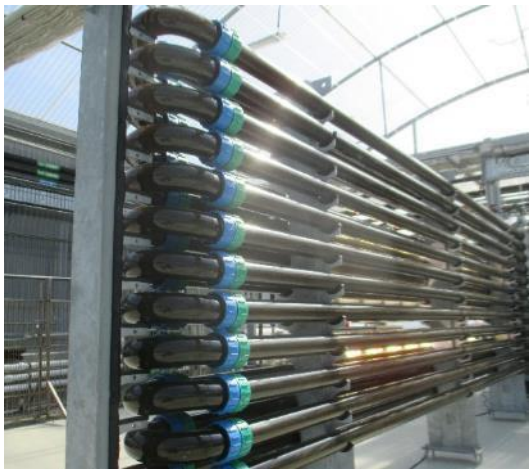
IPS Instituto Politécnico de Setúbal
Escola Superior de Tecnologia do Barreiro

INÊS ALVES COIMBRA

SUPPORT IN MICROALGAE PILOT SCALE PRODUCTION PLANT



Internship report of the Master's in Chemical and Biological Engineering

ADVISOR

Professor, PhD Ana Gabriela Gomes

CO-ADVISOR

Professor, PhD Ana Cláudia Coelho

SUPERVISOR

MSc Sara Pereira

November 2019

INÊS ALVES COIMBRA

SUPPORT IN MICROALGAE PILOT SCALE PRODUCTION PLANT

JÚRI

Presidente: Professora Doutora Maria de Lurdes de Figueiredo Gameiro, ESTBarreiro/IPS
Orientadora: Professora Doutora Ana Gabriela Gonçalves Neves Gomes, ESTBarreiro/IPS
Coorientadora: Professora Doutora Ana Cláudia Cavaco de Sousa Coelho, ESTBarreiro/IPS
Supervisora: Mestre Sara Patrícia Meira Fernandes Alves Pereira, A4F
Vogal: Professora Doutora Maria Manuela Regalo da Fonseca, IST, Universidade de Lisboa

November 2019

ACKNOWLEDGMENTS

I would first like to thank my advisors Dr. Gabriela Gomes and Dr. Ana Cláudia Coelho for all the commitment, encouragement and assistance shown along the elaboration of this thesis, for the confidence placed on my capabilities and for the strength given throughout the writing.

To A4F, for the opportunity to carry out my master's internship in order to develop my thesis, as well as the confidence given to better develop my skills. I would like to specially thank my supervisor at A4F, MSc. Sara Pereira for all the support and knowledge given through the internship and all the help on writing my report. I also would like to thank all my colleagues from the company for their collaboration and for providing a wonderful working environment, specially my everyday colleagues Inês Carvalho and Pedro Pereira for all the patience and friendship during and after the internship.

In addition, I would like to thank my parents for all the support and wise counsel, for giving me the tools to chase my dreams. Also, to my boyfriend and my friends that are always on my side and provided me guidance and focus on achieving my goals.

To all of you, a thank you for your understanding, patience and support. Without you finishing this thesis would have been harder and less happy.

ABSTRACT

The present work describes the activities developed at A4f (*Algae for Future*) during the Master's in Biological and Chemical Engineering curricular internship, as well as a brief proposal of the sterilization methodologies that can be used to avoid contaminations. The A4f company is dedicated to research and production of microalgae and develops the innovation research in a pilot scale production unit, where the internship took place. Microalgae production is achieved in photobioreactors that provide optimal growth conditions for these organisms. There are some systems that are indirectly linked to production, which are essential to this operation, such as thermoregulation, carbonation, aeration and effluent treatment systems. The production of microalgae has four main stages: inoculation, production, harvesting and processing where microalgae biomass is obtained (final product in the form of paste or powder). This biomass can be used for food, pharmaceutical and biofuel applications and is considered a product with numerous high value compounds. The internship lasted five months and aimed to learn and apply different methods in all procedures associated with the production of microalgae at the pilot scale. This project also allowed to research and propose alternative sterilization techniques for future implementation in the microalgae production unit. These proposals aim to achieve the microbiological safety levels required by food and pharmaceutical industry. The sterilization techniques suggested were: the use of an ozone system; the implementation of a continuous sterilizer for sterilization of culture medium, the use of absolute filters and the design of non-absolute filters for air sterilization.

All internship was a rewarding and interesting experience as it allowed the contact with innovation and product development in business environment as well as the daily work in a biological engineering industry.

KEYWORDS: Microalgae, Production, Photobioreactors, Sterilization, Optimization

RESUMO

Este relatório descreve as atividades desenvolvidas durante o estágio curricular integrado no Mestrado em Engenharia Biológica e Química, e apresenta uma breve proposta para a otimização dos métodos de esterilização utilizados numa unidade piloto da *Algae for Future* (A4F). A A4F dedica-se à investigação e produção de microalgas, contando com uma unidade de produção à escala piloto onde foi desenvolvido o estágio. A produção de microalgas é realizada em fotobiorreatores que fornecem condições ótimas de crescimento a estes organismos. Existem alguns sistemas que estão indiretamente ligados à produção, mas que são imprescindíveis ao seu bom funcionamento, como os sistemas de termorregulação, carbonatação, arejamento e tratamento de efluentes. A produção de microalgas conta com quatro etapas principais: inoculação, produção, colheita e processamento onde se obtém a biomassa das microalgas (produto final em forma de pasta ou pó). Esta biomassa é utilizada para inúmeras aplicações na área alimentar, farmacêutica e biocombustíveis, sendo considerada um produto com inúmeros compostos de valor acrescentado.

O estágio teve a duração de cinco meses e visou a aprendizagem e aplicação de diferentes metodologias em todos os processos associados à produção de microalgas à escala piloto. Este projeto permitiu ainda pesquisar e propor a otimização dos métodos de esterilização para futura implementação na unidade de produção de microalgas. Estas propostas focaram-se na possível implementação da biomassa das microalgas, na indústria alimentar e farmacêutica, sendo os métodos sugeridos a utilização de um sistema de ozono, a implementação de um esterilizador continuo para a esterilização de meio de cultura, a utilização de filtros absolutos e o dimensionamento de filtros não absolutos, para a esterilização do ar.

Todo o trabalho desenvolvido no estágio foi uma experiência muito enriquecedora e estimulante uma vez que permitiu o contacto com o meio empresarial, bem como com o dia a dia numa indústria de engenharia biológica.

PALAVRAS-CHAVE: Microalgas, Produção, Fotobiorreator, Esterilização, Otimização

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF EQUATIONS

LIST OF ABBREVIATIONS

A4F	Algae for future
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
CRW	Cascade Raceway
DBOT	Design, Build, Operation and Transfer
DHA	Docosahexaenoic acid
DO	Dissolved oxygen
DW	Dry weight
EPA	Eicosapentaenoic acid
FP	Flat-panel
G3P	Glycerate-3-phosphate
GM	Genetically modified
GMO	Genetically modified organism
GP	Glass-panel
HCl	Hydrochloric acid
LDPE	Low-density polyethylene
MHT-PBR	Multilayer Horizontal Tubular Photobioreactor
$NADPH_2$	Nicotinamide adenine dinucleotide hydrogen phosphate
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
NTU	Nephelometric Turbidity Unit
O_2	Oxygen
O_3	Ozone

PBR	Photobioreactor
PUFA	Polyunsaturated fatty acid
R&D	Research & Development
SD	Spray-dryer
TPBR	Tubular photobioreactor
UEL	Lisbon Experimental Unit
UHT-PBR	Unilayer Horizontal Tubular Photobioreactor

1. A4F – *ALGAE FOR FUTURE* AND INTERNSHIP AIMS

1.1. THE COMPANY

A4F – *Algae for future*, is a biotechnology-based company, located in Lisbon, Portugal, which focuses in microalgae production and Research & Development (R&D). A4F is specialized in the design, build, operation and transfer (DBOT) of commercial-scale microalgae production units, applying its accumulated knowledge to produce technologies that can be adapted to the costumers needs. This can be achieved by making a transposition from a pilot scale (using a pilot scale plant supported by a laboratory) to the commercial scale. The company develops operating protocols for optimized microalgae production, according to production goals and with the industry best practices. The main objective is to research and develop bioengineering projects for industrial production of microalgae, microalgae-based products and their applications. A4F provides consultancy services in all stages of microalgae industrial projects implementation. Several projects have been, or are currently being, carried out by the company, including large industrial projects (ALGAFARM, BIOFAT, ALGATEC) and R&D projects. The company's logo is presented in figure 1.

Figure 1 – A4F – Algae for future logo (1).

The Laboratory dedicates most of its work in services of client support and R&D activities at pilot scale and industrial production. Some of the activities carried out at the Laboratory are culture cultivation and scale-up, culture collection maintenance (of over 150 strains), analytical biochemistry and molecular biology procedures. At UEL (Experimental Unit of Lisbon) the main objective is also client support services and R&D projects at a pilot scale production, where all stages are followed, from the inoculum to the final product. UEL counts with a technological platform that replicates an industrial plant for microalgae production. Some of the working areas in UEL are culture scale-up to pilot scale production; microalgae

production in open and closed systems and harvesting, dewatering and processing technologies. Both units are also genetically modified organisms (GMO) compliant.

A4F provides consulting services in all stages of implementation of industrial microalgae projects. The sequence of activities and work performed in A4F is represented in figure 2.

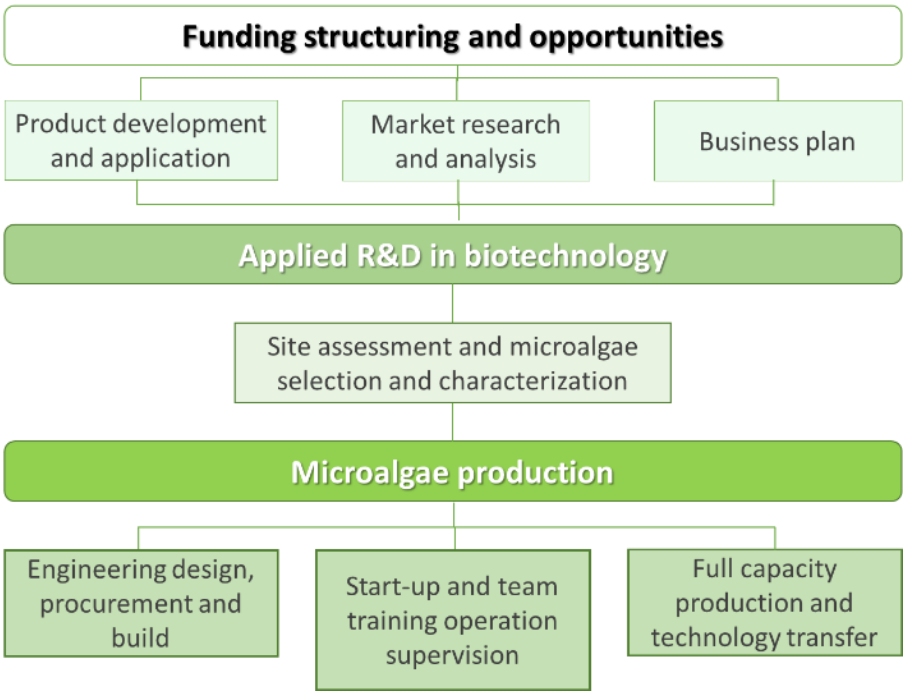


Figure 2 - Sequence of activities and work performed in A4F (adapted from (2)).

1.2. INTERNSHIP AIMS AND ACTIVITIES UNDERTAKEN

The master's internship was held in A4F's facilities in Lisbon, mostly in the experimental unit (UEL). In UEL all the activities performed were related to microalgae production, aiming to provide the intern with greater knowledge in biological engineering, more specifically in biological reactors and microalgae. This internship report will be based on the knowledge acquired throughout the activities developed and will stand for a Master Thesis in order to conclude a Master´s degree in Biological and Chemical Engineering, lectured at Escola Superior de Tecnologia do Barreiro (Instituto Politécnico de Setúbal). During the internship, several activities were performed, such as: assembly, preparation of microalgae culture and cleaning of bioreactors; control of the growth parameters in microalgae cultures (pH, temperature, volume, dissolved oxygen, turbidity, etc.). During the microalgae production, harvest and processing of the biomass was performed, as well as the development and

reviewing of relevant documentation for the pilot Unit operation (needed to guarantee that protocols are updated).

1.2.1. OBJECTIVES

The main objective of the present master curricular internship was to acquire skills and work methodologies in order to perform all the required activities of UEL, as well as the development of transversal competences in a business environment.

In table 1 are presented the specific objectives initially proposed by the company, as well as additional objectives that emerged during the internship for the development of this thesis.

Table 1 – A4f proposed Internship objectives

Initially proposed objectives
· Develop skills and work methodologies necessary for monitoring the activities performed in a microalgae pilot scale production plant
· Development of transversal competences in a business context
Other objectives
· Evaluate and propose new disinfection methodologies
· Verify the accuracy of the o-tolidine test results

1.2.2. ACTIVITIES DEVELOPED

With the objective to better understand the work developed at UEL several activities described in table 2 were performed.

Table 2 - Activities performed at UEL during the Master´s internship

Activities Developed
1. Monitoring and verification of microalgae cultures (pH, temperature, volume, dissolved oxygen, turbidity, etc.) (3x day)
2. General unit verifications (Verify the correct function of all unit equipment)
3. Material replacement, cleaning, storage, and equipment washing
4. Complete daily report sheets of the tasks developed
5. Disinfection of culture media, effluent tanks and water systems

6. Biomass processing: Harvesting and pre-concentration units
7. Biomass drying (Spray drying)
8. Preparation of process water (culture media)
9. Renewal of microalgae cultures
10. Inoculation of microalgae cultures in the bioreactors
11. Installation of culture systems (assembly of photobioreactors)
12. Review and improvement of operational procedures
13. Cascade Raceway (CRW) salinity control
14. Improvement of Safety Data Sheets
15. Quantitative biomass analysis – dry weight measurements
16. Equipment Calibration (pH probes; refractometer)
17. Research on disinfection methods to improve the present procedures used at A4f.

A chronogram was developed to schedule the activities and identify the logical order, duration and interdependence of the project tasks, which is represented in figure 3.

	Week																					
Activities	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1																						
2																						
3																						
4																						
5																						
6																						
7																						
8																						
9																						
10																						
11																						
12																						
13																						
14																						
15																						
16																						
17																						

Figure 3 - Internship chronogram, describing the activities developed at UEL and the time spent on each.

2. INTRODUCTION

2.1 MICROALGAE

Microalgae are microscopic organisms that can grow in salt or fresh water. These organisms have a photosynthetic mechanism like plants, but due to a simple cellular structure these organisms can convert solar energy in biomass more efficiently (3). Having one of the most efficient photosynthetic systems of converting solar energy into organic compounds, many of these species grow faster than terrestrial plants thus achieving higher yields and greater biomass productivity (4). They can be classified into divisions based on properties such as pigmentation, chemical nature of photosynthetic storage products, the organization of photosynthetic membranes and other morphological features. In total, eleven divisions exist, in which prokaryotic members are grouped into two divisions and eukaryotic members in nine divisions. The existing divisions are resumed in table 3 (3,5).

Table 3 - Classification of microalgae into groups of prokaryotes and eukaryotes (adapted from (5)).

Kingdom	***Division***
Prokaryota eubacteria	Cyanophyta
	Prochlorophyta
Eukaryota	Glaucophyta
	Rhodophyta
	Heterokontophyta
	Haptophyta
	Cryptophyta
	Dinophyta
	Euglenophyta
	Chlorarachniophyta
	Chlorophyta

Microalgae are mainly solitary cells, unicells with or without flagella, thus motile or non-motile. Also, others can exist as aggregates of several single cells held together loosely or in an organized way, as colonies (5).

These organisms can have phototrophic, heterotrophic and mixotrophic growth, as explained in table 4, which makes them adaptable to a wide range of environmental conditions.

Table 4 – Growth conditions of microalgae cultivation (adapted from (5,6))

Phototrophic	**Heterotrophic**	**Mixotrophic**
Most common growth condition where light, carbon dioxide (CO_2), nutrients and water are supplied. No need for external organic carbon source	Use of external organic carbon source for growth, present in the culture media. Leads to higher biomass productivity. Higher risk of contamination.	Light and external carbon source are supplied. Organisms can use organic and inorganic carbon sources simultaneously.

Because of their biodiversity, microalgae can produce a vast variety of high-value compounds such as carotenoids (β-carotene and astaxanthin), phycocyanin, polysaccharides, fatty acids, lipids, immune modulators and others, derived from the microalgae biomass (7,8). These compounds can have several applications for example in human nutrition, medical applications (pharmaceuticals), cosmetics, agrochemical industry (9), waste water treatment and biofuel production (10).

During the internship at A4F, four different types of microalgae were used, which will be described in the following sections of the introduction.

2.1.1 *DUNALIELLA* SP.

From the Chlorophyta division, *Dunaliella* sp. microalgae are biflagellate and pear-shaped cells (size varying from 5 to 25 µm in length and from 3 to 13 µm in width), as one can depict from a microscopic view of these cells represented in figure 4 (11–13). This microalgae lacks a rigid cell wall and has a type of glycocalyx (pericellular matrix) that involves the cell, becoming a sensitive microalgae to mechanical stress (14,15). These microalgae are halophilic species which can be produced in a range of 90-120 g/L of salt in water (1). *Dunaliella* sp. produces carotenoids of great significance, such as α-carotene, β-carotene, violaxanthin, neoxanthin, lutein and zeaxanthin. Microalgae of the genus *Dunaliella* sp. are considered the best natural source of commercial β-carotene. The shape of *Dunaliella* sp. varies according to salinity, due to the absence of cell wall. Its survival to hyper osmic shocks is the result of an osmoregulation mechanism, where glycerol is produced (about 50% (m/v) when produced at high salinity). Under hypersaline conditions *Dunaliella* sp. targets the osmoregulation mechanism and increases the photosynthetic production of glycerol, of which intracellular concentration varies in direct proportion to the extracellular salt concentration. This product also has great potential for industrial applications. In situations of hypo osmotic shock, the glycerol present in the cell is eliminated. The amount of intracellular glycerol and carotenoids is proportional to the salt concentration in the extracellular medium (13).

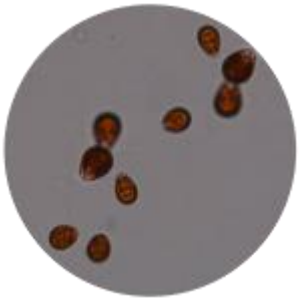


Figure 4 – Microscopic view of *Dunaliella* sp. cells (1).

The accumulation capacity of β-carotenes in *Dunaliella* sp. is induced by several stress factors in the cultivation, such as high light intensity, high saline concentration (> 27% (m/v) NaCl), extreme temperature (range of 5º to 40ºC) or cultivation with nitrate deficiency (16). In microalgae, carotenoids act as accessory pigments, being responsible for the absorption of energy in the blue region of the spectrum, transferring it to chlorophyll *a* (primary photosynthesize pigment) and play a major role in the protection of plants against photooxidative processes. Carotenoids have an antioxidant function and are produced in

response to stressful conditions where they act as photo-protectors, preventing the formation of oxygen reactive species (13,15,17). The main problem of cultivation of microalgae under stress conditions is that under these conditions, the number of cells per unit of culture volume is reduced, since these factors affect cell viability (17). In favorable conditions of growth (natural habitat) *Dunaliella* sp. presents a green color, but under stress conditions cells become orange due to the large quantity of β-carotene produced (1,12,13). The β-carotene is considered the most relevant carotenoid in the market and some species of *Dunaliella* sp. can produce up to 21 mg/g of β-carotene (in carrot it is only 0.058 mg/g) making the potential of microalgae in the production of this type of antioxidant on an industrial scale very appealing (13,18).

These microalgae have a great potential for industrial applications of β-carotene and glycerol production, described in table 5.

Table 5 – Products obtained by *Dunaliella* sp. production, respective application and the project involved (based on (1,17))

Products of Interest	Applications	A4F Project
・β-carotene ・Glycerol (by-product)	・Food supplement; Food dye; Animal feed supplement ・Biofuels; Chemical industry ・Cosmetics; Human health	*ABACUS* (19)

2.1.2 *TISOCHRYSIS SP.*

Tisochrysis sp. belongs to the Haptophyta division. Cells are round to oblong (3-7.5 µm diameter) and can be biflagellate with flagella of equal length and a short, scaly haptonema possessing a central swelling (20,21). In figure 5 a microscopic view of *Tisochrysis* sp. cells is represented.

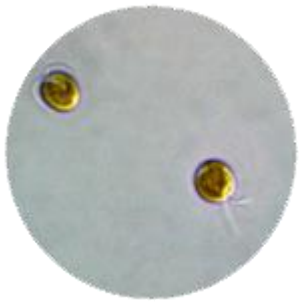


Figure 5 - Microscopic view of *Tisochrysis* sp. cells (1).

This species grows in marine water (salinity: 30 – 35 g/L) and its cultivation products for industrial applications are represented in table 6 (1). These cells are commercially attractive since they grow fast and storage lipids with high content of long-chain polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) , with great applications in nutrition (22,23). The contents of protein, lipid and carbohydrates are not significantly affected by growth conditions, but cell production is significantly increased under mixotrophic conditions. In these conditions and by adding glycerol to the culture media, the content of chlorophylls and carotenoids significantly increased, as well as biomass production (24).

Table 6 - Products obtained by *Tisochrysis* sp. production, respective application and the project involved (based on (12))

Products of Interest	Applications	A4F Project
・Whole Cell (Biomass) ・Eicosapentaenoic acid (EPA); Fucoxanthin (by-product)	・Aquaculture ・Human and Animal feed supplementation	*ABACUS* (19)

2.1.3. *NANNOCHLOROPSIS* SP.

Nannochloropsis sp. species are unicellular, planktonic, subspherical or cylindrical cells, belonging to Heterokontophyta division. The microscopic view of this microalgae is presented in figure 6 (25). They have an extremely strong cell wall, which makes them very resistant to a wide range of conditions with size varying from 2 to 5 µm in diameter (14,21,26).

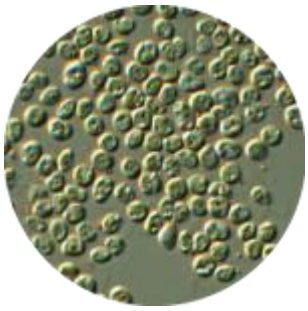


Figure 6 - Microscopic view of *Nannochloropsis* sp. cells (12).

This specie is cultivated in marine water (salinity: 20 - 40 g/L) (1) and has been industrially produced for several applications, resumed in table 7 below.

These microalgae have great commercial applications, mainly because they have a high fatty acid content, especially in the form of triglycerides. Also, the fatty acids of *Nannochloropsis* sp. contain a large proportion of polyunsaturated fatty acids (PUFAs), including the essential eicosapentaenoic acid (EPA) (27). The main application of this species is as aquaculture feed, although it is also considered for the production of EPA and biofuel (28–30). The production of lipid contents in *Nannochloropsis* sp. significantly increases as nitrogen supplementation decreases (31).

Table 7 - Products obtained by *Nannochloropsis* sp. production, respective application and the project involved (based on A4F, 2019)

Products of Interest	Applications	A4F Project
・Whole cell (Biomass) ・Eicosapentaenoic acid (EPA) (by-product)	・Aquaculture ・Food supplement	*ABACUS* (19)

2.1.4. *SYNECHOCYSTIS* SP.

Synechocystis sp. is a unicellular coccoid cyanobacteria (5) and belongs to the Cyanophyta division. The microscopic view of this organism is represented in figure 7.

Figure 7 - Microscopic view of *Synechocystis* sp. (GM) cells (1).

Several cyanobacterial species are very suitable for genetic modification. Important reasons for this to happen are that several cyanobacteria, including *Synechocystis* sp., are spontaneously transformable and can integrate efficiently foreign DNA into their genome by double homologous recombination. *Synechocystis* sp. has a wide variety of parallel physiological pathways, which can enable the organism to survive and thrive in a large spectrum of physiological conditions (34). Developments in synthetic biology have provided plenty of molecular biology tools to engineer *Synechocystis* sp. as a photosynthetic host for the production of diverse types of chemicals (35). At A4F two types of *Synechocystis* sp. are cultivated, wild types and genetically modified (GM) strains of this species, although during the internship only GM species were observed. Both GM and wild type grow in fresh water environments (1).

These microalgae have been studied for several industrial applications, summarized in table 8.

Table 8 - Products obtained by *Synechocystis* sp.(GM) production, respective application and the project involved (based on (1))

Products of Interest	Applications	A4F Project
· Phycocyanin · Other products from genetic modifications; Zeaxanthin (by-products)	· Food dye; Reagent (fluorescent tag) · Biofuels; Food dye	*PHOTOFUEL* (32)

2.2. MICROALGAE BIOMASS PRODUCTION AND PROCESSING

Microalgae are grown in different cultivation systems, in specific culture media, under illumination and CO_2 supply. The biomass is separated from the media, followed by biomass drying and disintegrated to be used in several applications mentioned before (*vide* section 2.1.). The general process is represented in figure 8. Depending on the application the final product can be obtained in each of these processes: biomass separation may produce paste, drying produces powder and from cell rupture is possible to obtain extracts and isolated compounds.

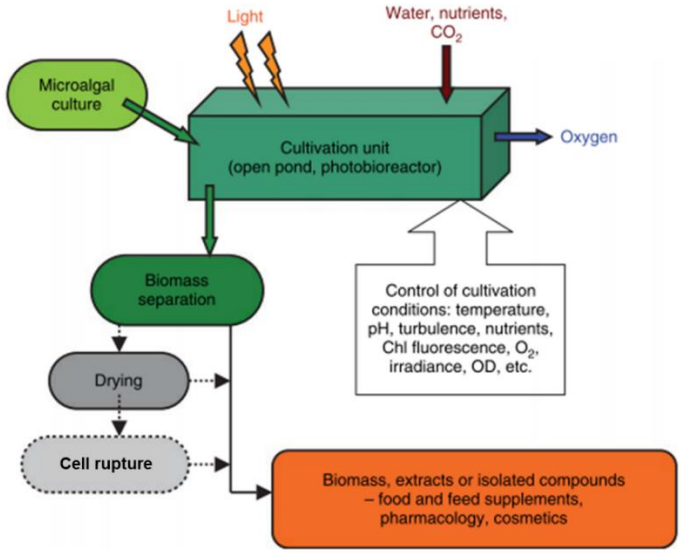


Figure 8 - Schematic diagram of microalgal biomass production and processing (adapted from (12).

Biomass production by microalgae is based on the photosynthesis mechanism, which requires carbon dioxide, water, nutrients (macro- and micronutrients) and light. Carbon dioxide is the natural carbon source for photosynthetic autotrophs. The photosynthetic process is divided into two main stages, the light reactions and dark reactions. The light reactions include the production of 2 moles of nicotinamide adenine dinucleotide hydrogen phosphate ($NADPH_2$), 3 adenosine triphosphate (ATP) and O_2. The dark reactions, represent the reduction of one mole of carbon dioxide and the synthesis of carbohydrates using the $NADPH_2$ and ATP produced in the light reactions (34). This process is represented in figure 9. Photosynthesis converts CO_2 into glycerate-3-phosphate (G3P) that is a starting material of storage compounds including lipids and carbohydrates (36).

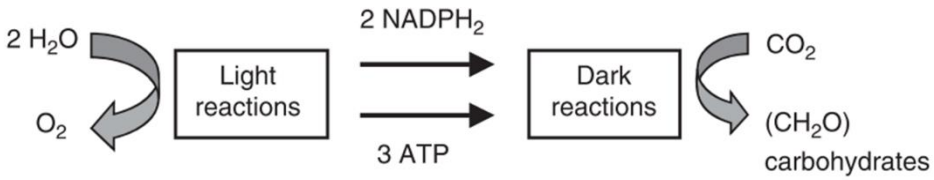


Figure 9 - Products of photosynthesis (adapted from (34)).

The growth of microalgae is affected and influenced by the conditions within the culture's environment, such as light intensity, pH, temperature, salinity, nutrients, CO_2 and others. Variations in these factors can cause immediate changes in growth and composition of microalgae, either increasing or decreasing their nutritional value, which may ultimately result in the production of non-desired products.

2.2.1. VISIBLE LIGHT

Light is one of the most important factors for microalgal growth, since it is required for microalgae photosynthesis. In this regard intensity, spectral quality, and photoperiod need to be considered (5,12,37). The quantity of photon energy received by each cell is a combination of several factors: photon flux density, cell density, length of optical path (thickness of culture layer), and rate of homogenization (12). The light received by microalgae can come from natural source as normal sunlight or artificial light source emitting either in the blue and/or the red-light spectrum, as these are the most active portions of the light spectrum for photosynthesis. Light intensity plays an important role, but the requirements greatly vary with the culture depth and the density of the algal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture. Low light intensity leads to poor growth by decreasing the photosynthetic rate whereas high light intensity can retard cell growth through inhibiting the photosynthesis process and may also burn the cells (5,6).

2.2.2. TEMPERATURE

Temperature is one of the most important parameters to measure and control in a microalgae culture. The growth of microalgae cells is temperature dependent and they typically tolerate a temperature range of 16 – 27°C (for most microalgae used in the industry) although this may vary with the composition of the culture medium, the species, and strain cultured. It is desirable to use external heat/cooling sources (heat exchangers) in combination with automated temperature control during the different year seasons to maintain optimal temperatures inside the reactors, therefore maintaining the productivity constant. Generally,

temperatures lower than 16ºC will slow down growth, whereas temperatures higher than 35ºC are lethal for several species (5,37,38).

2.2.3. SALINITY

Salinity (salt concentration) is another important parameter that influences algal growth. Marine algae can tolerate changes in salinity and most species grow best at a slightly lower salinity than in their natural habitat. For most microalgae species salinities of 20 – 24 g/L are optimal, but there are microalgae that can grow in hypersaline media which reduces the risk of contamination due to the harsh conditions which decreases competition species to grow. Salinity, in open and closed systems, can affect the growth and composition of microalgae cells. All microalgae have their optimum salinity range that can increase during hot weather conditions due to evaporation. The easiest way for salinity control is by adding fresh water or salt as required (5,6,38).

2.2.4. NUTRIENTS

Microalgae need a supply of macronutrients containing carbon, nitrogen and phosphorus and other micronutrients such as magnesium, iron, and other mineral and vitamins, in order to sustain their growth and produce the desirable metabolites. Cell growth and lipid composition are significantly affected by the nutrient's availability. Under nutrients limitation conditions, the cell division rate declines, but active fatty acid biosynthesis is maintained in certain species of microalgae under sufficient light and CO_2 for photosynthesis Consequently, it is essential that any microalgae cultivation system provides these nutrient demands in order to ensure suitable levels of biomass and/or other metabolites productivity (6,12,37).

2.2.5. PH, CO_2 AND AERATION

pH is used as an indirect measurement of the dissolved CO_2 and its range, for most microalgae species, is between 7 and 9. Nevertheless, there are still some species that live in more acidic/basic environments. A failure in maintaining a suitable pH can cause a complete collapse in the culture due to the disruption of the cellular processes. In the case of high-density algal culture, the addition of CO_2 allows to correct an increase in pH, which may reach limiting values of up to pH 9 during microalgae growth. Since microalgal cultures grow in dense

suspensions, aeration is required as a mixing strategy to expose cells to light and to allow an efficient mass transfer of nutrients and metabolism products. Mixing is achieved by air and CO_2 bubbling from the bottom (normally deriving from the same pipeline) (5,12,37).

2.2.6. BIOMASS PRODUCTION

Two cultivation regimes can be considered for the growth of microalgal cultures; batch and continuous. In a batch regime, the culture is inoculated and at a certain point of growth/productivity the biomass is harvested. In a continuous regime, the culture is continuously harvested according to its growth rate and fresh media is added to replace nutrients. In practice, semi-continuous or semi-batch regimes are usually adopted, that is, where a part of the culture is harvested at regular intervals so that the cultures don't get too saturated or contaminated (12).

Microalgae can be grown in open or closed photobioreactors (open and closed systems, respectively). Open ponds are most commonly used for commercial applications of microalgae production, because they require low costs of production and produce high biomass content (39).

Regardless of commercial production of microalgae being done in open ponds, efforts to prevent contamination and control culture conditions are very challenging. On the other hand, closed photobioreactors have attracted more interest given their potential uses in growing microalgae under controlled conditions. Closed photobioreactors are used for growing algae mainly for production of high-value compounds (40). Table 9 presents examples of each of the microalgae production systems and the main advantages and disadvantages between them.

Table 9 – Main examples, advantages and disadvantages between the microalgae production systems (open and closed) (based on (10,12,39))

	Open Systems	**Closed Systems**
Examples	Natural or artificial ponds, raceways and cascades raceways	Tubular Photobioreactors, Flat-panel and Fermenters
Advantages	· Cheaper than closed systems; · Ideal for species that grow rapidly, or under very selective/harsh conditions; · Easy maintenance and operation; · Suitable gas exchange with the atmosphere.	· Easy optimization; · Control of cultivation conditions; · Reduced risk of contamination; · Thermoregulation is possible; · Low CO_2 losses; · Smaller area requirements; · High productivity.
Disadvantages	· Limited to small number of species; · Limited control of cultivation conditions; · High probability of contamination · Culture affected by weather conditions; · Water loss by evaporation; · Large area required · Light limitation transfer if thick layers of microalgae are used · CO2 losses	· More difficulties in cleaning · System must be effectively cooled and degassed since excessive oxygen produced by the growing cultures can reduce growth · Expensive to build and operate

2.3. DOWNSTREAM PROCESSING

2.3.1. HARVESTING AND CONCENTRATION

The most suitable harvesting method for processing biomass depends on the characteristics of the microalgae specie, dimensions of the organism, state of culture, possible contaminants in the culture, specifications of the final product and its market and operational costs.

Harvesting methods include chemical, mechanical, physical, and biological methods, most of which aim at flocculation and sedimentation of the microalgae (41). The harvesting is usually performed by centrifugation, filtration, coagulation, flocculation, flotation, and sedimentation. Filtration is one of the most used processes in which algae can be filtered by applying pressure to pass through a membrane. The easiest form of filtration is dead-end filtration (6,12,42). For small, suspended microalgae tangential flow filtration is considered to

be more viable (39). Nevertheless, the method performed during the internship at A4F was centrifugation, which will be described in this chapter.

The centrifugation process consists on centrifugal forces higher than the gravity force, that are used to separate material based on density differences, which makes the solids move through the liquid. This method is considered to be the most rapid and reliable method of recovering suspended algae, which is easy to operate but requires high investment and operating costs (39). Centrifugation can produce a solid concentration after harvesting in a rage of 12–22% (m/v), while the supernatant composed mainly by culture media and cellular remains is discharged (41).

The equipment available for centrifugation is divided into rotating wall devices (sedimenting centrifuges) and fixed wall devices. A sedimenting centrifuge consists in a bowl into which a microalgae suspension is fed and rotates at high speed. The liquid (culture media) is discharged, while solids remain in the bowl (batch processing) or are continuously or intermittently removed from it (43–45).

In this process it is also possible to eliminate some contaminations. The size of bacteria (and other contaminants) range between 0,2 to 1,5 µm and microalgae size range between 3 and 15 µm, being significantly bigger. This is important to consider when choosing the centrifuge rotation velocity (*g* force), because if the g force is too strong it will discharge the microalgae along with some possible contaminating bacteria, but if a lower velocity is considered it can discharge the lighter particles (contaminants) with the culture media and allow the heavier particles (microalgae) to sediment on the centrifuge to be collected, as pellets, keeping in mind that the velocity chosen can't be too low in a point that no organism (contaminant or microalgae) will sediment.

This process allows the concentration of the microalgae culture (biomass) into a paste form which can be used as a final product.

2.3.2. DRYING

Drying is usually the final step in microalgae processing to attain a moisture content of 5-15% (m/m). By drying, the biomass is transformed into a stable storable product. Spray drying involves liquid atomization, gas/droplet mixing and drying of liquid droplets. The atomized droplets are usually sprayed downwards into a vertical tower through which hot gases pass descending. Drying is a fast process which is completed within a few seconds.

The product is removed from the bottom, and the gas stream is exhausted through a cyclonic dust separator (42,45,46). This process is represented in figure 10.

Spray drying has several objectives such as to avoid or eliminate moisture that can lead to degradation and decrease of product stability, improve or maintain material properties, reduce transportation costs of large volumes of material (liquids), making the product easier to handle and to maintain (46,47).

Spray drying is used in A4F as a process to obtain biomass powder, one of the forms of final product of microalgae.

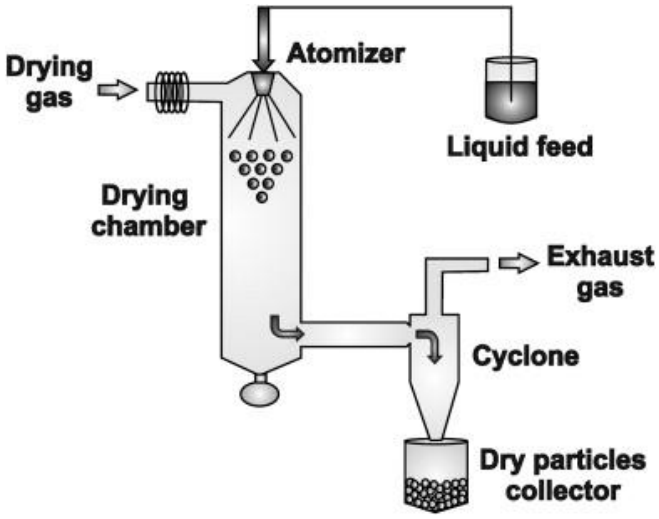


Figure 10 – Scheme of the components in a spray dryer equipment (47).

2.4. THE IMPORTANCE OF STERILIZATION AND DISINFECTION

Sterilization and disinfection methods and their validation are important in all stages of a biotechnological process. Disinfection is defined as a procedure that kills or reduces the number of pathogenic microorganisms in an environment or in a surface, which can't be mistaken with sterilization, which is the complete removal of all microorganisms and it will be better described later in this chapter (48). In microalgae production it is important to apply sterilization and/or disinfection methods in several steps of the process, such as represented in figure 11. To guarantee microbiological safety it is essential to sterilize/disinfect culture media, air and CO_2 streams, the photobioreactors itself and all the material that are released from them (gases) and into them (vessels, valves, pipes, probes, nutrients, antifoam) as well as all the transport lines. It is also relevant to ensure that the inoculum, to cultivate in the photobioreactors and all the downstream processes, are free of contaminants. In figure 11 are also represented several sampling points that should be used to validate or monitor the sterilization procedures. Microbiological tests (using appropriate culture media) and microscopic observations should be performed, to test sterilization and disinfection processes, indicating if the methods are being efficient or not (48–50).

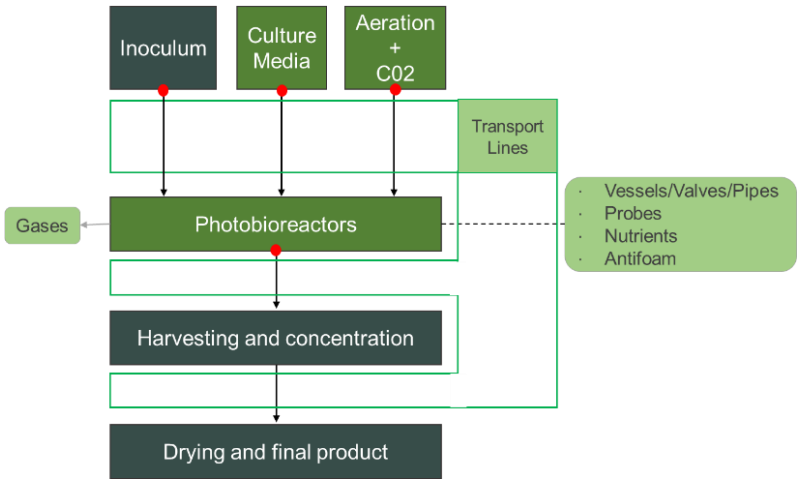


Figure 11 – Application of sterilization methods in microalgae cultivation process and validation points. In green are represented the areas where sterilization methods need to be applied, and in blue the points where it is important to maintain contaminant free. In red are represented the areas that should be controlled by taking samples and analyzed to ensure sterility or the absence of contaminants.

High-value compounds from microalgae are mainly obtained through production in closed systems, in order to control production conditions, cultivation variables and possible contaminations (39,51). Closed systems can operate continuously, requiring control of all

elements to prevent culture losses. In order to perform this kind of operation, the culture should be provided with sterilized elements like water, nutrients, air and carbon dioxide (CO_2) has to avoid contamination with competitor microorganisms (10). Regardless of being careful and using sterile techniques and closed systems operations it is possible that contaminations still occur, and in these situations it might be difficult to detect or identify the contamination source (48). To avoid this problem, it's important to constantly improve the sterilization methods applied and their control and validation.

2.4.1. CONTAMINATIONS

Contaminations can be defined as an entry of undesirable particles/ microorganisms in a controlled environment, which can compromise the desirable product production. Contaminations can be defined in several types such as: microbiological (bacteria, fungi and virus), particles (dust, crystals, powder, paper) and chemical (chemical substances) (52). Their source is from: the operators; the environment (air) or even from another cultures (present in the laboratory or in the pilot scale plant), usually from inappropriate cleaning procedures and reagents (28,27). Any reagent is also a potential carrier of contamination and when purchasing reagents or media for cell culture, it is important to determine whether they are sterile or will require sterilization (53). Also, some reagents cannot be sterilized by humid heat because of nutritional value loss, so other disinfection techniques must be performed. These might be ineffective and these reagents (such as vitamins) might become a contamination source.

All types of contaminations can cause significant changes and even loss of cell culture or product. Bacteria and fungi potentially increase the environmental contamination if not dealt with carefully. Fungi are especially difficult to eradicate since effective antifungal agents are often cytotoxic to cultures and their spores are likely to persist in cultures and in the environment (53).

A vast range of procedures can be adopted to prevent or eliminate and prevent these contaminations in order to avoid waste of resources and time.

Disinfection methods and the way that this methods are managed and operated, can have a very significant influence on the performance of disinfection (54) because each method as a specific application and different results, depending on contaminations and the subject to be disinfected, as will be describe in this chapter.

2.4.2. STERILIZATION

The removal or killing of all microorganisms is called sterilization and it consists in a process to establish aseptic conditions in the culture. It is not a difficult procedure, but precautions must be taken when working with sterilized material to avoid the risk of contamination. Sterilized material if not properly handled can easily become contaminated from the air. A cleaning routine and control methods (to verify sterility conditions) will help to control the levels of environmental contamination and thereby help to reduce the risks of day-to-day contamination (48,53).

Nowadays, there exist several ways to maintain a sterile environment when producing cell cultures, which can be applied to microalgae cultures. Some strategies include early harvesting of the product (before contamination) to avoid biomass loss (55), or the use of chemical and physical treatments which will be described later on, in this chapter (*vide* section 2.4.2.1. and 2.4.2.2.).

Sterilization techniques can be divided in two main categories, chemical and physical. Normally physical methods tend to be more effective but require high investments and normally complex equipment. Chemical methods are used to effect contaminants cells directly and normally require lower costs of operation, but some chemicals have toxicity risks. The most commonly used techniques for sterilization are summarized in figure 12 (sterilization techniques include both sterilization and disinfection methods).

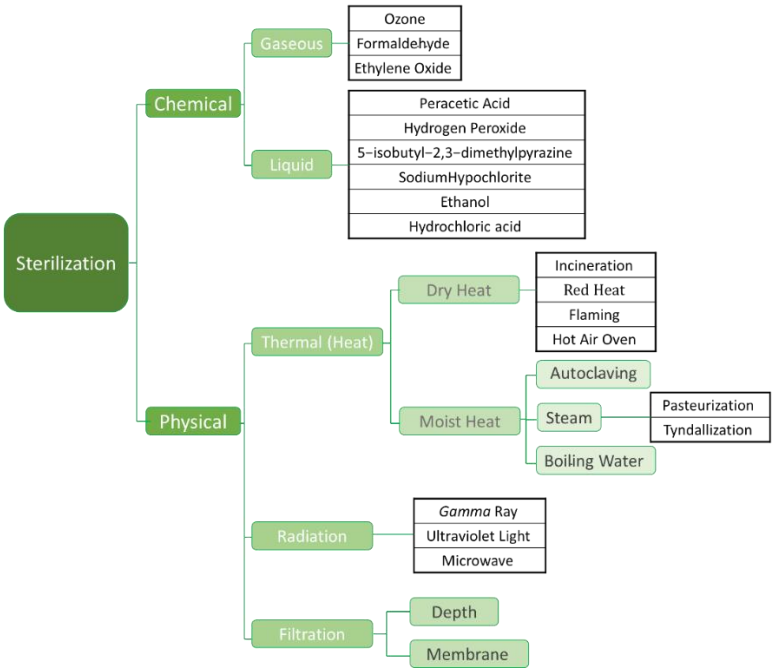


Figure 12 – Representation of the most commonly used sterilization techniques (include sterilization and disinfection methods) (5,48,54,56–58).

Not all the techniques can be applied in microalgae industrial production for different reasons (for example, high costs, toxicity, infrastructures and others). This chapter will only refer the techniques applied at A4F and the ones with potential to be applied.

2.4.2.1 Chemical methods

Several chemical solutions have been tested in microalgae industry, but not all of them are shown to be highly efficient. Liquid and gaseous solutions can be applied and different chemicals can be used (as showed in figure 11).

Chemical dosage is based on the deadly effect that a given chemical concentration and with a contact time (exposure of the microorganism to the chemical) has in contact with a given contamination. These chemical methods have been used for the purpose of sterilization, however, chemical traces may remain after the sterilization treatment, and those chemicals may be harmful to living microalgae, so it is important to neutralize them with the proper reagents. In this subchapter are described the most used chemical methods (48,54).

Sodium Hypochlorite (NaOCl)

Chlorine is the most common chemical disinfectant used for water treatment (used to treat culture media before microalgae cultivation and is generally obtained and used as either liquefied chlorine gas, as sodium hypochlorite solution or as an acidic solution, such as hydrochloric acid (37,47,54,59)

Microorganisms vary in their vulnerability to chlorine disinfection. Bacteria are generally most susceptible with a rising order of resistance from viruses, bacterial spores, to acid-fast bacteria and with protozoan cysts being the most resistant. Applying a chlorine dose that is effective against the more resistant will also be effective against the others (54).

When using NaOCl, it is important to note that the solution requires neutralization before placing microalgae cultures to grow. The culture media to treat needs to be in incubation with NaOCl solution for 24h without aeration, followed by aeration for 2-3h in order to remove residual chlorine. Addition of sodium thiosulfate to neutralize chlorine may be necessary if aeration fails to strip all the chlorine from the media (48,54). Nevertheless, the quantification of residual chlorine is usually performed.

Hydrochloric acid (HCl)

Hydrochloric acid (HCl) if often used to clean previously cultivated photobioreactors and eliminate all types of contaminants. In order to be effective, the bioreactor need to be immersed in a 10% (v/v) HCl solution for 1 day–1 week, and neutralization with a base, such as sodium carbonate, is required after that period of time (5,48,56,60).

2.4.2.1 Physical methods

Heat sterilization

Heat sterilization is the most common sterilization method and usually requires high temperatures, implying that the materials and media to be sterilized should resist high temperatures (60º-100ºC) (48). There are several types of heat sterilization in which the most commonly used are described in table 10.

Table 10 – Most common heat sterilization methods, including applications and limitations (48)

Sterilization method	Effective method	Application	Limitation
Autoclaving	High pressure and high temperature with moist heat; Time depends on the volume to sterilize	General use (Liquids, glass, metal and equipment)	Non-heat resistant materials, pH changes and metal contamination
Pasteurization	60-80ºC for at least 30min, followed by quick cooling (4-10ºC)	Liquid with heat-labile components	Doesn't make a complete sterilization
Tyndallization	High temperature (100ºC; 30min) followed by quick cooling; cycle repeated 3 times in 3 days	Liquid with heat-labile components	Requires time

In order to sterilize large volumes of culture media a continuous stream is used in a short-time, high-temperature treatment. Continuous sterilization can significantly reduce thermal damage to the media compared with batch sterilization, while achieving high levels of cell destruction. The continuous system includes a time period, during which the medium is heated to the sterilization temperature, a holding time at the desired temperature, and a cooling period to restore the medium temperature to enter the reactor. There are several designs for a continuous sterilizer, which must be adapted to the industrial plant and according the objectives of the project. One of the major advantages of continuous process is that a much

higher temperature may be used, thus reducing the holding time and reducing the degree of nutrient degradation. Also heating-up and cooling-down periods, are very small compared with those in a batch system since in this process small parts of medium are treated each time (59,61).

Filtration

Filtration is based on the principle that water is passed through a porous medium that doesn't allow particles of a given size to pass, while allowing solvents (57).

A diversity of filters is available that can vary on pore sizes, composition, color and geometry. Filters should have a pore size less than 0,1-0,2 µm, to prevent bacteria from going through, but viruses may pass through such pores. This method is normally used for liquids and gas when it contains fragile components that are destroyed by high temperatures (48).

There are two general types of filtration: Tangential filtration and Depth filtration.

Tangential filtration is the process of separating a material from a medium through which it can pass more easily than other materials in the same environment, in which membranes are used to remove unwanted suspended or dissolved substances. This type of filtration can be applied industrially in systems such as reverse osmosis, nanofiltration, ultrafiltration and microfiltration, depending on which substances are intended to be removed. These classifications are made based on the pore sizes, as represented in figure 13. Membranes are available in several formats: flat plate membranes (similar to filter paper); tubular membranes; hollow fiber membranes; capillary membranes; spiral membranes (62). Depending on their characteristics, if these filters can fully retain microorganisms, they are designated by absolute filters.

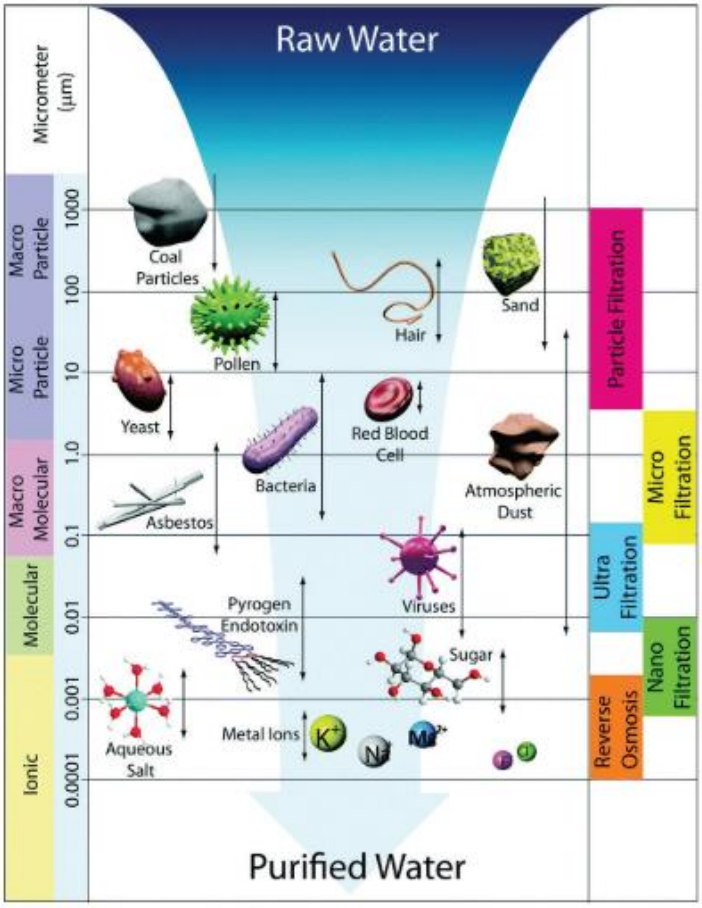


Figure 13 - Illustration of membrane filtration spectrum (57).

Depth filtration is based on deep-bed filters of fibrous material, such as glass wool and have been used extensively in the industry. Pores between fibers range between 2 to 10 pm, being much larger than the typical dimensions of bacteria and spores to be removed. For this reason, they are designated non-absolute filters. The particles present in the air penetrate the filtration bed at different depths and the production of sterile air depends also on the operative flow and the initial level of contamination. This type of filters perform badly if there are large fluctuations in the volumetric flow or if the air is wet, because the liquid condenses in the filter, promoting the formation of preferential channels of gaseous flow and provides a pathway for the microorganisms to pass through (57,62)

Regarding air sterilization, it is possible to design a deep filter considering that there is an uniform concentration of particles in the filter section perpendicular to the airflow according to the *Log penetration relationship* (Equation 1), where *K* is constant for a given filling material and for a certain linear velocity of air, N_0 is the average number of particles entering the filter,

N is the number of particles that abandon (probability of contamination) the filter and x is the depth of the filter (63).

$$\frac{dN}{dx} = -K.N \leftrightarrow N = N_0.e^{-K.x} \leftrightarrow ln\frac{N}{N_0} = -K.x \qquad (1)$$

These filters must be sterilizable by steam and should support several cycles of sterilization. In the case of deep filters, they retain a lot of moisture when sterilized, so it takes a long time to dry. However, they are relatively inexpensive (at least compared to the absolute filters). Absolute filters (membranes) have the advantage of not requiring drying after sterilization but are usually non reusable (57,62).

3. METHODOLOGIES

3.1. WATER AND CULTURE MEDIA TREATMENT, STORAGE AND DISTRIBUTION

Culture media are solutions made accordingly to the requirements of each microalgae specie, in order to simulate the natural habitat of microorganisms and with the aim of favoring their growth and/or specific metabolic pathways. The choice of the compounds to be used in media formulation is determined by parameters such as: required salinity, macro and micronutrient requirements of each specie (including vitamin needs) and industrial/local availability of the required compounds.

This subchapter briefly describes the steps involving preparation, storage and water decontamination, as well as the circuits linked to the microalgae production systems.

For microalgae biomass production, large volumes of culture media are required. At UEL there are four tanks for water storage and treatment, represented in figure 14. Two of them have capacity for 5 m^3 and the other two have capacity of 3.5 m^3. These tanks are used to prepare and store disinfection water and culture media.

Figure 14 - Tanks for water storage and treatment at UEL.

Each of the tanks is equipped with a centrifugal pump that allows the liquid to be recirculated inside it, for efficient homogenization.

There is a piping network responsible for making the distribution of water to all systems in UEL. This can be achieved through two pumps which can be fed by any of the four existing tanks. Downstream of the pumps there is a microfiltration system (down to 0.2 µm). After the microfiltration system, the piping network is divided into several lines that origin connection circuits to the photobioreactors, in order to perform operations such as transport of culture

media into the bioreactors. The circuits have manual valves along the piping lines, as well as electro valves in specific locations that allow control at a certain distance of the operation. In figure 15 is possible to see the microfiltration system.

Figure 15 - Microfiltration system.

Is essential to guarantee proper microbiological control of the prepared media and piping network, especially while they are not in use, since it is not desirable that they are contaminated with other organisms before they are inoculated with microalgae cultures in photobioreactors.

Sodium hypochlorite is used to prevent the growth of contaminant microorganisms in storage tanks (and in other disinfection containers used to decontaminate the material needed for certain operations carried out at UEL). This reagent is used because there are high volumes of solution to decontaminate and sterilization through autoclaving is not an economically viable option. NaClO is added with a concentration that ensures the elimination of contaminants and this concentration/presence of NaClO can be confirmed by the O-tolidine test. This test results in a colorimetric reaction that allows the determination of the presence of chlorine.

To help in the daily work at UEL a table was developed to assist in the O-tolidine test. This table presents the concentration of NaClO present in a solution, in ppm, and the color correlates with the chlorine concentration. When preparing the O-tolidine test it is immediately visible what is the concentration of NaClO present in the water. This table was prepared based on tests performed at A4F.

When culture media is not being used, it has to contain a defined percentage of NaClO. Every week a checkup is made to verify the concentration of NaClO in tanks, disinfection containers and in the pipe network so that disinfection throughout the process can be

guaranteed. If at some point the concentration needed to disinfect is not verified, NaClO must be added to the water to ensure their permanent decontamination.

In order to use water from the tanks to make a renewal in a photobioreactor, the chlorine that is present in the media must be neutralized. This means that all the NaClO present in the water must disappear, otherwise it will compromise the culture growth in the reactor. To neutralize the NaClO, thiosulfate is usually added. To evaluate if the water is neutralized, the O-tolidine test is again used, in which case the reaction must show no coloration. When this happens, the media is ready to be fed in the reactor but is also susceptible to contaminants. For this reason, it is important to ensure that the pipeline that guides the water to the reactors is always decontaminated in order to prevent future culture contaminations. After using the culture media from the tanks NaClO is added to the remaining volume in it, starting a new disinfection cycle.

3.2. AERATION AND CARBONATION

This subchapter briefly describes the aeration and carbonation system which is responsible for the injection of CO_2 (carbon dioxide) and aeration into the photobioreactor. Regarding CO_2 adding, the gas is derived from an external source of compressed CO_2 (CO_2 *rack*, figure 16) with the CO_2 in its pure state. This rack is connected to a distribution system composed by piping and valves that allows the manual control of gas injection into the cultures.

Figure 16 – CO_2 rack.

The CO_2 injection into the photobioreactors is continuously controlled, based on cultures pH, since the consumption of CO_2 by the cells raises the media pH. On the other hand, the CO_2 injection into the cultures will reduce the culture's pH and, in that way, it is possible to define a limit set-point for CO_2 injection.

All tubular photobioreactors and raceways have an automatic control for CO_2 injection that is activated depending of the culture's pH. While microalgae consume CO_2 in the photosynthesis process, the available quantity of this compound in the culture reduces, which is followed by a increases in pH, as referred previously. Thus, in these photobioreactors, a pH probe is installed and a signal converter to continuously monitor this parameter, which will indicate the need or not for CO_2 injection in the culture.

Every production system at UEL can be fed by an aeration flow in which the air comes from an aerifier installed at the plant, with the main purpose of ensuring culture agitation in the reactors. Agitation inside the reactors is essential to avoid biomass sedimentation at the bottom and to optimize mass and gaseous exchanges in de media.

The disinfection of the aeration and carbonation circuit is performed periodically and by parts before inoculating a system (the disinfection is made to the part that will be associated with the reactor). Since this system is directly linked to the cultures, it is also a possible source of contamination and is therefore essential to ensure its disinfection.

Before crossing the aerifier pump, atmospheric air is forced to pass through a particle filter and, after crossing the Aerifier, it is filtered by a 5–5000 µm particle filter before being distributed throughout the plant pipelines, which allows the retention of the larger contaminants.

A flowsheet representing this system and the thermoregulation system is presented in figure 17.

The disinfection method for the aeration pipeline consist in adding ethanol at the extremity of the pipe circuit to be disinfected, followed by the opening of the aeration circuit valves so that the ethanol goes through the pipeline and after some time evaporates. It is important to ensure that before the reactor inoculation all the required pipeline is ethanol free so that the cultures in the reactors aren't compromised.

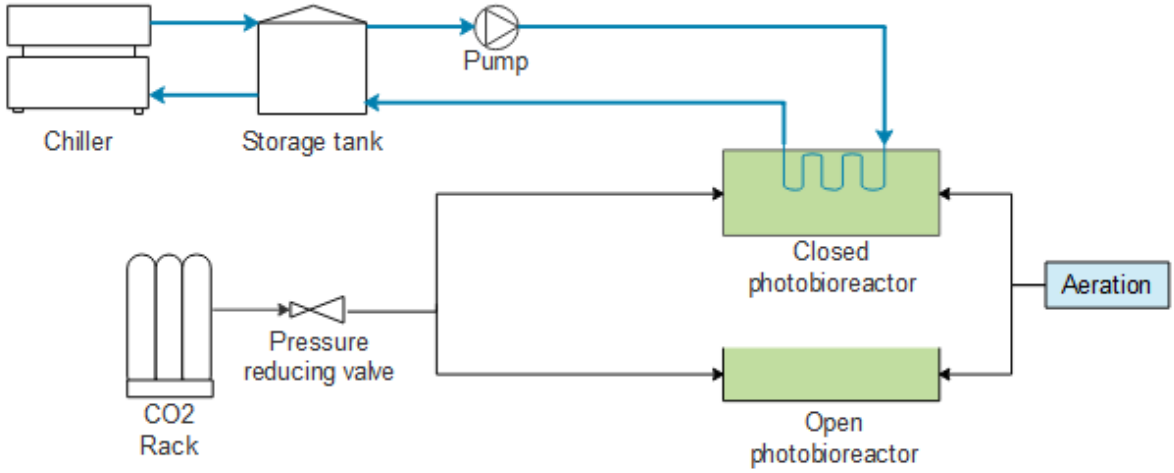


Figure 17 – Flowsheet of aeration and carbonation (thin and black flow) and thermoregulation (thick and blue flow systems.

3.3. THERMOREGULATION

In this subchapter the methods used to regulate the culture's temperature in the photobioreactors will be explained.

The thermoregulation system at the unit plant is a liquid stream circulating inside the photobioreactors which works as a heat exchanger, for cooling or heating depending on the culture requirements.

Refrigeration is carried out on a chiller (figure 18) whose fluid temperature (refrigerant fluid) is continuously measured at the process output. The refrigerant fluid is stored in a tank near the chiller before it is distributed to the culture systems.

Figure 18 – Chiller in thermoregulation system.

The thermoregulation of the unit plant works as a closed circuit, meaning that the refrigerant fluid is transported to the photobioreactors, drained through the coil inside and them brought back to the chiller to be cooled again.

The unit is also equipped with a boiler which allows the warming of the culture for the photobioreactors During the internship it was not possible to observe/deal with this procedure, because it took place in the spring/summertime.

In the case of vertical photobioreactors like Flat-panels, a coil is introduced inside the photosynthetic zone in order to be in direct contact with the culture. In tubular photobioreactors the coil is found inside de tanks and in raceways there is no thermoregulation system. It is possible to install aspersion points in which heat exchange is given by evaporation of water droplets on the surface of the reactor. In this case tap water is used instead of the refrigeration fluid. The system flowsheet was previously represented in figure 17.

The thermoregulation system control is based on a thermossensor placed inside the culture, which is linked to signal convertors (in some cases it controls both pH and temperature and in other cases only controls temperature; in both cases the control is made by opening an electro valve to the thermoregulation line) for temperature analysis and control being possible to define a set-point that activates the thermoregulation system. When the culture reaches the maximum temperature, a signal is sent to open the electro valve in the photobioreactor input and, therefore, the cooling water drains inside the coil. The temperature of the culture continues to be monitored, so that the electro valve is closed again when this value becomes lower than the set-point defined in the system.

3.4. PRODUCTION: PHOTOBIOREACTORS

To develop A4F's activities there are several photobioreactors installed in UEL, with different sizes and features to test different conditions in microalgae cultures. During the internship, it was possible to follow trials in several photobioreactors, as open (*Cascade Raceway*) and closed systems (*Flat Panel Photobioreactor*, *Unilayer Horizontal Tubular Photobioreactor* and *Multilayer Horizontal Tubular Photobioreactor*).

In each of them, the microalgae culture is exposed to solar radiation and depending on the availability of CO_2 and nutrients in the aqueous environment, to carry out photosynthesis.

The photobioreactors in the unit plant, allow the development of scale-up procedures and testing for future industrial applications, in which a photobioreactor is chosen to produce a certain microalgae culture considering the cultivation characteristics.

3.4.1. FLAT PANEL (FP)

FP photobioreactors are closed systems. FP's in UEL are used for both production tests and scale-up procedures. The scale-up process usually starts with the inoculation of a FP of smaller dimensions with culture from the laboratory, where the culture grows under controlled conditions and constant light. This FP is monitored until it reaches levels of growth/concentration that allows its use as inoculum for a larger FP or another type of photobioreactor. The scale-up process is represented in figure 19.

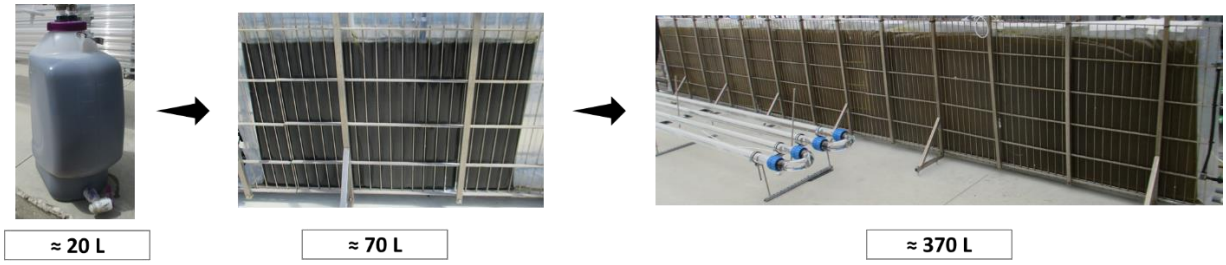


Figure 19 – Illustrative scale-up process in microalgae production.

The inoculum should be concentrated enough so that after its dilution in the new reactor there is no photoinhibition due to excess light. In order to avoid this problem, during FP'S inoculation a shade may be installed on the FP to avoid excessive solar radiation incident in the cells of the culture during the first cultivation days.

During the internship several operations of installation, inoculation, renewal and addition of nutrients in the FP's were performed.

All FP's in production at the unit plat are assembled at UEL. FP consist on a disposable transparent low-density polyethylene (LDPE) bag installed in a metal structure that supports microalgae culture inside. Depending on the test to be performed, the FP can be installed either inside the greenhouse or outdoors. The differences between the two are the isolation intended for the culture and control parameters such as temperature. In the case of outdoors FP-PBRs, the culture is exposed to the thermal amplitude of ambient air, and directly receives the solar lighting and the structure of the photobioreactor is in direct contact with the atmosphere, while inside the greenhouse rely on this as the first insulation layer.

To install a FP, it is necessary to place the LDPE bag in the structure defined and guarantee that all components that will compose the FP's structure will be disinfected. Apart from the LDPE bag, the components to add inside are coils, aeration diffusors, inoculation tube, a sampling system (syringe + silicon tube), thermossensor and pH probe (if intended).

The FP is full of culture media (90% of the bag capacity) and all the components are added inside. NaClO is added to ensure that no contaminates grow inside the FP until inoculation. Before inoculation it is important to disinfect the aeration pipeline as described before (*vide* section 3.2.).

Before inoculation, some procedures need to be carried out. Aeration is turned on a day before inoculation, adding positive pressure inside the FP that will prevent contaminant entry. Immediately before inoculation some media volume must be taken out in order to put the same volume of culture in. The remaining media must be NaClO free (neutralized), so NaClO concentration in the FP is measured (o-tolidine test) and if it still exits is neutralized with thiosulphate.

Inoculation can be provided from inoculum prepared at the laboratory or from a smaller FP. From the laboratory, the cultures come in a container that is directly connected to the inoculation tube (the procedure is pulverized with bleach in a proportion of 1:10). The container's valve is opened so that the inoculation of the FP begins. If the inoculum comes from another FP, a system of hoses is prepared, including a pump to transport the culture from a FP to another. After inoculation the FP is prepared for production, controlling all the parameters describe at chapter 3. 5..

The operation of a FP in production is quite simple. In the intel air and CO_2 enter the FP to prevent sedimentation and promote cell growth, respectively. Bleach or ethanol are used to disinfect the area in contact exposed to air. The thermossensor will indicate if the thermoregulation system is working properly. The transparent LDPE bag will allow microalgae photosynthesis culture during production.

Two cultures were followed in FP cultivation during the internship, namely *Dunaliella* sp. and *Tisochrysis* sp. represented in figure 20.

Figure 20 - *Dunaliella* sp. (A) and *Tisochysis* sp. (B) cultures followed in production in FP at UEL.

3.4.2. GLASS PANEL (GP)

At UEL exists a photobioreactor indicated for sensitive species including genetically modified (GM) microalgae. To work with this type of organisms, special care is required before, during and after production. This kind of photobioreactor provides the possibility of sterilization processes and works in a complete sealed system. This bioreactor is made of glass and stainless-steel compatible with steam sterilization.

Within the GP exists (as in FP) a coil that allows the thermoregulation of the culture and an aerator that injects air and CO_2 in the culture. The air inlets and outlets of the reactor are equipped with air filters to prevent the exit of microorganisms growing in the reactor. Both temperature and pH of the culture inside are measured by a probe connected to the signal converter. When reaching a certain value, a signal is sent to the opening of the electro valve of CO_2 or thermoregulation, and the valve allows the passage of fluid from the chiller to the coil installed inside the GP.

The sterilization method used in GP at UEL is steam sterilization. This procedure is carried out using the boiler that injects steam into the GP. The GP is filled with disinfection water before sterilization. Sterilization is completed after at least two cycles, in which each cycle consists in injecting steam from the boiler in the GP and let the water present in the GP boil (until it reaches 100ºC). After some hours a second steam cycle is applied, equal to the first cycle to ensure that all possible contaminants are eliminated.

Inoculation procedures are comparable to the ones made with a normal FP, where every inoculum comes from the laboratory, in figure 21 it's possible to see the GP before and after inoculation.

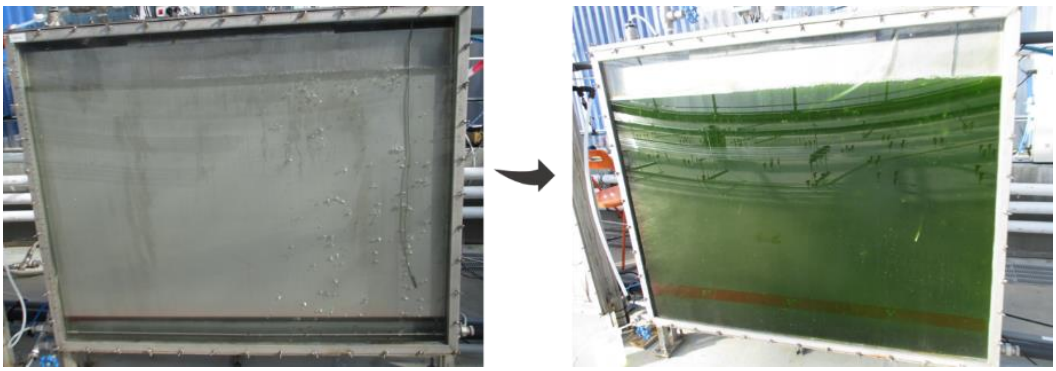


Figure 21 – GP before (left) and after (right) inoculation at UEL.

Synechocystis sp. (GM) was the only microalgae culture observed in production in this type of photobioreactor, during the internship.

3.4.3. TUBULAR PHOTOBIOREACTOR (TPBR)

Tubular Photobioreactors are one of the most used technologies for microalgae production. It is a closed system which allows a greater control of production conditions (pH, temperature, contaminants, etc.).

During the internship two types of TPBR's were in production specifically Unilayer Horizontal Tubular PBR (UHT-PBR) in cultivation with *Nannochloropsis* sp. (figure 22.a) and Multilayer Horizontal Tubular PBR (MHT-PBR) in cultivation with *Tisochrysis* sp. (figure 22.b). Although they share general characteristics, such as the introduction of CO_2 for pH control, the presence of a coil inside the tank for temperature control and the possibility of using aspersion in the tubes for thermoregulation, they differ on the tubes disposition.

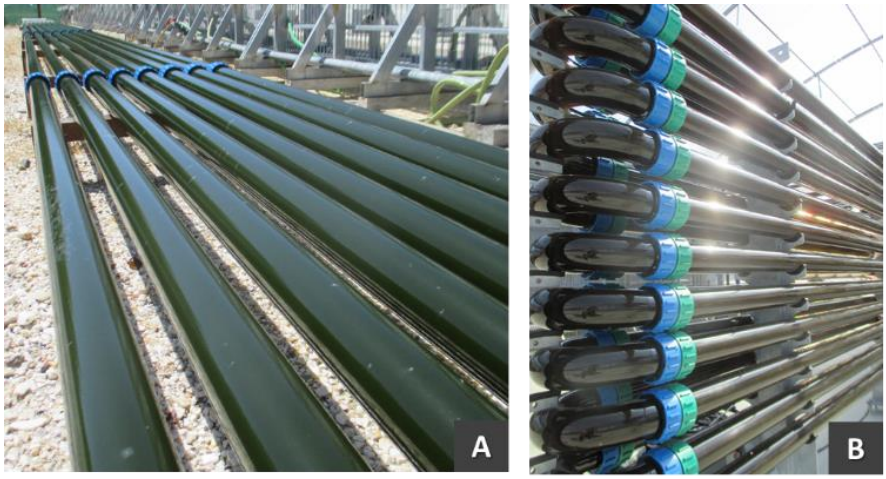


Figure 22 – TPBR'S in in production during the internship. UHT-PBR in cultivation with *Nannochloropsis* sp.(A) and MHT-PBR in cultivation with *Tisochrysis* sp. (B) ate UEL.

In MHT-PBR configuration there are several layers of tubes disposed horizontally and parallel to the ground, which means that it has a higher volume to area ratio. This type of reactor is presented schematically in figure 23. The layers being vertically disposed promote a shadow for the lower layer tubes, decreasing in that way the total photosynthetic area.

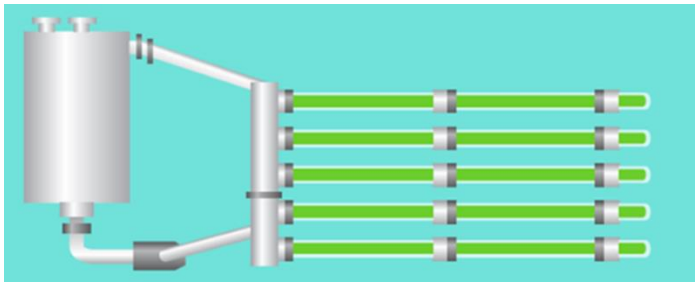


Figure 23 – MHT-PBR production scheme (64).

The UHT-PBR configuration comprises only one layer of tubes disposed horizontally and parallel to the ground, which maximizes the available radiation per cell, allowing a higher photosynthetic efficiency. This system is represented in figure 24.

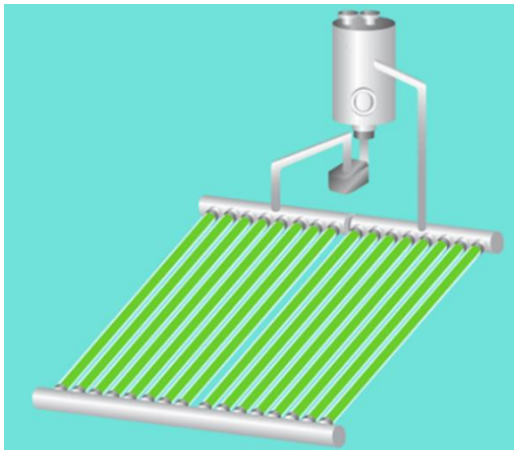


Figure 24 - UHT-PBR production scheme (64).

Regardless of the arrangement of the tubes the operation of both reactors is similar.

TPBR's have a tank where the culture goes after passing the photosynthetic area (tubes). At the end of each tube there are collectors that group a few tubes and redirect the culture to continue the production path. Between the tank and the collectors exists a centrifuge pump that allows the circulation of the culture through the reactor. Like in the GP, the tank can't be entirely full for optimal gaseous exchange and culture's mixture.

Being closed systems, disinfection became an important step before inoculation. TPBR's are cleaned with acid solutions (HCl) before every inoculation. Culture media is put inside the reactor and disinfected with NaClO until the inoculation day, to prevent contamination.

3.4.4. CASCADE RACEWAY (CRW)

The photobioreactor shown in figure 25 is a *Cascade Raceway* (CRW), a horizontal photobioreactor in which the microalgae culture drains along its ramps and is kept in direct contact with the atmosphere.

Figure 25 – CRW in cultivation with *Dunaliella* sp. at UEL.

The CRW has the particularity of being inclined. There are two ramps, which can be seen in figure 26. The angle in the ramps makes the fluid stream in opposite directions in each ramp and the flow is maintained by the effect of gravity. However, it is necessary to use a pump to make the culture circulate between the ramps.

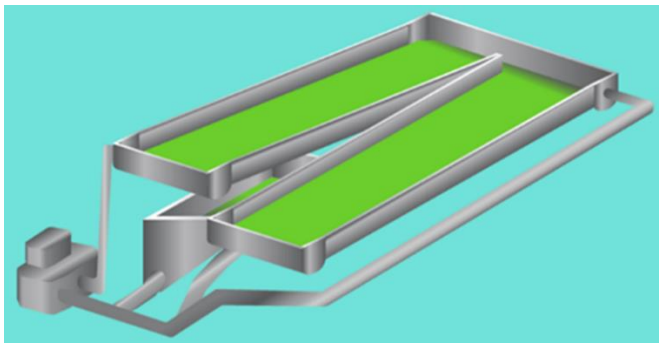


Figure 26 - CRW production scheme (64).

The injection of CO_2 is controlled by a probe and a signal converter, which injects CO_2 (electro valve). The thermoregulation of the system occurs mostly by the evaporation of the water present in the crop.

The main problem of this reactor configuration is the control of total media volume. Once it is exposed to the environment it can have uncontrollable volume additions (rain) as

well as volume losses (evaporation). Through the internship it was possible to improve the compensation methods for volumes evaporated.

Salinity is measured every morning in order to evaluate the evaporation occurred during a day. Depending on the concentration of salt measured in the CRW it's possible to know the total volume of the culture and based on this it's also possible to evaluate the total volume evaporation during the day. Knowing that during the summer evaporation rates are higher (due to higher temperatures), a pump was installed near the CRW to automatically (connected to a timer) add fresh water from a tank to the reactor so that the culture wouldn't be compromised by evaporation. Fresh water must be added to compensate the evaporation occurring during the day.

The microalgae culture produced in this type of photobioreactor is in constant contact with the atmosphere so there is a high number of contaminants inside. In this type of reactor, only sturdy enough species are cultivated to prevail over other populations that can grow in the cultivation media. In this reactor it was accompanied the production of a *Dunaliella* sp. culture. This is a good culture for cultivation in CRW because it is growing on stress conditions. The lack of nutrients and a hypersaline media prevent contaminants growth.

3.4.5. COMPARISON BETWEEN REACTORS

After the characterization of all reactors, the main advantages and disadvantages of their use was summarized in table 11, as well as the most common sterilization methods.

Table 11 – Advantages, disadvantages and sterilization methods of the photobioreactors (Flat-panel, Glass-Panel, Tubular PBR and Cascade Raceway)

	Advantages	**Disadvantages**	**Sterilization methods**
Flat-Panel	· Low investment · Easy operation · Flexibility and the capacity to be scaled-up · No civil construction required aside from a levelled and compacted site	· Risk of leaks	· Sodium Hypochlorite
Glass-Panel	· Reduced exposure to contamination · Ability to sterilize the materials contacting the culture · Production of highly sensitive species · Easy operation and capacity to scale-up	· Expensive to build and operate	· Hydrochloric acid · Steam/Boiling water
Tubular PBR	· Reduction of the reactor zero velocity areas/dead volumes · Constant velocity along all the PBR piping extension · Optimized global mass and energy balance · Efficient circulation pumps. · Control of culture parameters	· Cleaning · Expensive to build and operate	· Hydrochloric acid · Sodium Hypochlorite
Cascade Raceway	· Low operation costs · Easier CO2 control and lower O2 build-up · Culture continuously flows through a pipe (with pump) that provides higher mixing	· Limited of species; · Weather conditions and evaporation of water in the culture media · Less control of cultivation conditions · Susceptible to contamination from being exposed to the environment.	· Are not performed

3.5. CONTROL IN CULTURE PRODUCTION

An essential part in UEL's work is the monitoring of the cultures in production, namely all the parameters associated, such as pH, temperature, volume, dissolved oxygen, turbidity, etc. Every day, two times a day (in the morning, before any operations and at the end of the day) a verification of each bioreactor is made, including a verification of the general state of the cultures in production to make sure everything is in order. A more careful verification is made to each culture four times a day (9h, 12h, 14h30,17h) to better monitor de cultures development and to more rapidly identify any problems that may be occurring concerning the culture production.

In all systems pH, temperature and volume are analyzed. Some parameters are specific of each reactor, such as turbidity, dissolved oxygen (DO) and salinity.

The pH values are important to monitor and control in order to better understand the photosynthetic process of the culture, which is related with CO_2 decrease and with the carbonation system described above. pH values can be measured by a probe connected to signal convertors which can be seen on a monitor, for example the equipment presented in figure 27.a. In some occasions, it is necessary to remove a sample and make a manual measurement with a portable pH meter identical to the one in figure 27.b.

Figure 27 – Equipment used to measure pH. A – Equipment linked to a probe and a signal convertor that measures pH and temperature; B – Portable pH meter.

Temperature is directly linked with the thermoregulation system and as described before (vide section 3.3), the temperature is automatically controlled by thermossensors connected to signal convertors which will not only provide the values in a monitor but in case of reaching a set-point it will activate the thermoregulation system. This means that if the

temperature drops from the setpoint value, hot water will be supplied and on the other hand if the temperature increases from the setpoint value, cold water will be supplied to the culture in order to reestablish the setpoint conditions. This parameter is also essential to determine the functional production of microalgae since optimal conditions for culture growth must be established. In figure 28 is presented the most used temperature controller at UEL.

Figure 28 – Temperature control equipment.

The volume is a parameter that is controlled to recognize if something is wrong with the culture/reactor. By controlling this parameter, it is possible to know if evaporation is occurring, or even if there may be any problem with the reactor, such as a leakage. In the FP's this parameter is controlled by marks made in the beginning of the culture's trial that over time will not correspond to the actual volume at the FP (mostly because of sampling but also due to some evaporation). In the TPBR's a fixed volume is considered inside the tubes and to control the volume inside the tank a level probe is installed that shows volume measurement in a monitor.

The volume in CRW is determined by measuring the salinity of the culture as described before, and this is an important parameter to consider in CRW's and FP's. Salinity is significant to determine water losses or gains and to ensure the optimal growth conditions of the microalgae cultures. Salinity is measured by a refractometer (figure 29), which will indicate the percentage of salt in the water (%), that can be translated in grams of salt per liter of water (g/L). Then the volume can be corrected to the optimal conditions of a culture.

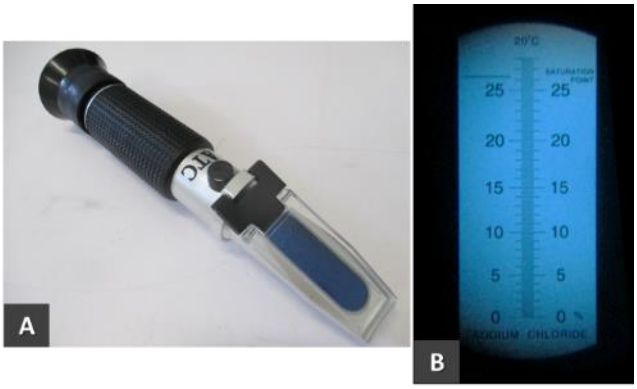


Figure 29 – Refractometer (A) and the respective measurement scale (B).

Regarding TPBR other parameters besides the ones mentioned above, where also measured, like turbidity and dissolved oxygen (DO).

The turbidity measurement is made by turbidity probes that are connected to a display where the turbidity value is visible (figure 30). Turbidity is measured in *Nephelometric Turbidity Unit* (*NTU*) and it is a physical property that quantifies the reduction of transparency in a fluid. This means that throughout the production of a culture the turbidity should raise over time, indicating that culture concentration is rising too.

Figure 30 – Turbidity display.

In a TPBR it is important to measure DO in the culture. Measurements about DO make more sense to be made in closed systems because those systems tend to accumulate oxygen concentrations that may damage the cultures. The equipment used measure is an oximeter, presented in figure 31, that measures que quantity of oxygen (O_2) dissolved in a certain fluid. In the oximeter it's possible to see the DO measured value in milligrams per liter (mg/L) and oxygen saturation in percentage (%), that normally refers to the same thing. The DO produced will peak during daylight hours and decline at night and oxygen saturation varies depending

on the temperature. During the day, cells should be producing oxygen and receiving CO_2 due to photosynthetic process. Having high concentrations of oxygen this oxygen needs to be expelled from the reactor. If oxygen is in high concentrations in the culture It means that the gas exchanges within the reactor aren't efficient, and degassing needs to be improved. A possible way to solve this problem can be a deposit where there is efficient turbulence of the culture releasing excess oxygen.

Figure 31 – Oximeter.

The control of all the parameters described above was carried throughout the internship and contributed to the correct production of all cultures. These controls are important to detect problems in cultures rapidly and efficiently troubleshooting them.

3.6. HARVESTING AND CULTURE PROCESSING

In this subchapter will be described the process associated with biomass harvesting and processing. This process consists on removing the culture from the photobioreactors and concentrating it in order to obtain biomass in its intended final form. There are two forms of final product obtained at UEL, depending on the process that is used: paste (centrifugation) or dry powder (spray drying).

For the culture harvesting, the process is similar in all photobioreactors, only differing in the pathway undertaken. For the TPBR there is a pipe network that connects the reactor to a harvesting tank that will feed the processing equipment. The culture is pumped through the pipeline by the TPBR centrifugal pump until it reaches the desired volume at the harvesting tank (the tank is equipped with a level probe). In the case of FP and *Raceways* the harvesting is carried out through a circuit linked to a pump that will transport the culture from the reactor

to the harvesting tank until the desired volume is harvested. During harvesting the reactor aeration is maintained in order to preserve the state of culture avoiding its possible sedimentation. The cultures can be harvested in total or just a part, and in that case after the harvesting the culture must be renewed with fresh media.

3.6.1. CENTRIFUGATION

The aim of centrifugation is to obtain a concentrated culture. The culture goes from the harvesting tank to the centrifuge where it is processed at a specified flow rate (determined for each operation). Within the centrifuge there is the separation of the liquid part (supernatant) from the solid biomass (paste, final product). The general operation procedure is described in table 12.

Table 12 – Centrifugation process carried out at UEL

1. Before starting centrifugation, it is necessary to assemble the centrifuge and turn on the oil pump, waiting for the equipment to heat up.
2. It is necessary to ensure that the valves connecting the tank to the centrifuge feed are properly open.
3. The centrifugal pump that feeds the centrifuge is switched on after the rotor is on (the frequency can be adjusted according to the trial). It is important to verify that the culture is passing through the transparent feed hose that feeds the centrifuge.
4. During centrifugation, the level in the harvesting tank must be controlled as well as the color that the supernatant acquires when it exits the centrifuge (permeate), in order to verify if there are significant losses of biomass;
5. When the entire culture of the harvesting tank has been processed the centrifugation is finished and the feeding is switched off, followed by switching off the rotor as well.
6. Biomass is collected from the centrifuge's drum for packaging.
7. The packaging, labelling and storage (at -20°C) of the biomass is made.

8. The process is finished by manual cleaning of all material and equipment, followed by a cleaning procedure with disinfection water of both the harvesting and the centrifuge.

In figure 32 is represented the final product obtained through the process, biomass in paste form.

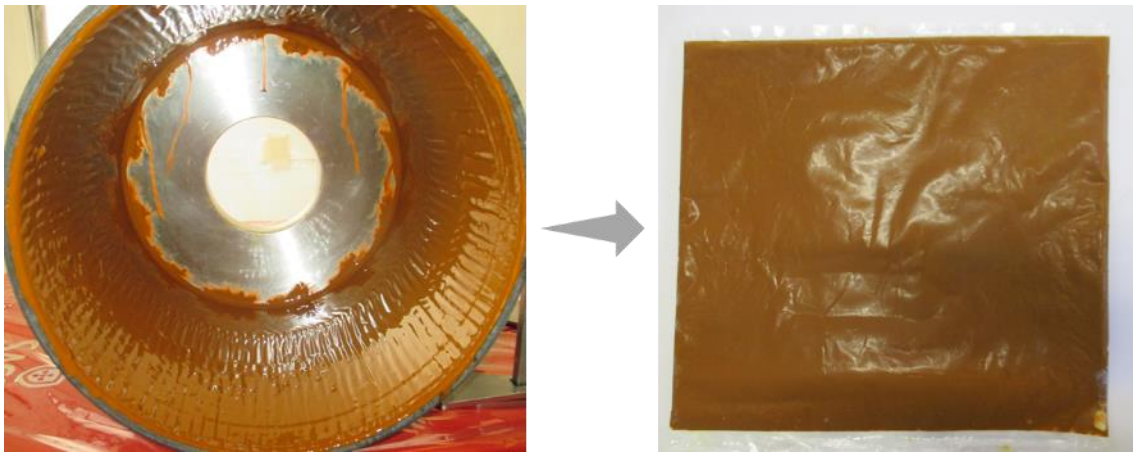


Figure 32 – Biomass processed thought centrifugation. Paste after centrifugation (left) and after packing (right).

3.6.2. SPRAY DRYING

Another way of biomass processing produced in this unit is drying with Spray Dryer (SD). The unit plant is equipped with a pilot scale spray dryer that allows to better concentrate the cultures and mimic an industrial process.

When exiting the SD, the mixture of hot and humid air and dry biomass powder (resulting from the drying process) enters the cyclone to be separated. The flow produces a vortex inside it, separating the powder (which comes out by the effect of gravity) from the air.

The biomass fed to the SD is previously prepared in a solution form.

The procedure carried out at UEL for drying biomass is described in table 13.

Table 13 -Spray drying process carried out at UEL

1. It is verified whether the SD and all the accessories, are clean and well assembled.
2. The equipment is switched on at the Control Panel, as well as the fan and the light in the drying chamber.

3. The heater is switched on and the inlet temperature is left to stabilize to the desired value.
4. It is necessary to establish a frequency for atomizer operation, and after being defined the atomizer is switched on.
5. The SD feed pump is linked to distilled water and the feed speed is adjusted.
1. After stabilizing the inlet and outlet air temperatures, the spray dryer is fed through a pump with concentrated culture, initiating the biomass drying process.
2. Dry biomass is collected in a cup located immediately after the cyclone. The cyclone separates dry biomass (sent to the cup) from the moist air (sent by the fan outlet).
3. The biomass is stored in a clean room for vacuum packaging. All packages are properly identified and stored in the freezer (freezer kept at -20°C).

In figure 33 is represented the SD installed at UEL.

Figure 33 – Spray Dryer at UEL.

3.6.3. QUANTITATIVE BIOMASS ANALYSIS – DRY WEIGHT

The measurement of dry weight (DW) is made in a humidity meter equipment (humidity balance), where the mass is measured in each powder sample after all the humidity present is removed. The value presented by the scale in grams allows to later determine dry matter content of microalgae in the culture through the process of mass measurement before and after processing. To determine the DW of biomass it is necessary to make a calculation, where the mass of biomass powder is divided after drying without water (*fm*) by the mass of biomass in wet powder (*im*), as presented in equation 2.

$$DW\ (\%) = \frac{fm\ (g)}{im\ (g)} \qquad (2)$$

3.7. EFFLUENT TREATMENT

This subchapter is related to the unit's plant outputs, more specifically the management of by-products of the biomass resulted from production processes.

In the unit plant is installed an effluent tank that collects all the water disposed to drain channels across UEL. This tank, when full is discharged into the municipal sewage system.

To prevent the risk of environmental contamination (due to the presence of toxic compounds in water) the fluid on the tank is disinfected before discharge. The normal procedure to disinfect de fluid is to add a certain volume of NaClO (the volume of NaClO added depends on de tank's volume and content). The tank remains in disinfection for at least two hours with recirculation of the volume (the tank is equipped with a centrifugal pump). Parameters like temperature, pH, turbidity and conductivity are registered (monitored inside the tank by a signal controller) at the beginning and in the end of the disinfection process. To finish the process, the fluid is discharged into a municipal wastewater network

4. PROPOSAL OF STRATEGIES FOR CONTAMINATION CONTROL

Several procedures can be applied in order to reduce contamination in microalgae cultures, and therefore in the final products. It's known that some methods can´t fully eliminate contaminants and for some industries only reducing the number of contaminates isn't enough. Examples of these are pharmaceutical and food industries which require more strict rules regarding sterilization methods.

Pharmaceutical and food industries are emerging markets for microalgae as they provide natural compounds of high nutritional value (fatty acids, carotenoids, vitamins, enzymes, and others). Having microalgae biomass such potential, it is mandatory to control the contamination level (improved sterilization methods) in order to reach these markets.

After analyzing existing sterilization techniques in a microalgae pilot scale unit, it is possible to propose some optimization procedures to the existing plant in order to produce microalgae products that can compete in the several emergent markets.

In this chapter it will be described tree different optimization proposals for sterilization of culture media and air, as these two are considered the main causes of contamination of microalgae cultures. To better sterilize the culture media methods, one suggests the use of ozone (*vide* section 4.1.) or continuous sterilization (*vide* section 4.2.) and for air sterilization the length of the non-absolute filter can be determined in order to obtain a given low probability of contamination or the use of absolute filters (*vide* section 4.3.).

4.1. OZONE

Ozone (O_3) is a very powerful oxidizing agent which is commonly used in water treatment. It can oxidize cell components of the bacterial cell wall, because of its high oxidation potential and has it entered the cell, it oxidizes all essential components (enzymes, proteins, DNA, RNA). There are essentially no harmful residuals from ozone use, as it undergoes a natural decomposition in water. It decays more rapidly than other disinfectants and therefore does not maintain a persistent residual value (54,65,66). Nevertheless, O_3 is a toxic, bluish, unstable, potentially explosive gas and is a hazard to plants and animals. It produces an irritation of the nasal passages in low concentrations, making it dangerous for human manipulations when not treated carefully. It is an unstable gas which must be generated as required on site. It is a more effective bactericide and virucide than chlorine. It is, however, an expensive disinfection technology in terms of capital and operating costs (10,54,66).

The generation of O_3 is made by an electrical discharge through dry air or pure oxygen and is generated onsite because it decomposes to elemental oxygen in a short amount of time. The use of oxygen enables ozone to be generated at higher concentrations, which is more energy efficient and beneficial for mass transfer but carries the additional cost of the oxygen. After generation, ozone is fed into a down-flow contact chamber containing the water solutions to be disinfected. From the bottom of the contact chamber, ozone is diffused into fine bubbles that mix with the downward flowing water. In figure 34 is represented a scheme of an air-fed ozonation system. Ozone is used as a primary disinfectant and should be coupled with a secondary disinfectant for a complete disinfection system, for example filtration (5 µm) to remove any by-products formed in this process (34,54,65,66).

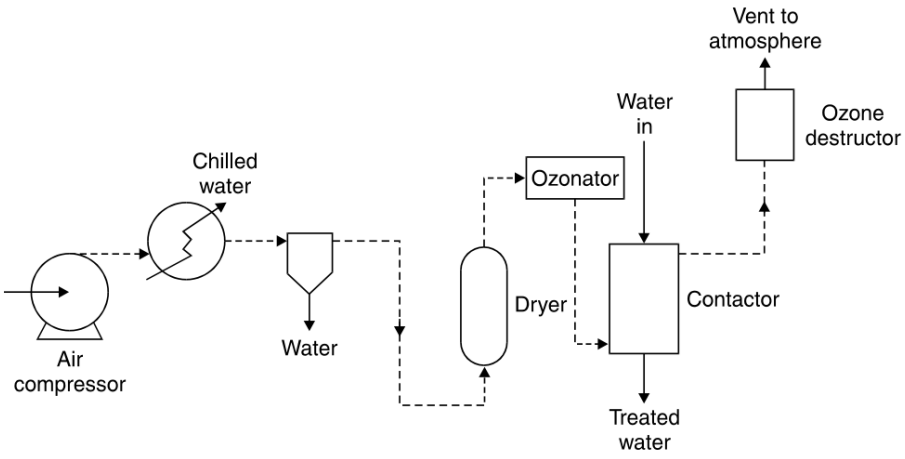


Figure 34 - Schematic of air-fed ozonation system (54).

This type of system can be applied in A4F's pilot unit plant, at a pilot scale and to perform culture media sterilization.

This reagent is widely used is several industries (water treatment, pharmaceutical, food) therefore being a very studied method with several information on how and where to apply.

The main advantages and disadvantages on using an ozonation system are described in table 14.

Table 14 – Advantages and disadvantages of ozonation (34,54,65,66).

Advantages	Disadvantages
· Strong oxidant and highly effective disinfectant compared with chlorine. · It is less sensitive to pH variation as a disinfectant than chlorine. · Provides no residual disinfectant.	· Complex, energy intensive and expensive equipment compared with other chemical disinfectants. · Ozone decays particularly at high pH levels. · Post process is usually required (filtration).

4.2. CONTINUOUS HEAT STERILIZATION

As seen before (*vide* section 2.4.2.1.) continuous sterilization can provide a secure and reliable sterilization and it can also be an alternative to the actual sterilization methods used at A4F's unit plant.

In the pilot unit the continuous sterilizer is placed between the culture media tanks and the reactors to be fed, and all the connections are made throw a pipeline (resistant to high temperatures between the sterilizer and the reactor). In figure 35 are represented schemes of a continuous sterilization plant that can be applied in A4F's unit, steam injection with flash cooling (figure 35.a) and heat transfer using heat exchangers (figure 35.b).

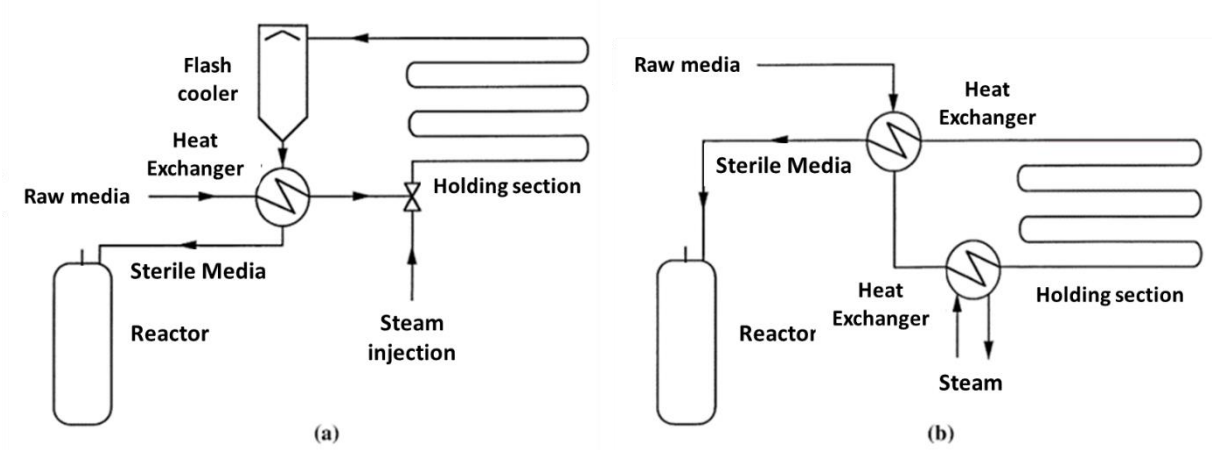


Figure 35 – Continuous sterilizers: (a) continuous steam injection with flash cooling and (b) heat transfer using heat exchangers (adapted from (61)). In the flash cooler or expansion chamber the sterilized medium is quickly cooled to the cultivation temperature or other heat exchanger is needed to provide that cooling step (less efficient).

A continuous sterilizer with steam injection delivers sterilized media to the fermenter. Culture media in the holding section of the sterilizer is maintained at 130ºC (61).

To apply this type of sterilization system in A4F's plant unit it is necessary to determine the flow required to feed the reactors. Also, this type of sterilization is normally applied to continuous processes, and the majority of UEL's projects are working in semi-continuous regimes. Nevertheless, this is a sterilization system to be considered once it can be adapted to that regime, working in cycles. The cycle begins by steam injection in all the sterilization system in order to prepare it for sterilization. Then the portion needed to feed the reactor enters the system, where it is steam injected and maintained in the holding section for complete sterilization (at a given temperature). After that period, after the flow is cooled (by flash cooler or heat exchanger) in order to enter the bioreactor at a suitable temperature. Whenever the

culture media needs to be sterilized (culture's renewal or new culture) the continuous sterilization systems begins a new cycle. After this process it is important that the system is properly secure and closed.

The main challenge on applying this type of system in UEL might be the equipment acquisition, considering that the unit has a boiler (heater) that can provide the steam required for these procedures and the energy cost.

In table 15 are named the main advantages and disadvantages by using continuous sterilization systems.

Table 15 – Advantages and disadvantages of continuous sterilization (67).

Advantages	Disadvantages
· Uniform steam throughout sterilization · Simplified process control · Shorter sterilization time leading to less thermal degradation of the culture media	· High demand for steam in a shorter period than batch heat sterilization · Concentration of media becomes dilute due to steam condensation · Since steam is dispersed in media, it must be clean to avoid contamination · Flash cooler: steam contacts directly with culture media

4.3. AIR STERILIZATION WITH ABSOLUTE AND NON-ABSOLUTE FILTERS

Filtration can provide effective elimination of contaminants as observed previously (*vide* section 2.4.2.1.) though absolute filters (pores in the filter are smaller than the particles which are to be removed) and non-absolute filters (pores are larger than the particles which are to be removed). In microalgae cultures air filtration plays an important role, being mandatory that the air entering the photobioreactors (cultures) is contaminant free, therefore continuously sterilized.

At UEL air filtration is well designed, were all the air that is fed to the bioreactors is previously filtered by non-absolute filters though the aerifier. Additionally, absolute filters are added when necessary for better sterilization, for example when working with GM microalgae.

An option to be considered in order to optimize air filtration though all photobioreactor in function at UEL is the use of absolute filters in each, which can significantly improve air sterilization. To use this type of filters it is important to have an air population characterization to better know what type of pore to choose for the filter, keeping in mind that this characterization varies throughout the year, with humidity levels and temperature. Although this type of filters is reported to be the most efficient in contaminant removal, using absolute filters has great costs and normally they are not reused.

Another step that can be taken toward sterilization optimization is to design a non-absolute filter to match required specifications, using *Log penetration relationship* (Equation 1). Assuming that when a particle touches a fiber, it remains attached to it, and that there is a homogeneous concentration of particles at any given depth in the filter, then each layer of the filter should reduce the population entering by the same proportion. Non-absolute filters are made of fibrous materials such as cotton, glass, slag or steel wool and in which each material have a constant *K* which is constant for a given filling material and for a certain linear velocity of air.

It is possible to calculate the depth of the filter required to remove 90% of the total number of particles entering the filter (X_{90}), where it can be also assumed that the N_0 is 10 (10% of the particles) and *N* is 1 (total number of organisms) and depth will depend on the material to be chosen (*K* constant):

$$ln\frac{\mathrm{N}}{\mathrm{N_0}} = -\mathrm{K.x} \leftrightarrow ln\frac{1}{10} = -\mathrm{K}.X_{90}$$

$$-2{,}30 = -\mathrm{K}.X_{90} \leftrightarrow 2{,}30 = \mathrm{K}.X_{90} \quad (3)$$

$$X_{90} = \frac{2{,}30}{\mathrm{K}}$$

Presented in table 16 are the advantages and disadvantages of both absolute and non-absolute filters.

Table 16 - Advantages and disadvantages of absolute and non-absolute filters in air filtration.

Filters	Advantages	Disadvantages
Absolute	· More efficient in retaining particles · Can be used for fine particles · Pore size is controlled	· Expensive · Non-reusable · Flow can be compromised by pore blocking by larger particles
Non-absolute	· Are reusable (must be sterilized) · Cheaper · Lower pressure drop	· Problems with wet air · Contaminants can grow within the filter matrix

6. CONCLUSIONS

Microalgae are already being used as food ingredients (animal and human) and as a source of high value compounds and proved to have great potential to reach pharmaceutical industries, biofuels and other markets. To produce biofuels, microalgae need to be cultivated at a large scale in economically viable systems, which are not available yet comparing to fossil sources. However, developments are being made towards new environmentally friendly cultivation technologies and mechanized harvest.

Most of the current microalgae cultivation is being done in open bioreactors, because they are economically more viable than closed photobioreactors. It is important for future work to improve closed photobioreactor design and operating costs to make closed systems as efficient as possible, since these reactors provide better control on culture parameters and contaminations, being only then possible to fill in the requirements to enter pharmaceutical and food industries - highly regulated markets. The choice to cultivate in an open or closed photobioreactor is related to the microalgae growth conditions. To produce a microalgae that can grow in a more hostile medium the photobioreactor can be opened (there will be no competition for any contamination). In addition, microalgae cultivation requires monitoring of several variables, such as temperature, aeration, pH, salinity, and others, to prevent contamination as well as to ensure that the product is intended.

There is a lot of room for improvement and optimization of all steps in microalgae production, from the inoculation to the harvesting procedure, where investigation and practice play an important role to better understand the potential for development of microalgae production. Optimal sterilization procedures throughout the production process makes a significant contribution on microalgae cultures and therefore on a high-quality product. Several methods are widely used in food and pharmaceutical industries and remain to be tested and validated for microalgae production at A4f the ozone, continuous sterilizer and absolute and non-absolute filters options for sterilization approaches.

In conclusion, there is still a lot of work and research to perform in the field of microalgae cultivation and production to make the most of this powerful type of microorganism.

7. REFERENCES

1. A4F. Produção Industrial - Experiência de Produção [Internet]. [cited 2009 Sep 5]. Available from: https://www.a4f.pt/pt/historico-microalgas

2. A4F. A nossa abordagem [Internet]. [cited 2019 Jul 20]. Available from: https://www.a4f.pt/pt/solucoes

3. Carlsson AS, van Beilen JB, Möller R, Clayton D. Micro and Macroalgae: Utility for industrial applications. In: Bowles D, editor. EPOBIO project. CPL Press; 2007.

4. Moreira M. Novas tecnologias de colheita de microalgas para a produção de biodiesel. Universidade de Lisboa - Faculdade de Ciências; 2013.

5. Barsanti L, Gualtieri P. Algae - Anatomy,Biochemistry, and Biotechnology. Taylor & Francis. Taylor & Francis, CRC Press; 2001. 1–289 p.

6. Gupta SK, Malik A, Bux F. Algal Biofuels: Recent Advances and Future Prospects. Springer Nature; 2017. 57–76 p.

7. Schlarb-Ridley B, Parker B. A Roadmap for Algal Technologies in the UK. Algal Bioenergy Special Interest Group. 2013.

8. Pulz O, Gross W. Valuable products from biotechnology of microalgae. In: Applied Microbiology and Biotechnology. 2004. p. 635–48.

9. Zittelli GC, Biondi N, Rodolfi L, Tredici MR. Photobioreactors for Mass Production of Microalgae. In: Richmond A, Hu Q, editors. Handbook of Microalgal Culture: Applied Phycology and Biotechnology. Secound. Blackwell Publishing Ltd.; 2013. p. 225–57.

10. Vieira V, Guerra L, Lapa J, Fonseca D, Costa L, Santos E. Microalgae Biotechnologies: Possible Frameworks from Biofuel to Biobased Products. In: Pereira L, editor. Algal Biofuels. Taylor & Francis, CRC Press; 2017. p. 1–29.

11. Tafreshi AH, Shariati M. Dunaliella biotechnology: methods and applications. J Appl Microbiol. 2009;107:14–35.

12. Masojídek J, Torzillo G. Mass Cultivation of Freshwater Microalgae. Ecol Eng. 2008;2226–35.

13. Tinoco NAB, Teixeira CMLL, Rezende CM. The Genus Dunaliella : Biotechnology and Applications. Rev Virtual Química. 2015;7(4):1421–40.

14. ALGATEC. Microalgas [Internet]. [cited 2009 Sep 5]. Available from: http://algatec.eu/microalgas/

15. Oren A. A hundred years of Dunaliella research : 1905 – 2005. Saline Systems. 2005;14:1–14.

16. Ye Z, Jiang J, Wu G. Biosynthesis and regulation of carotenoids in Dunaliella: Progresses and Prospects. Biotechnol Adv. 2008;26:352–60.

17. Lamers PP, Janssen M, Vos RCH De, Bino RJ. Exploring and exploiting carotenoid accumulation in Dunaliella salina for cell-factory applications. Trends Biotechnol. 2008;26(August):631–8.

18. Francavilla M, Trotta P, Luque R. Phytosterols from Dunaliella tertiolecta and Dunaliella salina: A potentially novel industrial application. Bioresour Technol. 2010;101(11):4144–50.

19. ABACUS [Internet]. [cited 2019 Sep 7]. Available from: http://www.abacus-bbi.eu/

20. Bendif EM, Probert I, Schroeder DC, de Vargas C. On the description of Tisochrysis lutea gen. nov. sp. nov. and Isochrysis nuda sp. nov. in the Isochrysidales, and the transfer of Dicrateria to the Prymnesiales (Haptophyta). J Appl Phycol. 2013;25(6):1763–76.

21. Heimann K, Huerlimann R. Microalgal Classification: Major Classes and Genera of Commercial Microalgal Species. In: Handbook of Marine Microalgae. Elsevier; 2015. p. 25–41.

22. Shi Q. Expression profiling of genes coding for abundant proteins in the alkenone body of marine haptophyte alga Tisochrysis lutea. BMC Microbiol. 2019;19:1–8.

23. Liu J, Sommerfeld M, Hu Q. Screening and characterization of Isochrysis strains and optimization of culture conditions for docosahexaenoic acid production. Appl Microbiol

Biotechnol. 2013;97:4785–98.

24. Alkhamis YA. Cultivation of Microalgae in Phototrophic , Mixotrophic and Heterotrophic Conditions. Faculty of Science & Engineering, Flinders University; 2015.

25. Borowitzka MA. Biology of Microalgae. In: Microalgae in Health and Disease Prevention. Elsevier Inc.; 2018. p. 23–72.

26. Kandilian R, Lee E, Pilon L. Radiation and optical properties of Nannochloropsis oculata grown under different irradiances and spectra. Bioresour Technol. 2013;137:63–73.

27. Huerlimann R, Nys R De, Heimann K. Growth , Lipid Content , Productivity , and Fatty Acid Composition of Tropical Microalgae for Scale-Up Production. Biotechnol Bioeng. 2010;107(2):245–57.

28. Guedes AC, Malcata FX. Nutritional Value and Uses of Microalgae in Aquaculture. Aquaculture. 2012;

29. Winwood RJ. Recent developments in the commercial production of DHA and EPA rich oils from micro-algae. Micro-organismes Prod lipides. 2013;20(6).

30. Passell H, Dhaliwal H, Reno M, Wu B, Ben A, Ivry E, et al. Algae biodiesel life cycle assessment using current commercial data. J Environ Manage [Internet]. 2013;129:103–11. Available from: http://dx.doi.org/10.1016/j.jenvman.2013.06.055

31. Huang X, Huang Z, Wen W. Effects of nitrogen supplementation of the culture medium on the growth , total lipid content and fatty acid profiles of three microalgae (Tetraselmis subcordiformis , Nannochloropsis oculata and Pavlova viridis). J Appl Phycol. 2013;25:129–37.

32. PHOTOFUEL [Internet]. [cited 2019 Sep 7]. Available from: http://www.photofuel.eu/home.php

33. BIOFAT [Internet]. [cited 2019 Sep 7]. Available from: biofat-project.eu

34. Tomaselli L, Masojídek J, Koblízek M, Torzillo G, Lee Y-K, Shen H, et al. Handbook of Microalgal Culture: Biotechnology and Applied Phycology. 1st ed. Richmond A, editor. Iowa, USA: Blackwell Science Ltd.; 2004. 1–479 p.

35. Yu Y, You L, Liu D, Hollinshead W, Tang YJ, Zhang F. Development of Synechocystis

sp. PCC 6803 as a Phototrophic Cell Factory. Mar Drugs. 2013;11:2894–916.

36. Bellou S, Moustogianni A, Makri A, Aggelis G. Lipids Containing Polyunsaturated Fatty Acids Synthesized by Zygomycetes Grown on Glycerol. Appl Biochem Biotechnol. 2012;166:146–58.

37. White D, Silkina A, Skill S, Oatley-Radcliffe D, Hende S Van Den, Ernst A, et al. Best Practices for the Pilot-Scale Cultivation of Microalgae. EnAlgae. 2015;1–16.

38. Martins A, Caetano NS, Mata TM. Microalgae for biodiesel production and other applications : A review. Renew Sustain Energy Rev. 2010;14:217–32.

39. Jerney J, Spilling K. Large Scale Cultivation of Microalgae : Open and Closed Systems. Methods Mol Biol. 2018;1–8.

40. Ugwu CU, Aoyagi H. Microalgal Culture Systems: An Insight into their Designs, Operation and Applications. Biotechnol 11. 2012;3:127–32.

41. Christenson L, Sims R. Production and harvesting of microalgae for wastewater treatment , biofuels , and bioproducts. Biotechnol Adv. 2011;29(6):686–702.

42. Show K, Yan Y, Lee D. Algal biomass harvesting and drying. In: Biomass, Biofuels and Biochemicals. Second. Elsevier B.V.; 2019. p. 135–66.

43. Gomes G. Separação Centrífuga. In: Sebenta de unidade curricular deProcessos de Separação IB. Escola Superior de Tecnologia do Barreiro, Instituto Politécnico de Setúbal; 2016.

44. Axelsson H, Madsen B. Centrifuges , Sedimenting. Encycl Ind Chem. 2012;1:494–519.

45. Shelef G, Sukenik A, Green M. Microalgae Harvesting and Processing : A Literature Review. 1984;(0).

46. Gomes G. Secagem e Liofilização. In: Sebenta de unidade curricular de Processos de Separação I B. Escola Superior de Tecnologia do Barreiro, Instituto Politécnico de Setúbal; 2016.

47. Sosnik A, Seremeta KP. Advantages and challenges of the spray-drying technology for the production of pure drug particles and drug-loaded polymeric carriers. Adv Colloid Interface Sci. 2015;1:1–70.

48. Kawachi M, Noel M-H. Sterilization and Sterile Technique. In: Andersen RA, editor. Algal Culturing Techniques. Elsevier Academic Press; 2005. p. 65–83.

49. Almeida A dos S. Validação do Processo de Esterilização de Refeições Prontas a Comer Enlatadas. Instituto Politécnico de Leiria; 2012.

50. Stanbury PF, Whitaker A, Hall SJ. Sterilization 5. In: Principles of Fermentation Technology. Third. Elsevier Ltd.; 2017. p. 273–333.

51. Carney LT, Lane TW. Parasites in algae mass culture. Front Microbiol. 2014;5(June):1–8.

52. Mendes A. Boas Práticas em Laboratórios de Microbiologia e Monitorização Ambiental. 2018. p. 2–59.

53. Stacey GN. Cell Culture Contamination. In: Cree IA, editor. Cancer Cell Culture: Methods and Protocols. secound. Springer Science+ Business Media; 2011. p. 79–91.

54. Monaghan S, Wall B, Page D, Fitzgerald J, McGuigan N, Kerr H, et al. Water Treatment Manual: Disinfection. 1st ed. Ireland: Environmental Protection Agency; 2011. 1–200 p.

55. Pulz O. Photobioreactors: production systems for phototrophic microorganisms. Appl Microbiol Biotechnol. 2001;57:287–93.

56. Sultana Y. Sterilization Methods and Principles. Pharm Microbiol Biotecnol. 2007;

57. Eyvaz M, Arslan S, Gurbulak E, Yulsel E. Textile Materials in Liquid Filtration Practices: Current Status and Perspectives in Water and Wastewater Treatment. In: Kumar B, Thakur S, editors. Textiles for Advanced Applications. 2017. p. 294–318.

58. Krug L, Erlacher A, Berg G, Cernava T. A novel , nature-based alternative for photobioreactor decontaminations. Sci Rep. 2019;(September 2018):1–10.

59. Ne CF. Bioreactor Engineering - Sterilization in Fermentation [Internet]. Available from: http://ocw.ump.edu.my/pluginfile.php/6878/mod_resource/content/2/Chapter 9.pdf

60. Coutteau P. Micro-Algae. In: Manual on the production and use of live food for aquaculture. 1996. p. 7–41.

61. Doran PM. Reactor Engineering. In: Bioprocess Engineering Principles. Secound.

Elsevier Ltd.; 2013. p. 761–852.

62. Gomes G, Santos C. Filtração com membranas. In: Sebenta de unidade curricular de Processos de Separação II B. Escola Superior de Tecnologia do Barreiro, Instituto Politécnico de Setúbal; 2016.

63. Gomes G. Esterilização. In: Sebenta de unidade curricular de Reatores Biológicos 2016- 2017. Escola Superior de Tecnologia do Barreiro, Instituto Politécnico de Setúbal; 2016.

64. A4F. Plataformas tecnológicas [Internet]. [cited 2019 Aug 2]. Available from: https://www.a4f.pt/pt/plataformas-tecnologicas

65. Botelho S, Luvielmo MDM, Geyer MC, Prá I. Potencialidades do uso do ozônio no processamento de alimentos. Ciências dos Aliment. 2011;32:659–82.

66. Ozone disinfection drinking water [Internet]. [cited 2019 Oct 1]. Available from: https://www.ozonetech.com/water-treatment/ozone-disinfection-drinking-water

67. Continuous Sterilization [Internet]. [cited 2019 Oct 9]. Available from: https://www.rpi.edu/dept/chem-eng/Biotech-Environ/Projects00/sterilize/continuous.html