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Development of an integrated process of membrane filtration for harvesting microalgae and recovery of high-value compounds

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Dedicada ao meu querido avô

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ABSTRACT

The work presented in this PhD thesis aims at developing an integrated method for harvesting *Dunaliella salina*, both in the non-carotenogenic and in the carotenoid-rich phases, using membrane processes and centrifuges. Additionally, the ultimate goal is the recovery of high-value compounds for application in the food, cosmetic and pharmaceutical industries. *Dunaliella salina* is a halotolerant green microalga, which under low nitrogen and high salinity conditions, produces carotenoids. This microalga is very fragile due to lack of a rigid cell wall and, consequently, can be easily ruptured when an excessive shear stress is applied.

Firstly, the pre-concentration of *Dunaliella salina* by membrane filtration prior to a final harvesting step by centrifugation was performed. The aim of the integrated harvesting process was to minimise the overall energy expenditure and reduce capital costs, while assuring a minimal loss of cell integrity. For the harvesting experiments the main parameters were optimised, namely: cross-flow velocity ($v_{cross-flow}$), transmembrane pressure (TMP), membrane molecular weight cut-off (MWCO), backflush protocol and permeate volumetric flux (J_V). The harvesting of non-carotenogenic *Dunaliella salina* by ultrafiltration, under controlled transmembrane pressure, allowed a final concentration factor of 5.9 and an average permeate flux of 31 L/(m^2.h). The harvesting of carotenoid-rich *Dunaliella salina*, under controlled permeate flux, reached a concentration factor of 10, at a permeate volumetric flux of 21 L/(m^2.h). The integrated process led to a reduction of Total Cost of Ownership (TCO) and energy consumption of 70% and 76%, respectively, when comparing with harvesting only with centrifuges (one-step approach).

Additionally, several oxidation processes were studied for treatment of the permeate recovered, after membrane harvesting, in order to assess its recycle as cultivation medium for *Dunaliella salina*. The permeate was treated either by ozonation or by UV radiation, and the different types of permeate were tested as cultivation media. The results obtained indicate that the permeate treated by UV with addition of H_2O_2 was best suited for the growth of carotenoid-rich *Dunaliella salina.* Nevertheless, the use of untreated permeate seems a reasonable alternative, considering that it might be the most cost efficient solution.

Finally, a *Dunaliella salina* biorefinery process was developed for the recovery and fractionation of added-value compounds, with a particular focus on the recovery of polar lipids. This work combined extraction with REACH solvents, a saponification reaction and organic solvent nanofiltration processes to obtain distinct fractions of compounds. Overall, high recoveries were obtained (85% of carotenoids, 86% of glycerol, 94% of polar lipids and 95% of proteins), as well as fractions with high product enrichment in polar lipids and in glycerol.

Keywords: *Dunaliella salina*, Membrane harvesting, Permeate recycling, Microalgae biorefinery, High-value products**,** Economic evaluation

RESUMO

O trabalho apresentado nesta tese de doutoramento tem como objetivo desenvolver um método integrado de colheita de *Dunaliella salina*, quer em fase não-carotenogénica como em fase rica em carotenoides, utilizando processos de membranas e centrífugas. O objetivo final é a recuperação de compostos de valor acrescentado para utilização nas indústrias alimentar, cosmética ou farmacêutica. A *Dunaliella salina* é uma microalga verde halotolerante que, sob condições de baixo teor em azoto e elevada salinidade, produz carotenoides. Esta microalga é muito frágil devido à ausência de parede celular rígida e pode ser facilmente rompida quando uma tensão de corte excessiva é aplicada.

Em primeiro lugar, foi realizada a pré-concentração de *Dunaliella salina* por filtração com membranas, de forma a facilitar a sua concentração final por centrifugação. O objetivo do processo integrado de colheita é minimizar os gastos de energia e reduzir os custos de capital, assegurando uma perda mínima de integridade celular. Os estudos de colheita celular foram realizados com o objetivo de otimizar os parâmetros operacionais mais relevantes, nomeadamente: a velocidade de escoamento ($v_{cross\text{-}flow}$), a pressão transmembranar (TMP), o limite de corte da membrana (MWCO), o procedimento de retro-lavagem *in-situ* e o fluxo volumétrico de permeado (J_V). A colheita de *Dunaliella salina* não carotenogénica por ultrafiltração, a pressão transmembranar controlada, atingiu um fator de concentração de 5.9 e um fluxo médio de permeado de 31 L/(m^2.h). A colheita de *Dunaliella salina* rica em carotenoides, realizada sob condições controladas de fluxo de permeado, atingiu um fator de concentração de 10, com um fluxo volumétrico de permeado de 21 L/(m^2.h). O processo integrado conduziu a uma redução do custo total de propriedade (TCO) e do consumo de energia de 70% e 76%, respetivamente, quando comparado com a colheita realizada apenas por centrifugação.

Adicionalmente, foram estudados vários processos de oxidação para o tratamento do permeado, após a colheita por membranas, de modo a avaliar a sua reutilização como meio de cultivo de *Dunaliella salina*. O permeado foi tratado alternativamente por ozonação e por radiação UV, e os diferentes tipos de permeado foram testados como meio de cultivo. Os resultados obtidos indicam que o permeado tratado por UV com adição de H_2O_2 foi o mais adequado para o crescimento de *Dunaliella salina* rica em carotenoides. No entanto, o uso de permeado não tratado parece ser igualmente uma alternativa razoável, visto ser a solução mais económica.

Finalmente, foi desenvolvido um processo integrado de biorefinaria de modo a recuperar e fracionar compostos de valor acrescentado, nomeadamente lípidos polares. Este trabalho combinou a extração com solventes REACH, uma reação de saponificação e processos de membranas resistentes a solventes de forma a obter frações enriquecidas em compostos distintos. No final, foram obtidos graus elevados de recuperação (85% de carotenoides, 86% de glicerol, 94% de lipídios polares e 95% de proteínas), além de frações com elevado enriquecimento em lípidos polares e em glicerol.

Palavras-chave: *Dunaliella salina*, Colheita por membranas, Reutilização do permeado, Biorefinaria de microalgas, Produtos de valor acrescentado, Avaliação económica

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Abbreviations

AFDW – Ash-free dry weight

AOPs – Advanced oxidation processes

BSA – Bovine serum albumin

CF – Concentration factor

CAPEX – Capital expenditure

Car/Chl ratio – Carotenoids/chlorophylls ratio

DG – Diglycerides

DPPH - 2,2-diphenyl-1-picrylhydrazyl

EFSA – European Food and Safety Authority

EPS – extracellular polymeric substances

FDA – Food and Drug Administration

FFAs – Free fatty acids

HPLC – High performance liquid chromatography

HPSEC – high performance size exclusion chromatography

ICP-MS – Inductively Coupled Plasma Mass Spectrometry

LC-MS – Liquid Chromatography with Mass Spectrometry

LP – Low-pressure

MBRs – Membrane bioreactors

MF – Microfiltration

MG - Monoglycerides

MP – Medium pressure

OPEX – Operational expenditure

Ozonation+H_2O_2 – Ozonation with the addition of hydrogen peroxide

PDA – Photodiode array

PES – Polyethersulfone

PS – Polysulfone

RI – Refractive index

SPT – Spiral plate technology

TCO – Total cost of ownership

TG - Triglycerides

UF – ultrafiltration

UV+H_2O_2 – UV with the addition of hydrogen peroxide

UV+pH9 – UV with an initial pH adjustment to 9

Variables

A – membrane area (m^2)

b – length of UV-cell (cm)

J_V – Volumetric permeate flux ($L/(m^2.h)$)

m_0 – initial mass (kg) or (g)

$m_{permeate}$ – mass of permeate (kg) or (g)

MWCO – molecular weight cut-off (kDa)

P – permeability ($L/(m^2.h.bar)$)

O_3 – concentration of ozone (mg/L)

Q_{feed} – feed flow rate (m^3/h) or (m^3/day) or ($m^3/year$)

Q_V – volumetric flow rate (L/min)

Re – Reynolds number (-)

TMP – Transmembrane pressure (bar)

$v_{cross-flow}$ – cross-flow velocity (m/s)

t – time (h) or (s)

T – temperature (ºC)

V – volume of the sample (L)

ΔAbs – variation of absorbance between the blank and the sample (nm)

Greek symbols

ρ - density (kg/m^3)

τ - shear stress (Pa)

1 BACKGROUND AND MOTIVATION

1.1 Background and Motivation

Microalgae are a promising feedstock as a source of biofuels, proteins and other bioactive compounds, which may contribute to address the problem of growing demand for renewable sources, in response to the increase of world population and the need for sustainable sources of energy and food. Microalgae are important photosynthetic organisms that convert sunlight, CO_2 and inorganic compounds, into food, feed, biofuels and other products, contributing to the sequestration of CO_2. Although microalgae have been mostly applied in biofuel and bioenergy processes, these fields are not yet economically viable nor competitive [1].

A considerable interest has been raising on their use for the production of compounds for nutraceutical, food, feed, chemical, pharmaceutical and cosmetic applications [2]. So far, the food industry is the largest application of microalgae. The Food and Drug Administration (FDA) and the European Food and Safety Authority (EFSA) have recognised several microalgae as safe for food use [3]. Animal feed and fertilizers are also fields of interest for microalgae.

Microalgae are able to growth in diverse environments under wide range of values of pH, temperature and nutrients. Also, microalgae may be found / cultivated in different ecosystems such as oceans, lakes and non-arable lands [4]. The main disadvantages of using microalgae are their low biomass productivity, low efficiency of harvesting processes and relatively high costs of production [5,6].

There is an huge potential in the exploitation of green halotolerant microalgae *Dunaliella salina,* which grows in extreme saline conditions, from 0.05 to 5.0 M NaCl [7]. This microalga is very fragile and shear sensitive due to lack of a rigid cell wall [8,9]. In hypersaline conditions, low nitrogen and high light intensity, *Dunaliella salina* presents a red colour, due to the accumulation of β-carotene, which may represent up to 10% of its dry weight [10]. The most abundant stereoisomers of β-carotene present are: all-*trans* and 9-*cis*-β-carotene. β-carotene is an essential nutrient and its market has increased due to the use as a natural colourant, food and vitaminic supplement and cosmetic additive [8]. The carotenoids' market was estimated at $1.24 billion in 2016 and it will reach $1.53 billion by 2021 [11].

This PhD thesis addresses the downstream processing of this microalga, which cost may represent up to 70-80% of the overall cost of production of a target compound [12,13].

The downstream processing comprises cell harvesting, cell disruption, extraction of target compounds and their fractionation and purification. The harvesting of microalgae should be economically viable and enabling for the recycling of the cultivation medium [14]. However, this process is commonly considered has a major bottleneck for the economic viability of microalgae production. It may represents up to 20-30% of the total production cost [15,16], due to the low cell concentration, small size and similar density to water [17]. Several mechanical, biological, chemical and electrical processes have been applied for microalgae harvesting, such as: flocculation, flotation, sedimentation, filtration and centrifugation [15,16,18–20]. For large scale applications, the most common and efficient method is centrifugation

[15–17,21], but it requires high capital and energy costs. The other harvesting methods are time consuming and require the use of chemical products, with the exception of membrane filtration. Table 1.1 presents the most common microalgae harvesting processes, their advantages and disadvantages.

Table 1.1. Description of the main microalgae harvesting processes, their advantages and disadvantages.

Harvesting method	Description	Advantages	Disadvantages	References
Flocculation	• Microalgae cells form aggregates, which can be separated by gravity sedimentation. • Technique used in wastewater treatment	• Simple method • Low or no energy required	• Use of chemicals •Might be toxic to microalgae • Difficult to apply in larger scales • Contamination of microalgae biomass • Interfere with food or feed applications	[17,22,23]
Sedimentation	• Microalgae in suspension settled through gravitational forces	• Simple and inexpensive • Energy efficient	• Time consuming • Biomass deterioration • Low concentration achieved • Requires large areas	[17,22,24,25]
Flotation	• Use air or gas bubbles as lifting force for cell/particle separation	• Low space required • Short operation time	• Not suitable for large scale • High cost and energy intensive	[26–28]
Electrical based processes	• Apply an electric field for the concentration of the algal cells	• No chemical addition • Environmental compatibility	• High power consumption • High electrode cost • Not suitable for large scale	[27,29]
Filtration	• Separation of algae cells through pressure-driven processes	• Easy to scale-up • Low energy required • Simple and efficient method • Separation of shear sensitive species	• Membrane fouling • Not suitable for high biomass concentration • Membrane replacement represent the major costs	[15,19,21,30]
Centrifugation	• Use centrifugal forces	• Very efficient • Simple method • Suitable for large scale application	• High energy cost • High capital cost	[15,24,31]

There is a key challenge on microalgae harvesting: the development of an efficient and economic process. Membrane technology may play an important role in microalgae harvesting [32–34]. This technology presents important advantages in relation to other microalgae harvesting processes: continuous and simple operation, environmental friendly and safe, and does not require the addition of chemical additives [35]. Also, the membranes market price is decreasing, turning this technique very appealing for microalgae harvesting at industrial level [36].

Most of the industrial systems use centrifugation for microalgae harvesting [37]. However, this process is only suitable for the recovery of high added-value compounds, due to the high investment and

operating costs. Therefore, it is worth to consider the possibility to combine membrane filtration, for pre-concentration of the dilute microalgae cultivation stream, with a final concentration step by centrifugation. In the first-step, most of the water is removed from the cultivation medium by membrane filtration and then, the concentrated stream is further processed by centrifugation allowing for achieving the required biomass concentration. Considering the volume reduction achieved in the first step, the number of centrifuges required for a given production capacity is significantly reduced, as well as the associated costs [21].

Besides the harvesting process, the cultivation costs represent a significant charge in the microalgae production, namely to the large amount of water and nutrients required [14]. The recycling of water and nutrients may represent a relevant saving, with a direct impact on the process economy and sustainability [38,39]. After the harvesting process, around 85% of water is discarded, which represents a high impact in terms of the water footprint [40]. The water removed during the harvesting is characterised by the presence of important nutrients for microalgae cultivation. Therefore, the recycling of the permeate recovered during the first stage of harvesting, by membrane filtration, may represent an attractive way to reduce the consumption of fresh water and nutrients [39,41]. The production of microalgae biomass has been estimated to consume about one metric ton of water per kg of biomass produced [39]. Moreover, 1 ton of microalgae biomass demands 200 ton of CO_2, 10 ton of nitrogen (N) and 1 ton of phosphorus (P) [42]. Yang et *al.* reported that 3.73 m^3 of water, 0.33 kg of nitrogen and 0.71 kg of phosphate are required for the production of 1 kg of microalgae biodiesel [40].

The recycling of culture medium after microalgae harvesting has been studied by several authors [40,43–45]. Harvesting by membrane filtration and recycling of the water recovered are advantageous because the water collected as permeate is free of chemicals, which is not true if flocculation or coagulation are used as harvesting processes [21]. Microfiltration and ultrafiltration membranes completely retain the microalgae cells [44,46], and produce a clear permeate which might be used directly / after post treatment as a recycled medium.

Previous work demonstrated that recycling medium can reduce the water demand and save nutrients. The recycling of cultivation medium for *Chlorella vulgaris* enables a reduction of 55% of nitrogen and phosphorous used [40]. Another work [47] reported that *Chlorella vulgaris* was able to grow in the recycled culture broth and the productivity of carbohydrates, proteins and lipids increased. A membrane photobioreactor (MBR) was tested for both harvesting and permeate recycling of *Chlorella vulgaris*, and this system enabled a reduction of 77% of water footprint, with no negative impact on microalgae growth [44]. Likewise, the halotolerant microalgae *Tetraselmis* could grow in recycled medium with no negative influence on biomass productivity [48].

Although the numerous studies performed with medium recycling, with a positive impact on microalgae cultivation, the excreted metabolites may hinder the process due to their progressive accumulation. The presence of extracellular compounds in the recycled medium may inhibit microalgae growth and potentiate the growth of other microorganisms, such as bacteria [43,49,50]. Therefore, besides microalgae cultivation with a direct water recycling, it is worth to assess the post-treatment of the recycled water and its impact. Membrane filtration, centrifugation and adsorption in activated carbon

have been proposed for water recycling treatment [43,44,51,52]. The permeate recovered from microalgae harvesting by membrane filtration is free from large molecular weight contaminants, which is advantageous from a recycling point of view. However, the permeate may contain other nutrients, cell debris and excreted organic compounds [43,53]. Consequently, the need for further treatment of the permeate before being recycled has to be evaluated.

Ozonation and UV radiation are the most common methods used for disinfection and water treatment [52,54,55]. Ozone is a very selective and powerful oxidant, that reacts with some water matrix components [56]. The main drawback is the formation of undesired by-products due to the reaction of matrix components with OH radicals and ozone. Depending on the water compounds, the main ozonation inorganic by-products identified are bromate, iodate, sulfate, chloride and nitrate [54,57]. Also, some organic by-products have been described, such as: alcohols, esters, ketones, carboxyl acids and aldehydes [57]. Previous studies demonstrated that ozonation may be applied for water recycling of *Nannochloropsis gaditana*, maintaining the quality of the biomass culture [52]. Regarding the UV application, it is mainly used for disinfection in water treatment plants, lowering the concentration of chlorine applied as final disinfectant and consequently the levels of chlorination by-products formed [55]. Besides disinfection, UV technology may also degrade organic compounds by direct and indirect photolysis. Additionally, advanced oxidation processes (AOPs) can be applied. These processes involve the formation of extremely reactive OH radicals, enhancing the reactivity and shortening the reaction times [54], which can be performed by increasing the pH or by addition of H_2O_2.To the best of our knowledge, there are no studies using advanced oxidation processes, aiming the recycling of the culture medium for *Dunaliella salina* cultivation.

Microalgae have a high potential to be used as feedstock for biorefineries, aiming the production of several compounds of interest. There is an important economic need to turn microalgae biorefineries into profitable processes, moving away from microalgae cultivation for solely biofuel, aiming a portfolio of products, including added-value compounds. The microalgae biofuel approach is not economically viable at the moment, unless other added-value compounds are produced [58].

Biorefinery is described as a process that transforms biomass and biomass wastes into commodities such as biofuel, energy, plastics, chemicals and also high-value products [30,59–61]. Microalgae biorefineries are emerging and there are several microalgae biorefineries described on the literature [1,2,5,62,63]. However, the downstream processing of microalgae greatly influences the sustainability and the efficiency of the biorefinery [2,64].

Microalgae can synthesise numerous bioactive compounds, such as: pigments (carotenoids and chlorophylls), lipids, proteins and carbohydrates, which may be applied in food, feed, cosmetic and pharmaceutical applications (see Figure 1.1) [5,59]. Nevertheless, the biochemical composition is dependent on the microalgae species and cultivation or environmental conditions, such as temperature, pH, salinity, light intensity and nutrients [58]. The cultivation conditions can be tuned in order to favour the content in specific target compounds [65].

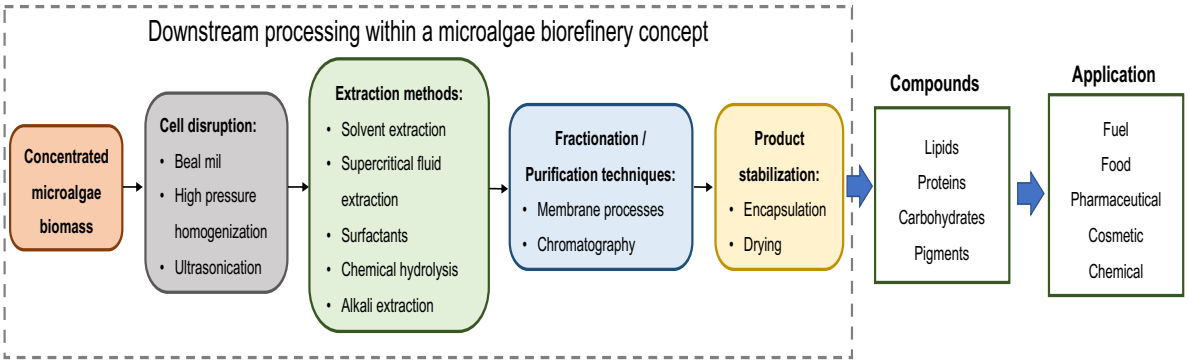

Figure 1.1. Schematic representation of the downstream processing of a microalgae biorefinery, including the main steps, compounds and applications.

For the development of a sustainable biorefinery, it is essential to focus on the main added-value compounds that could be recovered at relative abundance, in order to overcome the production costs [66]. The first step of the microalgae biorefinery is the cell disruption, for releasing of compounds, and then, the extraction and fractionation / purification of target compounds. For the fractionation of value-added compounds, one of the most promising techniques is membrane processing. Membrane technology is widely used in several areas and industries. However, the membrane application in biorefinery processes is a novel area and there are only a few examples of microalgae downstream processes using membranes [30,60,63]. In some biorefineries, there is a need to use organic solvents, therefore membrane processes can be applied, namely using organic solvent resistant membranes, which is an emerging technology.

It is therefore necessary to turn the microalgae biorefinery into more sustainable and economic processes, focusing on the recovery of added-value products, improving significantly the revenues of the microalgae industry. *Dunaliella salina* has been extensively studied due to the high production of β-carotene (up to 10% of dry weight) [10], and glycerol (50% of its dry mass) [67,68], to maintain the osmotic balance [8]. However, few studies are reported on the development of *Dunaliella salina* biorefineries [69–72]. Currently, *Dunaliella salina* is centrifuged, washed and spray-dried, for further solvent extraction (hexane). After extraction, membrane filtration is used to recover the hexane, and the retentate (rich in carotenoids) is processed by vacuum distillation to purify the β-carotene and enable hexane recycling [72]. This microalgae biorefinery is mainly focus on β-carotene and the remaining solid residue is used as fertilizer. Nevertheless, more valuable products should be recovered from this microalgae biorefinery, in order to turn the microalgae production economically viable.

1.2 Research strategy and Objectives

This PhD thesis was carried out within the context of an European Project entitled “D-factory: the microalgae biorefinery”. The aim was to set a world benchmark for a sustainable biorefinery based on *Dunaliella salina* biomass.

The research strategy of this PhD project comprises the development of a membrane filtration process combined with centrifugation, which enables the harvesting of both non-carotenogenic and carotenoid-rich *Dunaliella salina*. These strains present similar morphologic characteristics: small size (10-12 μm)

and lack of a rigid cell wall. However, they produce different valuable compounds: peptides and enzymes for non-carotenogenic *Dunaliella salina* and carotenoids and glycerol for carotenoid-rich *Dunaliella salina*. Furthermore, cells of carotenoid-rich *Dunaliella salina* are more sensitive to shear than non-carotenogenic cells.

Therefore, for non-carotenogenic *Dunaliella salina* a membrane harvesting process was studied for pre-concentrating cells under controlled transmembrane pressure conditions. In the case of carotenoid-rich *Dunaliella salina*, the harvesting process was performed under controlled permeate flux, using mild operating conditions suitable for these cells. The harvesting of carotenoid-rich *Dunaliella salina* by cross-flow ultrafiltration under controlled transmembrane pressure conditions was also performed but it showed a modest performance, due to a severe loss of cell integrity (data not shown in the thesis).

After optimisation of membrane harvesting, a large amount of permeate was produced. Therefore, the strategy was to use the permeate produced as a cultivation medium for carotenoid-rich *Dunaliella salina*. Several oxidation techniques were assessed for post-treatment of the permeate, due to the presence of organic compounds released during the harvesting step. The use of recycling medium can reduce the cultivation of microalgae costs and also reduce the water footprint.

Finally, a biorefinery concept based on carotenoid-rich *Dunaliella salina* is proposed, based on a downstream process where different compounds are targeted. Two main routes were established: an organophilic route and a hydroethanolic route. In the first one, through the combination of a saponification reaction and membrane processing, distinct fractions enriched in carotenoids and free fatty acids (FFA) were produced. Regarding the hydroethanolic route, two strategies were applied: 1) performing an organic solvent nanofiltration for the recovery of an enriched fraction of glycerol and 2) performing an acetone extraction for the recovery and purification of polar lipids.

This work demonstrates the importance of the harvesting process in order to reduce energy and capital costs, using an integrated process of membrane filtration coupled with centrifugation. This procedure also offers the potential advantage of minimising the loss of added-value compounds, using mild conditions during harvesting. Furthermore, the importance of reducing the harvesting costs is demonstrated. A strategy for the recycling of cultivation medium is presented in order to reduce the water footprint associated with microalgae production. Finally, this work discusses the potential of using *Dunaliella salina* as a feedstock for the production of different compounds, improving the revenues of the microalgae industry. Figure 1.2 shows the main processing steps discussed in this PhD work.

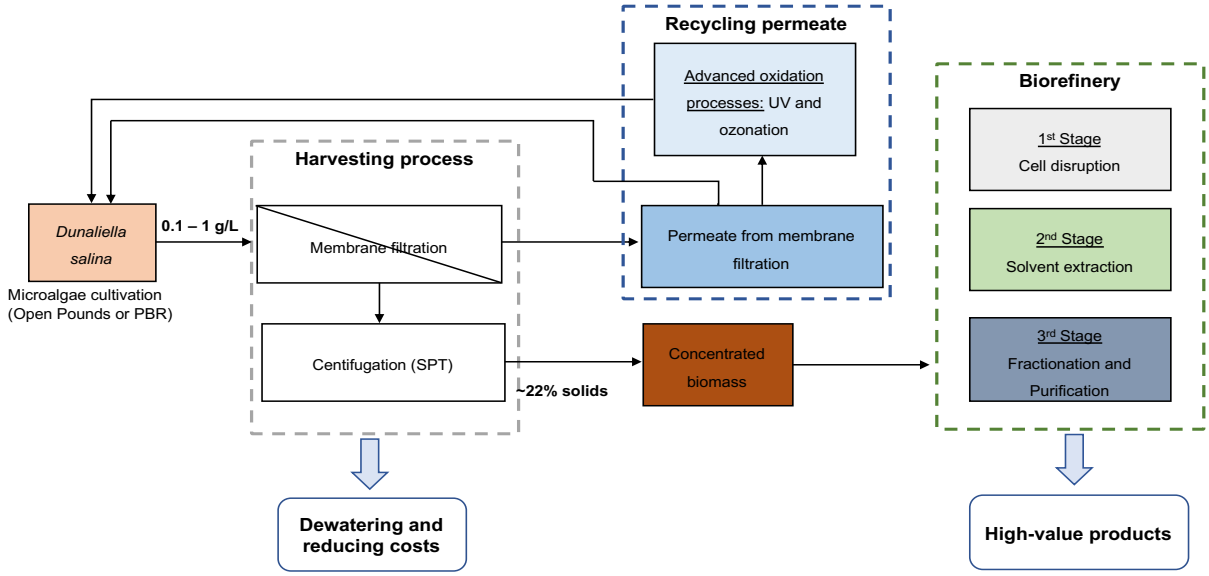

Figure 1.2. Schematic representation of several tasks involved in this PhD work.

The main objectives of this work are:

I. Study the pre-concentration of *Dunaliella salina* biomass (non-carotenogenic and carotenoid-rich) by membrane filtration prior to centrifugation, in order to reduce the overall energy and capital costs. Additionally, maximise the volumetric fluxes while maintaining cell integrity at high concentration factors.
II. Evaluate the permeate produced during harvesting of carotenoid-rich *Dunaliella salina* to be used as cultivation media, in order to reduce the microalgae production costs, specifically the high costs associated with the cultivation medium. Particularly, the assessment of the permeates to be recycled as cultivation media, comparing different ways of post-treatment.
III. Design and propose a sustainable biorefinery scheme, starting from carotenoid-rich *Dunaliella salina*, aiming the recovery of distinct fractions of compounds.

1.3 Thesis Outline

The work performed during this PhD is organised in the following way: a first chapter where the thesis background, motivation and outline is presented, followed by four chapters that correspond to scientific original papers accepted or submitted for publication, which describe the experimental work performed and the results obtained, and finally a chapter that presents the overall conclusions of the work and suggestions for future work.

Additionally, along this manuscript there are several denominations related with the microalga under study. The first part of the work was performed with non-carotenogenic *Dunaliella salina*, which for simplicity sometimes is named “green” *Dunaliella salina*, due to the low carotenoids’ content and the green colour presented. Then, carotenoid-rich *Dunaliella salina* was used, and for simplicity it is denominated “orange” *Dunaliella salina*, associated with the high concentration of carotenoids and the orange colour.

A summary of the content of each chapter is described below:

Chapter 1: Background and Motivation – Describes the background and motivation for this PhD, the main objectives and finally describes the thesis outline.

Chapter 2: Harvesting of non-carotenogenic *Dunaliella salina* by membrane filtration at pilot scale – Describes an integrated method using membrane filtration and centrifuges for the harvesting of "green" *Dunaliella salina* at pilot scale. For the optimisation of the membrane harvesting process, operating at constant transmembrane pressure, several parameters were evaluated, namely: cross-flow velocity, transmembrane pressure, backflush protocol and molecular weight cut-off of the membrane. Furthermore, a consecutive harvesting approach was assessed in order to reach higher concentration factors. Additionally, a techno-economic analysis of the integrated process was performed. The content of this chapter was published in "Separation and Purification Technology (2018), Vol. 190, 252-260".

Chapter 3: Development of an integrated process for harvesting carotenoid-rich *Dunaliella salina* at laboratorial and pilot scales – Reports the pre-concentration of carotenoid-rich *Dunaliella salina* ("orange") by membrane filtration prior to a final concentration step by centrifugation. The relevant operating parameters, namely: cross-flow velocity, molecular weight cut-off, relaxation protocol and volumetric permeate flux were assessed in order to select the optimal operating conditions. The harvesting of *Dunaliella salina* by membrane filtration under controlled permeate flux was performed at laboratorial and pilot scales. Finally, the comparison of the Total Cost of Ownership (TCO) and the consumption of energy were performed for the two-step approach (membrane processing prior to centrifugation) and the one-step approach (centrifugation). The content of this chapter was submitted to the journal "Separation and Purification Technology".

Chapter 4: Recycling of *Dunaliella salina* cultivation medium by integrated membrane filtration and advanced oxidation – Presents several oxidation processes for the treatment of permeate produced during the membrane harvesting of carotenoid-rich *Dunaliella salina*. The permeate was treated either with UV radiation or by ozonation, and the most relevant operating variables (namely the increase of pH and the addition of H_2O_2) were studied. Untreated and treated permeates were assessed as a cultivation medium for carotenoid-rich *Dunaliella salina*, evaluating the cell growth, the carotenogenesis process, and the chemical and microbiological quality of the resulting cultivation medium. The content of this chapter was published in "Algal Research (2019), Vol. 29, 101460", (available online).

Chapter 5: *Dunaliella salina* based biorefinery: sustainable recovery of carotenoids, polar lipids and glycerol – Describes a sustainable process for the production of several fractions produced from *Dunaliella salina* biomass, namely fractions rich in carotenoids, polar lipids, glycerol and proteins. The biorefinery scheme proposed integrates solvent extraction (REACH solvents) and membrane filtration, in order to recover different classes of compounds. Additionally, a techno-economic assessment of the biorefinery proposed was performed.

Chapter 6: Conclusions and Future Work - Presents the overall conclusions and results of this PhD project and suggestions for future work.

2 HARVESTING OF NON-CAROTENOGENIC *DUNALIELLA SALINA* BY MEMBRANE FILTRATION AT PILOT SCALE

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The author was involved in planning and performing the experiments, as well as on the discussion and interpretation of the results and preparation of the manuscript.

2.1 Summary

The microalga *Dunaliella salina* is industrially produced due to its high content in carotenoids induced by low nitrogen and high salinity conditions. *Dunaliella salina* with low carotenoids content also produces other added-value compounds, however its recovery has hardly been studied. This work aims to examine the potential of pre-concentrating *Dunaliella salina* by membrane processing prior to a final harvesting step by low-shear centrifugation. The aim is to minimise the overall energy expenditure and reduce capital costs, while assuring a minimal loss of cell integrity. This task is challenging, considering the sensitivity of *Dunaliella salina* to shear. Harvesting of *Dunaliella salina* by ultrafiltration allowed reaching a final concentration factor of 5.9, with an average permeate flux of 31 L/(m^2.h). The Total Cost of Ownership and energy consumption for harvesting are respectively 52% and 45% lower when applying a two-step approach with pre-concentration (ultrafiltration) compared to only harvesting by centrifugation.

2.2 Introduction

Dunaliella salina, a halophilic microalga, is a unicellular biflagellate [73] which grows in salinities ranging from 0.05M to 5M NaCl [68,74]. This microalga is very fragile due to lack of rigid cell wall [9], and can be easily ruptured when an excessive shear stress is applied. Dunaliella salina is produced industrially due to its high content in carotenoids, which market estimation is around $1428.12 million in 2019 [11]. The carotenoids of *Dunaliella salina* are induced by low nitrogen and high salinity conditions in a process that has been extensively studied [67,75,76]. Cells are commercialised as a dried algal supplement for food application [67], without fractionation and purification of any intracellular compound. Currently, during the harvesting of *Dunaliella salina*, its intracellular content is partially released into the aqueous cultivation medium due to the high shear conditions applied, including high value enzymes and, under high salinity, glycerol. The recovery of enzymes, peptides and other valuable compounds from *Dunaliella salina* has not been studied thoroughly. Carbonic anhydrase (involved in microalgae CO_2 uptake [9,77]) and glycerol 3-phosphate dehydrogenase (involved in the glycerol biosynthesis [78]) are commercially interesting enzymes present in *Dunaliella salina*. Carbonic anhydrase and glycerol 3-phosphate dehydrogenase are overexpressed in *Dunaliella salina* with low

carotenoids' content respectively at pH 8-9 [9] and high salinity (4.5 M NaCl) conditions [78]. For simplicity, *Dunaliella salina* with low carotenoids' content will be named green *Dunaliella salina* along this manuscript.

Microalgae harvesting may contribute to about 20-30% of the total cost of microalgae production [15,16]. In fact, microalgae harvesting involves high energy costs due to the low biomass concentration, their small size (3–30 μm) and similar density to water. Several harvesting techniques have already been used, such as centrifugation, flocculation, flotation, sedimentation and filtration [15,16,18–20]. Centrifugation is the fastest and most common and efficient harvesting technology [15–17,21], especially suitable for concentrated biomass. However, centrifugation requires high capital investment and energy costs. In order to increase the microalgae concentration up to 22% dry weight, the energy demanded reaches 8 kWh/m^3 [79]. Using a Spiral Plate Technology (SPT) a reduction in energy of centrifugation can be achieved. On average, a SPT centrifuge has an energy consumption of 2.5 kWh/m^3. Besides the lower energy consumption, the SPT centrifuge also reduces the shear on the algae. For very fragile algae like the *Dunaliella salina* strain this is a very important advantage [80]. Power consumption of SPT is built up of the energy for the pumps and the energy needed for acceleration of the water in the rotating drum. In the SPT, the feed flows into a rotating drum, the algae settle on the plates in the rotating drum due to centrifugal forces and the effluent flows out of the drum.

Membrane processes may play a relevant role in microalgae harvesting [32–34]. Membrane technologies are quite suitable for processing *Dunaliella salina*, as its culture (with around 0.1 g/L of dry weight of algae) is even more diluted than other common microalgae cultures, such as *Chlorella vulgaris* (0.4 g/L) or *Nannochloropsis oculata* (8.4 g/L) [20]. Membrane technologies can also be operated under gentle transmembrane pressure and moderate cross-flow velocity, in order to keep low shear conditions for fragile cells [15]. In Rose *et al.*, the harvesting of orange *Dunaliella salina* by cross-flow ultrafiltration was performed producing concentrates using a polyethersulfone membrane (with not specified pore size), although no results of cell integrity were shown [81]. Pumping energy required for cross-flow membrane filtration is in general 0.5 – 1.5 kWh/m^3 of algal medium [82]. Considering the difficulties of membrane processing to bring microalgae concentration from extremely low values up to a 22% dry weight, membrane technology should be regarded as a potential alternative for microalgae pre-harvesting prior to concentration by centrifugation. Integration of microalgae pre-harvesting and concentration by centrifugation may lead to a decrease of the overall energy demand and investment cost [20,21]. Energy consumptions of integrated membrane-centrifugation systems and of traditional centrifugation were estimated respectively as 0.169 kWh/kg and 0.5 kWh/kg of dry weight of algae [20]. The increase in microalgae concentration, achieved by harvesting with membranes, has no impact on the SPT power consumption.

In this work, the pre-concentration of green *Dunaliella salina* by membrane processing prior to centrifugation is evaluated. This procedure offers also the potential advantage of minimizing the loss of high added-value compounds present in green biomass, such as enzymes with commercial interest and bioactive peptides. This study aims at pre-concentrating green *Dunaliella salina* with reduced

energy expenditure, at high permeate fluxes and concentration factors, while maintaining cell integrity, which is challenging due to the shear stress sensitivity of *Dunaliella salina*.

Green *Dunaliella salina* was processed in a pilot membrane cross-flow unit. The relevant operating conditions were studied – cross-flow velocity ($v_{cross-flow}$), transmembrane pressure (TMP) and backflushing interval – as well as the molecular weight cut-off (MWCO) of the membrane. The overall efficiency of the process was quantified by the volumetric permeate flux (J_V) and loss of cell integrity. In addition, the pre-concentration of *Dunaliella salina* in three consecutive batches was also performed, with the optimised operating conditions previously determined, aiming at achieving cell integrity at higher concentration factors.

In order to compare Total Cost of Ownership (TCO) and energy consumption between harvesting with pre-concentration and harvesting with only centrifuges, extra measurements have been done, where a 14-membrane element unit (Liqoflux LF14AL22) was installed in combination with a Spiral Plate Technology centrifuge (Evodos 25 SPT).

2.3 Materials and Methods

2.3.1 Materials

Green *Dunaliella salina* was originally isolated from open ponds of NBT Natural Beta Technologies, Ltd (Israel) by MBA Marine Biological Association of the United Kingdom and it was scaled-up and provided by A4F - Algae for Future (Portugal). Fresh green algae cultures with high N concentration (corresponding to biomass with low carotenoids' content, particularly with a ratio of carotenoids/chlorophylls below 0.5), 1.5M NaCl and pH between 7 and 8 in the culture medium were sampled at different initial concentrations from $2x10^5$ to $3x10^5$ cells/mL, for the different experiments performed. In the case of consecutive harvesting batches, the initial algal concentration was $4x10^5$ cells/mL. For optimisation of the microalgae pre-concentration, a membrane unit from Liqoflux was used where two types of asymmetric polyethersulfone (PES) capillary membranes, with the active layer inside the fibres, were tested. The PES membranes, provided by Liqoflux, were an ultrafiltration membrane with a nominal molecular weight cut-off (MWCO) of 150 kDa and a microfiltration (MF) membrane with a nominal pore size of 0.1 µm. The membrane permeability of clean water was 320 and 1070 L/(m^2.h.bar) for ultrafiltration and microfiltration, respectively. Each type of membrane elements comprised 832 fibres with a length of 0.42 m, an internal diameter of 2.2 mm, and a total filtration area of 2.4 m^2. Filtration was performed inside-out under a controlled transmembrane pressure mode. For the comparison of the Total Cost of Ownership and energy consumption between harvesting with pre-concentration and harvesting with only centrifuges, a 14 element (Liqoflux LF14AL22) unit from Liqoflux with a nominal molecular weight cut-off (MWCO) of 150 kDa was used. A solution of 500 ppm of sodium hypochlorite, NaOCl, 13% active chlorine (Quicelplas, Portugal) was used for membranes cleaning.

2.3.2 Membrane experimental set-up and operation at pilot scale

Pre-concentration experiments were carried out, using a membrane pilot unit (Liqoflux, Turbin, The Netherlands), which experimental set-up is shown in Figure 2.1. The membrane filtration unit consists of a feed tank (1), a recirculation pump (2), a membrane module (3), a restriction valve (4), pressure gauges (5) and a permeate tank (6). The downstream side of the membrane pre-concentration unit consists of a recirculation pump (2) (a flexible impeller pump, suitable to process components sensitive to shear stress) automatically controlled by a frequency converter, and a backflush pump (7), which intervals and running time can also be controlled automatically.

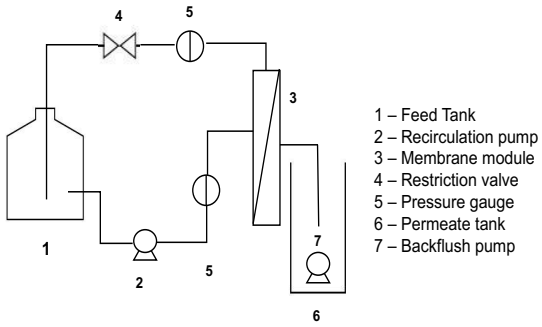


Figure 2.1. Representation of Liqoflux pilot unit, from Turbin.

The feed flow rate (and consequently the cross-flow velocity) and the transmembrane pressure (TMP) are set by defining the position of the feed restriction valve, manually controlled, and the frequency value of the recirculation pump. The permeate is collected in a recipient and the flux is monitored by acquisition of its weight during the course of the experiment, using an electronic balance with an accuracy of 0.01 g. The volumetric permeate flux, J_V (L/(m^2.h)), is measured from the permeate mass recovered during the experiments for a given permeation time, according to Equation 1:

$$J_V = \frac{m_{permeate}}{\rho \times t \times A} \quad (1)$$

where $m_{permeate}$ (kg) is the permeate mass, ρ (kg/m^3) is the density of the permeate medium, t (h) is the permeation time and A (m^2) is the membrane area. After each pre-concentration experiment, the membrane was chemically cleaned with a chlorine cleaning solution, at a cross-flow velocity of 0.4 m/s and a transmembrane pressure of 0.25 bar for 0.5 h followed by flushing with water until complete restoration of the hydraulic permeability corresponding to a clean membrane (also 0.5 h).

2.3.3 Operating conditions

Temperature in the feed tank during the pre-concentration experiments was 21ºC ± 2, except for the experiments at 0.50 bar, which was 26ºC. The pH value was 8.0 ± 0.5 in all experiments. Cell integrity may be affected by the increase of the cross-flow velocity (related to the increase of the shear stress) and by the permeation under controlled transmembrane pressure conditions. Therefore, preliminary tests were performed without permeation at increasing values of cross-flow velocity, in order to independently assess the impact of cross-flow velocity on cell integrity. Preliminary tests were

performed at 0.25 bar and at increasing values of cross-flow velocity, for assessing cell integrity during a short period of time (15 minutes). These experiments were carried out under different cross-flow velocities in a closed system without permeation, in order to study the influence of increasing cross-flow velocities on cell integrity.

The optimisation of the pre-concentration of *Dunaliella salina* by membrane processing was performed by evaluating the relevant operating parameters: the backflush intervals, the transmembrane pressure and the molecular weight cut-off (Table 2.1). For each membrane filtration experiment, fresh green biomass was introduced in the feed vessel and concentrated from 131 kg (one batch) to the minimum possible volume (dead volume of the membrane unit) of 13 kg. Therefore, the final biomass concentration factor was 10. The concentration factor, CF (-), was calculated along the harvesting process according with Equation 2:

$$CF = \frac{m_0}{m_o - m_{permeate}} \quad (2)$$

where m_0 (kg) is the mass in the feed vessel at the beginning of the experiment and $m_{permeate}$(kg) is the mass of permeate recovered up to a given instant.

Table 2.1 Operating parameters tested during the pre-concentration studies of green *Dunaliella salina* by membrane processing at pilot scale at cross-flow velocity of 0.6 m/s, previously optimised.

Operating parameters	**Type of study**		
	Pore size of the membrane	**Backflush interval (min)**	**TMP (bar)**
Pore size / MWCO of the membrane	150 kDa[(b)]; 0.1µm	150 kDa[(b)]	150 kDa[(b)]
Interval between consecutive backflush(min)[(a)]	20	6; 20	20
TMP (bar)	0.25	0.25	0.25; 0.50

[(a)] the runtime of backflush was kept constant at 3s
[(b)] MWCO, Molecular weight cut-off (molecules at this molecular mass are 90% retained)

A final harvesting study was carried out by membrane processing, in order to obtain the highest possible overall concentration factor, while keeping the highest possible cell integrity. This study comprised the pre-concentration of *Dunaliella salina* in three consecutive batches up to an overall concentration factor of 16.4 (see Figure 2.2). The parameters selected from the previous optimisation studies of pre-concentration in single batch (referred in Table 2.1), were used in this study.

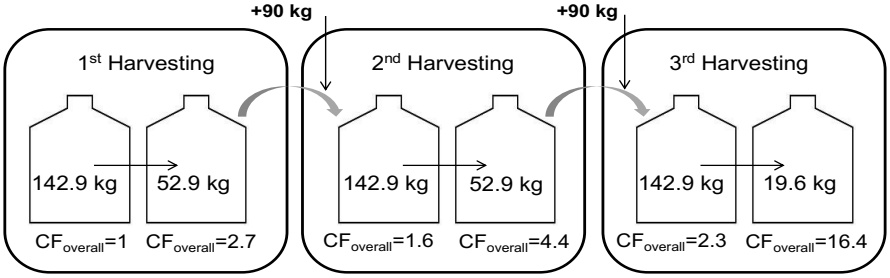


Figure 2.2. Schematic representation of the procedure used during the consecutive harvesting batches.

2.3.4 Characterisation of the biomass

The morphology and shape of the algal cells were observed by optical microscopy (Olympus, Tokyo, Japan). The total number of *Dunaliella salina* cells and their loss of integrity, observed during the harvesting experiments, were measured by a Muse® Cell Analyser equipment (Merck Millipore, Germany). This instrument is a flow cytometer where Propidium Iodide (PI, Sigma-Aldrich), a fluorescent dye, is used to assess the microalgal cytoplasmic cell integrity. If the microalgae membrane excludes the permeation of the dye it means that it keeps its functional integrity. Therefore, only injured cytoplasmic cells become stained. Muse® measures the total number of cells and the percentage of viable cells, which is calculated as the fraction of cells that keep their functional integrity.

2.3.5 Integrated membrane pre-harvesting with Spiral Plate centrifugation units

For the comparison studies of the Total Cost of Ownership and of the energy consumption, between harvesting with a Spiral Plate centrifuge and with an integrated membrane and Spiral Plate centrifuge system, a Liqoflux membrane pilot unit LF14AL22 (Liqoflux, The Netherlands) with 14 ultrafiltration modules and a total membrane area of 364 m^2 was used. The size of each UF membrane element is 26 m^2. All the 14 membrane elements have a separate housing and are pressurised. The membrane unit was operated under transmembrane controlled conditions. An Evodos 25 SPT centrifuge (Evodos, The Netherlands) was used for further concentrating the pre-harvested microalgae culture. The hydraulic capacity of the Evodos 25 is 4 m^3/h. In practice, the actual throughput of the Evodos 25 will depend on the separation efficiency required and the algae strain used. The nominal throughput of the Evodos 25 in this case was around 3 m^3/h.

2.4 Results and Discussion

Before starting the systematic studies aiming to define the optimal process operating conditions, it was necessary to identify the fluid flow conditions that allow for processing *Dunaliella salina* with non-observable impact on cell integrity. With this aim, a series of experiments was performed with increasing cross-flow velocity keeping the permeate valve closed. The microalgae culture was therefore exposed to the shear stress conditions imposed by circulation in the feed circuit but without permeation occurring.

2.4.1 Preliminary tests for evaluating the effect of cross-flow velocity on the pre-concentration of *Dunaliella salina*

Knowing that *Dunaliella salina* is sensitivity to shear stress [9], the cross-flow velocity is one of the most important parameters that needs to be optimised. The increase of cross-flow velocity leads to an increase of shear stress, which may result in cell damage. As a consequence, it may induce the loss of cell integrity and the release of cell debris, colloidal material and algal intracellular material, including extracellular polymeric substances (EPS), proteins and lipids, causing membrane fouling [36,83,84]. The effect of increasing the cross-flow velocity in each fibre of the membrane filtration element was

evaluated by performing short term harvesting experiments at 0.25 bar and observing the impact in cell integrity (see Table 2.2). The shear stress at the wall of each membrane fibre, τ_{wall} (Pa), can be calculated according to Equation 3 [85], which is valid for a laminar flow regime.

$$\tau_{wall} = 0.5 \times \frac{16}{Re} \times \rho \times v_{cross-flow}^2 \quad (3)$$

where ρ (kg/m^3) is the specific mass of the medium.

Table 2.2. Effect of the cross-flow velocity (preliminary tests) on the harvesting of *Dunaliella salina*: feed flow rates Q_{feed} (m^3/h), cross-flow velocities $v_{cross-flow}$ (m/s), Reynolds number Re (-) and wall shear stress τ_{wall} (Pa) at each fibre of the membrane. Cell integrity was characterised by optical microscopy observation.

Q_{feed} (m^3/h)	$v_{cross-flow}$ (m/s)	Re (-)	τ_{wall} (Pa)	Cell integrity
3.8	0.3	668	1.4	No damaged cells
5.1	0.4	896	1.9	No damaged cells
6.8	0.6	1194	2.5	No damaged cells
11.4	1.0	2003	4.2	Disrupted cells, no flagella
15.8	1.4	2775	5.9	Disrupted cells, no movement

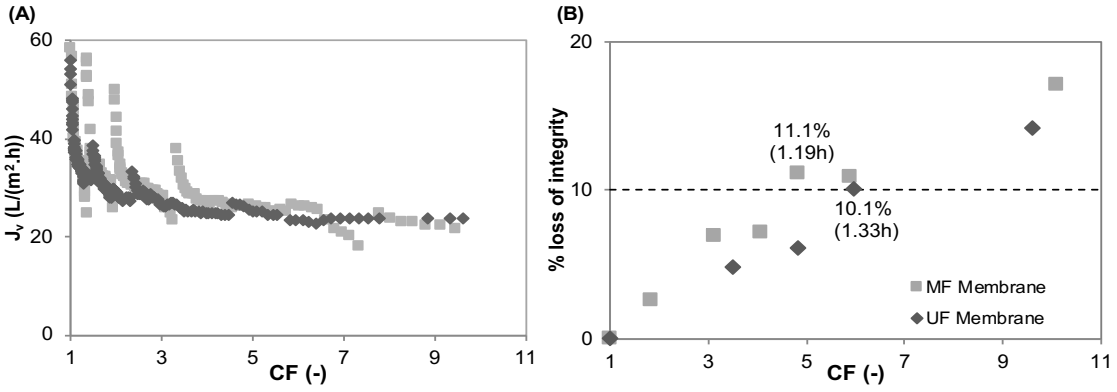
Table 2.2 shows the differences in microalgae cell integrity at imposed values of feed flow rate and corresponding cross-flow velocity. The optimised cross-flow velocity was considered to be the highest value (better fluid dynamics and external mass transfer conditions) without occurrence of cell disruption, which was found to be $v_{cross-flow}$ = 0.6 m/s, corresponding to an optimised feed flow rate of 6.8 m^3/h (for this particular rig). Considering the low values of shear stress at the membrane wall (Table 2.2) and that the pump used in this work is suitable for processing material sensitive to shear, it may not be excluded that the cause of cell disruption occurs at the upstream restriction valve, which has a rather small transversal area, imposing high local shear stress conditions.

2.4.2 Effect of different operating conditions on the pre-concentration of *Dunaliella salina*

The impact of the operating parameters, identified as most relevant, on the pre-concentration of green *Dunaliella salina*, was evaluated by monitoring the permeate flux and the cell viability given by the Muse® Cell Analyser, which is related to the functional cell integrity.

2.4.2.1 Effect of membrane pore size

The effect of membrane type (microfiltration *versus* ultrafiltration) on the pre-concentration of *Dunaliella salina* was tested using ultrafiltration and microfiltration membranes produced by the same manufacturer with the same type of material. The experimental conditions used were: TMP of 0.25 bar, cross-flow velocity of 0.6 m/s and backflushing with 20 minutes of interval (as shown in Table 2.1). Figure 2.3 shows the evolvement of the permeate flux J_V (L/(m^2.h)) (2.3.A) and the percentage of loss of cell integrity (2.3.B) plotted against the concentration factor, CF. The average operating temperature and permeate fluxes obtained for both microfiltration and ultrafiltration systems are shown in Figure 2.3.C. The global average fluxes were calculated taking into consideration the total permeate recovered in each situation and the corresponding operating time.



(C)

Membrane pore size	Microfiltration	Ultrafiltration
$T_{average}$ (ºC) [a]	19	21
J_{global} (L/(m^2.h)) [b]	33	31

[a] Average temperature
[b] Average volumetric flux at final concentration

Figure 2.3. Comparison of (A) volumetric permeate flux and (B) percentage of *Dunaliella salina* loss of integrity along the harvesting process, plotted against the concentration factor, CF. The dashed horizontal line on (B) refers to 10% of loss of integrity. (C) Average temperatures and global average fluxes for the microfiltration and the ultrafiltration studies.

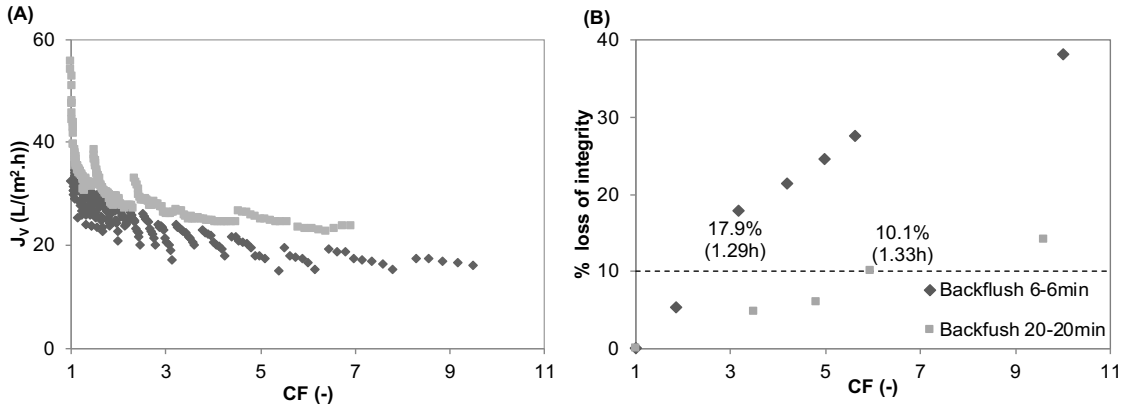
Although the MF membrane presents larger pores, the global average permeate fluxes obtained with both membranes are quite similar. This result is probably due to the fact that MF membranes are more prone to intrapore fouling, which becomes a problem in media where molecular foulants are present (in opposition with the situation when larger particles represent the main contribution to fouling). The rapid drop in permeate flux observed after each backflushing, when the MF membrane is used, supports the presence of an intrapore fouling effect. In contrast, when the tighter ultrafiltration membrane is used, flux declines but with a different pattern: after an initial steep decrease in flux, caused by fouling (partially intrapore fouling that cannot be totally ruled out and partially caused by pore blocking / cake formation), it can be observed that, although with a positive impact in flux, backflushing does not seem to be so relevant as the process evolves.

In Figure 2.3.B, the percentage of cell integrity loss was also quite similar, with a final loss of 17% for a concentration factor of 10. A loss of integrity of 10% was attained at a higher concentration factor of 5.9 when using the UF membrane and a CF of 4.8 when using the MF membrane, after respectively 1.33h

and 1.19h of operation. These results are quite encouraging, suggesting that both micro- and ultra-filtration membrane processes are suitable for *Dunaliella salina* harvesting with similar performances, as described for *Chlorella* [34]. Still, ultrafiltration may represent a wiser choice because it originates a clearer permeate, which makes its reuse easier due to the lower concentration of organic compounds; additionally, membrane cleaning is easier because severe intrapore fouling is reduced.

2.4.2.2 Effect of backflush intervals

The time interval between consecutive backflushes was initially set at 6 minutes (based on previous experience of the supplier). This time interval was then compared with a longer interval of 20 minutes. This comparison was performed for the ultrafiltration element, at a TMP of 0.25 bar and a cross-flow velocity of 0.6 m/s. Figure 2.4 shows the evolvement of the volumetric permeate flux J_V (L/(m^2.h)) (2.4.A) and of the percentage of cell loss of integrity (2.4.B) against the concentration factor. The overall average fluxes obtained at both backflush intervals are also shown in 2.4.C.



(C)

Backflush (for 3 seconds)	each 6 min	each 20 min
$T_{average}$ (ºC) [a]	23	21
J_{global} (L/(m^2.h)) [b]	25	31

[a] Average temperature
[b] Average volumetric flux at final concentration

Figure 2.4. Comparison of (A) volumetric permeate flux and (B) percentage of *Dunaliella salina* loss of integrity plotted against the concentration factor, CF. The dashed horizontal line on (B) refers to 10% of loss of integrity. (C) shows the average temperatures and the overall average flux for the two backflushing intervals (6 and 20 minutes).

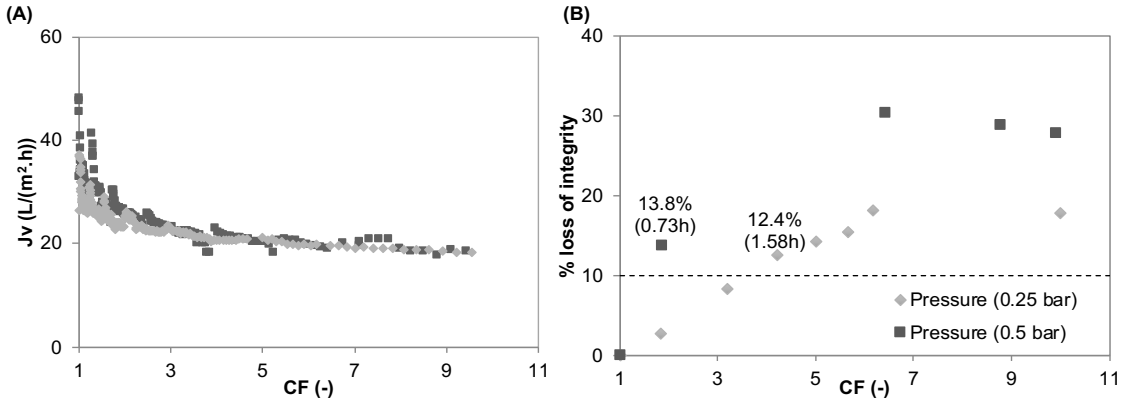
Improvements on filterability are expected when performing the backflushing process [86,87]. However, frequent backflushes decrease the actual working time and also the amount of permeate collected, since this process uses permeate in order to obtain the backflushing effect [36]. Higher (instant and average) volumetric permeate fluxes were attained with longer backflush intervals (Figure 2.4.A). These results could not be anticipated, since more frequent backflushes should assure a more effective removal of fouling components from the membrane and, consequently, lead to an increase of the volumetric permeate flux. However, frequent backflush may also promote cell disruption due to local high shear effects at the pores' mouth leading to the release of intracellular material and ultimately causing membrane fouling. A partial recovery of permeate flux was observed after each backflush,

which is more perceptive in the case of shorter backflush intervals. From Figure 2.4.B, it can be seen that the loss of cell integrity is significantly higher when a higher frequency backflushing procedure is used, probably associated with the frequent exposure of cells to high local shear stress conditions, as discussed above. In contrast, the use of a backflushing procedure with a time interval of 20 minutes allows for achieving a concentration factor of 5.9 with a total loss of cell integrity of 10%, when compared with the initial integrity. Therefore, a better process performance was observed with backflushing every 20 minutes, making possible to obtain higher volumetric permeate fluxes and lower losses of cell integrity.

2.4.2.3 Effect of transmembrane pressure

The effect of the transmembrane pressure on the pre-concentration of *Dunaliella salina* was tested with the ultrafiltration membrane element at 0.25 bar and 0.50 bar, maintaining constant the cross-flow velocity at 0.6 m/s and a backflushing time interval of 20 minutes (as shown in Table 2.1). A relatively low transmembrane pressure range was selected in order to minimise fouling effects, which are usually more relevant when the material retained by the membrane is strongly pushed towards the membrane surface compressing the fouling layer, which becomes less porous and permeable.

Figure 2.5 shows the volumetric permeate flux J_V (L/(m^2.h)) (2.5.A) and the percentage of cell loss of integrity (2.5.B) against the concentration factor, CF. In (2.5.C) the overall average fluxes obtained for a TMP of 0.25 bar and 0.50 bar are also shown.



(C)

TMP	0.25 bar	0.50 bar
$T_{average}$ (ºC) [a]	21	26
J_{global} (L/m^2/h) [b]	24	27

[a] Average temperature
[b] Average volumetric flux at final concentration

Figure 2.5. Comparison of (A) volumetric permeate flux and (B) percentage of *Dunaliella salina* loss of integrity plotted against the concentration factor, CF. The dashed horizontal line on (B) refers to 10% of loss of integrity. The average temperatures and overall average fluxes at the transmembrane pressures of 0.25 bar and 0.50 bar are shown in (C).

Operation with a TMP of 0.5 bar leads to an overall average permeate flux slightly higher than obtained with a TMP of 0.25 bar. Actually, the increase obtained is minimal and mostly due to a higher permeate flux during the initial period of operation (up to a concentration factor of 3). Doubling the driving force,

TMP, clearly does not pay back due to fouling effects. Additionally, in order to achieve operation at higher TMP, the valve in the upstream circuit is set in order to introduce a higher flow restriction, inducing a higher local shear stress, leading to cell disruption and release of intracellular material. From Figure 2.5.B, it can be seen that the loss of cell integrity started sooner at 0.50 bar, namely 10% of cell loss integrity was attained after 0.73h. In contrast, operating at 0.25 bar allows for achieving a concentration factor of 4.2 with a total loss of cell integrity of 10% after 1.58h. At the end of each experiment (CF of 10), the loss of cell integrity was 18% and 30%, respectively at 0.25 and 0.50 bar (Figure 2.5.B). Therefore, operation at a mild TMP, that assures a sufficient driving force, seems to be an adequate strategy to follow.

Our results are in accordance with studies reporting that when harvesting *Chlorella* there is an optimum pressure, after which the increase in TMP does not improve permeate flux [34]. At higher TMP, permeate flux declines faster due to significant fouling effects, which is common when compressible biological material is processed. Rossignol *et al*. also performed the harvesting of two marine microalgae: *Haslea ostrearia* and *Skeletonema costatum* using different TMP (0.2 to 3 bar). They conclude that permeate fluxes raise with increasing TMP, until reaching a maximum value [33].

2.4.3 Effect of harvesting of consecutive *Dunaliella salina* batches

The purpose of this study was to reach a higher concentration factor while keeping cell integrity at acceptable levels. The strategy of adding successively fresh *Dunaliella salina* culture, envisages to reduce the average time of exposure of the microalgae to the shear conditions associated with the concentration procedure (and, therefore, minimise, the impact of shear on the loss of cell integrity). Using this procedure, a total of 322.9 kg of fresh microalgae culture were concentrated into 19.6 kg. The first harvesting step took 1.36h, the second one took 1.60h and the third, 2.48h. However, due to the strategy of adding fresh microalgae culture in sequential steps, the average residence time of the microalgae during the harvesting process was only 3.62h. A final overall concentration factor of 16.4 (see Figure 2.6) was obtained, while keeping the cell integrity loss below 13%.

The operating conditions applied were previously optimised: use of the selected ultrafiltration membrane with a TMP of 0.25 bar, $v_{cross\text{-}flow}$ of 0.6 m/s and backflush every 20 minutes. Figure 2.6 shows the volumetric permeate flux J_V (L/(m^2.h)) evolvement (2.6.A) and the percentage of loss of cell integrity (2.6.B) against the concentration factor CF, during the 3 consecutive harvesting steps and the average fluxes (2.6.C).

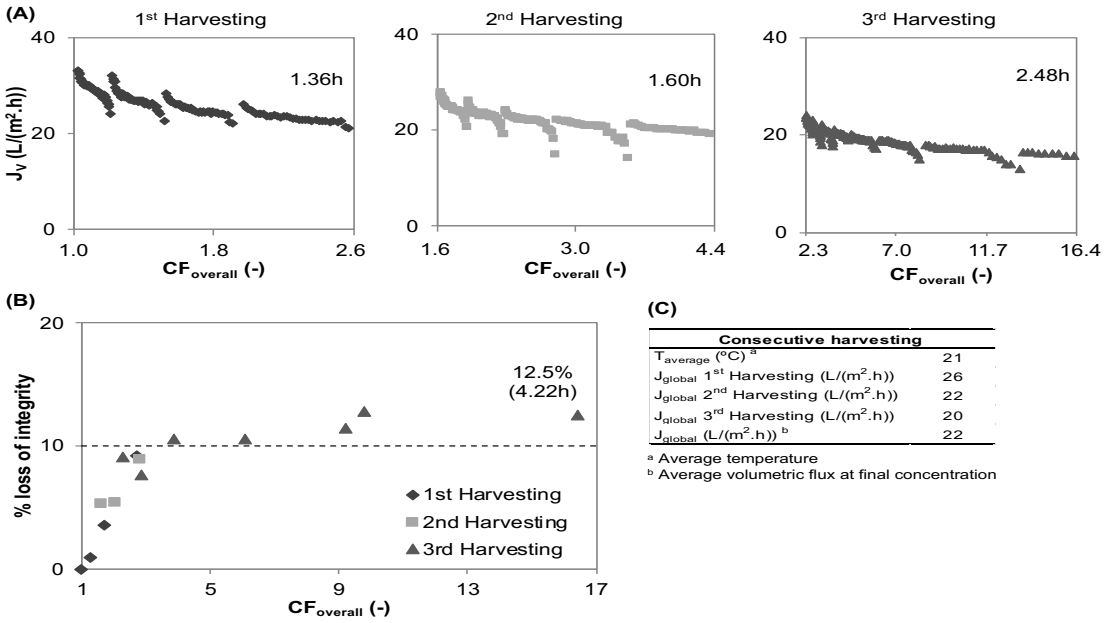


Figure 2.6. Consecutive green *Dunaliella salina* harvesting steps. (A) Evolvement of volumetric permeate flux and (B) of percentage of *Dunaliella salina* loss of integrity during the harvesting process, plotted against the concentration factor, CF. The dashed horizontal line on (B) refers to 10% of loss of integrity. (C) Average temperature and overall average permeate fluxes during the three consecutive harvestings.

In Figure 2.6.A, a lower initial flux was observed (35 L/(m^2.h)), when comparing with previous experiments under similar conditions (see 2.4.2.1), because the initial cell concentration in the fresh culture was almost two times higher than in the previous harvesting studies. The concentration of algal cells is one of the parameters affecting the permeate flux [17,30,33,61], as well as the concentration of extracellular compounds that may act as foulants. The average permeate flux of the overall harvesting process was 22 L/(m^2.h) (Figure 2.6.C), which is a rather positive result considering the compromise achieved between flux and cell integrity.

In Figure 2.6.B, a loss of integrity of 10% was observed at a concentration factor of 6.1 after 4.68h of operation time. After 3 consecutive harvesting steps, a loss of integrity of 13% was observed at the final overall concentration factor of 16.4. Therefore, under previously optimised operating conditions, *Dunaliella salina* harvesting using 3 consecutive batches produced biomass at a higher concentration factor than when using one harvesting batch, with an acceptable integrity loss of at minimum loss of 13%, as aimed.

2.4.4 Chemical cleaning

After each assay, the fouled membranes were cleaned chemically, in order to restore at least 95% of the original membrane clean water permeability. The membranes were chemically cleaned with 500 ppm of chlorine solution during 30 minutes at room temperature. After each cleaning procedure, membranes were flushed and the membrane permeability recovery was evaluated according to the initial pure water flux. In what concerns the assays using the ultrafiltration membrane, the permeability was restored after one cleaning cycle, indicating that fouling can be removed if an appropriate cleaning procedure is used. The clean water permeabilities were 320 ± 2 L/(m^2.h.bar) and 312 ± 8 L/(m^2.h.bar),

respectively before the ultrafiltration experiments and after the cleaning. In the case of the microfiltration membrane, two cleaning cycles were needed in order to restore the initial membrane permeability. The clean water permeabilities were 1070 ± 4 L/(m^2.h.bar), 1010 ± 5 L/(m^2.h.bar) and 1035 ± 3 L/(m^2.h.bar), respectively before the microfiltration experiments and after the first and second cleaning. The complete cleaning procedure took about 1h for the ultrafiltration membrane and 2h for the microfiltration membrane. Microfiltration membranes have larger pore sizes and, therefore, they are more prone to intrapore fouling, which makes the removal of foulants more difficult.

2.4.5 Comparison of energy and Total Cost of Ownership of an integrated membrane pre-harvesting with Spiral Plate centrifugation and of a stand-alone Spiral Plate centrifugation process

In order to compare Total Cost of Ownership (TCO) and energy consumption between harvesting with pre-concentration and harvesting with only centrifuges, results from a Liqoflux facility were used, where a membrane unit was installed in combination with a SPT Spiral Plate Technology centrifuge (Evodos 25).

The average power consumption of the centrifuge was 2.5 kWh/m^3 and the membrane unit used 0.87 kWh/m^3. When using pre-concentration plus centrifugation and assuming a maximum concentration factor of 5 for processing *Dunaliella salina,* an energy reduction could be accomplished compared to only using centrifuges. Recent developments of the ultrafiltration unit, by controlling the TMP with a suction pump on the permeate side, led to a decrease of the average energy consumption down to 0.35 kWh/m^3.

The Total Cost of Ownership (TCO) is calculated as the sum of CAPEX (investment) and the OPEX (energy and maintenance cost). For the two cases, the Total Cost of Ownership has been calculated. The microalgae concentration in the feed was 0.15 g/L. The required end concentration was 200 g/L. The economic study for the harvesting of *Dunaliella salina* is shown in Table 2.3. This process considers a feed of 320 m^3/day (112000 m^3/year). The one-step harvesting approach (only using Evodos 25 centrifuges) requires 5 centrifuges with a capacity of 3 m^3/h and a total investment of 625 k€ (5x125 k€). When the two-step harvesting approach is applied, one Liqoflux unit with 14 ultrafiltration elements is needed with a capacity of 1.1 m^3/h each and investment cost of 165 k€, plus one centrifuge with capacity of 3 m^3/h, with investment of 125 k€. A concentration factor of maximum 5 is assumed. The average annual maintenance cost as a percentage of the investment cost has been assumed for centrifuges at 5% and for membranes at 2%. Energy cost for centrifuges have been assumed at 2.5 kWh/m^3 and for membranes 0.87kWh/m^3. Depreciation period has been taken as 10 years for Liqoflux unit and Evodos 25, respectively. Interest is assumed at 3%. The lifetime of the membrane elements is considered to be 5 years.

Table 2.3. Economic evaluation of the harvesting process calculated for 350 days/year and 22h/day.

Capacity of processing algae biomass (m^3/year)		112000
One-step approach: 5 Evodos centrifuges		
Two-step approach: 1 Liqoflux membrane unit LF14AL22 with 14 membrane elements (installed membrane area = 364 m^2; Concentration factor= 5) + 1 Evodos centrifuge		
	One-step approach	**Two-step approach**
Investment costs		
Total equipment cost (€)	625000	290000
Capital costs (€/year) [a]	62500	34250
Total capital cost (€/year) [b]	73269	33997
Operating costs		
Operational Energy (kWh/year)	280000	153440
Energy price/year (€/year) (0.09 €/year)	25200	13810
Maintenance (€/year) [c]	31250	9550
Membrane replacement (€/year)	-	4900
Total Opex/year (€/year)	56450	28260
Total costs (capital+ total operation)	129719	62256
Total cost/m^3 (€/m^3)	1.16	0.56
Reduction of Total cost of Ownership		52%
Reduction of Energy		45%

[a] Based on a depreciation over 10 years for Liqoflux and Evodos 25
[b] Annual taxes of 3%
[c] Based on a 2% and 5% of total fixed cost for Liqoflux and Evodos 25, respectively

The Total Cost of Ownership for the one-step approach (only centrifuges) is 1.16 €/m^3, while for the two-step approach (pre-concentration with membranes and centrifuge) is 0.56 €/m^3. Therefore, pre-concentration of microalgae in a two-step process leads to a reduction of 52% of the Total Cost of Ownership and also an energy reduction of 45%.

2.5 Conclusions

A membrane harvesting process was evaluated for pre-concentrating *Dunaliella salina* under controlled transmembrane pressure conditions. Harvesting of *Dunaliella salina,* in one batch, allowed reaching a final concentration factor of 5.9, with an average permeate flux of 31 L/(m^2.h), while in consecutive steps allowed reaching a final concentration factor of 16.4, with an average permeate flux of 22 L/(m^2.h) and minimal loss of cell integrity of 13%.

Harvesting with pre-concentration led to a 45% reduction in energy when comparing with only centrifuges. The Total Cost of Ownership is reduced by 52% to 0.56 €/m^3 when applying an ultrafiltration pre-concentration harvesting step.

3 DEVELOPMENT OF AN INTEGRATED PROCESS OF MEMBRANE FILTRATION FOR HARVESTING CAROTENOID-RICH *DUNALIELLA SALINA* AT LABORATORY AND PILOT SCALES

Submitted as: Joana Monte, Jorge Bernardo, Marta Sá, Celina Parreira, Cláudia F. Galina, Luís Costa, Carlos Casanovas, Carla Brazinha, João G. Crespo, "Development of an integrated process of membrane filtration for harvesting carotenoid-rich Dunaliella salina *at laboratory and pilot scales", Separation and Purification Technology (2019).*

The author was involved in planning and performing the membrane filtration experiments at laboratorial scale, as well as on the discussion and interpretation of the results and preparation of the manuscript.

3.1 Summary

Dunaliella salina is an unicellular microalga, well-known for the production of carotenoids, especially β-carotene. This microalga is very fragile and shear sensitive due to the absence of a rigid cell wall and may easily rupture when high shear stress conditions are applied. This work proposes to study the pre-concentration of carotenoid-rich ("orange") *Dunaliella salina* culture by membrane filtration, preceding a final concentration using low-shear centrifugation. The goal is to minimise the total capital and energy costs, ensuring a low cell integrity loss and high permeate fluxes.

The harvesting process was performed under controlled permeate flux conditions at laboratory and pilot scale. At pilot scale, the harvesting of *Dunaliella salina* attained a final concentration factor of 10, at a volumetric permeate flux of 21 L/(m^2.h). When applying the two-step approach of membrane and centrifugation, the reduction of Total Cost of Ownership (TCO) and energy consumption were 70 and 76%, respectively, compared to harvesting with a one-step centrifugation approach.

3.2 Introduction

The unicellular *Dunaliella salina* is a green halophilic microalga, industrially produced due to its large amount of β-carotene [67,88,89] and other carotenoids. The carotenoids production is stimulated by high irradiance under high salinity and low nitrogen conditions [67,75,76]. These pigments are well-known for their antioxidant activity and colourant properties [89,90]. There is a high demand for carotenoids, more specifically, β-carotene for the pharmaceutical and the food industry, where more than 90% of the β-carotene is chemically synthesised [90]. However, natural and biosynthetic origin β-carotene reveals higher bioaccessibility in comparison with synthesised β-carotene, which does not present the cis-isomers [91,92]. Moreover, there is a consumer trend and trust market for products and ingredients with a natural origin and label.

The harvesting of microalgae requires substantial energy expenditure, due to their small size, low growth concentration and identical density to the aqueous medium [9]. The harvesting process may account for 20-30% of the total cost of algae biomass production [17]. When compared to other microalgae, the harvesting of *Dunaliella salina* is particularly challenging. In fact, *Dunaliella salina* does not present a rigid cell wall, making it more fragile and shear sensitive [9,89], being easily ruptured when submitted to high shear stress conditions. Moreover, in the carotenoid-rich cultivation medium, its concentration is typically lower than other microalgae (usually 0.1-1 g/L in open ponds) [93]. For microalgae harvesting, multiple techniques have been described, namely: centrifugation, flocculation, flotation, filtration and sedimentation [15,16,18–20]. The most common method is centrifugation [17,21]. However, this technique requires high capital investment and energy costs. The energy demand of a centrifugation (decanter bowl centrifuge) for microalgae harvesting can reach 8 kWh/m^3 [15,79]. A Spiral Plate Technology (SPT) centrifuge, already mentioned in [94], was developed to reduce the energy consumption (down to 2.5 kWh/m^3) and the shear applied on the algae cells. This feature is especially important for harvesting of fragile *Dunaliella salina* cells. Membrane technology has also been reported for microalgae harvesting [32,33,83], namely because it allows for a complete removal of microalgae cells from the cultivation media [83].

The harvesting of non-carotenogenic (“green”) *Dunaliella salina* by cross-flow filtration has already been reported [81,94]. Microfiltration and ultrafiltration membranes were tested under controlled transmembrane pressure conditions. Cross-flow ultrafiltration showed to perform better, producing a concentrated cell stream while maintaining their integrity [94]. On the other hand, the harvesting of carotenoid-rich *Dunaliella salina* by cross-flow ultrafiltration under controlled transmembrane pressure conditions was also performed but it showed a modest performance, due to a severe loss of cell integrity (data not shown).

Therefore, in this work, the harvesting of carotenoid-rich *Dunaliella salina* was performed under controlled permeate flux, using mild operating conditions suitable for these cells, which are more shear sensitive than the non-carotenogenic cells.

This study proposes an integrated process of pre-concentration of carotenoid-rich *Dunaliella salina* using membranes preceding a final concentration stage by low-shear centrifugation, in order to reduce energy and capital costs in comparison with harvesting solely by centrifugation. The effect of the operating parameters of the membrane filtration process (cross-flow velocity, molecular weight cut-off of the membrane, relaxation cycles and volumetric permeate flux) were assessed in terms of cell integrity loss, allowing for selecting the operating values which led to maximising of the volumetric permeate flux and the concentration factor, assuring a low loss of cell integrity.

The harvesting of carotenoid-rich *Dunaliella salina* at pilot scale was performed at optimised operating conditions settled at laboratory scale. Finally, the Total Cost of Ownership (TCO) and the consumption of energy was determined for the two-steps (membrane processing proceeded by centrifugation) and the one-step approach (centrifugation).

3.3 Materials and Methods

3.3.1 Culture of carotenoid-rich *Dunaliella salina*

Dunaliella salina (DF40) in the carotenoid-rich phase ("orange") was originally collected at the microalgae production plant at Monzón Biotech (Monzón, Spain), isolated by Marine Biological Association of the United Kingdom and scaled-up and produced by A4F - Algae for Future (Lisbon, Portugal). Carotenoid-rich fresh algae culture with 2M NaCl and pH between 7 and 8 were sampled for the several tests performed at different initial concentrations, ranging from $2.0x10^5$ to $5.7x10^5$ cells/mL. The strain used in the laboratory scale experiments (DF40) was provided by A4F and in the pilot scale experiments the microalgae culture samples were provided by Monzón Biotech.

3.3.2 Pre-concentration by membrane processing of carotenoid-rich phase *Dunaliella salina*

The microalgae pre-concentration optimisation at laboratory scale was performed using a membrane unit from GE Healthcare (Chicago, USA). Two asymmetric polysulfone (PS) capillary membrane modules were tested (operating inside-out), with a nominal molecular weight cut-off (MWCO) of 100 kDa and 500 kDa. Both membrane modules comprised 520 fibres, 0.5 mm of internal diameter, 0.64 m of length and 0.48 m^2 of total area. For the pilot scale experiments, a capillary membrane unit from Koch Membrane Systems (Massachusetts, USA) of asymmetric polysulfone was used with a MWCO of 100 kDa. The membrane element comprises 1765 fibres, 1.1 mm of internal diameter, 1.09 m of length and 6.1 m^2 of total area.

3.3.3 Membrane set-up for cross-flow experiments at laboratory and pilot scale

Laboratory and pilot scale pre-concentration tests were performed under controlled permeate flux conditions, using an inside-out filtration procedure (see scheme of membrane set-up in Figure 3.1). The feed (*Dunaliella salina* fresh biomass) was pumped from the feed tank through the membrane module, using a peristaltic pump, and recirculated to the feed tank.The permeate flux was defined by introducing a pump in the permeate circuit, which controls and defines the permeate flux, and collected in a permeate tank. The feed circulated inside the membrane tubes and the permeate flows through the membrane pores to the shell side. This operating mode allows to control and define the conditions of convective transport of material from the bulk retentate stream towards the membrane surface, making possible to operate under controlled mild permeate flux conditions. This mode of operation, inspired on the operation of membrane bioreactors (MBRs), minimises fouling and cell disruption. The membrane filtration equipment comprises a feed reservoir, a membrane unit, a recirculation pump, pressure gauges, a permeate pump (diaphragm pump) and a permeate reservoir.

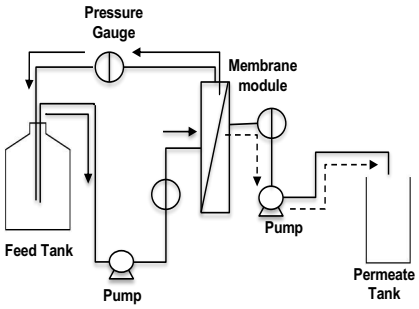


Figure 3.1. Scheme of laboratory and pilot scale membrane units used for carotenoid-rich *Dunaliella salina* harvesting (dark arrows represent the feed recirculation and dashed arrows correspond to the permeate flow).

The recirculation peristaltic pump set the recirculation flow rate, while the permeate pump set the volumetric permeate flux. For both pre-concentration experiments (laboratory and pilot scale), the permeate was collected in a recipient and the permeate flux was monitored by acquisition of its weight throughout the pre-concentration experiments, using an electronic balance. The volumetric permeate flux, J_V (L/(m^2.h)), was calculated through the mass of permeate collected along the harvesting tests, according to Equation 1:

$$J_V = \frac{m_{permeate}}{\rho \times t \times A} \quad (1)$$

where $m_{permeate}$ (kg) corresponds to mass of permeate, t (h) corresponds to time of permeation, A (m^2) corresponds to membrane area and ρ (kg/m^3) represents the density of the permeate (1070 kg/m^3 for a salinity of 120 g/L). After harvesting procedures, the fouled membranes were cleaned chemically with a sodium hydroxide solution (0.5M), during 30 min at 40 ºC, followed by cleaning with water until at least 90% of the initial membrane clean water permeability was restored. In what concerns the assays using the ultrafiltration membrane, the permeability was restored after one cleaning cycle, indicating that fouling can be removed if an appropriate cleaning procedure is used. In the case of the microfiltration membrane and no use of relaxation cycles, two or three cleaning cycles were needed, in order to restore the initial membrane permeability. Microfiltration membranes have larger pore sizes and, therefore, they are more prone to intrapore fouling, which makes the cleaning process more difficult. Also, the experiments with no relaxation cycles led to an increase of TMP and, subsequently, an increase of percentage loss of integrity, leading to a more severe fouling and a more difficult cleaning process.

3.3.4 Operating conditions

Feed reservoir temperature throughout the harvesting trials was 20 ± 2 ºC. The value of pH was 8.2 ± 0.5 in the experiments.

The raise of the cross-flow velocity ($v_{cross\text{-}flow}$) may affect *Dunaliella salina* integrity, related with the increase of the shear stress [94]. Therefore, preliminary experiments (recirculation without permeation) at laboratory scale were performed with the aim of independently evaluate the influence of cross-flow velocity on the integrity of the cell. These experiments were performed in a closed system (with no permeation) at several velocities of cross-flow, for assessing cell integrity during 120 minutes (average

time of a harvesting experiment). The membrane processing optimisation at laboratory scale of the harvesting of carotenoid-rich *Dunaliella salina* was performed by assessing the important operating factors: molecular weight cut-off, imposed volumetric permeate flux and the relaxation procedure (see Table 3.1). The relaxation procedure consists of 9 minutes of permeation and 1 minute without permeation (by stopping the permeate pump), which helps reducing fouling development. For each laboratory scale pre-concentration experiment, 20 kg of fresh carotenoid-rich *Dunaliella salina* culture was placed in the feed reservoir and concentrated to the minimum possible volume (corresponding to the membrane equipment dead volume) of approximately 1 kg. Thus, the final concentration factor was approximately 20. The concentration factor, CF (-), was measured during the pre-concentration procedure according to Equation 2:

$$CF = \frac{m_0}{m_o - m_{permeate}} \quad (2)$$

where $m_{permeate}$ (kg) represents the permeate mass collected up to a given time and m_0 (kg) represents the mass in the beginning of the trial in the feed reservoir.

Table 3.1. Operating factors evaluated in the harvesting experiments of carotenoid-rich *Dunaliella salina* at laboratory scale by membrane filtration, at a $v_{cross\text{-}flow}$ of 0.35 m/s, optimised previously (see section 3.4.1).

Type of Study	**Molecular Weight Cut-off (MWCO)**	**Relaxation cycles [(a)]**	**Imposed volumetric permeate flux (J_V)**
Operating parameters	100; 500 kDa [(b)]	Relaxation / No relaxation	10; 25; 30 L/(m^2.h)

(a) 10 minutes cycles consisting of 9 minutes of permeation + 1 minute of relaxation
(b) molecules with this, or higher, molecular mass are at least 90% retained (information provided by the manufacturer)

After optimising the pre-concentration of carotenoid-rich stage *Dunaliella salina* at laboratory scale, the harvesting process was tested at pilot scale conditions. At pilot scale, the pre-concentration set-up was operated under controlled permeate flux (using a value similar to the optimised value determined at laboratory scale), leading to a mild transmembrane pressure value, as required.

3.3.5 Characterisation of the biomass

In the laboratory scale pre-concentration experiments, Muse® Cell Analyser (Merck Millipore, Germany) was used to determine the total cell number and the cell integrity loss. This equipment is identical to a flow cytometer. A fluorescent dye, Propidium Iodide (PI, Sigma-Aldrich), was used to evaluate the microalgae cell integrity. If functional integrity is maintained, the microalgae membrane excludes the dye permeation. Therefore, solely damaged cells get stained. The equipment quantifies the total cell number and the fraction of cell integrity, determined through the fraction of cells with functional integrity (not stained) and total cells.

For the pilot scale experiments, the total number of cells was measured using a Neubauer counting chamber through optical microscopic observation [95]. At laboratory and pilot scale, an optical microscope (Olympus, Tokyo, Japan) was used to observe the algal cells morphology and shape.

3.3.6 Integrated harvesting process with membrane filtration and Spiral Plate centrifugation

The TCO and the consumption of energy required for harvesting using an integrated membrane filtration plus a Spiral Plate centrifugation was compared with the procedure using solely a Spiral Plate centrifugation. The biomass pre-concentrated with the pilot membrane unit installed at Monzón Biotech (with a 6.1 m^2 membrane area) was further concentrated using an Evodos 50 SPT (Spiral Plate Technology) centrifuge (Evodos, The Netherlands).

3.4 Results and Discussion

Previously to the optimisation of the operating conditions of microalgae pre-concentration, the effect of recirculation hydrodynamic conditions (quantified by the cross-flow velocity) on cell integrity was studied. This study aims at defining the highest cross-flow velocity while maintaining a high cell integrity. This study was performed by recirculating the *Dunaliella salina* culture at different cross-flow velocities, during 120 minutes, with the permeate valve closed.

3.4.1 Effect of cross-flow velocity

Since *Dunaliella salina* is shear stress sensitive [9], the velocity of cross-flow is one of the main factors that is required to be studied. The shear stress raise is a consequence of the increase of the cross-flow velocity, which may lead to cell damage. The loss of cell integrity may cause membrane fouling, not only due to the algal intracellular compounds released such as proteins and lipids, but also cell debris, extracellular polymeric substances (EPS), and colloidal material [36,83,84]. The increase of cross-flow velocity on the membrane module was studied by carrying out experiments under mild transmembrane pressure conditions and observing the influence on cell integrity using Muse® and microscopic observation (Table 3.2). Muse® and optical microscopy observation were used to determine the percentage of cell integrity.

Table 3.2. Summary of cross-flow velocities tested on the pre-concentration of carotenoid-rich *Dunaliella salina*. Feed flow rate Q_{feed} (mL/min), cross-flow velocity $v_{cross\text{-}flow}$ (m/s), number of Reynolds Re (-), wall shear stress tangential to the membrane surface τ_{wall} (Pa), cell integrity (%) and cell observation are presented.

Q_{feed} (mL/min)	$v_{cross\text{-}flow}$ (m/s)	Re (-)	τ_{wall} (Pa)	Cell integrity (%)	Cell observation
1960	0.32	171	5.2	95 ± 3	No damaged cells
2160	0.35	189	6.7	94 ± 2	No damaged cells
2280	0.37	200	6.0	77 ± 5	Disrupted cells

The shear stress, τ_{wall} (Pa), was determined according to Equation 3, valid for a tubular geometry at laminar flow regime [85]. As shown in Table 3.2, until a velocity of cross-flow of 0.37 m/s (corresponding to Re ~ 200), conditions of laminar flow are guaranteed.

$$\tau_{wall} = 0.5 \times \frac{16}{Re} \times \rho \times v^2_{cross-flow} \quad (3)$$

where ρ (kg/m^3) represents the density.

The cell integrity and cell observation are the most important parameters when selecting the cross-flow velocity to be used. The optimal cross-flow velocity should be the greatest value, in order to obtain good mass transfer and fluid dynamics conditions, while assuring the highest cell integrity and no cell damage (microscopic observation). This value was optimised to $v_{cross-flow}$= 0.35 m/s. This cross-flow velocity is lower than the optimised value found previously [94] for harvesting of non-carotenogenic *Dunaliella salina* ($v_{cross-flow}$ of 0.6 m/s), which is in line with the known fact that carotenoid-rich *Dunaliella salina* ("orange" *Dunaliella salina*) is even more sensitive than the non-carotenogenic ("green" *Dunaliella salina*) [10,67].

3.4.2 Selection of operational parameters on the membrane pre-concentration

The influence of the most relevant operating factors on the carotenoid-rich phase *Dunaliella salina* membrane harvesting was assessed by examining: the volumetric flux of the permeate, the transmembrane pressure and the cell integrity loss measured by the Muse® Cell Analyser. This work aims at selecting at laboratory scale the operating parameters values which lead to the highest possible permeate flux and concentration factor with a smallest fraction of cell integrity loss.

3.4.2.1 Selection of membrane molecular weight cut-off (MWCO)

The selection of MWCO on the membrane pre-concentration of carotenoid-rich phase *Dunaliella salina* was evaluated using two different membranes (100 and 500 kDa), made of the same membrane material (Polysulfone, PS) and produced by the same manufacturer (GE Healthcare, USA).

The experiments were conducted at: cross-flow velocity of 0.35 m/s (optimised previously), using relaxation cycles (9 minutes of permeation and 1 minute without permeation) and with a volumetric permeate flux controlled at around 25 L/(m^2.h). The average permeate flux obtained was 23 ± 1 L/(m^2.h), when using the 100 kDa membrane and 22 ± 1 L/(m^2.h), when using the 500 kDa membrane. Figure 3.2 presents the evolution of the TMP (bar) plotted against time and the percentage loss of integrity plotted against the concentration factor, CF.

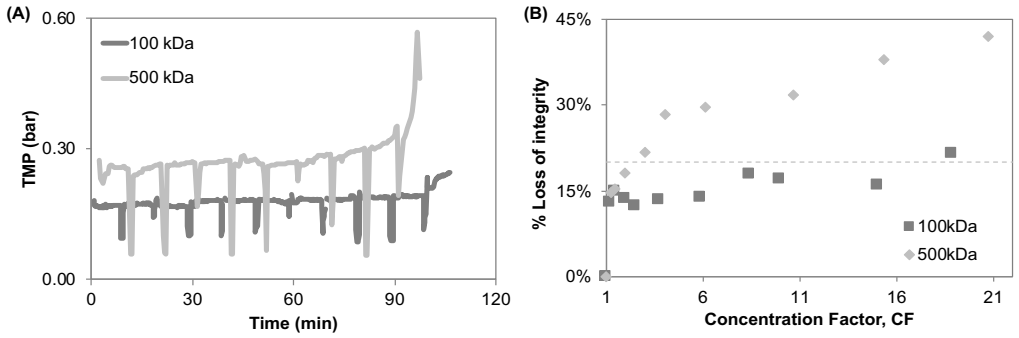


Figure 3.2. Graphical representation of (A) transmembrane pressure (TMP, bar) plotted against time and (B) percentage of loss of integrity during the membrane harvesting using membranes with different MWCO, plotted against the concentration factor, CF (-).

As shown in Figure 3.2.A, the transmembrane pressure was higher during harvesting with the 500 kDa membrane. The loss of integrity was not linear with the time of operation (data not shown). In what concerns cell integrity loss, when the small concentration factor of 1.25 was achieved, a fraction of cell integrity loss of 15% was observed for both cases (see Figure 3.2.B), with the release of some algal intracellular material namely the increase of glycerol content on the permeate (data not shown). This initial loss of integrity of 15% might be explained by a fraction of cells more susceptible to rupture than others. In the case of 100 kDa membrane, the loss of integrity was stable until 90 minutes of operation, and then started to increase. While for a 500 kDa membrane, the loss of integrity was more evident from 80 minutes of operation (CF = 3).

In the end of the membrane filtration experiments, an increase of fraction of cell integrity loss was observed (42% at a final CF of 20.7), along with an increase of the transmembrane pressure of 111% (from 0.27 to 0.57). This high increase of transmembrane pressure might be explained by the fact that the intracellular material released may block the membrane pores (fouling phenomena). Consequently, for maintaining the desired permeate flux, a significant increase of transmembrane pressure was observed. These results are in accordance with other *Dunaliella salina* studies [94], suggesting that membranes with tighter pores are more suitable to harvest *Dunaliella salina* leading to reduced intrafouling phenomena. The increase of fraction loss of cell integrity with the 500 kDa might be explained by the fact that a larger membrane pore induces a bigger suction from the permeate side. Therefore, the shear on the membrane pores' mouth is higher and consequently leads to an increase in the loss of cell integrity.

On the other hand, during the 100 kDa membrane experiment, the cell integrity loss remained roughly constant (21% at a final concentration factor of 18.6), and the transmembrane pressure increased only 36%. Compared to the increase of TMP when using a 500 kDa membrane, these values evidence a reduced fouling, due to a lower TMP increase and, accordingly, an easier membrane cleaning process. Therefore, the 100 kDa membrane was selected for harvesting carotenoid-rich *Dunaliella salina*.

3.4.2.2 Evaluation of relaxation cycles

The relaxation cycles consist of 9 minutes of permeation and 1 minute without permeation (relaxation) by stopping the permeate pump. This strategy was used to reduce the fouling of the membrane. The comparison of using relaxation cycles and no relaxation cycles was performed using the 100 kDa ultrafiltration membrane (previously selected – see section 3.4.2.1), at a $v_{cross\text{-}flow}$ of 0.35 m/s and imposing a permeate flux of 25 L/(m^2.h). Figure 3.3 presents the evolution of the TMP (bar) plotted against time and the percentage loss of integrity of *Dunaliella salina* plotted against the concentration factor, CF.

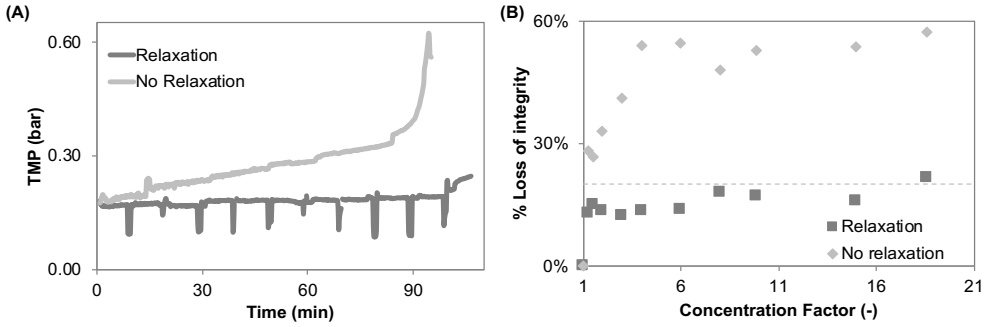


Figure 3.3. Graphical representation of (A) transmembrane pressure (TMP, bar) plotted against time and (B) percentage of loss of integrity during the membrane harvesting with 100kDa membrane with relaxation and without relaxation cycles, plotted against the concentration factor, CF (-).

As shown in Figure 3.3.A, there was an increase of transmembrane pressure since the beginning of the harvesting experiment with no relaxation cycles. On the other hand, the transmembrane pressure was constant when using relaxation. The average permeate flux obtained in the case of no relaxation cycles was 23 ± 2 L/(m^2.h) and in the case of using relaxation cycles the average permeate flux was 23 ± 1 L/(m^2.h).

In Figure 3.3.B, a lower cell integrity loss was observed when using relaxation cycles compared with experiments with no relaxation cycles. Regarding the fraction of cell integrity loss at the beginning of concentration process (CF=1.25), a loss of 15% was observed when using relaxation cycles and 30% when relaxation cycles were not applied.

During the experiments with relaxation cycles, the loss of integrity is maintained around 20% until the final concentration factor of 18.6, and the transmembrane pressure increased 36%. These results demonstrate a reduced formation of fouling because the algal intracellular compounds released to the media, which may block the membrane pores, are periodically removed during the relaxation cycles.

In contrast, when no relaxation is applied, the cell integrity loss was much higher: 57% at the final concentration factor of 18.6 and a transmembrane pressure increase of 265% (from 0.17 bar to 0.62 bar). Accordingly, the higher loss of integrity and the increase of transmembrane pressure might be explained by the accumulation of algal intracellular compounds which contribute to membrane fouling.

There is an increase in the transmembrane pressure, since the beginning of the experiments, when performing the harvesting with no relaxation cycles, along with an increase in the percentage loss of integrity. The fraction of loss of integrity was more noticed at transmembrane pressure higher than 0.30

bar. While using relaxation cycles, the transmembrane pressure was constant and an increase was only observed from 110 minutes. The loss of integrity was also maintained around 20% until the end of the experiment.

Hence, a better performance was noticed when using relaxation cycles, making possible to obtain higher concentration factors under lower transmembrane pressure and leading to a reduced loss of cell integrity.

3.4.2.3 Selection of the imposed volumetric permeate flux (J_V)

The selection of the imposed volumetric permeate flux (J_V) on the harvesting of *Dunaliella salina* with the 100 kDa membrane was evaluated by controlling its value at 10, 25 and 30 L/(m^2.h), while preserving the $v_{cross\text{-}flow}$ at 0.35 m/s and the relaxation procedure. Figure 3.5 presents the evolution of the TMP (bar) plotted against time and the percentage loss of integrity against the concentration factor, CF.

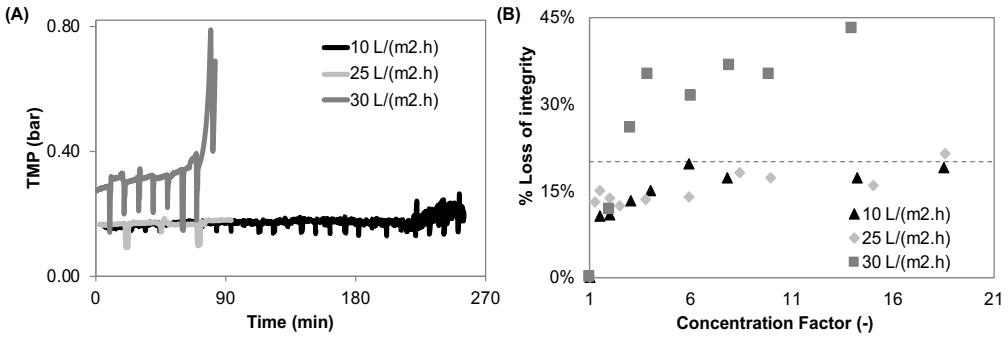


Figure 3.4. Graphical representation of (A) transmembrane pressure (TMP, bar) plotted against time and (B) percentage of loss of integrity during the membrane harvesting at different volumetric permeate fluxes (J_V = 10, 25 and 30 L/(m^2.h)), plotted against the concentration factor, CF (-).

As shown in Figure 3.4.A, the three volumetric permeate fluxes tested show different transmembrane pressure profiles during the harvesting experiments. The highest increase of transmembrane pressure was observed during the harvesting experiments at a permeate flux of 30 L/(m^2.h), and similar values of TMP were observed at 10 and 25 L/(m^2.h).

Regarding the loss of cell integrity, the fraction varied for the different volumetric permeate fluxes imposed (Figure 3.4.B). In general, the higher the flux, the higher might be the shear stress at the membrane pores' mouth and consequently the loss of cell integrity increases. Accordingly, at the highest flux tested of 30 L/(m^2.h), the loss of integrity was 26% at a concentration factor of 3.0. Moreover, at the final concentration factor (14.9), a high increase of transmembrane pressure and of cell integrity loss were observed, respectively 175% and 43 %. This high cell integrity loss and transmembrane pressure can be explained by the fact that algal intracellular compounds were released and accumulated in the membrane pores, leading to fouling.

When harvesting *Dunaliella salina* at lower permeate fluxes (namely 10 and 25 L/(m^2.h)), the cell integrity losses were reduced and similar. At the final concentration factor (18.6), the cell integrity loss was 19% and 22% for a permeate flux of 10 and 25 L/(m^2.h), respectively. In terms of transmembrane

pressure, it was observed an increase of 31% and 36% during the experiments with a permeate flux of 10 and 25 L/(m^2.h), respectively.

Since the permeate flux should be as high as possible, without damaging the *Dunaliella salina* cells, operation with a controlled permeate flux of 25 L/(m^2.h) was selected.

A summary of the results obtained in this study, performed at laboratory scale, is shown in Table 3.3 (following Table 3.1).

Table 3.3. Operating parameters optimised during the pre-concentration studies of carotenoid-rich Dunaliella salina by membrane processing at laboratory scale, at a cross-flow velocity of 0.35 m/s, previously defined.

Type of Study	Molecular Weight Cut-off (MWCO)	Relaxation cycles [a]	Imposed volumetric permeate flux (J_v)
Optimised operating conditions	100 kDa [b] (25 L/(m^2.h); Relaxation)	Relaxation (at MWCO 100 kDa; 25 L/(m^2.h))	25 L/(m^2.h) (at MWCO 100 kDa; Relaxation)
Results pre-concentrated biomass	*CF* = 18.6; 21% loss of integrity	*CF* = 18.6; 21% loss of integrity	*CF* = 18.6; 22% loss of integrity

(a) 10 minutes cycles consisting of 9 minutes of permeation + 1 minute of relaxation
(b) molecules with this, or higher, molecular mass are at least 90% retained (information provided by the manufacturer)

3.4.3 Pilot scale studies

Carotenoid-rich *Dunaliella salina* was successfully pre-concentrated by membrane processing at laboratory scale with a fraction of cell integrity loss of 22%. The optimised conditions using a 100 kDa ultrafiltration membrane were: operation with a cross-flow velocity, $v_{cross\text{-}flow}$, of 0.35 m/s, using relaxation cycles and controlling the permeate flux, J_V, at 25 L/(m^2.h) (23 ± 1 L/(m^2.bar)). After the promising pre-concentration results at laboratory scale, harvesting experiments of carotenoid-rich *Dunaliella salina* at pilot scale were carried out at the Monzón Biotech cultivation site.

The previously optimised conditions at laboratory scale were applied, except for the $v_{cross\text{-}flow}$, which was slightly lower (0.30 m/s), due to limitations of the pilot scale system.

At pilot scale, two harvesting experiments were performed, up to different concentration factors. During the experiment with a final concentration factor of 5, the average volumetric permeate flux was 22 ± 3 L/(m^2.h), which led to an expected low transmembrane pressure of 0.30 ± 0.15 bar and to cells which were intact, although without motility, due to a possible loss of their flagella (Figure 3.5.A). The temperature was kept at 21 ± 2°C. The *Dunaliella salina* culture was effectively concentrated from an initial concentration of 2.2x10^5 cells/mL to a final concentration of 1.1x10^6 cells/mL.

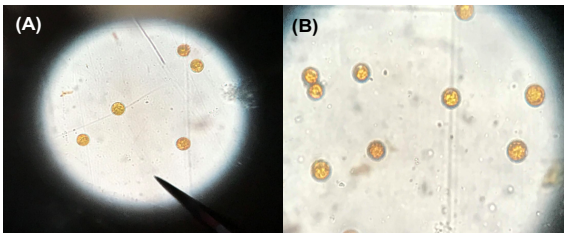


Figure 3.5. Microscopic observation of *Dunaliella salina* cells after pre-concentration experiments until a concentration factor of 5 (A) and until a concentration factor of 10 (B).

Regarding the harvesting experiment performed up to the final concentration factor of 10, the average volumetric permeate flux was 21 ± 3 L/(m^2.h), which led to a small transmembrane pressure of 0.30 ± 0.16 bar, and to cells which were also intact, although without motility (Figure 3.5.B). The temperature was 21 ± 3°C. In this experiment, carotenoid-rich *Dunaliella salina* was concentrated from an initial concentration of 2.8x10^5 cells/mL to a final concentration of 2.9x10^6 cells/mL.

In both experiments, cells had lost both flagella adopting a round shape, instead of the characteristic ellipsoidal shape of *Dunaliella salina*. At pilot scale, carotenoid-rich *Dunaliella salina* membrane pre-concentration, until a concentration factor of 10, was effectively performed with the selected ultrafiltration membrane (100 kDa) at a cross-flow velocity of 0.30 m/s, imposing a controlled permeate flux of 21 ± 3 L/(m^2.h) and using the relaxation procedure described above.

3.4.4 Comparison of the economic evaluation of the integrated process and the stand-alone Spiral Plate Technology centrifuge

An economic evaluation was performed to relate the Total Cost of Ownership (TCO) and consumption of energy for the two alternatives under study: 1 – one-step approach, using a stand-alone centrifugation; 2 - the integrated two-step approach that comprises pre-concentration by ultrafiltration followed by centrifugation. Figure 3.6 shows the flowchart for harvesting of *Dunaliella salina* using these two approaches for a project scenario of processing 9.94 x 10^5 m^3/year (or 151 m^3/h) of *Dunaliella salina* culture, obtained in a 20 ha plant in Monzón.

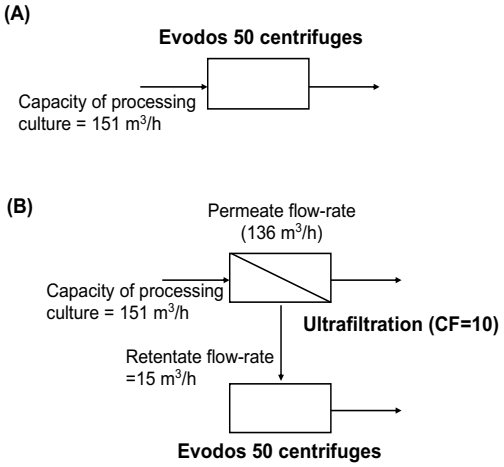


Figure 3.6. Flowchart of the harvesting of *Dunaliella salina*. Flow rates of the different streams for processing 151 m^3/h of culture using (A) a one-step approach with Evodos 50 centrifuges and (B) a two-step approach integrating membrane processing (with a CF of 10) and Evodos 50 centrifuges.

Aiming the comparison of the TCO and the consumption of energy of these two different procedures, experimental results obtained with the membrane unit installed at Monzón (with 6.1 m^2 membrane area) were used in combination with an Evodos 50 centrifuge (Spiral Plate Technology).

The TCO is measured by CAPEX (total investment) and OPEX (total maintenance and energy costs). A continuous operating regime was chosen with a working period of 300 days/year and 22h/day (2h of cleaning).

The economic study for the harvesting of carotenoid-rich *Dunaliella salina* is estimated in Table 3.4 for the concentration factor of 10 and an average J_V of 21 L/(m^2.h).

For the economic evaluation a feed of 3312 m^3/day (993600 m^3/year) was considered.

The one-step approach (operating with Evodos 50 centrifuges) requests an investment of 238 k€ for each centrifuge. In case of an integrated process (membranes and centrifuges). The total investment cost is 2259 k€ (for the membrane modules) and 1428 k€ (for the centrifuges). Specifically, 1058 membrane modules (with total area of 6454 m^2) are needed with a capacity of 151 m^3/h. A concentration factor of 10 and a permeate flux of 21 L/(m^2.h) are assumed. The pilot scale membrane module (capillary membrane unit from Koch Membrane Systems) has 6.1 m^2 of membrane area and the cost is 350 €/m^2, which includes the total equipment installation, comprising the recirculation and permeate pump, piping and instrumentation. Regarding the centrifuges, for the two-step approach, 6 centrifuges are needed (6 x 238 k€). The operating capacity of centrifuges and membrane is 3 m^3/h and 151 m^3/h, respectively. The energy expenditure was considered: 2.5 kWh/m^3 for the centrifuges and 0.35kWh/m^3 for the membranes [94,96].

Table 3.4. Comparison of economic analysis of the microalgae harvesting process with the different approaches presents.

Capacity of processing algae biomass (m^3/year)			9.94 x 10^5
Average permeate flux (L/(m^2.h)			21
One-step approach: 51 Evodos 50 centrifuges			
Two-step approach: 1 membrane unit with 1058 membrane elements (installed membrane area = 6454 m^2; Concentration factor= 10) + 6 Evodos 50 centrifuge			
	One-step approach	**Two-step approach**	**Observations**
Investment costs			
Total equipment cost (k€)	12138	3687	SPT centrifuge price - 238k€ (provided by Evodos)
Total capital cost (k€/year) [a]	1423	432	Based on equipment investment, interest taxes and depreciation time
Operating costs			
Operational Energy (kWh/year)	2.48 x 10^6	5.96 x 10^5	[94,96]
Energy price (k€/year) (0.09 €/kW.h)	224	54	-
Maintenance (k€/year) [b]	607	117	-
Membrane replacement (k€/year)	-	65	Based on 3 year membrane lifetime

Total Opex/year (k€/year)	830	235	Based on energy price, maintenance and membrane replacement
Overall costs (CAPEX+OPEX)) (k€)	2253	667	-
Overall cost/m^3 (€/m^3)	2.27	0.67	-
Reduction of Total cost of Ownership		70%	-
Reduction of Energy		76%	-

[a] Based on a depreciation over 10 years for the ultrafiltration system and Evodos 50 and annual taxes of 3%

[b] Based on a 2% and 5% of total fixed cost for the ultrafiltration system and Evodos 50, respectively

The TCO for microalgae harvesting with only centrifuges (one-step approach) is 2.27 €/m^3, while for the integrated process with membrane filtration and centrifuges (two-step approach) is 0.67 €/m^3. Hence, *Dunaliella salina* pre-concentration using an integrated process results in a reduction of TCO of 70% and also to a reduction of energy of 76%.

Indeed, the economic evaluation performed shows clearly that the harvesting of *Dunaliella salina* using a two-step approach integrating membrane processing with centrifuges is preferred over using solely centrifugation, in terms of capital and operational costs.

3.5 Conclusions

A membrane filtration harvesting method was examined for carotenoid-rich *Dunaliella salina* pre-concentration under controlled permeate flux conditions at laboratory and pilot scale. At pilot scale, carotenoid-rich *Dunaliella salina* was successfully harvested using an ultrafiltration membrane (100kDa) operated under controlled permeate flux conditions (average volumetric permeate flux of 21 L/(m^2.h)) until a concentration factor of 10 was reached, with no observed damaged cells.

The economic evaluation showed that harvesting with pre-concentration using membranes and centrifugation (two-step approach) led to a 76% energy reduction when comparing with harvesting only with centrifuges (one-step approach). The TCO was reduced by 70% to 0.67 €/m^3 when applying a two-step approach. Therefore, the economic evaluation performed has proven that harvesting of carotenoid-rich *Dunaliella salina* using a two-step approach is significantly more economic than harvesting with a single step using centrifugation.

4 RECYCLING OF *DUNALIELLA SALINA* CULTIVATION MEDIUM BY INTEGRATED MEMBRANE FILTRATION AND ADVANCED OXIDATION

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The author was involved in the harvesting and oxidation and photodegradation experiments, data discussion and interpretation, as well as, preparing and writing the manuscript.

4.1 Summary

β-carotene and other carotenoids are common food supplements industrially produced by *Dunaliella salina*. However, the cultivation production costs are substantial and determine the economic viability of production in large-scale systems. There is a strong interest in recycling the cultivation medium in order to reduce the microalgae production costs. This work aims to study several oxidation techniques for treatment of permeate produced during harvesting of microalga *Dunaliella salina* by membrane filtration. The treated permeate produced is assessed for reuse as cultivation medium of *Dunaliella salina*. The permeate produced during membrane harvesting of *Dunaliella salina* was recovered and treated by ozonation and UV radiation. The different types of permeate produced were tested as cultivation media. The results obtained indicate that the permeate treated by UV with addition of H_2O_2 was best suited for the growth of *Dunaliella salina* during carotenogenesis. Nevertheless, although not performing as well as the selected cultivation medium, the use of untreated permeate seems a reasonable alternative to it, as the untreated permeate has potential to be the most cost efficient solution.

4.2 Introduction

The halophilic microalga *Dunaliella salina* is produced industrially due to its high content in β-carotene [67,88] and other carotenoids, which have an estimated market value of $1428.12 million in 2019 [11]. The accumulation of carotenoids by *Dunaliella salina* is induced under high light exposure, low nitrogen and high salinity conditions [67,75,76]. This microalga also accumulates glycerol under high salinity conditions, up to levels that can exceed 50% of its dry mass [67,68].

Despite the high industrial potential of *Dunaliella salina*, the production costs are substantial and determine the economic viability of production in large-scale systems. The cultivation medium and harvesting stage represent significant running costs [50,53]. Harvesting represents up to 20-30% of the total cost of microalgae production [15,16]. Indeed, microalgae harvesting involves high energy expenditure due to the low biomass concentration of the culture (typically lower than 1 g/L), small cell

size (10-12 μm, in the case of *Dunaliella salina*) and similar density to the culture medium. The most common harvesting technique is centrifugation [15–17,21]. However, additional savings may be obtained when harvesting through a combination of pre-concentration by membrane filtration followed by centrifugation. Particularly, energy costs were reduced when harvesting *Nannochloropsis* [20]. Also, the authors found an OPEX and CAPEX reduction when harvesting *Dunaliella salina* by membrane filtration prior to centrifugation [94].

Membrane filtration may play an important role in *Dunaliella salina* harvesting because it is effective for processing dilute streams (pre-concentration step of harvesting). Moreover, it can operate under gentle operating conditions (low transmembrane pressure operation and moderate cross-flow velocity), assuring a low shear stress suitable for fragile cells with no rigid cell wall [15], such as *Dunaliella salina* [9]. Accordingly, successful microfiltration and ultrafiltration membranes were used for harvesting *Dunaliella salina* [81,94].

In order to reduce the microalgae production costs, specifically the high costs of the cultivation medium, some authors have already stated the importance of recycling the cultivation medium [43,44,50,53]. Water recycling is of extreme importance not only for reducing water demand, but also for saving of nutrients [39]. Microalgae biomass production has been estimated to consume about one metric ton of water per kg of biomass produced [39]. A recent study demonstrated that permeate recycling allows for a 77% reduction of the water footprint [44]. Therefore, the reuse and recycling of cultivation medium is a key parameter to reduce the nutrient costs and water footprint [44,50,53]. In this sense, microalgae harvesting by micro-/ultrafiltration allows for obtaining a cell-free permeate which may be used as a recycled medium if it provides the right conditions for cultivation. If successful, this approach will represent not only an economic way of pre-harvesting microalgae but, additionally, the possibility to reuse medium for cultivation with inherent savings in nutrients and water.

Recent studies reported the reuse of the permeate as cultivation medium, without any additional treatment. *Chlorella vulgaris* was successfully cultivated with recycled water after harvesting by microfiltration, nanoclays or centrifugation without significant influence on cell growth [47,50,97]. Spent broth after centrifugation of *Nannochloropsis* was also applied as culture medium with no influence on the growth [53]. Also, *Synechocystis* sp. shown promising results when using 50% of permeate harvested by microfiltration as cultivation medium, obtaining good biomass concentration and specific growth rate [98].

The permeate recovered from microalgae harvesting by membrane filtration may also be free from large molecular weight contaminants, which is advantageous from a recycling point of view. However, the permeate may contain other nutrients and dissolved substances excreted by microalgae and also cell debris [43,53], which needs to be taken into account, in a case by case approach, due to a high variability of combination between strain, medium, cultivation parameters and the harvesting and medium recycling process. Rodolfi *et al.*, demonstrated that particulate material present in the recycling media influences negatively the culture growth [43]. Consequently, the need for further treatment of the permeate before being recycled has to be evaluated.

The use of ozone in the treatment of drinking water has been studied around the globe [54,99–101]. Ozone is a very selective oxidant, that reacts with some water matrix components. Ozone is also characterised by its decomposition into hydroxyl radicals (OH·), which are extremely powerful oxidants [56]. However, the drawback is the formation of undesired by-products due to the reaction of matrix components with OH radicals and ozone. The stability of ozone depends on the water matrix, especially the pH, the organic compounds' type and load, and alkalinity [54].

Advanced oxidation processes (AOPs) are usually referred to processes involving the formation of highly reactive OH radicals as an oxidant [54,56]. The main advantages of AOPs are their enhanced reactivity and shorter reaction times when compounds resilient to ozone need to be degraded. Ozone decomposition can be promoted by increasing the solution pH or by addition of hydrogen peroxide (H_2O_2) [54]. Oxygen-based AOPs comprise the formation of OH radicals due to the reaction of ozone with ultraviolet light, ozone with hydrogen peroxide, hydrogen peroxide with ultraviolet light and the decomposition of ozone at high pH values.

Ultraviolet light (UV) irradiation is also a process widely used in Europe for disinfection of wastewater and drinking water [55]. Low- and medium-pressure (LP and MP) mercury lamps are the most frequently used. Low-pressure lamps emit monochromatic light at 254 nm, while medium-pressure lamps emit polychromatic light over a range of UV and visible wavelength [55,102]. The UV application for disinfection can lower the concentration of chlorine applied as final disinfectant in water treatment plants and thus lower the levels of the chlorination by-products formed [55]. Besides disinfection, UV technology may also degrade organic compounds by direct and indirect photolysis. Direct photolysis is a consequence of light absorption, while indirect photolysis is promoted by the addition of hydrogen peroxide (H_2O_2) [102].

The chemical analysis and the bacterial load of the medium recycled for cultivation of *Dunaliella salina* has never been described before, neither their impact on the growth of this microalga, and its behavior in terms of carotenoids' production. Moreover, to the best of our knowledge, the use of subsequent advanced oxidation processes, integrated with cell harvesting by micro-/ultra-filtration, has never been reported.

In this work, the use of several oxidation techniques is evaluated for treatment of the permeate produced during harvesting of microalga *Dunaliella salina* by membrane filtration. Finally, the treated permeate produced was assessed for reuse as cultivation medium of *Dunaliella salina*.

4.3 Materials and Methods

4.3.1 Materials

Dunaliella salina cultivation was conducted at laboratory and pilot scale at A4F – Algae for Future (Lisbon, Portugal) using fresh A4F Industrial Medium with a salinity of 120 g/L (measured with ATC-Zuzi analogic refractometer). Potassium iodide (KI), Sodium sulphite (Na_2SO_3), hydrogen peroxide (H_2O_2) and tert-butyl alcohol used for the oxidation treatments were purchased from Sigma-Aldrich

(Germany). Sodium hydroxide (NaOH) used for pH adjustments was purchased from Sameca (Portugal).

4.3.2 *Dunaliella salina* culture with fresh cultivation medium

Carotenoid-rich ("orange") *Dunaliella salina* DF40 was originally collected from open ponds of Monzón Biotech (Spain), isolated by Marine Biological Association of the United Kingdom and scaled-up and produced at pilot scale by A4F. Outdoor cultivation of *Dunaliella salina* in flat-panel photobioreactors was conducted at A4F with 120 g/L salinity under controlled conditions, such as solar irradiance, cell concentration and nitrogen concentration. This culture was periodically partially harvested (semi-continuous regime) providing culture for the harvesting trials with the following characteristics: $4.7x10^5$ cell/mL with a Car/Chl ratio (carotenoids/chlorophylls ratio) of 6.7 and a carotenoid content of 19.0 mg/L for the preliminary optimisation experiments of oxidation and photodegradation treatments; and $2.8x10^5$ cell/mL with a Car/Chl ratio of 4.5 and a carotenoid content of 10.7 mg/L for the permeate treatment intended for culture medium recycling experiments.

4.3.3 Harvesting of *Dunaliella salina*: pre-concentration by membrane processing

The microalgae pre-concentration experiments were carried out, under controlled permeate flux conditions, using the experimental set-up shown in Figure 4.1 A hollow fibre membrane module (GE Healthcare, UK), with capillary membranes of polysulfone (PS) with a molecular weight cut-off (MWCO) of 100kDa, was used. The membrane module comprised 530 fibres with a length of 63.5 cm, an internal diameter of 0.5 mm, and a total filtration area of 0.48 m^2. This small membrane filtration pilot unit consists of a feed tank, a recirculation pump (suitable to process shear sensitive cells), a membrane module, pressure gauges and a permeate tank. The downstream side of the membrane pre-concentration unit integrates a permeate pump (a diaphragm pump), which could be controlled at a defined flow rate.

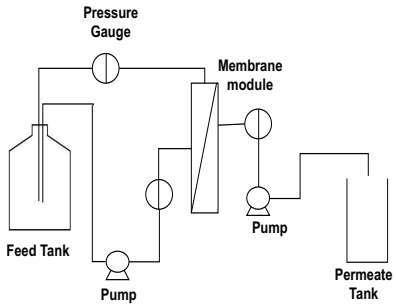


Figure 4.1. Representation of the small pilot membrane unit for *Dunaliella salina* harvesting.

The feed flow rate was set by the frequency of the recirculation pump, while the permeate flow rate was set by the permeate pump. The experiments were carried out under controlled permeate flux conditions. The permeate was collected in a recipient for further usage. Temperature in the feed tank during the pre-concentration experiments was 21 ± 2 ºC. The pH value was 8.0 ± 0.5 in all experiments. The pre-

concentration of carotenoid-rich *Dunaliella salina* by membrane processing was performed under gentle operational conditions, in order to not compromise cellular integrity. Preliminary studies under diverse cross-flow velocities ($v_{cross\text{-}flow}$) were performed to assess the integrity of *Dunaliella salina* cells. The cross-flow velocity of 0.35 m/s was selected because it corresponded to a high percentage of cell integrity (minimum of 94% after 120 minutes of recirculation) and also a low transmembrane pressure (TMP) of 0.32 ± 0.09 bar. The molecular weight cut-off of the membrane (MWCO) was 100 kDa, similar to the MWCO used in Monte *et al.* [94]. The volumetric permeate flux, J_V= 10 L/(m^2.h), was selected based on a typical value of permeate flux used in membrane bioreactors (MBR), where gentle operating conditions are also required. In order to minimise fouling, a relaxation procedure was implemented, as described by Monte *et al.* [94]. Specifically, this operating procedure consisted of repeated 10 minutes cycles, comprising 9 minutes of permeation and 1 minute with no permeation.

A volume of 20L of fresh biomass was introduced in the feed vessel and concentrated to the minimum possible volume (dead volume of the membrane unit, which was approximately 500 mL). The concentration factor, CF (-), was calculated during the harvesting process according to Equation 1:

$$CF = \frac{m_0}{m_o - m_{permeate}} \quad (1)$$

where m_0 (kg) is the mass in the feed vessel at the beginning of the experiment and $m_{permeate}$ (kg) is the mass of permeate recovered up to a given instant. The permeate, recovered up to a final concentration factor of 18, was further processed by different advanced oxidation processes.

A final harvesting study was carried out by membrane processing in three consecutives steps, similarly as performed in Monte *et al.*, 2018 [94]. The aim was to obtain the highest possible overall concentration factor without biomass damage, and also to recover higher amounts of permeate to treat at scaled-up conditions of advanced oxidation and produce sufficient recycled medium for cultivation experiments. This study comprised the pre-concentration of *Dunaliella salina* in three consecutive batches up to an overall concentration factor of 36.7 (see Figure 4.2). The optimised parameters selected for the single batch experiment were used in this study.

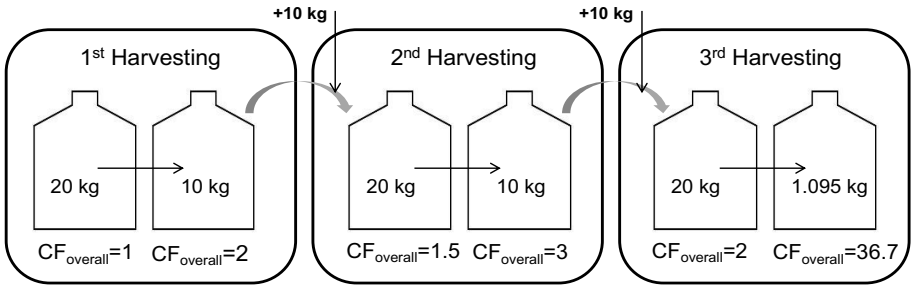


Figure 4.2. Schematic representation of the procedure used during the consecutive harvesting batches.

All permeates produced were frozen (-20ºC) until the oxidation process experiments were performed.

4.3.4 Oxidation and photodegradation treatments – small scale preliminary optimisation experiments

The oxidation treatments were performed on the permeate recovered from the single batch harvesting by ultrafiltration with a 100 kDa membrane, $v_{cross\text{-}flow}$= 0.35 m/s, with relaxation procedure and controlled permeate flux, J_V= 10 L/(m_2.h), at T= 25ºC. The accumulated permeate from a concentration factor of 2 to 18, which corresponds to the worst possible condition in terms of organic content (when comparing with the accumulated permeate with a concentration factor up to 2), was used for the different treatments in order to evaluate the potential for medium reuse in the worst-case conditions. The treatments tested were: ozonation and UV. For both types of treatment, three procedures were evaluated: 1- no pH correction; 2- no pH correction and addition of 120 mg/L H_2O_2 and; 3- pH correction to 9. The increase of pH and the addition of hydrogen peroxide may accelerate the ozone decomposition and the oxidation process [54]. The efficiencies of the treatments performed, both in this section and in section 4.3.5, were evaluated by the concentration of glycerol and the concentration of nitrate (potential by-product of the oxidation of N-compounds) in each treated sample. The glycerol content was monitored as a proxy of organic compounds present in the permeate. Indeed, glycerol is an intracellular organic compound in *Dunaliella salina*, which is accumulated in this microalga, up to levels that can exceed 50% of its dry mass under high salinity conditions [67,68]. Therefore, during the harvesting step, cell disruption may occur to a certain extent and, consequently, some intracellular organic compounds may be released, which may be related to the release of glycerol (due to its high abundance in *Dunaliella salina*).

4.3.4.1 Ozonation treatment

The membrane permeate samples were subject to ozonation in batches of 250 mL during 1 hour. The reaction was carried out in a reactor with the capacity of 500 mL and ozone was dispersed in the permeate solution using an ozone compatible fine pore (max pore size: 80 µm) rectangular diffuser (CD-1KB from Ozone Solutions, Inc.).The reactor where the ozone was bubbled was connected to two other reactors where a potassium iodide solution was placed to neutralise the excess ozone transferred to the air phase. Samples were taken every 20 minutes and placed in vials containing a sodium sulphite solution (24 mM) to quench the ozone residual [103]. Samples were also transferred along time directly into a UV-cell (without adding the quenching agent) to determine ozone spectrophotometrically using the indigo method [104,105]. The absorbance of the sample and the blank were measured at 600 nm. The concentration of ozone in the samples was measured according to equation 2:

$$O_3(\text{mg/L}) = \frac{100 \times \Delta\text{Abs}}{f \times b \times V} \quad (2)$$

where ΔAbs is the change of absorbance between the blank and the sample, *f* is a factor of 0.42, *b* is the length of the cell (in cm) and V is the volume of the sample. The factor *f* is based on a sensitivity

factor of 20000/cm for the change of absorbance (600 nm) per mole of added ozone per litre and corresponds to an absorption coefficient for aqueous ozone [105].

Permeate oxidation experiments were also conducted following the procedure described with the addition of 120 mg/L H_2O_2 to the sample and with adjustment of pH to 9 prior to ozone treatment, in order to increase the formation of the highly reactive and unselective hydroxyl radicals [54]. Samples were taken at different exposure times and placed in vials containing sodium sulphite (24mM) and tert-butyl alcohol (50mM) to quench any residual ozone and OH radical, respectively prior to analysis [103]. The concentration of glycerol and the concentration of nitrate in each treated sample were measured by HPLC and segmented flow analysis respectively. The pH and temperature were measured before and after treatment.

4.3.4.2 UV treatment

The photodegradation experiments were conducted in a double-walled petri dish kept at constant temperature (25 ± 1ºC) under a collimated beam UV source. For the optimisation of photodegradation experiments a medium pressure mercury lamp (UVH1019-Z6, UV-Technik Meyer GMBH, Germany) was used in a closed system. The UV assays were performed in batches of 50 mL during 3 hours. A stir bar was placed inside the Petri dish to ensure homogeneous exposure. Samples were taken every hour and placed in vials containing tert-butyl alcohol (50 mM) to quench any residual H_2O_2 (in samples with addition of 120 mg/L of hydrogen peroxide) prior to analysis. The concentration of glycerol and the concentration of nitrate in each treated sample were measured by HPLC and segmented flow analysis, respectively. The pH and temperature were measured before and after treatment.

4.3.5 Production of treated permeate with optimised conditions – scale-up experiments

After the optimisation of the ozonation and photodegradation treatments, a scale-up was conducted with the best results obtained in order to produce permeate for the cultivation medium assays. The treatments were performed with permeate recovered from the consecutive batches harvesting process described before. The permeate with higher organic content (accumulated permeate from concentration factor of 2 to 36.7) was used to perform the assays for the scale-up of oxidation treatments and cultivation medium experiments. For the treatment of the medium of *Dunaliella salina* to be recycled, ozone and UV with addition of H_2O_2 were performed in batches of 1.5 L. Besides the permeate treated by ozone coupled with H_2O_2, UV coupled with H_2O_2, untreated permeate and fresh cultivation medium (supplied by A4F) were used for recycling experiments as cultivation media for *Dunaliella salina*.

4.3.5.1 Ozone treatment with addition of H_2O_2

The experiments were performed as explained above (see section 4.3.4.1), however the exposure time of the samples in contact with ozone and hydrogen peroxide was increased to 6 hours due to a higher amount of organic compounds and permeate to treat. The same analyses were performed.

4.3.5.2 UV treatment with addition of H_2O_2

UV+H_2O_2 treatments were performed in 1.5 L batches during 6 hours for the production of media for cultivation experiments. For the scale-up of the process, the permeate was placed in a reactor and recycled in a closed system with a volumetric flow of (Q_v= 1L/min). For these assays, a Puro-Tap UV reactor with a low-pressure mercury lamp (PURO, Italy) was used. The analyses of glycerol and nitrate were performed as explained in section 4.3.7.1.

4.3.6 *Dunaliella salina* cultivation using differently treated recycling media

After ultrafiltration, three different samples of permeates (no further treatment; ozonation treatment with addition of H_2O_2; UV treatment with addition of H_2O_2) were used to perform a *Dunaliella salina* DF40 cultivation trial. This trial was set up under laboratory controlled conditions in triplicate for each condition: 1- fresh cultivation medium (negative control); 2- untreated permeate; 3- permeate treated with ozonation + H_2O_2; 4- permeate treated with UV + H_2O_2.

Bubble columns were inoculated in carotenoid-rich (“orange”) growth stage with an initial cell concentration of $5.0x10^5$ cell/mL for each condition and were subject to two volumetric renewals of 50%, which represents 3 successive cycles of cultivation for each condition. These systems were kept at 25ºC with a 0.5% CO_2 mixture in air bubbling under continuous one-side 100 µmol photons.m^{-2}.s^{-1}. All parameters were maintained stable, with pH between 7 and 8. All the measurements were performed in duplicate.

4.3.7 Analytical methods

4.3.7.1 Characterisation of the treated permeates

Glycerol was measured by reverse-phase high performance liquid chromatography (HPLC) (VWR Hitachi Organizer) with a refractive index detector (Merck Differencial Refractometer RI-71). The HPLC was equipped with a VARIAN Metacarb 87H column and a biorad aminex HPX -42A 125-0129 pre-column. 0.01 N sulfuric acid (H_2SO_4) was used as eluent, with a flow rate of 0.6 mL/min and an operating temperature of 50ºC. Nitrate and nitrite concentrations were measured by colorimetric methods implemented in a segmented flow analyser (Skalar 5100, Skalar Analytical, The Netherlands). The nitrite concentration during the experiments was not detected, meaning that its concentration was always below 2 ppm. The absorbance measurements to quantify the ozone concentration were

performed using an Ultrospec 2100 pro UV/Vis spectrophotometer (Biochrom, United Kingdom). All the measurements were performed in triplicate.

The total number of *Dunaliella salina* cells and their loss of integrity, quantified during the harvesting experiments, were measured with a Muse® Cell Analyser equipment (Merck Millipore, Germany). This instrument is a flow cytometer where Propidium Iodide (PI, Sigma-Aldrich), a fluorescent dye, was used to assess the microalgae cytoplasmic cell integrity.

4.3.7.2 Culture characterisation

Cultures' growth and quality were monitored through optical microscopic observation (Olympus BX53) and quantified using a Neubauer counting chamber. Carotenoids concentrations were determined by total wavelength spectrophotometric scan of the pigment solution obtained from biomass samples by extraction with bead beating and acetone. The measurements were performed in triplicate.

The elemental analyses were performed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

4.4 Results and Discussion

4.4.1 Optimisation of oxidation and photodegradation treatments at small scale – Ozonation and UV radiation

Before starting the studies of recycling the permeate as cultivation medium, it was necessary to identify the optimal conditions to treat the permeate. With this aim, a series of small scale experiments were performed with different conditions of oxidation and photodegradation of potential contaminants present in the permeate. Recycling the permeate is an important step to reduce the cost of microalgae cultivation. The permeate used for the optimisation experiments was collected during a single batch harvesting experiment, as explained in sections 4.3.3 and 4.3.4. The permeate collected from a concentration factor from 2 to 18, which corresponds to a retentate with a highest loss of cell integrity (21.5%), was used in this study.

Oxidation experiments were performed with the aim to achieve the degradation of glycerol and to ensure that other potential oxidation by-products, such as nitrate, were not formed, so that the treated media could be reused as cultivation media for *Dunaliella salina*. Glycerol was used as an indicator or a proxy of the organic compounds present in the permeate (resultant from some cell disruption which might occur during the harvesting step), since it can represent about 50% w/w of these compounds [67,68].

4.4.1.1 Evaluation of the ozone treatment at small scale conditions

For the preliminary experiments of ozonation, the treatment was performed following three different conditions: 1) permeate treated with ozone, 2) permeate treated with ozone and H_2O_2 and 3) permeate treated with ozone and an initial pH adjustment to 9. The temperature was maintained at 19 ± 1ºC

during the experiments of oxidation. However, the pH of the permeate decreases to acidic values and it therefore needs to be neutralised before being reused for the cultivation processes. When treating with ozone, ozone and H_2O_2, and ozone with pH adjustment, the initial pH was 7.1, 7.6 and 9.2 and decreased to 2.3, 1.7 and 1.5, respectively.

The ozone content (mgO_3/L) was measured in each treatment during the time of the experiments, as shown in Figure 4.3.

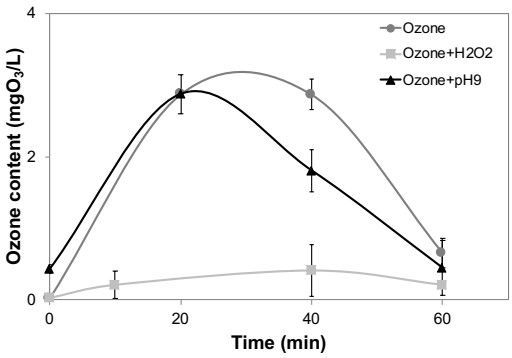


Figure 4.3. Ozone concentration (in mg O_3/L) in the permeates treated by: ozonation; ozonation with the addition of H_2O_2; ozonation after an initial pH adjustment to pH 9. Measurements were performed every 20 minutes. Data shown as mean ± standard deviation (n=3).

The maximum ozone dissolved quantified (2.8 mg/L) was obtained when treating the permeate with dispersed ozone only. Under the other two conditions, higher ozone decomposition was expected due to the formation of hydroxyl radicals. As expected, due to the high pH decrease, this effect was more notorious in the ozonation experiment conducted with hydrogen peroxide. Since ozone is unstable in water [54,106], the content of ozone decreased along all the experiments.

The concentration of glycerol and nitrate measured in the permeate samples are shown in Figure 4.4.

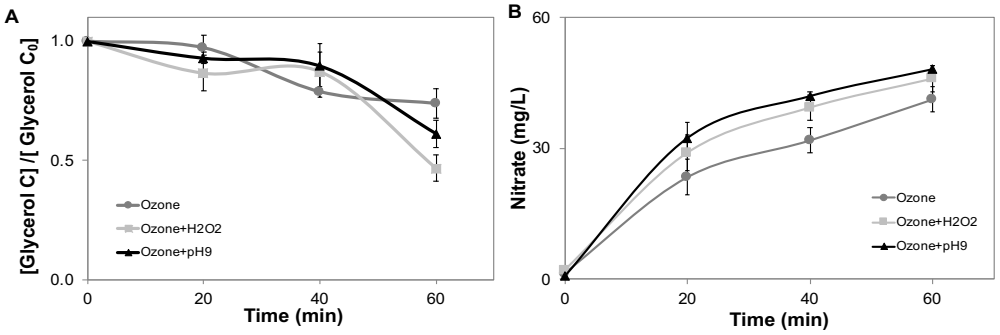


Figure 4.4. Glycerol normalised concentration (A) and nitrate content (B) (mg/L) measured in the permeates treated by ozonation, ozonation with the addition of H_2O_2 and ozonation after an initial pH adjustment to pH 9. Measurements were performed every 20 minutes. Data shown as mean ± standard deviation (n=3).

As shown in Figure 4.4, none of the ozone treatments led to a complete degradation of glycerol. All treatment efforts led to a partial reduction of glycerol concentration. The maximum reduction of glycerol content (53%) was obtained when permeates were treated by ozonation with addition of H_2O_2. Regarding the treatments by ozonation and ozonation with initial pH adjustment, there was a reduction of 26% and 39% in the glycerol content, respectively. However, oxidation by-products (such as nitrate)

increased to a maximum of about 50 mg/L after 1 hour of treatment. The ozone reacts with compounds present in the permeate, leading to the formation of nitrate upon oxidation with ozone, due to oxidation of ammonia or other nitrogen compounds to nitrate. The ozonation treatment with H_2O_2 and ozonation with adjustment to pH 9 resulted in the highest nitrate concentration. The pH of the water and the addition of hydrogen peroxide initiate ozone decomposition faster and contribute for the oxidation of ozone-resistant compounds [54], which may explain the higher concentration of nitrate observed. However, it is known that this concentration of nitrate does not negatively affect the growth of *Dunaliella salina*. Inorganic nitrogen is an essential nutrient for *Dunaliella salina* growth [107]. This microalga is able to grow under different concentrations of nitrogen [108]. Nevertheless, the nitrate availability affects the pigment content (chlorophylls and carotenoids) per cell [109,110] drastically. *Dunaliella salina* produces high amounts of carotenoids at low nitrate concentration and more chlorophyll at high nitrate concentration, so it becomes quite difficult to control the carotenogenesis process during microalgae cultivation. These preliminary tests led us to conclude that treatment by ozonation does not lead to the permeate quality required for microalgae cultivation, i.e. complete degradation of the organic compounds present, represented by glycerol, and no formation of relevant oxidation by-products, such as nitrate.

4.4.1.2 Evaluation of the UV treatment at small scale

The treatment of permeates at small scale was also performed using a medium pressure UV lamp under different conditions: 1) UV radiation, 2) UV radiation with addition of H_2O_2 and 3) UV radiation with an initial pH adjustment to 9. The temperature was maintained at 20 ± 1 ºC during the UV treatments. The pH of the solution does not present a significant variation, in contrast with the ozonation treatment. When treating with UV, UV and H_2O_2, and UV with initial pH adjustment, the initial pH was 8.0, 7.5 and 9.0, respectively. In this case, the permeates maintain their pH, which is required for reuse for the cultivation of *Dunaliella salina* (pH around 8).

The concentrations of glycerol and nitrate in each treated permeate obtained over time are shown in Figure 4.5.

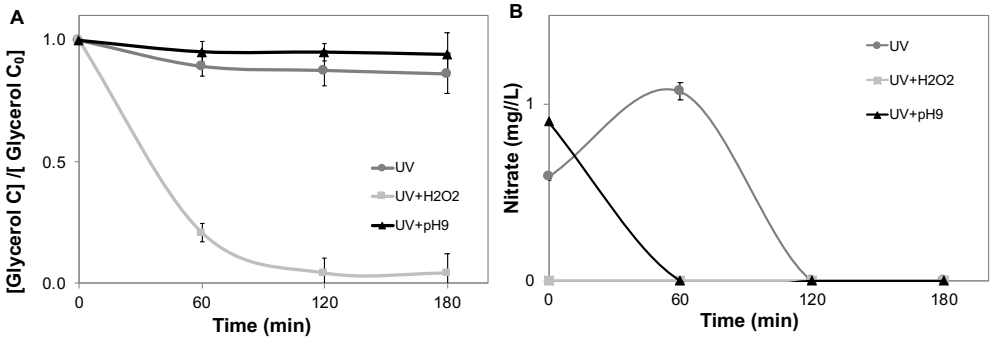


Figure 4.5. Glycerol normalised concentration (A) and nitrate content (B) (mg/L) measured in the permeates treated by UV, UV with addition of H_2O_2 and UV after an initial pH adjustment to pH 9. Measurements were performed every 1 hour. Data shown as mean ± standard deviation (n=3).

Regarding the results of glycerol oxidation, the treatment of UV with addition of H_2O_2 allows for eliminating glycerol in the medium after 1 hour of exposure to UV light. There was a reduction of 96% in the glycerol content regarding the initial value. Regarding the other UV treatments (UV and UV at pH 9), the glycerol content was maintained even after 3 hours of exposure. These results indicate that addition of hydrogen peroxide, and the subsequent formation of OH radicals, is essential for the photolysis of the organic compound glycerol in the permeate. As shown in Figure 4.5.B, the UV + H_2O_2 treatment did not lead to the production of nitrate, in contrast with the treatment by UV and UV with adjustment to pH 9. The maximum concentration of nitrate was 1.07 mg/L, accumulated when the permeated was treated by UV after 1 hour. Comparing the results obtained in the preliminary experiments conducted with ozone, it is possible to verify that the UV treatment seems to be more suitable to treat the permeate, due to the significant decrease of glycerol concentration without accumulation of oxidation by-products, such as nitrate.

4.4.2 Scale-up of the oxidation treatments – Ozonation with H_2O_2 and UV radiation with H_2O_2

The permeate used for the scale-up experiments of oxidation was collected during a three consecutive batches harvesting experiment as explained in sections 4.3.3 and 4.3.5. The permeate was characterised by a high organic carbon content, corresponding to a retentate where the biomass had a percentage of cell integrity loss between 5.8% (when the overall concentration factor was 2) and 6.6% (when the overall concentration factor was 36.7). The permeates treated by ozonation with H_2O_2 and UV radiation with H_2O_2, were recycled and used as cultivation media for *Dunaliella salina*.

The treatment of ozonation with addition of H_2O_2 decreased the pH of the permeate to acidic values (from 7 to 1.6). Regarding the temperature, it was maintained at 22ºC. In case of treatment with UV with addition of H_2O_2, the temperature increased (from 22 to 27ºC) which can be explained by the use of a pump to recirculate the volume of permeate in the system. The pH was maintained at 6.6 ± 0.6.

Figure 4.6 presents the results obtained in the two assays conducted, in terms of variation of the concentration of glycerol and nitrate (mg/L) with time, when the permeate with higher content of glycerol was treated.

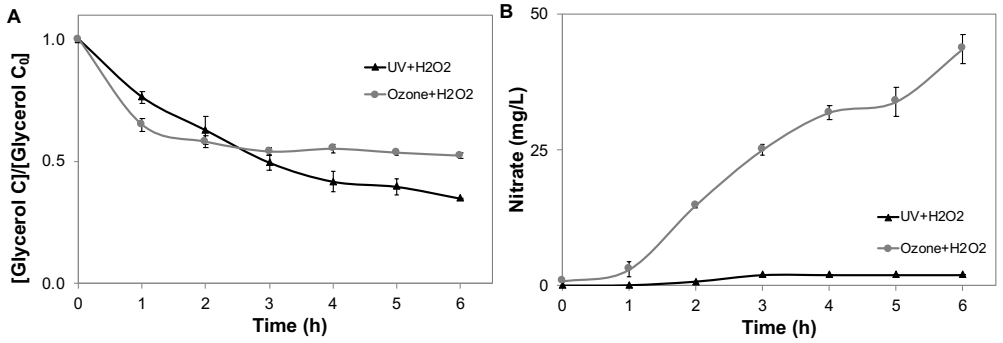


Figure 4.6. Glycerol normalised concentration (A) and nitrate content (B) measured in the permeates treated by UV with addition of H_2O_2 and ozonation with addition H_2O_2, during a 6 hour experiment at scale-up conditions. Measurements were performed every 1 hour. Data shown as mean ± standard deviation (n=3).

Under these scaling-up conditions, the concentration of glycerol decreases with time. Ozonation with H_2O_2 led to a higher content of glycerol at the end of the experiment. The permeates treated by UV with H_2O_2 and by ozonation with H_2O_2 reduced the glycerol content to 65% and 47% in relation to the initial value, respectively. Regarding the formation of nitrate, ozonation with H_2O_2 produced significantly higher concentrations. As stated before, the addition of hydrogen peroxide to the ozonation treatment, tends to increase the ozone decomposition and intensify the oxidation process [54]. As a consequence of oxidation, nitrate can be formed. The best result was once again attained with the UV + H_2O_2 treatment. Regarding the concentration of nitrate, the ozonation with H_2O_2 produced high quantities of by-products during the time of treatment. The production of nitrate is explained by the reactive OH radicals formed when using ozone coupled with H_2O_2 that react with nitrogen compounds present in the permeate. Although the formation of by-products is not suitable, the concentration of nitrate at this level is not negative for *Dunaliella salina* growth. In the case of UV radiation with H_2O_2, a LP lamp was used because the formation of nitrite is potentially higher when using MP lamps for disinfection [55]. Once again, our results only indicate the presence of a low concentration of nitrate in permeate treated with UV and H_2O_2. Nitrite was not detected.

4.4.3 *Dunaliella salina* cultivation using different recycled media after treatment

In order to examine a medium recirculation strategy for an industrial scale cultivation of *Dunaliella salina*, the untreated permeate and both UV + H_2O_2 and ozonation + H_2O_2 treated permeates were used for a *Dunaliella salina* recycled medium trial in orange growth phase, under laboratory controlled conditions.

Since the permeate treated by ozonation with addition of H_2O_2 showed a low pH (approx. 3.6), this parameter was corrected using NaOH to adjust pH to 7.3. Also, throughout the first cycle of cultivation, for all conditions, the increase of the cell density was followed by a decrease from the 4th day after inoculation. This observation allowed the optimisation of some operational strategies, namely the nitrogen concentration input and the period between renewals, in order to maintain increasing cell concentration in the cultures.

During the assay, culture pH and temperature remained within the desired range. *Dunaliella salina* growth and carotenogenesis were successful in all applied treatments and homogenous replicates were observed throughout the trial (Figure 4.7).

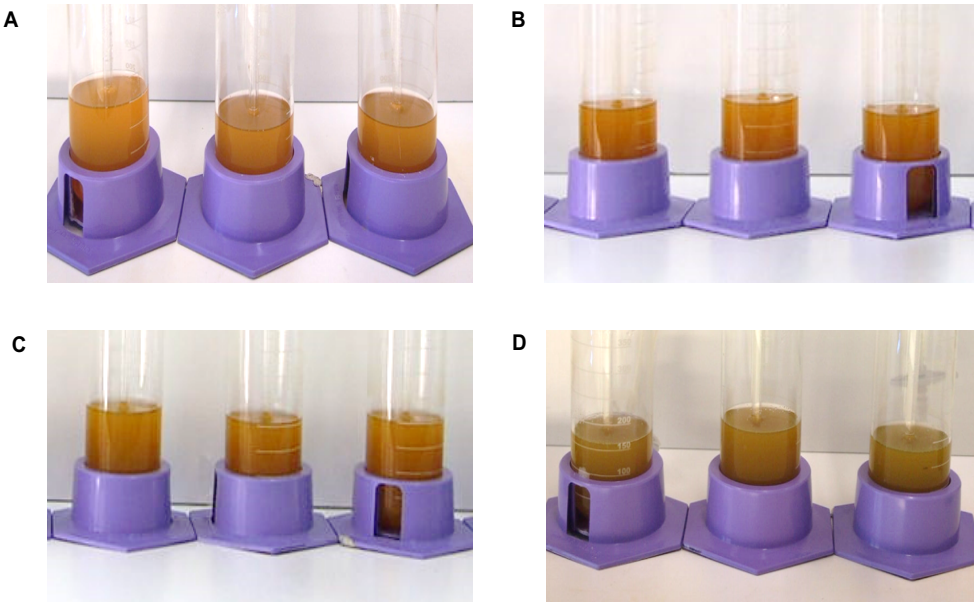


Figure 4.7. *Dunaliella salina* DF40 recycled medium trial in orange growth phase with (A) Fresh cultivation medium (negative control); (B) untreated permeate; (C) permeate treated by UV with addition of H_2O_2 and (D) permeate treated by ozonation with addition of H_2O_2. The recycling medium experiments were performed in triplicate.

Oxidation treatments applied to the permeate led to changes in the medium composition presented in Table 4.1. Due to complex organic matter oxidation, an increase in ammoniacal nitrogen occurred in both treated permeates. An increase in nitrate up to 4.5 mM was also observed in the permeate treated with ozonation + H_2O_2, making this permeate less suited to orange phase cultivations, as this amount of this nitrogen source makes the carotenogenesis process very difficult to control.

Table 4.1. Elemental analysis results of fresh and recycled media, with and without permeate treatment.

	Start of cultivation trial				**End of cultivation trial**			
Compound (mM)	**Fresh cultivation medium**	**Permeate without treatment**	**Permeate treated with ozonation + H_2O_2**	**Permeate treated with UV + H_2O_2**	**Fresh cultivation medium**	**Permeate without treatment**	**Permeate treated with ozonation + H_2O_2**	**Permeate treated with UV + H_2O_2**
Ammonia/N	0.064	0.010	0.161	0.126	0.008	0.014	0.039	0.004
Nitrate/N	0.200	0.000	3.693	0.000	0.000	0.000	2.093	0.000
Bicarbonate	1.803	2.590	0.459	2.032	2.639	2.885	3.081	2.770
K	2	5	5	3	4	5	5	4
P	0.017	0.004	0.010	0.007	0.005	0.006	0.007	0.004
Ca	0.965	1.544	1.676	1.157	2.282	1.585	1.735	1.655
Mn	0.002	0.002	0.003	0.002	0.000	0.001	0.001	0.002
Mo	0.001	0.002	0.002	0.001	0.002	0.002	0.002	0.002
S	54	50	54	56	39	38	40	35

Besides changes in the elemental composition caused by oxidation treatments, the characterisation of the elemental composition of the media after the *Dunaliella salina* recycled medium trial also points towards a potential for imbalance in the medium recipe in the case of Mn, which might lead to its depletion during cultivation. This result indicates that an adjustment of the concentration of this element ion in the fresh medium recipe might be in order. P concentration is also lower in the media at the end of the assay, but this is consistent with the rationed addition of nutrients done during orange growth stage.

The results of the elemental composition analysis were compared with the ones from *Dunaliella salina* cultivation trials in orange growth phase. As expected, using ozonation + H_2O_2 for permeate treatment did not allow the correct control of the nitrate concentration and, therefore, of carotenogenesis, which combined with high radiation also did not allow the growth of the culture. In addition to the stress conditions such as low nitrogen, carotenogenesis occurs with degradation of chlorophyll (Chl) entailing the reduction of photosynthetic apparatus in order to avoid photooxidative injury. Therefore, the Carotenoids/Chlorophylls ratio (Car/Chl ratio) values are evidence of the cell physiological condition for the ozonation treatment, whereas they achieved desired values for the remaining three conditions (Figure 4.8.A). The fresh medium culture achieved the best performance in the conditions tested (Figure 4.8).

Nevertheless, both untreated permeate and permeate treated with UV + H_2O_2 provided very promising results regarding culture growth and carotenoids production (Figure 4.8). Based on the results shown in Figure 4.8.B, the maximum volumetric productivity of carotenoids was also calculated: 6.93 ± 0.48 $mg.L^{-1}.d^{-1}$ for the untreated permeate and 5.98 ± 0.41 $mg.L^{-1}.d^{-1}$ for the treatment with advanced oxidation by UV+H_2O_2.

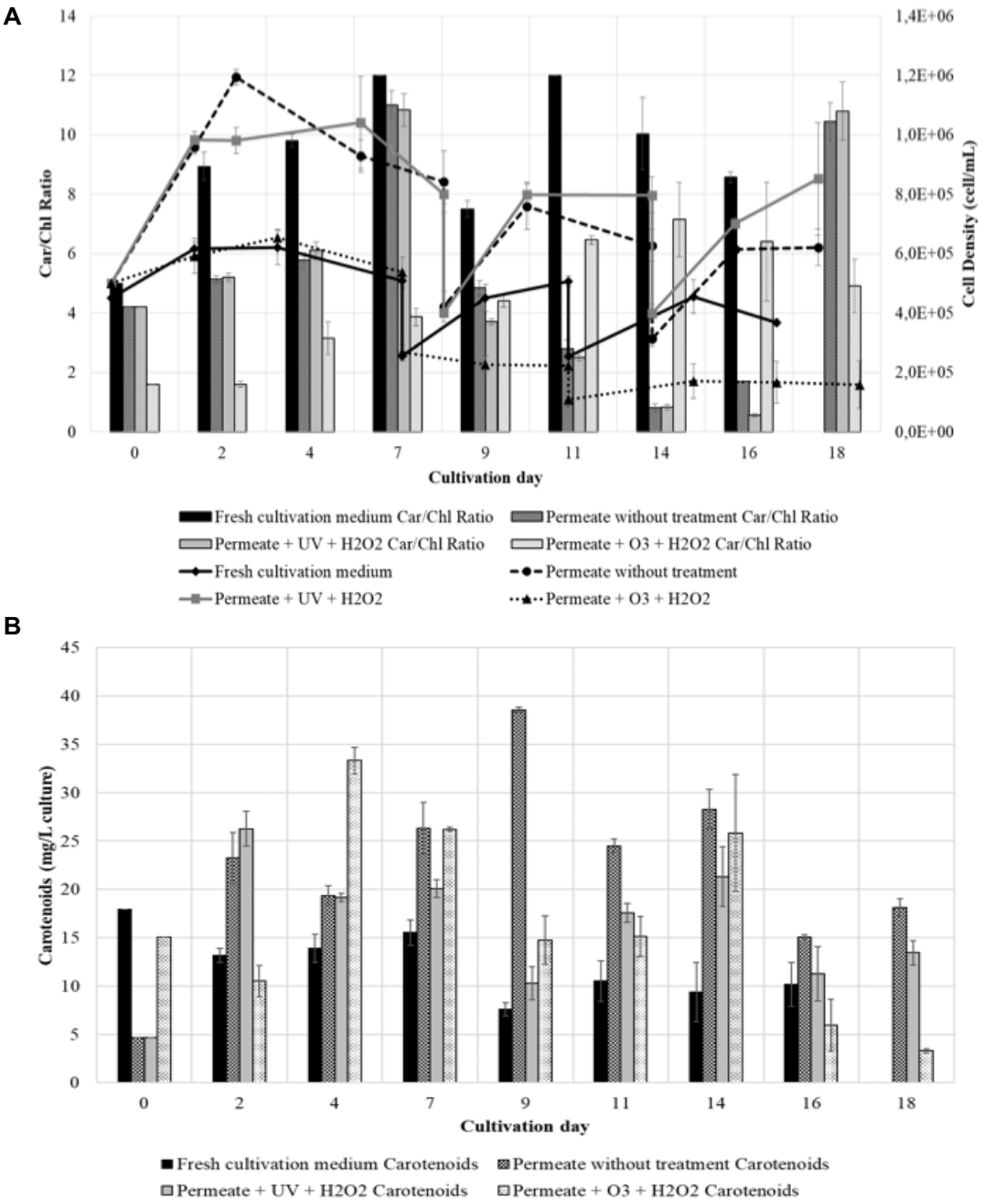


Figure 4.8. Overall (A) culture growth and the corresponding Car/Chl ratio and (B) carotenoid volumetric concentration for the Dunaliella salina DF40 recycled medium trial in orange growth stage. The bars correspond to (A) Car/Chl ratio and (B) carotenoids volumetric concentration and the symbols correspond to cell density. Data shown as mean Å} standard deviation (n=3).

Interaction between microalgae and bacteria are well known [111]. Microalgal-bacterial interactions are complex and recognised as competition, commensalism or parasitism [112]. The growth of microalgae depend on bacteria competition with algae for nutrients, such as nitrogen and phosphorus, and the possible release of inhibitory or stimulatory substances by bacteria [113]. Recent studies demonstrated that many bacterial strains present algicidal effects. *Flavobacterium* showed a decrease on algal cell density after bacterial inoculation [114]. Also, antagonistic relationships were reported between diatom growth and bacterial populations [115]. Due to the potential influence of bacteria on microalgae growth and on the quality of the harvested microalgal biomass, bacterial load was qualitatively analysed in order to compare the presence of bacteria during the cultivation trials with untreated and treated permeate. Throughout the cultivation trials, the qualitative bacterial load observed routinely by microscopy showed significant differences between the untreated permeate and the permeate treated with UV + H_2O_2, likely attributable to the elimination of organic carbon sources during UV + H_2O_2 application (Figure 4.9). Despite the merely indicative nature of the data, it strengthens the point that the treated permeate should be preferentially chosen for cultivation. Nonetheless, the qualitative bacterial load observed when using untreated permeate was always lower than when using the fresh cultivation medium. These results highlight the importance of treating the permeate from microalgae harvesting with advanced oxidation processes, especially with the addition of UV + H_2O_2 due to higher cell growth and lowest bacterial load.

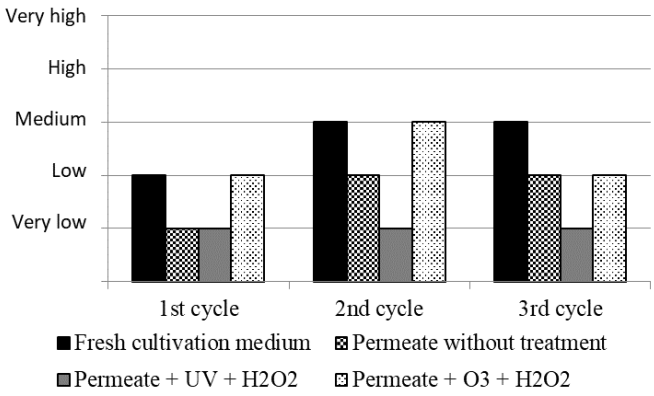


Figure 4.9. Qualitative bacterial load throughout the *Dunaliella salina* DF40 recycled medium trial in orange growth stage. Cultivation in each medium regime was conducted in triplicate and the observed bacterial load was consistent in each measurement of the replicates.

4.5 Conclusions

Permeate treatment using ozone and UV with advanced oxidation processes (AOPs) was proven to be a technically feasible solution, which has the potential to reduce production costs and the environmental impact. Culture medium treatment and recycling were assayed, with the permeate obtained from membrane filtration.

Several criteria were considered for the global assessment of the recycled media employed herein, which can impact on the quality of process and final product: chemical analysis of the medium,

carotenoid productivity during cultivation, Car/Chl ratio of the final biomass and qualitative bacterial load. The high amount of nitrate existent on the permeate after ozone + H_2O_2 treatment makes the carotenogenesis process difficult to control and, thus, this was considered an unrecommendable option in the conditions tested. According to chemical analysis and the *Dunaliella salina* recycled medium cultivation trial, the best treatment to be used for recycling medium is UV with addition of H_2O_2 due to the low content of nitrate formed and the highest degradation of organic compounds (glycerol) obtained. The results obtained indicate that the permeate treated with UV with addition of H_2O_2 was best suited for growth of *Dunaliella salina* in carotenogenesis, followed by the untreated permeate. In fact, recycling permeate treated with UV and H_2O_2 not only supported the growth rate of the microalgae, but also sustained the Car/Chl ratio and the low bacterial load, which evidence the high performance and quality of the culture. Reuse of the cultivation medium can be achieved using membrane harvesting by ultrafiltration combined with advanced oxidation processes.

The use of untreated permeate as cultivation medium led to a good growth rate of the microalgae and production of carotenoids similar to the permeate treated with UV and H_2O_2 but, on the other hand, to a corresponding chemical analysis and bacterial load at the end of the cultivation trial of inferior quality when compared to the selected cultivation medium. Nevertheless, the untreated permeate seems to be a feasible cultivation medium, because it has potential to be the most cost efficient solution.

Recycling medium can reduce the cultivation of microalgae costs and also reduce the water footprint.

5 BIOREFINERY OF *DUNALIELLA SALINA*: SUSTAINABLE RECOVERY OF CAROTENOIDS, POLAR LIPIDS AND GLYCEROL

To be submitted as: Joana Monte, Cláudia Ribeiro, Celina Parreira, Luís Costa, Lena Brive, Susana Casal, Carla Brazinha, João G. Crespo, "Biorefinery of Dunaliella salina*: sustainable recovery of carotenoids, polar lipids and glycerol", Bioresource Technology.*

The author was involved in planning and performing the experiments, as well as on the discussion and interpretation of the results and preparation of the manuscript.

5.1 Summary

Dunaliella salina is well-known for its high content in carotenoids and glycerol. Nevertheless, *Dunaliella salina* has also a high content in lipids, including polar lipids, which are suitable for nutraceutical/cosmeceutical applications.

This work proposes a sustainable process to maximise the potential of *Dunaliella salina* for the production of distinct fractions of carotenoids, glycerol, polar lipids and proteins, which may contribute to improve the revenues of the microalgae industry. In this work extraction with approved REACH solvents and organic solvent nanofiltration are integrated, in order to obtain added-value products and glycerol. Also, aiming to separate carotenoids from glycerides, a saponification process is proposed and studied. High overall recoveries were obtained for carotenoids (85%), glycerol (86%), polar lipids (94%) and proteins (95%). In order to evaluate the profitability of the proposed biorefinery, an economic assessment was accomplished. Both CAPEX and OPEX were calculated, likewise the Return of Investment (ROI).

5.2 Introduction

Microalgae are a promising feedstock as a source of biofuels, proteins and other bioactive compounds, which may contribute to address the problem of growing demand for renewable sources, in response to the increase of world population and the need for sustainable sources of energy and food. There is an economic need to turn microalgae biorefineries into profitable processes, moving away from microalgae processes solely focused on biofuel production. Biorefineries may be converted in a feasible cultivation for obtaining a portfolio of valuable products (e. g. biofuel, energy and chemical compounds) [30,59,60].

Dunaliella salina is industrially produced as dried algal cells (with no further processing for recovery and purification of target compounds) for use in food supplements, due to its high content in carotenoids, in particular β-carotene (up to 10-14% of dry weight) [76,110] known for its antioxidant properties [73,116]. Nevertheless, *Dunaliella salina* has also an important content in lipids (10 – 25 % dry weight)

[4,117]. 41% of the lipids present in *Dunaliella salina* are polar, suitable for nutraceutical/cosmetic applications [118]. Moreover, under high salinity cultivation conditions, *Dunaliella salina* can accumulate more than 50% of its dry mass as glycerol [10]. Proteins may represent 10-40% of the total dry weight [10,67] and are commonly used for feed applications. Also, carbohydrates represent up to 30% of the total dry weight [1]. Therefore, carotenoids, polar lipids, glycerol, proteins and carbohydrates may potentially be recovered and purified from *Dunaliella salina*.

Downstream processing has a high impact on the sustainability of a biorefinery [64]. The first stage of biorefinery processing is cell disruption, in order to release intracellular compounds. The most common cell disruption techniques are: bead mill, high pressure homogenisation and ultrasonication [119].

After cell disruption, the target compounds are recovered usually by solvent extraction [119]. The solvents selected should exhibit hydrophobic/hydrophilicity properties similar to the target compounds to be recovered. Accordingly, for the recovery of lipids, organic solvents or supercritical CO_2 are used [30,59] or, alternatively, a saponification process should be performed [120] . The aim of saponification is the transformation of glycerides into free fatty acids and glycerol in the presence of an alcohol or water and an alkali catalyst (NaOH or KOH) [14].

Finally, the target compounds are fractioned and purified from the extracts produced. Membrane processing is an emerging technology in algae biorefinery processes, but few examples of such processes are described [30,60,63]. Column chromatography is a well-known and established technique for the purification of all-trans-β-carotene and 9-cis-β-carotene [121,122], but it is much more expensive and it involves the use of non-REACH solvents.

So far, *Dunaliella salina* biorefineries have been focused on the recovery of carotenoids for food supplements, lipids for biodiesel and the residual biomass for biogas production [1,71,72]. Espada *et al.* reported a *Dunaliella salina* based biorefinery with the recovery of β-carotene using $scCO_2$ extraction and solvent extraction followed by saponification. The remaining biomass was anaerobically digested for biogas production and the solid residue was sold as fertilizer [71]. Other reports describe the extraction of β-carotene from *Dunaliella salina* biomass using organic solvents and membrane filtration, for further fractionation of the carotenoids, while biomass residues were used as fertilizer [72].

This work aims to propose a sustainable process scheme to maximise the potential of *Dunaliella salina* as a feedstock, focusing on the production of distinct fractions rich in carotenoids, polar lipids, glycerol and proteins. This integrated approach for the biorefinery of *Dunaliella salina* involves new concepts, namely the exclusive use of sustainable solvents (REACH approved) and technologies recognised as being low energy intensive (membrane processes). Additionally, to our knowledge, a process scheme for the production of such large diversity of compounds from *Dunaliella salina* has not been proposed and studied.

5.3 Materials and Methods

5.3.1 Materials

Analytical-grade n-heptane, tetrahydrofuran (THF), methyl tert-butyl ether (MTBE), methanol, concentrated sulphuric acid and phenol were purchased from Sigma-Aldrich (Germany). Ethanol absolute was purchased from VWR (Germany). Potassium hydroxide (KOH) was obtained from Merck (Germany). Analytical-grade standard substances monoglyceride olein (>99% purity), diglyceride olein (99.7% purity), triglyceride olein (99.6% purity), pentadecanoic acid (>99% purity), glycerol (≥99.5% purity), phenol (99.0% purity), glucose (≥99.5% purity) and DPPH (1,1-diphenyl-2-picrylhydrazyl radical) were purchased from Sigma-Aldrich. Bovine Serum Albumin (BSA) was obtained from Thermo Scientific (USA).

Four commercial organic solvent resistant nanofiltration membranes were selected for this work, namely: PuraMem S600, PuraMem 280, DuraMem 300 and DuraMem 500 from Evonik (Germany). The main characteristics of these membranes are shown in Table 5.1.

Table 5.1. Properties of the membranes tested in this work (provided by Evonik).

Membrane	MWCO (g/mol)	Membrane material	Maximum operating pressure [bar]
PuraMem 280	280 [(a)]	Polyimide	60
PuraMem S600	600	Polyimide	60
DuraMem 300	300	Modified Polyimide	60
DuraMem 500	500	Modified Polyimide	20

[(a)] molecules with this, or higher, molecular mass are at least 90% rejected (information provided by the manufacturer, Evonik).

5.3.2 Carotenoid-rich *Dunaliella salina* culture

The carotenoid-rich *Dunaliella salina* DF40 used in this work was collected from open ponds of Monzón Biotech (Spain), isolated by the Marine Biological Association (United Kingdom) and scaled-up and produced by A4F – Algae for Future (Portugal). For the biorefinery studies, the *Dunaliella salina* culture was harvested by centrifugation (5000 rpm for 10 minutes) and the cell pellet was lyophilised and used for the preparation of extracts.

5.3.3 Experimental procedure

The scheme of the different processing steps, performed within the biorefinery concept discussed in this work, is presented in Figure 5.1, where two downstream routes are proposed: an organophilic route and a hydroethanolic. The target products to be obtained in each route are also listed.

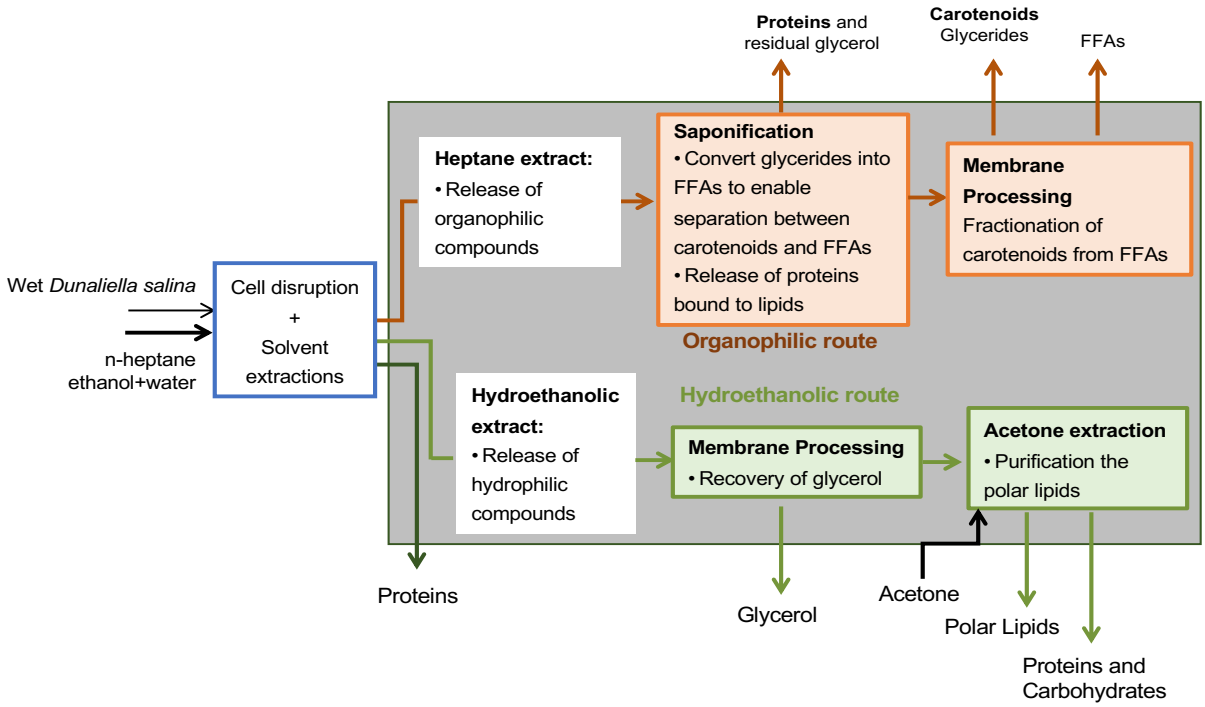


Figure 5.1. Scheme of the main processing steps of *Dunaliella salina*, considering the biorefinery scheme proposed in this work. Legend: FFAs (free fatty acids).

For the sake of simplicity, the detailed sequence of unit operations is not represented in Figure 5.1.

5.3.3.1 Cell disruption procedure

For the extraction of intracellular compounds from *Dunaliella salina*, a ultrasonication was used for cell disruption. At laboratorial scale, the ultrasonication equipment (Sonics VCX750) was used, applying 5 cycles of 20 seconds pulses with 5 seconds interval at 20% of amplitude (see Appendix A.1). However, at industrial scale, although bead milling has been proposed as a suitable cell disruption technology [2], on the economic assessment it was considered that, due to the fragility of *Dunaliella* cells, the rupture occurred concomitantly with the first step of the biorefining process (solvent extraction with n-heptane and ethanol + water).

5.3.3.2 Solvent extraction

Different solvent extraction processes were used to recover different target compounds. N-heptane was used for the extraction of carotenoids, glycerides and free fatty acids (FFAs), while more hydrophilic solvents (ethanol in water (68% v/v)) were applied for the extraction of glycerol, polar lipids, proteins and carbohydrates. As shown in Figure 5.1, n-heptane (ratio of 1:20 (mass of wet algae biomass/volume of solvent phase)), then ethanol in water (ratio of 1:14 (mass of wet algae biomass/volume of solvent phase)) were added to *Dunaliella salina*. Based on [123], an acetone extraction was also performed in the hydroethanolic route, aiming at recovering and purifying the polar lipids. The lipid residue was combined with acetone, using a ratio of 1:30 (mass of lipid residue/volume of solvent phase)).

5.3.3.3 Experimental procedure for saponification of the n-heptane extract from *Dunaliella salina*

After the solvent extractions performed, as described in section 5.3.3.2, the n-heptane phase was saponified using KOH as an alkaline agent. The saponification reaction was based on the procedure described by Viegas *et al.* [124] and Peterson *et al.* [125]. The n-heptane phase (10 mL) was combined with 5 mL of ethanol, 2 mL of H_2O and 0.4 mL of KOH (600 g/L) and incubated for 24 hours (see Appendix A.1), at room temperature, with constant agitation under dark conditions. After incubation, the two phases were separated and the organophilic phase was processed by solvent resistant nanofiltration (see section 5.3.3.4), while the hydrophilic phase was quantified in terms of proteins and glycerol.

5.3.3.4 Membrane processing

Membrane filtration experiments were performed in a stainless steel METcell unit (MET, United Kingdom). The extracts were processed in a dead-end mode, using a membrane with an effective area of 51.4 cm^2. The permeate was collected and the permeate flux was monitored by mass acquisition using an electronic balance (Kern 572, Kern, Germany). The feed reservoir was filled with 100 g of extract. The cell was pressurised at 15 bar (for DuraMem 500), 30 bar (for DuraMem300) and 35 bar (for PuraMem 280 and PuraMem S600), at 20 ºC. The organophilic and hydroethanolic fractions were processed using PuraMem and DuraMem membranes, respectively. After a reduction of 80% of the initial feed mass, that corresponds to a concentration factor of 5, samples of final retentate and final permeate were taken for analysis. The concentration factor reached, CF (-), was determined according to Equation 1:

$$CF = \frac{m_0}{m_o - m_{permeate}} \quad (1)$$

where m_0 (kg) is the mass of feed in the beginning of the experiment and $m_{permeate}$ (kg) is the mass of permeate collected up to a given time. The samples collected from the processing of both extracts were analysed, as explained in section 5.3.4.

5.3.4 Analytical methods

5.3.4.1 Quantification of Carotenoids

The carotenoids content was analysed by high performance liquid chromatography (HPLC). The HPLC used (Alliance e2695 model), supplied by Waters (Massachusetts, USA), was equipped with a degasser, autosampler and a photodiode array (PDA) detector. The chromatographic separation was performed with a YMC C30 Carotenoid column (Classical Analytical HPLC Column S-5µm, 250 x 4.6 mm, YMC) and a Guard cartridge YCM C30 pre-column (5µm, 10x4.0mm, YMC). Carotenoids were eluted using methanol:MTBE (methyl tert-butyl ether) (80:20) under isocratic conditions at a flow rate

of 1 mL/min and an operating temperature of 25ºC. The injection volume was 20 µL and the detector was set at 450 nm. Lutein, zeaxanthin, α-carotene, all-trans-β-carotene and 9-cis-β-carotene were quantified using calibration curves established with pure standards and expressed as micrograms per mL (µg/mL).

5.3.4.2 Quantification of glycerides and free fatty acids (FFAs)

The chemical characterisation of the organophilic samples was performed by high performance size exclusion chromatography (HPSEC). This technique permits the characterisation of samples in terms of the concentration of glycerides and free fatty acids in the same running samples. All samples from the organophilic fraction were further separated by HPSEC on a Jasco (Japan) HPLC system, equipped with a styrene-divinylbenzene copolymer R column (pore size 10 nm; 60 cm x 7mm) and refractive index (RI) detection (Gilson, USA). Samples were eluted using tetrahydrofuran at a flow rate of 1 mL/min and the injection volume was 20 µL. The quantification was achieved using commercial standards representative of each class, namely: FFAs, mono-, di- and triglycerides (MG, DG and TG).

5.3.4.3 Quantification of glycerol, proteins and carbohydrates

Glycerol was quantified by a spectrophotometric method, according to [126]. A volume of 1 mL of periodate reagent was added to a mixture of 0.2 mL of sample and 0.2 mL of H_2O in a test tube. This mixture was allowed to react for 5 minutes at room temperature. Immediately after reaction, acetylacetone (2.5mL) was added and mixed. The samples were placed in a water bath at 50ºC for 20 minutes. After cooling at room temperature, the absorbance was measured at 410 nm in a spectrophotometer. Glycerol was used as a standard for the calibration curve.

The protein content was measured using the Lowry method [127]. Briefly, an aliquot (1mL) of sample was mixed with 3 mL of an alkaline copper solution and allowed to react for 10 minutes. After reaction, 0.3 mL of diluted Folin-Ciocalteu reagent (1:2) was added to the mixture. After 30 minutes, the absorbance was measured at 750 nm. Bovine Serum Albumin (BSA) was used as standard for calibration and quantification.

Carbohydrates were estimated using a phenol-sulphuric acid method [128]. Briefly, 2.5 mL of concentrated sulphuric acid were added to 0.5 mL of sample and 0.5 ml of 5% phenol in water and allowed to react for 10 minutes. After reaction, samples were shaken and incubated in the dark during 30 minutes. The absorbance was measured at 490 nm. Glucose was used as standard for calibration and quantification.

5.3.4.4 Quantification of Polar Lipids

The total polar lipids were quantified based in the Bligh & Dyer method [129]. 2 mL methanol and 1 mL chloroform were added to 0.5 g of sample. The mixture was shaken vigorously for 2 minutes. Then, 1 ml of chloroform was added to the mixture and shaken again for 2 minutes. Distilled water (0.8 mL) was added and the mixture was vortexed for 2 minutes. The two phases were separated by centrifugation (2000 rpm for 10 minutes). The chloroform layer was transferred to a glass vial. A second extraction was performed with 2 mL of 10% (v/v) of methanol in chloroform. The mixture was shaken for 2 minutes and centrifuged for the separation of the two phases. The two chloroform phases were combined and evaporated. The lipid residue was weighted to determine the lipid content of the sample.

The different polar lipids were quantified by LC-MS at the laboratory of Life Sciences and Materials at Research Institutes of Sweden (RISE).

5.4 Results and Discussion

5.4.1 Design of a biorefinery based on carotenoid-rich *Dunaliella salina*

For the development of a biorefinery based on carotenoid-rich *Dunaliella salina*, a process scheme is proposed for the recovery of the target compounds defined previously: carotenoids, polar lipids, glycerol, proteins and carbohydrates. The flow chart of the biorefinery process is shown in Figure 5.2.

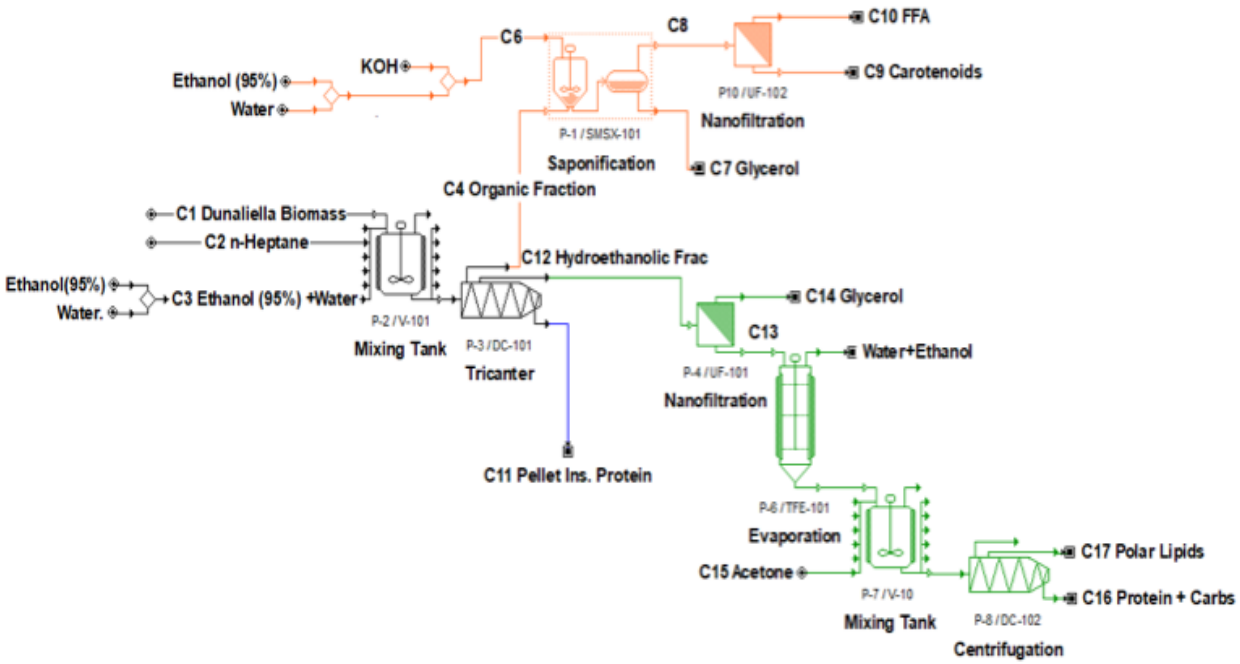


Figure 5.2. Flow chart of the biorefinery proposed for the production of target compounds from *Dunaliella salina* biomass (a detailed flow chart, which includes the process for solvent recovery is shown in the Appendix A.1).

The first step of the proposed scheme comprises a solvent extraction with n-heptane (organophilic route) and with ethanol in water (68% v/v) (hydroethanolic route). The aim is to use solvents with different polarities to extract compounds with different properties (more hydrophobic or more polar). N-

heptane was used for the extraction of carotenoids, FFAs and glycerides due to high affinity of this solvent towards these target compounds. The ethanol in water mix was chosen due to the affinity to more polar compounds, such as polar lipids, glycerol and some proteins. The solvents proposed are considered REACH, standard and harmless. The n-heptane route also comprises also a saponification reaction. The aim is to convert glycerides into FFAs, in order to enable the fractionation of carotenoids and glycerides, which present similar molecular weight. Also, solvent resistant nanofiltration membranes were integrated in the biorefinery process for the recovery of added-value compounds and for solvent recycling.

The proposed biorefinery scheme (see Figure 5.2) uses *Dunaliella salina* biomass that is extracted with n-heptane and ethanol in water (68% v/v) for the recovery of target compounds. Ultrasonication was used for cell disruption and two main routes were established: the organophilic route and the hydroethanolic route. In the organophilic route, a saponification reaction was additionally performed. Afterwards, carotenoids, FFAs and glycerides (MG, DG and TG) were fractionated using a solvent resistant membrane process (see section 5.4.2). Besides these organophilic compounds, also proteins and a small fraction of glycerol are recovered in the ethanolic phase, after the saponification reaction (C7). In the hydroethanolic route, membrane processing combined with a precipitation with acetone allows for the recovery of glycerol, polar lipids and proteins (see section 5.4.3).

5.4.2 Organophilic route for the recovery of carotenoids and free fatty acids (FFAs)

For the extraction of organophilic compounds (target carotenoids and also FFAs and glycerides), a highly nonpolar solvent (n-heptane) was chosen, due to its solubility of carotenes and the benignity of n-heptane, which is regarded as less harmful than tetrahydrofuran and hexane.

5.4.2.1 Optimisation of saponification of the n-heptane extract for the recovery of carotenoids and free fatty acids (FFAs)

Dunaliella salina glycerides are mainly in the form of triglycerides, which can be converted in FFAs, mainly in the form of palmitic (27%) and linoleic acid (23%). Linoleic acid is the most interesting compound, but represents less than one third of all FFAs formed. Triglycerides and carotenoids have similar molecular weights, which represents a challenge if their fractionation by nanofiltration is attempted. On the contrary, carotenoids and FFAs (200-300 g/mol) present significantly larger differences of molecular weight, which makes their fractionation by nanofiltration feasible. In order to solve this problem, the strategy proposed involves performing a saponification reaction, in order to hydrolyse triglycerides to FFAs. This reaction will enable for an easier fractionation of carotenoids from the glycerides (neutral lipids) that are present in the organophilic fraction. The conversion of glycerides was performed as described in section 5.3.3.3. The content in glycerides and free fatty acids (FFAs) is used as an indicator of the advancement of the saponification reaction. Besides the conversion of glycerides into FFAs and glycerol, it is necessary to assure that the carotenoids present maintain their

antioxidant activity and do not degrade during the saponification reaction. Therefore, after optimising the saponification reaction time (see Appendix A.1), the carotenoids were analysed by HPLC and DPPH. In Table 5.2, the results obtained concerning the carotenoids' concentrations and their bioactivity, expressed as percentage of inhibition of DPPH (1,1-diphenyl-2-picrylhydrazyl radical), are presented. The higher the percentage of DPPH, the stronger is the antioxidant activity, meaning a high availability for hydrogen donation. Compounds with antioxidant activity should be able to reduce the DPPH to 2,2-diphenyl-1-picrylhydrazil (yellow colour). The DPPH method is described in detail in the Appendix A.1.

Table 5.2. Concentration of carotenoids and DPPH radical scavenging effect (%) for the *Dunaliella salina* biomass (C1) and for samples before (C4) and after saponification (C8).

Sample	Total Concentration of carotenoids (mg/mL)	DPPH radical scavenging effect (%)
Biomass (C1)	0.85 ± 0.02	57% ± 2%
Before Saponification (C4)	0.84 ± 0.02	57% ± 1%
After saponification (C8)	0.80 ± 0.02	50% ± 1%

As shown in Table 5.2, the concentration of carotenoids was kept constant in all three streams, with only a slight reduction of values after the saponification reaction. Previous work also demonstrated the recovery of 95% of lutein (carotene) from microalgae biomass using an alkaline treatment [131]. The same results were obtained when measuring the antioxidant activity of the extracts before and after saponification (C4 and C8 process streams) (measured by DPPH radical scavenging effect). These results demonstrate that the saponification reaction does not degrade the carotenoids and has no impact on the antioxidant activity. However, it should be mentioned, under the conditions selected involving a mild saponification, not all glycerides are converted into FFAs. Actually, these reaction operating conditions were selected in order to assure that the carotenoids present are not affected during saponification. The high DPPH radical scavenging effect of carotenoids extracts was also reported in several studies [132–134]. The carotenoids are generally considered to be responsible for the high antioxidant activity observed in the extracts, due to their known antioxidant properties [73,116].

5.4.2.2 Separation of carotenoids from free fatty acids (FFAs) by organic solvent nanofiltration (OSN)

Membrane technology was used for the separation of carotenoids from the FFAs. The aim of fractionation using solvent resistant nanofiltration membranes is to obtain a retentate with a maximised concentration of carotenoids and a permeate rich in FFAs. Moreover, this organic solvent nanofiltration process allows the recovery and recycling of n-heptane, from the permeate stream, used in the extraction.

The organic fraction (C8) from the saponification reaction was processed by nanofiltration. Initially, two membranes were evaluated in order to characterise their behaviour concerning the retention of

carotenoids, glycerides and FFAs. These experiments were performed at 35 bar. Table 5.3 reports the global rejection and adsorption of carotenoids, FFAs and glycerides for each membrane experiment and the average flux obtained, J_v (L/m^2.h). The global rejection for each compound, R(%), is calculated using equation 2:

$$R(\%) = \frac{m_{feed} - m_{perm}}{m_{feed}} \times 100 \quad (2)$$

where m_{feed} is the mass of the compound in the feed stream.

The adsorption (%) is calculated according to equation 3:

$$Adsorption(\%) = 1 - \frac{m_{ret} + m_{perm}}{m_{feed}} \quad (3)$$

where m_{ret} is the mass of the compound in the retentate.

Table 5.3. Global rejection, R (%), and adsorptions (%) of carotenoids, glycerides and free fatty acids (FFAs) for the membranes tested at 35 bar. Average permeate flux J_V (L/(m^2.h)) obtained with PuraMem 280 and PuraMem S600.

	Carotenoids		Glycerides		Free Fatty Acids		
Membrane	***R* (%)**	**Adsorption (%)**	***R* (%)**	**Adsorption (%)**	***R* (%)**	**Adsorption (%)**	**Flux, J_v (L/(m^2.h))**
Puramem 280	70 ± 1	2 ± 1	83 ± 1	33 + 2	28 ± 2	10 ± 2	3.4 ± 0.7
Puramem S600	70 ± 1	0 ± 0	64 ± 4	37 ± 1	62 ± 3	38 ± 7	12.8 ± 1.6

A lower global rejection is observed for FFAs, when comparing with the rejections for carotenoids and glycerides: 28 ± 2% and 62 ± 3% when using membranes PuraMem 280 and PuraMem S600, respectively (see Table 5.3). This is explained by the lower molecular mass of these compounds. The global rejections for carotenoids (70 ± 1%) and for glycerides (> 64%) are higher for both membranes tested, confirming that most carotenoids and glycerides are retained by the membranes. As expected, the FFAs can be recovered in the permeate due to their lower molecular mass while the remaining glycerides (that were not saponified) are mostly retained, together with carotenoids. Since the main objective is the separation of carotenoids from FFAs, the membrane selected was PuraMem 280. Although the permeate flux obtained is modest and similar rejections were obtained for carotenoids with both membranes, the lowest rejection to FFAs and higher rejections to glycerides prevail in the choice of PuraMem 280. The PuraMem S600 membrane presents a relatively high rejection of FFAs (62 ± 3%), which was not expected due to its higher molecular weight cut-off (600 Da). This behaviour might be explained by the high values of adsorption of the glycerides and specially the FFAs, when the PuraMem S600 membrane is used, combined with a high retention of carotenoids and glycerides that may contribute for membrane fouling and increase the rejection of FFAs. Regarding the adsorption of the compounds, PuraMem S600 showed the highest values. Both membranes have the same chemical material; nevertheless it might be speculated that differences in the membrane surface rugosity can explain the differences in the adsorption observed. According to Ribeiro *et al*., polyamide/polysulfone membranes are effective in the fractionation of soyben oil/hexane extracts, composed by triglycerides

and FFAs, resulting in a low permeate flux (8.2 L/(m^2.h)) [135]. Another work, also demonstrated that lipid constituents (mono-, di- and triglycerides and FFAs) were effectively separated with dense polymeric membranes [136]. Previous work also evaluated the separation of steryl esters (~ 650 g/mol, similar molecular weight as carotenoids) from pesticides (300 g/mol, similar molecular weight as FFAs) using organic solvent nanofiltration, assuring rejections of oleic acid and steryl esters above 70% [137].

5.4.3 Hydroethanolic route for the recovery of glycerol, polar lipids, proteins and carbohydrates

For the extraction of hydrophilic compounds, a polar solvent (ethanol in water (68% v/v)) was chosen, due to high solubility of polar lipids and glycerol, which are the most important compounds in this route. In a first step, glycerol was separated from the proteins and carbohydrates through membrane processing. Subsequently, polar lipids were purified and separated by extraction with acetone.

5.4.3.1 Separation of glycerol from the proteins and carbohydrates by organic solvent nanofiltration (OSN)

Organic solvent nanofiltration membranes were used for the separation of glycerol from proteins and carbohydrates. The objective is to obtain the highest rejection to proteins and carbohydrates and the lowest rejection to glycerol. The hydroethanolic extract was processed with two different membranes: DuraMem 300 and DuraMem 500. Their performance was evaluated in terms of the global rejection and adsorption of glycerol, proteins and carbohydrates and the average permeate flux. Experiments with the DuraMem 300 and DuraMem 500 membranes were performed at 30 and 15 bar, respectively. The values of global rejection and adsorption for each compound and membrane are shown in Table 5.4.

Table 5.4. Global rejections, R(%), and adsorptions (%) of glycerol, proteins and carbohydrates for the membranes tested at 30 bar (DuraMem 300) and 15 bar (DuraMem 500). Average permeate flux, J_V (L/(m^2.h)), for the membranes tested.

	Glycerol		Proteins		Carbohydrates		
Membrane	***R* (%)**	**Adsorption (%)**	***R* (%)**	**Adsorption (%)**	***R* (%)**	**Adsorption (%)**	**Flux, J_v (L/(m^2.h))**
DuraMem 300	22 ± 1	3 ± 0	57 ± 4	21 ± 3	83 ± 1	1 ± 0	3.8 ± 0.9
DuraMem 500	26 ± 0	42 ± 4	50 ± 0	26 ± 5	73 ± 2	25 ± 8	11.6 ± 2.0

As shown in Table 5.4, among the compounds under study, glycerol has the lowest global rejection with both membranes tested (Table 5.4), explained by its lowest molecular weight (92 g/mol). Regarding proteins and carbohydrates, for DuraMem 300, the global rejections were higher: 57 ± 4% for proteins and 83 ± 1% for carbohydrates. When processing with DuraMem 500, the global rejections were reduced, as expected: 50 ± 0% for proteins and 73 ± 2% for carbohydrates. The higher rejections observed, using a DuraMem300 membrane, might be explained by the lower molecular weight cut-off of this membrane and the higher transmembrane pressure applied (usually, higher transmembrane

pressures lead to higher rejections). The exception is observed for glycerol, where its rejection with DuraMem 500 is slightly higher than the rejection with DuraMem 300, probably due to a much lower adsorption with DuraMem 300. In conclusion, and although with although with a modest permeate flux, DuraMem 300 presents the best compromise for the removal of glycerol, with high rejection for proteins and carbohydrates.

5.4.3.2 Separation and purification of polar lipids from the proteins and carbohydrates

Dunaliella salina comprises an important fraction of polar lipids, which had not been studied in a biorefinery concept. The characterisation of polar lipids in *Dunaliella salina* is presented in Table 5.5.

Table 5.5. Characterisation and identification of the main polar lipids present in Dunaliella salina biomass (dry weight), by LC-MS.

	Phosphatidylcholine (%w/w)	**Phosphatidylserine (%w/w)**	**Monogalactosyl-diacylglycerol (%w/w)**	**Total Polar Lipids (%w/w)**
Biomass	6%	7%	7%	20%

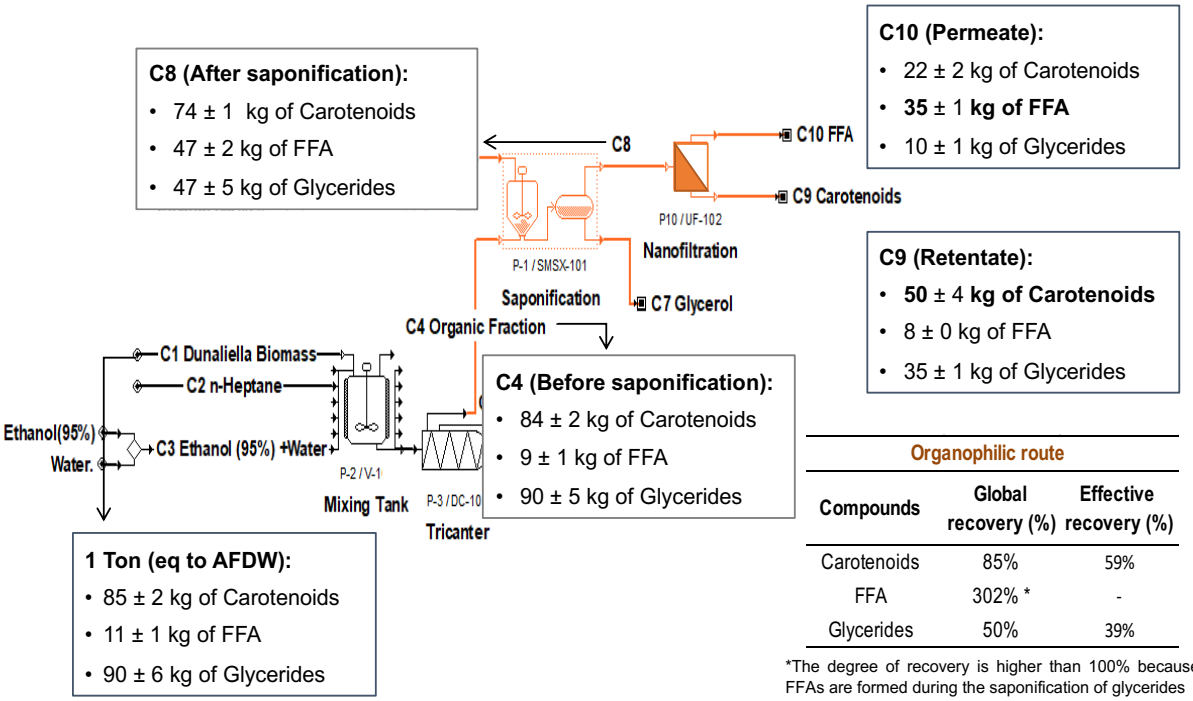
As shown in Table 5.5, *Dunaliella salina* has 20% (w/w) of polar lipids. The identified polar lipids were phosphatidylcholine, phosphatidylserine and monogalactosyl-diacylglycerol, which are important compounds for pharmaceutical applications. According to the Bligh & Dyer method, the total polar lipids quantified are 16% (w/w), which is similar to the value determined by LC-MS. Being a more common and simple method, this technique was used for further quantification. Polar lipids should be present in the hydroethanolic route due to their higher solubility in more polar solvents such as ethanol and methanol [138]. While all the other lipids (carotenoids, glycerides and FFAs) should partitioning to organic phase (organophilic route), due to their higher solubilities in heptane [139].

The nanofiltration process (presented and discussed in 5.4.3.1) allowed to obtain a retentate enriched in polar lipids, proteins and carbohydrates. However, it is also important to recover the polar lipids present. Based on previous works, a strategy to separate the polar lipids was implemented by extraction with acetone [123]. This strategy allowed also for the precipitation of the proteins and carbohydrates present in this fraction. Finally, after centrifugation, it was possible to obtain a pellet enriched in carbohydrates and proteins, and a supernatant rich in polar lipids (see section 5.4.4). The supernatant fraction, enriched in polar lipids, may found use in cosmetic and pharmaceutical applications.

5.4.4 Recovery of added-value compounds from *Dunaliella salina* biomass

After designing the biorefinery process, the chemical characterisation of each process stream and the degree of recovery of each compound was performed. The chemical characterisation of the process streams was obtained considering ash-free dry weight (AFDW) and the analysis performed to the extracts produced at laboratory scale. For this analysis, a base of 1 ton of *Dunaliella salina* (AFDW)

was assumed. In Figure 5.3, the characterisation of each process stream of the organophilic route is presented, as well as the overall and effective recovery yields obtained for each class of compounds. The global recovery was calculated considering all the process streams where the compound is present. The effective recovery was calculated considering the stream where the target compound was recovered in higher amount.



Organophilic route

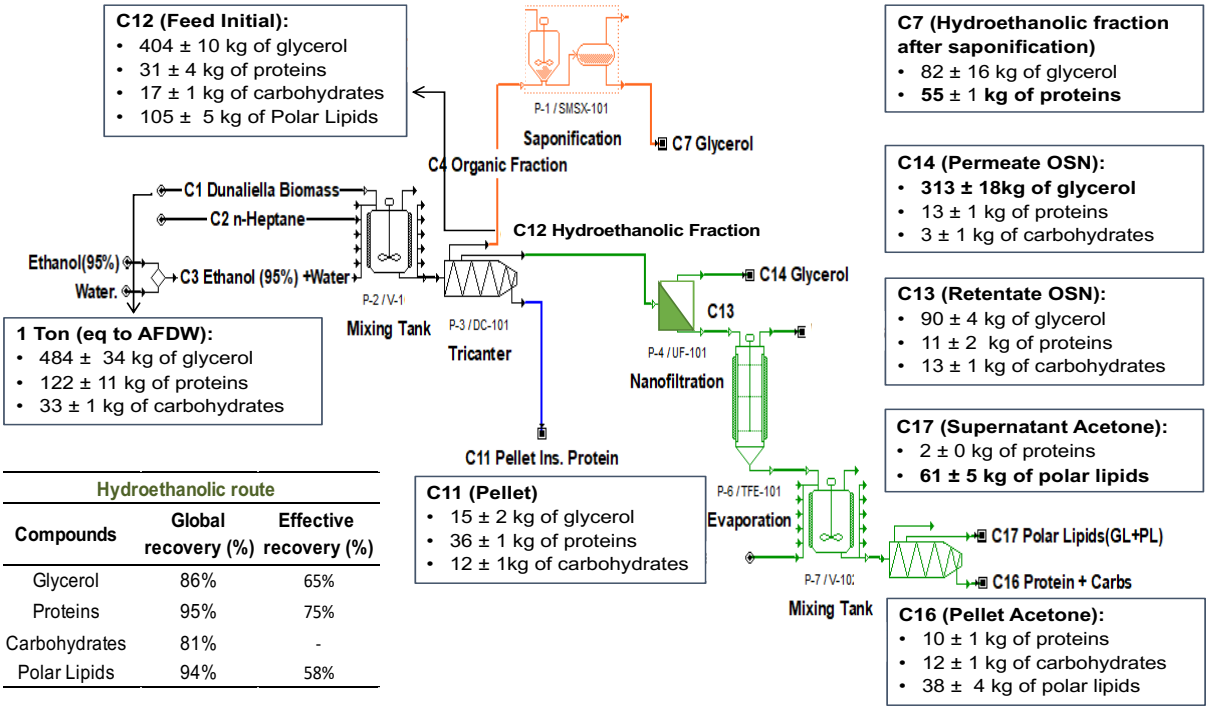
Compounds	Global recovery (%)	Effective recovery (%)
Carotenoids	85%	59%
FFA	302% *	-
Glycerides	50%	39%

*The degree of recovery is higher than 100% because FFAs are formed during the saponification of glycerides

Figure 5.3. Scheme of the proposed biorefinery, including a characterisation of the *Dunaliella salina* biomass and the composition of the process streams of the organophilic route in terms of carotenoids, FFAs and glycerides (mono-, di- and triglycerides). The global and effective yields of the recovery for each class of the compound are also presented.

In the organic fraction, 85% of the carotenoids were recovered, and 3 times more FFAs. This can be explained by the fact that during saponification, glycerides were transformed into FFAs and glycerol. Therefore, only 50% of glycerides were recovered, mainly in the retentate, due to a reduction of these compounds during saponification. At the end, 50 ± 4 kg of the carotenoids were recovered in the retentate (C9), representing an effective recovery of 59%, with some glycerides (35 ± 1 kg), which can be used as food supplement.

The same chemical analyses were performed to the process streams of the hydroethanolic route. The results obtained are presented in Figure 5.4.



Hydroethanolic route		
Compounds	**Global recovery (%)**	**Effective recovery (%)**
Glycerol	86%	65%
Proteins	95%	75%
Carbohydrates	81%	-
Polar Lipids	94%	58%

Figure 5.4. Scheme of the proposed biorefinery, including a characterisation of the *Dunaliella salina* biomass and the composition of the process streams of the hydroethanolic route. The global and effective yields of recovery for each class of compound are also presented.

Regarding the hydroethanolic fraction, it was possible to obtain a global recovery of 86% of glycerol, 95% of proteins and 81% and of carbohydrates. Carbohydrates are not a target class, considering that they are present in several process streams. Most of the glycerol (313 kg) was recovered in the permeate after membrane processing (C14), representing an effective recovery of 65%. This fraction, rich in glycerol can be applied in the food industry. The proteins are mainly present in the pellet (36 kg, corresponding to insoluble proteins), and the remaining proteins (soluble proteins), processed by nanofiltration, were recovered in the pellet of the acetone precipitation (10 kg). It was interesting to notice that a large portion of proteins was also recovered on the hydrophilic fraction after the saponification process (55 kg). Probably, this fraction is constituted by glycoproteins, which are linked to lipids and can only be recovered after saponification. These two fractions (C7 and C11) correspond to 75% of effective protein recovery. Finally, the polar lipids were mainly recovered in the supernatant (61 kg, corresponding to 58% of effective recovery), after extraction with acetone. This fraction can potentially be applied in the pharmaceutical and the cosmetic industries. The pellet from precipitation with acetone (C16) is mainly composed by polar lipids (38 kg) and smaller quantities of proteins and carbohydrates, which might be used for feed application. The remaining fractions can be applied as fertilizers due to the mix of chemical compounds present.

This biorefinery concept considers also the need for solvent recovery, after extraction and nanofiltration processing (see Appendix A.1). This process enables for a reduction in use of solvents and, consequently, energy and economic savings are expected to be achieved rendering the process more sustainable and economically competitive.

5.4.5 Economic evaluation

This process was simulated considering a *Dunaliella salina* dry biomass feed of 240 L/day (84 m^3/year), originated from a harvesting process fed with 112000 m^3/year of microalgal culture. A continuous operating regime was chosen with a working period of 350 day/year and 22h/day. The *Dunaliella salina* biorefinery overall flow chart with solvent recovery is shown in Appendix A.1 and includes: 3 mixing tanks, 1 tricanter, 2 centrifuges, 2 nanofiltration membrane units and 3 evaporators with an estimated total equipment investment of 191 k€. The average annual maintenance and consumables cost has been assumed as a percentage of 5% and 2% of the investment cost, respectively. Energy cost for mixing tanks were assumed at 10 kW/m^3_{tank}, for evaporators at 130 kWh/$m^3_{evaporated}$, for centrifuges at 2.5 kWh/m^3_{flux} and for membranes at 0.87 kWh/m^3_{flux}. Regarding the solvents cost calculation, a 90% recovery was assumed. Table 6 shows the equipment needed for the biorefinery, the solvents and reagents cost and the selling prices of the final products. For the calculation of the revenues, it was considered that all the final products were sold at market prices.

Table 5.6. (A) Equipment used in the biorefinery, sizing and cost of each equipment, (B) solvents and reagents cost and (C) final products selling price used for the economic assessment.

(A)

Equipment	IN (kg/h)	Sizing	CAPEX (€)
Mixing Tank 1	292.8	0.53 m^3	15 840 € *[1]
Tricanter	292.8	470 kg max	39 000 € *[2]
Saponification	301.0	0.76 m^3	19 200 € *[1]
Centrifuge 1	301.0	470 kg max	30 000 € *[2]
Evaporation 1	240.6	m3/h evaporated	24 000 € *[3]
Nanofiltration 1	60.5	0.64 m^2	6 400 € *[4]
Nanofiltration 2	140.7	1.40 m^2	14 000 € *[4]
Evaporation 2	112.3	0.06 m3/h evaporated	14 400 € *[3]
Mixing Tank 2	49.5	0.13 m^3	7 440 € *[1]
Centrifuge 2	49.5	60 kg max	21 000 € *[1]
Evaporation 3	38.4	0.04 m3/h evaporated	6 000 € *[3]

*[1] www.matche.com
*[2] Provided by A4F
*[3] www.alibaba.com
*[4] Considering 10000 €/m^2 of membrane

(B)

Solvents/ Reagents Cost	Unit price (€/kg)
n-heptane	0.684
Water	0.002
Ethanol (95%)	0.487
KOH solid	1.780
Acetone	1.200

(C)

Final products selling price	Unit price (€/kg)
Protein Fraction + Carbs	10
Carotenoids	800
Lipid Extract	60
Glycerol	0.125

A sensitive analysis for the case of the cost of the raw material was performed. In terms of raw material purchase cost (€/$kg_{biomass}$), with 8% increase of the cost of the raw material in the 2nd scenario. Two different scenarios are compared in order to attest that the biorefinery process proposed in this work is profitable and more economic than the current process, where only dried algal cells are produced from *Dunaliella salina* (Table 5.7).

Table 5.7. Economic assessment of the *Dunaliella salina* biorefinery. CAPEX (capital expenditure), OPEX (operational expenditure, ROI (Return of Investment), IRR (Internal Rate of Return) and pay-back (years) calculated for 350 day/year and 22h/day of biorefinery operation.

Economic evaluation of the biorefinery		
Microalgae Harvesting Capacity (m3/year)	112000	
Microalgae Biorefinery Capacity (m3/year)	84	
Raw Material Purchase Cost (€/kg biomass)	***50 €***	***54 €***
CAPEX		
Total equipment cost (€)	191 000	191 000
Capital costs (€/year)	19 100	19 100
OPEX		
Operational Energy (kWh/year)	360 000	360 000
Energy price (€/year)	55 000	55 000
(0,138 €/kWh)[*1]		
Maintenance (€/year)	10 000	10 000
Consumables (€/year)	4 000	4 000
Solvents/Reagents (€/year)	193 000	193 000
Biomass Purchase Cost (€/year)	850 000	918 000
Total OPEX/year	1.11 M	1.18
Total Cost (M€/year)	1.13	1.20
Revenue (€/year)	1.28	1.28
Revenue Biorefined products (€/kg biomass)	***75***	***75***
	+ 50%	*+ 39%*
IRR	*89%*	*53%*
ROI	*796%*	*440%*
Pay-back (years)	*1.3*	*2.3*

[*1]www.pordata.pt

For the proposed biorefinery process from *Dunaliella* biomass, and commercializing the products which were assumed to be valorised at a combined value of 75 €/kg of processed biomass, it is possible to envisage that the ROI of the operation is heavily dependent on the cost of the *Dunaliella* biomass purchased as raw material. In these scenarios, the first scenario, with a biomass purchase cost of 50 €/$kg_{biomass}$, shows an additional valorisation of biomass in 50% by commercializing refined products which in turn provides an appealing ROI of 796% and IRR of 89%. However, if the raw material purchase cost increases to 54 €/$kg_{biomass}$ (second scenario), the valorisation decreases to 39%. In terms of Return of Investment (ROI), considering the accumulated profit over 10 years, the second scenario

provides a ROI of 440% and an IRR of 53%. The economic evaluation performed shows that the designed biorefinery process is economically viable with a reasonable pay-back period of 1.3 years, in the best scenario.

5.5 Conclusions

An integrated process was proposed for the recovery and fractionation of a large diversity of compounds from *Dunaliella salina*, using REACH solvents and membrane processes. It is aimed the development of a sustainable process, economically competitive. The designed biorefinery allowed the recovery of 94% of polar lipids, 85% of carotenoids and 86% of glycerol and, in specific cases, it was possible to fractionate the valuable compounds in order to obtain streams with potential for direct application. Concerning the economic assessment, it was possible to valorise the biomass significantly and it was obtained a ROI of 796% and IRR of 89%

6 CONCLUSIONS AND FUTURE WORK

6.1 General conclusions

The work of this thesis is focused on the study of downstream processing of the halotolerant microalga *Dunaliella salina*, namely the harvesting and the extraction and fractionation / purification of added-value compounds. Additionally, a recycling process for the permeate was developed during this project, for reducing the associated costs of microalgae cultivation.

An integrated cell harvesting approach for both non-carotenogenic and carotenoid-rich *Dunaliella salina* was developed, by combining membrane filtration and centrifugation. Also, a new strategy for the recycling of cultivation media, after membrane harvesting, was proposed aiming to reduce the water footprint related to microalgae cultivation and, at the same time, reducing microalgae production costs. Finally, a *Dunaliella salina* biorefinery was designed combining REACH solvents and membrane technology.

Initially, the non-carotenogenic *Dunaliella salina* was assessed for the potential harvesting by pre-concentration with membrane filtration followed by a low-shear centrifugation (Spiral Plate Technology). The experiments were performed in a pilot scale membrane cross-flow unit and the main operating parameters were evaluated: cross-flow velocity ($v_{cross\text{-}flow}$), transmembrane pressure (TMP), backflush protocol and molecular weight cut-off (MWCO) of the membrane. Additionally, a comparison of the Total Cost of Ownership (TCO) and energy consumption using the two-step harvesting and using the harvesting solely by centrifugation, was performed. The harvesting of non-carotenogenic *Dunaliella salina* allowed reaching a concentration factor of 5.9, with an average volumetric permeate flux of 31 L/(m^2.h), and a loss of cell integrity of 10%. The harvesting strategy of the two-step process led to a reduction of 52% of the total cost of ownership and an energy reduction of 45%.

In what concerns the harvesting of carotenoid-rich *Dunaliella salina*, a membrane processing for pre-concentration under controlled permeate flux was examined, followed by a final concentration using low-shear centrifugation. The harvesting experiments were performed evaluating the following operating parameters: cross-flow velocity ($v_{cross\text{-}flow}$), molecular weight cut-off of the membrane (MWCO), use of relaxation cycles and volumetric permeate flux (J_V). After optimisation of the parameters at lab scale, the harvesting was performed at pilot scale and the Total Cost of Ownership (TCO) and the consumption of energy was determined. The carotenoid-rich *Dunaliella salina* was successfully harvested using a 100 kDa membrane, under controlled permeate flux conditions (J_V = 21 L/(m^2.h)), reaching a final concentration factor of 10, with no observed damaged cells. The economic evaluation showed that harvesting using a two-step approach was significantly more economic than harvesting solely by centrifugation. When applying the two-step approach, the reduction of Total Cost of Ownership (TCO) and energy consumption were 70 and 76%, respectively.

After membrane harvesting of carotenoid-rich *Dunaliella salina*, the use of advanced oxidation techniques was evaluated for the treatment of the permeate produced. Additionally, the treated and non-treated permeates were assessed for reuse as cultivation medium of *Dunaliella salina* under carotenogenesis conditions. The permeate treatment by ozone and UV proved to be a feasible solution, with high potential to reduce the microalgae production costs and environmental impact. The best treatment to be applied was found to be UV with addition of H_2O_2, due to the highest degradation of organic compounds and the lowest formation of oxidation by-products such as nitrate. Nevertheless, the untreated permeate might also be a feasible solution, due to the lower cost of its production. Recycling of culture medium has a significant impact on the cost of microalgae cultivation and also reduces the water footprint.

Finally, a sustainable process to maximise the potential of carotenoid-rich *Dunaliella salina* was proposed for the production of distinct fractions of carotenoids, glycerol, polar lipids and proteins. The work combined the extraction with REACH solvents and membrane processing (organic solvent nanofiltration) to obtain target added-value compounds. The compounds were extracted using two main routes: an organophilic process and a hydroethanolic process. In the organophilic route, an important fraction enriched in carotenoids was produced as well as proteins linked to lipids. Regarding the hydroethanolic route, fractions enriched in proteins and glycerol were produced. Additionally, a purified fraction of polar lipids was obtained. Overall, high recovery degrees were obtained, namely: 85% for carotenoids, 86% for glycerol, 94% for polar lipids and 95% for proteins.

The work developed in this PhD thesis constitutes a set of processing steps aiming an optimised downstream processing of *Dunaliella salina*. It has been proven that an integrated harvesting process (membranes and centrifuges) can reduce the overall energy and capital costs and, at the same time, produce a retentate characterised by a minimal loss of cell integrity and a clear permeate. It is also important to mention that a new method, suitable for growth of *Dunaliella salina* under carotenogenesis conditions, was implemented by recycling the previously harvested permeate.

Finally, the biorefinery scheme proposed represents a contribution for the design of a *Dunaliella salina* based biorefinery, allowing for the recovery of different classes of compounds (polar lipids, proteins and glycerol), in addition to the already common carotenoids. More interestingly, the biorefinery proposed uses wet biomass instead of lyophilised biomass. The removal of the evaporation step, while processing the *Dunaliella salina* biomass, represents a significant reduction in the cost of high-value compounds production.

6.2 Future Work

The present PhD thesis proposes a biorefinery process for the valorisation of the halotolerant *Dunaliella salina*. This process includes harvesting, the recycling of the permeate as a cultivation media and the recovery of high-value fractions from *Dunaliella salina* biomass, in order to reduce production costs and improve the revenues of the microalgae industry.

Shear stress on *Dunaliella salina* is a critical parameter, which needs to be studied in depth. Shear stress may induce morphological changes, loss of cell viability and cell lysis [140,141]. For better understanding the mechanisms related with the impact of shear stress and the loss of integrity of the cells, experiments should be performed in order to determine the ranges of tolerance to shear stress by *Dunaliella salina*, especially in the carotenoid-rich phase. In order to evaluate the shear stress effect individually, during a significant period of time, the cells should be recirculated and exposed to shear stress under laminar flow regime (taking into account that our harvesting experiments were conducted under this type of regime). These experiments can be performed using a shear stress device based on Poiseuille laminar flow [140]. Additionally, harvesting experiments should be performed adding 0.02% of carboxymethyl cellulose (CMC) to the cultivation broth, which help to protect the cells against shear-induced damage [142].

Regarding the recycling permeate experiments, the cost associated with the UV and ozone treatment should be studied and compared with the centrifugation treatment already applied in other microalgae [43]. Activated carbon is also an efficient adsorbent that can remove organic compounds and other contaminants in the recycling media. This process has proven to be beneficial for the removal of selective compounds such as nitrogen and organic carbon [143]. Therefore, this process should also be tested for production of recycling medium for *Dunaliella salina*, although an environmental impact analysis should be performed.

The biorefinery scheme proposed in this PhD thesis was designed for the recovery of several classes of compounds from *Dunaliella salina*. High overall recoveries and purified fractions of polar lipids and glycerol were obtained. The purification of other target compounds, such as carotenoids and FFAs, should be improved using other purifying techniques such as chromatography, crystallisation or diananofiltration. Then, a techno-economic analysis should be performed for the overall downstream processing scheme in order to assess its feasibility. In order to validate this biorefinery for application in the microalgae industry, other strains could be studied and some adjustments may be required, taking into account the major compounds of the microalgae strains.

A common saponification under alkaline conditions was proposed in this thesis. The saponification performed does not allow for a complete conversion of glycerides into FFAs, so a higher concentration of KOH should be tested. The selection of the KOH concentration to be used needs to take into consideration its potential impact on the carotenoids' antioxidant activity. Moreover, an enzymatic hydrolysis could also be performed as an alternative to alkaline saponification [144,145]. Additionally, the comparison of the efficiency of enzymatic and alkaline hydrolysis should be evaluated. Ultimately, a life cycle analysis is required in order to better define the most appropriate solutions.

Proteins are also an important class of compounds of interest, especially in the case of non-carotenogenic *Dunaliella salina*. The recovery and purification of proteins and enzymes should be tested, namely using aqueous two-phase systems. This technique is characterised by its high content in water (70-80% w/w) and low interfacial tension, providing gentle separation conditions and allowing for purification of high-value compounds, without denaturation and loss of biological activity [146]. This

process may increase the value of the enzymes recovered and potential for their use in pharma applications.

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APPENDIX

A.1. Supplementary Material for the biorefinery of *Dunaliella salina* (Chapter 5)

Quantification of inorganic compounds

The content in inorganic compounds of the liquid extracts produced at laboratorial scale was determined by pyrolysis at 550ºC during 3h. The content in organic compounds was calculated by the difference of the total dry weight and the content in inorganic compounds.

Optimisation of the cell disruption procedure

For cell disruption of *Dunaliella salina*, the method used combined ultrasonication and extraction with n-heptane. An ultrasonication equipment (Sonics VCX750) was employed, applying different cycles and amplitudes. The percentage of carotenoids released (%w/w) was used as an indicator of the disruption process. The conditions used for ultrasonication were: consecutive pulses of 3x20 seconds with 5 seconds interval at 20 % amplitude, which is indicated for the extraction of lipids or bulk proteins. Table A.1 shows the different ultrasonication procedures used and the percentage of carotenoids extracted.

Table A.1. Ultrasonication cycles tested and carotenoids recovered (%w/w) with extraction of biomass with n-heptane.

Ultrasonication	Carotenoids (%w/w)
Lipids and bulk proteins (3x20 sec)[a]	6.1 ± 0.4
4x20 sec[a]	6.9 ± 0.2
5x20 sec[a]	7.7 ± 0.6

[a] n x 20 s pulses with 5 s interval at 20 % amplitude, n is the number of cycles

As shown in the Table A.1, the ultrasonication procedure used (3x20 seconds) presented good percentages of extracted carotenoids. Increments of the number of cycles were also tested, in order to obtain the highest percentage of carotenoids. Accordingly, the obtained percentages of carotenoids were higher with repeated cycles. Therefore, the optimal ultrasonication procedure was found to be of 5x20 seconds pulses with 5 seconds interval at 20% of amplitude.

Optimisation of the saponification reaction time

The aim of saponification is the transformation of glycerides into free fatty acids. The conversion of triglycerides to fatty acids chains and glycerol occurs in the presence of an alcohol and a catalyst. The

reaction conditions such as time and temperature are critical parameters that determine the efficiency of the process. For optimisation of the saponification time, samples were prepared and collected at different times, namely: 0.5, 1, 2, 6, 9, 24 and 48h. The samples were analysed by HPSEC in terms of tri-, di- and monoglycerides and free fatty acids (FFAs). Table A.2 presents the summary of the glycerides and FFAs during the saponification reaction.

Table A.2. Percentage of triglycerides (TG), diglycerides (DG), monoglycerides (MG) and free fatty acids (FFAs) for different saponification reaction times.

Time of saponification (h)	TG (%wt)	DG (%wt)	MG (%wt)	FFAs (%wt)
0.5	29.7 ± 0.0	11.7 ± 0.1	2.2 ± 0.1	20.4 ± 0.3
1	25.4 ± 3.7	10.5 ± 1.6	1.4 ± 1.9	29.5 ± 6.2
2	27.9 ± 1.0	11.4 ± 0.1	2.6 ± 0.4	24.4 ± 1.4
6	23.1 ± 3.3	11.6 ± 1.1	3.8 ± 0.5	34.9 ± 4.7
9	17.5 ± 2.2	10.7 ± 0.2	2.3 ± 0.8	35.4 ± 4.2
24	9.6 ± 0.2	12.2 ± 0.0	3.6 ± 0.5	39.2 ± 0.5
48	6.4 ± 0.1	13.1 ± 0.2	4.0 ± 0.8	40.4 ± 6.7

TG: Triglycerides; DG: Diglycerides; MG: Monoglycerides; FFA: Free Fatty Acids.

The optimal saponification time was found to be 24h. This reaction time was selected due to the kinetics of reaction, which turns very slow and there is no significant decrease in the percentage of triglycerides converted into FFAs, between 24h and 48h. As shown in Table A.2, there is an effective reduction of TG with the increasing of time and an increase of FFAs, although the reaction is not complete.

Antioxidant activity of *Dunaliella salina* extracts

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed according to the method of Brand-Williams [147]. The DPPH method is a spectrophotometric method that evaluates the *in vitro* antioxidant properties of the extracts analysed, by measuring the free radical scavenging activity of the samples, through the inhibition percentage of a solution of 60 μM DPPH. The DPPH solution was prepared in methanol to obtain a solution with 60 μM of DPPH. Briefly, the extracts (0.5 mL) were allowed to react with 3.5mL of the DPPH working solution. After 30 minutes at room temperature in the dark, the absorbance was measured at 515nm. The antioxidant activity was determined spectrophotometrically by monitoring the decrease of DPPH concentration. Results are expressed in percentage of inhibition of DPPH. The ability to scavenge the DPPH radicals was calculated according to equation (1):

$$DPPH_{Inhibition}(\%) = \frac{Abs_{DPPH} - Abs_{sample}}{Abs_{DPPH}} x100 \quad (1)$$

where Abs_{DPPH} is the absorbance of the control and Abs_{sample} is the absorbance of the sample reaction.

In order to evaluate if membrane processing enhances the antioxidant activity of the saponified extracts, the DPPH method was performed in the feed and permeate samples (see Figure A.1)

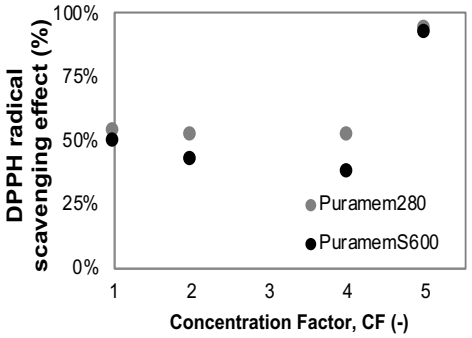

Figure A.1. Percentage of DPPH radical scavenging effect plotted against the concentration factor, CF (-), of the saponified extracts processed with Puramem280 and PuramemS600.

As shown in Figure A.1, all saponified extracts were able to inhibit DPPH solution in the feed solution before concentration (CF = 1). After membrane processing, the saponified extracts were measured to evaluate the DPPH radical scavenging effect, after concentration five times (CF = 5). The percentage of DPPH radical scavenging effect of the extracts was observed to be higher than that of the initial feed solution (CF = 1), both with PuraMem 280 or PuraMem S600. Therefore, the concentrated saponified extracts present a higher antioxidant activity after membrane processing, due to an increase of the carotenoids' concentration.

Characterisation of membranes by atomic force microscopy (AFM) and Fourier Transform Infrared (FTIR) analysis

The membranes used for the fractionation of the high-value compounds, namely PuraMem 280, PuraMem S600, DuraMem 300 and DuraMem 500, were analysed by FTIR and AFM.

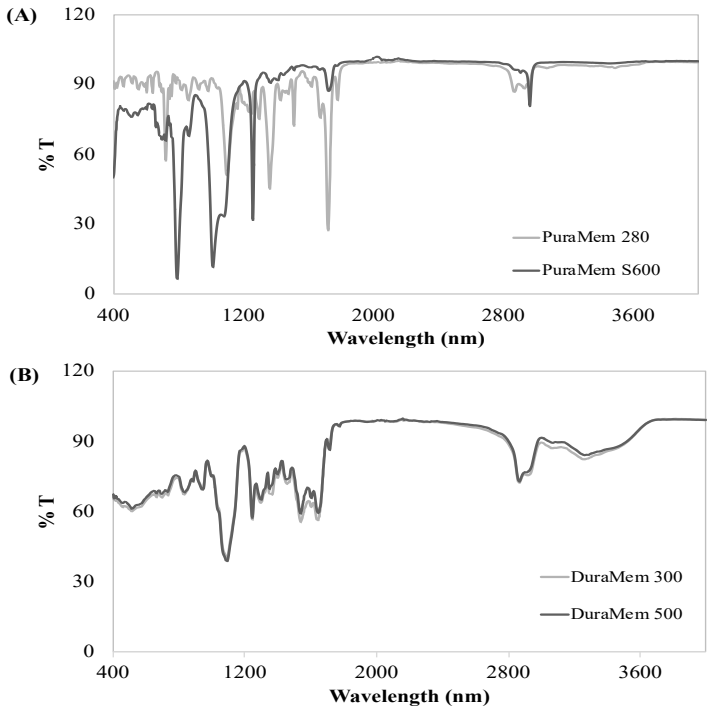

Figure A.2. Overlaping of FTIR analysis of (A) PuraMem 280 and PuraMem S600 and (B) DuraMem 300 and DuraMem 500.

In Figure A.2 it is shown the overlay of the FTIR analysis. Regarding the PuraMem membranes (Figure A.2 (A)), although both are made of Polyimide, the results obtained by FTIR show different profiles, meaning that there are some differences between the two materials. Some bands are characteristics of these membranes such as: 2960-2990; 1720 and 1090 (approximately). For PuraMem 280 the most intense bands and peaks are at the following wavelengths: 2980-2700; 1750; 1510; 1360; 1290; 1100 e 720 nm. These peaks might be related with presence of characteristic groups, such as: N-H (amine salt), C=O, N-O, C-N (aromatic amine), C-O (aromatic ester), C-O (secondary alcohol) and C=C. In the case of Puramem S600 (Figure A.2 (B)), the most intense peaks are at the following wavelengths: 2960; 1720, 1250, 1050, 1013 and 793 nm, representing the following groups: C-H, C=O, C-N, C-O, C=C and C-H, respectively. On the other hand, DuraMem membranes are both modified Polyimide and the FTIR results shown a complete overlap (Figure S1 (B)), confirming that the membranes' material is identical.

Afterwards, the membranes were analysed by AFM, in order to obtain the roughness of the membrane surface. The comparison of the surface of the membranes is shown in Figure A.3, performed by AFM.

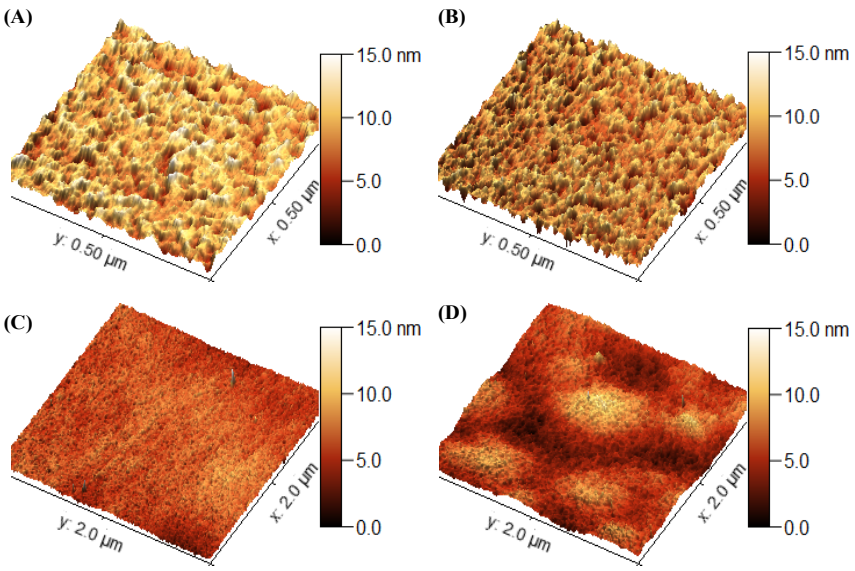

Figure A.3. 3D images of atomic force microscopy of the surface of (A) DuraMem 300, (B) DuraMem 500, (C) PuraMem 280 and (D) PuraMem S600.

Comparing the Figure A.3 (A) and (B), related with the DuraMem membranes, it is possible to observe a similar roughness between the membranes. However, DuraMem 500 seems to present a more pronounced roughness. Regarding the PuraMem membranes (Figure A.3 (C) and (D)), the roughness of the surface was higher in the PuraMem S600. These results might explain the higher adsorption obtained, when using the membranes with higher molecular weight cut-off (DuraMem 500 and PuraMem S600).

Comparison of wet biomass and lyophilised biomass for extraction of compounds from carotenoid-rich *Dunaliella salina*

The removal of an evaporation/drying step, at the beginning of the process, represents an advantage in the biorefinery scheme proposed, because it leads to a reduction of costs. Therefore, extraction of compounds using wet biomass was performed and compared with extraction from lyophilised biomass. The quantification of the main compounds extracted from wet and from lyophilised biomass is presented in Table A.3.

Table A.3. Comparison of the concentration of compounds extracted from wet and from lyophilised biomass.

	Compounds				
Biomass	**Carotenoids (mg/mL)**	**Glycerol (g/L)**	**Proteins (mg/L)**	**Carbohydrates (mg/L)**	**Total Lipids (%w/w)**
Wet	0.82 ± 0.12	4.56 ± 0.31	273.02 ± 12.66	424.98 ± 7.39	10 ± 1
Lyophilised	0.83 ± 0.04	4.13 ± 0.43	294.68 ± 25.96	423.32 ± 3.79	12 ± 2

As presented in Table A.3, the concentration of carotenoids, glycerol, proteins, carbohydrates and lipids are similar between lyophilised and wet biomass. Therefore, it was concluded that the process could be performed with wet biomass without significant losses in the concentration of the compounds of interest.

Flow chart of the biorefinery process

The overall flow chart of the biorefinery scheme for the recovery of target compounds from *Dunaliella salina* biomass is shown in Figure A.2. The solvent recovery of n-heptane, ethanol + water and acetone were also considered, in order to improve sustainability and reduce costs.

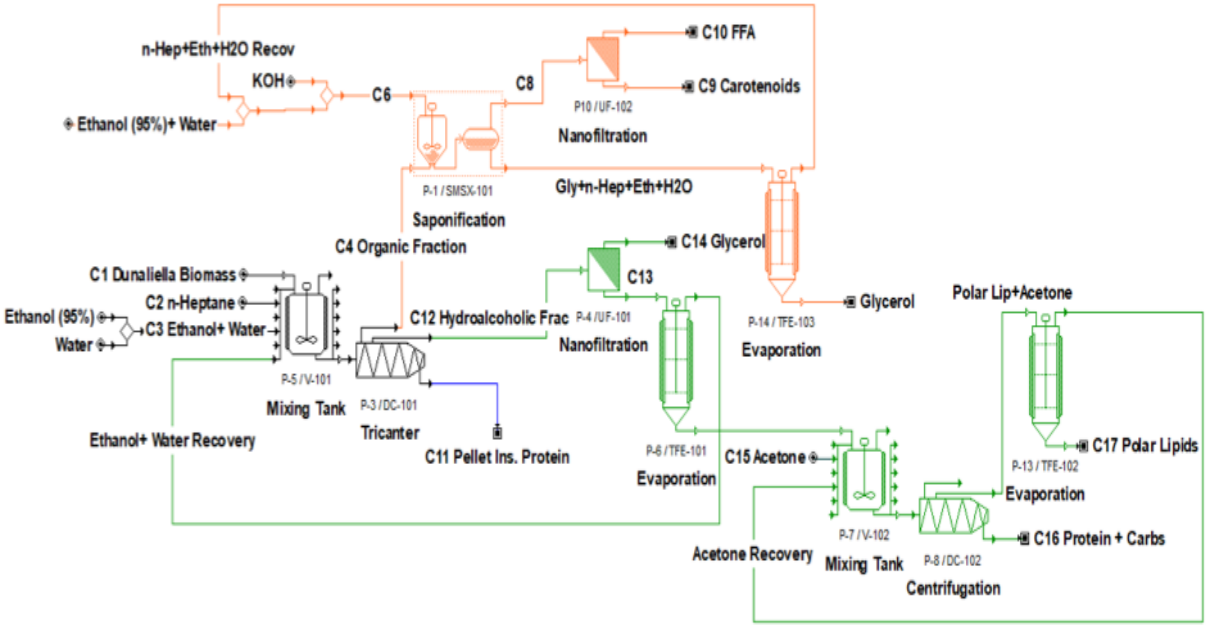

Figure A.4. Schematic flow chart of the biorefinery process for the recovery of target compounds from *Dunaliella salina* biomass. Solvent recovery after extraction and membrane processing is also shown.

The organic solvent nanofiltration operations allow for the recovery of n-heptane and etanol in water used for extraction of the compounds of interest. Also, this process enables a reduction in the amount of solvents used and energy savings in the evaporation step. Consequently, energy and economic savings are expected for the combination of an integrated membrane/evaporation process.