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**PAPER INDUSTRY EFFLUENTS TOWARDS THE DEVELOPMENT OF
ENERGY-DRIVEN BIOREFINERIES: AN EXPERIMENTAL AND TECHNO-
ECONOMIC APPROACH**

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Abstract

Humanity is facing some major global threats, namely: lack of environmental sustainability and energy crisis due to the unsustainable reliance on fossil fuels. Water scarcity is also one of the main problems for humankind, and it will be exacerbated with the rapid growth of urban areas. Excessive consumption of resources puts the future of the planet and man at risk.

However, with great challenges come even greater opportunities. It is of prime and utmost importance to develop new technologies and solutions, redesign systems and explore new sources of energy. Researchers have drawn their attention to microalgae. These photosynthetic microorganisms have the capability to play the dual purpose of wastewater remediation, as well as lipids accumulation to produce third-generation biofuels. Despite all the potential of these organisms, their production on a large scale has not yet been fully successful. Microalgal biorefineries arise as a solution to face this drawback, making the most of the potential of these organisms. However, further research is required on the design of these microalgal-based biorefineries and the economic viability and sustainability of these infrastructures.

In this study, it was evaluated the potential of the microalgae *Chlorella vulgaris* to: (i) successfully grow in different concentrations of a paper industry effluent (20% to 100%); and (ii) treat the industrial effluent, reducing phosphorus concentrations to values below the accepted legal limits. Additionally, a techno-economic assessment (TEA) was performed to study the viability of a *C. vulgaris* biorefinery to treat an industrial effluent and produce biofuels.

Results have shown that the paper industry effluent does not have an inhibitory effect on *C. vulgaris* growth. In fact, the best results regarding biomass growth were obtained for the assays with higher percentages of effluent: (i) the highest specific growth rate was determined for 80% of effluent ($0.33 \pm 0.07 \text{ d}^{-1}$); (ii) highest biomass concentration was observed for the percentages of 60% and 100% (495 ± 2 and $495 \pm 25 \text{ mg DW L}^{-1}$); and (iii) the highest average biomass productivity was determined in the 60% test ($26 \pm 1 \text{ mg DW L}^{-1} \text{ d}^{-1}$). Regarding phosphorus removal, the results were satisfactory for all the assays (20% to 100%). Final phosphorus concentrations ranged between 0.12 ± 0.01 and $0.5 \pm 0.3 \text{ mg P L}^{-1}$, which are below the legal limits imposed by the Portuguese Environment Agency (APA) to the paper industry (0.82 , or 0.50 mg P L^{-1} in occasional critical situations).

The TEA results have demonstrated that the proposed energy-driven biorefinery would be viable in six of the seven studied scenarios. The highest net present value was estimated as 15.4 million euros, in the scenario with a 3% photosynthetic efficiency, and the lowest as -7.4 million euros, in the scenario with a photosynthetic efficiency of 1%. Discounted payback time estimated for each scenario showed that, even in the best scenario, it would take 13 years until the biorefinery will start making profit. However, the proposed infrastructure promotes a series of benefits, of which some cannot be directly included in the economic viability study. Examples of these benefits are: (i) the remediation of paper industry effluents; (ii) CO₂ uptake for microalgal growth, contributing to a reduction of greenhouse gases emissions; (iii) the production of clean and renewable energy; (iv) contribution to soil regeneration; and (v) promotion of a circular economy. Despite being immeasurable in terms of process economics, all these benefits add value to the proposed project.

Keywords: Biorefinery; *Chlorella vulgaris*; Lipid accumulation; Microalgal production; Nutrients removal; Techno-economic assessment.

Resumo

A Humanidade tem vindo a enfrentar algumas ameaças globais, nomeadamente: a ausência de sustentabilidade ambiental e a crise energética decorrente da dependência não sustentável em combustíveis fósseis. A escassez de água é também um outro grande problema para todos os seres vivos e irá exacerbar-se com o rápido crescimento das áreas urbanas. O consumo excessivo de recursos irá colocar em risco o futuro do planeta e do Homem.

Contudo, grandes desafios trazem ainda maiores oportunidades. É primordial e da máxima importância desenvolver novas tecnologias e soluções, redesenhar sistemas e explorar novas fontes de energia. As microalgas têm vindo a chamar a atenção dos investigadores. Estes microrganismos fotossintéticos têm a capacidade de endereçar o duplo propósito da remediação de águas residuais e da acumulação de lípidos para produção de biocombustíveis de terceira geração. Apesar de todo o potencial destes organismos, a sua produção em larga escala não foi ainda totalmente bem-sucedida. As biorrefinarias de microalgas surgem como uma possível solução para fazer face a esse inconveniente, procurando retirar o máximo partido destes organismos, explorando vários produtos derivados dessa biomassa. Contudo, é necessária investigação adicional relativamente ao projeto de biorrefinarias e à viabilidade económica e sustentabilidade dessas infraestruturas.

Neste estudo, avaliou-se o potencial da microalga *Chlorella vulgaris* para: (i) se desenvolver com sucesso em diferentes concentrações de um efluente da indústria do papel (20% a 100%); e (ii) tratar o efluente industrial, reduzindo as concentrações de fósforo para valores abaixo dos limites legais. Adicionalmente, desenvolveu-se uma avaliação técnico-económica para estudar a viabilidade de uma biorrefinaria de *C. vulgaris* para tratar um efluente industrial e produzir biocombustíveis.

Os resultados mostraram que o efluente industrial não tem um efeito inibitório no crescimento da *C. vulgaris*. De facto, os melhores resultados relativamente ao crescimento de biomassa foram obtidos nos ensaios com maiores percentagens de efluente: (i) a maior taxa específica de crescimento foi determinada no ensaio dos 80% ($0,33 \pm 0,07 \text{ d}^{-1}$); (ii) a concentração de biomassa mais elevada ocorreu nos ensaios dos 60% e 100% (495 ± 2 and $495 \pm 25 \text{ mg DW L}^{-1}$); e (iii) a maior produtividade média de biomassa foi obtida no teste dos 60% ($26 \pm 1 \text{ mg DW L}^{-1} \text{ d}^{-1}$). No que diz respeito à remoção de fósforo, os resultados foram satisfatórios em todos os ensaios (dos 20% aos

100%). As concentrações finais de fósforo variaram de $0,12 \pm 0,01$ a $0,5 \pm 0,3$ mg P L⁻¹. Estes valores encontram-se abaixo dos limites legais impostos à indústria do papel pela Agência Portuguesa do Ambiente ($0,82$, ou $0,50$ mg P L⁻¹ em situações críticas excecionais).

Os resultados da avaliação técnico-económica demonstraram que a biorrefinaria proposta teria viabilidade em seis dos sete cenários estudados. O valor atual líquido mais elevado foi de 15,4 milhões de euros, no cenário com uma eficiência fotossintética de 3%, e o mais baixo foi -7,4 milhões de euros, no cenário com uma eficiência fotossintética de 1%. O período de retorno atualizado que foi estimado para cada cenário mostrou que, até no melhor cenário, seriam necessários 13 anos até a biorrefinaria começar a dar lucro. Contudo, a infraestrutura proposta promove uma série de benefícios, de entre os quais alguns não podem ser diretamente contabilizados no estudo de viabilidade económica. Exemplos desses benefícios são: (i) a remediação de efluentes da indústria do papel; (ii) a captura de CO₂ para o crescimento de microalgas, que contribui para a redução das emissões de gases com efeito de estufa; (iii) a produção de energia limpa e renovável; (iv) contribuição para a regeneração de solos; e (v) promoção de uma economia circular. Apesar de serem imensuráveis em termos de economia do processo, todos estes benefícios adicionam valor ao projeto.

Keywords: Biorrefinaria; *Chlorella vulgaris*; Acumulação de lípidos; Produção de microalgas; Remoção de nutrientes; Análise técnico-económica.

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Nomenclature

Acronyms

AD	Anaerobic digester
APA	' <i>Agência Portuguesa do Ambiente</i> ' - Portuguese Environment Agency
BOD	Biochemical oxygen demand
CCAP	Culture Collection of Algae and Protozoa
CEPCI	Chemical Engineering Plant Cost Index
CHP	Combined heat and power
CIT	Corporate income tax
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
DPP	Discounted payback-period
DW	Dry weight
EROEI	Energy returned on energy invested
FAS	Ferrous ammonium sulphate
HRP	High rate pond
IRR	Internal rate of return
LED	Light emitting diode
LOD	Limit of detection
LOQ	Limit of quantification
MR	Mass removal
NO ₃ -N	Nitrate-nitrogen
NPV	Net Present Value
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
PAR	Photosynthetically active radiation
PBR	Photobioreactor
PCI	Plant cost index
PE	Photosynthetic efficiency
PEF	Pulsed electric field
PO ₄ -P	Phosphate-phosphorus
PUFA	Polyunsaturated fatty acid
PVGIS	Photovoltaic Geographical Information System
RE	Removal efficiency

RR	Removal Rate
rpm	Rotations per minute
SDG	Sustainable Development Goals
TAG	Triacylglycerol
TEA	Techno-economic assessment
TP	Total phosphorus
USEPA	United States Environmental Protection Agency
VAYT	Value added tax
WWTP	Wastewater treatment plant

Notation

$CEPCI_1$	CEPCI value at a year in time at which index and cost value are known
$CEPCI_2$	CEPCI value at a year in time at which index value is known but cost is not
$Cost_1$	Known cost value (€)
$Cost_2$	Unknown cost value (€)
CF	Cash-Flow (€)
g	Gravitational acceleration constant ($m\ s^{-2}$)
h_b	180° bends head loss (m)
h_c	Channel straightway head loss (m)
h_f	Major head loss (m)
h_m	Minor head loss (m)
h_s	Sumps head loss (m)
i	A year in time
K	Kinetic loss coefficient
L	Channel Length (m)
M_{FAS}	FAS molecular weight ($g\ mol^{-1}$)
n	Data size
n_M	Manning's roughness coefficient
P_{avg}	Average biomass productivity ($mg\ DW\ L^{-1}\ d^{-1}$)
P_{max}	Maximum biomass productivity ($mg\ DW\ L^{-1}\ d^{-1}$)
P_x	Biomass Productivity ($mg\ DW\ L^{-1}\ d^{-1}$)
pH_{avg}	Average pH
pH_{max}	Maximum pH

pH_{min}	Minimum pH
r	Interest rate
R	Channel hydraulic radius (m)
R^2	Coefficient of determination
R_c	Average CO ₂ uptake rate (mg CO ₂ L ⁻¹ d ⁻¹)
R_N	Average nitrogen uptake rate (mg N L ⁻¹ d ⁻¹)
R_P	Average phosphorus uptake rate (mg P L ⁻¹ d ⁻¹)
S	Substrate concentration (mg L ⁻¹)
S_i	Phosphorus concentration in the initial instant of the cultivation time (mg P L ⁻¹)
S_f	Phosphorus concentration in the final instant of the cultivation time (mg P L ⁻¹)
$S_{P<0.5}$	Phosphorus concentration value lower than the limit for phosphorus discharge defined by APA for the paper industry company (mg P L ⁻¹).
t	Time (d)
t_0	Initial instant of the exponential growth phase (d)
t_1	Final instant of the exponential growth phase (d)
t_i	Initial instant of the cultivation period (d)
t_f	Final instant of the cultivation period (d)
$t_{P<0.5}$	Instant at which phosphorus concentration reached the limits for phosphorus discharge defined by APA (d)
t_z	Time at instant z (d)
t_{z+1}	Time at one more day after instant z (d)
T	Temperature (°C)
T_{avg}	Average temperature (°C)
T_{max}	Maximum temperature (°C)
T_{min}	Minimum temperature (°C)
v	Average mixing velocity (m s ⁻¹)
$V_{FAS; B}$	Volume of FAS used in the titration of the blank (mL)
$V_{FAS; S}$	Volume of FAS used in the titration of the samples (mL)
V_S	Volume of the sample (mL)
X	Biomass concentration (mg DW L ⁻¹)
X_0	Biomass concentration at the beginning of the exponential growth phase (mg DW L ⁻¹)
X_1	Biomass concentration at the end of the exponential growth phase (mg DW L ⁻¹)

X_i	Biomass concentration at the beginning of the cultivation period (mg DW L ⁻¹)
X_f	Biomass concentration at the end of the cultivation period (mg DW L ⁻¹)
X_z	Biomass concentration at instant z (mg DW L ⁻¹)
X_{z+1}	Biomass concentration one day after instant z (mg DW L ⁻¹)
X_{max}	Maximum biomass concentration (mg DW L ⁻¹)
$Y_{X/P}$	Specific biomass yield in terms of phosphorus (g _{DW} g _P ⁻¹)
μ	Specific growth rate (d ⁻¹)
σ	Standard deviation

Introduction and objectives

1.1. Scientific relevance and motivation

The booming world population, economic growth and improved standards of living are leading to an increase in the global demand for energy and natural and non-natural resources [1]. According to the United States Environmental Protection Agency (USEPA), electricity production alone generates, approximately, 25% of greenhouse gases (GHGs) at a global scale [2]. The largest anthropogenic source of these gases is the burning of fossil fuels (e.g., coal, natural gas and oil), mainly for electricity and heat production and transportation, having a major impact on global warming. GHGs also contribute to decreasing the oceans' pH, threatening coral reefs and aquatic organisms [3]. Moreover, fossil fuels' related sectors and other anthropogenic activities, such as agriculture, industrialisation and urbanisation, contribute to an increase in freshwater scarcity in terms of quantity and quality. All of these factors allied with the excessive consumption of other resources (soil, phosphorus, timber, etc.) pose a huge pressure on the environment [4].

These challenges can create great opportunities to develop sustainable solutions that take into consideration the needs of future generations [5]. It is of prime and utmost importance to redesign and create new systems, develop innovative technologies and investigate new sources, especially in the energy and transportation sectors, to reduce the environmental impacts caused by human-related activities [6,7]. Only then, a transition from a fossil-based linear economy to a circular-based (bio)economy will be possible, and this transition is one of the main requirements to achieve global sustainability [8,9].

Many alternative solutions are being studied, and some have already been implemented, in different areas of application, each one with its benefits and drawbacks. Regarding renewable energy sources, biofuels, (e.g., bioalcohols, biodiesel and biogas) have been studied and developed worldwide, as they seem to be good candidates for a long-term replacement of fossil fuels [3,10,11]. These fuel sources have characteristics very similar to those of fossil fuels and, yet, have a better environmental performance [12]. Biodiesel based on vegetable oil crops (e.g.,

rapeseed, soybean, palm and peanut) present many benefits when compared to oil fuel. However, they also present a lot of negative impacts, such as the use of pesticides and fertilisers, and the requirement of great areas that compete with resources needed for food security (e.g., arable land and freshwater) [1,13,14].

On the other hand, microalgae have drawn the attention of researchers because it seems to be a more attractive way to produce this renewable energy source. These microorganisms can play the dual purpose of wastewater bioremediation, as well as biomass generation and lipids accumulation, to produce biofuels. They have higher productivities than other crops, higher lipid contents (~30%), require less area, and cultivation can be carried out in places with less fertile or arid soils [3,13,15-17]. Chisti [18], compared corn oil with microalgal oil and concluded that, to meet 50% of the total US's transportation needs, it would be necessary 846% of the existing US cropping area for corn oil, and only 2.5% for microalgal oil. Furthermore, microalgae do not require freshwater. Indeed, these microorganisms are able to grow in wastewaters and treat them. Microalgae assimilate nutrients present in wastewaters (e.g., nitrogen and phosphorus), contributing to mitigating problems like eutrophication [9,19]. Also, when growing autotrophically, microalgae perform photosynthesis, sequestering CO₂ and releasing oxygen. Besides oils, microalgae also accumulate other products of commercial value [20].

Nevertheless, there are a few bottlenecks when scaling up microalgal production. To overcome these obstacles and enhance the economic viability of this process, it is necessary to fully exploit the potential of these microorganisms, which leads to a biorefinery approach [1,21]. These new infrastructures have a similar concept to the one from traditional oil refineries, being the main differences: (i) the raw materials used in each one, biomass or crude oil; and (ii) the applied technology. However, microalgal biorefinery success strongly depends on the final product sales price and on the price of competing products, and as of now, the industrial-scale production of microalgal products, such as biofuel, has not been very successful. Nevertheless, the overexploitation of fossil fuels led to an increased depletion of this energy source, which may increase oil prices in the future, opening opportunities for new solutions [18]. Therefore, more intensive research is needed on the design of this microalgal-based biorefinery and the economic viability and sustainability of these industrial plants [16,22-24].

1.2. Objectives

The main aim of this Thesis is to design and propose a microalgal production system (using the microalga *Chlorella vulgaris*), capable of reducing phosphorus concentration in an effluent from the paper industry. Additionally, it is also intended to study the viability and feasibility of a microalgal-based biorefinery for paper industry effluents remediation and biofuels production. For that purpose, this work was divided into three different, but interconnected stages:

(i) Evaluation of *C. vulgaris* growth in the paper industry effluent

To evaluate the behaviour of *C. vulgaris* in this culture medium, cultures were grown in flasks with the same volume but with different concentrations of the effluent, ranging from 20% to 100%. Growth parameters, such as specific growth rates and biomass productivities were determined for each assay.

(ii) Evaluation of the performance of *C. vulgaris* in the bioremediation of the industrial effluent

To assess the microalga ability to remove nutrients from the paper industry effluent, allowing its discharge in compliance with the legal limits, phosphorus concentration over time was studied. Due to restrictions imposed by the current pandemic, it was not possible to monitor nitrogen concentrations. Parameters, such as removal efficiency, removal rate, mass removal and specific biomass yield, were estimated for each assay.

(iii) Techno-economic assessment of a microalgal production system (biorefinery)

Taking into consideration the results obtained in the above mentioned laboratory steps, a techno-economic assessment (TEA) of a microalgal biorefinery for the production of biofuels and bioremediation of paper industry effluents, was performed. The main goal was to design biorefinery and conclude on the feasibility and sustainability of this project.

1.3. Thesis structure

The present work is divided into 5 different chapters. Chapter 1 presents the scientific relevance and main motivations for the development of this Thesis, as well as the main goals to be achieved with this work.

Chapter 2 presents the literature review, addressing: (i) the microalgal characteristics and major applications; (ii) the main systems and methods used for microalgal cultivation, harvesting and extraction of accumulated products; and (iii) the role of microalgae in nutrients removal from wastewaters, with a special emphasis on paper industry effluents remediation.

Chapter 3 defines the methodologies adopted to achieve the proposed objectives. In the first part, the laboratory methodology to evaluate biomass production and phosphorus removal from the industrial effluent is described. In the second part, the techno-economic assessment steps and structure are defined.

Chapter 4 is also divided into two parts: (i) laboratory-scale experiments results; and (ii) results obtained in the TEA. In the first one, it is shown and discussed how the paper industry effluent influences *C. vulgaris* growth and its ability to treat this industrial effluent. In the second part, the results obtained from the mass and energy balances, and the economic and sustainability assessments, are presented and discussed.

Chapter 5 provides a synopsis of all the developed work, presenting the main conclusions and key findings. Based on the research needs, some suggestions for future work are also proposed.

Literature review

2.1. Microalgae and major applications

Microalgae are microscopic organisms, representing an enormous variety of species, that can live in both aquatic and terrestrial ecosystems [25]. These microorganisms can live in harsh conditions (e.g., contaminated water, and thermal and glacial lakes) and grow fast, due to their unicellular or simple multicellular structure [3,26]. They are part of the first constituents of terrestrial microflora and are extremely important for the environment [18,27].

According to their morphologic characteristics, cell wall, pigmentation and other parameters, they can be classified into four groups: cyanobacteria (blue-green algae), rhodophytes (red algae), chlorophytes (green algae) and chromophytes (the other algae) [28,29]. The first group, cyanobacteria, consists of prokaryotic cells, without membrane-bound organelles, that are more similar to bacteria than algae. The remaining groups comprise eukaryotic cells, and do have these membrane-bound organelles that control the cell functions.

Typically, microalgae are photosynthetic autotrophic (photoautotrophic) organisms, but they can present other growth metabolisms depending on the microalgal species and available energy and carbon sources. When growing photoautotrophically, they require sunlight, carbon, and water (freshwater, marine water or wastewater). Approximately 50% of the dry weight of microalgal biomass consists of carbon [18]. This nutrient is mostly derived from carbon dioxide. Therefore, they can fix 1.83 kilogram of CO₂ per kilogram of produced dry biomass. Moreover, they are responsible for the production of half of the Earth's oxygen [25,27,30]. Part of the CO₂ can be supplied to microalgae from the flue gases released in power plants and industries. Hence, these microorganisms have a major role in the reduction of GHGs emissions to the atmosphere and, consequently, in the global warming mitigation [31]. Besides carbon, microalgae also require other nutrients, such as nitrogen, phosphorus and inorganic salts [18]. To obtain these nutrients, microalgae can grow in wastewater and industrial effluents and use their contaminants as nutrients source [32,33].

The wide variety of microalgal species, with distinct biochemical and physiological characteristics, make them capable of accumulating a lot of products [25]. Moreover, these photosynthetic microorganisms have a particular commercial interest because they efficiently convert carbon into compounds with market value, such as: lipids, pigments, proteins, carbohydrates, polyunsaturated fatty acids (PUFAs), antioxidants, vitamins, among others [27]. These bioactive compounds can be used in the manufacturing of several products, such as: (i) human food and animal feed; (ii) cosmetic products; (iii) pharmaceutical products; (iv) antiviral, antibacterial and anticancer drugs; (v) sterols for cardiovascular diseases treatment; (vi) biofertilisers; and (vii) biofuels (e.g., biogas, biodiesel and bioalcohols) [25,34-37]. Even so, there are still several microalgal metabolites that were not yet discovered [38-40]. Figure 2.1 shows a diagram with some of the main benefits and products derived from microalgae.

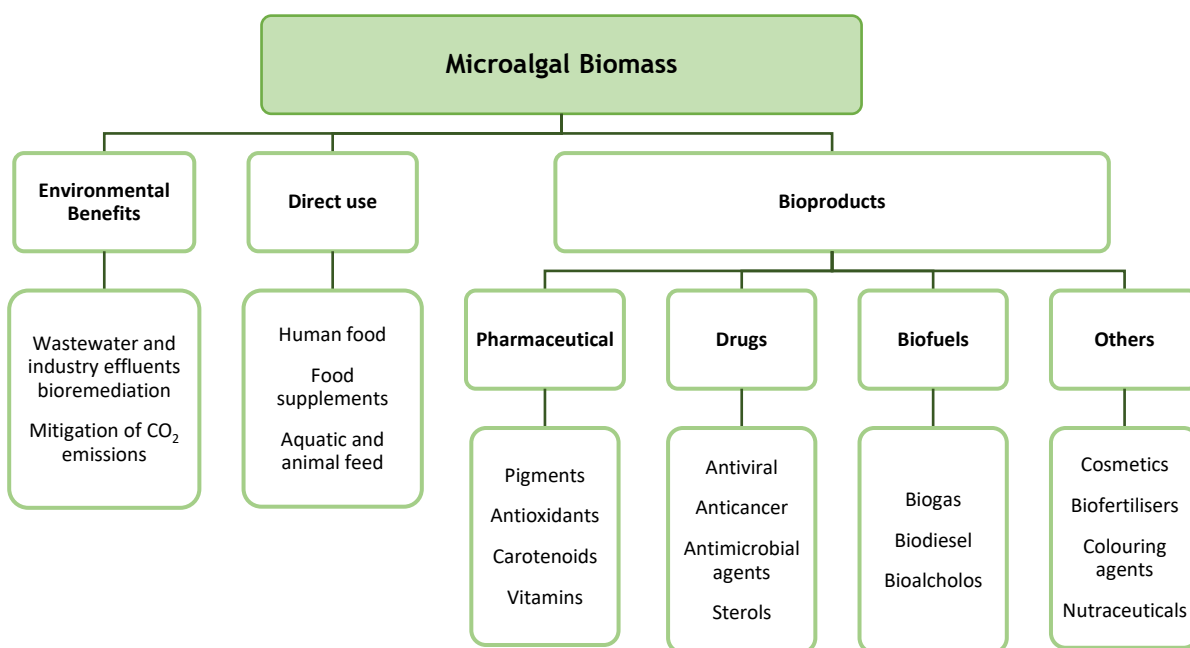


Figure 2.1. Diagram showing microalgal applications in different fields.

2.2. Microalgal biomass production and recovery of interest compounds

2.2.1. Cultivation and operation modes

Depending on the available energy and carbon sources, microalgae can adopt distinct growth metabolisms: photoautotrophic, heterotrophic, mixotrophic and photoheterotrophic [41]. In this Chapter, only the photoautotrophic metabolism will

be addressed, since it is the most explored and frequently used in the context of microalgal biorefineries. In this type of cultivation, microalgae perform photosynthesis, using light energy (e.g., sunlight) and inorganic carbon (e.g., CO₂) to produce organic matter (glucose) [42]. As these microorganisms can fix CO₂ from the atmosphere or from industrial emissions, they can play an important role in CO₂ capture and, consequently, on the mitigation of climate change. This makes autotrophic cultivation of microalgae even more attractive as a source of renewable and sustainable feedstock for industrial production [41,43,44]. However, photoautotrophic cultivation may also have some disadvantages, such as the low biomass production rates and low cell densities achieved. One of the main issues associated with this growth metabolism is capturing solar energy in the most efficient way throughout the year (taking into account the light intensity and temperature variations over the seasons), which strongly influences the photosynthetic activity of microalgae [45]. The fluctuation in biomass productivities during the year is also a challenge for downstream processing. To overcome this, the biomass achieved in high productivity periods could be stored to be processed in the low productivity ones. However, there is a risk of losing biomass quantity and quality due to inadequate storage. Moreover, artificial light may be required in some photoautotrophic cultivation systems, which can significantly increase biomass production costs and limit final applications of biomass to high-value products [41].

There are many cultivation systems for microalgal growth in photoautotrophic conditions. These systems can be divided according to: (i) the nature of microalgal growth (in suspension or attached/entrapped in a solid support); (ii) the type of reactor used (open or closed); and (iii) the biomass concentrations achieved (traditional or thin/intensive production). Moreover, microalgal growth can be carried out under different operation modes, namely batch, fed-batch, continuous and semi-continuous. Selection of the adequate cultivation system and operation mode is crucial for the success of a microalgal biorefinery and depends on the strain type and its metabolism, nutrients source, target products, and available investment cost [44].

2.2.1.1. Suspended vs Fixed

Regarding the different cultivation systems, conventional suspended systems, where microalgae grow as dilute suspensions, are the most frequently used [46]. However, microalgal growth in these systems generates relatively low biomass concentrations and requires an enormous volume of water [47]. According to Guieysse et al. [48], more than 1000 L of water are needed for each kilogram of biomass growing

in an open raceway reactor. Besides, harvesting techniques may be costly, due to the great difficulty in separating a dilute and stable microalgal suspension from the culture medium [46]. Harvesting techniques used for these systems may allow obtaining a solids content of 1% DW (dry weight basis) through sedimentation, 10% DW using chemical flocculation, and an improved percentage of 20% DW after centrifugation [49]. But the latter technique is costly, hindering the use of microalgal biomass for low-value applications. Nevertheless, these systems are still the most used, and efforts have been made to develop new suspended systems and to improve the existing ones to increase biomass productivities. An example of these are the thin culture systems discussed below.

On the other hand, recent advances have been made on fixed biomass cultivation methods as an alternative to traditional suspended systems. Since microalgae grow fixed/immobilised to substratum surfaces and higher algal densities can be achieved, it becomes easier to separate biomass from the culture medium [50]. For some fixed biomass systems, harvesting can be easily made by scrapping, and the resulting biomass typically presents a solids content of about 10% DW, without being necessary to use the cost- and energy-intensive centrifugation or filtration methods [51]. Besides, fixed biomass systems have few cultivation medium requirements, minimum water consumption, and reduced light limitations [50-52]. Indeed, they are predicted to have one of the highest light distributions within the known microalgal reactors, since there are no floating particles absorbing and reflecting light [53].

Fixed biomass systems for microalgal growth can be classified according to the relative position of the culturing medium and microalgal cells into: (i) enclosure methods, where biomass is encapsulated in a matrix; and (ii) non-enclosure methods, where biomass attaches to a surface, forming a biofilm [53]. Microalgal biofilms can be described as a microbial consortium (composed exclusively by microalgae or by microalgae and other organisms, such as fungi and bacteria) that form a complex structure in solid substrata [54]. Microalgal biofilms have higher acceptability than enclosure methods for microalgal biomass production, due to their advantages over the encapsulated technologies. For example, microalgal harvesting can be easily performed through scrapping and biomass processing does not require the disruption/disintegration of the encapsulation matrix. Moreover, it does not require an enclosure material, typically agar, alginate and carrageenan, which has a very high cost [53,55]. Based on substratum orientation, attached growth systems can be classified as horizontal, vertical, rotating and radial systems [50]. Horizontal systems have less than 10 degrees of inclination and microalgae are attached in a smaller area

than the footprint area. Vertical systems allow maximum utilisation of the cultivation area [50]. In rotating systems, algal cells are grown attached to a surface that rotates between the liquid medium and the surrounding gaseous phase [49]. Depending on substratum's shape, there may be several rotating systems with different footprint areas. Lastly, radial systems consist of a bioreactor where the supports for microalgal growth are submerged in the liquid medium and distributed in the radial direction [50]. Thus, different possible arrangements have been proposed to promote the attached growth of microalgae. Figure 2.2 shows two of the possible arrangements for attached biomass cultivation. Liu et al. [56] studied the productivity of *Scenedesmus obliquus* in a vertical-plate attached photobioreactor (PBR), where algal cells were allowed to grow on the surface of vertical supporting material (filter paper). Results showed that after nine days of indoor cultivation, the average areal productivity of $80 \text{ g m}^{-2} \text{ d}^{-1}$ could be obtained. Huang et al. [57] also showed promising results when growing *C. vulgaris* in BG11 medium and using polymethyl methacrylate cubes as bioreactors for biofilm formation. The authors compared biomass growth in both suspended and non-suspended systems and concluded that after seven days of cultivation, the areal density was 30.4% higher in the attached system. However, other studies showed less interesting results with attached cultivation reactors. The procedure of biofilm formation and development depends on the species that are part of it. Therefore, it is hard to take conclusions about the potential of the different biofilm systems already studied and tested [54]. Table 2.1 presents the most recent results on microalgal biomass production in attached growth systems.

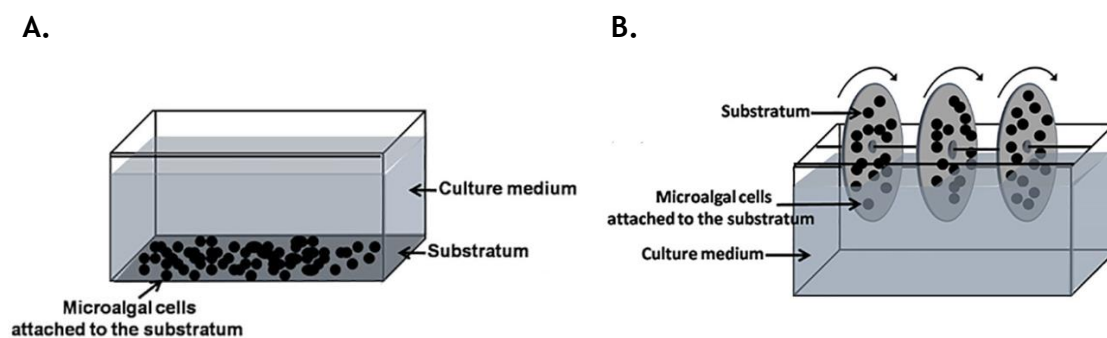


Figure 2.2. Schematic representation of two attached cultivation systems: (A) Horizontal; and (B) Rotating [54].

According to Wang et al. [58], although recent advances have been made on attached growth systems, with good results, the bioreactors reported for these systems are not yet feasible for mass cultivation of microalgae. They are far from being able

to promote efficient cultivation, suitable inoculation and harvesting simultaneously, and are not yet easy to scale-up. Therefore, more research should be done to: (i) develop improved PBRs for attached microalgal growth; (ii) better understand which supporting substratum materials lead to higher biomass productivities for each species; and (iii) increase knowledge on how microalgal cells behave in attached systems [58]. With these developments, it will be possible to consider attached growth systems for microalgal biomass production within the biorefinery approach.

Table 2.1. Biomass productivities and photosynthetic efficiencies reported for attached cultivation systems

Cultivation System	Microalgae	Culture Medium	Supporting Material	P _{areal} ^a (g m ⁻² d ⁻¹)	PE ^b (%)	Ref.
Flat-plate parallel horizontal PBR ^c	<i>Scenedesmus obliquus</i>	Unsterile secondary effluent + Synthetic medium	Glass	2.1	-	[59]
Multiple vertical-plate PBR prototype	<i>Botryococcus braunii</i>	Chu 13 medium	Filter paper and glass plates	49.1	14.9	[60]
Multiple vertical-plate PBR prototype	<i>Scenedesmus obliquus</i>	BG11 medium	Filter paper	70.9	11.0	[56]
Rotating biological contactor	<i>Chlorella sorokiniana</i>	M8a medium	Stainless steel woven rough mesh	20.1	-	[61]
Single-layer vertical-plate PBR	<i>Botryococcus braunii</i>	Chu13 medium	Filter paper and glass plate	5.5-6.5	-	[60]
Vertical algal biofilm - enhanced raceway	<i>Chlorella vulgaris</i> , <i>Oscillatoria</i>	BG11 medium	Coral velvet with long villus	10.5-14.7	-	[62]

^a P_{areal} - areal productivity; ^b PE - photosynthetic efficiency; ^c PBR - photobioreactor.

2.2.1.2 Suspended cultivation systems: open vs closed

Suspended cultivation systems for photoautotrophic production of microalgae can be classified as open or closed, depending on whether they are directly exposed to open air or not.

Open systems are the most traditional models for microalgal cultivation and include natural or artificial ponds, raceway ponds and inclined surface systems [63]. Compared to closed PBRs, these systems are easier to operate and scale-up and have a simpler construction, which makes them more attractive at an economic level [64]. However, they present several downsides [54,63,65,66]: (i) lower biomass productivities than closed PBRs; (ii) the need for a large area of land; (iii) reduced control of culture conditions (e.g., temperature, light intensity and CO₂ diffusion rate); (iv) significant evaporative losses; (v) high susceptibility to contaminations; (vi) high dependence on environmental conditions; and (vii) low biomass/water ratio, which leads to high water requirements and harvesting costs. Moreover, the poor control of culturing conditions and the risks of microbial contaminations limit the application of these systems to a few microalgal strains. Indeed, only robust species (e.g., *Chlorella* spp., *Scenedesmus* spp. and *Spirulina* sp.) are suitable to grow in open systems, since they can grow under unstable conditions and settle themselves as the main population in the culture [67]. Besides the dependence on culturing and environmental conditions, biomass productivities in open reactors may vary a lot with the selected microalgal strain. According to Vuppaladadiyam et al. [66], the volumetric productivity in an open pond may range from 0.05 to 0.32 g L⁻¹ d⁻¹, depending on the microalgal species. The reduced construction and operation costs of these systems, as well as the production of biomass with less predictable quality, make these systems more suitable for the production of low- and medium-value products, such as some proteins, carbohydrates and lipids, used for feed and fuel applications [22].

On the other hand, closed PBRs may offset some of the limitations of the open-air systems. These systems can be grouped into three main classes: tubular, flat-plate and column PBRs. Brennan and Owende [43] showed that biomass productivities in these systems could range from 0.1 to 3.8 g L⁻¹ d⁻¹, depending on the type of reactor and species used. Despite their differences, when compared to open systems, closed PBRs allow for higher biomass production rates, higher control of culture conditions, and better light penetration [54,63,66]. Besides, closed PBRs allow the cultivation of single-species of microalgae with low risk of contamination [18]. Since microalgae grown in closed PBRs are more protected from pollution and contamination, these systems are more suitable for the accumulation of high-value products, such as astaxanthin, β-carotene and other products that may be used in pharmaceutical and cosmetics industries [43,67]. Besides, PBRs have a more complex construction, are harder to operate and maintain and, therefore, have higher operation and investment costs. Hence, closed PBRs are a feasible option when target products have a high

market value. Figure 2.3 shows a schematic representation of the most common open and closed PBRs used for microalgal cultivation.

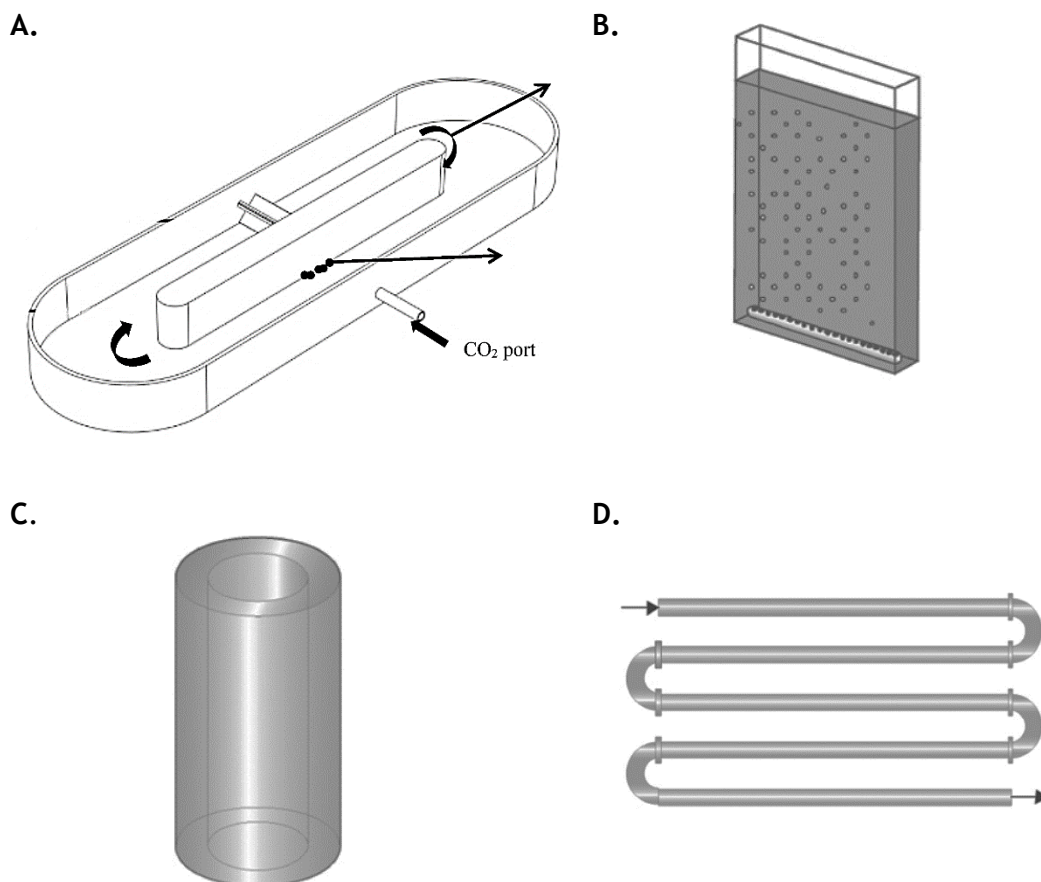


Figure 2.3. Most common reactors used for microalgal cultivation. Open: (A) raceway pond; Closed: (B) flat-plate, (C) bubble-column; and (D) tubular reactor [68, 69].

To make microalgal biorefineries profitable, one must choose between two approaches: exploitation of a few, or just one, high-value products, or many medium- and low-value products. For the first approach, a closed PBR is the most suitable option, since it protects biomass from possible contaminations and allows proper control of culture conditions. For the second route, an open system is more appropriate, as it works well to produce those target products and is less expensive. However, since microalgal biorefineries aim to fully exploit biomass potential, growth systems and strategies that allow biomass to accumulate multiple products, with different commercial values and market sizes, should be adopted [67].

2.2.1.3. Traditional vs Thin/Intensive

Conventional suspended systems (open or closed) still generate dilute cultures and are not yet profitable for mass cultivation of microalgae. Hence, new techniques have been proposed with the goal of promoting systems that allow higher photosynthetic efficiencies (PEs) and biomass yields. For instance, light supply may be increased by reducing layer thickness [70]. Therefore, researchers have drawn interest in thin culture systems (open or closed) as an alternative to traditional cultivation systems. Some of these systems may represent an improved version of the existing conventional suspended reactors. Figure 2.4 shows a schematic representation of one possible configuration of a thin reactor.

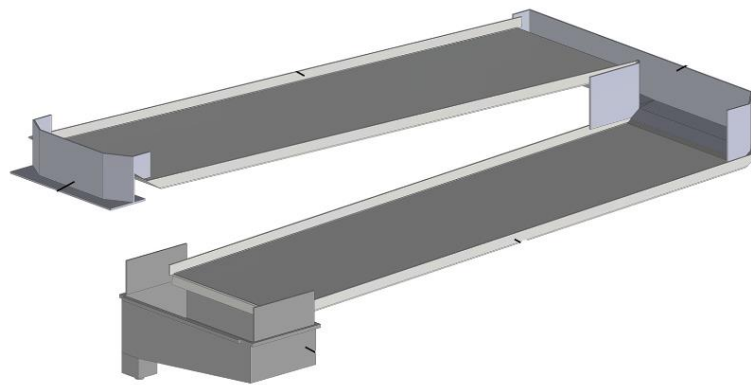


Figure 2.4. Open thin-layer cascade photobioreactor [71].

Thin reactors have a short optical path and reduced volume per unit of the irradiated area, which contributes to a decrease in the shadowing effect and to increase biomass productivity [72,73]. Increasing upstream biomass concentration contributes to an increase in downstream processes efficiency and, consequently, to a reduction in their costs. A few thin culture reactors have been proposed lately. Gifuni et al. [72] showed that a new ultra-flat PBR, with only a 3-mm thick layer, was able to generate 24 g L^{-1} of microalgal biomass in 80 h, reducing 10 times the harvesting costs, when compared to traditional systems. The main disadvantage of this reactor is the energy required to guarantee an efficient mixing [67]. Other thin PBRs have been proposed, showing promising results. For instance, cultivation of *Chlorella* spp. in the cascade system, proposed by Doucha and Lívanský [70] (with two sloped lanes and a thickness of 6-8 mm), resulted in biomass concentrations ranging between 15 and 35 g L^{-1} . Table 2.2 presents an overview of biomass productivities achieved in traditional (open or closed) and thin/intensive suspended cultivation systems.

According to Table 2.2, thin culture systems may allow for more intensive cultivation with higher productivities and lower downstream processing costs. Consequently, these systems may represent a possible solution for cost-effective mass production of microalgae in biorefineries. However, more research is needed to optimise thin systems already proposed or to develop new ones.

Table 2.2. Biomass productivities and photosynthetic efficiencies reported for traditional (open or closed) and thin/intensive suspended cultivation systems

Cultivation System		Microalgae	Optical Path (cm)	P _{areal} ^a (g m ⁻² d ⁻¹)	P _{volumetric} ^b (g L ⁻¹ d ⁻¹)	PE ^c (%)	Ref.
Suspended Open	Raceway pond	<i>Anabaena</i> sp.	30	9.4-23.5	-	-	[74]
		<i>Arthrospira platensis</i>	-	12.2	0.15	-	[75]
		<i>Graesiella</i> sp.	20	6.2-8.7	-	-	[76]
		<i>Haematococcus pluvialis</i>	12	15.1	-	-	[77]
		<i>Muriellopsis</i> sp.	30	8.0-20.0	0.04	0.97-0.69	[78]
		<i>Nannochloropsis</i> sp.	20	6.2-14.0	0.03-0.08	0.5-1.5	[79]
Suspended Closed	Flat-plate PBR ^d	<i>Nannochloropsis</i> sp.	10	10.0-14.2	0.21-0.27	-	[80]
	Flat-plate PBR	<i>Nannochloropsis</i> sp.	2	20.5-27.5	0.90-1.20	2.7-3.8	[79]
	Horizontal-tubular PBR	<i>Nannochloropsis</i> sp.	4.6	5.9-15.7	0.30-0.85	1.2-1.8	[79]
	Vertical-tubular PBR	<i>Nannochloropsis</i> sp.	4.6	10.6-24.4	0.31-0.71	2.4-4.2	[79]
		<i>Phaeodactylum tricornutum</i>	-	20.0	1.15	-	[81]
Thin	Thin-layer cascade PBR	<i>Nannochloropsis salina</i>	0.5-0.6	25.0	4.0	4.6	[71]
	Open thin-layer PBR	<i>Chlorella</i> sp.	0.6-0.7	38.2	4.3	7.05	[82]
	Ultra-flat PBR	<i>Chlorella sorokiniana</i>	0.3	31.2	11.28	-	[72]

^a P_{areal} - areal productivity; ^b P_{volumetric} - volumetric productivity; ^c PE - photosynthetic efficiency; ^d PBR - photobioreactor

2.2.1.4. Batch vs Continuous operation modes

The adopted operation mode for microalgal cultivation is a critical aspect while considering biomass productivity and products' accumulation [83]. The selected operation strategy influences the main accumulated products, since microalgae preferentially accumulate distinct components under different conditions and at different growth phases [67,84]. Figure 2.5 shows the main products accumulated in microalgal cells during the different growth phases.

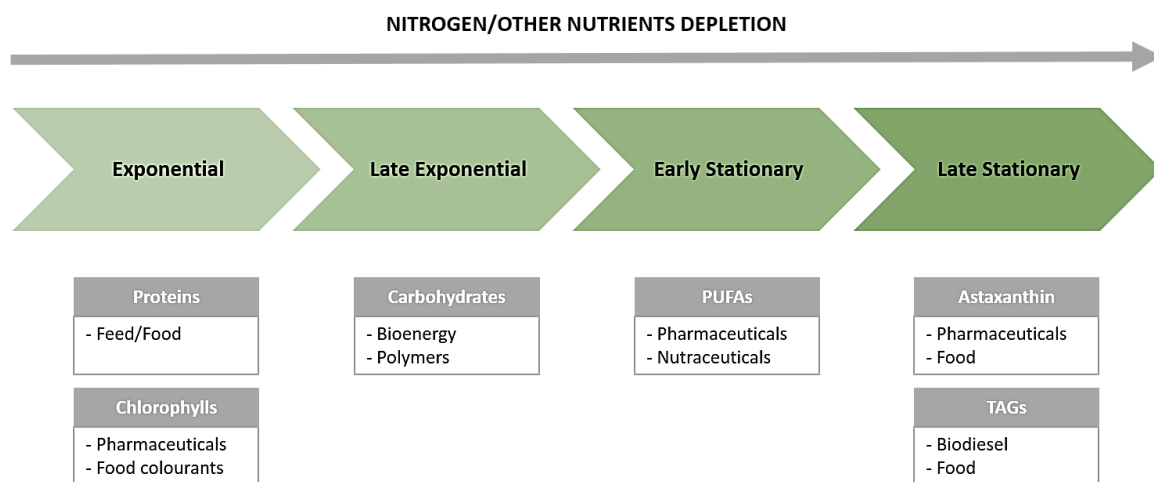


Figure 2.5. Main products accumulated by microalgae at different growth phases.

There are two main operation modes, batch and continuous, and some variations of these, such as fed-batch and semi-continuous ones. Definition of the adequate operation mode should consider the microalgal strain, bioreactor used and target molecules.

In batch mode, the bioreactor works as a closed system with no liquid inflow or outflow. There is an initial addition of inoculum and nutrients, without reposition over time. At the end of this sequence, all biomass is harvested [85]. Fed-batch can be described as a semi-closed system, since all biomass is introduced at the beginning of the sequence, but substrates/nutrients are fed step-by-step [86]. With this operation mode, some issues of the batch mode (e.g., growth inhibition due to high substrate levels at the beginning of the cultivation period and slow biological reaction rates due to limiting substrate concentrations at the end of the cultivation period) can be overcome [87]. In both batch modes, it is possible to distinguish different cell growth phases [3]: (i) lag phase; (ii) exponential growth phase (corresponding to the maximum growth rate); (iii) linear growth phase; (iv) stationary growth phase; and (v) decline/death phase.

On the other hand, the continuous mode has continuous inflow and outflow of liquid. Biomass and nutrients (supplied in a limited amount) are continuously added to the bioreactor, and a portion of the culture is harvested from the outgoing flow at the same rate (dilution rate). To avoid the continuous supply of new inoculum, biomass recycling can be promoted [85]. This applies mainly to the suspended cultivation systems, as in the attached ones, the continuous addition of biomass is not required. Semi-continuous is very similar to the continuous mode. The only difference is that a part of the culture is periodically taken away from the bioreactor and fresh medium is immediately added, until reaching the initial volume and nutrients' level [88]. In continuous cultures, it is not possible to observe the different biomass growth stages, as the physiological state is fixed and determined by the dilution rate.

Comparing the two main operation modes, it is possible to conclude that the continuous one allows for a higher level of control and enables the adjustment and regulation of cell growth rates. Besides, this mode avoids non-productive periods, which is an intrinsic characteristic of the batch mode (every time a sequence ends, it is necessary to harvest biomass, clean the reactor, prepare new medium and start biomass growth over again) [89]. Yet, not all microalgal strains are suitable for operation in continuous mode, since long growth periods increase the possibility of contamination, where the original strain may be overtaken by a more robust or fast-growing one [3,84]. Also, continuous modes are more complex to operate. On the other hand, batch is a simpler mode, but does not allow for high biomass productivities, since nutrients eventually run out, decreasing microalgal growth rates and increasing biomass decay. However, this operation mode allows microalgal growth in both non-limited and limited nutrients conditions, which can favour the accumulation of distinct compounds. Thus, with this cultivation mode, it is possible to harvest biomass in different growth stages, according to the target compounds. For example, proteins should be obtained by harvesting microalgal biomass during the exponential growth phase, whereas starch, triacylglycerols (TAGs), carotenoids and astaxanthin should be recovered in the early and late stationary growth phases (corresponding to substrate depletion conditions) [67,90-92]. Therefore, depending on the chosen biorefinery approach, some operation modes will be more suitable to increase target products accumulation than others [93].

For instance, on an energy-driven biorefinery, some of the products to be explored are lipids, such as TAGs and carbohydrates [94]. For that purpose, it is necessary to promote starvation conditions, typical of a late stationary growth phase. Therefore, the production of these molecules might be done using batch processes

[95,96]. However, the nitrogen starvation phase, which promotes the highest levels of TAGs accumulation, occurs simultaneously with a decrease in the photosynthetic activity [85,97,98]. Continuous and semi-continuous modes have been tested as an alternative to overcome the reduction in the photosynthetic activity while contributing to an increase in TAGs levels, as they can promote a continuous nitrogen depletion at low dilution rate. However, some studies reported that TAGs yields are still higher under batch conditions, since the continuous limitation of nutrients is not as effective as the final productivity of these molecules in batch mode [67,98]. Besides, it may be complex to achieve the optimal dilution rate and nutrients level in continuous mode when operating at large scale. For example, Remmers et al. [98] compared TAGs productivity of *S. obliquus* growing in a flat-panel airlift-loop reactor, in both batch and continuous modes. With this study, the authors concluded that TAGs productivity achieved under batch mode ($0.23 \text{ g L}^{-1} \text{ d}^{-1}$) was considerably higher than the one obtained in continuous mode ($0.11 \text{ g L}^{-1} \text{ d}^{-1}$).

Another possible solution to improve biomass concentration and high levels of molecules accumulated under starvation conditions is to adopt a two-stage culture approach. In this approach, microalgae are first grown in non-limiting conditions, for example in a reactor operating in semi-continuous or continuous modes, followed by a starvation phase, accomplished in batch or fed-batch modes. This technique is also feasible for biorefineries whose aim is to produce value-added products that are mainly accumulated under limiting conditions [99]. For example, Fábregas et al. [100] demonstrated the feasibility of this approach for the production of astaxanthin from *Haematococcus pluvialis*. In the first stage, biomass was produced under semi-continuous mode with daily renewal rates varying between 10 and 40%. In the second stage, microalgae were grown in batch mode to create the optimal conditions for the accumulation of astaxanthin. The authors concluded that the optimal renewal rate applied to increase the target molecule production was strongly dependent on the duration of the batch mode (accumulation) period. For a 12-day period, maximum astaxanthin productivity ($5.8 \text{ mg L}^{-1} \text{ d}^{-1}$) was obtained at a renewal rate of 20%. On the other hand, for a 15-day batch period, the maximum astaxanthin productivity ($9.6 \text{ mg L}^{-1} \text{ d}^{-1}$) was obtained at a dilution rate of 40%.

2.2.2. Biomass harvesting

After microalgal growth, it is necessary to harvest biomass for further extraction of the accumulated products. The harvesting process generally accounts for 20-30% of the total microalgal production costs [101]. The main characteristics of

biomass that hinder the harvesting process are the small size of microalgae, their growth in dilute cultures, with densities close to that of water, and the fact that microalgal cells are surrounded by algogenic organic matter. Also, their surface is negatively charged, which allows microalgae to remain stable in the dispersed state [102]. Currently, there is still no harvesting method that is simultaneously efficient and economically viable at an industrial scale. That is one of the major bottlenecks for the implementation of microalgal biorefineries [102,103].

An optimal harvesting method should work for most of the microalgal strains and allow to achieve high biomass concentrations, at low energy consumption levels. However, that is still not the case for the already studied harvesting techniques. Indeed, most of them present high operation costs or reduced harvesting efficiencies. Some may even decrease the quality of the end products [104]. Therefore, to increase the separation performance, it is essential to select the separation method taking into account: (i) the strain characteristics (i.e., density and size); (ii) the target products; and (iii) the cultivation system used [41,105].

Harvesting methods may be classified as chemical, mechanical, biological, or electrical-based, and they can be applied alone or combined, to achieve better results [102,103]. If more than one method is applied, the harvesting process is frequently split into two stages: (i) a first one, thickening, where biomass is separated from the bulk suspension, to obtain a slurry with 2-7% of total suspended solids; and (ii) a second one, dewatering, that aims to remove water and convert the algal slurry into a cake with 15-25% of total suspended solids [43,102]. For bulk harvesting stage, the main methods applied include coagulation/flocculation, auto- and bioflocculation, sedimentation, flotation, and electrical-based methods (e.g., electroflocculation and electroflotation). The thickening stage requires more energy-demanding methods, such as filtration and centrifugation.

Among the methods considered for biomass separation, centrifugation is the most frequently used and the one that allows better separation efficiencies. Besides, it is suitable for the processing of large culture volumes. However, it is one of the most energy-consuming and expensive methods, and cells can be harmed during this process, due to the high shear forces involved [1,106]. Moreover, when applied to diluted cultures, it can account for 20-25% of the total production costs [107]. Consequently, it is common to carry out a pre-concentration step using other methods (e.g., flocculation, filtration or flotation), following the two-stage harvesting approach. Nevertheless, this method is suitable for the exploitation of high-value

products (e.g., pigments, antioxidants, pharmaceuticals), as it contributes to low moisture levels in the end-product, and because the revenues of such high-value products compensate for the high energy costs [108].

Coagulation/Flocculation is a fast and straightforward method that can be easily implemented on a large scale. Chemicals coagulant/flocculant are added to the culture broth to promote cells' aggregation and flakes' formation, which facilitates sedimentation or flotation and, hence, biomass recovery [109]. It is suitable for a wide range of microalgal species [102]. However, some of the chemicals used (organic or inorganic) as coagulants/flocculant agents may contaminate microalgal biomass, limiting its final application to low-added value and non-food grade products [41,107]. Therefore, if potentially toxic coagulants/flocculants are used, this method may not be suitable for harvesting biomass intended for the extraction of high-value products or food and feed applications. Besides, those chemicals may also interfere with biomass processing on latter operations, reducing the extraction efficiencies of target products [110].

Bioflocculation and autoflocculation also promote flakes' formation to facilitate biomass recovery through gravity sedimentation or flotation. However, in bioflocculation a biologically-based/natural flocculant is added, whereas in autoflocculation cells' aggregation is induced by an increase in the pH of the algal slurry (generally through the addition of a basic substance, such as sodium or calcium hydroxide). Both methods represent simple, non-expensive, and non-toxic harvesting methods that do not require the addition of potentially harmful chemical agents [111]. However, in autoflocculation, due to the pH increase, some culture medium components may precipitate, leading to changes in the cell composition. Moreover, the natural flocculants used in bioflocculation (typically produced by bacteria, fungi or flocculating microalgae) can contribute to microbiological contamination of microalgae. Therefore, this method may not be suitable for food and feed applications [110]. Nevertheless, both methods may be a promising solution to harvest microalgae for further production of biofuels. In fact, the introduction of microorganisms in bioflocculation may even contribute to an increase in the lipid yields [102]. Also, according to Salim et al. [112], bioflocculation can be as effective as chemical flocculation, though more sustainable. Despite their advantages, these promising methods are still not proved to be economically viable for large-scale production.

Regarding sedimentation, it is a simple and inexpensive method, as it corresponds to biomass settling due to gravitational forces. Some microalgae have

specific characteristics that allow them to settle easily without the need for an external agent. For example, *Spirulina* is characterised by having a long spiral pattern that facilitates aggregation between cells and, hence, biomass sedimentation [113]. On the other hand, for strains that do not have settling ability, this method can be too time-consuming and may even lead to biomass deterioration, limiting its future applications to low-value products. To improve settling efficiencies and decrease harvesting time through sedimentation, it is common to apply a flocculation step before gravity sedimentation. The same happens for flotation; although this method works only by the action of gas bubbles, it usually requires the addition of a chemical flocculant to improve recovery efficiencies. Nevertheless, both methods are straightforward and low-cost; therefore, they can be an excellent solution to harvest microalgae that accumulate low-value products, such as TAGs [108].

Filtration is the most commonly used harvesting method at laboratory scale. At an industrial level, this method may not be so effective, due to membrane clogging, which determines the need to membrane cleaning or replacement. Therefore, this technique is more suitable for filamentous and large-colonial microalgal species [113,114]. Nevertheless, microfiltration can be applied when micrometre-size microalgae (e.g., *Scenedesmus*, *Dunaliella* and *Chlorella*) are harvested [106]. This method has been denoted as a good alternative for centrifugation, with less energy consumption and excellent harvesting performance [107,115]. Additionally, it is suitable for shear-sensitive species. Ahmad et al. [111] evaluated both centrifugation and microfiltration for the recovery of ~660 mL of a *Chlorella* sp. culture, concluding that the amount of biomass obtained by both methods was very similar: ~0.1174 and ~0.1145 g, respectively. Considering the overall costs of both processes, microfiltration seems to be a more interesting solution for large-scale biofuel production and microalgal biorefineries. However, the viability of this method on an industrial scale is not yet proven.

Recent research has carried out on electrical-based methods. The principle behind these methods is the negative charge of algal cells, which allows them to be concentrated by movement in an electric field [103]. If a sacrificial electrode is used, metal ions can be released during the process and aggregated with microalgae. However, the probability of microalgal contamination is low [102]. Therefore, most of these methods are suitable for harvesting biomass for food and feed applications. Still, they are not yet appealing for large-scale production, due to the significant energy requirements and electrodes costs [103].

However, the harvesting process success and cost strongly depends on other factors, such as the cultivation system selected. Ruiz et al. [116] concluded that when using a closed PBR for microalgal growth, centrifugation would only contribute to 5-7% of the total production cost. On the other hand, if an open raceway pond was selected as the cultivation system, centrifugation could represent 23% of the total costs.

2.2.3. Cell disruption and products extraction

In a biorefinery, the successful extraction of multiple products will typically depend on cellular disruption to isolate or fractionate the compounds of interest [22].

Depending on the microalgal species, and even on the growth stage, the cell wall structure may change and, therefore, it is essential to choose the most suitable disruption method, according to its characteristics. Most of the microalgal species present a rigid and robust cell wall, mainly composed by cellulose, capable of maintaining its integrity in diverse harsh conditions. Hence, in those cases, cell disruption is an essential downstream process step to maximise product recovery in the extraction step and to increase the bioavailability of algal constituents [117-119]. However, other species have a less complex or thinner cell wall. Some may even lack of cell wall, like *Dunaliella salina*, that has a thin and simple cell membrane instead. In this case, a cell disruption step is not required [120]. Products are generally inside globules or bound to the cell membrane, which makes this downstream process even more challenging [121]. Selection of an adequate cell disruption technique should consider the following parameters [117]: (i) the cellular structure of the selected species; (ii) the location of the compounds of interest within the cell; (iii) the efficiency and scalability of the method; and (iv) the associated costs. The different methods for cell lysis may be classified into four groups, according to Chew et al. [22]: mechanical, physical, biochemical and biological.

Mechanical methods usually present high extraction efficiencies and, consequently, require high-energy inputs. They also are easy to control and to operate at a large scale. These methods include bead milling and high-pressure homogenisation, which are the most common methods applied at an industrial scale [118,120,122,123]. Bead milling uses kinetic energy to make beads collide with each other and with the algal cells, destroying the microalgal cell wall. The main advantages of this process are the [124,125]: (i) high disruption efficiencies; (ii) high speed; and (iii) simple equipment requirements. However, the high-energy requirements for these techniques make them less favourable for the biorefinery concept [121]. Furthermore,

the high energy consumption, due to stirring intensity, results in a temperature increase that can damage some intracellular compounds [117].

Regarding physical methods, they may present lower efficiency on cell lysis and products extraction, but have low energy consumption. Examples of these methods are ultrasound treatment and pulsed electric field (PEF). The latter is attracting the attention of researchers lately, especially for lipids recovery. This non-thermal method consists of applying short electrical pulses (nano- to milliseconds) of high voltage to microalgae that are placed between two electrodes. The cell membrane polarisation leads to cellular tissues permeabilisation and disruption [126]. Even though it has resulted in lower yields, when compared to other techniques, such as the mechanical ones, it presents many benefits [126,127]: (i) low energy input; (ii) low operational costs; (iii) avoids quality loss and total cell wall disintegration; (iv) avoids the use of chemicals; (v) very short treatment time; (vi) does not require a dewatering pre-step (i.e., it is suitable to treat wet or dry microalgae); and (vii) seems to be a promising process for large-scale cell perforation [127-130].

Biochemical methods have high efficiencies and need low energy input. However, they have significant cell disruption and can degrade valuable compounds from the cell. Moreover, the costs of enzymes and chemicals are too high, which make them less attractive at an industrial scale [118]. There are several chemical materials that can be used (e.g., solvents, salts, acids and surfactants), and the suitability strongly depends on the composition and structure of the microalgal cell wall [131]. Concerning biological techniques, they require mild operating conditions and have low energy requirements. However, they are not so effective as the already described methods [131].

The ideal cell disruption technique should work effectively on wet microalgae, be energy efficient, inexpensive and easy to scale-up [132,133]. Moreover, in a biorefinery context, where it is necessary to exploit several products, the separation of the different fractions should be done without damaging the others. Therefore, the cell rupture must be done under mild conditions, since the structure of some cell compounds is quite fragile [118,127,134]. For example, water-soluble proteins have a high value because of their techno-functional properties. However, these compounds need to be in an intact form to be used; therefore, severe cell disruption conditions, such as chemical-based processes, should not be applied, as they affect the proteins' properties [135].

On the other hand, to release lipids from microalgae with a rigid cell wall, harsher techniques are used without endangering the quality of that compound. Therefore, selection of a disruption method for the recovery of more than one type of compound must be well planned because the chosen method can have different effects on the target products [123]. Hence, when products with different sensitivities to the disruption methods are envisaged, the mildest processes should be employed. Depending on the value of target products, methods with different energetic requirements could be used. For example, when the biorefinery aims the production of high-value compounds, energy-demanding methods, such as bead milling and high-pressure homogenisation can be applied, as profits from the target products will offset the energetic costs.

After cell wall disruption, the selection of an appropriate extraction method is essential to maintain the quality of the products extracted from microalgal biomass. Depending on the target compounds, different extraction technologies can be applied [136]. Usually, the most valuable compounds present in microalgal cells are carbohydrates, proteins, pigments and oils [22].

Organic solvents are used to separate both hydrophobic (lipids and pigments) and hydrophilic (proteins and carbohydrates) compounds. In this method, the hydrophobic components are extracted, whereas the hydrophilic fraction remains in the aqueous phase [134]. The organic solvents are usually applied for lipids and pigments extraction, but can promote protein denaturation, which become insoluble and lose their techno-functional properties [117]. Several studies related to lipids extraction from microalgae use mixtures of hexane and methanol or ethanol [117]. To increase lipids' extraction yields, it is common to dry microalgal biomass before solvent extraction. Dry extraction requires less solvent usage than the wet one, but it is more energy demanding and costly due to the drying step [117,134,137]. Even though the solvent methods are cheap and easy to perform, the use of organic solvents has some disadvantages [22,138]: (i) the environmental and health impacts of certain solvents used, especially those coming from fossil fuels resources; (ii) long extraction times; and (iii) difficulties in recovering the solvents, which affects the process economy.

To avoid the use of organic solvents, alternative techniques for lipids extraction should be considered, such as: (i) supercritical CO₂ extraction; (ii) liquid CO₂ extraction; (iii) extraction using ionic liquids; and (iv) extraction using switchable solvents. Supercritical CO₂ extraction is a solvent-free method, has high selectivity,

does not induce compounds degradation, and the used CO₂ can be easily recycled [1,137]. However, the capital and maintenance costs of this process are very high [139,140]. Therefore, it may be mainly used for the extraction of high-value products such as pigments (e.g., lutein and astaxanthin) [140]. Liquid CO₂ extraction has the same advantages as the supercritical CO₂ extraction, but does not require high temperature and pressure, which makes this approach more economical and feasible at an industrial scale [141]. Ionic liquids are organic salts, composed of cations and anions [142]. These compounds present thermal stability, low flammability, low toxicity, non-volatility, are recyclable and can be used in lipids extraction from wet biomass; however, the use of these ionic liquids is quite expensive [123,143,144]. Switchable solvents are liquids that can reversibly change their properties (from a hydrophobic solvent into a hydrophilic one or switch between a polar or nonpolar solvent) with the addition or removal of specific compounds, such as CO₂ [145,146]. However, their technical feasibility at large scale is still under research [147].

From a biorefinery perspective, cascade extraction can be used to extract a single target product (used in the case of high-value products) or to extract multiple products. The single-product valorisation strategy is based on the use of cost-effective techniques (e.g., supercritical CO₂ and ionic liquids) that enable high extraction efficiency of the target product [67]. In this approach, the costs of microalgal cultivation and extraction are compensated by the high-value products obtained (pigments, for example) and, at the same time, extraction of the residual fractions can be lucrative [1]. For instance, the residual biomass still contains proteins and carbohydrates, and these compounds can be further valorised, to produce biofuels, for example [67,139]. However, if the target high-value compounds are not damaged by the extraction of water-soluble proteins, then the high-value compounds can be extracted after proteins' extraction, thus increasing the range of functional products obtained from microalgal biomass [148].

In the multiproduct valorisation strategy, medium- to low-value compounds are extracted. According to the target compounds, different extraction techniques can be applied. However, this approach must take into account the fragility of the compounds; therefore, the selected techniques should be mild and with high selectivity, so that the functionality of all extracted compounds is not compromised [118]. Furthermore, the extraction order should be modulated, as extraction yields can be strongly influenced by the order by which the target compounds are extracted [148]. For instance, if the organic solvent lipid extraction is performed first, the protein fraction can be negatively impacted, as a large part of the soluble proteins will

lose their techno-functional properties [117]. Therefore, the most fragile compounds should be extracted first, following the order: soluble proteins > lipids/pigments > carbohydrates. From an economic point of view, the selection of an extraction technique that could be done in the same equipment is recommended; for instance, the use of supercritical extraction with a sequence of solvents with different polarities [149]. Despite being a promising alternative for a multiproduct biorefinery, the cascade extraction may promote the co-extraction of cell components presenting similar physical/chemical properties, requiring further separation of the compounds and increasing the extraction costs [67].

2.3. Microalgae in wastewater treatment

2.3.1. Nutrients removal by microalgae from different wastewater types

Large volumes of wastewater are generated daily during several anthropogenic activities, namely domestic and industrial ones [150]. Some of these waters are rich in nutrients, such as carbon, nitrogen and phosphorus. The human interventions in the global biogeochemical cycles have created a one-way flow of nutrients into the atmosphere and the environment: (i) nitrogen is taken from the atmosphere and moved into fields, rivers and forest; and (ii) phosphorus is obtained from rocks and disposed into farms, lakes and oceans. Therefore, this unilateral mobilisation, not only threatens the future abundance of these nutrients, but also the ecosystems, especially aquatic ones, leading to the formation of the so-called “dead zones” [150-152]. Regarding the future supply of these vital nutrients, the most alarming is phosphorus. Unlike nitrogen, it cannot be pulled from the atmosphere, and unlike carbon in the energy system, there has not been found any replacement. Reserves of this finite resource could disappear in the next 100 years [153]. Therefore, it is urgent to rethink anthropogenic interventions and promote the maximum reuse of these compounds in the context of the circular economy.

Wastewaters are considered secondary renewable sources of nutrients like nitrogen and phosphorus. Microalgae can grow in these effluents and have great potential to arise as an advanced biological treatment step for nutrients removal, resulting in several benefits [150,154-157]: (i) nutrients removal from the wastewater; (ii) decreased risk of eutrophication in coastal and inland water bodies; (iii) nutrients recovery and possible return to infertile soils; (iv) reclamation of water for reuse; (v) can be integrated with existing wastewater systems; (vi) reduced costs and

environmental impact of microalgal biomass production (as there is no need for freshwater and nutrients, usually fertilisers); and (vii) simultaneous accumulation of many added-value bioproducts and biofuels.

However, the growth behaviour and nutrients removal efficiencies differ greatly among microalgal species, the wastewater type chosen as culture medium, and other factors (e.g., light intensity, photoperiod, temperature, initial nutrients concentrations, N:P molar ratio and pH) [155,156,158]. Examples of different wastewaters that can be used as the culture medium for microalgal growth include [158-164]: agricultural wastewater; effluents from the industrial sector (e.g., paper and pulp; textile; brewery and winery); municipal wastewater and effluents from animal production (e.g., swine, poultry and dairy). Each of these waters has different compositions and characteristics that can significantly affect the growth, removal efficiency and products accumulation by microalgae.

Municipal wastewaters have low nitrogen and phosphorus concentrations. For example, Cai et al. [158] reported total nitrogen and total phosphorus concentrations in these effluents of 15-90 mg L⁻¹ and 5-20 mg L⁻¹, respectively. This wastewater type typically presents low contents of toxic substances, which make it suitable to support microalgal growth, and explains the reason why this effluent is the most frequently used in microalgal growth experiments [154,165]. However, when municipal wastewater has the contribution of industry effluents and urban runoffs, heavy metals (e.g., lead, zinc and copper) may be found in concentrations higher than those tolerated by microalgae, which may inhibit microalgal growth [166]. This residual water can be classified into three different types, depending on the degree of treatment [154,167]: (i) raw sewage; (ii) secondary effluent (after activated sludge treatment); and (iii) centrate, which results from the sludge dewatering process, and contains the highest concentration of nutrients.

Regarding livestock production, it generates large volumes of wastewater rich in N and P, mostly coming from manure-contaminated runoff [158]. For instance, according to Yetilmezsoy and Sakar [168], poultry production effluents may present a total nitrogen concentration of 802-1825 mg L⁻¹ and of total phosphorus between 50 and 446 mg L⁻¹. Swine effluents have higher concentrations: 1110-3213 mg L⁻¹ for total Kjeldahl nitrogen and 310-987 mg L⁻¹ for total phosphorus, according to Millmier et al. [169]. However, high concentrations of suspended solids, turbidity and ammonia can make this effluent inappropriate for microalgal growth, if directly used as culture medium [154]. If, for instance, the effluent undergoes an anaerobic digestion process,

then the resulting effluent may be already more adequate for microalgal biomass production. Another option is to dilute the manure effluent to lower chemical oxygen demand (COD), nitrogen and phosphorus concentrations [165,170]. Nevertheless, only a few microalgal strains are usually tested in animal wastewater treatment [171].

Industrial effluents show lower nitrogen and phosphorus concentrations than animal production effluents, but the concentrations vary a lot from industry to industry [158]. For instance, winery production generates effluents with an average of 110 mg L⁻¹ of total nitrogen and 52 mg L⁻¹ of total phosphorus, and textile industry has typical wastewater with 21 to 57 mg L⁻¹ of total nitrogen and 1.0 to 9.7 mg L⁻¹ of total phosphorus [172-174]. The feasibility of growing microalgae in an industrial effluent should be analysed case by case, considering other components that may be present in these wastewaters, such as heavy metals and other toxic elements [154]. Regarding the industries of olive oil, mill and paper industry, several authors have already proved the capability of different microalgae (e.g., *Chlorella*, *Ankistrodesmus* and *Scenedesmus*) to treat these industrial effluents [20,175].

Many studies have already reported good results when analysing nutrients removal from different wastewaters. Mennaa et al. [152] concluded that different microalgal species were able to remove more than 80% of the total dissolved phosphorus and 87% of total dissolved nitrogen from urban wastewater. Li et al. [176] studied the growth and nutrients removal performance of *Coelastrella* sp. in swine wastewater with different concentrations of zinc (Zn(II)). In this study, the authors concluded that maximum ammonium-nitrogen removal of 62.3% was obtained at 0.50 mg L⁻¹ of Zn (II), and maximum total phosphorus removal of 77.6% was obtained at the assay with 8.0 mg L⁻¹ of Zn (II). Hernández et al. [177] evaluated the growth behaviour and nutrients removal efficiencies of *Chlorella sorokiniana* in a potato-processing industry effluent. The results showed that this microalgal can remove 80.7% of soluble phosphorus and more than 95% of ammonium-nitrogen. Sometimes, microalgae-bacteria consortia are also used to treat wastewaters, showing promising results. Lee et al. [178] studied nutrients removal from municipal wastewater using the microalga *Scenedesmus* sp, plus a group of bacteria (*Flavobacteria* and *Shingobacteria*). This consortium achieved phosphorus and nitrogen removal efficiencies of 98.1% and 95.7%, respectively.

2.3.2. *The specific case of paper industry effluents*

Pulp and paper industry consumes large amounts of water, being one of the largest producers of industrial effluents at a global scale [179]. It is estimated that about 10-50 m³ of water are required to produce a tonne of paper [164]. Therefore, the treatment of these effluents, as well as their correct discharge concerning environmental limits, should be a major concern for companies in this sector.

On the other hand, with the advancement of the technological era, there has been a change in the demand for products from paper industries. Indeed, with the decline in newsprint usage and the increase of wood cost, paper production companies have to search for new solutions and opportunities to save the future of this business [180,181]. This is how the idea of microalgal biorefineries arises as a possible solution to solve the above-presented problems: the advanced treatment of the paper industry effluents and, simultaneously, the production of value-added products, to increase the competitive advantage of this business [180].

However, pulp and paper wastewaters are mostly deficient in nitrogen and phosphorus, concerning microorganismal nutritional needs. According to Slade et al. [182], typical concentrations of total nitrogen and total phosphorus in paper mill effluents are 1.1-10.9 mg L⁻¹ and 0.6-5.8 mg L⁻¹, respectively. Therefore, for microalgae to efficiently treat these wastewaters, sometimes the addition of supplementary nutrients is required. Moreover, pulp and paper mill wastewater can contain other compounds, such as lignin, humic acids, furans, and dioxins, that can slow down microalgal growth and, consequently, decrease bioproducts accumulation and exploitation [183]. All of these factors explain why the use of the pulp and paper effluent for microalgal cultivation is less explored than other wastewater types [164,184].

Nevertheless, several authors have already reported good results while treating paper industry effluents, especially in terms of nutrients removal. Usha et al. [164] evaluated the potential of a mixed culture of microalgae, with two *Scenedesmus* species, to treat this industrial effluent. Results have demonstrated that there were a nitrate-nitrogen and phosphate-phosphorus removal of up to 65% and 71%, respectively. Moreover, a COD removal of 75% and biochemical oxygen demand (BOD) removal of 82% were also observed. Gentili [184] studied the growth and lipid accumulation of different microalgal species on a mixture of a municipal wastewater and pulp and paper industry effluent. The author concluded that ammonia was reduced from 96% to 99% and phosphate from 91% to 99%, depending on the microalgal strain.

Another reason for pulp and paper industries to start thinking on the biorefinery solutions is the high demand for energy that these industries present. Therefore, it is of utmost importance to develop solutions that promote the production of green energy and optimise their energy sufficiency.

2.4. Biorefinery concept: energy- vs product-driven biorefineries

Biorefinery, by analogy with the petroleum refineries, tries to explore the vast portfolio of products that can be extracted from the raw material [185,186]. These new biomass refineries allow processing biomass into a series of marketable products that are chemical equivalent to existing petrochemical products [187].

Microalgal biorefinery has the following steps, already described in detail: (i) microalgae cultivation; (ii) harvesting; (iii) cell disruption; and (iv) product extraction. The design of microalgal biorefineries must consider the chemical composition of the selected species. Considering that microalgal biomass can be converted in energetic or non-energetic products, biorefineries can be classified in two main groups [188]: (i) energy-driven biorefinery (hereinafter called energy biorefineries); and (ii) product-driven biorefinery systems (hereinafter called chemical biorefineries or non-energy based biorefineries). Energy biorefineries have as main goal the large-scale production of energy products, such as biofuels, power and/or heat, and the residues can be used to extract biobased products [189]. This biorefinery has the following advantages: (i) it can be used to capture CO₂ and, consequently, contribute to mitigating GHGs emissions; (ii) microalgae can grow in a wastewater or industrial effluent, therefore, removing excess nutrients; and (iii) it promotes the improvement of energy efficiency. On the other hand, chemical biorefineries are specifically designed to obtain bioproducts and biomaterials from microalgal biomass, such as: chemicals, materials, food and feed. However, the resulting residues may be applied to produce bioenergy. The associated market volume and value also distinguish energy and chemical biorefineries: the first has low-value products with high market volume (e.g., biodiesel, biogas and fertiliser). In contrast, the second one has high-value products with low market volume (e.g., nutraceutical, cosmeceutical, and pharmaceutical applications) [189,190].

Regarding chemical biorefineries, these are still in progress, since some key technologies are in the research and development (R&D) stage. Although its potential is high, it is expected that with the increase in the production volume, the market

value of the products will decrease. Also, some of the products produced in these biorefineries, like biological ones, cannot be stored and released to the market when required.

Concerning energy biorefineries, they may have a key role in the transition from a fossil-based linear economy, to a circular based (bio)economy [188]. As it was previously mentioned, the largest anthropogenic source of GHGs is the burning of fossil fuels for the transport and energy sectors [2]. Therefore, new solutions for these sectors are of great relevance. Furthermore, the unsustainable exploitation of fossil fuels and the increase in fuel demand is leading to its depletion. With a lower volume available in the market, according to the law of supply and demand, the prices of these products will tend to increase, opening doors to new solutions. Besides, from a governance point of view, it is also essential for policymakers to encourage an environmentally friendly solution to meet established targets. Biofuels may be the solution, but its production efficiency is still lower than oil refining, increasing its costs. Accordingly, the development of energy biorefineries may optimise the energy, environment and monetary cost of the production of several fuels. More research is needed concerning the main challenges and bottlenecks that may exist during the biomass processing in biorefineries and optimisation of these processes.

Materials and methods

3.1. Paper industry effluent remediation using microalgae: laboratory-scale experiments

3.1.1. Microalgal strain and inoculum preparation

The microalga selected for the remediation experiments was *C. vulgaris*, as it is considered a robust specie, able to withstand harsh conditions and to establish itself as the main population in an open culture. Moreover, the growth of *C. vulgaris* in different wastewaters and its potential for nutrients removal have already been studied and proven [191]. Another interesting feature of this species is its ability to accumulate significant amounts of lipids, suitable for the production of biodiesel [192,193].

The strain used in this study, *C. vulgaris* CCAP 211/11B, was provided by the Culture Collection of Algae and Protozoa (CCAP, United Kingdom). Stock solutions were prepared in 100-mL Erlenmeyer flasks, with working volume of 50 mL, using a modified version of the OECD (Organisation for Economic Cooperation and Development) test medium [194], with the following composition (per litre): 250 mg NaNO₃; 12 mg MgCl₂·6H₂O; 18 mg CaCl₂·2H₂O; 15 mg MgSO₄·7H₂O; 45 mg KH₂PO₄; 0.08 mg FeCl₃·6H₂O; 0.1 mg Na₂EDTA·2H₂O; 0.185 mg H₃BO₃; 0.415 mg MnCl₂·4H₂O; 3 µg ZnCl₂; 1.5 µg CoCl₂·6H₂O; 0.01 µg CuCl₂·2H₂O; 7 µg Na₂MoO₄·2H₂O; e 50 mg NaHCO₃. Flasks were kept on an incubator with light supply and continuous agitation.

To evaluate the effect of this effluent on microalgal growth behaviour, cells were grown under different percentages of this effluent, varying from 20 to 100%.

3.1.2 Paper industry effluent characterisation

The effluent used in this study was obtained from a Portuguese paper company and collected after the secondary treatment step. Considering the sampling site, the effluent was characterised in terms of nitrogen (N), in the form of nitrate, phosphorus (P), in the form of phosphate, and chemical oxygen demand (COD). Analysis of each parameter was performed in duplicates, and the results are presented in Table 3.1.

To determine the nitrate-nitrogen concentration, the USEPA Approved Brucine Colorimetric Method [195] was used. Firstly, standards with concentrations ranging from 2.5 to 50 mg NO₃ L⁻¹ were prepared from a nitrate stock solution of 500 mg NO₃ L⁻¹. Then, 1 mL of brucine-sulfanilic acid reagent and 10 mL of sulphuric acid solution were added to standards and samples, which were further placed in the dark for 10 minutes. After this period, 10 mL of distilled water was added, and the samples and standards were returned to the dark. After 20 min, absorbance at 410 nm was measured using a UV/Vis spectrophotometer (GENESYS 10 UV, Thermo Scientific, USA). With the values determined for the standards, it was possible to obtain a linear regression between the absorbance measured at 410 nm and the nitrate concentration in mg NO₃ L⁻¹ (see Figure A.1), being it: $y = (0.01505 \pm 0.00009)x + (0.019 \pm 0.002)$; ($R^2 = 0.9992$). Nitrate-nitrogen concentration in effluent samples was then determined according to the established expression.

For phosphate-phosphorus quantification, the Spectroquant Phosphate Kit Test (Merck, Germany) was used. This method is analogous to the USEPA approved method for phosphorus determination 365.2+3, APHA 4500-P E, and DIN EN ISO 6878 [196], and lies on the following principle: in a sulphuric solution, orthophosphate ions react with molybdate ions to form molybdophosphoric acid. Then, ascorbic acid reduces this compound into phosphomolybdenum, which can be determined photometrically [197]. The determinations were carried out according to the instructions provided in the Kit Test. For each effluent sample, three drops of reagent PO₄-1 and one spatula of reagent PO₄-2 were added, and the mixtures were vigorously mixed. After the complete dissolution of reagent PO₄-2, a spectrophotometer (Spectroquant Prove 600, Merck, Germany) was used for phosphate-phosphorus quantification. Samples were read directly without establishing a calibration curve, as this method already provides an electronically-stored calibration curve [198]. It is also important to mention that total phosphorus (TP) was also determined for this effluent. The result obtained for TP was practically the same as for phosphate-phosphorus, with a negligible variation. These results were not surprising, since the analysed effluent from the paper industry was collected after the biological treatment stage, where it is expected that organic phosphorus is converted into inorganic, being phosphate the dominant form of inorganic phosphorus. Therefore, when analysing phosphorus concentration over time in the different assays (see 4.1.3), it was decided to always determine the values in terms of phosphate-phosphorus.

COD determination was done according to the 5220 C closed reflux titrimetric method from standard methods [199]. For that, 2.5 mL of the collected samples were

filled into the digestion vessels and were refluxed in a strongly acid solution of 3.5 mL of sulphuric acid reagent, with 1.5 mL of standard potassium dichromate ($K_2Cr_2O_7$) solution 0.01667 M. Then, the vessels were placed in a block heater, where digestion was performed at 150 °C for 2 h. After digestion, vessels were cooled to room temperature, and the remaining unreduced $K_2Cr_2O_7$ was titrated with ferrous ammonium sulphate (FAS) solution of 0.10 M, using ferroin as the indicator. The turning point was indicated by the colour change, from blue-green to red. The same procedure was done using distilled water instead of the paper industry effluent (blank) [200]. Organic matter was calculated in terms of oxygen equivalent, according to Equation 3.1:

$$COD = \frac{(V_{FAS;B} - V_{FAS;S}) \times M_{FAS} \times 8000}{V_S} \quad (3.1)$$

where, $V_{FAS;B}$ is the volume of FAS, in mL, used in the titration of the blank, $V_{FAS;S}$ is the volume of FAS, in mL, used in the titration of the samples, M_{FAS} is the molecular weight of FAS and V_S is the sample's volume, in mL.

Table 3.1. Characterisation of the collected paper industry effluent in terms of nitrate-nitrogen, phosphate-phosphorus and chemical oxygen demand

Parameter	Result
COD (mg O ₂ L ⁻¹)	323
NO ₃ -N (mg N L ⁻¹)	3.6 ± 0.6
PO ₄ -P (mg P L ⁻¹)	3.72 ± 0.07

According to Table 3.1, and considering the nutrients requirements for microalgal growth in terms of nitrogen and phosphorus concentration, it is possible to conclude that the selected effluent presents a low nitrogen concentration, which may be limiting for microalgal growth (N:P molar ratio determined for this effluent was about 2:1). According to Larsdotter, adequate N:P molar ratios for microalgal growth should be comprised between 5:1 and 30:1, with values below 5:1 indicating nitrogen limitation and values above 30:1 indicating a limitation by phosphorus. Therefore, to ensure an adequate nutrients supply to support *C. vulgaris* growth, the collected paper industry effluent was supplemented with an external nitrogen source, NaNO₃, trying to obtain a N:P molar ratio of 9:1. The selection of nitrate as the nitrogen source was because it is considered the most thermodynamically-stable form of inorganic nitrogen in aquatic systems [201].

3.1.3. Experimental setup

Experiments regarding microalgal growth in the paper industry effluent were performed in batch mode, using 1000-mL borosilicate bottles as cultivation system. To offset possible toxic effects of the paper industry effluent on microalgal growth, as well as light limitation due the effluent colour, different effluent concentrations were evaluated: 20%, 40%, 60%, 80% and 100%. These concentrations were prepared by diluting the nitrogen-supplemented effluent with distilled water. Besides these conditions, microalgal growth was promoted using the modified OECD test medium as the culture medium (positive control), and the raw effluent (100%) supplemented with nitrogen without microalgae was submitted to the same culturing conditions (negative control). The different effluent compositions and the positive control were inoculated with 40 mL of a previously centrifuged *C. vulgaris* inoculum, giving an initial biomass concentration of approximately 120 mg DW L⁻¹. Except for the positive and negative controls, all the experiments were performed in duplicates. After inoculation, the cultures were allowed to grow for 15 d under continuous light supply provided by LED lamps placed in parallel with the bottles, with photosynthetically active radiation (PAR) of 202.9 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The CO₂ necessary for microalgal photosynthesis was supplied to the cultures through the continuous injection of atmospheric air, previously filtered by 0.22- μm cellulose acetate membrane filters, at a flow rate of 1.5 L min⁻¹, using air pumps (AP-180, Trixie, Germany). Air injection was also performed to promote the cultures' mixing and avoid microalgal sedimentation. To keep the adequate temperature for microalgal growth in the coldest days, a heating tape was placed in the experimental facility (Heating tapes 265 W, J.P. Selecta, Spain). Figure 3.1 presents a picture of the experimental setup.

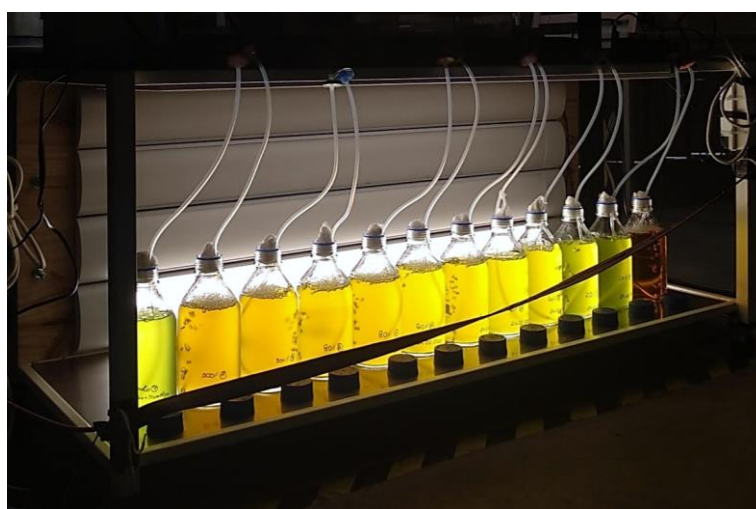


Figure 3.1. Experimental facility used for microalgal growth.

3.1.4. Microalgal growth monitoring and determination of growth parameters

Operational parameters, such as pH and temperature, were daily monitored using a single channel multi-parameter analyser (C6010, Consort, Belgium) and results are presented in Annex A.2.

Microalgal growth was also analysed daily, by measuring the optical density at 680 nm, OD_{680} , using a spectrophotometer (GENESYS 10 UV, Thermo Scientific, USA). Samples were always diluted to measure absorbance values lower than 0.8 (i.e., in the linear response of the method). Biomass concentration was obtained through a previously determined calibration curve that establishes the relation between OD_{680} , y , and the cell dry weight, x (see Annex A.3): $y = (0.0023 \pm 0.0002)x - (0.123 \pm 0.008)$; ($R^2 = 0.9758$).

With the biomass concentration values, the specific growth rates (μ , d^{-1}), maximum biomass concentration (X_{max} , $mg\ DW\ L^{-1}$) and biomass productivities (P_x , $mg\ DW\ L^{-1}\ d^{-1}$) were determined. Specific growth rates were obtained for each culture using a pseudo-first-order kinetic model (Equation 3.2). With the graphical representation of $\ln(X)$ versus time (t , d), it was possible to define the exponential growth phase and determine the specific growth rates for each tested condition:

$$\frac{dX}{dt} = \mu X \Leftrightarrow \ln \frac{X}{X_0} = \mu (t - t_0) \quad (3.2)$$

where X and X_0 are the biomass concentrations ($mg\ DW\ L^{-1}$) in the instant t and initial (t_0) instant of the exponential growth phase, respectively.

Biomass productivities were calculated for each pair of consecutive experimental points (X_z and X_{z+1}), according to Equation 3.3. With the results obtained, the maximum biomass productivity (P_{max}) was determined, which corresponds to the highest value from P_x set of values.

$$P_x = \frac{X_{z+1} - X_z}{t_{z+1} - t_z} \quad (3.3)$$

On the other hand, average biomass productivity was obtained from the ratio between the biomass produced during the assay and the elapsed experimental time, as defined in Equation 3.4:

$$P_{avg} = \frac{X_f - X_i}{t_f - t_i} \quad (3.4)$$

where X_f and X_i are the biomass concentrations (mg DW L⁻¹) in the final (t_f) and initial (t_i) instants of the cultivation period, respectively.

3.1.5. Phosphorus concentration in the culture medium and determination of nutrients removal kinetics

For nutrients concentration analysis, 20 mL samples of each culture were collected on days 0, 1, 2, 3, 4, 7, 9, 11 and 14. Then, the samples were centrifuged at a rotational speed of 4000 rpm, for 10 min, in an Eppendorf centrifuge (5819 R, Eppendorf, Germany). Phosphorus concentration in the supernatants was analysed using the Spectroquant Phosphate Kit Test, according to the procedure referred in Section 3.1.2. As previously mentioned, phosphorus was always analysed in terms of phosphate-phosphorus. Nitrogen concentration of the effluent samples during the cultivation period was not determined due to laboratory limitations resulting from the current pandemic situation.

To evaluate phosphorus removal under the studied conditions, removal efficiencies (RE (%)), mass removal (MR , mg P L⁻¹) and average removal rates (RR , mg P L⁻¹ d⁻¹) were calculated according to Equations 3.5, 3.6 and 3.7, respectively:

$$RE (\%) = \frac{S_i - S_f}{S_i} \times 100 \quad (3.5)$$

$$MR = S_i - S_f \quad (3.6)$$

$$RR = \frac{S_i - S_{P<0.5}}{t_{P<0.5} - t_i} \quad (3.7)$$

where S_i and S_f represent the phosphorus concentrations (mg P L⁻¹) at the initial (t_i) and final (t_f) instants of the cultivation period, respectively, and $S_{P<0.5}$ corresponds to the phosphorus concentration (mg P L⁻¹) at the instant at which phosphorus concentration reached the most demanding limit for phosphorus discharge defined by APA in the environmental permit of the paper industry company (0.5 mg P L⁻¹).

Also, with the values of average biomass productivity and average removal rate, both determined for the time interval at which phosphorus concentration reached 0.5 mg P L⁻¹, specific biomass yields ($Y_{X/P}$, g_{DW} g_P⁻¹) were calculated according to Equation 3.8:

$$Y_{X/P} = \frac{P_{tp < 0.5}}{RR} \quad (3.8)$$

This value represents the amount of biomass that is produced, per amount of substrate (i.e., phosphorus) consumed.

3.2. Techno-economic and sustainability assessment of a microalgal-based biorefinery

This study proposes a *C. vulgaris* biorefinery for biofuels production using a paper industry effluent as culture medium, thus promoting both biofuels production and wastewater treatment. To design the infrastructure and study the viability and sustainability of this project, a TEA was performed. This TEA framework is structured in the following steps:

- (i) Microalgal-based biorefinery location: in this step, the factors affecting microalgal growth are addressed, and the location characteristics are also presented to explain the rationale for site selection to construct this infrastructure.
- (ii) Process flowsheet description: the second step of this section presents the process flow diagram, describing all the production steps and input and output streams.
- (iii) Scenarios description: the base case scenario was firstly defined with respect to some of the parameters that mostly influence the biorefinery performance. Then, this section presents the other scenarios, highlighting the differences in relation to the base one.
- (iv) Mass and energy balances: the fourth and fifth stages of this TEA are the mass and energy balances of the process plant. An excel-based spreadsheet model was used to accomplish these balances.
- (v) Economic assessment: during this step, using the same excel spreadsheet, the economic feasibility of the project was assessed, taking into account all the revenues and costs associated with this project.

- (vi) Sustainability assessment: in this step, some environmental and sustainability indicators were determined, to better understand the contribution of this project to more sustainable development and to a bio-based economy.
- (vii) Sensitivity analysis: lastly, a sensitivity analysis was done to study the influence that each variable has on the project's viability.

3.2.1 Microalgal production facility: plant siting

As previously mentioned, microalgal growth can be affected by several factors. When growing autotrophically, microalgal growth depends on (i) nutritional factors, such as inorganic carbon source (e.g. CO₂), inorganic salts, and nutrients, like nitrogen and phosphorus; and (ii) environmental factors, such as light intensity, temperature and environment pH. If biomass is grown in open reactors, which is the case of the proposed microalgal biorefinery, then biomass productivities are much more dependent on environmental factors [202]. Therefore, the plant location must be strategically chosen, considering all these aspects. The plant site should be in an area that has considerable solar light irradiation throughout the whole year and an average temperature that enables microalgal growth and target compounds accumulation. Golzary [202] showed that the optimal temperature for *C. vulgaris* growth was 30 °C. On the other hand, Converti et al. [203] demonstrated that increasing temperature from 20 to 25 °C would lead to a double increase in the lipid content, whereas an increase from 25 to 30 °C would lead to a decrease of about 9% in the lipid content. Therefore, a balance between biomass productivity and lipid content is needed. According to Panahi et al. [204], the ambient temperature in open systems should be above 15 °C. Regarding light irradiation, if it is very intensive, it may decrease lipids and PUFAs accumulation and also damage microalgae, decreasing their productivity [205]. Another challenge that open ponds poses for cultivation, caused by their high dependence on environmental factors, is water loss due to evaporation. Evaporation may affect the concentration of different compounds in water, leading to a negative impact on microalgal growth [16].

To decrease transport costs and associated emissions, it is beneficial that the algal facility is close to the industry that supplies the culture medium (i.e., paper company). For the same reasons, it is also important that this infrastructure is close to the industries that might be interested in buying the products accumulated by microalgae (which will be extracted within the biorefinery unit).

To sum up, the site selection should take into account: (i) the weather conditions; (ii) the availability of a suitable culture medium (with all the nutrients and inorganic salts required for microalgal growth); and (iii) the existence of enough land to build the microalgal production unit [206]. Considering the above-referred aspects, the proposed location for the construction of this infrastructure is Setúbal (see Figure 3.2).



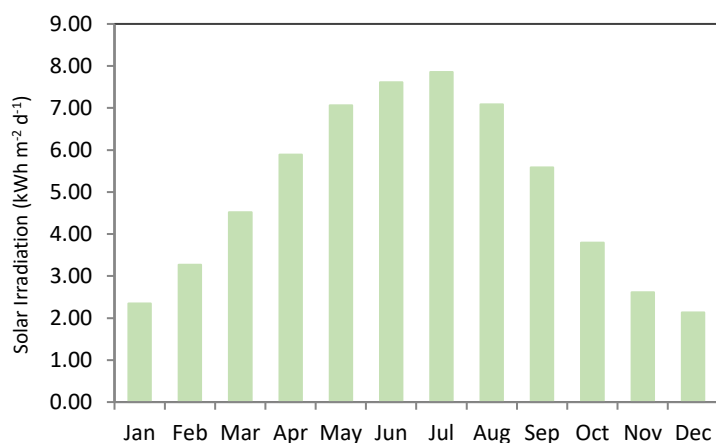
Figure 3.2. Location site proposed for the microalgal-based biorefinery (Retrieved from Google Earth).

Setúbal is a Portuguese municipality that belongs to Setúbal district and lies within the Lisbon metropolitan area. The municipality has a total area of 230.3 km² and a population density of 526.2 inhabitants per km², which leads to a total of 121 185 inhabitants, as reported in the last Portuguese census in 2011 [52]. According to data collected from the Photovoltaic Geographical Information System (PVGIS, European Commission), average horizontal solar irradiation in this area is approximately 4.98 kWh m⁻² d⁻¹ and average annual temperature is 16.8 °C. Horizontal solar irradiation per month, and maximum, minimum and average temperatures recorded per month in Setúbal, are shown in Figure 3.3. Regarding the risk of evaporation losses, Rodrigues [207] estimated evaporation rates in different reservoirs in southern Portugal, using various methods. From the nine reservoirs under study, *Pego do Altar*, *Alvito* and *Maranhão* are the closest to the site chosen for the microalgal biorefinery construction.

Through an energy balance, the author estimated the average monthly evaporation for each lagoon. With the values obtained in the three reservoirs, a weighted average was done, leading to an annual average evaporation rate of $0.075 \text{ m month}^{-1}$.

Besides the appropriate weather and light availability conditions, the plant site chosen has other advantages: (i) the presence of an agro-industrial company with a biodiesel production plant from oilseeds and to whom biofertilisers can be sold (located at less than 50 km from the biorefinery); (ii) the presence of a paper company that will supply the culture medium and CO_2 for microalgal growth and the sludge for anaerobic treatment (located at less than 5 km from the facility); (iii) proximity to water for the discharge of the treated effluent and, if necessary, to be used as an alternative culture medium (brackish water); and (iv) flat topography, which avoids the necessity for land preparation for the biorefinery construction.

A.



B.

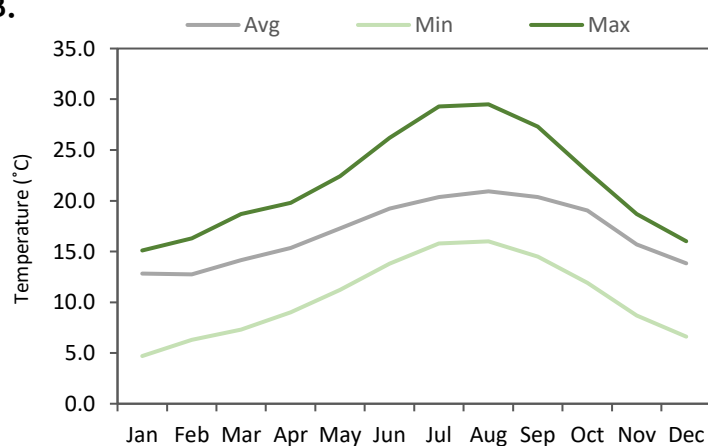


Figure 3.3. Monthly values recorded in Setúbal, Portugal, for: (A) Horizontal solar irradiation; and (B) Maximum, minimum and average temperature.

3.2.2 Process flow diagram

Despite all the drawbacks of growing microalgae in open reactors, these systems are less expensive than closed ones, are more suitable for large-scale production of microalgal biomass, and can achieve promising biomass productivities, when the selected strain is robust, and the environmental conditions are adequate for their growth (see Section 2.2.1). Taking into account these advantages, open systems were proposed in this project. As it was already mentioned, these biorefinery's main aims are: (i) to promote the treatment of an effluent resulting from the paper industry using the microalga *C. vulgaris*; and (ii) to produce lipids and biogas from the resulting biomass. Figure 3.4 shows the process flow diagram of the proposed biorefinery plant.

Microalgal cultivation

To ensure an adequate microalgal growth, it is necessary to supply the cultures with the essential nutrients for their growth, namely nitrogen, phosphorus and carbon. Nitrogen and phosphorus are available in the culture medium that enters the high rate pond (HRP) through S_{04} stream. This medium is a mixture of the effluent resulting from the paper company (S_{PE}) with the plant recycle effluent (S_{WR}). Carbon is supplied to the culture through the injection of flue gases resulting from: (i) the biomass plant of the paper company; and (ii) the combined heat and power (CHP) units from the proposed biorefinery and the paper company, where generators burn biogas to produce, simultaneously, electricity and heat. The carbon is assumed to be supplied as 100% CO_2 without any impurity that might negatively impact microalgal growth, such as sulphur oxides or nitrogen oxides [208].

Biomass harvesting

After microalgal growth, biomass leaves the HRP (S_{01}) to be harvested. The harvesting process follows the two-stage approach described in Section 2.2.2. First, there is a thickening step, flocculation, to make biomass settle faster in the clarifier and to separate it from the culture medium (S_{02}) more easily. Then, a dewatering step is followed, where biomass is centrifuged to end up with a concentration of approximately 20% (w/w) (S_{03}).

Cell disruption and lipids extraction

After biomass harvesting, lipids should be extracted from the biomass. Numerous cell disruption and products' extraction techniques, as well as their advantages and disadvantages, were presented in Section 2.2.3. For this project, PEF treatment was selected for microalgal cells disruption. This non-thermal technique was selected because it avoids the use of chemicals, has potential to be used at large

scale, does not require a dewatering process (i.e., it is suitable to treat wet or dry microalgae), has a very short treatment time, and has low energetic requirements and operational costs [129,130] (more information about this technique can be found in Section 2.2.3). Since lipids do not dissolve in water, after the cell disruption step, two phases are formed. Thus, it is possible to separate lipids from the rest of the algal slurry in a decanter. The lipids extracted (S_{05}) can then be sold to the above mentioned agro-industrial company that will transform microalgal lipids into biodiesel through transesterification.

Anaerobic digestion and cogeneration unit

The biomass resulting from the lipids' extraction step is then sent to an anaerobic digester (AD) to be stabilised (S_{06}). A part of the sludge from the paper industry wastewater treatment plant (WWTP), S_{09} , is also sent to the AD. As a result of this process, biogas (with an approximate composition of 60% CH_4 and 40% CO_2) and biofertilisers are produced. The biofertilisers stream (S_{08}) consists of stabilised biomass and effluent that can be sold to interested industries, such as the agro-industrial company that produces biodiesel. The biogas (S_{07}) is stored at a gasholder until being required at the cogeneration unit. In this unit, the biogas is burned to produce electricity and heat that can be further required to fulfil the biorefinery energy and heating needs.

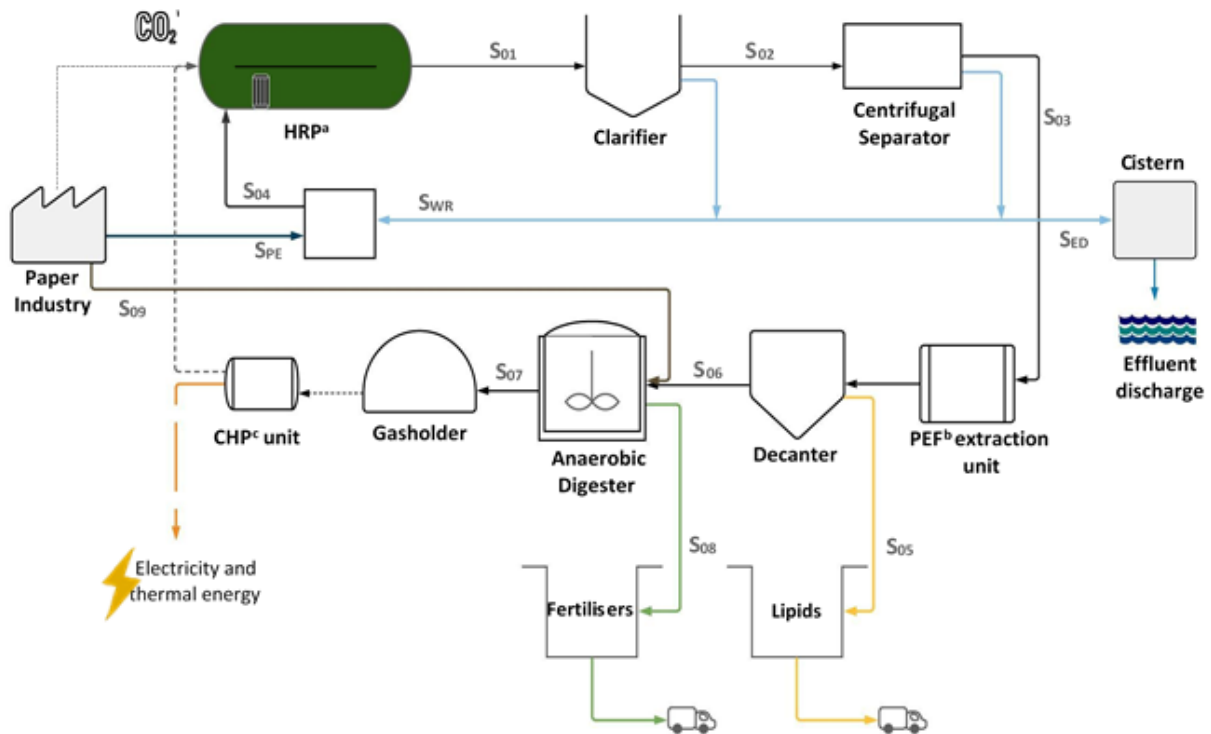


Figure 3.4. Process flow diagram; ^a High Rate Pond; ^b Pulsed electric field; ^c Combined heat and power (cogeneration) unit.

3.2.3 Scenarios description

This study considered seven different scenarios, which are characterised according to three important parameters for the biorefinery performance (Table 3.2). Despite their differences, all the scenarios share the same process diagram. Scenario 1 (defined as the base scenario) is characterised by a PE of 2%, which corresponds to a biomass productivity of $15.7 \text{ g m}^{-2} \text{ d}^{-1}$, a lipid extraction efficiency of 75% and an anaerobic digestion efficiency of 45%. Scenarios 2 and 3 are different from scenario 1 (base scenario) concerning PE, to evaluate the influence of this parameter on the biorefinery viability. In scenarios 2 and 3, PE was considered to be 1% and 3%, respectively. According to Carvalho et al. [209], in outdoor reactors, the PE values rarely exceed 6%. Other authors compared different outdoor reactors and determined that the highest PE obtained for open raceways ponds was 1.5% [79]. These values are typically low, due to different losses caused by reflection, photoinhibition, photon absorption, light saturation, among others [106]. Scenarios 4 and 5 are different from the base scenario regarding the efficiency of lipids extraction by the PEF unit. Lipids extraction efficiencies of 60% and 90% were defined in scenarios 4 and 5, respectively. Numerous studies have already presented this range of values (60-90%) for PEF extraction efficiency [210,211]. To evaluate the impact of the anaerobic digestion efficiency on the plant performance, this parameter was defined as 30% and 60% in scenarios 6 and 7, respectively. This range of values for anaerobic digestion efficiency has already been reported in the literature [212].

Table 3.2. Characterisation of the seven scenarios (Sc) evaluated in this study

Parameters	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
Photosynthetic efficiency (%)	2	1	3	2	2	2	2
Biomass productivity ($\text{g m}^{-2} \text{ d}^{-1}$)	15.7	7.80	23.5	15.7	15.7	15.7	15.7
Lipids extraction efficiency (%)	75	75	75	60	90	75	75
Anaerobic digestion efficiency (%)	45	45	45	45	45	30	60

3.2.4. Mass balance

The mass balance is a major step of the TEA. The process flow diagram and the explanation of the major steps and streams of this biorefinery plant were already described in Section 3.2.2. All calculations were performed in an Excel-based spreadsheet, and the results are presented in detail in Section 4.2.1.

Microalgal cultivation

The first step of the process, microalgal cultivation, is done in 25 equal HRPs with 0.3 m height, 60 m large and 690 m long (Figure 3.5). Therefore, the plant presents a total area of 100 ha and a total volume of 300 000 m³ for microalgal growth.

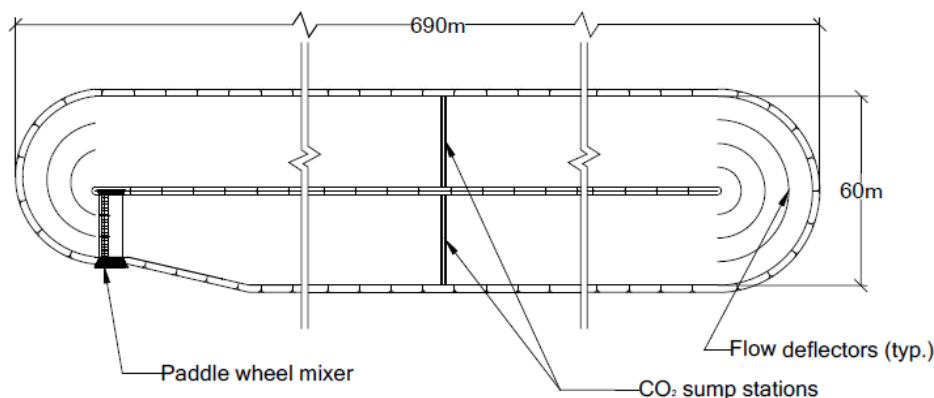


Figure 3.5. Plan view of an individual high rate pond of 4 ha [154].

Average biomass productivities estimated for these systems in each scenario are presented in Table 3.2. This parameter was calculated considering the average horizontal solar irradiation in the Setúbal area (Figure 3.3), microalgal energetic value and the PE for each scenario (1% to 3%). Microalgal energetic value was calculated assuming a lipid content of 25% (w/w), according to Chen et al. [141] and Dong et al. [132], and the remaining 75% (w/w) was assumed to correspond to carbohydrates and protein contents. Lipids energetic value was considered to be 37.5 MJ kg⁻¹ and carbohydrates and proteins energetic value 18 MJ kg⁻¹ [213]. The effluent output stream (S_{01}) was determined assuming the HRP as a continuous stirred-tank reactor (CSTR). A mass balance to this reactor was done considering stationary state and taking into account the estimated annual average biomass productivities, total cultivation area and the expected biomass concentration achieved by microalgal cultures grown in HRPs. According to Ación et al. [214], 0.5 g L⁻¹ is a typical value for this concentration in open reactors.

As previously referred, the input stream (S_{04}) consists of the paper industry effluent (S_{PE}) and the recycling effluent (S_{WR}) of the proposed biorefinery. According to data provided by the studied paper company, the effluent resulting from the secondary treatment step occurring in the company WWTP has an average concentration of nitrogen and phosphorus of 2.1 mg N L⁻¹ and 3.3 mg P L⁻¹, respectively (Annexe A.4). The environmental permit for effluent discharge for this paper industry,

admits the following values as limit values for emission: 7.0 mg N L^{-1} and 0.82 mg P L^{-1} (APA, Portuguese licensing entity, refers that there may be exceptional periods, depending on the meteorological conditions and/or quantitative and qualitative conditions of the effluent receiving environment, in which the limit value for phosphorus may even decrease to 0.50 mg P L^{-1}). According to these values, it is essential to highlight the need to develop a complementary treatment for this effluent that will allow its discharge, complying with the legal limits. However, the low nitrogen concentration present in this effluent limits its direct application as a culture medium for microalgal growth and phosphorus removal. Therefore, to ensure high biomass and target products' productivities, as well as high phosphorus removal efficiencies, it was considered that it would be necessary to add nitrogen to the paper industry effluent. In this study, the addition of fertiliser-grade ammonium nitrate (NH_4NO_3) was considered, to obtain a nitrogen concentration of approximately 36 mg N L^{-1} , which corresponds to a N:P molar ratio of 24:1.

In a first step, the input stream (S_{04}) was calculated considering the output stream (S_{01}) plus the evaporation flow, defined according to the average annual evaporation rate ($0.075 \text{ m month}^{-1}$), mentioned in Section 3.2.1. Then, the minimum concentrations of nitrogen, phosphorus and carbon required in the feed stream were calculated. To define these requirements, it was important to know the nutrients removal rates by microalgae, which were determined assuming that all the nutrients removed from the culture medium and the flue gas were integrated into the microalgal biomass. Therefore, the removal rates were determined considering the annual average biomass productivities and the molecular formula of microalgal biomass: $\text{CH}_{1.70}\text{N}_{0.10}\text{P}_{0.0004}\text{S}_{0.0009}$. This molecular formula was obtained according to the optimal nutrients ratios and molar ratios already reported in the literature [215,216].

In a second step, an optimisation process was done to determine the optimum volumes of the wastewater from the paper industry (S_{PE}) and the recycling water (S_{WR}), that is, the volumes that will allow inlet nutrients concentrations as close as possible to the minimum concentrations required for microalgal growth in the input stream (S_{04}). These flow rates can be adjusted to avoid culture dilution on rainy days.

Biomass harvesting

As mentioned in Section 3.2.2, a two-stage approach (thickening followed by dewatering) was proposed for microalgal harvesting. In the thickening step, a flocculant, sodium hydroxide (NaOH), is added to the microalgal slurry to promote pH-induced flocculation (autoflocculation). When adding NaOH , there is an increase in pH

that leads to the formation of precipitates. Then, these precipitates form large flocs with microalgal cells that can be easily separated from the medium by gravity sedimentation [208]. According to previous studies from the literature, it was assumed that 9 mg of NaOH per gram of biomass were required to induce flocculation at a pH of 10.8 [217]. Also, a concentration factor of 4 was considered for this step. Therefore, after flocculation, a biomass concentration of 2.0 g L^{-1} is expected.

The proposed dewatering step consists of centrifugation, which is assumed to lead to a concentration factor of 100. Thus, a final biomass concentration of 200 g L^{-1} (20% (w/w)) is expected after this step. Centrifugation is, indeed, the most expensive harvesting method. However, it is also one of the most suitable for large-scale processing and, when preceded by a pre-concentration step, costs can be significantly reduced (see Section 2.2.2). The overall harvesting efficiency was considered to be 95%, as reported by several authors in the literature [218,219].

With the harvesting efficiency, the biomass concentrations expected after the cultivation and harvesting steps, as well as microalgal effluent streams that leave the HRP (S_{01}), the sedimentation tank after flocculation (S_{02}), and the centrifugation unit (S_{03}) were determined for each scenario. By knowing the streams S_{02} and S_{03} , and the fraction of the recycling stream that enters again in the HRP, the water recycling stream (S_{WR}) and effluent discharge stream (S_{ED}) were also calculated.

With the above-mentioned streams already determined and knowing the paper industry effluent composition and nutrients removal rates, it was possible to determine the composition of the discharged effluent.

Cell disruption and lipids extraction

PEF method allows fast cell disruption and lipids extraction with low energy consumption (see Section 2.2.3). Knowing the flow rate that enters in the PEF unit (S_{03}), the technique efficiency (60-90%, depending on the scenario) and microalgal contents in lipids, it is possible to obtain the lipid extraction flow rate (S_{05}) and the anaerobic digestion input stream (S_{06}). Lipid content was assumed to be 25% (w/w), as previously mentioned.

Anaerobic digestion and cogeneration unit

After lipids separation, the remaining biomass follows to the AD. In this stage of the biorefinery plant, microorganisms break down organic matter, converting it into methane and carbon dioxide in the absence of oxygen. The process occurs in four different stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. When anaerobic digestion is used to break down more than one type of organic matter, co-

$$h_f = \frac{v^2 n_M^2}{R^{4/3}} \times L \quad (3.9)$$

digestion occurs. In this project, a co-digestion of microalgal biomass and sludge resulting from the paper industry wastewater treatment is proposed. Considering the input flow and its biomass concentration, as well as microalgal elemental composition after the lipids extraction ($\text{CH}_{1.70}\text{N}_{0.125}\text{P}_{0.005}\text{S}_{0.001}$) and the digestion efficiency (30-60%, depending on the scenario), nutrients removal rates were estimated. Knowing those values and the biogas composition, the resulting biogas stream (S_{07}) was determined for each scenario. Biogas was assumed to have a composition of 60% of CH_4 and 40% of CO_2 , as reported in several studies [220,221]. The remaining biomass, already stabilised, is rich in nitrogen and phosphorus. Therefore, it can be sold as a biofertiliser to interested industries. CO_2 formed in the CHP unit was determined, taking into account its fraction in the biogas stream.

3.2.5 Energy balance

Another major step of the TEA is the energy balance. As for the mass balance, all calculations were done in an Excel-based spreadsheet and results are presented in Section 4.2.2.

The stages of the biorefinery considered for this energy balance are microalgal cultivation, biomass harvesting, cell disruption and lipids extraction, for energy consumption, and the CHP unit, for energy production.

Microalgal cultivation

In this stage, only the following components are considered regarding energy consumption: open pond mixing, water pumping and blowers for flue gas injection.

The HRP is an open paddle wheel-mixed pond and is one of the most used commercial systems for microalgal biomass production. To determine the mixing energy required for microalgal growth, an average mixing velocity of 0.23 m s^{-1} was assumed, according to Lundquist et al. [222] and Milledge et al. [223]. Major and minor head losses were also calculated. Major head loss occurs due to friction resistance of the piping or open channel system [224]. In this HRP, major head loss accrues from the friction at the bottom of the pond (h_c - channel straightway head loss). Friction from the side walls of the HRP was not considered in this study. In an open channel flow, major head loss (h_f) can be determined according to Manning's equation, represented in Equation 3.9:

where v is the average mixing velocity, n_M is the Manning's roughness coefficient defined as 0.018 for clay channels [222], R is the channel hydraulic radius (m) and L is the channel length (m).

Minor head losses are local energy losses that occur due to abrupt variations in flow geometry and to the presence of appurtenances (e.g., valves, bends, inlets and pumps) [225]. For this open system, two minor head losses were taken into account: (i) head loss from flow around both 180° bends (h_b); and (ii) head loss caused by the two carbonation sumps (h_s) in each pond. These were estimated according to Equation 3.10, resulting from the Darcy-Weisbach equation (h_m - minor head loss):

$$h_m = \frac{Kv^2}{2g} \quad (3.10)$$

where K is the kinetic loss coefficient, assumed to be 1.5 for 180° bend and 4 for the carbonation sump, according to Kudela [226], and g the acceleration of gravity (9.81 m s⁻²). To determine the power required for the cultivation mixing, a paddle wheel efficiency of 40% and an average of 12 h diurnal and night period were assumed [227].

To ensure a continuous effluent supply, water has to be pumped into the ponds. The energy required for water pumping was determined taking into account the manometric head, the input flow (S_{04}), the specific effluent weight (which was assumed to be the same as water) and pump and motor efficiencies. Those efficiencies were considered to be 88% and 83%, respectively [222]. The flow leaves the ponds by gravity.

Based on the carbon requirements for microalgae in each scenario (calculated in the mass balance), and assuming a 7% (v/v) concentration of CO₂ in the flue gas, the energy required for the distribution of CO₂ was estimated [228]. The efficiency of the air blowers was considered to be 75% [229].

Biomass harvesting

The only energy requirement considered in this stage is the one for centrifugation. To determine the energy consumption, the input flow in the centrifuge (S_{02}), as well as its specific energy consumption, were considered. According to Milledge and Heaven [230], the centrifuge specific energy consumption is 1.4 kWh m⁻³.

Cell disruption and lipids extraction

Energy consumption in cell disruption stage corresponds to the energy required for the PEF unit. Specific energy consumption for this equipment was considered to be

4 kW m⁻³, as reported by Flisar et al. [231]. Knowing this value and the input stream resulting from the harvesting process (S₀₃), the energy requirement was calculated.

CHP unit

In this cogeneration unit, thermal and electrical energy are produced from the biogas formed in the AD. Energy production estimations were performed considering the gas flow rate that enters the CHP unit (S₀₇), and the biogas calorific value. According to the literature, a normal cubic meter of CH₄ has a calorific value of 10 kWh [232]. Therefore, assuming a biogas composition of 60% of CH₄, the expected calorific value is 6 kWh m⁻³ [233]. The energetic output was considered to be 40% for electrical energy and 45% for thermal energy [234].

3.2.6 Economic assessment

In this stage of the TEA, the feasibility of implementing this biorefinery plant was studied. For that, the net present value (NPV) was estimated for each scenario by taking into account annual estimated capital investment, annual production costs and the expected annual revenues from the explored products and services provided by the algal facility. Discounted payback period (DPP) and internal rate of return (IRR) were also determined for each scenario.

Capital Investment

Capital investment, or fixed capital, corresponds to the necessary investment to create the biorefinery plant. This capital includes major equipment purchase, as well as all the direct and indirect costs associated with these. All main equipment was considered in this study: the HRPs, air blowers, clarifier, centrifuge, decanter, PEF unit, AD and CHP unit. Equipment costs were determined based on values reported in the literature and updated using euro values for the year of 2019 using the Chemical Engineering Plant Cost Index (CEPCI), according to Equation 3.11:

$$\text{Cost}_2 = \text{Cost}_1 \times \frac{\text{CEPCI}_2}{\text{CEPCI}_1} \quad (3.11)$$

where 1 represents a year in time at which cost and the index value are known, and 2 represents a year in time at which index value is known, but the cost is not. Total equipment acquisition cost was assumed to account for 85% of total investment costs.

Direct costs included in this assessment are the piping system, yard improvements, equipment installation, instrumentation and control, support buildings,

switchboards, and service facilities. The indirect costs include the construction expenses, the contractor's fee, contingency, and engineering and supervision [206]. For each of these investment components, a fraction of the total equipment purchase cost was assumed. Table 3.3 shows these factors, according to information retrieved from the literature.

Production Costs

Besides major equipment costs and associated expenses, it is also important to consider costs associated with microalgal biomass production. Those can be divided into variable, fixed and other costs.

Variable costs include costs that may vary throughout the year, depending on seasonal productivity. These expenses include raw materials, the miscellaneous materials, energy required for the plant operation, the amount of flocculant (NaOH) needed for biomass thickening step, the nitrogen source (NH_4NO_3) needed to guarantee an adequate N:P molar ratio for microalgal growth and shipping and packaging. Fixed costs do not depend on productivity fluctuation during the year. These expenses include equipment maintenance, operating labour, laboratory costs, supervision, plant overheads, insurance, local taxes and royalties. For this project, other costs, such as research and development associated expenses and sales costs, were considered negligible. As for the investment costs, a percentage of the fixed capital or other costs was attributed to each of the mentioned components (Table 3.3).

Revenues

In the proposed biorefinery the following revenues were assumed: (i) lipids extracted from microalgal biomass, and sold to biodiesel production industries at 1 € kg^{-1} ; (ii) treatment of the paper industry effluent with a credit of 2.40 € per kg of phosphorus removed [235,236]; (iii) steam and electricity production in the CHP unit and sale at 0.14 € kWh^{-1} [236,237]; (iv) treatment of the sludge from the paper industry WWTP at 25 € ton^{-1} ; (v) biofertiliser production in the anaerobic digestion stage, sold at 0.40 € kg^{-1} [238] and (vi) CO_2 uptake for microalgal growth with a credit of 30 € t^{-1} [236,239].

Viability

After determining expenses and incomes, the project investment analysis was done considering a project time of 30 years. Inflation, company taxes and working capital needs were taken into account. Inflation was assumed to be 1.5%, according to the last values reported in PORDATA [240], corporate income tax (CIT) 21%, value-added tax (VAT) 10%, working capital needs 5%, and cost of capital 6% [241-243]. NPV

was calculated by adding the present values of annual cash-flows, according to Equation 3.12:

$$NPV = \sum_{i=0}^n \frac{CF_i}{(1+r)^i} \quad (3.12)$$

where, CF_i is the cash flow in the year i , and r is the interest rate. If the NPV is positive, then the project is viable because costs are lower than the net income. DPP corresponds to the number of years that takes to break even that is when the net cash flows that are generated cover the initial investment of the proposed project. In this case, the DPP must be less than 30 years for the project to be viable. However, high payback periods are typically not desired. Moreover, the payback period calculation does not take into account other factors such as risk, financing and other considerations that may be important for certain investments. IRR is the annual rate of growth that an investment is estimated to generate. This parameter is determined using the same concept as the NPV, but in this one, the NPV is set to zero. The project is viable if IRR is higher than the interest rate, so that there are more incomes than outcomes. The higher the IRR, the more attractive the project is for investors.

3.2.7 Sustainability assessment

Two sustainability parameters were determined in this project. With the energy consumption and production values, it is possible to do an energy balance for each scenario, to understand if it is required more energy than the one produced by the microalgal facility (energy obtained from biogas cogeneration and extracted lipids). Knowing the energy produced to energy consumed ratio, it is possible to calculate the energy returned on energy invested (EROEI), according to Equation 3.13:

$$EROEI = \frac{\text{Energy produced in the microalgal facility}}{\text{Total energy required}} \quad (3.13)$$

When EROEI index is higher than one, then there is more energy produced in the facility than the one consumed. Therefore, the biorefinery is energy self-sustained, which means that it does not need to buy energy from the network.

Table 3.3. Factors for estimation of project fixed capital and production cost

Item	Detail	Factor/Cost	Ref.
Investment Capital			
1	Major Equipment	0.85	
Direct Costs			
2	Installation	0.20	[238]
3	Instrumentation and control	0.15	[238]
4	Piping	0.20	[238]
5	Electrical	0.10	[238]
6	Buildings	0.15	[238]
7	Yard improvements	0.05	[238]
8	Services facilities	0.20	[238]
Indirect Costs			
9	Engineering and supervision	0.30	[238]
10	Construction expenses	0.05	[238]
11	Contractor's fee	0.03	[238]
12	Contingency	0.08	[238]
Production Costs			
Variable Costs			
1	Raw materials	Negligible	-
2	Miscellaneous materials	0.10 of Maintenance	[244]
3	Utilities	Energy values from flowsheet	-
4	NaOH (flocculant)	0.33 € kg ⁻¹	[208]
5	NH ₄ NO ₃ (Nitrate source)	0.26 € kg ⁻¹	[245]
6	Shipping and packaging	Negligible	[244]
Fixed costs			
7	Maintenance	0.05 of Fixed capital	[244]
8	Operating Labour	$\text{N}^{\circ} \text{ Operators} \times \text{Salary} \times \text{social tax} \times \text{N}^{\circ} \text{ Months}$	
9	Laboratory costs	0.20 of Operating labour	[244]
10	Supervision	0.20 of Operating labour	[244]
11	Plant overheads	0.50 of Operating labour	[244]
12	Capital charges	Negligible	-
13	Insurance	0.01 of Fixed capital	[244]
14	Local taxes	0.02 of Fixed capital	[244]
15	Royalties	0.01 of Fixed capital	[244]
Other Costs			
16	Sales expenses	Negligible	-
17	General overheads	Negligible	-
18	Research and Development	Negligible	-

Net CO₂ balance was also determined. For that, sources of consumption and production of CO₂ were determined. On the one hand, microalgae uptake CO₂ for their growth. This requirement was calculated taking into account carbon removal rate, total cultivation area and CO₂ capture efficiency, which was assumed to be 80% [222,246]. On the other hand, CO₂ is released in the CHP unit. With these two values already determined, it is possible to estimate the net CO₂ balance. If it is negative, it means that microalgae consume more CO₂ than the one that is released in the algal facility. If it is positive, the biorefinery releases more CO₂ than the one that is consumed by microalgae, which means that this project does not contribute to mitigating carbon dioxide emissions.

There are more sustainability parameters. Some measurable, but not considered in this work, and others unmeasurable. Thus, in the discussion of the results, Sustainable Development Goals (SDG) that this biorefinery will help to promote and achieve will be addressed.

Results and discussion

4.1. Paper industry effluent remediation using microalgae: laboratory-scale experiments

4.1.2 Biomass growth

The daily analysis of microalgal growth is fundamental to understand the biomass behaviour over time and to determine a set of parameters that help to characterise its growth. Those parameters can be then compared with the ones in the literature, to comprehend if the obtained results are similar to the ones reported in analogous studies. Figure 4.1 shows the biomass concentration over time for *C. vulgaris*, grown under different culture medium compositions (i.e., different paper industry effluent concentrations).

It is possible to observe that *C. vulgaris* has successfully grown in all different culture medium compositions, which indicates that the evaluated paper industry effluent did not have an inhibitory effect on biomass growth. For all cultures, the microalgal lag phase was either inexistent or very short (up to one day). However, the duration of this phase increased with the increase in the effluent percentage, being, therefore, more notorious in the cultures grown with 80% and 100% of effluent. These results seem to indicate that, although the paper industry effluent was not inhibitory for microalgal growth, a longer adaptation period was required for higher effluent loads, which may be due to [247-249]: (i) the higher colour intensity of the concentrated effluent (that can limit microalgal access to light); and (ii) the presence of lignin, humic acids, furans, dioxins, aluminium and manganese, that can slow down microalgal growth and increase the lag period. Regarding the exponential growth phase, all cultures achieved the end of this phase before day 5 of the experiment. At the end of the experiment, almost all cultures were in the deceleration or stationary growth phase.

In the assays with higher effluent percentage (60%, 80% and 100%) it is possible to observe that higher biomass concentrations were achieved. Moreover, *C. vulgaris* growth behaviour in these assays was similar to the one observed for the positive

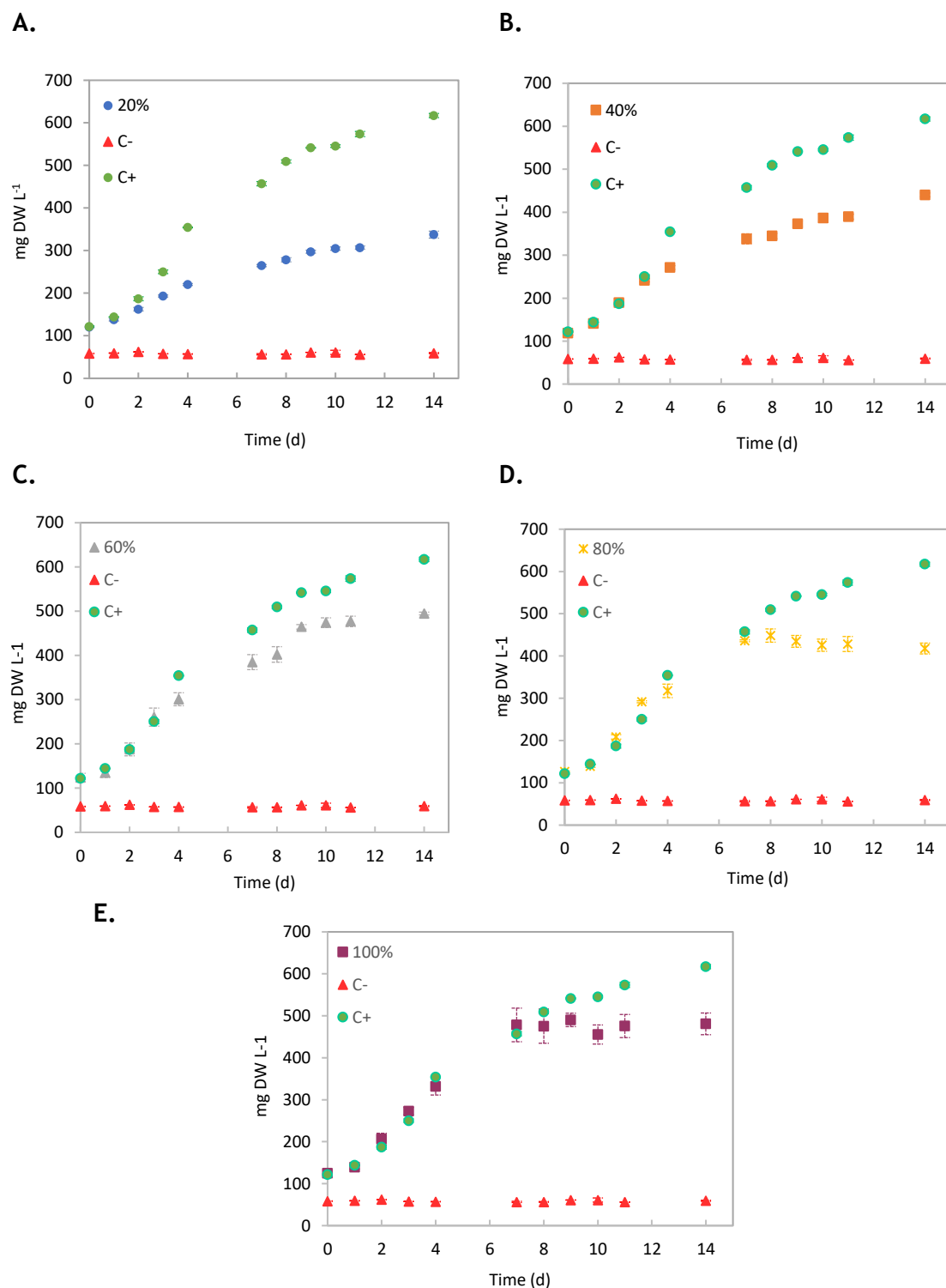


Figure 4.1. Growth curves obtained for *C. vulgaris* grown in the positive and negative controls and under different paper industry effluent compositions: (A) 20% effluent; (B) 40% effluent; (C) 60% effluent; (D) 80% effluent; and (E) 100% effluent. Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

control assay (C+). The higher biomass concentrations achieved in these three assays may be explained by the fact that these culture medium compositions have higher

nitrogen and phosphorus concentrations. With more nutrients available, microalgae grow and reproduce more and faster. For the assays with a reduced concentration of effluent from the paper company - 20% and 40% - a lower microalgal biomass concentration was achieved. This behaviour may be a result of the low nitrogen and phosphorus concentrations supplied in these conditions, as these nutrients are considered two of the main essential nutrients for microalgal growth. Nitrogen is known for their influence in the amino acids supply and protein synthesis, improving the photosynthetic, growth and respiratory rates. On the other hand, phosphorus is involved in the nucleic acid synthesis, controlling protein synthesis and cell metabolism. Therefore, the limitation of these nutrients can have a great negative effect on microalgal biomass productivity [201].

To accurately evaluate *C. vulgaris* growth in the studied effluent compositions, Table 4.1 shows the main growth parameters (μ , $X_{\max}$, $P_{\max}$, P_{avg}) determined for *C. vulgaris* cultures. Specific growth rates were determined to analyse the growth speed of the culture, under the different studied conditions (Annex A.5). This value ranged from 0.155 ± 0.005 to $0.33 \pm 0.07 \text{ d}^{-1}$, being the lowest growth rate observed for the 20% assay, and the highest for the 80% effluent assay. The specific growth rate determined for the positive control (modified OECD medium) was 0.299 d^{-1} . This parameter was not determined for the negative control (C-) because this assay was not inoculated with biomass and, as expected, no microalgal growth was observed. The specific growth rates obtained in these laboratory experiments are in line with the range of values reported in other studies from the literature. For example, Frumento et al. [250] obtained specific growth rates between 0.107 ± 0.002 and $0.289 \pm 0.027 \text{ d}^{-1}$ for *C. vulgaris* growing in a laboratory-scale tubular PBR using Bold's Basal Medium (with CO_2 , NaNO_3 and NaHCO_3 supply). Also, Sarker and Salam [251] reported values varying from 0.10 to 0.25 d^{-1} for *C. vulgaris* growing in outdoor and indoor PBRs using wastewater resulting from a WWTP as culture medium.

Regarding the maximum biomass concentration results, the assays with 60% and 100% of effluent were the ones presenting the highest value of $X_{\max}$ (495 ± 2 and $495 \pm 25 \text{ mg DW L}^{-1}$, respectively). On the other hand, the lowest value of this parameter was registered for the 20% effluent assay ($337 \pm 9 \text{ mg DW L}^{-1}$). These results demonstrate that low concentrations of nutrients (mainly nitrogen and phosphorus) limit microalgal growth, leading to lower biomass concentrations. For the positive control, the highest biomass concentration achieved was $617 \pm 5 \text{ mg DW L}^{-1}$, which shows that, although *C. vulgaris* grew well in the paper industry effluent, better results can be achieved when biomass is grown in a synthetic growth medium, such as the modified OECD test

medium. The highest biomass concentration achieved in the negative control was 63 ± 2 mg DW L⁻¹. If there were no interference in the absorbance readings and no experimental errors, it would be expected that the maximum biomass value recorded for the negative control assay would be zero. Therefore, the result obtained in this assay indicates that there is an interference in the optical density readings in the spectrophotometer that must be related to the effluent colour. Thus, it is understood that all biomass concentration values determined in this study might be slightly above the real value of absorbance, especially in the experiments with higher effluent loads (i.e., the ones that present the strongest colour).

Table 4.1. Growth parameters obtained for *C. vulgaris* grown under different effluent compositions

Assay	μ (d ⁻¹)	$X_{\max}$ (mg DW L ⁻¹)	$P_{\max}$ (mg DW L ⁻¹ d ⁻¹)	P_{avg} (mg DW L ⁻¹ d ⁻¹)
20%	0.155 ± 0.005	337 ± 9	31 ± 2	15.5 ± 0.5
40%	0.220 ± 0.007	440 ± 3	53 ± 2	23.0 ± 0.5
60%	0.27 ± 0.01	495 ± 2	73 ± 7	26 ± 1
80%	0.33 ± 0.07	448 ± 19	83 ± 8	20.8 ± 0.9
100%	0.288 ± 0.002	495 ± 25	74 ± 2	25 ± 2
Positive Control (C+)	0.299	617 ± 5	104 ± 3	35.4 ± 0.2
Negative Control (C-)	-	63 ± 2	4.6 ± 0.9	0.06 ± 0.05

Concerning maximum biomass productivity values, the highest value obtained in these experiments was 83 ± 8 mg DW L⁻¹ d⁻¹ for the 80% effluent assay, as it would be expected, given that it was also in this assay that the highest specific growth rate was achieved. Still, the $P_{\max}$ value is slightly lower than the one obtained for the positive control, 104 ± 3 mg DW L⁻¹ d⁻¹, as a result of the colour and presence of potentially inhibitory substances in the experiments dealing with real effluent compositions. Experiments with 60% and 100% effluent also showed high maximum biomass productivities (73 ± 7 and 74 ± 2 mg DW L⁻¹ d⁻¹, respectively), due to the high nutrient's availability observed in these culturing conditions. The 20% effluent test was the one that presented the lowest value for this parameter (31 ± 2 mg DW L⁻¹ d⁻¹), which is in line with the results presented so far. Different studies have reported similar values for the microalga used in these experiments [252,253]. For example, Silva et al. [254] determined a maximum biomass productivity range between 33 ± 1 and 106 ± 3 mg L⁻¹ d⁻¹ for *C. vulgaris* growth in a synthetic medium with different N:P molar ratios.

Considering the average biomass productivity, the obtained values range from 15.5 ± 0.5 to 26 ± 1 mg DW L⁻¹ d⁻¹. Contrary to what was observed for specific growth rates and maximum biomass productivities, the assays with 60% and 100% of effluent have registered the highest values of average biomass productivity: 26 ± 1 and 25 ± 2 mg DW L⁻¹ d⁻¹, respectively. Since the 80% assay has registered the highest values of specific growth rate, it reached the stationary and death phases faster, leading to negative productivities at the end of the experiment and, consequently, to lower average biomass productivities, when compared to the 40%, 60% and 100% assays. On the other hand, the result obtained for the 20% assay is consistent with the rest of the analysed results, as it was the lowest value of average biomass productivity achieved. In the positive control, there was still a significant increase in biomass concentration at the end of the 15-days experiments, meaning that *C. vulgaris* did not reach the stationary growth phase during this period. Therefore, *C. vulgaris* average biomass productivity in this control was significantly higher than those obtained in the 60% and 100% assays (35.4 ± 0.2 mg DW L⁻¹ d⁻¹).

In summary, the results obtained for the five different culture medium compositions, and the positive and negative controls, show that a culture medium with low nitrogen and phosphorus concentrations will not allow such a high and rapid biomass growth, as will a medium with higher nutrients availability. Therefore, culture media with lower effluent concentrations result in lower biomass productivities, which makes them less attractive from the perspective of biomass proliferation and accumulation of interest products. These conditions also contribute to lower specific growth rates, which leads to longer exponential and deceleration phases, preventing microalgae from achieving high concentration values, as it happens in growth media with higher nutrients concentrations.

Regarding the experiments performed with higher percentages of the effluent, it is understood that the higher availability of nitrogen and phosphorus makes microalgae grow and reproduce more and faster. Therefore, shorter exponential and deceleration phases were observed, and the stationary phase was attained earlier, due to the scarcity of the essential nutrients, such as nitrogen and phosphorus. However, there is a particularity in the obtained results. Although the highest values of μ and P_{max} were registered for the 80% effluent assays, the experiments with 60% and 100% effluent registered higher X_{max} and P_{avg} values. Considering the obtained growth curves and the calculated parameters, which demonstrated a promising growth performance without evidence of strong inhibitory effects, together with the requirements for treating the paper industry effluent, it is possible to infer that a culture medium

consisting of 100% of paper industry effluent would be suitable for *C. vulgaris* production in the studied conditions.

4.1.3 Nutrients Removal

Time-course evolution of phosphorus concentration in the different tested conditions is shown in Figure 4.2. Measurements were done based on the evaluation of phosphate-phosphorus concentrations in the culture medium throughout the cultivation experiments. Analysis of this figure demonstrates that *C. vulgaris* has successfully removed phosphorus from all tested culture medium compositions. At the end of the 15th day, the final phosphorus concentrations varied between 0.12 ± 0.01 and 0.5 ± 0.3 mg P L⁻¹. Since these values are lower than the discharge limit for phosphorus established by APA (0.8 mg P L⁻¹, or 0.5 mg P L⁻¹ at exceptional periods of the year), it is possible to confirm the potential of this species for the treatment of secondary-treated effluents resulting from the paper industry. Although the value established by APA refers to TP, it was previously mentioned that, in the effluent under analysis, TP corresponds practically only to phosphates. That is why it is possible to conclude about the potential of these microorganisms to treat this effluent in terms of phosphorus, by analysing only phosphate-phosphorus concentrations. The lowest value of phosphorus registered at the end of the experiments was obtained for the 100% effluent assay, whereas the highest was obtained at 20%. These values are in agreement with microalgal growth results. In the 20% assays, it was verified that the culture did not reach the stationary growth phase during the experimental period. However, it is known that in batch mode, biomass grows until the depletion of nutrients and that the stationary phase typically coincides with the run out of nutrients. Therefore, as biomass in 20% experiments was still growing at the end of the cultivation period, phosphorus concentrations were expected to be higher than in the assays where such phase was reached. This also explains the low phosphorus concentration registered at the end of the 100% effluent experiments. The assays with the highest concentrations of the paper industry effluent - especially the 80% and 100% - showed higher variations in phosphorus concentration over time due to the higher nutrients loads and, consequently, to the higher biomass concentrations achieved. Cultures grown in 100% effluent presented an initial phosphorus concentration closer to the one used in the positive control. This effluent property explains the similarities between the growth and phosphorus removal curves obtained for this effluent composition and the positive control.

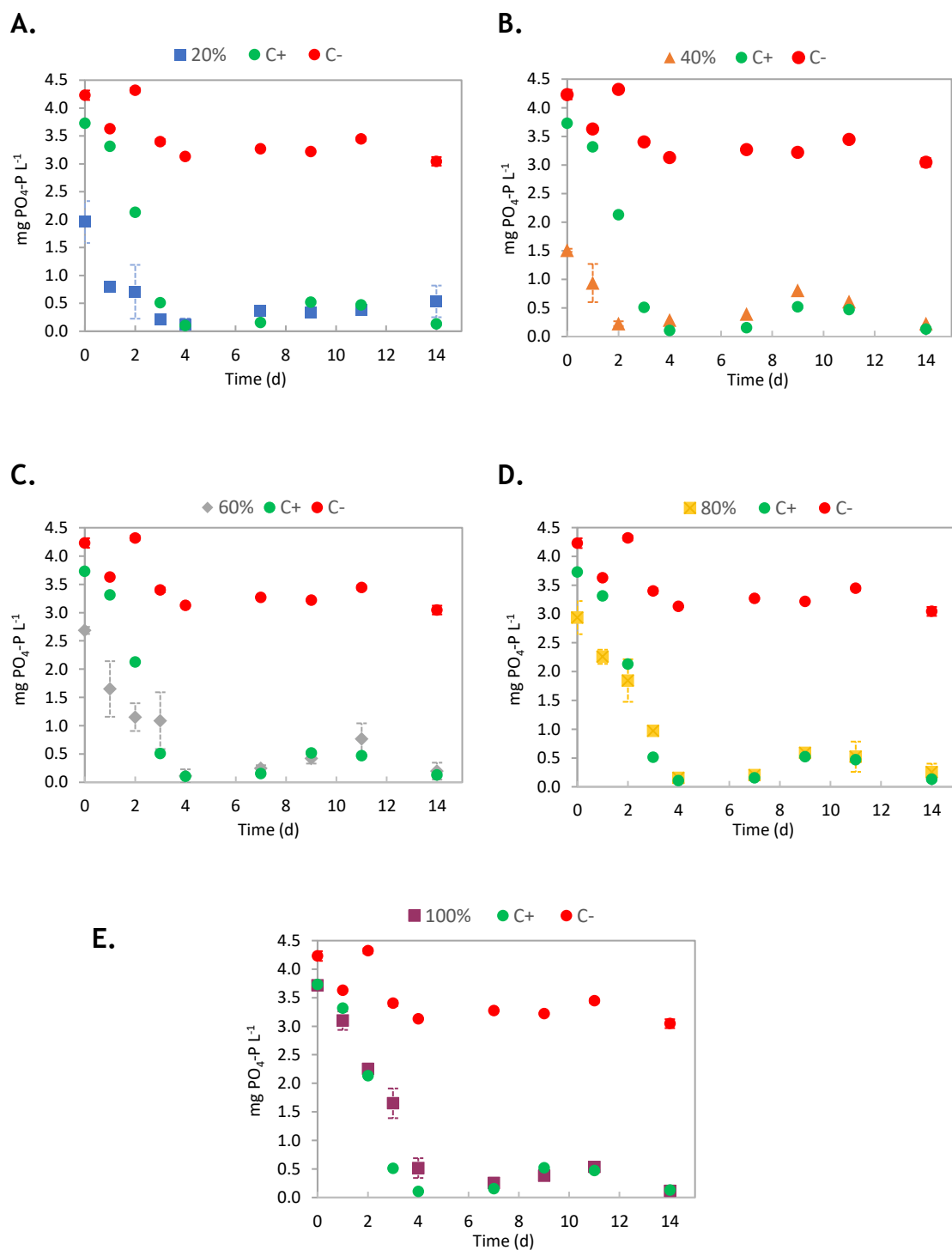


Figure 4.2. Phosphorus concentration variation over time for *C. vulgaris* grown in the positive and negative controls and under different paper industry effluent compositions: (A) 20% effluent; (B) 40% effluent; (C) 60% effluent; (D) 80% effluent; and (E) 100% effluent. Error bars correspond to the standard deviation of the mean obtained from two independent experiments.

For all assays, a similarity in the pattern of the phosphorus concentration was found. It decreases until day 4 and then there are small variations until the last day of the experience. However, the variations observed can be considered not significant,

meaning that phosphorus concentration remains approximately constant after this period.

For more accurate quantification of the potential of *C. vulgaris* for phosphorus removal from a paper industry effluent, some removal parameters were determined: RE (%), RR (mg P L⁻¹ d⁻¹), MR (mg P L⁻¹) and Y_{X/P} (g_{DW} g_P⁻¹). The results are presented in Table 4.2.

Regarding the removal efficiencies, the obtained values range between 71.6±0.2 and 96.908±0.003%, indicating that there is a substantial phosphorus removal, particularly in the experiments performed with the highest effluent loads: the highest removal efficiency was obtained for the 100% effluent assay, being this value very close to the one achieved in the positive control (96.5%). These results are in agreement with the results obtained for the growth parameters and biomass growth curves, and with the literature [255]. In general, there is a more efficient phosphorus uptake when nutrients availability is higher.

Analysing the phosphorus removal rates, achieved values range between 0.6±0.3 and 0.80±0.04 mg P L⁻¹ d⁻¹, being this range below the range reported by Silva et al. [254] (0.48-2.62 mg L⁻¹ d⁻¹). The highest value was determined, again, for the 100% effluent assay, but the lowest value for the 80% assay. The lowest value would be expected to be registered for the 20% assay. However, the range of values obtained for this parameter is relatively small, meaning that all values determined in the different culture conditions are very similar. Besides, the analysis was not made considering the initial and final instant of the experiment. Instead, it was considered the initial instant and the instant at which phosphorus concentration reached a value lower than the limit for phosphorus discharge defined by APA. For the positive control, the phosphorus removal rate was slightly higher than the one obtained for the 100% assay: 0.91 mg P L⁻¹ d⁻¹.

In what concerns the mass removal values, a higher removal of phosphate-phosphorus was observed in the experiments carried out with 100% effluent (3.60±0.06 mg P L⁻¹), being this value the same as the one registered for the positive control. These results are not surprising, due to the higher phosphorus concentrations supplied in these experiments. The similar behaviour between both conditions was also expected because both nitrogen and phosphorus were supplied in similar amounts. As it was also predictable, the lowest values of mass removal were determined in the 20% and 40% effluent assays: 1.4±0.6 and 1.3±0.1 mg P L⁻¹, respectively.

Regarding the specific biomass yields, the range of values calculated, 34 ± 7 - 70 ± 4 $\text{g}_\text{x} \text{g}_\text{P}^{-1}$ is lower than those reported by Pereira et al. [256] (20 - 150 $\text{g}_\text{x} \text{g}_\text{P}^{-1}$) and Silva et al. [254] (37.0 - 150.2 $\text{g}_\text{x} \text{g}_\text{P}^{-1}$). This parameter determines the amount of biomass that is produced per amount of phosphate-phosphorus that is consumed. The result obtained for the test with 100% paper industry effluent (64 ± 3 $\text{g}_\text{x} \text{g}_\text{P}^{-1}$) was lower than the one obtained for the 80% assay, and practically the same as the one obtained in the 60% assay: 70 ± 4 and 65 ± 6 $\text{g}_\text{x} \text{g}_\text{P}^{-1}$, respectively. These results indicate that, for the same amount of phosphorus, the cultures grown with 80% of effluent produce more biomass, and cultures grow with 60% effluent produce practically the same biomass as the ones grown with 100% effluent.

More conclusions could be drawn if it had been possible to obtain the evolution of nitrogen concentrations in the different effluent compositions, since microalgae also require high concentrations of this nutrient to grow. However, for this specific study, it is more important to evaluate phosphorus removal, since the main objective of the proposed biorefinery is to remove this element, which typically persists after the secondary-treatment step of paper industry effluents and whose concentrations should be reduced to achieve the legal limits imposed by APA.

Table 4.2. Phosphorus removal parameters obtained for *C. vulgaris* grown under different effluent compositions

Assay	RE (%)	RR ($\text{mg P L}^{-1} \text{d}^{-1}$)	MR (mg P L^{-1})	$Y_{\text{x/p}}$ ($\text{g}_\text{x} \text{g}_\text{P}^{-1}$)
20%	71.6 ± 0.2	0.7 ± 0.1	1.4 ± 0.6	34 ± 7
40%	85.08 ± 0.07	0.64 ± 0.02	1.3 ± 0.1	59 ± 1
60%	92.57 ± 0.07	0.67 ± 0.03	2.5 ± 0.2	65 ± 6
80%	90.92 ± 0.07	0.6 ± 0.3	2.7 ± 0.5	70 ± 4
100%	96.908 ± 0.003	0.80 ± 0.04	3.60 ± 0.06	64 ± 3
Positive Control (C+)	96.515 ± 0.008	0.906 ± 0.002	3.60 ± 0.03	64.18 ± 0.05
Negative Control (C-)	28.018 ± 0.004	0.0846 ± 0.0005	1.185 ± 0.007	0.7 ± 0.5

4.2 Techno-economic and sustainability assessment of a microalgal-based biorefinery

4.2.1 Mass balance

All input and output streams of the proposed biorefinery, determined in the mass balance step, are shown in Table 4.3.

The HRP output streams (S_{01}) were determined through a mass balance to the reactor, assuming that it was a CSTR, as it was mentioned in Section 3.2.4. The values obtained vary from 1.6 to $4.7 \times 10^4 \text{ m}^3 \text{ d}^{-1}$, being the lowest value registered in Scenario 2 and the highest value in Scenario 3. This is because, in Scenario 2, a PE of 1% was assumed, while in Scenario 3, PE was considered as 3%. Therefore, biomass productivity is higher in Scenario 3, and lower in Scenario 2 (Table 3.2), which results in a higher input flow in Scenario 3 to satisfy the high nutrients requirements and, on the other hand, a lower input flow in Scenario 2. In all the other scenarios, where a 2% PE was assumed, a S_{01} value of $3.1 \times 10^4 \text{ m}^3 \text{ d}^{-1}$ was obtained. The input stream (S_{04}), corresponds to the outflow (S_{01}) plus the annual average daily evaporation flow, estimated as $2500 \text{ m}^3 \text{ d}^{-1}$.

Table 4.3. Mass balance results for each scenario

Streams Description	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
S_{01} -Biomass flow rate after cultivation ($\times 10^4 \text{ m}^3 \text{ d}^{-1}$)	3.1	1.6	4.7	3.1	3.1	3.1	3.1
S_{02} -Biomass flow rate after pre-concentration ($\times 10^3 \text{ m}^3 \text{ d}^{-1}$)	7.8	3.9	12	7.8	7.8	7.8	7.8
S_{03} -Biomass flow rate after centrifugation ($\text{m}^3 \text{ d}^{-1}$)	75	37	112	75	75	75	75
Evaporation flow rate ($\times 10^2 \text{ m}^3 \text{ d}^{-1}$)	25	25	25	25	25	25	25
S_{04} -Water (medium) input in the open pond ($\times 10^4 \text{ m}^3 \text{ d}^{-1}$)	3.4	1.8	5.0	3.4	3.4	3.4	3.4
S_{05} -Extracted lipids flow rate ($\text{m}^3 \text{ d}^{-1}$)	3.2	1.6	4.8	2.6	3.9	3.2	3.2
S_{06} -Biomass flow rate after lipids extraction ($\text{m}^3 \text{ d}^{-1}$)	71	36	107	72	70	71	71
S_{07} -Biogas flow rate after anaerobic digestion (t d^{-1})	4.9	2.5	7.4	5.1	4.7	3.3	6.5
S_{08} -Residue from the anaerobic digestion (biofertiliser) flow rate (t d^{-1})	66	33	99	66	66	68	64
S_{09} -Sludge from the paper industry flow rate (t d^{-1})	55	101	8.4	54	56	55	55
S_{PE} -Paper industry effluent flow rate required for microalgal growth ($\times 10^4 \text{ m}^3 \text{ d}^{-1}$)	2.5	1.2	3.7	2.5	2.5	2.5	2.5
S_{WR} -Recycling water flow rate required for microalgal growth ($\times 10^4 \text{ m}^3 \text{ d}^{-1}$)	0.93	0.59	1.3	0.93	0.93	0.93	0.93
S_{ED} -Flow rate of the discharged effluent ($\times 10^4 \text{ m}^3 \text{ d}^{-1}$)	2.2	0.98	3.4	2.2	2.2	2.2	2.2

S_{WR} and S_{PE} streams were calculated, taking into account the minimum nutrients requirements and according to the optimisation process already described in Section 3.2.4. As it was already expected, for both streams the highest values were obtained

in Scenario 3 ($S_{WR} = 1.3 \times 10^4 \text{ m}^3 \text{ d}^{-1}$, $S_{PE} = 3.7 \times 10^4 \text{ m}^3 \text{ d}^{-1}$) due to the higher nutrients requirements for biomass growth. In contrast, the lowest values of these streams were obtained in Scenario 2 ($S_{WR} = 0.59 \times 10^4 \text{ m}^3 \text{ d}^{-1}$, $S_{PE} = 1.2 \times 10^4 \text{ m}^3 \text{ d}^{-1}$). In the base scenario, a S_{WR} flow rate of $0.93 \times 10^4 \text{ m}^3 \text{ d}^{-1}$ and a S_{PE} of $2.5 \times 10^4 \text{ m}^3 \text{ d}^{-1}$ were determined.

These results are in line with the nutrients removal rates and input concentrations obtained and summarised in Table 4.4. The theoretical nitrogen, phosphorus and carbon removal rates in the base scenario are 0.82, 0.075 and $6.8 \text{ g m}^{-2} \text{ d}^{-1}$, respectively. Carbon removal rates are higher because carbon is the major constituent of microalgae, representing 43% of biomass dry weight, according to *C. vulgaris* elemental composition ($\text{CH}_{1.70}\text{N}_{0.10}\text{P}_{0.0004}\text{S}_{0.0009}$). The RRs obtained in Scenario 2 are half of the values obtained for Scenario 1, because the PE value assumed in the base scenario is two-times higher than the value assumed in Scenario 2. The same happened with the average productivity values: 15.7 and $7.8 \text{ g m}^{-2} \text{ d}^{-1}$ in Scenarios 1 and 2, respectively. In Scenario 3, the RRs were higher, but not double, since the PE assumed in this scenario was not 4%, but 3%. These results are also in line with what was discussed regarding laboratory results (Section 4.1). Indeed, if the culture medium has a higher initial nutrients concentration, it is expected that more biomass is produced and, therefore, more nutrients are removed from the medium, resulting in higher removal rates.

Table 4.4. Removal rates determined for nitrogen, phosphorus and carbon, and minimum concentrations of these nutrients required for microalgal growth in each scenario

Nutrients Removal rates and input concentration	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
$RR_N (\text{g m}^{-2} \text{ d}^{-1})$	0.82	0.41	1.2	0.82	0.82	0.82	0.82
N (mg L^{-1})	27	25	28	27	27	27	27
$RR_P (\text{g m}^{-2} \text{ d}^{-1})$	0.075	0.037	0.11	0.075	0.075	0.075	0.075
P (mg L^{-1})	2.4	2.3	2.5	2.4	2.4	2.4	2.4
$RR_C (\text{g m}^{-2} \text{ d}^{-1})$	6.8	3.4	10	6.8	6.8	6.8	6.8
C (t d^{-1})	31	16	47	31	31	31	31

Minimum input concentrations of nitrogen, phosphorus and carbon in the feed stream were calculated considering the nutrients removal rates. As expected, the values obtained were higher in Scenario 3, and lower in Scenario 2, when compared to the base scenario. However, there is no significant variation in the necessary concentrations of N and P, between the base scenario and Scenarios 2 and 3. The reason for this is that to calculate the required N and P concentrations (in mg L^{-1}), it

was necessary to take into account the value of the biomass concentration at the outlet of the reactor. This value was assumed to be 0.5 g L^{-1} in all scenarios, regardless of the productivity value obtained in each one.

Considering the streams resulting from the harvesting steps, S_{02} and S_{03} , the values determined for the base scenario were 7.8×10^3 and $75 \text{ m}^3 \text{ d}^{-1}$, respectively. The decrease in flow rate at the end of the dewatering step results from an increase in the biomass concentration from 2 to 200 g L^{-1} . Knowing the water that was collected in these two harvesting stages, and the water that is required to satisfy the nutrients input concentrations in each scenario, it was possible to calculate the value of the discharged (S_{ED}) stream, being $2.2 \times 10^4 \text{ m}^3 \text{ d}^{-1}$ in Scenario 1.

Concerning the lipids extraction stream (S_{05}), the values obtained in Scenarios 1, 4 and 5 range between 2.6 and $3.9 \text{ m}^3 \text{ d}^{-1}$. The base scenario presents a PEF efficiency for lipids extraction of 75%. Assuming an oil density of 0.86 kg L^{-1} , the oil extraction flow rate was estimated as $3.2 \text{ m}^3 \text{ d}^{-1}$ [257]. The lowest value from this range was registered in Scenario 4 that presented a PEF efficiency of 60%. On the other hand, a higher value was obtained in Scenario 5, when compared to the other two scenarios, where a 90% extraction efficiency was assumed, leading to the logical conclusion that a higher PEF efficiency results in a higher quantity of lipids extracted, and vice-versa. Considering all the scenarios, the highest value was obtained in the third scenario ($4.8 \text{ m}^3 \text{ d}^{-1}$), due to the higher biomass productivity and, consequently, to the greater accumulation of the target product. The lowest value was determined in the second scenario, due to the opposite reason ($1.6 \text{ m}^3 \text{ d}^{-1}$). It is also possible to observe that lower PEF efficiencies, which result in lower lipids extraction, lead to higher biomass flows after the lipids extraction (S_{06}).

For the anaerobic digestion step, three different efficiencies of this process were evaluated: 45, 30 and 60% in Scenarios 1, 6 and 7, respectively. The results of the mass balance step show that the biogas flow rate was higher in Scenario 7 (6.5 t d^{-1}) and lower in Scenario 6 (3.3 t d^{-1}). Indeed, higher anaerobic digestion efficiencies result in higher biogas production and vice-versa. However, regarding the biofertiliser production (i.e., residue from the AD), the conclusions are the opposite: in scenarios with a more efficient anaerobic digestion, there is more organic matter conversion into gases and, therefore, there is less formation of residues resulting from this process (i.e., less biofertiliser). The values obtained from this flow rate, S_{08} , in Scenarios 1, 6 and 7 were, respectively, 66, 68 and 64 t d^{-1} .

It is important to highlight the limitation of this Thesis. Although the sludge from the paper industry flow rate, S_{09} , has been estimated, and the profit from its treatment accounted in the financial analysis step, this stream was not considered in the mass balance. The reason for this is that the composition and characteristics of the sludge from the paper industry are not known. Without knowing that, it is not possible to estimate the conversion rates of biomass into biogas accurately. An anaerobic reactor of 4000 m³ was considered in all seven scenarios, regardless of the value of the AD input stream (S_{06}). The amount of sludge coming from the paper industry in each scenario, was estimated through the difference between the volume occupied by the sludge produced in the biorefinery process, assuming a retention time of 30 d, and 85% of the maximum volume of the reactor (15% as a safety margin). Thus, the cost of the reactor was taken into account, as well as the revenue from sludge treatment. However, the revenue from the production of biogas and biofertilisers, due to the sludge input from an external source, has not been considered. The daily sludge mass values from the paper industry that can be treated in the anaerobic reactor in each scenario are shown in Figure 4.3. Since the same reactor volume was considered in all scenarios, in Scenario 3, the reactor has a smaller capacity to receive sludge from other sources, whereas Scenario 2 has a greater capacity. This is because in Scenario 2 the value of the S_{06} stream is lower, resulting in a lower input of biomass in the anaerobic reactor. Therefore, there is more room to receive external sludge.

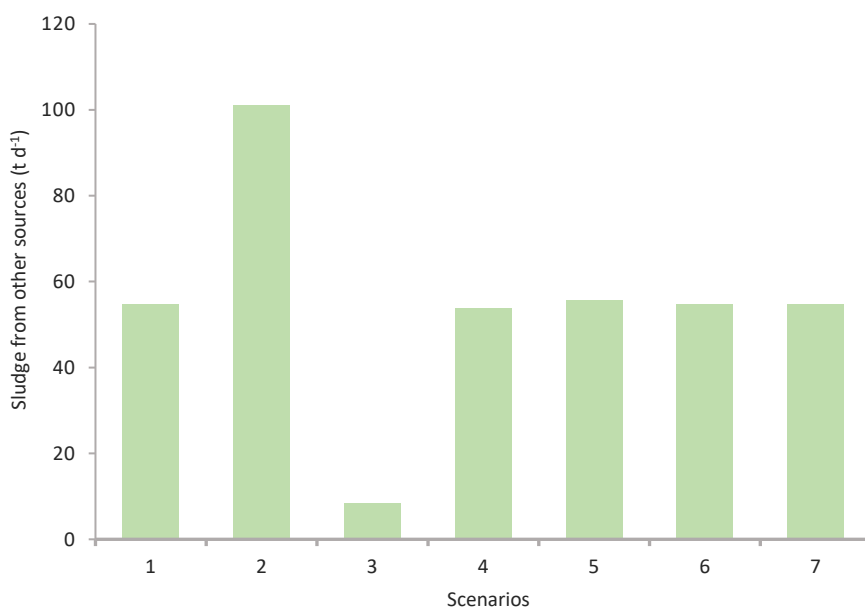


Figure 4.3. Amount of sludge that each scenario has the capacity to receive from an external source (e.g., paper industry) per day.

4.2.2 Energy balance

The microalgal facility steps assumed to require more energy, as well as the methodologies and assumptions made in the energy balance, were already mentioned in Section 3.2.5. This step of the TEA allows evaluating the electrical requirement of each of the studied scenarios and which processes have a bigger impact in terms of energy consumption. Results are shown in Table 4.5 and in Figure 4.4, for an easier visual analysis.

Table 4.5. Energy balance results for each scenario

Energy Streams	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
ER ^a in the cultivation step (x10 ³ kWh d ⁻¹)	5.8	5.0	6.5	5.8	5.8	5.8	5.8
ER in the harvesting step (x10 ³ kWh d ⁻¹)	11	5.5	16	11	11	11	11
ER in the lipids extraction step (x10 ³ kWh d ⁻¹)	7.1	3.6	11	7.1	7.1	7.1	7.1
EO ^b from the extracted lipids (x10 ⁴ kWh d ⁻¹)	3.0	1.5	4.4	2.3	3.5	3.0	3.0
Electrical EP ^c in the CHP ^d unit (x10 ⁴ kWh d ⁻¹)	0.97	0.49	1.5	1.1	0.93	0.65	1.3
Thermal EP in the CHP unit (x10 ⁴ kWh d ⁻¹)	1.1	0.55	1.6	1.2	1.0	0.73	1.5

^a Energy required; ^b Energy obtained; ^c Energy produced, ^d Combined heat and power

Analysing the obtained results, the energy requirements for the cultivation, harvesting and lipids extraction steps are the same for Scenarios 1, 4, 5, 6 and 7, being 5.8, 11 and 7.1 x10³ kWh d⁻¹, respectively. These requirements are higher in Scenario 3, and lower in Scenario 2 for the same reasons already presented in the discussion of mass balance results (Section 4.2.1). Although the PEF unit efficiencies are different between Scenarios 1, 4 and 5, no variation was observed between the results of the energy consumption in the extraction step, because the value only depends on the unit input flow (S₀₃) and on the specific energy consumption of the equipment, which was considered to be the same in all the studied scenarios.

However, regarding the energy obtained from the lipids extracted in the facility, the values in these three scenarios are already different, with a positive and negative variation of 20% between the results in Scenarios 1 and 5, and 1 and 4, respectively. The reason for that is the fact that the value of this energy requirement, in kWh per day, depends on the extracted lipid stream (S₀₅), which is higher in the scenario with greater extraction efficiency, and lower in the Scenario with reduced

efficiency. Concerning the results obtained for the electrical and thermal energy produced in the CHP unit, in Scenarios 1, 6 and 7, it is possible to conclude that there is greater energy production (electrical and thermal) in the scenario with higher anaerobic digestion efficiency (1.3 and $1.5 \times 10^4 \text{ kWh d}^{-1}$), and less energy formed in the scenario with lower efficiency (0.65 and $0.73 \times 10^4 \text{ kWh d}^{-1}$).

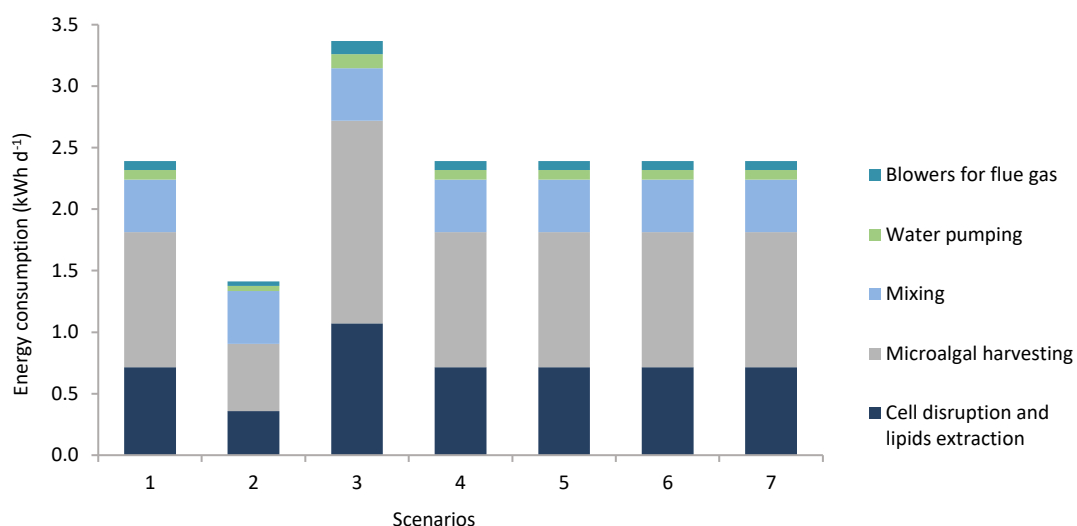


Figure 4.4. Electrical requirements in each scenario for different processes of the biorefinery.

Analysing Figure 4.4, it is easy to understand that the harvesting step, which includes the energy consumed during centrifugation, is the one with the greatest weight in terms of the energy consumed in the biorefinery, representing 46, 39 and 49% of all the energy consumed in Scenarios 1, 2 and 3, respectively. Therefore, these results confirm that, effectively, the harvesting stage represents one of the bottlenecks for microalgal biorefineries, as mentioned in the literature review (Chapter 2). The PEF unit for cel's disintegration and lipids extraction represents the second stage of the process with higher levels of energy consumption, having a weight that varies between 25 and 32% in the studied scenarios. Furthermore, it is also possible to conclude that the three stages of the microalgal cultivation step together have a lower energy consumption value, than the remaining stages considered. Therefore, the cultivation stage has the least impact at the energy consumption level, being the CO_2 supply (blowers for flue gas) and the water pumping stages the ones that require less energy, representing each 3% of the total energy required, in all the seven scenarios.

4.2.3 Economic assessment

The main constituents of the economic assessment were mentioned in Section 3.2.6. Concerning the capital investment, the acquisition costs of the equipment considered in this study are shown in Table 4.6. The total equipment cost, considering that it accounts for 85% of the total purchase cost, ranges between 7.2 and 8.4 million euros. The lowest value was determined for Scenario 2 and the highest for Scenario 3. The costs of the AD, CHP and PEF unit were considered to be the same in all the studied scenarios, regardless of the differences in biomass productivity and in the streams flowrates. This is because it was assumed that a single PEF and CHP unit would have the capacity to treat the flow ranges determined in these scenarios. Regarding the AD, the same volume was also considered, as it was previously explained in Section 4.2.1.

Table 4.6. Acquisition costs of each of the major equipment (in k€) and total purchase costs for each scenario.

Equipment	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7	Ref.
HRPs ^a	3 518	3 518	3 518	3 518	3 518	3 518	3 518	[222]
Air blowers	210	107	314	210	210	210	210	[238]
Clarifier	314	157	471	314	314	314	314	[222]
Centrifuges	514	294	808	514	514	514	514	[244,258]
Decanter	47	47	94	47	47	47	47	[238]
AD ^b and PEF ^c unit	1 414	1 414	1 414	1 414	1 414	1 414	1 414	[259]
CHP ^d unit	560	560	560	560	560	560	560	[260]
Total	6 578	6 097	7 180	6 578	6 578	6 578	6 578	-
Total purchase cost	7 739	7 173	8 447	7 739	7 739	7 739	7 739	-

^a High rate ponds; ^b Anaerobic digester; ^c Pulsed-electric field; ^d Combined heat and power

Figure 4.5 shows the graphs obtained with the values of the percentage cost of each equipment in Scenarios 1, 2 and 3. By examining these results, it is possible to conclude that the HRP is the equipment with the highest cost, having an impact of 49 to 58% on the total purchase cost. On the other hand, the decanter is the equipment with lower impact in terms of capital investment, corresponding to only 1% of the total acquisition costs in all three scenarios.

To estimate the total capital investment, direct and indirect costs associated with the equipment and physical plant construction had to be determined, according to the factors presented in Table 3.3. Results of those costs are summarised in Table 4.7. Total capital investment ranges between 18.0 and 21.2 million euros.

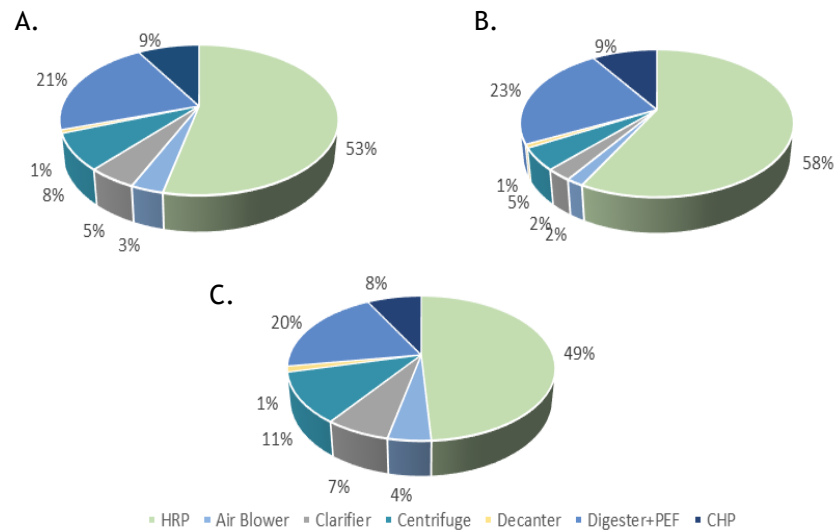


Figure 4.5. Percentage cost of each equipment for: (A) Scenario 1; (B) Scenario 2; and (C) Scenario 3.

Table 4.7. Total capital investment (in k€) determined for each scenario

Capital investment	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
Direct costs							
Total purchase cost	7 739	7 173	8 447	7 739	7 739	7 739	7 739
Equipment installation	1 548	1 435	1 689	1 548	1 548	1 548	1 548
Instrumentation and control	1 161	1 076	1 267	1 161	1 161	1 161	1 161
Piping	1 548	1 435	1 689	1 548	1 548	1 548	1 548
Electrical	774	717	845	774	774	774	774
Buildings	1 161	1 076	1 267	1 161	1 161	1 161	1 161
Yard improvements	387	359	422	387	387	387	387
Service facilities	1 548	1 435	1 689	1 548	1 548	1 548	1 548
Indirect costs							
Engineering and supervision	2 322	2 152	2 534	2 322	2 322	2 322	2 322
Construction expenses	387	359	422	387	387	387	387
Contractor's fee	232	215	253	232	232	232	232
Contingency	619	574	676	619	619	619	619
Total (k€)	19 426	18 004	21 202	19 426	19 426	19 426	19 426

This value is predominantly affected by the costs associated with the equipment acquisition and installation, piping, service facilities and engineering and supervision.

Due to the higher PE assumed in Scenario 3, this scenario presents the highest capital investment, whereas Scenario 2 presents the lowest. These results show that there is a relative increase of 20% in the total capital investment of Scenario 3, and a relative decrease of 7% in the result obtained in Scenario 2, concerning the base scenario.

Expenses associated with microalgal production were also calculated, and the results are presented in Table 4.8, being divided into variable and fixed costs. Assumptions made in the calculations were mentioned in Section 3.2.6. From all the variable and fixed costs associated with the production process, utilities present the highest impact on the value of the total expenses. That is in line with what

Table 4.8. Annual production costs, variable and fixed, (in k€) in each scenario

Production costs	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
Variable costs							
Raw materials	0	0	0	0	0	0	0
Miscellaneous materials	97	90	106	97	97	97	97
Utilities	1 221	721	1 721	1 221	1 221	1 221	1 221
Flocculant (NaOH)	17	9	26	17	17	17	17
Nitrogen source (NH ₄ NO ₃)	227	113	340	227	227	227	227
Shipping and packaging	0	0	0	0	0	0	0
Fixed costs							
Maintenance	971	900	1 060	971	971	971	971
Operating labour	32	32	32	32	32	32	32
Laboratory costs	6	6	6	6	6	6	6
Supervision	6	6	6	6	6	6	6
Plant overheads	16	16	16	16	16	16	16
Insurance	194	180	212	194	194	194	194
Local taxes	389	360	424	389	389	389	389
Royalties	194	180	212	194	194	194	194
Total (k€)	3 372	2 615	4 163	3 372	3 372	3 372	3 372

was expected, since the utilities in this study account for the electrical energy required for the well-functioning of the biorefinery. On the other hand, laboratory and supervision costs represent the smallest fraction of the production costs in all the seven scenarios. The annual production costs obtained vary from 2.6 to 4.1 million euros, being 3.4 million euros in the base Scenario.

Regarding the biorefinery revenues, the main aim of this facility is to treat the industrial effluent and produce lipids and energy. However, other profits were considered, as mentioned in Chapter 3. The results of this revenue assessment are presented in Table 4.9, and in a graphic form in Figure 4.6. By examining these results, it is noticeable that the treatment of the industrial effluent, the sale of accumulated lipids, and the sale of biofertilisers, represent the products/services that generate the most profit to the biorefinery, in almost all scenarios. Scenario 2 is an exception, with the sludge treatment representing the largest revenue. This happens due to what was previously explained in Section 4.2.1: in Scenario 2 the anaerobic reactor has more free space to receive sludge from other sources, which results in a higher profit resulting from sludge treatment. CO₂ capture represents the profit with the least impact in the annual revenues of the facility in all scenarios.

Table 4.9. Annual revenues (in k€) determined for each scenario

Revenue source	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
Lipids extraction (€/yr)	1 019	509	1 529	815	1 223	1 019	1 019
Electricity (k€/yr)	497	249	746	520	474	331	663
Steam (k€/yr)	559	280	839	585	534	373	746
Industrial effluent treatment (k€/yr)	1 148	574	1 723	1 148	1 148	1 148	1 148
CO ₂ capture (k€/yr)	186	93	279	182	190	215	157
Biofertilisers (k€/yr)	1 022	511	1 533	1 069	975	1 270	774
Sludge treatment (k€/yr)	499	922	77	492	507	499	499
Total (k€/yr)	4 932	3 138	6 725	4 812	5 051	4 857	5 007

Although Scenario 3 has the highest value of total annual revenues, and Scenario 2 the lowest, as expected, there are also small variations in the revenues generated between Scenarios 1, 4, 5, 6 and 7, resulting from the different values of the AD or PEF unit efficiency assumed. It is important to mention that the same did not happen with capital investment and production costs values.

Firstly, the annual revenue value is slightly higher in Scenario 5, when compared with Scenarios 1 and 4, due to a higher profit generated by the lipids sales, resulting from the greater amount of lipids extracted. The opposite happens in Scenario 4. The variance between the annual revenues of the base scenario and Scenarios 4 and 5 are, respectively, -2.4% and 2.4%. It should be noted that, although Scenario 4 has less profit with the lipids extraction, there is a slight increase in the biofertilisers and biogas revenues, due to the higher concentration of biomass that is sent to the AD.

Secondly, it is also possible to observe that Scenario 6 presents a lower annual revenue value when compared to Scenarios 1 and 7. The reason for that is the lower anaerobic digestion efficiency assumed in this scenario (30%), leading to a decrease in the production of electrical and thermal energy. However, an increase in the biofertilisers revenue is observed, which is in line with the conclusions already drawn in Section 4.2.1. Scenario 7 has higher annual revenue, according to the same conclusions presented above. There is a relative decrease of 1.5% in the total revenues obtained in Scenario 6, and a relative increase of 1.5% in the result obtained in Scenario 7, concerning the base scenario.

From all these five scenarios (1, 4, 5, 6 and 7), Scenario 5 is the one with the highest value of annual revenues, leading to the conclusion that the variable PEF unit efficiency has more impact in the final revenue result than the AD efficiency. Further findings on this topic are approached in the sensitivity analysis presented in Section 4.2.5.

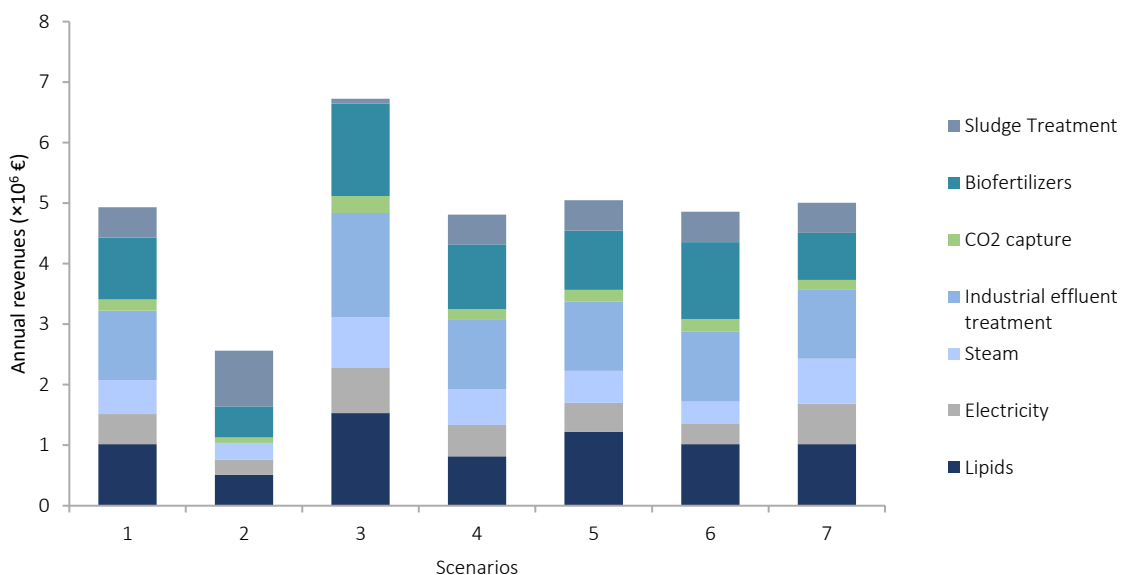


Figure 4.6. Graphical representation of the annual revenues in each scenario.

Regarding the viability analysis, the values assumed for the different financial variables were mentioned in Chapter 3 and are summarised in Table 4.10. The results of this analysis are presented in Table 4.11 for the seven scenarios.

Table 4.10. Financial parameters assumed in the viability assessment

Parameter	Assumed value
VAT ^a (%)	10
CIT ^b (%)	21
Inflation (%)	1.5
Cost of Capital (%)	6
Working Capital needs (%)	5
Project lifetime period (yr)	30

^a Value added tax; ^b Corporate income tax

Table 4.11. Economic viability results for each scenario

Parameter	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
NPV ^a (k€)	5 070	-7 411	15 375	3 699	6 440	4 210	5 929
IRR ^b (%)	8	3	12	8	9	8	9
PP ^c (yr)	13	29	9	14	12	13	12
DPP ^d (yr)	23	>30	13	27	20	25	21

^a Net present value; ^b Internal rate of return; ^c Payback time; ^d Discounted payback time

Analysing the results obtained in the economic viability evaluation, it is perceptible that from all the studied scenarios, only Scenario 2 presents a negative NPV. On the other hand, Scenario 3 shows the highest NPV, with a relative increase of 203% concerning the base scenario. As previously mentioned, if the NPV is negative, it means that the net income is inferior to the total costs, leading to an economically unviable project, and vice-versa. Nevertheless, to conclude about the feasibility of the project, it is important to consider other financial parameters.

Concerning the cost of capital, it was assumed to be 6%, as explained in Section 3.2.6. However, this variable is hard to define, since it must consider factors such as investment risk. The risk of investing in the proposed project may be considered high since it is a 30-year project. Hence, in such a long period, a lot can change, and a

project that today seems to be viable, may not be in the future if unpredictable events or sudden changes in the economy occur. These evaluation limitations must be considered when concluding on the viability of the biorefinery project.

Regarding the payback time results, PP is always lower than DPP because, in the first one, the returns required by investors (cost of capital) are not taken into account. Therefore, the analysis of these results will be done, considering only the DPP values. By analysing these results, Scenario 3 is the one in which the project is paid in a shorter period (13 years) and, in contrast, in Scenario 2, the biorefinery is not even paid over the project lifetime (>30 years). From the other five scenarios, Scenario 4 is the one that presents the worst estimate for the time needed to pay the initial investment (27 years), as expected considering that it was also in this scenario that the lowest value of annual revenue generated was recorded. On the other hand, Scenario 5 shows the best result being paid 3 years earlier than the project defined in the base scenario. Nevertheless, although the NPV was positive in 6 scenarios, the time necessary to pay the project can be considered too long. Even in the best scenario, it would take 13 years for the project to start making profit, which, from an investor's point of view, can make the project less appealing and riskier.

With regard to IRR values, as previously stated, the project is feasible if the IRR is higher than the defined cost of capital, meaning that the annual revenues are enough to pay the capital of investment and the required return. Only Scenario 2 showed an IRR value lower than the cost of capital (3%). Scenario 3 presents the highest rate of return, being double the cost of capital (12%).

After analysing the results of these financial parameters, it can be concluded that this biorefinery project would be viable in all analysed scenarios, except for Scenario 2, under the conditions that were established in this study. Yet, it is understood (by the number of years required for the project to start making profit, by the project lifetime, and by the internal rates of return determined) that, even in the best scenario, the project may not be attractive enough from an economic point of view for possible shareholders.

4.2.4 Sustainability assessment

The results of the two sustainability parameters are shown in Table 4.12.

Concerning the EROEI results, they can be analysed in two different ways: (i) considering only the energy produced in the biorefinery; and (ii) considering the energy

that is produced in the industrial plant, as well as the energy that can be obtained from the main exploited product, lipids.

Table 4.12. Net CO₂ balance and energy returned on energy invested results for each scenario

Parameter	Sc 1	Sc 2	Sc 3	Sc 4	Sc 5	Sc 6	Sc 7
CO₂							
CO ₂ required in the cultivation step (t d ⁻¹)	31	16	47	31	31	31	31
CO ₂ resulting from CHP ^a generation (t d ⁻¹)	7.9	4.0	11.9	8.3	7.6	5.3	10.6
Net CO ₂ balance (t d ⁻¹)	-17	-8.5	-26	-17	-17	-20	-14
Energy balance							
Energy produced in the CHP unit (x10 ⁴ kWh d ⁻¹)	2.1	1.0	3.1	2.2	2.0	1.4	2.8
Total energy produced from the biorefineries exploited products (x10 ⁴ kWh d ⁻¹)	5.0	2.5	7.6	4.6	5.6	4.3	5.8
Energy consumed (x10 ⁴ kWh d ⁻¹)	2.4	1.4	3.3	2.4	2.4	2.4	2.4
EROEI ^b	0.87	0.73	0.92	0.91	0.83	0.58	1.2
EROEI ^c	2.1	1.8	2.3	1.9	2.3	1.8	2.5

^a Combined heat and power; ^b Energy returned on energy invested, considering only the energy produced in the CHP unit; ^c Energy returned on energy invested, considering energy produced in the CHP unit and energy obtained from the extracted lipids

Looking at the results of the first case, the value of this parameter is lower than 1 in all scenarios, except for Scenario 7, due to the higher efficiency of the anaerobic digestion assumed (60%). Therefore, the proposed project would only be energetically efficient in one of the studied scenarios, promoting a self-sustained biorefinery. In the other scenarios, it would be necessary to buy energy from the network to fulfil the energy needs of the plant. In the worst scenario, Scenario 6, where a low efficiency of the AD was assumed (30%), approximately 10 000 kWh from the energy network would be needed per day.

Regarding the second case, a more favourable situation was observed. Although the obtained results do not allow to conclude about the project's self-sufficiency or energetic performance, EROEI values determined in this case were higher than 1 in all studied scenarios. These results demonstrate that this biorefinery can promote the production of clean energy in greater quantities than what it consumes, even in Scenario 2, which always presented the worst results in the other assessments. Scenario 7, with an EROEI of 2.5, demonstrated to have the best capacity to promote energy production. The reason why the results of this second case are not so accurate is that the energy obtained from the extracted lipids is being accounted, but the

energy consumed outside the biorefinery boundaries in the transesterification of lipids into biodiesel is not.

Concerning the net CO₂ balance, this parameter is negative in all the studied scenarios, meaning that there is more consumption of CO₂ by microalgae than the one that is released in the biorefinery processes. The scenario with the highest PE, Scenario 3, has higher biomass productivity and, therefore, requires more CO₂, resulting in a higher value of the net CO₂ balance in absolute terms. Since all scenarios have a negative value, an external source of carbon is required to satisfy the biomass carbon needs. In this case, the flue gases resulting from the paper industry biomass power plant were considered as the external source of carbon dioxide.

For the calculation of this parameter, the CO₂ released by microalgae during the night, when there is no light to carry out photosynthesis, was not considered. That is because, for the calculation of biomass productivity, only the daytime period was considered. Thus, it was assumed that if the productivity in the absence of sunlight was not counted, then the CO₂ released in the night period would not be counted either. Moreover, the biorefinery also needs to consume energy from the network in 6 of the 7 scenarios, as mentioned above. The CO₂ released from burning fossil fuels in the electricity production process was also not considered for the calculation of this net balance. Though, these are limitations of this study.

From a sustainability point of view, it is also important to highlight other benefits of this project that may be intangible and/or unmeasurable, but add value to it. Projects like this, in which less carbon dioxide is released than consumed, and where clean energy is produced, are essential to mitigate climate change and help meet the targets set in the Roadmap for Carbon Neutrality 2050 [261]. They also contribute to a more circular economy, where the waste from one industry is the raw material for another: wastewater from the paper industry becomes the culture medium for microalgae, and the sludge generated in the two industries becomes the raw material for the production of biogas and biofertilisers. These fertilisers, with a high concentration of nitrogen and phosphorus, return these nutrients to the soil, promoting their regeneration. Furthermore, this project solves an environmental problem by allowing the discharge of the industrial effluent within legal limits. Thus, avoiding an excessive increase in the concentration of phosphorus in the surrounding aquatic environment and, consequently, preventing eutrophication.

The local community also benefits from the construction of this microalgal facility, since it promotes the creation of new jobs with reasonable remuneration

conditions: 1000 euros per month, 14 months a year. Besides, this infrastructure would not pose a risk to the community or environment health, unlike oil refineries [262]. With all the above mentioned advantages, it can be concluded that this industrial plant would enhance the impact on the SDGs and help reach the proposed targets established by the United Nations [263]. Figure 4.7 shows the seven goals that this biorefinery would help to promote, being them:

- SDG 7: Ensure access to affordable, reliable, sustainable, and modern energy for all;
- SDG 8: Promote sustained, inclusive and sustainable economic growth, full and productive employment and decent work for all;
- SDG 9: Build resilient infrastructure, promote inclusive and sustainable industrialisation and foster innovation;
- SDG 11: Make cities and human settlements inclusive, safe, resilient and sustainable;
- SDG 12: Ensure sustainable consumption and production patterns;
- SDG 13: Take urgent action to combat climate change and its impacts;
- SDG 14: Conserve and sustainably use the oceans, seas and marine resources for sustainable development.



Figure 4.7. Sustainable Development Goals that the proposed project will help to enhance.

4.2.5 Sensitivity analysis

Considering the NPV obtained in each scenario, a sensitivity analysis was done to study the influence that each of the three variables has on the final result of the project's viability. The results are shown in Figure 4.8.

Analysing the graph, it is perceptible that PE is the variable that has the greatest impact on the project NPV. The value of this parameter ranges between -7.4 to 19.5 million euros, for PEs of 1 and 3%, respectively.

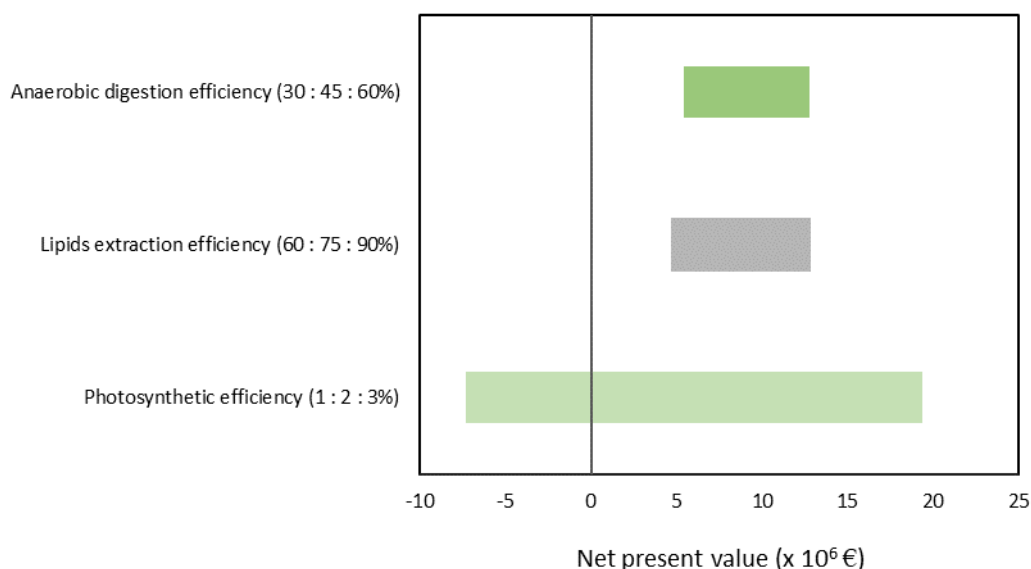


Figure 4.8. Sensitivity analysis of the proposed biorefinery, considering the variables changed between the studied scenarios.

With regard to the other two variables, lipids extraction efficiency has a slightly greater influence on the economic viability of the project, than the anaerobic digestion efficiency. Indeed, a greater oscillation in the NPV was observed in the scenarios assuming a PEF unit efficiency of 60, 75 or 90%: 4.7 to 8.2 million euros. On the other hand, for anaerobic digestion efficiencies between 30 and 60%, the NPV varies from 5.3 to 7.5 million euros. This conclusion had already been drawn when discussing the annual revenues of each scenario in Section 4.2.3. For instance, Scenario 5, with a higher PEF extraction efficiency, presented higher annual revenues than Scenario 7, with the highest anaerobic digestion efficiency.

Concluding remarks and future work

5.1 General conclusions

The main aim of this Thesis was to project and study the viability of an energy-driven microalgal biorefinery to remove excess phosphorus from a paper industry effluent and produce biofuels. The study was divided into two parts: (i) a laboratory step to evaluate the growth behaviour of the microalga *C. vulgaris* in the industrial effluent, as well as its ability to remove nutrients from this culture medium (to obtain concentrations under the limits imposed in legislation); and (ii) a techno-economic analysis to design the industrial plant and conclude on the feasibility of the project.

In the laboratory step, the growth of *C. vulgaris* was evaluated in different percentages of the paper industry's effluent (varying between 20 and 100%). The results showed that the industrial effluent did not have an inhibitory effect on *C. vulgaris* growth. In fact, the assays with higher concentrations of the industrial effluent, showed the best results: (i) in the 80% test, biomass reached the highest specific growth rate ($0.33 \pm 0.07 \text{ d}^{-1}$); (ii) in the 60% and 100% tests, the highest biomass concentration values were registered (495 ± 2 and $495 \pm 25 \text{ mg DW L}^{-1}$, respectively); and (iii) the highest average biomass productivity value was also recorded in the 60% test ($26 \pm 1 \text{ mg DW L}^{-1} \text{ d}^{-1}$). Nevertheless, a longer adaptation phase was observed in the assays with higher effluent loads, and better results were determined for the positive control assay, leading to the conclusion that the paper industry effluent may have characteristics that slow down microalgal growth. On the other hand, in the 20% assay, the lowest values of the growth parameters under analysis were determined, leading to the conclusion that culture media with low nutrients concentrations may limit microalgal growth.

In terms of phosphorus removal, the results were satisfactory for all the assays. Phosphorus final concentrations on each assay ranged between 0.12 ± 0.01 and $0.5 \pm 0.3 \text{ mg P L}^{-1}$. These values are below the legal limits imposed by APA for the paper industry (0.82 , or 0.50 mg P L^{-1} in occasional critical situations), showing that the microalga *C. vulgaris* can efficiently treat this effluent.

Regarding the removal efficiencies, the results varied between 71.6 ± 0.2 and $96.908 \pm 0.003\%$, increasing with the increase of the industrial effluent in the culture medium. In terms of phosphorus mass removal, the highest value was also determined in the 100% assay ($3.60 \pm 0.06 \text{ mg P L}^{-1}$), whereas the lowest value was determined in the 40% assay ($1.3 \pm 0.1 \text{ mg P L}^{-1}$). Therefore, it is possible to conclude that culture media with higher initial phosphorus concentrations promote higher biomass productivities and, hence, higher nutrients uptakes.

From this experimental study, the most important conclusions to draw are: (i) the paper industry effluent does not actually have a toxic or inhibitory effect on the microalga *C. vulgaris*; (ii) this effluent can effectively serve as a culture medium in the proposed biorefinery; and (iii) *C. vulgaris* can effectively treat this effluent, complying with the legal limits imposed to the company.

In the TEA step, the energy-driven biorefinery was dimensioned, through the mass and energy balances, and the economic viability of the project was studied, as well as its performance at a sustainability level. Seven possible scenarios were considered in this assessment, where different efficiencies in some of the processing stages were assumed. Analysing the economic viability of the project, considering only the NPV and IRR values, only one of the seven studied scenarios was not considered feasible. This was the Scenario 2, where a PE of only 1%, a PEF extraction efficiency of 75%, and an anaerobic digestion efficiency of 45% were assumed, resulting in a NPV of - 7.4 million euros, and an IRR of 3% (which was lower than the established cost of capital, 6%). On the other hand, Scenario 3 showed the best economic results, with a NPV of 15.4 million euros and a 12% IRR. In this scenario, a 3% PE, a PEF extraction efficiency of 75%, and an anaerobic digestion efficiency of 45% were considered. With the sensitivity analysis, it was understood that the variable that has the most influence on the final value of NPV is the PE, followed by the efficiency of the PEF unit and, finally, the anaerobic digestion efficiency.

There are, however, other financial and non-financial parameters essential to take into consideration, when concluding about the viability of a project. Firstly, the proposal was projected for a period of 30 years. The fact that it is such a long period, which increases the likelihood of unpredictable events and significant changes in the economy, increases the investment risk for shareholders.

Secondly, the obtained DPP results are not very satisfactory. Scenario 2, as expected, presents the worst result, where the initial investment is not possible to pay during the estimated project lifetime, making it completely unfeasible. In contrast,

Scenario 3, shows the shortest period, 13 years, required to pay the capital investment and start making profit. Yet, even in this Scenario the payback time may be considered too long, making the project less appealing for possible shareholders.

Nevertheless, there are also a series of benefits that this biorefinery presents, especially at the sustainability level, that add a lot of value and interest to it. Some of these benefits are not possible to count directly in the financial assessment, such as in the NPV calculation, as they are intangible and/or immeasurable. Regarding the net CO₂ balance, despite the limitations presented, it was negative in all scenarios, ranging from -8.5 to -26 t d⁻¹, in Scenario 2 and 3, respectively. These results show that there is less release of CO₂ in the CHP unit, than consumption in microalgal ponds. In the scenario with the best results, Scenario 3, there is an uptake of 1.7x10⁴ t of CO₂ per year. This is one of the greatest advantages of this project at an environmental level: the contribution to decarbonisation and, consequently, the reduction of GHGs emissions and the mitigation of the effects of climate change.

Additionally, this industrial plant also promotes the production of clean and renewable energy, helping to minimise the consumption of energy derived from fossil fuels, at a Nacional level. Although the value of the EROEI parameter, considering only the energy produced in the biorefinery, is lower than 1 in six of the seven scenarios, when the energy that will be produced from the extracted lipids is also taken into account, this value is already greater than 1 in all scenarios. Therefore, the biorefinery promotes the generation of more energy than the one that is consumed. Scenario 7 is the most efficient in terms of energy, obtaining an EROEI value of 1.2, even when only the energy produced in the CHP unit is taken into consideration. Scenario 3, however, shows the highest values of energy produced: 1.1x10⁷ kWh yr⁻¹ in the CHP unit, and 1.6x10⁷ kWh yr⁻¹ from the extracted lipids (1.7x10³ m³ yr⁻¹).

The proposed project also promotes a more circular economy, with the production of biofertilisers from sludge, which returns nutrients to the soil (recovery), and with the use of wastewater as a culture medium. In the best studied scenario, 3.5x10³ t of biofertilisers are produced, and 1.3x10⁷ m³ of industrial effluent are treated annually. The biorefinery capacity to treat paper industry effluent allows discharging the effluent into the environment without harming aquatic living beings and without contributing for environmental issues, such as eutrophication.

To sum up, the viability of a project should not be evaluated with a financial analysis alone. The proposed biorefinery presented a positive NPV in 6 of the 7 scenarios, but payback time values are over 13 years. Yet, all the innumerable benefits

presented, some immeasurable, show that this is an innovative and sustainable project that would certainly attract the attention of many shareholders, as well as the Portuguese government and European Union who may possibly contribute with incentives.

5.2 Research needs

This study has demonstrated the ability of the microalga *C. vulgaris* to grow in a paper industry effluent and to efficiently remove phosphorus from this culture medium. To broaden the laboratory approach of this study, some further analysis could be developed, namely: (i) evaluate nitrogen concentrations throughout the experimental process and determine the removal parameters for this nutrient; (ii) run a similar experience, but with different microalgal species, to compare the growth kinetics and nutrients removal parameters and determine which species shows best results in this culture medium; (iii) develop similar experiences, but adding more parameters to the analysis, such as COD concentration and lipid content over time; and (iv) evaluate the influence of different operational conditions (e.g., light intensity, different N:P molar ratios, and different CO₂ concentrations in the injected air) on microalgal growth and nutrients uptake performance.

Regarding the TEA, more scenarios may be developed, based on the ones presented in this Thesis, to further improve the analysis. To study possible economies of scale, at least one scenario would be interesting to analyse, namely with a larger area, for example 400 ha, with the capacity to treat a larger volume of effluent. The harvesting step was the processing stage with the greatest impact in terms of energy consumption. Therefore, an alternative approach, through a different dewatering method, with lower energy consumption than centrifugation (e.g., filtration) could be considered [101]. To assess the impact of the limitation considered in the net CO₂ balance, it would be important to have another scenario, in which the productivity would be calculated for a 24-hour period (considering day and night), and the carbon dioxide released at night by microalgae would be considered in the CO₂ balance. By including these new scenarios, a complete sensitivity analysis may be achieved.

To further improve and complete the TEA done in this study, different digesters' volumes could be considered for each scenario, depending on their volumetric treatment capacities. Furthermore, the flow of sludge from external sources should be included in the mass balance.

The sustainability assessment included in this work can also be further improved with the determination of more sustainability indicators and/or the application of environmental decision tools. When carefully chosen, indicators may help policymakers to support decision-making and track progress [264]. Sustainable performance is probably the most important component to positively distinguish microalgal biorefineries from traditional oil refineries. Therefore, indicators, such as N&P loadings, water safety, employment extent, and sustainable land-use, should be calculated and analysed [265]. Finally, to compare the performance of the proposed biorefinery with other existing infrastructures, a life-cycle assessment (LCA) of the oil produced in this biorefinery could be done, and then compared with the LCA of the oils produced in other biomass refineries and in the traditional oil refineries.

References

1. Koyande, AK, Show, P-L, Guo, R, Tang, B, Ogino, C and Chang, J-S, *Bio-processing of algal bio-refinery: a review on current advances and future perspectives*. Bioengineered, 2019. 10: p. 574-592.
2. EPA. *Global Greenhouse Gas Emissions Data*. 2014 [cited 2020 22 of May]; Available from: <https://www.epa.gov/ghgemissions/global-greenhouse-gas-emissions-data>.
3. Mata, TM, Martins, AA and Caetano, NS, *Microalgae for biodiesel production and other applications: A review*. Renewable and Sustainable Energy Reviews, 2010. 14: p. 217-232.
4. Owusu, P and Asumadu Sarkodie, S, *A Review of Renewable Energy Sources, Sustainability Issues and Climate Change Mitigation*. Cogent Engineering, 2016. 3: p. 1167990.
5. OECD, *Green Growth Studies - Energy*. 2011, OECDpublishing.
6. Aydın, M, Uslu, S and Bahattin Çelik, M, *Performance and emission prediction of a compression ignition engine fueled with biodiesel-diesel blends: A combined application of ANN and RSM based optimization*. Fuel, 2020. 269: p. 117472.
7. Demirbas, MF, *Biofuels from algae for sustainable development*. Applied Energy, 2011. 88: p. 3473-3480.
8. Venkata Mohan, S, Hemalatha, M, Chakraborty, D, Chatterjee, S, Ranadheer, P and Kona, R, *Algal biorefinery models with self-sustainable closed loop approach: Trends and prospective for blue-bioeconomy*. Bioresource Technology, 2020. 295: p. 122128.
9. Ribeiro, LA and Pereira da Silva, P, *Technoeconomic Assessment on Innovative Biofuel Technologies: The Case of Microalgae*. ISRN Renewable Energy, 2012. 2012: p. 173753.
10. Simsek, S and Uslu, S, *Comparative evaluation of the influence of waste vegetable oil and waste animal oil-based biodiesel on diesel engine performance and emissions*. Fuel, 2020. 280: p. 118613.
11. Venu, H, Subramani, L and Raju, VD, *Emission reduction in a DI diesel engine using exhaust gas recirculation (EGR) of palm biodiesel blended with TiO₂ nano additives*. Renewable Energy, 2019. 140: p. 245-263.

12. Scaldaferri, CA and Pasa, VMD, *Production of jet fuel and green diesel range biohydrocarbons by hydroprocessing of soybean oil over niobium phosphate catalyst*. Fuel, 2019. 245: p. 458-466.
13. Lardon, L, Hélias, A, Sialve, B, Steyer, J-P and Bernard, O, *Life-Cycle Assessment of Biodiesel Production from Microalgae*. Environmental Science & Technology, 2009. 43: p. 6475-6481.
14. Viccaro, M, Cozzi, M, Rocchi, B and Romano, S, *Conservation agriculture to promote inland biofuel production in Italy: An economic assessment of rapeseed straight vegetable oil as a self-supply agricultural biofuel*. Journal of Cleaner Production, 2019. 217: p. 153-161.
15. Chisti, Y, *Biodiesel from microalgae beats bioethanol*. Trends in Biotechnology, 2008. 26: p. 126-131.
16. Liang, Y, Kashdan, T, Sterner, C, Dombrowski, L, Petrick, I, Kröger, M and Höfer, R, *Chapter 2 - Algal Biorefineries*, in *Industrial Biorefineries & White Biotechnology*, A. Pandey, et al., Editors. 2015, Elsevier: Amsterdam. p. 35-90.
17. Dillschneider, R, Steinweg, C, Rosello-Sastre, R and Posten, C, *Biofuels from microalgae: photoconversion efficiency during lipid accumulation*. Bioresource Technol, 2013. 142: p. 647-54.
18. Chisti, Y, *Biodiesel from microalgae*. Biotechnology Advances, 2007. 25: p. 294-306.
19. Renuka, N, Sood, A, Prasanna, R and Ahluwalia, A, *Phycoremediation of wastewaters: A synergistic approach using microalgae for bioremediation and biomass generation*. International journal of Environmental Science and Technology, 2014. 12.
20. Rawat, I, Ranjith Kumar, R, Mutanda, T and Bux, F, *Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production*. Applied Energy, 2011. 88: p. 3411-3424.
21. Dias, M, Cavalett, O, Filho, R and Bonomi, A, *Integrated first and second generation ethanol production from sugarcane*. Chemical Engineering Transactions, 2014. 37: p. 445-450.
22. Chew, KW, Yap, JY, Show, PL, Suan, NH, Juan, JC, Ling, TC, Lee, D-J and Chang, J-S, *Microalgae biorefinery: High value products perspectives*. Bioresource Technology, 2017. 229: p. 53-62.
23. de Souza, MP, Hoeltz, M, Gressler, PD, Benitez, LB and Schneider, RCS, *Potential of Microalgal Bioproducts: General Perspectives and Main Challenges*. Waste and Biomass Valorization, 2019. 10: p. 2139-2156.

24. Khoo, CG, Dasan, YK, Lam, MK and Lee, KT, *Algae biorefinery: Review on a broad spectrum of downstream processes and products*. Bioresource Technology, 2019. 292: p. 121964.
25. Rizwan, M, Mujtaba, G, Memon, SA, Lee, K and Rashid, N, *Exploring the potential of microalgae for new biotechnology applications and beyond: A review*. Renewable and Sustainable Energy Reviews, 2018. 92: p. 394-404.
26. Li, Y, Horsman, M, Wu, N, Lan, CQ and Dubois-Calero, N, *Biofuels from microalgae*. Biotechnology Progress, 2008. 24: p. 815-820.
27. Cezare-Gomes, EA, Mejia-da-Silva, LdC, Pérez-Mora, LS, Matsudo, MC, Ferreira-Camargo, LS, Singh, AK and de Carvalho, JCM, *Potential of Microalgae Carotenoids for Industrial Application*. Applied Biochemistry and Biotechnology, 2019. 188: p. 602-634.
28. Morais Junior, WG, Gorgich, M, Corrêa, PS, Martins, AA, Mata, TM and Caetano, NS, *Microalgae for biotechnological applications: Cultivation, harvesting and biomass processing*. Aquaculture, 2020. 528: p. 735562.
29. Mobin, S and Alam, F, *Some Promising Microalgal Species for Commercial Applications: A review*. Energy Procedia, 2017. 110: p. 510-517.
30. Singh, A, Nigam, PS and Murphy, JD, *Mechanism and challenges in commercialisation of algal biofuels*. Bioresource Technology, 2011. 102: p. 26-34.
31. Wang, B, Li, Y, Wu, N and Lan, CQ, *CO₂ bio-mitigation using microalgae*. Applied Microbiology and Biotechnology, 2008. 79: p. 707-718.
32. Barroso Soares, R, Ferreira Martins, M and Franci Gonçalves, R, *Experimental investigation of wastewater microalgae in a pilot-scale downdraft gasifier*. Algal Research, 2020. 51: p. 102049.
33. Feng, X, Chen, Y, Lv, J, Han, S, Tu, R, Zhou, X, Jin, W and Ren, N, *Enhanced lipid production by *Chlorella pyrenoidosa* through magnetic field pretreatment of wastewater and treatment of microalgae-wastewater culture solution: Magnetic field treatment modes and conditions*. Bioresource Technology, 2020. 306: p. 123102.
34. Suganya, T, Varman, M, Masjuki, HH and Renganathan, S, *Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach*. Renewable and Sustainable Energy Reviews, 2016. 55: p. 909-941.
35. Moreno-Garcia, L, Adjallé, K, Barnabé, S and Raghavan, GSV, *Microalgae biomass production for a biorefinery system: Recent advances and the way*

- towards sustainability. *Renewable and Sustainable Energy Reviews*, 2017. 76: p. 493-506.
36. Priyadarshani, I and Rath, B, *Commercial and industrial applications of microalgae-A review*. *J Algal Biomass Utln*, 2012. 3: p. 89-100.
 37. Khan, MI, Shin, JH and Kim, JD, *The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products*. *Microbial Cell Factories*, 2018. 17: p. 36.
 38. Gavrilescu, M and Chisti, Y, *Biotechnology - A sustainable alternative for chemical industry*. *Biotechnology Advances*, 2005. 23: p. 471-499.
 39. Singh, S, Kate, BN and Banecjee, UC, *Bioactive compounds from cyanobacteria and microalgae: An overview*. *Critical Reviews in Biotechnology*, 2005. 25: p. 73-95.
 40. Spolaore, P, Joannis-Cassan, C, Duran, E and Isambert, A, *Commercial applications of microalgae*. *Journal of Bioscience and Bioengineering*, 2006. 101: p. 87-96.
 41. Chen, C-Y, Yeh, K-L, Aisyah, R, Lee, D-J and Chang, J-S, *Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review*. *Bioresource Technology*, 2011. 102: p. 71-81.
 42. Huang, G, Chen, F, Wei, D, Zhang, X and Chen, G, *Biodiesel production by microalgal biotechnology*. *Applied Energy*, 2010. 87: p. 38-46.
 43. Brennan, L and Owende, P, *Biofuels from microalgae – A review of technologies for production, processing, and extractions of biofuels and co-products*. *Renewable and Sustainable Energy Reviews*, 2010. 14: p. 557-577.
 44. Chew, KW, Chia, SR, Show, PL, Yap, YJ, Ling, TC and Chang, J-S, *Effects of water culture medium, cultivation systems and growth modes for microalgae cultivation: A review*. *Journal of the Taiwan Institute of Chemical Engineers*, 2018. 91: p. 332-344.
 45. Richmond, A, *Open systems for the mass production of photoautotrophic microalgae outdoors: physiological principles*. *Journal of Applied Phycology*, 1992. 4: p. 281-286.
 46. Xu, X-Q, Wang, J-H, Zhang, T-Y, Dao, G-H, Wu, G-X and Hu, H-Y, *Attached microalgae cultivation and nutrients removal in a novel capillary-driven photo-biofilm reactor*. *Algal Research*, 2017. 27: p. 198-205.

47. Yang, J, Xu, M, Zhang, X, Hu, Q, Sommerfeld, M and Chen, Y, *Life-cycle analysis on biodiesel production from microalgae: Water footprint and nutrients balance*. Bioresource Technology, 2011. 102: p. 159-165.
48. Guieysse, B, Béchet, Q and Shilton, A, *Variability and uncertainty in water demand and water footprint assessments of fresh algae cultivation based on case studies from five climatic regions*. Bioresource Technology, 2013. 128: p. 317-323.
49. Gross, M, Henry, W, Michael, C and Wen, Z, *Development of a rotating algal biofilm growth system for attached microalgae growth with in situ biomass harvest*. Bioresource Technology, 2013. 150: p. 195-201.
50. Wang, J-H, Zhuang, L-L, Xu, X-Q, Deantes-Espinosa, VM, Wang, X-X and Hu, H-Y, *Microalgal attachment and attached systems for biomass production and wastewater treatment*. Renewable and Sustainable Energy Reviews, 2018. 92: p. 331-342.
51. Gross, M, Jarboe, D and Wen, Z, *Biofilm-based algal cultivation systems*. Applied Microbiology and Biotechnology, 2015. 99: p. 5781-5789.
52. Berner, F, Heimann, K and Sheehan, M, *Microalgal biofilms for biomass production*. Journal of Applied Phycology, 2015. 27: p. 1793-1804.
53. Katarzyna, L, Sai, G and Singh, OA, *Non-enclosure methods for non-suspended microalgae cultivation: literature review and research needs*. Renewable and Sustainable Energy Reviews, 2015. 42: p. 1418-1427.
54. Mantzorou, A and Ververidis, F, *Microalgal biofilms: A further step over current microalgal cultivation techniques*. Science of The Total Environment, 2019. 651: p. 3187-3201.
55. Hameed, MSA and Ebrahim, OH, *Biotechnological potential uses of immobilized algae*. International Journal of Agriculture and Biology, 2007. 9: p. 183-192.
56. Liu, T, Wang, J, Hu, Q, Cheng, P, Ji, B, Liu, J, Chen, Y, Zhang, W, Chen, X, Chen, L, Gao, L, Ji, C and Wang, H, *Attached cultivation technology of microalgae for efficient biomass feedstock production*. Bioresource Technology, 2013. 127: p. 216-222.
57. Huang, Y, Xiong, W, Liao, Q, Fu, Q, Xia, A, Zhu, X and Sun, Y, *Comparison of Chlorella vulgaris biomass productivity cultivated in biofilm and suspension from the aspect of light transmission and microalgae affinity to carbon dioxide*. Bioresource Technology, 2016. 222: p. 367-373.

58. Wang, J, Liu, W and Liu, T, *Biofilm based attached cultivation technology for microalgal biorefineries – A review*. Bioresource Technology, 2017. 244: p. 1245-1253.
59. Schnurr, PJ, Espie, GS and Allen, DG, *Algae biofilm growth and the potential to stimulate lipid accumulation through nutrient starvation*. Bioresource Technology, 2013. 136: p. 337-344.
60. Cheng, P, Ji, B, Gao, L, Zhang, W, Wang, J and Liu, T, *The growth, lipid and hydrocarbon production of Botryococcus braunii with attached cultivation*. Bioresource Technology, 2013. 138: p. 95-100.
61. Blanken, W, Janssen, M, Cuaresma, M, Libor, Z, Bhaiji, T and Wijffels, RH, *Biofilm growth of Chlorella sorokiniana in a rotating biological contactor based photobioreactor*. Biotechnology and Bioengineering, 2014. 111: p. 2436-2445.
62. Zhang, Q, Li, X, Guo, D, Ye, T, Xiong, M, Zhu, L, Liu, C, Jin, S and Hu, Z, *Operation of a vertical algal biofilm enhanced raceway pond for nutrient removal and microalgae-based byproducts production under different wastewater loadings*. Bioresource Technology, 2018. 253: p. 323-332.
63. Xu, L, Weathers, PJ, Xiong, X-R and Liu, C-Z, *Microalgal bioreactors: Challenges and opportunities*. Engineering in Life Sciences, 2009. 9: p. 178-189.
64. Larsdotter, K, *Wastewater treatment with microalgae - A literature review*. Vatten, 2006. 62: p. 31-38.
65. Ugwu, CU, Aoyagi, H and Uchiyama, H, *Photobioreactors for mass cultivation of algae*. Bioresource Technology, 2008. 99: p. 4021-4028.
66. Vuppaladadiyam, AK, Prinsen, P, Raheem, A, Luque, R and Zhao, M, *Microalgae cultivation and metabolites production: a comprehensive review*. Biofuels, Bioproducts and Biorefining, 2018. 12: p. 304-324.
67. Gifuni, I, Pollio, A, Safi, C, Marzocchella, A and Olivieri, G, *Current Bottlenecks and Challenges of the Microalgal Biorefinery*. Trends in Biotechnology, 2019. 37: p. 242-252.
68. Posten, C, *Design principles of photo-bioreactors for cultivation of microalgae*. Engineering in Life Sciences, 2009. 9: p. 165-177.
69. Suparmaniam, U, Lam, MK, Uemura, Y, Lim, JW, Lee, KT and Shuit, SH, *Insights into the microalgae cultivation technology and harvesting process for biofuel production: A review*. Renewable and Sustainable Energy Reviews, 2019. 115: p. 109361.
70. Doucha, J and Lívanský, K, *Productivity, CO₂/O₂ exchange and hydraulics in outdoor open high density microalgal (Chlorella sp.) photobioreactors*

- operated in a Middle and Southern European climate. Journal of Applied Phycology*, 2006. 18: p. 811-826.
71. Apel, AC, Pfaffinger, CE, Basedahl, N, Mittwollen, N, Göbel, J, Sauter, J, Brück, T and Weuster-Botz, D, *Open thin-layer cascade reactors for saline microalgae production evaluated in a physically simulated Mediterranean summer climate. Algal Research*, 2017. 25: p. 381-390.
 72. Gifuni, I, Pollio, A, Marzocchella, A and Olivieri, G, *New ultra-flat photobioreactor for intensive microalgal production: The effect of light irradiance. Algal Research*, 2018. 34: p. 134-142.
 73. Souliès, A, Legrand, J, Marec, H, Pruvost, J, Castelain, C, Burghilea, T and Cornet, J-F, *Investigation and modeling of the effects of light spectrum and incident angle on the growth of Chlorella vulgaris in photobioreactors. Biotechnology Progress*, 2016. 32: p. 247-261.
 74. Moreno, J, Vargas, MÁ, Rodrı, x, guez, H, Rivas, J, x and Guerrero, MG, *Outdoor cultivation of a nitrogen-fixing marine cyanobacterium, Anabaena sp. ATCC 33047. Biomolecular Engineering*, 2003. 20: p. 191-197.
 75. Pushparaj, B, Pelosi, E, Tredici, MR, Pinzani, E and Materassi, R, *As integrated culture system for outdoor production of microalgae and cyanobacteria. Journal of Applied Phycology*, 1997. 9: p. 113-119.
 76. Wen, X, Du, K, Wang, Z, Peng, X, Luo, L, Tao, H, Xu, Y, Zhang, D, Geng, Y and Li, Y, *Effective cultivation of microalgae for biofuel production: A pilot-scale evaluation of a novel oleaginous microalga Graesiella sp. WBG-1. Biotechnology for Biofuels*, 2016. 9: p. 123-134.
 77. Huntley, ME and Redalje, DG, *CO₂ mitigation and renewable oil from photosynthetic microbes: A new appraisal. Mitigation and Adaptation Strategies for Global Change*, 2007. 12: p. 573-608.
 78. Blanco, AM, Moreno, J, Del Campo, JA, Rivas, J and Guerrero, MG, *Outdoor cultivation of lutein-rich cells of Muriellopsis sp. in open ponds. Applied Microbiology and Biotechnology*, 2007. 73: p. 1259-1266.
 79. de Vree, JH, Bosma, R, Janssen, M, Barbosa, MJ and Wijffels, RH, *Comparison of four outdoor pilot-scale photobioreactors. Biotechnology for Biofuels*, 2015. 8: p. 215-226.
 80. Cheng-Wu, Z, Zmora, O, Kopel, R and Richmond, A, *An industrial-size flat plate glass reactor for mass production of Nannochloropsis sp. (Eustigmatophyceae). Aquaculture*, 2001. 195: p. 35-49.

81. Acien Fernández, FG, Fernández Sevilla, JM, Sánchez Pérez, JA, Molina Grima, E and Chisti, Y, *Airlift-driven external-loop tubular photobioreactors for outdoor production of microalgae: assessment of design and performance*. Chemical Engineering Science, 2001. 56: p. 2721-2732.
82. Doucha, J and Lívanský, K, *Outdoor open thin-layer microalgal photobioreactor: Potential productivity*. Journal of Applied Phycology, 2009. 21: p. 111-117.
83. Benvenuti, G, Bosma, R, Ji, F, Lamers, P, Barbosa, MJ and Wijffels, RH, *Batch and semi-continuous microalgal TAG production in lab-scale and outdoor photobioreactors*. Journal of Applied Phycology, 2016. 28: p. 3167-3177.
84. Benvenuti, G, Bosma, R, Klok, AJ, Ji, F, Lamers, PP, Barbosa, MJ and Wijffels, RH, *Microalgal triacylglycerides production in outdoor batch-operated tubular PBRs*. Biotechnology for Biofuels, 2015. 8: p. 100.
85. Remmers, IM, Wijffels, RH, Barbosa, MJ and Lamers, PP, *Can We Approach Theoretical Lipid Yields in Microalgae?* Trends in Biotechnology, 2018. 36: p. 265-276.
86. Wang, S-J and Zhong, J-J, *Bioreactor Engineering*, in *Bioprocessing for Value-Added Products from Renewable Resources: New Technologies and Applications*, S.-T. Yang, Editor. 2011, Elsevier: United Kingdom. p. 131-161.
87. Liu, J, Sun, Z and Chen, F, *Heterotrophic production of algal oils*, in *Biofuels from Algae*, A. Pandey, et al., Editors. 2014, Elsevier: The Netherlands. p. 111-142.
88. Ashokkumar, V, Agila, E, Salam, Z, Ponraj, M, Din, MFM and Ani, FN, *A study on large scale cultivation of Microcystis aeruginosa under open raceway pond at semi-continuous mode for biodiesel production*. Bioresource Technology, 2014. 172: p. 186-193.
89. Blunt, W, Levin, DB and Cicek, N, *Bioreactor operating strategies for improved polyhydroxyalkanoate (PHA) productivity*. Polymers, 2018. 10: p. 1197.
90. Gifuni, I, Olivieri, G, Pollio, A and Marzocchella, A, *Identification of an industrial microalgal strain for starch production in biorefinery context: The effect of nitrogen and carbon concentration on starch accumulation*. New Biotechnology, 2018. 41: p. 46-54.
91. Johnson, EM, Kumar, K and Das, D, *Physicochemical parameters optimization, and purification of phycobiliproteins from the isolated Nostoc sp.* Bioresource Technology, 2014. 166: p. 541-547.

92. Minhas, AK, Hodgson, P, Barrow, CJ and Adholeya, A, *A review on the assessment of stress conditions for simultaneous production of microalgal lipids and carotenoids*. *Frontiers in Microbiology*, 2016. 7: p. 546-564.
93. Geada, P, Vasconcelos, V, Vicente, A and Fernandes, B, *Microalgal biomass cultivation*, in *Algal Green Chemistry: Recent Progress in Biotechnology*, R.P. Rastogi, D. Madamwar, and A. Pandey, Editors. 2017, Elsevier: The Netherlands. p. 257-284.
94. De Farias Silva, CE, Sforza, E and Bertucco, A, *Continuous cultivation of microalgae as an efficient method to improve carbohydrate productivity and biochemical stability*, in *25th European Biomass Conference & Exhibition*. 2017, European Commission: Stockholm, Sweden.
95. Benvenuti, G, Lamers, PP, Breuer, G, Bosma, R, Cerar, A, Wijffels, RH and Barbosa, MJ, *Microalgal TAG production strategies: why batch beats repeated-batch*. *Biotechnology for Biofuels*, 2016. 9: p. 64.
96. Rodolfi, L, Chini Zittelli, G, Bassi, N, Padovani, G, Biondi, N, Bonini, G and Tredici, MR, *Microalgae for oil: Strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor*. *Biotechnology and Bioengineering*, 2009. 102: p. 100-112.
97. Breuer, G, Lamers, PP, Martens, DE, Draaisma, RB and Wijffels, RH, *The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains*. *Bioresource Technology*, 2012. 124: p. 217-226.
98. Remmers, IM, Hidalgo-Ulloa, A, Brandt, BP, Evers, WAC, Wijffels, RH and Lamers, PP, *Continuous versus batch production of lipids in the microalgae *Acutodesmus obliquus**. *Bioresource Technology*, 2017. 244: p. 1384-1392.
99. Nagappan, S, Devendran, S, Tsai, P-C, Dahms, H-U and Ponnusamy, VK, *Potential of two-stage cultivation in microalgae biofuel production*. *Fuel*, 2019. 252: p. 339-349.
100. Fábregas, J, Otero, A, Maseda, A and Domínguez, A, *Two-stage cultures for the production of astaxanthin from *Haematococcus pluvialis**. *Journal of Biotechnology*, 2001. 89: p. 65-71.
101. Singh, G and Patidar, SK, *Microalgae harvesting techniques: A review*. *Journal of Environmental Management*, 2018. 217: p. 499-508.
102. Barros, AI, Gonçalves, AL, Simões, M and Pires, JCM, *Harvesting techniques applied to microalgae: A review*. *Renewable and Sustainable Energy Reviews*, 2015. 41: p. 1489-1500.

103. Christenson, L and Sims, R, *Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts*. Biotechnology Advances, 2011. 29: p. 686-702.
104. Danquah, MK, Gladman, B, Moheimani, N and Forde, GM, *Microalgal growth characteristics and subsequent influence on dewatering efficiency*. Chemical Engineering Journal, 2009. 151: p. 73-78.
105. Olaizola, M, *Commercial development of microalgal biotechnology: from the test tube to the marketplace*. Biomolecular Engineering, 2003. 20: p. 459-466.
106. Williams, PJB and Laurens, LML, *Microalgae as biodiesel & biomass feedstocks: Review & analysis of the biochemistry, energetics & economics*. Energy & Environmental Science, 2010. 3: p. 554-590.
107. t Lam, GP, Vermuë, MH, Eppink, MHM, Wijffels, RH and van den Berg, C, *Multi-Product Microalgae Biorefineries: From Concept Towards Reality*. Trends in Biotechnology, 2018. 36: p. 216-227.
108. Molina Grima, E, Belarbi, EH, Acien Fernández, FG, Robles Medina, A and Chisti, Y, *Recovery of microalgal biomass and metabolites: process options and economics*. Biotechnology Advances, 2003. 20: p. 491-515.
109. Shah, JH, Deokar, A, Patel, K, Panchal, K and Mehta, AV. *A comprehensive overview on various method of harvesting microalgae according to Indian perspective*. in *International Conference on Multidisciplinary Research & Practice*. 2014. Gujarat, India.
110. Vandamme, D, Foubert, I and Muylaert, K, *Flocculation as a low-cost method for harvesting microalgae for bulk biomass production*. Trends in Biotechnology, 2013. 31: p. 233-239.
111. Ahmad, AL, Yasin, NHM, Derek, CJC and Lim, JK, *Comparison of harvesting methods for microalgae Chlorella sp. and its potential use as a biodiesel feedstock*. Environmental Technology, 2014. 35: p. 2244-2253.
112. Salim, S, Bosma, R, Vermuë, MH and Wijffels, RH, *Harvesting of microalgae by bio-flocculation*. Journal of Applied Phycology, 2011. 23: p. 849-855.
113. Tan, XB, Lam, MK, Uemura, Y, Lim, JW, Wong, CY and Lee, KT, *Cultivation of microalgae for biodiesel production: A review on upstream and downstream processing*. Chinese Journal of Chemical Engineering, 2018. 26: p. 17-30.
114. Schenk, PM, Thomas-Hall, SR, Stephens, E, Marx, UC, Mussgnug, JH, Posten, C, Kruse, O and Hankamer, B, *Second generation biofuels: High-efficiency microalgae for biodiesel production*. BioEnergy Research, 2008. 1: p. 20-43.

115. Gerardo, ML, Zanain, MA and Lovitt, RW, *Pilot-scale cross-flow microfiltration of Chlorella minutissima: A theoretical assessment of the operational parameters on energy consumption*. Chemical Engineering Journal, 2015. 280: p. 505-513.
116. Ruiz, J, Olivieri, G, de Vree, J, Bosma, R, Willems, P, Reith, JH, Eppink, MHM, Kleinegris, DMM, Wijffels, RH and Barbosa, MJ, *Towards industrial products from microalgae*. Energy & Environmental Science, 2016. 9: p. 3036-3043.
117. Pr  at, N, Taelman, SE, De Meester, S, Allais, F and Dewulf, J, *Identification of microalgae biorefinery scenarios and development of mass and energy balance flowsheets*. Algal Research, 2020. 45: p. 101737-101747.
118. Garcia, ES, Olivieri, G, Sijtsma, L, Vermu  , MH, Barbosa, M, Reith, JH, van den Berg, C, Eppink, MH and Wijffels, RH, *Integrated biorefineries for algal biomolecules*, in *Grand Challenges in Algae Biotechnology*, A. Hallmann and P. Rampelotto, Editors. 2019, Springer: Switzerland. p. 293-317.
119. Waghmare, A, Nagula, K, Pandit, A and Arya, S, *Hydrodynamic cavitation for energy efficient and scalable process of microalgae cell disruption*. Algal Research, 2019. 40: p. 101496.
120. D'hondt, E, Martin-Juarez, J, Bolado, S, Kasperoviciene, J, Koreiviene, J, Sulcius, S, Elst, K and Bastiaens, L, *Cell disruption technologies*, in *Microalgae-Based Biofuels and Bioproducts*, C. Gonzalez-Fernandez and R. Mu  oz, Editors. 2017, Elsevier: The Netherlands. p. 133-154.
121. G  n  rken, E, D'Hondt, E, Eppink, MHM, Garcia-Gonzalez, L, Elst, K and Wijffels, RH, *Cell disruption for microalgae biorefineries*. Biotechnology Advances, 2015. 33: p. 243-260.
122. Monte, J, Ribeiro, C, Parreira, C, Costa, L, Brive, L, Casal, S, Brazinha, C and Crespo, JG, *Biorefinery of Dunaliella salina: Sustainable recovery of carotenoids, polar lipids and glycerol*. Bioresource Technology, 2020. 297-306: p. 122509.
123. Vanthoor-Koopmans, M, Wijffels, RH, Barbosa, MJ and Eppink, MH, *Biorefinery of microalgae for food and fuel*. Bioresource Technology, 2013. 135: p. 142-149.
124. G  n  rken, E, D'Hondt, E, Eppink, M, Garcia-Gonzalez, L, Elst, K and Wijffels, RH, *Cell disruption for microalgae biorefineries*. Biotechnology Advances, 2015. 33: p. 243-260.
125. Karim, A, Islam, MA, Khalid, ZB, Faizal, CKM, Khan, MMR and Yousuf, A, *Microalgal cell disruption and lipid extraction techniques for potential biofuel*

- production, in *Microalgae Cultivation for Biofuels Production*, A. Yousuf, Editor. 2020, Elsevier: The Netherlands. p. 129-147.
126. Leonhardt, L, Käferböck, A, Smetana, S, de Vos, R, Toepfl, S and Parniakov, O, *Bio-refinery of Chlorella sorokiniana with pulsed electric field pre-treatment*. Bioresource Technology, 2020. 301: p. 122743.
 127. Vanthoor-Koopmans, M, Wijffels, RH, Barbosa, MJ and Eppink, MHM, *Biorefinery of microalgae for food and fuel*. Bioresource Technology, 2013. 135: p. 142-149.
 128. Buchmann, L, Brändle, I, Haberkorn, I, Hiestand, M and Mathys, A, *Pulsed electric field based cyclic protein extraction of microalgae towards closed-loop biorefinery concepts*. Bioresource Technology, 2019. 291: p. 121870.
 129. Joannes, C, Sipaut, C, Dayou, J, Yasir, S, Mansa, R, Author, C, Rachel and Mansa, F, *The Potential of Using Pulsed Electric Field (PEF) Technology as the Cell Disruption Method to Extract Lipid from Microalgae for Biodiesel Production*. International Journal of Renewable Energy Research-IJRER, 2015. 5: p. 598-621.
 130. Eing, C, Goettel, M, Straessner, R, Gusbeth, C and Frey, W, *Pulsed Electric Field Treatment of Microalgae—Benefits for Microalgae Biomass Processing*. Plasma Science, IEEE Transactions on, 2013. 41: p. 2901-2907.
 131. Lee, SY, Cho, JM, Chang, YK and Oh, Y-K, *Cell disruption and lipid extraction for microalgal biorefineries: A review*. Bioresource Technology, 2017. 244: p. 1317-1328.
 132. Dong, T, Knoshaug, EP, Pienkos, PT and Laurens, LML, *Lipid recovery from wet oleaginous microbial biomass for biofuel production: A critical review*. Applied Energy, 2016. 177: p. 879-895.
 133. Papachristou, I, Silve, A, Jianu, A, Wüstner, R, Nazarova, N, Müller, G and Frey, W, *Evaluation of pulsed electric fields effect on the microalgae cell mechanical stability through high pressure homogenization*. Algal Research, 2020. 47: p. 101847.
 134. Vermuë, M, Eppink, M, Wijffels, R and Van Den Berg, C, *Multi-product microalgae biorefineries: from concept towards reality*. Trends in Biotechnology, 2018. 36: p. 216-227.
 135. Schwenzfeier, A, Wierenga, PA and Gruppen, H, *Isolation and characterization of soluble protein from the green microalgae Tetraselmis sp.* Bioresource Technology, 2011. 102: p. 9121-9127.

136. Bueno, M, Gallego, R, Mendiola, JA and Ibáñez, E, *Downstream green processes for recovery of bioactives from algae*, in *Grand Challenges in Algae Biotechnology*, A. Hallmann and P. Rampelotto, Editors. 2019, Springer: Switzerland. p. 399-425.
137. Patel, A, Mikes, F and Matsakas, L, *An overview of current pretreatment methods used to improve lipid extraction from oleaginous microorganisms*. *Molecules*, 2018. 23: p. 1562-1583.
138. Pieter't Lam, G, *Harvesting and cell disruption of microalgae*. 2017, Wageningen University.
139. Sankaran, R, Show, PL, Nagarajan, D and Chang, J-S, *Exploitation and biorefinery of microalgae*, in *Waste Biorefinery*, B. Bhaskar, et al., Editors. 2018, Elsevier: United Kingdom. p. 571-601.
140. Cuevas-Castillo, GA, Navarro-Pineda, FS, Rodríguez, SAB and Rivero, JCS, *Advances on the processing of microalgal biomass for energy-driven biorefineries*. *Renewable and Sustainable Energy Reviews*, 2020. 125: p. 109606-109625.
141. Chen, Z, Wang, L, Qiu, S and Ge, S, *Determination of Microalgal Lipid Content and Fatty Acid for Biofuel Production*. *BioMed Research International*, 2018. 2018: p. 1-17.
142. Soccol, CR, Faraco, V, Karp, SG, Vandenberghe, LP, Thomaz-Soccol, V, Woiciechowski, AL and Pandey, A, *Lignocellulosic bioethanol: Current status and future perspectives*, in *Biofuels: Alternative Feedstocks and Conversion Processes for the Production of Liquid and Gaseous Biofuels*, A. Pandey, et al., Editors. 2019, Academic Press: United Kingdom. p. 101-122.
143. Mubarak, M, Shaija, A and Suchithra, T, *A review on the extraction of lipid from microalgae for biodiesel production*. *Algal Research*, 2015. 7: p. 117-123.
144. Garcia, ES, Van Leeuwen, J, Safi, C, Sijtsma, L, Eppink, MH, Wijffels, RH and van den Berg, C, *Selective and energy efficient extraction of functional proteins from microalgae for food applications*. *Bioresource Technology*, 2018. 268: p. 197-203.
145. Du, Y, Schuur, B, Kersten, SR and Brilman, DW, *Opportunities for switchable solvents for lipid extraction from wet algal biomass: An energy evaluation*. *Algal Research*, 2015. 11: p. 271-283.
146. Orr, VC and Rehmann, L, *Ionic liquids for the fractionation of microalgal biomass*. *Current Opinion in Green and Sustainable Chemistry*, 2016. 2: p. 22-27.

147. Sati, H, Mitra, M, Mishra, S and Baredar, P, *Microalgal lipid extraction strategies for biodiesel production: A review*. Algal Research, 2019. 38: p. 101413-101424.
148. Ansari, FA, Shriwastav, A, Gupta, SK, Rawat, I and Bux, F, *Exploration of microalgae biorefinery by optimizing sequential extraction of major metabolites from Scenedesmus obliquus*. Industrial & Engineering Chemistry Research, 2017. 56: p. 3407-3412.
149. Gilbert-López, B, Mendiola, JA, van den Broek, LA, Houweling-Tan, B, Sijtsma, L, Cifuentes, A, Herrero, M and Ibáñez, E, *Green compressed fluid technologies for downstream processing of Scenedesmus obliquus in a biorefinery approach*. Algal Research, 2017. 24: p. 111-121.
150. Nagarajan, D, Lee, D-J, Chen, C-Y and Chang, J-S, *Resource recovery from wastewaters using microalgae-based approaches: A circular bioeconomy perspective*. Bioresource Technology, 2020. 302: p. 122817.
151. Elser, J and Bennett, E, *Phosphorus cycle: A broken biogeochemical cycle*. Nature, 2011. 478: p. 29-31.
152. Mennaa, FZ, Arbib, Z and Perales, JA, *Urban wastewater treatment by seven species of microalgae and an algal bloom: Biomass production, N and P removal kinetics and harvestability*. Water Research, 2015. 83: p. 42-51.
153. Cooper, J, Lombardi, R, Boardman, D and Carliell-Marquet, C, *The future distribution and production of global phosphate rock reserves*. Resources, Conservation and Recycling, 2011. 57: p. 78-86.
154. Li, K, Liu, Q, Fang, F, Luo, R, Lu, Q, Zhou, W, Huo, S, Cheng, P, Liu, J, Addy, M, Chen, P, Chen, D and Ruan, R, *Microalgae-based wastewater treatment for nutrients recovery: A review*. Bioresource Technology, 2019. 291: p. 121934.
155. Fan, H, Wang, K, Wang, C, Yu, F, He, X, Ma, J and Li, X, *A comparative study on growth characters and nutrients removal from wastewater by two microalgae under optimized light regimes*. Environmental Technology & Innovation, 2020. 19: p. 100849.
156. Markou, G and Georgakakis, D, *Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: A review*. Applied Energy, 2011. 88: p. 3389-3401.
157. Roostaei, J and Zhang, Y, *Spatially Explicit Life Cycle Assessment: Opportunities and challenges of wastewater-based algal biofuels in the United States*. Algal Research, 2017. 24: p. 395-402.

158. Cai, T, Park, SY and Li, Y, *Nutrient recovery from wastewater streams by microalgae: Status and prospects*. Renewable and Sustainable Energy Reviews, 2013. 19: p. 360-369.
159. De Andrade, CJ and Andrade, L, *Microalgae for bioremediation of textile wastewater: An overview*. MOJ Food Processing & Technology, 2018. 6.
160. Chitra, J, Das, A, Goel, M and Kanapuram, R, *Microalgae Application for Treatment of Textile Effluents*. Research Journal of Pharmaceutical, Biological and Chemical Sciences, 2013. 4: p. 1602-1606.
161. Amenorfenyo, DK, Huang, X, Zhang, Y, Zeng, Q, Zhang, N, Ren, J and Huang, Q, *Microalgae Brewery Wastewater Treatment: Potentials, Benefits and the Challenges*. International journal of environmental research and public health, 2019. 16: p. 1910.
162. Tarlan, E, Dilek, FB and Yetis, U, *Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater*. Bioresource Technology, 2002. 84: p. 1-5.
163. Wang, Y, Ho, S-H, Cheng, C-L, Guo, W-Q, Nagarajan, D, Ren, N-Q, Lee, D-J and Chang, J-S, *Perspectives on the feasibility of using microalgae for industrial wastewater treatment*. Bioresource Technology, 2016. 222: p. 485-497.
164. Usha, MT, Sarat Chandra, T, Sarada, R and Chauhan, VS, *Removal of nutrients and organic pollution load from pulp and paper mill effluent by microalgae in outdoor open pond*. Bioresource Technology, 2016. 214: p. 856-860.
165. Pittman, JK, Dean, AP and Osundeko, O, *The potential of sustainable algal biofuel production using wastewater resources*. Bioresource technology, 2011. 102: p. 17-25.
166. Spiegel, S, Farmer, J and Garver, S, *Heavy metal concentrations in municipal wastewater treatment plant sludge*. Bulletin of environmental contamination and toxicology, 1985. 35: p. 38-43.
167. Wang, L, Min, M, Li, Y, Chen, P, Chen, Y, Liu, Y, Wang, Y and Ruan, R, *Cultivation of Green Algae Chlorella sp. in Different Wastewaters from Municipal Wastewater Treatment Plant*. Applied Biochemistry and Biotechnology, 2010. 162: p. 1174-1186.
168. Yetilmezsoy, K and Sakar, S, *Development of empirical models for performance evaluation of UASB reactors treating poultry manure wastewater under different operational conditions*. Journal of Hazardous Materials, 2008. 153: p. 532-543.

169. Millmier, A, Lorimor, J, Hurburgh C, Jr., Fulhage, C, Hattey, J and Zhang, H, *Near-infrared sensing of manure nutrients*. Transactions of the American Society of Agricultural Engineers, 2000. 43: p. 903-908.
170. Zhu, L, Wang, Z, Shu, Q, Takala, J, Hiltunen, E, Feng, P and Yuan, Z, *Nutrient removal and biodiesel production by integration of freshwater algae cultivation with piggery wastewater treatment*. Water research, 2013. 47: p. 4294-4302.
171. Zhou, W, Hu, B, Li, Y, Min, M, Mohr, M, Du, Z, Chen, P and Ruan, R, *Mass cultivation of microalgae on animal wastewater: a sequential two-stage cultivation process for energy crop and omega-3-rich animal feed production*. Applied biochemistry and biotechnology, 2012. 168: p. 348-363.
172. Chinnasamy, S, Bhatnagar, A, Hunt, RW and Das, KC, *Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications*. Bioresource Technology, 2010. 101: p. 3097-3105.
173. Şen, S and Demirer, GN, *Anaerobic treatment of real textile wastewater with a fluidized bed reactor*. Water Research, 2003. 37: p. 1868-1878.
174. Mosse, KPM, Patti, AF, Christen, EW and Cavagnaro, TR, *Review: Winery wastewater quality and treatment options in Australia*. Australian Journal of Grape and Wine Research, 2011. 17: p. 111-122.
175. Bhatnagar, A, Chinnasamy, S, Singh, M and Das, K, *Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters*. Applied Energy, 2011. 88: p. 3425-3431.
176. Li, X, Yang, C, Zeng, G, Wu, S, Lin, Y, Zhou, Q, Lou, W, Du, C, Nie, L and Zhong, Y, *Nutrient removal from swine wastewater with growing microalgae at various zinc concentrations*. Algal Research, 2020. 46: p. 101804.
177. Hernández, D, Riaño, B, Coca, M and García-González, MC, *Treatment of agro-industrial wastewater using microalgae-bacteria consortium combined with anaerobic digestion of the produced biomass*. Bioresource Technology, 2013. 135: p. 598-603.
178. Lee, CS, Oh, HS, Oh, HM, Kim, HS and Ahn, CY, *Two-phase photoperiodic cultivation of algal-bacterial consortia for high biomass production and efficient nutrient removal from municipal wastewater*. Bioresource Technology, 2016. 200: p. 867-875.
179. Mandeep, Kumar Gupta, G and Shukla, P, *Insights into the resources generation from pulp and paper industry wastes: Challenges, perspectives and innovations*. Bioresource Technology, 2020. 297: p. 122496.

180. Kouhia, M, Holmberg, H and Ahtila, P, *Microalgae-utilizing biorefinery concept for pulp and paper industry: Converting secondary streams into value-added products*. Algal Research, 2015. 10: p. 41-47.
181. Pätäri, S, Kyläheiko, K and Sandström, J, *Opening up new strategic options in the pulp and paper industry: Case biorefineries*. Forest Policy and Economics, 2011. 13: p. 456-464.
182. Slade, AH, Gapes, DJ, Stuthridge, TR, Anderson, SM, Dare, PH, Pearson, HGW and Dennis, M, *N-ViroTech® - a novel process for the treatment of nutrient limited wastewaters*. Water Science and Technology, 2004. 50: p. 131-139.
183. Ali, M and Sreekrishnan, TR, *Aquatic toxicity from pulp and paper mill effluents: a review*. Advances in Environmental Research, 2001. 5: p. 175-196.
184. Gentili, FG, *Microalgal biomass and lipid production in mixed municipal, dairy, pulp and paper wastewater together with added flue gases*. Bioresource technology, 2014. 169: p. 27-32.
185. Khoo, CG, Dasan, YK, Lam, MK and Lee, KT, *Algae biorefinery: Review on a broad spectrum of downstream processes and products*. Bioresource Technology, 2019. 292: p. 121964-121976.
186. Pires, JCM, Alvim-Ferraz, MCM, Martins, FG and Simões, M, *Carbon dioxide capture from flue gases using microalgae: Engineering aspects and biorefinery concept*. Renewable and Sustainable Energy Reviews, 2012. 16: p. 3043-3053.
187. Cherubini, F, *The biorefinery concept: Using biomass instead of oil for producing energy and chemicals*. Energy Conversion and Management, 2010. 51: p. 1412-1421.
188. Bose, A, O'Shea, R, Lin, R and Murphy, JD, *A perspective on novel cascading algal biomethane biorefinery systems*. Bioresource Technology, 2020. 304: p. 123027-123044.
189. LNEG. *Biorefineries*. [cited 2020 15 of Juned]; Available from: <https://www.lneg.pt/en/area/energy/bioenergy/biorefineries/>.
190. Zhu, L, *Biorefinery as a promising approach to promote microalgae industry: An innovative framework*. Renewable and Sustainable Energy Reviews, 2015. 41: p. 1376-1384.
191. Singh, R, Birru, R and Sibi, G, *Nutrient Removal Efficiencies of Chlorella vulgaris from Urban Wastewater for Reduced Eutrophication*. Journal of Environmental Protection, 2017. 08: p. 1-11.

192. Gifuni, I, Pollio, A, Safi, DC, Marzocchella, A and Olivieri, G, *Current Bottlenecks and Challenges of the Microalgal Biorefinery*. Trends in Biotechnology, 2018. 37.
193. Safi, C, Zebib, B, Merah, O, Pontalier, P-Y and Vaca-Garcia, C, *Morphology, composition, production, processing and applications of Chlorella vulgaris: A review*. Renewable and Sustainable Energy Reviews, 2014. 35: p. 265-278.
194. OECD, *Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test*. 2011.
195. EPA, *Method 352.1: Nitrogen, Nitrate (Colorimetric, Brucine) by Spectrophotometer*. 1971.
196. EPA, *Method 365.3: Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Two Reagent)* 1978.
197. Supelco, *Instructions for Use - 114848 Spectroquant Phosphate Test*. 2019, Merck KGaA.
198. *Operating Manual Spectroquant Prove*. Version 2.0 ed. Spectroquant Prove Spectrophotometer 100 - 300 - 600, ed. M. KGaA. 2018, Darmstadt, Germany. 216.
199. Baird, R and Bridgewater, L, *Standar methods fot the examination of water and wastewater*. 23rd ed. Vol. 1. 2017, Washington, D.C.: American Public Health Association.
200. Gonçalves, A, *Microalgal cultivation for biomass production, carbon dioxide capture and nutrientes uptake*, in *LEPABE, Department of Chemical Engineering*. 2017, Faculty of Engineering, Universtiy of Porto.
201. Barsanti, L and Gualtieri, P, *Algae: anatomy, biochemistry, and biotechnology*. 2014: CRC press.
202. Golzary, A, *Investigation of optimal condition for Chlorella vulgaris microalgae growth*. Global Journal of Environmental Science and Management, 2017. 113: p. 217-230.
203. Converti, A, Casazza, AA, Ortiz, EY, Perego, P and Del Borghi, M, *Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production*. Chemical Engineering and Processing: Process Intensification, 2009. 48: p. 1146-1151.
204. Panahi, Y, Yari Khosroushahi, A, Sahebkar, A and Heidari, HR, *Impact of Cultivation Condition and Media Content onChlorella vulgaris Composition*. Advanced pharmaceutical bulletin, 2019. 9: p. 182-194.

205. Amini Khoeyi, Z, Seyfabadi, J and Ramezanpour, Z, *Effect of light intensity and photoperiod on biomass and fatty acid composition of the microalgae, Chlorella vulgaris*. Aquaculture International, 2012. 20: p. 41-49.
206. Gonçalves, A, Alvim-Ferraz, M, Martins, F, Simões, M and Pires, J, *Integration of Microalgae-Based Bioenergy Production into a Petrochemical Complex: Techno-Economic Assessment*. Energies, 2016. 9: p. 224.
207. Rodrigues, CMM, *Cálculo da evaporação de albufeiras de grande regularização do sul de Portugal*, in Departamento de Engenharia Rural. 2009. p. 269.
208. Kang, S, Heo, S and Lee, JH, *Techno-economic Analysis of Microalgae-Based Lipid Production: Considering Influences of Microalgal Species*. Industrial & Engineering Chemistry Research, 2019. 58: p. 944-955.
209. Carvalho, AP, Meireles, LA and Malcata, FX, *Microalgal Reactors: A Review of Enclosed System Designs and Performances*. Biotechnology Progress, 2006. 22: p. 1490-1506.
210. Zbinden, M, Sturm, B, Nord, R, Carey, W, Moore, D, Shinogle, H and Stagg-Williams, S, *Pulsed Electric Field (PEF) as an intensification pretreatment for greener solvent lipid extraction from microalgae*. Biotechnology and bioengineering, 2013. 110.
211. Joannes, C, Sipaut, C, Dayou, J, Yasir, S and Mansa, R, *Review Paper on Cell Membrane Electroporation of Microalgae using Electric Field Treatment Method for Microalgae Lipid Extraction*. IOP Conference Series: Materials Science and Engineering, 2015. 78.
212. Kinnunen, V, Craggs, R and Rintala, J, *Influence of temperature and pretreatments on the anaerobic digestion of wastewater grown microalgae in a laboratory-scale accumulating-volume reactor*. Water Research, 2014. 57: p. 247-257.
213. Tran, KC, Mendoza Martin, JL, Heaven, S, Banks, CJ, Acien Fernandez, FG and Molina Grima, E, *Cultivation and anaerobic digestion of Scenedesmus spp. grown in a pilot-scale open raceway*. Algal Research, 2014. 5: p. 95-102.
214. Acién, FG, Molina, E, Reis, A, Torzillo, G, Zittelli, GC, Sepúlveda, C and Masojídek, J, *1 - Photobioreactors for the production of microalgae*, in *Microalgae-Based Biofuels and Bioproducts*, C. Gonzalez-Fernandez and R. Muñoz, Editors. 2017, Woodhead Publishing. p. 1-44.
215. Gonçalves, AL, Rodrigues, CM, Pires, JCM and Simões, M, *The effect of increasing CO₂ concentrations on its capture, biomass production and*

- wastewater bioremediation by microalgae and cyanobacteria. *Algal Research*, 2016. 14: p. 127-136.
216. Porto, B, Gonçalves, A, Esteves, A, Guelli Souza, S, Souza, A, Vilar, V and Pires, J, *Microalgal Growth in Paper Industry Effluent: Coupling Biomass Production with Nutrients Removal*. *Applied Sciences*, 2020. 10: p. 3009.
 217. Vandamme, D, *Flocculation Based Harvesting Processes for Microalgae Biomass Production*, in *Faculty of Bioscience Engineering*. 2013, Katholieke Universiteit Leuven, Belgium.
 218. Fasaeei, F, Bitter, JH, Slegers, PM and van Boxtel, AJB, *Techno-economic evaluation of microalgae harvesting and dewatering systems*. *Algal Research*, 2018. 31: p. 347-362.
 219. Najjar, YSH and Abu-Shamleh, A, *Harvesting of microalgae by centrifugation for biodiesel production: A review*. *Algal Research*, 2020. 51: p. 102046.
 220. Jankowska, E, Sahu, AK and Oleskowicz-Popiel, P, *Biogas from microalgae: Review on microalgae's cultivation, harvesting and pretreatment for anaerobic digestion*. *Renewable and Sustainable Energy Reviews*, 2017. 75: p. 692-709.
 221. Klassen, V, Blifernez-Klassen, O, Wibberg, D, Winkler, A, Kalinowski, J, Posten, C and Kruse, O, *Highly efficient methane generation from untreated microalgae biomass*. *Biotechnology for Biofuels*, 2017. 10: p. 186.
 222. Lundquist, T, Woertz, I, Quinn, N and Benemann, J, *A Realistic Technology and Engineering Assessment of Algae Biofuel Production*. *Energy*, 2010. October: p. 1.
 223. Milledge, J, Nielsen, B, Maneein, S and Harvey, P, *A Brief Review of Anaerobic Digestion of Algae for Bioenergy*. *Energies*, 2019. 12: p. 1166.
 224. Khare, V, Khare, C, Nema, S and Baredar, P, *Chapter 4 - Optimum Sizing and Modeling of Tidal Energy Systems*, in *Tidal Energy Systems*, V. Khare, et al., Editors. 2019, Elsevier. p. 189-241.
 225. Annan, M and Gooda, EA, *Effect of minor losses during steady flow in transmission pipelines - Case study "water transmission system upgrade in Northern Saudi Arabia"*. *Alexandria Engineering Journal*, 2018. 57: p. 4299-4305.
 226. Kudela, H, *Hydraulic losses in pipes*. Wroclaw University of Science, 2012.
 227. Li, Y, Zhang, Q, Wang, Z, Wu, X and Cong, W, *Evaluation of power consumption of paddle wheel in an open raceway pond*. *Bioprocess and Biosystems Engineering*, 2014. 37: p. 1325-1336.

228. Vitázek, I, Klúčik, J, Mikulová, Z and Vereš, P. *Effects on concentration of selected gaseous emissions at biomass combustion*. in *AIP Conference Proceedings*. 2016. AIP Publishing LLC.
229. Programme, UNE, *Electrical Energy Equipment: Fans and Blowers*, in *Energy Efficiency Guide for Industria in Asia*. 2006.
230. Milledge, JJ and Heaven, S, *Disc stack centrifugation separation and cell disruption of microalgae: A technical note*. Environment and Natural Resources Research, 2011. 1: p. 17.
231. Flisar, K, Meglic, SH, Morelj, J, Golob, J and Miklavcic, D, *Testing a prototype pulse generator for a continuous flow system and its use for E. coli inactivation and microalgae lipid extraction*. Bioelectrochemistry, 2014. 100: p. 44-51.
232. AB, SGC, *Basic data on biogas*. Sweden, 2012.
233. energypedia. *Biogas Basics*. 19 September 2018 [cited 2020 22 July]; Available from: https://energypedia.info/wiki/Biogas_Basics.
234. Pöschl, M, Ward, S and Owende, P, *Evaluation of energy efficiency of various biogas production and utilization pathways*. Applied Energy, 2010. 87: p. 3305-3321.
235. Fux, C and Siegrist, H, *Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitrification/anammox: Environmental and economical considerations*. Water science and technology : a journal of the International Association on Water Pollution Research, 2004. 50: p. 19-26.
236. Zamalloa, C, Vulsteke, E, Albrecht, J and Verstraete, W, *The techno-economic potential of renewable energy through the anaerobic digestion of microalgae*. Bioresource Technology, 2011. 102: p. 1149-1158.
237. PORDATA. *Preços da eletricidade para utilizadores domésticos e industriais (Euro/ECU)*. 2019 [cited 2020 24 of July]; Available from: [https://www.pordata.pt/Europa/Pre%C3%A7os+da+electricidade+para+utilizadores+dom%C3%A9sticos+e+industriais+\(Euro+ECU\)-1477-313029](https://www.pordata.pt/Europa/Pre%C3%A7os+da+electricidade+para+utilizadores+dom%C3%A9sticos+e+industriais+(Euro+ECU)-1477-313029).
238. Acién, FG, Fernández, JM, Magán, JJ and Molina, E, *Production cost of a real microalgae production plant and strategies to reduce it*. Biotechnology Advances, 2012. 30: p. 1344-1353.
239. Declercq, B, Delarue, E and D'Haeseleer, W, *Impact of the economic recession on the European power sector's CO2 emissions*. Energy Policy, 2011. 39: p. 1677-1686.
240. PORDATA. *Taxa de Inflação (Taxa de Variação do Índice de Preços no Consumidor): total e por consumo individual por objetivo*. [cited 2020 2

- August]; Available from:
<https://www.pordata.pt/DB/Portugal/Ambiente+de+Consulta/Tabela>.
241. Hoffman, J, *Techno-economic assessment of micro-algae production systems*. 2016.
 242. Areias, R, Figueiredo, J and Verschoor, L. *Corporate - Taxes on corporate income*. 2020 30 July 2020 [cited 2020 2 August]; Available from:
<https://taxsummaries.pwc.com/portugal/corporate/taxes-on-corporate-income>.
 243. Damodaran, A. *Cost of Capital by sector (US)*. 2020 [cited 2020 20 of July]; Available from:
http://people.stern.nyu.edu/adamodar/New_Home_Page/datafile/wacc.htm
 244. Towler, G and Sinnott, R, *Chemical engineering design: principles, practice and economics of plant and process design*. 2012: Elsevier.
 245. AHDB, *GB Fertiliser Price Market Update*. 2020.
 246. Sayre, R, *Microalgae: The Potential for Carbon Capture*. BioScience, 2010. 60: p. 722-727.
 247. Tao, R, Kinnunen, V, Praveenkumar, R, Lakaniemi, A-M and Rintala, JA, *Comparison of Scenedesmus acuminatus and Chlorella vulgaris cultivation in liquid digestates from anaerobic digestion of pulp and paper industry and municipal wastewater treatment sludge*. Journal of Applied Phycology, 2017. 29: p. 2845-2856.
 248. Polishchuk, A, Valev, D, Tarvainen, M, Mishra, S, Kinnunen, V, Antal, T, Yang, B, Rintala, J and Tyystjärvi, E, *Cultivation of Nannochloropsis for eicosapentaenoic acid production in wastewaters of pulp and paper industry*. Bioresource Technology, 2015. 193: p. 469-476.
 249. Alam, MA and Wang, Z, *Microalgae biotechnology for development of biofuel and wastewater treatment*. 2019: Springer.
 250. Frumento, D, Casazza, AA, Al Arni, S and Converti, A, *Cultivation of Chlorella vulgaris in tubular photobioreactors: A lipid source for biodiesel production*. Biochemical Engineering Journal, 2013. 81: p. 120-125.
 251. Sarker, NK and Salam, PA, *Indoor and outdoor cultivation of Chlorella vulgaris and its application in wastewater treatment in a tropical city—Bangkok, Thailand*. SN Applied Sciences, 2019. 1: p. 1645.
 252. Melo, RGd, Andrade, AFd, Bezerra, RP, Correia, DS, Souza, VCd, Brasileiro-Vidal, AC, Viana Marques, DdA and Porto, ALF, *Chlorella vulgaris mixotrophic*

- growth enhanced biomass productivity and reduced toxicity from agro-industrial by-products*. Chemosphere, 2018. 204: p. 344-350.
253. Blair, MF, Kokabian, B and Gude, VG, *Light and growth medium effect on Chlorella vulgaris biomass production*. Journal of Environmental Chemical Engineering, 2014. 2: p. 665-674.
 254. Silva, NFP, Gonçalves, AL, Moreira, FC, Silva, TFCV, Martins, FG, Alvim-Ferraz, MCM, Boaventura, RAR, Vilar, VJP and Pires, JCM, *Towards sustainable microalgal biomass production by phycoremediation of a synthetic wastewater: A kinetic study*. Algal Research, 2015. 11: p. 350-358.
 255. Gonçalves, AL, Pires, JCM and Simões, M, *A review on the use of microalgal consortia for wastewater treatment*. Algal Research, 2017. 24: p. 403-415.
 256. Pereira, SFL, Gonçalves, AL, Moreira, FC, Silva, TFCV, Vilar, VJP and Pires, JCM, *Nitrogen Removal from Landfill Leachate by Microalgae*. International journal of molecular sciences, 2016. 17: p. 1926.
 257. Schlagermann, P, Göttlicher, G, Dillschneider, R, Rosello-Sastre, R and Posten, C, *Composition of Algal Oil and Its Potential as Biofuel*. Journal of Combustion, 2012. 2012: p. 285185.
 258. Green, DW and Perry, RH, *Perry's Chemical Engineers' Handbook/edición Don W. Green y Robert H. Perry*. 2019.
 259. Benemann, J and Oswald, WJ, *Systems and Economic Analysis of Microalgal Ponds for Conversion of CO₂ to Biomass, Final Report to the US Department of Energy*. Pittsburgh Energy Technology Center. 1996.
 260. AB, G, *ECOMAX - The most recognized modular CHP package solution in the world*, C.w. AB, Editor.
 261. Portuguesa, R, *Roteiro para a Neutralidade Carbónica 2050*, A.e.T. Energética, Editor. 2019.
 262. Pulster, EL, *Assessment of public health risks associated with petrochemical emissions surrounding an oil refinery*. 2015.
 263. Nations, U, *The Sustainable Development Goals Report*. 2020.
 264. Singh, RK, Murty, HR, Gupta, SK and Dikshit, AK, *An overview of sustainability assessment methodologies*. Ecological Indicators, 2012. 15: p. 281-299.
 265. Sacramento-Rivero, JC, Navarro-Pineda, F and Vilchiz-Bravo, LE, *Evaluating the sustainability of biorefineries at the conceptual design stage*. Chemical Engineering Research and Design, 2016. 107: p. 167-180.

Annexes

A.1. Calibration curve for nitrate quantification

Figure A.1:1 shows the calibration curve of absorbance measured at 410 nm *versus* nitrate concentration in mg L⁻¹. Table A.1:1 shows the validation parameters for this calibration curve.

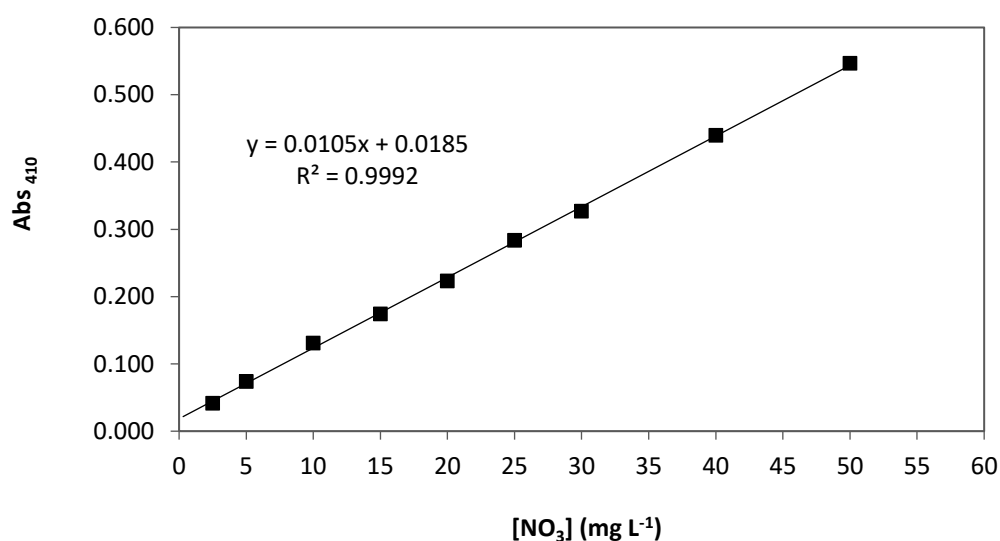


Figure A.1:1. Calibration curve of absorbance measured at 410 nm as a function of nitrate concentration in mg L⁻¹.

Table A.1:1. Validation parameters of calibration curve for nitrate quantification

Validation Parameters			
$a \pm s_a$	$b \pm s_b$	LOD (mg L ⁻¹)	LOQ (mg L ⁻¹)
0.0105 ± 0.0001	0.019 ± 0.002	0.6730	2.243

LOD- limit of detection; LOQ- limit of quantification

A.2. Temperature and pH values for the different culture medium conditions and positive and negative control

The maximum, minimum and average temperature and pH obtained throughout the *C. vulgaris* growth experiments using different effluent compositions are presented in the Table A.2:1:

Table A.2:1. Temperature and pH values determined over the cultivation period for the different experiments

Assay	T _{avg} (°C)	T _{min} (°C)	T _{max} (°C)	pH _{avg}	pH _{min}	pH _{max}
20%	18 ± 1	15.9	20.6	8.3 ± 0.3	7.3	8.6
40%	18 ± 1	16.1	21.6	8.9 ± 0.5	7.4	9.5
60%	19 ± 2	15.8	22.4	9.2 ± 0.6	7.5	9.7
80%	20 ± 2	15.3	23.4	9.3 ± 0.6	7.6	10.1
100%	20 ± 2	15.1	23.8	9.2 ± 0.6	7.5	9.8
Positive Control (C+)	18 ± 2	15.5	20.6	9.2 ± 0.7	7.5	10.1
Negative Control (C-)	17 ± 1	15.2	19.1	8.6 ± 0.4	7.5	8.7

A.3. Calibration curve for biomass quantification

Figure A.3:1 shows the calibration curve of OD₆₈₀ *versus* biomass concentration, in mg DW L⁻¹.

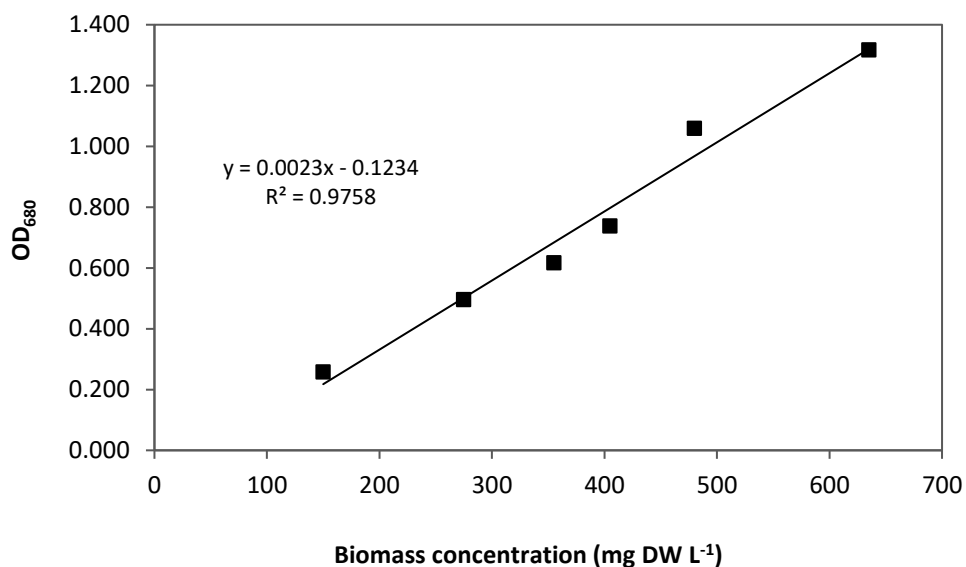


Figure A.3:1. Calibration curve of OD680 versus biomass concentration in mg DW L⁻¹.

Table A.3:1. Validation parameters of calibration curve for biomass quantification

Validation Parameters			
$a \pm s_a$	$b \pm s_b$	LOD (mg DW L ⁻¹)	LOQ (mg DW L ⁻¹)
0.0023 ± 0.0002	-0.123 ± 0.008	98.14	327.1

R²- coefficient of determination; LOD- limit of detection; LOQ- limit of quantification

A.4. Paper industry effluent characteristics

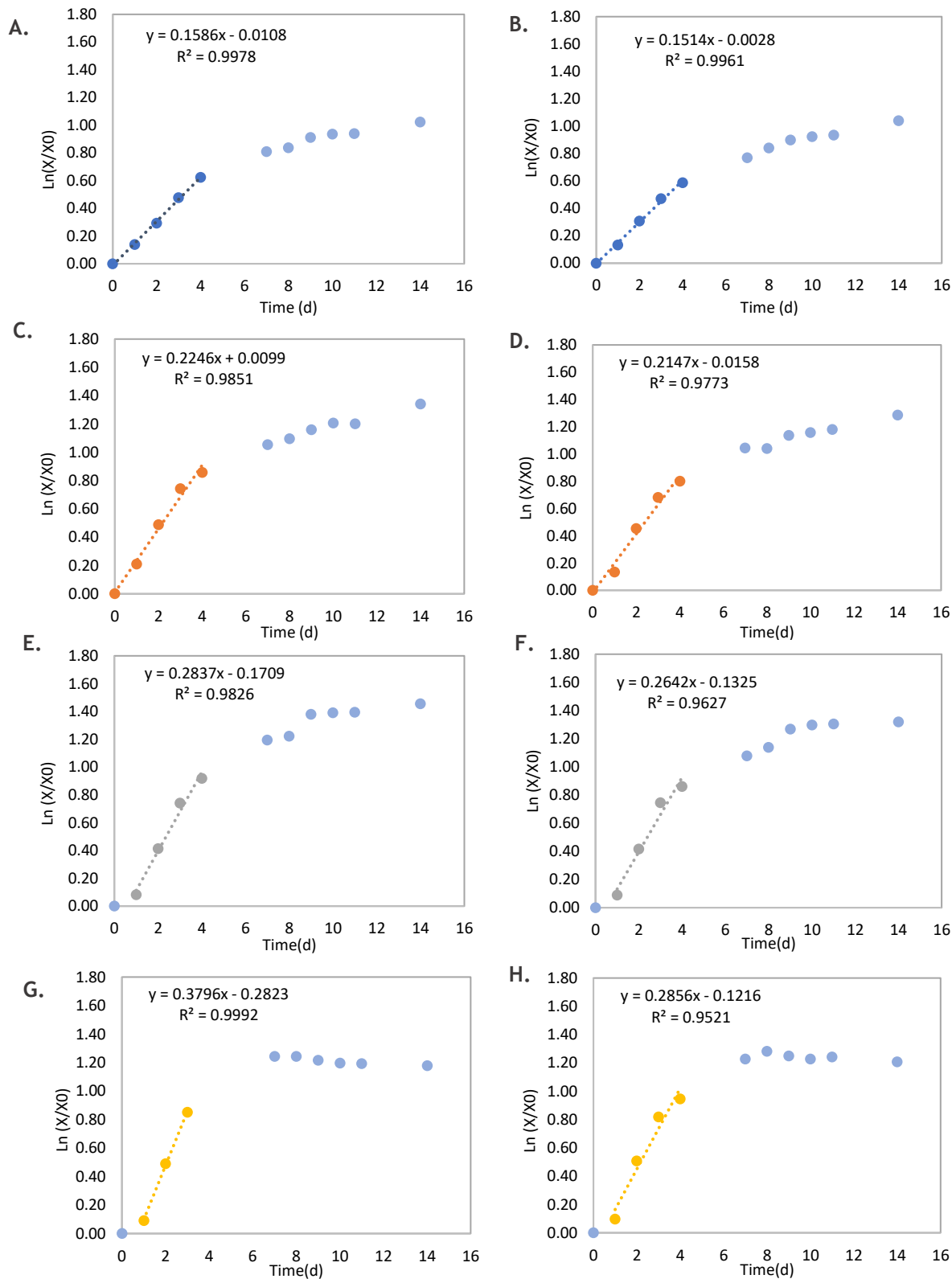
Table A.4:1 presents the values of the monthly flows of treated effluent, as well as the values of nitrogen and phosphorus concentrations in the treated effluent from the paper industry. All data presented are for the year of 2019 and were provided by the company itself.

Table A.4:1. Nitrogen and phosphorus concentrations in the paper industry effluent after biological treatment on the company WWTP (Data from 2019)

Month	Flow (m ³)	N (mg L ⁻¹)	P (mg L ⁻¹)
January	1 220 179	2.8	3.9
February	1 128 272	2.0	4.2
March	1 260 679	2.4	2.3
April	90 5031	2.3	1.9
May	1 268 557	2.4	3.0
June	1 210 138	1.6	4.5
July	1 272 890	2.5	1.4
August	1 319 607	2.1	3.5
September	1 278 195	1.8	3.9
October	1 321 312	1.6	3.9
November	1 361 827	2.2	3.5
December	1 324 695	1.9	3.0
Average	1 239 282	2.1	3.3

A.5. Graphical representation of $\ln(X/X_0)$ as a function of time for the different culture medium conditions and positive control

Figure A.5:1 presents the Graphical representation of $\ln(X/X_0)$ as a function of time for different culture medium conditions.



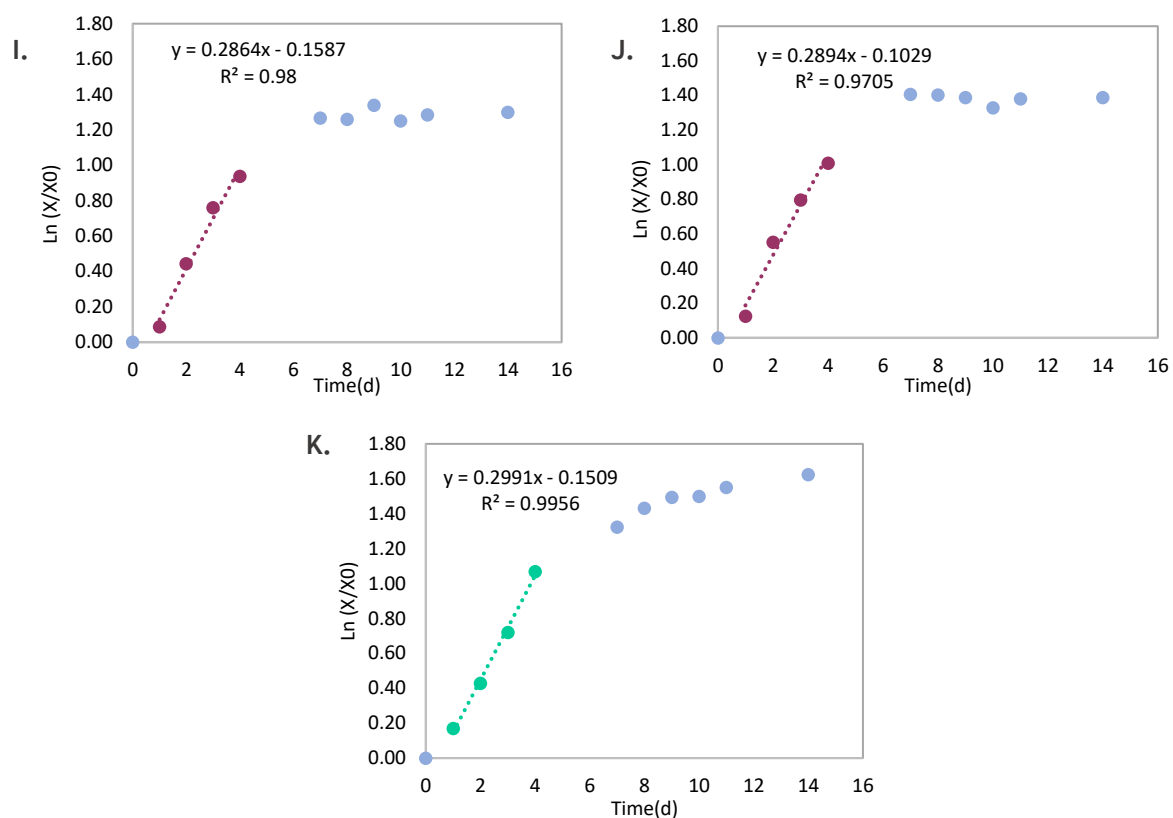


Figure A.5:1. Graphical representation of $\ln(X/X_0)$ as a function of time for different culture medium conditions: (A) 20%, assay 1; (B) 20%, assay 2; (C) 40%, assay 1; (D) 40%, assay 2; (E) 60%, assay 1; (F) 60%, assay 2; (G) 80%, assay 1; (H); 80%, assay 2; (I) 100%, assay 1; (J) 100%, assay 2; and (K) Positive control.