Exploring the potential resource recovery from pot ale utilising microalgae and novel LED photobioreactors.

MCNERNEY, C.C.

2019

Exploring the potential resource recovery from pot ale utilising microalgae and novel LED photobioreactors.

Calum Carter McNerney

A thesis submitted in partial fulfilment of the requirements of the Robert Gordon University for the degree of Doctor of Philosophy

June 2019

Table of content

Declaration

I declare that the work presented in this thesis is my own, except where otherwise acknowledged, and has not been submitted in any form for another degree or qualification at any other academic institution.

Information derived from published or unpublished work of others has been acknowledged in the text and a list of references is given.

Calum C. McNerney

Dedication

I dedicate this work to my parents John and Alison McNerney, whose love and support have made it all possible.

Acknowledgements

I am extremely grateful for the constant support, guidance, encouragement and excellent mentorship of my supervisory team, Professor Christine Edwards and Professor Linda Lawton. It was truly a pleasure to learn from them during this PhD. I would also like to extend my thanks to the Industrial Biotechnology Innovation Centre (IBioIC) and the school of Pharmacy and Life Sciences at Robert Gordon University for providing the funding that enabled this project.

I would also like to thank Dr. Douglas Mackenzie and all the team at Xanthella. Ltd for providing the photobioreactors and much needed technical assistance throughout the course of this PHD. I am also very grateful for the support provided by Dr Kenneth MacGregor and Dr Frances Jack at the Scottish Whisky Research Institute (SWRI), for their vital contribution to the pot ale characterisation study. I would also like to thank all the staff Angus Dundee Distillers Plc for providing me with pot ale samples throughout the project.

I am also extremely grateful for the support of the Cyansol research group: Aakash, Mahalaksmi, Julia, Josh, Len and Carlos for their help and friendship during my time working alongside them.

Finally, I would like to thank my friends and fellow PhD college:

Dr Matteo Scipioni, Dr Franziska Pohl, Dr Tesnime Jebara, Zoi Papadatou, Josh Burns, Hazel Ramage, Mhairi Paul, Cameron Campbell, Teodora Stoyanova, Martin Corsie, Dorothy McDonald and Andrea MacMillian, who made my time in Aberdeen an experience that I will never forget.

List of Abbreviations

AU: Arbitrary units

AD: Anaerobic digestion

AMD: Age related macular degeneration

COD: Chemical oxygen demand

DDG: Distillers dark grains

IbioIC: Industrial Biotechnology innovation centre.

IC: Ion chromatography

ICP-OES: Inductively coupled plasma – optical emission spectrometry

HPLC: High performance liquid chromatography

LOQ: Limit of Quantification

LOD: Limit of Detection

PBR: Photobioreactor

PLA: Poly lactic acid

PLE: Pressurised liquid extraction

PPDD: Photosynthetic photon flux density

PSU: Photosynthetic unit

SEPA: Scottish environmental protection agency

SWRI: Scottish Whisky Research institute

TOC: Total organic carbon

UASB: Up flow anaerobic sludge blanket digestor

List of Tables

List of Figures

Abstract

Pot ale is a protein rich, high carbon and acidic (pH 3.5) by-product of the whisky industry. With 4.9 million tonnes of pot ale being produced per annum in Scotland alone, it presents a difficult disposal challenge for the industry. Pot ale has high levels of organic carbon and concentrations of toxic metals, therefore direct discharge into marine or land environments is highly regulated. Traditionally pot ale has been utilised to produce animal feed due to its high protein content. However, only approximately 50% of pot ale that is produced is utilised for animal feed production which presents an opportunity for further resource recovery. A characterisation audit of pot ale was carried out in order to gain a detailed understanding of the chemical composition of pot ale and how it varies. This audit sampled pot ale from 22 whisky distilleries and highlighted significant variation in the components of pot ale between the distilleries. Significant concentrations of lactic acid (1675 mg/L) were identified which presents an opportunity for further resource recovery. It was demonstrated by this investigation that lactic acid can be successfully recovered from pot ale using ion exchange chromatography with high yields of 95%.

In this investigation the potential recovery of the nitrate and phosphate content of pot ale is explored using microalgae. Currently the industrial scale production of microalgae is not economically viable in many instances due to high operational costs. To reduce these costs alternative nutrient sources from waste streams such as pot ale can be utilised instead of traditional growth media. In this investigation pot ale was utilised as a nutrient source for the growth *Synechocytis sp.* PCC 6803, *Chlorella sorokiniana, Microcystis aeruginosa* PCC 7813 and *Nodularia harveyana* PCC 7804. Despite attempts to overcome some of the challenges of utilising pot ale in this manner such as pH and nitrate content, successful growth was not achieved. In order to improve the economic viability of microalgal production, photobioreactor technology must also be improved. In this study a range of novel LED photobioreactors designed by industrial partners Xanthella Ltd were utilised to produce high density cultures containing concentrations of high value carotenoid pigment zeaxanthin. It was observed in this study that the production of zeaxanthin and echinenone by *Synechocystis*

sp. was successfully upregulated by 35% and 141%, respectively when using light tracking conditions. *C. sorokiniana* displayed the highest concentrations of zeaxanthin (4.58 mg/L) when cultivated in the 1 litre Micro-Pharos PBR and was therefore selected for scale-up. *C. sorokiniana* was successfully cultivated in the 700 litre Pandora photobioreactor producing zeaxanthin concentrations of 2.62 mg/L which demonstrates the feasibility of industrial scale production.

Chapter 1

Introduction

1.1 Scotch whisky

Scotch whisky is a spirit that is produced using malt barley or cereal grain along with water and yeast. No other ingredients are permitted by law. This law was first defined in the UK in 1909. The current legislation related to Scotch whisky is defined in the Scotch Whisky Regulations (2009). The Scotch whisky industry is essential to the Scottish economy. It is currently valued at over £4 billion and employs approximately over 10,600 people (Barrena et al. 2018, Arnison & Carrick 2015). Recent figures by the Scottish whisky association show that total production is up by 300 % between 2010 and 2015 (Table 1.1). There are two primary varieties of Scotch whisky, malt and grain. Malt whisky utilises malted barley as the sole feedstock whereas grain whisky production uses a mixture of unmalted cereal in addition to malted barley.

Table 1.1. Total available stocks of whisky as of December 2015 (Scottish Whisky Association, 2015)

Year produced	Original litres of alcohol produced (Malt distilleries)	Original litres of alcohol produced (Grain distilleries)
2010	118,515,470	62,368,325
2011	155,162,491	123,955,955
2012	216,150,371	199,919,218
2013	273,660,107	335,866,885
2014	285,125,991	328,428,899
2015	277,323,482	269,031,667

1.2 Malt whisky production process

The batch production of malt whisky can be divided into four main stages which are malting, mashing, fermentation and distillation (Figure 1.1). Prior to malting the barley is screened to remove any foreign particulates eg. stones and the barley is soaked for up to 3 days in tanks of water. The barley must then be allowed to germinate (Russel, 2014). The process of germination causes the barley to secrete the enzyme diastase which increases the solubility of the barley (Yu et al., 2018). Traditionally this is achieved by spreading the barley out onto the distillery floor where typically germination times can be 8 – 12 days. However, as distilleries have modernised germination is typically controlled by drum malting systems. During malting the barley may be dried with peated smoke or hot air depending on the distiller's preferences. Due to the expansion of the industry larger distilleries will now outsource the malting stage to centralised malting companies that will supply several distilleries (Russel, 2014). After malting the malt is ground in a mill and then mixed with water in a large circular vessel called the mash tun. The mashing process allows the enzymes present in the mash such as amylase and diastase to breakdown the starch present in barley into fermentable sugars (Chisti, 2018).The resulting sugary liquid is known as the wort. This process is accelerated by continual mixing and increasing the temperature to 80°C (Russel, 2014). The wort is then allowed to cool before being passed into the 1st fermentation vessel. The solid component that remains in the mash tun is a co-product known as draff which is utilised to produce animal feed (Figure 1).

The yeast in the fermentation vessel then convert the fermentable sugars in the wort (Glucose / maltose) into ethanol and carbon dioxide. The yeast that distillers utilise are typically isolated or genetically modified stains of *Saccharomyces cerevisiae.* The fermentation time can vary greatly between distilleries (36 – 100 hours) (Russel,2014). The resulting liquid wash is then distilled twice in traditional copper pot stills. Distillation separates the alcohol from the liquid by increasing the temperature to 78°C. The vapour containing ethanol then rises up the still and is passed into the cooling plant where it is condensed back into liquid state. In order to further increase the alcohol concentration, the resulting distillate known as the "low wines" is then passed

into an additional distillation vessel where the process is repeated (Russel, 2014) The first distillation process generates large volumes of the co-product known as pot ale which traditionally is combined with draff to produce animal feed (Traub, 2015) (Figure 1.1).

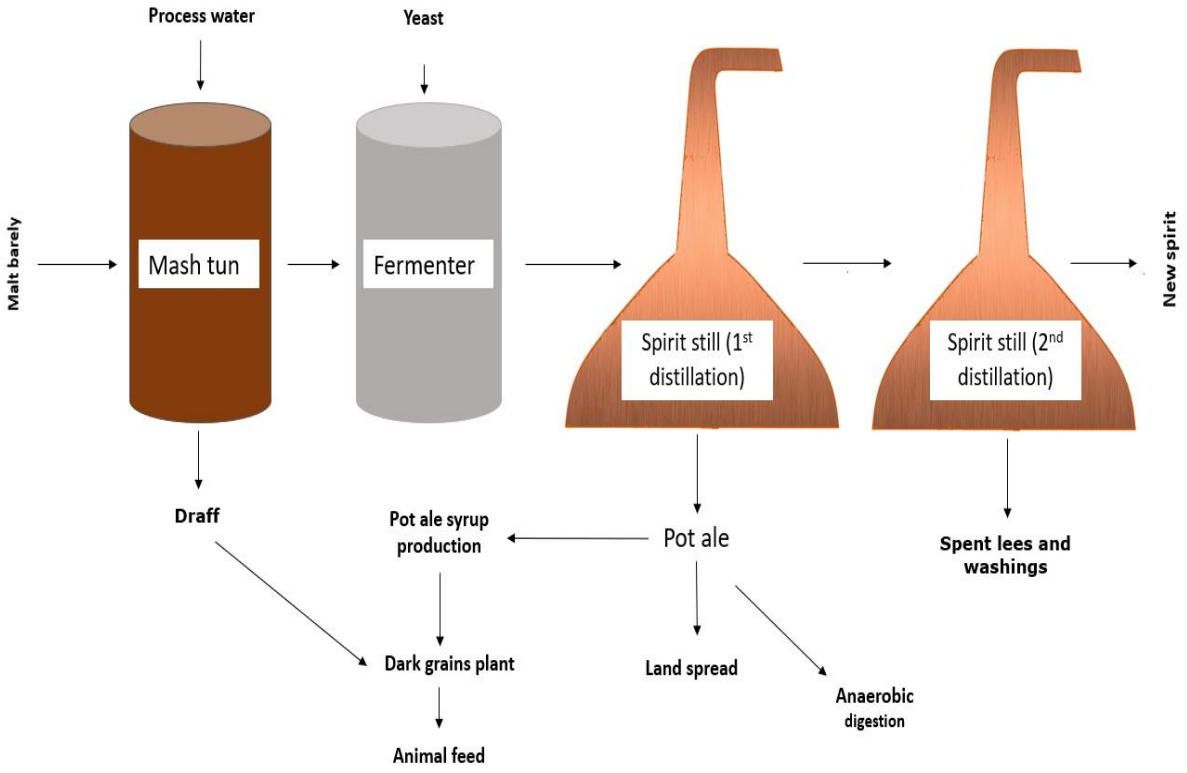


Figure 1.1. Flow diagram of the malt whisky production process with possible routes for pot ale use.

1.2.1 Grain whisky production process

In grain whisky production any cereal can be used as the starch substrate rather than just malted barley, however traditionally only one cereal type is used at a time due to processing differences. Relatively smaller quantities of malted barley are also typically added to the mash to provide the enzymes necessary to breakdown the starch into fermentable sugars (Agu, 2006).

Unmalted cereals are processed separately and are cooked using pressurised steam to break down the grain starch to increase its solubility. Similarly, to the production of malt whisky, the wort is then fermented by adding yeast. The distillation phase is carried out in column stills which are constructed from stainless steel rather than copper (Russel, 2014). The fermented liquid (wash) is pumped into the rectifier as it flows down through the coil, it is heated by the vapours rising up the rectifier. After the wash it is heated, and it enters the analyser where it encounters hot steam and is vaporised. The resulting vapor then rises up the analyser and is transferred via the vapor pipe to the bottom of the rectifier. As the vapor rises up the rectifier it is cooled by the pipe carrying the cool wash and condenses (Agu, 2006). The resulting condensate is collected by the spirit receiver (Figure 1.2). This is a continuous process unlike pot stills traditionally used to produce malt whisky which are a batch process. The process produces a co-product known as spent wash which is the equivalent of pot ale.

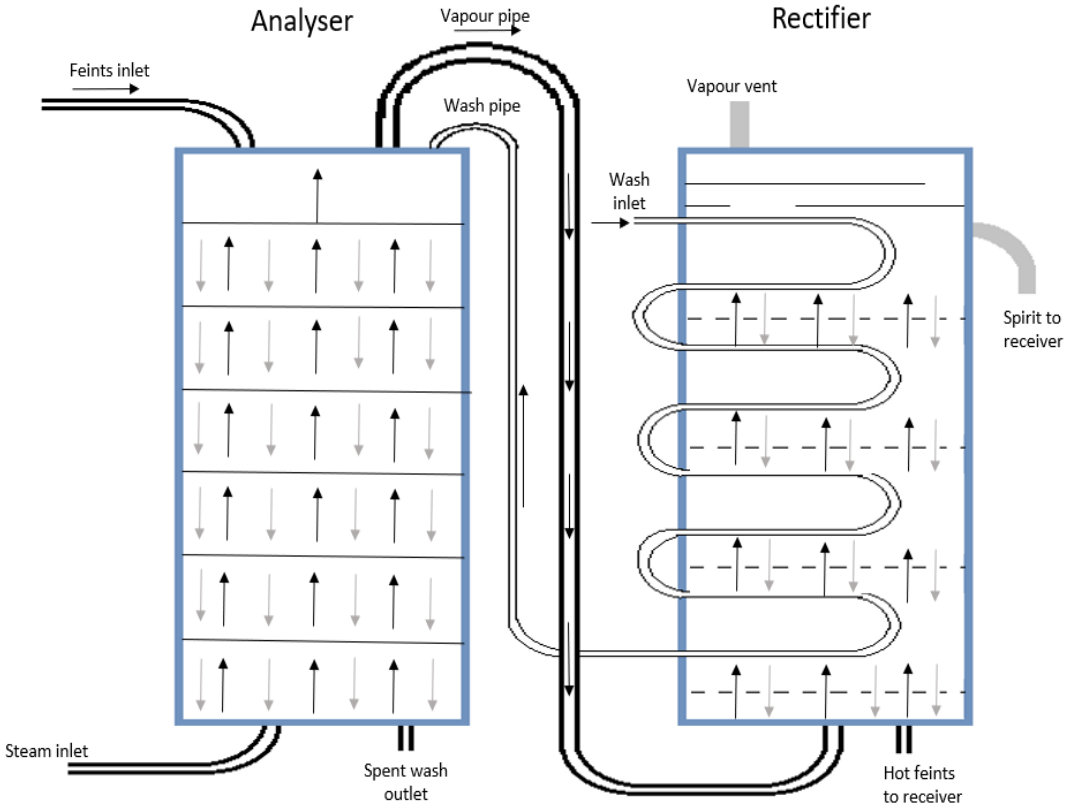


Figure 1.2. Flow diagram of a continuous Coffey still which is commonly used to produce grain whisky

1.3 Scotch whisky by products

Scotch whisky produces a number of by-products (Table 1.2). Of these by-products pot ale and spent wash are the most significant due to the large volumes produced and opportunities for valorisation. Hereafter, for the purpose of this report spent wash will also be referred to as pot ale. For every litre of scotch whisky produced approximately 9 litres of by-products remain. (Arnison and Carrick, 2015). This equates to an estimated production of 4.9 million tonnes of pot ale as the per the 546,355,149 litres of scotch whisky produced in 2015 assuming that the density of pot ale is 1 kg/L (Scottish Whisky Association, 2015). A previous study by Traub et al,(2015) estimated that the annual production of pot ale from malt whisky distilleries was 2 – 3 million tonnes annually (Traub et al. 2015). Spent lees refers to the remaining liquid after the second distillation phase of malt whisky. Spent lees has a similar composition to pot ale but is much more dilute, therefore options for valorisation and resource recovery are limited. Spent lees are typically treated and discharged as waste (Arnison and Carrick, 2015). Draff refers to the leftover wet grains from the mashing process which are rich in carbohydrates, protein and fibre (Traub et al, 2015).

Table 1.2. By-products produced by malt whisky distilleries**.**

Malt distillery (By product)	Grain distillery (By product)	Description
Draff	Spent grain	Spent grains with a moisture content of 80 %. These grains are rich in carbohydrates, fibre and protein.
Pot ale	Spent wash	Liquid waste from the first distillation stage with a solid content of around 5 % containing high levels of protein as well as copper.
Spent Lees		Liquid residue from the second distillation stage in malt distilleries. Spent Lees have similar properties to pot ale but are far more dilute.

1.3.1 Pot ale

Pot ale is defined in literature as being a light brown turbid liquid with an average pH of 3.5 and a solid content of approximately 5 % (Graham et al., 2012; Mallick et al.,2010). These solids largely consist of yeast cells, yeast residues, carbohydrates and insoluble protein. A study by Mallick et al, (2010) found that the solid content of pot ale largely consists of intact yeast cells, staining with methylene blue confirmed that the cells were dead although the cells walls were intact. Pot ale also contains high concentration of organic acids and other organic material which is reflected by its high COD (Table 1.3, Table 1.4). Pot ale also has a high BOD concentration of 30,000 mg/L (Tokuda et al., 1999). A study which characterised pot ale from a single malt whisky distillery found that acetic acid was the most abundant acid present followed by lactic acid and propanoic acid respectively (Graham *et al.*, 2012). An earlier study reported that lactic acid was the most abundant acid present by an order of magnitude (Tokuda et al.,1998).

A previous study by Priest and van Beek,(2002) which determined that lactic acid is the most abundant acid present in pot ale with concentration of 0.5 g/L in comparison to acetic acid (0.02 g/L). It has been previously determined that lactic acid bacteria are present during the mashing process and are introduced into the fermentation stage (Priest and van Beek, 2002). This explains the high concentrations of lactic acid observed in pot ale. It has been reported that there is a significant but variable amount of copper and other heavy metals present in pot ale. Graham et al, (2012) reported a copper concentration of 2 – 6 ppm and Quinn et al, (1980) reported a concentration of 2.1 - 2.3 mg/L. The heavy metals in pot ale including copper are also thought to be largely bound to solids (Quinn, Barker and Marchant, 1980; Graham *et al.*, 2012).The presence of copper in pot ale can be attributed to the dissolution of Cu (II) from fractional copper pot stills (Lu and Gibb, 2008; Julio Enrique Traub *et al.*, 2015).

Table 1.3. Physiochemical characterisation of pot ale (Barrena *et al.*, 2018)

Parameter	Unit	Value
pH	n/a	3.85
Total solids (TS)	% w/w	4.22
Total COD	g / L	54.8
Carbohydrate	g / L	14.55
Soluble protein	mg / L	353
Crude protein	g / L	9.4
Suspended solids	mg / L	2925
Cu, total	µg / L	1341
Nitrate	mg / L	23.6
Total N	mg / L	1495
Phosphate (total)	mg / L	330

Table 1.4. Organic compounds in pot ale and spent wash (Tokuda, Fujiwara and Kida, 1999).

Organic compounds and organic matter (% w/v)	Pot ale	Spent wash
Maltose	0.15	0.06
Fructose	0.08	0.09
Glucose	0.18	0.18
Dextrins	2.1	1.08
Lactic acid	0.61	0.42
Acetic acid	0.06	0.06
Propionic acid	0.03	0.12

1.3.2 Pot ale syrup

Pot ale syrup is pot ale that has been concentrated by evaporation. Pot ale syrup has a higher solid content than pot ale as it is approximately 10 times more concentrated and is therefore easier to transport (Traub et al.,2015). The protein content of pot ale syrup is of course also more concentrated which along with the higher solid content makes it more for optimal animal feed applications (Dionisi et al., 2014). However, the evaporation process is extremely energy intensive and has become increasingly economically inefficient, as the whisky industry has expanded over the years (Traub et al.,2015).

1.4 The use of whisky co-products as animal feed and concerns surrounding copper content.

Traditionally pot ale syrup has been combined with draff to produce a low-grade animal feed known as distillers' dark grains (DDG). The ratio is approximately 2.7 tonnes of draff to produce 1 tonnes of DDG and 9 tonnes of pot ale to produce 1 tonnes of DDG (Arnison and Carrick, 2015). In 2013 the production of 254 k tonnes of DDG was reported which approximately equates to 2.3 million tonnes of pot ale which represents approximately 50 % of the total pot ale volume produced that year. Due to high concentrations of copper, animal feed derived from distillery by products cannot be given to sheep as they are highly sensitive to copper poisoning (Wainman and Dewey, 1982). A study investigating acute copper poisoning in sheep determined that toxicity begins at 20 mg/L and that concentrations of 50 mg/L are 100 % fatal. The copper content of pot ale is approximately 2–6 mg/L (Graham et al., 2012) at this concentration it would be nontoxic, however, it is concentrated by a factor 9 to produce DDG. Therefore, the copper concentration of DDG could range from 18-54 mg/L.

Copper is an essential element for life. It plays an essential role in the aerobic respiration of all eukaryotes due to its diverse roles in electron and oxygen transportation (Cotruvo *et al.*, 2015). In food products minimum and maximum copper levels are set to negate and minimise negative effects ie. toxicity in humans and animals as well as environmental concerns (EFSA, 2013). Table 1.5 shows the maximum limits of copper in animal feed diets set by European

regulations (Commission Regulation (EC) No 1334/2003). Due to its high copper concentration DDG is mainly utilised as cattle and pig feed, although special care must also be taken with bovine before rumination (calves). DDG and pot ale syrup is typically not utilised as a feed for sheep due to toxicity concerns (Wainman and Dewey, 1982).

Table 1.5 Maximum permitted copper content of the feedstuff. (Commission regulation (EC) No 1334 / 2003)

Animal	Maximum limit (mg / kg)
Pre-ruminating bovines	15
Other bovine	35
Piglets up to 12 weeks	17
Other pigs	25
Crustaceans	50
Ovine	15
Fish	25
Other species	25

1.5 Environmental concerns and the treatment of distillery effluent

The whisky industry is primarily regulated under the water environment-controlled activities act (Scottish government regulations, 2011). Excess pot ale that is not processed into DDG is often spread onto land as low grade fertiliser, which is permitted under strict SEPA control (The Scotch Whisky Association, 2012). Distilleries may also apply for permits in order to dispose of pot ale directly into the sea for example distilleries located on islands or other remote areas (Sepa, 2018).

There are many environmental concerns surrounding the disposal of pot ale primarily due to its high COD, BOD, heavy metal content and high levels of inorganic compounds (Table 1.3). Studies have shown that distillery effluent including pot ale can have a negative effect on both ground water by altering the pH and nutrient composition due the leaching down of organic and inorganic ions

(Singh et al., 2003; Jain et al., 2005). An excess of nutrients such as nitrate and phosphate can lead to the eutrophication of water courses which can be detrimental to aquatic life. The use of distillery by-products in agriculture is controversial. It has been reported that distillery by-products can have beneficial effects on crop yield such as providing nitrate and phosphate. However, there can also be detrimental effects such as the increase of soil salinity (Pathak et al., 1999) due to the leaching of heavy metals such as copper and manganese (Sing et al.,2003). The heavy metal content of pot ale also poses a risk to soil and marine environments. Copper is highly toxic to microalgae as well as aquatic life and can disrupt food chains when present in high concentration in the environment (Grosell et al., 2007).

Due to these environmental concerns there has been great interest in the treatment of pot ale to remove pollutants. Globally there have many physio-chemical methods which have been utilised for the treatment of distillery waste such as flocculation, coagulation, reverse osmosis and oxidation. These methods primarily remove colour and reduce turbidity by concentrating the waste into sludge or by the partial or complete breakdown of the organic molecules. The main disadvantage of these physiochemical disposal methods is that they generate high volumes of sludge which subsequently produces disposal problems. They also tend to have high installation and operational costs which precludes smaller distilleries (Mohana et al, 2013). There has, therefore, been a greater emphasis on the development of biologically based treatment options such as anaerobic digestion in recent years (Mallick el al., 2010; Barrena et al., 2018)

1.6 The treatment of pot ale utlising anaerobic digestion

Anaerobic digestion (AD) is defined as the breakdown of organic material by micro-organisms in the absence of oxygen. AD produces valuable methane biogas which can be used to produce electricity, a nutrient rich digestate also remains which can be used as a fertiliser. Pot ale has a high COD content and is readily biodegradable which makes it an attractive feedstock for anaerobic digestion (Tokuda et al., 1999; Goodwin, Finlayson and Low, 2001). As the whisky distilleries have increased in scale the traditional pot ale valorisation

method of producing DDG is becoming less attractive due to high production costs (Traub et al. 2015). The earliest research into the use of pot ale as an AD feedstock occurred in the late 1980‘s and 1990‘s, however, the traditional production of DDG was more economically attractive due to the small size of traditional whisky distilleries (Goodwin and Stuart, 1994).

Despite the potential economic benefit and attractiveness of pot ale as an AD feedstock there has been a lack up of uptake by the whisky industry (Barrena *et al.*, 2018). This lack of uptake can primarily be attributed due to certain physiochemical properties of pot ale that negatively impact the efficiency of AD. Yeast cells which contribute to the COD of pot ale can sink to bottom of AD reactors which can lead to difficulties with digestion stability (Goodwin and Stuart, 1994). The high protein content of pot ale can also lead to problems for AD. Studies have shown that protein breakdown in AD reactors can lead to a build-up of ammonia which can have a inhibitory effect on methanogenesis (Ariunbaatar et al., 2015; Mahdy et al., 2017).

Several studies have investigated this problem which mainly focus on varying pre-treatment processes for pot ale prior to AD. A study by Mallick et al,(2010) investigated the enzymatic pre-treatment of yeast cells to break them down and make them more available for digestion (Dionisi et., 2014). Another study focused on the removal of protein from pot ale using ion exchange chromatography prior to AD. However, the methane yield of deproteinated pot ale was 20.6 % lower when compared to un-treated pot ale (Barrena *et al.*, 2018).

1.7 The utilisation of whiksy co-products in the aquaculture industry

Currently global aquaculture production is approximately 70 million tonnes. The aquaculture industry in the UK is primarily focused on the production of farmed Atlantic Salmon (*Salmo salar*) and is largely based in Scotland. In 2017 the production volume was 189 K tonnes which was worth over £1 billion to the Scottish economy (Scottish Government 2017).

Aquaculture feeds have traditionally relied on fish meal and fish oil as their primary protein and carbohydrate source. Feeding wild fish protein to farmed fish has clear sustainability issues. Therefore, there has been great interest in

developing more sustainable protein sources. Alternatives sources such as soybeans are an economical alternative with good nutritional properties i.e. high in protein and good amino acid profile. However, soybeans and other plant sources can contain significant concentrations of saponins and glucosinolate, which can be toxic and can have a negative effect on digestibility (Hardy, 2010). A study by Knudsen et al, (2008) demonstrated that soya saponins plays an important role inducing enteritis in Atlantic salmon.These compounds are not destroyed by processing or pelleting and therefore have to be mitigated by supplementation (Hardy, 2010).

Pot ale has a high protein content (Table 1.3) and a desirable amino acid profile. A previous study successfully developed an ion exchange method to recover protein from the soluble portion of pot ale for the purpose of producing aquaculture (Traub et a.,2015) This process is less energy intensive than concentrating the protein content of pot ale using evaporation and also has the benefit of being highly selective, allowing for higher quality products. (Traub et al., 2015).

1.8 The current market for pot ale

The current market for pot ale is almost entirely focused around the agriculture industry. Pot ale syrup when marketed as a liquid animal feed typically sells for £80 – 100 per tonne (Traub et al. 2015). DDG from malt distilleries and DDG from grain distilleries are typically marketed at £ 191 per tonne and £ 161 per tonne, respectively. The relative value of animal feeds is typically set by factors such as energy and protein content. However, other factors such as viscosity and suspended solids are also factors that can affect the market price when concerning liquid feeds.

Other industrial processes that employ fermentation can also produce DDG or similar co-products. A large bio-ethanol plant operated by Vivergo recently opened near Hull in England (The Guardian, 2013). Bio-ethanol plants produce a co-product that is similar to pot ale that can be utilised to produce DDG. The Vivergo plant is set to be the UK largest single consumer of wheat and is set to produce 500,000 tonnes of by-product which it aims to sell to the animal feed

market (The Guardian, 2013). This exceeds the estimated 450,000 tonnes production of the Scottish whisky sector (Arnison and Carrick, 2015). However, the Vivergo plant recently ceased production due to legislative delays by the British government, to increase the bioethanol content of petrol to 10% from 5% (BBC, 2018). Globally the production of DDG in the last 10 years expanded rapidly with large increases in corn processing for bio-ethanol production (Traub *et al.*, 2015). This rapid increase in production will inevitably cause supply and demand problems which raises questions about the economic feasibility of DDG derived from the whisky industry, therefore, there is a need to investigate alternative valorisation methods.

1.9 Potential for further valorisation

Currently most of the valorisation efforts for pot ale are focused on its protein content. With approximately 50 % of pot ale not being directly processed into DDG there is still huge potential for further valorisation. Components of pot ale that could be potentially exploited further include its high phosphate content, nitrate content and its high concentrations of organic acids.

1.9.1 Phosphate recovery

Phosphate is heavily utilised by the agricultural industry to produce inorganic fertiliser. Mined phosphate is a finite resource therefore the recovery of phosphate from waste streams such as pot ale is desirable. Pot ale contains concentrations of phosphate up to 0.5 g/L (Dionisi et al., 2014). Several methods could be potentially employed to recover phosphate from pot ale including precipitation and absorption (Shepherd et al.,2016; Dionisi et al., 2014; Rahman et al., 2014).

A recent study investigated the potential of using anaerobically digested sewage sludge to produce biochar via pyrolysis (Shepherd et al., 2016). Biochar has a high affinity for aqueous phosphorus. The biochar could then be integrated into the treatment plant to recover phosphate from the waste streams such as pot ale. With an increasing focus on the development of anaerobic digestion systems for pot ale, there is potential to investigate the use of similar processes for the recovery of phosphate from pot ale. The recovery of phosphates from waste

water using precipitation to form struvite has also been investigated by several studies (Rahman et al., 2014; Kataki et al., 2016). Struvite ($NH_4MgPO_4.6H_2O$) is a crystalline phosphate mineral that can be used to produce agricultural fertiliser. Struvite can be produced from phosphate rich waste streams via precipitation with NaOH and Mg $(OH)_2$. Although more commonly utilised with municipal waste streams. Successful struvite recovery has been reported from several industrial waste streams including: Tannery waste (Tünay *et al.*, 1997) food processing waste (Moerman *et al.*, 2009), textile waste (Huang *et al.*, 2012) and waste from yeast production (Uysal and Demir, 2013). To the authors knowledge struvite production from distillery waste streams has yet to be investigated.

1.9.2 Organic acid recovery

As detailed previously in section 1.3.1 pot ale contains high concentrations of organic acids with lactic acid being the most abundant (Tokuda et al, 1998) with concentrations of approximately 0.5 g/L (Priest and van Beek, 2002). If lactic acid is present in pot ale in high concentrations, then there is potential for further valorisation. Lactic acid is a valuable industrial chemical and is widely used as a food preservative and is a key component of Polylactic acid (PLA) which has a wide range of applications which include surgical sutures, disposable plastic packaging and drug delivery systems (Melnicki et al., 2013). The bioplastic market is projected to grow by 20% annually due to the increasing demand for biodegradable single use plastic products (Ou et al. 2016; Madhaven-Nampoothiri et al. 2010). Lactic acid could be recovered from pot ale using ion exchange chromatography as this method is frequently used as a downstream processing step in lactic acid fermentation (Thang and Novalin, 2008). A previous investigation achieved the separation of lactic acid from grass silage using ion exchange chromatography. The investigation achieved a recovery yield of 97% with a lactic acid purity of 94% (Thang and Novalin, 2008). A similar technique could be applied to pot ale in order to recover its lactic acid content.

1.9.3 Nitrate and phosphate recovery using microalgae

Microalgae encompass a large range of photosynthetic organisms which include eukaryotic species from the Protista kingdom and prokaryotic cyanobacteria. Microalgae require nutrients such as nitrate/phosphate and sunlight to grow and can be found in all habitats across the globe. Microalgae can be cultivated at an industrial scale to produce a wide variety of products including biofuels, pigments, fatty acids, biomass, aqua culture feed, polysaccharides and phycobilin's (Pulz and Gross, 2004; Markou and Nerantzis, 2013).

Despite the great biotechnological potential of using microalgae as cell factories to produce high value products, there are key issues associated with industrial scale-up. Currently there are very few microalgal based products that are economically viable when produced at an industrial scale. These products include *Chlorella* sp, which is produced and sold as a health supplement and Astaxanthin, which is carotenoid pigment, produced from *Haematococcus* sp (Norsker et al., 2011). Astazanthin is sold a food colourant with its primary use being to colur farmed salmon (Olaizola, 2003).

The cultivation of microalgae at an industrial scale has high operational costs largely due to the energy required for lighting and aeration, large volumes of water and nutrients are also required (Lopes and Reis, 2002; Lam and Lee., 2013). These high operational costs have largely stifled attempts to economically produce high volume/low value products from microalgae such as bio-ethanol (Singh and Gu, 2010; Lam and Lee, 2013). Algal biotechnology, however, is still in many regards still in its infancy. Recent advancements in reactor design and synthetic biology could propel the industry forward and increase the range of products that are economically viable (Lam and Lee., 2013).

Another approach to increasing the economic viability of microalgal production is to reduce operation costs. The estimated cost to cultivate microalgae at a scale of 100 tonnes per annum is approximately $3000/ton (Das,2015). The cost of the inorganic nutrient/nitrate source used in cultivation media has been estimated to amount to 80% of the total media cost (Abreu *et al.*, 2012). This cost can be significantly lowered by using alternative carbon and nitrate sources. Wastewater and other industrial waste sources are promising sources of cheap

readily available nitrate and phosphate sources for the industrial cultivation of microalgae. Agricultural and municipal wastewaters contain many important macronutrients which are vital for the growth of high-density cultures of microalgae. These macronutrients include nitrates, ammonium and essential trace elements (Pittman *et al.,* 2011). Waste waters that have previously been tested as nutrient sources for microalgae include municipal and dairy waste waters (Pant and Adholeya, 2007; Abreu et al., 2012). A study previously investigated the mixotrophic growth of *Chlorella vulgaris* using hydrolysed cheese whey as a carbon source achieved a growth rate of 0.43 μ d^{-1} which was 3.5 times higher than the growth rate when using inorganic media. The final biomass achieved using hydrolysed cheese whey was 3.58 g/L which was significantly higher than the biomass achieved using inorganic media 1.22 g/L. (Abreu et al., 2012). Cheese whey contains high concentrations of sugars such as lactose, galactose and glucose which makes it a good carbon source for microalgae. Microalgae have been considered for application in tertiary municipal waste water treatment due to their ability to utilise the nutrients present and reduce the COD of the waste (Pant and Adholeya, 2007; Mohana *et al.*, 2013). However, these applications are primarily concerned with the removal of pollutants and do normally consider the production of microalgal products.

Pot ale is a nutrient rich co-product which to date, has yet to be considered for the use in the cultivation of microalgae. However, the use of pot ale as nutrient source for microalgae could present significant challenges. The copper concentration of pot ale could prove to be an issue as copper is toxic to many species of microalgae when present in significant concentrations. Previous studies have shown that concentrations of 0.75 mg/L are enough to reduce the growth rates of certain microalgal species growth (Giner-Lamia *et al.*, 2012). Pot ale is also acidic (pH 3.5), therefore the pH would have to be neutralised if it is to be applied as nitrate/phosphate source. These issues will have to be addressed if micro-algal cultivation is be successful. Any treatment of pot ale to enhance microalgal growth must also be economically viable if it is to be competitive with traditional inorganic growth media.

1.10 Photobioreactors

Photobioreactors are specialised culture vessels designed for the cultivation of photosynthetic microalgae. They differ from traditional bioreactors in that they must incorporate light and this fact heavily influences their design philosophy.

The first cultivation systems that were designed to grow microalgae at an industrial scale were raceway ponds. These ring channel systems first began seeing widespread use in the 1940s and were largely constructed from poured concrete (Wang et al.,2012). Raceway ponds are typically mixed by a paddle wheel which also enhances gas exchange (Alcántara et al., 2015).The large surface area of raceway ponds allows for efficient light penetration by sunlight which is essential in order to reach high density cultures. The crucial limitation of raceway ponds is that they are entirely open to the environment and therefore are subject to quality and purity issues. They are also entirely reliant on natural sunlight which severely limits their application to limited geographical areas (Wang et al., 2012). These limitations make raceway ponds ill-suited to produce high value more specialised products that will be key to algal biotechnology industry going forward.

Advanced modern photobioreactors (PBRs) are closed systems which are designed to cultivate microalgae in a highly controlled environment. The reactor vessels are usually constructed from transparent plastic or glass. Processing parameters such as air/ carbon dioxide supply, temperature and mixing rate can all be externally controlled by the operator (Wijffels and Barbosa, 2010). This allows the operators to have full control of the environmental conditions within the PBR and the ability to adjust parameters to better optimise the growth of individual microalgal species (Olivieri & Marzocchella, 2014). Photobioreactors are less vulnerable to contamination than open systems however, sanitation must still be considered. This can be achieved by manual cleaning with disinfectant in the case of smaller systems, larger PBRs may utilise automated sterilisation systems (Walter *et al.*, 2003). Light can either be provided by natural sunlight, artificial light or a combination of both.

A factor which can limit the productivity of photobioreactors is the self-shading effect. Self-shading occurs when microalgal cultures become dense and light

penetration into the culture volume begins to decrease. This limiting factor can be partially mitigated by having efficient mixing. Another way to alleviate this problem is to design reactors that have large surface areas with a narrow light path, in order to achieve this many PBR styles have been developed.

The most popular photobioreactor design types are flat panel reactors and tubular reactors. Flat panel reactors are flat rectangular vessels, the culture depth will typically vary from 1 to 20 cm. The reactors are aerated (≤1 L/min), designs may incorporate baffles to aid mixing (Wang et al., 2012; Yen et al., 2013). Tubular photobioreactors are constructed from transparent tubing with the culture circulated at velocities of typically 0.5 m/s. Tubular reactors can be configured into many different orientations; single vertical columns, horizontal tubes arranged in a single plane or vertically stacked horizontal tubes (fence systems). The diameters of the tubes can vary but are typically 3 cm to 10 cm (Yen *et al.*, 2013)**.** The design of photobioreactors focus heavily on the maximisation of surface area which leads to a reduced culture volume when compared to traditional bioreactors. This can result in photobioreactor systems requiring a large land area and therefore low areal productivities. Single reactor units rarely exceed a volume > 1,000 litres which can be problematic when scaling-up production (Huang *et al.*, 2017). To overcome this issue large scale operations typically employ modular designs consisting of numerous smaller reactors that are linked together (William and Cove, 2016).

Internally illuminated photobioreactors (Figure 1.3.) are a relatively recent development that can potentially overcome some of the issues surrounding PBR scale-up. Internally illuminated PBRs lower the light path, as they shorten the distance that the light has to travel and provide a larger illumination area as the culture is illuminated volumetrically, rather than just on the surface (Pegallapati and Nirmalakhandan, 2013). This lighting method can allow for the development of larger volume PBR as light penetration becomes less of an issue if an array of lights can be evenly distributed within the reactor (Pegallapati and Nirmalakhandan, 2013). The main negative aspect of internally illuminated reactors is that they can be more expensive and difficult to design than more traditional PBR configurations. Internal lighting can present a number of technical challenges such as waterproofing the electronics and ensuring that the lights, do not transfer to0 much heat into the culture volume (Schulze et al., 2014)

Figure 1.3. An internally illuminated 20 Litre vertical column PBR (Xanthella Ltd).

1.11 The utilisation of LEDs for the illumination of photobioreactors

Currently the most promising artificial light source for photobioreactors are light emitting diodes (LEDs). LEDs have number of characteristics which make them ideally suited for use in photobioreactor systems such as; long life expectancy, low heat generation, high conversion efficiencies and relative ease to program and control with software. (Melnicki *et al.*, 2013). A potential issue of cultivating microalgae with LEDs is that LEDs typically have a narrow emission spectrum when compared to fluorescent lights and natural sunlight. Microalgae need a balanced mix of wavelengths for normal growth. Due to their evolutionary history microalgae have varying pigment compositions that are designed to absorb light across a spectrum of wavelengths (Schulze *et al.*, 2014) Therefore, biomass

productivity when utilising LEDs can be dependent on the pigments present. The best performing LEDs for biomass production are currently cool white phosphor-converted-LEDs which have the highest photosynthetic photon flux density (PPFD) per input wattage (PPFD/W), in comparison to other white LEDs (Schulze et al., 2014). This is due to their blue emission peak (440-460 nm) which is a good match for the blue absorption spectrum of many algae (Figure 1.4)(Schulze et al.,2014 ; Abiusi *et al.*, 2014). LED lighting units can be designed to utilise numerous LEDs of different wavelengths in order to maximise photosynthetic performance (Figure 1.5) This can also allow for an element of adaptability as the emitted wavelength can be changed to fit operational requirements i.e.to upregulate targeted metabolites.

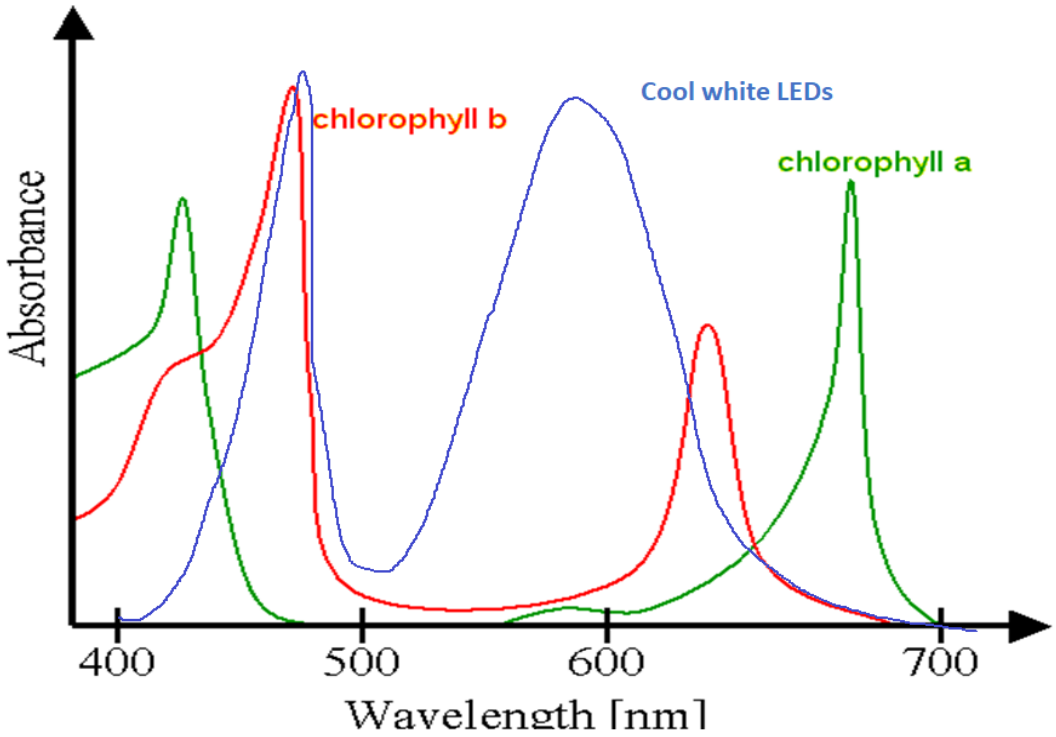


Figure 1.4. Spectral absorbance of chlorophyll a, chlorophyll b and cool white LEDs.

Figure 1.5. The goldilocks® LED unit (Xanthella Ltd) is a light unit designed for the internal illumination of a 20 L photobioreactor. The unit is capable of emitting light in three different wavelengths (white, blue and red), which can be independently controlled.

Previous studies have suggested that the wavelength of light that a species is exposed to can greatly affect the production of certain metabolites for example pigments. An investigation by Kuo et al., (2012) found that illumination with blue LEDs enhanced the carotenoid production of *Rhodopseudomonas palustris*. The wavelength of light has also been shown to increase the lipid production of microalgae. A study observed that *Tetraselmis sp*. and *Nannochlorpsis sp.* displayed a higher growth rate and oil production when cultured using blue LEDs (457nm), compared to white fluorescent bulbs of the same light intensity (Teo et al., 2014).

LEDs can also be programmed to flash intermittently in windows of milliseconds. The flashing light effect occurs when cells are periodically exposed to light and darkness. This can also be achieved by intentionally designing PBRs to have light and dark zones with a mixing system that facilitates the movement of cells through these zones.

The effect can also be achieved by the intermittent flashing of light elements (Abu-Ghosh *et al.*, 2016). It was first reported by Emerson and Arnold, (1933) that algal cells grown under flashing light increased the maximum carbon dioxide uptake and oxygen production rates. This results in energy saving and enhanced algal productivity when utilising optimal flashing light parameters. However recent advancements in this field have observed that sub optimal flashing frequencies can result in less biomass productivity (Vejrazka *et al.*, 2012). It has also been observed that lipid production can be increased when grown under continuous light due to light stress (Combe *et al.*, 2015). Therefore, the benefits of flashing light are highly dependent on flashing frequency and may not be beneficial to produce certain metabolites.

1.11 Thesis objectives

This study aims to characterise pot ale from numerous whisky distilleries from across Scotland with a view to enhance resource recovery and explore novel valorisation opportunities utilising microalgae. The rationale behind the three key objectives of this study are detailed below, followed by the key objectives.

There is increasing interest surrounding the better utilisation of whisky co-products such as pot ale. However, there is limited literature pertaining to the physiochemical characterisation of pot ale. If pot ale is to be further valorised, then it is imperative to have an accurate understanding of its chemical composition of. In previous studies little consideration has been made to the variance pot ale from different distilleries and how processing parameters and storage conditions may affect its composition.

Objective 1

The characterisation of pot ale from a wide range of malt whisky distilleries using a suite of analytical techniques. The investigation of how varying storage conditions effect the chemical composition of pot ale, in order to better inform current and future resource recovery.

There is need to reduce the operational costs of large scale microalgal cultivation. One approach to achieve this to replace traditional growth media with nutrient rich waste streams such as pot ale. Microalgae have already been utilised for the territory treatment of municipal wastewater as a low cost environmentally friendly method of nitrate and phosphate reduction. The phosphate and nitrate of pot ale is also high and presents a significant disposal issue due to eutrophication concerns. If pot ale can be successfully utilised as a nutrient source for the growth of microalgae, then there is potential for the simultaneous reduction of N + P and valorisation via microalgal products. This study will investigated the growth of cyanobacteria/microalgae in pot ale in order to determine if microalgal solutions can be applied to the exploitation of pot ale.

Objective 2

The utilisation of pot ale as a nutrient source for the cultivation of microalgae was investigated. Several species were trialled including *Synechocystis sp.* PCC 6803, *Chlorella sorokiniana*, *Microcystis aeruginosa* PCC 7813 due to their production of high value carotenoids. The growth of the nitrogen fixing species *Nodularia harveyana* PCC 7804 in pot ale was also investigated.

The commercialisation of microalgal products is also dependent on continual innovation of photobioreactor design. This study will investigated the use of a range of novel LED photobioreactors that are manufactured by industrial partners Xanthella Ltd. The systems designed by Xanthella utilise software which allows for light tracking which is a novel approach that manages light intensity within the reactor. Light tracking software automatically adjusts the light intensity of LEDs as the available light decreases within the reactor. This helps to minimise the effects of self-shading and photo limitation. This study will focus on the production of high value carotenoid pigments from microalgae using a range of photobioreactors of increasing volume (1 – 700 litres). Carotenoids such as zeaxanthin are powerful antioxidants with applications in macular degeneration treatment as well as anti-tumour and anti-cancer properties.

Objective 3

The optimisation of light tracking software to up regulate the production of high value carotenoids by *Synechocystis* PCC 6803, *Chlorella sorokiniana* and *Microcystis aeruginosa* PCC 7813. Initial investigations will be carried out at a volume of 1 litre before being scaled up to 16 litres and finally 700 litres, in order to assess the feasibility of industrial scale production.

1.12 References

Abiusi, F., Sampietro, G., Marturano, G., Biondi, N., Rodolfi, L., D'Ottavio, M., Tredici, M.R., 2014. Growth, photosynthetic efficiency, and biochemical composition of Tetraselmis suecica F&M-M33 grown with LEDs of different colours. Biotechnology and Bioengineering 111, 956–964. https://doi.org/10.1002/bit.25014

Abreu, A.P., Fernandes, B., Vicente, A.A., Teixeira, J., Dragone, G., 2012. Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. Bioresource Technology 118, 61–66. https://doi.org/10.1016/j.biortech.2012.05.055

Abu-Ghosh, S., Fixler, D., Dubinsky, Z., Iluz, D., 2016. Flashing light in microalgae biotechnology. Bioresource Technology 203, 357–363. https://doi.org/10.1016/j.biortech.2015.12.057

Alcántara, C., Posadas, E., Guieysse, B., Muñoz, R., 2015. Microalgae-based Wastewater Treatment. Handbook of Marine Microalgae: Biotechnology Advances 439–455. https://doi.org/10.1016/B978-0-12-800776-1.00029-7

Ariunbaatar, J., Scotto Di Perta, E., Panico, A., Frunzo, L., Esposito, G., Lens, P.N.L., Pirozzi, F., 2015. Effect of ammoniacal nitrogen on one-stage and two-stage anaerobic digestion of food waste. Waste Management 38, 388–398. https://doi.org/10.1016/J.WASMAN.2014.12.001

Arnison, R., Carrick, R., 2015. Sector Study on Beer , Whisky and Fish Final Report.

Barrena, R., Traub, J.E., Gil, C.R., Goodwin, J.A.S., Harper, A.J., Willoughby, N.A., Sánchez, A., Aspray, T.J., 2018. Batch anaerobic digestion of deproteinated malt whisky pot ale using different source inocula. Waste Management 71, 675–682. https://doi.org/10.1016/j.wasman.2017.06.025

BBC, 2018. Vivergo; Biofuels plant in Hull to stop production. Acsessed online 31 July 2019. https://www.bbc.co.uk/news/uk-england-humber-45435022

Chisti, Y., 2018. Fermented foods and beverages…, Biotechnology Advances. https://doi.org/10.1016/j.biotechadv.2018.06.006

Combe, C., Hartmann, P., Rabouille, S., Talec, A., Bernard, O., Sciandra, A., 2015. Long-term adaptive response to high-frequency light signals in the unicellular photosynthetic eukaryote Dunaliella salina. Biotechnology and Bioengineering 112, 1111–1121. https://doi.org/10.1002/bit.25526

Cotruvo, J.A., Aron, A.T., Ramos-Torres, K.M., Chang, C.J., 2015. Synthetic fluorescent probes for studying copper in biological systems. Chemical Society Reviews 44, 4400–4414. https://doi.org/10.1039/c4cs00346b

Dionisi, D., Bruce, S.S., Barraclough, M.J., 2014. Effect of pH adjustment, solid–liquid separation and chitosan absorption on pollutants' removal from pot ale wastewaters. Journal of Environmental Chemical Engineering 2, 1929–1936. https://doi.org/10.1016/j.jece.2014.08.013

Emerson, R Arnold, W., 1933. A seperation of the reactions in photosynthesis by means of intermittent light. The Journal of General Physiology 3, 391–420.

Food, E., Authority, S., 2013. Scientific opinion on the safety and efficacy of iron compounds (E1) as feed additives for all species: iron chelate of amino acids, hydrate, based on a dossier submitted by Zinpro Animal Nutrition Inc. EFSA Journal 11, 3287. https://doi.org/10.2903/j.efsa.2013.3287

Giner-Lamia, J., Lopez-Maury, L., Reyes, J.C., Florencio, F.J., 2012. The CopRS Two-Component System Is Responsible for Resistance to Copper in the Cyanobacterium Synechocystis sp. PCC 6803. Plant Physiology 159, 1806–1818. https://doi.org/10.1104/pp.112.200659

Goodwin, J.A.S., Finlayson, J.M., Low, E.W., 2001. A further study of the anaerobic biotreatment of malt whisky distillery pot ale using an UASB system. Bioresource Technology 78, 155–160. https://doi.org/10.1016/S0960-8524(01)00008-6

Goodwin, J.A.S., Stuart, J.B., 1994. Anaerobic digestion of malt whisky distillery pot ale using upflow anaerobic sludge blanket reactors. Bioresource Technology 49, 75–81. https://doi.org/10.1016/0960-8524(94)90175-9

Government, T.S., 2017. Scottish Fish Farm Productuion Survey 2017.

Graham, J., Peter, B., Walker, G., Wardlaw, A., 2012. Chapter 43 Characterisation of the pot ale profile from a malt whisky distillery 1–8.

Grosell, M., Blanchard, J., Brix, K. V., Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquatic Toxicology 84, 162–172. https://doi.org/10.1016/j.aquatox.2007.03.026

Hardy, R.W., 2010. Utilization of plant proteins in fish diets: Effects of global demand and supplies of fishmeal. Aquaculture Research 41, 770–776. https://doi.org/10.1111/j.1365-2109.2009.02349.x

Huang, H., Song, Q., Wang, W., Wu, S., Dai, J., 2012. Treatment of anaerobic digester effluents of nylon wastewater through chemical precipitation and a sequencing batch reactor process. Journal of Environmental Management 101, 68–74. https://doi.org/10.1016/j.jenvman.2011.12.035

Huang, Q., Jiang, F., Wang, L., Yang, C., 2017. Design of Photobioreactors for Mass Cultivation of Photosynthetic Organisms. Engineering 3, 318–329. https://doi.org/10.1016/J.ENG.2017.03.020

Jain, N., Bhatia, A., Kaushik, R., Kumar, S., Joshi, H.C., Pathak, H., 2005. Impact of post-methanation distillery effluent irrigation on groundwater quality. Environmental Monitoring and Assessment 110, 243–255. https://doi.org/10.1007/s10661-005-7695-6

Julio Enrique Traub, 2015. Protein recovery from whisky by - products : a study of using ion exchange chromatography for the recovery of proteins from pot ale by Julio Enrique Traub Modinger Submitted for the degree of Doctor of Philosophy Heriot - Watt University Institute of Biol. Waste Management 177, 675–682. https://doi.org/10.1002/pmic.200600033

Kataki, S., West, H., Clarke, M., Baruah, D.C., 2016. Phosphorus recovery as struvite from farm, municipal and industrial waste: Feedstock suitability, methods and pre-treatments. Waste Management 49, 437–454. https://doi.org/10.1016/j.wasman.2016.01.003

Knudsen, D., Jutfelt, F., Sundh, H., Sundell, K., Koppe, W., 2008. Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (Salmo salar L .) 120–129. https://doi.org/10.1017/S0007114507886338

Lam, M.K., Lee, K.T., 2013. Scale-Up and Commercialization of Algal Cultivation and Biofuel Production, Second Edi. ed, Biofuels from Algae. Elsevier B.V. https://doi.org/10.1016/B978-0-444-59558-4.00012-7

Lopes, T., Reis, A., 2002. Scale-up Problems for the Large Scale Production of Algae 125–149. https://doi.org/10.1007/978-3-319-22813-6

Lu, S., Gibb, S.W., 2008. Copper removal from wastewater using spent-grain as biosorbent. Bioresource Technology 99, 1509–1517. https://doi.org/10.1016/j.biortech.2007.04.024

Mahdy, A., Fotidis, I.A., Mancini, E., Ballesteros, M., González-Fernández, C., Angelidaki, I., 2017. Ammonia tolerant inocula provide a good base for anaerobic digestion of microalgae in third generation biogas process. Bioresource Technology 225, 272–278. https://doi.org/10.1016/j.biortech.2016.11.086

Mallick, P., Akunna, J.C., Walker, G.M., 2010. Anaerobic digestion of distillery spent wash: Influence of enzymatic pre-treatment of intact yeast cells. Bioresource Technology 101, 1681–1685. https://doi.org/10.1016/j.biortech.2009.09.089

Markou, G., Nerantzis, E., 2013. Microalgae for high-value compounds and biofuels production : A review with focus on cultivation under stress conditions. Biotechnology Advances 31, 1532–1542. https://doi.org/10.1016/j.biotechadv.2013.07.011

Melnicki, M.R., Pinchuk, G.E., Hill, E. a., Kucek, L. a., Stolyar, S.M., Fredrickson, J.K., Konopka, A.E., Beliaev, A.S., 2013. Feedback-controlled LED photobioreactor for photophysiological studies of cyanobacteria. Bioresource Technology 134, 127–133. https://doi.org/10.1016/j.biortech.2013.01.079

Moerman, W., Carballa, M., Vandekerckhove, A., Derycke, D., Verstraete, W., 2009. Phosphate removal in agro-industry: Pilot- and full-scale operational considerations of struvite crystallization. Water Research 43, 1887–1892. https://doi.org/10.1016/j.watres.2009.02.007

Mohana, S., Acharya, B.K., Madamwar, D., 2013. Bioremediation Concepts for Treatment of Distillery Ef fl uent. https://doi.org/10.1007/978-81-322-0876-1

Olaizola, M., 2003. Commercial de v elopment of microalgal biotechnology : from the test tube to the marketplace 20, 459–466. https://doi.org/10.1016/S1389-0344(03)00076-5

Olivieri, G., Salatino, P., Marzocchella, A., 2014. Advances in photobioreactors for intensive microalgal production: Configurations, operating strategies and applications. Journal of Chemical Technology and Biotechnology 89, 178–195. https://doi.org/10.1002/jctb.4218

Pant, D., Adholeya, A., 2007. Biological approaches for treatment of distillery wastewater : A review 98, 2321–2334. https://doi.org/10.1016/j.biortech.2006.09.027

Pegallapati, A.K., Nirmalakhandan, N., 2013. Internally illuminated photobioreactor for algal cultivation under carbon dioxide-supplementation: Performance evaluation. Renewable Energy 56, 129–135. https://doi.org/10.1016/j.renene.2012.09.052

Pittman, J.K., Dean, A.P., Osundeko, O., 2011. The potential of sustainable algal biofuel production using wastewater resources. Bioresource Technology 102, 17–25. https://doi.org/10.1016/J.BIORTECH.2010.06.035

Pulz, O., Gross, W., 2004. Valuable products from biotechnology of microalgae. Applied Microbiology and Biotechnology 65, 635–648. https://doi.org/10.1007/s00253-004-1647-x

Quinn, J.P., Barker, T.W., Marchant, R., 1980. Relationship Between Free and Complexed Forms of Copper in Malt Whiskey Distillery Spent Wash. Journal of the Institute of Brewing 86, 274–276. https://doi.org/10.1002/j.2050-0416.1980.tb06880.x

Rahman, M.M., Salleh, M.A.M., Rashid, U., Ahsan, A., Hossain, M.M., Ra, C.S., 2014. Production of slow release crystal fertilizer from wastewaters through struvite crystallization – A review. Arabian Journal of Chemistry 7, 139–155. https://doi.org/10.1016/J.ARABJC.2013.10.007

Schulze, Peter S C, Barreira, L. a., Pereira, H.G.C., Perales, J. a., Varela, J.C.S., 2014. Light emitting diodes (LEDs) applied to microalgal production. Trends in Biotechnology 32, 422–430. https://doi.org/10.1016/j.tibtech.2014.06.001

Schulze, Peter S.C., Barreira, L.A., Pereira, H.G.C., Perales, J.A., Varela, J.C.S., 2014. Light emitting diodes (LEDs) applied to microalgal production. Trends in Biotechnology 32, 422–430. https://doi.org/10.1016/J.TIBTECH.2014.06.001

Scottish whiksy association, 2015. Stasttical report

Sepa, 2018. Supporting Guidance (WAT-SG-75) Sector Specific Guidance: Construction Sites.

Shepherd, J.G., Sohi, S.P., Heal, K. V., 2016. Optimising the recovery and re-use of phosphorus from wastewater effluent for sustainable fertiliser development. Water Research. https://doi.org/10.1016/j.watres.2016.02.038

Singh, A.B., Biswas, A.K., Ramana, S., 2003. Effect of distillery effluents on plant and soil enzymatic activities and groundnut quality. Journal of Plant Nutrition and Soil Science 166, 345–347. https://doi.org/10.1002/jpln.200390053

Singh, J., Gu, S., 2010. Commercialization potential of microalgae for biofuels production. Renewable and Sustainable Energy Reviews 14, 2596–2610. https://doi.org/10.1016/j.rser.2010.06.014

Teo, C.L., Atta, M., Bukhari, A., Taisir, M., Yusuf, A.M., Idris, A., 2014. Enhancing growth and lipid production of marine microalgae for biodiesel production via the use of different LED wavelengths. Bioresource Technology 162, 38–44. https://doi.org/10.1016/j.biortech.2014.03.113

Thang, V.H., Novalin, S., 2008. Green Biorefinery: Separation of lactic acid from grass silage juice by chromatography using neutral polymeric resin. Bioresource Technology. https://doi.org/10.1016/j.biortech.2007.08.045

The Guardian, 2013. Biofuels plant opens to become UK's biggest buyer of wheat. Acsessed online, 31 July 2019. https://www.theguardian.com/environment/2013/jul/08/biofuels-plant-wheat-vivergo-hull

The Scotch Whisky Association, 2012. Scotch Whisky Sector Plan.

Tokuda, M., Fujiwara, Y., Kida, K., 1999. Pilot plant test for removal of organic matter, N and P from whisky pot ale. Process Biochemistry 35, 267–275. https://doi.org/10.1016/S0032-9592(99)00063-1

Tokuda, M., Ohta, N., Morimura, S., Kida, K., 1998. Methane fermentation of pot ale from a whisky distillery after enzymatic or microbial treatment. Journal of Fermentation and Bioengineering 85, 495–501. https://doi.org/10.1016/S0922-338X(98)80068-8

Tünay, O., Kabdasli, I., Orhon, D., Kolçak, S., 1997. Ammonia removal by magnesium ammonium phosphate precipitation in industrial wastewaters. Water Science and Technology 36, 225–228. https://doi.org/10.1016/S0273-1223(97)00391-0

Uysal, A., Demir, S., 2013. Struvite Pyrolysate Recycling for Removing Ammonium from Baker ' s Yeast Industry Wastewater. Icoest 2013.

Vejrazka, C., Janssen, M., Streefland, M., Wijffels, R.H., 2012. Photosynthetic efficiency of chlamydomonas reinhardtii in attenuated, flashing light. Biotechnology and Bioengineering 109, 2567–2574. https://doi.org/10.1002/bit.24525

Wainman, F.W., Dewey, P.J.S., 1982a. The energy value to ruminants of malt distillers' draff, and of a mixture of draff and pot ale syrup. Animal Production 34, 325–328. https://doi.org/10.1017/S0003356100010278

Wainman, F.W., Dewey, P.J.S., 1982b. The energy value to ruminants of malt distillers' draff, and of a mixture of draff and pot ale syrup. Animal Production 34, 325–328. https://doi.org/10.1017/S0003356100010278

Walter, C., Steinau, T., Gerbsch, N., Buchholz, R., 2003. Monoseptic culti v ation of phototrophic microorganisms * de v elopment and scale-up of a photobioreactor system with thermal sterilization 20, 261–271. https://doi.org/10.1016/S1389-0344(03)00068-6

Wang, B., Lan, C.Q., Horsman, M., 2012. Closed photobioreactors for production of microalgal biomasses. Biotechnology Advances 30, 904–912. https://doi.org/10.1016/j.biotechadv.2012.01.019

Wijffels, R.H., Barbosa, M.J., 2010. An outlook on microalgal biofuels. Science (New York, N.Y.) 329, 796–799. https://doi.org/10.1126/science.1189003

William, E., Cove, H., 2016. Modular algal aquaculture system and method. US 2016/0029579 A1.

Yen, H.W., Hu, I.C., Chen, C.Y., Chang, J.S., 2013. Design of Photobioreactors for Algal Cultivation, Second Edi. ed, Biofuels from Algae. Elsevier B.V. https://doi.org/10.1016/B978-0-444-59558-4.00002-4

Yu, W., Quek, W.P., Li, C., Gilbert, R.G., 2018. Effects of the Starch Molecular Structures in Barley Malts and Rice Adjuncts on Brewing Performance. https://doi.org/10.3390/fermentation4040103

Chapter 2

Evaluation of high–performance LED photobioreactors to produce high value carotenoid pigments.

2.1 Chapter outline

In this chapter a range of novel light tracking photobioreactors (1 L, 16 L and 700 L) will be tested and optimised for the production of high value carotenoid pigments from selected species of cyanobacteria and microalgae.

In addition, the effect of light tracking technology on the carotenoid production of *Synechocystis sp.* PCC 6803 was also assessed.

2.1.1 Introduction

Photobioreactors are typically limited in scale in comparison to traditional bio reactors and fermentation vessels, this is primarily due to the requirement for light. As the culture density increases in photobioreactor light penetration decreases, therefore designs that maximise surface area to volume ratio are preferred.

To overcome the production scale issue modular systems consisted of many small PBR can be designed (Mahoney et al., 2018). Despite these design considerations the large-scale industrial production of microalgae is often not economically viable due to the large associated production costs. Therefore, there has been a shift in focus to produce lower volume, high value products such as pigments (Gerardo *et al.*, 2015).

To date only a handful of microalgae compounds have been commercialised. Perhaps the most notable product being astaxanthin which is a carotenoid pigment that has applications in the aquaculture industry as a colourant. Carotenoids are a class of pigments that are synthesized by both photosynthetic organisms and non-photosynthetic organisms. In photosynthetic organisms they are present in the photosynthetic membrane and are essential to the photosynthetic apparatus. Their primary role is to protect against oxidative stress. Carotenoids can absorb and dissipate light energy which protects the photosynthetic apparatus from light damage in conditions of excess light energy.

Absorbed light energy can also be transferred to the photosynthetic apparatus therefore carotenoids also perform an accessory role to chlorophyll in the harvesting of light (Lagarde et al., 2000). Carotenoids can be largely split into two main groups, carotenes and xanthophylls. Carotenes and xanthophylls are both long chain poly-unsaturated hydrocarbons with 40 or more carbon atoms, however, xanthophylls also contain oxygen atoms which increase their polarity (Jeanmonod, Rebecca and Suzuki, 2018).

Zeaxanthin is a xanthophyll that is produced by many higher plant and microalgae. Similarly, to astaxanthin, zeaxanthin has applications as a food colourant (Liu et al.,2015). Zeaxanthin is preferred over other carotenoids such as lutein for the enhancement of pigmentation in poultry due to its ability to deposit the colour evenly in the flesh (Sajilata et al.,2008). Zeaxanthin also has application in the health and pharmaceutical industries. It is sold along with other carotenoids as a dietary supplement. Carotenoid rich diets have been epidemiologically linked to a lower risk of cancer due to their strong anti-oxidative properties (Sharoni et al., 2012). It is also thought that zeaxanthin plays a role in the prevention of age – related macular degeneration (AMD) which is a leading cause of irreversible blindness in adults. AMD is the result of degenerative changes that occur in the retina and macula. Zeaxanthin can accumulate in macular tissue and prevent damage by absorbing harmful high energy blue light and preventing photo-oxidative damage (Sajilata et al.,2008; Liu et al., 2015)

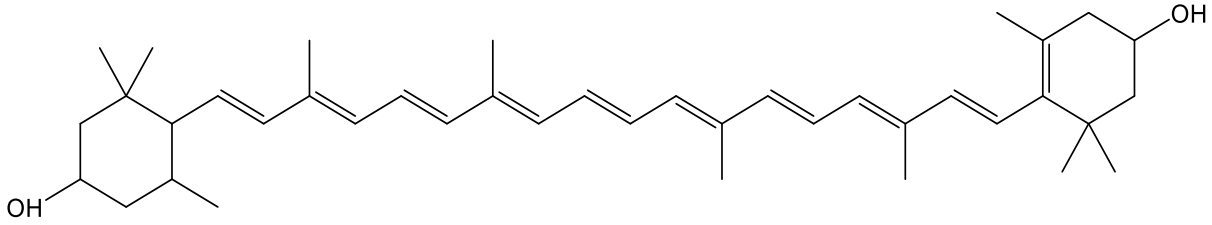


Figure 2.1. The chemical structure of Zeaxanthin

The main photo-protective system in the majority of photosynthetic organisms is the xanthophyll cycle which is a light induced process of enzymatic reactions of epoxidation and de-epoxidation of xanthophyll pigments (Figure 2.2).

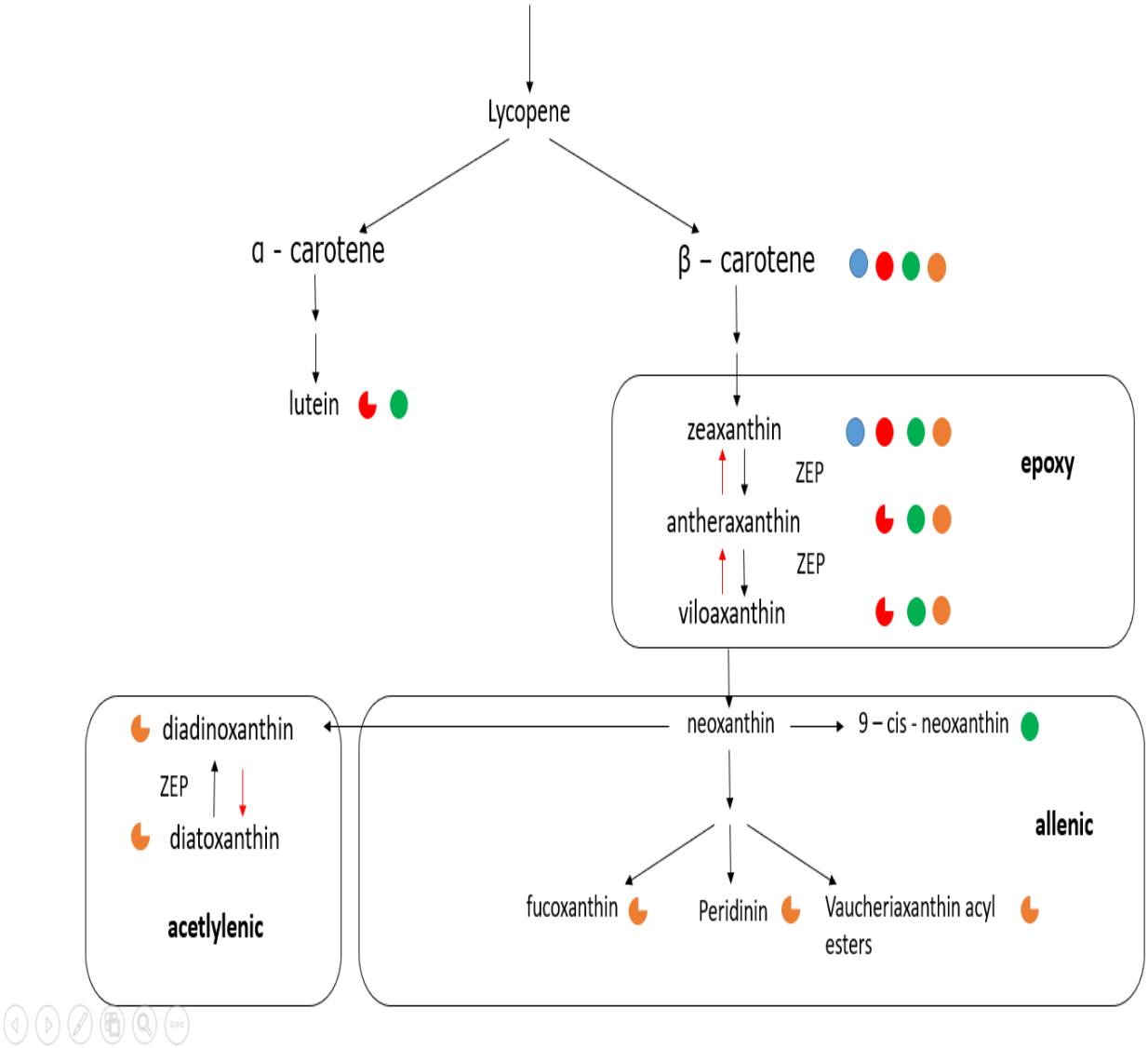


Figure 2.2. The occurrence and biosynthesis of the major carotenoids within the epoxy, acetylenic and allenic groups in photosynthetic eukaryotes. Steps that are characterised by zeaxanthin epoxidases are labelled with ZEP. De – epoxidation steps that are catalysed by violaxanthin de-epoxidase under light stress, are indicated with a red arrow. The filled circles indicate the general occurrence in Viridplantae (green), Glaucophyta (blue), Rhodophyta (red) and Chromalveolata (orange). Incomplete circles indicate that the carotenoid is not present in every species in a given taxon. Figure adapted from (Dautermann and Lohr, 2017)

These reactions lead to cyclic conversions between several pigments including primarily violaxanthin, antheraxanthin and zeaxanthin as well as others. It is known that in microalgae the zeaxanthin content in regulated by light irradiance as a product of the xanthophyll cycle. Cyanobacteria lack a xanthophyll cycle due to their prokaryotic nature (Rakhimberdieva *et al.*, 2004). Therefore, high light

conditions should upregulate the production of zeaxanthin in these microalgae due to de–epoxidation reactions.

Select heterotrophic bacteria such as *Paracoccus zeaxanthinifaciens* are also capable of producing zeaxanthin (Table 2.9). *Paracoccus zeaxanthinifaciens* produces both intracellular and extracellular zeaxanthin, with the extracellular zeaxanthin being excreted into the media in lipophilic vesicles (Joshi and Singhal, 2016) whereas, microalgae typically only produce intracellular zeaxanthin. Heterotrophic bacteria typically grow significantly faster than microalgae and cyanobacteria with some species having doubling times of only several hours. They also have the advantage of not requiring light for growth which results in considerably easier scale up.

Recent advancements in LED technology have made them an attractive option for modern photobioreactor designs due to improvements in cost and energy efficiency (Yeh, Ding and Yeh, 2015). LED lights also have the advantage of being easily programable which allows for advanced features such as wireless communication and light tracking. They also come in a wide variety of wavelengths and their light intensity output can also be precisely controlled. This control makes them ideally suited to produce high value carotenoids.

There is also the potential to combine LED light arrays with a light sensor which allows for light tracking. The concept of light tracking involves maintaining the available light within a reactor at a constant level. This elevates the growth limiting problem of light penetration as light intensity can be increased to match changes in cells density. A similar concept called solar tracking has previously been employed in outdoor photobioreactors where the reactors are rotated to be in optimal alignment with the sun (Hindersin et al.,2014). However light tracking using artificial lights and internal illumination is something that has not been extensively investigated in the existing literature.

This study has three primary aims

1. To assess the performance of a range of high-performance LED photobioreactors.
2. To ascertain the effect of light intensity on the pigment content of *Synechocystis sp*. PCC 6803 utilising light tracking in order to provide continual high light conditions
3. The scale up of the production of high value carotenoids using a 16 L and a 700 L internally illuminated photobioreactor.

2.2 Materials and Methods

2.2.1 Culture maintenance

Synechocystis sp. PCC 6803 and *Microcystis aeruginosa* PCC 7813 were obtained from the Pasteur culture collection (Paris, France). *Chlorella sorokiniana* was obtained from CPI.

Routine cultivation of all cultures was performed using 100 ml volumes contained within 250 ml Erlenmeyer flask. Each 100 ml maintenance culture was prepared by inoculating approximately 10 ml of stock culture into 100 ml of freshly prepared BG-11 contained in a 250 ml Erlenmeyer flask. Maintenance cultures were exposed to 10 ±1 μmol m^{-2} s^{-1} (Li-Cor Intelligent light meter Li-250) provided by artificial light in a temperature-controlled growth chamber. (Filotron growth chamber – Weiess Technik, UK) at a constant temperature of 21 °C. A light dark cycle of 12 hours light and 12 hours dark was also employed.

For experiments that required a larger inoculation volume, the 100 ml-maintained cultures were used to inoculate 1.8 L of fresh media in a 2 L Erlenmeyer flask. The 2 L cultures were sparged with filtered ambient air (0.22 μm; Millipore, UK) using an air pump (230 V, 50Hz; Fisherbrand, UK). Cultures were illuminated with 20±3 μmol m^{-2} s^{-1} of artificial light provided by cool white fluorescence tubes (58 W, Osram) and maintained at 22±2 ^{o}C in a temperature-controlled room. BG -11 media stock solutions were prepared using an electronic

balance and diluted to the required concentration in distilled water (Elga, Veolia water, UK) (Table 2.1). The stock solutions were then added to a desired amount of distilled water in a flask (Table 2.1). The flask was then sealed using a cotton wool bung and autoclaved at 121°C for 20 minutes.

Table 2.1. Composition of BG-11 media (Stanier,1971)

Component	Final concentration (g/L)
Sodium nitrate ($NaN0_3$)	750
Dipotassium phosphate (K_2HPO_4)	0.04
Magnesium sulphate septahydrate ($MgSO_47H_2O$)	0.075
Calcium chloride dihydrate ($CaCl_22H_2O$)	0.036
Sodium carbonate (Na_2CO_3)	0.020
Citric acid ($C_6H_8O_7$)	0.006
Iron sulphate septahydrate ($FeSO_4$ $7H_2O$)	0.006
EDTA (di sodium)	0.001
Trace element solution	1 ml/L
Trace element solution:	
Boric acid (H_3BO_3)	2.86
Manganese chloride tetrahydrade ($MnCl_2.4H_2O$)	1.81
Zinc sulphate septahydrate ($ZnSO_4.7H_2O$)	0.222
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.390
Copper sulphate pentahydrate ($CuSO_45H_2O$)	0.079
Cobalt nitrate hexahydrate (Co $(NO_3)_2.6H_2O$)	0.049

2.2.2 Monitoring of growth

2.2.2.1 Cell counting with coulter counter

The cells/ml within samples were measured using the Coulter counter Multisizer® 4 (Beckman Life Science, Indianapolis, USA). Samples were dilute in Isotone II prior to analysis. The dilution factor was dependent on the density of each culture. The measuring probe had a 100 µm aperture and the measuring range was between 1.9 µm and 60 µm. The measurement is based on Coulters principle which can enumerate and measure the size of non-conducting particles suspended in a fluid. Measurements were taken in triplicate unless otherwise stated. The instrument was calibrated with nominal 10 µm latex beads (Beckman life Science).

2.2.2.2 Enumeration using optical density

Cell growth was quantified by reading the optical density at a wavelength of 730 nm using a Spectrophotometer (Novaspec 11, Pharmacia Biotech Inc, USA). A calibration curve was created by performing a serial dilution of a 2 week of old culture of *Synechocystis sp.* with a cell density of 15.5 $x10^6$ cells/ml as measured by Coulter counter. There is a linear relationship between the cells/ml and $OD_{730\ nm}$.

2.2.2.3 Manual counting with a Haemocytometer

A 1 ml sample of the culture was taken under aseptic conditions and placed in a 1.5 ml microcentrifuge tube. The sample was then diluted with de-ionised water in another 1.5 ml microcentrifuge tube and thoroughly vortexed. In order to obtain an accurate count, there must be greater than 100 but less than 1000 cells. A cover slip was then placed on a haemocytometer (Neubauer) and 10 µl of the diluted sample was then pipetted into the haemocytometer chamber. The haemocytometer was then viewed under a light microscope at magnification x 200. Haemocytometers consist of 9 large squares divided into differently sized cross sections. Five large squares of the haemocytometer chamber were used

per counting. The number of cells/ml was then calculated using the following equation.

$$\frac{(number\ of\ cells\ counted)(dilution\ factor)}{(number\ of\ large\ squares\ counted)(volume\ of\ 1\ large\ square)}\ x\ 10{,}000$$

2.2.2.4 Dry weight measurements

To determine the biomass of microalgal cultures the dry weight was calculated by first pre-drying three 47 mm GF/C filters (Whatman, UK) which were placed on petri dishes and dried in an oven at 80ºC for 12 hours. The filters were then removed from the oven and placed in a desiccator for 6 hours to ensure that they were completely free of moisture content. The filters were numbered and then weighed using an electronic balance.

Empty 15 ml falcon tubes x 3 were then numbered and weighed using an electronic balance. These tubes were filled with 5 ml of algal sample each and then weighed again. The difference between these two measurements was recorded in order to precisely determine the volume of algal sample that would be poured through the GF/C filters. Each sample tube was then poured through the corresponding GF/C filter utilising a vacuum pump to ensure that all the algal biomass was retained by the filter paper. The GF/C's were then dried in an oven at 80 ˚C for 24 hours and subsequently placed in a desiccator for 6 hours before being weighed.

The biomass concentration of each sample expressed in grams/litre could then be calculated using the following equation

$$Cx\ (g_{bio}\ L^{-1}) = \frac{W2\ (g) - W1\ (g)}{x\ (ml)\ x\ 0.001}$$

Where W2 is the weight of the filter with biomass and W1 is the weight of the same filter without biomass. X represents the volume of sample that was filtered.

2.2.2.5 Nitrate analysis

To determine the nitrate utilisation of microalgal species during a 14-day cultivation period the nitrate content was analysed using Ion Chromatography (IC). The analytes were separated on an AS11-HC column (4 mm x 250 mm long, 4 µm particle size) equipped with an AG11 guard column (4 x 50 mm). Data acquisition and the analysis of chromatograms was performed using Chromeleon 7.1 chromatography software (Dionex). The mobile phase was prepared by the automated dilution of potassium hydroxide (KOH) with ultrapure water. The gradient was generated by the elution generation system with a possible concentration range of 1.5 mM to 60 mM. There was a 7-minute equilibration step before each run at 1.5 mM KOH. The gradient used for each analysis was as follows; 0 – 8 minutes (1.5 mM), 8 – 18 minutes (15 mM), 18 - 23 minutes (15 mM), 23 – 24 minutes (24 mM) and 24 – 30 minutes (60 mM). All separations were performed at a flow rate of 0.38 ml/min. The injection volume was 2 µl. The conductivity of the mobile phase was supressed using an Aers 500 suppressor. Sample injection started when the background conductivity was < 1 µS. The methodology was developed with the aid of the virtual column online tool provided by (Thermo fisher scientific, UK).

2.2.3 Pigment extraction and analysis

To assess the extraction efficiency of pigments, three different solvents (methanol, ethanol and acetone) were evaluated. The pigment extraction method was adapted from a study by Henriques et al.(2007). The pigments extractions were prepared by centrifuging 1 ml of 17-day old *Synechocystis sp* (PCC6803) for 5 minutes at 13,000 g. The supernatant was then discarded, and the pellet was placed in a -21 °C freezer for 24 hours. The pellets were then allowed to defrost at room temperature before being re-suspended in 1 ml of solvent. The solvents were evaluated at both 100% and 90% plus 10% water (n=3). Samples were extracted after 6 hours, centrifuged to remove cell debris then analysed by UPLC-MS.

2.2.3.1 UPLC conditions

The analytical conditions for the UPLC-MS^E were adapted from Fu et al., (2012) and Henry et al., (2014). The UPLC-MS^E analysis was performed using an Acquity H-class UPLC (Waters, USA) coupled to a QTof mass spectrometer (Xevo, Waters, USA). The column used for analysis was a Waters Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm). The system was operated under the following gradient elution program: solution A (H_2O with 0.01% formic acid) and solution B (Acetonitrile with 0.01% formic acid) at a flow rate of 400 µL/nin as follows: 0-0.5 min, 70% B; 0.5-3.0 min, 70% -100% B; 3.0-11.0 min, 100% B; 11.0-13.0 min, 70% . The column and auto sampler were maintained at 25°C and 4°C respectively. The scan time was set to 0.5 s and the data was collected using the MS^E function in centroid mode with a collision energy ramp of 20-40 V and a collision energy of 6 V. The mass spectrometer was calibrated with 0.5 mM sodium iodide before analysis. The data was analysed using the Mass Lynx version 4.0 software.

Analytical standards for each carotenoid pigment were purchased from Sigma. Calibration curves were generated for each carotenoid using a range of 1 mg/L – 50 mg/L and were quantified using a photodiode array set to 450 nm. The R^2 of all calibration curves were > 0.99 (Figure 4A in the appendix).

2.2.4 Micro–Pharos (1 litre) photobioreactor setup and specifications

The Micro-Pharos photobioreactor (Xanthella, Ltd) features two LED panels that are fitted to the external surface of the PBR using magnets (Figure 2.3). The maximum current that they can be driven on is 450 ma which equates to an approximate maximum light intensity of 1250 $umol.m^2s^1$ however, the manufacturer recommends to limit overheating, that the current should not exceed 400ma this equates to a light intensity of approximately 1150 $umol.m^2s^1$.

The Micro-Pharos photobioreactor has a built in cooling system so the temperature within the reactor can be set by the operator. This is facilitated by a cooling fan that is magnetically attached to the back of the reactor and an internal temperature probe that measures temperature within the reactor.

Aeration is achieved by sparging via an air stone, with a flow rate of 1.5 L/min to ensure mixing (Figure 2.3).

Light intensity and temperature setting were controlled by the Zeus II control unit. The control unit also allows light tracking which enables the reactor to maintain the available light within the reactor at a constant level. This is achieved by an internal light sensor measuring the available light within the reactor and feeding this information back to the Zeus II control unit. The control unit then displays this information in arbitrary units (AU). The user can then set a desired value and it will be maintained for the duration of the growth period. The Zeus II control unit allows for two PBRs to be connected in parallel, thus enabling replication

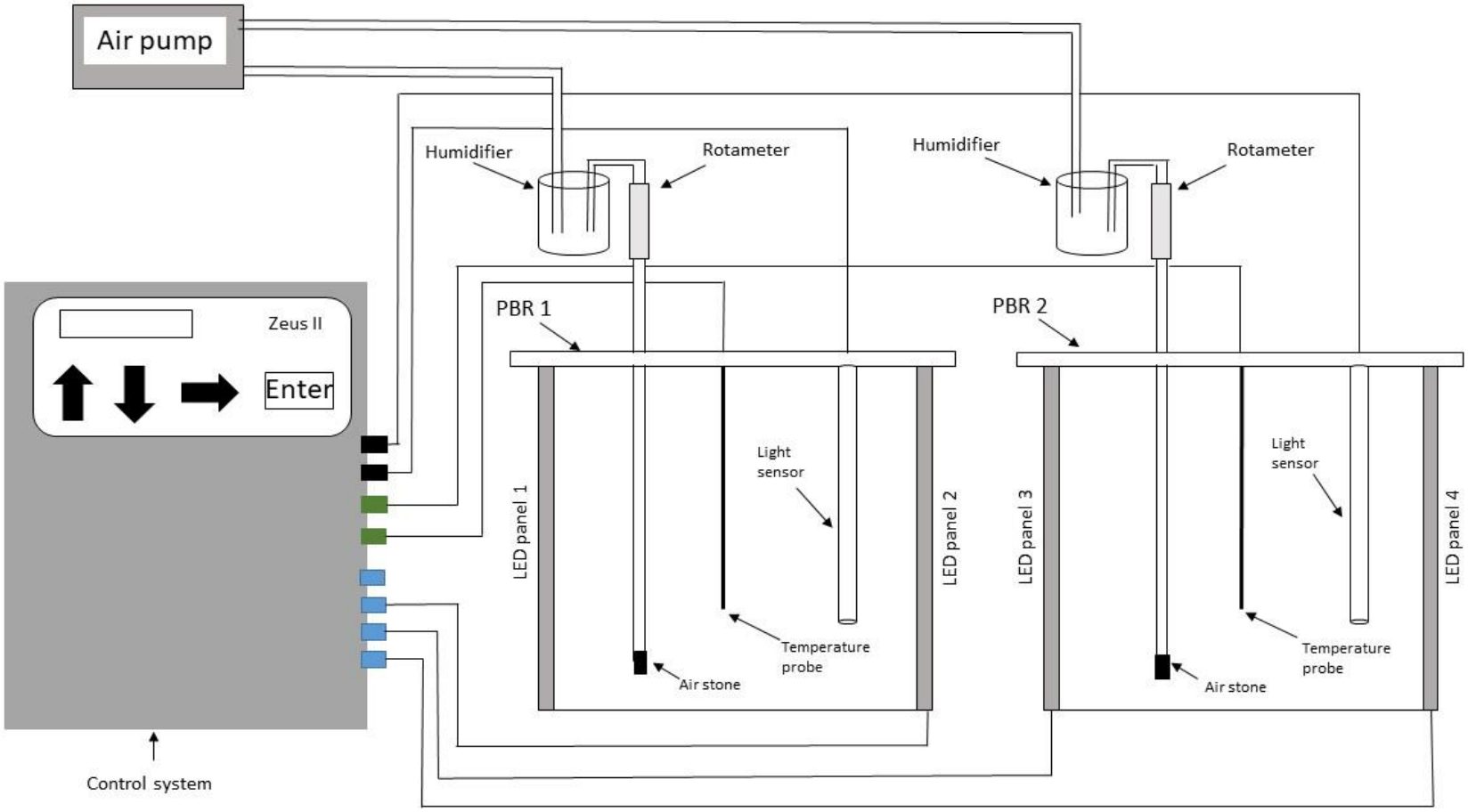


Figure 2.3. Diagram detailing the setup of the Micro-Pharos photobioreactors

2.2.4.1 Evaluation of the performance of light tracking capacity in the Micro-Pharos (1 litre PBR)

To determine an optimum light tracking value for growth of microalgae and cyanobacteria in the Micro-Pharos photobioreactor, the light intensity during the cultivation of *Synechocystis sp.* was manually adjusted and the growth rate was assessed. The light regime was also tested by turning one of the LED panels off in PBR 2. This was done in order to assess the effect of having a light and dark zone within the reactor (Figure 2.4) as light/dark zones have previously been shown to increase growth rates in other reactor types (Cheng *et al.*, 2018). The Micro-Pharos PBRs were inoculated with cultures of 2-week-old *Synechocystis sp.* PCC 6803. The inoculation density was 2.5 x 10^6 cells/ml. The temperature and air sparging rate were maintained at a constant 21 °C and 1.5 L/min, respectively. For the purposes of the investigation the light tracking functionality of the PBR's was turned off and light intensity was increased manually by increasing the current supplies to the LEDs. Adjustments in current were made according to the daily point to point growth rate observed in each reactor. After each adjustment in current, the light intensity of the panels was measured with a light meter (LI – 250 A, LI – COR biosciences, United Kingdom).

The growth of *Synechocystis sp.* PCC 6803 in each reactor was then assessed by cell counting using the Multisizer® 4 (section 2.2.2.1). The point to point growth rate was then determined using the following equation.

$$(\mu.\ d^{-1}) = \ln(C2 - C1)/(T2 - T1)$$

Where C2 is the cells/ml of the PBR being tested and C1 is the cells/ml of that PBR 24 hours prior.

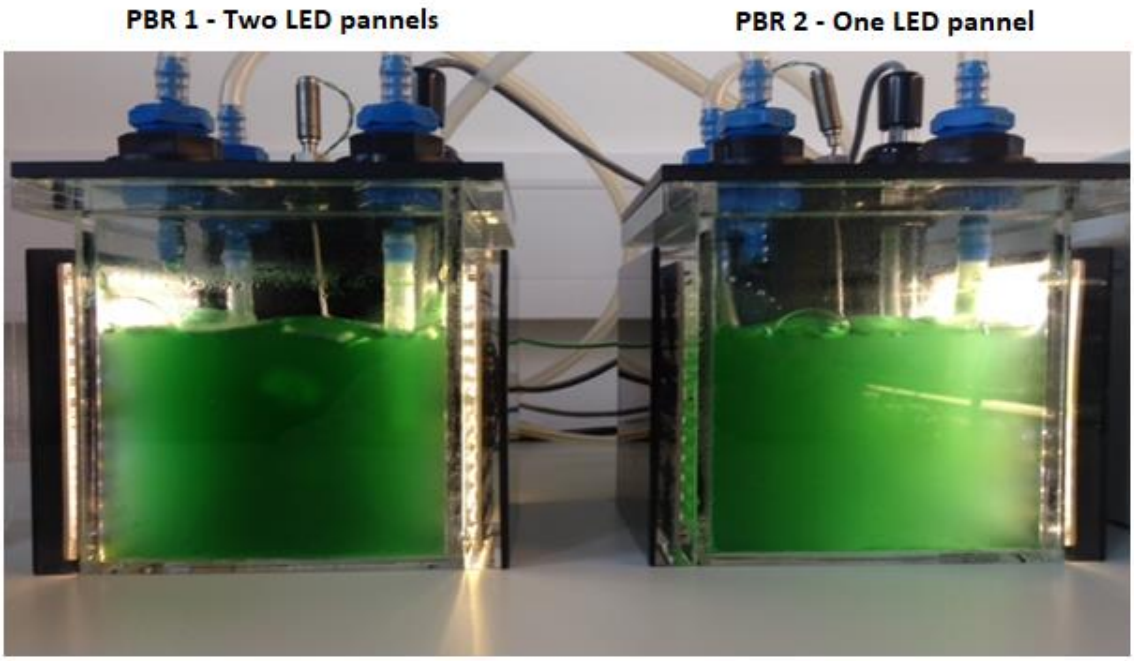


Figure 2.4. The Micro–Pharos photobioreactors inoculated with Synechocystis sp. PCC 6803.

2.2.5 Evaluation of the Micro-Pharos for growth and carotenoid production of cyanobacteria and microalgae

The Micro-Pharos PBR's were filled with 1 litre of autoclaved BG-11 media and inoculated with *Synechocystis sp* PCC 6803 to produce a final cell density of 2.5 x 10^6 cells/ml. The light tracking was turned on and set to a value of 300 AU. The temperature was maintained at a constant 21 °C and the flow rate was set to 1.5 L / min. The duration of the experiment was 2 weeks. Daily samples (1 ml) were taken for cell number, nitrate concentration and pigment content. Samples (5 ml) were also taken for dry weight every 24 hours. All samples were taken in triplicate resulting in a total daily sampling volume of 24 ml. This experiment was repeated with *Chlorella sorokiniana* and *Microcystis aeruginosa* PCC 7813, with all parameters kept the same.

2.2.6 Cyclops (16 L) photobioreactor setup and specifications

The Cyclops photobioreactor (Xanthella Ltd.) is a 16-litre tubular bubble lift photobioreactor. The PBR is internally illuminated via a tubular LED array that sits within the main reactor tube. The LED unit (Goldilocks, Xanthella Ltd.) is tri colour and has cool white, blue and red LEDs embedded into its internal surface which results in an illuminated surface area of 0.13 m^2 (Figure 2.5)

The key design principle of the reactor is that the air stone at the base of the reactor will create a bubble lift mixing through the illuminated tube of the LEDs. The cells will then flow down the sides of the reactor that are non-illuminated before being picked up in the air flow again, which in theory facilitates good light to dark zone mixing (Figure 2.5)

Similarly, to the 1 litre Micro–Pharos PBR parameters such as light and temperature are controlled by the Zeus control box. The Cyclops PBR also has a light tracking feature which operates using a similar principle.

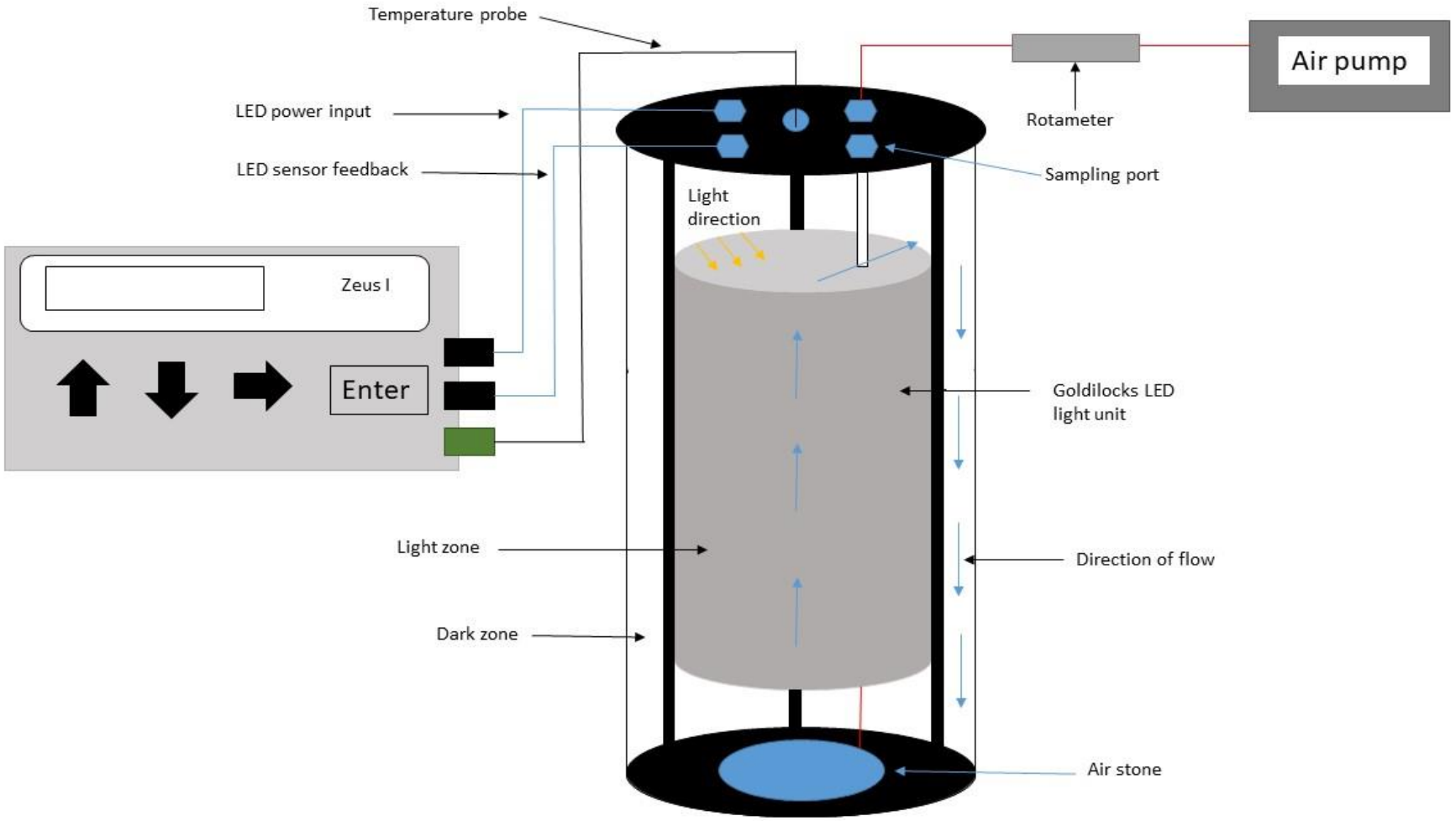


Figure 2.5. Diagram detailing the setup of the 16 litre Cyclops photobioreactor (Not to scale).

2.2.7 Evaluation of the light tracking capability and performance of the Cyclops 16 litre internally illuminated PBR.

The light conditions within the Cyclops PBR are different than the Micro–Pharos PBR as it is an internally illuminated reactor with a circular light unit (Figure 2.5) Due to these different light conditions the optimal light tracking value is likely to be different. This investigation aimed to determine a light tracking value that functions well with the Cyclops 16 litre PBR. The methodology was the same as the previous light tracking experiment with the Micro–Pharos reactor (section 2.2.4.1), where the light intensity is gradually increased, and the light tracking value is recorded daily.

Table 2.4. The current setting and corresponding light intensity values for each reactor during the Cyclops PBR (16 litre) light tracking experiment.

Day	Current PBR 1 (ma)	Light intensity (µmol.m^{-2}/s)	Current PBR 2 (ma)	Light intensity (µmol.m^2/s)
0	300	140	300	143
1	315	145	315	145
2	375	152	380	155
3	400	185	400	185
5	450	215	450	215
6	475	236	470	230
7	550	305	550	307
8	550	310	575	328
9	525	290	550	312
10	500	293	530	305
11	500	292	515	300
12	490	281	500	295
13	400	279	455	289
14	390	271	400	272

2.2.8 The scale up of carotenoid pigment production using the Pandora (700 litre)

In order to assess the feasibility of industrial scale production the production of zeaxanthin from *C. sorokiniana* was assessed at a scale of 700 litres. The Pandora reactor is currently under development as of July 2019 by Xanthella Ltd. The system was designed for pilot scale and industrial scale production. A 700-litre prototype was utilised in where the reactor was internally illuminated with 8 LED light tiles which represent an illuminated surface area of 6.4 m^2. The reactor was mixed by air/CO_2 injection at the bottom of the tank with an air curtain distributing air bubbles uniformly across the reactor. Temperature, light intensity and light tracking are controlled by a Zeus control unit.

The reactor was inoculated with *Chlorella sorokiniana* with all sampling and growth analysis carried out at Xanthella Ltd (Oban, Scotland). Samples were freeze dried and analysed using UPLC–MS in order to determine zeaxanthin content (section 2.2.3)

Table 2.5. Current supplied to the Pandora LED panels and corresponding light intensity readings for each day of cultivation.

Time (d)	Current (mA)	Light intensity (µmol/m^2/s)
0	1160	50
3	1160	50
4	6023	260
5	16540	713
6	20000	862
7	20000	862
11	20000	862
12	20000	862
13	20000	862
14	20000	862
17	20000	862
18	20000	862
19	20000	862
20	20000	862
24	20000	862

Figure 2.6. The Pandora 700 litre photobioreactor. A) Inside the photobioreactor. B) The outside of the photobioreactor including the control unit.

2.3 Results and discussion

2.3.1 Optimisation of the light tracking functionality of the Micro-Pharos (1 litre) photobioreactor.

This investigation aimed to optimise the light tracking functionality of the Micro-Pharos photobioreactor. Light tracking is controlled by the Zeus II control unit and is set by adjusting the light tracking value which is recorded in arbitrary units (AU). When utilising light tracking the cell density of the culture is directly corelated with light intensity (Figure 1A in appendix). A high AU value translates to a high level of available light within the reactor. In order to optimise the light tracking value, the light intensity was adjusted manually and the resulting light tracking value (AU) was recorded. As carotenoid pigments are the targeted product of this research the light intensity was purposely kept high, with the aim of light stressing the organism to promote the production of xanthophyll pigments without causing photo inhibition.

The number of cells observed at the end of the experiment was higher in PBR 2 when compared to PBR 1, with PBR 1 reaching 9.96 $x10^7$ and PBR 2 reaching 3.36 $x10^8$ cells/ml (Figure 2.7). A significant decrease in cells/ml in PBR 1 was observed between days 10 and 12 (Figure 2.7). This is likely due to photo-inhibition as the light reached over saturated levels and caused damage to the cell structure of *Synechocystis sp*. The current was then lowered by 25ma which allowed for an increase in growth rate (Figure 2.7).

The growth rate in PBR 2 decreased significantly after day 10 this is likely due to the light level in the PBR not being increased high enough to match the growth rate which results in cell shading (Figure 2.7). This suggests that *Synechocystis sp*. PCC 6803 can tolerate light levels of > 675 $umol.m^{-2}/s$ after the culture surpasses 1.5 x 10^8 cells/ml. This indicates that faster growth rates could have been achieved with PBR 2 if the light was better optimised, which will be considered in future studies.

The highest growth rates in PBR2 occurred when the light sensor was reading values of 300-330 (Figure 2.7). A growth rate of 0.54 (μ/day) was also observed on day 7 in PBR 1, however, a negative growth rate was recorded after 4 days at

this light intensity. This suggests that the culture became over saturated with light resulting in photo-inhibition and damage to cellular structures. A light sensor reading of 401 AU was recorded at the end of the experiment in PBR 2, the culture was shown to be still growing at this light intensity however at a reduced rate (Figure 2.7). It can be summarised from this data that *Synechocystis sp.* PCC 6803 is at its most productive when the available light is within the 300-330 AU range and photo-inhibition begins after the light sensor reads approximately 400 AU+. Going forward the light tracking was set with a value of 320 which will be automatically maintained by the reactor as the culture grows. Theoretically this light tracking value should increase productivity as manually increasing light intensity can lead to poorly optimised growth as observed in (Figure 2.7), however future studies are needed to verify this.

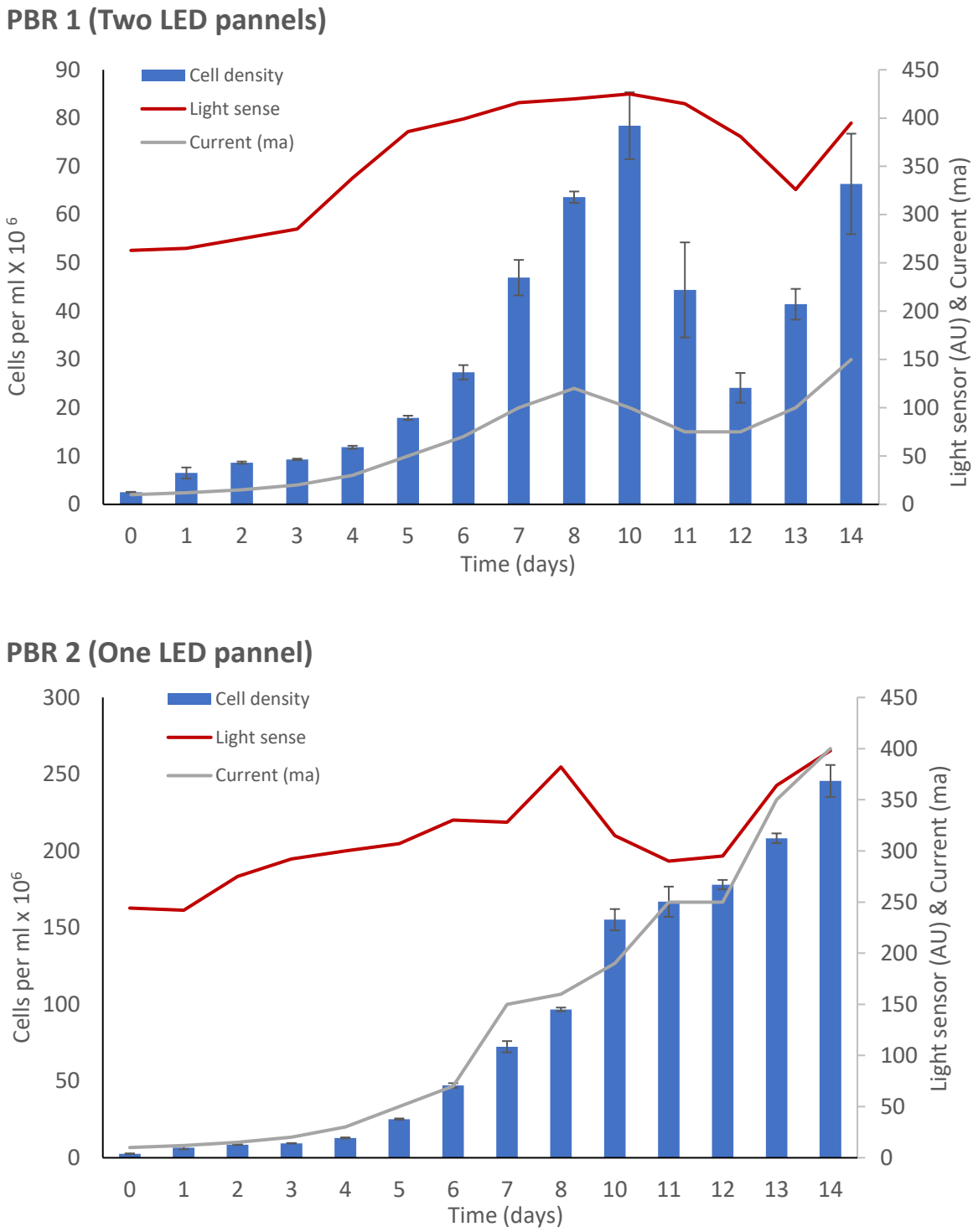


Figure 2.7. The growth of *Synechocystis sp.* PCC 6803 in PBR 1 and 2 over time. The errors bars are representative of standard deviation from the mean of technical samples (n=3). Data from the PBR's internal light sensor is plotted on the secondary y axis. A higher light sensor value (AU) correlates to higher available light within the reactor. The PBR was kept at a temperature of 21˚C and under constant illumination.

2.3.2 Evaluation of the performance of the Micro-Pharos photobioreactor

2.3.2.1 The growth of *Synechocystis sp.* PCC 6803, *Chlorella sorokiniana* and *Microcystis aeruginosa* PCC 7813 in the Micro-Pharos photobioreactor

Synechocystis sp. PCC 6803, *C. sorokiniana* and *M. aeruginosa* PCC 7813 were selected due to their known concentrations of the targeted pigment zeaxanthin. After prior screening of the culture collection. The light tracking value was set to 320 AU in all experiments and no manual adjustment of light intensities were made throughout.

Each of the organisms was successfully cultured in Micro-Pharos PBR and reached high cell densities. *Synechocystis sp.* PCC 6803 displayed the highest mean growth rate of 0.33 μ/day and reached a mean maximum cell density of 253 x 10^6 cells/ml. In comparison *Chlorella sorokiniana* and *Microcystis aeruginosa* PCC 7813 had mean growth rates of 0.25 μ/day and 0.27 μ/day, reaching cell densities of 94 x 10^6 cells/ml and 72.5 x 10^6 cells/ml, respectively. Despite each species being cultured in identical conditions and inoculated at the same density of 2.5 x 10^6 cells/ml from the same inoculum, there were differences observed between the reactors (Figure 2.8). The difference between the growth rate of *Synechocystis sp* and *Microcystis aeruginosa* in PBR 1 and PBR 2 were not statistically significant with p values of 0.38 and 0.08 respectively. A statistical difference, however, was noted when comparing the growth rate of Chlorella *sorokiniana* in each reactor with PBR 2 displaying a significantly slower growth rate ($P < 0.05$). P values were calculated via t test assuming unequal variances.

Synechocystis sp. PCC 6803 cultured in the Micro-Pharos without light tracking (section 2.3.1) achieved a mean growth rate of 0.28 μ/day and mean maximum cell density of 162 x 10^6 cells per ml compared to the 0.33 μ/day and 253 x 10^6 cells/ml that was achieved with light tracking. Light tracking, therefore, directly increased the productivity of *Synechocystis sp.* PCC 6803.

The trends observed for the dry weight of each the cultures are largely similar to the trends observed for cells/ml, however, the relationship between cells/ml and

dry weight was not entirely linear. *Synechocystis sp.* reached a maximum dry weight of 1.71 g/L and *C. sorokiniana* and *M. aeruginosa* PCC 7813 reached a maximum of 0.98 and 1.19 g/L respectively (Figure 2.9).

A recent study by Ajayan et al, (2019) tested the performance of 200 ml bench top PBR that was externally illuminated by LEDs. The study compared the growth of *Chlarmydomonas reinhardtii* under different wavelengths of light at a constant light intensity of 35 μmol/m^2/s for 14 days of growth. The culture grown under white light reached a concentration of 50 x 10^6 cells/ml after 14 days of growth which is significantly lower than the cell density achieved by the three organisms tested in this study. The study by Ajayan et al, (2019) however, observed a significant increase in cell density when *C. reinhardtii* was grown under blue light (77.5 x 10^6 cells/ml) which was lower that the cell density achieved by each organism in this study.

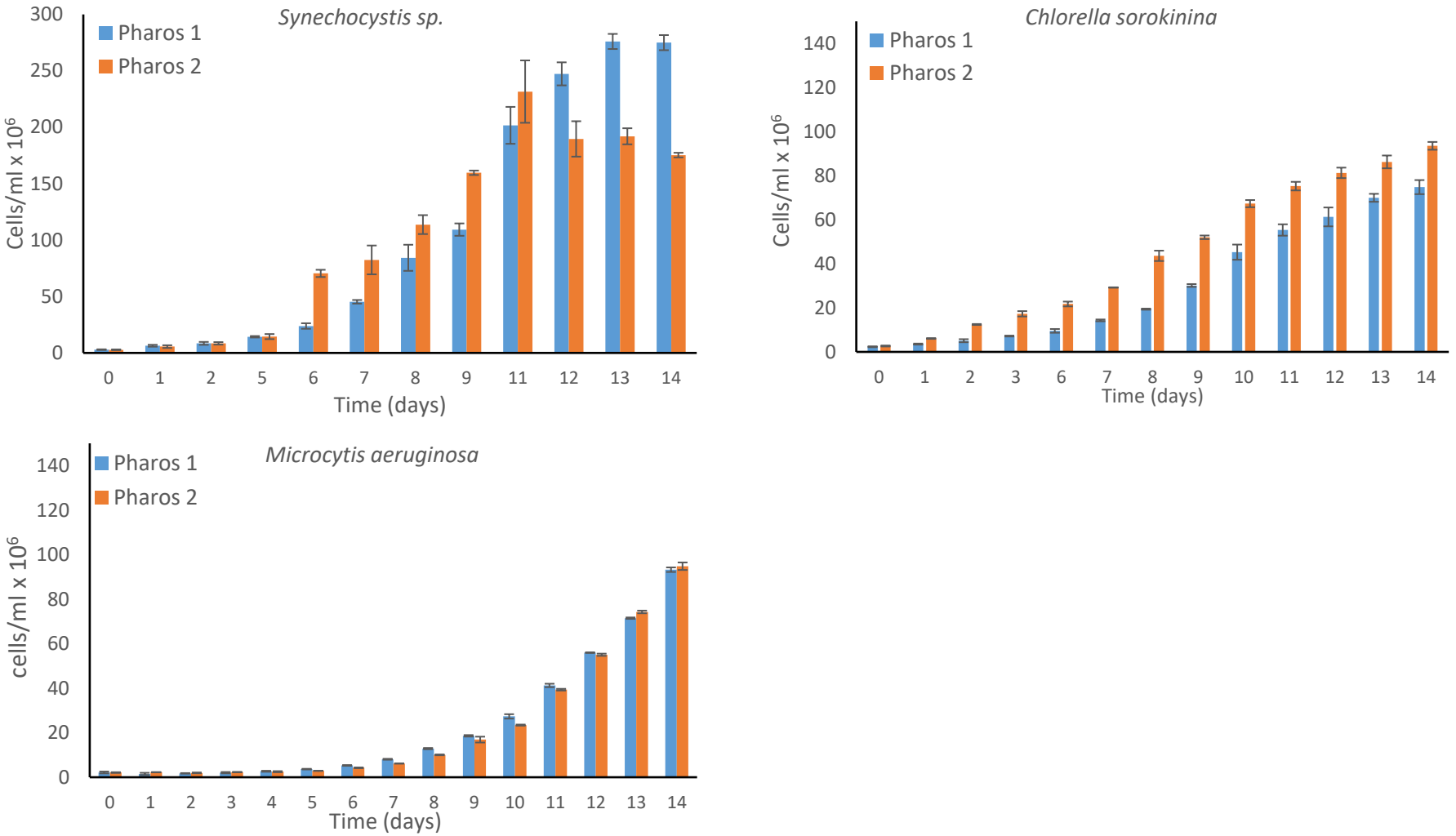


Figure 2.8. The number of cells per ml of each culture in each PBR over time. The error bars are representative of the standard deviation from the mean, n=3. The temperature was kept at a constant 21 °C throughout the experiment. The PBRs were sparged at a rate of 1.5 L/min and the light tracking value was set to 320 AU.

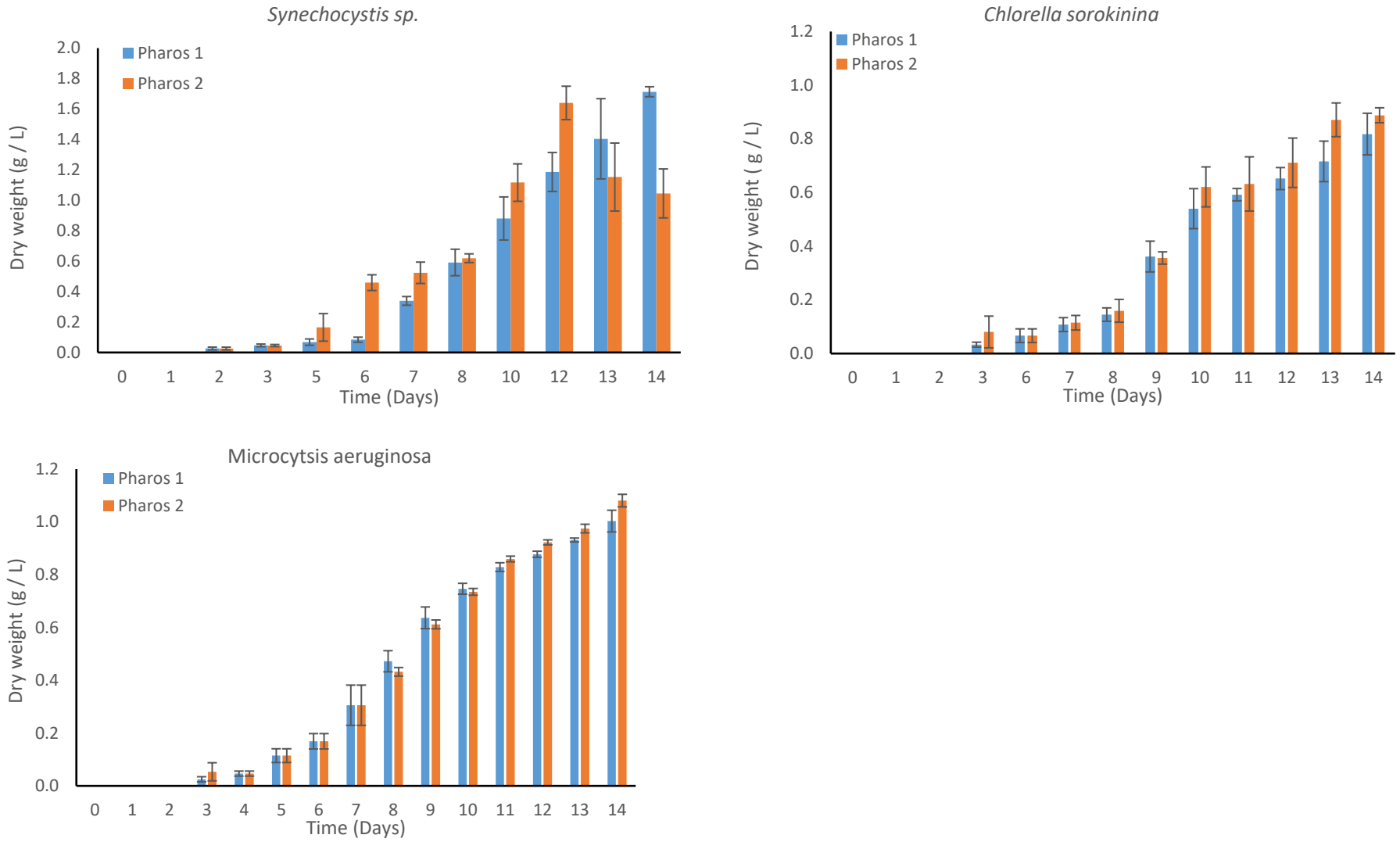


Figure 2.9. The dry weight of each culture in each PBR over time. The error bars are representative of the standard deviation from the mean, n=3. The temperature was kept at a constant 21 °C throughout the experiment. The PBRs were sparged at a rate of 1.5 L/min and the light tracking value was set to 320 AU.

BG-11 media used here contains approximately 750 mg / L of sodium nitrate ($NaNO_3$). The nitrate depletes steadily as the cultures grow (Figure 2.10) with an increased depletion rate after 8 days in *C. sorokiniana* and *M. aeruginosa*. After 15 days of growth the nitrate concentration of the *Chlorella sorokiniana* culture in PB2 was non-quantifiable. The LOQ of this method is approximately 0.5 mg/L. The nitrate concentration of the other species also reached concentrations of ~ 100 mg / L after 14 days (Figure 2.9). This suggests that the cultures were becoming nutrient depleted and the low nitrate concentration may be a limiting factor on continual growth.

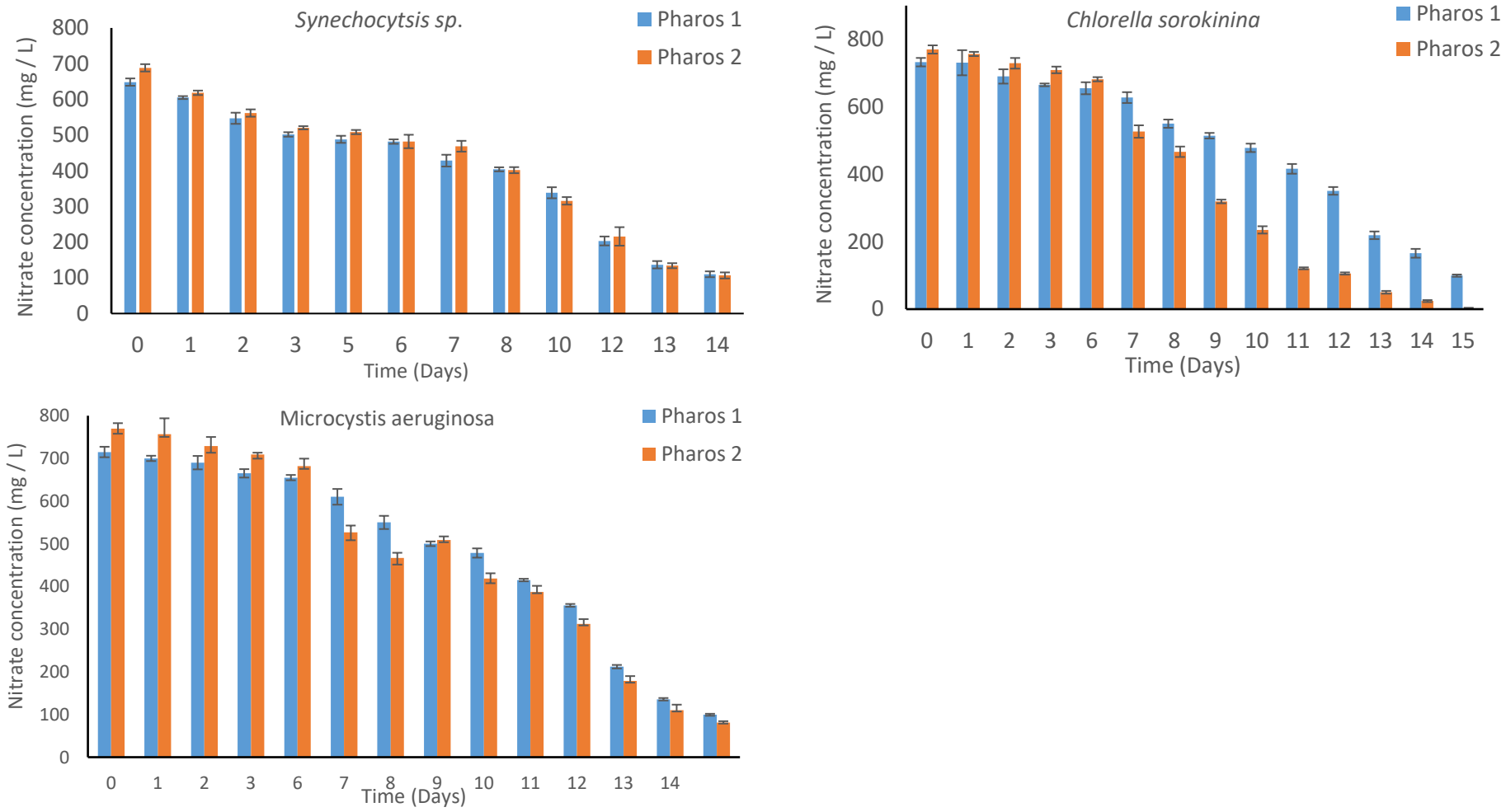


Figure 2.10. The nitrate concentration of each culture in each PBR over time. The error bars are representative of the standard deviation from the mean, n = 3. The temperature was kept at a constant 21 °C throughout the experiment. The PBRs were sparged at a rate of 1.5 L/min and the light tracking value was set to 320 AU

2.3.2.2 Light tracking performance

Evaluation of light tracking performance indicated that light tracking enhances the growth rate of *Synechocystis sp.* PCC 6803 (section 2.3.2.1). The light intensity gradually increased as the cells per number increases in each experiment. Although the relationship is not entirely linear there is a clear trend. This indicates that the light tracking feature is functioning well at maintain an available light level that facilitates a high growth rate.

Synechocystis sp. grew at a significantly faster rate than the other organisms that were tested. After 12 days of growth the current supplied to the LED panels reached 400 ma which supplies their maximum combined light intensity of approximately 1120 µmol/m^2/s. Due to their similar growth rates *C. sorokiniana* and *M. aeruginosa* PCC 7813 the light intensity also increased at a similar rate reaching a maximum of 852 µmol.m^2/s and 845 µmol/m^2/s, respectively (Figure 2.10).

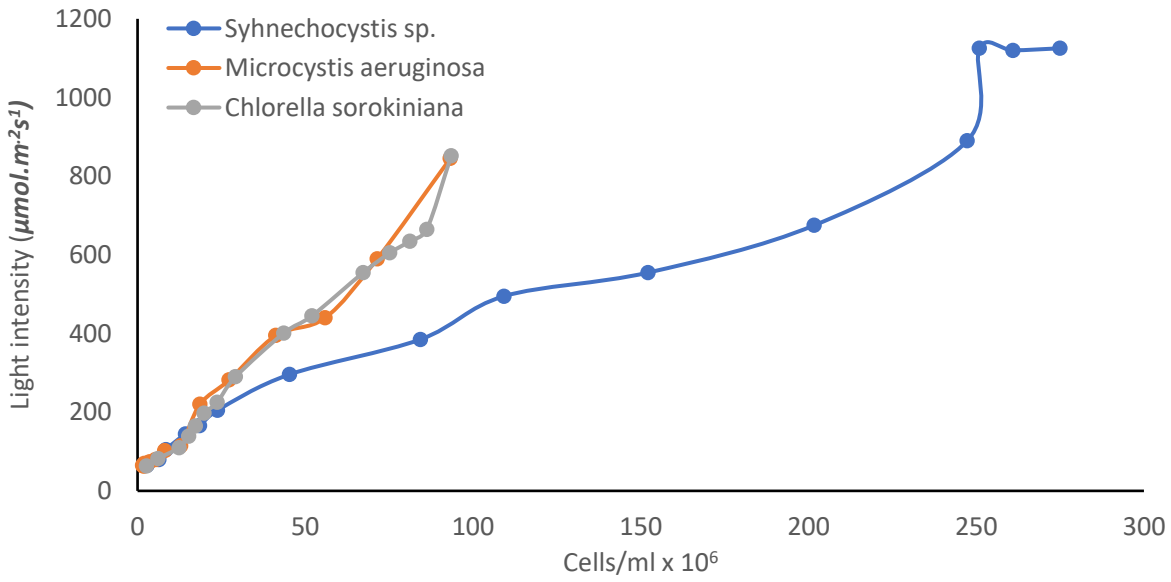


Figure 2.11. The relationship between light intensity and culture density at a light tracking value of 320 AU. Cells/ml is representative of the mean cell density of the PBRs. n=2.

2.3.2.3 The pH of the cultures throughout the investigation

The pH was not controlled throughout the Micro-Pharos experiments. The pH increased from pH 7 to > pH 9 in all experiments. The highest pH was observed in *Synechocystis sp*. cultures which is potentially a result in their high biomass concentration after 14 days of cultivation (Figure 2.11).

The performance could also be increased by aeration with an air / CO_2 mix rather than just air. Microalgal cultures tend towards alkalinity as they grow due to the fixing of CO_2 during photosynthesis leading to the production of HCO_3. The pH of the cultures reached pH values in excess of pH 9 towards the end of the 14-day cultivation period. It has been shown that high pH values can have an inhibitory effect on the growth of microalgae (De Farias Silva, Sforza and Bertucco, 2017).

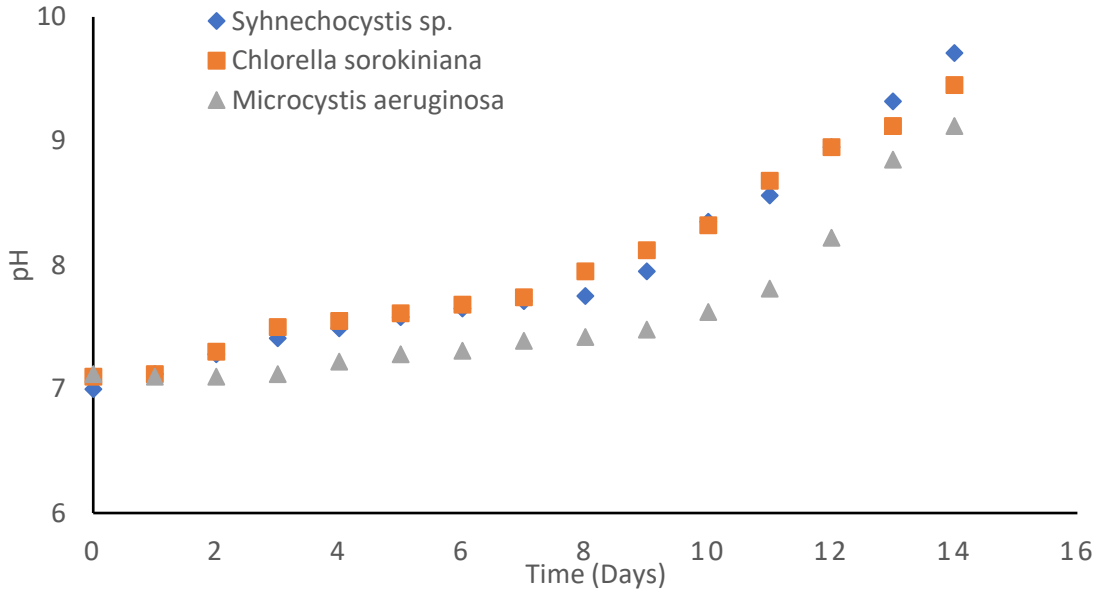


Figure 2.12. The increase in pH observed in the Micro-Pharos experiments overtime. The values are representative of the mean from both photobioreactors N = 2.

2.3.2.7 Evaluation of performance

The Micro-Pharos photobioreactor performed well with high cells densities being reached by each of the species that were cultivated. The light tracking value of 320 AU facilitated fast growth rates without the need for manual adjustments to be made to light intensity.

Synechocystis sp. PCC 6803 was the best performing organism in the Micro–Pharos PBR achieving the highest cell density (275 x 10^6 cells/ml) and biomass (1.73 g/L) after 14 days of cultivation. A study utilising a 10-litre flat panel PBR (achieved a doubling rate of 5.13 hours which is among the fastest ever recorded doubling rates for *Synechocystis sp*. PCC 6803. (Zavřel et al., 2015). In comparison the overall doubling rate achieved in this study was greater than 24 hours. The study utilised red LED's with a wavelength of 585–670 nm at intensities increased between 55 and 660 µmol/m^2/s. *Synechocystis sp.* PCC 6803 contains high concentrations of the blue pigments phycocyanin and allophycocyanin which absorb light in the 585-670 nm range (Zavřel et al., 2015). The use of tailoring LED wavelengths to fit the pigment profile of different organisms could therefore dramatically increase growth rates. The Micro–Pharos PBR could easily be modified to utilise LED panels that output different wavelengths of light.

In order to further increase the performance of the Micro-Pharos 1 litre PBR, the pH could be controlled. This could be achieved by sparging with an air / CO_2 mix rather than purely air. Additional CO_2 reduces the pH as it dissociates in water which forms a free H^+ ion as well as a $HCO3^-$ ion which results in a net pH reduction (Goldman, Dennett and Riley, 1982; De Farias Silva, Sforza and Bertucco, 2017).

The performance could further be enhanced by increasing the temperature to 30 -35°C which is the optimal temperature range for *Synechocystis sp.* PCC 6803 and many other species of microalgae (Zavřel *et al.*, 2015). The Micro-Pharos reactor has the ability to maintain this temperature range however significant evaporation was observed with a total volume loss of 180-200 ml observed in preliminary testing at 21˚C. Increasing temperature to 30°C, therefore, would have likely exacerbated this issue. Evaporation can also be limited by using

humidified air. Higher temperatures are also more viable in photobioreactors with larger operational volumes as evaporation has less impact on biomass yield.

The limiting factor in many photobioreactor designs is photo limitation due to self-shading. Photo-limitation, however, is likely less to be a factor during the experiments with the Micro-Pharos PBR as the light intensity did not reach its maximum after 14 days of cultivation. The light tracking functionality also helps to reduce self-shading, however if the LED panels reach their maximum intensity it could be a factor.

In this investigation the limiting factor on growth is potentially nitrate depletion (Figure 2.10) or increasing pH (Figure 2.12). The BG-11 media could be supplemented with additional nitrate or other nutrient sources such as urea to further enhance biomass production.

Overall the PBR performs well as a bench top photobioreactor providing the user with excellent control over the light regime. Improvements could be made to the mixing by using a different sparger shape that disperses air into the corners. The corners of the reactor tend to become dead zones with significant biomass build observed during the later stages of cultivation. The reactor design could also benefit from the use of fluid modelling software. The light tracking capability and high-power LEDs ensure that high light intensities can be maintained throughout the entire cultivation period. It also provides the ability to maintain light stressing conditions without causing photo-inhibition. This is favourable for the production of certain light protective pigments (Dautermann and Lohr, 2017).

2.3.3 Pigment extraction: a comparison of solvents

The pigments were identified in each extraction by comparing the mass and retention times with the pigment standards (Figure 2.13). The mean concentration of echinenone and β–carotene was significantly higher in the extractions that used acetone when compared to the other solvents (Figure 2.13).

There was little difference observed between the same solvents at 90% strength with the exception that β–carotene was not detected when extracted with 90 % methanol (Figure 2.13). A previous study also reported that there was significant increase in pigment recovery when echinenone was extracted using acetone when compared to extraction using methanol (Hagerthey et al. 2006).

The method could further be enhanced with the addition of sonication. Sonication mechanically agitates the cells using high frequency sound waves while they are suspended in a solvent. This has been shown to increase the extraction efficiency and reduce extraction time. Utilising freeze drying instead of freezing has also been shown to increase the extraction efficiency of hydrophobic pigments such as β–carotene (Sartory & Grobblear, 1984). An alternative to the additional solvent extraction method is supercritical fluid extraction using CO_2. A study reported a total carotenoid yield of 1.5 mg/ml using supercritical carbon dioxide extraction. In comparison the same study reported a yield of 1.35 when using traditional solvent extraction (Montero et al. 2005). Due to the results of this study all future extractions were carried out using Acetone.

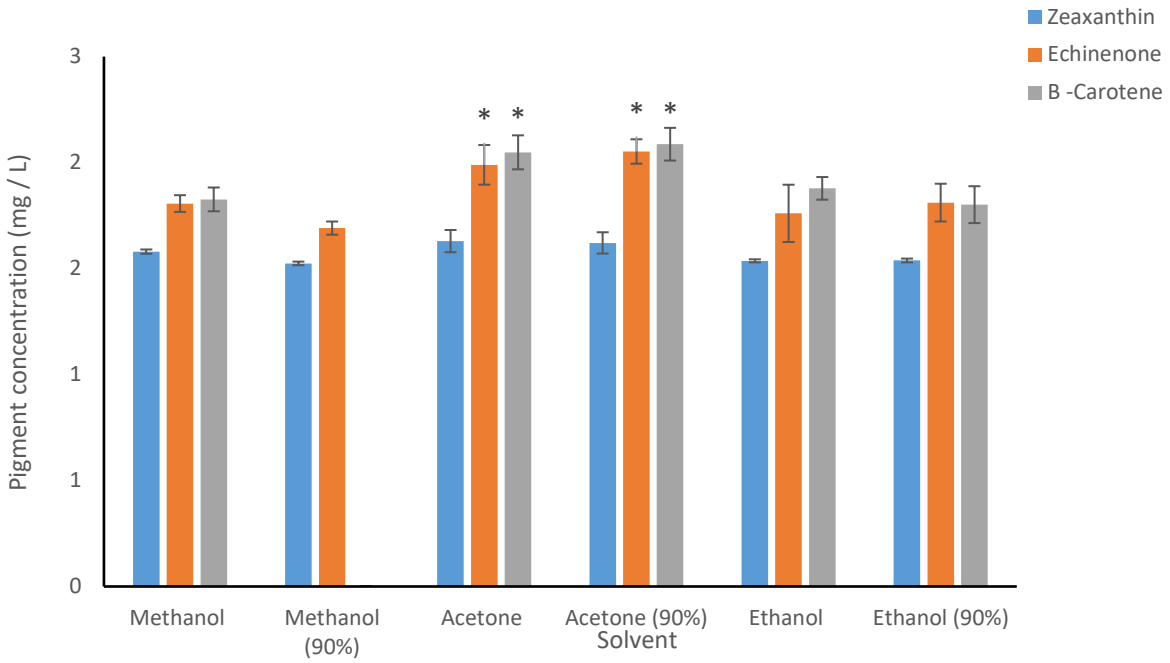


Figure 2.13. The mean concentration of pigments identified in a 4-week-old culture of *Synechocystis sp*, extracted with different solvents. The culture was incubated in a growth chamber at a constant temperature of 21°C and a light intensity of 10 µmol.m^{-2}s^{1}, with a light / dark cycle of 12 hours. The error bars are representative of the standard deviation from the mean. * indicates $p < 0.05$ N = 3.

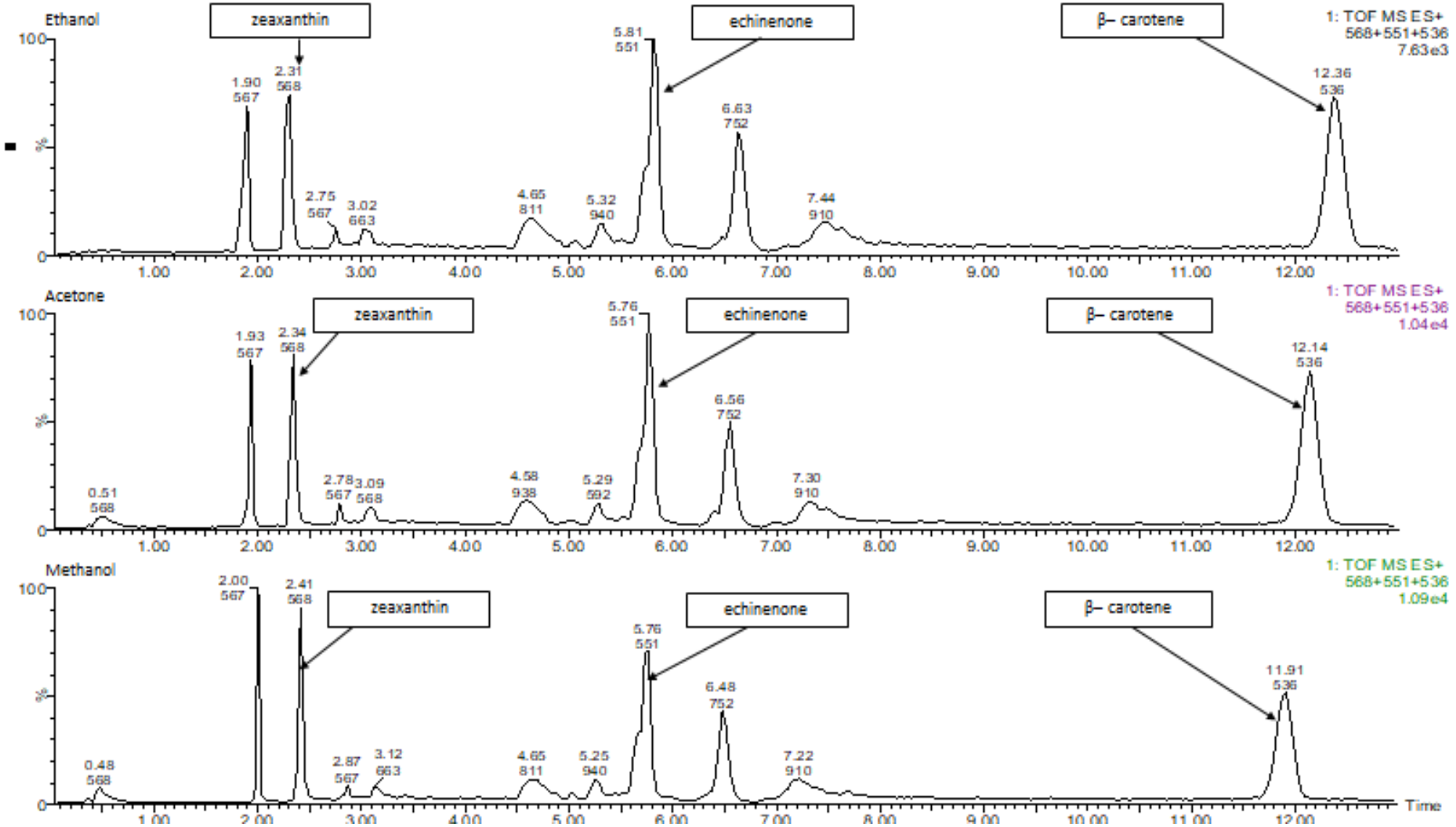


Figure 2.14. Chromatograms of extractions using each solvent. Identifiable peaks have been annotated.

2.3.4 The identification of pigments in *Synechocystis sp.* PCC 6803

The xanthophyll pigments zeaxanthin, echinenone and β–carotene were successfully identified in a 3-week-old culture of *Synechocystis sp.* PCC 6803. The culture was grown in conditions with a relativity low light intensity of 10 μmol/m^2/s (Fig. 2.15 - 2.19). The pigment profile of *Synechocystis* sp. has been well characterised in other studies, with the predominant carotenoids being identified as zeaxanthin, β–carotene and echinenone (Steiger, Schäfer and Sandmann, 1999; Lagarde, Beuf and Vermaas, 2000). Zeaxanthin was also successfully identified in *C. sorokiniana* and *M. aeruginosa*. Zeaxanthin and echinenone are known to be photo protective pigments therefore culture conditions using higher light intensities may upregulate their production in *Synechocystis sp.* PCC 6803 as well as other species.

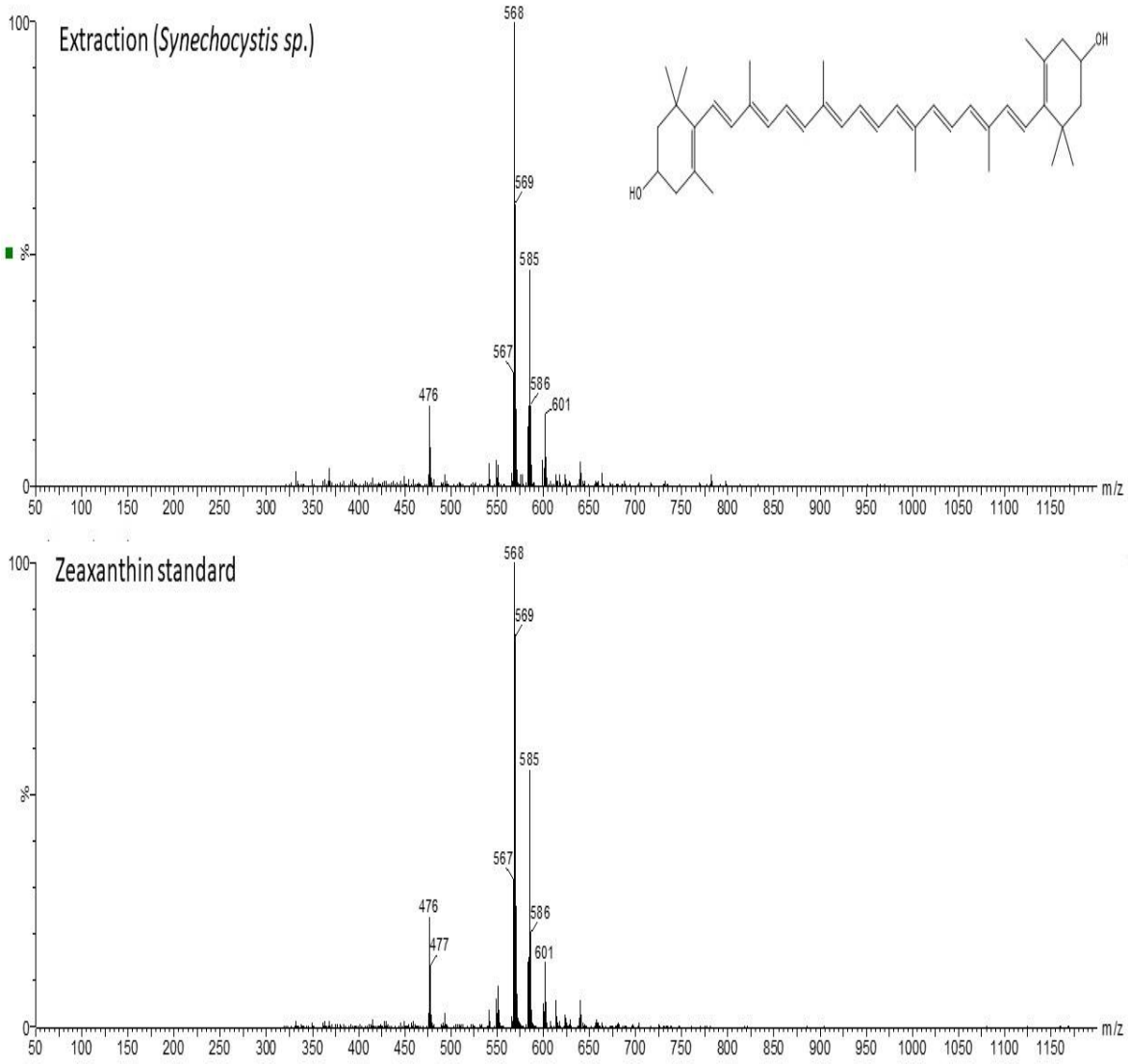


Figure 2.15. Comparison of the mass profiles of the zeaxanthin detected in a acetone extraction of *Synechocystis sp.* PCC6803 (top) and the zeaxanthin standard (bottom).

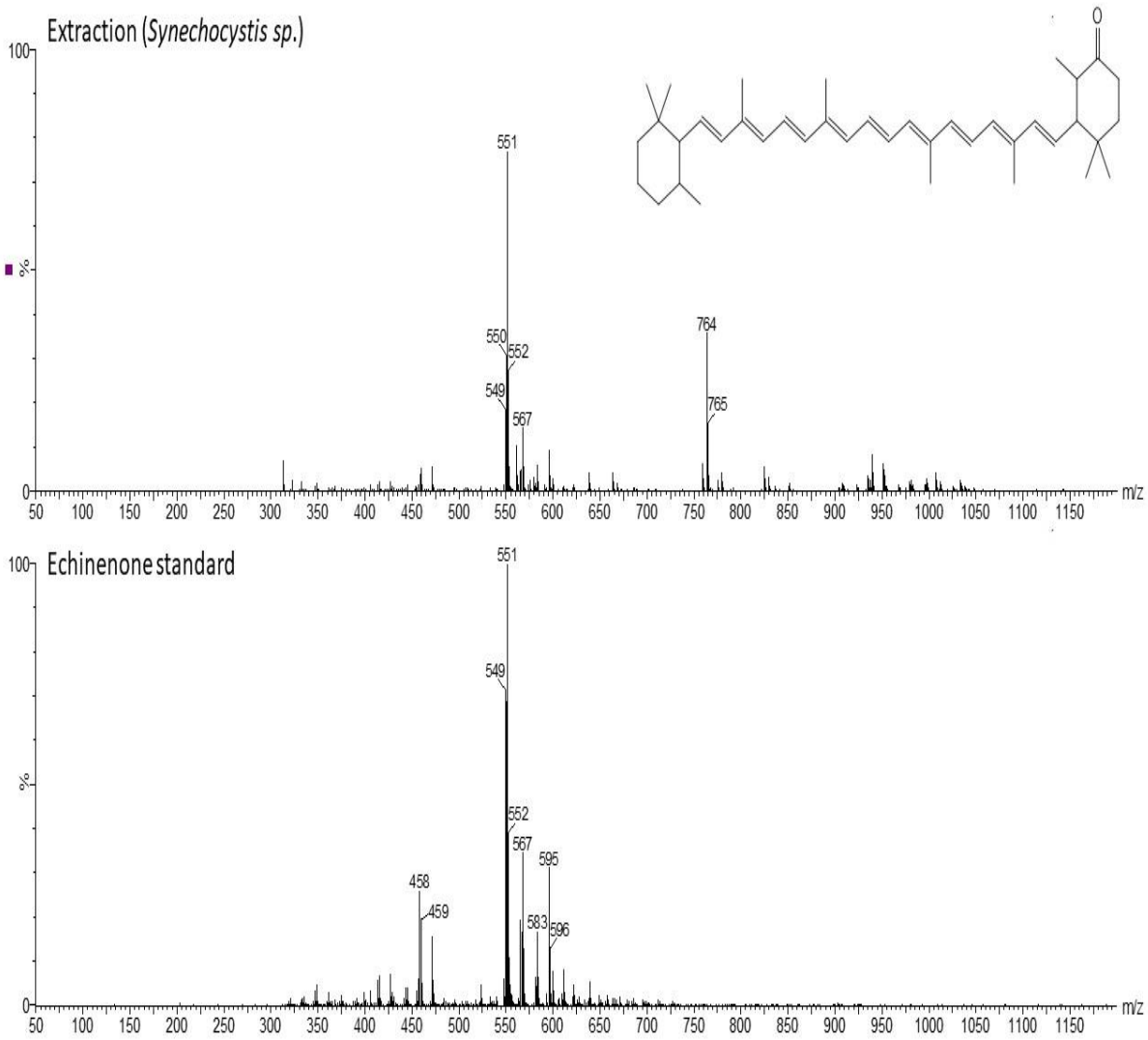


Figure 2.16. Comparison of the mass profiles of the echinenone detected in a acetone extraction of *Synechocystis sp.* PCC6803 (top) and the echinenone standard (bottom).

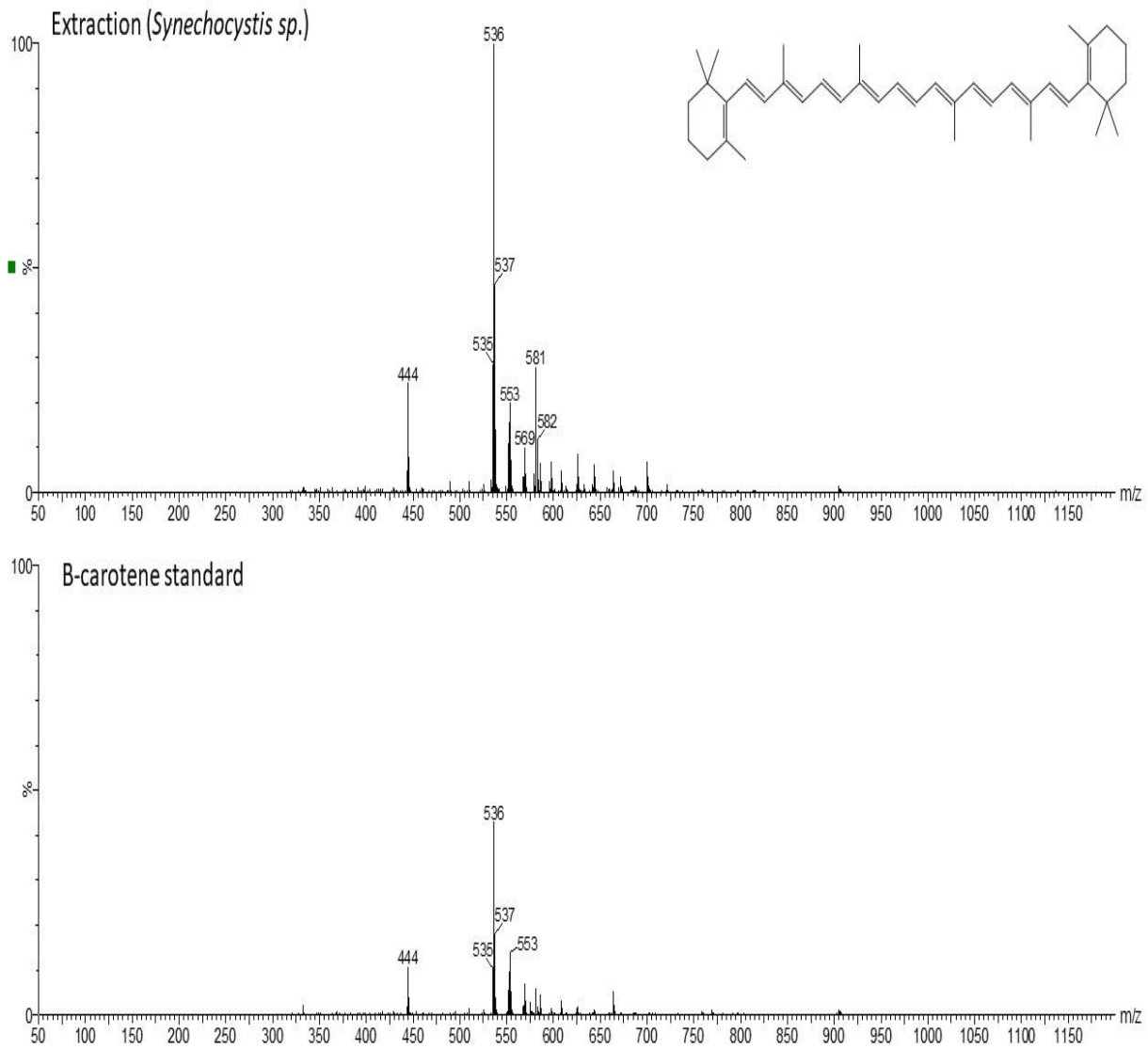


Figure 2.17. Comparison of the mass profiles of the β – Carotene detected in an acetone extraction of *Synechocystis sp.* PCC6803 (top) and the β – Carotene standard (bottom).

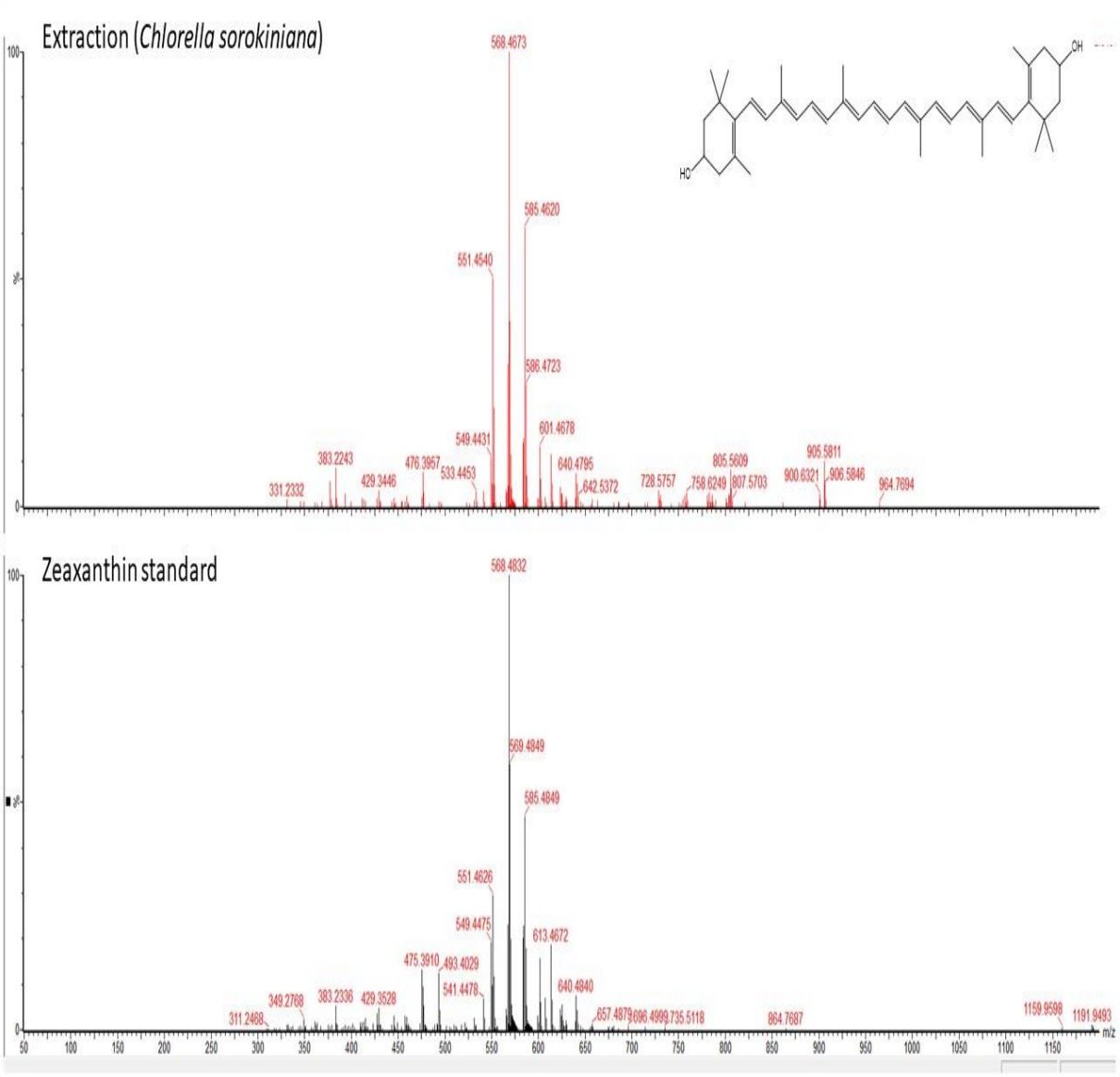


Figure 2.18. Comparison of the mass spectra of a Zeaxanthin standard (top) and the Zeaxanthin present in an extraction of *Chlorella sorokiniana* (bottom).

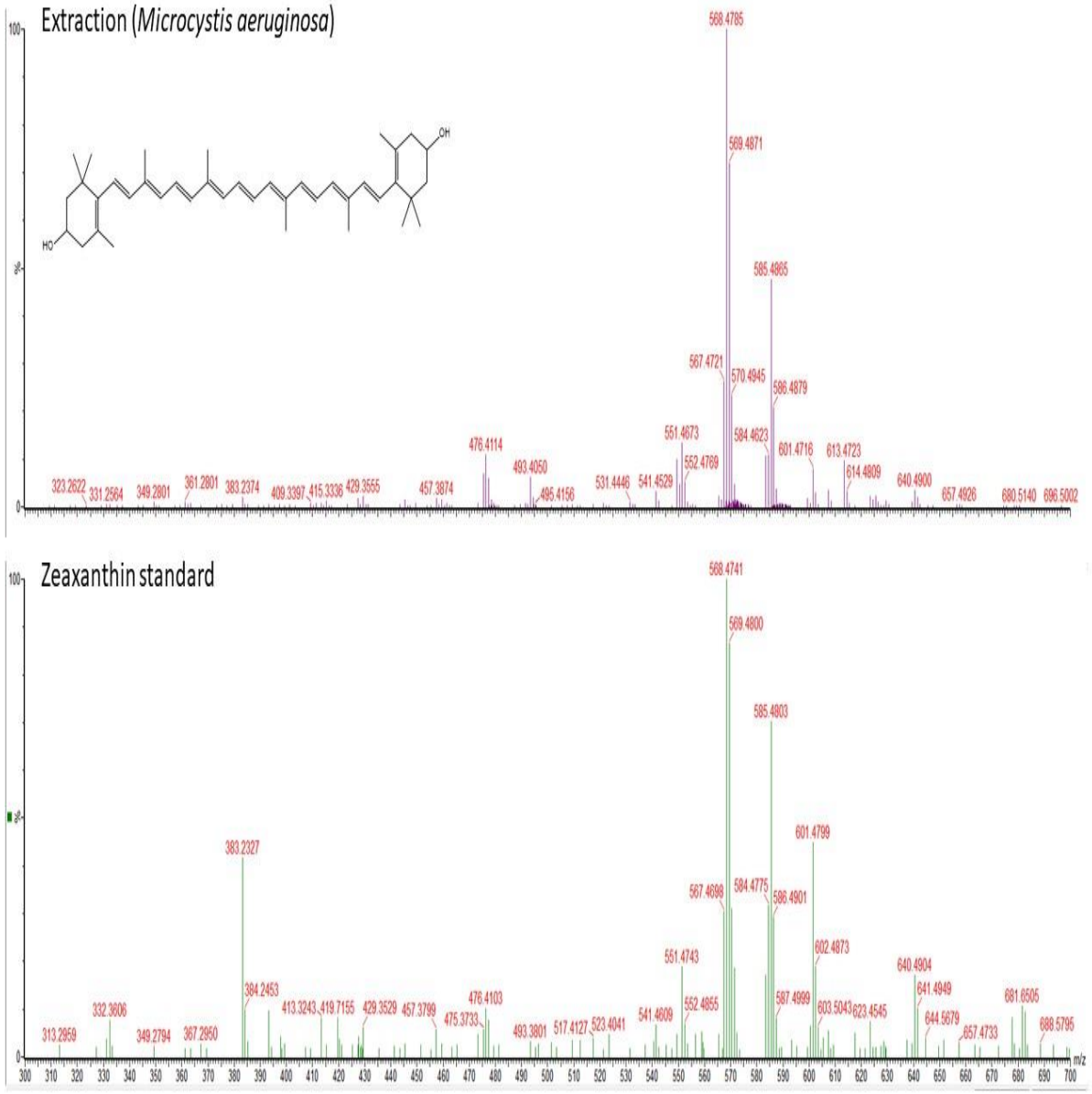


Figure 2.19. Comparison of the mass spectra of a zeaxanthin standard (top) and the zeaxanthin present in an extraction of *Microcystis aeruginosa PCC 7813*(bottom).

2.3.5 The effect of light intensity on the pigment profile of *Synechocystis sp*. PCC 6803

It was previously observed that light tracking increased the growth rate of *Synechocystis sp.* in the Micro-Pharos PBR (section 2.3.2.3). In this investigation it was determined that light tracking also had a significant effect on pigment production of *Synechocystis sp*.

It was observed that light tracking growth conditions resulted in a significant reduction in β – carotene concentration when compared to lower light conditions, after 12 days of growth (Figure 2.21, Figure 2.22). The concentrations of zeaxanthin and echinenone were also significantly increased, after 12 days of growth when Synechocystis sp PCC 6803 was cultured in light tracking conditions (Figure 2.21, Figure 2.22). The difference in cell density the between light tracking and low light cultures will account for some of this increase. However, the pigment production rates for zeaxanthin and echinenone in light tracking conditions are higher than their production rates in low light conditions. The inverse is true for β–Carotene which has a higher production rate in lower light conditions (Table 2.7).

β–Carotene is an accessory pigment which can absorb light energy at wavelengths outside the range of chlorophyll (Lagarde et al., 2000).Similarly, to xanthophylls β–Carotene also has a photo protective function and is involved in the quenching of singlet oxygen free radicals. In *Synechocystis sp*. the xanthophylls zeaxanthin and echinenone are synthesised from β – Carotene by the enzymes β–Carotene hydroxylase (*crtR*) and β – carotene ketolase (*crtO*), respectively (Figure 2.19). The results of this experiment suggest that there is light dependent up-regulation of these enzymes taking place which leads to the observed significant increase in zeaxanthin and echinenone concentrations. Although further experimentation would be required to confirm this hypothesis.

A study by *Steiger* et al, (1999) observed a decrease in zeaxanthin and β - carotene in *Synechocystis sp*. when grown under high light. Similar, to the results presented here echinenone was shown to be upregulated under high light intensities. However, the experiment only allowed for 30 hours of cultivation time

and the decrease in concentration observed was only significant at one time point.

The growth conditions especially with respect to light intensity were also different with *Synechocystis sp* grown at two fixed light intensities, low light (35 μmol/m^2/s) and high light (550 μmol/m^2/s) (Steiger et al., 1999). It is possible that photo–inhibition occurred due to the exposure of low-density cultures to relatively high light intensities. Light tracking technology circumvents this issue by gradually increasing light intensities as the culture grows.

Table 2.7. The pigment production rate for each PBR in low light and light tracking conditions

	Zeaxanthin (μ. /day)	Echinenone (μ. /day)	β – Carotene (μ/day)
PBR 1 (10 μmol/m^2/s)	0.0732	0.1434	0.1611
PBR 1 (Light tracking)	0.1194	0.1960	0.0560
PBR 2 (10 μmol/m^2/s)	0.0051	0.0392	0.2073
PBR 2 (Light tracking)	0.1628	0.1070	0.1305

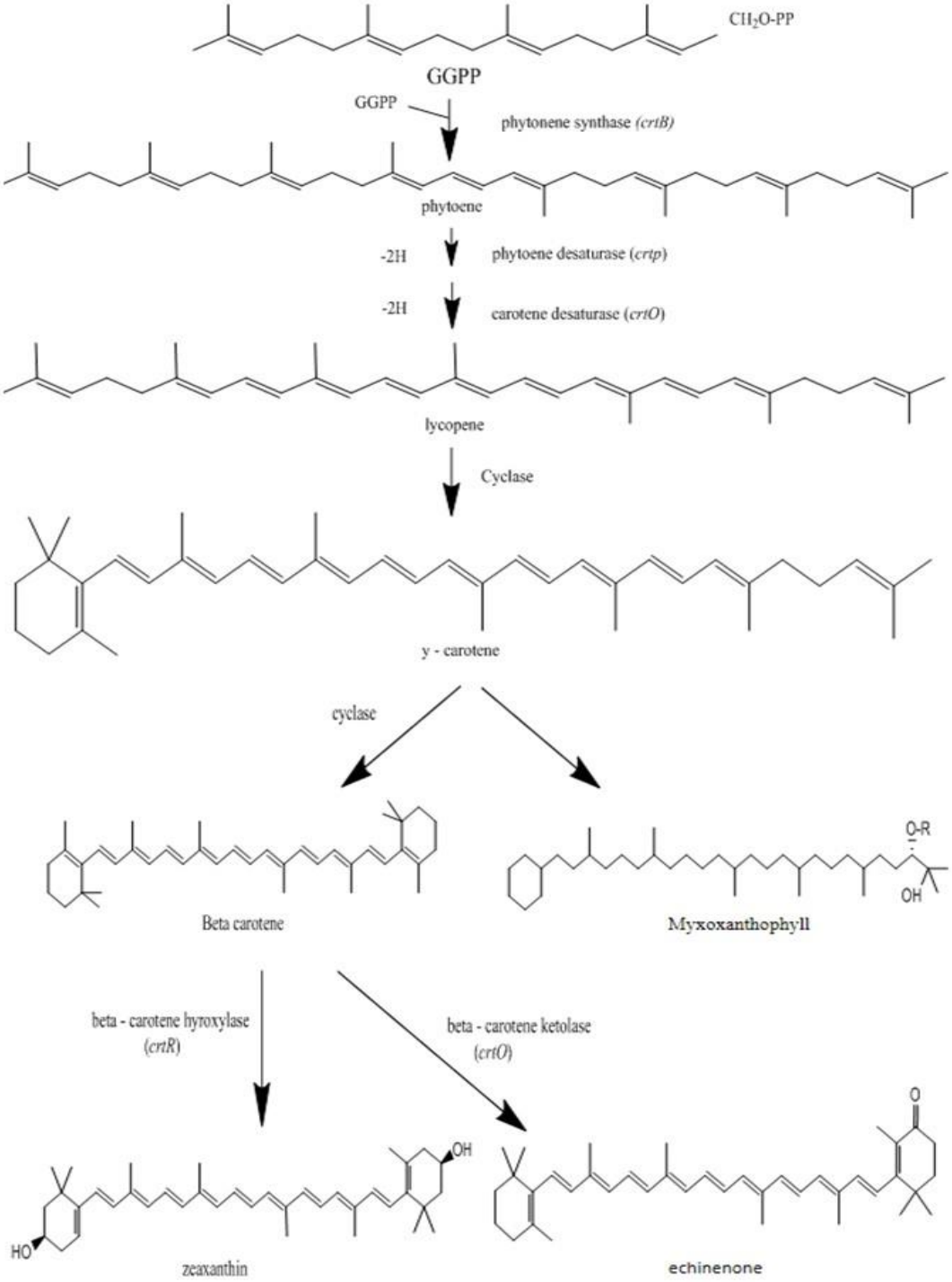


Figure 2.20. The synthesis pathway of carotenoid pigments in *Synechocystis sp* PCC 6803**.**

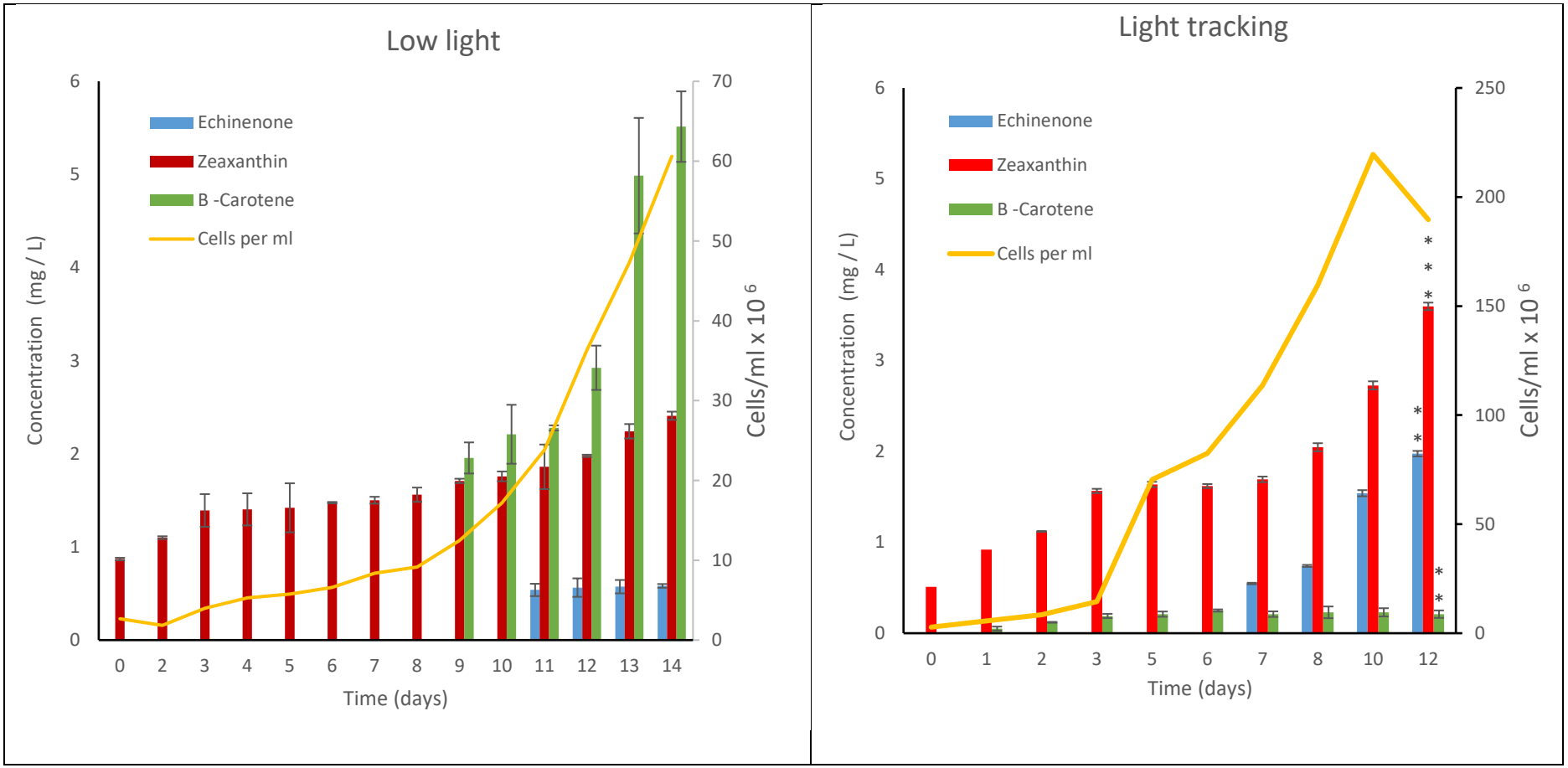


Figure 2.21. The pigment profile of *Synechocystis sp.* PCC 6803 in low light conditions compared to high light intensity conditions in PBR 1. The low light cultures were grown in a 1 litre photobioreactor at a constant light intensity of 10 µmol.m^2/s. The light / dark cycle was set to 12 hours. The high light conditions were cultured in a 1 litre light tracking PBR. All cultures were grown at 21 °C. P value < 0.01 (* *), P value < 0.001 (***). N =3

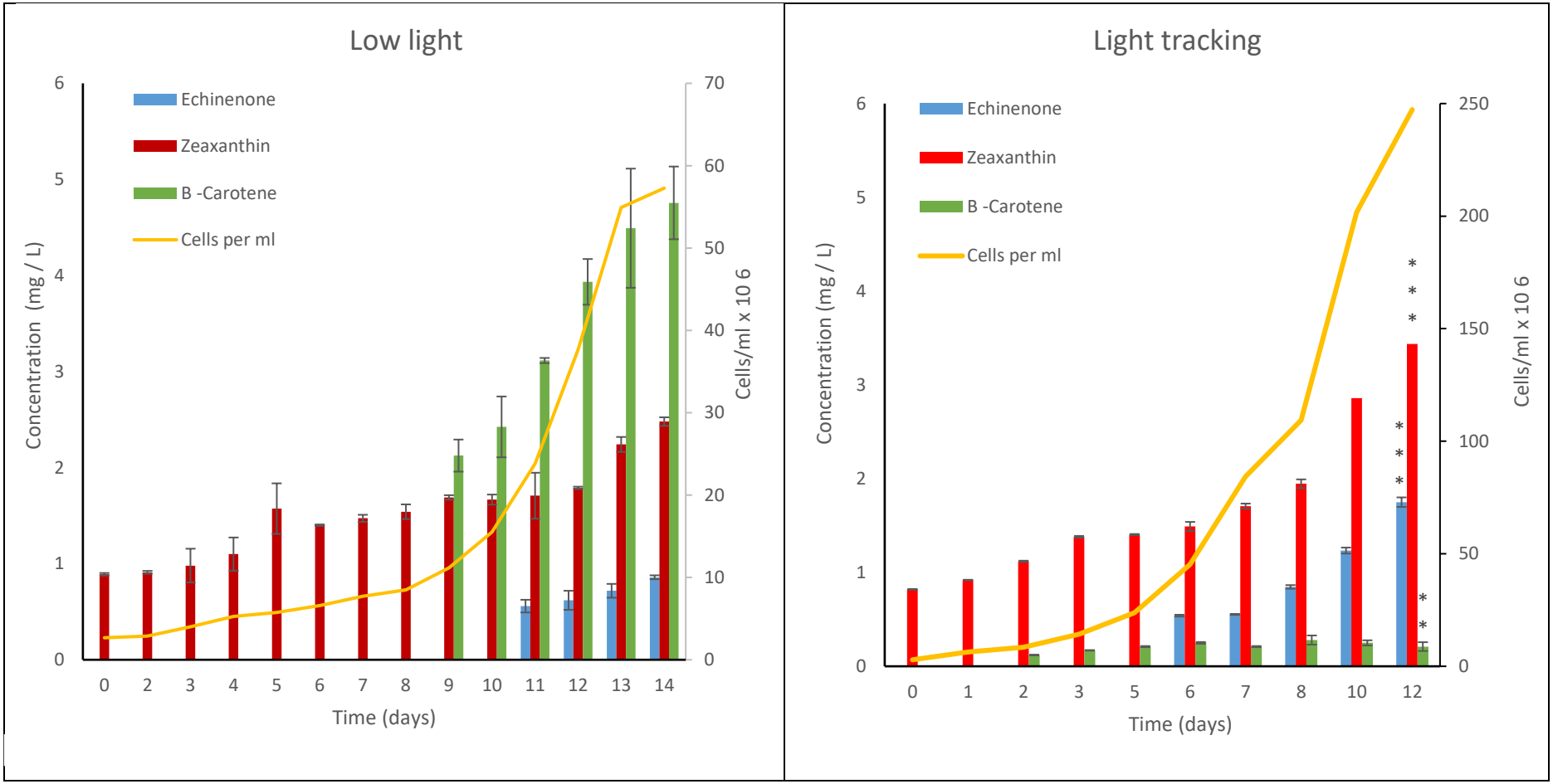


Figure 2.22. The pigment profile of *Synechocystis sp.* PCC 6803 in low light conditions compared to high light intensity conditions in PBR 2. The low light cultures were grown in a 1 litre photobioreactor at a constant light intensity of 10 µmol.m^2/s. The light/dark cycle was set to 12 hours. The high light conditions were cultured in a 1 litre light tracking PBR. All cultures were grown at 21 °C. P value < 0.01 (* *), P value < 0.001 (***). N =3

2.3.6 The production of high value pigments using the Micro–Pharos PBR

C. sorokiniana has the highest concentration of zeaxanthin (4.58 mg/L) after 14 days of cultivation when compared to *Synechocystis* (3.25 mg/L) and *M. aeruginosa PCC 7813* (1.99 mg/L). The lowest density was also observed in *Chlorella sorokiniana*. If the productivity of *C. sorokiniana* was further enhanced, it could prove to be a good candidate for the scale up of zeaxanthin production (Table 2.8).

Table 2.8. Comparison of the productivity and pigment production of different species in the Micro–Pharos PBR. n = 2. n/d = not detected

	Synechocystis	*Chlorella*	*Microcystis*
Mean Cells/ml x 10^6	275.00	93.50	94.80
Dry weight (g/L)	1.71	0.96	1.19
Zeaxanthin *concentration* (mg /L)	3.25	4.58	1.99
Echinone concentration (mg /L)	1.4	n/d	n/d
*Zeaxanthin production rate (μ/*day*)*	0.09	0.115	0.06

Another *Chlorella* species *Chlorella ellipsoidea* was shown to produce zeaxanthin with a yield of 4.26 mg/L (Cezare-gomes et al., 2019). This is comparable to the yield of 4.58 mg/L that was achieved in this study with *Chlorella sorokiniana.* The study with *C. ellipsoidea* utilised an advanced extraction technique known as pressurised liquid extraction (PLE) to extract zeaxanthin from dried *C. ellipsoidea* powder. PLE uses pressurised vessels and heat (> 100°C) to extract metabolites from samples suspended in a solvent (Koo et al., 2012). The study by Koo et al, (2012) tested the extraction efficiency of zeaxanthin when using hexane, isopropanol and ethanol at range of temperatures (60 - 156°C). The highest extraction efficiency of 4.58 mg/L was achieved when using ethanol at a temperature of 115°C.

If advanced LED photobioreactors and sophisticated extraction techniques could be combined cost effectively then the yield of carotenoids and other high value metabolites could be increased.

Table 2.9. Zeaxanthin production by non-modified naturally zeaxanthin producing micro-organisms (Zhang et al., 2018).

	Strain	Cultivation method and nutrients	Yield
Bacteria			
	Flavobacterium multivorum ATCC 5528	30 °C, Shake flask (50 rpm)	10.65 mg/L
	Mesoflavibacter zeaxanthinifaciens TD-ZX30^{T}	Marine agar, 30 °C,48 hr	910 (µg g^{-1})
	Paracoccus zeaxanthinifaciens	30 °C, 72h	11.63 mg l^{-1}
	Muricauda flavescens JCM 1182 ^{T}	Marine broth 32 °C, 72 h	4.4 mg/L
	Muricauda lutanensis CC – HSB-11^{T}	Bioreactor, 40 °C, 150 rpm, pH 7.4, 72 h	3.12 mg/L
Microalgae			
	Dunaliella salina (mutant)	28 °C, under low light	6 mg/L
	Synechococcus sp. (PCC 7942)	BG-11 with 30 µg ml^{-1}	1.7 mg/L
	Chlorella saccharophila	20 °C, pH 6.5, 8 days	11.2 mg/L
	Chlorella pyrenoidosa	N. A	2170 mg/L

Genetic modification can also be utilised to increase zeaxanthin production in a variety of organisms. A mutant of *Dunaliella salina* was shown to reach a zeaxanthin concentration of 6 mg per g of dry weight which was substantially higher than the wild type (0.2 mg/g) (Cezare-gomes et al., 2019).

2.3.7 The performance of the Cyclops photobioreactor

Due to its high zeaxanthin content *C. sorokiniana* was selected as a good candidate for scale up going forward. In comparison to the Micro – Pharos PBR the growth rate observed in the 16 litre Cyclops was significantly lower when grown in similar culture conditions i.e. temperature (21 °C), sparging rate (1.5 L/min) and light tracking value. When grown using a light tracking value of 320 AU there was a noticeable lag phase of 48 hours where there was extremely limited growth. The doubling rate was approximately 5.5 μ/day. The culture then reached its peak of 6 x 10^6 cells per after 8 days and then began to decline (Figure 2.22). This impaired growth suggests that there may be a problem with the light regime and either photo-inhibition or photo-limitation is taking place. The starting light intensity was 140 μmol/m^2/s which is significantly higher than the 20 μmol/m^2/s in the Micro–Pharos experiments, although it dispersed through a larger reactor volume.

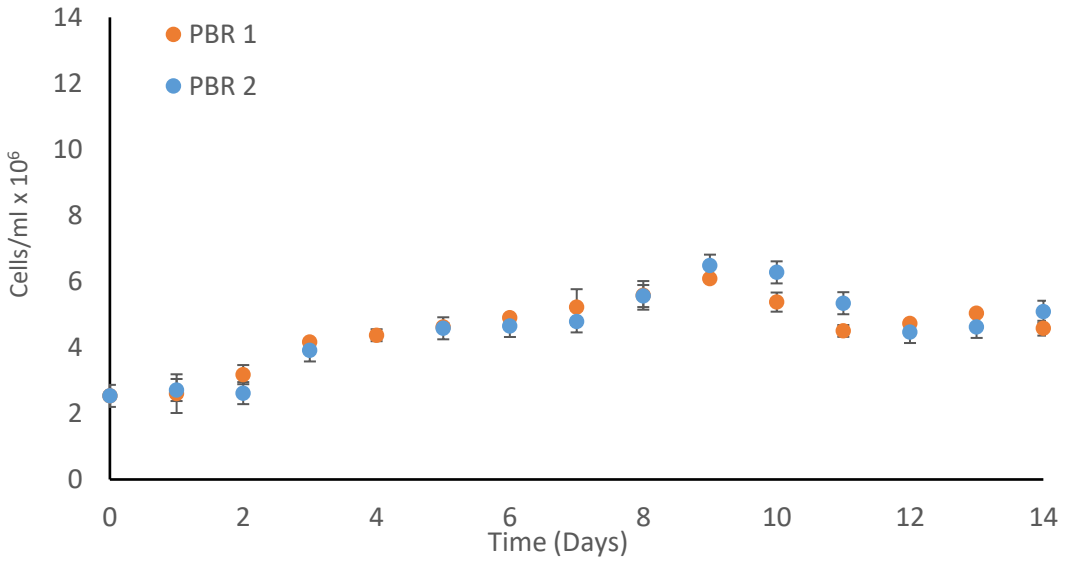


Figure 2.23. The growth of *Synechocystis sp*. in the Cyclops 16 litre photobioreactor. The error bars are representative of the standard deviation from the mean. The PBR was mixed using air at a flow rate of 1.5 L / min. The light tracking was enabled and set to a value of 320 AU. n = 3

Table 2.10. Growth rate and light sensor values for each day of the light tracking experiment with the Cyclops PBR. The light sensor measures light in arbitrary units (AU).

Time (Days)	Growth rate (μ/day)	Light sense (AU)	Growth rate (μ/day)	Light sense (AU)
1	0.058	309	0.008	309
2	0.170	310	0.116	311
3	0.058	310	0.097	317
4	0.016	311	0.089	316
5	0.013	305	0.061	319
6	0.141	315	0.074	312
7	0.060	318	0.074	310
8	-0.021	320	0.063	314
9	-0.033	320	0.052	315
10	-0.059	325	0.039	310
11	0.028	305	0.046	316
12	-0.005	305	0.041	314
13	-0.014	307	0.040	310
14	-0.098	305	0.036	312

In the next experiment the inoculation volume was increased to 5.5 x 10^6 cells/ml and light tracking was disabled, in order to reduce photo–inhibition. However, no significant increase in growth rate was observed and the growth rate stagnated after 8 days of cultivation (Table 2.10). The stagnation of growth suggests that the *Chlorella* are photo limited, i.e. not receiving enough light to grow. The reactor vessel is made from PVC plastic and blocks light from the surrounding environment (Figure 2.4) which in theory allows for short light / dark cycles within the reactor. Short light / dark cycles have previously been shown to improve photosynthetic efficiency in several reactor designs (Abu-Ghosh et al., 2016; Cheng et al., 2018). If the dark zone, however, is too large and the residency time of the cells in the illuminated area is not long enough then photo-limitation could occur.

In a previous study with an early version of the Cyclops PBR, the reactor used a transparent Perspex outer reactor vessel rather than a closed one utilised in this study. In this study the productivity of the reactor was significantly higher. Although the study used *Desmodosmus subspicatus* and further enhancements

were made to the PBR, which included a heating coil and an additional power supply that could boost the current supplied to the light unit. The study achieved a concentration of approximately 16.5 x 10^6 cells/ml after 8 days of growth which is significantly higher than the maximum density observed in this study (Figure 2.23) (McNerney et al., 2015). This suggests that reducing the inbuilt dark zones within the reactor may increase the productivity of *C. sorokiniana.*

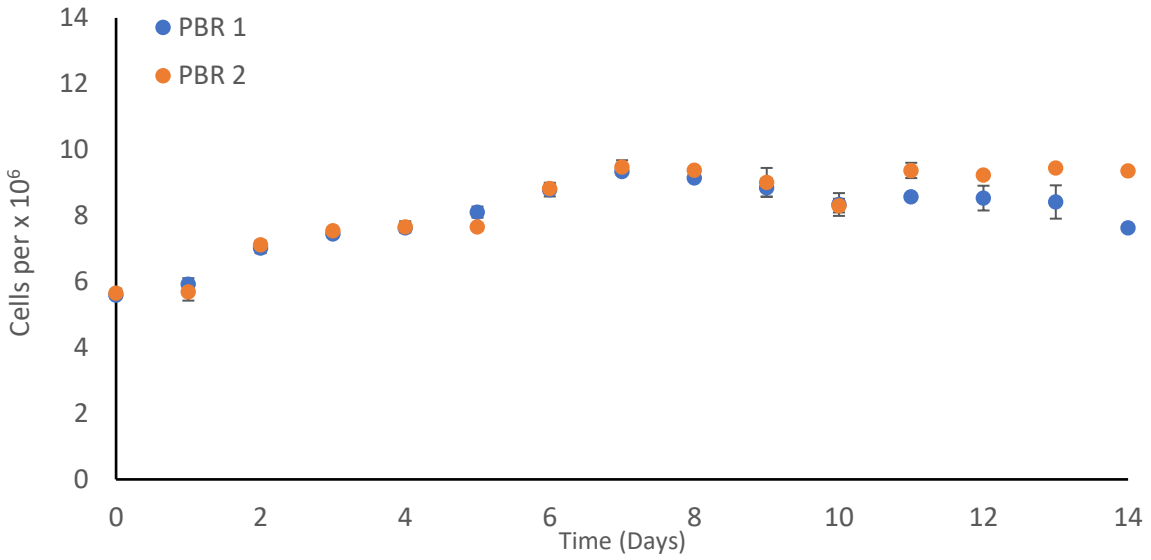


Figure 2.24. The growth of *C. sorokiniana* PCC 6803 in the Cyclops 16 litre photobioreactor. The error bars are representative of the standard deviation from the mean. The PBR was mixed using air at a flow rate of 1.5 L/min. Temperature was 21°C. n=3

To investigate the impact of external additional light the Cyclops PBR was modified to have a transparent Perspex outer vessel. The maximum cell density achieved was 14.5 x 10^6 cells/ml after 14 days of growth (Figure 2.24) which was significantly higher than the 9.36 x 10^6 cells/ml achieved in the closed vessel with the same growth conditions (Figure 2.23).

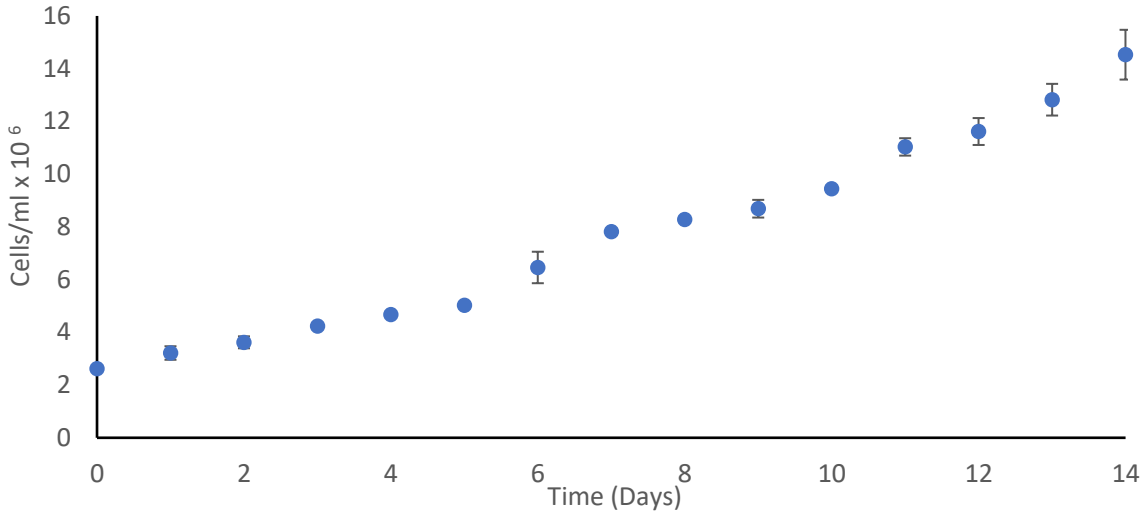


Figure 2.25. The growth of *Chlorella sorokiniana* in the Cyclops 16 litre photobioreactor in a clear tube. Light tracking disabled. Temperature 21˚C. The error bars are representative of the standard deviation from the mean. The PBR was mixed using air at a flow rate of 1.5 L / min.

The increase in growth rate suggests that the *Chlorella sorokiniana* grown in the closed vessel version of the PBR was light limited. Although there was an increase in growth rate when using a transparent culture vessel, the productivity of the Cyclops PBR is still significantly lower than the productivity observed in the Micro-Pharos PBR (Figure 2.8).

The internal light unit of the cyclops can be driven with a maximum current of 900 ma which results in a light intensity of approximately 825 µmol/m^2/s which is lower in comparison to the Micro-Pharos LED panels (1250 µmol/m^2/s). The Micro–Pharos LEDs are also more energy efficient with only 450 ma required to reach their maximum light intensity. Overall the LED panels in the Micro-Pharos system are therefore superior to the Cyclops internal LED unit. This difference in performance may account for the large productivity differential between the two PBR designs.

The illuminated surface area of the Cyclops light unit is 0.13 m^2. Comparatively the total illuminated surface area of the Micro–Pharos system is 0.024 m^2. This

results in an illuminated surface area to volume ratio of 8.13 m^2 per cubic metre for the Cyclops and 24 m^2 per cubic meter for the Micro–Pharos reactor. The low ratio observed in the Cyclops reactor is likely contributing to the low growth rates that were observed. Surface area to volume ratio is an important factor to consider when designing photobioreactors as a higher ratio allows for better light penetration within the reactor (Wolf et al., 2016). This presents a problem when scaling up several PBR design types. Traditional externally illuminated tubular reactors can prove difficult to scale up as the tubes have to be made wider to facilitate a larger culture volume which ultimately reduces light penetration. Internally illuminated tubular reactors such as the Cyclops PBR attempt to mitigate this problem as the availability of light within the reactor is typically increased, provided there is sufficient mixing (Aoyagi et al., 2008).

The performance of the Cyclops reactor could be ehanced by increasing the illuminated surface area of the light unit. The previous study working with the Cyclops reactor achieved a 68 % increase in growth rate when using a prototype double-sided light unit which effectively doubles the illuminated surface area of the reactor (McNerney et al., 2015). Another potential solution would be to increase the length of the internal light unit.

In conclusion the current iteration of the Cyclops photobioreactor is not optimal for the scale up of zeaxanthin production due to its limited biomass productivity, although there is potential to further optimise internal illumination.

2.3.8 Scale up of pigment production with the Pandora 700 litre PBR.

The Pandora PBR reached its maximum cell density of 208 x 10^6 cells per ml after 18 days of growth (Table 2.11).The average daily growth rate for the first 7 days of cultivation was 0.68 μ/day, the growth rate then significantly slowed for the remainder of the experiment (0.03 μ/day). The output of the LED panels reached 826 μmol/m^2/s after 6 days of cultivation. Therefore, the reduction in growth rate may be due to photo-limitation (Figure 2.25). The utilisation of light tracking could result in a more consistent growth rate and a higher final biomass concentration, as this was observed in the Micro–Pharos reactor (Figure 2.9).

Table 2.11. Summary of key parameters and performance of the Pandora 700 litre (PBR).

PBR volume (L)	700
PBR footprint area (m^2)	1
Illuminated surface area (m^2)	6.4
Cultivation time (Days)	17
Maximum cells/ml x 10^6	208
Growth rate (µ/day)	0.29
Harvest biomass dry weight (g/L)	1.17
Maximum biomass productivity (g/L/d)	0.07
Maximum areal productivity ($g/m^2/d$).	11.78
Total power usage for illumination ($kW\ h\ Kg^{-1}$)	1432

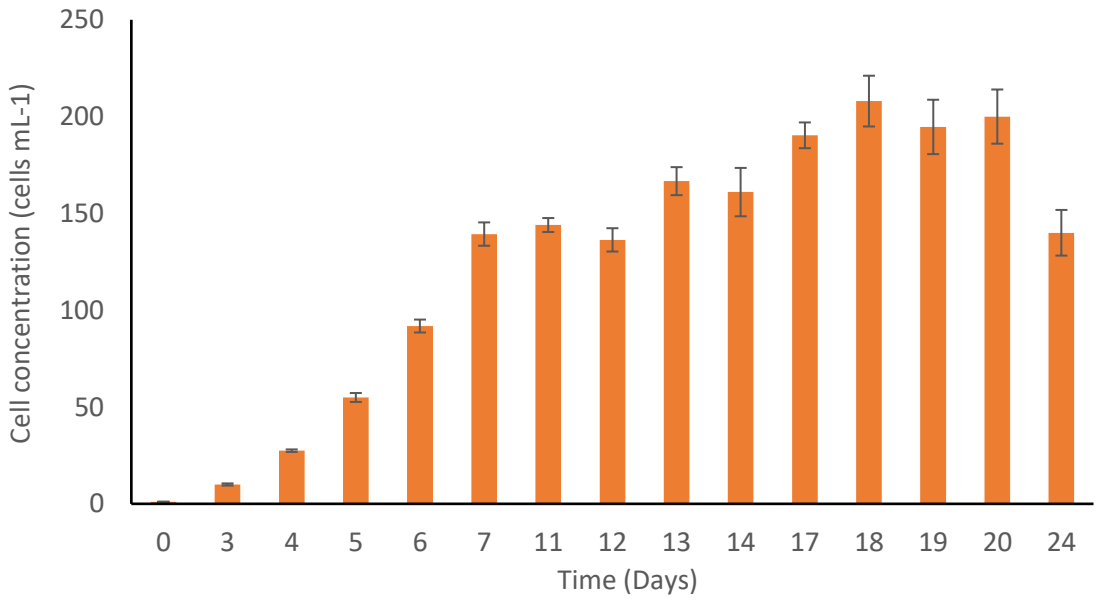


Figure 2.26. The growth of *Chlorella sorokiniana* in the Pandora 700 litre PBR. Error bars are representative of the standard deviation from the mean. Light dark cycle 15/9 (L/D). Sparging = 45 L/min air with an additional 2 L/min pulse of CO_2 for pH control. Temperature = 39°C. n = 3.

In comparison to the Micro–Pharos PBR the Pandora reactor reached a significantly higher cells/ml after 14 days of cultivation, 93.5 x 10^6 and 165 x 10^6 cell density respectively. The Pandora reactor also reached a cell density of 91 x 10^6 which is similar to the maximum cell density achieved by the Micro-Pharos reactor, after only 6 days of cultivation.

The increased productivity observed in the Pandora reactor could be attributed to differing processing parameters such as temperature (39 °C) and nitrogen source (Urea). Urea has been shown to increase the growth rate of several microalgae including *Chlorella* species when compared to inorganic nitrogen sources (Danesi *et al.*, 2002; Hsieh and Wu, 2009b). Urea is also advantageous for the industrial cultivation of microalgae due to its lower cost and ease of transportation in large quantities, as it is less explosive when compared to its inorganic counterparts (Erratt, Creed and Trick, 2018).

Despite achieving a significantly higher growth rate and cell density the zeaxanthin concentration observed in samples taken after 14 days of in cultivation in the Pandora were significantly lower than the Micro-Pharos (Table 2.12). Zeaxanthin production in eukaryotic photosynthesising organisms such as *Chlorella sorokiniana* is controlled by the xanthophyll cycle which is highly light dependent. When the photosynthetic unit (PSU) is exposed to high light intensities the production of zeaxanthin is upregulated. The Micro-pharos PBR therefore provides more favourable conditions for zeaxanthin production due to its higher illuminated surface area to volume ratio and more powerful LED panels (Table 2.12). The Pandora reactor, however, can produce high yields of up to 1.8 g/L zeaxanthin per run due to its large reactor volume. This could potentially be enhanced by using light tracking as demonstrated in section 2.3.6. The effect of light tracking, however, could be potentially limited by the light intensity output of the LED panels which is lower in comparison to LED panels used in the Micro–Pharos reactor (Table 2.11).

Table 2.12. Comparison of the Micro–Pharos and Pandora photobioreactors.

	Micro – Pharos PBR	Pandora PBR
Cultivation time (Days)	14	13.9
Cells/ml (x 10^6)	93.5	165
Zeaxanthin concentration (mg/L)	4.56	2.62
Volume (L)	1	700
Illuminated surface area/volume (S/V)	24	9.14
Maximum light intensity (µmol/m^2/s)	1125	826

Unlike the Pandora reactor, most of the large scale photobioreactors currently in operation are outdoor systems that rely on natural sunlight. By relying on natural sunlight operational costs are reduced. Raceway ponds were among the first photobioreactor design types. Raceway ponds typically maximise their illuminated surface area by spreading out a low depth culture over a large surface area. This approach results in a high surface area to volume ratio which is favourable for light penetration. However, raceway ponds to have low productivity values as they are open systems and therefore cannot be temperature controlled. The areal productivity of raceway ponds is also limited by their typically large footprints. A study by Chinnasamy et al, (2010) compared the productivities of several popular reactor types (Table 2.12). In comparison to raceway ponds the Pandora PBR has higher productivity's and illuminated surface area all within a smaller footprint (Table 2.11)(Table 2.12) (Chinnasamy et al., 2010).

The outdoor vertical tube reactor (100 L) investigated by Chinnasamy et al, (2010) achieved a significantly lower volumetric productivity than the Pandora PBR, 0.032 and 0.07 g/L/day respectively. However, its areal productivity of 20.3 g/m^2/d was higher (Table 2.12). Vertical tube reactors typically have comparitvely low footprints when compared to other design types which is one their main advantages (Singh and Sharma, 2012). Overall the Pandora reactor compares favourably to the large scale natural illuminated reactors that were compared by Chinnasamy et al, (2010). The Pandora displayed the highest

volumetric productivity which is likely the result of its larger illuminated surface area (Table 2.12). The cultivation time, however, took longer in the Pandora PBR when compared to the other reactor types.

Table 2.12. Comparison of outdoor PBR systems with the Pandora PBR (Chinnasamy et al., 2010).

Reactor type	Volume (m^3)	Reactor surface area			Productivity		**Time (D)**
		Foot print	Illuminated area (m^2)	SV	Volumetric productivity (g/L/day)	Areal productivity (g/m^2/day)	
Pandora	0.7	1	6.4	9.1	0.11	12	16
Raceway pond	0.95	3.1	3.1	3.3	0.015	4.42	10
Raceway pond	0.55	2.8	2.8	5.1	0.04	7.79	12
Raceway pond	0.5	2.8	2.8	5.6	0.057	10.36	8
Vertical tube reactor	0.1	0.16	0.43	10	0.032	20.3	8
Polybag	0.02	0.021	0.16	25	0.07	66.4	8

Artificially illuminated photobioreactors with working volumes of greater than 100 litres are still relatively uncommon with most designs being tested at laboratory scale. This is primarily due to the high running costs of supplying artificial light, however, rapidly improving LED technology will likely help to solve this issue.

A study evaluated the performance of 140 litre internally annular PBR achieved a biomass production rate of 0.1 g/L/day (Chini Zittelli, Rodolfi and Tredici, 2003). The highest biomass production rate achieved in this study was 0.07 g/L/day (Table 2.13). The PBR utilised in the study had an illuminated surface area of 9.3 m^2 which is significantly larger than the Pandora reactor (6.4 m^2). However, the study utilised fluorescent lights which only had an output of 89 µmol/m^2/s which is lower when compared to the Pandora (826 µmol/m^2/s). The fluorescent lighting also required a light energy of 1315 W/m^{-3} which is disproportional when compared to the 1733 W/m^{-3} that is required for the

Pandora's LED tiles. Despite the fluorescent lights utilised being largely inferior to the LED lights utilised in the Pandora reactor due to their light intensity, the productivity was still significantly higher. This suggests that illuminated surface area could have a greater effect on biomass production than light intensity. However, many other variables including species, nutrient source, volume and temperature etc. must be considered.

When compared to the other PBR designs in Table 2.13, the Pandora PBR has the lowest biomass production rate. However, its working volume was significantly higher. Maintaining high biomass production rates is a common issue in the scale up of PBRs as it becomes increasingly difficult to supply adequate light energy to the culture as the volume increases (Socher *et al.*, 2016).

Table 2.13. Comparison of PBR designs that utilise artificial illumination.

Type of PBR	Volume (L)	Illuminated surface area (m^2)	Light intensity ($\mu mol/m^2/s$)	Biomass production (g/L/day)	Light energy ($W\ m^{-3}$)	Reference
Annular	140	9.3	89	0.20	1315	(Chini Zittelli et al., 2003)
Annular	90	5.3	259	0.19	2554	(Chini Zittelli et al., 2003)
Bubble column	3	0.18	150	0.77	1923.5	(Jacob-Lopes *et al.*, 2009)
Bubble column	0.8	0.07	300	0.50	5385	(Chen *et al.*, 2007)
Flat plate	50	0.37	80	0.12	127.1	(Reyna-Velarde *et al.*, 2010)
Vertical tube	18	0.25	91	0.1	276	(Pegallapati et al., 2011)
Transparent rectangle chamber	18	0.05	660	0.3	399	(Hsieh and Wu, 2009)
Pandora	700	6.4	826	0.07	1733	This study

The total zeaxanthin yield after 14 days of cultivation in the Pandora reactor was approximately 1.8 g/L. The price of zeaxanthin is heavily dependent on the purity. Optically pure zeaxanthin (99%) is an extremely high value product with an average price of £400 per mg (Sigma, UK). However, such analytical standards are typically produced synthetically as reaching this level of purity from natural sources would require extensive downstream processing. Carotenoids from natural sources are preferred in the supplements industry for primarily marketing purposes (Twyman et al., 2014). Supplements containing zeaxanthin typically have low purity (5 -10%) and prices can range significantly depending on the brand. Lutigold a carotenoid supplement containing zeaxanthin that retails for £12 per 30 capsules (20 mg) each capsule contains approximately 2 mg of zeaxanthin. Therefore, the retail value of the zeaxanthin produced by the Pandora PBR is approximately £ 360 as the zeaxanthin produced by *C. sorokiniana* cultured in the Pandora PBR could be potentially utilised to produce 900 capsules. The Pandora PBR utilised 116.85 kWh of electricity to drive the 6 LED panels which roughly equates to £15 in total based on the current average UK energy tariff of 12.827 p per kWh (UK power, 2019). Electricity is also required for heating and mixing. Additional production costs include nutrients, CO_2 and transportation / shipping.

Xanthella Ltd. have a partnership with Ardnamurchan estates and are currently in the process of building a 32,000 litre microalgae production facility. The facility will consists of a modular array of 32 1000 litre Pandora PBR. The nearby Ardnamurchan distillery produces large volumes of excess CO_2 and electricity as the rural grid does not have sufficient capacity. Xanthella plan to utilise this excess energy and CO_2 to lower the cost of microalgal production. Production costs could also be further lowered by utilising pot ale as a nutrient source instead of traditional inorganic media (Shellcock, 2017). This will be investigated in Chapter 4.

2.4 Conclusions

The upregulation of zeaxanthin production in *Synechocystis sp.* has been successfully demonstrated when the organism was grown in a high light intensity environment in a 1 litre lab scale PBR. *C. sorokiniana* was selected as suitable candidate for scale up due to its high concentration of zeaxanthin. Scale up was unsuccessful when the volume was increased to 16 litres when using the Cyclops PBR. Future work is required to better optimise the Cyclops PBR. It was observed in this study that the growth rate was enhanced when the outer reactor vessel was made transparent. This change allowed for more light to enter the reactor and suggests that the available light within the current reactor design may not be sufficient. Another option to enhance the performance of the Cyclops is to double the light output by producing a Goldilocks® unit that is double sided. At industrial pilot scale (700 litres) the growth of *Chlorella sorokiniana* was successful and high cell densities were achieved resulting a total zeaxanthin yield of approximately 1.8 g. This level of production may have economic potential due to the increasing demand for naturally occurring carotenoids which is fuelled by the health supplements industry. However, future work is required in order to determine if the industrial scale production of zeaxanthin using the Pandora PBR systems is economically viable. The production of other high value products in the Pandora should also be investigated such as polyhydroxyalkonates (PHAs), fatty acids and phycobilin pigments. If a biorefinery approach could be adopted which incorporated the production of combination products then potential revenue could be greatly enhanced.

2.5 References

Ajayan, K. V., Anjula, K., Syamasurya, A.P., Harilal, C.C., 2019. Energy efficient technology for enhanced growth and lipid production in Chlamydomonas reinhardtii through additional reflector coated LED photo-bioreactor. Biochemical Engineering Journal 144, 81–88. https://doi.org/10.1016/j.bej.2019.01.010

Aoyagi, H., Uchiyama, H., Ugwu, C.U., 2008. Photobioreactors for mass cultivation of algae. Bioresource Technology. https://doi.org/10.1016/j.biortech.2007.01.046

Cezare-gomes, E.A., Mejia-da-silva, L.C., Pérez-mora, L.S., 2019. Potential of Microalgae Carotenoids for Industrial Application. Applied Biochemistry and Biotechnology 1–33. https://doi.org/10.1007/s12010-018-02945-4

Chaichalerm, S., Pokethitiyook, P., wenqiao, Y., Meetam, M., Sritong, K., Pugkaew, W., Kungvansaichol, K., Kruatrauchue, M., D., 2012. Culture of microalgal strains isolated from natural habitats in Thailand in various enreiched media. Applied Energy 296–302.

Chen, C.-H., Kuan, T.-C., Ong, S.-C., Chiu, S.-Y., Kao, C.-Y., Lin, C.-S., 2007. Reduction of CO2 by a high-density culture of Chlorella sp. in a semicontinuous photobioreactor. Bioresource Technology 99, 3389–3396. https://doi.org/10.1016/j.biortech.2007.08.013

Cheng, J., Xu, J., Lu, H., Ye, Q., Liu, J., Zhou, J., 2018. Generating cycle flow between dark and light zones with double paddlewheels to improve microalgal growth in a flat plate photo-bioreactor. Bioresource Technology 261, 151–157. https://doi.org/10.1016/j.biortech.2018.04.022

Chini Zittelli, G., Rodolfi, L., Tredici, M.R., 2003. Mass cultivation of Nannochloropsis sp. in annular reactors. Journal of Applied Phycology 15, 107–114. https://doi.org/10.1023/A:1023830707022

Chinnasamy, S., Bhatnagar, A., Claxton, R., Das, K.C., 2010. Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium. Bioresource Technology 101, 6751–6760. https://doi.org/10.1016/j.biortech.2010.03.094

Danesi, E.D.G., Rangel-Yagui, C.D.O., De Carvalho, J.C.M., Sato, S., 2002. An investigation of effect of replacing nitrate by urea in the growth and production of chlorophyll by Spirulina platensis. Biomass and Bioenergy 23, 261–269. https://doi.org/10.1016/S0961-9534(02)00054-5

Dautermann, O., Lohr, M., 2017. A functional zeaxanthin epoxidase from red algae shedding light on the evolution of light-harvesting carotenoids and the xanthophyll cycle in photosynthetic eukaryotes. Plant Journal 92, 879–891. https://doi.org/10.1111/tpj.13725

De Farias Silva, C.E., Sforza, E., Bertucco, A., 2017. Effects of pH and Carbon Source on Synechococcus PCC 7002 Cultivation: Biomass and Carbohydrate Production with Different Strategies for pH Control. Applied Biochemistry and

Biotechnology 181, 682–698. https://doi.org/10.1007/s12010-016-2241-2

Erratt, K.J., Creed, I.F., Trick, C.G., 2018. Comparative effects of ammonium, nitrate and urea on growth and photosynthetic efficiency of three bloom-forming cyanobacteria. Freshwater Biology 63, 626–638. https://doi.org/10.1111/fwb.13099

Fu, W., Magnúsdóttir, M., Brynjólfson, S., Palsson, B., Paglia, G., 2012. UPLC-UV-MSE analysis for quantification and identification of major carotenoid and chlorophyll species in algae. Analytical and Bioanalytical Chemistry 404, 3145–3154. https://doi.org/10.1007/s00216-012-6434-4

Gerardo, M.L., Van Den Hende, S., Vervaeren, H., Coward, T., Skill, S.C., 2015. Harvesting of microalgae within a biorefinery approach: A review of the developments and case studies from pilot-plants. Algal Research 11, 248–262. https://doi.org/10.1016/j.algal.2015.06.019

Goldman, J.C., Dennett, M.R., Riley, C.B., 1982. Effect of nitrogen-mediated changes in alkalinity on pH control and CO2 supply in intensive microalgal cultures. Biotechnology and Bioengineering 24, 619–631. https://doi.org/10.1002/bit.260240308

Henriques, M., Silva, A., Rocha, J., 2007. Extraction and quantification of pigments from a marine microalga : a simple and reproducible method. Applied Microbiology 586–593.

Hindersin, S., Leupold, M., Kerner, M., Hanelt, D., n.d. Key parameters for outdoor biomass production of Scenedesmus obliquus in solar tracked photobioreactors.

Hsieh, C.H., Wu, W.T., 2009a. Cultivation of microalgae for oil production with a cultivation strategy of urea limitation. Bioresource Technology 100, 3921–3926. https://doi.org/10.1016/j.biortech.2009.03.019

Hsieh, C.H., Wu, W.T., 2009b. A novel photobioreactor with transparent rectangular chambers for cultivation of microalgae. Biochemical Engineering Journal 46, 300–305. https://doi.org/10.1016/j.bej.2009.06.004

Jacob-Lopes, E., Scoparo, C.H.G., Lacerda, L.M.C.F., Franco, T.T., 2009. Effect of light cycles (night/day) on CO2 fixation and biomass production by microalgae in photobioreactors. Chemical Engineering and Processing: Process Intensification 48, 306–310. https://doi.org/10.1016/j.cep.2008.04.007

Jeanmonod, D.J., Rebecca, Suzuki, K. et al., 2018. Characterisation of Carotenoids Invloved in the Xanthophyll Cycle. Intech open science 2, 64. https://doi.org/10.5772/32009

Joshi, C., Singhal, R.S., 2016. Modelling and optimization of zeaxanthin production by Paracoccus zeaxanthinifaciens ATCC 21588 using hybrid genetic algorithm techniques. Biocatalysis and Agricultural Biotechnology 8, 228–235. https://doi.org/10.1016/j.bcab.2016.10.004

Koo, S.Y., Cha, K.H., Song, D.G., Chung, D., Pan, C.H., 2012. Optimization of pressurized liquid extraction of zeaxanthin from Chlorella ellipsoidea. Journal of Applied Phycology 24, 725–730. https://doi.org/10.1007/s10811-011-9691-2

Lagarde, D., Beuf, L., Vermaas, W., 2000. Increased production of zeaxanthin and other pigments by application of genetic engineering techniques to Synechocystis sp. strain PCC 6803. Appl Environ Microbiol 66, 64-72. https://doi.org/10.1128/AEM.66.1.64-72.2000

Liu, R., Wang, T., Zhang, B., Qin, L., Wu, C., Li, Q., Ma, L., 2015. Lutein and zeaxanthin supplementation and association with visual function in age-related macular degeneration. Investigative Ophthalmology and Visual Science 56, 252–258. https://doi.org/10.1167/iovs.14-15553

Mahoney, E, A., Garcia, L, A., Bacchetta, B, C., 2018. Modular photobioreactors system for the cultivation of algae. US 2018/0216056 A1.

McNerney, C., 2015. The Cyclops photobioreactor, Performance and Characterisation of algal growth.

Pegallapati, A.K., Nirmalakhandan, N., 2011. Energetic evaluation of an internally illuminated photobioreactor for algal cultivation. Biotechnology Letters 33, 2161–2167. https://doi.org/10.1007/s10529-011-0691-8

Rakhimberdieva, M.G., Stadnichuk, I.N., Elanskaya, I. V., Karapetyan, N. V., 2004. Carotenoid-induced quenching of the phycobilisome fluorescence in photosystem II-deficient mutant of Synechocystis sp. FEBS Letters 574, 85–88. https://doi.org/10.1016/j.febslet.2004.07.087

Reyna-Velarde, R., Cristiani-Urbina, E., Hernández-Melchor, D.J., Thalasso, F., Cañizares-Villanueva, R.O., 2010. Hydrodynamic and mass transfer characterization of a flat-panel airlift photobioreactor with high light path. Chemical Engineering and Processing: Process Intensification 49, 97–103. https://doi.org/10.1016/j.cep.2009.11.014

Sajilata, M.G., Singhal, R.S., Kamat, M.Y., 2008. The Carotenoid pigment Zeaxanthin - A Review. Comprehensive Reviews In Food Science And Food Safety 7, 29–49. https://doi.org/10.1111/j.1541-4337.2007.00028.x

Schulze, P.S.C., Barreira, L.A., Pereira, H.G.C., Perales, J.A., Varela, J.C.S., 2014. Light emitting diodes (LEDs) applied to microalgal production. Trends in Biotechnology 32, 422–430. https://doi.org/10.1016/J.TIBTECH.2014.06.001

Sharoni, Y., Linnewiel-Hermoni, K., Khanin, M., Salman, H., Veprik, A., Danilenko, M., Levy, J., 2012. Carotenoids and apocarotenoids in cellular signaling related to cancer: A review. Molecular Nutrition and Food Research 56, 259–269. https://doi.org/10.1002/mnfr.201100311

Shellcock, C., 2017. Algal Solutions for Local Energy Economy. http://aslee.scot/

Singh, R.N., Sharma, S., 2012. Development of suitable photobioreactor for algae production - A review. Renewable and Sustainable Energy Reviews 16, 2347–2353. https://doi.org/10.1016/j.rser.2012.01.026

Socher, M.L., Löser, C., Schott, C., Bley, T., Steingroewer, J., 2016. The challenge of scaling up photobioreactors: Modeling and approaches in small scale. Engineering in Life Sciences 16, 598–609. https://doi.org/10.1002/elsc.201500134

Steiger, S., Schäfer, L., Sandmann, G., 1999. High-light-dependent upregulation of carotenoids and their antioxidative properties in the cyanobacterium Synechocystis PCC 6803. Journal of Photochemistry and Photobiology B: Biology 52, 14–18. https://doi.org/10.1016/S1011-1344(99)00094-9

Stanier, R.Y., Kunisawa, R., Mandel, M., Cohen-Bazire, G., 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). Bacteriological reviews 35, 171–205.

Twyman, R.M., Farré, G., Zhu, C., Zorrilla-López, U., Christou, P., Capell, T., Sandmann, G., Berman, J., 2014. Nutritionally important carotenoids as consumer products. Phytochemistry Reviews 14, 727–743. https://doi.org/10.1007/s11101-014-9373-1

Ukpower, (2019) *Gas and Electricity Tariff Prices per kWh.* Assessed online 15/06/19 [https://www.ukpower.co.uk/home_energy/tariffs-per-unit-kwh]

Wolf, J., Stephens, E., Steinbusch, S., Yarnold, J., Ross, I.L., Steinweg, C., Doebbe, A., Krolovitsch, C., Müller, S., Jakob, G., Kruse, O., Posten, C., Hankamer, B., 2016. Multifactorial comparison of photobioreactor geometries in parallel microalgae cultivations. Algal Research 15, 187–201. https://doi.org/10.1016/j.algal.2016.02.018

Yeh, N., Ding, T.J., Yeh, P., 2015. Light-emitting diodes' light qualities and their corresponding scientific applications. Renewable and Sustainable Energy Reviews 51, 55–61. https://doi.org/10.1016/j.rser.2015.04.177

Zavřel, T., Sinetova, M.A., Búzová, D., Literáková, P., Červený, J., 2015a. Characterization of a model cyanobacterium Synechocystis sp: PCC 6803 autotrophic growth in a flat-panel photobioreactor. Engineering in Life Sciences 15, 122–132. https://doi.org/10.1002/elsc.201300165

Zavřel, T., Sinetova, M.A., Búzová, D., Literáková, P., Červený, J., 2015b. Characterization of a model cyanobacterium Synechocystis sp: PCC 6803 autotrophic growth in a flat-panel photobioreactor. Engineering in Life Sciences 15, 122–132. https://doi.org/10.1002/elsc.201300165

Zhang, Y., Liu, Z., Sun, J., Xue, C., Mao, X., 2018. Biotechnological production of zeaxanthin by microorganisms. Trends in Food Science and Technology 71, 225–234. https://doi.org/10.1016/j.tifs.2017.11.006

Chapter 3

The Characterisation of Pot ale and identification of novel valorisation opportunities.

3.1 Chapter outline

In this chapter the findings of a pot ale characterisation encompassing 22 distilleries from across Scotland are presented.

In addition, a study investigating the stability of pot ale overtime in varying storage temperatures was also undertaken. The pot ale used in this study was sourced solely from the Glencadam distillery, Brechin, Scotland.

3.1.1 Introduction

The Scotch Whisky industry is essential to the UK economy. It provides £5.5 billion in gross value added to the UK economy and employs 42,000 people including 10,500 directly in Scotland (SWA, 2018). The production of malt and grain whisky in 2017 exceeded 550 million litres of pure alcohol (mLPA)(Gray, 2018) However, for every litre of malt whisky produced, approximately 9 litres of residue, known as pot ale, remains after the distillation process (Arnison and Carrick,2015). In 2014 an estimated 4.4 million tonnes of pot ale was produced by Scottish malt whisky distilleries (Table 3.1). Globally the total production can be estimated to be 15.7 million tonnes.

Table 3.1. Estimated pot ale production of the leading whisky producing countries. (Russel & Stewart, 2014)

Region	Pure Alcohol (Millions of litres)	Pot ale (Millions of tonnes)
Scotland	550	4.4
N. America	333	2.6
Ireland	64	0.5
India	936	7.5
Japan	90	0.7
Total	1973	15.7

Similarly, for the production of grain whisky, eighteen litres of a comparable residue, spent wash, also remains (ZWS, 2015). Using annual spirit production

data reported by Gray, (2018), a total of 7.65 million tonnes of pot ale and spent wash is produced per year. A significant portion of this is converted into pot ale syrup (PAS) or distillers' dark grains and used as animal feed. Although this process can be time consuming and expensive, reducing the aqueous fraction of these by-products will have environmental and cost benefits. For example, since pot ale is only 5% solid, reducing its aqueous volume by a factor of ten to produce PAS will decrease the number of tankers required for off-site transportation of by-products. In addition to producing animal feed, distillery by-products are also used by agriculture as a fertiliser, where the nutrient deficient soils can be remediated by its application to land. Due to environmental and economic factors, this traditional practice has declined in recent decades where distilleries are choosing other means to utilise their co-product. There are environmental concerns surrounding the disposal of pot ale primarily due to its high carbon oxygen demand (COD), biological oxygen demand (BOD), heavy metals and levels of inorganic compounds. Studies have shown that distillery by-products can have negative effects on both ground water, by altering the pH and nutrient composition, by leaching organic and inorganic ions (Singh et al.,, 2003; Jain et al., 2005). In some instances, distilleries may have little choice but to dispose of their by-product to sea, where its discharge and subsequent dilution is heavily regulated by the Scottish Environment Protection Agency.

Whilst well established in principle, the production of bioenergy from anaerobic digestion (AD) is the most commonly used contemporary application for distillery by-products. Given the high COD (30,000-50,000 mg/L) in pot ale for example (Barrena et al., 2018), and the greater efficiencies being obtained in AD plants, it is easy to understand why they have become so popular for whisky producers. Furthermore, the reduction in greenhouse gas emissions when by-products are used for AD when compared to traditional methods is another important factor (Leinonen et al., 2018). In 2014, the House of Lords released a study detailing the potential value chains that could be established from stimulating the bioeconomy (House of Lords, 2014). The report highlighted the need to focus on developing the circular bio-based economy and greatly improve the use of by-products and waste streams. Since the whisky fermentation process only utilises a proportion of carbohydrates present in barley grains (i.e. monosaccharides) (Leinonen et al., 2018), further valorisation of pot ale and spent wash is needed

to understand the remaining, potentially high value chemicals, left after distillation. With the Industrial Biotechnology sector in Scotland expected to grow to approximately £900 m by 2025 (Gray, 2018), emerging biotechnology hopes to utilise pot ale and spent wash for higher value chemicals either to replace or complement current distillery practices. For example, recent studies have developed novel by-product processing that can recover protein from pot ale or can produce omega 3 using specific algae strains (Traub et al., 2015, Ricardo energy and Zero Waste Scotland, 2017).
The application of novel technologies, alongside distillery pot ale, could be used to produce a more sustainable, environmentally friendly, high quality aquaculture feedstock (Traub et al., 2015).

Pot ale has been previously characterised in a study by Graham *et al*, (2012). The study analysed pot ale from a single whisky distillery. The study identified concentrations of copper (2-6 ppm) and high but variable concentrations of organic acids (4,000-10,000 ppm) in pot ale. A recent study by Barrena et al., (2018) characterised pot ale from four whisky distilleries which highlighted the varability of the physiochemical compostion of pot ale. In current and future efforts to valorise pot ale it is imperative to understand to what extent the compostion of pot ale varies and what factors if any, affect its compostion.

This study examines pot ale and spent wash collected from twenty-two distilleries that were representative, at the time of the study, of the 128 distilleries in production in Scotland. This study also examines the stability of pot ale at varying temperatures over time and assesses the effects of filtering on four key analytes; lactic acid, acetic acid, nitrate and phosphate. If pot ale is to be effectively valorised, then it is crucial to understand how transportation and storage may affect its chemical stability.

The primary aim of this study was to: (i) quantify concentrations of organic acids, inorganic anions, total organic carbon, metals, amino acids and carbohydrates in pot ale and spent wash; (ii) assess its variability in chemical composition; (iii) determine the production factors that may influence the components of pot ale and spent wash; (iv) assess the stability of pot ale and; (v) review the circular economy opportunities for pot ale and spent wash using novel biotechnology.

3.2 Materials and Methods

3.2.1 Pot ale stability assessment

Samples were taken from a single Scottish malt whisky distillery. Samples were collected in sterile 15 ml centrifuge tubes (Corning®). Half of all samples were filter sterilised using single use sterile 0.2 µm hollow fibre syringe filters (MediaKap plus,Repligen), the other half were left unfiltered. Samples were then stored at three different temperatures at 21°C in a temperature-controlled room at 4 °C (fridge) and at – 20 °C (freezer). The aim was to assess the influence of filtration and storage temperature on the stability of pot ale. Stability was assessed by monitoring the changes in pot ale composition over time by the analysis of nitrate, phosphate, acetate and lactate content, using ion chromatography (section 3.2.4)

3.2.2 The sampling protocol and sample collection process for the pot ale characterisation audit

Single use 0.2 µm hollow fibre syringe filters were provided to participating distilleries. A total of 6 samples were collected from each distillery, 3 filtered samples and 3 unfiltered samples. All sampling was carried out on site by distillery operators. The correct operation of the 0.2 µm filters was instructed by a supplied protocol (Figure 3.1). Each participating distillery was also supplied with a sampling form which was developed in collaboration with the Scottish whisky research institute. The purpose of the form is to survey key processing parameters such as barley variety, malt peating level, wort clarity, yeast strain and length of fermentation. To ensure anonymity each distillery was given a unique identifier code (A – V).

Sample collection was undertaken with the aid of the Scottish whisky research institute (SWRI) (Edinburgh, Scotland). In total 25 Scottish whisky distilleries were contacted by SWRI and agreed to participate in the study, 22 distilleries responded with results in a response rate of 88 %. As of 2018 there 128 active whisky distilleries in Scotland therefore this audit encompasses 17 % of the industry (Scottish whisky assocation, 2019).

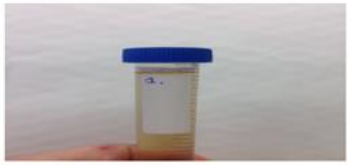
Following the evaluation of pot ale stability (3.2.1) a sampling protocol which includes filter sterilisation was developed in order to enhance pot ale stability during transit

RGU CyanoSol IBioIC

Pot ale sampling procedure

1) First draw off 2 buckets of pot ale to ensure that the lines are clear. Draw off a sample from the main pot ale waste tank (>500ml) and mix/shake thoroughly.

2) Please fill the provided 3 plastic tubes with 10ml of pot ale **This sample does not have to be filtered.** Please label each sample with the **time and date** of sampling and the **initials NF.**

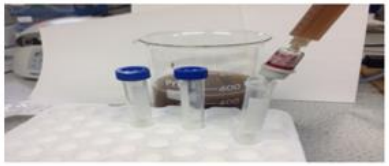


3) From the bucket of pot ale, completely fill the provided syringe.

4) Open the bag containing the filter cap, ensuring that you **do not handle** the tip of the filter directly.

4) Attach the filter cap to the syringe. A **new filter cap** is required for each sample.

5) Expel the contents of the syringe into the provided sample tube. **Do not exert excessive force onto the syringe**. The pot ale should slowly drip into the sample tube. **Please place the spent filter caps in the provided press-lock bag after use. (1 per bag).**



6) The resulting pot ale sample should be light in colour and not cloudy. Repeat the filtering process another two times for a total of **3 samples. Please label each sample with the time and date of sampling.**

7) You should now have a total of **3** filtered 10 ml samples and **3** 10 ml non filtered samples. Please place the samples, along with the filters and **completed survey** in the provided return envelope. A prepaid postage label is provided.

Many thanks for participating in this study.

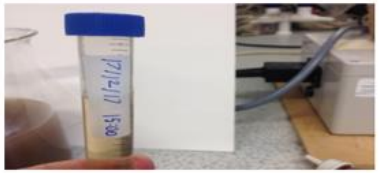


Figure 3.1. The sampling procedure instructing the proper use of the syringe filters. This form was sent to all participating distilleries.

3.2.3 Processing and Storage of survey pot ale samples

Upon receipt of the samples, the time in transit of each sample was recorded. The 3 unfiltered samples were then immediately stored at - 21 °C. From each of the 3 filtered samples aliquots of 1 ml (x 3) were taken under sterile conditions, to give a total of 9 aliquots. Of these aliquots 6 were stored at -21 °C. The remaining 3 aliquots were immediately analysed using Ion Chromatography.

3.2.4 The analysis of pot ale samples using Ion Chromatography

The analysis of the lactate, acetate, succinate, phosphate, nitrite and nitrate content of pot ale samples were performed by IC (Dionex Intergrion HPIC). The analytes were separated on an AS11-HC column (4 mm x 250 mm long, 4 µm particle size) equipped with an AG11 guard column (4 x 50 mm). Data acquisition and the analysis of chromatograms was performed using Chromeleon 7.1 chromatography software (Dionex). The method was the same as detailed previously in section 2.2.25. Calibration curves were produced using analytical standards (Sigma, UK) with a range of 5 – 100 mg/L (Figure 7A and 8A in the appendix). Pot ale samples were diluted 1/100 with ultrapure water prior to analysis, to ensure that they were within the calibration range.

The purpose of quality control spiked recoveries were determined by spiking pot ale samples with a known concentration of each analytical standard (10 mg/L) and comparing the percentage difference against an equivalent unspiked sample of pot ale and a 10 mg/L analytical standard. All samples were analysed in triplicate (Table 3.2). Limits of quantification were estimated by continually diluting analytical standards by 10 % starting from a concentration of 0.5 mg/L (Table 3.2).

Table 3.2. The retention time of each analyte, limits of quantification (LOQ) and spiked recoveries. All analysis was carried out in triplicate and the values displayed are representative of mean values. n = 3.

Analyte	Retention time (minutes)	LOQ (mg/L)	Spiked recovery (%)
Lactate	7.03	0.05	98
Acetate	7.57	0.06	95
Chloride	16.10	0.05	97
Nitrite	17.60	0.06	103
Nitrate	22.40	0.03	95
Succinate	22.90	0.08	99
Carbonate	23.73	0.23	97
Sulphate	25.28	0.14	98
Phosphate	27.20	0.04	95

3.2.5 Microwave digestion for the total metal analysis in pot ale.

To analyse the total metal content of pot ale a microwave digestion technique was utilised. Each unfiltered pot ale sample was vortexed for 2 minutes. A 1 ml aliquot of each sample was added to 10 ml of Aqua regia acid (1-part HNO3, 3 parts HCl). The samples were then subjected to microwave digestion at a temperature of 180 °C for 90 minutes (Ethos EZ -Milestone, UK). After the digestion process was complete the samples could cool at room temperature before being diluted with 15 ml of deionzed water prior to analysis which resulted in a total volume of 26 ml.

3.2.6 Quantification of metals in soluble and insoluble fraction of pot ale

Concentrations of Cd (228.802 nm), Cr (267.716 nm), Cu (327.393 nm), Fe (238.204 nm), Ni (231.604 nm), Mg (285.213 nm), Mn (257.610 nm), Pb (220.353 nm) and Zn (206.200 nm) in pot ale digests were determined by inductively coupled plasma optical emission spectroscopy (ICP–OES) using a PerkinElmer Optima 8000 DV instrument coupled to a S10 PerkinElmer autosampler (PerkinElmer, UK). Using individual 10,000 mg/L stock solutions (Fisher Scientific, UK) and ultrapure water, calibration standards were prepared at a concentration range of 0.1 to 10 mg L^{-1} in triplicate (Figure 9A and 10A). The argon flow rates were; Plasma 15 L/min, Auxiliary 0.2 L/min and Nebulizer 0.8 L/min. The ICP-OES was re calibrated and blanked using ultrapure water prior to each batch of analysis. Samples were injected at a flow rate of 1.5 ml/min. Calibration curves obtained for all elemental analysis were ≥ 0.995 with % RSDs of < 5%. Prior to analysis pot ale samples were diluted 1/10 with ultrapure water (Elga, UK) to be within calibration range.

Table 3.3. The wavelength that the optical emissions spectrometer used to quantify each analyte and their limits of quantification. Limits of quantifications are listed as estimations as per manufacturer data.

Analyte	Wavelength (nm)	LOD (mg/L)
Mg (I)	285.213	0.002
Mn (II)	257.610	0.001
Fe (II)	238.204	0.005
Ni (II)	231.604	0.015
Cu (I)	327.393	0.010
Zn (II)	206.200	0.006
Pb (II)	220.353	0.042
Cr (II)	267.716	0.007
Cd (I)	228.802	0.003

3.2.7 Carbon analysis

The total organic carbon (TOC) content was determined by wet chemical combustion using a Shimadzu TOC-VCPH total organic carbon analyser connected to a Shimadzu ASV-V autosampler (all supplied by Shimadzu, UK). The furnace temperature was set at 750°C with an oxygen carrier gas flow of 150 ml min^{-1}. A total of 25 µl of sample was injected and hydrolysed with phosphoric acid (25% w/w). Samples were analysed in triplicate. The instrument was calibrated using potassium hydrogen phthalate (Sigma-Aldrich, UK) and calibration curves ranged from 100 mg L^{-1} to 1000 mg L^{-1}.

3.2.8 Determination of free carbohydrates in pot ale

Carbohydrate and monosaccharide analysis were carried out at Rothamsted research institute (Hertfordshire, UK). Rothamsted were sent a set of 1 ml aliquots taken from the pot ale samples collected by the pot ale audit. Data analysis was carried out by the author.

For the analysis of free monosaccharides (arabinose, rhamnose, galactose, glucose and xylose), aliquots of resuspended pot ale were filtered through 0.45 µm PVDF filters and diluted to 0.15 mg/L using ultrapure water. Samples were analysed by high-performance anion-exchange chromatography with pulsed amperometric detector (HPAEC-PAD) using a Dionex ICS-5000+ chromatography system (Thermo Scientific, Hemel Hempstead, UK) at a column oven temperature of 30°C. The analytical column used was a CarboPac PA20 (3 x 150 mm) with CarboPac PA20 guard column (3 x 30 mm). A KOH mobile phase was used at a flow of 0.5 ml/min. The gradient elution programme was as follows; 0 – 14.5 min (4 mM KOH), 14.5 – 15.0 min (linear increase to 100 mM KOH), 15 – 18 min (hold at 100 mM KOH), 18 – 18.5 min (linear decrease to 4 mM KOH) and 18.5 – 23.5 min (hold at 4 mM KOH). Data was analysed using Chromeleon 7.2 (Thermo Scientific).

3.2.8.1 Determination of hydrolysed carbohydrates in pot ale

Monosaccharides (arabinose, galactose, glucose, mannose and xylose) were determined following acid hydrolysis, as described in Freeman et al. (2017). Briefly, freeze-dried pot ale samples were resuspended to a concentration of 5 mg mL^{-1} with water. Aliquots equivalent to 1 mg of pot ale were then dried by vacuum centrifuge. Using 2 M trifluoracetic acid (Merck, UK), 400 µl was added to the samples and then incubated for 1 hour at 120°C and left to cool in ice. Hydrolysed samples were again dried by vacuum centrifuge, washed with 500 µl of water and dried again (to remove residual trifluoracetic acid). The samples were resuspended in 1 ml of ultrapure water, centrifuged at 13400 x *g* for 2 minutes and the supernatant filtered through 0.45 µm PVDF filters. A set of monosaccharides standard sugars were treated in exactly the same manner and analysed alongside a set of standards by HPAEC-PAD as described in section 3.2.8

3.2.10 Amino acid analysis

The analysis of amino acid content was carried out at Scottish whisky research institute. SWRI received a set of 1 ml aliquots collected by the audit for both unfiltered and filtered pot ale samples.

Mixed calibrations standards (0.1 — 5.0 mg/L) were prepared from 20 individual amino acids (alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) (Sigma, USA) with *o*-Methyl Tyrosine (1.0 mg/L) used as an internal standard. All analysis was completed on calibration curves with correlation coefficients of > 0.990. Samples were diluted in a 5% ethanol solution prior to analysis. Standards and samples were derivatised prior to analysis using an AccQTag™ derivatisation kit (Waters, USA). Quantification was carried out by LC-MS employing a Waters Acquity LC coupled to an Agilent (California, USA) 6150B single quad MS on single ion monitoring detection mode. Retention and

separation of all derivatised amino acids was achieved using a Waters Acquity T3, (2.1 mm ID x 150 mm long, 1.6 µm particle size) column at a flow rate of 0.5 ml min^{-1} and an injection volume of 0.5 µl. Mobile phases consisting of 0.4 % formic acid in water (A) and 0.4 % formic acid in acetonitrile (B) were used in a 20-minute gradient elution. The gradient was as follows; 0 – 1 min (100% A), 1 – 20 min (99 % A), 20 – 22 min (80% A), 22 – 25 min (0% A) and 25 min (99% A). For quality control, a laboratory prepared wort was spiked with known concentrations of each amino acid standard. The laboratory wort (n = 12) gave typical amino acid accuracy and precision within 10 % of the target concentration and an RSD ranged from 3.77% (tryptophan) to 19.8% (hydroxyproline). Spiked wort recoveries ranged from 83.5% (cysteine) to 119% (arginine). All samples were analysed in triplicate and were above the limit of detection.

3.2.11 Statistical analysis of the compositional data

Principal Component Analysis (PCA) was used to summarise the compositional data. In this method, derived variables known as principal components (PCs) were constructed in order to express a large proportion of the total variance of the original multivariate data with a smaller number of variables. By plotting the PCs, interrelationships between the compositional variables could be viewed along with sample (distillery) patterns, groupings, similarities or differences. This allowed relationships between the process parameters and composition to be explored. The analysis was carried out using JMP software, V 14.3.0.

3.3 Results and discussion

3.3.1 Pot ale stability

The concentration of lactic acid in unfiltered samples was significantly lower after 10 days of storage at 21°C and 4°C. However, the downward trend was not consistent on a day to day basis (Figure 3.2). Overall filtration had little effect on the stability of lactic acid as concentrations fluctuated from 22,880 mg/L (time zero) - 16,407 mg/L (day 10) with a significant increase observed at time point 6. There was also a downward trend observed with the non-filtered samples, 25,256 mg/L (time zero)–10,892 mg/L (day 10). This could potentially be the result of microbial action. A similar trend, however, was also observed with filtered samples so this is unlikely. When stored at 4°C the lactic acid concentrations were also unstable in both filtered and non-filtered sample (Figure 3.2). The stability of lactic acid, however, was increased when the samples were stored at - 21°C with no significant differences observed between filtered and non-filtered samples over the 10-day time period (Figure 3.2). The trends observed for acetic acid were similar to that of lactic acid, with filtration having no significant effect on stability and lower temperatures having a somewhat positive effect (Figure 3.3).

A significant decrease in lactic acid, however, was observed from time zero–day 1 in both sample types (Figure 3.2). This suggests the freezing of the pot ale is impacting the lactic acid concentration. A previous study investigating the organic acid concentration in cheeses after freezing also observed a significant decline in lactic acid concentration (Park et al., 2010). There is known to be significant concentrations of lactic acid bacteria present during whisky fermentation (Priest, 2004). Therefore, it is possible that they are also present in pot ale despite its low pH of 3.5. Although this pH can be inhibitory several strains of lactic acid bacteria such are *L. oenos* known to tolerate pH levels below pH 3 (Munoz and del las Rivas, 2011). Therefore, freezing pot ale will result in the death of any lactic acid present and cause a reduction in lactic acid concentration. However, future is required to verify this hypothesis. The trends observed for acetic acid were similar to that of lactic acid, with filtration having

After 10 days of storage at 21 °C the phosphate concentration of unfiltered samples was significantly lower than that of filtered samples (Figure 3.5). A similar trend can be observed with the concentration of nitrate (Figure 3.4). This reduction in phosphate and nitrate concentration can be potentially attributed to microbial action. At 4°C and -21 °C the observed difference between filtered and non-filtered samples was less significant with regards to the concentration of nitrate and phosphate. Again, this can likely be attributed to the colder temperatures reducing microbial activity. The stability of the phosphate content was also greatly improved at – 21 °C with little to no variance observed between samples (Figure 3.4).

Overall filter sterilisation improved the stability of nitrate and phosphate in samples stored at 21ºC with significant reductions observed in the concentrations of non-filtered samples. The observed concentrations, however, were highly variable with large standard deviations from the mean observed (Figure 3.4-3.5). Filtration, however, had little effect on the concentration of lactic and acetic acid. Storage at -21°C significantly improved the stability of lactic acid and phosphate. Pot ale is a highly complex medium and there could also be chemical and enzymatic reactions taking place which impact stability. Future work would have to be carried out to truly understand these interactions.

This investigation highlights that pot ale is unstable at room temperature, especially with regards to concentrations of nitrate and phosphate. This can be improved by using filtration. Although the overall stability was improved at -21°C this is impractical for the transportation of samples as specialised resources would be required to transport pot ale in a frozen sate.

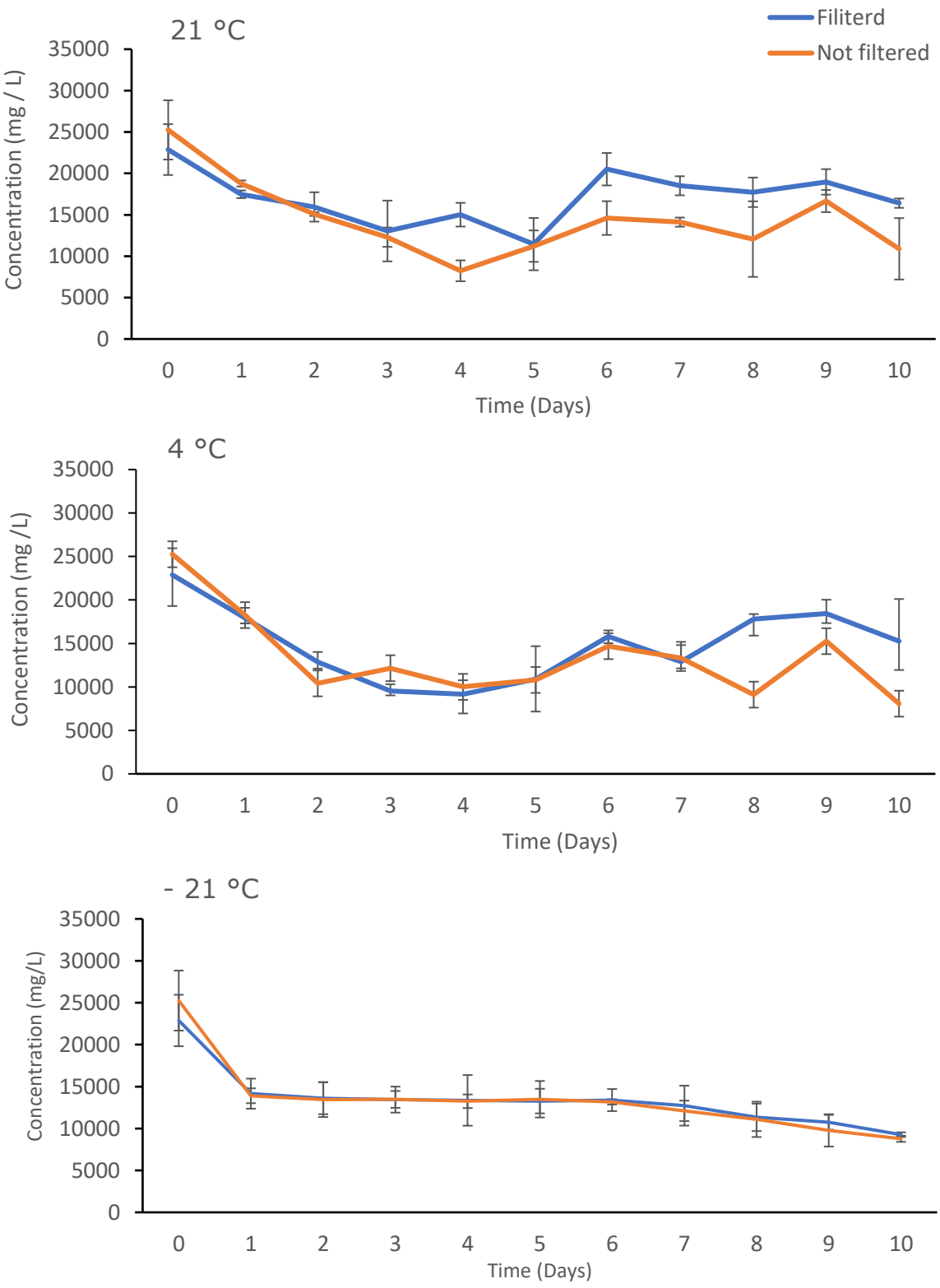


Figure 3.2. The stability of lactate in pot ale under different storage conditions. The error bars are representative of the standard deviation from the mean. n = 3

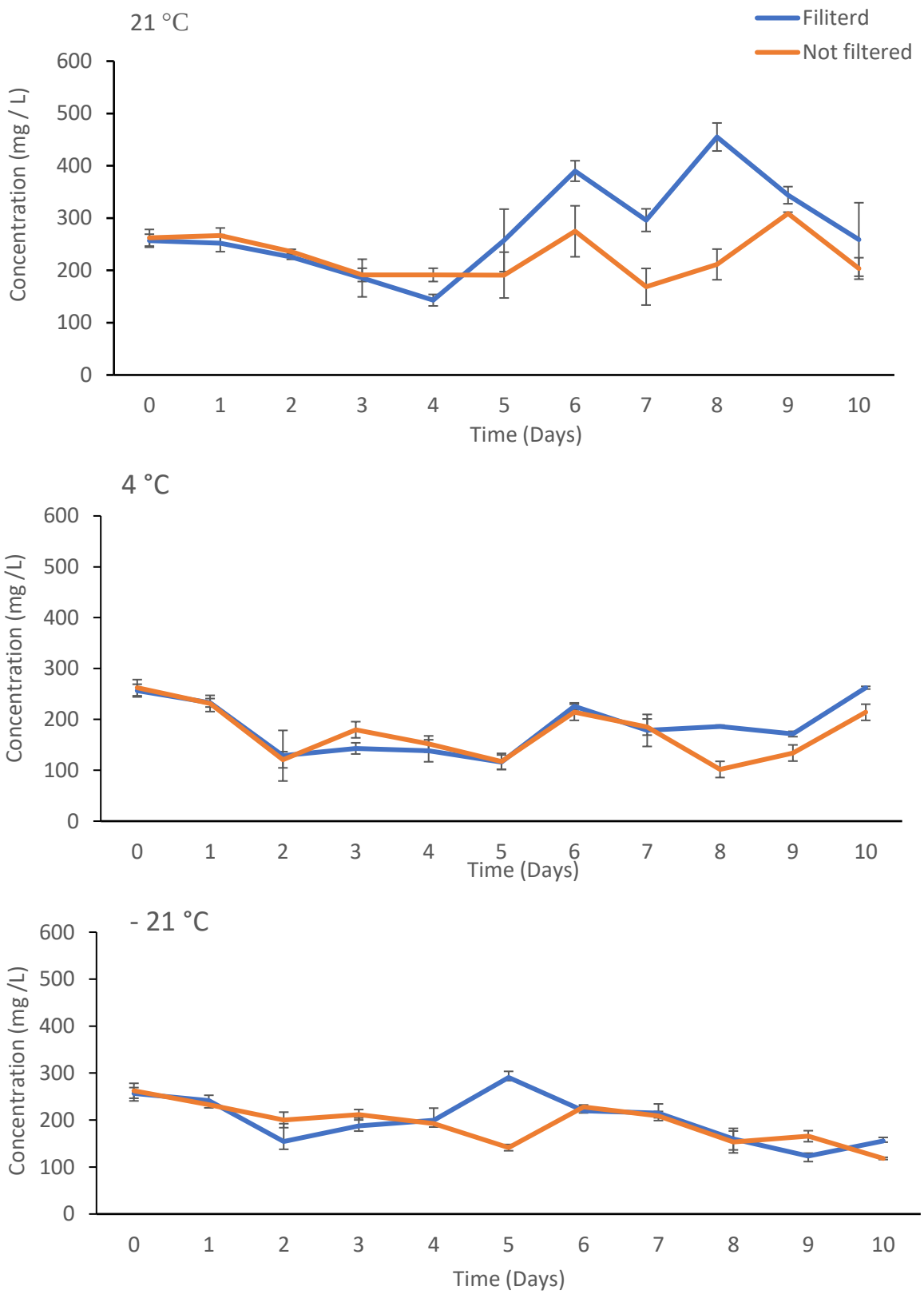


Figure 3.3. The stability of acetate in pot ale under different storage conditions. The error bars are representative of the standard deviation from the mean. n = 3

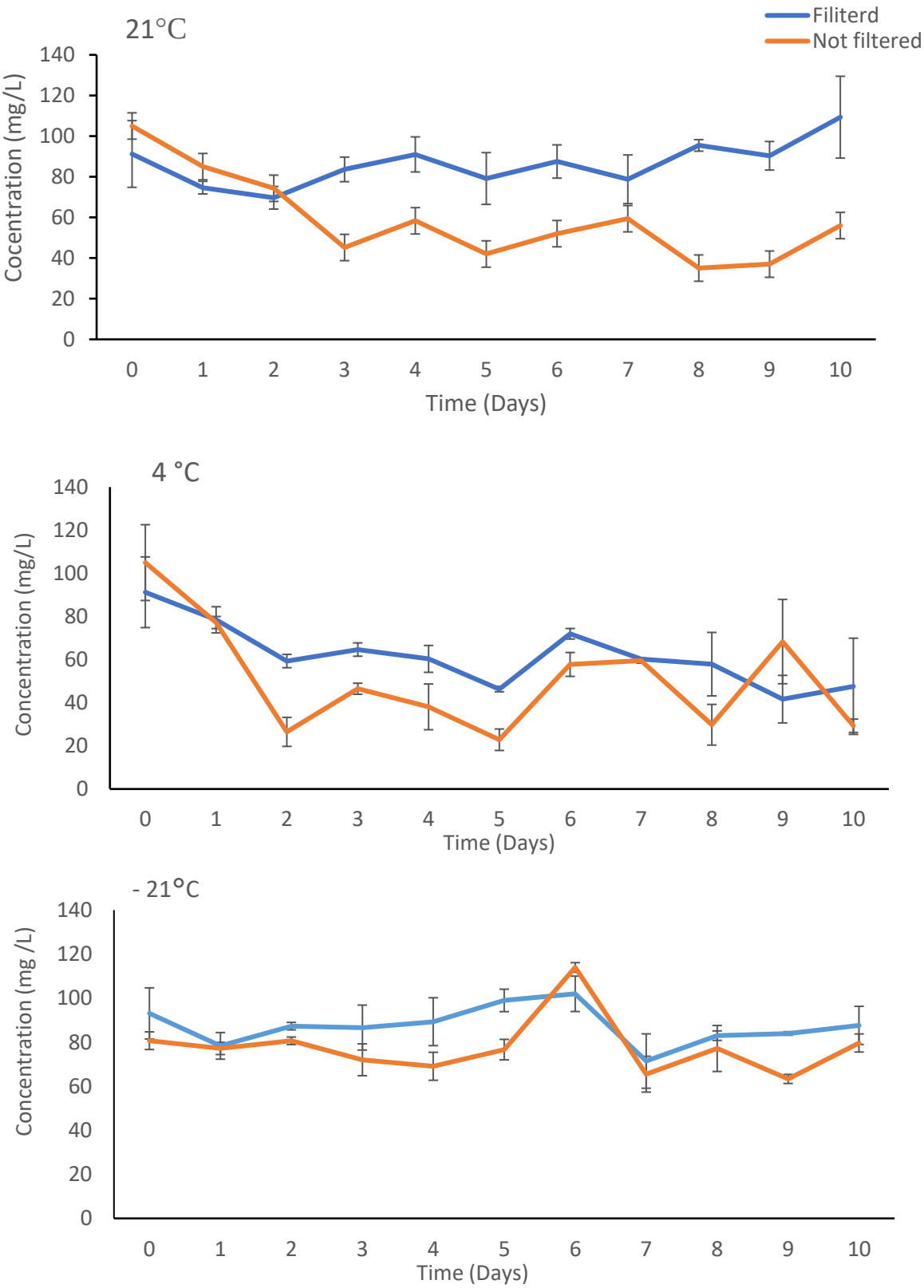


Figure 3.4. The stability of nitrate in pot ale under different storage conditions. The error bars are representative of the standard deviation from the mean. n = 3.

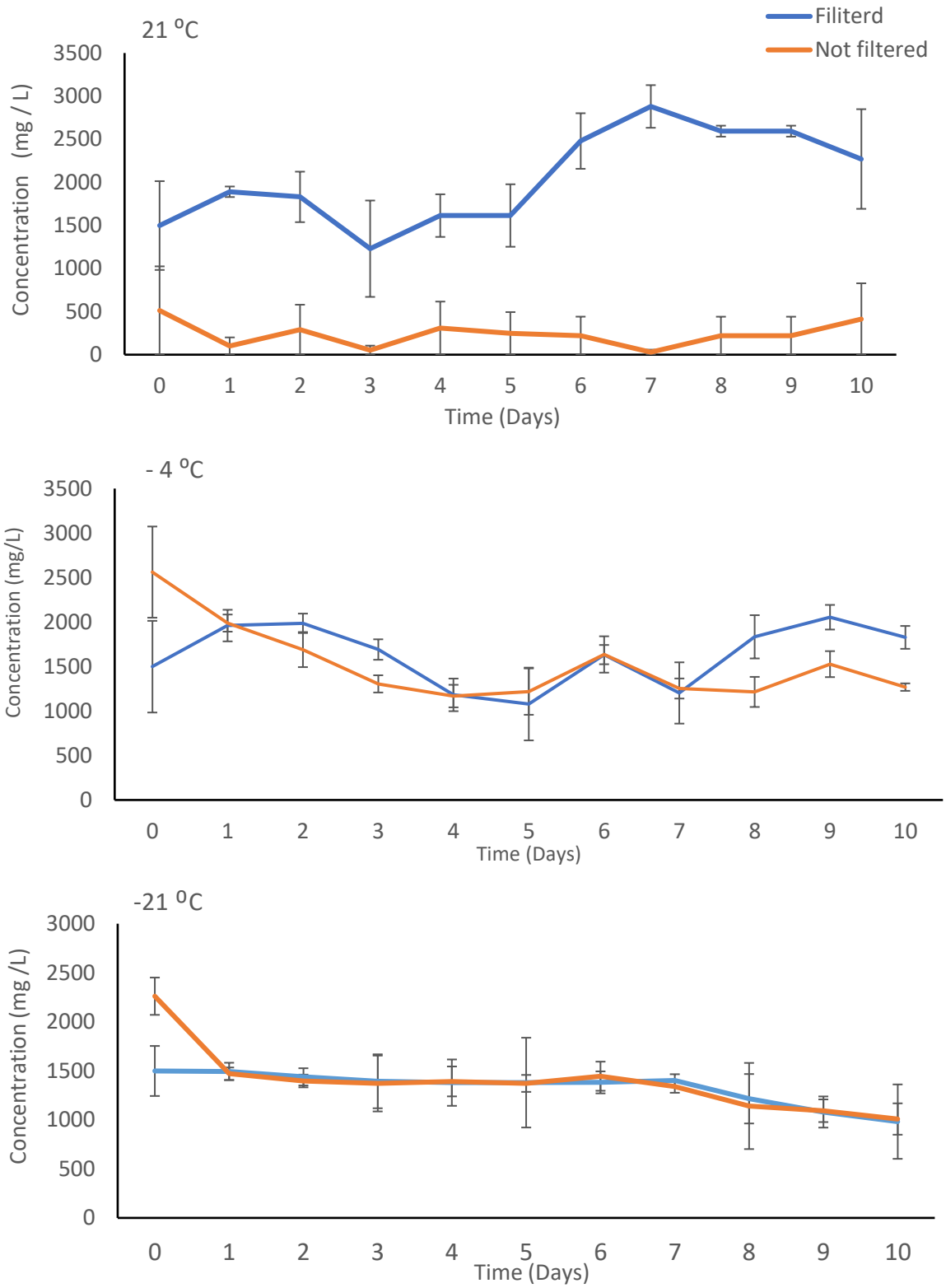


Figure 3.5. The stability of phosphate in pot ale under different storage conditions. The error bars are representative of the standard deviation from the mean. n = 3.

3.3.2 Findings of a Scotland wide pot ale survey

The response rate of the audit was 88 % with samples received from 22 distilleries that received a sampling kit. The key parameters (barley variety, malt peating level, wort clarity, yeast strain, length of fermentation) that may influence the chemical composition of pot ale was collected from all distilleries (Table 3.3). The data indicates that the pot ale sampled gave a good representation of the variation likely to be present in this Scotch whisky by-product. Two of the samples were from grain whisky distilleries, while the remaining twenty were from malt whisky distilleries. Of these malt distilleries, twenty were processed from Concerto barley, the key variety used at the time of the study. The other two distilleries used Sienna (Distillery A) and Laureate (Distillery K). This provided an opportunity to examine if the barley variety was likely to have an impact on pot ale composition. There was a good spread of distilleries using unpeated versus peated malt. Wort clarities were predominantly clear, with only three distilleries (Distilleries C, D and G) using a cloudy wort (Distilleries L, N, U and V did not give a response). A range of yeast strains had been used, with the industry favouring Mauri and Kerry strains at the time. Nine distilleries used the Kerry M strain (Distilleries D, G, K, L, Q, R, S, U and V) with seven others using the equivalent Mauri Pinnacle strain (Distilleries B, C, E, H, J, N, O and P). A further five distilleries (Distilleries A, F, M, N and T) used the Mauri Pinnacle MG+ yeast strain marketed as being more effective at utilising carbohydrates present in the wort (Distillery I did not state their yeast strain used). There was significant variation in the fermentation time between distilleries, with the length of fermentation ranging from 48 – 114 hours. Data was also collected on the time in transit between sampling and date of receipt. This ranged from 1 – 8 days.

Table 3.4. Results of a Scotland wide pot ale survey

Distillery code	Barley variety	Malt peating level	Wort clarity	Yeast strain	Length of fermentation (hours)	Sample date	Date of receipt	Time in transit (days)
A	Sienna	unpeated	clear	Mauri MG +	62	06/04/2018	12/04/2018	6
B	Concerto	unpeated	clear	Mauri pinnacle	70	13/03/2018	19/03/2018	6
C	Concerto	unpeated	cloudy	Mauri pinnacle	50	01/05/2018	04/05/2018	3
D	Concerto	unpeated	cloudy	Kerry MSI Cream	70	13/04/2018	19/04/2018	6
E	Concerto	unpeated	clear	Mauri	60	13/03/2018	19/03/2018	6
F	Concerto	unpeated	clear	Mauri MG+	56	15/03/2018	19/03/2018	4
G	Concerto	unpeated	cloudy	Kerry M strain	85	14/03/2018	19/03/2018	5
H	Concerto	heavily peated	clear	Mauri pinnacle	53	13/04/2018	17/04/2018	4
I	Concerto	heavily peated	clear	n/d	109	13/03/2018	19/03/2018	6
J	Concerto	heavily peated	clear	Mauri	59	11/04/2018	17/04/2018	6
K	Laureate	unpeated	clear	Kerry MSI cream	60	15/03/2018	19/03/2018	4
L	Concerto	unpeated	unknown	Kerry MSI Cream	60	28/03/2018	03/04/2018	6
M	Concerto	unpeated	clear	Mauri MG+	56	19/03/2018	27/03/2018	8
N	concerto	unpeated	unknown	Mauri pinnacle	48	07/03/2018	12/03/2018	5
O	Concerto	unpeated	clear	Mauri pinnacle	51	21/03/2018	22/03/2018	1
P	Concerto	unpeated	clear	Mauri pinnacle	65	06/04/2018	12/04/2018	6
Q	wheat			Kerry	57	13/03/2018	19/03/2018	6
R	wheat			Distillery yeast M cream	91	19/03/2018	27/03/2018	8
S	Concerto	unpeated	clear	Kerry	114	20/03/2018	22/03/2108	2
T	Concerto	unpeated	clear	Mauri MG +	68	22/03/2018	27/03/2018	5
U	Concerto	medium peated	unknown	Kerry	n/d	11/04/2018	15/04/2018	4
V	Concerto	medium peated	unknown	Kerry M strain	98	14/03/2018	20/03/2018	6

3.3.3 The occurrence of organic acids in pot ale

The most abundant organic acid present in all samples except for sample M was lactic acid. Additional organic acids that were present in all samples were identified as acetic acid and succinic acid (Figure 3.5). The median concentrations of the organic acids were as follows lactic acid–1675 mg/L, acetic acid–165 mg/L and succinic acid 225-mg/L. A previous study has also identified lactic acid and acetic acid in pot ale in similar proportions to the concentration observed in this investigation (Tokuda et al., 1998). Another previous study reported an acetic acid concentration of approximately 1000 mg/L and a lactic acid concentration of approximately 500 mg/L (Graham et al., 2012). However, it was noted that the acetic concentration may have increased as the pot ale sample had soured due to microbial action.

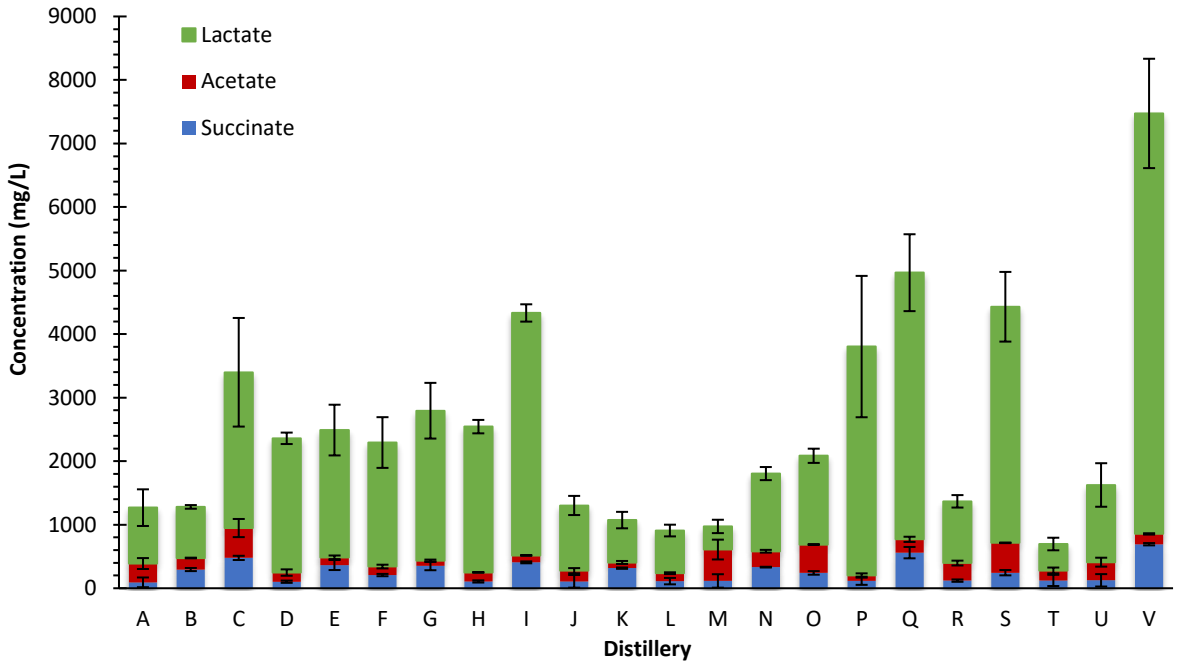


Figure 3.6. The concentration of organic acids found in pot ale across the distilleries. The error bars are representative of the standard deviation from the mean (n =3). Lactic acid (green), Acetic (red) and Succinic acid (blue).

Distillers yeast (*Saccharomyces Cerevisae*) does not produce lactic acid (CH_3-CHOHCOOH) during fermentation. The significant lactic acid concentration observed in pot ale can be attributed to the presence of lactic acid producing bacteria in the fermentation stage (Beek and Priest, 2002). It is known that *Lactobacillus* strains can survive the mashing process, the maximum temperature of mashing is typically (70 °C) (Priest and Pleasants, 1988). These bacteria are subsequently introduced along with the wort into the fermentation vessel (Wilson, 2014). Typically, the lactobacilli will begin flourishing after the yeast reach their stationary phase and will grow on autolysing yeast cells and residual nutrients (Figure 3.7) (Priest,2004). Therefore, they do not typically directly compete with the yeast for nutrients, however, if high concentration of lactobacilli ($> 10^6$ cells/ml) enter the fermentation they will begin competing with nutrients and can reduce the ethanol yield (Beek and 2002). The growth of lactobacilli is encouraged by some distillers as many believe that they have a positive effect on the flavour of the final spirit. This can be achieved by having longer fermentation times (Priest, 2004), however, no statistically significant relationship could be found between fermentation time and lactic acid concentration (Figure 12A). It is possible that differences in mashing procedures such as temperature, washing procedure and mixing rate would influence lactic acid concentrations, as they could affect the initial concentration of lactobacilli in the wort prior to fermentation.

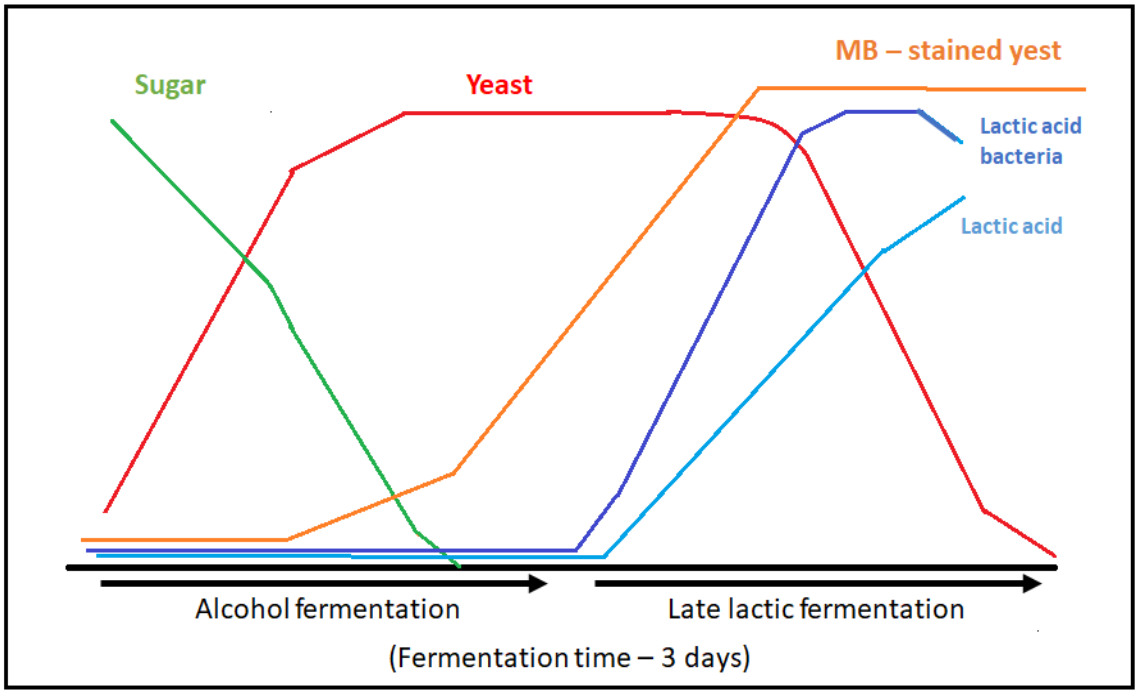


Figure 3.7. The general progress of malt whisky fermentation. MB – stained refers to yeast cell that have been stained with methylene blue and therefore not viable. Adapted from (Priest, 2004).

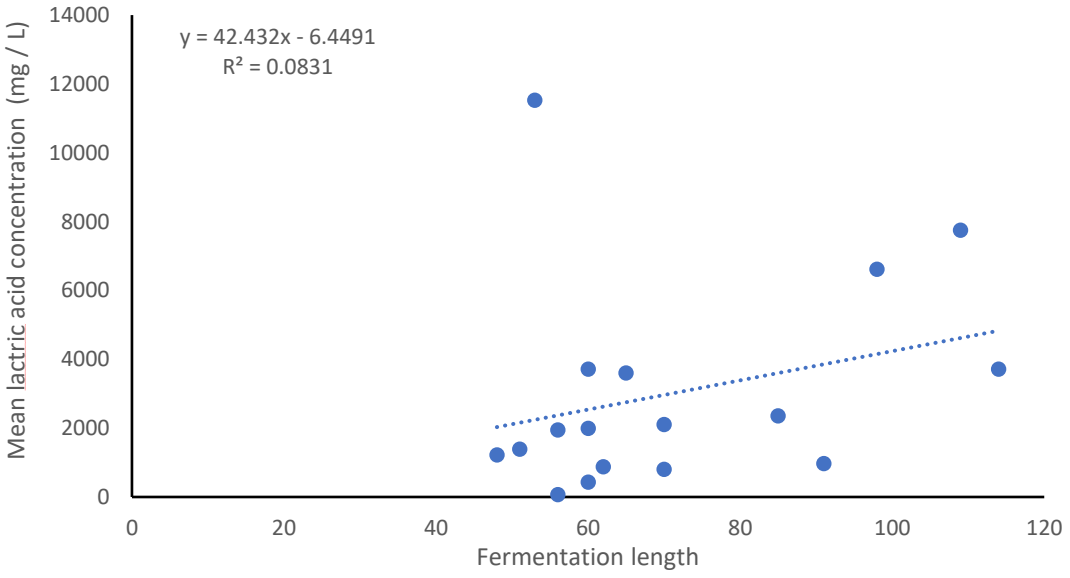


Figure 3.8 The relationship between mean lactic acid concentrations and fermentations times for each pot ale sample.

The observed acetate and succinate concentrations were also variable across the sampling sites (Figure 3.5) and no statistically significant relationship could be found between their concentrations and the surveyed processing parameters, after principal component analysis (Figure 11A, Figure 12A). Pot ale is a complex matrix and there is inherent variability, so it is possible a multitude of factors affect the concentration of organic acids. Lactic acid is a valuable industrial chemical and is widely used as a food preservative and is a key component of Polylactic acid (PLA) which has a wide range of applications which include surgical sutures, disposable plastic packaging and drug delivery systems (Melnicki *et al.*, 2013). There is potential to recover the lactic acid present in pot ale using ion exchange chromatography (Chapter 5).

3.3.4 Nitrate and Phosphate content of soluble pot ale

Pot ale is known to have high concentrations of phosphate but comparitvely lower concentrations of nitrate (Tokuda, 1999; Barrena, 2018) There is potential for valorisation as these nutrients could be used for microalgae growth or in the case of phosphate directly recovered. It is also important to gain a better understanding of the nutrient content of pot ale due to environmental concerns. Waste pot ale is typically spread on land, these nutrients therefore have the potential to pollute water courses and cause eutrophication.

The nitrate and phosphate concentrations are also highly variable with large variability between sampling sites. The observed ranges for nitrate and phosphate were 10 – 123 mg/L and 859 – 3198 mg/L, respectively (Figure 3.9). The median concentration of nitrate was 45 mg/L while phosphate was 1580 mg/L which results in a median N:P ratio of 1:30.

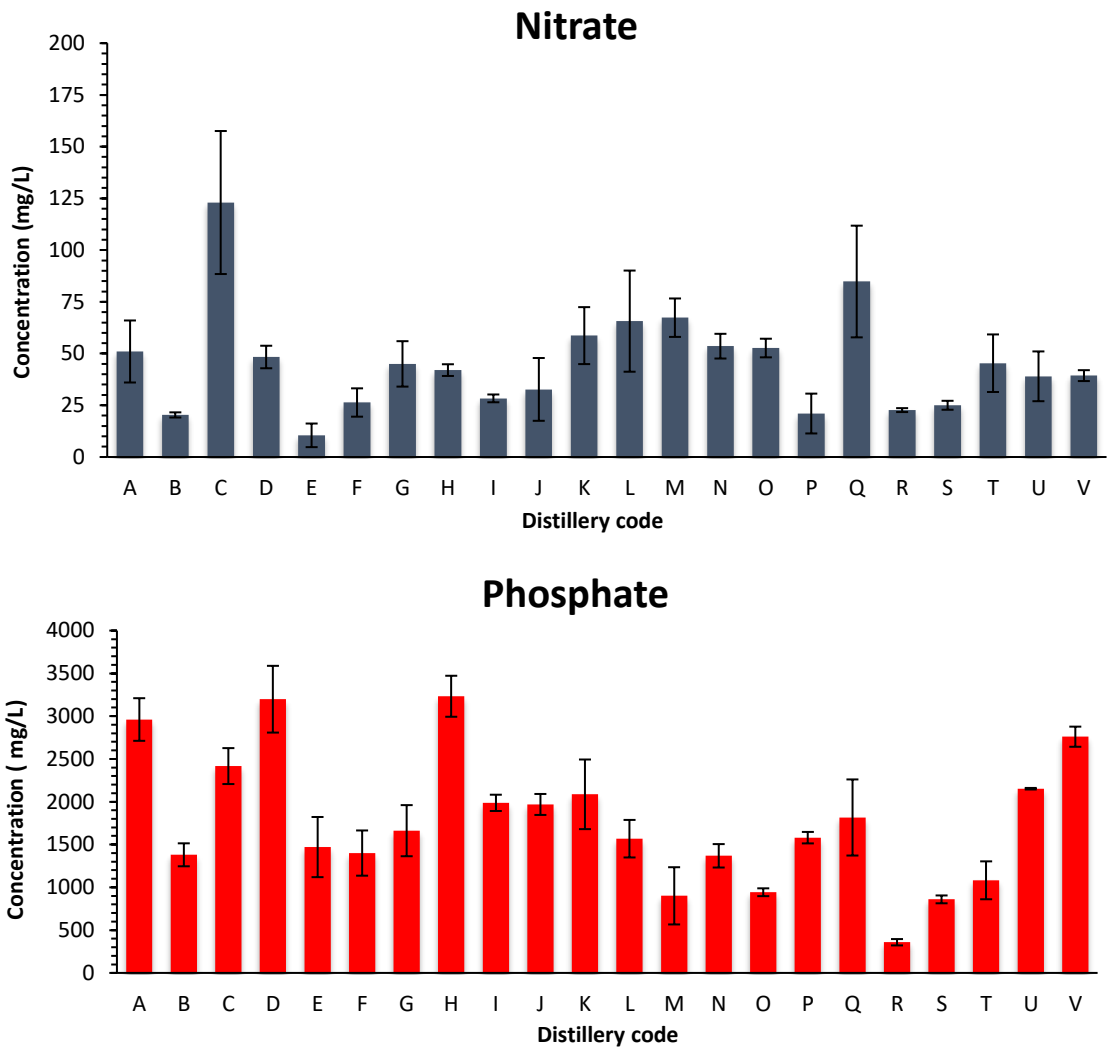


Figure 3.9. The mean concentration of nitrate and phosphate found in the pot ale samples from each distillery represented the audit. The error bars are representative of the standard deviation from the mean. n=3

The high concentrations of phosphate present in pot ale causes significant environmental concern largely due the potential of eutrophication. Several previous studies have investigated the removal of phosphates from pot ale (Dionisi, et al. 2014; Tokuda et al. 1999). An investigation by Tokuda et al, (1999) removed phosphate using a magnesium and phosphate ($MgNH_4PO_4$) reactor which converts the phosphate present in pot ale into struvite via the addition of magnesium salts. The process achieved a phosphate removal efficacy of 90 %. (Tokuda, Fujiwara and Kida, 1999). Struvite is a phosphate rich fertilizer and is an effective alternative to mined phosphate for use in agriculture (Rahman et al., 2014). Tokuda et al., (1999) also investigated the removal of

nitrate from pot ale using biological denitrification. The process utilised a 65 L fluidized – bed reactor which included denitrifying bacteria that were immobilised on cellulose based flotation carriers. Similarly, this process also achieved a removal rate of 90 % (Tokuda, Fujiwara and Kida, 1999). A more recent study carried out by Dionisi et al, (2014) investigated the removal of phosphate from pot ale using chitosan absorption. The removal efficiency was highly pH dependent with the highest phosphate reduction of approximately 60 % rate occurring at pH 9, the pH was adjusted using NaOH. The depletion of geological phosphate rock which is a finite resource is currently a global concern and the popularity of alternative fertilizers such as struvite is likely to increase as the availability of mined phosphate diminishes (Scholz *et al.*, 2013b). Phosphate is a critically important nutrient, as current agricultural practices are heavily reliant on large quantities of phosphate for both use as supplements in animal feed and as a crop fertiliser. Estimates of the total global phosphate reserves vary as new offshore deposits are found and extraction technology advances but the fact remains that phosphate is a finite resource. Therefore, the recovery of phosphate from waste such as pot ale will become increasingly important (Scholz et al. 2013; Dawson & Hilton. 201).

3.3.5 The metal content of pot ale and the impact of filtration on concentration

The total and soluble metal concentrations found in pot ale was determined (Table 3.5). Except for Mg (- 1.5 %) and Zn (57 %) most metals that were analysed were found in the total fraction largely bound to organic solids (Table 3.5). The mean soluble copper concentrations were approximately 90 % lower than the values obtained from the digested sample. Previous studies have also shown that the copper concentration of pot ale is largely bound to solids. Graham et al. (2012) reported a copper concentration of 2 – 5 mg L^{-1} with 50 % being soluble whilst Quinn et al. (1980) reported copper concentrations of 2.1–2.3 mg L^{-1}, with up to 70 % being bound to solids.

Table 3.5. Summary of the soluble metal and total metal concentrations found in pot ale. n/d = not detected due to being under detection limits (n=22).

		Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
Maximum (mg/L)	Soluble	234	4.8	1.7	n/d	2.9	15.9	0.16	0.50	n/d
	Total	304	6.3	36.6	4.9	18.6	18.6	5.72	7.84	0.14
Median (mg/L)	Soluble	195	n/d	0.28	n/d	0.10	0.95	n/d	n/d	n/d
	Total	182	1.50	4.27	1.14	3.30	3.83	3.59	0.83	n/d
Minimum (mg/L)	Soluble	89.9	n/d	n/d	n/d	n/d	n/d	n/d	n/d	n/d
	Total	126	n/d	2.01	n/d	0.63	n/d	3.59	n/d	n/d
Mean (mg/L)	Soluble	185	0.46	0.39	n/d	0.52	2.10	0.03	0.02	n/d
	Total	188	2.18	8.65	1.79	5.62	4.91	2.93	1.35	0.02
Standard deviation (mg/L)	Soluble	37.8	1.14	0.48	n/d	0.84	3.64	0.05	0.10	n/d
	Total	45.1	1.58	8.08	1.27	5.02	4.85	1.68	1.85	0.04
% Bound to solids		1.5	79.0	95.5	100	90.7	57.3	99.2	98.3	100

The copper concentration of pot ale has long been a concern due to its toxicity. Copper is present in pot ale due to the use of traditional copper stills. Animal feed derived from distillery by products cannot be given to sheep as they are highly sensitive to copper poisoning (Wainman and Dewey, 1982). A study investigating acute copper poisoning in sheep determined that toxicity begins at 20 mg/L and that concentrations of 50 mg/L are 100 % fatal. The median total concentration of copper detected across all sampling sites was 3.330 mg/L, although there was significant variance with a maximum concentration of 18.75 mg/L and minimum concentration of 0.63 mg/L. Therefore, all of the pot ale samples are under the toxicity threshold for sheep. However, if the pot ale was concentrated in order to produce feed products for example pot ale syrup, then the resulting copper concentrations for some of the distilleries, will likely be in excess of the toxicity threshold for sheep. A study carried out by Murphy et al installed a reed bed system at Scotch whisky distillery, for the purpose of copper removal from distillery effluent (Murphy, Hawes and Cooper, 2009). The reed bed system removed copper at a rate of 61 mg/m^2/d. It has also been reported by Jack et al., (2014), that some malt whisky distilleries have ion exchange systems installed for copper removal from distillery waste streams. Sample L in this study had a significantly lower total copper concentration of 0.63 mg/L when compared to the median concentration of 3.33 mg/L. It is possible that this distillery is utilising a process which removes copper (Table 1A and 2A in the appendices).

The most abundant metal found in all samples was magnesium with a median concentration of 182 mg/L and range of 126 to 304 mg/L (Table 3.5). Cereal grains including barley are known to contain high concentrations of magnesium (Venn and Mann, 2004). A previous study by Barrena et al. (2018) reported a mean magnesium concentration of 192 mg/L for centrifuged pot ale, which concurs with the concentrations found here. Significant concentrations of iron (Fe) and zinc (Zn) were also detected with median concentrations of 4.27 mg/L and 3.83 mg/L respectively although there was variation between samples (Table 3.5).

The results of this study suggest that other toxic heavy metals found in pot ale such as Cu, Mn, Fe, Pb, Cr, Ni and Cd, are also largely bound to solids (Table 3.5). This indicates that a large portion of these undesirable metals can be removed via filtration.

3.3.6 The total carbon content of pot ale

The median total organic carbon content (TOC) of pot ale across all sampling sites was 13027 mg/L with a standard deviation of 1579 mg/L. There is significant variation between sampling sites (Figure 3.10). A study carried out by Tokuda which analysed the TOC of pot ale from four Japanese malt whisky sites observed a mean TOC 16,415 mg/L (Tokuda et al., 1999).

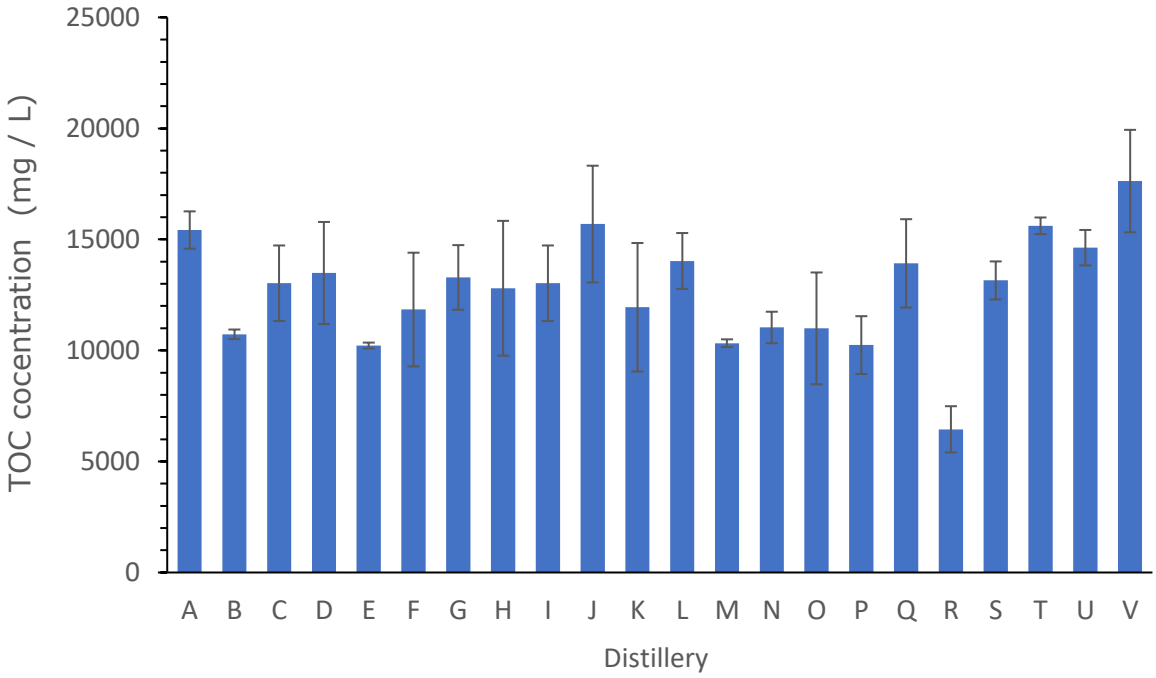


Figure 3.10. The concentration of total organic carbon (TOC) in each sample. The error bars are representative of the standard deviation from the mean (n = 3).

The high concentrations of organic carbon present in pot ale can likely be attributed to the presence of organic acids and other organic molecules such as proteins, amino acids and sugars (Traub et al. 2015). Using data from this investigation it can be estimated that organic acids are responsible for on average approximately 20 % of total organic carbon concentration of pot ale. Substances that have a high TOC concentration will also have high COD concentrations as COD is measurement of the available oxygen that can be consumed during reactions.

Therefore, COD is frequently used to determine the organic content of water. The relationship between the COD and TOC is typically linear although the precise ratio is dependent on the organic components of the given material (Bernardo et al., 2012). Using existing literature and the TOC data gathered in this investigation it can be estimated that the COD/TOC ratio of pot ale is approximately 3:1 (Tokuda et al., 1999; Julio Enrique Traub et al., 2015; Barrena *et al.*, 2018).

The high TOC of pot ale is a serious threat to marine and fresh water ecosystems and its disposal is highly regulated by Scottish environmental protection agency (Goodwin, Finlayson and Low, 2001). Current disposal methods include spreading on land as a low-grade fertiliser or in select cases dilution and direct discharge into the sea (Dionisi et al. 2014). SEPA guidelines state that trade effluent being discharged into the environment should not have a COD of > 500 mg/L (SEPA, 2014). The anaerobic digestion of pot ale to produce methane gas is an increasingly popular alternative disposal method for pot ale (Barrena et al. 2018; Goodwin et al. 2001). Successful anaerobic treatment of ethanol and grape wine distillery wastewaters has been demonstrated in several previous studies (Satayawali & Balakrishnan. 2008; Bustamante et al. 2005). A study by Goodwi*n* et al. (2001) achieved a COD removal rate of 70-90 % from diluted pot ale using a up flow anaerobic sludge blanket digestor (UASB) at a mean organic loading rate of 5.46 kg COD/m^3/day (Goodwin et al., 2001). Comparitvely a study by Tokuda et al, (1999) achieved a COD removal rate of 70-80% at a loading rate of 20 kg COD/m^3 day. Something which may limit the organic loading rate of the anaerobic digestion of pot ale is its high protein content. The presence of high concentrations of protein in AD systems can lead to build up of ammonia which at significant concentrations can

have an inhibitory effect on methanogenic activity (Ariunbaatar *et al.*, 2015). A study by Barrena et al. (2018) investigated the batch anaerobic digestion of deproteinated pot ale. The study deproteinated pot ale from 4 independently owned malt whisky distilleries and anaerobically digested them using 2 different inoculums sourced from AD plants. No significant differences in methane yield between un-treated and deproteinated pot ale was observed. However, the deproteination process reduced the COD of pot ale by up to 35 %, therefore there is a possibility of the process being a beneficial wastewater treatment process for pot ale (Barrena et al. 2018).

3.3.7 Concentrations of free and hydrolysed monosaccharides in pot ale.

There were low concentrations of free monosaccharides present with a median concentration of 0.028 g/L for total monosaccharides. The median value of total monosaccharides significantly increased after hydrolysis to 0.820 g/L. This indicates that most of the monosaccharides in pot ale form part of more complex carbohydrates (Table 3.6). Glucose was the most abundant sugar present in all hydrolysed samples. However, this is likely due to the hydrolysis process breaking down more complex polysaccharides e.g. cellulose into smaller monosaccharides such as glucose. Future work is required to determine what complex polysaccharides are present.

Table 3.6. Median carbohydrate content of the pot ale samples analysed using IC. (n = 22)

	Non-hydrolysed mean (g/L)	**Hydrolysed mean (g/L)**
Glucose	0.015	0.648
Arabinose	0.005	0.046
Galactose	0.002	0.017
Xylose	0.005	0.067
Mannose	0.001	0.043
Total monosaccharides	0.028	0.820

Differences were observed in carbohydrate composition from pot ale collected from grain and malt distilleries.Pot ale sampled from grain distilleries (Q & R) had far greater mean concentrations of arabinose (94.2 mg/L), galactose (33.4 mg/L) and xylose (170 mg/L) when compared to values observed for malt distilleries (arabinose: 41.94 mg/L; galactose: 15.66 mg/L and xylose: 58.22 mg/L). With exception of distilleries P, S and V, pot ale collected from malt distilleries generally had greater concentrations of glucose present when compared to grain distilleries (Table 3A and 4A in appendix). Although it would be desirable to have a better representation of data from grain distilleries.

Carbohydrate rich waste streams can be utilised as feedstocks for the fermentative production of second-generation biofuels. Second generation biofuels can be defined as biofuels derived from food waste or lignocellulosic feedstocks. These are preferential to the first-generation biofuels as they do not require arable land and do not compete with food sources. Other carbohydrate rich feedstocks that have been utilised to produce second-generation biofuels include cheese whey, potato starch and instant noodle waste, as well as others (Zhang et al.,2016). Similar to pot ale these feedstocks generally must be hydrolysed in order to make their carbon sources more bioavailable for fermentation. (Table 3.7). In comparison to these feedstocks, pot ale has lower concentration of potentially fermentable sugars present, largely due to its dilute form. A reduction in the pot ale aqueous fraction would improve its potential to produce second-generation biofuels.

Pot ale and draff are already utilised as a feedstock for fermentative processes using *Clostridium sp.* which produce biobutanol (Zero Waste Scotland, 2017). Therefore, there may be further potential to exploit pot ale and other similar distillery/brewery waste streams to produce biobutanol and bioethanol.

Table 3.7. Summary of liquid biofuels from food processing wastes (Zhang et al., 2016).

Biofuel	Food processing waste (g/L)		Pre-treatment	Oleaginous microorganism	Microbial alcohol concentration (g/L)
	Type	Carbon source			
Biobutanol	Potato starch	60 starch	-	*Clostridium acetobutylicum*	9.9
	Inedible dough	50 starch	-	*Clostridium. beijerinckii*	9.26
	Cheese whey	50 glactose	-	*Clostridium acetobutylicum*	7.25
Bioethanol	Instant noodle waste	167 starch	Enzymatic hydrolysis	*Saccharomyces cerevisiae*	61.1
	Potato peel	6% potato peel	Enzymatic hydrolysis	*S. cerevisiae*	19.6
	Cheese whey	150	Dilution	*S. cervisiae*	63.3
	Whey permeate	50 – 108	-		-

3.3.8 Amino acid analysis

Amino acids have been produced on an industrial scale by microbial fermentation for more than 50 years. Essential amino acids such as lysine and methionine form a large proportion of the market and are commonly used to fortify the nutritional properties of food stuffs and animal feed. Amino acids such as glutamine are also used to produce flavour enhancers in the food industry. (Leuchtenberger et al. 2005); (Friedman and Finot, 1990); (Friedman and Brandon, 2001). There could be potential for valorisation if significant concentrations of essential amino acids are identified in pot ale. There was considerable variation observed in the amino acid concentrations between the distilleries, however, there was no observable relationship between amino acid concentrations and processing parameters following PCA analysis (Figure 12A). An investigation assessing fermentative methods for the production of L–lysine found a maximal yield of 22.8 g/L (Razak and Viswanath, 2015). In comparison the median concentration found in pot ale by this study was 93 mg /L. It is therefore unlikely that there is any potential for valorisation of amino acids from pot ale due to the low concentrations observed (Table 3.8).

Proline was the most abundant amino acid present in all samples with a median concentration of 705 mg/L and higher than the other detected amino acids (Figure 3.9). There was also large variance in amino acid concentrations across the distilleries. The range of total amino acids was 2737 – 195 mg/L with a median value of 1515 mg/L. The lowest values were observed in sample R (Table 3.8).

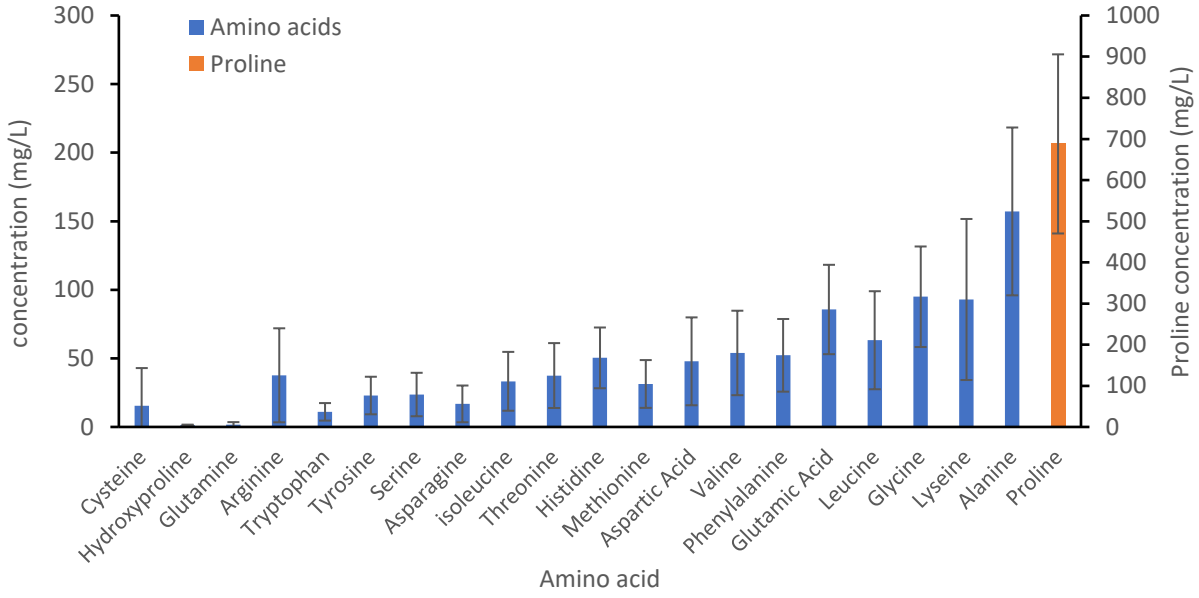


Figure 3.10. The median concentration of amino acids across all distilleries. The error bars are representative of the standard deviation from the mean. The concentration of proline is displayed on the secondary y axis. n = 22

Proline is common in many higher plants and acts as an osomoregulator, to protect against osmotic stress (Kishor *et al.*, 2005). Proline is the second most abundant amino acid present in barley grains after Glutamine (Jaikaran et al. 2018). During fermentation yeast will actively use free amino acids for the synthesis of proteins (Jones and Pierce, 1964). A study by Lekkas et al found that there was no uptake of proline during fermentation (Lekkas *et al.*, 2007). Proline metabolism requires oxygen and a functioning electron transport chain. As whisky production employs anaerobic fermentation this could explain the significantly higher proline concentration in comparison to other free amino acids. Due to the low concentrations of desirable amino acids observed in this study (Table 3.8), the further valorisation of the amino acids from pot ale would require the adoption of a significant concentration step, which would have to be proven to be financially viable.

Table 3.8. The mean concentration of analysed free amino acids in each distillery sample. Units = mg/L. (n=3).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
Cysteine	1	1	2	2	1	2	1	2	2	2	85	51	86	1	1	2	1	4	3	30	1	1
Hydroxyproline	2	2	1	1	1	1	1	1	2	1	1	1	2	1	1	1	1	0	1	1	2	1.5
Glutamine	2	3	1	1	1	0	0	0	3	1	1	1	1	0	0	2	6	8	4	1	0	0.3
Arginine	116	13	14	33	18	15	14	13	13	14	102	88	93	22	59	13	9	9	13	71	21	61
Tryptophan	21	16	14	5	9	11	2	14	24	12	11	7	9	8	7	14	8	1	27	6	8	7.4
Tyrosine	40	27	20	10	14	21	6	24	62	24	32	12	17	12	17	29	23	1	50	18	21	24
Serine	41	41	31	9	16	32	0	27	64	29	21	10	19	11	11	18	38	5	54	13	13	16
Asparagine	10	42	20	7	7	6	6	31	48	39	25	24	6	3	4	10	7	5	24	21	21	4.5
Isoleucine	53	50	40	15	28	42	9	38	82	31	29	12	30	25	24	47	26	2	93	16	16	25
Threonine	62	55	38	20	29	42	7	35	84	41	35	21	33	27	27	59	21	3	108	20	26	29
Histidine	92	58	43	43	41	45	34	65	83	75	40	46	45	38	37	55	17	3	101	45	58	42
Methionine	55	62	47	22	33	41	9	30	50	31	25	16	39	29	23	41	18	1	70	13	16	18
Aspartic Acid	103	63	65	38	50	30	4	22	106	41	34	22	56	46	53	53	16	4	132	20	27	68
Valine	85	79	60	26	43	66	18	63	122	62	50	24	49	40	39	76	43	4	134	29	33	42
Phenylalanine	88	79	61	29	45	61	18	63	105	52	52	25	55	41	37	70	55	2	112	27	36	36
Glutamic Acid	100	81	62	75	58	89	33	92	142	98	66	113	64	57	59	110	113	46	183	87	82	75
Leucine	105	105	76	31	54	82	14	77	138	62	63	19	59	48	45	85	74	4	140	26	37	48
Glycine	136	113	95	68	82	106	65	120	157	141	89	83	83	75	75	109	38	3	168	102	108	73
Lysine	120	203	116	75	103	33	42	26	241	118	59	46	64	109	89	122	26	11	195	52	93	101
Alanine	229	212	164	103	113	168	105	230	271	249	180	158	119	99	103	177	96	32	247	114	171	118
Proline	1218	771	554	696	629	610	527	759	886	832	669	727	893	663	733	764	288	45	692	671	715	791
Total amino acid content	2737	2076	1524	1308	1374	1504	914	1730	2683	1955	1669	1504	1819	1356	1445	1859	923	195	2552	1380	1505	1587

3.4 Conclusions

It was observed by this investigation that the composition of pot ale degrades over time therefore it is important to consider this when attempting to valorise pot ale. Colder temperatures and treatment options such as filter sterilisation were shown to significantly improve pot ale stability.

The composition of pot ale is highly variable across the distilleries, however, no statistically significant relationship between processing parameters and pot ale composition could be identified. Sample R had significantly lower phosphate, TOC and total amino acid concentration when compared to the other distilleries. This distillery could potentially be treating the pot ale by either removing components or by dilution.

Toxic heavy metals such as copper that are often a concern for agricultural applications of pot ale were identified. However, the concentrations of these metals were relatively low and can be significantly reduced by filtration.

There were high concentrations of phosphate observed in pot ale across the distilleries which could present opportunities for valorisation either by direct recovery or by utilisation as a nutrient source. This investigation also identified that there is a high concentration of fermentable sugars present in pot ale after hydrolysis. These sugars could be utilised as feedstocks to produce second generation biofuels such as biobutanol and bioethanol.

Perhaps the most interesting resource recovery option highlighted in this audit is lactic acid recovery. Although the concentration of lactic varied significantly across the distilleries there were still high concentrations observed. The market demand for lactic acid is rapidly increasing due to the growing bioplastic industry. The lactic acid content of pot ale could be recovered at low cost using ion exchange technology. There is potential to compete with the growing lactic acid fermentation industry by providing a lower cost and thus a more sustainable option for the PLA bioplastic market.

3.5. References

Ariunbaatar, J., Scotto Di Perta, E., Panico, A., Frunzo, L., Esposito, G., Lens, P.N.L., Pirozzi, F., 2015. Effect of ammoniacal nitrogen on one-stage and two-stage anaerobic digestion of food waste. Waste Management 38, 388–398. https://doi.org/10.1016/J.WASMAN.2014.12.001

Barrena, R., Traub, J.E., Gil, C.R., Goodwin, J.A.S., Harper, A.J., Willoughby, N.A., Sánchez, A., Aspray, T.J., 2018. Batch anaerobic digestion of deproteinated malt whisky pot ale using different source inocula. Waste Management 71, 675–682. https://doi.org/10.1016/j.wasman.2017.06.025

Beek, S. V, Priest, F.G., Priest, F.G., 2002. Evolution of the Lactic Acid Bacterial Community during Malt Whisky Fermentation : a Polyphasic Study Evolution of the Lactic Acid Bacterial Community during Malt Whisky Fermentation : a Polyphasic Study. Applied and Environmental Microbiology 68, 297–305. https://doi.org/10.1128/AEM.68.1.297

Bernardo, M., Vespeira, C., Cantinho, P., Minhalma, M., Santos, A., 2012. Cork industry wastewater characterization: assessment of the biodegradability, reuse and of the relationship between BOD, COD and tannins with TOC. Journal of Water Reuse and Desalination 2, 33–39. https://doi.org/10.2166/wrd.2012.047

Delgadillo-Mirquez, L., Lopes, F., Taidi, B., Pareau, D., 2016. Nitrogen and phosphate removal from wastewater with a mixed microalgae and bacteria culture. Biotechnology Reports 11, 18–26. https://doi.org/10.1016/j.btre.2016.04.003

Dionisi, D., Bruce, S.S., Barraclough, M.J., 2014. Effect of pH adjustment, solid–liquid separation and chitosan absorption on pollutants' removal from pot ale wastewaters. Journal of Environmental Chemical Engineering 2, 1929–1936. https://doi.org/10.1016/j.jece.2014.08.013

Duncan, S.H., Louis, P., Flint, H.J., 2004. Lactate-Utilizing Bacteria, Isolated from Human Feces, That Produce Butyrate as a Major Fermentation Product. 70, 5810–5817. https://doi.org/10.1128/AEM.70.10.5810

Friedman, M., Brandon, D.L., 2001. Nutritional and Health Benefits of Soy Proteins. Journal of agricultural and food chemsitry 49, 1070–1086. https://doi.org/10.1021/jf0009246

Friedman, M., Finot, P., 1990. Nutritional Improvement of Bread with Lysine and 7-Glutamyllysine. Journal of agricultural and food chemsitry 38, 2011–2020. https://doi.org/10.1021/jf00101a005

Goodwin, J.A.S., Finlayson, J.M., Low, E.W., 2001. A further study of the anaerobic biotreatment of malt whisky distillery pot ale using an UASB system. Bioresource Technology 78, 155–160. https://doi.org/10.1016/S0960-8524(01)00008-6

Graham, J., Peter, B., Walker, G., Wardlaw, A., 2012. Chapter 43 Characterisation of the pot ale profile from a malt whisky distillery 1–8.

Gray, A. S. 2018. The Scotch Whisky Industry Review. 41st Edition. Pagoda Scotland.

House of Lords. 2014. Waste or resource? Stimulating a bioeconomy. HL Paper 141.

Available at: https://publications.parliament.uk/pa/ld201314/ldselect/ldsctech/141/141.pdf

Jaikaran,S., Hsu,H., Prommer,E,A., Recinos Diaz, G., 2018. Measuring Amino Acids in Western Canadian Hulled and Hulless Barley.

Jones, M., Pierce, J.S., 1964. Absorption of Amino Acids From Wort By Yeasts. Journal of the Institute of Brewing 70, 307–315. https://doi.org/10.1002/j.2050-0416.1964.tb01996.x

Julio Enrique Traub, 2015. Protein recovery from whisky by - products : a study of using ion exchange chromatography for the recovery of proteins from pot ale by Julio Enrique Traub Modinger Submitted for the degree of Doctor of Philosophy Heriot - Watt University Institute of Biol. Waste Management 177, 675–682. https://doi.org/10.1002/pmic.200600033

Kida, K., Morimura, S., Mochinaga, Y., Tokuda, M., 1999. Efficent removal of organic matter and phosphate from pot ale by a combination of methane fermentation and biological denitrification and nitrification processes 34, 567–575. https://doi.org/10.1016/S0032-9592(98)00129-0

Kishor, P., Sangam, S., Amrutha, R., Laxmi, P., 2005. , Uptake and Transport in Higher Plants: Its Implications in Plant Growth and Abiotic Stress. Curr. Sci 34, 5–18. https://doi.org/http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.471.372&rep=rep1&type=pdf.

Lekkas, C., Stewart, G.G., Hill, A.E., Taidi, B., Hodgson, J., 2007. Elucidation of the role of nitrogenous wort components in yeast fermentation. Journal of the Institute of Brewing 113, 3–8. https://doi.org/10.1002/j.2050-0416.2007.tb00249.x

Melnicki, M.R., Pinchuk, G.E., Hill, E. a., Kucek, L. a., Stolyar, S.M., Fredrickson, J.K., Konopka, A.E., Beliaev, A.S., 2013. Feedback-controlled LED photobioreactor for photophysiological studies of cyanobacteria. Bioresource Technology 134, 127–133. https://doi.org/10.1016/j.biortech.2013.01.079

Munoz, R, del las Rivas, B., 2011. Learn more about Lactic Acid Bacteria Lactic Acid Bacteria. Molecular Wine Microbiology 1, 103–167. https://www.sciencedirect.com/topics/food-science/lactic-acid-bacteria

Murphy, C., Hawes, P., Cooper, D.J., 2009. The application of wetland technology for copper removal from distillery wastewater: A case study. Water Science and Technology 60, 2759–2766. https://doi.org/10.2166/wst.2009.729

Özogul, F., Hamed, I., 2018. The importance of lactic acid bacteria for the prevention of bacterial growth and their biogenic amines formation: A review. Critical Reviews in Food Science and Nutrition 58, 1660–1670. https://doi.org/10.1080/10408398.2016.1277972

Park, Y.W., Lee, J.H., Lee, S.J., 2010. Effects of Frozen and Refrigerated Storage on Organic Acid Profiles of Goat Milk Plain Soft and Monterey Jack Cheeses. Journal of Dairy Science 89, 862–871. https://doi.org/10.3168/jds.s0022-0302(06)72150-6

Priest, F.G., Pleasants, J.G., 1988. Numerical taxonomy of some leuconostocs and related bacteria isolated from Scotch whisky distilleries. Journal of Applied Bacteriology 64, 379–387. https://doi.org/10.1111/j.1365-2672.1988.tb05094.x

Rahman, M.M., Salleh, M.A.M., Rashid, U., Ahsan, A., Hossain, M.M., Ra, C.S., 2014. Production of slow release crystal fertilizer from wastewaters through struvite crystallization – A review. Arabian Journal of Chemistry 7, 139–155. https://doi.org/10.1016/J.ARABJC.2013.10.007

Raspor, P., Goranovič, D., 2008. Biotechnological applications of acetic acid bacteria. Critical Reviews in Biotechnology 28, 101–124. https://doi.org/10.1080/07388550802046749

Razak, M.A., Viswanath, B., 2015. Comparative studies for the biotechnological production of l-Lysine by immobilized cells of wild-type Corynebacterium glutamicum ATCC 13032 and mutant MH 20-22 B. 3 Biotech 5, 765–774. https://doi.org/10.1007/s13205-015-0275-8

Russel, I., S.G., 2014. Whisky. Production, Technology and Marketing., 2nd ed. Academic Press. https://doi.org/10.1016/B978-0-12-401735-1/00024-6

Scholz, R.W., Ulrich, A.E., Eilittä, M., Roy, A., 2013. Sustainable use of phosphorus: A finite resource. Science of The Total Environment 461–462, 799–803. https://doi.org/10.1016/J.SCITOTENV.2013.05.043

Scotch Whisky Association. 2019. Economic Impact Report. Available at: https://www.scotch-whisky.org.uk/media/1591/final-2018-economic-impact-report.pdf

Tokuda, M., Fujiwara, Y., Kida, K., 1999. Pilot plant test for removal of organic matter, N and P from whisky pot ale. Process Biochemistry 35, 267–275. https://doi.org/10.1016/S0032-9592(99)00063-1

Tokuda, M., Ohta, N., Morimura, S., Kida, K., 1998. Methane fermentation of pot ale from a whisky distillery after enzymatic or microbial treatment. Journal of Fermentation and Bioengineering 85, 495–501. https://doi.org/10.1016/S0922-338X(98)80068-8

Venn, B.J., Mann, J.I., 2004. REVIEW Cereal grains, legumes and diabetes. European Journal of Clinical Nutrition 58, 1443–1461. https://doi.org/10.1038/sj.ejcn.1601995

Wainman, F.W., Dewey, P.J.S., 1982. The energy value to ruminants of malt distillers' draff, and of a mixture of draff and pot ale syrup. Animal Production 34, 325–328. https://doi.org/10.1017/S0003356100010278

Wang, J., Tang, H., Zhang, X., Xue, X., Zhu, X., Chen, Y., Yang, Z., 2018. Mitigation of nitrite toxicity by increased salinity is associated with multiple physiological responses: A case study using an economically important model species, the juvenile obscure puffer (Takifugu obscurus). Environmental Pollution 232, 137–145. https://doi.org/10.1016/J.ENVPOL.2017.09.026

Wilson, N., 2014. Chapter 8 – Contamination: bacteria and wild yeasts in a whisky fermentation, Second Edi. ed, Whisky. Elsevier Ltd. https://doi.org/10.1016/B978-0-12-401735-1.00008-8

Zero Waste Scotland, 2017. Biorefining Potential for Scotland. Biorefining potential for Scotland.

Zhang, Z., O'Hara, I.M., Mundree, S., Gao, B., Ball, A.S., Zhu, N., Bai, Z., Jin, B., 2016. Biofuels from food processing wastes. Current Opinion in Biotechnology 38, 97–105. https://doi.org/10.1016/j.copbio.2016.01.010

Chapter 4

The utilisation of pot ale as microalgal growth media

4.1 Chapter outline

This chapter investigates the growth of microalgae in pot ale. Laboratory scale growth experiments were conducted in order to determine the effect of various experimental pot ale medias on the growth of *Synechocystis sp* PCC 6803.

4.1.1 Introduction

Pot ale has high concentrations of phosphate and contains lower concentrations of nitrate with a N:P ratio of approximately 1:30 (section 3.3.4). The presence of significant concentrations of phosphate and nitrate in pot ale presents a disposal challenge for the industry as there is a risk of eutrophication. A large portion of Scotland's whisky distilleries are located on islands and remote coastal regions which limits options for disposal. Many distilleries discharge their pot ale directly into the sea under strict SEPA control (SEPA, 2017), therefore, nutrient recovery options could add value to the industry as well as lessening the environmental impact of pot ale disposal.

The cultivation of microalgae using pot ale as a nutrient source is a potential method of resource recovery. Large scale microalgal cultivation is known to be costly primarily due to the large volumes of light energy and water required. To date, industrial scale production of microalgae is only economically feasible when using natural sunlight, therefore, production at high volume is limited to geographical areas with sufficient incidences of natural sunlight. Nitrate and phosphate are also a large input cost that must be carefully managed for economically viable production. A prior study estimated that the cost of conventional media would be £ 2400/tonne for a production of 100 tonnes of biomass per annum (Lopes *et al.*, 2015). The high cost of conventional media limits its microalgae production to high value lower volume products such as pigments. To produce high-volume lower value products such as biofuels and feed supplements, alternative nutrient sources are required. Domestic and industrial wastewaters are an attractive option due to their low cost and availability. Many wastewaters contain the macronutrients that are required for microalgal growth, such as nitrate, phosphate, urea, ammonium and essential trace elements (Taziki *et al.*, 2016).

Municipal waste is a good option due to its favourable pH and dissolved CO_2 concentration. Microalgae are also commonly used in waste treatment plants as a way of reducing nitrate and phosphate via the removal of nutrients. High-rate algal pond photobioreactors (HRAPs) can be installed as part of tertiary waste water treatment installations (Alcántara *et al.*, 2015). HRAPs are largely based on the traditional raceway pond design and have large surface areas to improve light penetration. Wastewater can be continually pumped into the reactor as a nutrient source (Alcántara *et al.*, 2015).

Microalgae as a treatment option is also somewhat unique as it is possible to simultaneously add value whilst treating the waste. Wastewater can be integrated into microalgal production systems as a low-cost nutrient source, however, the downside to this approach is that pre-treatment of the waste is often required in order to optimise microalgal growth. Common pre-treatment methods for microalgal cultures include centrifugation and dilution. These pre-treatment methods are required to reduce the microbiological load and improve the transparency of the wastewater. This ensures that microalgae are not out competed by heterotrophic bacteria as there is sufficient light penetration for phototrophic growth (Alcantara et al., 2015)

The objective of this investigation is to assess if pot ale can be utilised for microalgal growth. Pot ale has a low pH of 3.5 due to the presence of high concentrations of organic acids. The pH is inhibitory to the majority of cyanobacterial and microalgal species except for rare extremophiles (Wang *et al.*, 2011), therefore, approaches to balance the pH will be addressed. The colouration of pot ale is another potential issue as it may limit light penetration. Pot ale also contains concentrations of copper and other metals which are toxic to microalgae when present in sufficient concentrations, therefore, metal toxicity is another potential issue that will be investigated.

4.2 Materials and Methods

4.2.1 Spectral analysis of pot ale dilutions

In order to assess if the colouration of pot ale has a negative effect on photosynthesis the absorbance of pot ale dilutions was tested alongside *Synechocystis sp.* PCC 6803. Dilutions of pot ale; 10%, 25% and 50% were prepared and aliquoted into 2 ml cuvettes. The dilutions were then analysed in triplicate using a scanning absorbance spectrometer (Thermo Helios α - Fisher Scientific, UK). The spectrometer was blanked using ultra-pure water (Elga, UK). The absorbance of *Synechocystis sp.* PCC 6803 was also tested in triplicate using a blank of BG-11 media (section 2.21) as the blank.

4.2.2 The effect of increasing concentrations of magnesium on the growth of *Synechocystis sp.* (PCC 6803).

Previous investigations have indicated that there are high concentrations of soluble magnesium in pot ale (89 - 234 mg/L) (Table 3.5). Magnesium in an essential element for photosynthetic life as Mg is required for the synthesis of chlorophyll (Rahman *et al.*, 2014). Magnesium is present in BG-11 in low concentrations (7.3 mg/L). The investigation utilised standard BG-11 as a control and BG-11 augmented with increasing concentrations of $MgSO_4 7H_2O$ in order to determine the effect, if any, of Mg on the growth of *Synechocystis sp*. PCC 6803. The concentrations of Mg that were tested were approximately 15, 30, 60, 120, and 240 mg/L. This was achieved by adding an additional 150, 300, 600, 1,200 and 2,400 mg/L of $MgSO_4 7H_2O$ to 100 ml of BG-11 (lacking magnesium) Table 4.1).

Table 4.1. Concentrations of additional Mg added to BG-11 media in the investigation.

Concentration of $MgSO_4 7H_2O$ added to BG-11 (mg/L)	Approximate additional Mg content of the BG-11 (mg/L)
150	15
300	30
600	60
1200	120
2400	240

The BG-11 media and BG-11 media containing additional Mg were prepared (100 ml in 250 ml Erlenmeyer flasks) and autoclaved at 121°C, 15 psi (Astell, UK).

The BG-11 and BG-11 + Mg media was then aseptically transferred to 24 well plates, 2 ml each well, and were then inoculated with 3-week-old *Synechocystis sp.* to produce a final cell density of 2.5 $x10^6$ cells/ml, in triplicate. A total of 7 plates were inoculated to produce sampling time points (0 – 6 days). The plates were incubated in a temperature control growth chamber (21°C, 12 hour light/dark cycle) as detailed in section 2.2.1. Each well plate was sampled in triplicate, daily using a Coulter counter (Multisizer® 4, Beckman Life Science, USA) as detailed in section 2.2.2.1.

4.2.3 The heterotrophic and phototrophic growth of *Synechocystis sp.* PCC 6803 in pot ale.

The turbidity and colouration of pot ale has the potential to limit light penetration which could inhibit phototrophic growth. *Synechocystis sp.* PCC 6803 can grow in both heterotrophic conditions and phototrophic conditions therefore it is well suited for this investigation (Osanai *et al.*, 2005).

Freshly collected pot ale was sourced from a single distillery and was filtered using Whatman GF/C filter paper (Sigma, UK). The filtered pot ale was then diluted to three concentrations (1/10, 1/20, 1/50) using ultra-pure water (Elga, Veolia water, UK). The diluted pot ale was then decanted into 250 ml Erlenmeyer flasks. BG-11 media was also prepared in 250 ml Erlenmeyer flasks as a control. A total of 6 replicate flasks were prepared for each pot ale dilution and the BG-11 control. The flasks were then autoclaved at 121°C, 15 psi (Astell, UK). Prior to inoculation half of the flasks were covered with tin foil to prevent light penetration and favour heterotrophic growth.

The pot ale media dilutions and BG-11 media were then inoculated with 3-week-old *Synechocytis sp.* to produce a final cell density of 10 x 10^6 cells/ml. A higher density inoculation volume was selected to test if it would enhance the productivity of *Synechocytis sp.* in pot ale. The cultures were then incubated in a temperature (21°C) and light (12/12 hr light dark) controlled growth chamber. The cultures were enumerated in triplicate, daily for 7 days using a Coulter counter (Multisizer® 4).

4.2.4 The growth of *Synechocytis sp.* PCC 6803 in pH adjusted pot ale using sea water.

The low pH of pot ale is a barrier to microalgal growth as the majority of microalgal and cyanobacterial species have severely limited growth in acidic environments. The use of pH buffers such as sodium bicarbonate and sodium hydroxide at a large scale would be either hazardous or create additional expenses. Sea water which is naturally more alkaline (pH 8.3) when compared to freshwater due to the presence of bicarbonate and carbonate ions, was therefore selected as a practical alternative as many distilleries are located in coastal regions. The pot ale utilised in this investigation was freshly obtained from a single whisky distillery. The sea water was obtained from Stonehaven harbour following high tide (Stonehaven, Scotland 56.9647° N, 2.2066° W).

The initial study (4.2.3) indicated that some growth occurred in 1/50 diluted pot ale hence this rate of dilution used here. The pH of the pot ale media was measured using a pH meter (Melter Toledo, UK). The pH of pot ale diluted 1/50 with sea water was pH 5.5. A BG-11 control was prepared using the same sea water. All medias were prepared in 250 ml Erlenmeyer flasks, in triplicate. The flasks were then autoclaved at autoclaved at 121°C, 15 psi (Astell, UK).

The pot ale media dilutions and BG-11 media were then inoculated with 3-week-old *Synechocytis sp. PCC 6803* to produce a final cell density of 2.5 x 10^6 cells/ml and incubated in the growth chamber. After inoculation three of the flasks were covered with tin foil in order to facilitate heterotrophic growth conditions. The cultures were sampled in triplicate daily using a Coulter counter (Multisizer® 4 (Beckman Life Science, Indianapolis, USA). This duration of the investigation was 7 days.

4.2.5 The growth of *Synechocytis sp.* PCC 6803 in pH adjusted pot ale using sodium bicarbonate

Following the investigation using sea water (section 4.2.4) further pH modification was evaluated using sodium bicarbonate. An additional control of ultrapure water inoculated with *Synechocystis sp.* was used as a negative control as it lacked any nutrients.

A 5 M solution of sodium bicarbonate (Sigma, UK) was prepared by dissolving 420 g of sodium bicarbonate in 1 L of ultra-pure water (Elga, Veolia water, UK). Dilutions of pot ale (1/20 and 1/50) were prepared to a volume of 350 ml, in 500 ml Erlenmeyer flasks. The pH of the pot ale dilutions was then adjusted to pH 7 by slowly dripping approximately 2.5 ml of 5M sodium bicarbonate into the pot ale whilst it was being stirred using a magnetic stirrer. The pH adjusted pot ale dilutions (100 ml) were then decanted into 250ml Erlenmeyer flasks, in triplicate

The pH adjusted pot ale dilutions were then autoclaved along with a BG-11 control and water control. The pot ale media dilutions and control media were then inoculated with 3-week-old *Synechocytis sp.* PCC 6803 to produce a final

cell density of 2.5 x 10^6 cells/ml and placed in the growth chamber. The cultures were then sampled daily in triplicate for 7 days and cell density was determined using a Coulter counter.

4.2.6 The effect of increasing concentrations of copper on the growth of *Synechocystis sp.* PCC 6803.

Pot ale is known to contain significant concentrations of copper which originates from the traditional copper stills used to produce malt scotch whisky. The existing literature reports the copper concentration of pot ale to be 2 – 6 mg / L (Quinn, Barker and Marchant, 1980; Graham *et al.*, 2012). Copper is toxic to microalgae when present in high concentrations. A previous study investigating the effects of copper on the cyanobacterium *Chroococcus sp*. PCC 9106 and the microalgae *Spirulina sp*. PCC 6313 observed that the inhibition of growth began at a concentration of 2μM (Seder-Colomina *et al.*, 2013)

BG-11 contains low concentrations of copper as its trace elements solution contains 0.08 mg/L of $CuSO_4.5H_2O$ with results in a Cu concentration of 0.02 mg/L. In order to create a BG-11 control media lacking in copper a trace element

solution was prepared which did not include $CuSO_4.5H_2O$. This solution was then added to the standard BG-11 recipe (section2.2.1). The BG-11 media minus Cu (100ml) was then decanted into 3 250ml Erlenmeyer flasks

Stock solutions of $CuSO_4.5H_2O$ (100 ml) were then prepared at the following concentrations 0.25 g/L (1 mM), 0.5 g/L (2 mM) and 0.75 g/L (3 mM) which equates to 0.06 g/L, 0.12 g/L and 0.18 g/L of Cu, respectively. These stock solutions (0.1 ml) were then added to 100 ml of BG-11 media contained in 250 ml Erlenmeyer flasks which resulted in final copper sulphate concentrations of 0.25 mg/L, 0/5 mg/L and 0.75 mg/L in BG-11. Each concentration was prepared in triplicate.

All flasks were autoclaved at 121°C, 15 psi (Astell, UK) then aseptically inoculated with 2.5 x 10^6 cells/ml of a *Synechocystis sp* PCC 6803 culture that was 2 weeks old. The flasks were placed in a growth chamber set at 10 µmol of white light with a 12-hour day light cycle and a temperature of 21 °C (Filotron growth chamber – Weiss Technik, UK). The cultures were enumerated by measuring optical densities at 730 nm using a spectrophotometer (Thermo spectromic, Thermo-Scientific). The spectrophotometer was blanked using BG-11 media. Each culture was sampled in triplicate.

4.3 Results and discussion

4.3.1 The analysis of pot ale dilutions using scanning spectrophotometry

Evaluation of pot ale to determine its suitability for algal growth first determined the influence of the pot ale colour/turbidity.
The 50 % pot ale displayed an absorbance of greater than 4 in the range of 200 – 350 nm. The 10 % pot ale displayed a similar pattern of absorbance, however its absorbance dropped sharply at 275 nm rather than 350 nm when compared to the 50% dilution (Figure 4.1). Organic material including organic acids typically have absorptions of 200 – 260 nm. A previous investigation determined that an absorbance of 254 nm is strongly corelated with the presence of up to 13 organic matter isolates in water and performing spectrospy at this wavelength can be an accurate measuring tool for the TOC content of water (Weishaar *et al.*, 2003). As previously determined in section 3.3.6 pot ale has a high TOC concentration of 13,000 mg/L which can explain the observed absorbance spectra for pot ale (Figure 4.1).

The absorbance spectra of *Synechocystis sp.* PCC 6803, showed three distinct peaks at approximately 435 nm, 620 nm and 676 nm (Figure 4.1). The peak at 435 nm corresponds to chlorophyll a, the peak at 620 nm corresponds to the phycobililsome and the peak at 676 nm corresponds to chlorophyll a (Hasunuma *et al.*, 2013). In scanning absorbance spectra there is also typically a peak at 450 nm – 500 nm which corresponds to carotenoids which are absent from (Figure 4.1), it is possible that this is indistinguishable from the background, due

to low concentrations of carotenoids. The absorbance of the 50% pot ale at 435 nm was approximately 0.7, giving it the potential to partially absorb light before it reaches chlorophyll a. The absorbance of the 10% pot ale at 435 nm was minimal. In conclusion pot ale must be diluted to improve light penetration if it is to be utilised as a nutrient source for microalgae, however, this reduces the nutrient content of the pot ale.

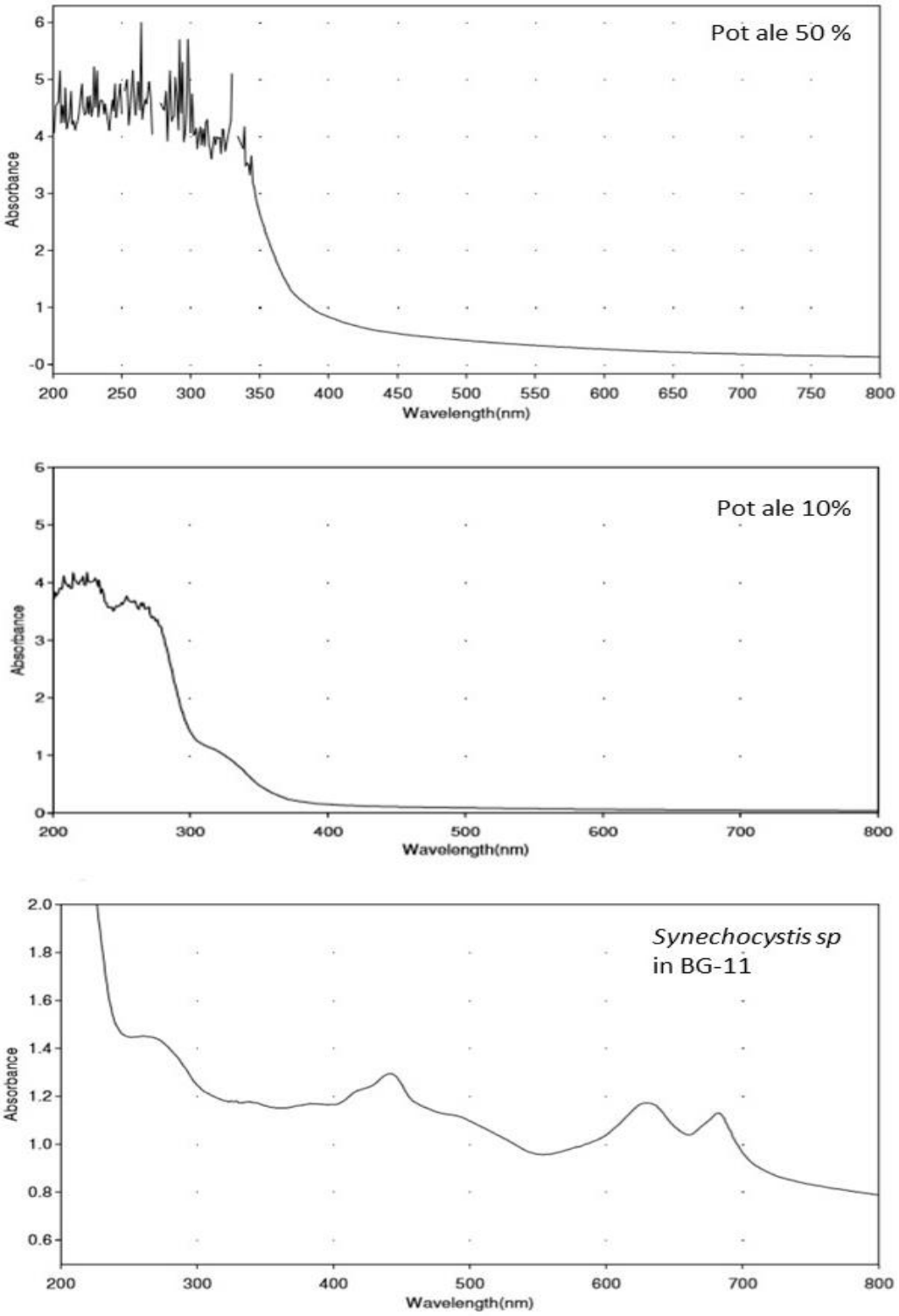


Figure 4.1. Absorbance of pot ale dilutions compared to Synechocystis sp.

4.3.2 The effect of increasing magnesium concentrations on the growth of *Synechocystis sp*. PCC 6803

Increasing magnesium concentrations were observed to have no substantial effect on the growth rate of *Synechocytis sp* after 7 days of cultivation, in this investigation (Figure 4.2.). Statistical analysis using t tests showed no significant difference ($p > 0.05$) from the control by any magnesium concentration, at each time point. The magnesium concentration of pot ale has a range of 126 – 304 mg/L with a median of 195 mg/L (section 3.3.5). Therefore, the magnesium content of pot ale should not have a negative effect on the growth rate of *Synechocytis sp.* PCC 6803.

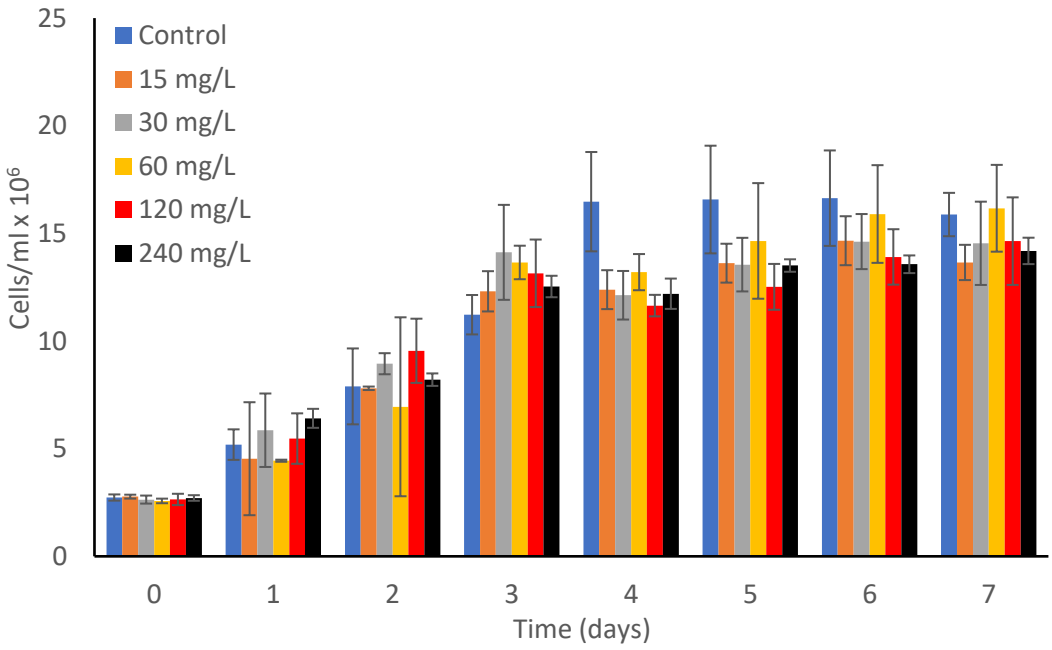


Figure 4.2. The growth of *Synechocytis sp.* PCC 6803 in BG-11 augmented with increasing concentrations of additional magnesium. The error bars are representative of the standard deviation from the mean (n=3).

Flocculation was observed in the wells containing 240 mg/L magnesium (Figure 4.3). Magnesium hydroxide has been shown to promote flocculation in previous studies (Vandamme *et al.*, 2012; García-Pérez *et al.*, 2014). Flocculation is utilised in the algal biotech industry to harvest large volumes of microalgae typically used prior to centrifugation to pre-concentrate the biomass. Common

coagulants that are utilised include alum ($KAl(SO_4)_2$ and iron chloride (FeCl) (García-Pérez *et al.*, 2014), however, large volumes of coagulants are required, and metal contamination of the biomass can occur. Alternative methods of flocculation have been investigated which include electro-coagulation and auto–flocculation via pH adjustment (Vandamme *et al.*, 2011). If the magnesium content of pot ale promotes flocculation, then there are potential costs savings to be made if pot ale can be utilised to produce microalgal biomass. The dewatering of algal biomass is reported as a significant bottleneck in advancing large scale processing (Uduman et al., 2010).

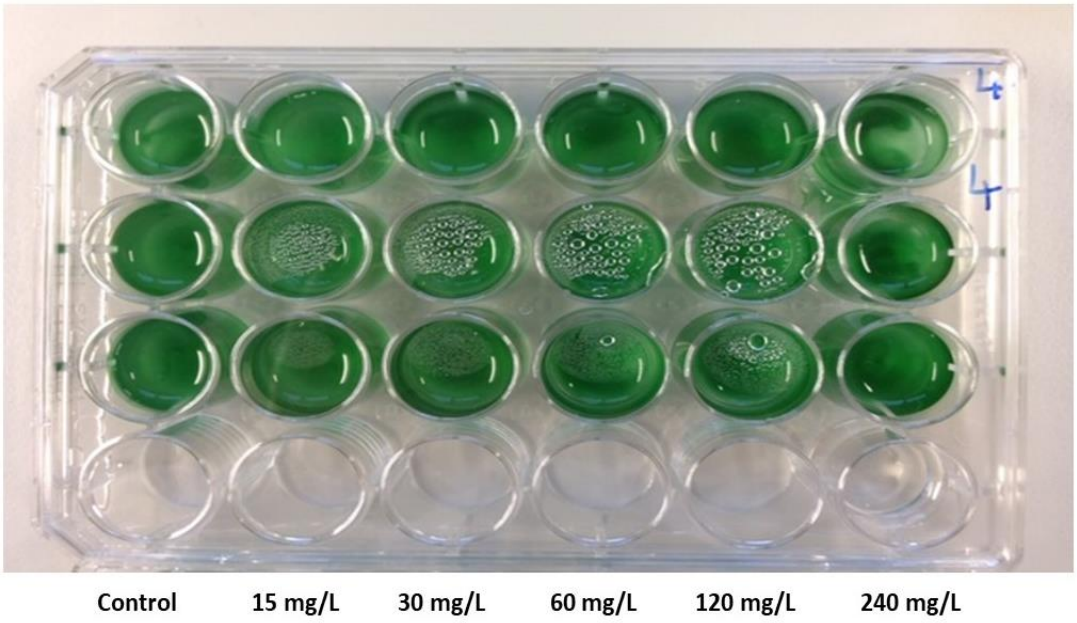


Figure 4.3. The growth of *Synechocytis sp.* PCC 6803 across a range of magnesium concentrations after 7 days of cultivation.

4.3.3 The growth of *Synechocytis sp*. PCC 6803 using pot ale as a nutrient source in phototrophic and heterotrophic growth conditions.

The *Synechocytis sp.* PCC 6803 grown in BG-11 media outperformed the pot ale dilutions in terms of growth rate and cell density (Figure 4.4). In the first 3 days of cultivation there was no significant growth observed in the pot ale treatments, with growth remaining around the inoculation density of 10 x 10^6 cells/ml. After

4 days of cultivation there was growth observed in each of the pot ale dilutions with all reaching a cell density of > 13 x 10^6 cells/ml (Figure 4.6). The most successful dilution of pot ale was 1/50 which reached a maximum cell density of 22.4 x 10^6 cells/ml in comparison to 16.2 x10^6 (1/10) and 17.6 x10^6 (1/20).

The pH of pot ale is a major limiting factor of cyanobacterial growth. The pH of 1/50 pot ale was slightly higher (pH 4) than the 1/10 dilution and the 1/20 dilution which had pH values of 3.5 and 3.6 respectively. This difference in pH could contribute to the difference in growth observed between the 1/50 dilutions and other pot ale dilutions. After 6 and 7days cultures grown in 1/50 pot ale had significantly higher ($P < 0.05$) cell densities when compared to the other pot ale dilutions. The 1/50 dilution being the most dilute also afforded the best light penetration so this could also be a factor.

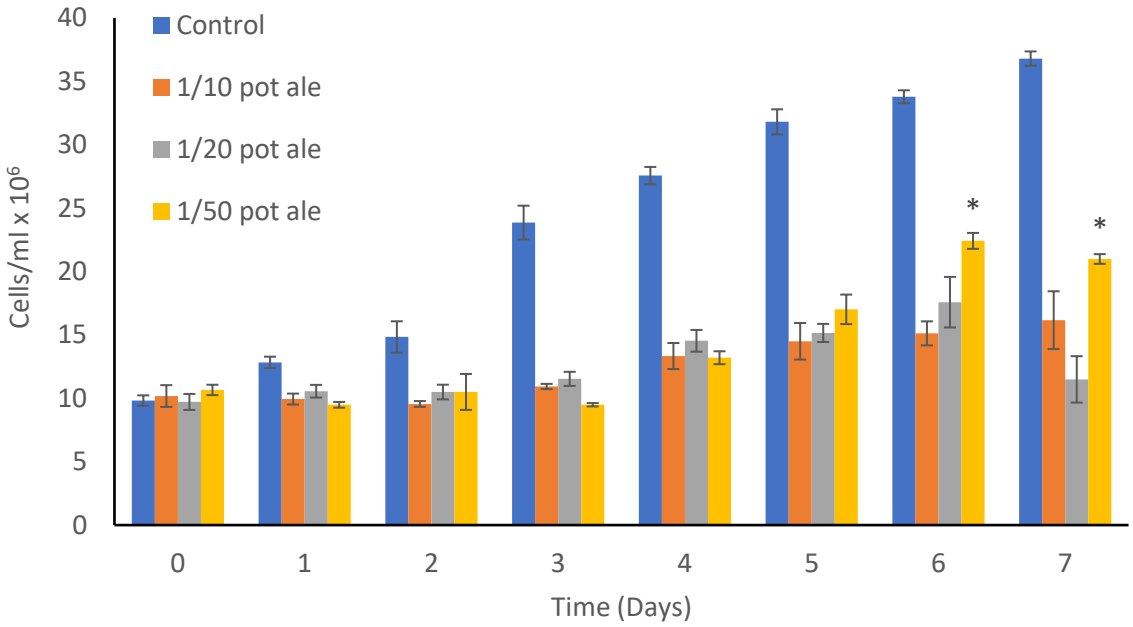


Figure 4.4. The phototrophic growth of *Synechocytis sp.* PCC 6803 in dilutions of pot ale. The error bars are representative of the standard deviation from the mean. $P < 0.05$ (*). N = 3

When cultivated under heterotrophic conditions no was growth observed in cultures grown in BG-11 media (Figure 4.5), which would be expected. However, each of the pot ale dilutions displayed growth over the 7-day cultivation period.

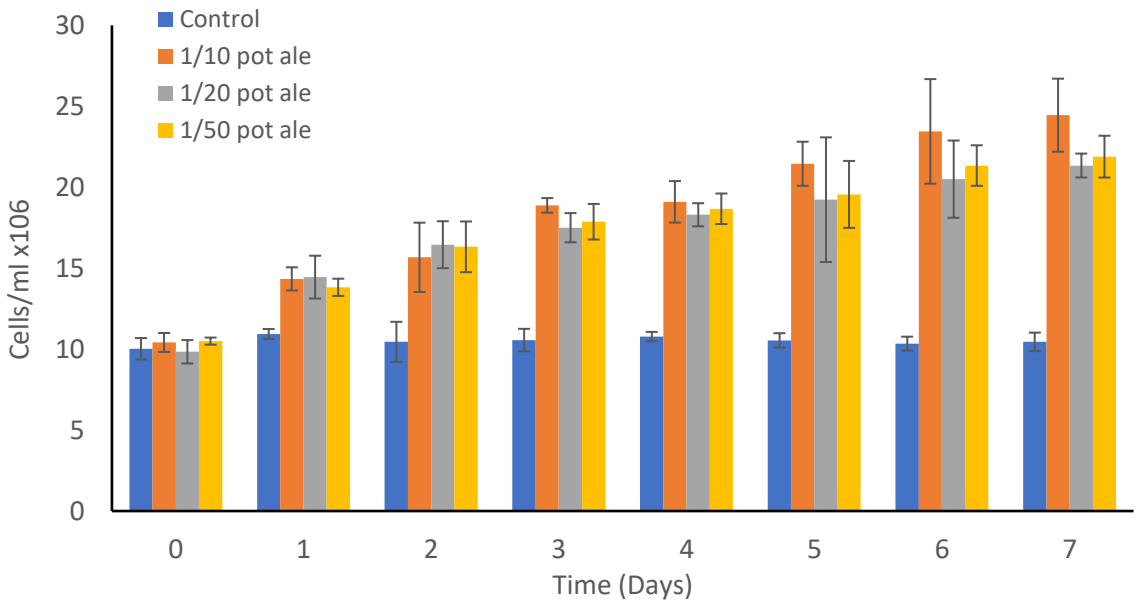


Figure 4.5. The heterotrophic growth of *Synechocytis sp*. in dilutions of pot ale. The error bars are representative of the standard deviation from the mean. n = 3.

Pot ale contains 300 mg/L of soluble monosaccharides (section 3.3.7) consisting of sugars such as glucose, galactose and xylose. *Synechocytis sp.* can utilise sugars for heterotrophic growth which may account for the growth observed in this investigation. However, the dilution of pot ale will have significantly reduced the concentrations of freely available sugars that are present. Several studies report that *Synechocystis sp.* PCC 6803 is only capable of light activated heterotrophic growth (Anderson and McIntosh, 1991; Kong, Xu and Hu, 2003). This means that the culture must be provided with a brief pulse of light 5 – 10 minutes for each day of cultivation, although since all cultures were sampled daily this may have inadvertently provided the short bursts of light that were required.

It is also possible that the heterotrophic cultures containing pot ale were contaminated with additional heterotrophic bacteria. In order to determine if the

cultures were contaminated, samples were taken from each culture after 7 days of growth. These samples were then observed through a light microscope at x 400 magnification (Figure 4.6). Several clusters of rod-shaped bacteria can be observed in the heterotrophic culture when compared to the control. Contamination was not observed in the phototrophic cultures or control. These unidentified bacterial strains could have been counted during the daily enumeration of *Synechocystis* sp. which resulted in inaccurate cell density data.

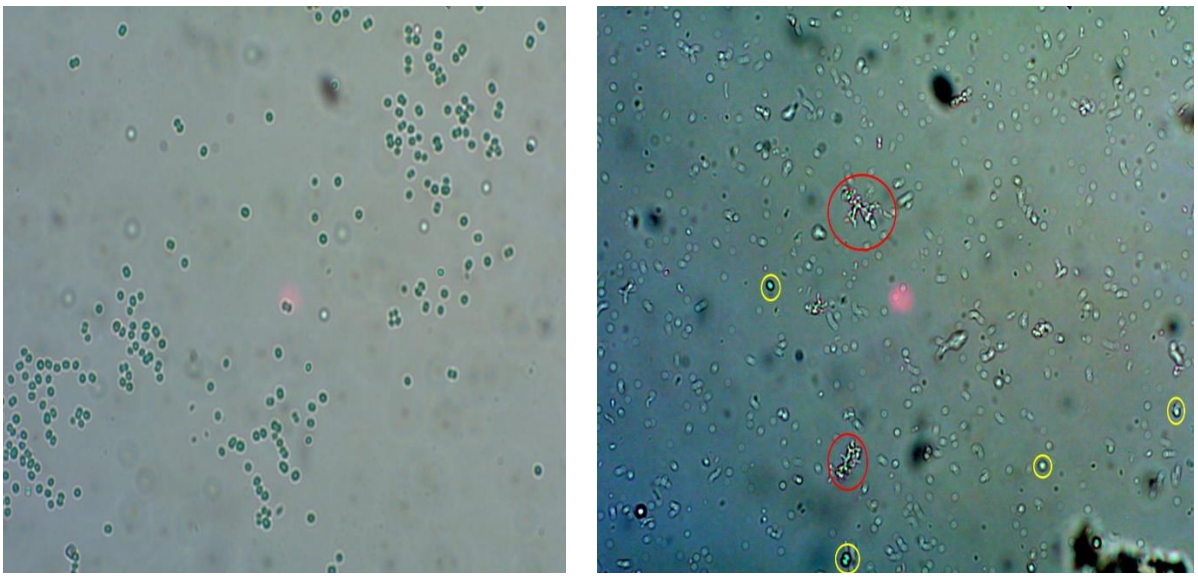


Figure 4.6. Images of the BG-11 control culture (left) and Heterotrophic culture (right) at x400 magnification. *Synechocytis sp*. circled in yellow, potential contaminants circled in red.

4.3.4 The pH adjustment of pot ale using sodium bicarbonate and sea water and the effect on the growth of *Synechocystis sp.* PCC 6803.

Due to its performance in the previous investigation (section 4.33) a dilution of 1/50 was also utilised in this investigation. In order to assess the effect of pH adjustment on the performance of pot ale as a microalgal growth medium, pot ale was pH adjusted using sea water and sodium bicarbonate.

The pH of pot ale diluted 1/50 with sea water was 5.6. Despite the increase in pH there was slower growth rates obsereved in both the phototrophic and heterotrophic cultures when compared to the BG-11 control (Figure 4.7). The

cell density of the heterotrophic cultures was lower than that of the phototrophic cultures at all stages of cultivation (Figure 4.7).

The phototrophic growth rate in this investigation was 0.18 µ/day. This was higher than the growth rate (0.02 µ/day) observed in phototrophic cultures grown in 1/50 pot ale in previous investigation (Figure 4.4) (section 4.3.3). This difference in growth rate could be attributed to the pH difference between the two experiments. However, the inoculation density was also lower in this experiment (2.5 x 10^6 cells/ml) versus (10 x 10^6 cells/ml) in the previous experiment (Figure 4.4). This could have impact on the observed difference in growth rates.

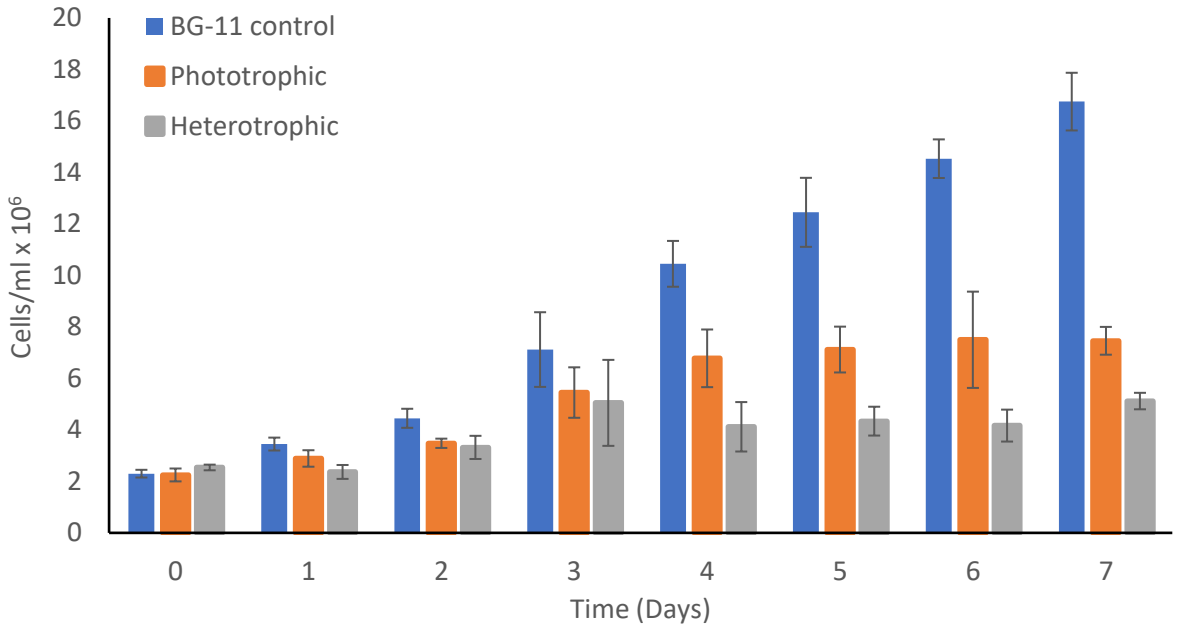


Figure 4.7. The cell density of *Synechocytis sp.* grown in dilutions of pH adjusted (pH 5.6) pot ale (1/50) using sea water. The error bars are representative of the standard deviation from the mean (n=3). Light intensity = 10 µmol/m^2/s with 12-hour light/dark cycle, Temperature = 21 °C.

The cell density of the *Synechocystis sp.* PCC 6803 when grown as a control in BG-11 was significantly higher than all other treatments reaching 16.75 x10^6 cells/ml after 7 days of growth (Figure 4.8) In contrast, the cell density in both water and pot ale demonstrates very low growth < 5.52 x 10^6 after 7 days (Figure 4.8). The results of the investigation indicate that despite pH balancing to pH 7, there are other factors limiting the growth of *Synechocystis sp.* in pot ale.

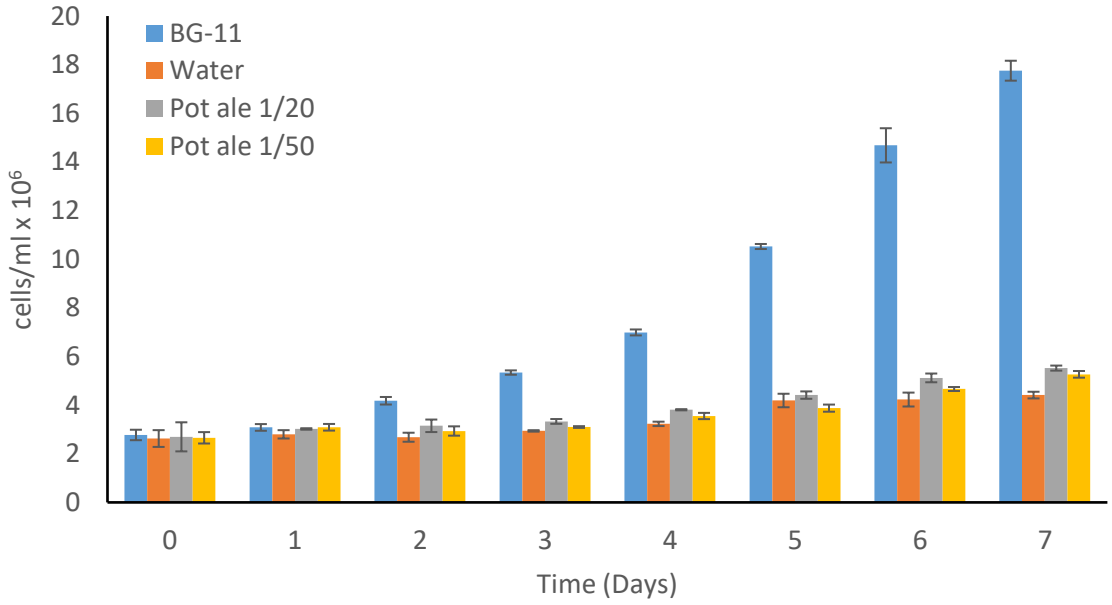


Figure 4.8. The cell density of *Synechocytis sp*. grown in dilutions of pH adjusted (pH 7) pot ale using sodium bicarbonate. The error bars are representative of the standard deviation from the mean. n = 3. Light intensity = 10 µmol m^{-2} s^{-1} with 12-hour light/dark cycle, Temperature = 21°C.

4.3.5 The effect of increasing copper concentrations on the growth of *Synechocystis sp.* PCC 6803

To determine if copper in the pot ale could be a limiting factor, a copper sulphate concentration of 0.25 mg/L was shown to have no effect on the growth of *Synechocystis sp.* with no significant difference observed when compared to the BG-11 control. However, concentrations of 0.5 mg/L. and 0.75 mg/L showed reductions in growth after 3 and 4 days respectively (Figure 4.9). After 14 days of cultivation the growth of *Synechocystis sp.* in 0.75 mg/L copper sulphate showed there was a significant reduction in growth when compared to the control (Figure 4.9). The IC50 value after 14 days was 0.68 mg Cu L^{-1}. The results of this

experiment are largely consistent with the investigation carried out by Seder-Colomina et al., (2013) in that there was no inhibition observed at 1μM with increasing inhibition observed as the Cu concentration increased. A concentration of 3 μM (0.75 mg/L) of copper sulphate has previously been shown to reduce the growth of *Synechocystis sp.* PCC 6803 with a concentration of 5 μM (1.25 mg/L) of copper sulphate completely inhibiting growth (Giner-Lamia *et al.*, 2012).

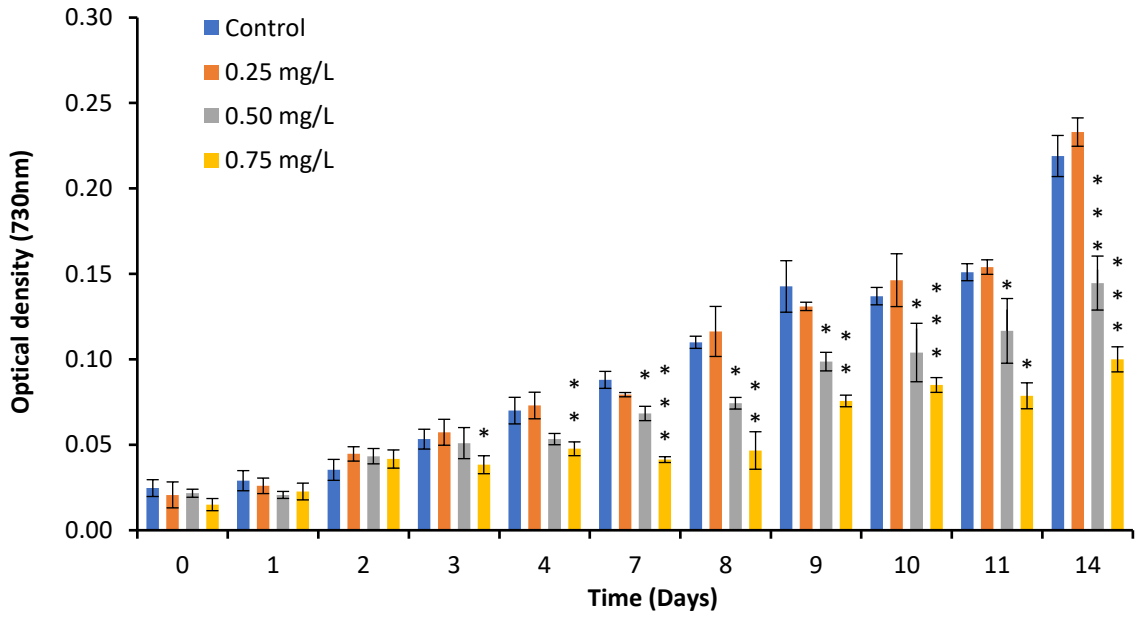


Figure 4.9. The growth of *Synechocytis sp.* PCC 6803 in BG-11 augmented with increasing concentrations of additional copper. The error bars are representative of the standard deviation from the mean. Statistical analysis performed using t test assuming unequal variances, $p < 0.05$ (*), $p < 0.01$ (* *), $P < 0.001$ (***). N =3

It was previously determined that the median soluble copper concentration of pot ale is 2.97 mg/L (section 3.3.5). The median concentration, however, was only 0.10 mg/L with a standard deviation of 0.84 mg/L. Therefore, the copper concentration of pot ale has the potential to be toxic to *Synechocytis sp*. PCC 6803.

Copper sulphate is used as the active ingredient in many commercial algicides such as Clearigate and Cutrine–Plus with typical application rates in the range of 0.1 – 1.0 mg/L (Murray-Gulde et al., 2002). However, the increasing use of

copper as an algicide can lead to the proliferation of copper resistant mutants. A previous study cultured *Microcystis aeruginosa* on selected media containing copper sulphate in order to select for copper resistance. These resultant mutants were then successfully cultured in copper sulphate concentrations of up to 10 μM (2.5 mg/L) whereas the wild type strain could not tolerate 3 μM of copper sulphate. Previous investigations have identified a novel copper binding protein CopM that is involved in the copper resistance of *Synechocytis sp.* PCC6803 (Giner-Lamia et al., 2015, 2012). Therefore, it may be possible to create resistant strains of *Synechocystis sp*. PCC 6803 using a similar technique that are tolerant to 2.5 mg/L of copper which is close to the maximum soluble copper concentration observed in section 3.3.5.

Another possibility would be to remove the copper from pot ale using ion exchange chromatography. A number of distilleries already utilise ion exchange to remove copper from their waste streams in order to lessen environmental impact (Jack *et al.*, 2014). It may also be possible to simultaneously add value by producing copper nanoparticles from the Cu that is removed from pot ale. Copper nanoparticles can be utilised to produce specialised printer ink (Lee *et al.*, 2009). Therefore, there may be potential for valorisation.

4.4 Conclusions

It proved challenging to successfully culture *Synechocytis sp.* PCC 6803 in pot ale. The requirement to dilute reduces the already limited nitrate content that is present. The growth of cyanobacteria/microalgae in pot ale may require additional nitrate to supplement the existing concentrations. However, in an industrial setting the addition of large volumes of nitrates would be expensive and defeat the cost saving purpose of utilising pot ale as an alternative nutrient source to traditional media. Alternatively, nitrogen fixing species could be assessed using pot ale as nutrient source. The effect of adding additional nitrate and the growth of a nitrogen fixing species (*Nodularia harveyana*) in pot ale was assessed in Chapter 5. Despite efforts to neutralise the acidic pH reliable growth and productivity was not achieved. This is potentially due to toxic concentrations of copper being present in the pot ale. Further studies are required to optimise the growth of microalgae in pot ale which may require a period of gradual copper acclimatisation, to achieve successful growth.

4.5 References

Alcántara, C., Posadas, E., Guieysse, B., Muñoz, R., 2015. Microalgae-based Wastewater Treatment. Handbook of Marine Microalgae: Biotechnology Advances 439–455. https://doi.org/10.1016/B978-0-12-800776-1.00029-7

Anderson, S.L., McIntosh, L., 1991. Light-activated heterotrophic growth of the cyanobacterium Synechocystis sp. strain PCC 6803: A blue-light-requiring process. Journal of Bacteriology 173, 2761–2767. https://doi.org/10.1128/jb.173.9.2761-2767.1991

García-Pérez, J.S., Beuckels, A., Vandamme, D., Depraetere, O., Foubert, I., Parra, R., Muylaert, K., 2014. Influence of magnesium concentration, biomass concentration and pH on flocculation of Chlorella vulgaris. Algal Research 3, 24–29. https://doi.org/10.1016/j.algal.2013.11.016

Giner-Lamia, J., López-Maury, L., Florencio, F.J., 2015. CopM is a novel copper-binding protein involved in copper resistance in Synechocystis sp. PCC 6803. MicrobiologyOpen 4, 167–185. https://doi.org/10.1002/mbo3.231

Giner-Lamia, J., Lopez-Maury, L., Reyes, J.C., Florencio, F.J., 2012. The CopRS Two-Component System Is Responsible for Resistance to Copper in the Cyanobacterium Synechocystis sp. PCC 6803. Plant Physiology 159, 1806–1818. https://doi.org/10.1104/pp.112.200659

Graham, J., Peter, B., Walker, G., Wardlaw, A., 2012. Chapter 43 Characterisation of the pot ale profile from a malt whisky distillery 1–8.

Hasunuma, T., Kikuyama, F., Matsuda, M., Aikawa, S., Izumi, Y., Kondo, A., 2013. Dynamic metabolic profiling of cyanobacterial glycogen biosynthesis under conditions of nitrate depletion. Journal of Experimental Botany 64, 2943–2954. https://doi.org/10.1093/jxb/ert134

Jack, F., Bostock, J., Tito, D., Harrison, B., Brosnan, J., 2014. Electrocoagulation for the removal of copper from distillery waste streams. Journal of the Institute of Brewing 120, 60–64. https://doi.org/10.1002/jib.112

Kong, R., Xu, X., Hu, Z., 2003. A TPR-family membrane protein gene is required for light-activated heterotrophic growth of the cyanobacterium Synechocystis sp. PCC 6803. FEMS Microbiology Letters 219, 75–79. https://doi.org/10.1016/S0378-1097(02)01205-3

Lee, B., Kim, Y., Yang, S., Jeong, I., Moon, J., 2009. A low-cure-temperature copper nano ink for highly conductive printed electrodes. Current Applied Physics 9, e157–e160. https://doi.org/10.1016/j.cap.2009.03.008

Lopes, T., Silva, D., Reis, A., Da Silva, T.L., Reis, @bullet A, 2015. Scale-up Problems for the Large Scale Production of Algae. https://doi.org/10.1007/978-3-319-22813-6_6

Murray-Gulde, C.L., Heatley, J.E., Schwartzman, A.L., Rodgers, J.H., 2002. Algicidal effectiveness of Clearigate, Cutrine-Plus, and copper sulfate and margins of safety associated with their use. Archives of Environmental Contamination and Toxicology 43, 19–27. https://doi.org/10.1007/s00244-002-1135-1

Osanai, T., Kanesaki, Y., Nakano, T., Takahashi, H., Asayama, M., Shirai, M., Kanehisa, M., Suzuki, I., Murata, N., Tanaka, K., 2005. Positive regulation of sugar catabolic pathways in the cyanobacterium Synechocystis sp. PCC 6803 by the group 2 σ factor SigE. Journal of Biological Chemistry 280, 30653–30659. https://doi.org/10.1074/jbc.M505043200

Quinn, J.P., Barker, T.W., Marchant, R., 1980. Relationship Between Free and Complexed Forms of Copper in Malt Whiskey Distillery Spent Wash. Journal of the Institute of Brewing 86, 274–276. https://doi.org/10.1002/j.2050-0416.1980.tb06880.x

Rahman, M.M., Salleh, M.A.M., Rashid, U., Ahsan, A., Hossain, M.M., Ra, C.S., 2014. Production of slow release crystal fertilizer from wastewaters through struvite crystallization – A review. Arabian Journal of Chemistry 7, 139–155. https://doi.org/10.1016/J.ARABJC.2013.10.007

Seder-Colomina, M., Burgos, A., Maldonado, J., Solé, A., Esteve, I., 2013. The effect of copper on different phototrophic microorganisms determined in vivo and at cellular level by confocal laser microscopy. Ecotoxicology 22, 199–205. https://doi.org/10.1007/s10646-012-1014-0

SEPA, 2017. Isle of arran distillery. Propose long sea outfall.pdf.

Taziki, M., Ahmadzadeh, H., A. Murry, M., R. Lyon, S., 2016. Nitrate and Nitrite Removal from Wastewater using Algae. Current Biotechnology 4, 426–440. https://doi.org/10.2174/2211550104666150828193607

Uduman, N., Qi, Y., Danquah, M.K., Forde, G.M., Hoadley, A., 2010. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Journal of Renewable and Sustainable Energy 2. https://doi.org/10.1063/1.3294480

Vandamme, D., Foubert, I., Fraeye, I., Meesschaert, B., Muylaert, K., 2012. Flocculation of Chlorella vulgaris induced by high pH: Role of magnesium and calcium and practical implications. Bioresource Technology 105, 114–119. https://doi.org/10.1016/j.biortech.2011.11.105

Vandamme, D., Pontes, S.C.V., Goiris, K., Foubert, I., Pinoy, L.J.J., Muylaert, K., 2011. Evaluation of electro-coagulation-flocculation for harvesting marine and freshwater microalgae. Biotechnology and Bioengineering 108, 2320–2329. https://doi.org/10.1002/bit.23199

Wang, X., Hao, C., Zhang, F., Feng, C., Yang, Y., 2011. Inhibition of the growth of two blue-green algae species (Microsystis aruginosa and Anabaena spiroides) by acidification treatments using carbon dioxide. Bioresource Technology 102, 5742–5748. https://doi.org/10.1016/j.biortech.2011.03.015

Weishaar, J.L., Aiken, G.R., Bergamaschi, B.A., Fram, M.S., Fujii, R., Mopper, K., 2003. Evaluation of specific ultraviolet absorbance as an indicator of the chemical composition and reactivity of dissolved organic carbon. Environmental Science and Technology 37, 4702–4708. https://doi.org/10.1021/es030360x

Chapter 5

The recovery of lactic acid from pot ale using ion exchange chromatography.

5.1 Chapter Outline

This chapter investigates the removal of lactic acid from pot ale via ion exchange. The resulting pH adjusted pot ale was also utilised in a final pot ale growth assay. The growth assay investigates species investigated in previous chapters as well as *Nodularia Harveyana* PCC 7804 which is a nitrogen fixing cyanobacteria.

5.1.1 Introduction

Lactic acid is a valuable industrial chemical and is widely used as a food preservative and is a key component of Polylactic acid (PLA) which has a wide range of applications which include surgical sutures, disposable plastic packaging and drug delivery systems (Melnicki *et al.*, 2013). However, perhaps the most interesting aspect of PLA going forward is its use in producing biodegradable polymers. In 2013 the estimated global market for lactic was 725,000 tonnes around half of this demand can be attributed to the bioplastics industry. The bioplastic market is projected to grow by 20% annually due to the increasing demand for biodegradable single use plastic products (Ou et al. 2016; Madhava-Nampoothiri et al. 2010). Currently lactic acid can be produced synthetically or through bacterial fermentation. The most popular chemical synthesis approach is based on the hydrolysis of Lactonitrile by strong acid which produces a racemic mixture of L and D lactic acid (John and Nampoothiri, 2007). The microbial production of lactic acid can be carried out using a variety of carbon sources such as pure sugars; lactose, sucrose, lactose or from starch-based materials largely derived from arable crops such as tapioca, wheat, corn and barley. The resulting cost of production is largely based on the cost of the feedstock (Juturu and Wu, 2016).

The current first-generation feedstocks are also in direct competition with the food and animal feed markets. With a rapidly increasing population and limited arable land there is considerable pressure not to utilise farmland to produce industrial feedstocks. This is commonly referred to as the food versus fuel debate (Graham-Rowe, 2011).Therefore, there has been increasing interest in using industrial / agricultural waste products or residues that do not directly

compete with food products. Many of these alternatives are also cheaper than crops and refined sugars. The fermentative production of lactic acid also produces optically pure L (+) or D (-) lactic acid, this high product specificity is a distinct advantage over synthetic production (Madhavan-Nampoothiri et al. 2010).

Significant concentrations of lactic acid were detected in pot ale by previous characterisation investigations (Section 3.3.3). The lactic acid produced during whisky production is also likely to be optically pure as it is also produced as a by-product of fermentation. Other advantages of fermentative production over synthetic production include lower production temperatures, lower energy consumption and low costs of substrates (Ahring *et al.*, 2016). Typical yields of fermentative production techniques can be in excess of 100 g/L (Cubas-Cano *et al.*, 2018). The highest lactic acid concentration observed in this study was 6.8 g/L. Despite the wide range of applications and the advantages of fermentative production, PLA struggles to compete economically with petro-chemically derived plastics due to high production costs. Downstream processing is currently responsible for 30 – 40 % of the total production costs of PLA. Therefore, the majority of production costs relates to feedstocks and upstream processes such as fermentation (John and Nampoothiri, 2007). However, the purification of lactic acid from pot ale would have the benefit of being an entirely downstream process. This would result in significantly lower operational costs than traditional fermentative production. Across the major whisky producing countries there is an estimated total of approximately 2.2 kilo tonnes per annum of lactic acid that could be potentially recovered from pot ale (Table 3.4). This far exceeds the current market demand hence the question is not availability, but if the recovery process can be made cost effective and scalable.

Table 5.1. Estimated total lactic yield from pot ale across the major whisky producing countries. Calculated using the average lactic acid concentration of 2035 mg/L found in this study (Russel., 2014).

Country	Estimated lactic acid in pot ale (Kilo tonnes)
India	1034
Scotland	607
North America	368
Japan	99
Ireland	71
Total	**2181**

This investigation assesses the treatment of pot ale using ion exchange chromatography. By removing the organic acid content using ion exchange the pH will increase. The low pH of pot ale is one of the primary barriers that inhibit microalgal growth. The growth of several microalgal and cyanobacterial species in ion exchange treated pot ale was investigated. As the organic acid contributes to the TOC content of pot ale, the ion exchange should also significantly reduce the TOC.

5.2 Materials and Methods

5.2.1 Ion exchange resin preparation

Amberlite IRA 96 (Sigma Aldrich, USA) was selected as the anion exchange resin for this investigation. Amberlite IRA 96 is a weak base macroreticular anion exchange resin with a high capacity for organic acids. Amberlite IRA 96 has been shown to provide a high recovery yield of lactic acid when compared to other resins (Chendake and Kharul, 2014). Other advantages of Amberlite IRA 96 include its ease of regeneration and maximum porosity which is higher than its gel resin counterparts. These aspects increase throughput and ease of use which makes Amberlite IRA 96 a good candidate for large scale operation. The binding of the lactate ion and the Amberlite resin occurs via the equation below. The Amberlite resin was also selected as it is a food grade resin and therefore the recovered lactic was also food grade and could be approved for pharmaceutical, food and nutraceutical applications.

$$R^+Cl^- + L^- \longrightarrow R^+L^- + Cl^-$$

Where R is the stationary matrix containing the functional group and L denotes the lactate ion. During loading the weak base Cl^- contained in the matrix is continually exchanged with the lactate ions present in the mobile phase which act as counter ions. As the equilibrium is shifted the lactate ions are reversibly absorbed onto the resin. During elution and regeneration of the column this process is reversed when Cl^- ions are introduced into the resin via elution with HC. Amberlite resin (150 g) was soaked in 500 ml of ultrapure water for 24 hours prior to being packed under gravity into a 120 g cartridge (Biotage,UK). Prior to utilisation the resin was washed with 1N HCL (Sigma, UK) in order to convert into its Cl^- form as it could be obtained in its OH^- form. The resin was then sequentially washed with 1 N HCL solution, ultrapure water (Elga, UK) and 1 N NaOH solution, ultrapure water, 1 N HCL solution and ultrapure water, with the intent on reaching pH 7 as described by Bishai et al., (2015).

Increasing the pH to 7 proved to be difficult as the final wash step of ultrapure water would not elute enough of the H^+ ion that remained in the resin after washing with 1 N HCL. A pH of 6 was achieved after washing with 10 litres of glass filtered water.

5.2.2 Preliminary recovery testing with a lactic acid standard.

A 15 mg/L standard solution was created by dissolving sodium lactate salt (Sigma, UK) in 333 ml of ultrapure water (Elga, UK). The resulting solution was then adjusted to pH 5 by adding 10 M NaOH as lactic acid adjusted to pH 5 was shown to have the highest binding efficiency with the Amberlite resin (Bishai et al., 2015).

The lactate concentration was verified by analysis using ion chromatography (section 3.2.4). The resulting concentration was 12345 mg/L which is in line with the expected concentration of 12,000 mg/L as sodium ions represent 20 % of the total mass of sodium lactate. This lactate concentration was selected for preliminary testing as this concentration was the observed median lactate concentration in previous analysis of pot ale from the same whisky distillery.

The pH adjusted lactate solution was then loaded onto the Amberlite resin at a flow rate of 10 ml/min using an Isolera one flash chromatography system (Biotage, UK). Fractions were manually collected every 25 ml until a total volume of 300 ml was eluted, resulting in 12 fractions of 25 ml. The fractions were analysed using ion chromatography to determine lactate content. This was done in order to establish a breakthrough curve and the binding efficiency of the resin. The lactate bound to the resin was then eluted with 300 of 1 N HCL at a flow rate of 10 ml/min. Fractions were manually collected after every 25 ml and analysed using ion chromatography.

5.2.3 Removal of organic acids from pot ale via ion exchange chromatography.

The pot ale used in this investigation was sourced from a single malt whisky distillery and sampled on site (Aberdeenshire, UK). The pot ale (1 litre) was then filtered sterilised using sterile 0.2 µm hollow fibre syringe filters (MediaKap plus, The Netherlands). This was done to remove particulates and improve stability. The filtered pot ale (500 ml) was then adjusted to pH 5 using 10 ml of 10 M NaOH.

The pot ale (400 ml) was then loaded onto the Amberlite resin at a flow rate of 10 ml/min. Manual fractions were collected to establish a breakthrough curve. Due to blockages in the column which caused problems with the flow rate, the fractions were not of equal volumes. A total of 15 fractions were collected with an average volume of 29 ml. The fractions were subsequently analysed for lactic acid and acetic acid content.

The Amberlite resin was then eluted using a gradient of HCL ranging from 0.1 M to 1 M. A total of 1 L of HCL was passed through the column with the concentration of HCL being increased by 0.1 M after every 100 ml of eluent. Fractions (2 x 50 ml) were manually collected after each step, resulting in 20 fractions of 50 ml. These fractions were then subsequently analysed by ion chromatography (Dionex HPIC, Thermo scientific, UK).

5.2.5 The evaluation of microalgal growth in ion exchange treated pot ale.

The three main barriers to successful microalgal growth in pot ale are turbidity, pH and low nitrate concentration. Previous investigations in chapter 4 have addressed the pH and turbidity issues by diluting and pH buffering. This investigation used an assay approach to test various pot ale media against a positive and negative control. Pot ale that has been treated using ion exchange was augmented with nutrients to create a range of experimental pot ale media. These pot ale media were then tested against a positive control (BG-11 media) and a negative control unaltered pot ale.

The species selected for this investigation were *Synechocystis sp*. PCC 6803, *Chlorella sorokiniana*, *Microcystis aeruginosa* PCC 7813 and *Nodularia harveyana* PCC 7804. *Synechocystis sp*. was selected to provide comparative data as it has already been utilised in previous growth experiments. It is also a very popular organism within the algal biotechnology sector due to its fast growth and synthetic biology applications. *C. sorokiniana* was selected due to its capability to produce high concentrations of zeaxanthin*. M aeruginosa* PCC 7813 was selected for its production of zeaxanthin as well as its production of the class of toxins known as microcystins which have a high value. *N. harveyana* was selected as it is a nitrogen fixer giving it the capability of growing in low nitrate environments which potentially includes pot ale.

The rationale behind the experimental media used in this investigation is to test the effectiveness of pot ale that has been treated by ion exchange (neutralised pot ale) this pot ale will then be further augmented with additional sodium nitrate, trace elements and sodium nitrate + trace elements (Table 5.1). This was done in order to test each augmentation to the pot ale in isolation and test their combined effects.

Table 5.2. The media used in the assay investigating the performance of pot ale treated with ion exchange, as microalgal growth medium. The standard deviation from the mean is listed in parenthesis (N=3).

Condition	Nitrate concentration (mg/L)	Phosphate concentration (mg/L)	pH
BG-11	750 (27)	80 (4)	7 (0.2)
Pot ale	6 (0.5)	115 (11)	3.5 (0.1)
Neutralised pot ale	6 (0.4)	115 (12)	6.7 (0.2)
Neutralised pot ale + trace elements	20 (1)	115 (10)	6.7 (0.1)
Neutralised pot ale + 500 mg/L sodium nitrate	520 (22)	115 (11)	6.8 (0.2)
Neutralised pot ale + 500 mg/L nitrate + trace elements	520 (19)	115 (9)	6.8 (0.1)

Stock cultures that were 3 weeks old were numerated using a Coulter counter Multisizer® 4 (Beckman Life Science, Indianapolis, USA). Aliquots (1.5 ml) of organism were then taken and centrifuged at 10,000 rpm for 10 minutes. The supernatant was then discarded and re-suspended in the desired culture media. This was done in order to limit the amount of carryover from BG-11 media.

Each experimental media (100 ml) was filter sterilised using 2 µm sterile 0.22 µm stericups (Plus membrane, Millipore,UK). The media was then transferred to sterile 25 ml falcon tubes under aseptic conditions. Each media was then inoculated at a concentration of 2.5 x 10^6 cells/ml with the selected microalgae.

Of the resulting 20 ml cultures 4 ml was used for time zero sampling. For day 3 sampling 8 ml of each species and media combination was aseptically transferred to 2 ml vials (quadruplicates) which were placed in sterile 25 ml beakers, covered with sterile petri dish lids. This process was repeated in order to produce a sample set for day 7 sampling. The day 3 and day 7 sample sets were then placed in a temperature and light controlled growth chamber (Filotron growth chamber – Weiss Technik, UK)**.** The temperature was 21 °C and the light intensity was 10 µmol/m^2/s with a light dark cycle of 12 hours light / 12 hours dark (Figure 5.1).

The *Synechocystis sp*. PCC 6803, *Chlorella sorokiniana*, *Microcystis aeruginosa* PCC 7813 cultures were enumerated using the method described in section (2.2.2.1). The filamentous structure of *N. harveyana* leads to considerable difficultly when counting using a haemocytometer. Therefore, the cell density of *N. harveyana* cultures was determined using optical density at a wavelength of 720 nm. The cells/ml of *N. harveyana* were then approximated using the linear correlation between absorbance at OD 720nm and cells numbers (Figure 13A in the appendices), which were manually counted via haemocytometer using the method described in section 2.2.2.1.

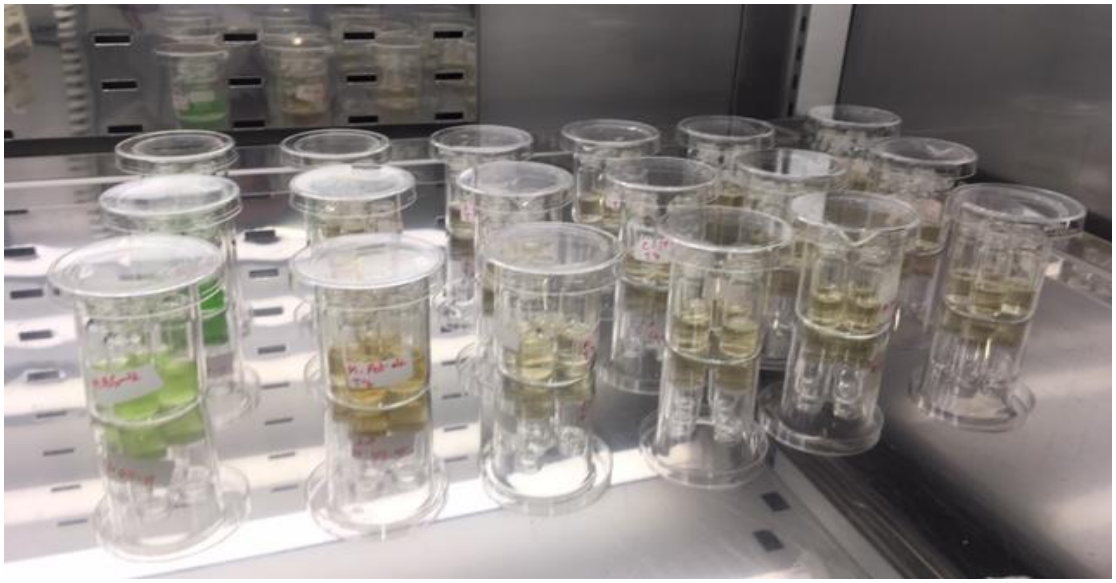


Figure 5.1. Pot ale media assay being incubated in the (Fitotron growth chamber – Weiss Technik, UK

5.3 Results and discussion

5.3.1 Evaluation of the lactic acid removal capability of the Amberlite ion exchange resin

A total of 3700 mg of lactate was loaded onto the resin with a total of 320 mg breaking through in the loading fractions which resulted in a binding efficiency of 91.3 %. No lactate was detected in the loading fractions until 200 ml had been loaded. After this point lactate was detected in increasing concentrations as the resin reached saturation (Figure 5.2).

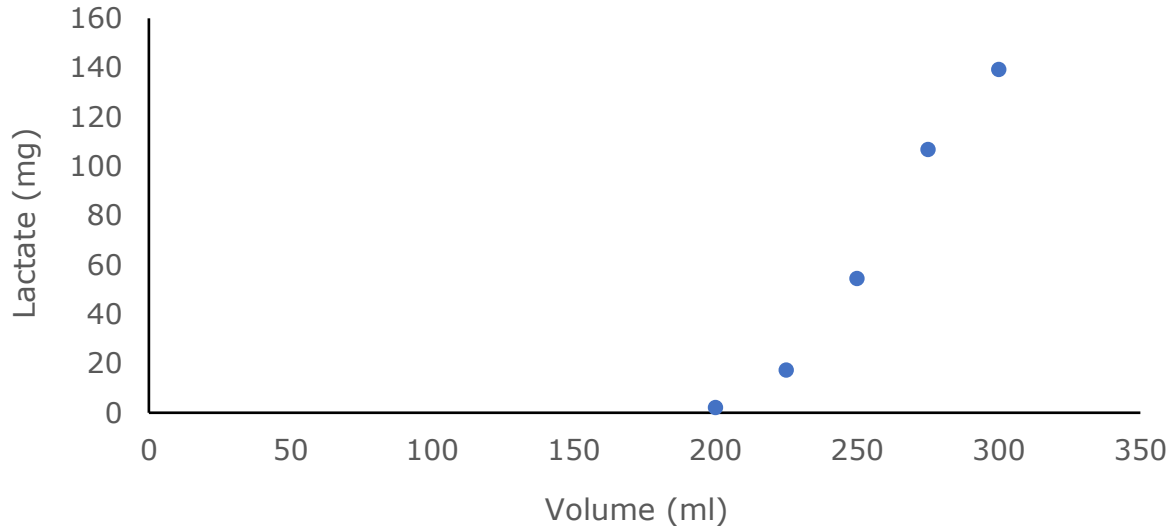


Figure 5.2. Breakthrough curve of lactic acid at pH 5 for anion exchange resin Amberlite resin IRA 96 at a flow rate of 10 ml/min**.**

The Amberlite resin was initially eluted with 0.1M HCL as this concentration was shown to achieve the highest recovery rate in a previous study (Bishai *et al.*, 2015). However, no lactic acid was observed in any of the 25 ml fractions after eluting with this concentration of HCL.

The concentration was increased to 1M HCL which resulted in a successful elution (Figure 5.3). A total of 2744 mg of lactate was recovered after eluting with 1M HCL with the majority being recovered after 175 ml and 200 ml of eluent had passed through the resin (Figure 5.3), suggesting that the dead volume of the column is approximately 150 ml. The overall recovery was

calculated as 81 %. The elution could be further optimised by using a gradient of HCl rather than an isocratic approach. Bishai et al., (2015) utilised a gradient ranging from 0.1 – 1M HCl, and although 93 % of the lactic acid was eluted at 0.1 M there were still small amounts eluted after increasing the molarity of HCl.

Previous studies have reported a higher lactic acid recovery when using Amberlite resin. Bishai et al. (2015) reported a recovery of 93%. Joglekar et al. (2006) reported a recovery of 92 % using Amberlite IRA-92 resin. Cao et al. (2002) achieved recovery of 97% using an Amberlite IRA-400 which is a similar weak anion exchange resin. This study also reported that the lactic acid recovery was significantly affected by the concentration of the chosen eluent. Bishai *et al*. (2015) tested the effect of 5 different HCL concentrations ranging from 0.05 – 1 N. The highest concentration and lactic acid purity were achieved at 0.1 N any increase in HCL concentration was shown to have a negative effect. Going forward a gradient of HCL will be tested in order to determine the optimal HCL concentration for the recovery of lactic acid. Overall the Amberlite IRA 96 resin was shown to be effective at removing a sodium lactate standard from water with a binding efficiency of 91%. The elution could be potentially further optimised by utilising a gradient of HCL.

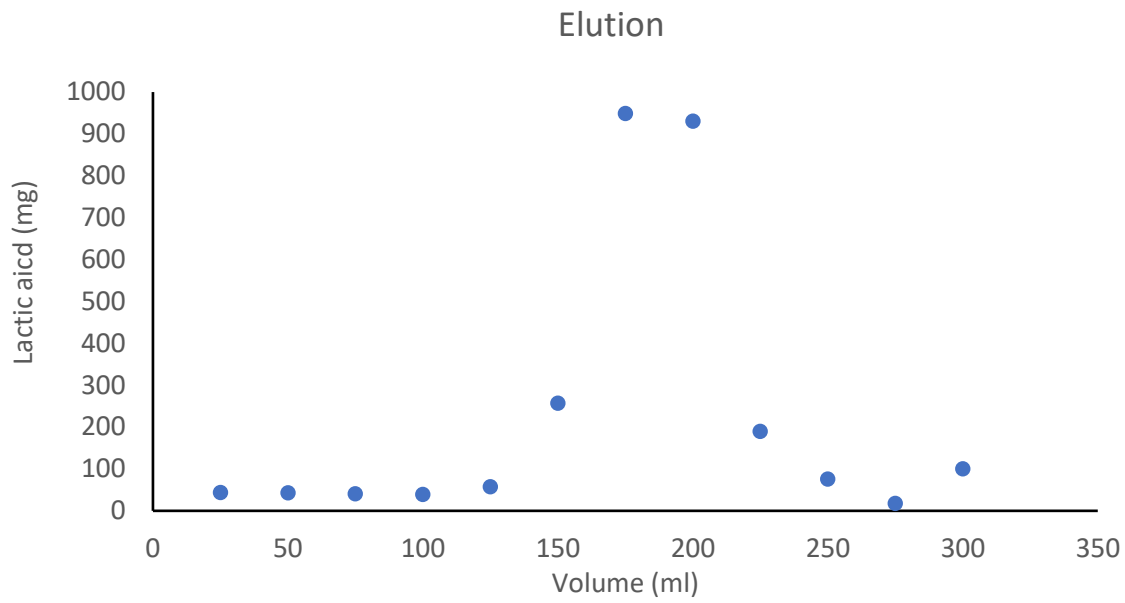


Figure 5.3. The elution of lactic acid from the Amberlite ion exchange resin using 1N HCL.

5.3.2 Treatment of pot ale using ion exchange chromatography

After filtering to remove the solid content of pot ale, the pH of the pot ale was adjusted from pH 3.5 to 5 using 10 M NaOH. This pH adjustment had the unintended effect of causing significant flocculation (Figure 5.4). A previous study also noted that pH adjustment of pot ale using sodium carbonate resulted in significant flocculation of yeast (Goodwin, Finlayson and Low, 2001), however, although the pot ale used in this investigation had been previously filtered (0.22 µm) it is unlikely that the observed precipitant contains substantial quantities of yeast. It is more likely that the precipitant primarily consists of the freely available protein content in the pot ale. Proteins are commonly precipitated from microbiological media by salting or by increasing pH (Nandakumar *et al.*, 2003; Wong, Ariff and Stuckey, 2018).

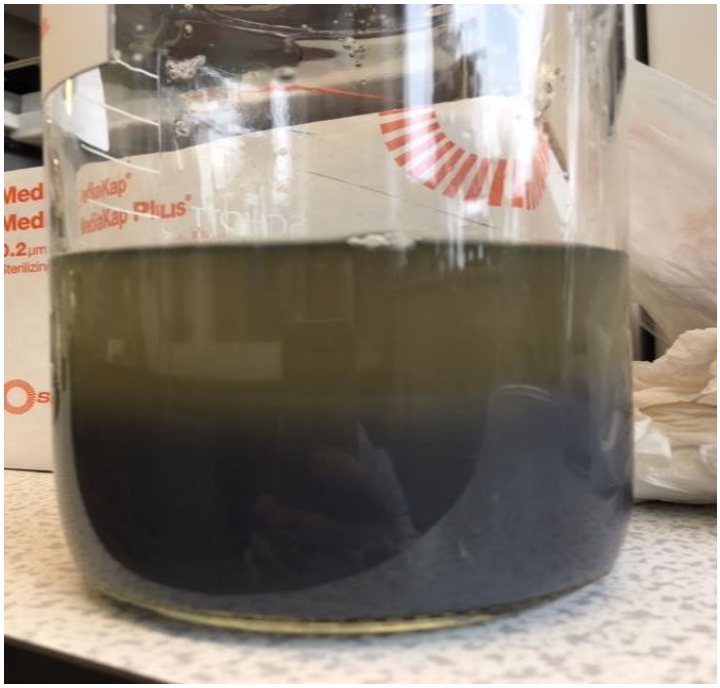


Figure 5.4. Flask of filtered pot ale showing precipitation after pH adjustment with NaOH.

Pot ale (440 ml) was loaded onto the Amberlite resin at a flow rate of 10 ml/min. The total starting amount of lactic acid was 1405.5 mg. A total of 33.2 mg of lactic acid was detected in the loading fractions (figure 5.5) which resulted in a binding efficiency of 97.6 %. The binding of acetic acid was also similar with limited breakthrough (Figure 5.5) observed and a binding efficiency of 97.8 %.

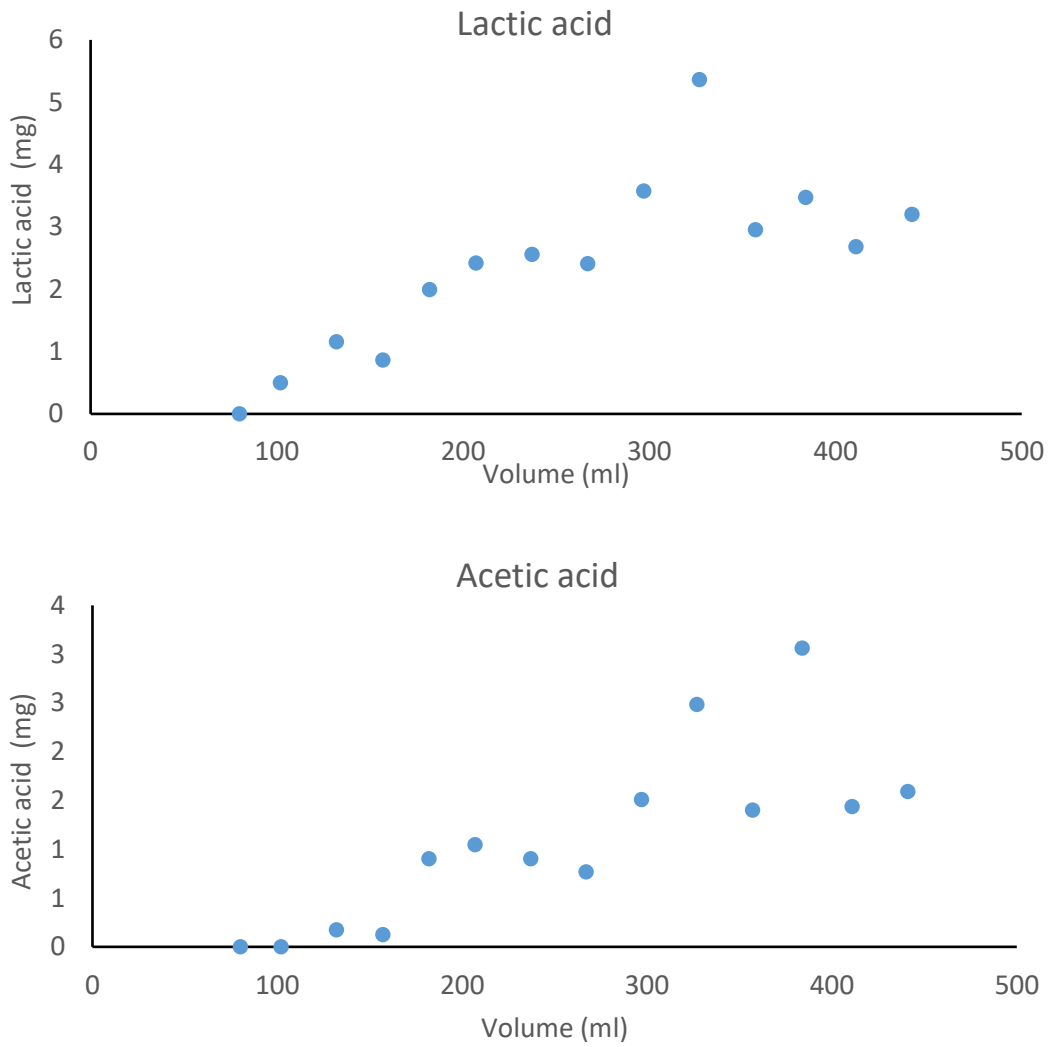


Figure 5.5. Breakthrough curves for the lactic acid and acetic acid content of pot ale fractions that were loaded onto Amberlite resin at a flow rate of 10 ml/min.

After being loaded with 500 ml of filtered pot ale the Amberlite resin was eluted with a gradient of HCL (0.1 – 1 M). A total of 1372 mg of lactic acid was recovered which represents 97.6 % of lactic acid that was loaded onto the resin. The concentration of recovered lactic acid was 3.1 g/L. The majority of the lactic acid was eluted when the concentration of HCL was 0.6M. Acetic acid also had a high recovery rate of 104 %. There is likely a degree of error that can be attributed due to slight variations in fraction size which resulted in a small degree of error. The elution of acetic acid was similar to lactic acid with the majority being eluted with 0.6 M of HCL.

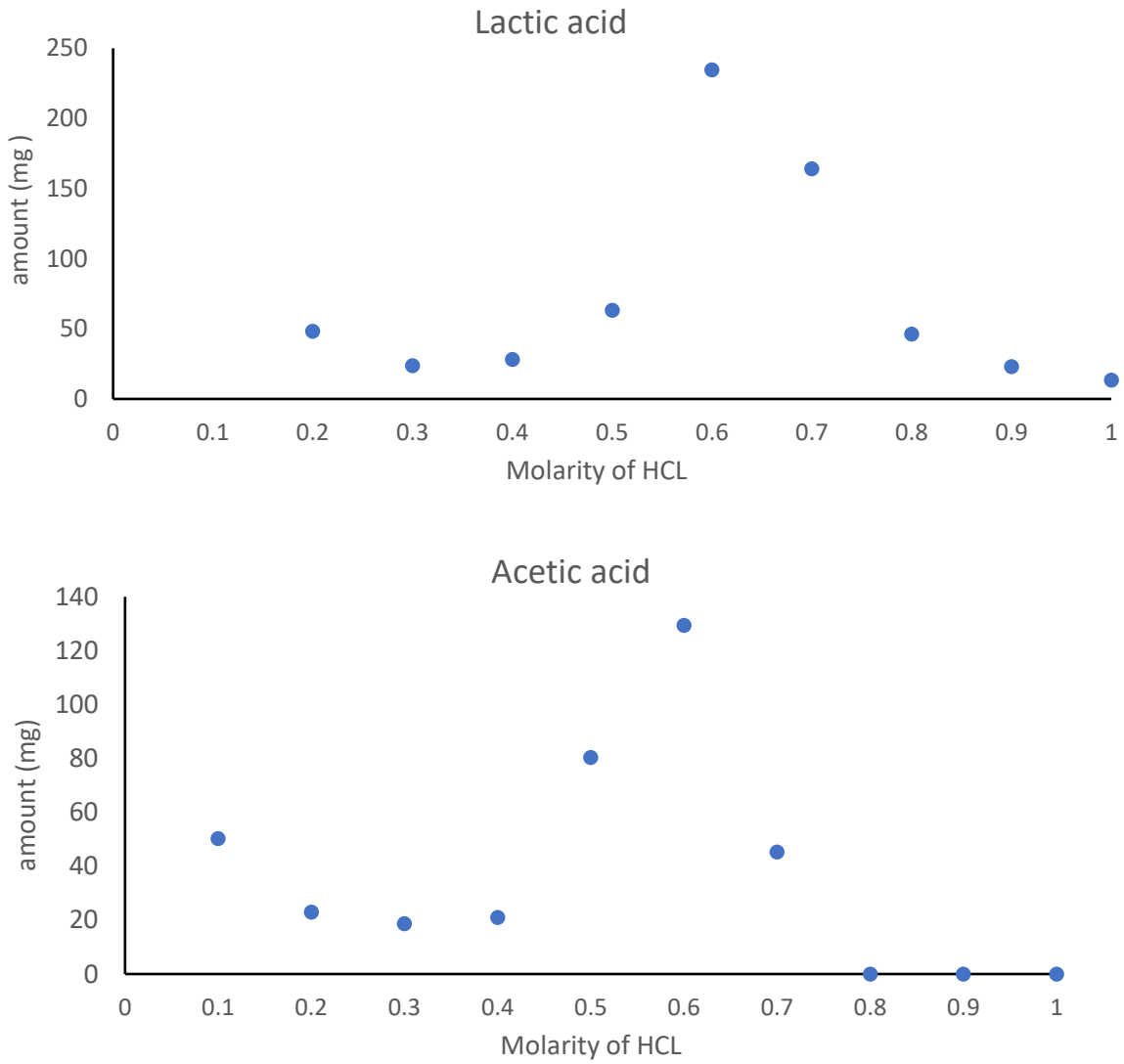


Figure 5.6. Elution of the Amberlite resin using a gradient of HCL at a flow rate of 10 ml/min.

The removal of the organic acid portion of pot ale was successful with greater than 98 % recovery rates observed in both lactic and acetic acid. These recovery rates are comparable with existing studies on the recovery of organic acids via ion exchange (Joglekar *et al.*, 2006; Bishai *et al.*, 2015; Komesu *et al.*, 2017). The downstream processing of organics acids from fermentation broths represents approximately 50-60% of the total cost during industrial scale production of organic acids (Qian-Zhu *et al.*, 2016). One of the commonly used methods of purification employed by industry is precipitation. Precipitation can be considered as a classical method as it has been used to recover organic acids from fermentation broths since the last century. Precipitation can recover organic acids from broth efficiently and is often considered as a good candidate

for preliminary purification in a downstream processing chain. Common precipitants include calcium salts ($CaOH_2$, $CaCO_3$) and ammonia. A previous study that utilised calcium precipitation to recover lactic acid from a fermentation broth reported yields of 92% under optimal conditions (Min *et al.*, 2011). The downside to calcium precipitation is that lactate is recovered as calcium lactate, therefore further downstream processing is required (Figure 5.7). This includes further precipitation to remove the calcium and acidification with H_2SO4 to return the lactate to its H^+ form. This process generates large quantities of calcium sulphate which can result in environmental concerns (Joglekar *et al.*, 2006). Therefore, ion exchange may be a more favourable option.

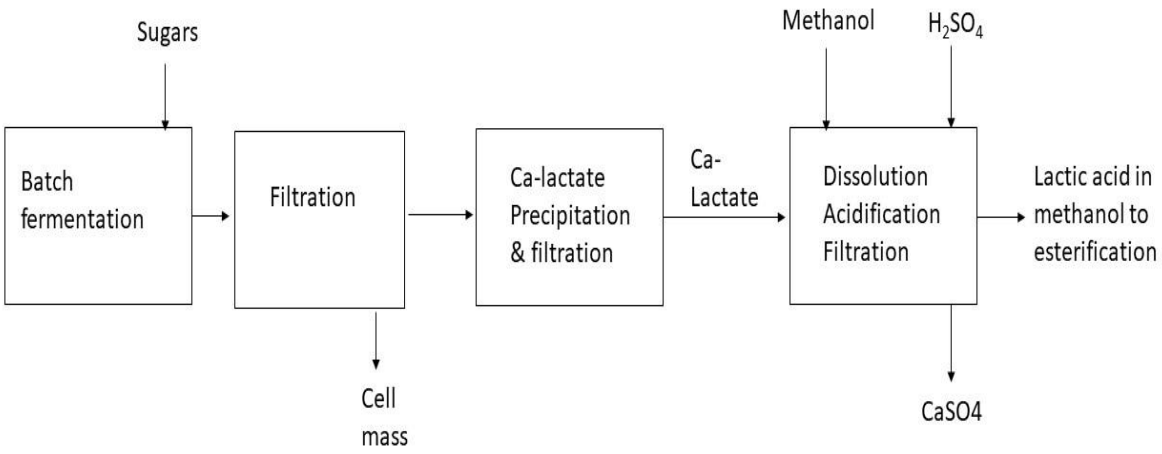


Figure 5.7. Example of a traditional downstream processing pathway for lactic acid production (Joglekar et al., 2006)

If lactic acid is the targeted product after the treatment of pot ale using ion exchange, then further purification steps will be required. The removal of organic acid impurities remains one the biggest challenges for the downstream processing of lactic acid at an industrial scale. Several methods including HPLC processes (Nam, Lim and Mun, 2012) and membrane separation (Cho, Lee and Park, 2012) have been successfully employed at lab scale. However, esterification and distillation remains one of the few methods that has been successfully utilised at industrial scale to remove organic acid impurities

(Joglekar *et al.*, 2006). Ion exchange could be incorporated into a downstream processing pathway that recovers lactic acid from pot ale (Figure 5.8).

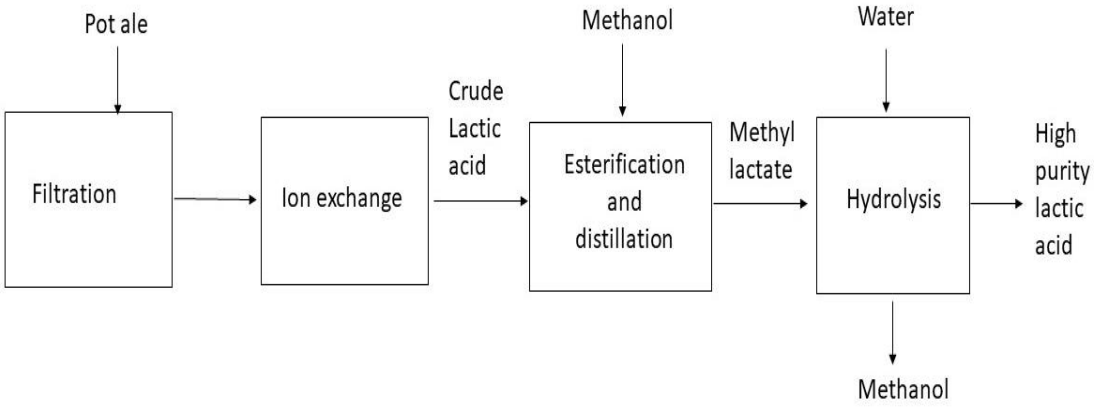


Figure 5.8. Proposed downstream processing pathway to purify lactic acid from pot ale.

The downside to this pathway is that large scale esterification and distillation is expensive due to the large volumes of solvents and high temperatures that are required. Therefore, further work is required to investigate more cost-effective methods for the separation of organic acids.

5.3.3 Economic viability of purifying lactic acid from pot ale

The fermentative production of lactic acid is well established in industry with sugars derived from cereal crops being among the most popular feedstocks (Garde *et al.*, 2002). Due to the variability of fermentation and losses during downstream processing the fermentative efficiency and lactic acid yield can be variable (Garde *et al.*, 2002). Previous studies have achieved 81.5% from glucose (Tokuhiro *et al.*, 2008), 78% from cellobiose (Garde *et al.*, 2002) and 69% from xylose (Turner *et al.*, 2015). Yields are typically greater than 0.7 g/L (Turner *et al.*, 2015). A previous study produced 80.95 g/L of purified racemic lactic acid from a fermentation using *L.delbrueckii*. (Orozco and Zuluaga, 2014). This yield is significantly higher than the 3.1 g/L of lactic acid that was recovered from pot ale in this investigation. Due to low yields the recovery of lactic acid

from pot ale may not be competitive with fermentative production despite cost savings by being an entirely downstream process. In order to establish the economic viability of this approach of lactic acid recovery a full techno-economic analysis would have to carried out which is not within the scope of this investigation.

In terms of availability there is approximately 607 kilo of available lactic acid in pot ale from Scottish malt whisky distilleries (Table 5.1). The lactic acid market, in 2013 was 750 kilo tonnes, therefore Scottish pot ale alone could make up 81% of the market demand in that year. As the demand for lactic acid has dramatically increased primarily due to increasing demand for PLA bioplastic the total demand for lactic is projected to increase to 1,844 kilo tonnes in 2019 (Zaini et al., 2019). This simultaneously increases the demand for carbon-based feedstocks for lactic acid fermentation putting further strain on agricultural land use, therefore, there is a need for research into the use of alternative non-first-generation feedstocks.

There have been previous studies that investigated the use of carbon-based waste streams as feedstocks for lactic acid fermentation. Ohkouchi & Inoue, (2006) investigated the fermentative production of lactic acid from food waste. A yield of 38.3 g/L of lactic acid was achieved from 200 g of food waste which is competitive with some lower end industrial fermentations and significantly higher than the yield achieved by this investigation. A similar study by Pleissner et al. (2017) achieved 52.4 g/L of lactic acid from the saccharification and fermentation of mixed restaurant food waste. Increasing concerns over stability may push the lactic acid industry into using second generation feedstocks such as food waste, this transition is encouraged by the promising results of research in this field and large availability of food waste worldwide (Djukić-Vuković *et al.*, 2019).

There has been little research investigating the direct recovery of organic acids from waste streams. A recent study by Reyhanitash et al. (2017) investigated the recovery of volatile fatty acids from fermented wastewater. The wastewater that was investigated was in some respects similar to pot ale with high but variable concentrations of organic acids and phosphates whilst also containing trace concentrations of heavy metals. The study utilised unspecified cation and

anion absorbents to recovery an array of compounds including lactic acid, acetic acid, butyric acid, pyruvic acid, phosphate and sulphate. Following desorption, all the VFAs were condensed into a single condensate and analysed via IC and HPLC. The lactic acid concentrations were low with less than 1 wt% observed in 6.15g of absorbent. Comparitvely large quantities of butyric acid were recovered (7.27 wt%). Similar to the findings of this research the study by Reyhanitash et al. (2017) recovered low concentrations of organic acids in comparison to traditional fermentative approaches. The multi-target approach adopted by Reyhanitash et al. (2017) could be investigated with pot ale, however, this may further complicate the downstream processing.

This investigation has demonstrated that it is possible to recover lactic acid from a distillery waste stream using ion exchange chromatography. However, future work is required in order to determine if this approach is economically viable and can be competitive with traditional industrial lactic acid fermentation.

5.3.4 The effect of ion exchange on the total organic carbon content and pH of pot ale.

The removal of organic acids from pot ale using ion exchange chromatography resulted in a significant 25% reduction in total organic carbon concentration (Figure 5.7). The pH of the untreated pot ale was pH 3.5, treatment with ion exchange increased the pH to 6.3 which presents a more favourable environment for microalgal growth. Therefore, the treatment of pot ale using ion exchange simultaneously decreases the negative environmental impact of pot ale whilst potentially adding value. The low pH of pot ale is also an issue for applications involving anaerobic digestion (Goodwin, Finlayson and Low, 2001). The treatment of pot ale using ion exchange could therefore potentially improve its performance as an AD feedstock.

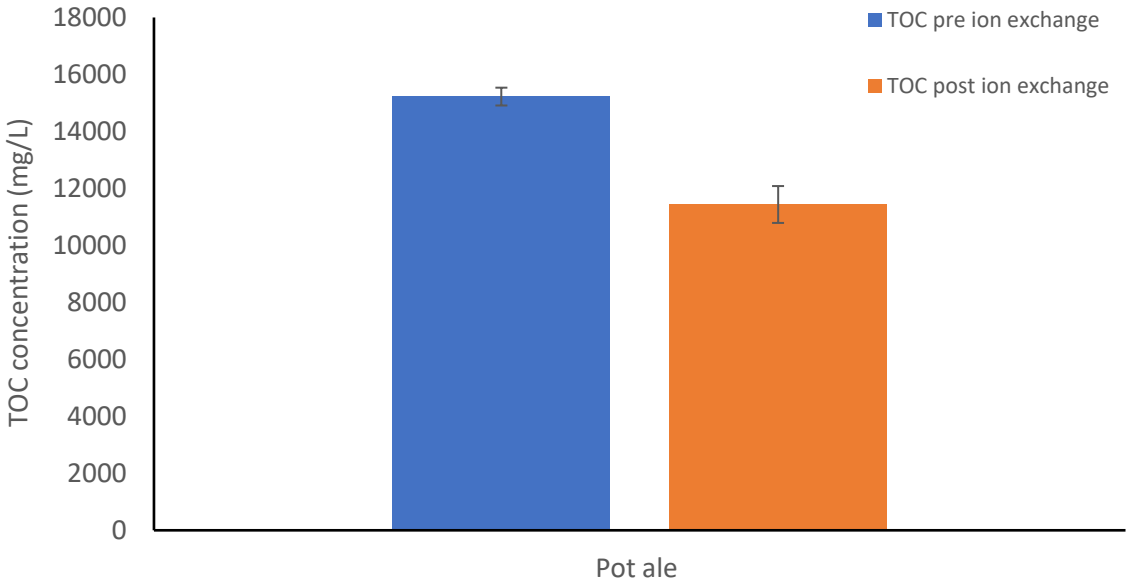


Figure 5.9. The total organic carbon content of pot ale, pre and post ion exchange chromatography using Amberlite IRA 96 resin**.**

5.3.3 The growth of microalgae in ion-exchange treated pot ale

To evaluate the potential benefits of pre-treating pot ale with ion exchange, the growth of a range of microalgae was evaluated. All species when grown in the BG-11 control had a significantly higher cell density when compared to the pot ale media treatments. *N. harveyana* PCC 7804 demonstrated the highest cell densities after 7 days of growth in the nutrient augmented pot ale medias. After 7 days the cell density of *N. harveyana* in all augmented pot ale medias was > 4.2×10^6 (Figure 5.10)

Synechocytis, Chlorella and *Nodularia* cultured in treated pot ale (pH adjusted via ion exchange) and treated pot ale supplemented with additional nutrients, resulted a significant increase in cell density when compared to an unaltered pot ale control (Figure 5.10). This shows that pH adjusting the pot ale via ion exchange and adding additional nutrients had an overall positive effect on growth. Cultures of *Microcystis* grown in treated and nutrient supplemented pot ale displayed no significant difference in cell density when compared to the pot ale control. This suggests that successful growth in pot ale could be species dependant.

The results of this investigation suggest that even after increasing the pH to 6.3 via ion exchange and fortifying the nutrient content of pot ale, it still failed to support microalgal growth. Therefore, there must be another aspect of pot ale that is limiting the growth of microalgae. It is possible that the copper content of pot ale is inhibiting growth as discussed previously in section 4.3.5. Pot ale is a very complex medium and it is also possible that the combined effects of some of its chemical components are having a negative culminative effect on growth. Future work is required to screen a wider range of microalgae

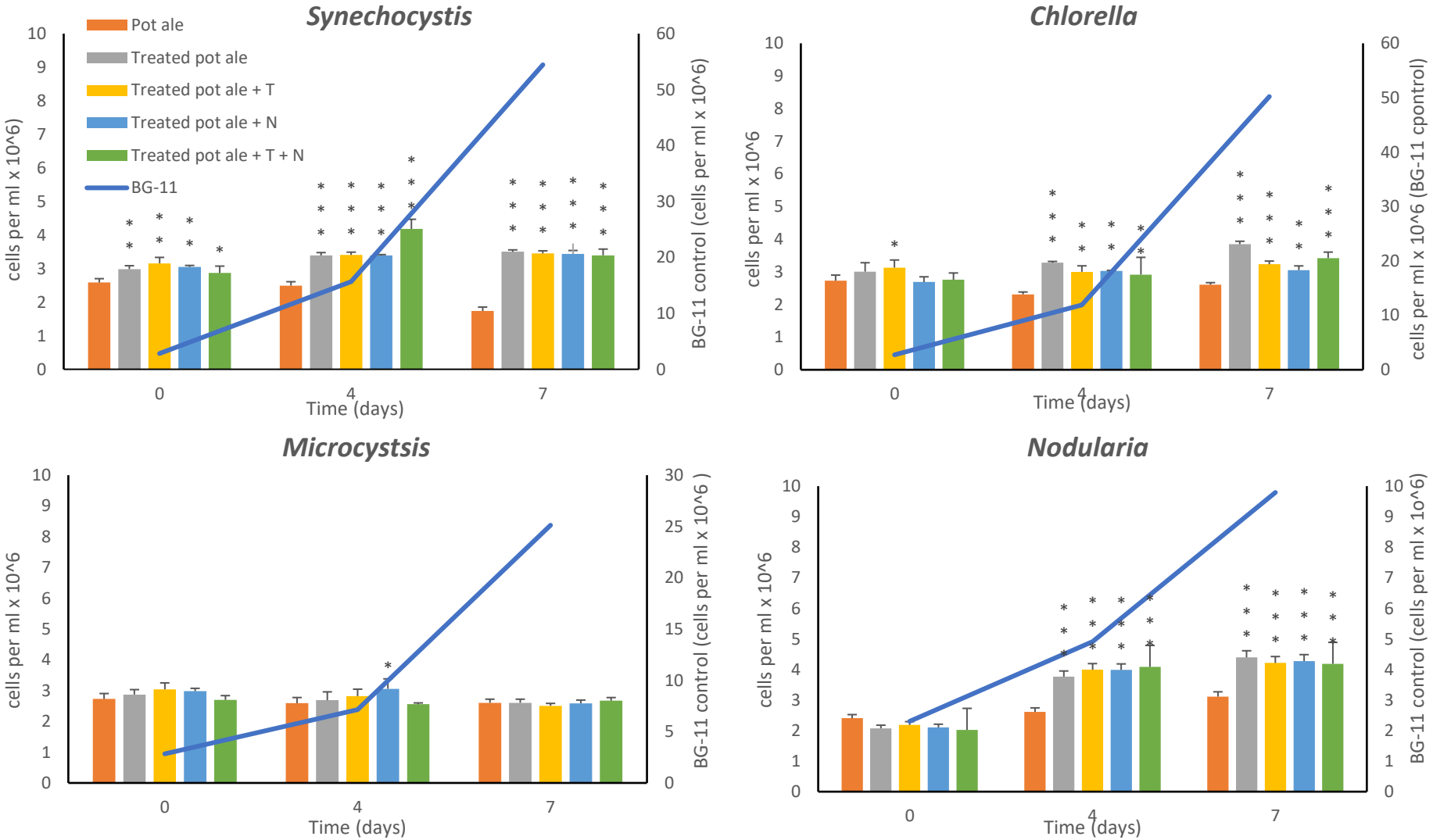


Figure 5.10. The growth of microalgal and cyanobacterial species in experimental pot ale media. The error bars are representative of the standard deviation from the mean. T = Trace elements, N = Nitrate. Statistical analysis performed using t tests assuming unequal variances, $p < 0.05$ (*), $p < 0.01$ (* *), $P < 0.001$ (***). n = 4

5.4 Conclusion

It was successfully demonstrated that lactic acid can be removed from pot ale using ion exchange. This process also increases the pH and reduces the TOC content. Therefore, the environmental impact is also minimised whilst also providing added value. Future work is required in order to determine if the purification of lactic acid from pot ale is economically viable on a large scale. Despite the removal of lactic acid from pot ale there was still limited microalgal growth observed even when heavily augmenting the pot ale with additional nutrients. This suggests that there are toxic elements in the pot ale that are inhibiting microalgal growth. Future work could aim to remove these elements for example utilising cation exchange resin to remove copper. However, this may prove to be too costly to make pot ale a viable replacement for traditional media.

5.5 References

Ahring, B.K., Traverso, J.J., Murali, N., Srinivas, K., 2016. Continuous fermentation of clarified corn stover hydrolysate for the production of lactic acid at high yield and productivity. Biochemical Engineering Journal 109, 162–169. https://doi.org/10.1016/j.bej.2016.01.012

Bishai, M., De, S., Adhikari, B., Banerjee, R., 2015. A platform technology of recovery of lactic acid from a fermentation broth of novel substrate Zizyphus oenophlia. 3 Biotech 5, 455–463. https://doi.org/10.1007/s13205-014-0240-y

Cao, X., Shik, H., Koo, Y., 2002. Recovery of l- (+) -lactic acid by anion exchange resin Amberlite IRA-400. Biochemical Engineering Journal 11, 189–196.

Chendake, Y.J., Kharul, U.K., 2014. Transport of organic acids through polybenzimidazole based membranes by "Chemodialysis." Journal of Membrane Science 451, 243–251. https://doi.org/10.1016/j.memsci.2013.10.014

Cho, Y.H., Lee, H.D., Park, H.B., 2012. Integrated membrane processes for separation and purification of organic acid from a biomass fermentation process. Industrial and Engineering Chemistry Research 51, 10207–10219. https://doi.org/10.1021/ie301023r

Cubas-Cano, E., González-Fernández, C., Ballesteros, M., Tomás-Pejó, E., 2018. Biotechnological advances in lactic acid production by lactic acid bacteria: lignocellulose as novel substrate. Biofuels, Bioproducts and Biorefining 12, 290–303. https://doi.org/10.1002/bbb.1852

Djukić-Vuković, A., Mladenović, D., Ivanović, J., Pejin, J., Mojović, L., 2019. Towards sustainability of lactic acid and poly-lactic acid polymers production. Renewable and Sustainable Energy Reviews 108, 238–252. https://doi.org/10.1016/j.rser.2019.03.050

Garde, A., Jonsson, G., Schmidt, A.S., Ahring, B.K., 2002. Lactic acid production from wheat straw hemicellulose hydrolysate by L. pentosus and L. brevis.pdf. Bioresourse Technology 81, 217–223.

Goodwin, J.A.S., Finlayson, J.M., Low, E.W., 2001. A further study of the anaerobic biotreatment of malt whisky distillery pot ale using an UASB system. Bioresource

Technology 78, 155–160. https://doi.org/10.1016/S0960-8524(01)00008-6

Graham-Rowe, D., 2011. Agriculture: Beyond food versus fuel. Nature 474, S6–S8. https://doi.org/10.1038/474s06a

Joglekar, H.G., Rahman, I., Babu, S., Kulkarni, B.D., Joshi, A., 2006. Comparative assessment of downstream processing options for lactic acid. Separation and Purification Technology 52, 1–17. https://doi.org/10.1016/j.seppur.2006.03.015

John, R.P., Nampoothiri, K.M., 2007. Fermentative production of lactic acid from biomass : an overview on process developments and future perspectives. Applied Microbiology and Biotechnology 524–534. https://doi.org/10.1007/s00253-006-0779-6

Juturu, V., Wu, J.C., 2016. Microbial production of lactic acid: the latest development. Critical Reviews in Biotechnology 36, 967–977. https://doi.org/10.3109/07388551.2015.1066305

Komesu, A., de Oliveira, J.A.R., Martins, L.H. da S., Maciel, M.R.W., Filho, R.M., 2017. Lactic acid production to purification: A review. BioResources 12, 4364–4383. https://doi.org/10.15376/biores.12.2.4364-4383

Madhavan Nampoothiri, K., Nair, N.R., John, R.P., 2010. An overview of the recent developments in polylactide (PLA) research. Bioresource Technology 101, 8493–8501. https://doi.org/10.1016/J.BIORTECH.2010.05.092

Melnicki, M.R., Pinchuk, G.E., Hill, E. a., Kucek, L. a., Stolyar, S.M., Fredrickson, J.K., Konopka, A.E., Beliaev, A.S., 2013. Feedback-controlled LED photobioreactor for photophysiological studies of cyanobacteria. Bioresource Technology 134, 127–133. https://doi.org/10.1016/j.biortech.2013.01.079

Min, D.J., Choi, K.H., Chang, Y.K., Kim, J.H., 2011. Effect of operating parameters on precipitation for recovery of lactic acid from calcium lactate fermentation broth. Korean Journal of Chemical Engineering 28, 1969–1974. https://doi.org/10.1007/s11814-011-0082-9

Nam, H.G., Lim, G.W., Mun, S., 2012. Separation of acetic acid, formic acid, succinic acid, and lactic acid using adsorbent resin. Journal of Chemical and Engineering Data 57, 2102–2108. https://doi.org/10.1021/je201065u

Nandakumar, M.P., Shen, J., Raman, B., Marten, M.R., 2003. Solubilization of

trichloroacetic acid (TCA) precipitated microbial proteins via NaOH for two-dimensional electrophoresis. Journal of Proteome Research 2, 89–93. https://doi.org/10.1021/pr025541x

Ohkouchi, Y., Inoue, Y., 2006. Direct production of l(+)-lactic acid from starch and food wastes using Lactobacillus manihotivorans LMG18011. Bioresource Technology 97, 1554–1562. https://doi.org/10.1016/j.biortech.2005.06.004

Orozco, F.G., Zuluaga, F., 2014. Lactic Acid Yield Using Different Bacterial Strains, Its Purification, and Polymerization through Ring-Opening Reactions. Intefnational journal of Polymer Science 2014.

Pleissner, D., Demichelis, F., Mariano, S., Fiore, S., Navarro Gutiérrez, I.M., Schneider, R., Venus, J., 2017. Direct production of lactic acid based on simultaneous saccharification and fermentation of mixed restaurant food waste. Journal of Cleaner Production 143, 615–623. https://doi.org/10.1016/j.jclepro.2016.12.065

Qian-Zhu, L., Xing-Lin, J., Xin-Jun, F., Ji-Ming, W., Chao, S., Hai-Bo, Z., Mo, X., Hui-Zhou, L., 2016. Recovery Processes of Organic Acids from Fermentation Broths in the Biomass-Based Industry. Journal of microbiology and biotechnology 26, 1–8. https://doi.org/10.4014/jmb.1505.05049

Reyhanitash, E., Kersten, S.R.A., Schuur, B., 2017. Recovery of Volatile Fatty Acids from Fermented Wastewater by Absorption. ACS Sustainable Chemistry and Engineering 5, 9176–9184. https://doi.org/10.1021/acssuschemeng.7b02095

Russel, I., S.G., 2014. Whisky. Production, Technology and Marketing., 2nd ed. Academic Press. https://doi.org/10.1016/B978-0-12-401735-1/00024-6

Tokuhiro, K., Ishida, N., Kondo, A., Takahashi, H., 2008. Lactic fermentation of cellobiose by a yeast strain displaying β-glucosidase on the cell surface. Applied Microbiology and Biotechnology 79, 481–488. https://doi.org/10.1007/s00253-008-1454-x

Turner, T.L., Zhang, G.C., Kim, S.R., Subramaniam, V., Steffen, D., Skory, C.D., Jang, J.Y., Yu, B.J., Jin, Y.S., 2015. Lactic acid production from xylose by engineered Saccharomyces cerevisiae without PDC or ADH deletion. Applied Microbiology and Biotechnology 99, 8023–8033. https://doi.org/10.1007/s00253-

015-6701-3

Wong, F.W.F., Ariff, A.B., Stuckey, D.C., 2018. Downstream protein separation by surfactant precipitation: a review. Critical Reviews in Biotechnology 38, 31–46. https://doi.org/10.1080/07388551.2017.1312266

Zaini, N.A.B.M., Chatzifragkou, A., Charalampopoulos, D., 2019. Microbial production of d-lactic acid from dried distiller's grains with solubles. Engineering in Life Sciences 19, 21–30. https://doi.org/10.1002/elsc.201800077

Chapter 6

Conclusions and future work

6.1 Conclusions

There is potential for further resource recovery from pot ale as it is currently a underutilised co-product that presents a significant disposal challenge to the industry. Pot ale has a high COD and contains toxic heavy metals, therefore, there are environmental concerns surrounding its disposal. If pot ale can be further valorised, then its environmental impact can be minimised. There is currently a desire to create a circular bioecomony in Scotland and beyond hence, if pot ale can be better utilised then a significant contribution can be made towards that goal (Arnison and Carrick, 2015).

This investigation aimed to recover the nutrient component of pot ale by utilising it as a carbon/N + P source to produce microalgae. Despite the concentrations of nitrate, phosphate and sugars present successful cultivation of microalgae using pot ale was not achieved in this study. Pot ale was significantly modified by removing the organic acid component via ion exchange chromatography and by the addition of nutrients. Despite these alterations there was little growth observed in the four species tested. This suggests that there may be toxic elements in the pot ale that are inhibiting growth or that it is lacking components necessary for growth It was observed that pot ale can contain toxic concentrations of copper which may be the cause of this inhibition.

An extensive characterisation audit carried out in this investigation sampled pot ale from 22 distilleries. This is the largest such audit carried out on pot ale to date. The aim of the audit was to highlight potentially novel valorisation opportunities and to gain a better understating as to what extent the chemical components of pot ale varied. The audit observed that the chemical components of pot ale vary greatly, however, no statistically significant relationship between

the observed variation and whisky processing parameters could be identified by PCA analysis. The characterisation audit also identified large concentrations of lactic acid in pot ale. This present an opportunity for valorisation as the demand for lactic acid is increasing due to the rising bio-plastics market. The investigation demonstrated that lactic acid can be removed from pot ale utilising ion exchange chromatography, achieving a yield of 98 %.

Another aim of this investigation was to optimise the light tracking capability of the Micro-Pharos photobioreactor in order to produce high value pigments. The light tracking software utilised in the Micro-Pharos PBR was successfully optimised and utilised to produce high density cultures of *Synechocystis sp.* PCC 6803, *Chlorella sorokiniana* and *Microcystis aeruginosa* PCC 7813. It was demonstrated that light tracking conditions increased the zeaxanthin and echinenone production rate of *Synechocystis* sp. PCC 6803 by providing light stressing conditions. It was identified that *C. sorokiniana* was the best candidate for scale up by screening the available cultures. The production of *C. sorokiniana* was successfully scaled up to 700 litres using the Pandora LED photobioreactor. However, the concentration of zeaxanthin was approximately 50% lower than the concentration observed in the Micro-Pharos PBR. This is potentially due to the difficultly in providing enough light to achieve light stressing conditions in a 700-litre culture volume. However, the total potential zeaxanthin yield of 1.8 g/L could result in significant profit if operational cost can be reduced.

The key and most impactful findings of this investigation were as follows.

- The data generated by the characterisation audit which informs existing valorisation efforts and highlights novel options for resource recovery.
- The lactic acid content of pot ale can be removed using ion exchange chromatography which adds value whilst simultaneously reducing the environmental impact of pot ale.
- Photobioreactors utilising LED light tracking technology can be utilised to produce high value carotenoids at scale of 700 litres which highlights the potential for industrial scale production.

6.2 Future work

The characterisation audit highlighted further opportunities for resource recovery from pot ale such as the high concentrations of phosphate that were observed. Phosphate is a finite resource and is heavily utilised due to the demand for inorganic fertiliser. Future work could investigated the removal of phosphate from pot ale by using techniques such as biochar and chitosan absorption and struvite precipitation. Future work is also required to determine if extraction of lactic acid from pot ale can be made economically viable. Cost effective methods of purification are required if the recovery process is to be competitive with the fermentative production of lactic acid.

Regarding the growth of microalgae in pot ale future work could investigate the removal of copper from pot ale using cation exchange chromatography. The pot ale – Cu could then be utilised for microalgal growth investigations. Another approach would be to acclimatise microalgae to pot ale over a longer period or to investigate the use of Cu tolerant extremophiles.

To advance the production of zeaxanthin from *C. sorokiniana* using the Pandora PBR to the next stage a viable means of large-scale extraction and purification must be identified. A full techno-economic analysis of the production process must also be carried out in order to determine economic viability. Interest in photobioreactor design and applications will continue to develop with demands for 'green chemistry' as many more solutions become available through LEDs and nutrient recycling. Simple modular systems will transform the range of applications and types of products available for microalgae.

Appendices

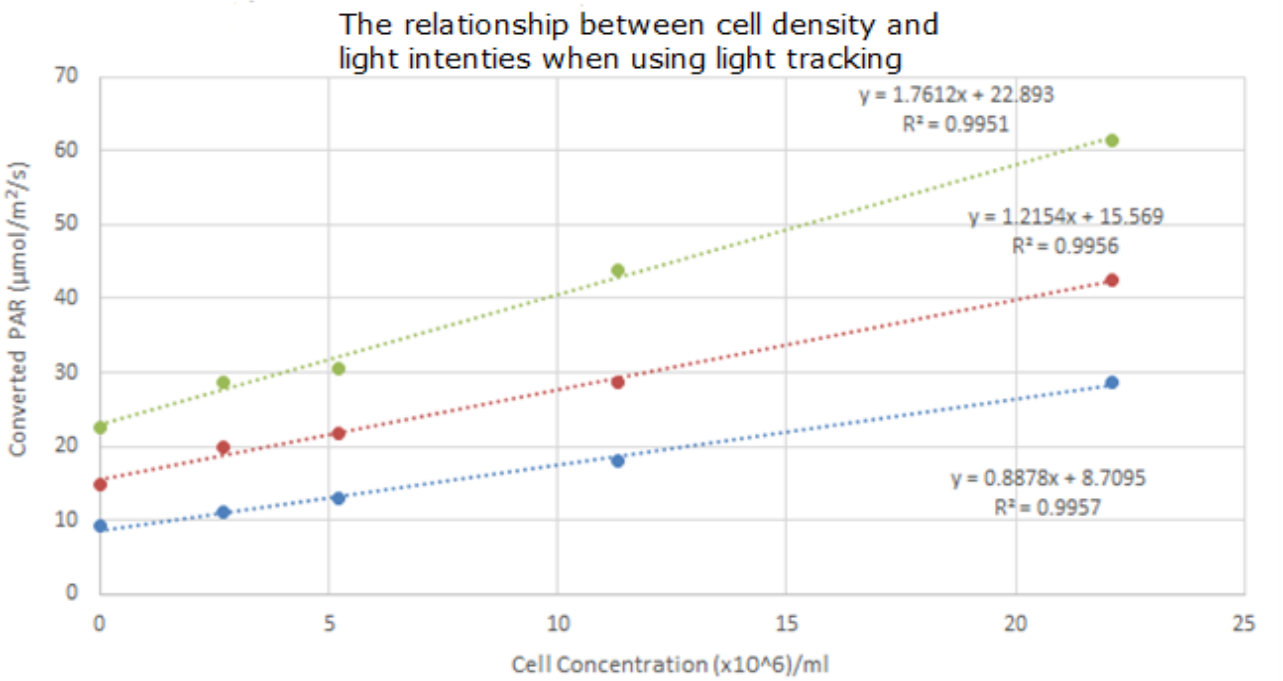


Figure 1A. The green line 400 AU tracking, the red line 350 AU tracking and the blue line 300 AU tracking value. Showing the positive correlation $R^2 > 0.99$ between cell density and light intensity when using light tracking.

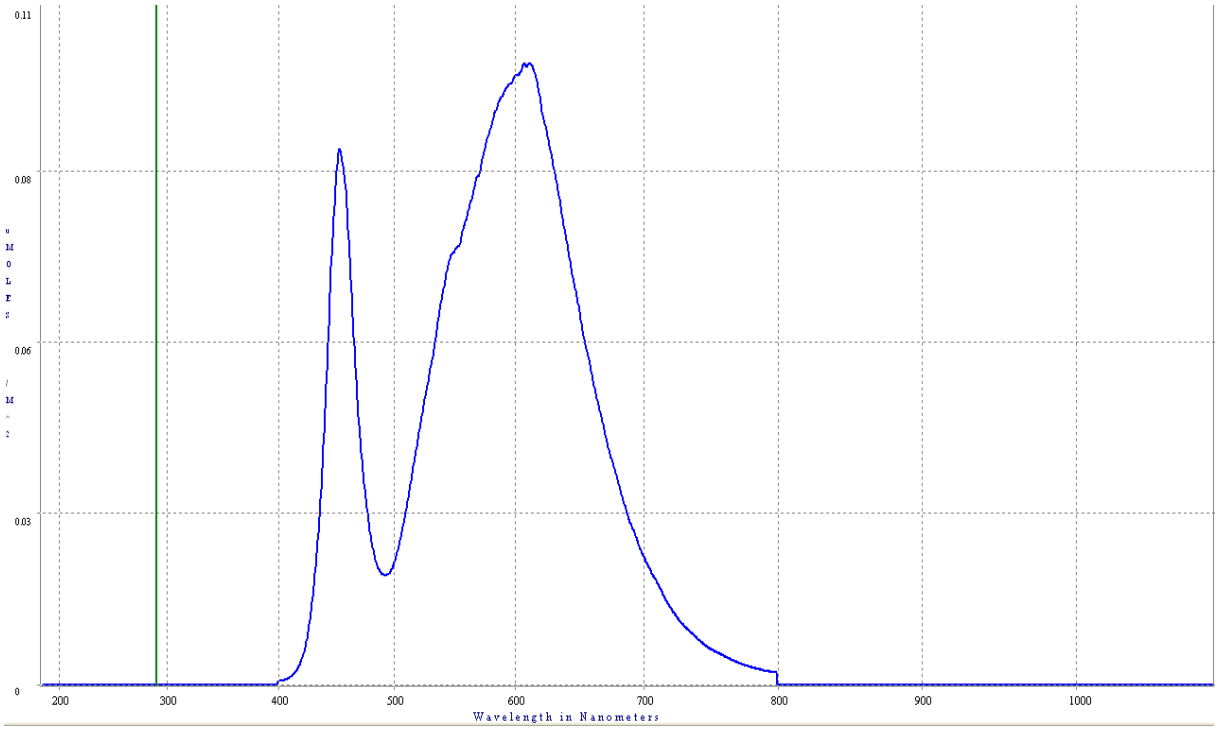


Figure 2A. Spectral output of the white LED panels used in the Micro-Pharos 1 litre PBR at a current of 140ma.

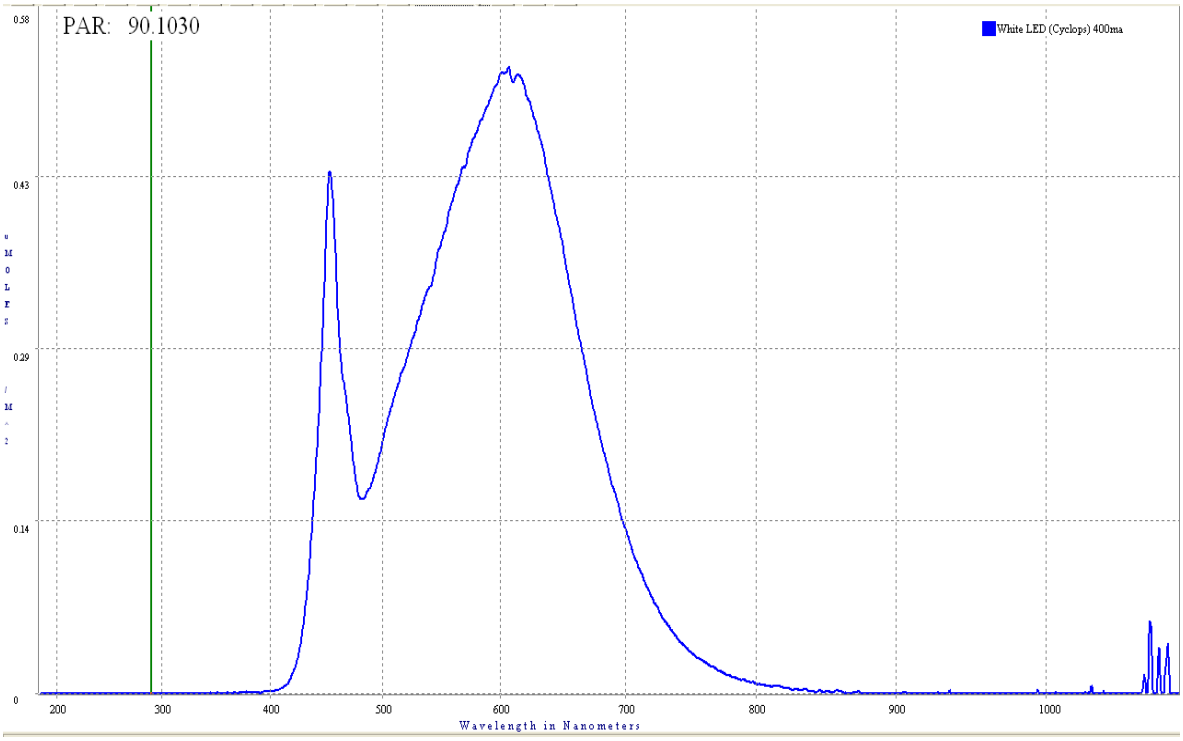


Figure 3A. Spectral output of the white LEDs in the Goldilocks light unit utilised by the Cyclops 16 litre PBR at a current of 400ma.

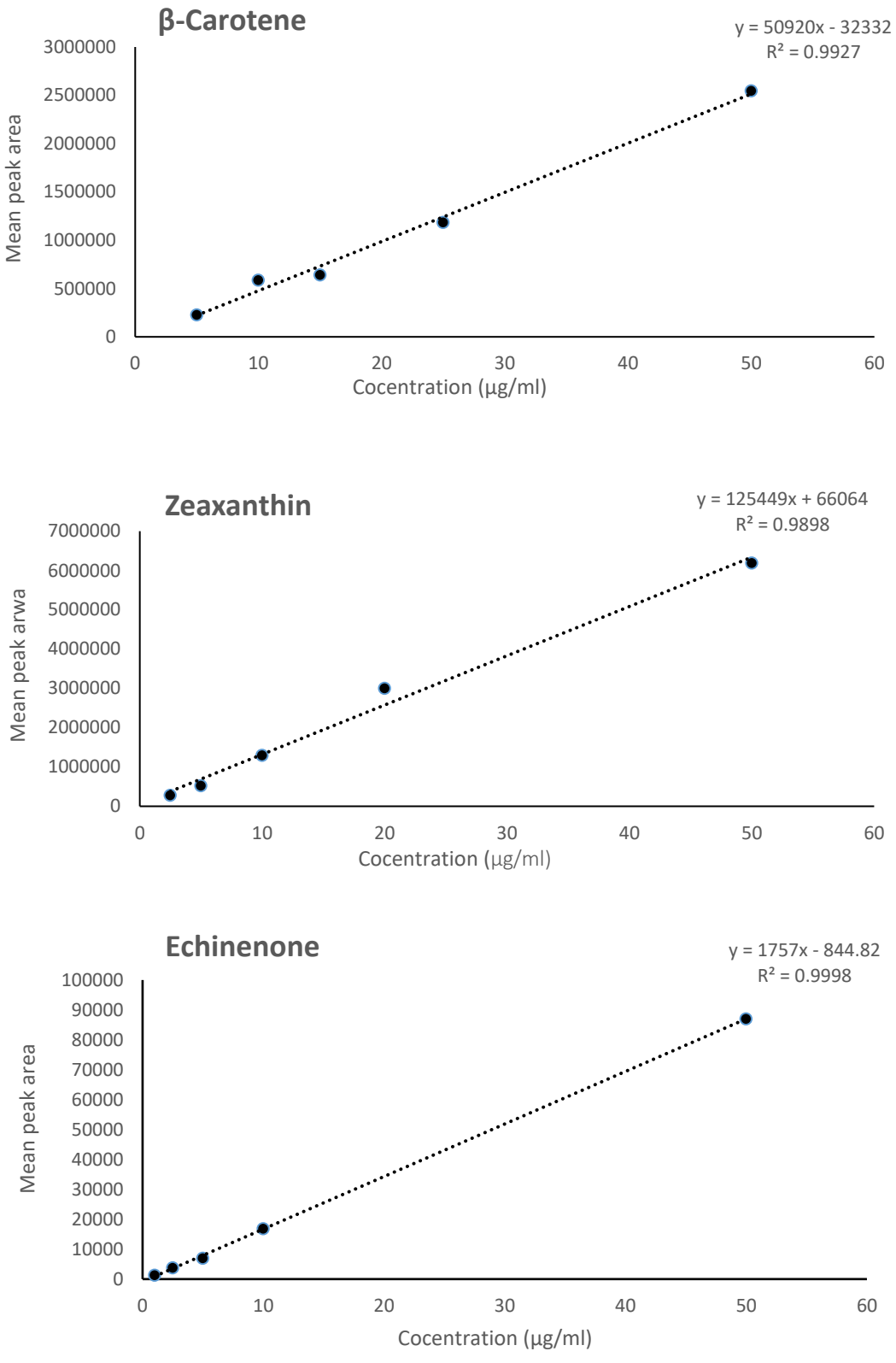


Figure 4A. Calibration curves of the absorption (452nm) of the carotenoid standards.

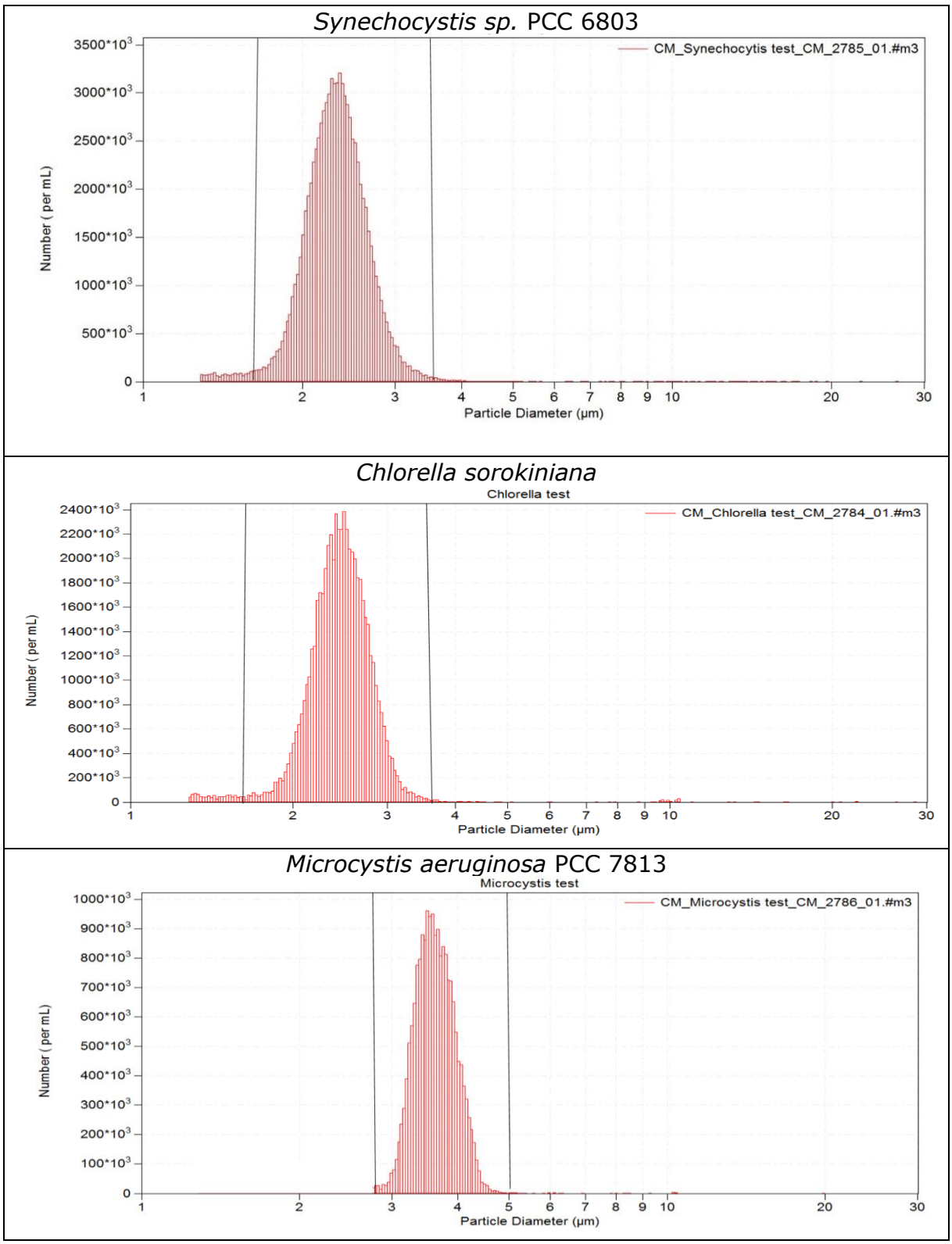


Figure 5A. Coulter counter data for each of the species utilised in this study. The vertical lines indicate the size window used for counting.

Characterisation Sampling Form

Please complete this information and return with your samples

Distillery code:
This is a numeric code provided via email by SWRI to ensure confidentiality of the data (please email kenneth.macgregor@swri.co.uk if you can't find this information).

Barley variety:

Malt peating level (delete as applicable): unpeated / lightly peated / medium peated/
heavily peated

Wort clarity (delete as applicable): clear / cloudy / unknown

Yeast strain:

Length of fermentation (hours):

Date of sampling:

Any other comments:

Date of receipt (to be completed by Robert Gordon University):

Figure 6A. Pot ale sampling form that was sent to distilleries participating in the pot ale characterisation audit.

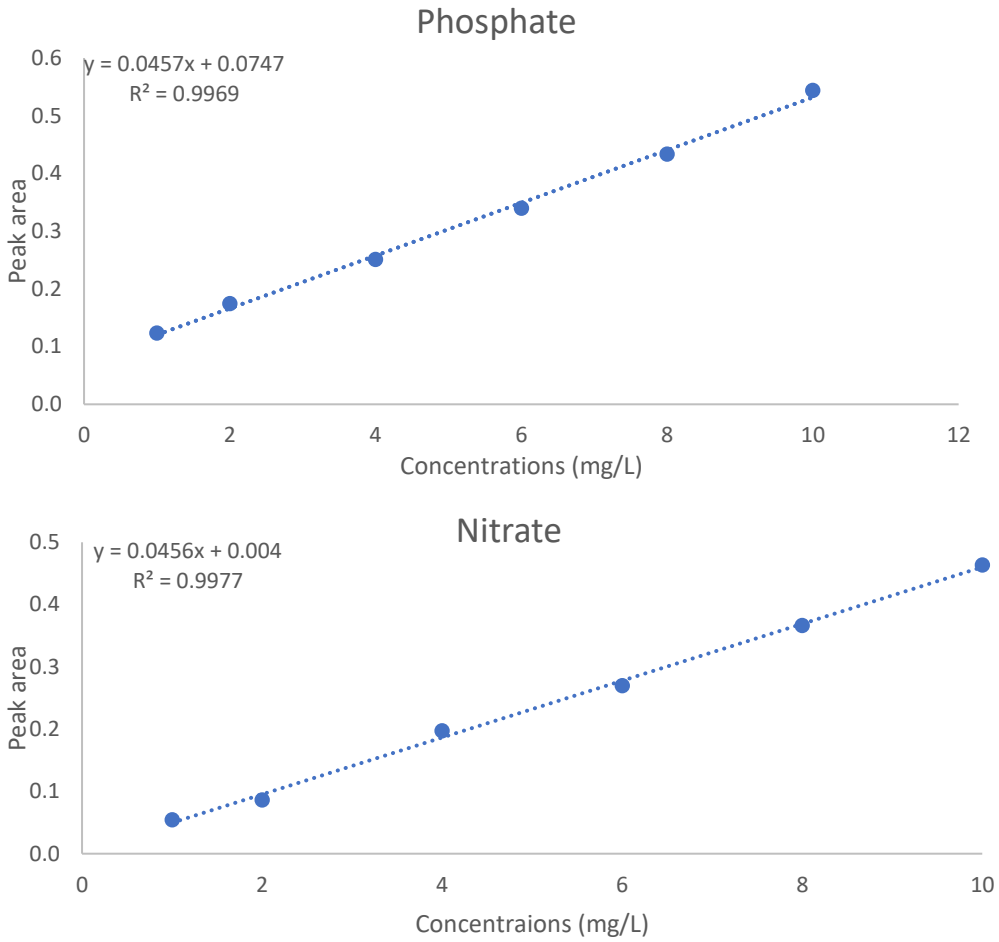


Figure 7A. Calibration curves for the Phosphate and Nitrate standards as measured by ion chromatography.

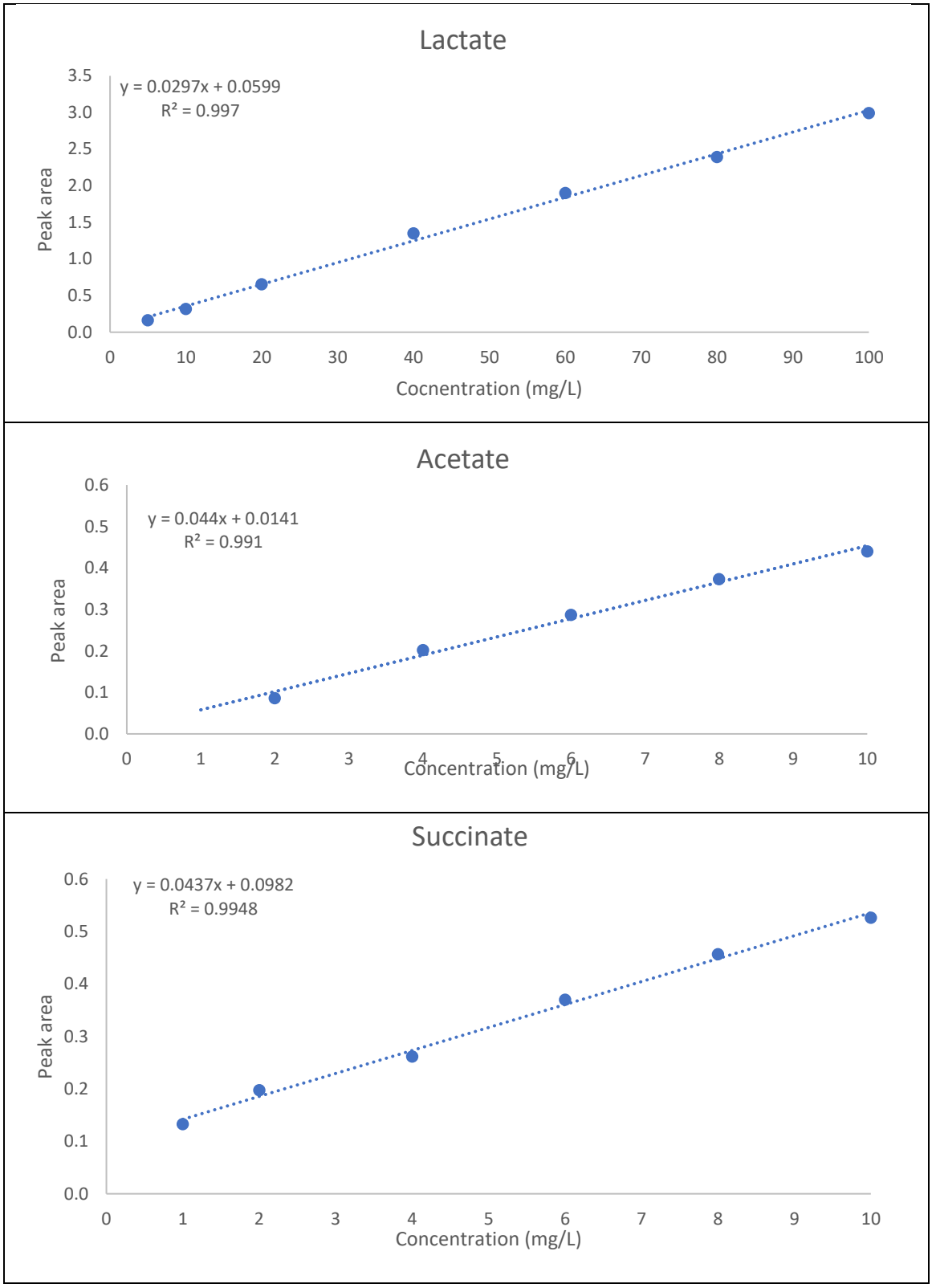


Figure 8A. Calibration curves for the Lactate, Acetate and Succinate standards as measured by ion chromatography.

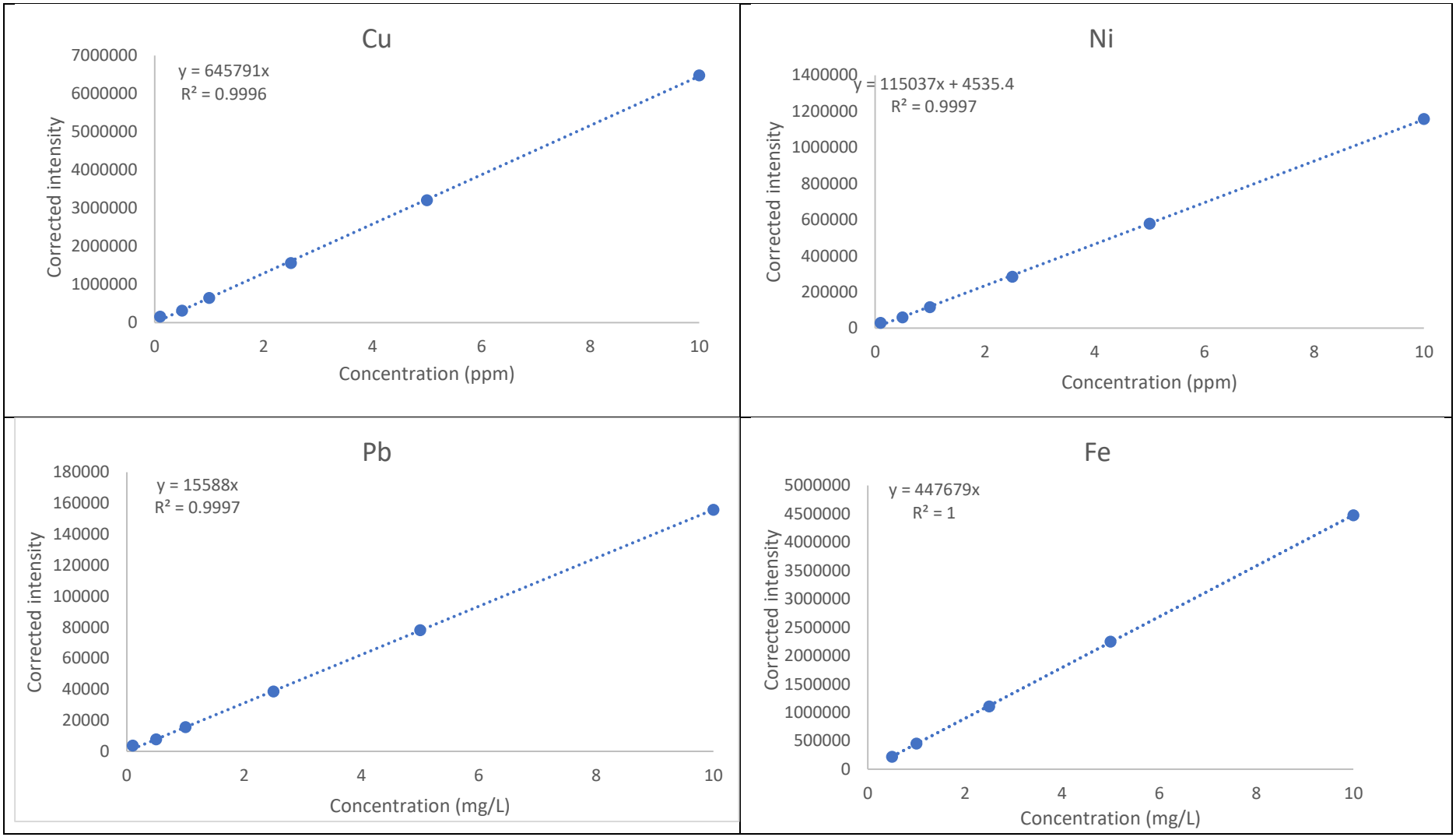


Figure 9A. Calibrations curves generated by the standards used to quantify the metal content of pot ale

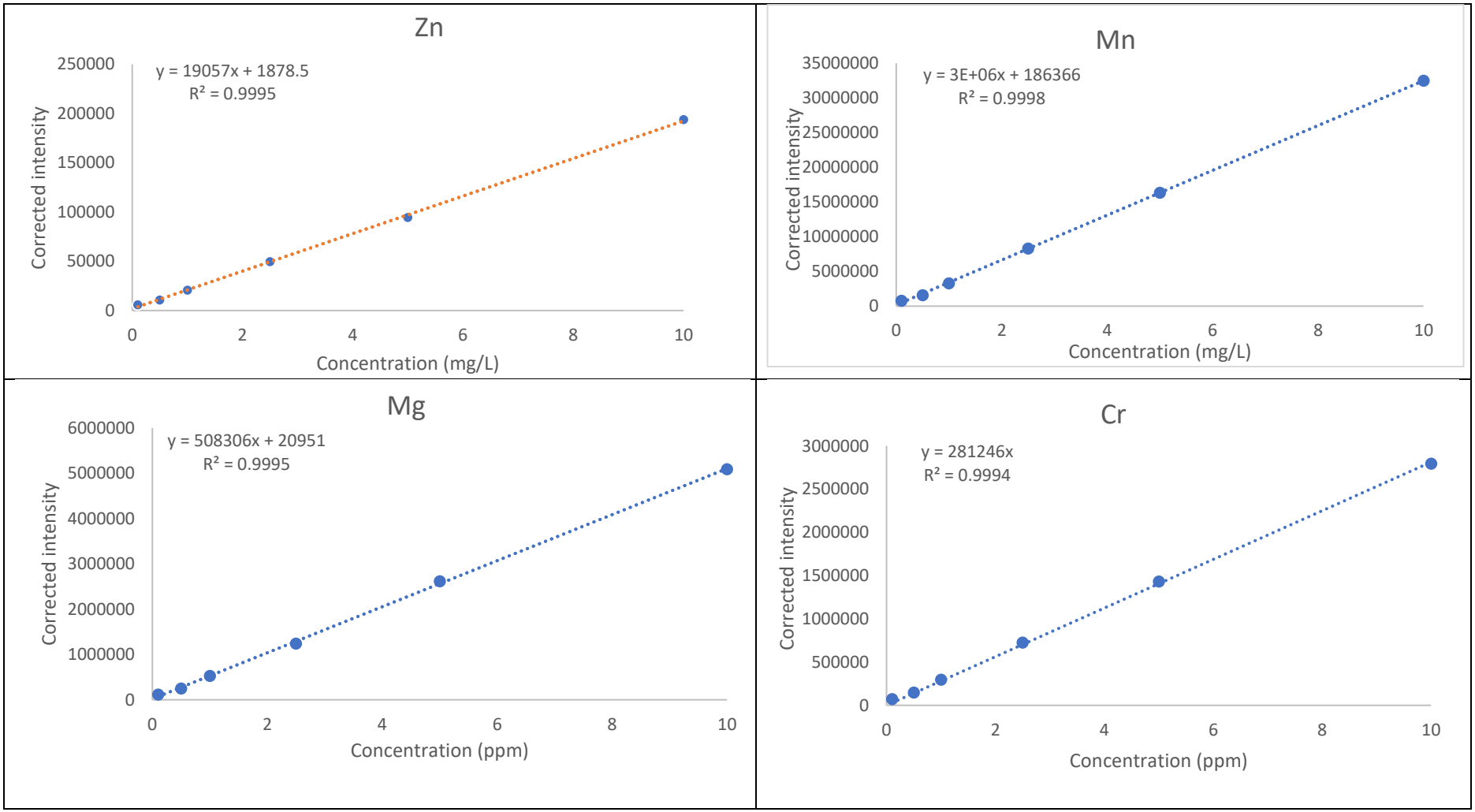


Figure 10A. Calibrations curves generated by the standards used to quantify the metal content of pot ale

Table 1A. The soluble metal content of each distillery represented in the pot ale audit. Units = mg/L Dilution factor = 10

Distillery A	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	21.35	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00
2	21.20	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00
3	23.16	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00
Mean	21.90	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.00
Corrected	219.03	0.00	0.19	0.00	0.01	0.00	0.00	0.00	0.00
SD	0.89	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SD corrected	8.91	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00

Distillery B	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	16.59	0.02	0.03	0.00	0.02	0.22	0.00	0.00	0.00
2	20.57	0.03	0.04	0.00	0.02	0.27	0.00	0.00	0.00
3	23.43	0.03	0.05	0.00	0.02	0.23	0.00	0.00	0.00
Mean	20.20	0.02	0.04	0.00	0.02	0.24	0.00	0.00	0.00
Corrected	201.97	0.24	0.38	0.00	0.18	2.39	0.00	0.00	0.00
SD	2.80	0.00	0.01	0.00	0.00	0.02	0.00	0.00	0.00
SD corrected	28.05	0.05	0.06	0.00	0.02	0.22	0.00	0.00	0.00

Distillery C	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	20.23	0.00	0.05	0.00	0.12	0.00	0.00	0.00	0.00
2	19.81	0.00	0.04	0.00	0.05	0.00	0.00	0.00	0.00
3	19.12	0.00	0.02	0.00	0.11	0.00	0.00	0.00	0.00
Mean	19.72	0.00	0.04	0.00	0.09	0.00	0.00	0.00	0.00
Corrected	197.20	0.00	0.37	0.00	0.93	0.00	0.00	0.00	0.00
SD	0.46	0.00	0.01	0.00	0.03	0.00	0.00	0.00	0.00
SD corrected	4.58	0.00	0.12	0.00	0.31	0.00	0.00	0.00	0.00

Distillery D	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	22.21	0.00	0.05	0.00	0.12	0.00	0.00	0.00	0.00
2	21.45	0.00	0.05	0.00	0.07	0.00	0.00	0.00	0.00
3	19.14	0.00	0.04	0.00	0.08	0.00	0.00	0.00	0.00
mean	20.93	0.00	0.05	0.00	0.09	0.00	0.00	0.00	0.00
corrected	209.33	0.00	0.47	0.00	0.90	0.00	0.00	0.00	0.00
SD	1.31	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
SD corrected	13.05	0.00	0.05	0.00	0.22	0.00	0.00	0.00	0.00

Distillery E	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	16.20	0.00	0.00	0.00	0.17	0.19	0.02	0.00	0.00
2	13.44	0.00	0.00	0.00	0.14	0.13	0.02	0.00	0.00
3									
Mean	14.82	0.00	0.00	0.00	0.16	0.16	0.02	0.00	0.00
Corrected	148.20	0.00	0.00	0.00	1.59	1.62	0.16	0.00	0.00
SD	1.38	0.00	0.00	0.00	0.02	0.03	0.00	0.00	0.00
SD corrected	13.80	0.00	0.00	0.00	0.16	0.28	0.01	0.00	0.00

Distillery F	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	15.11	0.00	0.00	0.00	0.01	0.06	0.04	0.00	0.00
2	15.77	0.00	0.01	0.00	0.01	0.08	0.00	0.00	0.00
3	16.71	0.00	0.00	0.00	0.01	0.07	0.00	0.00	0.00
Mean	15.86	0.00	0.00	0.00	0.01	0.07	0.01	0.00	0.00
Corrected	158.63	0.00	0.02	0.00	0.05	0.69	0.12	0.00	0.00
SD	0.66	0.00	0.00	0.00	0.00	0.01	0.02	0.00	0.00
SD corrected	6.57	0.00	0.02	0.00	0.00	0.07	0.16	0.00	0.00

Distillery G	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	21.55	0.03	0.00	0.00	0.62	0.14	0.00	0.00	0.00
2	19.52	0.03	0.00	0.00	0.11	0.11	0.00	0.00	0.00
3	21.81	0.03	0.01	0.00	0.10	0.13	0.00	0.00	0.00
Mean	20.96	0.03	0.00	0.00	0.28	0.12	0.00	0.00	0.00
Corrected	209.60	0.28	0.03	0.00	2.76	1.25	0.00	0.00	0.00
SD	1.02	0.00	0.00	0.00	0.24	0.01	0.00	0.00	0.00
SD corrected	10.24	0.02	0.04	0.00	2.40	0.09	0.00	0.00	0.00

Distillery H	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	20.71	0.00	0.04	0.00	0.00	0.00	0.00	0.00	
2	17.49	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00
3	21.26	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00
Mean	19.82	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00
Corrected	198.20	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00
SD	1.66	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
SD corrected	16.63	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00

Distillery I	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	18.64	0.00	0.01	0.00	0.00	0.12	0.00	0.00	0.00
2	22.43	0.00	0.01	0.00	0.00	0.14	0.00	0.00	0.00
3	22.41	0.00	0.01	0.00	0.00	0.14	0.00	0.00	0.00
Mean	21.16	0.00	0.01	0.00	0.00	0.13	0.00	0.00	0.00
Corrected	211.60	0.03	0.09	0.00	0.02	1.31	0.00	0.00	0.00
SD	1.78	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
SD corrected	17.82	0.01	0.03	0.00	0.01	0.11	0.00	0.00	0.00

Distillery J	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	20.21	0.00	0.04	0.00	0.02	0.00	0.00	0.00	0.00
2	19.12	0.00	0.05	0.00	0.12	0.00	0.00	0.00	0.00
3	18.74	0.00	0.03	0.00	0.14	0.00	0.00	0.00	0.00
Mean	19.36	0.00	0.04	0.00	0.09	0.00	0.00	0.00	0.00
Corrected	193.57	0.00	0.41	0.00	0.93	0.00	0.00	0.00	0.00
SD	0.62	0.00	0.01	0.00	0.05	0.00	0.00	0.00	0.00
SD corrected	6.23	0.00	0.08	0.00	0.52	0.00	0.00	0.00	0.00

Distillery K	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
111A	14.54	0.00	0.00	0.00	0.01	0.11	0.00	0.00	0.00
111B	22.43	0.00	0.01	0.00	0.00	0.14	0.00	0.00	0.00
111C	22.41	0.00	0.01	0.00	0.00	0.14	0.00	0.00	0.00
Mean	19.79	0.00	0.01	0.00	0.00	0.13	0.00	0.00	0.00
Corrected	197.93	0.02	0.07	0.00	0.04	1.29	0.00	0.00	0.00
SD	3.71	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00
SD corrected	37.15	0.02	0.05	0.00	0.03	0.15	0.00	0.00	0.00

Distillery L	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	17.23	0.00	0.04	0.00	0.01	0.00	0.00	0.00	0.00
2	16.12	0.00	0.05	0.00	0.02	0.00	0.00	0.00	0.00
3	15.23	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Mean	16.19	0.00	0.04	0.00	0.01	0.00	0.00	0.00	0.00
Corrected	161.93	0.00	0.37	0.00	0.10	0.00	0.00	0.00	0.00
SD	0.82	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00
SD corrected	8.18	0.00	0.12	0.00	0.08	0.00	0.00	0.00	0.00

Distillery M	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	19.72	0.00	0.00	0.00	0.02	0.09	0.00	0.00	0.00
2	19.54	0.00	0.00	0.00	0.01	0.09	0.00	0.00	0.00
3	17.92	0.00	0.00	0.00	0.02	0.09	0.00	0.00	0.00
Mean	19.06	0.00	0.00	0.00	0.01	0.09	0.00	0.00	0.00
Corrected	190.60	0.00	0.00	0.00	0.15	0.93	0.00	0.00	0.00
SD	0.81	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SD corrected	8.09	0.00	0.00	0.00	0.01	0.02	0.00	0.00	0.00

Distillery N	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	17.36	0.00	0.00	0.00	0.01	0.17	0.02	0.00	0.00
2	22.15	0.00	0.00	0.00	0.01	0.22	0.02	0.00	0.00
3	10.74	0.00	0.00	0.00	0.01	0.04	0.01	0.00	0.00
Mean	16.75	0.00	0.00	0.00	0.01	0.14	0.02	0.00	0.00
Corrected	167.50	0.00	0.00	0.00	0.09	1.39	0.16	0.00	0.00
SD	4.68	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.00
SD corrected	46.78	0.00	0.00	0.00	0.02	0.75	0.01	0.00	0.00

Distillery O	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	18.61	0.00	0.00	0.00	0.02	0.05	0.00	0.00	0.00
2	19.71	0.01	0.00	0.00	0.02	0.06	0.00	0.00	0.00
3									
Mean	19.16	0.00	0.00	0.00	0.02	0.06	0.00	0.00	0.00
Corrected	191.60	0.04	0.03	0.00	0.20	0.58	0.00	0.00	0.00
SD	0.55	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
SD corrected	5.50	0.01	0.01	0.00	0.01	0.05	0.00	0.00	0.00

Distillery P	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	23.01	0.02	0.09	0.00	0.00	0.32	0.04	0.05	0.00
2	23.16	0.01	0.09	0.00	0.00	0.31	0.00	0.05	0.00
3	24.04	0.01	0.09	0.00	0.00	0.36	0.00	0.05	0.00
Mean	23.40	0.01	0.09	0.00	0.00	0.33	0.01	0.05	0.00
Corrected	234.03	0.14	0.88	0.00	0.00	3.31	0.12	0.50	0.00
SD	0.45	0.00	0.00	0.00	0.00	0.02	0.02	0.00	0.00
SD corrected	4.54	0.01	0.02	0.00	0.00	0.18	0.16	0.00	0.00

Distillery Q	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	25.73	0.55	0.10	0.00	0.33	1.76	0.00	0.00	0.00
2	22.44	0.46	0.08	0.00	0.28	1.49	0.00	0.00	0.00
3	20.92	0.43	0.08	0.00	0.28	1.53	0.00	0.00	0.00
Mean	23.03	0.48	0.08	0.00	0.30	1.59	0.00	0.00	0.00
Corrected	230.30	4.80	0.83	0.00	2.97	15.93	0.00	0.00	0.00
SD	2.01	0.05	0.01	0.00	0.02	0.12	0.00	0.00	0.00
SD corrected	20.08	0.51	0.09	0.00	0.23	1.21	0.00	0.00	0.00

Distillery R	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	10.20	0.13	0.10	0.00	0.00	0.40	0.00	0.00	0.00
2	9.79	0.12	0.08	0.00	0.00	0.37	0.00	0.00	0.00
3	7.00	0.08	0.08	0.00	0.00	0.31	0.00	0.00	0.00
Mean	9.00	0.11	0.08	0.00	0.00	0.36	0.00	0.00	0.00
Corrected	89.95	2.23	1.69	0.00	0.00	7.24	0.00	0.00	0.00
SD	1.42	0.02	0.01	0.00	0.00	0.04	0.00	0.00	0.00
SD corrected	14.21	0.21	0.09	0.00	0.00	0.38	0.00	0.00	0.00
Distillery S	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	21.85	0.00	0.00	0.00	0.01	0.11	0.00	0.00	0.00
2	19.89	0.00	0.02	0.00	0.00	0.09	0.00	0.00	0.00
3	14.93	0.00	0.00	0.00	0.01	0.09	0.00	0.00	0.00
Mean	18.89	0.00	0.01	0.00	0.01	0.10	0.00	0.00	0.00
Corrected	188.90	0.00	0.08	0.00	0.11	0.98	0.00	0.00	0.00
SD	2.91	0.00	0.01	0.00	0.00	0.01	0.00	0.00	0.00
SD corrected	29.12	0.00	0.07	0.00	0.05	0.09	0.00	0.00	0.00
Distillery T	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	22.58	0.00	0.02	0.00	0.03	0.00	0.00	0.00	0.00
2	21.33	0.00	0.02	0.00	0.05	0.00	0.00	0.00	0.00
3	23.38	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
Mean	22.43	0.00	0.01	0.00	0.03	0.00	0.00	0.00	0.00
Corrected	224.30	0.01	0.12	0.00	0.35	0.00	0.00	0.00	0.00
SD	0.84	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00
SD corrected	8.44	0.02	0.09	0.00	0.12	0.00	0.00	0.00	0.00

Distillery U	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	17.23	0.00	0.04	0.00	0.01	0.00	0.00	0.00	0.00
2	16.12	0.00	0.05	0.00	0.02	0.00	0.00	0.00	0.00
3	15.23	0.00	0.02	0.00	0.00	0.00	0.00	0.00	0.00
Mean	16.19	0.00	0.04	0.00	0.01	0.00	0.00	0.00	0.00
Corrected	161.93	0.00	0.37	0.00	0.10	0.00	0.00	0.00	0.00
SD	0.82	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.00
SD corrected	8.18	0.00	0.12	0.00	0.08	0.00	0.00	0.00	0.00
Distillery V	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	10.20	0.13	0.10	0.00	0.00	0.40	0.00	0.00	0.00
2	9.79	0.12	0.08	0.00	0.00	0.37	0.00	0.00	0.00
3	7.00	0.08	0.08	0.00	0.00	0.31	0.00	0.00	0.00
Mean	9.00	0.11	0.08	0.00	0.00	0.36	0.00	0.00	0.00
Corrected	89.95	2.23	1.69	0.00	0.00	7.24	0.00	0.00	0.00
SD	1.42	0.02	0.01	0.00	0.00	0.04	0.00	0.00	0.00
SD corrected	14.21	0.21	0.09	0.00	0.00	0.38	0.00	0.00	0.00

Table 2A. The total metal content of each distillery represented in the pot ale audit. Units = mg/L Dilution factor = 25

Distillery A	**Mg**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**	**Cr**	**Cd**
1	9.17	0.03	0.08	0.04	0.06	0.00	0.04	0.04	0.00
2	9.35	0.03	0.07	0.03	0.06	0.00	0.04	0.04	0.00
3	7.17	0.03	0.13	0.04	0.06	0.00	0.05	0.06	0.00
Mean	8.56	0.03	0.09	0.04	0.06	0.00	0.05	0.05	0.00
Corrected	214.08	0.80	2.33	0.90	1.45	0.00	1.13	1.14	0.00
SD	0.99	0.00	0.03	0.00	0.00	0.00	0.00	0.01	0.00
SD corrected	24.70	0.00	0.63	0.07	0.00	0.00	0.12	0.20	0.00
Distillery B	**Mg**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**	**Cr**	**Cd**
1	6.67	0.05	0.13	0.05	0.12	0.21	0.23	0.03	0.00
2	7.26	0.05	0.11	0.05	0.11	0.21	0.21	0.03	0.00
3	7.06	0.06	0.14	0.05	0.11	0.20	0.21	0.03	0.00
mean	7.00	0.05	0.13	0.05	0.11	0.21	0.21	0.03	0.00
corrected	174.89	1.32	3.19	1.18	2.83	5.19	5.31	0.75	0.00
SD	0.24	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.00
SD corrected	6.10	0.05	0.27	0.02	0.20	0.14	0.22	0.04	0.00
Distillery C	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	11.10	0.14	0.52	0.12	0.42	0.34	0.23	0.00	0.00
2	10.81	0.11	0.63	0.23	0.51	0.33	0.19	0.00	0.00
3	10.51	0.09	0.49	0.24	0.65	0.29	0.19	0.00	0.00
Mean	10.81	0.11	0.55	0.20	0.53	0.32	0.20	0.00	0.00
Corrected	270.17	2.83	13.67	4.92	13.17	8.00	5.08	0.00	0.00
SD	0.24	0.02	0.06	0.05	0.09	0.02	0.02	0.00	0.00
SD corrected	6.02	0.51	1.50	1.36	2.37	0.54	0.47	0.00	0.00

Distillery D	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	10.23	0.07	0.41	0.12	0.21	0.42	0.03	0.00	0.00
2	10.45	0.08	0.43	0.14	0.24	0.32	0.04	0.02	0.00
3	10.85	0.00	0.45	0.15	0.27	0.47	0.02	0.00	0.00
mean	10.51	0.05	0.43	0.14	0.24	0.40	0.03	0.01	0.00
corrected	262.75	1.27	10.77	3.42	6.00	10.08	0.75	0.17	0.00
SD	0.26	0.03	0.02	0.01	0.02	0.06	0.01	0.01	0.00
SD corrected	6.42	0.87	0.39	0.31	0.61	1.56	0.20	0.24	0.00
Distillery E	**Mg**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**	**Cr**	**Cd**
1	6.12	0.16	0.12	0.11	0.31	0.25	0.12	0.03	0.00
2	5.98	0.14	0.15	0.12	0.35	0.27	0.15	0.05	0.00
3	6.23	0.12	0.21	0.15	0.65	0.24	0.17	0.02	0.00
mean	6.11	0.14	0.16	0.13	0.44	0.25	0.15	0.03	0.00
corrected	152.75	3.53	4.00	3.17	10.92	6.37	3.68	0.83	0.00
SD	0.10	0.02	0.04	0.02	0.15	0.01	0.02	0.01	0.00
SD corrected	2.56	0.42	0.94	0.42	3.79	0.29	0.52	0.31	0.00
Distillery F	**Mg**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**	**Cr**	**Cd**
1	5.21	0.05	0.09	0.04	0.07	0.07	0.16	0.06	0.01
2	4.50	0.05	0.08	0.04	0.05	0.04	0.14	0.06	0.01
3	5.43	0.06	0.07	0.04	0.06	0.05	0.16	0.06	0.01
mean	5.05	0.05	0.08	0.04	0.06	0.05	0.15	0.06	0.01
corrected	126.18	1.35	2.01	0.93	1.49	1.27	3.83	1.45	0.13
SD	0.40	0.00	0.01	0.00	0.01	0.01	0.01	0.00	0.00
SD corrected	9.90	0.02	0.20	0.04	0.17	0.36	0.21	0.05	0.00

Distillery G	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	6.11	0.07	0.10	0.04	0.16	0.10	0.14	0.06	0.01
2	6.66	0.08	0.12	0.04	0.07	0.07	0.14	0.06	0.01
3	6.33	0.08	0.25	0.05	0.16	0.08	0.15	0.02	0.00
mean	6.36	0.08	0.16	0.04	0.13	0.08	0.14	0.05	0.00
corrected	159.06	1.90	3.92	1.10	3.22	2.06	3.49	1.21	0.12
SD	0.23	0.00	0.07	0.00	0.05	0.01	0.00	0.02	0.00
SD corrected	5.65	3.09	1.66	0.02	1.13	0.27	0.10	0.45	0.03

Distillery H	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	7.51	0.12	0.76	0.06	0.52	0.63	0.13	0.20	0.00
2	7.52	0.05	0.78	0.08	0.69	0.89	0.14	0.24	0.00
3	7.43	0.00	0.71	0.05	0.48	0.71	0.23	0.21	0.00
mean	7.49	0.06	0.75	0.06	0.56	0.74	0.17	0.22	0.00
corrected	187.17	1.42	18.75	1.58	14.08	18.58	4.17	5.42	0.00
SD	0.04	0.05	0.03	0.01	0.09	0.11	0.04	0.02	0.00
SD corrected	1.01	1.23	0.74	0.31	2.28	2.72	1.12	0.42	0.00

Distillery I	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	8.52	0.03	0.10	0.04	0.06	0.00	0.03	0.05	0.00
2	10.20	0.03	0.10	0.04	0.06	0.00	0.05	0.05	0.00
3	10.35	0.03	0.14	0.04	0.06	0.00	0.06	0.06	0.00
mean	9.69	0.03	0.11	0.04	0.06	0.00	0.04	0.05	0.00
corrected	242.25	0.82	2.79	0.92	1.55	0.00	1.08	1.27	0.00
Sd	0.83	0.00	0.02	0.00	0.00	0.00	0.01	0.01	0.00
SD corrected	20.74	0.01	0.55	0.04	0.05	0.00	0.27	0.17	0.00

Distillery J	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	6.32	0.04	0.52	0.14	0.32	0.15	0.21	0.00	0.00
2	7.14	0.02	0.45	0.25	0.35	0.14	0.14	0.00	0.00
3	7.21	0.06	0.53	0.12	0.36	0.18	0.12	0.00	0.00
mean	6.89	0.04	0.50	0.17	0.34	0.16	0.16	0.00	0.00
corrected	172.25	1.00	12.50	4.25	8.58	3.92	3.92	0.00	0.00
SD	0.40	0.02	0.04	0.06	0.02	0.02	0.04	0.00	0.00
SD corrected	10.10	1.00	0.89	1.43	0.42	0.42	0.96	0.00	0.00

Distillery K	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	5.698	0.063	0.08	0.042	0.09	0.13	0.162	0.061	0.006
2	5.808	0.064	0.119	0.045	0.09	0.062	0.181	0.073	0.005
3	5.829	0.062	0.095	0.04	0.084	0.087	0.125	0.065	0.006
mean	5.78	0.06	0.10	0.04	0.09	0.09	0.16	0.07	0.01
corrected	144.46	1.58	2.45	1.06	2.20	2.33	3.90	1.66	0.14
SD	0.06	0.00	0.02	0.00	0.00	0.03	0.02	0.00	0.00
SD corrected	1.44	0.02	0.40	0.05	0.07	0.70	0.58	0.12	0.01

Distillery L	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	7.54	0.03	0.11	0.04	0.06	0.00	0.04	0.05	0.00
2	7.54	0.03	0.18	0.05	0.00	0.03	0.07	0.00	0.00
3	7.23	0.04	0.15	0.03	0.02	0.00	0.02	0.00	0.00
mean	7.44	0.04	0.14	0.04	0.03	0.01	0.04	0.02	0.00
corrected	185.92	0.88	3.59	0.97	0.63	0.28	1.11	0.45	0.00
SD	0.15	0.00	0.03	0.01	0.02	0.02	0.02	0.03	0.00
SD corrected	3.65	0.11	0.67	0.16	0.57	0.40	0.54	0.64	0.00

Distillery M	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	7.21	0.00	0.18	0.00	0.22	0.03	0.02	0.00	0.00
2	7.33	0.00	0.16	0.00	0.22	0.04	0.05	0.00	0.00
3	7.54	0.00	0.16	0.00	0.23	0.02	0.02	0.00	0.00
mean	7.36	0.00	0.17	0.00	0.22	0.03	0.03	0.00	0.00
corrected	183.98	0.00	4.14	0.00	5.51	0.82	0.77	0.01	0.00
Sd	0.14	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00
SD corrected	3.46	-	0.24	-	0.10	0.18	0.36	0.01	-

Distillery N	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	7.24	0.03	0.11	0.04	0.06	0.00	0.04	0.03	0.00
2	7.94	0.01	0.18	0.05	0.02	0.03	0.08	0.00	0.00
3	7.13	0.04	0.15	0.03	0.01	0.00	0.02	0.02	0.00
Mean	7.44	0.04	0.14	0.04	0.03	0.01	0.04	0.02	0.00
Corrected	185.92	0.88	3.59	0.97	0.63	0.28	1.11	0.45	0.00
SD	0.36	0.01	0.03	0.01	0.02	0.02	0.03	0.01	0.00
SD corrected	8.97	0.30	0.67	0.16	0.47	0.40	0.65	0.35	0.00

Distillery O	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	7.45	0.23	0.21	0.05	0.71	0.35	0.18	0.00	0.00
2	7.23	0.25	0.13	0.03	0.81	0.45	0.17	0.00	0.00
3	8.23	0.28	0.14	0.01	0.73	0.28	0.16	0.00	0.00
Mean	7.64	0.25	0.16	0.03	0.75	0.36	0.17	0.00	0.00
Corrected mean	190.92	6.33	4.00	0.75	18.75	9.00	4.25	0.00	0.00
SD	0.43	0.02	0.04	0.02	0.04	0.07	0.01	0.00	0.00
SD corrected	10.73	0.51	0.89	0.41	1.08	1.74	0.20	0.00	0.00

Distillery P	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	4.98	0.04	0.17	0.06	0.13	0.16	0.15	0.05	0.00
2	5.21	0.05	0.20	0.06	0.13	0.14	0.16	0.05	0.00
3	4.98	0.04	0.17	0.06	0.13	0.16	0.15	0.05	0.00
mean	5.05	0.05	0.18	0.06	0.13	0.15	0.15	0.05	0.00
Corrected mean	126.36	1.13	4.40	1.41	3.29	3.75	3.78	1.19	0.00
SD	0.11	0.00	0.01	0.00	0.00	0.01	0.01	0.00	0.00
SD corrected	2.80	0.12	0.35	0.05	0.01	0.21	0.15	0.06	0.00
Distillery Q	**Mg**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**	**Cr**	**Cd**
1	6.53	0.21	0.25	0.04	0.16	0.48	0.22	0.04	0
2	7.61	0.23	0.23	0.04	0.17	0.59	0.25	0.03	0
3	7.61	0.23	0.25	0.04	0.18	0.58	0.21	0.03	0
mean	7.25	0.23	0.25	0.04	0.17	0.56	0.23	0.03	0.00
corrected mean	181.31	5.72	6.20	1.09	4.37	13.89	5.72	0.83	0.00
SD	0.51	0.01	0.01	0.00	0.01	0.05	0.02	0.00	0.00
SD corrected	12.66	0.29	0.25	0.01	0.22	1.19	0.42	0.11	0.00
Distillery R	**Mg**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**	**Cr**	**Cd**
1	6.19	0.11	1.50	0.15	0.07	0.00	0.00	0.36	0.00
2	7.45	0.12	1.42	0.15	0.15	0.00	0.00	0.25	0.00
3	6.23	0.14	1.48	0.15	0.15	0.00	0.00	0.34	0.00
mean	6.62	0.12	1.47	0.15	0.12	0.00	0.00	0.31	0.00
corrected mean	165.57	3.08	36.66	3.71	2.98	0.00	0.00	7.84	0.00
Sd	0.59	0.01	0.03	0.00	0.04	0.00	0.00	0.05	0.00
SD corrected	14.63	0.31	0.85	0.06	0.95	0.00	0.00	1.22	0.00

Distillery R	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	6.19	0.11	1.50	0.15	0.07	0.00	0.00	0.36	0.00
2	7.45	0.12	1.42	0.15	0.15	0.00	0.00	0.25	0.00
3	6.23	0.14	1.48	0.15	0.15	0.00	0.00	0.34	0.00
mean	6.62	0.12	1.47	0.15	0.12	0.00	0.00	0.31	0.00
corrected	165.57	3.08	36.66	3.71	2.98	0.00	0.00	7.84	0.00
Sd	0.59	0.01	0.03	0.00	0.04	0.00	0.00	0.05	0.00
SD corrected	14.63	0.31	0.85	0.06	0.95	0.00	0.00	1.22	0.00
Distillery S	**Mg**	**Mn**	**Fe**	**Ni**	**Cu**	**Zn**	**Pb**	**Cr**	**Cd**
1	7.12	0.12	0.81	0.01	0.45	0.32	0.19	0.00	0.00
2	7.71	0.12	0.75	0.11	0.55	0.45	0.17	0.00	0.00
3	6.98	0.14	0.82	0.09	0.56	0.29	0.16	0.00	0.00
Mean	7.27	0.13	0.79	0.07	0.52	0.35	0.17	0.00	0.00
Corrected	181.75	3.17	19.83	1.77	13.00	8.83	4.33	0.00	0.00
SD	0.32	0.01	0.03	0.04	0.05	0.07	0.01	0.00	0.00
SD corrected	7.91	0.24	0.77	1.06	1.24	1.74	0.31	0.00	0.00

Chapter 2 Distillery U	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	8.12	0.23	0.42	0.12	0.04	0.42	0.02	0.05	0.00
2	7.45	0.14	0.51	0.09	0.09	0.36	0.04	0.02	0.00
3	7.23	0.05	0.67	0.10	0.12	0.12	0.09	0.00	0.00
mean	7.60	0.14	0.53	0.10	0.08	0.30	0.05	0.02	0.00
corrected mean	190.00	3.50	13.33	2.58	2.08	7.50	1.25	0.58	0.00
SD	0.38	0.07	0.10	0.01	0.03	0.13	0.03	0.02	0.00
SD corrected	9.46	1.84	2.58	0.31	0.82	3.24	0.74	0.51	0.00

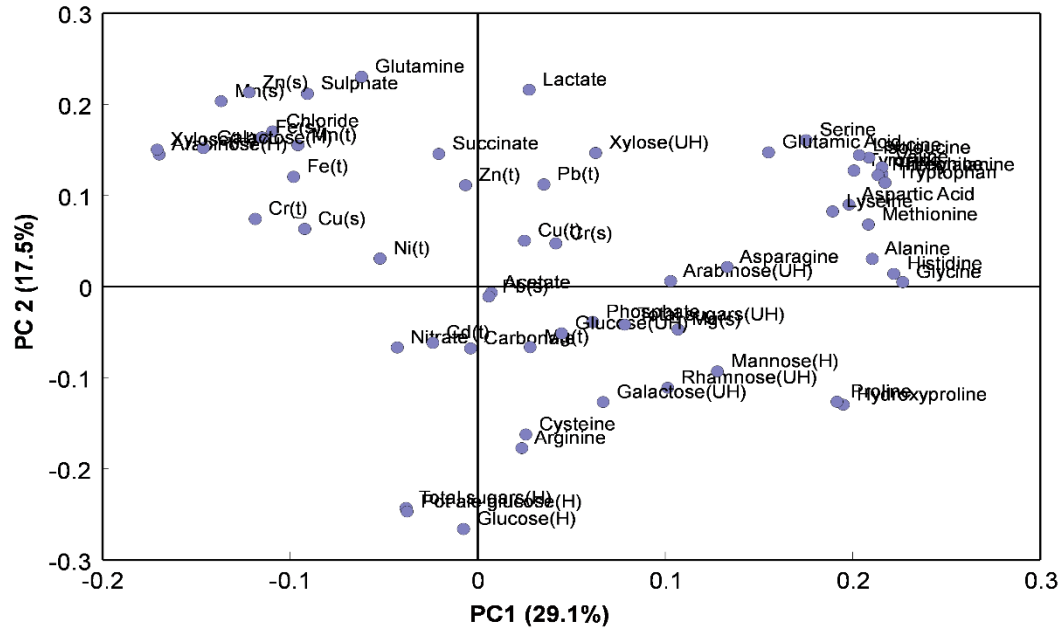
Distillery V	Mg	Mn	Fe	Ni	Cu	Zn	Pb	Cr	Cd
1	6.31	0.16	0.31	0.04	0.12	0.25	0.06	0.06	0.00
2	4.07	0.12	0.28	0.04	0.15	0.09	0.13	0.06	0.00
3	6.21	0.15	0.25	0.04	0.13	0.15	0.12	0.05	0.00
mean	5.53	0.14	0.28	0.04	0.13	0.16	0.10	0.06	0.00
corrected mean	138.33	3.53	6.96	1.02	3.32	4.06	2.49	1.43	0.00
SD	1.03	0.02	0.03	0.00	0.02	0.07	0.03	0.01	0.00
SD corrected	25.86	0.44	0.69	0.08	0.38	1.72	0.73	0.13	0.00

Table 3A. The monosaccharides detected in unhydrolyzed pot ale. < = under detection limits. (n=3).

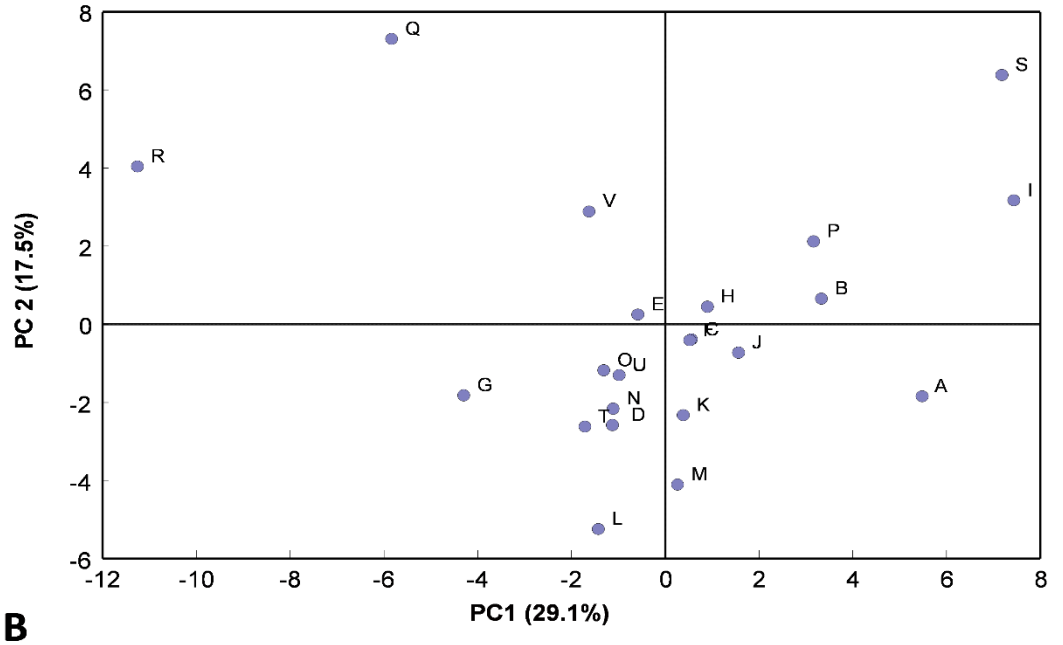
Distillery	*Rhamnose*	*Arabinose*	*Galactose*	*Glucose*	*Xylose*	*pot ale*
	g/L	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**
A	0.32	2.71	1.59	4.39	3.13	12.14
B	0.19	1.28	1.06	15.66	1.95	20.14
C	0.26	0.75	0.47	11.79	2.82	16.08
D	0.21	3.52	0.61	29.90	2.08	36.32
E	0.10	4.30	<	6.15	3.12	13.66
F	0.23	2.77	1.40	3.91	2.95	11.25
G	0.11	1.60	<	0.29	1.53	3.53
H	0.17	1.85	1.21	4.59	2.43	10.25
I	0.14	4.03	1.07	5.36	2.52	13.11
J	0.19	1.08	1.47	3.38	1.87	7.98
K	0.19	2.85	1.61	4.24	3.15	12.04
L	0.14	2.76	1.33	4.95	1.04	10.22
M	0.21	2.92	1.30	2.37	2.45	9.24
N	0.18	3.46	1.52	6.52	2.94	14.62
O	0.17	3.06	1.26	3.46	2.97	10.92
P	0.13	6.42	1.90	6.62	4.51	19.58
Q	0.21	1.35	0.18	0.73	4.15	6.62
R	<	0.67	0.97	1.23	1.47	4.34
S	0.11	3.83	0.62	5.41	3.33	13.31
T	0.19	2.95	1.65	2.36	2.78	9.92
U	0.15	3.05	1.55	8.05	2.63	15.43
V	0.13	4.10	0.01	6.25	3.50	13.99
Mean	0.18	2.79	1.14	6.26	2.70	12.94
		2.79	1.14	6.26	2.70	12.94

Table 4A. The monosaccharides detected in unhydrolyzed pot ale. < = under detection limits. (n=3).

Distillery	Arabinose	Galactose	Glucose	Xylose	Mannose	pot ale
	g/L	**g/L**	**g/L**	**g/L**	**g/L**	**g/L**
A	20.0	10.15	403	25.2	24.0	482
B	24.9	9.38	345	34.5	23.7	438
C	22.2	7.69	465	34.2	21.1	551
D	18.0	8.31	351	23.4	22.3	423
E	23.3	5.74	224	31.9	35.6	320
F	26.7	9.74	362	40.4	30.9	470
G	21.3	6.55	340	30.8I	23.6	423
H	18.4	7.18	293	25.5	18.9	363
I	22.2	8.40	206	30.9	27.3	295
J	20.3	7.34	363	31.4	19.6	441
K	18.5	8.50	330	24.8	22.1	404
L	21.1	7.95	691	30.4	21.6	773
M	22.2	8.07	545	31.7	27.2	634
N	26.4	9.23	414	36.7	27.6	514
O	23.4	8.29	365	30.1	21.7	448
P	11.2	6.68	78	12.4	22.4	131
Q	47.0	17.31	135	84.7	13.5	298
R	50.8	19.30	201	90.8	10.7	372
S	19.5	8.28	135	25.3	25.6	214
T	26.1	9.94	388	34.1	23.4	482
U	19.0	8.95	379	24.6	19.5	451
V	22.1	8.46	111	28.1	21.8	191
Mean	22.1	8.46	111	28.1	21.8	414.4



A



B

Fig 11A. Loadings of the compositional parameters (A) and corresponding scores for the distilleries (B) across PCs 1 and 2 of the Principle Components Analysis of the data from all 22 distilleries

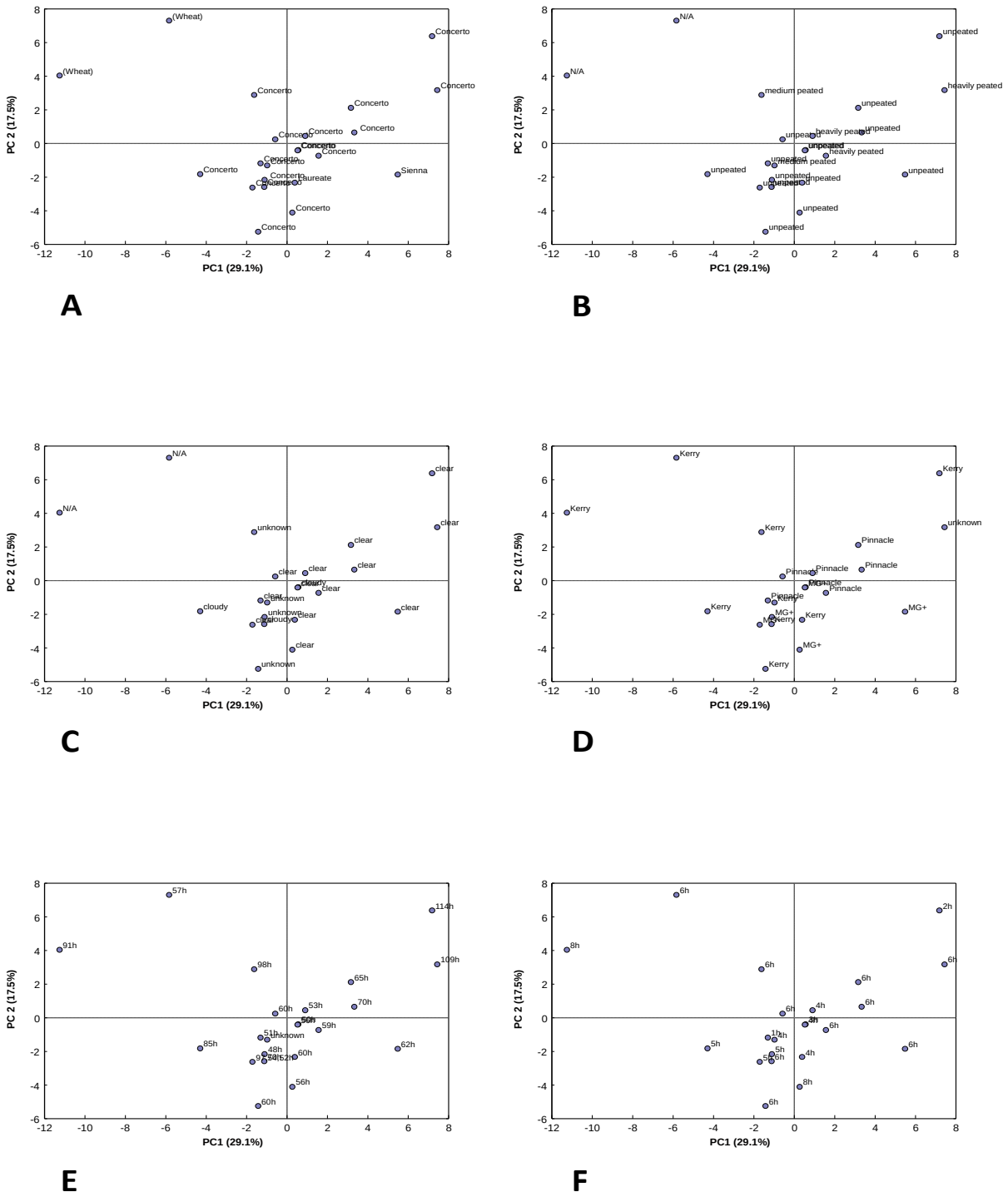


Figure 12A: Scores plot for the distilleries across PCs and 2 from the Principle Components Analysis of the data from all 22 distilleries, recoded to show distribution in relation to process parameters (A – barley variety, B – malt peating level, C – wort clarity, D – yeast strain, E – fermentation time and F – time in transit)

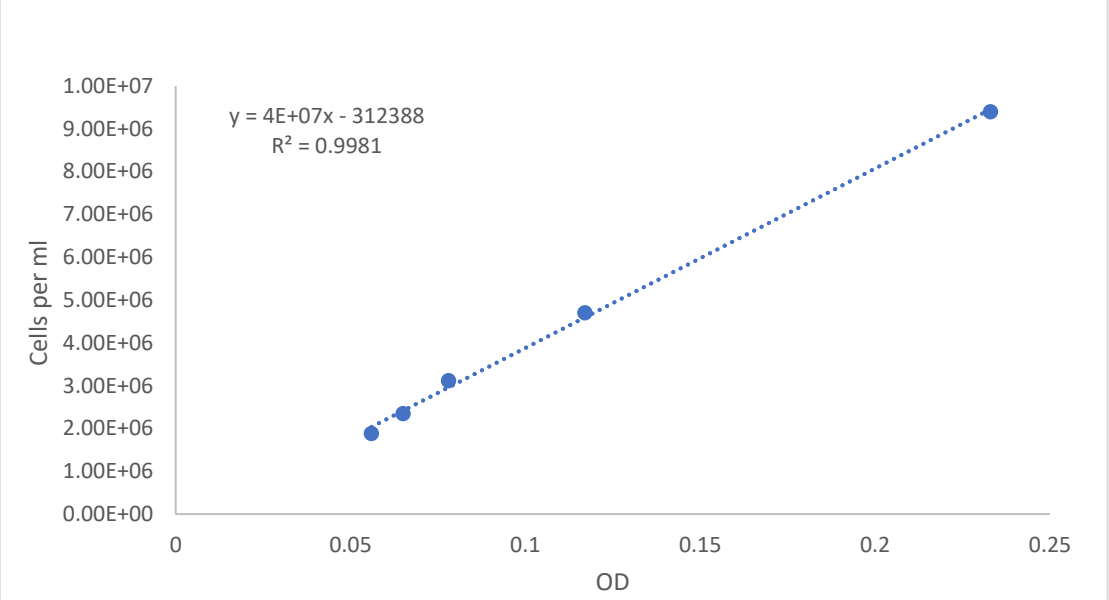


Figure 13A. The relationship between the cells per ml and absorbance at 720 nm of *Nodularia Harveyana* PCC 7804.