UNIVERSIDADE D
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Pedro Rafael Martins Pedroso

BIOGAS UPGRADING THROUGH A MICROALGAE PLATFORM

Master's Dissertation in Chemical Engineering, Specialization in Process, Environment and Energy, supervised by Engineer Andre Retief and Professor Doctor Jorge Pereira, presented to the Department of Chemical Engineering, Faculty of Sciences and Technology of the University of Coimbra

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FACULDADE DE CIÊNCIAS E TECNOLOGIA DA UNIVERSIDADE DE COIMBRA

DEPARTAMENTO DE ENGENHARIA QUÍMICA

BIOGAS UPGRADING THROUGH A MICROALGAE PLATFORM

CURRICULAR INTERNSHIP AT ALGAEMENTUM

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Supervisors:

Eng. Andre Retief, *Algaementum*

Prof. Dr. Jorge Pereira, Department of Chemical Engineering, Faculty of Sciences and Technology of the University of Coimbra

Coimbra

September 2023

(…)

Qual é o teu sonho?

Protege-o bem

Para não esqueceres

Ou a vida vai levá-lo

Sem te aperceberes

E eu quis sonhar mais alto

(…)

Mas tenho de continuar a sonhar

- *João Batista Coelho*, artista português

ACKNOWLEDGEMENTS

This is my last written chapter as a chemical engineering student, which I will write, wholeheartedly, in my mother language.

Ser estudante de Coimbra era um objetivo desde uma visita de estudo, no oitavo ano, ao pólo I da Universidade de Coimbra, com uma palestra no departamento de Química, e quis o tempo que chegasse ao Departamento de Engenharia Química em setembro de 2017. Hoje, levo de Coimbra tudo aquilo que ela promete: o sentimento, a saudade, as memórias e os meus piores momentos, todos eles esquecidos quando penso nos melhores. Estudar na Universidade de Coimbra permitiu-me fazer o que sempre quis, tudo. Vivi e desfrutei, nesta cidade, de tudo aquilo que ela tem para oferecer: um bom curso, a cultura, a academia, o desporto e as pessoas. E daqui saio, comprovando que, de facto, ela é uma lição, de sonho e tradição.

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É impossível olhar para trás e não ver a diferença de quem era quando fiz a matrícula na UC, um dia que nunca esquecerei, pois foi quando conheci os meus padrinhos da faculdade. Engenheiros João e Sofia, tenho saudades vossas, obrigado por tudo o que fizeram por mim, pelos momentos que partilhámos e por me incentivarem a nunca baixar a cabeça neste curso. Desejo-vos o melhor do mundo.

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Foram muitas semanas, às vezes meses, sem ir a casa, e continuo a esquecer-me de ligar quando chego. *Daqui para a frente é outro desafio*. Obrigado por gostarem tanto de mim, eu também gosto muito de vocês.

working on a bright future,

PP

RESUMO

As emissões globais de gases de efeito de estufa aumentaram nas últimas décadas, sendo o setor energético o principal responsável. Aumentar a quota de energia renovável utilizando novas tecnologias mais verdes é um dos principais focos da União Europeia para as próximas décadas, com o grande objetivo de atingir emissões-zero até 2050. O REPowerEU é uma das estratégias para aumentar a produção de energias renováveis e a poupança de energia, reduzindo ainda a independência energética do gás proveniente da Rússia. Uma das iniciativas propostas é o aumento da produção de biogás e a sua conversão para biometano, para injeção nas redes de gás já existentes, até 2030. As microalgas têm vindo a surgir como uma potencial tecnologia de conversão do biogás devido à sua elevada atividade fotossintética, consumindo CO_2 enquanto geram biomassa que pode originar produtos de valor acrescentado.

Esta dissertação centrou-se na melhoria de biogás utilizando uma plataforma de microalgas, que consistiu numa cultura de *Chlorella vulgaris* NIVA CHL-108, num depósito IBC (*International Bulk Container*). O processo consistiu no arejamento da cultura com biogás proveniente de outro IBC, que funcionou como um digestor anaeróbio. Este sistema piloto foi criado pela *Algaementum* e instalado na Monte Silveira Bio, uma quinta com certificação biológica desde 1999. A matéria-prima da digestão anaeróbia consistiu em estrume de vaca e a monitorização do biogás produzido pelo sistema iniciou no dia 23 e terminou no dia 51. Os resultados mostraram um valor máximo de produção de 52.8% de CH_4 no biogás bruto, sendo os restantes valores inferiores a 40%. O sistema mostrou não satisfazer o processo de melhoria do biogás, tendo todo o biogás proveniente da digestão anaeróbia solubilizado na cultura, devido à falta de fluxo inerente à operação do sistema. Foram realizados alguns ensaios em contentores mais pequenos e utilizando outras matérias-primas (*Opuntia Ficus Indica* e cultura de microalgas).

Realizou-se ainda a caracterização final da biomassa de microalgas, que mostrou diferenças na cultura entre os dias 28 e 40 de monitorização. O teor de proteínas aumentou de 19.15% para 25.91%, enquanto o teor de lípidos aumentou apenas de 1.05% para 1.45%. A concentração de clorofila no dia 40 foi de 9.42 mg/L, aumentando desde o dia 28, em que registou uma concentração de 4.32 mg/L.

Este novo sistema de cultivo de microalgas mostra, assim, algum potencial, visto que as propriedades da cultura de microalgas foram melhoradas utilizando um IBC, cujos custos, comparados aos de um fotobiorreator, são muito inferiores.

Palavras-chave: Pacto Ecológico Europeu, Agricultura, Melhoria de Biogás, *Chlorella vulgaris*, Estrume de gado

ABSTRACT

Global greenhouse gas emissions have increased in the last decades and the energy sector is the main responsible. Thus, the need to pursue new green technologies and expand the share of renewable energy is one of the main focuses of the European Union for the next few decades, with the grand objective of reaching net-zero emissions by 2050. The REPowerEU is one of the strategies to increase renewable production and energy saving, and seeks energy independence from Russian gas, following recent events. One of the initiatives proposed is the increase in biogas production, and its upgrading to biomethane, for injection in the already existing gas grids, until 2030. Microalgae have been surging as a potential biogas upgrading technology because of its high photosynthetic activity, capturing CO2 whilst growing biomass that can generate high value-products.

This dissertation focused on the upgrading of biogas using a microalgae platform, which consisted in an *International Bulk Container* (IBC) filled with a *Chlorella vulgaris* NIVA CHL-108 culture. The process consisted in aerating the culture with biogas. Another IBC was set up, working as an anaerobic digestor. The biogas resulting from the anaerobic digestion (AD) also served as a carbon source for microalgae culture in the process.

This pilot system was created by *Algaementum* and was set up in Monte Silveira Bio, a certificated organic farm since 1999. The AD feedstock consisted in the farm's cattle manure. Biogas monitorization was initiated on the 23rd day following the pilot setup. Results showed a peak production value of 52.8% CH_4 raw biogas, with the remaining values below 40%. The system operation was unsuccessful regarding the biogas upgrading process, as all the biogas solubilized in the culture, due to low AD biogas flux. Some trials were performed in smaller containers and using other feedstocks (OFI and microalgae culture).

A final characterization of the microalgae biomass was performed, which showed differences in the culture between days 28 and 40 of the monitorization period. Protein content increased from 19.15% to 25.91% and lipids content increased slightly, from 1.05% to 1.45%. Chlorophyll *a* concentration was 9.42 mg/L in day 40, an increase from day 28, when the value was 4.32 mg/L.

Thus, this method of cultivating microalgae offers great potential, since there was an improvement in the microalgal biomass properties, in an IBC system, and the costs involved in IBC processes are much lower than those of photobioreactors (PBRs).

Keywords: European Green Deal, Agriculture, Biogas Upgrading, *Chlorella vulgaris*, Cattle Manure

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS

AD – Anaerobic Digestion

APHA – American Public Health Association

BIP – Biogas and Biomethane Industrial Partnership

BMP – Biochemical Methane Potential

CAP – Common Agricultural Policy

CAPEX – Capital Expenditures

CEAP – Circular Economy Action Plan

CEI – Centro de Empresas Inovadoras

COD – Chemical Oxygen Demand

CoO – Certificate of Origin

CSTR – Continuous Stirred Tank Reactor

EGD – European Green Deal

EIT – European Institute of Innovation and Technology

ERGAR – European Renewable Gas Registry

EU – European Union

FAMEs – Fatty Acid Methyl Esters

FAs – Fatty Acids

F-Gas – Fluorinated Gas

FID – Flame Ionization Detector

GDP – Gross Domestic Product

GHG – Greenhouse Gases

GoO – Guarantees of Origin

GP – Gas Production

GVA – Gross Value-Added

HASL – Hybrid Anaerobic Solid Liquid System

HRT – Hydraulic Retention Time

IBC – International Bulk Container

ICT – Information and Communications Technology

IPCC – Intergovernmental Panel on Climate Change

ISO – International Organization for Standardization

LED – Light-Emitting Diode

MF – Microfiltration

NGO – Non-Governmental Organization

OFI – Opuntia Ficus Indica

OLR – Organic Loading Rate

PBR – Photobioreactor

pH – Hydrogen potential

PoS – Proof of Sustainability

PS – Polysulfone

PVC – Polyvinyl Chloride

PVDF – Polyvinylidene Difluoride

RE – Removal Efficiency

SRT – Solids Retention Time

T – Temperature

TDS – Total Dissolved Solids

TP – Total Protein

TRL – Technology Readiness Level

TS – Total Solids

TSS – Total Suspended Solids

UAA – Utilized Agricultural Area

UASB – Upflow Anaerobic Sludge Blanket

UF – Ultrafiltration

UN – United Nations

VFAs – Volatile Fatty Acids

VS – Volatile Solids

1. INTRODUCTION

1.1. Work Motivation

Over the years, climate change has become an urgent global challenge and one of today's most pressing challenges, with far-reaching impacts on ecosystems and society. It is primarily driven by increased greenhouse gas (GHG) emissions to the atmosphere, especially carbon dioxide (CO_2), of which, since the 2000's, the total emitted tons are over 35 Gton, as shown in Figure 1. In 2019, CO_2 emissions accounted for approximately 74.1% of all GHG emissions to the atmosphere, with methane (CH_4), nitrous oxide (N_2O), and fluorinated gases (F-Gas) being responsible for the other 17.3%, 6.2%, and 2.4%, respectively, making a total of 49.8 $GtCO_2$-equivalents (a unit based on the global warming potential of each relative gas to that of carbon dioxide) [1].

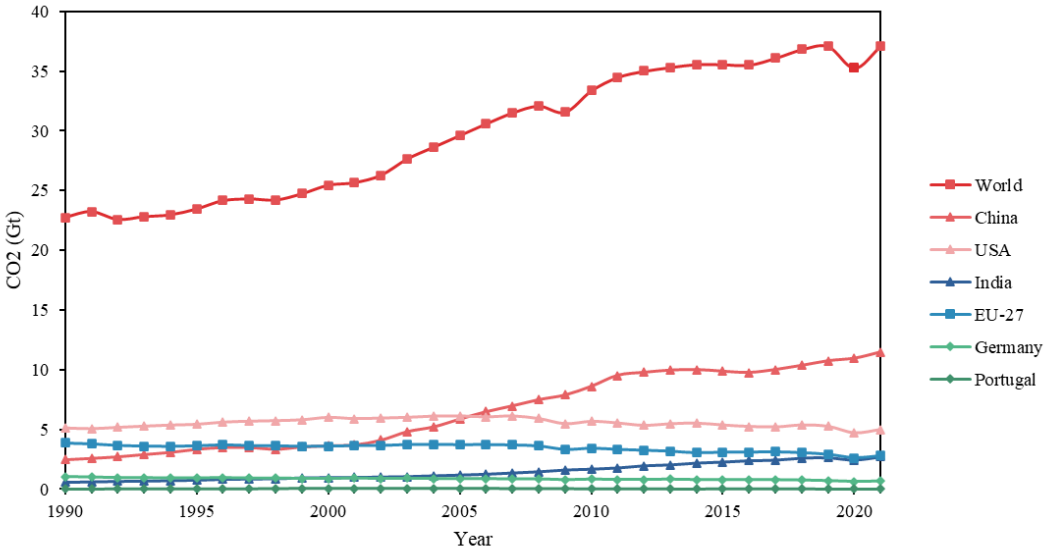

Figure 1. Annual CO2 emissions in the world, the main polluting countries (China, USA, India), the EU-27, Germany (the most polluting EU country) and Portugal (Adapted from [2])

This increase in global GHG emissions has been of utmost importance to key entities in the world since 1992, when the United Nations (UN) Framework Convention on Climate Change was signed, by 154 nations, at the Conference of the Parties held to review developments in tackling climate change. Further agreements have been signed since then, such as the Kyoto Protocol (1997) and the Paris Agreement (2015). The latter main goal is to keep the increase of global temperatures below 2°C, and preferably with a maximum of 1.5°C above pre-industrial levels. This threshold value would indicate severe and irreversible consequences for the planet if exceeded, such as extreme hot days, sea-level rise, the loss of more than half of the viable

habitat for 8% plants and 4% of vertebrates, and a decrease in annual global fisheries catches by 1.5 million tons, according to the Intergovernmental Panel on Climate Change (IPCC) [2].

Currently, despite the progress made in the reduction of GHG emissions, according to the UN, "*there is a 66% likelihood that the annual average near-surface global temperature between 2023 and 2027 will be more than 1.5ºC above pre-industrial levels for at least one year*", and it will take substantial efforts across all sectors of the economy to meet the European Union (EU)'s 2030 target of a 55% net reduction in GHG emissions, and even more so to achieve a climate neutral economy by 2050. Also, for a 50% chance of limiting warming to 1.5ºC, GHG emissions should peak by 2025, which is far from the reality, as national emissions commitments are still not sufficient to keep the planet within the target. In (Annex A), an estimation to meet these targets is presented, according to the Summary for Policymakers Report (IPCC) [3]. Thus, it is very important not only to continue working towards this goal, but also improving strong efforts across all sectors to limit GHG emissions. To have an overall picture of this scenario, in Figure 2 are shown the GHG emissions by sector and the activities responsible for those emissions within each sector. As presented, the energy supply is still the most prominent, followed by agricultural and industrial processes.

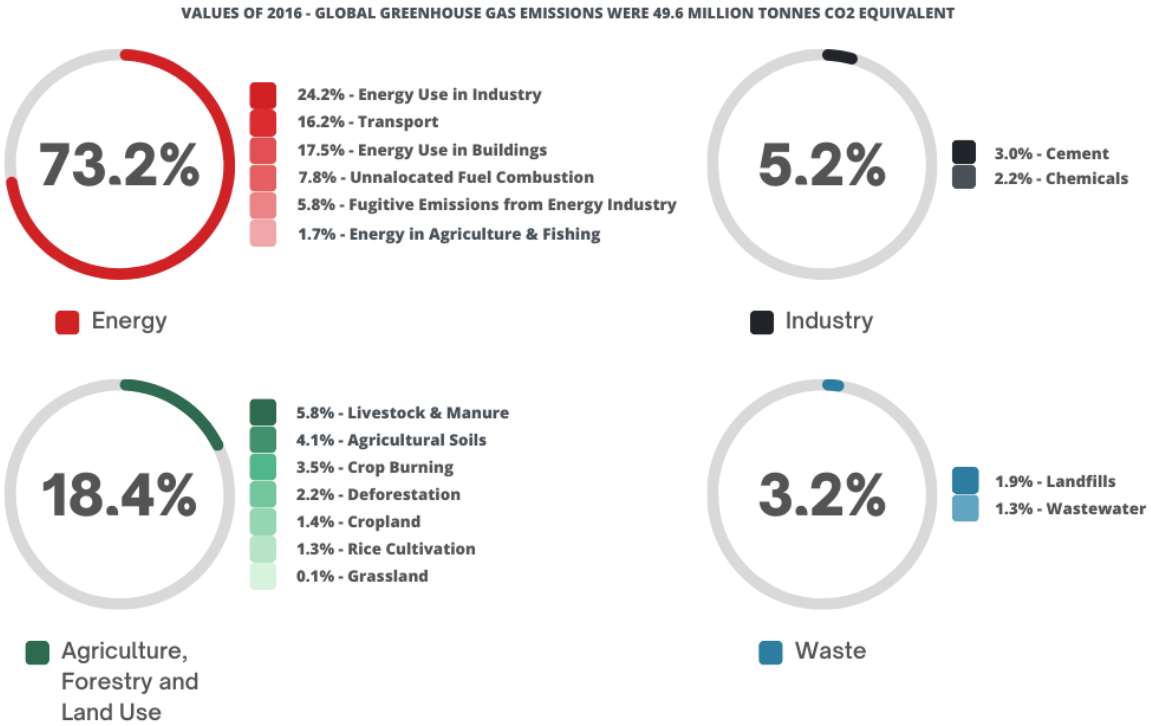

Figure 2. Emissions by sector in 2016. The energy sector was responsible for 73.2% total GHG emissions followed by 18.4% in the agricultural sector, out of 49 Mton CO_2-eq. total emitted (Adapted from [5]).

In the last couple of years, the EU has come around with many incentives to meet emissions targets, especially in the energy sector. The conflict in Ukraine has also made the EU reassess

its energy strategies because it relied on Russian natural gas, accelerating efforts to diversify energy sources, enhance energy efficiency, and ensure secure, affordable, and sustainable energy between its member states. The concept of a circular economy has gained momentum as a sustainable approach to resource management by developing innovative solutions that can not only generate clean and renewable energy, but also utilize waste streams and byproducts to their fullest potential. This will minimize waste and maximize resource efficiency by promoting the reuse, recycling, and repurposing of the materials through the implementation of efficient and sustainable circular processes [4].

Anaerobic digestion is a biological process that breaks down organic materials, such as agricultural waste, food scraps, and sewage. In the absence of oxygen, it allows the generation of biogas, a mixture of CH_4 and CO_2, which can be employed as a renewable energy source for the production of heat and electricity. This process also generates a nutrient-rich byproduct known as digestate, which can be used as a natural fertilizer, completing the circular loop by returning valuable nutrients to the soil. Upgrading biogas to biomethane by removing impurities (i.e., CO_2 and H_2S) gives it a greater calorific value, making it usable as a transport fuel and ready to be injected into existing gas grids and, then, contributing to close circular loops [5]. Microalgae offer a promising way to upgrade biogas, mainly, because of the microalgae' high photosynthetic efficiency and remarkable ability to convert CO_2 into biomass using sunlight. In addition, these microorganisms can grow in diverse environments, including wastewater and brackish water, utilizing nutrients and capturing CO_2 during the process. This makes them excellent candidates for biofuel production and for the generation of valuable chemicals and food additives. By harnessing the potential of microalgae, it is possible to simultaneously reduce GHG emissions, recycle nutrients, and create new renewable energy sources [6].

Integrating circular economy principles into new energy production solutions, such as anaerobic digestion and microalgae cultivation, represents a significant step towards mitigating climate change and building a sustainable future. These innovative technologies not only reduce dependence on fossil fuels but also promote efficient resource utilization and waste management [5].

Embracing these solutions holds the promise of creating a resilient and regenerative energy system that aligns with the principles of sustainability, helps combat climate change, and fosters a circular economy for the benefit of the present and future generations. In addition, incorporating a system that combines all these elements with the agricultural sector, which is one of the most important sectors in the world and the second most polluting sector, can become one step further toward achieving the 2050 EU's targets, reducing waste, and generating value-added products.

1.2. Objectives

The main objective of *Algaementum*'s pilot system is to produce a standard upgraded biogas under 15 €/MWh, with much more affordable equipments than the literature reported photobioreactors (PBRs). This dissertation focused on verifying the system's ability to upgrade biogas, whilst promoting microalgal growth. First, it's necessary to prove that the biogas leaving the digestor has the required properties; then, that the microalgae platform is capable of removing impurities to obtain a ~96% CH_4-rich biogas. The produced biomass characterization is also of interest, regarding value-added products.

The specific objectives of this study were as follows:

1. Characterization of the selected feedstock;
2. Weekly evaluation of gas production in digestor;
3. Algae cultivation;
4. Assessment of CO_2 and H_2S removal using the microalgal platform;
5. Evaluation of microalgal biomass growth during biogas production;
6. Comparison between using different feedstocks in the AD process: first trial using only manure, second using both manure and *Opuntia Figos Indica* (OFI), and a last trial recirculating microalgae culture;
7. Characterization of the system's biomass in different days of the monitorization period to assess the lipid and nutrient content.

1.3. Document Structure

This dissertation document is divided into six sections. Chapter 1 summarizes the work motivation and objectives. Chapter 2 presents the state of the art and background of the work developed, which is essential to its understanding. Chapter 3 presents an overview of the topics and a presentation of *Algaementum*'s project. Chapter 4 describes the materials and methods used in this study. Chapter 5 describes the results obtained during the monitorization of the pilot system, and presents a critical discussion of such system. Finally, Chapter 6 presents conclusions and suggestions for future work.

2. STATE OF THE ART

2.1. EU's Current Environmental and Climate Perspective

The EU approved, in 2019, the European Green Deal (EGD), an ambitious environmental plan for the promotion of sustainable growth. The EGD aims to make the EU climate-neutral by 2050 through initiatives such as increasing the share of renewable energy, improving energy efficiency, and promoting sustainable transport and the circular economy [7]. This can only be achieved by implementing a series of legislation combined with adequate funding and taxation policies. One of the most important short-term objectives established was that by 2030, the net GHG emissions are reduced by at least 55% (compared to 1990 levels). It is necessary to keep revising the EGD and its measures because the reduction plans for emissions in 2030 are currently off track [8]. The Intergovernmental Panel on Climate Change (IPCC) has stated the anthropogenic global warming is currently increasing at 0.2ºC per decade, due to past and ongoing emissions, and in 2052 it is likely that the global warming reaches 2ºC and stays at between 1.5ºC and 2ºC at least until 2100 [2]. The commission has been proposing new initiatives and measures, presented in (Annex B), e.g., a zero-emissions target by 2030 for new city buses and a 90% reduction in emissions for new trucks by 2040.

In order to reduce pressure on natural resources, the EU launched the Circular Economy Action Plan (CEAP) in 2020, as part of its EGD's strategies, seeking to reduce waste and that the resources used are kept in the EU economy for as long as possible, halting biodiversity loss. It has become a a prerequisite to achieving the EU's 2050 climate neutrality target [9]. Sustainable products, circular production processes, and the empowerment of consumers and public buyers will affect sectors such as electronics and information and communications technology (ICT), batteries, packaging, plastics, textiles, construction and buildings, and food [9]. The new regulation outlines certain requirements to guarantee that the products being created are now more durable, repairable, recyclable, and suitable for remanufacturing [7].

In addition, at the heart of the EGD, it is found the Farm to Fork Strategy, a plan that has several objectives: to ensure that Europeans have access to healthy, affordable, and sustainable food; to tackle climate change; to protect the environment and preserve biodiversity; to ensure a fair economic return in the supply chain; and to increase organic farming. A series of targets for 2030 have been set; among others, action will be taken to reduce the use of chemical and more hazardous pesticides by 50%, to reduce fertilizer use by at least 20% and, to aim for 25% of total farmland being used for organic farming. Food systems currently account for nearly one-third of global GHG emissions, consume large amounts of natural resources, result in

biodiversity loss and negative health impacts. Thus, this strategy combined with the Common Agricultural and Fisheries Policies represent key tools to support a *Just Transition* [10]. The EU introduced this transition mechanism as a way to provide financial and technical support to the regions most affected by the move towards a low-carbon economy, i.e., some member states are reliant on fossil fuels or have carbon-intensive industries that employ a significant number of people, making the energetic transition more difficult. As such, at least €55 billion will be mobilized between 2021-2027 to ease employment opportunities and reskill, improve energy-efficient housing, and fight poverty, making the transition to low-carbon technology attractive for investment, providing financial support and investment in research and innovation (R&I), investing in new green jobs, sustainable public transport, digital connectivity, and clean energy infrastructure [7].

Portugal has aligned itself with the EU's climate targets and has set its national targets for carbon neutrality. An overall analysis of the evolution of the Electric Energy Sources in Portugal, from 2000 to 2022, is represented in Figure 3.

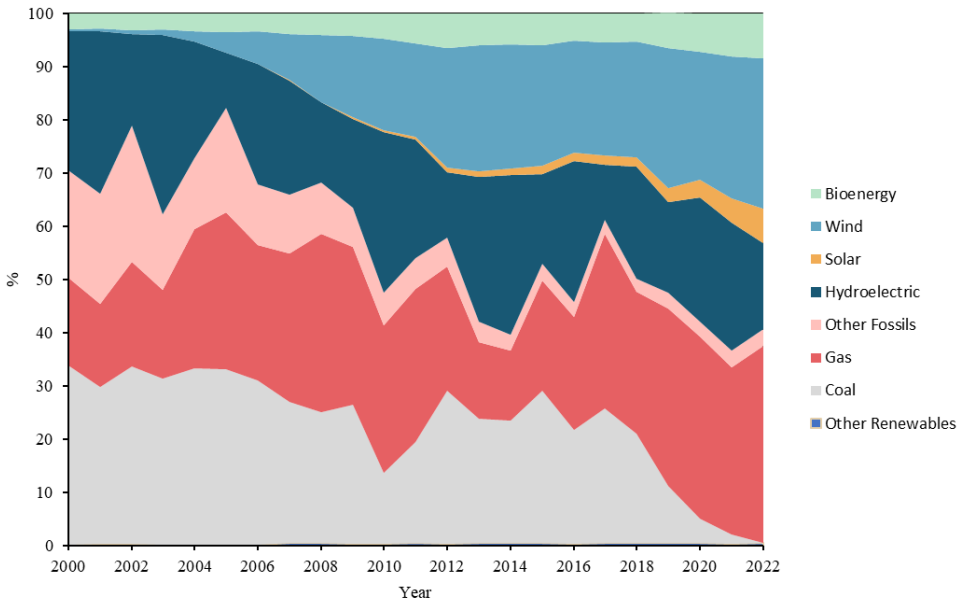

Figure 3. Evolution of electric energy sources in Portugal. The graphic shows the elimination of coal as an energy source and the increase, the progressive reduction of other fossils and the increase in wind energy, slight increase in bioenergy and natural gas (Adapted from [11]).

The country relies on wind energy production and solar power as renewable energy, which accounted for over 54% of total energy production in 2020. In April 2023, the record for solar energy production was recorded, accounting for 15.13% of total production. Wind power also accounted for 35.71% of power generation. In April 2020, energy from the water was around 36% of the total Portuguese value, whereas in the same month of 2023, the value decreased to 14%, mainly due to droughts. Although 40% of Portuguese energy comes from fossil fuels,

recent data shows that the country *"is on the right track for an energy transition*". Portugal was the fourth European country to achieve progressive elimination of coal in Europe. Furthermore, "*it is one of only four EU countries with a target of 100% clean energy by 2030, having substantially increased its ambition in response to the invasion of Ukraine*" [11].

The EU might already be on a path further away from fossil fuels, but the gas crisis and invasion of Ukraine undoubtedly accelerated the process significantly. "*The two 'twin' crises have brought into focus the major risks to energy security and stability of dependence on fossil fuels, forcing governments to take swift action to accelerate the adoption of renewable energy*" [11]. This led to the creation of the REPowerEU plan, another important EGD strategy launched in 2022, which made possible to significantly reduce the imports of Russian gas from 41% in August 2021 to 8% in September 2022 in all gas pipelines. This plan also reinforces the need for affordable energy supplies, new gas storage rules, energy savings, and investments in renewables [4].

Biomethane can play an important role in achieving the REPowerEU plans of diversified gas supplies and reducing the EU's dependence on Russian fossil fuels, while simultaneously decreasing the exposure to volatile natural gas prices. As a renewable and dispatchable energy source, scaling up the production and use of biomethane also helps address the climate crisis. Therefore, biomethane production must reach 35 billion cubic meters (bcm) annually by 2030. The proposed actions aim to support production of a sustainable potential volume of biogas to further upgrading to biomethane and to direct biomethane production from waste and residues, avoiding the use of food and feed feedstocks leading to land use change issues. These actions should also create preconditions for the sustainable upgrading and safe injection of biomethane into the gas grid. In addition, by 2024, EU countries will have to collect organic waste separately, which will be an opportunity to upscale the production of sustainable biomethane and create income opportunities for farmers and foresters [12].

To achieve the biomethane targets set in the REPowerEU plan, one of the key proposed actions is the creation of the Biogas and Biomethane Industrial Partnership **(**BIP**)**, launched in September 2022, to promote the sustainable production and use of biomethane. The BIP will promote engagement between EU countries, industry representatives, feedstock producers, academic organizations, and NGOs through incentives for upgrading biogas into biomethane, which will help reduce the costs linked to biomethane production for individual economic operators. R&I of biomethane in the EU are supported by the Horizon 2020 and Horizon Europe programs for the development and demonstration of innovative biomethane technologies and market uptake measures [13].

As a sustainable energy source that can be transported across the existing natural gas transport system, biomethane can make Europe's energy grid greener and more independent. Doing so will require increased use of the European common biomethane market, which will also enable investments and promote cross-border trade. REGATRACE, a project created by the EU, provides an efficient trade system based on the issuing and trading of biomethane gas certificates. These provide Guarantees of Origin (GoO) and Proof of Sustainability (PoS) for biomethane and other renewable gases. Between end-2021 and mid-2022, the amount of biomethane transferred via the ERGaR CoO Scheme (European Renewable Gas Registry Certificate of Origin) increased from 30 GWh to 159 GWh. This ten-fold increase over current production is drawn from a range of sources. Upgrading all existing biogas facilities to produce biomethane is expected to contribute 8 bcm, while the remainder is generated by increasing the collection and processing of feedstocks, such as woody biomass, organic matter, and wastewater. Innovative technologies will shape the exact contribution of each element to the 2030 target [13]. All the information relatively to the Innovative Biomethane for the REPowerEU Report can be found in Annex C.

Bioenergy should play an essential role in reaching targets to replace petroleum-based transportation fuels with a viable alternative and in reducing long-term CO_2 emissions, if environmental and economic sustainability are considered carefully. The world continues to increase its energy use, brought about by an expanding population and desire for a greater standard of living. This energy use, coupled with the realization of the impact of CO_2 on the climate, has led to the reanalysis of the potential of plant-based biofuels. This term refers to liquid or gaseous fuels for the transport sector, produced from all types of biomass, since the organic fraction of almost any form of biomass (from plants, algae, and other microorganisms), including sewage sludge, animal waste, and industrial effluents, can be broken down into CH_4 and CO_2 [14].

2.2. Anaerobic Digestion

Biogas is receiving increased attention with the intensification of renewable energy interests, and the application of anaerobic digestion (AD) as a core treatment technology in biogas plants should be highlighted, as this process combines wastewater treatment and the generation of bioenergy, based on the conversion of the organic fraction of materials to a methane-rich gaseous mixture that may meet the energetic demands of biogas plants [15].

AD is the process by which organic materials are biologically treated in the absence of oxygen by naturally occurring bacteria to produce biogas, a mixture typically consisting of 40–75% CH_4, 25–60% CO_2, 0.005–2% H_2S, and other trace constituents such as H_2, O_2 or N_2 at trace levels [16]. The production of biogas as a renewable energy carrier through AD has been shown to contribute significantly to the mitigation of GHG emissions when replacing traditional fossil fuels. Upgraded biogas with a methane concentration greater than ~96% is suitable for the substitution of natural gas, which can be injected into the gas grid or compressed for use as fuel in transportation [16].

A large variety of wet organic feedstocks, including energy crops, agricultural and livestock residues, and industrial organic waste, can be converted to biogas via AD. Thus, from an environmental and resource-efficient perspective, biogas has several advantages over other biofuels because of the organic materials and microorganisms required for its synthesis [17].

Digestate (*i.e.*, digester effluent) is the main waste byproduct of the anaerobic digestion of agricultural waste. Its use as a fertilizer is limited by seasonal application and finite arable land. Excessive irrigation of raw digestate can cause environmental problems, such as human exposure to pathogens, ammonia emission, antibiotic resistance genes, and other micropollutants in crops and soils, and the migration of excess nutrients into water bodies. Traditional physicochemical methods and nitrification–denitrification biotechniques are not always sustainable, particularly for high digestate flows with high nitrogen concentrations [17].

2.2.1. Principles

AD is a multi-step process that occurs in a sequential order as defined by the dominant microbial population of the digester. The four major stages of AD include hydrolysis, acidogenesis, acetogenesis and methanogenesis, as schematized in Figure 4.

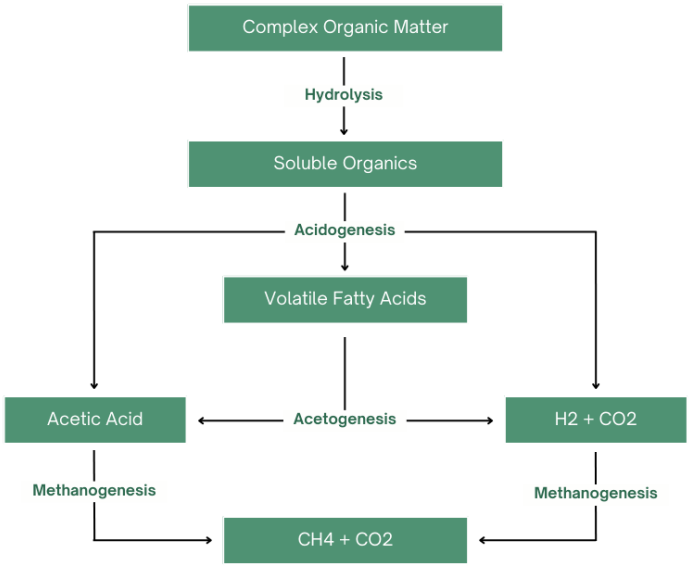

Figure 4. Anaerobic Digestions Steps (Adapted from [14])

The first step, hydrolysis, corresponds to the disintegration of complex organic matter into monomers which will be then converted by fermentative bacteria. Carbohydrates, proteins, and lipids are broken down into monosugars, long-chain fatty acids (FAs), and amino acids for further utilization by the bacteria. Acidogenesis is the principal fermentation phase for the conversion of monomeric compounds to short/long chain FAs with the simultaneous evolution of gaseous CO_2 and H_2. Acetogenesis is the metabolic link between the major fermentation products of the acidogenesis and methanogenesis phases. Acetogens convert higher organic acids and alcohols into acetate and H_2. Acetogenesis is thermodynamically favorable only under low partial H_2 pressures, leading to a syntrophic relationship between H_2-evolving acetogens and H_2-consuming methanogens, which defines the biomethane production efficiency along with effective hydrolysis. At high H_2 concentrations, autotrophic homoacetogens consume H_2 for the reduction of CO_2 to acetate, thereby reducing CH_4 generation potential. The final phase of AD is methanogenesis, which is dominated by methane evolving/methanogenic archaea that can utilize diverse substrates such as acetate, CO_2, H_2, formate, and methylated carbons as a source of energy and carbon. H_2 is consumed during methanogenesis, and acetoclastic methanogens decarboxylate acetate and evolve into methane and hydrogen. However, very few methanogenic bacteria are acetoclastic, and hydrogenotrophic methanogens also utilize CO_2/H_2 as a substrate to generate CH_4 [18].

2.2.2. Operating Conditions

In an AD process, a special care should be taken to ensure that methanogens outcompete homoacetogens and methanotrophs for favorable methane production via careful control of

operating parameters [18]. The performance of biogas plants can be controlled by monitoring parameters such as total solids (TS), volatile solids (VS), volatile fatty acid (VFA) concentration, carbon to nitrogen ratio (C/N), inoculum to substrate ratio (ISR), temperature (T), pH, alkalinity, and mixing intensity [19].

The C/N ratio is one of the most important parameters in AD, as carbon is the source of energy for bacteria and nitrogen is a growth nutrient. If the nitrogen content in the substrate is low, the population of bacteria grows slowly, and it takes longer to decompose the substrate; however, a high nitrogen content causes the production of excessive ammonia gas, which hinders bacterial development. The rate of carbon digestion in anaerobic systems is 30-35 times faster than the rate of nitrogen conversion, for which the ideal C/N ratio is 30:1–35:1.

Temperature and pH have a greater impact on the hydrolysis and acidogenesis processes than biological factors. In most anaerobic experiments, mesophilic (35-37 ºC) and thermophilic (55-60 º C) environments are selected because a faster rate of hydrolysis is observed, which results in more biogas production. The ideal pH also varies from phase to phase, as the hydrolysis pH is around 6.0, for acetogenesis is between 6.0 and 7.0 and the methanogenic bacteria prefer a pH between 6.5 and 7.5. The biochemical reactions can change the medium pH without sufficient buffering capacity. Alkalinity in the reaction mixture should be between 2500-5000 mgCO_3/L to avoid large fluctuations due to the formation of VFAs.

Concerning the influence of the ISR (inoculum to substrate ratio), a high ISR boosts the degradation rate and buffer capacity, but it also means that fewer substrates can be handled. An ISR of 2 is recommended for most substrates, whereas 4 can be applied to rapidly degradable substrates (e.g., glycerol and food waste) and 1 for slowly biodegradable substrates (e.g., lignocellulosic materials).

Microbial activity inhibition may occur because microorganisms are sensitive to several compounds present in the reaction mixture or produced during the degradation process, such as ammonia (NH_3), sulfide (S^{2-}), heavy metals, and oxygen (O_2); hence, it is important to control the selected parameters appropriately. The values reported in the literature are listed in Table 1.

Table 1. Inhibitory compounds concentration for methane formation (Obtained from [19])

Compound	**Effect**	**Inhibitory Concentration (mg/L)**
Oxygen	The presence of O_2 does not inhibit the facultatively anaerobic acidifying bacteria activity, but methanogenic bacteria are obligatorily anaerobic.	0.1
Sulfur	H_2S is formed in the stage before methane formation and the sulfides toxicity may be problematic for different groups of microorganisms, besides competition for the substrate, leading to unwanted H2S formation, which is also associated with corrosion problems in the reactor or downstream equipment.	50 (H_2S) 100 (S^{2-}) 150 (Na_2S)
Fatty Acids and Amino Acids	Present in the substrate and degraded during methanogenesis. High concentration may rise the culture's acidity and disturb the digestion phase.	6000 (VFA) 50 ($C_4H_8O_2$)
Ammonia	NH_3 and NH^{4+} may result from the degradation of nitrogen compounds. Ammonia is beneficial to AD at low concentrations because nitrogen is an essential nutrient; in high concentrations it can be toxic to microorganisms.	80 (NH_3) 1500 (NH^{4+})
Heavy Metals	Trace elements allow stimulation of bacterial activity in low concentrations, working as nutrients; high concentrations may also result in toxic effects.	28-300 (Cr) 10-300 (Ni) 40-300 (Cu) 400 (Zn) 70-600 (Cd) 8-340 (Pb) 1500 (Mn)

2.2.3. Technologies

Anaerobic digesters can be built as single-phase or two-phase reactors to produce biogas, depending on the type of substrate, as well as economic and technical issues.

In a single-phase digester, all steps (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) proceed in a single reactor. This system provides advantages such as higher sludge stabilization, simple operation, and low capital cost over a two-phase reactor. However, VFAs and other organic acids produced during acidogenesis may impair the performance of methanogenic bacteria. Furthermore, a single-phase reactor can be operated in both the batch and continuous modes. In a single-phase batch reactor, substrates are placed at the beginning and removed completely after a certain period and can also be operated continuously using, for

example, a continuous stirred tank reactor (CSTR) or an up-flow anaerobic sludge blanket (UASB).

In a two-phase reactor, the effluent from the acidification reactor provides a substrate (mainly VFAs) for methanogenesis. VFAs pumped into the methanogenesis reactor are quickly consumed by methanogenic bacteria without accumulating in the reactor or inhibiting the reaction. However, when the organic load is too high or hydrolysis is too fast, the methanogenesis reactor still has the potential to be inhibited, but since there are two favorable environments for microorganisms. Anyway, these are theoretically more efficient. To stabilize two-phase anaerobic digestion, a hybrid anaerobic solid-liquid (HASL) digestion system for food waste was built. The acidification reactor and leachate were diluted by recycling the effluent from the methanogenic reactor.

For appropriate operation of AD equipment, parameters such as tank design, mixing, solids retention time (SRT), hydraulic retention time (HRT), organic loading rate (OLR), and volatile solids reduction must be considered, in addition to the feedstock parameters. A summary of the main AD digesters and corresponding process parameters are summarized in Table 2 [19].

Table 2. Description and process parameters for anaerobic digesters (Adapted from [19])

Equipment	Description	Parameters
Single Stage AD System	Standard-rate digestion process – batch digesters are unheated, unmixed and fed intermittently; supernatant and digested sludge are withdrawn periodically.	T = 20-30 (ºC) pH = 6.8-7.2 SRT = 30-60 (d) OLR = 0.6-1.6 (kgVS/m^3/d) VSR = 30-40 (%)
	High-rate digestion process – digester is heated and completely mixed; solids loading considerably higher than that of standard-rate; the digester feed and sludge withdrawal are done on a regular schedule each day.	T = 30-38 (ºC) pH = 6.8-7.2 SRT = 15-20 (d) OLR = 1.6-4.8 (kgVS/m^3/d) VSR = 40-60 (%)
Staged AD System	A high rate mesophilic (CSTR/UASB) and standard anaerobic digesters are arranged in series; the standard digester stores digested sludge, clarifies the supernatant and provides additional digestion and gas recovery.	T = 30-38 (ºC) pH = 6.8-7.2 SRT (1st reactor) = 7-10 (d) SRT (2nd reactor) = variable OLR = 0.5-1.6 (kgVS/m^3/d) VSR = 40-60 (%)

Table 2 (cont). Description and process parameters for anaerobic digesters (Adapted from [19])

Two-Phase AD System	Two digesters are operated in series at different temperatures. The first or second phases are either the thermophilic acidogenic anaerobic digester or mesophilic acidogenic anaerobic digester.	T (mesophilic) = 30-38 (ºC) T (termophilic) = 50-57 (ºC) pH (both reactors) = 6.8-7.2 SRT ($1^{st}/2^{nd}$ thermophilic acid phase) = 3-5 / 7-15 (d) SRT ($1^{st}/2^{nd}$ mesophilic acid phase) = 7-10 / >5 (d) OLR = 4.8-6.4 ($kgVS/m^3/d$) VSR = 50-65 (%)

2.2.4. Co-Digestion

A co-digestion system consists of combining substrates as feedstock for AD and has been proven to be more efficient in terms of biogas productivity, as well as other benefits, such as the enhanced balance of nutrients, synergetic effects of bacteria, and improved process stability. It should be noted that a high organic fraction of sole food waste would result in a high C/N ratio and too low buffer capacity, caused by the high biodegradability of the substrate. To solve these problems, co-digestion of food waste with livestock manure, sewage sludge, or green waste has been proposed [19]. Currently, livestock manure is considered an optimal co-substrate, which has a high nitrogen content, high buffering capacity, and a wide range of nutrients required by methanogens. Green waste is another popular co-substrate with food waste; however, green waste may be rich in lignin content and may reduce the biodegradability rate of food waste, as lignin is hardly hydrolyzed by bacteria [19]. A summary of the substrates reported in the literature is presented in Table 3. This summary only included substrates that were considered or closely related with this dissertation study.

Table 3. Biogas output using different manure feedstocks, OFI and different working anaerobic digesters

Feedstock	Parameters/Observations	Biogas Output	Work
Cattle manure, solid manure, including bedding material (i.e., straw), and poultry manure.	Active digestor volume of 1000 m^3, running at 38 ºC. Average OLR of 2.7 kgVS/m/d and HRT of 30 days. Fe supplement added to the liquid manure. ISR set to 3:1. Inoculum from a wastewater treatment plant was used, with TS content of 3.1% wet weight and VS content of 2.0%.	BMP = 189.6 ± 2.7 NmL/gVS 50% BMP after 2d 100% BMP after 10d T = 52 ºC pH = 7.8 GP = 6.983 mL/d VFA = 0.18 g/L H_2S = 31 ppm CH_4 = 64%	[20]
Liquid and solid cattle manure with high dry matter content, including straw used as bedding material.	Total working volume of 1300 m^3. Average OLR of 3.5 kgVS/m3/d and HRT of 22 days. Fe supplement applied to the solid fraction. Inoculum from a wastewater treatment plant was used, with TS content of 3.1% wet weight and VS content of 2.0%.	BMP = 169.0 ± 8.7 NmL/gVS 50% BPM after 4d 100% BMP after 8d T = 52 ºC pH = 7.71 GP = 5.745 mL/d VFA = 0.29 g/L H_2S = 281 ppm CH_4 = 57%	[20]
Raw cattle manure	Digesters volume of 500 mL, operated for 117 days. Average OLR of 4gVS/L/d and HRT of 28 days.	BMP = 181 ± 30 L/kgVS T = 35 ºC pH = 7.4 ± 0.7 TS = 61 ± 5.3% VS = 46.1 ± 6.4%	[21]

Table 3 (cont). Biogas output using different manure feedstocks, OFI and different working anaerobic digesters

Slaughterhouse residual effluent (SWW) and *Opuntia Figus Indica (OFI)* cladodes	Total working volume of 6 L (8 L capacity). Ideal mixture of 75% SWW + 25% OFI. Operation temperature of 38 ºC and pH 7.4. OLR of 64 gVS/L/d; fresh manure was prepared with water in a 1:10 ratio to boost the biogas production and a previously prepared anaerobic media served as inoculum. TS 6.17%, VS 84.59%, COD 14.960 mg/L, C/N = 20	Yield in 45 days: (SWW + OFI) = 87 L (SWW) = 0.8 L (OFI) = 37 L CH_4 content (v/v): (SWW + OFI) = 57% (SWW) = 24% (OFI) = 33%	[22]

2.3. Microalgae

Algae are the primary producers in aquatic ecosystems. Many species of algae are present, such as green, red, and brown algae belonging to the Chlorophyta, Rhodophyta, and Phaeophyta groups, respectively. Algae are a diverse group of predominantly aquatic photosynthetic organisms of tremendous ecological importance because they are the beginning of the food chain for other animals. Algae provide the basis for the aquatic food chain, and they play a substantial role in maintaining the CO_2 of the carbon cycle via photosynthesis in all biogeochemical cycles [14]. Algae also play an important role in the self-purification of contaminated natural water and offer an alternative for advanced nutrient removal from water or wastewater. The idea of incorporating microalgae as an agent for bioremediation was first proposed by Oswald and Gotaas in 1957; the recovered biomass was converted to methane, which was considered a major source of energy.

Among the potential sources of biogas, photosynthetic microalgae are among the most efficient producers of biomass. Photosynthetic pigments, including chlorophyll, are of upmost importance because they provide oxygen and are a source of energy for all living organisms. Plant and algal growth are affected by photosynthesis speed, which depends on the availability of CO_2. Biological CO_2 fixation by algae is another form, in which sunlight is used to reduce CO_2 to carbon. Therefore, a promising approach seems to be the use of fast-growing algae species for anaerobic fermentation to produce biogas, which can then substitute natural gas resources. Moreover, since algae is a key primary producer global-wide, algae biomass is considered an essential biological natural resource, playing an important role in nutrients, food, fertilizer, pharmaceutics and biofuel [14].

2.3.1. Growth and Harvesting

The main advantages of culturing algae as a source of biomass are: (1) specificity for CO_2 sequestration with high photosynthetic yields; (2) the best growth rate among plants, in fresh, salt, and wastewater; (3) high oil content; (4) the ability to produce non-toxic and biodegradable biofuels; and (5) high value of algal biomass, including feed, food, nutrition, pharmaceutical chemicals, fertilizer, and aquaculture [14].

In general, there are four main conditions for microalgae: photoautotrophic, heterotrophic, mixotrophic, and photoheterotrophic. Under photoautotrophic cultivation, microalgae can trap light energy as an energy source and assimilate CO_2 as a carbon source. Heterotrophs utilize organic compounds (e.g., glucose, acetate, and glycerol) as both energy and carbon sources in the dark, whereas light is required to use organic compounds as carbon sources for photoheterotrophic processes. Mixotrophy is broadly defined as a growth regime in which CO_2

and organic carbon are simultaneously assimilated and both respiratory and photosynthetic metabolism operate concurrently [23].

To achieve optimal microalgae growth, high microalgae productivity, and maximizing their potential for the desired application, several important aspects and parameters need to be carefully controlled, such as light intensity, salinity, nutrient concentration, temperature, mixing and aeration, pH, and CO_2 supply. Choosing the right strain for the intended application is also crucial, since different species have varying growth rates, nutrient requirements, and tolerance to environmental conditions. The selection of cultivation system is also important. All these aspects need to be carefully and continuously monitored. After cultivation, the microalgae biomass has to be separated from the growth medium and recovered through a series of downstream processing operations. Algae grow in dilute suspensions (<0.5 kg/m^3 dry biomass), and the negative surface charge results in dispersed stable algal suspensions, which turns microalgae harvesting a major hurdle in industrial scale processing. It is estimated that the latter can account for 20–30% of the total biomass production costs [23]. A summary of microalgae processing, together with some data from literature, is shown in Table 4.

Table 4. Microalgae growth and harvesting parameters and processes (Obtained from [18])

Parameters	Working Mode or Technique	Considerations	Remarks	Work
Light	Light is required for synthesis of ATP and NADPH, which drive the dark reactions of photosynthesis that produce carbon skeletons.	Light duration and intensity affect microalgae photosynthesis, has influence on its biochemical composition and biomass yield. Appropriate light penetration and uniform distribution, as well as duration, is necessary in bioreactors for microalgae to avoid photooxidation and growth inhibition [24]. Light reactions of photosynthesis: $2NADPH + 2H_2 + 3ADP + 3Pi \rightarrow 2NADPH_2 + 3ATP + O_2\uparrow$ Dark reactions: $CO_2 + 4H^+ + 4e^- \rightarrow (CH_2O) + H_2O$	Mata et al. [25] reported that an aerated culture of microalgae under 12,000 lx intensities for 12 h of daylight produced a higher biomass yield, whereas biomass decreased when the light intensity was reduced. Khan et al. [26] reported that *microcystis aeruginosa* give maximum biomass and carbohydrates productivity at red LED light of about 5000 lx. Daliry et al. most recently reported the maximum growth rate and lipid production by *Chlorella vulgaris* at light intensities of 5000–7000 lx [27]. The optimum level of light intensities for most of the microalgae species are about 200–400 μM photons/m2/s [28].	[18], [24], [25], [26], [27], [28]
Temperature	Increasing temperature to the optimum range exponentially increases algal growth, but an increase or decrease in the temperature beyond the optimal point retards or even stops algae growth and activity.	Growing microalgae cultures at non-optimal temperatures will result in high biomass losses, particularly in outdoor culture systems. Low temperatures affect photosynthesis by reducing carbon assimilation activity, whereas too-high temperatures reduce photosynthesis by inactivating the photosynthetic proteins and disturbing the balance of energy in the cell. Higher temperature also reduces cell size and respiration.	Converti et al. [29] found that a culture of *Chlorella vulgaris* produced more carbohydrates and lipids if grown at 25 °C than at 30 °C. Kitaya et al. [30] found that temperatures between 27 and 31 °C were optimum for several microalgae species.	[29], [30]

Table 4 (cont). Microalgae growth and harvesting parameters and processes (Obtained from [18])

pH	Increasing the pH will increase the salinity of the culture media, which is very harmful for algae cells.	Most algae species are pH sensitive and few can endure a range of pH as broad as that tolerated by *C. vulgaris*.	*C. vulgaris* maximum growth rate and biomass productivities are reported at pH 9–10.	[24]
Nutrient Concentration	Nitrogen, phosphorus and carbon form the backbone of microalgae ($CH_{1.7}O_{0.}4N_{0.15}P_{0.0094}$) and are classified as macronutrients required for algal growth.	Nitrogen limitation in the microalgae culture, can reduce growth and biomass productivity although they increase production of carbohydrates and lipids. The micronutrients Mo, K, Co, Fe, Mg, Mn, B, and Zn are only required in trace amounts but have a strong impact on microalgae growth, as they influence many enzymatic activities in algal cells.	It has been reported the growth of *Chlorella* declined when the concentrations of nitrogen and phosphorus reduced from 31.5 and 10.5 mg/l respectively. 0.5 g/L nitrogen has been proved to be optimum concentration for *Chlorella vulgaris* at which it produces 3.43 g/L biomass.	[27]
Mixing and Aeration	Provide uniform distribution of nutrients, air, and CO_2 in microalgae culture. They also enable the penetration and uniform distribution of light inside the culture and prevent the biomass from settling and causing aggregation.	If all the other requirements are met but there is no mixing, biomass productivity will be lowered significantly. Thus, microalgae cultures must be continuously mixed to keep all cells in suspension with free access to light. A proper mixing system in a photo-bioreactor not only enables nutrient dissolution and light penetration into the culture but also provides for efficient gaseous exchange.	-	[31]

Table 4 (cont). Microalgae growth and harvesting parameters and processes (Obtained from [18])

CO_2 supply And Strain Selection	Microalgae are potential CO_2 bio-mitigation agents with their high CO_2 fixation ability and photosynthetic efficiencies.	Microalgae are tolerant to high concentrations of CO_2. Some strains are tolerant to up to 100% CO_2. The increase in CO_2 concentration is also related to increased lipid accumulation and polyunsaturated FAs. Acidification of the culture medium and disruption in pH homeostasis of the microalgae cells is another feature.	*Chlorella sp.* TISTR 8263 has shown a specific growth rate of 0.466 d^{-1} (μ) at 50% CO2 with 25% lipid content and 13.9 mg/L/d lipid productivity [32]. *Chlorella vulgaris* FACHB-31, *Scenedesmus obliquus* FACHB-416, and *Neochloris oleabundans* UTEX-1185 have grown well in the presence of 45-55% CO2 in the presence of activated sludge, with only a slight decrease in biomass productivity (0.138-0.153 g/L/d) [15]. In *chlorococcum littorale* photosynthesis has been inhibited due to intracellular acidification, which didn't occur in *Chlorella sp.* UK00 in the same conditions, since the latter is highly CO_2 tolerant	[15], [32]
Cultivation System	Open/Raceway Ponds	Suspension is mixed with a paddle wheel - relatively easy and cheap to operate. Low biomass productivity and strongly limited by contamination (of other algae or bacteria) and climatic conditions (very difficult to maintain an open algal culture in tropical weather).	Over 90% of the world's microalgae biomass production	[33]
	Closed Photobioreactors (PBRs)	Tubular, Flat Plate or Column Reactors (Bubble or Airlift) - efficiency and biomass productivity highly dependent on the construction materials, hydrodynamics, efficient mixing, mass and light transfer, heating/cooling, CO_2 supply and oxygen removal.	Less prone to contamination (not immune); growth parameters are better controlled; high surface-to-volume (S/V) ratio leading to higher volumetric productivities and cell concentrations; reduced evaporation. More expensive and difficult to scale up.	

Table 4 (cont). Microalgae growth and harvesting parameters and processes (Obtained from [18])

Harvesting	Centrifugation	Solid-liquid separation is driven by a much greater force (gravity) to promote accelerated settling of microalgae cells	Used for almost all types of microalgae reliably and without difficulty. Only feasible if the targeted metabolite is a high-value product because the process is highly energy intensive, which is problematic at a large-scale level due to power consumption and consequent increased production costs.	[34]
	Filtration	Physical separation process by filter (membrane), characterized by their efficiency, reliability and safety for solid-liquid separations. Microalgae cells are separated from the culture medium and water recycling is possible. Different membrane materials and pore sizes are used – polyvinylidene fluoride (PVDF), polyvinyl chloride (PVC) and polysulfone (PS) in both microfiltration (MF) and ultrafiltration (UF).	PVDF polymer membrane and UF show superior results and better fouling resistance.	[35]
	Chemical Flocculation	Addition of flocculants to counter the surface charge on the algae. Inorganic coagulants – aluminium and ferric salts such as aluminium sulphate ($Al_2(SO_4)_3$), ferric sulphate ($Fe_2(SO_4)_3$) and ferric chloride ($FeCl_3$); these inorganic multivalent metal salts however, are toxic and expensive when commercial-scale used. Non-toxic organic polymers – polyacrylamide copolymers, chitosan and cationic starch; negative for later processing as residues are left in the algal biomass, culture medium recycling, and still not economically feasible due to high prices.	Optimum flocculation of marine *Chlorella sp.* with 143 mg($FeCl_3$)/L, pH 8.1 and settling time 40 min. Harvesting efficiency of *Nannochloris oculata* of 96% using 0.0016 ng ($AlCl_3$)/cell, pH 5.3.	

Table 4 (cont). Microalgae growth and harvesting parameters and processes (Obtained from [18])

Drying	Solar Drying	Low capital costs without use of fossil fuel energy. Slow process that requires large areas of land and is highly dependent on weather conditions.	-	[34]
	Spray Drying	Rapid drying process, high drying efficiency and direct drying into powder. High capital and operational costs, with significant deterioration of microalgal pigments.		
	Freeze Drying	Operates under vacuum, protecting some substances easily oxidized (such as lipids) and presents high cell recovery. High capital and operational costs.		

2.3.2. Biogas Upgrading

Biogas purification is a process in which impurities, such as sulfides and ammonia, are removed. Biogas upgrading, on the other hand, is a process that removes CO_2 and the end product is biomethane. Biogas requires cleaning for two main reasons: to improve the calorific value of the product gas and to reduce the chance of damaging downstream equipment due to the formation of harmful compounds. CO_2 in raw biogas decreases the specific heating value and increases the energy demand for gas compression and transportation; CO_2 accounts for 25-50% of the biogas volume basis and a decrease in its content will result in lower transportation costs. H_2S (0-2%v/v) is also highly corrosive, toxic, and malodorous. In addition to the reduction in GHG emissions and other environmental benefits, upgraded biomethane is suitable for injection into the national gas grid or to be used as a vehicle fuel [36].

CO_2 biofixation using photosynthetic microorganisms is the most important and most effective carbon sequestration method on earth; they convert inorganic carbon for growth; hence they can convert CO_2 to biomass. Microalgae have a higher photosynthetic efficiency, higher biomass production and faster growth rates compared to other energy crops, thus, the scale up of microalgae technologies has potential to reduce carbon emissions and ease energy supply, while generating value-added products in the process [32].

Apart from the aforementioned primary determinants of effective CO_2 biosequestration in microalgae-based systems, there are other major factors: PBR illumination, gas flow and biomass concentration in the medium, as well as the methods used for stirring, for ensuring optimal gas retention in the biosequestration zone, and for maintaining interphase contact. The type of culture system, and the mechanism used to supply CO_2-rich gas, are factors cited as the most important [18]. Some process examples are shown in Table 5.

Table 5. Process examples of biogas upgrading using different microalgae strains (Obtained from [18])

Strain	Process Description	Remarks	Work
Microalgal consortium comprised of *Chlorella vulgaris*, *Stigeoclonium tenue*, *Nitzschia closteirum* and *Navicula amphora*	Outdoor horizontal hybrid tubular PBR: 11.7 m^3 capacity, connected to an external 45L absorption column; raw biogas from microalgal biomass. AD: 86.2% CH_4, 13.7% CO_2, 0.1% H_2S; agricultural wastewater as nutrient medium.	RE: > 91% Upgraded biogas: 94.1-98.9% CH_4 High alkalinity favours CO_2 absorption, the liquid to gas ratio influences biogas CO_2 solubility.	[37]
Chlorella sp.	Raw biogas from pilot scale UASB AD treating vinasse with: 58.9–81.8% CH_4, 11.6–38.1% CO_2, 0.4–0.8% H_2S. Transparent polyethylene bag as PBR: initial biomass 877.68 mg dry weight, 25 °C, light intensity 800 µmol/m^2/s, light dark cycle as 12h:12h, mixed LED light with red:blue at 5:5, mixing by shaking the bag thrice a day. Filtered and UV sterilized biogas slurry; desulfurized raw biogas with 64.21% CH_4, 31.38% CO_2, 3.79% H_2O, 0.68% O_2, <50 ppm H_2S.	pH 8.1 RE: 62% Biomass production: 494.23 mg/L Nutrient RE: 78.91% COD, 73.05% TN, 67.54% TP Upgraded biogas: 93.68% CH_4, 1.57% CO_2, 3.8% H_2O, 0.99% O_2, <50 ppm H_2S	[38]

Table 5 (cont). Process examples of biogas upgrading using different microalgae strains (Obtained from [18])

Chlorella vulgaris FAHCB 31	Transparent polyethylene bag as PBR: initial biomass 0.068 g/L, 25 °C, light intensity 150 µmol/m^2/s, light dark cycle as 14h:10h, mixing by shaking the bag thrice a day. Filtered and UV sterilized AD slurry; desulfurized biogas from AD treating piggery wastewater with 61.38% CH_4, 32.57% CO_2, 5.52% H_2O, 0.54% O_2, <0.005% H_2S.	Biomass production: 0.139 mg/L/d Nutrient RE: 64.76% COD, 55.67% TN, 53.84% TP Upgraded biogas: 83.46% CH_4,	[39]
Chlorella vulgaris, Scenedesmus obliquus	Closed glass PBRs: 16.8L capacity, 25 ºC, continuous illumination with 200 µmol/m^2/s, light dark cycle as 12h:12h. Desulfurized biogas: 61.23% CH_4, 34.69% CO_2, 3.22% H_2O, 0.84% O_2, <0.005% H_2S.	RE: 63.48% Biomass production: 0.1 g/L/d Nutrient RE: 79.86% COD, 80.23% TN, 89.37% TP Upgraded biogas: 83.24% CH_4 Microalgae co-culture with nitrifying-denitrifying sludge proved to work better than mono algal culture or co-culture with fungi.	[40]

2.3.3. Value-Added Products

In addition to biodiesel, various high-value chemical compounds such as pigments, antioxidants, β-carotenes, polysaccharides, vitamins, and biomass can be extracted from microalgae, and they are largely used as bulk commodities in different industrial sectors (e.g., pharmaceuticals, cosmetics, nutraceuticals, and functional foods) [41], as schematized in Figure 5.

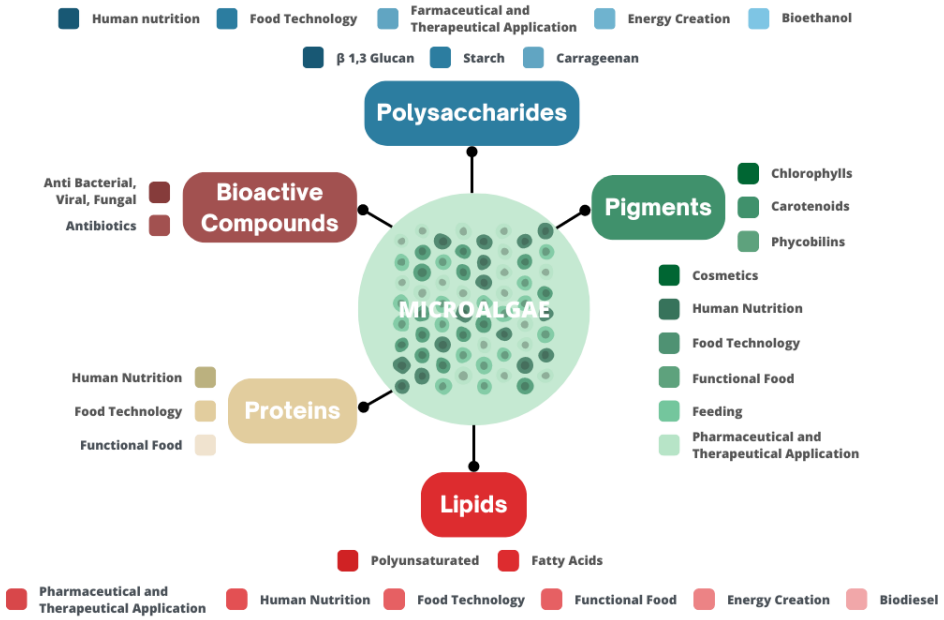

Figure 5. Main value-added microalgae products and applications (Adapted from [41])

β-Carotene, a vitamin A precursor in health foods, was the first high-value product commercially produced from *Dunaliella bardawil*. The biomass of microalgae as sun-dried or spray-dried powder is the predominant product in microalgal biotechnology, for the human food market. Bioactive compounds such as hydrocolloids, alginate, agar, and carrageenan are also valuable sources in microalgae [42].

Due to its high levels of polysaccharides, proteins, and lipids, microalgal biomass can also be used to produce bioplastics and environmentally friendly materials, as they do not increase the CO_2 pool and are quicker to biodegrade. Biocement production using microalgae is another method of long-term CO_2 capture, as is carbon fixation into the soil using microalgae, which may provide long-term CO_2 storage to promote sustainable agriculture, and serve as a biostimulant to improve crop production and reduce chemical fertilizer application [42].

2.4. EU's Agricultural Waste

Waste is defined in the Waste Framework Directive (Directive 2008/28/EC) as any substance or object that the holder discards, intends, or is required to discard. This Directive helps to ensure proper waste management practices, resource efficiency, and environmental protection. The first objective of any waste policy is to minimize the negative effects of waste generation and management on human health and the environment. The waste policy should also aim to reduce the use of resources and favor the practical application of the waste hierarchy. Waste prevention should be the priority of waste management, and reuse and material recycling should be preferred for energy recovery from waste, where and insofar as they are the best ecological options [43].

One key focus of EGD is promoting sustainable agriculture by reforming the EU's Common Agricultural Policy (CAP), aligning it with the objectives of the Green Deal. The reformed CAP aims to support sustainable farming practices, agroecology, and climate-friendly agriculture, which can not only help minimize the generation of organic waste and promote sustainable land management, but also enhance waste management systems and promote recycling across the EU [44].

The European Commission plans to cut food waste by half by legally binding targets by 2030 [45]. The Farm to Fork Strategy is another key focus of EGD, and it specifically aims at making food systems more sustainable, healthy, and resilient. It addresses various aspects of the food chain, including reducing food waste and ensuring the availability of safe and nutritious food, thereby promoting sustainable agricultural practices. The EGD emphasizes the transition to a circular economy, where resources are used more efficiently and waste generation is minimized. This approach encourages the reduction, reuse, and recycling of organic materials, including food waste and agricultural byproducts, to prevent their disposal in landfills and promote their valorization; hence, the Circular Economy Action Plan is updated [45].

Agriculture, forestry and fishing, as well as the processing of food, beverage and tobacco (F&B processing), generated 56.1 million tons of waste across the EU in 2020, a small decrease in the last years, at it can be seen in Figure 6.

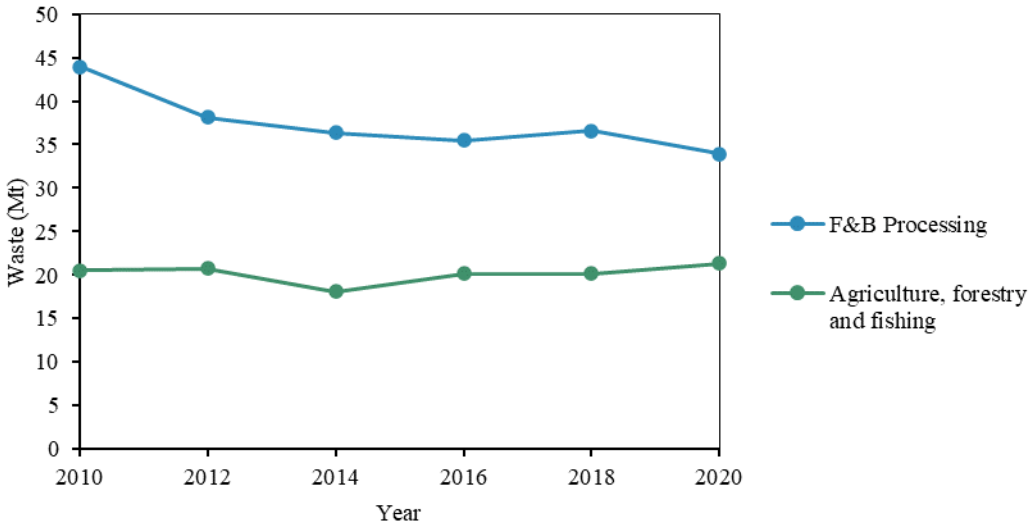

Figure 6. Developments of Waste Generation in the EU (Obtained from [10])

Together these activities accounted for 2.9% of all waste from productive activities (of a total 2 billion tons). EU waste generated by F&B processing fell by a little more than one-fifth (down 21.4% overall) between 2010 and 2020. The level of waste from agriculture, forestry and fishing was relatively stable, other than a short-lived contraction in 2014, with an overall increase of 4.6% between 2010 and 2020. About 60% of all the waste from F&B processing was animal and vegetal wastes, which include animal and plant-tissue waste, sludge, greases and oils, and biodegradable waste [45].

In 2020, agricultural processes in the EU produced 382 million tonnes GHG CO_2-eq. From 2010 to 2021, there was an overall increase in agricultural emission levels of 1.5%. To date, the largest GHG emissions from agriculture are CH_4 and N_2O (nitrous oxide). Agriculture is the largest source of emissions of these gases; in 2020, 55.4% of all CH_4 emissions and 80.1% of N_2O emissions in the EU were from the agricultural sector [10].

Three principal greenhouse gases are related to agricultural processes: carbon dioxide (CO_2), methane (CH_4), and nitrous oxide (N_2O). To compare and combine the emissions of these different gases, each gas is expressed in tons of CO_2-equivalents (a unit based on the global warming potential of each gas relative to that of CO_2; for example, CH_4 is 25 times more potent as a greenhouse gas than carbon dioxide), as shown in Figure 7.

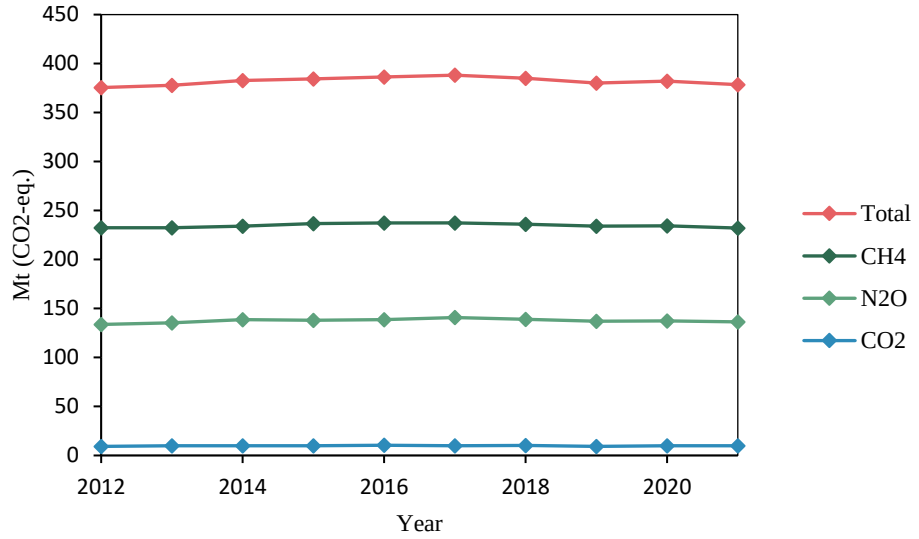

Figure 7. Main GHG emissions in the agricultural sector of the EU (Adapted from [46])

Emissions from manure management are approximately two-thirds CH_4 and one-third N_2O. Emissions from enteric fermentation made up more than two-fifths (42.9%) of all GHG emissions from agriculture in the EU in 2020, somewhat higher than the share (38.4%) for managed agricultural soils; the third largest contributor to agricultural GHG gas emissions was manure management (14.8%) [45].

2.4.1. Agricultural Sector Performance

Agricultural production in the EU by the millions of predominantly small farms adds up to a big business, even without considering its importance as the key building block for the downstream food and beverage processing industry, as schematized in Figure 8 [45].

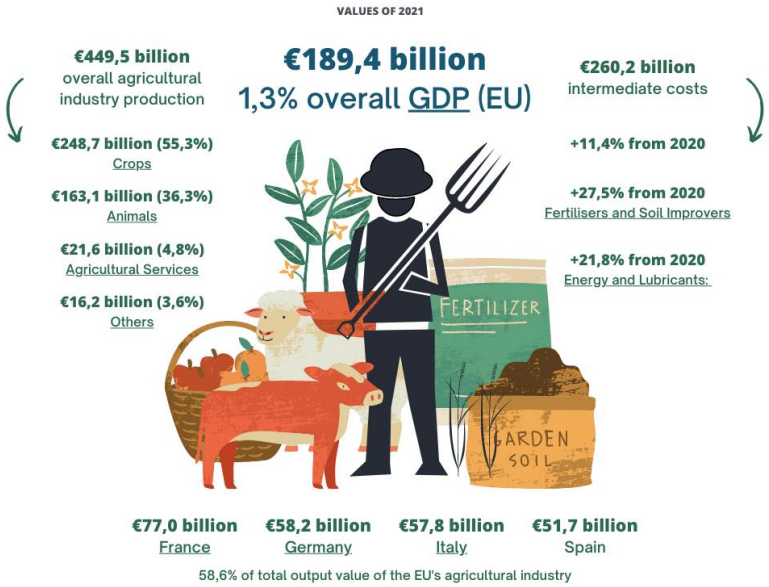

Figure 8. Schematic representation of the GDP in the EU in 2021 (Adapted from [45])

The agricultural sector contributed €189.4 billion towards the EU's overall gross domestic product (GDP) in 2021 (1.3% of the overall GDP), which represents the difference between the value of agricultural output and the value of various input costs built up in the production process, adjusted for taxes and subsidies on products. It is therefore interesting to examine the structure and composition of the value of agricultural production and the various inputs used [45].

The value of everything that the EU's agricultural industry produced in 2021 was an estimated €449.5 billion; this includes the output values of crops (€248.7 billion; 55.3%), animals (€163.1 billion; 36.3%) and agricultural services (€21.6 billion; 4.8%), as well as some goods and services that were not strictly agricultural but which could not be separately measured (€16.2 billion; 3.6%).

More than half (58.6%) of the total output value of the EU's agricultural industry came from four countries: France (€77.0 billion), Germany (€58.2 billion), Italy (€57.8 billion), and Spain (€51.7 billion).

However, producing all this output incurs costs. Farmers have to make purchases of goods and services to be used as inputs in the production process, such as seeds, fertilizers, animal feeding, fuel (for tractors), and veterinary services, among other things. These input costs are termed 'intermediate consumption' in an accounting context. Intermediate consumption costs for the agricultural industry came to a total of €260.2 billion for the EU as a whole in 2021, a value 11.4% higher than in 2020. The substantial input price costs were recorded for fertilizers and soil improvers (up 27.5%) and for energy and lubricants (up 21.8%) [45]. In Annex D, the distribution of farms and farmland by size, and the distribution for gross output for the agricultural industry is presented.

The volume of mineral fertilizers, nitrogen, and phosphorus consumed by agriculture also remained high from 2007 to 2018; an estimated 11.3 million tons were used in 2018. When the nutrients used in agriculture are not absorbed by crops, their use is considered excessive and linked to environmental issues such as water pollution, climate, and reduced biodiversity [10].

2.4.2. Organic Farming

The organic farming percentage increase proposed by the EGD holds the premise of reducing GHG emissions and increasing carbon sequestration in soils. On average, the climate protection performance of organic materials results in 1082 kg CO_2-eq per hectare per year. 2020 data on

organic farming in the EU are also presented in Annex D, and the evolution of the occupied organic farming area and the total utilized agricultural area are presented in Figure 9.

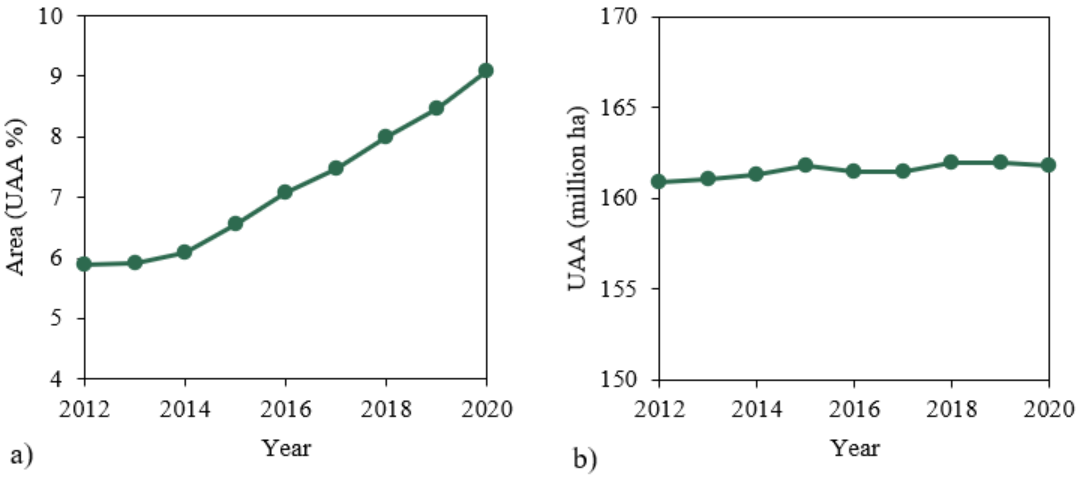

Figure 9. a) Evolution of Occupied Organic Farming Area in terms of UAA%; b) EU's Utilized Agricultural Area (Obtained from [45])

It is possible to observe that while the UAA remains the same for some years, the area that is now occupied for organic farming practices has been increasing, which is aligned with the EGD's goals.

Instead of being dependent on external fertilizer inputs, organic farming relies on seeking to close nutrient cycles through natural fixation of nitrogen, the recycling of organic manures and minimizing nitrogen losses. This helps to optimize available nutrients resulting in generally lower nitrogen levels on organic farms. Nitrogen fixating legumes, such as clover grass leys, and the use of organic manure to recycle nutrients help to build up soil fertility. Studies also show a reduction of 40% less nitrous oxide emissions per hectare for organic systems [47].

The number of animals allowed per hectare is also limited in organic farming, with the objective of not exceeding the holding capacity of the land. A reduced number of animals lowers the emissions, connected with synthetic fertilizer in particular, as well as manure management, reduces the nitrogen application rate and avoids over-fertilization of land. 42% of organic land are pastures and meadows mostly used for grazing organic livestock. Improved techniques, such as manure composting is often used in organic agriculture and it can reduce nitrous oxide by 50% and methane emissions by 70% [47].

Reducing emissions from manure management aims for limiting the anaerobic generation of methane or using closed storage to capture methane and use it for instance as biogas. However, in order for biogas production to be sustainable and not negate its benefits through indirect land use changes, it has to exploit waste and residues, and not rely on the large-scale cultivation of energy crops (e.g., maize) [47].

Overall, organic agriculture also shows a lower energy use per hectare and per unit product, with studies suggesting that around 15% less energy is consumed in organic agriculture per unit produced [45].

2.4.3. Portugal's Agricultural Potential

Portugal's Utilized Agricultural Area (UAA) is about 3.6 million hectares, 39% of its land surface; 30% are occupied by temporary crops, 20% by permanent crops and 50% by permanent grassland. 15% of the UAA are suitable for irrigation, representing 58.5% of total farm holdings. 2018 data on the number of farms and agricultural production is shown in Figure 10.

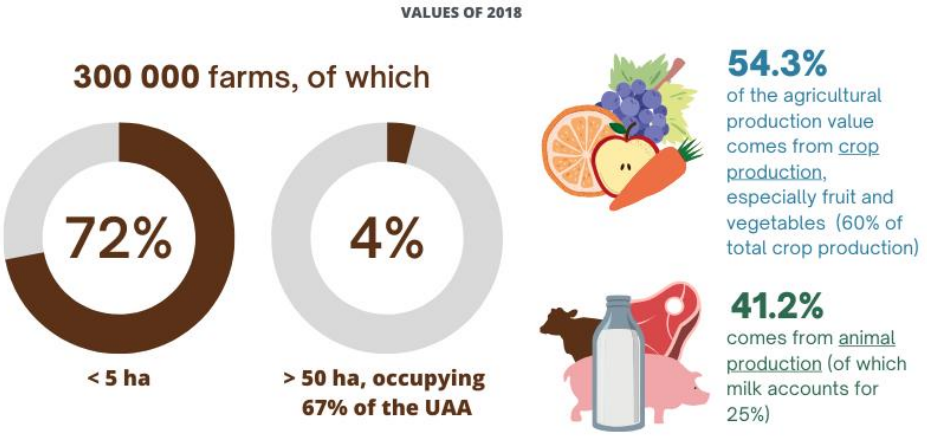

Figure 10. 2018 farm and agricultural production data, evidencing most farms are of small size and that the agricultural production highest value comes from crops (Adapted from [48])

Over one third of the country's territory is covered by exploited or exploitable forests, making forestry and the related value chains (pulp and paper, wood and cork) key segments of the primary sector. Current forest area totals 3.22 million hectares, with a great diversity of species, predominantly native species. The three most relevant tree species are eucalyptus, maritime pine and cork oak; oak (in particular evergreen oak) and chestnut are also present but have a lower economic impact.

The agro-food industry is the largest sector among the processing industries in terms of turnover and gross value added (GVA), which are better explained in (Annex E), and the second in terms of employment [48]. Figure 11 show the recent evolution of agricultural production in monetary terms.

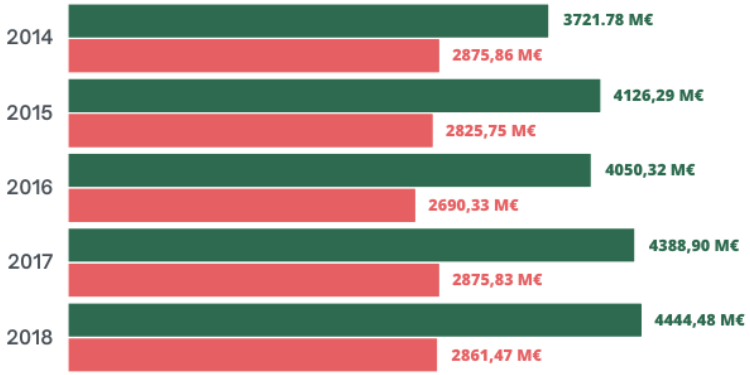

Figure 11. Agricultural production values (Adapted from [48])

Bio-based industries require sustainably produced and supplied biomass feedstock for conversion into value-added products and services. It works intimately with the primary sectors to jointly add value to available and unused biomass, side streams, by-products, and residual streams (waste) from these sectors. This interaction includes returning nutrients to the soil and lowering or eliminating soil, water, and air pollution. It will thus help increase food and feed production, support sustainable forestry, and make their value chains more efficient and competitive by adding higher economic value to biomass streams that today find no or low value only. These are all topics included in organic farming methodology, and the data for 2020 in Portugal are shown in Figure 12.

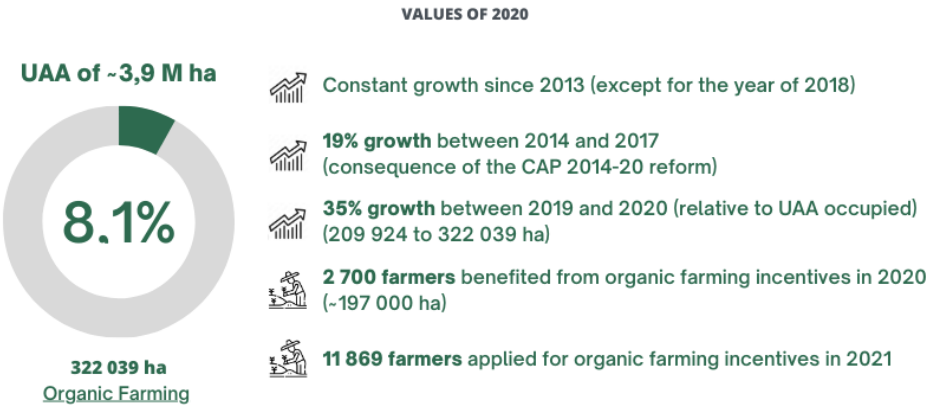

Figure 12. 2020 organic farming data in Portugal (Adapted from [49])

For the bio-based industry it is therefore of interest to explore availabilities of unused and residual streams from the agricultural, forestry and marine/aquatic sectors in Portugal, given their size and strengths. In addition, relevant and attractive feedstock for the bio-based industry can come from the food and feed processing industries, wood-based industries, other bio-based industries (such as breweries), municipalities, and relevant gaseous sources. Figure 13 shows the waste generated by the region in 2014.

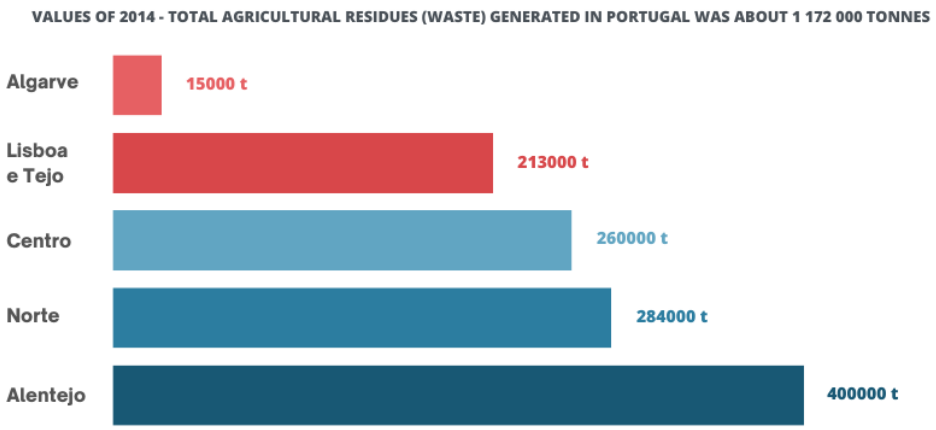

Figure 13. Waste generated in Portugal, by region, in 2014 (Adapted from [48])

For a sustainable bio-based industry it is essential to create new value chains that cross the boundaries of the various and distinctive industrial and academic sectors for synergies in areas of feedstock, technology and market [48].

3. ALGAEMENTUM

3.1. Overview

Algaementum is a Portuguese start up incubated in the Centro de Empresas Inovadoras (CEI), which is in the city of Castelo Branco. It subscribes to the importance and value that a Circular BioEconomy can provide for the EU, and, thus, specializes in microalgae-based products and services, not only contributing to circular bioeconomy but also clear water saving.

Initially dedicated to microalgae cultivation, it is now also developing high-protein food and feed solutions with unique functional ingredients, as well as accessing the viability of applying microalgae as bio-fertilizers to enhance the soil microbiome. Algae-derived products, such as whole plant proteins, omega-3, and functional phytoceuticals, may improve the health and wellbeing of humans. Moreover, including these same functional ingredients in the diets of farm-reared animals can possibly result in increased muscle mass and high milk volume and quality, including the overall improved health of the animals, reducing the amount of traditional medications.

Since 2023, *Algaementum* is committed to the establishment of specialized cultivation sites in central-eastern Portugal, where an abundance of sunshine, pristine water, and qualified human capital were combined to provide a unique opportunity for algaeculture [50]. In 2020, *Algaementum* presented a project in the 5th Cleantech Camp, an Acceleration Program. The pilot proposed the production of biomethane through modular and decentralized carbon cultivation platforms, which is the key differentiation of the company [51]. In 2023, the same project was presented at the EIT Food Accelerator Network, which seeks to accelerate the market launch of 10 projects from European agri-food startups, focusing on the challenge of achieving zero-emissions agriculture [52]. One of the proposed challenges was avoiding the excess water use in agriculture through random irrigation and animal effluent runoff. *Algaementum*'s pilot proposed that by intercepting animal-effluent-runoff, it is possible to recover and retain valuable nutrients on a farm, keeping them out of local rivers. Decentralized, onsite treatment of agri-waste-waters through an algae-platform results in clear water and fertilizer cost reductions to the farmer. Microalgae from agri-wastewater treatment can also be used as a soil amendment or biofertilizer, increasing the soil organic carbon and reducing water needs [53]. This directly complements the EU Farm to Fork Strategy and other related environmental objectives of the EGD [54].

3.2. Project Design

Algaementum's project pilot was awarded by Enagás Emprende in the 5th Cleantech Camp in 2020. The pilot is a TRL 7 (Technology Readiness Level) project. This index is a method to understand the technical maturity of a technology and scales from 1 to 9, with the last level representing an actual system proven in an operational environment. TRL 7 corresponds to prototype system demonstration in an operational environment [55].

The system has been operating in Monte Silveira Bio, a certified organic farm since 1999, located in Ladoeiro, Idanha a Nova, Castelo Branco, Portugal. Focused on regenerative agriculture and planned animal management, they are capable of producing nutrient-dense food while nourishing and regenerating soil health and fertility, whilst respecting biodiversity and ecosystem boundaries. Of the approximately 700 ha, 500 are occupied by a silvopastoral system of holm and cork oak (a *montado* ecosystem), where *Adaptive Multi-Paddock* grazing of cattle, sheep, pigs, and horses is carried out. The remaining 200 ha are dedicated to the cultivation of cereals, legumes, fodder, and olive groves, as well as regenerative practices with animal integration. In addition to contributing to a more prosperous and healthier diet, there is an environmental duty to sequester more carbon, reconstitute organic matter, promote biodiversity, and improve nutrient and water cycles, which are all critical factors for the sustainability and resilience of the ecosystem and planet [56].

The pilot facility of *Algaementum*'s project consists in 2 Intermediate Bulk Containers (IBC), which work in series, one functioning as an anaerobic digestor (AD-IBC) and the second one as the algae cultivation and upgrading platform (ALG-IBC). The pilot conceptual design is represented in Figure 14.

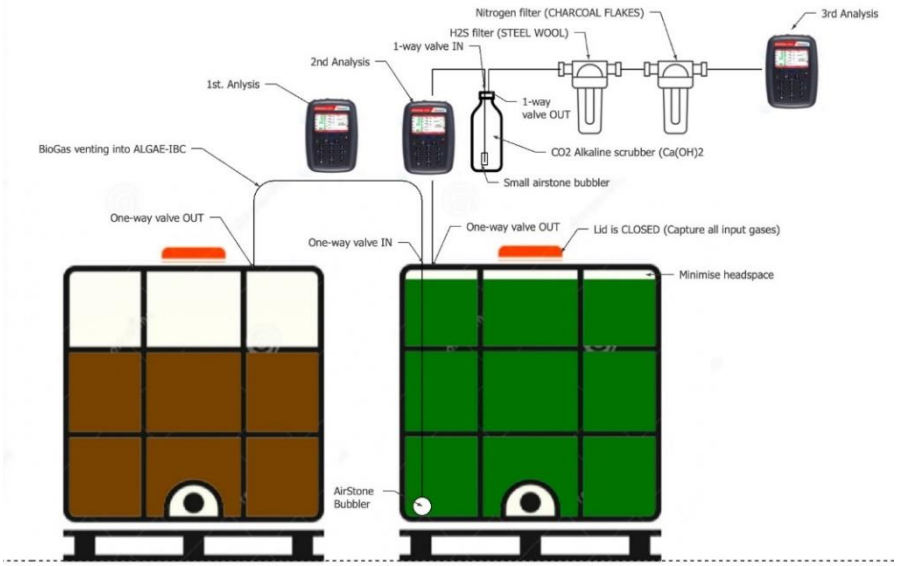

Figure 14. Schematics of the pilot system design

A full project timeline is presented in Figure 15, while Annex F shows a more detailed on-site decision-making report.

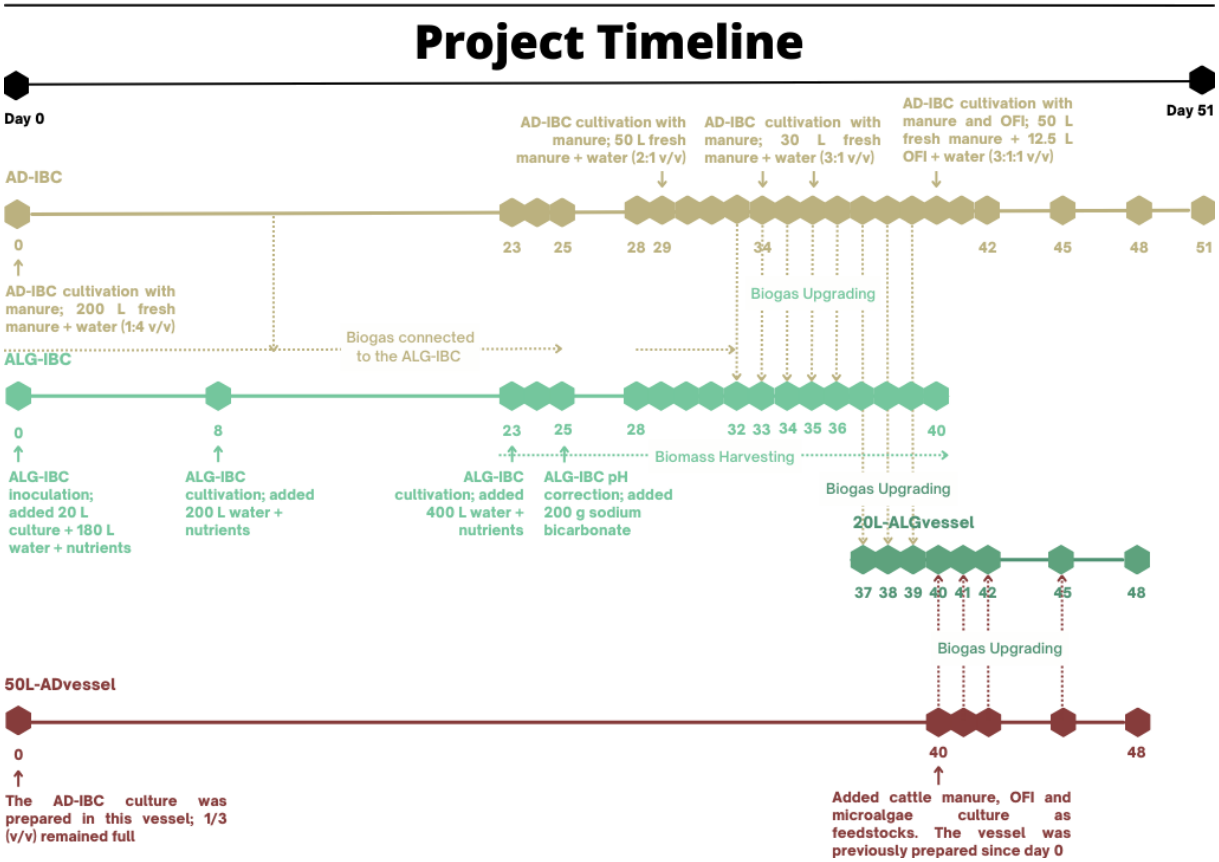

Figure 15. Project timeline

Initially, the AD-IBC was fed once a week for 3 weeks, with only cow manure and water from the farm. The gas analyzer, a Geotech BIOGAS 5000 analyzer, of which details are presented in Annex G, only available after the second week, was used to measure the CH_4 content inside the IBC. After some days, without reaching 40% (v/v), the AD-IBC was fed every three days. The CH_4 content decreased for some days, which could be due to microorganism adaptation/climatization. After this period, the content reached a maximum of 54% (v/v), with most days near 36% (v/v). Biogas analysis were carried out for 21 days.

During the same period, microalgae were cultivated in the ALG-IBC with nutrient feeding once a week and aeration inside the container using an airstone, which was powered by a solar panel. Since this container didn't have a mixing system, the microalgae biofilm was accumulating in the walls; scrubbing of the walls was carried out using a broom. Microalgae monitorization was carried out for the 21 days, and samples were collected in days 4, 9 and 16, to analyze in the lab for characterization.

The ALG-IBC was only filled after three weeks; in the first week, it was added the inoculum, 200 L water and nutrients (200 g sodium bicarbonate, 200 g sea salt and 100 g

VALAGRO fertilizer, which is a mixture of 20:20:20 nitrogen, phosphorus and potassium, serving as the macronutrients needed for growth), the same amount in the second week, and then 400 L and nutrients in the third week. After this period, the ALG-IBC was filled to the top of the container, leaving a minimum headspace of approximately 5 cm inside it, where the inlet and outlet valves were connected, to allow the gas to sit in the headspace.

Upgrading trials started one week after starting the biogas quantification. This process consisted in connecting an airstone to the AD-IBC gas outlet; initially, the airstone was sitting in the bottom of the container, and the biogas would be aerated into the container through the culture, working as a passive scrubbing process (no energy use). This way, the microalgae could naturally capture the CO_2. However, the pressure in the system was not sufficient for this process; other trials included using the airstone at different heights inside the IBC (knowing that there would be less water pressure closer to the surface, but also less area of transfer for the scrubbing process). This was carried out from the 7^{th} to the 12^{th} day of monitorization. After day 12^{th}, a 20 L vessel (20L-ALGvessel) was set up, with the same microalgae culture, and connected to the AD-IBC, because the flux needed to obtain an upgraded biogas would be lower than in the 1 m^3 container. However, no biogas upgrading was obtained because of the low biogas flux, as the bubbles of the scrubbing process did not reach the gaseous headspace, which means all the biogas was dissolved in the culture. This process was monitored from days 13 to 16.

On day 16, a new feedstock, cattle manure and *Opuntia Ficus Indica*, was tested to boost the biogas production and increase the system's gas flux. At the same time, a 50 L vessel (50L-ADvessel), which was used to dilute the feedstocks for the AD-IBC, was connected to the 20L-ALGvessel, because the biogas flux in this container would be higher, as the pressure inside was higher. This downscale still didn't offer better results regarding the upgrading process in the remaining days of monitorization (16 to 21), but made it possible to verify the recirculation of microalgae to raw biogas production was viable, as the 50L-ADvessel was fed with cattle manure, *Opuntia Ficus Indica* and microalgae culture.

4. MATERIALS AND METHODS

The first experimental analysis focused on the initial characterization of feedstocks, namely, fresh cow manure and water, and the algal culture used to inoculate the ALG-IBC.

The cattle manure was collected from the closest site to the system, where the cows are prepared for transportation to the slaughterhouse, and where they are fed straw.

The water used in the system was analyzed because the farm is in a calcium-rich soil area. The ALG-IBC was first inoculated with a 20 L culture, from which 10 g of biomass was collected, lyophilized and further characterized.

Biogas and biomethane analysis were performed using the Geotech BIOGAS 5000 Gas Analyser (Geotechnical Instruments).

Approximately 800 mL of algae samples were harvested for 16 days and frozen at -18 ºC before transportation to the laboratory for biomass growth quantification. Three samples, of days 28, 33 and 40, were then properly characterized to evaluate if there are differences in the algae biomass properties during the biogas upgrading.

Lipids content, chlorophyll *a*, proteins, pH and elemental analysis were performed in the microalgae biomass. Manure characterization consisted in moisture, total solids, volatile solids, total suspended solids, total dissolved solids, chemical oxygen demand, phosphorus, pH and elemental analysis. Manure quantification was carried out in triplicates, as well as biomass growth monitoring assays, while the biomass characterization in terms of oil and chlorophyll were carried out in duplicate.

4.1. Analytical Methods

4.1.1. Determination of Lipids Content

Total lipids were determined by using the Soxhlet extraction methodology, which was partially adapted from Dasan Y. et al. [57]. The harvested biomass (6000 rpm for 10 min) was freeze dried for 72 h by a freeze dryer (MRC, FDL-10N-80-TD-MM). Then, 200 mg of microalgae biomass were placed in a cellulose extraction thimble along with 210 mL chloroform (99+%, J.T. Baker) + methanol (99+%, Fischer Chemical) (2:1 vol ratio); the solvent mixture was refluxed for 4 h at 65 ºC, at a siphon rate of 2 cycles per hour, using water as refrigeration fluid. The crude lipid in the balloon was then recovered using a rotary evaporator for 30 min at 65 ºC, after which the balloon was autoclaved for 12 h to remove the remaining moisture content before esterification. Total lipid yield was calculated using Equation (1):

$$Y\ (\%) = \frac{W_{LE}}{W_{BS}} \times 100 \quad (1)$$

where, W_{LE} is the weight of the extracted lipids and W_{BS} is the initial weight of the lyophilized biomass sample (g).

Fatty acid methyl esters (FAMEs) contents analysis was performed using a partially adapted procedure described in the ISO 12966-2 (2014). The previously extracted crude lipid was dissolved in 3 mL of methanol, passed onto a 10 mL vial and evaporated using gaseous nitrogen. Then, 1.8 mL of methanolic sodium hydroxide solution (0.5 M) were added and heated in an agitated glycerin bath (250 rpm, at 77 ºC for 10 min), after which 3.2 mL of a methanolic sulfuric acid solution (0.6 M) was added to the vial, and the sample was heated in the same bath. After cooling at ambient temperature, 3 mL hexane and approximately 3 mL saturated sodium chloride solution were added. The mixture was agitated in a vortex mixer and formed two distinct phases after settling. The organic phase (top phase) was transferred to a 20 mL vial and the hexane was evaporated using gaseous nitrogen. The methyl esters obtained were then analyzed by gas chromatography in a gas chromatograph (Shimatzu, GC-2014), with a Split injector, flame ionization detector (FID) and column (SH-Stabilwx-DA, L × I.D. 30 m × 0.25 µm, df 0.25 µm); injector and detector temperatures were 250ºC, with split injection mode (1:10) and nitrogen as a gas carrier, at a flow rate of 0.55 mL/min. The initial column temperature was 60 ºC, reaching 210 ºC (heating rate of 20 ºC/min), and remaining at 210 ºC for 7 min. The temperature was then raised to 250 ºC (20 ºC/min) and maintained for 14 min, resulting in 21 min total analysis time.

The quantity of individual FAMEs, C_{FAME} (%) were calculated based on Equation (2):

$$C_{FAME}\ (\%) = \frac{A_{peak}}{A_T} \times 100 \quad (2)$$

where, A_{peak} is the peak area of each individual component existing in the FAME profile and A_T is the sum of the total peak area (mV).

4.1.2. Chlorophyll a

Total chlorophyll *a* (Chl *a*) concentration was determined spectrophotometrically by measuring the maximum absorbance of the extract at a wavelength range of 650-675 nm and at room temperature, using a P9 double-beam UV-Vis spectrophotometer. The prepared samples were dilutions of the crude lipid extracted by the Soxhlet method using the same solvent (chloroform:methanol), in a 1:200 factor (20 µL sample and 3.98 mL solvent). Before obtaining the absorbance spectrum of the samples, the respective blanks measurements were recorded.

Chl *a* concentration was then obtained using a previously calculated calibration curve, presented in Annex H, which was performed by measuring the maximum absorbance of different concentrations (1, 2, 3, 5, 10, 15 and 20 ppm) of a liquid chlorophyll supplement (Liquid Chloropheal, Alkalinecare) with 140 mg of chlorophyll in 60 drops.

The linear regressions obtained for Chl *a* quantification is presented in Equation (3):

$$A = 0.031[\mathrm{Chl}(a)] - 0.0019 \ , \ R^2 = 0.9999 \quad (3)$$

where, A is the maximum absorbance near 660 nm and $[\mathrm{Chl}(a)]$ is the correspondent chlorophyll *a* concentration.

4.1.3. Proteins

Total protein was calculated by direct conversion of the total elemental nitrogen obtained, multiplying the value by the factor of 5.04, as reported by Chambonniere et al. [58].

4.1.4. Moisture and Total Solids

Approximately 5 g of the cattle manure sample were weighed in a crucible and dried in an oven at 105 °C until constant weight (~24 h), according to APHA (American Public Health Association, 1998). Sample moisture content (moist. (%)) is given by the difference between the initial and final weights according to Equation (4):

$$\text{moist. (\%)} = \frac{m_{fs} - m_{ds}}{m_{fs}} \times 100 \quad (4)$$

where, m_{fs} is the mass of fresh sample (g), and m_{ds} is the mass of the dried sample (g).

The total solids (TS) value is equivalent to the % (w/w) of dried solids in the sample and can be determined using Equation (5):

$$\text{TS (\%)} = 100 - \text{moist. (\%)} \quad (5)$$

4.1.5. Volatile Solids

Volatile solids (VS) were determined using the sample previously dried at 105 °C and calcinated at 550 °C until constant weight (~3h), using equation (6):

$$\text{VS (\%TS)} = \frac{m_{ds} - m_{cs}}{m_{ds}} \times 100 \quad (6)$$

where, m_{cs} is the weight of the sample after calcination at 550 ºC (g).

4.1.6. Total Suspended Solids and Total Dissolved Solids

Total suspended solids (TSS) were determined by filtration of a known volume of sample (~ 2 g of sample in 50 ml of distilled water). The filter was then dried in an oven at 105 °C until a constant weight was obtained. The TSS was then determined using Equation (7):

$$TSS\ (g/L) = \frac{m_{pfds} - m_{pf}}{V_{sample}} \quad (7)$$

where, m_{pf} and m_{pfds} are the masses of the dried paper filter before filtration (g) and dried paper filter after filtration (g), respectively. V_{sample} is the volume of the filtered sample (in L).

The total dissolved solids (TDS) were determined by drying the filtered sample in an oven at 105 ºC until a constant weight was obtained, using Equation (8):

$$TDS\ (g/L) = \frac{m_{bfs} - m_{afs}}{V_{sample}} \quad (8)$$

where, m_{bfs} and m_{afs} correspond to the mass of the solids obtained after drying an aliquot of the filtrate at 105 ºC (g).

4.1.7. Chemical Oxygen Demand

Chemical oxygen demand (COD) determination was performed by combining the sample with acid and digestion solutions and quantifying it by spectroscopy using a calibration curve for both liquid and solid samples.

All vials were digested in an ECO25 thermoreactor (VELP Scientific) for 2 h at 150 °C. Then, the absorbance of each solution in the vials was read in a photometer PhotoLab S6 (WTW) at 605 nm.

- Liquid samples

For total chemical oxygen demand (COD_{total}) and soluble COD ($COD_{soluble}$), 1.2 mL of digestion solution, 2.8 mL of the acid solution, and 1 mL of sample were added to each vial.

Approximately 2 g of the sample was used and passed to a 50 cL bottle, which was then automatically agitated for 2h. The 1 mL sample was diluted again using a dilution factor of 5. The $COD_{soluble}$ was determined in a liquid sample previously filtered through a 1-3 µm filter.

- Solid samples

The APHA standard methods (1992) was used to determine the chemical oxygen demand in solids (COD_{solids}).

15 g of dried sample (for 24h at 105 °C) were first milled and filtered, and 3 mg was passed to each analysis vial; before digestion, 197 mg of distilled water, 1.8 mL of the acid solution, and 1.8 mL of digestion solution were added to the vial. After 2h and another hour of cooling to room temperature, the absorbance was read.

4.1.8. Phosphorus

Total phosphorous (P) was determined according to standard methods (365.3 EPA) (Baird et al., 2017). 50 mL of the sample, 1 mL of sulphuric acid (11 N) and 0.4 g of ammonium persulphate ($(NH_4)_2S_2O_8$) were added to the digestion tube. Digestion was performed at 100 ºC for 30 min (DKL Fully Automatic Digestion Unit from VELP Scientifica).

After cooling, the sample was filtered and 4 mL of antimony-phospho-molybdate complex and 2 mL of ascorbic acid were added. After 5 min, the absorbance was read at 650 nm using a spectrophotometer T60 UV-Vis. The quantification was performed using a calibration curve, presented in Annex H.

4.1.9. pH

For the determination of pH, the CRISON GLP21 pH meter with an adequate electrode was used in the laboratory, while on site, Combur-Test (Roche) strips were used.

4.1.10. Elemental Composition

The elemental composition of each residue, C, N, H and O was determined on the equipment *Elemental Analyzer NA 2500*. The oxygen content was obtained by subtracting the sum of C, N, and H content from the VS content.

5. RESULTS AND DISCUSSION

5.1. Feedstock and Microalgae Inoculum Characterization

As stated in Section 3.2, the feedstocks for AD included diluted cattle manure and *Opuntia Figos Indica* (OFI), the first used since the beginning of the pilot assay, while the second was only added in the last days cultivation in the AD-IBC and 50L-ALGvessel (day 40). In addition to these two feedstocks, a microalgae sample was used as inoculum for the ALG-IBC. The physicochemical characterization of the two AD feedstocks and microalgae inoculum was carried and the corresponding results are shown in Table 6.

Table 6. Physicochemical characterization of AD feedstocks and microalgae inoculum.

Parameter	Dried Cattle Manure	OFI*	Microalgae (Inoculum)
Moisture (%)	8.92	91.65 ± 1.33 [59]	n.d.
TS (%)	91.08 ± 2.93	15.7 [60]	n.d.
Ash (%TS)	2.22 ± 0.75	20.4 ± 0.83 [59]	n.d.
VS (%TS)	97.78 ± 0.75	73.95% [61]	n.d.
VS ($g/g_{amostra}$)	0.89	-	n.d.
TSS (g/L)	5.56 ± 1.35	-	n.d.
TSS (%)	29.41 ± 6.75	13.05 – 15.63 [60]	-
TDS (g/L)	9.25 ± 0.27	-	n.d.
C (%TS)	43.74 ± 0.28	63.74 ± 1.23 [61]	17.55 ± 0.03
H (%TS)	6.19 ± 0.11	-	4.31 ± 0.16
N (%TS)	2.21 ± 0.02	2.08 ± 0.20 [61]	3.80 ± 0.01
O (%TS)	45.62 ± 0.35	43.56 ± 0.14 [59]	n.d.
P ($mg/g_{amostra}$)	2.00 ± 0.00	0.41 ± 0.01 [59]	n.d.
S (ppm)	≤ 100	-	≤ 100
Proteins (%TS)	n.d.	4.48 ± 0.01 [62]	19.15
Lipids (%TS)	n.d.	1.06 ± 0.30 [62]	1.05 ± 0.08
CODsolids (mgO2/gTS)	1738.17 ± 488.48	-	-

*values obtained from the literature

*n.d. – not determined

As shown in Table 6, the collected cattle manure had a very high content of total solids (91.08%), and was mainly composed of VS (97.78 %TS), which indicates that the manure's organic matter had a very high potential to be converted to CH_4 and CO_2 in the AD process. The manure was picked from the ground and analyzed in the same day. In addition, the sample didn't contain a large proportion of bedding material (e.g., straw), which could lower the concentrated organic content. The values obtained for TS are higher than the previously

reported by Rosenberg et al.[63], with cattle manure ranging from 13-18%, while VS (%TS) content is slightly lower, ranging from 76-82%.

The elemental analysis values obtained, in a dry basis, were of 43.74 ± 0.28% C, 6.19 ± 0.11% H, 2.21 ± 0.02% N and 45.62 ± 0.35 O. Ashraf et al. [64] report values of 35-43% C, 2-8% H, 1-4% N and 25-55% O, thus, this dissertation results are well within the range.
OFI weren't characterized due to time restrictions, but literature reports values of 91.65% moisture [59], 73.95% VS and 63.74% total carbon [62], which indicates the potential of using it as an AD feedstock because of the high organic matter content.

Lyophilized microalgae was characterized by determining the oil (total lipids) and chlorophyll content, as it is expected that these values can vary during the microalgae growing and biogas upgrading. The lyophilized cellular biomass is composed of 1.05% total lipids and 16.24 mg/L of chlorophyll *a*. An elemental analysis of the biomass was also performed, obtaining values of 17.55% carbon, 4.31% hydrogen, and 3.8% nitrogen, with a protein content of 19.15%. These values are much lower than the one's reported by Klassen et al. [65] at low nutrient medium, of 46.4% C, 21.4% total lipids, 28% proteins and a chlorophyll concentration of 19.305 mg/L reported by Fakhri et al. [66].

5.2. Biogas Monitorization

Methane content was monitored for 21 days using a Geotech BIOGAS 5000 gas analyzer, which obtains values for CH_4, CO_2 and O_2 in percentage, plus CO and H_2S in concentration (ppm); this analyzer also measures the *bal* value, which corresponds to an approximation of the components remaining in the current to close out the total balance in the systems outlet. It should be noted that onsite decisions and adaptations were made during the monitoring period. These decisions followed the more detailed decision-making report presented in Annex F, which will be important for understanding the discussion in the following chapters, as the biogas produced in the AD-IBC is the main restriction in its upgraded results. As shown in the timeline presented in Section 3.2., due to analyzer availability, the first analysis occurred 3 weeks after loading of the AD-IBC and the biogas evolution during the monitoring period according to all these parameters was determined. The corresponding results are presented in Figure 16.

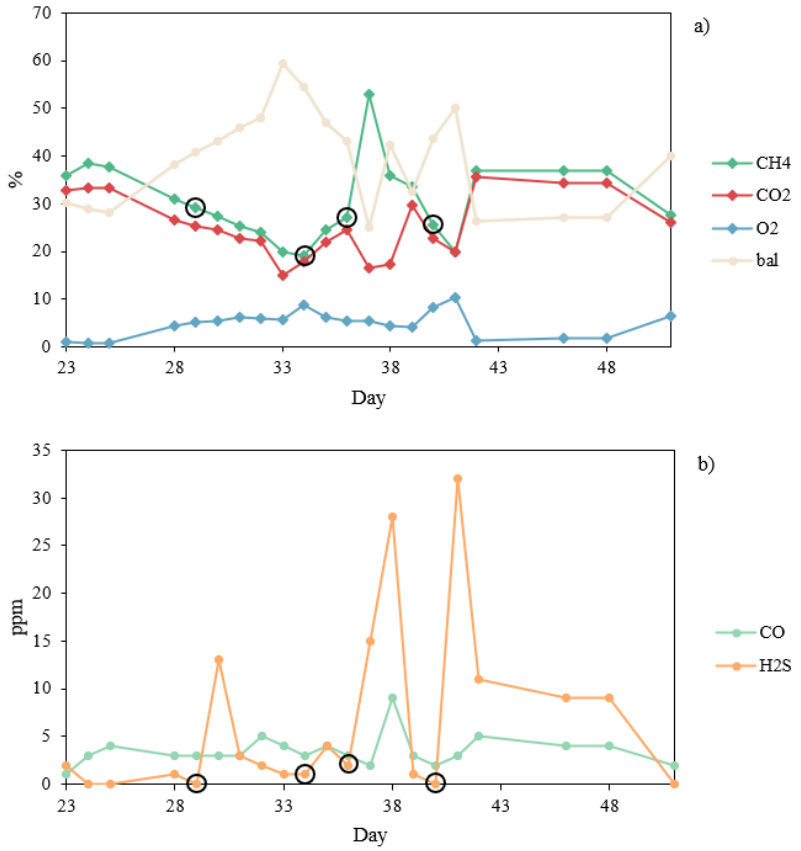

Figure 16. a) Methane (CH_4), Carbon Dioxide (CO_2) and Oxygen (O_2) evolution inside the AD-IBC, from day 23 to day 49 of the pilot working time (values in %); b) Carbon Monoxide (CO) and Sulfuric Acid (H_2S) evolution inside the AD-IBC, in the same period (values in ppm).◯ indicate when feedstock was added to the AD-IBC.

From the analysis of the results, in Figure 16.a) we can observe that 3 weeks after charging the AD-IBC with 40 L diluted cattle manure (*i.e.*, 1:4 volume dilution with water), the CH_4 content inside AD-IBC was 35.8%, which is lower than the minimum biogas CH_4 content of 40% previously reported by Demirer et al. [16]. Interestingly, our results are in line with those obtained by Panizio et al. [22], which reported also low production of CH_4 (~33%) for AD using cattle manure as single feedstock.

Day 29 recorded the third day of CH_4 values dropping; it was decided to feed the AD-IBC again with 50 L diluted cattle manure (2:1 volume dilution of water), which caused an even bigger drop in production due to microorganism acclimatization. Subsequently, a 5-days consecutive dropping of CH_4 production was observed, achieving a minimum of 19.1% CH_4 on the 34th day. This decrease could also be due to the lack of mixing in the system and quick sedimentation of the feedstock. Considering the low and unexpected performance observed in the AD-IBC, it was decided to proceed with an alternative approach, in which AD-IBC was fed more regularly and with smaller amounts of feedstock (fed-batch approach), as well as to promote mixing in the system whenever feedstock was added by mechanical stirring. Then, 30

L of diluted cattle manure (2:1 in water) were added on days 34 and 36, which led to a sudden boost in biogas production on day 37, where a production maximum value of 52.8% was achieved. This reports a high value for CH_4 content in biogas, using only cattle manure as feedstock, compared to the reported values of Panizio et al. [22].

On the 40th day, the value recorded was 25.5%; the AD-IBC was charged with a 50 L mixture of diluted cattle manure once again, but also adding OFI (75/25% as reported by Panizio et al [22]) and adjusting the feedstock's pH. This time it took only two days instead of five for the system to acclimate; CH_4 production dropped to 19.9% in day 41, before returning to stable values of 36.9% in days 42 to 48, a similar profile to the first three days of analysis, suggesting there is a stabilization of the system after 15 days of higher loading rates. Figure 16.b) also shows the effect of increasing H_2S concentration after the addition of feedstock and the CH_4 content starts to increase.

At the same time in the 40th day, it was experimented to recirculate microalgae culture from the ALG-IBC as a biogas feedstock, but since there was risk of stalling the AD-IBC due to too high alkalinity and bacterial activity, this was experimented in a 50 L vessel (50L-ADvessel), which would also allow a higher biogas flux. Figure 17 shows the results of the biogas evolution in this vessel.

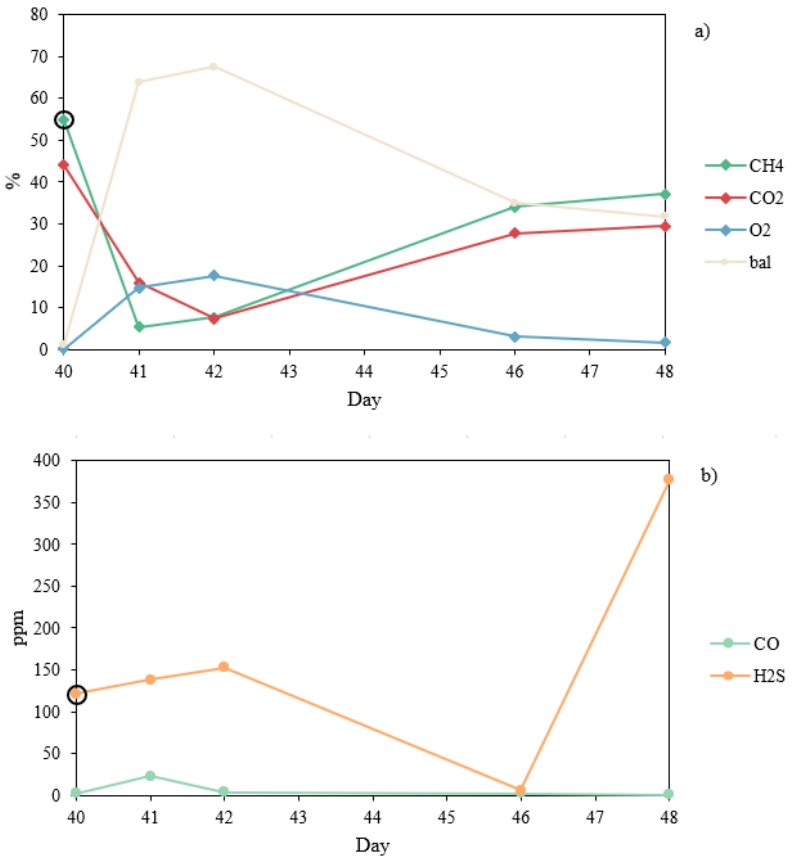

Figure 17. a) Methane (CH_4), Carbon Dioxide (CO_2) and Oxygen (O_2) evolution inside the 50L-ADvessel, from day 40 to day 48 of the pilot working time (values in %); b) Carbon Monoxide (CO) and Sulfuric Acid (H_2S) evolution inside the 50L-ADvessel, in the same period (values in ppm). ○ indicate when feedstock was added to the AD-IBC.

The initial CH_4 content of the previously prepared vessel in day 40 was 54.7%; this value dropped to 5.4% in only one day, but it also recorded a 30% improvement between days 41 and 46, reaching 37.2% CH_4 with a slight growing tendency. The H_2S concentration also spiked during this period, as shown in Figure 17.b), with the addition of feedstock and increase in CH_4 generation.

Microalgae are reported in the literature as being able to produce biogas with up to a maximum 60 to 70% CH_4 yield, co-digested with wastewater, and depending on the media composition, strain, and type of photobioreactor (PBR) selected in its cultivation [67]. Although the monitorization period ended on day 48, there was an increasing profile in the CH_4 production inside the 50L-ADvessel, which indicates recirculation of biomass might be interesting as feedstock for biogas production.

5.3. Upgraded Biogas Monitorization

Upgraded biogas tests were started on the 32nd day, because the ALG-IBC was being cultivated since day 0, as explained in the project timeline. The container was only filled on day 31, leaving minimal headspace in the inside, where the upgraded gas could accumulate and the pressure inside the container would push it to the gas outlet. The results of the upgraded biogas in the ALG-IBC are shown in Figure 18.

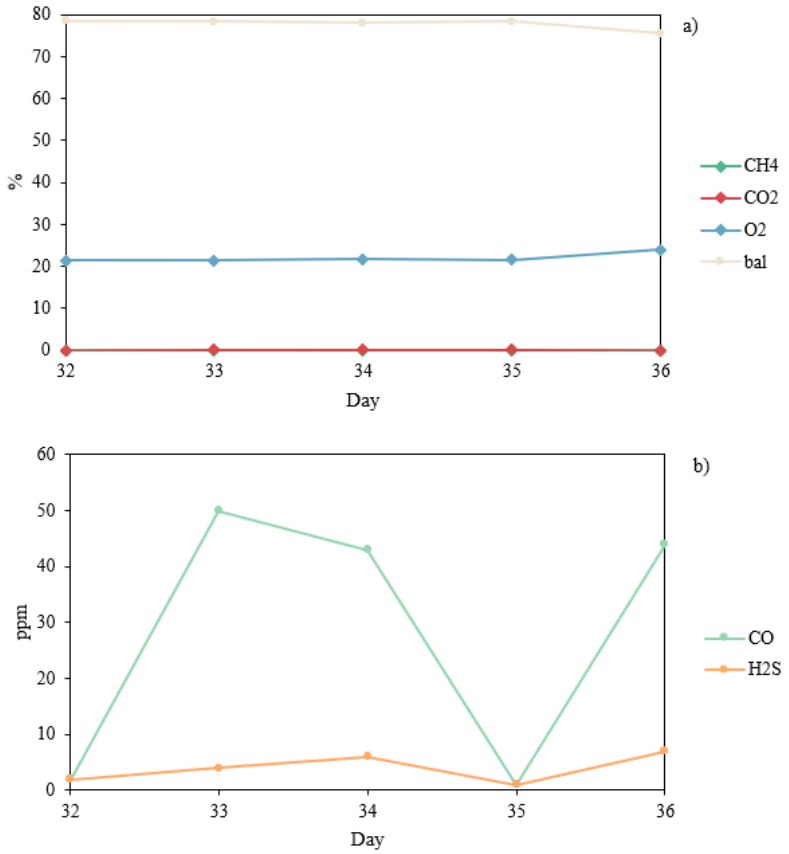

Figure 18. a) Methane (CH_4), Carbon Dioxide (CO_2) and Oxygen (O_2) upgraded biogas evolution in the ALG-IBC, from day 32 to day 36 of the pilot working time (values in %); b) Carbon Monoxide (CO) and Sulfuric Acid (H_2S) evolution inside the ALG-IBC, in the same period (values in ppm).

As shown in Figure 18, the percentage of gas present in the ALG IBC was O_2, i.e., there was no presence of biogas, which means that the CO_2 and H_2S of the AD-IBC biogas were dissolved in the cultivation media and/or accumulated by microalgae cells. This result was unexpected but after through an analysis of the pilot facility design some assumptions were drawn. In this pilot apparatus, the biogas outlet was connected to an airstone at the bottom of the ALG-IBC, working as a passive scrubbing process with the objective of bubbling biogas through the height of the container until it reached the available minimum headspace. Therefore, with this design, we believe that the process failed because the biogas flux entering the ALG-IBC was not

sufficient to obtain a proper scrubbing process and, consequently, the biogas components eventually were solubilized in the cultivation media, and used as a substrate for the growth of the microalgae cells.

Trials to obtain an upgraded gas included experimenting different airstone heights inside the container, because the water pressure in the bottom of the container is higher and it would be of greater difficulty for the bubbles to reach the surface without sufficient flux. The tube connected to the airstone was cut 30 cm each following day until it reached the surface. However, having the airstone closer to the water surface would mean a less efficient scrubbing process, due to lower transfer surface area in the microalgae culture. Currently, there are no works reporting on a passive microalgae biogas scrubbing technology. This technology is still very related to small PBRs with pressurized gas inlets; Bahr M. et al., have reported on a minimum flow of 50 mL/min in a 50 cm height × 4.5 cm internal diameter bubble column [36].

In order to overcome the flux limitations, from days 37 to 48, a next trial was performed by bubbling the gas from the AD-IBC in a 20 L vessel (20L-ALGvessel) containing microalgae culture that was taken from the ALG-IBC on the same day (37). The 20L-ALGvessel was sealed using Teflon tape and silicon. In that case, since the volume of cultivation medium was lower, the flux needed to bubble the biogas would also be lower. The results of the gas analysis obtained in this second trial are shown in Figure 19.

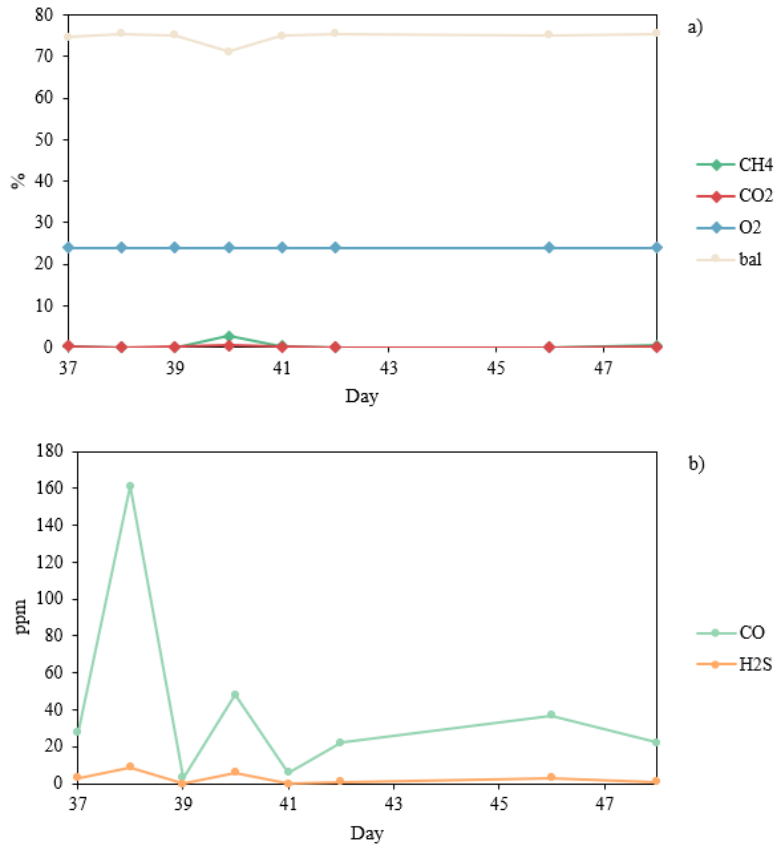

Figure 19. a) Methane (CH4), Carbon Dioxide (CO2) and Oxygen (O2) upgraded biogas evolution in the 20L-ALGvessel, from day 37 to day 48 of the pilot working time (values in %); b) Carbon Monoxide (CO) and Sulfuric Acid (H_2S) evolution inside the 20L-ALGvessel, in the same period (values in ppm).

As shown in Figure 19, regardless of the different airstone heights tested, only a maximum of 2.6% CH_4 and 0.4% CO_2 were registered in day 40, at the maximum height (closer to the lid). In addition, that day corresponds to the day with maximum biogas flux coming from the 50L-ADvessel, assuming the pressure in the smaller container was higher, resulting in higher flux. Apart from this exception, all the other results showed no significant biogas presence, *i.e.,* 0.1 to 0.4% levels for both CH_4 and CO_2, while the O_2 levels maintained at 24%. This last value is explained by the fact the container was well sealed and cell respiration was occurring, accumulating O_2 in the available headspace.

Several reasons can be appointed for the unsuccess of the upgrading process:

1) Cell density – biomass growth and cell distribution are the major factor for a successful upgrading process, since CO_2 is captured for cell growth.
2) Inadequate mixing – the ALG-IBC had a small airstone installed, which might have been too small to promote system's homogeneity.

3) Strain selection – there are no reports regarding biogas upgrading using *Chlorella vulgaris* NIVA-CHL 108. Studies indicate *Chlorella vulgaris* FAHCB 31 as the most interesting *Chlorella sp.* strain for this process [18].
4) Inefficient biogas fluxing - the biogas flux needs to increase so that the bubbles reach the surface during the scrubbing process. The CO_2 mass transfer efficiency was also reported to increase with increasing liquid flow [15].
5) Variable biogas composition – because the biogas composition varies significantly every day, it is more difficult for the culture to adapt, stabilize growth, and perform a standardized upgrading process.

The number of works dealing with the upgrading of biogas using microalgae cultures and well-developed PBRs under strictly controlled conditions is still limited. Besides the medium composition, which will be better discussed in the next chapter, the key parameters for reported upgraded biogas with >90% CH_4 are a very high CH_4 rich raw biogas (>80%), with a microalgae culture stabilized for 30-50 days using 10-30% CO_2 as carbon source. In addition, a 50 mL/min and 2.2 $gCO_2/L/h$ flux in bubble columns with a biogas/liquid flow rate ratio of 1 has been studied, but in a much lower size than the ALG-IBC, of only 0.8 L [36].

Unfortunately, the current pilot tests did not allow to obtain data for biogas flux and, consequently, a critical comparison with literature values. Anyway, few initial tests theoretically seem to indicate that upgrading can be implemented using this approach. Specifically, the pH analysis of the culture using test strips on the first day of monitoring confirmed that the pH was slightly acidic at a total culture volume of 400 L. The biogas outlet of the AD-IBC was bubbled through the airstone into the ALG-IBC, which means that the carbon source (CO_2) was dissolved in the culture and that there was biogas upgrading due to the combined acidic pH and carbon uptake. Unfortunately, due to the insufficient biogas flux there was no standard CH_4 biogas release. It only took the microalgae a couple of days to adjust the pH, and after filling the ALG-IBC, carbon uptake was much slower. On the other hand, biomass growth, as discussed in the next chapter, also indicates that CO_2 was absorbed by the microalgae, despite the use of bicarbonate to ensure that the culture did not run out of a nutrient source.

5.4. Microalgae Growth Monitorization

Microalgal biomass growth was monitored by calculating the change in dry biomass during cultivation. For that purpose, aliquots of 600 mL of cultivation culture were collected from the ALG-IBC every day, for 16 days (from day 23 to 40 with exceptions on days 26 and 27) of the pilot monitoring period. All 16 samples were centrifuged and lyophilized. The final lyophilized biomass of triplicate assays was weighed. The corresponding biomass concentration (g/L) and productivity yields were determined. Although the experiments were performed in triplicate to minimize errors, the biomass and productivity yields showed considerable variation over the days, due to sampling issues. Figure 20 shows the cell growth monitoring as the biomass concentration (g/L), while in Table 7 these values are presented together with pH values of the culture and productivity yields.

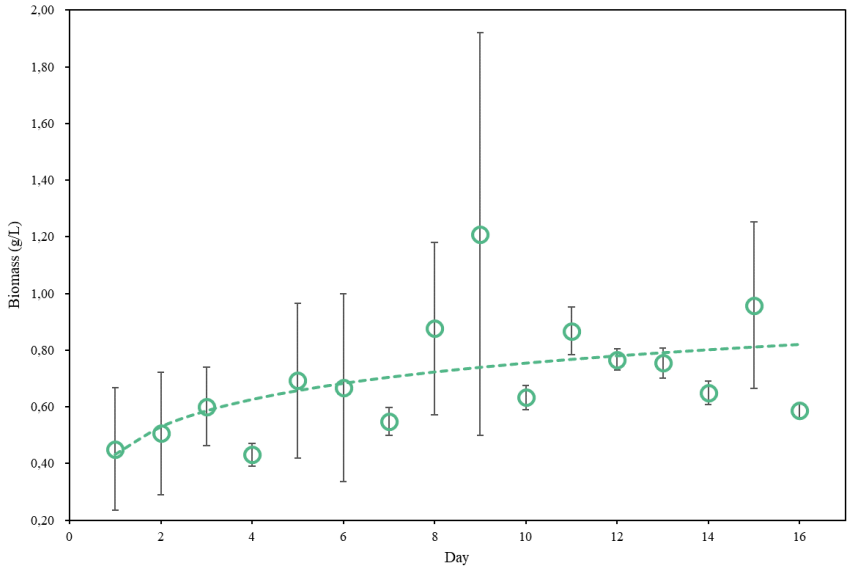

Figure 20. Microalgae biomass growth curve (g/L)

Table 7. Microalgae biomass concentration in the 16 days of harvesting

Day	pH	Biomass (g/L)
1	10.12	0.45 ± 0.22
2	9.15	0.51 ± 0.23
3	9.21	0.60 ± 0.14
4	9.67	0.44 ± 0.04
5	9.73	0.69 ± 0.27
6	7.9	0.67 ± 0.33
7	9.42	0.55 ± 0.05
8	9.48	0.88 ± 0.31
9	9.52	1.21 ± 0.71
10	9.67	0.63 ± 0.04
11	9.57	0.87 ± 0.08
12	9.57	0.77 ± 0.04
13	9.68	0.76 ± 0.05
14	9.74	0.65 ± 0.04
15	8.57	0.96 ± 0.29
16	9.71	0.59 ± 0.03

Unexpectedly, a lot of days registered negative productivity values, whilst showing a maximum variation in total biomass concentration of ~400 mg (relative to the day before). The growth profile shows a slightly increasing biomass concentration, already starting to stabilize by the end of this monitorization period, at 0.80 g/L. This represents a very interesting result since it is very closed to 0.90 g/L of biomass concentration found by [18] for non-specialized *Chlorella vulgaris* production. However, the ideal growth rates in biogas upgrading should be just above 1 g/L and lower than 1.50 g/L, the rate at which bacterial contamination might start occurring, leading to nutrient competition and the growth of other unwanted species [6]. The negative productivity values can be explained by the difficulty in an ideal harvesting of the sample in a non-homogeneous culture.

pH was measured on site using test strips, and some of the values reported in Table 7 indicate the culture adjusted its pH between sampling and laboratory analysis.

In the first 3 days, slightly acidic pH values between 6 and 7 were observed, meaning the slow carbon uptake was occurring for microalgae growth, as the biogas outlet was connected

to the airstone in the bottom of the ALG-IBC. However, productivity values were low, compared to literature ranges reported by Nagajaran et al. [18] (minimum 0.1 g/L/d for *Chlorella sp.*), which means the biogas flux to the ALG-IBC should increase, as this was the main carbon source to promote biomass growth.

Chlorella vulgaris is also reported to grow more rapidly at a pH range of 8-9 [18]. To try to boost this growth rate, the biogas was disconnected from the ALG-IBC to allow pH adjustment (restricted because of CO_2), and nutrients were added to the culture (sodium bicarbonate). Day 4 exbihited a pH increase to >9, suggesting the culture rapidly adjusted its pH, but the productivity value dropped. The biogas was then reconnected to keep CO_2 flowing to the ALG-IBC.

Day 9 recorded the highest biomass production value of 1.21 ± 0.71g/L, a value higher than expected considering the values of the days immediately before and after, 0.88 ± 0.31 g/L in day 8 and 0.63 ± 0.04 g/L in day 10. This translates in a 37.5% increase in biomass from days 8 to 9, followed by an immediate reduction of 52% from days 9 to 10. Interestingly, in days 6 and 7, nutrients were added to the culture because the ALG-IBC was filled in day 6, and the pH registered in day 7 was <7; thus, the sudden boost in biomass concentration might be due to the fast sodium bicarbonate dissolution in the culture. After this day, all pH values reported were >9, both on site and in the laboratory, in the 1 m^3 total occupied volume of the ALG-IBC.

These results are interesting considering the low CAPEX costs involved in IBC processes, compared to expensive CAPEX of photobioreactors. If productivity is optimized, this unique method of culturing microalgae is a very promising business case.

5.5. Final Microalgae Characterization

To evaluate the potential of microalgae biomass valorization, specially, for pigments and lipids production, some aliquots were collected during the cell growth and the respective characterization properly conducted. The characterization of the lyophilized microalgae was performed at days day 28, 33 and 40 of the experiment (days 4, 9 and 16 of the microalgae growth profile), being analyzed the lipid and chlorophyll concentration, as well as an elemental analysis for obtaining a C:H:N:S profile of the cells.

5.5.1. Elemental Analysis

To understand how the biomass properties varied throughout the growth monitorization period, an elemental analysis was performed. The results are presented in Table 8.

Table 8. CHNS biomass analysis results for days 4, 9 and 16 of monitorization.

Parameter	Day 28	Day 33	Day 40
C (%TS)	15.50 ± 0.53*	10.23 ± 0.29*	28.34 ± 0.26*
H (%TS)	3.02 ± 0.13	2.72 ± 0.02	4.59 ± 0.29
N (%TS)	2.55 ± 0.21	1.64 ± 0.04	5.14 ± 0.12
S (ppm)	≤ 100	≤ 100	≤ 100
C/N ratio	6.11 ± 0.46	6.25 ± 0.04	5.52 ± 0.127
Proteins (%)	12.85	8.24	25.91

*These values are much lower than the reported in the literature (~50%) and shouldn't be used; the analysis was performed by another lab and, due to time restrictions, it wasn't repeated

Carbon is the fundamental element in microalgal cells, accounting for nearly 50% of biomass [15]. The C values obtained from elemental analysis were considerably lower than those reported in literature; Sun et al. [15] report values of 51% total C in microalgal cells, while this work's highest value was reported in day 40, of only 28%. However, the value does represent a small increase in total biomass, since days 28 and 33 had a total C value of 15.50% and 10.23%, respectively.

Days 28 and 33 reported C/N ratios of 6.11 ± 0.46 and 6.25 ± 0.04, respectively, while the mean value in previous literature findings was 5.6 ± 0.2, for *Chlorella sp* [68]. Day 40 showed a C/N ratio of 5.52, which is in agreement with these findings. An increase in the C/N ratio can be related to the carbon assimilation of the microalgae culture. Carbohydrates increase with a gradual increase in CO_2 concentration and phosphorus limitation, depleting protein content, which was verified in this project since the AD-IBC biogas outlet was connected to the ALG-IBC, i.e., on days 28 and 33. On day 40, the C/N ratio dropped, since the biogas outlet was not

connected to the ALG-IBC for 4 days. In this case, the AD-IBC was connected to the 20L-ALGvessel, as explained in Section 5.1.

Other studies have reported that a C/N ratio between 5:1 and 19:1 for *Chlorella vulgaris* is more beneficial for biomass productivity (0.90 g/L/d), as the reducing sugar consumption rate in the medium is faster and the consumption of carbon and nitrogen sources is balanced. At a C/N ratio of 19:1, nitrogen starvation occurs, inhibiting biomass production. However, it has been demonstrated that at this ratio, the mean diameter of *Chlorella vulgaris* cells is smaller (the values range from 2 to 6.9 μm). A smaller cell has a larger surface area to volume ratio, facilitating the assimilation of nutrients and minimizing the sedimentation effect, the latter which can be minimized by adjusting the mixing/aeration [68].

In addition to the analysis of C/N ratio, a good indicator of a balanced system is related with the increase in protein content to 25.91%. Despite being below literature values of 28 to 44%, as per Klassen et al. [65], there was a 45% increase in protein content between days 33 and 40, which is correlated with better photosynthesis activity, CO_2 and N uptake.

5.5.2. Lipids Content

The total lipid extracted and FAME composition from microalgae biomass was quantified and compared with the starting ALG-IBC inoculum. The results are shown in Table 9 and the GC-MS fatty acid profiles depicted in Figure 20.

Table 9. Total Lipid yield and FAMEs composition of the analyzed microalgae

Parameters (%)	Inoculum	Day 28	Day 33	Day 40
Total Lipids Yield (%)	1.05 ± 0.08	1.37 ± 0.13	1.52 ± 0.37	1.45 ± 0.01
C16:0 (Palmitic)	41.31	39.33	38.29	36.15
C16:1 (Palmitoleic)	3.22	4.01	5.34	5.10
C18:0 (Stearic)	7.59	5.65	8.09	7.41
C18:1 (Oleic)	6.66	4.85	4.44	5.64
C18:2 (Linoleic)	3.19	19.69	22.07	17.59
C18:3 (Alpha-linoleic)	8.04	9.75	9.75	9.77
C19:0 (Nonadecanoic)	12.27	4.38	2.27	2.49
C20:0 (Eicosanoic)	15.87	12.34	9.75	15.84
C22:0 (Docosanoic)	1.85	-	-	-

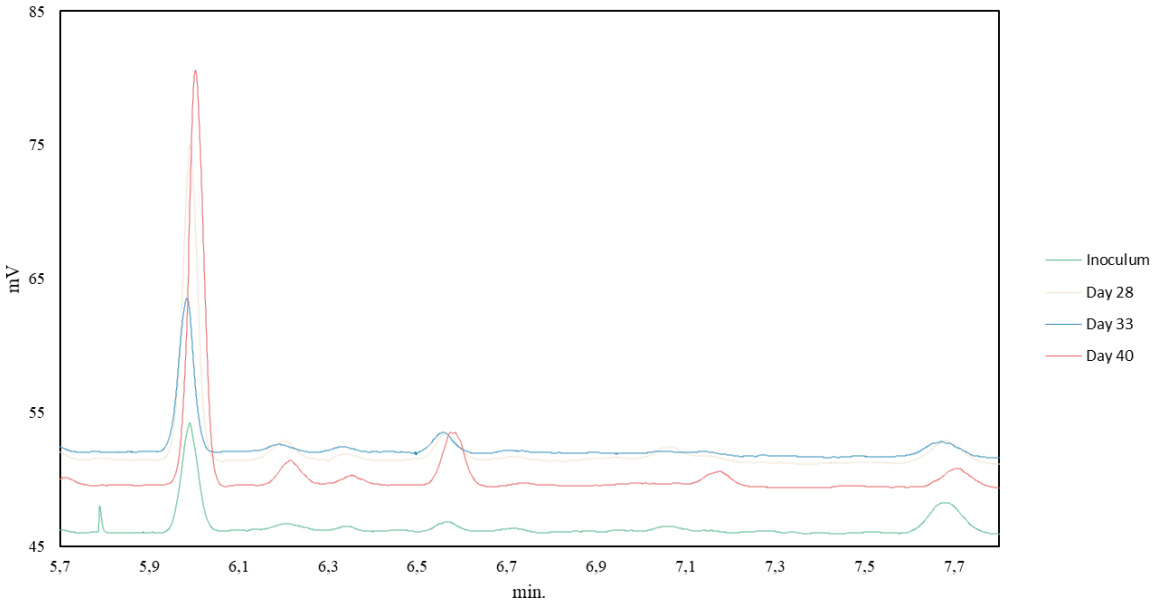

Figure 21. GC-MS FAMEs profile of each sample

The total lipid content is related to biomass growth, as lipid formation is related to the depletion of nutrients in the culture medium; if the culture is provided with the necessary amount of a carbon source for growth. Long-chain fatty acids (FAs) are specifically formed as a shutdown mechanism, by oxidative cell stress, mainly caused by nitrate limitation, which induces lipid accumulation. This work evidences the fact that *Chlorella vulgaris* favors the accumulation of saturated FAs, mainly palmitic acid (41.31% on day 0, 39.33% on day 28 and 36.15% on day 40, decreasing slightly) [57]. This also evidences that it occurred the accumulation of monounsaturated and polyunsaturated FAs, since the linoleic acid content increased from 3.19% in day 0 to 19.69% on day 28 and 17.59% on day 40, which means palmitic acids were converted to longer-chain FAs, caused by oxidative stress; this also means the culture might've entered a slight nutrient depletion state, despite the values being within the obtained in other studies (35% total saturated and 40% polyunsaturated acids) [57]. Also, the fact total lipid yield increased is indicative of biomass growth, and that day 33 was in fact the day the culture was denser, since it registered the highest lipid yield of 1.52%.

5.5.3. Chlorophyll

The UV-Vis Spectroscopy profiles for chlorophyll, in the samples of days 28, 33 and 40, are shown in Figure 22.

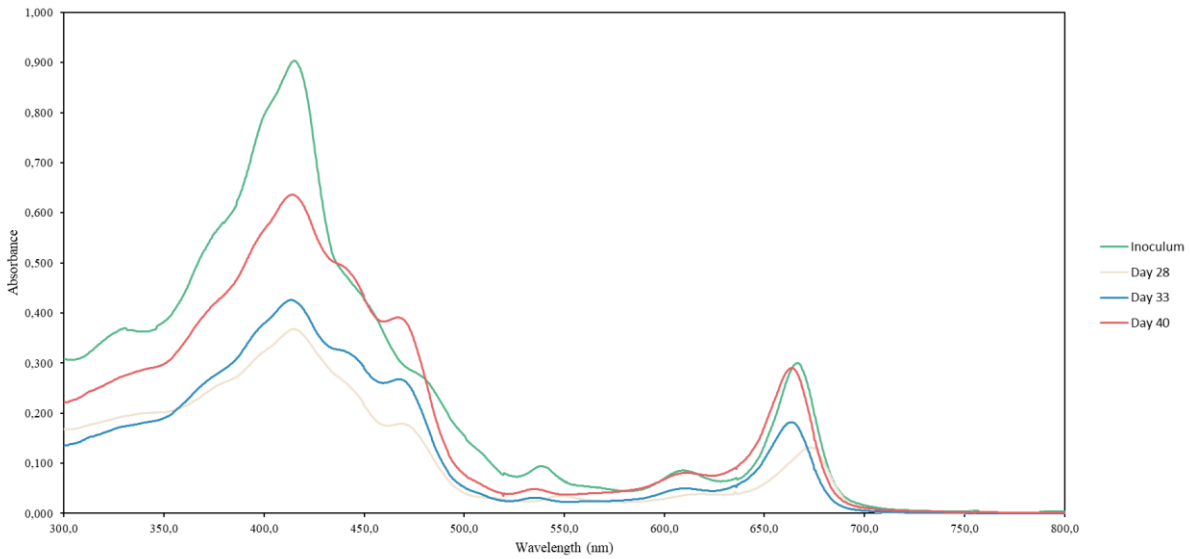

Figure 22. Chlorophyll sample's absorption spectrum

Chlorophyll *a* and *b* (Chl *a* and *b*) absorption maxima are in the spectral ranges near 428 and 453 nm for blue light and 661 and 642 nm for red light, respectively [69]. In this study, only Chl *a* was quantified, in a 650 to 675 nm range, as other pigments, such as carotenoids and xanthophylls, overlap in the spectral regions in the lower wavelengths and a proper thermogravimetrical analysis (TGA) should be done to properly quantify these pigments [69]. Thus, Chl *a* quantification was performed by estimating the maximum absorbance in the 650-675 nm wavelengths, using a calibration curve built with a standard liquid solution to estimate the total Chl *a* concentration. The chlorophyll concentrations of the samples are shown in Table 10, and the calibration curve in Annex H.

Table 10. Chlorophyll concentration of the inoculum on days 4, 9 and 16 of monitorization.

Parameter	Abs	Chl *a* (mg/L)
Inoculum	0.30 ± 0.00	9.66 ± 0.08
Day 28	0.13 ± 0.02	4.32 ± 0.75
Day 33	0.18 ± 0.05	5.93 ± 1.48
Day 40	0.29 ± 0.02	9.42 ± 0.76

Chlorophyll accumulation in Chlorella vulgaris is mostly dependent on the optimum light:dark cycle, light intensity (blue/red colored), temperature, culture medium nutrients, type of cultivation (where mixotrophic is ideal), and proper stirring/aeration to promote homogeneity and cell distribution [70].

The values obtained were lower than in other studies since the culture was inside a container without direct sunlight or artificial LED lights; Fakhri et al. [66] reported values of 19.305 mg/L

minimum Chl *a* concentration in a nutrient depleted medium. This is also representative of a lower CO_2 (or other carbon source) solubilization in the system, because the carbon uptake for growth is lower if the photosynthesis activity is reduced, which is evidenced by the low chlorophyll concentration [70]. This also has direct influence in the upgrading process, as the culture's density is one of the major restraints to obtain a successful upgraded biogas.

However, there was a slight Chl *a* increase between days 28 and 40 of the monitorization, from 4.32 to 9.42 mg/L, representative of biomass growth. Thus, it is interesting to increase the amount of light entering the system, since it will boost chlorophyll and biomass production significantly.

6. CONCLUSION AND FUTURE WORKS

This dissertation focused on verifying the system's ability to upgrade biogas, whilst promoting microalgal growth, using cattle manure as a feedstock for AD, in a decentralized organic farm (Monte Silveira Bio, Ladoeiro, Castelo Branco, Portugal). The equipments used were IBC's, for biogas generation and microalgal cultivation, due to their low costs and simple processing techniques.

The upgrading process was tested using a *Chlorella vulgaris* NIVA-CHL 108 microalgae passive platform (with 1 m^3 volume), as no energy except for an airstone connected to a solar panel was being used. This would meet the EGD's perspective of a sustainable step towards the mitigation of GHG emissions, contributing to carbon fixation by reducing agricultural emissions, which accounted for 55.4% of all CH_4 emissions in the EU in 2020, whilst generating value-added products by culturing microalgae in the process. It would also contribute to the REPowerEU plan of increasing biomethane production until 2030.

The pilot system was set in 17/07/2023 (day 0); biogas monitorization started on 09/08/2023 (day 23) and was carried for a total 21 days, ending on 06/09/2023, with ambient temperatures varying from 18 to 43 ºC. The analysis were performed in the AD-IBC all 21 days and on a 50L-ADvessel for the last 5 days of monitorization to experiment different feedstocks, while upgraded biogas analysis were done in the ALG-IBC for 5 days (32 to 36) and on a 20L-ALGvessel for 8 days (37 to 48).

The AD-IBC CH_4 production peaked at 52.8% in day 37, but all other results were below 40%, reaching the lowest production of 19.1%. The first three days of monitorization reported values > 35%, which were obtained again 2 weeks after; loading rates were changed during the experiment and became more frequent after day 34. The results in the ALG-IBC and a downscaled 20L-ALGvessel showed there was no upgraded biogas due to a low biogas flux entering these containers, which didn't allow the aerated biogas to flow through the microalgae platform, and it eventually dissolved in the culture. The maximum CH_4 value obtained in the ALG-IBC was 2.4%.

However, the high CO_2 solubility in water made possible the microalgae culture growth, which shows potential for microalgae culturing in the pilot's simple system if optimized. It is also important to note that the CO_2 solubility is in itself indicative of biogas upgrading, as the CO_2 was being captured by the culture, but the CH_4 didn't reach the gas phase in the ALG-IBC (nor the 20L-ALG-vessel).

To test a co-digestion system, which is reported to boost CH_4 production, OFI were added as feedstock in day 40, which showed interesting results, especially regarding the acclimatization time of the system, which was faster than the addition of the same quantity of cattle manure as single a feedstock (40 L, 3 days).

In the end, three samples of biomass were characterized, showing differences in their properties; oil and protein content increased, while chlorophyll content decreased probably due to insufficient light. This is an interesting microalgae culturing system to optimize, since the CAPEX costs involved in IBC processes are much lower than the reported in PBRs. Since the IBC is a closed container, nutrient competition by other species will be reduced, and bacterial contamination is less likely to occur.

The project will continue research towards obtaining an upgraded biogas, exploring different options to maximize the microalgae platform's culture conditions. Future works will include:

- Increasing the aeration in the ALG-IBC to increase the culture's homogeneity;
- Incorporating LED lights to promote photosynthetic activity;
- Increasing the biogas flux leaving the AD-IBC by trying different OLR's and feedstocks (e.g., Sudan grass), as OFI are a seasonal fruit, which usually grows in Portugal only in the dry weather of august and beginning of September.
- Minimizing the AD-IBC biogas variation of CH_4 and CO_2, which represents a major constraint in the adaptation and stabilization of the ALG-IBC, since the biogas is the main carbon source of the microalgae culture besides the bicarbonate feeding once a week.

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ANNEXES

Annex A | Summary for Policymakers (SPM) Report

Annex A1 | Cumulative emissions of CO_2 estimations until 2100 (page 6 of the SPM report)

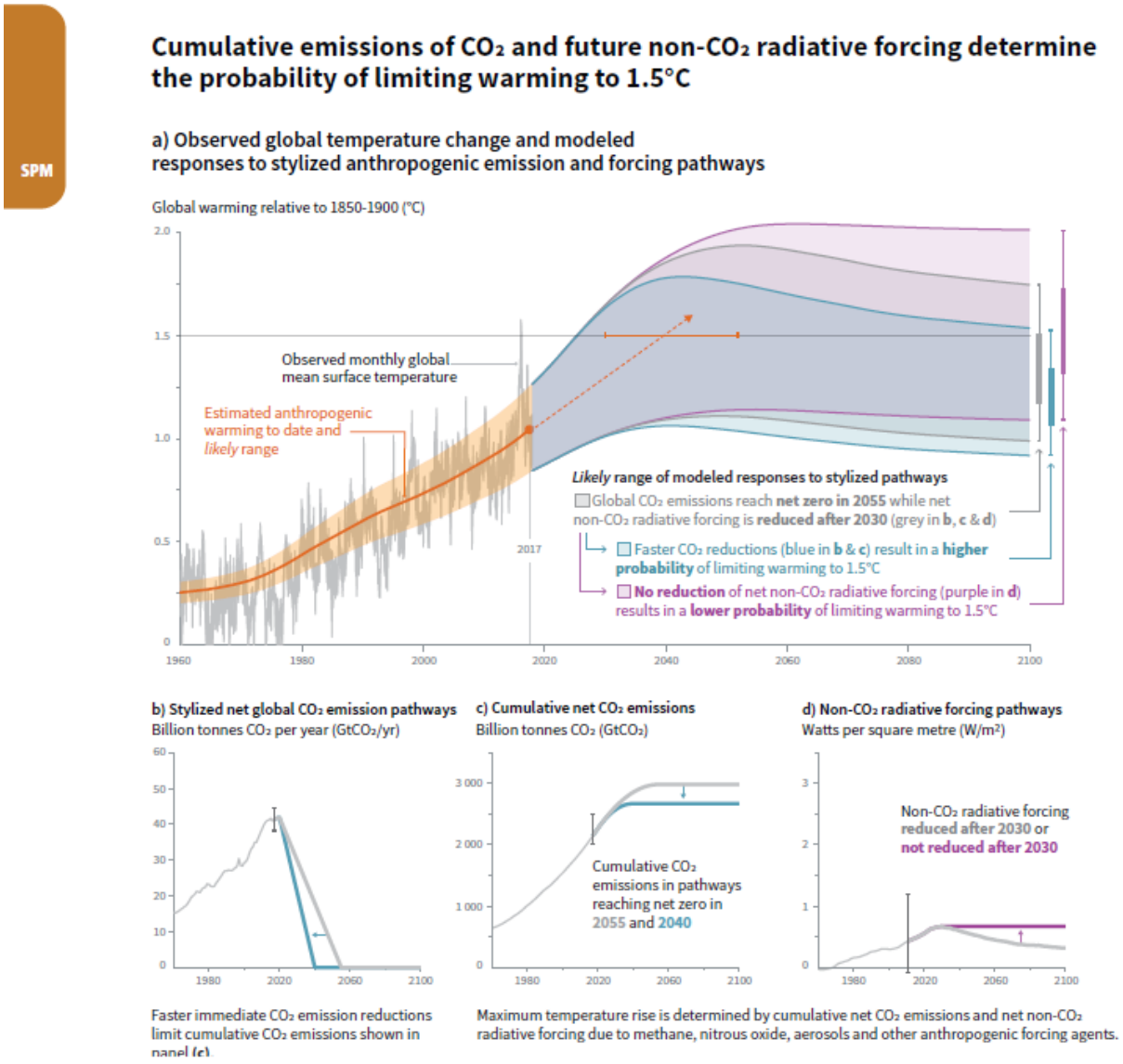


Figure SPM.1 | Panel a: Observed monthly global mean surface temperature (GMST, grey line up to 2017, from the HadCRUT4, GISTEMP, Cowtan–Way, and NOAA datasets) change and estimated anthropogenic global warming (solid orange line up to 2017, with orange shading indicating assessed *likely* range). Orange dashed arrow and horizontal orange error bar show respectively the central estimate and *likely* range of the time at which 1.5°C is reached if the current rate of warming continues. The grey plume on the right of panel a shows the *likely* range of warming responses, computed with a simple climate model, to a stylized pathway (hypothetical future) in which net CO_2 emissions (grey line in panels b and c) decline in a straight line from 2020 to reach net zero in 2055 and net non-CO_2 radiative forcing (grey line in panel d) increases to 2030 and then declines. The blue plume in panel a) shows the response to faster CO_2 emissions reductions (blue line in panel b), reaching net zero in 2040, reducing cumulative CO_2 emissions (panel c). The purple plume shows the response to net CO_2 emissions declining to zero in 2055, with net non-CO_2 forcing remaining constant after 2030. The vertical error bars on right of panel a) show the *likely* ranges (thin lines) and central terciles (33rd – 66th percentiles, thick lines) of the estimated distribution of warming in 2100 under these three stylized pathways. Vertical dotted error bars in panels b, c and d show the *likely* range of historical annual and cumulative global net CO_2 emissions in 2017 (data from the Global Carbon Project) and of net non-CO_2 radiative forcing in 2011 from AR5, respectively. Vertical axes in panels c and d are scaled to represent approximately equal effects on GMST. (1.2.1, 1.2.3, 1.2.4, 2.3, Figure 1.2 and Chapter 1 Supplementary Material, Cross-Chapter Box 2 in Chapter 1)

Figure A1. Cumulative emissions of CO_2 estimations until 2100 (Obtained from [2])

Annex A2 | Global emissions reduction estimation to reach net-zero (page 13 of the SPM report)

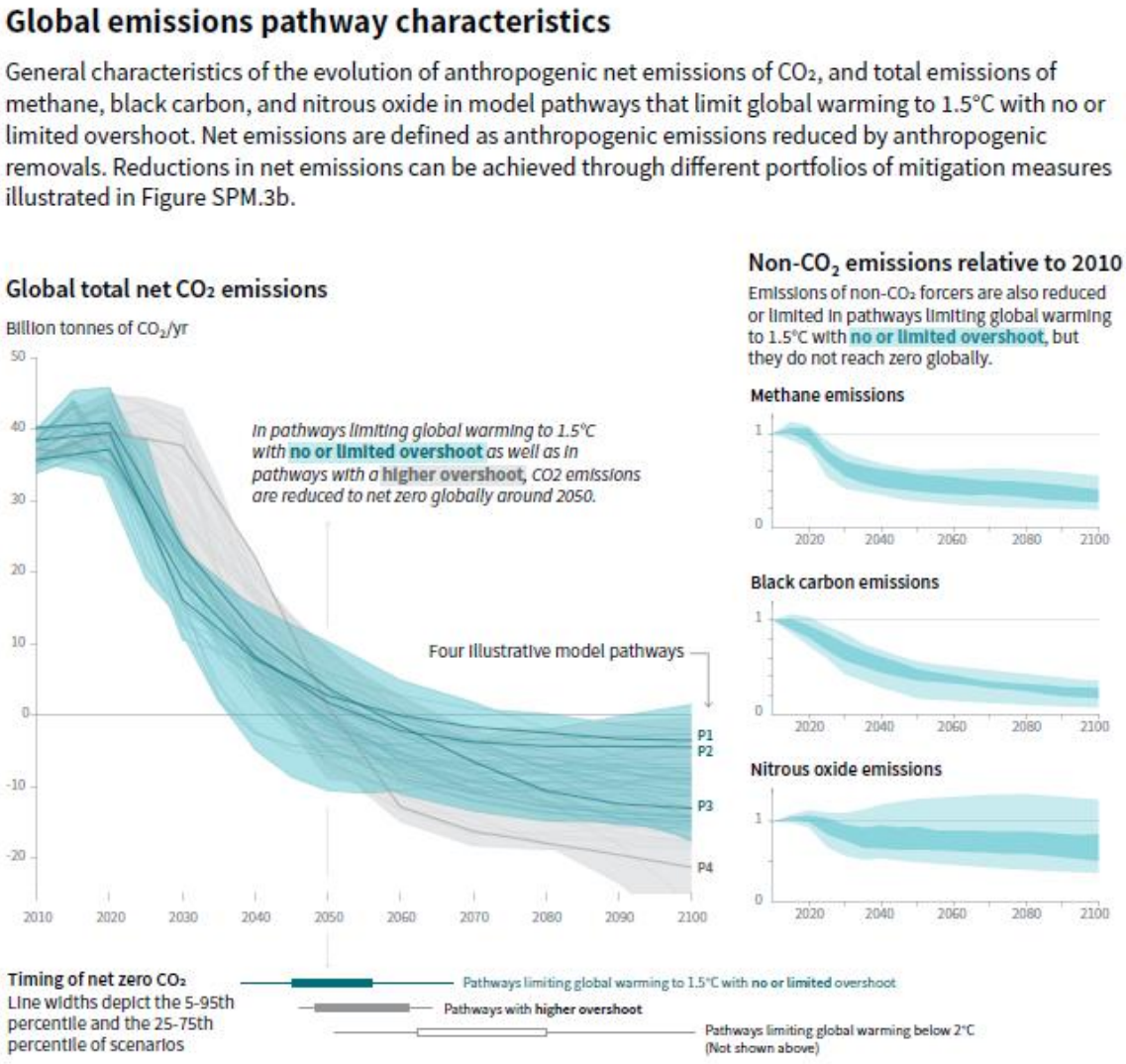


Figure SPM.3a | Global emissions pathway characteristics. The main panel shows global net anthropogenic CO_2 emissions in pathways limiting global warming to 1.5°C with no or limited (less than 0.1°C) overshoot and pathways with higher overshoot. The shaded area shows the full range for pathways analysed in this Report. The panels on the right show non-CO_2 emissions ranges for three compounds with large historical forcing and a substantial portion of emissions coming from sources distinct from those central to CO_2 mitigation. Shaded areas in these panels show the 5–95% (light shading) and interquartile (dark shading) ranges of pathways limiting global warming to 1.5°C with no or limited overshoot. Box and whiskers at the bottom of the figure show the timing of pathways reaching global net zero CO_2 emission levels, and a comparison with pathways limiting global warming to 2°C with at least 66% probability. Four illustrative model pathways are highlighted in the main panel and are labelled P1, P2, P3 and P4, corresponding to the LED, S1, S2, and S5 pathways assessed in Chapter 2. Descriptions and characteristics of these pathways are available in Figure SPM.3b. (2.1, 2.2, 2.3, Figure 2.5, Figure 2.10, Figure 2.11)

Figure A2. Global emissions reduction estimation to reach net-zero (Obtained from [2])

Annex B | European Green Deal (EGD) recent initiatives

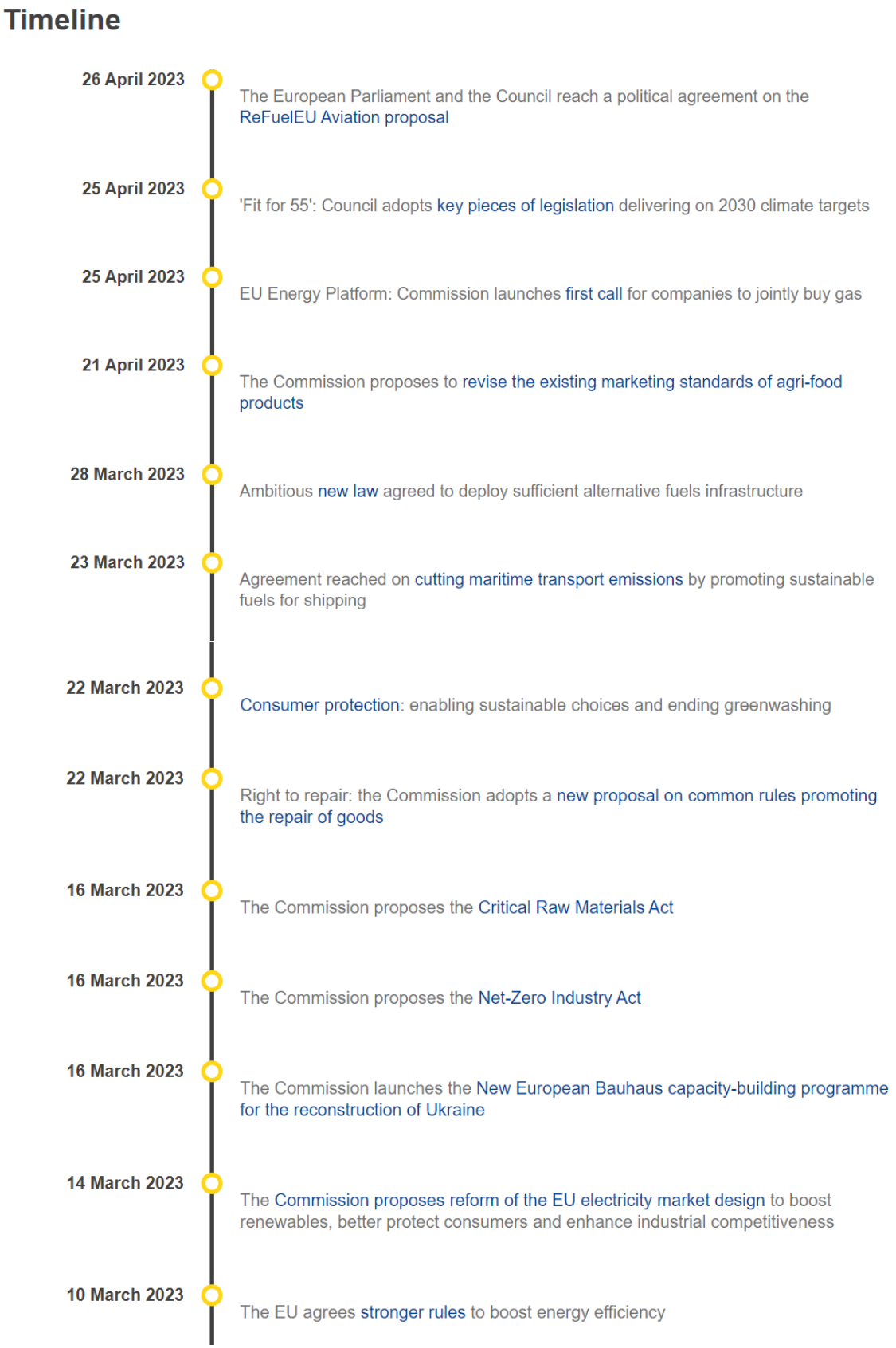


Figure B. 2023 EGD's initiatives (Obtained from [71])

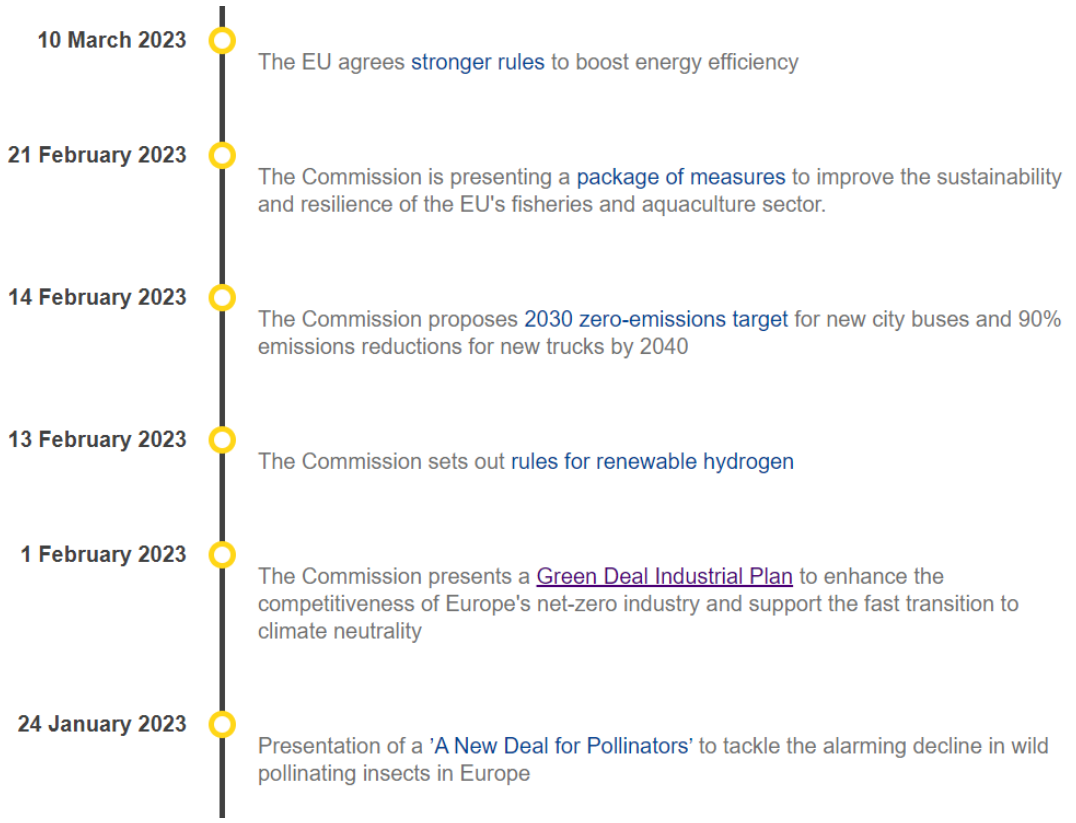


Figure B(cont.). 2023 EGD's initiatives (Obtained from [71])

Annex C | Innovative Biomethane for RePowerEU

Annex C1 | Fuelling Innovation (pages 6-7)

Figure C1. The gas network concept (Obtained from [13])

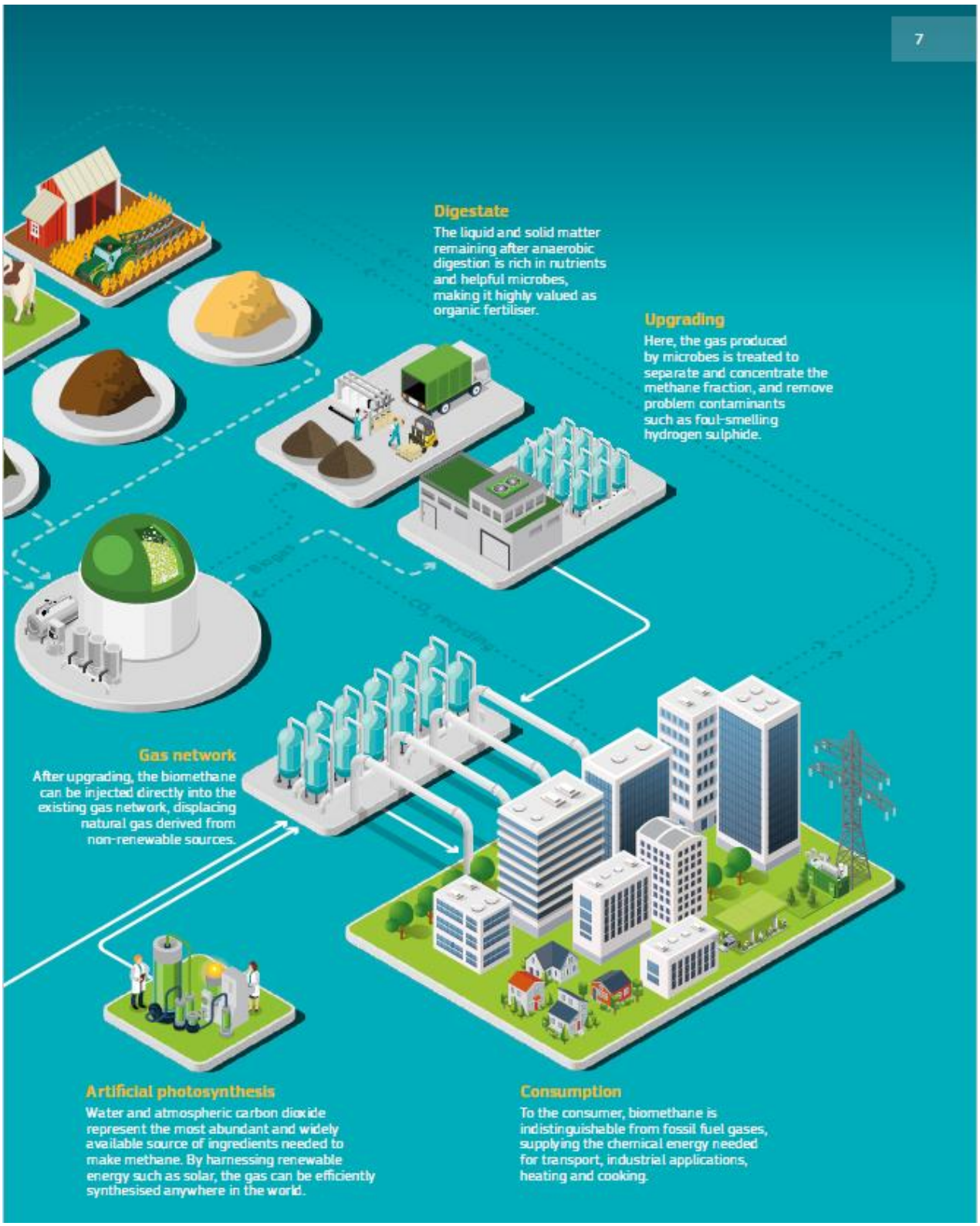


Figure C1 (cont.). The gas network concept (Obtained from [13])

Annex C2 | Projects (page 11)

Bin2Grid: Turning unexploited food waste into biomethane supplied through local filling stations network

Over 88 million tonnes of food are thrown away in the EU every year. The Bin2Grid project promoted the collection of food waste, and its conversion to biogas and upgrading into biomethane, supplying stations in Zagreb, Skopje, Malaga and Paris.

To bridge the gaps between waste management and renewable energy production, the project investigated technologies related to biowaste separation and treatment, biogas production and upgrading, and economic tools to boost profitability of the concept.

Project dates:
1 January 2015 – 31 December 2017

Coordinated by:
Zagrebacki Holding in Croatia

Funded under:
Horizon 2020-ENERGY

CORDIS factsheet:
cordis.europa.eu/project/id/646560

Total budget:
EUR 709 468

EU contribution:
EUR 709 468

BiogasAction: Promotion of sustainable biogas production in EU

The BiogasAction project developed the European biogas sector across 14 European regions by focusing on the removal of non-technical barriers to widespread production from manure and other organic waste.

As well as a comprehensive biomethane market web portal, the project created a guidance document for investors on financing biogas and biomethane projects, and advice for policymakers and local authorities on improving national framework conditions for biogas and biomethane deployment.

Project dates:
1 January 2016 – 31 December 2018

Coordinated by:
Energy Consulting Network in Denmark

Funded under:
Horizon 2020-ENERGY

CORDIS factsheet:
cordis.europa.eu/project/id/691755

Total budget:
EUR 1 999 885

EU contribution:
EUR 1 999 885

BIOSURF: BIOmethane as SUstainable and Renewable Fuel

By harmonising biomethane registration, labelling, and certification, we can streamline cross-border trade in biomethane. The BIOSURF project extended national registries of biogas injection to the whole of Europe, enabling movements of biomethane through the European natural gas infrastructure.

It also developed a calculation to quantify the greenhouse gas emissions of biomethane that is compliant with both the RED framework and the EU Emissions Trading System.

Project dates:
1 January 2015 – 31 December 2017

Coordinated by:
Institute of Studies for the Integration of Systems (I.S.I.S), Cooperative Society in Italy

Funded under:
Horizon 2020-ENERGY

CORDIS factsheet:
cordis.europa.eu/project/id/646533

Project website:
biosurf.eu/en_GB

Total budget:
EUR 1 872 912

EU contribution:
EUR 1 872 912

ISABEL: Triggering Sustainable Biogas Energy Communities through Social Innovation

Sustainable biogas technologies have been slow in catching up with community energy developments.

Founded on the principles of Social Innovation, the ISABEL project carried out work in Germany, Greece and the United Kingdom to pave the way for the transition from traditional supply chains to community ownership, allowing citizens to take full advantage of the ample societal benefits of local community-driven biogas systems.

Project dates:
1 January 2016 – 31 December 2018

Coordinated by:
Q-Plan International Advisors in Greece

Funded under:
Horizon 2020-ENERGY

CORDIS factsheet:
cordis.europa.eu/project/id/691752

Total budget:
EUR 1 897 437

EU contribution:
EUR 1 897 437

Figure C2. Projects financed by the HORIZON programme (Obtained from [13])

Annex C3 | Filling the tank (page 14)

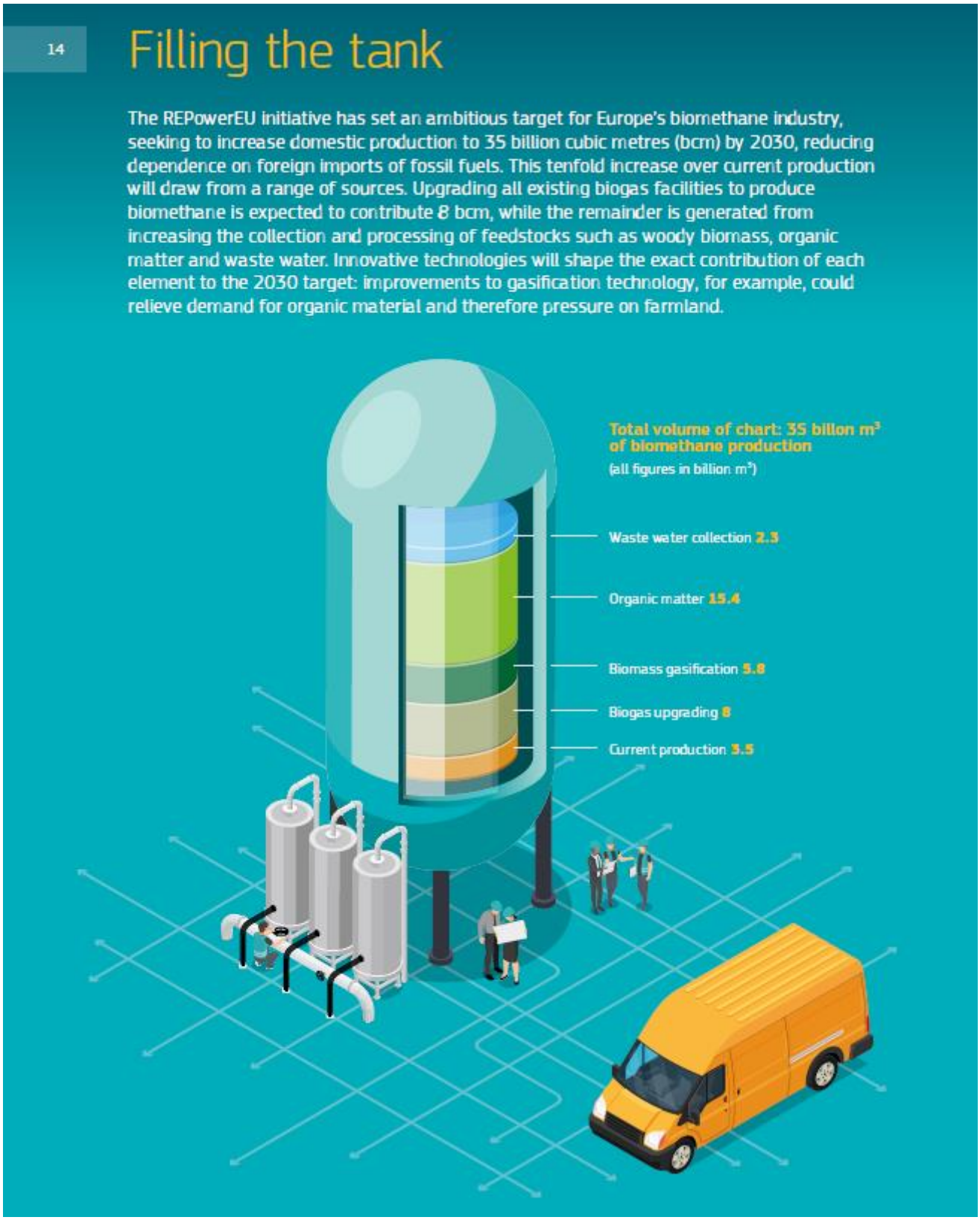


Figure C3. Projection for the increase in biomethane production until 2030 (Obtained from [13])

Annex D | Key Figures on the European Chain

Annex D1 | Farms (page 11)

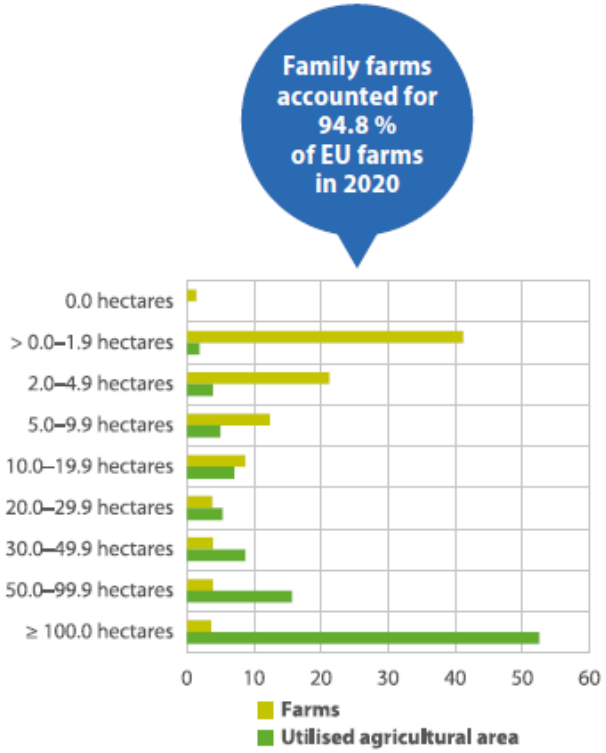
Farms

Distribution of farms and farmland by farm size

(% share of total, EU, 2020)

There were 9.1 million agricultural holdings (simply referred to as farms) in the EU in 2020. Almost one third (31.8 %) of these were located in Romania, with more than one tenth in each of Poland (14.4 %), Italy (12.5 %) and Spain (10.1 %).

The average (mean) size of a farm in the EU in 2020 was 17.4 hectares. However, almost two thirds (63.8 %) of the EU's farms were less than 5.0 hectares in size, while just over one tenth (11.4 %) of the farms in the EU had 30.0 hectares or more. The largest size category of farms, those with at least 100.0 hectares, accounted for 3.6 % of the total number of farms, but collectively had slightly more than half (52.5 %) of the total area used for agricultural production in the EU. As such, there were very many semi-subsistence farms in the EU and only a few particularly large ones.

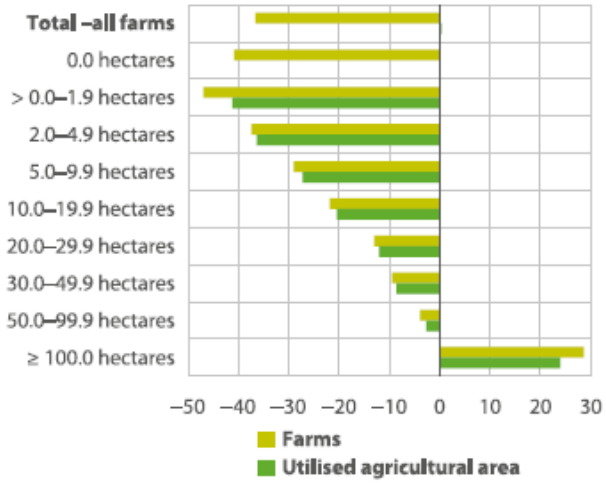


Source: Eurostat (online data code: ef_m_farmleg)

Overall change in farms and farmland by farm size

(%, EU, 2005–2020)

There were about 5.3 million fewer farms in the EU in 2020 than in 2005, a decrease of 37 % (1). The vast majority of the decrease in farm numbers concerned farms smaller than 5.0 hectares in utilised agricultural area; there were 4.6 million fewer farms in this category during the period under consideration. The only category of farms for which an increase in farm numbers was observed was for those with at least 100.0 hectares. As the overall area used for agricultural production in the EU hardly changed between 2005 and 2020 (an increase of 0.3 %), the falling number of farms among all size categories except for the largest reflects mergers or takeovers of farms.



Note: 2005 includes 2007 data for HR.

Source: Eurostat (online data code: ef_m_farmleg)

(1) Some of this observed change may reflect methodological difference in the statistics for 2005 and 2020 (in particular changes in survey thresholds). Note also that the EU figure for 2005 includes 2007 data for Croatia.

Figure D1. Distribution of farms and farmland by size (Obtained from [45])

Annex D2 | Gross Output and Intermediate Consumption (pages 38-41)

Figure D2. Distribution of gross output for the agricultural industry (Obtained from [45])

Gross output and intermediate consumption

Distribution of gross output for the agricultural industry

(€ billion, values at basic prices, EU, 2021)

Among other objectives, the EU's *Farm to Fork Strategy* aims to generate fairer economic returns and foster competitiveness of the EU supply sector. The economic performance of the agricultural sector matters directly for farms, farmers and farm workers as well as indirectly for upstream and downstream activities, rural communities, and final consumers of products derived from agricultural output.

The term agricultural industry is used to describe all agricultural holdings (farms) involved in agricultural production, groups of producers (co-operatives) that make wine and olive oil, and specialised agricultural contractors. The value of the gross output produced by the EU's agricultural industry was €449.5 billion in 2021.

This includes crop output (€248.7 billion; 55.3 % of the total), animal output (€163.1 billion; 36.3 %), agricultural services (€21.6 billion; 4.8 %) and some inseparable non-agricultural goods and services (€16.2 billion; 3.6 %).

At a more detailed level, the largest categories of the EU's agricultural output in 2021 were cereals (€64.1 billion; 14.3 %), vegetables and horticultural products (€61.7 billion; 13.7 %), milk (€58.0 billion; 12.9 %), pigs (€35.8 billion; 8.0 %) and fruits (€31.7 billion; 7.1 %). A majority of the cereals produced in the EU are used for animal feed, with the remainder for human consumption and use within non-food/feed industries, such as biofuels.

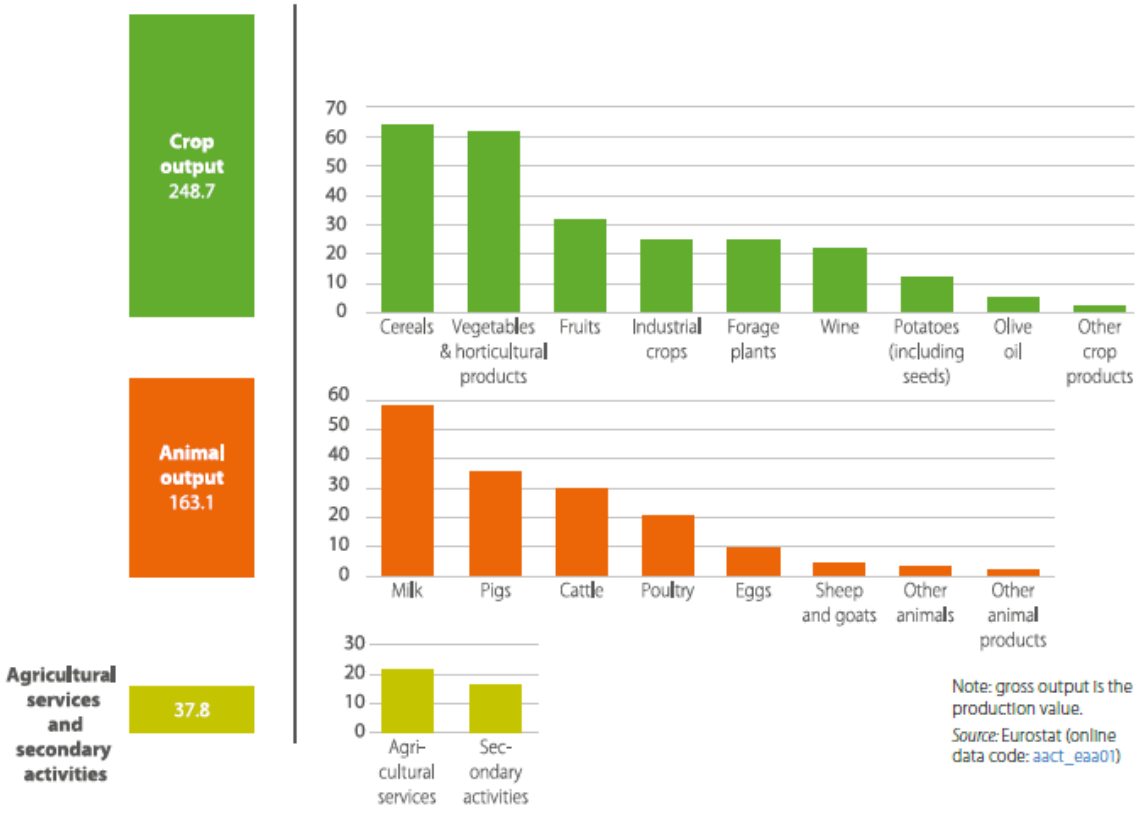
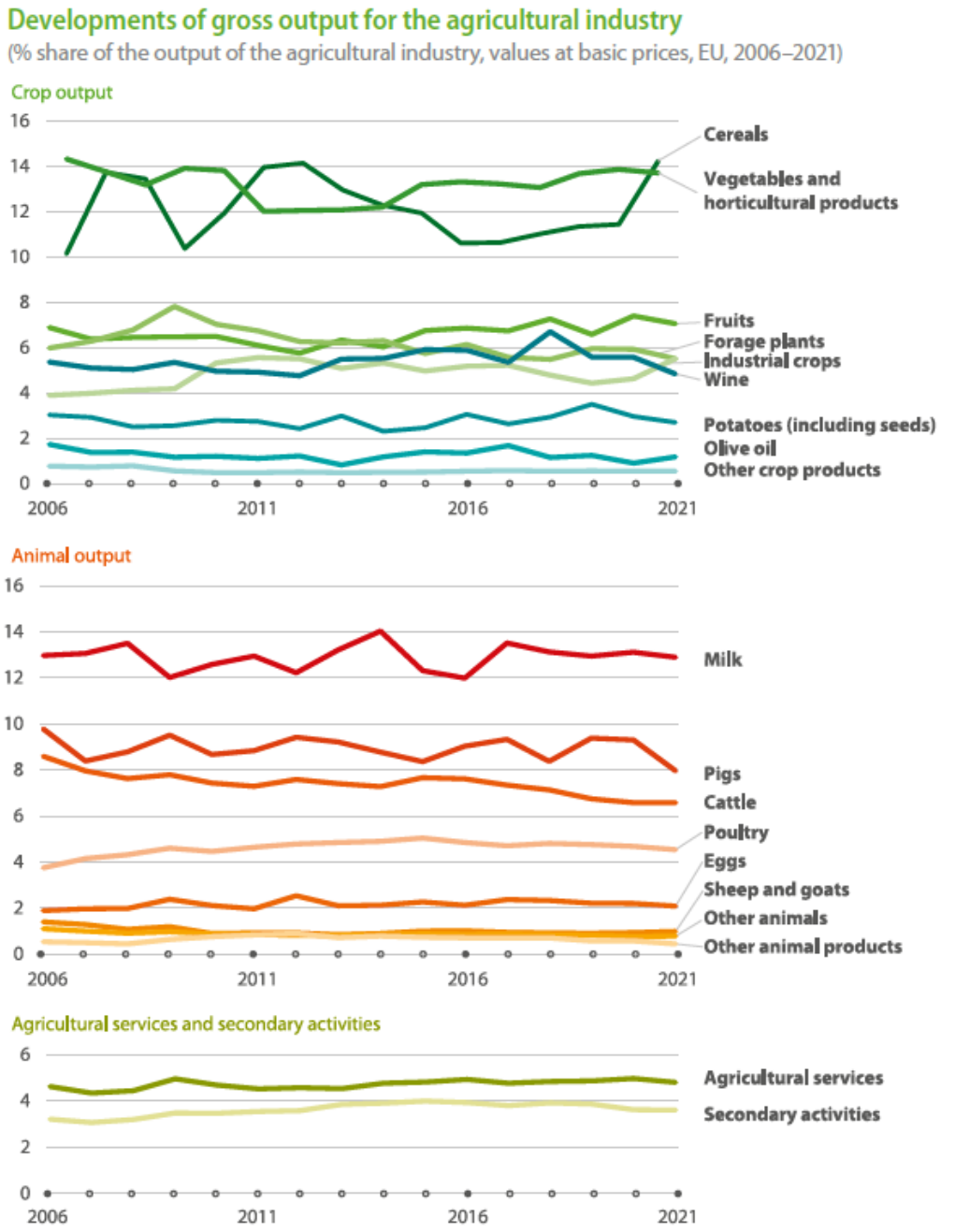


Figure D2 (cont.). Distribution of gross output for the agricultural industry (Obtained from [45])



The relative share of crops in the gross output of the EU's agricultural industry rose by 2.1 percentage points between 2020 and 2021, while there was a decrease of similar magnitude for animal output (down 1.9 points). The gains recorded for crop output were principally driven by an increase in the gross output of cereals (whose share of the agricultural industry's output was up 2.8 points), along with smaller gains for industrial crops (up 0.9 points) and olive oil (up 0.3 points).

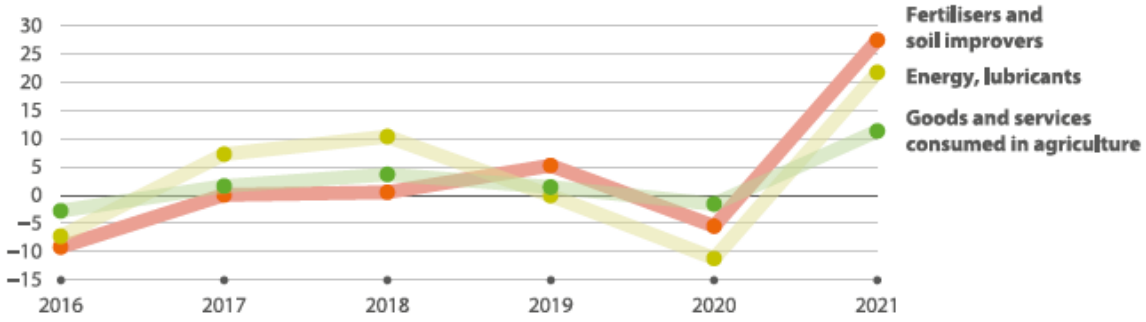
Source: Eurostat (online data code: aact_eaa01)

Annual rate of change of input price indices for the agricultural industry

(%, EU, 2016–2021)

Since the start of Russia's large-scale military invasion of the whole of Ukraine in February 2022, there has been considerable pressure from rising energy prices. These increases have also impacted a number of downstream/related activities; within the context of agriculture, one of the main impacts has been on the price of fertilisers. Although data covering this period are not yet available, there was already considerable pressure on prices in 2021.

Input price indices cover the intermediate consumption of goods and services (for example, fertilisers, pesticides, seed or energy) and gross fixed capital formation (for example, machinery and equipment). There was a rapid increase in input prices for the EU's agricultural industry between 2020 and 2021, as the overall price of goods and services rose 11.4 %. More substantial input price developments were recorded for fertilisers and soil improvers (up 27.5 % in 2021) and for energy and lubricants (up 21.8 %).

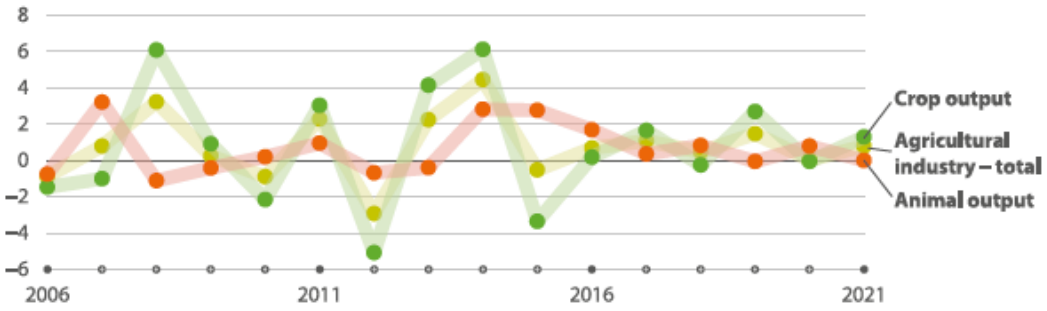


Note: based on indices compiled with 2015 = 100.
Source: Eurostat (online data code: apri_pi15_ina)

Annual rate of change of volume indices for the agricultural industry

(%, basic prices, EU, 2006–2021)

Changes in the volume indices of output reflect a change in the value of output after removing any price changes (inflation or deflation); this is broadly synonymous with a change in constant prices. With an 8.3 % increase in the value of output and a slightly lower increase (up 7.5 %) in output prices of agricultural goods and services, the volume index of output for the EU's agricultural industry rose 0.8 % in 2021. This increase reflected a rising volume index for crop output (up 1.3 %), while there was no change in the volume index for animal output (0.0 %).

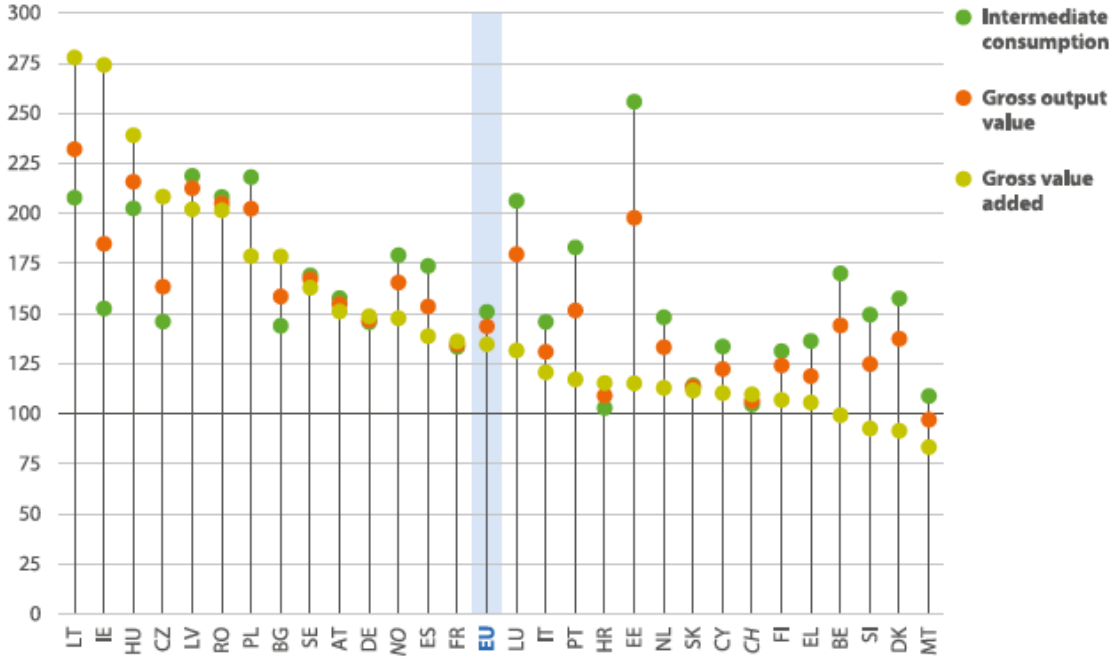


Note: based on indices compiled with 2015 = 100.
Source: Eurostat (online data code: aact_eaa05)

Figure D2 (cont.). Distribution of gross output for the agricultural industry (Obtained from [45])

Developments of output and consumption for the agricultural industry

(2006 = 100, values at current basic prices, 2021)



Note: Indices originally compiled with 2015 = 100; rescaled to 2006 = 100. Ranked on the change in value added.

Source: Eurostat (online data code: aact_eaa05)

The total output value of the EU's agricultural industry (in basic prices) was €449.5 billion in 2021. Inputs of products that are used up (consumed) in a production process, such as fertilisers, pesticides, seed, animal feed, energy and veterinary services, are referred to as intermediate consumption. The cost of these inputs for the agricultural industry totalled €260.2 billion across the EU. The difference between the output value and the cost of intermediate consumption is the value added at basic prices, in other words, the value that has been added through production (in this case agricultural) processes. In 2021, gross value added for the EU's agricultural industry was €189.4 billion.

Between 2006 and 2021, gross value added in the EU's agricultural industry increased overall by 34.7 % in current price terms, reflecting a 43.7 % increase in the value of output offset to some extent by a 51.1 % increase in the costs of intermediate consumption.

Six of the EU Member States – Lithuania, Ireland, Hungary, Czechia, Latvia and Romania – recorded value added in their agricultural industries at least doubling in current price terms between 2006 and 2021. In Poland, Bulgaria, Sweden and Austria, value added increased by at least 50 % during the period under consideration. By contrast, value added was lower in 2021 than in 2006 in Belgium, Slovenia, Denmark and Malta. The output of the agricultural industry and the cost of intermediate consumption were both higher in 2021 than in 2006 for each of the EU Member States, except for Malta (where the output of the agricultural industry fell by a modest amount).

Figure D2 (cont.). Distribution of gross output for the agricultural industry (Obtained from [45])

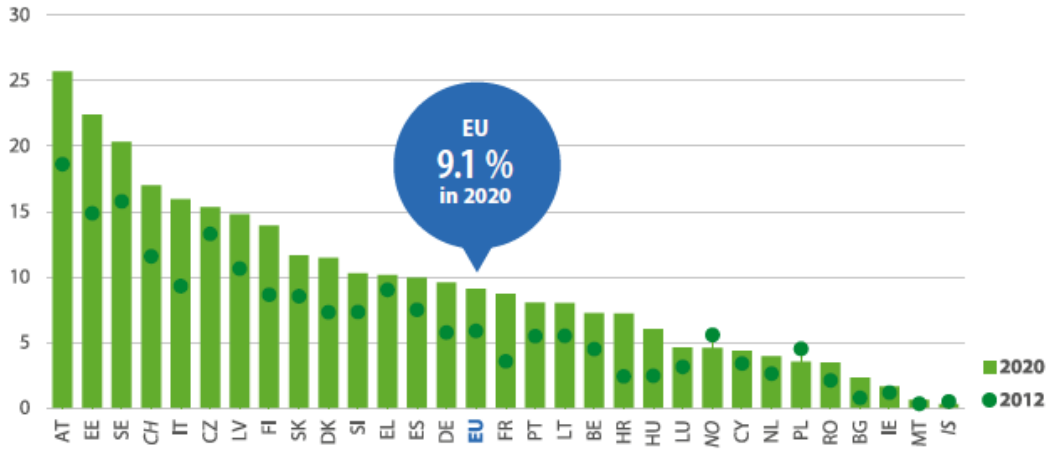
Annex D3 | Organic Farming (pages 14-15)

Figure D3. Organic Farming area in %UAA (Obtained from [45])

Organic farming

Organic area

(% share of total utilised agricultural area, 2012 and 2020)



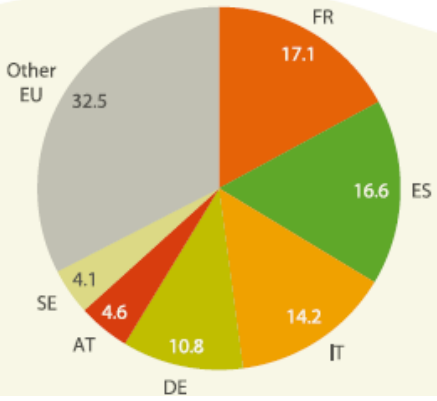
Note: Includes fully converted areas and areas under conversion.
IS: 2013 instead of 2012.

Source: Eurostat (online data code: org_cropar)

Organic farming is a method that aims to use natural substances and processes and to do so in a more sustainable way than conventional farming. The EU's *Farm to Fork Strategy* set an objective that at least 25 % of the EU's agricultural land should be farmed using organic processes by 2030.

In 2020, the area used for organic agricultural production within the EU was 14.7 million hectares. The total organic area in the EU increased by 5.3 million hectares between 2012 and 2020, equivalent to a rise of more than one half (55.7 %). The share of the total utilised agricultural area that was organic increased from 5.9 % in 2012 to 9.1 % in 2020. During this period, the share of the agricultural area used for organic farming increased in all EU Member States except for Poland.

In 2020, the highest shares of organic farm areas within the total utilised agricultural area were in Austria (25.7 %), Estonia (22.4 %) and Sweden (20.3 %). By contrast, the share of organic farming was below 5.0 % in eight EU Member States, with the lowest shares in Ireland (1.7 %) and Malta (0.6 %).



Share of EU organic area

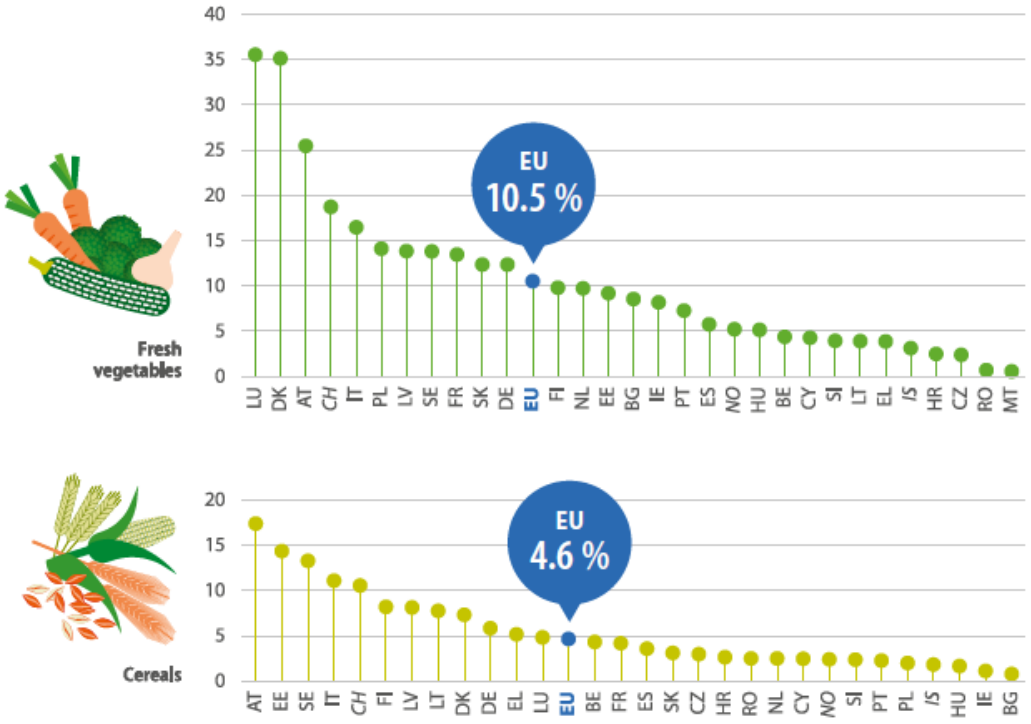
(%, 2020)

Nearly three fifths (58.7 %) of the EU's total organic area in 2020 was located in four EU Member States: France (17.1 %), Spain (16.6 %), Italy (14.2 %) and Germany (10.8 %).

Source: Eurostat (online data code: org_cropar)

Organic area for fresh vegetables and cereals

(% share of utilised agricultural area, 2020)



Note: fresh vegetables include melons and strawberries.
Cereals: MT, not available.

Source: Eurostat (online data codes: org_cropar and apro_cpsh1)

Cereals and fresh vegetables are among the main arable crops, along with root crops, green fodder and industrial crops.

The area used for the organic farming of fresh vegetables in the EU was 219 thousand hectares in 2020, equivalent to 0.1 % of all land used for agricultural production. About one tenth (10.5 %) of the land used for the production of fresh vegetables in the EU was farmed organically. Luxembourg, Denmark and Austria had notably higher shares of organic farming within their total area of land used for growing fresh vegetables.

The area used for the organic farming of cereals in the EU was 2.4 million hectares in 2020, equivalent to 1.5 % of all land used for agricultural production. Some 4.6 % of the land used for the production of cereals in the EU was farmed organically. Austria, Estonia and Sweden had the highest shares of organic farming within their total area of land used for growing cereals.

Figure D3 (cont.). Organic Farming area in %UAA (Obtained from [45])

Annex E | Mapping Portugal's Bio-Based Potential

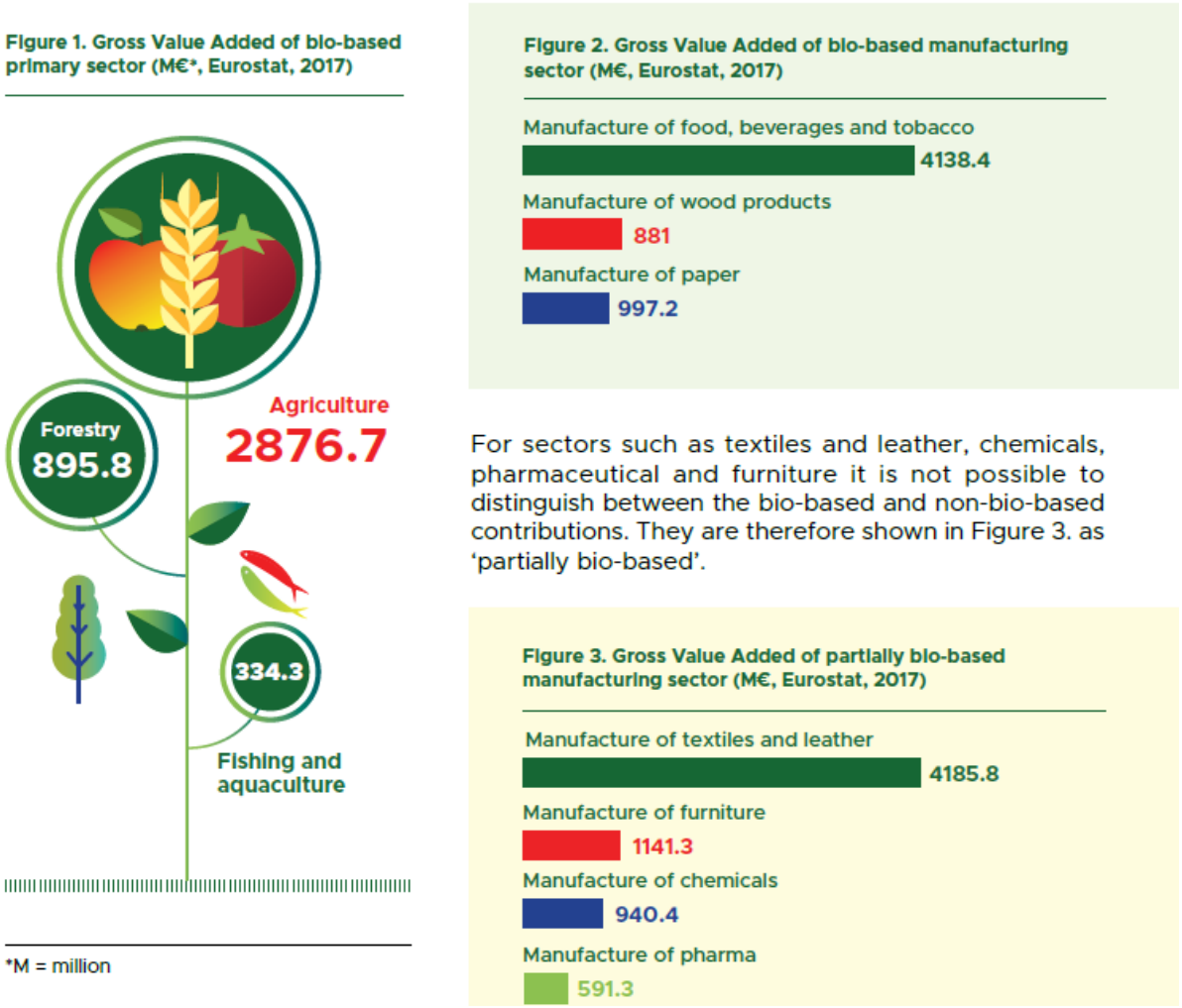


Figure E. Gross Value Added of the agricultural sector in Portugal (Obtained from [48])

Annex F | Project's On-Site Decision-Making Report

Project Timeline/Decision Making:

17/07 - Pilot preparation:

AD-IBC was filled with approximately 200L of fresh manure + water (1:4 (m/m))

ALG-IBC was inoculated with 20L of pre culture (1 g microalgae/L water) and 180L of water. Nutrients were added to promote growth - 200g NaHCO3, 200g Valagro (20:20:20), 100g NaCl, 20g MgSO4.

2 airstones are connected to the ALG-IBC, one bubbling air from a pump to promote water circulation through aeration and the other is connected to the gas outlet in the AD-IBC to promote algae growth through slow CO2 dissolution in the culture and assimilation.

25/07 - ALG-IBC Cultivation:

ALG-IBC was filled with approximately 200L of water (~400L total). Nutrients were added to promote growth - 200g NaHCO3, 200g Valagro (20:20:20), 100g NaCl, 20g MgSO4.

9/08 - ALG-IBC Cultivation + Biogas Monitorization:

ALG-IBC was filled with approximately 400L of water (~800L total). Nutrients were added to promote growth - 300g NaHCO3, 300g Valagro (20:20:20), 200g NaCl, 40g MgSO4.

ALG-IBC pH 6 - 7 (sampled with a test strip).

1st AD-IBC biogas reading (biogas analiser was available from this day on): CH4 ~ 35,8%

10/08 - Biogas Monitorization:

2nd AD-IBC biogas reading: CH4 ~ 37%

11/08 - Biogas Monitorization + AD-IBC Cultivation + ALG-IBC Cultivation:

3rd AD-IBC biogas reading: CH4 ~ 37,8%

ALG-IBC pH 6 - 7 (sampled with a test strip). Added 200g NaHCO3 for correction. The gas outlet tube connected to the airstone inside the ALG-IBC was also removed, as the CO2 uptake could decrease the culture's pH.

14/08 - Biogas Monitorization:

4th AD-IBC biogas reading: CH4 ~ 30,9%

ALG-IBC pH > 9 (sampled with a test strip). Reconnected the AD-IBC biogas to the ALG-IBC to promote growth.

15/08 - Biogas Monitorization + ALG-IBC Cultivation:

5th AD-IBC biogas reading: CH4 ~ 28,9%

Added 40 L fresh manure diluted with 2 parts of water to the AD-IBC to boost the methane content since the previous readings were below 40%.

16/08 - AD-IBC Cultivation + Biogas Monitorization:

6th AD-IBC biogas reading: CH4 ~ 27,4%

ALG-IBC was filled up (with approximately 200L of water), leaving the minimum headspace available so the gas could accumulate. Added 200g NaHCO3

17/08 - Biogas Monitorization + Biogas Upgrading:

7th AD-IBC biogas reading: CH4 ~ 25,3%

To set up the upgrading phase of the pilot, two valves were put in the ALG-IBC, the gas inlet port (coming from the AD-IBC and inside the ALG-IBC) and the upgraded gas outlet (going out of the ALG-IBC).

ALG-IBC pH < 7 (sampled with a test strip). Nutrients were added to promote growth - 200g NaHCO3, 200g Valagro (20:20:20), 100g NaCl, 20g MgSO4.

18/08 - Biogas Monitorization + AD-IBC Cultivation + ALG-IBC Cultivation:

8th AD-IBC biogas reading: CH4 ~ 23,9%

1st upgraded biogas reading: CH4 ~ 0,0%. First thought was there was no gas accumulated in the algae and the timespan to get a good reading would have to be discovered. However after a few tries, the result was always the same.

Figure F. Pilot Monitorization Decision Making Report

Figure F (cont.). Pilot Monitorization Decision Making Report

19/08 - Biogas Monitorization:

9th AD-IBC biogas reading: CH4 ~ 20,0%

2nd upgraded biogas reading: CH4 ~ 0,1%. Connected an airstone to the end of a tube inside the ALG-IBC, to establish the functioning depth of the airstone, working as a scrubbing process.

20/08 - Biogas Monitorization + ALG-IBC Cultivation:

10th AD-IBC biogas reading: CH4 ~ 19,1%

Decided to feed the AD-IBC more regularly and in smaller quantities. Added 30L manure diluted with 1 part of water.

3rd upgraded biogas reading: CH4 ~ 0,2%. Changed the inlet valve of the ALG-IBC and cut the airstone tube 30 cm.

21/08 - AD-IBC Cultivation + Biogas Monitorization:

11th AD-IBC biogas reading: CH4 ~ 24,6%

4th upgraded biogas reading: CH4 ~ 0,2%. Cut another 30 cm of the airstone tube.

22/08 - Biogas Monitorization + Biogas Upgrading:

12th AD-IBC biogas reading: CH4 ~ 27,1%

Added 30L fresh manure diluted with 1 part of water.

5th upgraded biogas reading: CH4 ~ 0,0%. Set up a 20L vessel to reduce the scale of the upgrading platform, as the biogas flux needed to reach the available headspace would be lower than in the ALG-IBC. The biogas outlet was also connected to an airstone inside the 20-ALGvessel.

23/08 - Biogas Monitorization + AD-IBC Cultivation + ALG-IBC Cultivation:

13th AD-IBC biogas reading: CH4 ~ 52,8%

1st 20L-ALGvessel upgraded biogas reading: CH4 ~ 0,3%.

24/08 - Biogas Monitorization:

14th AD-IBC biogas reading: CH4 ~ 27,1%

2nd 20L-ALGvessel upgraded biogas reading: CH4 ~ 0,0%. Cut the airstone tube inside the vessel and applied teflon tape to the lid.

25/08 - Biogas Monitorization + ALG-IBC Cultivation:

15th AD-IBC biogas reading: CH4 ~ 33,6%

3rd 20L-ALGvessel upgraded biogas reading: CH4 ~ 0,0%.

26/08 - AD-IBC Cultivation + Biogas Monitorization:

16th AD-IBC biogas reading: CH4 ~ 25,5%. Tested the CH4 content of a stored 50L vessel that was being used to dilute the cattle manure.

1st 50L-ADvessel biogas reading: CH4 ~ 54,1%

4th 20L-ALGvessel upgraded biogas reading: CH4 ~ 0,2%.

Added 40L diluted fresh manure to the AD-IBC to boost the methane content, plus *Opuntia Figos Indica* (75/25 % (w/w)). Also added 20L of the same mixture together with 5L of microalgae culture to the 50L-ADvessel. Both feedstocks' alkalinity were adjusted to reduce acclimatization period.

27/08 - Biogas Monitorization Biogas Upgrading:

17th AD-IBC biogas reading: CH4 ~ 19,9%.

2nd 50L-ADvessel biogas reading: CH4 ~ 5,4%

5th 20L-ALGvessel upgraded biogas reading: CH4 ~ 0,2%

28/08 - Biogas Monitorization + AD-IBC Cultivation + ALG-IBC Cultivation:

18th AD-IBC biogas reading: CH4 ~ 36,8%

3rd 50L-ADvessel biogas reading: CH4 ~ 7,6%

6th 20L-ALGvessel upgraded biogas reading: CH4 ~ 0,0%

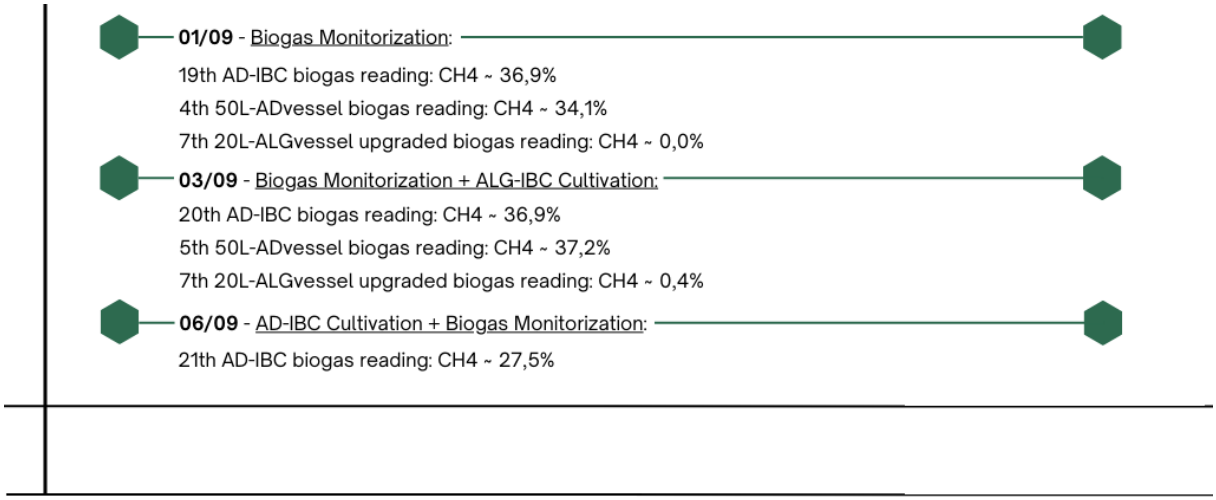


Figure F (cont.). Pilot Monitorization Decision Making Report

Annex G | Geotech BIOGAS 5000 Analyzer

Annex G1 | Equipment's Design

3.0 The BIOGAS 5000 gas analyser

3.1 The BIOGAS 5000

BIOGAS 5000 gas analyser

The BIOGAS 5000 gas analyser is designed for anaerobic digestion.

Benefits:

- Enables consistent collection of data for improved analysis and accurate reporting.
- No need for self-certification of anemometer.
- Easy to use and calibrate.
- User configurable operation.
- Helps check digester process is running efficiently.

Features:

- ATEX, IECEx certified.
- MCERTS (applied for).
- Robust design for market leading reliability.
- CH_4 and CO_2 accuracy ±0.5% after calibration.
- Measures % CH_4, CO_2 and O_2.
- H_2S to 0-500ppm or 10,000ppm.
- Modular and upgradeable.
- 3 year warranty.
- Stores and downloads readings.
- User selectable languages.
- Data logging.
- Up to 6 gases monitored.

Applications:

- Farm digester gas monitoring.
- Food processing biogas monitoring.
- Waste water biogas monitoring.
- Methane recovery.

Figure G1. BIOGAS 5000 Analyser

Annex G2 | Instructions for safe use

BIOGAS 5000 gas analyser OMBIO5KN4.1

2.2 Instructions for safe use

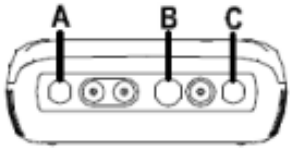
For ATEX and IECEx the 5000 series of gas analysers are certified to Hazardous Area Classification

II 2G Ex ib IIA T1 Gb (Ta = -10ºC to +50ºC)

It is vital instructions are followed closely. It is the responsibility of the operator to determine the protection concept and classification required for a particular application.

(Reference European ATEX Directive 94/9/EC, Annex II,1.0.6.)
The following instructions apply to equipment covered by certificate numbers SIRA 11ATEX2197X and IECEx Directive SIR 11.0089X:

- The equipment may be used with flammable gases and vapours with apparatus group IIA and temperature class T1.
- The equipment can contain gas sensing heads for the detection of particular gases. The inclusion of a sensor does not infer that the equipment is suitable for the use of gases with a temperature class of less than T1.
- The equipment is only certified for use in ambient temperatures in the range -10ºC to +50ºC and should not be used outside this range.
- The equipment must not be used in an atmosphere of greater than 21% oxygen.
- Repair of this equipment shall be carried out in accordance with the applicable code of practice.
- When used in a hazardous area only use GF5.2 temperature probe (SIRA 11ATEX2197X and IECEx SIR11.0089X). For connector C, the GF5.4 anemometer (BVS 04ATEXE194) for use with ATEX only. The analyser should not be connected to any other devices in the hazardous area including the GF-USB lead (connector A) or GF3.9 battery charger (connector B) supplied with the analyser.



Do not charge, recharge or open in a potentially explosive atmosphere.
In hazardous area only use "Temperature Probe GF5.2" in Connector B.
Connector C (Uo=10V,Io=5mA,Po=50mW,Ci=0,Li=0,Co=100uF,Lo=1000mH),
Connector B (Uo=5V,Io=6mA,Po=7mW,Ci=0,Li=0,Co=100uF,Lo=1000mH)

MAXIMUM NON-HAZARDOUS SUPPLIES:
Connector A - Um=6V Connector B - Um=10.1V

- The safe area apparatus that is to be connected to the USB Port shall be a Safety Extra Low Voltage (SELV) or Protective Extra Low Voltage (PELV) circuit.
- Only a Geotechnical Instrument battery pack part number 20087 or 2011113 is permitted as a replacement. This battery pack shall only be changed in a safe area by Geotech personnel or authorised distributors.
- Only Battery Charger type GF3.9 shall be used to recharge the batteries via Connector 'B'.
- If the equipment is likely to come into contact with aggressive substances, e.g. acidic liquids or gases that may attack metals, or solvents that may affect polymeric materials, then it is the responsibility of the user to take suitable precautions, e.g. regular checks as part of routine inspections or establishing from the material's data sheet that it is resistant to specific chemicals that prevent it from being adversely affected, thus ensuring that the type of protection

Figure G2. BIOGAS 5000 Analyser instructions for safe use

Annex H | Chlorophyll *a* calibration curve

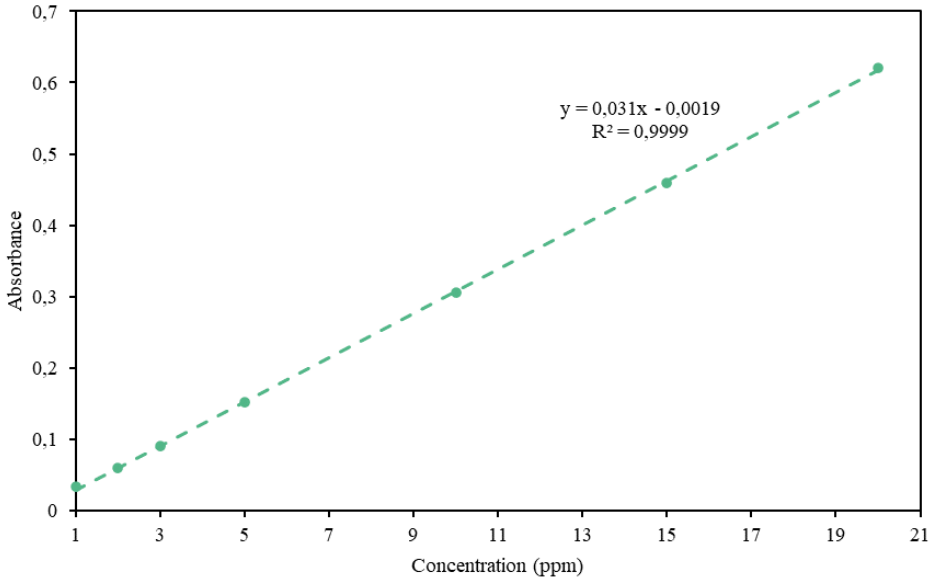


Figure H. Chlorophyll *a* calibration curve using a standard liquid solution at 1, 2, 3, 5, 10, 15 and 20 ppm

Annex I | Phosphorus calibration curve

Figure H. Phosphorus calibration curve