Development of an Eicosapentaenoic acid Production Bioprocess using an Indigenous Microalgal Isolate

A thesis submitted to the University of Cape Town in partial fulfilment of the requirements for the degree of Master of Science in Engineering

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PLAGIARISM DECLARATION

I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own.

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ABSTRACT

Eicosapentaenoic acid (EPA; 20:5) is an omega-3 polyunsaturated fatty acid of increasing interest as a nutraceutical. An indigenous microalgal isolate suitable for an EPA bioprocess was selected by screening monoalgal isolates from the Council for Scientific and Industrial Research (CSIR) micro-algal culture collection. A Cymbella diatom (A23.2) was selected for superior EPA production in both growth and stress conditions, using both fluorescent microscopy and flask studies. Studies investigated increasing biomass, improving EPA content, and optimising overall EPA productivity in a multi-stage bioprocess.

Growth studies found self-regulatory systems in both phosphate and nitrate metabolism. These mechanisms were absent in silicate and bicarbonate consumption, prompting their optimisation in the bioprocess medium. Cultivation pH was found to have a statistically modelled optimal value of 7.2 and a light intensity at a low range of 60 – 70 $\mu mol.m^{-2}.s^{-1}$ was found to be suitable. Nutrient and physicochemical parameters were assayed individually, and revealed cell productivities of between 2.0×10^8 to 3.0×10^8 $cell.L^{-1}.hr^{-1}$ in batch culture bioreactor studies. Further studies demonstrated the use of both nutrient stress and physicochemical stress to enhance EPA production.

These results informed the choice of operating parameters for a proof of concept, multi-stage raceway-based EPA bioprocess, consisting of a single growth pond and three stress ponds linked in series. The growth phase EPA productivity data of 0.68 $mg.L^{-1}.day^{-1}$, was higher than that of the stress phase, supporting its classification as a growth-associated product. Further, the EPA productivity in the raceway was more than twice that of initial batch culture screening. Once experimental limitations are addressed, a re-design of the bioprocess can be undertaken by optimising growth phase residence time, medium flow-rate and partial/complete elimination of the stress phase.

The EPA productivity of the diatom used in this work has the potential of reaching commercially viable values. The development of a commercial indigenous EPA producer has a dual impact, as it addresses various nutritional and medicinal market demands and improves the sustainability of the world's fish stocks.

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Acronyms

ADHD	Attention Deficit Hyperactivity Disorder
ALA	Alpha-linolenic acid
ASW	Artificial salt water
ATP	Adenosine triphosphate
BHT	Butylated hydroxytoluene
CeBER	Centre for Bioprocess Engineering Research (University of Cape Town)
CSIR	Council for Scientific and Industrial Research
C17-TAG	Glyceryl triheptadecanoate
C19-ME	Ethyl nonadecanoate FAME
DHA	Docosahexaenoic acid
DIC	Dissolved inorganic carbon
DNA	Deoxyribonucleic Acid
DPA	Docosapentaenoic Acid
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic acid
FAMEs	Fatty acid methyl esters
GC	Gas chromatography
GP	Growth Pond
HPLC	High performance liquid chromatography
HTGC	High temperature gas chromatography

Kcs	β-ketoacyl-coenzyme A synthase
NMR	Nuclear magnetic resonance spectroscopy
PMB	Phosphomolybdenum blue
PUFAs	Polyunsaturated Fatty Acids
RNA	Ribonucleic acid
SFAs	Saturated Fatty Acids
SP	Stress Pond
TAGs	Triacylglycerides
USA	United States of America

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction to omega-3 fatty acids

Omega-3 (ω−3) fatty acids are a group of long chain polyunsaturated fatty acids (PUFAs), classified as lipids, that have a final double bond in the n-3 position (Figure 1.1). Also referred to as essential fatty acids (EFAs), omega-3 and omega-6 fatty acids cannot be synthesized by humans, but must be consumed in their diet (Simopoulos 2002). The medically and nutritionally significant omega-3 essential fatty acids include α-linolenic acid (ALA; C18:3), docosahexaenoic acid (DHA; C22:6) and eicosapentaenoic acids (EPA; C20:5) (Simopoulos 2002; Kris-Etherton et al. 2003). In a formula to denote any essential fatty acid, the first numeral denotes the total number of carbon atoms in the chain and the second (one-digit) numeral denotes the degree of unsaturation, or number of double bonds.

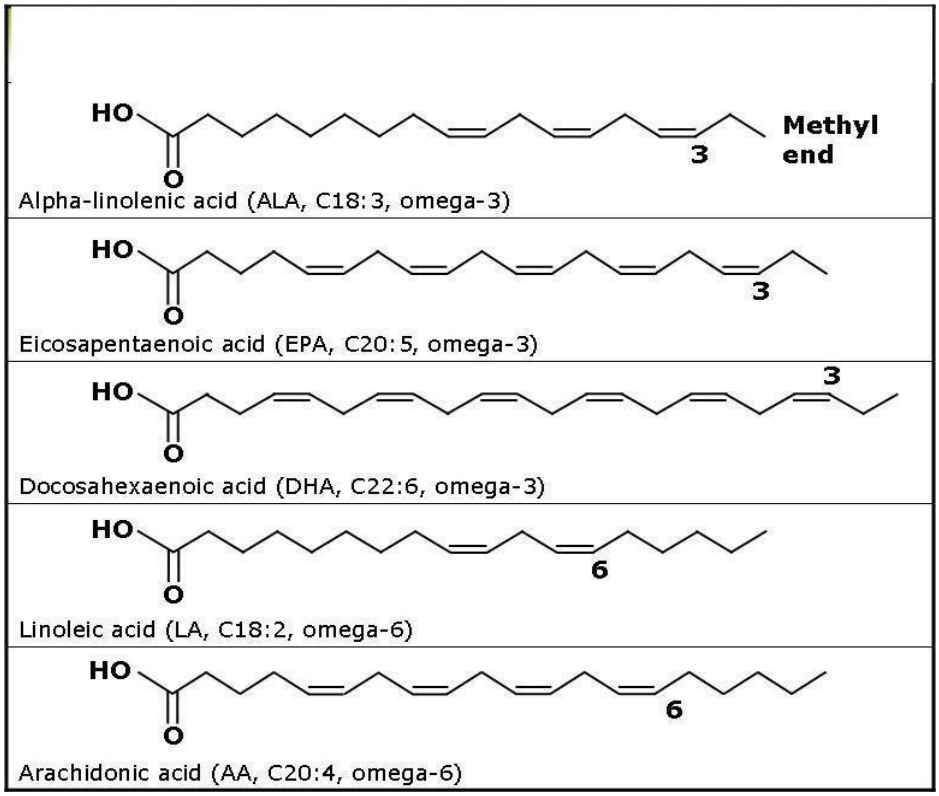


Figure 1.1 Chemical structures of Omega group fatty acids (Food Today 2008)

1.1.1. *General benefits of EPA and other omega-3 fatty acids*

The very long-chain polyunsaturated fatty acids EPA, DHA and arachidonic acid (AA) are known for their nutritional importance. They confer flexibility and selective permeability properties to cell membranes, have been shown to be vital for brain development, beneficial to the cardiovascular system and form important nutraceutical and pharmaceutical targets in both human and animal health (Ward and Singh 2005; Yongmanitchai and Ward 1989; Agostoni et al. 1995). Specific health benefits associated with these fatty acids include prevention of stroke, asthma, as well as rheumatoid arthritis prevention (Agostoni et al. 1995; Kris-Etherton et al. 2003; Ward and Singh 2005; Jude et al. 2006).

Attention to the health benefits of PUFAs first emerged when it was noted that populations deriving a substantial proportion of their food from fish had a much lower incidence of heart disease. The American Heart Association and the European Society for Cardiology recommend the intake of 1 g per day of the two omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for cardiovascular disease and sudden cardiac death prevention (Harris 2007). A number of changes in cell function are also observed upon the incorporation of EPA and DHA into the cell membrane. Among them are the modulation of the eicosanoid system towards vasodilatation, less inflammation and a lowering of blood triglycerides (Harris 2007). Ultimately, the beneficial effects of omega-3 fatty acids result from the competitive inhibition of inflammatory compounds produced from an omega-6 rich western diet.

EPA, in particular, is known to play an important role in mammals, as an agent for the reduction of blood platelet aggregation and blood cholesterol, as well as the prevention of other blood-circulatory diseases. The possible mechanisms by which omega-3 fatty acids reduce the risk of cardiovascular disease, summarised by Kris-Etherton et al. (2003), include:

- Ventricular arrhythmia reduction
- Mild hypotensive effects
- Nitric oxide induced endothelial relaxation promoted
- Retardation of atherosclerotic plaque growth
- Reduced adhesion, platelet derived growth factor
- Anti-inflammatory properties

Medical research frontiers are exploring EFA properties which can be used in psychiatric disorders, low birth weight and developmental differences such as autism and attention deficit hyperactivity disorder (ADHD).

There are a number of reports of significantly reduced levels of omega-3 EFA's in several psychiatric disorders. Reduced levels of linoleic acid, arachidonic acid, EPA and DHA were found in red blood cell membranes, compared to healthy controls in patients with schizophrenia (Emsley et al. 2003). A study of children with ADHD, reported reduced plasma concentrations of EPA, DHA and arachidonic acid (Burgess et al. 2000). In a study of boys with behaviour, learning and health problems, it was found that boys with lower omega-3 EFA levels had more behaviour problems, more temper tantrums and more sleep problems. All of these symptoms are commonly observed in autism (Burgess et al. 2000). The potential of EPA as an anti-tumour agent has also been documented (Jude et al. 2006).

1.1.2. *The omega-3:omega-6 ratio*

Excessive amounts of omega-6 polyunsaturated fatty acids (PUFAs) and a very high ratio of omega-6 to omega-3 promotes cardiovascular disease, cancer, and inflammatory and autoimmune diseases, whereas increased levels of omega-3 PUFAs, with a lower omega-6:omega-3 ratio, exert suppressive effects (Simopoulos 2002; Simopoulos et al. 1999; Hamazaki and Okuyama 2003). Eicosanoids are hormone-like substances, including prostaglandins, thromboxanes and leukotrienes (Simopoulos 2002; Hamazaki and Okuyama 2003). Eicosanoids made from precursor omega-3 fatty acids often have opposing functions to those made from omega-6 fatty acids (i.e. anti-inflammatory rather than inflammatory). If both are present, they "compete" for specific enzymes, so the ratio of omega-3:omega-6 directly affects the type of eicosanoids produced (Simopoulos 2002; Simopoulos et al. 1999). Where cellular proteins are genetically determined, the PUFA composition of cell membranes is, to a great extent, dependent on the dietary intake.

1.1.3. *Dietary sources of EPA*

Common foods which include EPA are flaxseed and cold water oily fish, such as eel, mackerel, herring, salmon and sardines. Six times richer than most fish oils in omega-3, flax or linseed (*Linum usitatissimum*), and its oil, are perhaps the most widely available botanical source. Other lesser known botanical sources include kiwi fruit, butternut, walnuts, strawberries and broccoli, amongst others (Gerster 1998). Most green leafy vegetables and

other organic products, is rich in alpha-linolenic acid (ALA), which is the "parent" fatty acid to DHA and EPA.

Humans can convert ALA to the functional forms EPA and DHA, provided they have the necessary vitamin and mineral co-factors required for enzyme activity. Recent evidence, however, shows that the capacity to produce EPA and DHA from ALA is inefficient and unlikely to supply nutritional requirements, especially in rapidly growing young children (Simopoulos 2002). The production of omega oils, using flax or any other vegetable source, necessitates the use of large tracts of arable land, fertilizer and water. Furthermore, a diet that is rich in trans-fatty acids, for instance, negatively impacts the metabolism of alpha-linolenic acid into EPA and DHA (Simopoulos 2002).

PUFAs are almost exclusively synthesized by plants. Animals can convert one form of PUFA to another through elongation and desaturation, but very few can actually synthesize these fatty acids (Brett and Müller-Navarra 1997). Animal oils and fats tend to be a mixture of all the fatty acids in the diet, as well as those that the animal makes itself. Unlike animal-derived oils, most plant oils are very simple in composition, since they contain only the fatty acids for which they have the biochemical and genetic means to produce. The plant based oils are also more stable, making it an attractive source for the supplement and nutraceutical industry (Belarbi et al. 2000).

1.1.3.1. *Fish derived omega-3 fatty acids*

Commercial sources of omega-3 fatty acids are almost exclusively limited to fish oils, due to the relative convenience and economics of bottling or encapsulation of low grade fish oil extracted from liver and tissue. However oceanic fish are a dwindling resource, due to overfishing, pollution and climate change (Guschina and Harwood 2006). Reliance on fish oil as the sole source of long chain omega-3 fatty acids is also complicated by taste, odour and stability (Ward and Singh 2005). Thus the supply of omega-3 fatty acids from fish is unlikely to meet future requirements. The quality of the oil depends on fish species, geographical location and quality of the food consumed. In addition, there is the ever increasing danger of contamination of lipids with environmental soluble pollutants such as mercury. The substantial consumption of certain fish or their oils by young children and by pregnant and nursing females is not recommended, due to the potential of the oils to accumulate hazardous, hydrophobic environmental contaminants, as well as possible allergic reactions (Ward and Singh 2005). The dominance of fish oils in the omega supplement market also marginalizes both vegetarians and vegans.

In fish, DHA and EPA are the major components of cell membranes (Sargent et al. 1999). In order to purify PUFAs from low grade crude fish oil, expensive and complex techniques, such as adsorption chromatography, fractional or molecular distillation, enzymatic splitting, low temperature crystallisation, super-critical fluid extraction and urea complexation, must be employed (Guschina and Harwood 2006).

Consumption of a substantial amount of fish is required to maintain a daily dietary intake of 1 g DHA/EPA per day, suggesting that demand could exceed supply. Consequently, there is medical, nutritional and commercial interest in finding alternative natural sources of easily isolated EPA. Fish, like all other vertebrates studied so far, require long chain polyunsaturated fatty acids for their normal growth and development, including reproduction (Sargent et al. 1999). These PUFAs are obtained via bioaccumulation in the food chain from phototrophic algae, (which are the primary producers of PUFAs). The fish eat zooplankton that have fed on microalgae (Ackmanet al. 1964). In fish, as in terrestrial mammals, DHA, EPA, and AA are all involved in maintaining cell membrane structure and function. Fungi, especially of the order, Mucorales, and bacteria of the genera, *Shewanella*, *Alteromonas*, *Flexibacter* and *Vibrio* can accumulate relatively large amounts of EPA (Yongmanitchai and Ward 1989). However, the ability of bacterial and fungal fermentations to compete economically with traditional sources of omega-3 fatty acids is limited by low productivities and excessively long production times (Barclay et al. 1994).

1.1.3.2. Algal derived EPA

Marine algae, the primary producers in the marine food chain, are also the primary synthesizers of omega-3 fatty acids, with only a few species of bacteria and fungi producing these highly unsaturated fatty acids (Yongmanitchai and Ward 1989). Considering the biodiversity of microalgae and recent developments in genetic and metabolic engineering, this group of organisms represents a promising source for new products and applications. Arachidonic and other polyunsaturated fatty acids with 20 or more carbon atoms occur in lipids of phytoflagellates and dinoflagellates, green and red microalgae and in diatoms (Radwan 1991).

Microalgae have properties that make them particularly suitable for industrial cultivation. Phototrophic microalgae are able to grow in many aquatic, and some non-aquatic, environments at high temperatures and in saline and alkaline waters (Griffiths and Harrison

2009). Some microalgae can potentially be grown on arid land or in the ocean (Griffiths and Harrison 2009). In addition, the purity of algal oil gives it a two-fold advantage in increased oxidative stability and greater purity, relative to animal-derived oil as mentioned in Section 1.1.3. Algae can potentially produce value added products, (including fuels such as biodiesel), in conjunction with bioremediation projects (Kshirsagar 2013). The ability of algae to fix carbon dioxide has been suggested as a method of removing carbon dioxide from power station flue gases, and thus, can be used to reduce greenhouse gas emissions.

Although the algal kingdom is extraordinarily diverse and may comprise more than 200,000 species, major algal products at present are based on only 10 to 20 algal species, almost all of them, macroalgae (Ward and Singh 2005). This gives support to the development of microalgal bioprospecting programs in order to assess biodiversity and discover potential commercial applications.

1.2. Microalgal fatty acid production and analyses

A fatty acid is specifically a carboxylic acid attached to a long hydrocarbon chain, and can be thought of as lipid building block. Lipids are usually defined as those components that are soluble in organic solvents (such as ether, hexane or chloroform). The microalgal lipid profile or total lipid content broadly includes triacylglycerides, diacylglycerides monoacylglycerides, free fatty acids, phospholipids, sterols, carotenoids and vitamins A and D. These lipids can be further divided into two main groupings: the nonpolar and polar lipids. Triacylglycerides (TAGs) fall under the nonpolar or neutral lipid group, playing a role in energy storage. This is of interest to the biofuel industry, whereas PUFAs mostly consist of omega group structural polar lipids within algal membranes (Berge et al.1995). These polar lipids are usually found as glycolipids or phospholipids within chloroplastic membranes (Berge et al. 1995). Microalgal species such as *Skeletonema costatum* and *Chlorella minutissima* can accumulate up to 45% of their total fatty acid as EPA, which falls into the polar lipid category (Radwan 1991). Average cell age also affects the lipid classes, producing changes in the amounts of nonpolar and polar lipids at different cultivation stages. In general, the content of polar lipids (which make up the PUFAs) tends to decrease with culture age (Wen and Chen 2003). It also varies with environmental factors and species of microalgae.

1.2.1. *Biochemistry of EPA synthesis*

The most regularly synthesized fatty acids in microalgae have chain lengths that range from C_{16} to C_{18}, longer chain lengths typical of omega group fatty acids are characteristically synthesized by the marine microalgae, as seen in Table 1.1. The conversion of saturated fatty acids (SFAs) to longer chain fatty acids is performed by a combination of desaturase and elongase enzymes, which are system-specific to most plants and algae. Some micro-organisms use a polyketide synthase-like system to produce EPA or DHA (Vrinten et al. 2007). Mammals lack the ability to introduce any double bonds in carbon atoms between C_9 and the terminal methyl group. This implies that such living systems cannot synthesize linoleic or linolenic acid, the parent fatty acids of the omega group (Radwan 1991).

Table 1.1 Proportions of Omega PUFAs in a selection of marine microalgae (adapted from Yongmanitchai and Ward 1989)

Organisms	**PUFAs (% total fatty acids)**		
	20:4 (AA)	20:5 (EPA)	22:6 (DHA)
Monochrysislutheri	1	19	-
***Pseudopedinella* sp.**	1	27	-
Coccolithushuxleyi	1	17	-
Cricosphaeracarterae	3	20	-
***Nannochloropsis* sp.**	-	27	-
Nannochloropsis salina	1	15	-
Hetermastrix rotundra	1	28	7
***Cryptomonas* sp.**	-	16	10

The biosynthetic routes for EPA have been elucidated by studies employing externally supplied fatty acids, as well as radio labelled fatty acid precursors (Khozin et al. 1997; Shiran et al. 1996). Exogenously supplied fatty acids were incorporated into Eustigmatophyte group eukaryotic microalgae and were metabolized along two different pathways, omega-3 and omega-6, shown in Figure 1.2. In the first (omega-6) path, c-linolenate is produced following the desaturation of linoleic acid. It is then elongated to dihomo c-olenate, subsequently desaturated to arachidonate and finally to EPA. In the second (omega-3) path, c-linoleate is first desaturated to a-linolenate, which is then converted to C18:4 (n-3), C20:4(n-3) and finally to EPA C20:5(n-3). The letter appearing before each organic molecule indicates is organic chemistry nomenclature indicting the position of the functional group on the carbon chain.

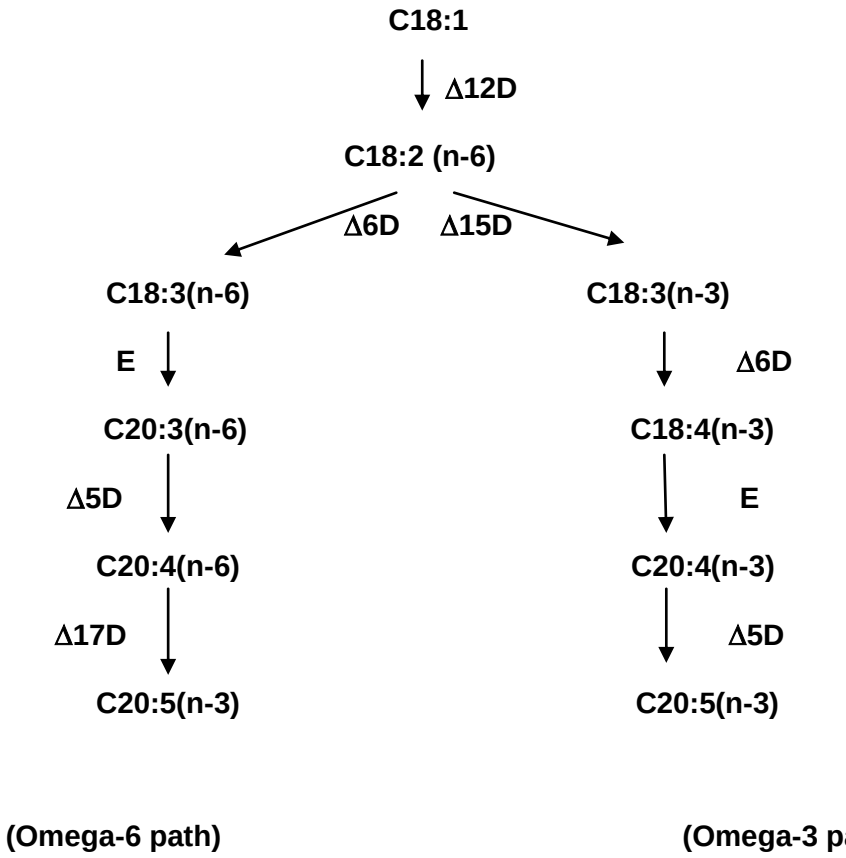


Figure 1.2 **Two metabolic pathways for the biosynthesis of EPA in eustigmatophytes via elongation and desaturation of fatty acid hydrocarbon chains (The most active route is shown with bold arrows, ΔD: varying desaturase E: elongase)**

(Guschina and Harwood 2006)

The lipid and fatty acid content and composition of microalgae vary, depending on culture conditions and strain. It has been found that, in some cases, lipid accumulation and composition can be enhanced by imposing a stress factor, such as nitrogen starvation, silicon deficiency, phosphate limitation, high salinity or heavy metal stress (Guschina and Harwood 2006). In one study, the supplementation of media with 100 ng of vitamin B12 per litre produced a 65% increase in yield of EPA per litre of *Phaeodactylum tricornutum* culture, compared with the control (Yongmanichai and Ward 1991). Different algal species tend to respond uniquely to environmental fluctuations, which warrant an investigation into enhancing EPA production in the candidate microalga.

1.2.2. *Environmental factors influencing fatty acid productivity*

In nature, algae are subjected to a wide variety of environmental stresses. Some, such as low temperatures, have a broad effect, temporarily slowing most aspects of metabolism. Where metabolic imbalance is created, some algal species respond by increasing their lipid content, as both an energy reserve and, where light stress occurs, a means of reducing photosynthesis by directly absorbing part of the incoming light (Thompson Jr. 1996). Subtle, localized fluctuations in lipid composition and the resultant changes in the physical behaviour of the cell membrane can have very significant physiological consequences. According to Thompson Jr. (1996), specific lipid types fulfil unique roles in regulating the metabolism of the cell, in addition to their more passive structural role. Lipid metabolism is key to adjusting the cell to both gradual and acute stress. Thus, cellular fatty acid content can be manipulated indirectly, by varying growth conditions.

1.2.3. *Nutrient stress*

A deficiency in mineral nutrients typically causes a steadily decreasing growth rate. Under these circumstances many algal species continue actively synthesizing fatty acids. Certain green algae can thereby more than double their lipid content, as a percentage of cellular dry weight (Thompson Jr. 1996). It is important to note that lipid productivity is a function of both cell and lipid production (Griffiths and Harrison 2009) and will be further addressed in Section 1.3. It has been speculated that fatty acid synthesis and accumulation in the form of triglycerides serves as an ideal mechanism for siphoning off the large amounts of ATP and NADPH, which, under different circumstances, might have been channelled into cell growth (Thompson Jr. 1996). Triglycerides (same as Triacylglycerides) can be temporarily stockpiled and utilized as an energy-rich carbon source when conditions for growth improve. In contrast, the lipid study by Arisz et al. (2000) found that the effect of nutrient-limitation on the lipid and fatty acid composition of *Chlamydomonas moewusii* mainly altered the fatty acid composition of the PUFA chloroplast or polar lipids.

Fluctuations in nitrogen concentration have been reported to cause a variety of cellular responses, depending on the species. Nitrogen starvation and very high levels of nitrogen (15 mM) increased total lipid content (as a percentage of dry weight) in *Ulva pertusa* (Floreto et al. 1996). Nitrogen starvation has been reported to enhance SFAs in many species of algae, but is generally accompanied by a sharp decrease in proportion of PUFA (Alonso et al. 2000; Chu 1943; Fidalgo et al. 1998; Floreto et al. 1996; Guschina et al. 2003; Illman et

al. 2000; Ohta et al. 1993). Under nitrogen stress, however, *Botryococcus braunii*, *Dunaliella bardawil* and *Dunaliella salina* produced a higher percentage of EPA than in nitrogen replete medium (Ben-Amotz et al. 1985). Generally changes in nitrogen concentration result in a change in the relative distribution of fatty acids (Sukenik 1991).

Increased EPA production by *P. tricornutum* caused by elevated CO_2 concentration was reported in (Yongmanitchai and Ward 1991). In a study using *Nannochloropsis* sp., the marked increase of EPA content in response to an elevated CO_2 concentration was temporary and the higher EPA content was not maintained (Hoshida et al. 2005). This can possibly be attributed to cells adapting to a higher CO_2 concentration after exposure. The data from this study (Table 1.2), demonstrated that brief CO_2 supply at the end of the cultivation phase yielded maximum EPA production, from standard CO_2 in ambient air. Therefore, manipulating available CO_2 concentration may be a useful parameter in controlling cellular EPA content before biomass harvesting.

Table 1.2 Effect of increasing medium CO_2 concentrations from initial ambient air on *Nannochloropsis* EPA production at various cultivation time periods (Hoshida et al. 2005)

CO_2 concentration and time of CO_2 alteration	**EPA concentration ($\mu g.L^{-1}$)**	**EPA productivity ($\mu g.day^{-1}$)**
Ambient air	167	62
0.3% (day 1.5)	161	60
2% (day 1.5)	55	20
0.3% (day 1.5), 2% (day 2.5)	230	86
2% (day 3.5)	340	126

It is generally accepted that phosphate-limitation causes the replacement of membrane phospholipids by non-phosphorus glycolipids. The phosphorus requirements for optimal growth differ considerably from species to species. Studies on the optimal phosphate concentrations for the growth of diatoms and green algae, under defined laboratory conditions, revealed that concentrations below 50 $\mu g.L^{-1}$ were limiting, those of about 20 $mg.L^{-1}$ were inhibitory and 0.1 to 2 $mg.L^{-1}$ was optimal (Chu 1943). A study by Guschina et al. (2003) found that algae maintained their phosphoglyceride synthesis under phosphate deficiency because there were significant endogenous phosphorus stores in the algae, as

revealed by X-ray probe electron microscopy. Yongmanitchai and Ward (1991) investigated the effect of phosphate concentrations on the EPA production of *P. tricornutum*. They found that the EPA yield was higher at a high phosphate concentration. However, in the culture of *Pythium irregulare*, a higher initial phosphate concentration resulted in a lower EPA yield (Stinson et al. 1991). Ohta et al. (1993) investigated the growth and EPA content of *Porphyridium purpureum* under different phosphorus concentrations. Their results showed that the optimal phosphorus concentration was 3 mM over the range of 0.3 to 30 mM phosphorus investigated. Omega-3 PUFAs such as EPA mainly exist in the cells in the form of polar lipids i.e. phospholipids, therefore the phosphorus level may significantly affect the cellular content of these fatty acids.

Diatoms are the predominant siliceous organisms in the marine environment, storing hydrated amorphous silica in their cell walls which mineralizes to form a structure called the frustule (Martin-Jezequel et al. 2000). Diatoms need silicon to form their frustules (cell walls composed of amorphous silica), thus investigations of EPA production by diatoms involve this essential nutrient. *Nitzschia laevis* was reported to yield higher EPA under silicon deficiency (Wen and Chen 2003). This in contrast to *Stephanodiscus minutulus,* when grown under silicon, nitrogen or phosphorus limitation showed an increase in triacylglycerols and a decrease of polar EPA rich lipids, expressed as a percentage of the total lipid profile (Lynn et al. 2000).

1.2.4. *Physicochemical Factors*

Factors influencing the optimisation of the synthesis of longer chain fatty acids would be those that promote the biosynthesis of cellular polar lipids, increase membrane fluidity, as well as enhance the rate of metabolism. With this in mind, physicochemical factors such as temperature, light, salinity and pH are also crucial.

1.2.4.1. *Temperature*

The effect of ambient temperature on the composition of intracellular fatty acids of two microalgae, *Chlorella vulgaris* and *Botryococcus braunii*, was studied under batch culture conditions. Both responded to the temperature regimes by similar changes in their intracellular fatty acid composition, showing a decrease in the relative content of the more unsaturated fatty acids when temperature was increased (Sushchik et al. 2003). In another study, lipids and fatty acids in cultures of the haptophyte, *Isochrysis galbana*, grown at temperatures of 15 and 30°C, were analyzed and compared. Total lipids accumulated at a

higher rate at 30°C due to a higher growth rate, with a slight decrease in the proportion of non-polar lipids to polar lipids (Zhu et al. 1997). A fatty acid assay of four tropical Australian microalgal species, a diatom *Chaetoceros* sp., two cryptomonads, *Rhodomonas* sp. and *Cryptomonas* sp. and an unidentified prymnesiophyte, cultured at a range of 5 - 10°C, have reported similar increases in total lipid content (Renaud et al. 2002). EPA was identified in all species with the highest amount in the prymnesiophyte, where its percentage decreased at higher temperatures. All species had lower percentages of DHA at higher temperatures. The results of an earlier EPA optimisation study, using *Nannochloropsis* sp., also found that cellular EPA content decreased with increasing temperature (Sukenik 1991).

In Figure 1.3 using the fresh water *P. tricornutum*, optimal temperatures for enhanced EPA production fell between 20 and 25°C. However, the relative distribution of EPA had an optimal value at a temperature slightly below the optimum for growth. A temperature shift strategy seems to have been evolved by these microalgae to enhance overall omega-3 PUFA production, as optimal temperature for growth may have been beyond the limits for efficient PUFA metabolism (Wen and Chen 2003). Such a phenomenon has been observed in many different algal species, including *Nannochloropsis* sp. (Sukenik 1991) and *P. irregular* (Stinson et al. 1991).

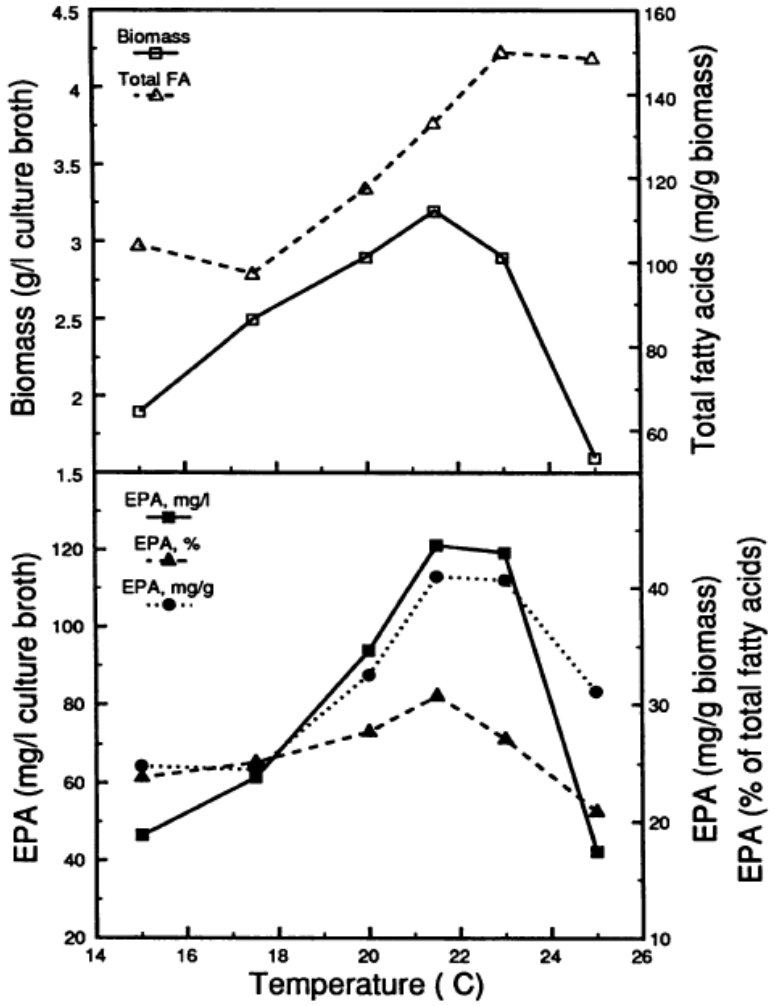


Figure 1.3 Effect of temperature on growth and production of EPA and total fatty acids by *P. tricornutum* mg.L^{-1}, mg.g^{-1} biomass and % of total fatty acids (Yongmanitchai and Ward 1991)

It is known that C_{20} PUFAs are associated with the chloroplast and membrane constituents, such as phospholipids and galactolipids. Thus, factors that increase membrane fluidity at sub-optimal temperatures may increase the biosynthesis of these fatty acids (Radwan 1991). Temperature is thus a crucial factor in EPA metabolism and the optimal temperatures for cultivation and EPA synthesis must be determined for a commercial microalgal EPA producer. This optimal temperature range will govern the selection of either a closed cultivation or an open pond system.

1.2.4.2. *Light cycling and intensity*

Illumination (both natural and artificial) is dependent on low light penetration, which decreases with increasing culture density (Langley et al. 2012). Mixing is the most practical

way to dilute radiation evenly to all cells in the culture, improving the light regime. This can be done by supplying an adequate air flow rate or mechanical agitation. A growth aspect study by Rocha et al. (2003) found that continuous illumination of *Nannochloropsis* sp. yielded faster growth, when compared to diurnal light/dark cycles in a sparged vessel. The strain in Figure 1.4 showed good adaptation to continuous illumination.

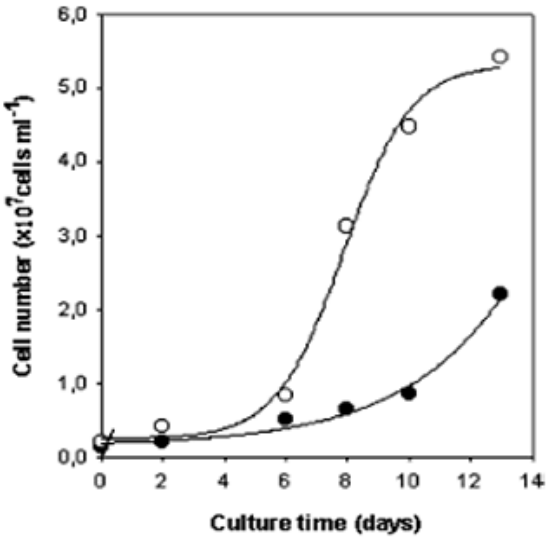


Figure 1.4 Effect of illumination cycles on *Nannochloropsis* sp. cell growth in Erlenmeyer flasks (Rocha et al. 2003)

(o) continuous illumination (•) light/dark cycles (12 h: 12 h)

A light-intensity study by Sukenik (1991) found that *Nannochloropsis* sp. growth was light-saturated at a photon flux above 200 µmol photon.m^{-2}.s^{-1}. Along with this plateau in growth came an exponential decrease in the relative abundance of EPA. Brown et al. (1996) performed studies of light exposure with a marine diatom, in which cells grown under 100 µmol photon m^{-2}.s^{-1} continuous light showed a high accumulation of triacylglycerides (TAGs) and a reduced percentage of polar lipids (Brown et al.1996). A light intensity of 100 µmol photon m^{-2}.s^{-1} is exponentially lower than bright sunlight which can surpass 1000 µmol photon m^{-2}.s^{-1}. The study found that the contents of both storage and structural lipids were affected by light intensity; however, the content of the most unsaturated acid, EPA, was slightly higher in the algae under low light. When light was limiting, cells increased chloroplast membranes to maximise light utilization. These membrane lipids are composed of polar lipids, which are highly unsaturated, possibly resulting in an increase of EPA cellular content (Brown et al. 1996). Light intensity assays using the candidate EPA producer should

determine the intensity at which EPA synthesis is optimal, this would govern cultivation light intensity in a closed cultivation system and govern location selection of an open pond system.

1.2.4.3. *Inorganic carbon speciation and pH*

For photosynthetic organisms, CO_2 is one of the most important factors that affect growth and metabolism. Inorganic carbon sources have a special role in intensive microalgal cultivation, with carbon supply usually being either in the form of CO_2-enriched air or as bicarbonate salts. The ability to use both sources may be a competitive advantage over phytoplankton using CO_2 exclusively, when other essential nutrients or light are not rate-limiting for growth. Supplementation of media with sodium bicarbonate was reported to increase final cell concentration, as well as EPA production per cell in the marine microalga *Glossomastix chrysoplasta* (Hsiao and Blanch 2005). Higher terrestrial plants usually depend entirely on CO_2 as their only source of inorganic carbon for photosynthesis. Marine microalgae are able to supplement this with the carbon from dissolved inorganic carbon (DIC) in sea water, in various ionic forms (Nimer et al.1997).

Variation in culture pH can affect algal growth and lipid metabolism metabolically by changing the distribution of inorganic carbon species and thus carbon availability; it can alter the availability of trace metals and essential nutrients through effects on solubility and speciation; and at extreme pH levels, it can potentially cause direct negative physiological effects on enzyme function (Chen and Durbin 1994). In general, changes in pH levels in marine systems are the result of changes in temperature, dissolved oxygen and algal biomass production.

The relative concentrations of carbonate ions CO_2, HCO_3^-, and CO_3^{2-} of the carbonate system and the pH of culture medium are closely linked. As microalgae metabolise dissolved CO_2, there is an equilibrium trend for the pH to increase with increasing cell density. As culture pH tends to increase, carbonate increases and bicarbonate and molecular CO_2 decrease (Chen and Durbin 1994). This pH drift can be controlled by using buffers, by the addition of gaseous CO_2 or inorganic acids (Rocha et al. 2003).

Some algae are capable of growing under quite extreme conditions of pH, prompting many studies to see whether moderate pH changes can affect lipid metabolism in non-extremophiles. The relative percentage of TAGs in the total lipid content of *Chlamydomonas*

sp. grown in medium at pH 1 was higher than that in cells grown at higher pH (Tatsuzawa et al. 1996). It was further suggested that the increase in saturation of fatty acids in the membrane lipids of *Chlamydomonas* may represent a possible adaptation mechanism for low pH, in order to decrease membrane lipid fluidity (Tatsuzawa et al. 1996). Figure 1.5 illustrates the EPA content of polar lipids in *Glossomastix chrysoplasta*, in a narrower more neutral pH range, peaking at a pH of 7.2. A neutral pH is supported by many studies as the most conducive to EPA productivity and growth, since most metabolic enzymes are stable at this pH (Cohen et al. 1988; Hsiao and Blanch 2005; Nuutila et al. 1997; Tatsuzawa et al. 1996).

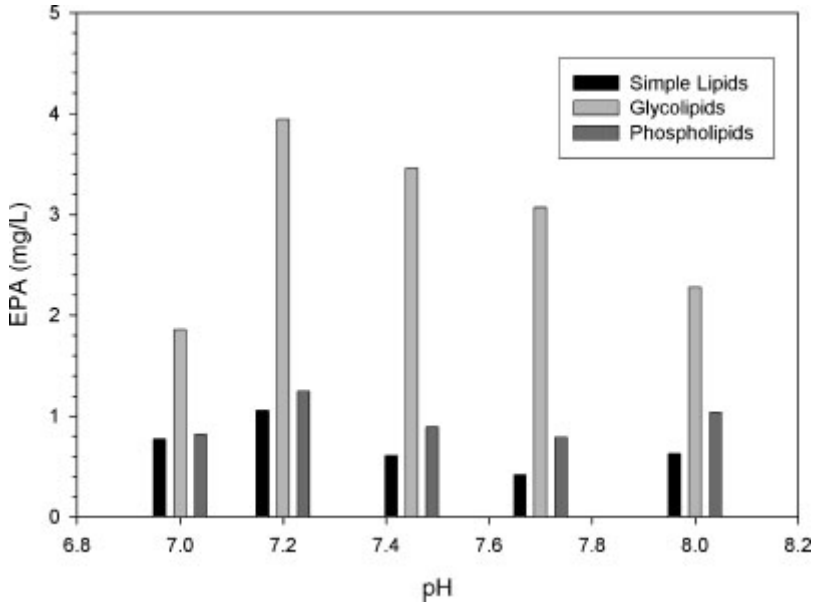


Figure 1.5 Effect of pH on steady-state EPA concentration in three polar lipid classes: simple lipids, glycolipids, and phospholipids in *Glossomastix chrysoplasta* (Hsiao and Blanch 2005)

1.2.4.4. *Salinity*

Not only are some algae well adapted to extreme pHs, they can also tolerate high salt levels. Azachi et al. (2002) showed that the expression of β-ketoacyl-coenzyme A synthase (Kcs), (which catalyses the first and rate-limiting step in fatty acid elongation), increased in *Dunaliella salina* cells transferred from 0.5 to 3.5 M NaCl. The salt-induced Kcs, jointly with fatty acid desaturases, were proposed to allow for intracellular membrane compartments to function in the high internal glycerol concentrations, used to balance external osmotic pressure (Azachi et al. 2002).

Ultimately, when one assesses the literature on manipulation of lipid metabolism by microalgae in its entirety, cognisance must be taken of the fact that these environmental effects are species specific. Having stated this, the various responses of EPA metabolism to cellular stress strongly suggest the investigation of a stress phase step in an EPA production process. When EPA content is considered, one should first determine the lipid classes which are specifically enriched with this particular fatty acid and then define the response of those lipids to variations in environmental conditions. However, this must also be balanced with adequate cellular growth to maintain high EPA productivity.

1.2.4.5. *Review of methods for fatty acid analysis*

In order for lipid-bound fatty acids to be analysed, the fatty acids have to be split from their glycerol backbone and converted into derivatives with lower boiling points (Figure 1.6), such as alcoholic esters (Eder 1995). Formerly, fatty acids were split off by an initial saponification step with sodium hydroxide or potassium hydroxide, and then methylated, creating very long reaction times (Eder 1995). Problems encountered in preparation of esters for analysis include: (1) incomplete conversion of lipids into FAMEs (fatty acid methyl esters); (2) changes in fatty acid composition during transesterification; (3) formation of artifacts which can be wrongly identified as fatty acids or overlap with FAME peaks; (4) contamination and subsequent damage to separation columns, resulting from traces of esterification reagents and (5) incomplete extraction of FAMEs after transesterification (Eder 1995).

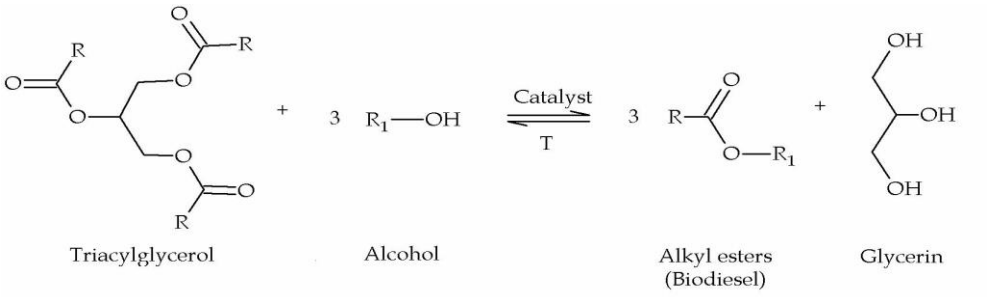


Figure 1.6 Reaction diagram showing lipid esterification in the presence of a catalyst (Feltes et al. 2011)

Two major methods of esterification include both acid- and base-catalysed reactions. The former involves heating with a large excess of anhydrous methanol and an acidic reagent, (hydrochloric or sulphuric acid), as a catalyst. Base-catalysed procedures are simpler and are more rapid, involving anhydrous methanol in the presence of a basic catalyst, usually sodium methoxide, which facilitates the exchange between glycerol and methanol (Christie

1993). This method requires less heating but is much more sensitive to water as well as reagent quality (Christie 1993). Most analysts have used acidic media for simultaneous extraction and transesterification, since these are affected less by small amounts of water (Griffiths et al. 2010). An internal standard, usually an odd-chain fatty acid, can be added to enable fatty acid profiling; this can also compensate for any partial hydrolysis that may occur (Christie 1993). Accuracy of extraction and esterification of lipids can be checked by comparing them with those of commercially available, individually purified FAME standards, processed prior to microalgal samples. Quantification of FAMEs in the case of gas chromatography (GC) is commonly carried out using peak areas in the chromatogram, after calibration with internal standards containing known amounts of FAMEs.

High performance liquid chromatography (HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify and quantify compounds. Retention time of fatty acids vary, depending on the interactions between the stationary phase, the molecules being analysed and the solvent(s) used. A major advantage is that HPLC operates at ambient temperature, so there is relatively little risk to sensitive functional groups (Matsukawa et al. 1999).

Silver ion (or "argentation") chromatography, is a technique that utilises the property of silver ions to form polar complexes reversibly, with unsaturated centres of organic molecules such as lipids (Adlof and Emiken 1985). A method used by Teshima et al. (1978) used a silver nitrate-impregnated silica gel column to separate EPA and DHA from squid-liver oil, after forming methyl esters, the authors were able to isolate a purity of 85 - 96% EPA.

Another emerging area in the field of lipid science is that of Nuclear Magnetic Resonance spectroscopy (NMR). Here algae are grown and exposed to a radiolabelled carbon isotope, such as ^{13}C. Algal biomass is carbonized and the content analysed using a hand-crafted, high-precision isotope ratio mass spectrometer (Beal et al. 2010). The advantages of NMR include: (i) the ability to analyse intact algae, (avoiding lengthy extraction procedures which have the potential of altering the sample), (ii) analysing many algal samples at once, and (iii) the ability to distinguish between types of lipids and types of fatty acids in lipids (Beal et al. 2010). Thus, NMR can be a useful complementary analysis tool to supplement data provided by solvent extraction and chromatography.

Samples of polyunsaturated fatty acids should be handled under nitrogen whenever possible and antioxidants such as 2,6-di-*tert*-butyl-4-methylphenol (Butylated hydroxytoluene) may be

added to solvents and reagents to minimize auto-oxidation (Christie 1993). A requirement of this study is to determine the EPA content in different microalgal isolates and in isolates cultured under various environmentally controlled parameters. To minimize the labour involved and potential sources of error, there have been many attempts to combine extraction and methylation of the lipids directly from microalgal biomass for chromatographic analysis (Montaini et al. 1995; Carvalho et al. 2006; Fajardo et al. 2007).

1.3. Properties of candidate EPA producer and growth optimisation

"*A reduction in the cellular growth rate may reduce the overall yield of the specific desired product, unless the relative increase in the cellular content of that compound is far greater than the reduction in growth rate*" (Sukenik 1991). Increased oil production under stress occurs during the cessation of cell division; however, the increased oil content of the algae does not necessarily lead to increased overall productivity of oil, since the rate of production of all cell components is lower under nutrient starvation or other stress. Environmental conditions that affect cellular EPA content may influence cell growth differently. Consequently, the sets of conditions that are required to maximize EPA content may differ to the sets that are needed for the maximization of EPA productivity. The cellular content of EPA may also be controlled by a different set of conditions to those regulating the accumulation of saturated or neutral lipids (Guschina and Harwood 2006; Sukenik 1991; Yongmanitchai and Ward 1991).

To obtain a higher yield of EPA from algae, culture conditions that increase EPA content per cell, but maintain optimum growth, have to be determined, and are ultimately dependent on the microalgal species selected. Alternatively, a bi-phasic process can be employed, for example, the biomass might be produced in the first phase at a relatively high optimum temperature, and in the second phase, the harvested cells might be chilled to increase their EPA content.

1.3.1. *Growth optimisation and media development*

In the development of a high-yield EPA production process by microalgal cultures, optimisation of medium components and environmental factors is vital as they can affect EPA yield and volumetric productivity significantly, as first mentioned in Section 1.2.2.

Statistically based experimental design was used as one of the many approaches to optimise EPA productivity of *N. laevis*, in which concentrations of NaCl, $CaCl_2$, pH and temperature were identified to be significant variables (Wen and Chen 2001). The experimental data were correlated by a second-order polynomial model, which was used to identify optimal values of these significant variables. This optimisation strategy led to an EPA yield of 280 $mg.L^{-1}$ and an EPA productivity of 28 $mg.L^{-1}.day^{-1}$. Like any other species, multiplication rate is highly dependent on environmental conditions. In an EPA production process, the goal should be to favour increasing growth rate as much as possible and to push the metabolic route to follow one direction.

The achievement of high lipid productivities of vegetative cells requires the formulation of a suitable culture medium. There is a lack of data comparing growth across various nutrient concentrations and species of microalgae under standard conditions. Table 1.3 is a summary adapted from Griffiths et al. (2011), comparing the growth of different high lipid producing microalgae in appropriate media, under standard conditions. The makeup of each medium is detailed in Appendix B.

Table 1.3 Growth performance of different microalgal isolates cultivated in various media (Griffiths et al. 2011)

Media	**Zarrouk**	**F/2**		**Walne's**			
Species	Spir	Nan	Ts	Cf	Pt	Pav	Iso
Max biomass conc (X) ($g.L^{-1}$)	2.7	1.9	2.4	1.5	1.7	1	2.5
Max ave inst biomass productivity (Qx) ($g.L^{-1}.day^{-1}$)	0.29	0.24	0.49	0.4	0.3	0.2	1.23
Max specific growth rate (mμ) (day^{-1})	0.43	0.76	1.2	0.7	0.7	0.6	1.32
Max doubling time (T_d) (days)	1.6	0.9	0.6	1	1	1.1	0.5

Spir - *Spirulina,* Nan - *Nannochloropsis*, Ts - *Tetraselmis suecica*, Cf - *Cylindrotheca fusiformis*

Pt - *Phaeodactylum tricornutum*, Pav - *Pavlova*, Iso - *Isochrysis* C4

1.3.2. *Selection of potential isolates for an EPA bioprocess*

With the development of detailed culture and screening techniques, microalgal biotechnology has the potential to meet the high demands of the food, energy and pharmaceutical industries. The ability to screen for algal strains on a large-scale for commercial EPA production requires the identification of high-yielding algal strains, and

identifying the optimal methods and systems to cultivate them. The use of an indigenous strain may circumvent vulnerability to both contamination and local conditions. A summary of the desirable characteristics for mass culture are provided in Table 1.4.

Table 1.4 Characteristics of microalgae for mass culture (Griffiths and Harrison 2009)

Characteristic	***Advantages***
Rapid growth rate	Competitive advantage over competing species, reduces culture area
High product content	Higher value of biomass (Note: use of metabolic energy leads to slower growth)
Large cell size	Reduces harvesting and downstream processing cost
Wide tolerance of environmental conditions	Less control of culture conditions required. Growth over range of seasons
CO_2 tolerance and uptake	Greater potential for CO_2 sequestration and use of waste CO_2
Tolerance of shear force	Allows cheaper pumping and mixing methods

A single algal species is unlikely to excel in all aspects detailed in Table 1.4; therefore, priority should be given according to available resources, culture system and environmental conditions (Griffiths and Harrison 2009). The use of an indigenous strain is, thus, highly desirable. Selection of fast growing strains encourages high biomass productivity, which, in turn, increases yield of product per harvest volume. The risk of contamination is also lowered, as a fast growing strain has the ability to outcompete other species of microalgae (Griffiths and Harrison 2009).

Green and fresh water algae rarely seem to contain the C_{20} and C_{22} polyunsaturated fatty acids that establish certain other algal divisions as the key foundation of the marine food chain. However, a number of marine species were found to produce these PUFAs, such as the EPA producing *P. tricornutum*. One practical advantage of green algae is that they generally grow faster than other algal classes, thereby offsetting, in part, the lower concentrations of PUFAs that they often contain (Thompson Jr. 1996).

It is known that brown and red algae generally contain higher proportions of EPA and DHA fatty acids than other taxa. Diatoms also represent a potential source of C_{20}-polyunsaturated

fatty acids. Although these unicellular algae are usually poor in arachidonic acid, they normally contain major proportions of eicosapentaenoic acid; this provides a strong motivation for the screening of diatoms for suitable EPA production.

Species selection is critical to any bioprocess, and properties of productivity, adaptability and robustness are identified to be the most important criteria for selection. The selection of an indigenous strain can not only satisfy the above requirements, but also remove the expense of using already commercialised genetically enhanced microalgae. Lipid and growth studies on specific algal classes, which display similar properties and responses to environmental fluctuations, are necessary to simplify screening programs. The immense biodiversity within South Africa is an added advantage for both commercial and scientific research and dynamic bioprospecting libraries will prove to be an invaluable asset in this regard.

1.3.3. *Commercial growth systems*

Open ponds can be categorized into natural waters (lakes, lagoons, ponds) and artificial ponds or containers, which most commonly include tanks, circular ponds and raceway ponds (Harun et al. 2010). Due to their limited control, operating conditions in open system ponds are a function of the local climate, thus, the location significantly contributes to the success of cultivation. Raceway systems use paddlewheels for agitation, thereby reducing cell shading, enhancing gas-liquid mass transfer and distributing nutrients for maximal algal growth. Lee (2001) reported that only a limited range of microalgae species are suited to being cultured in open ponds, as a severe culture environment is desirable to reduce contamination by other species (e.g *Dunaliella* - high salinity, *Spirulina* - high alkalinity and *Chlorella* - high nutrition).

It is well known that both the capital and operating costs of cultivation ponds are significantly less than those of closed systems (Table 1.5). It is important to note that limitations in open ponds include light availability, temperature control, evaporative losses, contamination and the requirement of large areas of land, although scope exists to enhance the design and associated productivity of open systems without increasing expense or sacrificing large land area.

Table 1.5 Comparison of open and closed cultivation systems relative to each other (adapted from Griffiths et al. 2011)

Factor	Open	Closed
Control over process parameters	Low	High
Contamination risk	High	Low
CO_2 loss	High	Low
O_2 build up	Low	High
Area required	High	Low
Productivity	Low	High
Consistency and reproducibility	Low	High
Weather dependence	High	Low
Cost (Operational and Capital)	Low	High
Energy requirements	Low	High

The construction, operating and maintenance costs of cultivation ponds are generally lower than photobioreactor options, being less technical in design and more scalable. The EPA productivity data of various EPA production process studies, using various cultivation systems and techniques, are summarised in Table 1.6.

Table 1.6 Comparison of EPA productivity of microalgae under various culture conditions

Organism	Culture vessels	Culture mode	EPA productivity *(mg.L^{-1}.day^{-1})*	Reference
Porphyridium cruentum	Pond	continuous	8.0	Ratledge 1997
P. tricornutum	Glass tanks	continuous	25.1	Yongmanitchai and Ward 1991
***Nannochloropsis* sp.**	Tubular PBR	continuous	23.0	Zittelli et al. 1999
Trachydiscus minutus	Flasks surrounded by external light tube panel	batch	88.0	Rezanka et al. 2010

1.4. Current applications and market

Omega-3 fatty acids are found in many different product applications, including infant milk formulas, adult dietary supplements, animal feed, food additives and pharmaceutical precursors. These applications represent an extensive market for PUFAs. The world wholesale market for infant formula alone is estimated to be about $10 billion per annum (Ward and Singh 2005). Current products are tailored mainly for the infant and elderly market, due to the cognitive benefits associated with the lipids (Belarbi et al. 2000). Other markets for omega-3 fatty acids include dietary supplements for pregnant and nursing women and applications in cardiovascular health.

Marine algae and rotifers containing PUFA's have been used as a source of enriched EPA in commercial fish cultivation and aquaculture, another niche market for algal nutraceutical oils (Sukenik et al. 1994). The results of a study by Brett and Müller-Navarra (1997) found that EPA as a single fatty acid, and not the omega-3 fatty acid family, was most strongly related to *Daphnia* growth. Therefore a high aquaculture growth rate may only be attainable when direct dietary sources of EPA are available.

Companies reported to be researching, developing, manufacturing or marketing single cell PUFA's and associated products include Aventis, Hoffman-LaRoche A.G, Martek Inc., Nestle, Novartis and Walmart (Ward and Singh 2005), some products are listed in Table 1.7. The former Martek Biosciences Corporation, now a part of Royal DSM, as of the end of 2010, received clearance for DHA inclusion in infant formulae in the United States of America (USA). The DHA for this application comes from the alga, *Crypthecodinium cohni* and contains 40–50% DHA but no EPA (Marvelous Israel-Magnetika Interactive 2009). In 2012, DSM was the primary supplier of algal-based DHA omega-3 fatty acid for the U.S. market (with approximately 80% market share). DSM is also the market leader in Europe and most of Asia (not including China) (Algae Industry Magazine 2014). A report from Packaged Facts, a market research website, reported that foods with added omega-3 fatty acids approached $4 billion in sales in 2010. In 2006, this figure stood at $2 billion, with the market still very much dominated by small entrepreneurial companies (Packaged Facts 2011). The current market price of microalgal EPA ethyl ester (95% pure) in bulk quantities is about $650/kg and any new source would need to compete with that price (Belarbi et al. 2000).

Table 1.7 Microalgal based Omega-3 fatty acid supplements and additives currently on the market

Company	Product Name	Fatty Acid	Reference
Royal DSM	Life's Omega	EPA / DHA	Algae industry Magazine (2014)
Cellana	ReNew Omega-3	EPA	Cellana (2014)
Deva	Vegan Omega-3	DHA	Deva (2014)

1.5. Project definition

1.5.1. *The current knowledge base*

Growth and lipid accumulation are often augmented by differing operating conditions, requiring a compromise or a bioprocess consisting of multiple stages, in order to optimise lipid productivity, the product of growth rate and lipid content. The impact of the ratio of carbon, phosphorus and nitrogen provided by the macro- and micro- nutrients on growth and EPA production must be determined. The physicochemical optimisation in literature was focused on light, temperature, salinity and pH. Environmental stress and its role in lipid accumulation have been demonstrated in many studies. Nitrogen deficiency was a common lipid trigger; however, physicochemical lipid triggers were species specific.

1.5.2. *Project aims and objectives*

1. Identify a suitable indigenous microalgal EPA producer to be used in an EPA production bioprocess from the CSIR microalgal culture collection.
2. Develop and optimise a growth stage medium, based on a standard fresh or saltwater medium from literature.
3. Optimise physicochemical parameters for cultivation to maximise biomass productivity.
4. Investigate the stress responses that enhance cellular EPA content and determine the associated EPA productivity.
5. Demonstrate a proof of concept EPA production process in a raceway pond system. Determine the average productivity of such a system, the role of process stress and identify key factors to be considered in the subsequent optimisation of this process.

The aim of the project is met by the following experimental objectives:

1. Perform GC profiles on indigenous high lipid producing microalgal isolates under balanced growth and nitrogen deficient stress conditions in flask studies, comparing growth and EPA production of positive hits to a known industrial strain. This objective is centred on pre-screening of these isolates using fluorescent microscopy.
2. Determine statistically optimum initial concentrations of impacting nutrients within growth media.
3. Determine statistically optimum physicochemical growth parameters of pH, temperature, salinity (if applicable) and light intensity of selected isolate.
4. Investigate physicochemical stress intensities to determine any significant increases in EPA yield.
5. Perform growth and EPA production studies using a raceway based EPA production process.

1.5.3. *Research Questions*

Research questions which guided the development of the bioprocess included:

1. What are the optimum environmental parameters for growth?
2. After optimising growth, is a stress phase step necessary in which greater lipid accumulation is triggered? If so, what stress conditions are best to apply?
3. What are the optimum environmental parameters for maximum EPA productivity?
4. What potential problems are likely to be encountered when upscaling to a raceway cultivation system?
5. What biomass productivity and EPA productivity can be achieved with this system?
6. What arrangement of raceway ponds will provide best EPA production: growth ponds only or a combination of growth and stress ponds?

1.5.4. *Research Approach*

The initial stages of this study involved qualitative evaluation of a number of indigenous microalgal isolates for EPA production. This progressed to a more quantitative evaluation of both EPA and biomass productivity in the final selection of a candidate organism for an EPA

bioprocess, an outline is shown in Figure 1.7. The hypothesis of the study that followed was that EPA productivity can be enhanced with an initial growth stage followed by cellular stress, rather than optimal cultivation alone, as demonstrated for neutral lipid groups in literature. The study focused mainly on optimisation of the process, with regard to inputs and cultivation environment in both growth and stress phases. These optimised operating parameters were combined within a cascading multi-stage raceway EPA production system, at a 100 L scale, to explore maximum productivity of EPA attained and the relative value of the stress stage.

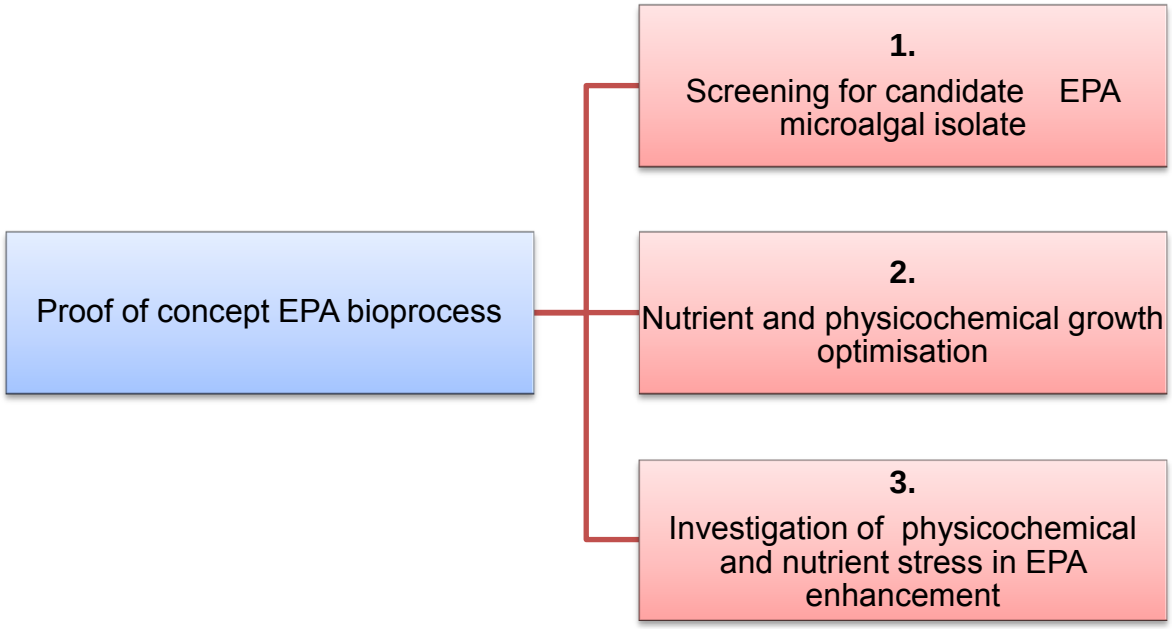


Figure 1.7 Research approach outlining the development of an EPA production bioprocess from organism screening to proof of concept study

2. GENERAL MATERIALS AND METHODS

The biodiversity of the South African coastline is remarkable; the western coast upwelling of the Benguela current supports an ecosystem of seaweed, fish, penguins and seals, with the warm east coast having even greater species diversity. The primary producers of both these rich marine ecosystems are algae (Branch et al. 1994). The microalgal bioprospecting programme within the CSIR bioprocess division was launched in order to isolate, purify and characterise indigenous lipid producing microalgae. The aim of the project was to establish an algal technology development platform, within the Bioscience Division of the CSIR. The team isolated over 50 microalgal strains, highlighting the need for long term maintenance of a working database of organisms, especially with regard to declining biodiversity across the world. Qualifying isolates populating the database underwent preliminary lipid screening, which consisted of an initial qualitative areal fluorescent microscopy method, using Nile Red dye, followed by semi-quantitative fluorescent spectroscopy. The database was used as a resource, with the objective of selecting a suitable EPA producer for studies in the development of an EPA bioprocess. Species selection is key in influencing reactor design, culture conditions, harvesting method and product range (Pulz and Gross 2004).

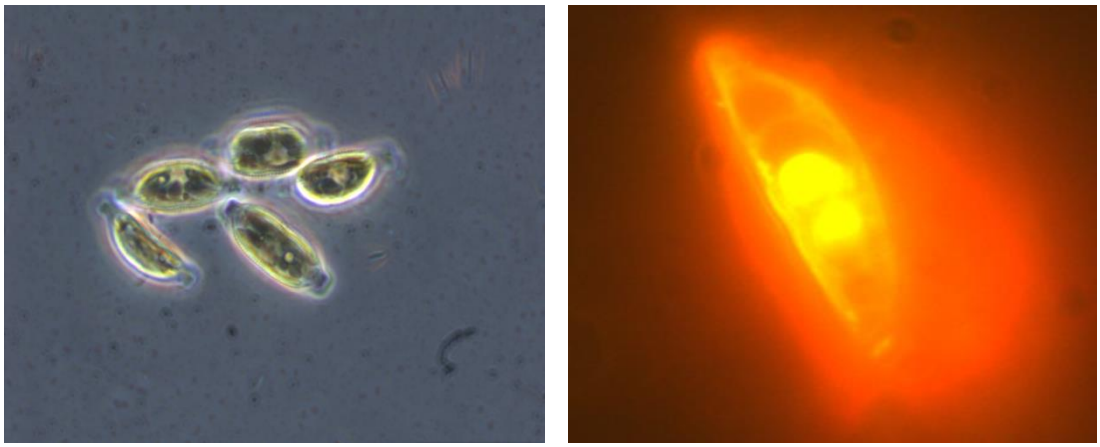
2.1. Screening of Micro-organisms

Twelve isolates from the CSIR bioprospecting database with Nile red fluorescent areal lipid content greater than 20%, were selected as candidate organisms for this study under nutrient replete conditions. Previously isolated mono-algal cultures were obtained from maintenance tissue culture flasks. Maintenance cultures were approximately six months old and grown under low light, with the incident light intensity determined to be approximately 1 - 4 µmol.photons.m^{-2}.s^{-1} restricting growth. Cultures were grown in either artificial salt water (ASW) or artificial fresh water (AF6) medium, depending on their sampling origin (Appendix A).

2.1.1. *Qualitative screening methods*

Initial screening used a Nile Red staining protocol in which a solution of Nile Red N3013 powder (Sigma-Aldrich, USA), was made up to a concentration of 250 mg.mL^{-1} using acetone, and stored in a lightproof bottle at 4 °C. Microalgal colonies from tissue culture

flasks were resuspended in 500 µL distilled water and adjusted to similar cell concentrations before 100 µL of the colony suspension was dispensed onto a viewing slide along with a drop of Nile red solution. These slides were then covered with a coverslip and viewed at an excitation wavelength of 450 - 490 nm using an Olympus BX45 fluorescent microscope (Olympus, Japan) equipped with a digital camera and AnalySIS software. The software allowed the calculation of areal lipid content, comparing the yellow lipid zone to the total cell area. Micrographs were viewed using an ocular of 10x magnification and an objective lens of 100x magnification unless stated otherwise. Figure 2.1 shows the siliceous cell wall of isolate A23.2, as well as the large lipid reserve when stained with Nile Red dye (yellow region).



(a) **(b)**

Figure 2.1 Microalgal diatom A23.2 visualised under (a) normal light (100 x magnification (b) stained with Nile red under fluorescent light (1000 x magnification)

Isolates having an areal lipid content of over 20% were selected for further screening for the presence of EPA. EPA presence was screened using direct transesterification of algal lipids and their extraction with hexane, followed by gas chromatographic (GC) analysis (see Section 2.3.4). *Phaeodactylum tricornutum*, a known EPA producing isolate, was supplied by the University of Hawaii and used as a positive control (Yongmanichai and Ward 1991). The qualitative results, displayed in Table 2.1, demonstrate the presence of omega-3 fatty acids, EPA and DHA in the positive control isolate and in isolate A23.2, while isolates A3.2 and A26.1 only revealed the presence of EPA. None of the other lipid-positive organisms produced these fatty acids. The first letter of the isolate name denotes the sample batch, the

second number before the decimal, the sampling location of the culture, and the last numerical, the isolate number.

Table 2.1 Qualitative results of EPA, DHA and DPA in lipid positive isolates through initial direct transesterification of lipids and then qualitative GC analysis

CULTURE	EPA	DHA	DPA
A4.1	-	-	-
A15.1	-	-	-
A15.2	-	-	-
A23.1	-	-	-
A23.2	+	+	-
A26.1	+	-	-
A26.2	-	-	-
A41.1	-	-	-
A11.1	-	-	-
A11.4	-	-	-
A10.3	-	-	-
A3.2	+	-	-
Phaeodactylum tricornutum	+	+	-

Based on the qualitative results obtained from the GC analyses, further studies were warranted on the EPA positive producers (A23.2, A26.1, A3.2). Considering that the production of different types of lipids by phytoplankton is influenced by the stage of growth (Sheehan et al. 1998), an evaluation of EPA produced at each growth stage, from the lag phase to the stationary phase and beyond, into the stress phase, under standard environmental parameters followed.

2.2. Reactors

Batch culture profiles (reported in Chapter 3) and lipid stress studies (reported in Chapter 6) were studied in round-bottomed flasks, sealed with cotton wool. Batch flask cultures (including inoculum cultures) were agitated on magnetic stirrer plates (Heidolph Instruments, Germany) using stirrer bars approximately 5 cm in length and 1 cm in diameter (increasing to 10 cm in length with volumes above 2 L).

Nutrient and physiological optimisation experiments, reported in Chapters 0 and 5, were performed in glass stirred tank bioreactors (B. Braun Biostat, Germany) as seen in Figure 2.2. The bioreactor allowed precise control of culture conditions (including pH and temperature) and representative sampling.

Figure 2.2 Glass bioreactor and experimental setup for microalgal cultivation surrounded by light rigging

(A. Agitator B. pH/Temp electrode C. Air filter D. Acid buffer)

Lighting for both batch flask and bioreactor experiments were provided by fluorescent light bulbs (Philips Master TL-D 90 Delux, Holland) mounted on light rigs. Incident light intensity measured at the flask/bioreactor surface ranged from 100 - 140 $\mu mol.m^{-2}.s^{-1}$; light intensity for bioreactor studies was measured at the centre of an empty reactor with the water jacket filled, resulting in an approximate range of 60 - 100 $\mu mol.m^{-2}.s^{-1}$. The light path length (to vessel surface) ranged from 15 – 20 cm in both reactor and batch culture studies. Experimental blocks are described in Table 2.2.

Table 2.2 Experimental blocks describing inoculum, standard operating parameters and reactors used

Experimental Block	Inoculum Preparation	Standard Operating Conditions and Media	Reactor
Selection of candidate EPA isolate (Chapter 3)	Vegetative culture flasks (500 mL), in ASW/AF6 media grown at 1 - 4 $\mu mol.m^{-2}.s^{-1}$ light intensity 23 ± 3°C. 40% ($v.v^{-1}$) inoculum used in study.	Cultures were grown in AF6/ASW media incubated at 23 ± 3°C, with lighting set at an average intensity of 100 - 140 $\mu mol.m^{-2}.s^{-1}$ and agitation set at 500 rpm.	2 L round-bottom flasks
Growth stage media development (Chapter 4) **Physicochemical growth optimisation study (Chapter 5)**	Round bottom flask (5 L) grown in Aquil medium (Appendix B) and maintained at 23 ± 3°C with 500 rpm agitation. Exposed to 100 - 140 $\mu mol.m^{-2}.s^{-1}$ light intensity with a 40% ($v.v^{-1}$) inoculum used in both studies.	Culture grown in Aquil medium at 25 ± 1°C and exposed to 60 - 100 $\mu mol.m^{-2}.s^{-1}$ light intensity. The pH was kept at 7.00 by 0.5% ($v.v^{-1}$) HCl titration and impeller speed set to 100 rpm.	5 L Bioreactor (B. Braun Biostat, Germany)
Investigation of physicochemical stress impacts on EPA production (Chapter 6)	Same inoculum used in Chapters 4 and 5 above.	Culture grown in Aquil medium at 23 ± 3°C with lighting set at an average intensity of 100 - 140 $\mu mol.m^{-2}.s^{-1}$ and agitation set at 500 rpm.	1 L round-bottom flasks

Results from experimental blocks described in Table 2.2 were used to develop a proof of concept, multi-stage, raceway pond bioreactor, under optimised environmental conditions. The system was designed to resemble an outdoor industrial system. An example of a general indoor raceway pond reactor is shown in Figure 2.3.

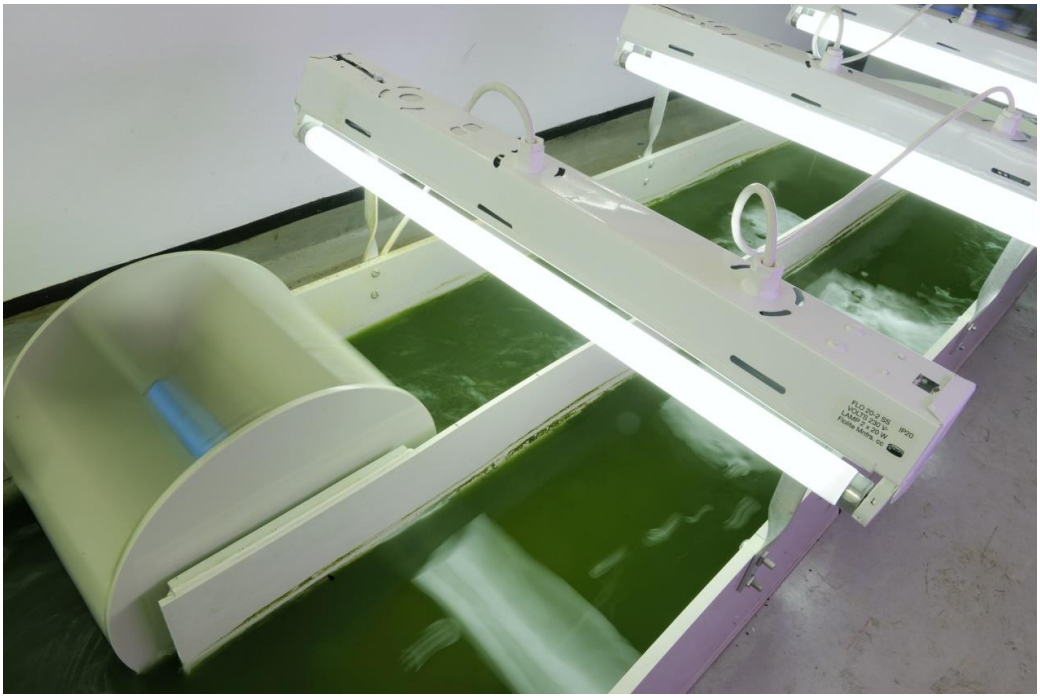


Figure 2.3 Prototype raceway pond microalgal cultivation system used at CSIR Modderfontein facility

A 4 L starting inoculum for the raceway was maintained in Aquil medium (Appendix B) under parameters of 23 ± 3^{o}C temperature, at 500 rpm agitation and exposed to 80 - 100 µmol.m^{-2}.s^{-1} light intensity. The raceway inoculum culture was grown until it reached light limitation (indicated by the accumulation of nutrients and levelling or decline in cell counts). Thereafter, this culture was used as an inoculum source for the next scale (10 L); where the same growth conditions occurred in order to keep the system as stable as possible. The scaling up of the culture took place until a volume of 20 L was reached (Figure 2.4). The 20 L culture was then used to inoculate the raceway pond up to a 100 L volume. The schematic and operating conditions of the multi-stage raceway pond system developed are detailed in Chapter 7.

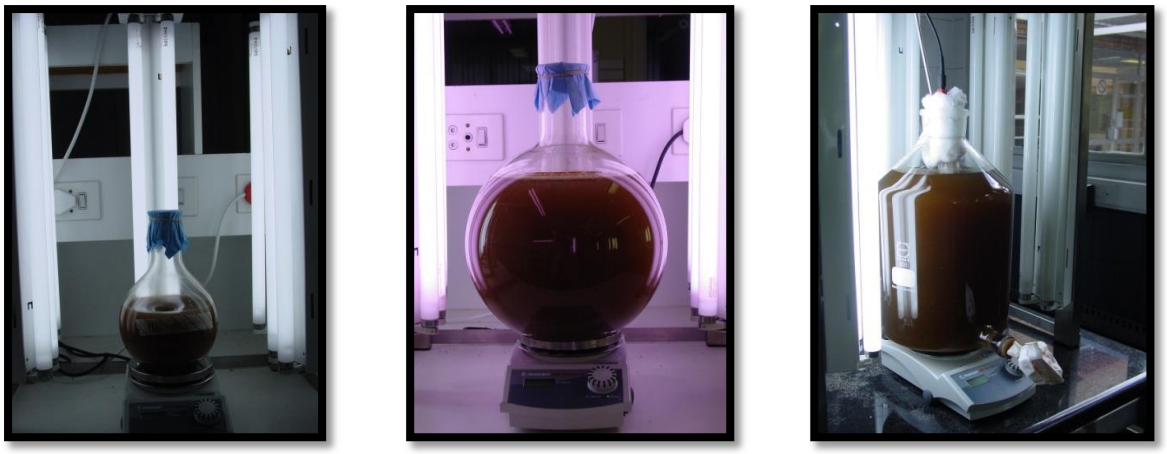


Figure 2.4 Raceway inoculum development from a 4 L round-bottom flask (left) to a 10 L round-bottom flask (centre) and then to a 20 L barrel (right) maintained under the same conditions until light limitation

2.3. Analyses

2.3.1. *Physicochemical and nutrient analysis*

The pH and temperature readings were recorded by a Master controller pH meter (Mettler Toledo, USA). Salinity was measured using a refractometer (Model 300052, Sper Scientific, USA) and light measured using a LI-COR inc. LI-1400 data-logger with attached LI190- SA quantum sensor (Nebraska, USA) (note measured but not controlled).

Nitrate, phosphate and silicate assays were performed using a Merck Nova 60 photometric Spectroquant® (Merck, New Jersey, USA) along with corresponding nutrient test kits. These tests involved the reaction of culture supernatant with specific kit reagents resulting in various colorimetric assays (Table 2.3).

Table 2.3 Merck Spectroquant® Nutrient Test Kit methods detailed

Nutrient Test kit and Catalogue No.	Principle	Interferences	Detection Limit and Coefficient of variation
Nitrate (fresh water) 114773	In concentrated sulphuric acid, nitrate ions react with a benzoic acid derivative to form a red nitro compound that is determined photometrically.	Nitrites	2.2 – 88.5 $mg.L^{-1}$ (1.6%)
Nitrate (sea water) 114942	In concentrated sulphuric acid, in the presence of chloride nitrate ions react with resorcinol to form a red-violet indophenol dye that is determined photometrically.	Nitrites	0.9 – 75.3 $mg.L^{-1}$ (1.4%)
Phosphate 114848	In sulphuric acid, orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Ascorbic acid reduces this to phosphomolybdenum blue (PMB) that is determined photometrically.	Reducing ions	0.2 – 15.3 $mg.L^{-1}$ (1.2%)
Silicate 114794	In sulphuric acid, silicate ions react with molybdate ions to form a yellow heteropoly acid. This is reduced to silicomolybdenum blue that is determined photometrically.	Sample turbidity	0.21 – 10.7 (1.3%)
Sulphate 114564	Sulfate ions react with barium ions to form slightly soluble barium sulfate. The resulting turbidity is measured in the photometer (turbidimetric method).	Sample turbidity	100 – 1000 $mg.L^{-1}$ (1.9%)

Carbonate concentration was determined by titration of a filtered 50 mL culture sample with 0.1 M HCl, carbonate concentration was calculated using Equations 2.1 and 2.2:

$$\text{Equiv. } CO_3^{2-} \text{ mM} = \frac{[\text{Vol. HCl (mL) pH 4} - \text{Vol. HCl (mL) pH 7.5}] \times 0.1 \text{ M}}{50 \text{ mL}} \times 1000 \qquad (2.1)$$

$$g.L^{-1}\ CO_3^{2-}\ \text{present} = \frac{(\text{Equiv. } CO_3\ \text{mM}) \times (g.L^{-1}\ CO_3)}{\text{mM } CO_3} \qquad (2.2)$$

$g.L^{-1}\ CO_3$ needed = 2.0

mM CO_3 needed = constant (24)

2.3.2. *Biomass productivity analysis*

Biomass quantification was first attempted using dry cell weight. Dry cell weight assays were conducted on each of four isolates discussed in Chapter 3. In the dry cell weight assay, filter papers were prepared by drying and pre-weighing. Culture samples (5 mL) were filtered through prepared 0.45 µm cellulose nitrate filters (Sartorius Stedim) and dried at 80°C overnight. The initial filter paper weight was subtracted from the final weight, to provide a measure of the mass of cells. Data were recorded in triplicate on each day and is summarised below in Figure 2.5.

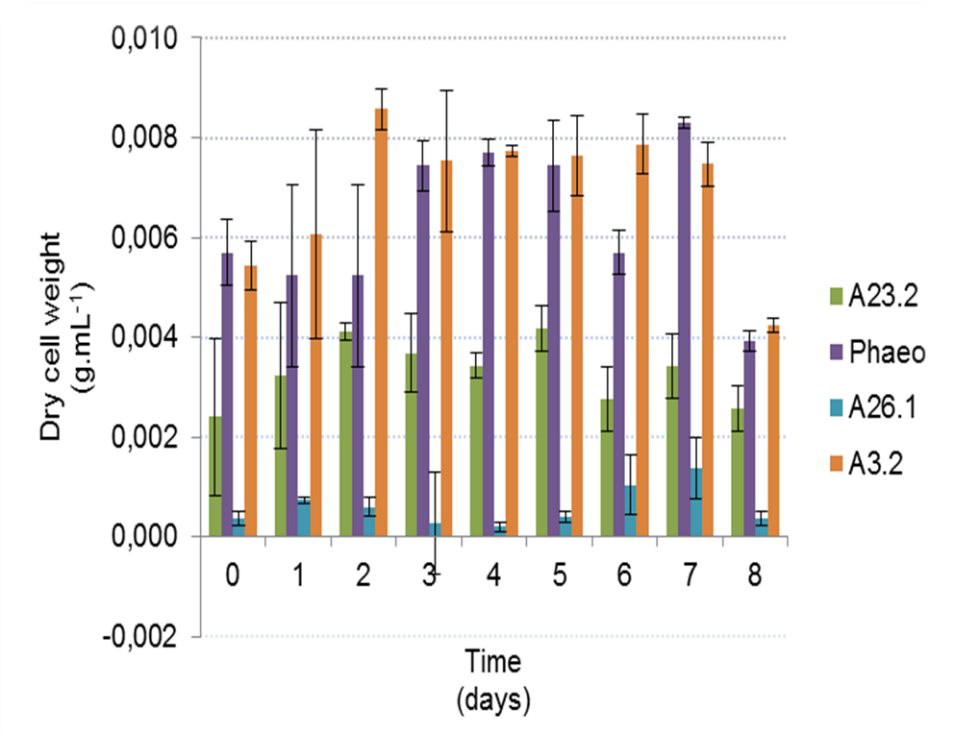


Figure 2.5 **Mean dry cell weight data recorded on each day for each isolate with corresponding standard deviation**

Standard deviation across replicates was highly variable; in addition, the coefficient of variance was also unacceptably high, sometimes exceeding 50% (data not shown). Data variance may have been owing to cell size differences, silicate content, the tendency of some cells to clump, pigment concentration and/or media salts (Griffiths et al. 2011). Cell counting was therefore chosen as the preferred method of biomass quantification for this project.

Microscopic cell counts throughout the project were prepared using a double Neubauer haemocytometer and slides were viewed using an Olympus BX45 fluorescent microscope (Olympus, Japan), with a digital camera and AnalySIS software to determine cell size and morphology. Cell counts were performed in triplicate on the same isolates assayed for dry cell weight, with mean data shown in Figure 2.6.

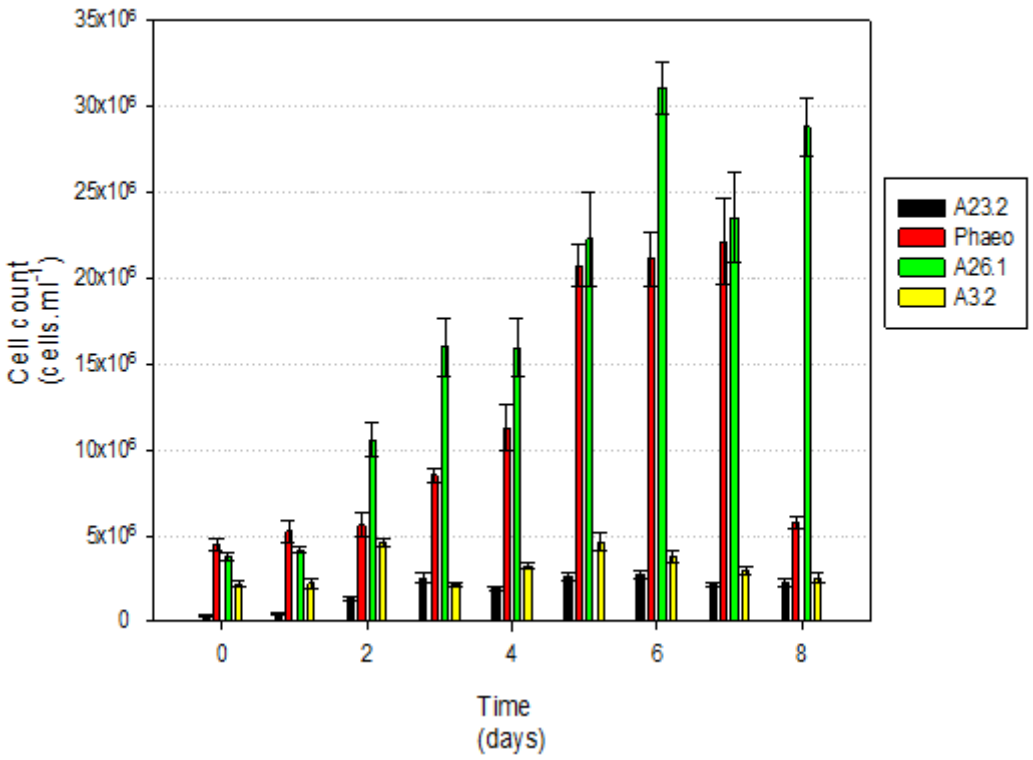


Figure 2.6 **Mean cell count data recorded on each day for each isolate with corresponding standard deviation**

Cell count data showed more consistency relative to dry cell weight, as seen in the comparative size of the error bars as well as the growth profile. Diatom A23.2 was subjected to pre-treatment with Tris-HCl (1M, pH 8) and then vortexing, to reduce clumping and

facilitate counting. According to McBride (1988), chloride neutralises electrostatic charges on suspended particles and reduces aggregation.

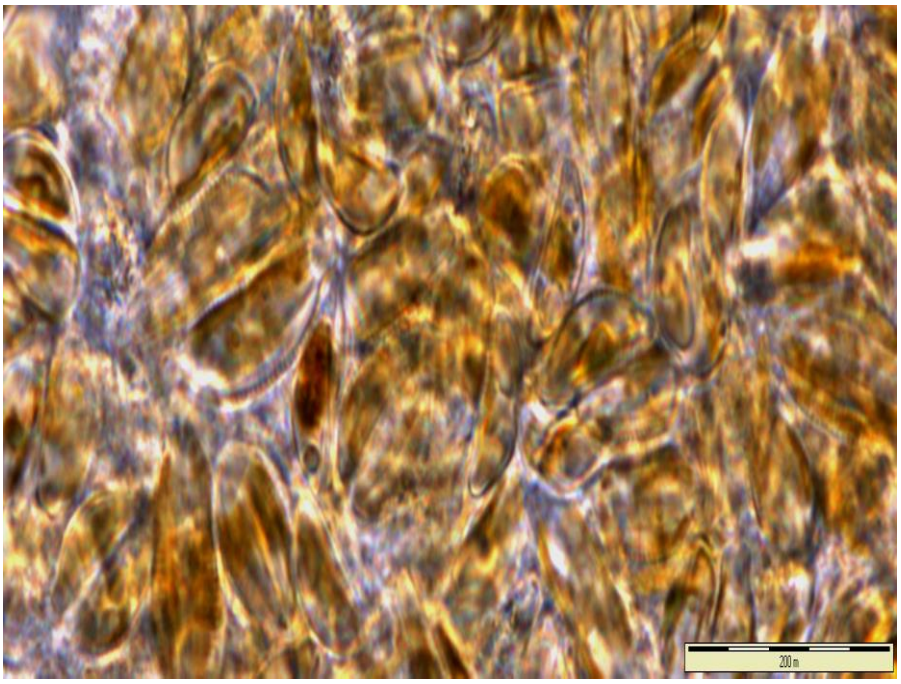


Figure 2.7 Clumped cells of microalgal diatom A23.2 prior to Tris-HCl treatment (100x magnification)

Specific growth rate (μ) was then determined by using data points of cell concentration conforming to an exponential trend, leading to high linearity of a plot of *ln* cell number against time. Doubling time (t_d) was calculated as (Equation 2.3):

$$t_d = \ln 2 / \mu \qquad (2.3)$$

2.3.3. *Extraction of lipids*

The methods for extraction of lipids from algae are diverse, depending on whether total lipid or a specific fatty acid is the desired product. Algal cell walls are extremely tough and difficult to break and a variety of methods have been employed to do this, these include:

- Bead beating
- Acid hydrolysis
- French press
- Homogenisation
- Ultra-sonication

Solvents used to extract lipids must demonstrate a high solubility for all lipid compounds and, more importantly, be sufficiently polar to remove EPA lipids from their association with cell membranes and lipoproteins. Chloroform/methanol mixtures are recognised and adapted by Bligh and Dyer (1959) for this purpose, resulting in an often-modified standard method for total lipid determination used for over 40 years. The extraction procedure, briefly, is as follows:

A methanol and chloroform solvent mixture is added to the sample, followed by mixing for a pre-determined time period. This mixing is repeated after adding a second portion of chloroform and after further addition of deionised water. After centrifugation, the organic phase is isolated using a Pasteur pipette, the solvent evaporated and the lipid weight recorded. This concept has been further developed and automated, in what is now called a Soxhlet extraction apparatus, in which a cellulose thimble containing the sample is immersed in solvent and refluxed over a set period of time. The solvent containing the lipid is then collected and evaporated, and the retained lipid weighed.

2.3.4. *EPA analysis*

For the scope of this project, specific lipid analysis of microalgae is needed, as opposed to the mapping of an entire lipid profile. The specific lipid analysis area has been mainly dominated by chromatographic techniques and these assays have advanced with regard to their target products.

2.3.4.1. *Method selected for direct transesterification of lipids*

For fatty acid screening of microalgae by GC, crude lipid must be transesterified to fatty acid methyl esters, such that they are volatile enough to enter the gaseous phase. Direct transesterification of lipids is a far more rapid and efficient process, with hydrolysis and esterification taking place concurrently (Griffiths et al. 2010). However, in order for accurate quantitative results to be obtained, the process must be optimised. The direct transesterification method used for this project is adapted from Griffiths et al. (2010) and is as follows:

i. Samples of 15 mL were first collected and centrifuged for 15 minutes at 4500 rpm, using an Allegra X-22R centrifuge (Beckman Coulter, USA). The supernatant was then discarded ensuring no loss of biomass; minimum wet biomass was 0.1 g.
ii. The resultant pellet was re-suspended in 0.8 mL of MilliQ distilled water and transferred (~1 mL) to a labelled 2 mL screw-cap vial (Simport). The sample was

then re-centrifuged at 12 000 rpm for 5 minutes, the supernatant discarded and excess water drained on a paper towel. Once again ensuring no loss of biomass.

iii. 250 µL of toluene, 500 µL of 0.5N NaOH in methanol and 10 µl of internal standard one was then added to the pellet. Internal standard one, Glyceryl triheptadecanoate (C17-TAG) is a quantitative standard, which is added to the sample vial prior to the start of the reaction. This standard is not transesterified and is used to quantify the efficiency of the transesterification process.

iv. The sample vial was then vortexed for 1 minute and placed on an incubating/shaking platform (Eppendorf® Thermomixer comfort, Germany) set at 950 rpm at 80ºC, for 20 minutes.

v. The sample vial was removed and placed on a -20ºC cooling block (Eppendorf®) for 5 minutes. 500 µL of 5% HCl in methanol was then dispensed into the reaction vial and the process in step iv, above, repeated.

vi. After the second incubation, another cooling step was performed for 5 minutes, as above. Subsequent steps were performed on the cooling block to prevent evaporation.

vii. 200 µL of distilled water (dH_2O) was dispensed to stop the reaction, 300 µL of hexane and 10 µL of internal standard 2 were added next. Internal standard two is an Ethyl nonadecanoate FAME (C19-ME) and is added during the final solvent extraction step; the standard is purchased as a FAME and is used to establish efficiency of extraction into the organic hexane layer.Following this the sample was vortexed for 1 minute and centrifuged at 12 000 rpm to aid phase separation, seen in Figure 2.8.

viii. 250 µL of the top organic layer was transferred into a pre-labelled 500 µL Eppendorf® tube, containing approximately 2 mg of anhydrous $MgSO_4$.

ix. The 500 µL Eppendorf® tube was vortexed for 1 minute and then centrifuged at 12 000 rpm for 2 minutes.

x. Aliquots of 200 µL of the organic hexane phase was then dispensed into a GC vial containing a glass insert and sealed.

Figure 2.8 The phase layer separation after direct transesterification of microalgal culture. Esterified lipid is seen at the top of the tube, dissolved in a hexane solvent with the microalgal cells at the bottom.

The assay was conducted in a -20°C block to prevent evaporation and consequent altering of standard and reagent concentrations. All reagents used were of chromatography standard and internal standards (Sigma-Aldrich, USA) were made up to a concentration of 1 $\mu g.mL^{-1}$ in hexane and stored at -10ºC.

2.3.4.2. *Method selected for gas chromatography analysis and quantification of EPA*

High temperature gas chromatography (HTGC) is a very common analytical method used for the determination of triglycerides, FAMEs and other organic and inorganic compounds present in fats and oils. Generally gas chromatographic analysis consists of fatty acid esterification, injection and separation, based on retention times within a column. Lipid extracts are first transmethylated to volatilize samples and then run on a GC column, before being compared to a mixture of known standards containing the target FAME, in order to identify and quantify specific fatty acids, as seen in Figure 2.9.

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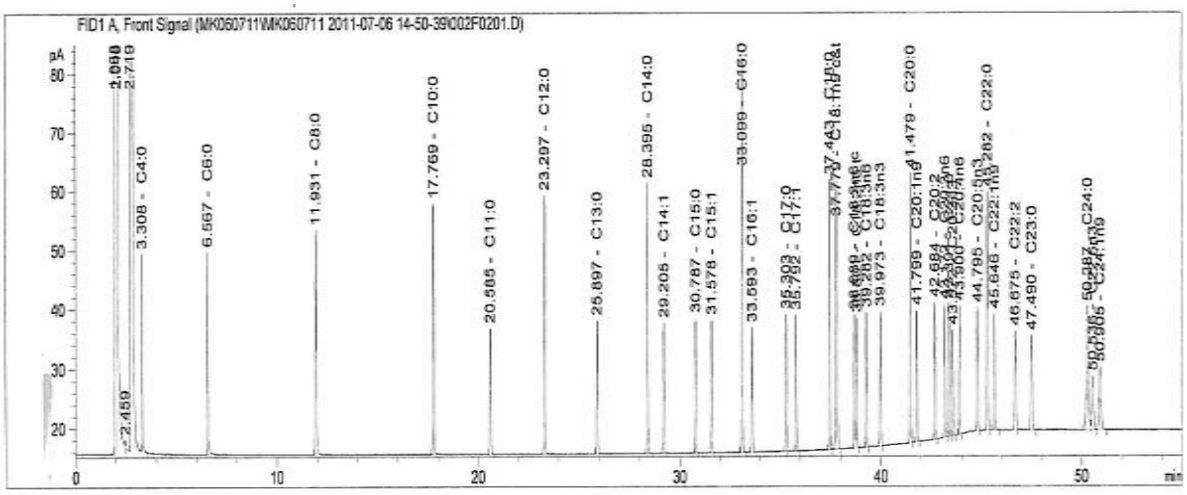


Figure 2.9 Typical gas chromatogram of a FAME standard mix

The GC instrument setup is shown in Table 2.4, peaks were identified by retention time using Supelco 37 Component FAME and C14:0 to C22:0 FAME mixtures.

Table 2.4 Gas Chromatograph program setup for microalgal EPA analysis

Instrument	HP5890 Hewlett Packard
Column	Omegawax 320 fused silica capillary column (30 m X 0.32 mm X 0.25 µm film thickness)
Carrier gas	Hydrogen
Carrier gas flow rate	0.5 $mL.sec^{-1}$
Detector Flame Ionisation	Yes (FID)
Injection port temperature	250ºC
Detector temperature	260ºC
Injection volume	1.0 µL
Split ratio	100:1
Temperature program	
Initial oven temperature	160ºC
Initial hold time	5 min
Ramp rate	4ºC.min^{-1}
Final oven temperature	240ºC
Final hold time	10 min
Total run time	35 min

Peak areas were used to quantify EPA relative to the internal standard. EPA culture content (P) was calculated by multiplying the EPA oil fraction concentration by 0.3 mL (within the

inorganic solvent phase) after GC analysis and then dividing this value by 15 mL (culture sample volume), as seen in the Equation 2.4.

$$P = (\text{GC concentration value} \times 0.3) / 15 \quad (2.4)$$

EPA productivity is expressed as concentration over time. Cell productivity (E) was calculated by subtracting maximum cell concentration from initial cell concentration and dividing this value by the age point where the concentration of the cells was at maximum (Equation 2.5). Overall EPA productivity (Y) was calculated similarly, by dividing the change in EPA culture content by its maximum age point (Equation. 2.6). Specific EPA productivity (Q) is the amount of EPA produced per cell, in a specific period of time and was calculated by dividing EPA productivity by cell productivity (Equation 2.7).

$$E = (C_{fx} - C_{ix}) / T_f \quad (2.5)$$

$$Y = (C_{fQ} - C_{iQ}) / T_f \quad (2.6)$$

$$Q = Y / E \quad (2.7)$$

The assay was found to be accurate and reproducible to 5% above 0.5 mg and 15% above 0.05 mg dry sample weight. The assay is able to maintain accuracy up to a total water reaction volume of 10%.

2.4. Statistical data analysis

Methods employed for optimisation of a particular bioprocess includes one-at-a-time, statistical and mathematical, fuzzy logic and genetic algorithms. The one-at-a-time method keeps the levels of all factors constant except one, the level of this factor is then changed within a desired range (Wen and Chen 2003). This strategy is simple and has been widely employed for optimizing EPA production (Yongmanitchai and Ward 1991). Once raw growth data in a design table is obtained, it can be converted into models or three dimensional plots, to determine the optimal levels of the factors investigated (Wen and Chen 2003).

All statistical analysis was performed using the response surface design function of Design Expert-8® v8.0.7 software (Stat-Ease, Minneapolis, USA). The two-factor, three-level

(lower and upper limit) full factorial design function used responses of specific growth rate and cell productivity when plotted against either a nutrient concentration or physiological range. These data were first analysed statistically using the analysis of variance (ANOVA) test. This determined whether a particular nutrient concentration or physiological value significantly affected a specific growth response, therefore allowing appropriate fits to polynomial models. Criteria for optimisation focused only on significant growth responses, using an optimisation function described by Myers et al. (2009). The software allowed the tool of response surface methodology to find significant responses in growth, caused by a specific nutrient concentration or physiological value and translating this into an optimal statistical range, ensuring maximum growth.

3. GROWTH AND PRODUCTIVITY STUDY OF CANDIDATE EPA MICROALGAL ISOLATES UNDER GROWTH AND STRESS CONDITIONS

3.1. Introduction

One of the most important decisions in a microalgal lipid production process is the choice of species to use (Griffiths and Harrison 2009). For the commercial production of EPA or any other lipid based product from microalgae, the selected strain must have suitable lipid productivity with regard to both growth and EPA content (Griffiths and Harrison 2009). Other key factors include the extractability and yield of product, vulnerability of strain to local conditions, as well as its capability for large-scale conversion of total algal oil into EPA.

Producing EPA on a large scale requires selection of microalgal strains that have the ability to synthesize and accumulate large quantities of lipid rapidly i.e. high lipid productivity. High lipid content also helps improve the oil extraction process (Griffiths and Harrison 2009). Factors which contribute to an economically feasible and environmentally desirable process include increasing productivity in large-scale cultures, minimising predation and contamination by other algal species, decreasing overall energy and cost requirements, and incorporating harvesting and downstream processes (Borowitzka 1992; Chisti 2007; Lardon et al. 2009; Mata et al. 2010; Rodolfi et al. 2009; Sheehan et al. 1998). A fast growing, high lipid producing indigenous isolate will address all of these factors.

In order to find an indigenous EPA producing microalga, the best high lipid producing monoalgal isolates, obtained from the bioprospecting library of the CSIR bioprocess division, were qualitatively screened for EPA production as stated in Section 2.1.1. The results of the study identified 3 potential EPA producers for further research and analysis. These isolates were those designated A3.2, A23.2 and A26.1. The aim of this part of the study was to observe growth and EPA productivity of these isolates during growth and stress phases, the latter having potential to increase productivity of lipids in some cases. Comparison of key growth and EPA production indicators would allow selection of the best isolate to be exploited towards realising a proof of concept stage for algal EPA production.

3.2. Materials and Methods

3.2.1. *Inoculum*

Previously selected monoalgal cultures, designated A3.2, A23.2 and A26.1, were evaluated. *Phaeodactylum tricornutum*, a known EPA producing isolate, was used as a positive control throughout the study (Yongmanichai and Ward 1991) to benchmark growth and EPA production. Inocula were obtained from vegetative 500 mL culture flasks. Inoculum and growth stage culture medium consisted of nutrient-replete artificial salt water (ASW) for isolates A3.2 and A23.2, and artificial fresh water medium (AF6) for isolate A26.1 (Appendix A). Inoculum cultures were made up of 200 mL of the vegetative culture and 300 mL of culture medium and then maintained under low light, with the incident light intensity determined to be 1 - 4 $\mu mol.m^{-2}.s^{-1}$.

3.2.2. *Growth stage*

For each isolate, 1600 mL of culture medium, nutrient-replete artificial salt water (ASW) for isolates A3.2 and A23.2, and artificial fresh water medium (AF6) for isolate A26.1 (Appendix A), was placed in a 2 L round-bottom flask. Medium pH was adjusted to 7.0 with 0.5% ($v.v^{-1}$) HCl, before autoclaving at 121^{o}C for 15 minutes. Each flask was inoculated with 400 mL of inoculum, using each respective culture. The culture was maintained at room temperature at approximately 23 ± 3^{o}C, with lighting set at an average intensity of 100 - 140 $\mu mol.m^{-2}.s^{-1}$ (measured as the incident light intensity at the flask interface) for ten days, resulting in stationary phase cultures. Cultures were agitated as described in Section 2.2

3.2.3. *Stress stage*

Once the stationary phase was reached, as indicated by three successive 'flat' or approximately equal cell count data points, a 1200 mL aliquot of culture was harvested by centrifugation using an Allegra^{TM} X-22R centrifuge (CA, USA) at 4500 rpm for 20 minutes. The culture pellets were washed with nitrogen-free stress medium, re-centrifuged and then re-inoculated into 2000 mL nitrogen deficient ASW / AF6 medium. The cultures were maintained under the same operating conditions as the growth stage until decline in EPA content was observed.

3.2.4. *Sampling and analysis of growth and stress experiments*

Each flask was aseptically sampled once per day at approximately the same time every day, for the 10 day duration of the experiment. Approximately 35 mL of culture was collected in a 50 mL Falcon tube (BD Biosciences, San Jose, California, USA) from each flask on a daily basis. The pH and temperature (Mettler Toledo, USA) of the culture was measured at the same time. Biomass and EPA data were measured, as described in Sections 2.3.2 and 2.3.4.2 respectively. Microscopic observations of each isolate were made and the morphology recorded. Nitrate and phosphate levels were monitored during the stress phase, as described in Section 2.3.1.

3.3. Results and Discussion

3.3.1. *Morphological characterisation of EPA-producing micro-organisms*

Isolate A3.2 (Figure 3.1) a unicellular green alga, consisted of large round and oval cells. All cells were granulated. Green algae generally grow faster than other algal classes, offsetting the lower concentrations of PUFAs that they often contain and thereby increasing PUFA productivity (Thompson Jr. 1996).

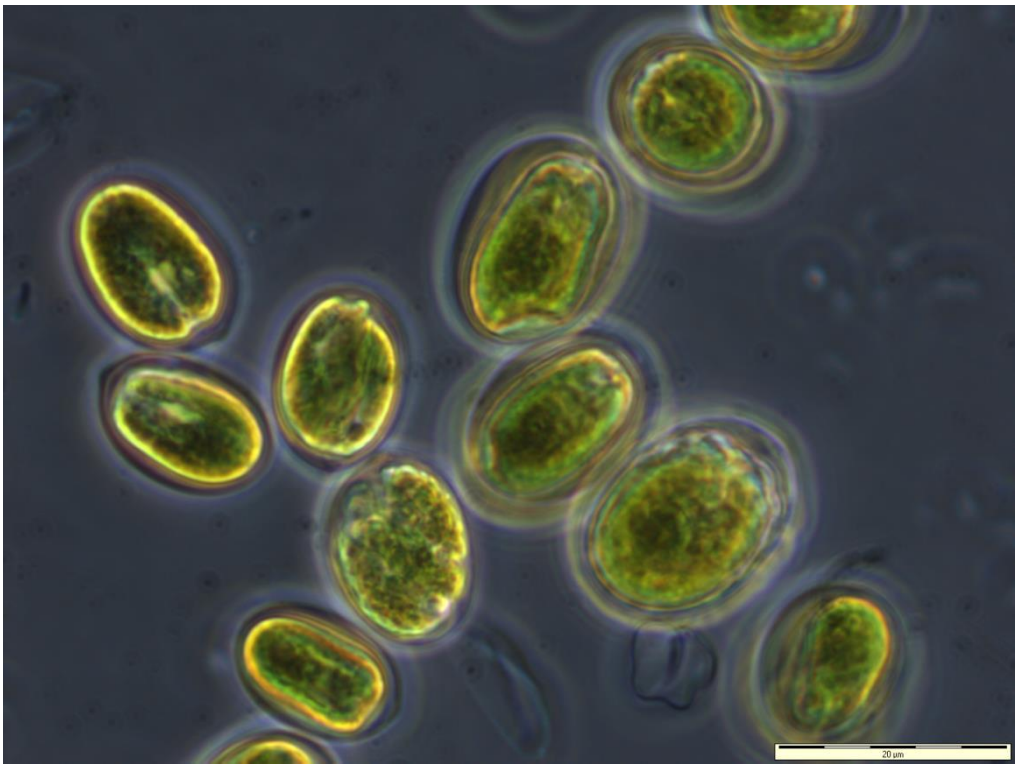


Figure 3.1 Light micrograph of isolate A3.2 using 1000 x magnification

Isolate A23.2 (Figure 3.2) a diatom, displayed a classically pennate form (bilaterally symmetric) and was tentatively identified as a *Neidium* species (Cox 1996). Organisms tended to aggregate in clumps and the frustule (outer siliceous shell) was fenestrated. These frustules show a wide diversity in form, but usually consist of two asymmetrical sides, with a split between them. Diatoms are abundant in most aquatic habitats and are considered the most important primary producers within marine food chains; phytoplankton seasonal blooms are dominated by these microalgae (Guschina and Harwood 2006). They are also extensively used in the mariculture industry, as feed for the larvae of crustacea and molluscs.

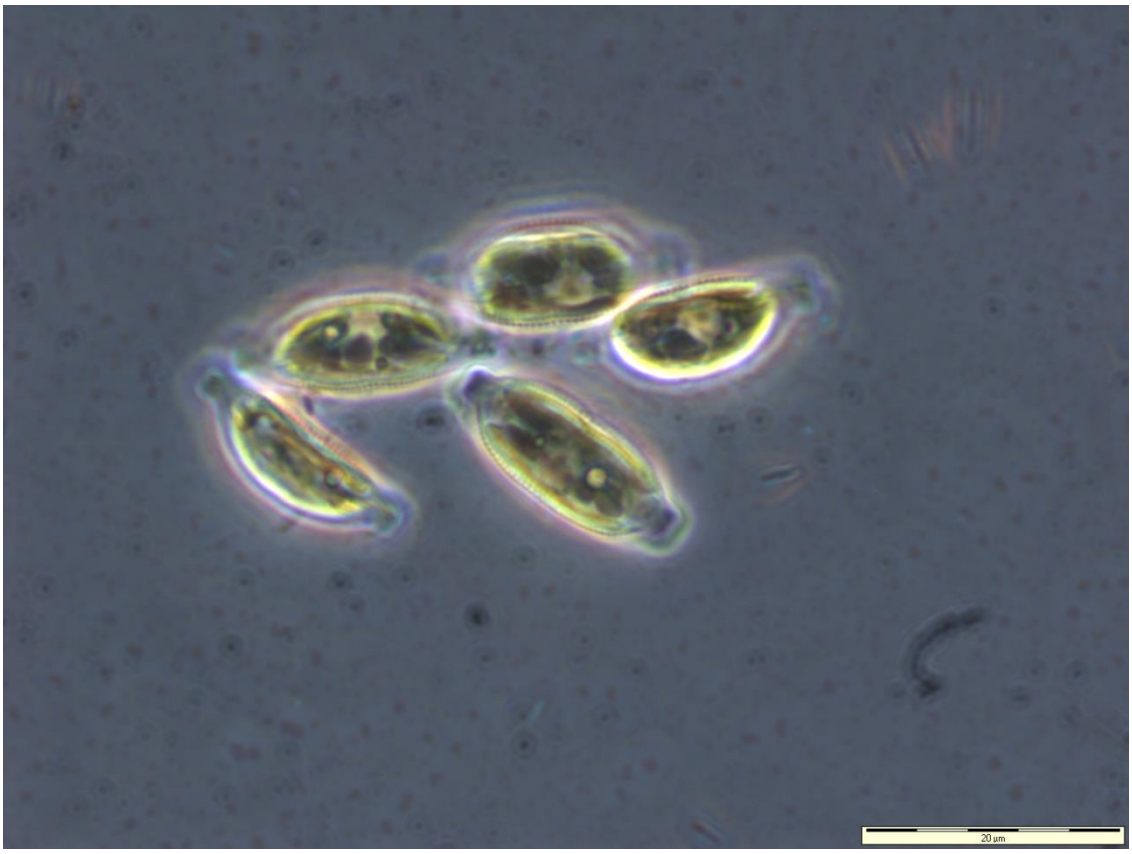


Figure 3.2 Light micrograph of isolate A23.2 using 1000 x magnification

Isolate A26.1 (Figure 3.3) was another unicellular green alga with a cell wall, having either a rod, rectangular or oval shape, depending on the age of the cell. Qualitative lipid analysis using the neutral lipid fluorescent dye Nile Red, showed that the cells contained round lipid deposits, similar to that of the *P. tricornutum* control, with many joined to form a stalk-like structure. Younger, newly divided cells were oval or square in shape. As the cells aged, they had a tendency to become more rectangular.

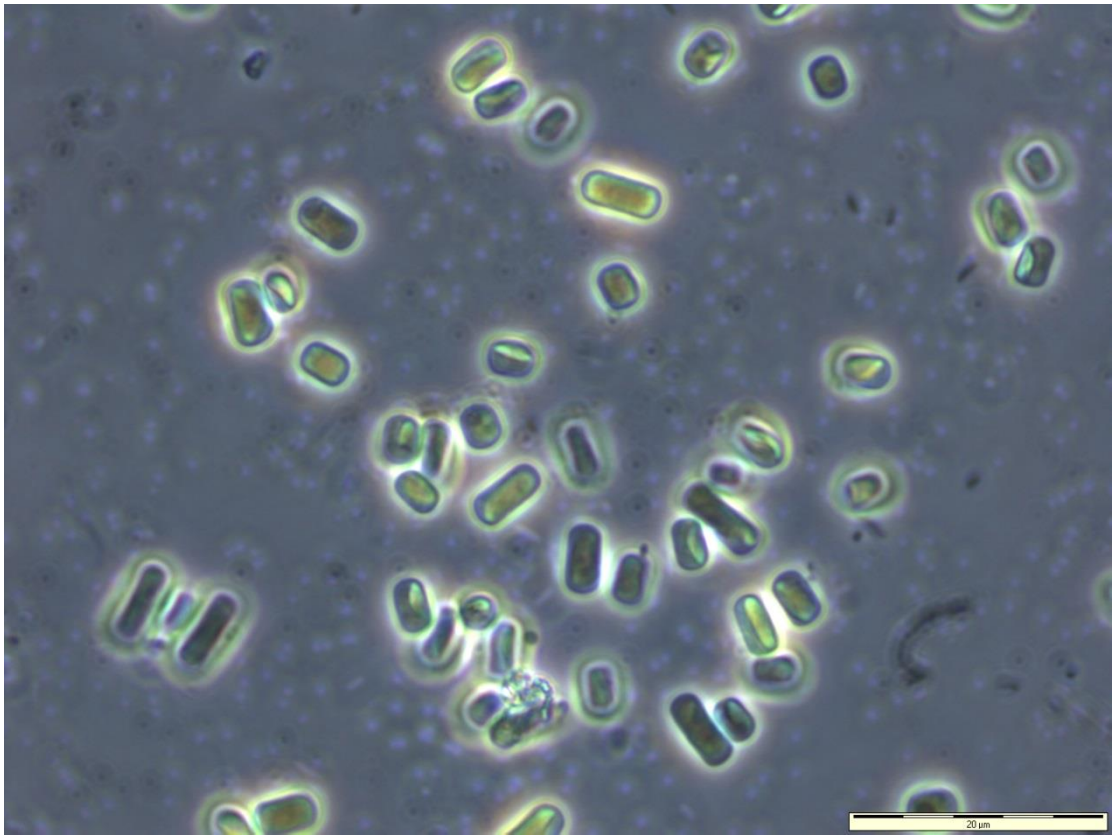


Figure 3.3 Light micrograph of isolate A26.1 using 1000 x magnification

P. tricornutum seen in Figure 3.4 (the lipid positive control), was supplied by the University of Hawaii and is a spindle shaped diatom distributed around Europe and North America (Kim et al. 2004). Unlike other diatoms, *P. tricornutum* can exist in different morphotypes such as fusiform, triradiate and oval, in which changes in cell shape can be stimulated by environmental conditions (De Martino et al. 2007).

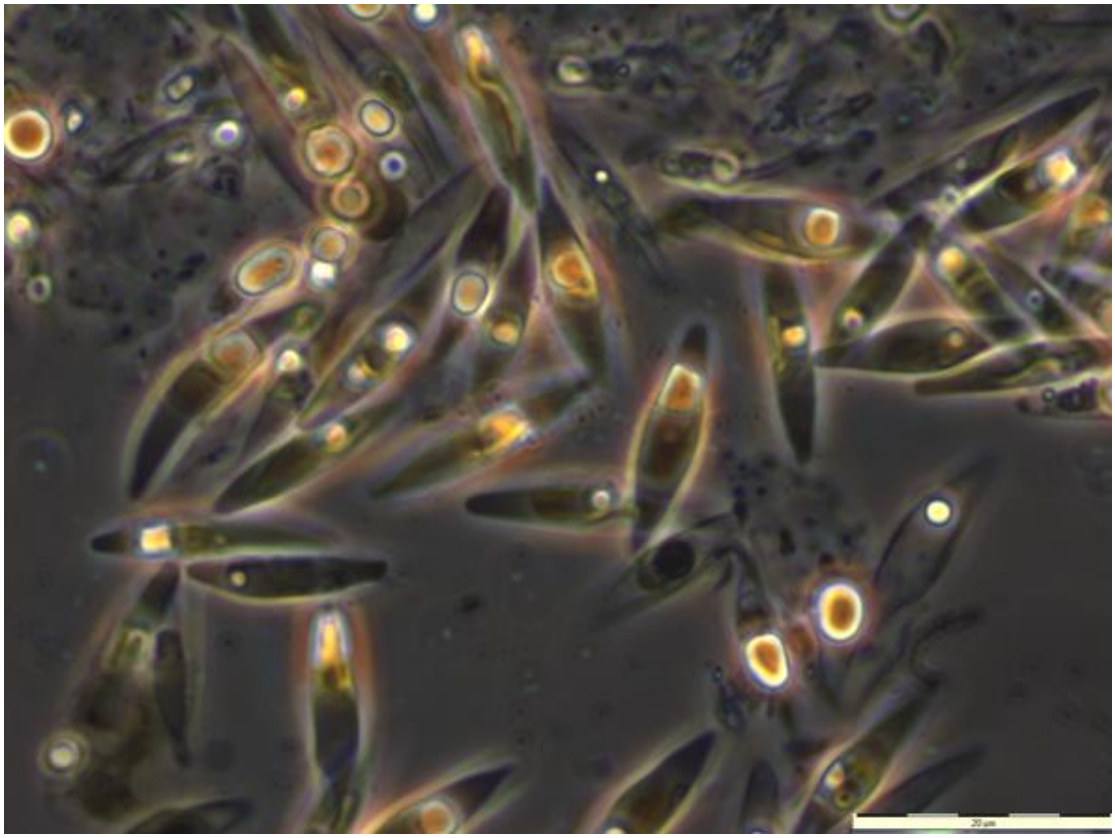


Figure 3.4 Light micrograph of *Phaeodactylum tricornutum* using 1000 x magnification

3.3.2. *Growth and EPA production of test isolates in growth stage*

Analysis of Figure 3.5 shows that isolate A26.1 reached the most favourable cell density in comparison with the other isolates, with overall cell density for both A3.2 and the diatom, A23.2 extremely low in relation to the positive control (*P. tricornutum*).

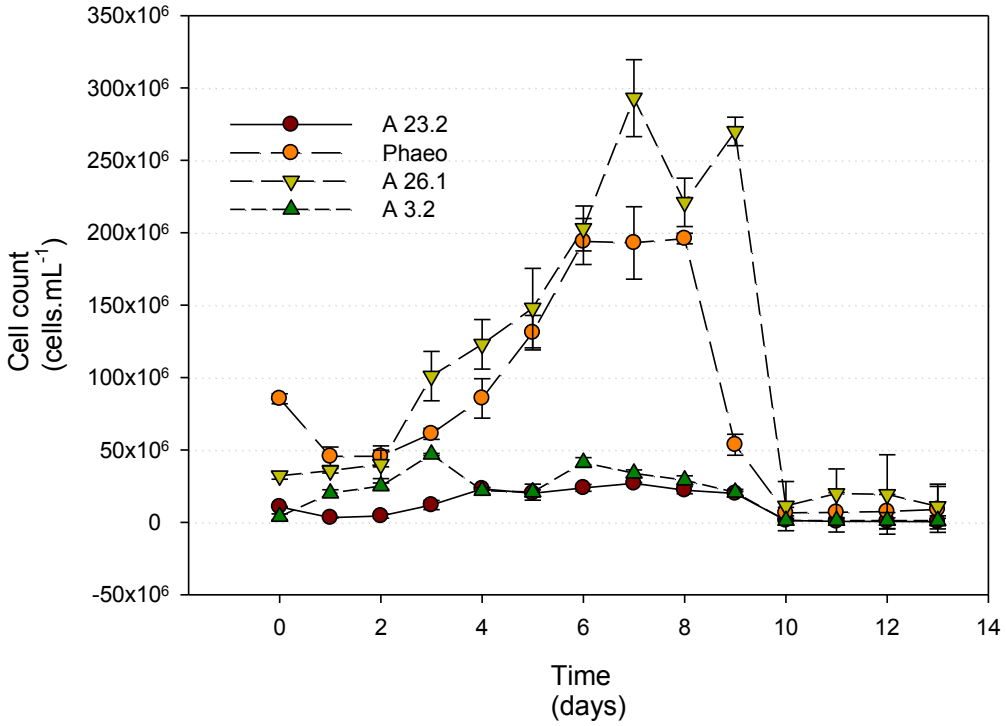
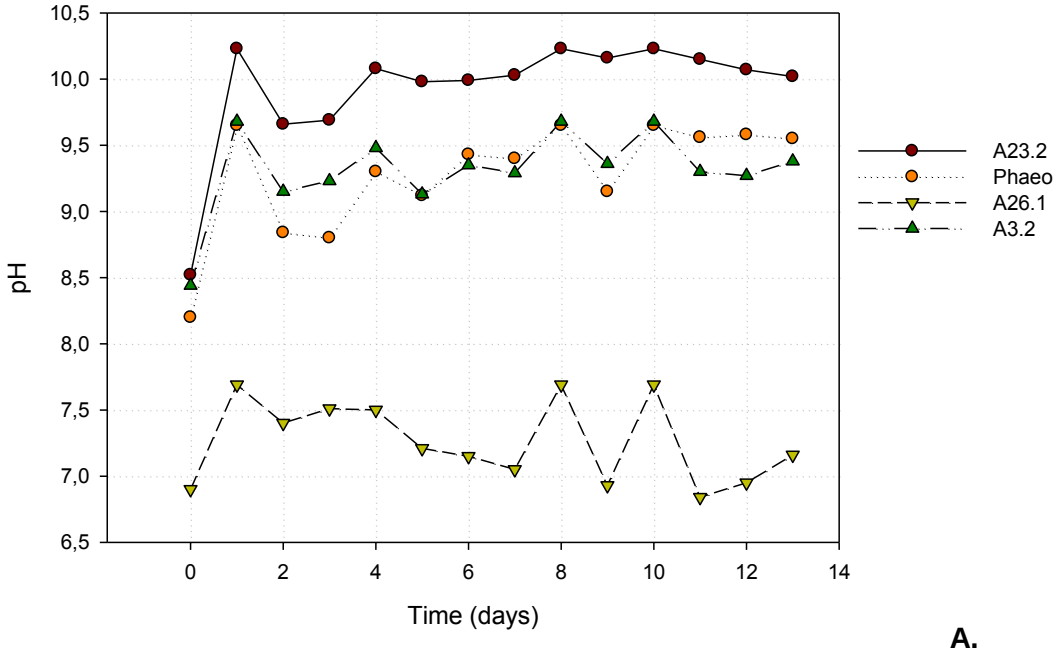


Figure 3.5 **Time profile of cell concentration of isolates in growth stage versus positive control *P. tricornutum* cultivated in respective media at approximately 25 °C**

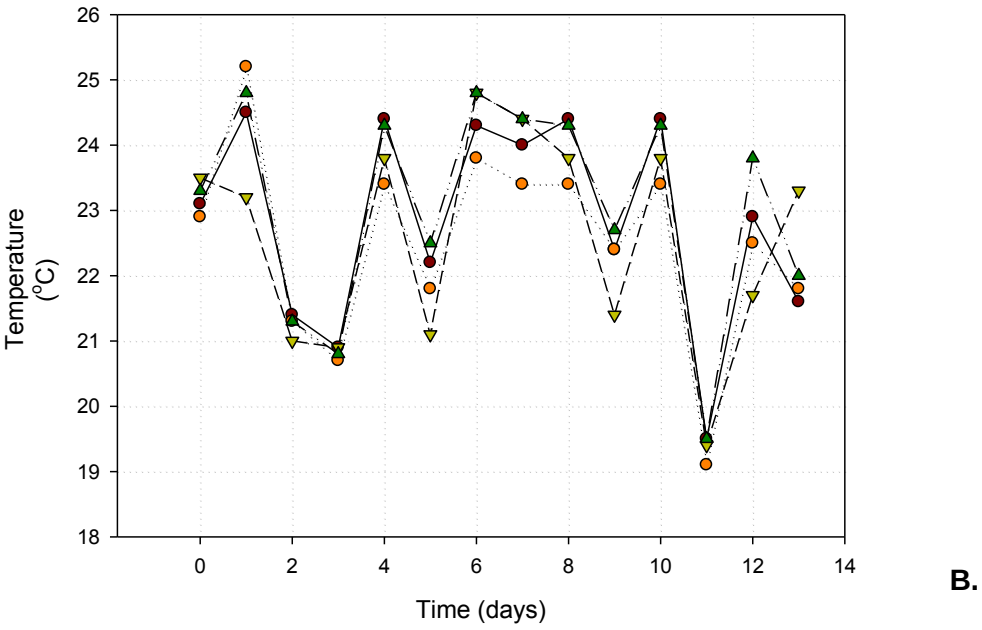
Isolates A3.2 and A23.2 showed higher initial maximum growth rates over 4 days as compared to A26.1 and the control, but lower overall cell densities (Table 3.1). When observing the morphology of these two isolates, they are relatively larger than A26.1 and *P. tricornutum*, which increases the likelihood of cell shading and reduced access of cells to light, restricting growth. Although isolate inocula were prepared and maintained similarly, the starting cell concentration of both A26.1 and the *P. tricornutum* control were substantially higher than the other candidate isolates likely due to in part to less cell shading.

A clear distinction is seen when comparing the starting pH of A26.1 with the other isolates (Figure 3.6). The elevation of pH is associated with CO_2 metabolism according to Rocha et al. (2003), the green isolate A26.1, most likely from the Chlorophyta division, showed good growth while still maintaining a stable neutral pH, possibly having a more efficient photosynthetic system which regulated carbon metabolism (Zelitch 1971). High pH

generation beyond that of 9.0 may have been a determining factor in limiting cell densities, which indicated a strong requirement for pH control in following studies.



A.



B.

Figure 3.6 **Time profiles of A. pH and B. temperature during the growth stage of various microalgal isolates growth in respective fresh or saltwater media**

Although A26.1 achieved roughly similar cell concentrations to the positive control, no omega-3 lipids were detectable across the 13 day culture under nitrogen replete conditions (< 0.5 $mg.L^{-1}$ in all 13 samples) (Table 3.1). The false positive in the selection reported in Section 2.1 could possibly have been attributed to cross contamination or the production of artefacts during transesterification in the previous screening trial. This isolate was discarded from any further analyses.

Data from Table 3.1 shows growth phase EPA concentration was poor in A3.2. The focus, however, should encapsulate both EPA concentration as well as cell productivity, as their product governs lipid productivity (Griffiths and Harrison 2009). Despite the higher relative growth rate of A3.2, a similarly low EPA productivity was reported. Isolate A23.2 in contrast was also severely growth limited, resulting in poor biomass productivity, however the maximum recorded growth rate, and to a greater extent specific EPA productivity, was higher than the control. Growth limitation of A23.2 did not substantially affect specific EPA productivity and its high lipid concentration had the potential to improve extraction efficiency (Rodolfi et al. 2009).

Table 3.1 EPA productivity data of isolates cultured in nutrient replete medium

Isolate	Max Growth rate	Doubling time	Max culture EPA	Cell productivity	EPA productivity	Specific EPA productivity
	($\mu.day^{-1}$)	*(days)*	*($mg.L^{-1}$)*	*($cells.mL^{-1}.day^{-1}$)*	*($mg.L^{-1}.day^{-1}$)*	*($mg.cell^{-1}.day^{-1}$)*
Phaeo Control	0.37	1.9	1.160	$1.81x10^{7}$	0.033	$1.83x10^{-9}$
A23.2	0.69	1.0	1.366	$0.31x10^{7}$	0.277	$89.20x10^{-9}$
A 3.2	0.77	0.9	0.308	$1.43x10^{7}$	0.044	$3.07x10^{-9}$
A26.1	0.36	1.9	-	$3.73x10^{7}$	-	-

Growth rate was recorded over the time period where linearity was at its maximum, EPA productivity was selected according to the maximum value recorded over the duration of the experiment. The EPA profile generated from the three isolates is illustrated in Figure 3.7.

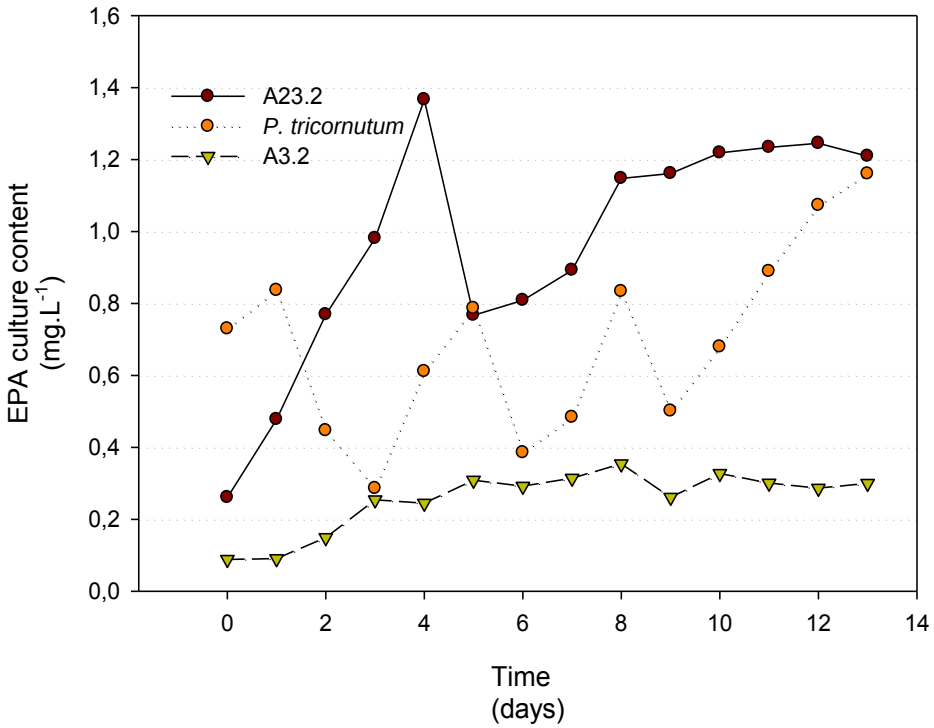


Figure 3.7 Volumetric EPA culture concentration recorded for three microalgal isolates under standard conditions and respective media over 13 days

Isolate A3.2 showed the lowest overall EPA content, the control *P. tricornutum* showed more erratic EPA content, stabilising after day 9. Isolate A23.2 showed initial increase in EPA content before a plateau was reached at day 8, showing relative consistency relative to the control. Overall EPA productivity data indicates positive commercialization potential for A23.2, considering that the control is an organism commonly used in lipid production because of its high lipid content and growth rate (Yongmanichai and Ward 1991). A maximum growth rate of 0.69 day^{-1} was recorded when nutrients were sufficient and pH close to neutrality during the initial stages of the growth phase. Isolate A23.2 showed a consistent increase in EPA concentration in the initial stages of growth before later attenuation.

3.3.3. *Growth and EPA production of selected isolates under stress conditions*

Nitrogen deficient medium stress is reported to increase lipid production in many species (Rodolfi et al. 2009) and was used in the observation of EPA productivity of A3.2, A23.2 and

the *P. tricornutum* control. In this stress phase a gradual pH decline was observed along with a consequent decrease in metabolic activity, which was expected due to a decline in cell productivity relative to the growth phase of the experiment (data not shown). Table 3.2 shows a substantial increase in EPA production across all EPA producing isolates, including the control, under nitrogen stress conditions, substantiating the hypothesis of nitrogen stress as a general induction mechanism for lipid production (Sheehan et al. 1998; Rodolfi et al. 2009).

Table 3.2 Isolate EPA productivity data in stress phase

Isolate	**Max EPA in culture** ***(mg.L^{-1})***	**EPA productivity** ***(mg.L^{-1}.day^{-1})***	**Specific EPA productivity** ***(µg.cell^{-1})***
P. tricornutum	4,378	0,233	2.12x10^{-7}
A23.2	4,072	0,300	3.19x10^{-7}
A 3.2	0,749	0,073	8.07x10^{-7}

EPA productivity was best in A23.2, which exhibited a slightly higher productivity than the control. Isolate A3.2 was higher than the other isolates with regard to specific EPA productivity per cell. EPA volumetric culture concentration of A23.2 increased to approximately 4 mg.L^{-1}, a threefold increase in EPA under nitrogen stress, which mirrored the *P. tricornutum* control. Although this EPA content took a longer time to reach, overall EPA data was more stable across all isolates under nitrogen stress (Figure 3.8). Increases in EPA seen in the control and A23.2 from day 6 levelled off after day 8 (data not shown).

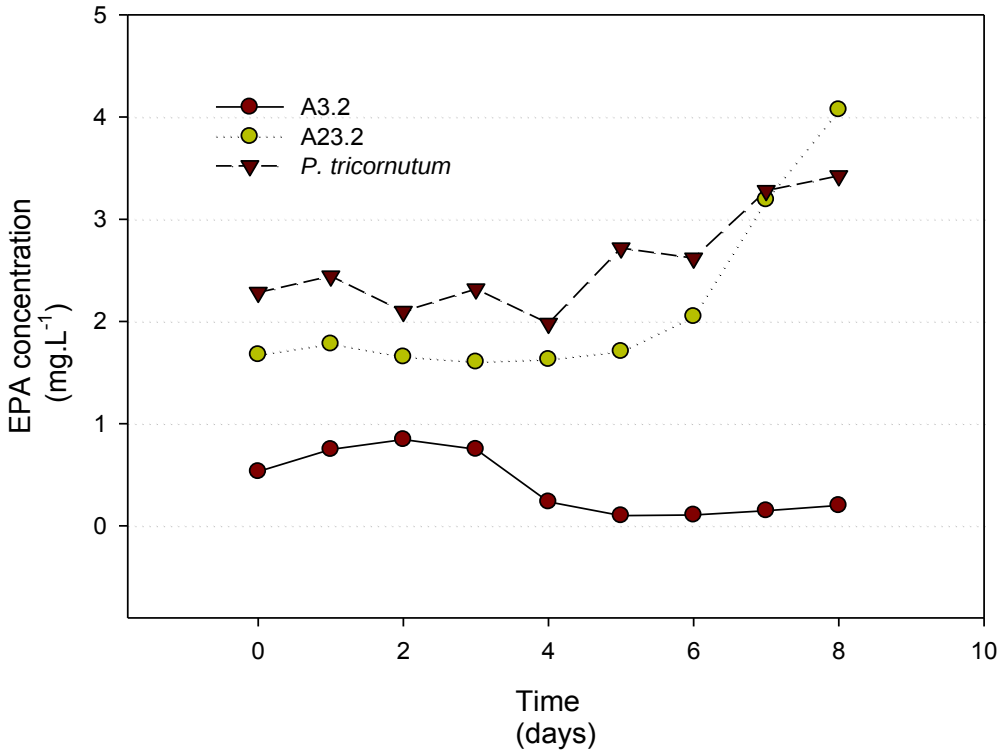


Figure 3.8 Volumetric EPA culture concentration recorded for three microalgal isolates cultured in respective nitrogen deficient media over 8 days

It was interesting to note a decrease in culture pigment or 'culture bleaching' of isolate A23.2 under stress conditions, which can be explained by the lack of available nutrients for pigment production. The dense siliceous cell wall and large size of the diatom drastically reduced culture settling time, a further advantage from a harvesting perspective (Figure 3.9).

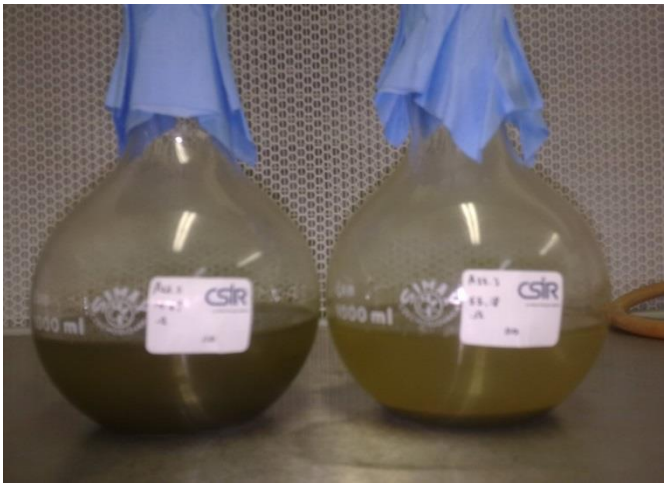


Figure 3.9 **Culture flasks showing the settling of isolate A23.2 from turbulent immediately removed from agitation (left) to still after approximately 5 minutes (right)**

3.4. Conclusion

The data obtained in this study showed that the production of EPA by phytoplankton is influenced largely by the stage of growth and nutrient limitation. Nitrogen deficient medium stress, is a bioprocess strategy that may be used successfully to enhance lipid productivity, as confirmed in literature (Rodolfi et al. 2009; Stephenson et al. 2010). According to Griffiths et al. (2011), microalgal species have unique characteristics. The location, culture system, resources and goals of the project determine final selection of species. EPA productivity of the indigenous diatom A23.2 under both nutrient-replete and stress conditions suggests that it is an ideal candidate for EPA production, having the potential to surpass even the productivity of *P. tricornutum* (Table 3.1 and Table 3.2). In addition, its property of observed rapid settling, make it a favourable candidate for use in a bioprocess.

In order to enhance EPA productivity of diatom A23.2, the optimisation of growth regarding both medium development and physicochemical conditions needed to be addressed. These parameters are considered individually to assess their effect on the maximum growth rate. Chapter 4 focuses on medium development for this diatom, identifying specific limiting nutrients in order to obtain higher cell densities and consequent potential for higher EPA productivity on exposing cells to lipid-enhancing environmental stress.

4. DEVELOPMENT OF GROWTH STAGE MEDIA FOR CULTIVATION OF THE EPA PRODUCING DIATOM A23.2

4.1. Introduction

Isolate A23.2 was collected off the Slangrivier river, at GPS co-ordinates 34° 08.882' S, 20° 52.065' E in the Western Cape of South Africa. The diatom was further identified using 18S RNA sequencing, at the UCT Centre for Bioprocess Engineering Research (CeBER). The results yielded the closest match to the genus *Cymbella* (Figure 4.1). Algal species belonging to the genus *Cymbella* have been reported to be benthic, producing mucilaginous stalks and considerable amounts of EPA (Jagannathan et al. 2010). It is for this reason that these species have been used in hatchery systems as feed supplements, enhancing the growth and survival of *Panaeus monodon* post-larvae, amongst others (Khatoon et al. 2009). Reproduction among diatoms is primarily asexual by binary fission. No evidence of auxospore formation and sexual reproduction was detected in A23.2.

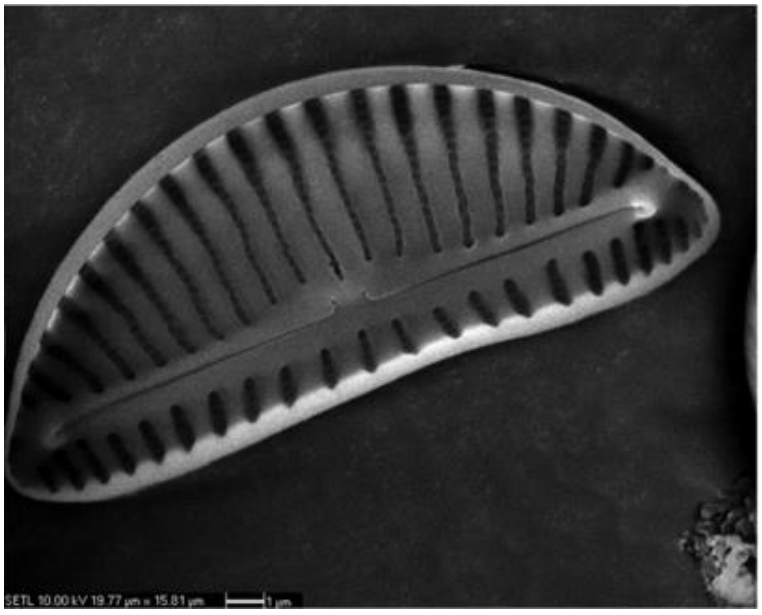


Figure 4.1 Electron micrograph of *Cymbella* sp. (Zhang et al. 2012)

High productivities of vegetative microalgal cells are essential for any production process (Fábregas et al. 2000); this requires the formulation of a suitable culture medium, supporting both high cell concentration and a rapid specific growth rate. In previous batch growth

culture studies of the diatom A23.2, conducted at the CSIR Bioprocessing unit, (Modderfontein, South Africa), four macronutrients were identified as having been consumed: phosphate, silicate, nitrate and bicarbonate (in order to reduce experimental costs bicarbonate was favoured over gaseous CO_2). Nitrate, phosphate and bicarbonate all play essential roles in cellular structures, metabolism and replication, with their required ratios varying from species to species. These varied nutrient requirements between species of microalgae may form the basis for competitive exclusion or coexistence (Yull 1978).

The determination of optimal concentrations of key nutrients, therefore, ensures maximum biomass productivity, a key requirement for an industrial bioprocess. Unused medium components result in a negative environmental impact and the need for waste water treatment. Production costs also improve with more efficient utilization of nutrients. There is a lack of data comparing growth across various nutrient concentrations and species of microalgae under standard conditions.

In this chapter, a modified version of Aquil medium (Appendix B) was chosen, due to a lower relative nutrient concentration and consequent chemical adsorption, to other saltwater media, as well as its close imitation of natural conditions (Morel et al. 1979). The range of nutrient concentrations selected could therefore be robust, ranging from 50% to 200% of base case Aquil nutrient concentrations. The study to determine optimum initial charge nutrients for diatom A23.2, in terms of the macronutrients: phosphate, silicate, nitrate and bicarbonate, are reported. The selection of medium composition was based on the biomass concentration, productivity and specific growth rate achieved. Further, the potential for these nutrients to become limiting within 12 hours was considered.

4.2. Materials and Method

4.2.1. *Inoculum*

A vegetative stock culture of A23.2 was selected for the detailed study (Figure 4.2). The culture was maintained at 23 ± 3^{o}C, with an average light intensity of 100 µmol.m^{2}.s^{-1}, in a 5 L round-bottom flask. A 40% (v.v^{-1}) inoculum in medium was made up with 400 mL of the stock culture transferred aseptically to inoculate 600 mL of sterile medium, contained in a 2 L glass bioreactor.

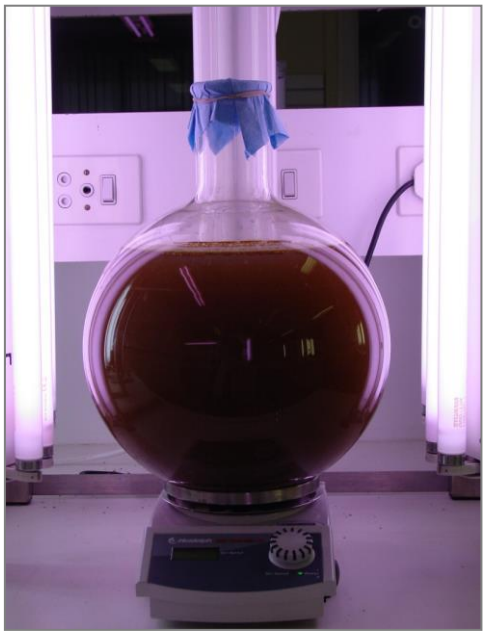


Figure 4.2 Setup of vegetative stock inoculum

4.2.2. *Growth Medium*

A modified Aquil diatom medium (Appendix B) was selected as growth medium for both inoculum and experimental studies, based on Anderson (2005), owing to the stoichiometric basis of the macro-nutrients. The modified Aquil medium used in this study had an approximate stoichiometric ratio of C: Si: N: P = 12: 3: 2: 1. A summary of the ion concentration ranges tested are presented in Table 4.1.

Table 4.1 Nutrient ion concentration ranges tested in study

Nutrient	**Lower limit** ***(g.L^{-1})***	**Standard Medium** ***(g.L^{-1})***	**Upper limit** ***(g.L^{-1})***
Phosphate	0.03	0.05	0.14
Nitrate	0.14	0.18	0.37
Silicate	0.07	0.13	0.27
Bicarbonate	1.09	1.45	2.89

Nutrients were added drop-wise into distilled water while mixing on a stirrer plate set at 250 rpm to minimise precipitation. This was particularly important for the silicate stock, as it tended to precipitate. The pH was adjusted to 7.00 using 0.5% (v.v^{-1}) HCl, before

autoclaving within the bioreactor (B. Braun Biostat, Germany, described in Section 2.3) at 121^{o}C for 20 min.

4.2.3. *Cultivation conditions*

Cultures were incubated within the bioreactors at 25 ± 2°C under constant illumination, at 60 - 100 µmol.m^{-2}.s^{-1} intensity (measured at the centre of an empty bioreactor with the water jacket filled). An impeller speed of 100 rpm was used to provide mixing of nutrients and repeated exposure of the algal cells to light, while minimising shear, bioreactors were sparged with air at approximately 1 vvm. The pH was maintained at 7.00 using 0.5% (v.v^{-1}) HCl by automated titration.

4.2.4. *Sampling*

Samples of approximately 10 mL or 1% culture volume, were taken aseptically every hour for 11 hours, and vortexed. Algal growth was monitored by immediate cell counting (Section 2.3.2). End point nutrient concentration tests were performed at the end of the 11-hour period, using the supernatant of samples (20 mL) centrifuged at 4500 rpm (Heidolph Instruments, Germany). Batch assays performed on different days showed slight variations in starting cell counts.

4.2.5. *Data Analysis*

Average cellular yield per unit nutrient (cells.mg^{-1}) was calculated using Equation (4.1), by expressing net biomass (difference between maximum and minimum cell count), as a ratio of net nutrient consumption (difference between initial medium and final nutrient ion concentrations). Percentage nutrient consumption was calculated as a ratio of net nutrient consumption over starting nutrient concentration.

$$\text{Ave. cell yield (cells.mg}^{-1}\text{)} = \text{Net biomass formation / Net nutrient consumption} \quad (4.1)$$

Optima were based on statistical polynomial fits. These provided an indication of a suitable concentration range of a particular nutrient for improved growth as compared to standard medium concentration. Lipid productivity is dependent on both biomass productivity and lipid content (Griffiths and Harrison 2011) and therefore the inclusion of cell productivity and growth rate are used as key growth responses in this study. Average cell yield on

milligram of ion in medium was calculated to give a better understanding of feed rates for future scale-up studies.

The experimental approach used in the current study was to vary single nutrient concentrations within the tested ranges during the initial growth phase (11 hours), to determine its requirement and effect, minimizing light limitation and ensuring that the other nutrients would not be limiting. No replicates were performed for the different nutrient concentrations; however repeat assays were done if triplicate cell counts showed excessive fluctuation in standard deviation data.

4.3. Results and Discussion

4.3.1. *Phosphate medium concentration and effects on growth*

Growth data were collected across phosphate concentrations of 0.034 to 0.138 $g.L^{-1}$. The concentration of phosphate in standard Aquil medium was 0.05 $g.L^{-1}$. Cell concentration was measured over 11 hours and is presented as a function of time in Figure 4.3. (Set 1: 0.034 $g.L^{-1}$, 0.052 $g.L^{-1}$, 0.138 $g.L^{-1}$ and Set 2: 0.069 $g.L^{-1}$, 0.083 $g.L^{-1}$, 0.103 $g.L^{-1}$, 0.120 $g.L^{-1}$).

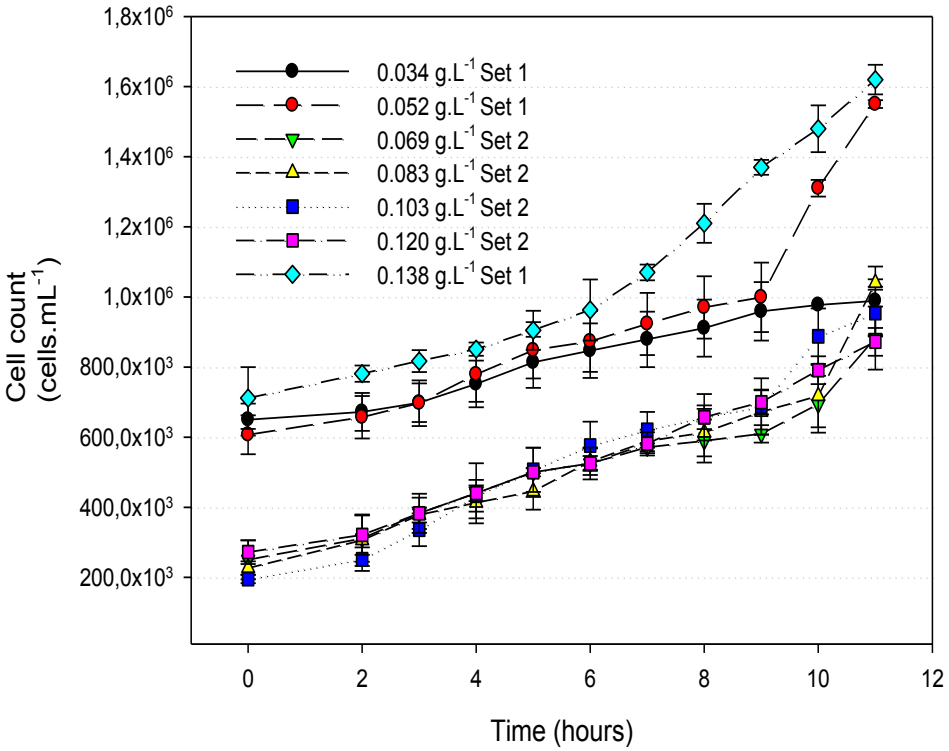


Figure 4.3 Growth profile of A23.2 cultured in a stirred tank bioreactor in Aquil medium with various phosphate concentrations

Similar growth trends for A23.2 cultured in different phosphate concentrations were observed in both sets of experiments. Specific growth rate for each concentration was calculated with each experimental set separately entered into Design-expert®. The optimisation function incorporating growth rate data did not find any significant impact of phosphate on growth. Cell productivity modelling could not be performed because of fluctuating data, productivity values ranged from 3.08 x 10^7 cells.L^{-1}.hr^{-1} to 8.57 x 10^7 cells.L^{-1}.hr^{-1}.

The average cell yield, with respect to phosphate, (consumed over 11 hours) displayed a pattern of decreasing biomass formation per unit phosphate consumed, with increasing concentration of phosphate supplementation (Figure 4.4). The concentration of phosphate in standard Aquil medium of 0.05 g.L^{-1} was not inhibitory and found to be a suitable concentration for the growth phase of the bioprocess.

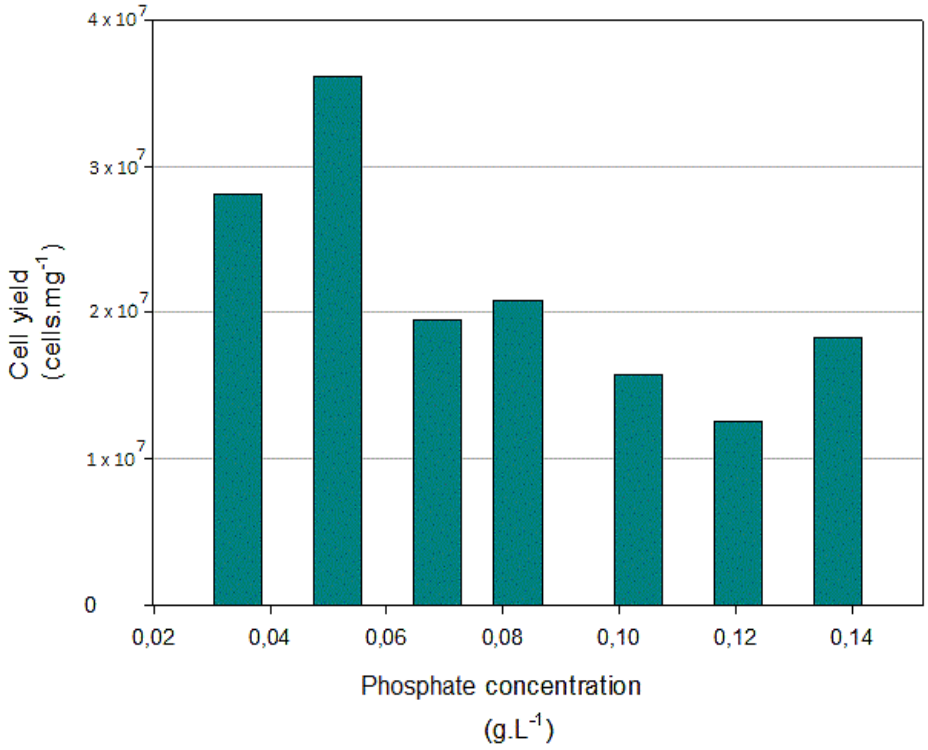


Figure 4.4 **Average cell yield per milligram of phosphate consumed at different phosphate concentrations over 11 hours**

Decreasing cell yield with increasing phosphate concentration beyond that of 0.05 $g.L^{-1}$ parallel findings in literature, which state that synthesis of the phosphatase enzyme is inhibited under phosphate excess conditions (Perras and Kuenzler 1965; Jansson 1988). Phosphatase enzymes in microalgae are generally bound near the cell surface, high phosphate concentrations may trigger a shutdown of phosphatase synthesis at this interface, resulting in reduced phosphate utilization (Perras and Kuenzler 1965). Studies by Chu (1943), revealed that phosphate concentrations of approximately 0.02 $g.L^{-1}$ were inhibitory to most diatoms and green algae. A23.2, in contrast, had a peak cell yield at more than twice this concentration.

4.3.2. *Silicate medium concentration and effects on growth*

Growth data were collected across silicate concentrations of 0.069 to 0.268 g.L^{-1}, with a standard Aquil medium concentration of 0.134 g.L^{-1}. Growth profiles under varying silicate concentrations are shown in Figure 4.5.

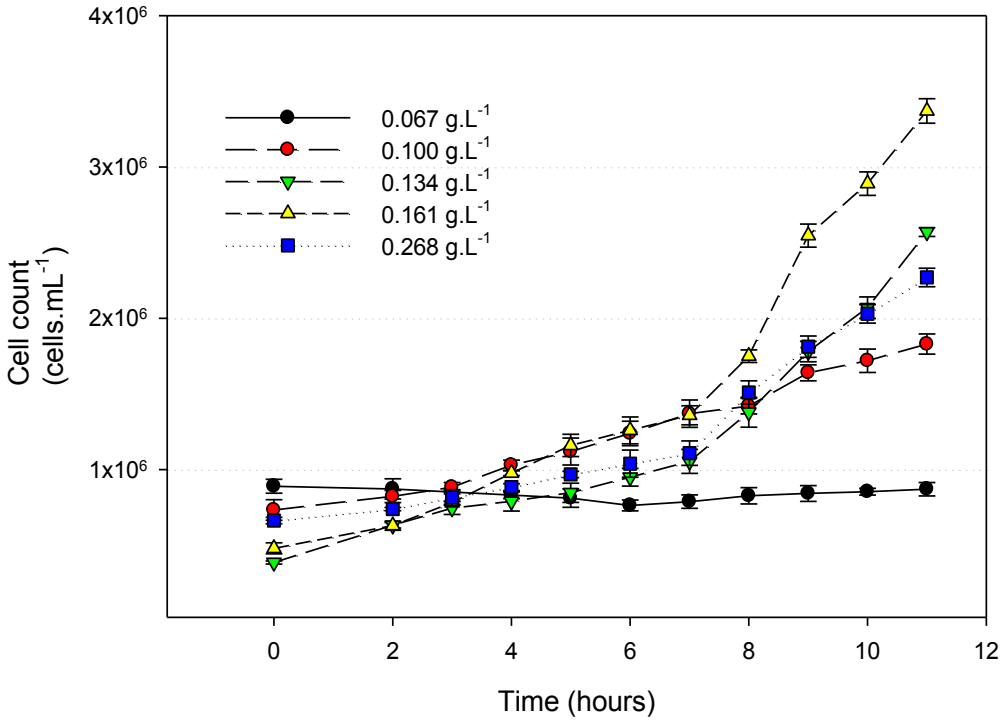


Figure 4.5 Growth profile of A23.2 cultivated in a stirred tank bioreactor in Aquil medium with various silicate concentrations

The maximal specific growth rate across the tested range of silica concentrations occurred at the approximate silicate concentration of 0.16 g.L^{-1} (Figure 4.6), modelled with a quadratic expression. Using Design-expert®, a cubic model was proposed for cell productivity as a function of silicate concentration. The optimisation model of Design-expert® found both specific growth rate ($p<0.0200$) and cell productivity ($p<0.0003$) to be significant functions of silicate concentration at the 95% confidence level. The predicted optimal silicate concentration was calculated to be at 0.2 g.L^{-1}, maximising biomass at a cell productivity of 3.07 x 10^{8} cells.L^{-1}.hr^{-1}. The closeness of the experimental maximum value of 0.16 g.L^{-1} to the statistically calculated maximum of 0.2 g.L^{-1} gave further evidence for diatom A23.2 having optimal growth at this silicate concentration range. Since diatoms are well known to supersaturate their cell walls, the statistical value can be justified as an upper limit (Martin-Jezequel et al. 2000).

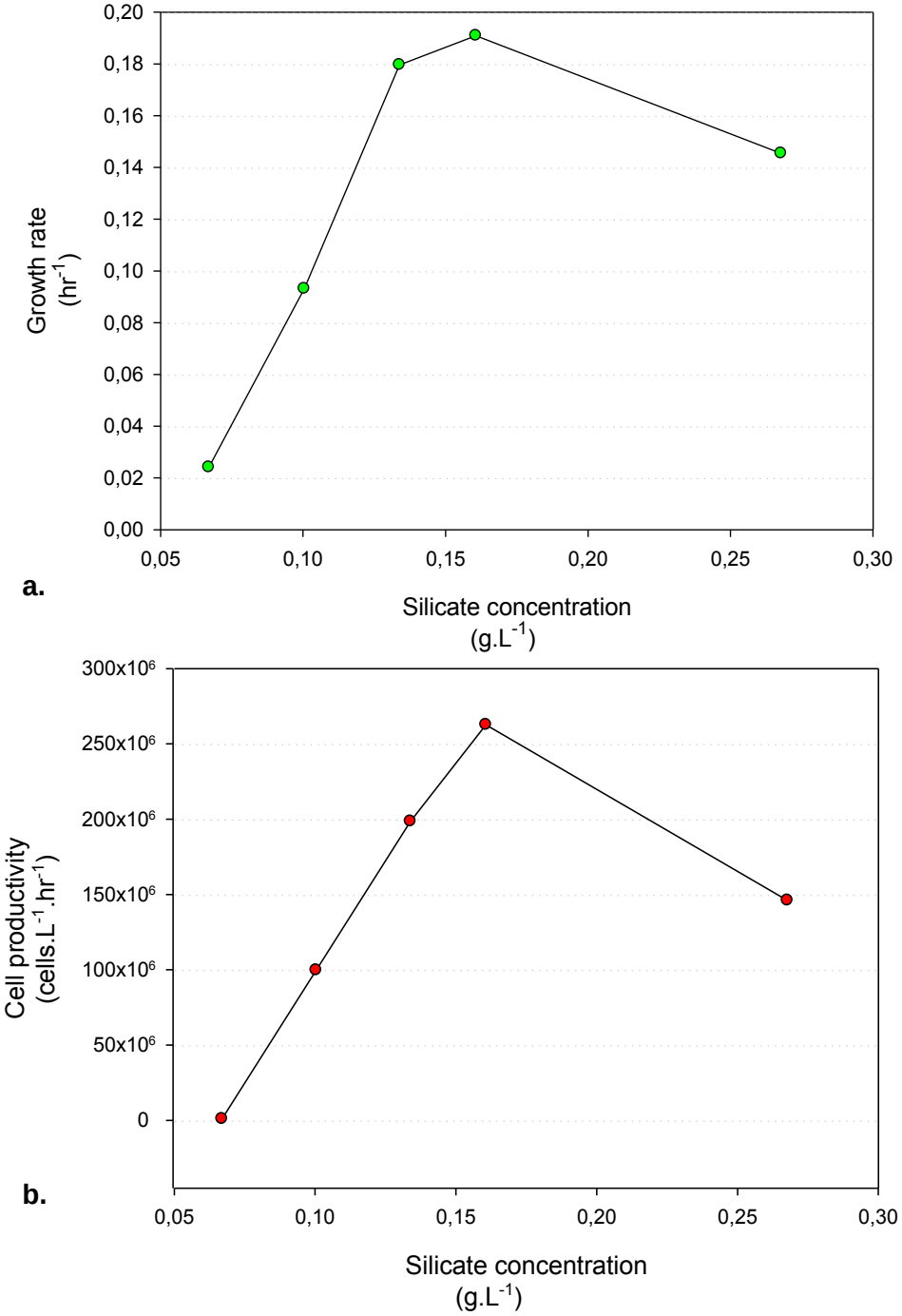


Figure 4.6 **Effect of silicate concentration on a) specific growth rate and b) cell productivity of A23.2 in a stirred tank bioreactor using Aquil medium (agitation 100 rpm, pH maintained at 7)**

Egge and Aksnes (1992) showed that at non-limiting silicate concentrations, diatoms have a relatively higher inherent growth rate when compared to other microalgae cultivated under the same growth conditions. This optimal value should be strictly maintained within the

growth stage of the production system, maximising bio-available silicate. Silicate consumption and cell yield data are presented in Table 4.2, in which cell numbers were observed to increase with increasing silicate concentration, except at the highest concentration tested. Further, the cell yield on silica was largely consistent, except for the last data point. At all concentrations, more than 95% usage of silicate was observed, inclusive of silicate precipitated out of medium.

Table 4.2 Reactor silicate consumption and cell yield of A23.2

Silicate medium conc. ($mg.L^{-1}$)	Net consumption ($mg.L^{-1}$)	% Consumption	Net biomass ($cells.L^{-1}$)	Cell yield ($cells.mg^{-1}$)
67	64.39	96	$0.01x10^{9}$	$0.01x10^{7}$
100	97.03	97	$1.10x10^{9}$	$1.13x10^{7}$
134	130.49	97	$2.18x10^{9}$	$1.67x10^{7}$
161	152.63	95	$2.89x10^{9}$	$1.89x10^{7}$
268	258.28	96	$1.61x10^{9}$	$0.62x10^{7}$

Saturation of the cell with silicate at unnatural concentrations could inhibit other nutrient transport mechanisms and alter cell buoyancy (Martin-Jezequel et al. 2000). This indirectly affects sinking rates and consequent light/nutrient exposure, likely resulting in the slower growth and cell yield seen at 0.268 $g.L^{-1}$, which is twice the standard Aquil medium concentration of 0.134 $g.L^{-1}$. Residual silicate was observed to precipitate out of solution, restricting cellular access; fed-batch silicate feed is therefore a necessity in the production process. At the highest silicate concentration tested, precipitation was observed within the medium, further restricting both light and silicate availability. The method of addition of silica salt to media should therefore be standardised, as rapid addition without mixing resulted in increased precipitation. The ability of diatoms to maintain saturated levels of soluble silicate within their cells is a probable evolutionary tool to maximise a scarce yet essential resource. Deposition of silicate in the frustule is also an obligatory step before the division of vegetative cells (Martin-Jezequel et al. 2000), creating a close coupling between the cell cycle and silicate metabolism. Hydrated silicon is thus a major limiting nutrient and controlling factor in primary cell diatom growth.

4.3.3. *Nitrate medium concentration and effects on growth*

Growth data for A23.2 were collected across a range of nitrate concentrations from 0.137 to 0.365 $g.L^{-1}$, where 0.182 $g.L^{-1}$ is present in the standard Aquil medium. Growth profiles,

presented in Figure 4.7, showed similar trends to each other across the nitrate concentrations tested, with the possible exception of 0.274 $g.L^{-1}$ nitrate in medium, where A23.2 showed elevated growth after 3 hours. Parameters of specific growth rate and cell productivity did not result in a statistically significant relationship as a function of nitrate concentration at the nitrate levels tested. A statistically significant relationship between growth parameters and nitrate concentration was a requirement for the Design-expert® optimisation model. Average growth rate ranged from 0.05 to 0.13 hr^{-1} in the study, with cell productivity ranges from 1.3 x 10^7 $cells.L^{-1}.hr^{-1}$ to 1.8 x 10^8 $cells.L^{-1}.hr^{-1}$.

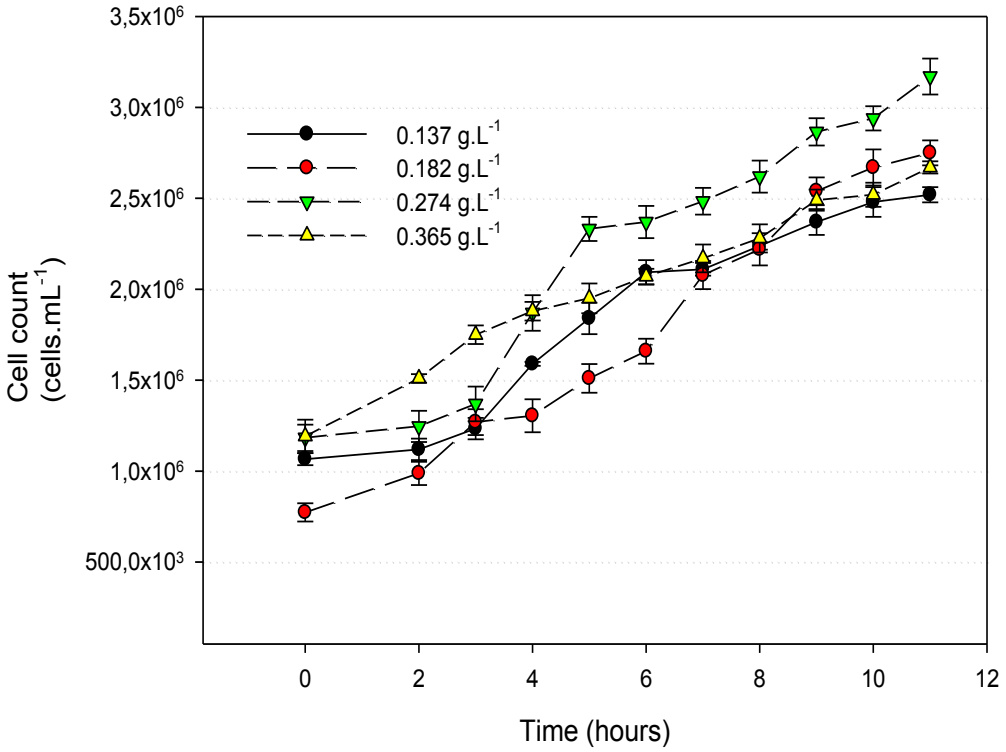


Figure 4.7 Growth profile of A23.2 cultivated in a stirred tank bioreactor in Aquil medium with various nitrate concentrations

The nitrate cell yield (Figure 4.8) decreased with increasing nitrate concentration, indicating that a low nitrate concentration range of 0.13 – 0.18 $g.L^{-1}$ is suitable for cultivation.

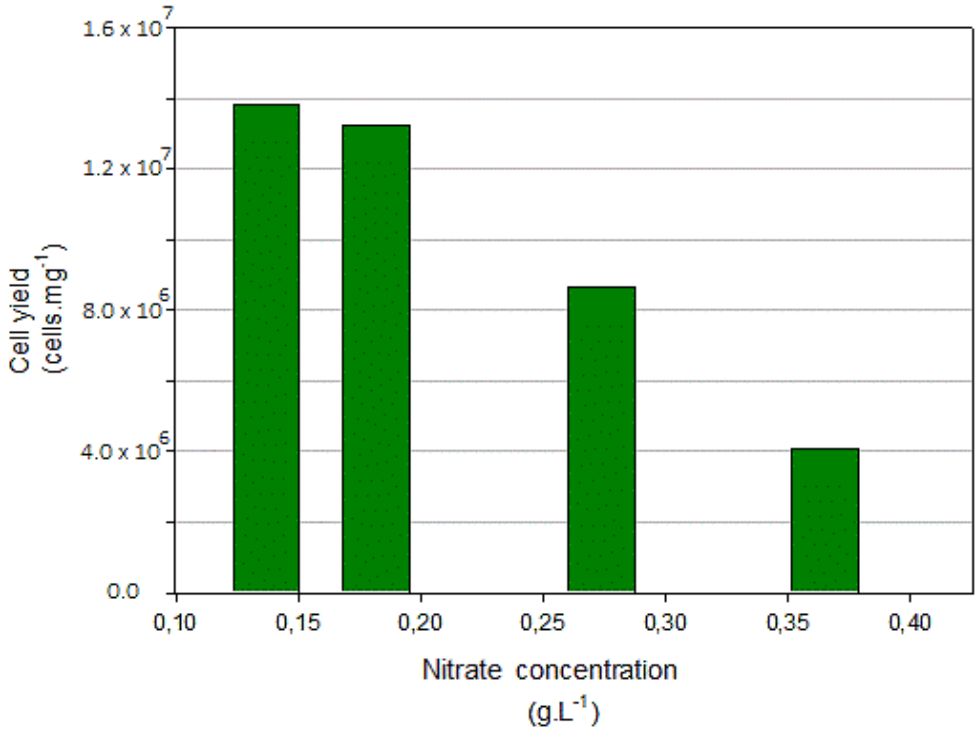


Figure 4.8 Average cell yield per milligram of nitrate consumed at different nitrate concentrations

Percentage consumption was observed to increase with increasing nitrate concentration (data not shown). Nitrate and nitrite reductase enzymes are responsible for the metabolism of nitrogen in the cell; however, it is documented that an ammonium intermediary compound is responsible for repression of the synthesis of both these enzymes (Flores et al. 1980). This ammonium ion intermediate in A23.2 may possibly play a feedback role in the nitrogen regulating mechanism, allowing storage of assimilated nitrates at higher concentrations but preventing them from being metabolised.

4.3.4. *Bicarbonate medium concentration and effects on growth*

Growth data were collected across bicarbonate concentrations of 1.09 to 2.89 $g.L^{-1}$ (standard Aquil medium: 1.45 $g.L^{-1}$) and are presented in Figure 4.9, showing distinct patterns of increasing growth at higher bicarbonate concentrations, up to 2.0 $g.L^{-1}$.

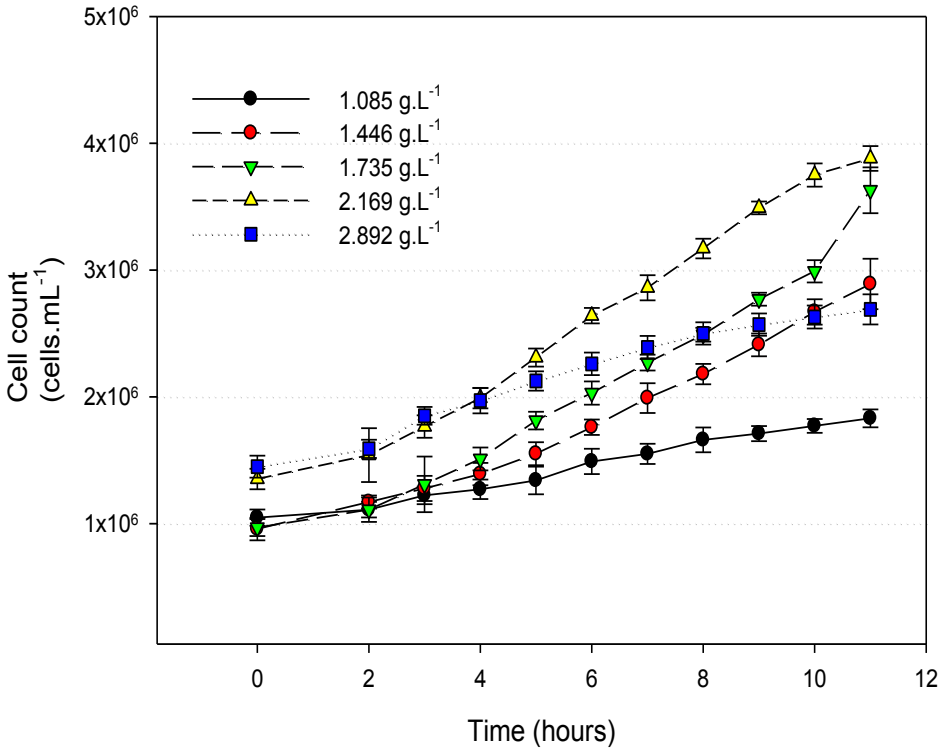


Figure 4.9 Growth profile of A23.2 cultivated in a stirred tank bioreactor in Aquil medium with various bicarbonate concentrations

The input of specific growth rate and cell productivity data into the quadratic optimisation model of Design-Expert®, revealed specific growth rate and cell productivity to be significant functions of bicarbonate concentration, with $p<0.0475$ and $p<0.0163$ respectively. Growth rate and cell productivity data when expressed as a function of bicarbonate concentration revealed maximum values at approximately 1.8 $g.L^{-1}$ (Figure 4.10). A statistical optimal bicarbonate concentration of 2.0 $g.L^{-1}$ was calculated by Design-Expert® to give a projected growth rate of 0.12 hr^{-1} and a maximal cell productivity of 2.42 x 10^8 $cells.L^{-1}.hr^{-1}$.

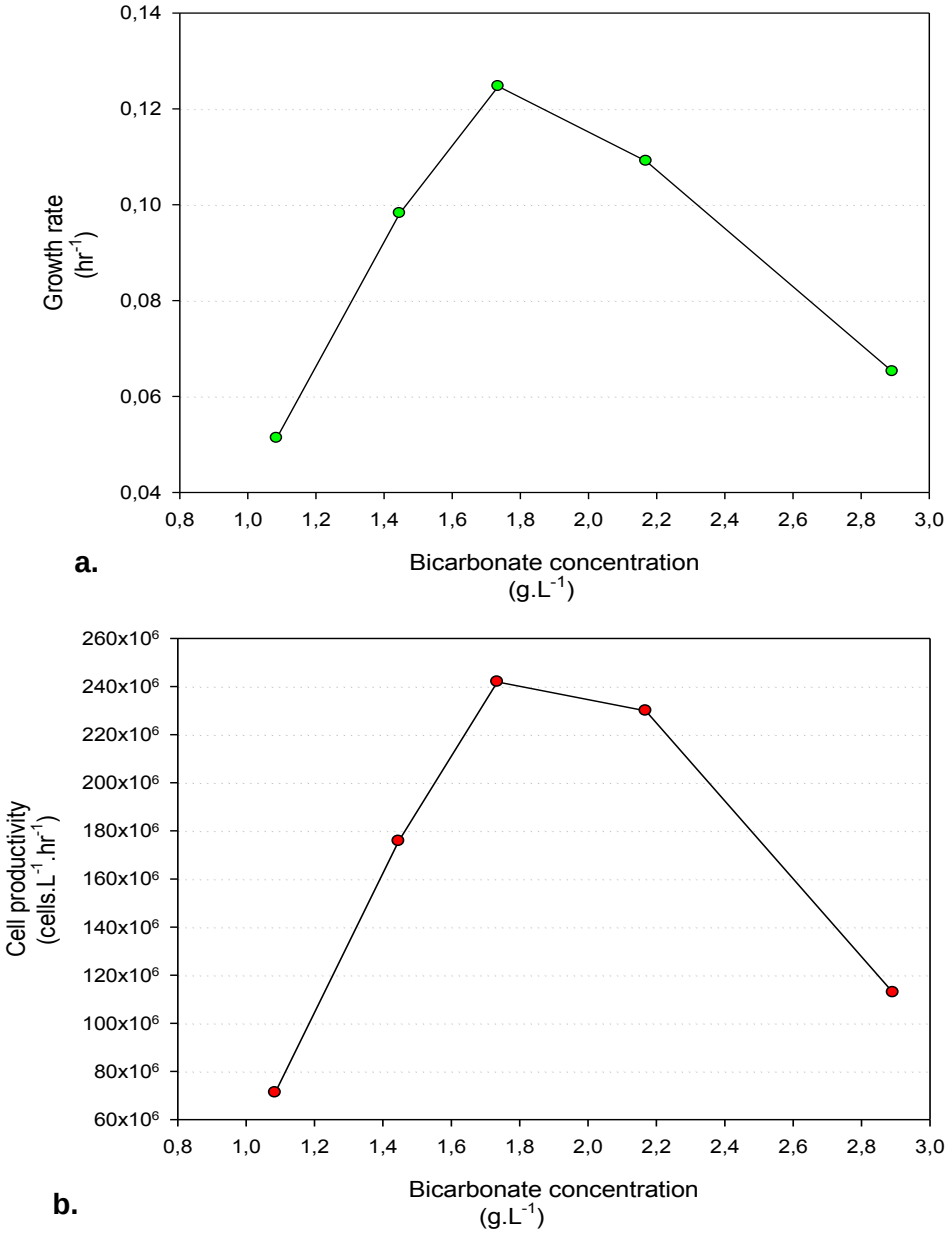


Figure 4.10 Effect of bicarbonate concentration on a) growth rate and b) cell productivity of A23.2 in a stirred tank bioreactor using Aquil medium (agitation at 100 rpm, pH maintained at 7)

The average cell yield trend (Figure 4.11) mirrors the growth response trends, indicating greater biomass yield at the midpoint of bicarbonate concentration range tested. Variations in pH, temperature and salinity govern the complex equilibrium between the four forms of carbon in media, i.e. CO_2, H_2CO_3, HCO_3^- , and CO_3^{2-}. The utilisation of HCO_3^- can either occur by direct uptake or by carbonic anhydrase, catalysing the conversion of HCO_3^- to CO_2

and OH^-, increasing culture pH (Nimer et al. 1997). These two mechanisms likely work in an independent way, constantly assimilating environmental dissolved inorganic carbon (DIC).

In Section 1.2.4.3 the distribution of carbon species as a function of pH was discussed. Although there is a higher ratio of HCO_3^- at pH 7, increasing concentrations of bicarbonate salt increased the buffering capacity of the medium, resulting in more HCl entering the culture and negatively impacting growth beyond 1.8 $g.L^{-1}$ bicarbonate. The negative effect on growth observed at 2.857 $g.L^{-1}$ bicarbonate could be a response to osmotic stress, pH drift or both.

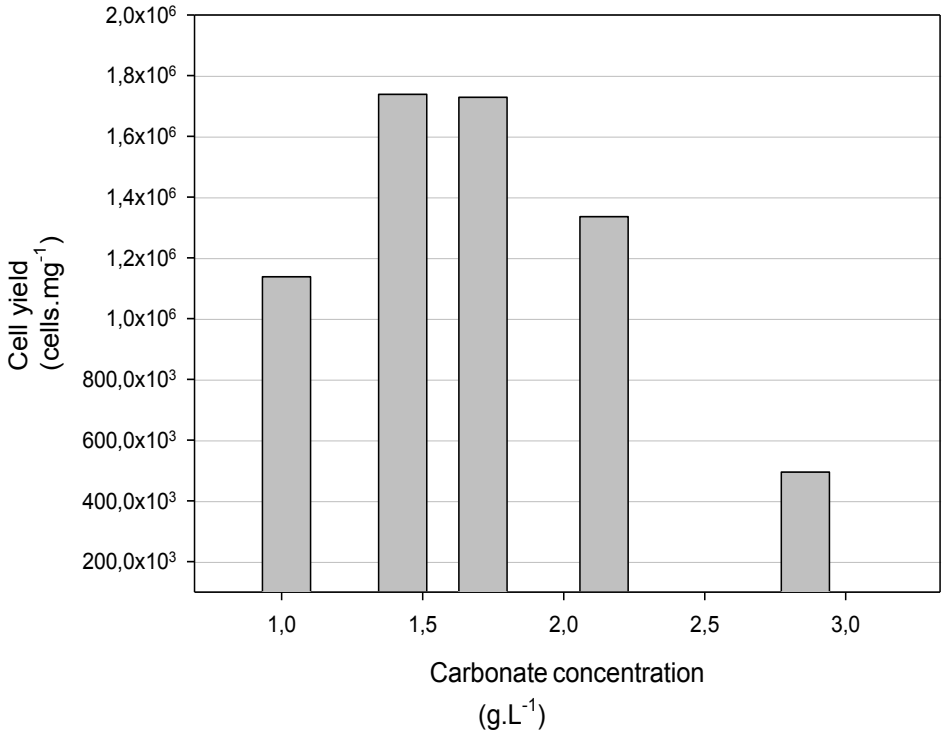


Figure 4.11 Average cell yield per milligram bicarbonate consumed at different bicarbonate concentrations

At the highest bicarbonate concentration tested, increased precipitation of salts was observed, which may have been a factor contributing to poor growth. This doubling in bicarbonate compared with the standard medium concentration triggered excessive salt precipitation, which may have contributed to a combination of pH drift, nutrient unavailability and sluggish growth, ultimately resulting in culture death after 12 hours (Figure 4.12).

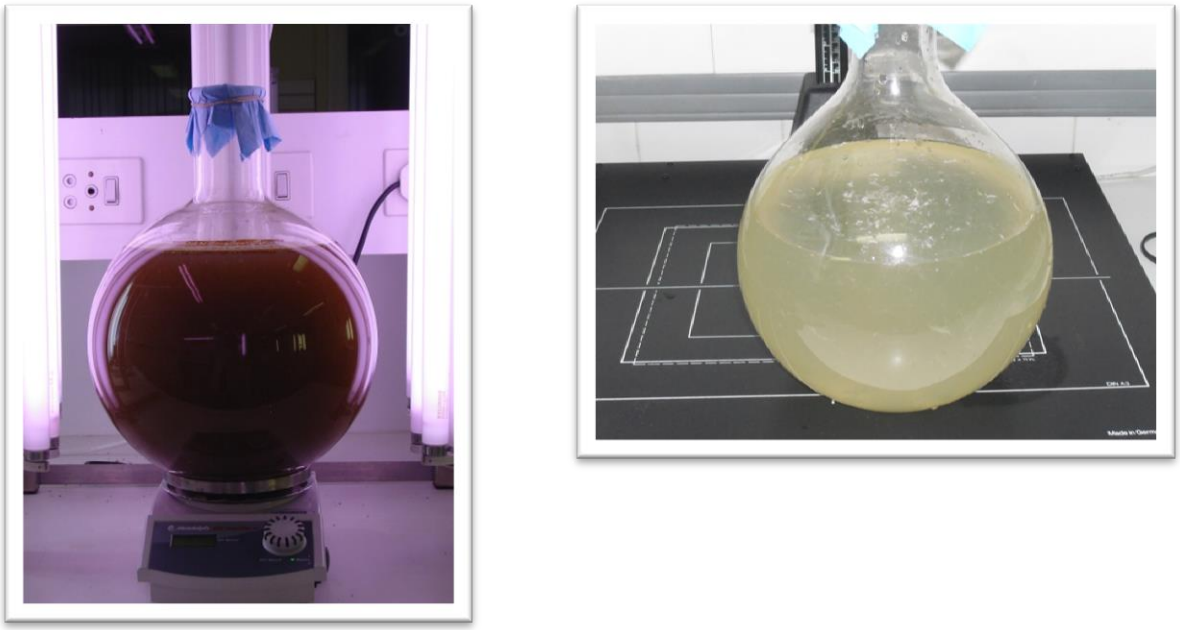


Figure 4.12 Healthy culture of A23.2 (left) and culture death at bicarbonate concentration twice that of standard Aquil medium (right)

4.4. Conclusion

Variation in phosphate and nitrate concentrations had a negligible effect on growth and productivity across the range of concentrations tested, in which growth data revealed metabolic regulation of these two nutrients. Decreasing cell yield beyond 0.05 $g.L^{-1}$ phosphate concentration suggested an inhibition of the phosphatase enzyme bound at the cell surface. Repression of nitrate reductase enzymes by an intermediate ammonium ion may have caused the decreased cell yield at increasing nitrate medium concentrations. Increasing nitrate consumption rates at higher nitrate concentrations likely resulted in more intermediate ammonium ions being produced and further nitrate metabolic inhibition. Standard Aquil medium phosphate concentration along with a nitrate level of 0.14 – 018 $g.L^{-1}$ was found to maintain satisfactory growth without any inhibitory effects. Phosphate input above optimal levels are wasted, not significantly improving cell growth, whereas excess nitrate concentration inhibits cell growth, increasing process costs and turnaround time from biomass to product.

Silicate and bicarbonate levels, in contrast, when increased beyond standard medium levels, were found to increase biomass formed and specific growth rate. A ceiling in metabolism was however, reached, due to precipitation and osmotic effects, allowing optima to be calculated. Diatoms are known to supersaturate their walls with hydrated silicon and this was evident from consumption rate data. Silicate salt tended to precipitate, restricting availability and light penetration, therefore silicate concentration should be maintained by adding silicate solution drop-wise when making up the growth medium, to prevent precipitation and resultant restriction in availability to cells.

Marine phytoplankton. can be found in diverse ecophysiological conditions that necessitate different and adaptable mechanisms for dissolved inorganic carbon (DIC) utilization. Consequently they are found in almost any body of marine water, including estuarine, coastal, and oceanic conditions. Although bicarbonate salt was used as a carbon source, it is suggested that gaseous CO_2 should be used, with the added property of maintaining a pH of 7. Greater amounts of HCl buffer were required at higher bicarbonate medium concentrations increasing cellular osmotic stress. Table 4.3 summarises optimal nutrient concentrations for A23.2 modelled on cell growth data and is therefore proposed for the growth stage of the bioprocess.

Table 4.3 Optimum recommended nutrient concentrations proposed for growth of A23.2

Nutrient ion	Optimum recommended findings ($g.L^{-1}$)
Bicarbonate	2.00
Phosphate	0.05
Nitrate	0.10 – 0.20
Silicate	0.20

5. IMPACT OF PHYSICOCHEMICAL PARAMETERS OF PH, SALINITY, TEMPERATURE AND LIGHT INTENSITY ON GROWTH OF DIATOM A23.2

5.1. Introduction

In Chapter 4, nutrient concentrations which supported maximum growth of isolate A23.2 were determined. In this chapter, the major physicochemical variables of pH, salinity, temperature and light are investigated in order to evaluate their effect on growth rates and cell productivity of diatom A23.2. The range of physicochemical parameters investigated extended beyond those experienced in most natural conditions (with the exception of temperature), to detect any specific adaptation or growth niche. The pH range tested was between 5.0 and 10.0, salinity from 20.56 to 71.46 $mS.cm^{-1}$, temperature from 15 to 30°C and light from 26 – 122 $\mu mol.m^{-2}.s^{-1}$.

Another physiological variant in marine ecosystems is salinity, which affects diatoms directly by exerting osmotic stress. Variation in salinity may also impact diatoms indirectly via interaction with other factors, such as nutrient dynamics, in terms of nutrient availability and uptake rates due to changes in osmolarity (Saros and Fritz 2000).

Temperature ranges for maximum growth vary with diatom species, as temperature is known to affect metabolism and reproductive rates. This effect may either be direct, by changing enzyme efficiency, or indirect, by altering water viscosity and dissolved oxygen (Werner 1977). The sampling location of A23.2 allows for a moderate range of temperatures to be tested, in which the most suitable for enhancing cellular growth was taken forward.

The effects of light intensity are species specific, depending on the habitat of the isolate in question. Vertical transport of diatoms and their corresponding light exposure is governed both by turbulence and sinking rate (Post et al. 1984); they can thus be transported from low light intensities in deeper layers, to surface light intensities on a time scale of a few hours. Examples include low light species that are found in caves or those that prefer abundant light originating from shallow littoral zones (Werner 1977). A light intensity assay was thus

conducted in order to guide current diatom experimental design. The assay focused on light intensity exposure, to establish an approximate range of light intensity suitable for cultivation.

5.2. Materials and Method

5.2.1. *Inoculum*

A vegetative stock culture of 40% $v.v^{-1}$ inoculum was used to inoculate a 2 L glass bioreactor by sterile transfer, in the same procedure as described in Section 4.2.1.

5.2.2. *Standard cultivation conditions*

The culture was incubated within glass bioreactors (Section 2.2) and operated at the same physicochemical parameters as described in Section 4.2.3.

5.2.3. *Experimental Design*

Specific physicochemical conditions sampled each hour over an 11 hour period are summarised in Table 5.1. Growth responses measured included cell concentration; specific growth rate and cell productivity were determined from these cell growth profiles. The relationship between specific growth rate or productivity and the physicochemical parameter was analysed statistically by DesignExpert®, which integrated significant growth responses into the optimisation model.

Table 5.1 Ranges of physicochemical intensities investigated over 11 hours

pH	**Temperature (°C)**	**Salinity ($g.L^{-1}$)**	**Approx Light intensity ($\mu mol.m^{-2}.s^{-1}$)**
5.0	15	12.3	26
6.0	20	18.4	45
7.0*	25*	24.5*	65*
8.0	27	30.7	72
9.0	30	49.1	116
10.0			122

***Constant physicochemical parameters in which only one parameter was varied at a time

Growth assays of A23.2 cultivated under varying medium pH was conducted in two sets. Set 1 consisted of pH 5, 9 and 10 and set 2 of pH 6, 7 and 8. NaCl concentrations of 50% $w.v^{-1}$, 75% $w.v^{-1}$, 100% $w.v^{-1}$, 125% $w.v^{-1}$ and 200% $w.v^{-1}$ of standard modified Aquil medium were tested. Cultivation temperatures were adjusted using the water jacket temperature set-point on the bioreactor. Light intensity was measured using a LI-COR inc. LI-1400 data-logger, with attached LI190- SA quantum sensor (Nebraska, USA). Light intensity tested ranged between 26 and 122 $\mu mol.m^{-2}.s^{-1}$, with an accepted fluctuation of 5 $\mu mol.m^{-2}.s^{-1}$. This was measured as the average light intensity within the centre of the bioreactor itself, with the water jacket filled, prior to addition of medium or culture. These intensities were created by varying the number of lamps illuminated on the light rig surrounding the bioreactor. Light assays were conducted in two sets, Set 1 at 26 – 72 $\mu mol.m^{-2}.s^{-1}$ and Set 2 at 116 and 122 $\mu mol.m^{-2}.s^{-1}$. Light intensity range was chosen around standard cultivation intensity used in previous experiments, at 65 $\mu mol.m^{-2}.s^{-1}$. Biomass growth was determined by cell counts using a Neubauer haemocytometer, as described in Section 2.3.2.

5.3. Results and Discussion

5.3.1. *The effect of cultivation pH on growth*

Growth profiles as a function of pH, based on cell concentration measured over 11 hours, are summarised in Figure 5.1, showing a preference of A23.2 to a pH range of 6.0 to 8.0. An environmental pH of between 6.0 and 8.0 yielded the highest relative specific growth rates, cell productivity as well as cell concentration after 11 hours.

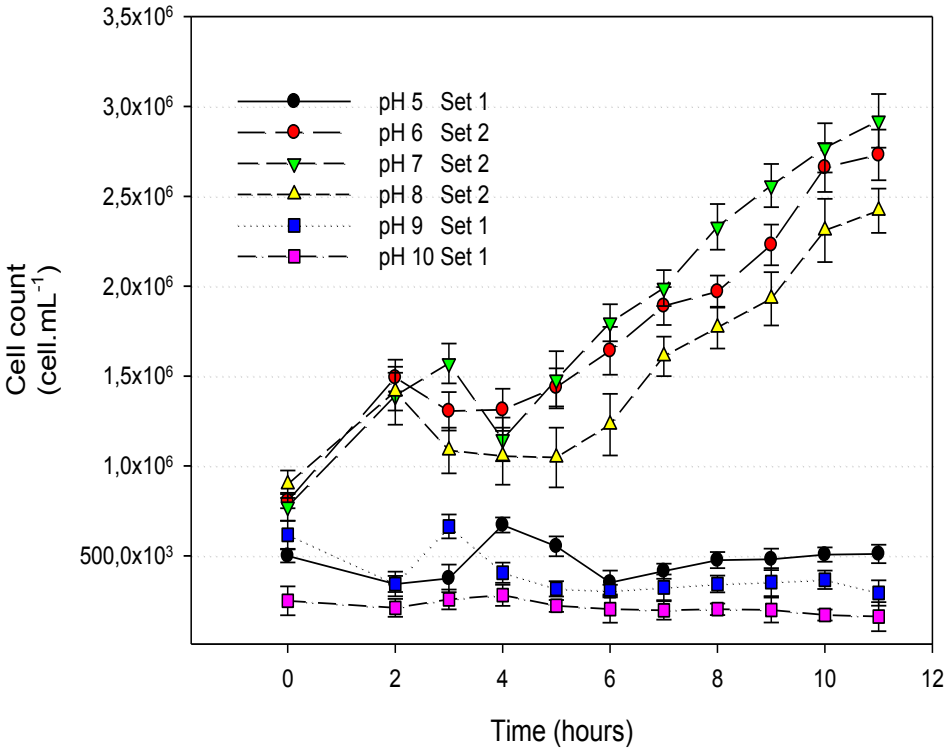


Figure 5.1 Growth profile of A23.2 cultivated in a stirred tank bioreactor in Aquil medium at various pH

When growth parameters were anaysed as a function of medium pH, a clear maximum peak was observed at approximately pH 7 (Figure 5.2). A statistically significant response to pH was thus attained for both specific growth rate ($p<0.0191$) and cell productivity ($p<0.0155$). Using the Design-Expert® optimisation model, a maximum specific growth rate of 0.14 hr^{-1}, with a corresponding cell productivity of 2.06 x 10^8 $cells.L^{-1}.hr^{-1}$ at pH 7.2 was estimated. The evaluation of these cell growth trends showed that environmental pH of 5, 9 and 10 were inhibitory.

Different inorganic carbon species exist at different ratios depending on medium pH, as discussed in Section 1.2.4.3. Bicarbonate ions tend to increase with increasing pH starting at pH 5, reaching a plateau at the approximate range of pH 6 to pH 8, according to the Bjerrum plot of the carbonate system in seawater at 25 °C (Anderson 2002). Figure 5.2 suggests greater bicarbonate availability at this pH range and therefore a peak in cell growth. At higher pH the carbonate ion dominates, preventing access of cells to bicarbonate.

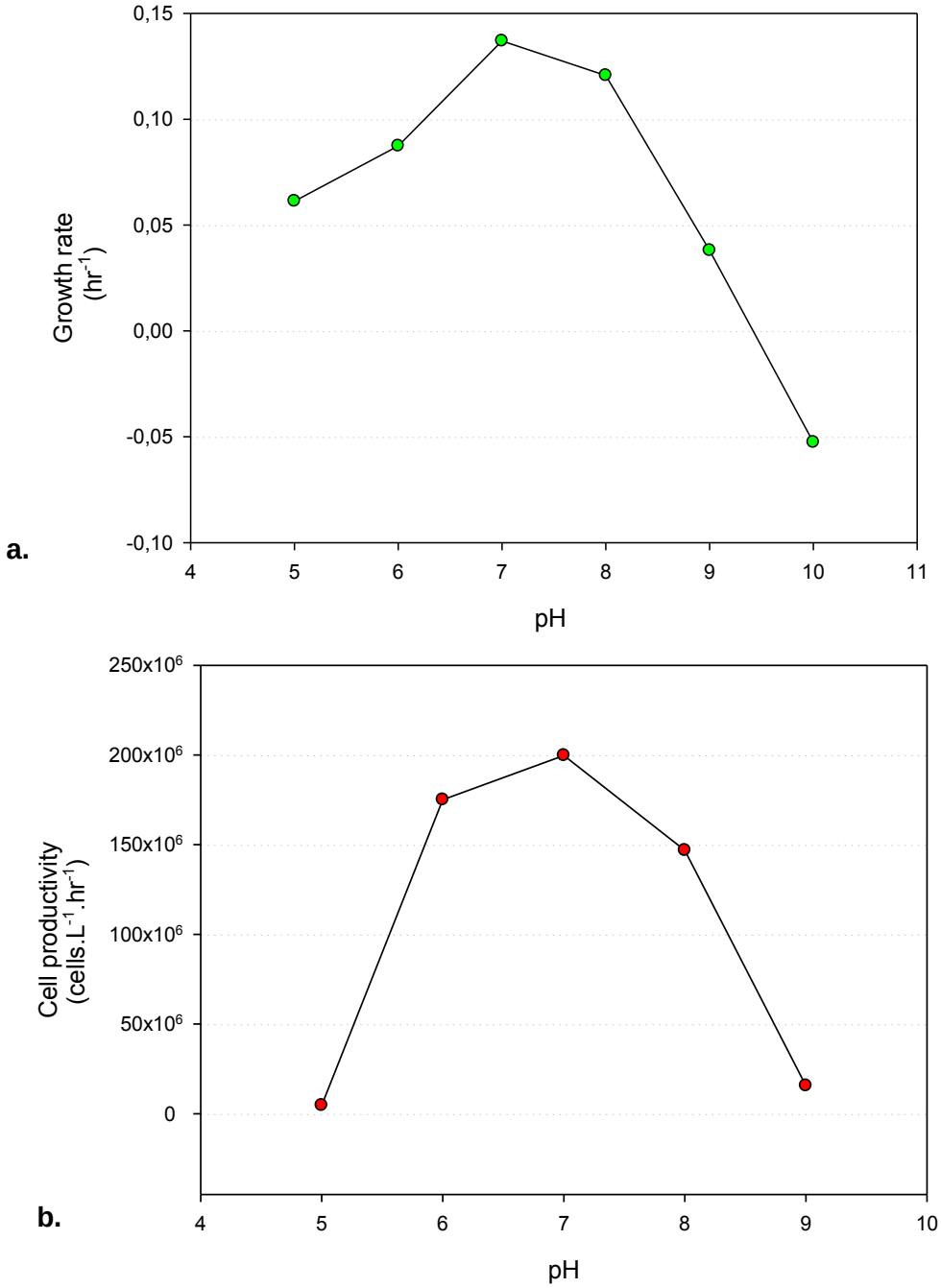


Figure 5.2 Effect of culture pH on a) growth rate and b) cell productivity of A23.2 in a stirred tank bioreactor using Aquil medium (agitation at 100 rpm)

5.3.2. *The effect of medium salinity concentration on growth*

The physicochemical study tested the growth of diatom A23.2 under different NaCl concentrations. Cell concentration data under various concentrations of NaCl were captured

and presented in Figure 5.3. These values were chosen around the standard Aquil medium concentration.

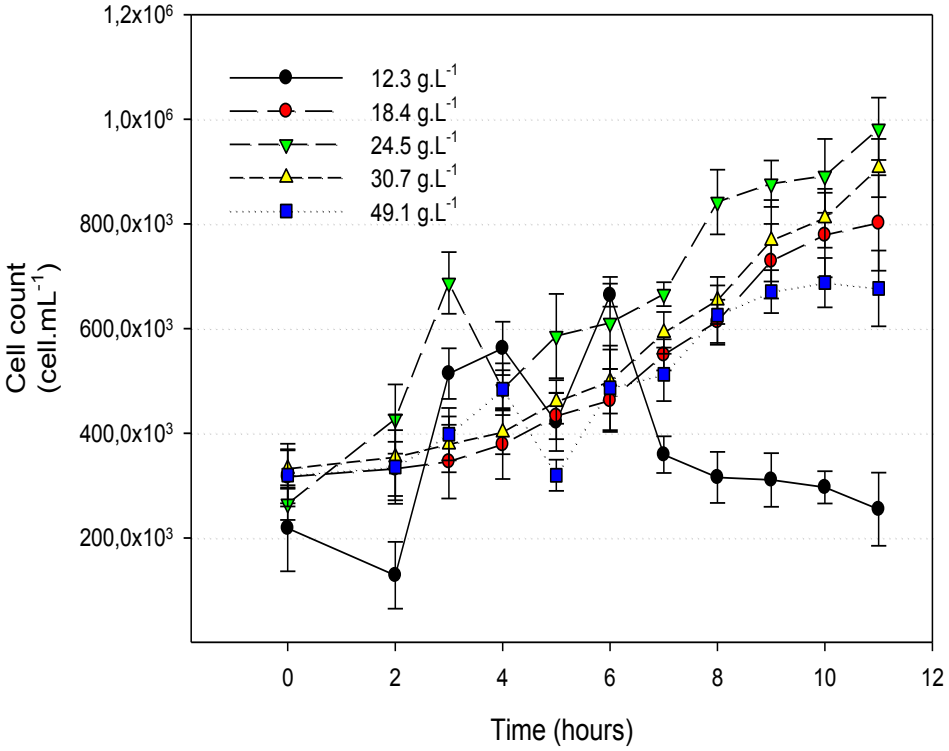


Figure 5.3 Growth profile of A23.2 cultivated in a stirred tank bioreactor in Aquil medium at various concentrations of increasing salinity

Growth data indicated that base case NaCl concentration (24.5 $g.L^{-1}$) showed better growth in terms of overall cell concentration, when compared to other NaCl concentrations, over a 11 hr period. At 50% of the base condition (12.3 $g.L^{-1}$), a negative growth rate resulted, possibly due to hypo-osmotic stress, as the cells absorbed excess water from a dilute environment. This was clearly seen from the large number of ghost or dead cells in which only the outer cell wall was discernible (Figure 5.4). Doubling the concentration of NaCl with respect to the standard medium resulted in a decrease in cell concentration. Osmotic stress exerted in the form of a more concentrated saline medium; has shown to result in excess water leaving the cell, upsetting metabolic activity and consequent nutrient uptake (Saros and Fritz 2000).

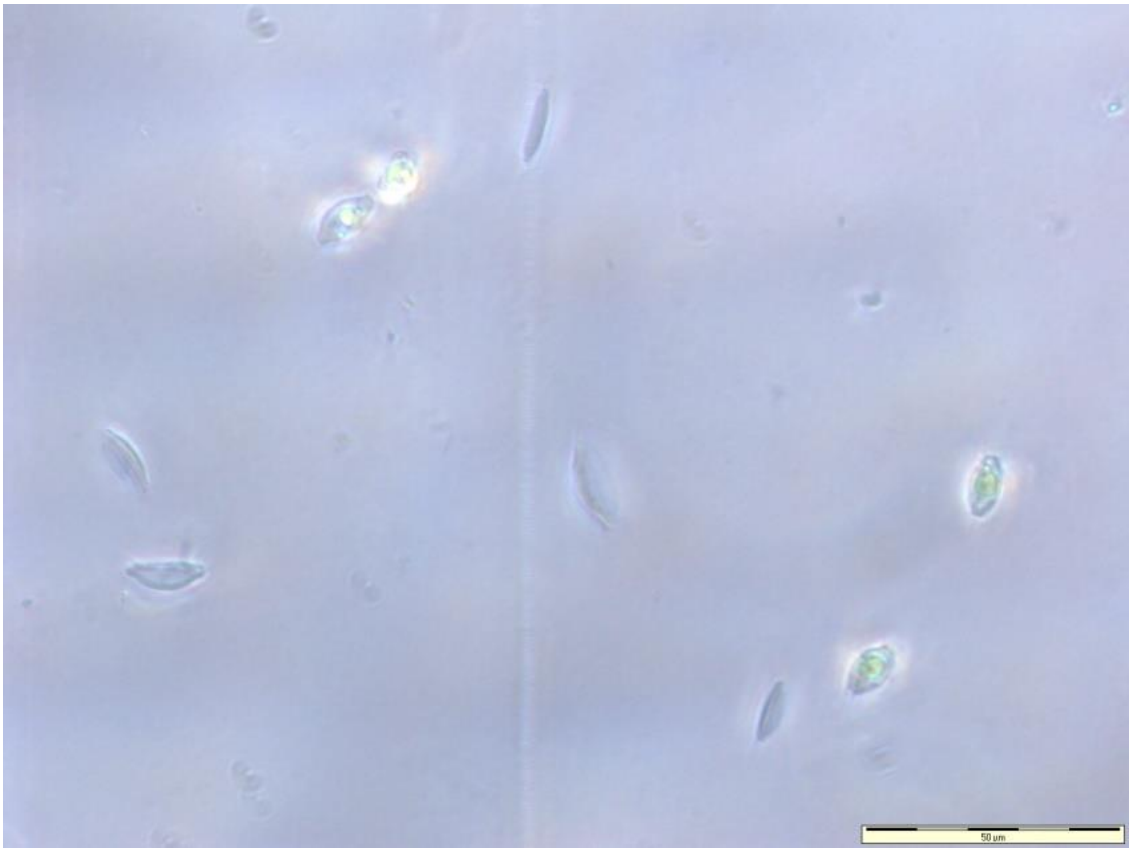


Figure 5.4 Light microscope image of dead cells at half medium salt concentration (100x magnification)

Salinity growth rate and productivity data of A23.2 had poor fits to polynomial models and therefore revealed no significant relationship to salinity at the range of 75% to 200% base medium value when entered into the statistical optimisation model. Data indicated that medium NaCl concentration of 24.54 $g.L^{-1}$ to 30 $g.L^{-1}$ was suitable and fit for growth of A23.2.

5.3.3. *The effect of cultivation temperature on growth*

Temperature affects diffusion rates of chemicals into the cell, cell metabolism, and dissolved oxygen. Metabolic enzymes have a specific operating temperature range thus, temperature is a particularly important factor to consider in proof of concept studies performed in outdoor cultivation systems. Diatoms have optimum ranges of temperature for growth, related to their habitat of origin and governing the diversity of diatom species during different seasons (Werner 1977). Growth profiles of A23.2 comparing cell concentration and cultivation temperature are shown in Figure 5.5. Higher growth is observed between a temperature range of 15 – 30 °C, consistent with the diatoms sampling location.

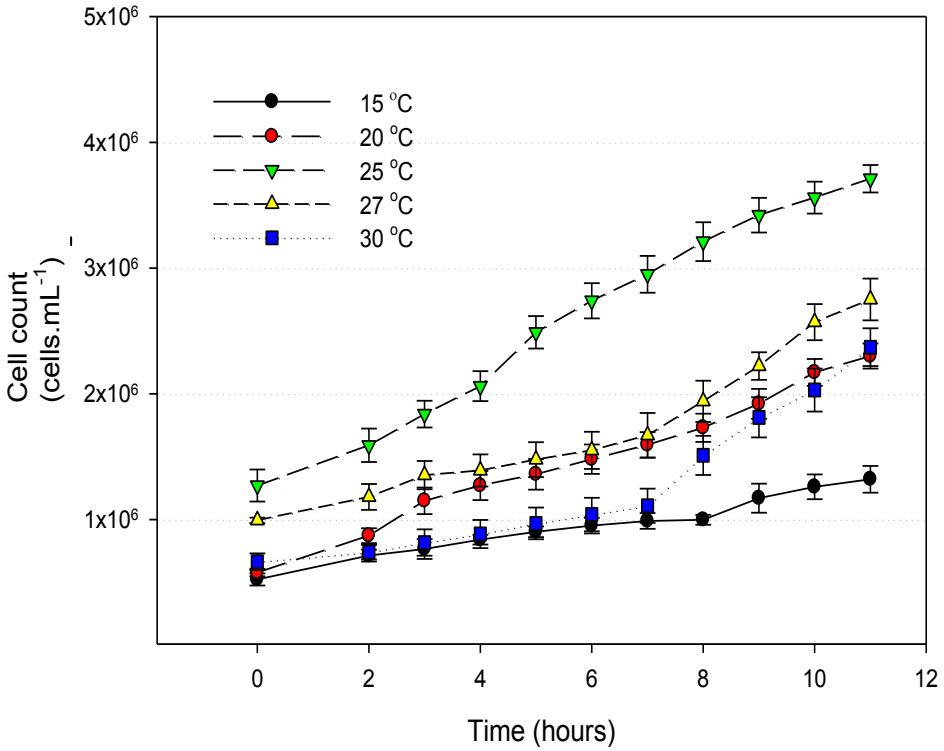


Figure 5.5 Growth profile of A23.2 as a function of time at different temperatures and standard cultivation conditions in a stirred tank bioreactor

Growth rate and productivity data showed poor fits to polynomial models, and therefore no value could be calculated by the Design-Expert® optimisation software. Climate selection is important for location of large scale raceway ponds or photobioreactors, in order to find the most stable temperature range possible. Suitable growth at a temperature range between 20 – 27 °C may be advantageous, in that it mirrors the average minimum and maximum temperatures of Heidelburg near the sampling location.

5.3.4. *The effect of light intensity on growth*

Light intensity affects the level of cell production and is the core regulator of photosynthesis (Watson et al. 2004); therefore, growth rates of diatoms should theoretically increase with increasing light intensity, up to levels of light saturation. Growth results based on light intensity are summarised in Figure 5.6. Growth below 65 µmol.m^{-2}.s^{-1} light intensity resulted in lower overall cell densities, due to less light penetration. Light intensity above tested 72 µmol.m^{-2}.s^{-1} resulted in much lower overall biomass, due to possible light saturation. This results when more photons are absorbed than the photosynthetic apparatus can actually

utilize, damaging the chloroplast and resulting in photoinhibition (Benemann 2013). The cultures exposed to the two highest light intensities showed a peak at the end of the experimental period, suggesting further work into the adjustment of light intensity as a function of biomass density.

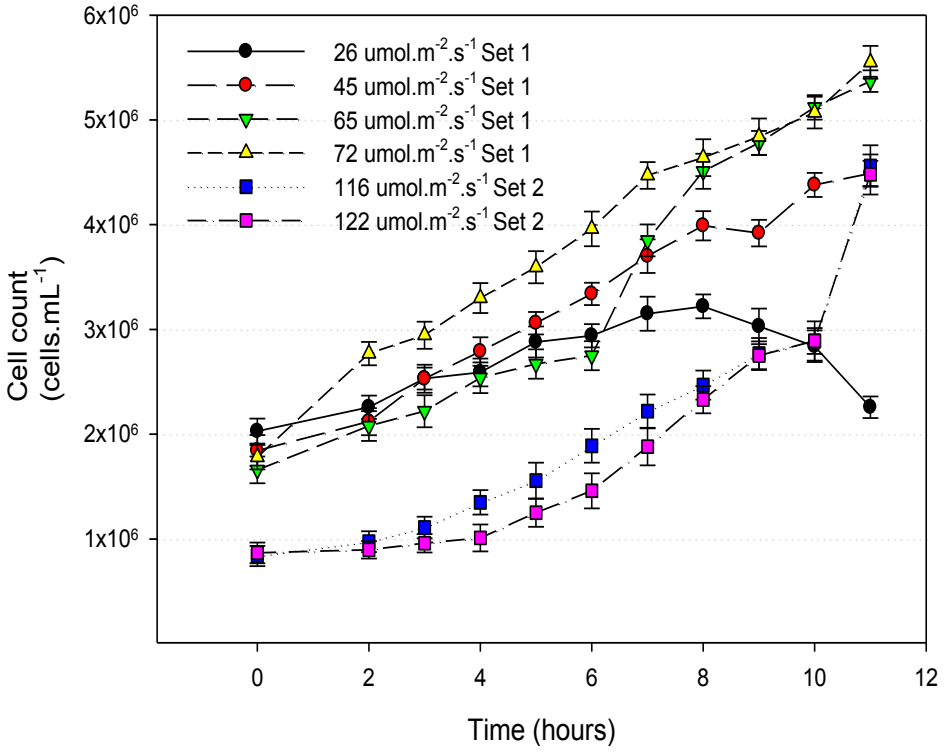


Figure 5.6 Growth profile of A23.2 at increasing light intensities and standard cultivation conditions in a stirred tank bioreactor

Cell productivity of isolate A23.2, can only manage a linear relationship with increasing light intensity, suggesting light limitation, as a plateau is observed in Figure 5.7. As this study was conducted indoors under laboratory conditions, these observations need to be verified outdoors under natural sunlight, in which intensities can exceed 1000 µmol.m^{-2}.s^{-1} (Thimijin and Heins 1982). The quantity of light and, more importantly, actual light penetration, has a direct impact on growth of the photoautotroph. In a study by Post et al. (1984) diatoms were capable of optimizing exposure to higher irradiance levels by rapidly increasing their division rate. The hurdle of cell shading regulating growth in A23.2 is made clear from the data; and can be used to justify an indoor light intensity range of approximately 60 – 70 µmol.m^{-2}.s^{-1}. This range should ideally be dynamic, in terms of obtaining greater light penetration into the culture without excessive heating or damage to the photosynthetic system. Ultimately

greater light penetration is critical to achieving commercial biomass densities, warranting further research into new ways of internal culture illumination. The effect of light intensity on EPA productivity is determined via stress batch studies in Section 6.3.1.

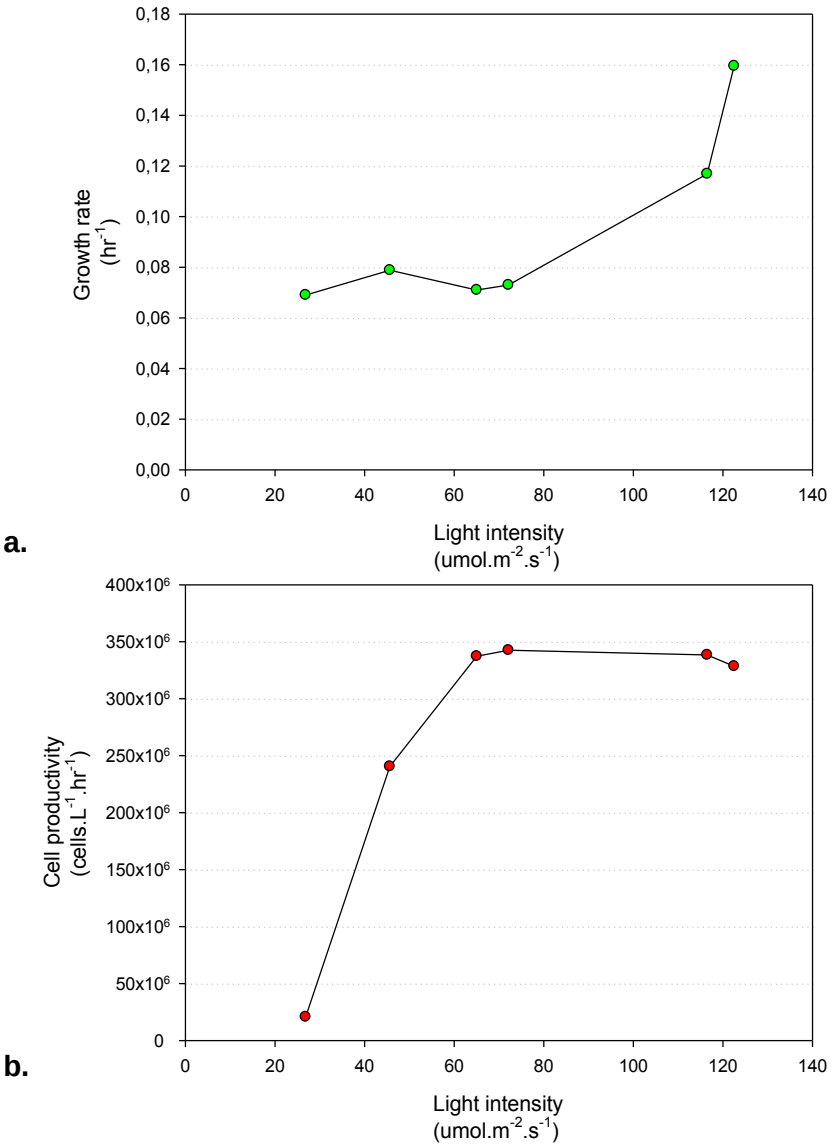


Figure 5.7 Effect of culture light intensity on a) growth rate and b) cell productivity of A23.2 in a stirred tank bioreactor using Aquil medium (agitation at 100 rpm)

5.4. Conclusion

A statistically optimal cultivation pH of 7.2 was a reflection of the neutral conditions diatoms inhabit. The pH in a large scale system should therefore be strictly controlled.

Environmental pH also effects carbon speciation and hence availability. The pH was the parameter tested with most significant effect on growth, likely due to the dual effect on enzyme function and carbon speciation.

Cell productivity was found to increase with increasing light intensity, until a plateau was reached, showing evidence of shading at light intensities above 72 $\mu mol.m^{-2}.s^{-1}$, where cell division and consequent cell shading was more rapid. This prompted a tentative indoor cultivation light intensity of between 60 – 70 $\mu mol.m^{-2}.s^{-1}$. A further study on this diatom is recommended at greater light penetration methods beyond those tested here, simulating outdoor radiation to determine quantitative cell shading limits.

Salinity and anion composition are strongly correlated with algal species distribution (Saros and Fritz 2000). The salinity range of 18 – 30 $g.L^{-1}$, had a limited impact on growth, with maximum recorded cell numbers at Aquil medium-defined salinity of 24.54 $g.L^{-1}$. Temperature growth data indicated a temperate origin of the isolate, in which maximum growth occurred at 25 °C. Growth stage physicochemical operating parameters taken forward are summarised in Table 5.2.

Table 5.2 Optimum recommended nutrient concentrations proposed for growth of A23.2

Physicochemical parameter	Optimum recommended findings
pH	7.2
Salinity	24.54 $g.L^{-1}$
Temperature	25 °C
Light intensity	60 – 70 $\mu mol.m^{-2}.s^{-1}$

6. INVESTIGATION INTO THE IMPACT OF PHYSICOCHEMICAL STRESS ON THE EICOSAPENTAENOIC ACID PRODUCTION OF DIATOM A23.2

6.1. Introduction

Under optimal growth conditions microalgae synthesize proteins and nucleic acids required for growth. Under a variety of stress conditions, many of these cells accumulate storage products or stress response molecules. The conditions under which microalgae are cultured, therefore, impact their biochemical composition and hence fatty acid content (Behrens and Kyle 1996; Brown et al. 1996). Response to stress is unique to the specific stress factor and the microalgal isolate.

Environmental stress factors such as light and UV radiation (Brown et al. 1996; Alonso et al. 1996; Wen and Chen 2000; Fábregas et al. 2002; Tzovenis et al. 2003), salinity (Saros and Fritz 2000), temperature, and nutrient availability (Kitano et al. 1998; Hu and Gao 2003; Hoshida et al. 2004) alter cellular composition due to the accumulation of lipids, carbohydrates or other secondary metabolites (Lv et al. 2010). In microalgae that accumulate EPA in response to stress conditions, the environmental conditions that affect cellular EPA content may influence cell growth differently. For example, a reduction in cellular growth due to stress may be accompanied by an increase in EPA content; depending of the magnitude of these effects and the overall cell concentration. This may either reduce or increase the overall yield of EPA and EPA productivity. The conditions that are required to maximize EPA content may differ to those needed for the maximization of EPA productivity (Hoshida et al. 2004; Griffiths and Harrison 2009).

It was previously demonstrated (Section 3.3.3) that environmental stress had a beneficial effect on EPA production and a negative effect on cellular growth in isolate A23.2. This suggested that a biphasic EPA production process would be favoured to first maximise biomass production and its resultant concentration and thereafter, to stress cells to increase their EPA content. In order to achieve this, various environmental factors were investigated to determine their effects on EPA content. In this study the response of EPA production to different physicochemical stress parameters was evaluated in the brown diatom A23.2. The

numerical range for each physicochemical condition was chosen to straddle the standard values found in Section 5.2 for optimal growth. The EPA production data generated for each stress factor and its corresponding intensity were evaluated. From these results, operational conclusions were drawn to increase the yield of eicosapentaenoic acid at the stress stage of a model production system. These parameters were tested in the raceway system, as reported in Chapter 7.

6.2. Materials and Method

6.2.1. *Inoculum and Medium*

The vegetative stock culture of diatom A23.2, with an approximate cell count of 1.2×10^7 cells.mL^{-1}, outlined in Section 4.2.1, formed the inoculum for this trial. A culture volume of 1 L was passed through a 45 µm filter and the resulting paste was used to inoculate 500 mL of sterile Aquil medium, contained in a 1 L glass round-bottomed flask, previously autoclaved at 121°C for 15 min, bringing the cell count up to 2.4×10^7 cells.mL^{-1} before the start of growth. This was done for each physiological parameter tested. The modified Aquil diatom medium used throughout as substrate, was adapted from Anderson (2005) (Appendix B), unless otherwise stipulated.

6.2.2. *Standard cultivation conditions*

Unless stated otherwise, the batch flask cultures were incubated at 25 ± 2°C under constant illumination by a surrounding light rig during the course of the trial, at 100 – 140 µmol.m^{-2}.s^{-1} (measured at the incident surface of the cultivation flask). The pH was monitored but not adjusted, salinity was not monitored. Standard conditions for pH and salinity were pH 7.0 and 24.51 g.L^{-1} respectively. Agitation on stirrer plates was less effective than bioreactor impellers; flask agitation was therefore increased to 500 rpm.

6.2.3. *Conditions for the stress study*

6.2.3.1. *Light study*

Although light intensity at peak daylight can reach levels of over 1000 µmol.m^{-2}.s^{-1}, this value varies substantially in the ocean due to scattering and algal depth (Thurman and Elizabeth 2001). Stress light intensities were chosen around values in Section 5.3.4, in which a plateau in growth productivity data was observed. Six separate, increasing light intensities

were used to illuminate each of the flasks in the light study, with the seventh flask kept in constant darkness as a control. Light intensity was measured using a LI-COR inc. LI-1400 data-logger, with attached LI190- SA quantum sensor (Nebraska, USA), at the incident surface of each flask. The intensities used were 15, 55, 80, 120, 130 and 180 $\mu mol.m^{-2}.s^{-1}$ with a ±10 $\mu mol.m^{-2}.s^{-1}$ fluctuation and continuous illumination.

6.2.3.2. *Salinity Study*

Varied NaCl concentrations, ranging at 50 – 200 % of standard Aquil medium salinity, was tested in standard conditions (Section 6.2.2), with ranges given in Table 6.1.

Table 6.1 Salinity concentrations used to evaluate corresponding effects on EPA production

Salinity wrt std medium (%)	NaCl ($g.L^{-1}$)
50	12.3
75	18.4
100	24.5
120	29.5
150	36.8
200	49.1

6.2.3.3. *pH Study*

The growth medium was prepared as above (Section 6.2.1), however before autoclaving each flask, its pH was adjusted. Starting pH values of 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 were tested in this experiment. The pH was not maintained in each flask for the duration of the 10 day assay after inoculation.

6.2.3.4. *Temperature Study*

Cultures were inoculated into standard Aquil medium and one flask incubated at each of the following temperatures: 5, 10, 15, 20, 25, and 30°C under conditions described in Section 6.2.2.

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6.2.4. *Sampling and EPA assay*

Cultures were sampled daily (15 mL volume) at approximately 9.00 am for 10 days. A direct transesterification of the lipids within the culture was first performed according to the

standard operating procedure outlined in Section 2.3.4.1. The esterified lipids were separated into their various fatty acid components, using gas chromatographic analysis described in Section 2.3.4.2, after which EPA data were plotted as a function of time. Overall EPA productivity data were expressed using Equation (5), described in Section 2.3.4.2.

6.2.5. *Nutrient stress confirmation study*

Concentrations of nutrient salts which were not removed were based on Aquil medium concentrations. A qualitative nutrient stress study, reported by Zulu and Lalloo (2011), found that nitrate, phosphate, sulphate and silicate deficiencies enhanced EPA content. This was determined by looking at relative gas chromatographic peaks. Their findings, summarised in Table 6.2, were used to form a base stress medium devoid of nitrate, phosphate, sulphate and silicate salts. This stress medium was used for experiments in which data needed confirmation, by observing any minor increase in EPA beyond nutrient stress.

Table 6.2 **Assessment on the effect of key nutrient deficiency on EPA productivity in batch flask culture under standard cultivation conditions** (Zulu and Lalloo 2011)

Key nutrients and combinations	**Approximate EPA productivities ($mg.L^{-1}.day^{-1}$)**
-SO_4^{2-}	0.3
-PO_4^{2-}	0.1
-NO_3^-	0.1
-SiO_3^{2-}	0.2
-HCO_3^-	0.3
Tap water	0
-NO_3^- PO_4^{2-}	0.5
-NO_3^- PO_4^{2-} SO_4^{2-}	0.4
-NO_3^- PO_4^{2-} CO_3^-	0.1
-NO_3^- PO_4^{2-} SO_4^{2-} SiO_3^{2-}	0.8

If more than one stress intensity showed spikes in volumetric EPA concentration trend over time, the experiment was re-evaluated in triplicate. This confirmation trial was conducted under standard cultivation conditions described in 6.2.2, with the exception of using a nutrient stress culture medium. This stress medium was based on the Aquil medium (Appendix B), but salts of Na_2SO_4, $NaH_2PO_4.H_2O$, $NaNO_3$ and $Na_2SiO_3.9H_2O$ were omitted. To obtain average productivity data, the highest EPA volumetric concentration value for each

re-tested parameter was divided by the time at which this was achieved. These productivity data were then subjected to a statistical T-test, in order to determine if EPA productivity was significantly different between the levels tested at the 95% confidence interval.

6.3. Results and Discussion

6.3.1. *The effect of light intensity on EPA production*

In their natural environments, microalgae are acclimatized to the circadian light:dark cycle (Fábregas et al. 2002). Algal cell division occurs during the dark period, while other metabolic processes, such as pigment, lipid and fatty acid synthesis, including accumulation of omega-3 fatty acids, take place during the light period, fuelled by energy captured photosynthetically (Brown et al. 1996). The light study performed here was designed to observe the approximate continuous light intensity at which EPA cellular productivity reached a peak, using a 'dark' culture as a control.

Fatty acids are consumed to a greater degree compared to carbohydrates and proteins during the dark cycle (Fábregas et al. 2002; Lv et al. 2010; Sukenik 1991). Volumetric EPA production of A23.2 as a function of time at different light intensities is shown in Figure 6.1. The control culture kept in the dark was consistent in the decline of EPA production as expected, with peak stable EPA content observed between days 2 and 4 of the batch culture experiment at light intensities above 14.89 $\mu mol.m^{-2}.s^{-1}$.

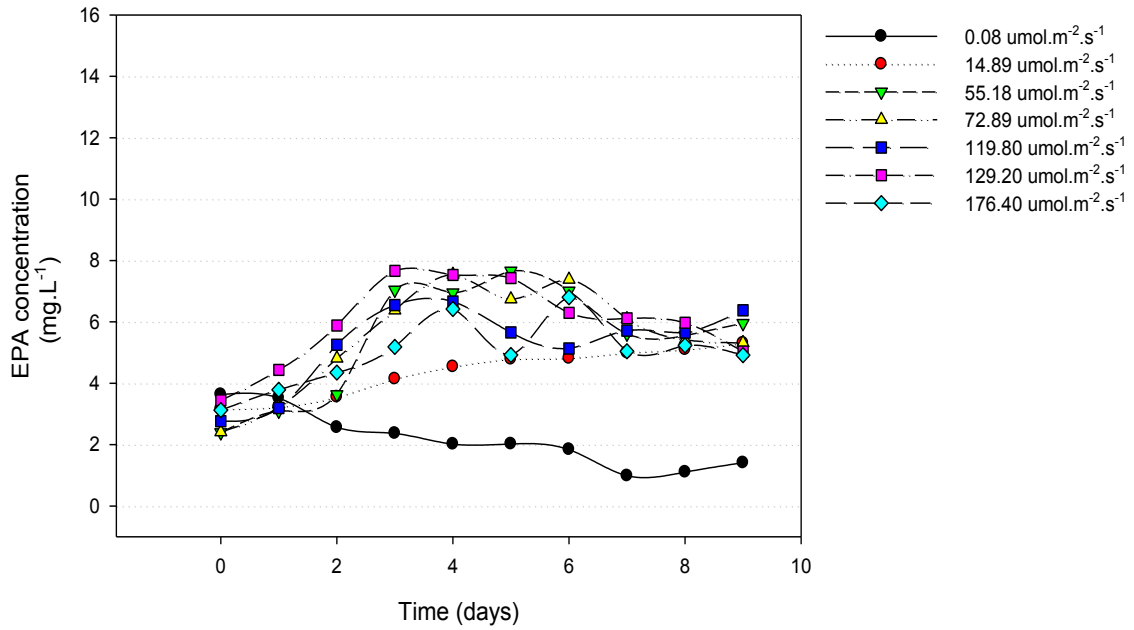

Figure 6.1 The volumetric EPA concentration of isolate A23.2 as a function of time and culture light intensity at standard cultivation conditions

Overall EPA productivity was plotted as a function of light intensity in Figure 6.2, in order to examine the relationship between them. The overall EPA productivity of A23.2 increased initially with increasing light intensity, a plateau in productivity was observed at approximately 70 – 130 µmol.m^{-2}.s^{-1}, and a decline in productivity observed at the higher intensity.

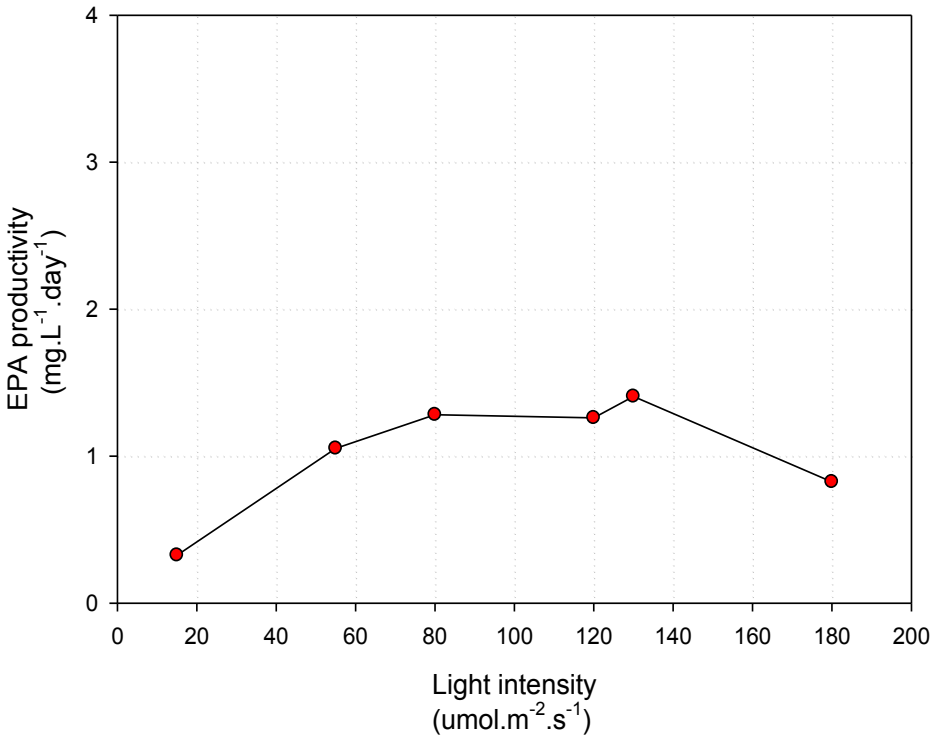

Figure 6.2 Overall EPA productivity of isolate A23.2 as a function of light intensity during 24 h illumination

Brown et al. (1996) found that low light intensities typically induce the EPA based polar lipids associated with the chloroplast, whereas high light intensity increases growth rate (Figure 5.7) and the amount of neutral lipids, mainly triacylglycerols. These two factors combine to suggest an optimal light intensity range, for cell and EPA productivity at 60 - 80 $\mu mol.m^{-2}.s^{-1}$ when considering growth trends in Section 5.3.4. At this range light intensity is able to sustain cell productivity in both the growth and stress phases, while being low enough to maximise EPA production.

6.3.2. *EPA production and medium salinity*

Culture EPA profiles at varying salinity is shown in Figure 6.3. The trends in EPA concentration were similar (with the exception of the declining EPA concentration at a salinity of 49.1 $g.L^{-1}$) and showed stability between days 2 and 5. A greater elevation in EPA concentration was also observed at 18.4 $g.L^{-1}$ salinity.

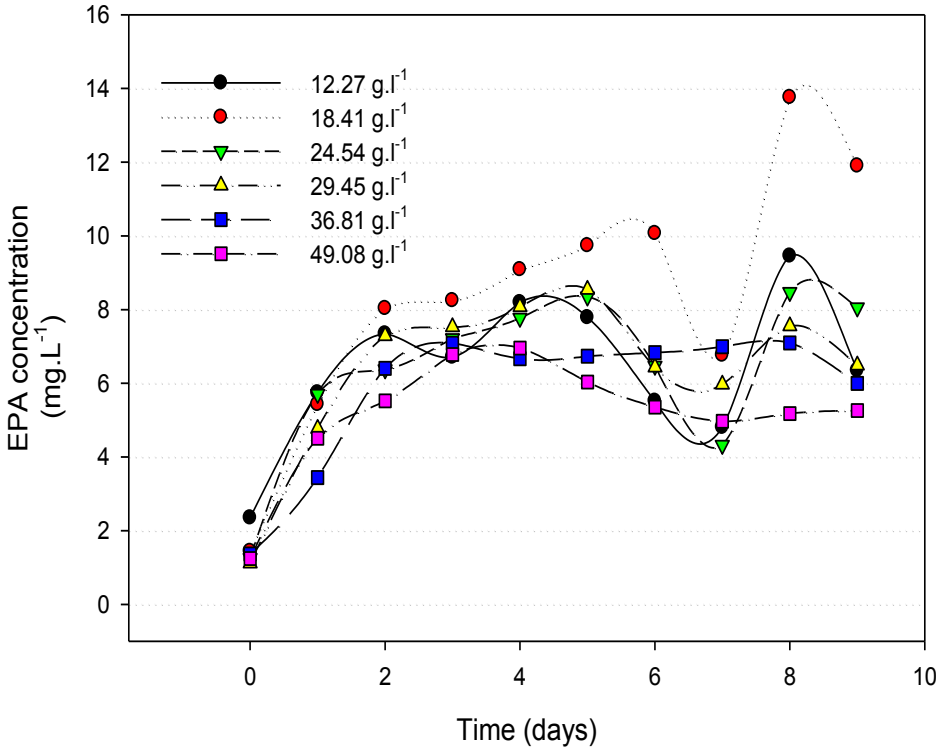

Figure 6.3 The volumetric EPA concentration of isolate A23.2 as a function of time and culture salinity under standard cultivation conditions

The overall EPA productivity is given as a function of salinity in Figure 6.4. Peaks are observed both at 18.4 g.L^{-1} and 29.5 g.L^{-1} NaCl, (75% and 150% w.v^{-1} standard modified Aquil medium salinity respectively), suggesting that osmotic stress triggers an increase in EPA production above and below standard medium concentration. This osmotic stress can be deduded from growth data in Section 5.3.2, in which a decline in growth is observed at these saline concentrations. Since EPA is part of the lipid structural component of cell membranes (Thompson Jr. 1996), fluctuations in salinity within this range may have resulted in an increase of these lipids and a spike in EPA productivity, to manage osmotic imbalance. The expression of the fatty acid elongating Kcs enzyme discussed in Section 1.2.4.3, may have caused the second productivity peak in a more saline environment. Increasing the NaCl concentration below 18.4 g,L^{-1} and above 29.5 g.L^{-1} may have resulted in a lower EPA productivity, as this directly affected the cells physiologically, as observed in Section 5.3.2.

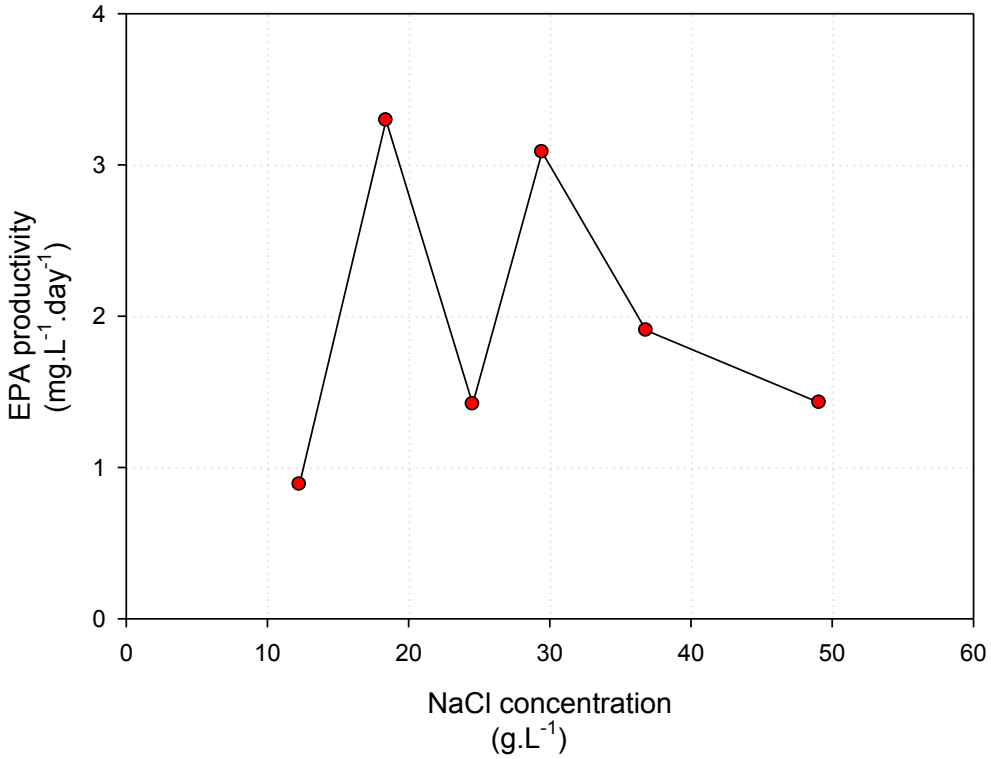

Figure 6.4 Overall EPA productivity of isolate A23.2 cultured at different saline concentrations

In Section 5.3.2 it was shown that salinity was not a significant driver of growth, therefore an elevated salinity approaching 36 $g.L^{-1}$ would be useful in a stress stage to further enhance EPA accumulation. The spike in EPA productivity is only slightly below that 18.4 $g.L^{-1}$ and will be considerably less expensive if allowed to occur naturally via evaporation, dependent on the choice of configuration for the bioprocess reactor.

6.3.3. *Effect of medium starting pH on EPA production*

The solution pH influences the aqueous ion equilibria of inorganic carbon, solubility products of these compounds and the resultant mass transfer rates (Azov 1982; Chen and Durbin 1994). Hence, pH is important in regulating the metabolism of EPA (Yoo 1991; Hinga 1992), both through impact on metabolism and on carbon supply. The effect of cultivation pH on the volumetric concentration of EPA in the culture is presented in Figure 6.5.

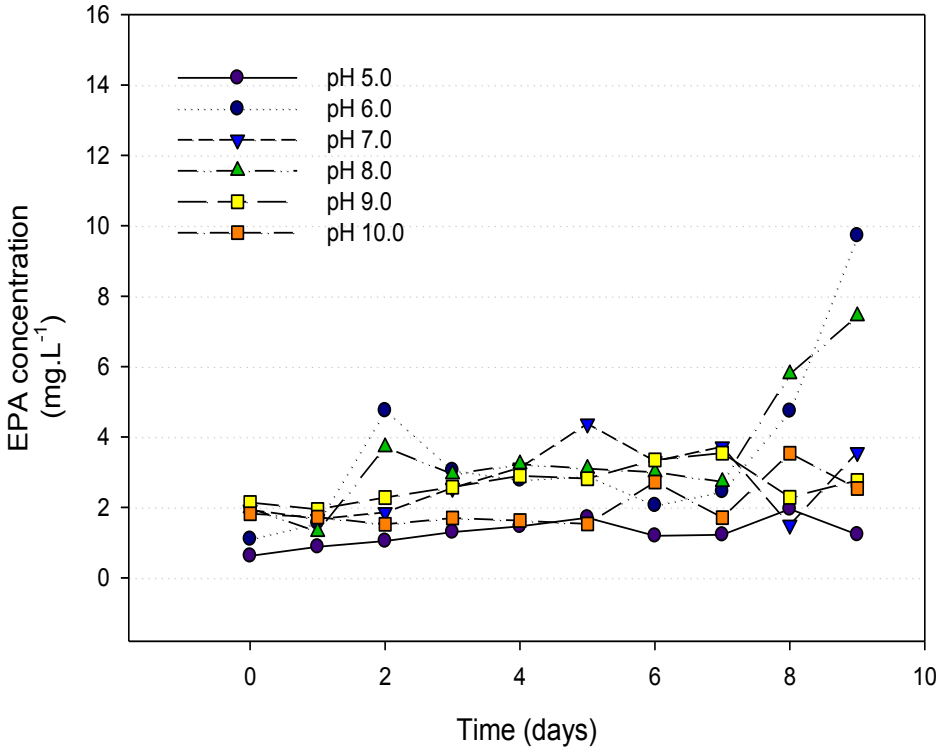

Figure 6.5 **The volumetric EPA concentration of isolate A23.2 as a function of time and medium starting pH at standard cultivation conditions**

The initial trial revealed unusually low EPA volumetric concentration data relative to the other assays tested, with precipitation of salts noted above pH 8.0 and culture death visually noted in loss of pigment at pH 10.0. Maximum EPA concentrations were 7.538 $mg.L^{-1}$ and 8.345 $mg.L^{-1}$ for base case studies of light and salinity, respectively. Low volumetric EPA concentration and anomalies of exponential increases in daily EPA concentration data at both pH 6.0 and 8.0 after day 7, justified a confirmation trial. The results of the confirmation study are summarised in Table 6.3.

Table 6.3 **EPA productivity data summarized for A23.2 cultured at 6.0, 7.0 and 8.0 starting pH in triplicate (n = 3)**

pH	***Maximum Ave EPA productivity*** ***($mg.L^{-1}.day^{-1}$)***	***Day***	***Std Dev.***	***CV*** ***(%)***
6	1.274	7	0.049	3.9
7	1.349	7	0.072	5.3
8	1.556	6	0.051	3.3

In the confirmation study no spikes in EPA concentration were observed, although average instantaneous EPA productivity was seen to increase from pH 6.0 to 8.0. The results indicate that increasing starting pH from 6.0 to 8.0, triggered a stress response that likely geared cells to greater EPA production to fortify cellular membranes. An alternate explanation could be that EPA productivity could have been related to a higher bicarbonate concentration (HCO_3- availability), at higher pH in these batch studies. The statistical significance of these pH values and their relationship to each other with regard to EPA productivity is shown in Table 6.4.

Table 6.4 Statistical T-test results showing the significance of EPA productivity between starting pH values of 6, 7 and 8

pH	p-value
6 and 7	0.0313
6 and 8	0.0196
7 and 8	0.0457

A significant effect is seen in the relationship of EPA productivity to changing starting pH, contributing to higher EPA production from marginal acidic to alkaline medium. Cell growth data in Section 5.3.1 however reveals that pH 7 is only marginally better than a pH of 8. Thus, one can conclude that from an operational perspective, cultivation at neutral pH is suitable for growth and in contrast, a more alkaline pH of 8.0 may be utilised for stress stage EPA enhancement.

6.3.4. *Cultivation temperature and effects on EPA production*

Temperature has a major effect on the types of fatty acids produced by microalgae. In general, there is an increase in lipids in all of the lipid classes when cells are grown at lower temperatures (Werner 1977; Yongmanitchai and Ward 1991). Temperature affects diffusion rates of chemicals into the cell through membrane fluidity, water viscosity and dissolved oxygen content. To control the intra-cellular environment, a change in membrane composition is triggered. Enzymes are characterised by specific operating temperature ranges, which may differ for enzymes geared to growth or to lipid metabolism (Werner 1977).

Comparison of the relationship between temperature and cellular EPA concentration revealed relatively low EPA content values. At incubation temperatures of 10 °C and 25 °C, a relatively elevated volumetric EPA content was observed. This observation, in combination with data fluctuation at 25 °C, warranted a confirmation study, focusing specifically on EPA productivity at the above two incubation temperatures. The results of this secondary study are summarized in Table 6.5. Data from the initial temperature stress study showing volumetric EPA concentration as a function of time is presented in Figure 6.6.

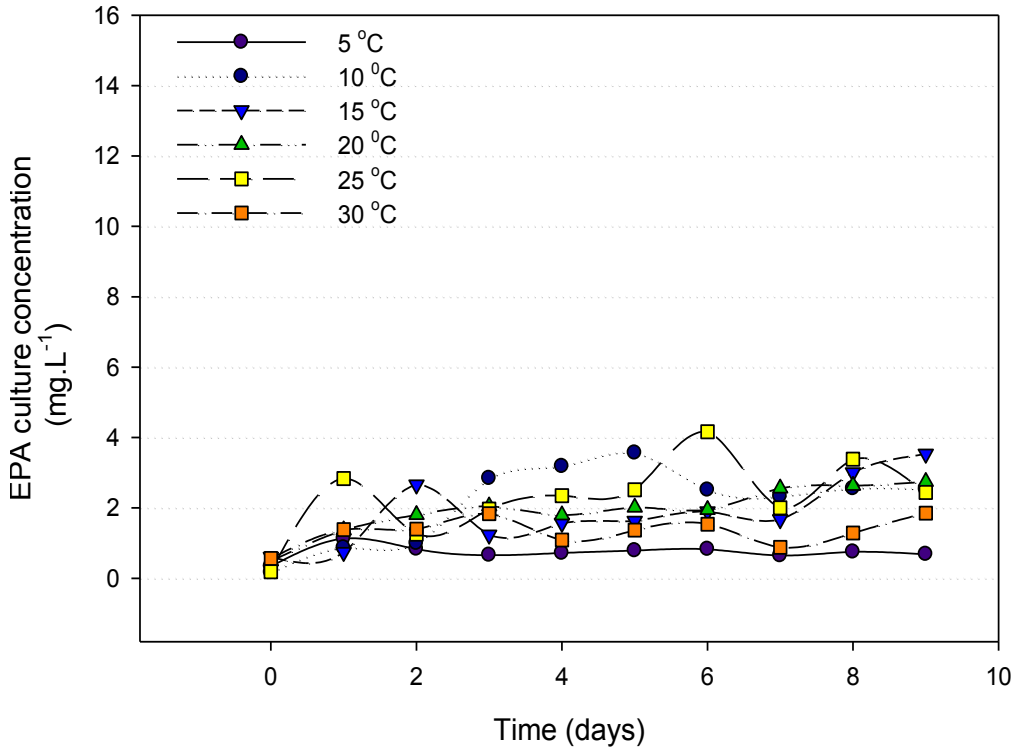

Figure 6.6 **Initial temperature stress study showing volumetric EPA content as a function of time**

The base case incubation temperature of 25 °C is shown to be a more significant driver of EPA productivity than a colder incubation temperature of 10 °C, when the data are subjected to a statistical T-test. Isolate A23.2 shows metabolic resilience to temperature fluctuation. A temperature of 25 °C is thus, suitable in terms of both growth and EPA production. The temperate origin of A23.2 is of great value, as strains that produce EPA only at low temperatures are a disadvantage from a process productivity point of view, specifically with regard to cost and energy, balancing growth and EPA production.

Table 6.5 Instantaneous EPA productivity data summarized for A23.2 incubated at 10 °C and 25 °C in triplicate (n = 3)

Temp (°C)	*Maximum Ave EPA productivity ($mg.L^{-1}.day^{-1}$)*	*Day*	*Std Dev.*	*CV (%)*	*p-value*
10	1.343	7	0.176	13.1	0.0099
25	1.842	5	0.124	6.8	

6.4. Conclusion

An EPA production process using isolate A23.2 should include a growth and stress stage, as some physiochemical parameters conducive to growth and EPA production are antagonistic. EPA production was found to occur during the light cycle as the control culture placed in the dark showed minor EPA content relative to all other culture flasks exposed to light. Light intensity should preferably be kept moderate at approximately 60 - 80 $\mu mol.m^{-2}.s^{-1}$ as confirmed by the growth study (Section 5.3.4) and overall EPA productivity (Section 6.3.1) data. Further investigation is still needed to confirm photoinhibition and EPA productivity in more innovative bioreactors, which allow internal irradiation, enabling greater light intensities to be used without compromising ambient temperature Another approach would be to ensure more efficient penetration of light by improving agitation, for example by the use of sparging.

Diatom A23.2 displayed two EPA productivity peaks at 18.4 $g.L^{-1}$ and 29.5 $g.L^{-1}$ salinity. This suggests that slight saline fluctuations from standard medium salinity (24 $g.L^{-1}$) triggered an increase in EPA-rich membrane lipids, countering osmotic imbalance. Overall saline concentration should therefore be allowed to increase marginally to 29 $g.L^{-1}$ or actively diluted to a threshold of 18 $g.L^{-1}$ in a stress enhancement phase.

A significant difference in EPA production was found from a starting pH 6.0 to pH 8.0. Diatoms favour slightly acidic conditions for growth (Section 5.3.1); however a pH of 8 should be maintained in the stress phase of an EPA production process. Greater EPA production at this pH may be a response to greater bicarbonate availability or to pH itself as a physiological stress trigger. The production of EPA and cellular growth was found to be suitable at a temperature of 20 – 27 °C. The eastern and northern coastal climate for the

location of large scale raceway ponds will thus be ideal. During light intensity and salinity stress experiments, volumetric EPA data was shown to stabilise between days 2 to 5, this indicates a stress stage residence time of approximately 48 hours.

7. PROOF OF CONCEPT DEMONSTRATION USING A LABORATORY SCALE CASCADE RACEWAY SYSTEM

7.1. Introduction

Mass cultivation of microalgae originated from open pond development, in which the oldest and simplest methods involve culturing under conditions identical to the natural environment (Wen and Chen 2003). The major photoautotrophic systems that have been developed for microalgal cultivation include open ponds; closed photobioreactors using natural sunlight and closed photobioreactors with artificial illumination (Apt and Behrens 1999). A typical microalgal production process is illustrated in Figure 7.1, in which its waste efficiency and variety of spin off uses are highlighted. The by-products of the bioprocess have a variety of applications, which include nutraceuticals, animal feed, fertiliser and energy generation. These applications along with the recycling of greenhouse gases are advantageous in generating increased investor interest, aligning with trends in sustainable technologies.

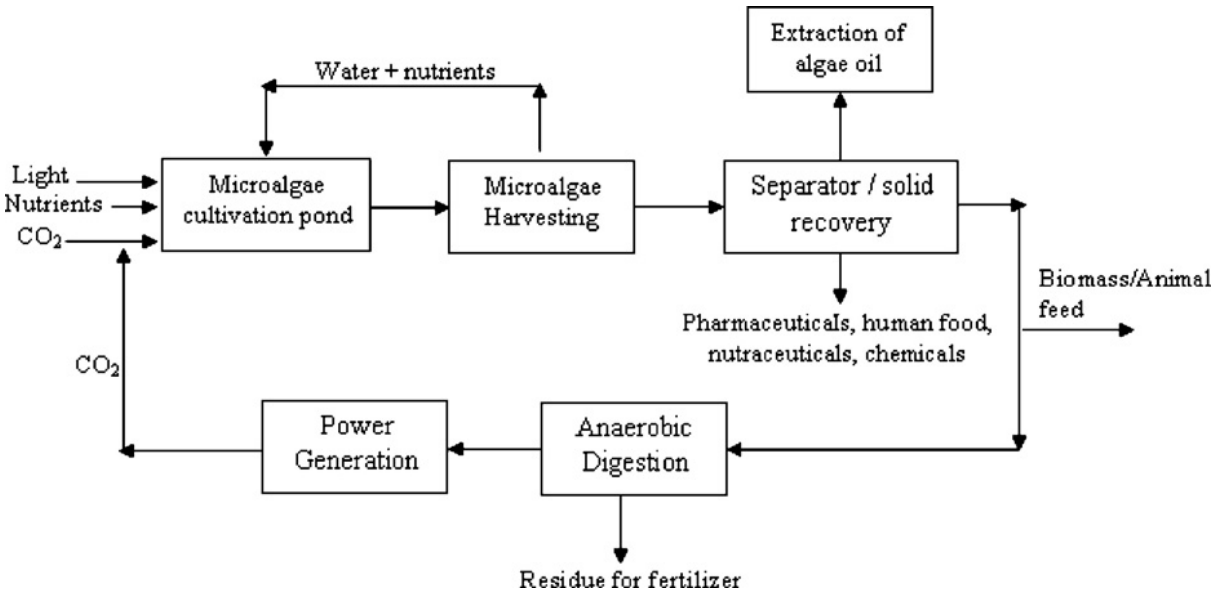

Figure 7.1 Diagram of a typical microalgal production process (Harun et al. 2010)

A raceway pond design was chosen as the preferred reactor type for this study due to it being less capital and energy intensive than closed bioreactors, particularly when scaled up to industrial level. These raceway systems can each be operated using fed-batch,

continuous or perfusion methods of cultivation. In the first phase of the project, the diatom A23.2 was selected as an indigenous candidate organism for EPA production mainly based on its properties of EPA accumulation and resistance to contamination. The diatom was found to accumulate EPA under both nutrient and physiological stress, allowing the use a cascading multi-phase raceway bioprocess in the final proof of concept study, using optimal parameters calculated by statistical polynomial analysis of batch, laboratory scale experiments. The raceway system was used simultaneously for various other research projects, in which variable system harvesting reduced the consistency of data, allowing only indicative productivity data to be calculated.

The bioprocess was designed as a sequence of ponds, starting under optimal growth conditions in the first pond and moving to stress conditions in the following ponds. Both cell and EPA productivity data were determined separately for the growth phase, stress phase and the overall system. The limitations of the chosen EPA production system are addressed, and recommendations for further research are included.

7.2. Materials and Method

7.2.1. *Multi-stage raceway fed-batch system set-up and operating parameters*

The laboratory scale process comprised one Growth Pond (GP) and three cascading Stress Ponds (SP1, SP2, and SP3) in series as seen in the flow chart in Figure 7.2. The three stress ponds were used in order to increase the retention time of the stress phase.

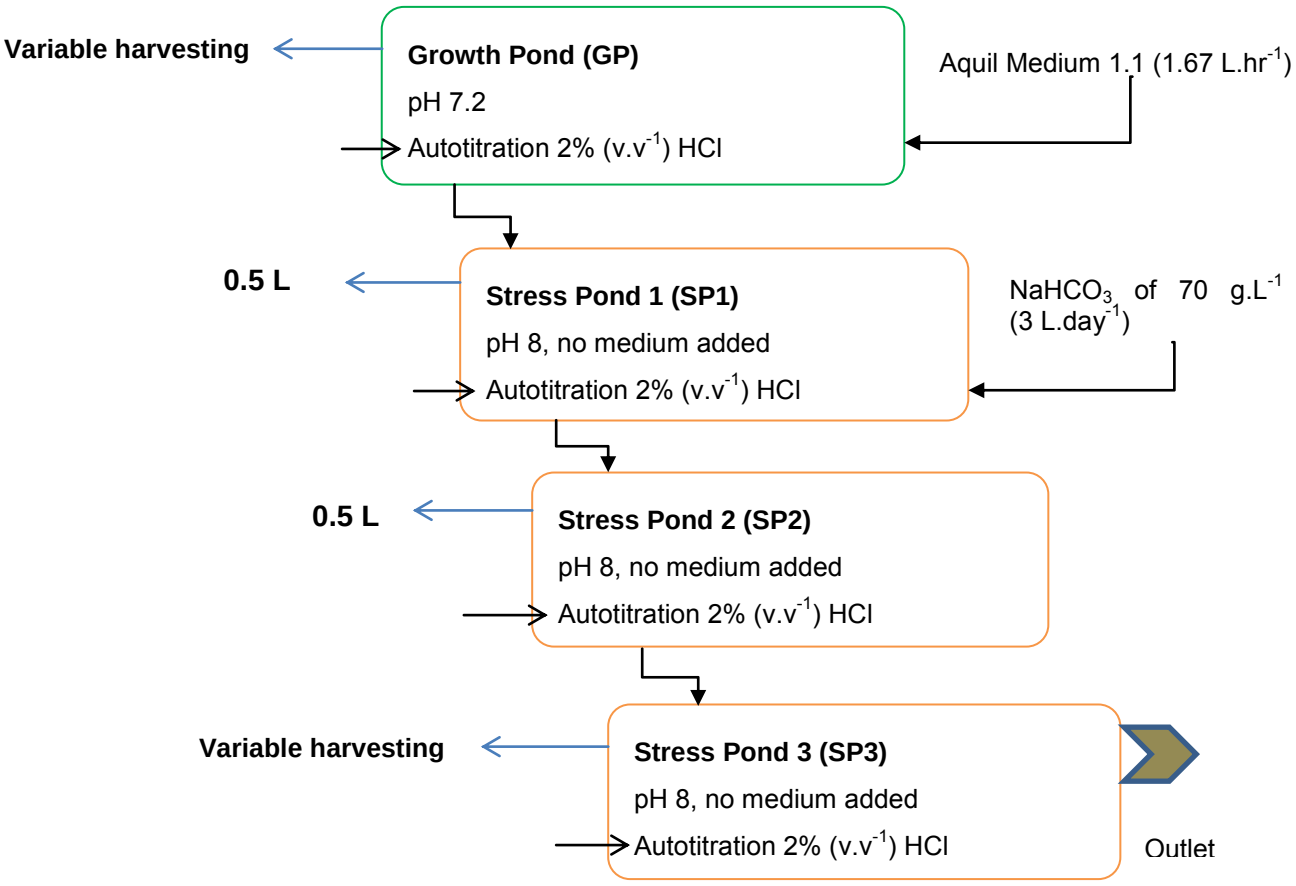

Figure 7.2 Process block flow diagram of multi-stage EPA production system

Dimensions of each pond were uniform with an approximate length of 150 cm, width of 60 cm and depth of 15 cm, with each pond maintained at a volume of 100 L. Lighting throughout the system was provided by fluorescent light bulbs (Philips Master TL-D 90 Delux, Holland) mounted on light rigs, which delivered an average light intensity of 60 - 70 $\mu mol.m^{-2}.s^{-1}$ measured at the culture surface. The lighting on the ponds was set to a day-night cycle in which the culture was exposed to light and darkness for 12 hours respectively. The Growth Pond was maintained at pH 7.2 and the Stress Ponds at pH 8.0 by titrating with 2% ($v.v^{-1}$) HCl, using a Master controller pH meter, which also continuously recorded temperature (Professional instruments, SAGA). The volume of acid solution added was recorded. Agitation in the raceway ponds was provided by rotating paddle wheels (100 rpm) and ambient temperature was maintained at ~25^{o}C, via air-conditioning. Salinity was not controlled, but measured at the end of the stabilisation period by a salinity probe (B. Braun Biostat, Germany). To minimize contamination, evaporation and heat loss, all the ponds were covered by a transparent plastic barrier (Figure 7.3). The pond system was operated

for two weeks following complete system setup (i.e. all ponds filled and operational), before data collection started (Day 0), to reach the most stable-state system possible. Data were then recorded for a period of 10 days.

Figure 7.3 The cascading multi-stage EPA Raceway production system

7.2.1.1. *Growth Stage (Growth Pond – GP)*

A 4 L starting inoculum of diatom A23.2 was scaled up to a volume of 20 L using the cultivation conditions described at the end of Section 2.2. The 20 L culture was then used to inoculate the GP Raceway containing 80 L of modified Aquil medium version 1.1 (Appendix C), resulting in a total raceway operating volume of 100 L. The cells in the GP were allowed to reach stationary phase, (three consecutive days at the same cell count), before a continuous medium feed was started at an initial rate of 2.10 $L.hr^{-1}$ (Aquil medium version 1.1) for 12 hours each day (during the light phase). This was adjusted to 1.67 $L.hr^{-1}$ after one day of feeding in order to reduce nutrients flowing into stress ponds. Variable algal biomass was harvested directly from GP (Appendix D); the remainder overflowed into the first stress pond, SP1.

7.2.1.2. *Stress stage (Stress – SP1, SP2, SP3)*

Once the Growth Pond was fed medium continuously, the culture was allowed to start overflowing (by gravity) into SP1 via a transfer port, which was situated at the liquid level

corresponding to a volume of approximately 100 L (~10 cm depth). SP1, SP2 and SP3 took about 5 days to fill up to a volume of 100 L, after which harvest pond SP3 was harvested by each day to maintain a depth below 11 cm. Following all ponds achieving their operating volume, a two week stabilisation period was allowed prior to data collection.

The pH of ponds SP1 to SP3 was maintained at pH 8.0 by titrating with 2% ($v.v^{-1}$) HCl, using a Master controller pH meter (Professional Instruments, SAGA). This is the pH most conducive to EPA production based on Section 6.3.3. The pH is recognised to impact the speciation of dissolved CO_2 / bicarbonate, by increasing its availability. A sodium carbonate solution (70 $g.L^{-1}$) was fed into SP1, to supplement inorganic carbon at a rate of 3 $L.day^{-1}$. Since the aim was to create stress conditions through nutrient limitation, no mineral medium was fed into any of the stress phase ponds. The conditions in the stress phase ponds 2 and 3 were similar to those in stress phase pond 1, except that there was no feeding of the carbonate solution. A working culture volume of 100 L (10 cm depth) was maintained in each pond, with an operating regime that allowed for a continuous system.

7.2.2. *Sampling and Analysis*

The Growth Pond and Stress Ponds were sampled each day for this and other separate research studies. Ponds SP1 and SP2 were sampled for this study only. Harvesting was performed manually on each pond, the volume harvested was recorded for GP and SP3. SP1 and SP2 had an approximate sample volume of 500 mL per day. The light phase and feeding was initiated at 8 am each day, for 12 hours. All ponds were sampled on a daily basis at around 9:00 am. Samples taken were analysed for nitrate, silicate and phosphate on every alternate day and for carbonate every day. All nutrient analysis tests including sulphate concentration, were carried out according to the methods outlined in Section 2.3.1.

Cell counts were performed and EPA quantified by GC (Sections 2.3.2 and 2.3.4.2 respectively). The level of each pond was recorded each day, and the non-agitated level of SP3 was kept below 11 cm throughout the study. In order to assess the approximate productivities of both cell and EPA production in the Growth Pond, the total volume of inputs such as acid and medium (V_{TOTAL}) was used to provide an estimate of the total output from each pond (mass balance with the assumption of negligible change in culture density). This input / output volume was multiplied by the cell concentration for that day, giving a value of $cells.day^{-1}$. The amount of EPA produced per day in GP ($mg.day^{-1}$), was calculated similarly

by multiplying V_{TOTAL} by EPA culture content for that day. These values were then divided by the 100 L pond volume to give an estimate of productivity in terms of amount of product per litre per day.

Each stress pond had multiple input volumes, including the overflow from the previous pond, acid added and, in the case of SP1, supplementary carbonate stock solution. Overflow volume (V_{IN}) was calculated from the previous pond, by subtracting harvest volume from V_{TOTAL}. The overflow also brought into the pond added cells and EPA from the previous pond. In order to estimate the amount of cells entering with overflow (V_{IN}*C_{IN}), the cell concentration measured in the outflow of the previous pond (C_{IN}) was multiplied by the overflow volume (V_{IN}). A similar calculation was performed with regard to EPA entering via overflow, to give a mg.day^{-1} value of EPA (V_{IN} *E_{IN}). The results of these calculations for each pond are summarised in Appendix D, with the assumption that there was no accumulation or attrition of cells in the ponds. Cell and EPA productivity in the stress ponds were calculated by the following equations:

$$Q_C \text{ (cells.L}^{-1}\text{.day}^{-1}) = (C_{OUT} \times V_{TOTAL}) - (C_{IN} \times V_{IN})/ 100 \quad (7.1)$$

Q_C = Cell productivity
C_{OUT} = Cell concentration in pond
V_{TOTAL} = Total input volume
C_{IN} = Overflow cell concentration entering the pond
V_{IN} = overflow volume entering the pond from previous pond
Pond volume = 100 L

$$Q_E \text{ (mg.L}^{-1}\text{.day}^{-1}) = (E_{OUT} \times V_{TOTAL}) - (E_{IN} \times V_{in})/100 \quad (7.2)$$

Q_E = EPA productivity
E_{OUT} = EPA culture concentration in pond
V_{TOTAL} = Total input volume
E_{IN} = Overflow EPA concentration entering the pond
V_{IN} = overflow volume entering the pond from previous pond
Pond volume = 100 L

7.3. Results and Discussion

Due to the random harvesting of both GP and SP3, steady state operation of the system was not achieved. There were no trends in terms of cell accumulation in the ponds, hence further work will be useful in delivering a more exact context for design decisions and reduce data variability. Productivities calculated from this work were an estimate of the productivity over each 24 hour period. Productivities were calculated for each pond, between growth and stress phases and overall throughout the system. These productivities were used to provide an indication of typical performance, to identify operational challenges and further research needed, with the ultimate aim of developing a more efficient EPA bioprocess and corresponding operational parameters.

Physicochemical parameters were maintained similarly across all ponds with the exception of pH, which was increased from pH 7.2 in the Growth Pond to pH 8.0 in the stress phase ponds. The selected *Cymbella* diatom used in this study showed resilience against contamination from other microalgal species, both at flask and raceway level. This is highly advantageous as it improves the EPA marketing value in terms of reduced antibiotics, herbicides and other biological control agents used in the production process.

7.3.1. *Growth Pond nutrient concentrations*

Nutrient concentrations recorded during the experimental period within the Growth Pond were not depleted i.e. nutrients were not consumed in their entirety and were present throughout the system. Table 7.1 compares nutrient ion concentrations of the macronutrients found to be optimal for growth, the modified feed version used for the raceway system, as well as average nutrient concentration recorded in the Growth Pond. Silicate and bicarbonate concentrations were increased beyond batch flask precipitation levels, due to their consumption rates being over 90% (Sections 4.3.2 and 0 respectively), with no precipitation observed in the Growth Pond.

Table 7.1 Comparison of concentration of nutrients found to be optimal for growth, to those used in the modified medium in the raceway Growth Pond, as well as actual average levels measured

Nutrient	Optimum recommended findings (Chapter 4)	Modified Raceway Medium (version 1.1)	Average Growth Pond nutrient concentration
	$g.L^{-1}$	$g.L^{-1}$	$g.L^{-1}$
Bicarbonate	2.00	21.30	0.21
Phosphate	0.05	0.04	0.05
Nitrate	0.10 – 0.20	0.13	0.06
Silicate	0.20	0.40	0.04

The first challenge appearing in the demonstration study was the maintenance of optimum nutrient levels in the growth phase, while limiting their presence in the stress ponds. Nutrient trends in the Growth Pond are plotted in Figure 7.4, where silicate concentration was well below the optimal recommended finding of 0.2 $g.L^{-1}$. The adjusted medium recipe used for the raceway system was initially based on concentrations in Chapter 4; however, due to the observed presence of nutrients detected in the stress ponds, phosphate and sulphate levels were reduced, due to them having a negligible effect on growth (Chapter 4). All nutrients measured declined as expected, from growth to stress, with the exception of these two nutrients. Sulphate and phosphate concentration only began decreasing after day 4, persisting in the system despite the reduction in medium concentration in the first week of the stabilisation phase. Further investigation is needed to find out whether significantly increasing silicate concentration and eliminating sulphate completely from the medium will impact growth.

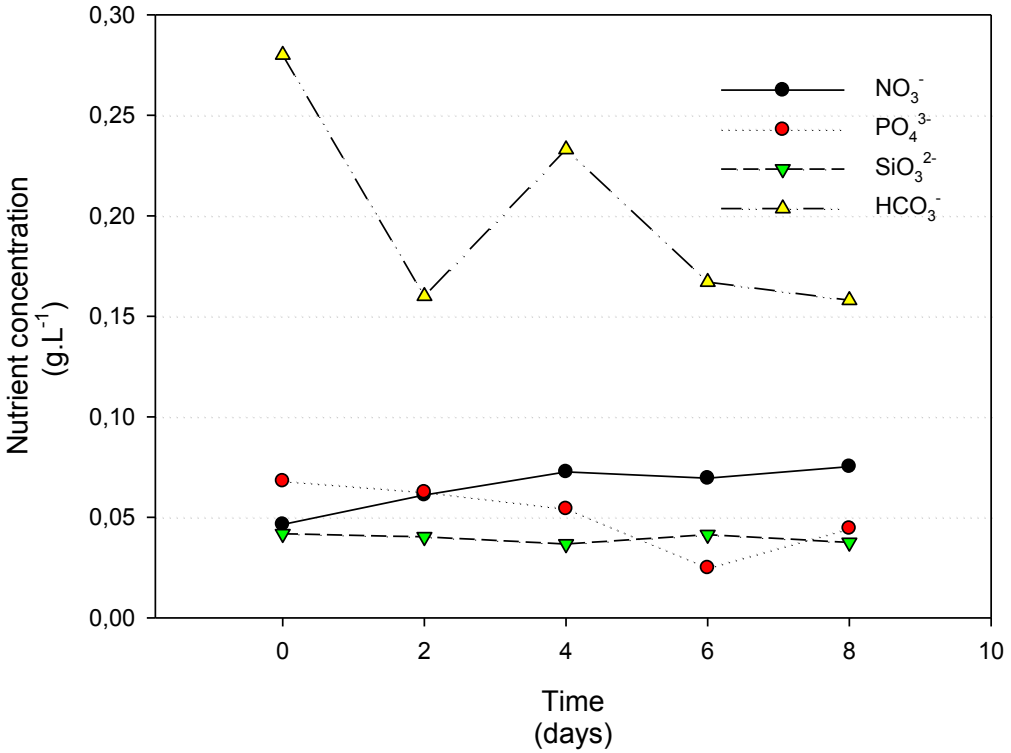

Figure 7.4 Concentration of macronutrients in the Growth Pond plotted over 8 days

7.3.2. *Bioprocess system cell productivity*

The average cell concentration from both growth and SP1 increased in the latter bioprocess ponds SP2 and SP3, however, it did not reach the desired concentration of the *P. tricornutum* control observed in Section 3.3.2, being approximately 2.0 x 10^{11} cells.L^{-1}. Figure 7.5 summarises average cell productivity for each pond, the overall stress phase as well as the overall system. The Growth pond had the highest average cell productivity as expected, however, SP1 was unusual in showing a marked reduction in cell productivity in relation to the other ponds, lowering the overall cell productivity of the system.

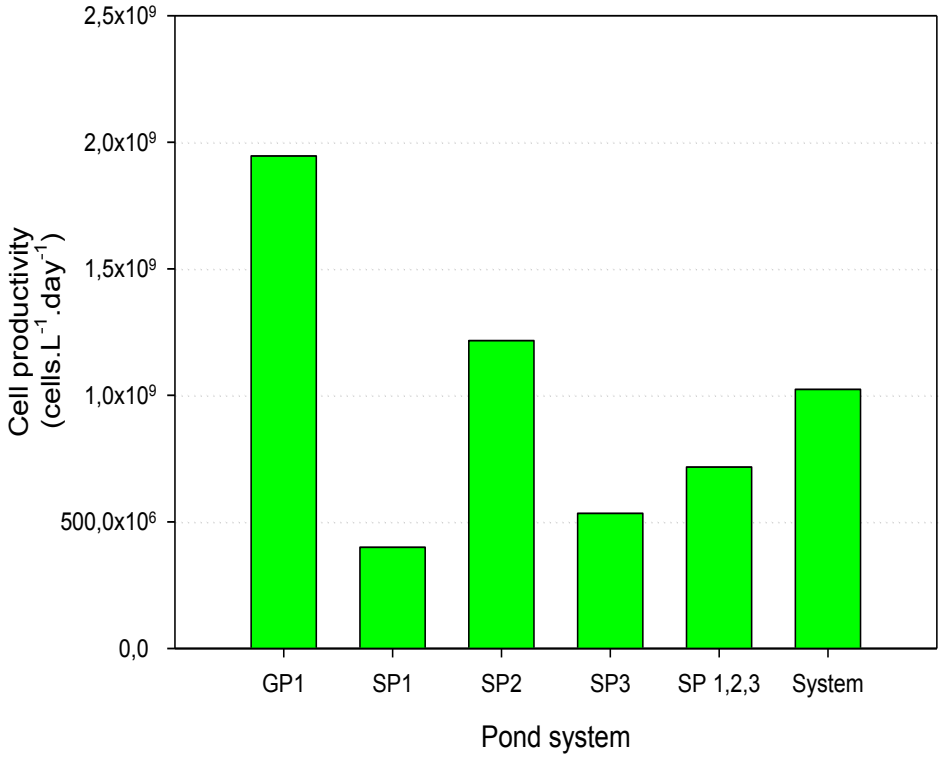

Figure 7.5 Average cell productivity expressed as a function of each pond from growth to stress and through different phases of the bioprocess

Since silicate and carbonate appeared to be non-limiting, overcoming light limitation was considered for further increasing biomass productivity. Although advantageous from a harvesting perspective, the 'sticky' nature of the diatom led to increasing adhesion along the length of the raceway and paddlewheel (Figure 7.6), becoming more pronounced over time and contributing to data variability. Owing to settling, water clarity was observed to increase moving away from the agitation; the increasing density of cells occupying a decreasing depth at the bottom of the pond would have increased cell shading further from the paddlewheel. One solution for this problem is improved culture mixing. Power consumption for culture mixing increases as a function of water velocity; guidelines state that a paddle moving at around 20 to 25 $cm.s^{-1}$ will have a reasonable power demand for a large scale bioprocess (Benemann 2013). The approximate horizontal velocity for the paddlewheel (15 cm radius) used in each raceway was 157 $cm.s^{-1}$. The approximate horizontal velocity for the paddlewheel, (15 cm radius), used in each raceway was 157 $cm.s^{-1}$. This velocity is extremely high relative to proposed guidelines, suggesting that the paddlewheel is an inefficient agitator for A23.2.

Light penetration and, the related, agitation are thus key drivers of growth; and should be enhanced as much as possible. There remain gaps in literature dealing with enhanced light penetration, especially in outdoor pond systems. In order to counter this constraint, innovative raceway designs using submerged light emitting diodes or rods can be used in conjunction with novel methods of mixing, reducing energy costs and making mass-transfer more efficient. Currently a high solar conversion rate is the core focus of microalgal research around the world; strategies of light dilution, intensive light flashing and light pigment attenuation are being debated (Benemann 2013).

Figure 7.6 Clumping and adhesion of isolate A23.2 around the edges and spokes of the paddlewheel

Temperature trends seen in Figure 7.7 are very low in relation to the optimum of temperature of 25 °C, this trend is compounded in the Stress Ponds with fluctuation in data of up to 4 °C between the ponds. The trend may have been attributed to cooling in the transfer ports when culture flowed downwards from one pond to the other. This resulted in average stress pond temperatures dropping to as low as 15°C, slowing both growth and EPA production and reducing EPA productivity. Room temperature was not measured, and therefore not

effectively maintained. Mitigation measures should include the selection of a warm, tropical, coastal location in the scale up to an outdoor bioprocess in which winter temperatures are relatively higher.

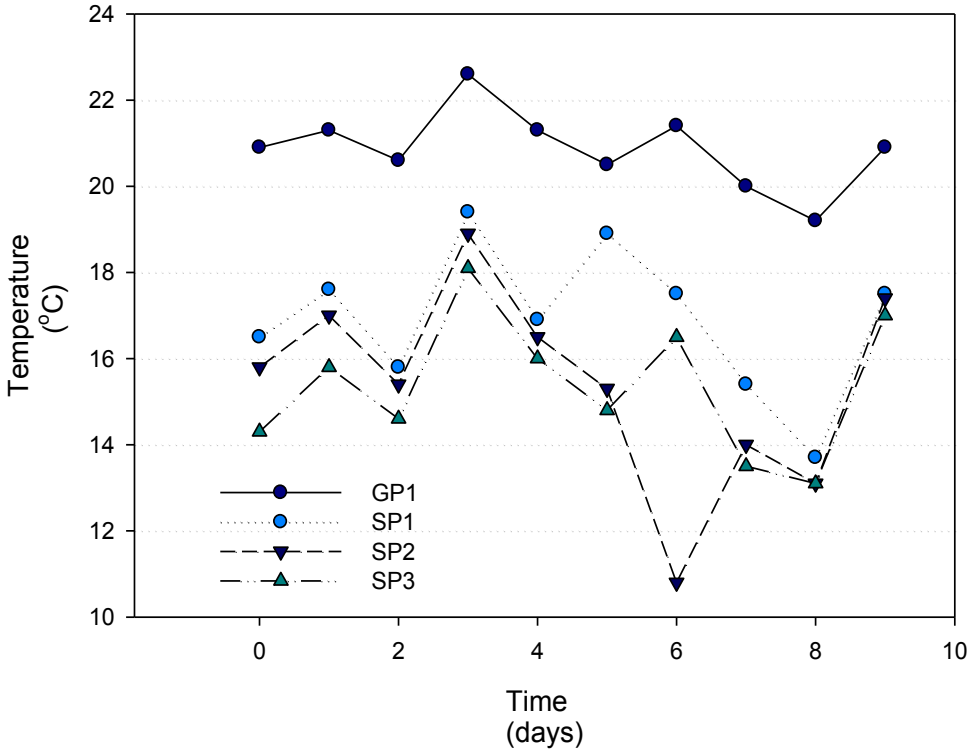

Figure 7.7 Temperature data plotted as a function of time in each pond from growth to stress

7.3.3. *Bioprocess EPA production*

Since all ponds were not harvested consistently, the system could not be maintained in steady-state; hence, daily productivities were recorded. Figure 7.8 shows EPA productivity from day 4 until day 9, in which an indicative range of EPA productivity in each pond was observed. Initial data showed fluctuation, in which the probability of error increased with the number of data points included. EPA productivity was seen to decrease from growth to stress, despite stress enhancing pH. In contrast to the other ponds, SP3 had the lowest EPA productivity overall as well as a low cell productivity.

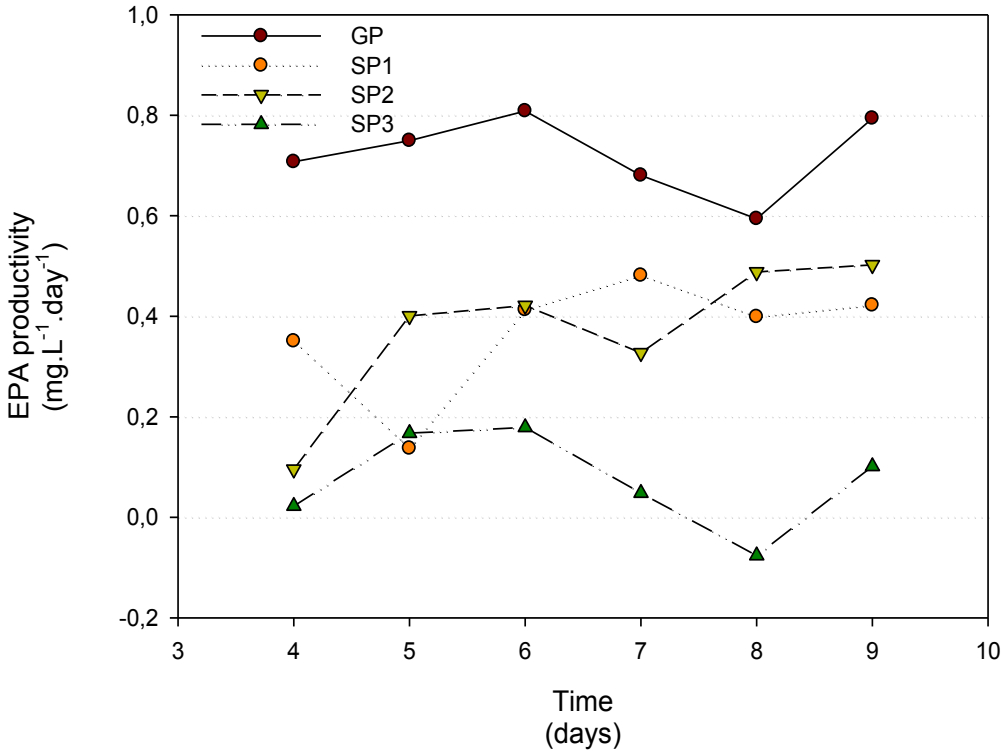

Figure 7.8 **EPA productivity for each pond expressed as a function of time from day 4 to 9**

Specific EPA productivity data for the raceway system is illustrated in Figure 7.9, where a marked elevation is observed in SP1. This data suggests that stress phase ponds SP2 and SP3 are ineffective in enhancing EPA productivity, driving up expense and residence time by an added 200 L system volume. A pre-harvest stress step using only a single stress pond should be investigated, combining nutrient and physicochemical stress, in order to obtain a more significant increase in EPA productivity from the data obtained in the Growth pond.

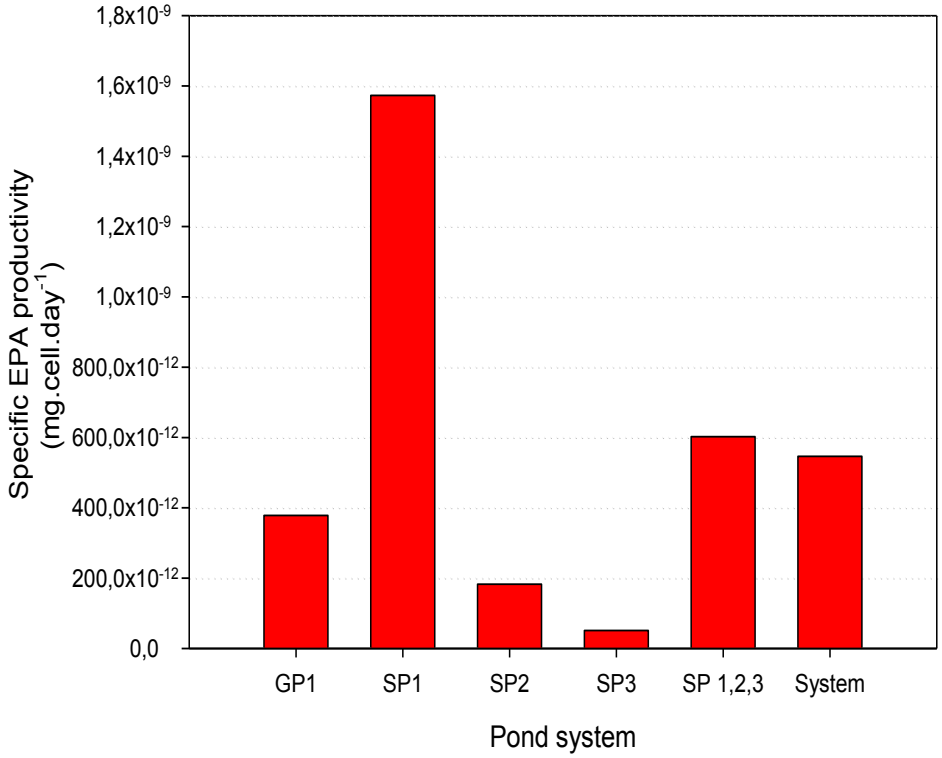

Figure 7.9 Comparison of average Specific EPA productivity in growth, stress and in the overall bioprocess raceway system

In Section 6.2.5 the qualitative results of a batch culture study found the most EPA enhancement in medium completely devoid of nitrate, silicate, phosphate and sulphate. Figure 7.10 shows the persistence of these nutrients throughout the system. The concentration of sulphate was found to be significantly higher than the other nutrients throughout the system, possibly being released from dead cells (Appendix D). A redesign of the bioprocess needs to be investigated, in order to address this challenge. The outcomes of this are crucial in determining whether a stress phase is viable in a continuous EPA bioprocess, in a trade-off between growth and EPA accumulation to achieve desired levels of productivity.

In photosynthetic organisms, EPA is mainly contained in galactolipids within the thylakoid, these amphiphilic lipids, of which EPA is a major structural component, allow for the formation of vesicles and play a fundamental role in metabolism and permeability (Sukenik et al. 1989; Hodgson et al. 1991). According to Hoshida et al. (2005), there is a marked increase in EPA content in response to elevated CO_2 concentration, which suggests that EPA content is controlled by available carbon concentration. This observation implies that a

pre-harvest carbonate shock step, in addition to standard CO_2 supplementation, may increase EPA yields and should be investigated. Mixotrophic strategies incorporating the use of supplementary micronutrients or EPA lipid precursors were reported in the literature to greatly increase EPA productivity. The addition of 100 $ng.L^{-1}$ vitamin B12 to a culture of *P. tricornutum* resulted in a 65% increase in EPA yield (Yongmanitchai and Ward 1991). Free fatty acids in media have been known to broadly suppress synthesis of fatty acid compounds in other micro-organisms (Lees and Korn 1966); however, omega group precursors, such as oleic, linoleic and linolenic acids, enhanced production of PUFAs, especially EPA in *Euglena gracilis* (Okumura et al. 1986). The investigation of adding these pre-cursors at different stages of growth and/or stress should be investigated to determine its effect on EPA productivity and whether EPA productivity enhancement negates supplement expense.

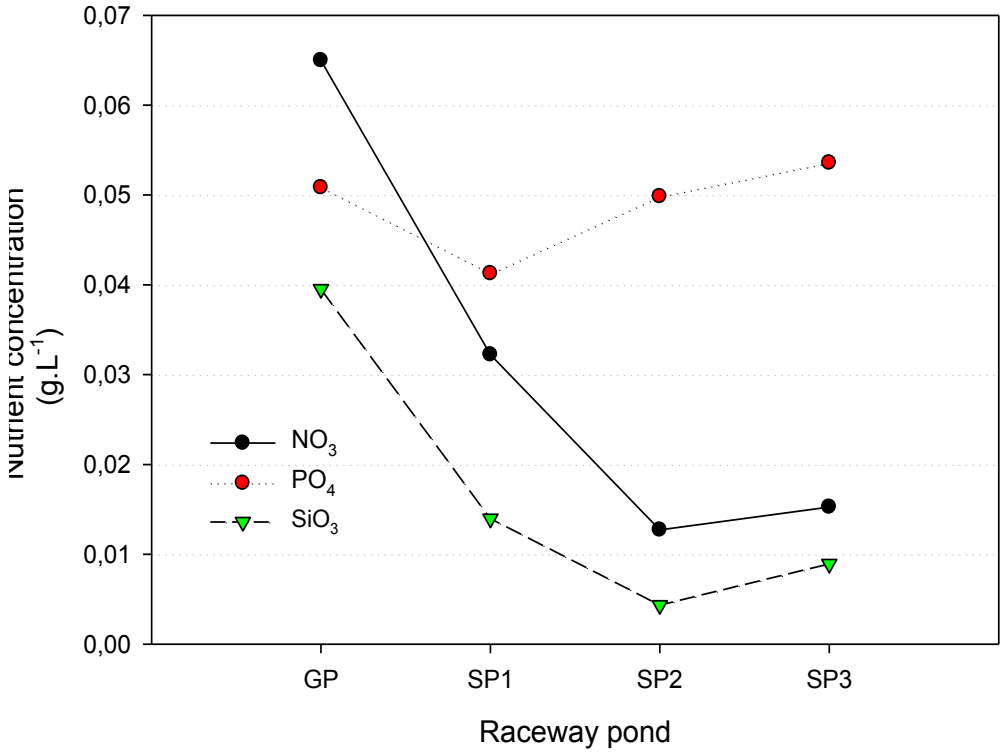

Figure 7.10 Average nutrient concentrations measured throughout the multi-stage raceway system

System pH trends are illustrated in Figure 7.11, in which GP and SP2 are relatively stable. This stability correlates to a more conducive environment to EPA production. The pH of the stress phase ponds showed fluctuation in SP1 and a decline in SP3. The observed anomaly in the data of SP1, was that of elevated pH and consequent acid volume added to SP1, averaging 2.0 $L.day^{-1}$, an approximate 4 times higher value than the average acid volume

added to SP2, with no increase in cell productivity. The decline of pH in SP3 after day 4 is expected, as cell metabolism declines, the final higher pH data point of SP3 may correlate to a temperature spike on day 9. The use of CO_2 gas in an industrial scale up of the bioprocess could provide a bio-available supplementary carbon source while maintaining optimum pH, without the expense and inhibition associated with acid addition, of which almost 10 L was added to the raceway system each day. This was nearly half of total daily medium feed.

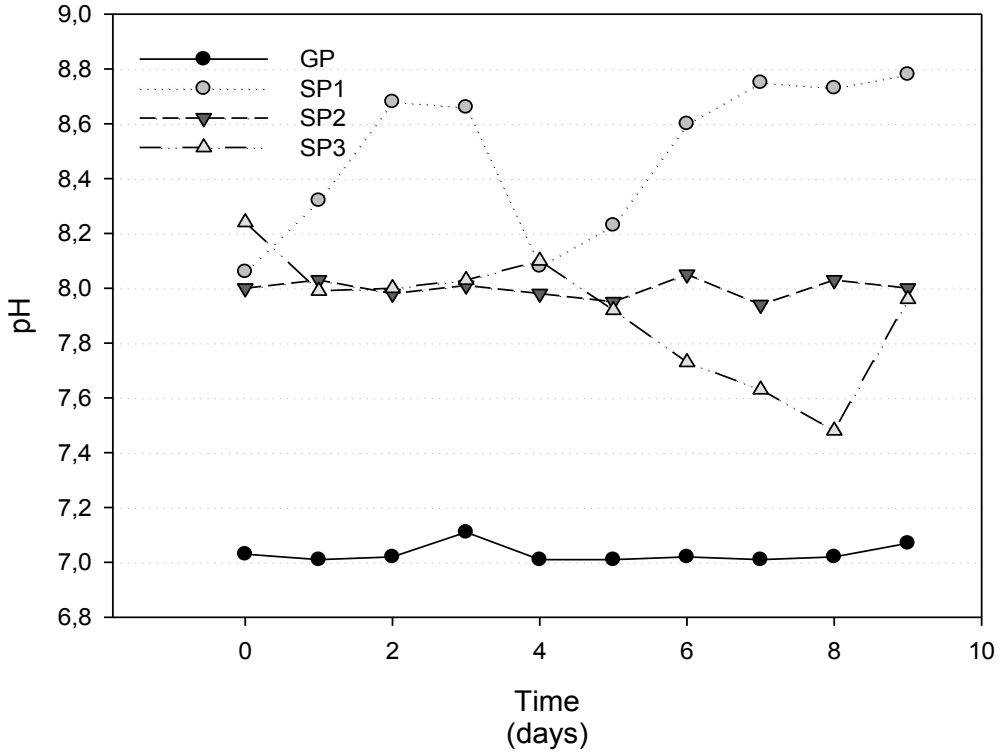

Figure 7.11 pH values expressed as a function of time in each pond system over 9 days

Salinity was another variable which affected EPA productivity positively. An initial approximate saline reading taken at the end of the stabilisation phase and the start of the study, at both GP and SP3, was found to be 25.6 $g.L^{-1}$ and 29.6 $g.L^{-1}$ respectively. The salinity concentration of both GP and SP3 closely matched the recommended findings in Sections 5.3.2 and 6.3.2. Salinity was not monitored for the duration of the study, as salinity stress can be incorporated passively outdoors, evaporation from growth to stress will provide a natural elevation, which should not exceed a maximum of 29 $g.L^{-1}$. Further research into active saline dilution at approximately 18 $g.L^{-1}$ in the stress ponds is recommended, as it produced marginally better EPA productivity in Section 6.3.2 .

7.3.4. *Multi-stage raceway system limitations and downstream processing recommendations*

The multi-stage raceway system was an experimental study in which various limitations were identified. These included:

- culture cooling from Growth Pond to Stress Pond 1
- large input volumes of acid
- inefficient agitation and therefore inefficient light penetration
- presence of nutrients throughout the system, reducing the stress

Indicative productivity data also suggested a re-design of the system, in which SP3 should be eliminated, reducing overall system residence time and increasing the dilution rate, while reducing energy inputs. The re-design should incorporate the results of future studies in which more efficient mixing, innovative light penetration and gaseous carbon dioxide to control pH are investigated. A quantitative investigation into the response of EPA metabolism to various nutrient levels will determine whether a nutrient stress phase step is necessary or can be removed from the bioprocess entirely.

The property of rapid settling of the diatom as observed in Chapter 3, is advantageous from a downstream processing perspective. Harvesting can be aided by settling tanks after stress phase EPA enhancement. This eliminates the need for expensive centrifugal dewatering technologies, while spent medium can be recycled into stress ponds, further reducing expense. The bioprocess, when scaled to an outdoor industrial system is environmentally sustainable, as effluent can be re-used or released into the environment with low risk of eutrophication or disruption to the ecosystem by the indigenous isolate, provided its nutrient loading and salinity meet discharge guidelines .

Extraction of EPA can employ the direct tranesterification techniques used in this study. This eliminates the expense and carbon footprint of dewatering and drying, while increasing extraction yield (Griffiths et al. 2010). Figure 7.1 illustrates the various spin-off uses of lipid extracted biomass, from fertiliser to substrate in anaerobic digestion, which in turn generates energy and CO_2.

8. CONCLUSIONS AND RECOMMENDATIONS

This study set out to select a potential indigenous microalgal EPA producer, with the aim of facilitating the development of a prototype EPA production bioprocess. The most efficient means of screening potential microalgal isolates for commercial lipid applications was to first assess lipid content qualitatively via fluorescence microscopy, followed by direct lipid transesterification to determine specific EPA fatty acid content. An indigenous diatom identified as belonging to the genus, Cymbella, was chosen as a candidate for the EPA bioprocess study, due to its property of high EPA content. The properties of A23.2 also allowed the use of a settling tank in downstream processing, reducing centrifugation or filtration requirements and thus decreasing harvesting costs.

8.1. Growth enhancement

The relatively low cell productivity of the diatom prompted a growth optimisation study. Concentrations of silicate and bicarbonate macronutrients were found to be limiting and statistically significant to growth. They were further increased from recommended findings of 0.2 $g.L^{-1}$ and 2 $g.L^{-1}$ respectively in batch culture, to 0.4 $g.L^{-1}$ and 21.3 $g.L^{-1}$ respectively, in the medium used for the final proof of concept raceway bioprocess.

Growth-optimised physicochemical parameters proved a challenge to maintain in the raceway system. These included a pH of 7.2, light intensity range of 60 – 70 $\mu mol.m^{-2}.s^{-1}$, a salinity of 24 $g.L^{-1}$ and a temperature of 25°C. Nutrient and physicochemical parameters were assayed individually. Cell productivities of between 2.0×10^8 to 3.0×10^8 $cell.L^{-1}.hr^{-1}$ in batch culture bioreactor studies were found.

8.2. EPA enhancement

Nutrient deficient medium devoid of nitrate, phosphate, silicate and sulphate salt was observed to stimulate EPA accumulation. Building on this observation, a physicochemical stress investigation revealed a significant increase in EPA productivity when cultivation pH was increased to pH 8. Increased EPA productivity was observed at both a salinity of 18 and 29 $g.L^{-1}$, likely due to osmotic stress. EPA productivity at optimal levels for both these specific parameters averaged 1.5 $mg.L^{-1}.day^{-1}$, improved from approximately 0.3 $mg.L^{-1}.day^{-1}$

in screening studies. Light intensity and temperature variation from standard conditions had no enhancement effects and were carried over to the operating regime of a proof of concept bioprocess.

8.3. Proof of concept bioprocess

The proof of concept study was undertaken in a multi-stage cascading raceway system. This consisted of a Growth Phase Pond and a succession of three Stress Phase Ponds, incorporating optimised parameters from growth and EPA stress studies, respectively. The aim of this investigation was to determine average cell and EPA productivities of A23.2 in the various ponds, guiding the development of future design and operating parameters, to ultimately achieve commercial levels of EPA production. A raceway pond was chosen due to being a suitable reactor for a bioprocess trial study, as well as ultimately less energy and capital intensive than many other options.

The pilot raceway system was used simultaneously for multiple research projects, resulting in variable amounts of harvesting. This allowed only approximate cell and EPA productivity to be determined. Although the bioprocess was designed to be a multi-stage system, both average cell (1.95×10^9 cells.L^{-1}.day^{-1}) and EPA (0.68 mg.L^{-1}.day^{-1}) productivity data for the growth pond surpassed that of the stress phase ponds as well as the overall system. Batch flask studies showed slightly elevated pH and cell productivities than the raceway system, which was expected due to greater light penetration and temperature control at small volumes. *P. tricornutum*, used in many commercial applications, showed a cell productivity of almost 2.0×10^{10} cells.L^{-1}.day^{-1} during initial screening studies and achieved over 20 mg.L^{-1}.day^{-1} in a continuous culture system in a study by Yongmanichai and Ward (1992), suggesting that improved productivity of the experimental system is required for a competitive local algal EPA producer.

Elevated pH and relatively high volumes of acid buffer required, in combination with lower overall cell and EPA productivity of the stress phase, suggests an investigation into bicarbonate supplementation in combination with acid buffer pH control. Specific EPA productivity in the first stress phase pond showed a marked elevation in relation to the other ponds. This finding suggests that the use of a single stress pond prior to harvesting will be more efficient in enhancing EPA productivity by reducing cost and residence time. The final two stress phase ponds were the worst performers in terms of both cell and EPA

productivity, confirming the need for investigation into reducing or removing the stress phase step.

8.4. Limitations of study and recommendations

The foremost limitation of the bioprocess proof of concept study was a lack of true steady-state conditions. It is recommended that culture temperature and (expense allowing) salinity, be strictly controlled in future studies. Temperature decrease from growth to stress was also noted, which deviated from the optimal temperature range, despite environmental control. This was most likely the result of inadequate temperature control in conjunction with the cooling of culture flowing downward from growth to stress ponds. A warmer environmental climate must be considered to counter this and increase growth rates in an outdoor system.

Growth Pond silicate concentration was well below the optimum finding, but persisted at low levels into the stress phase. Research into silicate metabolism and cell buoyancy will be a useful study, as this has a direct impact of culture exposure to light. This may explain the relatively thin silica cell walls of *P. tricornutum* and its superior biomass productivity when compared to the *Cymbella* species chosen for this project (Chapter 3). In comparison with A23.2, which had a higher cellular EPA concentration, *P. tricornutum* was microscopically observed to be weakly silicified, this had a knock-on effect of making the cell more slender and less prone to cell shading (Lewin et al. 1958), a vital structural property that allowed it to increase its cell densities beyond that of A23.2, with a possible trade-off in EPA production.

A practical way to help address the problem of cell shading in isolate A23.2 is by a re-design of the raceway pond, by the addition of more efficient culture agitating mechanisms. Further research should be conducted into other supplementary lighting options for A23.2 which increase in intensity with culture density. Enhanced light penetration technologies should be investigated, for example, submerged diodes or enhanced agitation strategies which off-set the clumping property of A23.2. In addition, the advantages and disadvantages of outdoor insolation must be assessed, and practical solutions (such as shade cloth covering) offered to counter inhibitory solar radiation levels should this prove to be an issue.

In nutrient deficient environments, metabolism is altered and energy diverted into lipid storage (Coombs et al. 1967). Another hurdle to greater EPA productivity in the raceway was the presence of nutrients in the stress phase, further work is needed to ascertain the effect on EPA metabolism in a zero nutrient medium versus low nutrient concentrations. Extending the residence time of the Growth Pond should be investigated to assess impacts on productivity, improve residual optimal nutrient concentrations and restrict nutrient flow into the stress pond.

Sparging of CO_2 directly into the culture has a triple effect of supplying a source of carbon, reducing pH without the use of acid, and increasing light exposure by improving agitation. However, the transfer of CO_2 into the medium is inefficient due to the short path length in the shallow raceway as well as leakage out of the pond. Nevertheless the introduction of CO_2 into the growth phase should be investigated. Supplementation of bicarbonate salts in the stress phase or pre-harvest, could also be tested for final EPA enhancement.

8.5. Future work

Using this study as an experimental framework to ramp up lipid productivity, other potential microalgal isolates can be used. Alternatively, a closed photobioreactor system can also be used, where operational parameters are more strictly controlled and potential EPA productivity may negate the greater expense. The proof of concept raceway bioprocess can be greatly improved, with further potential research into increasing the growth and consequent EPA productivity of A23.2 listed below:

- Investigate the effects of multi-factorial macronutrient concentrations on EPA metabolism, with the aim making the EPA bioprocess more efficient, either through improving the stress phase pond system or eliminating it completely. Research into effects of mixotrophic conditions on EPA production through addition of organic compounds, such as vitamin B_{12} and EPA pre-cursors, at various stages of the bioprocess may also prove to be beneficial.
- Research more efficient culture mixing and light penetration technologies.
- Explore the use of gaseous CO_2 to maintain pH and act as an added source of carbon and investigate a carbonate shock step prior to harvest to increase EPA culture concentration.

- Explore the redesign of the raceway system, or alternatively an adaptation of the model to a closed photobioreactor, where growth and stress phases are separated.
- Operate a steady state raceway pond system in which the impact of residence time in each stage, impact of growth and stress stages and their relative volumes as well as interaction between operating parameters can be assessed.
- Investigation of outdoor insolation and temperatures on growth and EPA productivity.

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10. APPENDIX A

10.1. Artificial fresh water medium (AF6)

Table 10.1 Trace Metal Stock Preparation

Compound	$g.L^{-1}$
$FeCl_3.6H_2O$	0.98
$ZnSO_4.7H_2O$	0.11
$CoCl_2.6H_2O$	0.02
$MnCl_2.4H_2O$	0.18
$Na_2MoO_4.2H_2O$	0.01
$Na_2EDTA.2H_2O$	5.00

Method: EDTA was dissolved in 950 mL of dH_2O followed by the other compounds.

Table 10.2 Method of preparation for Vitamin Stock

Compound	$mg.L^{-1}$
Thiamine-HCl	200
Biotin	1
B-12	1

Method: Primary stocks of B-12 (11.4 mg/10 mL) and biotin (10.4 mg/100 mL) were prepared initially. Then the biotin mixture was initially adjusted to a pH of 10 to dissolve. All stocks were adjusted to pH 4.5 and made up to final volume and stored at 4°C. The vitamin stock was then prepared by adding: 1 mL of biotin stock, 0.1 mL B-12 primary stock, and 20 mg thiamine HCl in 100 mL water and brought to a final volume of 1 L. Stock was filtered through a 0.22 µm filter.

Table 10.3 Artificial Fresh water Medium (AF6)

Compound	Quantity Used
MES*	2.000 g
Fe-citrate	0.010 g
Citric acid	0.010 g
$NaNO_3$*	0.700 g
$MgSO_4.7H_2O$	0.050 g
KH_2PO_4	0.050 g
K_2HPO_4	0.025 g
$CaCl_2.2H_2O$	0.050 g
Trace Metal Solution	5.000 mL
Vitamin Stock	5.000 mL

pH was adjusted to 6.6

Vitamin solution (0.5 mL) was added after sterilization to avoid degradation.

***Stress media was made up without $NaNO_3$ and MES buffer**

10.2. Medium Composition; Artificial Saline Water (ASW)

Table 10.4 Hydrous and Anhydrous Salt Preparation

Compound	($g.L^{-1}$)	Concentration in Final Medium (M)
Anhydrous Salts		
NaCl	13.98	2.39×10^{-1}
KCl	0.39	9.97×10^{-3}
$NaHCO_3$	0.20	2.38×10^{-3}
H_3BO_3	0.06	9.71×10^{-4}
Hydrous Salts		
$MgCl_2.6H_2O$	2.60	1.28×10^{-2}
$MgSO_4.7H_2O$	3.56	1.45×10^{-2}
$CaCl_2.2H_2O$	0.77	5.24×10^{-3}

Method: Anhydrous and hydrous salts were dissolved separately each in 300 mL of dH_2O, these two solutions were then combined (dissolved while continuously mixing using a magnetic stirrer). Each major nutrient was added in concentrations listed in Table 5, 0.5 mL of trace metal stock solution (Table 6), and 0.5 mL of Fe/EDTA stock (Table 7) was also added then autoclaved at 121°C for 15 min. Vitamin solution (prepared as for AF6 above) was added after sterilization to avoid degradation of the vitamins (0.5 mL).

pH was adjusted to 7.0

Table 10.5 ASW Major Nutrient Stock Solution Preparation

Compound	Stock Solution ($g.L^{-1}$ dH_2O)	Volume of stock used (mL)	Concentration in Final Medium (M)
$Na_2SiO_3.9H_2O$	60.82	5.0	1.05×10^{-3}
$NaNO_3$*	149.60	1.0	1.65×10^{-3}
NaH_2PO_4	8.80	0.5	3.33×10^{-4}

***Stress media was made up without $NaNO_3$ stock added**

Table 10.6 ASW Trace Metal Stock Preparation

Compound	g. 200 mL^{-1}
$CuSO_4.5H_2O$	3.92
$ZnSO_4.7H_2O$	8.80
$CoCl_2.6H_2O$	4.00
$MnCl_2.4H_2O$	72.00
$Na_2MoO_4.2H_2O$	2.52
$Na_2EDTA.2H_2O$	0.41

Method: Individual 200 mL stocks of the first 5 components were prepared; thereafter 250 µl of each of these stocks was added to 200 mL water. 0.41 g of Na_2EDTA was then added and this solution was boiled for 3 minutes, thereafter cooled to room temperature and adjusted to a volume of 250 mL. 0.5 mL of this solution was added to all media formulated. EDTA was stored away from the light at 4ºC.

Table 10.7 EDTA Stock Preparation

Compound	$g.L^{-1}$
$FeC_6H_5O_7$	5.71
$Na_2EDTA.2H_2O$	17.35

Method: Ingredients were dissolved in 800 mL of dH_2O and neutralized using 5 N NaOH. The stock was boiled to dissolve compounds, cooled, and then adjusted to a volume of 1 L. 0.5 mL of this solution was added to all media formulated.

11. APPENDIX B

11.1. Zarrouk Medium

Table 11.1 Zarrouk Base Medium Composition

Compound	$g.L^{-1}$
$NaHCO_3$	18.00
$NaNO_3$	2.50
K_2HPO_4	0.50
K_2SO_4	1.00
NaCl	1.00
$CaCl_2$	0.04
Na_2EDTA	0.08
$MgSO_4 \cdot 7H_2O$	0.20
$FeSO_4 \cdot 7H_2O$	0.01

Table 11.2 Trace Elements Stock Solution

Compound	$g.L^{-1}$
H_3BO_3	2.86
$(NH_4)6Mo_7O_{24}$	0.02
$MnCl_2 \cdot 4H_2O$	1.80
Cu_2SO_4	0.08
$ZnSO_4 \cdot 7H_2O$	0.22

Method: Trace element solution is made up to a 1 L stock solution and autoclaved, of which 1 mL is added per litre of base media.

11.2. F/2 Medium

Table 11.3 Trace Elements Stock Solution

Compound	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
$FeCl_3\ 6H_2O$	---	3.15 g	1.17×10^{-5} M
$Na_2EDTA\ 2H_2O$	---	4.36 g	1.17×10^{-5} M
$CuSO_4\ 5H_2O$	9.8 $g.L^{-1}$ dH_2O	1 mL	3.93×10^{-8} M
$Na_2MoO_4\ 2H_2O$	6.3 $g.L^{-1}$ dH_2O	1 mL	2.60×10^{-8} M
$ZnSO4\ 7H_2O$	22.0 $g.L^{-1}$ dH_2O	1 mL	7.65×10^{-8} M
$CoCl_2\ 6H_2O$	10.0 $g.L^{-1}$ dH_2O	1 mL	4.20×10^{-8} M
$MnCl_2\ 4H_2O$	180.0 $g.L^{-1}$ dH_2O	1 mL	9.10×10^{-7} M

Method: Prepared using 950 mL of dH_2O, components were added and brought to a final volume of 1 L with dH_2O.

Table 11.4 Vitamin Stock Solutions

Compound	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
Thiamine HCl (vit. B_1)	---	200 mg	2.96×10^{-7} M
Biotin (vit. H)	0.1 $g.L^{-1}$ dH_2O	10 mL	2.05×10^{-9} M
Cyanocobalamin (vit. B_{12})	1.0 $g.L^{-1}$ dH_2O	1 mL	3.69×10^{-10} M

Method: First, prepare primary stock solutions. To prepare final vitamin solution, begin with 950 mL of dH_2O, dissolve the thiamine, add the amounts of the remaining primary stocks and bring final volume to 1 with dH_2O. Store in refrigerator or freezer.

Table 11.5 F/2 Base Medium Composition

Compound	Stock Solution	Quantity	Molar Concentration in Final Medium
$NaNO_3$	75 $g.L^{-1}$ dH_2O	1 mL	8.82×10^{-4} M
$NaH_2PO_4\ H_2O$	5 $g.L^{-1}$ dH_2O	1 mL	3.62×10^{-5} M
$Na_2SiO_3\ 9H_2O$	30 $g.L^{-1}$ dH_2O	1 mL	1.06×10^{-4} M
Trace metal solution		1 mL	---
Vitamin solution		0.5 mL	---

11.3. Walne's medium

Table 11.6 Trace Elements Stock Solution

Compound	g.100 mL^{-1}
$ZnCl_2$	2.1
$CoCl_2.6H_2O$	2.0
$(NH_4)6Mo_7O_{24}.4H_2O$	0.9
$CuSO_4.5H_2O$	2.0

This solution is normally cloudy. Acidify with a few drops of concentrated HCl to give a clear solution.

Table 11.7 Vitamin Stock Solution

Compound	Quantity per 100 mL
Thiamine HCl (vit. B_1)	10 mg
Biotin (vit. H)	200 µg
Cyanocobalamin (vit. B_{12})	10 mg

Table 11.8 Nutrient Stock Solution

Compound	g.L^{-1}
$FeCl_3.6H_2O$	1.30
$MnCl_2.4H_2O$	0.36
H_3BO_3	33.60
EDTA (disodium salt)	45.00
$NaH_2PO_4.2H_2O$	20.00
$NaNO_3$	100.00

Method: Dispense nutrient stock (1 mL), Trace metal stock (1 mL) and vitamin solutions (0.1 mL) separately into 1 L of sterilised sea water.

11.4. Aquil medium adapted from Anderson (2005)

Version 1.0

Table 11.9 Base media recipe

Component	Conc in final medium ($g.L^{-1}$)	Conc in final medium (M)
Anhydrous Salts		
NaCl	24.54	4.20×10^{-1}
KCl	0.700	9.39×10^{-3}
KBr	0.100	8.40×10^{-4}
H_3BO_3	0.003	4.85×10^{-5}
NaF	0.003	7.15×10^{-5}
Hydrous Salts		
$MgCl_2.6H_2O$	11.100	5.46×10^{-2}
$CaCl_2 .2H_2O$	1.540	1.05×10^{-2}
$SrCl_2.6H_2O$	0.017	6.38×10^{-5}

pH adjusted to 7

Table 11.10 Nutrient Stock Preparation*

Component	Unit	Concentration in the Standard Medium ($g.L^{-1}$)	Stock Solution Concentration ($g.L^{-1}$)	Volume added to media (mL)
Na_2SO_4	$g.L^{-1}$	1.000	50	20
$NaHCO_3$	$g.L^{-1}$	2.000	100.0	20
$NaH_2PO_4.H_2O$	$g.L^{-1}$	0.100	5.0	20
$NaNO_3$	$g.L^{-1}$	0.250	12.5	20
$Na_2SiO_3.9H_2O$	$g.L^{-1}$	0.500	25.0	20

***Stress media was made up without highlighted nutrients**

Silicate stock is added slowly drop-wise while mixing media on a stirrer plate at 250 rpm.

Table 11.11 Vitamin Stock Preparation

Component	Conc in final medium ($g.L^{-1}$)	Stock Solution Conc ($g.L^{-1}$)	Amount added to stock solution	Conc in final medium (M)
Thiamine.HCl (Vit. B1)	1.00×10^{-4}		100 mg	2.97×10^{-7}
Biotin (Vit.H)	5.49×10^{-7}	5	1 mL	2.25×10^{-9}
Cyanocobalamin (Vit.B12)	5.02×10^{-7}	5.5	1 mL	3.70×10^{-10}

Method: Thiamine HCl is first dissolved into 950 mL dH_2O, thereafter 1 mL of each component is added to the total vitamin stock. Vitamin stock is then made up to 1 L, filter sterilized and added to starting inoculum.

Table 11.12 Trace Metal Stock Preparation

Component	Conc in final medium ($g.L^{-1}$)	Stock Solution Conc ($g.L^{-1}$)	Amount to be added to stock solution	Conc in final medium (M)
EDTA (anhydrous)	3.06×10^{-3}		29.20 g	1.00×10^{-5}
$FeCl_3.6H_2O$	2.70×10^{-4}		0.27 g	1.00×10^{-6}
$ZnSO_4.7H_2O$	2.29×10^{-5}		0.23 g	7.97×10^{-8}
$MnCl_2.4H_2O$	2.40×10^{-5}		0.02 g	1.21×10^{-7}
$CoCl_2.6H_2O$	1.20×10^{-5}		0.01 g	5.03×10^{-8}
$Na_2MoO_4.2H_2O$	2.42×10^{-5}		0.02 g	1.00×10^{-7}
$CuSO_4.5H_2O$	4.89×10^{-6}	4.9	1 mL	1.96×10^{-8}
Na_2SeO_3	1.73×10^{-6}	1.9	1 mL	1.00×10^{-8}

Method: The compounds below are dissolved in 900 mL dH_2O, $CuSO_4.5H_2O$ and Na_2SeO_3 are made separately and 1 mL of each is added to the trace metal solution. This is then made up to a litre. 1 mL added per litre media.

12. APPENDIX C

Modified Aquil medium used in cascading raceway cultivation system,

Version 1.1

Table 12.1 Controlled macronutrient composition

Component	Concentration in final Medium	Quantity to be added to 100 L media
	$g.L^{-1}$	g
Na_2SO_4	0.180	18.0
$NaHCO_3$	30.000	3000.0
$NaH_2PO_4.H_2O$	0.054	5.4
$NaNO_3$	0.180	18.0
$Na_2SiO_3.9H_2O$	1.440	144.0

Table 12.2 Nutrient Salt Composition

Component	Concentration in final Medium	Quantity to be added to 100 L media
	$g.L^{-1}$	g
NaCl	24.540	2454.0
KCl	0.700	70.0
KBr	0.100	10.0
H_3BO_3	0.003	0.3
$MgCl_2.6H_2O$	11.100	1110.0
$CaCl_2.2H_2O$	1.540	154.0
$SrCl_2.6H_2O$	0.017	1.7

Table 12.3 Vitamin Stock Solution

Component	Concentration in final Medium	Stock solution concentration	Quantity to be added to 100 L media
	$g.L^{-1}$	$g.L^{-1}$	mL
Thiamine.HCl (Vit.B1)	1.002×10^{-4}	1.000	10
Biotin (Vit.H)	$5.497E \times 10^{-7}$	0.005	10
Cyanocobalamin (Vit.B12)	5.019×10^{-7}	0.005	10

Table 12.4 Trace metals

Component	Concentration in final Medium	Stock solution concentration	Quantity to be added to 100 L media
	$g.L^{-1}$	$g.L^{-1}$	mL
EDTA (anhydrous)	3.063×10^{-3}	30.500	10
$FeCl_3.6H_2O$	2.703×10^{-4}	2.700	10
$ZnSO_4.7H_2O$	2.292×10^{-5}	0.229	10
$MnCl_2.4H_2O$	2.395×10^{-5}	0.239	10
$CoCl_2.6H_2O$	1.197×10^{-5}	0.120	10
$Na_2MoO_4.2H_2O$	2.420×10^{-5}	0.241	10
$CuSO_4.5H_2O$	4.894×10^{-6}	0.049	10
Na_2SeO_3	1.729×10^{-6}	0.017	10

13. APPENDIX D

Multi- Stage cascading raceway system bioprocess operating data

Table 13.1 GP operational data

Date May	Day	pH	Temp °C	NO3 mg/L	PO4 mg/L	SO4 mg/l	SiO3 mg/L	CO3 g/L	Level (stagnant) cm	Acid added L	Medium Flow L/h	Medium Fed L	Harvest Volume L	Cell count #/L	EPA ug/mL
19	0	7,03	20,9	46,48	68,07	794	41,78	0,28	9,5	5,0	2,1	25,2	32	6,08E+09	2,86
20	1	7,01	21,3					0,25	9,5	5,0	1,67	20	10,4	4,87E+09	4,407
21	2	7,02	20,6	61,09	62,55	902	40,3	0,16	9,5	6,0	1,67	20	20,4	7,70E+09	0,67
22	3	7,11	22,6					0,18	9,5	5,0	1,67	20	0,5	8,86E+09	1,37
23	4	7,01	21,3	72,59	54,27	900	36,7	0,23	7,5	6,5	1,67	20	21	9,80E+09	2,67
24	5	7,01	20,5					0,17	9,5	5,5	1,67	20	14	7,13E+09	2,94
25	6	7,02	21,4	69,49	24,84	642	41,4	0,17	9,5	6,0	1,67	20	11,2	7,25E+09	3,11
26	7	7,01	20					0,17	9,8	7,0	1,67	20	13,2	9,59E+09	2,52
27	8	7,02	19,2	75,25	44,46	580	37,45	0,16	9,8	7,0	1,67	20	15,2	7,01E+09	2,2
28	9	7,07	20,9					0,3	9,5	7,0	1,67	20	12,6	5,20E+09	2,94
ave		**7,031**	**20,87**	**64,98**	**50,84**	**763,6**	**39,526**	**0,21**	**9,36**					**7,35E+09**	**2,569**

Table 13.2 Input and output flow from GP to SP1

Total input (L)	cells formed = vol out *Cgp (cells/day)	EPA out = vol out * EPAgp (mg/day)	Cell prod. (cells/L/day)	EPA prod. (EPA/L/day)	Spec. EPA prod (mg/cell/day)		Overflow vol	cells to SP1 (cells/day)	EPA to SP1 (mg/day)
30,2	1,84E+11	86,372	1,84E+09	0,86372			-1,8		
25,0	1,22E+11	110,175	1,22E+09	1,10175	9,05E-10		14,6	7,11E+10	64,3422
26,0	2,00E+11	17,42	2,00E+09	0,1742	8,70E-11		5,6	4,31E+10	3,752
25,0	2,22E+11	34,25	2,22E+09	0,3425	1,55E-10		24,5	2,17E+11	33,565
26,5	2,60E+11	70,755	2,60E+09	0,70755	2,72E-10		5,5	5,39E+10	14,685
25,5	1,82E+11	74,97	1,82E+09	0,7497	4,12E-10		11,5	8,20E+10	33,81
26,0	1,89E+11	80,86	1,89E+09	0,8086	4,29E-10		14,8	1,07E+11	46,028
27,0	2,59E+11	68,04	2,59E+09	0,6804	2,63E-10		13,8	1,32E+11	34,776
27,0	1,89E+11	59,4	1,89E+09	0,594	3,14E-10		11,8	8,27E+10	25,96
27,0	1,40E+11	79,38	1,40E+09	0,7938	5,65E-10		14,4	7,49E+10	42,336
			1,95E+09	**0,681622**	**3,78E-10**				

Table 13.3 SP1 operational data

Date May	Day	pH	Temp °C	NO3 mg/L	PO4 mg/L	SO4 mg/l	SiO3 mg/L	CO3 g/L	Level (stagnant) cm	Acid added L	Medium Flow L/h	NaHCO3 Fed L	Harvest Volume L	Cell count #/L	EPA ug/mL
19	0	8,06	16,5	30,54	36,47	467	21,3	2,2	9,8	2,8		3,0	0,5	2,74E+09	4,46
20	1	8,32	17,6					1,93	9,8	2,0		3,0	0,5	3,84E+09	5,187
21	2	8,68	15,8	22,58	48,44	374	18,85	2,08	9,5	1,1		3,0	0,5	5,70E+09	4,55
22	3	8,66	19,4					2,33	9,5	0,7		3,0	0,5	7,13E+09	3,11
23	4	8,08	16,9	40,73	54,74	447	12,45	1,65	9,5	3,6		3,0	0,5	1,03E+10	4,11
24	5	8,23	18,9					1,78	9,5	2,0		3,0	0,5	8,05E+09	2,88
25	6	8,6	17,5	31,87	24,07	473	4,8	1,6	9,5	2,0		3,0	0,5	6,26E+09	4,41
26	7	8,75	15,4					1,72	9,2	2,0		3,0	0,5	1,28E+10	4,41
27	8	8,73	13,7	35,41	42,62	446	12,4	1,58	9,2	2,0		3,0	0,5	7,34E+09	3,92
28	9	8,78	17,5					2,15	9,2	1,3		3,0	0,5	7870000000	4,52
ave		**16,946**		**31,43**	**40,93**	**440,3**	**14,35**	**1,87**	**9,5**					**7,20E+09**	**4,115**

Table 13.4 Input and output flow from SP1 to SP 2

Incoming Overflow volume (L)	Total volume in (L)	cells to SP1 (cells/day)	EPA to SP1 (mg/day)	Cells out = vol out *Cgp (cells/day)	EPA out = vol out * EPAgp (mg/day)	Cell prod. (cells/L/day)	EPA prod. (EPA/L/day)	Spec. EPA prod. (mg/cell/day)		Outgoing Flow to SP2 (L)	Cells to SP2 (cells/day)	EPA to SP2 (mg/day)
-1,8	4,0			1,10E+10	17,84					3,5	9,59E+09	15,61
14,6	19,6	7,11E+10	64,3422	7,53E+10	101,6652	4,16E+07	0,37323	8,97E-09		19,1	7,33E+10	99,0717
5,6	9,7	4,31E+10	3,752	5,53E+10	44,135	1,22E+08	0,40383	3,32E-09		9,2	5,24E+10	41,86
24,5	28,2	2,17E+11	33,565	2,01E+11	87,702	-1,60E+08	0,54137	-3,38E-09		27,7	1,98E+11	86,147
5,5	12,1	5,39E+10	14,685	1,25E+11	49,731	7,07E+08	0,35046	4,95E-10		11,6	1,19E+11	47,676
11,5	16,5	8,20E+10	33,81	1,33E+11	47,52	5,08E+08	0,1371	2,70E-10		16,0	1,29E+11	46,08
14,8	19,8	1,07E+11	46,028	1,24E+11	87,318	1,66E+08	0,4129	2,48E-09		19,3	1,21E+11	85,113
13,8	18,8	1,32E+11	34,776	2,41E+11	82,908	1,08E+09	0,48132	4,44E-10		18,3	2,34E+11	80,703
11,8	16,8	8,27E+10	25,96	1,23E+11	65,856	4,06E+08	0,39896	9,83E-10		16,3	1,20E+11	63,896
14,4	18,7	7,49E+10	42,336	1,47E+11	84,524	7,23E+08	0,42188	5,84E-10		18,2	1,43E+11	82,264
						4,00E+08	**0,391227778**	**1,57E-09**				

Table 13.5 SP2 operational data

Date May	Day	pH	Temp °C	NO3 mg/L	PO4 mg/L	SO4 mg/l	SiO3 mg/L	CO3 g/L	Level (stagnant) cm	Acid added L	Medium Flow L/h	Medium Fed L	Harvest Volume L	Cell count #/L	EPA ug/mL
19	0	8,00	15,8	12,39	40,17	635	11,95	0,85	9,8	0,2			0,5	1,27E+10	6,18
20	1	8,03	17					0,72	9,8	0,1			0,5	1,62E+10	5,497
21	2	7,98	15,4	5,75	56,42	421	6,8	0,63	9,8	0,4			0,5	2,06E+10	4,51
22	3	8,01	18,9					0,63	9,8	0,4			0,5	2,11E+10	5,51
23	4	7,98	16,5	23,46	61,32	645	0,5	0,47	9,8	0,5			0,5	1,33E+10	4,73
24	5	7,95	15,3					0,28	9,8	0,6			0,5	1,62E+10	5,19
25	6	8,05	10,8	13,72	36,49	607	0,6	0,35	9,5	0,8			0,5	1,16E+10	6,33
26	7	7,94	14					0,32	9,5	0,7			0,5	1,47E+10	5,97
27	8	8,03	13,1	8,31	54,73	556	1,85	0,48	9,5	0,7			0,5	1,59E+10	6,63
28	9	8,00	17,4					0,28	9,5	0,7			0,5	2,34E+09	7,01
ave		**7,997**		**12,726**	**49,83**	**572,8**	**4,34**	**0,5**	**9,68**					**1,45E+10**	**5,756**

Table 13.6 Input and output flow from SP2 to SP 1

Input				Productivity						Output		
Incoming Overflow volume (L)	Total volume in (L)	cells to SP2 (cells/day)	EPA to SP2 (mg/day)	Cells out = vol out *Cgp (cells/day)	EPA out = vol out * EPAgp (mg/day)	Cell prod. (cells/L/day)	EPA prod. (EPA/L/day)	Spec. EPA prod (mg/cell/day)		Outgoing Flow to SP3 (L)	Cells to SP3 (cells/day)	EPA to SP3 (mg/day)
3,5	3,7		15,61	4,70E+10	22,866	4,70E+08	0,07256			3,2	4,06E+10	19,776
19,1	19,2	7,33E+10	99,0717	3,11E+11	105,5424	2,38E+09	0,064707	2,72E-11		18,7	3,03E+11	102,7939
9,2	9,6	5,24E+10	41,86	1,98E+11	43,296	1,45E+09	0,01436	9,88E-12		9,1	1,87E+11	41,041
27,7	28,1	1,98E+11	86,147	5,93E+11	154,831	3,95E+09	0,68684	1,74E-10		27,6	5,82E+11	152,076
11,6	12,1	1,19E+11	47,676	1,61E+11	57,233	4,15E+08	0,09557	2,31E-10		11,6	1,54E+11	54,868
16	16,6	1,29E+11	46,08	2,69E+11	86,154	1,40E+09	0,40074	2,86E-10		16,1	2,61E+11	83,559
19,3	20,1	1,21E+11	85,113	2,33E+11	127,233	1,12E+09	0,4212	3,75E-10		19,6	2,27E+11	124,068
18,3	19,0	2,34E+11	80,703	2,79E+11	113,43	4,51E+08	0,32727	7,26E-10		18,5	2,72E+11	110,445
16,3	17,0	1,20E+11	63,896	2,70E+11	112,71	1,51E+09	0,48814	3,24E-10		16,5	2,62E+11	109,395
18,2	18,9	1,43E+11	82,264	4,42E+10	132,489	-9,90E+08	0,50225	-5,07E-10		18,4	4,31E+10	128,984
						1,22E+09	**0,3073637**	**1,83E-10**				

Table 13.7 SP3 operational parameters

Date May	Day	pH	Temp °C	NO3 mg/L	PO4 mg/L	SO4 mg/l	SiO3 mg/L	CO3 g/L	Level (stagnant) cm	Acid added L	Medium Flow L/h	Medium Fed L	Harvest Volume L	Cell count #/L	EPA ug/mL
19	0	8,24	14,3	15,59	52,12	647	19,55	0,22	11	0,2			32	1,41E+10	5,96
20	1	7,99	15,8					0,28	9,5	0,2			10,4	1,78E+10	6,563
21	2	8	14,6	10,62	50,39	745	15,6	0,3	10,1	0,2			20,4	1,81E+10	4,88
22	3	8,03	18,1					0,22	10	0,2			0,5	2,66E+10	3,75
23	4	8,1	16	26,12	57,34	704	5,35	0,25	11	0,1			21	1,15E+10	4,88
24	5	7,92	14,8					0,13	9,5	0,1			14	1,80E+10	6,19
25	6	7,73	16,5	18,59	34,34	782	1,55	0,13	10	0,2			11,2	1,94E+10	7,17
26	7	7,63	13,5					0,12	10,5	0,3			13,2	1,31E+10	6,13
27	8	7,48	13,1	5,37	73,74	794	2,55	0,08	10	0,1			15,2	2,12E+10	6,13
28	9	7,96	17					0,15	10	0,1			12,6	8,98E+09	7,52
ave		**7,908**		**15,258**	**53,59**	**734,4**	**8,92**	**0,19**	**10,16**					**1,69E+10**	**5,917**

Table 13.8 Input and productivity data of SP3

Input				Productivity				
Incoming Overflow volume (L)	Total volume in (L)	cells to SP3 (cells/day)	EPA to SP3 (mg/day)	Cells out = vol out *Cgp (cells/day)	EPA out = vol out * EPAgp (mg/day)	Cell prod. (cells/L/day)	EPA prod. (EPA/L/day)	Spec EPA prod. (mg/cell/day)
3,2	3,4	4,06E+10	19,776	4,79E+10	20,264	7,30E+07	0,00488	
18,7	18,9	3,03E+11	102,7939	3,36E+11	124,0407	3,35E+08	0,212468	6,35E-10
9,1	9,3	1,87E+11	41,041	1,68E+11	45,384	-1,91E+08	0,04343	-2,27E-10
27,6	27,8	5,82E+11	152,076	7,39E+11	104,25	1,57E+09	-0,47826	-3,04E-10
11,6	11,7	1,54E+11	54,868	1,35E+11	57,096	-1,97E+08	0,02228	-1,13E-10
16,1	16,2	2,61E+11	83,559	2,92E+11	100,278	3,08E+08	0,16719	5,43E-10
19,6	19,8	2,27E+11	124,068	3,84E+11	141,966	1,57E+09	0,17898	1,14E-10
18,5	18,8	2,72E+11	110,445	2,46E+11	115,244	-2,57E+08	0,04799	-1,87E-10
16,5	16,6	2,62E+11	109,395	3,52E+11	101,758	8,96E+08	-0,07637	-8,53E-11
18,4	18,5	4,31E+10	128,984	1,66E+11	139,12	1,23E+09	0,10136	8,24E-11
						5,34E+08	0,0223948	5,09E-11