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Microalgae-based biodiesel: strategies to develop a sustainable biochemical process

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Elena Mazzocchi
Turin, April 30, 2026

Summary

After industrialization, human activities began to increase Green House Gasses (GHG) emissions with a clear effect on environmental and human health. The continuously increasing population and energy demand dramatically enhanced global warming. Right now, more than ever, we must face this urgent crisis by reducing emissions and replacing fossil-based energy with renewable alternatives. In this context, transport sector is responsible for majority of oil consumption and just 11.2% of energy used in this sector is renewable, far from the 29% target set by 2030. To achieve this goal new technologies are required.

Liquid biofuels mainly derive from crops, agricultural residues or cooking oils, but they are not enough to meet the growing demand for biofuels. Microalgae represent an alternative feedstock for biodiesel production. They are 20 times more productive than crops and do not require arable land utilization. However, nowadays microalgae-based biodiesel is not yet economically feasible. For these reasons, more efforts are needed to improve the production of this valuable biomass.

In this thesis three cultivation strategies to improve biomass and lipid production in microalgae have been employed. Overall, microalgae have been cultivated on industrial wastewater to reduce costs and increase sustainability. The utilization of streams from dairy industry resulted to be a valid solution for the cultivation of both *Chlorella sorokiniana* and *Tetradesmus obliquus*. When *C. sorokiniana* was cultivated in a balanced substrate obtained with 75% of dairy washing waters (DWW) and 25% of dairy exhausted sludge (DES) high biomass productivity ($0.052 \text{ gDW L}^{-1} \text{ d}^{-1}$) was achieved.

This substrate was used for the cultivation of *C. sorokiniana* in a multicultivator PBR under different light regimes to improve lipid productivity

and quality. A two-step cultivation process was designed, consisting of initial biomass accumulation followed by a shift in light wavelength to evaluate its effect on lipid fraction. Red light (660 nm) achieved the highest biomass productivity ($0.102 \text{ gDW L}^{-1} \text{ d}^{-1}$) and lipid content (40.75% of dried biomass). In contrast, blue light drove to an increase in biodiesel quality in terms of oxidative stability and ignition quality but showed poor low-temperature performances (high CFPP). Notably, when red light was implemented in the first step followed by blue light (R100-B300), CFPP was halved.

Nitrogen limitation is known to boost lipid productivity in certain microalgae, thus a two-step process involving different species in each stage was conducted. Mixed dairy effluent (MDE) from an Irish industry was used as substrate. The highest lipid content both in first and second steps were obtained when *T. obliquus* was cultivated in the first stage followed by *Nannochloropsis oceanica* (MDE-TET-NO) with 27.14% and 43.89% of lipid, respectively. Low oxidative stability was observed in the first step and low ignition performance in the second, thus by blending two biomasses higher quality of biodiesel is achieved. Coupling species with distinct lipid profiles is a promising strategy to improve the quality of final product.

Finally, *C. sorokiniana* was cultivated on a carbon-rich substrate derived from a valuable gas fermentation process. Here high biomass productivity ($0.295 \text{ gDW L}^{-1} \text{ d}^{-1}$) was obtained, together with high-quality biodiesel. High biomass productivity confirmed the feasible contribution of microalgae in valorising GFE. The cultivation of valuable biomass on GFE strongly improved the sustainability of the gas fermentation process, toward a circular process with zero-waste and high-value products.

Overall, biodiesel production was coupled with wastewater treatment. Nitrogen removal reached 50%, 99% and 100%, while phosphorus removal is 35%, 87% and 100% respectively for R100-B300, MDE-TET-NO and 50:50 DES:GFE cultivations. These results highlight the sustainability of these strategies and the adaptability of microalgae to polluted environments.

This thesis addresses the energy transition toward sustainable alternatives to fossil-fuels, contributing to advancing the knowledge necessary to make this biotechnological process economically viable.

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*I would like to dedicate
this thesis to my friends
and family*

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List of Abbreviations

3N BBM: Three-fold basal bold medium
ACP: Acyl carrier protein
ADP: Adenosine diphosphate
ATP: adenosine triphosphate
BG11: Blue-green 11
BOD: Biological oxygen demand
CFPP: Cold filter plugging point
Chl: Chlorophyll
CN: Cetane number
COD: Chemical oxygen demand
COP21: Conference of Parties
DES: Dairy Exhausted Sludge
DWW: Dairy Washing Water
FAS: fatty acids synthase
GFE: Gas fermentation effluent
GHG: Greenhouse gases
IV: Iodine Value
MDE: Mixed Dairy Effluent
MUFA: monounsaturated fatty acid
NADPH: Nicotinamide adenine dinucleotide phosphate
N/P: ratio nitrogen/phosphorus
OP: Open Pond
PAR: photosynthetic active radiation
PBR: Photobioreactor
PS I: Photosystem I
PS II: Photosystem II
PUFA: Polyunsaturated fatty acid
ROS: Reactive oxygen species
Rubisco: Ribulose-1,5-bisphosphate carboxylase/oxygenase
SC: Scotta
SDG: Sustainable Development Goal
SFA: Saturated fatty acid
TAG: Triglyceride
TOC: Total organic carbon
VFA: Volatile fatty acids
WWT: Wastewater treatment

Chapter 1

Research overview

1.1 Background of the Thesis

The current climate crisis has gained the interest of scientific community and governments, due to its harmful effects on human and environmental health.

Growing awareness of climate change and environmental issues led to increasing international efforts to mitigate greenhouse gases (GHG) emissions and limit global warming. Consequently, the Paris Agreement was adopted during the Conference of Parties (COP21) in 2015. The agreement aims to limit the increase in average global temperature to 1.5°C above pre-industrial levels (Schleussner et al., 2016). Moreover, it promotes the achievement of net-zero GHG emissions, meaning that the amount of GHG produced by human activities is balanced by the amount removed from the atmosphere. Furthermore, the United Nations Member States provided a detailed list of Sustainable Development Goals (SDGs) which rely on the necessity of end poverty, reducing inequality and improving health and education to reach the goals of Paris Agreement and to preserve natural environment while reducing climate change (United Nations, 2023).

In response to these global commitments, in 2019, the European Green Deal was introduced as an economic and political strategy to guide European Union in achieving goals established in the Paris Agreement. Its main objective is to reach climate neutrality by 2050, with an intermediate target of reducing GHG emissions by 55% by 2030 compared to 1990 (Kulovesi et al., 2024). To meet these goals a major transition across several sectors is needed, including industry, transport, agriculture and infrastructures, thereby modeling the European economic system toward a low-carbon strategy.

Despite these actions, ten years after the Paris Agreement, it has become evident that current efforts remain insufficient to meet the 1.5°C goal. Indeed, the *State of Climate Action 2025* report shows that none of the 45 indicators assessed

are in line to meet the 2030 targets. In particular, public financial support for fossil fuel continues to increase, as well as the transportation based on internal combustion engines (Schumer et al., 2025). These trends highlight the urgency of reducing emissions and accelerating the implementation of new low-carbon technologies.

Currently, biofuels used in the transport sector mainly include bioethanol and biodiesel from crops or oils. However, their contribution must rapidly increase to meet 2030 and 2050 targets. In this context, new strategies such as microalgae-based biodiesel are a promising solution to increase the role of biofuels and reduce reliance on fossil fuels.

Microalgae boast higher growth rate than conventional crops and do not require arable land, thus avoiding competition with food sector (Stansell et al., 2012). Despite these advantages, this biotechnological process still faces several limitations that affect both economic viability and sustainability. High energy consumption during cultivation and downstream processing remains the main bottleneck.

Nevertheless, with appropriate optimization and technological innovation, microalgae cultivation could become a viable alternative. Indeed, the high adaptability of microalgae to adapt to a wide range of environmental stressors, such as variable nutrient loads and pollutants in industrial wastewater, is a critical factor for advancing this technology. The utilization of polluted streams as nutrient source represents a key step toward reducing costs and improving sustainability. Moreover, when combined with controlled cultivation conditions aimed at enhancing biomass and lipid productivity, this approach can contribute to lowering overall energy requirements.

1.2 Aim of the Thesis

Microalgal biotechnologies have gained much attention due to the wide applicability of this biomass. Indeed, several sectors could take advantage from this process, from pharmaceutical, nutraceutical, animal feed, agricultural sectors for biostimulant production, wastewater treatment and biofuel production. For this reason, more efforts in improving these biotechnological processes and increasing knowledge on microalgal behavior are needed.

This thesis focuses on the improvement of microalgal cultivation for biodiesel production, thus aiming to boost both biomass and lipid productivity, with specific attention on the final composition of this valuable fraction. Indeed, the production of biodiesel from microalgal biomass has been widely investigated, but several challenges still need to be accounted.

In this framework, the present work wants to associate microalgal wastewater treatment, with strategies for the increase of lipid accumulation.

A primary screening of *Chlorella sorokiniana* and *Tetrademus obliquus* cultivated on different wastewater derived from a local dairy industry was performed to evaluate the biomass productivity on polluted environments and the capability of microalgae to grow on different kinds of wastewater. Subsequently, the utilization of proper wastewater substrates was coupled with different strategies to investigate lipid productivity and biodiesel quality.

The selected strategies include: (i) variation in light regime during the cultivation, (ii) application of two-step process with the second step of nutrient limitation, and (iii) the utilization of a novel stream rich in organic carbon.

The first strategy involved two stages where the first was conducted under white or red light to stimulate biomass productivity, while in the second red, blue or white light were applied. Differences in light wavelength is known to stimulate changes in cellular metabolism, thus the composition of biomass under tested regimes was evaluated.

The second strategy involved two steps with two different species, where in the first stage microalgae can grow fast and consume nutrients of the wastewater, while in the second stage *Nannochloropsis oceanica* is cultivated under nitrogen limitation to boost lipid accumulation. After cultivation, biomass harvested in both steps can be blended for the extraction of lipids, to improve the quality of biodiesel.

Finally, the effluent of the gas fermentation of *Clostridium carboxidivorans* was used for microalgae cultivation due to its content in organic acids that lead to mixotrophic cultivation which can improve lipid accumulation and quality.

The application of these strategies, coupled with wastewater treatments, wants to deeper understand the behavior of microalgae under different conditions. This screening will place new basis for biodiesel production with microalgal feedstock,

which is a future biotechnological process needed to meet the goals established with the Paris Agreement.

1.3 Structure of the Thesis

The present Thesis is structured as follows.

Chapter 1 provides the background of the Thesis and outlines the main objectives.

Chapter 2 presents the **Introduction**. It begins with an overview of energy transition toward renewable sources, highlighting the importance of developing new technologies for biofuel production. This is followed by a detailed discussion on the application of microalgae as feedstock for biodiesel production. Subsequently, strategies to decrease process costs, such as wastewater utilization as nutrient source, have been examined. Finally, approaches to enhance lipid accumulation have been explored, with particular attention to light induction and nitrogen limitation.

Chapter 3 describes the **Materials & methods** used in this work. Experimental design and procedures and analytical techniques are reported here.

Chapter 4 presents the **Results & discussion**, organized into following sections:

- **Chapter 4.1** Wastewater as substrate for microalgae cultivation.
This chapter presents an initial screening of *Chlorella sorokiniana* and *Tetrademus obliquus* grown on three dairy wastewaters. In parallel, the cultivation of *C. sorokiniana* on standard medium under phototrophic, mixotrophic and heterotrophic conditions was carried out as a reference condition. The optimal wastewater blend for the cultivation of the selected strains was defined using exclusively wastewater-based media. Biomass production and lipid composition were subsequently assessed.
- **Chapter 4.2** Harnessing light-induced lipid biosynthesis in microalgae grown on industrial wastewater.
Different light regimes were applied to *C. sorokiniana* cultivation on blended dairy wastewater. Biomass composition was analyzed both during and at the end of the cultivation to better understand metabolic changes induced by varying light wavelength.

- **Chapter 4.3** Two-step cultivation on Mixed Dairy Effluent, an overview of the context.

In the first step two strains (*C. sorokiniana* and *T. obliquus*) were cultivated, both as monoculture and in co-culture. Following biomass harvesting, the resulting effluent was reused to induce nutrient limitation in *Nannochloropsis oceanica*. This second step was designed to enhance lipid productivity. Furthermore, the two-step approach involving different microalgal species enables the improvement of biodiesel quality through the combination of biomasses with complementary lipid profiles.

- **Chapter 4.4** Valorization of a novel stream.

The effluent from an innovative gas fermentation process, rich in organic acids, was used as substrate for microalgal cultivation. This approach enables both wastewater treatment and the production of valuable microalgal biomass, contributing to the valorization of this process stream.

- **Chapter 4.5** Comparison between biodiesel obtained with different approaches.

Biodiesel quality resulting from the previously described strategies was comprehensively evaluated and compared to assess the applicability and potential for industrial scale implementation.

Chapter 5 presents the **Conclusions**, summarizing the main findings and achievements of this work, along with a future perspective and the importance of this study.

Chapter 2

Introduction

2.1 Energy transition: from fossil fuels to renewable energy

Global energy demand has been continuously increasing, largely driven by population growth and economic development (Jones & Warner, 2016). The Organisation of Petroleum Exporting Countries (OPEC) has predicted that fossil fuel demand could increase by up to 12% between 2021 and 2030, highlighting the continued central role of fossil fuels in global energy system (*Fossil Fuel Demand to Continue Expanding This Decade - Economist Intelligence Unit*, 2023).

In 2023 global population surpassed 8 billion people, and it is expected to continuously grow in coming decades (Jones & Warner, 2016). The rapid demographic expansion, combined with rising living standard and industrial development, is responsible for the increasing energy demand. Meeting this demand while ensuring environmental sustainability represents a challenge. In particular, the increased consumption of energy, still mainly based on fossils, leads to higher green-house gases (GHG) emissions, contributing to global warming and climate change. These effects are especially pronounced in urbanized areas, where pollution levels are associated with risks to human health.

In addition, the transition to non-fossil energy is also driven by geopolitical factors. Oil, gas and coal are present in limited number of countries, creating energy dependencies between states (Egli et al., 2025). These dependencies have historically contributed to geopolitical tensions and conflicts. In contrast, renewable energy sources are more widely distributed geographically, making them accessible to a larger number of countries. Their diversity allows them to be energy independent and potentially reduce geopolitical tensions. Furthermore,

increasing geopolitical risks may indirectly accelerate the reduction in fossil fuel consumption toward a greater adoption of renewable energy sources (Tabash et al., 2024).

In this context, the dependence on fossil fuels contributes not only to environmental concerns, but also to increasing energy costs, intensified by tensions among political actors. This represents an additional reason to accelerate the energy transition.

2.1.1 Energy consumption and demand in Europe and worldwide

The final energy consumption by fuel in Europe amounted 36566 PJ in 2023, corresponding to a 11.8% decrease compared with the peak recorded in 2006 (European Commission, 2025). The largest use of final energy is associated with oil and petroleum products, which account for 37.4% of the European total consumption in 2023, slightly lower than the global share of 40.1%. Electricity follows with 22.9% use in EU (Figure 1) and 21.2% at the global level. On the other hand, renewable energy and biofuels have gained relevance over decades, showing an increase from 4.3% to 12.6% from 1990 to 2023 in Europe, with a contribution to global consumption of 9.4% (European Commission, 2025; IEA, 2025b).

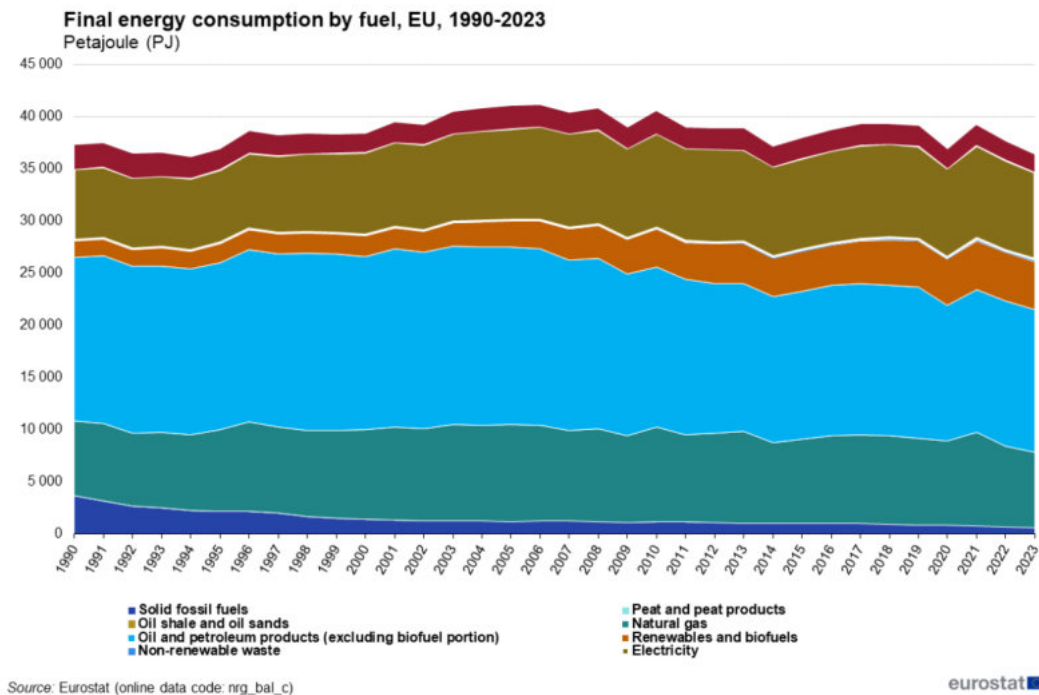


Figure 1: Final energy consumption by fuel in Europe from 1990 to 2023. Data reported from Eurostat analysis (online data code: nrg_bal_c).

The subdivisions of energy consumption into different sectors revealed that 30.1% is delivered to industries, 28.6% to transport and 19.6% to residential use worldwide (IEA, 2025b). In Europe a slight inversion of predominance is observed, with the highest consuming sector being transport (32%) followed by households (26.3%) and industry (24.6%) as shown in Figure 2 (European Commission, 2025).

Final energy consumption by sector, EU, 2023
(% of total, based on terajoules)

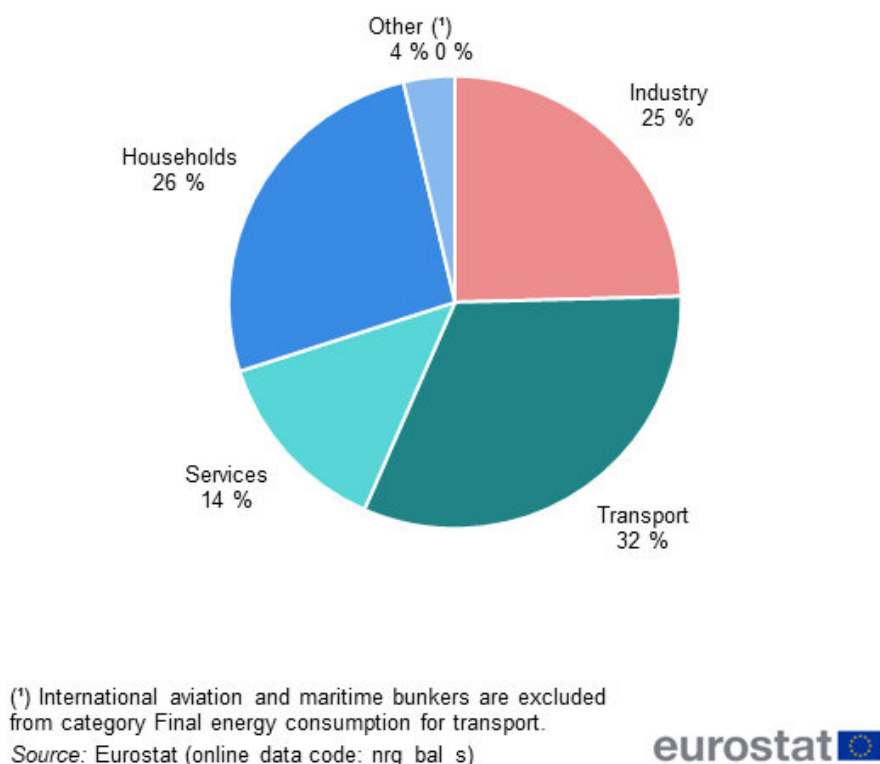


Figure 2: Final energy consumption in Europe in 2023 divided by sectors.

The transport sector accounts for 64.3% of total oil consumption, and oil represents 90.1% of the fuels used in this sector worldwide. Moreover, renewable energy covered 13.05% of total energy consumption in 2022 with transportation sector accounting for 11.7% of that total (IEA, 2025b). From this scenario it emerges that transport sector relies on oil-based fuels, having a significant impact on total oil consumption in the world. This reliance on fossil fuels has environmental consequences and highlights the urgent need of developing new technologies to decarbonize this sector.

Industrial efforts are increasingly focused on development of electric vehicles to reduce dependence on oil. However, the benefits of electrification depend also

on the energy mix, indeed electricity is not always sourced from renewables. Therefore, for decarbonization it is needed to simultaneously develop efficient vehicles and improve renewable electricity generation. Moreover, not all transport vehicles can be electrified in short-term. Aviation, for example, is the major responsible of oil consumption in transport sector in Europe (European Commission, 2025) and nowadays there are no alternatives to conventional fuels for it. For this reason and for the graduality of the energy transition, liquid fuels will continue to play an important role, placing renewable alternatives, such as biofuels, as crucial factors to reduce the carbon footprint.

In brief, to achieve the decarbonization of transportation sector a combination of different approaches is needed.

2.1.2 An overview of renewable energy worldwide

Renewable energy plays a key role in decarbonization, toward Net Zero Emission scenario. Renewable energy sources include solar Photovoltaic (solar PV), hydropower, geothermal, wind and bioenergy. Bioenergy comprises energy derived from biomasses, including bioelectricity and biofuels for transport and has shown continuous growth in recent years (Reid et al., 2020).

In 2022 several countries adopted new policies to support the energy transition, such as REPowerEU in Europe, China's 14th Five-Year Plan for Renewable Energy and the Inflation Reduction Act in United States (Zhukov & Reznikova, 2023). These policies contributed to significant increase in renewable energy production in 2023, with approximately 560 GW of additional annual capacity. This increase was driven mainly by solar and wind power, which contributed respectively with 7.7 EJ and 8.4 EJ in 2023, while hydropower remained the largest source of renewable electricity generation, with 15.3 EJ (Figure 3). (IEA, 2025a). Among these technologies, solar PV is currently the only renewable energy on track to meet the Net Zero Emissions by 2050, while wind, hydro, geothermal and bioenergy require further efforts to increase their share in global energy mix.

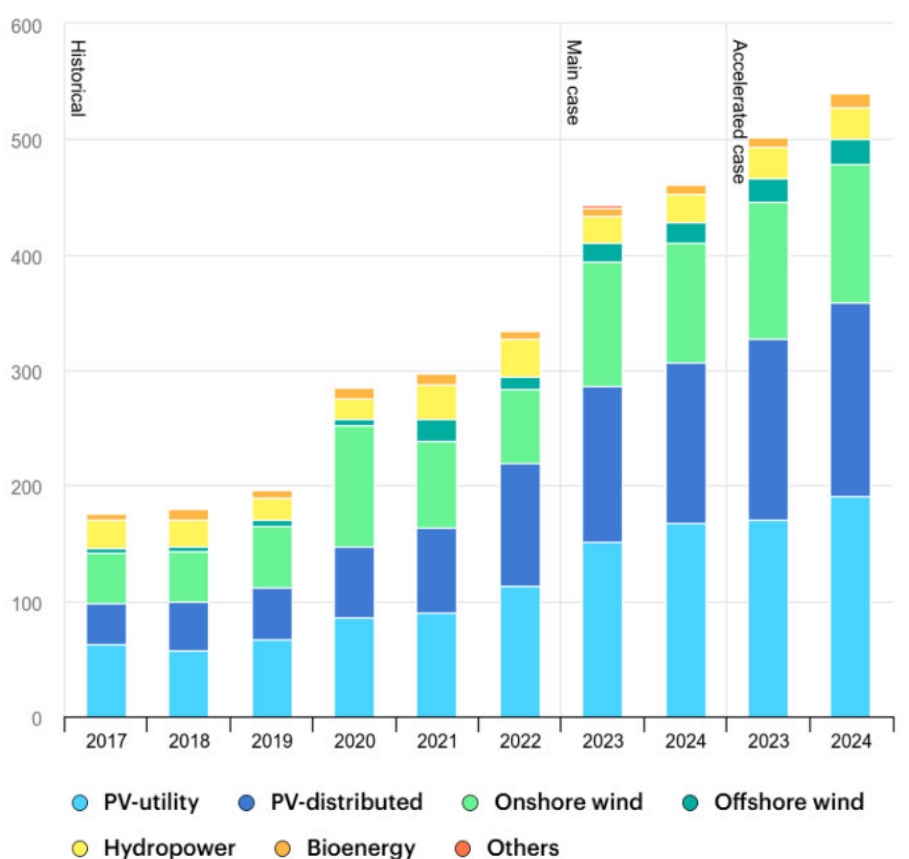


Figure 3: Net renewable electricity capacity by technology, 2017-2024. Values reported are electricity capacity measured in Gigawatt (GW). These data refers to electricity only, excluding heat and transport energy capacity. Source: IEA (2023)

2.1.3 Bioenergy: a focus on biodiesel

Among renewable energy, modern bioenergy derives from biomasses originating from organic matter, such as higher plants. Currently bioenergy represents the largest share, accounting for 55% of total renewable energy consumption, as it is widely used for heat and transport, and 6% of final energy supply, as reported by IEA in 2024 (IEA, 2024).

The major issue with these energy sources is their dependence on arable land, leading to competition with the food sector. The utilization of land not only competes with food production, but also threatens existing natural ecosystems, further driving conversion of land for human activities, at a time when it is crucial to preserve and, where possible, restore natural ecosystems. For these reasons, sustainable feedstocks, such as municipal streams or agricultural and forestry residues, can be exploited to avoid the use of dedicated land. In 2050, in line with Net Zero Emission scenario, 60% of the bioenergy capacity should come from these sources, to reduce utilization of land (IEA, 2024). Moreover, bioenergy is

considered a low-carbon and low-cost energy source, essential to mitigate the increase of global temperatures.

Bioenergy is mainly used for heat and electricity for industries, with minor contributions such as liquid biofuels for transport and biogases. To reach the goals of zero emissions in 2030 an annual rate of 9.3% increase in bioenergy production is required (IEA, 2024). Concerning liquid biofuels, which is the main focus of present thesis, they need to increase almost three times in 6 years.

The growth in biofuel production is driven by Brazil, where the Fuel of the Future law defines blending levels for biomethane, ethanol and biodiesel. It is estimate that half of the global increase in biofuels required by 2030 will be covered by Brazil production (Figure 4) (Ritchie et al., 2020). In detail, in 2023 Indonesia was the largest biodiesel producer with 14 billion liters from palm oil, higher than Europe which produced 13 billion liters from oil cooking and rapeseed, and Brazil with 8 billion liters from soybean (World Bioenergy Association, 2025). The utilization of liquid fuels for urban transportation is expected to be replaced by electric vehicles, however liquid fuels will remain necessary for aviation and maritime transport, which require systems with higher energy density fuels. Therefore, improving existing technologies for biofuels production and developing new ones, is essential to enable a greater supply of zero- or negative-carbon fuels, particularly for sectors where electric engines are not a viable option.

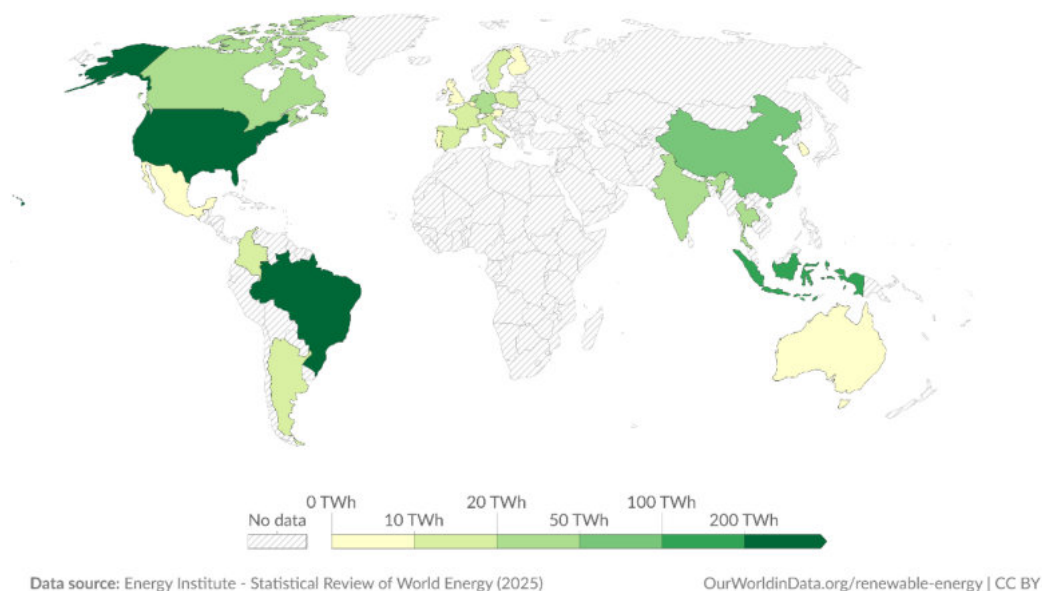


Figure 4: Biofuel production, including biodiesel and bioethanol, in 2024. Production is measured as terawatt hours⁻¹ per year. Source: Energy Institute - Statistical Review of World Energy (2025) (Ritchie et al., 2020)

2.1.4 Environmental impact: fossil fuels versus biofuels

Combustion of fossil fuels causes the production of not only carbon dioxide, which is the main GHG associated with global warming, but also nitrogen oxides, sulfur dioxide and particulate matter, which affects both human health and natural ecosystems. The total emissions of CO₂ from fossil fuels in Europe increased between 1960 and 2018, with a doubled share of oil combustion from 22.5% to 41.8% (Paraschiv & Paraschiv, 2020).

Furthermore, the fossil fuel industry is responsible for degradation of environments due to the extraction involving drilling and mining activities. To these collateral effects on environment must be mentioned the dispersion of oil while drilling it, like leaks of bigger spills occurring during transportation.

A promising approach to reducing emissions in transport sector is the use of biofuels, which can reduce environmental impact of both the combustion process and from biomass production. Biofuels comprise biogas, biodiesel, biomethane, methanol or butanol derived from different feedstocks such as crops and microorganisms or agricultural wastes (Awogbemi et al., 2021). When the life cycle of biodiesel is considered, a significant reduction in CO₂ emissions is observed. This is mainly due to the carbon uptake that occurs during biomass production through photosynthesis (from plants or photosynthetic microorganisms). As a result, the overall carbon footprint is significantly lower than that of fossil fuels (Ogunkunle & Ahmed, 2021). For example, it was demonstrated that soybean biodiesel drives to a reduction of 78% of CO₂ emissions (Milazzo et al., 2013).

In addition to carbon dioxide mitigation, biodiesel also contributes to the reduction of other atmospheric pollutants such as carbon monoxide, nitrogen oxides, sulfur oxides, hydrocarbons and particulate matter (Basha et al., 2009). This effect is mainly attributed to the higher combustion efficiency of biodiesel, which is promoted by the presence of oxygen. In contrast, the production of these pollutants from fossil fuels is associated with an incomplete combustion process (Wu et al., 2004). However, it was demonstrated that the production of NO_x by-products resultant from biodiesel combustion is higher compared to fossil fuels (Ogunkunle & Ahmed, 2021).

2.1.5 Biofuels from different feedstock

Different kinds of feedstock can be used for the production of biofuels. They can be classified into four generations depending on the biomass used (Gaurav et al., 2017).

First generation biofuels are derived from crops such as wheat, corn or sugarcane, which are used to produce biodiesel from oils or ethanol through fermentation. For this type of biofuel, the conversion process is technologically

feasible, simple and low-cost. Besides that, the utilization of edible crops and arable lands represents a major drawback due to competition with human food industry and the deforestation caused by expansion of agricultural lands (Kang et al., 2014).

For these reasons, second generation biofuels derive from agricultural residues or forestry wastes. In this way, waste materials are valorized, and there is no competition with agriculture or food chain. Exhausted animal fat, vegetable oils or lignocellulosic residues are used to produce biodiesel which is usually found in the market and blended biodiesel (for example 10% or 20% of biodiesel in the mixture to have B10 and B20 blends) (Naik et al., 2010).

Outside of agricultural sector, there are third-generation biofuels derived from microalgal biomasses. Microalgae and cyanobacteria use atmospheric carbon dioxide to produce biomass and metabolites (Chowdhury & Loganathan, 2019). They are generally used to produce biodiesel from lipids, as well as bioethanol, biohydrogen or syngas (Esipovich et al., 2025; Lakatos et al., 2019). The high photosynthetic rates and rapid biomass accumulation of microalgae are an important advantage. Moreover, non-arable lands and non-fresh water can be exploited. Generally, there is no competition with food and agriculture and there is a reduction in economic and environmental costs when cultivated on industrial wastewater. The issue faced by this kind of biodiesel is mainly related to downstream processes. Indeed, the harvest, drying and extraction of microalgal biomass need to be optimized to reduce energy consumption of the process (Tan et al., 2018).

A way to improve the biomass composition, growth rate and nutrient uptake of microalgae is the utilization of engineered microorganisms which show better performance. In this case the resulting biofuel is classified as fourth generation (Lü et al., 2011). The advantage of using engineered microorganisms is the increased tolerance to polluted or unfavorable environments, the higher reduction of carbon footprint and the enhanced final yield in biofuel. The main issue with fourth generation biofuels is the legislative and political barrier (Abdullah et al., 2019). In fact, the utilization of engineered organisms requires specific safety measures to be implemented, increasing also the costs of the process. A possible way to surpass this block is the random mutagenesis or adaptive evolution, which are not regulated as genetic modifications. Nevertheless, this biotechnological process is still far from industrial application due to existing legislative barriers (Onn et al., 2025).

From second to fourth generation biodiesel, a circular-economy perspective can be implemented, where wastes are valorized as energy sources. Indeed, in second generation biofuels agricultural wastes are used as feedstock, while in third and fourth generation biomass can be cultivated on industrial streams, reducing the utilization of fresh-water and valorizing wastewater.

2.1.6 Advantages of third generation biodiesel

Microalgae-based biodiesel is a renewable bioenergy source of considerable interest due to its low emissions and high-quality fuel properties. This technology offers several advantages, including the use of non-arable land, increased growth rate compared to higher plants, high lipid productivity and high-quality FAME profiles (Sarwer et al., 2022).

Compared to conventional crops, microalgae show exceptionally high biomass productivity per unit area and they are not constrained by seasonal cycles, enabling continuous and stable production over the year. It has been estimated that the annual areal energy yield for microalgae grown at $40 \text{ g}_{\text{DW}} \text{ m}^{-2} \text{ d}^{-1}$ (as hypothetical average growth rate) is 16-fold higher than that of corn, which is cultivated for only 3 months in a year (Subramanian & Sayre, 2022). This highlights the higher efficiency of microalgal system compared to traditional bioenergy crops.

In addition, the diversity of microalgal strains provides great flexibility for process optimization. Indeed, the selection of an appropriate strain or consortium represents a crucial step to maximize biomass production, lipid yield and fuel quality (Nashath et al., 2025). This selection is inherently linked to cultivation conditions, allowing us to tailor the process to specific operational and environmental conditions.

Another major advantage of microalgae relies on their adaptability. They are ubiquitous and can thrive in a wide range of environments, including extreme and polluted conditions. Their capacity of growing on industrial and municipal wastewaters represents a key opportunity to reduce production costs (Hamidian & Zamani, 2022b; Sarwer et al., 2022). The utilization of wastewater as a nutrient source leads to reduced freshwater consumption and remediation of streams, with consequent release of cleaner water that can be used for irrigation (Barreiro-Vescovo et al., 2021).

Despite the advantages of developing microalgal-based biodiesel as an alternative to fossil fuels, further effort is required to surpass economic and technological challenges of process scale-up. Continued research and development, along with supportive policies frameworks, are still necessary to ensure the economic viability of the process. From a technological perspective several strategies can be implemented in both the upstream and downstream processes. In this scenario, the intrinsic adaptability of microalgae, together with the wide range of available strains and cultivation systems, offers a unique opportunity to design processes tailored to diverse environmental and industrial conditions.

2.2 State of art microalgal biodiesel production

Physical and chemical properties of FAME biodiesel are comparable to those of conventional fossil diesel, making it a viable substitute for being used in diesel engines. Depending on its specific composition, biodiesel can be blended either with biodiesel derived from different feedstocks or with fossil fuel to meet fuel quality standards (Sandaka & Kumar, 2023).

Nowadays, biodiesel is produced through transesterification of vegetable oils, animal fat or waste oils yielding FAME which can be easily used in existing diesel engines. Depending on the regional availability of crops different feedstocks can be used, the most commercialized are produce in Indonesia from palm oil, and in Brazil from sugar cane (Nashath et al., 2025).

The production of first- and second-generation biofuels still relies on plant-based feedstocks, raising concerns related to competition with food industry and land use. In this context, alternative and more sustainable feedstocks are needed to ensure long-term scalability of biofuel production. Among these, microalgae emerge as promising candidates.

Several projects are currently active worldwide to investigate and demonstrate the industrial applicability of microalgae-based biodiesel production. In 2018, a Japanese biotechnology company, Euglena Co., launched a pilot-scale project for the production of biodiesel from *Euglena* biomass. Open ponds were used for biomass cultivation, while isoconversion was employed for biofuel production. This process consists of hydrothermal conversion and hydroprocessing of biomass for the production of high-quality biofuels (F&L, 2024). Notably, the biodiesel produced within this project was initially tested on a tugboat in the port of Nagoya and after this successful trial, in 2021 it was used to fuel an inspection aircraft for 2.5-hour flight (Euglena Co., 2021; PortNews, 2020). In 2024, after the success of pilot-scale project, Euglena Co. initiated the construction of a commercial plant in Malaysia for the production of Sustainable Aviation Fuels (Euglena Co., 2024). This example highlights the technical feasibility of producing high-quality biodiesel from microalgal biomasses and represents a benchmark for future industrial developments.

More recently, in 2022, Turkey established a 2500 m² facility equipped with both outdoor and indoor photobioreactors for microalgal cultivation aimed at bio-jet fuels production. To reduce the environmental impact and the operational costs, the facility is powered by wind energy, contributing to a potentially carbon negative process (World Biomarket Insight, 2022). This is part of the Turkey's Integrated Biorefinery Concept for Bioeconomy Driven Development (INDEPENDENT) project, based in Boğaziçi University in Istanbul.

A more recent project, launched in 2023 in Sarawak, Malaysia, involves the installation of 5 ha of flat-panel photobioreactors, named Chitose Carbon Capture Central (C4) (CHITOSE, 2023). In this case, a zero-waste biorefinery approach

has been adopted to improve economic viability. In addition to biofuels, the facility is designed to produce value-added products such as plastics, cosmetics and food, to diversify the products, while decreasing overall production costs. Furthermore, microalgae are cultivated using CO₂ derived from flue gases of a coal power plant, contributing to GHG mitigation. This integrated strategy is expected to reduce biomass production costs by up to five-fold (CHITOSE, 2023).

These ongoing projects provide a clearer perspective on the feasibility of microalgal biofuel production. In particular, they highlight key strategies to enhance sustainability and economic viability, including the integration of renewable energy sources (e.g. solar or wind power), utilization of industrial CO₂ emissions as carbon source and an integrated strategy that couples the biofuel production with valuable co-products.

Despite these advantages, challenges related to cost reduction and process scalability still hinder the large-scale deployment of microalgae-based biofuels production. Further research efforts are therefore required to improve the overall efficiency and economic viability of this process. Nevertheless, the applicability of microalgal systems has been widely demonstrated. Both the microalgal adaptability and the high quality lipid content are sufficient to support their potential as sustainable feedstock for biofuel production.

Consequently, increasing interests and investments have been directed toward innovation projects. In this context, Alagesol, a project launched in 2024 and funded by European Union's Horizon Europe Research and Innovation Action (RIA) program aims to produce sustainable fuels for shipping and aviation from microalgal biomass (ALGAESOL, 2024).

Another project funded under the Horizon Europe is SUN-PERFORM, which exploits nanotechnologies to enhance biomass accumulation. They propose the application of photoconversion films containing nanoparticles able to convert UV and blue light into red wavelength, which are more efficiently absorbed by microalgae, thereby improving photosynthetic performance (SUN-PERFORM, 2024).

In conclusion, considering the current global context, continued research is essential to improve each step of the overall process, from biomass cultivation to downstream processing. The present thesis aims to deepen the knowledge of microalgal bioprocess by implementing different approaches and identifying the limiting steps.

2.3 Overview on microalgal biology

Microalgae are photosynthetic microorganisms, both procaryotic and eukaryotic, accounting for 72500 catalogued species (Guiry, 2012). These microorganisms show an essential ecological role, being responsible for half of the carbon dioxide sequestration and generation of molecular oxygen and organic matter (Singh & Ahluwalia, 2013). Due to their high growth rate and their abundance in aquatic environments microalgae play a pivotal role in global carbon fixation, a function that is significant in this historical context of climate changes. Moreover, prokaryotic cyanobacteria are also able to perform nitrogen fixation (Borowitzka, 2018).

Microalgae display high morphological diversity, with sizes generally ranging between 0.5 and 200 μm . Depending on the species and growth phase they can undergo significant changes in both their morphology and sizes (Gualtieri & Barsanti, 2022). This plasticity enhances their ability to adapt to variations in environmental conditions.

To understand the biology and metabolisms of this microorganism a brief overview of cell organization is performed. Eukaryotic microalgae can be distinguished from procaryotic ones for the presence of cellular membrane-bound compartments which have different roles in microalgal metabolisms. These compartments, called organelles, include nucleus, chloroplasts, mitochondria, endoplasmic reticulum, Golgi apparatus and lysosomes (Borowitzka, 2018). Chloroplasts have an essential role in microalgae because here photosynthesis occurs. Indeed, they include thylakoid membranes where light-harvesting pigments are located. Another important organelle is mitochondria, where adenosine triphosphate (ATP) is produced as the energy source of biological systems (Gualtieri & Barsanti, 2022).

2.3.1 Growth conditions and metabolism of microalgae

Cultivation of microalgae for the production of high-valued compounds gained much attention due to their high growth rate and the potential applications of the biomass. Moreover, microalgal versatility enables them to grow under a variety of environmental conditions exploiting different energy sources. They primarily produce energy by converting sunlight and inorganic carbon into oxygen and organic matter. However, they can also activate respiratory metabolism, which consumes organic carbon and oxygen to produce energy (Kliphuis et al., 2011). This metabolic flexibility is fundamental, as it enables microalgae to regulate how energy is used between growth, cell division and accumulation of storage compounds.

The trophic mode in microalgae depends on the availability of light and carbon sources, whether organic or inorganic. Under phototrophic conditions,

energy is derived from photosynthesis. In the absence of light and presence of organic carbon, microalgae can shift to heterotrophy. When both organic carbon and light are present, they can grow mixotrophically, combining photosynthesis with respiration (Patel et al., 2019).

2.3.1.1 Phototrophic condition

Cyanobacteria are considered among the earliest organisms to have developed oxygenic photosynthesis, a process that had a profound impact on the evolution of life on Earth.

Photoautotrophic condition is the most used cultivation mode for microalgae. Photosynthesis occurs within chloroplasts and is divided into light-dependent reactions and carbon fixation reactions. During the first phase, light is absorbed by pigments organized in specialized structures called Photosystem I and II (PSI and PSII). In PSII water photolysis leads to the release of electrons, protons and oxygen. The electrons are transferred through an electron transport chain to PSI, where they are used for the reduction of NADP^+ (Nicotinamide Adenine Dinucleotide Phosphate) into NADPH. In the meanwhile, the generated proton gradient drives ATP synthesis (Blankenship, 2010).

In light-independent reactions, ATP and NADPH are used in the Calvin-Benson cycle to fix carbon dioxide into organic molecules through a series of reactions catalyzed by ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), ultimately leading to the synthesis of carbohydrates (Blankenship, 2010).

The products of photosynthesis are the primary source of energy and carbon of microalgal cells. They are then used for growth and cell division or for accumulation of storage compounds, depending on the cultivation condition.

2.3.1.2 Heterotrophic and mixotrophic conditions

Microalgae have gained significant interest in biotechnological applications due to their metabolic flexibility. They are able not only to perform photosynthesis, using atmospheric carbon dioxide as carbon source, but also to carry out cellular respiration by consuming organic carbon present in the medium to produce energy. Cellular respiration involves the oxidation of organic substrates leading to ATP production and the release of carbon dioxide, which can subsequently be then used for photosynthesis (Kliphuis et al., 2011).

Cellular respiration occurs through three main phases. First, glycolysis converts glucose into the three-carbon molecule pyruvate, producing energy in form of ATP (Mookerjee et al., 2015). Pyruvate then enters the mitochondria, where is transformed into Acetyl-CoA, which subsequently enters Krebs cycle (Wiskich, 1980). During this process carbon dioxide is released and electron carriers are generated. These electrons are transferred to the electron transport

chain, where oxidative phosphorylation leads to the production of water and the majority of ATP (Kliphuis et al., 2011).

This metabolic strategy is essential for microalgae grown under limited light or dark conditions (heterotrophic growth), allowing them to produce energy from organic carbon sources. However, these microorganisms are also able to simultaneously maintain both photosynthetic and respiratory metabolism under mixotrophic conditions, improving their growth rate and overall process stability. Indeed, mixotrophy provides greater adaptative capacity in stressful conditions such as in polluted environment. Moreover, it has been demonstrated that under mixotrophic conditions the effects of photoinhibition are reduced, leading to increased tolerance to high-light intensities (Chojnacka & Noworyta, 2004; Zhang et al., 2017).

Cellular respiratory metabolism is involved in essential pathways for the biosynthesis of cellular building blocks such as precursors of nucleic acids, amino acids and fatty acids, thus explained the increasing in biomass production under mixotrophic conditions (Qin et al., 2023).

2.4 Technological and economical challenges of microalgal cultivation

Despite the growing interest in microalgae cultivation for the production of food, feed, biofuels or other valuable products, several bottlenecks still need to be addressed to make this technology economically feasible (Ríos et al., 2013). Large-scale microalgal production for biodiesel is constrained by limitations affecting both the upstream and downstream phases of the process.

The upstream stage, which encompasses the production of high yield and lipids quality, depends on different factors. These include choice of strain and cultivation systems and the control of environmental conditions such as temperature, light exposure and nutrients and carbon dioxide supply. Moreover, the risks of contamination and the limited conversion of solar energy into biomass can significantly reduce productivity while increasing operational costs (Subramanian & Sayre, 2022).

On the other hand, downstream processing, comprising harvesting, drying and extraction of lipids from biomass, remains high energy-intensive and expensive, with efficiencies that vary depending on both the biomass characteristics and the technologies employed (Kim et al., 2013; Ríos et al., 2013). When considering the large volumes involved in industrial scale plants and the specialized infrastructures required both for upstream and downstream steps, these challenges become more critical.

For these reasons, improving upstream phase, particularly the bioprocess for biomass production, represents a key point to enhance the overall sustainability of the process. Increasing biomass productivity per unit area can effectively lower downstream processing burdens, reduce both economic and environmental costs and contribute to making microalgae-based biodiesel competitive on an industrial scale (Terigar & Theegala, 2014). Nevertheless, research needs to continue to improve downstream processes too, requiring an interdisciplinary effort to maximize the yield and reduce costs.

In this way it is possible to mitigate costs by increasing the yield of biomass per unit area and reducing economic and environmental impacts of the overall process and make microalgae-based biodiesel competitive at industrial scale.

2.4.1 Factors that influence biomass yield and composition

Biomass productivity and biochemical composition are influenced by several factors that can be modulated and controlled during cultivation process. The optimization of these conditions is essential to improve the overall process performance, redirecting the cultivation toward the metabolite of interest.

Operational parameters such as temperature, light intensity and quality, aeration, pH, nutrients availability and mixing are crucial to guide cellular growth

and metabolism. Moreover, the choice of the cultivation system is crucial in determining how effectively these parameters can be regulated.

In this context, an efficient cultivation strategy ensures biomass productivity and quality. For this reason, particular attention on optimization of each of these parameters is mandatory and is described in following sections.

2.4.1.1 Abiotic factors that influence microalgal cultivation

Environmental conditions such as temperature and light are fundamental for the cultivation of microalgae and strongly affects the growth performance and cellular activities (Gonçalves et al., 2016). Light is the primary parameter involved in photosynthesis and, consequently, in cellular energy production. Proper regulation of illumination supports regular growth of microalgae, whereas excessive light exposure may lead to photoinhibition or damage to light receptors (Nashath et al., 2025). On the other hand, temperature is crucial for the maintenance of functionality of proteins and enzymes as well as membranes fluidity, thus affecting cellular metabolic pathways (Ras et al., 2013).

Abiotic factors inherent to the growth medium, such as pH and nutrient composition, are also essential parameters that must be considered. In particular, pH level has a role in carbon and nutrient availability (Filali et al., 2021). At elevated pH levels, the precipitation of phosphates can occur, affecting their availability and uptake by microorganisms (Devine et al., 2024). Moreover, high pH conditions promote the formation of carbonates, reducing the dissolved inorganic carbon availability and consequently inhibit carbon utilization, negatively influencing the growth rate of microalgae (Qiu et al., 2017). Conversely, low pH levels can be dangerous for microalgal cultivation. Indeed, it may reduce cell wall permeability, having negative impacts on nutrients uptake and overall cellular metabolism (Lavoie et al., 2012). During microalgal cultivation, photosynthetic activity consumes dissolved carbon dioxide, leading to a progressive pH increase (Qiu et al., 2017). To overcome these effects, buffer systems are employed, mostly at laboratory scale, where their use is economically feasible. In alternative, CO₂ can be pumped in the substrate where it reacts with water to form carbonic acids, which contributes to decrease pH and maintaining more stable cultivation conditions (Agbebi et al., 2022).

In this context the composition of the substrate has an effect on microalgal growth and metabolisms. Several nutrients are needed for the cultivation of microalgae and are divided into two categories according to the concentrations: macronutrients are present in g L⁻¹, while micronutrients in mg L⁻¹. The former includes mainly nitrogen, phosphorus and sulfur. Nitrogen is an essential component of proteins, nucleic acids and other important components in microalgal cells, thus its supplementation has a huge influence on microalgal growth. Low concentrations of nitrogen, which can be supplied in different forms, drive to reduction in growth, indeed in phototrophic cultivations the complete

consumption of nitrogen generally leads to a stationary phase. In these conditions microalgae are able to consume cellular components which contain nitrogen to accumulate other storage compounds such as lipids (Fazeli Danesh et al., 2017; Mujtaba et al., 2012). It was also observed that the limitation of nitrogen also affects the assimilation of phosphorus, highlighting the importance of N/P ratio (Beuckels et al., 2015). Rapid uptake of phosphorus is generally observed, and intracellular polyphosphate bodies are produced, acting as protection from metals toxicity (Wang & Dei, 2006). Deprivation of phosphorus also leads to the reduction of growth, due to lower carbon dioxide fixation through photosynthesis, and results in starch accumulation (Dragone et al., 2011).

This section provided a brief overview of the main abiotic factors that influence biomass production and synthesis of metabolic compounds in microalgae cultivation. Biological systems require specific environmental conditions to function, which can represent a limitation or an advantage. By understanding the metabolic pathways of microalgae and how they respond to different environmental conditions, it become possible to strategically manipulate these factors toward the production of valuable biomass.

2.4.2 Scale-up cultivation systems

Cultivation systems for microalgae can be broadly divided into two main categories: open ponds and closed photobioreactors (PBRs). The choice between these systems depends on the application of microalgal biomass, including whether axenic conditions are required, as well as on the specific physiological needs of microalgae species.

2.4.2.1 Open pond cultivation systems

Open ponds (OPs) represent the simplest and most cost-effective cultivation systems. They are characterized by large volumes, minimal infrastructures and limited control over environmental parameters. Typically, OPs are illuminated by natural sunlight and lack temperature regulation. They can be closed loops or circular ponds mixed generally by a paddle wheel and with approximately 0.3 m of depth (Qin et al., 2019).

The simplicity of construction and ease of use and maintenance significantly reduce initial investment and operational costs (Chiaromonti et al., 2013). However, these advantages are offset by lower biomass productivity and high susceptibility to biological contaminations, which hinder the efficiency of these systems (Narala et al., 2016). Biomass productivity is limited by suboptimal light distribution due to self-shading and low surface to volume ratio. Additionally, environmental and technological factors such as temperature fluctuations and carbon dioxide loss further constrain growth (Narala et al., 2016; Sivakaminathan et al., 2020).

Light and temperature in OPs systems are strongly influenced by seasonal changes, weather conditions, geographic location and evaporation rates (Sarwer et al., 2022). Moreover, pond depth also plays a crucial role, as increased depth leads to reduced light penetration, with a consequent reduction in photosynthetic efficiency (Chowdury et al., 2020).

Temperature variability represents another major limitation. Both low and high temperatures may negatively affect the linear growth of microalgae (Chowdury et al., 2020). Low temperatures are particularly detrimental for microalgae as they reduce enzymatic activity, especially compromising the activity of Rubisco leading to a decrease in photosynthetic rate (Ras et al., 2013; Xiaogang et al., 2022). Conversely, temperatures above the optimal range can affect growth by causing the deactivation or denaturation of enzymes and proteins involved in key metabolic pathways such as photosynthesis. Moreover, elevated temperatures hamper CO₂ solubility, promoting the oxygenase activity of Rubisco and increasing photorespiration (by acting as oxygenase instead of carboxylase), with a consequent decrease in carbon fixation rate (Xiaogang et al., 2022). Teoh et al. (2004) demonstrated the effect of temperature on microalgal cultivation by testing six species. Here they observed that below and above a certain range there is an inhibition of the growth and affecting the composition of fatty acids.

Another challenge with OPs is the risk of contaminations, including bacteria, fungi, protozoa, or competing microalgal or cyanobacterial species (Lam et al., 2018). The presence of such biotic contamination can reduce cultivation efficiency, induce stress conditions, and, in severe cases, lead to culture collapse. A strategy to prevent and mitigate contamination is to establish selective environmental conditions (such as salinity, pH, or nutrient composition) that favor the target species, however this approach is not always applicable.

OPs are generally classified into two main designs: raceway ponds and circular ponds as shown in Figure 5 and Figure 6. The former are the most common configuration, they consist of looped channels mixed by a paddle wheel with baffles to direct the flow. Circular ponds, in contrast, use a rotating arm to maintain culture mixing. Due to their low cost and operational simplicity, these cultivation systems are particularly used in wastewater treatments (Laizu et al., 2025).



Figure 5: Examples of open ponds systems. Raceway on the upper image from U.S. Department of Energy National Renewable Energy Laboratory. In the lower photo a circular open pond system.



Figure 6: open pond raceway system (Savage, 2011)

2.4.2.2 Closed photobioreactors

To overcome the limitations associated with open ponds, closed photobioreactors have been designed to provide better control over microalgal cultivation conditions. These systems allow the maintenance of axenic cultures, high light exposure, controlled temperature, pH, nutrient availability and CO₂ dissolution. As a result, closed PBRs enable higher biomass productivity and

improved control of microalgae metabolisms (Rodolfi et al., 2009). Nevertheless, they are more expensive, both the infrastructures and the energy demand, making more challenging the industrial scale-up. The main strength of these systems lies in the enhanced biomass yield and metabolite productivity. For this reason, they are usually employed for the production of high-valued compounds, to maximize production and mitigate costs (Laizu et al., 2025).

Photobioreactors consist of transparent vessels designed to maximize light penetration into microalgal cultures. Aeration is generally introduced through spargers located at the bottom of the reactor, providing both gas exchange and mixing. Closed PBRs can be classified into columns, airlift or flat-panel PBRs (Qin et al., 2019). Primarily these configurations differ in vessel geometry and the aeration and mixing systems.

Column PBRs present sparger system at the bottom of the vessel which is responsible for both gas supply and mixing (Figure 7a). Airlift PBRs differ from column PBRs for the presence of two zones (riser and downcomer), which enable to improve mass transfer. Aeration is applied only in the riser, where the culture flows upward, before descendant through the downcomer. This design enhances mixing efficiency and reduces shear stress, minimizing energy consumption (Anas Al-Dailami et al., 2022).

Tubular PBRs include horizontal columns (Figure 7b), they consist in connected tubes with lower diameter than column PBRs and, depending on the design, may use pumps to guide the flow of the culture. The reduction of the radius of tubes helps to increase the surface to volume ratio and improve the exposure to light (Gupta et al., 2015).

Flat-panel PBRs offer improved light distribution through the culture thanks to limited depth of the vessel, ensuring higher photosynthetic efficiency (Figure 7c) (Anas Al-Dailami et al., 2022). They can be oriented vertically or on an angle, having more flexibility when implemented on industrial scale. However, a limitation of flat-panel systems is the possible deposit of biomass on the internal surface of the vessel, which can affect the light penetration and overall efficiency (Laizu et al., 2025).

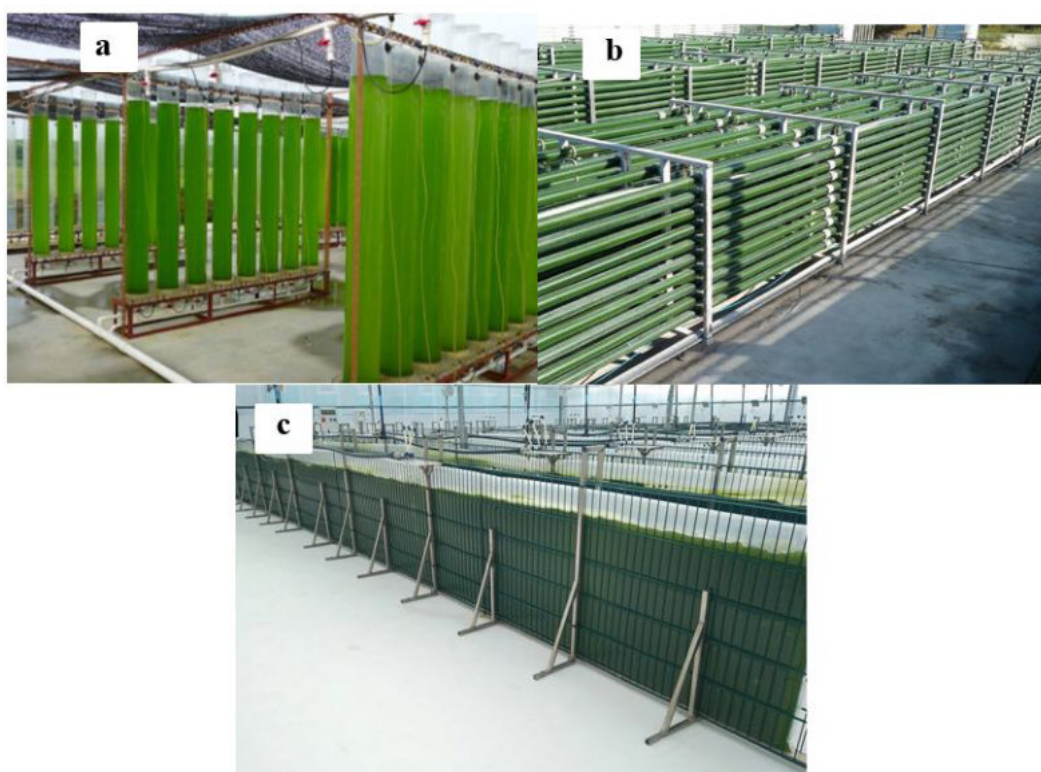


Figure 7: examples of closed PBRs: a) vertical column PBRs (Huo et al., 2018); b) horizontal tubular PBR and c) flat panel PBR (H. Pereira et al., 2018)

2.4.2.3 Comparison between open ponds and closed photobioreactors

Based on this overview, several key differences between OPs and PBRs can be highlighted. Thus, depending on the purpose of microalgal cultivation, it is possible to choose the correct system. One of the most impactful factors to be considered when discriminating against systems is the economic aspect. OPs are characterized by significantly lower expense of the infrastructures and the operational energy demand. Although they require large land areas, their construction and operations are relatively simple. Moreover, the utilization of sunlight and the absence of control of environmental conditions result in low energy demand. However, these economic advantages are contraposed by several limitations, including high evaporation, contamination risk, low carbon dioxide dissolution and inefficient light utilization (Kumar et al., 2015). Contamination with other microalgae, cyanobacteria or bacteria in the cultivation pond can lead to competition for nutrients and decreases in chosen microalga biomass production. In addition, the limited diffusion of carbon dioxide from atmosphere reduces the photosynthetic efficiency. This effect is boosted by the accumulation of dissolved O_2 , produced during photosynthesis, which can further inhibit photosynthetic efficiency (Kumar et al., 2015; Mendoza et al., 2013).

In contrast, closed PBRs have been specifically designed to overcome these limits. Indeed, they can operate both indoors and outdoors, using either natural or

artificial illumination, and allow precise control of key parameters, such as aeration, flow, mixing and temperature (Lee, 2001). While these features increase operational costs, they enhance cultivation performance by improving biomass productivity and process reliability. Closed systems also reduce evaporation and, through controlled aeration, improve carbon dioxide dissolution while facilitating the removal of excessive molecular oxygen, thereby improving photosynthetic efficiency (Mohsenpour & Willoughby, 2016). Moreover, the ability to regulate environmental parameters is essential for modulating microalgal metabolism and optimizing the production of the compound of interest.

In this context, the selection of appropriate system also depends on the robustness of microalgal species. Indeed, species with high resistance to environmental stress (such as temperature variability, low light exposure, high pH, high salinity or contaminations) are suitable for cultivation in OPs. Commonly used genera in OPs cultivation include *Chlorella* sp., *Spirulina*, *Dunaliella* sp. and *Haematococcus* sp (Laizu et al., 2025; Lee, 2001).

Due to their low cost, OPs are widely employed for wastewater treatment applications. For instance, Nwoba et al. (2016) compared the microalgal cultivation in Raceway open pond with a tubular PBR for wastewater treatment, observing similar removal efficiencies of main contaminants. However, closed PBR achieved approximately double the biomass productivity. Nevertheless, the biochemical composition of the biomass (lipids, carbohydrates and proteins) was comparable between two cultivation systems, making it suitable for applications such as animal feed or for biofuels production (Nwoba et al., 2016). These findings are consistent with other studies comparing OP raceway and horizontal tubular PBR for biodiesel production, where biomass productivity in closed PBR was around 1.5 times higher than in OP (Narala et al., 2016).

2.5 Wastewater valorization via microalgal cultivation

Water is an essential source for industries and human activities, and the increased population leads to increased freshwater demand and consumption. In this context, the reduction of water consumption and the reutilization of wastewater is mandatory. Microalgal cultivation requires large amounts of water, being costly and environmentally impactful, thus the utilization of wastewater as water and nutrient source gained high interests in scientific community (Daneshvar et al., 2019; Torres-Franco et al., 2021; Barreiro-Vescovo et al., 2021; Srimongkol et al., 2022).

Wastewater treatment (WWT) is fundamental for mitigating the environmental impact of industrial activities, as the production of polluted effluents is a critical issue. Industrial and municipal wastewaters typically contain significant amount of contaminants, including nitrogen, mainly in the form of $\text{NH}_4^+\text{-N}$ or $\text{NO}_3^-\text{-N}$, phosphorus as $\text{PO}_4^{3-}\text{-P}$ and organic carbon sources (Geremia et al., 2021). If discharged untreated into the environment, these streams can lead to eutrophication of receiving water bodies, threatening aquatic life and disrupting ecosystems balances by promoting the growth of algae and aquatic plants. Therefore, the removal of nitrogen and phosphorus, as well as carbon, is the main aim of WWT plants. To address this issue, legislative limits have been imposed. In Europe, the Directive 91/271/EEC sets limits for pollutant concentrations in discharged waters, requiring nitrogen content lower than 10-15 mg L^{-1} depending on the personal equivalent of the industry, and 1-2 mg L^{-1} of phosphorus (Geremia et al., 2021). In addition, organic matter is regulated through biological oxygen demand (BOD) and chemical oxygen demand (COD).

The recovery of wastewater is mandatory due to the water scarcity and the importance in valorizing waste and reducing environmental pollution. Nowadays primary, secondary and tertiary treatments are normally applied depending on the waste composition. They can be physical, chemical or biological treatments (Mohsenpour et al., 2021). Generally preliminary steps are made to eliminate the majority of solid contents by physical or chemical methods. Then, primary treatments are based on sedimentation of solids and reduction in bacterial count. Secondary treatments are generally biological, made with activated sludge and are followed by tertiary treatments with physical, chemical or other biological methods. This methods applied to wastewater are energy demanding and biological treatments are efficient to reduce concentration of pollutants but still require energy, produce GHG emissions and needs more treatments due to the production of high amount of sludge, which is high in bacterial content and organic matter. This sludge contains still N and P thus they could be used as fertilizer in agricultural sectors, nevertheless their implementation in this field is not possible due to heavy metal content and residual pathogens (Singh & Agrawal, 2008).

Conventional wastewater treatment is successfully applied worldwide, but still many concerns about energy consumption and emissions are reported, highlighting the urgency of new solutions with reduced consumption of energy and production of secondary wastes. In this context the utilization of microalgae for the reduction of pollutant concentrations is a valuable alternative. Microalgae indeed, can reduce nitrogen, phosphorus, sulfur and carbon contents, as well as many metal elements (Barreiro-Vescovo et al., 2021). Additionally, microalgae absorb carbon dioxide from the atmosphere, mitigating GHG emissions. Their capacity for growing in polluted environments and being adapted to several conditions make them a promising solution for wastewater treatments. Moreover, microalgal-bacteria consortium can be developed to increase the consumption of organic matter thanks to bacteria, by associating complementary metabolisms to increase the remediation rates (Dai & Wang, 2024; Jia & Yuan, 2016). Furthermore, a major advantage in using microalgae for these processes is the valuable biomass production which can be used in several sectors, such as biofertilizer, animal feed or energy production, further mitigating the economic impact of the process (Srimongkol et al., 2022).

Several kinds of wastewaters are produced by human activities, mainly municipal wastewaters are collected, but also industrial streams from textile, chemical or pharmaceutical industries, agro-industrial wastewaters from food, dairy or brewery industries, agricultural runoff or livestock wastewaters. Each one of these streams presents a distinct composition, which also varies during the years depending on industrial activities.

In the present thesis, for many reasons which will be explained in next section, dairy industry wastewater has been used for microalgal cultivation to reduce the cost of biomass production while remediating polluted streams and releasing cleaner waters.

2.5.1 Dairy industry

The dairy industry requires large amounts of water for milk processing and for equipment cleaning, pasteurization and sterilization processes. It has been estimated that between 1 and 10 m³ of water are consumed per m³ of processed milk (Boguniewicz-Zablocka et al., 2019). In 2019, Europe produced 167.4 tons of milk products, being the second largest producer (FAO, 2020), accounting for 192.5 10⁶ m³ of wastewater produced annually (Stasinakis et al., 2022). Notably, Italy is among the six countries contributing to 73% of total wastewater production from this sector, being third after Germany and France, with 18.9 10⁶ m³ in 2019, as shown in Figure 8 (Stasinakis et al., 2022). Milk can be used either as whole milk or skimmed milk depending on the final product. Both types are used for cheese, drinking milk, acidified products or powder products, while butter and cream derive exclusively from whole milk, since they are made from

the fat fraction of milk. Water consumption highly depends on the type of final product, in Europe fermented milk products require the highest water consumption, followed by cheese and milk powder production (Stasinakis et al., 2022). Moreover, both for drinking milk and cheese production the higher water consumption is associated with cleaning operations.

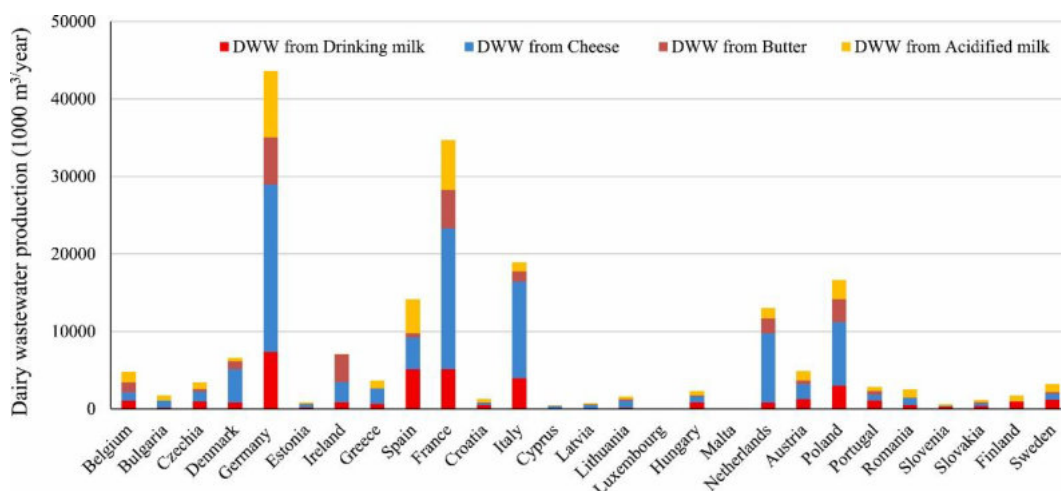


Figure 8: estimation of volumes of dairy wastewater (DWW) produced in Europe in 2019 divided into main products processes. Source: Stasinakis et al., (2022)

The composition of dairy wastewater varies depending on the specific processes but generally is characterized by high concentrations of organic matter, including fats, proteins, minerals and lactose from milk. Reported values include Chemical oxygen demand (COD) between 80-95000 mg L⁻¹ and Biological oxygen demand (BOD) between 40 and 48000 mg L⁻¹. Moreover, nitrogen accounts for 14-830 mg L⁻¹ while phosphorous 9-280 mg L⁻¹ (Chokshi et al., 2016; Daneshvar et al., 2019). The pH of these streams can vary from acidic to alkaline values, representing a challenge for some treatment processes, including microalgal cultivation.

During cheese production, milk coagulation and curd separation generate Cheese Whey, a by-product rich in phosphorus, nitrogen and organic carbon (Carvalho et al., 2013). Due to its composition it is widely exploited for biological valorization (Atra et al., 2005; Usai et al., 2024; Zandona et al., 2021). Nevertheless, in Italy it is still considered a valuable intermediate product for dairy industry, as is further processed into Ricotta cheese through further heating (Monti et al., 2018). This process produces another type of wastewater called Scotta (Sc), with low pH, high lactose content and high BOD and COD (Bergamaschi et al., 2016; Monti et al., 2018). Moreover, 75% of total water consumption in dairy sector is due to cleaning operations (Buabeng-Baidoo et al., 2017). Wastewater generated from washing procedures contains residual milk components, such as fats and lactose.

These primary streams are typically treated using conventional biological processes, such as activated sludge, which is the most widely applied strategy implemented for the reduction of organic matter and nitrogen and phosphorus concentrations (Mohsenpour et al., 2021). However, the generated excess sludge, is still rich in organic matter, nutrients, and metals, posing additional disposal and management challenges (Kaur, 2021; Lateef et al., 2013).

In this context, the utilization of microalgae for bioremediation of dairy effluents is a valid sustainable alternative to conventional treatments. The high concentrations of nitrogen, phosphorus and carbon in these effluents make them suitable for microalgal cultivation, enabling removal of pollutants and valuable biomass production. Several microalgal species have been investigated for this purpose including *Chlorella vulgaris*, *Chlorella sorokiniana*, *Spirulina platensis*, *Chlamydomonas polypyrenoideum*, *Scenedesmus acutus* and *Tetradesmus* sp. (Gupta et al., 2024; Hajri et al., 2024; Khalaji et al., 2023; Kothari et al., 2013; Kusmayadi et al., 2022a). These studies demonstrate the potential of microalgae-based systems to significantly reduce pollutants load. For example, *C. sorokiniana* was able to reduce COD, TN and TP of 87%, 94% and 81% respectively from dairy wastewater (Kusmayadi et al., 2022a).

Therefore, microalgae-based treatment addresses environmental impact mitigation through a circular process, where waste is converted into valuable resources. The development of key technologies for sustainable integrated wastewater management is a promising future perspective.

2.6 Lipid production in microalgae: classes and synthetic pathway

Lipids in microalgae are involved in different functions, depending on the class. Structural lipids include polar lipids, which are mainly involved in maintaining fluidity and stability of cellular membranes and include glycolipids, phospholipids or sterols. Neutral lipids are accumulated as energy source, usually found in lipid droplets and mainly composed of triacylglycerols (TAGs) (Solovchenko, 2012). TAGs also play an important role in cell protection from environmental stress which leads to the production of reactive oxygen species (ROS) (Klok et al., 2013). TAGs indeed, under oxidative conditions, can prevent excessive accumulation of electrons within electron transport chain in the chloroplast, working as electron sink (Morales et al., 2021). TAGs act also as energy sink under stress conditions and, when balanced environmental conditions are restored, they may be used for membranes and metabolite formations (Goncalves et al., 2013).

Lipid biosynthesis in microalgae follows two main pathways. The primary way is the *de novo* synthesis, which occurs in the chloroplast, while further elongation and lipid assembly take place in endoplasmic reticulum Figure 9. In addition, the acyl-CoA-independent pathway may occur through phospholipid remodeling. The *de novo* synthesis begins with the photosynthetic fixation of carbon dioxide into glyceraldehyde-3-phosphate, which is subsequently converted into Acetyl-CoA. Then the carboxylation of Acetyl-CoA occurs through enzymatic reaction to produce malonyl-CoA which, as Malonyl-ACP (Acyl carrier protein) enters the fatty acids synthase cycle (FAS) (Xin et al., 2024). Through a series of condensation reactions with acetyl-CoA, the acyl-chain is elongated, typically through seven cycles to produce C16-ACP (Proietti Tocca et al., 2024). The resulting Acyl-ACP are then hydrolyzed to release free fatty acids and transferred to the endoplasmic reticulum (Xin et al., 2024). Here, TAGs are synthesized via sequential acylation of glycerol-3-phosphate.

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thus their increase during stress condition is a defense strategy, to both respond to oxidative stress and reorganize cell metabolism to adapt to less favorable environmental conditions (Khozingoldberg & Cohen, 2006; Ratha et al., 2013).

Metabolic lipid accumulation in microalgal cells is primarily driven by the redistribution of intracellular carbon fluxes. Under optimal growth conditions, carbon fixed through photosynthesis is directed toward biomass production, including proteins, nucleic acids, and carbohydrates, to sustain cell division. In contrast, under stress conditions, cell growth is inhibited and the demand for protein synthesis decreases, leading to a redirection of carbon toward the synthesis of storage compounds, such as fatty acids and TAGs (Morales et al., 2021). Under excess carbon availability, such as elevated CO₂ concentrations or mixotrophic cultivation, this metabolic shift is enhanced, where the increased carbon supply cannot be fully utilized for growth and is stored.

In present work microalgae have been cultivated using industrial wastewater as liquid substrate, promoting mixotrophic metabolism that enhances both growth and lipid accumulation. However, when dealing with complex wastewater substrates, the application of nutritional stress strategies is not feasible. Although mixotrophic conditions can improve lipid production, they are insufficient to achieve high yields for economically viable biodiesel production. In this context, the implementation of an integrated approach combining wastewater treatment with controlled environmental stress conditions or optimized process design represents a promising strategy.

2.6.1.1 Nutritional stress: nitrogen limitation and starvation

Nitrogen is an essential component of nucleic acids, light-harvesting pigments and proteins, and is therefore involved in photosynthesis and cell division. Its limitation affects photosynthetic rate and biomass growth (Panyakampol et al., 2016). Under these conditions cells decrease the production of N-rich compounds such as pigments and proteins and, in severe conditions, degradation of proteins for nitrogen and carbon reallocation occurs. As a result, this condition leads to a dramatic decrease in growth rate and cell division, affecting both biomass and lipid productivity (Xin et al., 2024).

The increased TAG production may be due to different factors. Primarily, nitrogen limitation leads to reduced protein synthesis with a consequent shift of inorganic carbon fixation from carbohydrate production toward fatty acids synthesis, following the *de novo* fatty acids pathway (Msanne et al., 2012). In addition to this effect, nitrogen deprivation causes an imbalance in reduction and oxidation state of energy storage compounds (ATP versus ADP) and electron carriers (NADPH and NADP⁺) produced during photosynthesis. In detail, during photosynthesis electrons are transported to NADP⁺ and ADP to produce NADPH and ATP, which are in turn consumed during cell division. Under nitrogen deprivation, light-induced electron transport remains active, but the reduction in

nitrogen drives to reduction in cell division and thus lack of NADP⁺ and ADP availability. In this context, the fatty acid synthase (FAS) cycle consumes ATP and NADPH and restores the pool of electron acceptors (Fields et al., 2014). Moreover, under these conditions oxidative stress may lead to ROS accumulation, which usually leads to cell damage. The production of neutral lipids, mainly TAGs, is functional not only for the restore of electron acceptors, but also because they act as electron sink, having a protective effect against ROS (Khozingoldberg & Cohen, 2006).

Several species, mainly those known for their lipid accumulation capacity, have been investigated for the increased accumulation of lipids and TAGs under nitrogen deprivation or limitation. Species such as *Chlorella* sp., *Chlorella vulgaris*, *Nannochloropsis oceanica*, *Nannochloropsis gaditana*, *Neochloris oleabundas*, *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* have been cultivated under nitrogen limitation or deprivation with the result of increased lipid accumulation (Abedini Najafabadi et al., 2015; Breuer et al., 2012; Chu et al., 2019; Janssen et al., 2018; J. Jia et al., 2015; Msanne et al., 2012).

Lipid in microalgae can vary widely between 20 and 70% of dry biomass, depending on the species and on the specific cultivation conditions (Mujtaba et al., 2012). The species mainly selected for biodiesel, generally reach lipid content around 40-50% when accumulation is properly stimulated (Breuer et al., 2012; Illman et al., 2000; Jia et al., 2015; Roy et al., 2021). The increase in lipid content is mainly due to the TAG accumulation, which is the most relevant lipid class for biodiesel production.

A major drawback of this strategy is the limitation of biomass production, which is obtained in response to nitrogen deprivation. Nitrogen starvation happens when nitrogen is completely lacking and this condition leads to a rapid arrest of growth in favor of lipid accumulation. Although lipid content is higher under these conditions, overall lipid productivity is reduced due to the lower biomass yield. As an alternative, nutrient limitation can be applied, when nitrogen is present at suboptimal concentration, allowing continued biomass growth and simultaneously higher lipid accumulation. In this context, integrated approaches are often employed, involving an initial phase of biomass accumulation then shifted to nitrogen depletion conditions to induce lipid accumulation. This two-step strategy has been widely investigated (Benasla & Hausler, 2021; Chu et al., 2019).

2.6.1.2 Environmental stress: light regime modulation

Light is the primary energy source for the growth of photosynthetic organisms and several factors can modulate its effects on cells metabolisms. Indeed, not only the intensity of light, but also the quality - in terms of spectral composition or specific wavelength - and the photoperiod can influence biomass production and biochemical composition (Wahidin et al., 2013; Yan et al., 2013).

Light is absorbed by microalgal pigments between 400-700 nm wavelengths range, which corresponds to the Photosynthetic Active Radiation region (PAR) (Solovchenko, 2012). White light is commonly used for microalgal cultivation to cover the entire visible spectrum. However, it is possible to select a target wavelength to induce different metabolism responses in cells.

Under optimal irradiance conditions, microalgae exhibit stable growth with a balanced composition of carbohydrates, proteins and lipids. In contrast, under unbalanced light conditions cellular metabolism is reprogrammed to sustain growth and minimize photodamages. Indeed, under high light intensities, upon saturation levels, photooxidative stress may occur due to the overexcitation of photosystem II and the consequent production of ROS (Nashath et al., 2025). When ROS are present in limited concentrations, they act as signaling molecules that activate protective metabolism pathways. However, when light intensity is excessive, ROS production exceed their scavenging capacity and leads to oxidative damage (Sousa et al., 2012). Under these photooxidative conditions, microalgae shift their metabolisms to produce antioxidant compounds, including carotenoids and lipids to mitigate oxidative stress. Indeed, variations in biomass compositions include carotenoids, lipids, and composition of fatty acids. In this context the increase in carbon compounds represents a strategy to absorb the excessive light energy to avoid photooxidation. Clearly, this capacity is species-specific, due to different pigments composition and the efficiency in photochemical energy utilization. Similarly to what we observed with nitrogen limitation, exposure to excessive light can lead to increased synthesis of saturated fatty acids. Indeed, this process consumes ATP and NADPH contributing to the regulation of redox balance and avoiding photodamage (Amini Khoeyi et al., 2012a).

Light supply can be improved by properly designed PBR which increases the efficiency of light exposure of microalgae. The most commonly used sources of light are fluorescent lamps and Light Emitting Diodes (LEDs). The latter offer the advantage of emitting a specific wavelength or narrow spectral bands. Moreover, they are more efficient and thus energy-saving (Schulze et al., 2014).

In this scenario deeper knowledge on effects of light intensity and quality on different microalgal species is essential to develop optimized cultivation strategies that balance biomass productivity and the biochemical composition.

The production of biomass under selected light wavelength induces species-specific responses. For example, higher biomass production has been reported under blue and red light in *Tetradismus obliquus* (Jin et al., 2024), and *Rhodomonas* sp. (Latsos et al., 2021), while red light enhances biomass productivity of *C. sorokiniana* (He et al., 2023). In addition, in terms of lipid productivity *Chlorella vulgaris* and *Chlorella sorokiniana* drove to higher content under blue light (Sánchez-Saavedra et al., 2020; Singh Laura, 2019).

Species-specific responses to light conditions are largely determined by the composition and regulation of microalgal photoreceptors. Indeed, several classes of these macromolecules are present, including phytochromes which absorb in red and far-red light, cryptochromes, phototropins and aureochromes which are responsive to blue light, and rhodopsin-like proteins (Singh Laura, 2019). Depending on the composition of photoreceptors, microalgae exhibit different metabolic responses to specific wavelengths. For instance, red light has been associated with higher lipid accumulation probably linked to the activity of phytochrome-like proteins (Dong et al., 2023), whereas blue light can promote carotenoid synthesis, mediated by chryptochromes (Zhang et al., 2022). Moreover, phototropins regulate responses to blue light and in some species have been associated to higher biomass accumulation (Essen et al., 2017).

In this scenario the distribution and regulation of photoreceptors across different species and strains drives to metabolic adjustments under varying light regimes. This phenomenon further highlights the importance of selecting appropriate species in relation to biotechnological application. Moreover, the development of an efficient cultivation process requires a comprehensive understanding of microalgal physiology and biochemical composition.

2.7 Strain selection and biodiesel quality

In biotechnological processes for microalgal-biodiesel production, several key factors must be considered to select the most suitable species. A high growth rate is essential to ensure substantial biomass production and reduce the overall costs of the process (Song et al., 2013). In addition, lipid content plays a crucial role. When combined with growth rate it determines lipid productivity, which is a fundamental parameter in species selection (Banerjee et al., 2002). Indeed, high lipid content alone is not sufficient if not associated with high biomass production, because in that case lipid productivity remains low making the process economically unfeasible. For example, it has been observed in *Botryococcus braunii*, which records high lipid content, reaching 70% of dried biomass, but is also associated with a doubling time around 5-7 days (Banerjee et al., 2002; Li & Qin, 2005).

Beyond these aspects, lipid quality also influences the choice of the species. An optimal profile typically requires balanced proportions of SFA and PUFA (Stansell et al., 2012). Finally, another essential feature is the ability of the selected species to grow in polluted environments. The utilization of wastewater as cultivation substrate for microalgal is critical to developing a sustainable and cost-effective process for biodiesel production. In addition to the tolerance of polluted environments the selected species should exhibit strong competitiveness against biotic contamination. Indeed, the proliferation of undesired microalgal species or bacteria represents a major challenge in biotechnological microalgal cultivation.

Griffiths & Harrison (2009) compared literature results of biomass production and lipid productivity of 55 microalgal and cyanobacterial species and results revealed that *Amphora*, *Ettlia oleoabundans*, *Ankistrodesmus falcatus*, *C. sorokiniana* and *Tetraselmis suecica* are valuable species for biodiesel production, measuring lipid productivity between 97 and 160 mg L⁻¹ d⁻¹. This kind of research is fundamental as a primary screening based on widely available literature data and should be followed by experimental validation. However, the main limitation on literature-based screening lies in the variability of experimental conditions and recorded parameters, as environmental factors highly influence biomass and lipid productivity. For example, a study investigating ten microalgal species in terms of biomass and lipid productivity alongside with lipid quality identified five species suitable for biodiesel production, which are *Selenastrum capricornutum*, *Chlorella vulgaris*, *Scenedesmus obliquus*, *Phaeodactylum tricornerutum* and *Isochrysis sphacrica* (Song et al., 2013). Another widely implemented strategy is the selection of species from natural environments or wastewaters, to search for adapted strains. Between isolated strains from natural sources, *Chlorella vulgaris* and *Scenedesmus obliquus* stood out for their biomass and lipid productivity suitable for biodiesel production (Sadvakasova et al., 2019).

These screening approaches highlight that the most promising candidates are those combining high biomass and lipid productivity, lipid quality specific for biodiesel requirements and adaptability to several environmental conditions both physical (i.e. temperature and light) and chemical (composition of liquid substrate). Within this framework, the genus *Chlorella* has been widely investigated for biodiesel production, both *C. sorokiniana* and *C. vulgaris* resulted in high growth rate and lipid productivity alongside with high performance in wastewaters bioremediation (Asadi et al., 2020; Eladel et al., 2019; Hamidian & Zamani, 2022b; Talha et al., 2025; Usman et al., 2024). Similarly, *Tetradismus obliquus* has demonstrated to have high lipid content and a marked tolerance to polluted environments and resilience to environmental stress (Mofeed et al., 2025; Rajabi Islami & Assareh, 2019; N. Singh et al., 2020).

Recently, *Nannochloropsis* sp. has been widely investigated for biodiesel applications, with particular attention to *N. oceanica* (Ashour et al., 2019; Ma et al., 2014). This marine microalga requires saline conditions for optimal growth and has attracted interest due to its high lipid content, which can reach up to 60% of dried weight (Wan et al., 2013). However, despite its high lipid content, some limitations affect its suitability for biodiesel application. In particular, its lipid profile is rich in PUFA (especially eicosapentaenoic acid, EPA) which can reduce the oxidative stability of biodiesel (Ashour et al., 2019; Stansell et al., 2012). Moreover, although it has been demonstrated able to grow in wastewater, its marine nature may limit its cultivation in non-saline effluents, reducing its adaptability compared to other genera. Overall, these characteristics suggest that *Nannochloropsis* sp. is suitable for biodiesel production under specific process conditions.

Therefore, *C. sorokiniana* and *T. obliquus* represent promising candidates as freshwater microalgal species for biodiesel production, particularly for processes which involve wastewater treatments.

2.8 Biodiesel quality (FAME profiles of microalgae)

Biodiesel is produced via the transesterification of lipids extracted from microalgae, resulting in a mixture of fatty acids methyl esters (FAMES). Among various lipid classes, neutral lipids, particularly TAGs, are the most relevant fraction for biodiesel production, as they are directly converted into FAMES (Morales et al., 2021). The resulting FAMES composition determines the quality of biodiesel.

In detail, both the relative abundance of each FAME and its structural characteristic (chain-length and unsaturation level) significantly influence fuel performance. High-quality biodiesel is generally associated with high content in SFAs and MUFAs, and low concentrations of PUFAs (Stansell et al., 2012). Indeed, high PUFAs negatively affect ignition quality and performance, while also contributing to increase the viscosity and promoting sediment formation in biodiesel (Knothe, 2005). Moreover, longer carbon chains and lower levels of unsaturation improve ignition properties. Reduced unsaturation also enhances oxidative stability and functionality of fuel at low temperatures (Knothe, 2007). However, achieving an optimal and balanced FAME profile is challenging. Microalgae exhibit highly diverse and complex lipid profiles, which varies among species. In addition, cultivation conditions highly influence the composition of FAMES (Francisco et al., 2010). In this scenario high content in palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids is the ideal basis for improving biodiesel quality (Morales et al., 2021).

Overall, biodiesel quality is defined by key parameters that can be empirically or theoretically evaluated.

Indeed, the variation in carbon chain length, number and position of double bonds and percentage of each FAME determines the properties of biodiesel like oxidation stability, ignition time, engine performances and cold flow (Wu & Miao, 2014). Those characteristics are strictly regulated by International Standards, i.e. American standards American Society for Testing and Materials (ASTM D6751) and European Standards EN14214.

A central parameter for fuel quality is the Cetane Number (CN) which indicates the ignition and combustion performances of a diesel fuel. A low CN causes ignition delays and lower engine performances. CN depends on the hydrocarbon chain length and the presence of branches and unsaturation. Likewise, longer carbon chain with less unsaturation and branches make biodiesel more viscous and dense, raising the CN and thus the ignition quality (Knothe, 2007). A measurement scale for CN values was set by International Organization for Standardization (ISO 5165). The cetane scale ranges between 15 and 100, respectively associated to 2,2,4,4,6,8,8,-heptamethylnonane, a highly branched hydrocarbon, and hexadecane, which is long and unbranched hydrocarbon chain. According to ASTM D6751 the minimum CN is 47, while for EN14214 and

EN:590 (respectively referred to unblended and blended biodiesel) CN has to be more than 51 (Knothe, 2005). The level of unsaturation of fatty acids also affects the biodiesel performance at low temperatures. An increase in the number of double bonds determines a decrease in melting point, which means that the crystallization occurs at lower temperatures (Wu & Miao, 2014). The presence and accumulation of crystals and solids cause clogging of fuel pipes and filters. Consequently, the presence of high levels of saturated fatty acids, which solidify at higher temperatures, reduces the flow performances of biodiesel. The parameter that is used to calculate the lower temperature at which a biodiesel can perform is cold filter plugging point (CFPP) (Nascimento et al., 2013). To calculate CFPP another factor is primary measured, it is the Long-Chain Saturated Factor (LCSF) which considers only long chain SFA percentages.

The number and position of double bonds not only affect CN and CFPP, but also the Iodine Value (IV). IV is proportional to the number of unsaturation and high levels are responsible to lower oxidation stability, causing formation of deposits, corrosion and lubricity (Knothe, 2007). IV indicates the grams of iodine consumed by 100 g of biodiesel and EN14214 set the maximum limit of 120 g I₂ per 100 g of biodiesel.

Several microalgal genera have been identified as promising candidates for biodiesel production, including *Chlorella*, *Dunaliella*, *Phaeodactylum* and *Scenedesmus* (Francisco et al., 2010). Conversely, other species have been determined as less suitable for this application, due to low CN and high IV, such as *Scenedesmus dimorphus*, *Franceia* sp. and *Mesotaenium* sp. (Islam et al., 2013). Within this context, *C. sorokiniana* has been widely investigated and demonstrated to be suitable feedstock for biodiesel production, in fact resulted with biodiesel properties within legislative limits (Bharti et al., 2018; Menegazzo et al., 2022).

Biodiesel is currently produced from crops, however, these feedstocks display several limitations such as low yield, land utilization and competition with food sector. In this context, microalgae have shown potential as alternative feedstock, due to their high biomass yield and lipid productivity, and the FAME profile suitable for high-quality biodiesel, representing a sustainable alternative to conventional crop fuel (Nascimento et al., 2014).

Chapter 3

Material & methods

3.1 Microalgal strains and cultivation media

All trials were performed using *Chlorella sorokiniana* (CCAP 221/8k), *Tetradismus obliquus* (CCAP 276/3A) and *Nannochloropsis oceanica* (CCAP 849/10) that have been purchased by Culture Collection of Algae & Protozoa (Cambridge, UK).

Specifically, *Chlorella sorokiniana* (CCAP 221/8k) and *Tetradismus obliquus* (CCAP 276/3A) were maintained in standard medium. *C. sorokiniana* was cultivated on Three-fold Nitrogen Basal Bold Medium (3N BBM) while *T. obliquus* on Blue-Green Medium (BG11). The compositions of the media were obtained following the recipe proposed by CCAP. Both cultures were maintained in flasks at 130 rpm, 30 °C and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ in a shaking incubator (Eppendorf™ Innova™ S44i, Eppendorf, Hamburg, Germany).

Other experiments (related to Chapter 4.3) were conducted using a different strain of *T. obliquus* (CCAP 276/10) and one more species, which is *Nannochloropsis oceanica* (CCAP 849/10). Here, 3N BBM was chosen to maintain *C. sorokiniana* and *T. obliquus* while *N. oceanica* was cultivated with f/2. Here precultures were maintained in flasks at 100 rpm, 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at room temperature. Then each species was grown in 500 mL bottles, aerated with air pump (100% air) to prepare a consistent inoculum for the cultivation experiment.

More detailed, the composition of three media used in this thesis is as follow. 3N BBM was prepared with the following concentrations (mg L^{-1}): 750 NaNO_3 , 25 $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 75 $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 25 NaCl , 57 K_2HPO_4 , 175 KH_2PO_4 , 4.5 Na_2EDTA , 0.58 $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$, 0.24 $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$, 0.017 ZnCl_2 , 0.012 $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.024 Na_2MoO_4 . Moreover, BG11 in accordance with CCAP receipt is composed as follows (mg L^{-1}): 1500 NaNO_3 , 36 $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 24.1 Na_2EDTA 2

H₂O, 4.05 FeCl₃ 6 H₂O, 75 MgSO₄ 7 H₂O, 40 K₂HPO₄, 2.86 H₃BO₃, 1.81 MnCl₂ 4 H₂O, 0.22 ZnSO₄ 7 H₂O, 0.39 Na₂MoO₄, 0.079 CuSO₄ 5 H₂O, 0.049 Co(NO₃)₂ 6 H₂O. The last media implemented for the cultivation of marine water *N. oceanica*, is f/2 and is composed dissolving these chemicals into seawater (mg L⁻¹): 75 NaNO₃, 5.65 NaH₂PO₄ 2 H₂O, 4.16 Na₂EDTA, 3.15 FeCl₃ 6 H₂O, 0.01 CuSO₄ 5 H₂O, 0.022 ZnSO₄ 7 H₂O, 0.01 CoCl₂ 6 H₂O, 0.18 NaMoO₄ 2 H₂O, ND5 B12, ND5 Biotin and 0.1 Thiamine.

3.2 Wastewater treatments

In this study dairy industry has been selected as main source of streams. As primary wastes Scotta and Washing Waters (DWW, Dairy Washing-Water) were collected. After treatment with activated sludge, Exhausted Sludge' (DES, Dairy Exhausted Sludge) is produced as secondary wastewater from this industry. Moreover, from Irish dairy industry another kind of waste was purchased, the 'Mixed Dairy Waste' (MDE). Besides dairy industry, gas fermentation effluent (GFE) was tested for microalgal cultivation. This effluent derives from the fermentation of *Clostridium carboxidivorans* P7, which was obtained through Adaptive Laboratory Evolution and can hyper-produce hexanol growing with CO₂ and H₂ (Antonicelli et al., 2025). After the extraction of hexanol from the stream, the remaining effluent still contains organic carbon by-products such as acetate, butyrate, caproate, ethanol and butanol.

Prior cultivation of microalgae these streams need to be treated and sterilized to allow a controlled environment for microalgae. DWW, Whey and Scotta were subjected to a cycle of deproteinization in autoclave for 5 minutes at 115 °C and then cool down at room temperature before filtration. DES was centrifuged using a Sorvall RC6 Plus Superspeed Centrifuge (Thermo Scientific, Waltham, MA, USA) for 20 minutes at 7000 rpm to remove the solid organic waste and the supernatant was collected. Similarly, MDE was collected from the factory, freeze-stored and then maintained at 4°C to melt before use. It was centrifuged at 10000 rpm with a super-centrifuge SORVALL® RC 5C PLUS and supernatant was collected. Moreover, GFE was collected after the fermentations process, centrifuged at 4000 rpm to separate cells and the supernatant was collected. All these wastewaters were then sterile filtered using a vacuum system with polyethersulfone membranes with 0.22 µm pores size and stored at 4°C until use.

After that they have been chemically analyzed to unravel the composition of them and to speculate about the applicability to microalgal cultivation.

3.3 Ammonium tolerance

Considering that usually the nitrogen source in selected wastewaters is ammonia, the tolerance/toxicity of this form of nitrogen was tested for *C. sorokiniana* and *T. obliquus*. This was performed by using Erlenmeyer's flasks with a working volume of 30 mL with modified standard media. Both BG11 and 3N BBM were prepared by replacing sodium nitrate with ammonium chloride at different concentrations: 50 mg L⁻¹, 100 mg L⁻¹, 200 mg L⁻¹ and 500 mg L⁻¹. As for strain maintenance, BG11 was used for *T. obliquus* and 3N BBM for *C. sorokiniana*. The growth of each microalga was monitored daily by measuring optical density at 750 nm (OD₇₅₀). The test was conducted in triplicate for each condition. Flasks were maintained during the experiment in a shaking incubator (Eppendorf™ Innova™ S44i, Eppendorf, Hamburg, Germany) at 130 rpm, 30°C and with white light LED at 60 μmol photons m⁻² s⁻¹.

3.4 Screening of cultivation of microalgae on wastewaters

DWW, DES and Scotta have been experimentally tested as substrates for microalgal cultivation. This screening was conducted using 100 mL Erlenmeyer's flasks, with a working volume of 30 mL and maintained at 130 rpm, 30°C and 60 μmol m⁻² s⁻¹ in a shaking incubator (Eppendorf™ Innova™ S44i, Eppendorf, Hamburg, Germany). Each condition has been tested in triplicate with *T. obliquus* (CCAP 276/3A) and *C. sorokiniana* (CCAP 221/8k). All streams were diluted in the same way with standard media (3N BBM for *C. sorokiniana* and BG11 for *T. obliquus*) using 50%, 75% and 100% of the wastewater. After dilution, pH was adjusted to be around 7 for Scotta, while DWW and DES did not need any adjustment prior buffer addition. In all cases 25 mM of TES buffer (2-([Tris(hydroxymethyl)methyl]amino)ethane-1-sulfonic acid sodium salt) was added to maintain the pH around 8. In all tests the growth was monitored daily by measuring the optical density at 750 nm (OD₇₅₀).

3.5 Influence of light in cellular metabolism

This chapter will explore the effects of different wavelengths on microalgal metabolisms. Various qualities of light have been screened for *C. sorokiniana* cultivation and the composition of biomass during the experiment was monitored. During these experiments *C. sorokiniana* was cultivated on wastewater by blending two different streams, as described in the following section.

3.5.1 Blending of Dairy wastewaters

All dairy wastewaters need to be diluted prior microalgal cultivation, thus DWW and DES have been selected to be used in this experimental set up. To do so, three different ratios of wastewaters were tested using Erlenmeyer's flasks maintained at 30°C, 130 rpm and 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Here DWW and DES were mixed using 25%, 50% and 75% v/v of DWW diluted with DES (from now named as: 1:3 DWW:DES; 1:1 DWW:DES; 3:1 DWW:DES). Cultures were daily monitored to measure the OD₇₅₀ and pH. Each condition was performed in triplicate.

3.5.2 Experimental design: different light regimes

A Multi-cultivator Photobioreactor MC 1000-OD (PSI, Photon System Instruments, Drásov, Czech Republic) was used for the following experiments and is shown in Figure 10. The system is a laboratory-scale photobioreactor designed for the cultivation of photosynthetic microorganisms and consists of eight independent cultivation vessels with individually controlled illumination. It enables accurate control of cultivation parameters, including light intensity and quality, temperature, and aeration, allowing the simultaneous cultivation of biological replicates or the comparison of different experimental conditions.

For this set of experiment *C. sorokiniana* (CCAP 221/8k) was inoculated into a balanced mixture of wastewaters from Dairy industry. The substrate was obtained by mixing 75% of washing waters and 25% of exhausted sludge from dairy wastewaters (as a result of previous screening - see 0). Every light condition tested had the same temperature, aeration, and inoculum size: 30°C, 150 mL min⁻¹ of enriched air (1% CO₂), and an OD₇₅₀ of about 0.5 respectively. Experiments have been conducted in two stages, first biomass accumulation took place over two days under white light at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and it was the same for all the trials. After that, once reached an OD₇₅₀ of ~2, for the next 4 days the light was shifted to: white light at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; white light at 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; red light (660 nm) at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and blue light (450 nm) at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (from here referred as: W300, W600, R300 and B300 respectively) resulting in 4 different experiments lasting 6 days each. A further experiment was conducted by using red light at 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for the initial phase and then blue light at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for the second phase (R100-B300).

For all these experiments in order to monitor the physiological status of cultures, periodic sampling was carried out to analyse the pigments, carbohydrates and proteins. Prior the analysis, the pellet from each sample were obtained by

centrifuging 0.5 mL of sample at 10000 rpm and then the pellet was stored at -80°C until analysis. Moreover, OD₇₅₀ was measured daily while samples for the analysis of total carbohydrate, pigments and proteins were collected at different time points. Biomasses at the end of each test were centrifuged and freeze dried prior to lipid analysis.

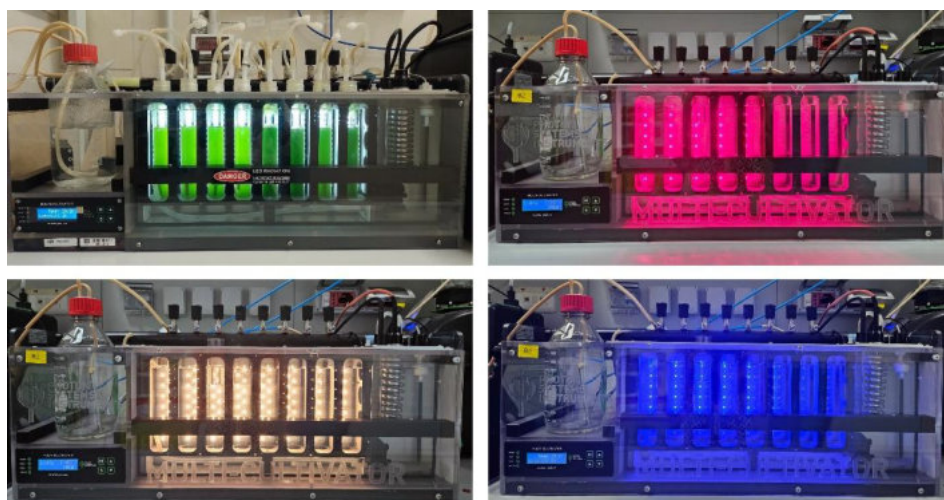


Figure 10: Multicultivator Photobioreactor MC 1000-OD (PSI, Photon System Instruments, Drásov, Czech Republic). Top-left photo reports 8 tubes with 80 mL of culture of *C. sorokiniana* as an example of the cultivation set up. Three different illuminations such as red light (top right), white light (bottom left) and blue light (bottom right) were used for microalgal cultivation. Temperature, aeration and illumination are precisely regulated by the software.

3.6 Two-step process: nutrient depletion for lipid accumulation

A two-step process was assessed with a first step of biomass accumulation and nutrient consumption followed by lipid accumulation using a species notable for its high lipid production: *Nannochloropsis oceanica*.

3.6.1 Experimental design

At Conway Institute, by UCD, Ireland, a two-step cultivation process was conducted using MDE as substrate for three microalgal species. For both steps, the cultivation conditions were kept constant: cultures were grown in 250 mL glass bottles at room temperature around 22°C, under continuous aeration with 100% air and illumination at 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ using LEDs. In the first step, *C. sorokiniana* (TCF-66g) and *T. obliquus* (CCAP 276/10) were cultivated separately and in co-culture in 200 mL of treated MDE for six days. The initial inoculum was adjusted to an OD₇₅₀ of approximately 0.3 for *C. sorokiniana* and 0.2 for *T. obliquus*; in the co-culture, the inoculum was prepared by mixing equal parts of each strain's monoculture inoculum. After cultivation, the biomass was harvested by centrifugation at 4000 rpm for 10 minutes, freeze-dried and stored for subsequent lipid analysis. The supernatant was collected and treated to adjust salinity (at 35 g L⁻¹) and pH (around 7.5) and then it was sterile-filtered to be used for the second step of the process. In the second step, *N. oceanica* was inoculated into each supernatant stream from the first step to reach an OD₇₅₀ of 2, and cultures were maintained for five days.

All conditions, for each step, were tested in triplicate. Daily samples were collected for flow cytometry analysis and for phosphate and ammonium quantification.

As reference condition, the standard medium BBM was prepared with modified concentrations of phosphorous and nitrogen to be equimolar to MDE concentrations. With the same conditions of working volume, light intensity, duration of the cultivation and inoculum size, the two-step process was performed using this modified BBM.

3.7 Valorization of a novel process stream

The utilization of microalgae for wastewater treatments is widely explored as we already discussed in previous chapters. Thus, to try something more innovative I selected a GFE derived from a fermentation process. Then, based on the composition of four wastewaters from dairy industry, the most suitable stream was blended to GFE and used for the cultivation of *C. sorokiniana* and *T. obliquus*.

3.7.1 Screening of alcohol tolerance and cultivation on GFE

Screenings of different ratio of wastewaters and toxicity of alcohols were conducted using Erlenmeyer's flasks with 30 mL working volume in a shaking incubator at 130 rpm, 30 °C and 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Eppendorf™ Innova™ S44i, Eppendorf, Hamburg, Germany). All tests were performed in triplicates.

The tolerance of *C. sorokiniana* (CCAP 221/8k) and *T. obliquus* (CCAP 276/3A) to alcohols present in the GFE, i.e. ethanol, butanol and hexanol (see Table 17), was tested by adding those alcohols to the standard media, maintaining the same ratios of the effluent (0.91 g L⁻¹ ethanol, 1.04 g L⁻¹ butanol, and 0.76 g L⁻¹ hexanol). At first, the complete set of alcohols was tested at the same concentration present in the effluent and doubled (from now referred as 1x and 2x respectively). After that, the mixture of only ethanol and butanol was tested at 1x and 2x concentrations. The inoculum size was set to obtain an initial OD₇₅₀ of 0.2 for both strains. Moreover, to ascertain whether microalgae can absorb alcohols and utilizing them for growth, we prepared two replicates of culture medium for each condition with same alcohol concentrations and put them in the same experimental set up without inoculating microorganisms, so that we could calculate evaporation and absorption where it occurred.

Another screening was performed in Erlenmeyer's flasks to evaluate the feasibility of the cultivation of microalgae on GFE. Increasing percentages of 50%, 75% and 100% v/v of GFE were diluted in deionized water, with pH adjustment at 6.75. As in previous tests, the inocula were performed to obtain an initial OD₇₅₀ of 0.2 for both strains.

3.7.2 Photobioreactor standard conditions and VFAs uptake

As comparative conditions, phototrophic, mixotrophic and heterotrophic cultivations have been tested using a Multi-cultivator MC 1000-OD (PSI, Photon System Instruments, Drásov, Czech Republic). With 80 mL of working volume per replicate, tubes were maintained at 30°C, aerated with CO₂ enriched air (1% CO₂) at 150 mL min⁻¹ with light intensity increased every 24 h from 60, 100, 200, 400 and 600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. As growth medium 3NBBM with 20 mM PIPES buffer (1,4-Piperazinediethanesulfonic acid) to maintain pH around 7 was

used and implemented with 1 g L⁻¹ of acetate (as sodium acetate) to obtain mixotrophic and heterotrophic metabolisms. To obtain heterotrophy, tubes were kept in darkness during the whole experiment. Precultures have been grown in the respective condition of phototrophy, mixotrophy and heterotrophy, in 100 mL Erlenmeyer's flasks, to obtain properly acclimated inoculum.

Additionally, *C. sorokiniana* was selected to perform a specific screening on the uptake of volatile fatty acids (VFAs) present in GFE. Here 50% of DES was diluted with 50% of 3N BBM and VFAs as acetate, butyrate and caproate were tested at 1.5 g L⁻¹, 0.75 g L⁻¹ and 1 g L⁻¹, respectively. Two pH buffers were used: 20 mM PIPES for pH 7 and 25 mM TES for pH 8. The addition of both acetate and butyrate was tested at both pH, while the entire mix, including caproate, was tested only at pH 7. Here the inoculum was performed to obtain an initial OD₇₅₀ of 0.2, and all experiments were tested in triplicates, with the overall parameters set at 30°C and constant light at 60 μmol photons m⁻² s⁻¹.

3.7.3 laboratory-scale up from flask to PBR with blended wastewater

A mixture made by 50% of GFE and 50% of DES (hereinafter referred to as 50:50 DES:GFE) was tested as cultivation medium for both microalgae. Firstly, the blend was tested for 9 days in 100 mL Erlenmeyer's flasks with 30 mL working volume in a shaking incubator set at 130 rpm and 60 μmol photons m⁻² s⁻¹ (Eppendorf™ Innova™ S44i, Eppendorf, Hamburg, Germany). The test was conducted in triplicates with an initial OD₇₅₀ of 0.2.

Then the same test was conducted only with *C. sorokiniana* in a MC1000-OD (PSI, Photon System Instruments, Drásov, Czech Republic) using the same temperature but implementing with enriched air (1% CO₂) and a gradual increase of light intensity from 60 to 600 μmol photons m⁻² s⁻¹. The increase was performed every 24 h as follow: 60, 100, 200, 400 and 600 μmol photons m⁻² s⁻¹ till the end of the experiment. The test was performed in triplicate.

3.8 Analytical methods

3.8.1 Determination of microalgal growth and biomass production

To create a correlation curve between dried biomass and optical density, 1L Erlenmeyer's flasks were used to cultivate in standard medium *C. sorokiniana* (CCAP 221/8k) and *T. obliquus* (CCAP 276/3A). For five sampling points between a wide range of OD₇₅₀ (from 0.5 to 3), optical density and dried weight were measured in triplicates. The resultant correlation curves were used to calculate the dried biomass during cultivation experiments. Thus, to monitor the growth of microalgae, samples for OD₇₅₀ measurement were collected and analyzed with a Jenway™ 315 spectrophotometer (Cole-Parmer Ltd., Staffordshire, UK). These data were used to calculate indirectly the dried biomass (gDW L⁻¹) using the following equations obtained from the correlation curves:

Eq. 1

$$g_{DW}L^{-1}/OD_{750} = 0.26 \pm 0.03$$

Eq. 2

$$g_{DW}L^{-1}/OD_{750} = 0.44 \pm 0.0$$

Eq. 1 was used to calculate the biomass production of *C. sorokiniana* while Eq. 2 for *T. obliquus*. To calculate the growth rate (d⁻¹) the trendline of the logarithmic plot of OD₇₅₀ was created, the slope corresponds to the Growth rate (μ).

To measure microalgal growth of *Chlorella sorokiniana* (TCF-66g), *Tetrademus obliquus* (CCAP 276/10) and *Nannochloropsis oceanica* (CCAP 849/10) the cell number was measured with flow cytometry. Samples were collected, fixed with paraformaldehyde (4% in phosphate buffered Saline, PBS) and stored at 4°C until analysis. Using Beckman Coulter CytoFlex flow cytometer (Beckman Coulter, USA) microalgae were analyzed based on their autofluorescence by exciting the samples with 350 nm laser and measuring the emissions at 450/40 nm. Populations of cells that showed autofluorescence were counted as microalgal cells and expressed as cell density: 10⁶ cell mL⁻¹.

3.8.2 Biomass composition: carbohydrate quantification

Around 1 mg of biomass for the determination of total carbohydrate composition, measured as glucose equivalent, was collected by centrifugation and stored at -4°C until analysis. The quantification was conducted as reported by (10.1016/j.ymben.2019.11.002). Briefly, cell pellet was resuspended with 200 μl of 3.5% v/v sulfuric acid and then boiled for 40 minutes. After 2 minutes of

centrifugation, 33 μ l of supernatant were transferred into a new tube and 117 μ l of 10% trichloroacetic acid were added. After 10 minutes of incubation at room temperature 750 μ l of 6% v/v 2-aminotoluene diluted in glacial acetic acid were pipetted into the tube. Samples were boiled for 10 minutes and then cooled down at room temperature prior absorbance read at 635 nm. A standard curve with glucose was prepared and used to calculate the concentration of carbohydrates of unknown samples.

3.8.3 Biomass composition: photosynthetic pigments quantification

The quantification of pigments (i.e. chlorophyll a, chlorophyll b and carotenoids) was conducted on 50-500 μ g of wet biomass by means of cold methanol extraction. Cell lysis was performed using a tissue-Lyser Retsch MM400. Cells were suspended with 1 mL of cold methanol and 200 mg of glass beads (0.25-0.50 mm) were added prior to perform three cycles of tissue-lyser of 5 min at maximum frequency (30 s⁻¹), tubes were kept in ice for two minutes and this step of tissue lyser was repeated a second time. After centrifugation at 12000 xg at 4°C the methanol was collected and the absorbance was recorder on Perkin Elmer UV/VIS Spectrometer Lambda 650 S between 800 and 400 nm. The pigments concentration was calculated using the following equations (Wellburn, 1994):

Eq. 3

$$Chl\ a\ (\mu g\ mL^{-1}) = 16.72 * (A_{665} - A_{770} - (9.16 * (A_{652} - A_{770}))$$

Eq. 4

$$Chl\ b\ (\mu g\ mL^{-1}) = 34.09 * (A_{652} - A_{730}) - 15.28 * (A_{665} - A_{730})$$

Eq. 5

$$Car(\mu g\ mL^{-1}) = 1000 * A_{470} - 1.63 * Chl\ a - \frac{104.96 * Chl\ b}{221}$$

3.8.4 Biomass composition: protein quantification

Protein content in biomasses was also quantified using Quantum Protein – Bicichoninic Protein Assay Kit was used (provided by Euroclone S.p.a., Italy) following the protocol provided by the manufacturer. In brief, 300 μ L of 0.2 M NaOH were added to around 0.5 mg of biomass and boil at 100°C for 30 minutes. Then 50 μ L of supernatant, obtained after centrifugation, were collected and 1 mL

of Working Reagent added. Samples were then incubated at 37°C for 30 minutes. After cooling down at room temperature the absorbance at 563nm was measured with a Jenway™ 315 spectrophotometer (Cole-Parmer Ltd., Staffordshire, UK). A standard curve was created with Bovine Serum Albumin and used to calculate the protein quantity in unknown samples.

3.8.5 Biomass composition: Lipid quantification

Lipid extraction, transesterification and analysis are conducted with slightly different protocols in different chapters, due to instruments availability.

The lipid profiles of *C. sorokiniana* (CCAP 221/8k) biomass grown at different light regimes were obtained after extraction, transesterification and GC-MS analysis, while other species were analyzed with GF-FID.

Extraction of lipid fraction from dried biomasses from Chapter 4.2 and 4.3 was conducted as follows. 1 mL of chloroform:methanol 4:5 v/v and 20 zirconium oxide beads (1.4 mm diameter) were added to a known amount of dried biomass (around 50 mg). 8 cycles of beat beater of 1 minute at maximum speed were performed with 2 minutes in ice between after every cycle. The total extract was poured into a 15 mL tube and the beat beater tube washed three times with 1 mL of chloroform:methanol 4:5 v/v solution. Tubes were vortexed and sonicated in a bath for 10 minutes. 2.5 ml of MilliQ water, containing 50 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) and 1 M NaCl, were added to each sample and another sonication of 10 minutes was performed. After that samples were vortexed and centrifuged at 4000 rpm for 10 minutes. After centrifugation three phases were separated. The upper aqueous phase is made of water and methanol and contains dissolved salts and water/soluble molecules. The intermediate phase is a pellet consisting of cell debris, while the lower organic phase is composed of chloroform extract. The upper phase was discarded while the lower phase was collected into a new tube. The extraction was repeated two times more. The extract was filtered with PTFE syringe filters to remove cell debris into a pre-weighed tube. The chloroform was completely evaporated under nitrogen gas stream at 40°C and the extract weighed.

The lipid extraction from biomass derived from Chapter 4.4 was performed following a different protocol modified from Blight and Dyer (DOI: 10.1139/o59-099). A mixture of chloroform:methanol:water 1:1:0.9 was added to around 20 mg of dried biomass, then the solution was mixed and vortexed. After centrifugation the lower phase was collected in a weighted tube. The extraction was repeated twice and the chloroform extract evaporated and weighted.

Samples of *C. sorokiniana* relative to chapter 4.2 and 4.4 were transesterified as follow. To obtain the Fatty acids methyl esters (FAME) profile, 300 µL of methanolic KOH 0.6 M were added to 10 mg of lipid extract. The solution was heated at 70°C for 10 minutes in water bath. After complete dissolution of the oil,

300 μL of 5% H_2SO_4 in methanol were added and the solution was heated at 70°C for 5 minutes. Then 2 mL of saturated NaCl solution and 2 mL of hexane were added. After centrifugation at 4000 rpm for 10 minutes the supernatant was collected and analysed with GC-MS. A gas chromatograph (Agilent 7890 A GC System, Agilent, Santa Clara, CA, USA) equipped with an Rxi-5 ms column ($30\text{ m} \times 0.25\text{ mm ID} \times 0.25\text{ }\mu\text{m}$; Restek, Bellefonte, PA, USA) and a quadrupole mass detector (Agilent 5975 C VL MSD, Agilent, Santa Clara, CA, USA) was used as previously reported (<https://doi.org/10.3390/microorganisms13050961>). The carrier gas was helium, set with a flow rate of 1 mL min^{-1} . The injection of $1\text{ }\mu\text{L}$ was performed in split mode with a ratio of 20:1 at 240°C . In the first 2 minutes the temperature of the column was set at 60°C and then increased of $10^\circ\text{C min}^{-1}$ until 200°C , then 5°C min^{-1} until 250°C and maintained for 7 minutes. The mass spectrometer operated under electron ionization at 70 eV in full-scan mode, covering a mass range from 50 to 600 a.m.u. Source and quadrupole temperatures were kept at 230°C and 150°C , respectively. Then FAMES were quantified using calibration curves developed using a FAME standard mixture (Sigma-Aldrich, St. Louis, MO, USA) and the ChemStation software (LTS 01.11) and NIST17 Library (<https://chemdata.nist.gov/>).

For the transesterification of samples derived from the two-step process (Chapter 4.3), round 300 mg of sodium sulphate anhydrous and 4 mL of internal standard solution ($\text{C:15 } 100\text{ }\mu\text{g mL}^{-1}$) in hexane were added to each tube. Once dissolved the extract, 4 mL of potassium methoxide in methanol 2M and 2 mL of HCl in methanol (20% w/w) were added. Tubes were thoroughly vortexed and transferred into a thermoblock at 70°C for 30 minutes. After that tubes are cooled down and centrifuged for 5 minutes at 4000 rpm. The upper phase was collected and analyzed with a gas chromatograph with a flame ionization detector (GC-FID) (Agilent Technologies, Ireland). Helium was utilized as the carrier gas in a 30-meter-long HP-5 capillary column (Agilent Technologies) with a split ratio of 10:1 and a flow rate of 2 mL min^{-1} . Using a four-step temperature method, which included a starting temperature of 50°C for two minutes, an increase to 150°C at $10^\circ\text{C min}^{-1}$ for two minutes, an increase to 185°C at $10^\circ\text{C min}^{-1}$ for two minutes, and a final climb to 300°C at $10^\circ\text{C min}^{-1}$ for two minutes, the FAMES were sorted according to their boiling points. The injection temperature was 280°C , while the detection temperature was set at 320°C . Retention-time comparison with a 37-component FAME standard mix (Sigma Aldrich, Ireland) was used to identify individual FAMES.

3.8.6 Nutrient consumption of MDE: analysis on supernatant

Samples of supernatant were analyzed for phosphate, ammonia and protein content using the following protocols. Reagent set for orthophosphate (PO_4^{3-}) determination was purchased by HACH. Briefly, $20\text{ }\mu\text{L}$ of reagent 1 (amino acid

reagent) and 20 μL of reagent 2 (molybdate reagent) were added to 200 μL of sample into a 1.5 mL tube. The tube was vortexed for 10 seconds and incubated at room temperature for 10 minutes. 200 μL of each reaction were pipetted into a 96-well plate and the absorbance at 530 nm read with a spectrophotometer. A standard curve was performed to calculate the concentration in each sample.

To quantify ammonia, HACH PERMACHEM® reagents were used: Ammonia Sylicate Reagent and Ammonia cyanurate. The powder of both reagents was dissolved in Millipore water and 100 μL of first reagent and 10 μL of second reagent were added to 200 μL of unknown samples and standard solutions appropriately prepared. The tubes were vortexed for 10 seconds and incubated for 15 minutes before transferring 250 μL of each into a 96-well plate. The absorbance was read at 655 nm.

API® nitrate kit test was used to quantify NO_3^- in the standard medium. To do so, 500 μL of unknown samples were transferred into a new 1.5 mL tube and 30 μL of Nitrate test solution 1 added. The tube was inverted several times and then 30 μL of Nitrate solution 2 were added and the tube thoroughly vortexed. After 5 minutes incubation at room temperature 250 μL of were transferred from each sample into a 96-well late reader and the absorbance measured at 545 nm. The standard Nitrate solution was opportunely diluted to prepare a standard curve.

The determination of Chemical Oxygen Demand (COD) was performed using the kit LCK514 (HACH), that has an interval of 100-2000 $\text{mg L}^{-1} \text{O}_2$. Cuvettes provided by the kit are gently inverted to suspend sediments. 2 mL of sample are added to the cuvette and then maintained for 2 hours at 148°C. After cooling down the cuvette, the result is measured with a spectrophotometer DR 1900.

3.8.7 VFAs and alcohol measurement via HPLC

The contents of ethanol, butanol, hexanol, caproic acid, butyric acid, and acetic acid of GFE were measured in accordance with Antonicelli et al. (2025). A Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) provided with a "Metab-AAC BF" series column (length = 300 mm; ID = 7.8 mm) (Isera, Düren, Germany) was used for quantification. The oven was set to 60 °C, and the elution buffer was 9 mM H_2SO_4 at a flow rate of 1 mL min^{-1} . UV-Vis (210 nm) and refractive index detectors were used to identify VFAs and alcohol, respectively. All of the calibration compounds were of HPLC quality and were acquired from Merck (Darmstadt, Germany).

3.9 Biodiesel quality

Fatty acids methyl esters profile was assessed to determine the quality of biodiesel through empirical calculations.

In this work the quality of biodiesel was determined by calculating those parameters using FAME profiles. IV, SV, CN, LCSF and CFPP were calculated for each sample using the following (Francisco et al., 2010; Nascimento et al., 2013):

Eq. 6

$$IV = \frac{\sum(254 * D * N)}{M}$$

Eq. 7

$$SV = \frac{\sum(560 * N)}{M}$$

Eq. 8

$$CN = 46.3 \frac{5458}{SV} - (0.225 * IV)$$

Eq. 9

$$LCSF = 0.1 * C16 + 0.5 * C18 + 1 * C20 + 1.5 * C22 + 2 * C24$$

Eq. 10

$$CFPP = (3.1417 * LCSF) - 16.477$$

Where IV is Iodine Value, SV is Saponification Value, CN is Cetane Number, LCSF is Long-Chain Saturated Factor, CFPP is Cold Filter Plugging Point, D is the number of double bonds, N is the percentage of a methyl ester of fatty acids and M is the molecular mass of each component. Moreover, LCSF is calculated using the percentage of each methyl ester of saturated fatty acid.

3.10 Statistical analysis

GraphPad Prism software (version 10.1.2; GraphPad Software, San Diego, CA, USA) was used for statistical One-way and Two-way analysis of variance (ANOVA). A statistically significant value was considered with a *p*-value < 0.05.

Chapter 4

Results and discussion

This section is organized into five Chapters that collectively report and discuss the main findings of the present thesis. First, Chapter 4.1 evaluates, by means of preliminary cultivation screening, how *C. sorokiniana* and *T. obliquus* respond to cultivation when dairy wastewater is used as nutrient source under real conditions. This chapter is fundamental in assessing the feasibility of using wastewater from this industrial sector as a cultivation medium, thus laying the groundwork for further studies. This aspect is particularly relevant, as all strategies investigated in this project (see Chapters 4.2, 4.3 and 4.4) have been performed by cultivating microalgae exclusively with these effluents, used either individually or as blends, without the addition of fresh water or chemical supplements.

Then three strategies have been assessed to evaluate lipid productivity and profile under different conditions. More in detail, Chapter 4.2 investigates how different light regimes influence growth, biomass composition and lipid biosynthesis in microalgae grown on selected wastewaters, with particular attention to biodiesel-relevant traits. Since physical parameters strongly influence microalgal behavior, understanding the impacts of such critical factors represents a significant advantage in biotechnological process development.

Chapter 4.3 introduces a two-step cultivation strategy separating biomass accumulation from lipid induction to enhance overall productivity and fuel quality. This experimental design is based on the assumption that one of the main factors that stimulate lipid accumulation is nitrogen limitation. Specifically, the aim of this approach was to decrease nitrogen and phosphate content of wastewater by initially growing *C. sorokiniana* and *T. obliquus* on MDE and then, after biomass harvesting, using the effluent for a N-depleted cultivation of *N. oceanica*.

Chapter 4.4 investigates the exploitation for algal growth of an innovative carbon rich-wastewater, i.e. gas fermentation effluent (GFE), examining microalgal tolerance, organic substrate assimilation, integration with complementary streams (dairy effluents) and scale-up performance. More in detail, GFE was assessed for cultivation of both *T. obliquus* and *C. sorokiniana* and the latter was chosen for subsequent experiments to optimize the biotechnological process (blend with dairy WWs), scale up the process and characterize the lipid profile. Part of these outcomes has been published in Mazzocchi et al., (2025).

Finally, Chapter 4.5 compares biodiesel quality obtained from the aforementioned cultivation processes, to identify the most effective route for high-performance biofuel generation.

4.1 Chapter 4.1: wastewaters as substrate for microalgal cultivation

4.1.1 Aim of the chapter

In this chapter, dairy exhausted sludge (here DES), dairy washing waters (DWW) and scotta (SC) collected by a local dairy industry have been treated, analyzed and screened for microalgal cultivation. The aim of the study was to determine which species is more suitable for biomass accumulation, between *Chlorella sorokiniana* and *Tetrademus obliquus* and select the best one for further experiments. A deep analysis and discussion on the composition of these wastes is carried out. This screening lets us understand which wastewater is better for different purposes. Moreover, the cultivation of *C. sorokiniana* was performed in three different metabolic modes (phototrophic, mixotrophic and heterotrophic) using standard medium, as references for biomass and lipid production under controlled conditions. Biodiesel quality was also evaluated and discussed.

4.1.2 Hydro-chemical analysis of three dairy wastewater and ammonium tolerance

The main macronutrient for microalgal growth is nitrogen, which is present as ammonium in tested streams measuring 370, 90, and 4.4 mg L⁻¹ NH₄⁺ respectively in DES, SC, and DWW. Considering that streams and standard media present different forms of nitrogen, is not correct to directly compare their concentrations. In fact, ammonium is more available to microalgae than nitrate, as it does not require enzymatic reduction prior to assimilation (González et al., 1997; Wang et al., 2016). Nevertheless, tolerance to ammonium is species-specific, and high ammonium levels can be toxic for many microalgal species. To deeper understand the behavior of the strains used in this study, the ammonium tolerance was tested (Figure 11). It was revealed that *C. sorokiniana* and *T. obliquus* grow well in the range 100-200 mg L⁻¹ of ammonium, while lower or higher concentrations cause a decline in biomass productivity. Previous studies from literature report that *C. pyrenoidosa* showed toxicity from 110 mg L⁻¹ (Zhao et al., 2019), while better resistance was observed with *C. vulgaris*, where a negative effect of ammonium was recorded at 350 mg L⁻¹ (De Lourdes et al., 2017). More in detail, in this assay 50, 100, 200 and 500 mg L⁻¹ of ammonium have been tested (Figure 11). *C. sorokiniana* (Figure 11a) showed good performances with 100 and 200 mg L⁻¹ of ammonium, while at 50 and 500 mg L⁻¹ the growth was slower and started the stationary phase from day 12 and 17 respectively. These findings are consistent with previous studies, where *C. sorokiniana* showed a slight decrease of growth rate as ammonium increases from 50 to 400 mg L⁻¹ (Ogbonna et al., 2000). Similarly, *T. obliquus* (Figure 11b) grows well with 100 and 200 mg L⁻¹ of

ammonium, producing higher biomass at 100 mg L⁻¹. With more than 100 mg L⁻¹ it grows slowly, but still more than the control condition with normal BG11, which contains sodium nitrate as nitrogen source (Figure 11). In summary, ammonium concentration of 50 mg L⁻¹ is limiting for both strains, while 500 mg L⁻¹ inhibits the algal growth if compared to 100 and 200 mg L⁻¹. Both strains, especially *T. obliquus*, are tolerant to high concentrations of ammonium, endorsing their suitability for wastewater remediation. Nevertheless, if we compare the test results with the effluent hydro-chemical analysis, it is evident that ammonium concentration of SC is near the optimal range, while DES and DWW are above and below it respectively, making the former potentially toxic and the latter limiting. Besides nitrogen, phosphorus is an important macronutrient for microalgal growth and should be considered in comparison with nitrogen as N/P ratio, which is well demonstrated that affects the growth, metabolisms and consequently the composition of biomass (Qian et al., 2024). The concentration of phosphates in wastewater is also highly variable, being potentially balanced for DES and DWW while particularly high for SC (Table 1). Nevertheless, the N/P ratio (molar) is lower than 1 for DWW and SC while DES measures 32.5, comparable to 3N BBM and BG11 with 8.8 and 17.6 respectively. In this case, the ratio is low for SC due to the high concentration of phosphorus while DWW has low concentration of nitrogen. In fact, it was determined a range in between microalgae can grow well, from 8 to 45, clearly it depends on the species (Qian et al., 2024; Whitton et al., 2016). Since nitrogen is essential for cell growth and protein synthesis, it has been shown that its uptake is independent of phosphorus accumulation. Conversely, the concentration of nitrogen affects phosphorus accumulation (Beuckels et al., 2015). Once again demonstrating their great adaptability and versatility, microalgae can alter their cellular composition in response to the availability of P and N in the medium (Whitton et al., 2016).

Total organic carbon (TOC) present in streams varies from 78, 803 and 22892 mg L⁻¹ for DES, DWW and SC, respectively. Organic carbon is an important source both for improving growth by activating mixotrophic metabolism, and to mitigate the high concentration of ammonium. In fact, it has been recently demonstrated that the presence of available organic carbon can reduce toxicity of ammonium (Sutherland, 2022), however too high TOC levels can inhibit growth. To confirm this, when cultivated on piggy wastewater, *C. sorokiniana* can grow and consume TOC at around 200 and 400 mg L⁻¹ while *Scenedesmus obliquus* consumed TOC only at the lower concentration (Godos et al., 2010). For instance, DES in this study has low TOC, while DWW has medium-high TOC which means that it can be more difficult to promote microalgal cultivation on DWW. Conversely, SC has extremely high TOC levels, which could be harmful to many species. Despite these findings, it must be mentioned that all these streams can be combined with others to add sources of nutrients and/or to dilute toxic components.

Table 1: Hydro-chemical analysis of dairy wastewaters compared with standard media BG11 and 3N BBM. The composition of Dairy Exhausted Sludge (DES), Scotta (SC) and Dairy Washing Waters (DWW) are reported. COD indicates Chemical Oxygen Demand and TOC stands for total organic carbon.

CHEMICAL	mg L ⁻¹				
	DES	SC	DWW	3N BBM	BG11
COD (mg L ⁻¹ O ₂)	/	49600	555	/	/
TOC	78.20	22892	803	/	/
NH ₄ ⁺	370	90	4.40	/	/
NO ₃ ⁻	<4.5	<4.5	8.18	547	1094
CLHORIDE	246	8370	256	27	19
SULFATE	24.8	129	<20	29	29
PO ₄	<60	1680	43.6	153	21.8
K	56	1110	9.5	75.8	17.95
Ca	46	960	36	12	9.8
Fe	0.196	0.083	0.163	0.12	0.84
Mg	11.70	115	7.7	7.40	7.39
Mn	<0.01	<0.01	<0.01	0.067	0.5
Mo	/	0.021	<0.01	0.011	0.16
Zn	/	0.786	0.044	0.008	0.05

Table 2: Analytical methods for chemical analysis on dairy wastewaters

CHEMICAL	METHOD
COD	ISO 15705:2002
TOC	UNI EN 1484:1999
NH₄⁺	UNI 11669:2017
NO₃⁻	UNI EN ISO 10304-1:2009
CLHORIDE	UNI EN ISO 10304-1:2009
SULFATE	UNI EN ISO 10304-1:2009
PO₄	UNI EN ISO 10304-1:2009
Ca	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Fe	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Mg	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Mn	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Mo	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Zn	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009

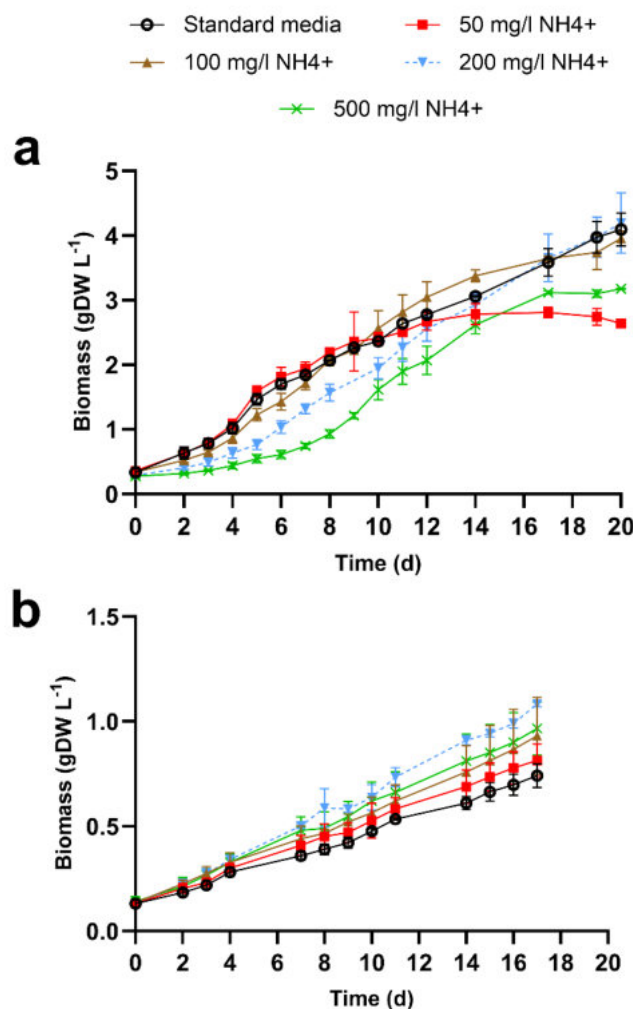


Figure 11: Growth curves of *C. sorokiniana* (a) and *T. obliquus* (b) at different ammonium concentrations. Each point is the average of three biological replicates, and error bars represent SD

4.1.3 Screening of cultivation on Dairy Exhausted Sludge

C. sorokiniana and *T. obliquus* were cultivated at different dilutions of exhausted sludge (DES). Both strains showed the highest biomass productivity at 50% (Figure 12). However, *C. sorokiniana* outperformed *T. obliquus*, reaching 0.45 gDW L⁻¹ after 8 days, exceeding the productivity achieved in standard media too. By contrast, *T. obliquus* reached 0.40 gDW L⁻¹, which was lower than in BG11 (Figure 12b). When DES concentration increased to 75%, *T. obliquus* growth was inhibited, whereas *C. sorokiniana* maintained growth performances comparable to the control, although with lower productivity than at 50%, indicating a great adaptability to DES. A similar trend was previously reported for *C. sorokiniana* cultivated in dairy wastewater, where maximum productivity occurred at 50% dilution and declined at higher concentrations (Kusmayadi, Lu,

et al., 2022), while *C. vulgaris* exhibited higher productivity at 75% in a recent study by Khalaji and coworkers (Khalaji et al., 2023). In the present study, biomass productivity at 50% and 75% of DES were 0.045 and 0.030 gDW L⁻¹ d⁻¹ for *C. sorokiniana*, and 0.030 and 0.010 gDW L⁻¹ d⁻¹ for *T. obliquus* (Table 3). Conversely, *Scenedesmus obliquus* has been reported to produce more biomass than *C. vulgaris* when cultivated on synthetic dairy sludge (de Mendonça et al., 2022). Both strains showed no growth cultivated on 100% of DES, suggesting that the stream contains either compounds that may inhibit or be toxic to algal growth at higher concentrations or insufficient nutrients. Based on the composition of DES, the high ammonium content (370 mg/L) is probably the primary inhibitory factor (Table 1). Ammonium levels in wastewater can, in fact, have a significant species-specific impact on microalgae growth, as demonstrated by a recent study in which various *Chlorella sorokiniana* strains displayed distinct behaviors (Song et al., 2024). Overall, these findings highlight that the optimal wastewater ratio strongly depends on its specific composition, which varies across countries and processing facilities. Consequently, process conditions must be adapted to each individual wastewater stream.

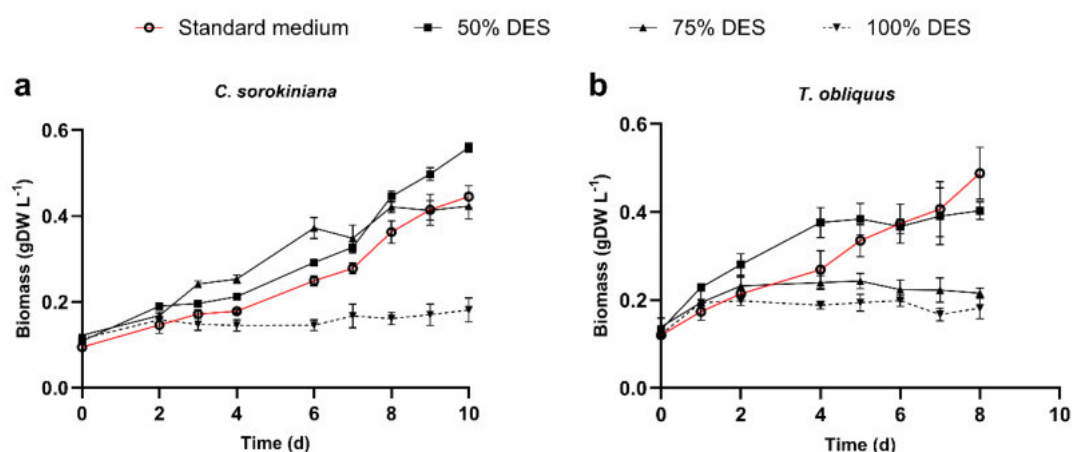


Figure 12: *C. sorokiniana* (a) and *T. obliquus* (b) cultivated on different dilutions of DES diluted with standard medium. Each point is the average of three biological replicates, and error bar represent SD.

Table 3: Biomass productivity of *C. sorokiniana* and *T. obliquus* cultivated on different dilutions of DES. Each value is reported and average of three biological replicates (Avg) and standard deviation (SD) is reported.

Substrate	g L ⁻¹ d ⁻¹			
	<i>C. sorokiniana</i>		<i>T. obliquus</i>	
	Avg	SD	Avg	SD
Standard media	0.035	0.003	0.046	0.006
50% DES	0.045	0.001	0.034	0.002
75% DES	0.030	0.003	0.010	0.002
100% DES	0.007	0.003	0.007	0.003

4.1.4 Screening of cultivation on DWW

The effect of different dairy washing-water (DWW) dilutions, performed with standard medium (3N BBM), on the growth of *C. sorokiniana* and *T. obliquus* has been evaluated by monitoring biomass production and pigment content. For both strains, the exploitation of DWW stimulated higher biomass production compared to standard media (Figure 13a,b) particularly *C. sorokiniana* biomass production was strongly influenced by DWW dilution rates. The highest biomass was achieved with 75% DWW, followed by 50% and 100%, which resulted in biomass accumulation similar to those obtained in control condition, performed in synthetic medium (3N BBM) (Figure 13a). After 10 days of cultivation, *C. sorokiniana* reached 0.51, 0.70, 0.82 and 0.54 gDW L⁻¹ of biomass when cultivated on 3N BBM, 50% DWW, 75% DWW and 100% DWW, respectively. Growth kinetics revealed different trends among dilutions. In 100% DWW *C. sorokiniana* grows faster during the first two days followed by an arrest of the growth, with a prolonged plateau phase for the rest of the cultivation, whereas other dilutions stimulated a slower but constant growth (Table 4; Figure 13a) In contrast *T. obliquus* showed the same productivity at different percentages and after 11 days of cultivation reached 0.92, 0.93 and 0.87 gDW L⁻¹ of biomass for 50%, 75% and 100% respectively (Figure 13b; Table 4).

Table 4: Biomass productivity of *C. sorokiniana* and *T. obliquus* grown on different ratio of DWW. Data are shown as average and standard deviation.

Substrate	g L ⁻¹ d ⁻¹			
	<i>C. sorokiniana</i>		<i>T. obliquus</i>	
	Avg	SD	Avg	SD
Standard media	0.029	0.000	0.036	0.005
50% DWW	0.043	0.003	0.069	0.008
75% DWW	0.053	0.002	0.071	0.002
100% DWW	0.030	0.002	0.066	0.007

Despite comparable biomass accumulation, clear physiological differences were observed for both strains when cultivated on 100% DWW. Cultures grown in undiluted DWW appeared visibly taken on a yellowish color as shown in Figure 13e,f, suggesting alteration in pigment composition. To investigate this effect, pigments content (i.e. chlorophyll a and b and carotenoids) was quantified at the beginning and at the end of cultivation. Chlorophyll a is the principal photosynthetic pigment for light harvesting in microalgae and its content is used as indicator of growth and parameter to evaluate photosynthetic activity (Arora & Philippidis, 2021).

Chlorophyll a concentration (µg mL⁻¹) varied markedly depending on the substrate. In fact, the biomass increase in standard media, 50% DWW and 75% DWW corresponds to higher chlorophyll a at the end of the cultivation (Figure 13c). On the other hand, with 100% DWW chlorophyll a content did not increase

in *T. obliquus* and decreased in *C. sorokiniana* (Figure 13c,d), despite biomass accumulation. The increased pigment production observed at 50% and 75% DWW suggests that dilution with standard medium provides a more balanced nutrient composition compared to 100% DWW, especially for nitrogen and phosphorous content, supporting the synthesis of photosynthetic pigments (Talha et al., 2025). In contrast, the decrease in pigment content in 100% DWW can be attributed to low nitrogen content - 4.4 mg L⁻¹ in DWW as reported in Table 1-, which is essential for pigments synthesis. Similar result was reported by Liu and colleagues when *C. vulgaris* was subjected to nitrogen limitation conditions resulting in lower pigment production with yellowish color of the cultures (Liu et al., 2022) such as happened in the present study (Figure 13e,f).

Despite low pigment production, biomass was accumulated, this phenomenon is likely due to the presence of organic carbon in DWW (Table 1). Organic carbon, in fact, stimulates mixotrophic metabolism enhancing biomass production while reducing photosynthesis compared to phototrophic metabolism, which explains the lower pigment production at 100% DWW (Arora & Philippidis, 2021; Deng et al., 2019). In fact, organic carbon is used instead of CO₂ fixation to produce biomass resulting in lower photosynthetic pigments production (Chapman et al., 2015). This behavior has been well demonstrated by Zheng and colleagues, who added increasing concentrations of sodium acetate to the substrate and observed higher biomass accumulation and lower Chlorophyll a content (Zheng et al., 2022). This scenario highlights that *C. sorokiniana* and *T. obliquus* could effectively assimilate exogenous organic carbon present in DWW to grow in biomass (Gupta et al., 2016; Kumar et al., 2018; Pereira et al., 2024; Wang et al., 2024). Furthermore, the reduction in photosynthetic pigments has been associated not only with the consumption of organic carbon, preferred over the assimilation of inorganic carbon through photosynthesis, but also with a reduction in the light availability, due to high biomass concentration and therefore the well-known self-shading effect (Yan et al., 2012). In this study self-shading cannot be the cause of low pigments accumulation because the biomass at the end of the cultivation of *C. sorokiniana* in 3N BBM and *T. obliquus* in 50% and 75% DWW, were comparable to the ones in 100% DWW, but only in 100% DWW low pigments accumulation occurred. In other words, the same biomass concentration, obtained with different substrates, did not affect the pigment production, thus low pigment concentration is not due to self-shading but to DWW composition.

In conclusion, biomass accumulation occurred in all dilutions tested, even at 100% DWW, with the best results at 75% DWW. The utilization of 100% DWW drove to lower biomass accumulation with lower pigments content, due to the presence of high COD and limitation of nitrogen. Indeed, high COD can trigger oxidative stress in microalgae by stimulating the accumulation of reactive oxygen species (ROS) (Hamed et al., 2017).

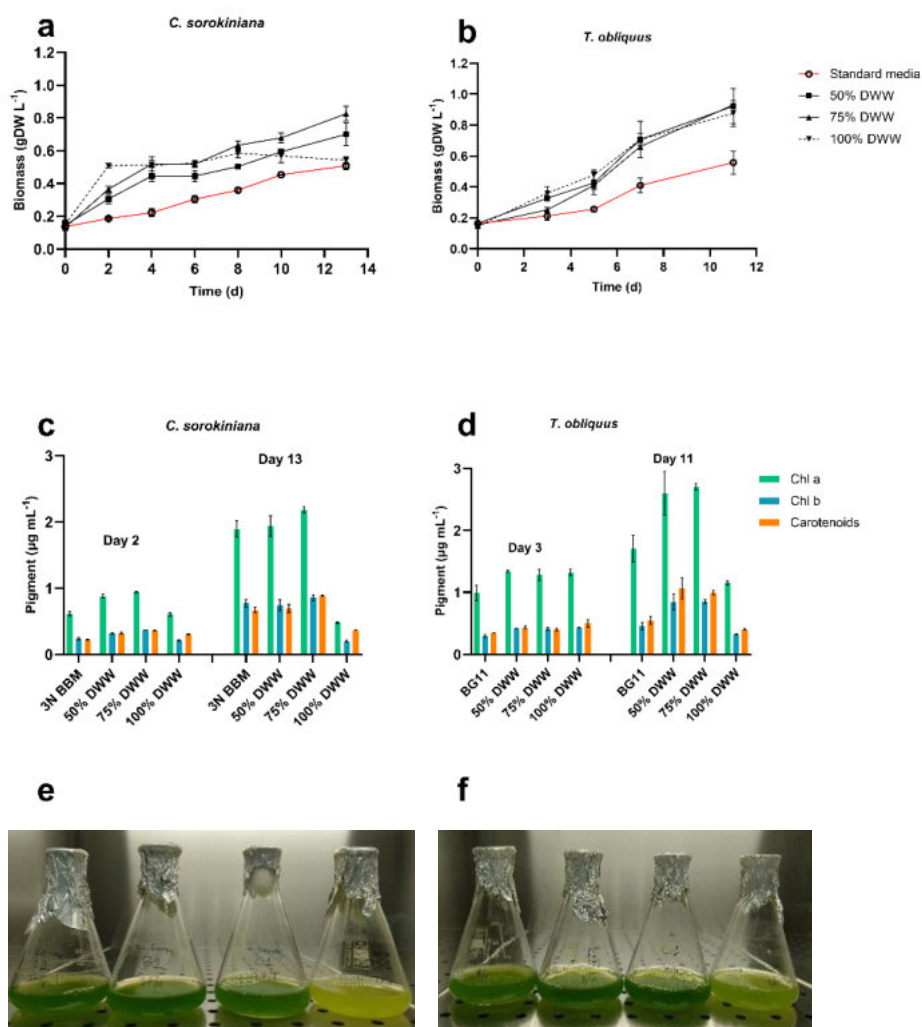


Figure 13: Biomass production of *C. sorokiniana* a) and *T. obliquus* b) grown on different percentages of DWW. Below graphs report chlorophyll a, b and carotenoids quantification, respectively for *C. sorokiniana* c) and *T. obliquus* d). e) and f) are pictures of one biological replicate for each dilution rate of DWW: from the left: control in standard medium, 50% DWW, 75% DWW and 100% DWW respectively for *C. sorokiniana* e) and *T. obliquus* f).

4.1.5 Screening of cultivation on SC

The cultivation of two strains was conducted on different concentrations of Scotta (SC). This specific effluent needs a prior pH adjustment to allow proper microalgal cultivation because it is around 5.5. After augmentation of pH around 7 the substrate showed an increased turbidity over the cultivation days, probably due to precipitation of salts. The presence of solids in the substrate may affect the growth of microalgae, due to reduction of both light penetration and nutrients availability. In this context, biomass accumulation and pigment content were

analyzed to better understand the effects of this wastewater on microalgal metabolism.

Similar biomass production trends were observed under 50% and 75% SC for both strains, with lower biomass values recorded at highest SC rates (Figure 14a,b). When 100% SC was supplied, biomass accumulation comparable to the control condition in standard medium was observed for both *C. sorokiniana* and *T. obliquus*. However, *T. obliquus* exhibited 3- and 2.2-times higher biomass production with 50% and 75% SC compared to BG11, highlighting distinct adaptation capacity in different strains (Figure 14a,b). This capacity of growing better on SC may be attributed to the great tolerance to COD of *T. obliquus*. In fact, SC recorded the highest COD of wastewater used, and even in 100% DWW, *T. obliquus* was found to be more resistant than *C. sorokiniana* (Figure 13).

Improvements in terms of biomass accumulation were reported by Ribeiro and colleagues, when *Chlorella protothecoides* was cultivated on 30% of scotta diluted in BG11 (Ribeiro et al., 2017). Here the higher biomass accumulation can be explained by both lower percentages of scotta (30% v/v), thus reducing the stress effect, and higher concentration of nitrogen in their wastewater, which was 0.11% (w/v), finding the optimized condition to cultivate *C. protothecoides* in scotta. Moreover, similarly to our results, the increase in SC percentages from 45% to 57% drove a slightly reduction in biomass accumulation of *Galdiera sulphuraira* (Russo et al., 2021). These differences highlight the variability of wastewater derived from different industries, even if the stream derives from the same industrial process. In general, these results confirm that the sustainable application of biotechnological processes requires targeted adaptation to the wastewater involved.

Table 5: Biomass productivity of *C. sorokiniana* and *T. obliquus* with different dilutions of SC. data are shown as average and standard deviations.

Substrate	g L ⁻¹ d ⁻¹			
	<i>C. sorokiniana</i>		<i>T. obliquus</i>	
	Avg	SD	Avg	SD
Standard media	0.037	0.009	0.041	0.003
50% SC	0.046	0.005	0.196	0.013
75% SC	0.037	0.004	0.156	0.006
100% SC	0.033	0.002	0.058	0.002

This wastewater stimulated different behaviors on both species, similarly to the observations with DWW. Figure 14e,f shows how higher concentrations of SC highly affected the metabolisms of microalgae, which clearly developed different pigment compositions. Thus, pigments have been quantified.

C. sorokiniana cultivated on 50% SC increased from 0.59 to 0.64 gDW L⁻¹ of biomass from day 2 to day 11, while at 75% SC decreased from 0.61 to 0.56 gDW L⁻¹ of biomass. These variations are consistent with Chl a which for 50% SC at the end of the cultivation is 1.5 times the amount of day 2, while almost no

variations were observed at 75% SC (Figure 14c). In contrast, with 100% SC biomass was 0.41 and 0.52 gDW L⁻¹, respectively at day 2 and 11, while Chl a content did not change (Figure 14a,c). The difference in chlorophyll content can be better understood when results in 3N BBM, as control condition, are compared to the ones in SC. In fact, Chl a content of *C. sorokiniana* at the end of the cultivation in standard medium is 1.5, 2.1 and 2.7 times higher if compared to biomass grown on 50%, 75% and 100% SC. Notably, the biomass at the end of the cultivation has the same values for all conditions. This means that at higher concentrations of SC, *C. sorokiniana* synthesizes less pigments.

T. obliquus recorded a different pigment production pattern, which is more stable within dilutions tested. At the end of the cultivation in BG11 and in 100% SC, a similar biomass and pigment content was measured, meaning that the color of the culture is likely due to the presence of precipitates, which contribute mainly affecting the turbidity (Figure 14d,f). Lower percentages of SC instead, drove to lower pigment content in biomass, more similar to the result of *C. sorokiniana*.

Moreover, for both strains, the higher the SC percentages, the lower the ratio Chl a/carotenoids. In detail, *C. sorokiniana* reported ratios of 2.9, 1.8, 1.4 and 1.3, respectively for 3N BBM, 50%, 75% and 100% SC. While *T. obliquus* measured 2.7, 2.4, 1.9 and 1.9, respectively for BG11, 50%, 75% and 100% SC. The decrease of the ratio is due to higher carotenoids content, which are antioxidant molecules responsible for the protection from ROS (Reactive Oxygen Species) which are produced under stress conditions like nutrient depletion (Arora & Philippidis, 2021). Thus, similarly to what observed with DWW, the increase in SC percentage drives to an induced stress condition, due to low nitrogen content and it is emphasized by salt precipitations.

The utilization of SC as growth substrate for microalgae resulted to be feasible but some aspects must be considered. In particular, nitrogen limitation affects the metabolism of microalgae. Furthermore, pH adjustment is required, because it is too low for microalgal cultivation. During pH adjustment, is important to pay attention to the potential precipitation of salts and saponification of fatty acids present in this specific wastewater, which can highly affect the turbidity of substrate.

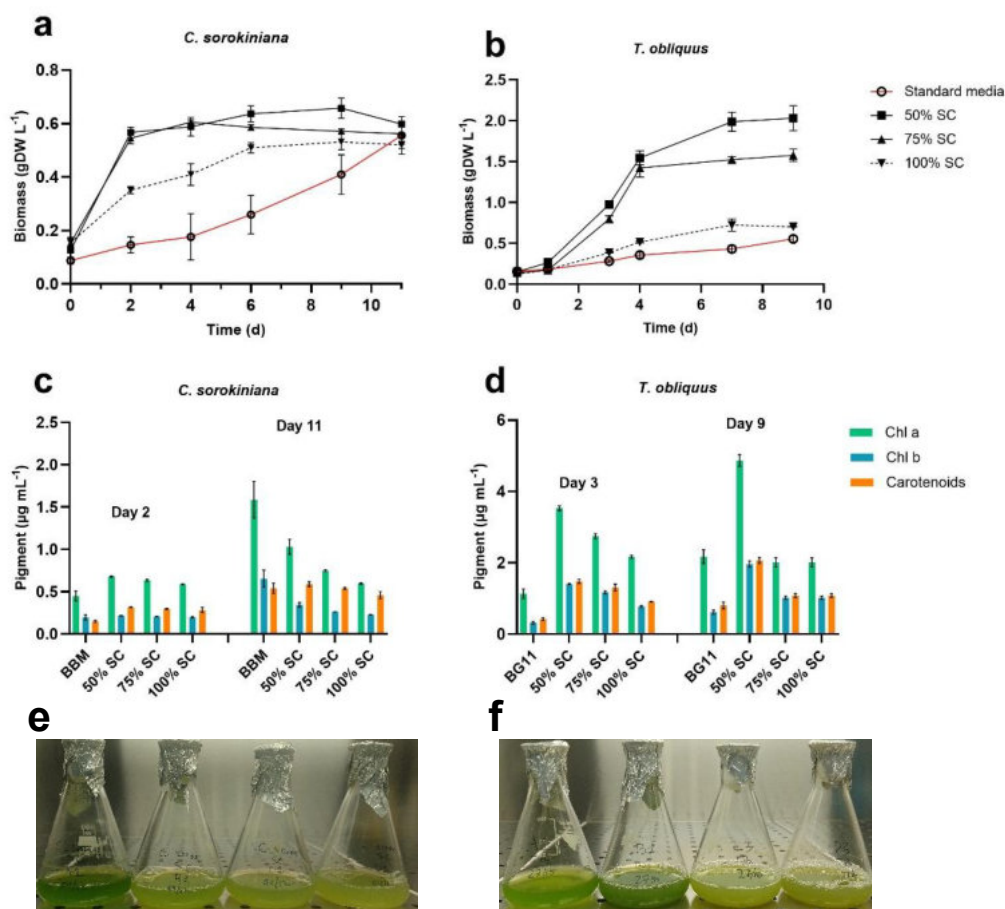


Figure 14: Growth curves of *C. sorokiniana* a) and *T. obliquus* b) on different dilutions of SC are compared to pigment contents at the beginning and at the end of the cultivation, for *C. sorokiniana* and *T. obliquus* respectively in c) and d). Picture of flasks for all conditions are reported in order: standard media, 50%, 75% and 100% SC, for *C. sorokiniana* e) and *T. obliquus* f) respectively.

4.1.6 Critical aspects of screened wastewater for microalgal growth: evaluation of most suitable stream

Three distinct streams from dairy industry were screened for microalgal cultivation and different behaviors were observed in two strains tested. Each wastewater, when properly diluted, is suitable for microalgal growth, although specific precautions were needed.

DES promoted a constant and linear growth pattern for both strains when properly diluted with standard medium. However, it resulted in the lowest biomass accumulation, if compared to SC and DWW. A key advantage of this stream is its pH level (around 8), which is suitable for most strains of microalgae. Moreover, no turbidity formation was observed during cultivation, which is important to avoid shading effect on microalgae, with the consequent reduction of

light availability. Nevertheless, the high levels of ammonium in DES require proper dilution before use.

Both DWW and SC exhibited turbidity development during cultivation, which was more pronounced in SC. This aspect is crucial when dealing with photosynthetic microorganisms, as it may reduce light penetration and consequently limit growth. Further investigation into the chemical mechanisms underlying this phenomenon would help to optimize cultivation conditions. Moreover, DWW promoted higher biomass production in *C. sorokiniana* compared to SC, with more constant increase, although SC led to faster initial biomass accumulation. In contrast, SC drove to the highest biomass accumulation in *T. obliquus*, demonstrating its ability to grow on high COD substrates. Nevertheless, the low nitrogen content of SC likely limits the long-term biomass accumulation, and may also affect the remediation efficiency, given that nitrogen is a key macronutrient required for the consumption of other compounds.

In this context DES and DWW appear more suitable for sustain microalgal cultivation, due to their balanced composition and transparency. Although SC can represent a valuable feedstock for certain species (i.e. *T. obliquus*), careful management of turbidity, pH and nutrient availability is necessary to endure stable growth. Moreover, SC was discarded for further analysis because it is currently valorized as source of fat and protein for animal feed, making the recovery and valorization of DES and DWW more strategically relevant.

4.1.7 *C. sorokiniana* cultivation on standard medium: screening of three metabolic conditions

Considering the previous screening and the different growth rates of two species, *C. sorokiniana* was selected for a screening to deeper understand the influence of growth conditions on biomass accumulation and lipid productivity and profiles. Here, phototrophy, mixotrophy and heterotrophy have been assessed. According to each metabolism organic (acetate) and/or inorganic (CO₂) carbon was used for cultivation.

Mixotrophic condition, where both organic and inorganic carbon sources are supplied, recorded the highest growth rate of 1.04 d⁻¹, while autotrophic and heterotrophic were similar to each other, with 0.86 and 0.87 d⁻¹ respectively (Figure 15a). This result is in accordance with literature (Castillo et al., 2021; Miazek et al., 2014), confirming that mixotrophic cultivation is the best condition for *C. sorokiniana*. A maximum biomass production of 1.15, 1.48 and 0.64 gDW L⁻¹ was obtained in autotrophic, mixotrophic and heterotrophic conditions respectively (Figure 15a).

Except for heterotrophic condition, the light intensity was increased every 24 hours, with a light regimen shown in Figure 15b. The abovementioned figure reports the light exposure as light intensity expressed as $\mu\text{mol photons s}^{-1} \text{ m}^{-2}$

normalized per OD₇₅₀ unit for both phototrophy and mixotrophy. In both conditions the highest value was observed at the beginning of the experiment, while during the cultivation, even though the light intensity was daily increased, the exposure remained constant between 200 and 100 $\mu\text{mol photon s}^{-1} \text{m}^{-2}$ per OD₇₅₀, due to the increase of cell density. The availability of light was similar in both conditions at the start and the end of the cultivation, with major differences at days 2 and 3. Indeed, in phototrophy an initial lag phase of two days characterized the growth curve of *C. sorokiniana*, responsible of lower biomass and thus higher light exposure, which was necessary to stimulate cell division and enter in exponential growth phase.

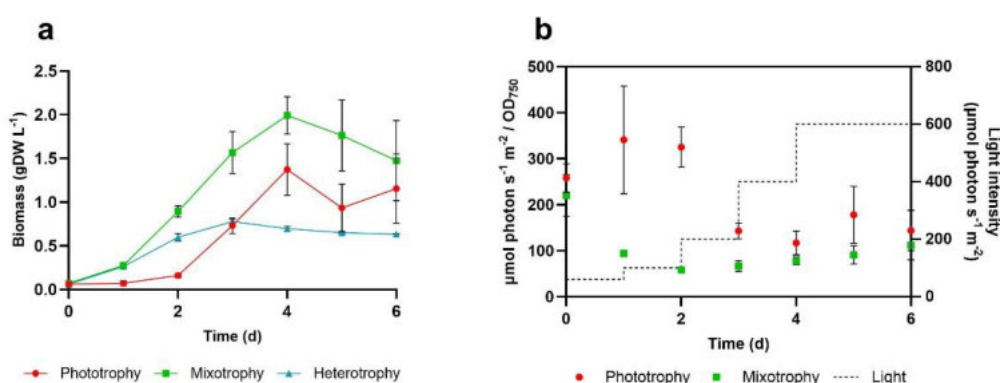


Figure 15: Growth curves of *C. sorokiniana* under phototrophy, mixotrophy and heterotrophy are reported as dried weight per liter (a). Light regime and light exposure expressed as $\mu\text{mol photons s}^{-1} \text{m}^{-2} / \text{OD}_{750}$ are depicted in graph b) for phototrophy and mixotrophy. Error bars represent SD.

4.1.8 FAME profile of *C. sorokiniana* cultivated under standard conditions

FAME profiles of *C. sorokiniana* biomass grown under different metabolic conditions are reported in Table 6 and Figure 16. Part of these data has been already published (Mazzocchi et al., 2025).

The results reveal significant variations in fatty acids composition depending on cultivation conditions. In all extracts the most abundant fatty acid was palmitic acid (C16:0), followed by linoleic (C18:2) and stearic (C18:0) acids, in agreement with previous studies (Banerjee et al., 2023; Papapolymerou et al., 2022a; Papapolymerou et al., 2022b). The saturated fatty acids, namely palmitic (C16:0) and stearic acids (C18:0), showed a similar trend, with the highest levels observed in phototrophy, followed by mixotrophy and heterotrophy. On the other hand, linoleic acid (C18:2), which contains two double bonds, was more abundant under mixotrophic and heterotrophic conditions than in phototrophic ones. This trend is further supported by higher content of oleic and elaidic acids (C18:1) recorded

under heterotrophic cultivation, where organic carbon is the sole carbon source confirmed from literature (Li et al., 2014; Yun et al., 2021). These findings suggest that the presence of organic carbon in the substrate leads to modified unsaturation levels, here an increase was observed. Nonetheless, the presence of different organic carbon sources influences lipid metabolism and FAME composition (Rodríguez-Rodríguez et al., 2016). Specifically, when increased concentration of acetate is supplemented its uptake and conversion is stimulated and carbon metabolism and lipogenesis are improved. Gluconeogenesis, citric acid cycle (TCA) and unsaturated fatty acids synthesis are enhanced under acetate supplementation (Gong et al., 2024).

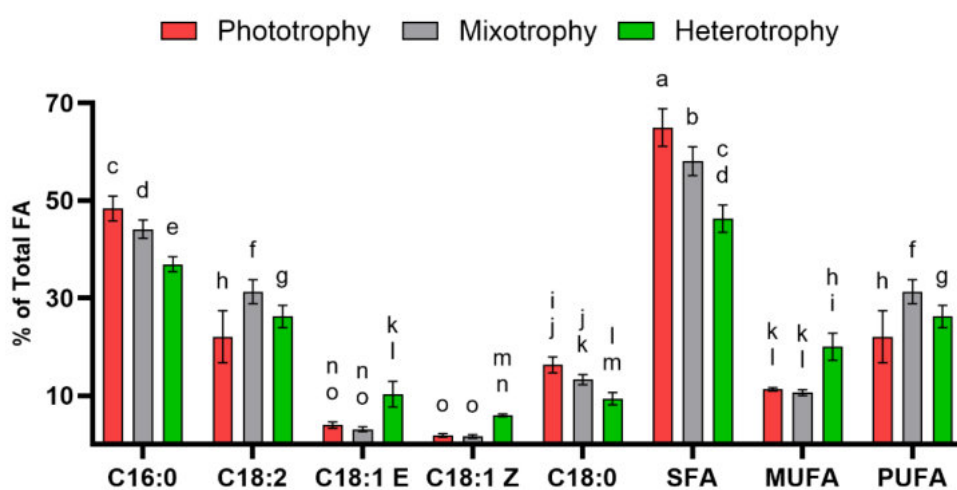


Figure 16: FAME profile of *C. sorokiniana* grown on standard medium in phototrophy, mixotrophy and heterotrophy. Data are reported as means of biological replicates and letters separate data into statistically different groups, with $p < 0.05$.

Moreover, this influence is more evident if dividing FAMES into classes: saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs). In this study, SFAs were more abundant in phototrophy compared to other conditions, whereas unsaturated fatty acids are increased. In particular, heterotrophy drove to the highest MUFAs and mixotrophy the highest PUFAs. This variations should be considered in relation to the specific experimental conditions, as strain and carbon sources can influence lipid metabolism. For instance, glucose supplementation was demonstrated to increase SFA and decrease PUFA (Li et al., 2014; Yun et al., 2021), while glycerol drove to lower SFA and enhance MUFA (Papapolymerou et al., 2022b). Moreover, when acetate was compared with glucose, SFA content grew while MUFA level was reduced (Gong et al., 2024). These different trends derive from the uptake and conversion pathways, which change in relation to the available carbon source.

Overall, the modulation of FAME composition under different cultivation modes represents a valuable strategy to tailoring lipid profiles toward specific high-value applications.

Table 6: FAME profile of *C. sorokiniana* cultivated in phototrophy, mixotrophy and heterotrophy. Means and standard deviations are reported for each FAME. SFA, saturated fatty acids, MUFA, monounsaturated fatty acids, PUFA, polyunsaturated fatty acids.

FAME	Phototrophy		Mixotrophy		Heterotrophy	
	Avg	SD	Avg	SD	Avg	SD
C16:1 (Δ^9 , Z)	1.65	0.22	2.80	1.00	3.78	0.64
C16:0	48.39	2.20	44.12	1.62	36.92	1.26
C17:1 (Δ^{10} , Z)	1.36	0.13	1.26	0.12	0.00	0.00
C17:0	0.34	0.34	0.73	0.05	0.00	0.00
C18:2 ($\Delta^{9,12}$ Z, Z)	22.05	4.62	31.29	2.14	10.31	2.15
C18:1 E (Δ^9 , E)	3.93	0.54	3.08	0.44	5.93	0.21
C18:1 Z (Δ^9 , Z)	1.78	0.31	1.56	0.32	5.93	0.21
C18:0	16.29	1.42	13.25	0.91	9.35	1.04
C24:1 (Δ^{15} , Z)	2.56	0.34	1.91	1.12	0.00	0.00
Σ SFA	65.03	3.34	58.10	2.56	46.27	2.28
Σ MUFA	11.29	0.31	10.61	0.51	20.03	2.26
Σ PUFA	22.05	4.62	31.29	2.14	26.21	1.85

4.1.9 Biodiesel quality of *C. sorokiniana* cultivated under standard conditions

In order to convert and use microalgal lipids as biodiesel, they must possess specific characteristics (See paragraphs 2.8 and 3.9), which allow us to analyze the quality of fatty acids. Cetane number (CN), which has a minimum limit of 51 and 47 respectively for EN14214 and ASTM D6751, is a fundamental factor that increases with longer carbon chains and lower unsaturation grade. CN indicates the ignition time and combustion performances. As the degree of unsaturation increases, the CN lowers, thus reducing biodiesel quality. Also, with the increase of unsaturation degree Cold Filter Plugging Point (CFPP) lowers, which enhances biodiesel performance at low temperature. Indeed, CFPP indicates the temperature at which FA can crystallize, causing deposit formations and pipe clogging. The last factor we investigated was Iodine Value (IV), which rises with the increase of unsaturation. IV should be lower than 120 g I₂/100 g to be adequate for biodiesel production, according to EN 14214 (Knothe, 2007). In this context, the main challenge in microalgal biodiesel relies on the balanced composition of FAME to obtain good quality, considering the trade-off between oxidative stability, ignition quality and cold flow properties.

Figure 17 reports results of IV, CN and CFPP of different biomasses. These results highlight the feasibility of implementing this species for biodiesel production, as it was already widely demonstrated (Hamidian & Zamani, 2022b; Talha et al., 2025). Indeed, iodine and cetane number are within European and American legislative limits. No differences have been evidenced between cultivation conditions for ignition quality, whereas oxidative stability is slightly improved in phototrophic biomass (Figure 17). Moreover, in present study CFPP was 24, 18 and 10 °C for phototrophic, mixotrophic and heterotrophic biomasses respectively (Figure 17). CFPP does not have a specific regulation because it depends on climate conditions of each country. Nevertheless, it is evident that CFPP of 18 or 24°C is too high for majority of climate conditions worldwide, thus a reduction of this parameter is mandatory to make this kind of biodiesel usable. To overcome this issue several methods can be proposed, such as blending with other diesel, the addition of chemical additives or biodiesel or winterization, that consists in fractionation of fatty acids to reduce high melting component of the mixture, resulting in lower CFPP (Edith et al., 2012).

These results are in line with previous works, where IV and CN were similar (Hamidian & Zamani, 2022b) with increased CN reported by Asadi and colleagues (Asadi et al., 2020a). Moreover, by using Amaral medium, *C. sorokiniana* biomass resulted in IV value exceeding the maximum legislative limit (Talha et al., 2025). These differences highlight the effect not only of the species but also the cultivation substrate used. Indeed, Amaral medium presents a different composition, especially different nitrogen source (urea) which may interfere with cellular metabolisms and lipid accumulation.

A balanced composition in FAME is required for developing biodiesel of good quality. The length of chains and the number of unsaturation are essential factors that influence biodiesel performances. Specifically, the longer the carbon chain is the higher CN is and ignition quality is improved. Moreover, when the level of unsaturation increases there is a decrease in CN and CFPP and increase of IV, corresponding to worse ignition performances and oxidative stability, while cold flow properties are improved. For these reasons an adequate increase in long-chain PUFAs could reduce the CFPP, however excessive increase could reduce ignition quality and oxidative stability.

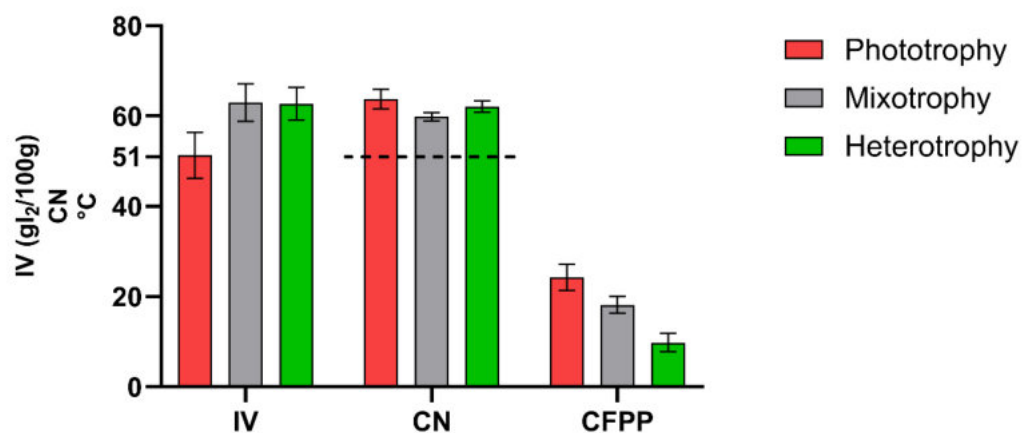


Figure 17: Biodiesel quality of *C. sorokiniana* grown under different culture conditions. Iodine Value (IV) Cetane Number (CN) and Cold Filter Plugging Point (CFPP) are reported as mean and bars show standard deviations. Dotted line indicates the European legislative lower limit of 51 for CN (EN 14214).

4.2 Chapter 4.2: Harnessing light-induced lipid biosynthesis in microalgae grown on industrial wastewater

4.2.1 Aim of the chapter

In this study, industrial wastewater was used as a source of carbon and nutrients for microalgal cultivation. Given the composition of this substrate, it was not feasible to modulate the organic carbon content to selectively activate specific metabolic pathways. However, light quality and intensity can still be controlled to influence microalgal metabolisms. Here, white, red (660 nm), and blue (450 nm) light were selected for the cultivation of *C. sorokiniana*.

The objective of this chapter was to evaluate how light wavelength influences biomass productivity and biochemical composition. The strategy adopted in this study involves an initial phase aimed at maximizing biomass accumulation, followed by a shift in light wavelength and intensity to induce lipid accumulation. Moreover, the shift in light wavelength from first to second step has not been deeply investigated but is reported to have huge impact on the metabolism of microalgae and thus composition of biomasses as already reported (Jungandreas et al., 2014a).

4.2.2 Selection and screening of wastewater for microalgal growth

Two wastewaters from dairy industry were selected for the cultivation of *C. sorokiniana*. Previous chapter highlighted how Dairy Exhausted Sludge (DES) and Dairy Washing-Waters (DWW) showed optimal performances when implemented at high percentages for microalgal cultivation. For this Chapter *C. sorokiniana* was selected for the screening due to its higher tolerance to DES, in fact it was able to grow on 50% and 75% of DES, outperforming control condition (Figure 12), while *T. obliquus* was inhibited when more than 50% of DES was applied.

However, *C. sorokiniana* demonstrated toxicity when cultivated on 100% of both DWW and DES (Figure 12a and Figure 13a). The selection of these two effluents was based on their complementary composition. Indeed, DES is characterized by high nitrogen content (370 mg L⁻¹ of ammonium), while DWW contains a significantly lower concentration (4.4 mg L⁻¹ of ammonium) (Table 1). Considering that nitrogen is an essential macronutrient for cellular metabolism, but may be inhibitory or toxic at high concentrations, blending DES and DWW represents a strategy to balance its concentration. Moreover, the organic load of DES is nearly 10 times higher than that of DWW, thus by mixing them it is

possible to obtain intermediate COD values, enabling better control of substrate composition to optimize microalgal cultivation.

For these reasons, a screening using a mixture of these streams was performed using 3:1, 1:1 and 1:3 DWW:DES in order to find a new substrate formulation that complementary combines the chemical features of both DWW and DES. All substrates tested allowed biomass production and the lack of an initial lag phase suggests that the inoculation into a wastewater mixture did not affect negatively the cell division, starting immediately with the exponential growth (Figure 18). Moreover, none of mixture tested caused growth inhibition, however, higher productivity was recorded with 3:1 DWW:DES, being comparable to the control condition in 3N BBM, measuring 0.052 and 0.049 gDW L⁻¹ d⁻¹ respectively against 0.032 and 0.031 gDW L⁻¹ d⁻¹ for 1:1 DWW:DES and 1:3 DWW:DES (Figure 18b). Similar results were obtained with *C. sorokiniana* cultivated on 50% of dairy wastewater, reaching 0.044 gDW L⁻¹ d⁻¹ (Hamidian & Zamani, 2022b). Working with wastewater involves dealing with variability in substrate composition. This variability is due to the specific industrial sectors involved, seasonal production patterns, the nature of the manufactured products, and the methods used for collecting wastewater flows, all of which can significantly influence their chemical characteristics. For this reason, several results can be observed in different publications, for example Tahla and colleagues recorded higher productivity of *C. sorokiniana* grown on 90% dairy wastewaters, reaching 0.47 gDW L⁻¹ d⁻¹ in six days (Talha et al., 2025). Additionally, from the observation of growth curves is possible to evince that with 1:1 and 1:3 DWW:DES *C. sorokiniana* begins earlier the stationary phase, maintaining barely the same biomass from day 7 onward (Figure 18a). Thus, the decrease of DWW ratio, from 3:1 to 1:1 and 1:3 DWW:DES, caused a lower biomass production, with a comparable growth rate but a shorter exponential phase. Similarly happened with *C. vulgaris* cultivated on 75% and 50% of dairy wastewater (Khalaji et al., 2023). A possible explanation of the reduced biomass production can be the high level of ammonium in DES, as already reported in the previous (Section 4.1.2 Hydro-chemical analysis of three dairy wastewater and ammonium tolerance; Figure 11). On the other hand, the condition with higher DWW, i.e. 3:1 DWW:DES, showed the highest biomass productivity. This condition resulted in 95.8 mg L⁻¹ of ammonium, a concentration within the optimal range for *C. sorokiniana* growth, and approximately 620 mg L⁻¹ of organic carbon. It is known that a successful nutrient removal requires a balanced C/N ratio, as the availability of organic carbon promotes nitrogen assimilation and biomass production (Zhu et al., 2019). For this reason, the supplementation of external sources of organic carbon has been proposed to increase nitrogen removal and biomass production (Carrera et al., 2004). In present study, however, the modulation of C/N ratio was achieved by blending DWW and DES. The resulting C/N ratios were 0.93, 2.35 and 6.49 C/N for 1:3, 1:1 and 3:1 DWW:DES, respectively. It was demonstrated

in previous study that the increase in C/N ratio from 2 to 8 enhances the growth rate of *C. sorokiniana* (Junttila et al., 2015). Consequently, the highest biomass accumulation was observed in 3:1 DWW:DES condition, which provided the most favorable C/N ratio. For these reasons, further experiments have been assessed by using 3:1 DWW:DES as cultivation substrate.

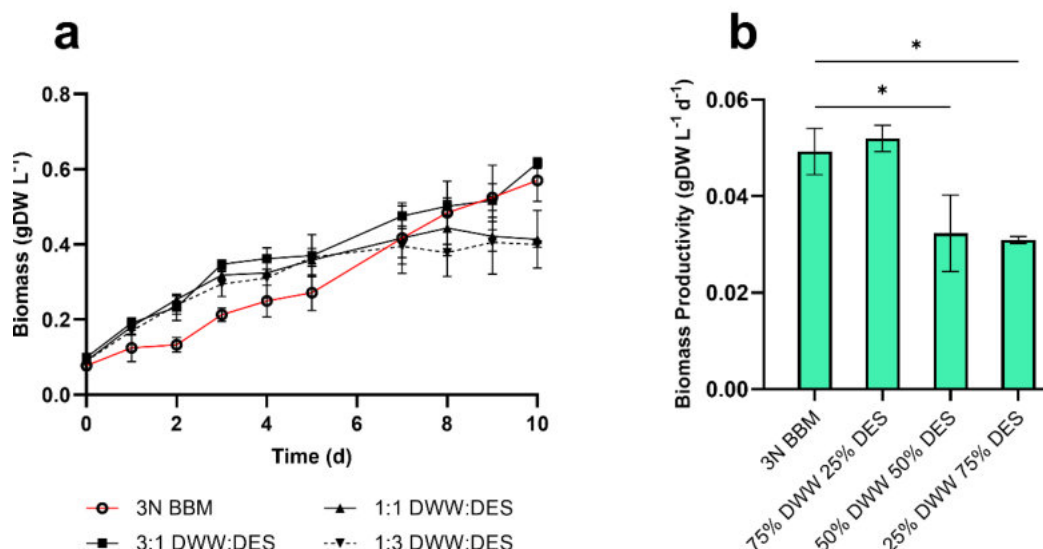


Figure 18: Cultivation of *C. sorokiniana* on different ratios of DWW and DES. Growth curves are represented on the left (a) measured as dried biomass, while b) reports biomass productivity. All tests were carried out in triplicates and error bars represent SD. Asterisks indicate statistical significance.

4.2.3 Biomass accumulation under different light regimes

The selected mixture of DWW and DES was used as substrate for developing a two-stage bioprocess under different light-regimes. This bioprocess was studied in a Multicultivator PBR, which allows us to select specific wavelengths. The first stage is dedicated to biomass production, while the second one was thought to induce lipids accumulation. In this study white, red (660 nm) and blue (450 nm) light have been selected for cultivation of *C. sorokiniana*. Red light is mainly absorbed by chlorophyll a, which is present in higher concentrations in microalgae, and is responsible for efficient photosynthetic rates, stimulating high biomass accumulation (Maltsev et al., 2021). On the contrary, blue light causes an increase in protein and lipid production (Zhu et al., 2024). White light, instead, covers a wider range of the visible spectrum, in both red and blue regions, stimulating biomass accumulation. *C. sorokiniana* has been selected as model organisms to screen the effects of different wavelengths on biomass composition when grown on wastewater. Given this context, the two-step process was performed with a first stage of biomass accumulation conducted under white light at moderate intensity and the second stage was characterized by a wavelength

shift into red (660 nm) and blue (450 nm) light, to stimulate lipid accumulation. Moreover, red light was also implemented in the first step due to its capacity on stimulating biomass accumulation, followed by a second step with blue light. The shift from red to blue light was already reported to stimulate lipid accumulation in *Phaeodactylum tricornutum* (Jungandreas et al., 2014b). About the intensities applied, in the first step 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were chosen, due to the low initial cell density, and it was increased in second step to 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ to enable a sufficient light exposition. Another condition was performed using only white light with an increased intensity in second step to 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Five conditions have been tested and are reported in Table 7.

Table 7: Experiment design: light intensities and wavelengths are reported in table below for two-step processes tested with *C. sorokiniana*.

Experiment	First step (100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$)	Second step (300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$)
W300	White light	White light
W600	White light	White light
R300	White light	Red light
B300	White light	Blue light
R100-B300	Red light	Blue light

Overall, the first phase under white light was comparable through experiments, while the first phase in R100-B300 resulted in higher biomass accumulation compared to white light. At the end of the first stage, biomass production under red light was 1.5 times higher than white light, in accordance with previous study with *C. vulgaris* under white and red light (Chang et al., 2022).

Moreover, in second stage 0.35, 0.25 and 0.13 gDW L^{-1} of biomass were produced respectively under red (R300), white (W300) and blue (B300) light. Furthermore, the lowest biomass production was observed with blue light which stimulated a shift to a stationary phase started in the second stage both in B300 and in R100-B300, measuring a production of biomass of 0.02 gDW L^{-1} in B300 and a decrease of 0.25 gDW L^{-1} in R100-B300 (Figure 19c, d). Similarly, in previous reported studies, the highest biomass accumulation was observed under red light and the lowest under blue light for both *C. sorokiniana* (He et al., 2023b) and *C. vulgaris* (Yan et al., 2013). Moreover, *Haematococcus pluvialis* showed the same result under blue light, which stimulated the lowest biomass accumulation, while the highest biomass production was reported under white light instead of red (WONG, 2016). In contrast, *Nannochloropsis* sp. showed higher biomass production under blue light followed by white and red light (Das et al., 2011). Depending on the species the effect of light on biomass production varies, mainly because of different pigment composition, indeed it has been

reported that *Arthrospira platensis*, *Scenedesmus obliquus* and *C. vulgaris* produced the same biomass weight under blue and white light (Sibi, 2019), in contrast with our result where under blue light almost no growth was observed. This species-specific response to light wavelengths is determined by the composition of photoreceptors (i.e. cryptochromes, phototropins, aureochromes, rhodospins and phytochrome-like proteins), which modulate photosynthesis and cellular metabolisms (Kianianmomeni & Hallmann, 2014). These photoreceptors are regulated by different wavelengths and are responsible for transcriptional regulation. Cryptochromes and phototropins are triggered by blue light and modulate cell growth and photosynthesis, indeed, some strains respond to blue light by increasing growth (Essen et al., 2017). Moreover, cryptochromes are demonstrated to be blue-light dependent and to be responsible for the biosynthesis of carotenoids such as astaxanthin and fucoxanthin in *Phaeodactylum tricornutum* and *Haematococcus pluvialis* (Zhang et al., 2022). Red light instead, can be perceived by phytochrome-like proteins and can modulate carbon flux and storage metabolism. Several species are associated with higher lipid accumulation under red light (Dong et al., 2023; Sharma et al., 2020). Overall, differences in response to light quality is the outcome of the composition of both pigments and photoreceptors, latter responsible for gene regulation involved in growth, storage compounds accumulation and pigment production.

In this study wavelength was varied while keeping the intensity, expressed as PPFD, constant. Since the same PPFD guarantees the same number of photons per second, and lower wavelength confers higher energy to photons, blue light provides more energy than red light (Das et al., 2011). This phenomenon must be considered when comparing growth under different light qualities since at the same intensity but lower wavelengths it can induce more easily photoinhibition (Matthijs et al., 1996). Consequently, the growth arrest observed in *C. sorokiniana* cultivated under blue light can be attributed to photoinhibition. To consider the major aspects that negatively affect biomass production, nutrient availability has been also investigated. Although limited nutrient availability, such as nitrogen, can inhibit growth, this scenario does not seem plausible in the present study. Indeed, biomass production occurred under red and white light from day 2, indicating that nutrients were still sufficient in those conditions. Consequently, nutrient limitation can be reasonably excluded as a cause of the growth inhibition observed under blue light.

On the other hand, white light can be absorbed by a wider group of pigments in respect to a single wavelength, because it covers between 400 and 700 nm. Here, under white light there are no differences between 300 and 600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, this can suggest that at the lower intensity, in this specific cultivation set up, was sufficient to enable stable growth of *C. sorokiniana*. The intensity of light was not deeper investigated, only with white light two intensities were tested. It is known that the quantity of light affects growth, with a direct

effect expressed as a logarithmic curve, showing an increase in growth rate at low intensities until a saturation point is reached with a plateau, followed by the photoinhibition effect at excessive light intensities (Williams & Laurens, 2010). Consequently, light is a limiting effect at low intensities but can be inhibitory if it is too high (Sibi, 2019). Here, we cannot say that photoinhibition happened, because a dramatic decrease in growth rate did not occurred, but we can suggest that in this set up 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ are sufficient to allow an efficient growth, similarly to what happened with *C. vulgaris*, which saw a decrease in cell density with light intensities higher than 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Gao et al., 2024).

The biomass measured at the end of the experiment was higher under W300 and W600, with 0.794 and 0.719 gDW L^{-1} , followed by R300, R100-B300 and B300, which measured respectively 0.666, 0.652 and 0.401 gDW L^{-1} (Figure 19). It is worth noting that the utilization of red light for the first step of cultivation (R100-B300), leads to an increase in the final biomass accumulated compared to B300.

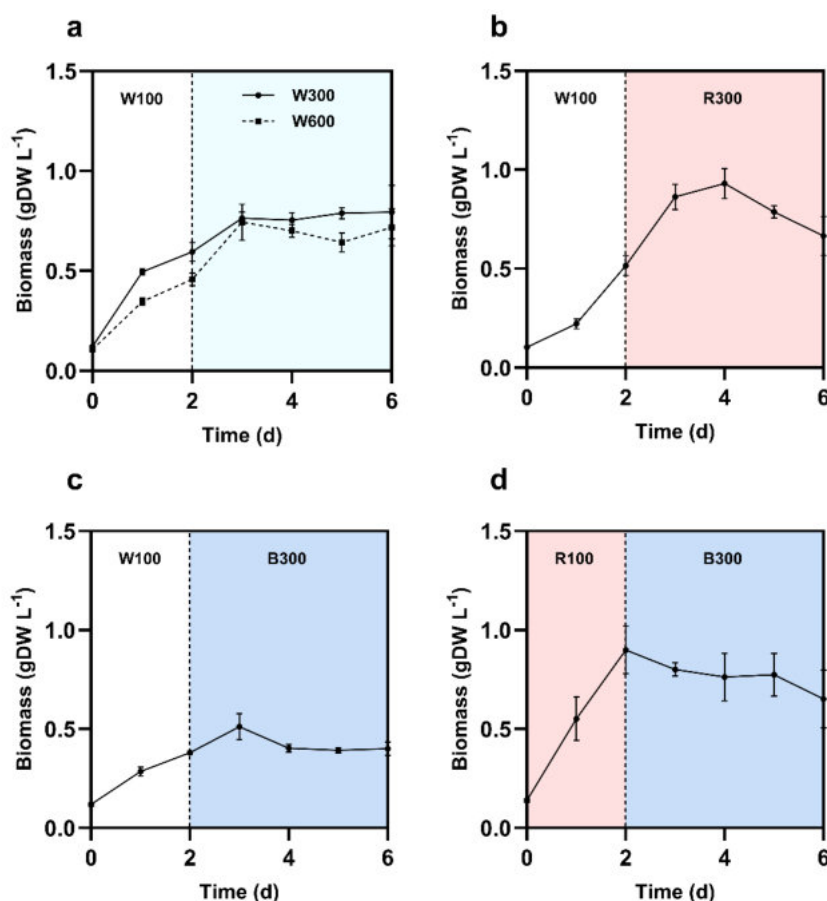


Figure 19: *C. sorokiniana* growth curves, in different light conditions, are shown. a) represents W300 and W600; b) is R300; c) B300 and d) R100-B300. At the end of the first phase a change in light wavelength or intensity was performed and depicted with colored boxes. All tests were carried out in triplicates. Data are shown as means and error bar are SD.

4.2.4 Photosynthetic pigments composition during cultivation under different wavelengths

Variations in quality of light can directly affect the composition of photosynthetic pigments of microalgae, thus, to deeper understand the effects of different wavelengths, they have been quantified during the experiments. Chlorophyll a and b (Chl a and b) and carotenoids have been quantified via spectrophotometric methods and results are reported in Figure 20 and Table 8. For all the studied light exposure conditions, pigments were quantified at the end of both first (2nd day) and second (6th day) stages, to better understand the effect and the physiological status induced by light within the cells (Luimstra et al., 2018). Under white and red light, a decrease of 64, 52 and 56% in Chl a was recorded for W300, W600 and R300 respectively, after the light shift, while blue light (B300 and R100-B300) showed different patterns, with an increase of 58% in B300 and a steady Chl a content for R100-B300 (Figure 20a, Table 8). Under white light the reduction of Chl a during the cultivation has been already observed for *C. vulgaris*, which showed a similar trend with our results, and in detail the increase of light intensity did not affect dramatically the content of Chl a when it was increased from 300 to 500 (Gao et al., 2024). Microalgae cells tend to absorb more efficiently blue and red light due to the specific wavelengths, which coincide with chlorophylls absorption ranges (Ra et al., 2018). Thus, microalgae under these light wavelengths should be able to accumulate more chlorophylls and increase the biomass production (Jin et al., 2024). Our results confirm the higher production of Chl a under blue light, which is about three times more compared to other conditions (14.12 mg gDW⁻¹) (Table 8: Pigment composition of *C. sorokiniana* grown under different light regimes at the end of the first and second stage of cultivation, measured as mg gDW⁻¹ ± SD Table 8), but it does not show an increase in biomass production, meaning that the higher photosynthetic rate may boost the allocation of storage compounds instead of cell division. Chl b under blue and red light did not change during the cultivation (Figure 20b).

Moreover, carotenoids are associated to a defense from stress, in this work carotenoids average was stable during the cultivation in all conditions except for B300, where we observed a slight increase, suggesting that under blue light *C. sorokiniana* is nearer to a stress condition (Figure 20c). This phenomenon, the increased production of carotenoids under blue light, not only for *Chlorella* sp., is well documented in literature (Duarte & Costa, 2018; Liu et al., 2021; McGee et al., 2020). The higher biosynthesis of pigments is also linked to an increase in storage compounds, such as Chl b and carotenoids which have been correlated to higher lipid production (Venkata Mohan & Prathima Devi, 2012; Xie et al., 2020; Zhang et al., 2013). Later I will discuss the production of lipids in comparison with other compounds.

Table 8: Pigment composition of *C. sorokiniana* grown under different light regimes at the end of the first and second stage of cultivation, measured as mg gDW⁻¹ ± SD. Chl a, chlorophyll a, Chl b, chlorophyll b. Experimental conditions are the following: white light at 300 µmol photons m⁻² s⁻¹ (W300); white light at 600 µmol photons m⁻² s⁻¹ (W600); red light at 300 µmol photons m⁻² s⁻¹ (R300); blue light at 300 µmol photons m⁻² s⁻¹ (B300); red light at 100 µmol photons m⁻² s⁻¹ followed by red light at 300 µmol photons m⁻² s⁻¹ (R100-B300).

Condition	Stage	mg gDW ⁻¹							
		Chl a		Chl b		Carotenoids		Chl a/Chl b	
		Avg	SD	Avg	SD	Avg	SD	Avg	SD
W300	I	13.09	0.99	4.92	0.70	4.04	0.49	2.70	0.3
	II	4.76	0.89	2.68	0.69	3.43	1.09	1.85	0.20
W600	I	14.05	1.16	5.96	0.60	4.56	0.88	2.36	0.10
	II	5.47	0.75	6.41	0.41	3.13	0.38	1.17	0.26
R300	I	11.66	1.08	5.34	0.06	3.34	0.60	2.18	0.20
	II	5.23	0.26	2.79	0.36	3.16	0.11	1.90	0.29
B300	I	8.96	0.44	4.89	0.98	3.00	0.60	2.07	0.50
	II	14.12	0.95	5.39	1.23	4.40	0.50	1.19	0.44
R100-B300	I	4.92	0.74	2.28	0.91	3.25	0.91	2.34	0.51
	II	5.20	0.87	2.15	0.24	3.82	1.15	2.41	0.28

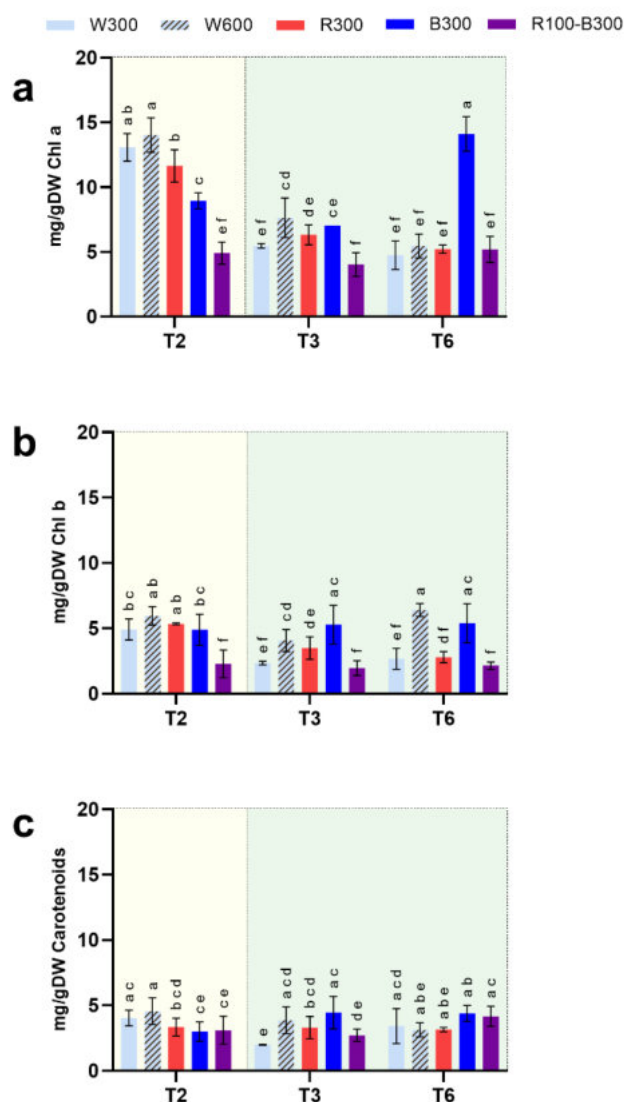


Figure 20. Pigments profiling of *C. sorokiniana* grown under different light regimes. Chlorophyll a (a), chlorophyll b (b) and carotenoids (c) are reported. Colored quadrants helps to distinguish between first and second stages. All tests were carried out in triplicates. Error bar represents SD. Two-way ANOVA was used for statistical analysis and letters indicate statistical significance, $p < 0.05$

4.2.5 Biomass composition: protein, carbohydrate and lipid fractions

Several stresses can boost the production of lipids or other compounds of interest in microalgal cells, such as starch (Zhu et al., 2014), carotenoids (Orosa et al., 2001; Sun et al., 2018) or antioxidant pigments (Gauthier et al., 2020). For lipid production it has been widely explored the nitrogen or phosphate limitation (Chu et al., 2013; Shen et al., 2015) or hyper-salinity (Xia et al., 2014). These stresses often implicate a reduced biomass production, which affects the overall lipid production, reducing the yield and increasing the costs of the process.

Instead, by modulating light intensity and quality is possible to maintain high photosynthetic efficiency while inducing lipid accumulation (He et al., 2015). The implementation of a two-step process is particularly advantageous for this purpose, as it enables the increase of biomass production in the initial phase before the induction of stress conditions for the production of a specific metabolite. The intensity of light has been deeply investigated (Amini Khoeyi et al., 2012; Chin et al., 2023; Nzayisenga et al., 2020), often in association with other stresses like nitrogen depletion. Moreover, the utilization of different wavelengths has huge potential for modulating microalgal metabolism. Here, we focused on the influence of light quality on biomass composition.

Light is primarily implicated in photosynthetic pathway, and an excess of light energy brings to an increased carbon fixed, generally stored as starch (T. Chen et al., 2015), utilized as an energy reserve. In this context, red light (450 nm), is absorbed by chlorophylls and thus stimulate photosynthesis and accumulation of carbohydrates, as already demonstrated (Esteves et al., 2025; Li et al., 2021; Six et al., 2024). Here, under red light, in comparison to white light, carbohydrate fraction was higher, being 37% of dried biomass, at the end of the first stage under red light (R100-B300) and 27% under white light (as mean of carbohydrate fraction at the end of the first stage for all other experiments) (Figure 21a,b,c,e). Moreover, blue light led to the lowest content of carbohydrate, both in B300 and R100-B300, being 29.8% and 29.5%, respectively (Figure 21d,e). Overall, a marked increase in carbohydrate fraction was observed after one day in second stage under both white and blue light conditions (Figure 21a,b), confirming that an increased light exposure leads to accumulation of this fraction (B. Chen et al., 2017). Overall, our findings are consistent with previous results achieved on *C. sorokiniana*, confirming either the content or the pattern of accumulation over the light exposure conditions, with a slight increase in carbohydrates under blue light and stability during the cultivation under red light (He et al., 2023a).

Meanwhile, protein content was around 40-50% at the end of first stage and then showed a marked decrease under white and red light during the second stage of the present bioprocess, while remained stable under blue light in both R100-B300 and B300 groups (Figure 21c,d), accounting for 50 and 40% respectively. These results are in accordance with data recorded in *C. vulgaris* and *S. obliquus* when subjected to blue light (Sibi, 2019). Conversely, under white and red light protein content decreased dramatically after the light shift between the first and second phases, and reached 32.23, 33.65 and 24.53 % of dried biomass for W300, W600 and R300, respectively (Figure 21a,b,c). Interestingly, Yuan and colleagues did not observe changes in protein content in *Chlorella* sp. under red light (Yuan et al., 2020), similarly in present study during the red phase no variations in proteins have been detected, while a reduction was observed due to the shift from white to red light (Figure 21c). On the whole, the variations in carbohydrate and

protein fractions describe a trend in which the increase in carbohydrate coincides with a decrease in proteins, while if one is steady the other is also stable, as shown in Figure 21.

The reduction of protein content observed under red light can be due to the reduction of nutrients due to the production of biomass. In fact, considering the initially low nitrogen availability and the high biomass production, it is plausible that nitrogen was depleted or dramatically reduced around day 4, coherently with the growth curves in Figure 19, where the stationary phase is observed across all the light exposure conditions. Under nitrogen depletion, protein synthesis is reduced and the fixed carbon is redirected to the synthesis of energy storage compounds, such as lipids (Mujtaba et al., 2012). In addition, nitrogen-rich compounds such as proteins and chlorophylls are degraded under nitrogen starvation conditions for a re-allocation of nitrogen in storage compounds (Fazeli Danesh et al., 2017). Consistently, the protein fraction of *C. sorokiniana* biomass cultivated under red light is the lowest, while the lipids fraction was the highest (Figure 21f). These data suggest that the protein fraction could be reduced and lipids synthesis induced. In detail, the R300 biomass exhibited the highest lipid content, reaching 40.8%, compared to 31.6%, 34.7%, and 31.0% observed in W600, B300, and R100-B300, respectively (Figure 21f). Lipid fraction, in terms of concentration per biomass and FAME profiles is discussed in the next paragraph.

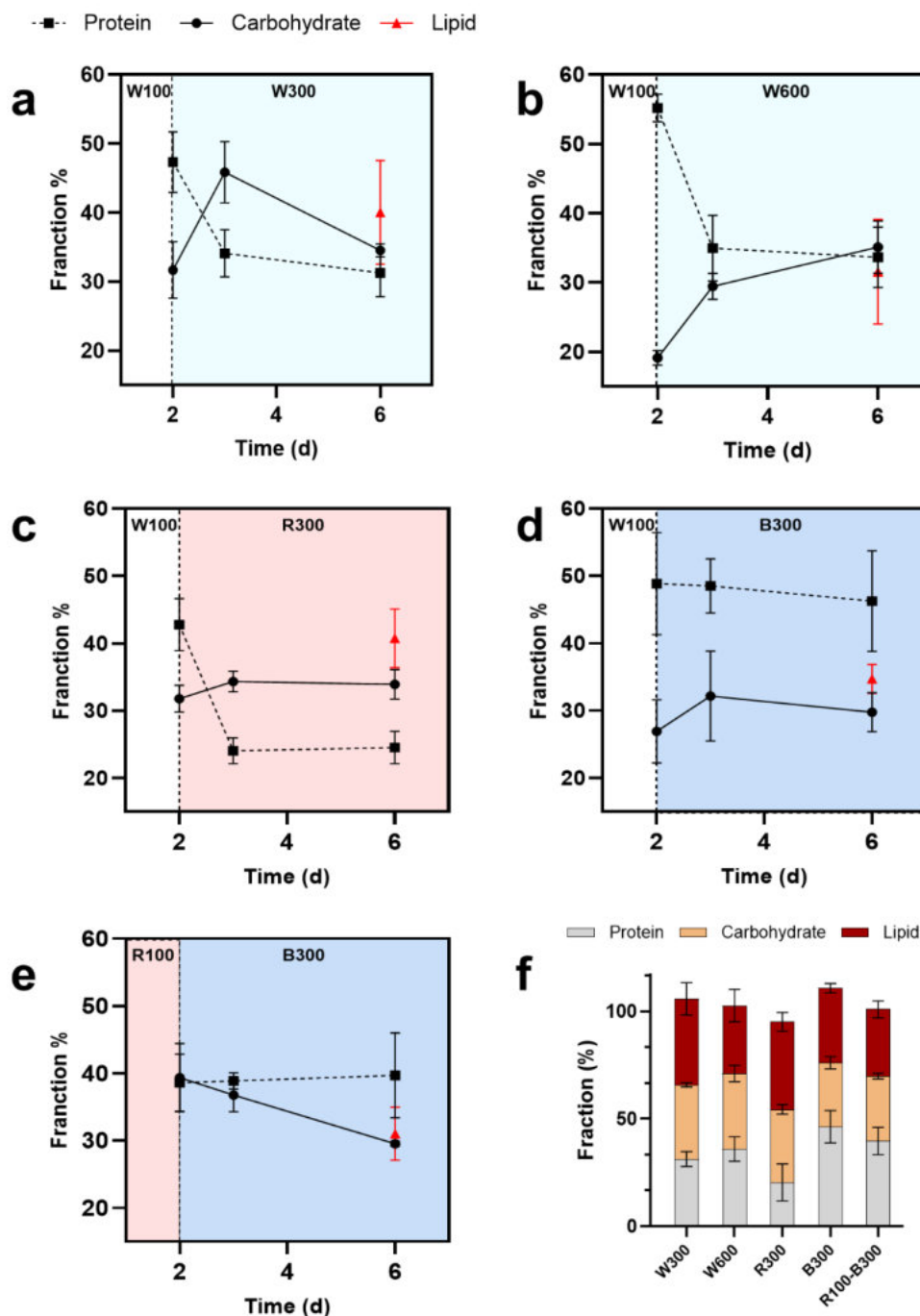


Figure 21: Composition of *C. sorokiniana* biomass grown under different light conditions: a) W300; b) W600; c) R300; d) B300 and e) R100-B300 at the end of first stage (day 2) and in the second stage (days 3 and 6) of the experiments. White, red and blue quadrants outline different steps of the process (first step from day 0 to 2 and second step from day 2 to day 7). Final composition of biomass, as protein, carbohydrate and lipid fractions is reported in graph f). All data are the mean of triplicates and error bars are SD.

4.2.6 Influence of light on lipid concentration and FAMES profile

Lipid productivity and quality is highly affected by culture conditions like temperature, nutrients and light (Chen et al., 2017). Depending on FAME profile and lipid productivity, microalgae can be used in different industrial fields, such as pharmaceutical and animal feed or for biofuels production (Gao et al., 2017; Remmers et al., 2017; Spolaore et al., 2006). While working with wastewater is more difficult to modulate the composition of substrate to boost the production of a specific metabolite, thus, as an alternative, it is possible to regulate environmental factors, such as light. Therefore, it is important to deeply understand how light affects the productivity and quality of lipid fractions, determining, consequently, the application fields.

Total lipid content, calculated as percentage of lipid on dried biomass, measured 40.03, 31.57, 40.75, 34.70 and 31.03% for W300, W600, R300, B300 and R100-B300, respectively (Table 9). Red and low white light conditions led to the highest lipid production, followed by B300 and R100-B300. Similarly, in another publication, *C. sorokiniana* grown under blue light accumulated 32% of lipids, while slightly reduced to 29% when grown under red light, differently from our results (Singh & Laura, 2020). It was demonstrated how lipid content highly varies between strains, indeed, three strains of *C. sorokiniana*, grown under the same environmental conditions, showed lipid production ranging from 20 to 50% of dried biomass (Zou et al., 2022).

Subsequently, FAME profiles have been analyzed to deeper understand the quality of lipids for potential application as biodiesel feedstocks, under varying light regimes. Fatty acids content under white light was three times higher at 300 than 600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Red light slightly reduced the FAME content while blue light was 1.69-fold lower than W300 biomass (Table 9). More in detail, the fractions of saturated, monounsaturated and polyunsaturated fatty acids (SFA, MUFA, PUFA) were calculated. SFA were higher under blue light (52.56%) compared to red light (42.24%), with middle values for white light (44.70% in W300 and 49.09% in W600) (Table 9). Interestingly, the utilization of red light followed by blue light (R100-B300) stimulated SFA production (46.23%) higher than red and lower than blue light (Figure 21b). W600 biomass contained the highest MUFA fraction, followed by R300 and B300, accounting for 20.74, 10.82 and 9.55%, respectively, while R100-B300 is comparable to W600 (19.27% and 20.74% respectively). Moreover, red light boosted the highest accumulation of PUFA (44.20%), while B300 and R100-B300 biomasses produced 37.89 and 34.13%, respectively (Table 9). In comparison to the literature, we obtained lower fractions of SFA, and higher fractions of MUFA and PUFA compared to other studies on *C. sorokiniana* (Singh & Laura, 2020; Tong

et al., 2026), although there is variability of these fractions between different conditions and studies (Papapanagiotou et al., 2024; Yun et al., 2020).

The quality of light highly affected the composition of fatty acids. As previously reported, C16 and C18 are the main fatty acids present in *C. sorokiniana* (Y. He et al., 2023a; Zou et al., 2022). In detail, palmitic (C16:0), linoleic (C18:2), oleic (C18:1), stearic (C18:0) and palmitoleic acids (C16:1) together account for more than 90% of total fatty acids (Figure 22a). Among these, C16:0 predominates in all conditions, except W300 and R300, where C18:2 is the most abundant. The highest percentage of C16:0 was recorded for B300 (40.92%), whereas the lowest one occurred in R300 (32.96%). According with the literature, He and colleagues reported higher concentration of C18:2 compared to other FAMES under all tested conditions (He et al., 2023a), whereas Singh *et al.* reported C16:0 as the most abundant FAME, except for C18:2 found to be the highest when biomass was exposed to red light (Singh & Laura, 2020). All these data, along with those from the present investigation, suggest a light-dependent fatty acid synthesis, indicating that light can induce the expression of specific pathways/enzymes. To clarify these observations, it is important to consider that fatty acids synthesis (FAS) occurs in the plastid, through cyclic elongation reactions of the acyl-chain, where each cycle adds 2 carbon atoms. Briefly, the elongation reaction involves the carboxylation of acetyl-CoA to produce three-carbon Malonyl-CoA, which is then transferred to Acyl Carrier Protein (ACP) to form Malonyl-ACP (Zhu et al., 2022). This condenses with Acyl-ACP to obtain 3-oxoacyl-ACP and this cycle can be repeated to obtain longer chain fatty acids.

Genes involved in FAS are regulated by light stimuli, thanks to promoter regions with light-responsive elements (Zhang et al., 2021; Zhu et al., 2024). In *Chlorella pyrenoidosa* both red and blue lights induced upregulation of 12 genes linked to FAS, although with different expression levels (Zhu et al., 2024). Blue light mainly enhanced the expression of genes involved in elongation steps, resulting in an increase in longer-chain fatty acids, whereas red light promoted the accumulation of C14 and C16 fatty acids due to higher expression of genes associated with the fatty acids release (Zhu et al., 2024). Indeed, in present study a marked increase in C16:1 was observed in R100-B300, whose production exceeded that under red and blue light, measuring 7.96, 1.77 and 0.98% respectively for R100-B300, R300 and B300 (Table 9). In accordance with this result, Ma and colleagues demonstrated that the cultivation under blue light of *Haematococcus pluvialis* stimulated the upregulation of genes involved in the elongation reactions, which are the first limiting steps for fatty acids synthesis (Ma et al., 2018). Moreover, *Tetraselmis chuii* under blue and red lights was demonstrated to overexpress genes involved in pyruvate synthesis, which is a precursor of fatty acids biosynthesis, with higher levels under red light (Patelou et al., 2025). These results confirm that light quality not only affects lipid productivity, but also FAME profiles by regulating biosynthetic genes.

Chapter 4.2 Harnessing light-induced lipid biosynthesis in microalgae grown on industrial wastewater

Table 9: Fatty acids profiles of *C. sorokiniana* grown under different light regimes are reported in this table. Data of each single FA is shown as percentage of total FAs, while total lipid content is calculated on dried biomass. Each data is indicated as average $\pm$ SD. ND stands for 'not detected' data.

FAME	Fatty Acids (% of total FA)									
	W300		W600		R300		B300		R100-B300	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
C8:0	0.156	0.074	0.504	0.022	0.215	0.135	0.191	0.014	0.396	0.039
C11:0	0.048	0.005	0.106	0.005	0.095	0.061	0.055	0.040	0.085	0.012
C12:0	0.089	0.063	ND	ND	0.348	0.185	0.224	0.015	ND	ND
C13:0	0.073	0.104	0.486	0.050	0.126	0.081	ND	ND	0.334	0.036
C14:1	0.183	0.221	0.373	0.148	ND	ND	ND	ND	0.427	0.224
C14:0	0.800	0.140	1.267	0.197	0.991	0.098	0.704	0.046	1.486	0.321
C15:1	0.155	0.219	0.893	0.033	0.020	0.028	ND	ND	0.679	0.062
C15:1	0.216	0.127	0.728	0.025	0.540	0.251	0.202	0.025	0.553	0.060
C16:1	1.161	0.086	8.277	1.708	0.559	0.237	0.981	0.084	7.959	1.527
C16:0	36.479	0.726	35.61 2	1.745	1.769	0.810	40.92 5	0.851	35.26 0	4.113
C17:1	0.388	0.008	1.108	0.059	0.332	0.022	0.647	0.105	0.639	0.133
C17:0	0.423	0.122	1.148	0.085	0.388	0.078	0.797	0.077	0.650	0.039
C18:2	43.795	6.472	26.83 3	4.279	47.84 2	0.578	37.34 8	0.951	35.40 0	1.242
C18:1	6.110	0.832	6.307	1.431	4.986	0.691	6.884	0.120	8.468	0.732
C18:0	5.404	0.962	8.693	0.237	5.614	1.161	7.463	0.530	6.609	0.480
C24:1	0.305	0.261	0.001	0.002	ND	ND	0.822	0.585	0.542	0.474
C24:0	0.728	0.308	0.301	0.186	ND	ND	1.649	0.380	0.221	0.112
C20:4	0.084	0.074	0.079	0.112	ND	ND	ND	ND	0.102	0.092
C20:5	0.121	0.060	0.335	0.058	ND	ND	ND	ND	0.247	0.035
C20:3	0.027	0.020	ND	ND	0.096	0.052	0.044	0.063	ND	ND
C20:2	0.163	0.017	0.147	0.032	0.481	0.262	0.319	0.016	0.213	0.062
C20:3	0.107	0.152	0.627	0.027	0.022	0.031	ND	ND	0.490	0.049
C20:0	0.299	0.048	0.513	0.021	0.496	0.273	0.271	0.021	0.824	0.470
C21:0	0.076	0.107	0.455	0.016	ND	ND	0.032	0.046	0.361	0.038
C22:6	0.148	0.083	0.528	0.023	ND	ND	0.059	0.084	0.409	0.041
C22:2	0.196	0.277	1.146	0.052	0.230	0.325	0.115	0.163	0.910	0.087
C22:1	ND	ND	ND	ND	0.007	0.005	0.016	0.012	ND	ND
C22:0	0.125	0.088	ND	ND	0.346	0.198	0.248	0.013	ND	ND
Σ SFA	44.70	1.54	49.09	1.11	42.24	2.68	52.56	0.90	46.23	3.72
Σ MUFA	10.58	4.29	20.74	4.37	10.82	3.66	9.55	0.87	19.27	2.19
Σ PUFA	44.64	5.89	29.69	4.30	44.20	7.62	37.89	0.98	34.13	5.38
TOT FA	9.03	1.82	3.20	0.33	7.68	0.65	5.36	0.37	4.91	0.16
TOT LIPID %	40.04	6.13	31.57	6.17	40.75	3.57	34.71	1.74	31.03	3.22

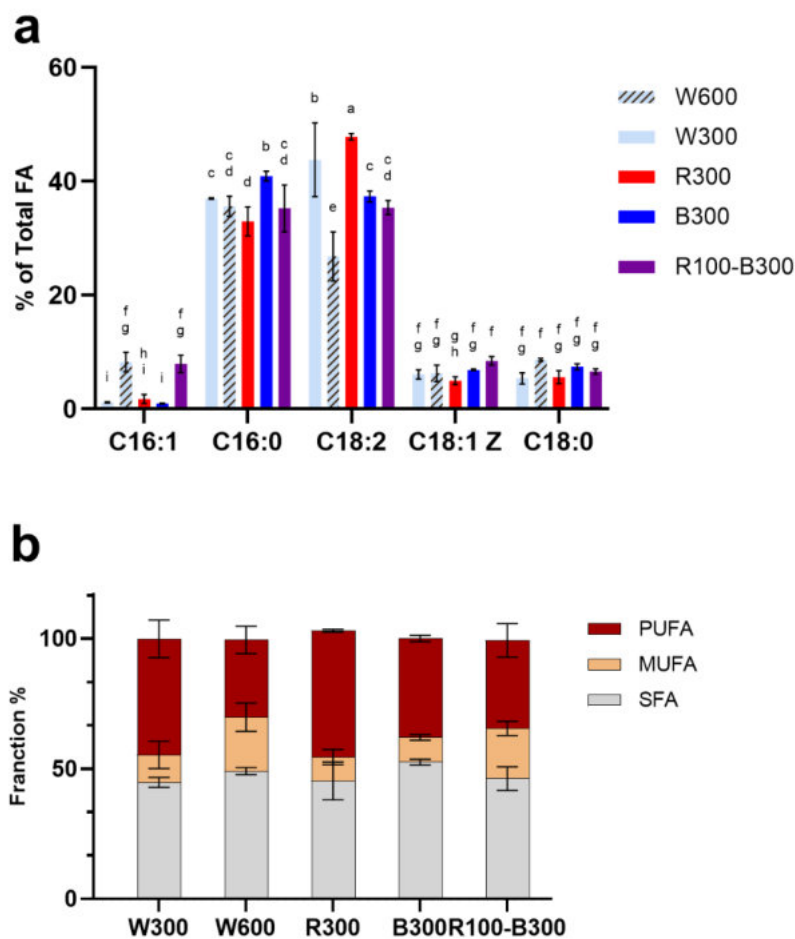


Figure 22: FAME profile of *C. sorokiniana* under different light conditions. a) represents the percentages of the main FAMES and b) shows the subdivision in SFA, MUFA and PUFA. All data are reported as average of three biological replicates. Two-way ANOVA test ($p < 0.05$) was conducted for statistical analysis. Lettering in graph a) refers to statistically significant results.

4.2.7 Evaluation of biodiesel quality

FAME profiles obtained from different biomasses have been used for the calculation of important parameters related to biodiesel quality. Figure 23 shows Iodine Value (IV), Cetan number (CN) and Cold Filter Plugging Point (CFPP) values. All these values respected standard limits: CN should be higher than 47 (ASTM D6751) or 51 (EN 14214) and IV lower than 120 g I₂/100 g. In detail, IV ranges from 72.75 (W600) to 92.41 (R300), while CN is between 54.60 (R300) and 59.69 (W300). These values give better biodiesel performances, in terms of ignition time and oxidative stability in comparison to similar microalgal biodiesel. Particularly, *Scenedesmus dimorphus* based biodiesel showed IV between 100 and 120 both in phototrophy and mixotrophy, while *Nannochloropsis* sp. had always

IV higher than 120 and *Chlorella* sp. draw a decrease in IV with the increase of organic carbon concentration (Manzoor et al., 2022). Moreover, their CN values were always lower than 51, demonstrating poor ignition and combustion performances, except for *Chlorella* sp. which in mixotrophy reported higher CN (Manzoor et al., 2022). In another study, *Scenedesmus obliquus* showed IV and CN around 70 g I₂/100 g and 57 respectively, while *Chlorella pyrenoidosa* reported IV of 130 g I₂/100 g and CN of 43 under different nitrate concentrations, the last resulting not suitable for biodiesel production (Wu & Miao, 2014). These studies demonstrate that it is possible to regulate the profile of FAs for high quality biodiesel production by changing nutrients composition but also it is important to first select a suitable species. Indeed, *S. obliquus* and *C. vulgaris* showed good performances both for ignition time and oxidative stability (Nascimento et al., 2013). Furthermore, *C. sorokiniana* lipids were of high quality compared to *C. vulgaris*, with markedly higher CN and lower IV (Asadi et al., 2020). Regarding *Chlorella* sp. it was demonstrated that different species of this genus have distinct FA profiles and, as a result, different biodiesel qualities highlighting the importance of strain selection. In this scenario, *C. sorokiniana* shows the best parameters for biodiesel quality within its genus, and maintains good performances while changing cultivation conditions, like composition of growth substrate and physical factors, such as temperature (Asadi et al., 2020; Hamidian & Zamani, 2022b; Li et al., 2013). Moreover, the FAME profile of *C. sorokiniana* is particularly advantageous for biodiesel production, with high levels of C16:0 and C18:0, which confers balanced levels of viscosity (Moser, 2014) and low content of volatile fatty acids with short chain. In addition, *C. sorokiniana* does not produce long-chain fatty acids with high levels of unsaturation, like eicosatetraenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) which highly affect oxidative stability of biodiesel and shelf-life (Moser, 2014). Another important parameter is CFPP, for which no specific limit is established by ASTM or EN 14214 standards, because it depends on the season and the climatic conditions of the country where biodiesel is employed. Although experimental methods are available to determine this value, a predictive correlation has been developed to estimate the CFPP when experimental measurement is not feasible. This approach allows us to calculate approximatively the low-temperature performances of biodiesel (Francisco et al., 2010). To calculate CFPP of microalgal oils, the formula includes long chain FA such as C16:0, C18:0, C20:0, C22:0 and C24:0 to have a more precise and accurate result, due to the composition of those oils (Eq. 9 for LCSF, Eq. 10 for CFPP). Despite that, several studies have reported low CFPP values, suggesting biodiesel with good cold-flow properties. However, closer examination reveals that many of these works employ a simplified version of the LCSF formula, which includes only C16:0 and C18:0 methyl esters (Dong et al., 2023; Nascimento et al.,; Wu & Miao, 2014), without considering other long-chain saturated fatty acids, such as

C20:0, C22:0 and C24:0, which play a significant role in determining the low-temperature performances of biodiesel. As a result, the calculated CFPP values tend to overestimate the actual quality of biodiesel at low temperatures. For this reason, in this work, CFPP are compared exclusively with those studies that applied the complete version of the LCSF equation. CFPP obtained in this study varies from 6.64 (R300) to 11.87 °C (W600), with a higher value under blue light which reaches 20.10 °C (Figure 23). The result of B300 is similar to *Scenedesmus* reported by Francisco and colleagues, while *Chlorella* and *Phormidium* showed a CFPP around 5°C, as our results under red light, while for other species it was lower than zero degrees (Francisco et al., 2010). Similarly, *Tetradesmus obliquus*, *Heterochlorella luteoviridis* and *Chlamydomonas reinhardtii* grown in mixotrophy reported CFPP around 5°C (Enwereuzoh et al., 2020). About *C. sorokiniana* biodiesel quality, in terms of cold-flow properties, our results are consistent with previously reported CFPP (Menegazzo et al., 2022). Therefore, this study evaluated the influence of light on biodiesel properties. CN shows slow fluctuations among light tested, always remaining between standard limits, such as IV, which is always lower than 120 g I₂/100 g but exhibits higher variations, from 72.75 (W600) to 92.41 (R300) (Figure 23). Here, red light negatively affected oxidative stability, while blue light improved it. Interestingly the utilization of both light wavelengths in sequence (R100-B300) led to an intermediate value. The effect of blue and red light on IV and CN have been already observed with *Phaeodactylum tricornutum*, whose IV increased while CN decreased compared to white light, noticing that CN remained always lower than 51, the minimum standard limit (Dong et al., 2023). Moreover, CFPP was also affected by wavelength used for the cultivation of *C. sorokiniana*. A decrease of three degrees Celsius was observed between W300 and R300, while conversely, B300 drove to a marked increase, doubling the value of W300 (Figure 23). Once again, we see how the subsequent use of both wavelengths (R100-B300) led to an intermediate value between R300 and B300, resulting respectively 8.96, 6.64 and 20.10 °C. *P. tricornutum* also had a decrease in CFPP under red light, while blue light did not affect this parameter (Dong et al., 2023).

In conclusion, a brief literature review confirmed that lipid profile of microalgae is a specie-specific trait. Moreover, the findings of the present work are consistent with previously reported and further extend the knowledge on this field by elucidating the influence of light quality on biomass and lipid production and compositions of a promising species for biodiesel production. Furthermore, the combination of red and blue light for cultivation of *C. sorokiniana* demonstrated a synergistic effect, resulting in an increase in biomass yield and quality of biodiesel. Specifically, biodiesel obtained from this light regime shows high oxidative stability and good ignition performances. The low temperature performance is better compared to other conditions, with the exception of red light

(R300), but can be still improved. Overall, all biomasses resulted in good oxidative stability and ignition performances, major differences were detected between cold flow properties.

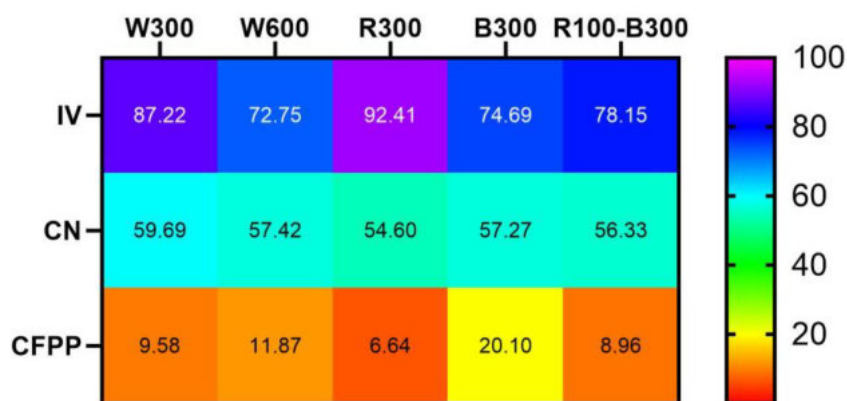


Figure 23: A heat-map visually demonstrates the variations of Iodine Value (IV), Cetane Number (CN) and Cold Filter Plugging Point (CFPP) among different light regimes. Each value is the average of three replicates.

Table 10: Summary table of major results of this chapter. Final biomass production, lipid production and biodiesel quality parameters (IV, iodine value, CN cetane number, and CFPP, cold filter plugging point) are reported as means and standard deviations (SD).

	Biomass (g L ⁻¹)		Lipid production (%)		IV		CN		CFPP	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
W300	0.794	0.13	40.04	6.13	87.22	5.73	59.69	6.62	9.58	0.61
W600	0.719	0.09	31.57	6.17	72.75	3.70	57.42	0.71	11.87	0.88
R300	0.666	0.1	40.75	3.57	92.41	3.24	54.60	2.50	6.64	0.47
B300	0.401	0.03	34.71	1.74	74.69	0.72	57.27	0.16	20.10	3.43
R100-B300	0.652	0.14	31.03	3.22	78.15	7.69	56.33	1.58	8.96	1.15

4.3 Chapter 4.3: Two-step cultivation on Mixed Dairy Effluent, an overview of the context

4.3.1 Aim of the chapter

In this chapter, a new approach is proposed to increase biodiesel quality and bioremediation levels through a two-step process. Specifically, distinct species have been employed in the two phases, selected according to their physiological behaviors and FAME profiles. In the first step, *C. sorokiniana* and *T. obliquus* were cultivated both individually and in co-culture due to their high adaptability to polluted environments, high growth rate, biomass and lipid accumulation. The second step employs only one species, *N. oceanica*, because it is known for its high lipid productivity under N-starvation conditions but characterized by lower tolerance to polluted substrates. The co-cultivation in the first step was investigated not only to evaluate its impact on nutrient removal kinetics but also due to its relevance in industrial, non-sterile systems. The choice of using different species in first and second step enabled us to obtain higher quality of biodiesel by mixing two biomasses with increased lipid productivity. Due to the different compositions of FAMES in *N. oceanica* compared to other species by mixing biomasses the oxidative stability and ignition performance of biodiesel are enhanced.

The research activity described in this chapter has been conducted at the University Collage of Dublin (UCD, Dublin, Ireland), under the kind supervision of Professor Ronald Halim.

4.3.2 Cell production on MDE

4.3.2.1 MDE composition

A brief description and discussion of composition of Mixed Dairy Effluent is reported to introduce the waste used in this study.

MDE results from all wastewaters of a dairy industry blended, containing washing waters from all plants (Figure 24). These washing water derive from different processes: butter processes, the evaporation of water from skim milk, coagulation of casein, nanofiltered raw whey and concentrated nanofiltered whey. They have different specific characteristics in terms of pollutant content and they result in a mixed effluent with 65 mg L⁻¹ of ammonia, 54 mg L⁻¹ of phosphates and a Chemical Oxygen Demand (COD) of 1526 mg/L O₂. COD is medium high if compared to Italian dairy wastewater used in the present thesis (See paragraph 4.1.2). Indeed, Dairy washing Waters (DWW) from Italian industry measured 555 mg/L O₂, while scotta (SC) is characterized by high COD of 49600 mg/L O₂ (Table 1). Nevertheless, the content of ammonia and phosphates are relatively

low, as the effluent consists of a mixture of streams, including plant washing waters which contribute to diluting the overall contaminant load. The resulting ratio N/P is 6.3 which is comparable to the one of 3N BBM, which is 8.8, meaning that MDE, in terms of N/P is balanced for microalgal growth, even with relatively low concentrations.

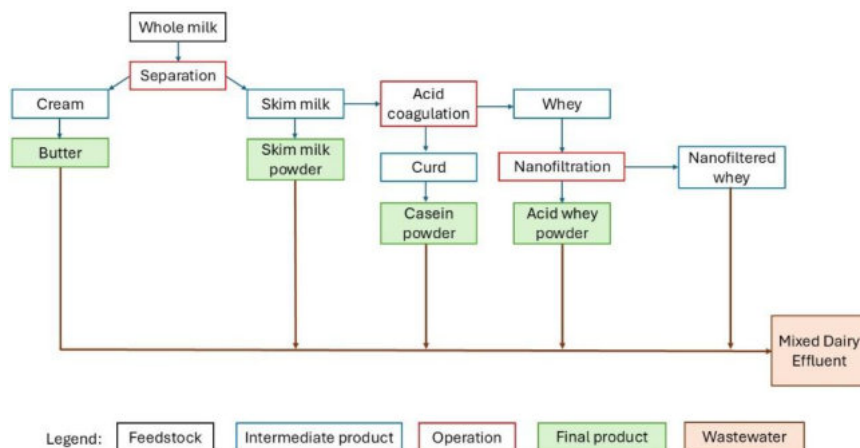


Figure 24: Dairy industrial process and wastewater generation.

4.3.2.2 Microalgal growth

Cell reproduction in dairy wastewater and in standard medium have been compared. Reference condition (i.e. modified-BBM) was obtained using the same initial concentrations of nitrogen and phosphorus as Mixed Dairy Effluent (MDE), which are 54 mg L⁻¹ of phosphates and 65 mg L⁻¹ of ammonia. The experiment was conducted for 6 days, after the complete consumption of nitrogen in control condition. Overall, higher cell production was observed for microalgae cultivated in MDE. Specifically, *C. sorokiniana* grew 1.4 times more in MDE compared to modified-BBM, *T. obliquus* 7.8 times and the co-culture 4.1 times (Figure 25). The major difference between two conditions is the presence of carbon sources derived from dairy production, which stimulates the mixotrophic metabolism, and the nitrogen source which is ammonium in MDE and nitrate in modified-BBM. Thus, the significant higher reproduction of microalgae using stream compared to standard media is not surprising, in fact the cultivation in standard media lacks organic carbon and it is known that the biomass accumulation is boosted in mixotrophic metabolism (Abreu et al., 2012). As evidence, *C. sorokiniana* grown in dairy wastewater showed two times cell production than standard media (Hamidian & Zamani, 2022a). Nevertheless, some studies reported that the utilization of 100% of streams can inhibit or decrease the growth of microalgae (Chandra et al., 2021; Sudhanthiran & Perumalsamy, 2024). Several factors are involved in this phenomenon, such as the complexity of wastewater, the tolerance of pollutants or the inoculum size. For instance, the inoculum size highly affects the initial lag phase and the overall growth of microalgae. To demonstrate that, in

a previous study *C. vulgaris* was cultivated on different dilutions of dairy effluent starting with two inoculum sizes, 13×10^6 cells mL⁻¹ and 26×10^6 cells mL⁻¹. With the lower inoculum size *C. vulgaris* was able to grow on 25% of effluent, while with double the inoculum size, growth was recorded even at 75% of dilution (Khalaji et al., 2023). In present work, the inoculum was performed around 1.5×10^6 cells mL⁻¹, which is more than 10 times lower than reported by Khalaji and colleagues, but it was enough to ensure a good growth of *C. sorokiniana* and *T. obliquus* on 100% MDE (Figure 25). Once again, the high variability when dealing with wastewater is emphasized. Compared to literature, the wastewater used in this study (MDE) was demonstrated to have a balanced nutrient composition for microalgal growth, allowing high growth rate without necessity of dilution prior cultivation, meaning that there are not pollutants at toxic concentrations. In addition, the selection of the species is also crucial, and our results confirm that *C. sorokiniana* and *T. obliquus* are high-performing species for wastewater treatment. In this context, Chandra and colleagues tested different dilution rates and species to screen best conditions for wastewater treatment (Chandra et al., 2021). They observed that *Chlorella minutissima* and *Scenedesmus abundans* grow better than *Nostoc muscorum* and *Arthrospira platensis* on wastewater for all dilutions tested, with the higher productivity at 70% of dairy wastewater.

In this chapter a different method to monitor the growth of microalgae was adopted due to the implementation of co-cultivation. The aim was to discriminate between *C. sorokiniana* and *T. obliquus* using flow cytometry, thus the growth was quantified in terms of cell production (cells mL⁻¹). By deeper analyzing data from the first step of cultivation, different behaviors were observed in two conditions where MDE limited cell production of *C. sorokiniana* compared to *T. obliquus* and co-culture, being 10.4 , 12.6 and 12.1×10^6 cells mL⁻¹, respectively. While in modified-BBM the opposite happened, with significantly higher cell production of *C. sorokiniana* followed by co-culture and *T. obliquus*, being 7.4 , 2.9 and 1.6×10^6 cells mL⁻¹, respectively (Figure 25). These results show that in MDE compared to standard medium, *T. obliquus* draw a more marked increase in cell production than *C. sorokiniana*.

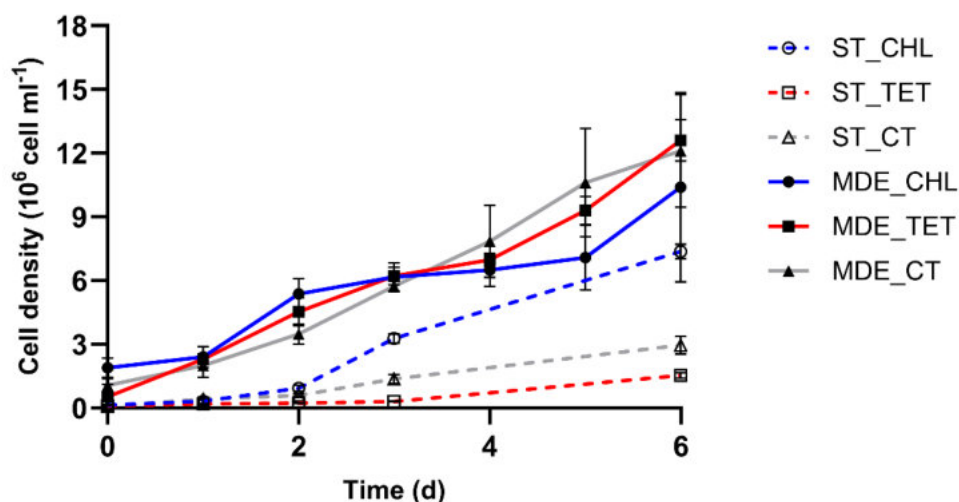


Figure 25: growth profiles in terms of cell number of *C. sorokiniana* (CHL), *T. obliquus* (TET) and co-culture (CT) in standard media (dashed lines, ST) compared to MDE (solid lines) in the first step of the process. Error bars refer to SD.

Co-culture was performed to understand the synergic nutrients consumption during cultivation of two species compared to single-species cultivation and the effects of final effluent on *N. oceanica* FAME profile. Based on the dimension and autofluorescence of the cells, two populations have been distinguished from one another (Figure 26). Firstly, whole cells have been selected by discarding cellular debris using FSC-A and then they have been divided into populations based on autofluorescence characteristics. To do so, the signals from R660-APC-A (which detects Chlorophyll fluorescence at 660 nm) and Y585-PE-A (which is typical for pigments fluoresce at 585 nm) channels have been compared. Monocultures were also used as a reference for more precise identification. Specifically, it was observed that on one hand *C. sorokiniana* showed slightly lower signal in both channels, appearing more defined and with less variations in dimension and pigments and it was identified with the blue gate in Figure 26c,f. On the other hand, *T. obliquus* showed higher variability in dimension and higher accessories pigment content and is represented by red gate in Figure 26c,f. Given the slight differences between the two species, the separation into two populations is approximative and it is not possible to discuss it accurately. Following these considerations, the distribution of two strains was measured at the end of the cultivation, when the separation is evident due to higher cell numbers. In this case, in modified-BBM *C. sorokiniana* accounted for 45% and *T. obliquus* for 50% of the cells while with MDE they are 36% and 62%, respectively. Besides that, due to the difficulties in clearly discriminating against two populations, co-cultures are considered only as total number of cells.

The combination of two species can be helpful to maximize biomass production when microalgae are cultivated in wastewater, in fact when a co-

cultivation of *C. minutissima* and *S. abundans* was performed, biomass production was almost doubled using 70% of dairy wastewater (Chandra et al., 2021). Moreover, another study used the consortium of *Chlorella* sp. and *Scenedesmus* sp. for the treatment of municipal wastewater obtaining higher biomass accumulation in all dilution tested compared to single-species cultivation and high remediation capacity (Koreivienė et al., 2014).

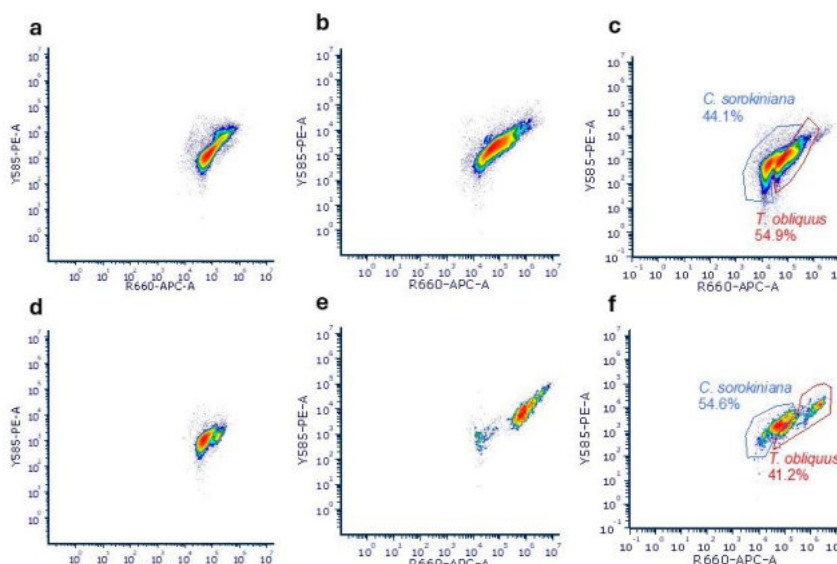


Figure 26: Flow cytometry plots at the end point of the first step of cultivation of *C. sorokiniana* (a, d), *T. obliquus* (b, e) and co-culture (c, f). a) MDE_CHL; b) MDE_TET; c) MDE_CT; d) ST_CHL; e) ST_TET; f) ST_CT.

4.3.2.3 Nutrient consumption

In this work nitrogen, phosphorous and COD were monitored during cultivation experiments. COD was measured at the beginning and at the end of the first stage, while after the cultivation of *N. oceanica* it was not possible due to the presence of salts in the medium, which interferes with the measurement.

The initial COD of MDE was 1526 mg L⁻¹ O₂ while at the end of the first stage 943, 551 and 401 mg L⁻¹ O₂ for MDE_CHL, MDE_TET and MDE_CT were respectively measured. The decrease of 38, 64 and 74% of COD in three tested conditions is significant, and a marked difference is observed for two species. It is evident how the co-culture drove to the highest reduction in COD, being an interesting candidate for remediation, followed by *T. obliquus* and *C. sorokiniana* single species cultivations. *Tetradesmus* sp. already demonstrated good performance in reduction of COD of dairy wastewater, specifically it reached 80% decrease when cultivated on whey (de Andrade et al., 2023) and 81% removal on pretreated digestate from dairy industry (Popovich et al., 2025). Moreover, Gupta and colleagues observed higher COD removal in *Scenedesmus obliquus* compared to *C. sorokiniana* (Gupta et al., 2016). Indeed, *C. sorokiniana* was able to decrease COD of wastewater with less efficiency. Indeed, in dairy

wastewater 57% reduction of COD was measured (Hamidian & Zamani, 2022a), and 65% when cultivated on 100% dairy wastewater (Kusmayadi, Lu, et al., 2022). Overall, the different capacity for COD reduction of two tested species is confirmed by literature, although present work had lower efficiency, especially for what concerns *C. sorokiniana*. These differences rely on the distinct composition of streams, even when derived from the same industrial sector.

Nitrogen and phosphorus removal from substrates during both phases of the process are reported in Figure 27. Interestingly, some differences were observed between inocula and substrates used. Nitrogen was consumed in the first step for both conditions in all inocula tested, with the difference that in modified-BBM it was completely consumed in 4 days, while in MDE in half the time (Figure 27). This is also in accordance with cell production, where in standard media an initial lag phase of two days was reported and an increase in cell production started from day 3 for all inocula (Figure 25). This phenomenon can be attributed to the different compositions of the substrate and mainly, to the different nitrogen sources. In fact, it was demonstrated that ammonia is preferred to nitrate and the uptake is faster because it requires less energy consumption for the assimilation (Lizzul et al., 2014; Ramanna et al., 2014).

Phosphorous showed major variations between experiments. Again, faster consumption was observed with MDE, where in two days the majority of P was consumed in the first step of cultivation for all conditions tested. In modified-BBM instead, steady consumption was observed until the end of the cultivation, with the difference that *C. sorokiniana* consumed it for three days and then remained constant (Figure 27). The higher consumption of phosphorous in mixotrophic mode is not new, in fact it is demonstrated that the presence of carbon sources in substrate can boost phosphorous consumption (Wu et al., 2021). In detail, phosphate at the end of the first stage was around 0.04 mM (i.e. 5 mg L⁻¹) with *T. obliquus* and co-culture in modified-BBM, 0.26 mM (i.e. 24 mg L⁻¹) with *C. sorokiniana* in modified-BBM, while in MDE it decreased to 0.07 mM (i.e. 7 mg L⁻¹) for all conditions (Figure 27a,b). Daily consumption of N and P is reported in Table 11. The bioremediation was successfully performed in all conditions, reaching more than 99% of nitrogen consumed in all conditions tested while phosphorous was decreased by 88%, 87% and 88%, respectively for *C. sorokiniana*, *T. obliquus* and co-culture in MDE. This result is comparable to modified-BBM where nitrogen was depleted for 99% too, while phosphorus was reduced by 53%, 92% and 89% respectively for *C. sorokiniana*, *T. obliquus* and co-culture (Figure 27a,b). These remediation levels are similar or higher than previously reported by other studies, where on average N and P were assimilated in the ranges of 70-50% and 80-67% with *C. sorokiniana* (Kusmayadi, Leong, et al., 2022; Melo et al., 2022) and 65% of N and 57% of P with *T. obliquus* (Ravi Kiran & Venkata Mohan, 2022).

A different behavior was observed by Talha et al., who cultivated *C. sorokiniana* in several dilutions of dairy wastewater (Talha et al., 2025). They reported complete P consumption and 50-75% N removal, with decreasing uptake at higher wastewater concentrations. This contrast can be explained by the different initial N concentrations of the two streams, which was 560 mg L⁻¹ in Talha and 51.2 mg L⁻¹ in MDE (65 mg L⁻¹ of ammonia). The lower N consumption observed by Talha et al. is likely due to inhibitory effects of high N concentrations and to an imbalanced N/P ratio, also demonstrated in present study in Figure 11 (Mazzocchi et al., 2025). In fact, in their study, P was the limiting nutrient, whereas in the present study N limitation occurred, highlighting the importance of both initial N/P ratio and the concentration of N.

The consumption of N/P is species-specific and derives from the stoichiometry of the strain, thus the remediation quality is highly affected by the chosen species (Chandra et al., 2021; Fernandes et al., 2017). In this context the utilization of consortium or co-cultures instead of single-species culture gives high advantages in pollutant removal. For this purpose, the consortium should use complementary species to improve biomass production and bioremediation (Cardinale, 2011; Fernandes et al., 2017; Wilkie, 2002). Previous study tested *C. minutissima* and *Scenedesmus abundans* on dairy wastewaters and, within 10 days, 56% of N and 83% of P were consumed, slightly higher than monocultures (Chandra et al., 2021). Our co-culture outperformed cited study with 99.7% and 87.7% removal of N and P, respectively.

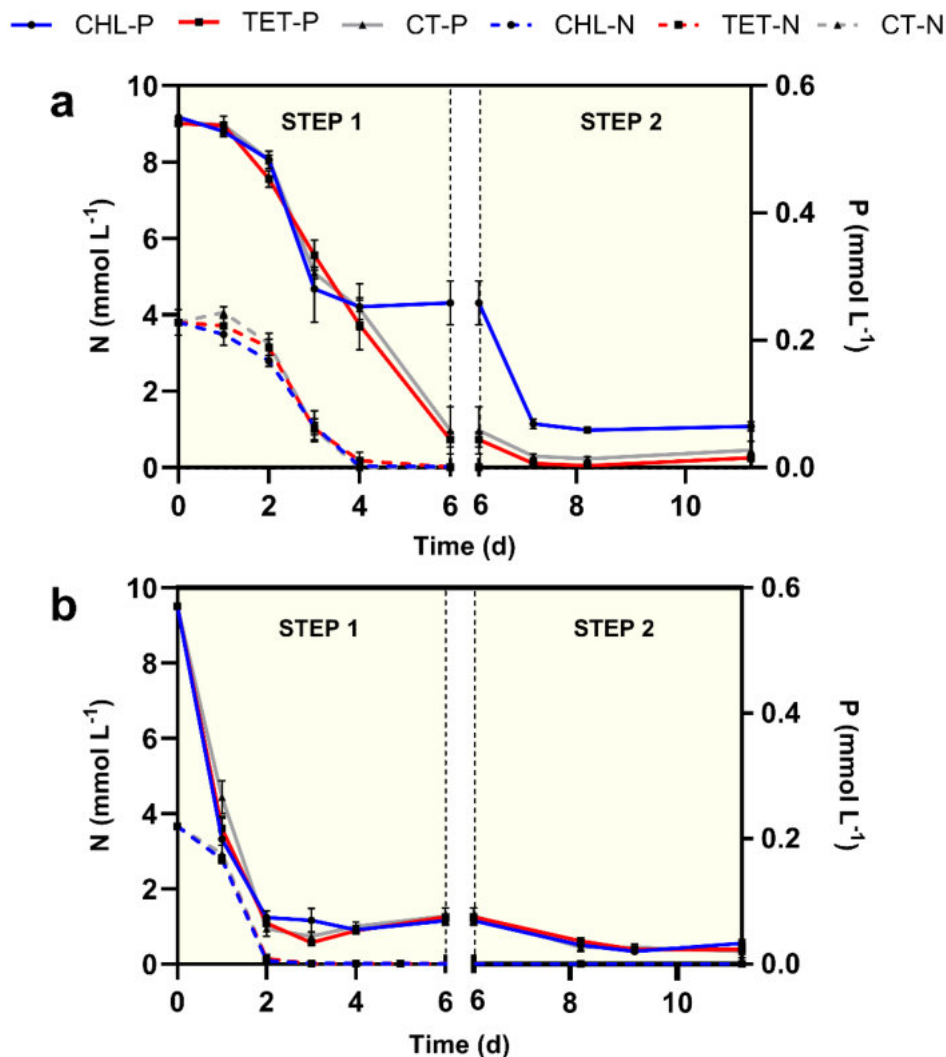


Figure 27: Nitrogen and phosphorous consumption is depicted in graphs. Graphs a) refers to cultivation conducted on modified-BBM, while b) to cultivation on MDE. The consumption during the two-step process is reported as mean of three biological replicates and error bars define standard deviations.

Table 11: kinetics of N and P removal in 3 days of cultivation on MDE of *C. sorokiniana* (CHL), *T. obliquus* (TET) and co-culture (CT). Data are reported as average and standard deviation.

Day	mmol N L ⁻¹ d ⁻¹						mmol P L ⁻¹ d ⁻¹					
	CHL		TET		CT		CHL		TET		CT	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
1	0.877	0.09	0.876	0.06	0.722	0.18	0.372	0.03	0.355	0.01	0.305	0.02
2	2.698	0.11	2.641	0.04	2.794	0.10	0.125	0.03	0.151	0.01	0.210	0.03
3	0.105	0.06	0.136	0.07	0.135	0.10	0.011	0.01	0.031	0.01	0.012	0.01

4.3.2.4 Second step: *N. oceanica* under nutrient depletion condition

To perform the second step of the process, *N. oceanica* was cultivated on standard medium f/2 for one week, then harvested to be inoculated into the supernatant derived from the first step. Here, cell density was monitored every day, and no significant differences among conditions were observed, with an increase of approximately 3×10^6 cells mL^{-1} in 5 days in all tests (Figure 28). As previously discussed, the first step drove to a strong depletion of phosphorous and nitrogen. The second step was designed to enhance lipid accumulation, therefore, strong cell accumulation was monitored but was not the primary focus. In this scenario, the 99% reduction of nitrogen operated by *C. sorokiniana* and/or *T. obliquus* in the first step, is expected to promote lipid accumulation in *N. oceanica* biomass in the second step. Moreover, phosphorus plays an essential role in both steps, enabling biomass accumulation in the first one and avoiding a double-stress, N and P, in the second one. Indeed, an adequate P concentration is required to maintain cells metabolically active during N-starvation, enabling efficient lipid accumulation (Chu et al., 2019). Thus, the presence of phosphorous in second stage is essential to sustain cellular metabolism and enable lipid accumulation (Wu et al., 2021). Accordingly, a further reduction of phosphorus was observed during this step in all conditions. In modified-BBM, during second step, phosphorus further decreased by 21.4%, 8.1% and 12.7% for *C. sorokiniana*, *T. obliquus* and co-culture respectively, whereas in MDE the reductions were 13.5%, 14.2% and 15.2% (Figure 27). These reductions drove to almost complete P removal in all cases, except for ST_CHL where 6 mg L^{-1} of PO_4 remained. These results will be compared to lipid productivity and FAME profiles to assess the impact of the different conditions of first step.

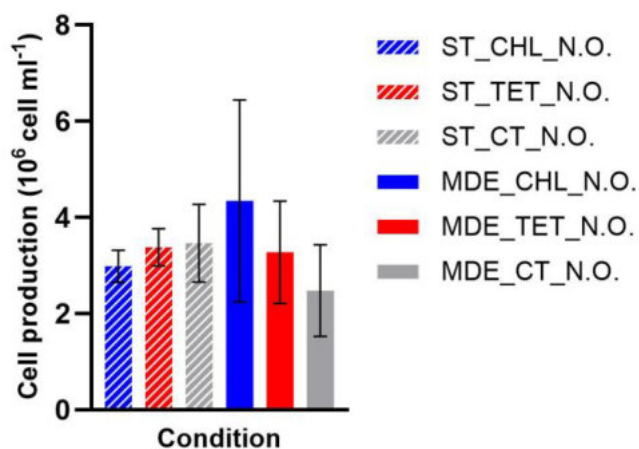


Figure 28: cell production of *N. oceanica* during second phase of the process. Striped bars represent cultures grown in modified-BBM (ST), while solid bars represent cultures grown in MDE. CHL, TET and CT refers to the first step of the cultivation. Error bars refer to SD.

4.3.3 Lipid production in first and second step of cultivation

Lipid production has been assessed for all biomasses accumulated at the end of each step and is reported in Table 12. In the first step, three different kinds of biomasses have been collected from single and co-cultivation. When *C. sorokiniana*, *T. obliquus* and the co-culture grew on MDE, 24.58, 27.14 and 23.37 % of lipids on dried biomass were measured respectively. While 26.88, 25.99 and 23.81% of lipids were observed when species have been respectively grown on modified-BBM (Table 12). These results highlight that there are no statistically significant differences between lipid accumulation within biomasses cultivated on modified-BBM compared to MDE, highlighting that the overall composition of MDE did not have negative impact on metabolism of microalgae. Moreover, lipid production of co-culture resulted to be lower than that one of single strain biomasses. A decrease in lipid production in mixed culture compared to single strain cultivation was already reported with *Scenedesmus obliquus* (Qu et al., 2019). In mixed cultures, competition mechanisms can be stimulated and, consequently, changes in metabolisms are activated. The co-cultivation of different microorganisms (for example microalgae and yeast or bacteria) shows the advantage of using different sources of nutrients and energy, resulting in less competition (Kumsiri et al., 2021; Padri et al., 2022). On the other hand, the same microorganism in co-cultivation competes for nutrients, such as nitrogen and phosphorus in the case of microalgae, or light for photosynthesis. This competition may lead to a reduction in biomass production and a modification of cellular composition (Lin et al., 2024; Zhao et al., 2014). However, this phenomenon is species-specific and microalgae may respond with higher lipid content or they can favor carbon flux toward cell growth. For this reason, the effect of co-cultivation of microalgae depends highly on the species, as well as the culture conditions. Lipid content in microalgae can be enhanced or reduced as energy storage compounds and the change in composition of FAME is related, for example, to different characteristics of cellular membrane, to improve nutrient uptake or light absorption (Zhao et al., 2014).

Similar result, in terms of lipid accumulation, was reported for *Chlorella pyrenoidosa* cultivated on standard medium, which produced 20% of lipid in six days (Chu et al., 2019), while higher production was observed by Asadi and colleagues, with 31 and 35% of lipids in *C. sorokiniana* cultivated on two dairy effluents for ten days (Asadi et al., 2020). In contrast with our data, another study observed an increase in lipid production of 13% of dried biomass of both *C. sorokiniana* and *T. obliquus* cultivated on dairy wastewater compared to standard medium (Gupta et al., 2016). However, different behaviors were observed in the present study, where lipid accumulation was comparable between cultures grown on MDE and on modified-BBM (Table 12). This is likely due to the equal availability of nitrogen and phosphorus in both conditions, as modified-BBM was

formulated to have the same molar concentrations of N and P present in MDE. As a result, similar overall quantity of nutrients was consumed in both substrates, i.e. MDE and standard medium, at the end of the cultivation, leading to comparable final lipid accumulation. Differently, in other studies, nutrient-rich conditions of standard media favor biomass growth over lipid storage, resulting in lower lipid production compared to wastewater. Indeed, in-depth analysis of lipid production demonstrated how microalgae increase the accumulation of lipids after nitrogen consumption, confirming that in nutrient-rich medium lipid accumulation occurs later (Li et al., 2015). Consistently, by increasing cultivation time to 28 days, *C. sorokiniana* could deplete nutrients and reach the stationary phase and thus exhibited similar lipid content when grown on wastewater or standard medium (Hamidian & Zamani, 2022a).

The cultivations of *N. oceanica* on effluents derived from the first step led to differences in lipid production. More in detail, the streams after *T. obliquus* and co-culture cultivated on MDE, compared to modified-BBM, lead to the highest lipid accumulation in *N. oceanica*, which were 43.89 and 43.56 %, compared to 39.66 and 40.33 % of lipid in modified-BBM (Table 12). Furthermore, in all tested conditions, the lipid content of *N. oceanica* increased after the second cultivation step under nitrogen starvation, compared to the initial lipid content of the biomass used as inoculum for this phase. Specifically, an increment of 8.5, 13.3 and 13.0% of lipid on dried biomass was recorded for MDE_CHL_N.O., MDE_TET_N.O. and MDE_CT_N.O., while 9.1, 9.1 and 9.7 % for ST_CHL_N.O., ST_TET_N.O. and ST_CT_N.O., respectively. The effect of nitrogen starvation on lipid productivity is well documented in *Nannochloropsis* sp., being the model photosynthetic microorganism for studying lipid synthesis (Jia et al., 2015; Pal et al., 2011; A. Solovchenko et al., 2014). *N. gaditana* produced 40% of TAG after 9 days of N-starvation (Janssen et al., 2018). Rahimi also noted that the total lipid content of *Nannochloropsis* increased from 30% to 35% and 45% of dried biomass respectively after 5 and 12 days of N-deprivation (Rahimi et al., 2025), indicating N as the strongest factor affecting lipid accumulation. Consequently, a reduction in protein and carbohydrates content while lipid increases is commonly observed (Villanova et al., 2022).

Table 12: Lipid content (% DW) of biomasses collected in the first (*C. sorokiniana*, *T. obliquus* and co-culture of them) and second step (*N. oceanica*). Data are reported as means and standard deviations.

First step						
Strain	<i>C. sorokiniana</i>		<i>T. obliquus</i>		Co-culture	
	Lipid content (%)					
	Avg	SD	Avg	SD	Avg	SD
MDE	24.58	1.45	27.14	0.93	23.37	1.69
Modified-BBM	26.88	1.28	25.99	1.29	23.81	0.63
Second step						
Strain	<i>N. oceanica</i>					
Substrate	CHL-effluent		TET-effluent		CT-effluent	
	Lipid content (%)					
	Avg	SD	Avg	SD	Avg	SD
MDE	39.09	1.63	43.89	0.72	43.56	0.72
Modified-BBM	39.74	0.81	39.66	0.89	40.33	1.43

4.3.3.1 FAME profiles: first step

FAME profiles of *C. sorokiniana* and *T. obliquus* biomasses derived from first step are reported in Figure 29. Distinct behaviors between microalgae grown on MDE and modified-BBM can be seen by classifying fatty acids into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). Fatty acids with low unsaturation ensure high quality biodiesel, influencing combustion and ignition time (Francisco et al., 2010). In biomasses collected in the first step, SFA ranges between 24.7% (*T. obliquus* on MDE) and 31.1% (Co-culture on MDE) of total FA, measuring only slight fluctuations between conditions. Conversely, MUFA levels showed greater variability and were higher across all examined cultures when microalgae were grown on MDE, reaching a maximum value of 18.5% with *C. sorokiniana* (Figure 29b). PUFAs represent the most abundant component among fatty acids, accounting for around 66% for *T. obliquus* and around 62% for co-culture without differences in two substrates, whereas *C. sorokiniana* measured a decrease from 59.1% in modified-BBM to 52.9% in MDE (Figure 29b). The lowest PUFA content was observed in *C. sorokiniana* on MDE, likely caused by the accelerated nitrogen depletion, which alters metabolism of *C. sorokiniana*. When grown on MDE cells consumed nitrogen in half the time compared to modified-BBM (Figure 27). Nitrogen depletion promotes lipid accumulation and membrane lipids rich in PUFAs, such as phospholipids and glycolipids, are degraded toward the synthesis of neutral lipids, mainly TAGs, which are composed of fatty acids with lower degrees of unsaturation, resulting in a reduction of PUFA content. The presence of long-chain PUFA is particularly relevant for biodiesel production because they improve performances at low temperatures by enhancing cold-flow properties (Knothe, 2007). However, more unsaturation negatively affects oxidative stability. Thus, a balance in PUFA content is required to have a final

product with positive low-temperature performances while maintaining resistance to oxidative degradation.

All fatty acids detected in this study are composed of 16 or 18 carbon atoms, in accordance with previous investigations (Daneshvar et al., 2019; Hena et al., 2015; Negi et al., 2016). Here, the highest portions are represented by linoleic (C18:2), linolenic (C18:3) and palmitic (C16:0) acid (Figure 29a, Table 13). Clear species-specific differences emerged between two species particularly for linoleic, linolenic and palmitoleic acids. Specifically, in *T. obliquus* biomass linoleic acid is the predominant fatty acid, accounting for 54.3% and 61.7% of total FAME in MDE and modified-BBM, respectively. In contrast, *C. sorokiniana* showed significant lower portions of linoleic acid (24.6% in MDE and 23.7% in modified-BBM), while linolenic acid was substantially higher reaching 28.3% and 35.4% in MDE and modified-BBM respectively, against 10.2% and 4.6% in *T. obliquus* (Figure 29a, Table 13). Moreover, palmitoleic acid is present in different portions between species, with higher contributions of *C. sorokiniana* (12.7% in MDE and 12.0% in modified-BBM) compared to *T. obliquus* (8.3% and 3.8%).

The FAME profile in *C. sorokiniana* is consistent with previous studies, in which the dominant fatty acids were linolenic acid, linoleic and palmitic acids (Negi et al., 2016). Moreover, this result is in accordance with previous analysis of present thesis with some differences. Indeed, *C. sorokiniana* cultivated on standard condition and under different light regimes with dairy wastewater showed abundance of linoleic and palmitic acids, even though linolenic acid was not detected (Figure 16, Figure 22). *T. obliquus*, instead, showed greater sensitivity to cultivation substrates, drawing clear variations between MDE and modified-BBM. This substrate-dependent effect is in line with findings of Darki work, who observed significant differences between three distinct media (Darki et al., 2017).

A more detailed comparison with literature data reveals that the composition of the substrate for *T. obliquus* mainly affects C16 fatty acids. In agreement with Girard et al., wastewater supplementation, with consequent increase in nitrogen content and shift to mixotrophic cultivation, brought to an increase in C16:1, while C16:0 remained unaffected compared to standard condition; moreover they reported no differences in C18:3 and an increase in C18:2 when *T. obliquus* was cultivated in wastewater (Girard et al., 2014). Conversely, in the present study, C18 fatty acids displayed opposite trends, with higher portions of C18:2 in modified-BBM compared to MDE, while C18:3 revealed higher values in MDE (Figure 29a). This partial divergence from trends in these studies may be due to the different overall composition of substrate, and to the cultivation conditions. Indeed, results obtained by Girard and colleagues are referred to the cultivation of microalgae in 40% wastewater diluted in BBM medium, thus 60% of the substrate derived from a balanced standard medium, giving nutrient support from a balanced medium. In this context both macro- and micro-elements availability

may influence microalgal metabolisms, including lipid profile. Except more deeply investigated macronutrients like nitrogen and phosphorous, variations in trace elements such as iron and zinc have been associated to increased MUFA and PUFA, suggesting their potential in modulating FAME profiles (Mandal et al., 2019; Sun & Huang, 2017). Furthermore, the rise of PUFA content when microalgae are cultivated on MDE is confirmed by FAME profiles. Indeed, *T. obliquus* and co-culture characterized by more C16:1 and C18:3 and less C16:0 and C18:2, while *C. sorokiniana* was more conservative between conditions, with differences limited to a reduction of C18:3 when cultivated on MDE (Figure 29). The increase in PUFAs during mixotrophic cultivation compared to phototrophic is well documented (Dubey et al., 2024; Enwereuzoh et al., 2020; Ruiz-Marin et al., 2018) but the metabolic mechanism under this phenomenon is often not mentioned. Considering that mixotrophy involves both photosynthesis and respiratory metabolism, cells are expected to incur structural and functional adjustments of membranes, including thylakoids and mitochondrial membranes. In this context, an increased appression of thylakoid under mixotrophic condition was demonstrated, suggesting membrane remodeling (Dubey et al., 2024). Therefore, it can be hypnotized that, when organic carbon is supplied, membranes are reorganized and may require more PUFAs to maintain fluidity and functionality, explaining the increment of this class of fatty acids in mixotrophic cultivation.

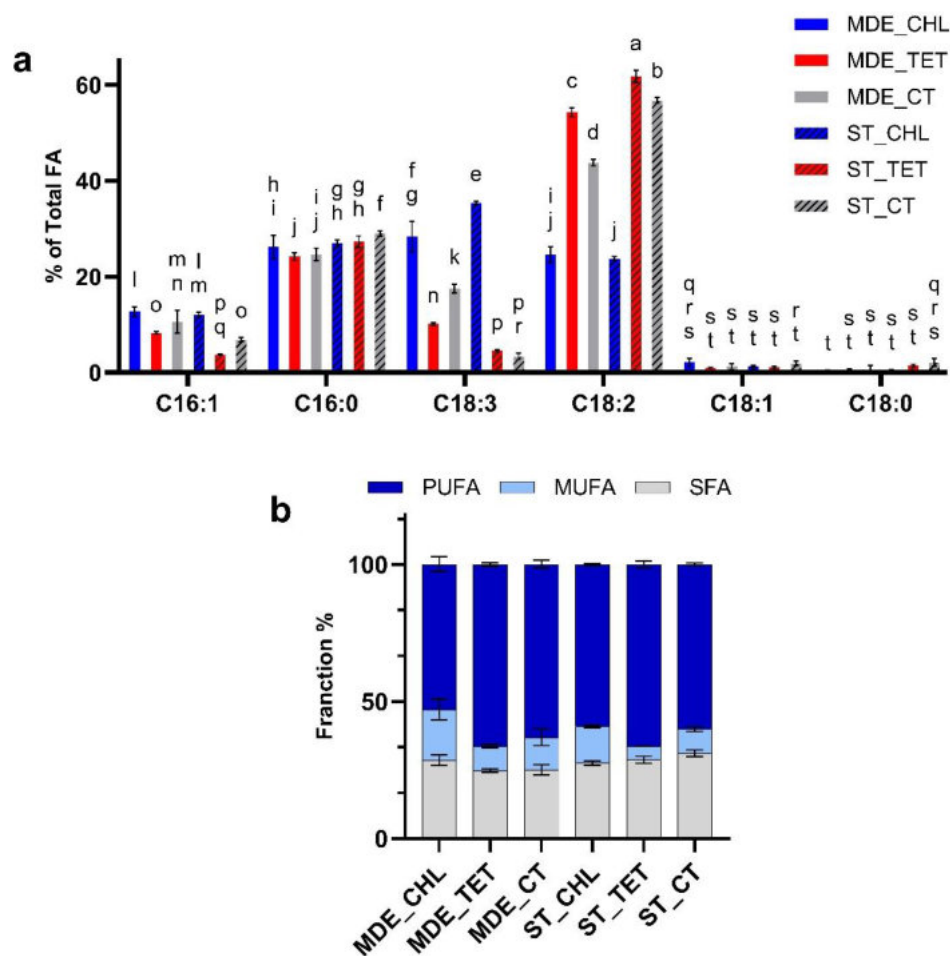


Figure 29: FAME profile of *C. sorokiniana* (CHL), *T. obliquus* (TET) and co-culture (CT) grown in MDE (solid bars) and modified-BBM (striped bars) (a). Fractions of SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids) are reported in graph b). Letters in graph a) separate data into statistically different groups, with $p < 0.05$.

Table 13: FAME profiles of *C. sorokiniana*, *T. obliquus* and co-culture cultivated both in MDE and modified-BBM. Percentage of total FAMES are reported as means and standard deviation.

FA	<i>C. sorokiniana</i>				<i>T. obliquus</i>				<i>C. sorokiniana</i> + <i>T. obliquus</i>			
	Mod.-BBM		MDE		Mod.-BBM		MDE		Mod.-BBM		MDE	
Formula	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
C14:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C16:1	12.04	0.39	12.73	0.83	3.77	0.12	8.28	0.22	6.83	0.39	10.61	1.97
C16:0	26.95	0.59	26.19	1.98	27.27	1.02	24.17	0.63	29.04	0.48	24.65	1.06
C17:1	0.00	0.00	3.66	1.78	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C17:0	0.00	0.00	2.07	0.75	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C18:3	35.38	0.34	28.34	2.64	4.56	0.21	10.16	0.21	3.35	0.56	17.51	0.77
C18:2	23.72	0.36	24.59	1.30	61.74	1.09	54.32	0.73	56.77	0.47	43.80	0.59
C18:1	1.35	0.13	2.13	0.70	1.17	0.16	1.03	0.02	1.94	0.39	1.25	0.52
C18:0	0.57	0.11	0.28	0.20	1.48	0.21	0.53	0.17	2.07	0.66	0.64	0.64
C20:4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C20:5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C24:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Σ SFA	27.52	0.65	28.55	1.59	28.75	1.04	24.70	0.48	31.11	1.01	25.08	1.52
Σ MUFA	13.39	0.43	18.52	3.10	4.94	0.05	8.97	0.57	8.77	0.76	11.86	2.48
Σ PUFA	59.09	0.32	52.93	2.23	66.31	0.99	66.33	0.55	60.12	0.34	63.06	1.15

4.3.3.2 FAME profiles: second step

The second step of cultivation involved a single strain, *N. oceanica*, cultivated under N-starvation on effluents derived from the first step. Species-specific differences in FAME profiles have been detected between *N. oceanica* and two strains used in first step. Actually, the most abundant fraction of FAME was represented by SFA for *N. oceanica*, while PUFAs prevailed in *C. sorokiniana* and *T. obliquus* biomasses (Figure 29a; Figure 30). Within the second cultivation step, effluents compositions significantly influenced FAME distribution in *N. oceanica*. Specifically, effluent after *C. sorokiniana* cultivation drove to a decrease in SFA and an increase in PUFA in *N. oceanica* compared to effluents from *T. obliquus* and co-culture. However, between MDE and modified-BBM some differences were detected. Indeed, enhanced MUFA and reduced SFA were detected in *N. oceanica* grown on MDE compared to modified-BBM (Figure 30).

Moreover, second step drove to a general reduction of PUFA and slight increase in MUFA and SFA when compared to *N. oceanica* composition before N-starvation. It was well demonstrated that the decrease in nitrogen, or the complete depletion of it, leads to an increment of SFA and MUFA, while PUFA decreases (Xiao et al., 2013). These positive accumulation of SFA and negative of PUFA were also observed when *N. oceanica* was cultivated on N-depleted dairy wastewater (whey-permeate) (Kiani et al., 2024). The decrease of PUFA is associated with a metabolic shift in microalgae under N depletion which stimulates the accumulation of storage compounds such as triacyl-glycerides (TAG). TAGs are mainly composed of SFA and MUFA, while PUFA are prevalent as structural lipids which contain nitrogen and therefore are no longer synthesized under N-limited condition (Simionato et al., 2013; Sreelakshmi &

Arunkumar, 2025). The increase in Neutral lipids (which include the majority of TAG) rich in SFA and MUFA under N-starvation, derives both from *de novo* synthesis of TAG and conversion of polar lipids, thus affecting the content of PUFA also (Xiao et al., 2013). Moreover, *de novo* synthesis focuses on the production of short chain fatty acids with low levels of unsaturation, due to the less energetic requirement for the synthesis of these kinds of FAs (Kiani et al., 2024).

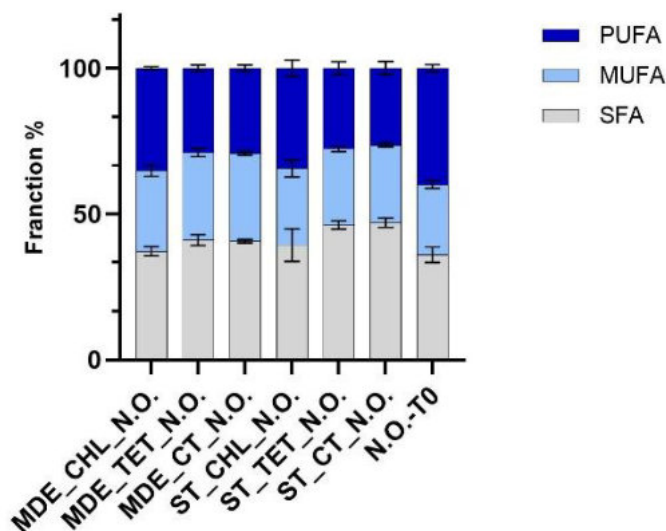


Figure 30: Fractions of SFA (saturated fatty acids), MUFA (monounsaturated fatty acids) and PUFA (polyunsaturated fatty acids) in *N. oceanica* grown on modified-BBM (ST) or MDE, after the cultivation of *C. sorokiniana* (CHL), *T. obliquus* (TET) or the co-culture (CT). Data are reported as means and standard deviation.

More in detail, *N. oceanica* FAME profile is mainly composed of palmitic and palmitoleic acids followed by linoleic and eicosapentaenoic acids (EPA). EPA is an essential fatty acid widely recognized for its nutraceutical and pharmaceutical application in human diet, particularly due to its cardio-protective effects (Rao et al., 2020; Uthaiah et al., 2025). EPA is primary found in seafood, however, its consumption does not meet the recommended dietary intake, thus requires supplementation. Microalgal-derived EPA represents a promising and more sustainable alternative to fish oil. *Nannochloropsis* sp. is known for its high EPA content, making it of significant industrial interest. The increase in EPA content in *Nannochloropsis* sp. is still a challenge for scientific community (Janssen et al., 2019; Schöler et al., 2017).

In this study, as part of PUFA, EPA and linoleic acids decreased during the second step, in comparison to the seed culture (Figure 31). When cultivated on MDE after the cultivation of *T. obliquus* and co-culture, palmitoleic acid has been accumulated more compared to modified-BBM, while palmitic acid has been reduced. Moreover, despite the general diminishing in EPA after N-starvation, it is higher in biomasses grown on MDE compared to modified-BBM. Specifically,

it measures 17.20, 12.04 and 12.64% of total fatty acids for MDE_CHL_N.O., MDE_TET_N.O. and MDE_CT_N.O. respectively, whereas in modified-BBM it is 14.89, 10.14 and 8.87% respectively (Figure 31, Table 14). This result agrees with previous study of N-depletion with *Nannochloropsis* sp.. Indeed, a decrease in nitrogen leads to a decrease in EPA content, linearly with the decrease of total PUFA (Sukenik, 1991; Xiao et al., 2013). Again, a decrease from 30.4 to 16.33% of EPA was observed with *N. oceanica* grown on dairy waste in nitrogen depletion (Kiani et al., 2024).

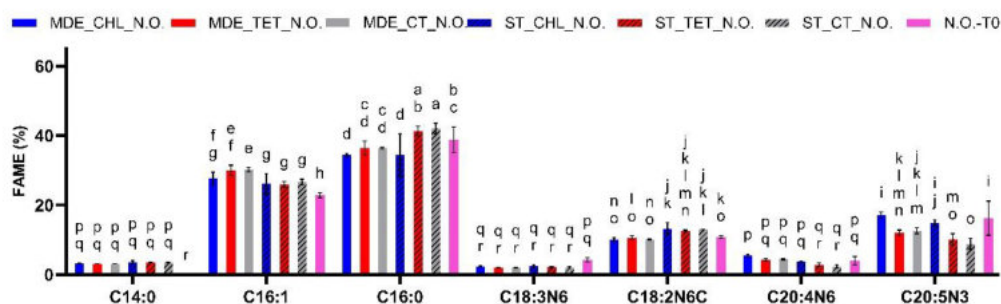


Figure 31: FAME profile of *N. oceanica* biomass is reported as percentage of each fatty acid on total FAME. MDE refers to the cultivation on Mixed Dairy Effluent, while ST on modified-BBM. *N. oceanica* was cultivated after the first step with *C. sorokiniana* (CHL), *T. obliquus* (TET) or the co-culture (CT). Data are the mean and standard deviation derived from three biological replicates. Letters indicate different statistical groups ($p < 0.05$).

Table 14: FAME profiles of *N. oceanica* cultivated on effluents of MDE and modified-BBM after the cultivation of *C. sorokiniana* and *T. obliquus*. Data are reported as average percentage of total FAME, with standard deviation (SD).

FA	<i>N. oceanica</i> Before N.-starvation		Effluent from <i>C. sorokiniana</i>				Effluent from <i>T. obliquus</i>				Effluent from <i>C. sorokiniana</i> + <i>T. obliquus</i>			
			Mod.-BBM		MDE		Mod.-BBM		MDE		Mod.-BBM		MDE	
Formula	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
C14:0	0.10	0.00	3.61	0.33	2.16	1.47	3.57	0.08	3.13	0.01	3.56	0.07	3.14	0.01
C16:1	22.87	0.60	26.08	2.38	27.59	1.53	25.99	0.66	30.02	1.18	26.59	0.69	30.15	0.53
C16:0	38.81	3.01	34.38	5.01	34.41	0.35	41.25	1.09	36.37	1.66	42.04	1.20	36.39	0.25
C17:1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C17:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C18:3	4.22	0.53	2.52	0.26	2.38	0.11	2.16	0.13	1.96	0.03	2.08	0.18	1.98	0.07
C18:2	10.85	0.30	13.20	1.44	10.05	0.46	12.55	0.22	10.62	0.42	13.01	0.03	10.11	0.12
C18:1	0.64	0.00	0.23	0.16	0.00	0.00	0.08	0.11	0.00	0.00	0.09	0.13	0.00	0.00
C18:0	1.09	0.48	0.89	0.14	0.43	0.03	1.19	0.11	0.84	0.10	1.17	0.08	0.90	0.11
C20:4	4.01	0.99	3.75	0.13	5.56	0.30	2.85	0.40	4.30	0.26	2.35	0.39	4.44	0.14
C20:5	16.23	4.01	14.89	0.66	17.20	0.60	10.14	1.34	12.04	0.63	8.87	1.30	12.64	0.69
C24:0	16.23	4.01	14.89	0.66	17.20	0.60	10.14	1.34	12.04	0.63	8.87	1.30	12.64	0.69
Σ SFA	41.61	5.76	39.34	4.52	37.21	1.30	46.23	1.14	41.07	1.55	47.02	1.31	40.68	0.47
Σ MUFA	23.08	0.34	26.31	2.42	27.59	1.53	26.07	0.71	30.02	1.18	26.68	0.60	30.15	0.53
Σ PUFA	35.31	5.50	34.35	2.28	35.20	0.41	27.70	1.84	28.92	0.95	26.30	1.86	29.17	0.94

4.3.4 Biodiesel quality estimation

FAME profile determines the quality of biodiesel derived from microalgal biomasses. Figure 32 reports three key parameters related to biodiesel performance: IV, CN and CFPP.

Cultivation on MDE changed the FAME profile, which had two opposite effects on different strains. In fact, ignition quality was raised in biomasses of *T. obliquus* and co-culture whereas it was reduced in biodiesel derived from *C. sorokiniana* (Figure 32a). However, the observed differences in CN (which is responsible for ignition performances) among substrates were limited to 1-2 units, reinforcing that the utilization of MDE did not have marked negative impact on quality of biodiesel. Additionally, oxidative stability was improved in *C. sorokiniana* grown on MDE (which changed from 146 to 133 gI₂ 100g⁻¹ on modified-BBM and MDE respectively) while it diminished in other biomasses. These opposite trends of *T. obliquus* and *C. sorokiniana* highlight once again the importance of selection of species for the specific process depending on the conditions tested, including the composition of substrate. Indeed, other studies observed different trends of *C. sorokiniana* biodiesel quality when grown on dairy wastewater. In fact, Hamidian & Zamani (2022b) reported a decrease of oxidative stability of biodiesel when *C. sorokiniana* was cultivated on undiluted dairy wastewater, while Talha and colleagues (2025) observed an increase of this parameter when biomass was grown on dairy wastewater, confirming our result with *C. sorokiniana* on MDE. In addition, ignition quality of 56 obtained on MDE is comparable with result of Hamidian and Zamani (57.5) and higher than what reported by Thala (51.9) (Hamidian & Zamani, 2022b; Talha et al., 2025).

Furthermore, the low-temperature performances of all characterized lipid extracts fall within acceptable range, with *C. sorokiniana* and *T. obliquus* showing better cold properties compared to *N. oceanica*. Specifically, the CFPP is lower than -4°C for *C. sorokiniana* and *T. obliquus*, while ranges between +1 and -5°C for *N. oceanica* depending on cultivation substrate (Figure 32a,b). *N. oceanica* exhibited a distinct behavior when grown on different substrates. Ignition quality was reduced when N-starvation was performed in MDE compared to modified-BBM.

In detail, CN calculated from the FAME profile of *N. oceanica* grown in standard medium prior N-starvation was 41, while after nutrient depletion CN increased to 46-52 in modified-BBM and 44-48 in MDE, indicating an overall improvement in the ignition quality of the resulting biofuel. Similarly, *N. oceanica* under N-starvation obtained good oxidative stability and ignition performances with values close to the limits (Touliabah & Almutairi, 2021).

After N-starvation of *N. oceanica*, biodiesel quality was changed. Indeed, oxidative stability and ignition quality are improved in all samples, being always within legislative ranges except for biomasses grown on effluents from *C.*

sorokiniana. In detail, oxidative stability raised in modified-BBM-grown cultures, compared to MDE, similarly to what was observed with other species in the first step. Moreover, ignition performances were enhanced after starvation, being always >47 (ASTM D6751), except for biomasses grown on effluents from *C. sorokiniana*, while biomasses cultured in modified-BBM are higher than 51, being acceptable for both European and American limits. This difference between modified-BBM and MDE is not surprising due to high variability of FAME profiles depending on the substrate. Indeed, a study comparing the quality of biodiesel produced from *N. oceanica* cultivated on four distinct wastewaters showed that only two of the tested streams were suitable for producing biodiesel. The authors measured IV ranging between 88 and 169 gI₂ 100g⁻¹ while CN between 38 and 56 (Mitra et al., 2016).

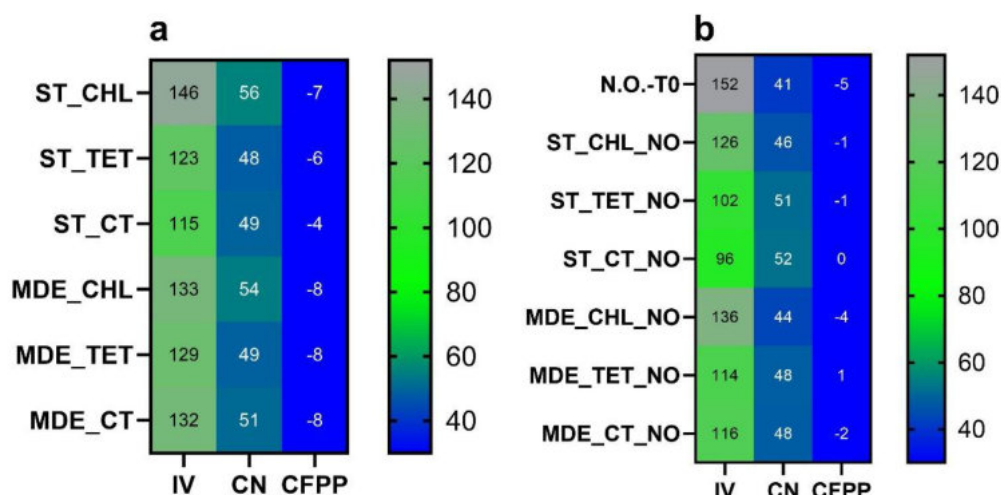


Figure 32: heat-map represents the variations of oxidative stability (IV), ignition performances (CN) and low-temperature performances (CFPP) for all biomasses. ST refers to microalgae that are cultivated on modified-BBM while MDE on Mixed Dairy Wastewater. Results are divided into first step a) and second step b).

Although MDE drove to a slight reduction in biodiesel quality, this effect is outweighed by the marked reduction of economic and environmental costs. In fact the utilization of wastewater reduces freshwater demand, nutrient addition and wastewater pollution, resulting in remediated stream and valuable biomass (Liu et al., 2017). Moreover, when microalgal FAME profile does not fully meet fuel standard requirements it can be blended with other biodiesel or diesel to obtain a final product which is compliant with quality specifications.

In this specific process two biomasses are produced and, in most cases, biomasses from the first step display IV higher than legislative limits, while from the second step biomasses are characterized by CN below the minimum European limit. In this scenario, these biomasses can be blended before lipid extraction, to obtain a final product of better quality (Figure 33). When *T. obliquus* was used for the first step of cultivation on MDE, the biodiesel quality derived from mixed

biomasses (*T. obliquus* and *N. oceanica*) displayed IV of 121.2 gI₂ 100g⁻¹ and CN of 48.5 (Figure 33) which are very close to the limits, making them suitable for blended biodiesel. Moreover, better quality of biodiesel was observed in modified-BBM compared to MDE, for *T. obliquus* and co-culture, where oxidative stability of mixed biomasses decreased under legislative limit (Figure 33a,b).

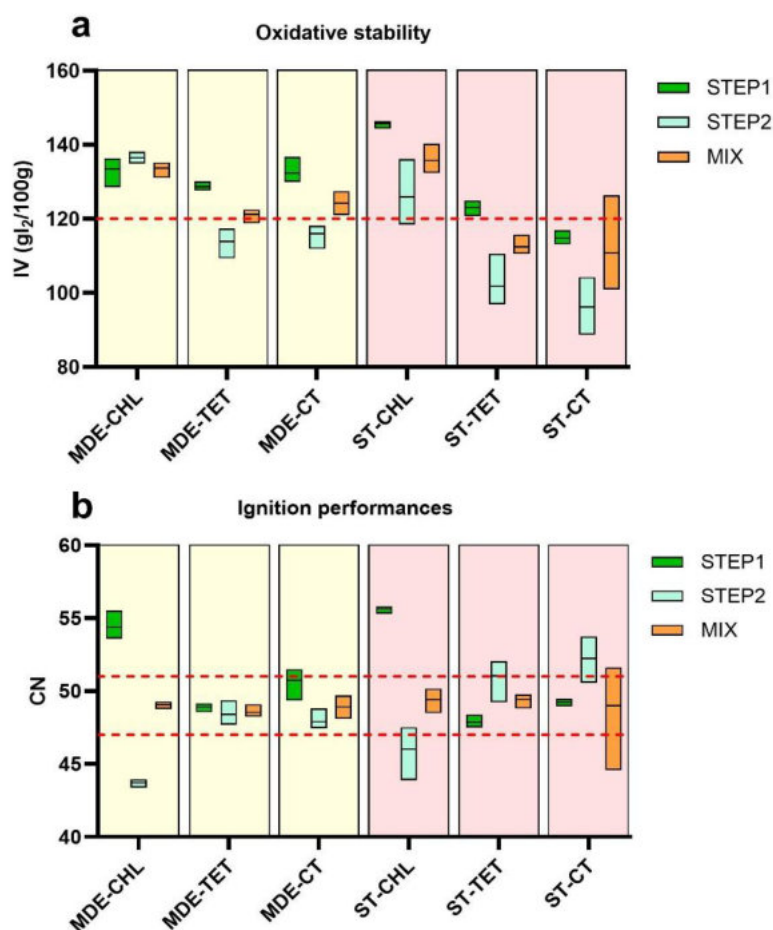


Figure 33: Oxidative stability a) and ignition performances b) of single and blended biomasses are reported in graphs. Each quadrant includes data from the same process, green boxes for biomass from step 1, light-blue boxed for biomass from step 2 and orange boxes for results of mixed biomasses. Yellow quadrants represent results conducted using MDE as substrate while pink ones are related to modified-BBM.

Table 15: Summary of main results of cultivation of three different strains in MDE

	CHL				TET				CT			
	First step		Second step		First step		Second step		First step		Second step	
	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD	Avg	SD
10⁶ Cell ml⁻¹	10.4	3.15	4.35	1.48	12.6	0.81	3.27	0.87	12.10	1.88	2.48	0.78
Lipid (%)	24.55	1.45	39.09	1.63	27.14	0.93	43.89	0.72	23.37	1.69	43.56	0.72
SFA (%)	28.55	1.59	37.21	1.30	24.70	0.48	41.07	1.54	25.08	1.52	40.68	0.47
MUFA (%)	18.52	3.10	27.59	1.53	8.97	0.57	30.02	1.18	11.86	2.48	30.15	0.53
PUFA (%)	52.93	2.23	35.20	0.41	66.33	0.55	28.91	0.95	63.06	1.52	29.17	0.94
IV	133.42	3.46	136.43	1.28	128.61	1.03	113.86	3.35	132.30	3.22	116.01	2.98
CN	54.37	0.82	43.70	0.24	48.92	0.27	48.41	0.71	50.73	0.97	47.91	0.62
CFPP	-7.80	0.78	-3.62	1.84	-8.05	0.11	0.79	0.85	-8.06	1.19	-2.01	1.32

Chapter 4.4: Valorization of a novel stream

4.4.1 Aim of the chapter

Until now we have explored various wastewaters to be implemented in microalgal cultivation selected for their composition, choosing that one that better fits with the final purpose of lipids accumulation suitable for biodiesel production. Here, we have tried a different approach, starting from the effluent of a gas fermentation process.

The implementation of microalgal cultivation after this fermentation process allows us to reduce the environmental impact of the process by reducing pollutants on gas fermentation effluent (GFE), and valorizing it into valuable products, aiming at a circular process development. Part of these findings have been published in (Mazzocchi et al., 2025).

4.4.2 Chemical characterization of gas fermentation effluent

Gas fermentation effluent (GFE) is carbon-rich stream with high nitrogen content and low pH. Due to its composition, it can be widely exploited for the cultivation of microalgae. Table 16 reports the chemical composition and the analysis method for GFE compared with the composition of standard media 3N BBM and BG11. Macronutrients like nitrogen and phosphorous are the most important components of a substrate for microalgal cultivation, followed by micronutrients present in traces like metal elements. Phosphate in GFE records concentration of 65 mg L^{-1} , falling between BG11 and 3N BBM, which are 22 and 157 mg L^{-1} respectively. Nitrogen is present in GFE as ammonium, which is a well available form of nitrogen, but can be toxic at high concentrations. GFE measures 219 mg L^{-1} of ammonium, which is 1.4 times more of 3N BBM and 13 times more of BG11 in terms of moles of N. If compared with 3N BBM and BG11, micronutrients (Mg, Mn, Ca, Na, Fe and K) are well balanced. Additionally, GFE contains 2530 mg L^{-1} of organic carbon, which is important to boost microalgal growth by shifting to a mixotrophic metabolism. It is worth noting that this high level of carbon is due to the addition of a buffer solution to modulate pH during gas fermentation process, thus it is not a carbon form available for respiratory metabolism in microalgae. For microalgal growth, another important parameter is pH, which should be around 7 for *C. sorokiniana* and 7.5-8 for *T. obliquus* (El-Sheekh et al., 2017; Qiu et al., 2017). GFE has a pH level of 5.5, lower than pH suitable for good growth performances, thus requires adjustment prior algal cultivation. During gas fermentation process, several carbon-based products (in present investigations: volatile fatty acids and alcohols) are produced and will be found in the final effluent. HPLC analysis revealed that GFE contains 0.21, 0.16 and 0.13 g L^{-1} for VFAs (acetate, butyrate and caproate,

respectively) and 0.91, 1.04 and 0.76 g L⁻¹ for alcohols (ethanol, butanol and hexanol, respectively) (Table 17). Hexanol is the main high-valued product of this process, thus after the fermentation it is chemically extracted from the medium, but, despite that, it is not completely removed, consequently 0.76 g L⁻¹ are still present in the effluent (Antonicelli et al., 2025). Alcohols (i.e. ethanol, butanol and hexanol Table 17), acetate and butyrate are well known to be used by microalgae for growth (Miazek et al., 2017; Tan et al., 2018; WISE, 1959; Xing et al., 2024), while caproate has never been tested.

Table 16: Hydro chemical analysis of GFE compared with BG11 and 3N BBM are shown in the table below. TOC stands for Total Organic Carbon.

Chemical	mg L ⁻¹			Methods
	3NBBM	BG11	GFE	
TOC	/	/	2530	UNI EN 15936:2012
NH ₄ ⁺	/	/	219	UNI 11669:2017
NO ₃ ⁻	547	1094	<4.5	UNI EN ISO 10304-1:2009
CLHORIDE	27	19	1250	UNI EN ISO 10304-1:2009
SULFATE	29	29	96.1	UNI EN ISO 10304-1:2009
PO ₄	153	21.8	65.8	UNI EN ISO 10304-1:2009
Ca	12	9.8	14.1	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Fe	0.12	0.84	0.67	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Mg	7.4	7.39	22.8	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Mn	0.067	0.5	2.94	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Mo	0.011	0.16	<0.01	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009
Zn	0.008	0.05	0.121	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009

Table 17: Main organic carbon sources of GFE measured with HPLC.

Organic Carbon	Concentration (g L ⁻¹)
Acetate	0.21
Butyrate	0.16
Caproate	0.13
Ethanol	0.91
Butanol	1.04
Hexanol	0.76

4.4.3 Alcohol tolerance of *C. sorokiniana* and *T. obliquus*

The tolerance of two strains to alcohol was tested using the same concentrations of GFE and double (Table 17). Both strains showed toxic effects when all three alcohols have been added to the media at the concentration present in GFE (1x). It was observed that there was a pronounced reduction in biomass production of 64 and 59% at 1x concentration and 90 and 89% reduction at 2x concentration for *C. sorokiniana* and *T. obliquus*, respectively (Figure 34). Then the same test was conducted with only ethanol and butanol, to better investigate the cause of this high inhibitory effect. Here, the same concentration of GFE did not affect the growth of both strains while a slight reduction of biomass production was observed at 2x compared to 1x. These results suggested that the inhibitory effect of the mixture of three alcohols was due to the presence of hexanol, while low concentrations of ethanol and butanol did not affect negatively the growth of these strains. Moreover, a look into literature reveals that ethanol can be used as carbon source by photosynthetic microorganisms to increase cell division and lipid accumulation (Bezerra et al., 2014; Wu et al., 2013). To confirm that, Abate and colleagues demonstrated that ethanol in concentrations up to 0.789 g L⁻¹ promotes growth of *C. sorokiniana* (Abate et al., 2024). But these results are not the same for all strains, in fact *Selenastrum capricornulum* and *C. vulgaris* exhibit toxicity at 0.39 g L⁻¹ of ethanol, with more damaging effects for *C. vulgaris* (Jay, 1996). Meanwhile, *Dunaliella tertiolecta* and *Isochrysis galbana* can tolerate 16-15 g L⁻¹ of ethanol, showing a growth inhibition of 50% (Okumura, 2001). In this study, two strains had different behaviors when cultivated with the addition of ethanol and butanol only. In fact, on one hand, *C. sorokiniana* biomass production was not affected by 0.91 and 1.04 g L⁻¹ of ethanol and butanol (1x) respectively but was slightly inhibited by double those concentrations (Figure 34a). On the other hand, *T. obliquus* showed a better response to alcohol at both the alcohol concentrations, recording more biomass than the control, with statistical difference only for 1x, being 75.1, 68.8 and 53.4 mgDW L⁻¹ d⁻¹ for 1x, 2x and control with no alcohols, respectively (Figure 34b). These results are coherent with the output obtained by HPLC analysis on consumption of alcohol. Indeed, in tests conducted with *C. sorokiniana* no alcohols consumption was detected, and consequently, even at 1x with ethanol and butanol, in which no toxicity was reported, no growth increase was observed, indicating that the microalga did not shift to a mixotrophic metabolism. Instead, *T. obliquus* was demonstrated to be able to consume ethanol and butanol at low concentrations, in fact at 1x the increase of biomass production coincides with the uptake of 95.5 mg L⁻¹ of ethanol and 98.6 mg L⁻¹ of butanol. Moreover, at 2x concentration *T. obliquus* could not consume alcohol and thus no biomass increase was recorded. These results, along with literature, highlight the variability of microalgae behaviors and the consequent need to widely uncover

those metabolisms. Studies on ethanol and butanol tolerance on microalgae can be found in the literature, while it is not the same for hexanol. Indeed, only few studies on toxicity (Aruoja et al., 2014; WISE, 1959) and production of hexanol by microalgae are available (Gupta et al., 2022). In the present study, even if *T. obliquus* responded better to ethanol and butanol, being even able to uptake them at low concentrations, both strains recorded hexanol inhibitory effects at 0.76 g L^{-1} . Moreover, these results highlight the necessity to improve the extraction methods of hexanol both for improving the yield and to make GFE less selective when used as microbial substrate.

In this scenario, the main obstacle to using GFE for microalgal cultivation is hexanol, which makes diluting GFE essential.

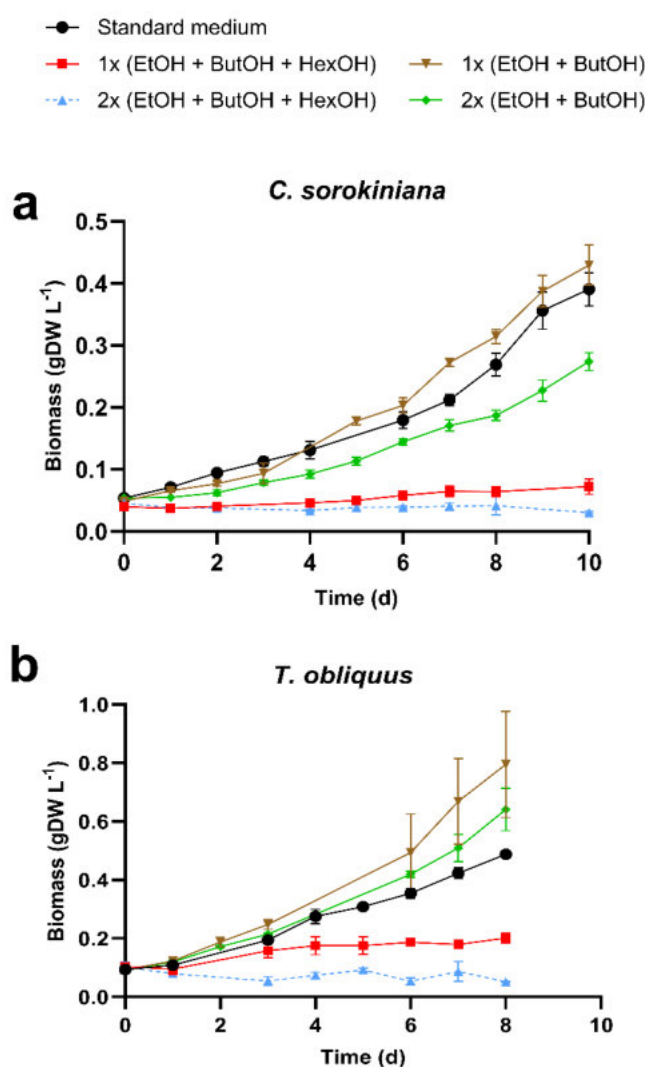


Figure 34: growth curves of *C. sorokiniana* (a) and *T. obliquus* (b) with the addition of ethanol, butanol and hexanol at different concentrations. Error bars refer to SD.

4.4.4 VFAs uptake and utilization for *C. sorokiniana* with different pH

Since GFE is quantified rich in organic acids, the effects of acetate, butyrate and caproate on *C. sorokiniana* growth have been investigated. These tests have been conducted at two different pH levels, 7 and 8, to understand how they affect the VFAs uptake.

C. sorokiniana grown at pH 7 outperformed the cultures at pH 8 (Figure 35a). The control conditions without VFAs reported growth rates 1.46-fold higher at pH 7 compared to pH 8, while no differences were observed in VFA-supplemented conditions. When no VFAs were added, after 7 days the maximum biomass production at pH 8 and 7 was respectively 0.42 and 0.71 gDW L⁻¹. At both pH levels the uptake of acetate was close to complete depletion, measuring 98% and 94% at pH 8 and 7, respectively (Figure 35d). Moreover, an increase of 29% of the uptake of butyrate, when added together with acetate, was observed at pH 7. This difference can be related to the pH-dependent protonation state of VFAs. There are two mechanisms for VFAs cell absorption: i) passive diffusion through membranes and it occurs when VFAs are undissociated; ii) active transporters for dissociated VFAs in their anionic form, which is dependent on the H⁺ concentration. Consequently, a lower uptake occurs when pH is higher due to the low availability of H⁺ ions (Lacroux et al., 2020, 2021). In addition, in presence of more than one VFA, microalgae favored the shorter carbon chain, in this case acetic, then butyric and third caproic acid. Specifically, other VFAs are absorbed only after the complete depletion of acetate, as confirmed by literature (Chalima et al., 2017; Chandra et al., 2015). Moreover, the toxicity of butyric acid at concentration higher than 1.25 g L⁻¹ (Baroukh et al., 2017; Fei et al., 2015) is an issue when trying to valorize these effluents (Baroukh et al., 2017; Hwang et al., 2014; Turon et al., 2015). In this scenario, butyric acid was tested alone at pH 7 to optimize the assimilation and it was observed a consumption of 68% after 8 days of cultivation. Besides that, when only butyrate was added there were no statistical differences in biomass production with the control without VFAs, so we can evince that butyrate did not affect the growth, neither positively nor negatively (Figure 35b). Consequently, the butyric acid consumed did not correlate with biomass accumulation, but it is, probably, implicated in other metabolic ways. Indeed, butyric acid can be oxidized after the absorption to acetyl-CoA through β -oxidation. Then acetyl-CoA can produce energy via tricarboxylic acid cycle, or produce lipids by producing triacylglycerols or, moreover, enter protein biosynthesis. Consequently, acetyl-CoA can be directly employed for fatty acids biosynthesis, reducing the number of steps required by other metabolic ways, such as throughout sugars (Bellou & Aggelis, 2012; Prathima Devi & Venkata Mohan, 2012).

In contrast, by adding caproate combined with acetate and butyrate, an increase of the growth of *C. sorokiniana* was observed, with the accumulation of 1.09 gDW L^{-1} , which is 1.5 times the biomass obtained when only acetate and butyrate were added. Here, the exponential phase of growth started later, with two days in lag phase and then showed a rapid increase of biomass, with consumption of 100%, 50% and 23% of acetate, butyrate and caproate, respectively. Instead, cultivations with acetate and butyrate only resulted in limited biomass accumulation and VFAs consumption, indeed 95% of acetate and 26% of butyrate were consumed at pH 7 and 98% and 6% were respectively consumed at pH 8 (Figure 35d).

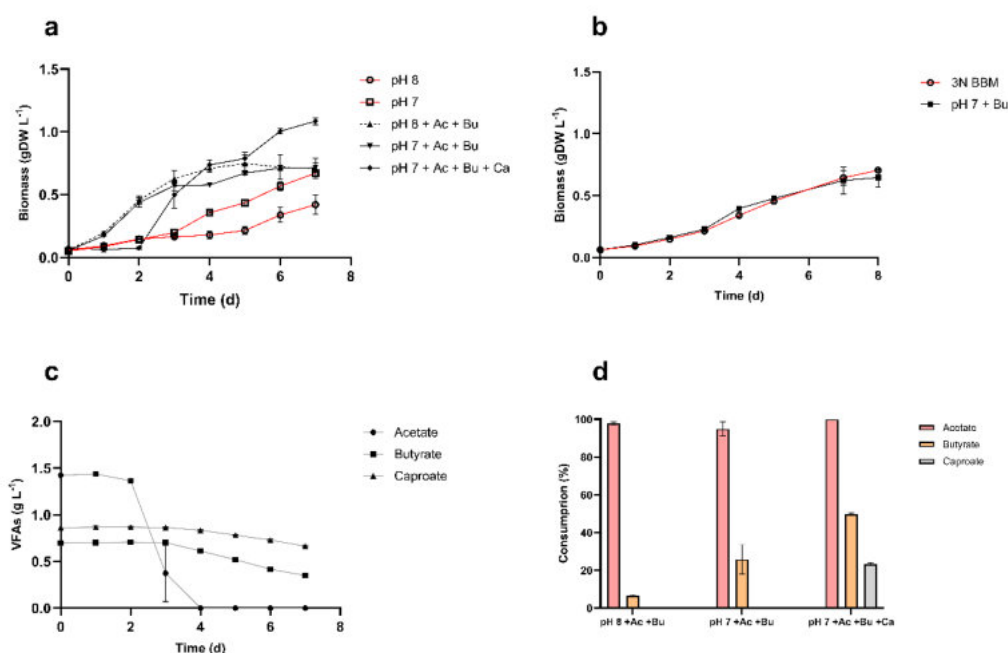


Figure 35: biomass production of *C. sorokiniana* under different pH levels with and without VFAs addition (a). Graph b) reports the effect of butyrate on growth of *C. sorokiniana* at pH 7. c) represents the consumption of VFAs when added contemporary at pH 7, while d) displays the final consumption of VFAs in different conditions tested.

4.4.5 Identification of complementary wastewater for nutrient balance

GFE must be diluted for microalgal cultivation, as demonstrated in the previous section (Figure 34). Thus, to avoid the utilization of fresh water for dilution, four wastewaters derived from dairy industry have been considered: exhausted sludge (DES), washing waters (DWW), and scotta. The related screening was largely discussed in Chapter 4.1, regarding hydrochemical analysis (Table 18) and growth performances of both *C. sorokiniana* and *T. obliquus* (Figure 12, Figure 13, Figure 14).

Briefly, among macronutrients present in streams, nitrogen is essential and should be between 100 and 200 mg L⁻¹ to avoid limitation or toxicity (Figure 11). For this reason, SC and DWW, which contains low ammonium concentrations, have been discarded, due to relative low nitrogen content. Moreover, phosphorous is essential for a balanced growth of microalgae and low concentrations are associated with high lipid accumulation (Anto et al., 2019; Liang et al., 2013), making DES the most suitable for this purpose. Moreover, N/P ratio of SC and DWW are lower than 1, while DES measured 32, being suitable for microalgal growth.

Based on the screening on dairy wastewaters performed in Chapter 4.1, DWW and DES seemed to be the best candidates. Particularly, DWW are poor of the majority of nutrients, even if rich in organic carbon sources. On the other hand, DES has higher concentrations of macronutrients and balanced micronutrients. Finally, being GFE rich in organic carbon, DES was selected as a complementary stream for GFE dilution.

Table 18: Chemical analysis conducted on four wastewaters derived from dairy industry: exhausted sludge (DES), scotta (SC) and washing waters (DWW).

Chemical	mg L ⁻¹			Method
	DES	SC	DWW	
NH ₄ ⁺	370	90	<2.5	UNI 11669:2017
PO ₄	<60	1680	<5	UNI EN ISO 10304-1:2009
TOC	78.20	22892	803	UNI EN ISO 15587-1:2002 (Annex A) + UNI EN ISO 11885:2009

4.4.6 Cultivation of both strains on DES and GFE

An initial screening of each single wastewater was conducted to evaluate the feasibility of their utilization for microalgal growth. Both strains have been cultivated on two wastewaters separately at different dilution rates. Detailed results for both strains cultivated on DES can be found in Chapter 4.1, here only a brief summary will be reported.

When 100% of DES was used for the cultivation of both strains an inhibition of growth was observed and the same result was obtained with 75% DES for *T. obliquus*. Instead, *C. sorokiniana* showed better performances when cultivated on 75% DES, having a fast growth at the initial phase, comparable to the control, but then decreased, starting earlier the stationary phase (Figure 36). The highest biomass productions were obtained with 50% DES for both strains. Here, *C. sorokiniana*, measured higher biomass productivity of the control after 10 days cultivation, being 0.045 and 0.035 g L⁻¹ d⁻¹ for 50% DES and 3N BBM, respectively (Figure 36c). Differently, *T. obliquus* grew slower in 50% DES

compared to BG11, with a productivity of 0.033 and 0.046 g L⁻¹ d⁻¹, respectively (Figure 36d).

Different results were obtained when microalgae have been cultivated on GFE. The effect of the utilization of this effluent is clearly pictured in Figure 36, which highlights that the utilization of 75% or 100% of GFE inhibits the growth of both strains. However, when GFE is properly diluted at 50% microalgae can grow even better than the standard medium. By producing 0.682 and 0.489 g L⁻¹ for *T. obliquus* and 1.074 and 0.496 g L⁻¹ for *C. sorokiniana*, with 50% GFE and standard medium respectively, they outperformed the control condition by 1.4 and 2.2 times as biomass production. The cultivation of *C. sorokiniana* on 50% GFE drove to higher biomass production compared to other studies where combinations of VFAs have been tested (Lu et al., 2024). Moreover, they demonstrated that VFAs, compared to other carbon sources such as glucose, are more effective for *C. sorokiniana* growth, highlighting the value of these kinds of wastewaters for microalgal cultivation.

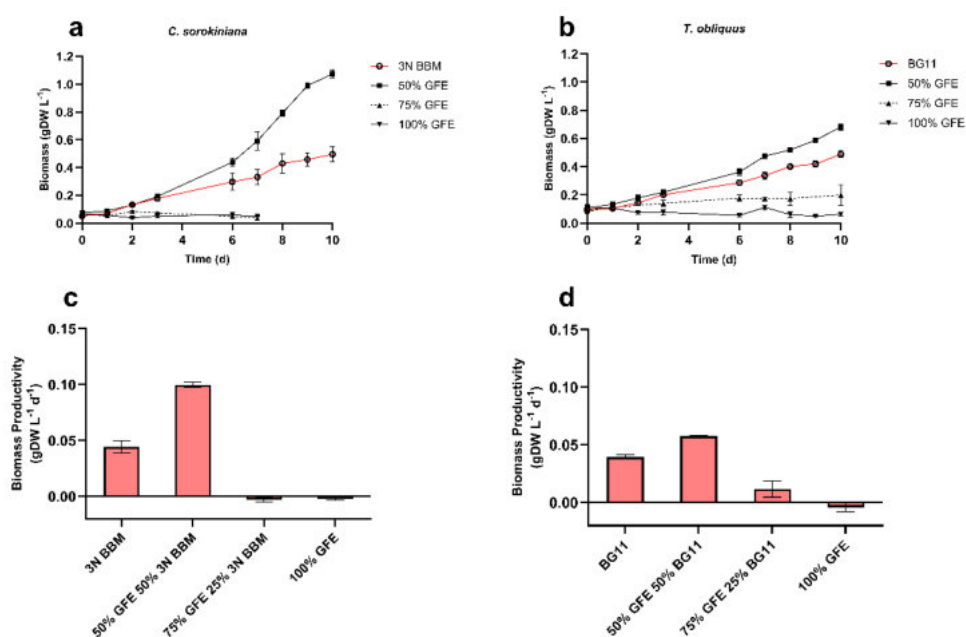


Figure 36: screening of *C. sorokiniana* a) and *T. obliquus* b) cultivated on different ratios of GFE. c) and d) reports respectively biomass productivity of *C. sorokiniana* and *T. obliquus*.

To avoid the utilization of freshwater and chemical compounds, to balance composition of wastewaters DES and GFE have been blended in a 50:50 ratio. After mixing DES and GFE the pH resulted in 6.7, thus suitable for microalgal growth without need to be adjusted.

Both strains cultivated on wastewaters mixture for 9 days showed better performances if compared to the standard medium. Even though, *T. obliquus* showed no statistical differences, measured a biomass productivity of 0.047 and 0.081 gDW L⁻¹ d⁻¹ in BG11 and 50:50 DES:GFE respectively, with growth rates

of 0.20 and 0.17 d⁻¹ respectively (Figure 37b). On the other hand, on 50:50 blend *C. sorokiniana* outperformed the control condition, growing at a rate of 0.24 d⁻¹ compared to 0.19 d⁻¹ in 3N BBM (Figure 37a). The high growth rate drove to a higher biomass productivity which quadrupled the control, with a final biomass, after 9 days, of 1.69 and 0.46 gDW L⁻¹ respectively. In both cases, when cultivated on 50:50, pH increased to 8.5 during the first 4 days and then stabilized at 7. Overall, *C. sorokiniana* accumulated 1.69 times biomass compared to *T. obliquus* (Figure 37a,b). Indeed, *C. sorokiniana* is known for its high growth rate while *T. obliquus* for efficient nutrient recovery in wastewater, thus the specific composition of the substrate can be selective for different species (Bulynina et al., 2023; Gomaa et al., 2026). actually, this result depends on the composition of wastewaters used for cultivation, indeed *Chlorella* outperformed *Tetradismus* in some cases (Capano et al., 2026) while other studies report opposite results (Tao et al., 2017).

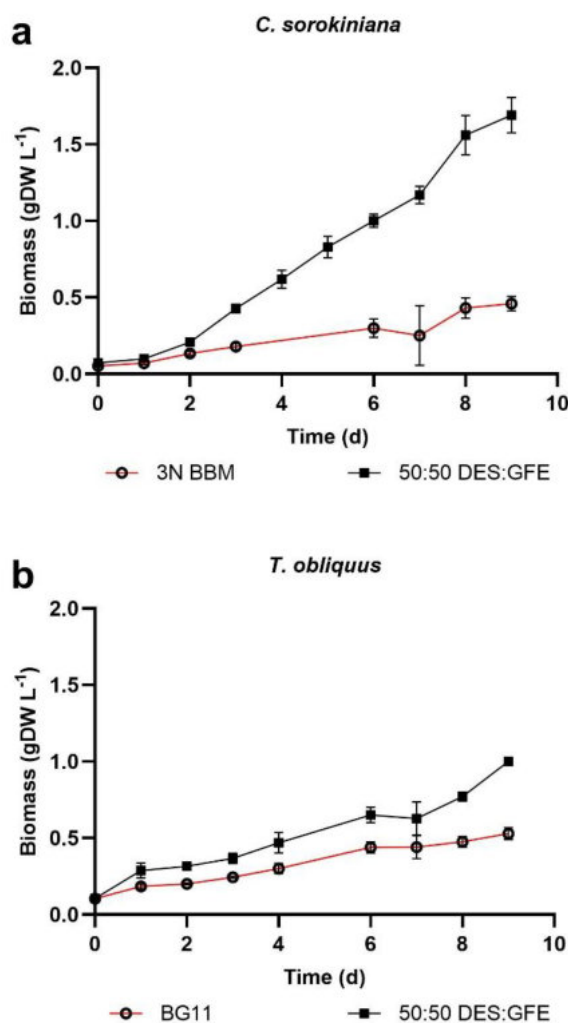


Figure 37: growth curves of *C. sorokiniana* (a) and *T. obliquus* (b) on 50:50 DES:GFE are compared to standard medium. Error bars represent standard deviation.

4.4.7 Laboratory scale-up of *C. sorokiniana* cultivation on industrial wastewater

Considering preliminary screening on 50:50, *C. sorokiniana* resulted to be the best candidate for further studies. Thus, it was selected for a laboratory scale-up in a multicultivator PBR under controlled parameters (temperature, light intensity regulation, gas bubbling), to monitor growth and lipid accumulation ability. Results of this screening are compared with *C. sorokiniana* cultivated under standard conditions in multicultivator, results are shown in paragraph 4.1.7.

The mixture 50:50 composed of DES and GFE was used as growth substrate in a MC PBR and inoculated with *C. sorokiniana*. *C. sorokiniana* reached a growth rate of 0.46 d^{-1} and a biomass accumulation of 1.96 gDW L^{-1} in only 6 days of cultivation (Figure 38a), even more than the cultivation on standard media supplemented with acetate (i.e. mixotrophy). Light exposure was higher at the beginning of cultivation for two days, due to lower biomass concentration, and then remained constant till the end of cultivation (Figure 38b). VFAs present in GFE were consumed by *C. sorokiniana* following this order: acetate (84.6% consumption), butyrate (99.4% consumption) and finally caproate (100% consumption), as previously reported (Turon et al., 2015) (Figure 38c). Chemical analysis of the mixed substrate conducted before and after the cultivation of *C. sorokiniana*, revealed the complete depletion of total nitrogen as Total N, Ammonium N and phosphorous (Table 19, Figure 38d). Moreover, a reduction in total carbon and sulphates of 61 and 68% respectively was measured after six days cultivation. Calcium and iron were also depleted, of 44 and 81% respectively (Figure 38d). Bioremediation levels are in accordance with literature results as *C. sorokiniana* is considered a robust strain in this field (Capano et al., 2026; Hamidian & Zamani, 2022a; Iliopoulou et al., 2022).

Our data thus suggest *C. sorokiniana* is an ideal platform for remediation of this mixture of wastewater, showing high removal capacity and valuable biomass accumulation in only few days of cultivation.

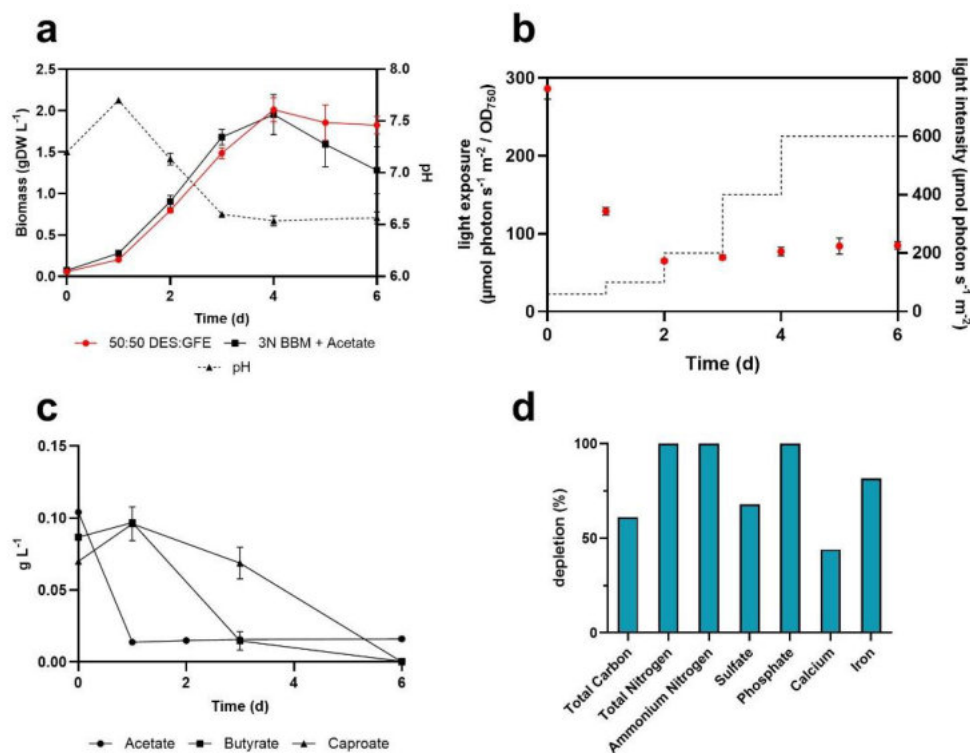


Figure 38: *C. sorokiniana* on 50:50 DES:GFE. Biomass production as dried weight per L is compared with standard mixotrophy obtained with the addition of acetate (a). light regime adopted and light exposure in relation to OD₇₅₀ are reported in graph b). Consumption of VFAs during the cultivation (c) and depletion of macronutrients (d) present in mixture of effluents are also represented.

Table 19: Chemical composition of 50:50 DES:GFE before and after the cultivation of *C. sorokiniana* are reported in table below as mg L⁻¹.

Chemical	mg L ⁻¹	
	Pre-treatment	Post-treatment
Total Carbon	4099.00	1591.60
Total Nitrogen	34.00	<5.0
Ammonium Nitrogen	294.50	<2.50
Nitric Nitrogen	<4.5	<1.50
Chloride	748.00	624.00
Sulfate	60.45	19.60
Phosphate	65.80	<15
Ca	30.05	16.80
Co	0.21	0.18
Fe	0.43	0.08
Mg	17.25	15.80
Mn	2.94	1.35
Cu	<0.01	0.02
Zn	0.06	0.06

4.4.8 FAME profile of *C. sorokiniana* grown on 50:50 DES:GFE

After cultivation in MC PBR using 50:50 DES:GFE, the biomass was analyzed to determine the profile of fatty acid methyl esters (FAMES) (Figure 39). Table 20 shows the FAME profiles of the biomass at the end of the cultivation, measured as percentages of each FAME, compared with biomass grown under phototrophic and mixotrophic standard conditions, (see paragraph 4.1.8). The most abundant is palmitic acid (C16:0) measuring 43, 44 and 48% for 50:50 DES:GFE, mixotrophy and autotrophy respectively (Table 20), followed by linoleic acid (C18:2) with 33, 31 and 22% respectively, consistently with literature (Banerjee et al., 2023; Kumar et al., 2014; Papapolymerou et al., 2022a). Overall, the profile of *C. sorokiniana* grown in the mixture is comparable to mixotrophic standard condition with minor fluctuations, as confirmed by statistical analysis. In detail, monounsaturated fatty acids with 18 carbon atoms (elaidic and oleic acid, C18:1) are more abundant in 50:50 compared to other conditions, measuring a total of 10.4, 4.6 and 5.7% in 50:50, mixotrophy and phototrophy respectively (Figure 39). Moreover, the saturated C18, stearic acid, is significantly lower in 50:50 DES:GFE if compared to other conditions, measuring half the percentage. Here the utilization of mixture of streams leads to an increased desaturation activity compared to standard mixotrophic condition.

On the contrary, the profile changed if compared to phototrophy and it is evident when studying the composition divided into saturated, monounsaturated and polyunsaturated fatty acids (SFA, MUFA and PUFA). Herein, when *C. sorokiniana* is grown on the wastewaters mixture there is an increase of MUFA and PUFA and a decrease of SFA compared to phototrophy and mixotrophy. More in detail, phototrophy drove to an increase in SFA due to higher C18:0 and C16:0, and lower C18:2 (Figure 39).

The decrease in SFA when mixture of wastewater is used can be attributed to different factors. For instance, it is noted that nitrogen starvation leads to an increase in SFA and MUFA (Papapanagiotou et al., 2024; Toumi & Politaeva, 2021) while it was observed an increase in unsaturated fatty acids under phosphorous limitation (Maltsev et al., 2018; Matsui et al., 2020). In the present study, 50:50 DES:GFE measures a N/P ratio of 24, meaning that there is a moderate deficiency of phosphorous, which is then completely depleted (Table 19). Generally, several factors have been associated to changes in FAME profiles of microalgae, such as CO₂ concentration, pH, light intensity, temperature or initial inoculum size (Qiu et al., 2017; Yun et al., 2020). This number of factors involved in modulation of FAME profiling represents a huge advantage for bioprocess development. Indeed, while working with wastewater is not convenient controlling substrate composition, however it is possible to manage external parameters to guide the final biomass composition toward desired FAME

profile. The purpose of the present investigation is to develop a process for the valorization of wastewater through valuable biomass production for biodiesel. In this scenario, the decrease of SFA leads to an improvement in cold flow properties of biodiesel, while oxidative stability decreases (Knothe, 2005).

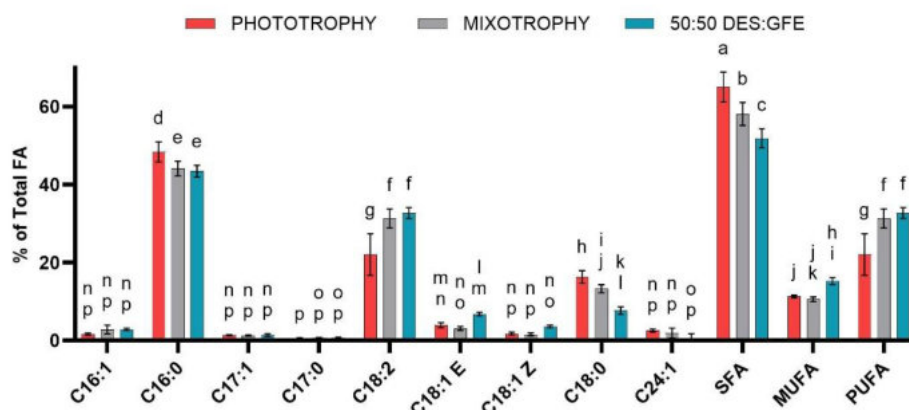


Figure 39: FAME profile of *C. sorokiniana* grown on standard medium in phototrophy and mixotrophy compared with biomass grown on 50:50 DES:GFE. Data are reported as means of biological replicates and letters separate data into statistically different groups, with $p < 0.05$.

Table 20: FAME profile of *C. sorokiniana* cultivated on 50:50 DES:GFE, mixotrophy and phototrophy. Means and standard deviations are reported for each FAME.

FAME	50:50 DES:GFE		Mixotrophy		Phototrophy	
	Avg	SD	Avg	SD	Avg	SD
C16:1 (Δ^9 , Z)	2.89	0.25	2.80	1.00	1.65	0.22
C16:0	43.45	1.25	44.12	1.62	48.39	2.20
C17:1 (Δ^{10} , Z)	1.41	0.26	1.26	0.12	1.36	0.13
C17:0	0.73	0.08	0.73	0.05	0.34	0.34
C18:2 ($\Delta^{9,12}$ Z, Z)	32.70	1.14	31.29	2.14	22.05	4.62
C18:1 E (Δ^9 , E)	6.78	0.38	3.08	0.44	3.93	0.54
C18:1 Z (Δ^9 , Z)	3.58	0.28	1.56	0.32	1.78	0.31
C18:0	7.70	0.78	13.25	0.91	16.29	1.42
C24:1 (Δ^{15} , Z)	0.62	0.88	1.91	1.12	2.56	0.34
Σ SFA	51.88	2.01	58.10	2.56	65.03	3.34
Σ MUFA	15.29	0.72	10.61	0.51	11.29	0.31
Σ PUFA	32.70	1.14	31.29	2.14	22.05	4.62

4.4.9 Biodiesel quality of *C. sorokiniana* cultivated on future valuable wastewater

FAME profiles of *C. sorokiniana* grown on different conditions have been analyzed to calculate three main parameters for the evaluation of biodiesel quality. In all biomasses tested IV and CN were within standard limits for EN 12414 and ASTM. Indeed, microalgae cultivated in phototrophy, mixotrophy and on 50:50 DES:GFE measured IV of 51, 63 and 70 g I₂ /100g, respectively (Figure 40). All these biodiesels are characterized by strong oxidative stability, because IV values are far away from the upper limit, which is 120 g I₂ /100g. Regarding CN, biomasses grown in phototrophy, mixotrophy and on 50:50 DES:GFE measure 64, 60 and 58, respectively (Figure 40). Once again, they are all above standard lower limits, thus exhibiting high ignition performances.

These results highlight the decrease in oxidative stability of biodiesel obtained with 50:50 DES:GFE compared to other ones, and a slight reduction of ignition quality too. Despite that, values are always acceptable for commercialization as B100 (meaning that this biodiesel can be used as it is, without necessity of blending). Moreover, the cold flow properties of biodiesels analyzed are 24, 18 and 9 °C respectively when phototrophy, mixotrophy or wastewater blending are used (Figure 40). This parameter does not have a neat legislative specification because it depends on season and country, anyway it is deducible that CFPP of these values are too high to be used in many countries. The CFPP recorded for biomass cultivated on 50:50 DES:GFE is half compared to mixotrophic biomass and almost one third of biodiesel derived from phototrophic cultivation. This finding makes this kind of biodiesel particularly advantageous, increasing its industrial application. A decrease in oxidative stability and improved cold properties have been already recorded with *C. sorokiniana* when cultivated in mixotrophy compared to phototrophy, and authors demonstrated also that by changing process conditions these values can vary (Kumar et al., 2014). Moreover, when compared quality of biomasses cultivated on standard medium with wastewater a reduction of oxidative stability is observed (Hamidian & Zamani, 2022b; Lee et al., 2023).

These results highlight how the implementation of this mixture of wastewater not only decreased costs of the process, but also improved the quality of biodiesel, making this process worthy of being valorized.

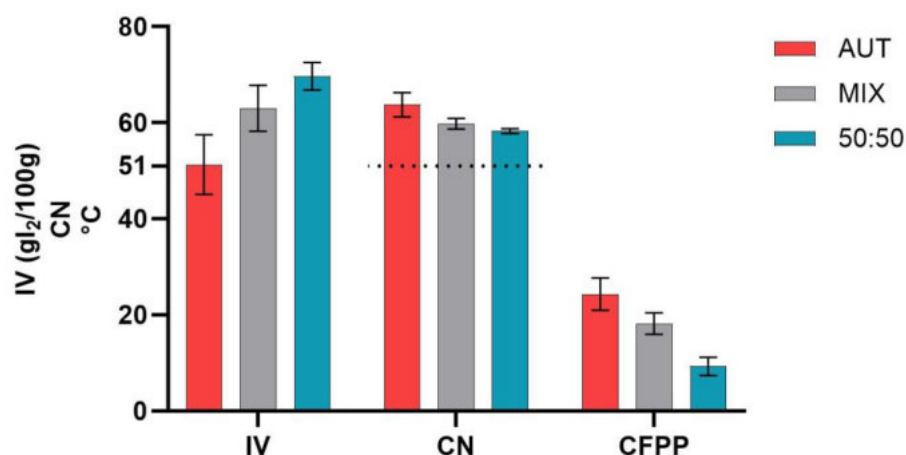


Figure 40: IV, CN and CFPP values are reported above for *C. sorokiniana* biomasses cultivated on different conditions. Dotted line on CN represents the lower limit set by EN 12414 legislation, which is 51. Data are reported as means of biological replicates and standard deviations.

Table 21: Summary of main data collected in this chapter compared with *C. sorokiniana* cultivated under standard conditions. Data reported are referred to experiments conducted on MC PBR.

	Phototrophy		Mixotrophy		50:50 DES:GFE	
	Avg	SD	Avg	SD	Avg	SD
Maximum biomass at day 4 (gDW L ⁻¹)	1.37	0.26	1.99	0.18	2.01	0.11
Biomass productivity (gDW L ⁻¹ d ⁻¹)	0.055	0.01	0.290	0.01	0.295	0.01
ΣSFA	65.03	3.34	58.10	2.56	51.88	2.01
ΣMUFA	11.29	0.31	10.61	0.51	15.29	0.72
ΣPUFA	22.05	4.62	31.29	2.14	32.70	1.14
IV (gI ₂ / 100g)	51.31	5.06	63.01	4.15	69.70	2.35
CN	63.76	2.18	59.81	0.94	58.29	0.39
CFPP (°C)	24.31	2.92	18.20	1.93	9.27	1.57

Chapter 4.5 Comparison between biodiesel obtained with different approaches

4.5.1 Aim of the chapter

To better understand the applicability of biodiesel production using microalgae as feedstock, a comparison between biodiesel obtained with three approaches and with literature data has been performed. Not only the feasibility of biodiesel production but also the advantages of biotechnological approaches proposed are evaluated in the present chapter.

4.5.2 Comparison between data of the present study: light induced lipid production, two-step process with N-starvation and novel stream utilization

In the present study, three different approaches have been proposed to enhance biomass and lipid production and to evaluate their effects on FAME profiles and, consequently, on biodiesel quality. The three approaches are based on the exploitation of diverse streams through combinations of different algal strains, i.e. *C. sorokiniana* growth on balanced dairy WWs with R100-B300 regime, two-step cultivation involving *T. obliquus* and *N. oceanica* cultivated on MDE and *C. sorokiniana* growth on 50:50 DES:GFE. The main biodiesel parameters obtained from each process are summarized in Table 22. At first sight, biodiesel derived from *C. sorokiniana* strain, which exhibits the highest quality, both for ignition performances, oxidative stability and cold flow temperatures. Comparable results were obtained with *C. sorokiniana* grown on the novel waste stream (50:50 DES:GFE) and under specific light regime (R100-B300).

When dealing with complex bioprocesses, it is essential to evaluate the entire system, considering all factors involved. In the context of biodiesel production, lipid content alone is not sufficient to assess overall process performance. Key parameters such as biomass productivity, cultivation time, and lipid productivity must be considered for determining the effective biodiesel yield. These aspects are particularly relevant for third generation biodiesel from microalgae, where volumetric productivity remains one of the main bottlenecks for large-scale implementation (Sarwer et al., 2022). In this study, the cultivation of *C. sorokiniana* on 50:50 DES:GFE drove to a lipid productivity four times higher than that of R100-B300, despite the latter having a higher lipid content per unit of dried biomass. Nevertheless, different light regimes influenced *C. sorokiniana* biomass and lipid productivity, thus the implementation of a balanced light regime using the novel stream 50:50 DES:GFE could be a solution to meet biomass and lipid productivity maintaining high biodiesel quality.

On the other hand, the two-step cultivation involving *T. obliquus* and *N. oceanica* produced biodiesel with distinct quality characteristics. The values reported in Table 22 represent the quality of biodiesel derived from blended biomasses from first and second steps. As discussed in Chapter 4.3, the utilization of the mixture of biomasses is advantageous for improving biodiesel quality, due to the complementary FAME profiles of the two selected strains. In this case the oxidative stability (IV) slightly exceeds legislative maximum limit, which is imposed at 120 g I₂ /100 g, indicating higher degree of unsaturation than recommended. Moreover, ignition performances comply with ASTM D6751 requirements, but do not fully meet European EN 14212 standards for B100 biodiesel. Nevertheless, this biodiesel boasts great cold flow performances, making it potentially suitable for colder climate conditions. Although the quality parameters do not allow direct commercialization as neat biodiesel (B100), a feasible strategy is to blend it with fossil diesel or other biodiesel to meet legislative standards.

4.5.3 Bioremediation levels

Regarding bioremediation performance, the two-step process with MDE and with 50:50 DES:GFE achieved the highest nutrient removal efficiencies with 99% and 100% of N removal and 87% and 100% of P removal, respectively. In contrast, cultivation under R100-B300 showed lower remediation efficiency, with only 49% removal of ammonium and 38% removal of phosphorous. From an industrial and economic perspective, nutrient removal is a crucial parameter, as it directly contributes to wastewater valorization and reduces treatment costs. The efficient performance observed with MDE and 50:50 DES:GFE processes reinforce the relevance of these biotechnological approaches from a circular-economy perspective, where wastewaters are converted into value-added products. In addition, the remediation capacity of *C. sorokiniana* grown under specific light regime could be optimized by modulating the first and second cultivation stages to stimulate cellular growth and nutrient consumption.

Typically, the trade-off between biomass productivity, lipid productivity, and lipid quality is a limiting factor for this emerging sector. Indeed, several investigations were conducted to enhance these parameters to achieve an overall improvement in microalgal biodiesel production through regulation of process conditions (Fernandes et al., 2017; Han et al., 2016; Singh Laura, 2019). In this context, the present work demonstrated that is possible to fine-tune all relevant parameters to reach a desired performance, even when operating in inherently environments such as industrial wastewater.

Table 22: summary of key parameters of three processes tested in this thesis. Bold characters highlight parameters outside legislative limits.

Microalgal species	Growth condition	Lip. Productivity mg/L/d	Lipid content (%)	IV (gI ₂ /100g)	CN	CFPP (°C)	N removal	P removal	Ref.
<i>C. sorokiniana</i>	R100-B300	23.92	31.03	78.15	56.33	8.96	49.50%	34.8%	Chapter 3.2
<i>T. obliquus</i> , <i>N. oceanica</i>	MDE-TET-NO	-	35.52	121.23	48.54	-3.63	99%	87%	Chapter 3.3
<i>C. sorokiniana</i>	50:50 DES:GFE	60.20	20.39	69.70	58.28	9.27	100%	100%	Chapter 3.4

4.5.4 Comparison of present work with literature data

An overview of the quality of microalgae-derived biodiesel based on recent literature is summarized in Table 23. The importance of microalgal strain selection stands out from these data. Indeed, among *Chlorella* species, *C. sorokiniana* carried out the best results in terms of biodiesel performances compared to *C. pyrenoidosa* and *C. vulgaris*, whether grown in standard medium or in wastewater (Asadi et al., 2020; Hamidian & Zamani, 2022b; Wu & Miao, 2014). Similarly, within the *Scenedesmaceae* family, *T. obliquus* showed better results than *Scenedesmus* sp. and *T. dimorphus* (Francisco et al., 2010; Ogbonna et al., 2026). In contrast, *Nannochloropsis* sp. has a distinctive FAME profile that results in lower oxidative stability and poorer ignition performance in the biodiesel, due to the presence of a higher proportion of polyunsaturated fatty acids (Mitra et al., 2016). When cultivated on wastewater, microalgae exhibit significant variations in their FAME profiles. Accordingly, a wide range of Iodine value (IV) and Cetane Number (CN) has been reported for *C. sorokiniana* grown on different dairy effluents (Table 23). The biodiesel obtained from *C. sorokiniana* in present study falls in the middle of these reported values. Its properties are comparable with those described by Hamidian and colleague, outperform those reported by Thala and colleagues but remain lower than the best-performing results documented by Asadi et al. (Asadi et al., 2020; Hamidian & Zamani, 2022b; Talha et al., 2025). This differences are mainly attributed to variations in FAMES composition, particularly in the proportions of SFAs, MUFAs and PUFAs. In this study, *C. sorokiniana* exhibited 34% of PUFAs when cultivated under red and blue light (R100-B300 condition) and 32% when cultivated in 50:50 DES:GFE. These values are similar to Hamidian (30%), while Thala displayed a higher PUFAs fraction of 55%, which negatively affected oxidative stability and ignition performance. In contrast, Asadi obtained the best biodiesel performances with the lowest portion of PUFAs (25%). These differences likely reflect the variability of streams composition, even if derived

from the same industrial sector, which influences microalgal metabolisms and FAME profiles. Moreover, the cultivation conditions, comprehensive of all parameters such as cultivation systems, light, temperature, mixing and aeration are fundamental and can influence the biodiesel quality, as it was demonstrated in present work.

Furthermore, *T. obliquus* resulted in producing biodiesel of slightly worse properties than reported in literature (Nascimento et al., 2013b; Wu & Miao, 2014). However, it should be emphasized that, in this study, it was cultivated on 100% wastewater, in contrast, the cited literature data were obtained under controlled conditions using standard media. Nevertheless, in our experimental design *T. obliquus* biomass was not considered to be used alone, but blended with *N. oceanica* prior lipid extraction, allowing mitigation of biodiesel quality parameters thanks to the complementary FAME profiles of the selected species. This process allowed the full exploitation of wastewater for the cultivation of microalgae while maintaining values of IV and CN in acceptable ranges for blended biodiesel.

Table 23: Data reported in literature for *Chlorella* sp., *Tetrademus* sp. and *Nannochloropsis* sp. biodiesel qualities are shown in table. Reported biodiesel quality parameters are: Iodine Value (IV), Cetane Number (CN) and Cold Filter Plugging Point (CFPP).

Feedstock	Growth condition	Lipid production (%)	IV (gI ₂ /100g)	CN	CFPP (°C)	Ref.
<i>Scenedesmus</i> sp.	Standard medium	14.1	68.2	56.1	20.8	DOI 10.1002/jctb.2338
<i>T. obliquus</i>	Standard medium	32.53	67.24	59.15	-4.11	http://dx.doi.org/10.1016/j.biortech.2014.08.017
<i>Scenedesmus obliquus</i>	Standard medium	16.73	35.38	63.63	-11.87	DOI 10.1007/s12155-012-9222-2
<i>Tetrademus dimorphus</i>	Standard medium	24.4	177.9	56.04	0.08	https://doi.org/10.1007/s10811-025-03745-0
<i>Chlorella</i> sp.	Standard medium	27	65	56.7	4.5	DOI 10.1002/jctb.2338
<i>C. pyrenoidosa</i>	Standard medium	28.58	133.16	43.88	-0.97	http://dx.doi.org/10.1016/j.biortech.2014.08.017
<i>C. sorokiniana</i>	Dairy effluent	35	62.81	108.31	-13.86	https://doi.org/10.1007/s40201-020-00483-y
<i>C. vulgaris</i>	Dairy effluent	36.67	95.93	83	-13.28	
<i>C. sorokiniana</i>	Dairy effluent	/	72.80	57.49	/	https://doi.org/10.1007/s12155-021-10338-5
<i>C. sorokiniana</i>	Dairy effluent 90%	40.33	134.89	51.92	/	https://doi.org/10.1007/s11356-025-36488-z
<i>N. oceanica</i>	Municipal wastewater	26.91	169.27	36.17	-1.13	http://dx.doi.org/10.1016/j.biortech.2016.07.083
<i>N. oceanica</i>	Standard medium	37.71	94.7	52.3	4.7	https://doi.org/10.1016/j.biombioe.2018.12.009

Chapter 5

Conclusions

The transition from fossil-based fuels to renewable alternatives is one of the most urgent needs of present time. In this context, this thesis aimed to improve biotechnological strategies for microalgal biomass accumulation for high-quality biodiesel production. To achieve this goal, innovative strategies are required, with microalgae emerging as a promising feedstock for the production of valuable biodiesel. Despite their high biomass productivity and the high-quality of lipid fractions, several challenges must be still addressed prior to industrial-scale implementation. High energy demand remains a critical limitation of this process, therefore, improving process efficiency by increasing biomass and lipid productivity could significantly enhance biodiesel yield while reducing overall costs.

In this work, to reduce the costs of the process and improve sustainability, microalgae have been cultivated exclusively on industrial wastewaters. The dairy industry was selected as source of effluents due to its elevated environmental impact. Indeed, large volumes of fresh water are implemented in milk processing generating $192.5 \cdot 10^6 \text{ m}^3$ of wastewater annually in Europe (Stasinakis et al., 2022). The composition of these wastewaters varies depending on the final product. A local dairy industry supplied us “scotta”, the by-product of ricotta production, washing waters, derived from cleaning operations, and exhausted sludge, from primary biological treatment with activated sludge. Notably, both *C. sorokiniana* and *T. obliquus* were able to grow on all the assessed wastewaters after proper dilution. Overall, one of the main growth inhibitors in these streams is ammonium, which is toxic for microalgae at high concentrations and represents the main nitrogen source present. However, through proper dilution and pH adjusting, microalgal growth can be successfully sustained. Among the tested effluents, DWW and DES resulted to be more suitable for microalgal cultivation

due to ammonium content, transparency and pH levels. For these reasons, they have been implemented in the cultivation of *C. sorokiniana* under different light regimes.

Indeed, a key strategy investigated in this study to modulate quality and quantity of lipids involved the application of specific light regimes during *C. sorokiniana* cultivation on 75%DWW and 25%DES. The utilization of different wavelengths enabled targeted modulation of lipid composition and thus influenced biodiesel quality. A key finding of this work is that the sequential application of red light and blue light improved low-temperature performances while maintaining high oxidative stability and favorable ignition performances. This condition enhanced biodiesel quality compared to blue or red light alone, while also improving biomass production compared to blue light. Light wavelength modulation thus represents a versatile strategy that can be extended to other microalgal species to tailor biomass composition toward a compound of interest. This approach is particularly valuable when combined with wastewater treatment, where substrate composition cannot be easily controlled.

Moreover, a two-step cultivation process implemented with a combination of different microalgal species in each stage was performed to induce nitrogen limitation during the second step and exploit the distinct lipid profiles to obtain blended biomasses for improved biodiesel quality. The highest cell density and lipid productivity were achieved when *T. obliquus* was cultivated in the first stage. Lipid content of 27% in *T. obliquus* and 44% in *N. oceanica* were recorded. *T. obliquus* showed high proportions of C18:2 and C18:3 fatty acids, which significantly increased the PUFA fraction driving to lower CN and higher IV. However, blending biomasses from both stages improved overall biodiesel quality, although values remains slightly outside standard limits. Further optimization could be achieved by adjusting the biomass mixing ratio or extending the cultivation time of the first step, as microalgae in the late growth phase typically increase SFA content and reduce PUFA content.

The last strategy explored the utilization of an effluent deriving from an innovative gas fermentation (GFE) process, which is involved in the carbon-negative production of a valuable organic alcohol (hexanol) widely used across several industrial sectors. To further valorize this process, the carbon-rich effluent was employed as a substrate for microalgal cultivation. In this study, high biomass productivity ($0.5 \text{ gDW L}^{-1} \text{ d}^{-1}$) and lipid quality for biodiesel were achieved using *C. sorokiniana* grown on 50:50 DES:GFE. The biodiesel obtained met IV and CN standards, making it suitable for direct use as pure biodiesel, without blending. This approach contributes to the development of a circular economy, promoting waste valorization and generation of valuable products.

Although these strategies were investigated independently, they could be integrated in future applications. For instance, the combination of optimized light regimes with the cultivation of microalgae on 50:50 DES:GFE could further enhance biomass productivity and lipid quality. This represents a promising application for future research based on findings of this work.

It is worth noting that all strategies led to the production of high-quality biodiesel, while cultivating microalgae on wastewaters, thereby simulating realistic industrial conditions. Additionally, significant nutrient removal was achieved in all cases, confirming the effectiveness of bioremediation process. The integration of microalgal wastewater treatment with enhanced biomass and lipid productivity contributes to developing feasible and cost-effective process.

Overall, this thesis provides valuable insights into upstream process optimization for microalgal biodiesel production. Specifically, it demonstrated that integrating wastewater valorization with cultivation strategies can improve microalgal biodiesel production. These findings contribute to the development of scalable and cost-effective biotechnological processes.

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