

**Development of a photobioreactor for the
cultivation of the freshwater microalga**

Haematococcus pluvialis.

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Abstract

Haematococcus pluvialis is a flagellated green alga that accumulates large quantities of the carotenoid astaxanthin. Astaxanthin is commercially important as a aquaculture pigment, and as a human health supplement. Currently the majority of astaxanthin used in aquaculture is synthetic. However recent advances in algal mass culture technology and growing consumer awareness about the origins of foodstuffs mean that the use of *H. pluvialis* for the production of astaxanthin may become a viable alternative to synthetically produced astaxanthin.

This study focused on the development of a scaleable photobioreactor and a methodology that could be used for the commercial production of *H. pluvialis*. Four successive air-lift driven novel tubular photobioreactors (termed TBR1, TBR2, AAPSTM1 and AAPSTM2) were developed for the photosynthetic cultivation of *H. pluvialis*. Physical assessment revealed that the four systems differed in terms of their mass transfer, gas hold-up, flow rate and photostage turbulence (Reynolds number). The photostage of each photobioreactor acted as the main area for light absorbance, (analysis of the spectral absorbance of the tubing revealed that it transmitted light of 320 - 820nm). The AAPSTM1 and AAPSTM2 had a manifold photostage, comprising of three windings. They also had a riser and downcomer of increased length when compared to TBR1 and TBR2. This increased the bulk density difference between these stages and resulted in an increased fluid flow rate and consequently improved turbulence within the photostage. As well as being longer, the riser of the AAPSTM systems also had an increased diameter. This, combined with the improved gas hold-up of the systems, increased the mass transfer of the AAPSTM1 and AAPSTM2. The manifold assembly also allowed the construction of a larger volume photostage while

frequently returning the cells to the riser/header tank where gas exchange occurred. This prevented the detrimental build-up of photosynthetic O₂.

The first two systems, termed TBR1 and TBR2, were sited indoors with artificial lighting. TBR1 failed to yield a *H. pluvialis* culture, whilst TBR2 produced a carotenoid-rich culture after 55 days. Problems with cell settling within these systems resulted in the development of the AAPS™1; further engineering developments resulted in the construction of the AAPS™2.

Three production methodologies were proposed for the cultivation of *H. pluvialis* in AAPS™, batch, single-phase continuous and two-phase continuous. In batch culture two media types, termed high and low [N], were used. The growth rate did not differ between the two media types, however growth in the high [N] media was sustained for a longer period. The low [N] media was found to produce a culture with a higher carotenoid content per cell but yielded a reduced carotenoid content per unit volume of media when compared to the high [N] media. This was due to the fact that the high [N] media produced a greater number of cells per volume of media. Under batch conditions the greatest concentration of biomass and carotenoids was produced during August and September 1999 in the AAPS™2. In the low [N] media a cell density of $20.50 (\pm 1.26) \times 10^4$ cells/ml ($n=4 \pm$ S.E.) was achieved, these cells had a carotenoid content of 127.73pg/cell (± 6.38 , $n=12 \pm$ S.E.) after 18 days. The culture contained 25.35mg/l (± 1.26 , $n=3 \pm$ S.E.) of secondary carotenoids. In the high [N] media the cell density was 70.00×10^4 cells/ml (± 5.43 , $n=4 \pm$ S.E.), these cells had a carotenoid content of 74.36pg/cell (± 3.14 , $n=12 \pm$ S.E.). The carotenoid content per unit volume of media was 51.13mg/l (± 1.44 , $n=3 \pm$ S.E.). This was achieved after 48 days of culture (nearly three times longer than the low [N] culture).

In two-phase continuous culture the biomass was produced indoors in a controlled environment using artificial illumination. Carotenogenesis was induced in a separate AAPS™ outdoors. In single-phase production (outdoors), growth was improved when compared to previous batch-grown cultures of *H. pluvialis*. This was due to the formation of aplanospores early in the culture, which proceeded to germinate. Germination of aplanospores was noted to produce as many as 16 zooids, although it was more typically eight. Division of zooids was only noted to result in two or four new cells. Batch cultivation typically produced $\sim 1.00 \times 10^4$ cells/ml/day. Continuous culture produced 83.64×10^4 cells/ml/day outdoors and 43.64×10^4 cells/ml/day indoors. *H. pluvialis* supported a continuous renewal rate of 10% (v/v) of the system operating volume. An increase to 20% was found to result in the 'wash-out' of the culture.

The AAPS™1 and AAPS™2 produced successive carotenoid-rich batch cultures of *H. pluvialis* ($\leq 3.40\%$ as dry weight w/v). The AAPS™2 was also used to produce carotenoid-rich cultures using single- and two-phase continuous culture ($\leq 1.80\%$ as dry weight w/v).

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Declaration

I declare that this is a report of the research carried out by myself from October 1997 until September 2000, in the school of Biological and Earth Sciences, Liverpool John Moores University, under the supervision of Prof. Andrew Young. It is entirely my own work and has not been submitted for any other degree.

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1. Introduction

1.1. Mass culture of microalgae

Microalgae have been a food source for over a 1000 years. One of the earliest recorded was the use of the prokaryote *Spirulina* (*Arthrospira*) by the Aztecs, who harvested it from Lake Texcoco (Vonshak 1990). *Spirulina* was also an important protein source for native Africans who harvested it from Lake Chad. The use of both prokaryotic and eukaryotic algae as a protein source has since been of increasing interest. As naturally occurring monocultures are rare, research turned to developing culture systems to produce large volumes of algae. The first major work published in this field concerned the mass culture of *Chlorella* by Burlew as recently as 1953.

1.1.1 Why culture microalgae?

In recent years there has been increasing interest in the production of microalgae as a protein source, a health food, as an animal feed component and also using it as a method for reducing CO₂ in the atmosphere. In general there are two main ways to utilise microalgae; in the first the whole algal cell is of interest, for example cultivation of single-celled *Chlorella* sp. for use as a protein-rich health food. Alternatively only a constituent of the algal cell is of interest, for example β-carotene extracted from *Dunaliella* sp.

A wide variety of microalgae have been investigated for possible products. One example is *Phaeodactylum tricornutum* for eicosapentaenoic acid (EPA) which is a dietary lipid for marine life and consequently of indirect nutritional importance to humans (Molina Grima *et al* 1994a,b). The most commercially successful venture in microalgal culture has been the cultivation of the marine alga *Dunaliella salina* for the

carotenoid β -carotene, which has been commercialised by Western Biotechnology Ltd and Betene Ltd, producing large amounts of β -carotene from shallow ponds in western Australia (Moulton *et al* 1987, Borowitzka and Borowitzka 1989). Another alga that is commercially produced is *Haematococcus* for the carotenoid astaxanthin by the Cyanotech Corp. (Lorenz and Cysewski 2000) and Aquaseach Inc. (Olaizola 2000) both of whom are based in Hawaii.

Both *Chlorella* sp. and *Spirulina* sp. are cultivated commercially in Japan and China as health food supplements (Lee *et al* 1987, Lee and Low 1994, Lee *et al* 1996). Products that can be extracted from algae are also of great commercial interest; Muller-Feuga *et al* (1998) examined the mass culture of *Porphyridium cruentum* for the production of fine chemicals and for use as a hatchery feed. Nelis and De Leenheer (1991) reviewed the use of the carotenoids β -carotene from *D. salina* as a food colourant, and lutein isolated from *Chlorella pyrenoidosa* for poultry pigmentation.

The production of fatty acids has been of increasing interest in recent years mainly for use as a supplement in animal feed. *Isochrysis galbana* has been extensively researched as a source of polyunsaturated fatty acids (Molina Grima *et al* 1994b, Molina Grima *et al* 1997, Zhu *et al* 1997). *Phaeodactylum tricornutum* and *Porphyridium cruentum* are also high in the fatty acid eicosapentaenoic acid (Otero *et al* 1997) and have received a great deal of research interest. Other algae that have been of interest as animal feed stuffs include *Ankistrodesmus falcatus* and *Scenedesmus incrassatulus* (Martinez-Jerónimo and Espinosa-Chávez 1994).

Another area of research where the large-scale culture of algae may be of importance is in the reduction of the CO₂ concentration in the atmosphere. Algae have a high photosynthetic capability and therefore could utilise CO₂ produced from power stations to produce energy rich biomass (Watanabe *et al* 1995a). Algae that have been

investigated for this particular application include *Spirulina platensis* (Watanabe and Hall 1995b, Watanabe and Hall 1996a,b) and *Chlorella* sp. (Hirata *et al* 1996, Watanabe and Saiki 1997) both of which were selected because of their high photosynthetic and growth rates (Watanabe *et al* 1998). Research into the use of marine microalgae to remove nitric oxide and CO₂ from flue gas has also been carried out (Yoshihara *et al* 1996).

1.1.2. Development of mass culture systems

The section above highlights just a few of the possible products that can be commercially produced from microalgae. However, the culture of sufficient quantities of microalgae to make production commercially viable has been the major problem in the commercialisation of any products. There are three main approaches to algal culture,

- **Autotrophic** culture uses light as an energy source through photosynthesis to convert CO₂ into organic material.
- **Heterotrophic** culture has no requirement for light. An organic carbon source is provided in the culture medium, for example glucose, which is utilised in place of CO₂.
- **Mixotrophic** culture uses light to convert CO₂ into organic material and is also able to utilise an organic carbon source.

These methods have been investigated for use in the large-scale culture of microalgae. As microalgae are photosynthetic organisms many are not adapted to growing on an organic carbon source and so must be cultured autotrophically. Autotrophic growth typically has a lower biomass yield than either mixotrophic or

heterotrophic growth (Johns 1994). However, the lack of an organic carbon source in the media enables the use of less sophisticated systems thereby reducing operating costs.

Algae that can be supported by heterotrophic growth tend to yield a higher level of biomass than when grown autotrophically as they have no dependence on illumination (i.e light is not a limiting factor at high cell densities, Margalith 1999). Another key advantage is that the microalgae can be grown in commercially available conventional fermentors, enabling axenic production and a high level of environmental process control (Johns 1994). A high level of control means that the quality of the product can be closely controlled which is important for products that must adhere to legislative controls (e.g. food or pharmaceutical products). However not all algae are capable of heterotrophic growth, and even those that are, may not necessarily produce the product of interest under these conditions. For example, the production of the carotenoid astaxanthin by *Haematococcus* sp. is a light-dependant process and only traces of astaxanthin are accumulated in heterotrophic (dark grown) cultures (Harker *et al* 1996a).

Mixotrophic culture involves the combined utilisation of an organic carbon source (thereby increasing the potential biomass yield of the algae), and also exposing the culture to light for photosynthesis. The combined use of light and an organic carbon source has been shown to improve yields. Ogbonna *et al* (1997), working with *Chlorella*, demonstrated that by growing the cells heterotrophically and then exposing the cells to a period of illumination both the protein and chlorophyll content of the cells could be improved.

As the use of an organic carbon source increases the risk of contamination by bacteria a great deal of care must be taken in the selection of the culture vessel.

Conventional fermentors are of little use due to the lack of illumination, however a closed system is vital to prevent contamination. A number of illuminated fermentors have been developed but the high capital and operational costs involved make mixotrophic production uneconomic for all but a small number of high value products, such as pharmaceuticals (Borowitzka 1994).

In the search for the ideal growth system for the cultivation of microalgae on a large scale two main categories of systems have been developed, open and closed. Open systems take the form of ponds or raceways, their use is restricted to autotrophic production; closed systems typically take the form of either tubular photobioreactors or bubble columns and, theoretically, can be used for autotrophic and mixotrophic growth. Conventional stirred tank fermentors (cstr) have been used for heterotrophic cultivation.

1.1.2.1. Ponds

Pond culture is the main type of large-scale culture system in use throughout the world (Pulz 1994). It is relatively cheap and unsophisticated enabling cost-effective production. Ponds can be operated as extensive or intensive systems. Extensive pond culture has been used for the culture of *Chlorella*, *Scenedesmus*, *Spirulina* and *Dunaliella* (Borowitzka and Borowitzka 1989). The best known example of a commercial extensive pond system is the Western Biotechnology Ltd and Betatene Ltd plant for the production of β -carotene from *D. salina* at Hutt Lagoon, Western Australia (Moulton *et al* 1987, Borowitzka and Borowitzka 1989). These ponds are unlined and cover an area of 5 ha, and are 500m long and 100m wide. They are 15-20cm deep and rely on wind for mixing (Borowitzka and Borowitzka 1989). Intensive or raceway ponds are more common, although much smaller ($\leq 1000\text{m}^2$) and are

currently used for the culture of *Chlorella*, *Spirulina* and *D. salina* in Taiwan, Japan, Thailand, China, the USA and Israel (Borowitzka 1994). These are often deeper than extensive ponds and are actively mixed using a paddle wheel (Figure 1.1). Another form of intensive pond is the circular stirred pond, which are used in Taiwan for the culture of *Chlorella* (Borowitzka 1994). The higher cost of construction and operation involved in intensive systems means that production costs of any product are higher than for an extensive system (Terry and Raymond 1985). However extensive systems are uneconomic where land costs are high unless the price of the product is also very high (Borowitzka 1994).

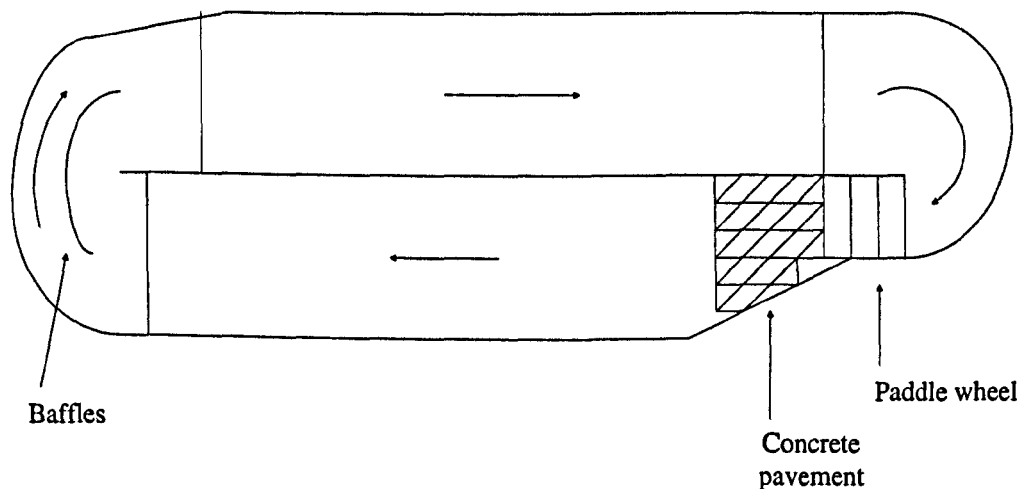


Figure 1.1. Diagrammatic representation of a raceway pond (taken from Borowitzka and Borowitzka 1989).

Although pond culture has been successfully used in the mass culture of a number of species of algae, it is only really successful in the culture of algae that grow in an extreme environment because this limits contamination and/or predation. For example *D. salina* has a high salt tolerance and by culturing in a solution with a high salt content, competitor species are excluded. *Spirulina* is cultured in an alkaline environment. *Chlorella*, although it does not tolerate extreme conditions, has a very fast growth rate and so is able to out-compete other microalgal species (Richmond and Vonshak 1978, Lee 1986, Margalith 1999). The development of pond culture for other species has often experienced problems. Microbio Resources (California, USA) found that freshwater protozoan, when established, could wipe out 90% of a *Haematococcus* culture in 72hrs (Bubrick 1991). For commercial production this is unacceptable, as there is no guarantee of product supply to the market place.

As well as contamination of the culture from other species, pond culture has a number of problems. To achieve a large ratio of surface area:volume the design of ponds tends to make them very large with a shallow algal culture. This means that evaporation becomes a problem, as does the loss of valuable CO₂ into the atmosphere (Pulz 1994). The large ratio of surface area:volume also means that large amounts of land are required. In some areas of the world where the cost of land is high, this has a significant impact of the cost of a production plant set up and consequently on the cost of any product. Another factor that acts to increase the cost of any product is harvesting. In any culture system, harvesting of the culture makes up a significant portion of the overheads, as it tends to be very energy intensive. As pond culture in general terms will only yield less than one gram of dry weight per litre (Borowitzka 1994) harvesting costs are high.

1.1.2.2. Bubble columns and airlift reactors

Bubble columns (Figure 1.2) are cylindrical vessels with air pumped into the base of the vessel, which acts to aerate the culture and also to provide mixing. Airlift reactors have the same basic design as the bubble column but the tube is separated into two sections by a baffle, forming a riser into which air is sparged and a downcomer. Fluid flows around the airlift due to different gas hold-up in the two sections, which produces a change in the bulk densities, causing the fluid to circulate around the system (Chisti 1989).

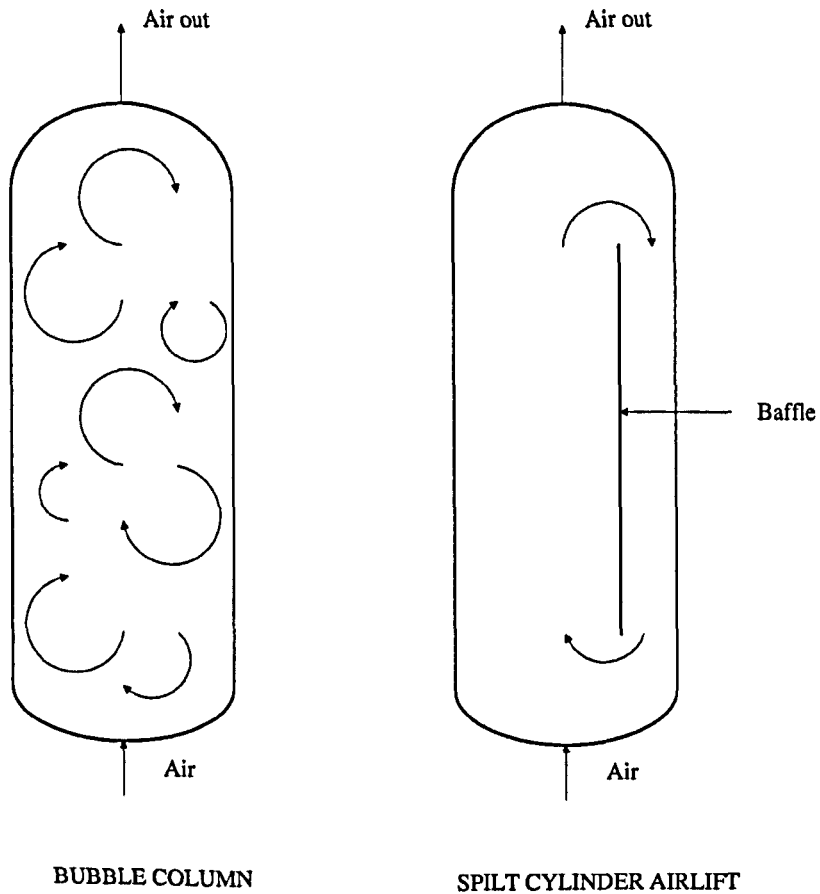


Figure 1.2. Diagrammatic representation of a bubble column and an airlift reactor (arrows show fluid direction and movement, based on diagrams taken from Chisti 1989).

There are a variety of airlift designs, which can be broadly split into two types, namely the internal and external loop. The internal design can be further divided into the split cylinder (as shown in Figure 1.2) and the concentric draft tube. In place of a baffle the concentric draft column has a second tube within the column with air sparged in at its base. The external loop has a separate riser and downcomer, which are joined at the top and bottom, by horizontal sections. An advantage of this type of system is that the airlift has very low shear, therefore causing little or no damage to the organism being cultivated (Chisti 1989). This is an important consideration for shear-sensitive organisms such as microalgae.

In all these designs the column tends to be transparent to allow maximal light penetration to the culture, and is typically made of glass or plastic. The materials of construction are selected to allow chemical or steam sterilisation as in general the size and shape of the vessel precludes autoclaving. The vessel can be kept sterile by filtering the air that is pumped into the system and by employing appropriate filters on the outlets. A major problem with the column reactor is that as it is a vertical system it is always at a large angle to the sun's rays (Lee 1986). Therefore unlike the pond design which is horizontal, the column is not very efficient at intercepting solar energy. Other design limitations, which preclude the scaling-up of these systems are the costs of fabrication, the weight of the materials (requiring extensive support) and the poor light penetration characteristics of large diameter vessels (Sánchez Mirón *et al* 1999).

1.1.2.3. Tubular photobioreactors

The tubular photobioreactor has developed from the systems described previously. Many variations have been developed, but the basic objectives of any tubular photobioreactor are to prevent contamination, maximise light-penetration and

volume by their mixing efficiency, and also by their poor light efficiency due to the depth (diameter) of the culture vessel (Sánchez Mirón *et al* 1999).

The tubular photobioreactor is not a new idea, and the first published information on this type of system was in the book edited by Burlew (1953). Development of the technology has resulted in the tubular photobioreactor taking a number of forms (Borowitzka 1999). The main variation is in the arrangement of the tubes that make up the photostage, the part of the system that is primarily used for light interception. These can be arranged horizontally along the floor, such as the photobioreactors described by Gudin and Chaumont (1991) and Molina Grima *et al* (1994b), or vertically in the form of a coil or a fence (Chrismadha and Borowitzka 1994, Watanabe *et al* 1995). A coiled photostage has an advantage over the fence design because the movement of liquid through a coil generates a secondary fluid motion, which improves mixing (Hoshino *et al* 1991, Borowitzka 1994, Carlozzi and Torzillo 1996). The choice of pump to circulate the algal culture is wide but this decision is primarily dependent on the species of algae to be cultured, as species vary considerably in shear tolerance. This subject shall be discussed in more detail below.

In the basic design of a tubular photobioreactor (Figure 1.3, using a helical photostage as an example) light penetration is primarily via the photostage which is made up of narrow-bore plastic tubing (in the case of a system constructed of tubing strips, it may be glass). Narrow-bore tubing maximises the ratio of surface area:volume thereby maximising the irradiance received per cell within the culture (Eriksen *et al* 1996, Kobayashi and Fujita 1997).

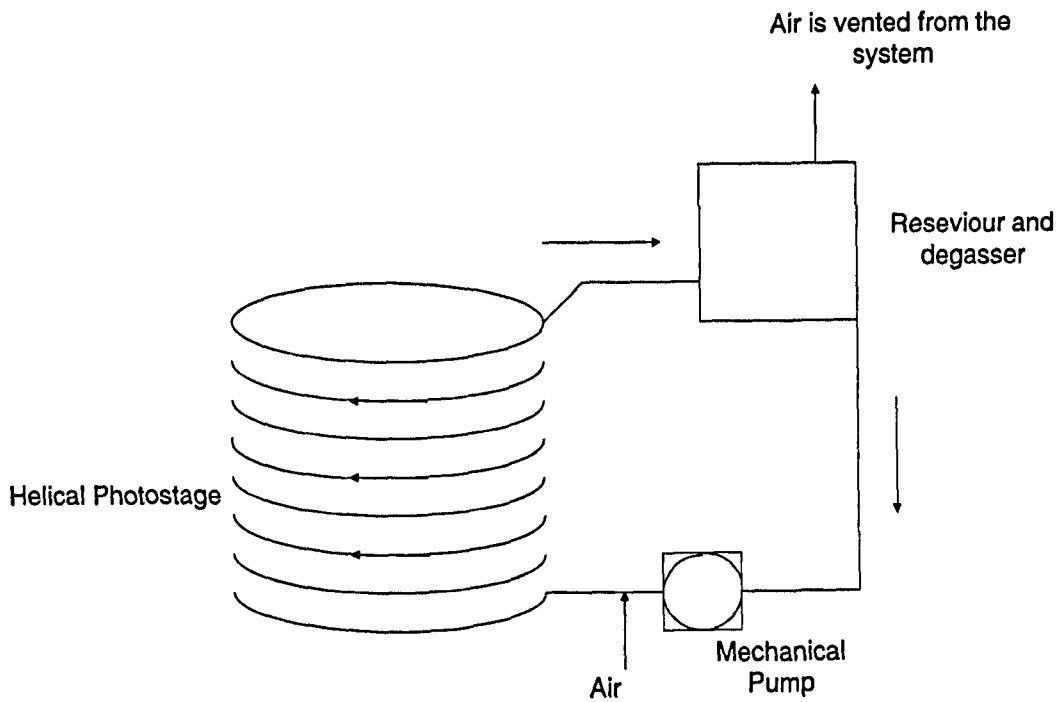


Figure 1.3. Diagrammatic representation of a tubular photobioreactor.

The algal culture is circulated through the tubes using a pump, this prevents settlement and the rate of pump operation allows control of the circulation time of the alga around the entire system, and hence the residence time in the photostage. The pump may be mechanical or take the form of an airlift (this is discussed in further detail below). In the case of an airlift, culture aeration and fluid circulation are achieved using the same mechanism. When a mechanical pump is employed, air (which may be supplemented with CO₂) must be injected separately to the pumping, normally at a point between the pump and the photostage (Figure 1.3).

Irrespective of the pumping method used, as the culture passes through the system it is enriched with oxygen from photosynthesis, this is then vented out of the system through a filter in the reservoir/degasser. The reservoir/degasser (and riser in airlift design) allows gas transfer, preventing the build up of toxic gases within the

culture (Chisti 1989, Borowitzka 1994). All gases in and out of the system are passed through 0.2µm microbial filters to maintain the sterility of the system. Sterilisation of the system is generally carried out with a chemical sterilising agent such as sodium hypochlorite. Effective sterilisation is vital as, in the production of high value products such as fatty acids and pigments it is important the product is prevented from contamination (García Camacho *et al* 1999).

The potential utilisation of tubular photobioreactors for the mass culture of a number of algal species has been assessed. Pirt *et al* (1983) constructed a tubular loop photobioreactor made of 52m of glass tubing of 1cm bore. The structure had a footprint of only 0.5m², and was developed for the production of algal biomass. The system had a number of advantages over pond culture, due to its small footprint it could be installed in any space and did not require flat land, it produced a higher concentration of biomass than pond cultures and minimised contamination. It also allowed complete control of CO₂ and O₂ mass transfer rates.

As with all microalgal culture systems the tubular photobioreactor has its limitations. The biggest problem in the design of the tubular photobioreactor is that the photostage is constructed of long lengths of tubing, which allows efficient photosynthesis, resulting in the uptake of CO₂ and the evolution of O₂. Therefore as the culture passes through the tubes there is a net build up of O₂ and a depletion of CO₂. This can result in photoinhibition and consequently death of the culture. This was the experience of a commercial production unit that was set up in Cartagena, Spain (Sánchez Mirón *et al* 1999). The tubes were several kilometres long, which resulted in oxygen accumulation in the culture; this was thought to be one of the reasons why the plant failed. It is therefore important that in the design of a photobioreactor a balance is

achieved which gives the system a maximal volume but has a minimal length of tubing so there can be no detrimental O₂ build-up.

- **Pumping systems for tubular photobioreactors**

In deciding on the pumping system for a tubular photobioreactor the main factor for consideration is the fragility of the alga to be cultured, as shear stresses imposed on the cell walls can provoke irreversible damage and limit, inhibit or totally prevent growth (Gudin and Thépenier 1986). Gudin and Chaumont (1991) found the best pump, in terms of reducing stress of the culture, to be the Archimedes screw pump with respect to *P. cruentum*, *D. salina* and *H. pluvialis*. They also found that volumetric pumps, which have a rotor revolution speed proportional to the culture flow rate, are less damaging than centrifugal pumps. This is because the centrifugal pump has a high rotation speed and a long retention time of the culture inside the pump.

The major factor in determining how delicate an alga is, is the presence or absence of a cell wall. For example, *D. salina* is a wall-less species that is very shear-sensitive. In the case of *H. pluvialis* it has two distinct life cycle phases, during the exponential phase of growth the cells are ovoid and green with two flagella. Gudin and Chaumont (1991) estimated the motility of the flagella to be 20µm/s. The flow rates achieved in photobioreactors is usually in the order of m/s, so it can be appreciated that this flow rate would put a high shear force on the algal flagella. The point where the flagella attach to the cell is slender and the polysaccharide envelope, which protects the cell, is thinner at this point, rendering it weak. If this area is eroded and the cells lose their flagella they stop growing and do not encyst, thus reducing the biomass yield of the culture. Encysted cells of this alga are, in contrast, much more resistant to shear.

When considering the pumping system for a tubular photobioreactor costs and scalability must also be taken into account. The airlift pump exhibits low uniform shear, has a low power input and has no moving parts, making it an ideal choice for microalgal culture, it also serves to provide a continuous supply of CO₂ (Chisti and Moo-Young 1987, Merchuck and Siegel 1988). However, the need for a high head pressure in order to achieve sufficient fluid flow in the reactor means that they are relatively costly to construct (Chisti 1989). The requirement for a tall airlift and downcomer may also limit the construction of very large volume systems.

1.1.2.4. Other mass culture systems

Other photobioreactors have been shown to be successful at laboratory scale for the culture of microalgae for specific, commercially-valuable, products. Immobilised cell photobioreactors act by holding the algal cells in a matrix, for example in calcium alginate beads or polyurethane foam (Gudin and Thépenier 1986, Ding and Lee 1994). This matrix is then placed into a suitable photobioreactor such as a fluidised or fixed bed and media is washed through it. This type of system is limited by the fact that the product of interest must be a substance than is excreted from the algal cells, and can be washed from the matrix, leaving the cells behind to continue production. It is therefore of little use in the production of products such as astaxanthin from *Haematococcus* as the product is accumulated within the cell itself. However work with this alga by Ding and Lee (1994) did find that although the entrapment process led to a long lag phase, once growth began the alga had a higher growth rate than when grown in free culture. Also the entrapment provided the cells with a stable microenvironment, which enabled growth to occur at a higher temperature. However they found that the alginate beads had a low stability which did not permit long-term culture.

Work has also been carried out on the development of illuminated fermentation systems in the hope that the already existing technology of fermentation vessels could be adapted to the production of photosynthetic organisms (Ogbonna *et al* 1996). Also under development are plate-type photobioreactors, which are of particular interest in the development of a system for hydrogen sulphide removal (Kim *et al* 1996) and for hydrogen production (El-Shishtawy *et al* 1997).

One of the more popular systems currently in use is the so-called 'polybag' or 'Milford' system. These systems are of a very basic design and therefore are easy to set up, require very little equipment or skilled labour, and are cheap to run (Martínez-Jerónimo and Espinosa-Chávez 1994). However they are not closed and therefore are prone to culture crashes due to contamination. Their design precludes little if any control of the culture or its environment, leading to relatively poor yields when compared to more developed intensive mass culture systems. Their design also makes scale up difficult, making production of anything more than a few hundred litres in a single system almost impossible.

1.1.2.5. Harvesting

In the development of an algal mass culture process, the most costly and technically difficult stage is the harvesting for the cells. Harvesting of algal cells is difficult because of their small size, which is typically only 5-20 μm in diameter. The problem is further complicated by their low concentration, with phototrophically grown algal culture achieving no more than, at the very best 5g/l and the fact that the cells tend to have a density that is close to that of water (Gudin and Thépenier 1986).

The methods traditionally used in the harvesting of algal biomass are

- Centrifugation
- Flocculation
- Autoflotation
- Vacuum filtration
- Ultrafiltration

Centrifugation is a reliable method of harvesting but is expensive both in terms of the initial lay out for equipment and also in running costs in terms of the energy requirement (Vonshak 1993). Flocculation can be successful with some algae by using either an inorganic flocculent such as lime, calcium chloride or ferric chloride or by using a organic method such as chitosan (Gudin and Thépenier 1986). However this causes the pollution of the harvested algae with the flocculent which may be unacceptable in terms of the algal products final use (Vonshak 1993). Autoflotation acts by causing the cells to aggregate on the surface of the holding tank, from where they can be skimmed off. This can be induced using air, CO₂ or micro-bubbles produced by electrolysis. Filtration is a reliable method of harvesting with equipment for methods such as tangential flow filtration readily available, however, like centrifugation, is also very expensive. With all the methods noted here care must be taken to ensure that the process itself does not damage the cells, resulting in the reduction in the value of the algal biomass or a loss of product.

1.2. *Haematococcus pluvialis*

Species and strains from the genus *Haematococcus* (Chlorophyta) have been investigated for a number of years as a possible source of the carotenoid astaxanthin (3,3'-dihydroxy- β , β -carotene-4,4'-dione). Astaxanthin is used commercially for the pigmentation of fish including salmonids and crustaceans (Spencer 1989). Currently the majority of astaxanthin used in aquaculture is synthetic. In recent years however, there has been a steady development in algal biotechnology with large-scale systems for the production of algae such as *H. pluvialis* becoming a reality. Two companies Cyanotech Corp. and Aquaseach Inc. are currently using pond culture and, in the case of Aquasearch Inc., photobioreactors to commercially produce *Haematococcus* (Lorenz and Cysewski 2000, Olaizola 2000). However pond culture has previously been shown to have little or no quality control, with the cultures prone to crashes due to protozoan predation (Bubrick 1991).

1.2.1. Life cycle

H. pluvialis (also known as *H. lacustris* Girod. Rostafinski) was first described in detail by Flotow in 1844 (Elliott 1934). At the turn of the last century *H. pluvialis* was also known as *Sphaerella lacustris* and was observed to be common in marble urns and shallow pools (Peebles 1909). Under these conditions the alga was found in its 'resting state' as a large red sphere, it was only in laboratory experiments by Hazen (1899) and others that the other stages of the life cycle became apparent.

Initial work by Hazen (1899) identified that the resting cell was formed by conditions termed 'unfavourable', but when the resting cells were transferred to favourable conditions the resting cells formed 'megazoids' which were motile. The

formation of microzooids was also observed, which Hazen believed to be gametes, though conjugation was not actually observed. Work by Wollenweber in 1908 concluded that there was no conjugation, that *H. pluvialis* multiplied asexually (Peebles 1909). It was Wollenweber who first named the resting cyst 'the aplanospore'.

Figure 1.4 shows a representation of the diagram produced by Elliot (1934) to demonstrate the life cycle of *H. pluvialis*. The life cycle of *Haematococcus* has two distinct phases. In vegetative growth the cells are green motile (zooid) cells (Elliot 1934). As the culture ages and becomes nutrient-limited, the cells form resting cysts with a sporopollenin-like cell wall (Burczyk 1987, Margalith 1999, Boussiba 2000). These cysts accumulate astaxanthin.

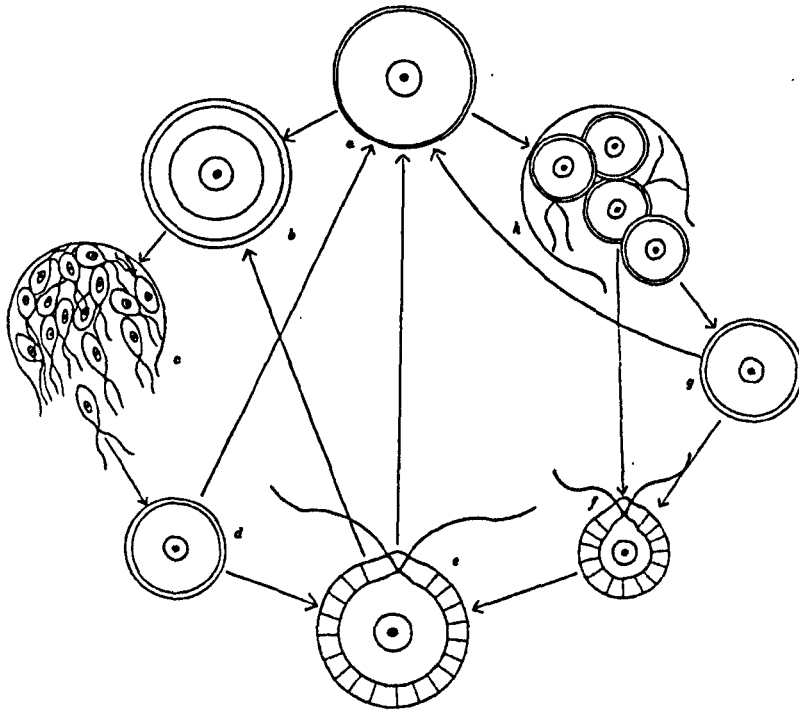


Figure 1.4. Life cycle of *Haematococcus pluvialis* (taken from Elliott 1934).

- a. palmella, b. aplanospore, c. microzooid, d. young palmella, e. macrozooid, f. young macrozooid, g. young palmella, h. palmella.

When conditions become favourable, for example with the addition of a nitrogen source, the aplanospores lose their heavy cyst walls and divide to produce daughter cells, which swim about inside the old cell membrane. These cells are macrozooids and appear red due to the astaxanthin they contain. The cell membrane then ruptures and the cells swim out into the media. The astaxanthin concentration slowly diminishes over the next 24-48hrs until the macrozooids appear green due to chlorophyll synthesis. The green swimming macrozooids continue to divide, increasing the population until the culture again becomes nutrient-limited. At this point the number of palmella cells in the population begins to increase, and as the culture ages aplanospores are produced. At this time the number of cells in the population decreases as not every macrozoid becomes an aplanospore. The reason for this is not clear. Microzoid cells are not typically found in a population. They only develop from the aplanospore (or heamatocyst) under certain conditions (Hazen 1899, Elliott 1934). They are cylindrical in shape and are $\leq 20\mu\text{m}$ in diameter, and, as such, differ from the macrozooids, which are 8-50 μm in diameter and pear shaped, although older forms may become spherical. Elliot (1934) also observed that the microzooids swim more actively than the macrozooids.

1.2.2. Cultivation

Determination of the optimum conditions for *Haematococcus* production is two-fold due to the complex cell cycle. Optimal conditions for biomass production are different to the conditions required for maximal astaxanthin accumulation. This section focuses on the conditions required to support biomass production. Astaxanthin production shall be discussed in section 1.2.3.

1.2.2.1. Nitrogen source

Extensive research on the nutritional requirements of *H. pluvialis* Flotow was carried out by Droop (1961). Droop determined that the alga was autotrophic with respect to nitrogen and carbon in the light. He also concluded that the alga had no requirement for vitamins and was able to utilise certain simple organic compounds as a nitrogen source, including urea, uric acid, guanine and tryptophane. *Haematococcus* is commonly found in temporary pools and in guttering, places that are likely to be enriched with avian guano; which may explain the alga's ability to utilise these organic nitrogen sources. The alga is also able to utilise nitrate and ammonia. However Droop (1961) did note that there were inter-strain differences, and not all strains were capable of utilising all the afore mentioned nitrogen sources.

Borowitzka *et al* (1991) found the best performance in terms of growth rate to be when the cells were grown on urea. Both ammonium chloride and nitrate resulted in slightly slower growth. Harker *et al* (1995) using response surface methodology demonstrated that the best nitrogen source was either urea or sodium nitrate. Gong and Chen (1997) concluded that the best nitrogen source was potassium nitrate. A comparison between nitrate, urea and ammonia carried out by Barberà *et al* (1993) found growth was unaffected by the nitrogen source. Based on information from the literature urea seems to be the best nitrogen source for growth. For commercial cultivation of the alga, other factors including cost, availability and ease of use should be considered.

1.2.2.2. Temperature

H. pluvialis is commonly found in cold climates such as the rocky shores of Scandinavian lakes (Pringsheim 1966). It therefore follows that it will have a low

temperature optimum. Borowitzka *et al* (1991) demonstrated the optimum to be 15°C though the cells were capable of growing up to a temperature of 25°C. Growth became strongly inhibited above 28°C, and at 35°C the culture died. This is supported by Harker *et al* (1995) who determined that the optimum temperature of *H. pluvialis* was 14-15°C. However, Fan *et al* (1994) found that their strain of *H. pluvialis* had an optimum temperature of between 25 and 28°C. Cell division was totally inhibited at 33°C. The differences in reported optimal temperatures for *H. pluvialis* is likely to be due to inter-strain differences.

1.2.2.3. Light

The light requirement of *H. pluvialis* is much lower for optimal growth than for astaxanthin synthesis. Fan *et al* (1994) reported the saturated irradiance (photon flux density; PFD) for growth was 90 $\mu\text{mol}/\text{m}^2/\text{s}$. Boussiba *et al* (1992) found that the optimal irradiance for growth was 85 $\mu\text{mol}/\text{m}^2/\text{s}$. Harker *et al* (1995) determined the optimum photo flux density for growth to be lower at 50-60 $\mu\text{mol}/\text{m}^2/\text{s}$. High irradiances are inhibitory for growth; Boussiba and Vonshak (1991) found that by increasing the culture irradiance from 85 to 170 $\mu\text{mol}/\text{m}^2/\text{s}$ growth was reduced (combined with reduced concentration of nitrate).

1.2.2.4. Organic carbon source

Haematococcus is able to grow on an organic carbon source both in the light and dark. Mixotrophic growth on acetate has been shown to improve the growth rate and the final cell yield, as well as stimulating the formation of astaxanthin (Borowitzka *et al* 1991). Chen *et al* (1997) found that the alga grew well on acetate, performing

better in mixotrophic than heterotrophic culture. Barberà *et al* (1993) identified that the algal growth rate was significantly enhanced by acetate as long as the light level was non-saturating. Moya *et al* (1997) noted that in heterotrophic conditions, acetate levels above 2.5g/dm³ inhibited growth, whereas in mixotrophic conditions up to 5g/dm³ enhanced growth as long as the irradiance was below 33 $\mu\text{mol/m}^2/\text{s}$.

1.2.2.5. pH

No published information is available concerning the optimal pH level for *Haematococcus*. McLachlan and Craigie (1965) did follow the growth and pH of *H. pluvialis* in unbuffered media and found that growth became depressed when the pH fell below 4.5. Most investigations, where a pH is given, quote a pH of 7-8 (i.e. Ding *et al* 1994, Harker *et al* 1996b, Boussiba 2000)

1.2.2.6. Salinity

Haematococcus is a freshwater microalgae and can not grow above a salinity of 1% (w/v) NaCl (Borowitzka *et al* 1991). This is one factor that makes this alga unfavourable for cultivation, as unlike *Dunaliella*, which is adapted to growth at a very high salinity, there is not the presence of salt to deter contaminating organisms. NaCl can be used to stimulate astaxanthin synthesis (Harker *et al* 1996b).

1.2.3. Accumulation of secondary carotenoids

Haematococcus was first identified in its astaxanthin-rich aplanospore stage. However it was not until 1944 that Tischer identified the red pigment as astaxanthin (Droop 1954). Conditions that trigger this accumulation of secondary carotenoids in the alga *Haematococcus* differ from the optimal conditions for growth. As discussed by

Droop (1954) work at the turn of the last century by Reichnow, Pringsheim, and Chodat and Mayer all identified that the two most important factors responsible for carotenogenesis were nitrogen deficiency and exposure to high irradiance.

1.2.3.1. Nitrogen and Phosphate

It is commonly accepted that the major trigger for carotenogenesis is a depletion of the nitrogen source of the culture. Transferring actively dividing cells into nitrogen-deficient media Zlotnik *et al* (1993) observed that aplanospores were rapidly formed. They observed a series of morphological changes to the culture: in the first week the cells were green and motile; the cells then began to encyst, losing their flagella and becoming spherical while increasing in diameter so that after four weeks they had doubled in size.

Harker *et al* (1996a) showed that by varying the nitrate concentration of a culture the cells could be manipulated both in terms of growth and astaxanthin formation. They also demonstrated that phosphate-limitation has a similar effect. Borowitzka *et al* (1991) found that ammonium chloride produced good growth but inhibited carotenogenesis. They also determined that although *H. pluvialis* grows very well on urea, carotenogenesis was slightly inhibited in cultures grown on urea when compared to cultures grown on a nitrate source.

It has been shown that nitrogen deficiency has a greater effect than high irradiance due to a very pronounced effect on blocking cell division (Fábregas *et al* 1998). Lee and Soh (1991) believed that the rate of astaxanthin accumulation in a culture was dependent on the nitrogen status of the original culture medium. However work by Boussiba and Vonshak (1991) has shown that astaxanthin accumulation in *H. pluvialis* has a requirement for nitrogen, in their experiments cells cultured under high

irradiance and high nitrogen produced a higher astaxanthin content than cells grown under high light and a lower nitrogen concentration. However they also noted that the rate of carotenoid accumulation was higher in the lower nitrogen media. They also found that cells that were phosphate-starved accumulated a high amount of astaxanthin. Maximal levels were obtained in cultures that also contained a high level of nitrogen.

1.2.3.2. Light

Exposure to high irradiance is seen as being synergistic with nitrogen limitation in causing rapid secondary carotenogenesis in *Haematococcus* (Fábregas *et al* 1998). The rate of carotenoid synthesis in a culture increases with an increase in irradiance. Kobayashi *et al* (1992) demonstrated that the carotenoid content of *H. pluvialis* could be correlated proportionally to the irradiance that the culture was exposed to. They also noted that the spectral quality of light had an effect on carotenoid formation, with carotenogenesis occurring more efficiently under blue rather than red light. Work investigating growth outdoors has shown that the carotenoid content of a culture changes over the course of the daily light/dark cycle (Maillard *et al* 1994, Chaumont and Thépenier 1995). Also continuous illumination of the culture is more effective for carotenoid formation than a 12/12 dark/light cycle (Kobayashi *et al* 1992).

Ding *et al* (1994) observed that the response of cells to high irradiance can be rapid. They noted that in the early stationary phase the cells began to accumulate astaxanthin in the central region of the cell. When these cells were exposed to a higher irradiance this centrally located area of carotenoid spread towards the periphery of the cell within only 20-60 minutes. When the illumination was switched off the astaxanthin migrated back to the centre of the cell within 1-2hours. During this time they noted no synthesis or degradation of pigments. Lee and Soh (1991) demonstrated in continuous

culture that the specific rate of astaxanthin accumulation was a function of the photon flux density that the cells were exposed to, not the absorbed light energy *per se*. They concluded that the rate of astaxanthin accumulation is determined by the PFD, but the cellular astaxanthin content is actually determined by the nitrogen status of the culture. Furthermore in non-growing cultures the rate of astaxanthin accumulation is determined by the nitrogen status of the original culture medium, though the actual capacity of the cells to accumulate astaxanthin is genetic.

1.2.3.3. Temperature

Exposure to high temperature induces a stress response in *H. pluvialis* bringing about carotenoid synthesis. When the alga was cultivated at 30°C, astaxanthin production was three times higher than at 20°C, although the higher temperature also resulted in a high cell mortality (Tjahjono *et al* 1994). Borowizka *et al* (1991) also determined that at temperatures above 28°C carotenogenesis and cell mortality increased. This has important implications for commercial productivity in terms of the necessity and cost effectiveness of cooling systems.

1.2.3.4. Organic carbon source

The addition of acetate (e.g. as sodium acetate) to a culture has been shown to induce carotenogenesis. As early as 1939, Chodat proposed that this was because of the change in the ratio of carbon:nitrogen within the culture (Droop 1954). Kakizono *et al* (1992) suggested that under a high ratio of carbon:nitrogen, despite vigorous acetate consumption, the algal cells reduced their nitrogen uptake. Donkin (1976) reported that during encystment (accompanied by carotenogenesis) that demand for carbon is greater than during vegetative growth. From this he concluded that synthesis *de novo* from an

exogenous carbon source (e.g. CO₂) is fundamental in the production of secondary carotenoids.

The effect of acetate on *Haematococcus* is such that it will induce carotenoid synthesis even in the dark (Droop 1954), although synthesis is at a much-reduced rate (Harker *et al* 1996a).

The addition of acetate in combination with iron to a culture has been shown to affect carotenogenesis (Kobayashi *et al* 1991, Harker *et al* 1996a). Although Kobayashi *et al* (1991) found it to be stimulatory to both growth and astaxanthin formation, Harker *et al* (1996a) found that iron only exerted an effect at relatively high concentrations and at the highest concentration used, growth was inhibited. This may be linked to an observation by Droop (1955) that a deficiency in iron inhibited encystment, whereas high iron concentrations seemed to encourage encystment.

1.2.3.5. Salinity

Droop (1955) reasoned that as encystment was caused by a deficiency then any agent that inhibited growth would induce encystment. Metals such as lead, copper and zinc failed to promote encystment. In general, the cells were either unaffected or killed depending on the concentration used. Following from this he observed that 0.4% (w/v) NaCl stopped cell division and caused a death rate of 6% per day after addition. Importantly this was coupled with the encystment of the culture. The response was concentration dependent: greater than 0.6% (w/v) NaCl resulted in high cell mortality and less than 0.3% (w/v) NaCl the cells were unaffected. These findings are supported by more recent work. Harker (1995) found that NaCl induced carotenogenesis in *Haematococcus* but also causes high cell mortality. The optimum level for encystment was determined to be 40mM NaCl. Other salts have been found to be more detrimental

to growth, Harker *et al* (1995) found that KCl caused high cell mortality even at low concentrations. Boussiba and Vonshak (1991) achieved astaxanthin accumulation in *H. pluvialis* with a irradiance of $170\mu\text{mol}/\text{m}^2/\text{s}$, phosphate starvation and salt stress, induced by 0.8% (w/v) NaCl. Droop (1955) determined that when cells were treated with NaCl they also required light for encystment (when the culture media did not contain acetate). In the presence of acetate encystment was achieved even in darkness.

Although nitrogen and phosphate deficiency, irradiance, temperature, organic carbon source and salinity all have an effect on carotenogenesis when treated separately they also act synergistically. For example, Tjahjono *et al* (1994) found astaxanthin synthesis was stimulated at 30°C and this was further enhanced by the addition of acetate to the media. They also found that high temperature combined with Fe^{2+} or H_2O_2 further increased the astaxanthin level when compared to high temperature alone, though the increase was not as great as obtained with the addition of acetate. Gong and Chen (1998) found that a high concentration of sodium acetate combined with a lower concentration of potassium nitrate could significantly enhance astaxanthin production. Kobayashi *et al* (1997) noted that astaxanthin accumulation was greater in the presence of Fe^{2+} combined with acetate rather than acetate alone.

As has been discussed above the two overriding factors in astaxanthin production are the level of irradiance and the nutrient concentration in the culture. Boussiba *et al* (1992) determined that under high irradiance the onset and rate of accumulation of astaxanthin was dependent on the nitrogen concentration of the media, with a higher concentration of nitrogen resulting in a delayed onset of accumulation. They also determined that the rate and onset of astaxanthin accumulation in phosphate-deprived cells was dependent on the irradiance. Fábregas *et al* (1998) concluded that

though nitrogen deficiency seemed to be the most important factor in triggering astaxanthin synthesis the use of both nitrogen deficiency and high irradiance was desirable for maximal productivity.

1.2.3.6. Role of astaxanthin in *Haematococcus*

It is accepted by a number of authors that astaxanthin is produced in response to high irradiance, probably acting as either a sunshade or passive filter (Yong and Lee 1991, Hagen *et al* 1993, Bidigare *et al* 1993). All photosynthetic organisms contain carotenoids within the thylakoid membrane; these play important photochemical roles. These carotenoids may therefore act to stabilise membrane systems and also to act as an oxidative reactions buffer (Hagen *et al* 1993). However, the astaxanthin accumulated by *Haematococcus* is accumulated outside the thylakoids, in the cytoplasm. It is therefore suggested that astaxanthin may simply act as a sunshade rather than being associated with photosynthesis. Secondary carotenoid accumulation is first seen surrounding the nucleus (Santos and Mesquita 1984). Yong and Lee (1991) demonstrated that under high irradiance secondary carotenoids can be seen dispersing to the periphery of the cell and moving back to the centre of the cell when light was discontinued. During this time they observed no quantitative or qualitative changes in the carotenoid profile. This behaviour was also noted by Hagen *et al* (1994) who believed it resulted in an increased shading of the cup-shaped chloroplast. Kobayashi and Okada (2000) exposed immature (astaxanthin-poor) and mature (astaxanthin-rich) cysts to UV-A and UV-B irradiation and found that the tolerance of mature cysts to UV-B radiation was 6 times higher than that of immature cysts. They determined that this indicated that astaxanthin functions as a protective agent against UV-B radiation.

However the action of astaxanthin as a photo-protectant has been questioned. The alga *D. salina* accumulates large amounts of β -carotene as a secondary carotenoid. However, β -carotene is accumulated in the inter-thylakoid spaces and can therefore fulfil the function of scavenging for oxygen radicals before they damage the photosynthetic apparatus. As *Haematococcus* accumulates astaxanthin in the cytoplasm, outside of the photosynthetic apparatus, it is of little use when oxygen radicals are generated during photosynthetic processes (Krishna and Mohanty 1998). Fan *et al* (1998) found that *Haematococcus* cells with a high astaxanthin content were as susceptible to stress brought about by high irradiance as green cells and that the addition of $^1\text{O}_2$ generators under non-inductive conditions resulted in the accumulation of astaxanthin. This process was reversed by the addition of a $^1\text{O}_2$ quencher. From these results they suggested that astaxanthin is in fact a result of the photo-protective process rather than the protection agent *per se*.

The suggestion that astaxanthin simply acts as a sunshade has also been questioned. Boussiba (2000) noted that the overlap between the absorption spectra of chlorophyll (Sonet peak) and astaxanthin is rather small and it is therefore difficult to imagine how astaxanthin could function as a filter to protect chlorophyll.

There is no definitive answer as to the nature of the function of astaxanthin in *Haematococcus*. It seems likely that there is a link with high irradiance, however it has also been suggested that it may simply act as storage compound during non-optimal growth conditions (Lee and Ding 1994). This, however, assumes that astaxanthin is only accumulated in non-dividing encysted cells.

Astaxanthin is found naturally in a number of forms. The hydroxy (OH) group on C3 and C'3 of the β -end group is a chiral centre and can have a different orientation when compared to the rest of the molecule (Figure 1.6). In the *R* configuration the hydroxy group points above the plane of the molecule, whereas in the *S* configuration it points below the plane. As astaxanthin has two hydroxy β -end groups it is capable of existing as *3S,3'S*; *3S,3'R* (meso form) and *3R,3'R* configurations.

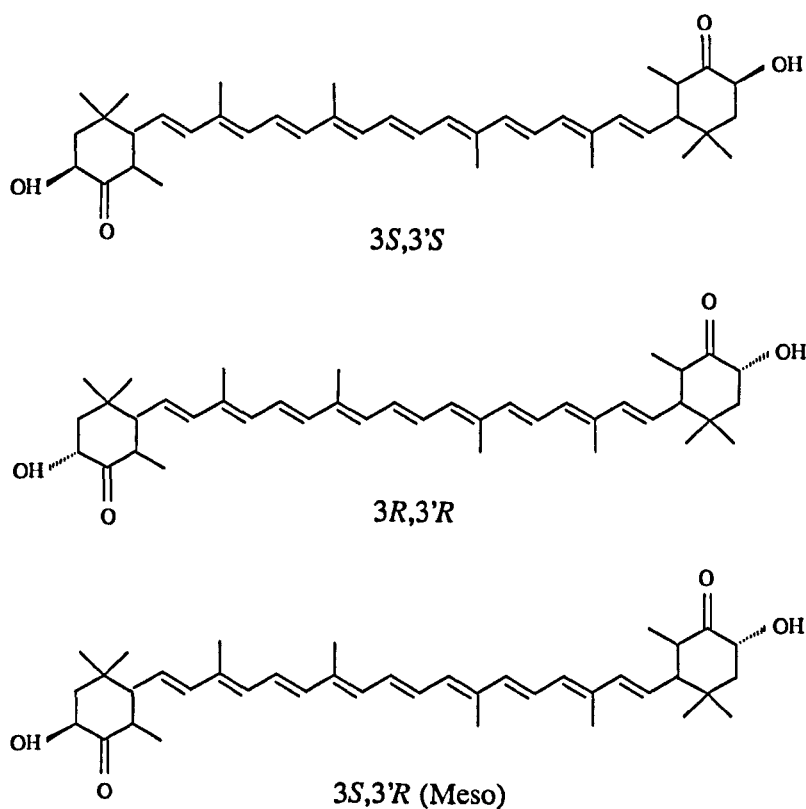


Figure 1.6. Chiral isomers of astaxanthin.

Astaxanthin can also exist in a number of geometrical forms termed *cis* and *trans*. The *cis* isomers have a bend in the polyene backbone, as shown in Figure 1.7. The *cis* isomer is named according to the carbon atom at which the bend occurs, for example Figure 1.7 shows 9-*cis*, 13-*cis* and 15-*cis* astaxanthin. In comparison the all-*trans* isomer is a linear molecule with no bend in the backbone.

Astaxanthin is often found in nature in its esterified form, with fatty acid moieties attached to the OH of the β -end groups. When a fatty acid molecule is attached to only one end group it is termed a monoester, a diester has fatty acids attached to both end groups.

1.3.2. Uses of astaxanthin

Carotenoids occur naturally in many foodstuffs such as carrots, tomatoes and peppers. They are also found naturally in animals such as lobsters and fish that are unable to synthesis carotenoids but rely instead on dietary intake via plants and plankton (Sinnott 1988). This has led to the commercial use of carotenoids by man for the pigmentation of many foods. This has included the use of β -carotene in margarine and ice cream; lutein and canthaxanthin in egg yolks and chickens; and red carotenoids (astaxanthin and canthaxanthin) in fish such as trout and salmon. The use of carotenoids has also been important in the colouring of pharmaceutical products and cosmetics (Bauernfeind 1981).

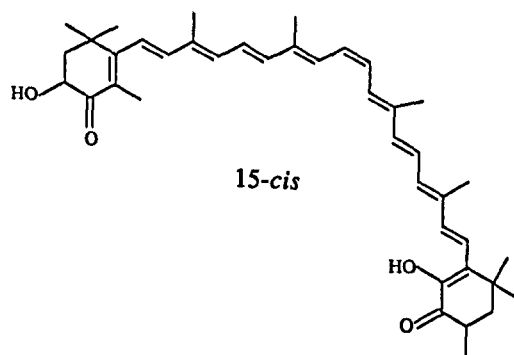
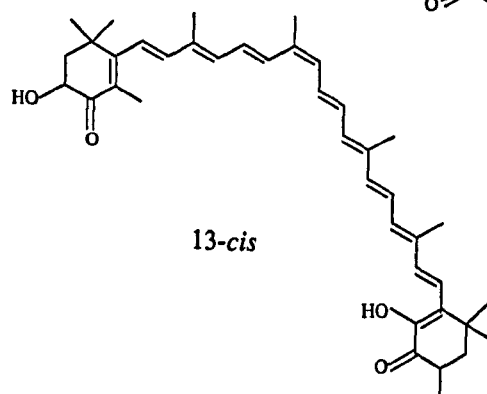
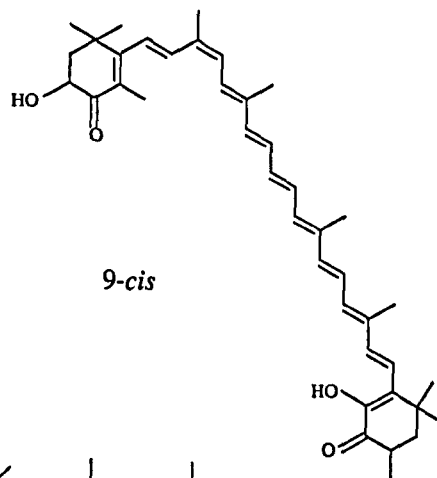
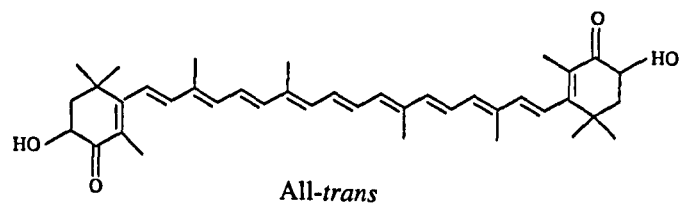


Figure 1.7. Geometric isomers of astaxanthin.

1.3.2.1. Aquaculture

Astaxanthin is widely used in the aquaculture industry for the pigmentation of salmonids. Wild fish have pink flesh due to the ingestion of carotenoids in their diet. Farmed fish are therefore given a diet containing a carotenoid supplement. Both canthaxanthin and astaxanthin are effective pigmentors, however feed studies have shown that astaxanthin is a superior pigmentor as it has a pinker tint and appears to be more efficiently absorbed and deposited in the flesh (Torrissen *et al* 1989). Astaxanthin has therefore become the preferred pigment despite the fact it is more expensive. Indeed it is one of the most expensive components of salmon feeds, comprising 10-15% of the total feed costs (Torrissen *et al* 1989).

Carotenoids are also used as a colouring agent for the skin of ornamental fish such as Koi carp. Again astaxanthin has been found to be particularly effective in this context (Sinnott 1988).

1.3.2.2. Poultry

Astaxanthin, as well as canthaxanthin and especially lutein, is used commercially for the pigmentation of poultry and eggs (Bauernfeind 1981).

1.3.2.3. Health

Astaxanthin is reported to be an effective anti-oxidant. Antioxidants are beneficial as they protect against excessive free radical formation, which would damage the cellular organelles and processes. Nishini (1998) found that lutein, zeaxanthin, lycopene, phytoene, fucoxanthin, peridinin and astaxanthin were all promising as cancer preventative agents. However in certain cases carotenoids may act as prooxidants, causing oxidative stress to the cell. Knowledge of the mechanism of

formation and action as a prooxidant is fragmented and there is a lack of direct evidence as to possible harmful effects (Palozza 1998).

Carotenoids have an important nutritional role in human health; for example β -carotene acts as a vitamin A precursor. They may also protect against cardiovascular and photosensitivity diseases, cataracts and age-related macular degeneration (Palozza 1998).

1.3.3. Sources of astaxanthin

1.3.3.1. Synthetic

Commercial production of synthetic carotenoids began in the early 1950's with the production of β -carotene by F. Hoffman La Roche (Basel, Switzerland). The production of synthetic carotenoids was essential for their industrial use, as natural sources could not produce the volume or consistency of supply that was necessary to make them commercially viable as pigments.

The synthesis and commercialisation of nature-identical β -carotene led to the synthesis of other carotenoids by F. Hoffman La Roche. The commercial production of these carotenoids led to four Carophyll® products.

1. **Carophyll® Yellow.** 10% (w/w) apocarotenoic acid ethyl ester for the pigmentation of broiler and egg yolks.
2. **Carophyll® Orange.** 5% (w/w) apocarotenoic acid ethyl ester plus 5% (w/w) canthaxanthin for the pigmentation of broiler and egg yolks.
3. **Carophyll® Red.** 10% (w/w) canthaxanthin for the pigmentation of broiler, egg yolks, fish and crustacean especially shrimps.

4. **Carophyll® Pink.** 8% (w/w) astaxanthin for the pigmentation of fish and crustacean especially shrimps.

These products are provided as free-flowing gelatine-coated beadlets with approximately 100,000 beadlets per gram. Irrespective of the degree of pigmentation the consumer always expects the colour to be consistent and the provision of the Carophyll® products as small spherical particles enables a good distribution of the carotenoids throughout the feed, ensuring equal pigmentation of all the animals. Another advantage of the synthetic product is that as a specific carotenoid is provided a specific colour is achieved in the flesh. The encapsulation of the carotenoid in gelatine beadlets means that the product is protected from oxidation, which lengthens its shelf life (Latscha, F. Hoffman La Roche).

Carophyll® Pink contains 8% astaxanthin, and has been produced by F. Hoffmann La Roche since 1984. The astaxanthin is in its free (non-esterified) form and is racemic containing the 3*S*,3'*S*, 3*S*,3'*R*, 3*R*,3'*R* isomers in a ratio of 1:2:1 (see section 1.3.1.). Free astaxanthin has been shown by some researchers to be a more efficient pigmentor of salmonids than esterified astaxanthin (Torrissen *et al* 1989, Storebakken *et al* 1987). It is proposed that the cleavage of the astaxanthin esters is a limiting step for pigmentation (Storebakken *et al* 1987) as only free astaxanthin is isolated from the flesh of salmonids. However a recent study by Bowen *et al* (2001) has demonstrated that esterified astaxanthin is an effective pigmentor of Rainbow trout.

BASF AG (Ludwigshafen, Germany) also produces synthetic astaxanthin, under the name Lucantin®Pink. Like Hoffmann La Roche they produce a range of carotenoids for use in the pigmentation of animals.

1. **Lucantin®Yellow.** 10% (w/w) C₃₀ ester used for egg yolk pigmentation.
2. **Lucantin®Red.** 10% (w/w) canthxanthin used for pigmentation of egg yolk, broiler skin and fish.
3. **Lucantin®Pink.** 10% (w/w) Astaxanthin used for fish pigmentation.
4. **Lucantin®CX forte.** 10% (w/w) citranaxanthin used for the pigmentation of egg yolks.
5. **Lucantin®10% feed.** 10% (w/w) β-Carotene used for fertility in farmed animals.

Lucantin®Pink is a free-flowing powder, which has a dark violet to aubergine coloured appearance. Again the particles are gelatine coated. It differs from Carophyll Pink® in that it contains 10% (w/w) astaxanthin rather than 8% (w/w). Little information is currently available as to the efficacy of this product.

1.3.3.2. Natural

Despite the advantages of the synthetic product there is an increased awareness amongst both farmers and the consumer about the use of synthetic chemicals in foodstuffs, despite the fact that many synthetic carotenoids are 'nature-identical'. In the case of astaxanthin, a number of sources have been identified for natural production

- **Algal sources**

As has already been discussed, *H. pluvialis* is an accumulator of large amounts of astaxanthin. This has been exploited commercially by a number of companies. It accumulates astaxanthin in the 3*S*,3*S* configuration, on analysis wild salmon contain

95% 3S,3'S (Schiedt and Leuenberger 1981), further confirming the natural properties of this source.

The Cyanotech Corporation (based in Hawaii, USA) use pond culture to produce their *Haematococcus* product Naturose™ (Lorenz and Cysewski 2000). This product is marketed for use in aquaculture feeds. They also market BioAstin™, which is a human dietary/antioxidant supplement rich in astaxanthin. Naturose™ is a spray-dried *Haematococcus* meal, which forms a fine dark red powder. It has been successfully used for the pigmentation of shrimp, rainbow trout, coho and Atlantic salmon, sea bream, poultry and various copepods (Naturose™ Technical Bulletin 6). Naturose™ has a variable carotenoid content normally providing between 15,000 (1.5% w/w) and 20,000 (2% w/w) ppm astaxanthin.

Aquasearch, also based in Hawaii, is currently in the process of launching a human health supplement rich in astaxanthin called AstaFactor™. Their production facility uses a combination of growth modules and ponds to culture astaxanthin-rich *Haematococcus* (Olaizola 2000).

- **Yeast**

The yeast *Xanthophyllomyces dendrorhous* (previous known as *Phaffia rhodozyma*) was first isolated in the 1970's from deciduous trees in Japan, Alaska and the USSR. The wild strain accumulates up to 500µg/g total carotenoid of which 40-95% is astaxanthin (Johnson and An 1991). *X. dendrorhous* has certain advantages over the alga *Haematococcus*, carotenoid accumulation is not light-dependent allowing the yeast to be grown in stirred-tank fermentors (i.e. there is no requirement for the costly development of novel culture systems). However, the yeast does require vigorous

aeration and when compared to other yeasts has a relatively slow growth rate, taking five days to reach a maximal biomass (Johnson and An 1991).

The main limitation in the commercialisation of astaxanthin from *X. dendrorhous* is the low carotenoid yield. Wild *X. dendrorhous* accumulates 500µg/g total carotenoid, in comparison *Haematococcus* can accumulate 2000-20,000 µg/g. Therefore, work focused on the isolation of *X. dendrorhous* mutants, and they have been developed to produce greater than 3,000µg/g (Johnson and Schroeder 1996). From this work Johnson and Schroeder (1996) have estimated that it is possible to achieve a total carotenoid concentration of 14,600 – 19,000 µg/g from *X. dendrorhous*.

Astaxanthin isolated from *X. dendrorhous* differs from other natural sources as it is free (not esterified) and mainly (92%) occurs as the 3*R*, 3'*R* configuration (Andrews and Starr 1976). This discovery led to a loss of interest in the development of *X. dendrorhous* as there was concern that, as the 3*R*, 3'*R* configuration is not normally found in nature, the Food and Drug Administration of the USA might reject it as unnatural. However subsequently both 3*R*, 3'*R* and 3*S*, 3'*S* optical isomers were isolated from a number of natural sources and interest in *X. dendrorhous* once again increased (Johnson and An 1991). The fact that the astaxanthin is present in its free form is of interest as some researches believe that esterified astaxanthin is poorly absorbed by salmonids (Torrissen *et al* 1989, Storebakken *et al* 1997). However for commercialisation, the astaxanthin yield of the yeast would have to be greatly improved. This can be achieved using mutants though it is questionable as to whether consumer reception to astaxanthin produced from a mutant would be any better than their reception to 'nature-identical' synthetic astaxanthin.

Despite these problems Igene Biotechnology Inc. (USA) have marketed AstaXin®, which is an inactivated dried product of *X. dendrorhous*. As with the

synthetic products it is produced as a free-flowing granular powder. Incorporation rates into the feed are higher than for a synthetic product due to the reduced astaxanthin content per gram of material.

- **Plants sources**

The higher plant *Adonis annua* accumulates 3*S*, 3'*S* astaxanthin mainly as mono- and di-esters in its petals (Renstrøm *et al* 1981). However, the levels that are accumulated are very low in comparison to the algal and yeast sources and it is unlikely that this source could become commercial.

- **Crustacean waste**

Crustacean wastes, from shrimp and krill, have traditionally been used in countries like Norway for the pigmentation of trout and salmon. However the carotenoid content is low and this means that in order to achieve satisfactory pigmentation the addition of 10-25% (w/w) of the extract to the diet is required (Torrissen *et al* 1989). The large volume of material that must be added to the feed to achieve the required carotenoid content may have a detrimental effect on the fish. For example the wastes tend to be high in calcium and this may result in a manganese deficiency within the fish (Meyers and Routledge 1971).

The crustacean waste is also processed to produce commercially-available krill oil. Mori *et al* (1989) found that there was no difference between the pigmentation of coho salmon fed the krill oil, which contained astaxanthin diester in the 3*R*,3'*R* configuration, or synthetic racemic astaxanthin. The use of the krill oil avoids some of the problems associated with a bulk dry waste product. However the processing

required dramatically increases the production costs, possibly making the process uneconomic.

Despite the apparent success of using crustacean waste products in the pigmentation of salmonids, their use is still limited.

- **Microbial sources**

The bacteria *Mycobacterium lacticola* and *Brevibacterium* sp. have both been reported to contain astaxanthin but the levels detected were very low. Fungi of the genus *Peniophora* have also been reported to contain low levels of astaxanthin (Johnson and An 1991, Nelis and De Leenheer 1991).

Other bacteria that have been isolated that accumulate astaxanthin include *Agrobacterium aurantiacum* isolated from seawater (Yokoyama *et al* 1996), *Paracoccus marcusii*, which is a soil bacterium with a low astaxanthin content (Harker *et al* 1998), and *P. carotinifaciens* (Tsubokura *et al* 1999).

Despite the isolation of a number of bacteria, none appear to have been identified so far that contain a commercially viable level of astaxanthin. It has been suggested that to isolate further biological accumulators of astaxanthin attention need to be focused in extreme environments.

1.4. Aims and objectives of the research program

The main objectives of the research program were concerned with the mass cultivation of the fresh water microalga *Haematococcus pluvialis* for astaxanthin production. Specifically, the main aims were,

- The development and characterisation of a photobioreactor to support the mass cultivation of *H. pluvialis*.
- The evaluation of the performance of *H. pluvialis* in a photobioreactor in terms of growth and astaxanthin biosynthesis/accumulation.
- The evaluation of the use of alternative production strategies for algal biomass and astaxanthin production (e.g. batch versus continuous, indoors versus outdoors).

2. Materials and Methods

2.1. Phycological methods

Haematococcus pluvialis (Strain 34/7) was obtained from the Culture Collection of Algae and Protozoa (CCAP, UK).

2.1.1. Cultivation of *Haematococcus pluvialis*

H. pluvialis was cultured in Bolds Basal Medium (BBM) modified with urea (Appendix 1a; Tsavalos 1995). All chemicals were obtained from Merck unless otherwise stated. Cultures were maintained in 100 ml of modified BBM in 250ml flasks sterilised by autoclaving (15 min, 121°C). Inoculation of the flasks, with 10% (v/v) of an exponentially-growing culture, was carried out in a laminar flow cabinet using aseptic technique. The flasks were cultivated on an orbital shaker (Lab Shaker, Adolf Kuhner A.G., Birsfelden, Switzerland) at 110 rpm, an incident irradiance of 60 $\mu\text{mol}/\text{m}^2/\text{s}$ (continuous) and a temperature of 18°C.

Larger volumes of algae required for inoculation of the tubular photobioreactors were cultured in a five litre aspirator, as shown in Figure 2.1. After sterilisation by autoclaving the aspirator was inoculated with 200 ml of an exponentially growing culture which had been cultivated in shake flasks, as above. Air was filtered (0.2 μm Hepavent, Merck) in and out of the system. The culture was stirred using a magnetic stirrer and stirring bar. Samples were taken by opening the clamp to the sample bottle and closing the condenser line.

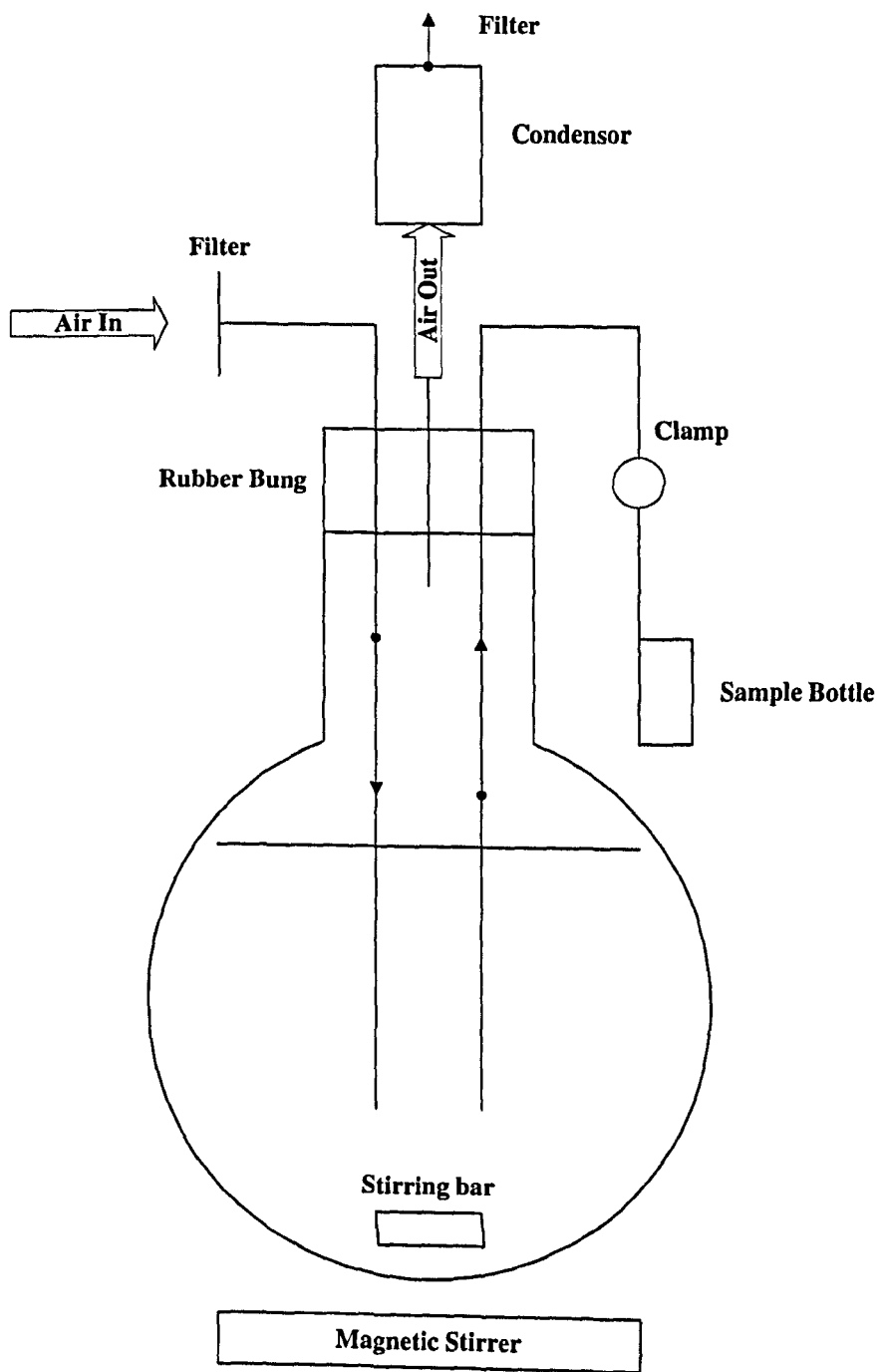


Figure 2.1. Schematic diagram of a five litre aspirator.

2.1.2. Analysis of growth

2.1.2.1. Cell count

The cell count of a *H.pluvialis* culture was determined using an Improved Neubauer Haemocytometer. The culture (1.0 ml) was fixed with 0.1 ml of 4% (v/v) formaldehyde. A small volume of the culture was pipetted onto the haemocytometer. Nine squares were counted at a magnification of 100 x. Four replicates were counted for all samples. To calculate the number of cells per ml, the following equation was used;

$$\text{Number of cells per ml} = \frac{(\text{number of cells counted})}{9} \times 1.1 \times 10^4 \quad \dots \text{eq2.1.}$$

2.1.2.2. Dry weight

A volume of culture (5.0 ml) was centrifuged (1,200g for 5 min), washed in distilled water and placed in a pre-weighed foil boat. The boats were then dried at 80°C to a constant weight (24 hours) and, after cooling in a desiccator, weighed.

2.1.2.3. Morphology and cell viability

The life cycle stage of the culture was noted at each sample point. The culture was identified to consist of zooid cells, palmella cells and/or aplanospores (Elliot 1934).

The viability of the cells was accessed using the Evans Blue staining method (Gaff and Okong'O-Ogola 1971, Crippen and Perrier 1974, Smith *et al* 1982).

2.1.2.4. Specific growth rate and doubling time

As *H. pluvialis* grows by cell division alone the specific growth rate and doubling time of the algal cultures was calculated using the typical methodology used for bacterial culture (Schlegel 1992). The division rate of the culture (v) was calculated as the gradient of a semi-log plot of cell density vs. time. The division rate of the culture was then used to calculate the specific growth rate (μ) and doubling time (t_d) as detailed in equations 2.2 and 2.3 below.

$$\mu = \ln 2 \times v \quad \dots eq.2.2$$

$$t_d = \frac{\ln 2}{\mu} \quad \dots eq.2.3$$

2.2. Pigment analysis

In addition to routine monitoring of the growth of each culture (see above), the chlorophyll *a* and *b* and total carotenoid contents of each algal culture was determined. During extraction and analysis care was taken to avoid exposure to light, oxygen, heat, acids and bases. Both chlorophyll and carotenoids are very susceptible to damage by these agents leading to the formation of artefacts.

2.2.1. Extraction of pigments

An aliquot of culture (typically 5 ml but 10ml for densities of less than 5×10^4 cells per ml) was washed with distilled water by centrifugation (1,200g) for 5 min and the supernatant discarded. The pellet was dissolved in the minimal volume of redistilled acetone and transferred to a bijou bottle two thirds full of glass beads (0.5cm diameter,

Merck). The bijou bottles were then homogenised (Bench Top Homogeniser, The Mickel Laboratory Engineering Co. Ltd., Gomshall, U.K.) for five minutes. The resulting sample was passed through a filter of absorbent cotton wool into a snap-top vial. The residue in the bijou bottle was rinsed using redistilled diethyl ether and also passed through the filter. The samples were then evaporated to dryness under oxygen-free nitrogen and, where necessary stored at -20°C.

2.2.2. UV/Vis spectroscopy

2.2.2.1. Procedure for green cultures

The concentration of chlorophyll *a* and *b*, and total carotenoids were calculated spectrophotometrically (Hewlett Packard 8453 spectrophotometer) by measuring the absorbance of the sample in acetone at 470, 661.6 and 644.8nm in a 1cm path length cuvette. The concentrations were determined using the equations shown below (Lichtenhaler 1987), where *X* = chlorophyll *a*, *Y* = chlorophyll *b* and *Z* = total carotenoid,

$$(11.24 \times A_{661.6}) - (2.04 \times A_{644.8}) = X \quad \dots eq2.4.$$

$$(20.13 \times A_{644.8}) - (4.19 \times A_{661.6}) = Y \quad \dots eq2.5.$$

$$[(1000 \times A_{470}) - (1.9 \times X) - (63.14 \times Y)] + 214 = Z \quad \dots eq2.6.$$

The samples were diluted to give an absorbance in the range 0.2 - 0.8. Values were corrected for the starting culture volume and any dilution factor to give the pigment concentration in µg/ml.

2.2.2.2. Procedure for carotenoid-rich cultures

In cultures of *H.pluvialis* that contained a high level of astaxanthin the method of calculation was slightly different. Although the equations shown above (eq 2.4.-2.6.) can be applied to such cultures (Tsavalos 1995) it is more accurate to use the extinction coefficient for astaxanthin. Initially in astaxanthin-rich cultures only the carotenoid concentration was calculated. Later, analysis involved the calculation of the astaxanthin concentration using the extinction coefficient and the calculation of the chlorophyll concentrations using equations 2.4. and 2.5. The pigment extract (prepared as above) was scanned to detect the absorbance at λ_{max} . The published extinction coefficient of astaxanthin was used ($A_{1cm}^{1\%} = 2100$ in hexane, Britton 1995) in order to determine the level of astaxanthin in the cells according to equation 2.7, where x = amount of carotenoid in g, A = absorbance, y = volume of sample (ml) and $A_{1cm}^{1\%}$ = extinction coefficient for astaxanthin.

$$x = \frac{A \times 1000 \times y}{A_{1cm}^{1\%} \times 100} \quad \dots eq2.7.$$

2.2.3. Thin layer chromatography

Thin layer chromatography (TLC) was used to determine the specific carotenoid composition of a culture. For analytical work, commercial plates were used (Silica gel 60 F₂₅₄, 0.25mm thickness, Merck). In preparative work plates were made in-house using silica gel (60G, Merck) as follows. Glass plates were prepared by washing and drying, followed by washing with acetone. The silica was thoroughly mixed with water (30g in 60ml of water) and then spread onto the plates at a thickness of 0.5mm. The

plates were placed into an oven at 130°C and allowed to dry for 2 hours. If they were not required immediately they were then stored in a cool, dry and dark place.

Prior to use the plates were pre-coated in a 2.5% (v/v) methanolic solution of citric acid and allowed to air dry. The acid coating prevented the tailing of astaxanthin on the silica (a common problem for carotenoids with a 3-hydroxy, 4-keto end group; A.Young, pers.comm.).

The pigment extract from the alga was prepared as described in section 2.2.1. The extract was applied (suspended in diethyl ether) near the base of the TLC plate using a drawn out glass pipette. The pigments were separated using a 3:7 mixture of acetone:hexane as the developing solvent. Typical R_f values of the main components are shown in Table 2.1.

Table 2.1. Typical R_f values of the main carotenoids of *H. pluvialis* in 3:7 acetone:hexane

Carotenoid	R_f
Lutein	0.31
Free astaxanthin	0.36
Astaxanthin monoesters	0.59
Adonirudin esters	0.68
Astaxanthin diesters	0.86
β -carotene	0.99

2.3. Physical characterisation of a novel tubular photobioreactor

Below is presented the methodology used in the development of the four stages of photobioreactor design. More detailed information of the design and development of the systems is given in Chapter 3. The four systems that were built were all of a coiled tubular design and from this point will be referred to as Tubular Photobioreactor 1 (TBR1), Tubular Photobioreactor 2 (TBR2) and Advanced Algal Production System 1 (AAPSTM1). The AAPSTM1 was further modified and is referred to as the Advanced Algal Production System 2 (AAPSTM2).

The total volume of each system was determined by calculation. For clarity each system was divided into four sections:

1. Header tank (resevoir)
2. Riser (including return manifold for AAPSTM)
3. Downcomer (including return manifold for AAPSTM)
4. Photostage (coil winding)

2.3.1. Flow characterisation

Flow characterisation of the photobioreactors was carried out with water as the density and viscosity of water and modified BBM are very similar (Tsavalos 1995). The flow rate in the photostage was measured using a particle with an equal density to water. The length of one coil in the photostage was measured and the time taken for the particle to travel around five coils of the winding was determined. This was repeated three times. The mean velocity of the particle in m/s was then calculated.

particle to travel around five coils of the winding was determined. This was repeated three times. The mean velocity of the particle in m/s was then calculated.

The bubble regime in the riser was also noted and characterised into one of four types (Hebrard *et al* 1996, see chapter 3 for further details),

- (i) perfect bubbly,
- (ii) imperfect bubbly,
- (iii) churn turbulent
- (iv) slug flow

This identified the airflow rate that gave a high fluid velocity in the winding without resulting in perturbed riser flow dynamics, i.e. churn turbulent. Perturbed riser flow dynamics would reduce the efficiency of mass transfer (Hebrard *et al* 1996).

2.3.1.1. Reynolds numbers

From the measurement of the winding flow rate Reynolds numbers were calculated using the following equation, where D = diameter of the tubing, v = liquid velocity (m/s), ρ = fluid density, and μ = fluid viscosity.

$$Re = D \cdot v \cdot \left(\frac{\rho}{\mu} \right) \quad \dots \text{eq2.8.}$$

The calculation of the Reynolds number can be used to determine whether the flow in the tubing is laminar or turbulent. There is some debate in the literature as to the definitive value at which point flow changes from laminar to turbulent. In this study the

figure of Carlozzi and Torzillo (1996) was used. A Reynolds number of less than 2680 is defined as laminar, whilst at values greater than 2680 flow is said to be turbulent.

2.3.2. Mass transfer

The mass transfer was measured using the dynamic method (Spriet and Botterman 1984, Hsium and Wu 1995, Gavrilesco and Tudose 1996), with a polarographic oxygen probe (Oxyprobe, Broadly James, Irvine, USA). The system was stripped of O₂ using N₂ via the sparger plate at the base of the riser. The N₂ was then switched off and ambient air applied at the selected air flow rate. The dissolved O₂ level (DO₂) was noted every 15 seconds until the system had saturated. Saturation was previously determined by filling the system with water and vigorously aerating it for at least 12 hours. The DO₂ was then determined. Mass transfer is affected by the temperature of the system (Vasquez and Heussler 1985) therefore all measurements were carried out in a temperature controlled room at 18°C.

The mass transfer coefficient (K_La) was calculated graphically, following the method of Gavrilesco and Tudose (1996). Correction of the time response of the probe was calculated following the method of Tribe *et al* (1995).

Each tubular photobioreactor can be considered to be a four-phase system in terms of mass transfer: (i) the riser, (ii) photostage (iii) downcomer and (iv) the header tank. The mass transfer of the system is dependent upon the mass transfer of the limiting section. As the riser is the aerated section of the system this would be expected to have the highest mass transfer. It is impossible to say, without testing, whether the winding or the header tank has the lower mass transfer. However the engineering of the system means that, the only port for the dissolved oxygen probe is situated in the

header tank. Therefore the mass transfer of the header tank was assumed to be representative of the entire system.

2.3.3. Gas hold-up

The gas hold-up in the riser/header tank was determined using the volume expansion method (Chisti 1989). The height of the fluid in the system was measured when there were no air bubbles within the system (unaerated). The air supply was then applied to the system and the height of the aerated liquid was measured. The gas hold-up (ϵ) was determined using equation 2.9, where h_D = height of gas-liquid dispersion upon aeration, h_L = height of unaerated, static liquid,

$$\epsilon = \frac{h_D - h_L}{h_D} \quad \dots eq2.9.$$

2.4. Operating procedures for large scale culture of *H. pluvialis* in a novel tubular photobioreactor

The section details the sterilisation and inoculation procedure for each of the four tubular photobioreactors. It also details the physical and biological measurements taken during the growth of *H. pluvialis* in the four systems.

2.4.1. Sterilisation of the system for batch operation

2.4.1.1. Tubular photobioreactors, version 1 and 2

Both the TBR1 and 2 were sterilised using the same procedure. Each system was connected to the air pump (Compton D2670, Pye Unicam Ltd, Ashborne, UK) by silicone tubing with an in-line air filter (0.2 μ m, Hepacap 36, Whatman) which had previously been autoclaved and dried for 12 hours at 30°C. During the sterilisation procedure the air-pump was left running continually. The airflow was controlled using a flow meter in-line (Caché Instruments, Wakefield, UK) between the compressor and the air filter.

The system was filled with tap water via the header tank and the concentrated sodium hypochlorite (12% available Cl⁻) was added to give a final concentration of 0.25% (v/v). This was the concentration determined by experiment (data not shown) required to kill bacteria in the system. The system was then allowed to circulate for two hours. Immediately after the system had been filled with the sterilising solution the air vent (0.2 μ m, Hepacap 75, Whatman) was connected to the header tank port. After 30 minutes of circulation the sample line was rinsed with 0.25% (v/v) sodium hypochlorite by drawing it through from the system using a syringe (Plastipak 60, Merck).

After two hours of circulation the system was slowly drained then filled with filter-sterilised tap water. All rinse water was sterilised in-line, using a Sartobran-P filter (0.2 μ m pore size 0.2m² area, Satorius, Goettingen, Germany). The photostage did not drain completely therefore each batch of sterile rinse water was circulated for 15 minutes prior to draining to ensure that the sterilising solution that remained in the photostage was fully distributed through the system. The system has successive rinses until the pH of the sterile water was neutral. This usually involved rinsing the system three times.

After the final drainage of tap water the system was filled with filter-sterilised distilled water, which was circulated for 15 minutes and then drained. Finally the system was filled with modified BBM. Both of these additions were carried out using a pump (EM40 Mini Vac Pump, Simer Company, Sedalia, USA) via the in-line filter (0.2 μ m pore size 0.2m² area, Satorius, Goettingen, Germany).

2.4.1.2. Advanced Algal Production System (AAPS™)

The sterilisation of the AAPS™ followed the same principle as described above. The system was first filled with tap water and sodium hypochlorite was added to give a final concentration of 0.25% (v/v). However, the air filter used was changed to a Polyvent 40 (0.2 μ m, Whatman) which provided improved moisture resistance. The air pump was also changed to an air compressor (SYCIO-1, Bell W. Cossett, Ashbourne, UK). A non-return valve performed the task of air venting from the header tank. A second Polyvent 40 filter (0.2 μ m) was attached to the header tank to allow sterile air to vent into the system during draining. AAPS™₂ incorporated CO₂ addition for pH control using a West 6100 pH controller (Brighton Systems, Hove, UK). CO₂ (from a cylinder) entered the system through a port in the return manifold. Media was prevented from leaving via this port by a second non-return valve (T50P0008, R.S.). At the start of sterilisation the non-return valve was purged with sodium hypochlorite (0.25% v/v) before an autoclaved filter (0.2 μ m Polyvent 4, Whatman) was attached.

All draining and filling was performed via the drain taps at the base of the system. After sterilisation, the system was filled with modified BBM (sterilised in-line) using a water pump (Model EM40 Mini Vac Pump, Simer company, Sedalia, USA). Initially water and media filtration was carried out using the Sartobran-P filter capsule. However due to the low throughput capacity of these filters, the filter system was

changed to a three-filter assembly comprising of a 5, 1, and 0.2 μ m filters in series (Osmonics, Filerder, Maidstone, UK). Unlike the Sartobran filter, which was sterilised by autoclaving, the three-filter assembly was chemically sterilised using sodium hypochlorite (0.25% v/v). When these filters were used the sodium hypochlorite solution was pumped from a holding tank into the photobioreactor through the filter assembly.

2.4.2. Sterilisation of the system for continuous operation

Only the AAPS™2 was used in continuous operation. The header tank was configured with three additional ports for the addition of media and distilled water, and the overflow of product. Sterilisation of the system was carried out using the same principles as for batch operation. The three-filter assembly was attached to the system using silicone tubing. Each cartridge was filled with 500ppm of available chlorine (manufactures recommendation) as sodium hypochlorite and allowed to soak for 30 minutes. Meanwhile the system was filled with 1200ppm of available chlorine as sodium hypochlorite in tap water and allowed to circulate. This was done via the glove port in the centre of the header tank; the port was then sealed with a neoprene glove (Industrial type 12-H, Marigold, Swindon, UK) which was attached using a jubilee clip.

After the bleach solution had circulated for 15 minutes the airline was removed from the non-return valve at the base of the riser and the non-return valve was purged with sodium hypochlorite (0.25% v/v) before attaching an autoclaved Polyvent 40 (0.2 μ m, Merck) filter. The airline was then reattached so that air entered the system via the filter. For continuous operation the system had CO₂ addition for pH control. This non-return valve (T50P0008, R.S.) was also purged with sodium hypochlorite (0.25% v/v) before an autoclaved filter was attached (0.2 μ m Polyvent 4, Merck). The header

tank Polyvent 40 filter (0.2µm, Merck) was attached as was the nutrient feed tubing and filter, both of which had been sterilised by autoclaving. When attaching the tubing to any port the port was first rinsed in 70% (v/v) ethanol.

After the filter assembly had soaked in the sodium hypochlorite solution for 30 minutes the assembly was attached to the mains water supply and 5l of tap water was passed through them and into the system. The sodium hypochlorite solution was washed out from the filters and acted to sterilise the silicone tubing between the filters and the system. The excess sodium hypochlorite solution that had passed into the AAPS™2 was allowed to flow out via the product line which also acted to sterilise it. The sample line was purged using sodium hypochlorite (0.25% v/v).

The sterilising solution was circulated for two hours. Periodically during this time the glove in the header tank was used to circulate the sterilising solution around the header tank to ensure all areas were sterilised. The system was drained and filled with tap water that had been passed through the three sterile filters. This was then allowed to circulate for 15 minutes. During this time the sample line was purged and the glove was used to circulate the filtered water around the header tank to ensure it was rinsed properly. Filtered water (five litres) was then allowed to flow through the product line and the remaining rinsing solution was drained through the taps at the base of the system. This procedure was repeated three times or until the pH of the system was neutral. The system was then filled with filter-sterilised modified BBM and left for at least 12 hours to circulate before inoculation.

After each rinse, samples of the rinse water were taken aseptically and plated onto tryptone soya agar plates (Oxoid, Basingstoke, UK), then incubated at 18°C to test the sterility of the system.

2.4.2.1. Continuous feed

For continuous operation the water and a concentrated media stock (which made up the media feed) were fed into the system separately. Distilled water was fed in via the three-filter assembly that was used to sterilise the system (after sterilisation the filters were left attached to the system). At the start of the sterilisation process the nutrient feed line was attached to a port on the header tank. The nutrient feed line consisted of a two litre glass bottle (Phillip Harris), connected to a length of peristaltic tubing which in turn was connected to silicon tubing which had an in-line media filter (0.2 μ m, Polyvent AS, Whatman). When continuous culture began the concentrated media stock was fed into the system using a peristaltic pump (101U, Watson Marlow Ltd., Falmouth, UK). Distilled water was fed in through the filters using a dosing pump (Alldos, Eichler, Germany). See Chapter 5 for further details.

2.4.3. Inoculation of the system

2.4.3.1. Tubular photobioreactors, version 1 and 2

Each system was inoculated with five litres of an exponentially growing culture of *H. pluvialis*. In TBR1 the algae was introduced by pouring it into the system via the header tank. In TBR2 the algae was introduced into the header tank through autoclaved silicon tubing (5mm bore, Watson Marlow Ltd, Falmouth, UK) using a peristaltic pump (500 series, Watson Marlow Ltd, Falmouth, UK).

2.4.3.2. AAPS™ Version 1 and 2

Due to the increased volume of the system the AAPS™ was inoculated with nine litres of exponentially growing *H. pluvialis*. This was introduced into the header tank using the peristaltic pump via autoclaved silicone tubing (as above).

For batch operation, the silicone tubing was attached to a suitable port in the header tank, which at all other times was blanked off. For continuous culture inoculation was initially performed via a t-piece on the nutrient line. However the culture of *H. pluvialis* was not axenic, which caused subsequent problems with bacterial growth (see Chapter 3). Therefore the inoculation point was changed to a t-piece on the tubing connecting the header tank inlet filter (0.2µm, Polyvent 40, Merck) to the system.

2.4.4. Parameters for measurement

All large-scale cultures were sampled at regular intervals and analysed in terms of cell number, dry weight and pigment content (see section 2.1.2.). In some cultures the urea and phosphate concentrations of the media were determined. Physical measurements of irradiance levels and temperature were also taken.

2.4.4.1. Urea analysis

The urea content of the media was determined using the method of HACH (Camlab, Cambridge, UK). Each sample was first centrifuged to remove the biological material (1,200g, 5 min), the supernatant was then filtered through a 0.2µm filter (Whatman). The total nitrogen content of the sample was then determined using the

total nitrogen Test 'N Tube method (HACH). The urea content of the sample was then calculated.

2.4.4.2. Phosphate analysis

The phosphate content of the sample was determined using the reactive phosphorus (PhosVer 3) method of HACH, using a media sample that had been prepared by centrifuging and filtering in the same way as for the urea assay (see above).

2.4.4.3. Physical measurements

- **Tubular photobioreactors, version 1 and 2**

TBR1 and 2 were run with *in situ* pH monitoring. The pH probe (Broadly James, Irvine, USA) was calibrated before it was put into the system using buffers at pH 4, 7 and 10 (Merck).

- **AAPS™ 1 and 2**

AAPS™1 had no *in situ* monitoring. AAPS™2 had pH control and culture temperature measurement. The pH probe (Combination pH probe, Russell, Auchtermuchty, UK) was calibrated before sterilisation of the system using buffers at pH 4,7 and 10 (Merck).

Both versions of the AAPS™ were sited outdoors. AAPS™2 had light and air temperature monitoring using a LiCor LI-1400 datalogger (Glen Spectra Ltd, London, UK). Photosynthetically active radiation (PAR) was measured using the LiCor LI-190SA quantum sensor and the total solar irradiance was measured using the Licor LI-200SA. Ambient temperature was measured using the LiCor 1400-102 air temperature sensor. The AAPS™2 also had an internal temperature probe (YSI 100, Yellow Springs

Instrument Company, Ohio, USA) which was also wired into the LI-1400 datalogger through a terminal block. Measurements were taken every 15 minutes, 24 hours a day. The data was accessed and downloaded using the LI-1400-501 communications software through a PC.

2.5. Harvesting and storage

At the end of a run each culture was gravity-drained into a barrel and then concentrated into a slurry. Concentration was carried out using a tangential flow filter system (HPK12, Millipore, Bedford, U.K.) with 2x0.5ft² of 0.45µm pore filter (Pellicon, HVL P000C5, Millipore, Bedford, U.K.) connected to a sliding carbon vane pump (Procon XX81, 4V2 30, Millipore, Bedford, U.K.) set at an outlet pressure of 10-12 psi. Before concentration the system was circulated with 0.1M sodium hydroxide (Merck) for 15 min, then rinsed with distilled water. After concentration the slurry was rapidly frozen and stored at -20°C.

3. Development of a tubular photobioreactor

3.1. Introduction

During this study a tubular photobioreactor was developed to support the cultivation of *Haematococcus* spp. (and subsequent astaxanthin accumulation) in collaboration with Addavita Ltd. (Chesterfield, U.K.). Tubular photobioreactors have a number of design advantages over other algal growth systems (e.g. open-ponds and raceways), (i) they provide a high ratio of surface area (for illumination) to volume (Lee 1986), (ii) as a closed system they can be established and maintained as an axenic system, preventing contamination and predation from external organisms (Pirt *et al* 1983); and, (iii) they permit a high level of process control to be employed (Pirt *et al* 1983). The development of the photobioreactor was undertaken in four stages, with each stage introducing improvements in photobioreactor design and/or operation.

The four systems incorporated a helical coil of narrow-bore PVC tubing (forming the photostage) and an external loop airlift (to provide aeration, mixing and the circulation of the culture around the photobioreactor). A more detailed description of each photobioreactor is provided later in this chapter. The airlift system has a number of advantages over other pumping systems: (i) the power input that is required for a given liquid flow rate is lower for an airlift than for other pumping mechanisms (Chisti 1989); (ii) airlifts have high gas absorption efficiency and a short mixing time (Merchuk and Siegel 1988); (iii) they have a low shear when compared to other pumps (Merchuck 1990) and (iv) perhaps most importantly, extended aseptic operation is possible in an airlift reactor as the operation is non-mechanical with no moving parts (e.g. invasive stirrer shafts, seals or bearings) (Pirt *et al* 1983, Chisti 1989). However one limitation of the airlift system is that once it has been constructed the main physical

parameter that can be varied is the air-input flow rate. This determines both the liquid flow rate and the mixing efficiency of the system. Thus it is very important that attention is given to the design of the airlift system so that it meets the requirements to support the cultivation of the target organism.

There are two basic designs of airlift, namely the internal loop and external loop. The former is basically a simple bubble column with an internal baffle, which splits the column into a riser and downcomer. This design can take the form of a simple dividing bar or a cylinder (Figure 3.1a shows the cylinder design). The external loop has a riser and downcomer as two separate tubes, connected at the top and bottom by horizontal sections (Figure 3.1b). The systems used in this study all employed external loop systems.

The airlift driven photobioreactor is a multiphase system that consists of four inter-connected sections (Figure 3.2):

- A. Riser**
- B. Header tank (reservoir)**
- C. Downcomer**
- D. Photostage (winding)**

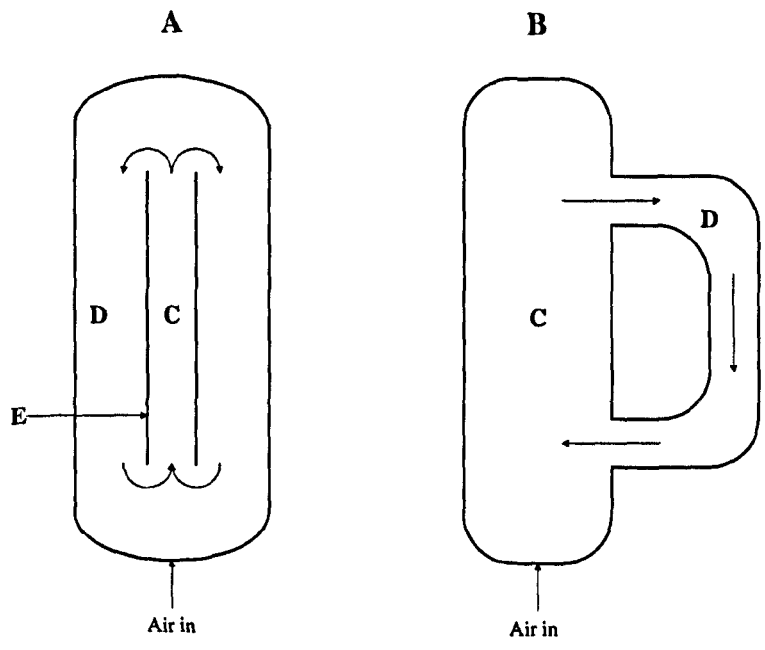


Figure 3.1. Schematic of an internal loop (concentric draft tube, A) and external loop airlift (B), C. Riser, D. Downcomer, E. Draft tube (the arrows show fluid direction).

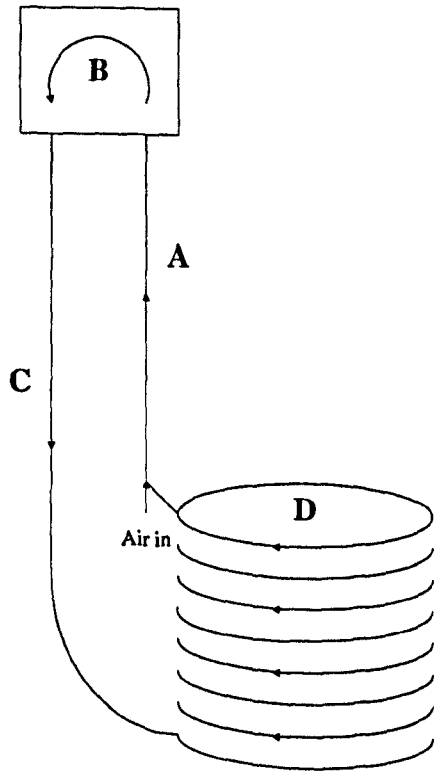


Figure 3.2. Schematic of idealised tubular photobioreactor (see text for details, the arrows show fluid direction).

The riser has gas injected into its base through a sparger, which in the case of the reactors developed in this study was a plate with a number of fixed diameter holes drilled in it. The air moves, as bubbles, through the riser and up into the header tank. The header tank has the function of gas separation and is connected to the riser and downcomer. The downcomer runs parallel to the riser, and in comparison with the riser it has a reduced gas hold-up. It is this difference in the bulk densities of the fluid in the riser and downcomer that causes the movement of liquid around the reactor (Chisti and Moo-Young 1987). The bottom of the downcomer and riser are connected, in the case of the reactors in this study, by the photostage (winding). This photostage holds the largest volume of culture, and acts as the major site for light transmission.

The four sections of an airlift are hydrodynamically different with different mixing, gas hold-up and mass transfer characteristics. Determination of these parameters for any system is essential so that there can be improved understanding of its operation and consequently improvements in design can be made. The mass transfer of a system is a measure of the rate of O₂ transfer from the gaseous stream into the liquid phase (Chisti 1989). The gas hold-up gives an indication of the fluid flow rate as well as impacting on the mass transfer of the system.

This hydrodynamic information is also essential for scale-up of any system. Ideally the mixing characteristics, gas hold-up and mass transfer should be calculated for each of the four sections separately.

3.1.1. Aims

The aims of this work were to characterise the four systems (configured for batch operation) in terms of,

- Fluid flow rate in the photostage
- Reynolds numbers in the photostage
- Gas hold-up of the riser/header tank.
- Mass transfer coefficient in the header tank

3.2. Tubular photobioreactor 1 (TBR1)

The system consisted of a coiled photostage with a tubing diameter of 22mm and a volume of 18 litres. The total working volume of the photobioreactor was 36 litres. Air was injected into the system at the base of the riser, which connected the top of the photostage to the header tank, entering at its side (Figure 3.3). The ratio of the downcomer to riser cross-sectional area was 0.25.

3.2.1. Physical characterisation

3.2.1.1. Flow characterisation

The photostage flow rate was measured at five airflow rates (2, 4, 6, 8 and 10 l/min) for a system working volume of 32, 34 and 36 litres. These three volumes correspond to three fill levels in the header tank. A working volume of 32 litres filled the system to just below the header tank side arm, a 36 litre system filled just above the side arm and the 34 litre system was level with the middle of the side arm entry (Figure 3.3). By characterising the system at the three working volumes the optimum operating parameters could be realised.

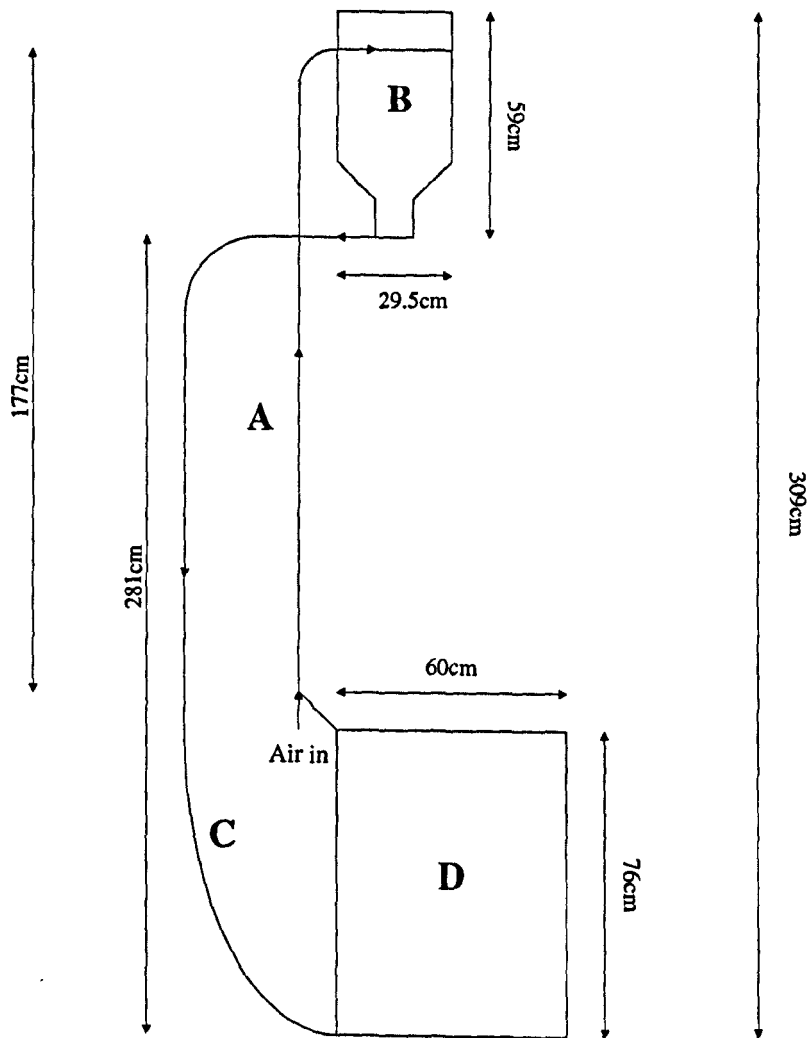


Figure 3.3. Schematic of Tubular Photobioreactor 1 (TBR1).

- A. Riser.** Internal diameter 4.4cm, volume of 2.7 litres
- B. Header tank.** Volume of 32-36 litres
- C. Downcomer.** Internal diameter 2.2cm, volume 1.1 litres
- D. Photostage.** Tubing diameter 2.2cm, 26 windings, volume 18 litres

The flow rate of the liquid in the photostage was determined as a function of the filtered air input rate via the sparger from the compressor. For TBR1 the target photostage flow rate was 0.25 m/s in order to avoid settlement of algae within the header tank or photostage (J. Harper, pers. comm.). This was achieved at an airflow of 4 l/min for the 34 and 36 litre systems and at 5 l/min for the 32 litre system (Figure 3.4). The relationship between the airflow rate and the liquid flow in the photostage was not linear.

The bubble regime in the riser was noted and characterised into one of four types as shown in Figure 3.5 (Hebrard *et al* 1996). This identified the airflow rate which gave a high fluid velocity in the photostage without resulting in perturbed riser flow dynamics (i.e. churn turbulent), avoiding conditions which would reduce the efficiency of mass transfer (Hebrard *et al* 1996).

The 36 litre system at an air flow of 4 l/min was chosen for further study as the flow in the riser was homogeneous perfect bubbly at a photostage flow rate of ~0.25m/s.

3.2.1.2. Reynolds numbers

At a working volume of 36 litres and an air input of 4 l/min the calculated Reynolds number was 4,931, indicating that flow in the photostage was turbulent (Gudin and Chaumont 1991, Carozzi and Torzillo 1996). It is essential that the flow in the photostage is turbulent as this will act to move the algal cells in and out of the light as well as preventing the cells from settling (Molina Grima *et al* 1999). It also acts to increase the rate of oxygen removal, through the gas-liquid interface (Weissmann *et al* 1988). Turbulence breaks down diffusion barriers between the cells and the bulk liquid thereby increasing the transfer of nutrients (Grobelaar 1989, 1991, 1994).

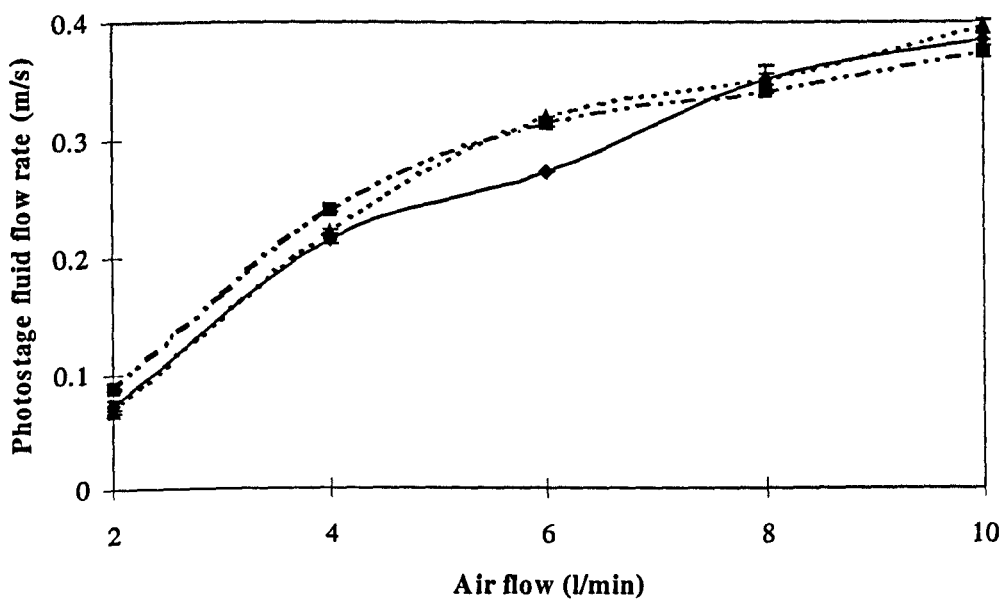


Figure 3.4. The effect of airflow on the photostage flow rate of the TBR1 for a range of system working volumes ($n=3$, \pm S.E.), 32 litres \blacklozenge , 34 litres \blacksquare , 36 litres \blacktriangle .

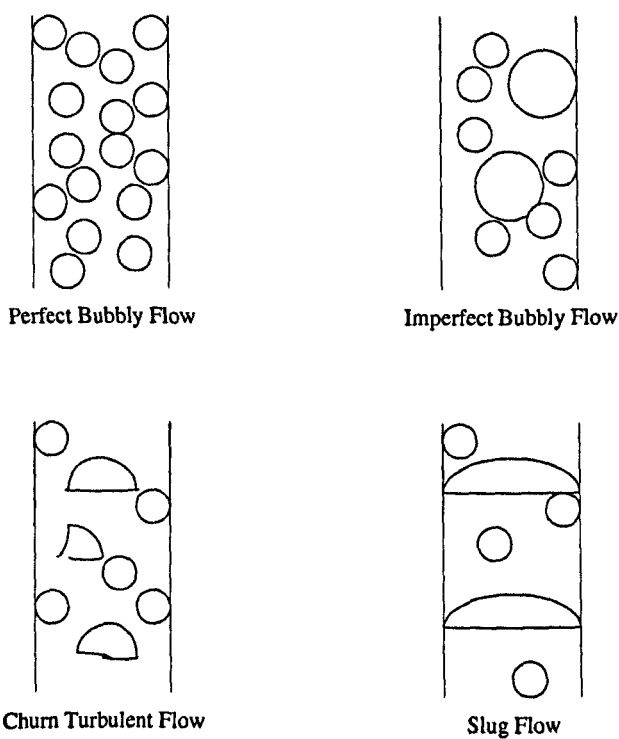


Figure 3.5. Riser Dynamics (see Hebrard *et al* 1996).

However high turbulence can affect shear-sensitive organisms causing a reduction in yield (Gudin and Chaumont 1991, Merchuk and Berzin 1995). Therefore a balance must be achieved between the degree of turbulence which optimises algal growth and the level that is so high that it causes shear damage to the cells. This critical level would be species dependent. In a traditional bubble column that has been previously used to cultivate *H. pluvialis* the Reynolds number was calculated to be 1680 (Tsavalos 1995). This flow is laminar rather than turbulent, which shows that turbulence is not essential for the production of *Haematococcus*.

3.2.1.3. Gas hold-up

The gas hold-up of an airlift system is an important criterion as it determines the residence time of the gas in the liquid, and in combination with the bubble size, influences the gas-liquid interfacial area available for mass transfer (Chisti 1989). It also determines the rate of flow around the system, as the difference in the gas hold-up of the riser and downcomer acts to move the liquid around the entire system. Ideally the gas hold-up of these two sections should be determined separately. However this represents considerable technical problems and in this study it is assumed that the gas separator (i.e. the header tank) is working efficiently and consequently the downcomer has no gas hold-up and therefore has no mass transfer (Fraser *et al* 1994).

The gas hold-up was measured in the header tank, at 36 litres volume and an air input of 4 l/min and was determined to be 0.0045.

3.2.1.4. Volumetric mass transfer coefficient

The volumetric mass transfer coefficient is a measure of the rate of movement of oxygen from the gaseous stream into the liquid phase (Chisti 1989). It was only

calculated under the optimal operating conditions (determined by the flow rates and Reynolds number; see above).

At a working volume of 36 litres and an air input of 4 l/min the mass transfer coefficient (K_{La}) was 9.36 h^{-1} (± 2.09 , $n=3$, \pm S.E.). The probe response was calculated according to the method of Tribe *et al* (1995), and was found to be less than the inverse of the K_{La} . Thus, no additional calculations were required. This was also found to be the case for all subsequent measurements.

In all aerobic fermentations the mass transfer rate of oxygen is of key importance for the design and scale up of the process. In algal culture however the mass transfer of CO_2 is of paramount importance. The ability of a system to transfer oxygen is directly related to its ability to transfer CO_2 (Augenstein 1987). As CO_2 is a more soluble gas than oxygen (Chisti 1989) it would be expected that the transfer of CO_2 from the gas to the bulk liquid would be higher than it is of O_2 . The rate of CO_2 transfer can be calculated for the rate of O_2 transfer. However whereas O_2 transfer is solely based on diffusion, CO_2 transfer can be affected by the presence of OH , H_2O and NH_3 in the liquid phase (Talbot *et al* 1991).

Using the penetration theory proposed by Higbie (1935), Contreras *et al* (1998) have proposed a factor for converting $K_{La}(\text{O}_2)$ into $K_{La}(\text{CO}_2)$. The $K_{La}(\text{CO}_2)$ is proportional to the square root of the diffusion ratio for CO_2 and O_2 . Therefore the $K_{La}(\text{CO}_2)$ can be calculated by multiplying the $K_{La}(\text{O}_2)$ by a factor of 0.91. From this the $K_{La}(\text{CO}_2)$ for the TBR1 was calculated to be 8.52 h^{-1} .

These characterisations set the operating conditions for the bioreactor (working volume of 36 litres and an air input rate of 4 l/min).

3.2.1.5. Assessment of photostage materials for light transmittance

Photosynthesis requires light of a wavelength between 400 and 700nm and efficiency can be affected by the spectral quality of light received (Senger and Bauer 1987). The PVC used in the construction of the photostage was spectrophotometrically (Hewlett Packard 8453 spectrophotometer) assessed to ensure that the plastic itself did not absorb between these wavelengths. No absorbency was measured between 320nm and 820nm (data not shown). This was the case for all plastics used in the construction of the photostages of the proceeding photobioreactors.

3.2.2. Biological production

The system was sterilised and filled with media as detailed in Chapter 2. It was then inoculated with an actively growing culture of *H. pluvialis* which gave a starting culture density of 1.06×10^4 cells/ml (± 0.30 , $n=4$, \pm S.E.). After a short lag phase the cells went into exponential growth reaching a cell density of 14.80×10^4 cells/ml (± 1.59 , $n=4$, \pm S.E.) after eight days. However growth then ceased and the cell number (and biomass) of the culture started to drop. From day eight to 11 the culture changed colour from green to yellow which corresponded to a reduction in the viability of the culture which was determined from microscopic examination of the cells using the Evans blue stain (see Chapter 2 for details). The subsequent death of the culture was thought to be due to poor mixing and mass transfer in the riser and/or header tank. The residence time of the algae in the photostage was measured to be 192 seconds, and it is possible that this length of time would result in a build-up of O_2 and a depletion of CO_2 , which would be detrimental to the culture (Sánchez Mirón *et al* 1999). This would particularly be the case if the mass transfer in the riser and header tank were not high enough to strip the photosynthetic O_2 completely from the system, resulting in a

gradual increase in the O₂ content of the culture over time. Unfortunately no data on DO₂ is available for this culture. A common cause of algal culture crash is a fluctuation in pH, however measurements of pH during the culture showed it to be constant at seven (data not shown). It was noted that at the culture velocity of 0.25m/s some settling of algal cells occurred, suggesting that a faster fluid flow rate was required. This would also reduce the residence time in the photostage.

Illumination of the system was provided by four fluorescent tubes (F18W/GROW, Growlux, 4 feet, Osram). The four tubes were positioned, equally spaced, around the inside of the photostage so that the mean irradiance was 60 $\mu\text{mol}/\text{m}^2/\text{s}$. However this configuration meant that the light around the photostage was not evenly distributed. The algal cells were exposed to much higher light levels directly in front of a light tube than at the mid point between two tubes, where the irradiance was measured to be practically zero. This may have caused limitations to the culture growth. As the density of the culture increased the cell shading in the culture would also have increased so that the light level intercepted by each cell would have been reduced. This would be expected to have a detrimental affect on photosynthesis (Märkl 1977, Qiang and Richmond 1996).

A second run with this photobioreactor configuration yielded very similar results. In this case an aliquot of culture was removed from the system and placed into sterile flasks and cultured at 18°C under an irradiance of 60 $\mu\text{mol}/\text{m}^2/\text{s}$. This resulted in normal growth of the alga indicating that the failure of the culture within the photobioreactor was not an intrinsic problem with the algae but a system design problem.

From the information gained from TBR1, TBR2 was developed with the dual aims of improving both the mass transfer and mixing of the bioreactor. It was thought

that the design of TBR1 resulted in the loss of energy from the riser into the side entry of the header tank (Figure 3.3). From this loss of energy a subsequent reduction in the mass transfer and mixing of the system relative to the energy input would be expected. The design of the header tank was such that it was intended to create a vortex, with the culture entering at the side of the tank and spiralling down to exit at the base of the tank through the downcomer. However the power of the airlift was not sufficient to produce a vortex making the header tank design of TBR1 unsuitable for this application. This type of header tank may however be more suited for use in a mechanically pumped system.

3.3. Tubular photobioreactor 2 (TBR2)

The second tubular photobioreactor had a photostage made up of 22mm PVC tubing with a volume of 17 litres (Figure 3.6). The total working volume of the system was 36 litres. The riser to downcomer cross-sectional area was 0.32. TBR2 adopted some of the design features highlighted during the operation of TBR1, especially in the configuration of the header tank and riser. As with TBR1 the riser started at the top of the photostage but entered the header tank at its base rather than side. It was thought that this design would maximise the use of the energy from the riser in mixing the culture in the header tank, whilst also maximising the difference in the bulk densities of the riser and downcomer so that a high flow rate in the photostage could be achieved.

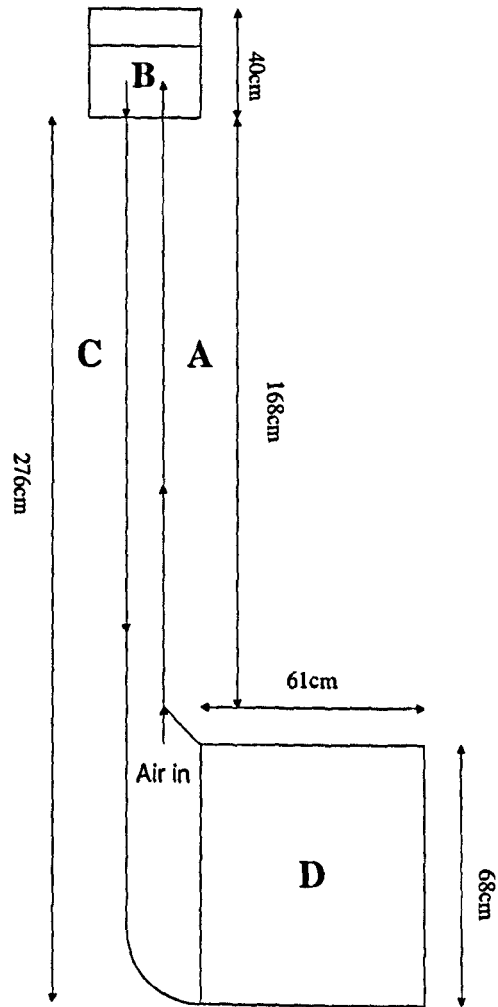


Figure 3.6. Schematic of Tubular Photobioreactor 2 (TBR2).

- A. Riser.** Internal diameter 4.4cm, volume of 2.6 litres
- B. Header tank.** Total volume 23.6 litres, working volume 10 litres
- C. Downcomer.** Internal diameter 2.5cm, volume 1.4 litres
- D. Photostage.** Tubing diameter 2.2cm, 24 windings, volume 17 litres

3.3.1. Physical characterisation

3.3.1.1. Flow characterisation

The target fluid flow rate of 0.25 m/s in the photostage of TBR1 resulted in the settling of *Haematococcus* cells during growth. Therefore in TBR2 the aim was to operate the system at as high a flow rate as the design would permit. The main limitation to flow in the photostage was the fluid dynamics of the riser.

Three system working volumes were investigated; 26, 31 and 36 litres. The 31 litre system was filled to half way up the header tank (10 litre header tank volume). The 26 and 36 litre systems had header tank volumes of five and 15 litres respectively. The air-input rate was set at 8 l/min as this provided a high density of bubbles in the riser. Higher air input rates for the 26 and 31 litre systems resulted in a churn turbulent riser and consequently a reduced mass transfer would be predicted (Figure 3.5, Hebrard *et al* 1996). For the 36 litre system a higher air input rate was required because of the increased header pressure associated with the increased volume in the header tank. A 36 litre system was therefore discounted due to limitations with the air pump.

The highest flow rate in the photostage was obtained for the 31 litre system at 8 l/min (Table 3.1). The calculated Reynolds numbers further supported the selection of the 31 litre system at 8 l/min as the operating conditions (section 3.3.1.2). This is actually comparable to the performance of the TBR1 at 8 l/min, which produced a flow rate of 0.35 m/s suggesting that if TBR1 had been operated at 8 l/min it may have performed in the same way as TBR2. Despite the apparent limitations of the side entry header tank it seems that at a comparable air input rate the two systems would have similar performance. However mixing in the header tanks would have been quite different, with the mixing in the header tank of TBR2 seen to be far more vigorous than in TBR1.

Table 3.1. Mean photostage flow rate of TBR2 at selected system working volumes at an air input rate of 8 l/min.

System working volume (litres)	Mean photostage flow rate (m/s, $n=3$, \pm S.E.)
26	0.29 (\pm 0.01)
31	0.31 (\pm 0.01)
36	0.08 (\pm 0.01)

The system circulation time was also determined by measuring the time it took for a neutral density particle to circulate around the entire system. At the operating conditions set (8l/min air, 31 litre volume) the system circulation was measured at 215 seconds. The flow rate in the photostage was 0.31m/s, which translates to a photostage residence time of 126 seconds. Therefore each cell of the culture would be resident in the photostage of TBR2 for 57% of the time.

The influence of the sparger plate design was also investigated in TBR2. Three plates were assessed, each containing holes of 0.32mm diameter, at a frequency of 21, 32 or 52. Changing the frequency of holes in the sparger plate was found to have no affect on the photostage flow rate, system circulation time or mass transfer (data not shown).

3.3.1.2. Reynolds numbers

The selected working volume of 31 litres at 8 l/min gave a higher Reynolds number than that calculated for TBR1 (at 4 l/min, section 3.2.1.2.). This shows that the fluid in the photostage is turbulent (Table 3.2).

Table 3.2. Reynolds numbers in the photostage of the TBR2 at selected system working volumes at an airflow rate of 8 l/min.

System working volume (litres)	Mean Reynolds number ($n=3 \pm \text{S.E.}$)
26	5700 (± 25.43)
31	6169 (± 22.22)
36	1689 (± 19.97)

3.3.1.3. Gas hold-up

The gas hold-up in the system (measured in the header tank) with an air input of 8 l/min was 0.0087. This is much higher than the hold-up of TBR 1 (section 3.2.1.3), indicating that the flow and mass transfer of the system would be improved as the volumetric mass transfer coefficient generally improves with increasing gas hold-up (Shamlou *et al* 1995).

3.3.1.4. Volumetric mass transfer coefficient

The mean mass transfer coefficient of TBR2 was calculated to be 16.55 h^{-1} (± 0.12 , $n=3$, $\pm \text{S.E.}$) at a working volume of 31 litres and an air input rate of 8 l/min, double the value from TBR1 (section 3.2.1.4). This is likely to be due to the higher air input rate into the system, and changes in the design of the header tank. Using the calculations of Contreras *et al* (1998) the mass transfer in terms of CO_2 , $K_{\text{La}}(\text{CO}_2)$, was 15.06 h^{-1} .

3.3.2. Biological production

The performance of TBR2 was an improvement of that seen for TBR1. The system was inoculated with the same density of an actively growing culture of *H. pluvialis* as for TBR1 (giving a starting cell density in TBR2 of 1×10^4 cells/ml), using the same media recipe and lighting arrangement. The system achieved a maximal cell density of 15.30×10^4 cells/ml (± 1.03 , $n=4$, \pm S.E.). The culture then went into stationary phase and formed carotenoid-rich cysts. This process took 55 days and resulted in a culture with a total carotenoid content of 51.88 pg/cell (± 1.34 , $n=12$, \pm S.E.).

Despite the change in the header tank design the header tank was still poorly mixed. As the culture aged and formed cysts, which are heavier than the green cells and also non-motile (Margalith 1999), the culture in the header tank had to be manually stirred at least once a week to resuspend settled material. Stirring helped to minimise the problem but obviously this settling indicated a severe problem in the long term for axenic production of the algae. It also suggested that the transfer of nutrients and gases between the gas and liquid phases was poor (Grobbelaar 1989). Settling and sticking also occurred in the photostage and cells collected at the bottom of the downcomer. Despite the obvious problem of poor mixing in the system it did produce an astaxanthin rich culture of *H. pluvialis*. The successful production of a carotenoid-rich culture in this system showed that although the lighting regime of the photostage was poor it provided sufficient illumination for the growth (and subsequent promotion of astaxanthin synthesis) of *H. pluvialis*. It also showed that it is possible to grow a large-scale culture of *H. pluvialis* in an external loop airlift device. However although the TBR2 performed successfully the cell density was well below the cell density that had previously been achieved in bubble column systems (25.00×10^4 cells/ml Harker *et al* 1996b) or in shake flasks (30.00×10^4 cells/ml). From this knowledge and the

experience gained from TBR1 and TBR2 the third tubular photobioreactor, the Advanced Algal Production System (AAPS™) was developed.

3.4. Advanced Algal Production System 1 (AAPS™1)

The AAPS™1 (Figure 3.7) was specifically designed for outdoor production (although it can be used indoors) and was situated on an open site on the roof of the Byrom Street campus building of Liverpool John Moores University. It had a photostage made of PVC tubing with an internal diameter of 25mm and a volume of 31 litres. The ratio of the downcomer to the riser cross-sectional area was 0.56. The total working volume of the system was 65 litres.

The system differed from the TBR1 and TBR2 in the configuration of the photostage. In the previous photobioreactors the photostage had consisted of a single coil of tubing. As systems are scaled up and their total volume increases a single length of tubing in the photostage becomes infeasible. This is because an excessive length of tubing results in a prolonged residence time of the algal cells in the photostage which allows a build-up of O₂ and a depletion of CO₂, which may result in the death of the culture (Borowitzka 1994). This was experienced in a large-scale algal growth facility in Spain (again a closed photobioreactor), despite the success of small-scale work, the large-scale facility proved to be unproductive due to the build-up of photosynthetic O₂ in the tubing mediating photoinhibition (Camacho Rubio *et al* 1999).

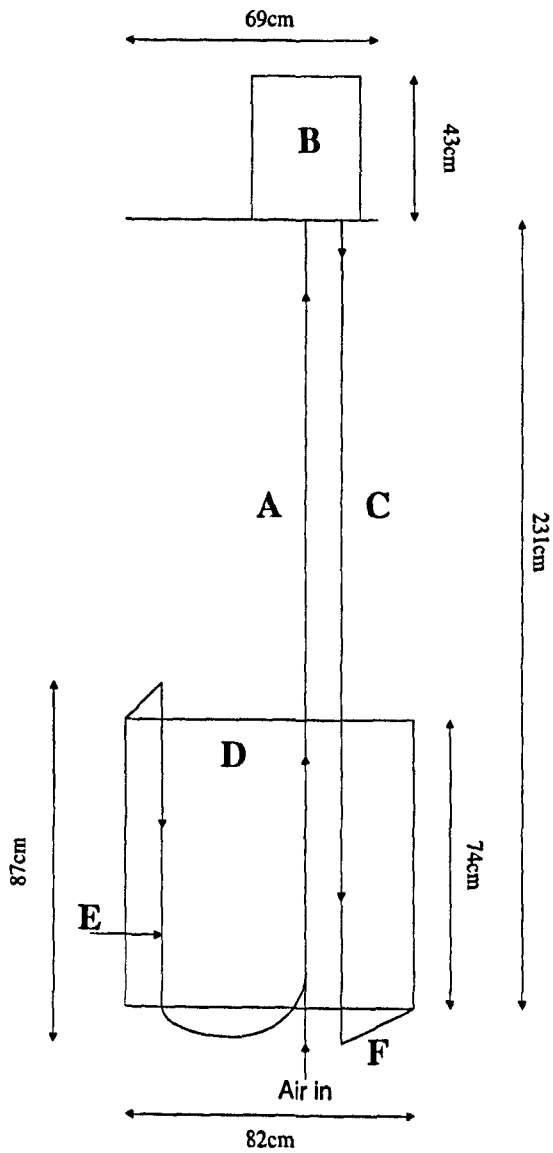


Figure 3.7. Schematic of Advanced Algal Production System 1 (AAPS™1).

- A. Riser.** Internal diameter 6.8cm, volume of 7.6 litres
- B. Header tank.** Working volume of 15.0 litres
- C. Downcomer.** Internal diameter 5.1cm, volume 3.8 litres
- D. Photostage.** Tubing diameter 2.5cm, 8x3 windings, volume 31.0 litres
- E. Return manifold.** Internal diameter 5.8cm, volume 7.0 litres
- F. Outlet manifold.** Internal diameter 5.1cm, volume 0.5 litres

Work by Richmond *et al* (1993) calculated that for a 5m high airlift with a velocity of 0.35m/s, the tubing could be 100m in length before losses due to pressure differential were experienced. Despite the physical ability of the system to operate with a 100m coil length they noted that at 0.35m/s the algal would take five minutes to move through a coil of that length, which might be too long in terms of O₂ build-up. As a result large-scale systems should ideally have several lengths of tubing wound together in parallel and joined at the top and bottom of the photostage via a manifold (Figure 3.8). This minimises the tubing length so the algal cells frequently return to the riser/header tank assembly where gaseous exchange occurs (Chisti 1989).

It is also the case that a single winding of tubing can only achieve a limited slope in the helix, which limits the ability to drain the tubing (as was found with TBR1 and TBR2). This, in turn, results in the need to use additional sterilising fluids to flush the system. It may also result in 'sagging' of the tubing in the photostage during extended operation causing the formation of air pockets. The use of a number of coils in parallel results in a greater slope in the helix, which enables the photostage to be drained completely and reduces the chance of 'sagging' or fouling (Addavita Ltd., UK Patent application GB 9818931.9).

AAPS™1 was therefore designed with a photostage that consisted of a coiled manifold assembly to optimise the operation of the system and also to make it more suitable for scale-up.

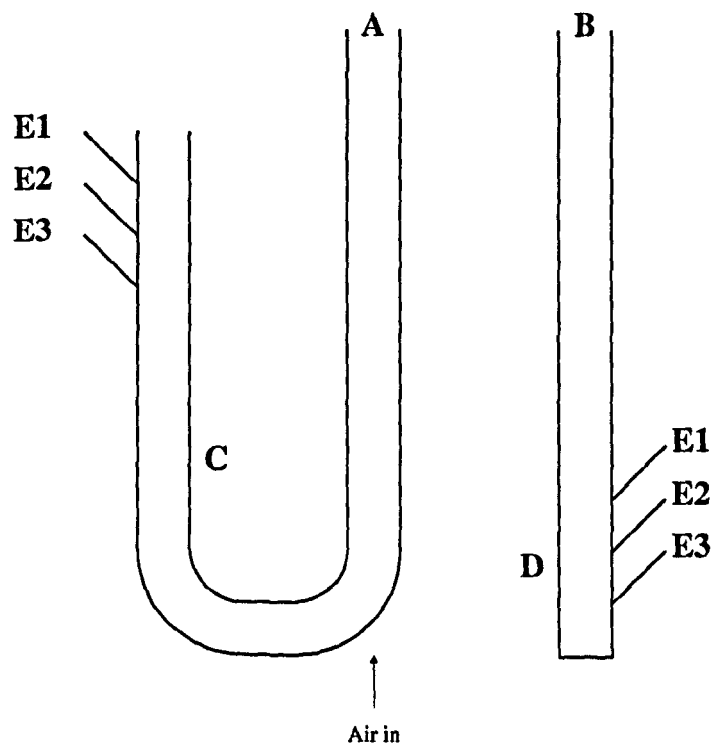


Figure 3.8. Schematic of manifold assembly of AAPS™.

A. Riser

B. Downcomer

C. Return manifold

D. Outlet manifold

E1-E3. Connections to the three windings of the photostage

3.4.1. Physical characterisation

In addition to the system design changes, the pump and air filtration system were changed. A water trap (WS/15, Domnik Hunter, Birtley, UK) and pre filters (AF2000-02D filter and AFD2000-02D micro mist separator, SMC, Manchester, UK) were assembled between the pump (Model 2D/197/10, double diaphragm compressor, Dawson, McDonald and Dawson, Ashborne, UK) and the microbial filter (0.2 μ m Polyvent 40, Whatman). An in-line flow meter (Caché Instrumentation, Wakefield, UK) was used to calibrate air intake rates as litres/hour and all subsequent airflow data will be presented using these units.

3.4.1.1. Flow characterisation

The flow rate in the photostage was measured as described in Chapter 2. The flow rate was measured at four air input rates, namely 60, 160, 200, 300 l/hr which were representative of the range of achievable flow rates. Figure 3.9 shows the fluid flow rates in the photostage that were attained at these air input rates. Flow was measured in each of the three coiled windings three times, there was no significant difference in the flow rate between the three (data not shown) so the mean of the nine flow rates are shown.

An airflow rate of 160 l/hr was chosen for the operation of the system as this provided a fluid flow rate in the photostage of 0.55m/s, which was nearly double that achieved in TBR2 (Table 3.1). At the same time flow in the riser remained perfect bubbly. At air flow rates higher than 200 l/hr the efficiency of energy transfer from the air input into fluid flow in the photostage was reduced (Figure 3.9). This was due to changes in the behaviour of flow in the riser. The fluid changed from perfect bubbly

flow to imperfect bubbly and then churn turbulent flow as the air-input rate increased above 200 l/hr.

At a photostage flow rate of 0.55m/s the residence time in a winding of the photostage was calculated to be 42 seconds. This is one third of the residence time of a particle in the winding of TBR2. As discussed above the length of the residence time in the photostage is critical for the health of the culture. The shorter the time the more frequently the cells are returned to the riser/header tank where gas exchange occurs. This facilitates the removal of photosynthetic oxygen so that the photosynthetic systems of the algal cells are not damaged (Camacho Rubio *et al* 1999, Boussiba 2000). However the photostage is also the site where the culture received irradiance therefore it is also important that the period of time spent in this section of the photobioreactor is not minimised to the detriment of the culture.

The time taken for a neutral density particle to circulate around the entire system was measured to be 123 seconds. As the residence time in the photostage was 42 seconds the culture spent 34% of its time in the photostage. This is nearly half of the time spent by a culture in the photostage of TBR2 (section 3.3.1.1.).

3.4.1.2. Reynolds numbers

The Reynolds numbers were calculated for the photostage as shown in Figure 3.10. All the tested air flow rates resulted in a Reynolds number that would indicate that flow in the photostage was turbulent rather than laminar. The selected air input rate of 160 l/hr gave a calculated Reynolds number of 13,700 which is more than double that achieved in the TBR2 (Table 3.2).

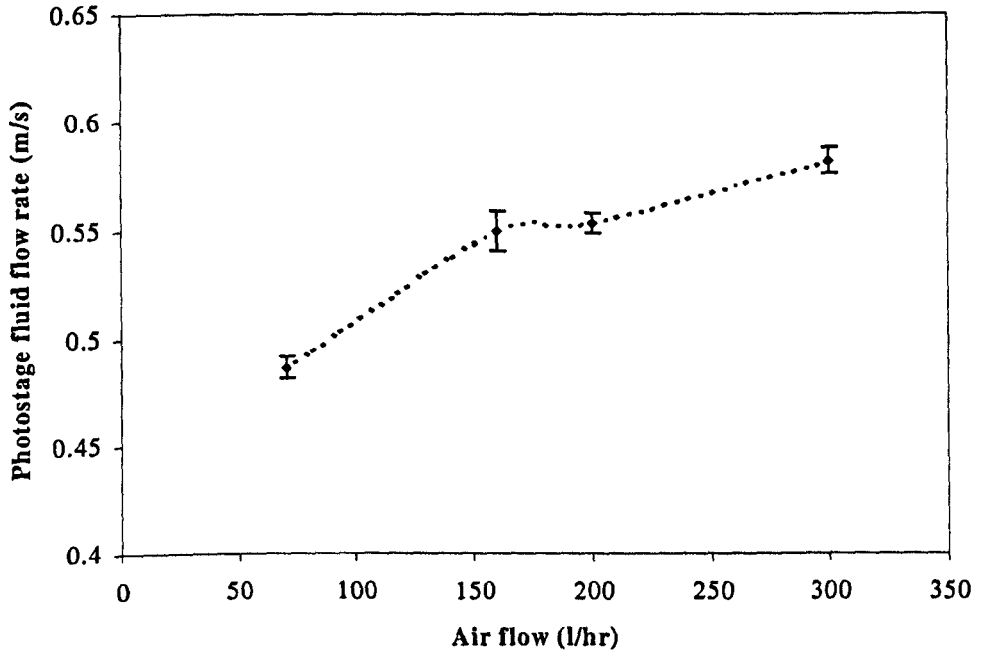


Figure 3.9. Flow rate in the AAPSTTM1 photostage at selected air input rates ($n=9 \pm$ S.E.).

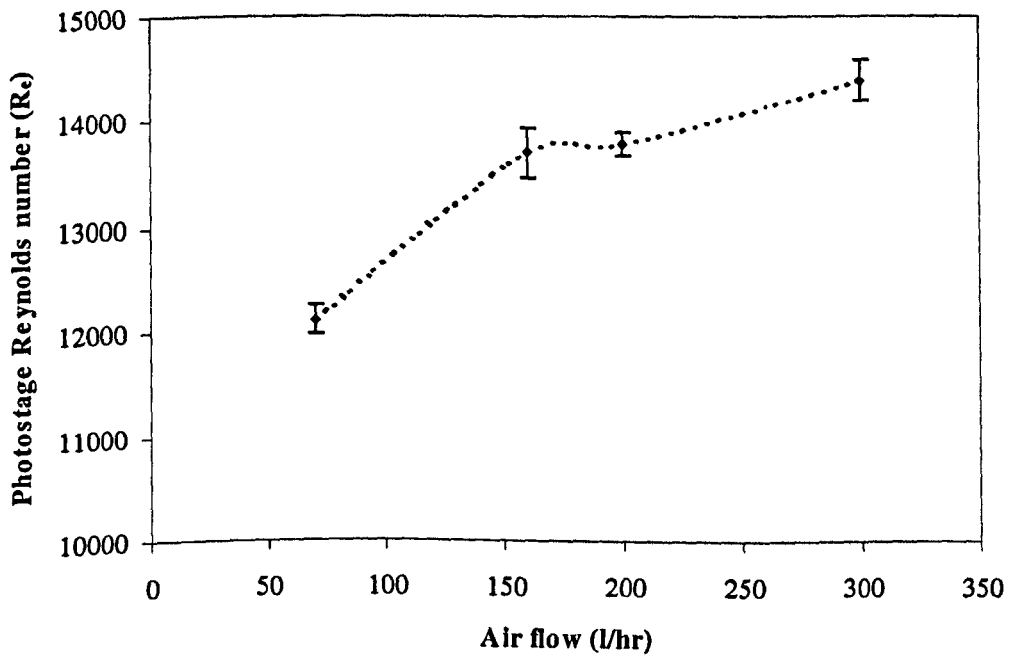


Figure 3.10. Calculated Reynolds numbers in the AAPSTTM1 at selected air input rates ($n=9 \pm$ S.E.).

It has been proposed by a number of authors that turbulent flow is beneficial to microalgae because of the 'flashing light effect'. However Grobbelaar (1994) proposed that this was a misinterpretation. For cells to benefit from the flashing light effect they must be exposed to high frequency fluctuations of 100ms (10Hz) or less. It is unlikely that turbulent flow in an algal growth system would expose cells to fluctuations this short, they are more likely to be medium frequency fluctuations of seconds to minutes (Grobbelaar 1989). Grobbelaar (1989, 1994) found that a modulating light environment in the order of seconds to minutes did not stimulate photosynthesis and suggested that the beneficial effect of turbulent flow was due to the influence on nutritional and gaseous gradients within the culture. This acted to decrease the boundary layer and therefore improve the rates of exchange of metabolites and nutrients between the cell and its environment, thereby enabling the cell to utilise light more efficiently. Despite disagreements between authors as to the action of turbulence, there is general agreement that turbulence increases the yield of an algal culture (Laws *et al* 1983, Terry 1986).

3.4.1.3. Gas hold-up

At 160 l/hr the gas hold-up was 0.0078. This is an improvement on the hold-up measured in TBR1 (section 3.2.1.3.) but lower than the gas hold-up in TBR2 (section 3.3.1.3.). This may be due to the difference in the fluid height of the two airlifts. The fluid height (length of riser plus fluid height in header tank) of TBR2 was 186cm whereas AAPSTTM1 was 254cm. Bentifraouine *et al* (1997) noted that for an external-loop airlift an increase in the height of the airlift resulted in an increase in the liquid circulation velocity because of the increased hydrostatic pressure difference between the riser and the downcomer. The increase in height also caused a fall in the gas hold-

up due to the decreased bubble residence time in the riser. Chisti (1989) noted that this also occurred in an internal-loop system.

3.4.1.4. Volumetric mass transfer coefficient

The mass transfer of the system was measured at 160 l/hr to be 47.10 h^{-1} (± 2.56 , $n=3 \pm \text{S.E.}$). The mass transfer coefficient of TBR2 was calculated to be 16.55 h^{-1} nearly three times lower than the AAPS^{TM1}. It therefore indicates that the AAPS^{TM1} is a much more efficiently mixed system, able to transfer from the gas to the liquid phase at a much greater rate than the TBR1 or TBR2. Efficient mixing was evident during the culture of *H. pluvialis* in the system, as no settling of cells occurred in the header tank. Using the factor determined by Contreras *et al* (1998) the $K_{La}(\text{CO}_2)$ was calculated to be 42.86 h^{-1} .

3.4.2. Biological production

The AAPS^{TM1} was successfully used to produce a number of astaxanthin-rich *H. pluvialis* cultures. This is discussed in detail in Chapter 4.

3.5. Advanced Algal Production System 2 (AAPS^{TM2})

The fourth tubular photobioreactor that was developed, AAPS^{TM2} (Figure 3.11) was a modification of AAPS^{TM1}. The photostage was made up of a three-coiled manifold system (Figure 3.8) and the riser was made of transparent ridged plastic as with the previous system. The material of the downcomer was changed to a ridged opaque plastic. The three previous systems had a downcomer that had been constructed of transparent PVC tubing, enabling a visual inspection of flow.

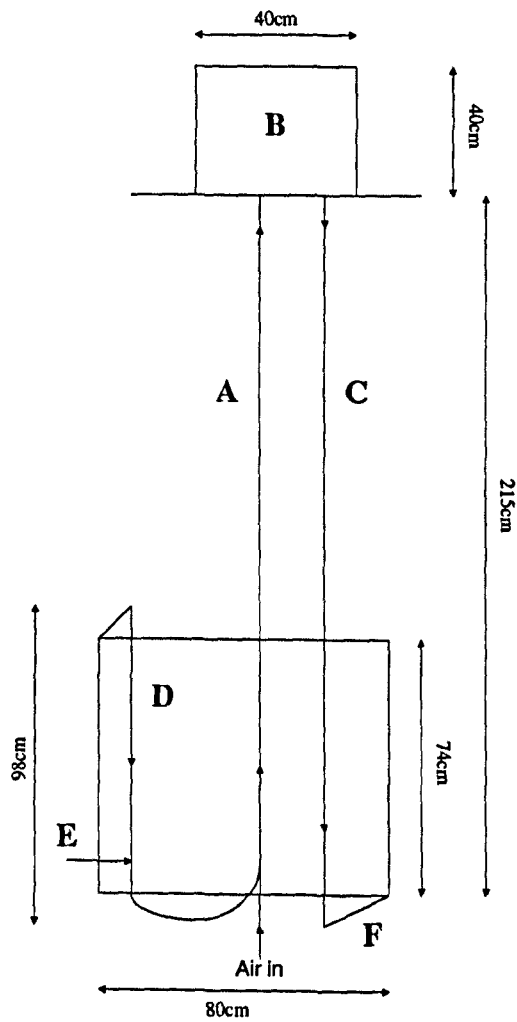


Figure 3.11. Schematic of Advanced Algal Production System 2 (AAPS™2).

- A. Riser.** Internal diameter 6.3cm, volume of 6.8 litres
- B. Header tank.** Working volume of 15.0 litres (total volume 25.0 litres)
- C. Downcomer.** Internal diameter 5.0cm, volume 4.2 litres
- D. Photostage.** Tubing diameter 2.5cm, 6x3 windings, volume 21.0 litres
- E. Return manifold.** Internal diameter 5.5cm, volume 7.0 litres
- F. Outlet manifold.** Internal diameter 5.0cm, volume 1.0 litres

The header tank design was also changed. It was manufactured as a tank rather than a cylinder (Figure 3.12 shows schematics of the three designs of header tank used during this study). It was seen as vital to have a transparent header tank at this stage of development in order to improve the understanding of the hydrodynamics of the system. Engineering difficulties with the materials available made it difficult to construct a transparent cylinder. Therefore a compromise had to be made and the square transparent header tank was provided.

The system design also differed from the other systems as it had a control box, through which pH and culture temperature could be monitored (details of which are given in Chapter 2, section 2.4.4.3.). The control box also enabled pH control via CO₂ injection when required.

The total working volume of the system was 55 litres with a ratio of downcomer to riser cross section area of 0.63.

3.5.1. Physical characterisation

AAPSTM2 was fitted with a control box, which incorporated an air flow meter. Though the system was still aerated using the air pump and filter/flow meter assembly that has been used for AAPSTM1 (section 3.4.1.). The air rate was controlled via the flow meter in the control box (calibrated in l/min, Caché Instruments, Wakefield, UK). Therefore all subsequent air measurements are in l/min.

3.5.1.1. Flow characterisation

The photostage flow rate was measured at 5, 10, 15, and 20 l/min. Higher air input rates resulted in slug flow in the riser. The photostage flow rate increased over the range of air input rates (Figure 3.13).

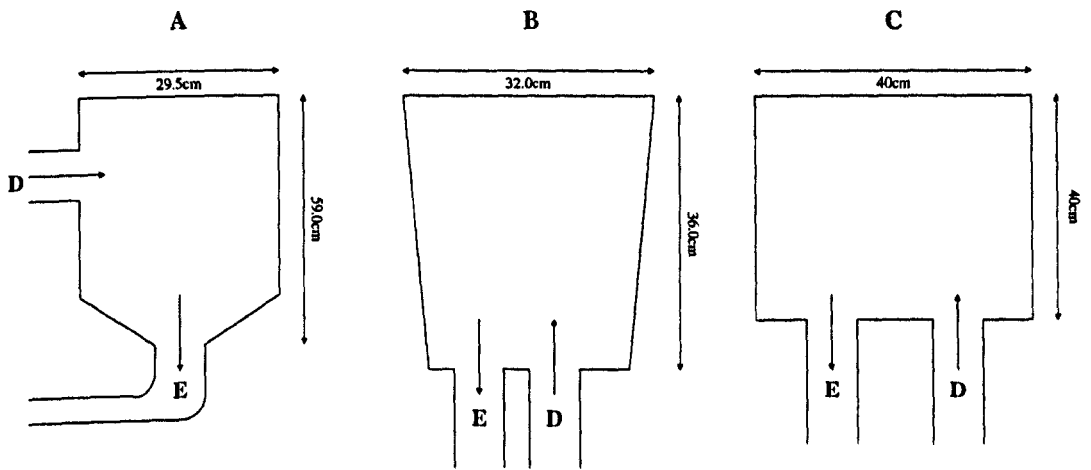


Figure 3.12. Schematics of the three designs of header tank developed for the tubular photobioreactors. A. TBR1, B. TBR2 and AAPS™1, C. AAPS™2 D. Riser entry point, E. Downcomer exit point.

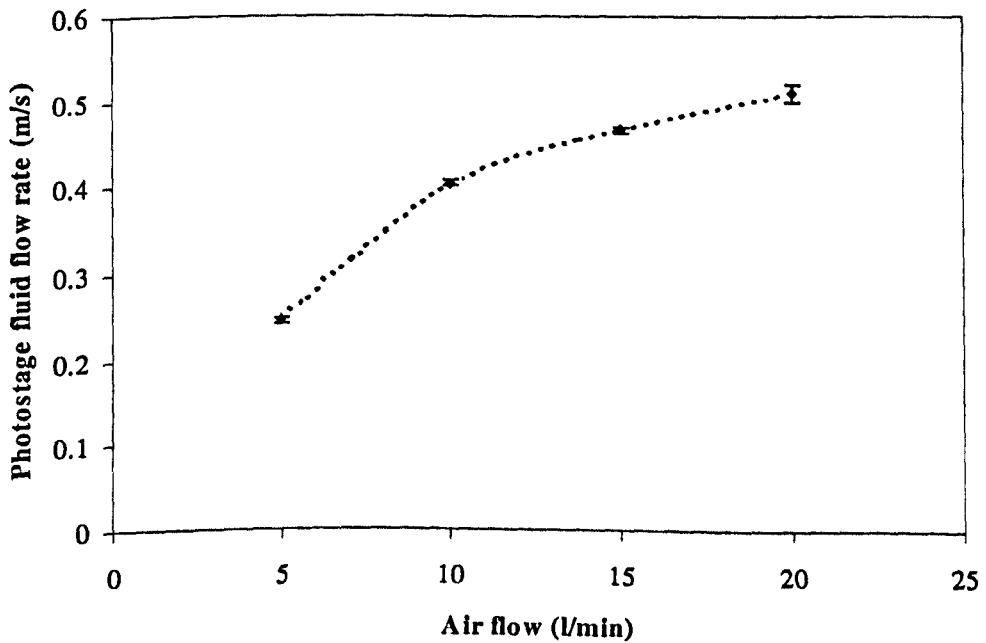


Figure 3.13. Flow rate in the AAPS™2 photostage at selected air input rates ($n=9 \pm \text{S.E.}$).

From these measurement an airflow rate of 15 l/min was identified as optimal as it gave a high photostage flow rate (0.47m/s) with dense perfect bubbly flow in the riser.

At a flow rate of 0.47m/s the residence time of a particle in the photostage was calculated to be 30 seconds, a further reduction on the residence time calculated for AAPS™1 or TBR2 (section 3.4.1.1.). The system circulation time was measured at 88 seconds. Therefore the residence time in the photostage of under the operating conditions discussed above was 34%, the same percentage as was calculated for the AAPS™1.

3.5.1.2. Reynolds numbers

The Reynolds numbers were calculated for the four air input rates (Figure 3.14). As with the previous AAPS™ system the flow in the photostage was turbulent. At 15 l/min the Reynolds number was 11,600 which was lower than the Reynolds number of the first AAPS™ (section 3.4.1.2.) but still highly turbulent, providing effective mixing in the photostage. The reduction in the Reynolds number was due to the reduced liquid flow rate in the photostage.

3.5.1.3. Gas hold-up

The gas hold-up of AAPS™2 at 15 l/min was 0.0061. This is a reduction on the hold-up of AAPS™1, which at its operating condition of 160 l/hr had a gas hold-up of 0.0078 (section 3.4.1.3.). The height of the liquid in each of these two systems was comparable and therefore may explain the reduced liquid flow rate in the photostage of AAPS™2 when compared to AAPS™1 (section 3.4.1.3.). This is because the flow rate

of an airlift is strongly determined by the gas hold-up. The reduced gas hold-up would also be expected to result in a reduction in the mass transfer within the system (Chisti 1989).

3.5.1.4. Volumetric mass transfer coefficient

For this system the $K_{La}(O_2)$ was calculated for the four air input rates as it was felt from the previous systems that this information was vital for selecting the correct operating conditions. The highest mass transfer was achieved at 15 l/min despite the fact that 20 l/min gave a higher flow rate in the photostage and what appeared to be a greater density of bubbles in the riser (Figure 3.15). The reduced mass transfer at 20 l/min is likely to be due to the density of bubbles causing collisions between themselves and the riser walls which in turn caused them to coalesce resulting in a reduced interfacial area and therefore reduced mass transfer (Benyahia and Jones 1997).

The calculated volumetric mass transfer coefficient of $32.66h^{-1}$ is a reduction when compared to the rate of AAPS™1 (section 3.4.1.4.) as indicated by the gas hold-up.

The $K_{La}(CO_2)$ was calculated to be $34.31 h^{-1}$, again a reduction on the figure calculated for AAPS™1. This corresponds to the reduced photostage liquid flow rate and gas hold-up of this system, indicating that the change in the header tank design of AAPS™2 may have been to the detriment of its physical performance.

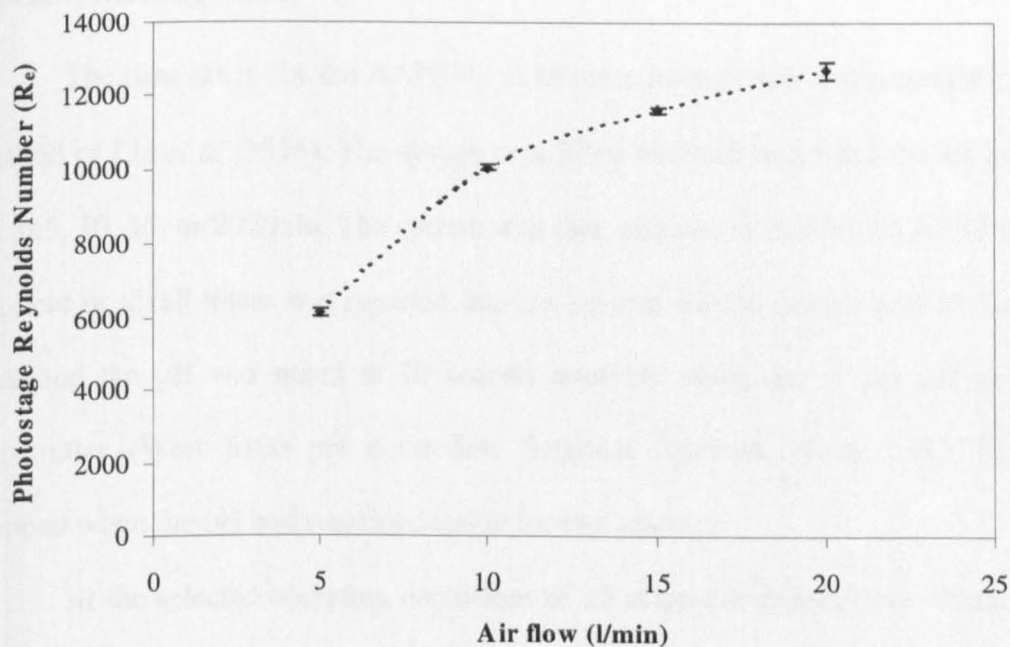


Figure 3.14. Calculated Reynolds numbers in the AAPS™2 at selected air input rates ($n=9 \pm$ S.E.).

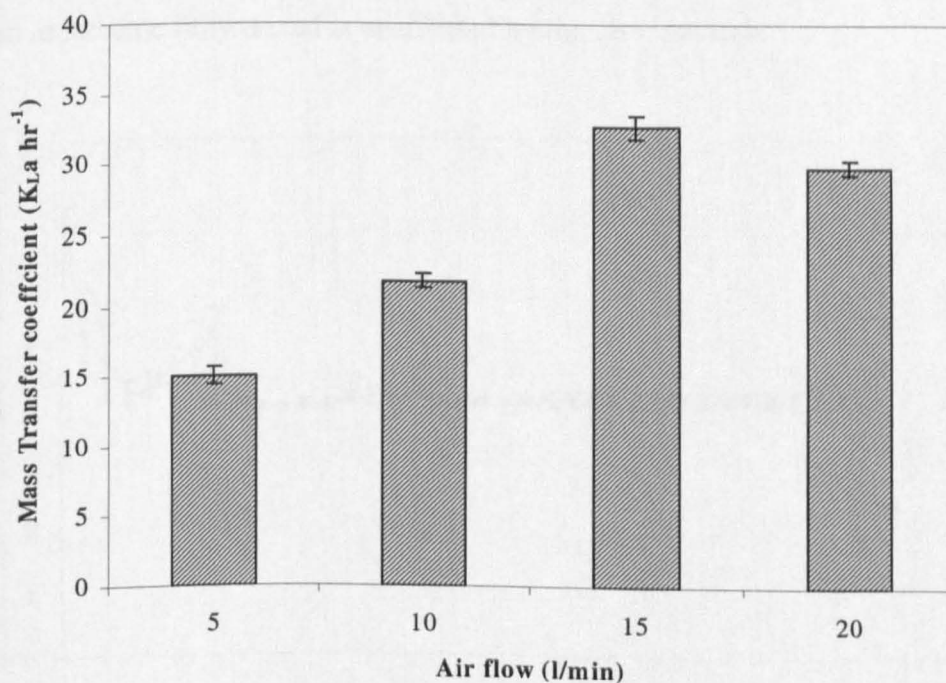


Figure 3.15. Calculated volumetric mass transfer coefficient in the AAPS™2 at selected air input rates ($n=3 \pm$ S.E.).

3.5.1.5. Mixing time

The time taken for the AAPS™2 to become fully mixed was assessed using the method of Lin *et al* (1976). The system was filled with tap water and the air input was set at 5, 10, 15, or 20 l/min. The system was then allowed to equilibrate for 15 minutes. An acid or alkali tracer was injected into the system via the sample port in the header tank and the pH was noted at 10 second intervals using the *in situ* pH probe and transmitter (West 6100 pH controller, Brighton Systems, Hove, UK). Recording stopped when the pH had remained stable for two minutes.

At the selected operating conditions of 15 l/min the system took ~90seconds to become fully mixed (Figure 3.16). At 5 l/min the system took ~140seconds, at 10 and 20 l/min the mixing time was ~100 seconds (data not shown). Indicating that once the system reaches a certain air input rate (between 5 and 10 l/min) the time taken for the system to become fully mixed is unaffected by the air input rate.

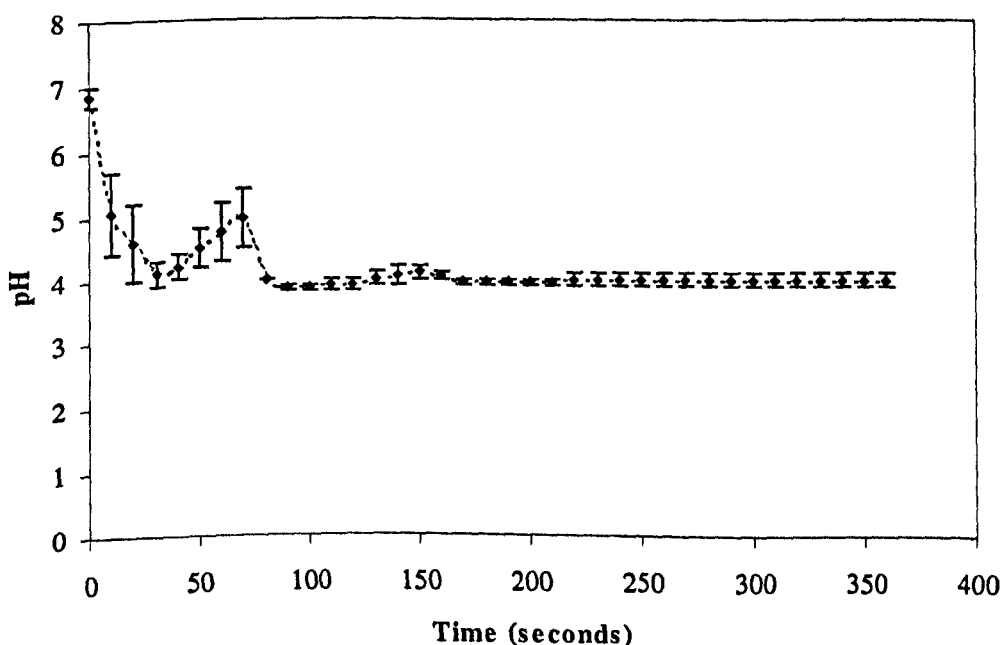


Figure 3.16. Mixing time in the AAPS™2 operated at an air input rate of 15 l/min ($n=3 \pm$ S.E.).

3.5.2. Biological production

The AAPS™2 successfully produced a number of astaxanthin-rich cultures of *H. pluvialis*. This is discussed in detail in Chapters 4 and 5.

3.6. Discussion

A summary of the physical data that has been collected for the four tubular photobioreactors is detailed in Table 3.3.

The first two tubular photobioreactors differed significantly from the subsequent AAPS™ designs. TBR1 and TBR2 though approximately the same height as the AAPS™ had a much shorter airlift as the riser began at the top rather than the bottom of the photostage (Figures 3.3 and 3.6). The two AAPS™ were designed with return and outlet manifolds so that the riser began at the base of the photostage thereby maximising its length (Figures 3.7 and 3.11). By making the riser as long as possible the difference in the hydrostatic pressure between the riser and the downcomer was increased, resulting in a faster flow rate around the photobioreactor (Chisti 1989).

The manifold design also reduced the time taken to pass through the system. Both AAPS™ had an increased volume when compared to TBR1 and TBR2 with a photostage made of a greater total length of tubing. For example TBR2 had a photostage consisting of 44m of tubing which the algae had to pass through. AAPS™1 contained 68m of tubing in its photostage but it was arranged as three windings of 23m. Therefore the algae only passed through 23m of tubing in any one circulation. This reduced the system circulation time of the AAPS™ and also the percentage of time that the algae spent in the photostage. In other words the cells were exposed to gas

exchange in the riser and header tank more frequently in the AAPSTTM units, thus preventing the build up of O₂ and the depletion of CO₂ in the photostage.

Table 3.3. Summary of tubular photobioreactor characterisation.

	TBR1	TBR2	AAPST TM 1	AAPST TM 2
Photostage diameter (mm)	22	22	25	25
Photostage volume (L)	18	17	31	21
Total working volume (L)	36	31	65	55
Ratio of photostage: total working volume (%)	50	55	48	38
Downcomer/riser cross sectional area	0.25	0.32	0.56	0.63
Air input rate	4 l/min	8 l/min	160 l/hr	15 l/min
Photostage flow rate (m/s, $n=9, \pm$ S.E.)	0.25 (\pm 0.00)	0.31 (\pm 0.00)	0.55 (\pm 0.01)	0.47 (\pm 0.00)
System circulation time (s, $n=9, \pm$ S.E.)	unknown	215.00 (\pm 25.66)	123.00 (\pm 16.98)	88.50 (\pm 13.86)
Residence time in photostage (s, $n=9, \pm$ S.E.)	192.16 (\pm 2.22)	126.13 (\pm 1.48)	42.00 (\pm 0.71)	30.78 (\pm 0.21)
Reynolds number ($n=9, \pm$ S.E.)	4,931 (\pm 33.33)	6,169 (\pm 47.80)	13,700 (\pm 231.20)	11,600 (\pm 83.00)
Gas hold-up ($n=3 \pm$ S.E.)	0.0045 (\pm 0.0001)	0.0087 (\pm 0.0005)	0.0078 (\pm 0.0001)	0.0061 (\pm 0.0000)
$K_{La}(O_2)$ (h^{-1} , $n=3 \pm$ S.E.)	9.36 (\pm 2.09)	16.55 (\pm 0.12)	47.10 (\pm 2.56)	32.66 (\pm 0.81)
$K_{La}(CO_2)$ (h^{-1} , $n=3 \pm$ S.E.)	8.51 (\pm 2.00)	15.06 (\pm 0.10)	42.86 (\pm 2.34)	29.72 (\pm 0.74)

Mixing and mass transfer was greatly improved in the AAPS™. This was probably a function of the increased diameter and length of the riser. The cross section area of TBR1 and TBR2 riser was 15.2cm² compared to 36.0cm² for AAPS™1. AAPS™1 had a riser length of 231cm, TBR 1 and 2 were 177 and 168cm long respectively. This enabled a larger volume of bubbles to be present in the riser, therefore improving the interfacial area for mass transfer in the riser and also the degree of mixing in the header tank (Chisti 1989). The improved mixing and mass transfer was also a function of the greater hydrostatic pressure of the system due to the longer riser. However the gas hold-up of these systems was reduced, because the higher liquid flow rate resulted in a decreased bubble residence time in the riser (Bentifraouine *et al* 1997).

The gas hold-up, mass transfer and photostage flow rate of the AAPS™2 was lower than that calculated for the AAPS™1. The two systems had the same design differing only in the volume of the photostage and the design of the header tank. The reduction in the gas hold-up, mass transfer and photostage flow rate of AAPS™2 may have been caused by a reduction in the efficiency of phase separation in the header tank (Bentifraouine *et al* 1997). If the gas was not disengaging from the liquid phase in the header tank and was consequently being carried into the downcomer there would be a reduction in the difference in the bulk densities of the riser and downcomer so the flow would be reduced. Unfortunately the flow in the downcomer of AAPS™2 could not be studied due to its opaque nature.

The internal diameter of the tubing in the photostage was increased in both AAPS™. This was simply due to the better commercial availability of 25mm tubing. Ideally the smaller the bore of the tubing the better to enable maximal light transmission to the culture. Kobayashi and Fujiya (1997) investigated the productivity

of a tubular photobioreactor with 16, 25, 41, and 50mm bore tubing. They found that the growth rate of *Chlorella* was reduced when the tubing diameter was increased. However a compromise must be reached, as a narrower tube requires a longer length for a set working volume. It is not believed, however, that the change from 22 to 25mm would have had a significant impact on the performance of the system, particularly at the relatively low cell densities.

The downcomer/riser cross section area was reduced in the AAPS™ design. In TBR1 and TBR2 the downcomer had a very small bore compared to the riser, whilst in the AAPS™ the bore was increased making it only slightly smaller than the riser. The ratio of riser to downcomer cross-sectional area as well as the reactor height have a major influence on the velocity of fluid circulation (Chisti *et al* 1988). However there is no general agreement in the literature as to the influence of this parameter on the circulation in the system (see Chisti 1989).

The failure of TBR1 to support a culture of *H. pluvialis* was thought to be due to the poor mixing of the system. However, compared to other fermentation organisms the growth rates of photosynthetic cells are relatively slow, so that even a low degree of mixing is often sufficient to avoid mass transfer limitations (Weissmann *et al* 1988). Weissmann *et al* (1998) suggested that in fact light limitation is the most commonly encountered problem in mass cultures. Efficient light supply is a very important factor; both TBR 1 and 2 were set up indoors with four fluorescent strips giving a mean irradiance of 60 $\mu\text{mol}/\text{m}^2/\text{s}$. The AAPS™1 and AAPS™2 were both used for culture outdoors where the irradiance was evenly distributed around the photostage and reached levels $>1000 \mu\text{mol}/\text{m}^2/\text{s}$. It is possible that the cultures in TBR1 and TBR2 were light-limited in addition to limitation caused by the poor mixing characteristics of

the system. Additional studies with the AAPS™2 have demonstrated the inefficiency of using this configuration of fluorescent tubes to illuminate the photostage.

3.7. Conclusions

The characterisation of the four tubular photobioreactors proved invaluable in determining the physical conditions required to sustain an algal culture and consequently the design adjustments that were required to optimise the process. Whereas the initial system, TBR1, failed to sustain a culture, TBR2 did produce a carotenoid-rich culture although the yield was poor. Further development resulted in AAPS™1 and then AAPS™2, both of which produced successive carotenoid-rich cultures (Chapters 4 and 5).

4. Batch growth of *Haematococcus pluvialis* in the Advanced Algal Production System

4.1. Introduction

The large-scale cultivation of microalgae can be approached in three main ways, as a batch, fed-batch or continuous operation. In batch growth the organism is inoculated into a volume of media in which it grows until growth becomes limited by nutrient exhaustion. There is no addition or removal from the culture during this period and the total volume is harvested when growth is exhausted. Fed-batch initially operates as a batch culture until the point just before growth becomes limited, at this time a volume of culture is removed and replaced with new media. In continuous culture the organism is inoculated into a volume of media and when it reaches the exponential phase of growth a nutrient feed is started. This generally takes the form of a constant renewal of selected nutrients or whole media. This prevents the growth of the organism becoming nutrient-limited, as occurs in batch culture. Without nutrient limitation the culture continues to grow for a much longer period than is seen in batch culture resulting in a much improved productivity.

The selection of the most appropriate production method for *H. pluvialis* is complicated as the alga only produces the product of interest, astaxanthin, under conditions of nutrient limitation and/or environmental stress (Harker *et al* 1996a, Kobayashi *et al* 1997, Fábregas *et al* 1998). These conditions may not necessarily exist in a continuous culture. This chapter describes a series of studies employing batch-mode production of *Haematococcus*.

4.1.1. Aims

The aim was to study variations in outdoor production (batch) of *H. pluvialis* in terms of biomass and carotenoid accumulation in the AAPS™.

4.2. Advanced Algal Production System 1 (AAPS™1)

Four separate runs were carried out in the AAPS™1, each at a different time of year (Table 4.1).

Table 4.1. Timing of *H. pluvialis* growth in the AAPS™1.

Culture one	September - October 1998
Culture two	November 1998 - January 1999
Culture three	April 1999 - May 1999
Culture four	June 1999 (abandoned due to contamination)

4.2.1. Culture one

This was the first outdoor grown *H. pluvialis* culture: the system was inoculated on 17th September and the culture was harvested on 20th October 1998.

4.2.1.1. Growth

Inoculation was made with an exponentially growing green culture (Chapter 2) to give an initial cell density of 1.65×10^4 cells/ml. The culture failed to exhibit a distinct lag phase but grew exponentially until day 18, reaching a cell density of 17.00×10^4 cells/ml (Figure 4.1). The specific growth rate of the culture during this period was 0.17 days, which corresponds to a culture doubling time of 4.08 days.

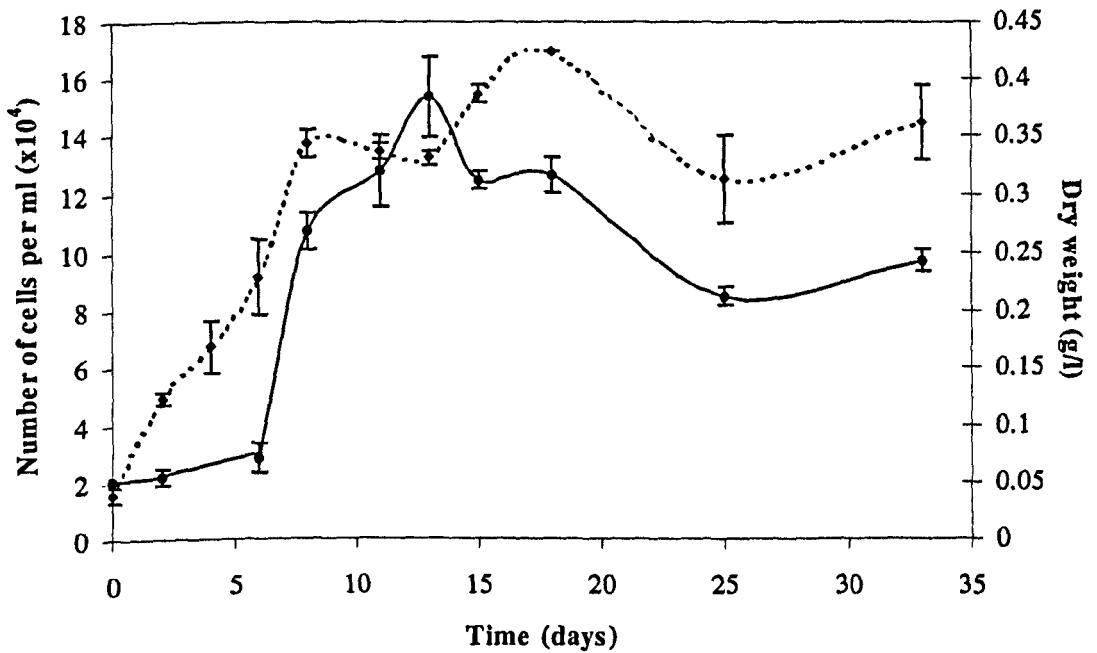


Figure 4.1. Cell density (---, $n=4 \pm$ S.E.) and dry weight (—, $n=3 \pm$ S.E.) of *H. pluvialis* (Culture one) grown in the AAPSTTM1 during September and October 1998.

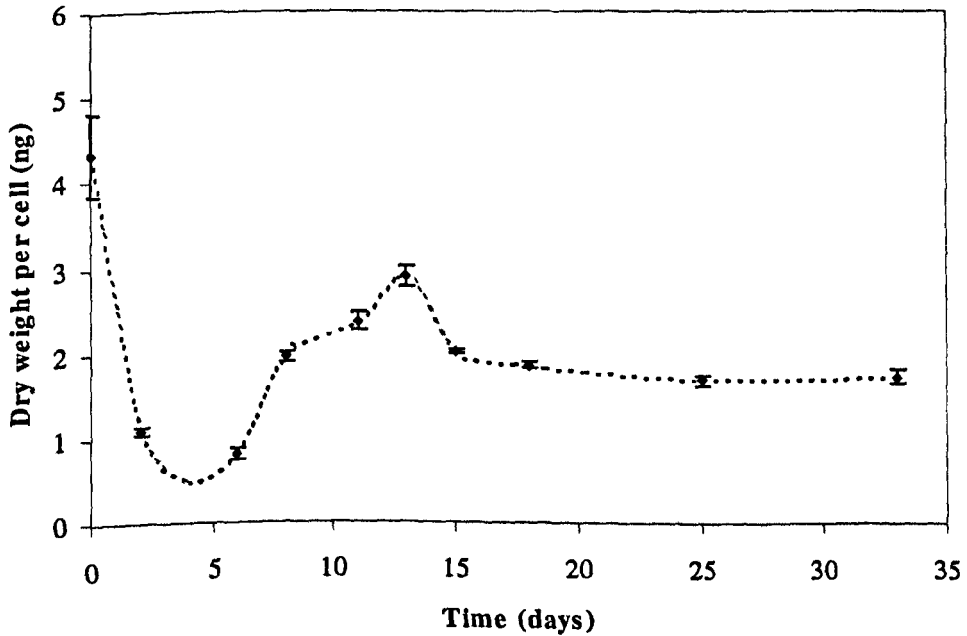


Figure 4.2. Dry weight per cell ($n=12 \pm$ S.E.) of *H. pluvialis* (Culture one) grown in the AAPSTTM1 during September and October 1998.

The dry weight of the culture increased until day 13, reaching a value of 0.38g/l, then progressively decreased (Figure 4.1). The decrease in cell dry weight from day 13 - 18 unexpectedly corresponded with an increase in the cell density of the culture. This reduction in the dry weight of the culture could be explained on the basis of changes to the cell type. From day seven - 13 the cell count dropped but the dry weight increased indicating that individual algal cells were enlarging. This is demonstrated in Figure 4.2, which shows the dry weight per cell of the culture. From day 13 - 18 the dry weight dropped and the cell count increased indicating perhaps that a proportion of the enlarged cells had divided to produce more, smaller cells. As no additions had been made to the culture this is most likely to have been caused by environmental conditions (e.g. changes in temperature, irradiance etc.). From day 18 both the cell density and the dry weight of the culture decreased. At this point the culture was beginning to change in cell type, forming encysted cells, but there was a net loss of cells from the culture. Loss of cells in sampling could have resulted from cell settling (e.g. in the opaque header tank) due to a loss of motility. Some settling was observed in the return and outlet manifolds but not in the photostage. Indeed the taps located at the bases of the manifolds contained a small volume of algal sludge. The pH of the culture remained constant at 7 (Figure 4.3), however there was no *in situ* measurement, all measurements were taken from samples used for growth analysis, and, as a result, possible fluctuations through the daylight cycle were not measured.

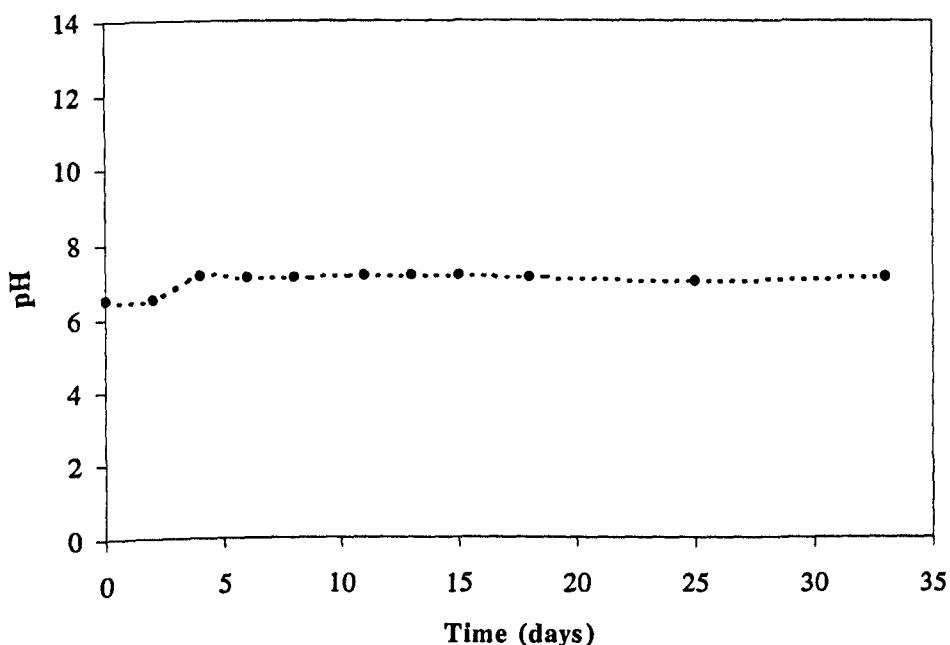


Figure 4.3. pH of the *H. pluvialis* culture (Culture one) grown in the AAPS™1 during September and October 1998 (All measurements were taken at 0900hrs).

4.2.1.2. Secondary carotenoid accumulation

From the time of inoculation the carotenoid content per volume of culture increased (Figure 4.4). Comparison of the carotenoid production per cell (Figure 4.5) with growth of the cells (Figure 4.1), reveals that carotenoid accumulation began shortly after the inoculation of the system, increasing exponentially until day 25. The accumulation of astaxanthin therefore occurred when there was no apparent nitrogen limitation in the culture (as indicated by active growth, Figure 4.1.). The cells were motile zooids, some of which were red due to the accumulation of carotenoids. Plates 4.1a-d and 4.2a and b show photomicrographs of the life cycle stages of *H. pluvialis* identified during this study. Plates 4.1a. and 4.1d show photomicrographs of the typical green zooid and the red zooid cells that were present in this culture.

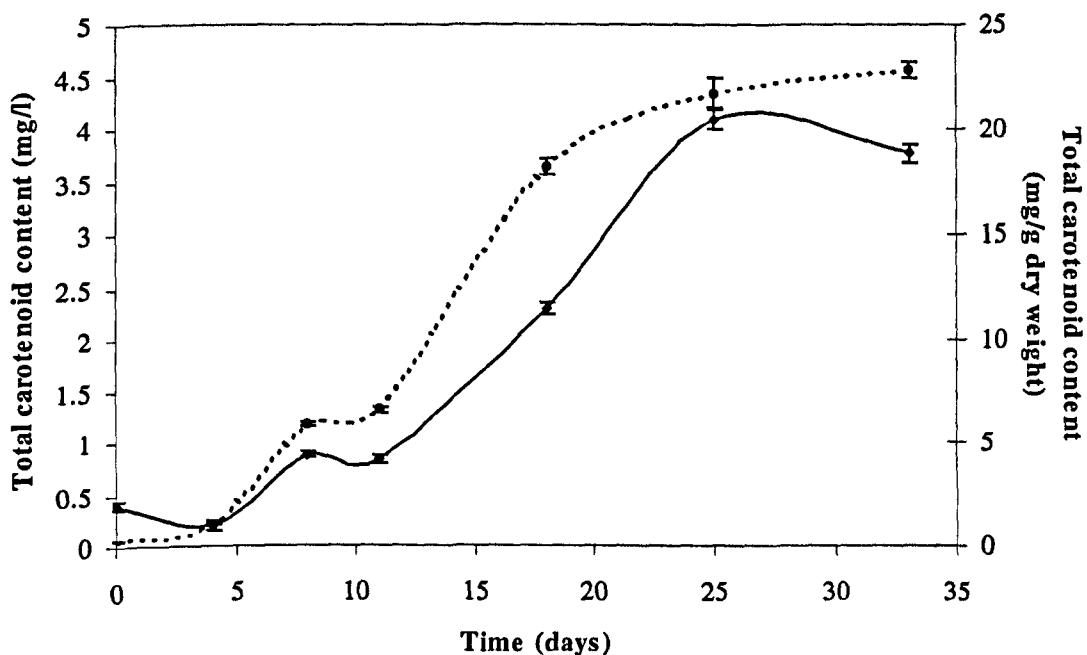


Figure 4.4. The total carotenoid content, calculated as mg/l (---, $n=3 \pm$ S.E.) and mg/g dry weight (—, $n=9 \pm$ S.E.) of *H. pluvialis* (Culture one) grown in the AAPS™1 during September and October 1998.

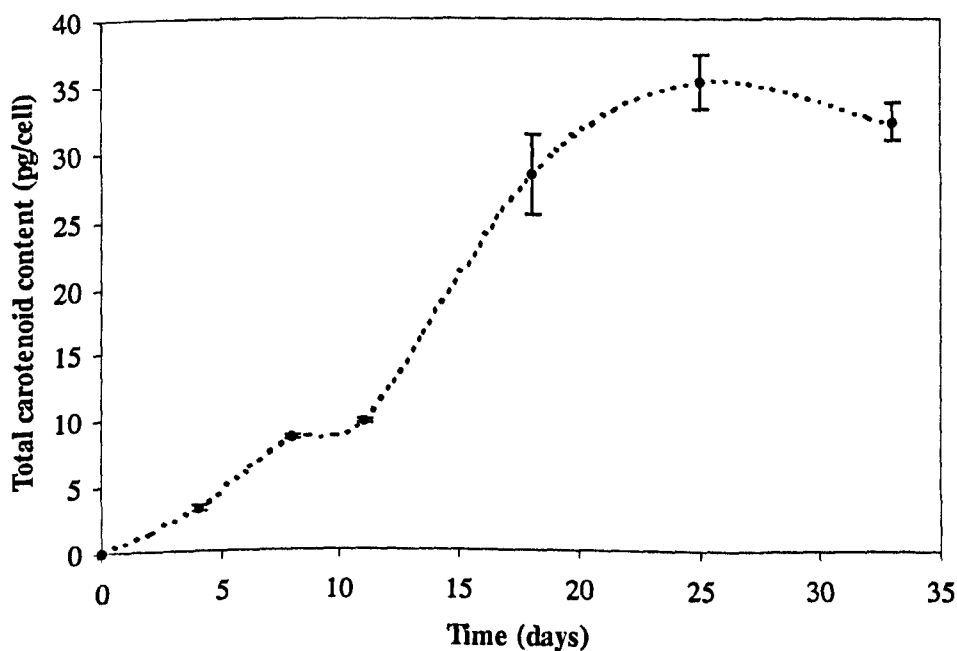


Figure 4.5. The total carotenoid content, calculated as pg/cell ($n=12 \pm$ S.E.) of *H. pluvialis* (Culture one) grown in the AAPS™1 during September and October 1998.

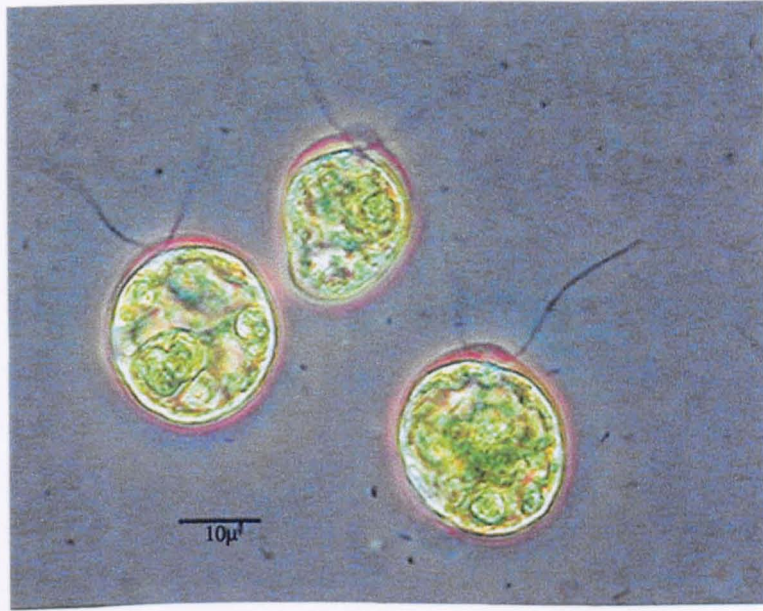


Plate 4.1a. Green zooid cells of *H. pluvialis*.

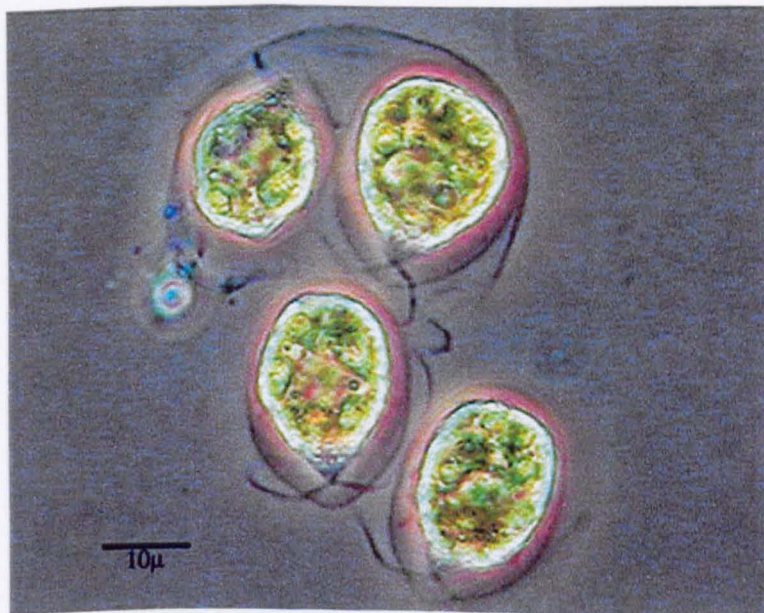


Plate 4.1b. Germinating green zooid cells of *H. pluvialis*.

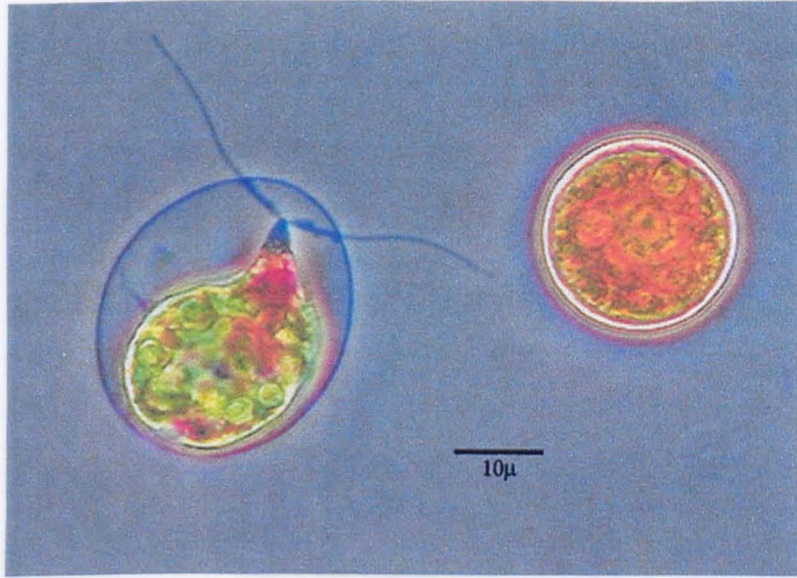


Plate 4.1c. An aplanospore and zooid cell of *H. pluvialis* (zooid cell shows localised carotenoid accumulation).

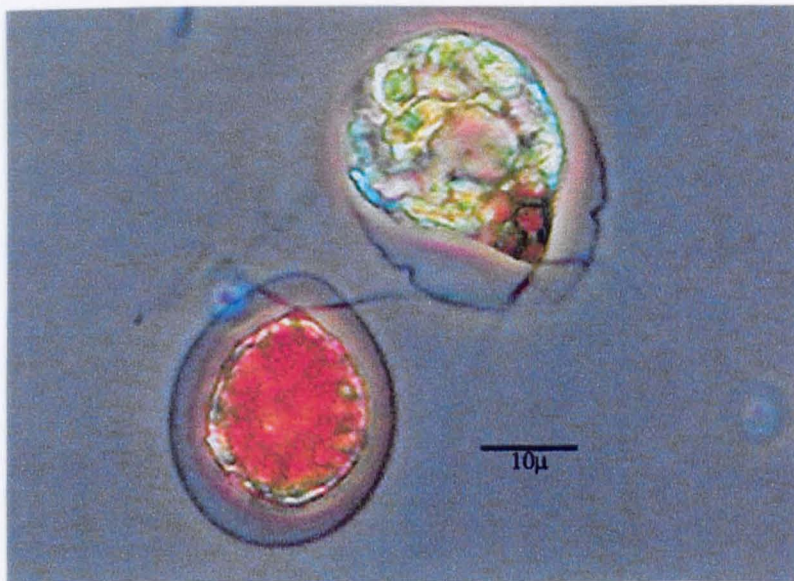


Plate 4.1d. Zooid cells of *H. pluvialis* with localised carotenoid accumulation.

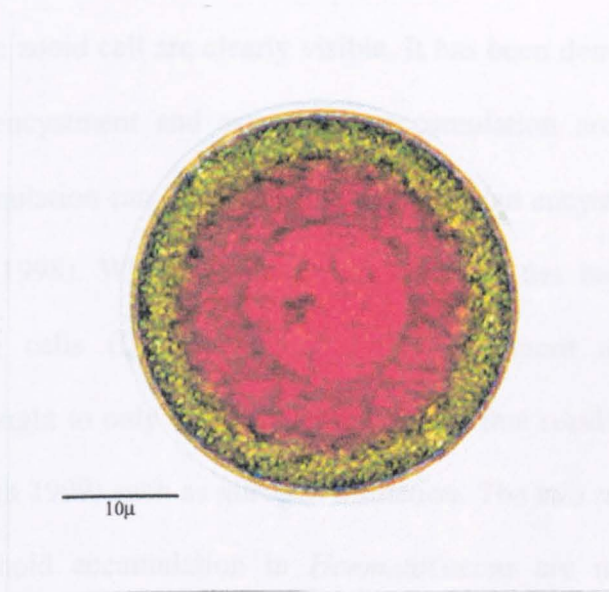


Plate 4.2a. A young aplanospore of *H. pluvialis*, with secondary carotenoids localised in the centre of the cell and chlorophyll visible around the periphery.

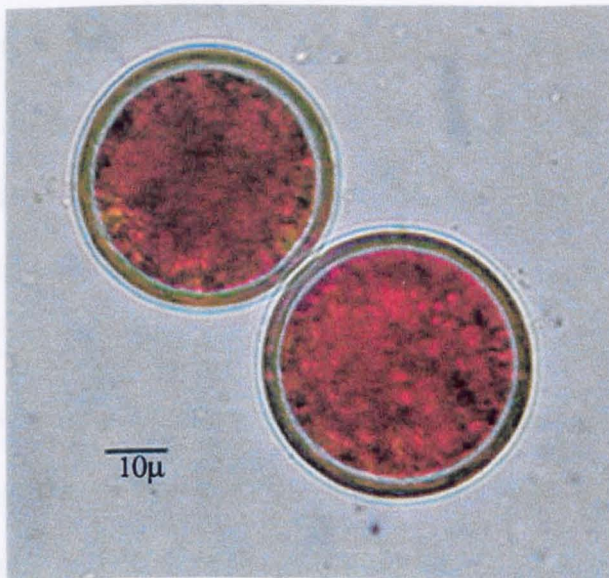


Plate 4.2b. Two highly encysted aplanospores of *H. pluvialis*.

The photomicrographs were taken under phase contrast and the two flagella at the anterior of the zooid cell are clearly visible. It has been demonstrated by a number of authors that encystment and astaxanthin accumulation are not linked, and that astaxanthin accumulation can occur in zooid cells without encystment (Grünewald *et al* 1997, Sun *et al* 1998). Whereas astaxanthin synthesis has been shown to occur in actively dividing cells (Lee and Ding 1994, Chaumont and Thépenier 1995), encystment is thought to only occur under conditions that result in the slowing of cell division (Margalith 1999) such as nitrogen limitation. The two major factors that cause secondary carotenoid accumulation in *Haematococcus* are nutrient limitation and exposure to high irradiances (Kobayashi *et al* 1992, Zlotnik *et al* 1993, Harker *et al* 1996a). Therefore it is reasonable to assume that light was the key factor inducing carotenoid accumulation in this culture and not nitrogen limitation.

At the point of harvest only approximately 50% of the cells were resting aplanospores, with the remainder as red zooid cells. Plate 4.1c. shows a typical aplanospore produced from an outdoor AAPS™ alongside a zooid cell with localised carotenoid accumulation. Further accumulation within the zooid cell would result in an overall red appearance as shown in plate 4.1d. The two cell types differ in a number of ways. The two flagella of the zooid cell are clearly visible as is the membrane which surrounds the cell, which is ovoid in shape. The aplanospore is spherical with a much thicker cell wall, which appears as a white ring around the cell. Plates 4.2a and 4.2b shown photomicrographs of typical aplanospores produced from indoor grown cultures. Carotenoid accumulation begins in the centre of the cell, with chlorophyll still visible around the periphery (Plate 4.2a). As the cells age further the level of carotenoid accumulation increased until the cells appear completely red (Plate 4.2b). The red colouration is much deeper than seen in the aplanospores produced from the AAPS™1.

4.2.1.3. Productivity

The culture produced 0.13mg carotenoid/l/day (± 0.00 , $n=9$, \pm S.E.) or 0.55mg carotenoid/g/day (± 0.01 , $n=81$, \pm S.E.). On a cellular basis the culture produced 0.94pg carotenoid/cell/day (± 0.01 , $n=144$, \pm S.E.). This corresponds to a yield, at the point of harvest on day 33 of 4.57mg carotenoid/l (Figure 4.4) or 32.25pg carotenoid/cell (Figure 4.5). In relation to the dry weight of the culture it yielded 18.82mg carotenoid/g (Figure 4.4) or 1.24% (w/v) carotenoid (± 0.08 , $n=9$, \pm S.E.).

The total yield from the culture (assuming a volume at harvest of 55l) was 0.25g of carotenoid. This translates to a yield of 0.46g per 100l.

The culture, on the day of harvest is shown in plate 4.3.

4.2.2. Culture two

The second culture in AAPS™1 was carried out using the same sterilisation procedure, media recipe and inoculation density of algae as the first culture. However the culture failed to produce a culture with a high cell density, after 27 days the culture had only increased from 1.30×10^4 cells/ml (± 0.37) to 5.96×10^4 cells/ml (± 0.05 , $n=4 \pm$ S.E.). A large number of 'ghost cells' were also present in the culture. These consisted of a cell wall with no visible internal organelles. As the system had successfully produced an astaxanthin-rich culture previously it was concluded that climatic conditions during November and December 1998 were probably too poor to enable a culture of *H. pluvialis* to grow.



Plate 4.3. A carotenoid-rich culture of *H. phuvialis* produced in the AAPS™1 during September and October 1998 (Culture one).

Unfortunately, limited climatic information was measured during the culture period, however data was obtained for Manchester Airport and is shown in Table 4.2. By comparing this information with the weather data obtained for September - October 1998 (duration of culture one in the AAPS™ 1), it is evident that the mean, maximum and minimum temperatures are all much lower during the second run. There were also fewer sun hours, as would be expected for the later time of year. The AAPS™ does not have any means of heating, only cooling under conditions of high temperature.

Table 4.2. Weather conditions at Manchester Airport from September to December 1998. Data from Reading University.

		Temperature (°C)			Sun hours	Total Rainfall (mm)
		Mean	Maximum	Minimum		
Culture 1	September 1998	14.8	23.5	6.1	133	44
	October 1998	10.5	18.3	0.7	76	179
Culture 2	November 1998	6.3	14.2	-1.9	56	71
	December 1998	5.4	14.9	-5.7	46	51

4.2.3. Culture three

The third culture of *H. pluvialis* was inoculated on 29th April 1999 and harvested on 19th May 1999.

4.2.3.1. Growth

The culture exhibited a short lag phase and then grew exponentially until day six (Figure 4.6), the culture doubled every 3.15 days during this period (specific growth rate 0.22 days). Growth then slowed although it did not reach a true stationary phase.

The culture achieved a maximal cell density of 1.70×10^5 cells/ml on day 20. The dry weight of the culture increased steadily over the course of the run reaching a density of 0.49g/l (Figure 4.6). The dry weight per cell of the culture (Figure 4.7) showed some fluctuation until the point of harvest on day 20. There was a marked drop between day four and six which corresponded with a period of rapid growth within the culture (Figure 4.6). The pH of the culture was a constant 7 (Figure 4.8) however as noted in culture one there was no *in situ* measurement therefore possible fluctuations during the daylight cycle were not detected as all measurements were taken at 0900hrs.

After harvest the system was filled with tap water and left circulating overnight. Approximately 19 hrs later the water in the system appeared red. Examination of the culture under the microscope revealed that it consisted primarily of red zooid cells at a density of 15.00×10^4 cells/ml (Figure 4.6). The dry weight of the culture was 0.16g/l (Figure 4.6). The dry weight per cell (Figure 4.7) was lower than it had been during the initial culture period. These cells probably germinated from aplanospores that had been left in the photostage, adhered to the walls, after harvest of the initial culture on the previous day. Monitoring of the culture over the next six days saw a slow decrease in cell number, a slight increase in the dry weight and a decrease in the carotenoid content per cell. At the same time the cells increased in weight (Figure 4.7). On day 26 the system was harvested for a second time with a cell density of 14.00×10^4 cells/ml and a dry weight of 0.17g/l.

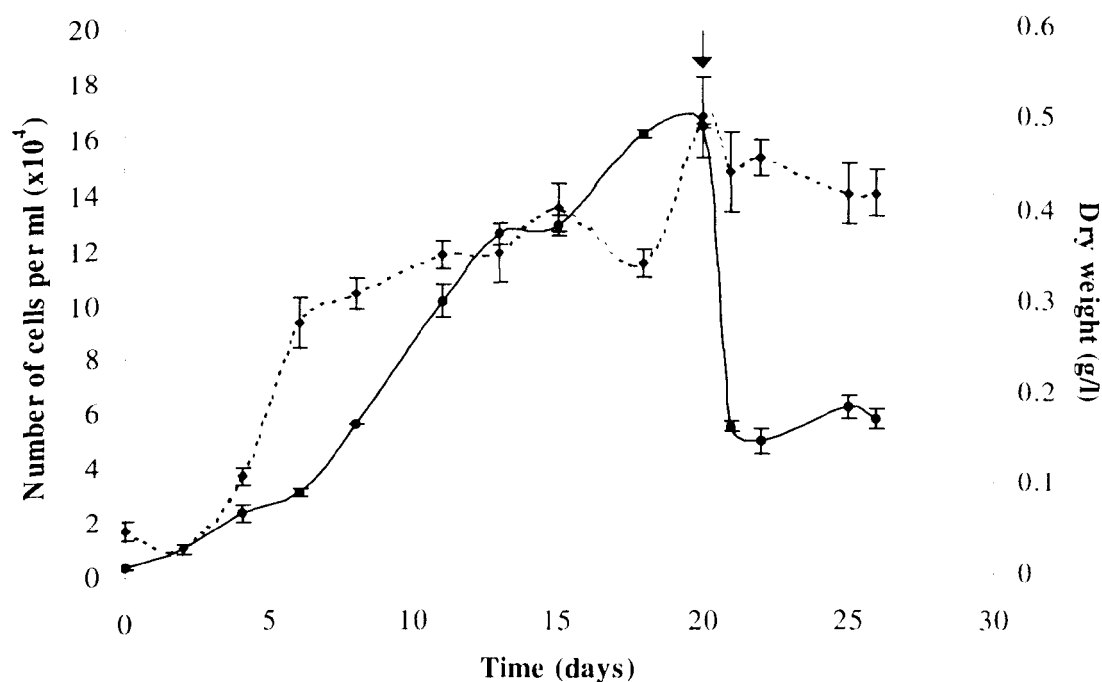


Figure 4.6. Cell density (---, $n=4 \pm$ S.E.) and dry weight (—, $n=3 \pm$ S.E.) of *H. pluvialis* (Culture three) grown in the AAPS™1 during April and May 1999. The culture was harvested on day 20 (→) and filled with tap water prior to cleaning (see text for details).

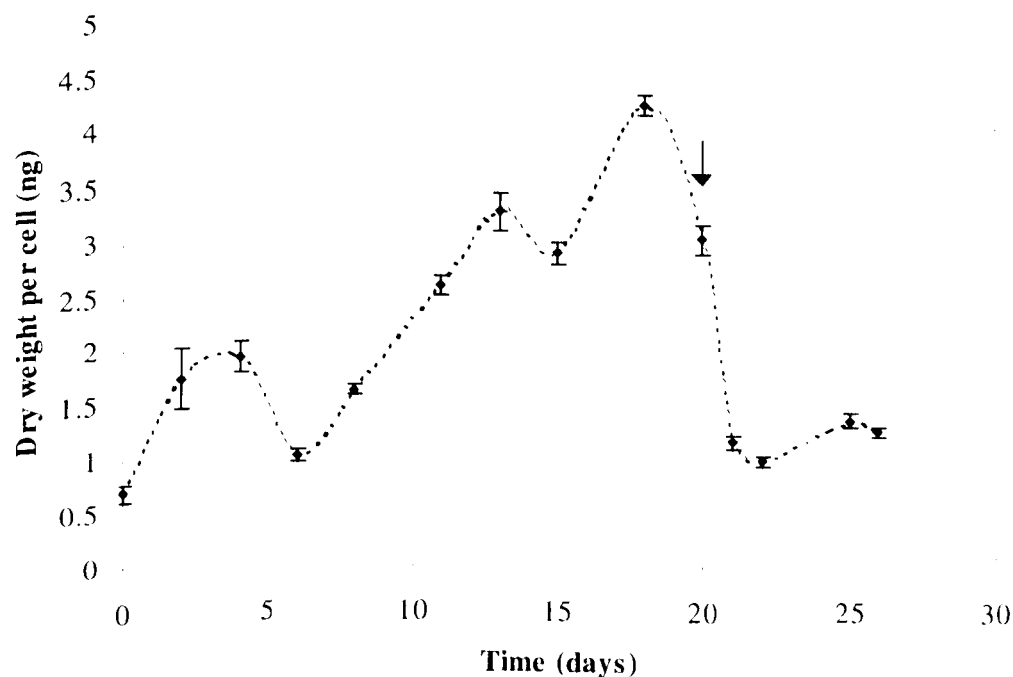


Figure 4.7. Dry weight per cell ($n=12 \pm$ S.E.) of *H. pluvialis* (Culture three) grown in the AAPS™1 during April and May 1999. The culture was harvested on day 20 (→) and filled with tap water prior to cleaning (see text for details).

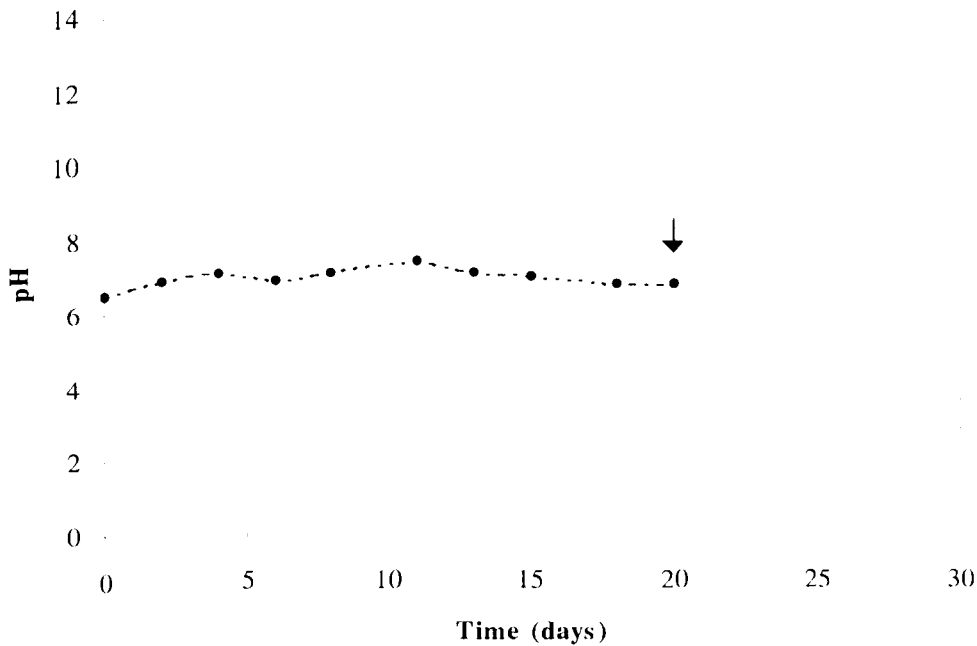


Figure 4.8. pH of the *H. pluvialis* culture (Culture three) grown in the AAPS™1 during April and May 1999 (All measurements were taken at 0900hrs). The culture was harvested on day 20 (→) and filled with tap water prior to cleaning, (see text for details), no pH data is available from day 20.

Early researchers had noted the germination of red zooid cells from aplanospores. Peebles (1909) termed them microzooids and noted that they rarely appeared in wild cultures but were frequently formed when aplanospores were exposed to intense sunlight. Her work also noted that they frequently died, which may explain the decrease in the cell number of this culture over time. Hazen (1899) also noted the formation of these red zooid cells, and demonstrated that by placing aplanospores into a dish of water and leaving them overnight, the following morning the cells were in the process of division, forming zooids which were rich in secondary carotenoids.

4.2.3.2. Secondary carotenoid accumulation

Unlike the first culture carotenoid accumulation was co-incident with the cessation of growth and started towards the end of the exponential phase of growth (Figure 4.9 and 4.10). The culture was harvested on day 20 (Plate 4.4 shows the culture on the day it was harvested). The culture mainly consisted of aplanospores rather than red zooid cells that had been present in the first culture (section 4.2.1.2). As both cultures were grown in the same media it seems likely that this difference in the developmental stage of the culture was an effect of climatic conditions.

A noticeable feature of this culture was the large reduction in the carotenoid content of the algae (from 127pg/cell to 85pg/cell, Figure 4.9) between day 18 and the harvest on day 20. This drop was mirrored by a reduction in the content when expressed as mg/l (Figure 4.10), although cell density remained fairly constant during this period (Figure 4.6). The reason for this drop in astaxanthin levels is unclear, although as no additions were made to the culture it seems likely that it was an effect of climatic conditions. However, no climate data on a day to day basis is available so it is impossible to draw firm conclusions.

The germination of *Haematococcus* aplanospores occurred on day 20 after the system was filled with tap water and left aerated overnight. Plate 4.5 shows the red zooid culture that was present in the system approximately 19 hours after refilling the system with tap water. This culture had a total carotenoid content of 35.98pg/cell (Figure 4.9), ~30% of the maximum content of the parent culture (harvested the previous day), but still comparable to the content per cell harvested in culture one (section 4.2.1.3.).

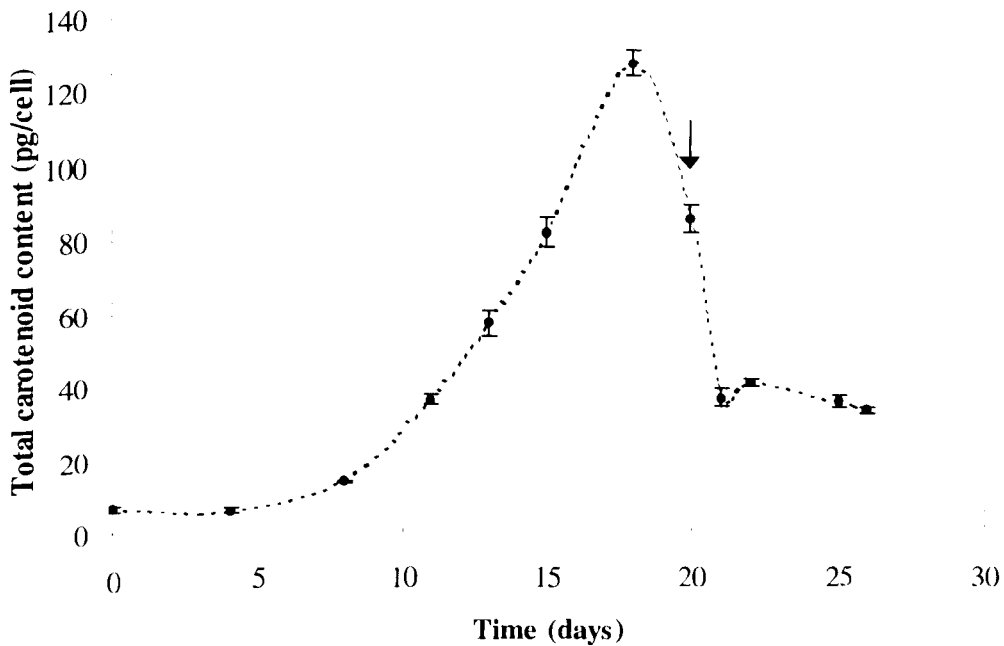


Figure 4.9. The total carotenoid content, calculated as pg/cell ($n=12 \pm \text{S.E.}$) of *H. pluvialis* (Culture three) grown in the AAPS™1 during April and May 1999. The culture was harvested on day 20 (→) and filled with tap water prior to cleaning (see text for details).

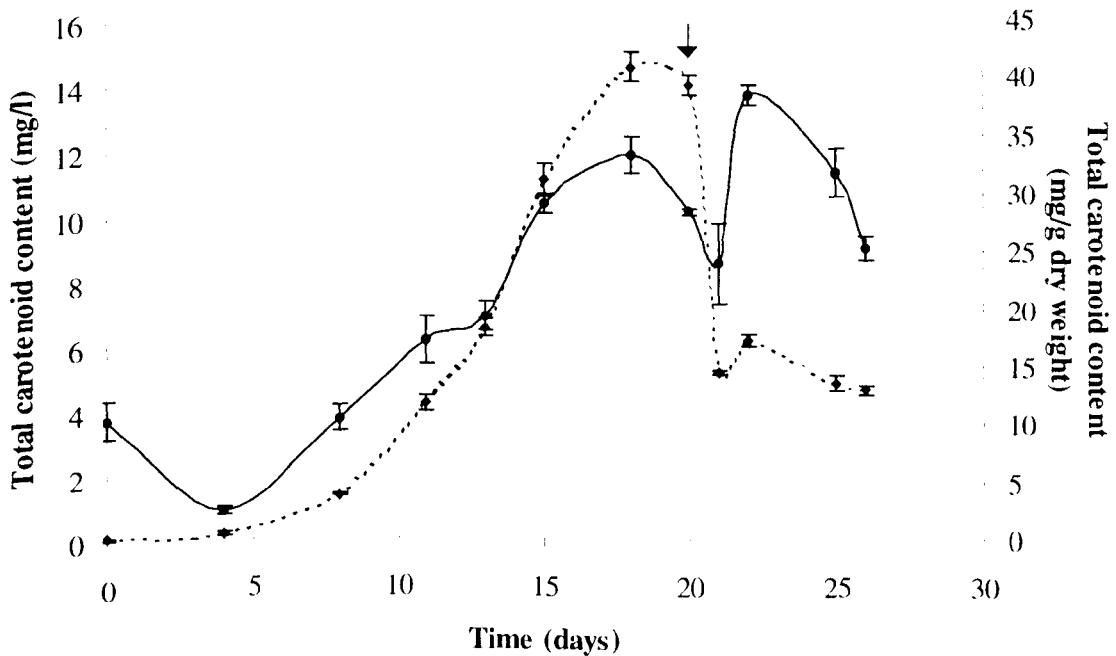


Figure 4.10. The total carotenoid content, calculated as mg/l (---, $n=3 \pm \text{S.E.}$) and mg/g dry weight (—, $n=9 \pm \text{S.E.}$), of *H. pluvialis* (Culture three) grown in the AAPS™1 during April and May 1999. The culture was harvested on day 20 (→) and filled with tap water prior to cleaning (see text for details).



Plate 4.4. A carotenoid-rich culture of *H. pluvialis* produced in the AAPS™1 during April and May 1999 (Culture three).



Plate 4.5. Appearance of the AAPS™1 the day after the harvest of culture three. The system contained tap water, the red colouration was given by the red zooids that germinated overnight from aplanospores that remained in the system after harvest (see text for details).

4.2.3.3. Carotenoid composition

The carotenoid composition of the culture harvested from the AAPS™1 was determined using TLC as detailed in Chapter 2 and is shown in Table 4.3.

Table 4.3. Carotenoid composition of *H. pluvialis* harvested from the AAPS™1 (Culture three).

Carotenoid	Carotenoid composition (%) ($n=3 \pm$ S.E.)
Lutein	1.31 (\pm 0.06)
Free Astaxanthin	0.75 (\pm 0.00)
Canthaxanthin	3.26 (\pm 0.08)
Astaxanthin mono-esters	71.65 (\pm 1.20)
Adonirubin esters	1.09 (\pm 0.19)
Astaxanthin di-esters	21.96 (\pm 0.94)
β -Carotene	0.74 (\pm 0.06)

4.2.3.4. Productivity

The productivity of the system to day 20 (the date of the first harvest) was 0.66mg carotenoid/l/day (\pm 0.01, $n=9$, \pm S.E.) or 0.90mg carotenoid/g dry weight algae/day (\pm 0.03, $n=81$, \pm S.E.). Per cell the culture productivity was 3.71pg carotenoid/cell/day (\pm 0.10, $n=144$, \pm S.E.).

The yield from the harvest was 13.94mg carotenoid/l (Figure 4.10) or 85.07pg carotenoid/cell (Figure 4.9). In terms of the dry weight of the culture it yielded 28.42mg carotenoid/g dry weight (Figure 4.10) or 2.80% (w/v) dry weight (\pm 0.07, $n=9$, \pm S.E.). The total yield from this run was 0.79g of carotenoid. The total astaxanthin content (including esters) of the culture was 94%. From the calculated yield of total

carotenoids from the culture, culture three yielded 0.74g of astaxanthin (mainly as monoesters), or 0.013g/l of culture. This translates to an overall yield of astaxanthin of 1.3g per 100l.

The productivity of this culture was much higher than the first; the first culture produced 0.94pg carotenoid/cell/day and after 33 days the culture had a total carotenoid content of 1.24% (w/v) dry weight. The media components of the two runs were identical and they achieved that same maximal cell count with similar cellular productivities. Therefore it is reasonable to assume that climatic conditions were a key factor in determining the productivity of these cultures.

4.3. Advanced Algal Production System 2 (AAPS™2)

The first set of outdoor cultures described in section 4.2 provided valuable information as to the behaviour of *H. pluvialis* when cultured in a large volume outdoors. Information was gained as to the behaviour of the alga in relation to its developmental stage during growth outdoors, as well as the rate of carotenoid accumulation. In addition, several important questions result. Firstly the importance of climatic data such as irradiance levels and temperature. Also the need for more than one AAPS™ unit to enable the more rapid development of the culture methodology as well as enabling the comparison of cultures under the same climatic conditions. With this in mind two AAPS™2 units were constructed and installed on the roof of the Byrom Street campus of Liverpool John Moores University. At the same time a weather station (LiCor LI-1400 datalogger, Glen Spectra Ltd, London, UK, see Chapter 2 for details) was also set up next to the two systems to enable continuous monitoring of irradiance and temperature.

Perhaps the most important point to arise from the culture of *H. pluvialis* in the AAPS™1 was the interaction between the nitrogen status of the culture and the irradiance that the cultures are exposed to when cultured outdoors. Therefore the importance of the nitrogen concentration of the media in the culture of *H. pluvialis* was assessed in the two AAPS™2. Before this the depletion of nitrogen was assessed in flask culture.

4.3.1. Aims

To compare the biomass and carotenoid productivities of the culture media with two different concentrations of the nitrogen source, firstly under controlled conditions in the laboratory and secondly outdoors in the AAPS™2.

4.3.2. Comparison of media with different nitrogen concentrations (in shake flask culture)

All the large-scale AAPS™ cultures up to this point had employed a growth media containing urea as the nitrogen source at a concentration of 0.75mM (low [N]). A second media had been developed which had a urea content of 3.00mM (high [N]) (Harker 1995). Previously, in flask culture, the high [N] produced a greater yield in terms of cell number and biomass, but the rate of accumulation of secondary carotenoids was slower due to apparent inhibition by the higher residual nitrogen content (Harker 1995). The performance of these two media was again assessed in flask culture in this project.

In flask culture, the low [N] media produced fewer cells per unit volume of culture (~30% of the cell density produced by the high [N] media, Table 4.4). Both

cultures had very similar yield on a dry weight basis, however the dry weight per cell of the culture produced by the low [N] media was much greater. The high [N] media had a higher cellular productivity both during the exponential phase of growth and through the entire culture. The culture grown in the low [N] media grew exponentially for 14 days with a specific growth rate (μ) of 0.17 days, corresponding to a culture doubling time (t_d) of 4.17 days. Over the first 14 days of growth the culture grown in the high [N] media had a doubling time of 4.55 days ($\mu=0.15$ days).

Table 4.4. Productivity of *H. pluvialis* in flask culture.

	Low [N]	High [N]
Final cell density ($\times 10^4$ cells/ml, $n=4 \pm$ S.E.)	30.00 (± 0.91)	117.50 (± 5.96)
Final dry weight (g/l, $n=3 \pm$ S.E.)	7.12 (± 0.58)	7.13 (± 0.11)
Final dry weight per cell (ng, $n=9 \pm$ S.E.)	32.15 (± 1.30)	6.48 (± 0.19)
Cell productivity during exponential growth ($\times 10^4$ cells/ml/day, $n=12 \pm$ S.E.)	1.91 (± 0.03)	3.10 (± 0.07)
Cell productivity over the whole culture period ($\times 10^4$ cells/ml/day, $n=12 \pm$ S.E.)	0.15 (± 0.03)	0.70 (± 0.05)

The carotenoid content of the two cultures, when expressed on a % dry weight basis, is very similar (2.61 and 2.89%, Table 4.5). However the concentration per cell is very different, 827pg/cell in low [N] compared to 188pg/cell in high [N]. This suggests that there is a difference in the level or degree of encystment between the two cultures. This is further supported by the increased dry weight per cell in the low [N] culture.

This may be because the low [N] culture would have used up the available nitrogen more rapidly (Figure 4.11). Therefore the cells would have begun the encystment process at an earlier stage giving them a longer period of time in which to encyst. This would result in the cells grown in the low [N] media being much larger and heavier (Table 4.4, Zlotnik *et al* 1993, Lee and Ding 1994). Also as the high [N] media produced more cells there would have been a higher degree of cell shading within the culture and consequently a lower irradiance per cell (Raven 1988, Eriksen *et al* 1996). Irradiance is known to have a strong affect on carotenogenesis (Kobayashi *et al* 1992, Harker *et al* 1995). However in this case the cultures were grown under an irradiance of 40 $\mu\text{mol}/\text{m}^2/\text{s}$ therefore nitrogen would be expected to have been the single major factor influencing the rate of carotenogenesis (Fábregas *et al* 1998, Sun *et al* 1998).

Table 4.5. Carotenoid productivity of *H. pluvialis* in flask culture.

	Low [N]	High [N]
Total carotenoid productivity		
mg/l/day ($n=9 \pm \text{S.E.}$)	1.33 (± 0.05)	1.67 (± 0.03)
pg/cell/day ($n=144 \pm \text{S.E.}$)	6.02 (± 0.12)	1.49 (± 0.05)
mg/g/day ($n=81 \pm \text{S.E.}$)	0.16 (± 0.01)	0.13 (± 0.00)
Total carotenoid yield after 150 days		
mg/l ($n=3 \pm \text{S.E.}$)	183.23 (± 13.60)	206.20 (± 3.27)
pg/cell ($n=12 \pm \text{S.E.}$)	827.38 (± 31.38)	187.37 (± 5.43)
mg/g dry weight ($n=12 \pm \text{S.E.}$)	26.06 (± 1.40)	28.93 (± 0.33)
% dry weight ($n=9 \pm \text{S.E.}$)	2.61% (± 0.14)	2.89% (± 0.03)

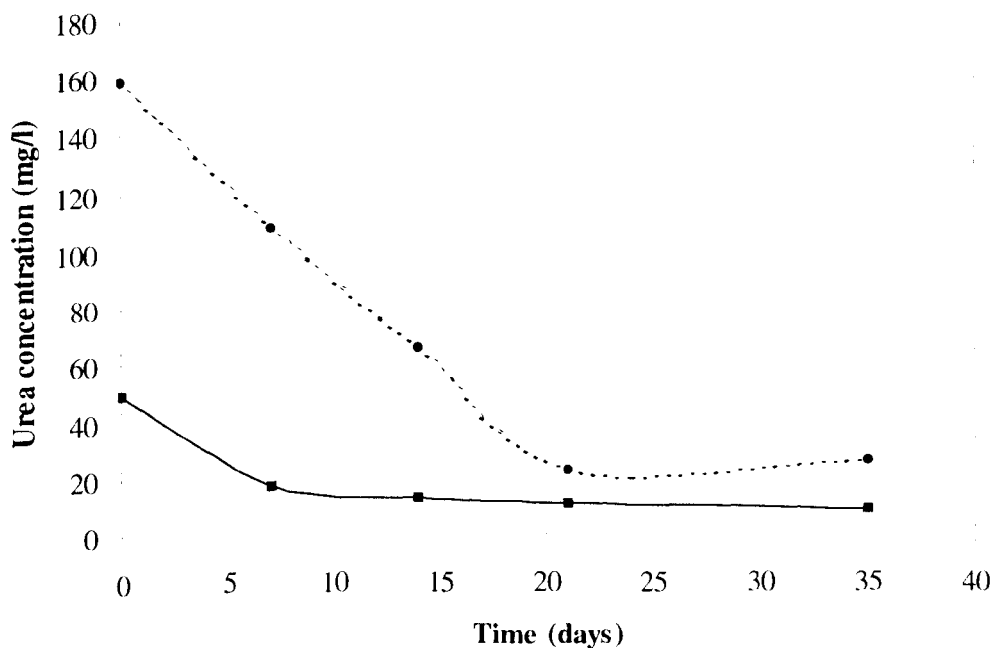


Figure 4.11. Urea concentration (mg/l, n=1) of flask cultures of *H. pluvialis* grown in the two media types (measurements were only taken until the end of the exponential phase of growth). Before the samples were analysed, three replicate analyses (of fresh autoclaved high and low [N] media) were carried out, the results did not differ by >3%. High [N] --- and low [N] —.

Each of the two media has its own advantages. The high [N] media produced more cells but the low [N] produced carotenoid-rich cells more rapidly (under identical conditions of irradiance), the low [N] media also accumulated carotenoids to a much higher concentration within the cell. A direct comparison between the two media was undertaken using two identical AAPSTTM2 systems operating outdoors. They were biologically commissioned at the same time under the same operating conditions. Both systems were given the same inoculation density (1×10^4 cells/ml) so that the only apparent difference between the two was the concentration of urea in the media.

4.3.3. Cultures five and six (AAPS™2)

A comparative study using the two media was carried out in the AAPS™2 from 27th August to 13th October 1999. Culture five was grown in the low [N] media, culture six was grown in the high [N] media (see Appendix 1a for details), in the AAPS™2 sited outdoors, adjacent to each other (but not in each others shadow).

4.3.3.1. Growth

The growth of the two cultures was comparable for the first 10 days (Figure 4.12), culture five (low [N] media) had a doubling time of 4.62 days, the culture grown in the high [N] media (culture six) doubled in 4.08 days. This compares favourably with the doubling times of flask grown cultures (section 4.3.2.).

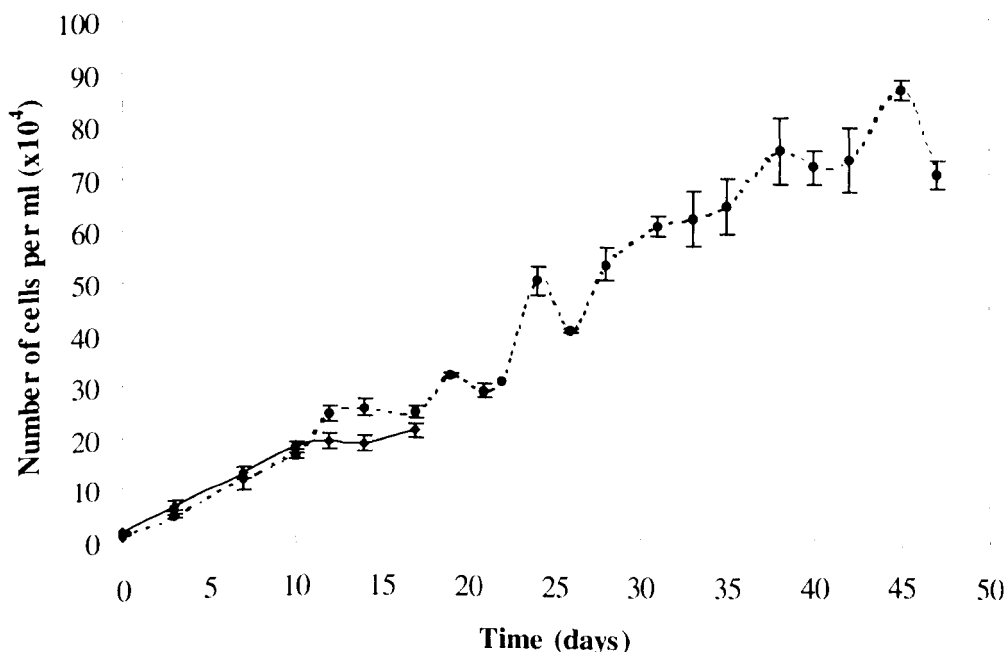


Figure 4.12. Cell density ($n=4 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) —, High [N] media (Culture six) - - - .

Cellular productivities were also similar during exponential growth. The low [N] cultures produced 1.46×10^4 cells/ml/day (± 0.48 , $n=16$, \pm S.E.), the high [N] culture had a slightly lower productivity of 1.30×10^4 cells/ml/day (± 0.26 , $n=16$, \pm S.E.). The cultures grew exponentially with no apparent lag phase. Overall the culture grown in high [N] media was more productive as it remained in exponential growth for nearly four-times longer than the culture grown in the low [N] media. The higher nitrogen content of the culture did not result in an increased growth rate *per se* but in a longer growth phase. In flask culture, the higher urea content resulted in a doubling of the length of the growth phase.

Measured over the full 47 days the cellular productivity in high [N] was 1.43×10^4 cells/ml/day (± 0.57 , $n=16$, \pm S.E.). Over its culture period of 17 days the culture grown in the low [N] the system produced 1.04×10^4 cells/ml/day (± 0.39 , $n=16$, \pm S.E.). The biomass, as dry weight, of both cultures steadily increased for the duration of the culture. After 17 days, the culture grown in the low [N] media contained 0.76g/l (Figure 4.13). In comparison the high [N] media yielded 1.70 g/l after 47 days, more than twice that of the low [N] media. Despite having an overall higher biomass yield the dry weight per cell of the high [N] culture was lower (2.44ng) than the dry weight per cell of the culture grown in the low [N] media (3.73ng, Figure 4.14). This was also noted in flask culture, although the difference was much more marked. The cells produced in flask culture in the low [N] media were 80% greater in mass than those produced in the high [N] media (Table 4.4). As discussed previously, this is probably due to the low [N] culture starting to encyst at an earlier stage than the high [N] culture.

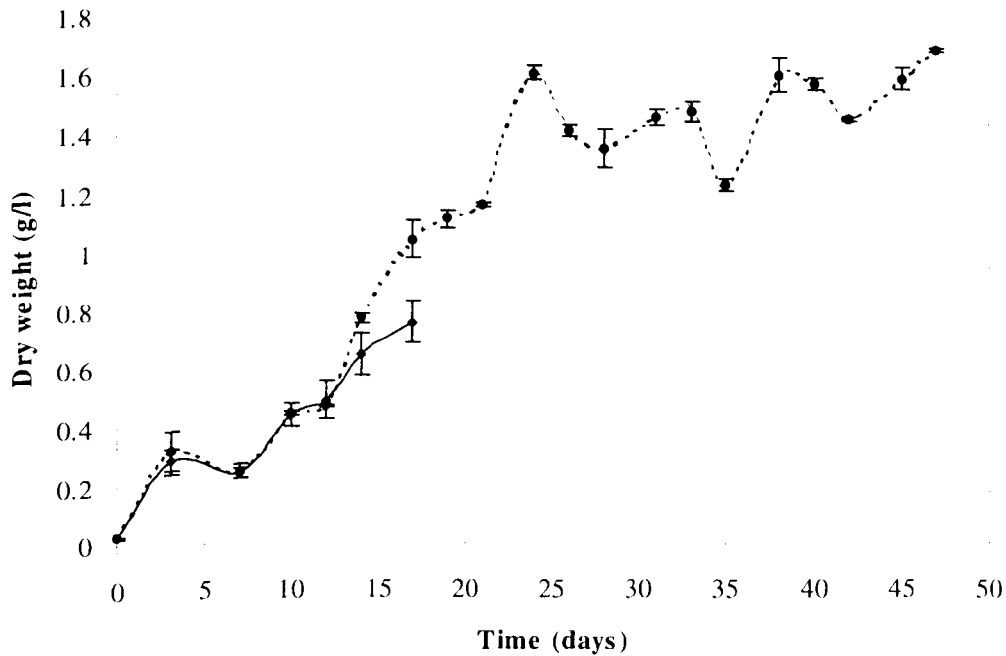


Figure 4.13. Dry weight ($n=3 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) —, High [N] media (Culture six) - - - .

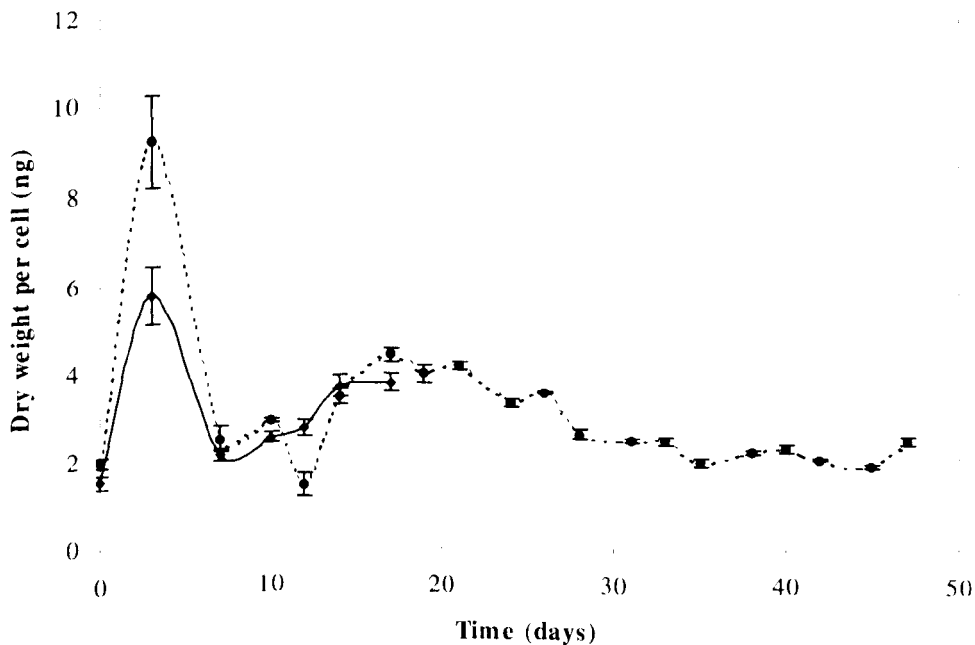


Figure 4.14. Dry weight per cell ($n=12 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) —, High [N] media (Culture six) - - - .

The irradiance that the culture was exposed to would have a significant effect on the photosynthetic efficiency of the culture and consequently growth as well as carotenogenesis (Raven 1988, Molina Grima *et al* 1997). Generally the growth rate of any alga increases with increasing irradiance until it reaches its maximal growth rate. Further increases in irradiance once the maximal growth rate has been reached may actually inhibit growth, causing photoinhibition (Molina Grima *et al* 1999). Photoinhibition is defined as the loss of photosynthetic capacity due to damage to the photosynthetic apparatus by excessive irradiance (Vonshak *et al* 1988, Henley 1993). Outdoor grown cultures are exposed to a changing light environment over the course of each day. Figures 4.15 and 4.16 demonstrate the diurnal fluctuation that the AAPS™2 cultures were exposed to on a cloudless and cloudy day. On a cloudless day the peak irradiance of nearly 1,400 $\mu\text{mol}/\text{m}^2/\text{s}$ was reached at 1.30pm. In contrast Harker *et al* (1995) demonstrated that the optimum irradiance for the growth of *Haematococcus* was 50-60 $\mu\text{mol}/\text{m}^2/\text{s}$. Photoinhibition would occur only when the irradiance increased beyond that which could be utilised by the photosynthetic apparatus, as this results in the energy received damaging photosystem II (PSII) (Henley 1993, Boussiba 2000). Typically photosynthesis is at its most efficient under early morning irradiance (Lee and Low 1992). Photoinhibition may occur toward noon as the irradiance increases. After the peak in irradiance, the photosynthetic apparatus does not fully recover and the photosynthetic rate of a culture is, typically, lower in the afternoon.

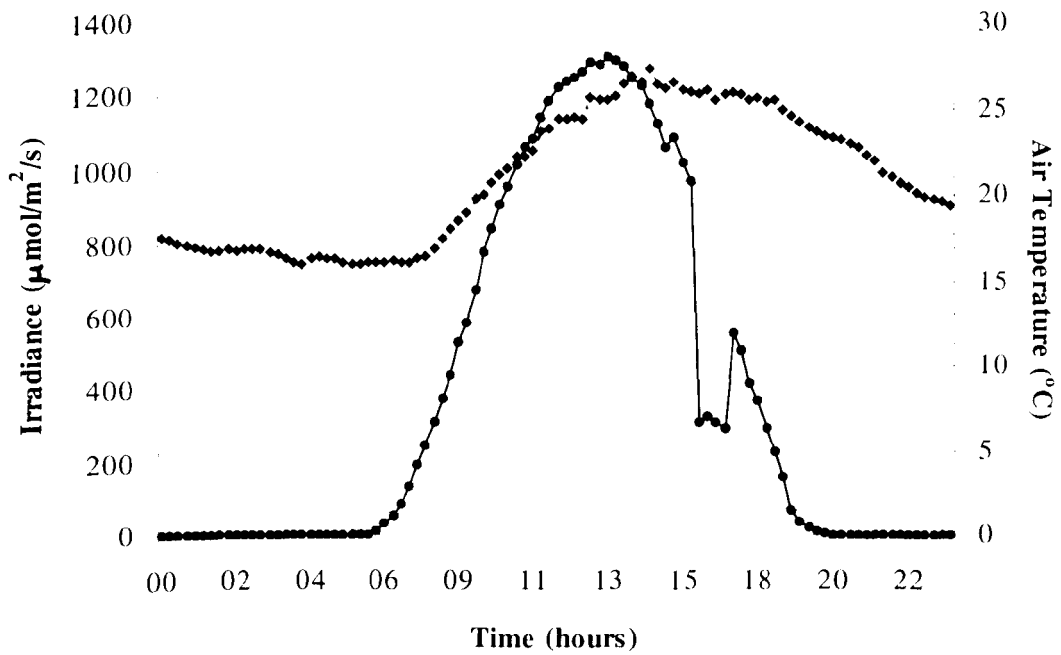


Figure 4.15. Changing irradiance and temperature adjacent to the two AAPS™2 on a cloudless day (5th September 1999, measurements were recorded every 15 mins). Irradiance ●, Air temperature ◆.

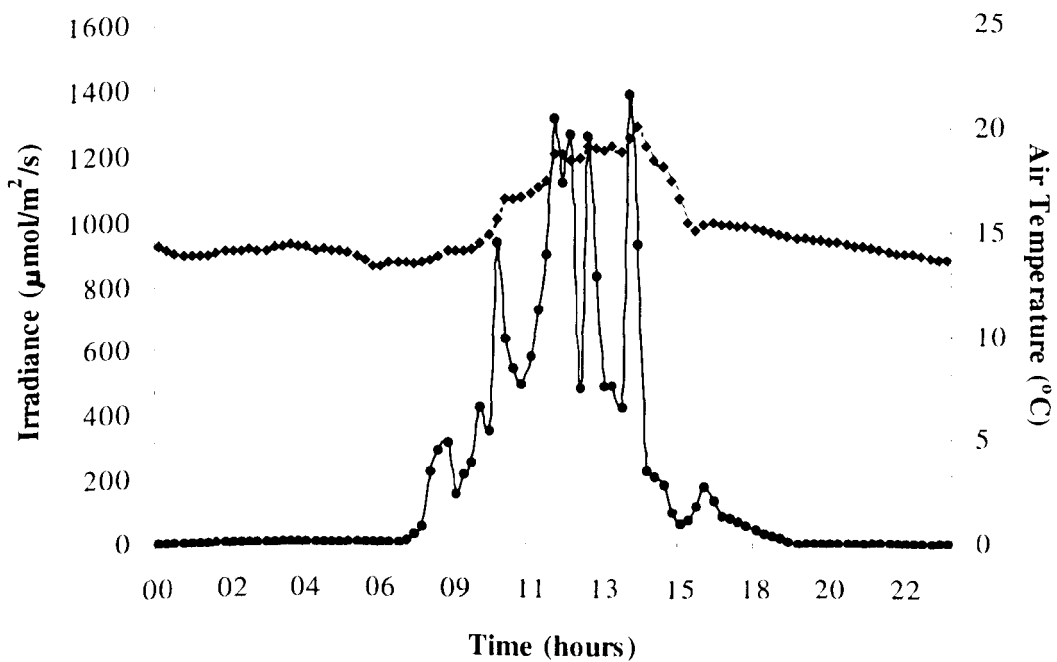


Figure 4.16. Changing irradiance and temperature adjacent to the two AAPS™2 on a cloudy day (25th September 1999, measurements were recorded every 15 mins). Irradiance ●, Air temperature ◆.

As with the previous cultures in the low [N] media no pH control was required, as the pH stayed constant at 7 (Figure 4.17). pH fluctuation was noted in the high [N] culture, it remained constant at 7 until day 10. It then increased, reaching a pH of 9.5 on day 17. The pH was brought back to 7 using 0.1M HCl, where it remained for the rest of the culture period. The pH was determined from aliquots removed from the AAPS™, as there was no *in situ* pH probe. All these samples were taken at 0900hrs, but subsequent work on an AAPS™ fitted with an internal pH probe used to culture *Haematococcus* in the high [N] media demonstrated that fluctuations in pH occurred throughout the daylight cycle. It is therefore unclear if by adjusting the pH to 7 with the addition of acid the pH would have remained constant throughout the day.

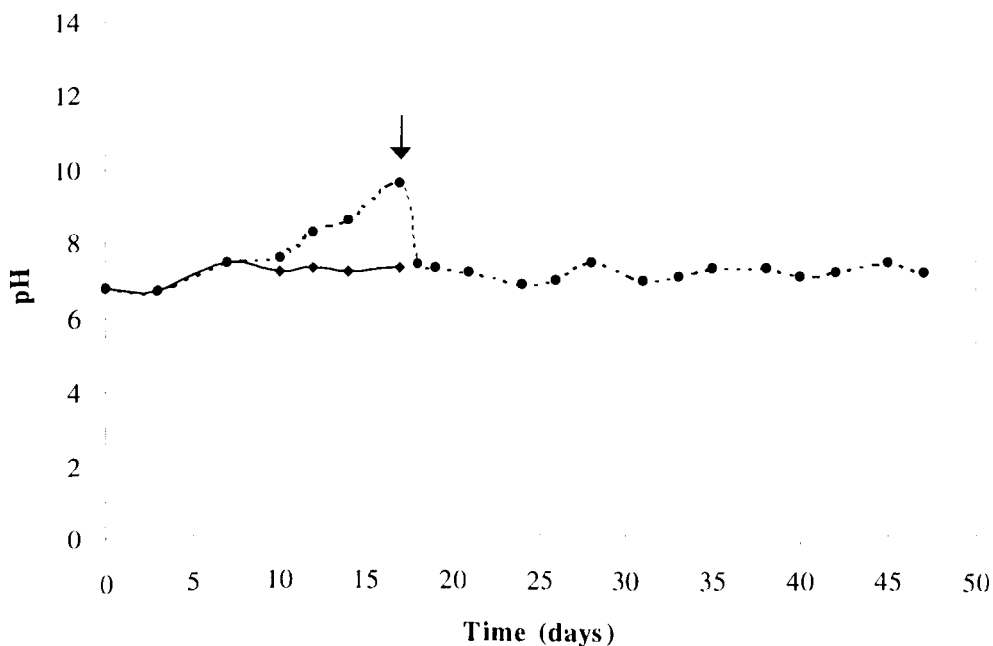
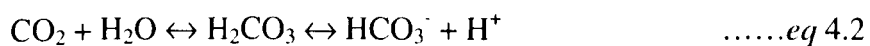


Figure 4.17. pH of the *H. pluvialis* cultures grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) —, High [N] media (Culture six) ---. All measurements were taken at 0900hrs, → indicates point of addition of HCl (see text for details).

Changes in the pH of algal cultures are due to the uptake of CO₂ by the culture (Camacho Rubio *et al* 1999). Nutrient uptake can also influence pH however urea is an uncharged chemical species and therefore has no effect on culture pH (Goldman *et al* 1982). CO₂ uptake influences pH as the dissolved CO₂ is in equilibrium with the carbonate and bicarbonate species in the culture medium. When CO₂ is taken up by the algal cells the loss is partially compensated for by regeneration from the carbonate and bicarbonate species as demonstrated by equations 4.1-4.3 below. Consequently there is a change in the culture pH (Camacho Rubio *et al* 1999).



4.3.3.2. Secondary carotenoid accumulation

Carotenoid accumulation (Figures 4.18, 4.19 and 4.20) began in both cultures on day seven. The chlorophyll *a* and *b* content per cell of the both cultures continued to increase for a further three days, until day 10 (Figure 4.21). The content per cell in the low [N] media then decreased, whereas the content in the cell of the culture grown in the high [N] media decreased only for a short period. The chlorophyll *a* and *b* content of the high [N] culture then increased again and continued to increase until day 21 (Figure 4.21). The content per cell of both chlorophyll *a* and *b* then decreased until day 35 where it remained stable until harvest. This behaviour was mirrored when the chlorophyll content was calculated on a per unit volume basis (Figure 4.22). The ratio of chlorophyll *a/b* of both cultures remained unchanged throughout the culture period.

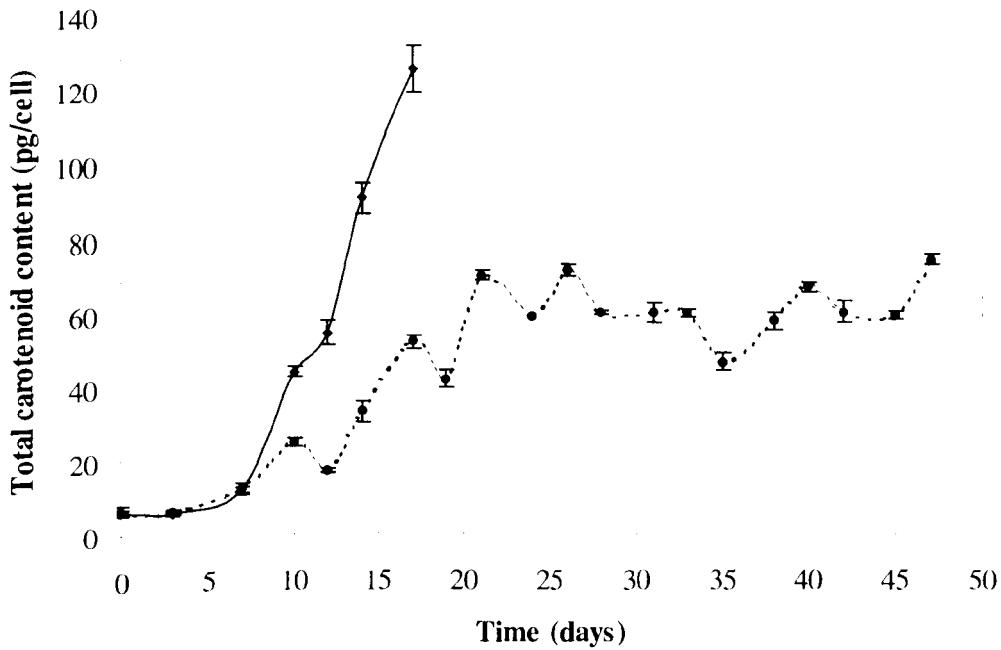


Figure 4.18. The total carotenoid content, calculated as pg/cell ($n=12 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) —, High [N] media (Culture six) - - - .

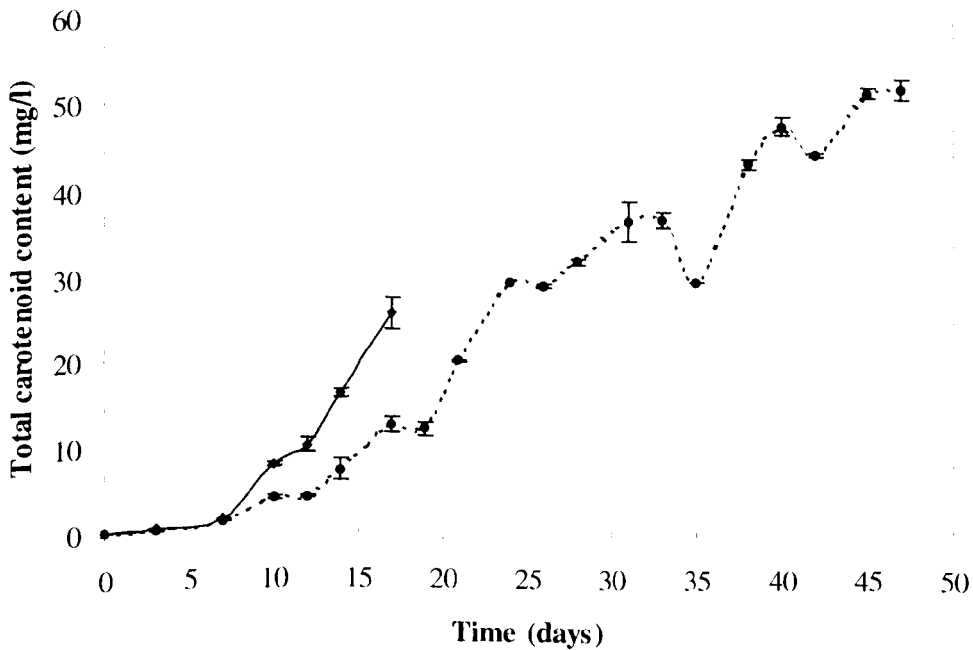


Figure 4.19. The total carotenoid content, calculated as mg/l ($n=3 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) —, High [N] media (Culture six) - - - .

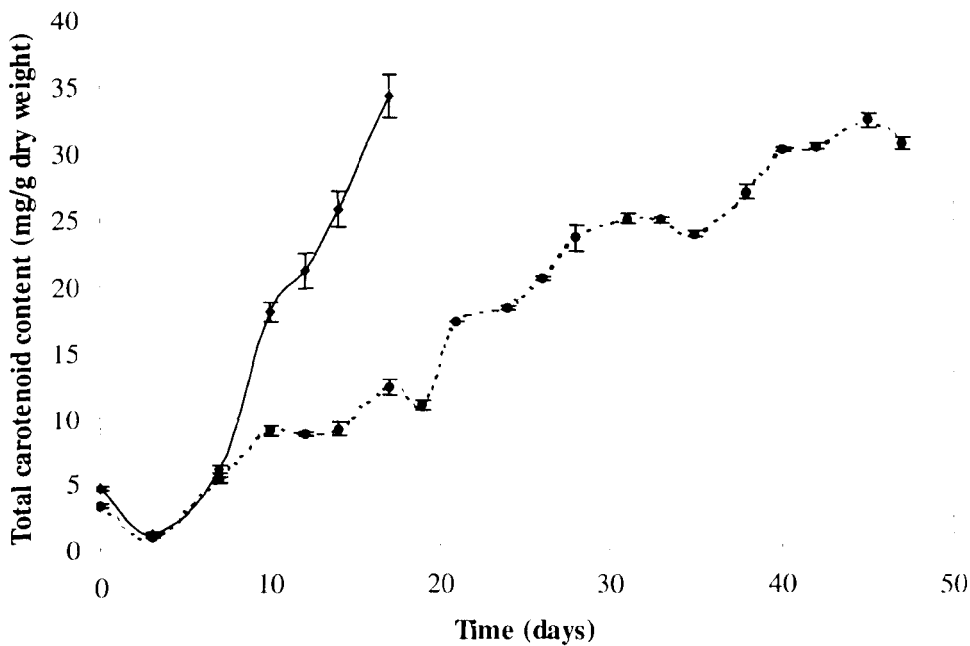


Figure 4.20. The total carotenoid content, calculated as mg/g dry weight ($n=9 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) —●—, High [N] media (Culture six) ---□---

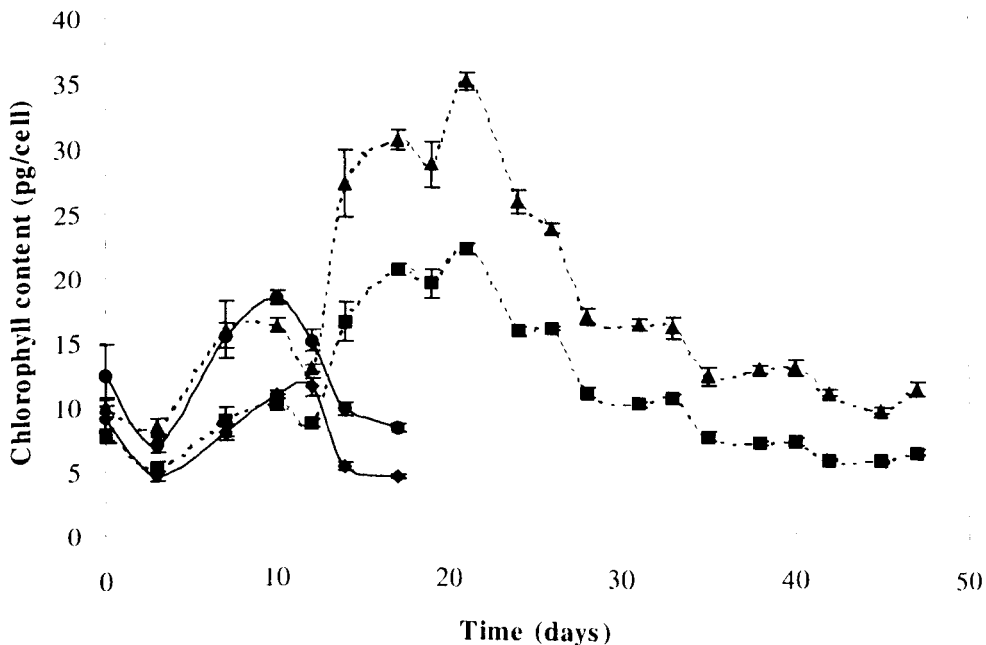


Figure 4.21. The chlorophyll *a* and *b* content, calculated as pg/cell ($n=12 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) chl *a* —●—, chl *b* —◆—, High [N] media (Culture six) chl *a* ---▲---, chl *b* ---■---

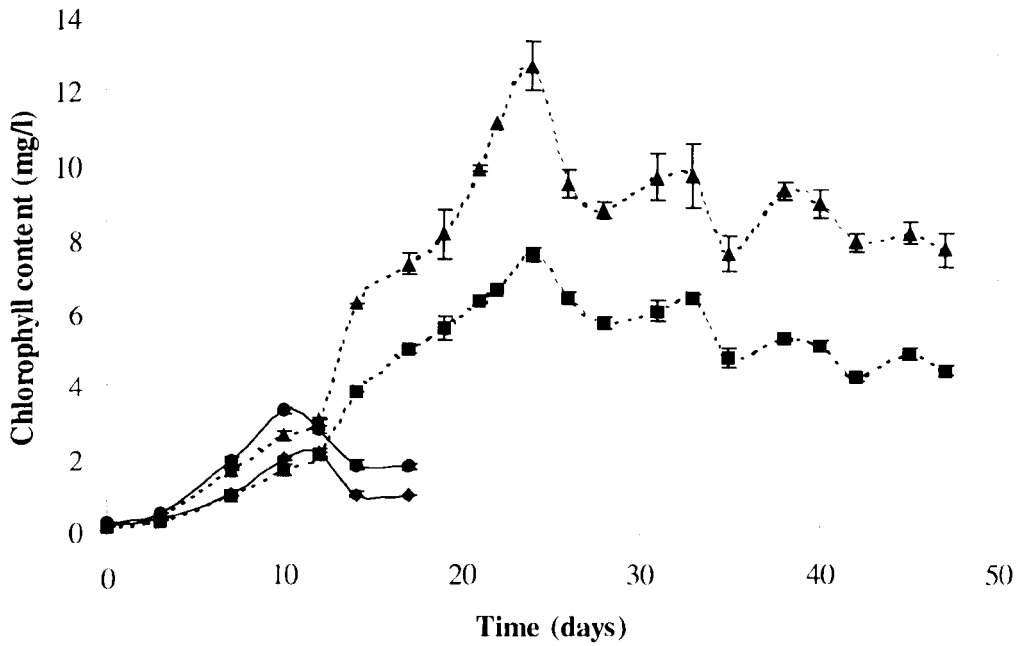


Figure 4.22. The chlorophyll *a* and *b* content, calculated as mg/l ($n=3 \pm$ S.E.) of *H. pluvialis* grown in the AAPSTM2 during September and October 1999. The culture grown in the low [N] media was harvested on day 17 (see text for details). Low [N] media (Culture five) chl *a* —●—, chl *b* —◆—, High [N] media (Culture six) ---▲---, chl. *b* ---■---.

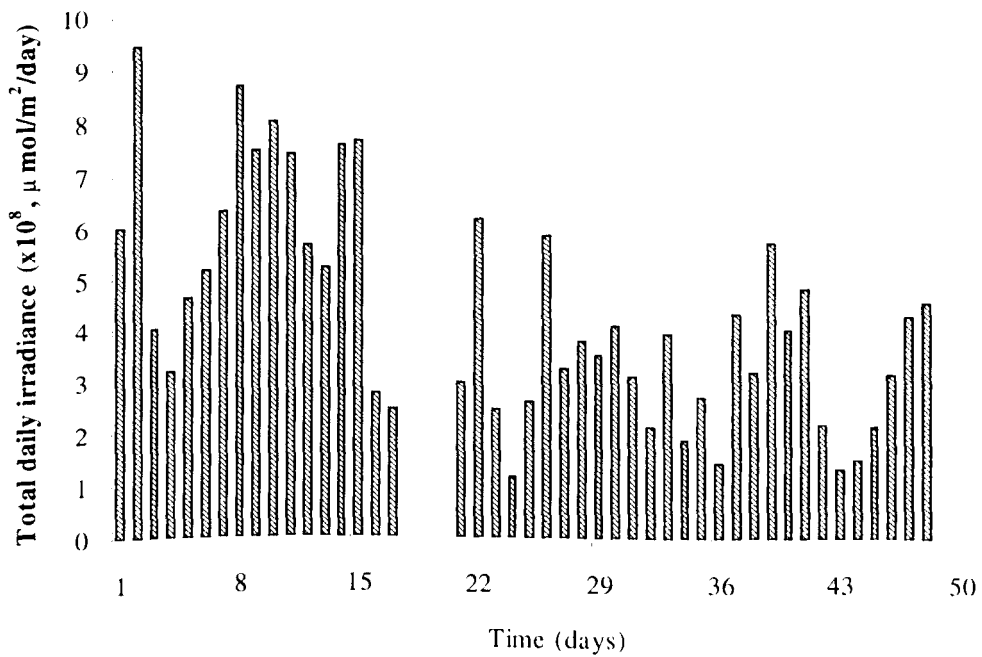


Figure 4.23. Total daily irradiance measured adjacent to the two AAPSTM2 during September and October 1999 (Cultures five and six). Due to technical problems the data from days 17-19 are not available.

The start of carotenoid accumulation corresponds with a period of relatively high irradiance when compared to the irradiance received over the whole culture period (Figure 4.23), and the end of the exponential phase of growth for the culture grown in the low [N] media (Figure 4.12). Increased irradiance is known to increase carotenoid biosynthesis in *H. pluvialis* (Droop 1955, Harker *et al* 1995, Kobayashi *et al* 1992). It has been proposed that carotenoid biosynthesis is a protection mechanism against photoinhibition. A number of researchers have determined that *H. pluvialis* cells rich in astaxanthin are more resistant to photoinhibition than carotenoid poor cells (Lee and Ding 1992, Bidigare *et al* 1993, Ding *et al* 1994). Lee and Ding (1992) also postulated that whereas other algae have developed effective repair mechanisms for the affects of photoinhibition *Haematococcus* uses secondary carotenoids as a protective mechanism.

The cultures were also exposed to temperatures higher than those determined by Harker (1995) to be optimal for growth (~14°C). In the first week the daytime air temperature reached as high as ~30°C (Figure 4.24). The culture temperature (not monitored) would be expected to be higher than this due to the added effect of solar irradiance. It is likely that the higher temperature was beneficial for carotenogenesis. Tjahjono *et al* (1994) demonstrated that when *H. pluvialis* was cultured at 30°C astaxanthin production was three times higher than at 20°C. Borowitzka *et al* (1991) also observed that a higher temperature resulted in increased carotenogenesis and aplanospore formation. It was also noted, by both studies, that the increased temperature resulted in an increased rate of cell death. As the cell density of both the high [N] and low [N] cultures increased constantly over the culture period (Figure 4.12) and few dead cells were visible microscopically it is unlikely that cell death was a great problem in the AAPS™2 cultures. Foam generation, also an indication of cell breakdown, was also minimal.

The scale-up of *Haematococcus* from flask culture (100ml) to the AAPS™ (~60l) had a detrimental affect on growth as both cultures had a reduced cellular productivity during exponential growth. Cellular productivity was, however, improved when calculated over the entire duration of the culture period. This is likely to be due to the reduced culture time of the two cultures in the AAPS™2. In the low [N] media the values for carotenoid productivity per cell per day were very similar: the AAPS™ culture had a productivity of 6.59pg carotenoid/cell/day (± 0.19 , $n=144$, \pm S.E.) between day seven - 17. However the final carotenoid yield per cell was only 15% of the flask culture, largely due to the much reduced culture period (17 rather than 154 days). The final yield after only 17 days cultivation was 127.73 pg carotenoid/cell (± 6.38 , $n=12$, \pm S.E.). Per volume of culture the yield was 25.35 mg/l (± 1.26 , $n=9$, \pm S.E.).

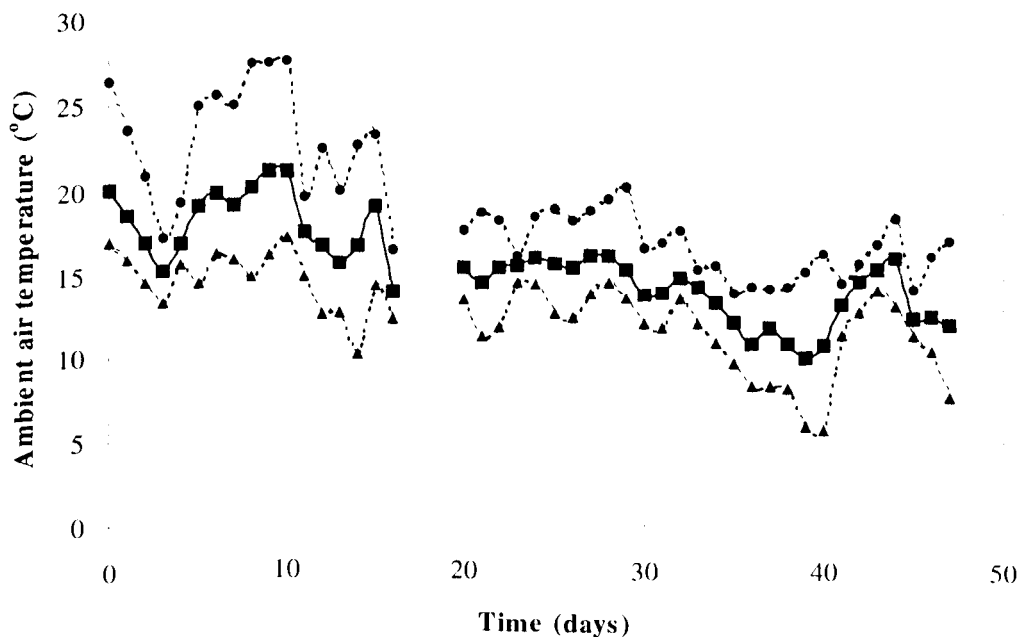


Figure 4.24. Air temperature adjacent to the two AAPS™2 during September and October 1999 (Cultures five and six). ▲ Minimum temperature, ● Maximum temperature, ■ Mean temperature. Due to technical problems the data from days 17 - 19 are not available.

In flask culture, under conditions of low irradiance ($\sim 60\mu\text{mol}/\text{m}^2/\text{s}$ and a constant temperature of 18°C) it took more than 40 days to achieve this level of secondary carotenoids. The optimum irradiance for astaxanthin synthesis in *H. pluvialis* (in flask culture) has been determined to be $1,600\mu\text{mol}/\text{m}^2/\text{s}$ (Harker *et al* 1995). On a dry weight basis the yield was $33.97\text{mg}/\text{g}$ dry weight (± 1.66 , $n=9$, \pm S.E.) or 3.4% (w/v) as dry weight (± 0.22 , $n=9$, \pm S.E.).

In contrast the culture grown in the AAPS™ in the high [N] media did not accumulate secondary carotenoids as rapidly. The culture contained a comparable number of cells on day seven as the culture grown in the low [N] media and was exposed to identical climatic conditions. The only difference between the two AAPS™ was the urea content. In flask culture, after seven days, the media still contained $108\text{mg}/\text{l}$ urea or 68% of the starting nitrogen level (the starting concentration was $180\text{mg}/\text{l}$, Figure 4.11). The total carotenoid content of the culture grown in the high [N] media on day 17 was only 51.80pg carotenoid/cell, less than half the content of the culture grown in the low [N] media. Differences were also seen in the developmental stage of the cultures (on day 17), the culture grown in the low [N] media contained a population of red aplanospores whereas the culture grown in the high [N] media contained young aplanospores and zooid cells. Within each of the zooid cells a red area was apparent but green chloroplasts were clearly visible around the periphery of the cell (plate 4.1c. shows an example of the green zooid with localised carotenoid accumulation).

Plate 4.6 shows the two AAPS™ on day 17. The difference in the carotenoid content of the cultures is clearly visible. The system containing the media with the higher urea content is in the foreground and still appears green, whilst the system behind (containing the low [N] media) is red.



Plate 4.6. The replicate AAPS™2 on day 17 of the culture period (September - October 1999). The system in the foreground contains the high [N] media (Culture six), the other system contains the low [N] culture (Culture five).

It is clear that although both AAPST[™] units have been exposed to identical climatic conditions the rates of carotenoid accumulation were very different. The carotenoid content per cell in the high [N] media, increased until day 21 reaching a carotenoid content of 70.23pg/cell (Figure 4.18). The content per cell then fluctuated around 70pg/cell until harvest on day 47 when the carotenoid content was 74.36pg/cell. At harvest the culture contained mainly aplanospores (75%) with some red zooid cells (25%) also present. Analysis on a per volume basis (Figure 4.19) revealed that the carotenoid content of the culture steadily increased from day seven (1.22mg/l) until harvest on day 47 (51.13mg/l). As there was no net increase in the carotenoid content per cell after day 21 (Figure 4.18) the rates of carotenogenesis and cell division in the culture must have been roughly equivalent. Figure 4.23 shows that the daily irradiance dropped by ~45% as the high [N] run progressed. However, a gradual decrease in the chlorophyll *a* and *b* content on the culture was measured (Figure 4.21). It has been concluded by some authors that chlorophylls are used as a carbon sink for carotenogenesis. This would indicate a breakdown in the photosynthetic systems during encystment. Other authors (Hagen *et al* 1993, Zlotnik *et al* 1993) have, however, questioned this, suggesting that aplanospores continue to function as photosynthetically active cells. It should be noted that during the culture of the cells in the high [N] media there was no change in the ratio of chlorophyll *a/b*.

H. pluvialis grown in the high [N] media is capable of achieving a higher total carotenoid content than was achieved in this culture, although lengthy time periods can be involved. In flask culture the media produced a culture with a total carotenoid content of 206pg/cell but this took 154 days (Table 4.5). However as this was cultured indoors the irradiance was very low compared with what is achievable outdoors.

4.3.3.3. Carotenoid composition

The carotenoid composition of both cultures was determined at the point of harvest using TLC as detailed in Chapter 2. The composition of carotenoids did not differ between cells cultivated in the two media as is shown in Table 4.6.

Table 4.6. Carotenoid composition (%) of *H. pluvialis* harvested from low [N] and high [N] media ($n=3$, \pm S.E.) in the AAPS™2.

Carotenoid	Low [N] culture	High [N] culture
Lutein	3.67 (\pm 0.13)	2.54 (\pm 0.37)
Free Astaxanthin	1.33 (\pm 0.09)	1.96 (\pm 0.24)
Astaxanthin Monoesters	49.9 (\pm 1.40)	50.81 (\pm 0.23)
Adonirubin Esters	6.64 (\pm 0.25)	9.93 (\pm 2.11)
Astaxanthin Diesters	36.6 (\pm 0.96)	32.62 (\pm 2.45)
β -Carotene	1.86 (\pm 0.24)	2.14 (\pm 0.14)

4.3.3.4. Carotenoid yield

After 17 days the culture grown in the low [N] media yielded 1.3g of total carotenoid, whilst the high [N] media yielded 2.6g after 47 days. In both cultures astaxanthin accounted for more than 80% of the carotenoids present, mainly as astaxanthin monoesters. From the calculated yield of total carotenoids detailed above the *H. pluvialis* grown in the low [N] media produced 1.14g of astaxanthin (0.11g astaxanthin/day). *H. pluvialis* grown in the high [N] media produced 2.22g of astaxanthin (0.06g astaxanthin/day).

The high [N] media yielded a much greater algal biomass, but it was not as productive in terms of carotenoid accumulation as the low [N] media. Although it produced twice the weight of carotenoid it took three times longer.



Plate 4.7. A carotenoid-rich culture of *H. pluvialis* (just before harvest) cultured in high [N] media in the AAPSTTM2 during September and October 1999 (Culture six).

It is however important to note that productivity in the culture grown in the high [N] media may have suffered because it lasted for a greater length of time. In the latter part of the culture period the irradiance and air temperature dropped when compared to conditions at the start of the culture period.

Plate 4.6 shows the culture grown in the low [N] media on day 17, Plate 4.7 shows the culture grown in the high [N] media after 47 days, just before harvest.

4.3.4. Cultures seven and eight (AAPS™2)

Despite the failure of AAPS™1 to produce a viable culture of *Haematococcus* in November/December 1998, the viability of growth outdoors in the winter months was further investigated in the AAPS™2. This also acted as a further comparison between the two media types. The two systems were set up containing the low [N] (culture seven) and high [N] media (culture eight), and were inoculated on the 22nd October 1999. Both cultures were harvested on 16th December 1999. Each system had pH control using CO₂ which acted to maintain the high [N] culture at pH 7 (Figure 4.25). The low [N] culture did not require pH control.

4.3.4.1. Growth

The growth and productivity of both cultures was much reduced when compared to the performance seen during September and October (section 4.3.3). The doubling time of the culture grown in the low [N] media (culture seven) was 11.55 days (culture five had a t_d of 4.62 days). The culture in the high nitrogen media (culture eight) had a better growth rate, it doubled in 8.66 days (compared with 4.08 days for culture six, section 4.3.3.1). Data from the culture grown in the low [N] media are limited as on day 31 there was combined air-line and non-return valve failure at the

base of the riser and 20l (36%) of the culture was lost overnight. To investigate the potential recovery of the culture from such a loss, 20l of fresh media was pumped into the system via a filter sterilisation unit. This resulted in a very small increase in the urea content of the culture (Table 4.7).

Table 4.7. Urea concentration (mg/l, $n=3$, \pm S.E.) of media during the growth of *H. pluvialis*

	Low [N] culture	High [N] culture
First day of culture	52.33 (\pm 2.03)	164.67 (\pm 2.33)
Day 31	25.67 (\pm 1.67)	-
Day 32	33.67 (\pm 1.67)	-
Day 57 (harvest)	19.67 (\pm 0.33)	29.00 (\pm 1.00)

The loss of culture appeared to have little affect on the growth of the culture (Figure 4.26) and it grew for a total of 41 days achieving a final cell density of 19.25×10^4 cells/ml. The culture grown in the high [N] media grew for 57 days, achieving a final cell density of 70.00×10^4 cells/ml. The cell density produced by both cultures was comparable with that achieved in the earlier runs, but lower than achieved in shake flask culture. Growth of *H. pluvialis* in shake flask culture in low [N] media yielded 30.00×10^4 cells/ml and 111.00×10^4 cells/ml in the case of the high [N] media.

The dry weight of each culture increased over the course of the run (Figure 4.27) though the dry weight per cell was erratic (Figure 4.28). This may have been due to changes in the developmental stage of cells within the culture. As a culture of *H. pluvialis* develops from zooids into aplanospores there is an increase in the cell volume and weight (Zlotnik *et al* 1993, Margalith 1999).

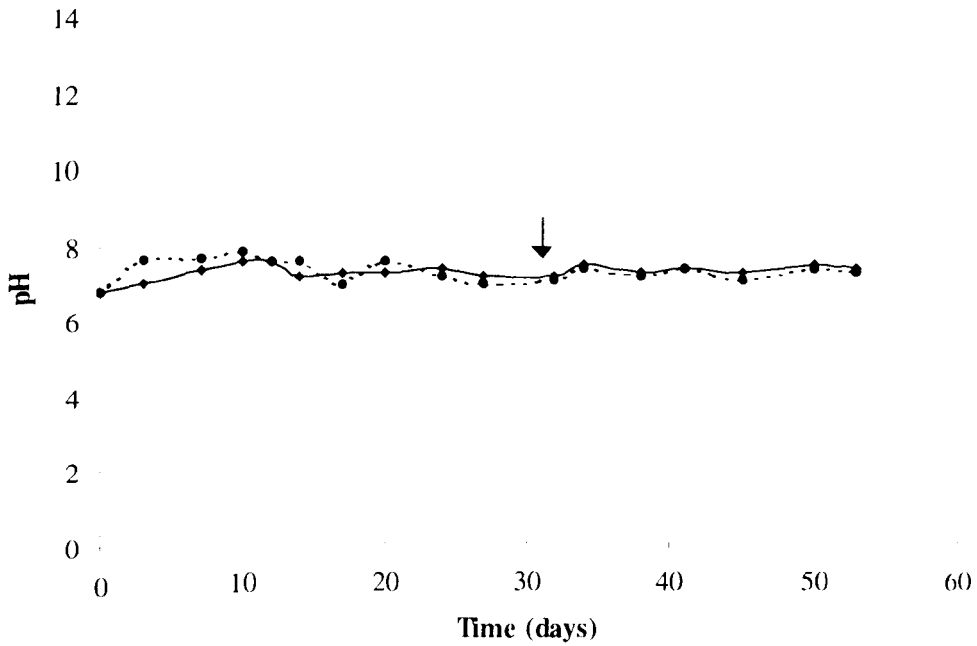


Figure 4.25. pH of the *H. pluvialis* cultures grown in the AAPS™2 during November and December 1999. Low [N] Urea (Culture seven) —, High [N] Urea (Culture eight) ---, → valve failure (see text for details).

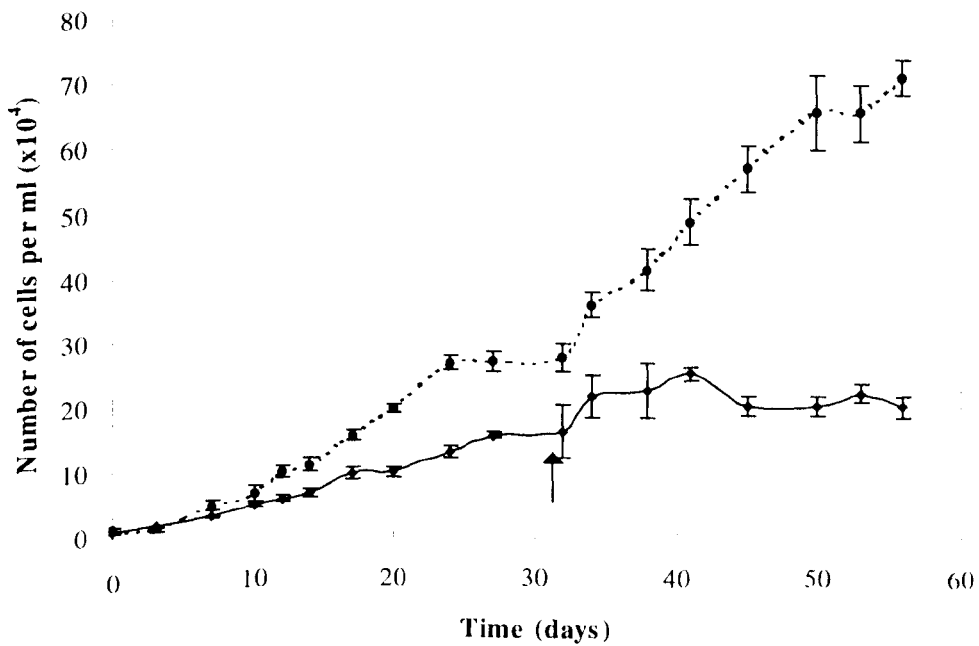


Figure 4.26. Cell density ($n=4 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during November and December 1999. Low [N] Urea (Culture seven) —, High [N] Urea (Culture eight) ---, → valve failure (see text for details).

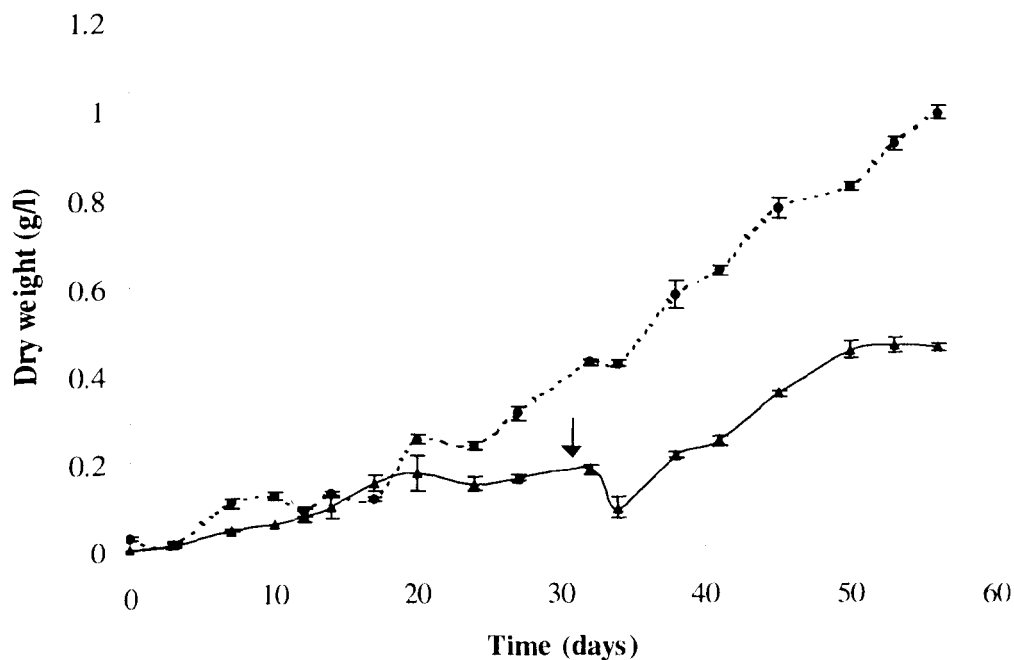


Figure 4.27. Dry weight ($n=3 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during November and December 1999. Low [N] media (Culture seven) —, High [N] media (Culture eight) ---, → valve failure (see text for details).

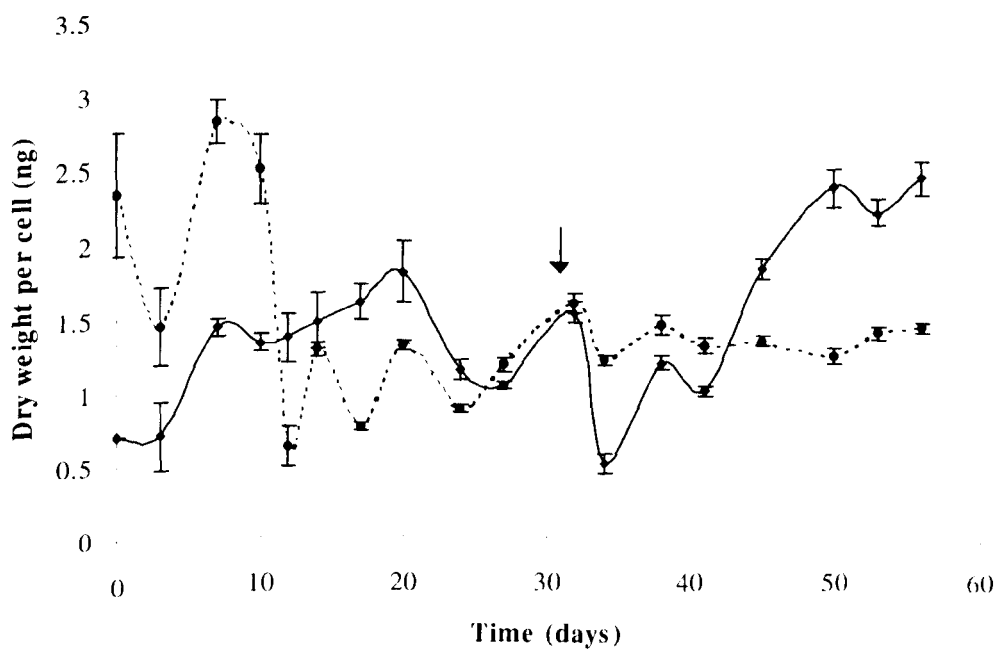


Figure 4.28. Dry weight per cell ($n=12 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during November and December 1999. Low [N] media (Culture seven) —, High [N] media (Culture eight) ---, → valve failure (see text for details).

Outdoor conditions may generate a mixed population, with both the aplanospores and zooid cells actively dividing (Lee and Ding 1995). This could result in changes in the dry weight per cell as shown in Figure 4.28. At the end of the culture period the culture grown in the low [N] media contained only aplanospores. The culture grown in the high [N] media was a mixture of aplanospores (60%) and zooids (40%).

Cultures five and six, which were cultured in September/October had productivities of $\sim 1.40 \times 10^4$ cells/ml/day. The cooler, duller weather conditions of late 1999 resulted in a reduced productivity for growth, in both media. The daily received irradiance of the culture (Figure 4.29) was reduced when compared to the conditions just one month earlier. The total daily irradiance was 1.03×10^8 $\mu\text{mol}/\text{m}^2/\text{day}$ during culture seven and eight, four times lower than the mean irradiance of the previous run (4.38×10^8 $\mu\text{mol}/\text{m}^2/\text{day}$). The air temperature was also greatly reduced when compared to the previous cultures and it did not rise above 15°C , and on two days dropped close to 0°C (Figure 4.30). The mean temperature over the culture period was 9°C , which is lower than the optimum for growth of $14\text{-}15^\circ\text{C}$ (Borowitzka *et al* 1991, Harker *et al* 1995). In comparison the mean temperature during cultures five and six was 15°C . Torzillo *et al* (1991) demonstrated that for *Spirulina*, when the morning temperature was below the optimum the alga was unable to fully utilise its photosynthetic capacity.

It is also important to note the change in the day length. Figures 4.31 and 4.32 show the irradiance and temperature on two days in this culture period (cloudless and cloudy days). By comparing them to Figures 4.15 and 4.16 not only is the irradiance received much lower but it is also received over a shorter period of time. The day length during cultures five and six was ~ 12 hours, whereas during cultures seven and eight it had decreased to ~ 7 hours.

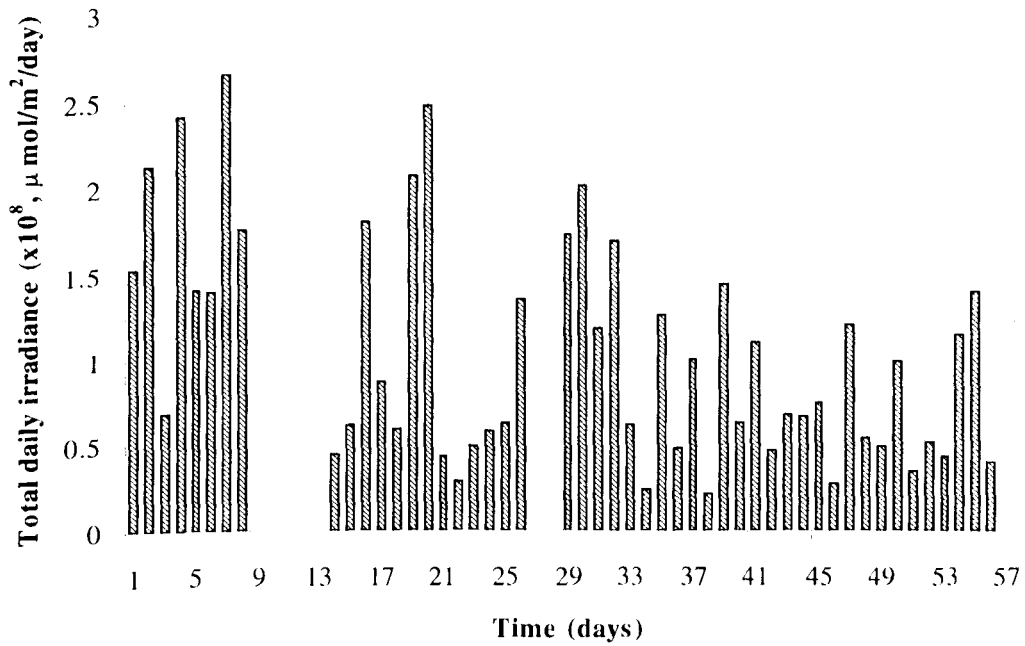


Figure 4.29. Total daily irradiance adjacent to the AAPS™2 during November and December 1999 (Cultures seven and eight). Due to technical problems the data from days 8-12, 26, 27 are not available.

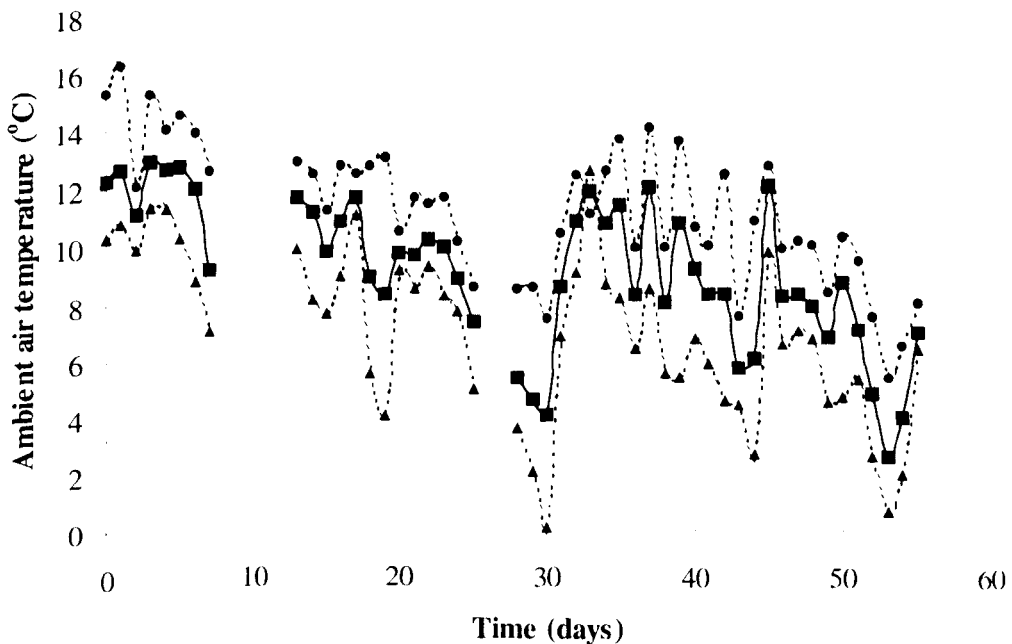


Figure 4.30. Air temperature adjacent to the AAPS™2 during November and December 1999 (Cultures seven and eight, measurements taken every 15mins over 24hrs). Due to technical problems the data from days 8-12, 26, 27 of the run are not available. Minimum temperature ▲, Maximum temperature ●, Mean temperature ■.

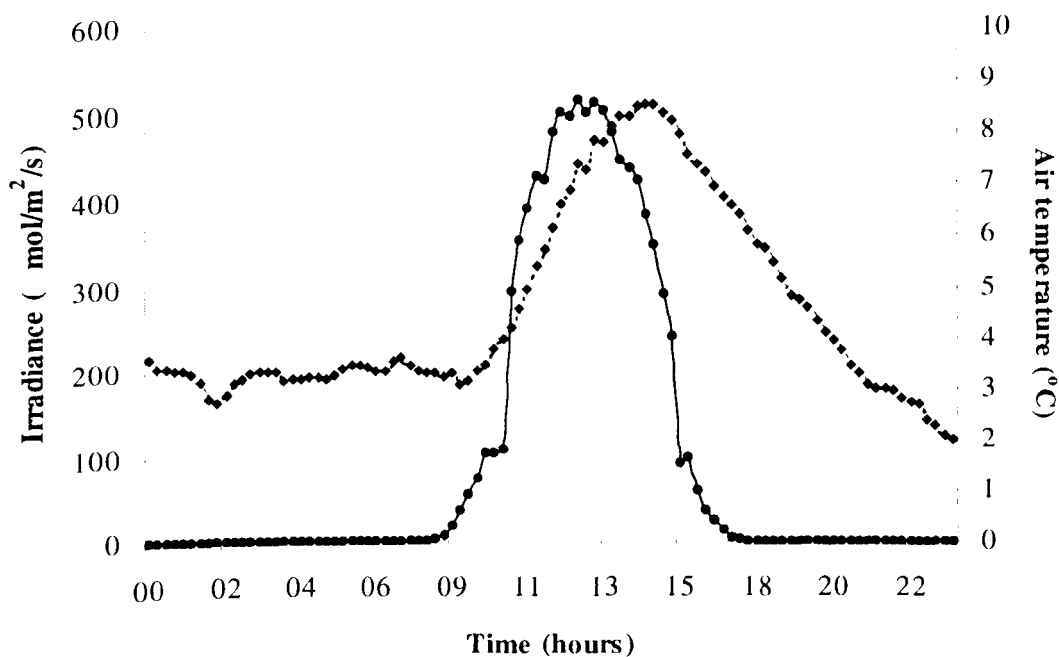


Figure 4.31. Changing irradiance and temperature adjacent to the two AAPS™2 on a cloudless day (20th November 1999, measurements were recorded every 15min). Irradiance ●, Air temperature ◆.

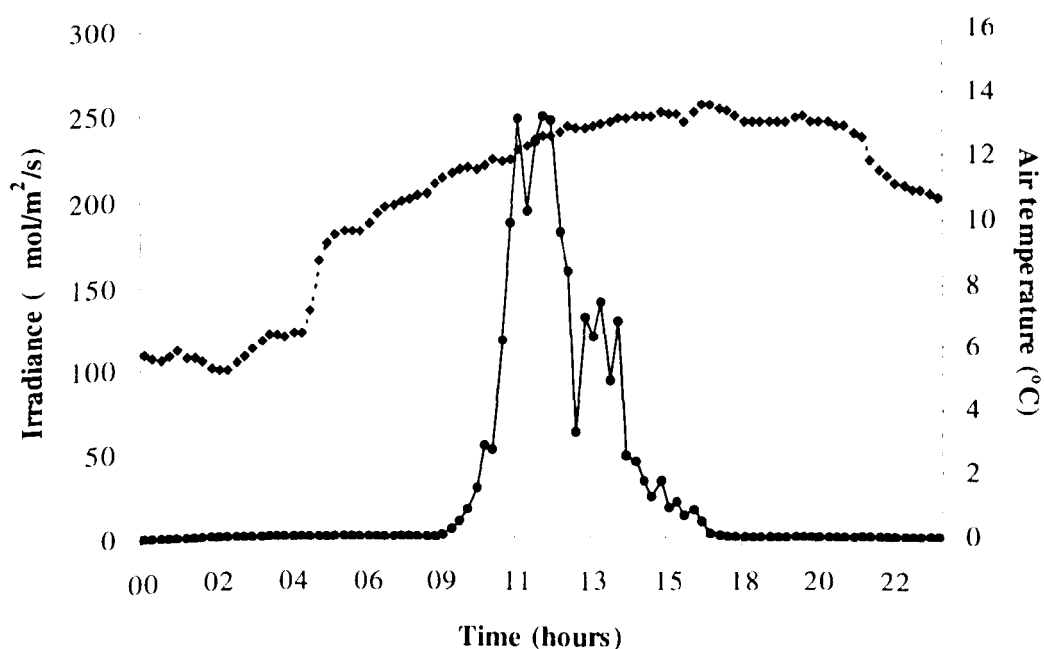


Figure 4.32. Changing irradiance and temperature adjacent to the two AAPS™2 on a cloudy day (30th November 1999, measurements were recorded every 15min). Irradiance ●, Air temperature ◆.

The culture grown in the low [N] media produced 0.33×10^4 cells/ml/day (± 0.16 , $n=16 \pm$ S.E.), much lower than had been achieved in previous cultures using this media (Table 4.4, and section 4.3.3.1). The high [N] media produced 1.24×10^4 cells/ml/day (± 0.40 , $n=4 \pm$ S.E.), which was comparable to the productivity of culture six, (also grown with this urea concentration during September and October 1999). As culture eight was grown under a lower irradiance and temperature than culture six it would be expected that its productivity would have suffered (as with cultures five and seven grown in the low [N] media). The productivity of culture eight may have been due to the CO₂ that was injected into the system for pH control (Figure 4.25). The previous culture grown in the high [N] media had no CO₂ addition (Figure 4.17). It is possible either the CO₂ improved the productivity of culture eight or the lack of pH control in culture six reduced its productivity. CO₂ injection could benefit culture growth as the CO₂ concentration at any point in a photobioreactor should not fall below a critical value, or the availability of the carbon source would limit photosynthesis (Camacho Rubio *et al* 1999). As the previous cultures had no CO₂ injection the only CO₂ available to the cells was that present in the air injected into the system, as well as generation from respiration. However as no data is available on the CO₂ concentration in any of the cultures it is impossible to say if the initial cultures were CO₂ limited. Conversely the elevated pH of culture six may have limited productivity by affecting the efficiency of CO₂ absorption, which would have also impacted on the photosynthetic efficiency of the cultures.

4.3.4.2. Secondary carotenoid accumulation

The accumulation of secondary carotenoids also appeared to be adversely affected by the climatic conditions. In the warmer, brighter conditions of September/October the low [N] media produced a carotenoid-rich culture after just 17 days with a carotenoid content of 127.73pg/cell (Figure 4.18). After 17 days, culture seven contained only 10.11 pg/cell (Figure 4.33) and after 56 days the total carotenoid content of the culture was only 28.99pg/cell, 5.35 mg/l (Figure 4.34) or 11.80 mg/g dry weight (Figure 4.35).

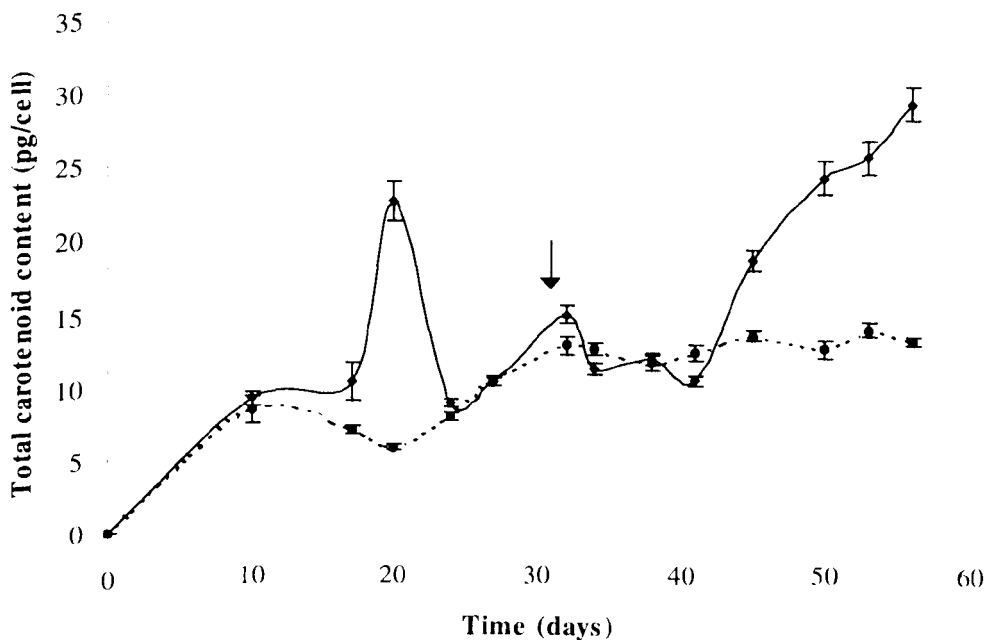


Figure 4.33. The total carotenoid content, calculated as pg/cell ($n=12 \pm$ S.E.) of *H. pluvialis* grown in the AAPSTTM2 during November and December 1999. Low [N] media (Culture seven) —, High [N] media (Culture eight) ---, —→ valve failure (see text for details).

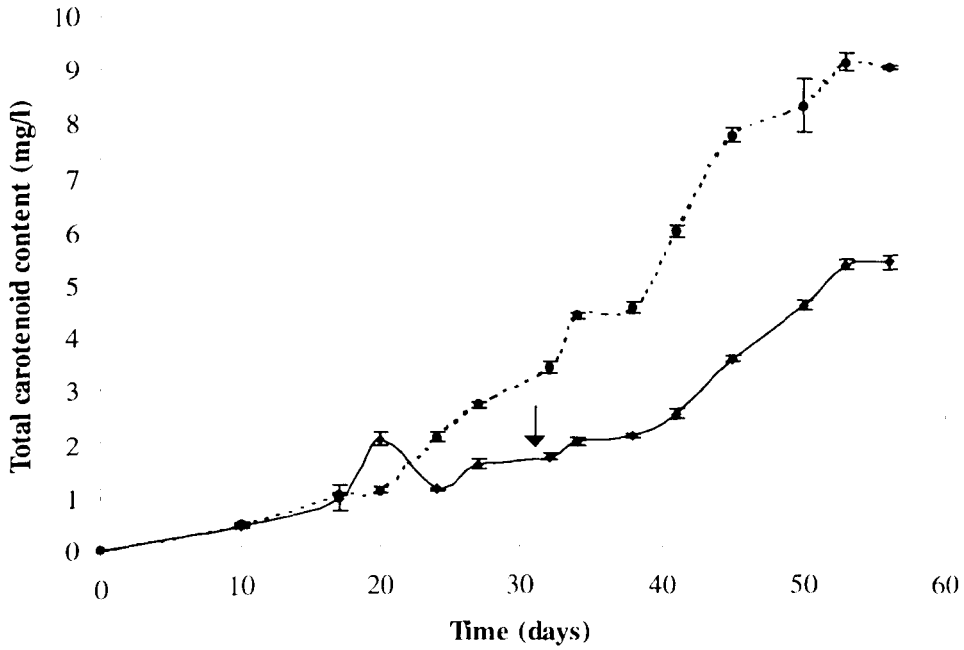


Figure 4.34. The total carotenoid content, calculated as mg/l ($n=3 \pm$ S.E.) of *H. pluvialis* grown in the AAPSTM2 during November to December 1999. Low [N] media (Culture seven) —, High [N] media (Culture eight) ---, \rightarrow valve failure (see text for details).

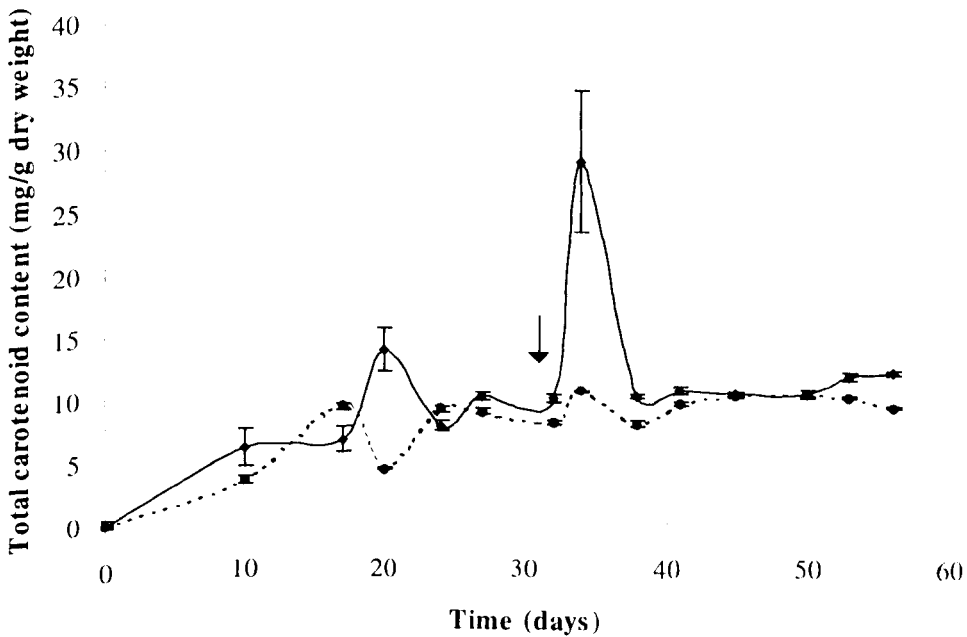


Figure 4.35. The total carotenoid content, calculated as mg/g dry weight ($n=12 \pm$ S.E.) of *H. pluvialis* grown in the AAPSTM2 during November and December 1999. Low [N] media (Culture seven) —, High [N] media (Culture eight) ---, \rightarrow valve failure (see text for details).

The culture produced in the high [N] media also proved to be less productive than previously, yielding only 8.98mg/l, 12.88 pg/cell or 9.12 mg/g dry weight. In fact, the culture appeared green to the eye and was made up primarily of green zooid cells and a small number of red aplanospores. The chlorophyll *a* and *b* content of both cultures increased over the course of the culture period (Figure 4.36). However some fluctuation in the content per cell of both cultures was noted (Figure 4.37). Particularly from day 41 there was a change in the ratio of chlorophyll *a/b* in the culture grown in the high [N] media, a drop in the concentration of chlorophyll *b* was measured. This indicates that the photosynthetic apparatus of the culture was stressed in some way.

As cultures seven and eight were grown using the same media as cultures five and six respectively (Appendix 1a), it seems likely that it was the climatic conditions that the cultures were exposed to that reduced their carotenoid productivity. The irradiance received by cultures seven and eight (mean daily irradiance $1.03 \times 10^8 \mu\text{mol/m}^2/\text{day}$, Figure 4.29) was reduced in comparison to culture five and six ($4.38 \times 10^8 \mu\text{mol/m}^2/\text{day}$, Figure 4.23). Harker *et al* (1995) determined that the optimum irradiance for carotenogenesis was $1,600 \mu\text{mol/m}^2/\text{s}$. During cultures seven and eight the irradiance rarely increased above $600 \mu\text{mol/m}^2/\text{s}$ (Appendix 2b). In comparison during cultures five and six the irradiance consistently reached $1,400 \mu\text{mol/m}^2/\text{s}$ (Appendix 2a). The irradiance received by the culture seven and eight was still in excess of typical irradiance used in the literature as high irradiance which are in the order of $100\text{-}300 \mu\text{mol/m}^2/\text{s}$ for indoor cultures (Borowitzka *et al* 1991, Fábregas *et al* 1998).

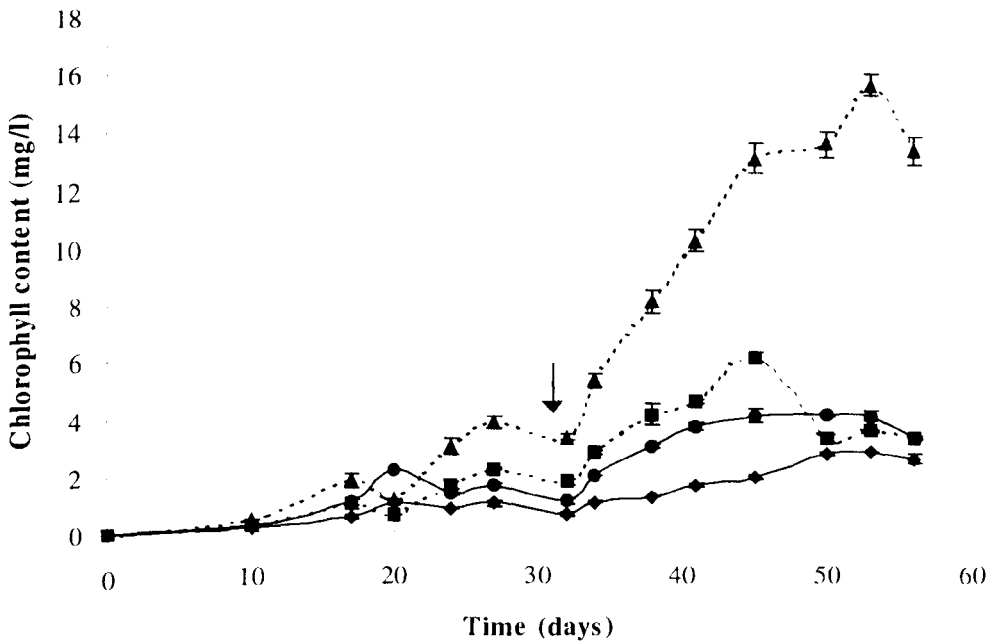


Figure 4.36. The chlorophyll *a* and *b* content, calculated as mg/l ($n=3 \pm$ S.E.) of *H. pluvialis* grown in the AAPS™2 during November to December 1999. Low [N] media (Culture seven) chl *a* —●—, chl *b* —◆—, High [N] media (Culture eight) chl *a* ---▲---, chl *b* ---■---, → valve failure (see text for details).

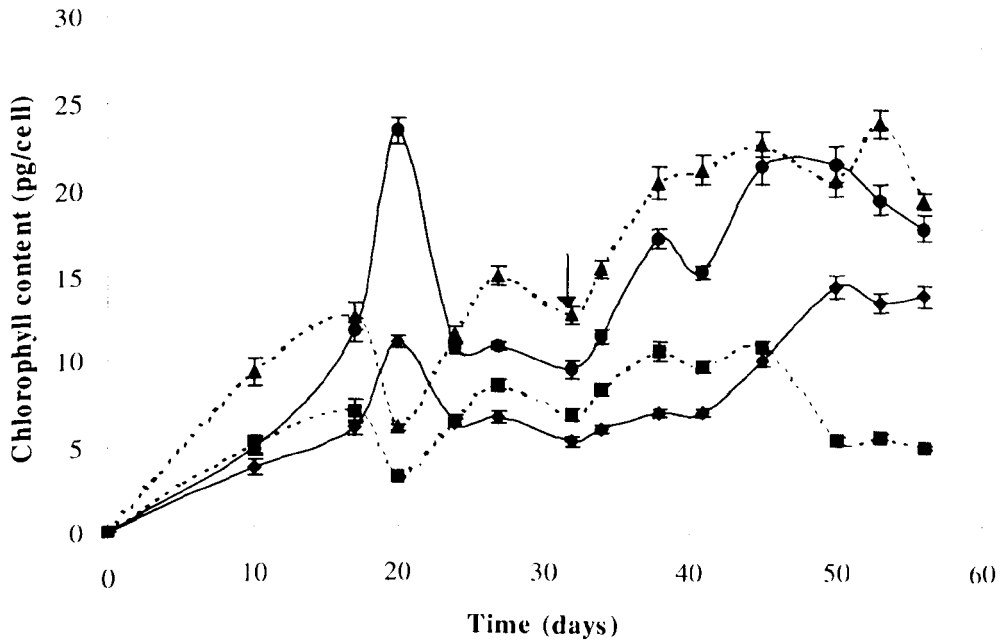


Figure 4.37. The chlorophyll *a* and *b* content, calculated as pg/cell ($n=12 \pm$ S.E.) of the *H. pluvialis* grown in the AAPS™2 during November and December 1999. Low [N] media (Culture seven) chl *a* —●—, chl *b* —◆—, High [N] media (Culture eight) chl *a* ---▲---, chl *b* ---■---, → valve failure (see text for details).

However it would appear that the reduced irradiance had a detrimental affect on carotenogenesis in the case of these cultures. This may have occurred in synergy with the reduced temperature that the cultures were exposed to. *Haematococcus* is commonly found in cold climates such as Scandinavian lakes (Pringsheim 1966) and hence has a low optimum temperature for growth. Borowitzka *et al* (1991) and Harker *et al* (1995) both determined the optimum to be ~15°C. However it is capable of growing at increased temperatures and higher temperatures have been shown to increase carotenogenesis (Borowitzka *et al* 1991, Tjahjono 1994). Therefore the reduced temperature experienced by cultures seven and eight could have contributed to the reduced carotenogenesis.

Despite the reduced carotenogenesis when *H. pluvialis* was grown in November/December the two media types behaved to a comparable way to the cultures produced in flask culture (section 4.3.1.) and also when cultured earlier in the year (cultures five and six). The high [N] media appeared more productive as it contained a greater carotenoid concentration per unit volume of media. However as previously, the carotenoid content per cell was much lower than achieved in the low [N] media.

4.3.4.3. Carotenoid yield

At the end of the culture period the cells that were harvested yielded 0.29g (1.18% w/v as dry weight) of total carotenoid from the low [N] media. This was only 22% of the carotenoid content produced in the previous low [N] AAPS™2 culture (culture five, section 4.3.3.4). This is especially low when it is considered that this culture was grown for 57 days whereas the previous low [N] culture was only cultured for 17 days. The high [N] culture produced 0.45g (0.91% w/v as dry weight) of total

carotenoid which was 17% of the yield of the previous high [N] culture (culture six, section 4.3.3.4).

4.4. Germination of aplanospores

Carotenoid-rich zooids (or red zooids) were produced in cultures one and three grown in the AAPS™1 in low [N] media. Culture one produced carotenoid-rich zooids during growth (section 4.2.1.), culture three yielded carotenoid-rich zooid cells from the germination of aplanospores after the culture had been harvested (section 4.2.3.). Information about the life cycle of *H. pluvialis* suggests that the red zooid cells produced in these two culture were in fact examples of different developmental stages. The red zooid cells that had germinated in culture three were likely to have been microzooid cells, which were first observed by Hazen (1899). Whereas the zooid cells that accumulated carotenoids during the growth of culture one were the typical macrozooids commonly seen as green cells in growing cultures. The microzooid has been observed to be smaller than the typical zooid cell and to swim more actively (Elliott 1934). Early postulations suggested that they were gametes (Peebles 1909) however this was never proven (Elliott 1934). In this study it is the germination behaviour of culture three, the formation of red microzooids that is of most interest.

Producing a culture with the secondary carotenoids contained in zooids rather than aplanospores has certain potential advantages. Aplanospores are formed under conditions that are unfavourable for growth (Zlotnik *et al* 1993, Chaumont and Thepénier 1995) causing a massive accumulation of the carotenoid astaxanthin and also the formation of a thick cell wall (sporopollenin-based, Grunewald *et al* 1997, Margalith 1999). This means that the manufacture of a commercial product must involve a step to fracture the cell wall (Bubrick 1991, Olaizola 2000). Carotenoid-rich

zooids would be commercially advantageous as the cells do not have the thick cell wall (Hagen *et al* 2001) and therefore it is likely that such processing could be avoided (J. Bowen, pers. comm.)

4.4.1. Aims

The aim of this study was to optimise the production of carotenoid-rich zooids from aplanospores of *H. pluvialis* under controlled environmental conditions. The availability of secondary carotenoids within a carotenoid-rich *H. pluvialis* culture was assessed in terms of the 'potential bioavailability' as detailed below.

4.4.2. 'Potential bioavailability'

A method was developed to assess the potential availability of secondary carotenoids within the cells of a *H. pluvialis* culture when used as a feed product (Bowen 2001). A 5ml sample was taken from the culture and pelleted by centrifugation (1,200g for 5 min) then resuspended in an equal volume of redistilled acetone (GPR, Merck) in a glass vial. The sample was then purged with N₂ (oxygen-free) and stored in the dark for 16 hours (to maximise the release of astaxanthin, Bowen 2001). After 16 hours the sample was filtered through absorbent cotton wool and the carotenoid content of the extract was calculated using the published extinction coefficient of astaxanthin (see Chapter 2). A second 5ml sample of the culture was extracted by homogenisation and analysed as detailed in Chapter 2. It was assumed that the homogenisation method obtained a 100% recovery of secondary carotenoids from the culture (Tsavalos 1995). By comparing the carotenoid content of this extract with that produced from soaking overnight in acetone the potential bioavailability of the cells was determined.

Using this method the potential bioavailability of the zooid cells produced after the harvest of AAPS™1 culture three was determined to be 91.50% (± 0.75 , $n=9 \pm$ S.E.). Aplanospores of a similar carotenoid content (3.00% as dry weight) had an ‘potential bioavailability’ of 0.41% (± 0.06 , $n=9 \pm$ S.E.). Indicating that the carotenoids contained within the zooids were more readily available, probably due to the absence of the thick sporopollenin wall.

4.4.3. Aplanospore germination under controlled conditions

The formation of the carotenoid-rich zooids in cultures one and three in the AAPS™1 occurred under uncontrolled conditions. In order to gain an insight into the conditions required for germination initial investigations into the formation of red zooids from aplanospores occurred at a small scale in flask culture under controlled environmental conditions (18°C, 40-60 μ mol/m²/s irradiance).

Red zooids can be produced when a culture of aplanospores is resuspended in fresh media, Lee and Ding (1994) demonstrated that motile zooids were released from aplanospores after 15 hours resuspension in fresh media. This however is not a viable method at a large scale because of the time and cost involved in dewatering and resuspending an aplanospore culture from an AAPS™ in fresh media. Therefore the focus of this study was to produce carotenoid-rich zooid cells from aplanospores through the addition of nutrients to the original culture.

4.4.3.1. Nutrient supplementation with BBM

Due to the effectiveness of resuspending aplanospores in fresh media to produce carotenoid-rich zooids (see above) the first approach was to add a concentrated stock of

modified BBM nutrients to a flask of aplanospores. A nutrient supplement of the modified BBM (Appendix 1b) was made up and 1ml was added to individual flasks containing 25ml of aplanospores, each flask had the same cell density (30×10^4 cells/ml, ± 1.29 , $n=12 \pm$ S.E.). Samples were then taken at three hour intervals and the total number of zooid cells in each sample was determined. For the purpose of this study successful germination was deemed to be reached when 70% of the cells within the culture were zooids. At this stage, the cells were harvested and their carotenoid content assessed (section 4.4.2).

Initial studies using this treatment yielded a population that was 72.57% zooids after 57 hours (experiment one, Table 4.8), the remaining 27.43% were aplanospores. Over the 57 hour period there was a steady release of carotenoid-rich zooids from aplanospores. 57 hours was much longer than had been indicated by the work of Lee and Ding (1994), Hazen (1899) or Peebles (1909). It is also a much greater time period than it had taken the aplanospores to germinate after the harvest of culture three (section 4.2.3). The increased time for germination in flask culture may have been due to the approach of adding nutrients to the old media rather than de-watering the culture and using fresh media. It is possible that secondary metabolites may be present in old media that have an impact on the behaviour of the culture.

The irradiance and temperature that the culture was exposed to may also have been important. AAPS™ culture three and the work of Hazen (1899) and Peebles (1909) all used natural daylight. The work of Lee and Ding (1994) used an artificial irradiance of $150 \mu\text{mol}/\text{m}^2/\text{s}$ and a temperature of 25°C . This study used a lower irradiance and temperature ($40\text{-}60 \mu\text{mol}/\text{m}^2/\text{s}$ and 18°C).

Table 4.8. Response of stationary phase (aplanospore) culture of *H. pluvialis* to the addition of a nutrient supplement of BBM (see text for details).

	Experiment one		Experiment two	
	Time 0 (before treatment)	57 hours after stock addition	Time 0 (before treatment)	55 hours after stock addition
Cell density ($\times 10^4$, $n=4 \pm$ S.E.)	30.00 (± 1.29)	57.00 (± 0.50)	28.50 (± 0.29)	79.00 (± 2.27)
Proportion of the population as zooid (%, $n=4 \pm$ S.E.)	0.00 (± 0.00)	72.57 (± 0.64)	0.00 (± 0.00)	85.75 (± 1.65)
Dry weight (g/l, $n=3$ \pm S.E.)	2.34 (± 0.05)	1.58 (± 0.07)	2.17 (± 0.06)	1.39 (± 0.10)
Dry weight per cell (ng, $n=12 \pm$ S.E.)	6.83 (± 0.72)	2.54 (± 0.20)	7.14 (± 0.50)	1.79 (± 0.06)
Potential bioavailability (%, $n=9 \pm$ S.E.)	4.71 (± 0.12)	50.39 (± 1.70)	0.43 (± 0.05)	74.33 (± 0.05)

It is widely accepted that irradiance (e.g. Kobayashi *et al* 1992, Harker *et al* 1995) and temperature (Borowitzka *et al* 1991, Tjahjono 1994) play an important role in the formation of aplanospores, it therefore follows that they may also be of importance in the subsequent germination of aplanospores to form zooids. It is also important to note here that the previous work has resulted in the synchronous release of carotenoid-rich zooids from aplanospores (Lee and Ding 1994, AAPS™1 culture three). The addition of the concentration modified BBM stock resulted in a staggered release over a prolonged time period. Again this may have been a function of the environmental conditions that the cells were exposed to (e.g. photoperiod).

The culture was characterised by an increase (2-2.5x) in the cell density, indicating that each aplanospore germinated into a number of carotenoid-rich zooids. The dry weight of the culture was noted to decrease (by ~ 40%) as did the dry weight per cell. At the point of harvest (after 57 hours) the carotenoid content per unit volume of the culture dropped, the chlorophyll *a* and *b* content remained constant (Figure 4.38a). The total carotenoid content per cell after germination was 58.74pg/cell (Figure 4.38b). However most of the carotenoids would still be contained in the aplanospores that remained in the culture after germination. Aplanospores accounted for 27.43% of the culture before the addition of the media stock and contained 141.43pg/cell (Figure 4.38b). Therefore assuming that the aplanospores in the culture still contained 141.43pg/cell, each zooid cell within the culture would only contain 25.83pg/cell, due in part to the division of cells but also to a possible loss of carotenoids in the zooids. The low carotenoid content of the zooid cells is demonstrated by a measure of the potential bioavailability of the cells. Only 50.39% of the carotenoids in the culture were present in the solvent after 16 hours (Table 4.8). The pigment content of the culture calculated as mg/g dry weight increased after the germination of the cells (Figure 4.38c). This was despite the fact that the carotenoid content per cell was lower after germination (Figure 4.38b). The increased amount on a per weight basis was due to the reduction in the dry weight per cell of the culture (Table 4.8) which was due to the change in the developmental stage of the culture (from aplanospore to zooid) and the loss of the thick cell wall.

The ratio of chlorophyll *a/b* remained unchanged (Table 4.9) but the ratio of chlorophyll/carotenoid decreased after germination largely because of the drop in the carotenoid content of the culture (Figure 4.38a)

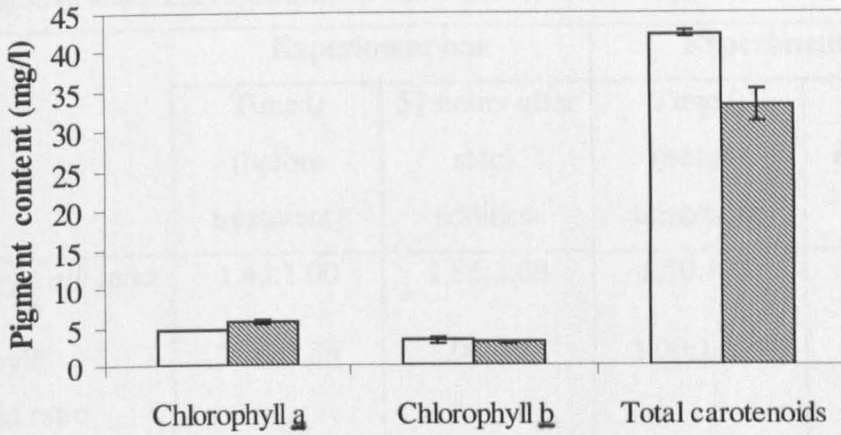
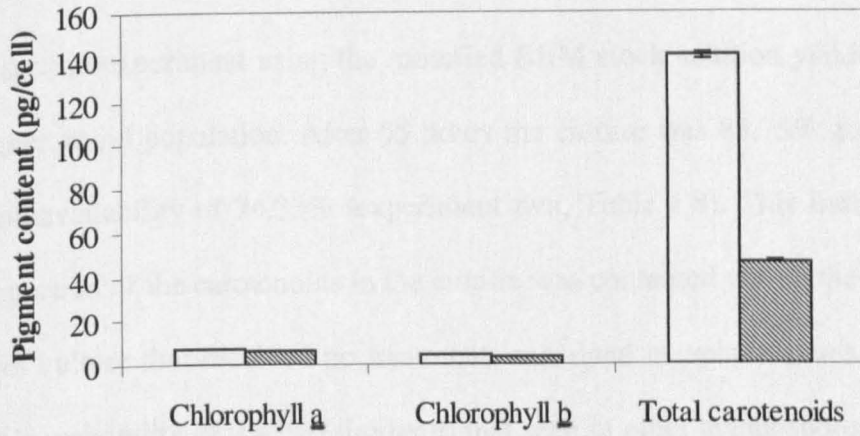
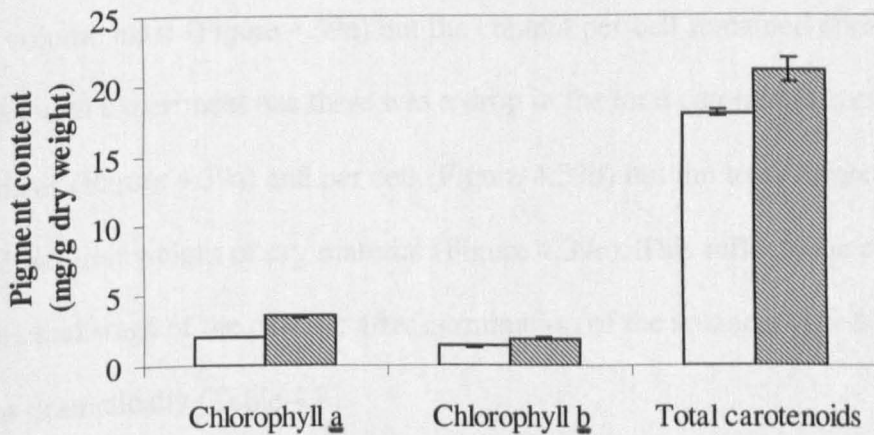
A**B****C**

Figure 4.38. Pigment content expressed as mg/l, (A, $n=3 \pm$ S.E.), pg/cell (B, $n=12 \pm$ S.E.) and mg/g dry weight (C, $n=9 \pm$ S.E.) of *H.pluvialis* before (□) and after (▨) the germination of the aplanospores with modified BBM (Experiment one, see text for details).

Table 4.9. Response of pigments in a stationary phase (aplanospore) culture of *H. pluvialis* after the addition of a nutrient supplement of BBM (see text for details).

	Experiment one		Experiment two	
	Time 0 (before treatment)	57 hours after stock addition	Time 0 (before treatment)	55 hours after stock addition
Chlorophyll <i>a/b</i> ratio	1.43:1.00	1.86:1.00	1.50:1.00	1.58:1.00
Chlorophyll/ carotenoid ratio	1.00:5.39	1.00:3.84	1.00:11.77	1.00:4.46

A second experiment using the modified BBM stock solution yielded a culture with a higher zooid population. After 55 hours the culture was 85.75% zooid with an potential bioavailability of 74.33% (experiment two, Table 4.8). This indicated that a higher proportion of the carotenoids in the culture was contained within the zooid cells. The control culture that received no treatments remained as aplanospores and had an potential bioavailability of 0.43% (similar to that seen in other aplanospores, J. Bowen, pers. com.). There was a slight increase in the chlorophyll *a* and *b* content of the culture on a per volume basis (Figure 4.39a) but the content per cell remained constant (Figure 4.39b). As with experiment one there was a drop in the total carotenoid content on a per volume basis (Figure 4.39a) and per cell (Figure 4.39b) but the total carotenoid content increased per unit weight of dry material (Figure 4.39c). This reflects the change in the developmental stage of the culture; after germination of the aplanospores the weight per cell drops dramatically (Table 4.8).

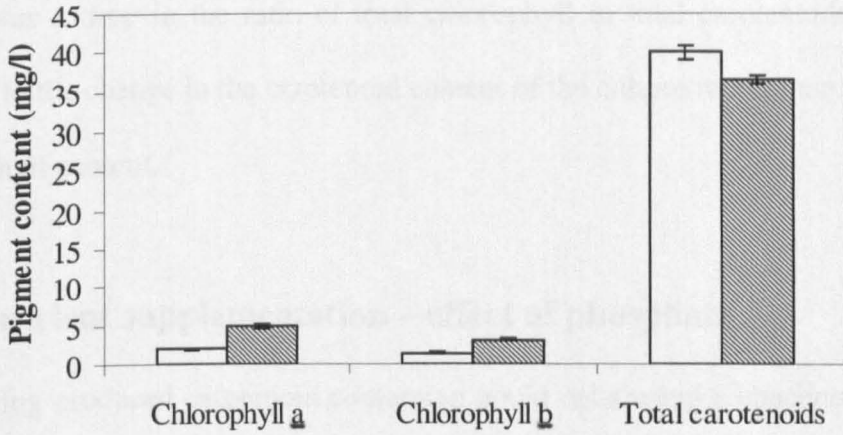
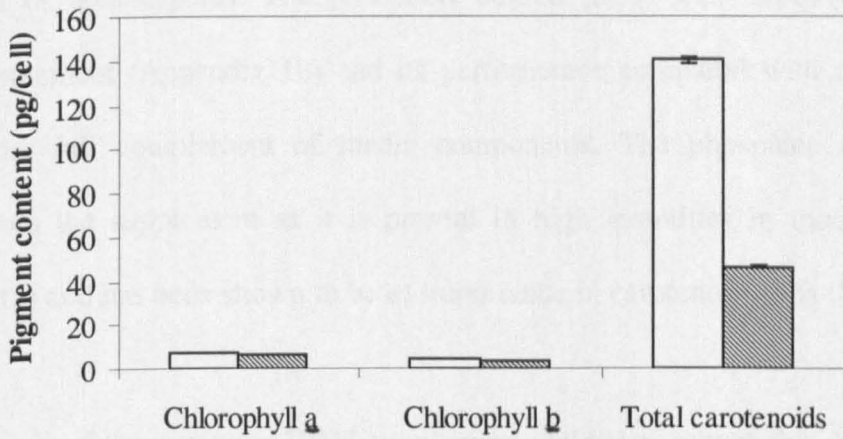
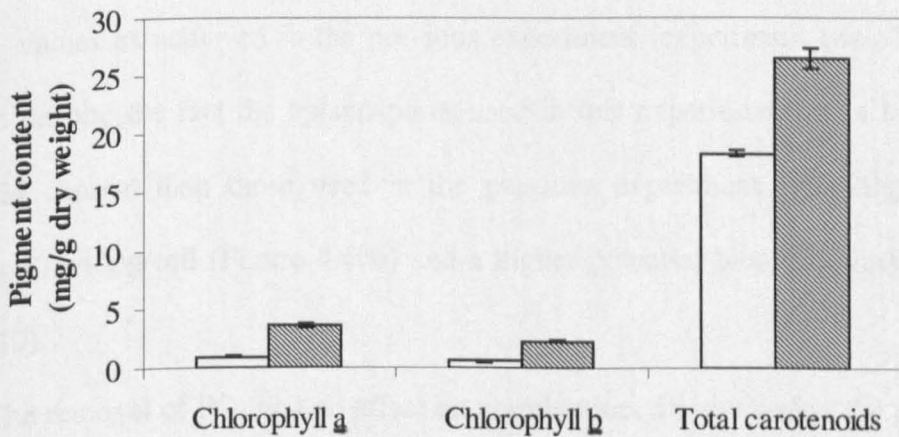
A**B****C**

Figure 4.39. Pigment content expressed as mg/l, (A, $n=3 \pm$ S.E.), pg/cell (B, $n=12 \pm$ S.E.) and mg/g dry weight (C, $n=9 \pm$ S.E.) of *H.pluvialis* before (\square) and after (hatched) the germination of the aplanospores with modified BBM (Experiment two, see text for details).

The ratio of chlorophyll *a/b* remained unchanged after germination (Table 4.9), and there was a drop in the ratio of total chlorophyll to total carotenoids. This was largely due to the change in the carotenoid content of the culture rather than a change in the chlorophyll content.

4.4.3.2. Nutrient supplementation – effect of phosphate

Having produced carotenoid containing zooid cells using a concentrated media feed the next stage was to investigate whether all the components were required for the germination of aplanospores. The phosphate source (PO_4) was removed from the nutrient supplement (Appendix 1b) and its performance compared with a stock that contained the full complement of media components. The phosphate source was removed from the supplement as it is present in high quantities in modified BBM (Appendix 1a) and has been shown to be of importance in carotenogenesis (Harker *et al* 1996a).

The use of the complete BBM supplement yielded a culture that was 83.75% zooid with a potential bioavailability of 74.89% (Table 4.10). These were practically the same values as achieved in the previous experiment (experiment two, Table 4.8). This was despite the fact the aplanospores used in this experiment had a higher total carotenoid content than those used in the previous experiment (50.42mg/l (Figure 4.40a) or 178.46pg/cell (Figure 4.40b) and a higher potential bioavailability (23.40%, Table 4.10).

The removal of PO_4 had no effect on germination, 58 hours after the addition of the nutrient supplement (- PO_4) the culture was 85.25% zooid and had an apparent bioavailability of 75.11% (Table 4.10). This demonstrated that there is no specific requirement for PO_4 to be added to an aplanospore culture for germination.

Table 4.10. Response of stationary phase (aplanospore) culture of *H. pluvialis* after the addition of a BBM nutrient supplement +PO₄ and -PO₄ (see text for details).

	Time 0 (before treatment)	58 hours after addition of BBM (+PO ₄)	58 hours after addition of BBM (-PO ₄)
Cell density (x10 ⁴ , <i>n</i> =4 ± S.E.)	28.25 (± 1.49)	85.00 (± 1.58)	95.50 (± 3.48)
Proportion of the population as zooid (%, <i>n</i> =4 ± S.E.)	0.00 (± 0.00)	83.75 (± 1.03)	85.25 (± 0.63)
Dry weight (g/l, <i>n</i> =3 ± S.E.)	3.00 (± 0.02)	2.25 (± 0.03)	2.23 (± 0.06)
Dry weight per cell (ng, <i>n</i> =12 ± S.E.)	9.28 (± 1.02)	2.42 (± 0.19)	2.10 (± 0.18)
Potential bioavailability (%, <i>n</i> =9 ± S.E.)	23.50 (± 1.78)	74.89 (± 2.14)	75.11 (± 1.30)

However it does not show that there is no requirement for PO₄ in the germination of aplanospores. Modified BBM contains a high concentration of phosphate, which remains at a high concentration even when the nitrogen source has been depleted and the culture is rich in aplanospores (data not shown).

The chlorophyll *a* and *b* content of the culture increased after germination on a per volume basis (Figure 4.40a), although the content per cell remained constant (Figure 4.40b). As with the previous experiments the content of chlorophyll *a* and *b* and the total carotenoids increased (when calculated on a per weight basis) after germination (Figure 4.40c).

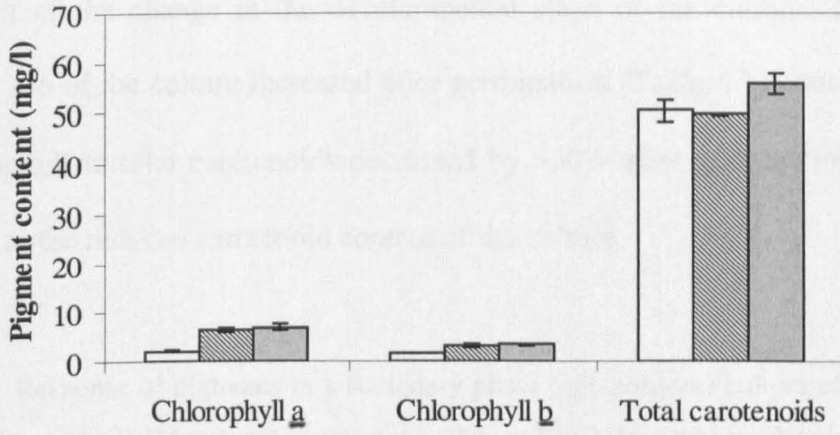
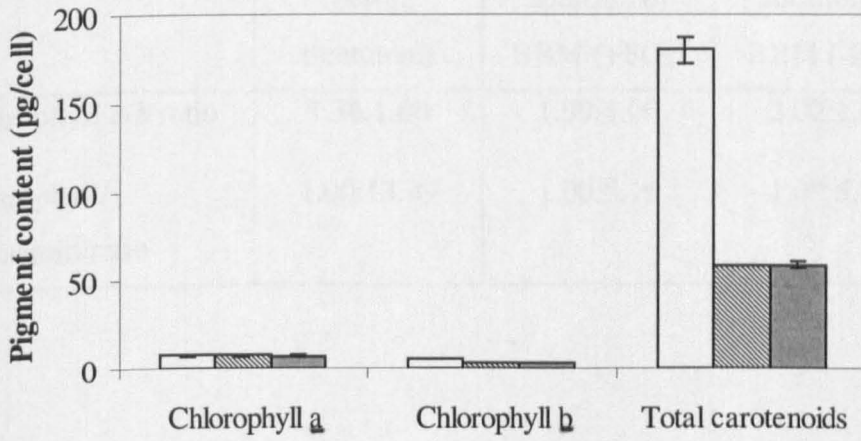
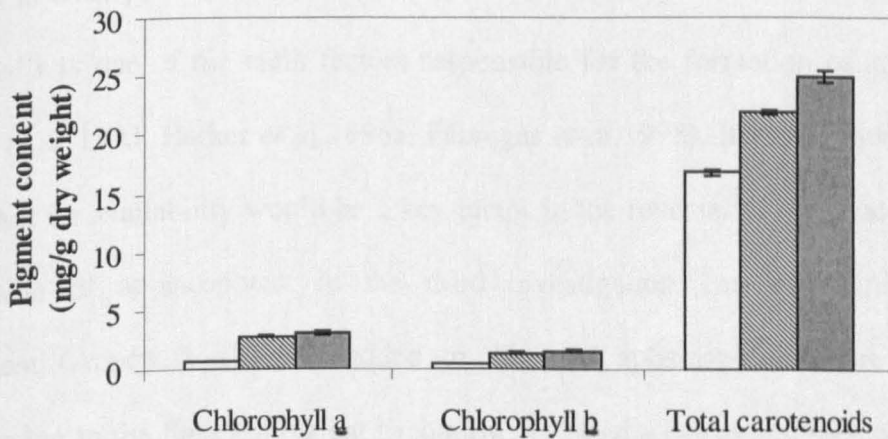
A**B****C**

Figure 4.40. Pigment content expressed as mg/l, (A, $n=3 \pm$ S.E.), pg/cell (B, $n=12 \pm$ S.E.) and mg/g dry weight (C, $n=9 \pm$ S.E.) of *H.pluvialis* before (□) and after the germination of the aplanospores using +PO₄ BBM (▨) and -PO₄ BBM (▩, see text for details).

This was due to the reduced weight per cell of the culture (Table 4.10), which was a result of the change in the developmental stage of the culture. The ratio of chlorophyll *alb* of the culture increased after germination (Table 4.11) but the ratio of total chlorophyll to total carotenoids decreased by >50% after germination. This was largely due to the reduced carotenoid content of the culture.

Table 4.11. Response of pigments in a stationary phase (aplanospore) culture of *H. pluvialis* after the addition of a BBM nutrient supplement +PO₄ and -PO₄ (see text for details).

	Time 0 (before treatment)	58 hours after addition of BBM (+PO ₄)	58 hours after addition of BBM (-PO ₄)
Chlorophyll <i>alb</i> ratio	1.36:1.00	1.99:1.00	2.02:1.00
Chlorophyll/ carotenoid ratio	1.00:13.89	1.00:5.19	1.00:5.35

4.4.3.3. Nutrient supplementation– effect of urea and trace elements

It is widely accepted that the depletion of the nitrogen source from a culture of *H. pluvialis* is one of the main factors responsible for the formation of aplanospores (Zlotnik *et al* 1993, Harker *et al* 1996a, Fábregas *et al* 1998). It would therefore seem logical that its availability would be a key factor in the reversal of the process, i.e. the germination of aplanospores. In the third investigation 1ml of a urea nutrient supplement (Appendix 1b) was added to 25ml of aplanospore culture to give a concentration in the flask equivalent to the low [N] media (assuming the concentration in the aplanospore culture was zero). The stock solution also contained trace elements at the same concentration as found in modified BBM (Appendix 1a). After 57h hours

this treatment yielded a culture that was 86.00% zooid with an apparent bioavailability of 91.29% (Table 4.12).

Table 4.12. Response of stationary phase (aplanospore) culture of *H. pluvialis* after the addition of a nutrient supplement of urea plus trace elements (see text for details).

	Time 0 (before treatment)	55 hours after nutrient supplementation
Cell density ($\times 10^4$, $n=4 \pm$ S.E.)	17.50 (\pm 1.32)	74.00 (\pm 5.24)
Proportion of the population as zooid (% , $n=4 \pm$ S.E.)	0.00 (\pm 0.00)	86.00 (\pm 1.08)
Dry weight (g/l, $n=3 \pm$ S.E.)	2.50 (\pm 0.05)	1.87 (\pm 0.04)
Dry weight per cell (ng, $n=12 \pm$ S.E.)	12.62 (\pm 1.40)	2.36 (\pm 0.20)
Apparent bioavailability (% , $n=9 \pm$ S.E.)	3.67 (\pm 0.43)	91.29 (\pm 0.96)

Of the three treatments tested this yielded the highest potential bioavailability of astaxanthin at 91.29%. The chlorophyll *a* and *b* content of the culture on a per volume basis increased after the germination of the cells (Figure 4.41a), there was also a small reduction in the total carotenoid content. The carotenoid content per cell dropped after germination as did the chlorophyll content (Figure 4.41b). There was an increase in the content on a per dry weight basis for both chlorophyll and carotenoids (Figure 4.41c), as had been seen in the previous germination cultures. Again this is likely to be due to the change in the developmental stage of the culture from aplanospore to zooid which results in a reduced weight per cell (Table 4.12).

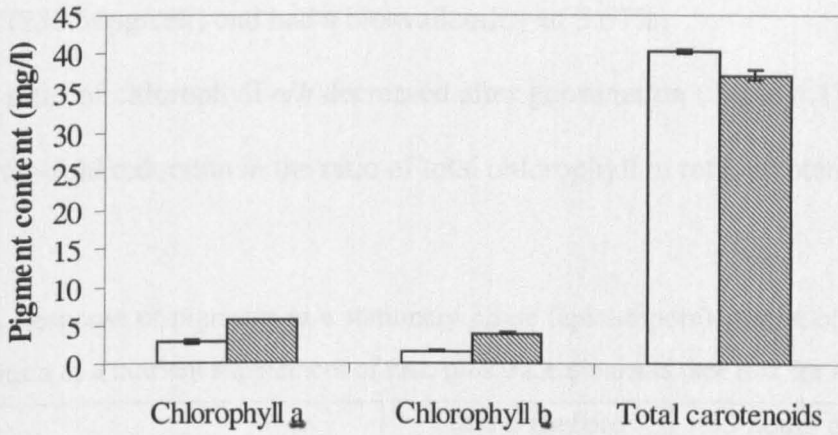
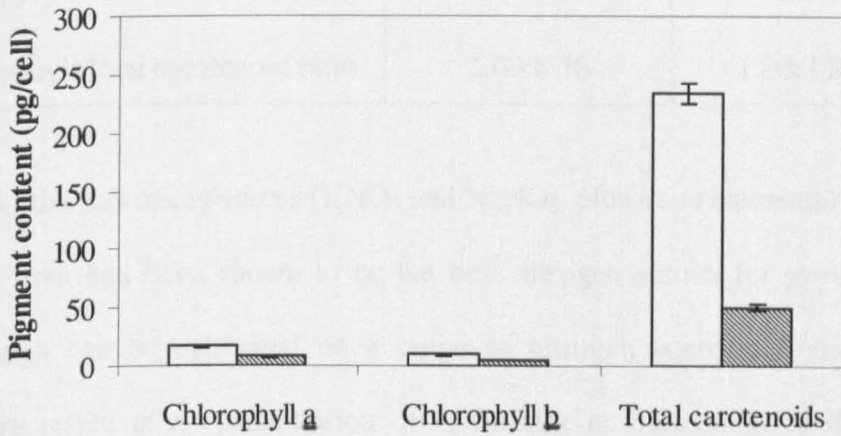
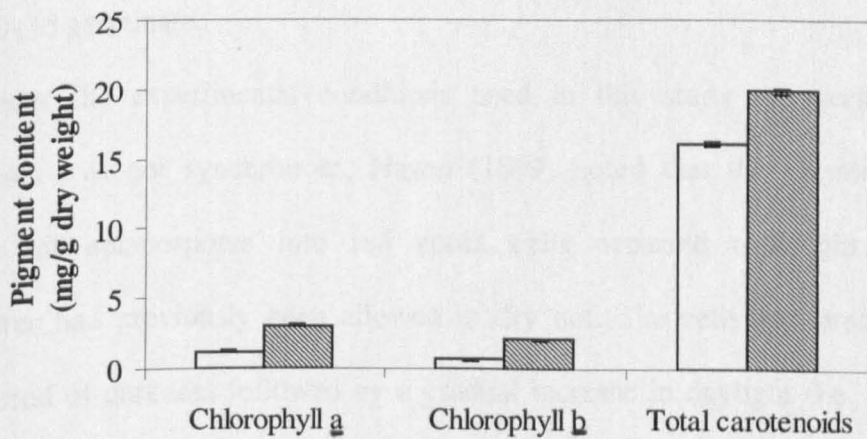
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Figure 4.41. Pigment content expressed as mg/l, (A, $n=3 \pm$ S.E.), pg/cell (B, $n=12 \pm$ S.E.) and mg/g dry weight (C, $n=9 \pm$ S.E.) of *H.pluvialis* before (□) and after (▨) the germination of the aplanospores with urea (+ trace elements, see text for details).

The aplanospores used for this experiment also contained the highest level of secondary carotenoids (230.56pg/cell) and had a bioavailability of 3.67%.

The ratio of chlorophyll *a/b* decreased after germination (Table 4.13) and there was also a two-fold reduction in the ratio of total chlorophyll to total carotenoids.

Table 4.13. Response of pigments in a stationary phase (aplanospore) culture of *H. pluvialis* after the addition of a nutrient supplement of urea plus trace elements (see text for details).

	Time 0 (before treatment)	55 hours after stock addition
Chlorophyll <i>a/b</i> ratio	1.76 :1.00	1.45:1.00
Chlorophyll/total carotenoid ratio	1.00:8.70	1.00:3.82

Two other nitrogen sources (KNO₃ and NaNO₃, plus trace elements) were tested as although urea had been shown to be the best nitrogen source for growth (Harker 1995) the alga can be cultivated on a range of nitrogen sources. Both alternative sources were tested at a concentration of 0.75mM (i.e. Equivalent to the low [N] media). In both cases it took a greater length of time for the cultures treated with KNO₃ and NaNO₃ to germinate.

Under the experimental conditions used in this study the germination of aplanospores was not synchronous. Hazen (1899) noted that the germination of a population of aplanospores into red zooid cells occurred overnight when the aplanospores had previously been allowed to dry out. The cells appeared to benefit from a period of darkness followed by a gradual increase in daylight (i.e. sunrise). In culture three grown in the AAPS™¹ the germination of the aplanospores occurred overnight resulting in a culture that was entirely made up of carotenoid-rich zooid cells. The cells had previously experienced a short dry period, after the harvest and before the

addition of tap water to the system. From this and the work carried out under controlled environmental conditions it appears that it is not just nutrient levels that are of importance in triggering the germination of aplanospores.

The synchronous release of red zooids is of importance because (as identified in AAPS™1 culture three), following germination there is a gradual decrease in the carotenoid content of the cells (section 4.2.3.1). For commercial production a consistent germination response is essential in order to obtain the highest carotenoid yields. A gradual decrease in the carotenoid content of germinated zooids over time was also noted by Lee and Ding (1994). During this study the cells started to germinate after 24 hours but it took a further 30+ hours before the culture reached 85% zooid. Those cells that germinated after 24 hours would have had a much reduced carotenoid content at the point of harvest (i.e. after 58 hours).

The indoor experiments were carried out under controlled conditions with a continuous irradiance of 40-60 $\mu\text{mol}/\text{m}^2/\text{s}$. The data and literature discussed above indicates that zooid production may benefit from a period of darkness which is sandwiched between two periods of illumination. Also Kobayashi *et al* (1992) determined that carotenoid formation was affected by the spectral properties of light, i.e. carotenoid formation was more efficiently enhanced under blue rather than red light. It is therefore possible that this is of importance in the germination of carotenoid-rich zooid cells from aplanospores.

4.5. Discussion

A summary of the biomass productivity and carotenoid yield of the *H. pluvialis* cultures produced by the AAPS™1 and AAPS™2 are detailed in Table 4.14 (cultures two and four are not presented as they failed to produce harvestable cultures). Four cultures were grown in media with an initial urea concentration of 0.75mM (low [N] media). Each had a different cellular productivity as well as final cell density and dry weight. As each culture was grown using the same media recipe the differences in performance are likely to be due to differences in the climatic conditions during growth. Differences in the physical performance of AAPS™1 and AAPS™2 could also have affected culture performance. Cultures one and three were grown in the AAPS™1, cultures five and seven in the AAPS™2. For example, the AAPS™1 was operated with a higher photostage flow rate and also a greater gas hold-up and mass transfer (Chapter 3, section 3.4.1 and 3.5.1) indicating that mixing and gas transfer would have been improved in the AAPS™1 (Chisti 1989). However no firm conclusion can be drawn as the AAPS™1 and AAPS™2 were not run together under the same climatic conditions.

Climatic measurements (irradiance and air temperature) were not made during the growth of cultures one and three. Culture five was produced under warmer and brighter conditions than culture seven. Irradiance is perhaps the most important factor affecting the growth of algal cultures. The irradiance received by cells within a culture will be dependent on the direct irradiance falling on the system as well as reflected and diffuse irradiance (Acién Fernández *et al* 1997). It would also be affected by the cell density (Eriksen *et al* 1996) and efficiency of mixing within the system (Grobbelaar 1991, 1994).

Table 4.14. Summary of production and yield of *H. pluvialis* from batch runs in the AAPS™ outdoors.

	AAPS™1 Culture one	AAPS™1 Culture three	AAPS™2 Culture five	AAPS™2 Culture six	AAPS™2 Culture seven	AAPS™2 Culture eight
Media	Low [N]	Low [N]	Low [N]	High [N]	Low [N]	High [N]
Length of run (days)	34	21	18	48	57	57
Cell productivity ($\times 10^4$ cells/ml/day, $n=16 \pm$ S.E.)	0.38 (± 0.02)	0.72 (± 0.03)	1.04 (± 0.04)	1.43 (± 0.06)	0.33 (± 0.01)	1.24 (± 0.04)
Cell yield ($\times 10^4$, $n=4 \pm$ S.E.)	14.50 (± 1.32)	16.80 (± 1.49)	20.50 (± 1.26)	70.00 (± 5.43)	19.25 (± 1.80)	70.00 (± 2.61)
Dry weight yield (g/l, $n=3 \pm$ S.E.)	0.24 (± 0.01)	0.49 (± 0.00)	0.76 (± 0.07)	1.68 (± 0.01)	0.45 (± 0.01)	0.98 (± 0.01)
Dry weight per cell (ng, $n=9 \pm$ S.E.)	1.72 (± 0.08)	3.00 (± 0.13)	3.73 (± 0.20)	2.44 (± 0.10)	2.41 (± 0.11)	1.41 (± 0.03)
Developmental stage of cells at harvest						
aplanospore (% , $n=4 \pm$ S.E.)	50	100	75	67	100	60
green zooid (% , $n=4 \pm$ S.E.)	0	0	0	0	0	40
red zooid (% , $n=4 \pm$ S.E.)	50	0	25	23	0	0
Carotenoid productivity						
mg/l/day ($n=9 \pm$ S.E.)	0.13 (± 0.00)	0.66 (± 0.01)	1.41 (± 0.05)	1.03 (± 0.00)	0.10 (± 0.00)	0.16 (± 0.00)
pg/cell/day ($n=144 \pm$ S.E.)	0.94 (± 0.01)	3.71 (± 0.10)	6.59 (± 0.19)	1.42 (± 0.04)	0.51 (± 0.02)	0.23 (± 0.00)
mg/g/dry weight ($n=81 \pm$ S.E.)	0.55 (± 0.01)	0.90 (± 0.03)	1.63 (± 0.05)	0.56 (± 0.01)	0.21 (± 0.00)	0.16 (± 0.00)
Carotenoid yield from harvest						
mg/l ($n=3 \pm$ S.E.)	4.57 (± 0.06)	13.94 (± 0.29)	25.35 (± 1.26)	51.13 (± 1.44)	5.35 (± 0.12)	8.98 (± 0.04)
pg/cell ($n=12 \pm$ S.E.)	32.25 (± 1.46)	85.07 (± 3.67)	127.73 (± 6.38)	74.36 (± 3.14)	28.99 (± 1.13)	12.88 (± 0.25)
mg/g/dry weight ($n=9 \pm$ S.E.)	18.82 (± 0.39)	28.42 (± 0.30)	33.97 (± 1.66)	30.43 (± 0.44)	11.82 (± 0.17)	9.13 (± 0.07)
% dry weight ($n=9 \pm$ S.E.)	1.24 (± 0.08)	2.80 (± 0.07)	3.40 (± 0.22)	3.05 (± 0.05)	1.18 (± 0.01)	0.91 (± 0.07)

Initially as each culture grown in the low [N] media had the same inoculation density the growth rate would have been determined by the irradiance received and the mixing of the system. As the cultures increased in density it is possible that cell shading would have begun to affect the growth rate by reducing the irradiance received per cell (Acién Fernández *et al* 1998). This would have impacted on the photosynthetic efficiency of the culture (Raven 1988). Without sufficient supply of CO₂ and removal of photosynthetic O₂ the photosynthetic apparatus would be unable to function effectively, which could lead to a reduction in growth (Borowitzka 1994, Molina Grima *et al* 1997). Also a build-up of photosynthetic O₂ could result in damage to the photosynthetic apparatus (Torzillo *et al* 1984, Boussiba 2000). Photosynthesis would have been affected by the daily cycle of irradiance in combination with temperature. If the morning temperature is low then the alga may be unable to make full use of its photosynthetic capacity (Torzillo *et al* 1991). Also if the irradiance level increased beyond that which was maximal for photosynthesis the culture would become photoinhibited with would affect growth and yield (Acién Fernández *et al* 1997).

The difference in the dry weight yields and the dry weight per cell could be due to differences in the developmental stage of the algal populations (Table 4.14). This is particularly the case when comparing culture one with culture three. Whereas culture three was made up of aplanospores, culture one was a mixture of red zooids (50%) and aplanospores (50%). This may account for the lower dry weight per cell of culture one. It may also be a function of the degree of encystment (see below).

The carotenoid productivity and carotenoid content at harvest of the four cultures grown in the low [N] media were very different. Culture five, which was grown in AAPS™2 in August/September 1999, proved to be the most productive. It had the highest productivity both in terms of the carotenoid concentration per cell and the

concentration on a per volume basis. It also yielded the highest carotenoid content as a percentage of the dry weight of the culture, over the shortest time period. A number of authors have clearly shown that carotenogenesis in *Haematococcus* is stimulated by high irradiance (Droop 1955, Kobayashi *et al* 1992, Chaumont and Thépenier 1995). Harker *et al* (1995) demonstrated that although the optimum irradiance for growth of *H. pluvialis* was 40-60 $\mu\text{mol}/\text{m}^2/\text{s}$ the optimum for carotenogenesis was 1,600 $\mu\text{mol}/\text{m}^2/\text{s}$. High temperatures have also been shown to stimulate carotenoid formation in *Haematococcus* (Tjahjono *et al* 1994). The mass transfer of the system and the photosynthetic activity of the culture may also have been important in carotenogenesis. Under conditions of O_2 partial pressures higher than air saturation the proportion of carotenoid-rich aplanospores in a *Haematococcus* culture have been found to increase (Lee and Ding 1995). $^1\text{O}_2$ may also be indirectly involved in astaxanthin accumulation in *Haematococcus* (Fan *et al* 1998). Therefore under conditions of poor mass transfer that would allow a build-up of O_2 , carotenogenesis may be enhanced. It has also been demonstrated that carotenoid rich *Haematococcus* cells are more resistant to photoinhibition (Lee and Ding 1992, Ding *et al* 1994). It has therefore been suggested that carotenoid accumulation in a response to photoinhibition experienced under high irradiance. There is a great deal of debate however as to whether secondary carotenoids in *Haematococcus* act as a passive sunshade (Yong and Lee 1991, Hagen *et al* 1994) or as an active $^1\text{O}_2$ quencher (Kobayashi and Sakamoto 1999, Tjahjono *et al* 1994).

Unfortunately although detailed weather data are available for culture five and seven, no such data are available for cultures one or three (in the AAPS™1). It is therefore difficult to draw strong conclusions as to the effect of irradiance on the productivity of carotenogenesis in the four cultures. It is highly likely that culture five has a faster accumulation rate than culture seven due to its higher received irradiance.

This combined with the fact that culture five had the fastest population doubling time which indicates that the culture would have used up its nitrogen source more rapidly and therefore become nitrogen limited at an earlier stage. Fábregas *et al* (1998) concluded that both nitrogen deficiency and high irradiance were desirable to maximise carotenogenesis in *Haematococcus*.

Two cultures were produced from the high [N] media. Culture six during September and October 1999 and culture eight, which was produced during November and December 1999. Both had similar culture lengths and produced similar cell densities. However the cells produced during September and October 1999 (culture six) were twice as heavy as those produced later in the year (culture eight) and also had a much greater carotenoid yield, producing nearly six times the carotenoid content per cell (Table 4.14). Comparison of the two cultures grown in low [N] media in September/October and November/December 1999 revealed a reduced cellular productivity as well as carotenoid productivity. It would appear that the affect of the poor climatic conditions in November/December 1999 did not have as detrimental an affect on the high [N] media (Table 4.14). As discussed previously (section 4.3.4.1) this may have been linked to the CO₂ feed for pH control, which was used in culture eight but not in culture six.

The yield from culture six was not as great as the yield of the low [N] culture produced at the same time in September and October 1999 (culture five). Culture five produced nearly twice the carotenoid content per cell, though it had a reduced content per gram of dry material. As both cultures were produced at the same time in comparable systems the difference in carotenoid productivities is likely to be due to the combined effects of an increased nitrogen content inhibiting carotenogenesis (Lee and Soh 1991, Harker *et al* 1996a) and the increased cell density. The increased cell density

would increase to cell shading thereby reducing the irradiance received per cell (Eriksen *et al* 1998). This would act to reduce the rate of carotenoid accumulation as irradiance is a major trigger of carotenogenesis (Harker *et al* 1995, Kobayashi *et al* 1997, Fábregas *et al* 1998).

Cultures grown in low [N] media produced an *Haematococcus* cell type that had not been previously seen in the laboratory. In the first culture grown outdoors the cells began accumulating secondary carotenoids while in the exponential phase of growth. This resulted in the production of a culture that was made up of carotenoid-rich zooids (Plate 4.1d.). As the culture aged and the carotenoid content increased the culture became a mixture of the typical red aplanospores (Plate 4.2b.) and the previously unseen red zooid cells. The factors that led to the formation of carotenoid-rich zooids are unclear as little work on this subject has been undertaken in the literature. Hagen *et al* (2001) noted that flagellates were capable of accumulating secondary carotenoids when exposed to nitrogen limitation and strong irradiance. Chaumont and Thépenier (1995) observed that in the continuous operation of their 50l tubular photobioreactor flagellates were produced that had a red area in their apical section. This phenomenon had also been noticed in the open pond cultivation of *H. pluvialis* (Bubrick 1991).

Red zooids were again produced in culture three in May 1999. Unlike the first culture, at the end of the run the algal population was made up entirely of red aplanospores. After harvest, but before cleaning, tap water was added to the system, which was left to circulate overnight. After 19 hours this system appeared red due to the production of red zooids in the tap water. These had germinated from aplanospores that had adhered to the photostage wall. Again the reasons for the formation of these zooids are unclear. Examination of the life cycle of *H. pluvialis* suggests that the red zooid cells produced at the end of culture three were very different from those produced

during the growth of culture one. The zooids in culture one were the typical zooid cells (described by Elliot (1934) as macrozooids) which normally appear green due to their lack of secondary carotenoids. However in the case of culture one environmental factors acted to cause the accumulation of secondary carotenoid within the cells while they were still actively growing. In the case of culture three the zooid cells did not synthesise the secondary carotenoids, they were formed from aplanospores that already contained the secondary carotenoids. The reason for their formation is unclear however they were not the typical macrozooid cells seen in actively growing cultures but microzooids. These microzooids have previously been described by a number of authors (Hazen 1899, Peebles 1909, Elliott 1934). They are smaller than macrozooids and swim more actively. It has been postulated that the purpose of the microzooid is to seek a more favourable environment, as they germinate from resting cysts and swim more actively than macrozooids as well as having a much shorter life-span (Elliott 1934). It had also been observed that they frequently die rather than divide (Hazen 1899).

Red zooid cells have been shown to readily germinate from resting aplanospores when the aplanospores are re-suspended in fresh media (Lee and Ding 1994). Therefore it may have been a synergistic effect between the nitrogen concentration of the tap water and the short-term irradiance of the culture. Hazen (1899) suggested that the production of red zooids from aplanospores might be a function of the irradiance they received. When aplanospores were exposed to a preparatory darkness and then received the natural gradual increase in daylight in the morning abundant red zooids were produced.

Following from the production of red microzooids in culture three, laboratory work was undertaken in an attempt to manipulate an aplanospore culture to produce carotenoid rich zooids (section 4.4). Germination of aplanospores was achieved with the addition of various nutrients to the cultures. However germination was never synchronous as was seen in culture three. This led to a reduction in the carotenoid concentration in the zooids that germinated first, while waiting to harvest until a large proportion of the cells were zooids.

The production of red zooids in the AAPS™ was not consistent. Subsequent cultures in the low [N] media did not exhibit red zooid cells in great numbers. Culture six, which grew in media with the high [N], produced a small number of red zooids in a culture that was primarily made up of aplanospores. However, these cells differed from the red zooids that had been produced in the low [N] media. Zooids in the low [N] media were completely red in appearance whereas those in the high [N] media were green on the periphery of the cell, with localised carotenoid accumulation (Plate 4.1c.). This may be a function of the combined effect of irradiance and nitrogen. High irradiance is stimulatory whilst high nitrogen is inhibitory towards carotenoid biosynthesis and subsequent accumulation (Lee and Soh 1991, Fabregas *et al* 1998).

The two cultures that were grown outdoors in November/December 1999 failed to produce a carotenoid-rich culture, whereas earlier work (September/October) in the same systems using the same media had resulted in carotenoid-rich cultures. This could be a result of two factors. Firstly the grow rate in the November/December cultures was much slower than the September/October cultures indicating that the nitrogen level in the media did not deplete as rapidly, therefore the onset of nutritional stress was delayed. Secondly the climatic conditions (irradiance, air temperature) were reduced in

the later cultures thereby reducing the environmental stress that the cultures were exposed to.

4.6. Conclusions

The AAPSTM1 and the AAPSTM2 both produced *H. pluvialis* with a high cell density and carotenoid content. The cell density, productivity and the carotenoid accumulation of the cultures was strongly dependant on the nitrogen content of the media used and the climatic conditions the cells were exposed to, particularly the irradiance.

Carotenoid accumulation can occur in *H. pluvialis* while the cells are actively dividing, i.e. nitrogen limitation is not essential for the accumulation of astaxanthin. When a culture that contained nitrogen was exposed to high irradiance carotenogenesis occurred. However the rate of accumulation is still dependent on the nitrogen content of the media. This was also demonstrated by Boussiba *et al* (1992) who determined that under high irradiance the astaxanthin accumulation rate was determined by the nitrogen status of the culture.

Both media recipes (low and high [N]) produced carotenoid-rich cultures although the medium with the lower nitrogen content had a higher productivity per cell when both were exposed to the same climatic conditions. Each medium has its advantages, the low [N] medium produces a higher total carotenoid content per cell, whereas cultures grown in the high [N] medium have a lower astaxanthin content per cell but produces far more cells, resulting in has a higher carotenoid yield per unit volume of media.

Having assessed the productivity of using batch culture to produce carotenoid-rich *H. pluvialis*, the next stage of the study was to assess the performance of *H.*

pluvialis in continuous culture to enable a conclusion to be made as to the best strategy for large-scale culture.

5. Continuous growth of *Haematococcus pluvialis* in the Advanced Algal Production System

5.1. Introduction

The studies shown in Chapter 4 demonstrated that carotenoid-rich *H. pluvialis* can be produced in the AAPS™ photobioreactor under batch conditions. As an alternative to batch cultivation of *Haematococcus* continuous cultivation may be viable. Continuous culture differs from batch culture in that when the culture reaches the exponential phase of growth it is supplemented with fresh media, which acts to sustain the growth of the culture. The culture remains at a constant volume throughout, with excess culture being harvested. This addition of media can take one of two forms. In the first, supplementation with fresh media occurs at discrete time intervals. A predetermined volume of culture is removed at a certain time each day and replaced with the same volume of fresh media. This is termed fed-batch, or semi-continuous culture. The second approach, involving the continuous feed of nutrients, is the method tested in the AAPS™2. This results in the continuous production of algal biomass. A dosing pump is used to provide a continuous, low-level addition of fresh media for up to 24hrs. In the case of a photosynthetic organism the length of this dosing period is dependent on the length of time that the cells are illuminated. In indoor culture, with artificial illumination, this may be continuously over 24 hours but for outdoor culture fresh media would only be fed into the culture during daylight hours. It is only when a photosynthetic culture is illuminated that the cells would be photosynthesising and therefore growing. A media feed into a non-growing culture would result in the 'washout' of the culture.

The cultivation of *H. pluvialis* is complicated by the fact that it is not sufficient to simply produce maximal biomass. The biomass must also be rich in the secondary carotenoid astaxanthin. In AAPS™ batch culture it was shown that astaxanthin synthesis can occur under non nutrient-limited conditions (Chapter 4, section 4.2.1.2.), however astaxanthin accumulation typically requires growth limiting conditions, especially nitrogen limitation (Lee and Soh 1991, Zlotnik *et al* 1993, Fábregas *et al* 1998). In continuous production the culture would not be nitrogen limited at any stage. There are therefore two possible methods for producing a carotenoid-rich culture (Figure 5.1).

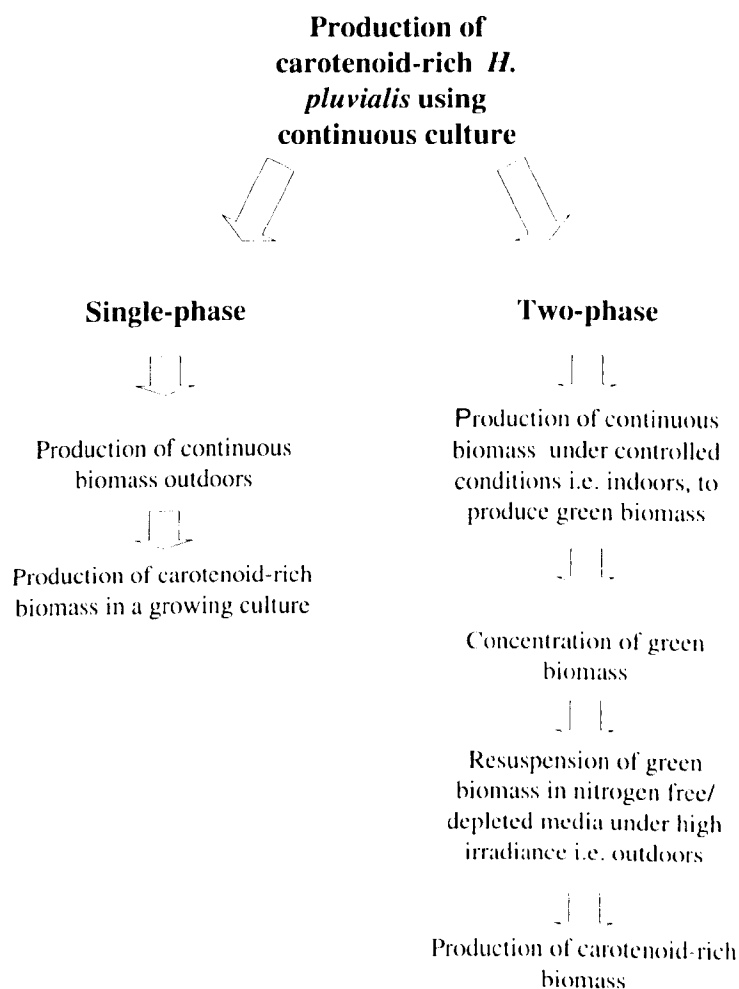


Figure 5.1. Single and two-phase strategies to produce carotenoid-rich *H. pluvialis* using continuous culture.

The first is a two-phase process. The continuous production of biomass would be set up under controlled conditions indoors optimised for growth. In this way the system would produce maximal algal biomass, however the resulting green biomass would not have accumulated astaxanthin. Therefore this culture would then be concentrated and/or washed to remove as much of the nitrogen as possible then resuspended in nitrogen-free/depleted media outdoors (in natural sunlight) to enable rapid carotenoid accumulation (Fábregas *et al* 1998). The second approach is a single-phase process in which the continuous culture would be operated outdoors where conditions may not be optimal for growth, but where secondary carotenoid accumulation could occur in the growing culture. This strategy is supported by the observation that *H. pluvialis* is capable in batch culture in the AAPS™, under high irradiance, of growth and secondary carotenoid synthesis in parallel (Chapter 4 section 4.2.1.2). Here it appears that exposure to irradiance much greater than can readily be achieved indoors overrides the requirement for nutrient-limited stimulation of carotenoid accumulation. Harker *et al* (1996a) found that the single most important factor in carotenogenesis was subjecting the *H. pluvialis* cells to a high irradiance. Fábregas *et al* (1998) studied the affect of nutrient limitation on *Haematococcus* when exposed to 'high' (230 $\mu\text{mol}/\text{m}^2/\text{s}$) and 'low' (40 $\mu\text{mol}/\text{m}^2/\text{s}$) irradiances. Their work determined that although nitrogen was the major trigger for astaxanthin synthesis the addition of 'high' irradiance further improved productivity. They concluded that for maximal synthesis both nitrogen limitation and high irradiance should be employed.

5.1.1. Aims

The aim of this work was to investigate the use of the AAPS™2 for the continuous production of *H. pluvialis*. Two-phase (indoor continuous with an outdoor

accumulation phase) and single-phase (outdoor continuous) methods of culture were assessed.

5.2. Two-phase production

The AAPS™2 was set up under controlled conditions in a growth room at 18°C. A lighting frame was positioned inside the photostage, which contained four fluorescent tubes (F18W/GRO, Grow-lux 4-feet, Osram). The measured mean irradiance at the internal surface of the photostage was 52µmol/m²/s, close to the irradiance determined optimal for growth (Harker 1995).

The system was sterilised as detailed in Chapter 2. The additional tubing and vessels required for the continuous culture were also sterilised and set up at this time in order to maintain the integrity of the system (Figure 5.2, also see Chapter 2). The system was operated containing media with a urea concentration of 3.0mM (termed high [N] media, see Appendix 1a); pH control was achieved by injecting CO₂ with a set point of 7.5.

5.2.1. Biomass production

The system was inoculated with an actively growing green culture of *H. pluvialis*, which gave a starting cell concentration of 3.68x10⁴ cells/ml. This was more than double the inoculum density of any previous batch culture and was used in order to minimise the lag phase and enable the commencement of the continuous feed as soon as possible after inoculation.

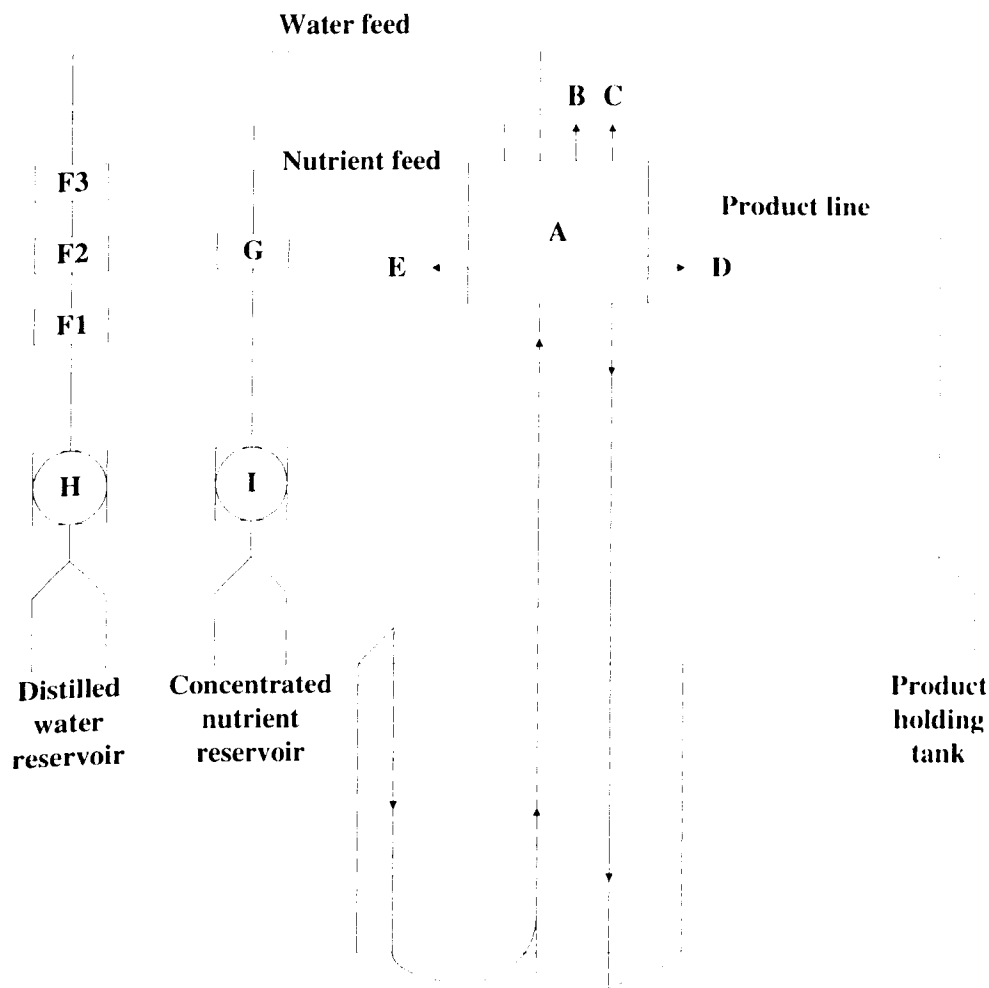


Figure 5.2 Schematic of the AAPS™2 for continuous operation.

- A.** Header tank
- B.** Non-return valve, air out (George Fisher)
- C.** Air filter (Polyvent 40, Whatman), air in during draining
- D.** Sample line
- E.** pH probe (Combination pH probe, Russell, Auchtermuchty, UK)
- F1-F3.** In-line water filters
(5, 1 and 0.1 μ respectively, Fieder, Maidstone, UK)
- G.** Nutrient filter (0.2 μ m, Merck)
- H.** Water dosing pump (Alldos, Eichler, Germany)
- I.** Nutrient dosing pump (101U, Watson Marlow Ltd., Falmouth, UK)

The culture did not exhibit any lag phase and grew exponentially until day 18 reaching a cell density of 31.25×10^4 cells/ml (Figure 5.3) when the media feed was started.

There was an initial drop in the dry weight per cell (Figure 5.4) which was likely to be due to germination and a period of rapid growth during the first six days of culture. Germination was observed as the inoculum was at the end of the exponential phase of growth and was noted to contain a small number of palmella and aplanospores (~5%). The productivity of this batch phase of growth, was calculated to be 1.45×10^4 cells/ml/day (± 0.04 , $n=16$, \pm S.E.), and was comparable to the productivity of batch cultures outdoors (Chapter 4, Table 4.14). However the doubling time of the cells was relatively slow ($t_d=6.60$ days, day 0-18) when compared to previous batch cultures grown outdoors (Chapter 4, section 4.3.3.1, and when compared to other algae, Clarkson *et al* 2001). In batch culture Zlotnik *et al* (1993) determined the doubling time (at an irradiance of $50 \mu\text{mol}/\text{m}^2/\text{s}$) to be approximately three days. As a result of the low growth rate the media renewal rate was initially set at 10% (v/v) to prevent the possible 'washout' of the culture. The working volume of the AAPS™2 was 55l. This translated to the addition of 5.5l of fresh media (with a urea content of 3.0mM) and the production of 5.5l of algae per day. As the system was illuminated 24 hours a day the media feed was dosed in on a continuous basis 24 hours a day. Plate 5.1 shows the system in continuous operation.

For the first three days of continuous feed (day 18 - 21), the culture continued to increase in cell number, while at the same time producing 5.5 litres of culture per day or 1.72×10^9 cells/day (± 0.08 , $n=4 \pm$ S.E.). The cell count then began to decrease (Figure 5.3) although the feed rate was unchanged.

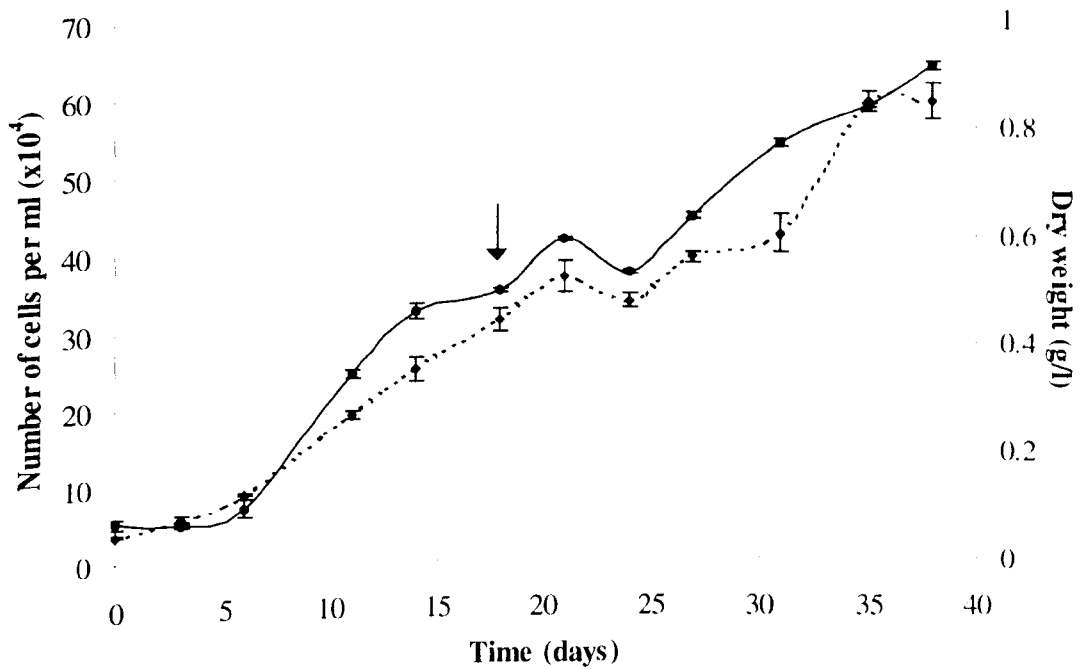


Figure 5.3. Cell density (---, $n=4 \pm$ S.E.) and dry weight (—, $n=3 \pm$ S.E.) of *H. pluvialis* grown in the AAPSTM2 under controlled environmental conditions (see text for details), \rightarrow start of 10% (v/v) media feed.

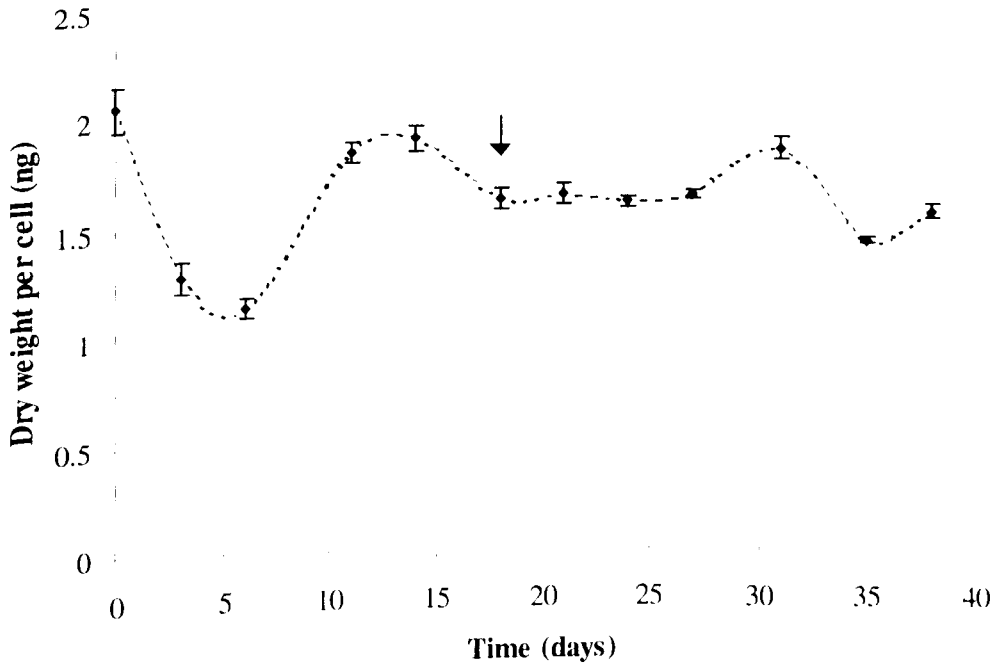


Figure 5.4. Dry weight per cell ($n=12 \pm$ S.E.) of *H. pluvialis* grown in the AAPSTM2 under controlled environmental conditions (see text for details), \rightarrow start of 10% (v/v) media feed.

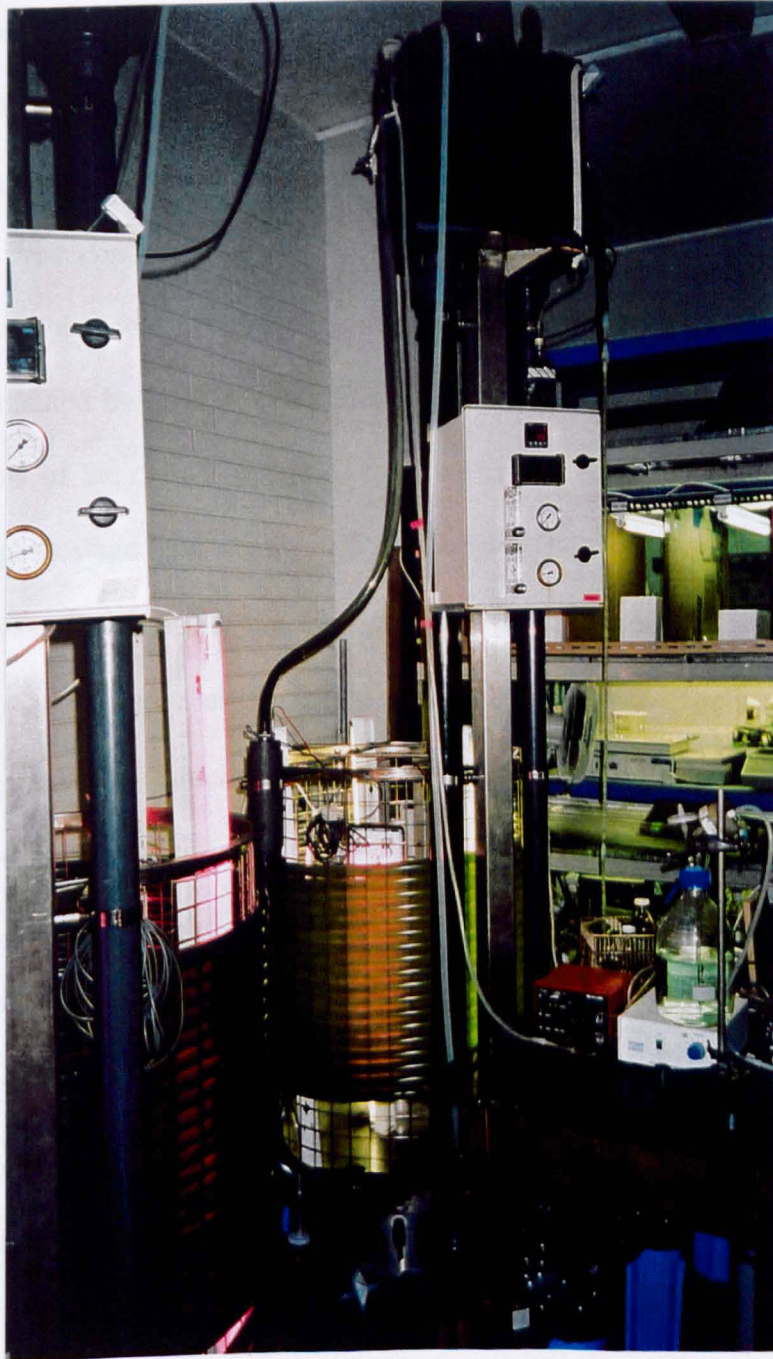


Plate 5.1. Continuous production of *H. phuvialis* biomass in the AAPSTTM2 indoors.

Possible explanations for this decrease in the cell count and dry weight of the culture include a change in the nutrient concentration of the media in the AAPS™2, contamination of the culture or a problem with the lighting regime around the photostage. The phosphate concentration of the media remained constant throughout the culture period (Figure 5.5). However the drop in the cell density corresponds with a drop in the urea concentration of the media to 37% (48.30 mg/l) of the starting concentration. This could indicate that the culture was reaching the stationary phase of growth as indicated by flask grown cultures in the high [N] media (chapter 4, section 4.3.2). The pH of the culture remained constant (Figure 5.6). The lighting regime of around the photostage was reassessed. Although the mean irradiance around the photostage was $52\mu\text{mol}/\text{m}^2/\text{s}$, the configuration of the lighting resulted in a large variation in the irradiance as the cells circulated around it. The irradiance varied between $150\mu\text{mol}/\text{m}^2/\text{s}$ (directly in front of a light tube) and $7\mu\text{mol}/\text{m}^2/\text{s}$ (at the point furthest between two lighting tubes). To give a more constant irradiance around the photostage eight further lighting tubes were added. This resulted in a mean irradiance of $94\mu\text{mol}/\text{m}^2/\text{s}$, and at no point around the photostage did the irradiance drop below $40\mu\text{mol}/\text{m}^2/\text{s}$.

Following the addition of further lighting the cell density of the culture steadily increased until day 35 reaching a cell density of 59.25×10^4 cells/ml. The urea concentration of the media remained constant (Figure 5.5) suggesting that the culture had been light limited. Over the course of the continuous feed (day 18 - 38) the system had a mean productivity of 2.40×10^9 cells/day (± 0.05 , $n=4 \pm \text{S.E.}$).

Light limitation could also explain the slow exponential growth rate (6.60 days) of this culture when compared to the outdoor grown batch AAPS™ cultures which had a doubling time in the order of four days (Chapter 4, section 4.3.3.1).

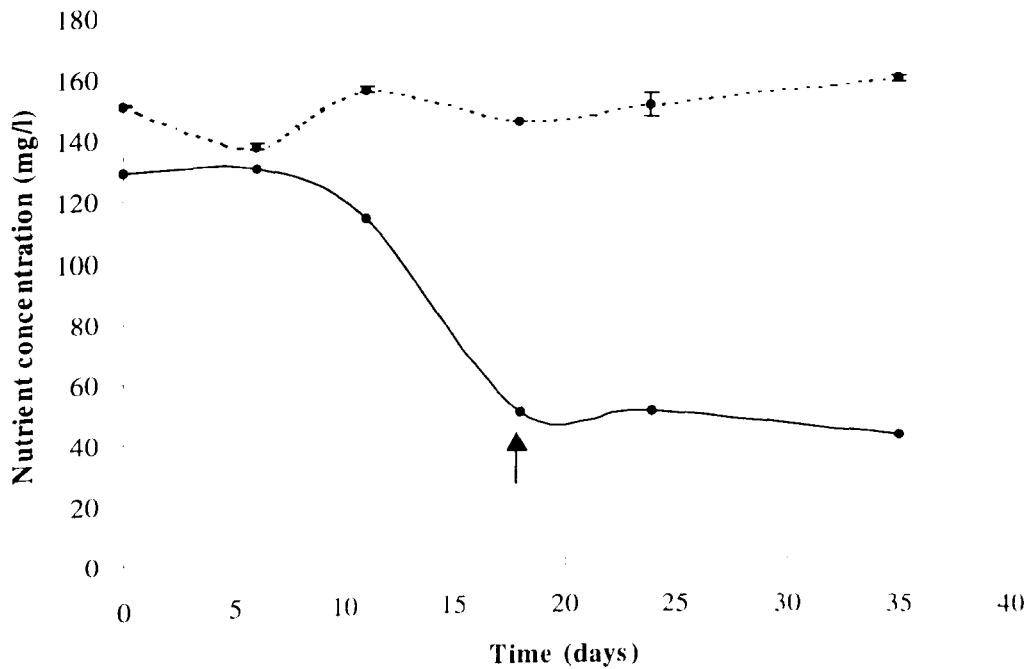


Figure 5.5. Media phosphate (---, $n=3 \pm$ S.E.) and urea (—, $n=1$) concentration during the growth of *H. pluvialis* in the AAPS™2 under controlled environmental conditions (see text for details), \rightarrow start of 10% (v/v) media feed.

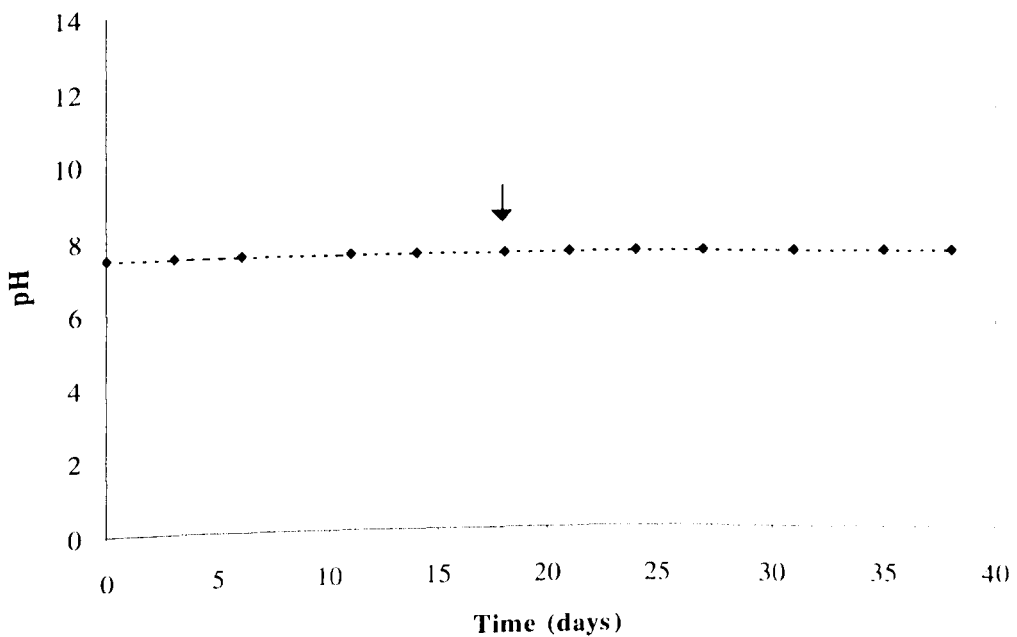


Figure 5.6. pH during the growth of *H. pluvialis* in the AAPS™2 under controlled environmental conditions (see text for details). \rightarrow start of 10% (v/v) media feed.

The light limitation would have limited the photosynthetic rate of the culture thereby limiting growth (Janssen *et al* 2000). Light limitation within the culture would have increased as the cell density increased due to the mutual shading affect (Pulz *et al* 1995, Eriksen *et al* 1996, Richmond and Zoh 1999) this may explain why the culture grew until it reached a cell density of 36.75×10^4 cells/ml (Figure 5.3).

The pigment concentration of the culture on a per volume basis (Figure 5.7) increased steadily over the course of the culture period even after the start of the 10% (v/v) media feed. The pigment concentration per cell (Figure 5.8) was relatively constant during the feed period (day 18-38) as was the concentration when calculated in terms of the dry weight of the culture (Figure 5.9). At all times the ratio of chlorophyll *a* to *b* remained constant.

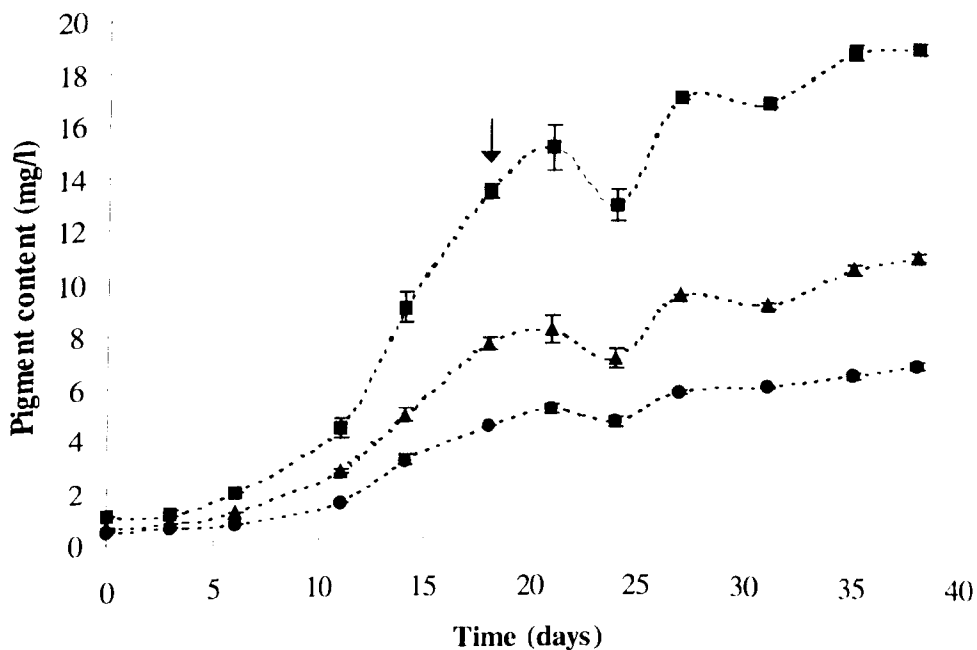


Figure 5.7. Pigment content of *H. pluvialis* (calculated as mg/l, $n=3 \pm$ S.E.) grown in the AAPSTTM2 under controlled environmental conditions (see text for details). Chlorophyll *a* ■, Chlorophyll *b* ▲, Total carotenoid ●, \rightarrow start of 10% (v/v) media feed.

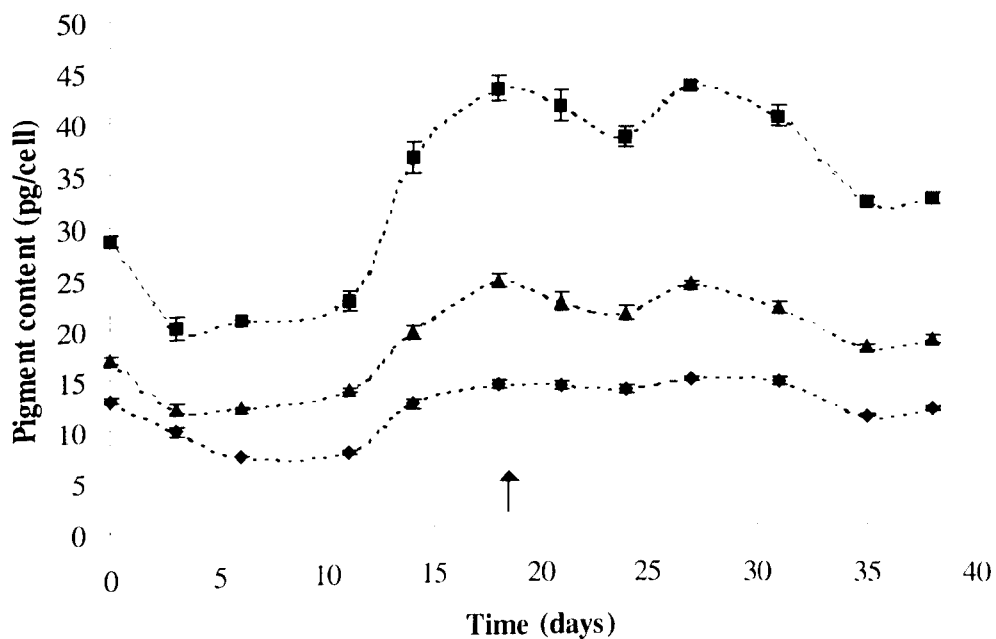


Figure 5.8. Pigment content of *H. pluvialis* (calculated as pg/cell, $n=12 \pm$ S.E.) grown in the AAPSTTM2 under controlled environmental conditions (see text for details). Chlorophyll *a* ■, Chlorophyll *b* ▲, Total carotenoid ◆, \rightarrow start of 10% (v/v) media feed.

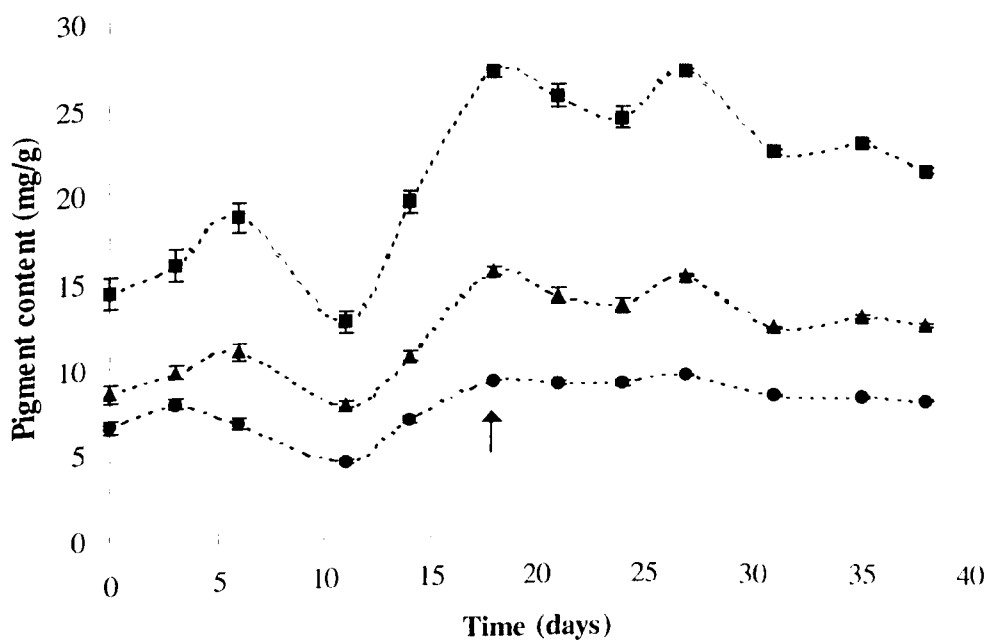


Figure 5.9. Pigment content of *H. pluvialis* (calculated as mg/g dry weight, $n=9 \pm$ S.E.) grown in the AAPSTTM2 under controlled environmental conditions (see text for details). Chlorophyll *a* ■, Chlorophyll *b* ▲, Total carotenoid ●, \rightarrow start of 10% (v/v) media feed.

5.2.2. Product (astaxanthin) formation

H. pluvialis produced from the indoor continuous culture was concentrated using a tangential flow filtration system (HPK12, Millipore, Bedford, UK, see Chapter 2 for details). Typically the original culture was concentrated three-fold as further concentration was found to result in the damage and death of the green zooids (Table 5.1). A three-fold concentration did not damage the flagella of the zooids. Gudin and Chaumont (1991) noted that with the loss of the flagella from the zooid form of *H. pluvialis* the cells were unable to turn into cysts.

Table 5.1. Effect of tangential flow filtration on green zooid cells of *H. pluvialis* (EC = encystment culture).

	EC1	EC2	EC3	EC4
Total number of cells concentrated ($\times 10^9$, $n=4 \pm$ S.E.)	5.41 (± 0.20)	6.05 (± 0.13)	6.90 (± 0.28)	13.3 (± 0.36)
Initial volume (litres)	15	15	15	15
Total number of cells in final concentrate ($\times 10^9$, $n=4 \pm$ S.E.)	3.65 (± 0.13)	5.95 (± 0.15)	7.99 (± 0.35)	14.99 (± 0.69)
Final volume (litres)	2	5	5	5
Concentration factor	8	3	3	3
Recovery of cells (% , $n=12 \pm$ S.E.)	67.21 (± 1.51)	96.96 (± 1.18)	100.00 (± 0.00)	100.00 (± 0.00)

The algal slurry was resuspended in tap water (total nitrogen content of $1.00 (\pm 0.27)$ mg/l ($n=4 \pm$ S.E.) in a second AAPS™2 situated on the roof of the Byrom Street campus building of Liverpool John Moores University. The system was aerated at 15l/min, which resulted in a culture flow rate in the photostage of 0.47m/s. The system was not sterilised prior to use.

5.2.2.1. Encystment of *H. pluvialis*

Initially two successive harvests from the indoor continuous culture of *H. pluvialis* were concentrated and resuspended in the AAPS™2 outdoors. They were termed encystment culture one (EC1) and encystment culture two (EC2). Both cultures took five days (8th-12th May and 12th-16th May 2000) to form a carotenoid-rich culture. A carotenoid-rich culture was defined as a culture of *H. pluvialis* containing at least 1.5% (w/w) total carotenoids as dry weight (Naturose™ Technical bulletin 006, Cyanotech Corp., Hawaii, USA). There was no change in the cell density of either culture over the culture periods, however, both the dry weight (g/l) and the dry weight per cell increased (Table 5.2). This was likely to be due to the encystment of the cells which causes an increase in the dry weight of the cells due to the production of a thick cell wall (Margalith 1999). EC1 was exposed to a higher mean irradiance per day than EC2 (Figure 5.10), it also received a greater total irradiance during the culture period (Figure 5.11). Both cultures were exposed to the same maximum and minimum temperature (Figure 5.13). EC1 also yielded a higher carotenoid content per cell than EC2 (Table 5.3).

EC2 contained nearly double the cell density of EC1 and therefore there would have been greater degree of cell shading in the culture. Due to the increased cell shading each cell in EC2 may have received a reduced irradiance when compared to

EC1. Combined with the fact that the total nitrogen content of EC2 was greater (Table 5.4) the cells of EC2 would have been expected to have a reduced carotenoid yield. Fábregas *et al* (1998) found that although nitrogen deficiency had the greatest affect on stimulating carotenogenesis in *H. pluvialis* the combined affect of nitrogen deficiency and high irradiance maximised productivity.

Table 5.2. Productivity of encystment cultures one (EC1) and two (EC2).

	EC1	EC2
Length of culture period (days)	5	5
Cell density on the first day of culture (x10 ⁴ cells per ml, n=4 ± S.E.)	6.50 (± 0.29)	10.75 (± 0.25)
Cell density at the end of the culture period (x10 ⁴ cells per ml, n=4 ± S.E.)	5.90 (± 0.55)	10.60 (± 0.33)
Dry weight on the first day of culture (g/l, n=3 ± S.E.)	0.08 (± 0.00)	0.11 (± 0.01)
Dry weight at the end of the culture period (g/l, n=3 ± S.E.)	0.18 (± 0.01)	0.23 (± 0.02)
Dry weight per cell the first day of culture (ng, n=12 ± S.E.)	1.29 (± 0.04)	1.03 (± 0.04)
Dry weight per cell at the end of the culture period (ng, n=12 ± S.E.)	3.21 (± 0.16)	1.89 (± 0.04)

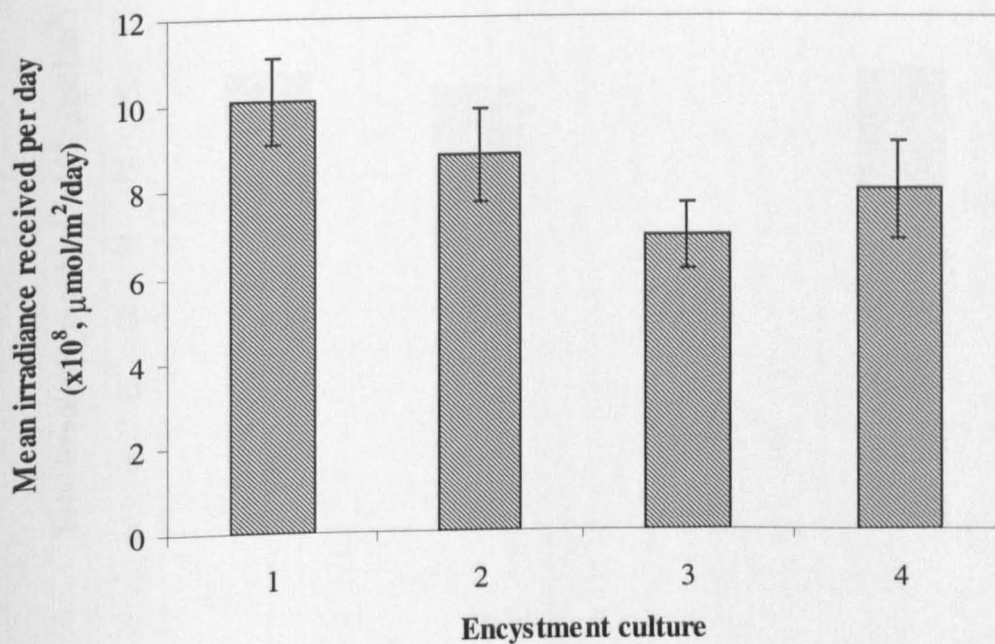


Figure 5.10. Mean daily irradiance ($\mu\text{mol}/\text{m}^2/\text{day}$) measured adjacent to the AAPSTM2 during the four encystment cultures (see text for details).

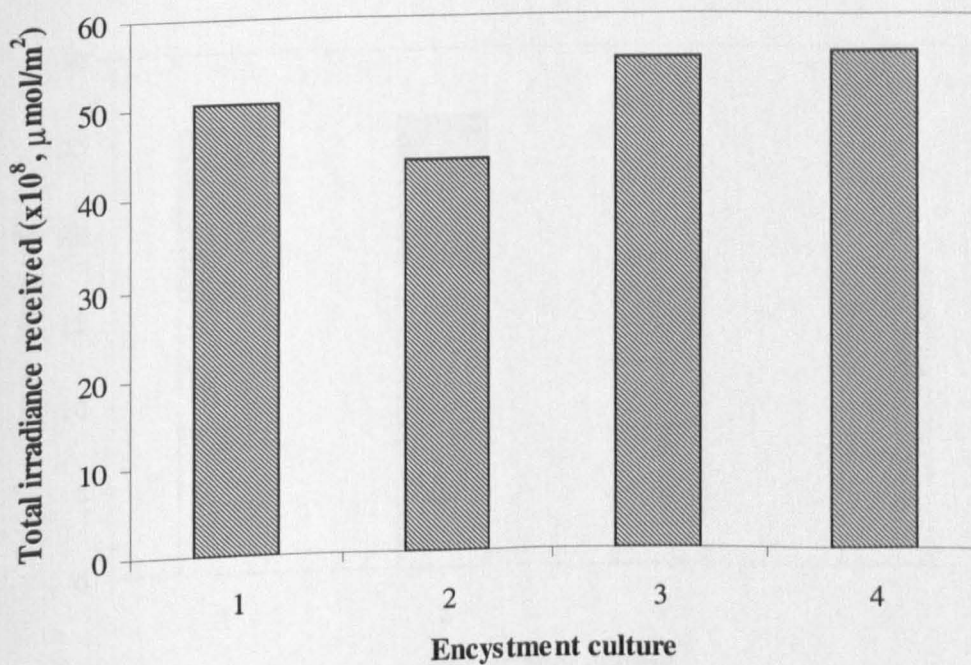


Figure 5.11. Total irradiance ($\mu\text{mol}/\text{m}^2$) measured adjacent to the AAPSTM2 during the four encystment cultures (see text for details).

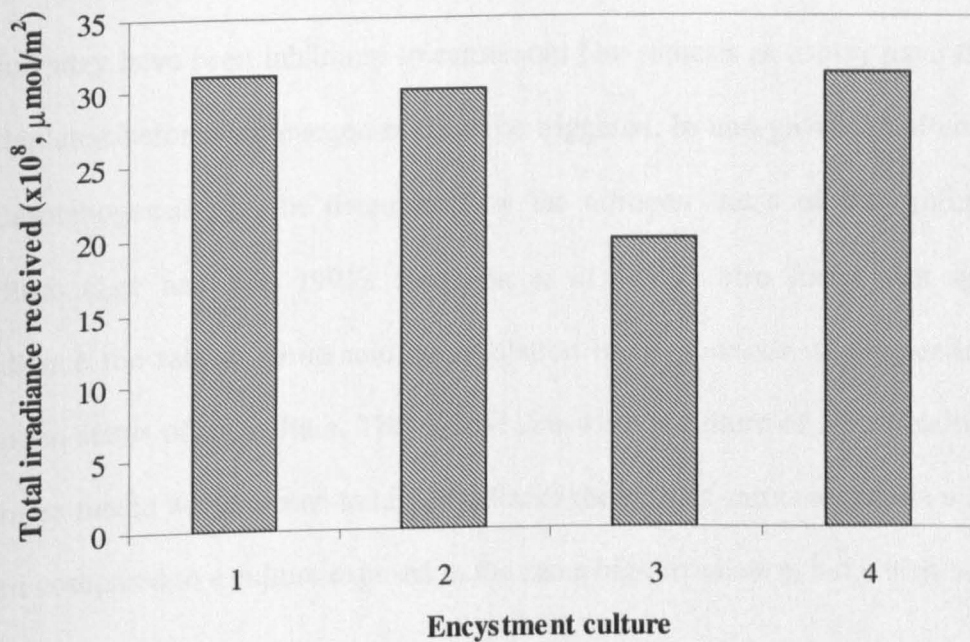


Figure 5.12. Total irradiance ($\mu\text{mol}/\text{m}^2$) measured adjacent to the AAPSTM2 during the first three days of each encystment culture (see text for details).

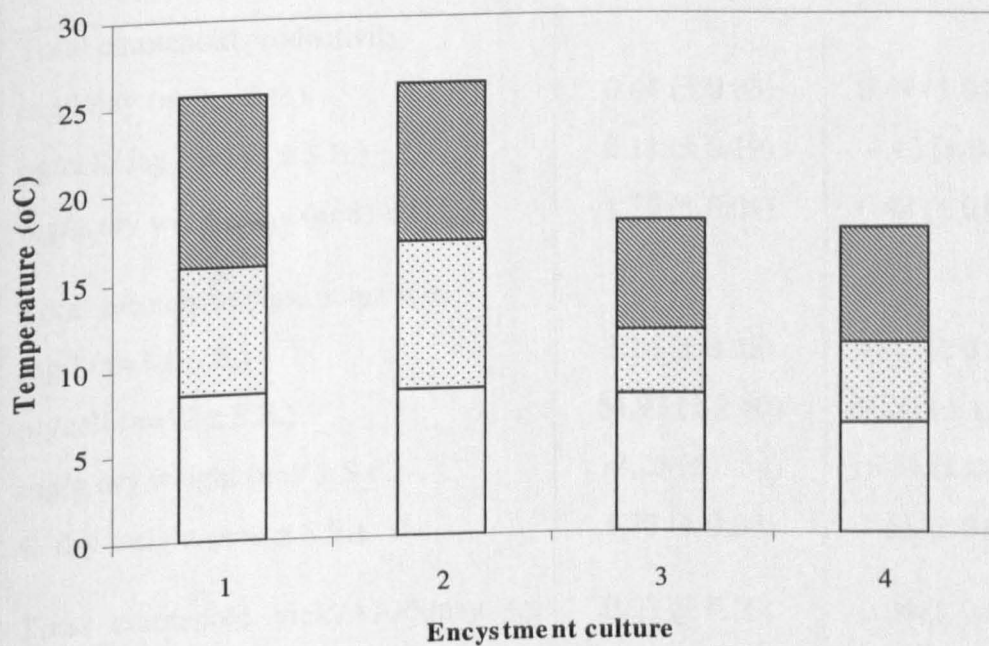


Figure 5.13. Mean (▣), maximum (▨) and minimum (□) air temperature adjacent to AAPSTM2 during the four encystment cultures.

The higher total nitrogen content of EC2 (Table 5.4) at the start of the culture period may have been inhibitory to carotenoid biosynthesis as it may have first had to be depleted before carotenogenesis could be triggered. In non-growing cultures the rate of carotenogenesis may be determined by the nitrogen status of the original culture medium (Lee and Soh 1991). Boussiba *et al* (1992) also found that under high irradiance the rate of carotenoid accumulation in *H. pluvialis* was dependent on the nitrogen status of the culture. They found that when a culture of *H. pluvialis* in a high nitrogen media was exposed to high irradiance the start of carotenogenesis was delayed when compared to a culture exposed to the same high irradiance, but which had a lower nitrogen content.

Table 5.3. Total carotenoid productivity of encystment cultures one (EC1) and two (EC2).

	EC1	EC2
Total carotenoid productivity mg/l/day ($n=9 \pm$ S.E.)	0.44 (\pm 0.03)	0.49 (\pm 0.03)
pg/cell/day ($n=144 \pm$ S.E.)	8.11 (\pm 0.19)	4.43 (\pm 0.1)
mg/g dry weight/day ($n=81 \pm$ S.E.)	1.20 (\pm 0.04)	0.43 (\pm 0.05)
Total carotenoid yield at harvest mg/l ($n=3 \pm$ S.E.)	3.14 (\pm 0.08)	4.00 (\pm 0.11)
pg/cell ($n=12 \pm$ S.E.)	54.93 (\pm 2.40)	36.53 (\pm 1.29)
mg/g dry weight ($n=9 \pm$ S.E.)	17.25 (\pm 0.54)	16.31 (\pm 0.30)
% dry weight ($n=9 \pm$ S.E.)	1.73 (\pm 0.05)	1.63 (\pm 0.08)
Total carotenoid yield/AAPS/day (g, $n=3 \pm$ S.E.)	0.03 (\pm 0.00)	0.04 (\pm 0.00)
Total carotenoid yield/AAPS (g, $n=3 \pm$ S.E.)	0.17 (\pm 0.01)	0.22 (\pm 0.01)

Table 5.4. Nitrogen status of encystment cultures one (EC1) and two (EC2).

	Nitrogen content (mg/l, $n=3$, \pm S.E.)	
	Initial	Final
EC1	3.00 (\pm 0.11)	0.30 (\pm 0.00)
EC2	9.00 (\pm 0.03)	0.90 (\pm 0.01)

Assuming a culture volume of 55l EC1 produced 0.17g of total carotenoid and EC2 produced 0.22g. Although the cells in EC1 had a higher carotenoid content, EC2 yielded a higher level of carotenoids on a per volume basis because the cell density of the culture was greater. This had been previously noted in batch cultures grown in low and high [N] media (Chapter 4, section 4.3). Cultures grown in a high [N] media produced more cells per unit volume but those cells had a reduced carotenoid content when compared to a culture grown in low [N] media under the same climatic conditions. The nitrogen content of the media is very important in determining the carotenoid content of the culture (Lee and Soh 1991, Boussiba *et al* 1992). Irradiance is also of key importance (Fábregas *et al* 1998), however the irradiance received per cell would be directly affected by the increased cell density in cultures grown in high [N] media (Eriksen *et al* 1996).

The carotenoid content in terms of mg/g dry weight was the same in both cultures as the cells produced from EC2 were not as heavy as those produced from EC1, indicating a reduced level of encystment (Table 5.3).

5.2.2.2. Encystment of *H. pluvialis* using sodium chloride

NaCl had been shown to accelerate the accumulation of secondary carotenoids in *H. pluvialis* (Droop 1955, Harker *et al* 1996b). Therefore the effect of NaCl on the second phase of continuous production was investigated. Two encystment cultures were prepared and inoculated into the roof AAPS™2 as detailed in the section above. In the first culture (encystment culture three (EC3), 16th -24th May 2000) 40mM NaCl (defined as optimal, Harker *et al* 1996b) was added to the AAPS™2 three days after inoculation of the system with EC3. For the first three days the culture had been exposed to a reduced level of irradiance (Figure 5.12) and temperature (Figure 5.13) when compared to EC1 and EC2, and still contained green zooid cells in majority (81.00%). Secondary carotenoid accumulation was visible (as droplets) within these cells (as demonstrated in Plate 4.1c.). EC3 had a higher cell density than previous cultures (Table 5.5), which would have resulted in a reduction in the irradiance received per cell (Eriksen *et al* 1996). The total nitrogen content of the culture at the point of inoculation into the outdoor AAPS™ (10mg/l) was also greater than either EC1 or EC2 (Table 5.7). The culture was harvested five days after the addition of the NaCl and contained carotenoid-rich aplanospores. There had been no increase in the received irradiance over this period when compared to the first three days. The addition of NaCl on day three appeared to have little affect on reducing the survivability of the algal cells (Table 5.5).

Upon harvest, after a total of nine days, the culture yielded 0.30g of total carotenoids (Table 5.6). This was an improvement on the total yield on a per volume basis of the previous cultures however EC3 was not as productive on a per cell basis. EC1 produced 8.11pg carotenoid/cell/day, EC2 produced 4.43pg carotenoid/cell/day (Table 5.3) whilst EC3 only produced 1.57pg carotenoid/cell/day (Table 5.6). The

productivity per cell dropped as the cell density of the culture increased, suggesting that for two-phase production a careful balance must be reached in the carotenoid production phase between using maximal biomass while permitting maximal irradiance per cell.

Table 5.5. Productivity of encystment cultures three (EC3) and four (EC4).

	EC3	EC4
Length of culture period (days)	9	7
Cell density on the first day of culture ($\times 10^4$ cells per ml, $n=4 \pm$ S.E.)	14.50 (± 0.65)	27.25 (± 1.25)
Cell density at the end of the culture period ($\times 10^4$ cells per ml, $n=4 \pm$ S.E.)	18.00 (± 0.16)	6.3 (± 0.06)
Dry weight on the first day of culture (g/l, $n=3 \pm$ S.E.)	0.25 (± 0.00)	0.317 (± 0.00)
Dry weight at the end of the culture period (g/l, $n=3 \pm$ S.E.)	0.37 (± 0.01)	0.286 (± 0.01)
Dry weight per cell on day on the first day of culture (ng, $n=12 \pm$ S.E.)	1.76 (± 0.04)	1.17 (± 0.03)
Dry weight per cell at the end of the culture period (ng, $n=12 \pm$ S.E.)	2.16 (± 0.04)	4.59 (± 0.16)

Table 5.6. Total carotenoid productivity of encystment cultures three (EC3) and four (EC4).

	EC3	EC4
Total carotenoid productivity		
mg/l/day ($n=9 \pm$ S.E.)	0.31 (± 0.05)	0.23 (± 0.05)
pg/cell/day ($n=144 \pm$ S.E.)	1.57 (± 0.07)	8.97 (± 0.16)
mg/g dry weight/day ($n=81 \pm$ S.E.)	0.49 (± 0.02)	1.41 (± 0.08)
Total carotenoid yield at harvest		
mg/l ($n=3 \pm$ S.E.)	5.17 (± 0.27)	4.62 (± 0.24)
pg/cell ($n=12 \pm$ S.E.)	29.83 (± 1.55)	73.74 (± 2.87)
mg/g dry weight ($n=9 \pm$ S.E.)	13.85 (± 0.40)	16.24 (± 0.49)
% dry weight ($n=9 \pm$ S.E.)	1.40 (± 0.04)	1.62 (± 0.05)
Total carotenoid yield/AAPS/day (g, $n=3 \pm$ S.E.)	0.03 (± 0.01)	0.04 (± 0.00)
Total carotenoid yield/AAPS (g, $n=3 \pm$ S.E.)	0.30 (± 0.01)	0.25 (± 0.01)

Table 5.7. Nitrogen status of encystment cultures three (EC3) and four (EC4).

	Nitrogen content (mg/l, $n=3 \pm$ S.E.)	
	Initial	Final
EC3	9.30 (± 0.15)	4.10 (± 0.16)
EC4	11.20 (± 0.20)	3.20 (± 0.05)

Encystment culture four (EC4, 24th -30th May 2000) was treated with NaCl at the point of inoculation into the outdoor AAPS™. This resulted in the loss of 75.00% of the culture by the seventh day (Table 5.5). The addition of NaCl on day three of EC3 did not result in a noticeable level of cell death. At the point of NaCl addition both EC3

and EC4 contained zooid cells in the majority. Although NaCl has been shown to increase the rate of carotenogenesis in the culture it also results in cell death of this freshwater algae. Droop (1955) found that the degree of NaCl-induced cell death was concentration dependent, the addition of greater than 0.6% (w/v) NaCl to the culture resulted in a high cell mortality. From the results of EC3 and EC4 it would appear that the additional stress that the NaCl inflicts on the culture is too great when the culture is also exposed to high irradiance for the first time, as in EC4. A two-stage approach, with the exposure of the cells to high irradiance to trigger carotenoid synthesis followed by the addition of NaCl, reduced the cell mortality (EC3).

The total nitrogen concentration at the start of EC4 (Table 5.7) was greater than any of the previous cultures. Again, as with the previous cultures, a 3.5 fold drop was measured at the end of the culture period although there was no evidence of growth.

Despite the loss of most of the cells from the culture, the productivity and yield of total carotenoid per cell from EC4 was greater than was demonstrated by the previous three cultures, at 73.74pg/cell (Table 5.6). EC1 accumulated the next highest carotenoid content, with 54.93pg/cell. As EC4 and EC1 received a very similar total irradiance (Figure 5.11) and the final cell density of EC4 was similar to EC1, the irradiance received per cell would be expected to be similar for these two cultures. As EC4 had a higher carotenoid content per cell this indicates that the addition of NaCl improved cellular carotenogenesis as previously shown (Droop 1955, Harker *et al* 1996a). However the culture period of EC4 was two days longer than that of EC1. EC4 yielded 0.25grams of total carotenoids which was not as great as the yield of EC3, but higher than the yield of either EC1 or EC2.

Each of the four encystment cultures contained cells from the same continuous culture, were concentrated using the same method and resuspended in the same

AAPS™2 in tap water (total nitrogen content 1.00 (\pm 0.27) mg/l, $n=4 \pm$ S.E.). Despite this each had a different starting total nitrogen content (Tables 5.4 and 5.7) and cell density (Tables 5.2 and 5.5), and each was also exposed to varying climatic conditions (Figures 5.10, 5.11 and 5.13). Each culture had a different cellular productivity of secondary carotenoids (Tables 5.3 and 5.6) but produced a very similar carotenoid yield per day, EC1 produced 0.03g, EC2 0.04g, EC3 0.03g and EC4 0.04g. Carotenoid productivity in these cultures was affected by a number of factors. The nitrogen status of the culture (Zlotnik *et al* 1993, Lee and Soh 1991, Harker *et al* 1996a), the cell density, and the irradiance received per cell (Kobayashi *et al* 1992, Boussiba *et al* 1992). The irradiance received per cell is a function of the irradiance incident on the photobioreactor surface, the cell density of the culture and the efficiency of mixing within the system (Terry 1986, Eriksen *et al* 1996). Mixing efficiency was assumed to be constant for the four encystment cultures as each was cultured in the same AAPS™2 under the same operating conditions. The temperature received by the cultures was also of importance in determining the rate of carotenogenesis (Tjahjono *et al* 1994), as was the addition of NaCl to EC3 and EC4 (Droop 1955, Harker *et al* 1995).

The above factors were all important in producing the carotenoid-rich encystment cultures. However, the initially slow productivity (in terms of carotenoid accumulation) of EC3 combined with the reduced irradiance it received per day (Figure 5.10) suggest that the irradiance received by the cultures was of key importance. EC3 was also exposed to a reduced air temperature which may also have affected carotenogenesis, especially as this was combined with the reduced irradiance. A temperature of 30°C has been shown to increase carotenogenesis in *H. pluvialis* (Tjahjono *et al* 1994). The addition of 40mM NaCl acted to increase the carotenoid

yield on a cellular basis however it also resulted in a high cell mortality thereby reducing the carotenoid yield per volume.

5.2.3. Summary of two-phase production

The productivity of the continuous culture of *Haematococcus*, in terms of cell density, in the AAPS™2 operated indoors far exceeded the productivity of any of the batch cultures. The system operated as a continuous culture with a 10% renewal rate for 21 days with a mean productivity of 1.72×10^9 cells per day (± 0.08 , $n=4 \pm \text{S.E.}$), batch production had a maximal productivity of 1.40×10^4 cells/ml/day (Chapter 4, Table 4.14). However the culture never reached a true steady state and was shut down after 44 days. Problems were experienced with the media feed method, which resulted in the contamination of the culture with unidentified small, green, unicellular alga (similar contamination has been seen in previous large-scale cultures of *Haematococcus*: A. Ince pers. comm.). The number of complete media replacements that have occurred in the system defines the steady state of a continuous culture. Lee and Ding (1995) considered that steady state had been reached in a continuous culture of *Haematococcus* when the cell density and dry weight had remained constant for four volume changes. However Molina Grima *et al* (1997) considered that a steady stage was reached in continuous culture when the cell count of the culture had remained stable for four days.

The media feed was a two-part operation, the nutrients were fed in as a concentrated stock via a nutrient filter, and distilled water was fed in separately via a three-stage filtering system (Figure 5.2). Problems were encountered as the nutrient filter easily became fouled with bacteria, causing it to block. This resulted in the frequent replacement of the filter, which may, in itself, have resulted in the

contamination of the culture. A further problem was noted when the system was shutdown for cleaning. The header tank had developed two cracks in the welding between the back wall and the roof of the tank. This may also have acted to introduce contamination into the culture.

Biomass produced from the continuous culture was successfully concentrated using the tangential flow filtration system. A three-fold concentration caused no significant damage to the cells (Table 5.1). The algal biomass was then resuspended in tap water in a second, outdoor, AAPS™2. High irradiance was used to hasten the encystment process. NaCl (40mM) was found to further increase the rate of secondary carotenoid accumulation although it could result in high cell mortality (Table 5.5 and 5.6).

Despite the difference in the cell densities and nitrogen concentrations of the four encystment cultures each produced a very similar yield on a daily basis (Table 5.3 and 5.6). The encystment culture took between five and nine days to produce cultures containing ~ 1.6% (w/v) carotenoids as dry weight. Aquasearch Inc. and Cyanotech Corp. both based in Hawaii USA, use a two stage production process for commercial production of astaxanthin from *Haematococcus*. In the induction stage they both routinely achieve >2.5% (w/v) carotenoids as dry weight (Olaizola 2000, Lorenz and Cysewski 2000).

5.3. Single-phase production

The previous section described the use of a continuous culture system to produce green biomass for use in a second carotenoid-induction phase. However this approach is man-hour and equipment intensive. A second strategy using a single production stage may be more efficient (Figure 5.1). This would involve the operation

of a continuous AAPS™2 outdoors to produce biomass that was rich in secondary carotenoids, namely astaxanthin, and did not require a second carotenoid-induction stage. In this approach the exposure to 'high' (outdoor) irradiances may counteract the relatively high residual [N] levels in the media (as previously seen in batch cultures, i.e. Chapter 4, section 4.2.1). Bubrick (1991), Lee and Ding (1994) and Chaumont and Thepénier (1995) also indicates that carotenogenesis can occur in a growing culture of *H. pluvialis*.

The AAPS™2 was set up and sterilised as detailed in Chapter 2 on the roof of the Byrom Street campus of Liverpool John Moores University. The feed bottle, pumps and water filtration system were all housed in the control room adjacent to the system (Figure 5.2). The culture was grown in media with a urea content of 3.0mM (high [N] media, Appendix 1) and pH control was achieved with CO₂ at a set-point of 7.5. After commencement of the continuous feed, samples were taken daily (0900 hrs) to assess the cell count, dry weight and pigment content of the cells (see Chapter 2).

5.3.1. Batch phase

The starting cell density of the culture was 2.63×10^4 cell/ml (Figure 5.14), this was provided from a 5l aspirator as detailed in Chapter 2. As before the culture did not exhibit a lag phase and grew exponentially until day 14 (to a cell density of 63.25×10^4 cells/ml) when the continuous feed was started. The dry weight of the culture (Figure 5.15) and the dry weight per cell (Figure 5.16) also increased exponentially.

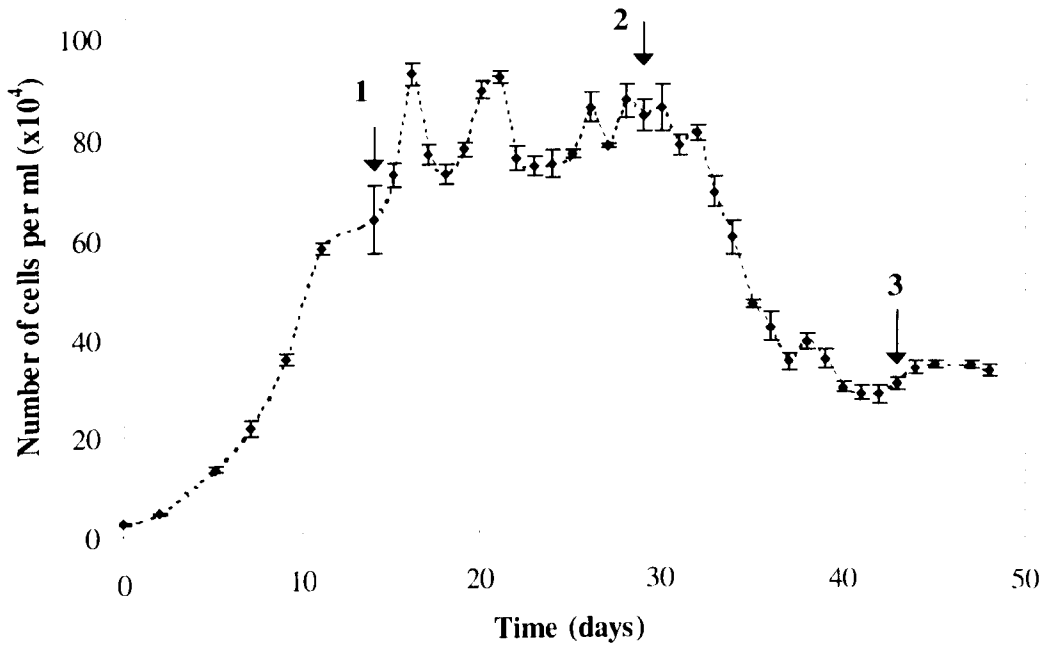


Figure 5.14. Cell density ($n=4 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

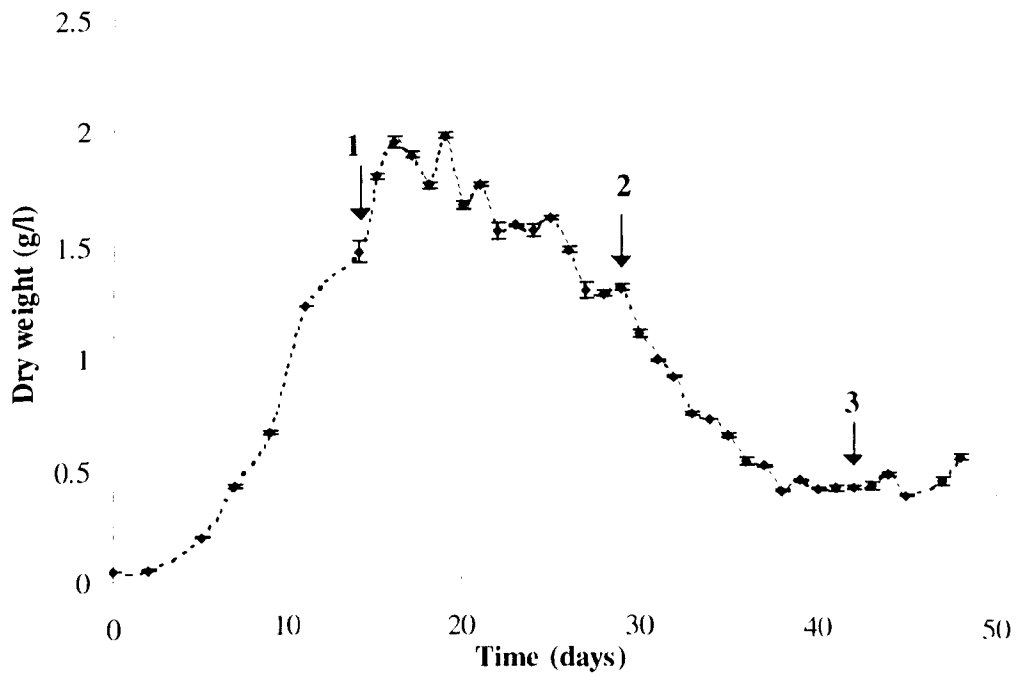


Figure 5.15. Dry weight ($n=3 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

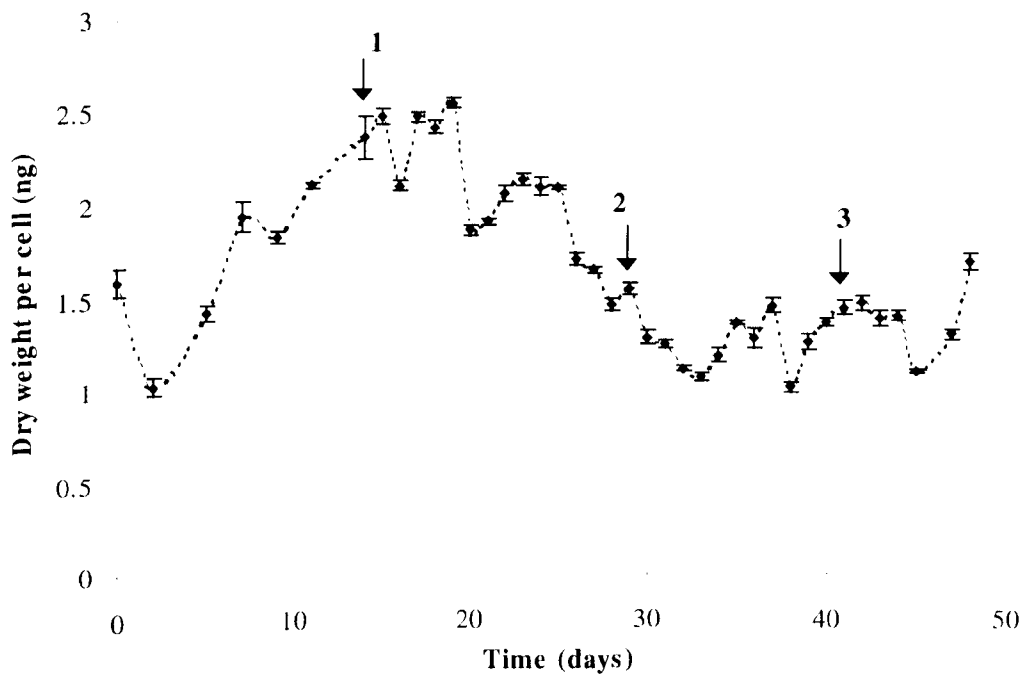


Figure 5.16. Dry weight per cell ($n=12 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

Over the course of the first 15 days the culture had a doubling time of 2.40 days, nearly three times faster than the indoor-grown continuous culture (section 5.2.1), and a productivity of 4.60×10^4 cells/ml/day (± 0.15 , $n=16$, \pm S.E.). From day five there was also an exponential increase in the carotenoid content per cell (Figure 5.17) which corresponded with a period of high irradiance when compared to the irradiance over the course of the entire run (Figure 5.18). The chlorophyll *a* and *b* concentration per cell remained constant (Figure 5.19).

On day 14, before the continuous feed was started, the culture consisted of a mixture of aplanospores (47.33%) and zooids (52.67%) (Figure 5.20), and had a total carotenoid content of 32.13pg/cell (Figure 5.17). This is a mean value for the algal population and microscopic examination revealed that the population was heterogeneous with a range of different life cycle stages represented.

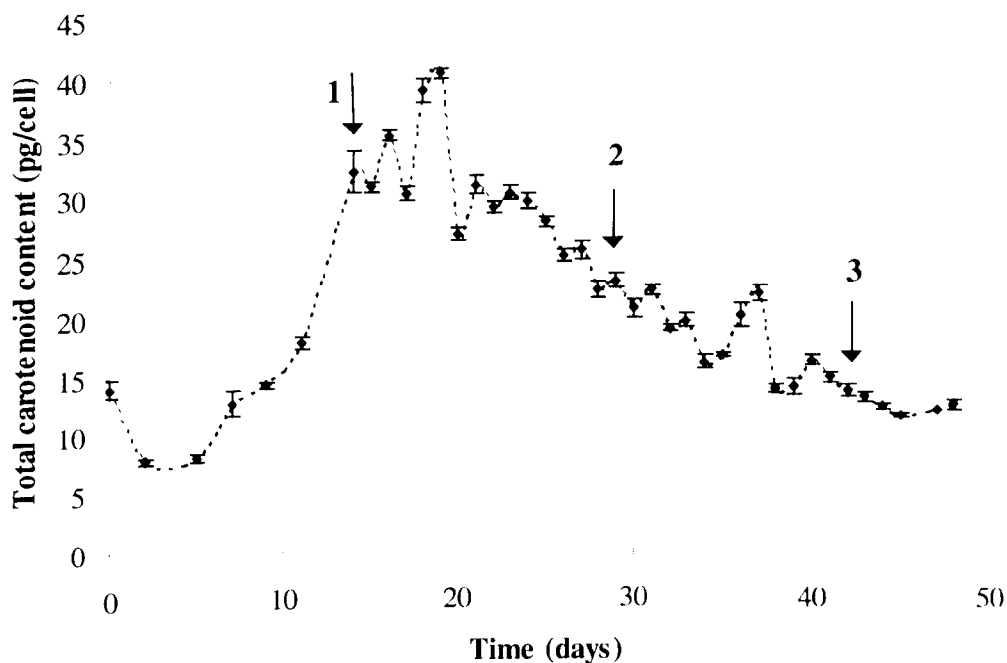


Figure 5.17. Total carotenoid content (calculated as pg/cell, $n=12 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

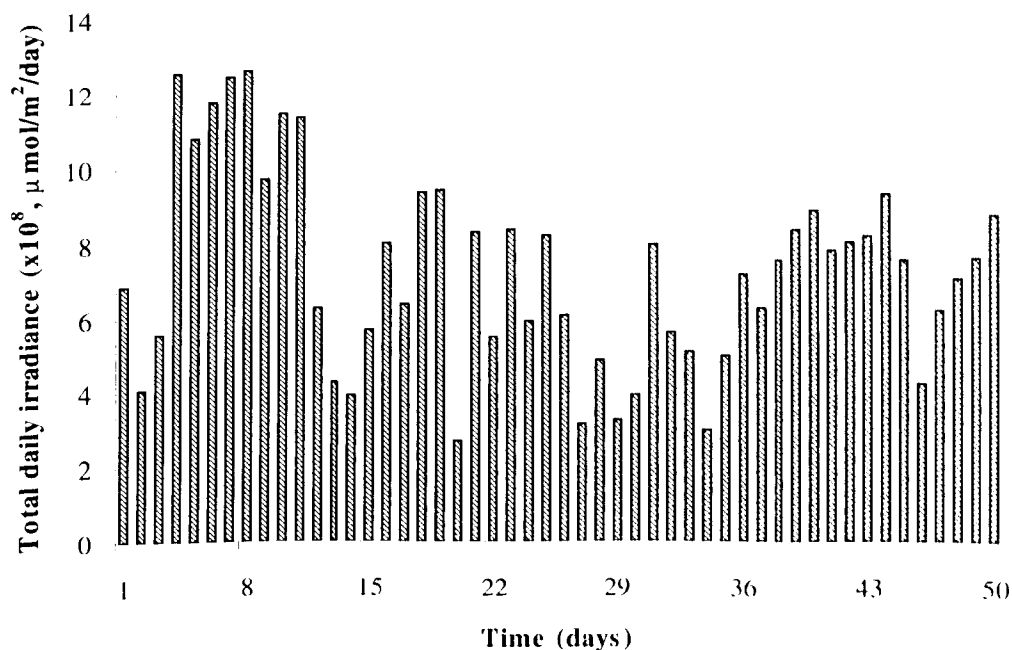


Figure 5.18. Total daily irradiance adjacent to the AAPS™2 during August and September 2000 (continuous outdoor culture).

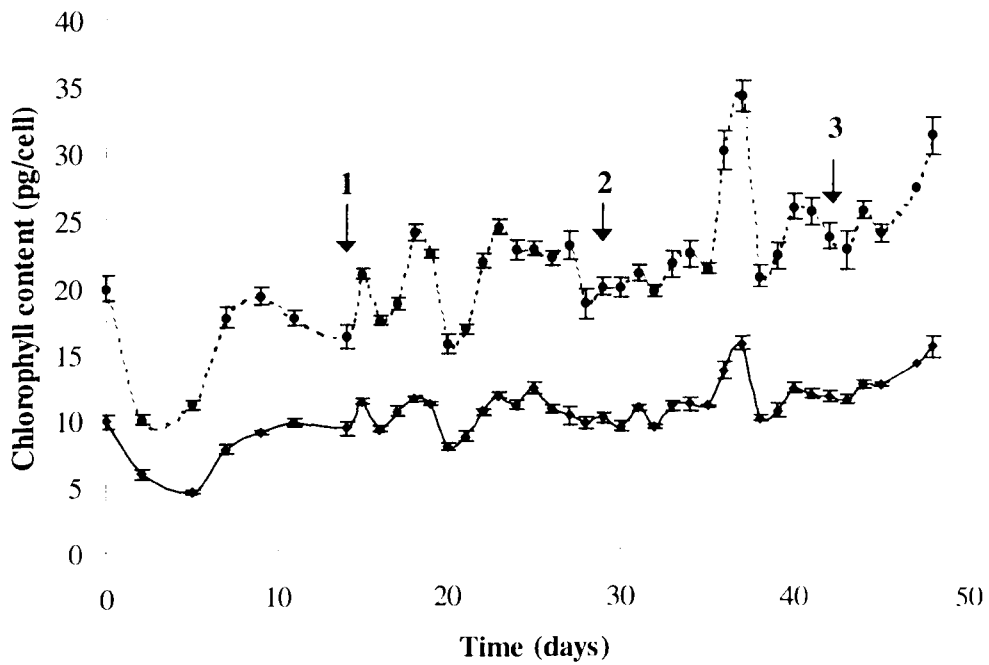


Figure 5.19. Chlorophyll content (calculated as pg/cell, $n=12 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed. Chl *a* ---, Chl *b* —.

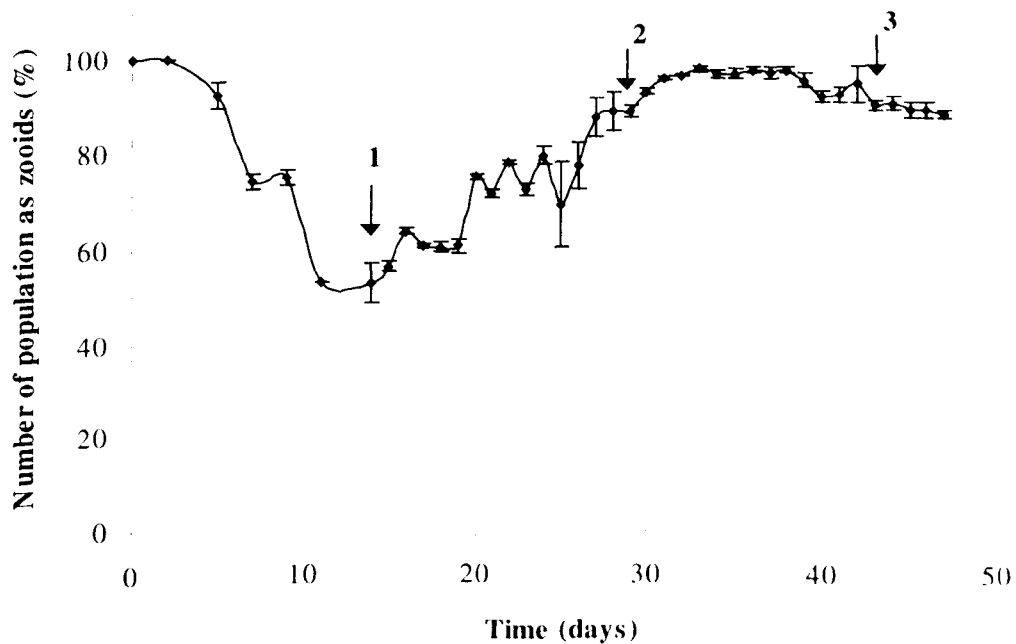


Figure 5.20. Developmental stage ($n=4 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (number of zooids shown, remainder are aplanospores, see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

The total carotenoid content per unit volume (Figure 5.21) and per gram of dry material (Figure 5.22) also increased exponentially from day five until the start of the continuous media feed on day 14. Correspondingly the chlorophyll *a* and *b* concentration per unit volume (Figure 5.23) increased exponentially until day 14. However the concentration per gram dry weight was relatively constant from inoculation until day 14 (Figure 5.24).

5.3.1.1. Carotenoid composition of culture upon commencement of continuous feed.

Analyses of the carotenoid composition of the culture on day 15 revealed that astaxanthin made up 81% of the total carotenoids present (Table 5.8).

Table 5.8. Carotenoid composition of the *H. pluvialis* produced at the start of the continuous culture (Day 15, 10% (v/v) renewal) in the AAPS™2.

Carotenoid	Carotenoid composition (%)
Lutein	7.95
Free Astaxanthin	5.55
Astaxanthin Monoesters	53.27
Adonirubin Esters	7.81
Astaxanthin Diesters	22.01
β-carotene	3.41

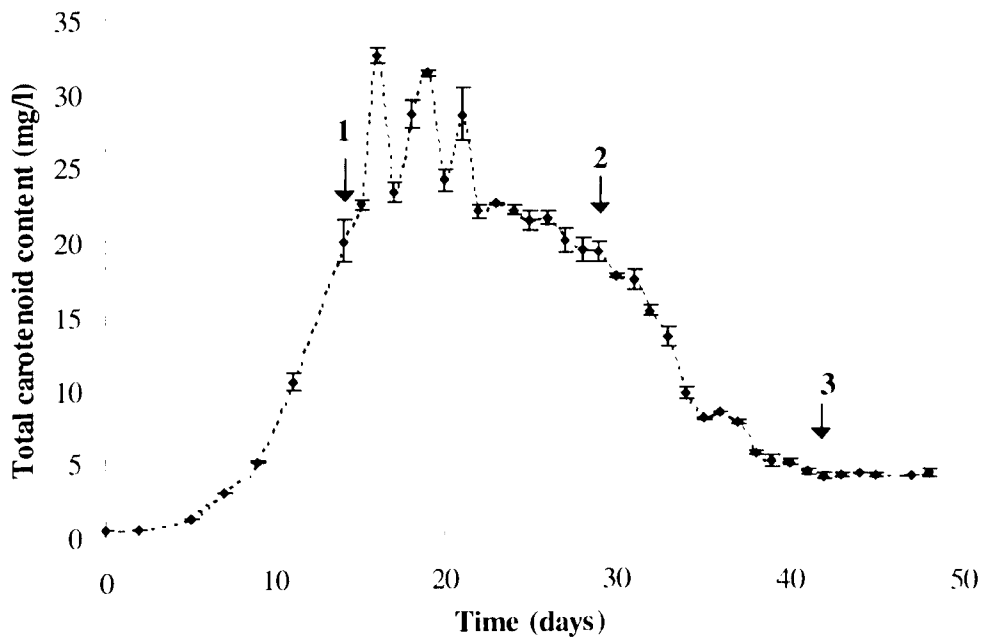


Figure 5.21. Total carotenoid content (calculated as mg/l, $n=3 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

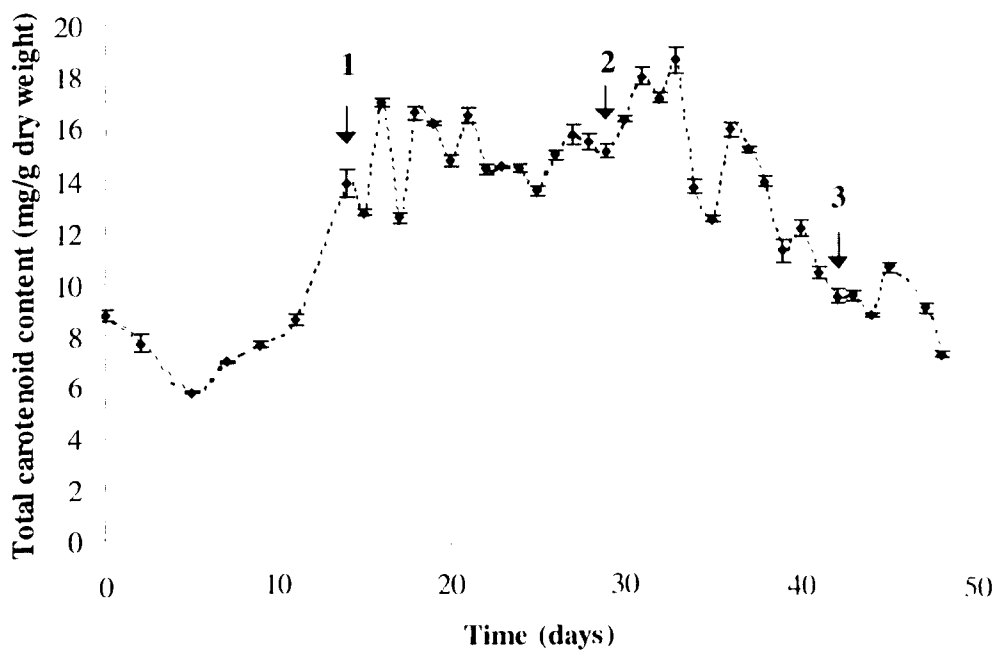


Figure 5.22. Total carotenoid content (calculated as mg/g dry weight, $n=3 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

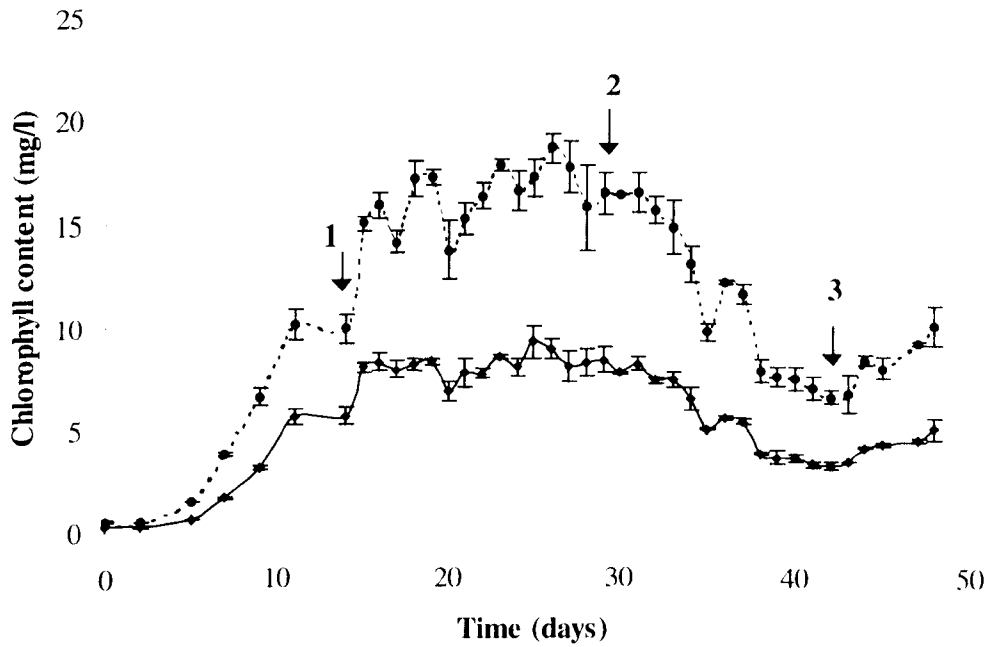


Figure 5.23. Chlorophyll content (calculated as mg/l, $n=12 \pm$ S.E.) of *H. pluvialis* cultured continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed. Chl *a* ---, Chl *b* —.

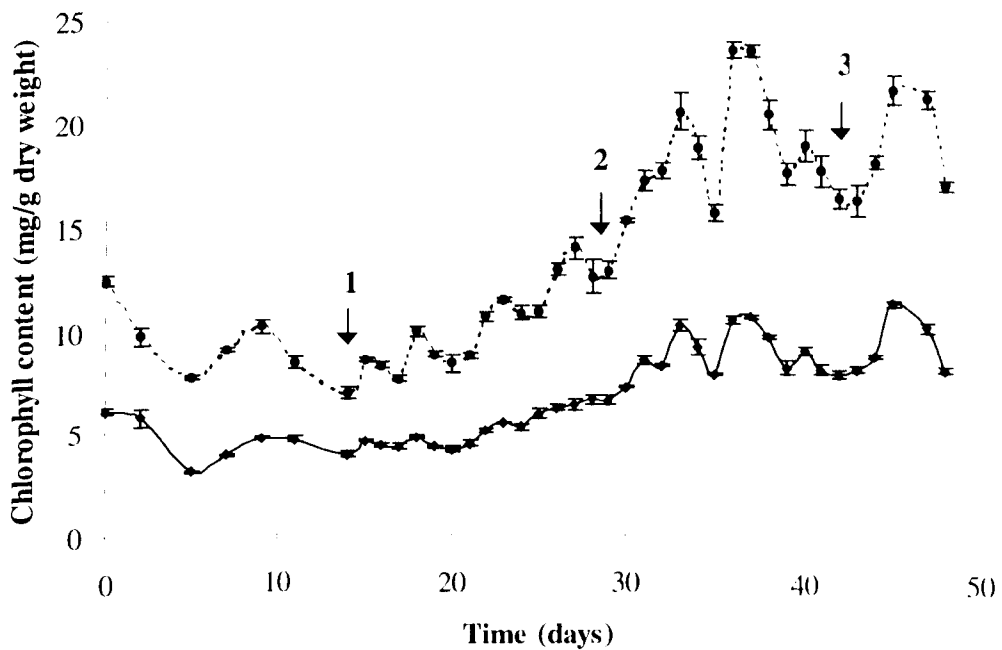


Figure 5.24. Chlorophyll content (calculated as mg/g dry weight, $n=12 \pm$ S.E.) of cultured continuously outdoors *H. pluvialis* in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed. Chl *a* ---, Chl *b* —.

The fast growth rate of the culture resulted in the reduction of the nitrogen content to only 20% of the starting concentration after 14 days (Figure 5.25) which combined with the high irradiance over that period may have resulted in the carotenoid accumulation within the culture (Figure 5.17). Figure 5.18 shows the total irradiance received per day over the course of the culture. The mean daily irradiance that the culture received over the course of the first 14 days was $8.82 \times 10^8 \mu\text{mol}/\text{m}^2/\text{day}$, whereas over the entire course of the culture the mean total daily irradiance was $6.59 \times 10^8 \mu\text{mol}/\text{m}^2/\text{day}$. The minimum and maximum air and culture temperature remained relatively constant throughout the culture period (Figure 5.26). Culture and air temperature were equal at night, however during daylight hours culture temperature increased above air temperature due to the effect of irradiance. The temperature of the culture did not drop below 11.59°C (night measurement). During daylight hours the culture temperature remained above air temperature and reached a maximum on day nine of 39.25°C . The maximum culture temperature typically fluctuated between 25 and 35°C . There is some debate in the literature as to the optimal temperature for growth of *Haematococcus* (which may be due to strain differences). Fan *et al* (1994) determined that the fastest growth rate was achieved at a temperature of $25\text{-}28^\circ\text{C}$, whereas the work of Tjahjono *et al* (1994) found that growth was much reduced at 30°C when compared to 20°C . Borowitzka *et al* (1991) also reported that *Haematococcus* could not grow above 28°C and that above this temperature the cells formed aplanospores. They determined the optimal temperature for growth to be 15°C . This is in agreement with the work of Harker *et al* (1995) who determined the optimum temperature for growth to be $14\text{-}15^\circ\text{C}$. The work of Borowitzka *et al* (1991) and Tjahjono *et al* (1994) suggests that temperature could affect carotenogenesis and aplanospore formation in this culture.

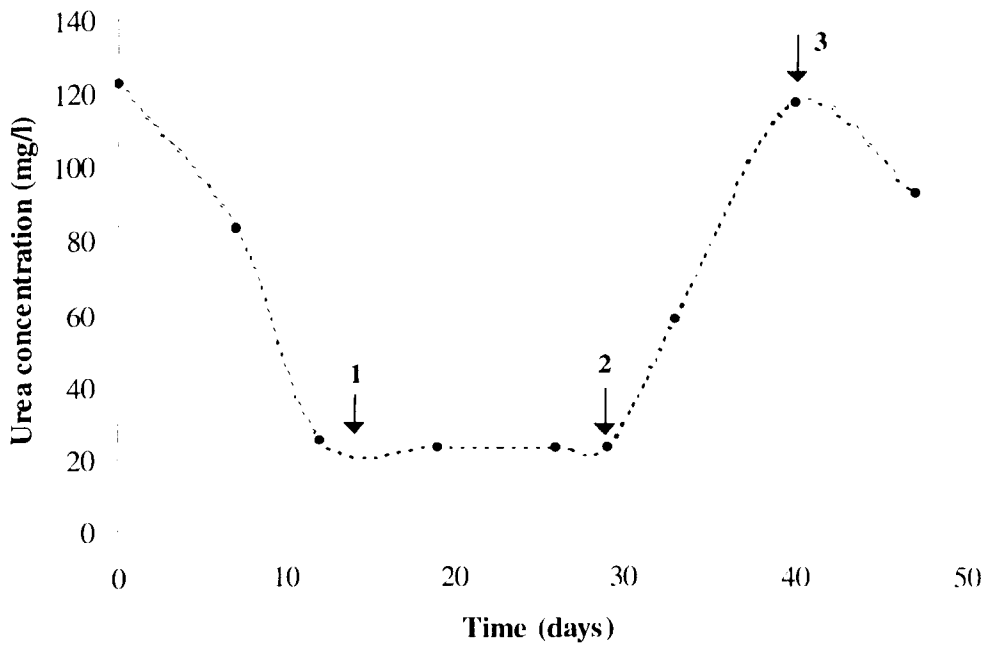


Figure 5.25. Urea concentration (mg/l, $n=1$) of media used to culture *H. pluvialis* continuously outdoors in the AAPS™2 (see text for details). **1** start of 10% (v/v) media feed, **2** increased to 20% (v/v) media feed, **3** reduced to 10% (v/v) media feed.

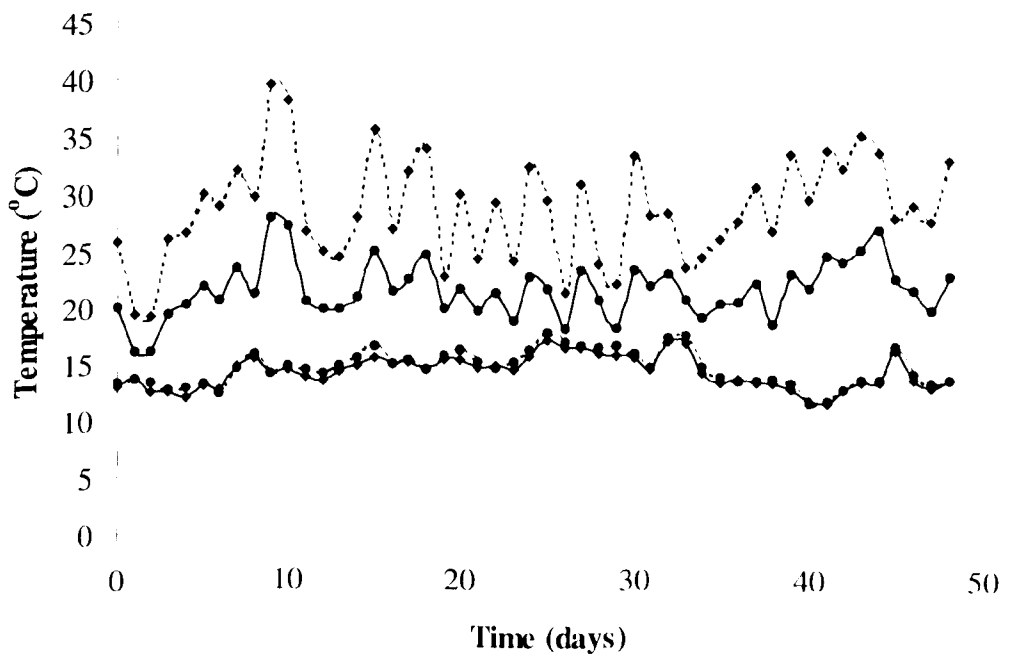


Figure 5.26. Minimum and maximum air and culture temperature adjacent to the AAPS™2 during August and September 2000 (continuous outdoor culture), culture temperature ---, air temperature —.

5.3.2. Continuous culture

On the 15th day of culture the continuous media feed was started at a renewal rate of 10% (v/v) per day. This value was selected as it was determined as most productive for the continuous culture of *H. pluvialis* (Clarkson *et al* 2001). Assuming a working volume of 55l, 5.5l of fresh media was fed into the system per day during daylight hours (0600hrs - 2100hrs). No additions were made to the culture at night. Plate 5.2. shows the system shortly after the commencement of the continuous feed.

5.3.2.1. Continuous operation with a renewal rate of 10% (v/v)

Continuous operation at a renewal rate of 10% (v/v) occurred from day 14-29 (26th July-10th August 2000). Daily sampling of the culture revealed fluctuations in both the algal cell density and the pigment content (per cell) of the culture (Figure 5.14 and 5.17 respectively). The culture reached a maximal cell density of 91.50×10^4 cells/ml. The mean productivity over the course of the 10% (v/v) media feed was 8.40×10^5 cells/ml/day (± 1.20 , $n=61$, \pm S.E.), which equated to a total production of 4.60×10^9 cells/day (± 0.07 , $n=61$, \pm S.E.). The culture reached a maximal carotenoid content on day 19 of 40.45 pg/cell (Figure 5.17). The mean carotenoid content per cell from day 14 - 29 was 27.32 pg/cell (± 1.28 , $n=192$, \pm S.E.). At a productivity of 4.60×10^9 cells/day and a total carotenoid content of 27.32 pg/cell the AAPSTM produced 0.13g/day (0.02 g/l/day) of total carotenoid, which assuming an astaxanthin content of 81% translates to an astaxanthin yield of 0.11g/day (0.02g/l/day). As the 10% (v/v) media feed operated for 15 days, the total astaxanthin production over the course of the 10% (v/v) media feed was 1.65g, mainly as a mixture of mono and diesters (Table 5.8).



Plate 5.2. Single-phase continuous production of *H. pluvialis* in the AAPS™2.

However Figure 5.17 shows that after the first five days of continuous production the carotenoid content per cell began to drop. Also at this time the proportion of the cells that were zooid increased from 52.67% (on day 14, at the start of the feed) to 88.25% on day 29 (Figure 5.20). The reason for this change in the developmental stage of the cells and the drop in the carotenoid content may be a function of climatic conditions. It was not a function of the nitrogen status of the culture, as the urea content of the media remained constant throughout the feed period (Figure 5.25). Also it is not a phenomena that has been seen indoors. The total daily irradiance received dropped to $6.17 \times 10^8 \mu\text{mol}/\text{m}^2/\text{day}$, two thirds of the level of the total irradiance measured during the first 14 days of culture before the continuous feed began (see above). The photosynthetic rate of the culture and the mass transfer efficiency of the system could also have affected the developmental stage of the culture. Lee and Ding (1995) cultured a mixed population of *Haematococcus* in continuous culture in which both aplanospores and zooids were noted to actively divide. The proportion of the culture that was zooid or aplanospore was found to be dependent on the partial pressure of DO_2 ($p\text{DO}_2$) of the culture. Above air saturation the culture contained a higher aplanospore content than when maintained with a $p\text{DO}_2$ below air saturation.

The continuous culture was noted to require a greater volume of CO_2 for pH control than previous cultures. During operation the CO_2 reservoir was emptied and there was no pH control, when this occurred the pH of the culture fluctuated during the daylight cycle. Early in the morning, at the point of sampling, the pH was close to 7 but by early afternoon had increased to pH10, the pH then started to decrease in late afternoon/evening (data not shown). This fluctuation in pH occurred due to the photosynthetic activity of the culture. As a result of photosynthetic generation the DO_2

concentration of the culture rises, whereas the DCO_2 concentration drops as it is consumed by photosynthesis. This causes the pH of the culture to rise (Camacho Rubio *et al* 1999). CO_2 is required to be injected into the system to prevent the pH of culture rising to a detrimental level. The higher the rate of photosynthesis the more frequently the CO_2 injections are required. Therefore as no problems were identified with the pH controller, the rapid use of CO_2 by this culture suggest that the culture had a high photosynthetic rate. This may explain the mixed population that was found in this culture as a high $p\text{DO}_2$ may result in a higher proportion of the cells as aplanospores within a continuous culture (see above, Lee and Ding 1995). The first culture to be grown with the high [N] media in the AAPS™2 (batch culture six, Chapter 4, section 4.3.2), had no pH control and despite the culture becoming rich in aplanospores, no germination was noted.

5.3.2.2. Continuous operation with a renewal rate of 20% (v/v)

On day 29 the media renewal rate was increased to 20% (v/v), which led to the production of 11l of culture per day. This renewal rate was operated until day 40. During this time the cell count of the culture steadily decreased and did not reach equilibrium (Figure 5.14), though it still remained more productive than the lower renewal rate of 10% (v/v) in terms of total cells produced per day. At the same time the urea content of the culture increased (Figure 5.25) and there was a corresponding reduction in the carotenoid content of the cells (Figure 5.17). There was also a gradual increase in the chlorophyll *a* and *b* content of the cells (Figure 5.19), between days 35 and 38 there was a sudden increase followed by a decrease. The chlorophyll *a* and *b* concentration on a per unit volume basis (Figure 5.23) decreased whereas the concentration calculated per g dry weight increased (Figure 5.24). Again a sudden

change was noted in both these figures between days 35 and 38. No change was noted in the ratio of chlorophyll *a/b*. The change in the chlorophyll *a* and *b* content of the cells over the culture period could indicate a response to the changing climatic conditions. Myers (1970), working with *Chlorella*, concluded that the chlorophyll content of the cells was an adaptive feature controlled by the irradiance received during growth so as to maximise the photosynthetic rate of the cells.

At the start of the 20% (v/v) media feed on day 29 the cell density was 83.40×10^4 cells per ml (Figure 5.14), with 88.25 % of the cells counted as zooid (Figure 5.20). The proportion of the culture that was zooid increased to 97.25 % by day 33 and then remained relatively constant. The cell density on day 33 had dropped to 68.25×10^4 cells/ml and continued to drop with time (Figure 5.14). Initially the cells were green with localised carotenoid accumulation (Plate 4.1c). As time progressed the cells lost the red segment and by day 33 appeared completely green (Plate 4.1a.). The system on day 33 is pictured in plate 5.3. This corresponded with a drop in the carotenoid content per cell to 19.55pg (Figure 5.17), approximately half of the content per cell on day 19. The change in the developmental stage and carotenoid content of the cells is a continuation of the behaviour of the culture towards the end of the 10% feed. The reduced carotenoid content of the cells is likely to be largely due to the reduced irradiance per day when compared to the irradiance received before feeding began (Figure 5.18). The increased nitrogen content of the culture, which had reached 117mg/l urea by day 40 would also have affected carotenogenesis (Boussiba *et al* 1992, Fábregas *et al* 1998).



Plate 5.3. Single-phase continuous production of *H. pluvialis* (20% feed (v/v)).

The urea concentration on day 40 had increased five-fold when compared to the urea concentration in the culture during the 10% (v/v) media feed (Figure 5.25). Reasons for the change in the developmental stage of the culture are unclear.

The gradual change from aplanospores to zooids (Figure 5.20) would suggest that conditions within the photobioreactor had become more favourable for growth as indicated by the increased urea content (Figure 5.25) and reduced irradiance (Figure 5.18). Lee and Ding (1994) demonstrated that when aplanospores were resuspended in fresh media with a urea content of 0.85 or 1.70 g/l they divided to produce a number of carotenoid-containing zooids. Germination from aplanospores was also seen to occur after the harvest of batch culture three from the AAPS™ (Chapter 4, section 4.2.3). Red zooids germinated from the aplanospores that had been left in the system after harvest, possibly due to the presence of nitrogen in the tap water.

By day 41 the cell density had dropped to 27.80×10^4 cells/ml and the culture was made up entirely of green zooid cells. This was largely due to a reduction in secondary carotenoids rather than from the increased synthesis of chlorophyll *a* and *b* (Figures 5.18 and 5.19). On day 41 at a production rate of 111 culture/day, the AAPS™ was producing 3.10×10^9 cells/day, in comparison to 4.60×10^9 cells/day at a renewal rate of 10% v/v.

The increased renewal rate resulted in an increase in the urea content of the culture, however, it did not act to increase the growth rate of the culture. Previously in batch culture in the AAPS™ (Chapter 4 section 4.3.2), the higher concentration of urea simply extended the growth phase.

5.3.3. Summary of single-phase production

The productivity of the culture before the start of continuous culture was far higher than any of the previous outdoor batch cultures. The culture produced 4.04×10^4 cells/ml/day. In comparison previous cell productivities had been in the order of 1.00×10^4 cells/ml/day irrespective of the urea content of the media. During this time there was also an exponential increase in the carotenoid content per cell (Figure 5.17). During the first 14 days secondary carotenoid production was 1.21pg/cell/day or 1.29mg/l/day, comparable with the productivities achieved in the batch cultures (Chapter 4, Table 4.14).

The reason for the improved growth of this culture when compared to previous cultures may have been due to the developmental stage of the cells. By day five the culture was a mixture of actively dividing zooids and germinating aplanospores. It was observed that the zooid cells divided into two or four cells, whereas the aplanospores divided into as many as 16 new cells, though more commonly eight. Although the majority of the population of the culture was zooid, the aplanospores would have had a significant impact on the growth rate of the culture because of the higher number of cells they produced from division. Mixed populations of zooids and aplanospores had been noted in previous cultures (e.g. Batch cultures one, six and eight; Chapter 4). This was, however, the first culture in which the aplanospores were present so early in the culture and were observed to be actively germinating. Germination of the aplanospores was noted to occur before the commencement of the 10% (v/v) feed, at a time when no addition of nitrogen was made to the culture but when pH control using CO_2 was intermittent which resulted in pH fluctuations through the daylight cycle. It is therefore possible that the photosynthetic rate of the culture and consequently the DO_2 content of the media affected the developmental stage of the culture (Lee and Ding 1995).

The nature of the climatic conditions that the culture was exposed to is an important factor in determining the developmental stage of the cells and the subsequent germination of the aplanospores. Both indirectly through photosynthesis (see above) and directly by affecting carotenogenesis. During the first 14 days the continuous culture received twice the daily irradiance of batch cultures five and six and 10 times the irradiance of batch cultures seven and eight (Table 5.9 and Chapter 4 section 4.3.3). The temperature that the culture was exposed to was similar to that received by batch culture five and six (Table 5.10 and Chapter 4 section 4.3.2) both of which produced carotenoid-rich cultures. Culture six was grown in the high [N] media and had a carotenoid productivity similar to the first 14 days of this continuous culture.

Table 5.9. Mean daily irradiance received by outdoor grown *H. pluvialis* cultures

Culture	Total daily irradiance ($\mu\text{mol}/\text{m}^2/\text{day}$)
Five/six	4.28×10^8
Seven/eight	1.03×10^8
Continuous	7.21×10^8

Table 5.10. Air temperature adjacent to outdoor-grown *H. pluvialis* cultures

Culture	Temperature ($^{\circ}\text{C}$)		
	Mean	Maximum	Minimum
Five/six	15	27	6
Seven/eight	9	16	0
Continuous	17	28	12

Batch cultures five and six did not exhibit aplanospore germination and were exposed to a very similar temperature range as the first 14 days of the continuous culture (Table 5.10). It is unlikely therefore that temperature had a major effect on the developmental stage of the continuous culture. The irradiance received by the continuous culture was in excess of that received by any of the batch cultures for which climatic conditions were measured (it should be noted that there is no climatic data available for culture grown in the AAPS™1). Irradiance has been shown to have a strong effect on carotenogenesis and encystment and it is therefore possible that the relatively high irradiance received by the culture during its first 14 days had a strong effect on its growth and life cycle. Such conditions are difficult to reproduce under controlled laboratory conditions.

The continuous feed commenced on day 14 providing a replacement of 10% (v/v) per day during daylight hours. This resulted in the production of 4.60×10^9 cells/AAPS™/day, mainly as zooids (with localised carotenoid accumulation, Figure 5.20). Up to 40% of the culture consisted of carotenoid-rich aplanospores. The culture yielded a total of 1.80g of astaxanthin between days 14-29, with a productivity of 0.15g carotenoid/AAPS™/day. This proved that the production of carotenoid-rich *H. pluviialis* is possible under continuous culture conditions outdoors. However production is apparently highly influenced by climatic conditions. After the first five days of continuous production the carotenoid content of the culture began to drop and the proportion of the cells present as zooids began to increase. As the urea content of the culture was constant from day 14 - 29 this was believed to be due to the climatic conditions that the culture was exposed to. Both irradiance (Boussiba and Vonshak 1991, Kobayashi *et al* 1992, Harker *et al* 1995) and temperature (Tjahjono *et al* 1994) have been shown to be of key importance in carotenogenesis. The total daily irradiance

was seen to drop after day 11 (Figure 5.18) which may have resulted in the reduction in carotenogenesis within the cells. However the carotenoid content of the cells did not begin to drop until day 19 (Figure 5.17).

To enable the production of a carotenoid-rich culture of *H. pluvialis* in a single phase the nitrogen content of the culture media must be maintained at as low a concentration as possible while remaining high enough to allow growth of the cells (Figure 5.25). Also critical for the production of carotenoid-rich *H. pluvialis* in a single phase is the irradiance that the culture receives. From the data presented here it is not possible to determine the level of irradiance that is the minimum required to produce a carotenoid-rich culture, in a single-phase continuous process. It is not known how the culture responds to constant (high) irradiance or whether the same results could be achieved from the period (be it hours or days) of extremely high irradiance followed by a period where the irradiance was significantly lower. What can be determined from this work is that the irradiance level is critical for the successful production of carotenoid-rich *H. pluvialis*, particularly in a single, continuous, phase.

As important as the actual irradiance incident on the surface of the photobioreactor is the ratio of surface area to volume, and the mixing efficiency. These are particularly important at high cell densities, when the cells shade each other, resulting in a heterogeneous pattern of light transmittance within the photobioreactor. As a result, cells experience different light conditions as they travel through the photobioreactor (Terry 1986, Ación Fernández *et al* 1998). This would be expected to have a direct effect on carotenogenesis in *H. pluvialis* grown outdoors unless mixing was optimised. Optimisation of mixing would be dependent on both the irradiance received by the photobioreactor and the cell density of the culture; consequently optimisation of mixing would be a complex task.

The increase in the media renewal rate to 20% (v/v) resulted in the 'wash-out' of the culture. During this feed period the cell density steadily dropped, until on day 41 the cell density was less than 50% of that produced during the 10% (v/v) renewal period, i.e. the system was no longer as productive. The cell count of the culture steadily declined because the growth rate of the culture was not high enough to maintain the loss of cells from the system. However, the initial calculation of the growth of the culture (day zero to 14) with a doubling time of 2.40 days, indicated that the culture would be able to sustain a renewal rate of 20% (v/v). However, at this time the culture was made up of a mixture of zooids and aplanospores, and as discussed above this resulted in an increased growth rate. During the 20% (v/v) renewal phase the culture contained 88.75% as zooids, which were only observed dividing into two or four new cells.

The combined effects of the increased urea content and the reduction in the irradiance received by the culture during the 20% (v/v) media feed period resulted in the production of a green zooid population. On day 41 the renewal rate was reduced to 10% (v/v) in an effort to recover the initial productivity of the system. This resulted in a slight increase in the cell count and the dry weight of the culture though the culture still remained 91.75% zooid (Figures 5.14, 5.15 and 5.20 respectively). The reduction in the media feed rate resulted in a slight reduction in the urea content of the culture (Figure 5.25). This was not enough to improve carotenoid accumulation and the total carotenoid content per cell (Figure 5.17) and per unit volume of the culture (Figure 5.21) continued to drop. The cell count of the culture failed to increase significantly after seven days at 10% (v/v) so the system was shut down. The reason for this lack of recovery is unclear however it was also noted to occur by Clarkson *et al* (2001) in small-scale chemostat continuous cultures of *H. pluvialis*. They found that when the

system was returned to its original operating state the culture failed to achieve the previously attained dry weight yield.

5.4. Discussion

Two approaches were used to assess the viability of using continuous culture to produce carotenoid-rich *H. pluvialis*. The use of continuous culture for *H. pluvialis* production is complicated because of the difference in the optimum conditions required for biomass and secondary carotenoid production. However, work with the batch production of carotenoid-rich *H. pluvialis* outdoors in the AAPS™ had shown that a culture is capable of accumulating secondary carotenoids while still in the growth phase (Chapter 4, section 4.2.1).

The first strategy was to produce the biomass under optimal growth conditions in an environmentally controlled growth room. The green zooid culture was then concentrated using tangential flow filtration and resuspended in tap water in a second AAPS™ outdoors. This was to allow the accumulation of secondary carotenoids as higher irradiances could be achieved outdoors. Harker *et al* (1995) determined that the optimum light level for carotenogenesis in *H. pluvialis* was $\sim 1,600 \mu\text{mol}/\text{m}^2/\text{s}$, far higher than could be achieved economically indoors. The growth rate of the biomass production phase was very slow with a doubling time >6 days. It is thought that the reason for this poor performance was due to the ineffective illumination of the culture. Previous cultures in the AAPS™ had been grown outdoors where the sun had provided illumination around the entire photostage. Prior to this, experience of large-scale indoor culture had been limited to the TBR 1 and 2. TBR1 failed to produce a viable *H. pluvialis* culture. TBR2 produced a carotenoid-rich culture of *H. pluvialis*, however the relatively small diameter of the photostage of TBR2 made it easier to achieve a

constant level of illumination around it using a minimal number of fluorescent lights. Despite this, problems with this arrangement of lighting had been identified (see Chapter 3). The same configuration of lighting that had been used for TBR 1 and 2 was used from the AAPS™2 due to the lack of a viable alternative. This resulted in the culture receiving high illumination close to the fluorescent tubes, but midway between two tubes the illumination reaching the culture dropped close to zero. This problem would have been exacerbated, as the irradiance incident on the surface of the photobioreactor would not have been constant through the tubing that made up the photostage, and would have decreased further towards the centre of the tubing (Ogbonna *et al* 1995). However, the turbulent mixing achieved in the photostage would have acted to move algal cells in and out of this dark zone thereby maximising the irradiance received by individual cells (Molina Grima *et al* 1996). The importance of an effective lighting arrangement was demonstrated when further lighting was added to the AAPS™2 which resulted in an increase in the minimum irradiance falling on the culture to 40 $\mu\text{mol}/\text{m}^2/\text{s}$, and a corresponding increase in the cell density of the culture.

The second strategy was to establish the continuous culture outside exposed to climatic conditions with no control over the irradiance received. The outdoor grown culture of *Haematococcus* had a much faster doubling time at 2.40 days. The productivity of the culture before the commencement of the continuous feed was 4.04×10^4 cells/ml/day, nearly three times greater than the culture grown indoors. The reasons for the improved productivity of the culture are probably a combination of the increased irradiance that the culture received and the fact that the culture was a mixture of zooids and aplanospore, (which for the reasons discussed above probably resulted in an increased growth rate). A comparison of the productivities achieved by adopting these two strategies is shown in Table 5.11.

Table 5.11. Productivity of continuous *H. pluvialis* culture.

	Two-phase production	Single-phase production
Cell productivity before start of continuous feed ($\times 10^4$ cells/ml/day, $n=16 \pm \text{S.E.}$)	1.45 (± 0.04)	4.04 (± 0.20)
Cell productivity during 10% (v/v) media renewal ($\times 10^9$ cells/day, $n=16 \pm \text{S.E.}$)	2.40 (± 0.12)	4.60 (± 0.15)
Maximal cell density reached ($\times 10^4$ cells/ml, $n=4 \pm \text{S.E.}$)	59.25 (± 2.29)	91.50 (± 1.26)
Developmental stage	Green zooid	Green zooid (with red section) and aplanospores
Secondary carotenoid production (g/day, $n=3 \pm \text{S.E.}$)	EC1 0.03 (± 0.00) EC2 0.04 (± 0.00) EC3 0.03 (± 0.01) EC4 0.04 (± 0.00)	0.15 (± 0.07)

N.B. The data shown here for single-phase production refers to the 10% renewal period only

The outdoor grown culture had an improved productivity when compared to the culture grown under controlled conditions. Both cultures were operated at a 10% (v/v) renewal rate. As shown in Table 5.11 the single-phase, outdoor culture reached a higher cell density than the two-phase system and produced, on average, twice as many cells per day. Combined with the increased number of cells produced by the outdoor system; it also produced cells that were rich in secondary carotenoids, whereas those produced through controlled culture required a second encystment stage to produce secondary carotenoids. The two-phase system produced green biomass with a urea content that

had been reduced to 37.44 % of the starting concentration by the growth of the cells. At the start of the continuous feed period in the outdoor system the urea content of the culture had dropped to 18.29 % of the starting concentration. This resulted in the production of a mixed population of aplanospores and zooids with a mean secondary carotenoid content of 27.32 pg/cell. As the culture ran at a 10% (v/v) renewal rate for 15 days, in total the culture produced 2.25g of secondary carotenoids, of which 1.80g was astaxanthin, mainly as a mixture of mono and di esters (Table 5.8)

The two-phase strategy resulted in the production of a series of carotenoid-rich cultures. Concentration of the green zooid cultures further reduced their urea content, so that upon resuspension in tap water the cultures contained 3-10mg/l total nitrogen. In total the four cultures took 26 days to produce carotenoid-rich cells and yielded a total of 0.95g of secondary carotenoids, which translates to 0.04g/day. The continuous culture produced 0.15g of secondary carotenoid/AAPS™/day making it nearly four times more productive. This is despite the fact that each of the two-phase encystment cultures received a higher daily irradiance (Figure 5.10) than the continuous culture (which received $6.17 \times 10^8 \mu\text{mol/m}^2/\text{day}$ during the 10% (v/v) renewal phase). The continuous culture was exposed to similar mean and maximum temperatures (Table 5.10) as the four encystment cultures (Figure 5.13). It differed in that the minimum temperature that it was exposed to was 14°C, whereas the encystment cultures were exposed to temperatures falling as low as 6°C. Higher temperatures have been shown to induce carotenogenesis in *Haematococcus* (Borowitzka *et al* 1991, Tjahjono *et al* 1994).

Although the continuous culture produced carotenoid-rich cells in a single phase system outdoors this was highly climate dependent. The figures given here are the mean productivities over the 15 days of the 10% (v/v) renewal period. In fact, after the

first five days of feeding the carotenoid content of the culture began to drop; although there was no increase in the urea content of the culture. This drop was co-incidental with a decrease in the irradiance received by the culture. The decrease in the proportion of aplanospores could also be due to the continuous nature of the culture. When the continuous feed started the culture was largely made up of aplanospores (Figure 5.20), which upon the commencement of the continuous feed were slowly removed from the culture. The culture was actively dividing to replace these cells with a mixture of aplanospores and zooids. It is therefore possible that if the culture were allowed to approach equilibrium this change in the culture dynamics would have continued until it reached a steady state population, irrespective of the climatic conditions.

5.5. Conclusions

Continuous culture of *H. pluvialis* produced carotenoid-rich cells using both the single-phase and two-phase approach. The culture of the alga outdoors resulted in a higher productivity and a greater yield of astaxanthin. However, it is highly likely that the biomass yield of an indoor grown culture could be improved by the optimisation of the lighting that the system used.

Both a 10% (v/v) and 20% (v/v) renewal regime were tested in the single-phase system. The data revealed that *H. pluvialis*, in the AAPS™, was only capable of sustaining production at <20% (v/v) and possibly as low as 10% (v/v). This is supported by the work of Clarkson *et al* (2001). They determined that in a small-scale continuous culture of *H. pluvialis* the highest cellular productivities were attained at a renewal rate of 10.80-12.96% (v/v). At a renewal rate of 17.28% (v/v) the cell density and productivity dropped.

Both strategies produced carotenoid-rich cultures of *Haematococcus*, although the single-phase production method was shown to be a more efficient system, producing twice the number of cells per day and four times the volume (mg/l) of secondary carotenoids. However, the outdoor method of production is limited by the climate that the culture is grown in and therefore may be better suited to conditions with a higher number of sunshine hours and total irradiance.

6. Conclusions

6.1. Introduction

The previous three chapters have detailed the development of a novel tubular photobioreactor, and an assessment of the viability of using the system to produce the alga *H. pluvialis*. This chapter summarises the performance of the tubular photobioreactor with relation to the production of carotenoid-rich *H. pluvialis*. Attention shall also be given to the potential for scale-up of this methodology for commercial production, and to suggestions for further work.

6.2. Development of the novel tubular photobioreactor

Four successive tubular photobioreactors were developed; each with a helically arranged photostage driven by an airlift pump. The configuration of the photostage as a helix had a number of advantages over other arrangements. A helix enables the use of a very long length of tubing in the photostage while minimising the area of land used and maximising the amount of irradiance falling on the tube (Borowitzka 1994, Lee 1986). The helical arrangement is also beneficial as it generates a turbulent flow pattern, which improves mixing (Borowitzka 1994, Carlozzi and Torzillo 1996). This results in the movement of the algal cells through the light regime within the photostage which maximises the amount of light that each cell is exposed to (Molina Grima *et al* 2000, Janssen *et al* 2000). Turbulent mixing also reduces the boundary layer between the algal cells and the media, which acts to increase the rate of exchange of nutrients and gases (Grobbelaar 1994). Another advantage of the helix is that there are no sudden changes in flow direction as the algae move through the photostage. In other tubing

arrangements sudden changes in flow can result in algal accumulation (Borowitzka 1994).

Problems with the initial photobioreactors (TBR1 and TBR2) in terms of their photostage flow rate and mixing led to the development of the AAPS™1. The design of this system differed from the first two in that it had a longer riser with a larger cross sectional area, which resulted in an improvement in the photostage flow rate and the mass transfer of the system (Merchuck and Siegel 1988, Bentifraouine *et al* 1997). Although the system had twice the volume of either TBR1 or TBR2 the length of each winding in the photostage was shorter. This was achieved by the introduction of a manifold assembly, which meant that the photostage was made up of three separate lengths of tubing. Therefore the cells were more frequently returned to the riser/header tank where gas transfer occurred than would have been the case if the photostage was made of a single tubing length (Borowitzka 1994, Sánchez Mirón *et al* 1999). This enabled the cells to be frequently replenished with CO₂ and prevented the build-up of photosynthetic O₂. The constant supply of CO₂ is vital to maximise the photosynthetic rate of the culture. O₂ must be removed rapidly to prevent growth inhibition (García Camacho *et al* 1999).

AAPS™2 was a further development of the AAPS™1: it differed in that the header tank changed shape from a cylinder to a rectangular box and pH control using CO₂ was added. The header tank design change was thought to have a detrimental affect on the physical performance of the system as it's presence resulted in a reduced photostage flow rate and mass transfer coefficient when compared to the AAPS™1. The differences in photostage flow rate and mass transfer would have resulted in different light/dark cycles within the photobioreactors as the degree of turbulence in each photostage would be different (Molina Grima *et al* 2000, Ogbonna and Tanaka 2000).

It would also have resulted in a difference in the size of the boundary layer between the cells and media (Grobbelaar 1994). Also as the AAPS™2 had a reduced mass transfer coefficient it would have had a reduced rate of CO₂ supply and photosynthetic O₂ removal (Chisti 1989). However the physical performance was still an improvement over that seen in TBR1 and TBR2.

The construction of the header tank of AAPS™1 and AAPS™2 also differed in that they were opaque and transparent, respectively. There was also a difference in materials between the two downcomers (AAPS™1 was transparent, AAPS™2 was opaque). This difference would have resulted in a change in the ratio of dark and light zones within the photobioreactors, which may have affected productivity (Janssen *et al* 2000). In this case however, it is unlikely to have affected the performance greatly as light transmission in the header tank of AAPS™2 and downcomer of AAPS™1 would have been expected to be negligible when compared to the photostage. Cultures grown in each of the AAPS™ units had the same percentage residence time in the photostage.

6.3. Production of *H. pluvialis*

The changes in the design of the photobioreactor, which resulted in improvements in the physical performance, also resulted in an improvement in the biological performance of the system. TBR2, AAPS™1 and AAPS™2 all sustained the cultivation of *H. pluvialis* and the subsequent accumulation of astaxanthin, however TBR2 produced a lower density culture. The reduced growth of the alga in TBR2 was thought to be caused by poor mixing in the system (as the cells frequently settled in the header tank) and light limitation caused by the configuration of lighting around the photostage. Poor mixing would have resulted in a poor rate of exchange between the

algae and the media in terms of nutrients and metabolites, which could have resulted in a reduced productivity (Grobbelaar 1994).

The two AAPS™ designs repeatedly produced cultures of *H. pluvialis* with a high cell density and carotenoid content. AAPS™1 and AAPS™2 differed from TBR2, as they were both sited outdoors where irradiance was not thought to be limiting. The irradiance incident on the photostage surface combined with the turbulent mixing regime in the photostage would have acted to supply each cell with an increased intercepted irradiance when compared to cells in TBR2. This could act to increase the photosynthetic efficiency of the cells (Terry 1986). However exposure to the high irradiances in outdoor cultures could also result in photoinhibition of the cultures (Pulz 1994, Vonshak *et al* 1988, Janssen *et al* 2000). It has been suggested that turbulent mixing within a photobioreactor would act to move the cells in and out of the high irradiance on the surface of the photostage thereby minimising any photoinhibitory effect (Janssen *et al* 2000). It would be further minimised by movement of the cells in and out of dark zones within the photobioreactor (Molina Grima *et al* 2000). The manifold construction of the AAPS™ photostage resulted in a reduction in the time spent by the cells in the photostage when compared to a photostage constructed of a single winding. Cultures grown in the AAPS™1 and AAPS™2 spent ~ 34% of their time in the photostage, in comparison cells in TBR2 were resident in the photostage for ~ 59% of the time. Increasing the residence time of the cells in the photostage may also limit the time cells would have to recover if exposed to photoinhibitory irradiance.

AAPS™1 was only used in batch operation, whilst AAPS™2 was used for both batch growth and continuous production of *H. pluvialis*. AAPS™2 proved more productive both in terms of biomass and secondary carotenoids when compared to the AAPS™1 in batch operation. This was despite the apparent detrimental effect of the

change in design on the flow rate and mass transfer of this system (section 6.2). This suggests that their level may not be critical once a certain threshold value is reached. It is also possible that the difference in performance was climatic, as each of the cultures was grown at a different time of year. Further work is clearly needed in order to elucidate the effect of short-term and long-term changes in certain environmental variables, e.g. irradiance, temperature, and day-length, on algal productivity in the AAPS™. Nevertheless it is clear that the AAPS™ can be used as a platform for the cultivation of *H. pluvialis*.

6.3.1. Batch production

H. pluvialis was cultured in modified BBM (Tsavalos 1995), with both a high [N] (3.00mM urea) and low [N] (0.75mM urea). The high [N] media produced a culture with a higher cell density but the carotenoid content per cell was much reduced when compared to cells grown in the low [N] media. The culture produced from the high [N] media however did contain a higher carotenoid content per volume of media due to the higher number of cells. The production of a greater number of cells could have protected the culture as a whole from becoming photoinhibited (Richmond 2000). A greater number of cells in the culture would result in a higher degree of cell shading, this would result in a heterogeneous irradiance within the photobioreactor (Ación Fernández *et al* 1998). It would also result in a reduction in the irradiance received per cell, which would consequently increase the irradiance the system could receive before the culture became photoinhibited.

The best performing low [N] culture proved to be 10x more productive in terms of secondary carotenoids but produced only ~30% of the cell biomass when compared to the best performing high [N] culture. Both were cultured outdoors at the same time

of year (Cultures five and six, Chapter 4, Table 4.14). This indicates that the increased nitrogen content of the high [N] media slowed the rate of carotenoid accumulation within the cells.

The yield of secondary carotenoids in the low [N] media was 3.80% (w/dw, after 18 days), in the high [N] media it was 3.00% (w/dw, after 48 days). These results compare favourably with published information on commercial ventures. Olaizola (2000) reports a carotenoid yield at harvest of 3% (w/dw) (Aquaseach Inc, Hawaii, USA), whereas Lorenz and Cysewski (2000) report yields of 1.5-3% (w/dw) (Cyanotech Corp. Hawaii, USA). The carotenoid yield is greater than other reported data (e.g. 1.4% (w/dw), Gudín and Thépenier 1986).

Typically, carotenoid accumulation occurs when the cells are in the stationary phase of growth (Droop 1954, Boussiba and Vonshak 1991, Zlotnik *et al* 1993). In the AAPS™1 sited outdoors it was noted to occur while the culture was still in the exponential phase of growth. Carotenogenesis began on day six while the cells were clearly still in the exponential phase of growth and resulted in the production of carotenoid-rich zooid cells. Initially the culture was a homogeneous population of red zooids (macrozooids, see Chapter 1 and Elliott 1934), as it aged and reached the end of the exponential growth phase the population became a mixture of red zooids and aplanospores. Unlike the outdoor continuous culture no germination of aplanospores was noted. The production of red zooids was thought to be due to the high irradiance that the cells were exposed rather than a lack of nitrogen (which would cause cessation of growth). It is widely accepted that one of the main triggers of carotenogenesis is high irradiance (Droop 1955, Kobayashi *et al* 1992, Fábregas *et al* 1998). In growing cultures it has been observed that the rate of accumulation within a *Haematococcus* culture is determined by the photon flux density (PFD) that the cells were exposed to

rather than the absorbed light energy (Lee and Soh 1991). Chaumont and Thépenier (1995) recorded red zooid cells in continuous culture when cultured outdoors. They noted that the carotenoid content doubled between 7-11am, the concentration then decreased with decreasing irradiance in the afternoon.

6.3.2. Continuous production

Continuous production of *H. pluvialis* proved to be a higher yielding system, in terms of biomass, than batch production. Two approaches to this form of culture were tested. The first was a two-phase method using an indoor AAPS™2 to produce the biomass and then transferring the cells outdoors for the carotenoid accumulation phase. In the second approach both cell and carotenoid production occurred outdoors.

6.3.2.1. Two-phase production

In two-phase production the algal cells were produced using an AAPS™2 that had been configured for continuous production. It was sited indoors using artificial lighting in a temperature controlled room. The culture was thought to be light-limited due to its slow growth rate (Acién Fernández *et al* 1998).

Cells produced from the system were concentrated (which removed a large proportion of the nitrogen rich media), and then resuspended in tap water in a second AAPS™2 that was sited outdoors. Four successive carotenoid-rich cultures were produced using this method, producing ~ 0.04g carotenoid/AAPS™/day. The carotenoid productivity of each culture was found to be affected by the irradiance received and temperature. The addition of NaCl (as a stimulant of carotenogenesis) was also found to result in extremely high cell mortality as had been previously shown by Droop (1955) and Harker *et al* (1996a).

Current commercial ventures based in Hawaii, USA, use the two-phase approach. Cyanotech Corp. produces vegetative cells under 'near optimal' growth conditions, carotenogenesis is then induced using nitrogen and phosphate deprivation, increased temperature and irradiance or by the addition of NaCl. Typically it takes three to five days to produce cells which contain 1.5-3% (w/dw) astaxanthin as dry weight (Lorenz and Cysewski 2000). Aquaseach Inc, also based in Hawaii, use a tubular photobioreactor to produce biomass which is then transferred to ponds where carotenogenesis is induced. After a five-day induction period the cells are harvested and typically contain >2.5% (w/dw) astaxanthin (Olaizola 2000).

6.3.2.2. Single-phase production

The operation of a continuous culture outdoors resulted in the continuous production of *H. pluvialis* cells that were rich in secondary carotenoids. This system was operated at the same renewal rate as the continuous stage of the two-phase system and resulted in a higher productivity both in terms of biomass (twice the number of cells per day) and carotenoids (nearly four times the weight per day). The improvement in cellular productivity when compared to the indoor continuous culture is thought to be due to the increased irradiance which when combined with the turbulent flow in the photostage maximises the irradiance received per cell (Acién Fernández *et al* 1998, Molina Grima *et al* 1996).

Carotenoid productivity in the continuous culture was highly climate dependent; a drop in the irradiance resulted in a drop in the carotenoid content. Chaumont and Thépenier (1995) also observed this in continuous culture over the course of a day-night cycle. They noted that the carotenoid content of a culture increased with

irradiance during the day. Towards evening when the irradiance began to decrease the carotenoid content of the culture was also seen to decrease.

6.4. Scale-up of *H. pluvialis* culture

In the scale up of a biological process it is important to realise that a process that has worked at laboratory scale may fail at a greater volume if attention is not paid to certain important design features. The liquid flow rate achieved in the photostage is of key importance. On scale-up a reduction in the flow rate within the photobioreactor can result in a reduced yield (Watanabe *et al* 1998). Also of importance is the maintenance of the culture depth and tubing diameter, changes in the culture depth could impact on light transmittance and consequently on culture productivity (Kobayashi and Fujita 1997). The mass transfer coefficient and the ratio of light to dark areas as well as the residence time in these sections of the photobioreactor should also be maintained (Chisti 1989), as should the ratio of surface area to volume of the photobioreactor (Tsygankov *et al* 1997).

The two AAPS™ used for this study had working volumes of 65l (AAPS™1) and 55l (AAPS™2). They represent the transition from laboratory to the first stage of scale-up. Commercial production would mean the operation of a plant containing many thousands of litres (Benemann 1990, Borowitzka 1994). Before systems of that volume are constructed it is important to construct systems at intermediate stages so that problems can be identified and tackled before a large monetary investment has been made. Borowitzka and Borowitzka (1989) have found that scale-up by a series of steps, each representing an approximate increase factor of 10, was the most manageable in biological and engineering terms, and yielded fewer surprises.

At each stage in the scale-up process attention must be paid to the key design features. As previously discussed in Chapter 3 perhaps the most important parameter is the length of tubing in the photostage. Larger volume systems require greater lengths of tubing in the photostage, this knowledge led to the development of the AAPS™ manifold systems, which improved the scalability of the system. Therefore careful attention must be paid to gas transfer as excessive lengths of tubing may result in a build up of photosynthetic O₂, which would cause photoinhibition in the culture. This was thought to be the cause of the failure of a large-scale growth facility in Spain (Camacho Rubio *et al* 1999). Awareness of this potential problem means that the scaled up system can be engineered to minimise the lengths of tubing in the photostage. This could be achieved by introducing further windings into the manifold; for example by increasing the three windings used in the AAPS™1 and AAPS™2 to five (I. Burbidge pers. comm.).

Another point for consideration, as mentioned above, is the fluid flow rate in the photostage and around the system. A reduced flow rate may result in cell settling (Terry and Raymond 1985) and reduced turbulence which would be detrimental for growth both in terms of the light received per cell (Eriksen *et al* 1996, Molina Grima *et al* 1999) and the rate of transfer of nutrients and gases from the culture medium to each cell (Grobbelaar 1994). Attention must be paid to the physical factors such as the gas hold-up differential between the riser and downcomer (Chisti 1989) and the bubble regime in the riser (Hsiun and Wu 1995) as they are of key importance in determining the fluid flow rate. Also of importance are the riser cross-sectional area and the bubble density, which is affected by the sparger design (Chisti *et al* 1988, Hebrard *et al* 1996).

A further factor that is important in the design of larger scale systems is the ratio of the photostage, riser and header tank volumes (Chisti 1989, Benyahia and Jones

1997). This ratio is important as the time spent by the culture in each of the zones will directly affect the growth and productivity of the culture. This is because each of the zones has an important function, the riser and header tank are important for gas transfer (Pirt *et al* 1983) whereas the photostage is the main site of light interception (Borowitzka and Borowitzka 1989, Lee and Low 1991, Watanabe *et al* 1995).

6.5. Further work

The work that leads from the research discussed in the previous chapters forms two major sections, (i) the further assessment of the hydrodynamics of the system and consequent design alterations and (ii) validation and development of the biological production of *H. pluvialis*.

6.5.1. Hydrodynamics of the photobioreactor

6.5.1.1. Sparger

In each system the sparger plate was positioned just below the point at which the return manifold (see Chapter 3) joined to the riser (Figure 6.1.). This however may not be the best positioning of the sparger because the fluid entering for the manifold causes a maldistribution of the bubble stream onto the wall of the riser, which reduces the interfacial area and consequently the mass transfer. Chisti (1989) found that using a sparger of the design shown in figure 6.1. the bubble stream could be optimised. Chisti also noted that no settling occurred around this type of sparger up to a solids density of 30g/l, though it would have to be tested using *H. pluvialis* to confirm this.

The type of sparger used could also be further developed. All four systems used a plate type sparger, which is a disc that has a number of holes of uniform size drilled

into it. There are a number of other sparger designs including the porous disc. The type of sparger design used has been found to have a significant effect on the hydrodynamics of a system (Hebrard *et al* 1996).

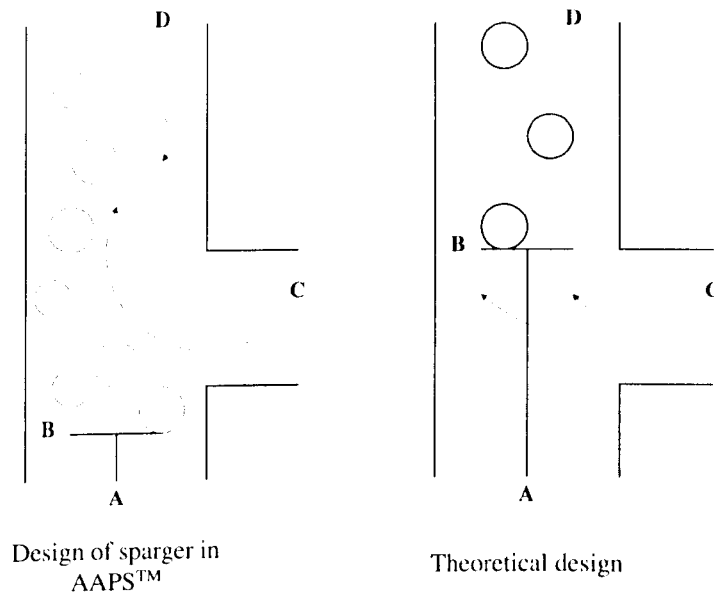


Figure 6.1. Sparger configuration (adapted from Chisti 1989)

- A** Air inlet
- B** Sparger plate
- C** Entry from return manifold
- D** Riser

In future work it would also be important to measure the pressure of air going into the system as well as the air flow rate.

6.5.1.2. Assessment of fluid flow

The fluid flow rate of each of the four photobioreactors was measured in this study, however for scale up further information on the behaviour of the fluid around the system would be invaluable. Methodology that could be employed includes the use of

dyes to visualise the flow patterns (Chisti and Moo-Young 1987) and the use of a conductivity probe to measure the flow in the downcomer (Bentrifraouine *et al* 1997). It would also be beneficial to measure the gas hold-up in the downcomer as this is of key importance in determining the flow rate around the system (Chisti 1989).

6.5.1.3. Mixing

As an airlift system, mixing in the AAPS™ was achieved by the addition of compressed air. Airlift systems can be difficult to control, as a change in the rheology of the media during growth results in a change in the riser dynamics from perfect bubbly to slug flow (see Chapter 3 and Hebrard *et al* 1996). This could have a serious effect on the physical performance of the system and consequently on the growth of the culture as there is little that can be done and the culture may be lost due to a reduction in the fluid flow rate and mass transfer (Chisti 1989, Watanabe *et al* 1998). Also for this reason anti-foaming agents cannot be used in this type of airlift design as they act by reducing bubble wall strength which increases coalescence thereby encouraging the formation of slug flow in the riser (Ghosh *et al* 1993, Al-Masry and Dukkan 1997). Coalescence in the riser of the AAPS™I was noted to occur after the addition of an anti-foam agent (data not shown).

6.5.1.4. Mass transfer

The most important further study that could be carried out in relation to mass transfer would be to alter the configuration of the present systems to allow the insertion of a dissolved oxygen probe in the riser, downcomer and photostage as well as the two manifolds. This would enable the determination of mass transfer at different parts of the system, rather than in the header tank as in this study. Benyahia and Jones (1997)

demonstrated that there can be up to a 20% variation in mass transfer depending on the point of measurement within an air-lift photobioreactor.

It would also be important to carry out mass transfer determinations with the algae present in the system to determine how the presence of cells affects mass transfer. Contreras *et al* (1998) found that $K_{L,a}$ declined with increasing solids concentration in a culture of *Phaeodactylum tricornutum*.

6.5.2. Biological performance

6.5.2.1. Photosynthetic efficiency and photoinhibition

Measurement of CO_2 and O_2 concentrations in the culture could be used to determine the photosynthetic efficiency of the culture. Continuous measurement of the CO_2 and O_2 levels in the out-gas in this study using a gas analyser was not possible due to the high rate of ambient airflow through the airlift. This effectively 'swamped' the expected changes in the CO_2 and O_2 levels due to the algal culture. Measurements could also be correlated with irradiance measurements to determine the irradiance at which *Haematococcus* becomes photoinhibited. CO_2 and O_2 measurements around the AAPS™ could also be used to determine the optimal point at which to inject CO_2 for maximal diffusion into the media. The data-logging of the CO_2 and O_2 concentrations would give a better understanding of the behaviour of *H. pluvialis* over the course of a day-night cycle and also over the course of the culture period with relation to the climatic conditions.

The DO_2 status of a culture may also be of importance in the accumulation of secondary carotenoids. Lee and Ding (1995) demonstrated that oxygen stress is a major factor in the induction of carotenogenesis. A high dissolved O_2 partial pressure ($p\text{DO}_2$) was seen to offer a non-destructive means to enhance astaxanthin productivity.

6.5.2.2. Lighting

The configuration of artificial lighting used to illuminate the photostages of the tubular photobioreactors indoors required further study. By developing a more efficient lighting system (constant illumination around the photostage) the optimum irradiance for growth in the AAPSTTM could be determined. Parallel studies (A. Ince, pers.com.) have developed a lighting rig that evenly illuminates the entire photostage at $\geq 120\mu\text{mol}/\text{m}^2/\text{s}$. The photostage light path (25mm) is similar to that seen in shake-flask culture. Light transmission properties may therefore be expected to be similar at any given density of algal culture, although due to mixing differences in the two vessels the proportion of time the cells are illuminated for may differ.

6.5.2.3. Data acquisition

The on-line monitoring of parameters such as the pH and rate and volume of CO₂ usage is an important consideration. CO₂ usage is of particular interest as the process is scaled-up as CO₂ is costly and minimising its use would reduce the total cost of production (Camacho Rubio *et al* 1999). The direct measurement of growth and pigment content in the reactor, using optical density sensors, could be used with climatic data to develop a computer model of the process (N. Clarkson, K. Jones, A.J. Young, pers. com). This would be of importance in process optimisation. The continuous monitoring of these parameters could also be used to assess the behaviour of a culture over a light cycle. Chaumont and Thépenier (1995) noted that the secondary carotenoid content of a culture changed with changing irradiance over the course of a day. This may have implications commercially in determining the time of harvest in order to maximise the yield.

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Appendix 1a

BBM recipe

Before sterilisation the pH of the media was adjusted to 7 using 4M NaOH.

Stock Solutions	Volume of stocks for 1 litre of modified BBM (made up to 1 litre with distilled water).
Urea $\text{CO}(\text{NH}_2)_2$ At 0.75mM (4.5g/l) At 3.0mM (18g/l)	10ml
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (7.5 g/l)	10ml
NaCl (2.5 g/l)	10ml
K_2HPO_4 (9.8g/l)	10ml
KH_2PO_4 (17.5g/l)	10ml
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (2.5 g/l)	10ml
Trace Element Solution (Autoclaved to dissolve) $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (8.82 g/l) $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.44 g/l) MoO_3 (0.71g/l) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.57g/l) $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.49 g/l)	1ml
EDTA-KOH Solution EDTA (50g/l) KOH (31 g/l)	1ml
Boric acid Solution H_3BO_3 (11.42 g/l)	1ml
Iron Solution $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (5 g/l) Citric acid (5g/l)	1ml

Appendix 1b

BBM supplementation for the germination of aplanospores

pH of the media was adjusted to 7 using 4M NaOH.

Urea $\text{CO}(\text{NH}_2)_2$	1.80g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.075g
NaCl	0.025g
K_2HPO_4	0.098g
KH_2PO_4	0.175g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.025g
Trace Element Solution	1ml
EDTA-KOH Solution	1ml
Boric acid Solution	1ml
Iron Solution	1ml
Distilled water	46ml

See Appendix 1a for solution recipes

BBM supplementation (-PO₄) for the germination of aplanospores

pH of the media was adjusted to 7 using 4M NaOH.

Urea CO(NH ₂) ₂	1.80g
MgSO ₄ .7H ₂ O	0.075g
NaCl	0.025g
CaCl ₂ .2H ₂ O	0.025g
Trace Element Solution	1ml
EDTA-KOH Solution	1ml
Boric acid Solution	1ml
Iron Solution	1ml
Distilled water	46ml

See Appendix 1a for solution recipes

Urea plus trace elements supplementation for the germination of aplanospores

Urea CO(NH ₂) ₂	1.80g
Trace Element Solution	1ml
Distilled water	49ml

See Appendix 1a for solution recipe

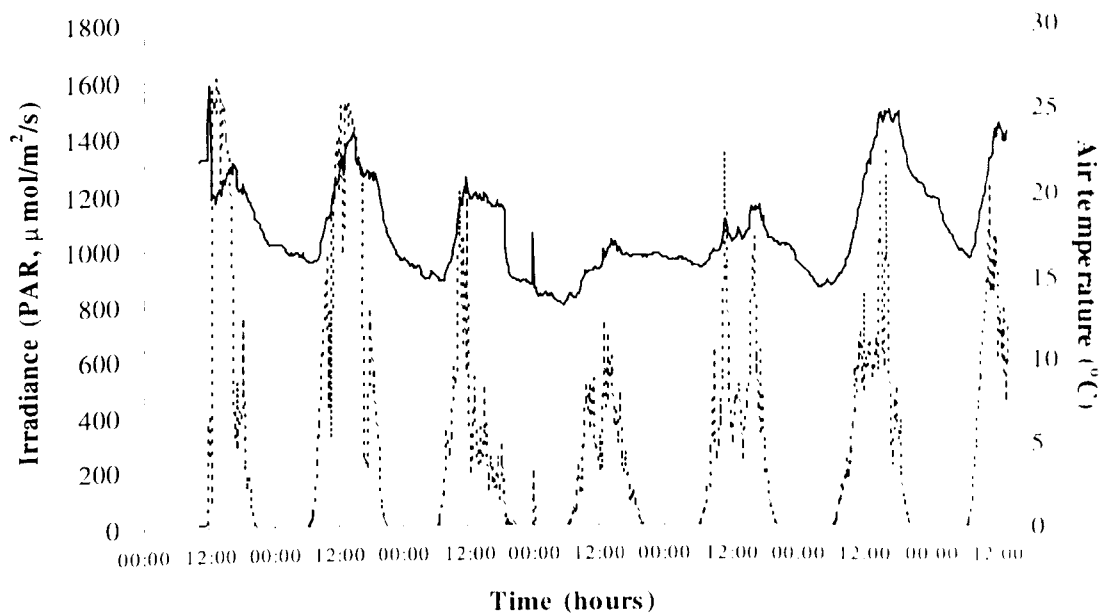
Appendix 2a. Climatic conditions

27th August to 13th October 1999.

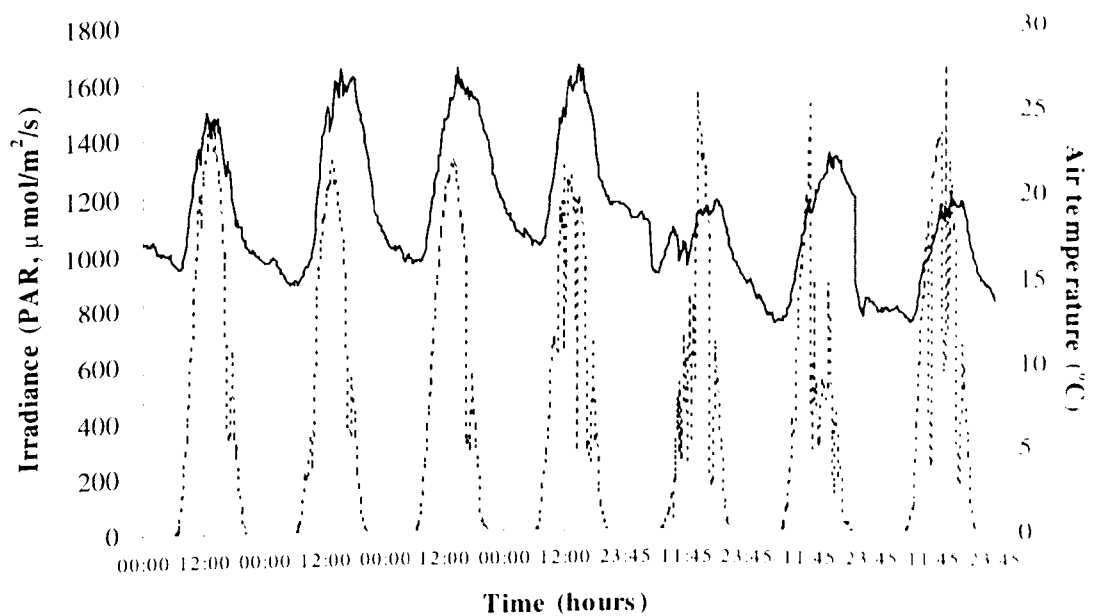
The graphs below show the irradiance (PAR) and air temperature (°C) measured during culture five and six in the AAPS™2. Measurements were taken every 15 minutes 24hours a day for the duration of the cultures.

Key. ----- Photosynthetically active radiation (PAR, $\mu\text{mol}/\text{m}^2/\text{s}$)
——— Air temperature (°C)

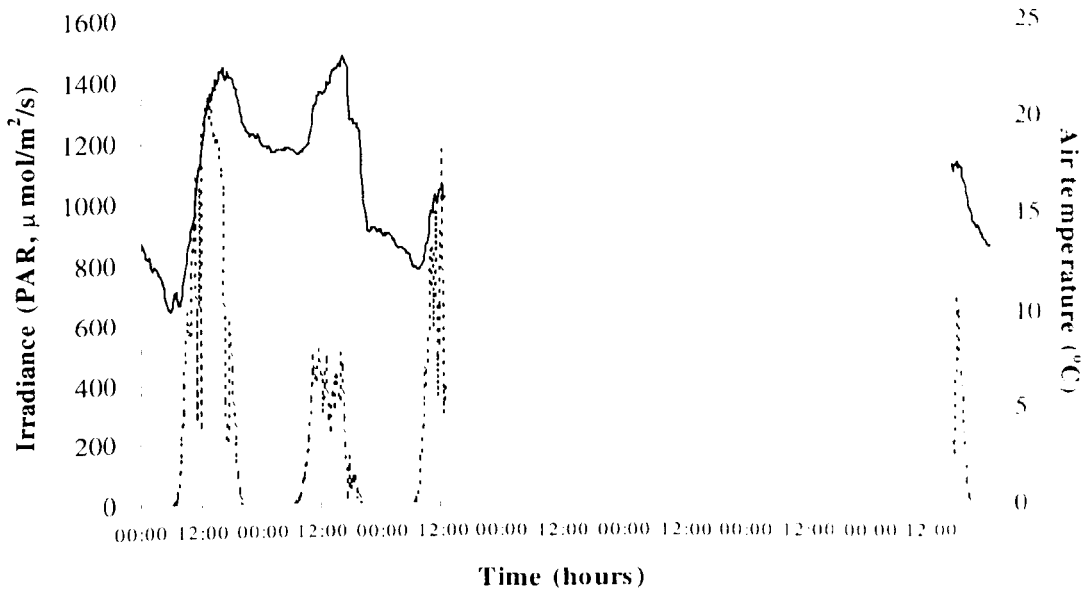
Day 0-6 (27/8/99-2/9/99)



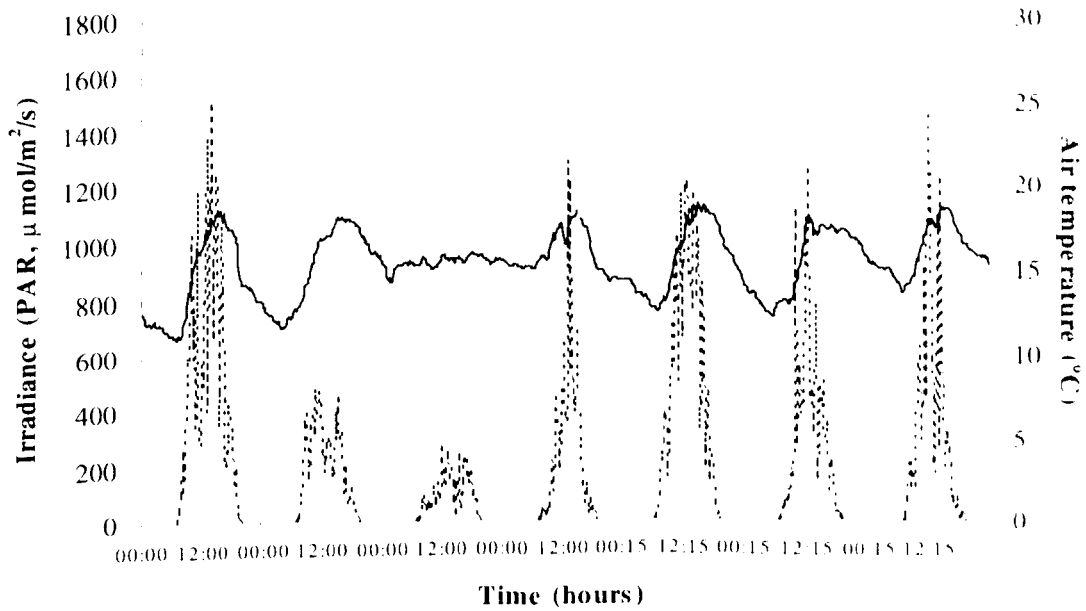
Day 7-13 (3/9/99-9/9/99)



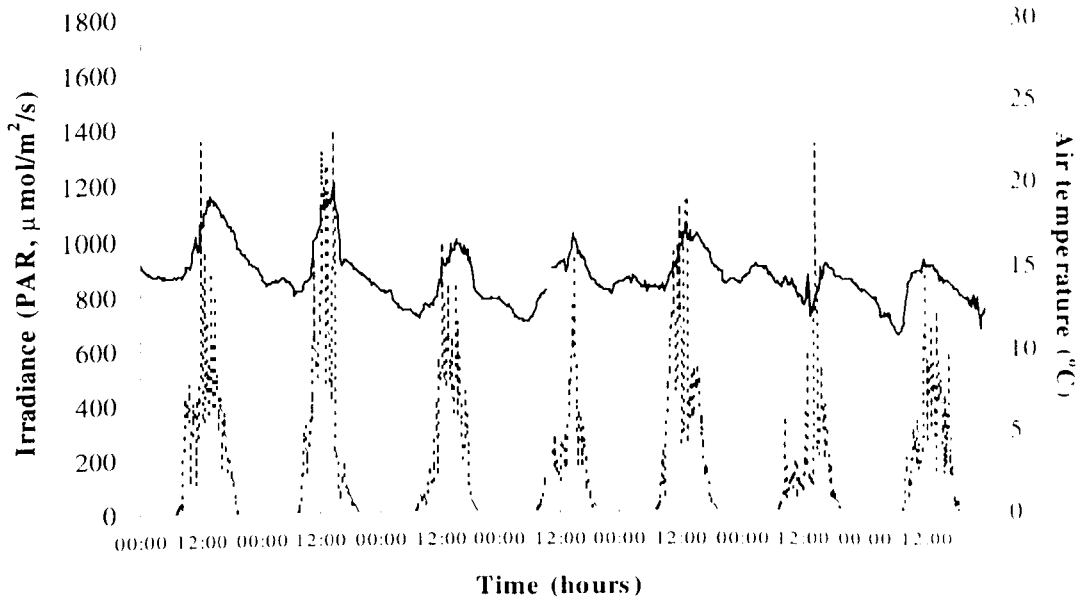
Day 14-20 (10/9/99-16/9/99)



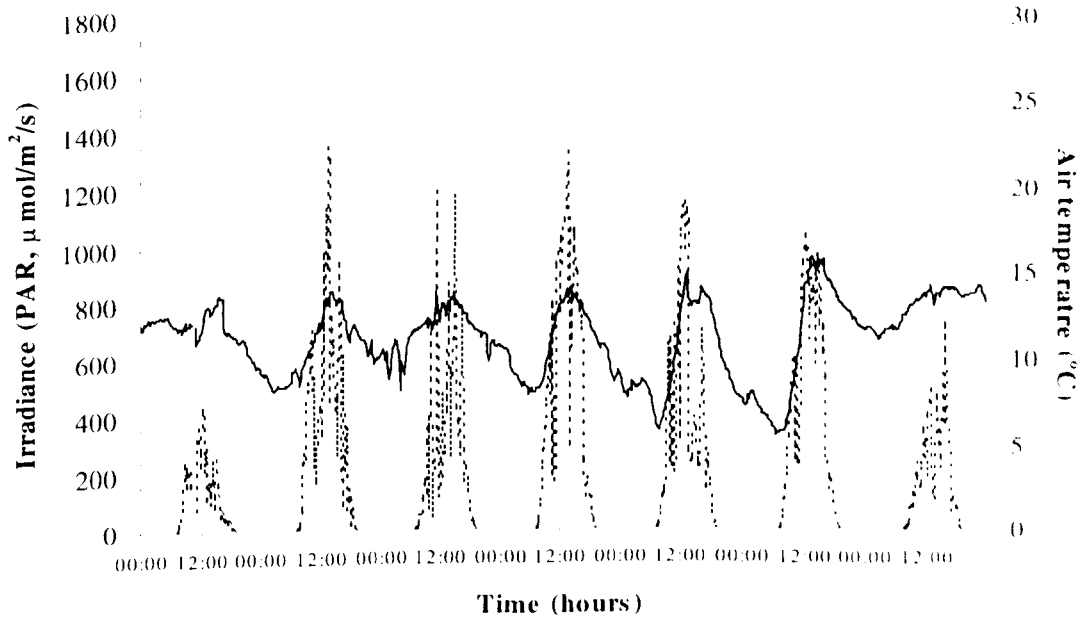
Day 21-27 (17/9/99-23/9/99)



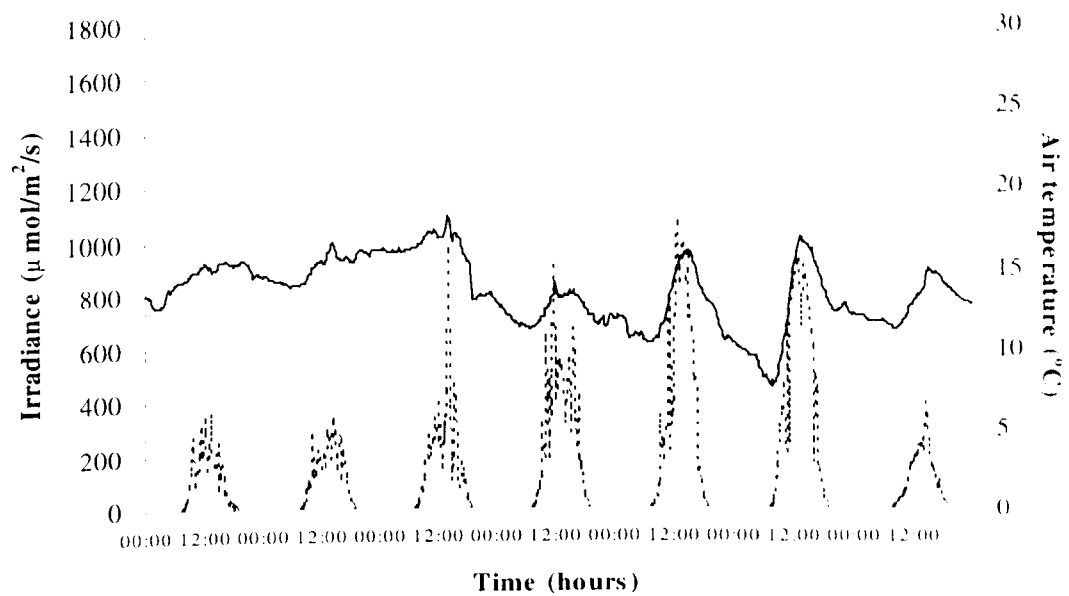
Day 28-34 (24/9/99-30/9/99)



Day 35-41 (1/10/99-7/10/99)



Day 42-48 (8/10/99-14/10/99)



Appendix 2b. Climatic conditions

22nd October to 16th December

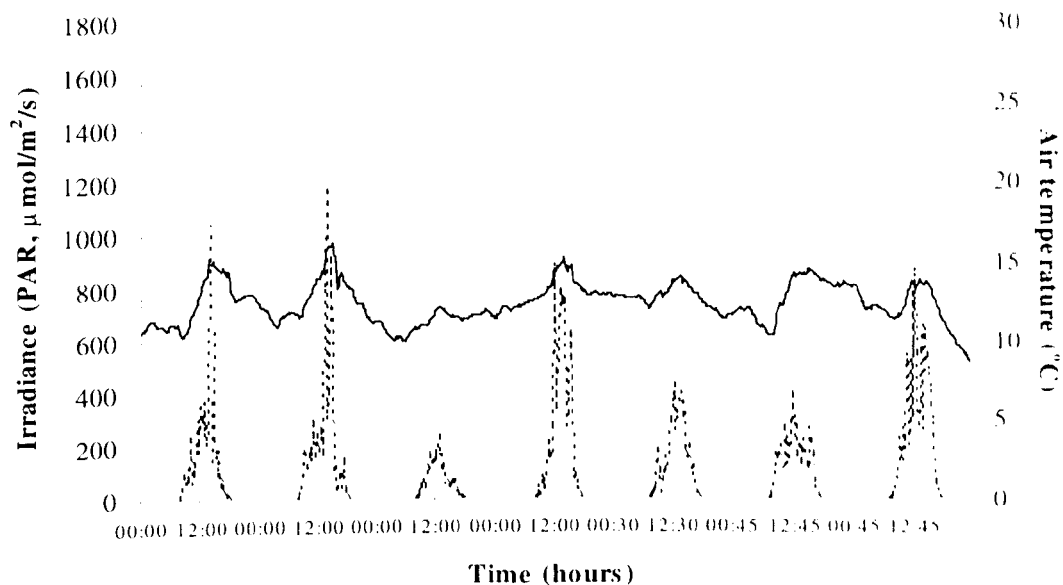
1999.

The graphs below show the irradiance (PAR) and air temperature measured during culture seven and eight in the AAPS™2. Measurements were taken every 15 minutes 24hours a day for the duration of the cultures.

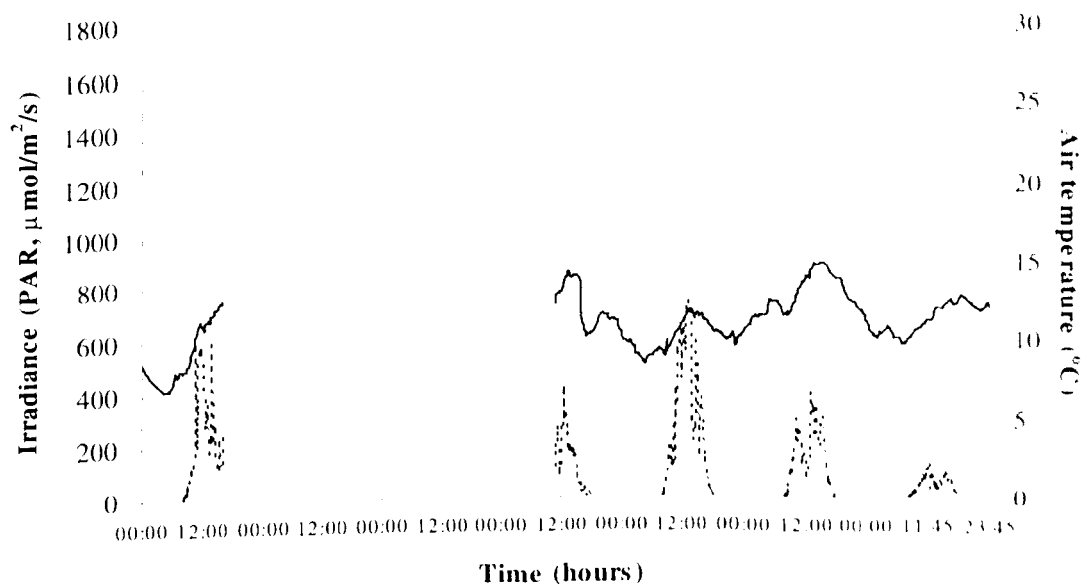
Key. ----- Photosynthetically active radiation (PAR, $\mu\text{mol}/\text{m}^2/\text{s}$)

—— Air temperature ($^{\circ}\text{C}$)

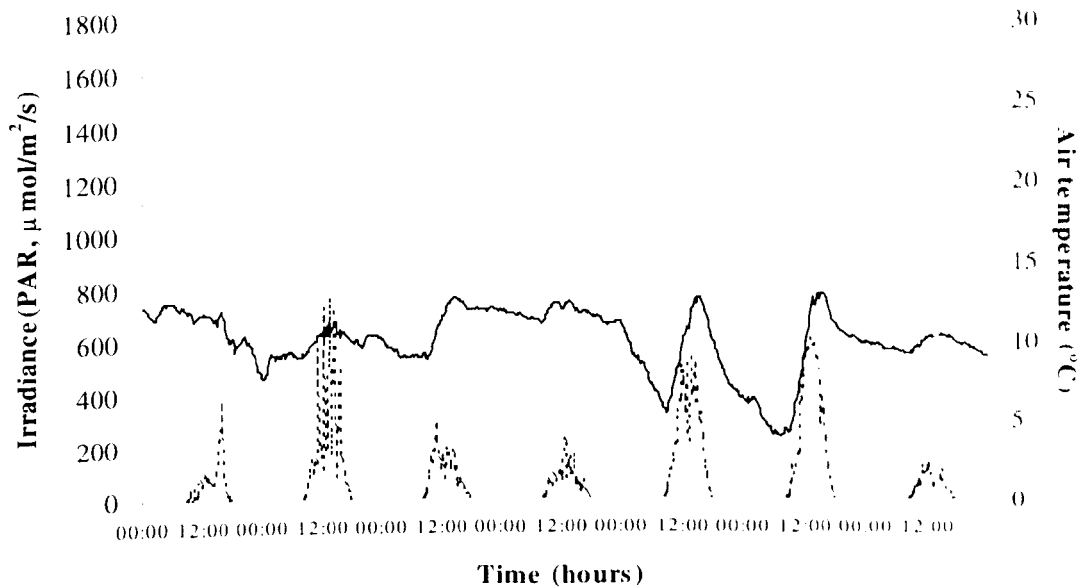
Day 0-6 (22/10/99-28/10/99)



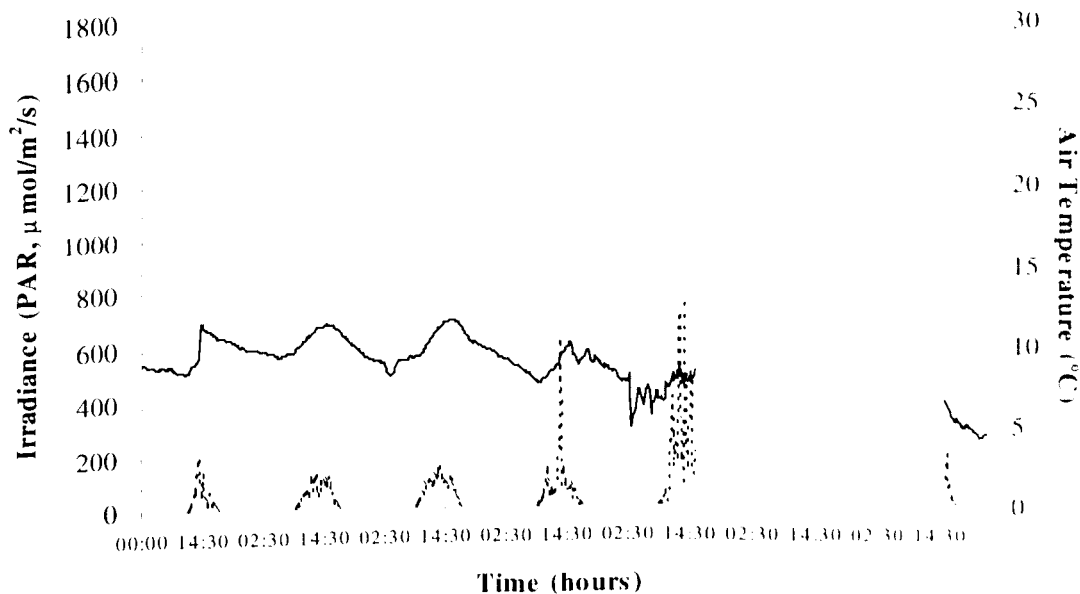
Day 7-13 (29/10/99-4/11/99)



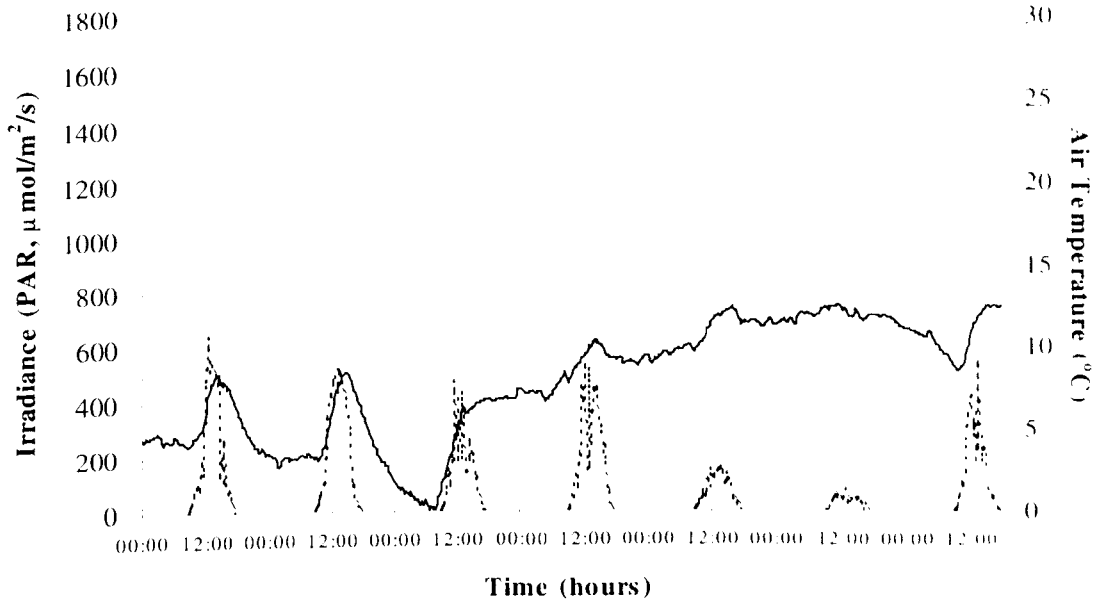
Day 14-20 (5/11/99-11/11/99)



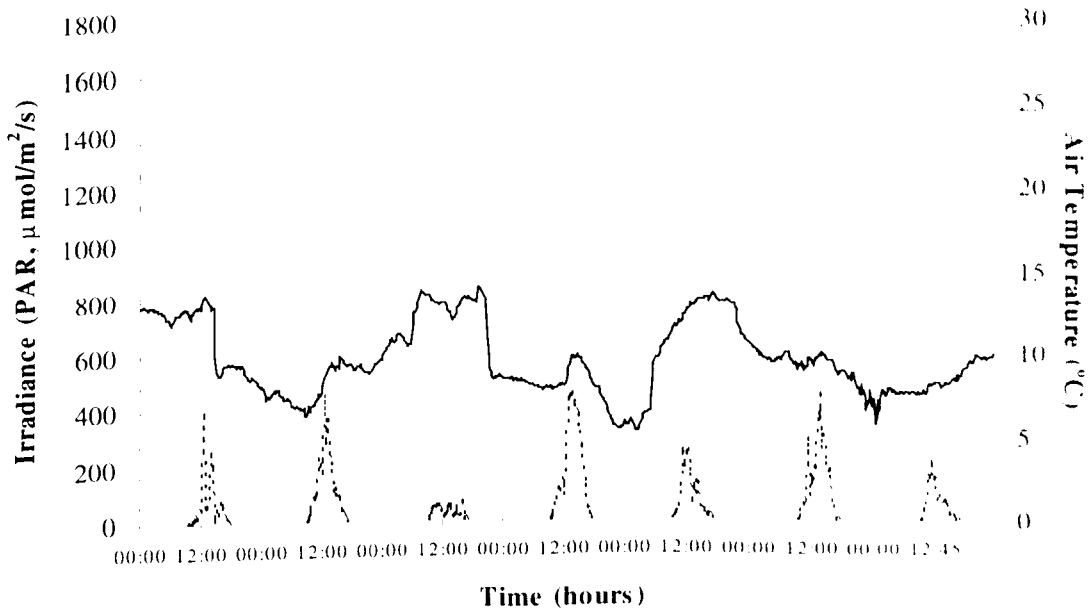
Day 21-27 (12/11/99-18/11/99)



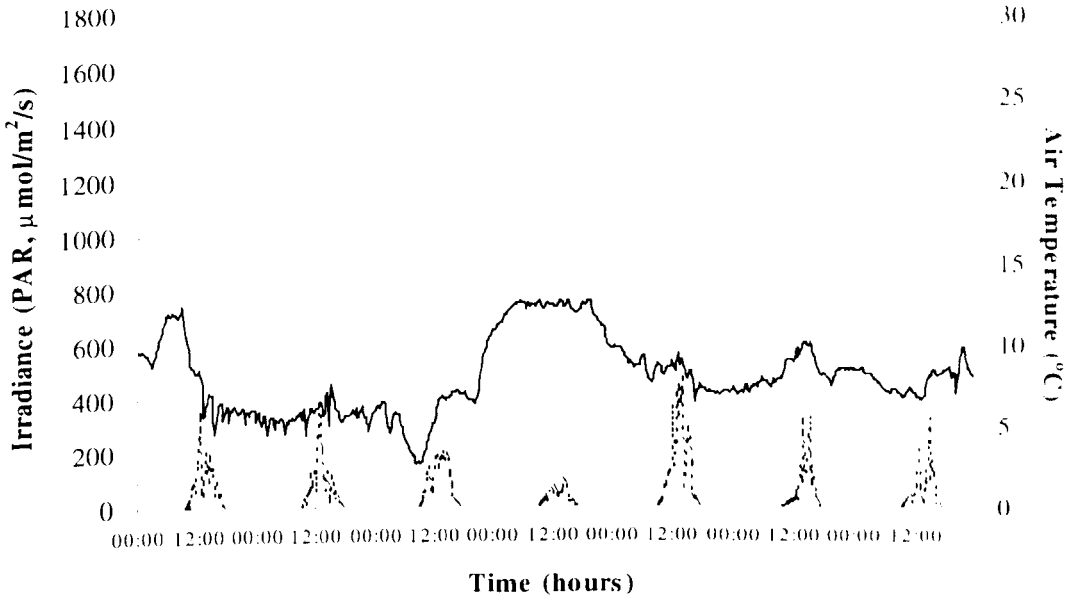
Day 28-34 (19/11/99-25/11/99)



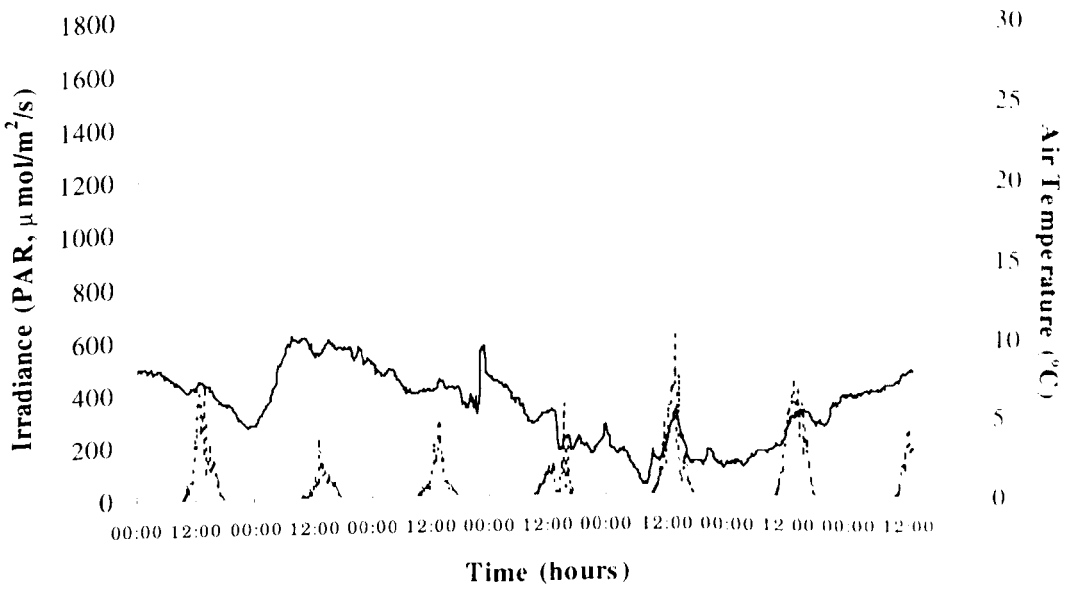
Day 35-41 (26/11/99-2/12/99)



Day 42-48 (3/12/99-9/12/99)



Day 49-55 (10/12/99-16/12/99)



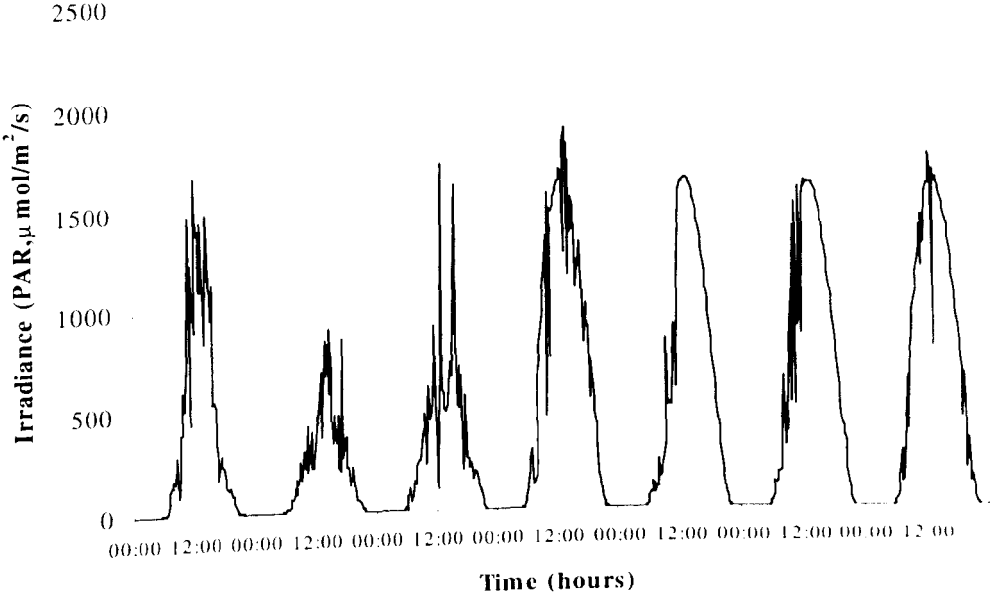
Appendix 2c. Climatic conditions

12th July until 29th August 2000.

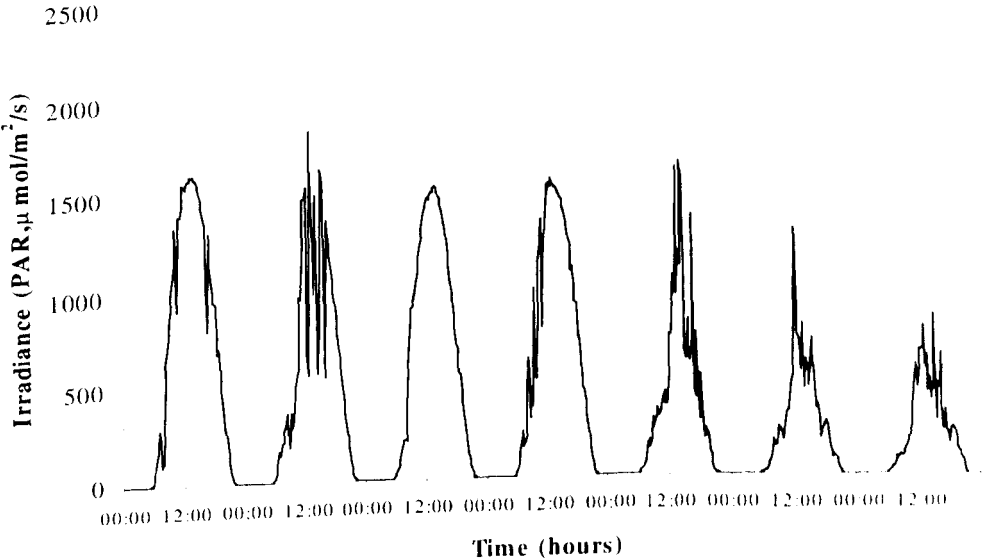
The graphs below show the irradiance (PAR, $\mu\text{mol}/\text{m}^2/\text{s}$), air temperature and culture temperature ($^{\circ}\text{C}$) measured the single-phase continuous culture of *H. pluvialis*. Measurements were taken every 15 minutes 24hours a day for the duration of the cultures.

Irradiance (Photosynthetically active radiation, PAR, $\mu\text{mol}/\text{m}^2/\text{s}$)

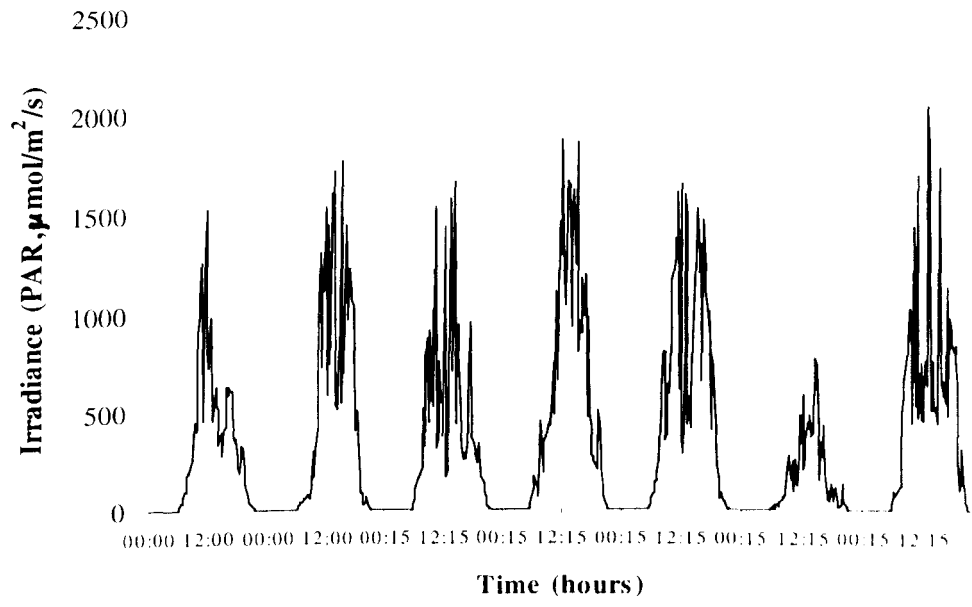
Day 0-6 (12/7/00-18/7/00)



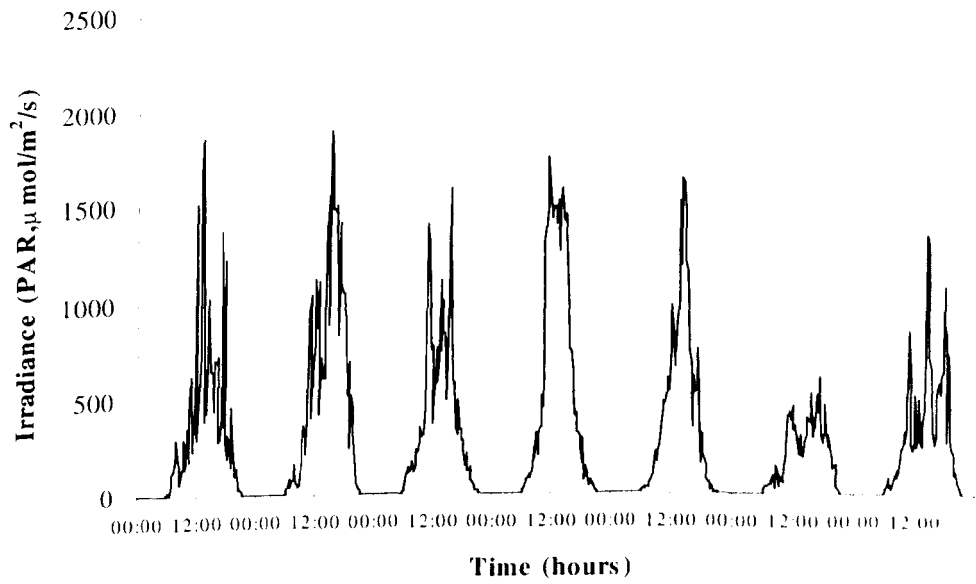
Day 7-13 (19/7/00-25/7/00)



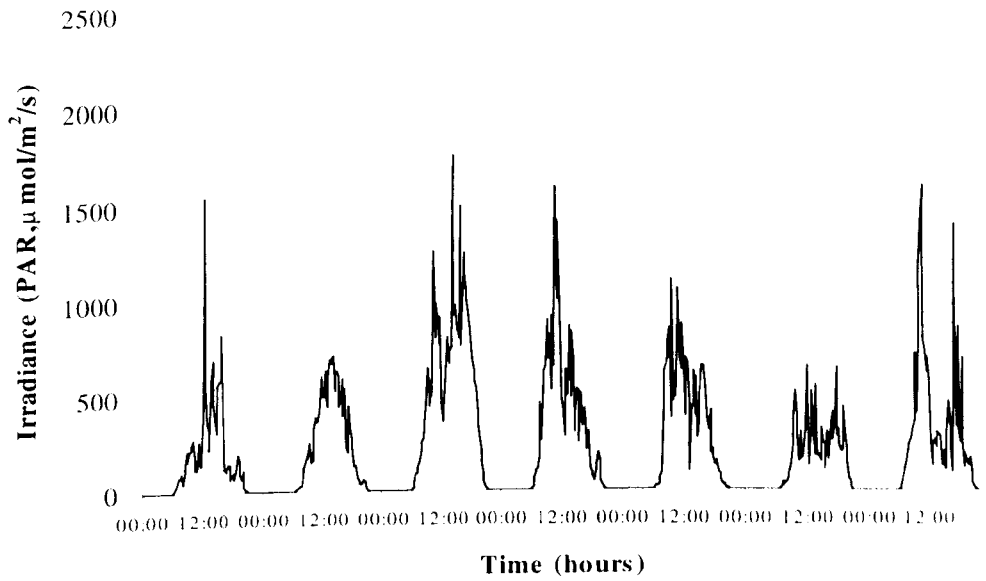
Day 14-20 (26/7/00-1/8/00)



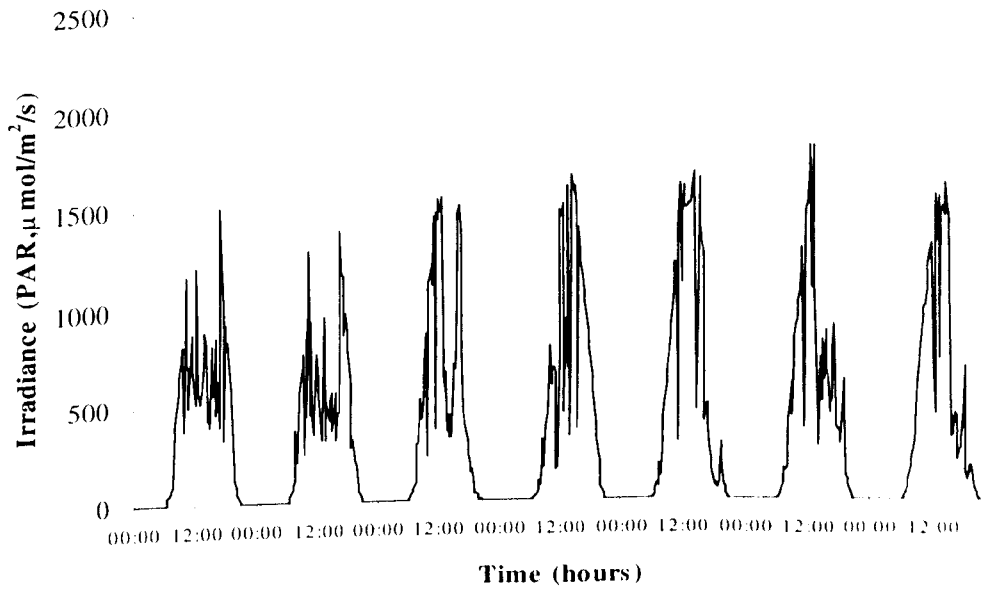
Day 21-27 (2/8/00-8/8/00)



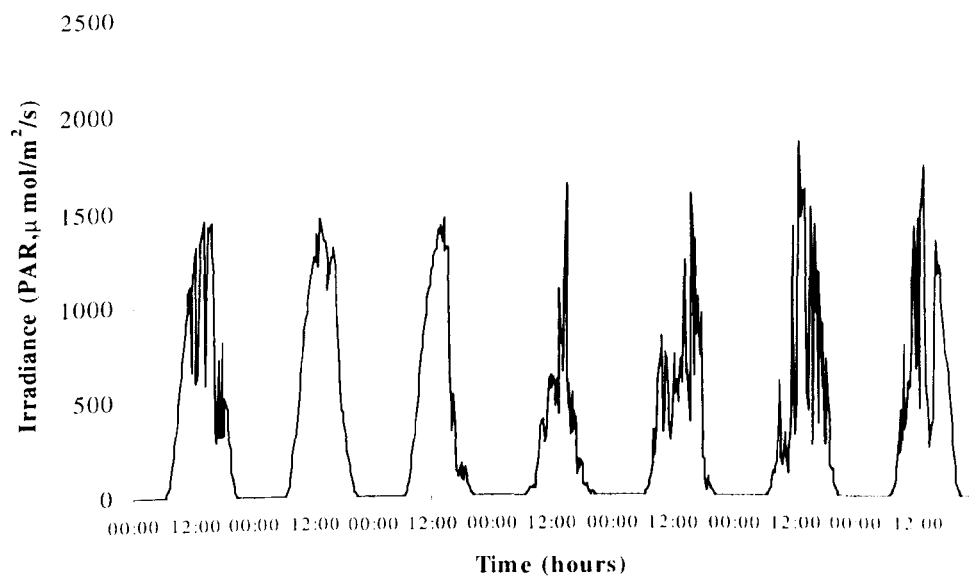
Day 28-34 (9/8/00-15/8/00)



Day 35-41 (16/8/00-22/8/00)



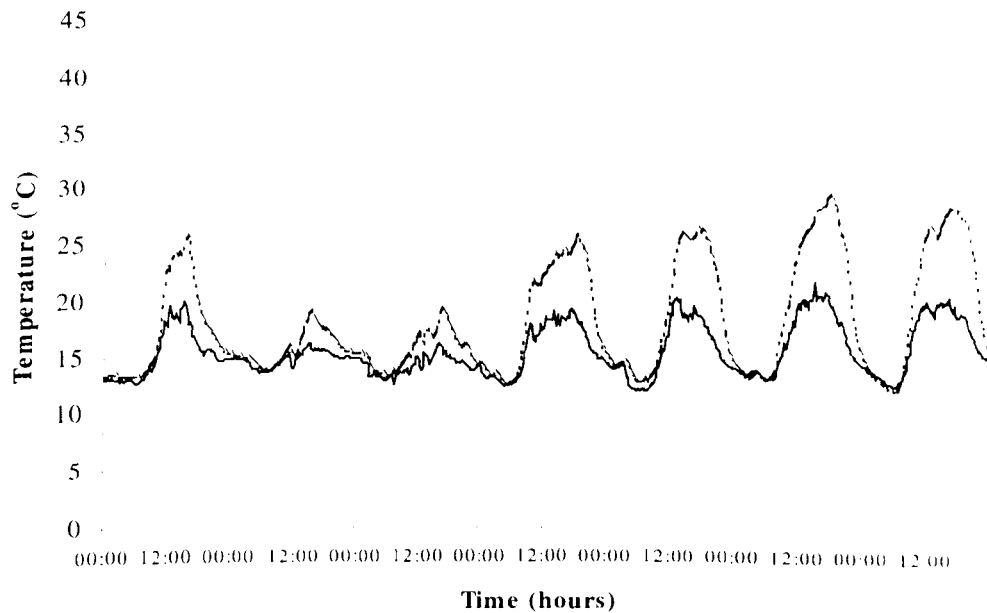
Day 42-48 (23/8/00-29/8/00)



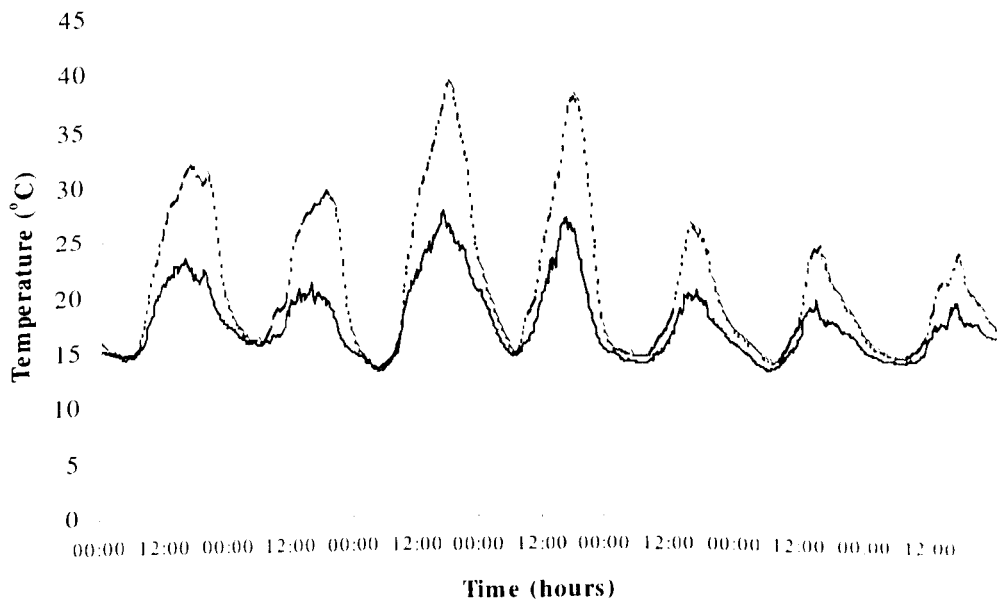
Temperature. ----- Culture temperature (°C)

———— Air temperature (°C)

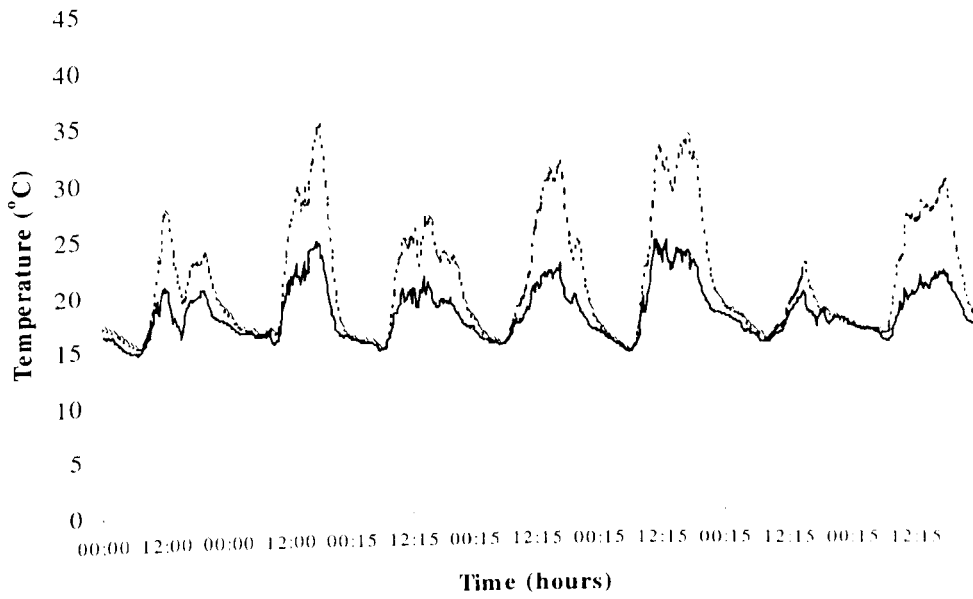
Day 0-6 (12/7/00-18/7/00)



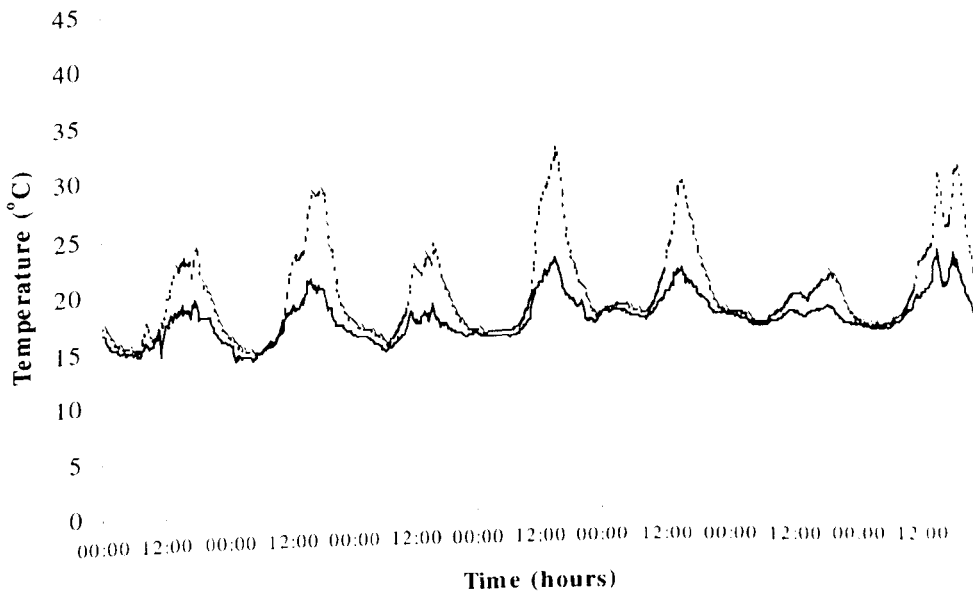
Day 7-13 (19/7/00-25/7/00)



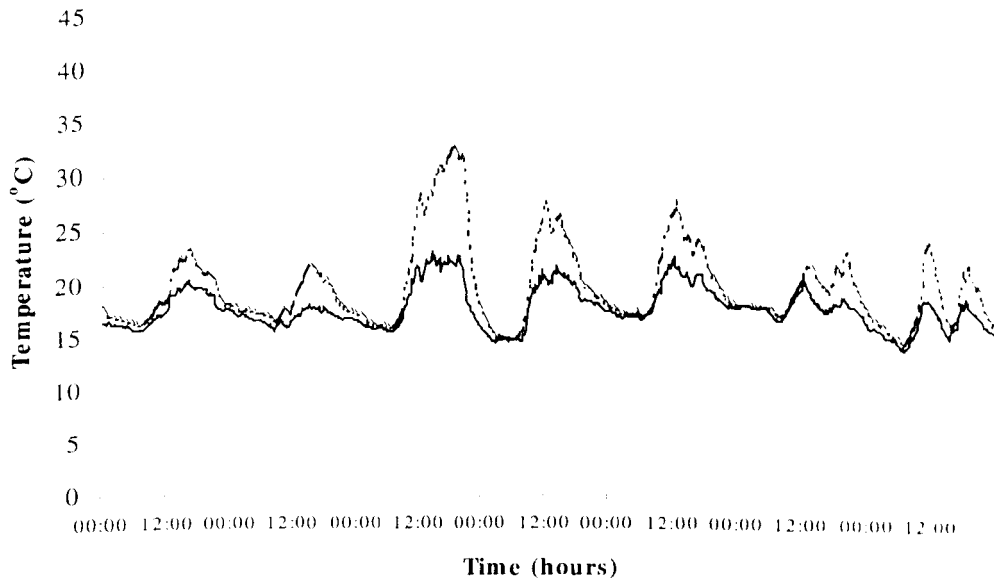
Day 14-20 (26/7/00-1/8/00)



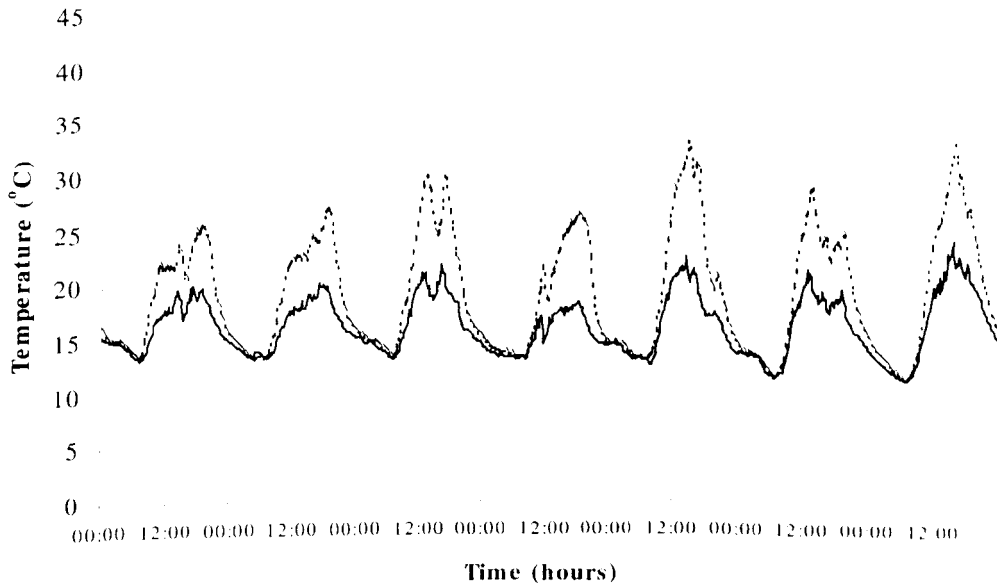
Day 21-27 (2/8/00-8/8/00)



Day 28-34 (9/8/00-15/8/00)



Day 35-41 (16/8/00-22/8/00)



Day 42-48 (23/8/00-29/8/00)

