NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF SCIENCES AND TECHNOLOGY OF BIOMASS

BIOREMEDIATION OF AGRO-INDUSTRIAL EFFLUENTS MEDIATED BY MICROALGAE

CATARINA VIEGAS DE SOUSA

BSc Agronomic Engineering

Master in Energy and Bioenergy

DOCTORATE IN BIOENERGY

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To my mother.

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Abstract

Every year, large amounts of agro-industrial effluents are produced all over the world and its sustainable management is still a technological challenge. This thesis addresses the remediation of four agro-industrial effluents (aquaculture, cattle, swine, and poultry) and an industrial effluent (landfill leachate) by treatment with biomass ash and microalgae. The pre-treatment with biomass ash allowed the partial precipitation of total solids, reduction of the turbidity and microbial load of the effluents, resulting in a partially treated effluent and a precipitate rich in mineral and organic components. The aqueous effluents were treated in batch and semi-continuous modes with the microalgae *Chlorella vulgaris* (Cv), *Auxenochlorella protothecoides* (Ap), *Tetradesmus obliquus* (To), *Isochrysis galbana* (Ig), *Microchloropsis salina* (Ms), and *Spirulina major* (Sm). Maximum biomass yields were reached for microalgae Cv (193.6 to 879.8 mg L^{-1} day^{-1}) and To (236.7 to 811.7 mg L^{-1} day^{-1}) in agro-industrial effluents. The remediation of effluents allowed reaching discharge values mandatory by law for total nitrogen and total phosphorus, COD, BOD_5, and total solids. The precipitate obtained in the pre-treatment of the effluents and the algal biomass showed positive effects as biostimulants for the germination of watercress (*Nasturtium officinale*) and wheat (*Triticum aestivum*) seeds. The microalgae biomass was characterised and evaluated as a food supplement for mussels (*Mytilus edulis*) for 45 days, with changes in the contents of lipids, carbohydrates, and ash of the mussels being observed. The torrefaction of algae biomass and its mixtures with lignocellulosic biomass made it possible to obtain biochars with potential for energy recovery, use as biostimulants for seed germination, or as adsorbents for cationic pigments. The work carried out allowed to demonstrate the feasibility of treating the studied effluents by chemical precipitation and bioremediation with microalgae and to suggest different ways of valuing the solid by-products generated.

Keywords: microalgae bioremediation; physicochemical pre-treatment; torrefaction; agro-industrial effluents; fertilizer; biostimulant.

Resumo

Anualmente, grandes quantidades de efluentes agroindustriais são produzidas em todo o mundo e a sua gestão sustentável constitui, ainda, um desafio tecnológico. Esta tese aborda a remediação de quatro efluentes agroindustriais (aquacultura, gado bovino, gado suíno e aviário) e um efluente industrial (lixiviado de aterro) por tratamento com cinzas de biomassa e microalgas. O pré-tratamento com cinzas de biomassa permitiu provocar uma precipitação parcial dos sólidos totais, reduzir a turvação e a carga microbiana dos efluentes, originando um efluente parcialmente tratado e um precipitado rico em componentes minerais e orgânicos. Os efluentes aquosos foram tratados em modos descontínuo e semi-contínuo com as microalgas *Chlorella vulgaris* (Cv), *Auxenochlorella protothecoides* (Ap), *Tetradesmus obliquus* (To), *Isochrysis galbana* (Ig), *Microchloropsis salina* (Ms) e *Spirulina major* (Sm). Foram atingidas produtividades máximas de biomassa para as microalgas Cv de 193,6 a 879,8 mg L^{-1} dia^{-1} e To de 236,7 a 811,7 mg L^{-1} dia^{-1} em efluentes agroindustriais. A remediação dos efluentes permitiu atingir valores de descarga obrigatórios por lei para azoto total, fósforo total, CQO, CBO_5 e sólidos totais. O precipitado obtido no pré-tratamento dos efluentes e a biomassa algal apresentaram efeitos positivos como bioestimulantes da germinação de sementes de agrião (*Nasturtium officinale*) e de trigo (*Triticum aestivum*). A biomassa microalgal foi caracterizada e avaliada como suplemento alimentar de mexilhões (*Mytilus edulis*) durante 45 dias, tendo-se observado alterações nos teores de lípidos, hidratos de carbono e cinzas dos mexilhões. A torrefação de biomassa algal e das suas misturas com biomassa lenhocelulósica permitiu obter biocarvões com potencial para valorização energética, utilização como bioestimulantes na germinação de sementes ou adsorventes para pigmentos catiónicos. O trabalho realizado demonstrou a viabilidade do tratamento dos efluentes analisados por precipitação química e biorremediação com microalgas e sugerir diferentes vias de valorização dos subprodutos sólidos gerados.

Palavras-chave: biorremediação por microalgas; pré-tratamento físico-químico; torrefação; efluentes agroindustriais; fertilizante; bioestimulante.

Résumé

Chaque année, de grandes quantités d'effluents agro-industriels sont produites partout dans le monde et leur gestion durable reste un défi technologique. Cette thèse porte sur la dépollution de quatre effluents agro-industriels (aquaculture, bovins, porcs et volailles) et d'un effluent industriel (lixiviat de décharge) par traitement avec des cendres de biomasse et des microalgues. Le prétraitement aux cendres de biomasse a permis la précipitation partielle des solides totaux, la réduction de la turbidité et de la charge microbienne des effluents, résultant en un effluent partiellement traité et un précipité riche en composants minéraux et organiques. Les effluents aqueux ont été traités en mode batch et semi-continu avec les microalgues *Chlorella vulgaris* (Cv), *Auxenochlorella protothecoides* (Ap), *Tetradesmus obliquus* (To), *Isochrysis galbana* (Ig), *Microchloropsis salina* (Ms) et *Spirulina major* (Sm). Des rendements maximaux de biomasse ont été atteints pour les microalgues Cv (193,6 à 879,8 mg L^{-1} $jour^{-1}$) et To (236,7 à 811,7 mg L^{-1} $jour^{-1}$) dans les effluents agro-industriels. La dépollution des effluents a permis d'atteindre les valeurs de rejets imposées par la loi pour l'azote total et le phosphore total, la DCO, la DBO_5 et les solides totaux. Le précipité obtenu lors du prétraitement des effluents et de la biomasse algale a montré des effets positifs en tant que biostimulants pour la germination des graines de cresson (*Nasturtium officinale*) et de blé (*Triticum aestivum*). La biomasse de microalgues a été caractérisée et évaluée en tant que complément alimentaire pour moules (*Mytilus edulis*) pendant 45 jours, avec des modifications des teneurs en lipides, glucides et cendres des moules. La torréfaction de la biomasse algale et ses mélanges avec la biomasse lignocellulosique ont permis d'obtenir des biochars à potentiel de valorisation énergétique, utilisables comme biostimulants pour la germination des graines ou comme adsorbants pour les pigments cationiques. Les travaux réalisés ont permis de démontrer la faisabilité du traitement des effluents étudiés par précipitation chimique et bioremédiation aux microalgues et de proposer différentes voies de valorisation des sous-produits solides générés.

Mots-clés: bioremédiation avec microalgues; prétraitement physico-chimique; torréfaction; effluents agro-industriels; fertilisant; biostimulant.

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Abbreviations

ACOI	*Algoteca da Universidade de Coimbra, Portugal* - Coimbra Collection of Algae
AFDW	Ash-free dry weight
Ap	*Auxenochlorella protothecoides*
ar	As received
Ash	Ash content
ASTM	American Society for Testing and Materials
ASW	Artificial seawater medium
BOD_5	Biochemical oxygen demand (5 days)
CCAP	Culture Collection of Algae and Protozoa, Scottish Marine Institute, U.K.
CHNS	Carbone, hydrogen, nitrogen and sulphur
CI	Condition Index
CITRI	*Centro Integrado para o Tratamento de Resíduos Industriais, S.A.*
COD	Chemical oxygen demand
Cv	*Chlorella vulgaris*
cw	carcass weight
daf	Dry ash free basis
db	Dry basis
DNS	Dinitro salicylic acid
dw	Dry weight
EU	European Union
FC	Fixed carbon
FT-IR	Fourier Transform Infrared Spectroscopy
GC-FID	Gas Chromatography – Flame Ionization Detector
GC-MS	Gas Chromatography coupled with Mass Spectrometry
GHG	Greenhouse gas
GI	Germination index
HHV	High heating value
HRAP	High rate algal ponds
HRT	Hydraulic retention time
HTC	Hydrothermal carbonization
ICP-AES	Inductively Coupled Plasma – Atomic Emission Spectrometer
Ig	*Isochrysis galbana*
INETI	*Instituto Nacional de Engenharia e Tecnologia e Inovação*
Lc	Lignocellulosic material
LNEG	*Laboratório Nacional de Energia e Geologia, LNEG, Portugal*
MB	Methylene blue
Ms	*Microchloropsis salina*
OD_{540}	Optical density at 540 nm
OMW	Olive-oil mill wastewater

P+A	Piggery effluent with ash
P+A+O	Piggery effluent with ash and olive-oil mill wastewater
PE	Poultry effluent
PE+A	Poultry effluent with ash
PEE	Process energy efficiency
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
R1	Reactor 1
R2	Reactor 2
R3	Reactor 3
RP	Raw piggery effluent
RSM	Response surface methodology
SD	Standard deviation
Sm	*Spirulina major*
TN	Total nitrogen
To	*Tetradesmus obliquus*
TP	Total phosphorus
UBB	*Unidade de Bioenergia e Biorrefinarias*
UTEX	Culture Collection of Algae at the University of Texas at Austin, USA
VM	Volatile matter
VT	Transference volume

Symbols

C/N	Carbon-nitrogen ratio
C/P	Carbon-phosphorus ratio
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C16:2	Hexadecadienoic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
C18:3	Linolenic acid
C22:0	Behenic acid
C24:0	Lignoceric acid
Cf	Final concentration
Ci	Initial concentration
CLA	Conjugated linoleic acid
Cp_{H2O}	Specific heat of water (4.138×10^{-3} MJ Kg^{-1} K^{-1})
DHA	Docosahexaenoic acid
H/C	Hidrogen-carbon ratio
Inc.	Incorporation
L	Latent heat of water vaporization (2.26 MJ Kg^{-1})

m	Mass
M	Moisture
O/C	Oxigen-carbon ratio
q	Adsorption capacity
Q_1	Energy needed to heat the feedstock
Q_2	Energy needed for heating the water
Q_3	Latent heat of water vaporization
Q_4	Heat of reaction for the torrefaction process
Q_5	Thermal losses
Q_{input}	Total energy requirements
Q_{output}	Energy contained in the process products
T	Temperature
t	Time
ΔT	Temperature variation
(v/v)	Volume per volume
wt	Weight

Units

cm	Centimetre
g	Gram
h	Hour
J	Joule
K	Kelvin
kg	Kilogram
L	Litre
m	Metre
m^3	Cubic metre
min	Minute
Mj	Mega joule
mL	Millilitre
mS	Micro Siemens
nm	Nanometre
°C	Degree Celsius
s	Second
µE	Micro Einstein
µm	Micrometre

Chapter 1

Introduction

1.1 Framework and relevance of the study

The treatment of effluents, waste and end-of-life materials is currently one of the greatest challenges facing science globally because of the huge amounts of resources and energy required and the vast impacts on the environment.

Livestock for food production, such as manure, slurry, poultry, and aquaculture, generates large amounts of aqueous waste, with washing water from production units and animal remains such as skins and viscera. These agro-industrial effluents are a considerable source of pollution for marine and freshwater ecosystems due to their high organic load, requiring physicochemical treatments before they can be discharged, mostly involving stabilization ponds and anaerobic digestion reactors. The treatments in the stabilization ponds take time and involve a lot of energy on the aeration for bacteria oxidation processes, the loss to the atmosphere of CO_2, and several compounds by degradation, mainly CH_4 and H_2S. The use of anaerobic digestion to decompose these effluents is expensive and complex, time-consuming, and limited to certain ranges of chemical and organic load. In addition, a digestate is obtained which should be rid of. In less developed countries these agro-industrial effluents are mostly discharged into water courses, rivers, groundwater, and soils, without any treatment with tremendous impact on the environment and people's health. More than 80% of wastewater worldwide is discharged into water bodies without any treatment (United Nations, 2021). Thus, in addition to potentially being a source of pollution, its full potential of use is not being extracted, namely concerning its energy or intrinsic composition. The removal of the organic load and the high levels of nitrogen and phosphorus in these effluents can be mediated by microalgae, instead of resorting almost exclusively to the use of physicochemical methods. Hence, the potential for eutrophication of water bodies can be reduced, and the available nitrogen and phosphorus can be efficiently recovered and recycled to produce algal biomass. There are very robust and versatile microalgae with an extraordinary capacity to develop and remedy

agro-industrial effluents. Their needs are reduced, and handling is not complex. These microorganisms originate biomass rich in protein, carbohydrates, and lipids, which can be used in several applications such as feed, fertilization, and energy applications. This approach allows the subsequent integration of the produced biomass in the formulation of agricultural fertilizers and animal feed, reducing the carbon footprint of the agri-food industries. Still, it contributes to a circular economy by reusing the residuals produced in these industries, targeting zero waste, transforming them into products with added value.

European Union guidelines and strategy for waste management, presented in Directive 2008/98/EC, state that "the following waste hierarchy shall apply as a priority order in waste prevention, management legislation and policy: prevention, preparing for re-use, recycling, energy recovery and disposal" (European Commission, 2015). This hierarchy ranks valorisation higher than disposal methods such as landfilling.

Microalgae are organisms with extremely attractive nutritional characteristics due to their balanced composition and abundance in proteins, with a good profile in essential amino acids and oils, and with important polyunsaturated fatty acids. Its incorporation in animal feed diets would greatly increase the nutritional value of these diets and, therefore, healthier animals, potentially originating a richer meat. Today's consumers are increasingly aware of these issues and value the way animals are produced, besides the healthy concerns, which, from a marketing perspective, would also be an advantage.

The present thesis presents a way of treating several agro-industrial effluents using simple and inexpensive remediation techniques. The innovation of this approach is the use of a pre-treatment with biomass ash, which leads to the precipitation of dissolved and suspended compounds due to the rise in pH between 12 and 12.5, decreasing the nitrogen, phosphorus, and chemical oxygen demand (COD) loads. It is intended to be used in effluents with high densities and reduced light penetrability, without using high dilution rates. Mostly projected to be applied by small agro-industrial companies that do not have large economic or technical resources but aim to be more sustainable and maximize their available resources, for a circular economy concept. The choice of torrefaction as a thermochemical conversion process for the microalgae biomass is proposed to be less energy demanding since it can work without pressure, as opposed to hydrothermal liquefaction or pyrolysis, but also because it is a simple process to perform that does not require great technology or specialized knowledge. In addition, the main product is a char that can be used directly without the need for a pre-treatment, as in the case of bio-oil.

1.2 Research Goals

This work aims to contribute to the study and implementation of solutions for the treatment of several agro-industrial effluents, with the use of microalgae and subsequently the use of the obtained add value biomass for several applications, to reduce the carbon footprint of the agri-food industries and foster a circular economy concept. In particular, it is intended to develop an integrated treatment system, able to remediate effluents with large contents of suspended solids, dark coloration, high values of chemical oxygen demand, and presence of recalcitrant organics without resorting to high dilution rates as a strategy to overcome these negative characteristics. The next figure (Figure 1.1) comprises all stages of the practical work developed in the present thesis.

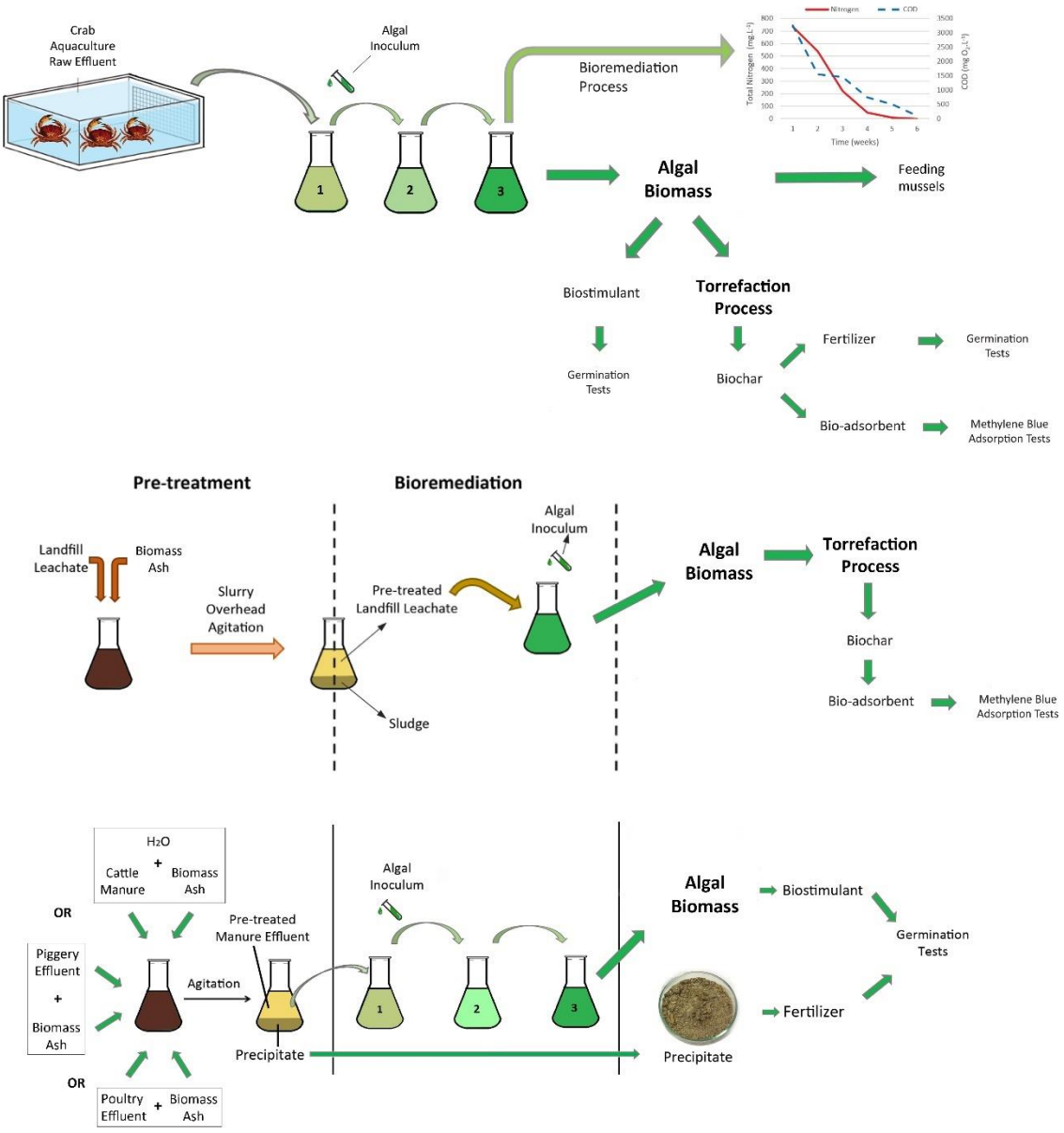

Figure 1.1 – Graphical abstract of the developed work.

It is intended to evaluate the remediation of poultry, piggery, cattle, and aquaculture effluents and landfill leachate using physicochemical pre-treatment processes with forestry biomass ash and then using the pre-treated effluents as microalgae growth media. Enhance the use of algal biomass obtained in the bioremediation processes, namely by testing it as biostimulant and studying its composition to integrate into animal feed, but also as char for energy purposes or as activated carbon for the purposes of filtration and cleaning effluents with dyes.

Finally, this work also encompasses a bioenergy perspective, by applying the torrefaction process to microalgae biomass with different incorporation rates of lignocellulosic material, using different temperatures, residence times, and moisture rates, to evaluate the energy efficiency, mass yield, and energy yield of the process.

1.3 Thesis outline

- **Chapter 1 – Introduction**

This chapter defines the framework of the thesis, and the relevance of the study as well as the main objectives and the thesis outline.

- **Chapter 2 – State of the art**

Chapter 2 represents the knowledge up to now on the topics of this thesis, namely wastewater remediation by conventional treatments, but also with microalgae; the valorisation of algal biomass as an integral part of animal feed, as a fertilizer, and as an energy vector through its conversion into liquid and solid biofuels.

- **Chapter 3 – Methodology**

Chapter 3 describes the entire methodology used in effluent characterization and pre-treatment, of the different bioremediation assays, the characterization of algal biomass and precipitate, within the potential of them on fertilization and biostimulation effects, and finally the biomass torrefaction process and the characteristics of the obtained biofuels.

- **Chapter 4 – Pre-treatment evaluation and precipitate characterization**

In this chapter, the pre-treatment carried out with biomass ash for several effluents and the resulting yield in the precipitate and aqueous phase were analysed. The different precipitates obtained were also characterised in this chapter.

- **Chapter 5 – Biomass productivity and wastewaters bioremediation process**

This chapter discusses the results obtained for all the tested effluents in terms of algal biomass productivity attained for the different microalgae used. It also examines the effluents' bioremediation process both in batch and semi-continuous mode proposed.

- **Chapter 6 – Biomass characterization**

Chapter 6 assesses the different microalgal biomass obtained for all the tested effluents in terms of protein, carbohydrates, lipids, methyl esters of fatty acids, and ash content.

- **Chapter 7 – Microalgae biomass applications**

In chapter 7 the different applications were tested using the produced algal biomass. In terms of animal feed, this evaluation is made from a theoretical point of view by comparison of the obtained biomass composition and the feed needs of the respective animal. In this chapter, it was evaluated the use of microalgae biomass produced in aquaculture effluent in the mussels' feeding. The algal biomass precipitate obtained from the treatment of agro-industrial effluents was also tested as a biostimulant in the germination of wheat and watercress seeds.

- **Chapter 8 – Precipitate applications**

In this chapter, the precipitate was tested as fertilizer in the germination of wheat and watercress seeds.

- **Chapter 9 – Microalgae co-torrefaction experiments**

In chapter 9 the co-torrefaction of commercial algal biomass with lignocellulosic material was studied for different parameters: temperature, residence time, microalgae moisture content, and incorporation rate of lignocellulosic material. After studying the torrefaction products and evaluating the performance of the process, the torrefaction conditions were optimized. Finally, tests were carried out with algal biomass grown in the aquaculture effluent and landfill leachate.

- **Chapter 10 – Microalgae biochar applications**

Chapter 10 characterizes the products of the torrefaction of algal biomass grown in wastewaters and analyses the possibility of using the biochars as an energy vector, as a fertilizer in seed germination, and as an adsorbent for cationic dyes was tested.

- **Chapter 11 – Process integration and production potential**

In chapter 11 it is presented an integration of the remediation process at an industrial scale with the production inflows and outflows.

- **Chapter 12 – Conclusions and future perspectives**

This last Chapter refers to the main results achieved in the developed work and what should be explored in the future to complement the current thesis.

The following papers and conference proceedings were based on chapters 4, 5, 6, 7, 8, 9, 10, and 11.

Papers:

Viegas, C., Nobre, C., Correia, R., Gouveia, L., Gonçalves, M., 2021. Optimization of biochar production by co-torrefaction of microalgae and lignocellulosic biomass using response surface methodology; Energies, 14 (21), 7330; https://doi.org/10.3390/en14217330

Viegas, C., Gonçalves, M., 2021. Integrated Treatment of Pig Production Wastewaters Using Pre-treatment with Biomass Ash and Bioremediation by Microalgae. Acta Sci. Agric. 5, 44–57, ISSN: 2581-365X.

Viegas, C., Gouveia, L., Gonçalves, M., 2021. Evaluation of microalgae as bioremediation agent for poultry effluent and biostimulant for germination. Environmental Technology & Innovation. https://doi.org/10.1016/j.eti.2021.102048

Viegas, C., Gouveia, L., Gonçalves, M., 2021. Bioremediation of cattle manure using microalgae after pre-treatment with biomass ash; Bioresource Technology Reports 14; 100681; https://doi.org/10.1016/j.biteb.2021.100681

Viegas, C., Gouveia, L., Gonçalves, M., 2021. Aquaculture wastewater treatment through microalgal. Biomass potential applications on animal feed, agriculture, and energy; Journal of Environmental Management 286; 112187; https://doi.org/ 10.1016/j.jenvman.2021.112187

Viegas, C., Nobre, C., Mota, A., Vilarinho, C., Gouveia, L., Gonçalves, M., 2021. A circular approach for landfill leachate treatment: Chemical precipitation with biomass ash followed by bioremediation through microalgae, Journal of Environmental Chemical Engineering 9; 105187; https://doi.org/10.1016/j.jece.2021.105187

Conference Proceedings:

Viegas, C., Gonçalves, M., 2021. Integrated Treatment of Pig Production Wastewaters Using Pre-treatment with Biomass Ash and Bioremediation by Microalgae. In: Costa Sanches Galvão J.R. *et al.* (eds.) Proceedings of the 1st International Conference on Water Energy Food and Sustainability (ICoWEFS 2021). Springer, Cham. https://doi.org/10.1007/978-3-030-75315-3_29

Viegas, C., Gonçalves, M., Gouveia, L, 2019. Bioremediation of seafood production effluents using microalgae; 5th International Conference - WASTES: Solutions, Treatments and Opportunities, Caparica, Portugal, 4-6 September.

Viegas, C., Gonçalves, M., Jorge, V., Mendes, B., 2018. Bioremediation of bovine wastewater using a pretreatment and microalgae, WasteEng2018 - Proceedings - 7th International Conference on Engineering for Waste and Biomass Valorisation; ISBN: 979-10-91526-07-4 -, Prague (Czech Republic), 2nd - 5th July 2018.

Chapter 2

State of the art

Current technologies for the treatment of agro-industrial and urban wastewaters are complex and expensive procedures. However, these treatments are absolutely necessary since these effluents contain a high organic load and represent an important source of nutrients and pollutants in fresh and saltwater ecosystems (Mo and Zhang, 2012).

The poultry, piggery, and cattle industries produce a huge amount of wastes with significant environmental impact, including manure, and effluents from cleaning activities and processing dead animals (Martinelli *et al.*, 2020). A vast amount of residual effluents are also produced, which includes leachates (Cherubini *et al.*, 2015). In traditional productions and with low herds the mixture of urine, feces, and wastewater are discharged into the soil as fertilizer, but with the increase of intensive explorations, this option is no longer feasible (European Commission, 2015).

The environmental impacts associated with these aqueous effluents are mainly related to their eutrophication potential (Oryschak and Beltranena, 2020). In addition, the direct use of agro-industrial effluents as a fertilizer has negative impacts, such as the emission of unpleasant odours, the contamination of soils with pathogenic microorganisms, and groundwater with compounds from these effluents (Markou *et al.*, 2018). An alternative option may be the use of these effluents as a substrate/culture medium to produce microalgae biomass and to be valorised either as an additive for animal feed or as a biofertilizer (A. Ferreira *et al.*, 2019; Gramegna *et al.*, 2020).

Microalgae have enormous potential as agents for converting solar energy into chemical energy since they have high rates of biomass production, much higher than those of vascular plants. In addition, as they have reduced cultivation needs, cultures can be implanted in degraded lands (*e.g.*, in deserts or off-shore structures) and no need of irrigation water (*e.g.*, in saltwater, brackish, or wastewater) not competing with the food sector (Demirbas, 2011; Suganya *et al.*, 2016).

Microalgae can use nitrogen, phosphorus, and other mineral components present in the wastewaters for their growth, reducing its potential to eutrophicate water bodies and producing a product (algal biomass) with commercial value. In the case of agro-industrial effluents, with high levels of nutrients but not contaminated with heavy metals or other potentially toxic elements, algal biomass can be incorporated in different stages of the industrial process, in a circular economy strategy (Viegas *et al.*, 2021a).

Additionally, these microorganisms can be used in the remediation of agro-industrial effluents, also contributing to the mitigation of CO_2. The fixation of atmospheric CO_2 by microalgae has an efficiency ten times greater than that of terrestrial plants and its biomass can be used to produce a huge variety of products used in human and animal food and in the production of fertilizers and biofuels. These microorganisms often produce other compounds in reduced quantities with high interest for several industries (namely pharmaceutical and cosmetic) and can be exploited exclusively due to these compounds (Fernández *et al.*, 2021). Applications of microalgae biomass (Figure 2.1) include supplements for animal feed (Gouveia *et al.*, 1996; Jacob, 2013; Niccolai *et al.*, 2019; Saeid *et al.*, 2016), biofertilizers for animal feed crops (Deepika and MubarakAli, 2020; Ferreira *et al.*, 2019; Navarro-López *et al.*, 2020) and raw material for the production of biofuels (Ellersdorfer, 2020; Ferreira *et al.*, 2020).

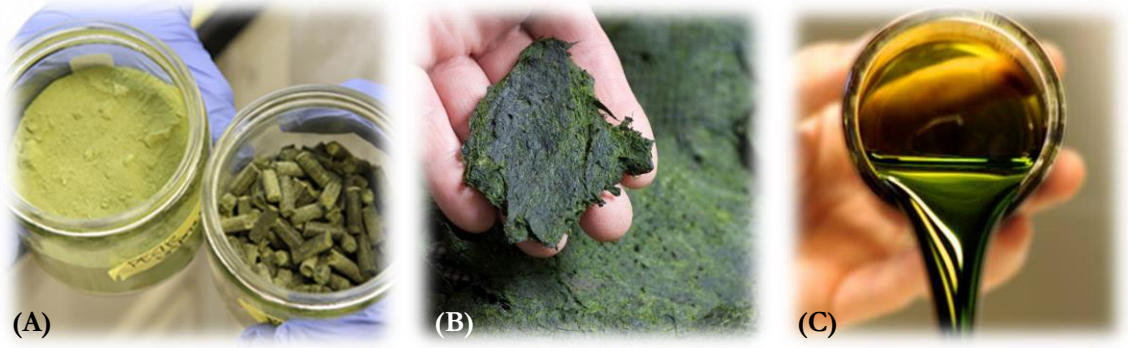

Figure 2.1 – (A) Microalgae powder processed into pellets to feed livestock (animalscience.tamu.edu); (B) Microalgae agglomerate for organic fertilizers production (www.popsci.com); (C) Biodiesel produced from microalgae (www.sapphireenergy.com).

2.1 Wastewater pollution and treatment

Wastewater, whether agro-industrial or urban, is a very significant source of surface water bodies' pollution when discharged into the aquatic environments. In general, these effluents contain many nutrients, such as nitrogen and phosphorus, which will directly

affect aquatic ecosystems due to the eutrophication and degradation they induce (Gouveia *et al.*, 2016), as well as the subterraneous waters.

Conventionally, chemical or electrochemical processes can be used in the treatment of effluents. Traditional wastewater treatment uses a series of processes designed to remove specific compounds. As they were initially intended for the treatment of urban wastewater, they must be improved or modified to be able to treat agro-industrial effluents. It is currently known that the activated sludge-based wastewater treatment (WWT) plants are not sustainable due to their resource-, energy-, and environmental footprints. These processes involve energy to bubble O_2 so that bacteria oxidize organic compounds, the non-recovery of nutrients from wastewater, the release of greenhouse gases, and the production of a sludge without application, which is a problem (Delanka-Pedige *et al.*, 2020). The use of electrochemical processes for the treatment of wastewaters has been increasing in the last two decades. These techniques are mainly used with good rates of degradation in very complex and saline industrial matrices, containing refractory organic pollutants, high toxic organic load, and low biodegradability, but also in tertiary treatment of effluents with micro-contaminants (Salmerón *et al.*, 2021). These industrial effluents result from the petrochemical, textile dyeing, paper mill, and tannery industries, as well as the urban and domestic wastewaters (Garcia-Segura *et al.*, 2018). The main benefits of its use include durability, automation, energy efficiency, selectivity, fewer chemicals used, and environmental compatibility. The process is simple, just applying an electric current or electrode potential and a wide spectrum of compounds can be eliminated. The efficiency of the pollutant removal process depends largely on the type of anode electrodes' material and on the electrochemical parameters, such as current density, time, and agitation (Salmerón *et al.*, 2021; Suman *et al.*, 2021). There are different electrochemical methods, such as electrochemical oxidation, electrochemical reduction, electrocoagulation, indirect electrooxidation with strong oxidants, electrodeionization, capacitive deionization, photo or ultrasound-assisted electrochemical methods, and electrokinetics (Sillanpää and Shestakova, 2017). Despite the efficiency of these processes, the effluents are degraded, and their nutrients are not recycled, nor used for any usable product generated.

The integration of conventional treatments with bioremediation driven by the growth of microalgae has economic and environmental advantages, having been considered the most viable alternative to produce liquid fuels and renewable energy, but mainly and in a short term for agriculture purposes. The idea of using microalgae in wastewater treatment

is not new, but it is still limited on a large scale. Presently, there is a tendency towards more integrated systems for effluents treatment, in a circular economy approach (Figure 2.2). Compared to the conventional WWT, microalgae immediately have the advantage that through photosynthesis they supply the oxygen necessary for bacteria to oxidize organic compounds and, in turn, the bacteria supply the necessary carbon dioxide to microalgae (Delanka-Pedige *et al.*, 2020).

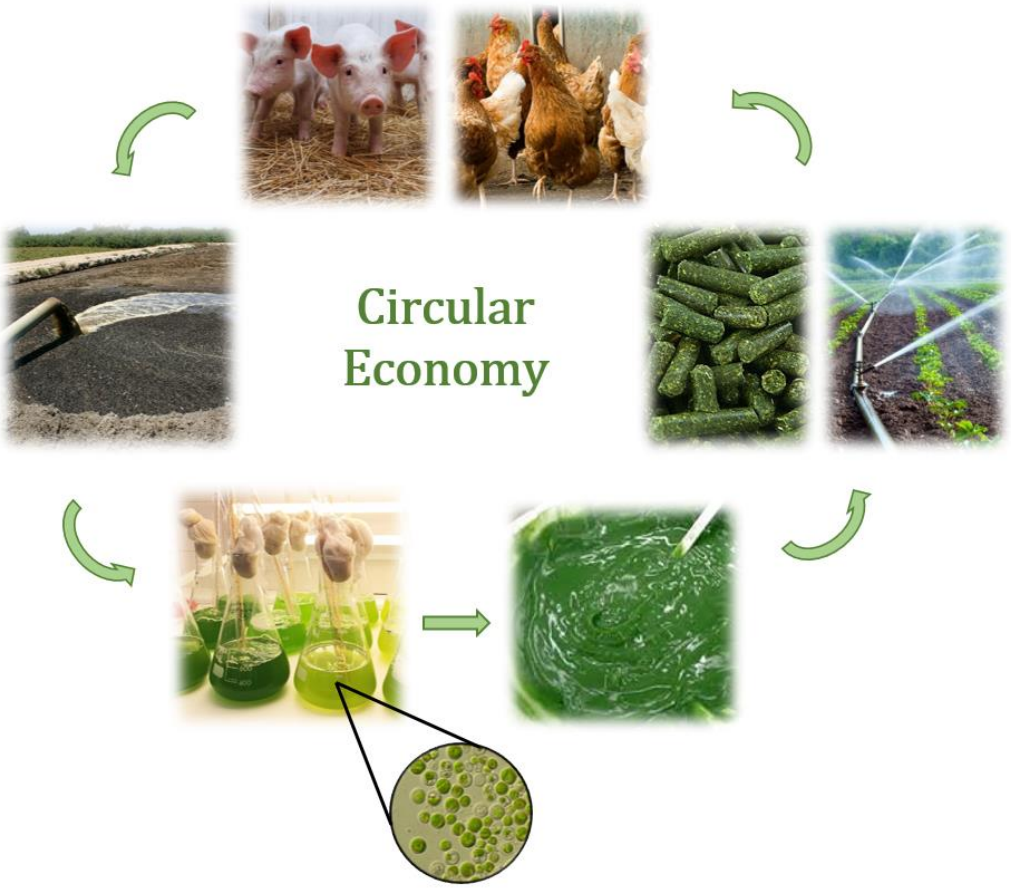

Figure 2.2 - Circular economy diagram applied to poultry and piggery effluents with the production of algal biomass for animal feed or biofertilizers.

The removal of phosphorus from wastewater is particularly difficult, but microalgae are very efficient in this removal, as well as nitrogen and heavy metals, which is why they can play a very important role in this remediation (Acién Fernández *et al.*, 2018; Kalra *et al.*, 2020). However, this strategy is not yet fully studied, since the use of wastewater is difficult for laboratory-scale cultures and increases its contamination with bacteria, fungus, and

viruses (Maryjoseph and Ketheesan, 2020). Still, several genera of microalgae have been successfully tested in the bioremediation of effluents to produce biofuels and biofertilizers with the resulting biomass, including *Scenedesmus* sp., *Chlorella* sp., *Chlamydomonas* sp., *Botryococcus* sp., *Micractinium* sp., *Actinastrum* sp., *Heynigia* sp., *Hindakia* sp., *Pediastrum* sp., *Dictyosphaerium* sp., *Coelastrum* sp., *Phormidium* sp., *Chlorococcum* sp., *Ourococcus* sp., *Nitzschia* sp., *Micractinium* sp., *Microspora* sp., *Rhizoclonium* sp., *Pleurochrysis* sp. (Aliyu *et al.*, 2021a; Ganesan *et al.*, 2020; Hussain *et al.*, 2021). The great real example of microalgae-based wastewater treatment, with a positive life cycle analysis, is Aqualia company, in Spain, with a biomass production of 40-60 tonnes $year^{-1}$ to be used as biofertilizer and 13,000 kg $year^{-1}$ of biogas, allowing the run of cars and bus for 325,000 Km $year^{-1}$, by the treatment of 1000 m^3 of urban wastewater per day (Grupo FCC, 2021).

The autotrophic biological assimilation of nutrients from wastewater is four times less expensive, lower energy-demanding, more efficient, and ecologically safer than the conventional wastewater treatment (Acién *et al.*, 2016). The integration of the bioremediation of residual effluents with the production of biomass and energy could overcome the current unsustainability obstacles (Mendonça *et al.*, 2021).

Besides the useful biomass produced by phytoremediation, microalgae release oxygen into the wastewater that induces aerobic degradation by other microorganisms, improving the levels of COD (chemical oxygen demand) and BOD_5 (biochemical oxygen demand) of the effluents and playing a crucial function in its tertiary treatment. These organisms also degrade the most persistent molecules such as hydrocarbons, antibiotics, and heavy metals, in addition to other components common in wastewater (Patel *et al.*, 2017; Suganya *et al.*, 2016).

Experiments were conducted with *Chlorella vulgaris* and *Tetradesmus obliquus* and nitrogen and phosphorus removal rates close to 100% were achieved in urban wastewater effluents (Gouveia *et al.*, 2016; Singh *et al.*, 2016). The ability of *Tetradesmus obliquus* microalga to degrade phenols and dichlorophenols was also reported (Papazi and Kotzabasis, 2013). Cyanobacteria have the ability to assimilate the amino acids glutamine, arginine, and asparagine from wastewater, using them as a nitrogen source for their needs (Patel *et al.*, 2017).

Using algal-bacterial systems appears to be the best solution to obtain the finest levels of remediation. Some important parameters in the use of these systems are the

balance between the organisms (microalgae and bacteria), the hydraulic retention time (HRT) and the organic load of the effluent to be treated. On the other hand, factors such as temperature, pH, mixing, and light intensity are other preponderant parameters for the success of the treatment (Zhong *et al.*, 2020). All these parameters influence the growth capacity of organisms, the balance between them, and their ability to remove nutrients from the medium. High Rate Algal Ponds (HRAP) are usually operated between 2-10 days HRT, however, if the conditions become more unfavourable (decrease in temperature, light intensity, CO_2 concentration) it will be necessary to increase HRT for the treatment to be effective. Increasing the pH of the medium to 9-11 leads to NH_3 volatilization and orthophosphate precipitation. The decrease in temperature usually leads to a decrease in the activity of microalgae and, consequently, a decrease in the efficiency of treatments. The luminous intensity should vary between 200-400 $\mu E\ m^{-2}\ s^{-1}$, for higher values may occur saturation of the photosynthetic apparatus (Muñoz and Guieysse, 2006; Yang *et al.*, 2020).

The wastewaters can have very different characteristics regarding its composition. When the effluents come from animal excreta, they usually have high levels of nitrogen (normally as ammonia). On the other hand, if they are industrial effluents, they may contain heavy metals or other specific constituents involved in the production processes (Amini *et al.*, 2020; Peng *et al.*, 2018).

In Portugal, wastewater effluents must comply with the parameters presented in Table 2.1, defined by Law Decree No. 236 (Portuguese Ministry of the Environment, 1998) for nitrogen, phosphorus, and COD, to be discharged.

Table 2.1 – Portuguese law decree for emission limit values in wastewater discharge (Portuguese Ministry of the Environment, 1998).

Parameter	Emission limit value
Chemical oxygen demand	150 mg O_2/L
Biochemical oxygen demand	40 mg O_2/L
Total phosphorus	10 mg P/L
Total nitrogen	15 mg N/L
Nitrates	50 mg NO_3/L
Total suspended solids	60 mg/L

2.2 Bioremediation of agro-industrial effluents

Effluents from agro-industries including animal production units contain large amounts of nutrients and microorganisms that have a huge potential to contaminate soils and eutrophicate water bodies (Oryschak and Beltranena, 2020). Poultry, piggery, and dairy farms daily produce massive quantities of manure and wastewaters from cleaning activities that cannot be drained into conventional wastewater treatment plants, nor can they be totally incorporated into the soil as fertilizer applications (Markou *et al.*, 2018). The treatment of these effluents generally requires multiple methods to efficiently decrease their COD and microbiological contamination thus constituting a significant economic load for animal producers.

Currently, the two most used solutions are the deposition of manure in open ponds and subsequent deposition in the soil, and the anaerobic digestion (Font-Palma, 2019). The deposition in the soil causes high risks of soil and groundwater contamination and is a source of considerable gaseous emissions. Anaerobic digestion implies tight control of the operating parameters and a high dilution ratio of the effluents, to avoid microorganisms' inhibition. In addition, the long-time of biogas production, and the digestate disposal, are factors that limited the use of this technology for processing large volumes of manure (Siddique and Wahid, 2018). However, anaerobic digestion is widely used for producing biogas (an important energy vector), also used to produce biomethane (a direct replacement for natural gas). The Portuguese directive - *Roteiro para a Neutralidade Carbónica 2050* (Resolução do Conselho de Ministros, 2019) indicates that the production of renewable gases will be the country's big focus until 2050.

Tests with various agro-industrial effluents revealed that microalgae have the capacity to treat these effluents, namely piggery effluents with *Chlamydomonas oblonga*, *Tetradesmus obliquus,* and *Chlorella vulgaris* (Abou-Shanab *et al.*, 2013; Viegas and Gonçalves, 2021), effluents from cattle in which were used *Chlorella* sp. and *Scenedesmus* sp. with promising results (Gramegna *et al.*, 2020; Labbé *et al.*, 2017; Ledda *et al.*, 2016; Viegas *et al.*, 2021b), effluents from poultry production (Ferreira et al., 2018; Markou et al., 2018; Viegas et al., 2021c), effluents from aquaculture production with *Tetradesmus obliquus* and *Chlorella sorokiniana* (Ansari *et al.*, 2017; Apandi *et al.*, 2019; Viegas *et al.*, 2021a) and effluents from oil extraction remedied by *Tetradesmus obliquus* (formerly known as *Scenedesmus obliquus*) (Hodaifa *et al.*, 2013).

Regarding the treatment of industrial effluents generally, there is a considerable load of heavy metals. Microalgae are sensitive to the toxicity of metals and, therefore, are used as biological indicators to detect the potentially toxic effects of metals. However, some of these organisms have developed ways to convert heavy metals into harmless forms. They have two mechanisms that allow them to maintain homeostasis and prevent intoxication by metals. One of the mechanisms in microalgae results from the ability to prevent absorption, the other is microalgae capacity to deal with high amounts of metals inside its tissues, which involves the absorption of metal ions and the formation of complexes. For this reason, they are often used to remedy large bodies of water containing low concentrations of metal ions (in the order of ppm). However, the most resistant microalgae species, which inhabit areas contaminated with metals, have an even greater capacity to accumulate metals (Singh *et al.*, 2021; Zada *et al.*, 2021).

Several studies of industrial effluent treatments with zinc, copper, and manganese have been conducted with very promising results for the microalgae *Chlorella*, *Tetradesmus*, *Spirulina*, *Oscillatoria,* and *Anabaena*. Trials with *Tetraselmis suecica* and *Chlorella vulgaris* also revealed the ability of these microalgae to remove cadmium (Kumar *et al.*, 2015). Other experiments with microalgae suggest that they can remedy effluents from acid mine drainage, which is one of the biggest environmental problems in the mining industry, with high removal rates for metals such as iron (95%), copper (79 to 97%), zinc (84%), lead (88%), cobalt (59 to 83%), nickel (22 to 62%) and manganese (28 to 45%) (Patel *et al.*, 2017). According to Kumar *et al.* (2015), the microalgae with the greatest potential for industrial effluents remediation are *Chlorella* sp., *Scenedesmus* sp., *Phaeodactylum tricornutum*, *Spirulina* sp., and *Chlamydomonas* sp..

The production of microalgae may be associated with industrial complexes, such as cement production, making it possible to capture the CO_2 produced in these locations. The combination of carbon dioxide fixation from flue gases with the removal of nutrients from wastewater in the production of microalgae is a very promising alternative, both from an economic and environmental point of view (Mendonça *et al.*, 2021).

2.3 Algal biomass valorisation

The biochemical diversity of microalgae includes a wide range of carbohydrates, lipids, and proteins with commercial value. Table 2.2 shows the lipid, protein, and carbohydrate content in the most studied microalgae species.

Table 2.2 – Lipid, protein, and carbohydrate content in some microalgae species.

Microalga	Protein content (% dw)	Carbohydrate content (% dw)	Lipid content (% dw)	Reference
Anabaena cylindrica	43–56	25–30	4-7	(Becker, 2007)
Arthrospira platensis (Spirulina)	50-68	10-14	10-15	(El-Kassas *et al.* 2015)
	52	8–14	4-9	(Becker, 2007)
	26-72			(Coca *et al.*, 2015)
Auxenochlorella protothecoides	48	25	25-35	(Krzemińska *et al.*, 2015)
			15	(Szabo *et al.*, 2013)
			58	(Cheng *et al.*, 2013)
Botryococcus braunii	17	32	17	(Ashokkumar and Rengasamy, 2012)
			29-75	(Chisti, 2007)
Chlorella vulgaris	51–58	12–37	14-22	(Becker, 2007);
			5-58	(Mata *et al.*, 2010)
Dunaliella salina	22-54	17-37	21-26	(Y. Chen *et al.*, 2015)
	57	32	6	(Becker, 2007)
Dunaliella tertiolecta		45	17-71	(Shin *et al.*, 2015)
	39	25	12	(Gorgônio *et al.*, 2013)
Euglena gracilis	39–61	14–18	14-20	(Becker, 2007)
Haematococcus pluvialis	17-23	37-40	7-21	(Lorenz, 1999);
			21-46	(Saha *et al.*, 2013)
Isochrysis galbana	4-45	25-57	43-50	(Valenzuela-Espinoza *et al.*, 2002)
	27	34	11	(Gorgônio *et al.*, 2013)
			7-40	(Mata *et al.*, 2010)
Microchloropsis salina	22-42	14-36	16-41	(Y. Chen *et al.*, 2015)
			60	(Mirsiaghi and Reardon, 2015)
Neochloris oleoabundans	13-35		14-28	(Baldisserotto *et al.*, 2016)
	17-44	27-44	34-52	(Morales-Sánchez *et al.*, 2014);
			20-56	(Gouveia *et al.*, 2009)
Porphyridium cruentum	28–45	40–57	9-14	(Becker, 2007)
Spirogyra sp.	6–20	33–64	11-21	(Becker, 2007)
Tetradesmus obliquus	50–56	10–31	12–14	(Becker, 2007);
			11–55	(Mata *et al.*, 2010);
			18	(Gouveia and Oliveira, 2009)
Tetraselmis suecica	39-53	5-27		(Michels *et al.*, 2014)
			15–23	(Chisti, 2007)

The application of microalgae could be in isolated bioactive compounds or in their whole form (Madeira *et al.*, 2017). There are several studies with its incorporation in the diet of cattle and other herbivores used in human nutrition. In general, the addition of 0.5 to 20% of microalgal biomass in the diet of these animals had positive effects on their health resulting in a meat of superior quality. In other animals such as pigs, poultry, and fish, experiments were carried out in which the whole diet was replaced by microalgae with several beneficial consequences on animals and without adverse effects founded (Medeiros *et al.*, 2021).

The use of algal biomass for aquaculture feed is a common practice. Some in vivo trials used the microalga biomass at the expense of soybean meal in gilthead seabream juveniles (Pereira *et al.*, 2020). However, there are few studies on the application of algae produced in wastewater as feed for aquaculture species. Li *et al.* (2019) used biomass of *Tetraselmis* sp. and *Phaeodactylum* sp. from aquaculture wastewater treatment in oysters' diet with good results.

Another application for algal biomass is its integration as an agricultural fertilizer and stimulant in seed germination, plant growth, resistance to stress (biotic and abiotic). Plant growth is influenced by phytohormones, that can be found in different extracts, namely microalgae. Those phytohormones include gibberellins, auxins, cytokinins, ethylene, and abscisic acid (Morais Junior *et al.*, 2020). Despite the few published studies on the effect of algae on seed germination, it is known bioactive compounds are necessary in very small quantities (Tarakhovskaya *et al.*, 2007). There are few studies on biostimulants using microalgae, yet, Navarro-López *et al.*, (2020) reported a 40% increase in watercress (*Lepidium sativum*) seed germination using *Scenedesmus* sp. produced in brewery effluents, as well as the application of *C. vulgaris* biomass produced in aquaculture effluent, which resulted in a 175% increase in germination index in watercress (Viegas *et al.*, 2021a). Other examples were done by Garcia-Gonzalez and Sommerfeld (2016) using *Acutodesmus dimorphus* to promote growth and flowering in Roma tomato plants, with good results. More efficient and sustainable use of resources can be achieved with the replacement of synthetic fertilizers by microalgae' biostimulants.

An additional application perspective is the use of algal biomass in the energy sector. Microalgae contain about 50% of carbon in their biomass, in most cases obtained through atmospheric carbon dioxide. For this reason, they have attracted interest as vehicles for carbon mitigation of industrial processes (Caporgno *et al.*, 2016; Suganya *et al.*, 2016).

Microalgae have also been the subject of many studies as they constitute an alternative raw material to produce biofuels, namely biodiesel and bioethanol, given their rapid growth and their ability to accumulate high amounts of lipids and carbohydrates, respectively, but also because they do not compete with food crops, arable land, and drinking water and fertilizers. However, there is still a huge limitation to the implementation of productive systems based on these organisms, due to their low volumetric efficiencies that still lead to costs that are too high compared to petroleum (Ananthi *et al.*, 2021). Figure 2.3 synthesizes the different processes of conversion of microalgae.

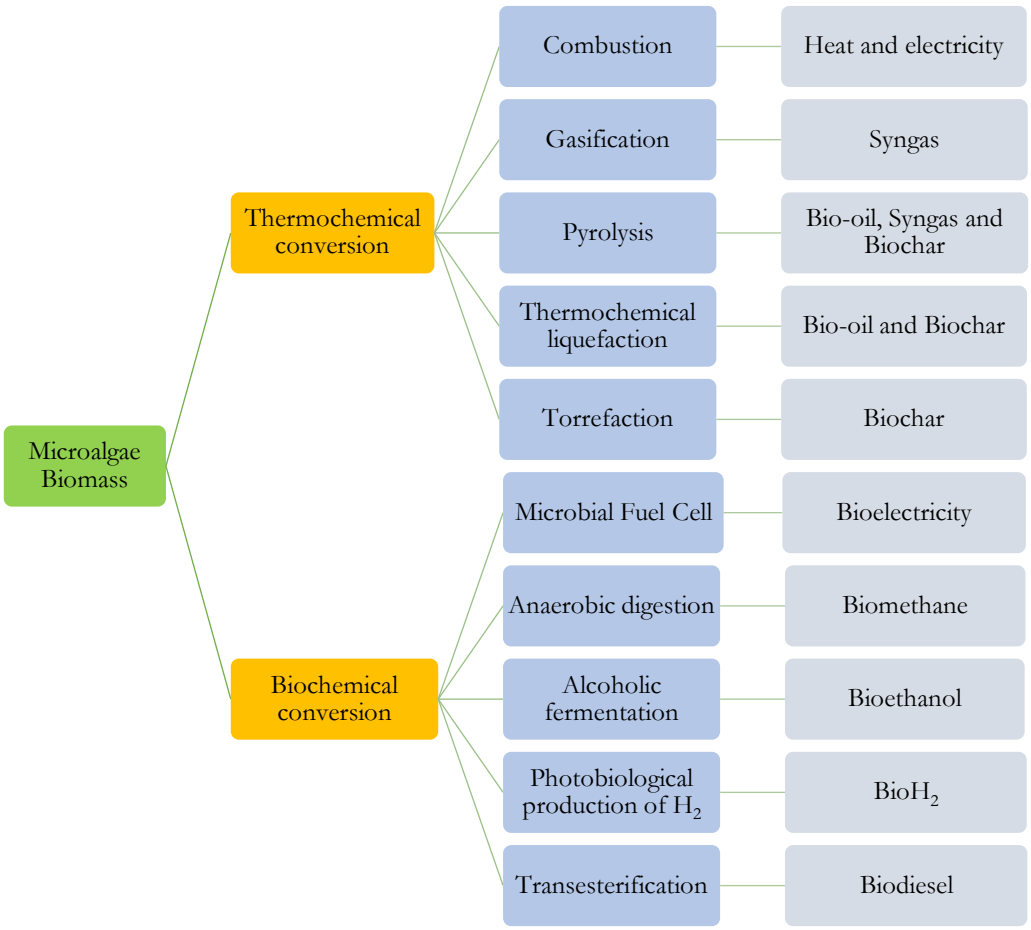

Figure 2.3 – Energetic processes for converting algal biomass, adapted from Aliyu, Lee, and Harvey (2021), Suganya *et al.* (2016), and Gouveia *et al.* (2014).

Energy and material applications are ways of using algal biomass. The thermochemical conversion process, such as torrefaction, can be used to produce chars for

coal fuel and bio-adsorbent from algal biomass (Gan *et al.*, 2018), to be used in soil amendment applications (Chu *et al.*, 2020), or as biostimulant (Ennis *et al.*, 2017).

Cyanobacteria and green microalgae are the only known organisms, to date, capable of carrying out both photosynthesis and hydrogen production (e.g., Marques *et al.*, 2011). These microorganisms produce different raw materials to generate energy: lipids to produce biodiesel, hydrocarbons, and isoprenoids to produce gasoline, and carbohydrates for the production of bioethanol or hydrogen. Furthermore, algal biomass can be processed to produce synthesis gas, whether or not followed by a Fischer-Tropsch synthesis for the production of liquid hydrocarbons; hydrothermal gasification or anaerobic digestion for the production of methane; pyrolysis to produce bio-oil and biochar; torrefaction or carbonization to produce biochars; microbial fuel cells to produce electricity; and the typical combustion for the production of electrical energy (Aliyu *et al.*, 2021; Ferreira *et al.*, 2017; Ganesan *et al.*, 2020).

Several researchers argue that these organisms can be used to produce biofuels in an economically and environmentally sustainable way, being possible to replace a substantial fraction of the use of fossil fuels in our society (Suganya *et al.*, 2016). According to Chisti (2008), all the US needs for transport fuels could be met by producing microalgae on less than 11% of that country's agricultural area.

Chapter 3

Methodology

The methodology used in the present thesis is described in this chapter and divided into two parts: materials used, and methods performed.

As the methodology used in the pre-treatment of the effluents, and in the study of the remediation of each effluent, microalgae productivity, characterization of the obtained microalgae, and application of algal biomass were identical, the author opted to present the methodology cohesively and uniquely. With this approach, the methodology section becomes more accurate and less repetitive.

3.1 Materials

Six microalgae species were selected to study the remediation of agro-industrial effluents: *Chlorella vulgaris* (INETI 58, LNEG_UBB, Portugal) (Cv), *Auxenochlorella protothecoides* (UTEX # 25) (known as *Chlorella protothecoides*) (Ap), and *Tetradesmus obliquus* (ACOI 204/07) (known as *Scenedesmus obliquus*) supplied by Coimbra University Culture Collection, Portugal (To). All the other microalgae cultures were purchased to the Scottish Marine Institute in Scotland, UK: *Isochrysis galbana* (CCAP 927/1) (Ig), *Microchloropsis salina* (CCAP 849/2) (known as *Nannochloropsis salina*) (Ms), and *Spirulina major* (CCAP 1475/3) (Sm). All microalgae were maintained in the appropriate culture medium. Cv, Ap, and To in BG-11 medium (UTEX, 2020), Ig and Ms in F/2 medium and Sm in ASW:BG11 medium (UTEX, 2020) at an average room temperature of 20°C, under artificial lighting with LED fluorescent lamps, at ± 200 μE m^{-2} s^{-1} with cycles of 12 h light/12 h dark.

Four agro-industrial effluents were studied: aquaculture, cattle, piggery, and poultry effluents. The aquaculture effluent was collected in March 2017, in October 2018, and in November 2020 from Pesca Verde Lda., a seafood hatchery supplier, located at Guincho (38°70'98.6" N, -9°48'45.3" W) – Cascais, Portugal. This effluent is produced by brown crabs (*Cancer pagurus*) that are transported in seawater by refrigerated truck from France to Portugal on a journey that lasts 24 hours. The cattle effluent used in this work was solid cow manure from a dairy farm located in Braga, Portugal, and collected in February 2018.

The piggery effluent from suckling sows, consisting essentially of pig urine, was from Raporal S.A., from a farm near Montijo (38°68'21.4" N, -8°93'98.6" W), Portugal and was collected in September 2017. The poultry effluent corresponds to boiled poultry remains (slaughterhouse), containing a high amount of fat. The effluent belongs to the company Avibom, S.A., in Ramalhal (38°15'553.6" N, -9°26'28.9" W) located in the Torres Vedras region, Portugal, and was collected in May 2018. All effluents were stored in 20 L plastic containers and stored at 4 °C, to minimize chemical and biological changes.

An industrial effluent, a landfill leachate was also studied. The landfill leachate was collected in April 2017 from CITRI S.A. (38°49'60.1" N, -8°81'73.1" W), an industrial non-hazardous solid wastes landfill (organic and recoverable materials in refuse derived fuel), located in Setúbal, Portugal. At the time of sample collection, the landfill was 6 years old, with a waste disposal rate of 100,000 tons $year^{-1}$. The average annual flow rate of leachate production was 3.4 m^3 h^{-1} (CITRI, 2018; Portuguese Ministry of the Environment, 2019). Leachate was collected from the buffer pond in 20 L plastic containers and stored at 4ºC.

The biomass ash was a consequence of the combustion process in the ceramics production industry and was supplied by Prélis Cerâmicas, Lda (39°70'12.3" N, -8°87'28.0" W), in Leiria, Portugal. Those industry furnaces use forestry biomass with a small percentage of polymeric residues as fuel. The olive-oil mill wastewater (OMW) came from Vila Velha de Rodão, in Beira Baixa, Portugal and was provided by Herdade da Tapada da Tojeira (39°65'72.9" N, -7°62'77.4" W), an organic olive oil producer.

The mussels (*Mytilus edulis*) used in this work were harvested on the groynes of São João beach, in Costa de Caparica, Portugal (38°65'47.4" N, -9°24'90.2" W) in April 2021.

The watercress (*Nasturtium officinale*) and wheat (*Triticum aestivum*) seeds came from organic farming, produced by Batllé and Biográ, respectively, and intended for cultivation.

For torrefaction tests, two biomasses were used initially: a commercial algal dry powder of *Chlorella vulgaris* (Allmicroalgae – Natural Products SA.) and a lignocellulosic material of an end-of-life pine from the furniture industry that was crushed and dried, used for pellet production and supplied by CMC Biomassa, S.A.. After the optimization of the torrefaction process, several experiments were made with other biomasses: *Chlorella vulgaris* and *Tetradesmus obliquus* both grown in aquaculture effluent, and *Chlorella vulgaris* grown in landfill leachate.

All the reagents used throughout this work were of analytical grade.

3.2 Methods

The tests for the remediation of agro-industrial effluents consisted of two parts, the first in batch mode until the consumption of nitrogen and phosphorus present in the medium and the decrease in the chemical oxygen demand (COD) parameter to levels below 150 mg O_2 L^{-1}, according to the legislation to allow the discharge of the effluent (Portuguese Ministry of the Environment, 1998). The second part was conducted to test microalgae growth in the semi-continuous mode, using a transfer method between reactors (explained in section 3.2.5) with different volume levels added. It was intended to do these remediations without using dilutions or pre-treatments as filtration, UV sterilization, or autoclaving.

The microalgae chosen for the batch mode for cattle, piggery, and poultry effluents were Cv, Ap and To. For aquaculture effluent, the batch mode was performed with five microalgae: Cv, To, Ig, Ms, and Sm. The semi-continuous mode for the four effluents was done with the two microalgae with the best performance in the batch mode of each effluent. In the test using aquaculture effluent reactors of 10 L to test the mussels' feed, the two microalgae selected were *Tetradesmus obliquus* and *Microchloropsis salina*.

The landfill leachate was performed only in batch mode with the microalgae Cv, Ap, To, Ig, Ms, and Sm.

The biomass ash used for the pre-treatment of each effluent (cattle, piggery, poultry, and landfill leachate) had a particle size < 500 μm and was analysed for its mineral composition and chemical composition through X-Ray fluorescence spectrometry (Philips X Unique II spectrometer). At the end of the pre-treatment, it was obtained a liquid phase (whose pH was neutralized with H_2SO_4) and a solid residue (precipitate) from ash and suspended solids.

3.2.1 Effluent characterization

Physicochemical parameters of the effluents (as received) such as optical density ($OD_{540\ nm}$), pH, COD, BOD_5, total nitrogen, nitrates, nitrites, total phosphorus, total suspended solids, and ash, were analysed using the standard methodology from Standard Methods for the Examination of Water and Wastewater (Rice *et al.*, 2017).

The ash of the microalgae treated effluents (and the raw effluent) were digested with HNO_3 (1:1) in a bath at 90ºC and the resulting solutions were diluted in distilled water to

100 mL and filtered (Whatman No. 42). The metals present in the effluents and their quantification were analysed by atomic absorption spectrometry (ZEEnit 700 - WinAAS, Analytik Jena).

Total phenolic compounds present in the landfill leachate were measured by the Folin-Ciocalteu method through an adaptation of the method described by Singleton *et al.* (1999), using gallic acid as a calibration standard.

Mineral composition (Al, B, Ba, Ca, Cr, Fe, K, Mg, Na, Ni, and Zn) was determined by ICP-AES (Horiba Jobin-Yvon Ultima) for the landfill leachate.

At the end of the experiments and after harvesting the microalgae, the treated effluents were analysed again for the same previous parameters, to determine the microalgae removal efficiency.

All the characterization analyses were conducted in triplicate, and the presented values correspond to average values (± standard deviation).

3.2.2 Microalgae growth media, conditions, and pre-treatment

Control tests were made for the batch growth experiments using synthetic culture medium adequate for each microalga.

The experiments with agro-industrial effluents were conducted in reactors (Erlenmeyer flasks) of 1 L working volume for batch and semi-continuous mode (except for aquaculture effluent where it was used 1.5 L in each reactor), agitated by an airflow of 15.2 L $L^{-1}_{culture}$ h^{-1} (air pump Stellar 380 D) that keeps the culture stirred preventing cells sedimentation and sealed with hydrophobic cotton. The microalgae grew at room temperature (23ºC ± 2ºC), under artificial lighting with LED fluorescent lamps at ± 200 μE m^{-2} s^{-1} (digital luxmeter ROLINE, model RO 1332A) with alternate 12 h cycles of light and dark.

A semi-continuous test was also carried out on a higher scale, using three 10 L flasks with the aquaculture effluent.

The inoculations were performed similarly independently of the microalga species, using 2% of stock solution (inoculum) calculated in order to have an initial optical density measured at 540 nm on the reactors around 0.2 (Gouveia *et al.*, 2016).

3.2.2.1 Aquaculture effluent experiments

The aquaculture effluent was slightly coloured and with low turbidity, with no need to be pre-treated with ash. In the semi-continuous growth, two tests were carried out with periodic transfers of 75 mL of raw effluent (2nd set of experiments) and 150 mL of raw effluent (3rd set of experiments). A fourth experiment was carried out in 10 L round bottom flasks reactors with periodic transfers of 1 L, in the same conditions of the other tests in semi-continuous mode.

The intention was to determine what was the maximum volume that could be transferred between reactors (every 48 h), obtaining an effluent treated in the last reactor.

The detailed raw effluent and culture medium transfer in semi-continuous tests, for configurations A and B (section 3.2.5) are described in Table 3.1.

Table 3.1 – Transfer of raw effluent, culture medium, and treated effluent used in Configuration A and B for the semi-continuous tests.

Set of Experiments		**Transferred Volume (mL/48 h)** *Configuration A*				**Transferred Volume (mL/week)** *Configuration B*	
		Reactor volume (mL)	Raw effluent addition to R1	R1 → R2 and R2 → R3	Treated effluent removal from R3	Raw effluent addition	Treated effluent removal
Aquaculture experiments	2nd test	1500	75	75	75	225	225
	3rd test	1500	150	150	150	450	450
	4th test	10000	1000	1000	1000	-	-
Cattle experiments	3rd test	1000	100	100	100	-	-
	4th test	1000	150	150	150	-	-
Piggery experiments	3rd test	1000	50	50	50	-	-
	4th test	1000	100	100	100	-	-
Poultry experiments	2nd test	1000	100	100	100	-	-
	3rd test	1000	200	200	200	-	-
	4th test	1000	300	300	300	-	-

3.2.2.2 Cattle effluent experiments

Cattle effluent (manure) for the batch mode was diluted 1:10 in tap water and the pre-treatment consisted in adding 80 g L^{-1} of biomass ash in the diluted effluent, with agitation by airflow for 1 h, to reach a pH of 12. After that, the effluent was separated from the precipitate by decantation and neutralized with sulfuric acid to pH 7. For the semi-continuous mode, the manure was diluted at 1:5, and the biomass ash added was 50 g L^{-1}.

The amount of ash added was tested so that the effluent became more transparent, and the precipitation of suspended solids occurred. In the semi-continuous test, a smaller dilution of water and addition of ash was tested. In these tests, two tests were conducted using transfer volumes of 100 and 150 mL.

3.2.2.3 Piggery effluent experiments

Piggery effluent was pre-treated with biomass ash with an amount of 120 g L^{-1} under agitation for 1 h, to reach a pH of 12. After that, the effluent was separated from the precipitate by decantation and neutralized with sulfuric acid to pH 7. Additions of smaller amounts of biomass ash did not lead to the precipitation of suspended solids in the effluent.

The 1st test, in batch mode, was performed testing two strategies, such as piggery effluents pre-treated plus ash (P+A) and piggery effluents pre-treated plus ash and combined with 2% of olive-oil mill wastewater (P+A+O).

In the semi-continuous mode, the transfer volumes used were 50 and 100 mL.

3.2.2.4 Poultry effluent experiments

The tests with this effluent were carried out using two different strategies, such as raw effluent (PE - poultry effluent) and raw effluent plus ash (PE+A - poultry effluent + ash). The pre-treatment was conducted adding 80 g L^{-1} of biomass ash in the effluent, with agitation by airflow for 1 h to reach a pH of 12. The effluent was separated from the precipitate by decantation and neutralized with sulfuric acid to pH 7.

In the semi-continuous mode, three tests were performed using three different transfer volumes: 100, 200, and 300 mL.

3.2.2.5 Landfill leachate experiment

The landfill leachate was pre-treated with 160 g L^{-1} of biomass ash in an overhead agitation for 96 h. The leachate was separated from the precipitate by decantation and neutralized with sulfuric acid to pH 7. Additions of smaller amounts of biomass ash and agitation with less time did not lead to the precipitation of suspended solids and discoloration of the leachate. The experiment was in batch mode and conducted in a 2 L graduated cylinder with a working volume of 0.82 L. The tests were carried out using pre-treated leachate diluted with deionized water (1:1) as a culture medium for the six algae and raw landfill leachate pre-treated with ash for *Chlorella vulgaris*.

3.2.3 Precipitate analysis

Concerning the precipitate obtained from the mixture of each effluent and biomass ash, it was determined its proximate and ultimate compositions. The moisture (M), ash content (Ash), and volatile matter (VM) were determined gravimetrically, following the methods prescribed in BS EN ISO 665:2020, 15403:2011, and 942-2007, respectively. The fixed carbon (FC) content was obtained as the difference between the full dry mixture and the ash plus volatile matter contents. The ultimate composition analysis was performed using a Thermo Finnigan elemental analyser (CE Instruments Model Flash EA 112 CHNS series). The oxygen content was obtained as the difference between the full dry ash free mixture and the C plus H plus N plus S contents. The mineral composition was determined through ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer Horiba Jobin-Yvon, Ultima). The conductivity and pH were determined using a Mettler Toledo MC226 conductivity meter and a Crison MicropH 2001 pH meter, respectively.

3.2.4 Microalgae growth monitorization

During the experiments, samples were collected from the bioreactors every other day, to analyse the pH medium (pH Tester PH-108), total nitrogen, total phosphorus and COD, and microalgae biomass growth by measuring the optical density at 540 nm (OD_{540}) (Gouveia *et al.*, 2016), using a spectrometer Biocrome S4 Libra. Samples were also taken every week, to access the microalgae biomass dry weight by filtering the samples through a Whatman GF/C 47 mm filter. All the determinations were performed in triplicates.

In the batch mode experiments when COD values reached the value that allows its discharge, the experiment ended. The culture was harvested by centrifugation at 7000 rpm for 5 minutes (Sigma 4K15) and the algal biomass was dried in an oven at 45°C for 48 h.

In semi-continuous experiments with periodic transfers, the tests lasted from 12 to 36 days and the cultures were harvested as previously described. At the end of that period, it was possible to know if the microalgae were able to semi-continuously treat the effluent under study adding the volume used in the transfer.

3.2.5 Transfer and supplementation processes

A method of periodic transfers of raw effluent was created and tested in the semi-continuous mode. The studied quantities for periodic transfers were two or three for each type of agro-industrial effluent. All effluents were tested for configuration A, but only aquaculture effluent was tested for configuration B. Figure 3.1 outlines the transfers scheme of configurations A (three reactors with 1.5 L each for aquaculture effluent and three reactors with 1 L each for cattle, piggery, and poultry effluent) and B (a single reactor with 4.5 L).

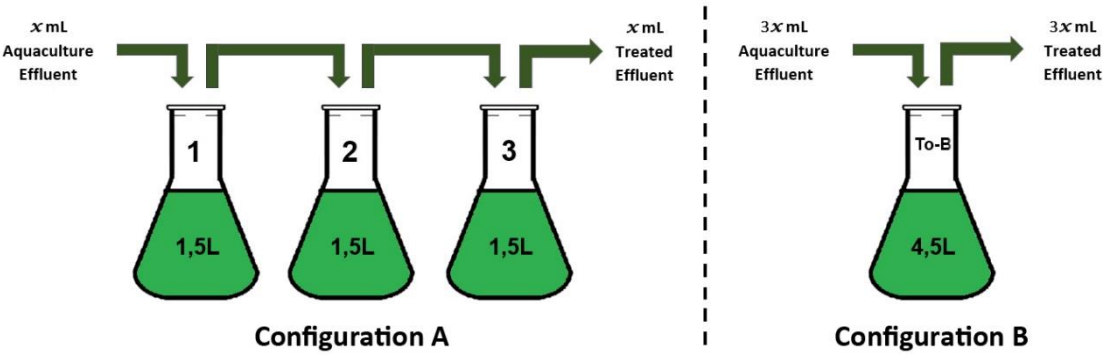

Figure 3.1 – Schematic representation of the semi-continuous tests for aquaculture effluent, showing the three reactors sequence and the effluent transfer process with configuration A and B. Configuration A – series of three reactors with 1.5 L with a transfer of *x* mL every 48 h; Configuration B - Single reactor with 4.5 L with a weekly transfer of 3*x* mL.

In configuration A, a series of three reactors were used and every 48 h, a given volume (*x*) of treated effluent was removed from reactor 3 being compensated by transfers of the same volume from reactor 2 to 3 and from reactor 1 to 2. The volume of reactor 1 was readjusted to 1.5 L (or to 1 L) by the addition of *x* mL of raw effluent. For the means of comparison in the aquaculture experiment, the semi-continuous mode was also

evaluated according to Configuration B, using a single reactor with 4.5 L of working volume inoculated with *T. obliquus*.

In the 3rd and 4th tests of the agro-industrial effluents reactors 2 and 3 were supplemented with aqueous $NaNO_3$ to a final concentration of 20 mg N L^{-1} in the culture medium, every 48 h (Ansari *et al.*, 2017), poultry effluent was also supplemented in the 2nd test. In cattle, piggery, and poultry effluents the medium was as well supplemented with KH_2PO_4 to a final concentration of 10 mg P L^{-1}.

3.2.6 Determination of productivity and remediation rates

The productivity of microalgae in different tests was determined using equation 1:

$$\text{Productivity (mg L}^{-1}\text{ day}^{-1}) = \frac{\text{microalgae dry weight} \times (1000 \div \text{filtered volume})}{\text{experiment's number of days}} \quad (1)$$

The removal efficiency of analysed parameters was established using equation 2:

$$\text{Removal efficiency (\%)} = \frac{C_i - C_f}{C_i} \times 100 \quad (2)$$

Where C_i is the initial concentration of the analysed parameter and C_f the final concentration of the parameter.

3.2.7 Determination of algal biomass composition

Before the biomass characterization, the microalgae were ground using a Retsch ball mill - model MM400 for 4 min at a speed of 25 s^{-1}.

The total nitrogen present in the biomass was quantified by the modified Kjeldahl method (AOAC, 2006). The total protein was calculated by multiplying the total nitrogen by the conventional conversion factor of 6.25 (Jones, 1931).

Sugar content was determined by quantitative acid hydrolysis using the literature (Miranda *et al.*, 2012) optimized for microalgae biomass sugars extraction followed by the method of the phenol-sulfuric reagent (Dubois *et al.*, 1956) for the total sugar content determination.

Lipid content was determined by Soxhlet extraction during 6 hours with solvent n-hexane and about 1 g of algae biomass.

The fatty acid methyl esters were prepared by adding equal parts of the sample and methanolic KOH (2N) (Nagappan *et al.*, 2019). The composition in terms of fatty acids was determined by GC-MS - Gas Chromatography coupled with Mass Spectrometry (Focus GC, Polaris Q - Thermo), equipped with a DB-5 capillary column (30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness). The fatty acids were injected in splitless mode, at 250°C and the GC temperature was programmed as follows: initial temperature of 40°C, held for 1 min, increased to 150°C at a rate of 10°C/min, held for 15 min, afterward, the temperature was increased 250°C at 5°C/min and lastly increased 280°C at 10°C/min and held for 10 min. The transfer line and ion source temperatures were 250°C and 230°C, respectively. The fatty acids present in the hexane solvent were identified by comparing their mass spectra with those in NIST and WILEY databases and with the retention time and mass spectra of corresponding standards.

After determining the ash content of algal biomass, the ash was digested in HNO_3 (1:1) in a bath at 90ºC and then the mineral composition was determined through ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer Horiba Jobin-Yvon, Ultima) for the microalgae biomass used in germination assays.

3.2.8 Feeding mussels with algal biomass

The mussels were brought to the laboratory where they were in an acclimation period for 7 days, being fed daily with *Tetradesmus obliquus* grown in Bristol synthetic medium (UTEX, 2020). They were cleaned of the shell attachments, staying in running seawater with aeration and artificial light in a light/dark cycle of 12/12 h, at 16 ± 1ºC. On day 1 (T1) a subsample of 10 mussels was used to determine, in triplicate, their condition index (Equation 3) and composition at the beginning of the experiment. Mussels were in starvation for the 24 h prior to the beginning of feeding experiments (Fidalgo *et al.*, 1994), and were randomly distributed across the aquariums.

The mussels' feeding experiment was conducted in 20 L parallelepiped aquariums, with 15 L of seawater (0.45 μm-filtered ultraviolet light treated seawater), with a salinity of 36 g kg^{-1} and with fifteen (15) mussels per aquarium. Six types of feeds were defined: *T. obliquus* and *M. salina* produced in aquaculture effluent and a mixture in equal parts of the two microalgae; as control, the same microalgae were used (alone and mixed), but grown in synthetic culture medium. The *M. salina* in this experiment was grown in Modified Artificial

Seawater medium that contains $NaCl_2$ (35.08 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (7.39 g L^{-1}), KNO_3 (1.0 g L^{-1}), K_2SO_4 (0.85 g L^{-1}), $CaCl_2 \cdot 2H_2O$ (0.8 g L^{-1}), and trace elements solution CHU (1 mL L^{-1}) and *T. obliquus* grown in Bristol medium that contains $NaNO_3$ (0.25 g L^{-1}), $CaCl_2 .2H_2O$ (0.03 g L^{-1}), $MgSO_4 .7H_2O$ (0.08 g L^{-1}), K_2HPO_4 (0.08 g L^{-1}), KH_2PO_4 (0.18 g L^{-1}) and NaCl (0.03 g L^{-1}) (UTEX, 2020).

To each aquarium, 15 mL of microalgae biomass in a concentration of 2.5 g L^{-1} was added daily (corresponding to 2.5 mg L^{-1} of algal biomass per aquarium), as Galimany *et al.* (2008). The temperature, pH, salinity, O_2 dissolved, and ammonia was daily evaluated, and the dead animals were removed. Weekly, ¾ of the total water volume in the aquariums was replaced.

The experiment ran for 45 days, being the mussels weighed and measured at day 1 (T_1), day 15 (T_{15}), day 30 (T_{30}), and day 45 (T_{45}). On day 30 seven mussels were dissected to analyse their condition index (Equation 3), shell length gain (Equation 4), weight gain (Equation 5), and composition in protein, carbohydrate, lipid, and ash content (using the methods previously defined in section 3.2.7). On day 45 the same procedure was done for the remaining mussels (7 or 8 mussels depending on the aquarium).

The condition index of mussels was determined using equation 3 (Gvozdenović *et al.*, 2020; Wyatt *et al.*, 2013):

$$\text{Condition Index (\%)} = \frac{\text{Dry meat weight}}{\text{Shell dry weight}} \times 100 \quad (3)$$

The mussels' growth was determined using the shell length gain (equation 4) and weight gain (equation 5):

$$\text{Shell length gain (\%)} = \frac{L_f - L_i}{L_i} \times 100 \quad (4)$$

$$\text{Weight gain (\%)} = \frac{W_f - W_i}{W_i} \times 100 \quad (5)$$

where L_f is the values of final shell length and L_i is the values of initial shell length. For the mussels' weight, W_f is the value of the final weight and W_i is the value of initial weight.

3.2.9 Germination assays

The germination of *Triticum aestivum* (wheat) and *Nasturtium officinale* (watercress) seeds using microalgae solutions and precipitate aqueous extracts were tested to evaluate the biostimulant activity of microalgae and the fertilizer effect of the precipitate, in the germination of the seeds. Wheat was chosen since it is a crop often incorporated into the cattle feed. Watercress was selected because it is a sensitive plant to toxic compounds in the soil and it is frequently used as a test plant in germination tests (Luo *et al.*, 2018).

To evaluate the biostimulant activity of microalgae, the germination index of wheat and watercress was used according to the methodology described by Zucconi *et al.* (1981), using the microalgae biomasses obtained in the last tests of the different effluents remediation at 0.2 and 0.5 g L^{-1} of concentrations.

The germination test seeds for precipitate extracts was adapted from Monteiro *et al.* (2011). The compost sample was (i) dried (80ºC), (ii) sieved (2 mm), (iii) mixed with distilled water at 60ºC, in the proportions of 0 (control), 5, 10, 20, and 40%, and subsequently (iv) stirred with a magnetic stirrer for 3 hours. This procedure was also applied to the ash used in the pre-treatment, in proportions of 5 and 10%. The aqueous extracts were then filtered through filter paper (Whatman 2).

In the germination tests, 3 mL of microalga cultures, precipitate extracts, or ash extracts were pipetted into 90 mm diameter Petri dishes lined with sterile absorbent paper. 50 watercress and 50 wheat seeds were put in each box (with 3 replications per treatment), which was then sealed with parafilm and placed in an incubator at 28ºC and for 5 days without light. The germination index, recorded after the fifth day, was determined through:

$$\text{Germination index (\%)} = \frac{G \times W}{G_C \times W_C} \times 100 \qquad (6)$$

where *G* is the number of germinated seeds, *W* is the seedling weight – *Gc* and *Wc* are the values of these same parameters in the control case (distilled water).

3.2.10 Co-torrefaction experiment

For the torrefaction tests, two types of biomasses were used, microalgal and lignocellulosic biomasses. The algal biomass was a *Chlorella vulgaris* dry powder obtained from a commercial source (Allmicroalgae – Natural Products SA). For the final tests, after

optimizing the process, the microalgae biomass chosen were *Chlorella vulgaris* and *Tetradesmus obliquus* obtained from the remediation of the aquaculture effluent (3rd test) (Viegas *et al.*, 2021a) and *Chlorella vulgaris* grown in landfill leachate (Cv-1:2) (Viegas et al., 2021d). The lignocellulosic material (Lc) was composed of waste pine biomass used for pellet production. The Lc sample was further milled in a coffee grinder (Bosch TSM6A011W) and sieved by a 10 mesh (= 2 mm) sieve. Both biomasses were oven-dried until a moisture content of 5%.

The torrefaction tests were performed on a glass reactor placed in a gas chromatography furnace (Thermo Finnigan Trace GC with FID), under oxygen-limited conditions, as described by Şen *et al.* (2020). For each experiment, selected masses of dry algal biomass, lignocellulosic material, and added water were introduced in the reactor to achieve the values of Lc incorporation rate and feed moisture defined in the experimental design, as shown in Appendix 1. A total of 39 initial compositions were considered, with incorporations of 0, 25, 50, 75, and 100% of lignocellulosic material and feed moisture contents of 5, 15, 30, 45, and 70%. The samples were placed in 250 mL glass flasks and heated up to the final torrefaction temperatures (200, 225, 250, 275, or 300°C), the conditions were kept isothermal for 15, 30, 45, or 60 min. In the end, the furnace was cooled to 35°C, with a cooling rate equal to the heating rate that varied between 11 and 14°C/min. The liquid phase was collected in a cooling trap and the mass yields of biochar and liquid phase were determined with an analytical scale (Mettler Toledo AB204-S). The gas yield was determined by difference. Biochar samples were stored in dry conditions until further analysis and liquid phase samples were stored at -4°C to avoid chemical and biological degradation.

3.2.10.1 Characterization of the biomass feedstocks and torrefaction products

Proximate analysis, namely ash content (Ash), volatile matter (VM), and moisture (MC) were determined gravimetrically, for both biomass materials and biochars, according to the methods described in BS EN 15403:2011, 942-2007, and ISO 665:2020, respectively. Fixed carbon (FC) was determined by difference, on a dry basis (db). Ultimate analysis (carbon, hydrogen, nitrogen, and sulfur contents) was performed using an elemental analyser (Thermo Finnigan – CE Instruments Model Flash EA 112 CHNS series). Oxygen content was determined by difference, on a dry ash free basis (daf).

High heating value (HHV) of the feedstocks was calculated using a correlation established by Huang and Lo (2020) based on elemental composition data (equation 7):

$$HHV\ (MJ\ Kg^{-1}, db) = 0.3443C + 1.192H - 0.113O - 0.024\ N + 0.093S \quad (7)$$

The HHV of the produced biochars was determined through the correlation established by Parikh *et al.* (2005) - equation 8:

$$HHV\ (MJ\ Kg^{-1}, db) = 0.3536\ [FC] + 0.1559\ [VM] - 0.0078\ [Ash] \quad (8)$$

where FC, VM, and Ash are fixed carbon, volatile matter, and ash content of the char, respectively, expressed in wt. %, db.

The aqueous phase of the torrefaction process was analysed for pH (Crison MicropH 2001 m), conductivity (MC226 Conductivity meter Mettler Toledo), COD through the high range dichromate method (Hanna instruments Kit tests), total phenolics content (TPC), and reducing sugars (RS). Total phenolics were quantified by the Folin-Ciocalteu method, as proposed by Singleton *et al.* (1999) and total reducing sugars content was determined by the DNS method, as defined by Miller (1959). All the analyses were performed in triplicate, and the presented results correspond to average values.

3.2.10.2 Process performance

The mass and energy yields of the produced biochars were calculated using equations 9 and 10, respectively:

$$\text{Mass yield (\%)} = \frac{m_{biochar}}{m_{raw\ material}} \times 100 \quad (9)$$

$$\text{Energy yield (\%)} = [\text{Mass yield} \times \frac{HHV_{biochar}}{HHV_{raw\ material}}] \times 100 \quad (10)$$

where $m_{biochar}$ and $HHV_{biochar}$ are the mass (kg) and high heating value of biomass biochar (MJ kg^{-1}); $m_{raw\ material}$ and $HHV_{raw\ material}$ are the mass and high heating value of raw biomass material.

Process energy efficiency (PEE) compares the energy contained in the biochars with the sum of the energy in the original raw biomass plus the energy required for the torrefaction process. This parameter was calculated through equation 11:

$$PEE\ (\%) = \frac{m_{biochar} \times HHV_{biochar}}{m_{raw\ material} \times HHV_{raw\ material} + Q_{input}} \times 100 \quad (11)$$

where Q_{input} is the total energy requirements of the torrefaction process, in MJ. Q_{input} is determined as the sum of the different energy requirements of the process, as follows:

$$Q_{input} = Q_1 + Q_2 + Q_3 + Q_4 + Q_5 \quad (12)$$

$$Q_1 = m_{raw\ material} \times Cp_{raw\ material} \times \Delta T \quad (12.1)$$

$$Q_2 = m_{H_2O} \times Cp_{H_2O} \times \Delta T \quad (12.2)$$

$$Q_3 = m_{H_2O} \times L \quad (12.3)$$

Where Q_1 represents the energy needed to heat the feedstock from room temperature to process temperature (ΔT). The specific heat of the biomass sample ($Cp_{raw\ material}$) was evaluated using the values of 1.58 KJ Kg^{-1} K^{-1} for algal biomass (Wibawa *et al.*, 2018) and of 1.7 KJ Kg^{-1} K^{-1} for lignocellulosic material (Delrue, 2018), and taking into account their relative concentrations in the initial feedstock.

Q_2 represents the energy needed for heating the water present in the reactor (m_{H2O}) from room temperature to 100ºC (ΔT), considering the specific heat of the water as Cp_{H2O} = 4.14 KJ Kg^{-1} K^{-1}.

Q_3 is the latent heat of water vaporization (L = 2.26 MJ Kg^{-1}), corresponding to the energy needed for water evaporation at 100ºC.

Q_4 corresponds to the heat of reaction for the torrefaction process itself. This parameter was inferred from the values obtained by Ohliger *et al.* (2013), for different temperatures, residence times, and moisture (Table 3.2).

Table 3.2 – Values attained for the heat of reaction (MJ) of the torrefaction process for different process conditions.

Temp. (ºC)	Residence Time (min)							
	15	15	30	30	45	45	60	60
	Moisture (%)							
	≤ 25	≥ 50	≤ 25	≥ 50	≤ 25	≥ 50	≤ 25%	≥ 50
200	1000	1500	900	1400	800	1300	700	1200
225	950	1500	850	1400	750	1300	650	1100
250	900	1350	800	1200	700	1100	600	1000
275	850	1200	750	1000	650	900	550	800
300	850	1200	750	1000	650	900	550	800

Q_5 are the thermal losses connected to the diffusion losses through the reactor walls and heat loss from the biochar and produced gas exiting the reactor. These thermal losses were assumed to be 45% at 300ºC, 60 min and 25% at 200ºC, 15 min, the other values for all the combinations of temperature and residence time were interpolated (Table 3.3) (Nobre *et al.*, 2019).

Table 3.3 – Thermal losses (MJ) of the torrefaction process for different process conditions.

Temperature (ºC)	Residence Time (min)			
	15	30	45	60
200	25	30	32.5	35
225	27.5	32.5	35	37.5
250	30	35	37.5	40
275	32.5	37.5	40	42.5
300	35	40	42.5	45

3.2.10.3 Response surface methodology

The response surface methodology (RSM) has been widely applied to process improvement and optimization, reducing the number of experimental runs needed to evaluate the influence of several independent variables in the critical properties to be studied (Igwegbe *et al.*, 2019).

For the study, four independent variables (temperature, residence time, incorporation rate of lignocellulosic material, and moisture content of algal biomass) were considered, using four or five levels for each variable (Table 3.4).

Table 3.4 – The independent variables and its experimental range and units. Lc - lignocellulosic material.

Independent Variables	**Unit**	**Variable Range**				
Temperature	ºC	200	225	250	275	300
Residence time	min	15	30	45	60	
Moisture content	%	5	15	30	45	70
Incorporation rate of Lc	%	0	25	50	75	100

The selection of the experimental conditions was based on RSM model, software Design Expert ® Software version 12-Stat-Ease (12.0.0.6) yielding a total of 39 experimental runs including 29 trials for model determination, five trials for Lack of fit, and five replicates for pure error estimation. The operational variables of the process were optimized considering the results obtained.

The p-value confidence level used to assess the model terms was 95%. To verify the adequacy of the model, the value of the determination coefficient (R^2) was compared to the adjusted value of R^2. The three-dimensional surface graphics generated by the software served to evaluate the interaction between the process variables and their effect on the output response.

3.2.10.4 Biochar as fertilizer

To determine the possible fertilizing effect of microalgae biochar, germination tests were carried out identical to those done for algal biomass cultures. The microalga biochar chosen was *Tetradesmus obliquus* grown in aquaculture effluent (3rd test). Three biochars were used for the germination test: *Tetradesmus* (To), *Tetradesmus* + Lignocellulosic Material (To + Lc), and Lignocellulosic Material (Lc), at two different concentrations (0.2 g L^{-1} and 0.5 g L^{-1}). The same plant seeds (*Triticum aestivum* and *Nasturtium officinale*) were used and the germination index was determined by Equation 6.

The mineral composition of the biochars was evaluated through ICP-AES (Inductively Coupled Plasma – Atomic Emission Spectrometer Horiba Jobin-Yvon, Ultima).

3.2.10.5 Adsorption experiment

Methylene blue (MB), a synthetic dye often present in industrial wastewaters was used as a model compound to evaluate the biochar adsorption capacity towards cationic contaminants. The adsorption of MB by the produced biochars was measured with a quick adsorption method based on the work of Correia *et al.* (2017). The biochars' samples were milled and sieved to less than 500 μm diameter before using in the adsorption experiments. A small mass of each sample (25 mg) was added to a test tube containing 5 mL of a MB aqueous solution (100 mg L^{-1}) and the tube was shaken for 3 s (Heidolph top shaker) and then centrifuged at 3000 rpm for 5 min (Hettich EBA 20). The supernatant was transferred

to another tube and the concentration of dye was determined by UV–VIS spectrophotometry (Biochrom Libra S4) at 664 nm. The adsorption capacity (q_{MB}) was determined using equation 13:

$$q_{MB}\ (\%) = \left(\frac{C_i - C_f}{C_i}\right) \times 100 \qquad (13)$$

where C_i and C_f are the initial and final concentrations (mg L^{-1}) of dye in the aqueous solution.

3.2.11 Statistical analysis

Duplicate tests were performed for the microalgae growth and the triplicate ones were performed for the germination tests and for all the characterization analysis. Data were reported as mean ± standard deviation (SD). Parameters such as the productivity, biomass composition, or germination index were compared using the ANOVA analysis of variance with one-way, carried out using the IBM SPSS statistical 23 software. The mean values obtained were compared using the Tukey HSD test and the correlation observed was deemed statistically significant when $p < 0.05$.

Chapter 4

Pre-treatment evaluation and precipitate characterization

The pre-treatment with biomass ash was used for all the studied effluents, except for aquaculture since it had a much lighter colour and less turbidity than all the other ones.

Conducting a physicochemical pre-treatment in the agro-industrial effluents leads to an almost sterilization of the effluent, as well as the precipitation of several particles in suspension allowing better light penetration and its use as culture medium, namely for microalgae.

Chemical precipitation is a widely applied pre-treatment for wastewater treatment that aims to remove ammonia-nitrogen, heavy metals, and other non-biodegradable organic compounds (Barbosa Segundo *et al.*, 2019; Li *et al.*, 2017). This process involves the combination of metal cations and some soluble anions to form insoluble species that precipitate and are subsequently removed by sedimentation or filtration. Several chemical precipitating agents can be used in this process, such as lime (CaO), hydrated lime ($Ca(OH)_2$) and combinations of magnesium oxide (MgO) and phosphates (PO_4^{3-}) (Abiriga *et al.*, 2021; Barbosa Segundo *et al.*, 2020).

The advantages of chemical precipitation, when compared to other methods, are its simplicity and low implementation costs. But the constant consumption of the chemical agent and the need to eliminate the generated sludge may increase operating costs, impairing economic viability (Kurniawan *et al.*, 2006). Thus, minimizing the costs of this process entails finding low-cost precipitating agents, with suitable chemical characteristics that are available in significant amounts. Biomass ash is an inorganic by-product of solid biofuels' combustion that is generated in large amounts in industrial boilers or thermal power plants. Usually, this by-product contains high concentrations of silicon, aluminium, iron, calcium, and magnesium oxides, and is known for its precipitating capacity (Ondrasek *et al.*, 2021). There is an increasing number of thermal power plants operating on forestry biomass or biomass wastes worldwide, generating around 480 million tons of ash (Silva *et al.*, 2019). This ash may be used for soil amendment or incorporated in construction

materials. However, new valorisation processes should be proposed in order to manage such large quantities following environmentally friendly and sustainable criteria (Fořt *et al.*, 2021; Ondrasek *et al.*, 2021). Chemical precipitation using fly or bottom ash is not very well documented in the literature and the works are mostly focused on the removal of metallic species from industrial wastewater (Park *et al.*, 2020). Although chemical precipitation allows the removal of several organic and inorganic contaminants, the process also involves the extensive dissolution of ash components in the aqueous medium, namely calcium or magnesium cations, hydroxide ions, or phosphates. Those soluble components yield high COD values and high pH values to the treated effluent. Consequently, the effluent must be acidified to return to neutrality and subjected to reverse osmosis or ion exchange processes to achieve regulated COD values (Renou *et al.*, 2009).

The chemical composition of the biomass ash used in the pre-treatment of the cattle, piggery, poultry, and landfill leachate is reported in Table 4.1. This mineral waste was mainly composed of CaO (65.9%) and contained several other water-soluble components such as MgO or Fe_2O_3; the alkalinization potential of this ash is expressed by its pH of 13.0, which corresponds to the equilibrium pH in an aqueous solution. This biomass ash had some heavy metals such as copper, chromium, manganese, and zinc, but the amounts present are almost vestigial.

Table 4.1 – Main chemical composition of biomass ash (wt. %).

Parameter	Value	Parameter	Value
pH	13.00 (± 0.03)	SO_3	0.920 (± 0.010)
CaO	65.90 (± 0.20)	P_2O_5	0.770 (± 0.010)
Cl	11.50 (± 0.04)	Na_2O	0.560 (± 0.010)
SiO_2	6.60 (± 0.06)	MnO	0.170 (± 0.008)
Al_2O_3	4.01 (± 0.04)	BaO	0.160 (± 0.002)
MgO	3.20 (± 0.02)	ZnO	0.088 (± 0.001)
TiO_2	2.51 (± 0.06)	SrO	0.086 (± 0.001)
Fe_2O_3	2.30 (± 0.03)	Cr_2O_3	0.068 (± 0.006)
K_2O	1.20 (± 0.03)	CuO	0.058 (± 0.003)

4.1 Cattle effluent

The dilution of the raw cattle manure with tap water was an essential step to isolate soluble components and to allow the action of the biomass ash. The 1:10 dilution ratio and addition of 80 g L^{-1} of biomass ash resulted in 85% (wt.) of pre-treated liquid effluent and 15% (wt.) of the precipitate. When the dilution ratio was 1:5 and ash concentration was 50 g L^{-1}, the following fractions were obtained: 77% (wt.) pre-treated effluent and 23% (wt.) precipitate. As it can be seen, reducing the dilution volume by half did not result in a 50% reduction of the liquid phase and a 50% increase of the precipitated solids, which indicates that solubility limits for components of cattle manure or biomass ash were not reached.

The raw cattle manure, the liquid effluent obtained after dilution with tap water, and the liquid effluent obtained after dilution in tap water and treatment with biomass ash were evaluated and presented in Table 4.2.

Table 4.2 – Characterization of the initial cattle manure, diluted manure, and pre-treated manure effluent used in the batch (Manure 1:10 + 80 g L^{-1} of ash) and semi-continuous mode (Manure 1:5 + 50 g L^{-1} of ash).

Parameter	Units	Crude Manure	Batch mode tests		Ash removal efficiency (1:10 dilution) (%)	Semi-continuous mode tests		Ash removal efficiency (1:5 dilution) (%)
			Manure 1:10	Manure 1:10 + ash (80 gL^{-1})		Manure 1:5	Manure 1:5 + ash (50 gL^{-1})	
pH	-	-	7.2	12.6	-	7.4	12.3	-
Total N	mg N L^{-1}	4741.7	474.2	91.30	73.6	963.3	454.9	35.7
Kjeldahl N	mg NH_3 L^{-1}	4740.7±35.6	474.1±12.2	91.30±6.20	**73.6**	948.1±7.7	442.1±16.0	36.1
Nitrates	mg NO_3^- L^{-1}	0.27±0.00	N.d.	N.d.	-	13.6±2.3	11.6±0.2	14.5
Nitrites	mg NO_2^- L^{-1}	0.73±0.00	0.07±0.00	N.d.	-	1.5±0.1	1.1±0.0	27.6
Total P	mg P L^{-1}	810.5±88.7	81.1±7.2	4.10±0.30	**94.9**	162.1±30.7	13.3±1.0	**91.8**
O.D.	540nm	-	1070±38	0.36±0.01	**99.9**	5350±145	0.645	**99.9**
COD	g O_2 L^{-1}	43.86±1.03	4.39±0.40	2.70±0.20	38.4	8.77±0.56	4.74±0.09	46.0
BOD_5	g O_2 L^{-1}	23.50±0.61	2.35±0.13	0.88±0.03	**62.6**	4.70±0.12	4.30±0.24	8.5
Total solids	g L^{-1}	118.37±25.20	8.72±0.20	19.1±1.1	-119.0	17.5±2.3	19.2±0.5	-10.0
Ash content	g L^{-1}	19.0±0.5	1.56±0.00	16.1±0.8	-934.0	3.1±0.2	14.8±0.5	-374.7

N.d. – Not detected.

The values in bold are the ones with the greatest reductions.

Raw manure had an extremely high COD, high nitrogen and phosphorus contents, as expected for this type of residue. The dilution with water and the pre-treatment with ash resulted in a decrease in these three parameters, however, not enough to allow its discharge. The physicochemical pre-treatment significantly reduces the optical density of the medium, through the deposition of suspended solids. After dilution and immediately after ash addition, the liquid medium had a dark and turbid appearance but, as the pH of the liquid medium increased by reaction with biomass components, it was visible a progressive precipitation of components and clarification of the solution. At a pH of 12 or higher, proteins denaturate and other molecules also suffer structural changes, which contributes to break micellar structures and reducing the solubility of many components in the liquid medium. On the other hand, the change in the solution pH showed that a relevant fraction of ash components were dissolved in the liquid medium, and this high pH value also contributes to the death of microorganisms, reducing this type of contamination and decreasing competition with the microalgae during the bioremediation step. After the settling period, the manure or ash components that were insoluble in water or that reached their solubility limits accumulate in the bottom of the flask, forming a precipitate layer, leaving on the top a liquid phase less turbid than the diluted manure. The liquid phase was recovered after filtration through a cotton cloth and used for microalgae inoculation. The dilution followed by the physicochemical pre-treatment had a very significant effect in reducing the levels of total nitrogen, phosphorus, and COD from the crude effluent (manure) to the effluent used for microalgae growth. Regarding the biomass ash treatment, reduction of total nitrogen, phosphorus, and COD were 35.7%, 91.8%, and 46%, respectively for the 1:5 diluted effluent and 73.6%, 94.9%, and 38.4% for the 1:10 diluted effluent. Final COD values were (4736.8 and 4386.0 mg O_2 L^{-1}) reflecting the dissolution of mineral components and were much higher than the limit value allowed for discharge: 150 mg O_2 L^{-1} (Portuguese Ministry of the Environment, 1998), thus requiring an additional remediation step. Total nitrogen concentration had low final values after pre-treatment (35.7 and 91.3 mg L^{-1}) which justified the need to supplement the culture medium with this element during the semi-continuous tests (3^{rd} and 4^{th}) to avoid growth inhibition effects.

The composition of the precipitate recovered after the treatment of the diluted manure with biomass ash is presented in Table 4.3.

The precipitate had a slightly alkaline pH, low moisture (17.5%), and an organic matter content of (22.8%), corresponding to the organic components coagulated from the

diluted manure. This precipitate may be combined with soil and organic waste (such as the original manure) to produce a compost soil with moisture in the range of 30 and 60% (Wang *et al.*, 2020), and organic matter higher than 50% (USDA, 2010), appropriate characteristics for soil amendment. The precipitate is rich in cations relevant for plant nutrition, in particular, Ca, Mg, and K, often present in low concentrations in Portuguese soils. The precipitate also had relatively high N and P contents, 4.8 and 2.3 mg g^{-1}, respectively, but also high C/N and C/P ratios indicating that mineralization of these components is complex, thus they have a poor bioavailability (Deng *et al.*, 2020).

Table 4.3 – Analytical characterization of the cattle precipitate (mean ± SD, n = 3).

Parameter	Units	Cattle precipitate	Parameter	Units	Cattle precipitate
Moisture	%	17.5 ± 0.5	C/N	-	33.6
Ash content	%	51.7 ± 0.4	C/P	-	158.4
Volatile Matter	%	42.3 ± 2.4	Nitrogen	mg g^{-1}	4.8
Organic matter	%	22.8 ± 1.9	Phosphorus	mg g^{-1}	2.3
pH	-	8.7	Calcium	mg g^{-1}	114.1
Electrical conductivity	$dS.m^{-1}$	6.8	Magnesium	mg g^{-1}	10.2
Bulk density	m/v	2.42 ± 0.06	Potassium	mg g^{-1}	1.1

Concerning micronutrients and heavy metals, their concentration in the precipitate is much lower than the maximum concentrations admitted for wastewater sludge or other sludges from organic products processing, that are allowed for agricultural use, in law decree 276/2009 (Portuguese Ministry of the Environment, 2009).

4.2 Piggery effluent

The piggery effluent was treated with 120 g L^{-1} of biomass ash. This treatment had a yield of 89.8% (wt.) of pre-treated liquid effluent and 10.2% (wt.) of the precipitate.

The raw piggery effluent, the piggery effluent pre-treated with ash (P+A), and the piggery effluent pre-treated with ash plus olive-oil mill wastewater (P+A+O) were evaluated and had the characteristics presented in Table 4.4.

Table 4.4 – Characterization of the raw piggery effluent (RP), piggery effluent pre-treated with ash (P+A), and piggery effluent pre-treated with ash and olive-oil mill wastewater (P+A+O).

Parameter	Units	Batch mode tests			Ash removal efficiency (%)	Semi-continuous mode tests		Ash removal efficiency (%)
		RP	(P+A) (120 g L^{-1})	(P+A+O)		RP	(P+A) (120 g L^{-1})	
pH	-	6.9 ± 0.1	13.1 ± 0.2	12.8 ± 0.2	-	6.8 ± 0.2	12.5 ± 0.2	-
Total N	mg N L^{-1}	1138.7 ± 15.1	913.7 ± 6.9	913.1 ± 11.5	19.8	860.1 ± 4.3	690.0 ± 5.1	19.8
Kjeldahl N	mg NH_3 L^{-1}	1137.3	912.5	911.7	19.8	860.0	690.0	19.8
Nitrates	mg NO_3^- L^{-1}	7.78 ± 0.82	1.10 ± 0.10	0.71 ± 0.01	85.9	0.046± 0.010	0.007 ± 0.000	84.8
Nitrites	mg NO_2^- L^{-1}	0.00 ± 0.00	0.33 ± 0.14	0.01 ± 0.00	-	0.003 ± 0.000	0.052 ± 0.008	-1633.3
Total P	mg P L^{-1}	13.2 ± 1.0	6.2 ± 1.4	6.8 ± 0.3	53.0	26.2 ± 0.1	6.4 ± 0.0	75.6
O.D.	540nm	1.830 ± 0.036	0.766 ± 0.015	1.067 ± 0.022	**58.1**	1.593 ± 0.043	0.198 ± 0.009	**87.6**
COD	mg O_2 L^{-1}	2150.0 ± 31.3	1043.4 ± 58.3	2100.5 ± 63.2	**51.5**	2300.0 ± 91.4	1171.4 ± 40.4	**49.1**
BOD_5	mg O_2 L^{-1}	1050.0 ± 26.6	800.0 ± 11.3	970.0 ± 35.3	**23.8**	1250.0 ± 70.7	970.0 ± 14.1	**22.4**
Phenols	mg L^{-1}	25.8 ± 2.4	17.2 ± 0.3	87.2 ± 4.0	33.3	n.d.	n.d.	-
Total solids	g L^{-1}	10.1 ± 0.5	27.7 ± 0.3	29.9 ± 0.1	-174.3	6.3 ± 0.0	14.3 ± 0.1	-127.0
Ash content	g L^{-1}	6.2 ± 0.2	24.0 ± 0.4	24.0 ± 0.2	-287.1	4.0 ± 0.1	10.9 ± 0.1	-172.5

n.d. – not determined.

The values in bold are the ones with the greatest reductions.

The conducting of a physicochemical pre-treatment led to an increment of the pH to 12.5, as well as the precipitation of several particles in suspension allowing better light penetration and its use as a culture medium, namely for microalgae. The pre-treatment with ash resulted in an effluent with a COD and optical density at least 50% lower and a BOD_5 decrease of about 23%, making this effluent much less organically charged. Conversely, the pre-treatment considerably increased the total solids of the effluent, as well as its ash content.

The precipitate obtained after the pre-treatment of the piggery effluent with biomass ash is mainly composed of ash and some suspended solids that were found in the effluent. The composition of the precipitate is presented in Table 4.5.

The precipitate had an extremely high alkaline pH and low moisture (27.5%). The organic matter was quite low (5.2%) however, most agricultural soils have levels of 1 to 2%. The presence of organic matter generates benefits to the soil as it conserves moisture and soil aggregation (USDA, 2010). The presence of calcium, magnesium, and potassium cations are important as they are essential for the development of plants. The C/N ratio is used to check the stability of nitrogen in the compost.

Table 4.5 – Analytical characterization of the piggery precipitate (mean ± SD, n = 3).

Parameter	Units	Piggery precipitate	Parameter	Units	Piggery precipitate
Moisture	%	27.5 ± 0.8	C/N	-	69.8
Ash content	%	81.2 ± 0.3	C/P	-	44.5
Volatile Matter	%	17.0 ± 0.9	Nitrogen	mg g^{-1}	1.5
Organic matter	%	5.24 ± 0.70	Phosphorus	mg g^{-1}	5.2
pH	-	12.3	Calcium	mg g^{-1}	233.2
Electrical conductivity	$dS.m^{-1}$	6.2	Magnesium	mg g^{-1}	20.5
Bulk density	m/v	1.29	Potassium	mg g^{-1}	2.3

Composts with low C/ N ratios (< 15) indicate that their decomposition can provide high amounts of nitrogen, high ratios indicate nitrogen is stable in the compost and it will be less accessible to be assimilated by plants (Deng *et al.*, 2020). The precipitate can be considered as a source of P, as it had a high content of this nutrient (5.2 mg g^{-1}), and since the C/P ratio was not very high, phosphorus mineralization was not too complex. The precipitate had a low amount of micronutrients and heavy metals in its constitution, so its incorporation in the soil would never exceed the legal limits allowed for sludge applications (Portuguese Ministry of the Environment, 2009).

4.3 Poultry effluent

The poultry effluent was treated with 80 g L^{-1} of biomass ash. This treatment resulted in 93.2% (wt.) of pre-treated liquid effluent and 6.8% (wt.) of a precipitate.

The compositions of the poultry effluents, raw poultry effluent, poultry effluent without (PE), and with (PE+A) ash, used in the different tests are given in Table 4.6. Although the effluents used do not have high nitrogen and phosphorus load, the initial COD load is very significant (6140 mg L^{-1} and 5630 mg L^{-1}).

Similar to what was described for the previous effluents, the pre-treatment with ash led to a significant decrease in COD, BOD_5, and optical density of the raw effluent, above 70%. In this case, nitrogen and phosphorus were also reduced by 50% after pre-treatment.

Table 4.6 – Compositions of the poultry effluents without (PE) and with (PE+A) ash in the batch mode and semi-continuous mode tests.

Parameter	Units	Batch mode tests		Ashremoval efficiency (%)	Semi-continuous mode tests		Ash removal efficiency (%)
		PE	PE + A		PE	PE + A	
pH	-	7.2 ± 0.4	12.3 ± 0.3	-	7.2 ± 0.2	12.3 ± 0.3	-
Total N	mg N L^{-1}	205.0 ± 19.3	104.8 ± 11.0	48.9	371.3 ± 24.1	186.3 ± 13.8	49.8
Kjeldahl N	mg NH_3 L^{-1}	204.8	104.0	49.2	369.0	185.5	49.7
Nitrates	mg NO_3 L^{-1}	0.2 ± 0.0	0.8 ± 0.0	-300.0	2.07 ± 0.05	0.69 ± 0.02	66.7
Nitrites	mg NO_2 L^{-1}	0.00 ± 0.00	0.05 ± 0.00	-48.7	0.27 ± 0.01	0.09 ± 0.00	66.7
Total P	mg P L^{-1}	61.4 ± 8.3	4.9 ± 0.0	92.0	58.5 ± 6.6	11.3 ± 1.3	80.7
O.D.	540 nm	0.75 ± 0.04	0.03 ± 0.00	**96.1**	0.95 ± 0.03	0.02 ± 0.00	**97.9**
COD	g O_2 L^{-1}	6.32 ± 0.88	1.31 ± 0.14	**79.3**	5.63 ± 0.27	1.52 ± 0.08	**73.0**
BOD_5	g O_2 L^{-1}	1.35 ± 0.07	0.04 ± 0.00	**97.0**	2.40 ± 0.05	0.35 ± 0.01	**85.4**
Total solids	g L^{-1}	3.5 ± 0.5	15.6 ± 1.6	-345.7	5.7 ± 0.2	17.2 ± 0.8	-201.8
Ash content	g L^{-1}	1.0 ± 0.1	13.1 ± 0.7	-1210.0	2.6 ± 0.2	15.7 ± 0.2	-503.8

The values in bold are the ones with the greatest reductions.

The characterization of the precipitate obtained after pre-treating the poultry effluent with biomass ash is presented in Table 4.7.

Table 4.7 – Analytical characterization of the poultry precipitate (mean ± SD, n = 3).

Parameter	Units	Poultry precipitate	Parameter	Units	Poultry precipitate
Moisture	%	11.0 ± 0.2	C/N	-	122.4
Ash content	%	69.8 ± 0.2	C/P	-	44.9
Volatile Matter	%	28.2 ± 0.4	Nitrogen	mg g^{-1}	0.8
Organic matter	%	6.0 ± 0.2	Phosphorus	mg g^{-1}	4.8
pH	-	10.5	Calcium	mg g^{-1}	208.0
Electrical conductivity	dS.m^{-1}	5.8	Magnesium	mg g^{-1}	20.2
Bulk density	m/v	1.48	Potassium	mg g^{-1}	0.6

The precipitate had an alkaline pH and extremely low moisture (11%), since the ideal would be between 30 and 40% (Wang *et al.*, 2020) however, it should also be pointed out that higher moisture leads to higher transportation costs, as it affects the soil bulk density.

Organic matter consists of the carbon-based amount present in the compost and, ideally, its percentage should be higher than 50%. The precipitate considered contains a substantially lower value (6.0%) which, depending on the precipitate proportion incorporated into the soil, may compromise the soil aggregation and/or moisture retention (USDA, 2010). The precipitate had bases in its composition, mainly calcium and magnesium, which is beneficial because they are essential elements for plant nutrition. The high C/N ratio of the precipitate shows that nitrogen is less accessible to be assimilated by plants and, therefore, the incorporation of nitrogen fertilizers would be required (Deng *et al.*, 2020). In view of this knowledge, the value 122.4 indicates that the nitrogen in the compost was practically inaccessible to the plants. Because the precipitate had a high phosphorus content (4.8%) it constitutes a significant source of this nutrient – however, it was found that the organic P mineralization becomes more difficult as C/P increases. Regarding micronutrients and heavy metals, the application of the precipitate incorporates in the soil amounts much lower than the maximum values allowed and prescribed by the Portuguese authorities for the agricultural use of sludge originated from the processing of organic products (law decree L276/2009 – (Portuguese Ministry of the Environment, 2009)).

4.4 Landfill Leachate

Table 4.8 presented the chemical analysis of the landfill leachate as received and pre-treated with ash and the removal rate obtained after the biomass ash pre-treatment is also presented.

After the chemical precipitation treatment, the leachate's dark brown colour turned into a light translucid yellow that enables light penetration, a condition that is necessary for microalgae growth. Moreover, the COD and BOD_5 values of the treated leachate decreased by 81.1% and 92.0%, respectively, representing the removal of a significant fraction of the oxidizable compounds present in the original leachate. Nevertheless, it should be noted that the biodegradability of the leachate was not improved by the precipitation process. The process caused a decrease in BOD_5/COD ratio from 0.12 to 0.05, indicating that a large fraction of the precipitated species was biodegradable while more recalcitrant species remained in the treated leachate.

Table 4.8 - The main characteristics and composition of the landfill leachate used.

Parameter	Units	Landfill Leachate	Landfill Leachate pre-treated with ash	Removal Rate (%)
pH	-	8.1 ± 0.3	12.7 ± 0.1	-
Conductivity	$mS.cm^{-1}$	33.7 ± 0.9	53.8 ± 0.3	-59.6
Total nitrogen	$mg\ L^{-1}$	3295 ± 438	2205 ± 63	33.1
Kjeldahl N	$mg\ NH_3\ L^{-1}$	3266 ± 435	2184 ± 40	33.1
Total phosphorus	$mg\ L^{-1}$	22.9 ± 0.1	12.5 ± 2.4	45.4
COD	$mg\ O_2\ L^{-1}$	9600 ± 571	1818 ± 286	**81.1**
BOD_5	$mg\ O_2\ L^{-1}$	1150 ± 70	92.1 ± 14.0	**92.0**
Total solids	$g\ L^{-1}$	33.66 ± 1.10	39.96 ± 0.27	-18.7
Fixed solids	$g\ L^{-1}$	25.29 ± 0.72	37.81 ± 0.58	-49.5
Total phenolics	$mg_{\text{Gallic acid equivalents}}.L^{-1}$	742.2 ± 27.6	76.6 ± 7.40	**89.7**
Chlorine	$mg\ L^{-1}$	10497 ± 289	22749 ± 1037	-116.7
Al	$mg\ L^{-1}$	13.3 ± 1.7	23.6 ± 0.1	-77.4
Ca	$mg\ L^{-1}$	318.2 ± 30.4	621.7 ± 77.1	-95.4
K	$mg\ L^{-1}$	430.7 ± 40.8	640.1 ± 34.7	-48.6
Mg	$mg\ L^{-1}$	31.3 ± 6.9	12.8 ± 0.9	59.1
Na	$mg\ L^{-1}$	624.4 ± 5.3	660.5 ± 26.7	-5.8

The values in bold are the ones with the greatest reductions.

The fixed solids of the treated leachate increased by 49.5%, because of the partial dissolution of ash components. This ash dissolution effect was also reflected by the increase of pH, conductivity, and concentration of some inorganic components, such as chlorine, aluminium, calcium, potassium, and sodium. Nevertheless, Kjeldahl nitrogen, total phosphorus, and total phenolics suffered considerable reductions (31.1%, 45.4%, and 89.7%, respectively) following the pre-treatment, achieving values that are more adequate for the subsequent remediation step. The pre-treatment process removes efficiently organic contaminants that contribute to the leachate colour, but it was also a source of additional inorganic compounds that were transferred from the biomass ash into the leachate. Some components that were absent in the original leachate (Mn, Cu, Cd, Pb, or Cr) were also not detected in the treated leachate, confirming that these components were not dissolved by contact with the biomass ash. Overall, biomass bottom ash showed the ability to act as a precipitating agent with significant improvements, particularly regarding colour, total suspended solids, and COD, which were limiting factors for microalgae growth (Yin *et al.*,

2020). As such, this mineral waste has the potential to be used as an efficient, sustainable, and cost-effective alternative to the commonly applied precipitating agents in landfill leachate pre-treatment.

One of the main drawbacks of chemical precipitation includes the high dose of precipitant that is required. These very significant amounts of precipitating agents generate sludge (ash + precipitated material), which needs further management or valorisation. For that purpose, preliminary tests of mortar production were done, incorporating the sludge produced after chemical precipitation of landfill leachate (Viegas et al., 2021d).

4.5 Final considerations

The pre-treatment with biomass ash was efficient in all effluents tested, which has led to a significant reduction in the levels of an oxidizable material, supported by the COD and BOD_5 values achieved after the treatment. Nevertheless, these levels would in no case allow the discharge of the respective effluents into body streams or municipal collectors, requiring additional treatment. However, this pre-treatment resulted in an increase of dissolved minerals in the effluents, which were expected to promote the subsequent microalgae growth.

The three precipitates obtained (from cattle, piggery, and poultry) showed quite distinct characteristics, mainly regarding the organic matter and the amount of nitrogen. The precipitate with the best agronomic attributes comes from the treatment of cattle effluent, being the one with the highest amount of organic matter and moisture, the least extreme pH, and the best proportion of essential minerals for plant development.

Chapter 5

Biomass productivity and wastewater bioremediation process

The aim of studying the bioremediation of different effluents with microalgae is to optimize the remediation process concomitant to the maximization of biomass production. A semi-continuous transfer system was developed and the addition of different volumes of effluent to the system was studied, to test the maximum additional volume for which the microalgae are able to treat each of the effluents. The ability of microalgae to remedy the various effluents is different between effluents and between microalgae. Additionally, it was intended to do these remediations without using dilutions or pre-treatments that involved high costs. So, it was not used the typical filtration, UV sterilization, or autoclaving, to decrease nitrogen, phosphorus, and COD concentrations.

5.1 Aquaculture effluent

Intensive or semi-intensive aquaculture increases the concentration of nutrients in the aqueous medium. This accumulation is due to feed residues and excrements of the aquatic species produced, which stimulates the growth of several microorganisms, some of them pathogenic. To prevent the development of diseases in animals in aquaculture, antibiotics and other antimicrobial agents are added. These agents of control and prevention of the proliferation of microorganisms may not be completely metabolized or excreted, thus bioaccumulating in the species produced and being transposed to the food chain could constitute a consumers' health risk (Rosa *et al.*, 2020).

The regular discharge of nutrient-rich effluents into adjacent water bodies can also lead to eutrophication phenomena due to the uncontrolled proliferation of algae (micro and macro). This problem is particularly critical when these effluents with high organic and inorganic loads are discharged into aquatic environments with low dispersion rates, such as lakes or estuaries (Fonseca *et al.*, 2021).

Thus, the search for alternative and sustainable methods to control the excessive accumulation of nutrients and microorganisms in these growth mediums of aquatic species

or the corresponding effluents has been an area of growing interest (Lin *et al.*, 2020; Paul *et al.*, 2021).

Bioremediation of aquaculture effluents has been tested with several microalgae. The remediation rates of total nitrogen and total phosphorus from an aquaculture tank of the shrimp *Penaeus vannamei* using *Chlorella vulgaris*, in continuous mode, were 86.1% and 82.7%, respectively (Gao *et al.*, 2016). Nile tilapia (*Oreochromis niloticus*) effluent in batch mode using *T. obliquus* removed 88.7% and 100%, respectively, and *C. sorokiniana*, 98.2%, and 100%, respectively. It is important to note that this effluent had a very low COD value (96 mg L^{-1}) with a 42% remediation for *T. obliquus* (Ansari *et al.*, 2017). Batch mode flathead grey mullet *Mugil cephalus* effluent using *Tetraselmis suecica*, *Dunaliella tertiolecta* and *Isochrysis galbana* removed 94.4% and 96.0%, 95.4% and 91.2%, 66.0% and 91.9% for total nitrogen and total phosphorus, respectively (Andreotti *et al.*, 2017). A wet market wastewater mainly from fish and seafood entrails (diluted 50% in deionized water) was remediated with *Scenedesmus* sp. achieving removal rates of 87% and 91% for total N and total P (Apandi *et al.*, 2019). Nevertheless, several of these works were performed with aquaculture effluent previously treated with ultraviolet light (Andreotti *et al.*, 2017), filtered using 0.45 μm filter papers followed by autoclaving prior to microalgal inoculation (Ansari *et al.*, 2017), settling overnight, and filtration (Gao *et al.*, 2016), or filtered through a membrane filter of 0.45 μm (Apandi *et al.*, 2019).

In the studied case, the aquaculture company receives brown crabs weekly and needs to carry out the treatment of the transport effluent of these animals, before releasing it. This effluent is highly charged with nitrogen and presents a remarkably high COD. The objective was to optimize a semi-continuous remediation approach, that allows the effluent's remedy, considering the volumes received weekly by the company. A system consisting of a bioreactor made up of three independent sequential containers was compared to a system using only a single container.

5.1.1 Biomass Productivity

The composition of aquaculture effluent used in the tests is presented in Table 5.1. For the 1st test aquaculture effluent, 1 was used, for the 2nd and 3rd ones it was used the aquaculture effluent 2, and for the 4th test, it was used the aquaculture effluent 3. The

hydraulic retention time in the 2nd test was 20 days and, in the 3rd and 4th tests was 10 days. The pH of all effluents was 7.3 and the conductivity was between 38 and 40ms/cm.

Table 5.1 – Composition of aquaculture effluents (mean ± SD, n = 3).

	Total nitrogen (mg N L^{-1})	**Total phosphorus (mg P L^{-1})**	**COD (g O_2 L^{-1})**	**BOD_5 (g O_2 L^{-1})**	**Total solids content (g L^{-1})**	**Total ashes content (g L^{-1})**	**Optical density (540 nm)**
Aquaculture effluent 1	168.3 ± 1.3	32.9 ± 1.0	1.25 ± 0.07	0.68 ± 0.18	39.2 ± 0.7	33.1 ± 0.2	0.21 ± 0.02
Aquaculture effluent 2	737.8 ± 4.7	22.1 ± 1.0	5.20 ± 0.14	3.05 ± 0.21	40.9 ± 0.7	33.7 ± 0.4	1.87 ± 0.12
Aquaculture effluent 3	210.0 ± 5.3	18.4 ± 0.2	0.76 ± 0.05	0.62 ± 0.03	39.0 ± 1.3	33.0 ± 0.1	0.17 ± 0.01

The experiments demonstrated that microalgae could grow in these aquaculture effluents. The 1st test used the aquaculture effluent 1 and was in batch mode, lasted for 33 days, and ended when all nitrogen and phosphorus available were consumed and a decline in COD (<150 mg O_2 L^{-1}) was observed. The objective of this experiment was to determine the two algae species that had the best performance in terms of remediation and biomass production. Microalgae *C. vulgaris* and *T. obliquus* managed to achieve the remediation after 22 days, in contrast to the other microalgae that only reached remediation after 33 days. Biomass concentration reached after 22 days was significantly higher for the microalga *Chlorella vulgaris* grown in aquaculture effluent (3.22 ± 0.04 g L^{-1}) when compared with the one grown in the synthetic medium (1.36 ± 0.39 g L^{-1}) (Figure 5.1). The same situation occurred for *Tetradesmus obliquus*, 2.20 ± 0.21 g L^{-1} in aquaculture effluent and 1.60 ± 0.03 g L^{-1} in synthetic medium.

I. galbana had similar growth for synthetic medium (as control) and aquaculture effluent: 1.09 ± 0.02 g L^{-1} and 1.03 ± 0.03 g L^{-1}, respectively after 33 days. Lower growth was found in aquaculture effluent when using *M. salina* than in synthetic medium: 0.69 ± 0.06 g L^{-1} and 1.47 ± 0.13 g L^{-1}, respectively.

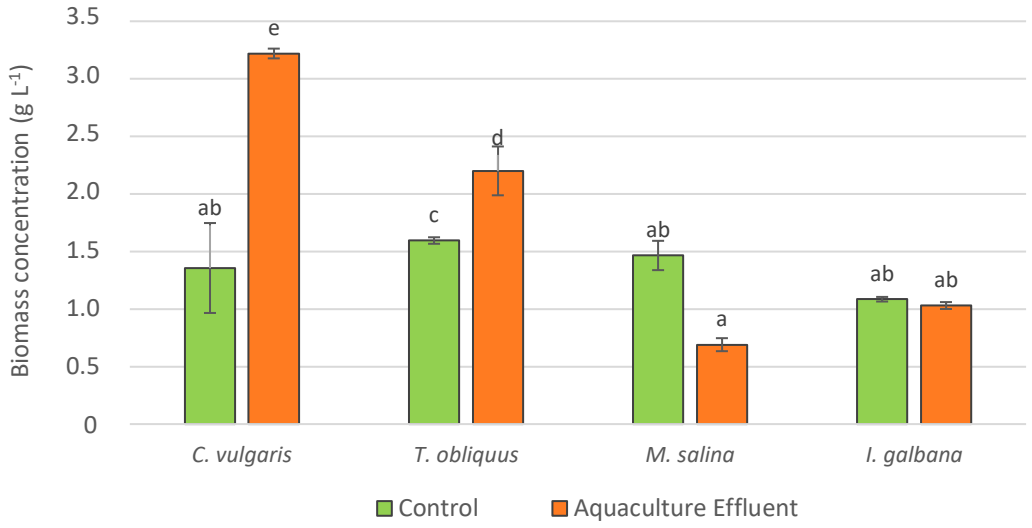

Figure 5.1 – Biomass concentration of the microalgae grown in synthetic medium and aquaculture effluent in the 1st test (mean ± SD, n = 3) after 22 days (*Chlorella vulgaris* and *Tetradesmus obliquus*) and after 33 days for the remaining microalgae of the test. The values with different index letters show significant differences with $p < 0.05$.

The 2nd and 3rd tests were in semi-continued mode and lasted 36 and 34 days, respectively, and it was used the aquaculture effluent 2. The two microalgae selected were *Chlorella vulgaris* and *Tetradesmus obliquus* for the 2nd and 3rd tests because they were the ones who, in addition to being able to remedy the initial effluent in less time, achieved higher biomass concentration. *T. obliquus* was also chosen for the test with the configuration B (reactor with triple effluent volume: To-B) because in preliminary tests it had demonstrated a better ability to remedy these aquaculture effluents, especially COD.

The microalga *T. obliquus* stood out during the 2nd test, but *C. vulgaris* performed better in the 3rd test, as shown by Figure 5.2, which explains the evolution of productivity in the Cv3-A, To3-A, and To-B reactors, over the weeks for the two tests.

The yield on the last day of the 2nd and 3rd tests for the various reactors is shown in Figure 5.3, where there was an evident trend towards higher productivity in the final reactors, Cv3-A and To3-A.

In the 2nd test, *T. obliquus* was the microalga with the best performance with productivity of 427.7 mg L^{-1} day^{-1}. Although, in the 3rd test *C. vulgaris* had productivity of 879.8 mg L^{-1} day^{-1} higher than *T. obliquus* in the two configurations: A - 3 reactors of 1500

mL with 811.7 mg L^{-1} day^{-1} (To3-A); B - one reactor of 4.5 L (To-B) with 731.0 mg L^{-1} day^{-1}.

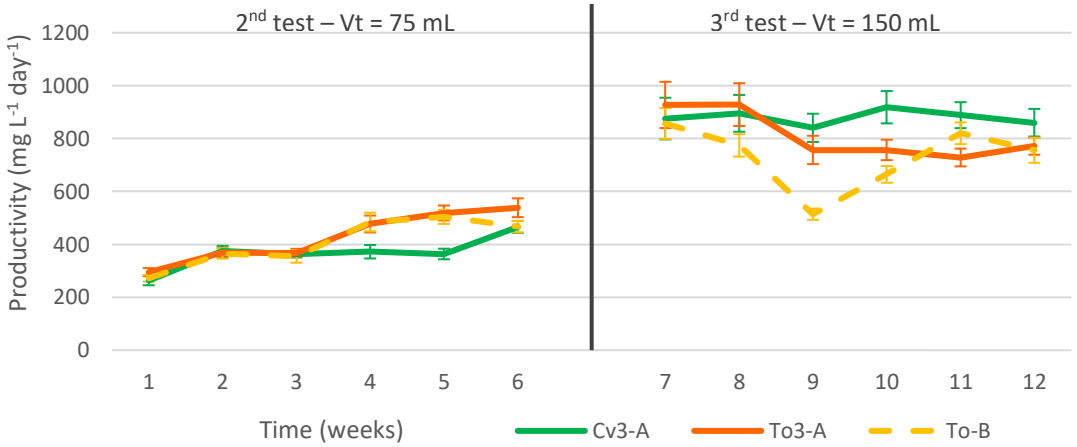

Figure 5.2 – Progress of biomass productivity in aquaculture effluent, for the last reactor, for the 2nd and 3rd tests (mean ± SD, n=3). Vt – transference volume; Cv – *Chlorella vulgaris*; To – *Tetradesmus obliquus*; A - configuration A (three reactors with 1.5 L each): B - configuration B (single reactor with 4.5 L).

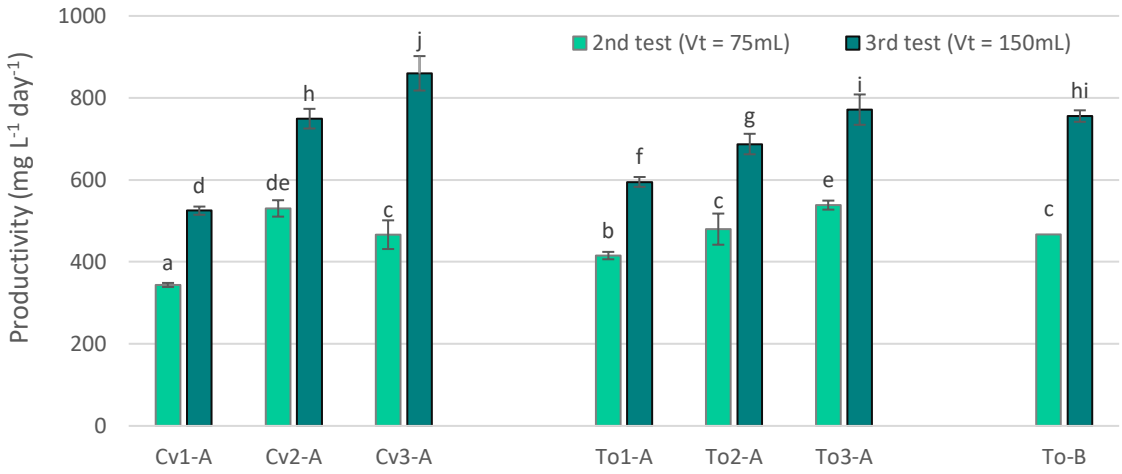

Figure 5.3 – Biomass productivity in the 2nd and 3rd tests for all reactors (Vt – transference volume; Cv – *Chlorella vulgaris*; To – *Tetradesmus obliquus*; A – configuration A (three reactors with 1.5 L each); B – configuration B (single reactor with 4.5 L)). The values with different index letters show significant differences with $p < 0.05$.

Gao *et al.* (2016) grew *C. vulgaris* in shrimp aquaculture wastewater with a lower concentration of nutrients (6.8 mg L^{-1} for total nitrogen and 0.5 mg L^{-1} for total phosphorus) in a membrane photobioreactor and attained a biomass yield of 7.3 and 42.6

mg L^{-1} day^{-1} for batch mode and continuous mode, respectively, these values are significantly lower than those obtained in the present study (146.4 mg L^{-1} day^{-1} in batch mode and 879.8 mg L^{-1} day^{-1} in semi-continuous mode for Cv). In another study, with aquaculture wastewater containing lower nutrient concentrations (60 mg L^{-1} for TN, 6.8 mg L^{-1} for TP, and 112 mg O_2 L^{-1} for COD), the microalga *Chlorella* sp. reached biomass productivity of 243 mg L^{-1} day^{-1} (Kuo *et al.*, 2016). Apandi *et al.* (2019) grew *Scenedesmus* sp. in wet market wastewater mostly from fish and seafood entrails (with 480 mg L^{-1} for TN, 87 mg L^{-1} for TP, and 1754 mg O_2 L^{-1} for COD) achieving maximum productivity of 98.54 mg L^{-1} day^{-1}. Higher productivity of microalgae was expected in the present study since the effluent used was richer in nutrients.

The 4th test was also in semi-continuous mode using the aquaculture effluent 3 and lasted for 35 days. The purpose of this test was to study the effect of the transfer method on a larger volume reactor and to obtain sufficient algal biomass to conduct a mussel feed test. For this reason, two microalgae with different profiles were chosen: *Tetradesmus obliquus* and *Microchloropsis salina*. Both microalgae had a good development reaching, respectively, yields of 194.3 and 156.4 mg $L^{-1}day^{-1}$. The effluent used had a lower nutrient load compared to the one used in 2nd and 3rd tests, however, it was decided to supplement with half the dose of nitrogen and phosphorus used in the previous case. This is probably the reason why the productivity achieved for To is not as high as in the 2nd and 3rd tests.

5.1.2 Bioremediation process

The remediation rate in the aquaculture effluent for total nitrogen and total phosphorus was 100% for all selected microalgae (Table 5.2). Regarding COD, the remediation rate was higher than 72% for all algae and higher than 97% for BOD_5. Therefore, there were no microalgae that stood out significantly from the rest.

Table 5.2 – Remediation rates of aquaculture effluent by microalgae in the 1st test (n = 3).

		Removal efficiency (%)				
		Total Nitrogen	**Total Phosphorus**	**COD**	**BOD_5**	**Ash content**
1st test	*C. vulgaris*	100	100	86.6 ^{bc}	98.0 ^{a}	11.8 ^{a}
	T. obliquus	100	100	90.6 ^{bc}	96.5 ^{a}	19.5 ^{b}
	S. major	100	100	94.0 ^{c}	97.6 ^{a}	16.4 ^{ab}
	M. salina	100	100	71.8 ^{a}	98.4 ^{a}	18.7 ^{b}
	I. galbana	100	100	83.9 ^{b}	97.6 ^{a}	18.9 ^{b}

Note: The values with different index letters show significant differences with $p < 0.05$.

Concerning the removal efficiency of the two microalgae selected for 2nd and 3rd tests, it became evident that in the 2nd test the available nitrogen and phosphorus were also consumed, but the COD in the last reactor did not reach the concentration needed to be released: lower than 150 mg O_2 L^{-1} (Portuguese Ministry of the Environment, 1998). In the 3rd test, it was decided to add a nitrogen supplementation of 20 mg N L^{-1} every other day in the 2nd and 3rd reactors so that the culture could lower COD levels. In these reactors, the nitrogen levels were too low to allow the culture to grow, a situation that had already been verified by Bona *et al.* (2014) and Markou *et al.* (2016).

The remediation rate in the aquaculture effluent for total nitrogen and total phosphorus was also 100% for the two tests and the two microalgae (Table 5.3). Regarding the COD, the removal efficiency was higher than 88% in the 2nd test and it was verified that with the nitrogen supplementation in the 3rd test, the microalgae were able to remedy more than 90% (Figure 5.4). The BOD_5 was remediated in the 2nd test between 91 and 96%, but when nitrogen supplementation was added, the results approached the total removal both for Cv and To.

Table 5.3 – Remediation rates for aquaculture effluent for *C. vulgaris* and *T. obliquus* in the 2nd and 3rd tests and *T. obliquus* and *M. salina* in the 4th test (mean ± SD, n = 3). The values with different index letters show significant differences with $p < 0.05$.

		Removal efficiency (%)				
		Total Nitrogen	**Total Phosphorus**	**COD**	**BOD_5**	**Ash content**
2nd test (75 mL)	*C. vulgaris*	100	100 ^{c}	91.5 ^{ab}	95.1 ^{b}	13.3 ^{c}
	T. obliquus	100	100 ^{c}	92.2 ^{ab}	96.3 ^{b}	12.9 ^{c}
	T. obliquus (To-B)	100	100 ^{c}	88.4 ^{a}	91.1 ^{a}	12.4 ^{c}
3rd test (150 mL)	*C. vulgaris*	100	96.5 ^{a}	96.2 ^{b}	99.7 ^{c}	10.3 ^{c}
	T. obliquus	100	98.6 ^{b}	98.1 ^{b}	99.7 ^{c}	1.6 ^{b}
	T. obliquus (To-B)	100	99.3 ^{c}	90.4 ^{ab}	99.3 ^{c}	1.9 ^{b}
4th test (1000 mL)	*T. obliquus*	100	100 ^{c}	91.0 ^{ab}	98.4 ^{bc}	-6.3 ^{a}
	M. salina	100	100 ^{c}	86.7 ^{a}	100 ^{c}	-9.3 ^{a}

In the 4th test, the remediation was remarkably high for all the parameters analysed, except for ash content, for To and Ms, which showed an increase in the amount of salts in the medium.

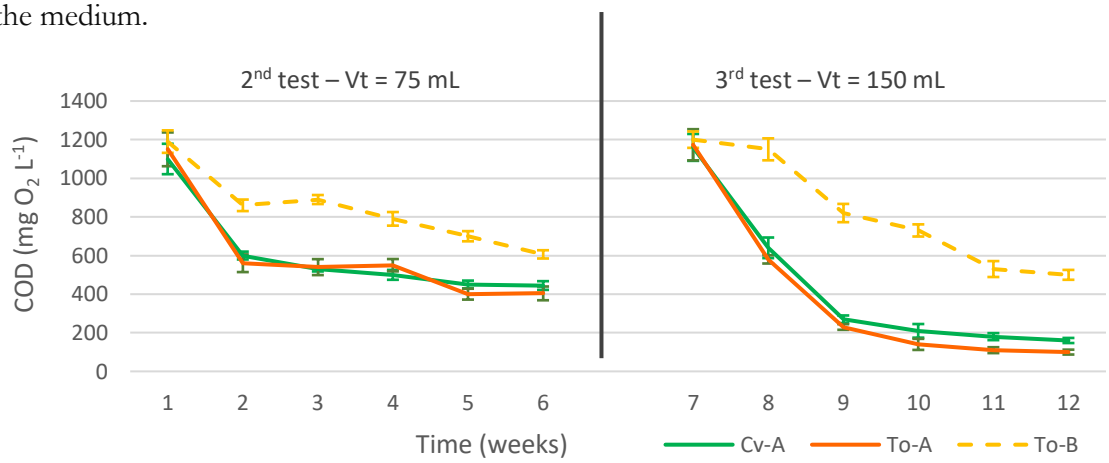

Figure 5.4 – Progress of COD in aquaculture effluent for the 2nd and 3rd tests (mean ± SD, n = 3). Between the 2nd and 3rd tests, 1 week passed, for the culture to rebalance. (Vt – transference volume; Cv – *Chlorella vulgaris*; To – *Tetradesmus obliquus*; A – configuration A (three reactors with 1.5 L each); B – configuration B (single reactor with 4.5 L)).

Comparable studies with aquaculture effluents from Nile Tilapia obtained remediations of 99.8% for nitrate and 99.7% for phosphate for the microalga *Chlorella vulgaris* (Tejido-Nuñez *et al.*, 2019). The microalgae *T. obliquus* and *C. sorokiniana* were able to remediate 88.7% and 100% for total nitrogen and 98.2% and 100% for total phosphorus respectively, concerning COD, *T. obliquus* and *C. sorokiniana* removed respectively 42 and 69% (Ansari *et al.*, 2017). In another study with aquaculture wastewater from *Mugil cephalus*, the microalga *Isochrysis galbana* was able to reduce 66% of the total nitrogen and 80% of the total phosphorus in the effluent (Andreotti *et al.*, 2017). The best COD remediation in the present study occurred in the case of microalgae that did not immediately consume all the existing nitrogen.

5.2 Cattle effluent

Effluents from agro-industries including animal production units contain large amounts of nutrients and microorganisms that contaminate soils and eutrophicate water bodies (Zouboulis *et al.*, 2015). Dairy farms produce massive quantities of manure and wastewaters daily from cleaning activities that cannot be drained into conventional

wastewater treatment plants, nor be totally incorporated into the soil as fertilizer applications (Markou *et al.*, 2018). The treatment of these effluents generally requires multiple methods to efficiently decrease their chemical oxygen demand, ammonia, and microbiological contamination thus constituting a significant economic load for animal producers.

Currently, the two most used solutions are the deposition of manure in open ponds and subsequent deposition in the soil, and the anaerobic digestion of the produced effluents (Font-Palma, 2019). The deposition in the soil causes high risks of soil and groundwater contamination and is a source of considerable gas emissions. Anaerobic digestion implies tight control of the operating parameters and a high dilution ratio of the effluents so that microorganisms are not inhibited, being a limited solution for processing large volumes of manure (Siddique and Wahid, 2018).

Microalgae have been used in the bioremediation of effluents as an alternative to more complex and expensive conventional treatment (Ferreira *et al.*, 2018; Patel *et al.*, 2017; Suganya *et al.*, 2016). Nevertheless, the high solids content of manure and its high degree of microbial contamination require the use of adequate pre-treatment processes before microalgae could be successfully grown.

The productivity and remediation of microalgae in dairy effluents were determined by several authors. Ferreira *et al.*, (2018) used the *Tetradesmus obliquus* microalga to treat dairy wastewater in a flat panel airlift during a 12-day batch, obtaining productivity of 183 mg L^{-1} day^{-1} and remedies of 70% for COD, 78% for phosphorus, and 99% for nitrogen. Biswas *et al.* (2021) also treated dairy effluents with a mixed consortium of microalgae, achieving remedies of 93% for COD, 87% for nitrogen, and 100% for phosphorus. Both studies started with effluents with high CODs (greater than 1800 mg O_2 L^{-1}).

To date, only biofuels have been produced when cattle effluent remediated by microalgae were used (Beevi and Sukumaran, 2014; Gramegna *et al.*, 2020; Hena *et al.*, 2015; Labbéa *et al.*, 2017). Although algal biomass from cattle treatment could have different applications such as crop fertilization or as biostimulant for germination, that is simpler to apply. The inclusion in cattle feed would require more complex studies (Kusmayadi *et al.*, 2021; Wells *et al.*, 2017).

As previously mentioned in the methodology section, the manure was subject to dilution, pre-treated with biomass ash, and phase separation by filtration. The liquid

effluent was then bioremediated using three different microalgae species, separately. The removal of nitrogen, phosphorus, and other components contributing to COD and turbidity were evaluated. The tested process enabled the production of microalgae that could be used as fertilizer and/or feed and from the ash-rich precipitate, as fertilizer. The water used in the dilution step was treated by the microalgae and could be recirculated for dilution of another batch of manure. To the author's knowledge, this is the first application of microalgae in the bioremediation of raw cattle manure.

This strategy could be a sustainable solution for the treatment of raw cattle effluents, using a pre-treatment with a residue (biomass ash) and the subsequent growth of microalgae as an alternative or complement to the existing methods of manure processing by composting, or by anaerobic digestion.

5.2.1 Biomass Productivity

In the 1st experiment the three microalgae were able to grow in the pretreated effluent and reached productivities of 64, 68 and 67 mg L^{-1} day^{-1} for Cv, Ap, and To, respectively, on the last day of the test (Figure 5.5).

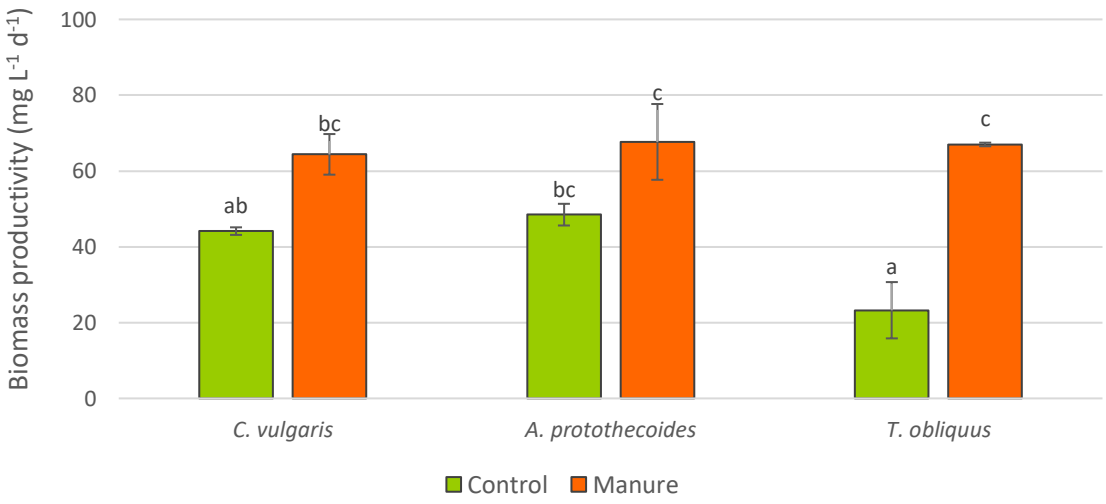

Figure 5.5 – Productivity in synthetic medium and manure effluent for the three algae on the last day of 1st experiment (mean ± SD, n = 2). The values with different index letters show significant differences with $p < 0.05$.

The objective of this test was to select the best two algae species, in terms of effluent remediation's performance and biomass growth. This experiment ended after 12 days when

the effluent reached the parameters N, P, and COD to levels that could be legally discharged.

The growth of the three microalgae was not significantly different between them, however, the growth in the effluent was higher than in the synthetic medium, for the three algae. Similar productivity of 53 mg L^{-1} day^{-1} was achieved by Beevi and Sukumaran (2014) for *Chlorococcum* in fifteen days of cultivation in dairy effluent. However, Hena *et al.* (2015) could attain productivities of 144 mg L^{-1} day^{-1} for *C. vulgaris* grown in dairy farm effluents for 14 days and Franchino *et al.* (2016) achieved 195 mg L^{-1} day^{-1} for *C. vulgaris* grown in digestate from anaerobic digestion of agriculture wastes diluted in tap water 1:10 for eleven days.

All experiments (1st, 2nd, 3rd, and 4th) were conducted for 12 days. The 3rd test was sequential to the 2nd test (after homogenization of the entire culture volume between both duplicates), and the 4th test was sequential to the 3rd. The 4th test was identical to the 3rd test, but the volume added, transferred, and removed from the reactors was increased from 100 to 150 mL. The hydraulic retention time in the 3rd test was 10 days and in the 4th test was 6.7 days.

In the 2nd, 3rd, and 4th experiments productivity increased (Figure 5.6), however, this is also due to the use of $NaNO_3$ supplementation in reactors 2 and 3 of each microalga. Supplementation with phosphorus wouldn't be necessary, since the N:P molar ratio is less than 30:1, according to several authors ratios higher than this are associated with the inhibition of microalgal growth by limiting phosphorus (Porto *et al.*, 2021). However, as the nitrogen supplementation was done, to a final concentration of 20 mg N L^{-1}, supplementation of 10 mg P L^{-1} with KH_2PO_4 was also done in the culture medium, to avoid inhibition of microalgae growth by depletion of those elements (Ansari *et al.*, 2017).

The microalga To had productivity slightly higher than that of Ap during the three tests. There is a tendency for greater productivity in the final reactors, Ap-3 and To-3 in the 3rd experiment, reaching 320.95 and 338.10 mg L^{-1} day^{-1}, respectively. In the 4th test, with transfers of 150 mL of effluent, the productivity in the Ap-3 and To-3 reactors were 522.86 and 554.29 mg L^{-1} day^{-1}, respectively. This result confirms the great capacity of To to treat the effluent, which was already described by several authors (Apandi *et al.*, 2019; A. Ferreira *et al.*, 2019; Viegas *et al.*, 2021a).

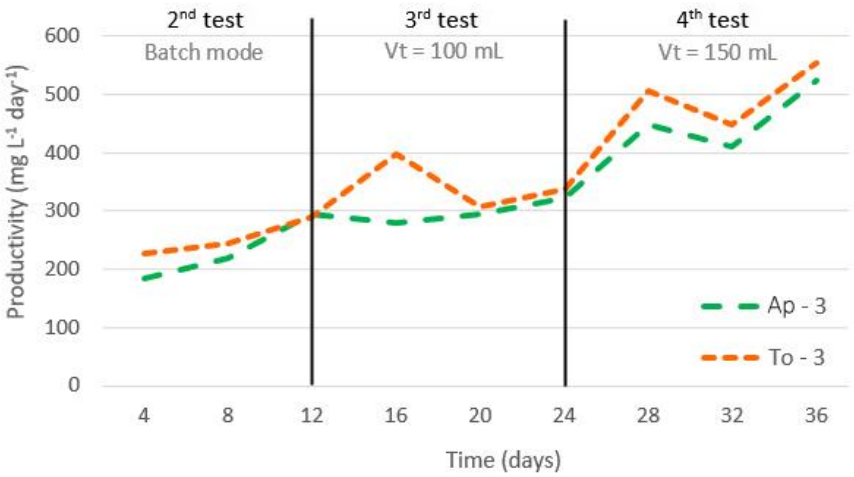

Figure 5.6 - Progress of biomass productivity in manure effluent for the 2nd, 3rd, and 4th tests (mean, n = 2). Vt – transference volume, Ap-3 – *Auxenochlorella protothecoides* in the 3rd reactor, To-3 – *Tetradesmus obliquus* in the 3rd reactor.

The pH of pre-treated cattle effluent for the growth of microalgae was gauged at 7.5. Having a little increase along with the cultures' growth up to 8.5, 8.6, and 9.0 for Cv, Ap, and To, respectively.

5.2.2 Bioremediation process

The growth of microalgae leads to a significant increase in optical density (0.645 ± 0.021 to 3.989 ± 0.093) along 12 days, however, after the culture centrifuged the treated effluent becomes more transparent than before microalgae treatment (0.015 ± 0.003) by adsorption or consumption of suspended materials (Figure 5.7).

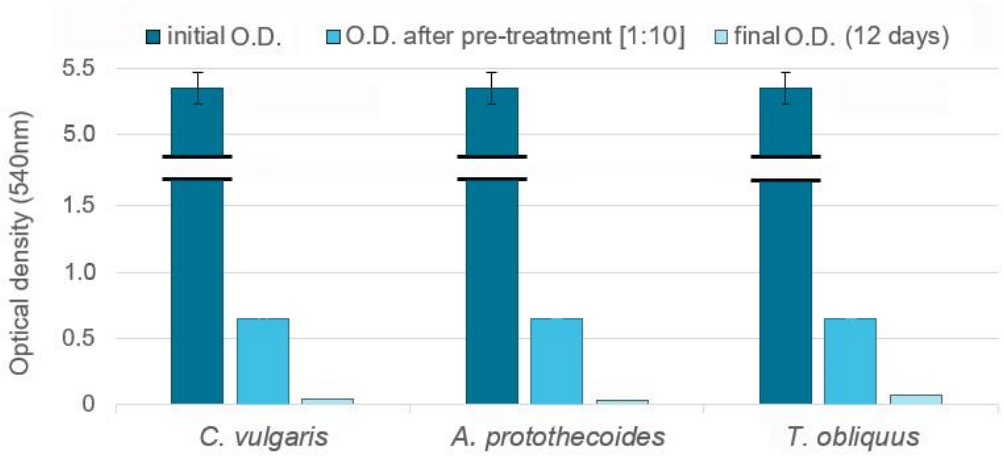

Figure 5.7 - Optical density at the beginning (manure [1:10]), after pre-treatment, and at the end of the 1st experiment for manure effluent in the three algae (after decantation of microalgae) (mean ± SD, n = 3).

The most significant decrease in optical density was through pre-treatment with ash (5.350 ± 0.117 to 0.645 ± 0.021).

In Figure 5.8 it is possible to observe the evolution of the manure effluent throughout the remediation process.

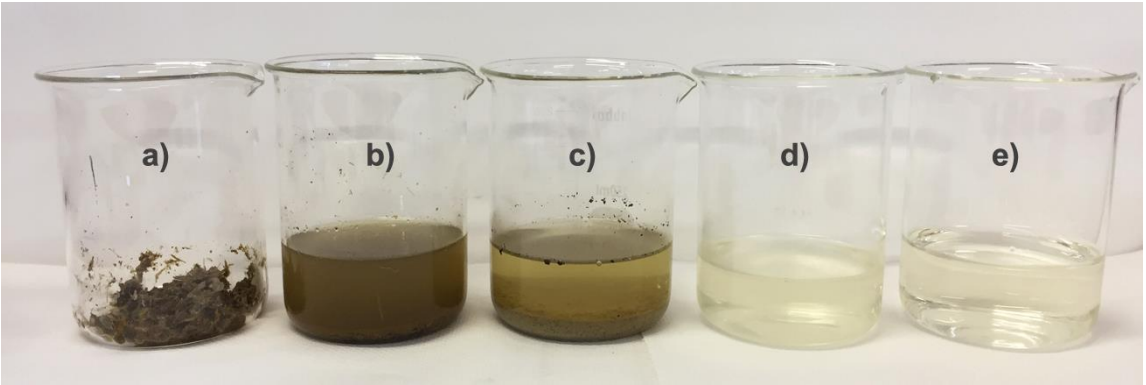

Figure 5.8 – Visual evolution of manure effluent during the remediation process: *a*) cattle manure; *b*) manure diluted (1:10); *c*) manure diluted (1:10) pre-treated with ash; *d*) manure filtered after pre-treatment; *e*) treated effluent after *A. protothecoides* growth.

The concentration of microalgal biomass tends to increase until nutrients are consumed. Table 5.4 shows the removal efficiency of microalgae in the different tests. In the 1st test, the removal efficiency was 100% for nitrogen and that may be a limiting factor to algae growth since they were depleted of nitrogen at a certain moment.

Table 5.4 – Remediation rates for the 1st, 2nd, 3rd, and 4th tests of manure effluent (n = 3).

		Removal efficiency (%)				
		Total Nitrogen	**Total Phosphorus**	**COD**	**BOD_5**	**Ash content**
1st test	*C. vulgaris*	100 ^{a}	29 ^{a}	76 ^{b}	99 ^{a}	54 ^{c}
	A. protothecoides	100 ^{a}	64 ^{c}	91 ^{c}	99 ^{a}	48 ^{b}
	T. obliquus	100 ^{a}	37 ^{a}	77 ^{b}	97 ^{a}	49 ^{b}
2nd test (Batch)	*A. protothecoides*	100 ^{a}	51 ^{b}	97 ^{c}	98 ^{a}	53 ^{c}
	T. obliquus	99 ^{a}	65 ^{c}	95 ^{cd}	98 ^{a}	44 ^{a}
3rd test (100 mL)	*A. protothecoides*	100 ^{a}	83 ^{d}	97 ^{d}	100 ^{a}	56 ^{c}
	T. obliquus	100 ^{a}	79 ^{d}	98 ^{c}	100 ^{a}	57 ^{c}
4th test (150 mL)	*A. protothecoides*	98 ^{a}	100 ^{e}	72 ^{ab}	100 ^{a}	51 ^{bc}
	T. obliquus	98 ^{a}	100 ^{e}	68 ^{a}	100 ^{a}	55 ^{c}

Note: The values with different index letters show significant differences with $p < 0.05$.

The removal efficiency for phosphorus was 29, 37, and 64% for Cv, To, and Ap, respectively. The three tested microalgae have a high potential to remove the nitrogen and total solids, although Ap has a better ability to remove phosphorus. According to Franchino *et al.* (2016), a 64% ammonium reduction and a 93% reduction for phosphate was achieved for *C. vulgaris* grown in digestate from anaerobic digestion of agriculture wastes diluted in tap water 1:10.

The dilution followed by the physicochemical pre-treatment has a very significant effect in reducing the levels of total nitrogen, phosphorus, and COD from the crude effluent (manure) to the effluent used for microalgae growth (manure [1:10] with ash).

Regarding total nitrogen and phosphorus, microalgae treatment should be necessary since the levels are about 9 times higher than the discharge limits (10 mg L^{-1}), however the COD concentration (2105 mg O_2 L^{-1}) is much higher than that allowed for discharge: 150 mg O_2 L^{-1} (Portuguese Ministry of the Environment, 1998), which is why this treatment is mandatory. The biomass ash dissolved in the liquid fraction during the pre-treatment was absorbed by the microalgae at removal rates from 48% to 54%.

The COD reduction was higher than 75% for all algae although Ap was the one with better performance (91%). For BOD_5 the efficiency of removal was nearly 100% for them all. Beevi and Sukumaran (2014) had reported an analogous reduction for COD (93%) and an 82%, for BOD_5 in dairy effluent for *Chlorococcum*.

The nitrogen remediation in the 2nd, 3^{rd,} and 4th tests is extremely high for the tested microalgae. The consumption of phosphorus present in the effluent was also high, however, the initial value was close to the discharge limit and the final values reached were close to zero (0 to 6.77 mg P L^{-1}).

Regarding COD, the cultures were able to lower the levels of this parameter when the addition of effluent had a rate of 10% of the total volume of the reactor. The microalga To was able to immediately lower the COD levels, never exceeding the discharge limits in the 3rd test. However, when the rate was increased to 15% (4th test), its capacity for total nitrogen, phosphorus, and BOD_5 remediation remains or increases (phosphorus case), but the COD levels do not fall in the same proportion, as it can be seen in Figure 5.9. Consequently, the discharge limit values of 150 mg O_2 L^{-1} were not reached.

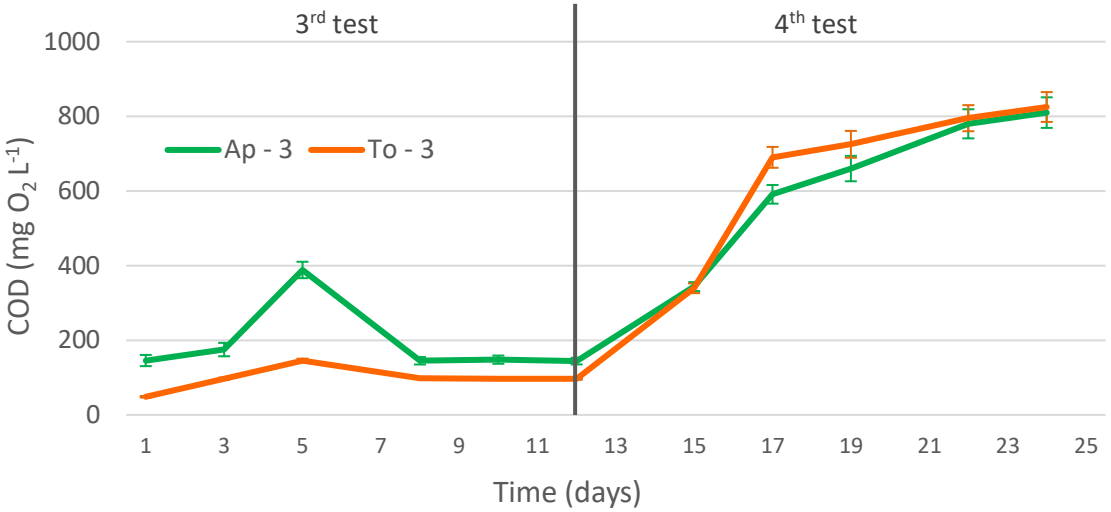

Figure 5.9 – Progress of COD in the last reactor of manure effluent for the 3rd and 4th tests, Ap - 3 – *Auxenochlorella protothecoides* in the 3rd reactor, To - 3 – *Tetradesmus obliquus* in the 3rd reactor (mean ± SD, n = 3).

5.3 Piggery effluent

Worldwide, the sector that contributed most to meat production in 2019 was pork, with 32.4% of the total 339 million tonnes of carcass weight (FAO, 2020). In 2018 the meat production sector in the European Community (EU28) amounted to 172,076.32 million EUR (animal output valued at basic prices) (Eurostat, 2018). In that year, the EU produced 47.8 million tonnes of meat, one-half of which was from pigs (23,846,360 tonnes of pork), corresponding to 259,316,600 pigs. In Portugal, around 361,510 tonnes of pork were produced in 2018 (Eurostat., 2019). The pig sector has annual emissions of 668 million tonnes CO_2-eq., 27% of which correspond to emissions from manure management. The total manure and feed emissions in industrial pig production systems was 5.2 kg CO_2-eq/kg cw (Macleod *et al.*, 2013). Each pig is estimated to produce 5.3 kg of feces and urine per day, corresponding to 38 g N $head^{-1}$ day^{-1} (Gale, 2017). The pigs are slaughtered at 145 to 171 days of life and 125 kg live weight (Cherubini *et al.*, 2015). According to data on pig production in Europe and knowing that the density of pig manure is 0,886 kg dm^{-3} (Kowalski *et al.*, 2013), it can be assumed that about 566 million cubic meters of manure are produced in Europe annually. In Portugal, it is estimated that around 12 million cubic meters of manure are produced annually. The pig farm effluents are mainly composed by the natural production of feces and urine and the water from cleaning the facilities.

Normally, these effluents are mainly sent to stabilization tanks for composting and for anaerobic digestion.

Remediation studies with *Tetradesmus obliquus* were conducted in piggery effluents reaching remedies of 98% for nitrogen and 60% for COD, starting from effluents with an initial load of 3.2 g N L^{-1} and 14.2 g O_2 L^{-1} previously diluted in tap water to 5% (v/v). Another study with *Chlorella vulgaris* and *Tetradesmus obliquus* reached biomass concentrations of 0.53 and 0.49 mg L^{-1}, respectively, after 20 days of cultivation. The achieved remediation rates were 58 and 50% for nitrogen and 28 and 27% for COD, with *C. vulgaris* and *S. obliquus*, respectively. In this case, the effluent had a relatively low COD (276 mg O_2 L^{-1}) and 56 mg N L^{-1} (Abou-Shanab *et al.*, 2013).

The pig effluent remediation experiment was also used to test the incorporation of another agro-industrial effluent: olive-oil mill wastewater. The olive-oil mill wastewater (OMW) is a wastewater that is produced in large quantities in olive oil producing countries like Portugal. This residue is originated from the olive-oil extraction process and generates 1 to 1.6 m^3 of wastewater per tonne of olive fruit processed (Markou *et al.*, 2012). It constitutes a significant environmental problem mainly due to its high chemical oxygen demand and difficult biodegradability related to its antibacterial activity induced by the high quantity of polyphenols and tannins. In 2018 olive oil production in Portugal was 135,000 tonnes, resulting in a production of 725,000 to 1,161,000 m^3 of OMW (Eurostat., 2020; Instituto Nacional de Estatística, 2019). The OMW has been previously tested for the growth of the microalga *Arthrospira platensis*, however, they were used after a sodium hypochlorite treatment (NaClO) which significantly decreases the concentration of phenols and turbidity of the medium but also raises environmental problems (Markou *et al.*, 2012).

5.3.1 Biomass Productivity

The 1st and the 2nd tests ran for 12 days because after this time the discharge limits for total N, P, and COD were reached for poultry effluent plus ash (P+A). The 1st test demonstrate that the three microalgae were able to grow in the control (synthetic medium) and tested effluents, achieving average productivities of 18.9, 30.9, and 16.1 mg L^{-1} day^{-1} for Cv, Ap, and To, respectively in P+A+O, and 15.8, 11.4, and 19.2 mg L^{-1} day^{-1} for Cv, Ap, and To, respectively, in P+A (Figure 5.10).

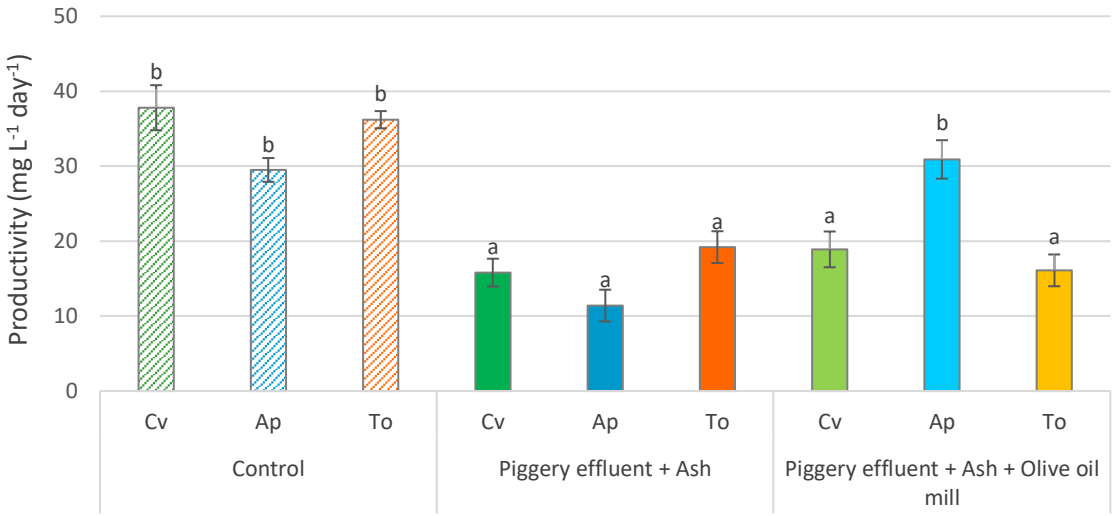

Figure 5.10 – Average productivity for the 1st test in 12 days (mean ± SD, n = 3). (Synthetic medium; Piggery effluent + ash; Piggery effluent + ash + olive oil mill wastewater; Cv – *Chlorella vulgaris*; Ap – *Auxenochlorella protothecoides*; To – *Tetradesmus obliquus*). The values with different index letters show significant differences with $p < 0.05$.

The addition of a reduced quantity of OMW led to an increase in the productivity of microalga *A. protothecoides*. The remaining microalgae do not present significant differences between P+A and P+A+O. Although, microalgae growth was higher for P+A+O, than for P+A, except for *T. obliquus*.

For the 2nd, 3rd, and 4th tests, the two algae with the best growth and remediation performance were selected, therefore *C. vulgaris* and *T. obliquus* were chosen.

The hydraulic retention time in the 3rd test was 20 days and in the 4th test was 10 days.

In the 3rd and 4th experiments, a supplementation of $NaNO_3$ and KH_2PO_4 in reactors 2 and 3 of each microalga was used. Since the N:P molar ratio of the piggery effluent used in the semi-continuous mode was 108:1, which indicates that phosphorus concentration may be limiting for microalgal growth, it would be necessary to supplement the medium with phosphorus (Porto *et al.*, 2021).

In the semi-continuous mode, the 3rd and the 4th tests last for 20 days. The highest productivity was reached in the 3rd test with 258.2 ± 7.1 and 236.7 ± 8.9 mg L^{-1} day^{-1} for *C. vulgaris* and *T. obliquus* respectively (Figure 5.11).

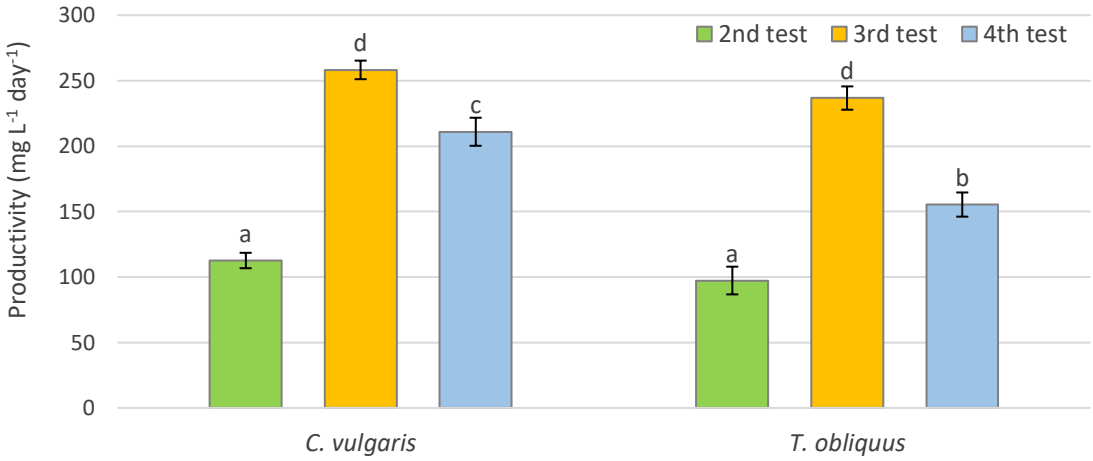

Figure 5.11 – Average biomass productivity of the 2nd (12 days), 3rd (20 days), and 4th (20 days) tests for *Chlorella vulgaris* and *Tetradesmus obliquus* (mean ± SD, n = 3). The values with different index letters show significant differences with $p < 0.05$.

5.3.2 Bioremediation process

The growth of the microalgae led to the effective removal of nitrogen and phosphorus and other components contributing to COD (Table 5.5).

Table 5.5 – Remediation rates for the microalgae (n = 3)(Cv – *Chlorella vulgaris*, Ap – *Auxenochlorella protothecoides*, To – *Tetradesmus obliquus*) in the four tests (Control and Piggery effluents).

			Removal efficiency (%)						
			Total Nitrogen	Total Phosphorus	COD	BOD_5	Ash content	Phenols	Optical density
1st test (Batch)	Control	Cv	99 c	34 c	-	-	27 b	100 d	51 c
		Ap	99 c	43 d	-	-	26 b	100 d	34 b
		To	100 c	42 d	-	-	29 b	100 d	4 a
	P+A	Cv	91 b	77 e	88 c	99 a	61 c	65 c	95 e
		Ap	93 b	100 g	82 bc	100 a	59 c	59 c	98 e
		To	89 b	100 g	89 c	99 a	61 c	33 a	99 e
	P+A+O	Cv	98 c	91 fg	73 b	98 a	58 c	45 b	90 e
		Ap	99 c	100 g	76 b	99 a	56 c	40 b	90 e
		To	100 c	86 f	76 b	99 a	57 c	45 b	90 e
2nd test (Batch)		Cv	100 c	100 g	90 c	100 a	15 a	-	96 e
		To	100 c	100 g	91 c	100 a	14 a	-	99 e
3rd test (50 mL)		Cv	99 c	22 b	89 c	99 a	28 b	-	97 e
		To	99 c	38 cd	91 c	99 a	23 b	-	99 e
4th test (100 mL)		Cv	78 a	6 a	40 a	98 a	12 a	-	91 d
		To	77 a	30 c	39 a	97 a	10 a	-	94 e

Note: The values with different index letters show significant differences with $p < 0.05$.

The removal efficiency was superior to 82% for N and more than 77% for P in the batch mode. The three tested microalgae have a high potential to remove the N, P, and total solids. Regarding COD removal, microalgae in P+A were shown to be effective in reducing COD. In the case of P+A+O, the microalgae were able to significantly reduce N and P, but the COD was not reduced to levels that would allow its discharge. The raw effluent P+A+O had a higher COD (2100.5 mg O_2 L^{-1}) and the values attained were 511.3 and 574.9 mg O_2 L^{-1}, for To and Cv, respectively.

In the 3rd test the remediation to allow the effluent release was reached after 8 days of transfers. Although remediation rates are low for phosphorus (22 and 38%), the discharge value had already been reached before remediation (6.4 mg P L^{-1}). In the 4th test, the COD remediation never achieved the required levels for discharge (150 mg O_2 L^{-1}). When the rate was increased to 10% (4th test), its capacity for total N, P, and BOD_5 remediation remains, but the COD levels do not fall in the same proportion, as can be seen in Figure 5.12. Consequently, the discharge limit values of 150 mg O_2 L^{-1} were not reached.

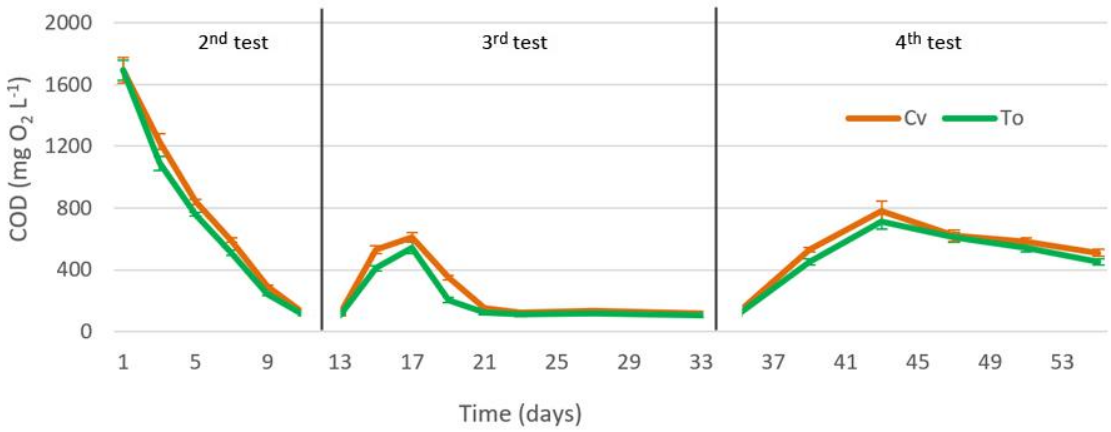

Figure 5.12 – Progress of COD in the reactors of the 2nd test and the last reactor of 3rd and 4th tests, Cv – *Chlorella vulgaris*, To - *Tetradesmus obliquus* (mean ± SD, n = 3).

Another study with piggery effluents reached biomass yields of 0.53 g L^{-1} and 0.49 g L^{-1} for *T. obliquus* and *C. vulgaris*, respectively, higher than those achieved in the present study. However the starting effluent had only 1/20 of the total nitrogen of the current study, and the removal rate for nitrogen and phosphorus was only 49% and 18% for *C. vulgaris* and 58% and 24% for *T. obliquus,* respectively (Abou-Shanab *et al.*, 2013).

5.4 Poultry effluent

The poultry sector is currently the second-largest contributor to global meat production, being responsible for 39% of the total of 339 million tonnes carcass weight produced in 2019 – chicken meat represents 89% of the poultry tonnage (FAO, 2020). The poultry effluents are produced, in large volumes, in agro-industrial farms and slaughterhouses worldwide. They contain a significant organic load, including phosphorus and nitrogen compounds, emulsified fats, and particulate matter.

Usually, the organic residues of poultry slaughterhouse effluents are homogenized, thermally treated, and sent to a solid-liquid separator. While the decanted solid waste is used to produce pet diets, the liquid phase, with high-fat content, is subjected to a sequence of operations, such as flocculation, intended to reduce the organic load. The aqueous part goes for anaerobic digestion or to a water treatment plant, whereas the solid one, with high-fat content, goes to the landfill. The whole process entails high costs and a negative environmental impact and is associated with potential high eutrophication (Martinelli *et al.*, 2020; Oryschak and Beltranena, 2020). Some authors assessed the possible assembly of effluents stemming from multiple sources in a joint treatment using anaerobic co-digestion, and their findings were quite promising (Asses *et al.*, 2019; Latifi *et al.*, 2019).

Conventional effluent treatments require large amounts of chemicals and energy and are responsible for high greenhouse gas emissions and sludge with no potential use/benefit, while the use of microalgae reduces all these drawbacks. Microalgae provide the O_2 used by heterotrophic and autotrophic microorganisms to oxidize and/or assimilate organic carbon, as well as nitrogen and phosphorus (Moreno-Garcia *et al.*, 2017; Patel *et al.*, 2017). Still, those same microorganisms provide the CO_2 that ensures efficient microalga growth.

The poultry effluents can be used as a culture medium for microalgae growth, thus contributing to their remediation through the consumption of organic and inorganic nutrients, with the ensuing biomass production. This approach has the advantage of producing algal biomass that can be incorporated in different stages of the agro-industrial process, as well as used as feed-in animal production (Dineshbabu *et al.*, 2019; Saeid *et al.*, 2016), as biofertilizers or biostimulants (Navarro-López *et al.*, 2020) or in a variety of other by-products of several industries, such as the pharmaceutical and cosmetic ones (Levasseur *et al.*, 2020; Yarkent *et al.*, 2020). Markou *et al.* (2016) studied the use of raw poultry litter leachate, with dilutions of 10x, 15x, 20x, and 25x, using the cyanobacteria *Arthrospira platensis* and microalga

Chlorella vulgaris. The authors found that the *C. vulgaris* had the best performance, being able to grow at 10x dilution, producing between 1.76 and 1.87 g L^{-1} in 11 days, and exhibiting a superior consumption of nutrients (i.e., a higher bioremediation). The initial COD load of the effluent was 6542 mg O_2 L^{-1}, with the dilutions it was between 261 and 654 mg O_2 L^{-1} and *C. vulgaris* had the ability to remedy between 45 and 82%. Sing *et al.* (2011) reported a remediation of diluted poultry litter from anaerobic digester, for the total N and P, of about 65% and 85%, with the *Chlorella minutissima* (*Mychonastes homosphaera*), and 70% and 88%, with the *Scenedesmus bijuga* f. *irregularis*. In a 29-day study involving *Tetradesmus obliquus* grown in slaughterhouse poultry effluent, productivities of 100 mg L^{-1} day^{-1} and remediations of 100% for total N and P were achieved (Ferreira *et al.*, 2018).

5.4.1 Biomass Productivity

The results show that microalgae could grow in the two poultry effluents: poultry effluent (PE) and poultry effluent plus ash (PE+A).

The batch mode experiment ran for 10 days – at the 10^{th} day the nitrogen and phosphorus available fell below their discharge limits and the COD in the medium PE+A reached the discharge limit of 150 mg O_2 L^{-1} (Portuguese Ministry of the Environment, 1998). Figure 5.13 displays the biomass productivities obtained with the three microalgae considered grown in the control medium and poultry effluents PE and PE+A. It is observed that the highest and lowest biomass productivities were obtained with the microalgae *Tetradesmus obliquus* grown in the ashless effluent PE (94.9 ± 2.8 mg L^{-1} day^{-1}) and control medium (50.0 ± 5.3 mg L^{-1} day^{-1}), respectively. On the other hand, the microalgae *C. vulgaris* and *A. protothecoides* grew better in the effluent PE+A than in its PE counterpart (76.2 vs. 65.1 and 72.0 vs. 61.2 mg L^{-1} day^{-1}, respectively). It is also noted that the biomass productivity in the control medium is the intermediate one in both cases.

Calixto *et al.* (2016) grew *Chlorella* sp. in a bio-compost of chicken excrements and in raw chicken manure, attaining biomass productivities of 6.8 mg L^{-1} and 4.3 mg L^{-1} day^{-1}, respectively, in 17 days – these values are considerably lower than those obtained in the present study. However, it should be noted that Markou *et al.* (2016) grew the microalga *Chlorella vulgaris* in diluted poultry litter leachate during 11 days, achieving productivities ranging from 160 to 169 mg L^{-1} day^{-1}. Moreover, Ferreira *et al.* (2018) grew *Tetradesmus obliquus* in slaughterhouse poultry effluent (COD of 3.7 g O_2 L^{-1} and 122.7 mg N L^{-1}) for 29 days and achieved productivity of 100 mg L^{-1} day^{-1}.

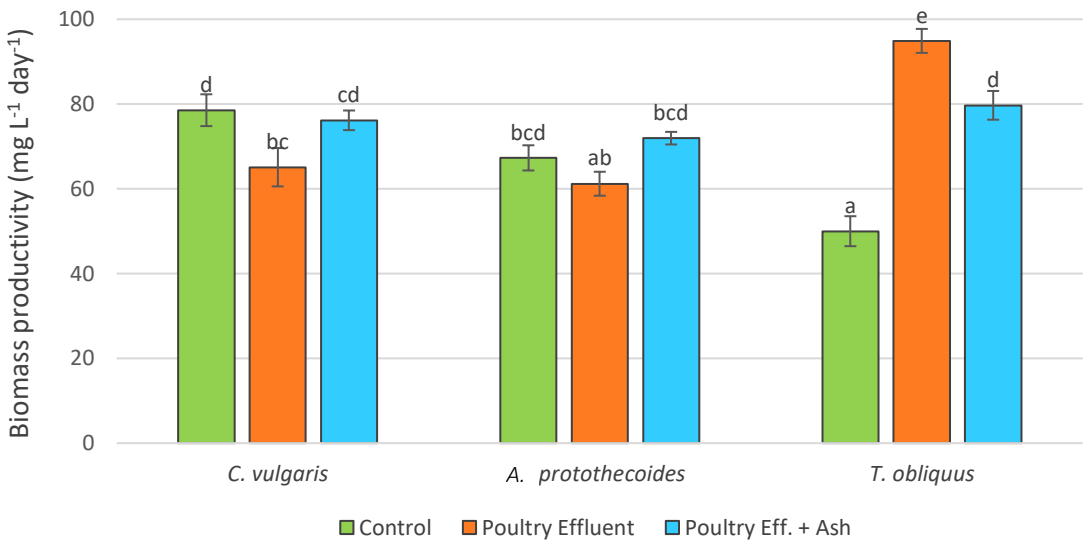

Figure 5.13 – Biomass productivity (mean ± SD, n = 3) of the three microalgae considered in the control (synthetic medium) and poultry effluent with ash and without. The values with different index letters show significant differences with $p < 0.05$.

The semi-continuous mode tests ran for 28 days with microalgae *C. vulgaris* and *T. obliquus*. The hydraulic retention times were 10, 5, and 3.3 days, in the second, third, and fourth test sets, respectively. The highest biomass productivity was obtained for *T. obliquus* (244.5 ± 5.1 mg L^{-1} day^{-1}) in the second test with PE+A, while the second highest one was obtained for the same microalga with PE and also in the second test – indeed, *T. obliquus* always outperformed *C. vulgaris* concerning productivity and bioremediation. The algal productivity during the semi-continuous mode tests increased over time (28 days) in the 2nd and 3rd tests, and it decreased in the 4th test where it always decreased, further showing that cultures were not able to multiply at a sufficient rate. Figure 5.14 shows the average productivity in the semi-continuous tests.

It should be noted that, in the semi-continuous tests, reactors 2 and 3 were supplemented weekly with aqueous $NaNO_3$ and KH_2PO_4, to achieve final concentrations in the culture medium of 20 mg N L^{-1} and 10 mg N L^{-1} (Ansari *et al.*, 2017; Markou *et al.*, 2016).

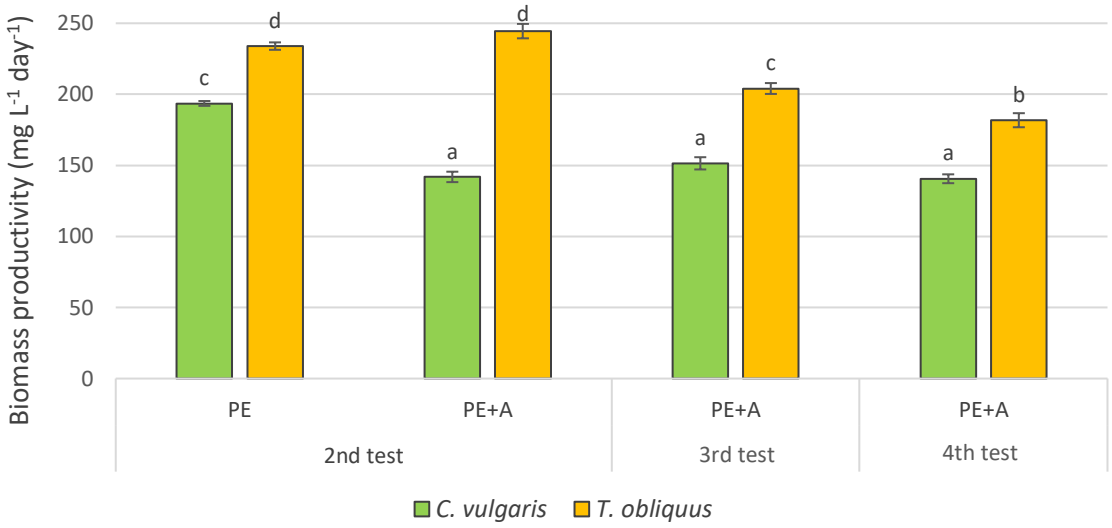

Figure 5.14 – Average biomass productivity (mean ± SD, n = 3) of microalgae *Chlorella vulgaris* and *Tetradesmus obliquus* in the poultry effluents without (PE) and with (PE+A) ash in the semi-continuous mode tests. The values with different index letters show significant differences with $p<0.05$.

5.4.2 Bioremediation process

In the batch mode tests, the remediation capacity for total nitrogen was 100% in all variants. Regarding phosphorus, the microalgae grown in PE+A completely remedied it, whereas in PE the remediation was 82.0 ± 2.5%. Similar studies with poultry effluents also found remediation close to 100%, for total nitrogen and phosphorus (Ferreira *et al.*, 2018). However, the remediation achieved with diluted poultry litter anaerobic digester, for total nitrogen and phosphorus, was of about 65% and 85%, for *Chlorella minutissima* (*Mychonastes homosphaera*), and 70% and 88%, for *Scenedesmus bijuga* f. *irregularis* (Singh *et al.*, 2011). For the poultry effluent PE, the COD remediation was above 93% with the three microalgae, whereas for the PE+A this remediation varied between 70% (Cp) and 83% (To). While the BOD_5 was remediated to almost 100% by the three microalgae in PE, in the case of PE+A the Cp microalga exhibited significantly better performance: a remediation rate of 75%, compared with 39% (Cv) and 47% (To). Despite lower COD levels in the diluted poultry litter leachate, Markou *et al.* (2016) attained lower remediation rates (between 45 and 82%).

The amount of suspended solids before and after remediation in batch mode by microalgae can be seen in Figure 5.15, where it is clear the effect of the pre-treatment and the remediation of microalgae on suspended solids, by monitoring the optical density (OD_{540}).

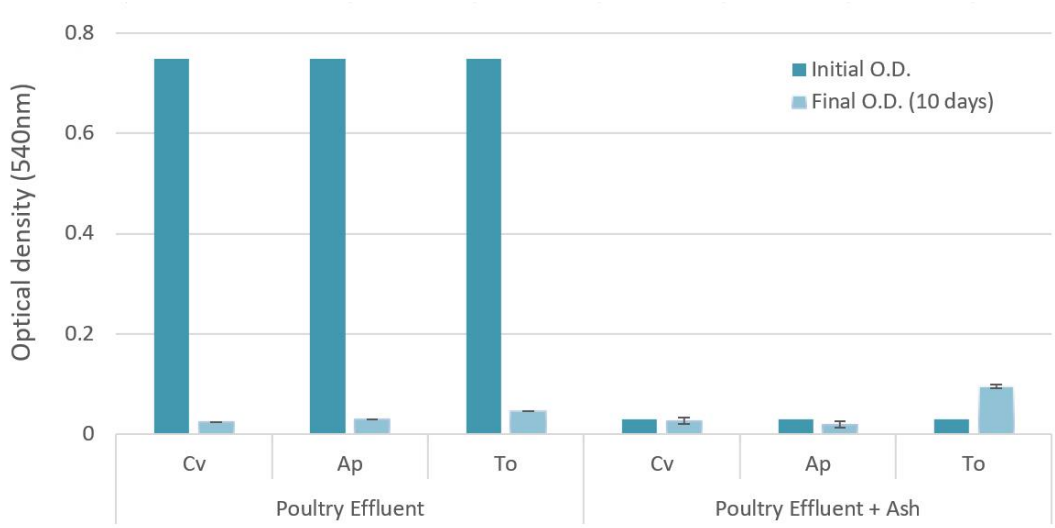

Figure 5.15 – Optical density at 540 nm (initial and final) for the three microalgae (Cv – *C. vulgaris*, Ap – *A. protothecoides* and To - *T. obliquus*) in poultry effluent (PE) and poultry effluent plus ash (PE+A) in the 1st test (mean ± SD, n = 3).

Table 5.6 shows the average algal biomass productivities during the different tests and the remediation rates for each microalga and experiment.

Table 5.6 – Average biomass productivities and final bioremediation rates (n = 3) for the Cv (*Chlorella vulgaris*), Ap (*Auxenochlorella protothecoides*), and To (*Tetradesmus obliquus*) microalgae in the first to fourth test (PE - poultry effluent; PE+A - poultry effluent with ash).

				Removal efficiency (%)			
		Microalga	Biomass productivity (mg L^{-1} day^{-1})	Total Nitrogen	Total Phosphorus	COD	BOD_5
1st Test (Batch mode)	PE	Cv	65.1 ^{bc}	100 ^{c}	86 ^{ef}	96 ^{d}	100 ^{e}
		Ap	61.2 ^{ab}	100 ^{c}	82 ^{e}	94 ^{d}	99 ^{e}
		To	94.9 ^{e}	100 ^{c}	82 ^{e}	93 ^{d}	99 ^{e}
	PE + A	Cv	76.2 ^{cd}	100 ^{c}	100 ^{g}	75 ^{bc}	39 ^{a}
		Ap	72.0 ^{bcd}	100 ^{c}	100 ^{g}	70 ^{b}	75 ^{c}
		To	79.7 ^{d}	100 ^{c}	100 ^{g}	83 ^{c}	47 ^{a}
2nd Test (100 mL)	PE	Cv	193.6 ^{h}	72 ^{a}	35 ^{ab}	94 ^{d}	99 ^{e}
		To	234.0 ^{i}	96 ^{c}	41 ^{b}	96 ^{d}	99 ^{e}
	PE + A	Cv	141.9 ^{f}	77 ^{ab}	66 ^{d}	93 ^{d}	100 ^{e}
		To	244.5 ^{i}	98 ^{c}	81 ^{e}	100 ^{e}	95 ^{d}
3rd Test (200 mL)	PE + A	Cv	151.5 ^{f}	95 ^{c}	28 ^{a}	94 ^{d}	99 ^{e}
		To	204.1 ^{h}	96 ^{c}	97 ^{g}	94 ^{d}	99 ^{e}
4th Test (300 mL)	PE + A	Cv	140.6 ^{f}	71 ^{a}	61 ^{c}	55 ^{a}	99 ^{e}
		To	181.8 ^{g}	82 ^{b}	89 ^{f}	56 ^{a}	93 ^{d}

Note: The values with different index letters show significant differences with $p < 0.05$.

In the 4th test, no microalga managed to reach the required limits for effluent discharge for the COD parameter (664.0 ± 6.4 and 676.2 ± 8.5 mg O_2 L^{-1}) and total nitrogen (34.2 ± 2.1 and 53.7 ± 2.8 mg N L^{-1} – the first and second values concern *T. obliquus* and *C. vulgaris*, respectively).

Figure 5.16 displays the evolution of COD in the last reactor (Cv-3 and To-3) over the three semi-continuous mode tests. The figure shows that, in the 2nd test, the discharge limit for COD is reached after 8 days and remains fairly constant. In the 3rd test the discharge limit is reached after 12 days, while in the 4th test no discharge limit is ever reached for the COD – thus, it can be concluded that the microalgae considered do not have the capacity to remedy such a large addition volume (300 mL of poultry effluent) every other day. To ensure that the treatment is effective (i.e., the effluent can be released from the last/third reactor), the additional volume should not exceed 200 mL at a time, which means 600 mL weekly or 20% of the total volume.

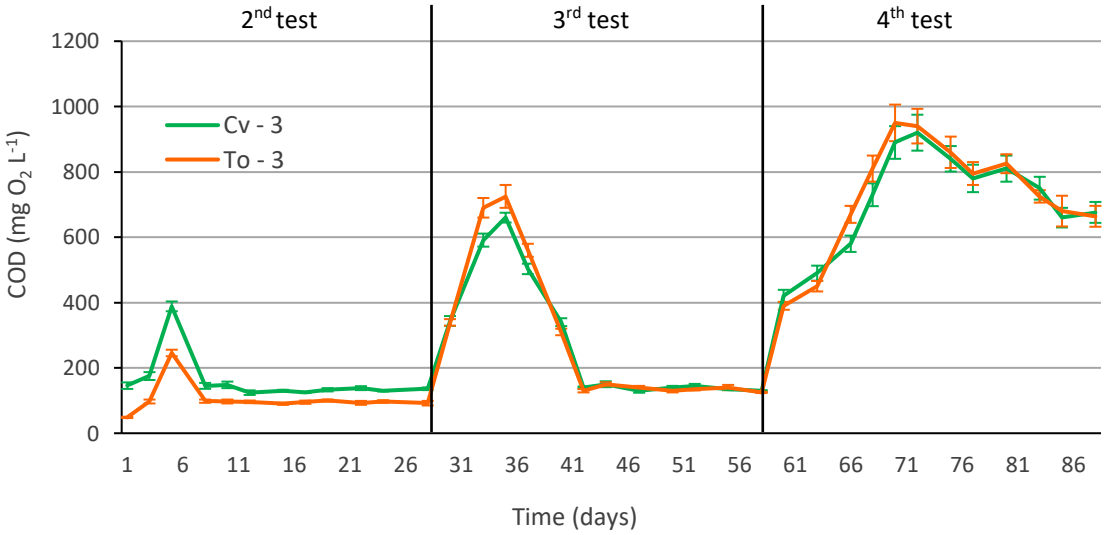

Figure 5.16 – COD evolution in the last reactor of PE+A (poultry effluent + ash) in the second, third, and fourth tests, for Cv-3 (*Chlorella vulgaris* in the 3rd reactor) and To-3 (*Tetradesmus obliquus* in the 3rd reactor) (mean ± SD, n = 3).

Supplementation of nitrogen and phosphorus was found to be necessary for the 2nd test, which involved only the addition of 100 mL of effluent every other day – in this test it was necessary to add $NaNO_3$ twice and KH_2PO_4 once, during the 28 days. This supplementation was not necessary for the 3rd and 4th tests, probably because the addition of a larger effluent volume met the microalga culture needs.

5.5 Landfill Leachate

One of the most significant aspects of landfill management is the production of highly complex leachate, which represents a very serious pollution problem affecting soils, water bodies, and human health. Landfill leachate is an aqueous solution of organic and inorganic components produced by infiltration of rain water into the layers of waste deposits exposed to environmental conditions and subject to processes of aerobic and anaerobic decomposition by the local microbiome (Deng *et al.*, 2020). The composition of leachate is highly variable depending mostly on the type of waste in the landfill, landfill age, climate conditions, and geochemical characteristics of the landfill site (Danley-Thomson *et al.*, 2020). These complex effluents are characterised by a dark colour, bad odour, and significant values of COD, ammonia-nitrogen, and heavy metals (Barbosa Segundo *et al.*, 2019). Among the different leachate categories, the treatment of stabilized leachate is very difficult to achieve, largely due to the presence of refractory substances, such as humic and fulvic acids, which are not easily degraded (Reshadi *et al.*, 2020; Sruthi *et al.*, 2018). To eliminate these refractory organic materials found in stabilized landfill leachate, it is necessary to use complementary remediation techniques, such as membrane technologies, ion exchange, adsorption by activated carbon, flocculation-coagulation, chemical oxidation, or advanced oxidation processes (Mahtab *et al.*, 2020; Reshadi *et al.*, 2020). The combination of different treatment processes has been proposed by several authors in order to achieve high treatment efficiencies and maximize the removal of organic and inorganic contaminants (Baiju *et al.*, 2018; Deng *et al.*, 2020).

Bioremediation with microalgae may also be used as a final step to reduce the concentration of dissolved components after chemical precipitation, due to the capacity of those microorganisms to absorb inorganic compounds (Paskuliakova *et al.*, 2018). There are also some studies on landfill leachate bioremediation using microalgae, but they are mostly performed with significant dilutions (between 1:5 and 1:10) to reduce colour and allow the diffusion of light into the effluent (Porto *et al.*, 2020) or using membrane reactors, where there is no direct contact between the microalgae and the leachate. This system allows for nutrient reclamation, such as nitrogen and phosphorus, but not a complete removal of other leachate components (Chang *et al.*, 2018). The use of high dilution rates or membrane filtration also allows reducing the concentrations of components with high toxicity for microalgae, and of the organic components that contribute to the opacity of the leachates. Nevertheless, large dilutions will require significant amounts of water and land space,

making this solution less sustainable for large-scale applications (Nair *et al.*, 2019). The association between leachate pre-treatment techniques and bioremediation with microalgae may be an alternative approach to overcome these limitations and achieve appropriate removal efficiencies. Nair *et al.* (2019) used coagulation with aluminium sulphate and air-stripping to reduce the concentration of organic compounds, colour, and ammonia-nitrogen in landfill leachate followed by bioremediation using *Chlorella pyrenoidosa* (*Auxenochlorella pyrenoidosa*). Their approach was intended to complete the removal of species contributing to the high initial COD value (1800 mg O_2 L^{-1}). The final concentration of microalgae biomass reached a value of 2.9 g L^{-1} while consuming carbon dioxide at the rate of 0.26 g L^{-1} day^{-1} and achieving a COD removal of 74%. Another study with remediation of microalgae *C. vulgaris* and *T. obliquus* in urban waste landfill achieved reductions of 42 to 43% for nitrogen (initial 637 mg N L^{-1}) and 33 to 34% for phosphorus (initial 8 mg L^{-1}). It should be noted that the leachate was used with microalgae in dilutions of 25%, 20%, 15%, 10%, and 5% (v/v) and that the initial COD value was 663 mg O_2 L^{-1} (Porto *et al.*, 2021). Paskuliakova *et al.* (2016) used *Chlamydomonas* sp. to treat numerous landfill leachates using several dilutions in order to have no total nitrogen greater than 250 mg L^{-1} and no COD greater than 530 mg O_2 L^{-1}.

The conducted research intends to study a sustainable approach to the treatment of landfill leachate, by combining chemical precipitation with a low-cost precipitation agent (biomass ash), followed by bioremediation through microalgae. To the best of the author's knowledge, this is the first study where the pre-treatment with biomass ash is coupled with microalgae bioremediation to achieve adequate treatment conditions for highly loaded and poorly biodegradable landfill leachates. Moreover, potential applications for the obtained algal biomass were addressed. The evaluation of the process by-products intends to assess the overall reduction in emissions whilst improving the sustainability of the global process.

5.5.1 Biomass Productivity

The batch growth experiments were performed using the pre-treated leachate, as received or after dilution with distilled water (1:2), to evaluate the behaviour of different microalgae (*Chlorella vulgaris*, *Auxenochlorella protothecoides*, *Tetradesmus obliquus*, *Spirulina major*, *Microchloropsis salina*, *Isochrysis galbana*) in this effluent when compared to a synthetic growth medium.

The obtained results indicate that Cv was able to grow in the pre-treated undiluted landfill leachate. The other tested microalgae were also able to grow in the pre-treated landfill leachate with dilution (1:2) achieving biomass concentrations that varied from 0.43 ± 0.16 g L^{-1} (Ms) to 1.13 ± 0.00 (To), corresponding to 29.5% to 79.8% of the values obtained with the synthetic culture medium, respectively. These differences may be due to the low phosphorus concentration in the pre-treated leachate (12.5 mg L^{-1}) and the presence of some inorganic components that may present some toxicity, namely chlorine (22.8 g L^{-1}) or aluminium (23.6 mg L^{-1}).

To (1.13 ± 0.00 g L^{-1}), Cv (1.08 ± 0.05 g L^{-1}) and Ap (0.88 ± 0.04 g L^{-1}) stood out in terms of biomass concentration in the diluted leachate (Figure 3). For these three microalgae, the growth in the diluted leachate was not significantly different from the growth in the culture medium. The higher value of biomass concentration (1.23 ± 0.11 g L^{-1}) was obtained with Cv grown in the undiluted leachate, which may indicate that the diluted effluent contained insufficient nutrients. However, in preliminary tests using lower volumes of pre-treated undiluted leachate Sm, Ms, and Ig microalgae cultures did not develop. These were the microalgae chosen for the preliminary tests because they are theoretically the most adapted to media with high salt content (Al Dayel and El Sherif, 2020; Bezerra *et al.*, 2020; Li *et al.*, 2021). The biomass concentration in these 27 days, expressed as the final concentration in the culture medium is presented in Figure 5.17.

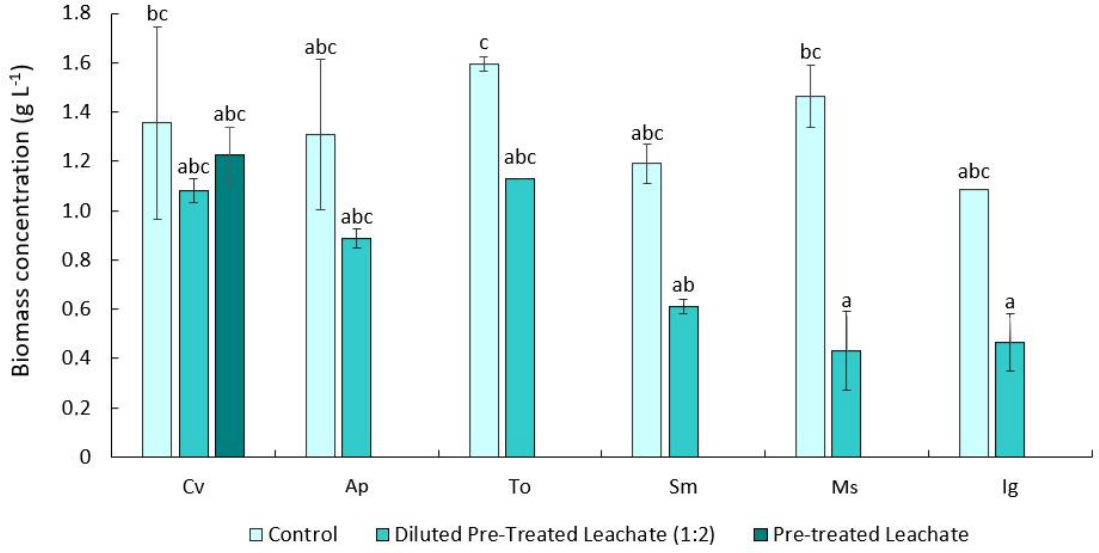

Figure 5.17 – Biomass concentration of the six microalgae in pre-treated landfill leachate and control after 27 days of culturing (mean ± SD, n = 2). Cv – *Chlorella vulgaris*; Ap – *Auxenochlorella protothecoides*; To – *Tetradesmus obliquus*; Sm – *Spirulina major*; Ms – *Microchloropsis salina*; Ig - *Isochrysis galbana*. The values with different index letters show significant differences with $p < 0.05$.

5.5.2 Bioremediation process

Regarding the microalgae bioremediation, the batch growth experiments ended after 27 days, corresponding to the achievement of COD levels below the legal discharge limits. Microalgae took a long time to develop in this effluent due to its characteristics (low degradability, chlorine, and phenolic content). The removal efficiency for several critical parameters is presented in Table 5.7 for these seven batch growth experiments.

Table 5.7 – Removal efficiency for bioremediated leachate with the six microalgae (Cv – *Chlorella vulgaris*; Ap – *Auxenochlorella protothecoides*; To – *Tetradesmus obliquus*; Sm – *Spirulina major*; Ms – *Microchloropsis salina*; Ig - *Isochrysis galbana*).

Sample	Removal efficiency (%)					
	Total nitrogen	**Total phosphorus**	**COD**	**BOD_5**	**Total phenolic compounds**	**Fixed solids**
Cv - leachate (1:2)	59.2 ^{a}	16.9 ^{a}	70.3 ^{ab}	67.4 ^{a}	56.1 ^{a}	41.4 ^{ab}
Cv - leachate	62.9 ^{a}	96.0 ^{b}	79.7 ^{cd}	78.3 ^{ab}	70.4 ^{b}	82.2 ^{c}
Ap - leachate (1:2)	53.3 ^{a}	15.3 ^{a}	71.4 ^{ab}	78.3 ^{ab}	58.5 ^{a}	42.0 ^{ab}
To - leachate (1:2)	60.7 ^{a}	100 ^{b}	64.8 ^{a}	79.3 ^{ab}	56.7 ^{a}	55.7 ^{b}
Sm - leachate (1:2)	60.3 ^{a}	100 ^{b}	72.5 ^{bc}	89.1 ^{ab}	56.2 ^{a}	51.4 ^{b}
Ms - leachate (1:2)	52.5 ^{a}	88.3 ^{b}	85.7 ^{d}	100 ^{b}	58.4 ^{a}	31.8 ^{a}
Ig - leachate (1:2)	58.9 ^{a}	100 ^{b}	84.6 ^{d}	89.1 ^{ab}	55.3 ^{a}	43.8 ^{ab}

Note: The values with different index letters show significant differences with $p < 0.05$.

Removal of total nitrogen in the leachate was similar among the 6 microalgae, between 52.5 (for Ms-leachate 1:2) and 62.9% (for Cv-leachate). Phosphorus was completely or almost completely removed by To, Sm, Ig, and Cv-leachate. In contrast, Cv-leachate (1:2) and Ap-leachate (1:2) practically did not remediate phosphorus. Since phosphorus concentration was already low in the original leachate (22.9 mg L^{-1}) and this component was widely removed during the pre-treatment step, the final concentration in the treated leachate was only 12.5 mg L^{-1}, constituting a limiting nutrient for microalgae growth.

According to Porto (2021), since the N:P molar ratio is 176:1, it would be necessary to supplement the effluent with phosphorus to avoid limiting algal growth due to insufficiency of this nutrient. In these conditions, the low phosphorus removal efficiency observed for Ap and Cv - leachate (1:2) should correspond to some release of this element to the leachate by microalgae, since their cultures reached the onset of the death phase. Regarding COD, the removal efficiency in the leachate was between 64.8% (To) and 85.7% (Ms). COD removal obtained in the present study would allow discharging the effluent treated by the Ns and Ig algae. These removal efficiencies are in line with the work of Quan *et al.* (2020) with *Tetradesmus obliquus* microalga in landfill leachate. The removal efficiency of BOD_5 was higher than 67% for all microalgae, with Ms reaching 100%, which is a very promising result. All microalgae remediated around 52.2 and 58.5% of phenols, with Cv-leachate reaching the highest removal efficiency (70.4%). Removal of fixed solids varied between 31.8 and 55.7%, for the pre-treated diluted leachate (1:2). For the pre-treated leachate without dilution, fixed solids removal reached a value of 82.2% (with Cv microalga). Sodium, potassium, magnesium, and calcium are quite common metals in leachate, remaining in high concentrations even after pre-treatment, but did not interfere with microalgae growth.

5.6 Final considerations

The productivity of microalgae varied depending on the effluent and microalga. However, productivity was higher (in some cases exceeding those obtained for synthetic media) for agro-industrial effluents compared to experiments conducted by other authors. The higher productivities were obtained for the semi-continuous experiments. The greater productivity was achieved for aquaculture effluent (880 and 812 mg L^{-1} day^{-1} for Cv and To) followed by cattle effluent (554 and 523 mg L^{-1} day^{-1} for To and Ap) then by piggery effluent (258 and 237 mg L^{-1} day^{-1} for Cv and To) and at last by poultry effluent (245 and 193 mg L^{-1} day^{-1} for To and Cv). The productivity in the landfill leachate and batch mode (46 and 42 mg L^{-1} day^{-1} for Cv and To) was lower and the microalgae took longer to adapt to these conditions, nevertheless, it is important to note that this medium had a low phosphorus load (there was no supplementation), which certainly conditioned the growth of the microalgae. The remediation rates achieved by microalgae were particularly good for most microalgae in all tested effluents, allowing them to reach discharge limits.

The tested transfer system, made up of three containers, proved to be more efficient than when using only one container. This conclusion was reached because, in the situation of 450 mL weekly transfers of aquaculture effluent, microalgae managed to remedy the effluent in the last container of the set of three but were unable to do so when there was only one container.

The transfer system does not allow all effluents to be treated at the same speed, however, it admits transfers of 10% of total volume for aquaculture and cattle effluents, 5% of total volume for piggery effluent, and 20% of total volume for poultry effluent.

Chapter 6

Biomass characterization

The characterization of algal biomass is important to assess the potential of future applications. In the case of biomasses rich in proteins and lipids, without contaminants, their incorporation in animal feed does not present contraindications. In contrast, the biomasses with some level of contaminants, but rich in sugars and lipids, can be directed to the production of biofuels.

6.1 Aquaculture

The algal biomass produced from aquaculture effluents was evaluated by quantifying the protein, carbohydrate, lipid, and ash contents at the end of the 1st experiment (Figure 6.1).

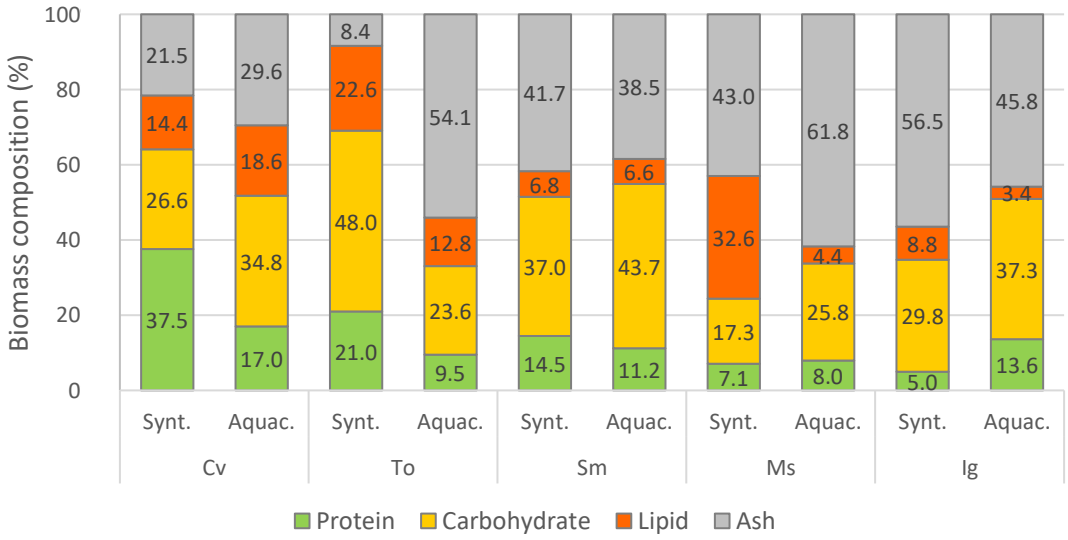

Figure 6.1 – Biomass composition (% dw) for the five microalgae in the 1st test (mean, n = 3). Cv – *Chlorella vulgaris*; To – *Tetradesmus obliquus*; Sm – *Spirulina major*; Ms – *Microchloropsis salina*; Ig - *Isochrysis galbana*; Synt. – Synthetic medium (control); Aquac. – Aquaculture effluent.

In the 1st test, the higher protein content was achieved in synthetic medium (37.5 ± 0.4% for Cv and 21.0 ± 0.2% for To). The algae grown in the effluent had 11.8 ± 3.5% of protein content. In terms of carbohydrates, the To grown in the control medium had 48.0 ± 3.5% and in the effluent 23.6 ± 0.8%, while the other algae grown in the effluent had 35.4 ± 7.4% of carbohydrates. Related to lipids the microalga that stood out was Ms grown in the control medium with 32.6% while Cv and To grown in the effluent present 18.6% and 12.8%, respectively. The ash content in the microalgae is significantly higher for To and Ms grown in the effluent, although a high value was found for *I. galbana* and *M. salina* grown in synthetic medium because F/2 is essentially salt water. A study with *Scenedesmus* sp. grew in wet market wastewater found a composition of algal biomass with higher protein content (48.7%), even compared to the control, and a lipid content also superior (27.1%) to that obtained in the present study. The major fatty acid compound was oleic (C18:1) with 34.6% (Apandi *et al.*, 2019), as in the current study.

At the end of the 3rd and 4th experiments, the microalgal biomass was quantified for protein, carbohydrate, lipid, and ash content (Figure 6.2). In the 3rd test, the composition of the microalgae was very homogeneous among the three of them (Cv-A, To-A, and To-B), although the microalga with the highest protein content was To-A with 35.2 ± 2.3% followed by Cv-A (30.6 ± 2.1%). The highest carbohydrate content belongs to Cv (39.4± 2.7%) and the highest lipid content was for To-A with 7.9± 0.1%. In the 4th test, there was a considerable difference between the To produced in the synthetic culture medium and the aquaculture effluent, mainly in terms of the amount of carbohydrates (43.5% for To-Synth. to 30.4% in To-Aquac.) and the ash in the biomass (8.1% for To-Synth. to 27.2% in To-Aquac.). About proteins, both microalgae present a greater amount in the synthetic medium compared to the aquaculture effluent.

In further studies, with *Chlorella vulgaris* in aquaculture effluents, biomasses were obtained with similar protein levels, about 44 to 46% (Daneshvar *et al.*, 2018), compared to those obtained in the present study of 40% and 48% (AFDW) for Cv-A and To-A, respectively. As regard carbohydrates, Daneshvar *et al.* (2018), obtained a lower value, around 18% for *Chlorella vulgaris*, while in the present case the values obtained were 52 and 41% (AFDW) for Cv-A and To-A, respectively.

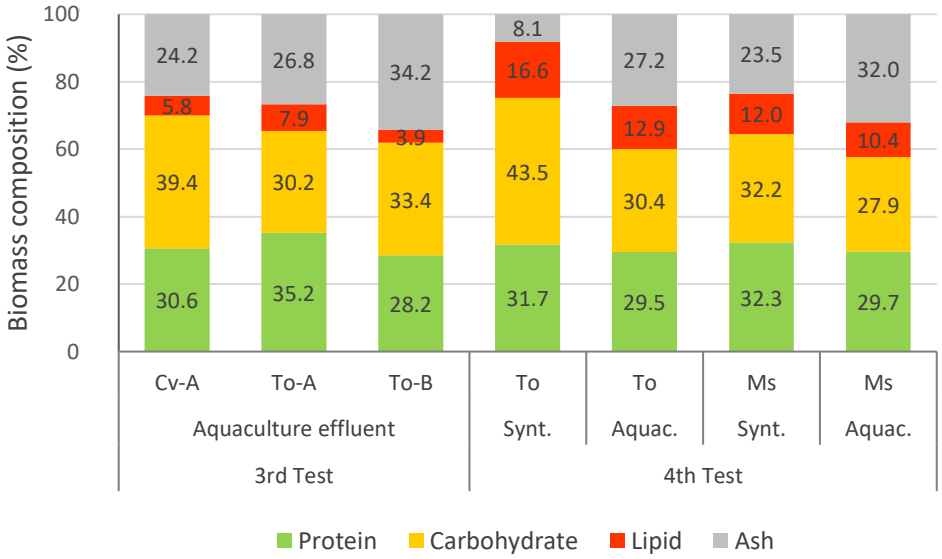

Figure 6.2 – Biomass composition (% dw) for the microalgae in 3rd and 4th experiment (mean, n = 3). Cv – *Chlorella vulgaris*; To – *Tetradesmus obliquus*; Ms – *Microchloropsis salina*; Aquac. – Aquaculture effluent; Synth. - Synthetic medium; A – configuration A (three reactors with 1.5 L each); B – configuration B (single reactor with 4.5 L).

Concerning the amount of lipids and profile, in general, the microalgae have a balanced fatty acid composition for animal feed, as they have a high content of monounsaturated and polyunsaturated fatty acids (Zhang *et al.*, 2019). It is noted that there is a greater incidence of unsaturated fatty acids in the biomass of microalgae in semi-continuous growth, compared to those in batch growth, especially for *T. obliquus* (7.9 ± 0.5%). Daneshvar *et al.* (2018) and Kuo *et al.* (2016) obtained higher amounts of lipids in the microalgae obtained in aquaculture effluents (8 to 23%), still with an identical proportion between saturated and unsaturated fatty acids compared to the present thesis. The ash content in the microalgae was, again, significantly higher because of the dissolved salts in the aquaculture effluents of seawater, 34.2 ± 0.5% for To-B followed by To-A with 26.8 ± 0.7% and Cv-A (24.2 ± 0.4%).

The analysis of the mineral composition of the treated effluents revealed the presence of sodium, magnesium, calcium, potassium, and iron in similar proportions to those detected for saltwater and raw aquaculture effluent. Reduced amounts of zinc (0.40 ± 0.02 mg L^{-1}), cadmium (0.30 ± 0.00 mg L^{-1}), and aluminium (3.65 ± 1.01 mg L^{-1}) were detected in the effluents treated by the microalgae, yet smaller than those of the raw effluents (0.60 ± 0.18, 0.67 ± 0.40 and 16.5 ± 6.04 mg L^{-1}, respectively) and saltwater (0.38

mg Cd L^{-1} and 14.32 mg Al L^{-1}). This leads to assuming a certain absorption of these elements by the microalgae. However, very small amounts of these metals are involved. No traces of lead, chromium, copper, and manganese were detected in the effluents. Given these data, the use of microalgal biomass would not be limited by the presence of heavy metals.

Regarding the fatty acid composition of the microalgae biomass, in this study, there was a predominance mixture of unsaturated fatty acids including palmitoleic (C16:1), hexadecadienoic (C16:2), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and conjugated linoleic acid (CLA), as well as saturated fatty acids including palmitic (C16:0), stearic (C18:0), behenic (C22:0) and lignoceric (C24:0). Figure 6.3 presents the variations of fatty acids in the microalgae grown in aquaculture effluent used.

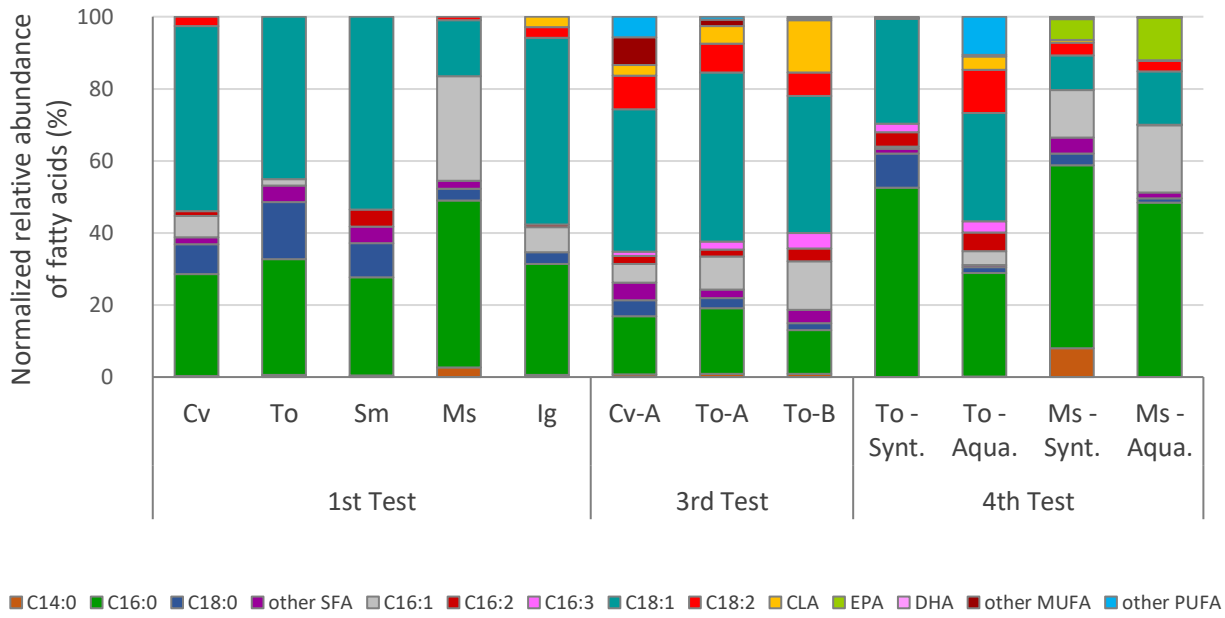

Figure 6.3 – Fatty acids in the microalgae grown in the aquaculture effluent for 1st, 3rd, and 4th tests (Cv - *C. vulgaris*, To – *T. obliquus*, Sm – *S. major*, Ms – *M. salina*, Ig – *I. galbana*, A – configuration A (three reactors with 1.5 L each), B – configuration B (single reactor with 4.5 L); Cont. – Control; Aqua. – Aquaculture effluent).

The fatty acid with the greatest expression in all microalgae was oleic acid followed by palmitic acid, except for *Microchloropsis salina*, which is palmitic followed by palmitoleic acid. For growth in semi-continuous mode (3rd and 4th test), a larger number of peaks was detected in the chromatograms (some unsaturated fatty acids), but with less concentration

of each one, as C12:0, C15:1, C16:4, C16:3, C18:4, C18:3, C20:1; C22:1. The microalga *M. salina* also shows a higher amount of palmitoleic acid in the 4th test than *T. obliquus*. Zhang and collaborators produced *Chlorella sorokiniana* in mariculture wastewater (filtering through 0.22 µm membranes) and obtained biomass composition with 43% proteins and 34 to 43% lipids, composed predominantly of C16:0 and C18:2 fatty acids (Zhang *et al.*, 2019). The quality of the fatty acids in the studied algal biomass showed an interesting application in animal feed. It is known that the increase in polyunsaturated fatty acids in fish feed is beneficial for their growth, development, as well as an intrinsic quality of the product (Han *et al.*, 2019).

6.2 Cattle

The characterization of the algal biomass produced in cattle effluent in terms of main nutrient components (protein, carbohydrate, lipid, and ash content) and the fatty acid profile of the lipid fraction are presented in Figures 6.4 and 6.5, respectively.

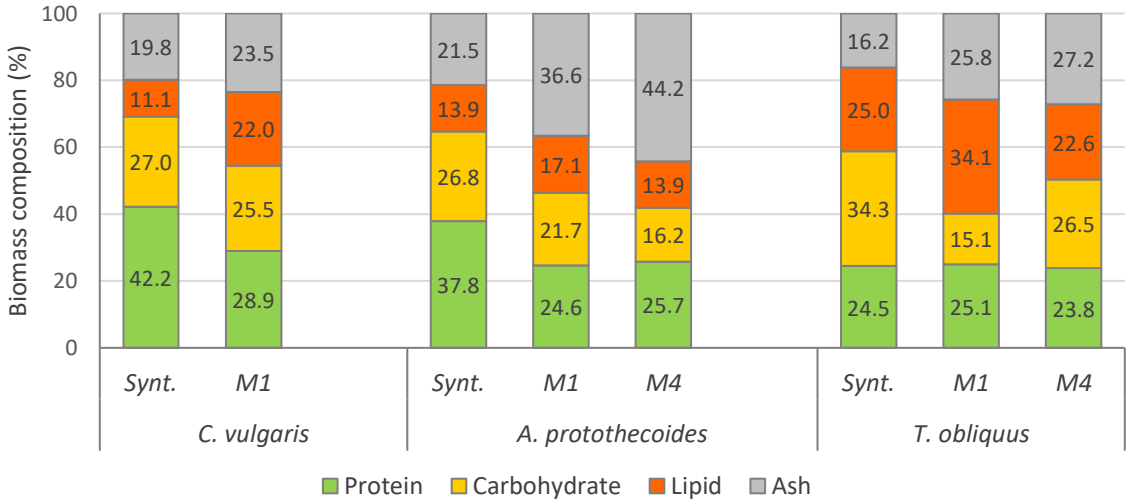

Figure 6.4 - Characterization of algal biomass (% dw) for synthetic medium (Synt.), 1st and 4th tests (mean, n = 2). M1 – Manure Effluent 1st test; M4 – Manure Effluent 4th test.

Considering the algal biomass produced during the 1st experiment, it was observed that protein content was significantly higher for *Chlorella* species grown in synthetic medium (42.2% for Cv and 37.9% for Ap), but both had lower protein content when grown in the effluent, probably due to the nitrogen limitations of this culture medium. The To biomass had lower protein content (24.5% for control) but was similar when grown in the effluent (25.1%). The carbohydrate content had a similar pattern and was higher in

synthetic mediums (34.3% for To, 27.0% for Cv, and 26.8% in Ap), compared to the cattle effluent it varies between 15.1% (To in 1st test) and 26.5% (To in 4th test).

The lipid content was higher for To biomass grown in the effluent (34.1%), while Cv and Ap controls and Ap grown in effluent had substantially lower values (11.1%, 13.9%, and 17.1%, respectively).

Concerning the semi-continuous experiments, biomass harvesting, and characterization was performed only at the end of the 4th test. To biomass presented the higher values of protein and lipids, while Ap had a higher amount of carbohydrates. Ash concentration was similar for both microalgae and higher than the values obtained in the 1st experiment, although ash concentration in the effluent was lower (14.8 mg L^{-1} for the 4th test and 16.1 mg L^{-1} for the 1st test), because of the cumulative effect of the successive effluent additions and culture medium transfers.

The fatty acid profile of the lipid fraction (Figure 6.5) showed a predominance of oleic and linoleic acids (C18:1 and C18:2) and palmitic acid (C16:0), which may be the result of the combination of lipid molecules produced during microalgae metabolism and adsorption of lipid molecules present in the manure effluent.

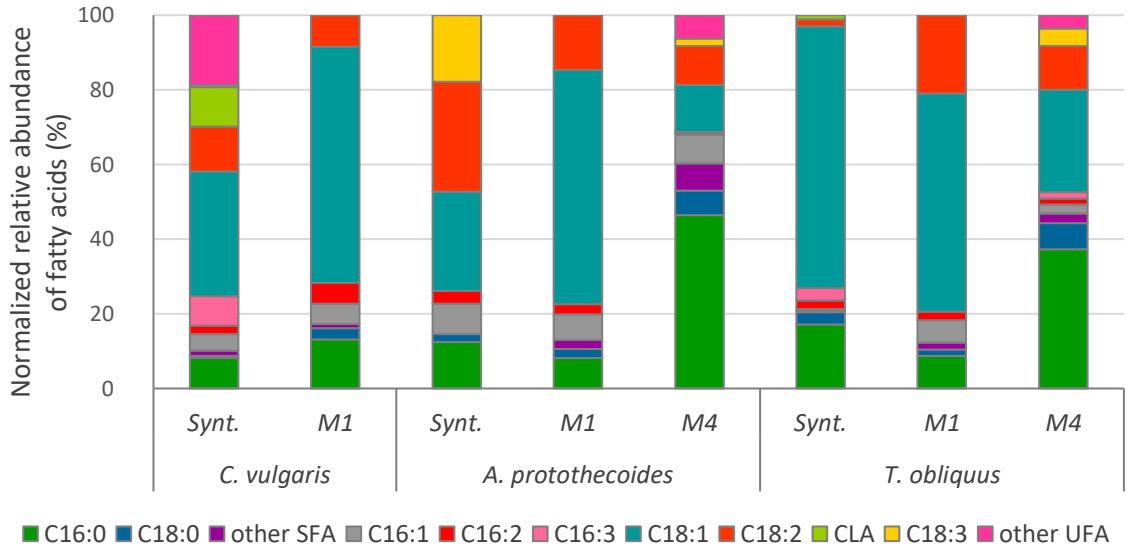

Figure 6.5 – Characterization of fatty acids of microalgae grown in synthetic medium (Synt.), 1st and 4th tests. M1 – Manure Effluent 1st test; M4 – Manure Effluent 4th test.

High content of oleic acid (18:1) was observed in all microalgae produced in batch mode from manure effluent (1st test), reaching 63.5% for Cv, 62.7% for Ap, and 58.4% for

To. The microalgae grown in the 4th test showed a decrease of oleic acid concentration (31% for Ap and To), at the expense of an increase in palmitic acid (40.3% for Ap and 38.3% for To). This variation may be the result of the higher adsorption of animal fat lipids typically richer in saturated fatty acids, more available due to the successive effluent additions. Beavi & Sukumaran (2014) also detected a predominance of palmitic acid (57%) and oleic acid (27%) in *Chlorococcum sp.* biomass cultivated mixotrophically in dairy effluent.

On the other hand, the lipid fraction of the microalgae produced in the 4th test also presented higher relative amounts of C16:3, C18:3, and other unsaturated fatty acids, suggesting that microalgae metabolism was probably less affected by nutrient restrictions than in the 1st test.

Several studies refer to the importance of incorporating unsaturated fatty acids in the diets of cattle (Medeiros *et al.*, 2021; Neofytou *et al.*, 2020). This topic will be further discussed in chapter 7 (7.1 – As animal feed).

6.3 Piggery

The characterization of the algal biomass from piggery effluents was made in terms of protein, sugar, lipid, and ash contents (Figure 6.6).

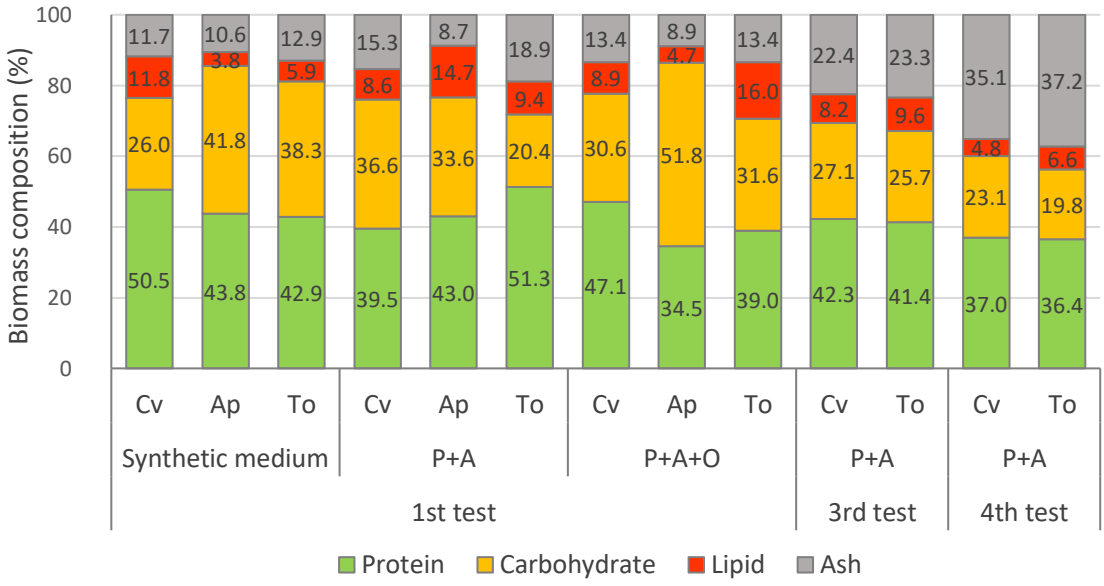

Figure 6.6 – Characterisation of the biomass (% dw) for the 1st, 3rd and 4th tests (mean, n = 3). (Synt. – synthetic medium; (P+A) – piggery effluent + ash; (P+A+O) - piggery effluent + ash + olive oil mill wastewater; Cv – *C. vulgaris*; Ap – *A. protothecoides*; To – *T. obliquus*).

Protein is the most abundant content, independently of the microalga species or culture conditions. There was a tendency of an ash increase from the 1st to 4th test. The microalga with the highest protein content was *T. obliquus* (51.3%) in the (P+A), the one that had the highest sugar content was *A. protothecoides* (51.8%) in (P+A+O) and the highest lipid content was again To (16.0%) in (P+A+O).

The lipidic fraction composition is presented in Figure 6.7 and as it can be seen, there is a clear predominance of unsaturated fatty acids, mainly oleic acid (C18:1), presumably conjugated linoleic acid (CLA), and linoleic acid (C18:2). The most representative saturated fatty acid is palmitic (C16:0). In semi-continuous tests, both microalgae showed a tendency to have a higher amount of palmitic acid, mainly due to the reduction of CLA, which in the case of the 4th test, it does not exist at all. CLA is a term that corresponds to a set of isomers of linoleic acid with conjugated double bonds, it is a fatty acid typically present in ruminant meat and dairy products. However, it was likely detected in the mass spectrometry chromatograms of the microalgae profiles according to the databases used. Other identification studies will be subsequently conducted to confirm this classification. Though, according to Witkamp (2010), there are other sources of CLA such as seeds and seaweeds.

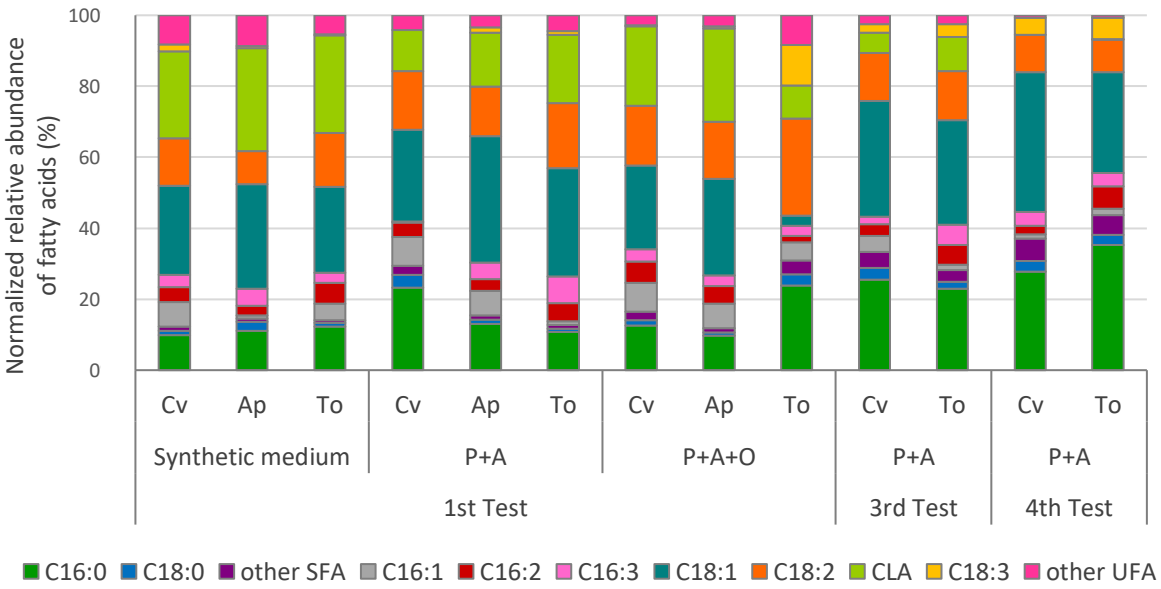

Figure 6.7 – Characterisation of microalgae fatty acids for the 1st, 3rd and 4th tests (mean, n = 3). (Synt. – synthetic medium (control); P+A – piggery effluent + ash; P+A+O - piggery effluent + ash + olive oil mill wastewater; Cv – *C. vulgaris*; Ap – *A. protothecoides*; To – *T. obliquus*).

6.4 Poultry

The compositions of the algal biomass produced in the poultry effluent, obtained by quantifying the protein, carbohydrate, lipid, and ash contents at the end of each test (batch or semi-continuous mode) is presented in Figure 6.8. In this figure, it is provided the biomass percentage composition, in dry weight, for the first test (batch mode) involving the three microalgae considered in the synthetic medium (control), poultry effluent (PE), and poultry effluent plus ash (PE+A).

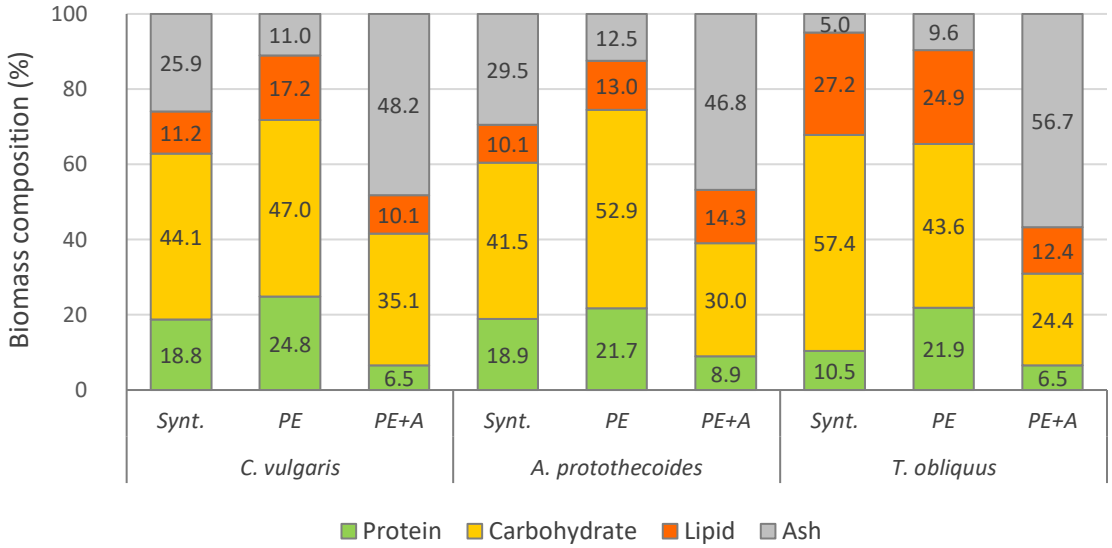

Figure 6.8 – Biomass percentage composition (% dw) for the 1st test (batch mode) involving the microalgae *Chlorella vulgaris*, *Auxenochlorella protothecoides,* and *Tetradesmus obliquus* in the synthetic medium (Synt.) and poultry effluent without (PE) and with (PE+A) ash (mean, n = 3).

The microalgae with the highest protein content were those grown in PE and also the Cv and Ap grew in their control media. The remaining cases showed significantly lower amounts of protein, namely all those involving microalgae grown in PE+A. Concerning the sugar contents, the To grown in the BG-11 medium had 57.4%, while the microalgae grown in PE had an average of 47.8 ± 4.7%, and those grown in PE+A had a much lower value (29.8 ± 5.4%). Regarding the lipid content, To was the microalga with the highest lipid content: 24.9% in PE and 27.2% in the control medium. The remaining cases had lower values with relatively small differences between them: their average was 12.6 ± 2.5%. The algae ash contents are significantly higher in the PE+A cases since this medium contains much more dissolved salts – To has the highest value, followed by Cv and Ap. In the remaining cases, the ash content varies considerably, between 29.5 ± 2.1% (Ap in the control medium) and 5.0 ± 0.3% (To in

the control medium). Comparatively, the *T. obliquus* grown in the same kind of effluent (slaughterhouse poultry) shows lower values of sugars (36.2%) and lipids (19.8%), protein, and ash content were not determined (Ferreira *et al.*, 2018).

Figure 6.9 provides similar results for the three semi-continuous mode tests but involving only the microalgae *Chlorella vulgaris* (Cv) and *Tetradesmus obliquus* (To) in poultry effluent without (PE) and with (PE+A) ash.

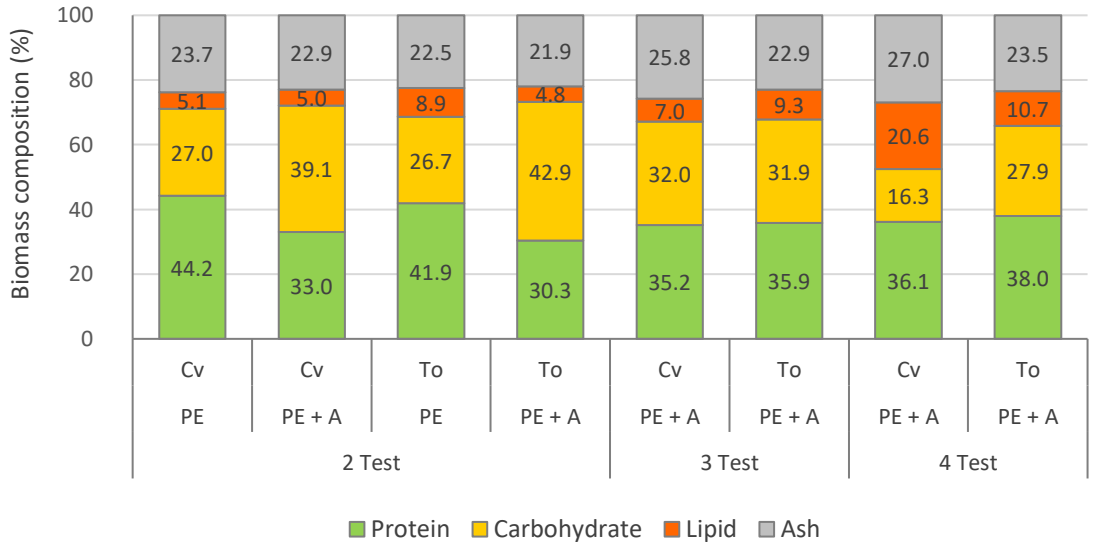

Figure 6.9 – Biomass percentage composition (% dw) for the three semi-continuous mode tests involving the microalgae *Chlorella vulgaris* (Cv) and *Tetradesmus obliquus* (To) in poultry effluent without (PE) and with (PE+A) ash (mean, n = 3).

The comparison between Figures 6.8 and 6.9 makes it possible to conclude that the compositions of the microalgae grown in the semi-continuous mode tests were very similar to those observed in the batch mode tests. It is clear that a pre-treatment with ash leads to microalgae with higher sugar and lipid contents, and fewer proteins.

Figure 6.10 shows the variation of the fatty acids present in the microalgae grown in PE and PE+A poultry effluents (and also in the control medium, in the batch mode tests). Regarding the fatty acid composition of the microalgae biomasses, it consisted of a mixture of (i) unsaturated fatty acids, including C16:1, C16:2 and oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and conjugated linoleic acid (CLA), and (ii) saturated fatty acids, including palmitic (C16:0), stearic (C18:0), and lignoceric (C24:0), regardless of the algae species and growth medium.

High content of oleic acid (C18:1) was observed in all the microalgae, particularly for *T. obliquus*: 60.3% and 59.6%, respectively in PE+A and PE – *C. vulgaris* also has a fairly high content when grown in PE+A (48.2%). Note that oleic acid is the predominant methyl ester in olive oil and is beneficial in preventing heart problems in humans, as it lowers blood cholesterol levels and regulates insulin and blood pressure (Jones *et al.*, 2015).

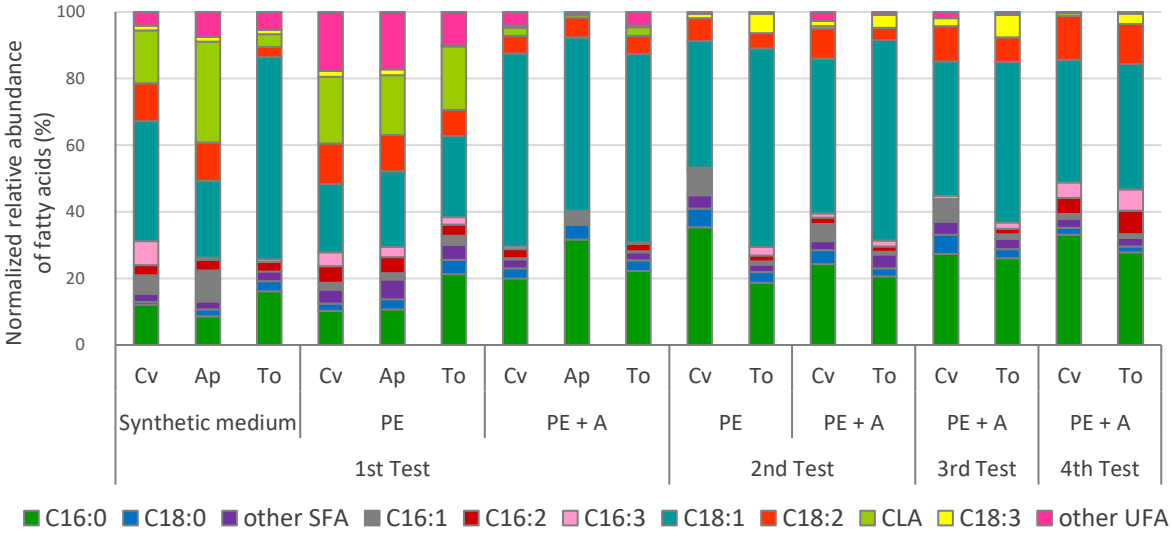

Figure 6.10 – Fatty acids percentage composition in the microalgae (Cv - *Chlorella vulgaris*, Ap - *Auxenochlorella protothecoides,* and To - *Tetradesmus obliquus*) grown in the synthetic medium (1st test) and the poultry effluent without (PE) and with (PE+A) ash (all tests).

Linoleic acid (C18:2), mostly, and conjugated linoleic acid (CLA) are two methyl esters exhibiting also reasonable contents in the analysed algae, mainly in the batch mode tests in the control media and PE. It is worth noting that linoleic acid consistently appears in all tests for the three microalgae considered – although its percentages do not differ considerably, it is fair to say that the highest values are obtained for *Chlorella vulgaris*. On the negative side, it must be recognised that, as also reported by Calixto *et al.* (2016), palmitic acid also appears in all tests for the three microalgae, with the highest percentages occurring in the semi-continuous mode tests involving *C. vulgaris*.

6.5 Landfill leachate

The algal biomass produced in the landfill leachate was also evaluated by quantifying the protein, carbohydrate, lipid, and ash content at the end of the experiment, to evaluate the influence of the growth medium in the physiological condition of the microalgae (Figure 6.11).

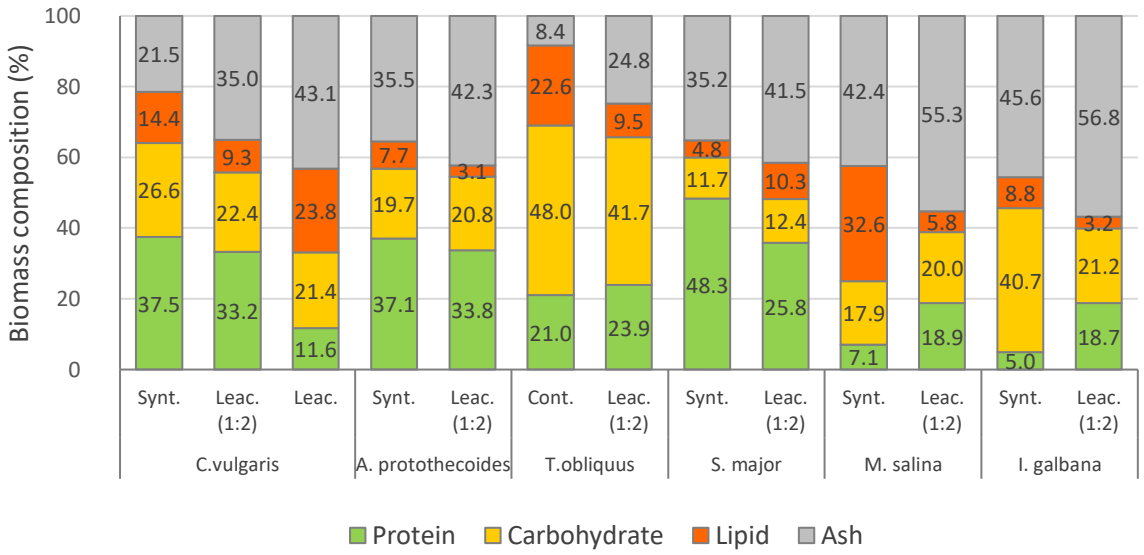

Figure 6.11 – Biomass composition (% dw) for the six microalgae in pre-treated leachate (Leac.) and synthetic medium (Synt.) as control (mean, n = 3).

The most notorious effect of the use of landfill leachate was the increase in the ash content of the microalgae, reflecting the capacity of these microorganisms to remove mineral components that are abundant in the pre-treated leachate. All microalgae contained a higher amount of ash when grown in the leachate when compared to the same algae grown in the control medium. In particular, the ash content of Cv grown in the pre-treated leachate (Cv-leachate) was higher than that of the same microalga grown in the diluted leachate, (Cv-leachate, 1:2), suggesting that adsorption of mineral components from the culture medium was concentration-dependent. The microalgae with the highest protein content were Sm (48.3 ± 1.2%), Cv (37.5 ± 1.6%) and Ap (37.1 ± 1.1%) controls followed by Sm, Ap and Cv-leachate (1:2) with 35.8 ± 2.4%, 33.8 ± 1.8% and 33.2 ± 3.1%, respectively. These results indicate that the pre-treated leachate had a minimum of nutrients adequate for protein production almost equivalent to the control medium. Nevertheless, the remaining microalgae grown in the leachate originated higher protein content than the ones grown in the control. For carbohydrates, To grown in the control medium had a concentration of 48.0 ± 3.8%, followed by To-leachate with 41.7 ± 2.2%

and by Ig-control (40.7 ± 1.3%). The remaining microalgae had a value of 19.4 ± 4.2% for this parameter. Accumulation of carbohydrates in algal biomass is generally related to nutrient restriction namely during feast and famine cycles (Cheng *et al.*, 2017). Nevertheless, the carbohydrate levels of the microalgae grown in the pre-treated leachate with or without dilution were comparable or lower than the same microalgae grown in the control medium, indicating that nutrient availability was not a relevant stress factor during these experiments. Concerning lipid concentration, Ms-control presented a value of 32.6 ± 1.9%, Cv-leachate had 23.8 ± 0.8% and To-control had 22.6 ± 1.4% while other microalgae showed a value of 7.7 ± 3.3%.

According to the literature, the composition of microalgae varies depending on several factors, especially microalgae strain, light (intensity and period), temperature, pH, culture medium, and aeration conditions (Yin *et al.*, 2020). However, the composition of the studied algae is within the average observed by other authors (Daneshvar *et al.*, 2019; Hernández-García *et al.*, 2019; Niccolai *et al.*, 2019).

Regarding the fatty acid composition of the analysed microalgae biomass, there is a predominant mixture of unsaturated fatty acids including palmitoleic (C16:1), hexadecadienoic (C16:2), oleic (C18:1), linoleic (C18:2), linolenic (C18:3) and conjugated linoleic acid (CLA). It also includes saturated fatty acids such as palmitic (C16:0), stearic (C18:0), behenic (C22:0), and lignoceric (C24:0). Figure 6.12 shows the fatty acid profile found in the studied microalgae.

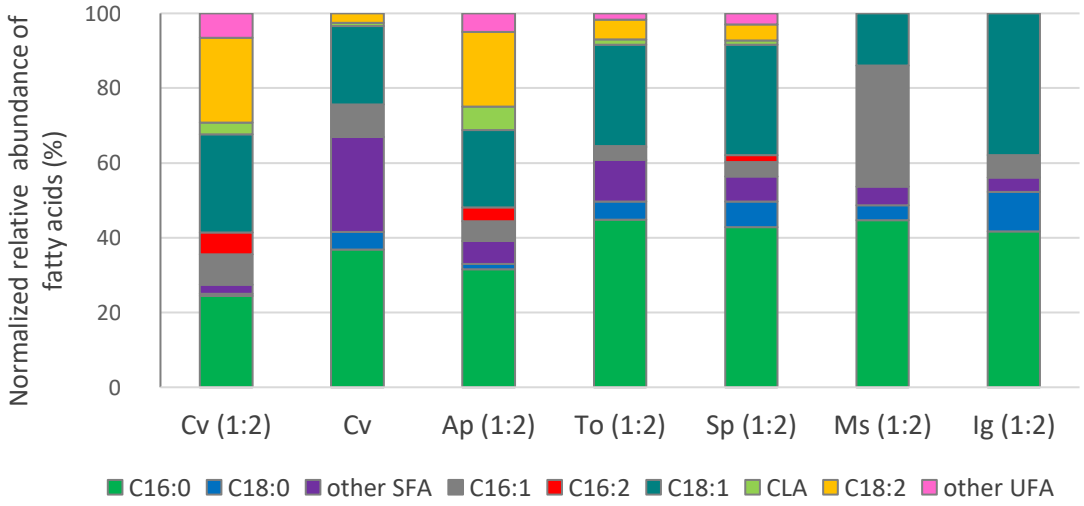

Figure 6.12 – Composition in fatty acids of microalgae grown in landfill leachate (Cv – *Chlorella vulgaris*; Ap – *Auxenochlorella protothecoides*; To – *Tetradesmus obliquus*; Sm – *Spirulina major*; Ms – *Microchloropsis salina*; Ig - *Isochrysis galbana*).

The tested microalgae did not develop the same proportions of fatty acids. Nonetheless, all microalgae have the highest relative abundance for palmitic acid (except for Cv (1:2) that has a higher abundance of oleic acid), followed by oleic acid (except Ms).

Chang *et al.* (2019) also investigated the development of *Chlorella vulgaris* in landfill leachate without direct contact with the leachate and concluded that the most abundant fatty acids were saturated, unsaturated, and polyunsaturated, namely C16:0, C16:1, C16:2, C18:1, and C18:3. Other authors who analysed *Chlorella* sp. and *Tetradesmus* sp. also concluded that the main fatty acids in these algae were C16:0, C18:2, and C18:1 (Hernández-García *et al.*, 2019; Zhang *et al.*, 2019). Mitra & Mishra (2018) studied the microalga *M. salina* and found the same proportion of fatty acids found in the present study. An experiment with *I. galbana* grown in a medium with nitrogen starvation also concluded that the main fatty acids found in this microalga were C16:0 and C18:1 (Zarrinmehr *et al.*, 2020).

The obtained algal biomass could be used to produce biofuels, like bioethanol with *Tetradesmus obliquus* microalga, since it had about 41% of sugars in its composition. It could also be used to produce animal feed since Cv and Sm had a balanced composition and a considerable amount of protein. And lastly, pigment extraction could also be an alternative, because pigments are very interesting as high added-value products.

6.6 Final considerations

The composition of the microalgae obtained in the different effluents varied considerably. Protein levels in the semi-continuous mode were higher in microalgae grown in piggery and poultry effluents (36-42% and 35-38%, respectively), followed by the ones grown in aquaculture effluent (29-35%), bovine effluent (24-26%) and finally (in the batch mode) by landfill leachate (12-36%). The amount of protein in the microalgae is directly related to the amount of nitrogen present in the effluents used for its growth, except for the landfill leachate. The effluent with the highest total nitrogen content was the piggery effluent. In addition, pigs' diets contain large amounts of protein, and their excess is excreted in the form of nitrogen compounds. As for carbohydrates, the microalgae that showed the greatest amount in the semi-continuous mode were those grown in aquaculture effluent (30-39%), followed by the others with more dispersed values (12-42%, 16-32%, 20-27%, and 16-27% for landfill leachate in batch mode, poultry, piggery, and cattle,

respectively). The increase in carbohydrates in the composition of microalgae is greatly influenced by the stress to which they were subjected. The growth of microalgae in media with a high salt content (aquaculture effluent and landfill leachate) may have led to this increased stress, resulting in higher sugar content. Concerning lipids, they were higher in microalgae grown in semi-continuous mode from cattle effluent (14-23%), in landfill leachate (batch mode) (3-24%), and poultry effluent (7-21%), followed by those grown in piggery effluent (5-10%) and aquaculture effluent (4-10%).

Regarding the content in fatty acids, these were more constant among the microalgae produced in the various effluents, with a predominance in oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2), and conjugated linoleic acid (CLA).

Chapter 7

Microalgae biomass applications

7.1 As animal feed

The use of microalgae in human and animal nutrition is not new. However, for its use in animal feed, the production costs of microalgae must be reduced. One of the approaches to achieve this reduction is to combine the production of microalgae with the effluent treatment produced by the animals, in a perspective of a circular economy.

Several studies have shown that microalgae can successfully replace fish meal and fish oils in fish diets, as well as improve the growth and quality of meat in pigs, poultry, and rabbits. In the different ruminants, the dietary inclusion of marine microalgae improved the fatty acid profile of milk and meat obtained. The bioactive compounds of microalgae have also been studied, showing that they lead to an increase in the defence activity of the organism and health status in several animal species, providing protection to tissues and antioxidant effects, also affecting pigmentation in fish.

7.1.1 Aquaculture animals

The algal biomass from aquaculture wastewater treatment can have different applications. The use of algal biomass for aquaculture feed is a common practice. However, there are few studies on the application of algae produced in wastewater as feed for aquaculture species. Li *et al.* (2019) used biomass of *Tetraselmis* sp. and *Phaeodactylum* sp. from aquaculture wastewater treatment in oysters feed with good results.

For feeding applications of aquatic animals, the high protein content is crucial to have high economic value. The aquatic animals need about 40% protein in their feed to have a normal and balanced growth (Zhang *et al.*, 2019). However, other researchers focus their attention on the amount of PUFA's, antioxidants, and in counteracting the immunosuppressive effect *(Ayyat et al.*, 2018; Dotta *et al.*, 2018; Lazo *et al.*, 2020). There are also some studies showing that the introduction of microalgae in the diet of fish and other marine animals leads to the appearance and maintenance of more appealing skin coloration

(Cunha *et al.*, 2020; Knutsen *et al.*, 2019), in terms of human's acceptance and consumption, namely for salmon, rainbow trout, seabream, shrimps, crabs, and lobsters (Kiron *et al.*, 2012; Kousoulaki *et al.*, 2016). The colour is also crucial for ornamental species (Gouveia *et al.*, 1996).

The microalgae grown in aquaculture effluent obtained a good proportion of protein, with a good profile of amino acids essentials, but also a balance composition of fatty acids. Since no heavy metals were detected in the biomasses obtained through remediation, it seems that they could be a good choice for feeding aquatic animals.

7.1.1.1 Microalgae from aquaculture as a source of mussels' feed

The mussels were the animals model used to test the application of different microalgae as a diet. These filter bivalve molluscs inhabit the intertidal zones and feed on phytoplankton and detritus filtered from the surrounding seawater (Chapman *et al.*, 2020). These animals are mostly produced in aquaculture for human consumption, around 94% worldwide representing over one-third of the European Union's aquaculture production (Avdelas *et al.*, 2021).

The 190 mussels collected from their natural and uncontaminated environment had an average total weight of 5.91 ± 0.28 g and an average shell length of 37.8 ± 1.1 mm at the beginning of the experiment (day 1). The feeding experience with the mussels ran for 45 days, with four sampling points: day 1 (T_1), day 15 (T_{15}), day 30 (T_{30}), and day 45 (T_{45}).

The average weight and length gain of mussels subjected to different diets over time (T_{15}, $T_{30,}$ and T_{45}) are shown in Figure 7.1 (regarding measurements on T_1). The accounting is not cumulative, reporting earnings every 15 days.

On the fifteenth day, the average weight of mussels increased similarly in all treatments varying between 0.51 and 0.98% relative to T_1 mussels. Despite, in the To+Ms Aq. treatment the increase in weight was superior (0.98%), the differences between them were not significant except for To+Ms synthetic. This synthetic diet stands out for the noticeable lower gain. After 30 days the effect of the To aquaculture diet on weight gain was more representative (0.91%), however also no significant differences between diets except, again, for To+Ms synthetic. For the length, the growth is similar between the several diets, however, the mixed diets show a lower growth.

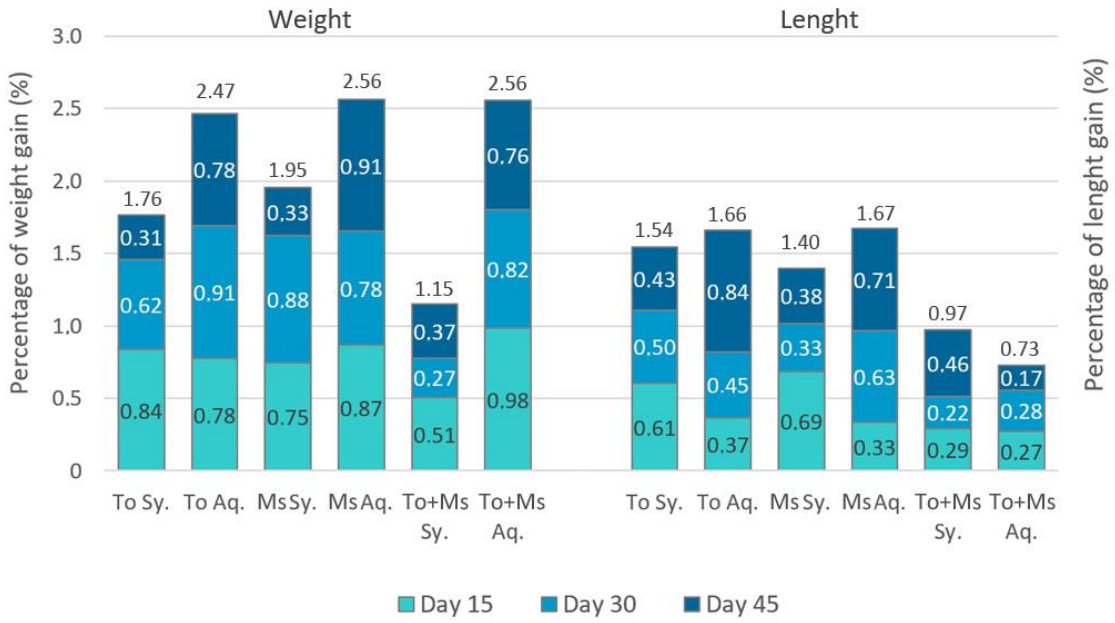

Figure 7.1 - Average weight and shell length gain of mussels by type of diet at day 15, 30, and 45 (mean = 2). The values at the top of each column indicate the total gains for each diet. (To – *Tetradesmus obliquus*; Ms – *Microchloropsis salina*; Aq. – Aquaculture Effluent; Sy. – Synthetic medium).

In the final evaluation, T_{45}, the difference among weight gains between aquaculture and synthetic medium diets was more evident, with significant differences between them. Regarding shell length, the greatest growths were found for the two pure culture diets grown in aquaculture with significant differences for all others. The To+Ms Aq. mixture was the one that showed the worst performance.

In general, there were significant differences between weight gain for microalgae aquaculture diets (2.47, 2.56, and 2.56% for To-Aq., Ms-Aq. and To+Ms Aq., respectively) compared to microalgae synthetic diets (1.76, 1.95 and 1.15% for To-Sy., Ms-Sy. and To+Ms-Sy, respectively), suggesting that feeding mussels with algal biomass produced in aquaculture effluents led to a greater increase in weight. Concerning the shell length, the differences in the growth were not significant between pure culture diets (To-Aq., Ms-Aq., To-Sy and Ms-Sy), however, the mixed diets showed significantly lower growth rates.

Concerning condition index (CI) it was found that the average of the individuals from T_1 was 9.7 ± 1.63 (ranging from 7.6 to 12.7). The CI at day 30 and day 45 is shown in Figure 7.2 for all the different diets.

The CI variation throughout the year in the wild is natural and it is due to several factors such as temperature, salinity, oxygen concentrations, food availability, and changes in the reproductive cycles (Gvozdenović *et al.*, 2020). CI tends to be higher at the end of winter, presenting its lowest value in the months of August and September, when most individuals are at the resting/inactive stage due in large part to the increase in temperature in the summer which also leads to higher mortality in mussels and consequently lower CI (Gvozdenović *et al.*, 2020).

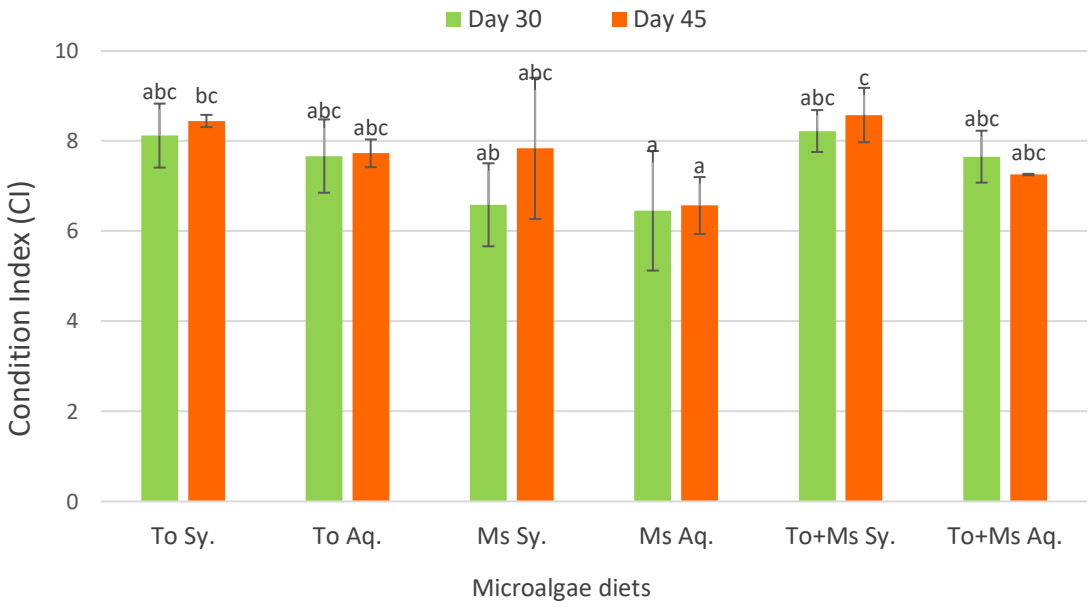

Figure 7.2 - Average condition index (CI) of mussels at day 30 and 45, for all the different diets (mean ± SD, n = 2) (To – *Tetradesmus obliquus*; Ms – *Microchloropsis salina*; Aq. – Aquaculture Effluent; Sy. – Synthetic medium). The values with different index letters show significant differences with $p < 0.05$.

In the present assay, after one month of feeding with the several diets, there was no mortality to report. Only one mussel died during the entire assay, in the period between 30 and 45 days, in the To+Ms synthetic diet. It is evident that individuals on T_1 had a higher CI than individuals on any of the tested diets, either after 30 days or 45 days. There were no significant differences over time in the different diets studied, but there was an increase between T_{30} and T_{45} for all diets (except To+Ms-Aq). Still, the Ms-Aq. diet had a lower CI than the other diets. The decrease was more emphasised in diets of *M. salina*. These results may be due to differences in cell size, cell wall thickness, digestibility, toxic metabolites, fatty acid content, nutrient composition, and various trace elements among the diets

(Fidalgo *et al.*, 1994). The microalgae *M. salina* has a smaller size compared with *T. obliquus*, which may influence the filtration/absorption capacity of mussels. Nevertheless, the ash composition of this microalga is higher than in *T. obliquus*, and the carbohydrates and fatty acid content are lower (Guimarães *et al.*, 2021; Santhakumaran *et al.*, 2020). It can also be verified that mussels grown on synthetic medium diets have a higher condition index than those grown on aquaculture effluent. Wyatt *et al.* (2013) also evaluated mussels in terms of their condition index and for freshly caught mussels the CI was 16.86 ± 4.81, however, the mussels studied had a commercial length, 58.83 ± 5.35 mm, which was much larger than the size of the mussels used in the present study (37.8 ± 1.1 mm) and the harvest of these mussels was carried out in June. Still, the study focused that storage in wet-holding facilities could reach two months and might cause an increase in mussels stress response and consequently a decrease in condition and meat yield, with a significant decrease in the CI to 5.89 ± 1.46 (Wyatt *et al.*, 2013).

Regarding the mussels' composition, the protein, carbohydrate, lipid, fatty acid profile, and ash content were analysed in the edible organism. The nutritional composition of mussels for the 6 treatments at T_1, T_{15}, $T_{30,}$ and T_{45} are presented in Table 7.1.

Table 7.1 – Nutritional composition of mussel biomass by type of microalgae diet at day 1 (T_1), 30 (T_{30}) and 45 (T_{45}) (mean ± SD, n=2) (To – *Tetradesmus obliquus*; Ns – *Microchloropsis salina*; Aquac. – Aquaculture Effluent; Synt. – Synthetic medium).

	Biomass Component	**Sampling Day**	**Mussel Biomass Samples**					
			To - Synt.	To - Aquac.	Ms - Synt.	Ms - Aquac.	To+Ms - Synt.	To+Ms - Aquac.
Concentration (% db)	Protein	T_1	55.7±4.9 ^{a}					
		T_{30}	54.4±3.3 ^{a}	51.7±0.3 ^{a}	57.9±0.7 ^{a}	53.9±5.8 ^{a}	53.1±2.7 ^{a}	48.3±0.1 ^{a}
		T_{45}	**58.3**±0.8 ^{a}	54.7±0.1 ^{a}	56.6±1.8 ^{a}	55.5±1.4 ^{a}	58.2±2.3 ^{a}	54.3±0.7 ^{a}
	Lipid	T_1	2.1±0.6 ^{a}					
		T_{30}	3.3±0.2 ^{abc}	3.6±0.2 ^{abcd}	3.0±0.3 ^{ab}	3.1±0.1 ^{ab}	3.1±0.5 ^{abc}	3.8±0.1 ^{abcd}
		T_{45}	**5.2**±0.1 ^{d}	4.1±0.1 ^{bcd}	5.0±0.4 ^{cd}	4.9±1.0 ^{bcd}	4.3±0.2 ^{bcd}	4.7±0.5 ^{bcd}
	Carbo-hydrate	T_1	30.7±3.1 ^{c}					
		T_{30}	23.0±1.8 ^{abc}	24.9±0.5 ^{abc}	18.7±2.1 ^{ab}	22.5±2.8 ^{abc}	22.0±0.6 ^{ab}	**25.5**±0.3 ^{bc}
		T_{45}	16.4±1.1 ^{a}	19.5±1.1 ^{ab}	19.5±1.4 ^{ab}	20.6±0.6 ^{ab}	18.8±2.2 ^{ab}	21.3±0.1 ^{ab}
	Ash	T_1	11.5±1.2 ^{a}					
		T_{30}	19.3±1.6 ^{b}	19.8±0.4 ^{b}	20.5±1.1 ^{b}	20.5±2.9 ^{b}	21.8±2.8 ^{b}	**22.5**±0.1 ^{b}
		T_{45}	20.1±1.7 ^{b}	21.7±1.2 ^{b}	18.9±0.8 ^{b}	19.1±0.2 ^{b}	18.7±0.3 ^{b}	19.7±1.2 ^{b}

Note: The highest values are in bold

The composition of mussels at 30 and 45 days of exposure to diets presented significant differences in relation to T_1 organisms, mainly in the amount of ash, carbohydrates, and lipids. Over time, there was a decrease in mussels' carbohydrates and an increase in lipids and ash content in their biomass. The protein content of mussels grown on synthetic diets was slightly higher than that of mussels on diets with microalgae from aquaculture effluent. The opposite is true for carbohydrates and ash content.

Figure 7.3 shows the evolution over time of the average concentration of mussels' constituents for the groups of diets.

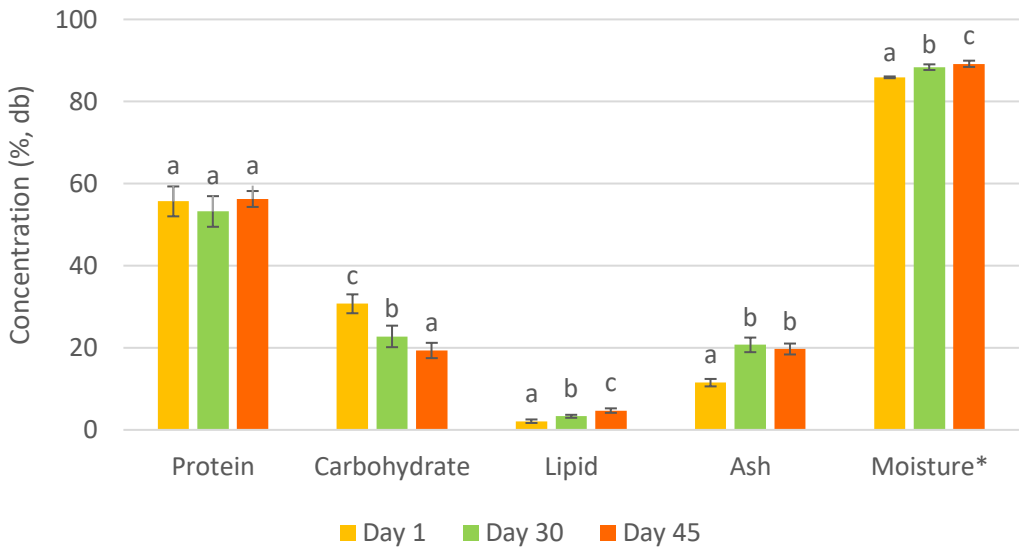

Figure 7.3 – Mussels' average concentration of protein, carbohydrate, lipid, and ash content (in db) and moisture content (*as received) at day 1, 30, and 45 (mean ± SD, n=3). The values with different index letters show significant differences with $p < 0.05$.

According to the analysis of Fidalgo *et al.* (1994), wild mussels are composed of 52.9% protein, 11.7% carbohydrates, 8.3% lipids, and 14.6% ash. According to the *Instituto Nacional de Saúde Doutor Ricardo Jorge*, raw mussels are composed of 69.1% protein, 11.4% carbohydrates, 8.6% lipids, and 10.9% ash (INSA, 2017). It is conceivable that the amount of lipids found in the mussels in the present study is less since they are younger animals that have not yet reached sexual maturity, so their lipid content is naturally lower (Pettersen *et al.*, 2010). According to Gallardi *et al.* (2014), mussels take between 12 and 24 months to reach the appropriate size (between 50-75 mm) to be captured and traded.

Regarding the fatty acid profile, there was a slight increase in the amount of palmitic acid and a decrease in the palmitoleic acid in the various diets, compared to the T_1 profile group. The fatty acids present in mussels grown in different diets are given in Figure 7.4.

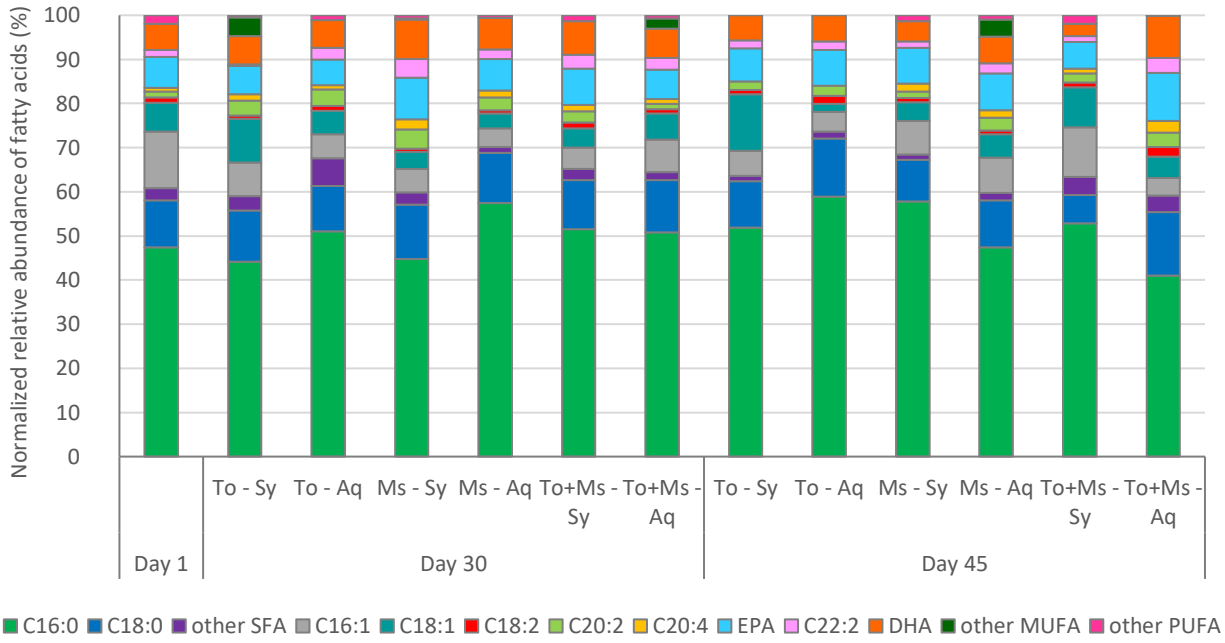

Figure 7.4 - Characterization of fatty acids of mussels at day 1 and fed with different microalgae diets at day 30 and 45 (To – *Tetradesmus obliquus*; Ms – *Microchloropsis salina*; Aq – Aquaculture Effluent; Sy – Synthetic medium).

The fatty acid profile showed a predominance of palmitic acid (C16:0) with 47.2 ± 1.2%, followed by palmitoleic acid (C16:1) with 12.9 ± 0.3% and stearic acid (C18:0) with 10.6 ± 0.2%. There are no significant differences among the various diets. Regarding the fatty acids profile of mussels, other authors also mention the predominance of these fatty acids for mussels fed with microalgae (Nevejan *et al.*, 2007; Pettersen *et al.*, 2010).

Pettersen *et al.* (2010) studied the feeding of mussel larvae (*Mytilus galloprovincialis*) concluding that the inclusion of the diatom *Chaetoceros calcitrans* was important in the survival of the molluscs at this stage of development. Apparently, the balance between fatty acid ratios of DHA, EPA, and ARA is directly related to the larvae's ability to undergo successful settlement and metamorphosis. Another study with juvenile mussels of the same species fed with 3 different algae (*Tetraselmis suecica*, *Dunaliella tertiolecta,* and *Phaeodactylum tricornutum*) or in a two-by-two mixture concluded that the mixture *T. suesica* + *P. tricornutum* led to the highest growth rates followed by *T. suecica*. However, Chen *et al.* (2021) tested the

feeding of oysters (*Hyriopsis cumingii*) fed with *Tetradesmus dimorphus* that showed a high growth rate. In the present study, the results were similar for feeding mussels with To Aquac. and To+Ms Aquac. mixture in terms of weight gain, however the To-Aquac. diet had better results for increasing shell length.

The mineral composition of mussels' biomass was analysed and is shown in Figure 7.5.

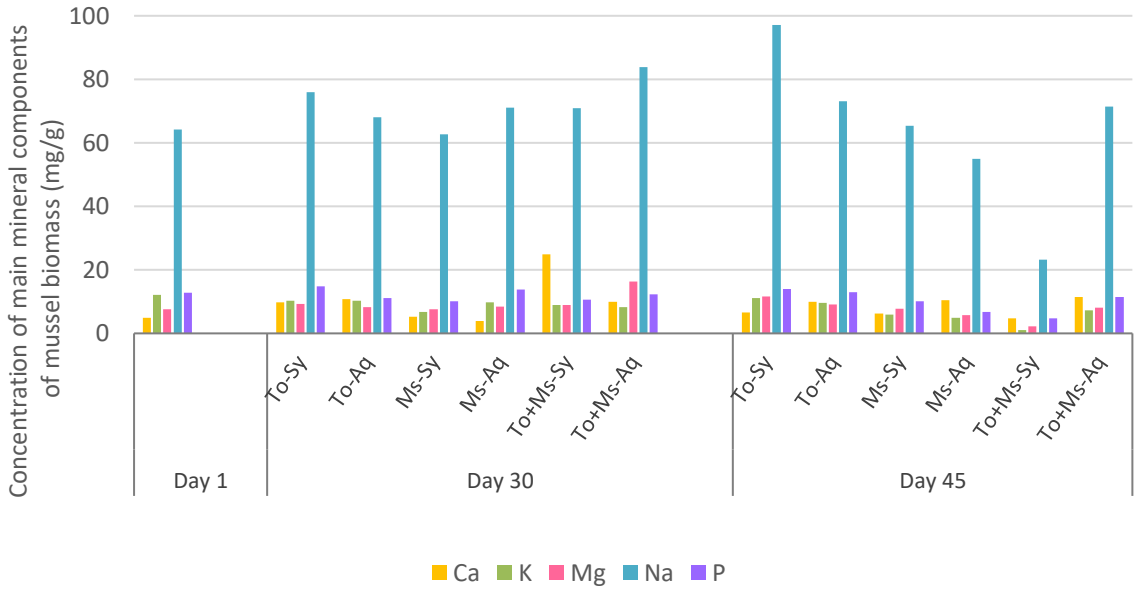

Figure 7.5 - Composition of the mineral fraction from mussel biomass (mg g^{-1}), at day 1, 30, and 45 for different microalgae diets (To – *Tetradesmus obliquus*; Ms – *Microchloropsis salina*; Aq – Aquaculture Effluent; Sy – Synthetic medium). Undetected elements: Ag, As, Bi, Cd, Co, Cr, Hg, Li, Mo, Pb, Sb, Se, Sn, Tl, V, W, Zr.

Although total ash content of mussels showed significant differences ($p<0.05$) between day 1, 30, and 45, the qualitative composition of the mineral fraction did not show a clear variation trend related to the sampling day, the nature of the microalgae that fed the mussels, or the type of culture medium used to produce the microalgae. According to Santhakumaran *et al.* (2020), the mineral composition of *T. obliquus* has as main constituents sodium, potassium, magnesium, calcium, and phosphorus (9.87, 6.43, 1.62, 1.39, and 0.99 mg g^{-1}, respectively), the same as the analysed mussel biomass. Also, the microalga *M. salina* shares the same main minerals as the mussel biomass, except for sulphur (Guimarães *et al.*, 2021).

It can be concluded that all algae diets led to the growth of mussels without mortality. Additionally, it is important to verify that microalgae diets led to an increase in weight and shell length, with a higher increase for diets with microalgae grown in aquaculture effluent. Therefore, it becomes evident the advantage of feeding aquaculture mussels with microalgae that have already treated aquaculture effluents. The results obtained represent great potential in the supplementation of feed for aquaculture mussels. Although there are some differences in the composition of mussels, there were no negative consequences. However, it would be important to carry out more tests with longer periods of feed time and even analyse the potential incorporation in the biomass of mussels some important minerals for human health, such as selenium and iodine.

7.1.2 Cattle animals

The inclusion of microalgae in cattle diet was mainly evaluated through the effect on milk production and its composition, meat composition, and animal growth. These ruminants have the advantage of being able to metabolize the non-protein nitrogen and digest the cell wall of microalgae. Studies on the inclusion of microalgae in the diet of these animals go back to the 1950s, with the aim of replacing part of the protein source with algae, but also as a way of supplying some important lipids such as DHA (docosahexaenoic acid) (Valente *et al.*, 2021).

By supplementing cow diets with olive cake, rich in oleic acid, there is a direct increase in this fatty acid in the milk produced (Neofytou *et al.*, 2020). Oleic acid is widely used in cosmetics and it is beneficial in preventing heart problems in humans as it lowers blood cholesterol levels and regulates insulin and blood pressure (Jones *et al.*, 2015).

This balance between saturated and unsaturated fatty acids is a relevant parameter for the potential use of algal biomass for cattle feed. It is known that an increase in the polyunsaturated fatty acid content of the animal feed is beneficial for their growth, as well as for the quality of their meat (Díaz *et al.*, 2017).

There are some studies with algal biomass incorporation in the diet of cattle and other herbivores relevant for human nutrition. In general, the addition of 0.5 to 20% of microalgal biomass in the diet of these animals had positive effects on their health resulting in meats with superior quality (Holman *et al.*, 2012; Kulpys *et al.*, 2009). The application of microalgae components could be done with the whole biomass or with isolated bioactive

fractions (Madeira *et al.*, 2017). In other animals such as pigs, poultry, and fish, complete replacement of the whole diet by microalgae biomass was evaluated and no adverse effects were found (Medeiros *et al.*, 2021).

However, there are some inconsistent effects of including microalgae in the diet of animal cattle, often without specific effects on animals (Valente *et al.*, 2021).

7.1.3 Piggery animals

Swine compete directly with the human food chain because they eat mainly cereals and soybeans, so there is a strong interest in introducing microalgae in pig feed (Valente *et al.*, 2021). Several studies are showing the benefit of introducing microalgae in the pigs' diet, namely regarding the daily weight gain, to average daily feed intake and feed conversion ratio. These studies with durations varying between 2 and 8 weeks, introduced in the pigs' diet (piglets, female pigs, weaned piglets, and weaned castrated male swine) between 0.1 (*Chlorella vulgaris*) to 5.5% (*Schizochytrium* sp.) of microalgae (Madeira *et al.*, 2017). The microalgae *Arthrospira maxima* and *Arthrospira platensis* were also tested with positive results regarding the pigs' daily weight gain. It was reported that *Arthrospira platensis* may increase up to 15–26% of the average daily gain, with no effect on backfat thickness (Šimkus *et al.*, 2013). In addition, a beneficial effect on the intestinal development of these mammals was also detected, particularly for *Chlorella*, enhancing the control of mild digestive disorders, without compromising the digestibility of nutrients (Madeira *et al.*, 2017). Other studies with additions of 10 and 15% of algal biomass in pig feed for 28 and 42 days could enhance growth and decrease plasma uric acid concentrations (Ekmay *et al.*, 2014).

There is scant information about the effect of adding microalgae to the diet of sows and gilts and how it affects the reproductive cycle. However, microalgae are a source of DHA and protein, two major components of the sperm membrane. As a result, these two compounds are added to boar diets in order to improve sperm quality (Valente *et al.*, 2021). A study with the addition of 150 g *Schizochytrium* sp./Kg points to an improvement in the mobility of sperm (Andriola *et al.*, 2018). On the other hand, the existing information shows the added value of DHA ingestion during gestation and lactation due to the immunomodulatory properties of DHA to improve the immunological status of piglets before and after birth (Valente *et al.*, 2021).

7.1.4 Poultry animals

The main food of poultry animals is corn and soy, which require an extensive amount of land to grow. With the growing environmental and sustainability concerns of modern society, with the increase in the human population and the demand for animal-derived products, it is foreseeable that it will be unsustainable to allocate these cultures around the globe (Madeira *et al.*, 2017). Microalgae may have a higher amount of protein than soy, in addition to being a source of other compounds such as essential fatty acids, minerals, vitamins, and antioxidants, constituting a very appealing alternative. In addition, the microalgae in feeds serve as a pigmentation agent in the poultry industry for meat and egg yolk. Two of the constraints to its wider use are still the price and its availability (Valente *et al.*, 2021).

In chicken and hen production, diets rich in oleic acid were found to improve the meat and egg quality (Toomer *et al.*, 2020, 2019). Several studies mention the benefits of CLA concerning animal and human health, as it stimulates the immune function with protective effects against cancer, obesity, diabetes, and atherosclerosis in both animal studies and different human cell lines (Yang *et al.*, 2015). With respect to chicken feed, it was also found that even a small CLA increase in the basal diet had a noticeable influence in reducing abdominal fat and cholesterol in the liver and eggs of laying hens (Wang *et al.*, 2019). The advantages of incorporating microalgae into the diet of laying hens have been known for a long time. Its addition leads to an increase in the levels of natural carotenes in eggs, beneficial to the health of consumers, but also a marketing asset because the orangeness eggs are more appealing (Gouveia *et al.*, 1996). Because consumers like the egg yolks quite orange, artificial additives are normally used in chicken feeds so that the eggs show these tones. Some studies have also shown that microalgae can be used to increase beneficial microflora in the digestive tract of poultry animals and improve immune function. Furthermore, the addition of 1% fresh liquid *Chlorella* to the feed of broiler chickens resulted in a significantly higher *Lactobacillus* population in the ceca, a considerably higher concentration of IgA and IgM plasma and an improved body weight gain (Valente *et al.*, 2021).

7.2 As biostimulant

The application of algal biomass as a biostimulant is understudied, though *Tetradesmus obliquus* biomass obtained from brewery wastewater treatment was tested as a biostimulant in seed germination with increased germination of 40% (Navarro-López *et al.*, 2020). Other

examples were done by Deepika and MubarakAli (2020) using *Chorococcum* sp. to promote growth in *Cucumis sativus*, *Solanum lycopersicum*, *Capsicum annuum*, and *Vigna radiata*, with good results. Grzesik et al (2017) also studied the effect of applying a triple foliar spray of intact cells of *Chlorella* sp. and concluded it improved the growth of willow plants (*Salix* sp.). Agwa *et al.* (2017) had positive results when using *C. vulgaris* for *Hibiscus esculentus* development and its role in enhancing soil fertility. Likewise, Marks *et al.* (2017) applied a liquid slurry to soil of live cells of *Chlorella* sp. from wastewater treatment concluding there is an enhancement of soil fertility.

Plant growth is influenced by phytohormones and amino acids, among other compounds that can be found in different sources, namely microalgae. Those phytohormones include gibberellins, auxins, cytokinins, ethylene, and abscisic acid (Morais Junior *et al.*, 2020). To establish the biostimulating capacity of microalgae in seed germination, a complete chemical analysis including the content of amino acids and phytohormone profile should be performed. However, it is simpler to directly test the effect of microalgae biomass on seed development through germination tests. The potential biostimulant activity was evaluated by determining the germination index (GI) of the control, with distilled water (100%), and the GI obtained with microalgae biomass of Cv or Ap and To obtained in the last test of each of the tested agro-industrial effluents. If the GI is higher than 100% it is considered that there is a biostimulating activity.

Figure 7.6 shows the results of the tested microalgae cultures, at two concentrations (0.2 and 0.5 g L^{-1}) on the germination of the two species of seeds (wheat and watercress).

All microalgae had a positive effect on seed germination, except To (0.5 g L^{-1} grown in aquaculture and poultry effluents), Cv (0.5 g L^{-1} grown in poultry effluents), and Ap (0.2 g L^{-1} grown in cattle effluent) for watercress seeds, probably a toxicity effect due to the high concentration. The microalga *T. obliquus* has a higher amount of proteins and lipids in its composition, a factor that can lead to an inhibitory effect when in higher concentrations (Puglisi *et al.*, 2020). This situation is no longer registered when using the 0.2 g L^{-1} concentration, except for watercress with Ap grown in cattle effluent.

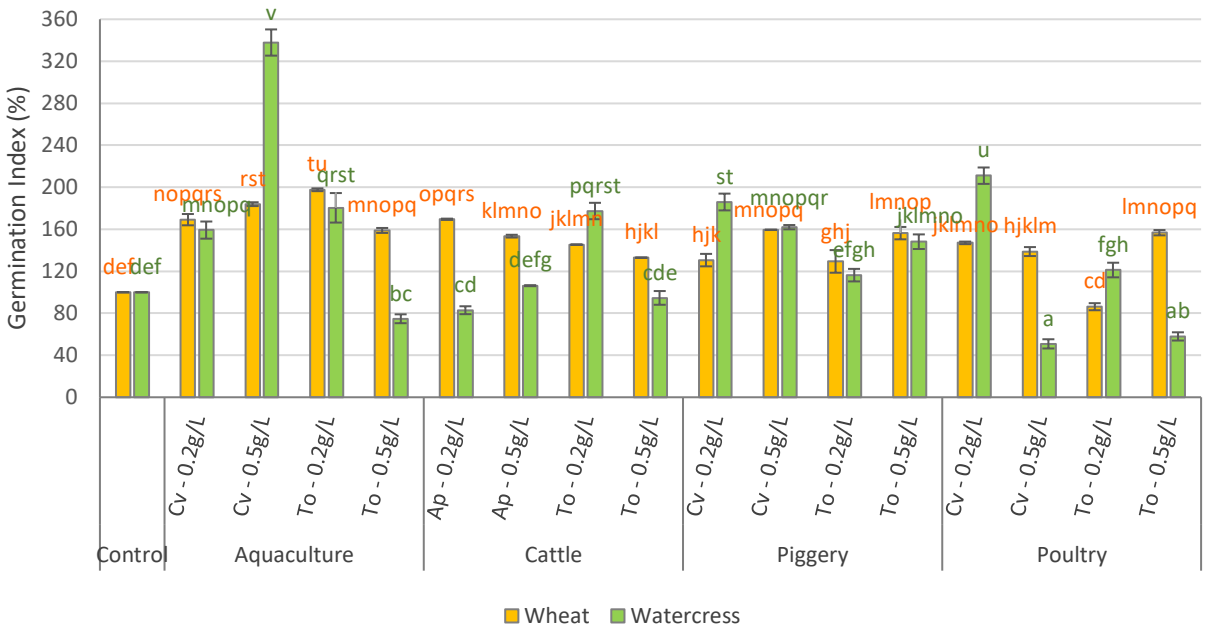

Figure 7.6 – Germination Index (%) for the control and the two microalgae cultures (Cv – *Chlorella vulgaris* or Ap - *Auxenochlorella protothecoides* and To – *Tetradesmus obliquus*) in the four effluents (Aquaculture, Cattle, Piggery, and Poultry) and two concentrations (0.2 and 0.5 g L^{-1}) (mean ± SD, n = 3). The values with different index letters show significant differences with $p < 0.05$.

The highest GI for watercress was reached for the biomass of Cv - 0.5 g L^{-1} from aquaculture with 337.9 ± 12.5%, meaning a huge increase of 238% when compared with the control (distilled water), followed by Cv - 0.2 g L^{-1} from poultry with an increase of 111%.

The highest GI for wheat was achieved for the biomass of To - 0.2 g L^{-1} from aquaculture with 197.6 ± 1.3%, which corresponds to almost 100% more than the control. The biomass of different microalgae had some important macro and micronutrients from the point of view of plant nutrition, namely potassium, phosphorus, calcium, magnesium, and sodium (biofertilizer effect) (Table 7.2). Probably some of the negative effects of applying the higher dosage (0.5 g L^{-1}) of *C. vulgaris* grown in poultry effluent were due to the existence of some metals in higher concentrations compared to other microalgae, namely aluminium and zinc.

Promising results had already been achieved by Navarro-López *et al.* (2020) in watercress with microalga *T. obliquus*. Still, the results attained were 40% higher than the control. The addition of ash to the effluent may have also contributed to the positive effect

on seed germination, as it contributes with essential minerals to plant development. However, a toxic effect with reduced germination index had already been noticed for concentrations below 1 g L^{-1} by Navarro-López *et al.* (2020). Although the number of germinated seeds is lower, the average weight of each seedling is greater than that of the control. Despite the few published works on the effect of algae on seed germination, it is known that bioactive compounds are necessary in very small amounts (Chhaya *et al.*, 2021).

Table 7.2 – Chemical characteristics of microalgae biomass used in germination tests, presented in mg g^{-1} (Cv – *Chlorella vulgaris*, Ap – *Auxenochlorella protothecoides*, To – *Tetradesmus obliquus*).

	Al	**B**	**Ba**	**Ca**	**Cu**	**Fe**	**K**	**Mg**	**Mn**	**Na**	**P**	**Si**	**Sr**	**Zn**
Aquac - Cv	1.64	0.04	0.10	13.97	0.04	0.25	**4.24**	**7.26**	0.01	**5.27**	1.13	0.16	0.12	0.13
Aquac. - To	0.31	0.04	0.01	11.61	0.09	0.40	**4.90**	**6.53**	0.01	**5.32**	0.00	0.15	0.10	0.09
Cattle – Ap	0.80	0.01	0.41	**135.06**	0.06	0.20	**4.39**	2.22	0.08	0.88	1.05	0.18	0.11	0.14
Cattle – To	0.36	0.01	0.27	**103.57**	0.05	0.14	2.73	0.52	0.03	0.46	0.23	0.13	0.08	0.08
Piggery – Cv	0.36	0.01	1.03	8.34	0.04	0.09	**6.28**	2.60	0.03	2.63	0.00	0.23	0.29	0.09
Piggery – To	1.27	0.01	**3.82**	3.42	0.14	0.50	3.29	0.26	0.09	3.51	0.00	0.66	0.39	0.23
Poultry – Cv	**4.37**	0.07	0.54	4.92	0.07	**2.09**	**4.49**	**9.69**	0.26	1.10	**2.93**	**2.96**	0.15	**1.82**
Poultry - To	0.44	0.03	0.14	9.48	0.11	**2.44**	0.87	3.42	0.48	0.58	0.00	0.45	0.13	**1.73**

Note: The presence of Ag, As, Bi, Cd, Co, Hg, Mo, Sb, Sn was not detected in the microalgae biomass. The elements Cr, Li, Pb, Se, Ti, Tl, W, and Zr were only detected vestigially.

The results demonstrated the potential of microalgae resulting from the treatment of agro-industrial effluents to be used as stimulants, being a way to replace the use of synthetic fertilizers, contributing to more efficient and sustainable use of resources and agriculture practice.

In this investigation, the results achieved provide clear evidence about the benefits of using microalgal biomass produced in agro-industrial effluent as biostimulant for seed germination.

7.3 Final considerations

Microalgae have been studied, among other applications, for animal feed, namely regarding their effect on the production and meat quality of livestock (ruminants, pigs, birds, poultry, and rabbits) and aquaculture species, mainly for protein, fatty acids, and

colouring purposes. Microalgae have a variable nutrient composition, depending on the species chosen, on the production conditions, on the formulation of algal biomass (used in isolated bioactive fractions or whole), among other factors.

Regarding the study conducted with mussels fed with microalgae grown in aquaculture effluent and synthetic medium, it can be concluded that the mussels gained more weight when fed with microalgae grown in effluent than in synthetic medium. Furthermore, there were no significant differences in mussel constitution or fatty acid profile, suggesting that there is no problem with feeding mussels with microalgae grown in this type of effluent, meaning saving costs and increasing blue circular economy.

The study on the biostimulating activity of microalgae in seed germination has generally shown an extraordinarily positive effect. The algal biomass with the most evident effects on the germination of both seeds was produced in aquaculture effluent (238% increase in watercress seeds and 97% increase in wheat seeds). These good results could be due to the presence of phytohormones and amino acids, besides a more balanced set of mineral salts for seed germination. However, the algae grown in the other three effluents have also been shown an increase between 60 and 110% in the germination index of wheat and watercress seeds.

Chapter 8

Precipitate applications

8.1 Precipitate as fertilizer

The precipitates obtained in the three agro-industrial effluents (cattle, piggery, and poultry) were tested as fertilizer for the germination of wheat and watercress seeds.

Watercress seeds were used since the plant is very susceptible to toxicity due to excess mineral elements or organic phytotoxic substances (Luo *et al.*, 2018). These phytotoxic substances can be present when the compost has not yet reached the degree of maturity, considering they are formed during composting (Liu *et al.*, 2019; Muscolo *et al.*, 2018). Wheat seeds were also chosen for the germination test since this cereal integrates human food and animal diets. The germination index (GI) of watercress seeds is shown in Figure 8.1 and for wheat seeds in Figure 8.2, for the prepared precipitate extracts, the biomass ash extracts, and the control.

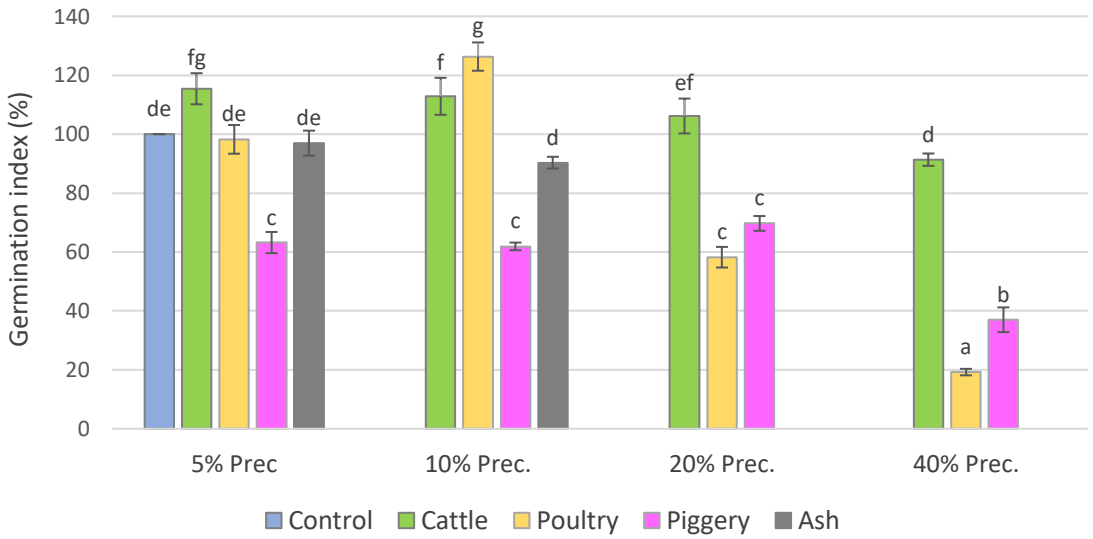

Figure 8.1 – Germination Index (%) for watercress seeds (mean ± SD, n = 3) for control and different aqueous extract of precipitate and biomass ash (Prec.: Precipitate aqueous extract; Ash: Ash aqueous extract). The values with different index letters show significant differences with $p < 0.05$.

The results showed that the watercress seeds developed better with the poultry precipitate, but only at a concentration of 10%, with an increase of 26% compared to the control. Nonetheless, the cattle precipitate has shown to have a positive effect even at a high concentration of 20%. According to Zucconi *et al.* (1981), who developed the procedure used, germination breaks in watercress below 10% compared to the control are not significant and suggest that the precipitate has an adequate degree of maturation.

The results obtained for the piggery precipitate demonstrate that it presents some toxicity for the watercress since the germination index shows a decline close to 40% compared to the control.

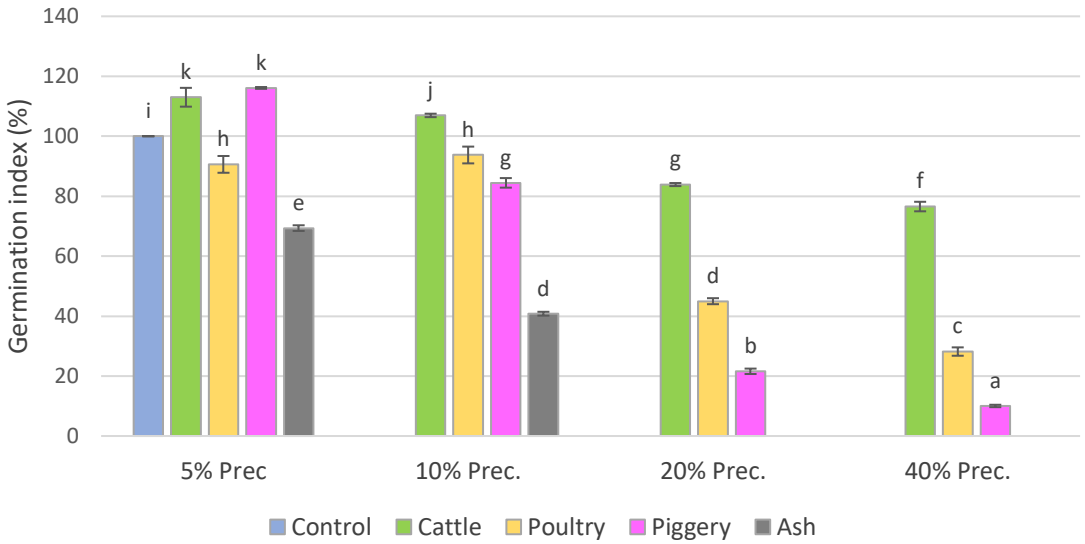

Figure 8.2 – Germination Index (%) for wheat seeds (mean ± SD, n = 3) for control and different aqueous extract of precipitate and biomass ash (Prec.: Precipitate aqueous extract; Ash: Ash aqueous extract). The values with different index letters show significant differences with $p < 0.05$.

Regarding wheat seeds tests, the incorporation of more than 10% cattle aqueous precipitate extract or the application of aqueous ash extract leads to a breakdown in the germination index. Concerning piggery precipitate, in the case of wheat, there was an increase in the germination rate of 16.2% for incorporations of 5% of precipitate like the effect of cattle precipitate. Although, the incorporation of piggery precipitate on the soil could have different behaviour. The precipitate has a higher concentration of minerals than the ash, namely aluminium, boron, barium, copper, iron, manganese, sodium, titanium, and zinc, and this can positively affect the germination of the tested seeds.

Furthermore, it is evident that the incorporation of the precipitate extract (not exceeding 10%) resulted in a germination index slightly superior to the control (in both tested species). Therefore, it could be inferred that the incorporation of the cattle precipitate in the soil, in concentrations between 5 and 10% would be beneficial for soil fertility. In the case of the poultry precipitate, results show that wheat is more susceptible to the presence of this precipitate or ash than watercress, verifying a positive effect for incorporations of 10% of poultry precipitate.

All the remaining cases exhibit negative effects, such effects are, with one exception (5% piggery precipitate), more pronounced in the wheat seeds. According to Zucconi *et al.* (1981) criterion, meaningful toxicity is only present in watercress seeds when 20% or 40% of the precipitate is incorporated. Nevertheless, such toxicity is only absent in wheat seeds when 5% or 10% of the precipitate is incorporated – in particular, the incorporation of biomass ash is always toxic, regardless of the amount. Finally, it should still be pointed out that the incorporation of the precipitate in the soil may alter their expected behaviour, due to the interaction with the soil nutrients.

Chapter 9

Microalgae co-torrefaction experiments

Microalgae are efficient agents for the bioremediation of animal production effluents due to their high capacity to remove nitrogen and phosphorus, two important contributors to the chemical oxygen demand of such effluents (Ferreira *et al.*, 2018; Viegas *et al.*, 2021b; Viegas and Gonçalves, 2021). The robustness of microalgae makes them suitable to treat recalcitrant effluents with high levels of organic or inorganic contaminants, that are difficult to remediate with aerobic or anaerobic bacteria (Porto et al., 2020; Viegas et al., 2021a, 2021d). After bioremediation, the algal biomass must be separated from the treated effluent and ideally valorised as raw material for fuels and/or specialty chemicals. Separation of the algal biomass has been studied using membrane technology (Loulergue *et al.*, 2019), electrocoagulation (Lucakova *et al.*, 2021; Visigalli *et al.*, 2021), radiofrequency (Carvalho *et al.*, 2020), and coagulation with polymers (Rao *et al.*, 2018), but these techniques involve complex equipment and additional costs that are compatible only with large scale systems with the subsequent valorisation of the algal biomass in multiple applications. A possible alternative is the decantation of the sedimented biomass, in the form of an algal sludge that can be further dried or mixed with other biomass feedstocks to be used in energy or material applications. For example, mixing wet algal biomass (80% moisture) with yard waste has been proposed as a stabilization technique to preserve the algal biomass and avoid or facilitate the drying step (Wahlen *et al.*, 2020).

The torrefaction is a thermochemical process that applies moderate pyrolysis conditions, with temperatures between 200 and 320°C, under an inert atmosphere. It is an effective process of upgrading biomass by removing moisture and reducing the content of the volatile component while promoting chemical reactions that involve the constituting polymers (Chen *et al.*, 2019; Chen *et al.*, 2015b). This process results in the densification of the torrefied material, an increase in its heating value and carbon content, and a decrease of its H/C and O/C atomic ratios, reinforcing its hydrophobic nature (Arias *et al.*, 2008; Chen *et al.*, 2018). Depending on the specific torrefaction conditions, namely temperature and residence time, different yields of biochar, condensates, and gas products will be obtained.

The composition of the raw material also greatly influences the composition and relative concentrations of the torrefaction products (Yu *et al.*, 2017a). The thermochemical co-processing of different biomass feedstocks allows obtaining bio-oils and biochars with specific characteristics, different from those obtained when processing the individual materials (Azizi *et al.*, 2018; Chen *et al.*, 2017; Tang *et al.*, 2019). Torrefaction has already been tested for numerous biomasses, however, the use of microalgae is a very promising option, especially if algal biomass is obtained through the remediation of residual effluents (Viegas *et al.*, 2021a; Wu *et al.*, 2012; Yu *et al.*, 2017a). For instance, the torrefaction of several microalgae (*Chlamydomonas* sp., *Tetradesmus obliquus*, *Chlorella sorokiniana,* and *Chlorella vulgaris*) at temperatures between 200 and 300°C for 15 to 60 min, was carried out by several authors aiming to evaluate the influence of the conversion conditions in the biochar yield and properties. High biochar yields (86 - 91%) were obtained at 200°C and 1 h, while at 300°C and 1 h the biochar yield was strongly dependent on the type of micro-algae used, with values of 38% for *Chlorella sorokiniana* (Chen *et al.*, 2014a), 41% for *Chlamydomonas* sp. (Chen *et al.*, 2014a), 53% for *Chlorella vulgaris* (Chen *et al.*, 2015a) and 63% for *Tetradesmus obliquus* (Chen *et al.*, 2014b). The previous experiments were all conducted in a nitrogen atmosphere, with the objective of preventing oxidation of the biochar during torrefaction. The torrefaction using a non-inert gas is being implemented recently in order to save nitrogen gas and energy (Chen et al., 2015a; Zhang et al., 2019). This thermochemical process is the most reliable process to produce high-quality biochar as a consistent and reliable product for agriculture use. Torrefaction allows the retention of up to 50% of the carbon in the biomass in stable biochar. The biochars produced at low temperatures can be easily mineralized by microorganisms compared to biochars produced at higher temperatures (Yu *et al.*, 2017b).

Microalgae produced in contaminated effluents are not suitable for food applications, therefore their valorisation in the energy conversion process has been presented in the literature (Choi *et al.*, 2019; Hosseinizand *et al.*, 2018; Lee *et al.*, 2019). Microalgae can be grown as lipid or carbohydrate-rich raw materials with a view to their subsequent extraction and conversion into biofuels of hydrocarbon or alcohol type (J. Wang et al., 2019; Wu et al., 2012). More recently the processes of integral conversion of algal biomass, namely through fermentation (Sambusiti *et al.*, 2015), anaerobic digestion (Marin-Batista *et al.*, 2019), hydrothermal liquefaction (Heilmann *et al.*, 2010; Liu *et al.*, 2019), pyrolysis or gasification (Pecchi and Baratieri, 2019; Xie *et al.*, 2019) have been approached for their

efficiency and simplicity. Using these biological or thermochemical processes the algal biomass is converted to gas or liquid products with a high calorific value that may be used as fuels either directly or after some upgrading step.

The combination of algal biomass with other materials or biomass residues has also been addressed by some authors who have studied co-pelletization of *Chlorella vulgaris* biomass with pine sawdust (Hosseinizand *et al.*, 2018), co-carbonization of coal mixed with algae in different proportions (He *et al.*, 2012), co-liquefaction of *Tetraselmis sp.* biomass in the presence of ethylene glycol or isopropyl alcohol (Han *et al.*, 2019). The co-liquefaction of microalgae *Chlorella pyrenoidosa* (*Auxenochlorella pyrenoidosa*) and rice husk in subcritical water showed synergistic effects that decreased acidity and nitrogen content of bio-crude oils (Gai *et al.*, 2015). Biomass is a very abundant material and can be converted into energy in several methods, namely by thermochemical and biochemical processes. With fossil fuels concern, new raw materials and forms of conversion have emerged and been explored (Chiaramonti *et al.*, 2015). The choice of the torrefaction technology mostly relates to its less demanding energy features when compared to other thermal processes (Gan *et al.*, 2018). In addition, the main product obtained in torrefaction is a char that can be used directly in various energy or material applications without requiring further processing, as is the case of pyrolysis bio-oil, which usually requires upgrading treatments (Azizi *et al.*, 2018; Gai *et al.*, 2015). Hydrothermal carbonization (HTC) also produces biochar that can be used as a fuel, as activated carbon, or as a fertilizer for soil correction and amendment. However, the HTC process also produces high volumes of a liquid phase that must be treated or valorised to decrease costs and increase the sustainability of this technology. Furthermore, scaling up the HTC technology requires expensive high-pressure reactors and accessory equipment capable of withstanding corrosive media (Aguado *et al.*, 2020; Atallah *et al.*, 2021).

This study evaluates the co-torrefaction of microalgae sludge with lignocellulosic biomass, as a simple and low-cost technology to process algal sludges obtained from bioremediation of industrial or agro-industrial effluents. The co-torrefaction process was also selected as a technology to promote the elimination of nitrogen, therefore improving the fuel quality of algal biomass, by decreasing its potential to generate NOx emissions. The influence of process parameters (carbonization temperature, residence time, concentration of lignocellulosic biomass in the feed, and the moisture of the feed) in the yields of carbonization products and properties of the biochars were studied using a series

of experiments defined by RSM (response surface methodology). Moreover, this work also tested the co-torrefaction of microalgae used in bioremediation of an aquaculture effluent and landfill leachate as examples of real case scenarios and provides insights on the use of the produced biochars as low-cost adsorbents and fertilizers (Viegas *et al.* 2021e).

9.1 Biomass feedstock characterization

The characterization of the original feedstocks and the mixtures of microalgae and lignocellulosic biomass used in the torrefaction tests was made by determining their proximate and elemental analysis as well as their HHV (Table 9.1).

Table 9.1 – Proximate analysis, ultimate analysis, and heating value of raw materials and mixtures used in the torrefaction tests (Cv – *Chlorella vulgaris*, Lc - Lignocellulosic material).

Biomass	Cv	Lc	75% Cv + 25% Lc	50% Cv + 50% Lc	25% Cv + 75% Lc
Proximate analysis (wt.%, db)					
Volatile matter	86.46 ± 0.74	78.41 ± 3.89	84.45 ± 0.85	82.44 ± 0.56	80.43 ± 1.89
Fixed carbon	6.01 ± 0.73	19.06 ± 3.97	9.27 ± 1.03	12.53 ± 0.45	15.79 ± 0.54
Moisture *	6.35 ± 0.52	9.28 ± 0.84	7.08 ± 0.68	7.81 ± 0.54	8.54 ± 0.67
Ash	7.53 ± 0.09	2.53 ± 0.08	6.28 ± 0.02	5.03 ± 0.04	3.78 ± 0.07
Elemental analysis (wt.%, daf)					
C	51.29 ± 0.09	50.10 ± 0.16	50.99 ± 0.04	50.70 ± 0.12	50.40 ± 0.10
H	7.31 ± 0.42	6.21 ± 0.09	7.04 ± 0.14	6.76 ± 0.24	6.49 ± 0.08
N	9.05 ± 0.00	1.10 ± 0.08	7.06 ± 0.08	5.08 ± 0.02	3.09 ± 0.07
S	0.24 ± 0.04	0.00 ± 0.00	0.18 ± 0.00	0.12 ± 0.02	0.06 ± 0.01
O **	32.11 ± 0.10	42.59 ± 0.04	34.73 ± 0.02	37.35 ± 0.01	39.97 ± 0.04
O/C ratio	0.47	0.64	0.51	0.55	0.60
H/C ratio	1.71	1.49	1.65	1.60	1.54
HHV (MJ Kg^{-1}) (db)	15.54 ***	18.94 ***	16.39 ***	17.24 ***	18.09 ***

* As received; ** By difference; *** HHV calculated based on the formula developed by Huang and Lo (2020), which uses the ultimate analysis of biomass.

Samples Cv and Lc presented significant differences in their proximate and ultimate compositions: Cv presented higher ash, nitrogen, and volatile matter contents while Lc showed higher concentrations of fixed carbon and oxygen. The lower ash content of Lc

may be related to a higher HHV, and its lower nitrogen content reduces the potential for harmful NOx emissions in case of energy recovery. Han *et al.* (2020) determined the elemental analysis of the *C. vulgaris* biomass, obtaining values (C: 47.4%; O: 27.9%; N: 10.9%; H: 9.9%, and S: 0.7%) in line with those obtained in the present study, and confirming the tendency of high nitrogen concentrations in algal biomass.

The advantage of mixing lignocellulosic biomass with algal biomass is primarily to decrease the ash and nitrogen contents of algal biomass, improving its fuel quality. Furthermore, in real conditions, algal biomass can be obtained in the form of a decanted sludge, with high moisture content and the mixing with dried biomass residues can facilitate the process of sludge dispersion and drying, even at atmospheric conditions. Microalgae coming from effluent treatments are not typically used in food or cosmetic applications therefore do not undergo the same treatments as commercial microalgae (spray drying). Therefore, in this work, different mixtures of Cv and Lc biomasses were considered as a substrate for co-carbonization, and the moisture of the biomass mixtures was adjusted to controlled values by the addition of selected volumes of water. The purpose of this approach is to simulate the mixing of dried lignocellulosic biomass and decanted microalgae sludge and the direct use of the mixture as feed for biochar production. As seen in Table 9.1, additions of 25%, 50%, 75%, and 100% of Lc to the feed were considered, and this approach had a significant effect on the ash content and nitrogen content of the feed, reaching values of 3.78% for ash content and 3.09% for nitrogen content for the mixture with 75% Lc.

9.2 Characterization of torrefaction products

The conditions of the co-torrefaction experiments were selected according to a response surface methodology, to evaluate the influence of the following parameters: torrefaction temperature, residence time, incorporation of lignocellulosic biomass in the feed, and moisture of the feed. A total of 39 experiments were performed and the solid and liquid products obtained were characterised for composition and relevant properties.

9.2.1 Biochars

The appearance of the biochars obtained by torrefaction of the microalgae and lignocellulosic biomass mixtures, with 50% Lc, at different temperatures, residence times, and moisture contents is shown in Figure 9.1.

The variation in colour intensity is clearly a function of the final torrefaction temperature. At the temperatures of 200°C, regardless of the residence time, there were no significant changes in the colours of biochars that were similar to the colour of the feed mixture. At such mild conditions, this suggests that the torrefaction process had minor effects on feedstock composition and structure, with changes restricted to the loss of water and volatile matter.

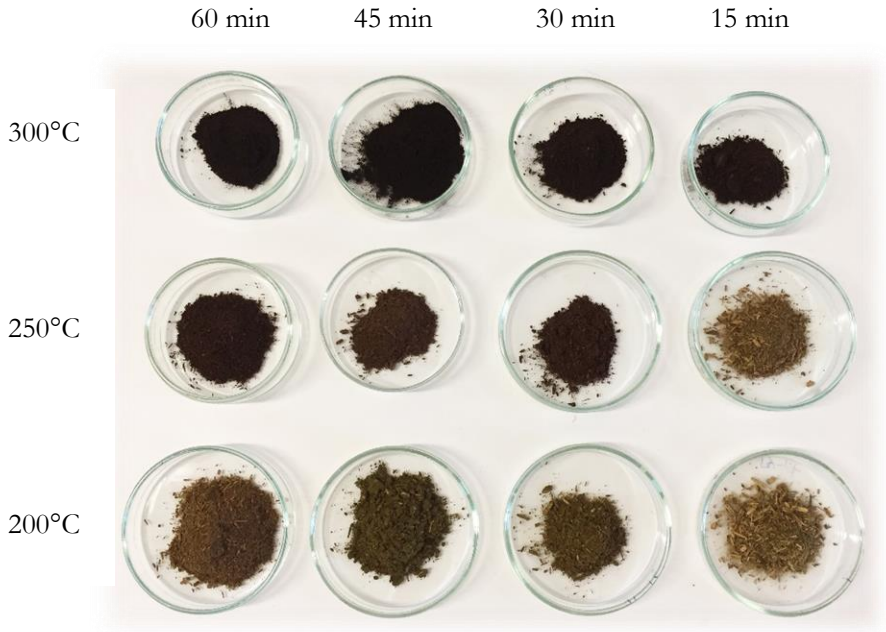

Figure 9.1 – Biochars obtained for a 50% incorporation of lignocellulosic biomass with a variation of temperature and residence time.

Higher temperatures and longer residence times yielded darker biochars, indicating some degree of decomposition and rearrangement of the feedstock. The colour of the biochars produced at 250°C was influenced by the residence time while all biochars produced at 300°C presented a homogeneous black colour. As such, torrefaction temperature showed a larger impact on biochar colour than torrefaction time, thus being responsible for a higher degree of decomposition and molecular rearrangement of the tested samples.

The proximate and ultimate compositions of the biochars were determined, as well as their HHV and methylene blue (MB) adsorption capacity, to evaluate their suitability for energy or material applications (Table 9.2).

Table 9.2 – Proximate and ultimate composition, HHV, and MB adsorption capacity of the 39 biochars.

Run	T (°C)	time (min)	M (%)	Inc. (%)	Proximate analysis (wt.%, db)			Ultimate analysis (wt.%, daf)					HHV (MJ Kg^{-1}, db)	q_{MB} (%)
					Ash	VM	FC	N	C	H	S	O		
1	300	60	30	50	20.9	43.2	35.9	4.7	68.9	5.5	0.3	20.7	19.3	13.0
2	300	60	15	25	15.0	56.0	29.0	9.4	69.8	6.2	0.4	14.2	18.9	20.5
3	300	60	15	25	16.6	55.0	29.4	9.9	71.9	6.3	0.4	11.5	18.9	18.9
4	300	45	45	0	12.6	64.6	22.9	12.3	68.1	6.7	0.7	12.3	18.0	17.0
5	300	45	5	100	6.5	58.6	34.9	1.5	70.2	5.1	1.2	21.9	21.4	15.6
6	300	45	5	50	16.8	53.5	29.8	7.9	71.0	6.1	0.4	14.6	18.7	17.9
7	300	45	5	0	12.3	62.6	25.1	12.1	70.1	6.7	0.5	10.6	18.5	15.3
8	300	30	30	50	10.5	63.0	26.6	5.0	61.2	6.0	0.2	27.6	19.1	18.3
9	300	15	45	25	9.5	75.0	15.5	7.0	55.0	6.5	0.3	31.3	17.1	52.1
10	300	15	45	0	9.0	77.0	13.9	11.2	59.6	7.0	0.5	21.8	16.9	58.9
11	300	15	5	50	8.6	69.9	21.6	6.5	61.5	6.4	0.2	25.5	18.4	19.6
12	275	60	70	0	11.1	68.6	20.3	12.6	66.6	6.9	0.5	13.4	17.8	18.1
13	275	45	30	25	9.3	67.4	23.3	8.1	64.8	5.9	0.7	20.5	18.7	17.8
14	250	60	15	0	9.6	75.3	15.5	12.0	61.9	7.0	0.4	18.7	17.2	38.8
15	250	60	5	50	10.9	71.0	18.1	7.4	59.0	6.5	0.2	26.9	17.4	33.5
16	250	45	30	50	19.2	66.5	14.3	4.8	56.8	5.6	0.0	32.8	15.3	34.2
17	250	45	30	25	13.4	69.8	16.9	8.9	59.4	6.6	0.3	24.8	16.7	37.5
18	250	45	30	25	12.6	71.7	15.7	9.5	58.7	6.6	0.3	25.0	16.6	37.4
19	250	45	15	25	9.5	75.1	15.4	9.3	58.1	6.7	0.3	25.6	17.1	38.4
20	250	45	15	25	9.7	76.2	14.1	8.6	57.7	6.5	0.6	26.7	16.8	39.0
21	250	30	45	25	11.4	75.0	13.6	5.7	54.5	6.7	0.3	32.8	16.4	63.0
22	250	30	45	25	11.3	76.6	12.1	4.9	53.4	6.8	0.2	34.8	16.1	62.1
23	250	30	15	50	14.5	68.4	17.1	6.0	59.0	6.8	0.2	28.1	16.6	52.6
24	250	30	5	25	7.5	75.9	16.6	7.6	55.5	6.2	0.7	30.1	17.6	69.9
25	250	15	30	50	9.6	75.4	15.1	6.1	51.2	6.6	0.2	36.0	17.0	78.8
26	250	15	5	0	8.0	79.3	12.7	9.8	54.6	7.1	0.3	28.2	16.8	87.9
27	250	15	5	0	8.0	77.7	14.3	10.6	56.2	7.6	0.4	25.3	17.1	88.4
28	225	60	5	25	7.4	78.0	14.6	8.8	56.4	7.3	0.3	27.1	17.3	88.8
29	225	45	45	0	8.5	84.4	7.1	10.5	56.2	7.6	0.5	25.2	15.6	86.0
30	225	15	15	25	10.5	87.4	2.2	8.7	52.0	7.4	0.5	31.6	14.3	94.4
31	200	60	45	25	10.6	77.8	11.7	7.7	52.3	6.8	0.4	32.8	16.2	50.8
32	200	60	30	50	15.6	71.4	13.0	4.2	55.0	6.8	0.0	34.0	15.6	80.7
33	200	60	5	0	8.0	77.1	14.9	10.0	54.4	7.3	0.7	27.6	17.2	90.2
34	200	30	15	25	10.0	75.8	15.3	8.0	51.2	7.2	0.5	33.2	17.1	91.5
35	200	30	15	25	11.0	75.0	14.0	8.4	51.6	7.1	0.6	32.3	16.6	95.4
36	200	30	5	50	7.6	75.3	17.1	7.6	50.8	6.8	0.3	34.5	17.7	91.3
37	200	15	70	0	8.9	87.7	3.4	9.8	51.2	7.1	0.3	31.6	14.8	60.4
38	200	15	30	50	8.4	78.2	13.4	5.1	47.3	6.7	0.0	40.9	16.9	75.4
39	200	15	30	25	8.1	77.9	14.0	5.0	47.1	6.7	0.0	41.2	17.0	91.1

Note: T – Temperature; M – Moisture; Inc.- Incorporation of lignocellulosic material; q_{MB} – methylene blue adsorption capacity.

The biochars obtained at higher temperatures and residence times presented a higher amount of fixed carbon and less volatile matter. The incorporation of lignocellulosic biomass decreased the ash content of the biochars (12.6 to 4.8%) because of the higher ash content of the Cv sample.

The HHV of biochars presented the lowest and highest values of 14.8 and 21.4 MJ Kg^{-1}, for 0% Lc and 100% Lc, respectively. This parameter was significantly improved for samples subjected to more severe torrefaction conditions and lower for a greater amount of water in the feedstock. In particular, HHVs higher than 18 MJ Kg^{-1} were obtained for mixtures with the incorporation of Lc from 0% to 50% at the temperatures of 275°C and 300°C. Similar conclusions were drawn by Chen et al (2015b) for biochars produced from sawdust, wheat straw, pine, and microalgae. In general, the biochars with better fuel properties were those subjected to a torrefaction process at temperatures above 275°C and residence time above 45 min, showing that nitrogen elimination and deoxygenation reactions played a major effect on the biochar final properties (Mao *et al.*, 2020).

The variation of the elemental composition of the biochars relatively to the feedstocks expressed as their H/C and O/C atomic ratios is shown in a van Krevelen diagram (Figure 9.2).

As it can be seen, the biochars produced at 200°C and 225°C evidenced some degree of deoxygenation, expressed as a decrease of the O/C ratio relative to the raw materials Cv and Lc, but no significant structural rearrangements are detected since the H/C ratio is comparable to the feedstocks. As the torrefaction temperature increases the deoxygenation reactions become more relevant leading to biochars with O/C ratios between those of lignite and anthracite, demonstrating the upgrading effect on fuel quality (Barskov *et al.*, 2019). For torrefaction temperatures higher than 250°C, a temperature-dependent decrease of the H/C ratio also occurs indicating that the rearrangement of the carbonaceous structure takes place with the increased formation of aromatic structures (Cao *et al.*, 2021).

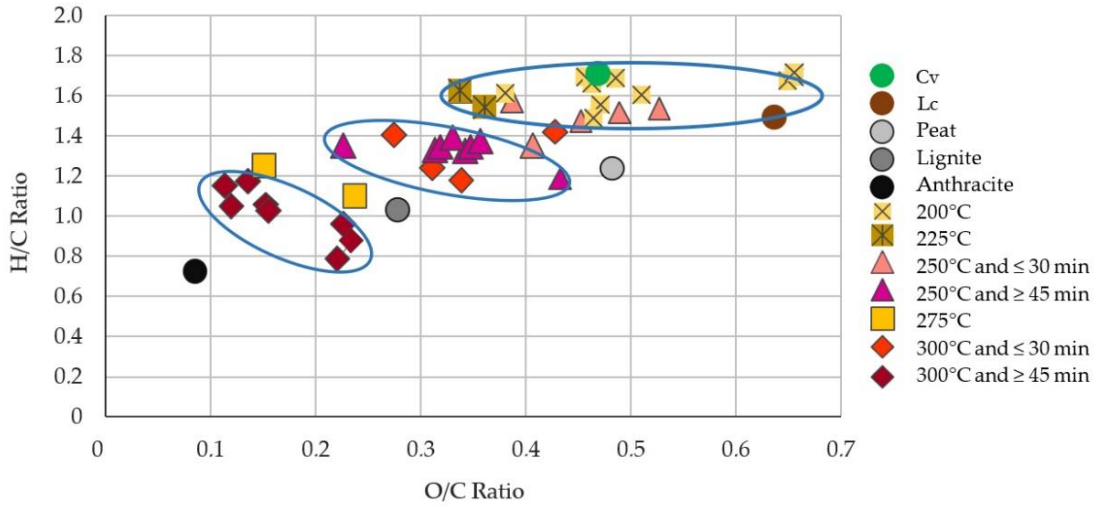

Figure 9.2 – van Krevelen diagram for samples Cv (algal biomass), Lc (lignocellulosic material), and the obtained biochars for different fossil fuels (Zhang *et al.*, 2018).

In contrast, the adsorption capacity for methylene blue (q_{MB}) was negatively influenced by the torrefaction capacity since q_{MB} values higher than 60% were obtained for biochars produced at 250°C or lower (Table 9.2). Since methylene blue is a cationic dye, this behaviour agrees with the adsorption of the dye due to ion exchange, hydrogen bonding, and electrostatic interactions with the carboxyl groups from the feedstock, that are partially retained in the biochar surface, if the carbonaceous structure is not strongly modified, that is for the lower torrefaction temperatures (Correia *et al.*, 2017). Severe torrefaction conditions lead to more significant rearrangement in the lignocellulosic structure of the biochars with Lc, altering the surface porosity, by removing the binding –OH groups (Nobre *et al.*, 2019). The biochars with the highest adsorption capacity were obtained at 200°C, 30 min, 25% of lignocellulosic material incorporation, and 15% moisture, reaching 95% adsorption. Some biochars showed a better adsorption efficiency than the original biomasses feedstocks (59.2% for Cv and 18.3% for Lc) which may be the result of an increase of hydroxyl and carboxyl groups generated on the biochar surface by hydrolysis reactions at low torrefaction temperatures. Relatively to the raw biomasses, biochars have also the advantage of being less susceptible to chemical or microbial degradation, because of their lower moisture content (Binda *et al.*, 2020).

The q_{MB} capacity of the biochars was not influenced by the percentage of Lc incorporation, probably because both feedstocks present anionic groups in their surface

that may be involved in the adsorption process (Huang *et al.*, 2021; Landin-Sandoval *et al.*, 2021).

The adsorption capacity of a commercial activated carbon is close to 100% for several contaminants, including MB, because it involves porous adsorption, a more effective and extensive adsorption mode. Nevertheless, activated carbon production is expensive, energy-demanding, and generates contaminated effluents thus its use should be reserved for specific high-value applications, while low-cost biochars may be used for the preliminary treatment of highly contaminated effluents produced in high volumes (Jagodzińska *et al.*, 2019). Thus, by choosing the operational conditions is possible to obtain biochars with characteristics more adequate for use as an alternative adsorbent for cationic species or to incorporate in solid fuels. The incorporation of Lc biomass is more relevant for the energy application, allowing the reduction of the moisture of the feedstock mixture and improving the composition of the biochar product.

9.2.2 Aqueous phase

The torrefaction process yields a liquid by-product mainly composed of water recovered from the feedstock moisture and dehydration reaction. This aqueous phase contains various organic products produced during the decomposition of the feedstock and distilled at the reaction temperature. Those volatile organic products are either dissolved in the aqueous phase (if they have moderate to high dipole moment) or in the form of suspended droplets or particles (if they are non-polar compounds with low solubility in water). In some of the torrefaction conditions, the concentration of non-polar organic products was high enough to form a water-immiscible organic phase (bio-oil) that separated from the aqueous phase upon cooling of the condensated liquid products. This bio-oil product was separated by decantation prior to the characterization of the aqueous phase. The presence of dissolved or suspended organic compounds confers specific properties to the aqueous phase, namely, influences its pH, conductivity, total phenolic components, total sugars, and chemical oxygen demand. Characterization of the aqueous phases from all torrefaction tests was performed to evaluate the influence of operational parameters in these properties and is presented in Table 9.3.

Table 9.3 – Characterization of the aqueous phase of the 39 torrefaction tests.

Run	T	time	M	Inc.	**pH**	**Conductivity**	**TPC**	**COD**	**RS**
	(ºC)	(min)	(%)	(%)		mS cm^{-1}	(mg L^{-1})	(g O_2 L^{-1})	(mg L^{-1})
1	300	60	30	50	4.0	1.1	7940	39.6	14500
2	300	60	15	25	6.8	19.9	5215	41.3	4500
3	300	60	15	25	8.0	19.9	5758	39.8	2500
4	300	45	45	0	9.0	34.6	3075	40.0	2750
5	300	45	5	100	4.2	3.8	40547	766.0	54000
6	300	45	5	50	5.0	37.6	9631	68.4	6000
7	300	45	5	0	9.0	24.1	7108	67.0	3750
8	300	30	30	50	4.0	19.6	6586	40.0	5750
9	300	15	45	25	6.0	10.0	1256	23.6	2500
10	300	15	45	0	6.5	19.2	1104	40.0	1250
11	300	15	5	50	5.5	29.0	6318	43.0	2500
12	275	60	70	0	8.5	16.7	861	24.6	750
13	275	45	30	25	6.8	11.2	7944	40.1	n.d.
14	250	60	15	0	7.3	40.0	3223	40.4	750
15	250	60	5	50	5.0	13.3	7141	42.1	3000
16	250	45	30	50	4.5	10.2	2191	38.5	1000
17	250	45	30	25	6.0	19.5	2133	41.3	2500
18	250	45	30	25	5.5	19.2	2059	39.6	2200
19	250	45	15	25	5.8	19.1	4763	40.1	4000
20	250	45	15	25	6.2	18.2	2543	39.8	3000
21	250	30	45	25	6.8	19.9	411	15.5	n.d.
22	250	30	45	25	6.2	8.6	340	14.3	n.d.
23	250	30	15	50	5.1	18.6	2414	39.8	3500
24	250	30	5	25	6.1	11.2	7682	38.5	n.d.
25	250	15	30	50	6.9	4.0	263	24.7	n.d.
26	250	15	5	0	6.0	18.0	6887	40.5	2000
27	250	15	5	0	6.0	18.5	6783	40.4	1980
28	225	60	5	25	6.2	17.0	5653	39.6	1000
29	225	45	45	0	6.1	9.8	549	28.8	n.d.
30	225	15	15	25	6.5	8.5	225	21.8	500
31	200	60	45	25	9.0	5.5	73	6.7	2500
32	200	60	30	50	8.3	6.2	182	13.8	500
33	200	60	5	0	7.1	5.5	1474	37.9	n.d.
34	200	30	15	25	6.9	4.2	248	22.3	n.d.
35	200	30	15	25	7.5	8.2	187	18.5	n.d.
36	200	30	5	50	7.1	2.2	506	39.5	n.d.
37	200	15	70	0	6.2	1.1	22	0.8	n.d.
38	200	15	30	50	7.1	2.2	780	5.1	1000
39	200	15	30	25	7.0	2.1	35	5.1	1100

Note: T – temperature, M - moisture, Inc. – incorporation of lignocellulosic material, TPC – total phenolic compounds, COD - chemical oxygen demand; RS – reducing sugars; n.d. – not detected.

As expected, torrefaction trials with higher temperature and residence time led to aqueous phase samples with a greater load of organic components, resulting in higher COD (766 g O_2 L^{-1}), reducing sugars (54 g L^{-1}) and total phenolics (40.5 g L^{-1}). The increase in phenolic compounds concentration is largely due to the degradation of lignin and hemicellulose that occurs to a greater extent at higher temperatures, such as 275 and 300°C (Jagodzińska *et al.*, 2019). Moreover, at temperatures of 250°C and higher, cellulose suffers decomposition reactions leading to the formation of acids and alcohols, aldehydes, and ketones such as acetic acid, oxalic acid, acetone, acetaldehyde, propanoic acid (Atallah *et al.*, 2021).

The increase in moisture led to a significant decrease in the total phenolic content (from 40547 to 22 mg L^{-1}), COD (from 766 to 0.8 g L^{-1}), and reducing sugars (from 54000 mg L^{-1} to n.d.) potentially related with a dilution effect (Atallah *et al.*, 2021).

In contrast, decreasing the incorporation of lignocellulosic material resulted in a reduction in total phenolics and reducing sugars, but a slight increase in COD, which may be related to the fact that microalgae may yield lower amounts of reducing sugars and phenolic compounds than lignocellulosic biomass. The decomposition of microalgae generates other organic analytes such as simple sugars, organic acids, and amino acids, that contribute to the COD value (Atallah *et al.*, 2021).

Conductivity and pH were also higher in the situations of greater reaction severity, because of the dissolution of polar and acidic organic products. According to Cahyanti *et al.* (2020), biomass torrefaction condensates produced below 300°C, are potential inhibitors of microbiological processes due to their concentrations in phenolic compounds, furans, but also polycyclic aromatic hydrocarbons (PAHs), whose concentration increases with the severity of the torrefaction process. For this reason, the use of the aqueous phases of the torrefaction process has been studied in agriculture as an herbicide and pest repellent (Cahyanti *et al.*, 2020). This product is also known as pyroligneous acid, and it is known for its effectiveness as an antimicrobial agent, insecticide, antioxidant, and for having properties to promote seed germination and plant growth. It mostly consists of aromatic, aliphatic, and naphthenic hydrocarbons and other oxygenated compounds such as aldehydes, alcohols, furans, ketones, acids, phenols, and ethers (Grewal *et al.*, 2018).

9.3 Torrefaction process performance

The torrefaction process converted the feedstocks to solid, liquid, and gas products at yields that were determined gravimetrically and shown in Table 9.4. The condensates included two immiscible phases (aqueous phase and bio-oils) that were separated by decantation for individual accounting and characterization.

Biochar yield decreased from 90.5 to 23.0% with increasing temperature or residence time due to a higher thermal decomposition of the raw materials, leading to an increase of the aqueous phase yields (0.7 to 57.5%), bio-oil (0 to 35.8%), and gas products (1.0 to 24.5%). As expected, biochar yields also decreased with the increasing water content of the feed due to the lower availability of carbonaceous components (Chen *et al.*, 2015a). Initial feed moisture had a positive correlation with the yields of the aqueous phase and gas products, because it contributes to a net water volume, and may increase the extension of some hydrothermal oxidation reactions (Frolov *et al.*, 2021).

Generally, high biochar yields indicate that most of the feedstock carbon was recovered in the solid products while high yields of condensates and gases maybe are related to their availability in the feed but also a higher degree of decomposition reactions. The loss of feedstock mass as volatile components was positively correlated with a decrease in the O/C and H/C ratios of the biochars, confirming an upgrading effect by the thermochemical process of torrefaction.

Bio-oil yield showed a linear correlation with operating temperature because of the increase in the formation of organic by-products through pyrolytic processes involving covalent bond breaking and deoxygenation processes (Grewal *et al.*, 2018). The influence of residence time in the bio-oil yield was more evident for tests performed at more than 30 min, probably due to kinetic limitations of the thermal degradation processes (Azwar *et al.*, 2022). At comparable temperatures, bio-oil yields were lower for higher moisture contents (>5%), which may be related to the increase of aqueous phase volume and consequent dissolution of higher amounts of bio-oil components such as aromatic hydrocarbons (*e.g.* naphthalene) and carboxylic acids (*e.g.* acetic acid) (Sun *et al.*, 2020).

The incorporation of lignocellulosic material did not show a clear correlation with product yields because both feedstocks are susceptible to thermal degradation in the range of operating conditions used.

Table 9.4 – Product yields (biochar, condensates, and gas) from the 39 torrefaction tests.

Run	T	time	Moisture	Lc Incorporation	Biochar	Condensate		Gas
						Aqueous phase	Bio-oil	
	(°C)	(min)	(%)	(%)	(%)	(%)	(%)	(%)
1	300	60	30	50	36.5	46.0	6.0	11.5
2	300	60	15	25	46.0	33.5	11.5	9.0
3	300	60	15	25	46.0	34.0	10.0	10.0
4	300	45	45	0	31.0	47.0	8.0	14.0
5	300	45	5	100	52.5	0.7	35.8	11.0
6	300	45	5	50	55.0	23.5	13.0	8.5
7	300	45	5	0	58.0	16.0	19.0	7.0
8	300	30	30	50	51.0	39.0	5.0	5.0
9	300	15	45	25	42.0	51.5	2.0	4.5
10	300	15	45	0	41.5	44.0	10.0	4.5
11	300	15	5	50	70.0	10.0	2.5	17.5
12	275	60	70	0	23.0	57.5	1.0	18.5
13	275	45	30	25	40.5	45.5	8.0	6.0
14	250	60	15	0	59.5	29.5	10.0	1.0
15	250	60	5	50	76.5	13.5	3.0	7.0
16	250	45	30	50	53.5	30.0	1.5	15.0
17	250	45	30	25	52.0	39.2	4.8	4.0
18	250	45	30	25	51.0	38.1	4.9	6.0
19	250	45	15	25	66.5	18.0	2.0	13.5
20	250	45	15	25	66.5	18.0	2.5	13.0
21	250	30	45	25	45.0	50.0	2.5	2.5
22	250	30	45	25	46.0	49.0	3.0	2.0
23	250	30	15	50	73.5	20.0	0.5	6.0
24	250	30	5	25	80.5	14.9	1.1	3.5
25	250	15	30	50	70.0	18.0	0.0	12.0
26	250	15	5	0	85.5	5.0	0.0	9.5
27	250	15	5	0	86.0	6.0	0.0	8.0
28	225	60	5	25	85.0	9.5	2.0	3.5
29	225	45	45	0	47.5	33.5	0.0	19.0
30	225	15	15	25	78.5	20.5	0.0	1.0
31	200	60	45	25	47.0	28.5	0.0	24.5
32	200	60	30	50	70.5	24.0	0.0	5.5
33	200	60	5	0	90.0	9.0	0.0	1.0
34	200	30	15	25	79.5	15.5	0.0	5.0
35	200	30	15	25	78.0	14.5	0.0	7.5
36	200	30	5	50	90.5	4.5	0.0	5.0
37	200	15	70	0	61.0	32.0	0.0	7.0
38	200	15	30	50	69.5	25.0	0.0	5.5
39	200	15	30	25	69.5	25.0	0.0	5.5

Biochar yields higher than 80% were obtained for the tests performed with 5% of moisture, at 200 to 250°C and incorporation of at least 50% lignocellulosic material.

It is known that torrefaction increases the hydrophobicity of the torrefied material, as well as its energy density whilst reducing biomass tenacity (Cahyanti *et al.*, 2020). Torrefaction makes the product more energy-dense due to the elimination of oxygen in the pyrolytic degradation reactions. Zhang *et al.* (2019) studied the torrefaction of microalgae and assigned decarbonization, dehydrogenation, and deoxygenation levels of 20, 60, and 93%, respectively for the biochar product obtained at 300°C and 60 min, while at milder torrefaction conditions 200°C and 15 min, estimated values of 0.7, 10 and 17%, for the same thermal degradation processes.

To evaluate the performance of the torrefaction process, the energy yield, and the process energy efficiency (including process energy requirements) were determined and are detailed in Table 9.5.

The energy yield is the amount of energy contained in the raw material that is retained in the char, having reached its highest value (99.8%), at 200°C, 60 min, 5% moisture, and 0% lignocellulosic material incorporation. According to Zhang *et al.* (2018), the biochars produced by torrefaction of microalgae residue (1.8% moisture) had energy yields that ranged from 77% (300°C and 60 min) to 93% (200°C and 15 min) in a nitrogen environment. In the present study, comparable energy yields were obtained for feedstocks with 5% or 15% of initial moisture and temperatures up to 250°C. At higher temperatures, the biochar yields obtained were lower than 70%, probably because torrefaction was performed in oxygen-deficient conditions but not under a nitrogen flow, which limits autothermal decomposition reactions.

The determination of the heat requirements is challenging because there are considerable differences in the magnitude of thermal losses, in the specific heat of raw materials, and the heat of reaction, depending on the configurations and dimensions of the reactors (Sermyagina *et al.*, 2015). Q_1 (energy required for feedstock heating) increases with increasing process temperature. In the present study, there was also an increase in Q_1 when the feedstock was mostly lignocellulosic biomass, decreasing when algal biomass was incorporated because the *Cp* of lignocellulosic biomass is higher (*Cp* lignocellulosic = 1.7 KJ Kg^{-1} K^{-1} (Delrue, 2018)) than that of algal biomass (*Cp* microalgae = 1.58 KJ Kg^{-1} K^{-1} (Wibawa *et al.*, 2018)).

Table 9.5 – Energy yield, energy requirements, and process energy efficiency for the torrefaction of microalgae and lignocellulosic biomass.

Run	Process conditions				Energy yield (%)	Energy Requirements						PEE (%)
	T (°C)	time (min)	M (%)	Inc. (%)		Q_1 (MJ)	Q_2 (MJ)	Q_3 (MJ)	Q_4 (MJ)	Q_5 (MJ)	Q_{input} (MJ)	
1	300	60	30	50	40.8	0.31	0.10	0.75	0.80	0.89	2.85	40.4
2	300	60	15	25	53.0	0.38	0.06	0.41	0.55	0.63	2.02	52.6
3	300	60	15	25	52.9	0.38	0.06	0.41	0.55	0.63	2.02	52.6
4	300	45	45	0	36.0	0.24	0.15	1.08	0.90	1.01	3.37	35.6
5	300	45	5	100	59.4	0.47	0.00	0.00	0.65	0.47	1.59	59.1
6	300	45	5	50	59.7	0.45	0.01	0.06	0.65	0.50	1.66	59.4
7	300	45	5	0	69.2	0.43	0.02	0.11	0.65	0.52	1.73	68.8
8	300	30	30	50	56.6	0.35	0.07	0.54	1.00	0.79	2.75	56.1
9	300	15	45	25	43.8	0.23	0.16	1.14	1.20	0.95	3.68	43.3
10	300	15	45	0	45.0	0.24	0.15	1.08	1.20	0.93	3.60	44.5
11	300	15	5	50	74.9	0.45	0.01	0.06	0.85	0.48	1.84	74.5
12	275	60	70	0	26.3	0.14	0.21	1.51	0.80	1.13	3.78	26.0
13	275	45	30	25	46.1	0.27	0.11	0.78	0.90	0.83	2.89	45.7
14	250	60	15	0	65.6	0.28	0.07	0.54	0.60	0.60	2.10	65.2
15	250	60	5	50	77.2	0.37	0.01	0.06	0.60	0.41	1.45	76.9
16	250	45	30	50	47.4	0.25	0.10	0.75	1.10	0.83	3.04	47.0
17	250	45	30	25	53.1	0.25	0.11	0.78	1.10	0.84	3.08	52.6
18	250	45	30	25	51.8	0.25	0.11	0.78	1.10	0.84	3.08	51.3
19	250	45	15	25	68.3	0.31	0.06	0.41	0.70	0.55	2.02	67.8
20	250	45	15	25	67.6	0.31	0.06	0.41	0.70	0.55	2.02	67.2
21	250	30	45	25	45.0	0.19	0.16	1.14	1.20	0.94	3.62	44.6
22	250	30	45	25	45.3	0.19	0.16	1.14	1.20	0.94	3.62	44.8
23	250	30	15	50	70.8	0.33	0.04	0.27	0.80	0.50	1.95	70.4
24	250	30	5	25	86.6	0.36	0.01	0.08	0.80	0.44	1.70	86.2
25	250	15	30	50	69.0	0.29	0.07	0.54	1.35	0.68	2.93	68.4
26	250	15	5	0	92.4	0.36	0.02	0.11	0.90	0.42	1.80	91.9
27	250	15	5	0	94.6	0.36	0.02	0.11	0.90	0.42	1.80	**94.0**
28	225	60	5	25	89.5	0.32	0.01	0.08	0.65	0.40	1.47	89.1
29	225	45	45	0	47.6	0.17	0.15	1.08	1.30	0.95	3.65	47.1
30	225	15	15	25	68.5	0.27	0.06	0.41	0.95	0.46	2.15	68.0
31	200	60	45	25	46.3	0.15	0.16	1.14	1.20	0.92	3.56	45.8
32	200	60	30	50	63.8	0.22	0.07	0.54	1.20	0.71	2.75	63.3
33	200	60	5	0	**99.8**	0.28	0.02	0.11	0.70	0.39	1.49	**99.3**
34	200	30	15	25	83.1	0.24	0.06	0.41	0.90	0.48	2.08	82.5
35	200	30	15	25	78.8	0.24	0.06	0.41	0.90	0.48	2.08	78.3
36	200	30	5	50	93.1	0.29	0.01	0.06	0.90	0.38	1.63	92.7
37	200	15	70	0	58.1	0.10	0.21	1.51	1.50	0.83	4.14	57.3
38	200	15	30	50	68.0	0.20	0.10	0.75	1.50	0.64	3.19	67.4
39	200	15	30	25	73.8	0.19	0.11	0.78	1.50	0.65	3.23	73.1

Q_2 and Q_3, which are energy requirements for heating and evaporation of the water in the system (Nobre *et al.*, 2019), increased with moisture. Q_4 is the heat of reaction that decreases with the increasing temperature and residence time because the magnitude of exothermic reactions is increased by the process severity (Atallah *et al.*, 2021). Q_5 represents thermal losses, and it increases with the process temperature and residence time. The thermal losses were evaluated as Nobre *et al.* (2019). The PEE and energy yield were higher in mild torrefaction conditions because, under these conditions, less mass loss occurred. However, the HHV of these biochars was lower because of their high O/C and H/C ratios, and therefore their fuel quality was negatively affected.

The relation between the torrefaction process energetic efficiency (PEE, %) and the high heating value of the produced biochars (HHV, MJ Kg^{-1}) is represented in Figure 9.3.

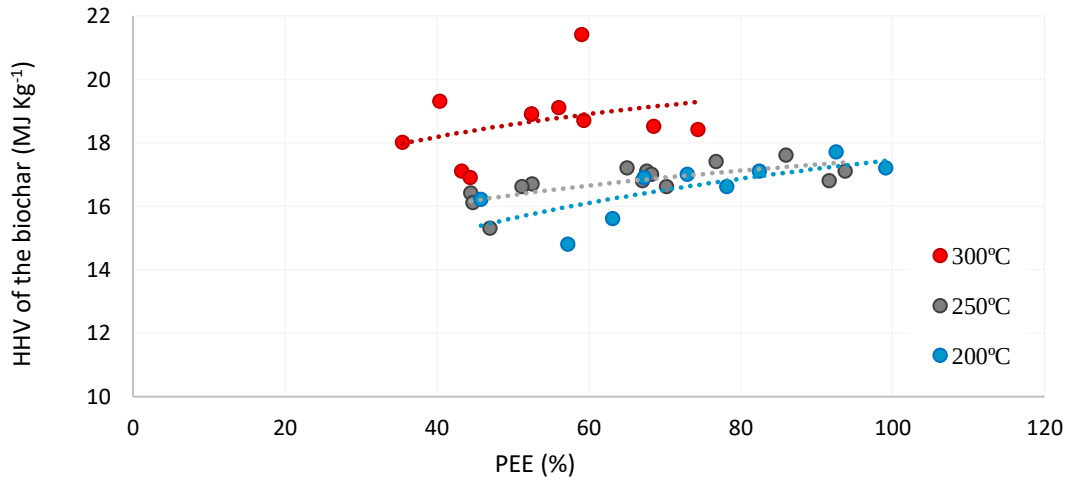

Figure 9.3 – High heating value of biochar (MJ Kg^{-1}) as a function of the process energy efficiency for experiments performed at different temperatures, residence times, initial moisture, and Lc incorporation rate.

For the energy valorization pathway to be viable it is necessary to choose conditions in which the process energy efficiency is higher than 50%. That occurs when the energy recovered in the products is higher than the sum of the process energy with the heat of combustion of the feedstock. Conversely, to produce biochar with a good fuel quality, process conditions that lead to biochars with the highest HHV should be selected.

As seen in Figure 9.3, the biochars produced at 200°C and 250°C presented HHVs lower than 18 MJ Kg^{-1}, regardless of the corresponding PEE value, while at 300°C, it was possible to obtain biochars with HHVs higher than 18 MJ Kg^{-1}, for most conditions tested and particularly for PEE values higher than 60%.

9.4 Response Surface Methodology (RSM) Analysis

To the quadratic model suggested by the RSM the non-significant terms, with p> 0.05 (except those that support the model - non-significant linear terms: A, B, C, D) were removed. This operation resulted in a reduced equation for each response, which describes the adjustment to the experimental data. The equations for product yields with significant terms are found in Appendix 2 and for biochar characterization in Appendix 3. The equations for process performance and HHV are presented in Table 9.6, except for Q_{input} and Q_{output} (Appendix 4).

Table 9.6 – Equations generated by RSM software, with R^2, Adjusted R^2, Predicted R^2, and Adequate Precision for HHV and energy and mass balances.

Parameter	Equation *	F-value	R^2	Adjusted R^2	Predicted R^2	Adequate Precision
HHV	$31.76 - 0.12\ T - 0.11\ t - 0.12\ M + 0.06\ I + 4.20\times10^{-4}\ (T \times t) + 3.34\times10^{-4}\ (T \times M) - 2.83\times10^{-4}\ (T \times I) + 5.28\times10^{-4}\ (t \times M) + 2.77\times10^{-4}\ (T^2)$	31.42	0.916	0.887	0.794	29.224
Mass yield	$47.22 + 0.91\ T - 0.73\ t - 2.07\ M - 0.52\ I + 6.03\times10^{-3}\ (T \times M) - 3.42\times10^{-3}\ (T \times I) - 9.73\times10^{-3}\ (t \times M) + 5.74\times10^{-3}\ (t \times I) - 2.06\times10^{-3}\ (T^2) + 3.31\times10^{-3}\ (t^2) + 8.25\times10^{-3}\ (I^2)$	778.33	0.998	0.997	0.992	95.136
Energy yield	$79.50 + 0.53\ T - 1.91\ t - 3.15\ M + 0.74\ I + 1.79\times10^{-3}\ (T \times t) + 5.78\times10^{-3}\ (T \times M) - 2.66\times10^{-3}\ (T \times I) + 3.83\times10^{-3}\ (t \times I) - 1.50\times10^{-3}\ (T^2) + 0.01\ (t^2) + 0.02\ (M^2) - 9.26\times10^{-3}\ (I^2)$	135.92	0.988	0.981	0.955	39.672
PEE	$80.40 + 0.51\ T - 1.49\ t - 2.58\ M + 0.29\ I + 4.28\times10^{-3}\ (T \times M) - 1.50\times10^{-3}\ (T \times I) + 5.59\times10^{-3}\ (t \times I) - 1.40\times10^{-3}\ (T^2) + 0.01\ (t^2) + 0.01\ (M^2)$	180.82	0.988	0.983	0.963	45.512

Note: p-value model is always 0.01%, and it means that there is only a 0.01% chance that an F-value this large could occur due to noise. The Lak of Fit is not significant (< 4).

* Where T, t, M, and I means temperature, residence time, moisture, and incorporation rate of lignocellulosic biomass, respectively.

For all the analysed responses, R^2, adjusted R^2, and predicted R^2 are high and the difference between adjusted R^2 and predicted R^2 is less than 0.2, which suggests a good fit

of the model to the obtained data. R^2 (coefficient of determination) represents the ratio of the total changes in the predicted response. The adequate precision measures the signal-to-noise ratio and has values well above 4, which is the minimum desirable value.

As it can be seen in Table 9.6, the description of the Energy Yield and PEE values include quadratic terms of temperature, time, and moisture, positive coefficients in the temperature and Lc incorporation terms, and negative coefficients in the time and moisture terms.

To study how the different variables interact in the process and their effects on the response (Energy Yield and HHV), three-dimensional (3D) response surface plots were made for any two independent process variables, keeping the others at their average level. Figures 9.4 and 9.5 present the 3D surface plots with the interactions between the energy yield and HHV and their respective output responses, respectively.

Energy yield was higher for lower temperatures and residence times (Figure 9.4), especially when associated with low values of feedstock moisture and Lc incorporation rate; this behaviour reflects the influence of the biochar mass yield on the energy yield. The negative correlation of the energy yield with initial moisture and Lc incorporation rate is corroborated in Figures 9.4-E and 9.4-F, for different torrefaction temperatures.

When looking at the influence of the different variables and the interactions between the variable pairs it is possible to conclude that the HHV of the biochar (Figure 9.5) is positively correlated with the torrefaction temperature and negatively correlated with the feedstock moisture, while time and Lc incorporation rate have less influence in this parameter.

Increasing temperature has the effect of increasing the thermochemical decomposition and of removing oxygen and nitrogen, favouring the formation of aromatic carbonaceous structures, but reducing biochar yield. In contrast, the increase in residence time favours the carbon content biochar yield because it benefits reactions of recombination and adsorption to the biochar itself. The water content always removes energy from the reaction, but it can have a hydrothermal effect as it is an oxidizing agent. Comparing Figure 9.4-A with 9.4-B, the negative effect of introducing more water is visible.

HHV increases with the incorporation of lignocellulosic biomass for low temperatures but follows an opposite trend at high temperatures. These different

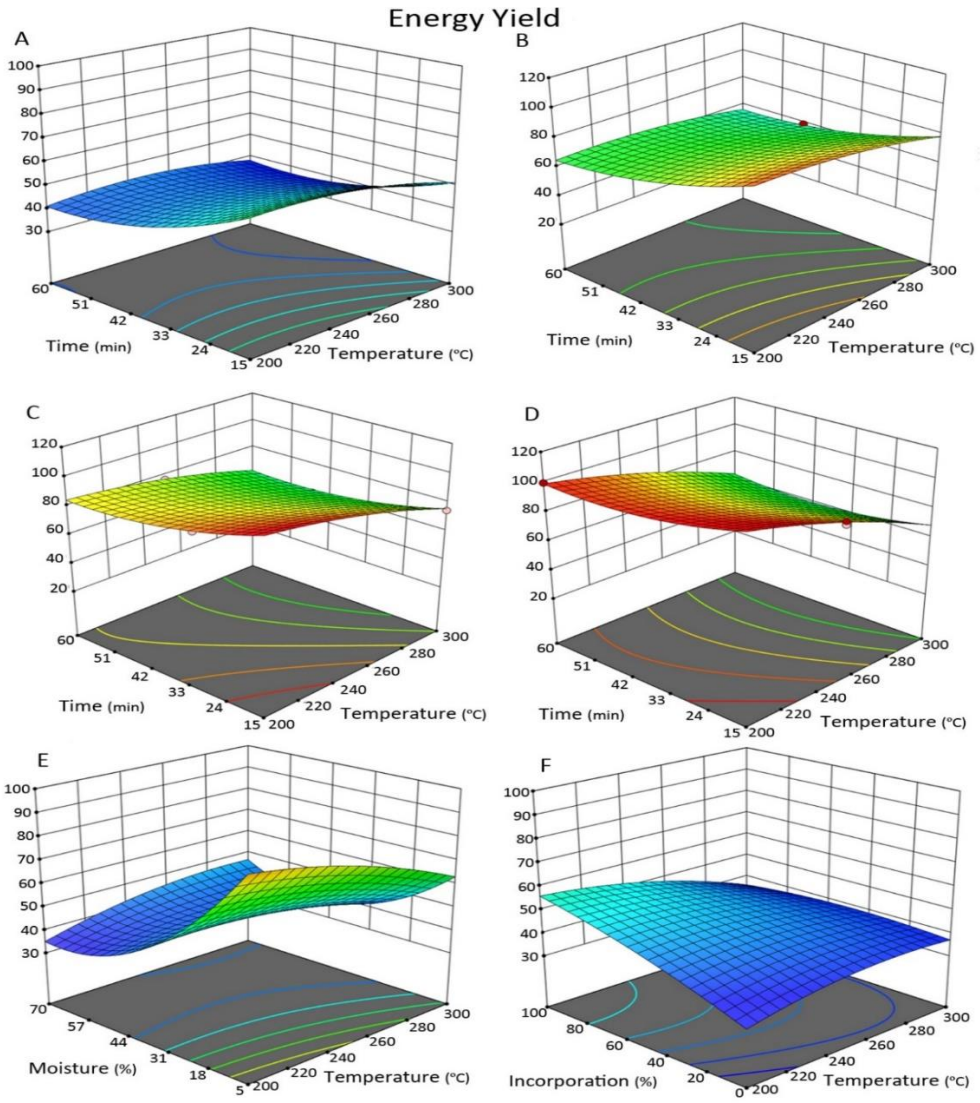

Figure 9.4 – Relation between Energy Yield and the interaction terms by 3D plot: A - interaction between T x t (M = 37.5% and I = 50%), B interaction between T x t (M = 5% and I = 100%); C interaction between T x t (M = 5% and I = 50%); D interaction between T x t (M = 5% and I = 0%); E interaction between T x M (t = 30 min and I = 50%) and F interaction between T x I (M = 37.5% and t = 30 min), T – *torrefaction temperature*, t – *time*, M – *feedstock moisture*, I – *Lc incorporation rate*.

behaviours may result from a higher degree of aromatization of the Lc biomass at temperatures above the cellulose and hemicellulose thermal decomposition threshold. The increase in water content decreases the HHV of the obtained biochar, but this decrease is only evident for the most severe torrefaction conditions (Figure 9.5 - A and B), in the case of low temperatures, regardless of residence time, the biochars HHV is not affected by the addition of water.

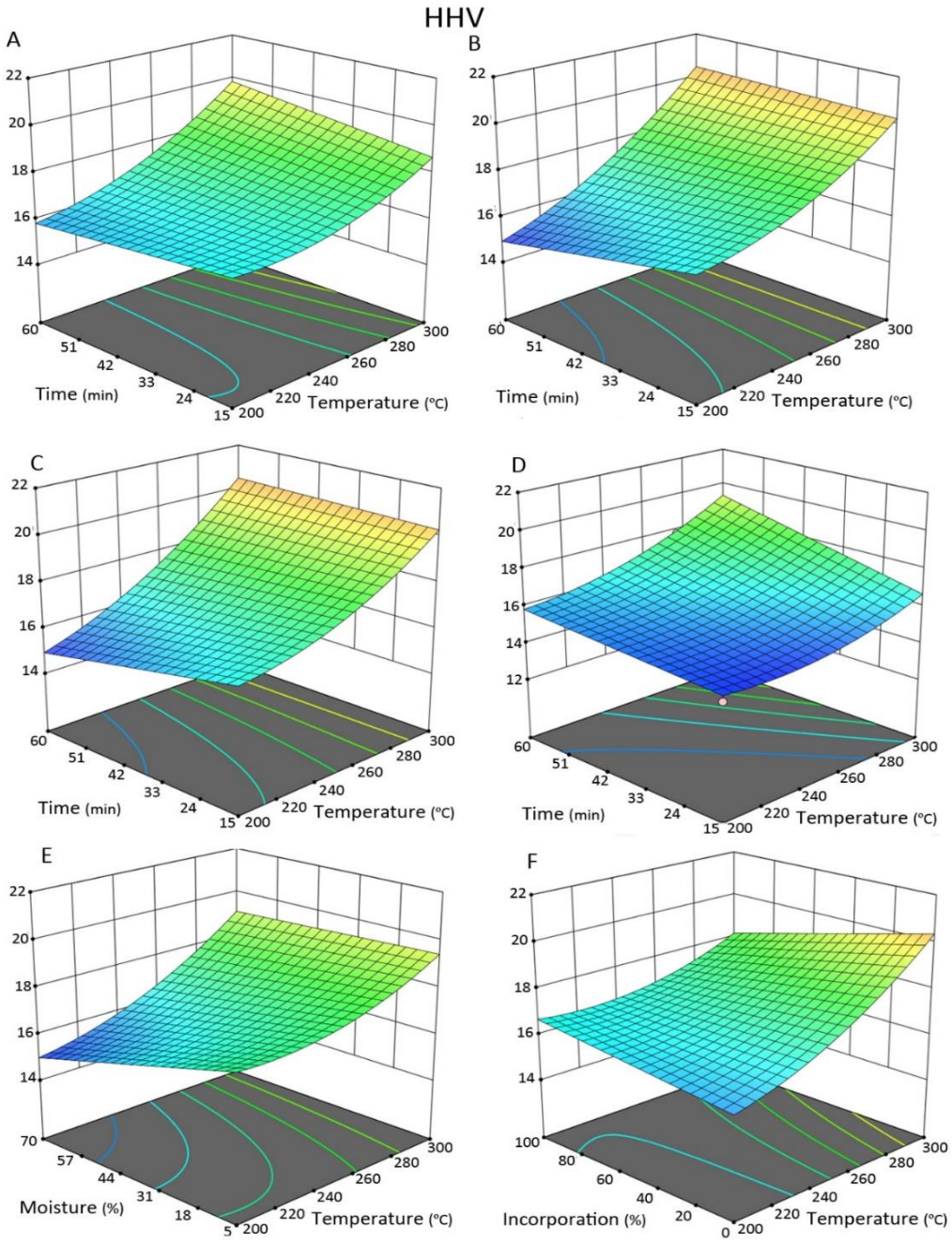

Figure 9.5 – Relation between HHV and the interaction terms by 3D plot: A - interaction between T x t (M = 37.5% and I = 50%), B interaction between T x t (M = 5% and I = 0%); C interaction between T x t (M = 37.5% and I = 100%); D interaction between T x t (M = 70% and I = 0%); E interaction between T x M (t = 30 min and I = 50%) and F interaction between T x I (M = 37.5% and t = 30 min), T – *torrefaction temperature*, t – *time*, M – *feedstock moisture*, I – *Lc incorporation rate*.

9.5 Biochars from wastewater microalgae biomass

To evaluate the effect of torrefaction on algal biomass produced in wastewaters effluents (aquaculture wastewater and landfill leachate), biomass samples from *Chlorella vulgaris* and *Tetradesmus obliquus* grown in these effluents were used as torrefaction feedstocks with and without the addition of lignocellulosic material. The torrefaction tests with algal biomass obtained from wastewaters were all conducted at 250ºC, for 60 minutes, using 10 grams of dry biomass (5% moisture).

For these experiments, the product yields were determined, and the biochar samples were characterised. Figure 9.6 shows the product yields of the torrefaction tests and Table 9.7 presents the proximate analysis, high heating values (HHV), and adsorption capacity of the produced biochar samples.

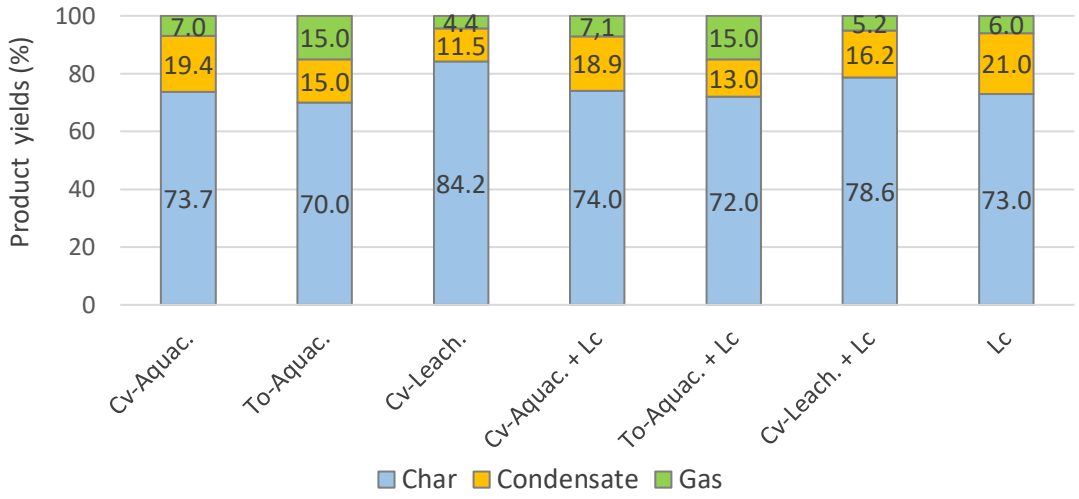

Figure 9.6 – Yields of the torrefaction process, using microalgae biomass produced in wastewater. (Cv-Aquac.: *C. vulgaris* grown in aquaculture effluent; To-Aquac.: *T. obliquus* grown in aquaculture effluent; Cv-Leach.: *C. vulgaris* grown in landfill leachate; Lc: lignocellulosic material).

Biochar yields varied in the range of 70.0% to 84.2% and were comparable for the torrefaction of *C. vulgaris* (Cv) and *T. obliquus* (To) biomass grown in wastewaters effluents, mixtures of those biomasses with 50% Lc or the lignocellulosic material alone (100% Lc). When the feed included Cv biomass produced in landfill leachate, the biochar yield was higher (79% and 84%) than for the tests using Cv or To grown in aquaculture effluents (70 and 74%) which may reflect the accumulation of non-volatile contaminants of the landfill leachate in the microalgae biomass.

On the other hand, condensate yield and gas products yields were higher for the tests including Cv biomass grown in aquaculture effluents or 100% Lc, indicating a higher abundance of organic volatile components in these feedstocks.

These observations agree with the high ash contents detected in Cv-leachate biochar, an indication that mineral components from the landfill leachate were bioaccumulated in the algal biomass. The high concentration of minerals associated with a low content of fixed carbon results in an HHV of only 5.6 MJ Kg^{-1} for this biochar.

Table 9.7 – Characterization of produced biochar by the torrefaction process.

	Cv-Aquac.	To-Aquac.	Cv-Leachate	Cv-Aquac. + Lc	To-Aquac. + Lc	Cv-Leac. + Lc	Lc
Ash content *(wt.%, db)*	33.5 ± 0.8	38.4 ± 0.2	65.5 ± 0.3	17.3 ± 0.8	24.6 ± 0.9	34.6 ± 0.4	3.8 ± 0.3
Volatile Matter *(wt.%, db)*	56.8 ± 1.1	54.7 ± 0.3	31.0 ± 0.5	66.1 ± 0.6	67.8 ± 1.5	56.1 ± 0.6	81.2 ± 1.1
Fixed Carbon *(wt.%, db)*	9.6 ± 1.4	7.0 ± 0.2	3.6 ± 0.1	16.6 ± 0.3	7.9 ± 0.6	9.3 ± 0.4	15.1 ± 0.9
HHV (MJ Kg^{-1}) (*db*)	12.0	10.7	5.6	16.0	13.1	11.8	18.0
O/C ratio (*daf*)	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
H/C ratio (*daf*)	1.3 ± 0.0	1.3 ± 0.0	1.7 ± 0.0	1.3 ± 0.0	1.15 ± 0.0	1.37 ± 0.0	1.3 ± 0.0
Adsorption 3 seconds	35.7 ± 0.7	39.2 ± 0.3	45.7 ± 0.9	32.8 ± 1.2	34.7 ± 0.4	30.5 ± 1.8	16.4 ± 1.7
capacity (%) 48 h	56.5 ± 0.5	62.9 ± 0.9	58.1 ± 2.1	52.3 ± 1.9	58.3 ± 0.8	46.5 ± 1.5	36.8 ± 0.8

Note: (Cv-Aquac.: 10 g of *Chlorella vulgaris* from aquaculture effluents; To- Aquac.: 10 g of *Tetradesmus obliquus* from aquaculture effluents; Cv-Leachate: 10 g of *C. vulgaris* grown in landfill leachate effluent; Cv-Aquac. + Lc: 5 g of *C. vulgaris* from aquaculture + 5 g of Lc; To-Aquac. + Lc: 5 g of *T. obliquus* from aquaculture + 5 g of Lc; Cv-Leach. + Lc: 5 g of *C. vulgaris* from leachate + 5 g of Lc; Lc: 10 g of lignocellulosic material).

The Cv aquaculture biochar had lower ash content and higher concentrations of volatile matter and fixed carbon mixture, which resulted in an HHV of 12.0 MJ Kg^{-1}, significantly higher than that of Cv-leachate biochar. These results indicate that not only the nature of the microalgae but also the composition of the effluent may have a strong influence on the composition of the algal biomass and consequently the yield and composition of the correspondent biochar. In both cases, the incorporation of 50% Lc material had a positive effect on the fuel quality of the biochars by reducing ash content and increasing volatile matter, fixed carbon, and HHV.

Microalgae produced in landfill leachate usually have a high amount of ash in their biomass (Viegas et al., 2021d). This occurs because these organisms tend to assimilate minerals from the environment inside their cells and landfill leachate has a high mineral

concentration (Hernández-García *et al.*, 2019). Also, microalgae produced in saltwater (or aquaculture effluents) tend to have a high ash content, as found by Fakayode *et al.* (2020).

The O/C and H/C ratios of raw materials (0.47 and 1.21 for algae and 1.71 and 1.51 for lignocellulosic biomass, respectively) were reduced with the torrefaction process, giving rise to biochar with characteristics close to lignite and better than peat. The torrefaction process is considered an upgrading process, because it increases the hydrophobicity and the energy density of the torrefied material, and reduces the need for biomass grinding energy (Cahyanti *et al.*, 2020). The production of biochar from microalgae has the advantage of greater stability and density of the final material, which also translates into lower transport costs (Fakayode *et al.*, 2020). Furthermore, it results in a material that can be easily used in agriculture as a soil amendment, releasing nutrients (N and P) slowly into the soil due to the presence of functional groups with oxygen on the surface of the biochar.

These biochars also reveal potential to be further explored as bioadsorbents reaching an adsorption capacity value for MB of 58.3%, without any activation process. Regarding the adsorption capacity of the biochars, there was a significant increase when compared to the biochars obtained from commercial algal biomass (33.5% to 52.3, 58.3 and 46.5% for Cv-Aquac. + Lc, To-Aquac. + Lc and Cv-Leach. + Lc, respectively), for similar torrefaction conditions. It was also observed that the increase in contact time from 3 seconds to 48 hours led to an increase in the adsorption capacity of up to 63% (To-Aquac. + Lc). This issue will be developed in section 10.2. Some experiments have revealed that torrefied algal biomass has a quite different structure than that from the original microalgae, becoming irregular and compact, which tends to increase its adsorption capacity (Wang *et al.*, 2013). In addition, the biomass that originated the biochars had already treated landfill leachate or an aquaculture effluent, thus this process can be integrated into a circular economy concept.

9.6 Final considerations

Torrefaction can be seen as a process with low energy requirements resulting in biochar, that is more stable and denser when compared to the original feedstock. Biochars have several applications, including energy valorization, soil amendment, and bioremediation of contaminated effluents.

In this work, RSM has proven to be a useful tool to optimize process parameters observed in the experimental data. A quadratic model has been recommended as a good model for predicting the production of biochar with high calorific value.

Moreover, the conditions that lead to greater biochar production with increased HHV were 250°C, 60 minutes of residence time, 5% moisture, and 50% lignocellulosic biomass as feedstock. However, the incorporation of lignocellulosic biomass had more influence on HHV and fixed carbon content rather than in biochar yield. Mixing algal biomass with lignocellulosic biomass was shown to be beneficial by improving the quality of the obtained biochars. Under optimum conditions, it is possible to obtain a biochar yield of 76.5% with an HHV of 17.4 MJ Kg^{-1}.

Results showed that it is feasible to lower the torrefaction temperature to 250°C without significantly affecting biochar yield and quality, provided that the residence time is maintained at 60 minutes. Alternatively, it is also possible to reduce residence time to 30 minutes, maintaining the temperature at 300°C, without significantly affecting biochar yields.

Adding water to the feedstock considerably reduces the efficiency of the torrefaction process. Therefore, for biomass with higher moisture levels, a greater amount of lignocellulosic biomass should be incorporated to compensate for this issue.

The evaluation of the torrefaction process performance showed that using moderate torrefaction conditions (temperature and residence time) leads to a better energy performance of the process with higher mass yields. However, biochars obtained under these conditions have lower HHV because of the high O/C and H/C ratios, so their fuel qualities were not enhanced.

Biochars produced in this experiment presented good adsorption capacities towards MB. Furthermore, the use of microalgae previously used in effluent treatment as feedstock in biochar production, also presents good adsorption capacities, compared to biochars obtained from commercial algal biomass. However, these algal biomass samples usually present high levels of salts (situation verified for microalgae produced in aquaculture effluent and landfill leachate), and as such, the obtained biochars presented high ash contents resulting in a decrease in their HHV. Even with lower HHV than the model biochar samples, these biochars can be used as low-cost adsorbents and further studies should be conducted to validate their use in soil amendment applications.

Chapter 10

Microalgae biochar applications

Energy and material applications are two additional ways of using algal biomass. The thermochemical conversion process such as torrefaction can be used in order to produce biochars for coal fuel and bio-adsorbent from algal biomass (Gan *et al.*, 2018), to be used in soil amendment applications (Chu *et al.*, 2020), or as biostimulant (Ennis *et al.*, 2017).

10.1 Biochar as fertilizer

The biochars obtained from *Tetradesmus obliquus* (To) biomass grown in aquaculture effluent and from lignocellulosic material (Lc) alone or mixed with To were assessed for their beneficial effect as fertilizer, compared with distilled water. The chosen seeds were the same (wheat and watercress) as used previously for the study of the bio-stimulating effect of algal biomass and the study of the effect of the precipitate as a fertilizer.

The germination index (GI) of wheat and watercress seeds is shown in Figure 10.1 for the three biochars (from To, To+Lc, and Lc) with two distinct concentrations (0.2 and 0.5 g L^{-1}) and for the control.

All the tested biochars had a positive effect on seed germination, except To+Lc (0.5 g L^{-1}) for wheat seeds, probably a toxicity effect due to the high concentration. The results were almost all better for the lower concentration (0.2 g L^{-1}).

The highest GI for wheat was achieved for the biochar To+Lc - 0.2 g L^{-1} with an increase of 40.4 ± 0.2%, similar to the biochar To - 0.2 g L^{-1}. The highest GI for watercress was also reached for the To+Lc - 0.2 g L^{-1} with an increase of 60.3 ± 1.7%, followed by the biochar To - 0.5 g L^{-1} with more 44% when compared with the control (distilled water).

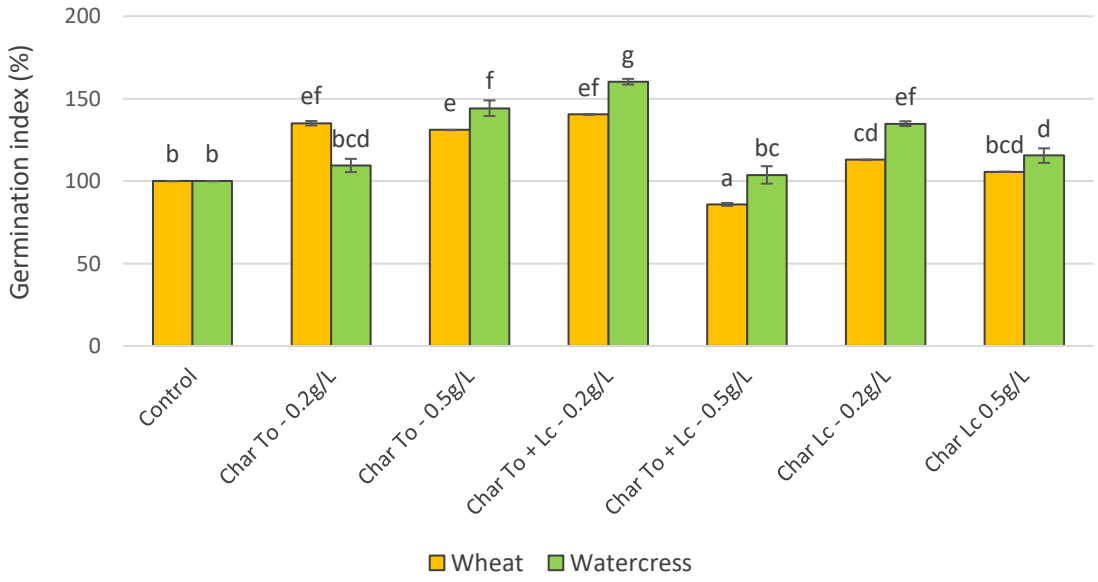

Figure 10.1 – Germination Index (%) for wheat and watercress seeds (mean ± SD, n = 3) for control and different biochar biomasses (To - *T. obliquus* grown in aquaculture effluent; To + Lc - *T. obliquus* plus lignocellulosic material; Lc: lignocellulosic material) and two concentrations (0.2 and 0.5 g L^{-1}). The values with different index letters show significant differences with $p < 0.05$.

The results show a positive effect of the use of biochars in the germination of the tested seeds. However, this effect is not as extraordinary as with the direct use of algal biomass without thermochemical treatment. This result was expected since the torrefaction process volatilizes some compounds, leaving others in the aqueous phase, thus the obtained biochar is poorer than the algal biomass that gave rise to it. In contrast, biochar is a more stable material, and much less likely to be contaminated microbiologically. The biochars tested had some important macro and micronutrients from the point of view of plant nutrition, namely calcium, magnesium, sodium, potassium, and iron as can be seen in Table 10.1.

Some studies have shown the interest in applying microalgae biochars as fertilizers since they tend to have a high level of nitrogen in their constitution. Furthermore, algal biomass produces biochars with a greater amount of minerals important for plant development, such as sodium, magnesium, potassium, calcium, and iron compared to biochar produced from lignocellulosic biomass (Yu *et al.*, 2017b). Conversely, *Spirulina* sp. has high carbon content in char compared to other algae which can be highly beneficial for carbon sequestration (Chaiwong *et al.*, 2013).

Table 10.1 – Chemical characteristics of biochars from microalgae biomass used in fertilization germination tests, presented in mg g^{-1}. (To - *T. obliquus* grown in aquaculture effluent; To + Lc - *T. obliquus* plus lignocellulosic material; Lc: lignocellulosic material).

	Al	**B**	**Ba**	**Ca**	**Cr**	**Cu**	**Fe**	**K**	**Li**	**Mg**	**Mn**
To	2.56	0.08	0.27	54.33	0.01	0.45	3.31	7.57	0.01	14.14	0.09
To + Lc	1.63	0.06	0.03	42.03	0.01	0.33	2.70	5.16	0.01	13.15	0.05
Lc	1.92	0.02	0.12	23.87	0.00	0.05	0.84	1.07	0.04	1.64	0.06
	Na	**Ni**	**P**	**Pb**	**Se**	**Si**	**Sr**	**Ti**	**Zn**	**Zr**	
To	5.18	0.02	0.57	0.00	0.01	0.63	0.21	0.03	0.83	0.07	
To + Lc	3.10	0.00	0.00	0.02	0.00	1.56	0.20	0.03	0.52	0.01	
Lc	0.25	0.00	0.00	0.03	0.00	2.11	0.09	0.05	0.20	0.00	

Note: The presence of Ag, As, Bi, Cd, Co, Hg, Mo, Sb, Sn, Tl, W, and V was not detected in the biochars.

10.2 Biochar as adsorbent

The biochars obtained from algal biomass grown in aquaculture effluent and in landfill leachate were tested for their adsorption capacity, compared with commercial chars. The removal efficiency of an activated commercial char is close to 100% for the adsorption with the cationic dye methylene blue. However, several negative factors are present in these chars production, namely the char activation, which is expensive and generates contaminated effluents, and the adsorption process is irreversible (Lashaki *et al.*, 2016).

Regarding the adsorption capacity of the biochars obtained (Table 9.7), it was found that this adsorption improved with the increased time from 3 seconds to 48 hours, in an almost proportional way for all biochars. The biochar with the highest adsorption capacity was To-Aquac., followed by To-Aquac.+Lc and Cv-Leachate, and the one with the lowest absorption capacity was from lignocellulosic material. These results were positive, mainly because the biochars obtained had not undergone any pre-treatment and its attainment does not require high energy expenses. In addition, the biomass that originated the biochars had already treated a landfill leachate or an aquaculture effluent. The surface of algal biomass biochar can be analysed for pore volume and porosity. Some experiments had revealed that torrefied algal biomass had a quite different structure from the original, becoming irregular and compact, which tends to increase its adsorption capacity. In contrast, torrefied lignocellulosic biomass tends to maintain its structure (Wang *et al.*, 2013). The measurement of microalgae biochar's ability to adsorb cationic nutrients can be done by analysing their cation exchange capacity. Thus, biochar with a high cation exchange capacity retains the cations avoiding their leaching in the soil. However, not all cation

nutrients were retained in the biochar and some preserved the high exchange capacity such as Ca, K, Mg, and Na (Roberts *et al.*, 2015). The cation exchange capacity is correlated with the ash content, as a result, it was proposed that alkaline and alkaline earth metals present in microalgal biomass favour the formation in the biochar of functional surface groups with oxygen (Yu *et al.*, 2017a). The increase in oxygen groups on the biochar surface is related to the absorption capacity of the methylene blue dye, such as pore channels and carboxyl groups generated in-situ by ion exchange and hydrogen bonding. The electrostatic interaction that occurs in the dye removal process between biochar and dye molecules is also crucial (Liu *et al.*, 2019).

10.3 Final considerations

The application of biochars in seed germination had shown to have a positive role, leading to increases in the germination of 60% for watercress and 44% for wheat. However, it is important to note that the direct use of microalgae as a biostimulant leads to an even more promising effect.

The results of using biochars as adsorbents were quite positive. If the purpose of using biochar is the adsorption of colouring compounds, it is possible to optimize the production of these biochars for lower temperatures with an increase in the energy efficiency of the process (PEE).

The use of algal biomass from effluents remediation with high salt load narrow its use in biochar production for energy purposes, as the ash content of the biochar increases considerably and, consequently the HHV decreases.

Chapter 11

Process integration and production potential

To make microalgae production for animal feed feasible and appealing, the economic evaluation of the production process must be profitable compared to feed alternatives.

Currently, about 56,500 tonnes of microalgae are produced per year worldwide (2019 values), mainly *Spirulina* sp. in raceway ponds (Cai *et al.*, 2021). The main disadvantages of these open systems are the loss of large amounts of water by evaporation and the contamination with biomass from different sources, which strongly limits the use of the biomass, however, they have lower operating costs. Closed systems (photobioreactors) reduce the loss of water, allow to operate with higher cell concentrations, and significantly reduce the risk of contamination (Sivakaminathan *et al.*, 2020). The choice of the production system will therefore depend on the purpose of the biomass that is intended to be produced.

One of the most significant aspects of this assessment is to consider an integrated system. When the objective lies only in the production of biomass, the cost of this biomass becomes higher and is only justified for high-value products. On the other hand, if there are several resources and products involved, in a concept of biorefinery and circular economy it is more likely that the system will become profitable.

If the economic and environmental costs of treating agro-industrial effluents are considered, the use of microalgae can be rewarding because, in addition to obtaining the treated effluent, it will be obtained an added value of algal biomass.

It is important to reflect on the fact that during the production of microalgae and respective effluents' treatment, significant amounts of atmospheric CO_2 are also absorbed to generate algal biomass.

11.1 Aquaculture effluent

The microalgae biomass produced from aquaculture effluent could be used as supplementation for feeding aquaculture species because microalgae had a good quantity of protein and a certain carotenoid content which contributes to the attractive colours of aquaculture animals (Zhang *et al.*, 2019).

According to the assessments developed in the laboratory and the need of the company Pesca Verde, Lda. to treat about 300 L of aquaculture transportation effluent every week, it would be possible to use the existing tanks to receive and remedy the weekly effluent. The tanks already have a water oxygenation system which also allows water agitation. It is possible to receive the 300 L weekly effluent and guarantee its treatment through transfers between tanks every other day, for one week, if starting from three contiguous tanks with 4 m^3 each, filled with only 0.25 m of water height (to allow light to enter the water column and obtain a stable microalgae culture and consequently reducing the capacity of the tanks to 25%). At the end of the week, 300 L of treated effluent with 0.26 kg of microalgal biomass could be released directly into a tank with small shrimp (krill type) that serve as feed for aquaculture animals produced by the company, such as brown crab, lobsters, and spinous spider crab. The algal biomass produced per week would be approximately 0.26 kg (dry weight) for *Chlorella vulgaris* and 0.23 kg for *Tetradesmus obliquus* (Figure 11.1).

Another option would be to concentrate the biomass by decantation (releasing the treated water) and process it into pellets serving as feed for aquaculture fish (Dineshbabu *et al.*, 2019; Milhazes-Cunha and Otero, 2017). In this case, a 4 m^3 tank would have a capacity for about 200 fish (with a 0.5 kg harvest weight), since the ratio is 25 kg m^{-3} (Craig and Helfrich, 2017). Aquaculture fish are typically fed 1 to 5% of their body weight per day (Opiyo *et al.*, 2018). According to the algal biomass obtained from *C. vulgaris*, it would be possible to provide 0.7 to 3.7% of the weekly feed of the fish growing in this tank with the biomass obtained in the effluent treatment. According to Shields and Lupatsch (2012), one of the reasons for not including more microalgae in aquaculture animals feed is due to the high cost it represents. From a nutritional and digestibility point of view, there would be no disadvantage, however, the microalgae species to be included in the diet of aquaculture animals would have to be studied due to the lower palatability of algal meal. Dineshbabu *et al.* (2019b) also state that food based on microalgae can be used in aquaculture alone or

combined with a regular feed. According to Oostlander *et al.* (2020), the main source of nutrients for larval and juvenile fish stages and all bivalve filter-feeder stages are microalgae.

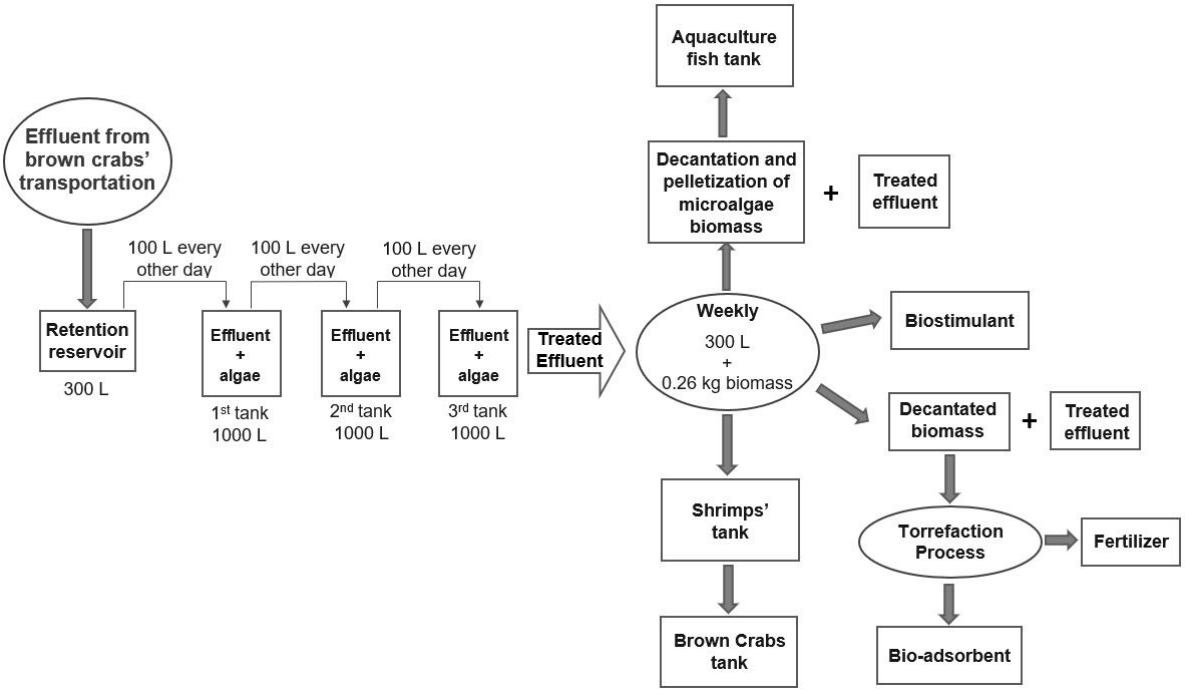

Figure 11.1 – Graphical representation of the aquaculture effluent treatment process with weekly quantity flows. The treated effluent with the microalgae biomass could be directly sent to shrimps' tank or decanted and sent to aquaculture fish tank, used as a biostimulant, bio-adsorbent or fertilizer after a torrefaction process.

The existing alternatives for effluent remediation and biomass production in these cases often involve the existence of membrane reactors, with the frequent problems of complexity, bridging, and cost (Kumar *et al.*, 2020). Finding alternative and integrated solutions for the treatment of aquaculture effluents can be the answer for the development of more sustainable processes. The possibility of obtaining different products with added value, in addition to the effluent treatment, is very promising.

11.2 Cattle effluent

According to the data obtained in this thesis for the cattle effluent and assuming a dairy farm with 100 cows (600 kg/animal), this would represent a daily production of 5 m^3 of manure (feces and urine) (United States Department of Agriculture, 1995). Designing a semi-continuous system consisting of three ponds of 500 m^3 each (with a usable height of

0.2m) with an input of 10 m^3 of manure (38,5 m^3 of pre-treated effluent) every 2 days, it would be possible to obtain every 2 days an output of: 26.03 kg of microalgal biomass (To); 38.5 m^3 of treated cattle effluent and 5500 kg of precipitate (Figure 11.2).

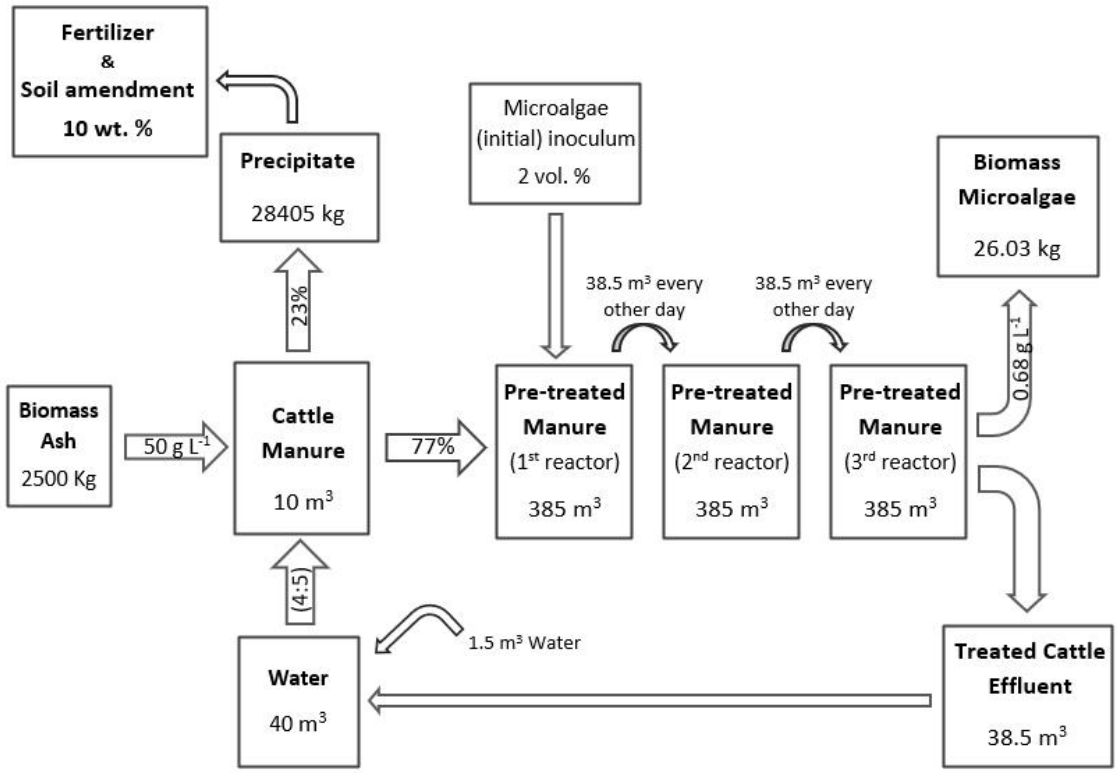

Figure 11.2 - Graphical representation of the cattle manure treatment process with quantity flows for every two days inputs.

To carry out the remediation, it would be necessary to introduce 2500 kg of biomass ash and 40 m^3 of water. At the end of each 2-day period, 38.5 m^3 of treated effluent would be obtained, which could be used in place of water for the initial dilution. If the option was to use microalga *A. protothecoides*, 24.71 kg of algal biomass would be obtained every 2 days.

Supposing the algal biomass production in the bovine exploration were 13 kg day^{-1} (26 Kg every two days), it would be possible to include 1% of the algal biomass in the 100 cow's diet, assuming that these animals consume about 2% of their daily weight in dry matter (Wright *et al.*, 2020).

11.3 Piggery effluent

Regarding the porcine sector, assuming an agro-industrial farm with around 1000 animals, which together produce daily 5.98 m^3 of manure (according to the data in point 5.3), this would represent a requirement of treating 12 m^3 of piggery effluent every 2 days (Figure 11.3).

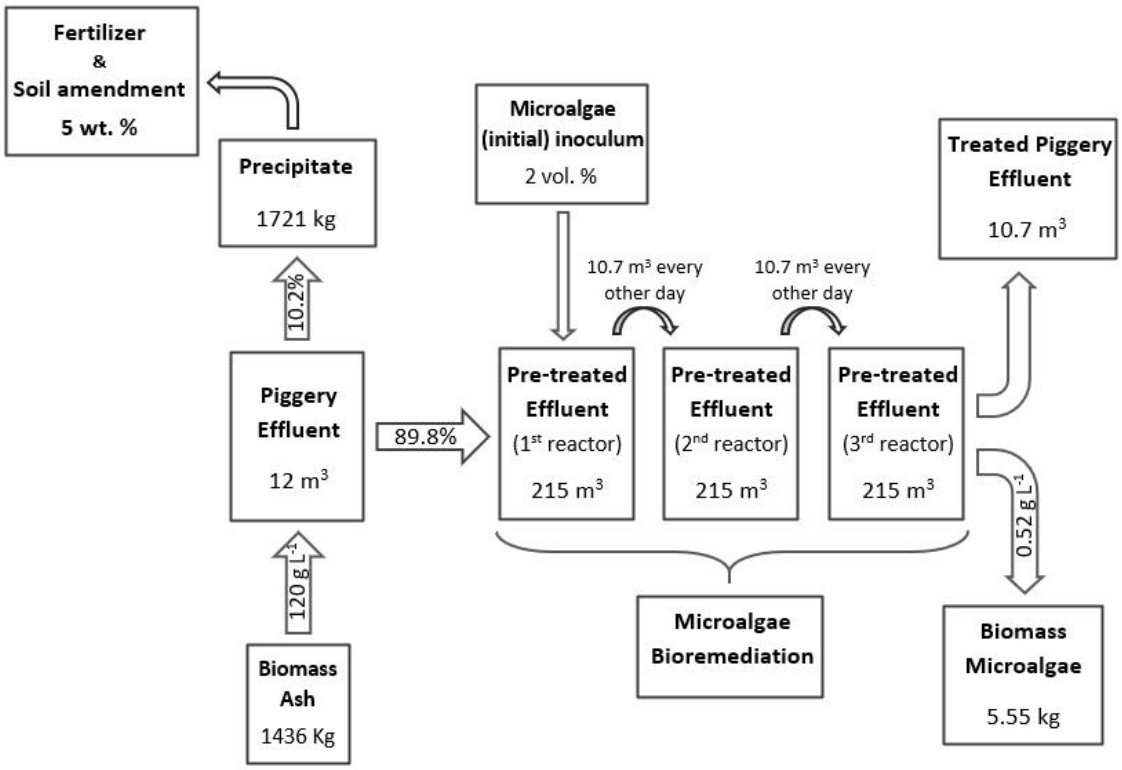

Figure 11.3 - Graphical representation of the piggery effluent treatment process with quantity flows for every two days inputs.

To carry out this pre-treatment, 1436 kg of biomass ash would be necessary, resulting in 10.7 m^3 of effluent to be bioremediated by microalgae and 1721 kg of precipitate that could be integrated into the soil as a fertilizer. In this system, 5.55 kg of algal biomass (Cv) would be obtained every 2 days and 10.7 m^3 of treated effluent, which could be discharged in body streams or used for washing and irrigation the cultures used for pigs' diet. However, since it is a reduced amount of algae biomass, it could only be used as a weekly supplement in the order of 20 g per animal. Alternatively, if the treated effluent were destined for agricultural irrigation, there would be no need to separate the algae from the medium and both products could be used for irrigation.

11.4 Poultry effluent

According to the results obtained in this thesis and the existing data (González-García *et al.*, 2014), and assuming a farm of broiler chicken production with 10,000 birds, where 450 chickens are slaughtered per day (22 days per month) after 34 days of growth, a farm with these dimensions would originate daily about 3.35 m^3 of slaughterhouse cooking water. To proceed with the pre-treatment of this effluent, approximately 605 kg of biomass ash would be needed every 2 days (Figure 11.4).

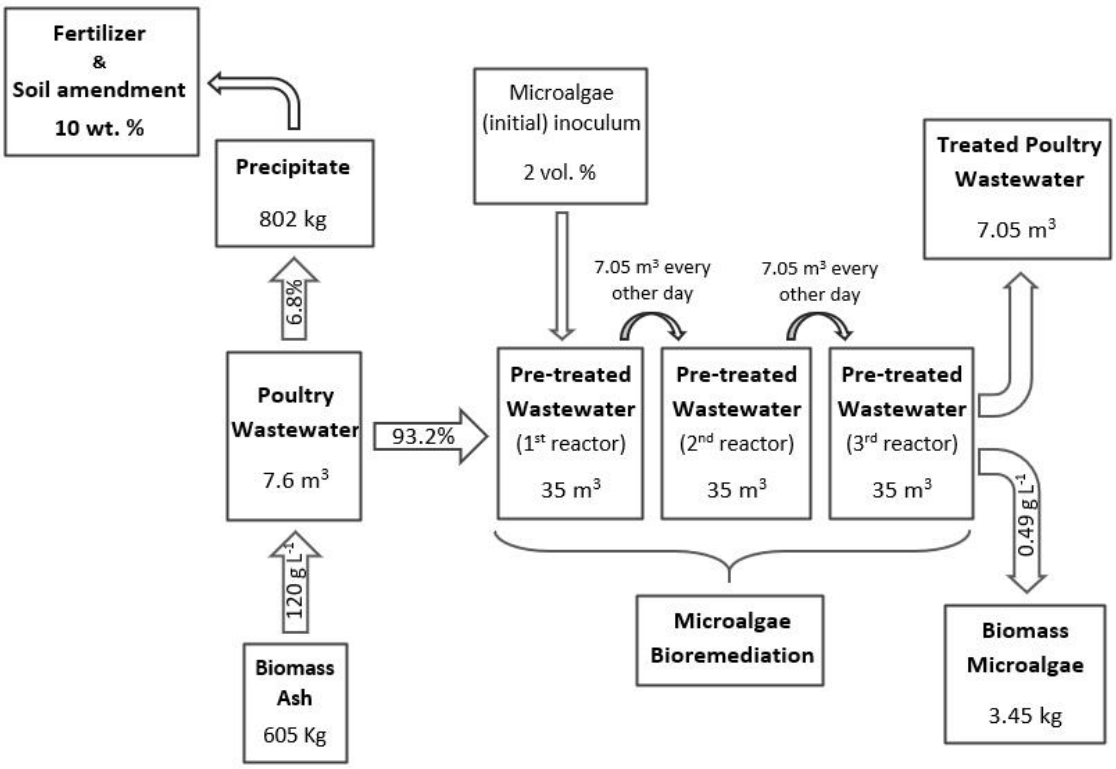

Figure 11.4 - Graphical representation of the poultry effluent treatment process with quantity flows for every two days inputs.

This process would result in 802 kg of precipitate, that could be integrated into the soil as a fertilizer, and 7.05 m^3 of effluent ready to be remediated by microalgae. At the end of the remediation process, 7.05 m^3 of treated effluent and 3.45 kg of *T. obliquus* algal biomass would be obtained (or 2.73 kg if *C. vulgaris* were chosen). As in the case of piggery effluent, the treated effluent could be used for irrigation and the algal biomass obtained every two days at the end of the process being reduced could only be used in the chicken feed as a weekly food supplement of 2% or integrated as a biostimulant in the production of chicken feed crops.

11.5 Landfill leachate

An annual diagram of the treatment of landfill leachate with the production of algal biomass and other by-products is presented in Figure 11.5, with the inflows and outflows, under the same conditions as in the present study, but on an industrial scale.

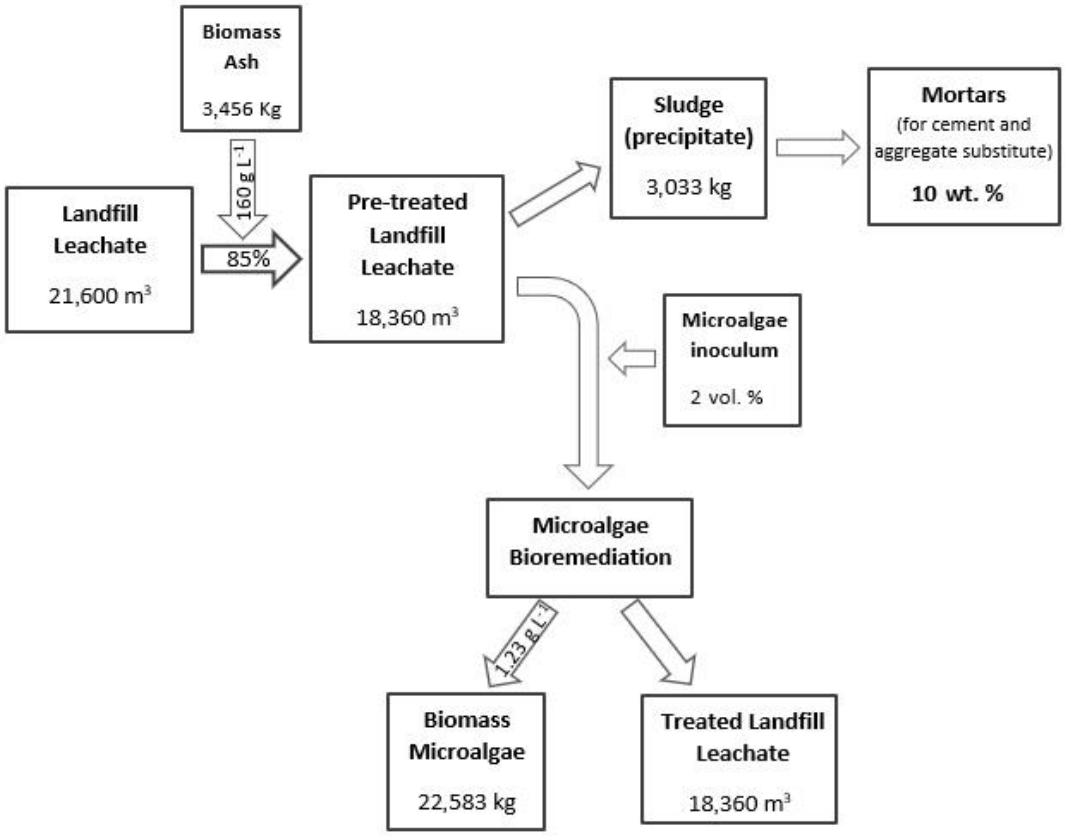

Figure 11.5 - Annual diagram of the potential for bioremediation of landfill leachate by microalgae on an industrial scale (based on the present study).

For this analysis, it was considered the amount of landfill leachate produced by CITRI, S.A. (on an annual basis). The data were collected according to its environmental license LA n.º20/2007 (Portuguese Ministry of the Environment, 2019). Considering only 6 months of leachate production at the landfill and taking into account the reduced rainfall during the Spring/Summer period, it is possible to obtain a maximum leachate volume of 21.6×10^6 L year^{-1}.

Applying a chemical pre-treatment with biomass ash, with a yield of 85%, results in a pre-treated leachate volume of 18,360 m^3. This volume could be sent to reactors where a bioremediation process with microalgae occurs. Considering a biomass concentration of 1.23 g L^{-1} in this leachate, it would be possible to obtain 22.6 tonnes of microalgal biomass.

Chapter 12

Conclusions and future perspectives

12.1 Conclusions

The main objective of the present thesis was to study the bioremediation of four agro-industrial effluents and an industrial effluent by microalgae and to evaluate the potential valorisation of the algal biomass produced.

All agro-industrial effluents presented high levels of total nitrogen and microbial contamination (microscopically observed), but the swine effluent and the bovine manure also presented high levels of suspended solids. The landfill leachate had a dark colour due to the presence of humic and fulvic acids produced during the decomposition of the lignocellulosic fraction of the materials deposited in the landfill. All these characteristics may constitute inhibition factors for the proliferation of microalgae, either by limiting the penetration of solar light in the culture medium or by the effect of inhibiting or competing factors. A possible strategy often used to overcome these limitations is the dilution of the effluent to decrease the influence of these negative characteristics but the use of clean water to dilute a contaminated effluent is not a very sustainable option.

In this work an alternative procedure was developed and proposed, involving the treatment of the effluent with biomass ash to induce an increase of the effluent pH to values higher than 11, eliminating most of the microbial contamination and causing precipitation of a large fraction of the suspended materials and coloured components, responsible by the effluent colour and turbidity. This treatment is a variant of the coagulation treatment of urban wastewaters using chemical additives such as calcium hydroxide, with the advantage of using biomass waste. The ashes from biomass combustion also possess a strong alkalinization capacity and high levels of calcium species.

This pre-treatment with biomass ash proved to be efficient in all tested effluents leading to a high removal of suspended solids (>58% of optical density reduction), a reduction of the nitrogen content and microbial load but with comparable COD because the removal of organic components was compensated by partial dissolution of the

inorganic components of the biomass ash in the effluent. This replacement of organic by inorganic species led to an effluent composition that is more suitable for microalgae bioremediation through autotrophic metabolism, than the original effluent with high concentrations of complex organic molecules. In this approach, the effluent required no dilution only a correction of the final pH, before inoculating the microalgae. In the laboratory experiments, this correction was made by adding a small volume of a concentrated mineral acid, but in real-life applications, this pH correction can be done by aeration of the treated effluent to promote the dissolution of carbon dioxide and the progressive acidification of the effluent until reaching a neutral pH, a procedure that has the advantage of promoting carbon dioxide capture.

Since biomass ash contains various components with variable solubility in water, a fraction of the added biomass ash is not solubilized and is recovered after the treatment, as a precipitate also containing the coagulated organic components.

The precipitates obtained in the treatment of the different effluents were found to be distinct from each other, with the one derived from diluted cattle manure being the richest in organic matter. Regarding their use as fertilizers, these precipitates could increase the germination of seeds when added to the liquid media at doses of 5 and 10% (v/v). Although, their application in soil amendment requires further studies, especially including field tests, to understand interactions with the soil structure and components, this type of alkaline precipitates, can be used in the correction of acidic soils or the adjustment of pH in composting units, two useful applications in the agro-industrial sector.

All tested effluents have been successfully remedied by microalgae, making it possible to discharge them into body streams or municipal collectors. The highest yields of biomass productivity were achieved for aquaculture effluent with 879.8 mg L^{-1} day^{-1} for *Chlorella vulgaris* and the lowest for landfill leachate with *C. vulgaris* with 47.0 mg L^{-1} day^{-1}. The remediation capacity of the microalgae varied from effluent to effluent and among the tested microalgae.

Remediation in semi-continuous mode, using three reactors of a certain volume and periodically transferring part of the culture medium between reactors proved to be more efficient than using only one reactor with the same total volume, for the same period of time. This difference may result from the dilution effect that occurs in the transfer between reactors, which enables the nutrient concentration to decrease from the first to the third

reactor and can also have a diluting effect in metabolite species that may influence the Removal efficiency. The fraction of volume that could be transferred between reactors was also optimized for each effluent, in order to obtain at the exit of the third reactor a treated effluent able to be discharged at every 48 h period. These transfer volumes were 5% for piggery effluent, 10% for aquaculture and cattle effluents, and 20% for poultry effluent, differences that are probably related to the nature and concentration of the contaminants present in each effluent and the capacity of the microalgae to remediate them in a given period of time.

The composition of the algal biomass obtained in the different effluents was quite heterogeneous. Comparing the results in the semi-continuous mode tests (except for landfill leachate), it can be concluded that protein levels varied between 42% (piggery) and 12% (landfill leachate), carbohydrates between 39% (aquaculture effluent) and 16% (cattle effluent) and lipids between 23% (landfill leachate) and 4% (aquaculture effluent). These differences show that the nature of the effluent strongly influenced the physiological condition of the microalgae cultures, affecting the concentrations of both structural components and energy storage metabolites. Regarding the fatty acids content, these were more constant among the microalgae produced in the different effluents, with a predominance in oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2), and conjugated linoleic acid (CLA).

In what concerns the applications of algal biomass studied, it can be concluded that all of them showed benefits in their application as fertilizer, however, the one that stood out was *C. vulgaris* biomass obtained from the treatment of aquaculture effluent, with 238% increment compared to the control, in the germination of watercress seeds.

The algal biomasses produced in agro-industrial effluents prove to be suitable for integration into animal feed due to their high protein content and the predominance of polyunsaturated fatty acids. On the other hand, the ash composition of these microalgae revealed the presence of some potentially critical compounds as aluminium and zinc, however in trace quantities. The use of microalgae produced in aquaculture effluent to feed mussels showed a higher weight gain when compared to feeding with the same microalgae grown in synthetic culture medium.

The torrefaction of mixtures of algal biomass and lignocellulosic biomass with different final moisture contents was evaluated to simulate the mixture of decanted or

centrifugated algal biomass, with a matrix with lower moisture content, and their co-conversion to biochars. The process proved to be effective in improving the properties of the original biomasses, decreasing the volatile matter content, and increasing the fixed carbon, homogeneity, grindability, and high heating value (14.8 to 21.4 MJ Kg^{-1}, db), mainly for biomasses subject to more severe torrefaction conditions. For this reason, it will be more efficient to redirect the biochars produced in more severe torrefaction conditions for energy use, and the biochars produced in milder conditions for soil amendment, where some nitrogen is preserved in the biochars. When using algal biomasses produced in effluents with high salt content (aquaculture and landfill leachate), the results for the high heating value of the biochars were reduced (5.6 to 16.0 MJ Kg^{-1}, db) due largely to the high ash content in their composition. In the case of algal biomass from effluents, the energy uses are not the most appropriate due to the low heating value of the obtained biochar. The use of these biochars as a fertilizer or adsorbent may be more beneficial. The germination of wheat and watercress seeds with algal biomass, allowed to obtain much more promising results than using the biochar obtained from the same biomass (algal biomass from aquaculture effluent), as was expected since the torrefaction process leads to the volatilization of some compounds, leaving others in the aqueous phase, thus the obtained biochar is poorer than the algal biomass that gave rise to it. However, biochar is a stable material and its incorporation into the soil usually has a beneficial effect as a soil corrector by improving its structure and releasing nutrients slowly in the soil.

As for the biochar adsorption capacity, it was found that those obtained from algal biomass from effluents proved to be more efficient than those from commercial algal biomass. Additionally, the increase in the contact time (3 seconds to 48 hours) of the biochars with the cationic dye methylene blue led to increases in adsorption between 24 and 68%.

The use of microalgae in the treatment of the tested agro-industrial and industrial effluents proved to be very promising, namely using simple pre-treatments with ash, making it possible to obtain biomass with excellent characteristics for integration in animal feed or crop fertilization. The use of different wastes becomes essential in a sustainable approach to the development of a circular economy.

This is a contribution to the crucial development of innovative approaches to solving global problems.

12.2 Future perspectives

The study developed aims to shed light on the use of simple and inexpensive processes in the treatment of agro-industrial effluents. Therefore, it can be applied by agricultural farms and/or companies without the need for large investments.

Considering the importance of reusing the nutrients contained in agro-industrial and industrial effluents, further studies on this theme are needed with methodologies that allow scaling for industrial applications, mainly being easy to apply and of reduced cost.

It is crucial to validate the application of pre-treatment with biomass ash for mixtures of agro-industrial effluents. In cases where there is a need to supplement the latest effluent reactors with nutrients (N and P), it would be important to make this compensation with other industrial effluents, instead of resorting to commercial mineral supplements.

It would be essential to study the application of algal biomass produced in agro-industrial effluents in animal feed on a continuous basis, to guarantee that it does not generate any kind of consequences in the most complex animals such as cows, pigs, and poultry in the long term, nor problems of bioaccumulation of toxic compounds.

Studying the application of precipitates that are formed during the pre-treatment of effluents with ash is also required, namely directly in the soil to analyse the interactions established or to develop other applications such as the integration in cement and mortar for the poorest precipitates.

To use algal biomass produced in effluents with reduced dissolved salts in torrefaction, in order to obtain biochars with lowered ash content, has a big potential for sustainable development, as a partial replacement of lignocellulosic biomass resources.

There are still some technological, regulatory, and market-related barriers but the results of this study and similar ones, emphasize a huge potential for renewable development. Only by overcoming the necessary economic, social, and environmental changes, it will be possible to achieve a more sustainable world.

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Appendix

Appendix 1 - Experimental design matrix using the RSM modelling technique (Cv – Microalgae sludge, Lc – Lignocellulosic biomass) and corresponding masses of Cv, Lc and water introduced in the reactor.

Run	Temperature (ºC)	Time (min)	Lc Incorporation rate (%)	Moisture content (%)	Cv (g)	Added water (g)	Lc (g)	Total water in feed* (g)	Total feed mass (g)
1	*300*	*60*	*50*	*30*	3.5	4.9	10.0	5.6	18.4
2	*300*	*60*	*25*	*15*	12.0	2.2	5.0	2.9	19.2
3	*300*	*60*	*25*	*15*	12.0	2.2	5.0	2.9	19.2
4	*300*	*45*	*0*	*45*	11.0	8.5	0.0	8.8	19.5
5	*300*	*45*	*100*	*5*	0.0	0.0	20.0	1.0	20.0
6	*300*	*45*	*50*	*5*	10.0	0.0	10.0	1.0	20.0
7	*300*	*45*	*0*	*5*	20.0	0.0	0.0	1.0	20.0
8	*300*	*30*	*50*	*30*	5.5	5.6	10.0	6.4	21.1
9	*300*	*15*	*25*	*45*	5.2	7.5	5.0	8.0	17.7
10	*300*	*15*	*0*	*45*	11.0	8.0	0.0	8.6	19.0
11	*300*	*15*	*50*	*5*	10.0	0.0	10.0	1.0	20.0
12	*275*	*60*	*0*	*70*	7.0	15.2	0.0	15.6	22.2
13	*275*	*45*	*25*	*30*	8.5	4.9	5.0	5.6	18.4
14	*250*	*60*	*0*	*15*	16.0	1.9	0.0	2.7	17.9
15	*250*	*60*	*50*	*5*	10.0	0.0	10.0	1.0	20.0
16	*250*	*45*	*50*	*30*	3.5	4.9	10.0	5.6	18.4
17	*250*	*45*	*25*	*30*	8.5	4.9	5.0	5.6	18.4
18	*250*	*45*	*25*	*30*	8.5	4.9	5.0	5.6	18.4
19	*250*	*45*	*25*	*15*	12.0	2.0	5.0	2.9	19.0
20	*250*	*45*	*25*	*15*	12.0	2.0	5.0	2.9	19.0
21	*250*	*30*	*25*	*45*	5.2	7.5	5.0	8.0	17.7
22	*250*	*30*	*25*	*45*	5.2	7.5	5.0	8.0	17.7
23	*250*	*30*	*50*	*15*	8.0	2.2	10.0	3.1	20.2
24	*250*	*30*	*25*	*5*	15.0	0.0	5.0	1.0	20.0
25	*250*	*15*	*50*	*30*	5.5	5.6	10.0	6.4	21.1
26	*250*	*15*	*0*	*5*	20.0	0.0	0.0	1.0	20.0
27	*250*	*15*	*0*	*5*	20.0	0.0	0.0	1.0	20.0
28	*225*	*60*	*25*	*5*	15.0	0.0	5.0	1.0	20.0
29	*225*	*45*	*0*	*45*	11.0	8.0	0.0	8.6	19.0
30	*225*	*15*	*25*	*15*	12.0	2.0	5.0	2.9	19.0
31	*200*	*60*	*25*	*45*	5.2	7.5	5.0	8.0	17.7
32	*200*	*60*	*50*	*30*	5.5	5.6	10.0	6.4	21.1
33	*200*	*60*	*0*	*5*	20.0	0.0	0.0	1.0	20.0
34	*200*	*30*	*25*	*15*	12.0	2.0	5.0	2.9	19.0
35	*200*	*30*	*25*	*15*	12.0	2.0	5.0	2.9	19.0
36	*200*	*30*	*50*	*5*	10.0	0.0	10.0	1.0	20.0
37	*200*	*15*	*0*	*70*	7.0	15.2	0.0	15.6	22.2
38	*200*	*15*	*50*	*30*	3.5	4.9	10.0	5.6	18.4
39	*200*	*15*	*25*	*30*	8.5	4.9	5.0	5.6	18.4

*Total water in feed corresponds to the sum of the added water and the residual moisture present in the Cv and Lc biomasses introduced in the reactor.

Appendix 2 - Equations generated by RSM software, with R2, Adjusted R^2, Predicted R^2 and Adequate Precision for product yields.

Parameter	Equation *	Model F-value	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
Char Yield	$86.52 + 0.40\ T - 0.62\ t - 2.10\ M + 0.14\ I + 3.98x10^{-3}\ (T \times M) - 1.52x10^{-3}\ (T \times I) - 1.25x10^{-3}\ (T^2) + 4.19x10^{-3}\ (t^2) + 5.35x10^{-3}\ (M^2) + 1.52x10^{-3}\ (I^2)$	130.00	0.980	0.973	0.950	40.754
Condensate Yield	$-1.10 + 1.14x10^{-2}\ T - 0.30\ t + 9.84x10^{-2}\ M + 9.70x10^{-5}\ I - 1.20x10^{-4}\ (T \times M) - 2.70x10^{-4}\ (t \times M) - 5.39x10^{-4}\ (M^2)$	26.19	0.859	0.827	0.698	16.900
Gas Yield	$-24.66 + 0.16\ T + 0.24\ t + 0.43\ M - 0.07\ I - 1.80x10^{-3}\ (T \times t) - 3.28x10^{-3}\ (T \times M) - 9.92x10^{-3}\ (t \times M) - 1.40x10^{-3}\ (M \times I)$	5.53	0.604[1]	0.595	0.340	9.911
Aqueous Phase Yield	$-1.87 + 3.05x10^{-3}\ T - 9.29x10^{-3}\ t + 0.05\ M + 0.08\ I + 7.50x10^{-5}\ (T \times t) - 2.20x10^{-5}\ (t \times I) - 1.30x10^{-5}\ (M \times I) - 4.42x10^{-4}\ (M^2) - 5.64x10^{-4}\ (I^2)$	30.58	0.908	0.878	0.611	27.606
Bio-oil Yield	$47.98 - 0.45\ T + 0.38\ t + 0.55\ M - 0.42\ I - 1.97x10^{-3}\ (T \times M) + 1.88x10^{-3}\ (T \times I) - 2.92\ (t \times M) + 8.98\ x10^{-4}\ (T^2) - 3.14x10^{-3}\ (t^2)$	18.98	0.864	0.818	0.700	18.463

Note: p-value model is always 0.01%, and it means that there is only a 0.01% chance that an F-value this large could occur due to noise.

The equations did not undergo any transformations except the Condensate and Aqueous phase equations with a natural log transformation.

The Lak of Fit is not significant (< 4), except for the parameter Bio-oil (Lak of Fit of 6.02).

* Where T, t, M and I mean temperature, residence time, moisture and incorporation rate of lignocellulosic biomass, respectively.

1 – a low R^2 suggests a dispersion of values that decrease the predictability of the model (Darvishmotevalli *et al.*, 2019).

Appendix 3 - Equations generated by RSM software, with R^2, Adjusted R^2, Predicted R^2 and Adequate Precision for char characterization.

	Equation *	Model F-value	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
Ash	18.09 - 0.16 T + 0.04 t + 0.02 M - 0.02 I - $1.53x10^{-4}$ (T x t) - $7.10x10^{-5}$ (T x M) + $9.40x10^{-5}$ (T x I) -$1.50x10^{-4}$ (t x M) - $5.90x10^{-5}$ (M x I) +$3.53x10^{-4}$ (T^2) - $3.03x10^{-4}$ (I^2)	57.80	0.962	0.946	0.886	23.239
Volatile Matter	-57.02 + 1.12 T - 0.06 t - 0.489 M + 0.39 I - $3.40x10^{-3}$ (T x t) + $3.68x10^{-3}$ (t x I) + $6.44x10^{-3}$ (M x I) - $2.26x10^{-3}$ (T^2) + $5.52x10^{-3}$ (t^2) - $3.38x10^{-3}$ (I^2)	41.07	0.943	0.920	0.874	27.313
Fixed carbon	116.76 - 0.80 T - 0.74 t - 0.54 M + 0.16 I + $3.12x10^{-3}$ (T x t) + $1.42x10^{-3}$ (T x M) – $9.18x10^{-4}$ (T x I) + $2.73x10^{-3}$ (t x M) + $1.66x10^{-3}$ (T^2)	80.69	0.964	0.952	0.920	39.412
O/C ratio	0.88 - $1.67x10^{-3}$ T + $3.46x10^{-3}$ t + $9.30x10^{-3}$ M - $1.98x10^{-4}$ I - $2.60x10^{-5}$ (T x t) - $9.50x10^{-5}$ (M x I)	60.89	0.921	0.910	0.871	26.910
H/C ratio	2.48 - $3.74x10^{-3}$ T - $5.96x10^{-3}$ t + $1.14x10^{-3}$ M + $1.85x10^{-3}$ I - $3.10x10^{-5}$ (T x t) + $1.13x10^{-4}$ (t^2)	102.92	0.954	0.944	0.923	35.734
Char adsorption capacity	273.18 - 0.80 T - 0.66 t – 2.74 M + 0.20 I + $9.55x10^{-3}$ (T x M)	38.40	0.857	0.835	0.786	19.010

Note: p-value model is always 0.01%, and it means that there is only a 0.01% chance that an F-value this large could occur due to noise.

The equations did not undergo any transformations except Ash equation with a natural log transformation. The Lak of Fit is not significant (< 4).

* Where T, t, M and I mean temperature, residence time, moisture and incorporation rate of lignocellulosic biomass, respectively.

Appendix 4 - Equations generated by RSM software, with R^2, Adjusted R^2, Predicted R^2 and Adequate Precision for Q_{input} and Q_{output}.

	Equation *	Model F-value	R^2	Adjusted R^2	Predicted R^2	Adeq. Precision
Q_{input}	$1.66 + 6.89x10^{-4}\ T - 4.70x10^{-3}\ t + 3.36x10^{-2}\ M + 6.367x10^{-3}\ I$	59.44	0.878	0.863	0.821	27.054
Q_{output}	$0.38 + 8.30x10^{-5}\ T - 1.94x10^{-3}\ t - 8.35x10^{-3}\ M + 2.19x10^{-3}\ I + 1.3x10^{-5}\ (T \times M) - 1.20x10^{-5}\ (T \times I) - 2.10x10^{-5}\ (t \times M) + 1.50x10^{-5}\ (t \times I) + 4.80x10^{-5}\ (M^2)$	193.09	0.988	0.983	0.972	49.376

Note: p-value model is always 0.01%, and it means that there is only a 0.01% chance that an F-value this large could occur due to noise.

The Lak of Fit is not significant (< 4).

* Where T, t, M and I mean temperature, residence time, moisture and incorporation rate of lignocellulosic biomass, respectively.