksibi, 2006). However, with the combination of either ozone, UV light, or iron, its effectiveness is greatly improved (Gogate and Pandit, 2004b).

While O_3 and H_2O_2 perform individually, combination of the two (i.e. O_3/H_2O_2 or peroxone) results in generation of very powerful free radicals with faster oxidation reaction, and is now regarded as an advanced oxidation process (Gogate and Pandit, 2004a). Advanced oxidation processes (AOPs) employ reactive oxidizing agents in the treatment of organic and inorganic substances in water (Achille and Yilian, 2010). The reaction of H_2O_2 with O_3 facilitates the formation of highly reactive radicals in water, thereby enhancing degradation of pollutants (Andreozzi *et al.*, 1999; Zhou and Smith, 2002; Gogate and Pandit, 2004b). Peroxone is widely used due to its simplicity and cost effectiveness in generation of radicals for oxidation of micropollutants, removal of odour, and colour from wastewater (Zhou and Smith, 2002).

IAPS was developed as a cost effective and ecofriendly means of sewage treatment. Therefore, any tertiary treatment unit to be incorporated must be chosen on this basis. Although other tertiary treatment systems such as a MPS, SSF, and CRF are suitable and have been reported for their efficiency in COD and TSS removal (Mambo *et al.*, 2014b), each has a very large footprint in terms of land requirement (Zhang, 2012; Gikas and Tsihrintzis, 2014). Therefore, incorporation of such large footprint units will increase the cost of implementing IAPS at commercial scale. In light of this concern, an advanced peroxone system with smaller land requirement was selected as a viable alternative. In this chapter, the effect of a peroxone generating system as a tertiary treatment unit for IAPS is investigated.

Furthermore, the ability of this system to specifically reduce COD, TSS, pathogens, and nutrient load in IAPS-treated water was examined.

2.2 Materials and Methods

2.2.1 IAPS Configuration

The IAPS used in this study is located at the Institute for Environmental Biotechnology Rhodes University (EBRU), Belmont Valley Municipal Wastewater Treatment Works, Grahamstown, South Africa (33° 19′ 07″ South, 26° 33′ 25″ East). The system continuously treats a maximum of 75 m^3 d^{-1} domestic sewage and comprises of an 840 m^2 advanced facultative pond (AFP), two 500 m^2 high rate algal oxidation ponds (HRAOPs) and two 12.5 m^2 algal settling ponds (ASPs) (Figure 2.1). The AFP incorporates a fermentation pit (225 m^3) with hydraulic retention time (HRT) of 20 d and 3 d respectively. Raw sewage enters the system through the fermentation pit (FP) some 6 m below water level, where anaerobic biodegradation of suspended and dissolved solids takes place. Effluent from AFP decants under gravity to the first HRAOP with 2 d HRT. Mixing and turbulent flow essential for nutrient uptake and biomass productivity are achieved by an eight-bladed paddlewheel powered by an electric motor (0.25 kW). Effluent from HRAOP A flows into ASP A where half of the effluent (37.5 m^3) is pumped back to HRAOP B after settling for 0.5 d. HRAOP B has HRT of 4 d before effluent flows by gravity to ASP B for another 0.5 d where biomass is recovered. Algae slurry is pumped into drying beds (DB) and treated water pumped back to the wastewater treatment works (WWTW).

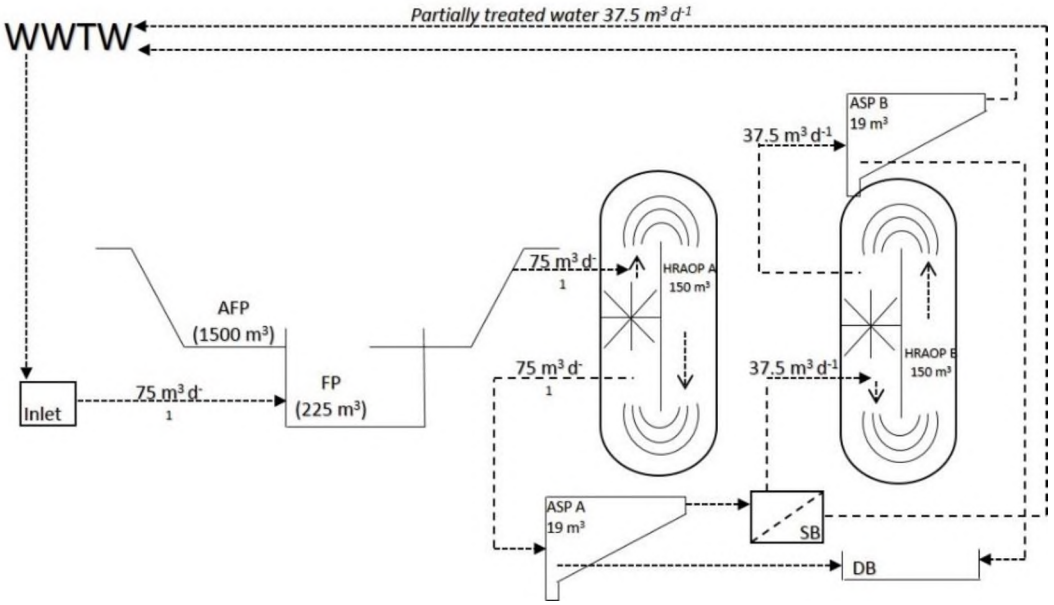

Figure 2.1: Configuration and flow diagram of the pilot scale IAPS at Belmont Valley wastewater treatment works. AFP=Advanced facultative pond; FP=Fermentation pit; HRAOP=High rate algal oxidation pond; ASP=Algal settling pond; SB=Splitter box; DB=drying bed.

2.2.2 Configuration of the peroxone system

Puricare® International, South Africa, supplied the advanced peroxone system used in this study (Figure 2.2). The system produces ozone by exposing filtered atmospheric air to UV light energy (254 nm; 55-90 Wm^{-2}) splitting O_2 into oxygen radicals or singlets (O^-). The generated O_3 is then coupled with H_2O_2 and infused into the water to be treated.

Treatment was in batch mode and IAPS-treated water collected into a reservoir using a 10,000 L Jojo tank. IAPS-treated water was pumped to the reservoir from the sump of the splitter box that collects and returns treated IAPS effluent to the Belmont Valley WWTW. The collected water was circulated using a high pressure pump (200 kPa: AquaDrive 1100, Speck Pumps South Africa) and H_2O_2 introduced at a rate of 13 ± 5 mL h^{-1} using a peristaltic pump (Pharmacia Fine Chemicals, Sweden) via the proprietary O_3 and H_2O_2 contact point of the Puricare® system for the periods specified in Results.

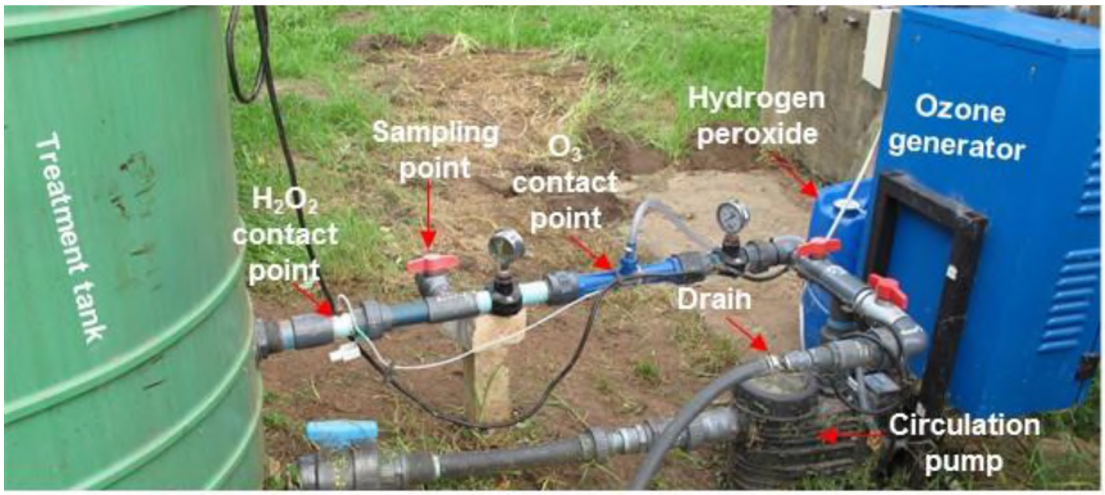

Figure 2.2: Configuration of O_3/H_2O_2 system for batch treatment of IAPS effluent.

2.2.3 Process optimization

To improve the efficiency of the peroxone system, volume of IAPS water to be treated was reduced to 1,500 L. A 1,500 L Jojo tank was connected to the peroxone system and treated under the same conditions as described in Section 2.2.2.

2.2.4 Water Sampling

IAPS-treated water was sampled from the point of discharge back to the WWTW and analysed for physicochemical parameters, COD, TSS, nutrient, and coliforms over a period of 5 months (May-September 2016). Peroxone batch treatment commenced with the 10,000 L IAPS water. After filling the tank, water was mixed for at least 5 min by recirculating the water without peroxone dosage before treatment commenced. Treatment was either for periods up to 6 h (short-term), 24 h (medium-term), or 8 d (long-term). Samples were collected at intervals specified in the Results from sampling point of the system (Figure 2.2), using 500 mL Duran Schott bottles and, parameters mentioned above were analysed. After the 10,000 L batches, 1,500 L batches were carried out for only up to 24 h (medium-term).

2.2.5 Analytical procedures

Physicochemical parameters including temperature, dissolved oxygen, pH and electrical conductivity were measured *in situ*. Temperature and electrical conductivity were measured using an EC Testr11 Dualrange 68X 546 501 detector (Eutech Instrument, Singapore). Dissolved oxygen was measured using an IP67 Combo (Water Quality Meter, China) while pH was measured using a Hanna HI 8424 microcomputer pH meter (Hanna Instrument, Romania). Colour removal was determined following the method of Muhammad *et al.* (2008)

and Garcia-Morales *et al.* (2013) by measuring sample absorbance at 465 nm using an Aquamate spectrophotometer (Thermo Spectronic, England) with distilled water as background.

Nutrient analysis was carried out according to APHA methods of wastewater analysis (APHA, 1998). Ammonium-nitrogen (NH_4^+-N) was analysed by the phenol-hypochlorite method (Method 4500 G), nitrate-nitrogen (NO_3^--N) by the sodium salicylate method and ortho-phosphate (PO_4^{2-}-P) by the ascorbic acid method (Method 4500 E). Grab samples were filtered and analysed immediately or kept at 4°C for not longer than 12 h.

Total suspended solids (TSS) was also analysed according to Standard Methods (APHA, 1998; Method 2540 B). A known volume of sample was filtered through previously dried Whatman glass microfiber filter discs of diameter 47 mm and pore size 0.45µm (grade GF/C; Merck Chemicals, South Africa). The filters were dried in oven at 105°C for 1 h or until a constant weight was achieved. The concentration of TSS was calculated using Equation 2.1.

$$TSS\ (mg/L) = [(W_2 - W_1) \times 1000] \div [\text{Volume of sample in mL}] \quad \textbf{Equation 2.1}$$

Where: W_2 is the sample + filter weight and
W_1, weight of the filter paper.

COD was analysed using a COD cell test kit (1.14541.0001) purchased from Merck Chemicals (South Africa) according to manufacturer's instructions. Microbial analyses were carried out after serial dilution using spread plate method (Mambo *et al.*, 2014b). *E. coli* and faecal coliform count were analysed using Chromocult and m-Fc Agar respectively (Merck Chemicals, South Africa). Grab samples of peroxone treated water were analysed immediately after collection by diluting 1 mL in 9 mL phosphate buffer saline (PBS). From this dilution, 1 mL was further diluted into another 9 mL PBS (i.e. 1:100). Aliquots (0.1 mL) of diluted samples were spread on prepared agar plates and incubated at 37°C and 45°C for Chromocult and m-Fc plates respectively for 24 h. Colonies on agar plates after incubation were counted and multiplied by their dilution factor for estimation of colony forming units (CFU).

2.2.6 Statistical analysis

All triplicate data were computed on Microsoft Excel 2016 to calculate the mean and standard error used in plotting graphs in Result section. A one-tailed distribution *t* test

(Microsoft Excel, 2016) at alpha level 0.05 was used to determine the level of significance between the mean concentrations at different treatment intervals for all data sets.

2.3 Results

2.3.1 Quality of IAPS-treated water

Results from analysis of IAPS-treated water carried out from May to September 2016 are presented in Table 2.2. There were variations in the water quality during the period of study due to varying weather conditions (see Appendix B, Figure B2). COD concentration ranged from 56.0 mg L^{-1} to 112.0 mg L^{-1} with an average of 81.3 ± 6.7 mg L^{-1}, and was therefore marginally higher than the 75 mg L^{-1} general standard. Similarly, TSS was also marginally higher than the 25 mg L^{-1} general standard and ranged from 16 mg L^{-1} to 46 mg L^{-1} with an average of 28.6 ± 3.4 mg L^{-1}. Very surprisingly, faecal coliform counts were also routinely above 1000 CFU 100 mL^{-1} general limit with an average of 1.6 ± 0.3 × 10^5 CFU 100 mL^{-1}. By comparison, nutrient concentrations were in accordance with general limits with mean values of 1.9 ± 0.4 mg L^{-1} for ammonium-N, 11.2 ± 1.9 mg L^{-1} for nitrate/nitrite-N, and 2.3 ± 0.2 mg L^{-1} for ortho-phosphate. Similarly, all physicochemical parameters measured were within the general standard for discharge during the sampling period with means of 8.1 ± 0.2, 7.3 ± 0.7 mg L^{-1} and 125 ± 5.6 mS m^{-1} for pH, dissolved oxygen and electrical conductivity respectively.

Table 2.2: Water quality of IAPS effluent over a period of 5 months. Data are presented as the mean ± SE of 9 samples collected between May and September 2016.

Parameter	Concentration
pH	8.1 ± 0.2
Dissolved oxygen (mg L^{-1})	7.3 ± 0.7
Electrical conductivity (mS m^{-1})	125.1 ± 5.6
COD (mg L^{-1})	81.3 ± 6.7
TSS (mg L^{-1})	28.6 ± 3.4
Nitrate/nitrite-N (mg L^{-1})	11.2 ±1.9
Ammonium-N (mg L^{-1})	1.9 ± 0.4
Ortho-phosphate (mg L^{-1})	2.3 ± 0.2
Faecal coliforms (CFU 100 mL^{-1})	1.6 × 10^5 ± 0.3 × 10^5

2.3.2 Effect of peroxone treatment on quality of IAPS water

Initially, the effect of peroxone on IAPS-treated water was investigated using 10 000 L batches and the results are summarized in Figures 2.3 – 2.6. There was no effect of peroxone on DO, pH or EC following short-, medium-, and long-term treatment (Figure 2.3a, b and c). A *t* test analysis (P>0.05) confirmed no significant difference in DO (8.9 ± 0.9 mg L^{-1}), pH (7.7 ± 0.3) or electrical conductivity (131 ± 5 mS m^{-1}) between IAPS water and water after short-, medium-, and long-term exposure to peroxone (P=0.10, 0.35, and 0.42 respectively). Generally, values for these physicochemical parameters were within the range for discharge to a watercourse.

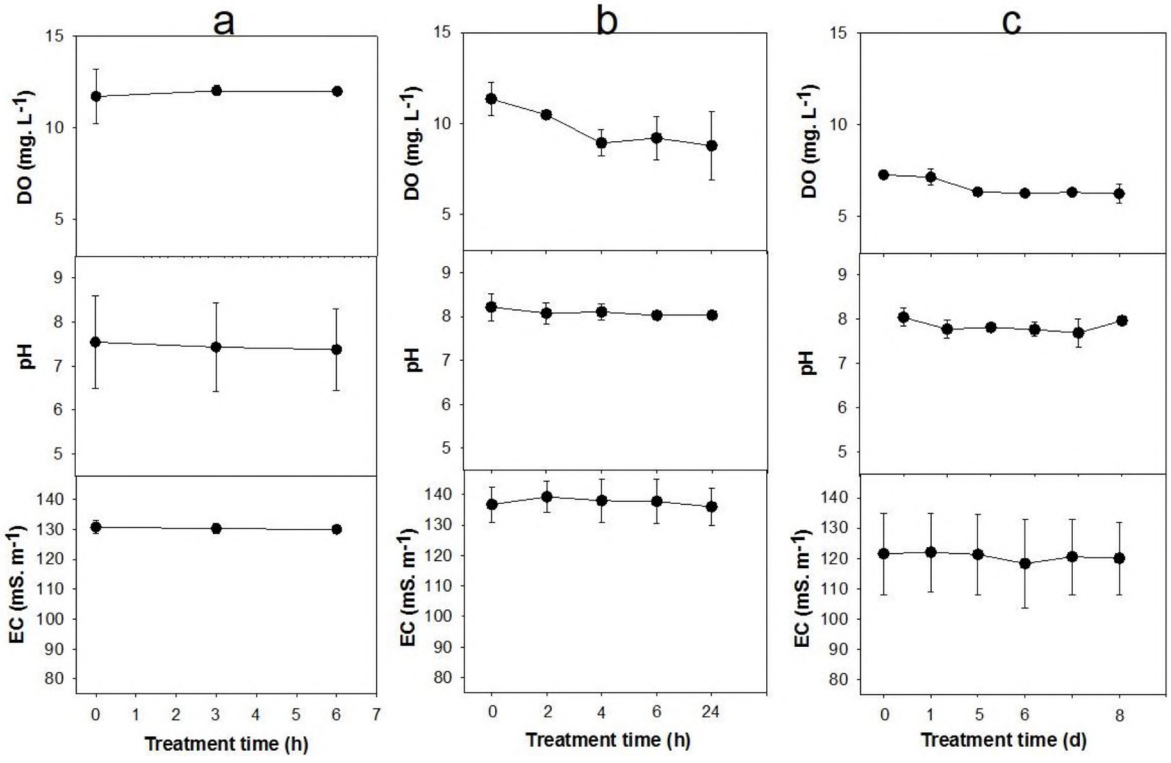

Figure 2.3: Effect of peroxone on physicochemical characteristics of IAPS-treated water following short-term treatment up to 6 h (a); medium-term treatment up to 24 h (b); and, long-term treatment up to 8 d (c) of 10,000 L batches. Data are presented as the average of two independent treatments. Bars indicate standard error.

Short- and medium-term exposure of IAPS water to peroxone did not reduce COD concentrations (Figure 2.4a and b). However, long-term treatment resulted in gradual reduction in COD up to 24% achieved after 8 d (Figure 2.4c). Statistical analysis (*t* test P>0.05) confirmed that a 22% COD reduction after 7 d was indeed significant (P=0.01). In contrast, a marked reduction of 25% was observed in TSS concentration within the short-term

treatment of 6 h (Figure 2.4a). A maximum of 58% reduction in TSS was achieved following a long-term treatment i.e. 8 d (Figure 2.4c).

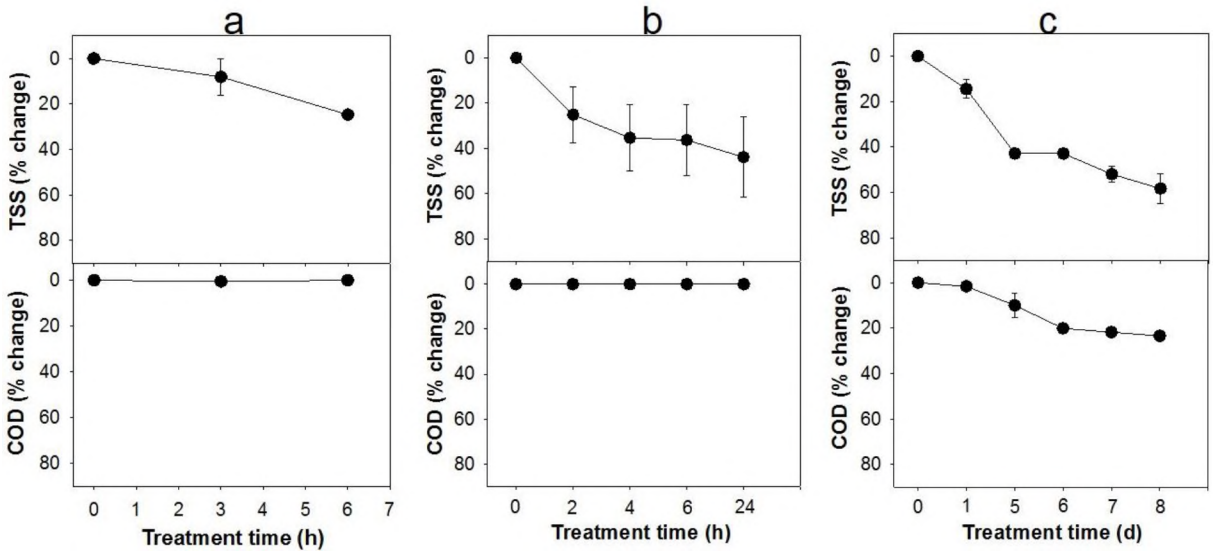

Figure 2.4: Effect of peroxone on COD and TSS of IAPS-treated water. Percentage change following short-term treatment up to 6 h (a); medium-term treatment up to 24 h (b); and, long-term treatment up to 8 d (c) of 10,000 L batches. Data are presented as the average of two independent treatments. Bars indicate standard error.

Nutrient concentration of IAPS-treated water was routinely within the general standard for discharge (see Table 2.2). Peroxone treatment of 10,000 L batches of IAPS water reduced ammonium-N concentration by 4% after medium-term treatment (Figure 2.5b), which was not significant (P=0.50). Long-term peroxone treatment however resulted in a linear reduction in ammonium-N, with maximum removal (i.e. 88% reduction) achieved after 8 d (Figure 2.5c). No change in nitrate and phosphate concentration was observed after the short- and medium-term peroxone treatment (Figure 2.5a & b). While ammonium-N concentration was reduced following long-term peroxone treatment of IAPS water, nitrate-N and phosphate concentration increased by 14% and 12% respectively (Figure 2.5c). However, these increases were not significant (P=0.15 and 0.06 respectively) suggesting that peroxone has little or no effect on the concentration of these nutrients in IAPS water.

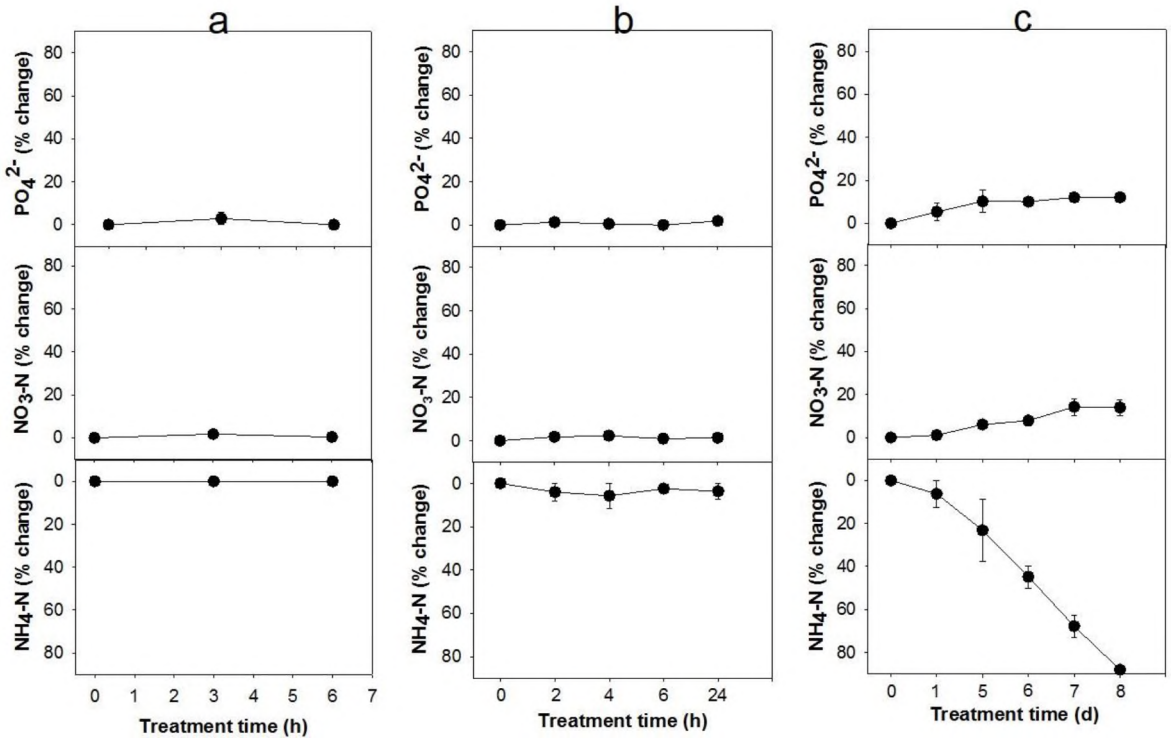

Figure 2.5: Effect of peroxone on nutrient concentration of IAPS-treated water. Percentage change following short-term treatment up to 6 h (a); medium-term treatment up to 24 h (b); and, long-term treatment up to 8 d (c) of 10,000 L batches. Data are presented as the average of two independent treatments. Bars indicate standard error.

Perhaps not unexpectedly, peroxone treatment impacted faecal coliform and *E. coli* cell counts substantially. As shown in Figure 2.6, peroxone treatment resulted in efficient removal of faecal bacteria in all treatments. Within 6 h of peroxone treatment, *E. coli* and faecal coliforms were reduced by 41% and 32% respectively (Figure 2.6a). Medium-term treatment up to 24 h resulted in 85% and 40% reduction (Figure 2.6b), while treatment for periods up to 8 d resulted in a 100% and 95% reduction of *E. coli* and faecal coliforms respectively (Figure 2.6c).

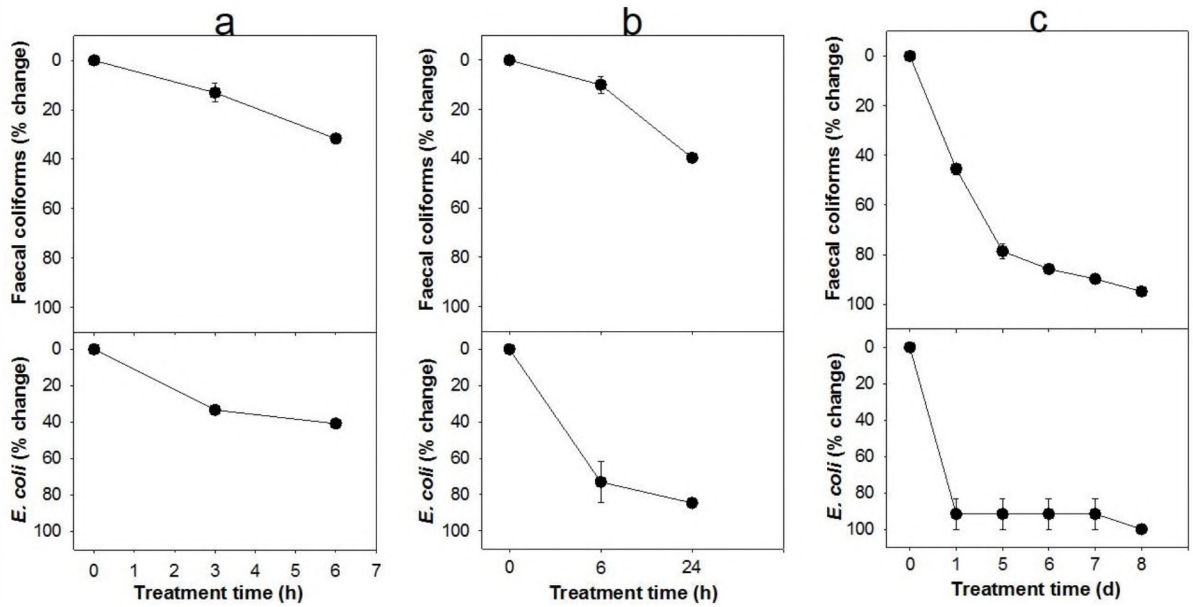

Figure 2.6: Effect of peroxone on percentage change in coliforms following treatment of 10,000 L IAPS water. (a) Short-term treatment up to 6 h; (b) medium-term treatment up to 24 h; and, (c) long-term treatment up to 8 d. Data are presented as the average of two independent batch treatments. Bars indicate standard error.

To determine whether the amount of IAPS water impacts treatment efficiency by peroxone, the batch volume was reduced to 1,500 L and results are shown in Figures 2.7-2.9. A noticeable and quantifiable change in the color of water samples taken at various intervals during the 24 h treatment period was observed, indicating increased decolourization in response to peroxone (Figure 2.7a). Furthermore, absorbance at 465 nm decreased gradually over treatment time, with a 40% reduction achieved within 24 h (Figure 2.7b).

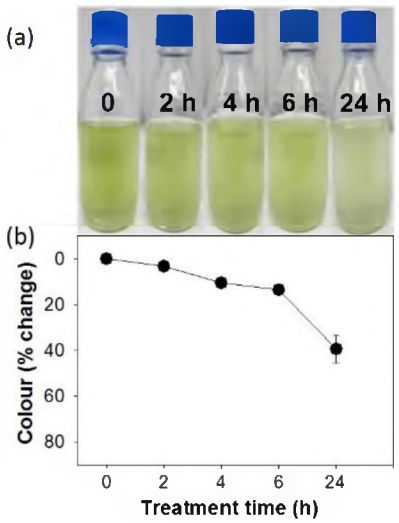

Figure 2.7: Effect of peroxone on colour of IAPS-treated water. Noticeable change in colour (a); and, percentage change at A_{465nm} (b) following treatment of 1,500 L batches for 24 h. Values are the average of two independent treatments. Bars indicate standard error.

No change in physicochemical parameters of IAPS-treated water was observed within the 24 h period of exposure to peroxone (Figure 2.9a). A *t* test analysis (P>0.05) confirmed no significant difference between values for IAPS and IAPS + peroxone treated water. However, average electrical conductivity (103 ± 0.8 mS m^{-1}), pH (7.8 ± 0), and dissolved oxygen (6.4 ± 0.1 mg L^{-1}) during the 24 h peroxone treatment period were within the specified range for discharge into watercourse. Furthermore, peroxone treatment resulted in slight change in nutrient concentration of the 1,500 L IAPS water at 24 h sampling interval (Figure 2.8b). As shown in Figure 2.8b, a 65% reduction in ammonium-N was observed at the 24 h sampling interval, while Nitrate-N increased slightly by 10%. However, whether this result represents a real effect of peroxone treatment is uncertain. Nevertheless, an insignificant 22% increase in phosphate concentration was also observed at 24 h sampling interval (P=0.15).

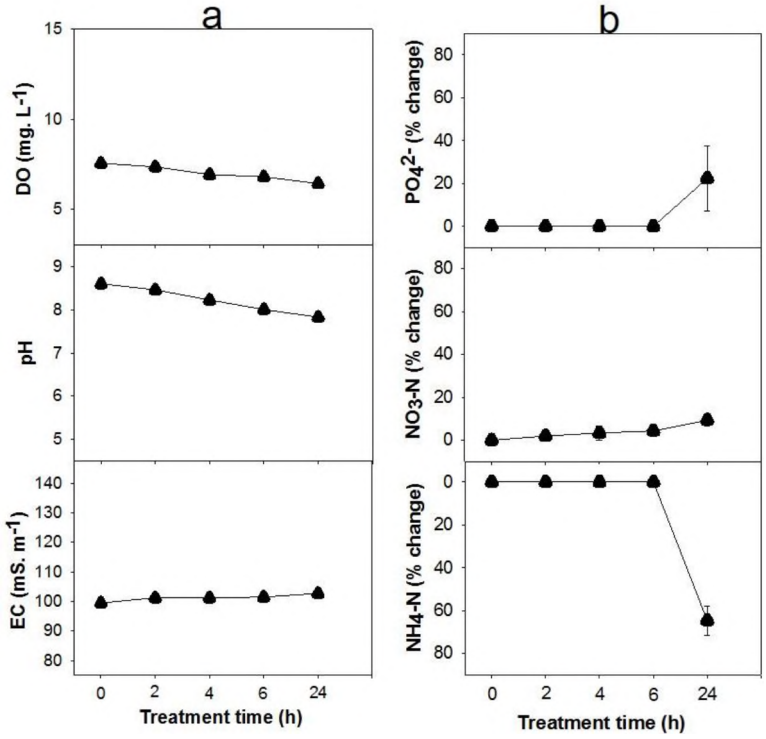

Figure 2.8: Effect of peroxone on the physicochemical characteristics of IAPS-treated water (a); and, percentage change in nutrient concentration (b). Water from the IAPS (1,500 L) was treated with peroxone for periods up to 24 h. Data are presented as the average of two independent batch treatments. Bars indicate standard error.

In response to peroxone treatment, COD and TSS of 1,500 L batches of IAPS water was reduced within 24 h (Figure 2.9a). The result contrasts with that obtained for 10,000 L batches of IAPS water and suggests that treatment volume is an important consideration. Thus, COD concentration of the 1,500 L batch reduced by 12% after 6 h of initiation of peroxone treatment and, by 24 h, had declined further to yield a significant 22% reduction

(P=0.04). Likewise, a significant reduction in TSS concentration of IAPS water was observed following onset of peroxone treatment (Fig. 2.9a). Within 2 h of treatment initiation, TSS declined by 11% and at 24 h, a 45% reduction was measured. Furthermore, 1,500 L batch treatment with peroxone resulted in a significant reduction in faecal coliform counts (70%) within 6 h, which was further reduced by 94% after 24 h (Figure 2.9b). The initial *E. coli* count were <1000 CFU 100 mL^{-1} and therefore, peroxone treatment resulted in complete removal of these bacteria within 6 h (Figure 2.9b).

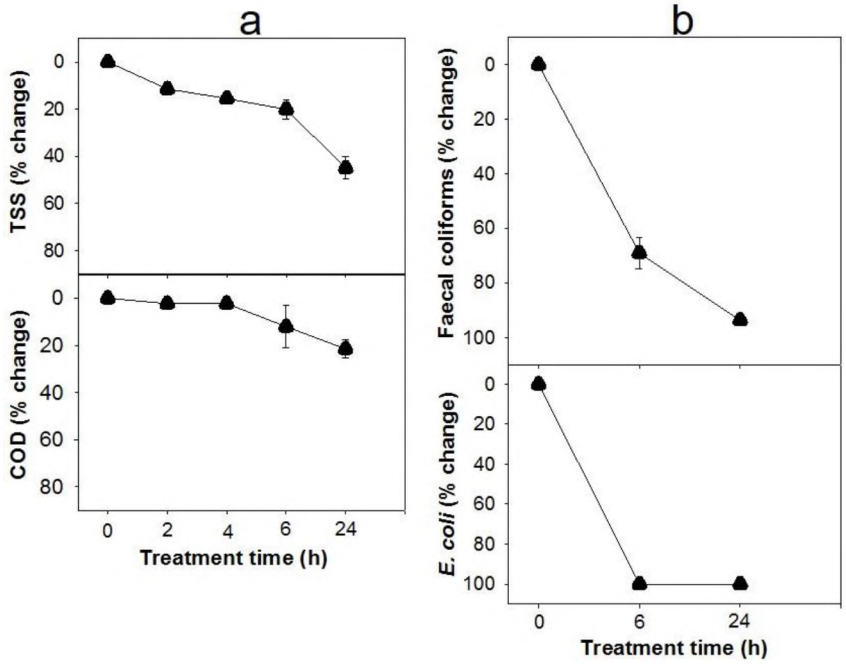

Figure 2.9: Effect of peroxone on percentage change in COD and TSS (a) and coliforms (b) of 1,500 L batches of IAPS water for periods up to 24 h. Data are presented as the average of two independent treatments. Bars indicate standard error.

It is evident from the above results that batch peroxone treatment at small scale (comparing 1,500 L and 10,000 L) is more efficient and yielded improved quality of IAPS water. In light of these results, water quality parameters of IAPS-treated water were adjusted to reflect the effect of peroxone as a tertiary treatment. Results were compared with the currently accepted General Authorization limits for discharge (DWA, 2013). As presented in Table 2.3, results show that peroxone treatment of IAPS-treated water for 24 h will indeed reduce COD, TSS, ammonium-N, nitrate-N, and ortho-phosphate concentrations to levels well within the South African limits for discharge to a watercourse. However, due to unexpected high coliform count in the IAPS water, the adjusted faecal coliform counts exceeded the standard limits for discharge. Nevertheless, the peroxone system was still effective in the destruction of faecal bacteria.

Table 2.3: Summary data describing the effect of peroxone on quality of IAPS-treated water. Results are presented to illustrate comparison of IAPS water quality with General Standard (DWA, 2013) after batch treatment with peroxone for 24 h. IAPS data collected over a period of 5 months ± SE (n=9). Adjustment in IAPS water quality calculated based on the % change following 24 h exposure to peroxone.

Parameter	IAPS	% change post peroxone	Adjusted IAPS + peroxone	DWS Standard
COD (mg L^{-1})	81.3 ± 6.7	-22 ± 4	63.4 ± 5.2	75
TSS (mg L^{-1})	28.6 ± 3.4	-45 ± 5	15.7 ± 1.9	25
Faecal coliforms (CFU 100 mL^{-1})	$1.6 \pm 0.3 \times 10^5$	-94 ± 1	$0.9 \pm 0.2 \times 10^4$	1000
Ammonium-N (mg L^{-1})	1.9 ± 0.4	-65 ± 7	0.7 ± 0.1	6
Nitrate-N (mg L^{-1})	11.2 ±1.9	+10 ± 3	12.3 ± 2.1	15
Orth-phosphate (mg L^{-1})	2.3 ± 0.2	+22 ± 15	2.8 ± 0.2	10

2.4 Discussion

The experiments described in this chapter were carried out to determine the effect of peroxone, administered using the proprietary Puricare® process as a tertiary treatment system, on quality of IAPS-treated water. Results show that peroxone has the potential to improve water quality to levels that allow for either irrigation or discharge into a water resource that is not a listed water resource for volumes up to 2 ML of treated wastewater on any given day. Except for coliform counts (unexpectedly higher in IAPS-treated water), other parameters including COD, TSS, pH, DO, EC, and N and P values were within the general limits after tertiary treatment. However, the peroxone system used in the present study was more effective at treating 1,500 L than 10,000 L suggesting that system optimization is essential to achieve the desired efficiency.

All wastewater treatment technologies require a tertiary treatment unit to achieve a final effluent that meets specified standards for discharge into the environment (Mambo *et al.*, 2014a). The combination of O_3 and H_2O_2 (peroxone) did not appear to impact physicochemical characteristics of IAPS-treated water after short-, medium-, and long-term treatment. This observation is similar to a report by Tripathi and Tripathi (2011), where ozonation of secondary treated water had no significant effect on physicochemical parameters. Even so, pH of IAPS water during the period of study was near neutral (between 7 and 8), which might have reduced the rate of hydroxyl radical formation by peroxone that is favored at higher pH (Andreozzi *et al.*, 1999; Klavarioti *et al.*, 2009; Michael *et al.*, 2013).

Where large volumes of water (i.e. 10 000 L) were batch-treated, there was no significant change in COD concentration until day 7 (22% reduction), indicating very low pollutant mineralization. Wu and Englehardt (2015) reported a 90% reduction in COD of secondary wastewater by peroxone within 24 h. Therefore, the low mineralization of COD in 10,000 L batches in the present study may be attributed to volume of water. In addition, the neutral pH of IAPS water might have contributed to limited formation of OH ions, further lowering mineralization potential during treatment (Muhammad *et al.*, 2008; Tripathi and Tripathi, 2011). Indeed, where small volume of water (i.e. 1,500 L) were batch-treated, peroxone reduced colour, and COD concentration significantly within 24 h, confirming water volume as a critical component in the peroxone treatment process.

TSS concentration of IAPS water was reduced effectively in the 10,000 L batch treatment. The highest reduction in TSS (58%) was achieved after day 8. Whereas for 1,500 L batches, a 45% reduction in TSS was achieved in 24 h. TSS reduction during perozone treatment was presumably due to destruction of residual microalgae, leading to oxidation of components by hydroxyl radicals. In addition, ozonation is known to enhance coagulation of suspended particles such as algae, leading to separation from medium (Show *et al.*, 2013). Therefore, reduction in TSS might be due to coagulation of suspended solids upon reaction with ozone or hydroxyl radicals during treatment.

There was no significant effect of peroxone on nutrient concentration in the short- and medium-term batches of 10,000 L. However, a gradual reduction in ammonium-N concentration observed could be attributed to the strong oxidative power of hydroxyl radicals, resulting in the direct oxidation of ammonium-N to various nitrogen compounds such as nitrogen oxides and nitrogen gas (Brito *et al.*, 2010). However, since the increase in nitrate-N concentration was not so high, it is likely that the ammonium-N decomposed directly to nitrogen gas instead of nitrate-N and nitrite-N (Kim *et al.*, 2005).

The peroxone system was efficient at removing coliforms particularly *E. coli* in the 10,000 L batches but more effective during 1,500 L batches. A complete removal of *E. coli* and 94% reduction of faecal coliforms was achieved within 24 h treatment. Even so, the removal efficiency measured in the present study using the peroxone system as configured is very low. Other reports indicate 99% removal within few minutes (Tripathi and Tripathi, 2011; Rizvi *et al.*, 2013). Generation of free radicals by O_3/H_2O_2 results in total destruction of the intracellular components of microorganisms (Ksibi, 2006; Rizvi *et al.*, 2013). It is suggested

that the large volumes of water used in the batch treatments probably contributed to low coliform removal. However, the reduction rate of *E. coli* was higher than for faecal coliforms in all treatments, probably due to less resistance of *E. coli* to O_3/H_2O_2 destruction (Rizvi *et al.*, 2013), or due to the initial low concentration of *E. coli* in IAPS-treated water.

In conclusion, a combination of ozone and hydrogen peroxide for batch treatment of 10,000 and 1,500 L of IAPS-treated water was studied. Peroxone was more effective at 1,500 L and resulted in water with COD, TSS and nutrient concentration within the limits for discharge to the environment. The further optimization, preferably to a continuous treatment process, may increase efficacy of the system and pave the way for its use as a tertiary treatment unit for final polishing of IAPS-treated water.

Chapter 3: Biomass Production in High Rate Algal Oxidation Ponds

3.1 Introduction

The use of microalgal biomass for production of useful products was first considered in Germany during World War II (Becker, 1994). Since then, there has been much interest in the production of biofuel from microalgae. Many studies have shown that algal biomass stores large amounts of energy, which can be converted into diesel, methane, ethanol, hydrogen etc. (Ho *et al.*, 2011; Prajapati *et al.*, 2013). For exploitation of the abundant energy stored, the harvested biomass can be converted using either thermo-chemical methods such as combustion, gasification, pyrolysis and liquefaction or biochemical methods such as anaerobic digestion and fermentation (Milledge and Heaven, 2014). Apart from biofuel, microalgae have applications as food, food supplements, and as a source of therapeutics, pharmaceuticals, and cosmetics (Milledge, 2011).

The benefits associated with HRAOPs such as eco-friendly, low cost operation and maintenance, efficient disinfection, and harvestable algal biomass production, as discussed in Chapter 1, make them a preferable wastewater treatment technology in developing and industrialized countries as well as small communities (Al-Shayji *et al.*, 1994; Pittman *et al.*, 2011). Algal biomass grows profusely in wastewater because of an ability to utilize nutrients such as carbon, nitrogen and phosphorus present in wastewater (Pittman *et al.*, 2011; Rawat *et al.*, 2011). Microalgae such as *Scenedesmus* sp., *Pediastrum* sp., *Micractinium* sp., *Chlorella* sp., *Actinastrum* sp., *Dictyosphaerium* sp., and *Coelastrum* sp. are typical of wastewater treatment HRAOPs.

Together with heterotrophic bacteria, these microalgae form aggregates known as microalgal-bacterial flocs (MaB-flocs). Thus, MaB-flocs typically consist of a consortium of microalgae, cyanobacteria and bacteria, and may include a number of rotifers, ciliates and precipitates (Van Den Hende *et al.*, 2011). Paddlewheel driven HRAOP maintain these MaB-flocs in suspension as biological aggregates that can easily be recovered by gravity sedimentation using ASPs (Park *et al.*, 2011). Recent studies have elaborated on the downstream uses of these MaB-flocs as substrates for methane production, CO_2 sequestration, fertilizer, and as feed in aquaculture, which emphasizes the value of this resource (Natrah *et al.*, 2013; Essam *et al.*, 2013; Wieczorek *et al.*, 2015; Arcila and Buitrón, 2016; Coppens *et al.*, 2016; Van Den

Hende *et al.*, 2016). Furthermore, productivity of 12-40 g m^{-2} d^{-1} is apparently achievable in HRAOP depending on the season, climate, and species composition (Park *et al.*, 2011; Davis *et al.*, 2011) and, provided an appropriate harvesting method is in place (Al-Shayji *et al.*, 1994).

Mixed liquor suspended solids (MLSS) is the concentration of biomass in an aeration basin treating wastewater (Cowan, 2014). Just like an aeration basin of an activated sludge system, HRAOPs of an IAPS are also aeration ponds and contain algal/bacterial biomass as the biocatalyst. The wastewater undergoing treatment in HRAOP is thus a mixed liquor, while MLSS is the concentration of all suspended solids, which is equivalent to MaB-floc concentration in the pond. Hence, it is proposed that MaB-floc concentration in HRAOPs can be measured and expressed as MLSS. In this chapter, studies were undertaken to investigate biomass production in the HRAOP of the Belmont Valley IAPS treating domestic sewage by describing and quantifying the MaB-flocs as MLSS. In addition, environmental parameters that influence biomass production in HRAOPs were also monitored. Molecular identification of a bacterium isolated from the pond is also described.

3.2 Materials and Methods

3.2.1 IAPS configuration and operation

The IAPS used in this study is located at the Belmont Valley municipal WWTW, Grahamstown, South Africa (33° 19' 07" South, 26° 33' 25" East) and has a design capacity of 75 m^3 d^{-1}. Details of configuration and operation were as described in Chapter 2 Section 2.2.1. For the purposes of this investigation all experiments were carried out using HRAOP B. Pond temperature and pH were measured *in situ* using an EC Testr11 Dual range 68 X 546 501 detector (Eutech Instrument, Singapore) and a Hanna HI 8424 microcomputer pH meter (Hanna Instrument, Romania) respectively. Daily solar radiation data at 14:30 for the period of sampling was obtained from South African Universities Radiometric Network (http://www.sauran.net/Data) and, data from Nelson Mandela Metropolitan University (NMMU) used as it is the closest station to Grahamstown with the most complete data (34° 00' 30.9" South, 25° 39' 54.9" East).

3.2.2 MaB-floc settleability and identification

Settleability of MaB-flocs was carried out by putting 10 mL MLSS sample in a cone-shaped test tube and allowed to settle for 2 h. Images of the test tube were captured at intervals

reported in Results section using a Canon PowerShot G12 (Canon Inc., Japan) digital camera. Composition and structure of the MaB-flocs were determined microscopically by placing a floc of the MLSS on a microscope glass slide and examined using a Zeiss Axiostar plus light microscope (Carl Zeiss, Jena, Germany) and the image was captured. The composition of the MaB-floc was identified by reference to published identification keys (Belcher and Swale, 1978; Cassie, 1983; Huynh and Serediak, 2006) and, previously identified species from the studied HRAOP (Johnson, 2011).

3.2.3 Isolation of microalgae and bacteria

Algae were isolated from MLSS of HRAOP by spread-plating 0.2 mL of sample on Bold 3N agar prepared by adding 12 g agar powder to 1 L Bold 3N medium (Appendix B, Table B1). Plates were incubated under continuous fluorescent light (70-90 µmol m^{-2} s^{-1}) in a growth room at 25°C until cells developed sufficiently. Upon development of colonies, single colonies were inoculated into 100 mL fresh sterilized Bold 3N medium until pure culture was achieved. The pure culture was maintained in 100 mL Bold 3N medium in the growth room. Sub-culturing was carried out regularly with 10% inoculum to avoid contamination. The isolated alga was identified by microscopic examination and by reference to published identification keys (Belcher and Swale, 1978; Cassie, 1983; Huynh and Serediak, 2006). Bacteria were isolated from MLSS by serial dilution of a 1.0 mL aliquot of sample and spread plating on nutrient agar plates (Merck Chemicals, South Africa). Subsequent sub-culturing was carried out by streaking on new agar plates until pure culture was achieved. The pure culture was kept on nutrient agar plates sealed with parafilm at 4°C for identification and further investigation.

3.2.4 DNA extraction

Only the isolated bacterium was identified by genomic DNA extraction and pyrosequencing. The alga was not subjected to molecular identification because they could be easily identified by microscopic examination and reference to the literature.

Extraction was carried out using the conventional Phenol-Chloroform method as described by Bond *et al.* (2000). Pure culture of the isolated bacterium was grown in Luria broth (Appendix B, Table B2) overnight. Culture (1.0 mL) was centrifuged at 10,000 rpm for 5 min and resuspended in 500 µL Tris EDTA (TE) buffer (10 mM Tris, 1 mM EDTA). Lysozyme stock solution (6 µL; 50 mg/mL) was added and incubated at 37°C for 3 h with occasional

mixing, followed by heating in boiling water bath for 1 min. Sodium dodecyl sulphate (50 µL of a 10% solution) and 2.5 µL Proteinase K stock solution (50 µg/mL) were added, thoroughly mixed and incubated at 37°C for 1 h. Cetyltrimethylammonium bromide (CTAB; 100 µL of 10% solution) and 200 µL of 5 M sodium chloride were added to the extract and incubated at 55°C for another 1 h. The extract (500 µL) was transferred to a fresh tube and equal amounts of buffer saturated phenol (purchased from Merck Chemicals) was added, mixed for 30 min and centrifuged at 10,000 rpm for 5 min. The upper aqueous layer was removed and an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1, v/v/v) added, mixed and centrifuged as above to separate the layers. Equal volume of chloroform:isoamyl alcohol (24:1, v/v) were added to the upper aqueous layer in a fresh tube, mixed and centrifuged. Finally, 2.5 volume of ice-cold ethanol (96%) was added to the upper layer and the DNA precipitated at -20°C for 12 h. DNA was recovered by centrifuging at 10,000 rpm for 20 min, dried and resuspended in 100 µL TE buffer. Following extraction, DNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific) and analysed by pyrosequencing (Inqaba Biotechnical Industries Ltd, South Africa).

3.2.5 Sequencing analysis

Following DNA extraction, 16S target was amplified using Dream Taq DNA polymerase (Thermo Scientific) with 16S-27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 16S-1492R (5'-CGGTTACCTTGTTACGACTT-3') primers. PCR products were gel extracted (Zymo Research, Zymoclean Gel DNA Recovery Kit), and sequenced in the forward and reverse directions on the ABI PRISM 3500xl Genetic Analyzer. Purified sequencing products (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit) were analysed followed by a BLAST search (NCBI).

3.2.6 MLSS measurement

Biomass concentration in HRAOP B was measured as MLSS in cycles over a period of 5 months (APHA, 1998; Method 2540 B). Each cycle represents a 4 or 5 d sampling interval. Samples were collected diurnally between 09:00-09:30 am ($MLSS_{am}$), 12:00-13:00 pm ($MLSS_{noon}$), and 16:00-16:30 pm ($MLSS_{pm}$) from August to November 2015. Samples of mixed liquor (200 mL) were from directly in front of the paddlewheel of HRAOP B and transferred to the laboratory for immediate MLSS estimation. Samples were in triplicate and mean MLSS concentration determined.

Where applicable, samples were first strained using a laboratory test sieve (pore size, 500 µm) to remove zooplankton. A known volume of well-stirred sample was then filtered using pre-dried and weighed (placed in desiccator prior to use) Whatman glass microfiber filter discs of pore size 0.45 µm (grade GF/C; Merck Chemicals, South Africa). The filters were oven dried at 105°C for 1 h and cooled in a desiccator for at least 30 min before determining the weight. The weight of the filters were recorded and MLSS concentration calculated using Equation 3.1.

MLSS (mg/L) = [(A - B) ×1000] ÷ [Volume of sample in mL] × 1000.....**Equation 3.1**

Where: A = sample + filter weight and

B = weight of the filter paper.

3.2.7 Biomass productivity

The areal productivity of HRAOP in kg ha^{-1} d^{-1} and g m^{-2} d^{-1} was calculated from the MLSS concentrations using Equations 3.2 (Al-Shayji *et al.*, 1994).

$P = 10 \times d/t \times n \times MLSS$.....**Equation 3.2**

Where: P is pond productivity (kg ha^{-1} d^{-1}),

d = pond depth (m),

t = hydraulic retention time of the pond (d),

MLSS = total mixed liquor suspended solids (mg) and

n = algae ratio in the MLSS (0.9-1.0 as estimated by Al-Shayji *et al.* (1994).

3.2.8 Statistical analysis

MLSS concentrations were measured in triplicate; therefore all data were presented as mean ± standard error calculated using Microsoft Excel 2016. A *t* test (alpha level 0.05) was carried out to determine the level of significance between mean $MLSS_{pm}$ and $MLSS_{am}$ for all sampling intervals using SigmaPlot version 11.2.

3.3 Results

3.3.1 HRAOP conditions and operation

Figure 3.1 shows the environmental and physicochemical parameters of HRAOP water measured during the course of sampling. Pond water temperature and pH varied diurnally with the lowest recorded in the morning while highest was in the afternoon. Morning

temperature ranged between 10°C in August to 22°C in November while afternoon ranged from 14°C to 27°C respectively. Pond pH increased diurnally with an average of 9 in the morning and 11 in the afternoon during sampling. However, this range did not vary across the sampling months. The daily solar radiation recorded ranged from 0.42 W. m^{-2} in August to 972 W. m^{-2} in October. The average daily pond water temperature, pH and solar radiation over the course of sampling were 17.5°C, 9.5, and 683 W. m^{-2} respectively.

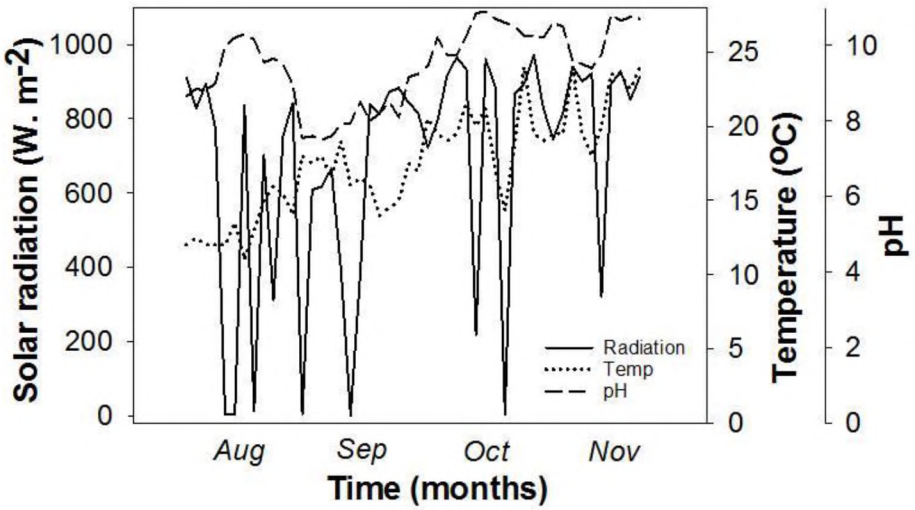

Figure 3.1: Average solar radiation, pond water temperature and pH measured in HRAOP B during MLSS monitoring (August to November 2015). Temperature and pH data are average of morning and afternoon values measured *in situ* while solar radiation data are average at 14:30 accessed online (http://www.sauran.net/Data).

3.3.2 MaB-floc structure and composition

An example of the settleability and composition of the MaB-flocs produced in HRAOP B of the pilot-scale IAPS treating municipal sewage during the course of this study are shown in Figure 3.2. MaB-flocs appear as discrete entities and the bulk of these flocs settle readily within 2 h (Figures 3.2a and b). Light microscope analysis of these MaB-flocs revealed recruitment of microalgae, diatoms, cyanobacteria, and bacteria presumably facilitated by production of extracellular polymeric substances (Figure 3.2 (c)). Among the more prominent species were the chlorophytes; *Pediastrum* sp., *Chlorella* sp., *Closterium* sp. and *Scenedesmus* sp. and the diatoms; *Cyclotella* sp., *Nitzschia* sp. and *Navicula* sp.

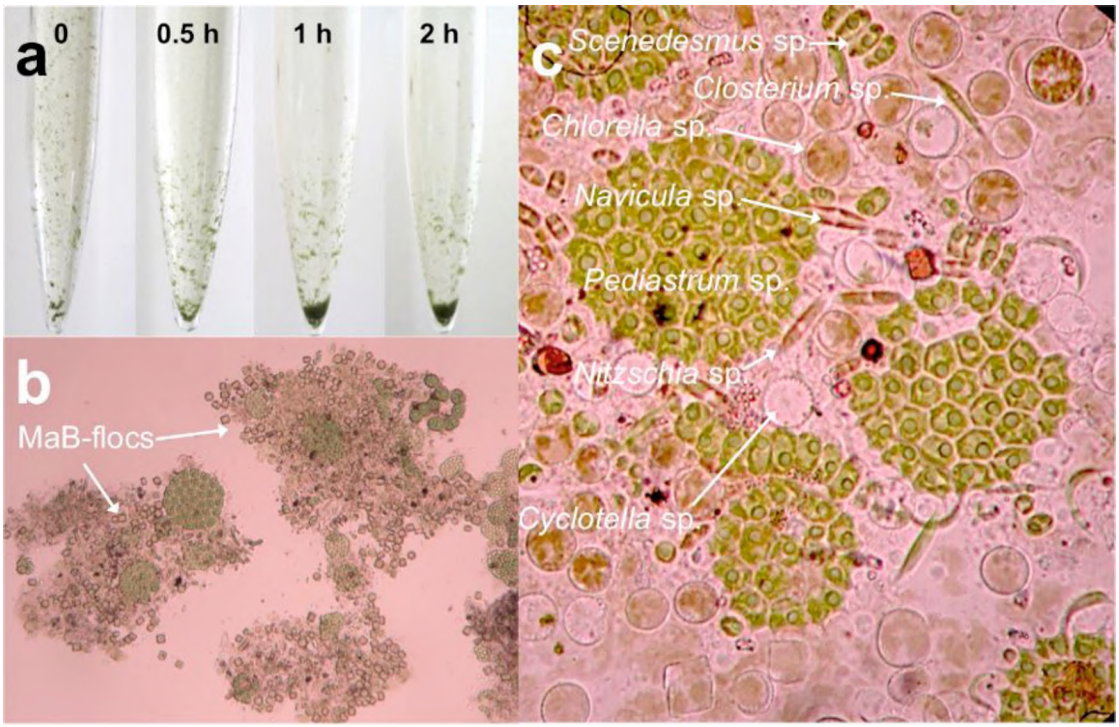

Figure 3.2: Settleability and representative light microscope analysis of MaB-flocs generated in high rate algae oxidation ponds of an integrated algae pond system treating municipal sewage. (a) Settleability, (b) low resolution (10 ×), and (c) high resolution (40 ×) light microscope images of the MaB-flocs.

3.3.3 Microbial composition and identification of MLSS

Growth observed using Bold 3N, nutrient, and potato dextrose agar in the present study revealed that HRAOP MLSS is composed of microalgae with few bacteria and no fungi. Microalgal species composition was highly dependent on season with dominance by *Pediastrum* sp., *Chlorella* sp., *Cyclotella* sp., *Micractinum* sp., and *Scenedesmus* sp. as season changed (Figure 3.3). Occasionally, species such as sp., *Closterium* sp., *Pyrobotrys* sp., *Actinastrum* sp., *Ankistrodesmus* sp., *Navicula* sp., and *Nitzchia* were also present. As grazing is inevitable in HRAOP, some zooplankton including *Daphnia* sp. was also noticed occasionally in the pond. These were removed during MLSS estimation to avoid interference with MLSS concentration.

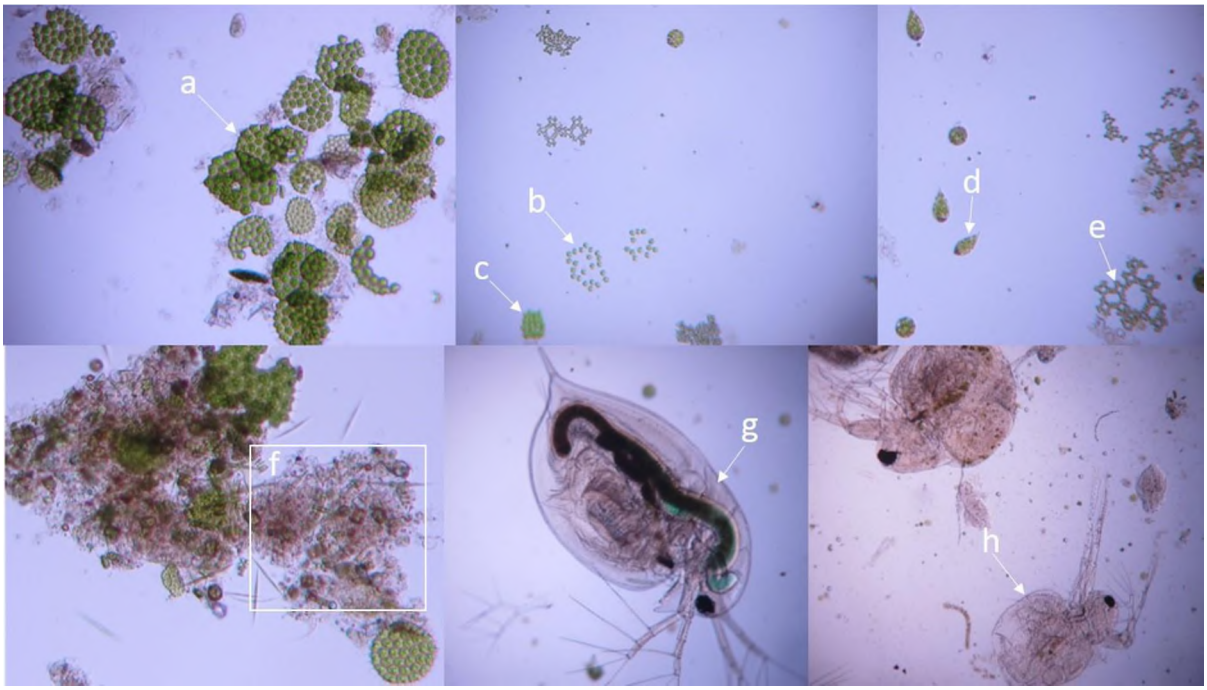

Figure 3.3: Examples of microalgal species and zooplankton in the MLSS from HRAOP B during the period of monitoring. *Pediastrum* sp. (a), *Chlorella* sp. (b), *Pyrobotrys* sp. (c), *Euglena* sp. (d), *Micractinium* sp. (e), Diatoms (f), side view of *Daphnia* sp. (g), and front view of *Daphnia* sp. (h).

Of the few bacterial colonies observed on nutrient agar after 24 h of incubation of MLSS sample, one appeared distinct from others as 1-2 mm orange round flat colony with smooth edges (Figure 3.4a) and was therefore isolated for identification. A 48 h incubation of the pure culture revealed a more intense orange colour of the colonies than 24 h incubation. For further identification of the organism, Gram stain analysis was carried out, which revealed the organism as a coccus shaped Gram-positive bacterium with cells arranged in chains as diplococci or streptococci (Figure 3.4b). For a complete identification, the DNA of the organism was extracted and 16S ribosomal sequence analysis was carried out. BLAST analysis confirmed the identity of the organism. The sequence was 99% matched to *Planococcus maitriensis* (Figure 3.5) (GenBank Accession number KC778380.1) and, typed strain deposited in EBRU culture collection as *Planococcus maitriensis* strain ECCN 45b.

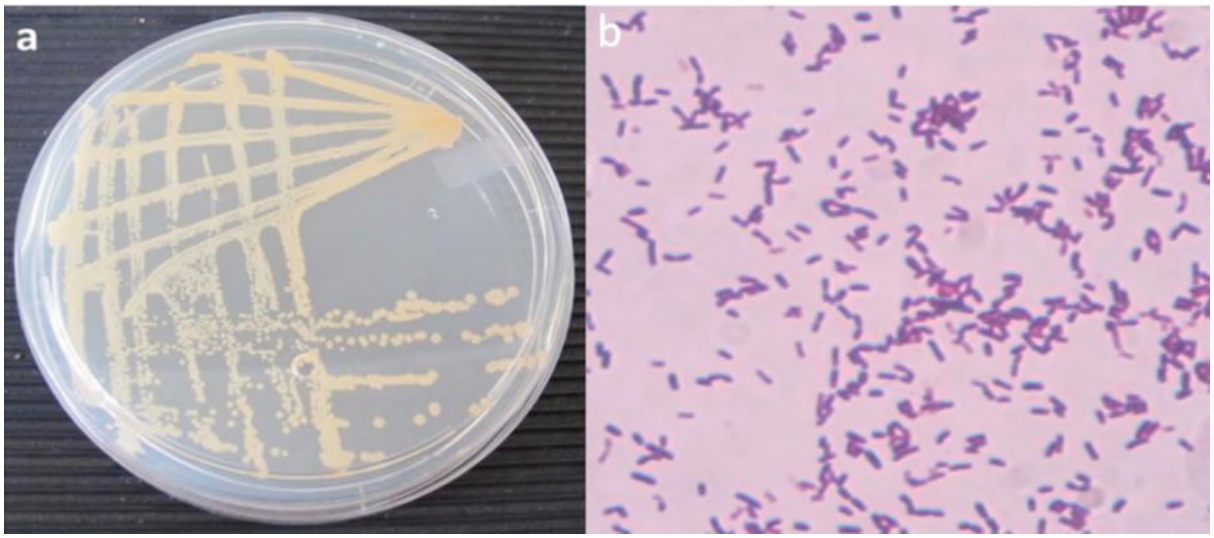

Figure 3.4: Pure culture of *Planococcus maitriensis* isolated from HRAOP after 48 h of incubation on nutrient agar (a) and light microscopic image of the Gram stained cells (b).

```
gb|KC778380.1| Planococcus maitriensis strain BGB15 16S ribosomal RNA gene, partial
sequence
Length=1340

Score = 1406.1 bits (1558), Expect = 0E00
Identities = 781/783 (99), Gaps = 0/783 (0)
Strand = Plus/Minus

Query 1   TGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACG 60
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 783 TGCGTTAGCTGCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACG 724

Query 61  GCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCA 120
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 723 GCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCA 664

Query 121 GTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCAC 180
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 663 GTTACAGACCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCAC 604

Query 181 CGCTACACGTGGAATTCCACTTTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACC 240
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 603 CGCTACACGTGGAATTCCACTTTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACC 544

Query 241 CTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGGAACCGCCTGCGCGCGCTTTA 300
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 543 CTCCACGGTTGAGCCGTGGGCTTTCACATCAGACTTAAGGAACCGCCTGCGCGCGCTTTA 484

Query 301 CGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAG 360
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 483 CGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAG 424

Query 361 TTAGCCGTGGCTTTCTGGTGAGGTACCGTCAAGGTACCAATAGTTAGTTGGTACGTGTTC 420
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 423 TTAGCCGTGGCTTTCTGGTGAGGTACCGTCAAGGTACCAATAGTTAGTTGGTACGTGTTC 364

Query 421 TTCCCTCACAACAGAGTTTTACGATCCGAAAACCTTCGTCACTCACGCGGCGTTGCTCCG 480
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 363 TTCCCTCACAACAGAGTTTTACGATCCGAAAACCTTCGTCACTCACGCGGCGTTGCTCCG 304

Query 481 TCAGACTTGCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCG 540
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct 303 TCAGACTTGCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCG 244

Query 541 TGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGTCGGCTACGCATCGTGGCCTTGGT 600
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 243 TGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGTCGGCTACGCATCGTGGCCTTGGT 184

Query 601 GGGCCGTTACCCCACCAACTAGCTAATGCGCCGCGGGCCCATCCTGCAGTGACAGCCGAA 660
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 183 GGGCCGTTACCCCACCAACTAGCTAATGCGCCGCGGGCCCATCCTGCAGTGACAGCCGAA 124

Query 661 ACCGTCTTTCCGTGAAGCCTCAGGWGAGGCTTCAAACTATTCGGTATTAGCACCGGTTTC 720
          |||||||||||||||||||||||| ||||| ||||||||||||||||||||||||||||
Sbjct 123 ACCGTCTTTCCGTGAAGCCTCAGGAGAGGCCTCAAACTATTCGGTATTAGCACCGGTTTC 64

Query 721 CCGGAGTTATCCCGATCTGCAGGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTA 780
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 63  CCGGAGTTATCCCGATCTGCAGGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTA 4

Query 781 AAC 783
          |||
Sbjct 3   AAC 1
```

Figure 3.5: BLAST analysis of *P. maitriensis* isolated from wastewater treatment HRAOP.

3.3.4 Variation in biomass concentration and productivity

Diurnal study of biomass concentration revealed gradual increase in MLSS concentration with highest attained in the afternoon (i.e. $MLSS_{pm}$) in most cases (Figure 3.6). However, the most fascinating discovery was that irrespective of MLSS concentration in the afternoon, a large portion (about 39%) was usually lost from the pond overnight, which significantly reduced biomass concentration in the pond every morning (Figure 3.6). Within the period of monitoring, MLSS concentration in HRAOP B ranged between 77 mg L^{-1} ($MLSS_{am}$ in September) and 285 mg L^{-1} ($MLSS_{pm}$ in November) with respective biomass productivity of 58 kg ha^{-1} d^{-1} (5.8 g m^{-2} d^{-1}) and 215 kg ha^{-1} d^{-1} (21.5 g m^{-2} d^{-1}) (Figure 3.7).

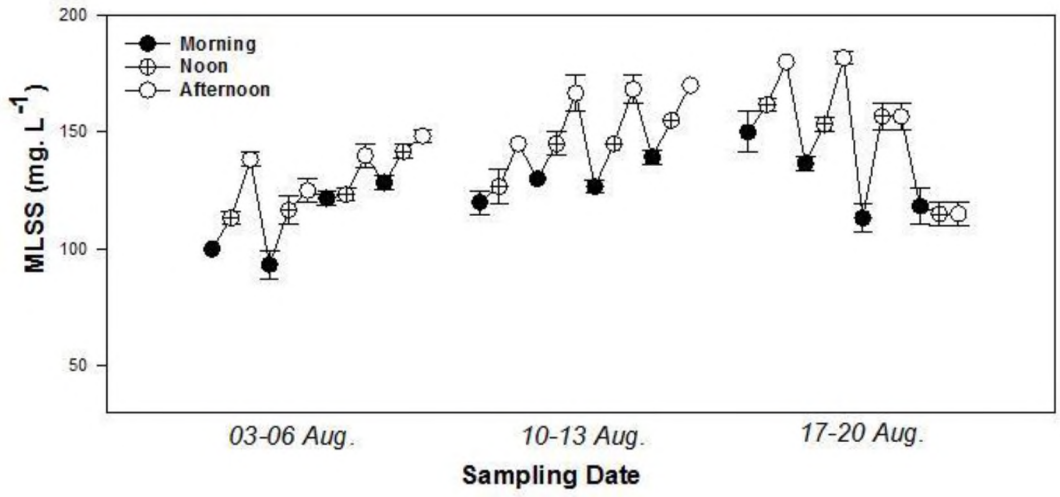

Figure 3.6: Diurnal change in biomass concentration measured as MLSS ± SE in HRAOP B in August 2015.

Figure 3.7 shows MLSS concentration, productivity, and water temperature of HRAOP measured routinely over 4 months. Results show that as temperature changed (i.e. from winter to summer), biomass concentration increased concomitantly, but diurnal biomass concentration remained low in the morning and high in the afternoon indicative of overnight biomass loss. Even so, biomass productivity was low in August and September (with an average of 110 ± 16 kg ha^{-1} d^{-1} (11 g m^{-2} d^{-1})), which increased in October and November (with an average of 167 ± 26 kg ha^{-1} d^{-1} (16.7 g m^{-2} d^{-1})). This indicates the influence of climatic conditions on HRAOP productivity.

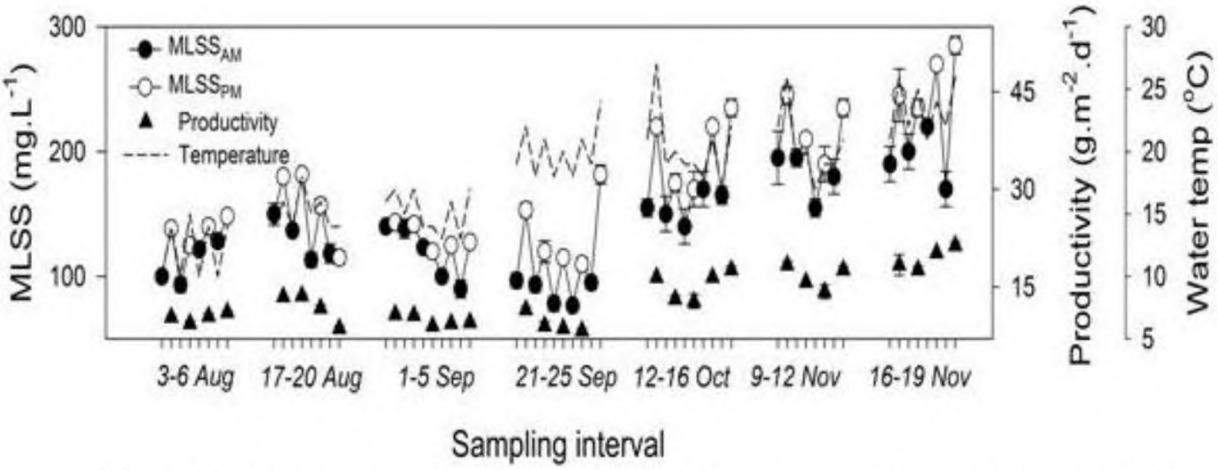

Figure 3.7: Time course of the change in MaB-flocs productivity, and diurnal MLSS and water temperature in HRAOPs of an IAPS treating municipal sewage. Data were captured from August through November 2015 and are presented as the mean ± SE.

3.3.5 Estimated and actual biomass loss

In an effort to account for biomass loss overnight from HRAOP B, the actual biomass loss based on the measured MLSS and estimated loss overnight into ASP based on designed flow rate and hydraulic retention time were calculated using Equations 3.4 and 3.5. The measured loss (36.70 ± 4.95 mg L^{-1}) in the present study was higher than the estimated loss (29.57 ± 1.60 mg L^{-1}) of biomass to ASP for all sampling intervals (Table 3.1). However, a *t* test analysis using alpha level 0.05 revealed that the values were not significantly different (P= 0.14), indicating loss is actually due to continuous passive flow into ASP.

$$\text{Measured loss} = MLSS_{pm} - MLSS_{am} \quad \textbf{Equation 3.4}$$

$$\text{Estimated loss} = (MLSS_{pm} \times V_P) - (\Delta t/24 \times V_D) \times MLSS_{pm}/V_P \quad \textbf{Equation 3.5}$$

Where: $MLSS_{pm}$ and $MLSS_{am}$ are consecutive afternoon and morning MLSS concentrations respectively (mg L^{-1}),

V_P = pond volume (L),

Δt = time difference between $MLSS_{pm}$ and consecutive $MLSS_{am}$ (h) and

V_D = volume displaced at Δt and (L).

Table 3.1: Diurnal fluctuation in MLSS concentration in the HRAOP of the IAPS treating municipal sewage. $MLSS_{PM}$ and $MLSS_{AM}$ concentrations are mean values ± SE for all sampling intervals (Figure 3.7). Loss of MLSS was quantified as the difference between consecutive $MLSS_{pm}$ and $MLSS_{am}$ determinations (i.e. $MLSS_{pm} - MLSS_{am}$) and is a mean value ± SE for all sampling intervals. Estimated loss of MLSS between evening and the following morning was calculated using the expression $[(MLSS_{pm} \cdot V_P) - (\Delta t/24 \cdot V_D) \cdot MLSS_{pm}]/V_P$ where: V_P = pond volume (L); Δt = time difference (h); V_D = volume displaced (L); and, is a mean value ± SE for all sampling intervals. ASP = algae settler pond.

MLSS	**mg L^{-1}**
$MLSS_{pm}$	172.08 ± 9.52
$MLSS_{am}$	135.48 ± 7.86
Measured loss of MLSS to ASP	36.70 ± 4.95 a
Estimated loss of MLSS to ASP	29.57 ± 1.60 a

3.4 Discussion

This chapter set out to describe the structure and composition of MaB-floc in HRAOP treating domestic sewage and investigate the productivity of the pond as MLSS under South African climatic conditions for its potential use in a biorefinery. Results show that the microbial composition of HRAOPs is mainly microalgae and bacteria, which are discreet aggregates that assemble to form MaB-flocs. Furthermore, the formation of these MaB-flocs aid settleability and harvestability of biomass. MaB-floc concentration and productivity in Belmont Valley HRAOP measured over a period of four months revealed that the pond is capable of producing substantial amount of biomass for use in biofuels, animal feed, composting, and fine chemicals production. However, biomass concentration was high at higher solar radiation, which increased pond water temperature. Thus, diurnal variation in environmental parameters influenced productivity of HRAOP.

Biomass from HRAOP in the present study appeared as stable aggregates in the form of flocs containing microalgae, diatoms and bacteria in their structure. MaB-floc formation are believed to be a consequence of EPS production by these organisms, which helps maintain stability, and acts as a source of nutrients when conditions are not favourable (Su *et al.*, 2011; More *et al.*, 2014; Ding *et al.*, 2015). Therefore, it is suggested that some or all the microbial components in the HRAOP are EPS producers. The MaB-flocs showed a good settleability

within 2 h, which is an advantage of IAPS for maximum biomass recovery for valorization. This corroborates previous findings that showed and emphasized the importance of MaB-flocs in wastewater treatment for simple separation and recovery of biomass from treated water (Van Den Hende *et al.*, 2011; Su *et al.*, 2011). Thus, MaB-floc formation in HRAOPs will provide quality water for discharge coupled with the cost effective recovery of biomass.

Algae species composition was similar to that previously reported for most wastewater treatment HRAOPs (Fallowfield *et al.*, 1999; Park *et al.*, 2011). Species composition in HRAOPs is dependent on environmental conditions and grazing (Mehrabadi *et al.*, 2016). Abundance of a particular species varied depending on the prevalent species. For example, *Pediastrum* sp. dominated the pond in September whereas the diatom, *Cyclotella* sp. were the most abundant in October. In addition to the usual algae composition, a marine bacterium was isolated from the HRAOP and, molecularly identified as *P. maitriensis*. This halophilic bacterium was first discovered in cyanobacterial mats in Antarctica by Alam *et al.* (2003). *Planococcus* sp. has been isolated from marine environments in the past (Junge *et al.*, 1998; Engelhardt *et al.*, 2001; Yoon *et al.*, 2003). Even so, there is no report yet in the literature on presence in wastewater. However, Seck *et al.* (2016) recently reported the isolation of *P. massiliensis* from human stool, indicating a possible source of *P. maitriensis* in HRAOP treating wastewater. Thus, further research is required to elucidate the origin, survival, and contribution of this bacterium to wastewater treatment.

Diurnal MLSS concentration in HRAOP was reduced by ~39% every consecutive morning (i.e. $MLSS_{am}$). This was attributed to passive settling in ASP. Nevertheless, the productivity achieved in this study corroborates previous findings and fits in the range reported of experimental, pilot and full-scale wastewater treatment HRAOPs (Al-Shayji *et al.*, 1994; Passos *et al.*, 2013; Sutherland *et al.*, 2013; Park *et al.*, 2011). The lowest productivity observed between August and September can be attributed to changes in climatic conditions particularly solar radiation and temperature (Sutherland *et al.*, 2015a; Chisti, 2016). In addition, grazing is a major limiting factor for biomass production in HRAOP (Park *et al.*, 2011). The low productivity observed in September was also coincidental with the invasion of *Daphnia* sp., indicating the negative impact of grazers on biomass production. A positive correlation was observed between the diurnal MaB-flocs concentration, temperature, irradiance, pH, and DO (see Figure 3.7 and Appendix B, Figure B1). Variation in these parameters is known to directly affect metabolism and productivity in HRAOP (Fallowfield

et al., 1999; Craggs *et al.*, 2004). Thus, explaining the reason for higher productivity between October and November.

In conclusion, the study of HRAOP treating domestic sewage revealed the recruitment of constituent microorganisms as MaB-flocs, which aid settleability by gravity. Results have shown that Belmont Valley HRAOP can achieve the biomass productivity expected of a typical wastewater treatment HRAOP. However, diurnal variation in environmental parameters has a great impact on the production and concentration of these MaB-flocs. Finally, isolation of *P. maitriensis* from HRAOP treating domestic sewage is novel to this study, requiring further investigation on its environmental and biotechnological applications.

Chapter 4: Extracellular Polymeric Substances in High Rate Algal Oxidation Ponds

4.1 Introduction

The basis of floc formation and water remediation in HRAOPs is the coexistence of microalgae and heterotrophic bacteria. The interaction between these two groups of organism affects the physiology and metabolism of each, during which improvement in biomass production and flocculation occurs (Barranguet *et al.*, 2005; Ramanan *et al.*, 2016). Floc formation aids settling and harvesting of biomass, which improves biomass recovery for downstream applications. As discussed in Chapter 1, extracellular polymeric substances (EPSs) are known for their crucial role in aggregation of cells to form flocs and biofilms (Sheng *et al.*, 2010; More *et al.*, 2014; Ding *et al.*, 2015). These substances are either released into the medium as soluble EPS (contributing to the organic matter composition of the medium), or attached to cells as bound EPS.

Apart from aiding MaB-floc formation, EPS protects cells from unfavourable conditions, aids symbiotic associations, and serves as a carbon reserve during starvation and stress avoidance (Parikh and Madamwar, 2006; Mishra *et al.*, 2011). Although there is not much published information on EPS production by algae, all living organisms from various niches are capable of secreting extracellular polymers provided sufficient organic substance and carbon/nitrogen is present (Singha, 2012). Blue green algae (cyanobacteria) EPS formation has been studied in some detail and applications in detoxification of heavy metals, soil conditioning to improve water holding capacity, bioflocculation, bioemulsification and biosurfactant chemistry have been reported (Mishra *et al.*, 2011; Parikh and Madamwar, 2006; Pereira *et al.*, 2011; Chug and Mathur, 2013; Khangembam *et al.*, 2016).

Although wastewater contains organic material that aids flocculation, the facultative pond of IAPS buffers and removes a significant portion before water passes to the HRAOPs (Mambo *et al.*, 2014a). Thus, it is very likely that microalgae and bacteria in HRAOPs are responsible for *in situ* floc formation (i.e. MaB-flocs) by synthesis and secretion of EPS. Park *et al.* (2013) speculated that environmental stressors such as darkness, low oxygen concentration, extreme pH and temperature might trigger EPS production in HRAOPs, which could aid in MaB-floc formation and settleability of biomass in ASPs.

Soluble EPSs released into the growth medium are known to contribute to COD and BOD concentration of treated wastewater (Barker and Stuckey, 1999; Laspidou and Rittmann, 2002). It is therefore distinctly possible that MaB-floc formation in the HRAOPs together with EPS formation is responsible for the relatively high COD (and TSS) concentration in treated water. This coupled with studies reported in Chapter 2 that showed peroxone-induced reduction in COD and TSS concentration of IAPS-treated water and, studies reported in Chapter 3 that showed the recruitment of microbial component of HRAOP prompted a study on EPS production. This chapter therefore carried out an investigation on the characteristics of EPS generated in HRAOP, to elucidate its importance as a potential high value product. In addition, experiments were carried out on EPS production by *Chlorella* sp. and *P. maitriensis* isolated from HRAOP, to give insight into the main EPS producers in the pond.

4.2 Materials and Methods

4.2.1 MaB-floc culturing for EPS production

Mixed liquor (500 mL) from HRAOP B containing mostly algae and bacteria was collected in Erlenmeyer flasks and the EPS extracted and quantified as described in Section 4.2.2. Where specified, flasks were incubated in a controlled environment either under continuous cool white fluorescent light (70-90 µmol m^{-2} s^{-1}) or in total darkness at 25°C on a rotary shaker at 100 rpm for 10 d. Samples were collected at intervals for EPS extraction, quantification and characterization.

In addition, EPS production by *P. maitriensis* and *Chlorella* sp. isolated from HRAOP B of the IAPS as described in Chapter 3 was investigated. For *Chlorella* sp., 200 mL of Bold 3N medium (Appendix B, Table B1) in Erlenmeyer flasks was inoculated with 20% culture and placed in a controlled environment under continuous cool white fluorescent light (70-90 µmol m^{-2} s^{-1}) at 25°C on a rotary shaker at 100 rpm for 12 d. For *P. maitriensis*, 100 mL mineral salt medium (Appendix B, Table B3) in Erlenmeyer flasks was inoculated with 5% culture and incubated at 37°C in a shaking incubator at 100 rpm (Labcon micro-processor controlled platform shaking incubator, South Africa) for 12 d. Growth and EPS production were measured every 3 d in both algal and bacterial cultures. Algal growth was monitored by measuring absorbance of culture medium at 760 nm using an Aquamate spectrophotometer (Thermo Spectronic, England) with Bold 3N medium as background, and, TSS quantified by filtration (APHA 1998). Bacterial growth was monitored by measuring absorbance of the

culture medium at 600 nm using an Aquamate spectrophotometer (Thermo Spectronic, England) with mineral salt medium as background.

4.2.2 EPS extraction

Only the EPS released into the medium (i.e. soluble EPS) was extracted. Algal and MaB-floc EPS were extracted according to the method described by Ahmed *et al.* (2012). Samples (100 mL) were centrifuged (Avanti® J-E centrifuge; Beckman Coulter Inc, USA) at 5000 × g for 20 min. Supernatant was filtered through 0.45 µm Whatmann filters (glass microfiber filters, grade GF/C), followed by 0.22 µm membrane filters. The filtrate was lyophilized, and mass of the residue determined and stored in a desiccator. For bacterial EPS, samples were extracted by centrifugation at 15,000 rpm for 20 min, reported not to cause cell lysis (Pan *et al.*, 2010) followed by filtration through 0.22 µm membrane filters. The filtrate was lyophilized, and mass of the residue determined and stored in a desiccator.

4.2.3 Biochemical analyses of EPS

Carbohydrate, protein and α-amino nitrogen content of EPS was analysed as follows; carbohydrate content was determined using the phenol-sulphuric acid assay as described by Dubois *et al.* (1956). EPS (2 mg) was dissolved in 0.5 mL distilled water, to these; 0.5 mL of phenol solution was added followed immediately by the addition of 2.5 mL concentrated sulphuric acid (reagent grade). The mixture was vortexed and cooled to room temperature. Absorbance was measured at 490 nm (UV mini 1240 spectrophotometer) against a background prepared with 0.5 mL distilled water and reagents. Sugar concentration in the samples was determined by interpolation from a standard curve prepared using a series of known concentrations of glucose (0.5 mg/mL) stock solution.

Protein analysis was carried using the Bradford dye-binding assay (Bradford, 1976). Bradford reagent (5 mL) was added to 3 mg of EPS suspension in 0.5 mL distilled water and mixed thoroughly. Absorbance was measured at 595 nm after 5 min against a background prepared with 0.5 mL distilled water and reagent using a UV-VIS mini 1240 spectrophotometer. Protein concentration was determined by interpolation from a standard curve prepared with a series of known concentrations of Bovine Serum Albumin (BSA; 2 mg/mL) stock solution.

Alpha amino nitrogen was analysed by the Ninhydrin method (Lie, 1973). Ninhydrin is an oxidizing agent that causes oxidative decarboxylation of α-amino acids to produce carbon dioxide, ammonia and aldehyde with one less carbon atom upon heating. The reduced

Ninhydrin reacts with the liberated ammonia to form a blue complex (Lie, 1973). To 2 mg of EPS dissolved in 2 mL distilled water, 1 mL of colour reagent (prepared by dissolving 100 g $Na_2HPO_4.12H_2O$, 60 g anhydrous KH_2PO_4, 5 g Ninhydrin, and 3 g fructose in 1 L distilled water, pH 6.7) was added and placed in boiling water for 16 min. The mixture was transferred immediately to another water bath at 20°C to cool for 20 min. Thereafter, 5 mL of dilution reagent (prepared by dissolving 2 g KIO_3 in 600 mL distilled water, which was then made to 1 L with 96% ethanol) was added to the tubes and mixed thoroughly. Absorbance was measured at 570 nm within 30 min (UV mini 1240 spectrophotometer) against a background prepared with 2 mL distilled water and reagents. Concentration was determined by interpolation from a standard curve prepared with a known concentration of glycine.

4.2.4 Fourier Transformed Infrared Spectroscopy (FT-IR)

FT-IR analysis of sub-samples of the extracted EPS (~1 mg) was carried out using a PerkinElmer Spectrum 100 instrument (PerkinElmer, Waltham, MA) with attenuated total reflectance (ATR) accessory eliminating the need for mixing of samples with potassium bromide. The ATR accessory, fitted with a diamond top-plate, has spectral range of 25000-100 cm^{-1}, refractive index of 2.4, and 2.01 μ depth penetration. FT-IR spectra were recorded in the range of 4000-650 cm^{-1}.

4.3 Results

4.3.1 Extraction and characterization of MaB-floc EPS in HRAOP

Extracted EPS appeared as white fluffy powdered material. Two independent sampling revealed an average of 116 ± 4 mg L^{-1} EPS is produced by MaB-flocs in HRAOP. Biochemical analyses revealed the EPS contained 58 ± 6 mg carbohydrate, 9 ± 1 mg protein and 3 ± 0.1 mg α-amino nitrogen, indicating the enrichment of the EPS in carbohydrate.

To further reveal the characteristic of the EPS, FT-IR analysis was conducted and the result interpreted by correlating with IR chart and other reports in the literature (Figure 4.1). The very broad stretch observed in the region 3400-3300 cm^{-1} was assigned to O-H (H-bonded) of carboxylic acid and N-H stretching of amines. The weak stretch in the region 2250-2100 cm^{-1} was assigned to C≡C of alkynes while another weak stretch in region 1660-1600 cm^{-1} could either be due to CO_2 adsorption (Nabiev *et al.*, 1976) or asymmetric stretching of –N=C=O- (Panda and Sadafule, 1996). The sharp bend in the region 1460-1380 cm^{-1} was assigned to C-C of aromatics while the region 1300-1000cm^{-1} was assigned to C-O, C-O-C corresponding

to the presence of carbohydrates and sugar derivatives (Sheng *et al.*, 2005; Bramhachari and Dubey, 2006; Mishra and Jha, 2009). Peaks observed in region 950-650 cm^{-1} were assigned to sp^2 C-H of alkenes and aromatics.

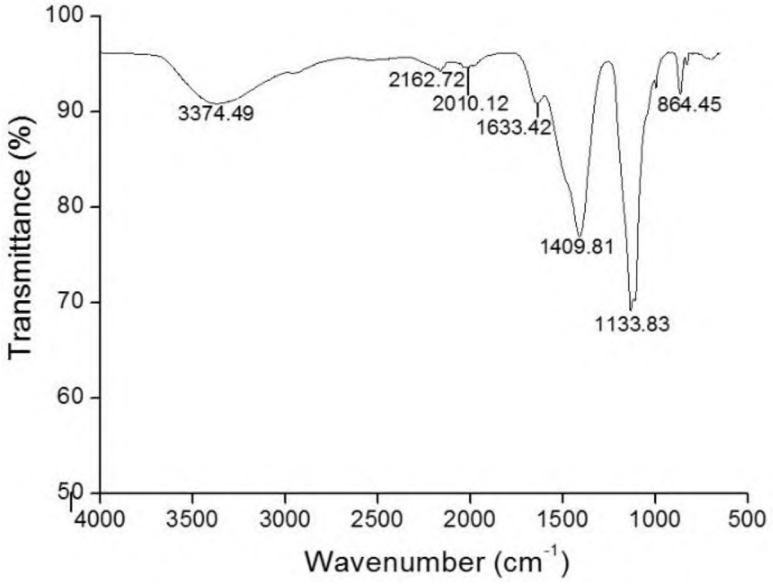

Figure 4.1: FT-IR spectra of MaB-floc EPS generated in HRAOP.

4.3.2 Diurnal changes in EPS in production in HRAOP

To establish the relationship between microalgal-bacterial biomass and EPS production in HRAOPs, MaB-floc concentration as MLSS and EPS were measured simultaneously. The EPS yield in HRAOP ranged from 0.5 g L^{-1} to 0.7 g L^{-1} in August 2016. Apparently, EPS production in HRAOP varied diurnally with MLSS concentration, which was concomitant with water temperature (Figure 4.2). Similar to diurnal MLSS pattern described in Chapter 3, diurnal EPS concentration was higher every afternoon than the measured value in the morning. This indicates a direct correlation between biomass and the EPS they produce, which might have a great influence on the formation and stability of MaB-flocs in HRAOP.

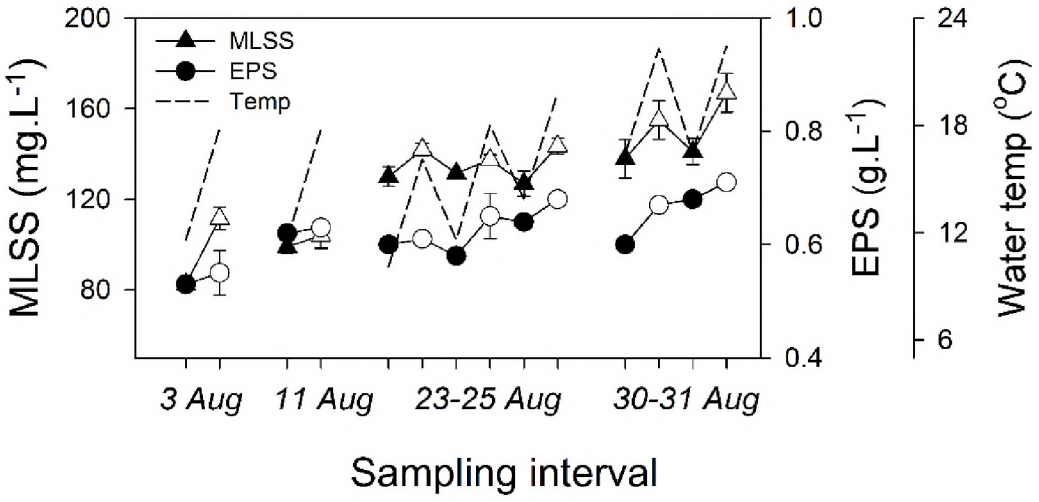

Figure 4.2: Diurnal change in MLSS, EPS, and water temperature in HRAOP B. data were captured in August 2016 and presented as the mean ± SE.

4.3.3 MaB-floc EPS production in flask cultures

MLSS containing MaB-flocs were collected in flasks and incubated under continuous light and total darkness for a period of 10 d. Accumulation of EPS was observed in both conditions. However, the accumulation was higher in continuous light (112-601 mg. L^{-1}) than in total darkness (112-254 mg. L^{-1}), depicting continuous illumination indeed has an effect on EPS production by MaB-flocs (Figure 4.3). Even so, the small increase in EPS concentration in total darkness indicates that EPS production is also achievable without photosynthesis provided carbon and nitrogen source.

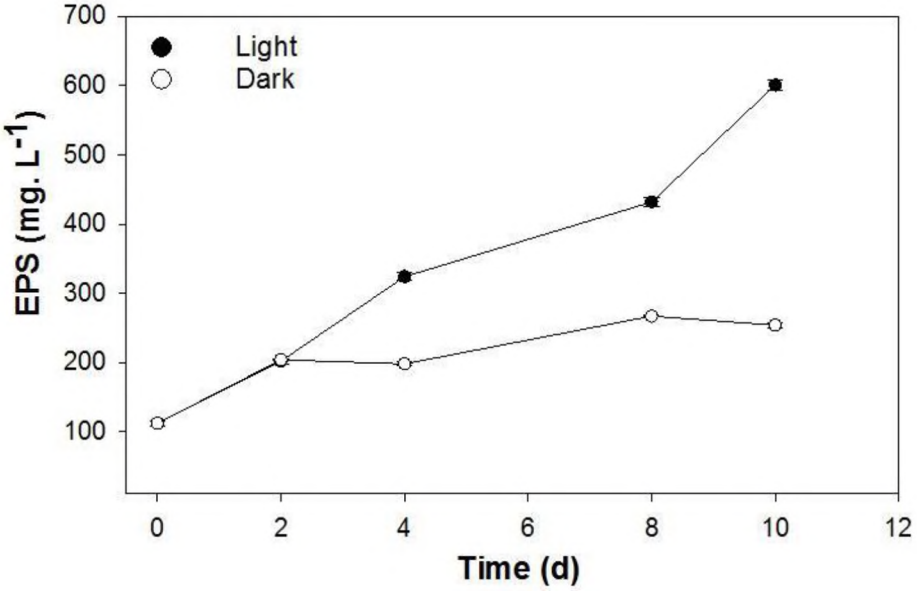

Figure 4.3: Accumulation of soluble EPS in MLSS from HRAOP incubated in continuous light and darkness over time.

4.3.4 Biochemical composition of MaB-floc EPS in flask cultures

Accumulation of EPS in light and dark was associated with reduction in the biochemical component over time (Figure 4.4). In the light incubated cultures, the carbohydrate content of the EPS reduced from 64 mg g^{-1} to 30 mg g-1, protein from 10 mg g^{-1} to 4 mg. g^{-1}, and α-amino nitrogen from 3 mg. g^{-1} to 1 mg g^{-1} after 10 d of incubation. However, the carbohydrate content in the dark incubated cultures reduced to 17 mg g^{-1}, protein to 3 mg. g^{-1}, and α-amino nitrogen to 1 mg g^{-1} after 10 d of incubation. Thus, result showed that the biochemical reduction in the dark was more pronounced and drastic than the light incubated cultures, indicating that the MaB-flocs were able to use EPS as carbon and nitrogen source heterotrophically in the absence of light (photosynthesis).

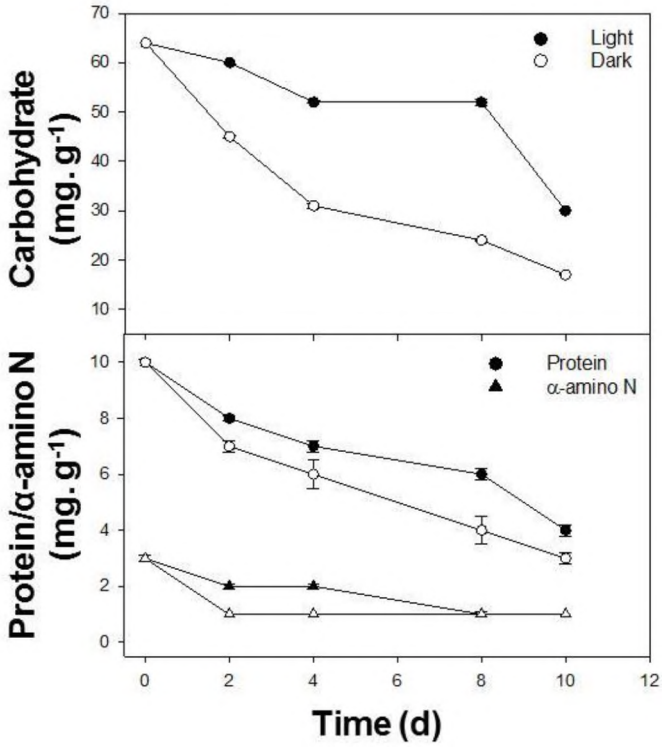

Figure 4.4: Change in biochemical composition of soluble EPS extracted from MLSS of HRAOP incubated in continuous light and darkness. Error bars indicate ± SE of two independent experiments.

4.3.5 FT-IR spectroscopy of the MaB-floc EPS in flask cultures

FT-IR analysis of the light and dark incubated EPSs further revealed the difference between the EPS generated in both conditions. The spectra of MaB-floc EPSs after incubation (Figure 4.5a and b) showed similar characteristics with that from HRAOP (Figure 4.1). However, there was a change in the intensity of the dark incubated EPS, which increased significantly in the regions corresponding to O-H of carboxylic acid, C-C and C-H of aromatics and, C-O,

C-O-C of carbohydrates (Figure 4.5b). The increased intensity in these regions was taken to be increased frequency of vibration possibly reflecting transition to heterotrophic growth.

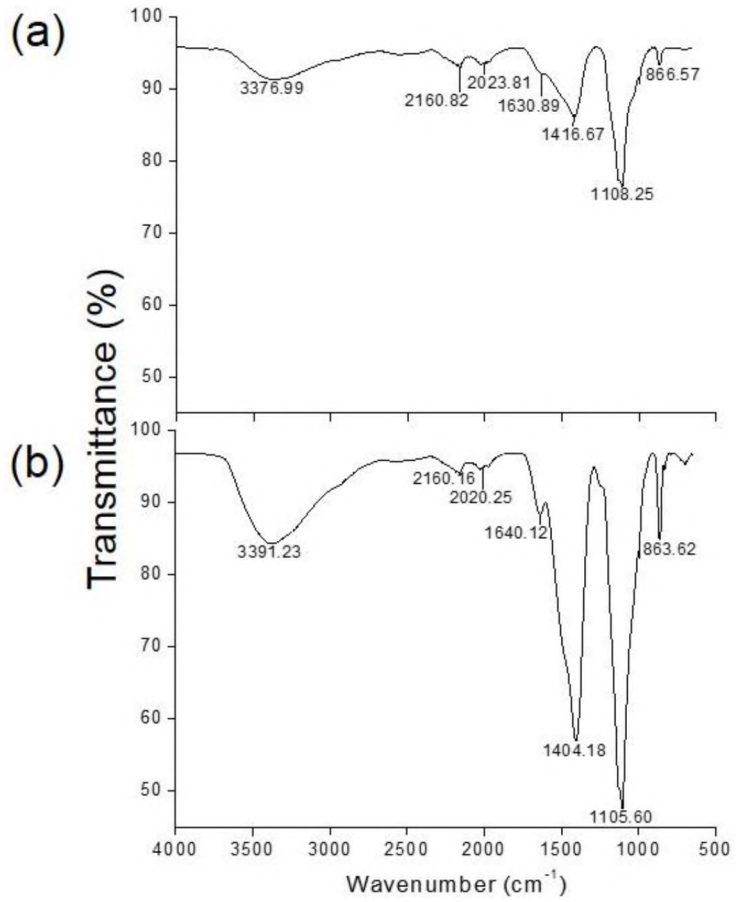

Figure 4.5: FT-IR spectra of soluble EPS accumulated in MaB-floc culture after incubation in continuous light (a) or total darkness (b).

4.3.6 Growth and EPS production by *Chlorella* sp. and *P. maitriensis*

Chlorella sp. and *P. maitriensis* isolated from HRAOP were cultured individually as described in Section 4.2.1 for 12 d to investigate their ability and contribution to EPS production. Growth of the organisms and EPS concentration in their respective cultures are presented in Figure 4.6. *Chlorella* sp. showed a typical sigmoid growth pattern from the beginning to the end of the experiment, with no occurrence of lag phase (Figure 4.6a). This is most likely because the cells have already adapted to the culture medium, as it was used for their initial isolation. Even so, maximum EPS production (0.31 ± 0.03 g L^{-1}) was attained within 3 d of incubation, after which concentration decreased for the rest of the incubation period (Figure 4.6a). In contrast, cell growth stopped completely after 3 d in *P. maitriensis* culture, whereby the absorbance of the culture declined precipitously for the rest of the 12 d incubation (Figure 4.6b). This was taken as the lack of carbon in the culture medium, which

resulted in cell death. Surprisingly, EPS concentration increased until 9 d of incubation (0.61 ± 0.03 g L^{-1}), with no visible decline over the period of incubation (Figure 4.6b).

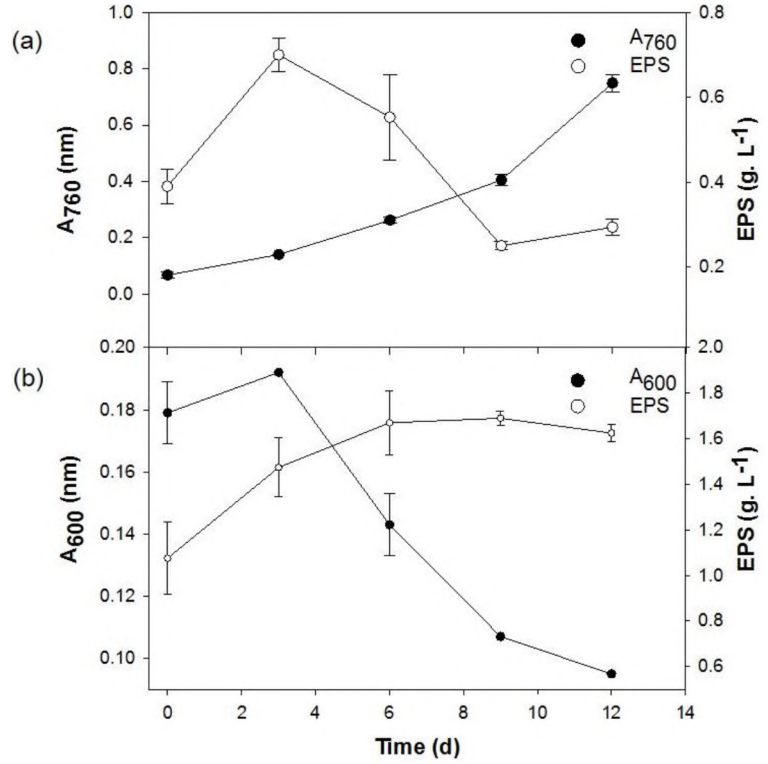

Figure 4.6: Biomass and EPS production in *Chlorella* sp. (a) and *P. maitriensis* (b) culture over time. Error bars indicate ± SE of duplicate samples.

4.3.7 Biochemical composition of *Chlorella* sp. and *P. maitriensis* EPS

Biochemical characteristics of *Chlorella* sp. and *P. maitriensis* EPS is presented in Table 4.1. Similar to MaB-floc EPS, the carbohydrate content was higher than protein and α-amino nitrogen in the two different EPSs. It was observed that the biochemical concentrations varied at different incubation time during the 12 d incubation. Even so, it appeared that at the end of incubation period, the carbohydrate content of *Chlorella* EPS increased by 2.3 mg. g^{-1}, protein by 1.6 mg. g^{-1}, and α-amino nitrogen by 0.2 mg. g^{-1}. A similar pattern of biochemical characteristics was observed of *P. maitriensis* EPS, where the carbohydrate content increased by 13.4 mg. g^{-1}, protein by 5.0 mg. g^{-1}, and α-amino nitrogen by 1.0 mg. g^{-1} after the 12 d incubation period. Thus, the biochemical content of *P. maitriensis* EPS was higher than the *Chlorella* sp. EPS, depicting the enrichment of the bacterial EPS.

Table 4.1: Biochemical characteristics (± SE) of soluble EPS extracted from *P. maitriensis* and *Chlorella* sp. incubated over time.

Time (d)	*P. maitriensis*			*Chlorella* sp.		
	Carbohydrate (mg. g^{-1})	Protein (mg. g^{-1})	α-amino N (mg. g^{-1})	Carbohydrate (mg. g^{-1})	Protein (mg. g^{-1})	α-amino N (mg. g^{-1})
0	1.0±0.1	0.3±0	3.6±0	11.3±1.0	0.9±0	0.1±0
3	6.9±0.3	4.4±0.2	3.4±0.3	13.1±0.4	1.3±0.1	0.1±0
6	6.7±0.4	4.0±0.1	3.1±0	11.3±0.7	1.3±0.1	0.2±0
9	11.1±0.5	6.9±0.2	5.1±0.1	12.7±0.9	2.1±0	0.1±0
12	14.4±0.4	5.3±0.3	4.6±0.1	13.6±0.7	2.5±0.1	0.3±0

4.4 Discussion

Investigation in this chapter describes the EPS generated in wastewater treatment HRAOP. The subject of interest was to quantify and characterize EPS associated with MaB-flocs as a potential high value product of biotechnological importance. Indeed, results show that a considerable amount of EPS is generated in HRAOP, which confirmed their involvement in recruitment of biomass into settleable flocs. The EPS generated varied diurnally with biomass concentration, suggesting that the amount of EPS produced in HRAOP greatly depends on the productivity of the pond. Biochemical and FT-IR analyses revealed characteristic carbohydrate enrichment of these polymeric substances. Furthermore, EPS production by HRAOP MaB-flocs was stimulated by continuous illumination while total darkness resulted in transition to heterotrophic metabolism. However, while accumulation of EPS was observed in the dark-incubated MaB-flocs, the biochemical components reduced markedly. With this, it was rationalized that total darkness resulted in a transition from phototrophic to heterotrophic metabolism.

EPS is regarded as a binding mechanism that facilitates the formation and stability of flocs in wastewater treatment systems (Su *et al.*, 2011). Sheng *et al.* (2006) have reported the importance of EPS concentration on the stability of flocs in wastewater treatment. Indeed, when MaB-floc concentration was low, EPS concentration was also low in HRAOP. It is therefore suggested that a direct correlation exists between EPS and MaB-floc formation in HRAOP (i.e. the more EPS produced, the more MaB-flocs will be formed in the pond).

With continuous illumination, EPS production was higher in comparison to the dark incubation. This is in accordance with a report by Pereira *et al.* (2009), where they emphasized that EPS in microalgae production is light dependent but the photoperiod does not affect the quality or composition of the EPS. Even so, incubation in total darkness also brought about a slight increase in EPS concentration, which was rationalized to have resulted from heterotrophic metabolism. Thus, in the absence of photosynthesis, carbon and nitrogen from the existing EPS were recycled to support growth. Hence the marked reduction in the biochemical content of the dark incubated EPS. Furthermore, it is most likely that the heterotrophic bacteria in the MaB-flocs were responsible for EPS production in the dark, but the contribution of microalgae cannot be overlooked, since they can also grow heterotrophically making use of carbon as their sole source of energy (Perez-Garcia *et al.*, 2011).

FT-IR analysis further confirmed the presence of carbohydrates, amines, alkyl groups and aromatic compounds in EPS produced in HRAOP. These functional groups are important adsorption sites, which determine the binding capacity of EPS (More *et al.*, 2014). The peaks observed are similar to those reported in literature of microalgal and cyanobacterial EPSs (Parikh and Madamwar, 2006; Mishra and Jha, 2009). Similar peaks were also detected in EPS extracted from wastewater (Zeng *et al.*, 2016). Nevertheless, heterotrophic metabolism of the MaB-flocs likely caused the increased intensity of some peaks in regions of aromatics and carbohydrates in dark incubated EPS. The increased intensity could also be attributed to accumulation of humic substances, which are major component of EPS from wastewater even though they are not secreted by the organisms (Sheng *et al.*, 2010). Since EPS contains charged functional groups such as aromatic, aliphatic and hydrophobic carbohydrates (More *et al.*, 2014), it is quite possible that unfavourable conditions such as darkness facilitates adsorption of humic acids by these functional groups and accentuate the more intense regions in the dark incubated spectrum.

Works in this chapter also reflect on the quantity and biochemical characteristics of EPS produced by *Chlorella* sp. and *P. maitriensis* described in Chapter 3. This was carried out to elucidate the biological origin of EPS produced in HRAOP. Results showed that *P. maitriensis* produced more EPS than *Chlorella* sp., suggesting that bulk of the EPS generated in HRAOP is likely to be of bacterial origin. With the pattern of growth and EPS yield in the present study, it was rationalized that carbon is essential for EPS production and, a careful

understanding of the optimum growth condition of the organism is required to achieve an optimum EPS yield. Studies have shown that addition of a source of carbon such as glucose stimulates EPS production (Yuksekdag and Aslim, 2008; Shahnavaz *et al.*, 2015). Results from the present study indeed confirmed this. Lack of carbon resulted in the death of *P. maitriensis* cells after day 3 of incubation. Thus, no direct correlation was found between cell growth and EPS yield as opposed to positive correlation reported in literature (More *et al.*, 2015; Nouha *et al.*, 2016). It was therefore rationalized that the EPS accumulation observed after day 3 was due to cell lysis. Conversely, where cell growth occurred in *Chlorella* culture, the decline in EPS after day 3 was likely caused by simultaneous production and degradation of the EPS. This might have contributed to the low biochemical contents of the EPS.

Overall, while EPS yield from both organisms was low, it is important to note that EPS production depends on the strain of organism, optimum growth conditions and medium composition (Sheng *et al.*, 2010; Shahnavaz *et al.*, 2015). Therefore, these conditions should be taken into consideration when analyzing the potentials of strains in EPS production. Furthermore, since both organisms were isolated from HRAOP, there is the possibility that heterotrophic bacteria contribute more to EPS production and floc formation in HRAOPs.

In conclusion, the EPS produced by MaB-flocs generated in HRAOP treating domestic sewage have been extracted and characterized. Biochemical and FT-IR analysis revealed the enrichment of the EPS in polysaccharides, proteins, amines, aromatic compounds and aliphatic alkyl groups, which are important binding sites in EPS. It was confirmed that EPS production is enhanced in the presence of light whereas, EPS from dark-incubated MaB-flocs showed increased vibration in aliphatic and aromatic functionalities relative to transition to heterotrophic metabolism. Finally, higher EPS yield by *P. maitriensis* relative to *Chlorella* suggests the likely biological origin of the EPS generated in HRAOP. Thus, this work has set a background for a comprehensive study on the structure of HRAOP EPS to evaluate its commercial use as natural flocculants.

Chapter 5: General Discussion and Conclusion

5.1 General Discussion

IAPS is an algal-based system that was developed for its cost effectiveness to remediate wastewater biologically by exploiting the interaction of algae and bacteria (Mambo *et al.*, 2014a). This coupled with an ability to produce novel by-products makes (re) investigation and evaluation of the technology important. However, IAPS typically produces treated water that contains COD and TSS, and in some cases coliforms, which exceed the limits set by regulatory authorities (Craggs *et al.*, 2012; Mambo *et al.*, 2014a). This can be remedied by inclusion of a tertiary treatment unit (e.g. MPS, SSF, or CRF) in the IAPS process, which results in water of a quality suitable for discharge (Mambo *et al.*, 2014b; Cowan *et al.*, 2016). Even so, while IAPS offers many advantages including efficient and simultaneous N and P removal, no requirement for additional chemicals, CO_2 mitigation, and a biomass with potential for valorization, the lack of technological advancement and particularly the requirement for large land area, has limited the reach of microalgal wastewater treatment at industrial scale. Indeed, the apparent need to include tertiary treatment in the IAPS process flow, which adds to the land area required, is distinctly disadvantageous and will further compromise IAPS as a viable municipal wastewater treatment technology.

Elevated COD in IAPS treated water has been described as persistent and, no change in concentration was evident following filtration using pore sizes smaller than 1.6 μm (Cowan *et al.*, 2016). One explanation is that the persistent COD in IAPS water arises due to production of microbial EPS. Indeed, the work presented in this thesis describes the isolation and partial characterisation of soluble EPS associated with MaB-flocs generated in HRAOP of an IAPS treating domestic sewage. Analysis by FT-IR revealed characteristic carbohydrate enrichment of these polymeric substances. Formation and accumulation of the EPS was stimulated by light. In contrast, FT-IR spectra of the EPS from dark-incubated MaB-flocs confirmed that these polymers contained increased aliphatic and aromatic functionalities relative to carbohydrates. These differences, it was concluded, were due to dark-induced transition from phototrophic to heterotrophic metabolism. Thus, EPSs formed by MaB-flocs in HRAOPs appear to serve in floc formation and as a store and source of carbon. It is the release of this EPS that likely contributes to the COD concentration of IAPS treated water (Barker and Stuckey, 1999; Laspidou and Rittmann, 2002).

South African regulatory authorities demand that treated wastewater destined for discharge meets the standard. For COD, this is 75 mg/L or less; and for TSS, values of 25 mg/L or less. Tertiary treatment processes such as a MPS or SSF have been demonstrated to reduce both COD and TSS of IAPS treated water (Mambo *et al.*, 2014b). Unfortunately, such systems result in a dramatic increase in land area required. For example, the success of a MPS depends on depth (1 m or less) and detention time, which is typically not less than 14 d (Cowan *et al.*, 2016). For the pilot plant system at EBRU (used in the present study) with capacity of 75 m^3/d, a MPS volume of at least 1000 m^3 would increase the land area requirement of the system by more than 50%. Thus, an alternative tertiary treatment system was sought. Specifically, a process that would target COD and TSS, and disinfect IAPS treated water was considered ideal. The proprietary Puricare® technology used in the present study is claimed to treat water to the highest standard by first exposing water to activated oxygen followed by UV sterilization i.e. peroxonation.

Generally, the results obtained in this thesis showed that peroxone treatment effectively reduced of COD, TSS and nutrient load of IAPS water, and without any significant impact on land area requirement. Indeed, a summary of data describing the effect of peroxone on quality of IAPS-treated water confirmed that it complies with the general limit values for either irrigation or discharge into a water resource that is not a listed water resource for volumes up to 2 ML of treated wastewater on any given day. Thus, use of a process like Puricare® that delivers peroxone treated water is potentially a suitable tertiary treatment unit for final polishing of IAPS-treated water. Ozone based advanced oxidation processes (AOPs) are gaining attention and are more practicable than other popular AOPs due to simplicity, high oxidation potential, non-toxic residues, and high-energy efficiency (Wu and Englehardt, 2015). Even so, pollutant mineralization was found to be low in the present study. Pollutant degradation with AOPs depends on the rate of hydroxyl radical generation, which is influenced by several factors including the nature of wastewater, reactor configuration, O_3/H_2O_2 ratio and radical scavengers (Wu and Englehardt, 2015; Tripathi and Tripathi, 2011). Unfortunately, knowledge about the concentration of ozone generated within the Puricare® system was regarded as proprietary and withheld by the supplier. Thus, the O_3/H_2O_2 ratio could not be adjusted to determine the optimum treatment condition. Further investigation is therefore needed to define more precisely the process and kinetic parameters for peroxonation as a tertiary treatment process. In addition, smaller treatment volumes gave better results

further supporting a need for process optimization studies. Future studies using peroxonation should perhaps focus on the effect of continuous treatment of IAPS water.

Characterization of MaB-floc EPS confirmed the identity of the soluble but persistent COD in IAPS treated water. Furthermore, continuous light and dark incubation of the MaB-flocs confirmed a relationship between photosynthesis and EPS production. Although not extensively investigated in the present study, a culture of *P. maitriensis* appeared to generate greater quantities of EPS than *Chlorella* sp., which confirms earlier results that indicated this bacterium to be an EPS producer (Kumar *et al.*, 2007). Indeed, the substantial amount of EPS produced in HRAOP appears to be the major cause of aggregation of biomass into settleable MaB-flocs. Thus, promotion of both the growth of settleable algal species or species that facilitate aggregation of MaB-flocs could greatly enhance the efficiency of wastewater treatment and biomass recovery from HRAOPs. With this, it was concluded that EPS recovered from HRAOPs might be an important product of IAPS-based wastewater treatment systems.

EPSs have found applications in wastewater treatment for flocculation and settling of suspended solids due to their adsorption and absorption properties (More *et al.*, 2014). Earlier report have shown that EPS can remove 85% TSS from wastewater (Deng *et al.*, 2003). Thus, EPS from HRAOP could serve as a flocculating and settling polymer in place of the conventional chemical polymers with no further pollution since it is biodegradable. In addition, establishing an algal biorefinery remains a challenge due to high cost of harvesting biomass (Lim *et al.*, 2013). Therefore, EPS also serve as flocculants for a cost-effective recovery of biomass. Apart from wastewater treatment sector, EPS has also found application in agriculture to improve water holding capacity of soil (Parikh and Madamwar, 2006). Thus, MaB-flocs containing EPS generated in HRAOPs will, in addition to its use as fertilizer, serve in soil conditioning. Furthermore, EPS from microalgae is a high value product which can find application in the industrial sector as a gelling and emulsifying agent to improve the texture of food products (Mishra *et al.*, 2011).

Results obtained over the period of monitoring showed that that a substantial amount of biomass typical of wastewater treatment HRAOPs was generated as MaB-flocs. Nevertheless, production was influenced by climatic conditions, with temperature and solar radiation being the major driving force of productivity in the pond. Thus, diurnal fluctuation resulted in higher productivity in the afternoon when temperature was higher. However, results only

captured productivity over the winter and spring months of South African weather, which means that productivity tends to be higher during summer, when temperature and solar radiation are high. With formation of MaB-flocs in HRAOP, settleability of the biomass was easy. Therefore, it appears that bulk of this biomass can be recovered for valorization, which will create an avenue for the establishment of an algae biorefinery with IAPS.

An important stage in a biorefinery system is the provision of a renewable, consistent and regular supply of feedstock (Cherubini, 2010). The concept involves the mild and inexpensive separation of various components of biomass for sequential production of various products without damaging any product fraction (Subhadra and George, 2010; Vanthoor-Koopmans *et al.*, 2013). If operated continuously therefore, as many valuable products as possible can be derived from biomass generated in HRAOP. For example, from the biomass, the oil content can be extracted for biodiesel; carbohydrate and protein content as food, pharmaceuticals, ethanol production, and fine chemicals. The residual biomass can then be digested anaerobically to generate biogas, or use as fertilizer and/or animal feed.

5.2 Conclusion

In conclusion, peroxonation was confirmed as an appropriate tertiary treatment process for use with IAPS. Concentration of COD, TSS, and faecal coliforms was reduced by 22%, 45%, and 94% respectively to yield water quality sufficient for discharge to river. A novel EPS was extracted from the associated MaB-flocs in HRAOPs, which increased biomass settleability and recovery. Together, these findings suggest that further development of IAPS to a full biorefinery will require management of EPS production to enhance efficiency of biomass recovery and its conversion to products of value.

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Appendices

Appendix A

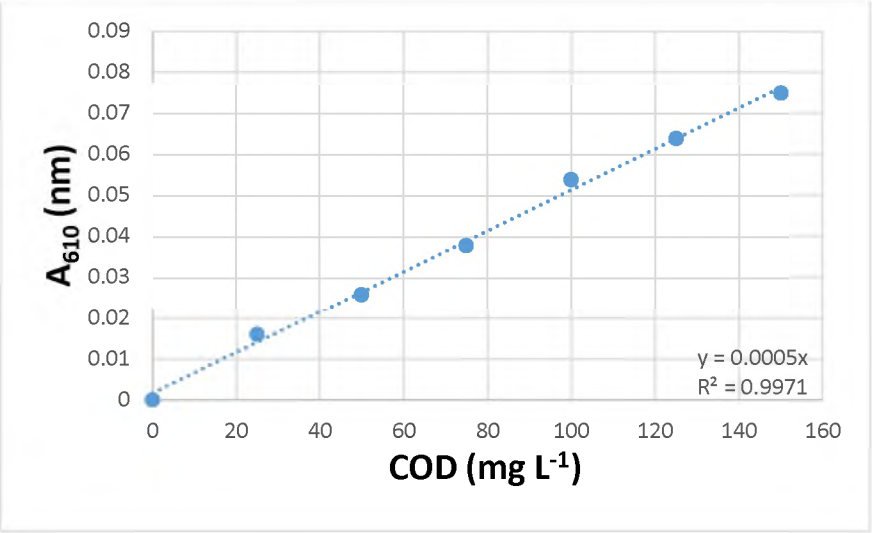

Figure A1: Graph illustrating the increasing concentrations of COD at a wavelength of 610 nm. The curve was used to determine unknown COD concentration in water samples.

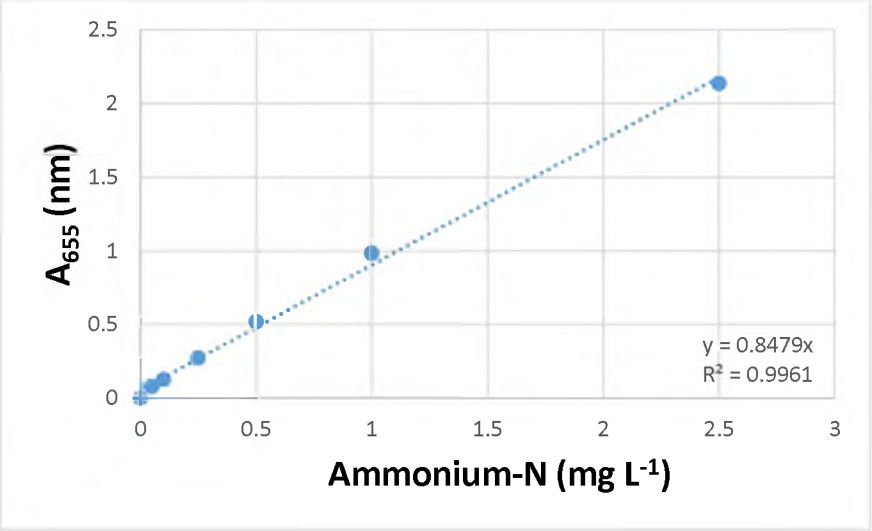

Figure A2: Graph illustrating the increasing concentrations of Ammonium-N at a wavelength of 655 nm. The curve was used to determine unknown Ammonium-N concentration in water samples.

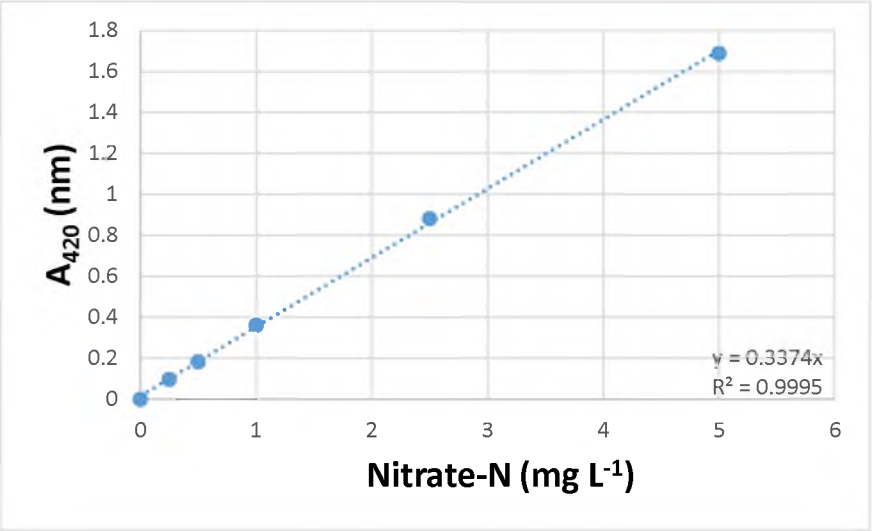

Figure A3: Graph illustrating the increasing concentrations of Nitrate-N at a wavelength of 420 nm. The curve was used to determine unknown Nitrate-N concentration in water samples.

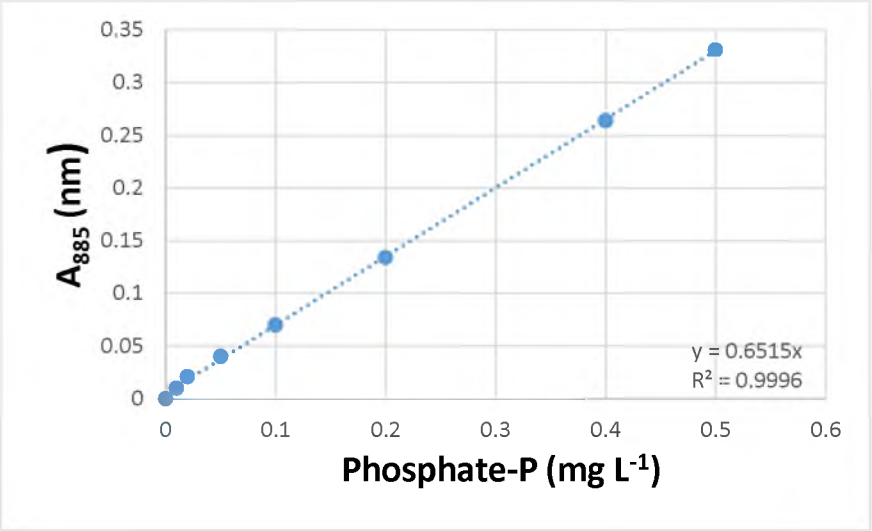

Figure A4: Graph illustrating the increasing concentrations of Phosphate-P at a wavelength of 885 nm. The curve was used to determine unknown Phosphate-P concentration in water samples.

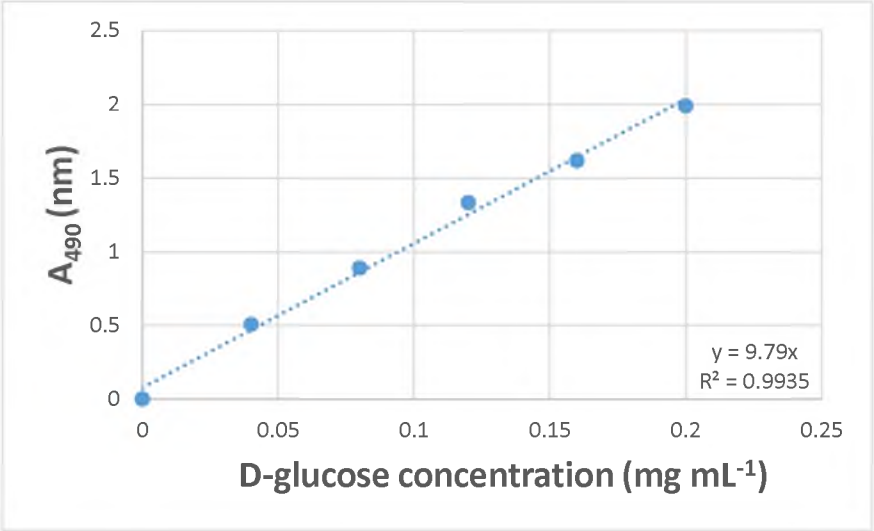

Figure A5: Graph illustrating the increasing concentrations of D-glucose at 490 nm using the Phenol-sulphuric acid assay. The curve was used to determine unknown carbohydrate concentration in EPS.

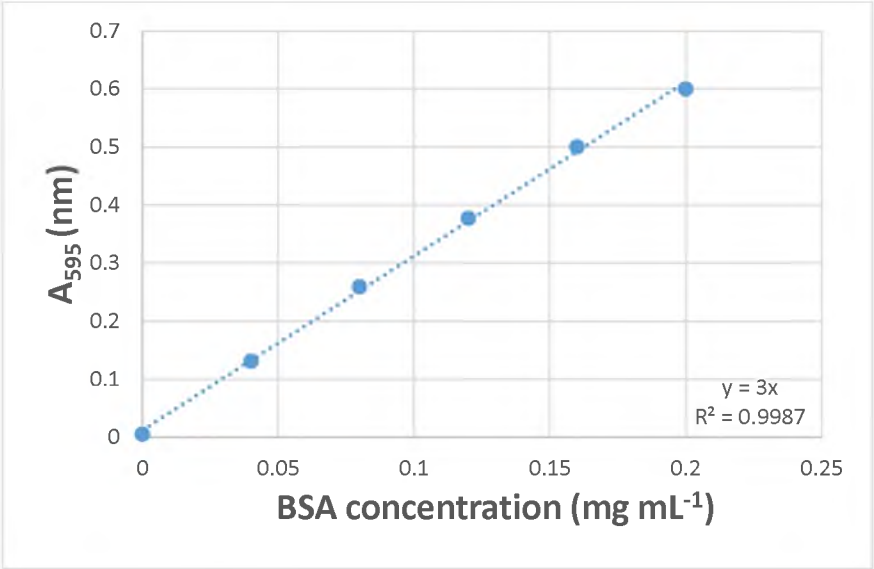

Figure A6: Graph illustrating the increasing concentrations of BSA at 595 nm using the Bradford assay. The curve was used to determine unknown protein concentration in EPS.

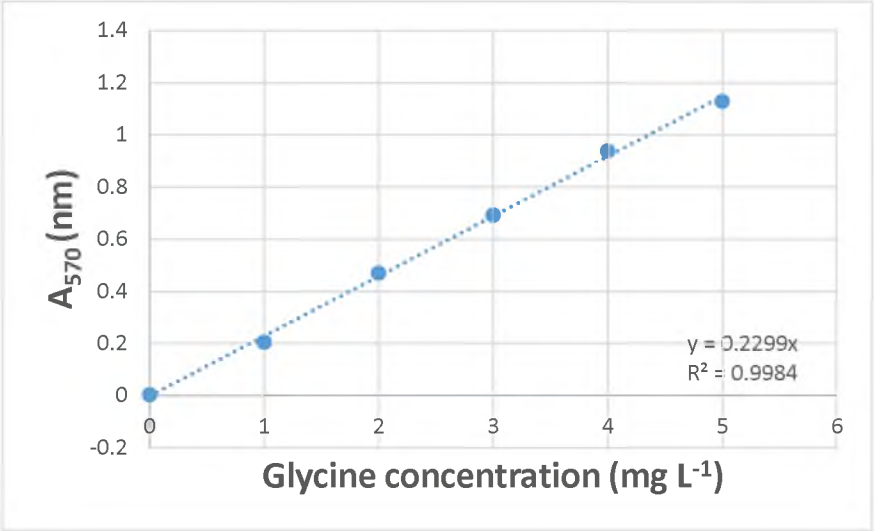

Figure A7: Graph illustrating the increasing concentrations of glycine at 570 nm using the Ninhydrin method. The curve was used to determine unknown α-amino N concentration in EPS.

Appendix B

Table B1: Bold 3N Medium composition

Stock Solution Concentration	Amount (per litre distilled water)
$NaNO_3$ (25 g. L^{-1})	30 mL
$CaCl_2$ (2.5 g. L^{-1})	10 mL
$MgSO_4$.$7H_2O$ (7.5 g. L^{-1})	10 mL
K_2HPO_4 (7.5 g. L^{-1})	10 mL
KH_2PO_4 (17.5 g. L^{-1})	10 mL
NaCl (2.5 g. L^{-1})	10 mL
P-IV metal solution	6 mL
Soil water	40 mL
Vitamin B_{12}	1 mL

Table B2: Luria Broth composition

Component	Amount (per litre distilled water)
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g

Table B3: Mineral Salt Medium composition

Component	Amount (per litre distilled water)
K_2HPO_4	1.71 g
KH_2PO_4	1.32 g
NH_4Cl	1.26 g
$MgCl_2 .6H_2O$	0.011 g
$CaCl_2$	0.02 g
Trace mineral solution	1 mL

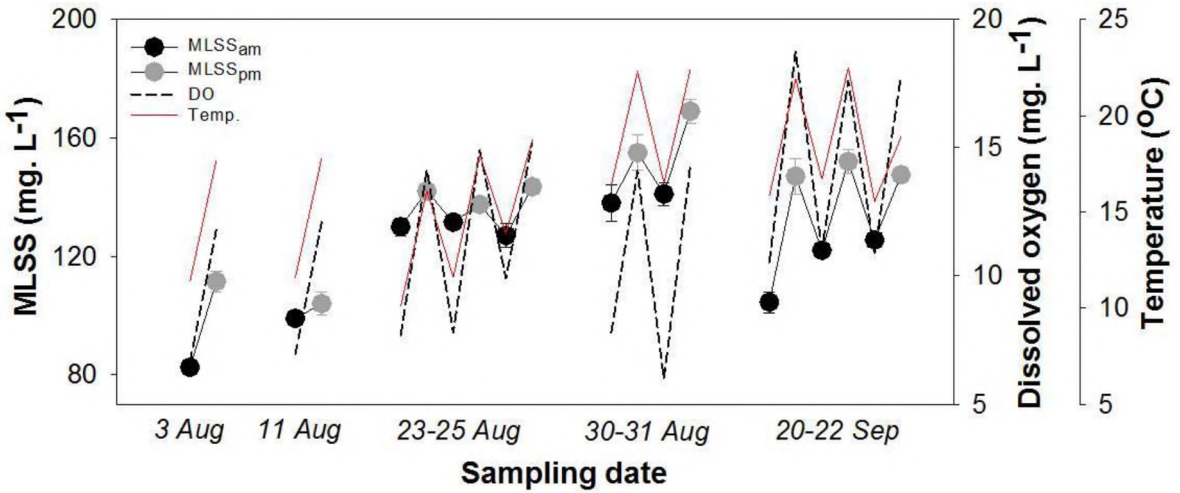

Figure B1: Diurnal change in MLSS, dissolved oxygen and water temperature in HRAOP B ± SE measured in August 2016.

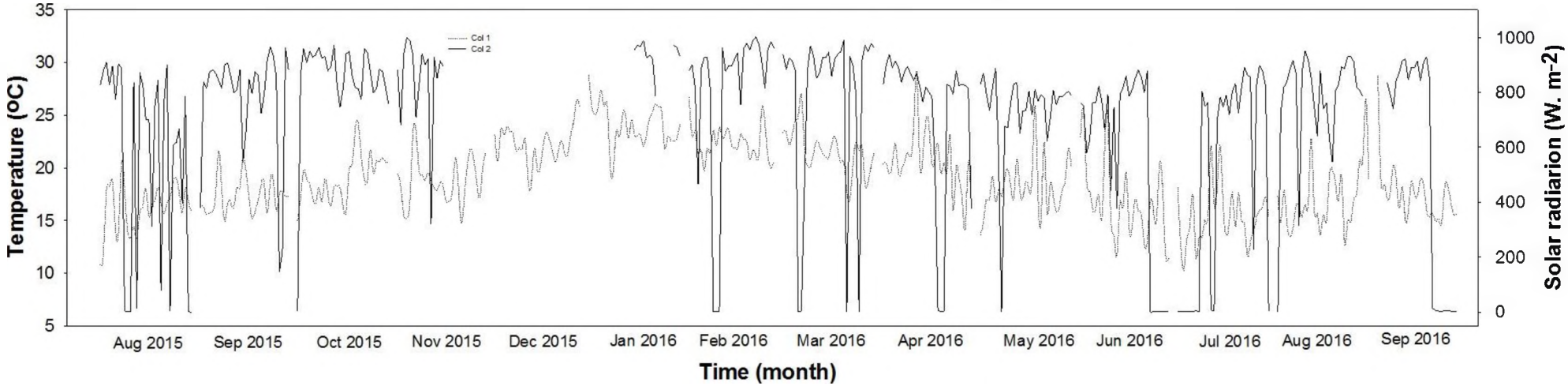

Figure B2: Average daily atmospheric temperature and solar radiation during the period of study (August 2015-September 2016). Data downloaded from http://www.sauran.net/Data.