Evaluation of the competitive performance of an indigenous eicosapentaenoic acid producing microalgal isolate

Presented by

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"Everything should be made as simple as possible, but not simpler"

- Albert Einstein

DECLARATION

I understand the meaning of plagiarism and declare that all work in the document, save for which is properly acknowledged, is my own and that I have not previously in its entirety or in part submitted it at any university for a degree.

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ABSTRACT

Omega-3 fatty acids are known to have positive effects on brain function, as well as cardiovascular disease and inflammatory diseases. They currently sold as fish oil concentrates. The fish oil extraction processes are unsustainable due to the diminishing fish stocks in open waters. In addition to this is the fact that the omega-3 fatty acid content in fish are directly dependent on the fish's diet. If the fish does not have access to nutrients containing omega-3s, the fish will not have assimilated the relevant fatty acids.

Currently, microalgae are a topic of interest regarding omega-3 fatty acid production because they are the ultimate *de novo* source of omega-3 fatty acids. In order to mitigate long-term fish resource depletion, it is imperative that the cultivation of edible fish becomes a priority. However, these aquaculture fish will not contain omega-3 fatty acids if this is not a part of their diet, either naturally or as a feed additive. Due to microalgae's *de novo* production of omega-3 fatty acids, this would be an ideal alternative to fish oil, as well as a source of omega-3 fatty acids for cultivated fish as a feed input.

South Africa is an ideal location for the commercialisation of microalgal technology, due to its biodiversity and climate. There is thus a desire to exploit the biodiversity of South Africa and over 750 microalgal isolates have been collected from across South Africa and are housed at the CSIR Biosciences and the Centre for Bioprocess Engineering (CeBER) at the University of Cape Town.

The main objective of this study was to investigate whether a locally selected isolate was a more suitable organism for omega-3 fatty acid production in an open raceway pond than a current species of choice. The objectives of the study were achieved by investigating the biomass, EPA production and response to reactor configuration and process conditions, with a view to assessing the potential to further scale up the bioprocess.

A literature review was conducted to identify a list of possible omega-3 producing candidates. The published data were analysed to reduce the overall list of potential organisms to six species. Initial experimental analysis indicated that *Phaeodactylum tricornutum* was a suitable control species against which to compare the locally selected isolate in terms of biomass and EPA production. Average specific growth rate and overall average EPA productivity were 0.24 d^{-1} and 10.85 $\mu g.d^{-1}$ respectively for *P. tricornutum*. The chosen CSIR species, WCA 23.2, had an average specific growth rate and overall average EPA productivity of 0.20 d^{-1} and 7.83 $\mu g.d^{-1}$ respectively. Molecular identification confirmed the identity of *Phaeodactylum tricornutum* and indicated WCA 23.2 most likely to be an *Amphora* species.

Abstract

Selected environmental factors, such as pH and different nutrient regimes were chosen to study the effect on growth rate, biomass production and EPA production under laboratory conditions. A study was devised to test the effects of pH control under maintained nutrient conditions. All studies were subjected to a period of nutrient deficiency for the last 4-6 days of the experiment to assess the effect on EPA induction. The pH studies resulted in similar average specific growth rates for both species under pH controlled *vs.* uncontrolled conditions with WCA 23.2 and *P. tricornutum* demonstrating growth rates of 0.20-0.22 d^{-1} and 0.30-0.33 d^{-1} respectively. Specific EPA productivity was negatively affected for both species in the absence of pH control, where the pH increased to above pH 9.4. For the pH studies, there was no observed increase in EPA content under nutrient (nitrate and silicon) deficient conditions. Subsequent experiments were performed with the pH controlled at pH 8.3 to ensure optimal biomass and specific EPA production.

To evaluate the impact of different nutrient addition regimes at a chosen pH, a study was designed to supply cultures with the same amount of nutrients where (1) the batch culture had all the nutrients supplied at the start and (2) a fed-batch regime where smaller amounts of nutrients were supplied every second day. Average specific growth rates were similar for both species under batch and fed-batch conditions with values ranging from 0.23-0.25 d^{-1}. Specific EPA productivities, however, were higher for both cultures when cultivated under the batch conditions with productivities of 0.412 $mg.g^{-1}d$ and 0.175 $mg.g^{-1}d$ for WCA 23.2 and *P. tricornutum* respectively.

To assess the impact of scale-up, biomass and specific EPA production was assessed in an open raceway pond system (50 L volume) where species were cultivated, in duplicate, under batch conditions at a pH of 8.3, relying on the environmental temperatures and natural sunlight. Biomass productivities for both cultures were significantly lower when compared to the productivities from the laboratory scale studies. Biomass productivity was 2.5 times lower for WCA 23.2 and five-fold lower for *P. tricornutum.* In terms of specific EPA production, WCA 23.2 suffered a 4.5 fold loss while specific EPA production in *P. tricornutum* was 20% higher.

While the locally selected isolate had a lower specific EPA production rate, it was quicker and easier to harvest. WCA 23.2 auto-flocculated and could be sieved within an hour, while *P. tricornutum* required an additional settling step to harvest the biomass, resulting in an overall harvesting phase that took two days.

The experimental data indicated that EPA productivity was higher in *P. tricornutum* than WCA 23.2, under the conditions tested. However, the ease of biomass recovery and regulatory advantages associated with using an endemic species mean that a more thorough economic evaluation is required to draw a definitive conclusion.

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CHAPTER I – INTRODUCTION

Overview

To date, over 500 000 articles have been published about the uses and benefits of omega-3 polyunsaturated fatty acids (PUFAs). These oils are claimed to have positive effects on diseases such as cardiovascular disease, inflammatory diseases as well as brain function (Adarme-Vega *et al.*, 2012). Omega-3 PUFAs are currently sold in fish oil concentrates that are obtained via a two-step process: oil extraction from raw material via wet pressing, and refining or concentration of omega-3 oils (Rubio-Rodríguez *et al.*, 2010). These processes have been reported to be unfeasible when omega-3 content is low in the fish itself (Rubio-Rodríguez *et al.*, 2010). This presents a problem, as the omega-3 content is directly dependent on the fish's diet. If the fish does not have access to nutrients containing omega-3s, the fish will not have assimilated the nutrient.

In addition to this, global catches have started to decline since the 1980s, causing the depletion of overfished stocks (AGDAFF, 2007; Worm *et al.*, 2006). Due to this, fish oil fluctuates in price and quality (Belarbi *et al.*, 2000). Furthermore, there are concerns regarding contamination by pesticides and heavy metals. There is therefore a need to find an alternative, sustainable source of omega-3 PUFAs.

Currently, microalgae are a topic of interest regarding omega-3 PUFA production. Current EPA yields, reported in the literature, range from 1 to 100 $mg.L^{-1}$ for EPA producing species, which are considered too low to make the process economically feasible (Vazhappilly & Chen, 1998; Duong *et al.*, 2012). It is therefore important to identify species that are capable of high EPA productivities and have the potential to overcome existing processing challenges.

South Africa has been identified as a potential location for the bioprospecting of a variety of microalgal species, due to its biodiversity and climate. There is a desire to explore the biodiversity of South Africa and over 750 microalgal isolates have currently been collected from across South Africa and are housed at the CSIR Biosciences and the Centre for Bioprocess engineering (CeBER) at the University of Cape Town.

This work investigated the growth characteristics and eicosapentaenoic acid (EPA, an omega-3 PUFA) producing capabilities of the microalga species, selected from the CSIR Biosciences culture collection. Chapter I gives a broad overview of omega-3 PUFAs and their production, as well as the aims and objectives of this project. Chapter II reviews the different materials and methods employed to generate results. Chapter III describes the evaluation of several culture collection species in terms of growth rates and EPA productivities. After the literature evaluation, the three best performing culture collection species were selected and experimentally compared to three

previously pre-screened CSIR isolates conducted during a phase prior to the work presented here. The two best performing candidates (one culture collection species and one from the CSIR culture collection) were selected for further experimental analysis. Chapter IV investigates the impacts of environmental factors on the selected isolates with the consequent identification of the parameters leading to optimal biomass and EPA producing capabilities. Chapter V evaluates the outdoor performance of the isolates in a raceway pond located in a greenhouse. The study employed the parameters identified in Chapter IV and investigated the effect of scale up along with other added environmental changes on the growth and EPA producing capabilities of the isolates.

1. OMEGA-3 POLYUNSATURATED FATTY ACIDS

1.1. AN INTRODUCTION TO FATTY ACIDS

Lipids, a major class of macromolecules, are essential components of all living organisms and are defined as "water insoluble organic compounds found in biological systems" (Horton *et al.*, 2006). These macromolecules have diverse biological functions; serving as intracellular energy stores and regulators of metabolic activities as well as serving a structural function as lipid bilayers surrounding cells and organelles. Fatty acids are a type of lipid that are composed of carbon (C), hydrogen (H) and oxygen (O) atoms with the general formula of R-COOH, where 'R' represents a hydrocarbon chain or a 'tail' (Figure 1) (Horton *et al.*, 2006; Sweet, 2011). These long 'tails' contribute unique structure, function and properties to each fatty acid.

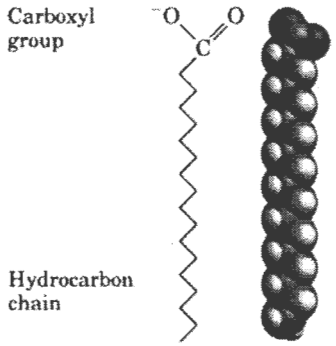


Figure 1: Structure of a fatty acid. Nelson (2000).

If the hydrocarbon tails contain no double bonds, the fatty acid is referred to as a 'saturated fatty acid' (SFA). Fatty acids containing one or more carbon-carbon double bond are referred to as 'monounsaturated fatty acids' (MUFAs) or 'polyunsaturated fatty acids' (PUFAs) respectively. PUFAs are named and numbered according to the number of carbon atoms and double bonds

contained per fatty acid. The naming of the molecule can be explained by the following notation Cx:y where x refers to the number of carbon atoms in the chain and y refers to the amount of double bonds in the carbon chain. The Greek letter ω (omega) specifies the carbon atom farthest from the carboxyl (-COOH) group.

The omega PUFAs can be subdivided into three major groups, based on molecular configuration: omega-3, 6 and 9 (Sweet, 2011), which contain double bonds starting at the third, sixth and ninth carbons respectively from the methyl (ω) end of the 'tail' (Figure 2).

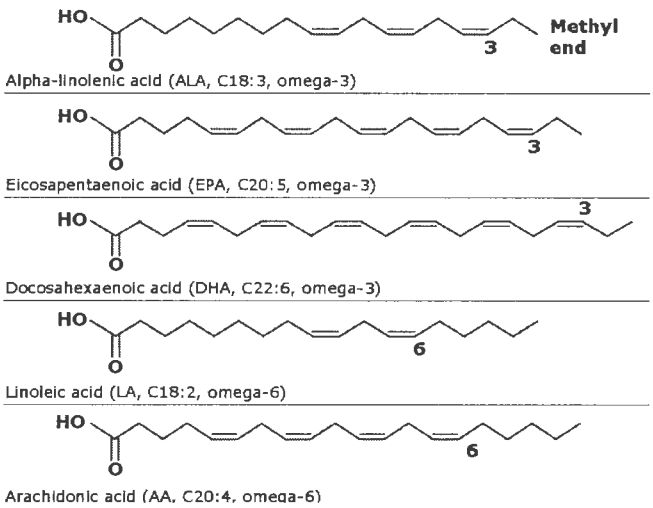


Figure 2: The omega-3 and 6 fatty acids (omega-9 fatty acids not shown here) (European Food Information Council, 2008).

The omega-3 PUFAs collectively refer to a group of fatty acids consisting of alpha-linolenic acid (ALA, C18:3), eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) also seen in Figure 2 (Sweet, 2011; Hull, 2011; Wall *et al.*, 2010). These PUFAs are of specific interest due to their ability to regulate blood pressure, body temperature and smooth muscle contraction (Horton *et al.*, 2006; Wall *et al.*, 2010) and will form the basis of this study.

1.2. HEALTH BENEFITS OF OMEGA-3 FATTY ACIDS

A recent article suggested that the omega-3 fatty acids are as, or more, important than the omega-6 and 9 fatty acids (Sweet, 2011). This statement is supported by the fact that they suppress and/or alleviate inflammation, which is thought to be the cause of certain debilitating conditions, such as cardiovascular disease (CVD), inflammatory bowel disease (IBD) and rheumatoid arthritis (RA) (Sweet, 2011; Wall *et al.*, 2010; Das, 2006). The anti-inflammatory nature of omega-3 fatty acids

(specifically EPA and DHA) can be ascribed to the fact that they are precursors of eicosanoids (lipid mediators) which have anti-inflammatory properties (Wall *et al.*, 2010; Das, 2006).

As such, EPA and DHA are able to directly or indirectly affect the immune response (Wall *et al.*, 2010). This ability has been described in several articles (Simopoulos, 1999; Kris-Etherton *et al.*, 2003; Wen and Chen, 2003; Cardozo *et al.*, 2007; Das, 2006; Domingo, 2007; Hull, 2007) and most recently by Barbosa-da-Silva *et al* (2014). The research described two mechanisms by which omega-3 fatty acids mediate an anti-inflammatory response. In the first mechanism, enzymes Cicloxygenase (COX) and lipoxygenase (LOX) convert EPA and DHA into eicosanoids, or inflammatory mediators, decreasing the levels of proinflammatory cytokines, via lipid-mediator-related and non-lipid-mediator related mechanisms. In the second mechanism, EPA and DHA are transported through the protein channels in the cell membranes where they directly influence transcription factors peroxisome proliferator-activated receptor (PPAR) and sterol regulatory element-bindingprotein-1c (SREBP-1c) (Figure 3) (Barbosa-da-Silva *et al.*, 2014).

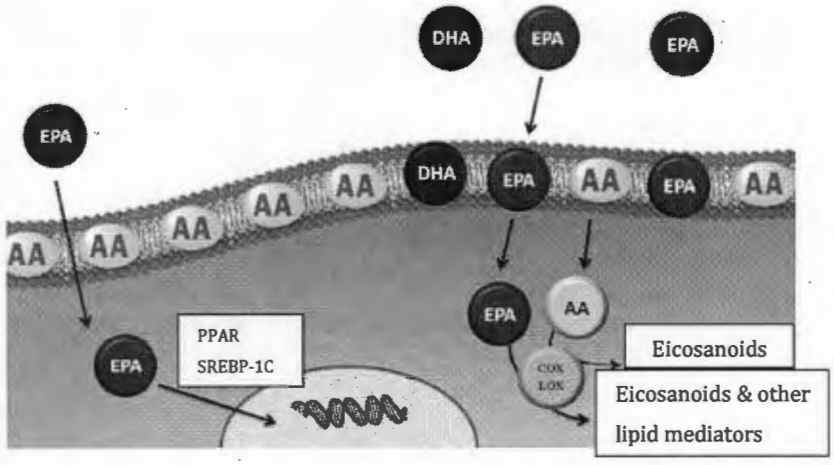


Figure 3: Simplified mechanism depicting anti-inflammatory action of long chain omega-3 fatty acids. AA= arachidonic acid; COX= cicloxygenase; DHA=docosahexaenoic acid; EPA= eicosapentaenoic acid; LOX= lipoxygenase; PPAR= peroxisome proliferator-activated receptor; SREBP-1c= sterol regulatory element-bindingprotein-1c. (Barbosa-da-Silva *et al.*, 2014)

The importance of omega-3 fatty acids to human health is well recognised. However, the human body is neither capable of synthesising the parent ALA, nor can it readily convert ALA to EPA or DHA (Sweet, 2011; Hull, 2011; Wall *et al.*, 2010). The consumption of large amounts of ALA will consequently not provide humans with sufficient amounts of EPA and/or DHA. Ingesting omegas already in EPA or DHA form is the only way to ensure sufficient levels. It is therefore imperative that humans include these fatty acids as part of the diet, and hence are they known as 'essential fatty acids'

1.3. EXISTING SOURCES OF OMEGA-3 PUFAS

The primary sources of dietary omega-3 fatty acids are from vegetables (green and leafy), vegetable oil (sunflower and rapeseed), certain types of nuts (walnuts) as well as certain fish products and fish oil supplements (Aurora Algae, 2013). While ALA is more commonly found in vegetable sources, EPA and DHA are found in cold-water fish such as sardines and mackerel (Hull, 2011; Wall *et al.*, 2010; Aurora Algae, 2013; Shahidi & Miraliakbari, 2004).

The American Heart Association (AHA) recommends that adults consume two servings (200 grams) of fatty fish per week (Kris-Etherton *et al.*, 2003; Domingo, 2007). However, the omega-3 content of the fish is rarely indicated and is significantly affected by the fish's diet, particularly in the case of ocean fish. In addition, the AHA also indicates that fish and seafood may represent a major source of human exposure to various environmental contaminants, particularly mercury (Domingo, 2007). An internet search was performed to investigate the type of information regarding 'good omega-3 sources', serving sizes and omega-3 content that is currently available to the average consumer (Table 1).

Table 1: Sources of omega-3s and their respective omega-3 contents. (Kris-Etherton *et al.*, 2000; Rahmawaty *et al.*, 2013)

Food portion	Omega-3 Content
Mackerel	1.8-5.3 (% by weight)
Salmon	1.0-1.4 (% by weight)
Tuna	0.5-1.6 (% by weight)
Shrimp	0.2-0.5 (% by weight)
Flaxseed	22.8 g per 100 g
Soybeans	3.2 g per 100 g
Oats, germ	1.4 g per 100 g
Avocados	0.1 g per 100 g
Egg	60 mg per 100 g
Milk	0.3 mg per 100 g

As can be seen from Table 1, fish contains the highest amount of omega-3s per portion. Nutraceutical companies have identified this as an opportunity to create a growing fish-oil supplement market, particularly targeting small children and senior adults.

1.4. THE CURRENT MARKET

In 2004, the PUFA market was estimated to be $700 million, with omega-3 having maximum growth potential compared to all other functional food ingredients. According to Global Organization for EPA and DHA Omega-3s (GOED) and Frost & Sullivan, the global market for EPA (and DHA) omega-3 oils was an estimated 85 000 metric tons in 2009 and is predicted to double in size by 2015 (Naidoo & Lalloo, 2011). At present, 97% percent of the market volume is attributed to marine fish oils with estimated revenue of $1.5 billion on the global market. According to Frost & Sullivan, the omega-3 market has been growing between 10-18% on an annual global scale (Naidoo and Lalloo 2011).

A more recent market study conducted by Grand Review Research (published in February 2014) indicated that the market for omega-3s is expected to reach USD 7.32 billion by 2020 (Grand Review Research , 2014). The vast growth of the market is thought to be the cause of growing consumer awareness regarding the health benefits associated with omega-3. This, coupled with the rising prevalence of coronary heart diseases, are expected to drive market demand over the next six years. Key findings from the study suggested that the global market for omega-3 ingredients was estimated to be 24.87 kilo tons in 2013, and is expected to grow at a compound annual growth rate (CAGR) of 13.7% from 2014 to 2020 (Grand Review Research, 2014). Companies are trying to obtain alternative sources for extracting omega-3 due to the tight demand supply situation in the global fish oil market.

Certain foods, such as fish fingers (I&J) and margarine (Flora) are enriched with omega-3 fatty acids. However, the market for omega-3 fatty acids is largely serviced by fish oils supplements, marketed in the form of oil-filled gel capsules.

While the supplements are marketed as fish oil, fish do not synthesise omega-3 fatty acids *de novo* (Cardozo *et al.*, 2007). Fatty fish acquire omega-3 fatty acids by feeding on microalgae that are able to synthesise omega-3 fatty acids, via a metabolic pathway unique to this group of organisms. Only plants, algae cyanobacteria and some protists and fungi are able to synthesise the parent ALA molecule of the ω-3 family. The ability to do so is due to the synthesis of the Δ15 desaturases responsible for the unsaturation of the fatty acid (Sperling *et al.*, 2003, Sayanova *et al.*, 2006; Alloatti and Uttaro, 2011).

Hence, the presence of omega-3 fatty acids in fish oil is solely dependent on whether the fish fed on an algal species capable of synthesising these fatty acids. Wild fish resources have come under increasing pressure from unsustainable fishing practices (Adarme-Vega *et al.*, 2012; Buchsbaum *et al.*, 2005; Naylor *et al.*, 2001; O'Boyle, 2012; Wildlife Trust). In addition, there is an increasing trend towards a vegetarian lifestyle in support of animal rights. The combination of expensive

supplements, decreasing fish stocks and an increase in vegetarianism provides the motivation for identifying an alternative source of EPA.

1.5. POTENTIAL ALTERNATE SOURCES

The interest in algae as a consumable product for humans began in the 1950s, due to an exponential increase in the world's population, which drove the identification of a product that had high nutritional value and low cost to mass-produce (Spolaore *et al.*, 2006).

Recent studies, aimed at identifying pharmaceutically active compounds from natural sources, identified algae as the source of a promising group of bio products (Cardozo *et al.*, 2007; Tringali, 1997; Burja *et al.*, 2001; Mayer & Hamann, 2005).

Compounds and products from algae include food, feed, fertilisers, hydrocolloids (carbohydrates), pigments, lipids, proteins and other bioactive compounds and have applications in the pharmaceutical, food and feed and energy sectors. Microalgae are able to produce and accumulate lipids to as much as 70% of their total dry weight (Spolaore *et al.*, 2006). These lipids include the valuable, essential omega-3 PUFAs (most notably EPA and DHA). The confirmation of algal omega-3 production led to the search for promising candidate species as potential producers (Table 2) (Spolaore *et al.*, 2006).

Table 2: List of microalgal omega-3 producers capable of producing the PUFA EPA, indicated as a percentage of total fatty acids produced.

Microalgal specie	**EPA content as a % of fatty acids**	**Reference**
Nannochloropsis sp.	20-30%	(Hu & Gao, 2003; Sukenik, 1991; Huerlimann *et al.*, 2010; Yan & Schenk, 2011; Vazhappilly & Chen, 1998)
Pavlova lutheri	20-30+%	(Vazhappilly & Chen, 1998; Carvalho & Malcata , 2005; Sakshaug & Holm-Hansen , 1977; Meireles *et al.*, 2003; Tatsuzawa & Takizawa , 1995)
Chlorella minutissima	30+%	(Vazhappilly & Chen, 1998; Yongmanitchai & Ward, 1991; Illman *et al.*, 2000; Seto *et al.*, 1984)

Dunaliella salina	20-30%	(Bhosale et al., 2010; Thompson, 1996; Griffiths & Harrison, 2009)
Isochrysis galbana	20-30%	(Vazhappilly & Chen, 1998; Yago et al., 2011; Molina Grima *et al.*, 1994; Griffiths & Harrison, 2009; Breuer *et al.*, 2012; Chisti , 2007)
Phaeodactylum tricornutum	20-30+%	(Vazhappilly & Chen, 1998; Griffiths & Harrison, 2009; Yongmanitchai & Ward, 1991; Yongmanitchai & Ward, 1992)
Monodus subterraneus	30+%	(Vazhappilly & Chen, 1998; Qiang *et al.*, 1997)
Porphyridium cruentum	20-30+%	(Vazhappilly & Chen, 1998; Griffiths & Harrison, 2009; You & Barnett, 2004; Razaghi *et al.*, 2014; Yan & Schenk, 2011; Breuer *et al.*, 2012; Chisti , 2007)
Skeletonema costatum	30+%	(Yan & Schenk, 2011; Chisti , 2007)

Currently, EPA from algae is considered as the primary source of omega-3s and hence the only known alternative to fish oils in such high content (Spolaore *et al.*, 2006). Algae are also an attractive alternative as they represent a vegetarian source, which is more sustainable than farmed fish, sardines, krill and engineered oil crops. The fish oil market is highly competitive, as seen by the basic market survey in Table 2, while the algal market is still in its infancy.

Currently, there are no algal omega-3 products on the market, except for a DHA product (life's DHA™) from Dutch State Mines (DSM) Nutritional Products, which currently dominates the algal market (DSM Nutritional Products, 2012). Therefore, EPA from algae is an attractive novel source of nutraceutical products. However, further investigation is necessary to assess the commercial potential.

2. ALGAE

2.1. GENERAL DESCRIPTION

The Glossary of Biotechnological Terms defines 'algae' as *a heterogeneous group of photosynthetic plants, ranging from microscopic single-cell forms to multicellular, very large macro forms such as seaweed. All of them contain chlorophyll and hence most are green, but some may be different colours due to the presence of other, overshadowing pigments* (Nill, 2002) .

Algae are primarily photosynthetic autotrophs that are able to convert energy from the sun into chemical energy, which drives the process of carbon dioxide (CO_2) fixation and the subsequent assimilation of fixed carbon into macromolecules such as proteins, carbohydrates and lipids (Harun *et al.*, 2010) (Figure 4).

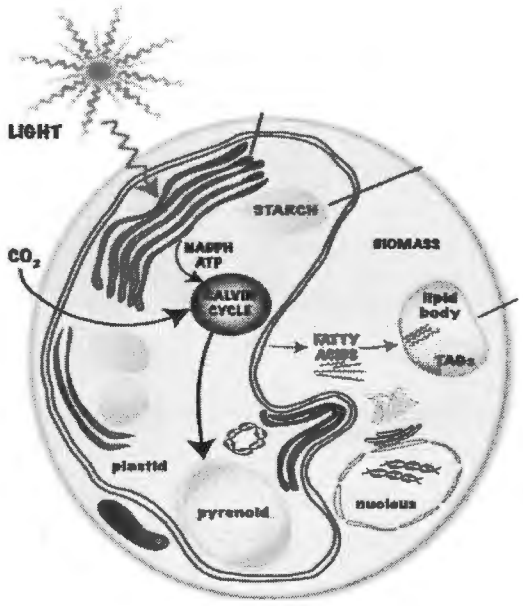


Figure 4: Simplified overview of the photosynthetic process in an algal cell. (Beer *et al.*, 2009)

Algae account for approximately 50% of the photosynthetic oxygen in the atmosphere, which makes them an indispensable natural resource (Anderson, 2005). Algal biomass has been cited as the original source of fossil carbon found in crude oil and natural gas. A certain type of algae (diatoms) account for up 40% of organic matter production in the ocean and hence supports most, if not all, of life in the ocean (Anderson , 2005; Obata *et al.*, 2013a; Obata *et al.*, 2013b). The focus from hereon will be exclusively on microalgae and not the more complex, larger counter parts, or macroalgae

2.2. FACTORS AFFECTING GROWTH AND PHYSIOLOGY OF MICROALGAE: GROWTH REQUIREMENTS AND DESIGN PARAMETERS

The productivity of microalgae is strongly influenced by culture conditions. For optimum growth, several environmental parameters should be kept within predefined physiological limits. These parameters can be grouped into chemical and physical factors (Figure 5). Reactor type, although a physical factor, is considered separately due to the size of the discussion.

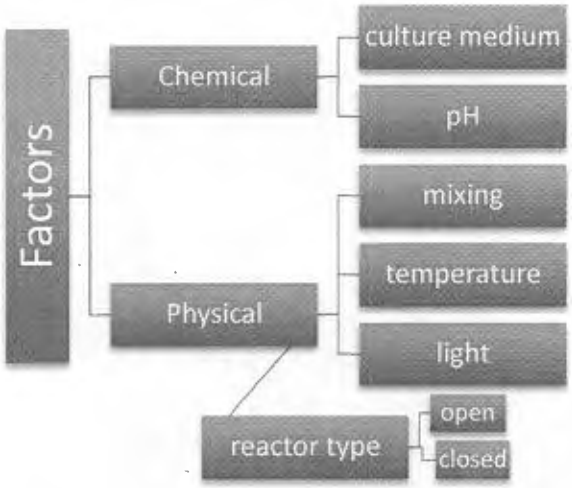


Figure 5: Factors governing the growth and physiology of microalgae.

2.2.1. Chemical factors

Nutrients

The reactor system in which experiments are conducted can be thought of as a 'synthetic environment' in which the microalgae grow. The purpose of microalgal culture medium is to simulate the natural environment, although typically with optimised nutrient concentrations, so that the effect of changing specific variables can be assessed under controlled conditions or to optimise productivity in an industrial setting..

Microalgae require approximately 4 macronutrient elements (carbon, nitrogen, phosphorous and silicon) as well as major ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^- and SO_4^{2-}) for the synthesis of macromolecules and the accumulation of biomass (Anderson, 2005). Several other elements are required as

constituents of enzymes and co-factors (Willey *et al.*, 2008). Microalgal culture media are specifically formulated to contain these elements (Anderson, 2005).

Essential macronutrients include carbon, nitrogen, phosphorous and silicon (for diatoms) and are typically provided as salts of bicarbonate (HCO_3^-), nitrate (NO_3^-), phosphate (PO_4^{3-}) and silicate (SiO_3^-). The primary metabolic functions of the macro elements are reported in Table 3.

Table 3: Macronutrients and their uses during microalgal growth. Taken from Willey (2008) and Shuler (1992). (Willey *et al.*, 2008; Shuler & Kargi, 1992)

Element	Physiological function
Nitrogen	Synthesis of amino acids, purines and pyrimidines
Phosphorous	Present in nucleic acids, ATP, cofactors and proteins
Silicon	Diatoms use silicate for the production of frustules in the cell wall
Carbon	Backbone of all organic molecules and used as an energy source

Microalgal growth not only depends on the macronutrients, but also on essential micronutrients. Essential micronutrients consist of trace metals and the three vitamins Thiamine, vitamin B_{12} and biotin (Anderson, 2005). Micronutrients are typically metal ions and include iron (Fe), manganese (Mn), zinc (Zn), cobalt (Co), copper (Cu) and molybdenum (Mb). These metals are added in trace amounts due to their toxicity at high concentrations (Anderson, 2005).

Micronutrients influence microalgal growth by playing a role in a variety of metabolic pathways that involve the utilisation of essential microalgal resources such as light, nitrogen, phosphorous and carbon dioxide (Anderson, 2005).

pH

The pH of the culture media is one of the most important factors affecting microalgal growth, physiology and reproduction (Visviki & Santikul, 2000). The pH of the culture medium is also closely linked to nutrient concentrations and mixing (Griffiths, 2013). The pH tolerance limits of algae are governed either by the chemical influence on the growth medium or by metabolic effects on the cells. Culture pH can fluctuate above, or below the optimum.

Acidic conditions are considered to be below optimum and nutrient uptake is negatively affected and can possibly induce metal toxicity, affecting microalgal growth (Anderson, 2005; Gensemer *et al.*, 1993; Juneja *et al.*, 2013). Visviki and Santikul studied the effects of continuous acidic conditions (pH 1.4-3.4), for a period of 5 days, on the growth of *Chlamydomonas* and observed no growth under these conditions (Visviki & Santikul, 2000).

It was explained that the exposure to acidic conditions caused an increase in the chemical gradient between the cytoplasm and medium. This ultimately resulted in a higher influx of hydrogen protons (H^+) into the cell. Although some of the protons can be neutralised by the buffering system inside the cell, most protons have to be removed to prohibit the interruption of normal metabolic processes. Maintenance under these conditions requires considerable energy expenditure to pump protons out of the cell, which could have been otherwise used for cell growth (Visviki & Santikul, 2000).

Basic conditions are considered to be above optimum and lower cell growth rates can be expected for neutrophilic algae (acidophilic and alkalophilic species can have optimal productivities under very acidic/alkaline pH, but are not considered here). At a culture pH of 9.0 and 9.4 Taraldsvik and Myklestad (2000) observed diminished daily divisions; on average 2.5 divisions day^{-1} (at pH 7.5) to 0.4 divisions day^{-1} (at pH 9.4) for the diatom species *Skeletonema costatum*. The decrease in growth rate at pH >9.0 was thought to be caused by a decrease in the rate of important biochemical reactions including a change in cell membrane properties. It was found that the cells contained 40% less cellular organic carbon in a culture with a pH of >9.0 than that of a more neutral pH of 8.0 (Taraldsvik & Myklestad, 2000). This indicates reduced uptake rates of the macronutrient carbon, suggesting a carbon limitation despite the supply of surplus nutrient.

This carbon limitation is presumed a pH effect and can be explained by investigating carbon speciation (Figure 6) as well the carbon concentrating mechanisms (CCM) microalgae use to assimilate carbon.

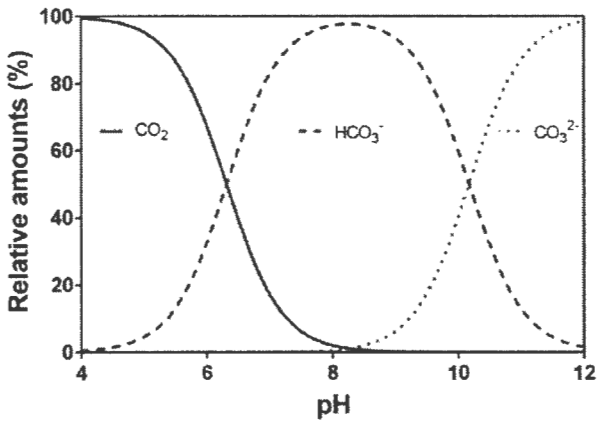


Figure 6: Impact of pH on carbon species distribution. CO_2 = carbon dioxide, HCO_3^-= bicarbonate and CO_3^{2-} = carbonate. Andersen (2002).

It is explained that the higher the pH, the lower the CO_2 concentration in the media. Hence, to cope with limited available CO_2, some microalgae have developed mechanisms to assimilate HCO_3^-

instead, as this is the next available carbon source (Reinfelder, 2011). There are two proposed CCMs: (1) a biophysical CCM which transports CO_2 and HCO_3^- as inorganic forms with the aid of carbonic anhydrases (CAs) and (2) a biochemical CCM involving the fixation of HCO_3^- into compounds, similar to terrestrial plants, as reviewed by Obata *et al* (Obata *et al.,* 2013a).

2.2.2. Physical conditions

Mixing

Mixing is important in algal cultivation for several reasons. Adequate mixing ensures that all cells are kept in suspension and prevents settling or sedimentation of the biomass. Good mixing ensures all cells are exposed to sufficient light and nutrients to maintain high productivity. Mixing is also a key factor in gas-liquid mass transfer, between the culture medium and the air. Good mass transfer is necessary to replenish carbon for photosynthesis and prevent the build-up of dissolved oxygen to inhibitory levels (Griffiths, 2013).

The type of mixing required depends on the size and configuration of the growth reactors. Manual agitation (shaking or stirring of flasks), aeration (bags, tanks, airlift reactors) or paddle wheels (ponds) are typically employed to achieve mixing.

Temperature

Temperature can play major role in microalgal productivity and, while it can be controlled in laboratory scale reactors, temperature control at commercial scale is typically not feasible. The optimal temperature for microalgal growth, while species specific, is generally in the region of 20°C to 30°C (Chisti, 2007).

Physiological responses to changes in temperature can include changes in concentrations of photosynthetic and respiratory enzymes, changes in cell number and nutrient uptake, as well as alterations in fatty acids and proteins (Juneja *et al.*. 2013)

Algal species can tolerate temperatures up to 15°C below their optimum, but this results in reduced growth rates. Low temperatures can result in (1) reduced electron transport due to slower rate of CO_2 fixation (2) inhibition of the active oxygen species resulting in the reduction of photo inhibition and (3) the inhibition of the D1 protein synthesis that is required for photo system II (PSII) repair (Vonshak & Torzillo, 2004). This can possibly result in cell death. Other effects of low temperatures include slower enzyme reaction kinetics that will ultimately have a negative effect on metabolic activity (Chen *et al.*, 2012).

Increasing temperature beyond the optimum (30-40°C) reduces protein synthesis and so consequently results in decreased growth rates and cell death due to protein denaturation

(Konopka & Brock, 1978; James & Boriah, 2010). Increases in temperature have also been reported to impact on the starch content of microalgal cells, leading to the degradation of the starch produced (Juneja *et al.*, 2013).

Yongmanitchai and Ward (1991) published research on the biomass productivity changes with a change in temperature, reporting decreased biomass concentrations of <2 $g.L^{-1}$ when grown at temperatures of 15°C and 25°C compared to a maximum biomass concentration of >3 $g.L^{-1}$ when cultivated at 21°.

It is important to take into account the differences in temperature regulation when a bioprocess is up-scaled, i.e. the switch from laboratory conditions (where temperature control is in place) to outside conditions where temperature can no longer be controlled and it therefore becomes a factor of the environment.

Light

Microalgae have the ability to fix CO_2 and to produce biomass and oxygen more efficiently than terrestrial plants (Darzins *et al.*, 2010). Since light is the driving mechanism for this reaction, light intensity, spectral quality and the photoperiod are important factors to consider when cultivating microalgae. Light can be supplied in its natural form (i.e. directly from sunlight), or artificially by incandescent, florescent or LED sources. Algal growth is limited by too little light, but over exposure to light can be more damaging (Anderson, 2005).

At the one end of the spectrum, phototrophs must receive sufficient light to exceed their light compensation point, to ensure growth, while at the other end of the spectrum, over illumination can result in a loss of viability (Carvalho *et al.*, 2011). This excessive exposure to light results in photo-oxidative stress (Anderson, 2005). Photo-oxidative stress ("photo-bleaching") is often observed in dilute cultures with a low cell concentration. However, in more dense cultures, a higher light intensity is essential to penetrate beyond the surface layer and hence becomes crucial to the productivity of the culture (Carvalho, 2011; Anderson, 2005).

Growth rate under increasing light intensity is a function of strain and culture temperature (Sorokin & Krauss, 1958). However, the growth rate of microalgae is maximal at saturation intensity (i.e. the point where the light harvesting complex is unable to harvest any more light. Linear, as opposed to exponential, growth is a sign of available light becoming the limiting factor (Langley *et al.*, 2012). An linear increase in biomass concentration is typically accompanied by a decrease in light penetration into the culture. This can be explained by the fact that the cells at the surface absorb the light and end up shading the cells below. Hence, the light intensity effectively decreases with culture depth with cells in the middle of the reactor receiving very little, or no light (Griffiths, 2013).

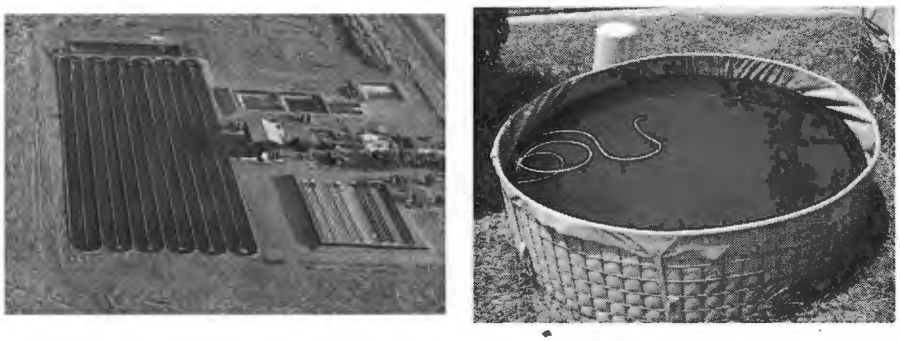
Light is therefore most often the principal limiting factor in the cultivation of photosynthetic microalgae. Reactor design is an important parameter to consider, as explained by Griffiths (2013). Factors contributing to light availability include culture depth and optical cross-section of the reactor, biomass concentration and areal density and turbulence induced by mixing (Griffiths, 2013).

2.3.3. Reactor type and configuration

Microalgal cells can be cultivated in a wide range of growth vessels or reactors, ranging from solid media in petri dishes to large open pond systems. Most importantly, reactors should provide optimal conditions for microalgal growth. Important aspects to keep in mind when considering a system include effective mixing and mass transfer, low system maintenance, energy input requirements and capital/operational costs (Griffiths, 2013). In industry, the two most commonly used approaches for the mass cultivation of microalgae are open pond systems and closed bioreactor systems.

Open pond systems

Commercial scale production of microalgae usually requires reactor volumes of 10,000 to 1,000,000 litres. Open pond systems are often the choice of the industry to mass produce a desired product (Harun *et al.*, 2010). Pond systems vary in shape and size and are the oldest cultivation systems, with the algae cultivated under conditions similar to the natural environment. Currently used models include raceway ponds, shallow natural ponds, circular ponds (Figure 7) and tanks as well as closed ponds. The location of the operation typically determines the type of pond selected, strain of microalgae and amount of light available for photosynthesis (Harun *et al.*, 2010).



(A) **(B)**

Figure 7: Examples of open systems. Open pond system (**A**) raceway and (**B**) circular for the cultivation of microalgae. Image (A) sourced from CSIR Biosciences and (B) Durban University of Technology (DUT) with permission.

However, due to the inability to exercise complete control over the environmental conditions, the pond typically becomes a function of the local climate (Harun *et al.,* 2010) and productivity is limited by the following parameters: light intensity (seasonal variation) and temperature (seasonal variation). Well-chosen locations are thus imperative and directly contribute to the success of the cultivation.

Additional challenges that need to be considered are contamination and predation. Contamination can limit the choice of microalgae to a strain that is able to grow under certain selective conditions (Harun *et al.,* 2010). To mitigate this problem, the isolates used need some sort of "selective advantage" that can be used to keep the culture as pure as possible. Predation by protists such as rotifers can significantly reduce productivity, particularly for slower growing cultures. Microalgae that have been successfully grown in outdoor ponds include *Dunaliella*, *Spirulina* and *Chlorella* (Harun *et al.,* 2010). The successful cultivation of these organisms can be due to selective advantages; i.e. *Dunaliella* that prefers a high salt concentration and *Spirulina* that prefers a high pH and salt concentration while Chlorella has a naturally high growth rate (Harun *et al.*, 2010). The advantages and disadvantages of open pond systems are presented in Table 4.

Table 4: Advantages and disadvantages of open pond systems compared to closed systems (Harun, *et al.* 2010).

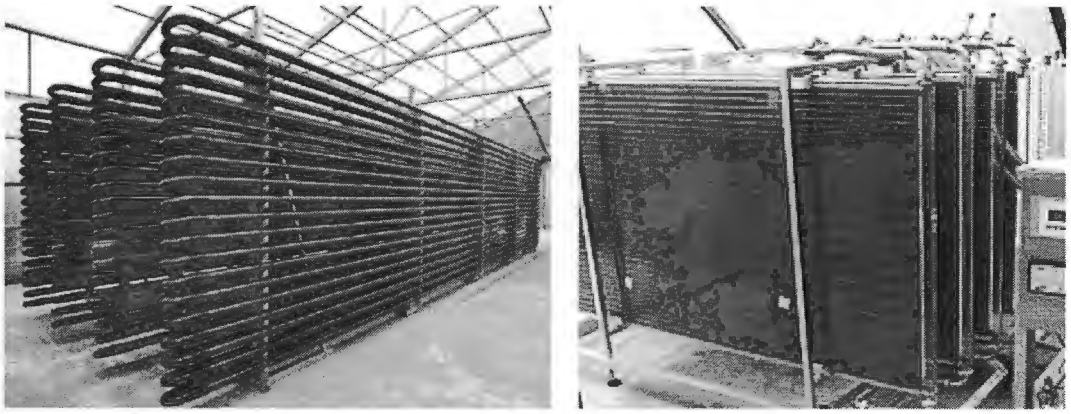
Advantages	Disadvantages
✓ Easier to construct than photobioreactors	× Poor light utilisation
✓ Easier to operate	× Evaporative losses
✓ Selective for robust organisms	× CO_2 diffusion
✓ Low production costs	× Contamination
✓ Low operating costs	× Inefficient stirring mechanism
✓ Large scale	× Poor mass transfer rate
	× Poor physiological control
	× Requires large area of land

Closed bioreactor systems

Closed systems were originally designed to overcome the problems associated with open pond systems (Wen & Chen, 2003) and to provide greater control over environmental parameters. Although the closed culture systems may reduce contamination, the success of the cultivation is still subject to variations in temperature and light intensity. These systems are made with transparent materials (glass and plastic) and can be placed indoors, either with or without artificial lighting, or outside (Figure 8) (Wen & Chen, 2003).

Two types of closed reactors can be distinguished: photobioreactors (PBR) for phototrophic production, where light energy is supplied via lights (Figure 8) and fermenters for heterotrophic production, where energy is supplied as an organic carbon source such as a sugar (Anderson,

2005). Fermenters will not be discussed further as the focus of this work is solely on photoautotrophic production of microalgae.



(A) (B)

Figure 8: Examples of closed photobioreactors (A) tubular glass photobioreactor and (B) plastic plate photobioreactor. Images obtained from Wikipedia as uploaded by IGV Biotech.

The design of a photoautotrophic PBR ultimately depends on the final product and its production requirements, which often escalates the cost of construction for these systems. For example if the final product is a high value, pharmaceutical the control and regulatory requirements would require a PBR with increased control specifications thus increasing the overall cost of the PBR. Types of PBRs include tubular, plate, bubble and bubble column reactors. Key sub components that a PBR employs include oxygen, CO_2, temperature, pH, light, and conductivity sensors as well as a recirculation, harvest and substrate pump. Other important components are a CO_2 injection valve and an oxygen release system. Due to all the control functions, these systems are normally more versatile than pond systems, but also more complicated and expensive to operate (Anderson, 2005).

Table 5: Advantages and disadvantages of closed bioreactor type 1: photobioreactors (Harun *et al.*, 2010)

Advantages	Disadvantages
✓ Controlled conditions	× High capital cost
✓ Large surface-to-volume ratio	× Difficulty in sterilising
✓ Better gas transfer	× Small scale
✓ Reduction of media evaporation	× Requires cooling
✓ Protection from contamination	
✓ Space saving	

2.3. OMEGA-3 PUFA PRODUCTION

This section will focus on PUFA production by autotrophic microalgae. Heterotrophic and mixotrophic production systems do potentially exist, but fall beyond the scope of this work and will therefore not be discussed.

2.3.1. Autotrophic metabolic pathways

Metabolism is broadly defined as the sum total of all chemical reactions occurring in a cell (Willey *et al.*, 2008) and all organisms can be defined metabolically in terms of their energy, carbon and electron source. Organisms produce different products as a result of differences in their metabolism (Shuler & Kargi, 1992).

The term "autotrophy" refers to the use of sunlight as an energy source to drive photosynthesis (Willey *et al.*, 2008). A broad overview of the metabolism involving lipid synthesis in autotrophic microalgae is presented in Figure 9. For phototrophs, the energy source is light; the carbon source is inorganic carbon (either CO_2 or HCO_3^-) and the electron source is water (Willey *et al.*, 2008).

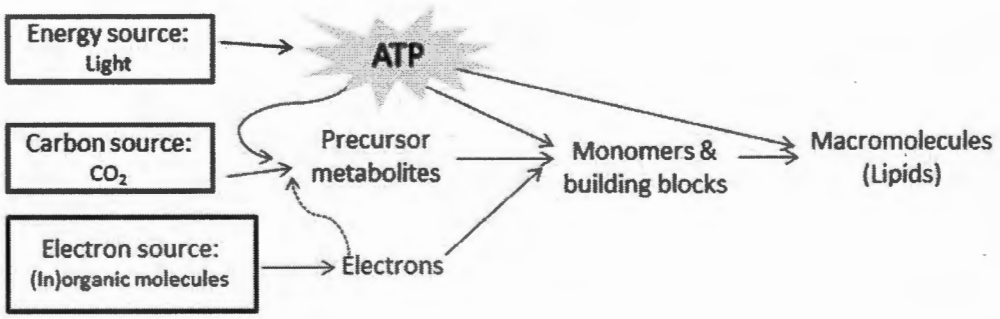
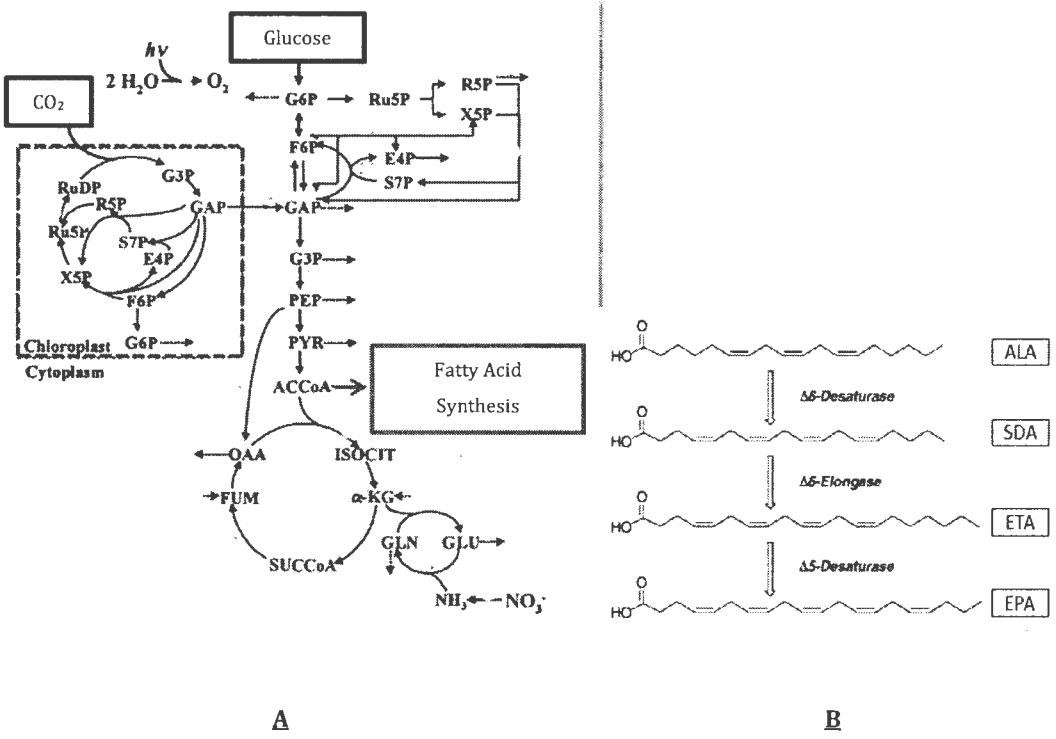


Figure 9: Broad overview of autotrophic metabolism for lipid production (Adapted from Willey, 2008).

In autotrophy, the precursor metabolites arise from CO_2-fixation pathways as seen in Figure 10A. Reducing power and ATP are consumed in the metabolic pathways. Monomers and other building blocks are the products of biochemical pathways that start with the precursor metabolites pyruvate and α-ketogluterate. Macromolecules are synthesised from the monomers (Willey *et al.*, 2008).



A B

Figure 10: Summarised metabolic pathways. Autotrophic metabolism indicated in red (**A**) and (**B**) fatty acid metabolism and the elongation of fatty acids to produce EPA (Horton *et al.*, 2006).

Fatty acid synthesis occurs in the chloroplast where the biosynthesis commences with the carboxylation of acetyl-coA (AcCoA) to form acetate or pyruvate, by the action of glycolytic enzymes (Figure 10A). Acetyl-CoA is converted into malonyl-CoA, which drives the condensation reaction to extend the acyl group to stearic acid (18:0) that is then desaturated to form oleic acid (18:1ω9). Oleic acid is then converted to linoleic acid (LA, 18:2 ω-6) and α-linolenic acid (ALA, 18:3 ω-3) (Figure 10B). Subsequent desaturation and elongation steps result in the formation of omega-3 PUFAs (Figure 10B)(Wen & Chen, 2003; Yang *et al.*, 2000; Cardozo *et al.*, 2007).

2.3.2. Factors affecting omega-3 production

Physiological factors affect the production of PUFAs by influencing metabolic activity. Consequently, these factors can be used to manipulate algal metabolism in order to produce, or over produce omega-3s. The control of metabolic pathways by chemical and physical regulation has thus become an important consideration in bioprocess engineering (Shuler & Kargi, 1992). The major influencing factors are nutrients, pH, temperature and light.

Nutrients

Nutrient supply strategies can be used to influence EPA production in two ways: (1) feeding regimes (batch *versus* fed-batch) and (2) elimination of certain nutrients.

(1) Feeding regimes: batch *versus* fed-batch

In this instance, 'batch' culture refers to adding sufficient nutrients to last a complete growth cycle to the culture medium prior to inoculation, while 'fed-batch' refers to a regime where a smaller amount of nutrients is supplied at discrete intervals, for example every second day over the duration of the growth cycle. Adequate concentrations for batch growth are determined by growing the culture at different nutrient loadings and measuring the growth response, productivity and residual nutrient concentration in order to determine the optimum initial nutrient concentration.

High initial concentrations may be detrimental to the microalgae under batch culture conditions. A previous study on surplus nutrients conducted in the CSIR laboratories revealed slower growth rates and phosphate uptake rates at high phosphate concentrations (Dickson & Lalloo, 2011a). It has been reported that high concentrations of phosphate caused inhibition of growth through enzyme inhibition and transport disruption (Perras & Kuenzler, 1965). At excessive phosphate concentrations, a shutdown of the phosphatase enzyme is triggered, causing the observed decrease in growth rate. A similar phenomenon was observed with surplus nitrate concentrations, where the enzymes responsible for the uptake of nitrate ions were repressed (Perras & Kuenzler, 1965). This study did not present lipid productivity data; however, from the information available on the impact of excessive nutrient load on growth, it is hypothesised that a similar effect will be apparent in relation to EPA content due to the direct relationship between lipid concentration and cell concentration.

(2) Elimination of nutrients

When the species is deprived of major nutrients (such as carbon, nitrogen, phosphorus or silicate) the cell tends to alter normal metabolism (Wen & Chen, 2003). The cells adapt their metabolism to the environment and could consequently undergo a change in cellular composition. Under nitrogen-limited conditions, where carbon is not limiting, many microalgae adapt their metabolism to channel carbon into storage products in the form of either carbohydrates or lipids (Anderson, 2005). Nitrogen limitation is the most frequently reported method used to enhance overall lipid content (Harding *et al.*, 2007).

Nitrogen depletion has been used to increase EPA production in *Phaeodactylum tricornutum*, with the lipid content increasing from 81.2 to 168.5 $mg.g^{-1}$ of culture dry weight (Yongmanitchai & Ward, 1991; Yongmanitchai & Ward, 1992). These 'stress' conditions, employed to enhance lipid and/or

EPA productivity, typically result in decreased growth. Roleda *et al.* (2012) showed a negative correlation between growth rate and fatty acid content when algae were grown under nitrate deplete conditions – an observation confirmed by several other studies (Illman *et al.,* 2000; Breuer *et al.,* 2012; Obata *et al.,* 2013a; Roleda *et al.,* 2013; Pal *et al.,* 2011; Converti *et al.,* 2009).

pH

The pH tolerance limits of microalgae are governed either by chemical influence on the growth medium or by metabolic effects on the cells. Culture pH directly influences the partitioning of carbon species as shown in the carbon distribution species plot in Figure 6. Without the ability to assimilate carbon, the organism will not be able to incorporate carbon into any cellular components, including fatty acids. Yongmanitchai and Ward reported good growth and EPA production in *P. tricornutum* cultures with initial pH values of between 6.4 and 8.4 resulting in EPA content ranging from 28.7 to 34.2 $mg.g^{-1}$ dry biomass. Optimum EPA production per unit volume of cell culture was reported to occur in a culture where the initial pH was 7.6 (Yongmanitchai & Ward, 1991).

Temperature and light

Other stimuli shown to increase omega-3 biosynthesis include temperature and light. Research using *Pavlova lutheri* and *Chlorella minutissima* demonstrated that relative EPA content increased from 20.3 to 30.3 mass % and 20.3 to 44.7 mass % when the culture temperature was reduced from 25°C to 15°C and 20°C respectively (Adarme-Vega *et al.,* 2012; Vazhappilly & Chen, 1998; Seto *et al.,* 1984).

In a similar study, higher EPA values were reported for *Phaeodactylum tricornutum* when the culture temperature was lowered from 25°C to 10°C for a period of 12 hours (Adarme-Vega *et al.,* 2012; Jiang & Gao, 2004). This increase in PUFAs is explained by the fact that, as these fatty acids have good flow properties and are predominantly used in the cell membrane to maintain fluidity at low temperatures. (Adarme-Vega *et al.,* 2012; Juneja *et al.,* 2013).

Sukenik (1991) showed that *Nannochloropsis* sp. growth was light saturated at a photon flux above 200 $\mu mol.m^{-2}\ s^{-1}$ causing a plateau in growth. In contrast to this, relative abundance of EPA increased exponentially with light intensity. Sukenik further demonstrated that the cellular EPA content in the same species decreased at night but the overall volumetric EPA concentration remained constant. This was attributed to the fact that secondary metabolite (such as EPA) production ceased at night while cell division continued (Sukenik, 1991).

3. BIOPROCESS CONSIDERATIONS

3.1. GENERIC PROCESS DEVELOPMENT TRAIN FOR THE OUTDOOR PRODUCTION OF MICROALGAL OMEGA-3 FATTY ACIDS

The previous sections discussed the potential of microalgae to produce a variety of products. Conditions affecting production of the desired product were also discussed. Considered together, this represents a 'bioprocess'. The term *'bioprocess'* is defined by the American Heritage® Dictionary simply as: *"A technique that produces a biological material for commercial use"* (Editors of the American Heritage Dictionaries, 2009). A simplified overview of a generic bioprocess tailored to using autotrophic microalgae to produce omega-3 fatty acids is provided by Figure 11.

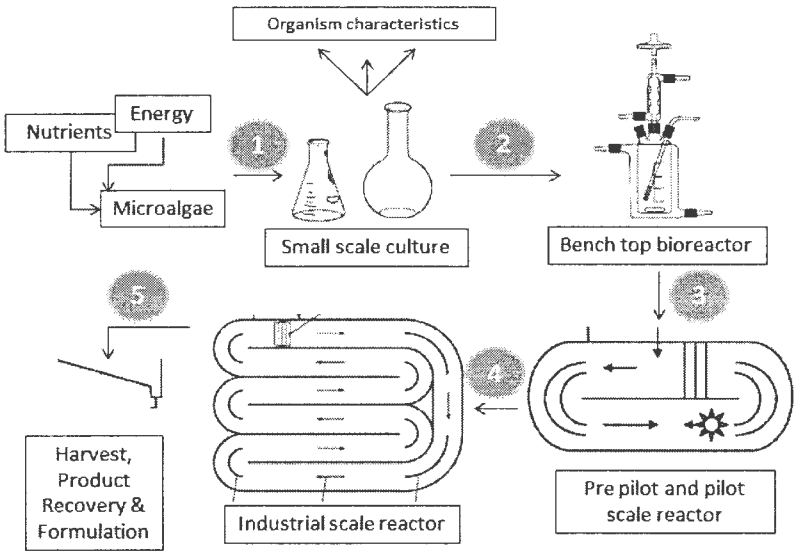


Figure 11: Broad overview of the development of products from microalgae. Adapted from Harun *et al.* (2010).

Substantial engineering input is essential in many aspects of microalgal bioprocessing, including the design and operation of vessels, reactors or ponds as well as product-recovery equipment, development of systems for process control, and efficient and safe layout of bioprocessing facilities. The following part in this section overviews the development of a generic bioprocess (adapted from Doran (1995)) tailored to the production of microalgal omega-3 PUFAs employing a photoautotrophic system.

Stage 1: Identification of the biomaterial

The process starts with the identification and culture of a unique algal strain that is capable of producing high levels of omega-3 fatty acids. Identifying a unique algal strain can be based on published information and the strain sourced from a culture collection, such as the Culture Collection of the University of Texas (UTEX), or a research group can embark on a bioprospecting program to isolate cultures from the environment.

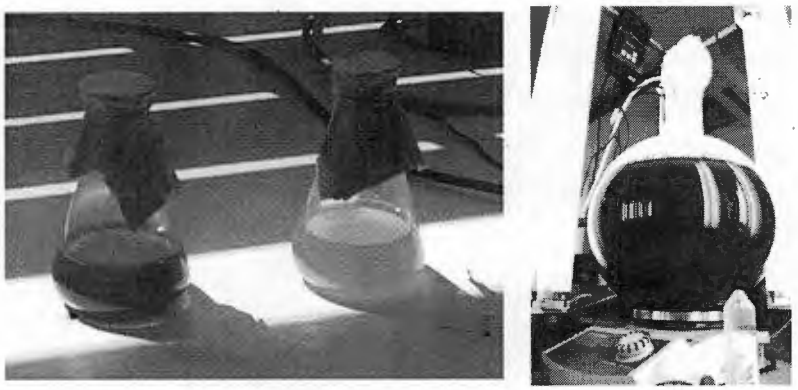
The level of expression of the desired product is confirmed before or during small-scale cultivation to test for product formation. This is typically achieved by a rapid screening method where qualitative and quantitative analyses are conducted to determine the level of expression. Once a decision has been made, long-term storage of the organism is also investigated. Microalgae are normally stored as dormant cultures on solid media (agar slants) or in liquid (test tubes) cultures (Figure 12).

(A) (B)

Figure 12: Long-term storage of microalgal cultures on (A) agar slants and (B) in test tubes. Image supplied by CSIR Biosciences.

Stage 2: Characterisation of the organism

The second stage of the bioprocess development train is the measurement of the growth and production characteristics as a function of culture environment. Characteristics include growth rate, sensitivity to pH and temperature, susceptibility to contamination, light and nutrient requirements. The data is used to determine the cell growth rate, specific productivity and product yield. Initial characterisation studies are typically conducted in simple bioreactors that can range from 96 well plates and 250 mL flasks (Fig.13A) to 10 L (Fig.13B) glass vessels under controlled laboratory conditions.



(A) (B)

Figure 13: Initial characterisation studies are conducted in stirred reactors ranging from (A) 250 mL to (B) 10 L. Image supplied by CSIR Biosciences.

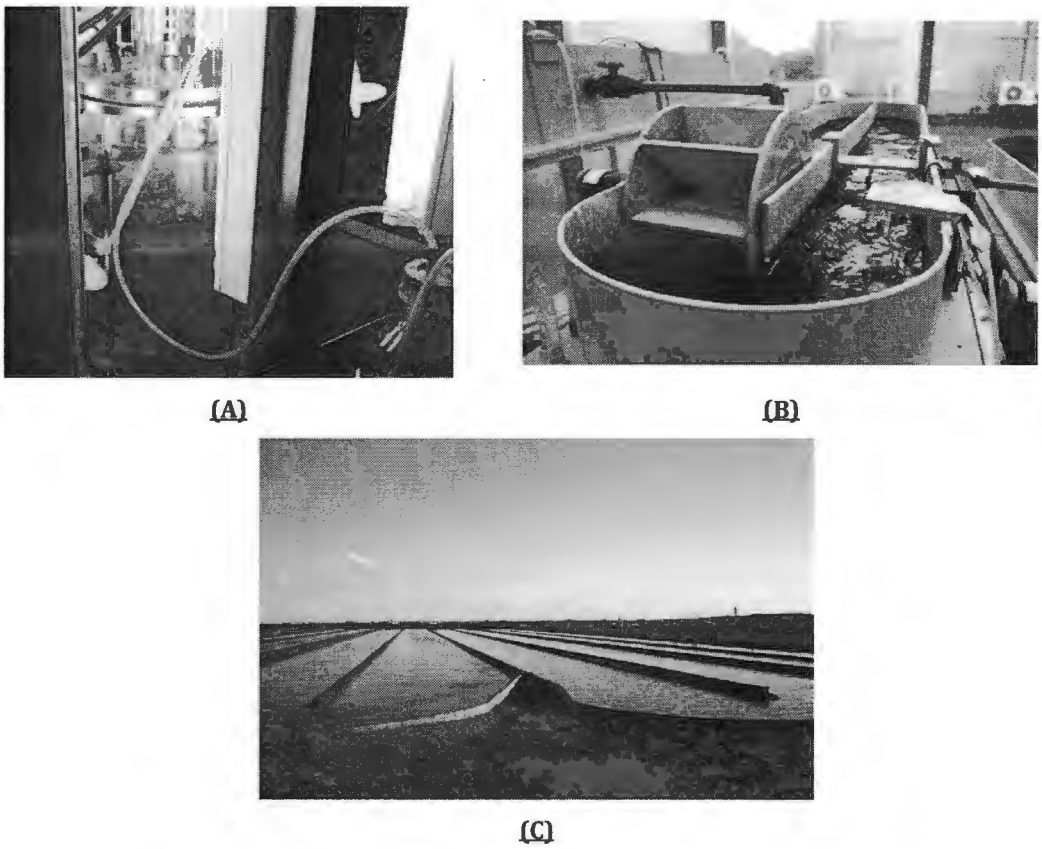
The calculated parameters will give an indication of the organism's performance. Key desirable traits, as identified by Griffiths and Harrison (2009) and Duong *et al.* (2012) for successful microalgal cultivation are presented in Table 6. Consideration of these factors when choosing a species to utilise in a commercial bioprocess is of utmost importance. Choosing the correct species can lead to higher efficiency, greater yields and lower costs. However, a single algal species is highly unlikely to possess all of the traits and hence prioritisation is required. Selection criteria are therefore defined around the factors favouring high growth and production of the metabolite of interest. Organisms that perform well according to the criteria can be considered for further evaluation.

Table 6: Desirable characteristics of microalgal culture.

Stage	Desirable Traits
Screening	High product (EPA) content. Rapid and synchronized lipid production. Radiation tolerance/pigment synthesis. Low starch contents. High protein contents.
Cultivation	Rapid growth rate. Wide tolerance of environmental conditions. Tolerance of contaminations. Flagella properties/possession. Sheering resistance.
Harvesting	(Large) cell size and cell wall properties amenable for auto flocculation. Sinking speed.
Extraction	Cell wall properties amenable for oil extraction. High lipid extraction efficiency

Stage 3: Scale up of the process

Once cultivation parameters have been determined, the cultivation is up-scaled to pilot (Figure 14A&B) and industrial (Fig.14C) sized reactors to evaluate the effect of scale up. During the scale up process, specific variables that can and cannot be controlled are identified. The effects of the uncontrolled variables can be used to assess the technical and economic viability of the process.



(A) (B)

(C)

Figure 14: Range of reactors used in up scaling includes (A) bench-top photobioreactors and (B-C) open pond systems. Images supplied by CSIR Biosciences.

Stage 4: Product recovery: harvest and product extraction

Once a system is in place for the optimal cultivation of microalgae, the next step is the recovery of biomass, followed by product extraction/recovery.

Biomass harvesting

The three methods most commonly reported for dewatering microalgae are flocculation, centrifugation and filtration (Harun *et al.*, 2010). Flocculation works based on counteracting the surface charges on the algae cell to facilitate clumping/coagulation. Centrifugation works based on the application of a centripetal acceleration to separate cells from the media. Filtration encompasses several techniques including microfiltration, ultra-filtration, pressure filtration and vacuum filtration (Harun *et al.*, 2010). Filtration involves running the culture through filters on which the algae will accumulate and allow the medium to pass through the filter. Flocculation and filtration varies in effectiveness while centrifugation is energy intensive and costly.

Product extraction

After dewatering of the culture, the resulting paste can be treated in wet or dry form. The oil (containing the omega-3 fatty acids) can be extracted from the biomass employing a number of techniques including oil press, solvent extraction, super critical fluid extraction and ultra sound. Each technique has its advantages and limitations as depicted in Table 7 (Harun *et al.*, 2010).

Table 7: Advantages and limitations of different oil extraction methods.

Extraction method	Advantages	Limitation
Oil press	✓ Easy to use ✓ No solvent involved	× Large amount of sample required × Slow process
Solvent extraction	✓ Relatively inexpensive ✓ Reproducible	× Solvents highly flammable × Recovery is expensive × Energy intensive
Supercritical fluid extraction	✓ Non toxicity (no organic solvents used) ✓ Non flammable ✓ Simple in operation	× Insufficient interaction between CO_2 and samples
Ultrasound	✓ Reduced extraction time ✓ Reduced solvent consumption	× High power consumption × Difficult to scale-up

The cost of biomass dewatering combined with product recovery is the ultimate determining factor of how economically feasible a new bioprocess will be as an extracted product requires an additional process step that has additional capital and operating costs. Therefore, the final cost of production, will be more.

4. CURRENT STATE OF THE MICROALGAL INDUSTRY

Food and feed industries produce a wide range of microalgal related products, such as fish feeds and protein supplements. Algae are rich in fibre, minerals, vitamins and antioxidants (Cardozo *et al.,* 2007). However, in recent years the focus has shifted from wild harvests to controlled, farmed cultivation in order to produce valuable products on an industrial scale (Cardozo *et al.*, 2007).

To date, there are only three key industry players in the area of vegetarian (from microalgae) PUFA production. These players are DSM Nutritional Products, Aurora Algae™ and AlgaeBio. The products available and technology used are summarised in Table 9. DSM Nutritional Products offers a product called *life'sDHA* consisting of refined "highly purified" DHA oil from microalgae (DSM Nutritional Products, 2012). It is a trusted source and used in over 99% of infant formula. This is a pure DHA product that does not contain EPA (Naidoo and Lalloo, 2011).

Aurora Algae™ currently offers a product calledA2 EPA Pure™ (crude oil product that contains 65% EPA) which "delivers exponentially higher potency EPA; the purest high quality ingredient for supplements and pharmaceuticals" (Aurora Algae, 2013). Aurora Algae™ uses open pond technology to cultivate the algae year round. This is a pure EPA product that does not contain DHA (Naidoo and Lalloo, 2011).

AlgaeBio (Algae Biosciences) are at the present a research based company with the goal to eventually commercialise algal derived products. The company's first target is to produce "ultra-pure omega-3 fatty acid oils for international customers in the nutraceutical and food additive sectors" (AlgaeBio, 2011) using a pond system as well (Naidoo and Lalloo, 2011).

Table 8: Summary of current market players, products and the technology used.

Company	Product	Technology
DSM Nutritional Products	*Life'sDHA*	Fermenter
Aurora Algae™	A2 EPA Pure™	Open pond
AlgaeBio	TBA (omega-3)	Open pond

6. PROJECT HISTORY AND CURRENT FOCUS

Since the launch of the CSIR Microalgal Bioprospecting Project in 2010, over 750 samples have been collected from seven provinces across South Africa: Kwazulu-Natal, Eastern Cape, Gauteng, Western Cape, Limpopo, North West, Mpumalanga, the Free State and the Northern Cape. These locations of the sampled sites are represented by the dots in Figure 15. The library collected through the bioprospecting project was used as the basis for bioprospecting/screening for local SA EPA producing species.

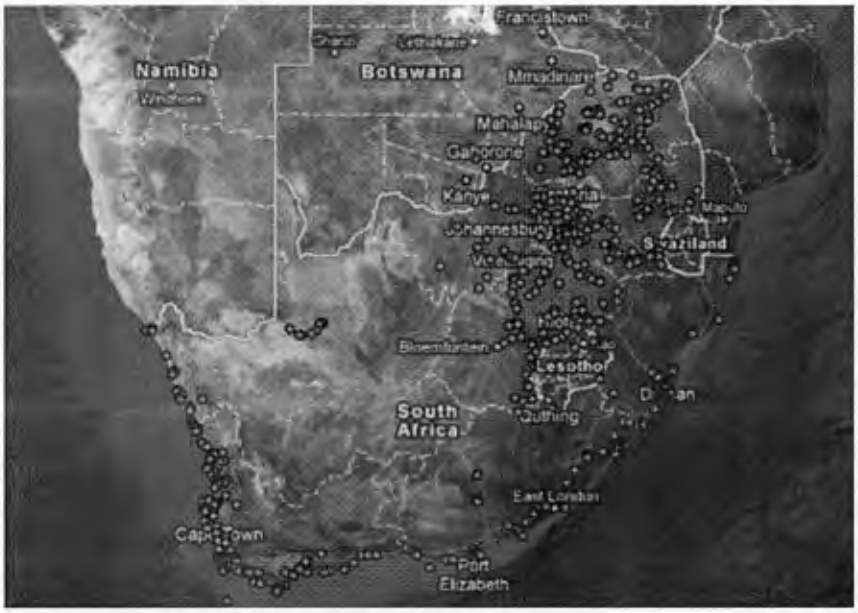


Figure 15: Sampling sites across South Africa where over 800 microalgal samples were collected. Image supplied by CSIR Biosciences.

The isolates are currently housed in the CSIR Biosciences algal cell bank on agar slants and are re-streaked on fresh agar every six months. A screening method was developed, using flow cytometry, in order to rapidly screen for lipid rich isolates. Ten isolates were selected for further studies and of these; one species was identified that contained a particular high lipid concentration. More specifically, the isolate produced high amounts of EPA.

Further studies were conducted on this species and are represented by the timeline in Figure 16. The research included a growth study, media optimisation study, a study to identify important process parameters, as well as upstream (USP) and downstream processing (DSP) studies.

For the purpose of this project, the organism (a diatom) will be referred to as "Isolate WCA23.2"; "WCA" referring to the region in the Western Cape from where it was isolated, "23" referring to the 23rd sample taken in the Western Cape and ".2" referring to the second species (isolate) isolated from the 23rd sample taken.

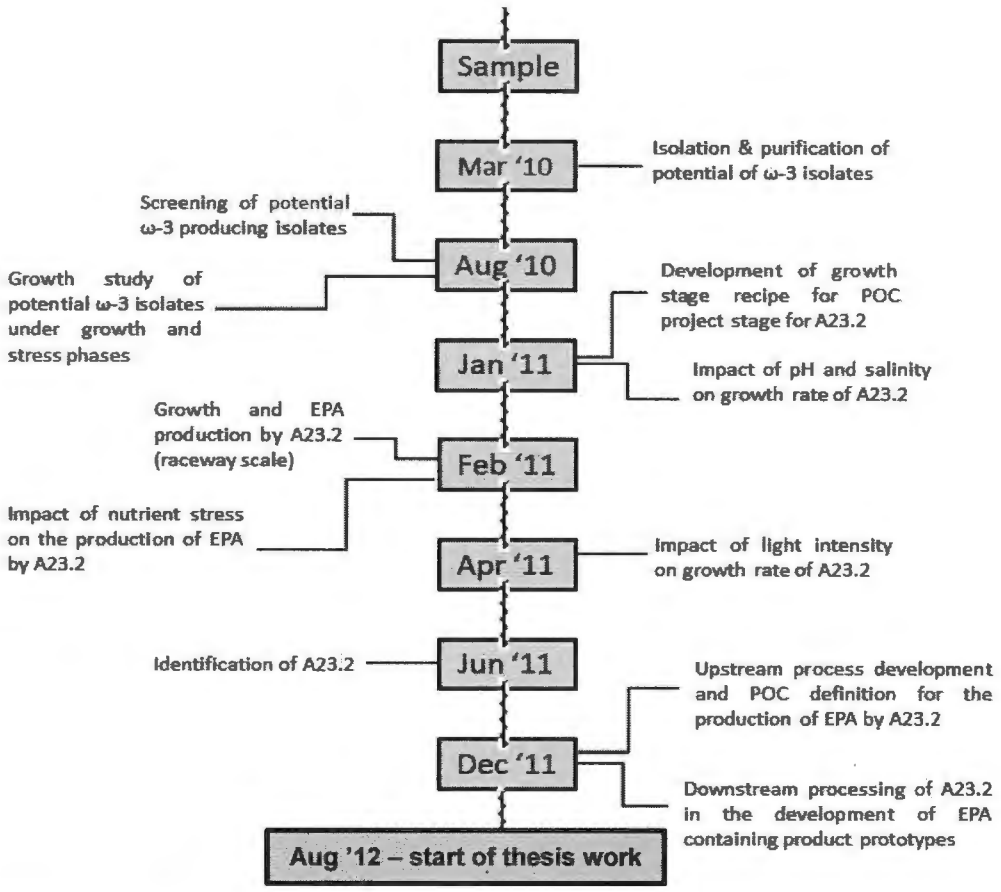


Figure 16: Chronological summary of the Microalgal Bioprospecting project at CSIR Biosciences and current focus.

The research represented in Figure 16 was conducted prior to the work presented in the following chapters. The figure is purely included to build a basis from which this work stems.

7. RESEARCH HYPOTHESIS, OBJECTIVES & KEY QUESTIONS

CSIR Biosciences have demonstrated the production of omega-3 oils in an open raceway system. The competitive performance of the organism and system have not been confirmed (benchmarked) against the cultivation of a proven, omega-3 producing species as reported in published literature.

Therefore, the research hypothesis is that a locally selected isolate is more suitable for omega-3 production in an open raceway pond than a current species of choice, based on its preference for local climatic conditions, capacity for induced EPA production and the ease of harvesting of the biomass.

To evaluate the hypothesis, the following project objectives and key questions were devised:

Research Objective 1: **Select one commercially available species and one uncharacterised locally selected microalgal isolate from the CSIR culture collection to compare in terms of growth and EPA production.**

Research Aims:

1 Select three characteristics to be used to evaluate published data.
2 Use published data and the selected criteria to rank known species in terms of EPA production potential.
3 Select locally sourced isolates based on previous biomass and lipid determinations.
4 Investigate and compare biomass and EPA production of pre-selected culture collection species and local isolates.
5 Confirm the identity of the selected strains using 18S rRNA gene sequence.

Research Objective 2: **Compare the effect of selected environmental factors on growth rate and EPA production under laboratory conditions in two chosen species.**

Research Questions:

1 Does pH control improve biomass growth and EPA production under nutrient sufficient and deficient conditions?
2 Is a fed-batch nutrient regime better than a batch regime in terms of biomass and EPA production under nutrient sufficient and deficient conditions?
3 What is the effect of nutrient deficiency (exclusion of nitrogen and silicon) on EPA production?

Research Objective 3: **Asses the growth and EPA production of a CSIR isolate and culture collection species in an open raceway pond system from a bioprocess perspective**

1 What is the effect of upscale on the biomass and EPA production focussing on scale changes and impact of uncontrolled variables?

CHAPTER II

CHAPTER II – MATERIALS AND METHODS

1. INTRODUCTION

This chapter presents the detail of the materials and methods used, including the microalgal cultures and their maintenance, media selection, reactor specification and design, cultivation conditions and general analytical methods. A data processing section is included to show general calculations. Further specific experimental details are presented in the relevant chapter.

2. CULTURES AND CULTURE CONDITIONS

2.1. MICROALGAL CULTURES AND GENERAL GROWTH CONDITIONS

The microalgal species cultivated, along with their taxonomy and origin are summarised in Table 9. Stock cultures were maintained on either artificial freshwater (AF6) or artificial saltwater (ASW) agar plates and slants. Liquid stock cultures of all species were maintained in 1 L Erlenmeyer flasks in a shaking incubator (20°C, YIH DER LM-530R) under constant illumination of 100 $\mu mol.m^{-2}s^{-1}$ provided by GRO-LUX fluorescent bulbs (Sylvania).

Inoculum for each study was prepared by scaling up stock cultures in 1 L flat bottom flasks under standard culture conditions for 7 to 10 days. The pH was adjusted to the desired levels on a daily basis, using 32% HCl. All experiments were inoculated at a cell concentration of 1×10^6 $cells.mL^{-1}$.

Table 9: Abbreviations, taxa, media specification and origins of the microalgal cultures used

Species	Abbr.	Medium	Taxa	Origin	Sampling Origin
P. tricornutum	P.t	ASW	Diatom	UTEX L642	Plymouth, UK
N. oculata	N.o	ASW	Green algae	UTEX 2164	Isle of Cumbrae, UK
C. minutissima	C.m	AF6	Green algae	UTEX 2219	Auckland, NZ
Isolate WCA 23.2	A23.2	ASW	Diatom	South Africa	Western Cape, ZA
Isolate WCA 27.2	A27.2	ASW	Brown algae	South Africa	Western Cape, ZA
Isolate WCC 39.3	C39.3	AF6	Green algae	South Africa	Western Cape, ZA

2.2. MEDIA

Part of this project was to compare the performance of six organisms (Table 9) in terms of growth and EPA production. To facilitate the comparison of the performance of different species, simple media were chosen to accommodate all six organisms; artificial fresh water (AF6) (Anderson, 2005) and artificial saltwater (ASW) (Brown, 2002; Sheenan *et al.*, 1998).

The freshwater species were grown on AF6 composed of: 0.400 $g.L^{-1}$ MES; 0.002 $g.L^{-1}$ Fe-citrate; 0.002 $g.L^{-1}$ citric acid; 0.022 $g.L^{-1}$ NH_4NO_3; 0.030 $g.L^{-1}$ $MgSO_4.7H_2O$; 0.010 $g.L^{-1}$ KH_2PO_4; 0.005 $g.L^{-1}$ K_2HPO_4 and 0.010 $g.L^{-1}$ $CaCl_2.2H_2O$.

The marine species were grown on ASW, composed of: 13.98 $g.L^{-1}$ NaCl; 2.60 $g.L^{-1}$ $MgCl_2.6H_2O$; 3.56 $g.L^{-1}$ $MgSO_4.7H_2O$; 0.77 $g.L^{-1}$ $CaCl_2.2H_2O$; 0.39 $g.L^{-1}$ KCl; 0.01 and $g.L^{-1}$ H_3BO_3.

A volume of 1.0 $mL.L^{-1}$ of trace elements solution composed of 19.60 $g.L^{-1}$ $CuSO_4.5H_2O$; 44.0 $g.L^{-1}$ $ZnSO_4.7H_2O$; 20.0 $g.L^{-1}$ $CoCl_2.6H_20$; 360.0 $g.L^{-1}$ $MnCl_2.4H_2O$ and 12.60 $g.L^{-1}$ $NaMoO_4.2H_20$ was added to the media.

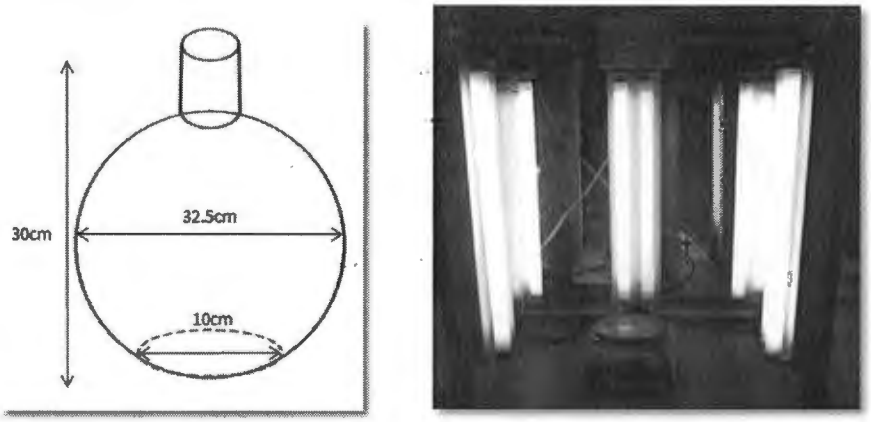
A volume of 1.0 $mL.L^{-1}$ of Fe/EDTA solution composed of 5.71 $g.L^{-1}$ $FeC_6H_5O_7$ and 17.35 $g.L^{-1}$ $Na_2EDTA.2H_2O$ was added to the media

A volume of 1.0 $mL.L^{-1}$ of the vitamin solution composed of 200.00 $mg.L^{-1}$ Thiamine-HCl; 1.0 $mg.L^{-1}$ Biotin and 1.0 $mg.L^{-1}$ B-12 was added to the media (after autoclaving).

Media components were weighed out and made up to the appropriate volume. Media was then sterilised for 20 minutes at 121°C. Filter sterilised Fe/EDTA (1 mL) and vitamin solution (1 mL) were added from the stock solutions.

3. REACTOR DESIGN AND OPERATION

All bench scale experiments were conducted in flat bottom glass flasks, while raceway studies were conducted in a raceway pond system located in a greenhouse. The flat bottom flask has a base diameter of 10 cm, a maximum width of 32.5 cm and height of 30 cm, with a working volume of 4 L (Figure 17A). Mixing of flask contents was achieved by a magnetically operated stirrer plate (Heidolph MR Hei-Mix D) and magnetic bar set to 500 RPM. The flasks were subjected to constant illumination of 140 $\mu mol.m^{-2}s^{-1}$ using a constructed light rig (Figure 17B) consisting of 10 fluorescent lights allowing light to shine on the flask from 3 angles. Light intensity at the surface of the flask was measured on a daily basis using a LI-1400 Data logger (LI-COR). Culture temperature and pH was monitored on a daily basis with a controller unit (Saga Master Controller). Flasks and media were sterilised by autoclaving at 121°C for 20 minutes prior to each study. Contents were harvested by centrifugation at 12 000 x*g* using a centrifuge (Du Pont Instruments).

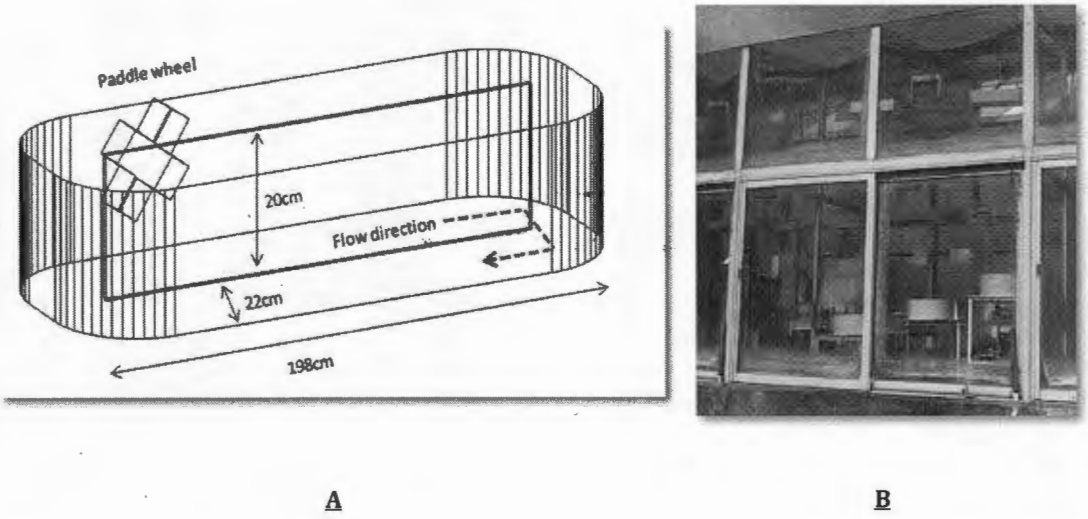


A **B**

Figure 17: Schematic depiction of flask for bench scale study (**A**) and light rig setup (**B**). Image supplied by CSIR Biosciences.

The raceway pond had a base diameter of 44 cm, a length of 198 cm and height of 20cm, with a working volume of 100 L (Figure 18A). A paddle wheel with 8 blades provided mixing of the raceway pond contents at 80 RPM. The raceway ponds were housed in a greenhouse (Figure 18B). Illumination of the culture was achieved using natural sunlight, so a diurnal light dark cycle prevailed. Light intensity was measured on a daily basis.

Culture temperature and pH was monitored on a daily basis with a Saga pH controller unit. Raceway ponds and media were not sterilised. The liquid volume was assessed for any increase/decrease using a ruler placed against the inside wall of the raceway pond, where 1 cm corresponded to approximately 10 L. Evaporation was adjusted for by topping up pond levels with tap water once a day.



A B

Figure 18: Schematic of a raceway pond (**A**) and an external view of a greenhouse (**B**). Image supplied by CSIR Biosciences.

4. ANALYTICAL METHODS

4.1. Quantification of biomass concentration

Optical density (OD) was used as a rapid method to estimate cell number and dry mass. A correlation between OD, dry mass and cell concentration was obtained by preparing a dilution series using a dense culture as the starting material. Each dilution was subjected to an OD reading, biomass quantification by dry weight and a cell number quantification by direct cell counting. Optical density was measured at 683 nm (Shuler and Kargi, 2005) using a DU 800 spectrophotometer.

The culture was diluted to generate an OD value of <1.0, ensuring a linear range of measurement. Dry mass was determined by transferring a 2 mL aliquot into a pre-weighed 2 mL Eppendorf tube and centrifuging for 5 minutes at 12,000 *g*. The supernatant was removed after which the pellet was thoroughly washed in deionised water. The sample was centrifuged again for 5 minutes at 12,000 *g*. The supernatant was discarded and the pellet washed with 0.1 M HCl, followed by a final spin, after which the supernatant was discarded and the pellet dried at 50°C over night. The Eppendorf tube was weighed again after drying to determine the weight of the pellet.

The resulting correlation curves were only set up for WCA 23.2 and *P. tricornutum* and are presented in Figures 19 and 20.

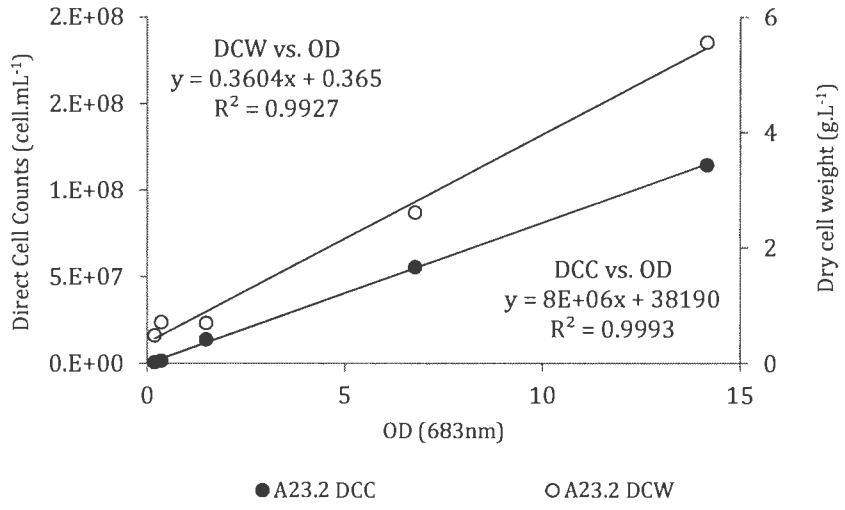


Figure 19: Correlation curves for Isolate WCA23.2. ● represents the relationship between DCC and OD; ○ represents the relationship between DCW and OD.

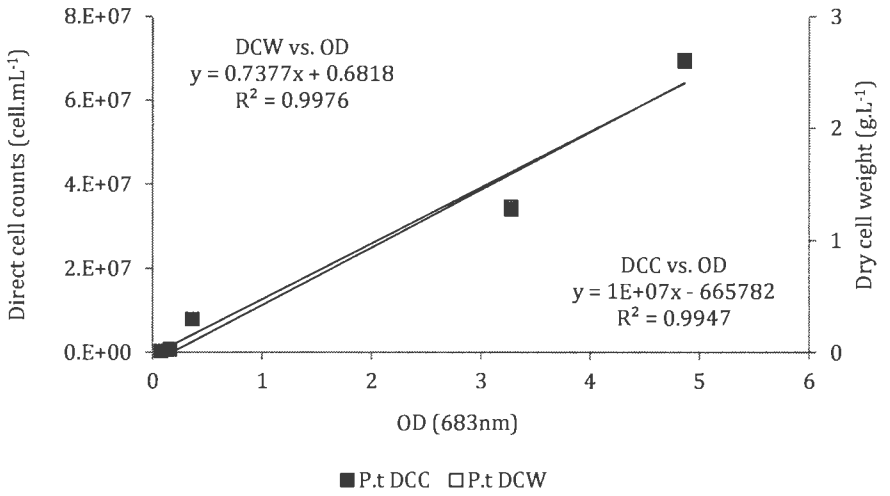


Figure 20: Correlation curves for *P. tricornutum* where ■ represents the relationship between DCC and OD; □ represents the relationship between DCW and OD.

Cell numbers were determined using a Neubauer counting chamber. A 5 mL sample was aseptically removed from the flask. Where cell aggregation was observed, the sample was diluted in 10% ammonium chloride (NH_4Cl) and sonicated using 5-10 pulses at a power setting of 0.5 with a sonicater (Hielscher). The sample was diluted to a maximum of 20 cells per counting block.

4.2. Quantification of media nutrient concentrations

Nutrient concentrations were determined for the following nutrients: nitrate, silicate, phosphate and carbonate. Testing kits from Merck Spectroquant were used to perform the analysis for nitrogen, silicate and phosphate. Carbonate was determined by using a pre-set up titration curve, as presented in Figure 21.

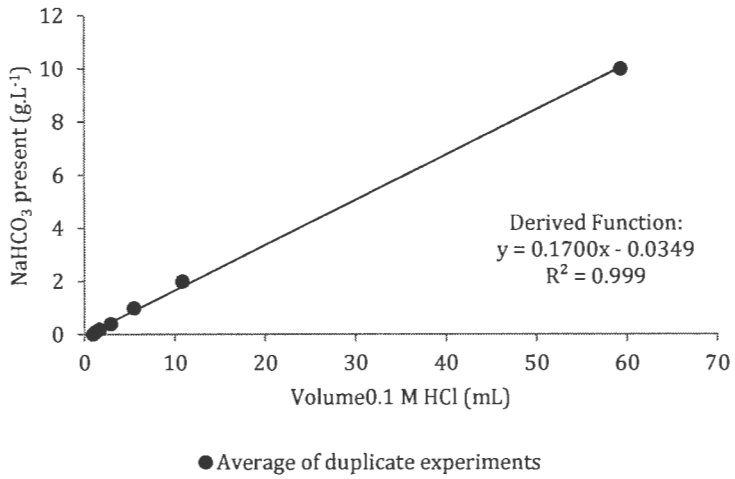


Figure 21: The titration of $NaHCO_3$ to its equivalent point and the derivation of a function to determine carbonate in a given sample.

4.3. Quantification of Omega-3 fatty acid content

The EPA content was measured by direct trans-esterification of lipids and gas chromatography, using centrifuged algal cell pellets. A volume of 15 mL sample was centrifuged and the pellets used for analysis. A two-step process, using a basic catalyst, followed by an acid catalyst converted the lipids to fatty acid methyl esters (FAMEs), as described by Griffiths *et al* (Griffiths *et al.*, 2010). Pellets were mixed with 250 µL toluene and 500 µLNaOH (basic catalyst) in methanol (alkylating agent) and incubated on a shaking heating block at 80°C for 20 minutes. After incubation, the vial was cooled on an icepack for 5 minutes. A volume of 500 µL of the acid catalyst, 5% HCl in methanol, was added to the vial, which was incubated on a shaking heating block at 80°C for 20 minutes. After incubation, the vial was cooled on an icepack for 5 minutes.

The reaction was stopped with distilled water and hexane was added to the vial to extract the FAMEs. The FAME extract was injected into an Agilent 6890N GC equipped with flame ionisation detector (FID) and a Supelco OmegaWax 320 column. Standard split/splitless injection was used with a split of 10 and the injector temperature was set at 280°C. The column temperature was 220°C. Helium (1.24 mL min^{-1}) was used as the carrier gas and the detector temperature was

280°C. Peaks were identified by retention time using a pure *cis*-8,8,11,14,17 EPA standard (Supelco). Peak areas were used to quantify each EPA peak relative to the pure EPA standard.

4.4. Molecular identification

Genomic DNA was extracted from the cultures using the NucleoSpin Soil Genomic DNA extraction kit. Biomass was harvested from approximately 20 mL of each culture by centrifugation (13,000 x *g* for 5 min at 4°C). The harvested biomass was resuspended in the MN lysis buffer and processed according to the manufacturer's instructions, which included a five minute bead beating step. The genomic DNA was eluted from the purification column and spectrophotometrically quantified, before being visually analysed for integrity and purity following electrophoresis on a 0.7% (w/v) TAE agarose gel.

The genomic DNA was diluted to approximately 20 $ng.\mu L^{-1}$ and used as the template in a PCR reaction to amplify the 18S rRNA gene, using a high fidelity DNA polymerase (Kapa Biosystems Hotstart HiFi DNA polymerase). Two sets of 18S rDNA-specific primers, namely Uni18SFwd and Uni18SRev and 18SUni_566Fwd and 18SUni_1200Rev were used. The first set amplified an approximately 1,000 bp portion of the 18S rRNA gene, while 18SUni_566Fwd and 18SUni_1200Rev amplify an approximately 600 bp region, internal to the Uni18SFwd and Uni18SRev amplicon. The PCR amplicons were analysed by electrophoresis on a 0.8% (w/v) TAE agarose gel. The single PCR product was excised from the gel, cloned into pJET1.2/Blunt and transformed into *E. coli* DH5α.

The presence of the expected cloned insert was confirmed by colony PCR, using the vector specific sequencing primers, pJET1.2 Fwd and pJET1.2 Rev, before sequencing of the recombinant pJET1.2/Blunt constructs with pJET1.2 Fwd and pJET1.2 Rev (Inqaba Biotec, Standard Sequencing Service). The sequences were manually edited using Chromas version 2.01 software (Technelysium Pty Ltd., Australia) and analysed using DNAMAN for windows version 4.13 (Lynnon Biosoft, Canada) software.

The sequences amplified using the two primer sets were compared and the consensus sequence subjected to further analysis. Homology and similarity searches of related DNA sequences were performed using the basic local alignment search tool (BLAST) programs (Altschul *et al.*, 1997; Marchler-Bauer *et al.*, 2005) as provided by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analyses were conducted using CLUSTAL X version 2.1 and MEGA version 5 and neighbour-joining trees constructed. Bootstrap values were based upon 1000 re-sampled data sets and only bootstrap values greater than 40% were considered.

5. DATA PROCESSING

Replicate experiments were conducted in all cases and mean values are reported, with the corresponding standard deviation. Table 10 summarises the derived variables used, calculated from the data collected during each study.

Table 10: Derived variables and their units as calculated in each study.

Measurable	Unit	Calculation
Specific growth rate (μ)	d^{-1}	$\mu = \frac{Ln\ cell\#1 - Ln\ cell\#2}{t2 - t1}$ The specific growth rate was calculated between the two points where the slope of the curve had a R^2 value of 0.999 (to obtain the average μ); or just taken as the highest individually plotted value over time to obtain μ_{MAX}. (Qiang *et al.*, 1996)
Biomass productivity (BP)	$cell.mL^{-1}d^{-1}$	Cell concentration ($cell.mL^{-1}$) was plotted against time (over the entire growth period) and the gradient obtained.
Biomass productivity (BP)	$g.L^{-1}\,d^{-1}$	Biomass concentration ($g.L^{-1}$) was plotted against time (over the entire growth period) and the gradient obtained.
Aerial biomass productivity (ABP)	$g.m^{-2}d^{-1}$	$g.L^{-1}d^{-1}$ biomass productivity was converted to $g\,d^{-2}$ and then further converted to $g.m^{-2}\,d^{-1}$ by dividing by pond surface area (0.89 m^2)
Cellular EPA accumulation	$\mu g.cell^{--1}$	Calculated by dividing EPA $\mu g.mL^{-1}$ by cell concentration $cell.mL^{--1}$
Cumulative EPA productivity (C-EP)	$\mu g.d^{-1}$	Cumulative EPA produced was plotted against time (over the entire growth period) and the gradient obtained.
Specific EPA productivity (S-EP)	$\mu g.g^{-1}d^{-1}$	$\frac{EPA\ \mu g}{Biomass\ g}$; plotted against time (over the entire growth period) and the gradient obtained.

6. IMPORTANT FACTORS TO BE CONSIDERED AT THE SCALE OF THIS PROJECT

Artificial illumination was used as the energy source for the bench top laboratory experiments. It must be noted that fluorescent light intensity is much lower than light intensity from the sun. This must be taken into consideration when assessing the outcome of the growth study.

The second phase of this project involved the outside cultivation of the organisms, in a greenhouse. While this mitigated the effect of temperature fluctuations to a degree, light intensity depended on the prevailing weather. Therefore, there was no guarantee that conditions would be similar across

replicate experimental runs. Therefore, sufficient light and temperature readings were necessary to allow the effect of fluctuations in light intensity and temperature on growth to be interpreted.

Contaminating organisms may be found everywhere, even inside the laboratory. It was noted that if a flask was left open inside the algal laboratory, a particular strain of filamentous algae contaminated the flask. This would severely affect the quality of subsequent data. Hence, the culture media was sterilised and flasks kept closed with a cotton wool plug. Measurements were taken as aseptically as possible.

Other precautions included daily disinfection, with 70% ethanol, of all surfaces and instruments used. The laboratory infrastructure (pH controller units, stirrer plates, acid feed bottles and pumps) allowed a maximum of eight flasks to be run at any time, so with four experimental conditions, only duplicate flasks were possible.

7. RESEARCH APPROACH

The overall research approach followed in this study is outlined below. Specific detail on how the individual sets of experiments were performed is provided in the relevant chapters.

The first objective was to select one well characterised species and one locally selected microalgal isolate (not previously characterised) to compare in terms of growth and EPA production. The approach for selecting the well characterised species involved a literature review to compile a list of well characterised species, that had been investigated for omega-3 fatty acid production. The review focussed on 15 species for which substantial published information was available, so while not exhaustive, the most extensively studied species were covered. The species were scored against three selected criteria, relating to biomass and lipid productivity. After the preliminary scoring, four candidate species were selected and ordered from UTEX. Stock cultures were grown up on the prescribed media and then adapted to the artificial salt or freshwater media selected for the study, to eliminate media composition as a variable.

The cultures (commercially available species as ordered from UTEX as well as 3 top performers from CSIR culture collection) were grown over a period of 8 days under nutrient replete conditions. Bench scale vessels used were 4 L flat bottom glass flasks (working volume of 3 L) placed in front of light rigs containing fluorescent lights as the light source. Mixing was achieved by a magnetically operated stirrer bar. Culture pH was monitored and maintained daily at a pH 7.2 via acid addition. A nutrient depleted phase was not included as this would have added another dimension to the selection, complicating the matter further.

The most promising species (both from literature and the local collection) were selected based on the preliminary laboratory tests. Three indigenous isolates were selected for similar evaluation, based on historical CSIR data obtained during the screening for lipid content and growth rate. One

isolate was selected for further study at the end of the preliminary tests. Molecular biology techniques, based on 18S ribosomal RNA gene sequence, were used to confirm the identity of the culture collection species and identify the indigenous isolate to genus level.

The effect of selected environmental factors on growth and EPA production was investigated under similar laboratory conditions, as explained previously, with the exception of the environmental parameters that were tested. Environmental factors collectively consist of chemical (media composition, pH) and physical (mixing, temperature, light, and reactor type) parameters, but only two (nutrients and pH) were selected for investigation. The chemical factors pH and nutrients were chosen as these were parameters that could be controlled even in outdoor conditions where environmental parameters (physical factors) such as temperature and light varied.

The pH study focussed on the effect of controlling the pH at 7.2 versus no control of pH. As and added investigation all experiments were subjected to a period of nutrient deficiency for the last 4-6 days of the study to demonstrate the EPA induction. The flasks were run for nine days under nutrient replete conditions, after which the biomass was recovered by centrifugation and re-suspended in media with no nitrogen and silicate and biomass and EPA content monitored for an additional six days.

The second factor investigated was the nature of nutrient addition, either as a single dose at the start of the experiment or in smaller amounts at regular intervals. The purpose of this study was to investigate whether adding smaller amounts (fed-batch) of the nutrients was better for growth and EPA production. Previous studies conducted at the CSIR suggested fed-batch nutrient addition was beneficial, but this introduces operational challenges at scale, requiring regular analyses of nutrient concentrations.

The final set of experiments were performed to assess the growth and EPA production of the two selected species in an open raceway pond system at a significantly larger scale (50 L) under less controlled conditions (i.e. natural light cycles and temperature). The study was conducted in a greenhouse located on the CSIR premises in Pretoria, South Africa during March and May 2014. The timing of the experimental runs was dictated by the completion of the preceding experiments, rather than to fit in with particular climatic conditions. However, the first set was conducted in late summer and the repeat run in autumn so the number of daylight hours and prevailing temperatures were different and these effects could be assessed. The pond studies were conducted in separate greenhouse cubicles to minimise the risk of cross contamination. Pond volumes were kept to a maximum working volume of 50 L. For the first 13 days the cultures were grown under nutrient replete conditions, after which the nitrogen and silicon sources were removed and the cultures were grown under nutrient deficient conditions for the final 10 days.

Chapter III

CHAPTER III - SELECTION OF EPA PRODUCING SPECIES AND COMPARATIVE TESTING

Research Objective:

Select one commercially available species and one uncharacterised locally selected microalgal isolate from the CSIR culture collection to compare in terms of growth and EPA production.

Research Aims:

1 Select three characteristics to be used to evaluate published data.

2 Use published data and the selected criteria to rank known species in terms of EPA production potential.

3 Select locally sourced isolates based on previous biomass and lipid determinations.

4 Investigate biomass and EPA production of pre-selected culture collection species and local isolates.

5 Confirm the identity of the selected strains using 18S rRNA gene sequence.

1. INTRODUCTION

Chapter III describes the process used to pre-screen the group of culture collection species, identified in Chapter I (Table 2) that have been reported to produce EPA. To do so in an objective way, three desirable traits were chosen from the selection criteria identified in Chapter I (Table 6). The species were ranked in terms of the selected criteria.

The most attractive culture collection species are discussed in terms of EPA production, growth rates and biomass productivities, using published information. Locally selected isolates were chosen based on previous lipid and growth rate determinations. Based on these analyses, shortlisted species were selected for experimental analysis at laboratory scale and the results are presented and discussed.

The top performing. local isolate and culture collection species were identified for further analysis, described in Chapter IV. The identity of the two selected strains was confirmed by 18s rRNA gene sequencing and the results are presented at the end of the chapter.

1.1. SELECTION CRITERIA FOR EVALUATING CANDIDATE SPECIES

The presence of EPA in marine fatty fish is due to their consumption of microalgae, currently the only known *de novo* producers of EPA in the marine food chain (Adarme-Vega *et al.,* 2012; Boelen *et al.,* 2013). The health benefits of EPA have been well documented (Adarme-Vega *et al.,* 2012; Harun *et al.,* 2010; Khozin-Goldberg *et al.,* 2011; Yongmanitchai & Ward , 1989) and the potential for commercial production is widely investigated (Spolaore *et al.,* 2006; Harun *et al.,* 2010; Pulz & Gross , 2004). The prevailing consensus is that current production processes are not economically viable, due to low lipid and/or EPA productivities and/or high downstream process costs (Adarme-Vega *et al.*, 2012).

It is well known that a species of microalgae targeted at lipid production needs to identified taking various aspects into account (e.g. high lipid content, ease of cultivation and harvesting) and needs to be retrofitted for the process and location. Therefore, a multi-faceted approach is required to select a high EPA producing species. Current EPA yields, reported in the literature, range from 1 to 100 $mg.L^{-1}$ for EPA producing species, which are considered too low to make the process economically feasible (Vazhappilly & Chen, 1998; Duong *et al.*, 2012). It is therefore important to identify species that are capable of high EPA productivities and have the potential to overcome existing processing challenges.

A high growth rate is advantageous from a bioprocess perspective. Growth rate is an important factor to consider for various reasons; faster growth ultimately results in a culture that reaches the

product recovery stage more quickly, which allows for a higher process frequency. Faster growth also results in a higher biomass productivity, which increases the yield per harvest volume (Griffiths & Harrison, 2009). A high growth rate can moreover reduce the contamination risk through competitive exclusion. Growth rates reported in the literature range from 0.1 to > 1.5 day^{-1} while biomass productivities range from 0.03 to 0.59 $g.L^{-1}$ day^{-1} under nutrient replete conditions, as calculated by Griffiths and Harrison (2009) .

Most industrial processes are designed to stimulate and optimise the expression of the product of interest. Production of EPA is not only dependent on the correct choice of species and metabolic conversions, but also the growth conditions (Adarme-Vega *et al.*, 2012). The lipid class and fatty acid compositions of microalgal cells can differ significantly and change with variations in culture conditions, such as nutrient status, temperature, salinity, pH, photoperiod, light intensity and light quality (Yongmanitchai & Ward, 1989; Dunstan *et al.*, 1993; Sharma *et al.*, 2012). The accumulation of lipid is considered an adaptive mechanism in response to severe conditions and is only possible as long as there is active photosynthetic carbon fixation (Adarme-Vega *et al.*, 2012; Thompson, 1996; Sharma *et al.*, 2012).

It is clear that possible candidates for commercial EPA production require high growth rates, EPA productivities, and evidence of EPA induction. The three major 'desirable traits' that were used as selection criteria are therefore:

1. High growth rates
2. High EPA content along with EPA productivity and
3. Ability to induce EPA production

The next section ranks the identified culture collection species according to how well they meet the selection criteria. In order to simplify the pre-screening, a points-based system was developed. The point allocations were set up using information and values gathered from the literature and is summarised in Table 11. Where an organism fell in both categories (for example, EPA content ranging from 20% to 30±%, both marks were allocated and the average taken, and will be indicated as **/***).

Table 11: Points based system used to score potential isolates during pre-screening.

Point Allocation	*	**	***
1. growth rate	<0.2 day^{-1}	0.2-1 day^{-1}	>1 day^{-1}
2. EPA content	<20% of total FA	20-30% of total FA	>30% of total FA
3. EPA production induction	<1 fold	1-2 fold	>3 fold

1.2. PRE-SCREENING OF CANDIDATE SPECIES BASED ON PUBLISHED DATA

There are an estimated 72 500 species of algae of which, 33 248 names have been processed and classified by the AlgaeBase (Guiry, 2012; Guiry & Guiry, 2014). The database does not specifically identify EPA producing strains, but rather the lipid content (typically 10-30%, but up to 70%) per dried mass of the various species (Ward & Singh , 2005). A wider consideration of published literature identified a number of organisms capable of producing EPA (Chapter I).

The results of the pre-screening exercise, ranking species for which several published studies are available, are presented in Table 12. This is by no means an exhaustive list of EPA producing microalgae. In addition, only studies that focussed on photoautotrophic production, in flask studies were considered. The reason for this approach is that the initial laboratory tests will be conducted in flasks in order to keep the testing conditions as similar as possible to literature studies, thus facilitating direct comparisons. The organisms listed below are ranked from highest to lowest scoring.

Table 12: Results of initial pre-screen to identify possible culture collection species. References used are listed in Table 2.

Microalgal specie	Abbreviation	Growth rate	EPA content	EPA fold increase	Total
Chlorella minutissima	C. m	**/***	***	**	7.5
Monodus subterraneus	M. s	***	***	*	7.0
Skeletonema costatum	S. c	***	***	*	7.0
Nannochloropsis oculata	N. o	**	**/***	**	6.5
Phaeodactylum tricornutum	P. t	**/***	**/***	*/**	6.5
Porphyridium cruentum	P. c	***	**/***	*	6.5
Dunaliella salina	D. s	***	**	*	6.0
Pavlova lutheri	P. l	**/***	*/***	*	5.5
Isochrysis galbana	I. g	**	*/**	*	4.5

Based on the overall ranking, the most promising potential candidates were *Chlorella minutissima*, *Monodus subterraneus* and *Skeletonema costatum*, followed by *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Porphyridium cruentum*. The species with the lowest scores were *Pavlova lutheri*, *Dunaliella salina* and *Isochrysis galbana* and were consequently not considered further.

Due to the relatively close scores, all top scoring six species could have been considered as potential candidates for further experimental analysis. However, some of the published information had not

been acquired at the time when candidate species were ordered from the UTEX culture collection. The potential of *Skeletonema costatum* only became apparent after the initial laboratory studies had been conducted, so could not be experimentally assessed. Similarly, *Monodus subterraneus* was not available from UTEX when the study commenced, so was therefore excluded from the list.

The cultures that were experimentally assessed were therefore *Chlorella minutissima, Nannochloropsis oculata, Phaeodactylum tricornutum* and *Porphyridium cruentum.* Micrographs of these organisms are presented in Figure 22.

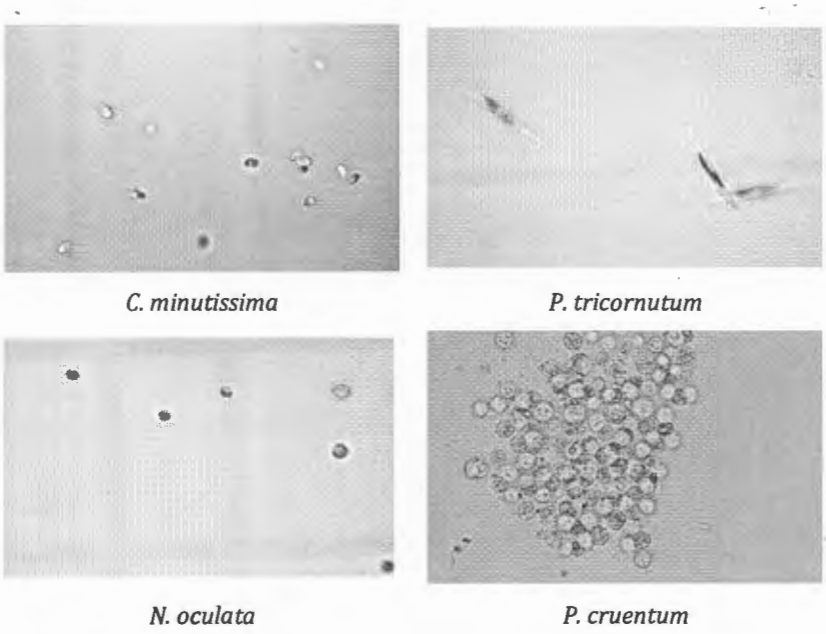


Figure 22: Micrographs of selected culture collection species. Images supplied by CSIR Biosciences.

This section presented a summary of relevant data, based on the three traits identified and discussed in the previous section. The final selection of the species for experimental evaluation was based on this comparison. A summary of published data on growth rate, biomass and EPA productivity is presented in Figure 23.

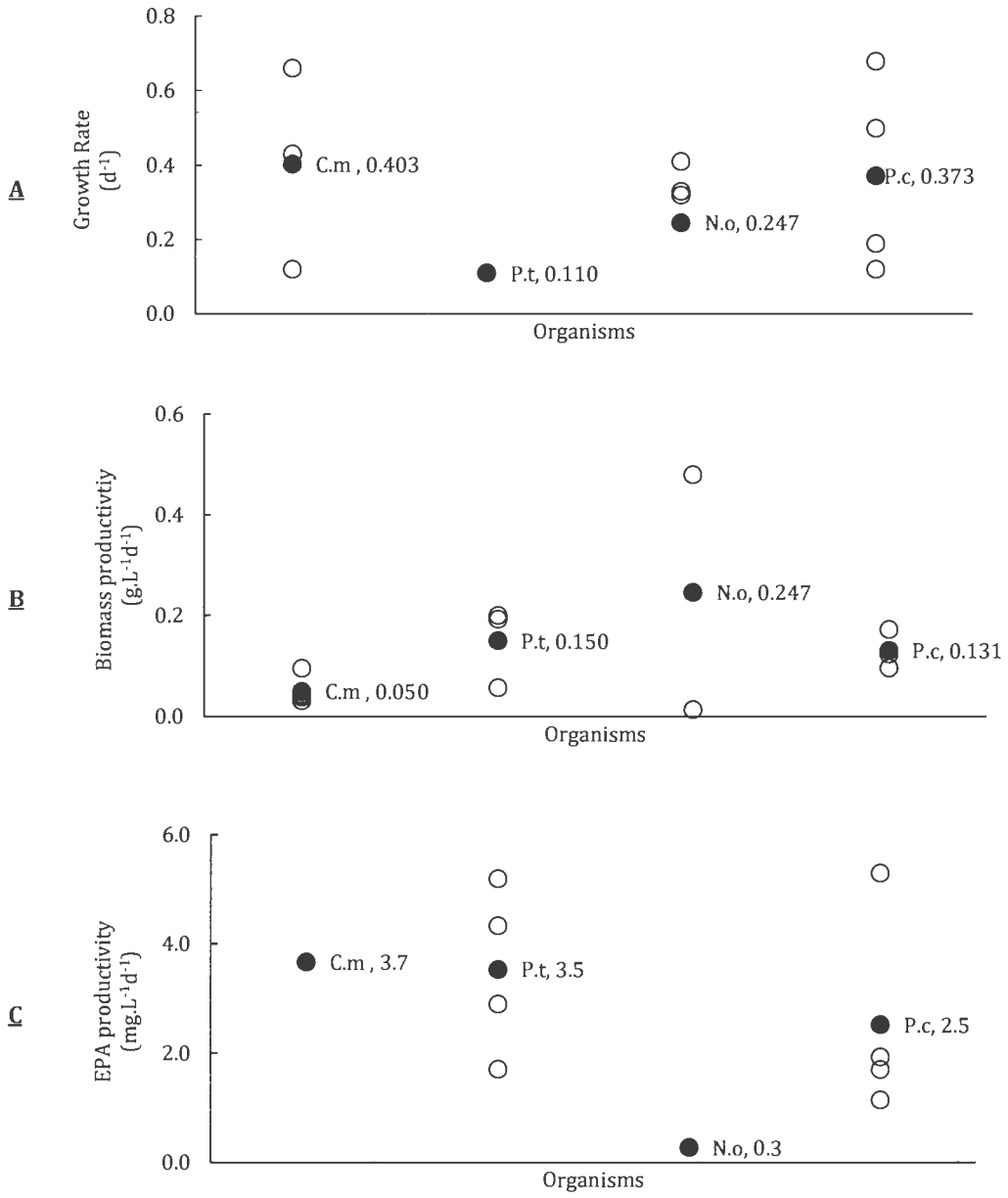


Figure 23: Published data for (A) growth rate, (B) biomass productivity and (C) EPA productivity for the pre-selected species. ○ represent data from individual studies, while ● represent the means of those data.

The variation between the values for each species can be attributed to difference in growth conditions, nutrient concentrations and light intensities. Only photoautotrophic cultures, cultivated in glass photo bioreactors were considered. Data for EPA production under heterotrophic conditions were not considered. For the sake of simplicity, the data are represented in comparative graphs (Figure 23A-C). A more complete summary of the values used and different culture conditions can be found in Appendix A. The mean values from Figure 23 are summarised in Table

14. Specific, or cellular, EPA productivity data was not typically reported, so an attempt was made to calculate the value(s). Specific EPA productivity provides a measure of the accumulation of EPA per unit biomass over the growth period. This is an important property, as average EPA productivity only refers to EPA accumulation over time and not EPA accumulation per cell or unit biomass. For the purpose of this study, specific EPA productivity was calculated as follow:

$$Specific\ EPA\ productivity\ (mg.g.^{-1}) = \frac{EPA\ productivity\ mg\ L^{-1}day^{-1}}{Biomass\ productivity\ g\ L^{-1}day^{-1}}$$

In Table 13, column[a] (Biomass Productivity) and column[b] (EPA Productivity) represent the average biomass and EPA productivities as calculated from Figure 23. Column[c1] (Specific EPA Productivity) represents the calculated cellular EPA productivity value from column[a,b] (no SD value available because cellular EPA value was calculated from the averages). Column[c2] (Specific EPA Productivity) also represents the calculated specific EPA productivity value – but from individual published articles were both biomass and EPA productivity values were reported. The reason for these double calculations was to compare the average values (Column[c1]) to actual values (Column[c2]).

Table 13: Summary of the comparative data, based on published data. All data represent mean values (except column c2).

Organism	μ d^{-1}	[a]BP $g.L^{-1}d^{-1}$	[b]EPA-P $mg.L^{-1}d^{-1}$	[c1]S-EPA-P $mg.g^{-1}$	[c2]S-EPA-P $mg.g^{-1}$
C. m	0.403 (SD± 0.27)	0.050 (SD± 0.03)	3.7	0.074	0.037 (Vazhappilly, 1998)
P. t	0.110	0.150 (SD± 0.08)	3.5 (SD± 1.55)	0.023	0.022 (Vazhappilly, 1998) 0.030 (Yongmanitchai, 1992) 0.015 (Yongmanitchai, 1992)
N. o	0.247 (SD± 0.12)	0.247 (SD± 0.36)	0.3	0.001	0.006 (Vazhappilly, 1998)
P. c	0.373 (SD± 0.26)	0.130 (SD± 0.13)	2.5 (SD± 1.88)	0.019	0.018 (Vazhappilly, 1998) 0.010 (Akimoto, 1998) 0.012 (Akimoto, 1998)

[c1]Calculated from literature averages in column[a] and column[b]

[c2]Calculated from articles that reported both biomass and EPA productivity

The discussion above compared two of the three selected criteria, namely growth rate and EPA productivity. While a qualitative assessment was made during the pre-screening, the range of methods used to induce EPA production was wide and the degree of success variable. As such, a quantitative comparison was of limited value. Furthermore, the decision was influenced by the overall objective of this study, to assess the potential for scale-up to an open pond system. As such, temperature and light intensity, which have been used to influence EPA production at laboratory scale, were excluded as control variables.

Due to variability in published data on growth rates and EPA productivities, as well as difficulty in quantifying induction of EPA production, it was decided to evaluate all 4 species experimentally.

All cultures were obtained from the culture collection of University of Texas (UTEX) and their respective UTEX identification numbers and original source location are presented in Table 14.

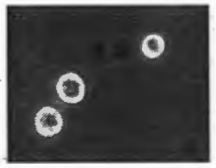
Table 14: Culture collection species as ordered from UTEX including identification numbers and original source locations.

Organism	UTEX ID	Origin
C. minutissima	UTEX 2219	Lake Pupuke, Auckland, New Zealand
P. tricornutum	UTEX L642	Plymouth, England
N. oculata	UTEX 2164	Isle of Cumbrae, Scotland
P. cruentum	UTEX 161	Basel, Switzerland

1.3. Selection of CSIR Isolates

The CSIR is host to a library containing over 750 isolates obtained by sampling across South Africa. A screening method, using flow cytometry, was developed in order to rapidly screen for lipid rich isolates, although complete physiological characterisation has not yet been concluded. Three isolates were selected, based on previous growth studies and rapid lipid screens (data not shown) and are presented in Table 15 (Dickson *et al.*, 2010). The organisms selected were done on the basis of lipid concentration as this is a rapid available method and the assumption that a high lipid content is a good starting point to look for EPA producers.

Table 15: South African isolates and their sampled locations, micrographs and species type.

Sample site	Designation	Micrograph	Description
Western Cape Region A Isolate 23.2	"WCA 23.2"		Unicellular brown diatom. Marine species
Western Cape Region A Isolate 27.2	"WCA 27.2"		Unicellular brown microalgae. Marine species
Western Cape Region C Isolate 39.3	"WCC 39.3"		Unicellular green microalgae. Fresh water species

2. Materials and Methods

The cultures obtained from UTEX were handled and stored according to the instructions. Each culture was initially grown on the UTEX recommended media, before switching over to the universal media used throughout this work. Culture collection species and CSIR isolates were grown on either ASW or AF6 media for a period of 7 days under nutrient replete conditions, using the standard experimental configuration described in Chapter II.

Nutrients were added in excess and concentrations were measured every two days. The pH was controlled at pH 7.2, by daily adjustment with 32% HCl ($m.v^{-1}$). Temperature was maintained at 23°C ± 2°C and light was supplied by 10 Sylvania GroLux lamps with an intensity of 140 $\mu mol.m^{-2}s^{-1}$ at the reactor's surface. Samples were taken daily and analysed for cell number, nutrient concentration and EPA content.

3. Results and Discussion

All species were able to grow on the universal media, although *P. tricornutum* repeatedly failed to grow in a stirred vessel, so the culture was transferred to a shaking incubator, where growth occurred. *P. cruentum* could not be scaled up beyond a volume of 100 mL, possibly due to the sensitivity of the organism to shear stress, as described by (Sobczuk *et al.*, 2006), where cellular damage was observed at certain agitation speeds. Due to the challenges in culturing of the *P. cruentum* and larger scale process implications for a sheer sensitive organism, this species was not pursued further.

Ultimately, a total of six species were experimentally evaluated. These were grouped into the culture collection species, *C. minutissima, P. tricornutum, N. oculata* and the CSIR isolates, WCA 23.2, WCA 27.2 and WCC 39.3. The two groups were considered separately, in terms of growth and EPA production, to facilitate the selection of the most attractive species from each group.

3.1. Growth characteristics of selected species

The resulting growth curves for the culture collection species (Figure 24A) and CSIR isolates (Figure 24B) are presented below. All species followed a characteristic growth curve, showing a lag, exponential, linear and in some cases, stationary phase, although the transition between phases was clearer in some cases. All data, for each group, are shown on one axis, in order to demonstrate the increase in cell number for organisms relative to each other. *N. oculata* and WCC 39.3 achieved the highest cell numbers in each group.

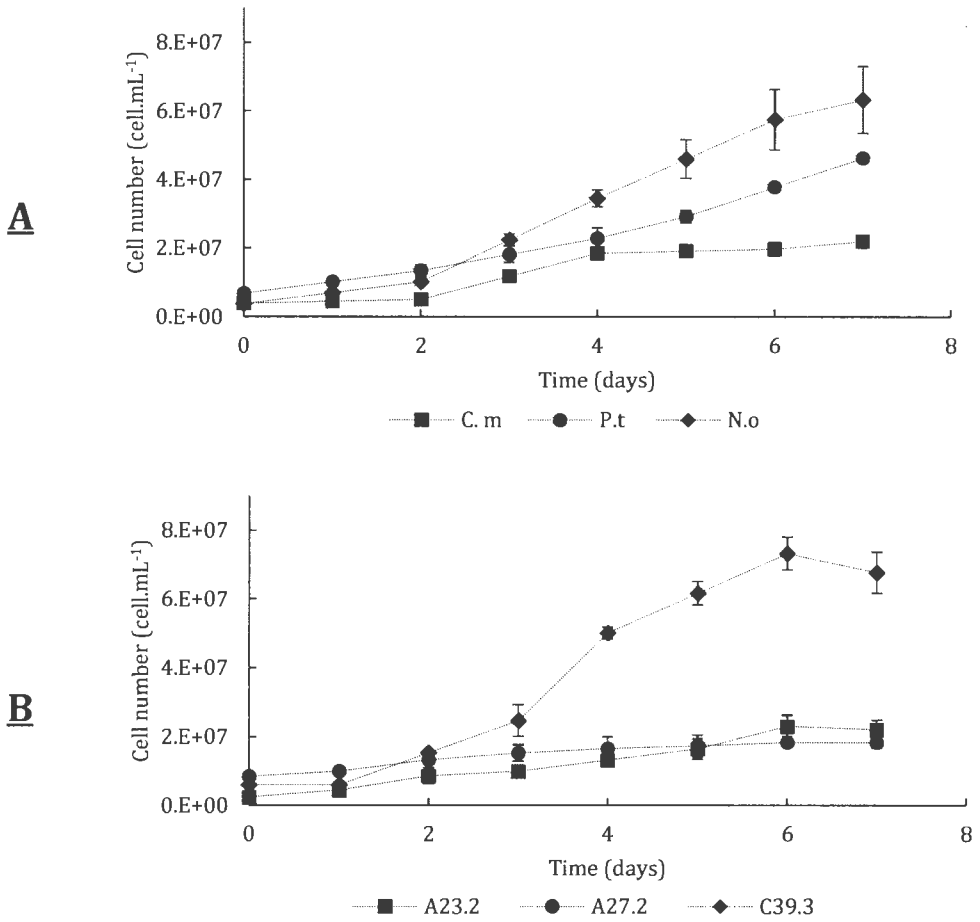


Figure 24: Growth curves of (**A**) culture collection species *C. minutissima, P. tricornutum* and *N. oculata* and (**B**) CSIR isolates WCA 23.2, WCA 27.2 and WCC 39.3. Each curve is the result of two replicates.

Microscopic evaluation of *N. oculata* and WCC 39.3 revealed that these organisms were much smaller (<5 μM in length) than the other members of their group (all 5-10+ μM in length). The observation suggests that smaller cells were capable of faster cell division, due to a higher cell number reached. This hypothesis was supported by work conducted by Geider *et al.* (1986), which discussed cell size and variability in maximum growth rate. The rates of metabolic processes and maximum growth rate have a size dependence that can be described by an equation taking into account the weight of the cell.

$$\text{metabolic rate}/\ \mu_m = aW^b$$

where "metabolic rate" is the specific rate of some metabolic process ($time^{-1}$) and "μ_m" is the maximum growth rate; "W" is the measure of organism mass and "a" and "b" are coefficients (Geider *et al.*, 1986; Peters, 1983). When metabolic rates are expressed using this equation, the finding is that specific metabolic rates decrease with increasing cell size. The idea is supported by

the observation that small phytoplankton are characterised by higher maximum rates of nutrient uptake than their larger counter parts (Banse, 1982; Banse, 1976).

To assess the validity of the hypothesis to the current study, a graph of cell size vs. average growth rate (Ln of cell.mL^{-1}) during the linear phase was plotted and is presented in Figure 25. The smaller cells had higher linear growth rates when compared to the larger cells, consistent with the work of Geider *et al*, supporting the hypothesis.

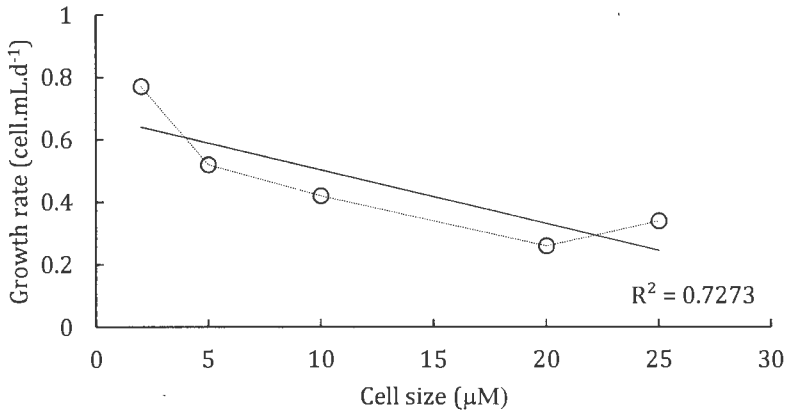


Figure 25: Correlation between linear growth rate and cell size.

The notion that the smaller cells are faster growers are most likely related to the fact that smaller cells have a bigger surface area to volume ratio given that transport of nutrients occurs over the entire cell surface. A greater surface area to volume ratio ultimately makes the nutrient uptake process more efficient.

Specific growth (µ) rate was investigated as it takes into account the cell concentration at the start of each measured interval and provides a more valuable means of comparison. This is particularly important during the initial phase of growth, where the culture is most likely to be in exponential phase. The specific growth rate for each organism is plotted in Figure 26. A general trend was observed, with all species reaching a maximum specific growth rate observed between Day 1-3, after which the specific growth rate dropped and continued to decrease towards the end of the growth period.

Calculated maximum specific growth rates (Figure 26) for the culture collection species were as follow: 0.85 (SD±0.03) d^{-1}, 0.40 (SD±0.04) d^{-1} and 0.79 (SD±0.02) d^{-1}, for *C. minutissima*, *P. tricornutum* and *N. oculata* respectively. Calculated maximum specific growth rates for the CSIR isolates species were as follows: 0.64 (SD±0.20) d^{-1}, 0.30 (SD±0.10) d^{-1} and 1.60 (SD±0.20) d^{-1}, for WCA 23.2, WCA 27.2 and WCC 39.3 respectively.

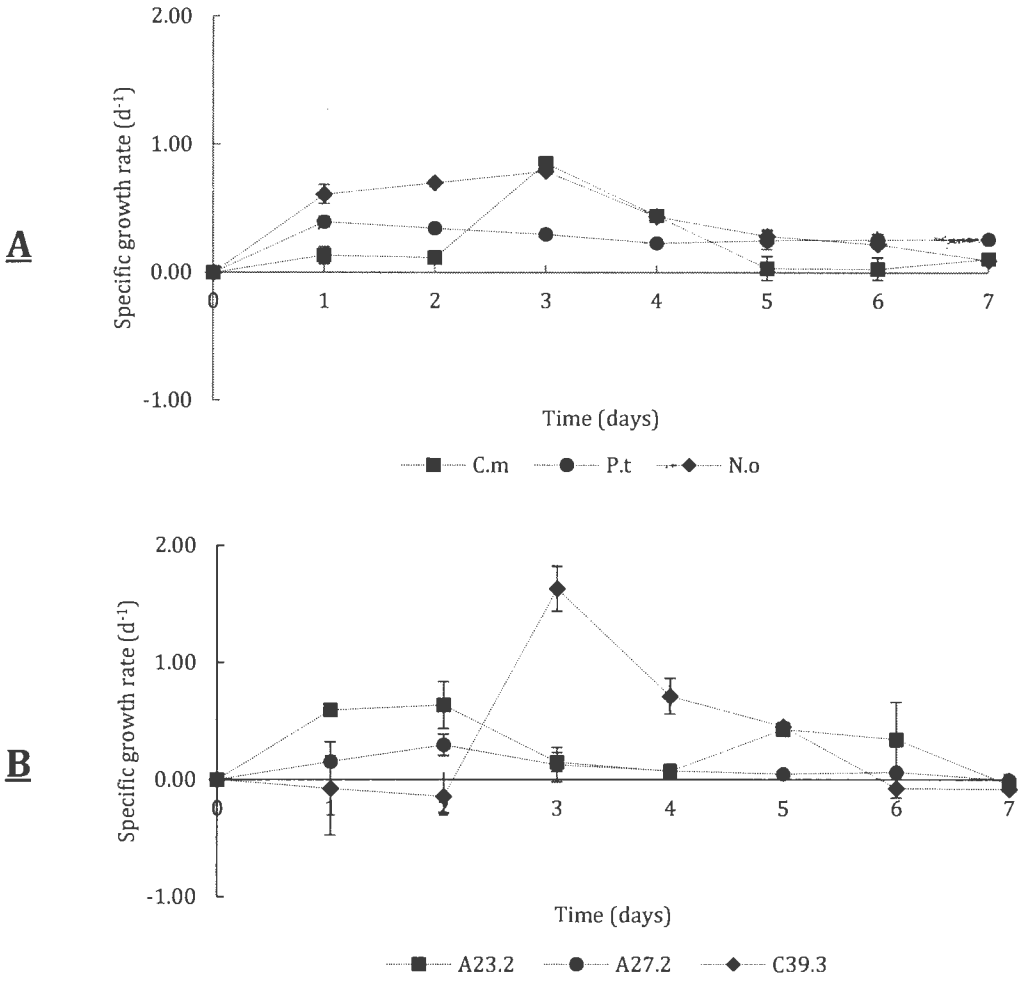


Figure 26: Specific growth rate of (**A**) culture collection species *C. minutissima, P. tricornutum* and *N. oculata* and (**B**) CSIR isolates WCA 23.2, WCA 27.2 and WCC 39.3. Data points represent the mean of two replicates.

From the curve, the species are most likely growing exponentially on Day 0-3 (hence the increase in specific growth rate) followed by a long linear growth phase (represented by the drop/decline in specific growth rate for the duration of the growth course). The drop/decline in specific growth rate indicates that one/or more of the growth factors have become limiting and is so preventing continued exponential growth. As nutrients were still in excess at this point in time, the limiting factor was possibly light.

3.2. EPA ACCUMULATION IN CULTURE SPECIES

Cellular EPA accumulation for culture collection species and CSIR isolates are presented in Figure 27. Cellular EPA accumulation was calculated as stated in Chapter II. It is evident that *C. minutissima* and WCC 39.3 produced very little EPA. Further investigation revealed that both

marine and freshwater strains of *C. minutissima* exist and the strain ordered from UTEX was the fresh water strain (UTEX ID 2219) and not the marine strain (UTEX ID 2349). The literature values used to select the organism during pre-screening was exclusively for the marine strain. A review by Vazhappilly (1998), confirmed the total EPA production for *C. minutissima* UTEX 2219 (as a percentage of total fatty acid production) was set at less than 5% (Vazhappilly & Chen, 1998). The difference only became apparent at the end of the first phase of experiments. At this stage, there was insufficient time to repeat the experiments with the marine strain without compromising the overall project timelines, so *C. minutissima* was not considered for the next phase.

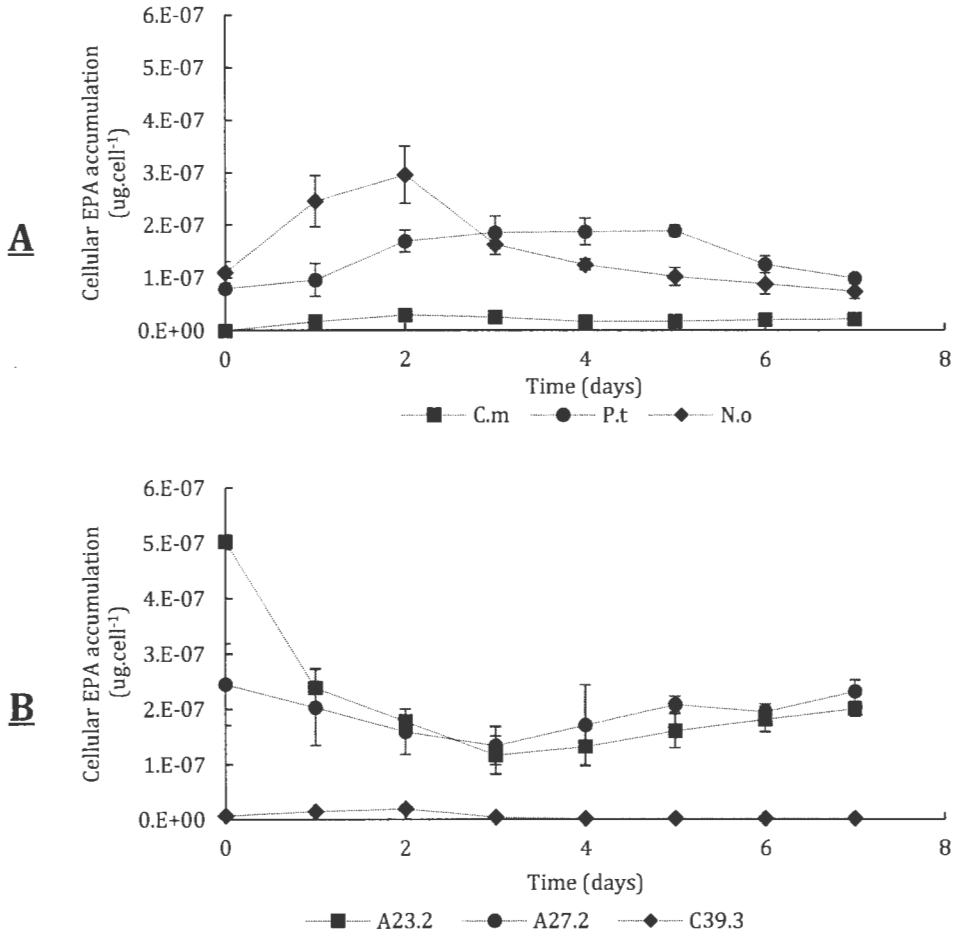


Figure 27: Cellular EPA produced by (**A**) culture collection species *C. minutissima, P. tricornutum* and *N. oculata* and (**B**) CSIR isolates WCA 23.2, WCA 27.2 and WCC 39.3. Each curve is the result of two replicates.

Although cellular EPA production by WCC 39.9 was low, additional GC analysis indicated the capability of producing high amounts of another omega-3 fatty acid, ALA, up to 32% of total fatty acids (data not shown). Despite the efficient production of ALA, WCC 39.9 was not considered for further study. This species, with a maximum specific growth rate of 4.2 day^{-1} is an excellent candidate to consider for the production of ALA and will be investigated further in a future study.

The remaining culture collection species, *P. tricornutum* and *N. oculata*, had similar EPA producing capabilities.

The data presented in Figure 27A are similar to trends reported in the literature. Initially, cellular EPA increased for both *P. tricornutum* and *N. oculata*. *P. tricornutum* continued to maintain its cellular EPA content into late growth phase after which it stopped. A similar trend was observed for *N. oculata*, except for the fact that cellular EPA production ceased significantly sooner after which it steadily declined. These trends are counter-intuitive; EPA is considered to be a secondary metabolite, so one would expect the EPA content to decrease (or stay the same) during the exponential growth phase, where the focus is on cell growth rather than lipid production. Okauchi (1990), however, confirmed that EPA production in *N. oculata* can occur during the linear or exponential phase in addition to the stationary phase (Okauchi *et al.*, 1990). Tonon (2002) reported a similar finding for *P. tricornutum* and indicated that more biochemical analysis was needed to fully understand whether EPA production is a time and/or species dependent process (Tonon *et al.*, 2002).

For species WCA 23.2 and WCA 27.2 the opposite trend was observed; cellular EPA did not increase until later in the growth phase after which it continued to increase. This is a more expected trend as all metabolic processes are directed towards growth, a primary process, and not towards secondary metabolite production as observed for the culture collection species.

3.3. SELECTION OF SPECIES FOR FURTHER ANALYSIS

The primary objective of this Chapter was to compare the performance of the most promising indigenous isolate to that of a well characterised species from the culture collection. The research presented in this Chapter was generated in order to inform the decision. The decision to evaluate only two species was based on available time and infrastructure. Demonstrating reproducibility at the pilot scale was considered more important than assessing a greater number of candidate species. A summary of the growth characteristics obtained in the experimental trials is presented in Table 16. The data allowed the organisms to be ranked in terms of maximum and average specific growth rate and cumulative EPA productivity. *C. minutissima* and WCC 39.3 were incapable of producing significant EPA, so these organisms were not considered for further experimental evaluation.

Table 16: Summary of maximum and average specific growth rates, cellular and cumulative EPA productivities* of selected organisms.

	Maximum μ	Average μ	Cumulative EPA productivity
	d^{-1}	d^{-1}	$\mu g.d^{-1}$
C. m	0.85 (SD±0.03)	0.22 (SD±0.00)	0.98 (SD±0.00)
P. t	0.40 (SD± 0.04)	0.24 (SD± 0.00)	10.85 (SD± 1.58)
N. o	0.79 (SD± 0.02)	0.35 (SD± 0.01)	9.21 (SD± 0.41)
WC A23.2	0.64 (SD± 0.20)	0.20 (SD± 0.00)	7.83 (SD± 0.32)
WC A27.2	0.30 (SD±0.10)	0.10 (SD±0.00)	5.34 (SD± 1.65)
WC C39.3	1.60 (SD± 0.30)	0.30 (SD± 0.00)	0.18 (SD± 0.15)

*Cumulative values used instead of cellular EPA accumulations (resulted in negative productivity values)

P. tricornutum had a lower maximum and average specific growth rate than *N. oculata,* but a higher EPA productivity. The data presented for *N. oculata* is a culmination of numerous failed attempts to culture this organism; this pointed to the susceptibility to prolonged exposure to outdoor conditions and was therefore excluded based on its perceived inability to be cultured in larger outdoor raceways. The EPA accumulation during the nutrient sufficient phase in combination with good growth was considered a more important parameter than fast growth, so *P. tricornutum* was selected instead of *N. oculata* for further study. WCA 23.2 had a higher maximum and average specific growth rate, as well as cumulative EPA productivity than WCA 27.2, making the selection more straightforward.

3.4. Molecular Identification

Cultures obtained from culture collections may occasionally be contaminated or the species may have been incorrectly classified, particularly since classification was historically based on physical characteristics, rather than DNA sequence analysis. Therefore, the decision was taken to use 18S ribosomal RNA gene sequence information data to confirm the identification of *P. tricornutum* and attempt to classify CSIR isolate WCA 23.2. DNA was successfully extracted from both cultures. The data are summarised in Table 17 below.

Table 17: Quantitation and quality control values for genomic DNA extracted from the diatom cultures.

Sample	Concentration ($ng.\mu L^{-1}$)	260/280	260/230
P. tricornutum	280.6	1.91	2.10
WCA 23-2	367.2	191	2.07

The extracted DNA was run on an agarose gel (Figure 28) to confirm the presence of genomic DNA and the fact that the DNA had not been degraded.

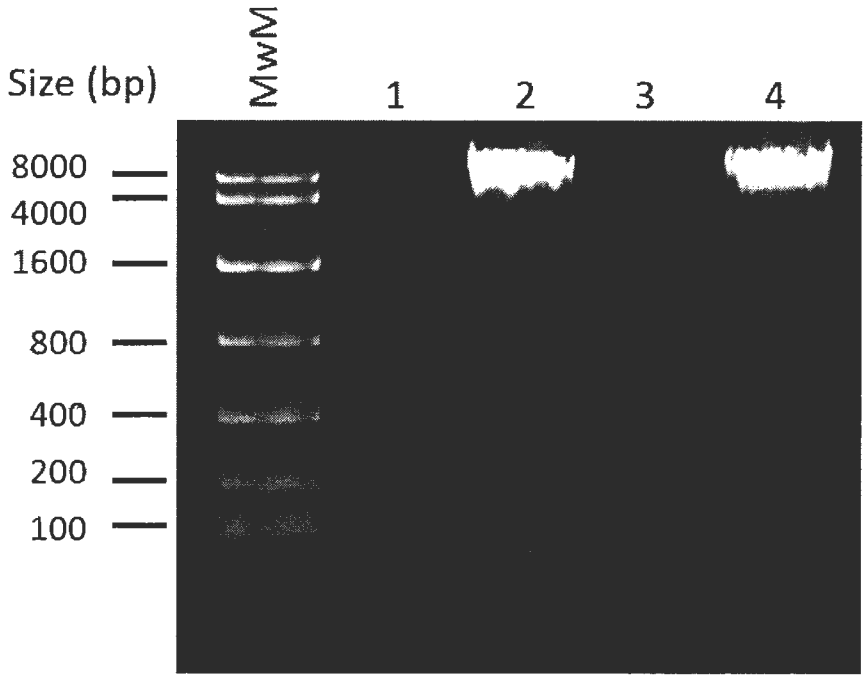


Figure 28: Agarose gel of total DNA extracted from the *P. tricornutum* (lane 2) and WCA 23-2 (lane 4) diatom cultures. Lanes 1 and 3 are empty. The molecular weight marker (MwM) is the Kapa Biosystems Express ladder and the sizes are as indicated.

The extracted DNA was used as a template for polymerase chain reaction using two different primer sets. The PCR products were run on an agarose gel (Figure 28) to confirm that the amplicons were the correct size. Both primer sets produced single bands of the correct size, confirming successful and specific amplification.

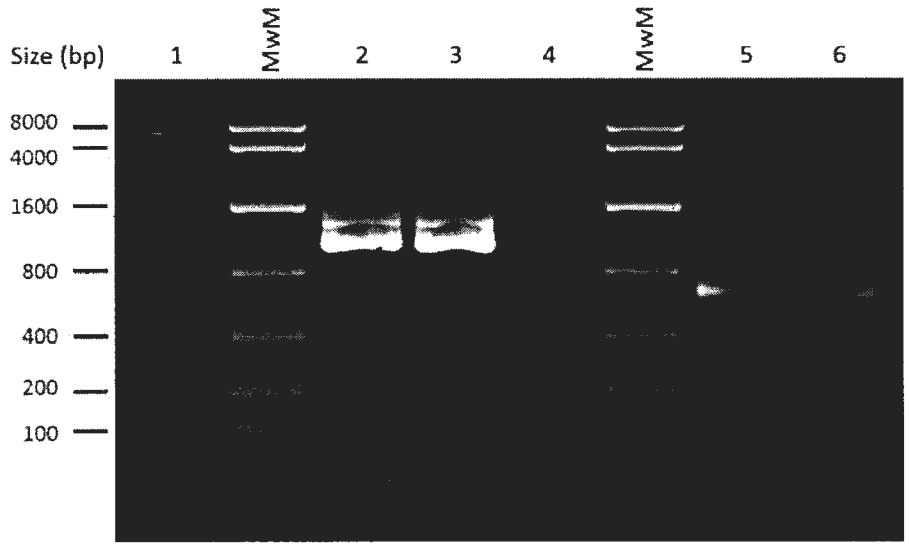


Figure 29: Agarose gel of the PCR amplification of the 18S rRNA gene(s) from genomic DNA extracted from the *P. tricornutum* and WCA 23-2 cultures. Lane 1, Uni18S No template control (NTC); lane 2, Uni18SFwd and Uni18S Rev amplified *P. tricornutum*; lane 3, Uni18SFwd and Uni18S Rev amplified WCA 23-2; lane 4, 18S NTC; lane 5, 18SUni_566Fwd and 18SUni_1200Rev amplified *P. tricornutum*; and lane 6, 18SUni_566Fwd and 18SUni_1200Rev amplified WCA 23-2. The molecular weight marker (MwM) is the Kapa Biosystems Express ladder and the sizes are as indicated.

The amplified gene fragments were cloned into a vector and sequenced. The sequence information was compared to available sequences on the NCBI database. On the basis of the amplified and sequenced 18S rRNA gene, the *P. tricornutum* culture grouped together with other *Phaeodactylum tricornutum* strains (Figure 29). In addition, a second 18S rRNA sequence was obtained from within the *P. tricornutum* culture, indicating the presence of an additional eukaryotic organism. The second sequence was closely related to *Bodo saltans,* a free-living non-parasitic species of kinetoplastid flagellate protozoan and did not represent an algal contaminant.

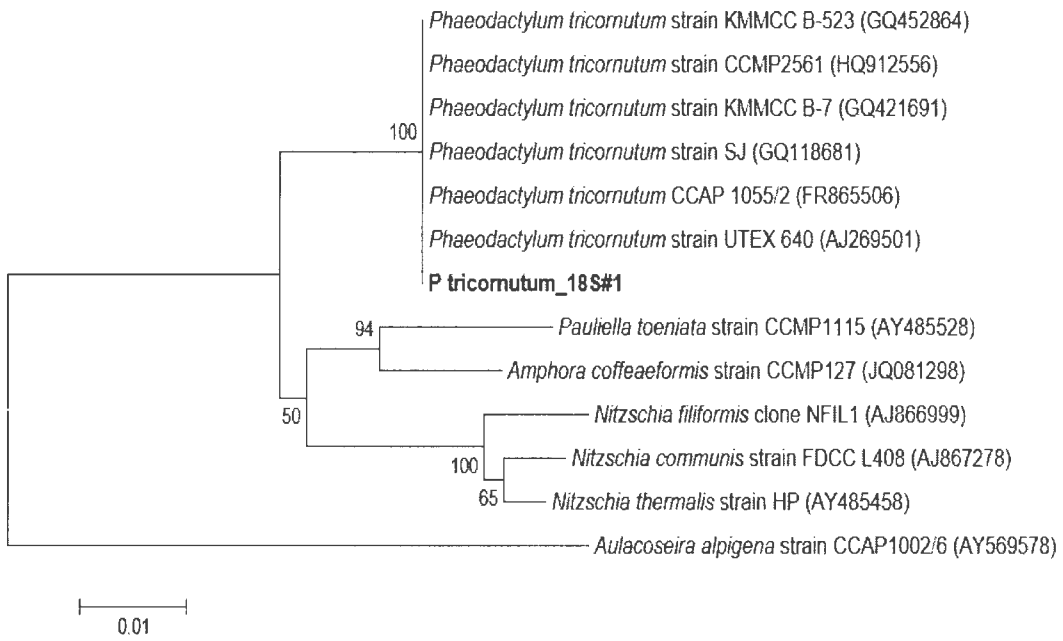


Figure 30: Unrooted phylogenetic tree of 18S rRNA clone (P tricornutum_18S#1) identified from the *P. tricornutum* diatom culture (highlighted in bold) and related sequences, based on the sequence alignment of a common length portion (970 nucleotides). The tree was constructed using the neighbour joining method. Bootstrap values are based upon 1,000 resampled data sets and only values of greater than 40% are indicated. *Aulacoseira alpigena* was included as an outlier. Accession numbers (where available) are shown in brackets. The bar represents 0.01 base substitutions per site.

The WCA 23-2 isolate culture also contained two eukaryotic sequences. The first, WCA 23-2_18S#1, was closely related to diatoms classified as *Amphora* species and those from division *Bacillariophyta* (Figure 30), while the second sequence, WCA 23-2_18S#2 was related to an *Aplanochytrium* species of unicellular protists.

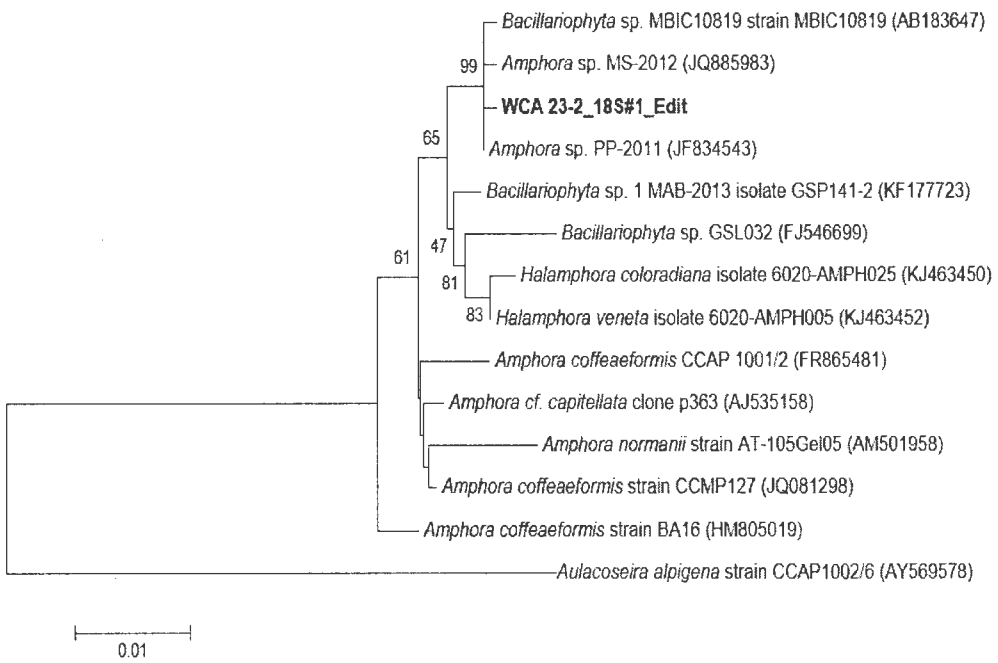


Figure 31: Unrooted phylogenetic tree of 18S rRNA clone (WCA 23-2_18S#1_Edit) identified from the WCA 23-2 diatom culture (highlighted in bold) and related sequences, based on the sequence alignment of a common length portion (970 nucleotides). The tree was constructed using the neighbour joining method. Bootstrap values are based upon 1,000 resampled data sets and only values of greater than 40% are indicated. *Aulacoseira alpigena* was included as an outlier. Accession numbers (where available) are shown in brackets. The bar represents 0.01 base substitutions per site.

The genetic characterisation confirmed the identity of the *P. tricornutum* culture, while the CSIR isolate most likely represents a member of the genus *Amphora* (Figure 31).

4. SUMMARY

The research objective for this chapter was to select one well characterised species, available from a culture collection and one locally selected microalgal isolate to compare in terms of growth and EPA production.

Published data were used to assess candidate species according to the selected criteria and assign a cumulative score to each species. The most promising potential candidates were *Chlorella minutissima*, *Monodus subterraneus* and *Skeletonema costatum*, followed by *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Porphyridium cruentum*. Due to unforeseen circumstances, the final cultures that were selected for further experimental analysis were *C. minutissima*, *N. oculata*, *P. tricornutum* and *P. cruentum*. Three CSIR isolates were selected based on previous growth studies and rapid lipid screens and included WCA 23.2, WCA 27.2 and WCC 39.3.

P. tricornutum had a lower maximum and average specific growth rate (0.40 d^{-1} and 0.24 d^{-1} respectively) than *N. oculata* (0.79 d^{-1} and 0.35 d^{-1} respectively), but a higher cumulative EPA productivity (10.85 $\mu g.d^{-1}$ vs. 9.21 $\mu g.d^{-1}$). The EPA accumulation was considered a more important parameter than fast growth, so *P. tricornutum* was selected instead of *N. oculata* for further study. WCA 23.2 had a higher maximum and average growth rate and cumulative EPA productivity (0.64 d^{-1}, 0.27 d^{-1} and 7.83 $\mu g.d^{-1}$ respectively) than WCA 27.2 (0.30 d^{-1} and 0.10 d^{-1} and 5.34 $\mu g.d^{-1}$ respectively), making the selection of WCA 23.2 straightforward.

The 16S rRNA gene sequencing confirmed the identity of the *P. tricornutum* culture, while the CSIR isolate was identified as a member of the genus *Amphora*.

CHAPTER IV

CHAPTER IV – INVESTIGATING THE IMPACT OF ENVIRONMENTAL FACTORS ON BIOMASS AND EPA PRODUCTION

Research Objective:

Compare the effect of selected environmental factors on growth rate and EPA production under laboratory conditions in two chosen species.

Research Questions:

1 Does pH control improve biomass and EPA production under nutrient sufficient and deficient conditions?

2 Is a fed-batch nutrient regime better than a batch regime in terms of biomass and EPA production under nutrient sufficient and deficient conditions?

3 What is the effect of nutrient deficiency (i.e. the exclusion of nitrogen and silicon) on EPA production?

1. INTRODUCTION

Environmental factors are those factors (physical or chemical) that influence the growth and metabolism of living organisms. These factors and their effects on growth and EPA production were reviewed in Chapter I. An outline of how each parameter can be varied in order to test the effects on growth and EPA production is presented in Table 18.

Table 18: Examples of how environmental factors can be varied to investigate the effect on growth and EPA production.

Factor	Range of variation	Parameters tested previously by Dickson (CSIR Biosciences)	How the current study aims to expand on Dickon's results
Temp	The effect of temperature can be investigated between 15 to 40°C	Effect of temperatures between 15 and 30°C were tested Optimal temperature range identified between 25 and 27°C. No EPA data reported.	As the cultures will be cultured in an open raceway setting, this factor was not further investigated.
Light	The effect of light intensity can be investigated by conducting a study at low light or high light intensities. Alternatively, the effect of light cycling can be investigated.	Effect of different light intensities (27 – 123 $\mu mol.m^{-2}.s^{-1}$) on the biomass production in WCA 23.2 was investigated. Results indicated optimum growth at a light intensity of 123 $\mu mol.m^{-2}.s^{-1}$.	As the cultures will be cultured in an open raceway setting, this factor was not further investigated.
pH	The effect of pH can be investigated by conducting a study at <pH6 to >pH9. Alternatively, the effect of pH control (as opposed to uncontrolled) can be investigated.	Effects of pH ranges 5-10 were tested on the biomass production in WCA 23.2. Results indicated slower growth above and below pH 7. No EPA data reported.	As pH ranges between 5 and 10 have been investigated, the aim here is to investigate whether pH control is actually necessary and what the effect if left uncontrolled – and its ultimate effect on EPA production
Nutrients	The effect of nutrient availability can be investigated under nutrient sufficient or deficient conditions. Batch and fed-batch nutrient regimes can also be investigated. Here the choice is made between supplying all the nutrients to the culture at the onset of the growth phase, or by adding smaller amounts of nutrients on a daily basis.	Effect of nutrient deficiency was tested to determine the impact on EPA production in WCA 23.2. Results indicated the exclusion of silicate, sulphate, nitrate and phosphate resulted in high EPA productivity.	Here batch and fed-batch nutrient regimes will be investigated and its effects on EPA production.

The environmental factors can be tested in isolation or a combination of factors can be tested. Previous studies conducted at the CSIR Biosciences laboratories investigated the impact of temperature, light, pH and nutrient stress on WCA 23.2 (Dickson, 2011a-c). Similar studies have been reported on for *P. tricornutum* (Yongmanitchai & Ward, 1991; Jiang & Gao, 2004; Liu *et al.*, 2009; Beerdall & Morris, 1976). There is therefore limited value, to the current study, in reinvestigating these factors, as optima have been established. This chapter will focus on the effect of pH control and the effect of different nutrient regimes.

2. Materials and Methods

Flask Study 1: Controlled vs. uncontrolled pH

The standard growth conditions have been described in Chapter II. The culture pH was adjusted to pH 7.2 before the addition of nutrients. For the controlled pH experiments, the pH was maintained at pH 7.2 with 0.5% HCl. For the uncontrolled experiments, the pH was initially adjusted to pH 7.2 and allowed to increase naturally for the duration of the experiment. Nutrients supplied to ASW media were 0.606 $g.L^{-1}$ $NaHCO_3$, 0.150 $g.L^{-1}$ $NaNO_3$, 0.900 $g.L^{-1}$ $NaSiO_3.5H_2O$ and 0.013 $g.L^{-1}$ $NaH_2PO_4.2H_2O$. Nutrients were supplied every second day in a fed-batch mode based on nutrient usage. At the end of the study, the biomass was centrifuged and used to inoculate nutrient deficient (nitrogen and silicate excluded) media.

Flask Study 2: Batch vs. Fed-Batch feeding regime

The pH was maintained at pH 8.3 (based on the conclusion from the pH study) with 0.5% HCl. Nutrients for the fed-batch regime were supplied to ASW media as 0.606 $g.L^{-1}$ $NaHCO_3$, 0.150 $g.L^{-1}$ $NaNO_3$, 0.900 $g.L^{-1}$ $NaSiO_3.5H_2O$ and 0.013 $g.L^{-1}$ $NaH_2PO_4.2H_2O$ every second day, according to a predetermined schedule. For the batch study 2 $g.L^{-1}$ $NaHCO_3$, 0.495 $g.L^{-1}$ $NaNO_3$, 2.970 $g.L^{-1}$ $NaSiO_3.5H_2O$ and 0.044 $g.L^{-1}$ $NaH_2PO_4.2H_2O$ were added at the outset, so that each study received the same amount of nutrients over the full duration of the experiment. At the end of the study, the biomass was centrifuged and used to inoculate nutrient deficient (nitrogen and silicate excluded) media.

3. Results and Discussion

Two sets of experiments were conducted to investigate the effect of the chosen environmental factors. The first experiment investigated the effect of pH on the growth and EPA production in WCA 23.2 and *P. tricornutum*.

The second experiment investigated the effect of two different feeding regimes, batch and fed-batch on the growth and EPA production in WCA 23.2 and *P. tricornutum*.

3.1. THE EFFECT OF PH CONTROL: CONTROLLED VS. UNCONTROLLED

3.1.1. Effect on growth

The resulting growth curves for WCA 23.2 and *P. tricornutum* under pH control during nutrient sufficient and nutrient deficient conditions are presented in Figure 32. It is important to establish the value of these interventions at laboratory scale, before scaling up a process, as there are costs associated with controlling pH in large volumes. If cultivation is possible with minimal or no pH control, it could have a positive impact on the financial viability of the process. To counter for the continuous acid addition (affecting the total flask volume of the pH controlled flasks), cell numbers were standardised by taking into account the volume added and then correcting for cell number so as to not falsely measure lower cell counts due to the dilution of the culture media.

The pH was controlled at pH 7.2, as previous studies confirmed good growth of both species between pH 7 and 8 (Yongmanitchai & Ward, 1991; Dickson & Lalloo, 2011c). The nutrient sufficient phase was Day 0-9 while the nutrient deficient phase was from Day 10-16.

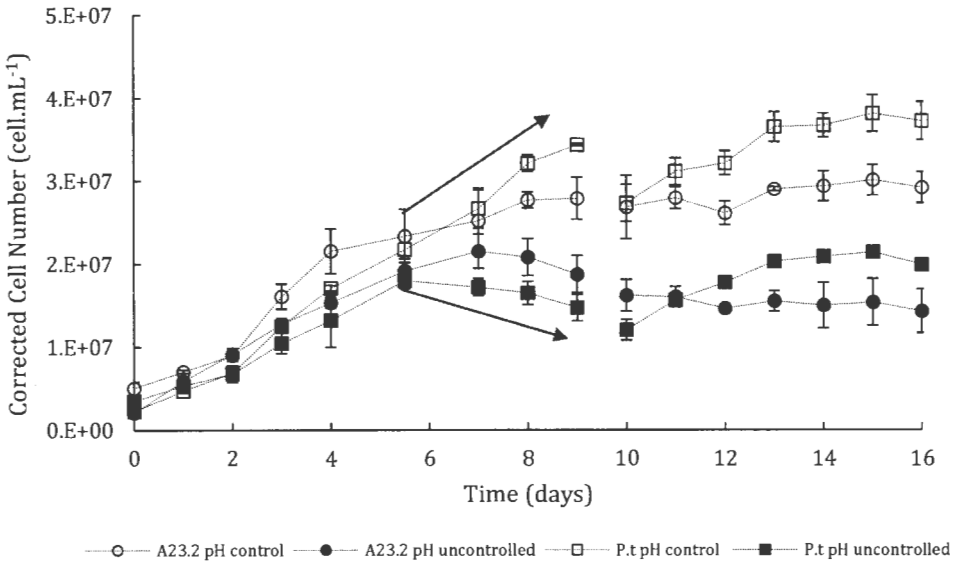


Figure 32: The effects of pH control on the growth of CSIR isolate WCA 23.2 (pH controlled ○ and uncontrolled ●) and *P. tricornutum* (pH controlled □ and uncontrolled ■) under nutrient sufficient (Day 0-9) and nutrient deficient (D10-16) conditions. Curves are the result of two replicates.

Calculated maximum and average specific growth rates during the nutrient sufficient phase were 0.36 (SD±0.07) d^{-1} and 0.20 (SD±0) d^{-1} under pH controlled conditions, and 0.50 (SD±0.03) d^{-1} and 0.22 (SD±0) d^{-1} under pH uncontrolled conditions for WCA 23.2. For *P. tricornutum* maximum and average specific growth rates during the nutrient sufficient phase were 0.50 (SD±0.02) d^{-1} and 0.30 (SD±0) d^{-1} under pH controlled conditions and 0.33 (SD±0.02) d^{-1} and 0.18 (SD±0) d^{-1} under pH uncontrolled conditions.

Average specific growth rates were only calculated for Day 0-4 as the rate started to decline after that. Calculation of the average specific growth rate over the entire period would therefore have resulted in a negative value. Hence, it is possible that the species grew the same (possibly better at pH uncontrolled) for the first 4 days. It might therefore be that the average specific growth rate was higher for uncontrolled conditions when from the graph; the cell number for WCA 23.3 (under uncontrolled conditions) was consistently lower than that under controlled conditions, and in fact declined from Day 6/7.

During the nutrient sufficient phase (Day 0-9); it appears that both species followed similar growth patterns in both the pH controlled and uncontrolled cultures for the first six days. However, from Day 6 onwards, the pH controlled and uncontrolled cultures diverged, with the controlled cultures maintaining linear growth, while the uncontrolled cultures entered stationary phase, as indicated by the red arrows in Figure 32. At this point, the mean pH in the uncontrolled cultures was pH 9.7 (SD±1.29) and pH 9.4 (SD±1.07) for WCA23.2 and *P. tricornutum* respectively. Further increase in the cellular concentration was not observed above these pH values.

This may suggest that the species is able to maintain growth during uncontrolled pH for a certain period of time after which it is unable to maintain further growth up until a pH of 9.7 and 9.4 respectively. This is consistent with previously published literature values (Hansen, 2002).

Hansen (2002) suggested that while some species cannot grow at pH 8-9, others are capable of growth at pH values up to pH 10, although of the species tested, non was able to sustain optimal growth rates above pH 9 (Hansen , 2002). To assess whether Hansen's observation supported the current study, daily growth rates were plotted against pH during the nutrient sufficient phase, as seen in Figure 33. The trend from the graph suggests a decrease in linear growth with an increase in pH.

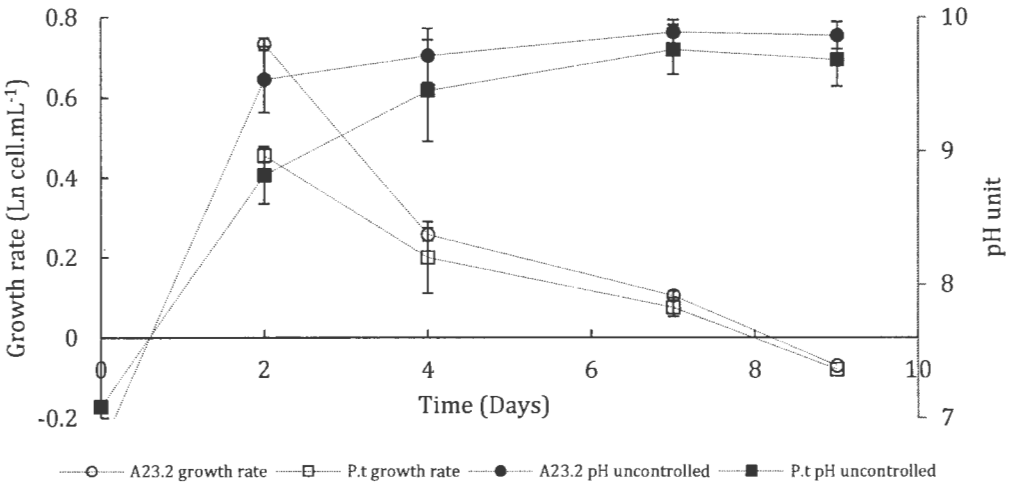


Figure 33: The effect of uncontrolled pH (A23.2 ● and P.t ■) on the linear growth rate of CSIR isolate WCA 23.2 ○ and *P. tricornutum* □ under nutrient sufficient conditions. Data points represent the mean of two replicates.

The relationship between growth rate and pH was negatively correlated (R^2>0.8), with a decrease in growth rate observed with an increase in pH (data not shown). The data are consistent with Hansen's assertion, with cell numbers decreasing as the culture pH increased beyond 9.5. A similar study by Chen and Durbin, on pH and its effect on growth rate, also reported a decrease in growth rate for diatoms as soon as pH increased above a value of pH 8.6 (Chen & Durbin, 1994).

Clearly, the reason for the decline in linear growth must be related to either light limitation, or (2) pH, as the organisms were not nutrient limited.

In the instance of light limitation, as soon as the cultures started to grow differently and reach different cell concentrations, cultures will experience different light conditions. Cultures were supplemented with nutrients (including bicarbonate, nitrate, phosphate and silicate) every second day. The decrease in growth rate with increasing pH can be attributed to either the change in inorganic carbon species distribution, resulting in a limited supply of available carbon for photosynthesis (Hansen, 2002) or it can possibly be due to the fact that the carbon assimilation apparatus of the species is not fully operational at such a high pH.

A limited supply of inorganic carbon ultimately restricts photosynthesis, the process directly responsible for biomass production. The relationship between pH and inorganic carbon speciation is depicted in Figure x (reference speciation figure from earlier on). Inorganic carbon is supplied as bicarbonate (HCO_3), in the form of $NaHCO_3$ which is the main source of carbon supplied to the organism for growth. It is understandable why overall growth for the controlled organisms was better when the results are related to the Figure 6 (shown in Chapter I).

During pH control at 7.2, the amount of HCO_3^- available was 90% as opposed to the 70% available during pH uncontrolled cultivation. It might be expected that an organism operating at 90% HCO_3^- availability grow better than an organism operating at 70% HCO_3^- availability. However, at a 70% bicarbonate species distribution, there is still plenty of bicarbonate available for organism growth.

Chen and Durbin reported low CCM functionality at high pH values of 9.4 and above for *P. tricornutum*. This is supported by the finding that there is a correlation between pH and carbon uptake rates, with a maximal uptake rate at pH ~8.2 and a decrease in carbon uptake rates at pH 8.5 and above, corresponding to a 10% carbon uptake rate at pH 9.4 (Chen & Durbin, 1994).

The reasoning is that at lower pH values, *P. tricornutum* was able to assimilate dissolved CO_2 freely available at low pH levels. A corresponding higher specific growth rate at low pH levels (vs. a lower specific growth rate at uncontrolled pH) supports the fact that *P. tricornutum* possibly does not have a well-developed CCM. Although it was not specifically discussed in the paper of Chen and Durbin, one can only speculate that at high pH values, a non-functional CMM leads to decreased bicarbonate uptake rates. This is ultimately a problem for the species as at this pH, the carbon is mainly in a bicarbonate form – the only form of bioavailable carbon present for use in photosynthesis.

In contrast to *P. tricornutum*, WCA 23.2 demonstrated better initial growth during the pH uncontrolled conditions (with a corresponding higher specific growth rate). This may indicate that WCA 23.2 has a CCM more suited / adapted to higher pH values than *P. tricornutum*.

3.1.2. Effect on EPA accumulation

Specific EPA produced under pH controlled and uncontrolled conditions are presented in Figure 34. Calculated specific EPA productivities ($mg.g^{-1}d^{-1}$ over the entire nutrient sufficient phase) was 0.057 $mg.g^{-1}\ d^{-1}$ (SD±0.043) under pH controlled conditions and 0.003 $mg.g^{-1}\ d^{-1}$ (SD±0.017) under pH uncontrolled conditions for WCA 23.2. For *P. tricornutum* specific EPA productivities was 0.154 $mg.g^{-1}d^{-1}$ (SD±0.058) under pH controlled conditions and 0.186 $mg.g^{-1}d^{-1}$ (SD±0.037) under pH uncontrolled conditions. This is in accordance with the theory presented in the previous section, that at high pH values the species are incapable of effectively assimilating bioavailable carbon for the use in growth, or cellular EPA production.

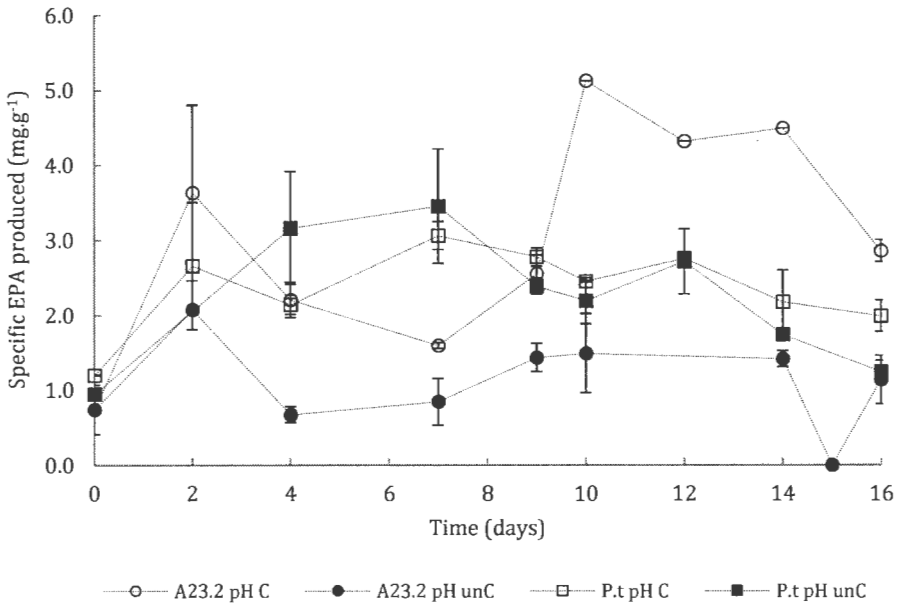


Figure 34: The effect of pH control on the specific EPA production of CSIR isolate WCA 23.2 (pH controlled ○ and pH uncontrolled ●) and *P. tricornutum* (controlled □ and uncontrolled ■) and under nutrient sufficient (Day 0-9) and nutrient deficient (D10-16) conditions. Curves are the result of two replicates.

Literature reported values for *P. tricornutum* grown under different pH conditions indicated that the species was able to produce up to 34.2 $mg.g^{-1}$ when grown under pH 7.6 conditions (Yongmanitchai & Ward, 1991). These values are higher than those observed in this study which indicated a reported specific EPA value of 3.06 $mg.g^{-1}$ on Day 7 of nutrient sufficient growth (Figure 35).

Under pH uncontrolled conditions, the specific EPA produced for *P.tricornutum* was highest on Day 2 at 3.16 $mg.g^{-1}$, but this can be due to the low pH still at this point in time before it naturally increased under pH uncontrolled conditions.

3.1.3. Comparison of pH control versus uncontrolled values

The experimental work conducted in this section gave rise to multiple values that are compared in bar graphs presented in Figure 35. Biomass productivities were higher under pH-controlled conditions. Specific EPA produced were also higher under pH controlled conditions. This experiment did not indicate any increase in specific EPA during the nutrient deficient phases.

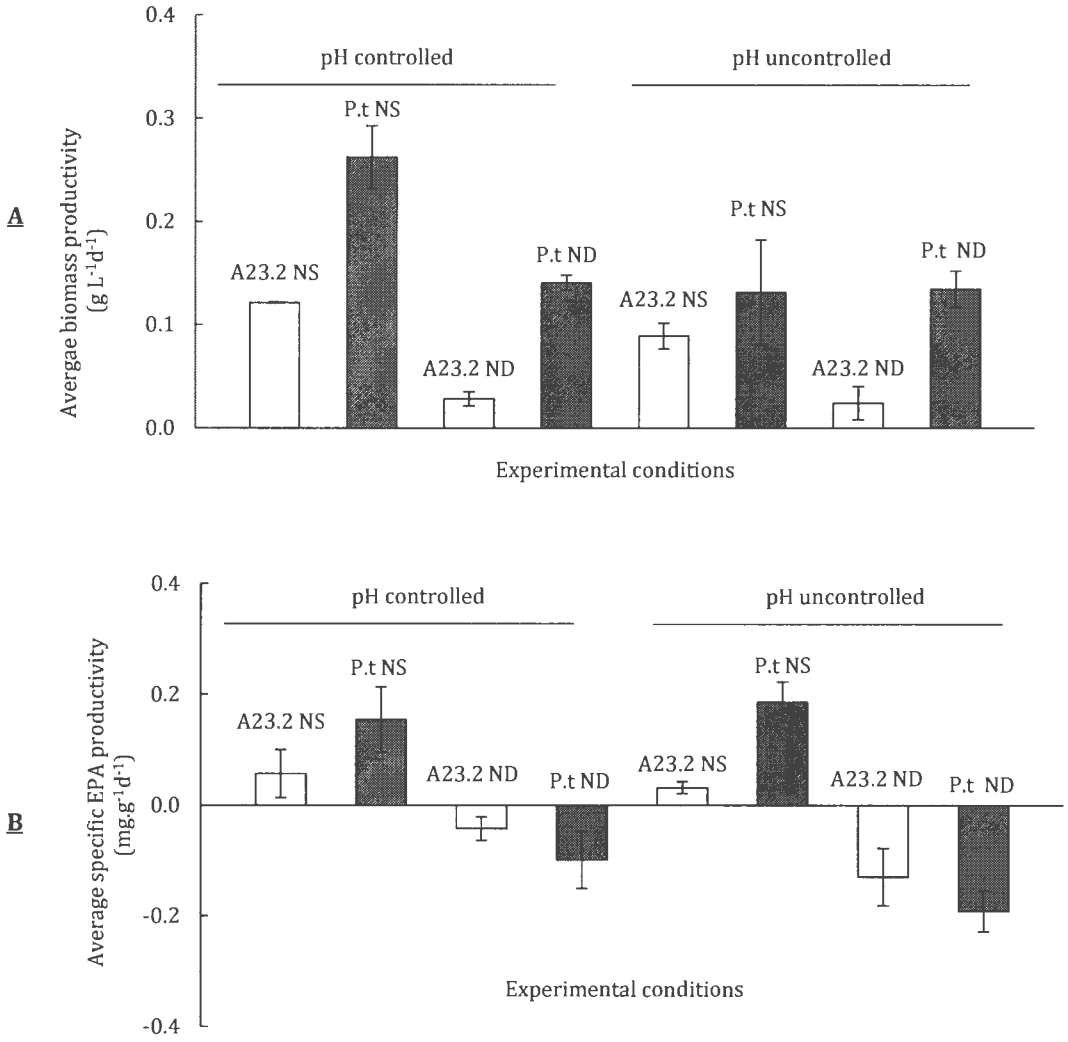


Figure 35: Summary of (**A**) average biomass and (**B**) specific EPA productivities obtained during the pH controlled vs. uncontrolled study. A23.2=WCA 23.2; P.t=*P. tricornutum*; NS=nutrient sufficient and ND=nutrient deficient.

The results and comparative graphs from the pH study indicate that both organisms prefer a controlled (and lower) pH in order to ensure maximum biomass and specific EPA production. However, literature indicated that *P. tricornutum* possible prefers to be controlled at a slightly higher pH than that of pH 7.2. For this reason, recommendations were made for the next section of the study: batch *vs.* fed-batch nutrient conditions.

3.1.4. Recommendations made for the next section of experimental work

From the results generated in this section, three recommendations were made:

1) pH should be controlled for optimal biomass production and maximal specific EPA production.

2) Assuming both species have efficient CCMs, pH should be controlled at a slightly higher value of 8.3 that will ensure maximal CCM operation and HCO_3^- availability. This was a particularly important recommendation as less acid is needed to maintain the medium at a higher pH, which can possibly lead to a lowered total cost of process operation. Although the growth rates for pH controlled and uncontrolled conditions in both species were similar (0.2-0.3 d^{-1} and 0.22-0.33 d^{-1} respectively) for the first 7 days, the impact on EPA productivity was negative for pH uncontrolled cultures. The proposition was made to control the culture at a slightly higher pH (pH 8.3) with the hopes that it may perform the same (or better) and use less acid to keep the pH at the set value.

3) The fed-batch nutrient concentrations used in this study resulted in good growth and was used in the next section. The amount added over the growing periods was computed and used as the starting batch concentration in the next section

3.2. The effect of Different feeding regimes: Batch vs. Fed-Batch

The work described in this section was performed under pH 8.3 controlled conditions, assuming a functional CCM and inorganic carbon primarily as HCO_3^-. The batch vs. fed-batch nutrient regimes were investigated to determine the impact of either regime on growth or more importantly, specific EPA production.

3.2.1. Effect on growth

The resulting growth curves, for WCA 23.2 and *P. tricornutum* grown under batch and fed-batch conditions are presented in Figure 36. Calculated maximum and average specific growth rates during the nutrient sufficient phase was 0.507 (SD±0.03) d^{-1} and 0.250 (SD±0.03) d^{-1} under batch conditions and 0.467 (SD±0.04) d^{-1} and 0.256 (SD±0.02) d^{-1} under fed-batch conditions for WCA 23.2.

For *P. tricornutum* maximum and average specific growth rates during the nutrient sufficient phase was 0.387 (SD±0.04) d^{-1} and 0.253 (SD±0.03) d^{-1} under batch conditions and 0.317 (SD±0.02) d^{-1} and 0.234 (SD±0.01) d^{-1} under fed-batch conditions.

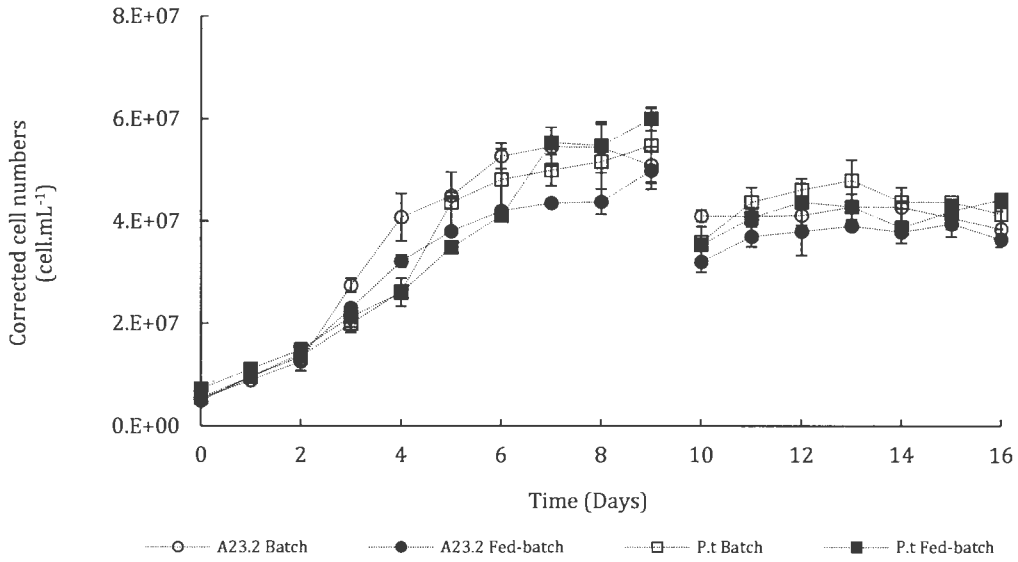


Figure 36: The effect of different feeding regimes on the growth of CSIR isolate WCA 23.2 (batch ○ and fed-batch ●) and *P. tricornutum* (batch □ and fed-batch ■) and under nutrient sufficient (Day 0-9) and nutrient deficient (D10-16) conditions. Curves are the result of two replicates.

From Figure 36, it is clear that the organisms followed normal growth. The results are similar to those obtained by Yongmanitchai and Ward, who investigated the growth of *P. tricornutum* over various nutrient concentrations (Yongmanitchai & Ward, 1991). Batch and fed-batch concentrations employed in the current study fell within the experimental range tested in the Yongmanitchai and Ward study.

In addition, previous studies on nutrient limiting conditions, conducted at the CSIR (Dickson & Lalloo, 2011b), suggested that algal growth was only negatively affected at phosphate

concentrations above 0.092 $g.L^{-1}$ and bicarbonate concentrations below 1.967 $g.L^{-1}$ (Dickson & Lalloo, 2011b). There is no difference in growth rates for WCA 23.2 (batch vs. fed-batch) nor for *P. tricornutum* (batch vs. fed-batch). It can be accepted that growth is similar when grown under either batch or fed-batch conditions.

3.2.2. Effect on EPA accumulation

Specific EPA produced under batch and fed-batch conditions are presented in Figure 37. Calculated specific EPA productivities ($mg.g^{-1}d^{-1}$ over the entire nutrient sufficient phase) was negative for all species during the nutrient sufficient conditions. Increased (positive) productivities were observed in the nutrient deficient phase. This was different to the pH study. The hypothesis is that the higher pH (8.3) conditions allowed this to happen. At these higher pH conditions, excess carbon was available and the species directed this to growth processes, and not secondary metabolite production. However, during the nutrient deficient phase, the species now directed available carbon to the production of structural membrane and storage lipids as a survival mechanism during nutrient deficient conditions.

Calculated specific EPA productivities ($mg.g^{-1}d^{-1}$) over the entire nutrient deficient phase were 0.412 $mg.g^{-1}d^{-1}$ (SD±0.023) under batch conditions and 0.122 $mg.g^{-1}d^{-1}$ (SD±0.085) under fed-batch conditions for WCA 23.2. For *P. tricornutum* specific EPA productivities were 0.175 $mg.g^{-1}d^{-1}$ (SD±0.052) under batch conditions and 0.032 $mg.g^{-1}d^{-1}$ (SD±0) under fed-batch conditions.

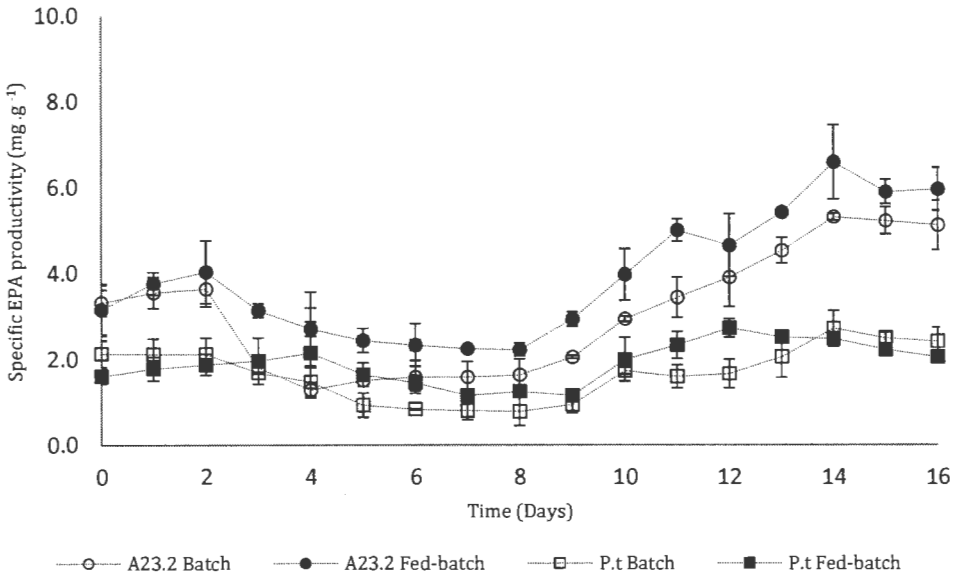


Figure 37: The effect of different feeding regimes on the specific EPA produced of CSIR isolate WCA 23.2 (batch ○ and fed-batch ●) and *P. tricornutum* (batch □ and fed-batch ■) and under nutrient sufficient (Day 0-9) and nutrient deficient (D10-16) conditions. Curves are the result of two replicates.

A higher specific EPA accumulation was observed for both species under batch conditions during the nutrient deficient phase. The fact that the fed-batch cultivated species performed worse than the batch cultivated species is thought to be the cause of the differing nutrient concentrations between the two regimes: the continued carbonate supplied (during the nutrient deficient phase) on every second day to the fed-batch cultures was now too little to meet organism nutrient demands due to increased growth. This may well be the case as carbonate was depleted on every second day for the fed-batch cultures as indicated by carbonate analysis.

The argument here is that under the new pH conditions (pH 8.3), the organism grew faster and hence used the carbon nutrient at an improved rate. During these experiments it became apparent that due to the improved growth rates (refer to pH section above) at pH 8.3, the carbon utilization increased thus making the calculated target amounts of carbonate insufficient at a growth pH of 8.3.

3.2.3. Comparison of Batch vs. Fed-batch values

The experimental work conducted in this section gave rise to multiple values that is compared in a bar graph, as seen in Figure 38. Biomass productivities were similar for batch and fed-batch conditions. Specific EPA productivity was higher for batch-cultivated organisms.

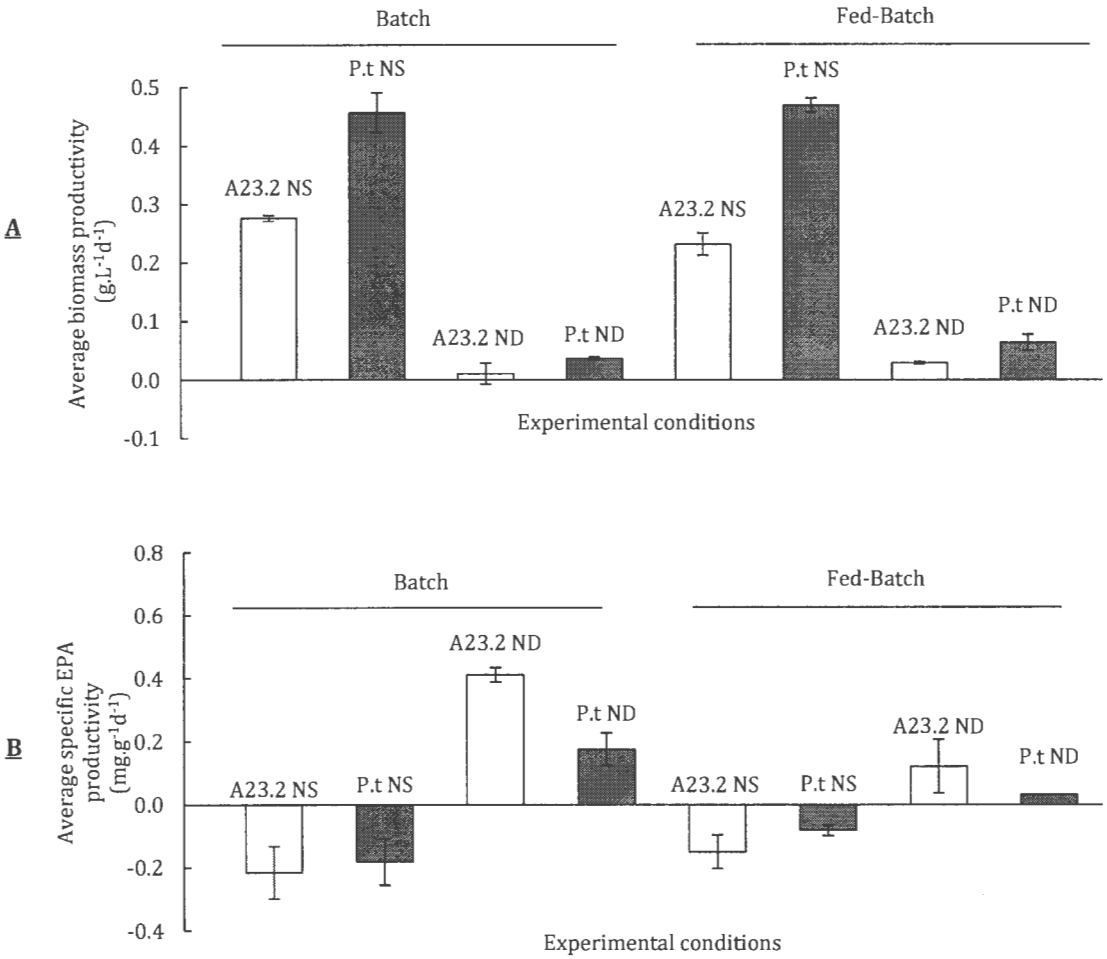


Figure 38: Summary of (**A**) average biomass and (**B**) specific EPA productivity values obtained during the batch vs. fed-batch feeding regimes. A23.2=WCA 23.2; P.t=*P. tricornutum*; NS=nutrient sufficient and ND=nutrient deficient.

4. SUMMARY

The primary objective of this section of the research was to compare the effect of selected environmental factors on the growth rate and EPA production of the two species under controlled, laboratory conditions.

The first aim was to determine whether pH control improved biomass and EPA production, under nutrient sufficient and deficient conditions. In the absence of pH control, the pH of the media rapidly increased, reaching a pH of over 9.4 for both species, by day 4. The increased pH did not have a significant effect on biomass productivity for the first six days, but thereafter growth in the uncontrolled flasks ceased and a decrease in cell concentration was observed from Day 7.

This was attributed to the relationship between pH and inorganic carbon speciation and the effect this had on the availability of carbon for photosynthesis. Above pH 8.3, the speciation shifts from bicarbonate to carbonate, which is not available for fixation, even if the organism has a well-developed CCM. Further research is necessary to characterise the CCM of both species. Specific EPA production was significantly reduced in A23.2 in the absence of pH control, but the effect was less significant for *P. tricornutum*.

Previous work at the CSIR suggested a fed-batch nutrient regime supported optimal growth. However, the data from the current study indicated that a fed-batch regime did not yield better biomass or specific EPA productivities. In fact, specific EPA production was higher in the batch fed cultures. The production of EPA was induced under nutrient (nitrogen and silicon) stress when the cultures were maintained at pH 8.3, which was consistent with published data in the batch cultures.

This was not the case when pH was controlled at pH 7.2 or allowed to increase to above pH 9.4 (no pH control). The reason for this is not obvious, but suggests that carbon assimilation was suppressed as no growth or significant lipid accumulation was observed.

CHAPTER V

CHAPTER V – COMPARING THE PERFORMANCE OF CSIR ISOLATE WCA 23.2 AND *P. TRICORNUTUM* IN A RACEWAY SYSTEM

Research Objectives: **Assess the growth and EPA production of a CSIR isolate and culture collection species in an open raceway pond system from a bioprocess perspective**

Research Questions: **1** What is the effect of upscale on the biomass and EPA production, focussing on scale changes and impact of uncontrolled variables.

1. INTRODUCTION

The laboratory scale characterisation of the chosen species was described in Chapter IV. The research objective was to compare the effect of selected environmental factors on growth rate and EPA production in WCA 23.2 and *P. tricornutum* under laboratory conditions. The next step, described in this chapter, involved a more than ten-fold increase in scale, to investigate the effect on growth and overall productivities, with a view to informing decisions with regard to the potential for commercial-scale production.

It is important to investigate if, and how, the performance of the system changes with scale as this impacts the commercial viability of the process. It has been reported that a scale change may be accompanied by certain complications, such as difficulty in maintaining homogeneity in large systems (for example a homogenous cell suspension in hectare ponds), changes in surface area to volume ratios as well as changes in cultures themselves, due to the increased length of culture time (Shuler & Kargi, 1992).

In addition to this, the effect of variables that can no longer be controlled efficiently, such as day light hours, light intensity as well as temperature cycles needs to be considered. The purpose of the work presented in this chapter was to assess the growth and EPA production of WCA 23.2 and *P. tricornutum* in an open raceway pond system.

2. MATERIAL AND METHODS

General growth conditions and the experimental setup were described in the Materials and Methods (Chapter II). The initial pond studies were conducted during March 2014 and repeated in May 2014. The ponds were housed in a greenhouse on the CSIR grounds in Pretoria. Two ponds were used in each study and species were kept in separate greenhouse rooms or "cubicles" to minimise possible contamination.

Pond volumes were maintained at 50 L during the course of the cultivation period. Pond volumes were measured by placing a standard ruler on the inside of the pond were 1 cm corresponded to approximately 10 L. Evaporation was compensated for on a daily basis by topping up of the pond volume to the 5 cm mark (~50 L) using tap water.

Nutrient concentrations, determined during the previous study, were achieved by a single addition of salts at the start of the experiment. The pH was controlled at 8.3 with 1% HCl ($v.v^{-1}$). Inoculums were grown from stock cultures and were used to inoculate the ponds to an initial concentration of approximately 1 x 10^6 cells.mL^{-1}. Samples and measurements were taken at noon on a daily basis and included pH readings, volume corrections, acid usage, temperature (outside the greenhouse,

inside the greenhouse unit as well as the water temperature) and light measurements (outside the greenhouse as well as at the water surface of the ponds).

Studies were conducted over a period of 23 days. Biomass was harvested, via settling and/or filtration, employing a screening pan fitted with 25-µM mesh. A simplified sketch is provided in Figure 39 to demonstrate the experimental setup of the raceway studies and provides an overview of the different temperature and light intensity measurements collected.

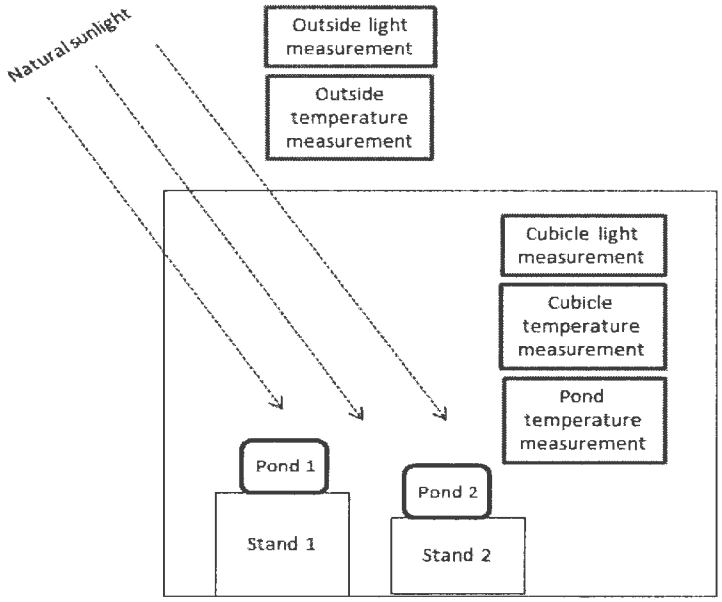


Figure 39: Raceway experimental setup inside the greenhouse cubicle as viewed from the side along with an indication of the measured values inside and outside the greenhouse cubicle.

3. Results and Discussion

Each study was conducted over a period of 23 days and included a nutrient sufficient (Day 0-13) and nutrient deficient (Day 14-23) phase. The March 2014 study was conducted at the end of the summer season while the May 2014 study was conducted at the start of the winter season. The transition from the laboratory to the greenhouse resulted in a number of the control variables changing and the changes are summarised in Table 19.

A number of factors were significantly different, with the pond study employing a longer cultivation period, larger culture volume, and uncontrolled light intensity supply and culture temperatures.

Table 19: Summary of the differing conditions as measured during the laboratory and greenhouse experiments.

	Laboratory conditions	**Greenhouse conditions**
Cultivation period	16 days Nutrient sufficient: Day 0-9 Nutrient deficient: Day 10-16	23 days Nutrient sufficient: Day 0-13 Nutrient deficient: Day 14-23
Culture volume	2-3 litres	50 litres
Nutrient supply	Batch	Batch
pH	8.3, 0.5% HCl	8.3, 1% HCl
Light intensity and supply	Artificial 140 $\mu mol.m^{-2}s^{-1}$ continuous	Natural 1000-4000 $\mu mol.m^{-2}s^{-1}$ 12:12 (March) and 11:13 (May) light-dark ratio
Environment temperature	20-25°C	3-28°C

Average high (day) and low (night) temperatures for the pond studies, measured outside the greenhouse cubicle, light intensity measurements, measured outside the greenhouse cubicle, as well as daily water (WT) and cubicle temperatures (CT) for the pond studies, measured inside the greenhouse cubicle, are shown in Figures 40-42.

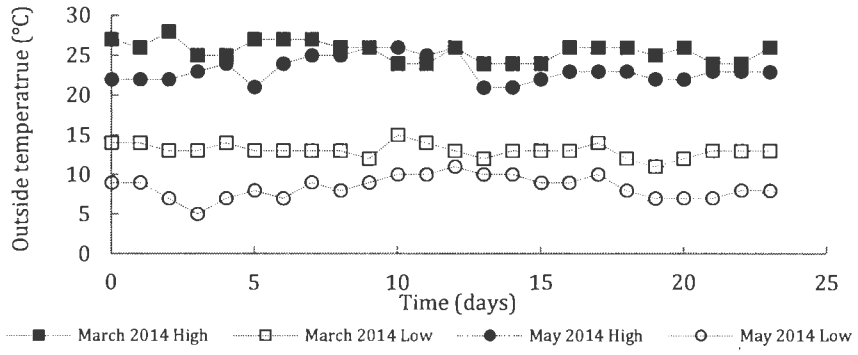


Figure 40: Average high (day) and low (night) temperatures for the pond studies as measured outside the greenhouse cubicle where the average high for March ■ was 25.5°C (±1.2°C), average low for March □ was 13.0°C (±0.9°C) average high for May ● was 23.2°C (±1.6°C) and average low for May ○ was 8.4°C (±1.4°C).

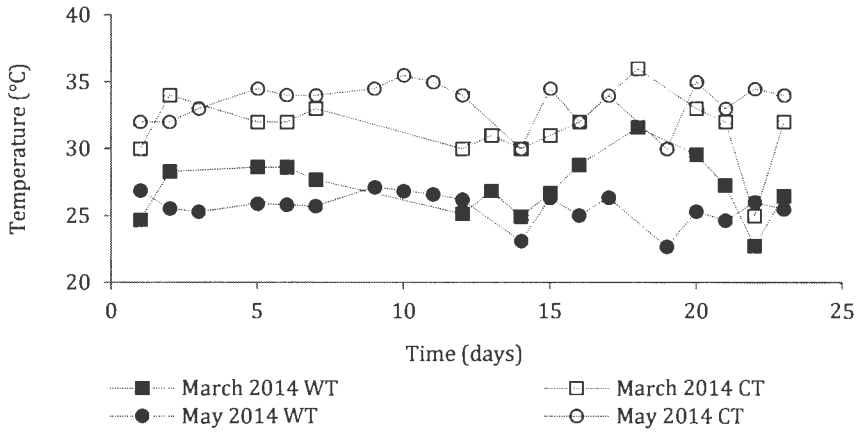


Figure 41: Daily water (WT) and cubicle temperatures (CT) for the pond studies as measured inside the greenhouse cubicle where March 2014 WT ■, March 2014 CT □, May 2014 WT ● and May 2014 CT ○.

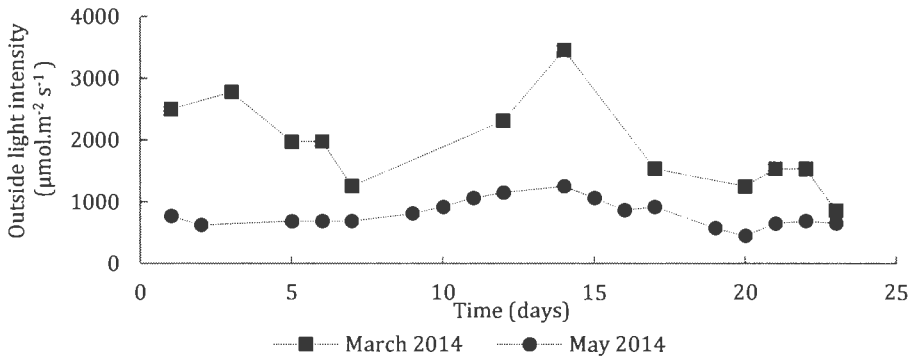


Figure 42: Light intensity measurements for the March 2014 and May 2014 pond studies as measured outside the greenhouse cubicle where the average for March 2014 ■ was 1919 µmol.m^{-2}s^{-1} (±741.02), and average for May 2014 ● was 811 µmol.m^{-2}s^{-1} (±216.78).

A summary of the average values recorded for the March 2014 and May 2014 pond studies is presented in Table 20, in order to facilitate a comparison between possible differences between the March 2014 and May 2014 studies. From the table, the May 2014 study had day: night cycling of 11:13 as opposed to the 12:12 day: night cycles recorded for the March 2014 study.

Although the May 2014 studies had slightly lower recorded average temperatures, overall recorded temperatures were similar across both studies. Average cubicle and water temperatures were also recorded to be similar across both studies. Average light intensity was however not the same across both studies, with the values recorded for March 2014 twice that of the values recorded in May 2014. Although solar radiance is typically high during the winter seasons in Pretoria, as there is no rainfall (i.e. cloudy conditions), the solar radiance presented in March were higher than May.

Table 20: Summary of temperature values as recorded for each pond study.

	Pond study 1	**Pond study 2**
Date	March 2014	May 2014
Season	Summer-autumn	Autumn-winter
Day: night cycles (alt. light: dark)	12:12 (SD±0.2)	11:13 (SD±0.1)
Average outside high (day) and low (night) temperatures	25.5°C (SD±1.2°C) 13.0°C (SD±0.9°C)	23.2°C (SD±1.6°C) 8.4°C (SD±1.4°C)
Average cubicle temperatures*	30.4°C (SD±3.6°C)	33.0°C (SD±2.5°C)
Average water temperatures*	26.1°C (SD±0.4°C)	25.4°C (SD±0.2°C)
Average outside light intensities*	1919 $\mu mol.m^{-2}s^{-1}$ (SD±741.0)	811 $\mu mol.m^{-2}s^{-1}$ (SD±216.8)

*Values as recorded at noon every day.

3.1. EFFECT ON GROWTH

The resulting growth curves of WCA 23.2 and *P. tricornutum* for the March 2014 and May 2014 pond studies are presented in Figure 43. The nutrient sufficient phase was Day 0-13 while the nutrient deficient phase was from Day 14-23. The dotted red line in Figure 43 separates the two nutrient phases.

Calculated average specific growth rates for WCA 23.2 during the nutrient sufficient phase was 0.271 (SD±0.06) d^{-1} and 0.191 (SD±0.02) d^{-1} during the March 2014 and May 2014 cultivations respectively. For *P. tricornutum* average specific growth rates during the nutrient sufficient phase was 0.239 (SD±0.01) d^{-1} and 0.196 (SD±0.01) d^{-1} during the March 2014 and May 2014 cultivations respectively.

The average specific growth rates were higher during the March 2014 cultivations for both species. The only factor that changed significantly between the two experimental runs was light intensity and slightly lower temperatures (Table 20).

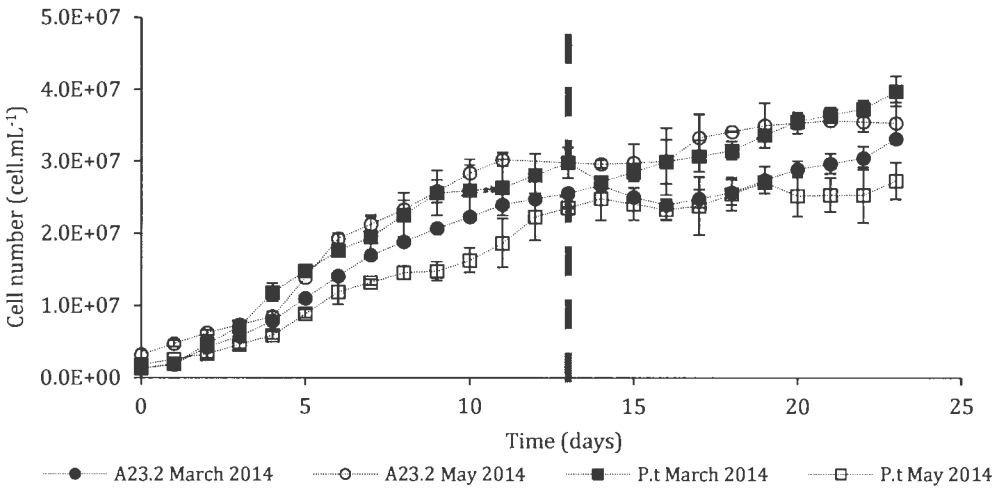


Figure 43: Growth curves of CSIR isolate WCA 23.2 March 2014 ●, May 2014 ○ and *P. tricornutum* March 2014 ■, May 2014 □ under nutrient sufficient conditions (Day 0-13) and nutrient deficient conditions (Day 14-23).

Published research by Watson *et al* (2004) states that light intensity affects the level of primary production and ultimately influences photosynthesis.. The growth rates of diatoms should therefore increase with increasing light intensity up to levels of light saturation (James & Boriah, 2010; Metz *et al.*, 2001). The lower growth observed for WCA 23.2 during the May 2014 experiment could therefore be due to the lower light intensity. However, the effects of light intensity is also species specific depending of the original habitat of the species (James & Boriah, 2010; Nelson *et al.*, 1979).

It was observed that WCA 23.2 experienced a much higher light intensity under greenhouse conditions (1919 µmol m^{-2} s^{-1} *versus* 140 µmol m^{-2} s^{-1}) and growth rates were lower during the pond cultivations. This is unexpected as more light was available to the culture for growth. However, it must be taken into consideration that the cultures are now cultivated outside (as opposed to inside) where parameters are no longer controlled such as temperature, light and water evaporation.

Converted values for aerial pond biomass productivities were calculated for both species. It was reported in the literature that biomass concentrations are normally in the region of 0.5 $g.L^{-1}$ corresponding to a biomass productivity of 10 to 25 $g.m^{-2}d^{-1}$ (Sheehan *et al.*, 1998; Griffiths, 2013).

Both species presented with much lower biomass concentrations. For WCA 23.2, converted biomass productivity was 5.62 $g.m^{-2}d^{-1}$ and 7.81 $g.m^{-2}d^{-1}$ during the March 2014 and May 2014 cultivations respectively. For *P. tricornutum*, it was 5.96 $g.m^{-2}d^{-1}$ and 4.44 $g.m^{-2}d^{-1}$ during the March 2014 and May 2014 cultivations respectively.

It was noted by Griffiths (2013) that at present, the only open system to achieve high cell densities was a cascade system employing *Chlorella* reaching concentrations of up to 10 $g.L^{-1}$ with a culture depth of 1 cm (Šetlík *et al.*, 1970). The culture depth in the current study was at least 5 cm and may be the reason for the lower biomass productivity due to less light reaching the cells at the bottom or due to poorer mixing in a higher volume.

3.2. Effect on EPA accumulation

The resulting specific EPA produced ($mg.g^{-1}$ biomass) curves of WCA 23.2 and *P. tricornutum* for the March 2014 and May 2014 pond studies are presented in Figure 44. The nutrient sufficient phase was Day 0-13 while the nutrient deficient phase was from Day 14-23. The dotted red line in Figure 45 separates the nutrient phases. Calculated average specific EPA productivities ($mg.g^{-1}d^{-1}$ produced over the entire nutrient sufficient phase) were 0.069 $mg.g^{-1}d^{-1}$ (SD±0.010) and 0.108 $mg.g^{-1}d^{-1}$ (SD±0.016) for WCA 23.2 and 0.112 $mg.g^{-1}d^{-1}$ (SD±0.027) and 0.206 $mg.g^{-1}d^{-1}$ (SD±0.045) for *P. tricornutum* during the nutrient sufficient March 2014 and May 2014 cultivation periods.

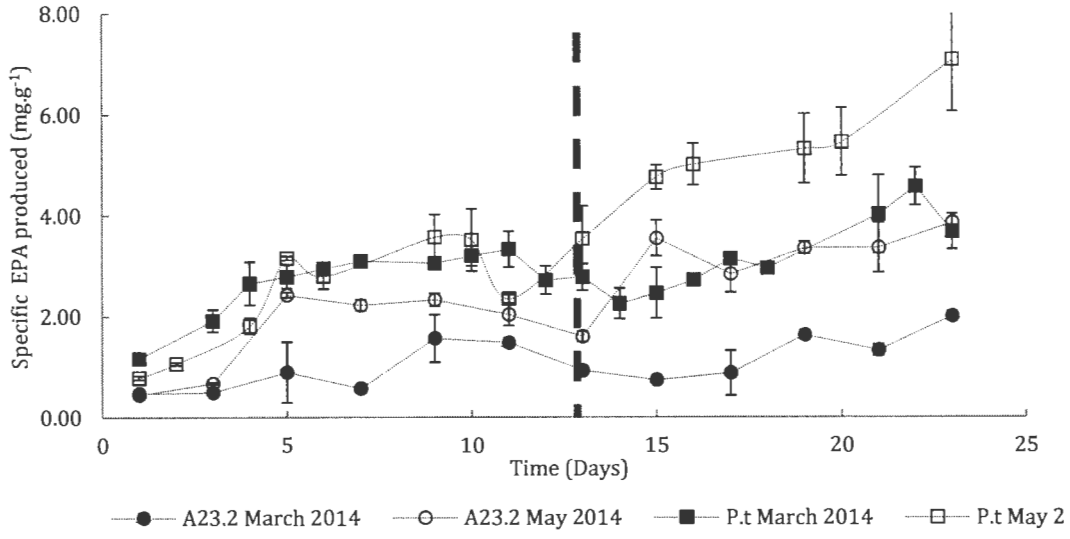


Figure 44: Effect of pond cultivation on the specific EPA production of CSIR isolate WCA 23.2 March 2014 ●, May 2014 ○ and *P. tricornutum* March 2014 ■, May 2014 □ under nutrient sufficient conditions (Day0-13) and nutrient deficient conditions (Day14-23).

Specific EPA productivity was higher during the May 2014 cultivations for both species. This can be because of two factors; (1) lower light intensities and (2) lower night time temperatures recorded for May 2014.

Olofsson (2012) reported maximal lipid contents during the autumn seasons in a study investigating the effects of seasonal changes on fatty acid accumulations, which is consistent with the cellular EPA produced in this study. They attributed the change to light and temperature changes/fluctuations. The hypothesis was that enhanced thylakoid stacking under reduced light conditions (consistent with the lower recorded light intensities in May 2014) resulted in the production of more polar (cell membrane) structural lipids. EPA production is known to occur when temperature decreases, as it is a mechanism used to facilitate membrane fluidity by the enhancement of structural lipids (Oloffson *et al.* 2012).

3.3. Comparison of March 2014 and May 2014 values

The experimental work conducted in this section gave rise to multiple values that are compared in a bar graph (Figure 45). Biomass productivities were similar for both species during the March 2014 cultivation. A drop in specific EPA productivity was observed for WCA 23.2 during the May 2014 cultivations during the nutrient deficient phase. The rate at which the WCA 23.2 species was actively producing EPA was higher during the March 2014 cultivations, while the actual EPA produced (EPA content) was higher during the May 2014 cultivations.

From the values reported here, *P. tricornutum* was able to sustain biomass production during both cultivations and produce up to double the amount of EPA when compared to WCA 23.2. This clearly indicates that *P. tricornutum* was able to outperform the CSIR species based on productivity values. The next section made the comparison between the bench scale and raceway scale studies to highlight the changes in productivity values.

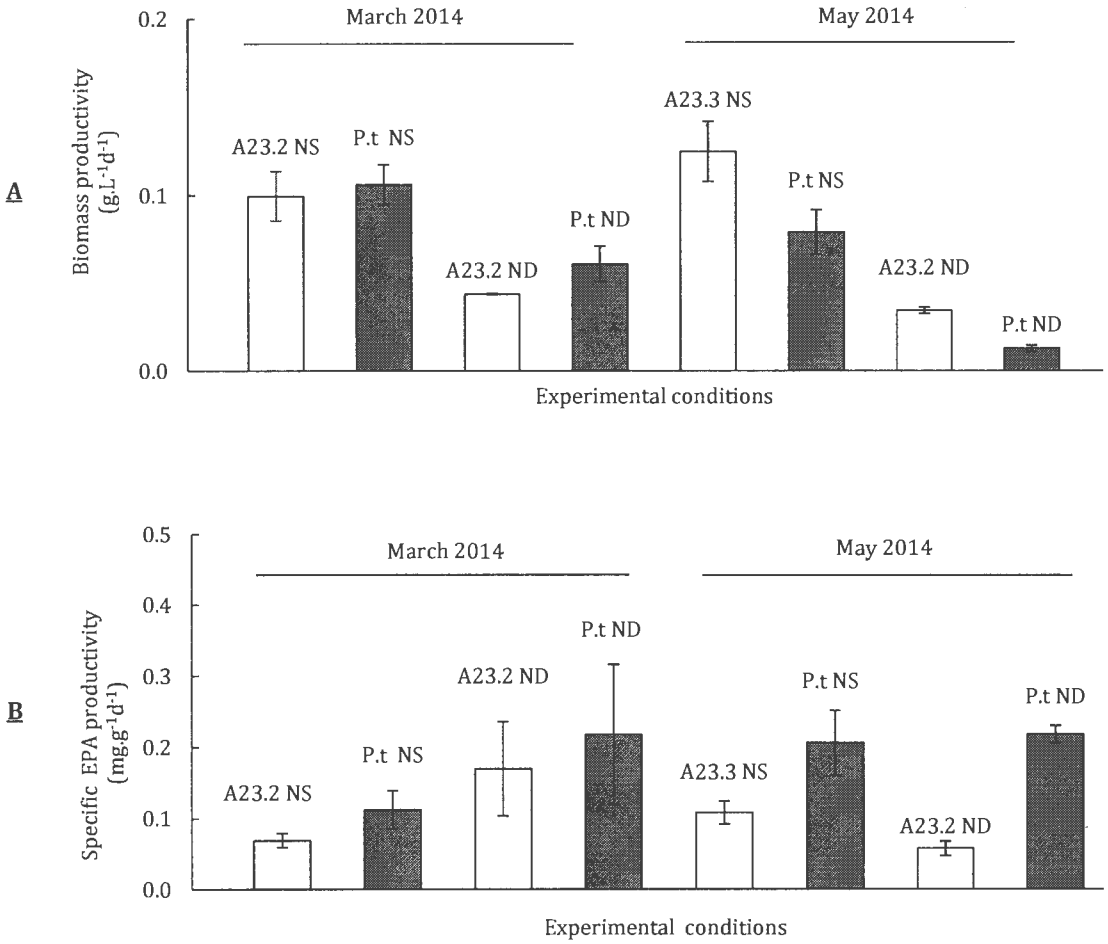


Figure 45: Summary of (**A**) biomass produced and (**B**) specific EPA productivity values obtained during the March and May cultivation periods. A23.2 = WCA 23.2; P.t = *P. tricornutum*; NS = nutrient sufficient and ND = nutrient deficient.

3.4. COMPARISON WITH BENCH SCALE STUDIES

A comparison was made between the bench scale and raceway scale studies and is presented in Figure 46. The figure exclusively shows biomass and specific EPA productivity during the bench scale batch pH 8.3 conditions and the March and May cultivations.

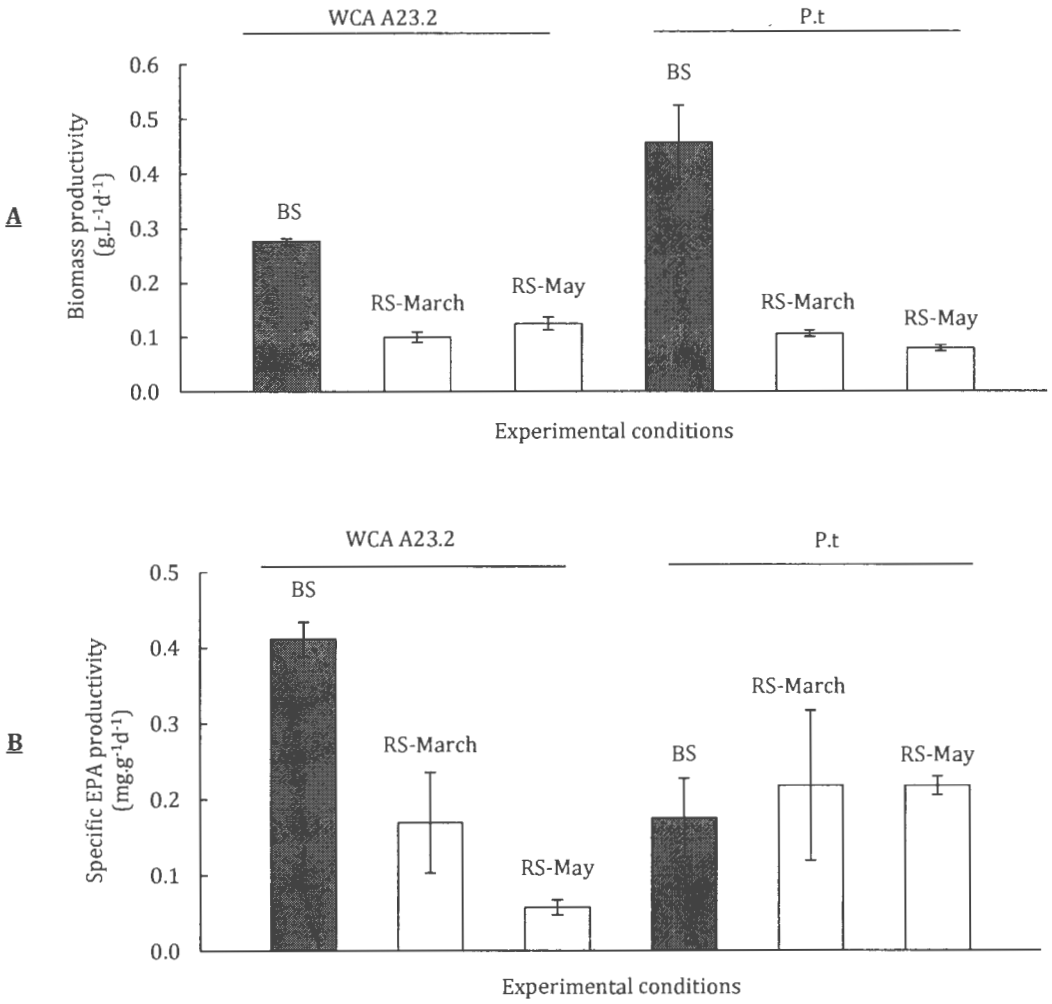


Figure 46: Comparison of bench scale and raceway scale biomass and EPA productivity values where (**A**) biomass produced and (**B**) is specific EPA productivity. BS = bench scale and RS = raceway scale

The data presented clearly shows the drop in biomass productivity going from a closed reactor in the laboratory to an open outside raceway pond. The drop in productivities are expected as various factors became uncontrollable such as light and temperature. Another factor potentially affecting productivity is the decrease in mass transfer. The magnetically operated stirrer bar achieved speeds of 500-600 RPM, whereas the paddlewheel only achieved a speed of 80 RPM maximum.

Poorer mixing directly influences the available sunlight that can reach the cells in the bottom of the raceway. This can be used to explain the lower biomass productivities. A significant drop was observed for specific EPA productivity in the organism WCA 23.2. It appears that *P. tricornutum* mostly maintained its ability to produce the same amounts of EPA going from bench scale to raceway scale, possibly indicating a more robust organism better suited to the process developed at the CSIR Biosciences.

3.5. HARVESTING

There are several methods currently employed to harvest biomass after a cultivation period, including sieving/filtration, settling, flocculation and centrifugation. A combination of sieving/filtration and settling was used to harvest the biomass during the March and May experiments. A settling study (Figure 47) was conducted to investigate the settling potential of both organisms. A volume of cells was taken from the pond and allowed to mix by inversion to ensure a homogenous mixture.

The suspension was allowed to settle by gravitation and samples were taken over a one-hour period to investigate settling. A final sample was taken at the 20-hour mark to compare to the previous day's findings. A curve of OD (sample taken) over time is presented in Figure 47 and shows the decrease in OD (reflective of settled cells) over time for WCA 23.2 and *P. tricornutum*. The settling study was conducted over a period of 1200 minutes (20 hours) but the last data point was left out as there was little change between the 60 minute *vs.* 1200-minute mark.

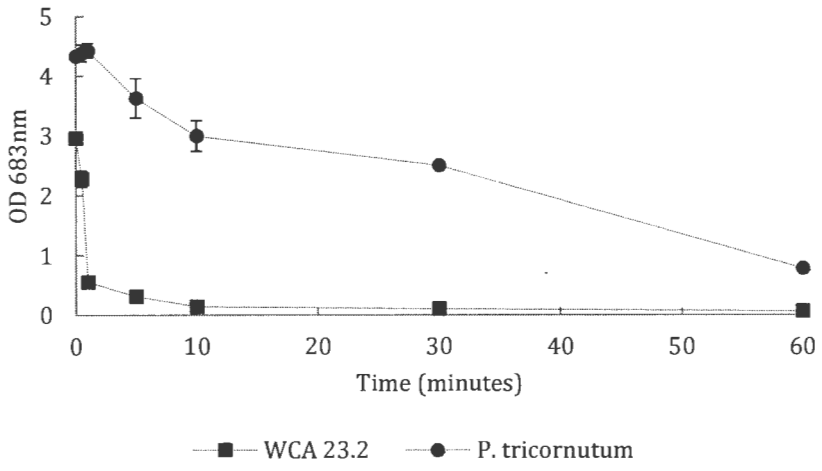


Figure 47: Settling time for WCA 23.2 and *P. tricornutum*.

The curve shows that WCA 23.2 is able to settle completely after 60 minutes with a corresponding OD of (0.066) whilst *P. tricornutum* still had at an OD of 0.786 at the 60-minute mark, indicating an unsettled culture. After 20 hours, the organism still did not settle completely as is reflected by an OD of 0.540 (not shown in Figure 47). In conjunction with the recorded OD values, pictures were taken at pre-determined intervals to visually demonstrate the settling of cells over time and presented in Figure 48.

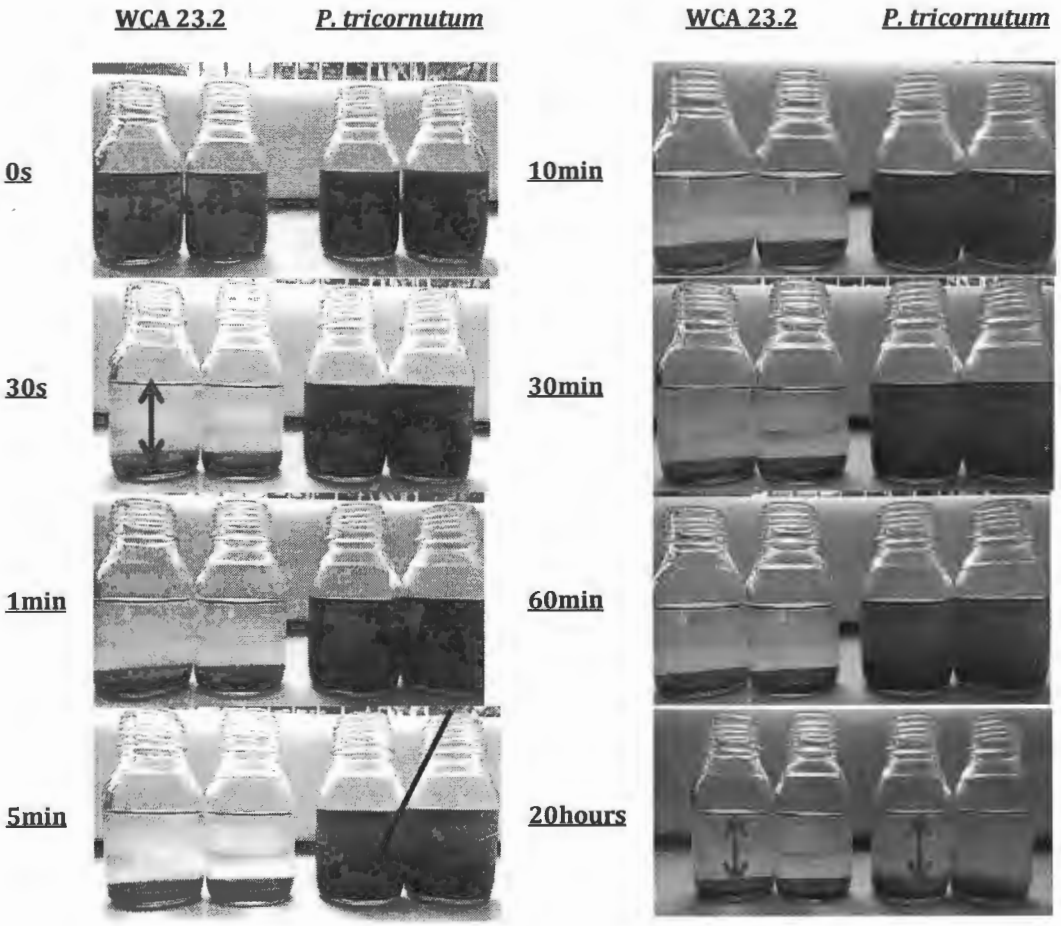


Figure 48: Visual depiction of the settling potential of WCA 23.2 **(LEFT)** and *P. tricornutum* **(RIGHT)**. Red arrows indicative of settled biomass. Images supplied by CSIR Biosciences.

The visual depiction of the settling study clearly shows that most of the WCA 23.2 cells had settled after 30 seconds, whereas *P. tricornutum* only started to settle after 5 minutes. The supernatant however still contained more than 83% of the cells in the suspension. After a 20-hour settling period the supernatant of WCA 23.2 contained less than 1% of the cells from the original cell

suspension whilst the supernatant of *P. tricornutum* contained 13% of cells, clearly indicating WCA 23.2's superiority in settling potential. The settling potential of WCA 23.2 can be ascribed to the ability to 'clump' or auto flocculate naturally. The mechanism behind auto flocculation was not clear at the time of processing. Despite the superior settling potential of WCA 23.2, settling as a harvesting method was also chosen for *P. tricornutum* as it was still capable of settling via gravity. This decision was supported by the fact that centrifugation is an expensive method and not always economically viable to a process. The harvesting of the ponds is depicted in Figure 49.

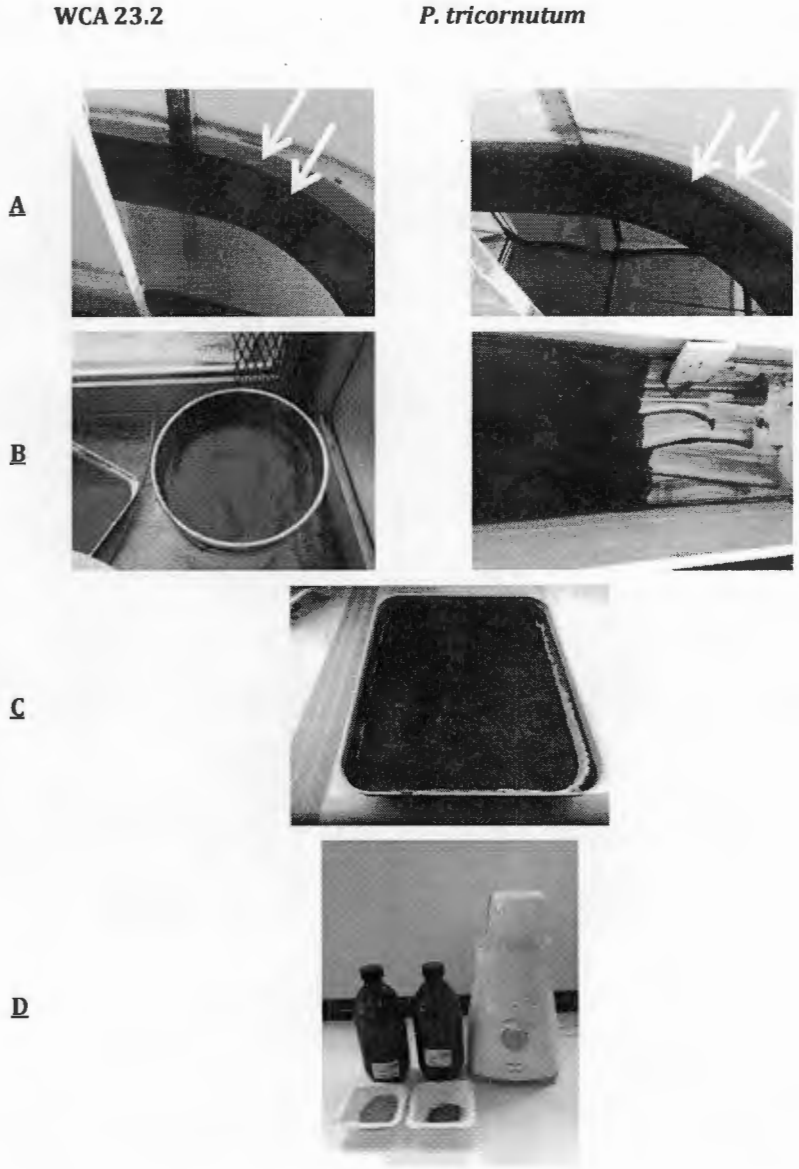


Figure 49: Figure depicting the train of events from pre harvest wet biomass to post harvest-dried biomass, where (A) settled biomass (B) sieved/filtered/dewatered biomass (C) harvested biomass and (D) dried and ground biomass. White arrows indicative of settled biomass. Images supplied by CSIR Biosciences.

Paddle wheels were switched off and the cells were allowed to settle via gravitation (Figure 49A left). The top media layer ('supernatant') for WCA 23.2 was drained after 60 minutes' settling. The wet biomass was pumped through a screening pan fitted with a 25-µM mesh (Figure 49B left) to achieve dewatered slurry, which was then loaded into a drying pan to dry for 48 hours at 50°C (Figure 49C).

The ponds containing *P. tricornutum* were left over night to settle after which the top media layer was drained in the same fashion. The wet biomass was pumped into large cylinders to facilitate further settling overnight. The eventual wet slurry was poured into drying pans (Figure 49C) and allowed to dry in the same manner. Complete harvesting for WCA 23.2 occurred within 1-2 hours of pond drainage, while complete harvesting for *P. tricornutum* occurred over a period of 48 hours of pond drainage. Dried biomass was ground using a homogeniser (Figure 49D).

The theoretical, actual and percentage losses as obtained for the March 2014 and May 2014 pond studies are show in Table 21. The theoretical harvest values were calculated by sampling and drying 50 mL of the pond culture prior to harvesting. The actual harvest values was obtained from the gross biomass collected, dried and ground during the harvesting period as seen in Figure 49D.

Table 21: Important harvesting values indicating theoretical and actual biomass yields and losses.

	March 2014		**May 2014**	
	WCA 23.2	*P. tricornutum*	WCA 23.2	*P. tricornutum*
Theoretical harvest	0.234 kg (SD±6.36)	0.353 kg (SD±1.41)	0.203 kg (SD± 5.48)	0.398 (SD±4.69)
Actual harvest	0.170 kg (SD±0.04kg)	0.268 kg (SD±0.07kg)	0.181 kg (SD±0.02kg)	0.331 kg (SD±0.17kg)
"% Loss"	27.74 (SD±15.49)	27.87 (SD±17.32)	10.83 (SD±12.98)	16.83 (SD±13.41)

It is evident from the table that the percentage losses were similar across the March 2014 and May 2014 studies. It must however be emphasised that *P. tricornutum* was harvested over two days as opposed to the 2-3 hours it took to harvest WCA 23.2. More efficient biomass harvest during the May 2014 cultivations can be due to the colder temperatures enhancing settling of biomass.

4. SUMMARY

The primary objective of the raceway study was to determine the effect of scale-up and reduced control of environmental factors on the growth and EPA productivity of the two species, specifically to evaluate the research hypotheses.

Published literature on raceway studies often reported biomass and EPA productivity as $g.m^{2}.d^{-1}$ – whereas in this work it is reported as $cells.mL^{-1}d^{-1}$ or $g.L^{-1}\ d^{-1}$. Data collected during the March 2014 and May 2014 cultivations was converted to a form where it was comparable to literature values. Converted values for aerial pond biomass productivities were calculated to for WCA 23.2 to be 5.62 $g.m^{-2}d^{-1}$ and 7.81 $g.m^{-2}d^{-1}$ during the March 2014 and May 2014 cultivations respectively. For *P. tricornutum*, it was 5.96 $g.m^{-2}d^{-1}$ and 4.44 $g.m^{-2}d^{-1}$ during the March 2014 and May 2014 cultivations respectively. Very little literature is available on actual commercial culture due to commercially sensitive process details. No literature was available for *P. tricornutum* cultivated in open raceways.

Another factor that played a role in this section was the seasonal weather changes between the pond studies, complicating the comparisons and final conclusions to be drawn. For both species, the biomass productivity was lower than that observed in the laboratory scale experiments. Both species was able to sustain a high specific EPA production across both pond studies.

The WCA 23.2 biomass auto flocculated and consequently settled relatively rapidly once the paddle wheel had been switched off, suggesting harvesting at a larger scale would be easy and inexpensive. The *P. tricornutum* biomass took a lot longer to settle, which would present a significant processing challenge at a larger scale. The impact of this on the overall viability of the process would need to be quantified through a full techno-economic analysis.

CHAPTER VI

CHAPTER VI – CONCLUSIONS AND RECOMMENDATIONS

The key objective of this research study was to select the most promising local isolates and literature species and to compare them on their performance under different laboratory conditions. As an ultimate comparison, the top performing isolates were cultivated in pilot-scale outdoor raceways designed to mimic actual commercial production conditions. This investigation has potential impacts on two levels; (1) a contribution towards research and development, that in turn (2) will have possible impact on the manufacturing sector for the production of microalgal omega-3 fatty acid production where the end user benefits the most as the products developed will support a healthy lifestyle. This thesis presents a rigorous and holistic approach to comparing the selected isolates in the applied field of bioprocess development. The key sub objectives identified at the commencement of the study were successfully achieved and the conclusions are presented below.

Selection of EPA producing species for comparative testing

The research objective for this chapter was to select one culture collection species and one locally selected microalgal isolate to compare in terms of growth and EPA production. A detailed survey of the literature and a critical analysis of this data identified *Chlorella minutissima, Monodus subterraneus* and *Skeletonema costatum,* followed by *Nannochloropsis oculata, Phaeodactylum tricornutum* and *Porphyridium cruentum* as the key organisms to consider for the study. Further refinement of the data based on EPA content, growth rate and EPA induction potential resulted in *Nannochloropsis oculata, Phaeodactylum tricornutum and Chlorella minutissima* being selected for further laboratory evaluations. Three local CSIR isolates were selected based on previous growth studies and rapid lipid screens and included WCA 23.2, WCA 27.2 and WCC 39.3.

The laboratory comparison studies concluded that *P. tricornutum* was a better process organism than *N. oculata* due to its superior EPA productivity (10.85 $\mu g.d^{-1}$). WCA 23.2 had a higher maximum and average growth rate as well as EPA productivity (0.64 d^{-1}, 0.27 d^{-1} and 7.83 $\mu g.d^{-1}$ respectively) than WCA 27.2 and was therefore selected as the local isolate of choice. The 16S rRNA gene sequencing confirmed the identity of the *P. tricornutum* culture, while the CSIR isolate was most likely a member of the genus *Amphora. C. minutissima* was eliminated from the study due to the correct strain not being available to the study at the time of experimental analysis, while WCC 39.3 did not produce any EPA.

Investigating the impact of environmental factors on growth and EPA production

The research objective for this chapter was to compare the effect of selected environmental factors on growth rate and EPA production under laboratory conditions. The series of experiments was designed to evaluate the impact of pH control on biomass growth and EPA production, under nutrient sufficient and deficient conditions. The possibility of growing a culture under pH uncontrolled conditions is quite attractive – this would mean that less capital is spent on the maintenance of the culture making the process more economically viable. However, as it is a living system, conditions do need to be monitored and controlled as was the finding in this particular study. The pH study concluded that controlling pH is imperative to specific EPA productivity.

Although the growth rates for pH controlled and uncontrolled conditions in both species were similar (0.2-0.3 d^{-1} and 0.22-0.33 d^{-1} respectively) for the first 7 days, the impact on EPA productivity was negative for pH uncontrolled cultures. The proposition was made to control the culture at a slightly higher pH (pH 8.3) with the hopes that it may perform the same (or better) and use less acid to keep the pH at the set value. This too will prove to be advantageous on a large scale.

Comparative studies to investigate the benefit of fed-batch concluded that a fed-batch regime did not yield better biomass productivities with reported values ranging from 0.276-0.457 $g.L^{-1}d^{-1}$ for batch conditions and 0.232-0.470 $g.L^{-1}d^{-1}$ for fed-batch conditions. However, contradictory to initial assumptions, the specific EPA production was higher under batch conditions as opposed to fed-batch conditions. Reported values for specific EPA productivity was 0.175-0.412 $mg.g^{-1}d^{-1}$ for batch conditions and 0.032-0.122 $mg.g^{-1}d^{-1}$ for fed-batch conditions. From a process point of view it makes sense to choose the former as a nutrient regime. This regime is more cost effective in the sense that it requires less man power and time than a fed-batch regime.

Comparing the Performance of CSIR Isolate WCA 23.2 and P. tricornutum in a raceway system

The research objective for this chapter was to assess the biomass and specific EPA production of a CSIR isolate and culture collection species in an open raceway pond system from a bioprocess perspective. As expected, there was a significant loss in biomass productivity because of scale up of the process from flask to raceway for the WCA 23.2 species. However, this productivity loss was significantly less for *P. tricornutum*. Considering specific EPA productivities, *P. tricornutum* produced on average 2.5 fold more EPA than WCA 23.2 reaching productivities of 0.218 $mg.g^{-1}.day^{-1}$.

This finding alone is not sufficient to conclude that WCA 23.2 is not a suitable process organism for EPA production in open raceways. The fact that biomass settling studies clearly demonstrated that WCA 23.2 settles naturally by means of auto-flocculation in a matter of minutes and *P. tricornutum* requires an additional process step for cell separation could have significant cost implications for the overall financial feasibility of the processes. It is therefore impossible to make a conclusion on

the comparison without conducting a detailed techno-economic evaluation on both processes, which is beyond the scope of this study.

The experimental data indicated that EPA productivity was higher in *P. tricornutum* than WCA 23.2, under the conditions tested. However, the ease of biomass recovery and regulatory advantages associated with using an endemic species mean that a more thorough economic evaluation is required to draw a definitive conclusion.

Recommendation for future research

The CSIR plays host to a microalgal isolate database consisting of more than 750 indigenous, locally selected isolates. These isolates have been rapidly screened for lipid content with the goal of finding a high lipid producing isolate. Lipids are only one of the many value-added products to be found in microalgae and hence the database is a promising resource to be screened for numerous other high value products. There seems to be a trade-off between the two organisms investigated; the higher EPA producer is difficult and time-consuming to harvest, while the other that produces less EPA is quick and easy to harvest. Here the question to answer is which is more beneficial to the process: more income from the high value product or less capital spent on the downstream processing? A detailed cost analysis can conclude the speculation.

A major challenge realised during the course of this work is going from bench to raceway and the effect on economies of scale. It was clear that there is a decrease in productivity with scale from lab to raceway ponds, however this reduction is productivity needs to be assessed in parallel to the economies scale that accompany a larger production facility and the implications on overall cost of production.

Capital cost reduction will require advances in cultivation system design and materials to reduce the cost per cultivation surface area while retaining the media and favouring healthy growth. This aspect warrants investigation in future work. The development of directed, rather than random, mixing to create light/dark cycles in cultivation systems will reduce energy consumption and increase photon utilization.

As a final recommendation, the isolates excluded from the initial comparisons should be evaluated, specifically the marine strain of *C minutissima*? It is also recommended that further screening of the local library should be conducted for high EPA productivity and perhaps combined with traits that would give high productivity and/or ease of harvesting? Other factors to be investigated include different downstream processing methods for extraction of EPA that are well established, in order to develop an economic process.

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APPENDIX A – SUPPLEMENTARY DATA FOR CHAPTER III

Table 22: Literature values and culture conditions for *C. minutissima*

Organism origin	μ (d^{-1})	BP ($g.L^{-1}d^{-1}$)	EPA	Conditions	Reference
UTEX 2341	0.43	0.032		2L stirred bioreactor, Guillard's, 25°C, 76 $\mu mol.m^{-2}.s^{-1}$, 8:16 LDLC, CO2 aeration	(Illman *et al.*, 2000)
France	0.12 (10°C) 0.66 (30°C)	-	-	Culture chamber, Dauta, 30-550 $\mu mol.m^{-2}s^{-1}$, 15:9 LDLC, CO2 aeration	(Aleya *et al.*, 2011)
UTEX 2341	-	0.099	36.7 $mg.L^{-1}$ yield on D10 (3.67 $mg.L^{-1}d^{-1}$)	250mL flasks, ASW media, 25°C, 60 $\mu Em^{-2}s^{-1}$, continuous, no aeration	(Vazhappilly& Chen , 1998)
Dr. C Kitakima	-	0.040		Flaska, Mann & Meyers, 20-25°C, 10 $J.m^{-2}s^{-1}$ fluorescent light, CO2 aeration	(Seto *et al.*, 1984)

Table 23: Literature values and culture conditions for *P. tricornutum*

Organism origin	μ (d^{-1})	BP ($g.L^{-1}d^{-1}$)	EPA	Conditions	Reference
UTEX 642	-	0.193	43.4 $mg.L^{-1}$ on D10 (4.34 $mg.L^{-1}d^{-1}$)	250mL flaks, LDM media, 25°C, 60 $\mu Em^{-2}s^{-1}$, continuous, no aeration	(Vazhappilly & Chen , 1998)
Own isolate	-	-	5.2 $mg.L^{-1}d^{-1}$	Tubular glass units, enriched seawater, 20°C, 152 $\mu mol.m^{-2}s^{-1}$, 12:12 LDLC, CO2 aeration	(Otero *et al.*, 1997)
UTEX 640	-	0.057 0.200	1.7 $mg.L^{-1}d^{-1}$ 2.9 $mg.L^{-1}d^{-1}$	Test tubes, Mann & Meyers, 20°C, 4000 lux , 16:8 LDLC, CO2 aeration	(Yongmanitchai & Ward , 1991)
Jinan University	0.11	-	-	250mL flaks, F/2 Guillard's, 20°C, 50 $\mu mol.m^{-2}s^{-1}$, 12:12 LDLC, no aeration	(Liu *et al.*, 2009)

Table 24: Literature values and culture conditions for *N. oculata*

Organism origin	μ (d^{-1})	BP ($g.L^{-1}d^{-1}$)	EPA	Conditions	Reference
Green (CS-179)	-	-	0.27 mg.L^{-1}d^{-1}	100l Poly-ethylene bags, F/2 media, 22°C, 100 μmol.m^{-2}s^{-1} , 12:12 LDLC, CO2 aeration	(Dunstan *et al.*, 1993)
NQAIF0101	0.41 0.33 0.32	13.4 4.4 g.m^{-2}d^{-1}		250mL flasks, L1, 24°C, 43 μmol.m^{-2}s^{-1} , 12:12 LDLC	(Huerlimann *et al.*, 2010)
UTEX LB 2164		0.014	0.08 mg.L^{-1}d^{-1}	250mL flaks, Erd-Schreiber media, 25°C, 60 μEm^{-2}s^{-1}, continuous, no aeration	(Vazhappilly & Chen , 1998)
Italy	0.13 0.10	-	-	2L flaks, F/2 media, 25°C, 70 μEm^{-2}s^{-1}, , no aeration	(Converti *et al.*, 2009)
NCTU-3	0.19	0.480		1L flaks, F/2 media, 26°C, 300 μmol.m^{-2}s^{-1}, CO2 aeration	(Chiu *et al.*, 2009)

Table 25: Literature values and culture conditions for *P. cruentum*

Organism origin	μ (d^{-1})	BP ($g.L^{-1}d^{-1}$)	EPA	Conditions	Reference
UTEX 161	-	-	5.3 mg.L^{-1}d^{-1}	Tubular glass units, enriched seawater, 20°C, 152 μmol.m^{-2}s^{-1}, 12:12 LDLC, CO2 aeration	(Otero *et al.*, 1997)
UTEX 161	-	0.096	1.7 mg.L^{-1}d^{-1}	250mL flaks, Porphyridium media, 25°C, 60 μEm^{-2}s^{-1}, continuous, no aeration	(Vazhappilly &Chen , 1998)
IAM R-3	0.50 0.68	0.124 0.173	1.14 mg.L^{-1}d^{-1} 1.93 mg.L^{-1}d^{-1}	1L flaks, ASW media, 23°C, 1.5klux, CO_2 aeration	(Akimoto *et al.*, 1998)
UTEX 161	0.19 0.12	-	-	Flasks, F/2 media, batch, 23°C, 7000 lux, 18:6 LDLC.	(Razaghi *et al.*, 2014)