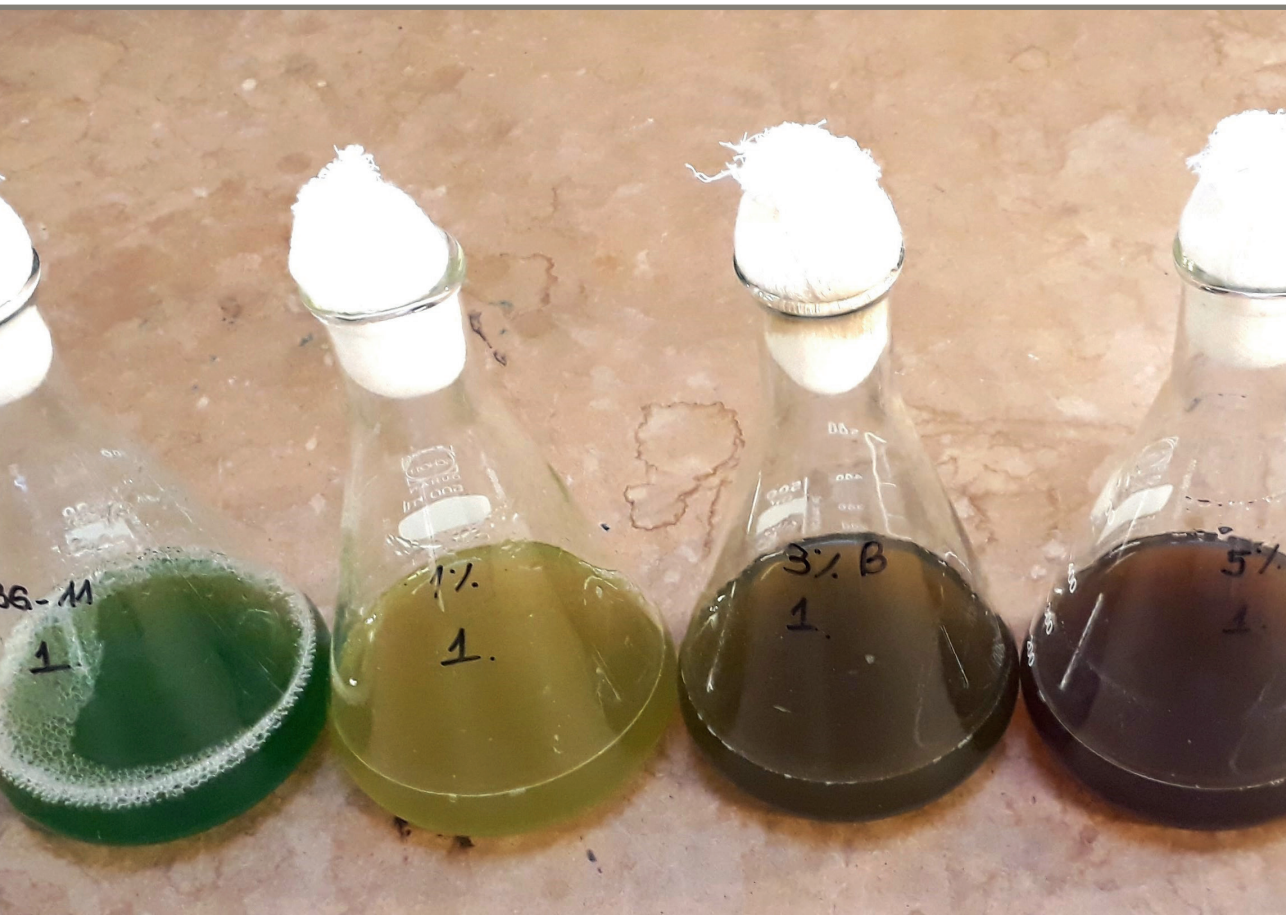
RIGA TECHNICAL UNIVERSITY

Baiba Ieviņa

INTEGRATION OF MICROALGAE CULTIVATION TECHNOLOGY IN BIOGAS PLANTS

Doctoral Thesis



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RIGA TECHNICAL UNIVERSITY
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INTEGRATION OF MICROALGAE CULTIVATION TECHNOLOGY IN BIOGAS PLANTS

Doctoral Thesis

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ANNOTATION

Humanity is facing an energy crisis due to depleting fossil resources, industrialization, and a growing world population, which has forced us to focus on finding alternative energy sources. Moreover, increasing carbon dioxide concentration in the atmosphere has led to climate change with severe consequences such as changing weather patterns and disruption of the balance of nature. In this context, microalgae have attracted global attention considering a potential solution for both sustainable energy and CO_2 sequestration. Microalgae biomass with its excellent qualities such as rapid growth rates, high carbon dioxide absorption capacity, resistance to harsh conditions, and the potential for continuous production throughout the year are superior to other traditional feedstocks considered for bioenergy. Despite the huge potential of microalgae biomass, its current use is limited to a few products and applications due to the low productivity and high production cost of biomass.

To offer a feasible solution, the current PhD thesis focuses on harnessing microalgae biomass as a sustainable resource for biogas production with potential applications in food, feed, nutraceuticals, cosmetics, and medicine industries when integrating a biorefinery concept. The PhD thesis aims to develop a novel improved microalgae biomass production system for biogas plants integrating biogas waste streams, namely digestate and flue gases as low-cost nutrients. The produced microalgal biomass is returned as anaerobic digestion feedstock closing the loop of nutrient circulation. This innovative approach seeks to integrate a circular economy model by utilizing waste streams from biogas plants, thereby transforming by-products and emissions into valuable resources for energy generation.

During the doctoral studies, an innovative technology for microalgae cultivation effective in colder climates was designed as a pilot, constructed and patented. This technology overcomes the limitations of existing cultivation methods by improving light access for the microalgae, minimizing land use with a stacked modular system, and integrating artificial LED lighting. Furthermore, potential microalgae species for low-temperature climates were selected based on the literature review and tested. Altogether three microalgae strains were selected for suitability for biomass production using digestate.

The PhD thesis consists of three main chapters: Literature review, Research methodology and Results and Discussion. In the literature review chapter, the factors affecting microalgae growth and biomass production are discussed, as well as state-of-the-art technologies for microalgae cultivation, potential biorefinery routes of microalgal biomass, digestate management and biomass harvesting techniques are reviewed. Moreover, the role of microalgae in the global bioeconomy is debated. In the Research methodology chapter methods applied are described. The obtained results are described and discussed in the Results and Discussion chapter.

The PhD thesis is based on seven scientific publications and presented in five international scientific conferences.

ANOTĀCIJA

Fosilo resursu izsīkšana, industrializācija un pieaugošais pasaules iedzīvotāju skaits ir izraisījis enerģijas krīzi, kas liek pievērsties alternatīvu enerģijas avotu meklēšanai. Turklāt pieaugošā oglekļa dioksīda koncentrācija atmosfērā ir novedusi pie klimata pārmaiņām ar smagām sekām tādām kā izmainīti laikapstākļi un izjaukts dabas līdzsvars. Šajā kontekstā mikroaļģes ir piesaistījušas pasaules uzmanību, izvirzot tās par potenciālu risinājumu gan ilgtspējīgas enerģijas, gan CO_2 sekvestrācijas jomā. Mikroaļģu biomasa ar savām izcilajām īpašībām, piemēram, strauju augšanu, augstu oglekļa dioksīda absorbcijas spēju, noturību pret skarbiem apstākļiem un iespēju nepārtraukti ražot biomasu visa gada garumā, ir pārāka par citām tradicionālajām bioenerģijas izejvielām. Neraugoties uz mikroaļģu biomasas milzīgo potenciālu, tās pašreizējā izmantošana ir ierobežota līdz dažiem produktiem un pielietojuma veidiem zemās biomasas produktivitātes un augsto ražošanas izmaksu dēļ.

Lai piedāvātu iespējamu risinājumu, šajā promocijas darbā galvenā uzmanība pievērsta mikroaļģu biomasas kā ilgtspējīga resursa izmantošanai biogāzes ražošanai ar potenciālu pielietojumu pārtikas, barības, uztura, kosmētikas un medicīnas nozarē, integrējot biorafinēšanas koncepciju. Promocijas darba mērķis ir izstrādāt jaunu uzlabotu mikroaļģu biomasas ražošanas sistēmu biogāzes stacijām, integrējot biogāzes atkritumu plūsmas digestāta un dūmgāžu veidā kā lētu barības vielu avotus. Saražotā mikroaļģu biomasa tiek nodota biogāzes ražošanai kā anaerobās fermentācijas izejviela, noslēdzot barības vielu aprites ciklu. Šīs inovatīvās pieejas mērķis ir integrēt aprites ekonomikas modeli, izmantojot biogāzes iekārtu atkritumu plūsmas, tādējādi pārveidojot blakusproduktus un emisijas par vērtīgiem resursiem enerģijas ražošanai.

Doktorantūras studiju laikā tika izstrādāta un patentēta inovatīva tehnoloģija mikroaļģu audzēšanai, kas ir piemērota aukstākam klimatam. Šī tehnoloģija pārvar pašreizējo audzēšanas sistēmu ierobežojumus, uzlabojot gaismas piekļuvi mikroaļģēm, samazinot nepieciešamo zemes platību, izmantojot saliekamu modulāru sistēmu un integrējot mākslīgo LED apgaismojumu. Turklāt, tika atlasītas un pārbaudītas potenciālās mikroaļģu sugas zemas temperatūras klimatam.

Promocijas darbs sastāv no trim galvenajām nodaļām: Literatūras apskats, Pētījuma metodoloģija un Rezultāti un diskusija. Literatūras apskata nodaļā ir aplūkoti mikroaļģu augšanu un biomasas ražošanu ietekmējošie faktori, kā arī apskatītas mikroaļģu audzēšanas tehnoloģijas, iespējamie mikroaļģu biomasas biorafinēšanas ceļi, digestāta apsaimniekošana un biomasas ievākšanas metodes. Turklāt tiek apspriesta mikroaļģu loma pasaules bioekonomikā. Pētniecības metodoloģijas nodaļā ir aprakstītas izmantotās metodes. Iegūtie rezultāti ir aprakstīti un apspriesti nodaļā "Rezultāti un diskusija".

Promocijas darba pamatā ir septiņas zinātniskās publikācijas un ziņojumi piecās starptautiskās zinātniskās konferencēs.

ACKNOWLEDGEMENT

The topic discussed in the thesis presented a significant challenge for me. Upon commencing my research, the microalgae topic was entirely new and uncharted territory for me. Therefore, I want to express my deepest gratitude to everyone who contributed to the development of my doctoral thesis.

First and foremost, I want to thank my supervisor, Professor Francesco Romagnoli, for his invaluable supervision, support and advice he invested in the development of this thesis.

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My appreciation also goes out to the rest of my family and friends for their encouragement and support throughout my studies.

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INTRODUCTION

The relevance of the doctoral thesis

The depletion of fossil resources, alongside industrial growth and a growing global population, set the ground for an energy crisis, forcing us to move to renewable energy alternatives. Additionally, rising levels of carbon dioxide in the air are causing climate change, leading to changes in weather and harming nature's balance. In this context, microalgae are considered a potential solution for both sustainable energy and CO_2 sequestration due to their superior qualities, such as fast growth rate, ability to absorb high concentrations of CO_2, and resistance to harsh conditions. In contrast to first-generation biomass such as corn or sugarcane, microalgae do not compete with food production because they do not require arable land for cultivation. Microalgae biomass can be converted to various types of energy, including biogas, biodiesel, and bioethanol. Moreover, they contain high-value compounds with high potential in food, feed, nutraceuticals, cosmetics, and medicine. In addition to already existing applications of microalgae, the potential of microalgae is being explored in other emerging areas, including wastewater treatment, biostimulants and biopesticides, and biochemicals [1].

Despite the vast potential of microalgae biomass, its current use is limited to a few products and applications due to substantial challenges of biomass production including high capital and operational costs, low biomass productivity, scale-up issues, and high costs of biomass harvesting and downstream processing [2]. Recently much effort has been focused on promoting the economic feasibility of microalgae cultivation including bioreactor design considerations [3], optimization of cultivation conditions [4]–[6], search for new more productive microalgal strains [7], [8] and new biomass harvesting techniques to decrease the costs and increase the harvesting efficiency [9], [10].

The application of various wastewaters as a low-cost nutrient source for microalgae growth has been studied extensively lately to further lower the cost of biomass production [11]. Digestate, a nutrient-rich by-product of anaerobic digestion, is currently used as fertilizer in agriculture; however, several challenges associated with digestate management limit land application. Moreover, the increasing number of biogas plants in Europe creates an overproduction of digestate resulting in environmental and human health risks. Coupling biogas production with microalgae cultivation can provide various benefits, including nutrient recycling from liquid digestate and CO_2 sequestration from flue gas.

To date, most large-scale microalgae cultivation is located in warm low-latitude regions such as Israel, Australia and the southern USA [12] whereas biomass production in Nordic regions remains a major challenge. Nevertheless, several recent studies prove that year-round microalgae cultivation in a low-temperature environment can be achieved if local strains adapted to the local climate are used [13]. However, studies on microalgae cultivation in high-latitude regions are scarce and no reports could be found on year-round cultivation of microalgae in Latvian climate conditions.

Objective and tasks

The doctoral thesis aims to develop a novel microalgae biomass production technology for biogas plants integrating biogas side waste streams. In order to achieve the goal, the following tasks were set:

1. Select potential microalgae species for the Latvian climate;
2. Assess the influence of factors affecting microalgae cultivation;
3. Test agricultural digestate as a low-cost nutrient source for microalgae;
4. Test the potential of increased CO_2 concentrations for enhanced biomass production;
5. Design a novel improved microalgae cultivation system;
6. Test the novel technology integrated into a biogas plant.

Scientific novelty

The scientific novelty of the Doctoral Thesis is related to several aspects linked to digestate management and microalgae biomass production. A new microalgae cultivation system was built to overcome the drawbacks of the existing ones, offering improved light availability to microalgae cells, a reduction in land use, and year-round cultivation. Microalgal species for cultivation in high-latitude climates were selected and tested, offering an opportunity for biomass production and wastewater treatment in the Latvian climate. Lower biomass production costs can be achieved by using waste products from biogas production, namely digestate and flue gases. It was demonstrated that selected microalgae can remove nutrients from agricultural digestate at low temperature with high efficiency, thus offering an alternative digestate management tool to traditional land application. To the author's best knowledge no other cultivation technology for microalgae year-round biomass production in Latvian climate conditions has been developed.

Practical significance

A novel microalgae cultivation system was designed and built, allowing biogas operators to potentially incorporate microalgae cultivation in biogas plant daily operations to increase biomass security, lower biomass transportation costs, and offer an alternative route of digestate management to deal with the overproduction issue.

The designed technology set the ground for a patent from the Patent Office of the Republic of Latvia, which was granted explicitly for developing a novel microalgae cultivation system.

Research framework

The research was framed in two blocks: (1) Laboratory tests and (2) Pilot race-way ponds and 7 stages namely, (1.1.) Microalgae strain selection, (1.2.) Impact of cultivation conditions, (1.3.) Digestate as a nutrient source, (1.4.) CO_2 as a carbon source, (2.1.) Design of cultivation

technology, (2.2.) Construction and integration of pilot into a biogas plant, and (2.3.) Testing of the novel cultivation system. The research framework is shown in Figure 1.1.

For each stage, a literature review was performed, and extensive laboratory tests were performed for stages 1.2., 1.3., and 1.4.

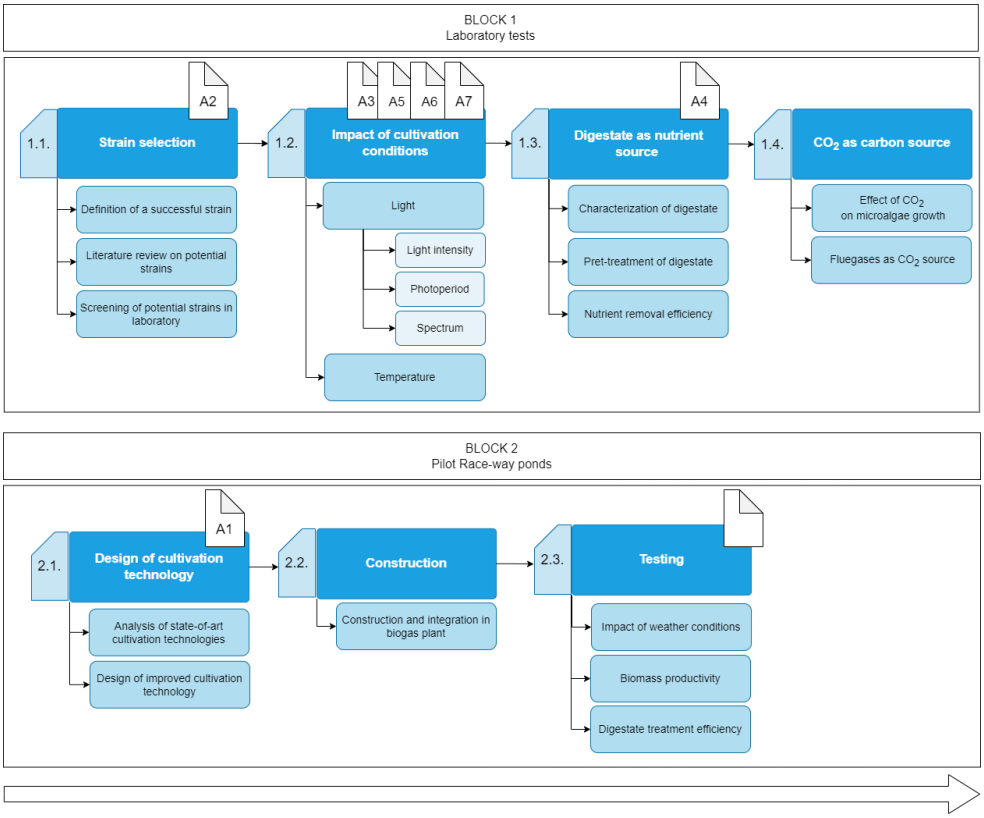


Figure 1.1. The research framework of the doctoral thesis.

Approbation of the research results

The thesis is based on seven scientific publications, three other scientific publications arose from the doctoral thesis but are not included in the thesis. Results have been presented at five international scientific conferences. The patent has been granted for the developed novel cultivation system from the Patent Office of the Republic of Latvia.

Scientific publications:

1. Romagnoli F., Ievina B., Perera W. A. A. R. P., Ferrari D. Novel Stacked Modular Open Raceway Ponds for Microalgae Biomass Cultivation in Biogas Plants: Preliminary Design and Modelling. Environmental and Climate Technologies, 2020, vol. 24, no. 2, pp. 1–19.

2. Ievina B., Romagnoli F. The potential of *Chlorella* species as a feedstock for bioenergy production: A review. Environmental and Climate Technologies, 2020, vol. 24, no. 2, pp. 203–220.
3. Ievina B., Romagnoli F. Effect of light intensity on the growth of three microalgae in laboratory batch cultures, 2020, European Biomass Conference and Exhibition Proceedings, pp. 169–174.
4. Ievina B., Mantovani M., Marazzi F., Mezzanotte V., Romagnoli F. Application of activated carbon treated agricultural digestate for microalgae cultivation, 2021, European Biomass Conference and Exhibition Proceedings, pp. 124–131.
5. Romagnoli F., Weerasuriya-Arachchige A. R. P. P., Paoli R., Feofilovs M., Ievina B. Growth Kinetic Model for Microalgae Cultivation in Open Raceway Ponds: A System Dynamics Tool. Environmental and Climate Technologies 2021, vol. 25, no. 1, pp. 1317–1336.
6. Ievina B., Romagnoli F. Microalgae *Chlorella vulgaris* 211/11j as a promising strain for low temperature climate. Journal of Applied Phycology, 2024, In press.
7. Ievina B., Romagnoli F. Unveiling the underlying factors for light spectrum preference for enhanced microalgae growth. (Algal Research. Under review).

Other scientific publications

1. Mezzanotte V., Romagnoli F., Ievina B., Mantovani M., Invernizzi M., Ficara E., Collina E. LCA of Zero Valent Iron Nanoparticles Encapsulated in Algal Biomass for Polishing Treated Effluents. Environmental and Climate Technologies 2022, pp. 1196 – 1208.
2. Romagnoli F., Thedy A., Ievina B., Feofilovs M. Life Cycle Assessment of an Innovative Microalgae Cultivation System in the Baltic Region: Results from SMORP Project. Environmental and Climate Technologies 2023, vol. 27, no. 1, pp. 117–136.
3. Romagnoli F, Spaccini F., Boggia A, Paoli R, Feofilovs M, Ievina B, Rocchi R. Microalgae cultivation in a biogas plant: Environmental and economic assessment through a life cycle approach. Biomass and Bioenergy 2024, 182: 107116.

Participation in scientific conferences

1. Romagnoli F., Ievina B., Perera W. A. A. R. P., Ferrari D. Novel Stacked Modular Open Raceway Ponds for Microalgae Biomass Cultivation in Biogas Plants: Preliminary Design and Modelling. CONECT 2019, May 15-17, 2019, Riga, Latvia.
2. Ievina B., Romagnoli F. The potential of *Chlorella* species as a feedstock for bioenergy production: A review. CONECT 2020, May 13-15, 2020, Riga, Latvia.
3. Ievina B., Romagnoli F. Effect of light intensity on the growth of three microalgae in laboratory batch cultures, 28th European Biomass Conference and Exhibition, July 6 – 9, 2020, Online.

4. Ievina B., Mantovani M., Marazzi F., Mezzanotte V., Romagnoli F. Application of activated carbon treated agricultural digestate for microalgae cultivation, 29th European Biomass Conference and Exhibition, April 26-29, 2021, Online.
5. Romagnoli F., Weerasuriya-Arachchige A. R. P. P., Paoli R., Feofilovs M., Ievina B. Growth Kinetic Model for Microalgae Cultivation in Open Raceway Ponds: A System Dynamics Tool. CONECT 2021, May 12-14, 2021, Riga, Latvia.

Patents

Romagnoli F, Dzikēvičs M, Ieviņa B. Atvērta tipa modulāra mikroaļģu kultivēšanas baseinu sistēma (Modular open microalgae cultivation pond system). Patent number 15742, 12.06.2023.

1. LITERATURE REVIEW

1.1. The biology and classification of microalgae

Microalgae are a diverse group of microorganisms comprising eukaryotic photoautotrophic protists and prokaryotic cyanobacteria. Microalgae are unicellular species that grow using carbon, water, and other nutrients through the process of photosynthesis [14]. Cyanobacteria are photosynthetic bacteria but are also referred to as microalgae due to their ability to perform photosynthesis and are sometimes called blue-green algae. Today 35 000 species of microalgae have been described [15], however, the actual number of species is much higher. It has been estimated that there are 70,000 to several million species of microalgae [14], [16]. Only a very small fraction has been investigated for biotechnological purposes.

Microalgae are ubiquitous organisms that are present in almost all ecosystems from extremely cold polar regions to dry deserts [17]. Although they can be found in diverse habitats such as soil, surfaces of rocks, tree trunks or walls of urban buildings, most microalgae are aquatic organisms and can be found in freshwater (ponds, rivers and lakes), brackish water (estuary) or seawater with varying degrees of salinity [18]. Some species of microalgae can be found in extremely saline environments such as the Great Salt Lake in the USA or Dead Sea in Israel [14], [16]. Although microalgae are generally free-living, they can be found in a symbiotic association with other organisms [19]. There is a huge diversity of microalgae species adapted to a specific environment.

Microalgae play a major role in ecosystems. They convert light energy and carbon dioxide into biomass through photosynthesis, producing carbohydrates, proteins, and lipids. With their fast growth and higher photosynthetic efficiency than terrestrial plants, microalgae contribute to around 50% of the planet's primary production and are an important source of biomass in food chains [20]. Moreover, together with macroscopic algae, they are the largest producer of oxygen, making them essential for carbon fixation. Microalgae can differ in size from 0.5 to 200 μm [21].

Microalgae are a highly diverse group of microorganisms that includes prokaryotes (cyanobacteria) and photosynthetic eukaryotes belonging to three kingdoms - Protozoa, Chromista, and Plantae [22]. Although different classifications are currently in use for algae, the classification into taxonomic groups is generally based on pigment composition, diversity of storage products and morphological features [22]. Moreover, microalgae classification is under constant revision at all levels following new molecular, genetic or ultrastructural evidence. Constant reclassification and moving from one division to another is not uncommon. Genetic data have added more complexity to the classification of algae. Recently the classification of microalgae is primarily based on their pigment composition [23], dividing them into nine classes. One prokaryotic linage is recognized: Cyanophyta (blue-green algae) and nine eukaryotes: *Prochlorophyta*, *Glaucophyta*, *Rhodophyta* (red algae), *Cryptophyta*, *Chlorophyta* (green algae), *Euglenophyta*, *Chlorarachniophyta*, *Pyrrophyta* (dinoflagellates), and *Chromophyta* (heterokonts) [24].

1.2. Microalgae and bioeconomy

The world population is estimated to exceed 10 billion people by 2050 [25]. The rapid increase in the world's population, growing consumption and associated industrialization have led to negative environmental impacts including depletion of fossil resources and water, increase in carbon emissions, and contamination of water bodies. The current situation presents profound challenges to meet future requirements and requires substantial changes in current practices in almost every aspect. The EU Bioeconomy Strategy aims to implement a sustainable and circular bioeconomy in Europe by balancing the growth of economic activities, the protection of natural resources and the needs of a growing world population [26]. In this context, microalgae are a renewable bioresource with various applications and an enormous potential to solve various future challenges including sustainable biofuel and agriculture, food security, clean water, and mitigation of climate change.

Microalgae are classified as third-generation feedstock and have evident advantages over other types of feedstocks, e.g. (1) fast growth rate, (2) growth in low-quality water, (3) high CO_2 fixation rate, (4) all-year-round production, (5) no competition with food production, (6) no arable land needed for cultivation and (7) production of a wide range of bioproducts with high market value [27]–[29]. These characteristics offer an enormous potential for microalgae to support the bioeconomy goals.

Although recognized as a promising source of sustainable energy already decades ago, microalgae have attracted more interest recently due to governmental policies and subsidies. Bioenergy from microalgae is also supported through the Renewable Energy Directive (EU 2018/2001) promoting the use of algae to achieve EU climate and energy targets. Due to the increased number of studies carried out in the last decade, scientific knowledge on microalgae culturing is continuously growing showing the enormous potential of microalgae biomass both for bioenergy and food and feed sector, as well as novel applications such as wastewater treatment and biofertilizers and biostimulants for increased sustainability of agriculture.

Despite the superior qualities of microalgae, the high cultivation cost of biomass is limiting the commercial use of microalgae as a feedstock for bioenergy production. Several strategies have been proposed to overcome the economic challenges of large-scale biomass production. Introduction of advanced approaches and technologies in microalgal cultivation including mixotrophic cultivation, co-cultivation of several microalgae species, cutting-edge designs of cultivation systems, and integration of wastewater and flue gases from industrial processes have been tested to increase the feasibility of microalgal bioenergy [30]–[32]. Out of these, wastewater and flue gas integration is seen as a highly promising direction to more sustainable and economically feasible microalgae biofuels [33]. The ability of microalgae to remove pollutants from various wastewaters can be exploited by integrating various wastewaters in cultivation thus providing a low-cost nutrient source at the same time performing wastewater treatment. Moreover, recycling of growth medium after treatment reduces water consumption and contributes to further reduction of cultivation costs.

Although there has been substantial technological progress in developing algae-based bioenergy in recent years [34], the scientific community has come to the agreement that the

current microalgae biofuel strategy is not viable. It is the consensus today that algal biofuels are not viable without the integration of the biorefinery concept where algal biomass is used to co-produce high-value compounds [35], [36]. A biorefinery approach that produces multiple high-value products from microalgae biomass is essential to fully exploit the vast potential of microalgae and enable economically viable coproduction of bioenergy. To shift from pure biofuel strategy to microalgal biorefinery requires cutting-edge technology developments to ensure the transition of the focus from algal-based bioenergy to high-value bioproducts.

Carbon dioxide is recognized as the primary greenhouse gas emitted through human activities. Various CO_2 sequestration technologies have been developed and proposed for the stabilization of emissions of various pollutants. CO_2 sequestration by photosynthetic microalgae shows good potential due to the high rate of CO_2 capture, high growth rate of microalgae and tolerance to high CO_2 concentrations [37]. Microalgae can be utilized as a part of the mitigation strategy for reduction CO_2 concentration in the atmosphere associated with greenhouse gases and global climate change. It has been reported that 1 kg of microalgae biomass can fix 1.83 kg of CO_2 [38] making microalgae utilization a promising strategy.

Microalgae can be cultivated commercially in open ponds or photobioreactors. Being a valuable source of proteins, lipids, carbohydrates and other value-added compounds microalgae biomass has demonstrated significant commercial potential in producing food, feed, biofuel, pharmaceuticals, cosmetics and other high-value products [20]. Currently, algae are used for a relatively small number of industrial applications [25]. There are a few species with a commercial interest: Spirulina (health), *Chlorella* (health), *Haematococcus* (astaxanthin), *Dunaliella* (beta-carotene), *Botrycoccus* (oil), *Phaeodactylum* (fucoxanthin, EPA omega-3) and Porphyridium. Only a few microalgal products are produced in large quantities and current applications include food, feed, health-related and cosmetic industries [1]. The price of microalgae biomass for these applications ranges from 5 to 500 € kg^{-1}, with a market size of up to 100 kt $year^{-1}$. However, new applications of algae biomass are currently being explored for bioremediation and biomonitoring [39], biofertilizer and plant biostimulant applications [26], biopolymers [40], [41], CO_2 sequestration [37], [42], and wastewater treatment [43], [44].

Currently 447 algae and cyanobacteria *Spirulina* spp. production units exist in Europe [26]. Algae production sites are spread between 23 European countries. More than 50% of these companies produce microalgae and/or Spirulina (*Arthrospira*) while macroalgae production depends more on wild harvesting.

A more detailed description of microalgae applications can be found in Chapter 1.4.

1.3. Bioenergy from microalgae

In the last decades, the scientific community's attention has been focused on the search for alternative fuels to replace the depleting fossil resources. Microalgae have been considered a sustainable feedstock for biofuel production due to their rapid growth rate, high biomass productivity and ability to produce high levels of lipids and carbohydrates. Moreover, microalgae possess no risks for competition with food crops for arable land and can be cultivated in wastewaters [28]. Consequently, microalgae have received great attention from

researchers, government officials and industrial owners as a novel biomass source for the generation of renewable energy.

Biofuels can be derived from a diverse range of feedstocks, including higher plants, microorganisms, organic waste, agricultural waste, and livestock manure. Generally, biomass is classified into four generations based on its origin. First-generation biomass is derived from edible feedstock, so-called energy crops, such as wheat, corn, sugarcane, and vegetable oils [45]. However, there are concerns that first-generation biomass is not sustainable due to competition with food production, the use of large quantities of fertilizers and water, and possible changes in land use patterns [45]. The use of energy crops as a feedstock for biogas production is no longer supported by the EU showing a tendency of reducing the share of energy crops in biogas production. However, first-generation biomass still represents a large part of the feedstock in some countries like Germany and Croatia [46].

Due to the drawbacks of first-generation biomass, efforts have been made to search for non-food materials. Second-generation feedstock utilizes non-edible residual and waste products such as agricultural and municipal residues, and waste oils. As a third-generation feedstock, microalgae are a promising alternative to conventional fossil fuels. The latest feedstock generation, termed fourth-generation biomass, encompasses the use of genetic engineering to increase the desired traits of organisms used in biofuel production [47]. A variety of traits such as higher growth rate, carbon fixation and accumulation of certain components or a tolerance to specific conditions have been targeted. Despite the high potential of the fourth-generation feedstock, its utilization is limited due to concerns about leaking genetically modified organisms into the environment [48].

The third and fourth-generation feedstocks are the potential sustainable source for the future production of biofuel. Microalgal biomass is a versatile feedstock that can be converted into various biofuels including biodiesel, biogas, biomethane, and bioethanol through various chemical processes such as transesterification, fermentation, pyrolysis, or anaerobic digestion (Figure 1.2.). A more focused literature review on biogas is provided below due to its direct connection with the topic of this thesis.

Biogas is produced by anaerobic digestion (AD) of organic feedstocks. The production of biogas has great environmental, economic, and socio-economic benefits including reducing landfills and promoting local energy independence, a circular economy, and employment in rural areas [49]. Biomass resources for biogas feedstock can be categorized as of animal or plant origin. According to the sector generating the feedstock, they can be divided as agricultural (animal manure, plant/vegetable by-products and agro-industrial residues, energy crops), industrial (residues and by-products from agro-industries, food industries, breweries), and municipal (sewage sludge, household biowaste, garden waste).

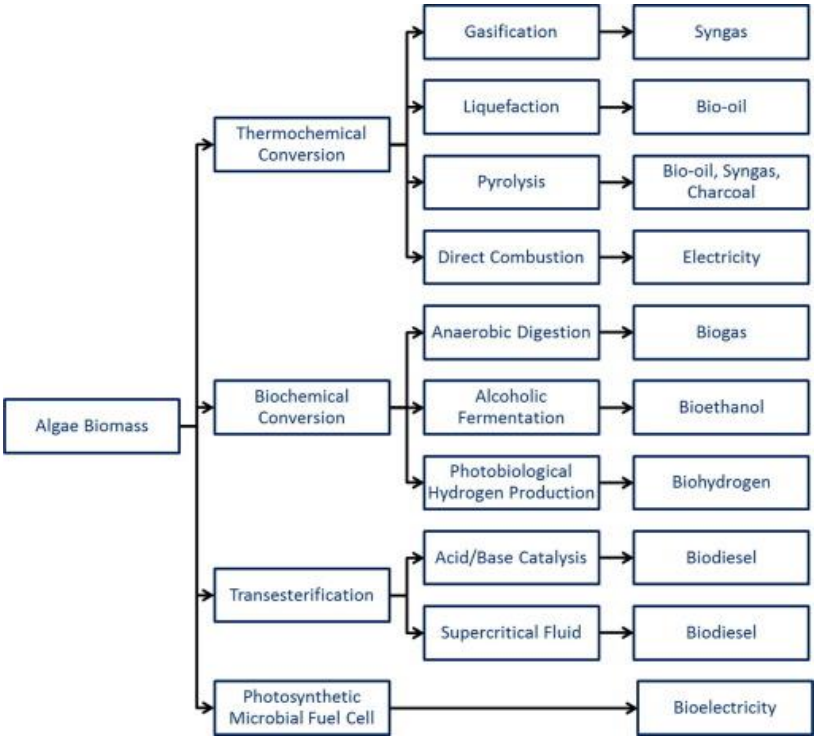


Figure 1.2. Algal biomass conversion processes for the production of biofuels [50].

Agricultural biomass is commonly utilized as a biogas feedstock. Animal manure is an excellent source for AD with a carbon-to-nitrogen ratio (C:N) of 25:1 and is rich in nutrients for the growth of anaerobic microorganisms [51]. Animal manure and slurry are characterized by differing dry matter content, composition based on animal species (e.g. pigs, cattle, horses, poultry) and the quality of animal feed. Solid manure has 10 to 30% dry matter, whereas liquid slurry <10% dry matter [51]. Manure contains various quantities of straw which is known as recalcitrant to anaerobic digestion due to high lignocellulose content. Therefore, animal manures are generally co-digested with other types of feedstock such as organic waste from various agro-industries, energy crops or sewage sludge [52].

Plant residues include a variety of agricultural by-products and harvest residues such as low-quality and spoiled crops, fruits and vegetables, and spoiled feed silage. Most plant residues need to be pretreated before anaerobic digestion to allow good digestion.

Microalgae biomass has been the focus of interest in AD during the last decade. Algae possess advantages over other types of feedstocks for AD such as fast growth rate, simultaneous carbon dioxide sequestration and wastewater treatment. Biogas production through anaerobic digestion is one of the most economically viable types of microalgae energy as it does not require highly concentrated biomass [53]. Methane production from microalgae has been reported in a range between 143 - 400 L-CH_4 $(kgVS)^{-1}$ depending on the cellular chemical composition of specific microalgae [54]. However, much higher methane yield has been

achieved with some species of microalgae. Mixing microalgae biomass with other AD feedstock (cattle slurry and maize silage) has been shown to improve biogas yield and composition [55]. Methane yield increased from 343 to 581 L-CH_4 $(kgVS)^{-1}$ in co-digestion of 40% *Arthrospira platensis* biomass.

Digestibility is a main AD feedstock parameter with a direct impact on methane production. Feedstock composition also determines the amount of time required to decompose a feedstock and thus the retention time of feedstock in a digester. For economic reasons, digesters are operated with the shortest retention times and the highest methane yields possible.

The properties and composition of biogas may vary depending on the type and structure of the feedstock, the biogas plant system, temperature, substrate retention time, volume load and other parameters [56]. Biogas is primarily composed of methane (50-70%) and carbon dioxide (30-50%). However, biogas contains small amounts of hydrogen sulfide and ammonia and is saturated with water vapor.

Anaerobic digestion is a complex process, which can be divided into four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Different microorganisms are involved in each step of decomposition with their own optimum range of conditions. The digestion process can take place in mesophilic (35–42 °C) or thermophilic (45–60 °C) temperature conditions. Temperature fluctuations are negatively affecting biogas production, therefore constant temperature during anaerobic digestion is required for optimal biogas production [57]. Anaerobic digestion by-product, digestate, is the decomposed feedstock rich in nutrients and is traditionally applied as a soil conditioner and fertilizer.

The number of biogas plants has been steadily growing over the last decade reaching 18 774 biogas plants throughout the Europe by the end of 2020 (Figure 1.3.) [46].

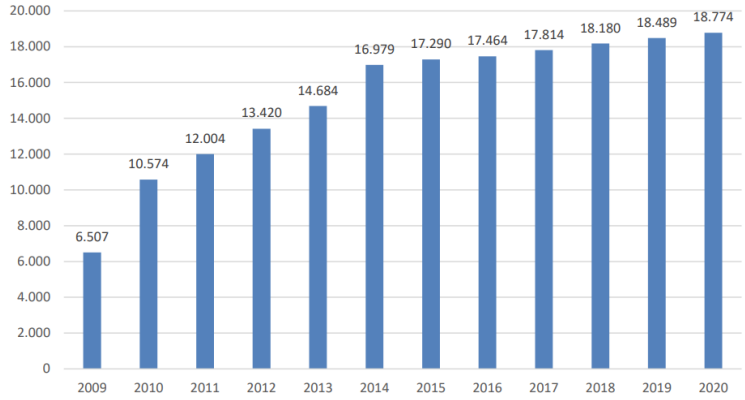


Figure 1.3. The number of biogas plants in Europe (EU27 + UK, + Switzerland, + Norway, +Serbia, + Iceland [46].

Biogas is an important source of heat and power in Europe. In 2020 the gross inland energy consumption of biogas reached 14.7 ktoe and represented 10% of the bioenergy consumption in the EU27. Moreover, biomethane production is increasing and is believed to continue to grow due to the versatility of biomethane as an energy source for transport, industry, power,

and heating [46]. An expansion of the biogas industry creates an overproduction of digestate leading to issues with its management (described in more detail in Chapter 1.8.).

1.4. Microalgae-based biorefinery

Although microalgae biomass has substantial potential for biofuel production, it is not viable in the current stage due to elevated production costs mainly associated with high energy demands and low productivity [2]. As mentioned before, the scientific community has agreed that algal biofuels are not viable without the integration of the biorefinery concept where algal biomass is used to co-produce high-value compounds. Biorefinery is seen as a promising option to reduce the production cost of microalgae biofuel [58]. Microalgae biorefinery is a concept of turning microalgae biomass into a range of valuable products, such as fuels, food, feed, chemicals, and other value-added products. This approach is analogous to the refinery of oil where a range of products is manufactured from a single feedstock to higher economic benefit. Microalgae biorefinery offers an innovative and sustainable way to utilize microalgae biomass, meanwhile making biofuel production economically viable. Microalgae biorefinery allows the use of resources efficiently and sustainably.

Lately, the microalgal biorefinery is receiving increasing interest. The commercial potential of microalgae biomass is still an untapped resource. Microalgae are a source of bioproducts such as pigments, proteins, lipids, carbohydrates, vitamins, and antioxidants with high commercial value. The extraction of these co-products is essential to improving the economic feasibility of microalgal bioenergy. The microalgae biorefinery concept is a new approach for better utilization of biomass potential, achieving higher viability and sustainability of bioenergy and moving towards “zero waste” production in a circular economy framework. Biorefinery results in a cost-effective simultaneous production of bioenergy and various valuable bioproducts. Moreover, besides economic benefits, it also minimizes the environmental impact with the more efficient use of resources and reduction of greenhouse gas emissions.

The current strategy applied for the extraction of high-value compounds is often commercially not viable due to the high cultivation costs, high energy requirements and low productivity of biomass and bioproducts [59]. To maximize the use of microalgae biomass, a comprehensive biorefinery strategy must be created. Several recent studies have been focusing on the concept of microalgae biorefinery offering various biorefinery routes [2], [33], [50], [60]–[63]. One example of microalgal biorefinery suggested by [2] is given below offering three potential biorefinery routes (Figure 1.4.).

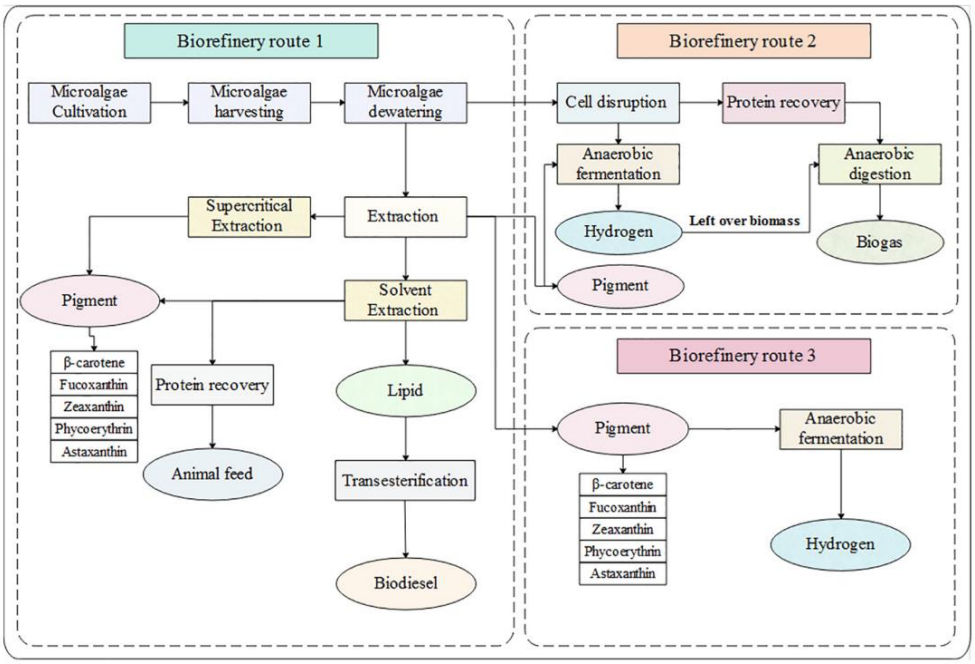


Figure 1.4. Process flow diagram of different potential microalgal biorefinery routes offered by [2].

To allow algal biomass to be sustainably processed into a range of bio-materials and products, optimization of material and energy use must take place. When microalgae-based wastewater treatment is considered, the energy consumption was reduced 10 times (0.2 kW h m^{-3}) when compared to the conventional wastewater treatment system (2 kW h m^{-3}) [58]. Therefore, algae-based wastewater treatment is energy efficient. Moreover, the utilization of wastewater as a nutrient source for microalgae reduces a considerable fraction of production cost, thereby making the biorefinery concept feasible.

Currently, microalgae are utilized in food, animal feed, health, and cosmetics sectors with emerging applications such as biofuels, fertilizers, biochemicals, wastewater treatment, and CO_2 biofixation showing a high potential (Figure 1.5.). Below a short description of current and emerging applications of microalgal biomass is given with a potential to establish viable biorefinery concepts.

Figure 1.5. Current and emerging applications of microalgae biomass [1]

Food and nutraceutical applications. Microalgae have high nutritional value and are considered a good source of macro- and micronutrients for human nutrition [64]. Furthermore, they are also rich in bioactive molecules with the potential to promote human health. Microalgae contain 40–70% proteins, 12–30% carbohydrates, 4–20% lipids, 8–14% carotene and substantial amounts of vitamins B1, B2, B3, B6, B12, E, K, and D [65], [66]. They are also an abundant source of biotin, folic acid, pantothenic acid, niacin, iodine, potassium, iron, magnesium and calcium [66]. High protein concentration with all the essential amino acids, lipids with a highly valuable profile, high content of omega-3 fatty acids, polysaccharides, antioxidants, including pigments are essential for human health [1]. Already widely established in Asia, algae biomass is gaining interest in the Western world due to its nutritional and therapeutic properties and the increasing demand for more sustainable and natural food sources [26]. Currently in the food industry microalgae are applied as whole biomass or extracted compounds in food products such as pasta, noodles, baked goods, plant-based milk, soups and many others [67].

Aquaculture and animal feed. Microalgae are used as feed in aquaculture for zooplankton, bivalve molluscs, crustaceans and some fish species due to the content of highly nutritional proteins and lipids in microalgae biomass [68]. Additional compounds, such as antioxidants, peptides, and fatty acids have additional benefits if incorporated into the daily diet.

The present production scale is still small when compared to other alternatives such as soy or fish oil and meal, which are commonly used in aquaculture [1]. Current microalgae production is estimated at around 25’000 t $year^{-1}$ with a market price of 20-50 € kg^{-1}. In contrast, soy oil and meal production exceed 200 million t $year^{-1}$ with a current price below 0.5 € kg^{-1} [1]. However, if microalgae production rose considerably then the price could be lowered to 5 € kg^{-1}.

Health industry. Microalgae contain various compounds with vast health benefits. The therapeutic properties of microalgae include cardioprotective, anticancer, anti-inflammatory, antioxidant, anticoagulant, antiviral, antibacterial, antifungal, anti-obesity and others [69].

Moreover, microalgal components are used to reduce blood cholesterol level, enhance the immune system, and prevent neurological disorders [1]. Microalgae biomass is marketed in the form of capsules, tablets, powders or liquids containing extracted compounds or whole biomass. Moreover, microalgae are able to produce bioactive compounds such as antibiotics, vaccines, monoclonal antibodies, hormones, enzymes, and other compounds with pharmaceutical and therapeutic applications, which are not easily synthesized using chemical methods [70]. Microalgae pigments also have health benefits such as the prevention of cancer, cardiac diseases, neurological disorders, and eye diseases. Microalgae are regarded as suitable hosts for production of recombinant proteins or peptides, such as monoclonal antibodies and vaccines due to their superior qualities such as high growth rate and relatively simple growing conditions.

Cosmetics. Use of microalgae in skin care products is widely applied and reported. Microalgal extracts can be found in anti-aging, refreshing, regenerating care products, as well as in sun-protection and haircare products [1]. Various pigments are used in care products as natural colorants. Moreover, due to their antioxidant activities they bring various other benefits and are used for skin aging, healing and preventing wrinkle formation [71].

Agriculture. Microalgae have beneficial effects on soil and plants. When applied to soil, microalgae are a source of organic carbon, improving soil quality. Moreover, microalgae influence soil microorganisms, produce phytohormones and other bioactive components that enhance plant growth and control pests and pathogens [18]. These properties of microalgae offer the opportunity to utilize microalgae biomass for various agricultural products with applications for soil improvement and plant growth stimulation and protection. Potential applications of microalgae biomass in agriculture are summarized in Figure 1.6. by [18].

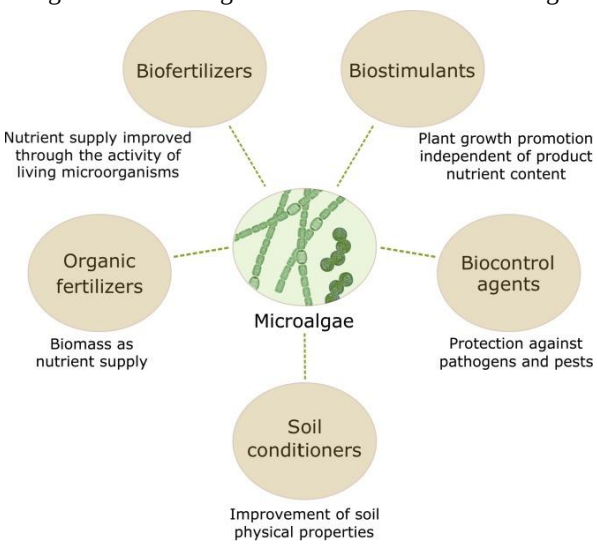


Figure 1.6. Potential agricultural products from microalgae for soil improvement, plant growth improvement and protection [18].

Biopolymers, Bioplastics, and Bulk Chemicals. The demand for plastic-based products has grown considerably in the last few decades, raising concerns about plastic pollution,

especially in marine ecosystems [25]. Biopolymers from microalgae can be used as an alternative to petrochemical-based plastics. Various microalgal biomass components such as starch, carbohydrates, and lipids can be converted into plastics. Three main approaches are currently used: (1) direct application of microalgae as bioplastics, (2) mixing of microalgae with petroleum-based plastics or bioplastics, and (3) genetic engineering of microalgae to produce bioplastic polymer precursors. Moreover, various other chemicals can also be produced from microalgae biomass offering sustainable substitution for fossil oil-based chemicals [25]. Acetic, propionic, (iso)butyris, (iso)valeric and caproic acids are volatile fatty acids traditionally obtained through a petrochemical pathway. These compounds can be used as building blocks in various fields including food additives, pharmaceuticals, adhesives, solvents and chemical intermediates [72].

1.5. Microalgae strain selection

The first step in developing a successful and commercially viable microalgae biomass production for biofuel or other applications is the selection of microalgae strains for cultivation. Suitable strains must be selected based on the intended application of biomass, cultivation technology and production scale. The selection of microalgae strains is a crucial factor for high productivity under the selected environment and for the overall success of large-scale biomass production. Although there are thousands of species, only a limited number of species have been described and characterized [73]. Moreover, a very small fraction of all species has been studied for large-scale biomass or bioenergy production and only some microalgae have been commercialized so far, e.g., *Haematococcus*, *Dunaliella*, *Chlorella*, and *Arthrospira* (Spirulina). Other commonly used species are *Nannochloropsis* spp., *Isochrysis* spp., *Thalassiossira* spp., *Tetraselmis* spp., and *Chaestoceros* spp. [74]. Microalgae are currently produced commercially mainly for high-value (>$10,000/t) human nutritional products [75].

Microalgae are known to occupy many different habitats characterized by various environmental conditions therefore, it is important to select the correct strain for the geographical location of interest in case of outdoor cultivation. Some strains are resistant to high temperatures, whereas others thrive in lower temperatures. Cultivation in outdoor facilities is associated with limited control of environmental conditions therefore highly robust and flexible microalgae are required for large-scale biofuel production due to dynamic weather and environment, especially strains that can tolerate high concentrations of inorganic carbon and have wide temperature tolerance [76]. The optimal temperature range for most microalgae is often reported between 20 and 25 °C [13], however, there are species thriving in both significantly higher and lower temperatures. In Southern regions receiving high insolation, strains resistant to high temperature and light intensity are crucial; however, finding an optimal strain for cultivation at higher latitude regions might be especially challenging due to dynamic weather conditions, changing seasons and increased cloud cover compared to more southern regions.

Moreover, specific conditions might be required for some species, such as low pH or higher salinity. For example, while most microalgae prefer a pH close to 7.0, the optimal pH for the

microalga *Dunaliela salina* is around 11.5 whereas for *Dunaliela acidophila* it is in a range of 0.0 to 3.0 [77]. If algal species is ubiquitous, it suggests that it can withstand a wide range of environmental conditions, which is another requirement for successful outdoor cultivation. For any commercial microalgae cultivation, high productivity of the target compound is a main prerequisite, therefore in addition to the overall growth rate, the productivity of the target compound must be evaluated.

The ideal strain for large-scale outdoor biomass production must have the following characteristics: rapid growth rate, wide temperature tolerance, high competitiveness, limited nutrient requirements, high CO_2 uptake, tolerance to shear force and to various contaminants in flue gas (e.g. NOx, SOx) and wastewater (e.g. heavy metals, ammonium), adaptation ability to fluctuating environmental conditions (light, pH, etc.) and source high-value co-products [27], [78]. Large differences between microalgae strains regarding their pollution removal capacity from wastewater have been reported pointing out the strain selection as a key aspect for successful microalgae application in wastewater treatment [79]. The desired characteristics of the ideal microalgal strain for large-scale biomass production are summarized in Table 1.1.

Extensive research has been carried out focusing on microalgal strains that can be cultivated for large-scale biomass production. Among microalgal strains, various *Chlorella* species have been studied extensively. The green microalga *Chlorella vulgaris* has received much attention and is probably the most studied microalga together with another green microalga *Chlamydomonas reinhardtii* which was the first microalga to be sequenced [80].

Table 1.1.

Desired characteristics for a microalgae species for biomass production, modified from [73].

Property	Reason
Rapid growth	Required for high productivity
High photosynthetic efficiency	Increases productivity
Shear tolerance	Must tolerate the shear created by paddle wheel
Broad temperature optimum	Required for high productivity in all seasons
Low temperature tolerance	Required for cultivation in cold seasons
High temperature tolerance	Reduces the risk of culture collapse due to high temperature
Ability to tolerate high irradiances	Reduces photoinhibition and photodamage at high irradiances
High CO_2 tolerance	Potential for CO_2 sequestration
Grows in a "selective" environment	Makes management of contamination easier
High lipid content (for biodiesel production)	Required for high lipid productivity for biodiesel
Contains high-value bioactive compounds with a commercial potential	Required for application of biorefinery approach
Weak or no cell wall	Required for easier extraction of compounds

In Nordic conditions when the cultivation system is located at high latitude, winter weather conditions present a great challenge. In outdoor cultivation, the weather conditions are dynamic

compared to nearly static laboratory conditions. Light availability varies greatly during the day as well as throughout the year: day length and light irradiance are especially limited in winter, while more than 17 hours of light per day with relatively high intensities are experienced during the summer months in Latvian climate conditions. Some algae have quite a narrow temperature optimum, whereas others exhibit good growth over a wide temperature range [73]. Although generally, the reported productivity is remarkedly lower during the winter, there are reports on species that can survive conditions when cultivation pond freeze over occasionally [81]. Therefore, successful microalgal cultivation at higher latitudes is assured by the use of local microalgae strains that are adapted to the local climate conditions and will yield higher biomass than foreign strains. Indeed, some reports can be found on successful microalgae cultivation in Nordic countries [13].

The ideal microalgal strain having all the properties mentioned above has not been identified so far. The rotation of microalgae species is suggested as a promising strategy to increase the viability of year-round cultivation [82]. The selection of microalgal strains for specific seasons based on weather conditions can increase annual biomass production in raceway ponds. Indeed, it was demonstrated that an increase in productivity up to 20% could be achieved by rotation of species compared to a monoculture of *Spirulina* and *Chlorella* [82]. Consequently, energy-efficient rotation of strains well adapted to the Nordic climate has the potential to provide high biomass yields throughout the year.

1.6. Microalgae cultivation systems

Two types of systems - open and closed, are currently widely used for microalgae biomass production. In closed photobioreactors, the culture is separated from the atmosphere by a transparent material, usually plastic or glass [1]. In contrast, the culture is in contact with the atmosphere in open reactors. Lately hybrid and other innovative systems have emerged due to the limitations of existing cultivation technologies. Currently, there is no single cultivation system that would be recommended as optimal as they all have their own advantages and disadvantages. Open systems are shallow open pond tanks of circular or raceway type [83], whereas many different designs of closed systems exist [84]. Closed systems are generally called photobioreactors (PBRs), the most popular being flat panel, horizontal tubular, vertical column and plastic bags [85].

The selection of the cultivation system depends on the overall goal of microalgae cultivation – high-value compound production, biofuels, bioremediation, as well as land and water availability, climate on the site, and accepted contamination risk [74]. Although there is no consensus on the most suitable cultivation vessel for microalgae biomass production, open raceway ponds are cheaper and more sustainable large-scale cultivation technology than most of PBRs for commercially viable microalgae bioenergy production [86]. Even though closed photobioreactors offer more flexibility in terms of the control of the culture conditions and higher biomass productivity per volume [83], high construction and maintenance costs limit their use as a large-scale biomass production technology. However, in cases such as for the

extraction of high-value pharmaceutical products, PBRs are also considered viable for biomass production.

An efficient commercial microalgae cultivation system should have the following features: [78], [85], [87].

- High surface area to volume ratio to efficiently capture solar radiation;
- Low shear stress on microalgae cells;
- Adequate mixing to avoid gradients and provide access of light to the cells;
- Easy maintenance/ simple operation procedure;
- Control of all crucial parameters (e.g. temperature, nutrients, light);
- Ability to achieve high growth rates;
- Minimal contamination risk;
- High land use efficiency;
- Low capital costs;
- Low operational costs.

Closed systems are generally more complex and have high capital costs as well as require improved technological skills to operate them [84]. On the other hand, closed systems generally provide better control of various crucial parameters leading to higher growth rates than those of open systems. Some major advantages of open systems are lower capital and operation costs compared to PBRs, and lower energy consumption for mixing [88]. However, open systems require large land areas, are prone to contamination and difficult to control due to outdoor conditions [88]. Moreover, other major drawbacks of closed systems are overheating, cleaning issues and build-up of high concentration of dissolved oxygen [88]. The pros and cons of the two types of cultivation systems are summarized in Table 1.2.

Table 1.2.

Pros and cons of open ponds and closed PBRs [74], [89], [90].

Parameter	Open systems	Closed photobioreactors
Process control	Low	High
Productivity	Low	High
Light utilization efficiency	Low	High
Temperature and pH control	Difficult	Easy
Evaporation	High	Low
Energy demand	Low	High
Contamination risk	High	Low
CO_2 diffusion to air	High	None
O_2 build-up	None	High
Species	Only selected species	Many species
Species control	Difficult	Easy
Construction costs	Low	High
Operational costs	Low	High

Space required	High	High
Life span	High	Low
Scale-up	Easy	Difficult

Although high productivity of various microalgae has been achieved under laboratory conditions, cultivation of microalgae in large-scale reactors is still a challenge. The choice of cultivation system greatly affects microalgae productivity, as well as suitability to a specific location. Resistance to shear stress can be a useful property of microalgae while generally, microalgae are sensitive to shear stress. Shear tolerance is greatest for green algae, followed by cyanobacteria, haptophytes, red algae and diatoms [91].

The choice of cultivation system will also determine whether the production of algae biomass is economically viable using the specific reactor. Generally, there is an agreement that closed photobioreactors are not suitable for the commercial-grade production of either biofuels or high-value compounds [75]. Because commercial-scale systems will require hundreds of hectares of land for individual PBR units, compared to a few multi-hectare raceway ponds.

Below the characterization of open and closed systems is given, and a short review on novel and hybrid technologies.

Open pond systems

Open pond systems include shallow ponds, tanks, circular ponds and raceway ponds [92]. Two common types of open ponds used for microalgae cultivation are shown in Figure 1.7. A raceway pond is by far the most often used. It is a shallow elongated pond with a typical depth of 20 to 40 cm [74] to allow light penetration but deep enough to allow the use of a paddle wheel and limit evaporation. Length-to-width ratio is an essential parameter in raceway ponds [92]. Too large width may result in slow circulation of a culture.

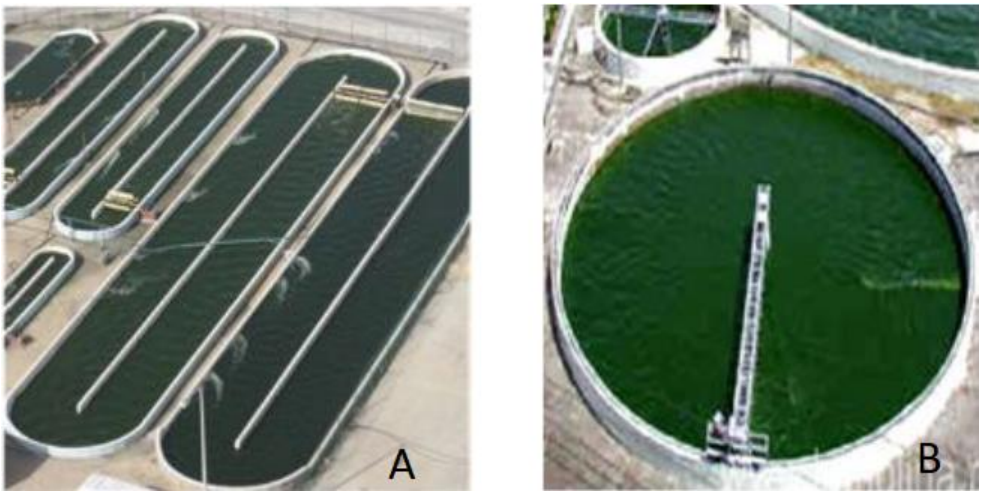


Figure 1.7. Open pond systems. A: Raceway pond, B: Circular pond [93].

Open raceway ponds are currently the most common systems used for outdoor commercial production of microalgal biomass [75]. Despite the disadvantages, more than 90% of the total microalgal biomass produced worldwide is obtained in open systems [83]. Open raceway ponds are also the most economical technology for large-scale biomass production [86], [94], [95] and are often regarded as economically viable systems for high-volume microalgae-based biofuel production [96]. Advantages of open ponds include lower construction, maintenance and operation costs compared to closed systems. Moreover, open systems usually have lower energy demand and are easier to scale up.

Mixing of raceway pond is required to rotate the culture to ensure light availability to cells and provide nutrients and CO_2. Mixing is generally less efficient in raceway ponds compared to PBRs, therefore baffles are usually installed to increase mixing efficiency, direct the flow and prevent sedimentation of cells [74]. Moreover, the installation of flow reflector baffles has been shown to increase the light time and the ratio of the light/dark cycle [97].

Temperature fluctuation in open ponds follows changes in outside air temperature. Generally cooling in open ponds is achieved by evaporation; however, it might not be sufficient in midday temperatures experienced in hot climates. Moreover, the evaporation rate can be significant in some places leading to considerable water loss.

Contamination with other microalgae species, bacteria and grazers is quite common in open pond cultivation [15]. Occurrences of rotifers and amoeba have been reported in cultures of *Tetraselmis*, *Chlorella*, *Nannochloropsis*, *Scenedesmus* and diatoms [88].

Various improvements have been proposed to overcome the drawbacks of open raceway ponds. Flow deflectors and wing baffles producing swirling flow were shown to reduce the dead zone, improve the flow velocity and increase productivity compared to raceway ponds without baffles [89].

Photobioreactors

One of the main principles for PBR design is a high surface area to volume ratio in order to utilize light efficiently [83]. Although various types of PBRs have been developed, only a few can be used for large-scale biomass production [85]. In PBRs, in contrast to open systems, a direct exchange of gases, liquids (e.g., rain), and particles between the culture and the atmosphere is strongly limited [78]. Closed PBRs are often chosen for the production of pharmaceutical compounds requiring the maintenance of pure axenic cultures [98].

The main drawbacks of all present PBRs remain the high capital and operating costs, the negative energy balance, and a limited possibility of being scaled up [78]. These limitations hamper the commercial application of PBRs for microalgae production. Several attempts have been made to develop simple, low-cost, easily scalable PBR designs in recent years, with some interesting new ideas or innovative applications of classic designs.

Many types of PBRs exist but classic designs include flat panel, horizontal tubular, vertical column and plastic bags [85]. Based on a mode of liquid flow, PBRs can be classified as stirred type, bubble column and airlift reactors (Gupta et al. 2015). Moreover, PBRs can be placed in

many configurations—horizontal, inclined, helical, vertical, rotating, submerged, floating etc. [75]. A short description of some of the common designs of PBRs is given below.

Flat panel PBRs are formed by two sheets of transparent material (typically glass, Plexiglas and polycarbonate) with a narrow light path of a few to 70 mm [99]. Illumination is provided from both sides resulting in a large surface providing a high surface area to volume ratio and thus high photosynthetic efficiency. The mixing of culture is performed by air circulation from a perforated tube at the bottom of PBR requiring low energy consumption. Flat panel PBRs are relatively cheap and easy to clean; however, the main drawbacks are associated with potential fouling and short light path leading to potential photo-inhibition at high irradiance. Moreover, a scale-up of the flat plate system is potentially difficult due to the increase in hydrostatic pressure with increasing volume (X. Zhang, 2015). Low efficiency in terms of mass production per unit of space is sometimes reported [100]. Examples of flat panel PBRs are shown in Figure 1.8.

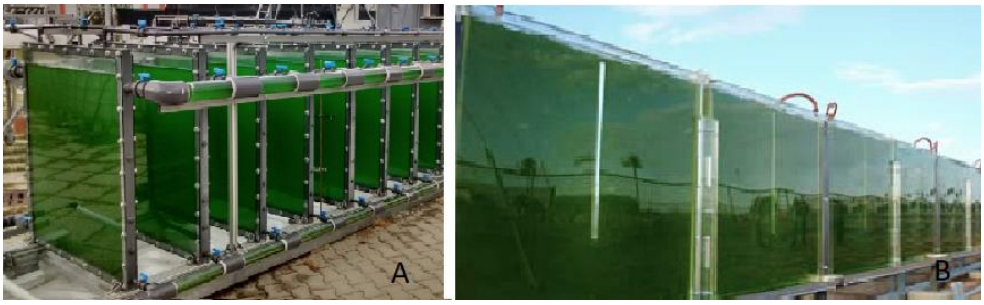


Figure 1.8. Examples of flat plate PBRs. A:[101], B: [102]

Tubular PBRs are both the most common design among closed systems and the most common type of PBRs developed at an industrial level [78]. These reactors are commonly constructed with horizontally placed glass or plastic tubes with small internal diameters to increase the penetration of light. The culture is circulated with pumps or airlift systems [103]. Horizontal tubular PBRs are placed horizontally in various designs and orientations, including parallel sets of tubes, a loop shape, an alpha shape, and an inclined tubular shape [100]. Some examples of tubular designs are shown in Figure 1.9.

Tubular photobioreactors are particularly suitable for outdoor cultivation, with the possibility of arranging reactors under an angle to the sun, providing effective capture of solar radiation [99]. The main factors affecting the performance of tubular PBRs are (1) the diameter of the tubing (2) the length of the tube, and (3) the mixing [104]. The internal diameter of tubes can be up to 0.1 m due to the limitation of the sun penetration into the deeper layers of high-density algae cultures; however, most commonly is from 10 to 60 mm [99]. The length of tubular PBRs can be several hundred meters.

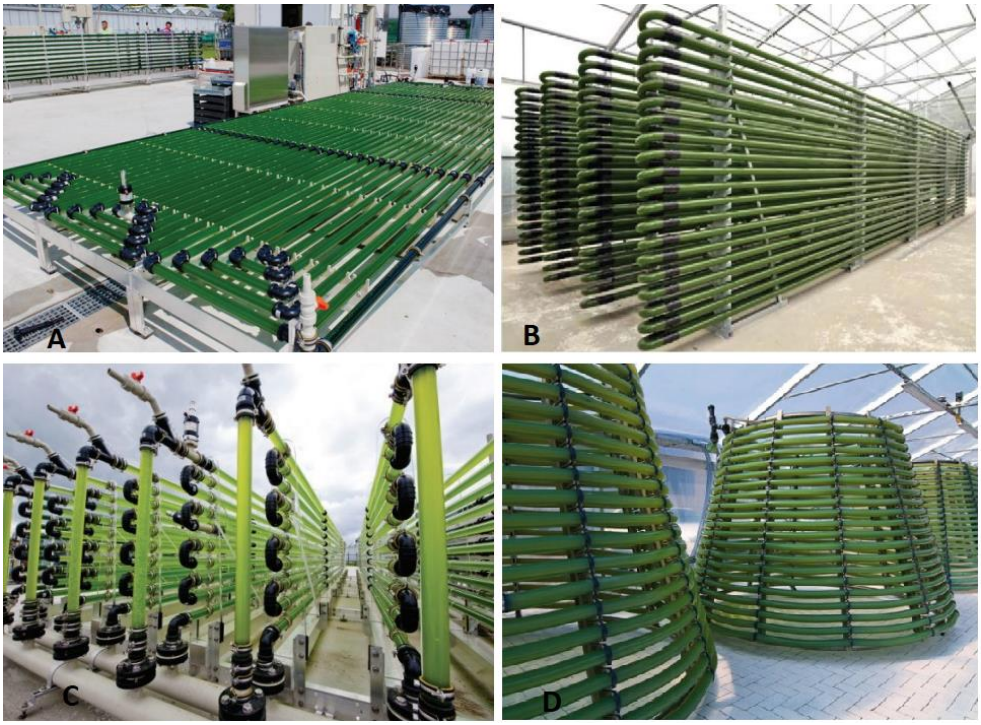


Figure 1.9. Examples of tubular PBRs. A: horizontal tubular [99], B: classic tubular PBR [100], C: stacked horizontal tubular PBR [99], D: conical helical tubular PBR [104].

Due to their high capital costs and energy input, the use of tubular PBRs is limited to the production of high-value compounds for various markets, such as human nutrition, cosmetics and the pharmaceutical industry [104]. Currently, large-scale tubular reactors are in operation in Germany and Israel for the production of *Chlorella* and *Haematococcus* [104].

Polyethylene bag PBRs are low cost and simple photobioreactors usually in the form of hanging vertical bags mounted on a support (Figure 1.10.). A circulation pump is used to feed the nutrients and air inside the bag. Although the cultivation of algae in polyethene bags is relatively common, there are certain drawbacks associated with the use of the material for a prolonged time as it gets brittle [99].

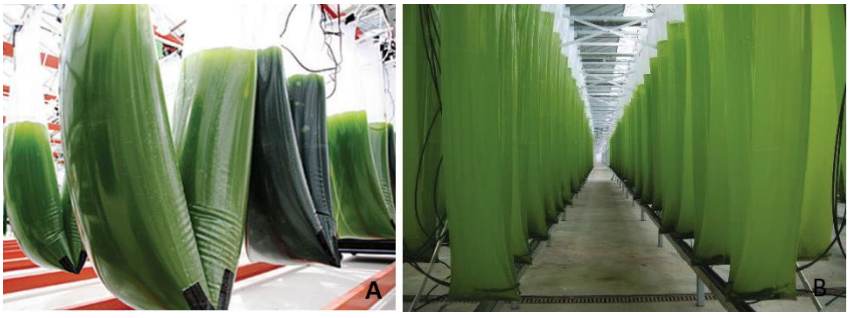


Figure 1.10. Microalgae cultivation in polyethylene bags, A: [104], B: [99].

Hybrid and innovative systems

Microalgae cultivation systems are continuously being modified and improved with the aim of reducing cultivation costs or adding some technological developments to enhance the microalgae growth rate [78]. Some technological configurations proposed include improved surface-to-volume ratio [105], light-to-biomass conversion efficiency [106], hydrodynamics [107] and power consumption [108]. Moreover, besides technological improvements of classic photobioreactors and race-way ponds, several new concepts have been proposed to overcome the disadvantages of the existing cultivation vessels. Hybrid technologies combining characteristics of different types of photobioreactors or even combining principles of both photobioreactors and open ponds have been developed [78].

Two-stage systems of algae cultivation have been proposed where initial cultivation takes place in closed PBR whereas outdoor open ponds are utilized subsequently. In such systems, pure high-density culture can be achieved at the first stage followed by the accumulation of target compounds under specific stress conditions applied during the second stage of cultivation [99].

A floating PBR, which aims at exploiting water bodies instead of land for algae cultivation, was created by Dogaris et al. [109] consisting of two plastic films forming the top and bottom surfaces of the horizontal raceway sealed to each other and connected to two vertical airlift units. The authors claimed the low cost and high productivity of the proposed system; however, scale-up of such a system remains challenging.

Biofilm-based algal cultivation has received increased attention recently representing an alternative to the conventional suspension-based systems [74]. Biofilms are a unique way to cultivate microorganisms usually consisting of complex microbial communities, including bacteria [110]. The main advantages of biofilms are resistance to growth stresses, high cell density and low harvesting and concentration costs. However, there are concerns over the scale-up and economic viability of such systems [111].

Pyramid PBR is another novel cultivation system which is a fully automated pyramid-shaped PBR made of acrylic. The main advantage of this type of PBR is the small land area required for installation when compared to other PBRs, however, they are still in an experimental stage [112].

Although many novel microalgae cultivation systems have been proposed, they all have certain disadvantages mostly associated with scale-up issues, construction or maintenance costs or high energy consumption showing a need for highly efficient and cost-effective novel design and technology. Moreover, it should be emphasized that many of the novel systems described in the literature have not reached the pilot scale and have been tested only at the laboratory level [113].

1.7. Microalgae cultivation conditions

The production of microalgal biofuel and marketable products requires a large amount of algae biomass at a low cost. The economic feasibility of microalgae biomass cultivation at a large scale depends on careful consideration of crucial factors affecting this process. The key

aspect is to maximize the algal biomass yield reducing the cost of production at the same time. Success on microalgae cultivation depends heavily on selecting the most suitable algal strain and setting up optimal growth conditions. It is crucial to determine parameters for maximum growth for the algae strain used and exploit the most suitable cultivation technology.

The growth rate of microalgae is determined by several abiotic and biotic, as well as technical factors. Light, temperature, availability of nutrients and pH are among the most important abiotic factors. Biotic factors, such as the presence of pathogens and interaction with other algae strains are also of high importance. Some technical parameters leading to biomass changes are mixing intensity, culture depth, and biomass harvesting frequency.

The success of microalgae biofuels requires extensive knowledge of microalgae culturing and harvesting relying on knowledge of microalgal biology and physiology. The response of microalgae to environmental factors such as light and temperature has been studied extensively, nevertheless, still, some knowledge gaps remain largely associated with microalgae response to low temperature and specific light spectrum. Maximal culture productivity can be reached only when all factors affecting productivity are in the optimum range of the species including nutritional requirements, temperature, and light.

Some very promising results have been shown reaching high growth rates and biomass productivity in laboratory experiments, however, it has been reported that such indices cannot be achieved in outdoor conditions. Although increasing in recent years, studies on microalgae performance in outdoor conditions under varying and extreme conditions are still limited in scientific literature.

Biomass yield is highly dependent on the cultivation system and plant location (Barsanti and Gualtieri 2018). The location of the microalgae cultivation plant has a major influence on biomass production due to environmental and climate conditions, such as light availability, light angle, daylight hours, temperature, rainfall, cloud cover etc. Moreover, light source and intensity, temperature, pH, nutrient and CO_2 availability, and mixing are the essential parameters affecting algae growth and must be considered when planning the cultivation of microalgae. The most important aspects of cultivation conditions with a high impact on growth rate and biomass production are described below.

Temperature

Temperature is one of the most crucial factors affecting microalgal productivity because of its direct influence on the photosynthesis rate. Temperature has a strong effect on chemical reactions within cells, e.g. uptake of nutrients and CO_2 [6]. It has been demonstrated that increasing water temperature enhances algae growth to a certain limit. When temperature exceeds or does not reach the optimal temperature for specific species, algal growth is decreased or even inhibited [114].

Temperature also affects cell size, biochemical composition and nutrient requirements. It has been observed that cell size decreases under high temperatures [115], [116]. Moreover, temperature above or below optimum results in changes in biochemical processes in algae cells, including changes in lipid synthesis and composition, and starch and protein content [117].

Furthermore, CO_2 solubility is affected by the temperature inside a pond, which decreases as the water temperature increases [114], [118].

Temperature in a natural environment is in constant variation, fluctuating both diurnal and seasonally. High temperature fluctuation requires the cultivation of algae strains with a broad optimum temperature range [119]. Different strains are accustomed to various temperatures. Moreover, some algae exhibit a narrow optimum temperature range and, therefore are not suitable for environments with highly fluctuating temperatures. Others can grow at a wide temperature range [15]. It is also essential to determine the temperature tolerance of the strain. Selected species might have outstanding performance and high productivity at optimal temperature but may exhibit very low productivity just a few degrees outside the optimum, thus considerably limiting their potential for outdoor cultivation.

Temperature varies primarily with latitude; therefore various requirements exist depending on the geographical location of the cultivation plant. Winter and summer temperatures vary significantly in most locations in Europe. Low productivity of microalgae during the winter season is generally observed [120]. Therefore, it is essential to utilize microalgal strains that are resistant to low temperatures for successful year-round cultivation. Moreover, it is vital to collect local climate data for the estimation of average temperatures in the potential location of the cultivation pond. The lowest and highest annual temperatures that might be experienced at the location must be considered. This will affect the choice of microalgae strain and the design and construction of the cultivation pond. Most microalgae prefer moderate to high temperatures with an optimum of approximately 27 – 30 °C [121], however, are capable of growing in a wider temperature range from approximately 15 to 30 °C [6]. Microalgae cultivation during winter might be even more challenging at higher latitudes due to lower temperatures, shorter days, declination angle of the sun and increased cloud cover [122]. In cooler climates, where winter temperatures decrease below the optimum of the strain, heating systems might need to be installed to maintain high productivity also during the winter period.

On the other hand, high temperatures in a pond are commonly observed during summer, especially in places with high insolation. During daylight hours, the temperature in a pond may exceed the maximum tolerance of the strain. Cooling in open ponds is generally provided by evaporation to a certain limit [15]. However, a cooling system or shading of the pond may be required in hot climates. In addition, night-time temperature is also an important factor to consider as respiration takes place during the night and is associated with considerable biomass loss reducing overall productivity [123], [124]. It has been demonstrated that an increase in night temperature increases biomass losses [124].

The temperature inside an open pond is affected not only by air temperature but also by insolation, depth of the pond and evaporation rate. The low depth of the culture in a shallow design race-way pond bioreactors makes cultures particularly vulnerable to changing environmental temperature. The shallower the depth of the culture the more evident the effect of temperature extremes [125]. Moreover, changes in external temperatures have a rapid impact on pond temperature due to the low volume of the culture. Water loss due to evaporation is considerable in open ponds and can reach up to 1 cm per day [126]. Consequently, water level

in open ponds must be constantly evaluated and controlled to maintain the required culture depth.

Furthermore, a strong interaction between light and temperature has been observed. At low light levels, high temperatures considerably decrease the photosynthetic rate. The same is true vice versa if too high light intensity is provided to cultures in low temperatures.

Light

Without a doubt, light is one of the most crucial aspects of microalgae cultivation because photoautotrophic microalgae depend on light for growth. Light is an energy source for photosynthesis reactions and has a direct impact on biomass production since the energy of light is used to convert carbon dioxide into sugars, the building blocks for biomass [127]. Consequently, light availability is critical to maintaining a high microalgae growth rate. Light is a complex system involving light intensity, duration, and spectrum that all are essential for optimum growth. Optimal light conditions ensure a high photosynthesis rate and therefore more rapid biomass accumulation. The availability and quality of light have been mentioned as the main factors limiting the productivity of microalgae large-scale cultures [128]. Both natural and artificial light can be used in microalgae cultivation. Without a doubt, sunlight is the most cost-effective light source as it is free, abundant and renewable. However, artificial light can provide better control over crucial parameters, moreover, in addition to optimized light intensity and length of light hours, also spectral quality of the light can be adjusted in algal cultivation by means of artificial lighting.

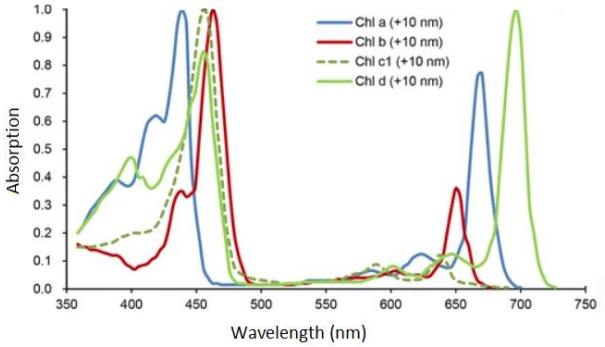


Figure 1.11. Absorption of major chlorophylls [129].

Only a fraction of solar radiation reaches the earth's surface, and an even smaller fraction can be used by photosynthetic organisms. Light with wavelengths between about 400 nm and 700 nm is called photosynthetically active radiation (PAR) and is used by microalgae and higher plants for light conversion processes in photosynthesis. Light energy is absorbed by pigments in microalgae cells and converted to chemical energy during photosynthesis. All photosynthetic organisms contain pigments to harvest light energy, however, the composition of pigments is different in various plants and microalgae. Each pigment has a unique absorption spectrum characterized by specific wavelengths of light it absorbs. Most photosynthetic organisms have a variety of different pigments allowing absorption of energy from a wide spectral range. This

distinctive set of light-harvesting pigments in a photosystem of different microalgae groups is responsible for the light spectral range these microalgae can absorb. Therefore, it is believed that pigments in microalgae play a crucial role in determining the light spectrum requirements.

Three major classes of pigments in microalgae are chlorophylls, carotenoids and phycobiliproteins [130]. The absorption maximum of chlorophylls is in the red and blue part of the light spectrum (Figure 1.11.). Due to chlorophylls, the light absorption of most microalgae is highest in the blue (approx. 460–490 nm) and red (approx. 630–700 nm) part of the light spectrum. While the middle part (490-570) of the PAR range containing mainly green light is hardly covered known as the "green gap" [131]. Nevertheless, this middle section is covered by accessory pigments carotenoids and phycobiliproteins (Figure 1.12.).

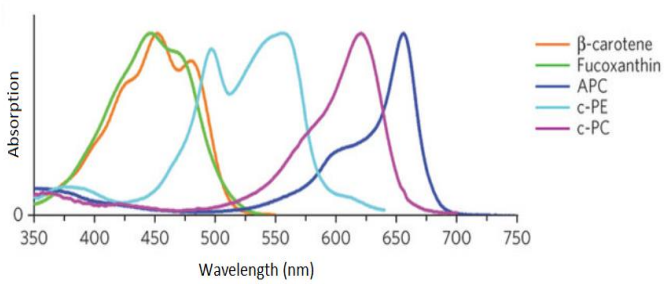


Figure 1.12. Absorption spectra of major phycobilins and carotenoids. APC - allophycocyanin; c-PC - phycocyanin; c-PE - phycoerythrin [132].

Light-emitting diode (LED) lights due to their various beneficial characteristics are fast gaining popularity as grow lamps over traditional lighting sources. Compared to fluorescent lamps, LEDs offer several advantages. In addition to lower heat dissipation and therefore lower energy consumption, they also have a longer lifetime, are mercury-free and have a narrow emission spectrum [133]. LED-based lighting not only is more energy efficient than conventional technologies but also enables better control of crucial parameters in microalgae cultivation. Due to their small size LEDs can be conveniently integrated with any cultivation vessel; and any type of lighting configuration can be easily achieved [134]. However, the greatest advantage of LED lighting is the ability to produce different colored light, making adaptation to specific needs possible. Therefore, LEDs have the potential not only to sustain good growth but improve it more than the capacity of conventional lights. The advances in the field of lighting and the development of LED technology make the exploitation of specific wavelengths of the light spectrum much easier, enabling the studies on the effect of various wavelengths to be more effortless, precise and widespread. Potentially the narrow band LEDs emission maximum can be exactly matched with the desired light absorption spectrum of the species offering a powerful tool for microalgae growth manipulation.

Light intensity

Cell growth of photoautotrophic microalgae is strongly affected by light intensity. Light availability can be divided into three categories: light limitation, light saturation and light inhibition. Increased photosynthesis rate is generally observed with increasing light intensity

leading to a higher growth rate. Therefore, an increase in light intensity enhances biomass production. However, algal growth is increasing up to a certain threshold; the maximum algal productivity is achieved at the specific light level known as the light saturation point [114]. Beyond this level a decline in algal productivity is observed. Excess light is damaging cells, slowing down photosynthesis and leading to photo-inhibition which results in a reduction of microalgal growth [117], [135]. Prolonged high irradiation can lead to growth inhibition. When light intensity is below the saturation point, algal growth is limited by light. Too low irradiance will slow down photosynthesis and reduce the biomass yield, creating a condition known as light limitation [136]. Microalgae species have various light requirements and optimal light intensity varies from strain to strain [137], hence optimal light conditions and light saturation point will depend on the algal strain used. Optimal irradiation for specific algal strains must be determined to reach rapid growth and maximum biomass yield.

In addition to irradiation, the amount of light received by algal cells depends also on the depth of the cultivation pond, density of the culture and mixing efficiency [85]. Successful bioreactor for microalgae cultivation requires a high surface-to-volume ratio to promote light penetration inside the culture. Therefore, open raceway ponds are commonly of a shallow construction to facilitate the availability of light. Even then, light intensity varies considerably inside a pond, being the highest at the surface and decreasing towards the bottom of the pond. At the top layer of the culture light intensity is high and the areal productivity of algae is high [138]. As the density of algal culture is increasing during the cultivation less light can penetrate the deeper layers of the pond. Consequently, at the lower levels of the pond algal growth is limited due to reduced light availability and light scattering caused by the shading effect of the top layer [114]. Moreover, algae near the surface are often exposed to excessive light while cells close to the bottom of the pond can experience severe light limitation conditions. Photo-inhibition can occur during the hours of the strongest irradiance, generally around midday. Commonly, algae experience extremely diverse lighting conditions in an open pond system, ranging from light deficiency to photo-inhibition. Good penetration and uniform distribution of light in a pond are essential to avoid photo-inhibition or light limitation and to maintain high growth rates and can be provided by means of appropriate design and efficient mixing.

Light limitation is a key limiting factor of large-scale microalgae cultures [125]. Sunlight is a free and abundant energy source for photosynthetic organisms however, it has some disadvantages like daily and seasonal fluctuation in irradiance level and day/night cycles [139]. Such limitations can be overcome by supplementing with artificial lighting. The addition of artificial lighting in outdoor cultivation systems can enhance algae growth during the conditions of light limitation. Low light conditions in an outdoor environment can occur on rainy or overcast days with a high cloud cover. Moreover, during suboptimal light hours, e.g. during winter months, supplementation of available daylight hours with artificial illumination will enhance the efficient use of available light. Consequently, efficient and cost-effective illumination is of particular importance to raise the economic feasibility of microalgal biofuels.

Photoperiod

Length of photoperiod or daylight hours is another important parameter affecting microalgae growth. The importance of photoperiod is well known for higher plants affecting

growth activity, flowering, and other seasonal activities [140]. Longer photoperiods have been frequently associated with higher growth rates in microalgae. Continuous illumination is often used to maximize biomass production; however, excess light can induce photoinhibition leading to cell damage and growth inhibition [141]. Moreover, it has been reported that a non-continuous illumination strategy with dark period after light is essential for microalgae biomass production. For example, Sasi et al. reported a 15% higher growth rate of *C. vulgaris* when an 8-hour dark period was applied compared to continuous illumination [142]. Therefore, appropriate light and dark periods are essential for optimum growth and biomass production and might be species-specific.

All light related parameters are closely linked. Light intensity and photoperiod are inversely correlated. Therefore, when light intensity is increased, the length of the photoperiod should be decreased. The duration of photoperiod influences the overall amount of light that microalgae culture receives in a 24-hour period. Therefore, light intensity and photoperiod should be matched accordingly. Most often 16:8 and 12:12 h light:dark periods are used for microalgae cultivation [134].

A novel illumination strategy has been proposed recently involving the application of flashing light or short light/dark cycles. Flashing light is intermittent light changing several times in a 24-hour period. Flashing frequency is a number of cycles per unit of time and can vary vastly. Maroneze et al. found that flashing light enhanced *Scenedesmus obliquus* growth rate and reduced electrical energy demand by 33% [143]. Another advantage of the application of short light flashes is the reduced risk of photoinhibition, moreover, the dark time is long enough to allow regeneration. Optimal flashing light conditions depend on the species of microalgae due to different reaction kinetics, properties of the linear electron transfer chain, energy dissipation, and storage mechanisms [5]. Flashing light might be a promising novel tool for efficient microalgae biomass production as well as reducing energy consumption compared with continuous light.

Light spectrum

Spectral composition of light has been recognized as an essential factor affecting the growth and productivity of microalgae [144], however, has been much less studied compared to other light related factors such as light intensity. Studies on the effect of the spectral composition of light on microalgae growth are more recent, however, a number of studies have raised sharply in the last decade. Some studies show that microalgae growth rate could be significantly enhanced by customizing the incident light spectrum transmitted to algal cultures. Therefore, a suitable spectral range becomes of paramount importance to the economic viability of microalgae mass culturing.

Although wavelength alteration is a promising strategy for growth improvement, it is still not completely understood. Published studies on the effects of distinct wavelengths on microalgae are inconsistent. Contradictory results are frequently reported showing that knowledge of the impact of different light wavelengths on microalgae growth and biomass production is still lacking.

Table 1.3.

Major pigments in microalgae and range of absorption [145]–[148].

Pigment group	Pigment	Pigment color	Absorption range, nm	Corresponding absorption color
Chlorophylls	Chlorophyll a	Blue-green	380 - 450, 600 – 670	Blue, red
	Chlorophyll b	Brilliant green	410 – 480, 600 – 685	Blue, red
	Chlorophyll c	Yellow-green	450	Blue
	Chlorophyll d	Brilliant/forest green	700	Far-red
	Chlorophyll f	Emerald green	700 - 750	Far-red
Carotenoids	β-carotene	Red-orange	400 - 500	Blue, blue-green
	Xanthophylls	Yellow	400 – 540	Violet and blue-green
Phycobiliproteins	Phycocyanin	Blue	600 – 640	orange
	Phycoerythrin	Red/pink	480 - 570	Blue-green, yellowish
	Allophycocyanin	Light blue (bluish-green)	620 – 660	Orange-red

The pigment composition of the light-harvesting complexes may provide information on the light requirements of microalgae within a taxonomic group [149]. Moreover, not only composition but also pigment quantitative content in microalgae is crucial since it affects the light spectrum requirements. Dominant pigments in green algae are Chl *a* and Chl *b*, giving the characteristic green color. Various carotenoids including β-carotene and several xanthophylls (e.g. astaxanthin, canthaxanthin, lutein and zeaxanthin) are also present in green algae [150], [151]. The composition of pigments in different microalgae groups is shown in Table 1.3. The main pigments found in red algae are phycobiliproteins (phycoerythrin and phycocyanin), Chl *a* and *d*; also various carotenes and xanthophylls are present [150], [151]. Interestingly, phycobiliproteins are the major light-harvesting pigments in red algae [152]. Diatoms contain Chl *a* and Chl *c*, xanthophylls and carotenes but lack phycobiliproteins [153]. Fucoxanthin is thought to be responsible for the golden brown color of diatoms resulting from its dominance over Chl *a* and *c* [154]. Although cyanobacteria contain also Chl *a*, phycobiliproteins are major pigments in cyanobacteria present in larger quantities than chlorophyll usually masking the chlorophyll pigmentation [151]. The presence of phycobiliproteins results in the characteristic blue-green color of cyanobacteria. Generally, algae with a high concentration of carotenoids appear yellow to brown. Those with a high concentration of phycocyanin appear blue but those with a high concentration of phycoerythrin appear red.

The effect of a specific wavelength on the growth rate of microalgae

Although it has been widely accepted that light quality has a considerable effect on biomass formation it is still poorly understood. Reports on the effects of the light spectrum on microalgae have increased significantly in recent years due to the advent of LED technology. Published

studies generally compare the effect of different monochromatic lights with a narrow emission spectrum with each other or with white light.

Impact of red light on microalgae growth

Red light (approx. 600 – 700 nm) is considered the most efficient wavelength based on the measurement of the quantum requirement for photosynthesis [155], thus, there are claims that red light is the most suitable for microalgae growth. Indeed, there are a number of studies reporting that red light enhances the growth of various microalgae species. [156], [157], [158] and [159] found that monochromatic red light is optimal for *Chlorella vulgaris* growth testing different monochromatic lighting (red, green, blue and yellow). Red light has been shown to enhance biomass production also in other microalgae species: green alga *Dunaliella salina* [160], red alga *Galdieria sulphuraria* [133] and cyanobacteria *Microcystis aeruginosa* [161]. However, inconsistent or contradictory results regarding the effect of red light on microalgae have been reported. Some reports indicate that monochromatic red light is not suitable for high biomass production [162], [163].

In addition to its role in photosynthesis, it is known that red light participates in the growth regulation and development of higher plants. For example, red light was shown to influence flowering in plants [164]. A lower photosynthetic rate in several crop plants grown under sole red light has been reported [165]. Moreover, studies demonstrate that plants grown under monochromatic red light do not develop normally having abnormal symptoms including stretched, elongated appearance and large and thin leaves [166] indicating that red light alone may not be sufficient to sustain normal growth. In microalgae, red light has been shown to escalate cell division resulting in smaller cells in algae cultivated under monochromatic red light [149], [167].

Impact of blue light on microalgae growth

Blue light is effectively utilized during photosynthesis but is also involved in several other physiological processes in cells and is known to affect metabolic pathways [149]. Blue light is involved in enzyme activation and regulation of gene transcription [168]. Moreover, blue light photoreceptors upregulate the genes involved in pigment biosynthesis [169]. Same as under red light, the cell size of microalgae is influenced by the application of blue light. Blue light receptors are thought to control the start of cell division inhibiting the division in small cells. The delay in cell division leads to an increased cell size commonly observed in microalgae cultivated under sole blue light. Consequently, in contrast to red light, the average cell size is larger under blue light compared to white light. This effect has been observed in several microalgae species e.g. *Chlamydomonas reinhardtii* [170], *C. vulgaris* [167], [171], *Chlorella kessleri* [172] and *Chlorella sorokiniana* [171].

Controversial studies have been published on the effect of blue light on the growth of microalgae. In a blue light-containing irradiance a higher biomass production and photosynthetic capacity of higher plants is generally observed [165]. Similar results have been demonstrated in microalgae. Higher growth rate under blue light was reported for *Chlorella ellipsoidea* [173], *C. vulgaris* [174], [175], *Chlorella pyrenoidosa* [176], *Nannochloropsis* sp.[177], [178], *Tetraselmis* sp. [178], *Chlorella* sp. [175] and *Scenedesmus* sp. [175]. In contrast, blue light yielded poor productivity of *C. vulgaris* in Yan's study [156]. Moreover,

cultivation under monochromatic blue LEDs resulted in the lowest growth rate in another green alga *Picochlorum* sp. compared to red, green or white [179]. The contrasting results demonstrate that the role of blue light is complex and most likely other factors are responsible for the results obtained.

Several studies in higher plants demonstrate that by increasing the fraction of blue photons above 5 to 10%, plant growth generally decreases [180]. Similar results were also obtained in microalgae. de Mooij et al. [163] demonstrated that blue light in small quantities (3.5%) is essential for mass cultures of *Chlamydomonas reinhardtii* possibly due to the role of blue light as a trigger for metabolic regulatory mechanisms. The significant role of blue light was supported also by another study of microalgae *Porphyridium purpureum* and *C. reinhardtii* [133]. It was demonstrated that the addition of blue light by up to 10% increased the biomass productivity of *C. reinhardtii*. Moreover, a decrease in growth of *P. purpureum* with an increasing fraction of blue light above 20% was observed [133]. These studies demonstrate the significant role of blue light in microalgae light spectrum requirements. In higher plants, blue light is involved in regulatory processes, such as phototropism, photomorphogenesis, stomatal opening and leaf photosynthetic functioning [165]. Similar mechanisms have been shown to be in place in microalgae. Blue light was required for photoprotection and acclimation to high light intensities in the marine diatom *Phaeodactylum tricornutum* [181].

Significant differences in sensitivity to blue light have been observed among higher plant species [182]. It has been speculated that response to blue light depends on the species of higher plants, some plant species being highly sensitive to blue light fraction whereas others have intermediate or low sensitivity [180]. If this is also true in microalgae, it could at least partly explain the contradictory results reported.

Impact of green light on microalgae growth

Perhaps the most puzzling is the role of green light in microalgae. Although main absorption is taking place in the red and blue parts of the spectrum, there are ongoing debates on the role of green light in photosynthesis. The green part of the light spectrum roughly spreading between 500 and 600 nm has been perceived inconsistently. Traditionally green wavebands have been considered to have very limited use in the process of photosynthesis since chlorophyll does not absorb light in this part of the spectrum [170] and are thought not to promote growth in higher plants [183]. Consequently, most often green wavebands are considered unsuitable for microalgae growth if applied without additional light sources[149]. It has been argued that green microalgae cannot use yellow and green light effectively due to the lack of phycobiliproteins [149]. However, there is strong evidence that green light takes part in photosynthesis and the regulation of physiological processes in plants [184], [185].

Exposure to green and yellow wavelengths alone (500 – 630 nm) generally leads to lower biomass production compared to either blue or red wavelengths [149]. There is only a limited number of studies on the effect of green light on microalgae since green light is often excluded from the light quality studies possibly due to the above-mentioned reasons. Nevertheless, the existing studies demonstrate that sole green light cannot sustain the growth of most algae [144], [169]. However, there is evidence that some species of microalgae are able to utilize the green part of the spectrum efficiently and grow well under green illumination [169], [173], [179],

[186], [187]. A relatively high growth rate of green microalga *Ettlia* sp. was achieved under green light showing that green wavebands could support *Ettlia* sp. growth; although the growth rate was lower than that under white light [187]. The same study revealed that the application of a green light might provide other advantages, e.g., better competitive ability. *Ettlia* sp. was able to out-compete *C. vulgaris* under white + green LEDs without losing productivity. Supplementation of white light with the green part of the spectrum could be potentially used as a tool to prevent invasion by other microalgae in mass cultures of *Ettlia* sp. This is an interesting aspect and should be further studied.

Probably the most important consideration is that green light is thought to increase the efficiency of sunlight use in deeper parts of a leaf and dense canopies since red and blue lights are preferentially absorbed by the upper layers of the leaf [185]. It could be argued that the same effect might be true for dense microalgae cultures in open ponds or photobioreactors. Indeed, the penetration of light into water is greatly affected by the absorption and scattering processes taking place within the water. Not only intensity but also the color of the light changes greatly with depth [188]. Upper layers of cells close to the top of the water surface absorb most of the red light, while blue light can penetrate deeper layers of water. Weakly absorbed wavelengths, such as green, have a high scattering coefficient and low absorbance coefficient [189]. Therefore, as culture density and water depth increase, red and blue wavelengths become less available to the algae than green wavelengths [149]. Consequently, green light penetrates deeper and can therefore be absorbed by algae cells at the lower levels of a pond being photosyntetically more efficient in high-density cultures. There is a lack of studies on the effect of green light on microalgae, however, few studies published, suggest that green light indeed is beneficial in high-density cultures. Mattos et al. [189] observed increased biomass production of the green alga *Scenedesmus bijuga* under green light in high-density cultures (2.9 g/L) compared to cultivation under monochromatic red light. Also, de Mooij et al. [163] demonstrated that weakly absorbed yellow light resulted in the highest biomass productivity in high-density *C. reinhardtii* cultures compared to deep red, orange red or blue light. These results demonstrate that the ability to use green light is a competitive advantage for high biomass production, especially under high-density cultures, long light pathways and high light intensity [179], [190].

The optimum wavelength for microalgae growth

Although many reports have stated that certain microalgae grow best under specific monochromatic wavelengths; the studies conducted using two or more wavelengths simultaneously, clearly indicate that the microalgae production rate is enhanced by a mix of different colors [133], [152], [191]–[194]. Red and/or blue lights are frequently reported as the most suitable lights for the maximum growth of different microalgae. This could be because of the corresponding absorption maximum of major light-harvesting pigments in this range. Nevertheless, there is growing evidence that other wavelengths than red and blue have a profound effect on microalgae cultivation and most likely a balanced mix of various wavelengths is required for optimal growth and biomass production. Although previously thought to be “ineffective” or “not required” parts of the spectrum, they are now gaining attention and are frequently called “weakly absorbed wavelengths” usually referring to green

and yellow regions. It has been suggested that the application of weakly absorbed wavelengths is not required for photosynthesis and should be avoided [144]. However, several recent studies have confirmed that regulatory effects of weakly absorbed wavelengths are required for optimal growth of microalgae [149], [169], [195]. The regulatory role of green and blue lights, stimulating other biochemical processes besides photosynthesis, was suggested by some authors [133], [169].

The effect of a combination of different single wavelengths on microalgae growth is less studied than the single wavelength and the data is lacking, as also pointed out by other authors [144], [196]. Furthermore, the ratio of different wavelengths applied also plays a significant role but is even less studied. It seems that high red to a lower percentage of blue light is required for optimal microalgae growth. The red:blue ratio of 0.7 was shown to be the most effective in higher plants resulting in appropriate plant development [197]. This suggests that similar red and blue ratios may also be suitable for green algae. Indeed, supplementation of red light with the blue part of the spectrum (75/25%) resulted in enhanced biomass productivity and carotenogenesis in *Dunaliella salina* as opposed to monochromatic red light [198]. More research must be performed especially on the effect of the combination of several wavelengths at different ratios in order to fully understand the effect of various light wavelengths on microalgae and to define optimum lighting conditions.

A large number of contradictory reports highlight that the light spectrum has a much more complex impact on physiology and morphology in microalgae than other light parameters such as light intensity or photoperiod. Different response of microalgae to the light spectrum demonstrates a species–specific behavior therefore, light requirements must be studied for the species of interest. Understanding how different microalgae respond to specific light spectrum conditions could contribute greatly to the industrial application of microalgae by improving cultivation conditions to maximize biomass production.

Furthermore, controversial results reported at the species level imply that not only light spectrum is species-specific but also cultivation conditions might have affected the preferred spectrum. The performance of microalgae under specific narrow wavelengths is influenced also by light intensity, illumination duration, culture density and mixing. Light intensity is closely interacting with spectral quality and has a major effect on the preferred spectral range. Moreover, the close interaction of light spectrum and intensity has been shown in several studies [163], [169], [181], [187], [199]. It was demonstrated that the preference for the spectral range of microalgae depends on the applied light intensity [169], [199]. It was shown that weakly absorbed green and yellow light might be used at high light conditions, whereas strongly absorbed red and blue wavelengths will be more suited for low light conditions. It can be concluded that several aspects work together in determining the spectrum requirements for the species: pigment composition and quantity within cells, environmental factors such as light intensity, temperature, nutrients and cultivation conditions and experimental setup such as cultivation vessel, culture depth and culture density are the main contributors.

It must be acknowledged that the light spectrum significantly affects not only the growth rate of microalgae but also the formation of specific compounds in cells. Particular wavelengths of light induce or suppress the formation of specific compounds, such as pigments [173], [186],

lipids [174], proteins [200] and polysaccharides [201]. Therefore, it is recommended to investigate the distinct light spectral composition required for the specific algae depending on the intended application of microalgae biomass.

Given the high potential of wavelength manipulation for microalgae growth enhancement, it becomes of utmost importance to the economic viability of microalgae mass culturing.

Nutrients

In addition to CO_2 and light, microalgae need nutrients to grow. Nitrogen and phosphorus are major macronutrients required for microalgae biomass formation [202]. After carbon, nitrogen is the most important nutrient for biomass production. It is mostly supplied as nitrate (NO_3^-), but often ammonia (NH_4^+) and urea are also used with similar growth rates [202]. Nitrogen is vital to algal growth as it is involved in the synthesis of proteins, amino acids and nucleic acids [203]. Phosphorus is the main component of nucleic acids and phospholipids in algal cells [203]. The ratio of N:P in a growth media is an important parameter as it has a great effect on algal growth and biochemical composition. The optimal ratio of N:P is 16:1 for algal growth; however, it can change according to the requirements for N and P of different microalgae species [204].

Other nutrients including Na, Mg, Ca, and K must be present in the cultivation medium. Moreover, micronutrients such as Mo, Mn, B, Co, Fe, and Zn are required at low concentrations. Other trace elements might also be required depending on the species [28].

Various culture media have been developed and used for the cultivation of microalgae. Some of them are widely applicable and can be used to grow many different groups of microalgae, others are more specialized for certain groups of microalgae due to their need for certain specific nutrients. When selecting a culture medium, the natural habitat of the species should be considered in order to determine its environmental requirements [68]. Rich organic media should be avoided unless the microalgae cultures are axenic to avoid heavy bacterial growth. For non-axenic cultures, mineral media should be used [68].

Large-scale production of microalgae biomass requires huge amounts of nutrients. Consequently, standard laboratory media are not suitable for the industrial level because their production is time-consuming and expensive, relying on the use of laboratory chemicals and procedures [74]. Alternatively, agricultural fertilizers have been used for large-scale microalgal production as they are relatively cheap and easily available and generally provide nutrients required for microalgae growth. Although agricultural fertilizers are cheaper than laboratory-grade chemicals, the costs of microalgae cultivation are still too high for large-scale biomass production to become economically feasible; therefore, alternative low-cost sources of nutrients have been of recent interest to researchers.

Various wastewater sources are explored for their suitability as nutrient source. Wastewater is a complex matrix containing significant concentrations of dissolved and suspended solids, various levels of nutrients, heavy metals and pollutants [74]. Nutrients present in wastewater can be used by microalgae for growth and biomass production simultaneously treating wastewater. Microalgae have shown effective removal and recovery of nutrients from various

wastewaters, e.g. industrial [205], municipal [206] and agricultural [207]. Thus microalgae are proving a sustainable approach to improved removal of nutrients and contaminants in wastewaters, while the treated water can be recycled to reduce the cost of microalgae cultivation. Microalgae have shown more effective removal of heavy metals and other harmful substances compared to conventional wastewater treatment. Moreover, it is more cost-effective and sustainable than traditional chemical processes applied in conventional wastewater treatment due to avoided use of harsh chemicals. Considering the benefits, microalgae cultivation coupled with wastewater treatment is a promising strategy to reduce cultivation costs and increase the economic viability and environmental sustainability of large-scale microalgae biomass production.

Carbon dioxide

Carbon is one of the most important nutritional elements for algal growth constituting half of the weight of dry biomass [208]. It is usually supplied in the form of carbon dioxide due to its high solubility and comparatively low price. For microalgae cultivation, CO_2 gas is usually aerated into the culture medium in the form of bubbles. The level of CO_2 in the air (0.036 %) is most often not sufficient to support the need for carbon for microalgae growth [118]. Although microalgae species have different carbon requirements, generally higher CO_2 level increases the growth rate and enhances biomass productivity, therefore additional CO_2 injection is usually required [209]. It has been estimated that the cost of carbon source can be as high as 27% of the total costs of microalgae biomass production [210]. Moreover, costs are highly affected by the CO_2 fixation efficiency therefore, highly effective CO_2 gas introduction into ponds is of high importance [211].

Carbon dioxide contributes up to 68 % of total greenhouse gas emissions worldwide [27]. In order to reduce the CO_2 load from the atmosphere, several technologies of carbon sequestration and storage have been developed, such as physicochemical, adsorption, membrane technology, cryogenic fraction, injection into deep oceans or geological formations [212]. However, most of these methods require considerable storage space and have high operational, monitoring, and maintenance costs; moreover, concerns about the CO_2 leakage also have been raised. Biological CO_2 fixation appears to be the only economical and environmentally viable technology of the future [213]. Plants and other photosynthetic organisms naturally capture and use CO_2 as part of their photosynthetic process. Due to a faster growth rate and higher CO_2 fixation efficiency than terrestrial plants, microalgae are able to sequester vast amounts of CO_2 from the atmosphere [118]. Microalgae ability to capture CO_2 with high efficiency has the potential of a novel approach to CO_2 mitigation. Bio-sequestration of atmospheric CO_2 and flue gases containing CO_2 by microalgae is a new promising strategy proposed to sustainably decrease CO_2 emissions and simultaneously increase microalgae productivity.

Waste gases from combustion processes are considered a potential source of CO_2 for microalgae growth. Flue gases consist of CO_2 ranging from 3 to 25 % of its volume [214]. For example, flue gases from fossil fuel power plants consist of 4–14 vol % of CO_2 [215], other

sources report even higher CO_2 content in waste gases - >15 vol % [212]. An optimal CO_2 concentration for microalgae is species-specific; however, is in a range of 2 to 10% for most microalgae [209].

Although the use of flue gases as a source of carbon for microalgae growth is a promising strategy, it has several drawbacks associated with excessive CO_2 concentration, the presence of toxic compounds and the high temperature of flue gases. Generally, CO_2 concentration above 5% is considered toxic to microalgae growth, however; in some reports, growth inhibition is mainly due to the presence of toxic compounds NO_x and SO_x in flue gas that acidify the cultivation medium [216]. Moreover, some microalgae strains can tolerate much higher CO_2 concentrations. Indeed, *Chlorococcum littorale*, a marine alga, showed exceptional tolerance to high CO_2 concentration of up to 40% [42]. Some strains of green algae from *Chlorella* spp. also can tolerate 40% CO_2 [42]. The concentration of CO_2 in a medium should be between the value which results in the maximum rate of cell growth and the microalgae tolerance threshold [217].

A high temperature of flue gases is another major concern. It can be as high as 250-450 ºC [214]. The decrease in growth when high temperature flue gas was injected was reported by Chiu et al. [218].

Despite the concerns, a direct utilization of flue gases from various sources has been demonstrated [211], [218]. Selection of NO_x and SO_x tolerant algal strains, the addition of $CaCO_3$ to keep pH at an optimum level, and the addition of NaOH to increase pH are some of the strategies applied to overcome the inhibitory effect of flue gas on the growth of microalgae [219].

Modes of cultivation

Microalgae have different metabolisms – autotrophic, heterotrophic or mixotrophic (Figure 1.13.). Most microalgae are autotrophic organisms and use light energy and atmospheric CO_2 as carbon sources for metabolism, whereas some microalgae can grow in the dark using organic carbon as an energy source, called heterotrophs. Mixotrophic microalgae can use both light energy and organic carbon sources simultaneously and both respiratory and photosynthetic metabolism operates concurrently [220]. Moreover, some species of microalgae are not true mixotrophs but can switch between phototrophic and heterotrophic metabolisms depending on environmental conditions [220]. There is only a limited number of microalgal species that can grow heterotrophically. More species are obligate autotrophs than facultative heterotrophs [220].

The production of biomass of photoautotrophic cultures is restricted by the availability of light. It has been reported that both heterotrophic and mixotrophic conditions increase biomass productivity [221]. It was reported that heterotrophic cultures could achieve biomass productivities ten times greater than that of autotrophic microalgae cultures [222]. On the other hand, mixotrophic cultivation can reach greater biomass productivity than heterotrophically grown cultures by overcoming the limitations of heterotrophic cultures [159]. Despite the high potential, heterotrophic cultures also have several limitations: (1) there is only a limited number of species that can grow heterotrophically, (2) increased cultivation costs due to organic carbon

source expenses, (3) bacterial contamination, (4) growth inhibition by excess of organic substrate, and (5) inability to produce light-induced metabolites [220].

Contamination is one of the main drawbacks of mixotrophic cultivation. Sometimes inorganic carbon is added continuously in small quantities to support microalgae growth but to prevent excessive bacterial growth. Moreover, organic carbon is usually added only during daytime hours to avoid faster-growing bacteria overgrowing microalgae under dark heterotrophic conditions [220].

Several *Chlorella* species such as *C. vulgaris*, *C. protothecoides*, *C. zofingensis*, *C. sorokiniana* and *C. minutissima*, as well as other species like *Tetraselmis* spp. and *Neochloris* spp. are capable of both autotrophic and heterotrophic growth [98], [223].

Naturally, all pigments are produced under autotrophic growth conditions; however, it has been demonstrated that synthesis of certain pigments is possible in the heterotrophic microalgae cultures [98]. Carotenes, xanthophylls and phycobiliproteins have been shown to accumulate also under dark conditions [220].

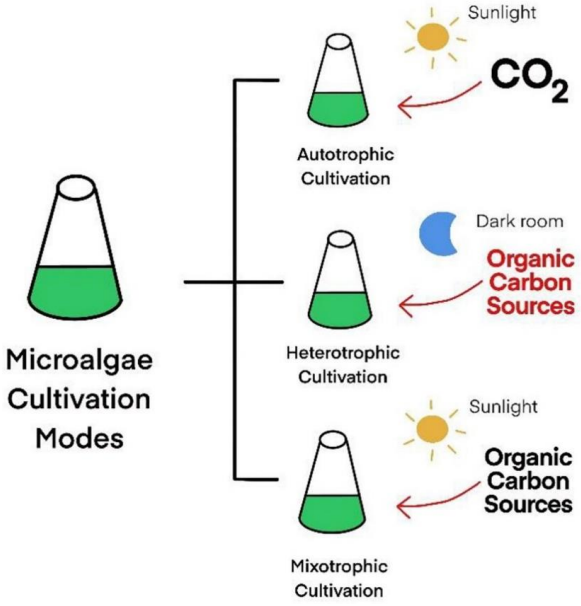


Figure 1.13. Microalgae modes of cultivation [224].

There are ongoing debates on the most economic cultivation mode of microalgae to enhance the microalgal biomass industry. Recently Ruiz et al. [222] made a comparison of the economic feasibility of photoautotrophic vs heterotrophic microalgae cultivation at an industrial scale. Calculations of costs revealed that heterotrophic production of microalgae of 4.00 €·kg^{-1} (dry weight as a centrifuged paste) is higher than that of photoautotrophic production in flat panel photobioreactor 3.50 € kg^{-1} (dry weight). Calculations were based on the production facility of 100 ha producing 6094 t of dry weight (paste) per year. Photoautotrophic cultivation of microalgae appears to be a more economical option; however, the cultivation system has to be taken into account as cultivation cost in other types of photobiorectors could be higher [225].

Other parameters

pH is an important factor in algal cultivation since it determines the solubility and availability of CO_2 and essential nutrients [117]. Therefore, the cultivation system must include a pH probe for close monitoring of pH fluctuation. Microalgae absorb CO_2 during growth, which causes the pH to rise. Therefore, the pH of microalgal cultures rises gradually during the day but decreases during a dark period. High pH limits the microalgae growth due to the limited availability of CO_2 [226]. Failure to maintain the optimum pH of the species can lead to culture collapse [68]. Methods for controlling pH include CO_2 injection, buffer addition, and acid or base adjustment [226].

Each microalgae species has its optimal pH range for maximum growth, which is usually narrow and species-specific [226]. The pH range of most microalgae species is between 7 and 9, with the optimum range of 8.2 – 8.7 (Barsanti and Gualtieri book). However, there are species that prefer a more acidic or more alkaline environment. Some algae prefer alkaline environmental conditions thus suppressing undesired biological contaminants naturally.

Mixing in a cultivation vessel should be gentile, however, must provide a reasonable mixing rate to create a uniform environment for microalgae culture. Mixing prevents algae from settling, ensures that cells are equally exposed to light and nutrients, and improves gas exchange between the culture medium and the atmosphere. The exchange of cells in the light-deficient bottom of culture with light-exposed surface cells is crucial for the optimal light–dark cycle frequency of algal cells. Moreover, high solar intensity can create a water temperature gradient in a pond, while mixing ensures uniform water temperature [227]. Furthermore, efficient mixing can enhance microalgae productivity.

There are various types of mixing of microalgal cultures. The mixing to be adopted depends on various factors such as the type of microalgal strain and culture system (open ponds or photobioreactors) and, the scale of culture systems (small or large-scale cultures). Mixing can be achieved using paddle wheels and jet pumps in open ponds. In PBRs mixing is generally done by bubbling directly with an air pump or indirectly, by an airlift system [68]. Not all algae can tolerate vigorous mixing, therefore shear resistant strains are preferred.

1.8. Digestate as a nutrient source

The European biogas sector has experienced an expansion in recent decades associated with favorable governmental measures promoting biogas industry. While the growth has not been that steep in the last few years, the number of biogas plants is still growing reaching 18 774 biogas plants throughout Europe by the end of 2020 [46]. The majority (63%) of biogas plants are working on agricultural residues [46] showing a close interaction between agriculture and biogas production. Digestate is a by-product of biogas production generated during biomass fermentation. It contains nutrients, such as nitrogen, phosphorus and potassium that are in a plant-available nutrient form and can be used for biomass formation. Consequently, the traditional use of digestate is the application on agricultural lands [228]. Although digestate is considered a valuable biofertilizer, several regulatory restrictions limit the land application. Not only nutrient input per hectare is restricted but also the period of application is limited to the

growing season allowing application only a few months per year. Excessive application of digestate on land can result in the oversupply of nutrients and lead to the leaching of nutrients into the groundwater.

Continuous increase in biogas production inevitably leads to an increased digestate load therefore, overproduction of digestate potentially triggering eutrophication is becoming a serious issue. Storage of digestate is one of the main concerns requiring large storage capacity and appropriate handling to avoid nutrient leaching and provide odor control [229]. Moreover, long-distance transportation of digestate is not viable, limiting the application to surrounding territories only. The management of digestate can present several environmental and health risks, if not handled properly, thus becoming a serious problem and creating the need for alternative digestate valorization routes.

The application of digestate as a nutrient source for microalgae growth has recently been proposed as one of the possible strategies to decrease cultivation costs and ensure cost-effective bioenergy production [230]. Wastewaters are complex mixtures with a variable composition depending on their origin. Generally, wastewater streams contain organic, inorganic and man-made compounds [44]. Microalgae are known to remove nutrients and heavy metals from various wastewaters, thus their application may be one of the best available strategies to decrease biomass production costs [230]. While microalgae are very conservative in their needs; they use sunlight and CO_2 as their energy sources and require some nutrients such as nitrogen and phosphorus for their growth, biomass production costs using current technologies are still too high to compete with other types of biomass. A liquid fraction of digestate from biogas production plants can be an excellent source of nutrients for microalgae cultivation at a minimal cost. Although the composition of digestate varies considerably mainly depending on the feedstock, source of inoculum and operating conditions of the biogas plant (Cai et al., 2013) generally, the nutrient level of diluted digestate is favorable for microalgae cultivation [231].

Wastewater use has multiple advantages for microalgae cultivation: (1) it is a source of nutrients for microalgae growth, (2) it provides a sustainable water source and (3) it is a source of organic carbon for heterotrophic and mixotrophic growth [232]. The main environmental issue of microalgae cultivation – the need for enormous amounts of freshwater thus could be mitigated, moreover, it reduces the expenses of nutrients required for microalgae cultivation.

Coupling digestate treatment with microalgae cultivation has the potential to significantly reduce the costs associated with the cultivation. Simultaneous nutrient removal and biomass production require microalgae species able to survive in specific conditions and reach high biomass yield. Species for wastewater treatment must exhibit good pollutant removal capacity mainly ammonium, nitrogen, phosphorus and heavy metals under specific environmental conditions. Due to large quantities of organic carbon in wastewaters, microalgae with heterotrophic metabolism are beneficial. Simultaneous use of carbon dioxide and organic carbon, known as mixotrophy, can more efficiently utilize the available light and organic nutrients from wastewater thus potentially enhancing microalgae growth. Recently, many studies have aimed at optimizing heterotrophic and mixotrophic cultivation to overcome the limitations of autotrophic growth such as light deficiency. Several studies have shown higher

efficiency in nutrient removal and biomass production in mixotrophic and heterotrophic cultivation modes compared to photoautotrophic conditions [233]–[235].

Although digestate presents great potential as a source of nutrients, certain obstacles hamper algae-based wastewater treatment from industrial-scale operation. Agricultural digestate is characterized by a very high nutrient load, strong turbidity and optical density, high chemical oxygen demand and a risk of bacterial contamination limiting the direct utilization of raw digestate for microalgae cultivation [236]. Until now, contradictory results have been demonstrated on the application of digestate for microalgae.

The main drawbacks of digestate application for microalgae cultivation are summarized below.

1. High turbidity due to dissolved and suspended matter.

Digestate is commonly characterized by high concentrations of total suspended solids causing high turbidity and decreasing light penetration in microalgae culture. Turbidity may be caused by impurities such as clay, silt, undigested organic matter or dyes [237]. Turbidity and the presence of solids decrease the light transmission in the microalgae culture and reduce the amount of light available for photosynthesis. Light is the most important energy source for microalgae growth therefore biomass yield can be severely reduced. Indeed, the inhibitory effect of turbidity on microalgae growth rate has been demonstrated [237].

2. High optical density.

Digestate is rich in humic substances and organic matter which creates the characteristic dark color of digestate [238]. The color intensity of the digestate depends on anaerobic digestion feedstock characteristics. Dark colour reduces the light availability to microalgae cells in the water and limits their growth. High optical density can seriously inhibit microalgae growth. Dilution with freshwater is usually suggested to decrease the optical density [239].

3. High ammonia concentration.

Nitrogen in digestate is found mainly in a form of ammonium [230]. Although ammonia is thought to be the preferred source of nitrogen for microalgae, there is a limit to ammonia tolerance and too high concentrations can cause toxicity to algae leading to growth inhibition. Indeed, studies show that high ammonia content can lead to limited microalgae growth [240]. Dilution of digestate is generally required for the cultivation of microalgae to decrease the effect of inhibitors [231], [241]. The dilution rate depends on the chemical composition and other properties of digestate, therefore chemical analysis of digestate is needed to understand the appropriate dilution rate.

4. High Chemical Oxygen Demand (COD).

COD is a parameter that measures the equivalent amount of oxygen required to chemically oxidize organic compounds in water. It is used as an indicator of water quality and wastewater strength. High COD means high content of organic matter in digestate and might limit microalgae growth.

5. Risk of contamination.

Species competitiveness is another important consideration for the assessment of species suitability for cultivation in wastewater. Wastewater contains biological contaminants such as bacteria and protozoa, therefore robust and fast-growing microalgae that can outcompete other

species are crucial for cultivation in wastewater. Wastewater treatment requires fast and efficient pollutant removal in the possibly shortest time therefore, in addition to fast growth successful algal strains must be tolerant to weather fluctuations and high nutrient concentrations.

Current studies suggest the dilution of digestate for the cultivation of microalgae to decrease the OD and the effect of inhibitors [231], [241]. Although this approach has shown a positive effect on microalgae growth, a large volume of freshwater is needed to decrease the OD and to dilute the inhibitors to a tolerable level for microalgae; on average 10 to 30-fold dilution is being reported most frequently [241]. A high dilution rate requires a large volume of freshwater resources limiting the economic and environmental benefit of such a system. Moreover, a high dilution rate limits the volume of digestate that can be fed into microalgae ponds for treatment, requiring a much larger area of cultivation ponds, a longer time to treat the digestate and thus higher energy demand. Decreasing the amount of freshwater is a key aspect of building a sustainable and economically viable system for digestate application for microalgae cultivation. Therefore, the removal of potential growth inhibitors by pretreatment of digestate may be a sought-after solution. The main challenge is to find an effective and low-cost pretreatment method however, only a few methods have been described so far [238], [239] indicating a lack of studies on available solutions. The most commonly applied pretreatment methods are centrifugation and filtration [242].

Activated carbon holds great potential as an efficient low-cost method to reduce turbidity, optical density and harsh chemicals in digestate due to the high capacity of adsorbing various substances. Activated carbon works as an adsorbent providing a large surface area where contaminants may be adsorbed providing the opportunity to reduce the optical density of digestate to a desirable level for microalgae and facilitate microalgae growth. Activated carbon may be produced from residual materials resulting in a low-cost and sustainable solution [239]. Although activated carbon has been applied for municipal wastewater treatment, it is a novel pretreatment method for digestate, and its actual potential is still unknown. It has shown some very promising results in a reduction of OD of digestate coming from a piggery farm in Italy [239].

1.9. Biomass harvesting

The cost-effective harvesting of microalgae biomass is considered to be one of the most problematic aspects of algal cultivation and commercialization [243]. It has been calculated that harvesting represents up to 20 – 30 % of microalgae biomass production costs [244]. While some other studies demonstrate considerably lower costs of 3 to 15% [9], cost-effective harvesting is a crucial aspect of biomass production. De-watering and harvesting costs largely depend on the harvesting technology applied, but also on the scale of cultivation and density of biomass culture.

Microalgae biomass contains high water content. Therefore, there is a need to remove large volumes of water to harvest the biomass. An ideal harvesting technique should be effective for most microalgal strains and result in high biomass concentration while requiring low costs of

operation, energy and maintenance [244]. Characteristics of microalgae species such as cell shape and size, as well as biomass density and cell age influence the selection of harvesting technology [21]. Moreover, the selection of an appropriate harvesting procedure must consider how the microalgal biomass will be further processed and the intended application. Generally, a harvesting technique yielding biomass with minimum moisture content is preferred. It is also recommended that the selected harvesting method allows the recycling of the culture medium thus increasing the sustainability [244].

Currently, algae harvesting involves physical, chemical, biological, electrical and magnet-based methods [21]. Various solid-liquid separation techniques are available for microalgae harvesting including coagulation, flocculation, flotation, centrifugation and filtration or a combination of several techniques [245]. No universal harvesting technique for microalgae exists which is both technically and economically viable. All available techniques have their advantages and disadvantages. A short description of common techniques is given below.

Sedimentation is a physical technique where gravitational forces ensure the setting of the suspended algal cells from the growth medium. However, sedimentation may be limited if the cell size or density difference is small [21]. The sedimentation rate varies among microalgae species. Furthermore, other factors such as cell age can also affect the process of sedimentation. Although the method is cost-effective, effortless and simple, it is time-consuming and not very effective; therefore, during the harvesting, sedimentation is generally used as a first step of harvesting [21].

Centrifugation uses the centrifugal force generated by the fast rotation of the algae suspension to spin out the algae cells and separate them from the growth medium. Centrifugation is one of the most applied microalgae harvesting techniques due to its high harvesting efficiency, often reported over 90% [10]. However, it is highly energy intensive [246]. Moreover, damage to microalgae cells can occur because of the high shear rates and high centrifugal forces. Cell harvesting by centrifugation highly depends on the microalgal species and the type of centrifuges [245]. Several types of centrifuges exist. Although it has a high operational cost, its many advantages such as rapid harvesting and high harvesting efficiency lead to its utilization [21]. Moreover, the harvested biomass can be safely used for high-value products because the chance of contamination is negligible with this technique [21].

During **flocculation**, the dispersed microalgal cells aggregate and form larger particles with higher sedimentation rate. Physical, chemical and bio-flocculation can be distinguished. Three common physical flocculation methods applied are ultrasound, electro-flocculation and magnetic separation [247]. Ultrasound flocculation has low efficiency, high energy consumption and requires special equipment [247]. On the other hand, electro-flocculation and magnetic particle flocculation have high removal efficiency and large-scale application prospects [247]. Chemical flocculation can be inorganic or organic based on the properties of chemical flocculants [247]. During the chemical flocculation chemicals called flocculants neutralizing the negative charge allow agglomeration of microalgae [245]. Flocculation is successfully used in wastewater treatment [248] and has been a focus of researchers in microalgae harvesting in the last few years as it is considered low-cost and highly effective. Most flocculants have the characteristics of efficient and rapid flocculation of microalgae;

however, biomass may be contaminated by metal salts requiring an additional step of purification to remove salts [247].

Bio-flocculation has recently attracted a lot of attention with low energy consumption and the potential to be a safe and sustainable technology [247]. Bio-flocculation is achieved in the presence of microorganisms or bio-flocculants released by these microorganisms. Various microorganisms, such as bacteria, algae, fungi and actinomycetes are considered producers of bio-flocculants [249]. Although bio-flocculation is promising it has been tested only on a small scale.

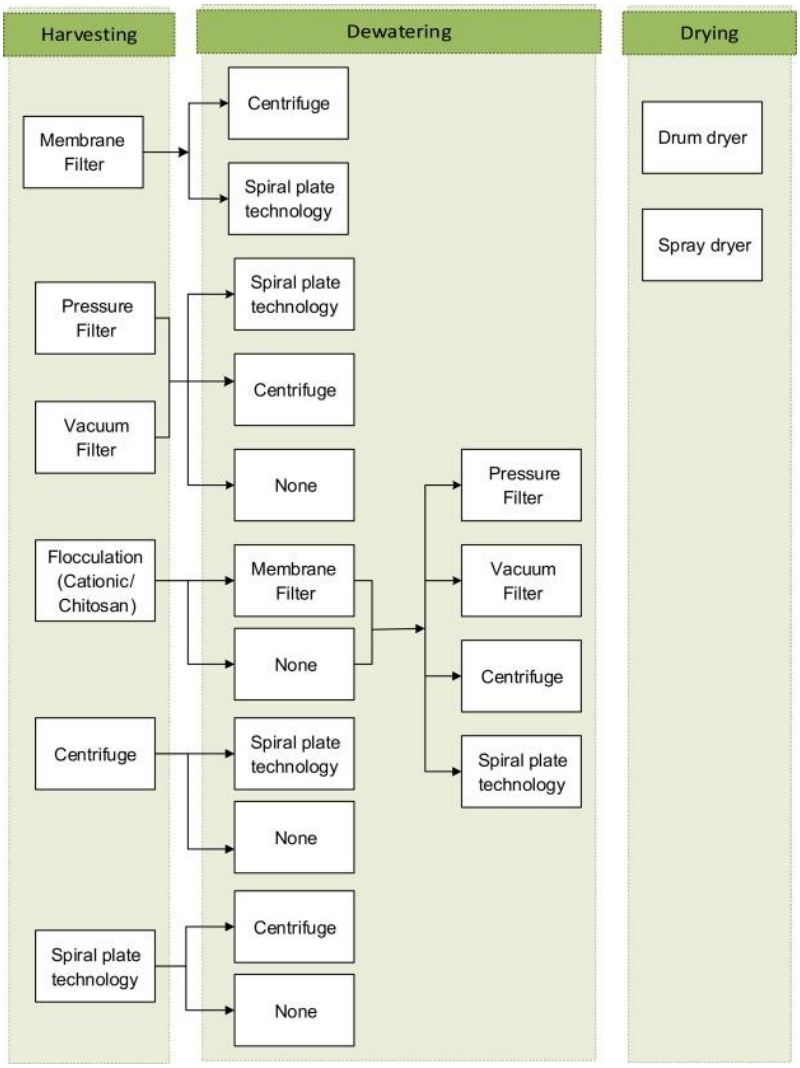


Figure 1.14. Combination of harvesting and dewatering techniques for large-scale microalgae harvesting [9].

In **membrane techniques**, algae culture passes through filters under gravity, pressure or vacuum force resulting in thick algae paste [245]. Microalgae cells are less disrupted compared to other harvesting techniques and no chemicals are required in membrane harvesting leading to good quality biomass. The disadvantages of this type of technology are low throughput and rapid fouling [245]. There is a wide variety of filter types, microfiltration (0.1 - 10 μm), macrofiltration (10 μm), dead-end filtration, ultrafiltration (0.02-0.2 μm), tangential flow filtration, vacuum filtration and pressure filtration [245].

Lately, it has been suggested that microalgal harvesting involves two concentration steps for more effective and energy-efficient biomass harvesting. Some combinations of techniques for harvesting and dewatering are suggested and presented in [9] (Figure 1.14.). Primary harvesting technologies as suggested by Fasaei and colleagues [9] are membrane filtration, chemical flocculation, vacuum and pressure filtration, centrifugation, and spiral plate technology. For the second dewatering step, membrane filtration, vacuum and pressure filtration, centrifugation, and spiral plate technology have been suggested.

2. RESEARCH METHODOLOGY

2.1. Design and construction of a novel cultivation system

An extensive literature review was conducted in order to uncover the advantages and disadvantages of existing cultivation systems. The design of a novel cultivation system was proposed to overcome the limitations of the existing cultivation technologies.

Planning and design of novel cultivation system included considerations on location and layout of facilities, pond size and configuration, hydraulics, paddle wheel design and materials for the construction. In design consideration, several aspects including geometrical design in terms of surface area-to-volume ratio, as well as light distribution, nutrient provision and gas transfer were studied. Mixing patterns and efficiently rely on paddlewheel design therefore scientific literature was studied to select the most effective type taking into account energy consumption and mixing efficiency.

An understanding of the morphology and physiology of specific microalgae is required for design considerations. Moreover, a knowledge of the complex interaction between biomass production and environmental parameters is essential [92].

The main aim addressed is to provide benefits towards: (1) the reduction of land use, (2) the increased light availability, and (3) the lower investment costs of the open systems compared to PBRs.

2.2. Laboratory scale tests

A range of laboratory tests were conducted to assess the influence of diverse environmental and cultivation conditions on the growth rate and productivity of candidate microalgae strains selected during the literature review, namely *Chlorella vulgaris* 211-11j, *Chlorella sorokiniana* 211-8k and *Chlamydomonas reinhardtii* 11-32b. The impact of temperature, light intensity, photoperiod, light spectral composition, and level of CO_2 on the microalgae growth rate was tested on a laboratory scale. Moreover, digestate as a nutrient source for microalgae growth was evaluated.

Microalgae strain selection and maintenance

The selection of potential microalgae strains for outdoor cultivation in Latvian climate conditions was based on an extensive literature review of published scientific research based on considerations described in the Chapter “Microalgae strain selection” in the Literature review section.

Three microalgae were selected for laboratory tests. Microalgae *Chlorella vulgaris* 211-11j, *Chlorella sorokiniana* 211-8k and *Chlamydomonas reinhardtii* 11-32b were obtained from the SAG Culture collection of algae at Göttingen University, Germany and The Culture Collection of Algae and Protozoa at Scottish Marine Institute, Scotland, UK.

Species were maintained in liquid BG-11 or TAP growth medium in Erlenmeyer flasks with baffles and 0,2 μm PTFE membrane screw caps (Duran, Germany) at 24 °C in low light

conditions on a rotary shaker at 150 rpm to avoid settling of cells. Sub-culturing was carried out approximately once per month to keep the algae cultures growing and in a healthy condition.

BG-11 medium was prepared according to [250], whereas Tris-Phosphate-Acetate (TAP) medium according to [251].

Growth assessment methods

Microalgae growth was assessed with several methods described below.

1. Cell counts with a hemocytometer.

A hemocytometer was used for manual cell counting. The daily count of microalgal cells in each culture flask using an improved Neubauer hemocytometer and a light microscope was performed for a specific experiment. The centre square of the hemocytometer was utilized for the cell counting following a standard procedure [252]. Microalgae cell density (cells ml^{-1}) was calculated according to the equation (1).

$$Cell\ density = \frac{Average\ number\ of\ cells\ per\ square\ *\ dilution\ factor}{Volume\ of\ the\ square\ (ml)} \quad (1)$$

2. Optical density measurements.

Optical density was used as a convenient indirect measurement of biomass concentration. Cultures were sampled daily for growth rate evaluation by optical density measurements. The readings were performed with a UV/VIS spectrophotometer (Thermo Scientific, USA) at 750 nm.

3. Specific growth rate (μ) is a widely used growth metric of microalgal cultures. The specific growth rate based on culture cell density was calculated during the exponential growth phase according to equation (2):

$$\mu = \frac{lnN_2 - lnN_1}{T_2 - T_1} \quad (2)$$

where N_1 and N_2 are microalgae culture densities (cells ml^{-1}) at the time T_1 and T_2 respectively.

4. Dry weight measurements.

Dry weight measurement was used for biomass yield calculation. 50 ml of homogeneous microalgae culture was collected from a culture flask in a pre-weighted 50 ml tube and centrifuged at 10`000 rpm for 10 minutes at a room temperature The liquid fraction was removed and the tube with biomass was dried at 80 °C in the oven until constant weight and weighed. Dry weight was calculated by subtracting the initial tube mass from the mass of tubes with the dry biomass. Biomass yield was calculated as grams of biomass per litre of growth medium (g L^{-1}).

Alternatively, the dry weight of the cultures was determined by vacuum filtering 200 ml of the culture through a pre-weighted 110 mm glass microfiber filter with a pore size 1,2 μm (Whatman GF/C). Filters were dried at 80 °C in an oven until constant weight and then weighed.

Dry weight (g L^{-1}) was calculated by subtracting the initial filter mass from the mass of filters with the biomass and expressed as g L^{-1}.

5. Biomass productivity.

Biomass productivity (g L^{-1} d^{-1}) was calculated according to the equation (3).

$$P = \frac{X_2 - X_1}{T_2 - T_1} \quad (3)$$

where X_2 is biomass dry weight (g L^{-1}) at the end of cultivation (T_2) and X_1 is the dry weight of the initial culture at the time (T_1).

All tests were done in triplicate and the Standard Deviation was calculated in Excel. A two-tailed t-test was performed to evaluate the significance of specific cultivation factors affecting microalgae growth.

Evaluation of a low-temperature strain

Chlorella vulgaris 2011-11j was selected as a potential low-temperature strain for outdoor cultivation in Latvian conditions in colder seasons. Microalga was cultivated in a wide temperature range from 8 to 32 °C to assess optimum cultivation temperature and lower and upper temperature limits. Cultures were grown in batch mode at 8, 12, 16, 20, 24, 28 and 32 °C for 10 days. Illumination was provided with natural white (4000 K) LED lights with light intensity ca. 2800 lux or 50 µmol photons m^{-2} s^{-1} and photoperiod of 16:8 h (light:dark). Light intensity was measured with a light meter (Testo, Germany). Cultures were cultivated in 500 ml Erlenmeyer flasks containing 200 ml BG-11 medium with an initial pH of 7.5. Aeration was provided with ambient air using an orbital shaker (Elmi, Latvia) at 150 rpm. The initial concentration of *C. vulgaris* cultures was approx. 2 x 10^6 cells ml^{-1}. Tests were performed in a benchtop incubated shaker (JeioTech 3075R, Korea) or refrigerated incubator (Friocell Eco line, MMM group, Germany) with manually installed LED lights. All the tests were conducted in triplicate.

Microalgal cell density was determined by the daily count of microalgal cells in each culture flask using an improved Neubauer hemocytometer as described previously. The mean value of triplicates was subsequently calculated for each cultivation temperature and standard deviation was determined. Specific growth rate (µ) based on *C. vulgaris* cell density in the culture was calculated as described previously. A two-tailed t-test was performed to evaluate the significance of cultivation temperature. The dry weight of the cultures was determined and biomass productivity (g L^{-1} d^{-1}) was calculated as described previously. Productivity was also expressed as a percentage for easier comparison between various cultivation temperatures, 100% was attributed to the highest productivity gained.

Daily pH readings were collected manually with a pH meter (Hanna, USA) to monitor the microalgae growth and to evaluate the effect of non-controlled pH on the growth of microalgae.

Light intensity tests

The effect of light intensity on microalgae growth and biomass production was assessed under various irradiances from 30 to 200 μmol photons m^{-2} s^{-1}. Specifically, light intensity of 30, 50, 100, 150 and 200 μmol photons m^{-2} s^{-1} was applied. Natural white (4000 K) linear 10W LED lights (V-TAC, Samsung) were manually installed inside an incubator (Friocell Eco line, MMM group, Germany). Specific light intensity was achieved by adjusting the number of LED lights and their distance from culturing flasks. Installed light intensity was measured with a light meter (Testo, Germany). Microalgae *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* were cultivated in 500 ml Erlenmeyer flasks with cotton plugs containing 200 ml BG-11 (*C. vulgaris*, *C. sorokiniana*) or TAP (*C. reinhardtii*) medium. Aeration was provided with ambient air using an orbital shaker (Elmi, Latvia) at 150 rpm. Algae were grown at a constant temperature of 24 °C under a photoperiod of 16:8 h (light:dark) for 10 days under batch cultivation mode. The initial optical density (OD) of all cultures was approx. 0.05. The initial pH of the BG-11 medium was 7.5-7.7 and 7 for the TAP medium.

Experiments were conducted in two rounds. In the first setup light intensity of 30, 50, 100 and 150 μmol m^{-2} s^{-1} was tested: *C. vulgaris* and *C. sorokiniana* were cultivated in BG-11 medium while *C. reinhardtii* in a TAP medium. A slower growth rate of *Chlorella* species was detected compared to *C. reinhardtii*. Moreover, a sharp rise in pH during the cultivation was observed that might have affected the biomass productivity of *Chlorella* spp. In the second round *Chlorella* species were cultivated in a TAP medium in order to understand if a change of medium can improve the growth rate of *Chlorella* spp. In the second round, also light intensity was adjusted and was set to 50, 100, 150 and 200 μmol m^{-2} s^{-1}. All tests were conducted in triplicate.

Cultures were sampled daily for growth rate evaluation by optical density measurements at 750 nm. Moreover, daily pH readings were collected manually with a pH meter (Hanna, USA) to monitor the microalgae growth. Biomass production was evaluated based on the cell dry weight at the end of the batch cultivation as described previously. The specific growth rate (μ, d^{-1}) was calculated as described previously.

Light photoperiod tests

During the literature review, a photoperiod of 16:8 h light:dark was found to be mentioned as the optimum for high growth rate and biomass yield. However, 24:0 h or continuous illumination has resulted in the maximum growth rate in several microalgae. Therefore, to select the most suitable lighting conditions, microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Chlamydomonas reinhardtii* were cultivated in 16:8 and continuous illumination. Furthermore, as light intensity and photoperiod are closely linked, two light intensity settings were also tested., e.g. 50 and 100 μmol photons m^{-2} s^{-1}.

Lighting was provided with natural white (4000 K) linear 10W LED lights (V-TAC, Samsung) manually installed inside an incubator (Friocell Eco line, MMM group, Germany). Microalgae *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* were cultivated in 500 ml Erlenmeyer flasks with cotton plugs containing 200 ml TAP medium. Aeration was provided with ambient

air using an orbital shaker (Elmi, Latvia) at 150 rpm. Algae were grown at a constant temperature of 24 °C for 10 days under batch cultivation mode. All tests were conducted in triplicate.

Cultures were sampled daily for growth rate evaluation by optical density measurements at 750 nm. Moreover, daily pH readings were collected manually with a pH meter (Hanna, USA) to monitor the microalgae growth. Biomass production was evaluated based on the cell dry weight at the end of the batch cultivation as described previously. The specific growth rate (μ, d^{-1}) was calculated as described previously.

Light spectrum tests

Green microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Chlamydomonas reinhardtii* were used for light spectrum tests. To find the optimal light spectrum for the growth of the selected microalgae, the red+blue LED spectrum was compared to full spectrum white LED lights. A mix of red and blue lights was selected based on the literature review as a promising spectral combination often reported to enhance microalgae growth. Red+blue 5W linear LED lamps with a ratio of 3:1 (red:blue) were used. Full spectrum white 5W linear LED lamps were used for comparison (as a control). Moreover, the impact of light intensity on the preferred spectrum was also tested since light intensity is known to affect the optimal spectral composition. Three different light intensities were tested by adjusting the distance of culturing flasks to the light source. Actual light intensity measured with PAR light sensor as $\mu mol/m^2/s^{-1}$ varied due to the different spectral composition of the LED lights, therefore for simplicity light intensity in results is converted to the level of intensity; 1. level – 40 cm apart from the light source, 2. level – 30 cm apart from the light source and 3. level – 20 cm apart from the light source. Cultivation was carried out at 28 °C under a photoperiod of 16:8 h (ligh:dark) in 500 ml flasks with a working volume of 200 ml. TAP medium was used as a nutrient source. Aeration was provided with ambient air using an orbital shaker at 150 rpm. Microalgae were cultivated in batch conditions for 10 days in triplicate.

The daily growth rate was measured by OD readings at 750 nm. Moreover, pH readings were collected daily. At the end of the cultivation biomass yield was determined for all cultures based on a dry weight.

Carbon dioxide tests

The impact of elevated CO_2 concentration was assessed on the growth of the potential candidate strains of green microalgae *Chlorella vulgaris*, *Chlorella sorokiniana* and *Chlamydomonas reinhardtii*. Three different settings were used: (1) cultivation without extra CO_2 supply, (2) 5% CO_2 mix (3) 10% CO_2 mix. CO_2 was mixed with air and fed in microalgal cultures at a rate of 0.1 L per minute. Cultivation was carried out at 24 °C and 50 μmol photons m^{-2} s^{-1}. LED illumination under a photoperiod of 16:8 h (ligh:dark) in 500 ml flasks with a working volume of 200 ml. TAP growth medium was used as a nutrient source. Aeration was provided with ambient air using an orbital shaker at 150 rpm. Microalgae were cultivated in batch conditions for 8 days.

The daily growth rate was measured by OD readings at 750 nm. Moreover, pH readings were collected daily. At the end of the cultivation biomass yield was determined for all cultures based on a dry weight.

Digestate as a growth medium

Pretreatments of digestate

Liquid digestate after separation of solid fraction was obtained from SIA "Agro Iecava" biogas plant located in Iecava, Latvia. Due to very high turbidity created by suspended solids and high OD, raw digestate was not suitable for microalgae cultivation. Various pretreatment methods were employed prior to use for microalgae cultivation for the removal of suspended particles of the digestate to reduce turbidity and allow light penetration.

Several pretreatment methods were applied and tested to improve digestate properties:

(1) Centrifugation at 10'000 rpm;

(2) Vacuum-filtration through a 1.6 μm microfiber filter;

(3) Filter centrifugation at 10'000 rpm;

(4) Adsorption on activated carbon (described in the next subchapter)

Centrifugation was carried out in a laboratory centrifuge (MegaFuge 16R, Thermo Scientific) in 50 ml flasks at 10'000 rpm for 10 min. The liquid fraction was then transferred to a new tube and centrifugation was repeated. The final supernatant was collected and stored in a fridge until the start of the growth tests. For vacuum filtration pretreatment, centrifuged digestate was vacuum filtered through a 1.6 μm glass microfiber filter (Whatman) to further reduce the total solids content of digestate. Filter centrifugation was performed at 10'000 rpm with Hermle centrifuge (Hermle sieva, Germany) as a more viable option for large scale pretreatment of digestate. Sterilization of digestate by autoclaving was carried out to remove any microorganisms that might be present in a digestate. Autoclaving was performed at 120 °C for 20 min.

Characterization of digestate was done subsequently for each of the pretreatment methods including the determination of suspended solids, total nitrogen, total phosphorus, ammonia nitrogen, nitrates, and chemical oxygen demand (COD) to assess the pretreatment efficiency. Analyses were purchased as an external service from the Vides audits laboratory.

Pretreated digestate was thereafter used for microalgae growth tests. For the first round of growth tests, green microalga *Chlorella vulgaris* 211-11j was cultivated in sterilized (1) centrifuged and (2) vacuum-filtered digestate diluted to 1%, 3% and 5% with distilled water. 5% dilution was used only for vacuum-filtered digestate (pretreatment 2.) because pretreatment with centrifugation at 5% digestate was not suitable for microalgae growth due to excessively high OD. Standard BG-11 medium was used as a control. Cultivation was carried out at 28 °C and 150 μmol photons m^{-2} s^{-1} white LED illumination under a photoperiod of 16:8h (light:dark) in 500 ml flasks with a working volume of 200 ml. Aeration was provided with ambient air using an orbital shaker at 150 rpm. Microalgae were cultivated in batch conditions for 10 days.

The daily growth rate was assessed by optical density measurements at 750 nm. Moreover, pH readings were collected daily. All tests were conducted in triplicate. At the end of each cultivation, biomass yield was determined for all cultures based on a dry weight as described previously. Furthermore, suspended solids, turbidity, COD and nutrient levels in digestate after microalgae cultivation were analysed and the nutrient removal rate was calculated to assess the potential of microalgae for digestate treatment.

Liquid digestate pretreatment with activated carbon

Tests described in this chapter were performed at the Department of Earth and Environmental Sciences of the University of Milano-Bicocca, Italy during the Erasmus^{+} exchange.

Although the applied pretreatment methods described in the previous chapter resulted in significantly improved properties of digestate in terms of total solids and turbidity, the main issue remained the dark color limiting digestate application. To reduce the OD, pretreatment with adsorption on activated carbon was performed.

Digestate characterization

Chemical composition, pH, turbidity, optical density and content of solids were analyzed in a liquid agricultural digestate collected from Agro Iecava biogas plant in Latvia and the level of potential inhibitors was assessed before the treatment. Raw liquid digestate was centrifuged at 10,000 rpm to decrease the amount of solids and kept at +4 °C before use. The levels of total nitrogen, phosphate, nitrate, ammonium, COD, turbidity, and OD were then determined in the liquid digestate. Commercial cuvette test kits (Hach Lange, Germany) were used for spectrophotometric quantification of phosphate (PO_4-P), total nitrogen (TN), ammonium (total ammonia nitrogen (NH_4-N), nitrate (NO_3-N), and chemical oxygen demand using DR3900 spectrophotometer (Hach Lange, Germany) according to Hach standard methods. Samples were filtered through a 0.45 μm filter before the analysis of TN, NH_4-N, NO_3-N, PO_4-P and COD. Turbidity was determined spectrophotometrically with a DR3900 spectrophotometer by using pre-set turbidity parameters. OD was measured at 680 nm using a spectrophotometer. Total solids, suspended solids and volatile solids in digestate were analyzed according to the standard methods [253].

Pretreatment with activated carbon

To test the potential of the application of activated carbon to reduce the optical density of digestate, several activated carbon concentrations and various adsorption durations were tested to find the most effective conditions. Activated carbon (Chemviron, UK) concentrations of 3, 10, 20 and 40 g per liter were tested. Liquid digestate was incubated with activated carbon on a rotary shaker at 200 rpm for 5, 10, 30 and 180 minutes, and then centrifuged at 13,000 rpm to remove activated carbon particles. The OD was measured after the pretreatment and the reduction rate was calculated. The best-performing activated carbon concentration and adsorption time combination was then selected for digestate pretreatment for microalgae growth tests based on the most efficient OD reduction.

Set-up of growth tests

Thereafter, the activated carbon pretreated digestate was tested as a growth medium for microalgae cultivation. Tests were carried out in parallel with a raw and activated carbon pretreated digestate as a growth medium in four plexiglass column photobioreactors with 10 cm diameter and a working volume of 1.5 L (IDEA Bioprocess Technology Srls, Italy) (Figure 2.1.).

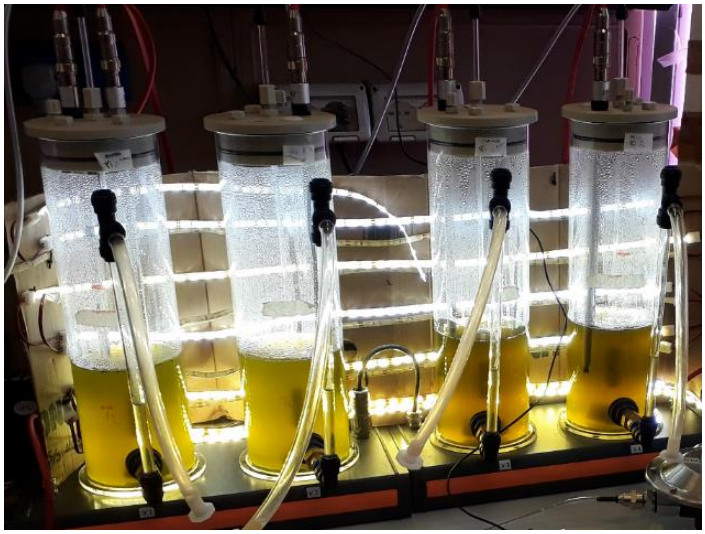


Figure 2.1. Experimental setup with photobioreactors at the beginning of growth tests.

Microalgae culture containing mainly *Chlorella* sp. was obtained from Istituto Spallanzani (Rivolta d'Adda, CR, Italy) and was acclimated to growing in digestate by culturing in diluted liquid digestate at room temperature under white LED lights (12:12h light-dark cycle) on a magnetic stirrer for approximately two weeks before the growth tests in photobioreactors.

The untreated digestate was diluted down to OD 0.1 corresponding to 1% dilution with distilled water to increase the light availability and decrease the nutrient load. Digestate pretreated with activated carbon 3 g L^{-1} and an adsorption time of 10 minutes was then diluted to OD 0.1 to match the OD of the untreated digestate. Microalgae cultivation was performed with two replicates for each condition; in PBR 1 and PBR 2 untreated diluted digestate was used, and in PBR 3 and PBR 4 – activated carbon pretreated digestate. PBRs were inoculated with microalgae culture dominated by *Chlorella* sp. The initial OD of the algal culture was 0.1 in all PBRs. PBRs were mixed with magnetic stirrers at 250 rpm. pH was controlled automatically by CO_2 injection in the system when the pH moved out of the set range. The optimal pH was set between 7 and 7.8. Lighting was provided by white LED lights under a 12:12h light-dark cycle at an average light intensity of 50 µmol m^{-2} s^{-1}. The cultivation was carried out in batch cultivation mode at room temperature of approximately 24 °C for 14 days.

Nutrient removal and biomass accumulation

The initial nutrient concentration, OD, pH and COD were determined in PBRs and thereafter were monitored regularly during the cultivation. Phosphate (PO_4-P), total nitrogen

(TN), ammonium (total ammonia nitrogen, NH_4-N), nitrate (NO_3-N), and COD were determined with Hach Lange DR 3900 spectrophotometer as described previously. Samples were analyzed in two replicates from each PBR. Initial nutrient concentrations in treated and untreated PBRs varied due to different dilution rates of digestate. Nutrient concentrations in PBR 3 and 4 (pretreated) were higher than those of PBR 1 and 2 (untreated). Nutrient removal rates were subsequently calculated at the end of cultivation and compared between treated and untreated digestate. Microalgae biomass accumulation was measured based on OD, cell counts and suspended solids content. Microalgal cell counts were carried out using a hemocytometer (Marienfeld, Germany) and an optical microscope 40X (B 350, Optika, Italy). Cell counts were performed every three days during cultivation to evaluate the growth of microalgae, changes in species composition and the presence of potential predators. *Chlorella* spp., *Scenedesmus* spp. or other species were identified according to their morphological characteristics and counted. Microalgae productivity in each PBR was calculated based on the determination of cell dry weight. Optical density was measured by a spectrophotometer (DR3900 Hach Lange) at a wavelength of 680 nm. The specific growth rate (μ, d^{-1}) was calculated as described previously.

Cell viability test

Furthermore, a viability test was performed to assess the condition of microalgal cultures during the cultivation test. Nucleic acid stain Sytox (Thermo Scientific) was used according to the manufacturer's instructions to determine the viability of cells using a Zeiss fluorescence microscope Axio 170 Scope HBO 50 at the wavelength of 450–490 nm. Sytox can only penetrate the damaged cell walls of dead microalgae which can be detected by their bright green fluorescence. Living cells are red due to the autofluorescence of chlorophyll [254]. 1 mL of each microalgal suspension was centrifuged for 10 minutes at 10,000 rpm. The supernatant was discharged while the biomass was resuspended in 1 mL of distilled water. Finally, Sytox label was added (0.5 μL) for the staining process and the samples were kept in the dark for ten minutes before the observation. For each sample, an average of 300 cells were counted, assessing the proportion between dead and living cells.

Photosynthetic efficiency

Photosynthetic performance measured with pulse amplitude modulated (PAM) fluorometry was used to evaluate the effect of digestate on the growth of microalgae. PAM is a valuable technique to evaluate the physiological stress of microalgae caused by the potential toxicity of the growth medium that can be seen from photosystem II performance [255]. Microalgal culture samples were collected from all bioreactors and diluted to OD 0.1 at 680 nm. Samples were kept in the dark for 20 minutes. Algal cultures from bioreactors were compared with algae grown in a synthetic medium. PHYTO-PAM-II (Heinz Walz, Germany) was used for the analysis.

F_v/F_m represents the maximum photochemical quantum yield of photosystem II (Equation 4), where F_o is the minimum fluorescence level excited by very low intensity of measuring light, F_m is the maximum fluorescence level elicited by a pulse of saturating light.

$$\frac{Fv}{Fm} = \frac{(Fm - Fo)}{Fm} \quad (4)$$

Moreover, different groups of photosynthetic organisms can be detected with PAM readings as differentiation of 4 different pigment types and therefore detection of green algae, cyanobacteria, diatoms/dinoflagellates and phytoerythrin containing organisms can be made.

2.3. Growth tests in pilot raceway ponds

Microalgae cultivation was performed to test the novel cultivation technology in a real environment integrated into a working biogas plant.

Liquid digestate was collected from the Iecava biogas plant and used as a nutrient source for microalgae cultivation in the novel algae cultivation system. Digestate for cultivation was prepared by centrifugation using a filtration centrifuge (Hermle, Germany) at 10'000 rpm, and liquid fraction was collected in containers and kept at +4 °C until inoculation of algae pond. Chemical analysis of digestate was performed before the inoculation to assess the level of nutrients and contaminants at the beginning of microalgae cultivation. Total nitrogen, total phosphorus, ammonia nitrogen, nitrates and chemical oxygen demand were determined in an external laboratory.

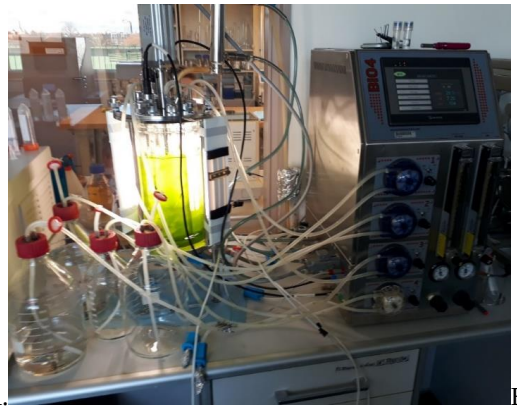
A.  B. 

Figure 2.2. Set up of 5L cultivation bioreactor (A) and LED light lamps attached to the walls of the photobioreactor (B).

Microalgae culture for inoculation of the algal pond was grown in a 5L photobioreactor (Bio4, Biotehniskais centrs) in the RTU Biosystems laboratory (Figure 2.2. A). TAP medium was used as a nutrient source. *C. sorokiniana* was cultivated at 26 °C, rotation was set to 150 rpm, bubbling with air at the rate of 50L per minute. pH was automatically controlled at the optimum level by the addition of 2M HCl acid when the pH exceeded pH 7.9. The light was provided with 3 pieces of 10W linear white LED lamps attached to the outside of the reactor (Figure 2.2. B). The light-dark cycle was set to 16h light and 8h dark.

The microalgal pond was filled with tap water at 20 cm depth, 2 L of pretreated digestate was thereafter applied and pre-cultivated *C. sorokiniana* biomass at the rate of ~ 1.5% was added (Figure 2.3. A and B).

CO_2 introduction in the pond was implemented by bubbling of flue gas captured from the motor chimney. Mixing was provided with an electrical motor-driven paddlewheel at the frequency of 10 Hz, resulting in a paddlewheel speed of approximately 2.6 rpm and water speed of 10 cm s^{-1}.

Probes with temperature, pH, and PAR sensors were installed and used to record the cultivation conditions. The temperature in the pond, in the greenhouse and outdoors was recorded. Light intensity in PAR at the water level was recorded. The temperature of flue gas pumped inside the pond was recorded. Samples for nutrient removal and biomass analysis were taken every 3 days. Additional measurements were done with a multiparameter reader (HI 9829, Hanna instruments), including total dissolved solids, turbidity and conductivity. Samples were analyzed for nutrient content, suspended solids and optical density. Analysis of total nitrogen, total phosphorus, ammonia nitrogen, nitrates and chemical oxygen demand were performed in an external laboratory, other analyses were done in the RTU Biosystems laboratory.

A.  B. 

Figure 2.3. Prepared inoculum (A) and pretreated liquid digestate (B) for the inoculation of the cultivation pond.

Cultivation was performed in batch cultivation mode, no nutrients were added during the cultivation period and no biomass was removed, therefore it was possible to calculate nutrient removal of digestate during the cultivation test.

Microalgae cultivation was carried out from 21.04.2021. – 06.05.2021. and lasted for continuous 16 days.

3. RESULTS AND DISCUSSION

3.1. The concept and design of the novel cultivation system

Coupling the anaerobic digestion process with microalgae cultivation may contribute to nutrient bioremediation from liquid digestate as well as CO_2 capture from biogas. The main concept of the created system is shown in Figure 3.1. Scenario 1 shows the traditional biogas production and digestate management route, whereas the system with biogas waste streams, namely, digestate and flue gases, integrated into microalgae cultivation is shown in Scenario 2. The traditional practices involve storage of produced digestate and field application when possible. Flue gases created during the combustion of biogas are commonly released into the atmosphere, and electrical and thermal energy created is sent to the public network. When microalgae cultivation is integrated within a biogas production process, digestate is applied for microalgae cultivation as a source of nutrients and flue gases as a source of CO_2. Furthermore, the electrical energy produced is used to maintain the operations of microalgae cultivation ponds, whereas the heat can be used for heating microalgal ponds during cold seasons.

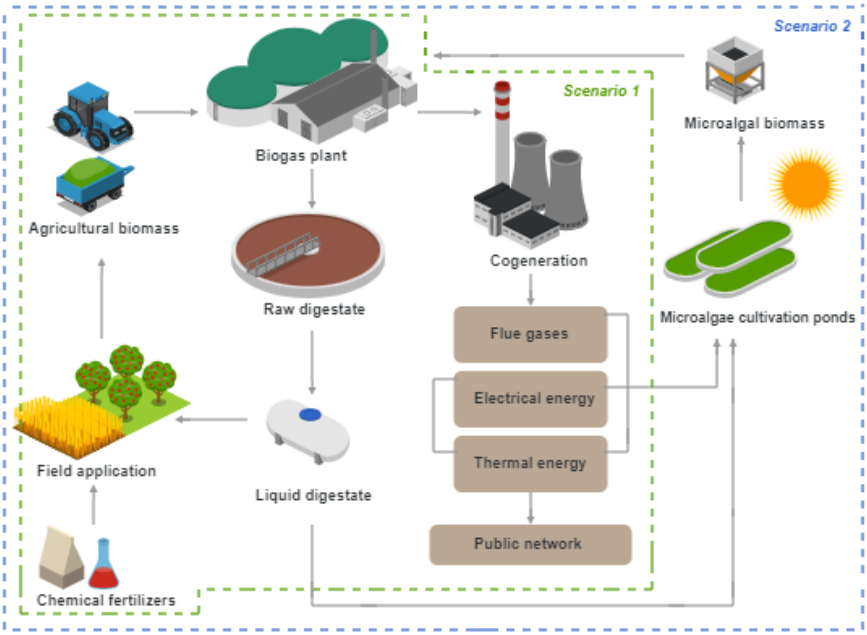


Figure 3.1. Simple schematic representation of the concept of integration of microalgae cultivation into biogas plant. Scenario 1 shows a traditional biogas plant; the integration of microalgae cultivation is depicted in Scenario 2.

This cutting-edge technology has been integrated into a biogas plant, utilizing biogas byproducts namely liquid digestate and flue gases as nutrient sources for growing microalgae. Microalgae uptake nutrients such as phosphorus and nitrogen from digestate for growth while simultaneously removing other contaminants such as heavy metals,

pharmaceuticals, and personal-care products. Consequently, alongside biomass production, wastewater treatment occurs concurrently, thereby reducing costs associated with both microalgae nutrients and wastewater treatment. Moreover, microalgae uptake carbon dioxide from flue gas coming from biogas combustion utilizing it as a carbon source for growth, therefore presenting an opportunity for carbon dioxide biosequestation. The produced microalgal biomass is directed to anaerobic digestion, creating a loop of nutrient use. This integrated approach not only lowers the costs associated with microalgae biomass production by utilizing waste streams as low-cost nutrients but also increases microalgae productivity through improved cultivation conditions. Furthermore, it provides an alternative route for digestate management and facilitates carbon dioxide sequestration. In the novel SMORP system microalgae acts as a biofilter for the treatment of the liquid digestate and flue gases from the cogeneration unit in a biogas plant offering alternative management method of biogas production waste streams. This solution creates a transformation of the main environmental drawbacks from the anaerobic digestion related to the storage and disposal of the digested biomass and high CO_2 emissions in a valuable closed-loop technological system. The overall technological scheme of the SMORP pilot creates a closed loop which enables a biogas operator to produce energy from microalgae biomass creating benefits from the management of waste products and emissions (i.e. digestate and CO_2). At the same time, microalgae biomass production benefits from low-cost nutrients from biogas waste streams. The pilot concept offers a solution for the issue of digestate storage and transport offering an alternative digestate valorization route. This can significantly contribute to reducing the energy cost in the overall plant management and operational system.

The microalgae production unit was integrated into an existing biogas plant and microalgae-based system and its harvesting can be considered as a side-stream processing module. The main challenge is the development of a mass microalgae cultivation system with high productivity and low energy requirements at the same time. The current research and studies in the field have shown major problems related to the regulation of optimal microalgae growing conditions as well as extensive land use for the open raceway ponds. A novel type of microalgae cultivation system was created during doctoral studies, named Stacked Modular Open Raceway Ponds (SMORP). The principle of SMORP is to combine the advantages of existing systems creating a hybrid between open and closed cultivation systems. The novel technology is based on a traditional open raceway pond design but features of closed photobioreactors are added such as artificial lighting, heating, and cooling. Potential limiting factors experienced in open ponds such as light and temperature limitations were overcome with the novel design. The main concept of SMORP is the stacked design allowing to save space which is considered as one of the main limitations of existing designs. Moreover, with a supplemental artificial lighting system, modular design and use of transparent material, the proposed technology has significant advantages over the currently available ones.

Three microalgae cultivation ponds are arranged in a pyramid shape by placing the 3rd tank on top of the 2 bottom tanks overlapping half of each bottom tank. The use of transparent material and additional LED lighting help to mitigate the shadowing effect. The proposed concept considers a combined sunlight and artificial lighting system with low power-consuming

LEDs and a proper light wavelength to balance the light variation and shadow made by the upper ponds, in turn compensating with a higher biomass yield. Furthermore, technological advances including the integration of flue gases and digestate as CO_2 and nutrient sources significantly contribute to the environmental and technological feasibility of microalgae biomass production.

The search for the most appropriate material for ponds was one of the major aspects. Open ponds are typically constructed as concrete, clay, or plastic-lined ponds [256]. Whereas, glass, fiberglass, PVC, polyethylene, polycarbonate, HDPE polymer, Plexiglas or acrylic have been used as the basic materials for the construction of closed PBRs [257]. Nowadays plastic materials are used more often than glass due to lower costs, higher light transmission, ease of transportation, lower maintenance, durability, and better mechanical properties. Each of the materials has its advantages and limitations. Pond material must be transparent enough to allow light penetration, though durable at the same time, affordable, easy to clean, avoid sticking to walls and UV resistant. Based on the desired characteristics, acrylic was selected for the SMORP pilot. Acrylic material has the capability to be easily shaped for rounded geometry. Using a transparent material, the effect of natural light can be maximized, increasing light penetration through the system, in contrast to conventional open pond designs. Acrylic is a transparent material which allows light to pass through (transparency of 92 %).

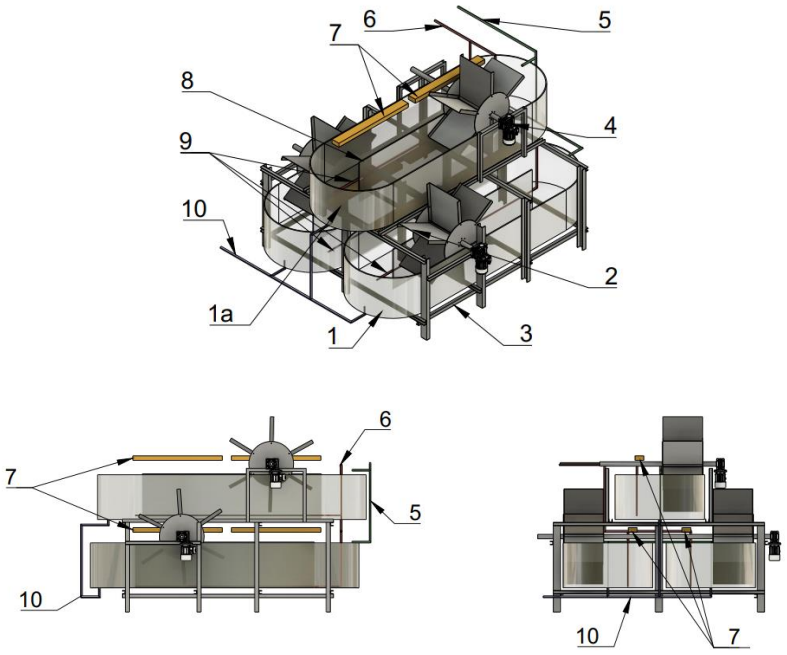


Figure 3.2. Schematic representation of novel design open raceway cultivation ponds.

Each pond is one module, they can be arranged in an unlimited number of levels to form a modular microalgae cultivation pond system. Modular design allows ease of construction and

flexibility for scaling up by adjusting the number of single modules per construction unit. The single modular pond is an oblong-shaped shallow pond having a length-to-width ratio equal to 3 (i.e. length = 3 m, width = 1 m), an area of 3.6 m^2, and a height of 50 cm. Total volume depends on culture depth. The sides are made from 15 mm acrylic sheets, the bottom is 20 mm thick, and the internal walls are made of 10 mm sheets. The parts are bent and fixed by glueing.

The design of the SMORP pilot is presented in Figure 3.2. The cultivation of microalgae takes place in transparent, oval open ponds (1) arranged in a pyramid shape on top of each other. The ponds (1) are arranged on the support structure (3). The support structure is constructed in a way that minimises the shading of the ponds using metal grids to let the light through. The microalgae cultures are continuously mixed with a paddlewheel (2) that is driven by a geared motor (4). Microalgae cultures are fed nutrients automatically or manually using a nutrient supply (5). Flue gases containing carbon dioxide are introduced into the ponds via a carbon dioxide supply (6). LED cultivation lamps (7) are located above each pond. The flow of microalgae cultures is restricted by an acrylic separating wall (8) placed in the middle of each pond. The gas is evenly distributed in each pond using a perforated carbon dioxide diffuser (9) at the bottom of the pond. This also ensures good solubility of carbon dioxide in water. For biomass harvesting, there are openings and inlets (10) for biomass collection at the bottom of the ponds.

The motor and reductor are manufactured by Motovario. Motor is OMEC OMT4 632-4 IM B5 1310 rpm 0.18kW 230/400 V IP55 and reductor is Motovario NMRV050 i=100 PAM63 B5. To ensure the rotation control a frequency a 230V 0.4kW controller (Santerno Sinus N 001 2S XIK2 AC 1PH) is used. Two sets of motors, reductors and controllers are used – each set for one of the mixing axes. The maximum frequency is 400 Hz, which would produce 52 rpm.

The SMORP technological scheme is shown in Figure 3.3. and the main components are reported below.

− Liquid digestate as a nutrient source: digestate discharge from the biogas plant is stored in a continuously stirred holding tank. The digestate can be fed to the pond by an automatically controlled peristaltic pump or manually. Feeding volume depends on the characteristics of digestate. Critical characteristics of digestate such as pH, oxidation-reduction potential, turbidity, and temperature are continuously monitored.

− Flue gas as a carbon source: flue gas emitted from the biogas cogeneration unit is used as a carbon source for the growth of microalgae biomass. Gas is fed to the system through microporous tubular diffusers installed at the bottom of each pond. The flue-gas when it exits the engine has a temperature of 400 °C. When there is a heat load, the flue gas is cooled by the use of a heat-exchanger to a temperature of 100 °C. 10 m long and 20 mm in diameter metal pipe is used to transport the gas. The metal pipe works as a heat-exchanger and provides close to ambient temperature gas at the outlet. The gas cooling is realized by the use of copper tubing for gas transport and the use of low flow rates which ensure that gas has a lot of time to cool when travelling down the pipe. Additionally, there are by-passable cooling loops which are placed in a forced air-flow location. The pump can provide 10-15 litre min^{-1} of gas for each pond. The mixing of flue gas with ambient air is possible to avoid growth inhibition by

excessive CO_2 content. At the time they were tested, flu gases contained 14% CO_2, 8% O_2, 265 ppm N_{ox} and had a temperature of 522 °C.

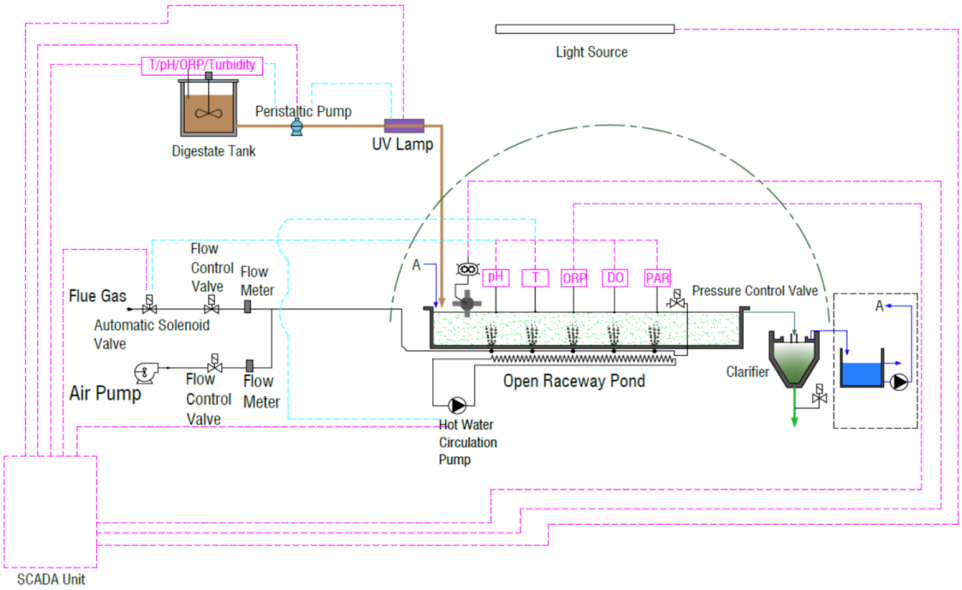


Figure 3.3. SMORP technological scheme.

− Mixing mechanism of microalgae culture: adequate mixing is necessary to ensure a suspended state of the microalgae cells, gas exchange between the culture and air, and even light access to the microalgae cells. Mixing in ponds is provided by paddle wheels consisting of flat blades. Since power consumption is greatly affected by the intensity of mixing, it is necessary to maintain the minimum turbulence required in terms of the energy efficiency of the system. The paddlewheel is driven by a geared motor. Paddles are fixed to the horizontal axis. The bottom axis has 2 sets of paddles and one motor, while the top motor works for a single paddlewheel. Each set of paddlewheels is run by a motor through a redactor. The paddle length and width are made so that they would fit the pond with minimal gaps at the bottom and sides to ensure optimum mixing. The paddles are made out of an aluminium sheet that is cut to the size and bent to the U shape.

− Light Source: energy efficient LED lights are installed into the pilot allowing supplemental illumination to ensure year-round optimal light conditions. LED light panels are attached in a way to overcome the sharding created by the upper pond. IP65 protection grade 125 W LED grow lights with dimensions 1,500 x 68 x 36 mm and photon flux PAR output 240 - 280 μmol s^{-1} were purchased from Ambra Elettronica (Ambra Light, Italy) emitting blue light at 450 nm, red at 630 and 660 nm, and far-red at 735 nm. Due to the combined (sunlight and artificial) lighting system, it is possible to optimize the diurnal and annual lighting cycle. Although the incorporation of artificial lighting adds to the total capital expenditures and cultivation costs, it may be justified by increased biomass productivity. Moreover, supplemental LED lights are used only when necessary, providing optimal light conditions to

maximize microalgae growth and maintain consistent biomass production. It can be used to mitigate the natural daily and seasonal fluctuations e.g. low light intensity on overcast days, short daylight hours during the winter season or highly dense microalgae cultures.

− Monitoring of key parameters: sensors are installed in the pond to measure crucial parameters which affect the growth of microalgae such as pH, PAR, temperature (outside temperature, air temperature in greenhouse and ponds' temperature), and dissolved oxygen. For the monitoring of physiochemical parameters and measurement data acquisition, Aranet remote data logging system with wireless sensors for temperature, light, pH and DO was installed. Some of the parameters were not supported by the wireless sensor manufacturer, therefore portable multiparameter meter (HI 9829, Hanna instruments) was used for manual measurements of turbidity and ORP. Later additional remote monitoring capabilities were added to remotely control mixing motors and flue gas pump and monitor heating temperatures. Additionally, a web camera was installed to provide remote visual observation possibilities.

− Greenhouse: the ponds with the structure are placed inside a greenhouse made form transparent polycarbonate sheets. The greenhouse is 6 m long, 4 m wide and 2.7 m at the highest point. The roof is round-shaped to withstand snow. The greenhouse has doors at one end and multiple windows for ventilation during warm days. A simulation performed by Pessi et al. showed that temperature control inside the greenhouse by simple air exchange with the outside can be effective enough to greatly improve productivity compared to a greenhouse without temperature management [82].

The greenhouse provides protection from unfavorable weather conditions (wind, rain) and contamination (dust, pollens, microorganisms) and maintains the optimal temperature to ensure year-round production. Greenhouse helps to limit the potential biological contamination such as bacteria, viruses and rotifers often reported to lead to culture collapse [258]. It also serves as a shelter from the environment to protect equipment and reduce the wear of the materials although all the components are chosen to withstand water. However, the greatest benefit of the greenhouse in high-latitude regions remains the possibility to heat or cool down the environment depending on the season. Additional costs in terms of energy can be justified by increased productivity. Light attenuation by greenhouse can be an advantage in high solar irradiation conditions, whereas during winter can limit the available light.

It has been reported that the productivity of certain microalgae can be improved by cultivation under a greenhouse. The productivity of *Spirulina platensis* under the greenhouse was increased by more than 80% during winter and by more than 20 % over the whole year [82].

− Heating is required during winter months and uses waste heat that comes from cooling cogeneration engines in the plant. Heating is realized by using an air blower heat exchanger. Hot water is pumped by use of a circulating pump to the greenhouse. Then it goes through a heat exchanger equipped with an air blower.

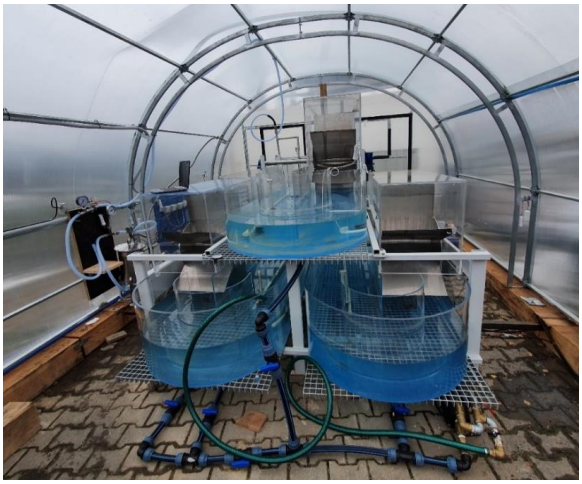


Figure 3.4. Construction of SMORP pilot and integration into the Iecava biogas plant.

The construction of the SMORP pilot took place during the winter season of 2020/2021. Then the system was tested and adjusted. The final set-up of SMORP pilot ponds and the greenhouse integrated into Iecava Biogas plant is shown in Figure 3.4.

3.2. Microalgae strain selection

Microalgal species were selected based on considerations described in the Literature review chapter Microalgae strain selection. After an extensive literature review, three microalgae were selected as candidate strains for mass biomass production at Latvian climate conditions: *Chlorella vulgaris* 211-11j, *Chlorella sorokiniana* 211-8k and *Chlamydomonas reinhardtii* 11-32b.

Chlorella species are found to be between predominant strains occurring naturally in wastewater ponds [259], [260] and can survive in various wastewater streams showing great potential to adapt to various environmental conditions [206], [237], [259], [261]. Recent studies show the suitability of various *Chlorella* species for wastewater treatment. Oberholster demonstrated that a combination of *C. vulgaris* and *C. protothecoides* is effective in nutrient removal from wastewater stabilization ponds (75 % total phosphorus and 43% total nitrogen removal). In the same study *Chlorella* spp. stayed dominant after inoculation of ponds, moreover, other microalgae species coexisted with *Chlorella* spp. in treatment ponds [262].

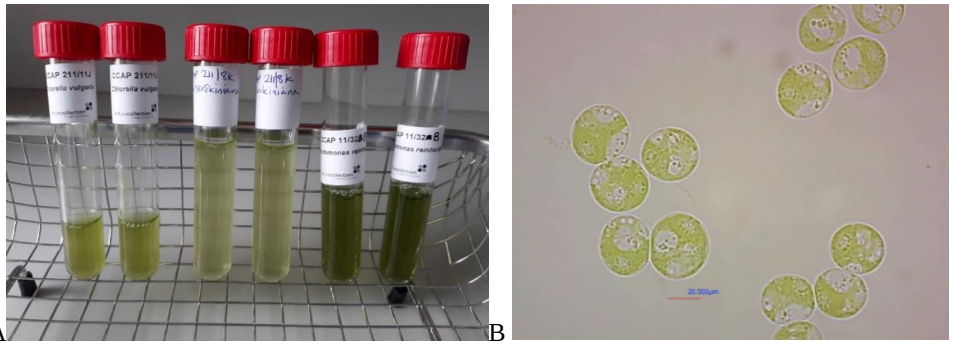
It has been demonstrated that *Chlorella* species are capable of growing in autotrophic, heterotrophic and mixotrophic conditions performing photosynthesis as well as ingesting organic materials such as glucose [86], [263]. When cultivated in wastewater, *Chlorella* is able to switch from phototrophic to heterotrophic or mixotrophic growth showing universal and flexible properties.

Exploring waste stabilization ponds Palmer found that the most abundant and frequent genera were *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium* and *Golenkinia* [264]. Furthermore, Palmer published another study with the

results of extensive research covering 165 studies and reported that *Chlorella* is between the top eight pollutant-tolerant genera [260]. Moreover, in another study, screening the top 17 strains with the best performance in wastewaters collected locally from natural freshwater habitats and wastewaters, it was found that 60% belongs to *Chlorella* spp. [259] demonstrating the superiority of *Chlorella* over other microalgae and indicating its potential for wastewater treatment.

Chlorella spp. have shown superior resistance to high ammonium concentrations compared to other species [265]. *Chlorella* spp. have been used in numerous studies and have shown good nitrogen and phosphorus removal rates. Generally, *Chlorella* spp. can remove 23 – 100% nitrogen, while phosphorus removal efficiency is 20-100% [230].

Two species were selected from the genus *Chlorella* as potential microalgae for mass culturing using digestate as a nutrient source. *Chlorella vulgaris* is a single-cell green microalga belonging to the division *Chlorophyta*. Freshwater microalga *C. vulgaris* was selected after an extensive literature review as one of the most promising species for large-scale outdoor cultivation due to its flexibility in cultivation conditions, capability to absorb high CO_2 concentrations and high specific-growth rate. *C. vulgaris* strain 211-11j was selected due to its northern origin in Sweden with high potential for cultivation at high latitude regions. Very few scientific reports could be found on this strain of *C. vulgaris*, therefore it was necessary to evaluate the optimal cultivation conditions of this species including both optimum growth temperature and minimum and maximum temperature resistance in order to assess its potential for cultivation in Latvia.



A B

Figure 3.5. Microalgae cultures from CCAP culture collection (A), *C. reinhardtii* in light microscope (B).

Chlorella sorokiniana is a green microalga from the genus *Chlorella* which has shown outstanding performance in wastewater treatment [79]. Moreover, it has demonstrated better adaptability to physiological stresses than some other green microalgae species [266]. Its usefulness can be particularly appreciated during high-temperature conditions that can be experienced during the summer time as *C. sorokiniana* has been shown to be resistant to temperatures up to 42 °C [267]. *C. sorokiniana* was selected for this study due to its resistance to high cultivation temperatures and high irradiation commonly experienced during cultivation in summer.

Chlamydomonas reinhardtii is a photosynthetic biflagellate microalga that has been studied as a model for basic and applied physiology and biochemistry for more than 30 years and is one of the most studied microalgae [80]. It is one of the most characterized algal species. Moreover, *C. reinhardtii* was the first green microalga to be sequenced [80] giving the opportunity to use it for genetic manipulations [268]. *Chlamydomonas* species have also been commonly found in wastewaters [264] indicating their suitability to resist harsh conditions and ability to utilize nutrients from wastewaters.

Microalgae strains *Chlorella vulgaris* 211-11j, *Chlorella sorokiniana* 211-8k and *Chlamydomonas reinhardtii* 11-32b were obtained from reference culture collections in CCAP and SAG (Figure 3.5. A), examined under the microscope (Figure 3.5. B) and used in laboratory tests. Various aspects of cultivation were evaluated during laboratory tests including the effect of temperature, light, CO_2, and ability to grow and remove nutrients from liquid agricultural digestate.

3.3. Evaluation of a low-temperature strain

C. vulgaris 211-11j was selected as a potential species for cultivation in low temperatures. The strain was grown in batch cultures at temperatures ranging from 8 to 32 °C to evaluate the optimal temperature range as well as both minimum and maximum temperature tolerance. Cultures exhibited good growth at all temperatures tested except at 32 °C (Figure 3.6.). The growth of microalgae was very limited at 32 °C (μ 0.024 d^{-1}) with very little cell division occurring (Table 3.1.). The highest specific growth rate of 0.224 d^{-1} was observed at 20 and 24 °C. Microalgal cell density increased with increasing the cultivation temperature from 8 to 20 °C. Growth started to decrease at temperatures exceeding 24 °C. The concentration of cells at 20 and 24 °C was comparable at the end of the 10-day cultivation, 1.501 x 10^7 and 1.495 x 10^7 cells mL^{-1}, respectively. Moreover, the t-test revealed no significant differences between these cultivation temperatures at the 95% confidence level (p = 0.878). Furthermore, the growth of microalgae was slow at 8 °C with the specific growth rate of 0.157 d^{-1}.

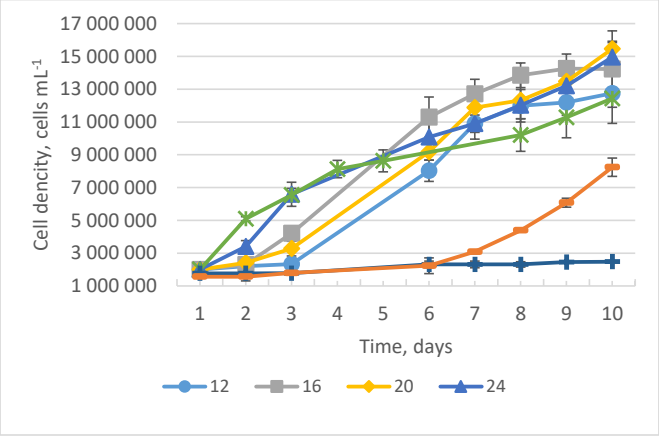


Figure. 3.6. *C. vulgaris* culture cell density and growth pattern at various temperatures. Error bars indicate standard deviation (n=3).

Although the highest microalgal cell number was observed at 20 and 24 °C, the highest biomass accumulation (dry weight, g L^{-1}) was achieved when cultures were grown at 28 °C, 0.228 g L^{-1} (Figure 3.7.). Whereas the dry weight of microalgae at 20 and 24 °C was 0.208 and 0.210 g L^{-1}, respectively. Microalgae grown at 8 °C and 32 °C had comparable dry weight, 0.130 and 0.136 g L^{-1}, respectively, whereas cell density was much higher for cultures under 8 °C, 8.24 x 10^6. In contrast, the number of cells in the 32 °C cultures was 2.48 x 10^6 (Table 3.1.).

Table 3.1.

Effect of cultivation temperature on *C. vulgaris* growth kinetics and productivity. The standard deviation of three replicates is shown in brackets where applicable.

Cultivation t, °C	Cell density, cells mL^{-1} (± SD)	μ, day^{-1}	Dry weight, g L^{-1} (± SD)	Productivity, g L^{-1} d^{-1}	Cell weight, $x10^{-11}$ g
8	8.24 x 10^6 (± 5.59 x 10^5)	0.157	0.130 (±0.003)	0.014	1.58
12	1.28 x 10^7 (± 1.84 x 10^6)	0.206	0.184 (±0.004)	0.020	1.43
16	1.42 x 10^7 (± 5.57 x 10^5)	0.218	0.194 (±0.006)	0.022	1.37
20	1.50 x 10^7 (±1.09 x 10^6)	0.224	0.208 (±0.006)	0.023	1.38
24	1.50 x 10^7 (±9.67 x 10^5)	0.224	0.210 (±0.001)	0.023	1.41
28	1.2 x 10^7 (±5.41 x 10^5)	0.203	0.228 (±0.009)	0.025	1.83
32	2.48 x 10^6 (±2.36 x 10^4)	0.024	0.136 (±0.002)	0.015	5.47

Cultures grown at 28 °C had the highest biomass productivity per day (g L^{-1} d^{-1}) of 0.025 (Table 3.1.). Biomass productivity at 24 and 20 °C was 92.5 and 91.1% of the maximum productivity observed at 28 °C (Figure 3.7.). However, the productivity of cultures cultivated at 12 and 16 °C reached 80.7 and 85.4% of the maximum productivity, respectively. Relatively low accumulated biomass was observed at 8 °C, reaching just 57% of the maximum productivity.

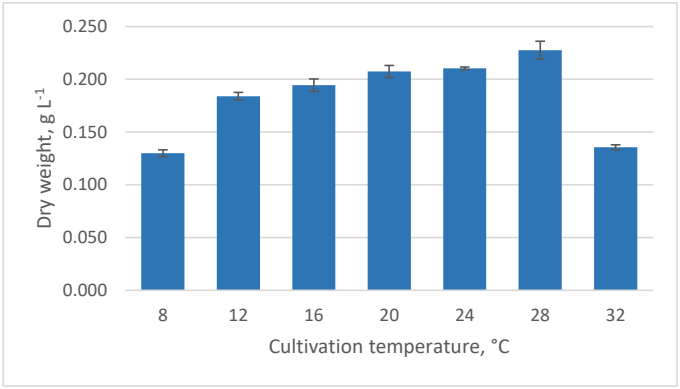


Figure 3.7. Biomass yield of *C. vulgaris* at different cultivation temperatures at the end of the cultivation. Error bars indicate standard deviation (n=3).

The fastest initial growth was observed at 28 and 24 °C, reaching the highest growth rate on day 2 and day 3, respectively, followed by cultures at 16 °C (Figure 3.8). The slowest initial growth was noted at 8 °C. This culture did not reach the stationary growth phase after the 10-day cultivation, and the growth rate continued to increase. Due to the longer acclimation phase at the beginning of cultivation, cultures at low temperatures require cultivation longer than 10 days to reach the stationary phase and maximum productivity.

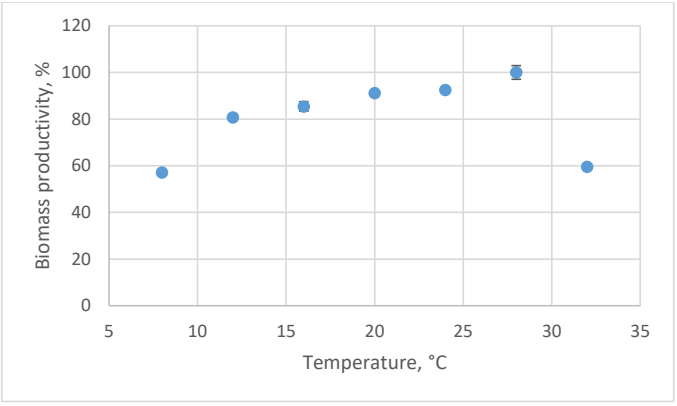


Figure 3.8. *C. vulgaris* biomass productivity at different cultivation temperatures expressed in the percentage of maximum productivity. Error bars indicate standard deviation (n=3).

Although cell density was higher at 20 and 24 °C, higher biomass productivity was observed in cultures cultivated at 28 °C that might be attributed to the smaller size of the cells at 20 and 24 °C. Indeed, the calculation of cell weight of dry biomass showed that cell weight was higher at 28 °C than at 20 or 24 °C. The highest cell weight was of microalgae cultivated at 32 °C whereas the lowest was observed at 16, 20 and 24 °C, indicating that cells of *C. vulgaris* 211-11j were larger at high temperatures compared to average cultivation temperatures. An increase in cell weight was observed again in lower temperatures (12 and 8 °C).

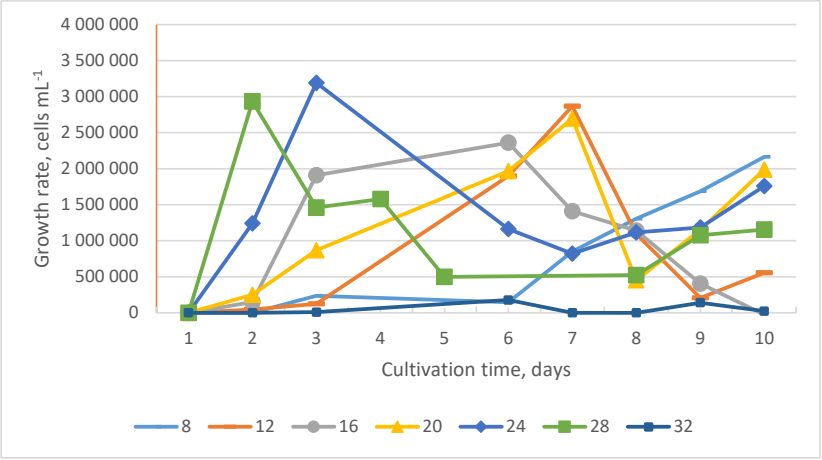


Figure 3.9. Growth rate of *C. vulgaris* per day at different cultivation temperatures.

The maximum biomass yield of *C. vulgaris* 211/11j was observed at 28 °C, therefore this temperature is suggested as optimal for cultivation for this strain at the given experimental setup. Furthermore, temperatures from 20 to 28 °C can be considered the optimal range for the cultivation of this strain as no significant difference in productivity was observed. The data reported in the literature on the optimal cultivation temperature of green microalga *Chlorella vulgaris* vary widely, generally ranging from 25 to 32 °C [137], [269]–[272]. The findings of this study are consistent with those of a study by Serra-Maia et al. [272], who observed the highest growth rate (number of cells) at 25 °C when cells were grown at 20, 25, 28 and 30 °C. Similar to this study, they reported no significant differences between the growth rates at 24 and 28 °C. Moreover, the growth rate at 25 °C only slightly increased when compared to that at 20 °C. Similarly, the authors observed a decrease in the growth rate from 25 to 28 °C. However, Barghbani et al. [269] reported 30 ±2 °C as the optimum temperature when testing *C. vulgaris* growth at 20, 25, 30 and 35 °C. The observed differences are most likely due to the different strain of *C. vulgaris* used in their study. Microalgae were obtained from the Shahriar River near the city of Tehran (Iran); therefore this strain could be more adjusted to hot climates than the strain used in the present study. Some other studies have reported higher optimum temperatures than those observed in the present study. For example, also Chinnasamy [273] reported optimal growth at 30 °C at elevated CO_2 level (6%); however, the *C. vulgaris* stain used was ARC1 originally isolated from the oxidation pond system at Delhi (India) most likely being responsible for the higher optimum growth temperature observed. *C. vulgaris* strain 211-11j is rarely studied; only one report was found analyzing the optimal growth conditions [274] and is discussed below.

We observed that the *C. vulgaris* 211-11j growth by means of cell density was higher below the optimum rather than above the optimum temperature. An increase in temperature of just four degrees above 28 °C resulted in a more than eightfold decrease in the growth rate. At the same time, the growth rate below the optimum decreased gradually. The characteristic of algae

that the lethal temperature is only a few degrees above the optimum temperature is well known [73] and has been reported often [125], [270], [275].

It seems that temperature has a strong effect on the cell weight of this microalgae strain. Generally, not all microalgae show a positive correlation between cell size and temperature. In this study, the maximum biomass yield observed at 28 °C was due to an increased size of the cells, rather than the number of cells. This finding suggests that *C. vulgaris* cells tend to grow larger in size at high temperatures but are not actively dividing. The largest cells were observed at high cultivation temperatures (28 and 32 °C) with a maximum weight at 32 °C. Our finding is in agreement with the study by Dai et al. [276] who reported that the cell size of *Chlorella pyrenoidosa* was increased under high culture temperature. On the other hand, other studies have reported that cell size decreased at high temperatures in various microalgae [115], [116]. These reports confirm that the response of cell weight to temperature is species-specific.

While there are many studies assessing the optimum and maximum growth temperature for *C. vulgaris*, only a few studies considering low temperatures can be found. In the present study, the growth of *C. vulgaris* at low temperatures (16, 12 and 8 °C) was studied. While the growth rate decreased by nearly 43% at 8 °C, compared to the maximum productivity at 28 °C, productivity was still near 85% and 81% of the maximum at 16 °C and 12 °C, respectively, showing the good ability of this strain to grow in moderate temperatures and substantial resistance to low temperature. Although microalgae cultures cultivated at 8 °C did not result in high biomass yield at the end of the cultivation, cells were actively dividing resulting in increasing culture density after the long adaptation phase of 6 days. At the end of the batch cultivation, cultures at 8 °C were still increasing their density, therefore a longer cultivation time is needed to fully assess the potential of this strain at very low temperatures. Nevertheless, these results are very promising, showing that cultures cultivated at 8 °C can reach a good growth after the low-temperature acclimation.

Moreover, this strain exhibits lower optimum cultivation temperature than some other *C. vulgaris* strains showing an advantage over other strains for outdoor cultivation in cooler climates and therefore could be selected as a candidate stain for biomass production in Latvia. The results of the present study are consistent with the findings by Maxwell, who reported that this strain of *C. vulgaris* was able to grow at 5 °C [274]. The authors demonstrated that *C. vulgaris* is capable of acclimation to low temperatures by adjusting the photosynthesis apparatus and exhibits a similar pattern to high light acclimation. In another study, it was demonstrated that this strain of *C. vulgaris* could be successfully used for lutein production at low temperatures [277] suggesting the potential application of harvested biomass supporting the biorefinery concept.

This study suggests that *C. vulgaris* strain 211/11j has a great advantage in colder climates. The optimal temperature range for biomass production was 20 to 28 °C, with maximum biomass productivity reached at 28 °C. It was demonstrated that *C. vulgaris* strain 211/11j has a wide optimum temperature range that is also lower than that of other *C. vulgaris* strains reported in the literature suggesting that this strain prefers cooler environment. It was demonstrated that *C. vulgaris* 211-11j can grow effectively in moderate temperatures and exhibits good resistance to low temperatures. Tolerance to low temperatures makes *C. vulgaris* 211-11j a potential

candidate for the production of biomass under cooler weather conditions. Moreover, a wide optimum temperature range is suitable for highly variable outdoor conditions often experienced in higher latitude regions where fluctuations in diurnal temperatures even during summer may be high.

3.4. Effect of light intensity on microalgae growth

To find optimal illumination conditions of three candidate microalgae strains, namely *C. vulgaris* 211-11j, *C. sorokiniana* 211-8k and *C. reinhardtii* 11-32b, growth rate and biomass production were evaluated at five different light intensities: 30, 50, 100, 150, 200 μmol m^{-2} s^{-1}. When *Chlorella* species were cultivated in BG-11 medium that is commonly used for *Chlorella* spp. cultivation, species exhibited low growth rate compared to *C. reinhardtii* and a linear growth curve was observed for all light intensities tested. Consequently, cultures did not reach a stationary phase during the 10-day batch cultivation (Figure 3.10. A and B).

On the other hand, *C. reinhradtii* cultivated in TAP medium showed a high growth rate and reached maximum culture density on day 4 to day 5 of the cultivation (Figure 3.10. C). The uncommon growth curve of *Chlorella* spp. suggested that cultures are not under optimal growth conditions and that some limiting factors exist inhibiting the growth. Furthermore, daily pH measurements revealed a high pH of *Chlorella* spp. cultivated in BG-11 media reaching pH 11 at the end of the cultivation (Figure 3.11.). However, the pH of *C. reinhardtii* cultures did not exceed pH 8.48.

The slow growth rate of *Chlorella* species observed suggested that cultivation conditions must be improved therefore, a second round of experiments was carried out. pH during the first cultivation exceeded the optimum pH range of both *C. vulgaris* and *C. sorokiniana. C. vulgaris* has a wide optimum pH range of approx. 7 to 10, whereas the optimum for *C. sorokiniana* is approx. pH 6 to 7.5 [278]. Therefore, the actual pH during the cultivation was significantly higher than the optimum which might have affected the growth rate.

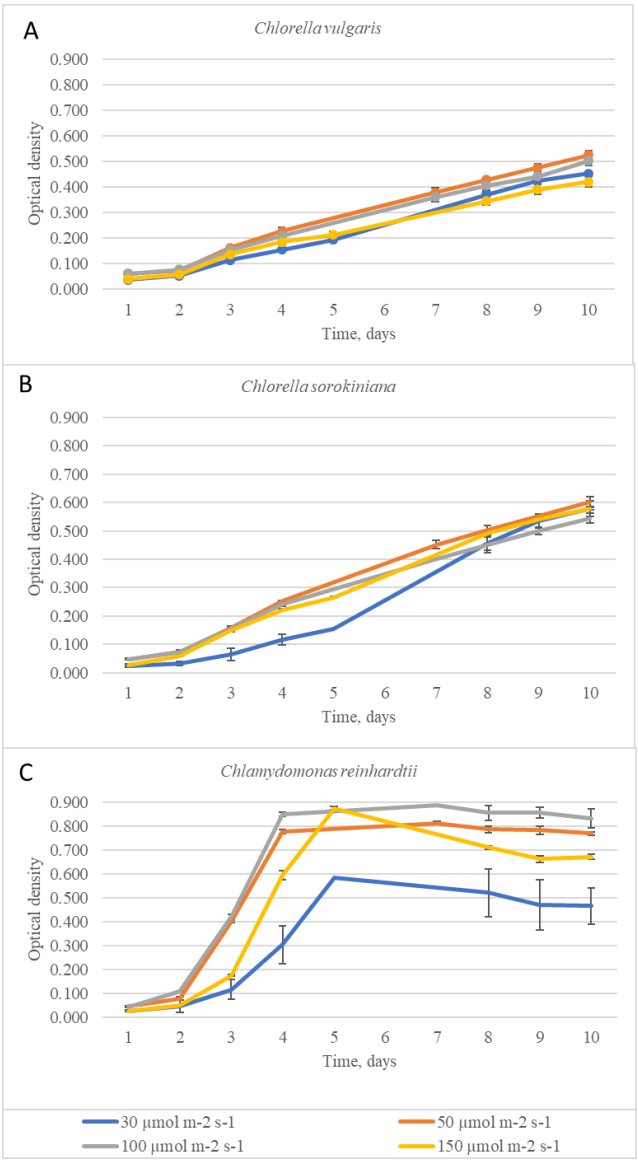


Figure 3.10. Growth curves of *C. vulgaris* (A), *C. sorokiniana* (B) and *C. reinhardtii* (C) at 30, 50, 100 and 150 µmol m^{-2} s^{-1} cultivated in BG-11 medium (*Chlorella* spp.) and TAP (*C. reinhardtii*). Each dot represents the average of three replicates, error bars indicate standard deviation.

During the subsequent round of experiments, all species including *Chlorella* spp. were cultivated in a TAP medium. Furthermore, the first set of experiments showed that irradiation of 30 µmol m^{-2} s^{-1} was too low for high biomass production for all species therefore it was excluded from the second set of experiments. Moreover, maximum irradiation was extended to 200 µmol m^{-2} s^{-1}. Microalgae were cultivated at 50, 100, 150 and 200 µmol m^{-2} s^{-1}. Other

cultivation parameters were not changed. The growth curves of three microalgae during the second set of experiments are shown in Figure 3.12. *C. vulgaris* and *C. sorokiniana* exhibited much higher growth rate in TAP compared to BG-11 medium suggesting that TAP is a more suitable medium for fast biomass accumulation of *Chlorella* species.

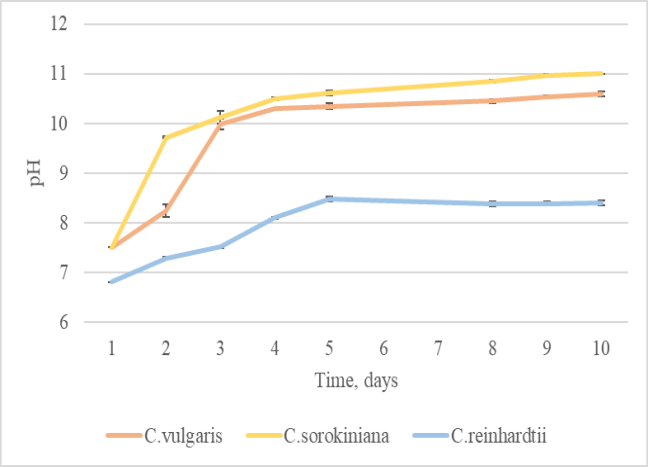


Figure 3.11. pH range during the cultivation of *C. vulgaris* (BG-11), *C. sorokiniana* (BG-11) and *C. reinhardtii* (TAP) under illumination of 150 µmol m^{-2} s^{-1}.

All strains exhibited an exponential growth starting from the second day (Figure 3.12.). The lower growth rate at the start of cultivation could be explained by the adaptation of cultures to the new growing conditions. *C. vulgaris* reached maximum culture density at 100 µmol m^{-2} s^{-1} light intensity (OD 2.3). *C. sorokiniana* exhibited comparable growth rate at 100, 150 and 200 µmol m^{-2} s^{-1} of OD 2.3, 2.4 and 2.4, respectively. Whereas growth was slightly lower at light intensity of 50 µmol m^{-2} s^{-1} (OD 2.1). *C. reinhardtii* exhibited lower biomass density at OD 750 nm than other two species in all light intensities showing the highest density of OD 1.28 at 200 µmol m^{-2} s^{-1}. The lowest growth rate was detected at 50 µmol m^{-2} s^{-1} for *C. sorokiniana* and *C. reinhardtii* and at 150 and 200 µmol m^{-2} s^{-1} for *C. vulgaris.* After days 5 to 6 growth rate started to slow down supposedly due to scarcity of nutrients. Growth of *C. sorokiniana* started to decrease after day 4 but the growth curve did not show a flat stationary phase and was still growing slowly suggesting that also other factors than lack of nutrients could have affected the growth kinetics. The increased culture density at the end of the cultivation period could have limited the light availability to cells due to the cell shading effect leading to the light limitation conditions.

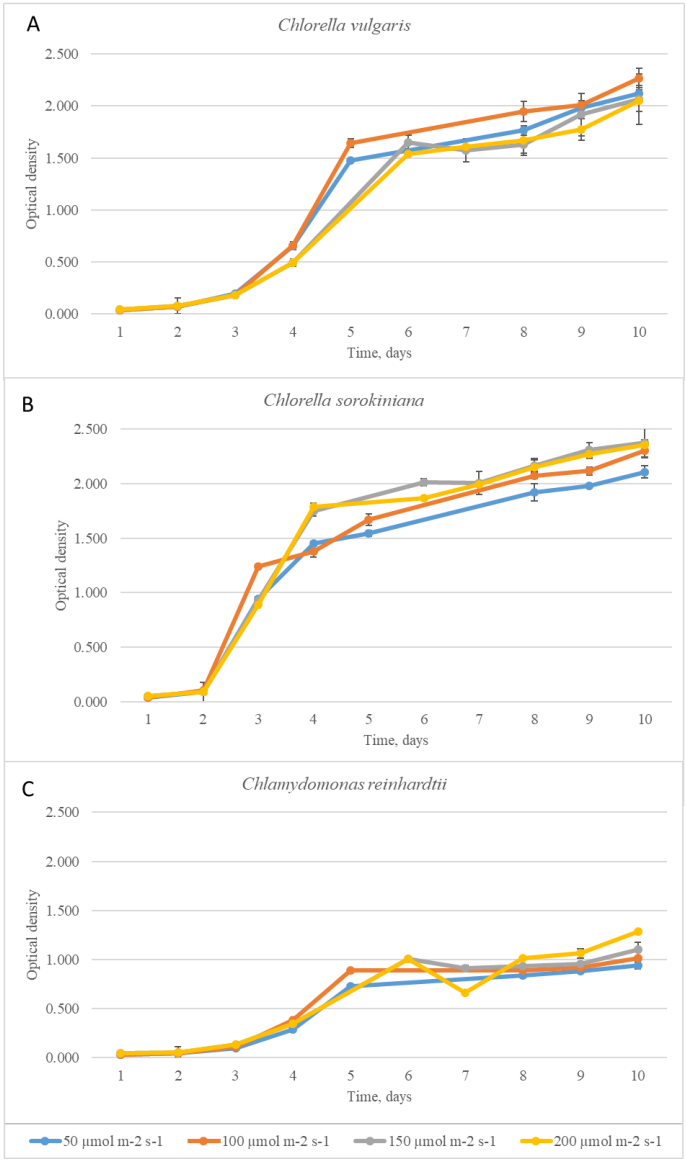


Figure 3.12. Growth curves of *C. vulgaris* (A), *C. sorokiniana* (B) and *C. reinhardtii* (C) at 50, 100, 150 and 200 µmol m^{-2} s^{-1} cultivated in TAP medium. Each dot represents the average of three replicates, error bars indicate standard deviation.

Maximum biomass yield (g L^{-1}) was calculated from the dry weight of microalgae cultures at the end of the 10-day batch cultivation (Figure 3.13.). Biomass yield was comparable among all the microalgae strains studied. Although *C. reinhardtii* exhibited significantly lower optical density at 750 nm compared to other microalgae strains, biomass production was comparable to other strains. Biomass increased with the increasing light intensity up to 150 µmol m^{-2} s^{-1} for *C. vulgaris* and *C. reinhardtii* and up to 200 µmol m^{-2} s^{-1} for *C. sorokiniana*.

The highest biomass yield for *C. sorokiniana* was recorded at a light intensity of 200 µmol m^{-2} s^{-1} (1.13 g L^{-1}). Whereas *C. vulgaris* and *C. reinhardtii* produced the most biomass when cultivated at 150 µmol m^{-2} s^{-1}, 1.05 and 1.06 g L^{-1}, respectively. The lowest biomass yield was recorded at light intensity of 50 µmol m^{-2} s^{-1} for all three microalgae strains studied, 0.75 g L^{-1} for *C. reinhardtii* and *C. sorokiniana* and 0.82 g L^{-1} for *C. vulgaris*.

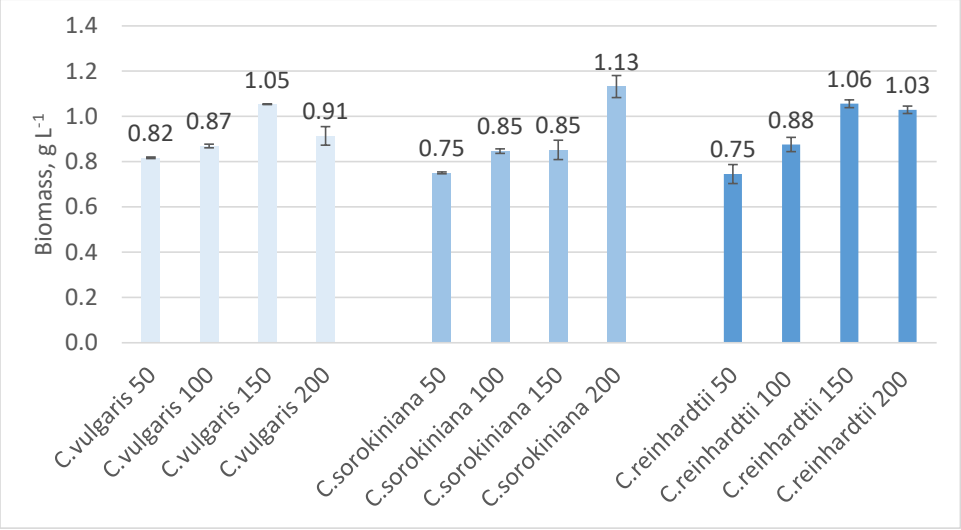


Figure 3.13. Maximum biomass yield of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* under various light intensities 50 – 200 µmol m^{-2} s^{-1}. The number next to the species name indicates light intensity (µmol m^{-2} s^{-1}).

Results of the biomass production suggest that optimal light intensity for *C. vulgaris* and *C. reinhardtii* is around 150 µmol m^{-2} s^{-1} while higher light intensity of approximately 200 µmol m^{-2} s^{-1} is more suitable for *C. sorokiniana*. These results confirm other reports as *C. sorokiniana* is known to be a high light intensity tolerant alga [279]; consequently, optimal light intensity requirements are higher than those of other microalgae. *C. vulgaris* biomass decreased at 200 µmol m^{-2} s^{-1} showing that this light intensity might be too high, and the photo-inhibition process might have been initiated during the cultivation at 200 µmol m^{-2} s^{-1}.

The specific growth rate (µ, d^{-1}) was calculated for each species at the specific light intensity (Figure 3.14.). All species exhibited comparable growth rates varying from 0.467 to 0.552 d^{-1}. The highest growth rate was observed for *C. vulgaris* at light intensity of 100 µmol m^{-2} s^{-1} (0.552 d^{-1}), followed by *C. vulgaris* at 50 and *C. sorokiniana* at 200 µmol m^{-2} s^{-1}, 0.540 and 0.535 d^{-1}, respectively.

Optimal light intensity for *C. vulgaris* reported in the literature varies widely from 62.5 µmol m^{-2} s^{-1} [280] and 80 µmol m^{-2} s^{-1} [281] to 2000 µmol m^{-2} s^{-1} [156]. However, most often light intensity around 200 µmol m^{-2} s^{-1} is proposed [137], [282]. *C. vulgaris* strain used in the present study exhibits lower light intensity requirements that could be attributed to its Nordic origin and might be well adjusted to lower light intensity conditions as experienced at high latitudes.

On the other hand, Yan et al. observed that much higher light intensities are needed in synthetic wastewater [156]. Irradiation with 2000 µmol m^{-2} s^{-1} was found to yield the maximum biomass of *C. vulgaris*. Moreover, a novel strategy for improving light availability in high-density cultures suggesting that higher light intensity must be provided to microalgae cultures as the culture density is increasing during the cultivation was also suggested. The authors recommended using 800 µmol m^{-2} s^{-1} at the beginning of cultivation and increasing the light intensity to 1600 µmol m^{-2} s^{-1} at the final stage of cultivation.

Reported variance in optimal light intensity stresses the importance of the impact of other cultivation parameters on the light requirements. Therefore, optimal light intensity must be determined taking into account the specific cultivation conditions, especially day/night length, light spectrum and temperature that are known to be interdependent.

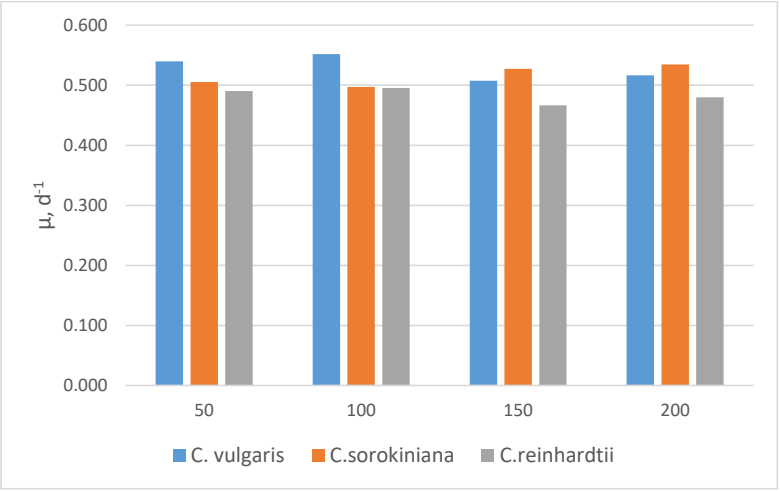


Figure 3.14. Specific growth rate (µ, d^{-1}) of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* under various light intensities.

pH was measured daily during the cultivation period. pH reached very high values during the first set of experiments in BG-11 medium exceeding the optimum range of *C. vulgaris* and *C. sorokiniana* thus could have contributed to the slow growth observed in BG-11 medium. During the second set of experiments, when all microalgae strains were cultivated in a TAP medium, pH did not increase more than 8.4, not exceeding the optimum pH range of species (Figure 3.15.). pH showed a steep rise during the first days of cultivation, rising from 7 to about 8.4. The pH can be seen as an indicator of culture condition, a rise in pH indicates a growth of microalgae cultures due to the uptake of carbon by cells during the day [226]. pH of cultures levelled out from day 4 to 6 and stayed constant during the second part of the cultivation period.

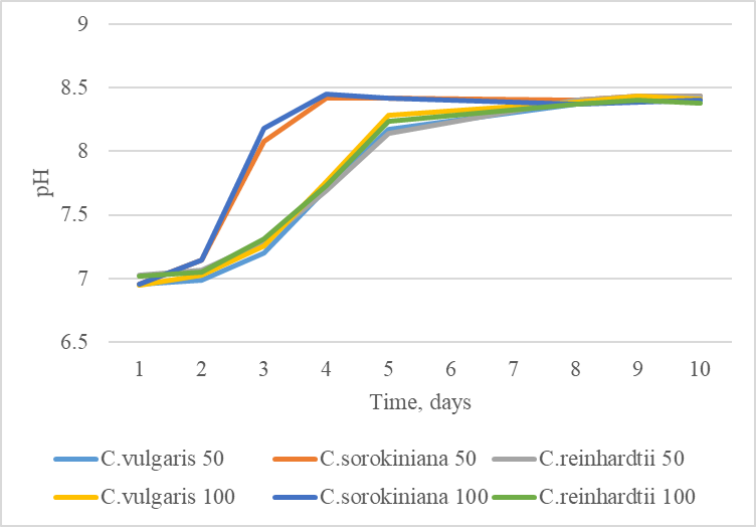


Figure 3.15. pH of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* during the cultivation under 50 and 100 µmol m^{-2} s^{-1} light intensity. The number next to the species name indicates light intensity (µmol m^{-2} s^{-1}).

The current study was carried out to investigate the effect of light intensity on the growth rate and biomass production of three microalgae strains intended for cultivation in outdoor raceway ponds supplemented with artificial LED illumination. The study has shown the impact of light intensity on the growth of microalgae having a major role in biomass production. All species tested exhibited similar growth rate and biomass productivity under selected light intensities and specific cultivation conditions. It was shown that light intensity of 30 and 50 µmol m^{-2} s^{-1} is too low to maintain the maximum growth rate for microalgae strains studied. Nevertheless, *C. vulgaris* was superior to other strains at low light conditions (50 µmol m^{-2} s^{-1}), exhibiting a potential for cultivation at limited light settings which may be particularly useful in Nordic countries. On the other hand, the results suggest that *C. sorokiniana* has higher light requirements when compared to *C. vulgaris* and *C. reinhardtii*, which offers advantages at high light conditions, e.g. at mid-summer in high latitude regions. The highest biomass yield was produced at a light intensity of 150 µmol m^{-2} s^{-1} for *C. vulgaris* and *C. reinhardtii* and at 200 µmol m^{-2} s^{-1} for *C. sorokiniana*.

Moreover, the choice of growth medium was shown to have a large effect on microalgae growth. Cultivation in the TAP medium resulted in a higher growth rate of *C. vulgaris* and *C. sorokiniana* compared to the BG-11 medium.

3.5. Effect of light photoperiod

The impact of daylight length (16 h and 24 h) on biomass yield for three potential microalgae strains was tested at four different light intensities (50, 100, 150 and 200 µmol m^{-2} s^{-1}). The obtained results show that continuous illumination with 24-hour daylight resulted in an increased growth rate of all microalgal strains tested (Figure 3.16.). The highest increase in

biomass yield was observed for all strains cultivated at low light intensities, e.g. 50 and 100 µmol photons m^{-2} s^{-1}. This could be explained by the total amount of light received by microalgae cells over the 24-hour period. Microalgae cultivated at 50 and 100 photons m^{-2} s^{-1} were light-limited therefore an increase in daylight length leads to enhanced biomass productivity. At more optimal light conditions when the light intensity is close to or at the saturation limit (150 and 200 µmol photons m^{-2} s^{-1} in the case of *C. vulgaris* and *C. reinhardtii*), the effect of increased daylight length resulted in a smaller increase in biomass production. It is not completely clear why a decline in *C. sorokiniana* biomass was observed at 200 µmol photons m^{-2} s^{-1} when continuous illumination was provided. Being a high-light-resistant species, it has a better tolerance to high light conditions but in the present study, it exhibited symptoms of photo-inhibition. These conditions should be re-tested to draw any conclusions.

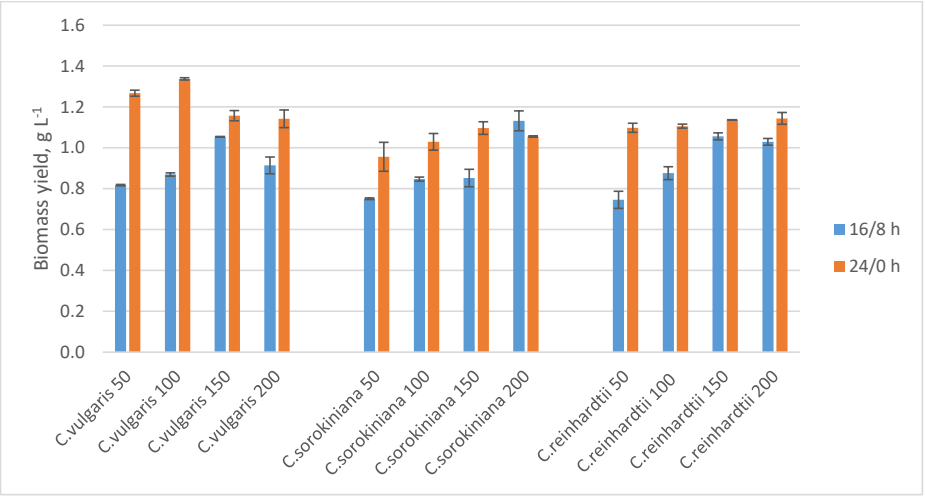


Figure 3.16. Effect of illumination duration on biomass productivity of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* at various illumination intensities of 50, 100, 150 and 200 µmol m^{-2} s^{-1}.

Appropriate light and dark periods are essential for maximum growth and biomass production. Longer photoperiods have been commonly associated with higher growth rates in microalgae. Whereas continuous illumination is reported as controversial. Continuous illumination is frequently used to maximize biomass production; however, excessive light can induce photoinhibition leading to cell damage and even growth inhibition. Moreover, light intensity and photoperiod are inversely correlated. Therefore, when light intensity is increased, the length of photoperiod should be decreased accordingly. Indeed, Atta et al. 2013 reported that the optimum photoperiod for *C. vulgaris* under 100 µmol m^{-2} s^{-1} blue light was 24:0 h (light:dark) while an increase in light intensity up to 200 µmol m^{-2} s^{-1} reduced the optimum photoperiod to 12:12 h (light:dark). The duration of photoperiod influences the overall amount of light that microalgae culture receives in a 24-hour period. Therefore, light intensity and photoperiod should be matched accordingly to reach maximum biomass productivity.

3.6. Effect of light spectrum

Red and blue spectral ranges of visible light have been frequently reported to enhance biomass production of green microalgae compared to white light. Therefore, red + blue wavelengths were used for microalgae cultivation and compared to full spectrum white light for the selected candidate strains. Daily optical density measurements were performed to inspect the growth of the cultures at various light intensities under blue+red and full spectrum LED light. All three green microalgae species, *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* exhibited similar growth trends at the same growth conditions. No significant differences in culture density between blue+red LED illumination and full spectrum light were observed for *C. vulgaris* (Figure 3.17.), as well as for *C. sorokiniana* (Figure 3.18.) and *C. reinhardtii* (Figure 3.19.).

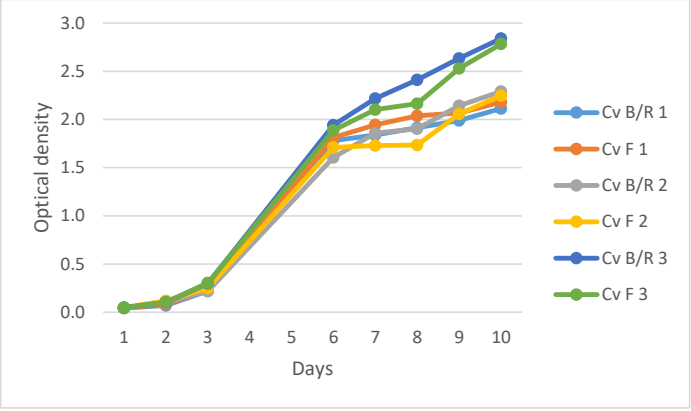


Figure 3.17. The optical density of *C. vulgaris* cultures cultivated at blue+red (B/R) and full (F) spectrum light and 3 levels of light intensity.

The culture density was affected more by light intensity than by light spectrum, with all species reaching higher density at the highest light intensity. Both *Chlorella* species showed comparable culture density, however, *Chlamydomonas* reached a lower density based on the optical density measurements at 750 nm. Although frequently reported as optimal for growth the mix of red and blue wavelengths did not result in higher growth rates of selected microalgae in this study.

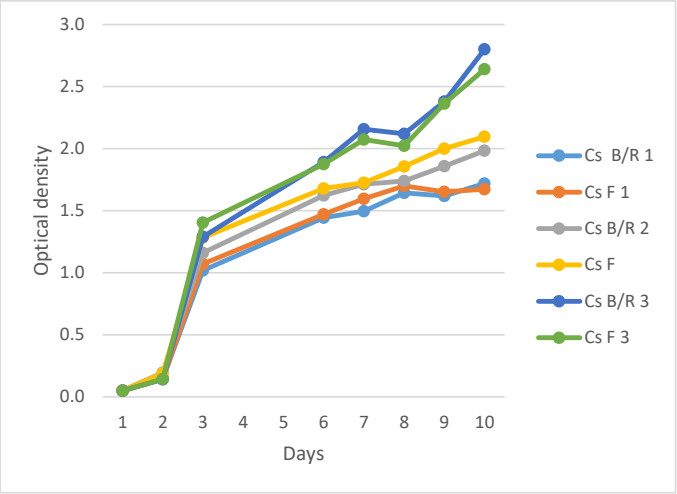


Figure 3.18. The optical density of *C. sorokiniana* cultures cultivated at blue/red (B/R) and full (F) spectrum light and 3 levels of light intensity.

Regarding biomass production, no significant differences were found between cultivation at blue+red spectrum or full spectrum LED lights, however light intensity had a great impact on total biomass yield (Figure 3.20.). Maximum biomass yield was observed at the highest light intensity for microalgae species tested.

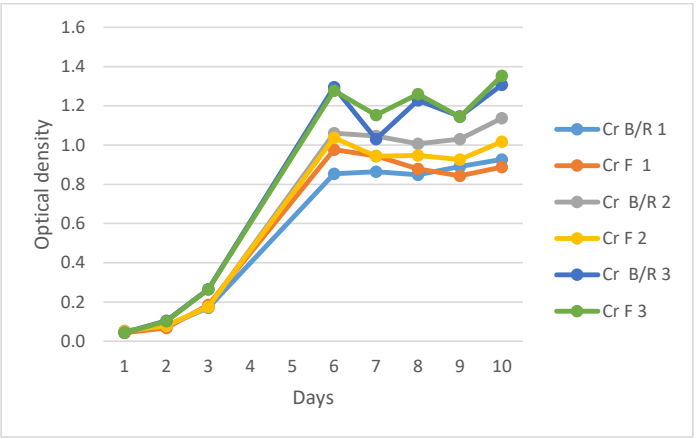


Figure 3.19. The optical density of *C. reinhardtii* cultures cultivated at blue/red (B/R) and full (F) spectrum light and 3 levels of light intensity.

The current study revealed that the growth rate and biomass production of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* were not influenced significantly by part of the solar spectrum applied but more by the light intensity. Both, the combination of blue and red lights and the full spectrum white light, resulted in a high growth rate and productivity of all microalgae tested.

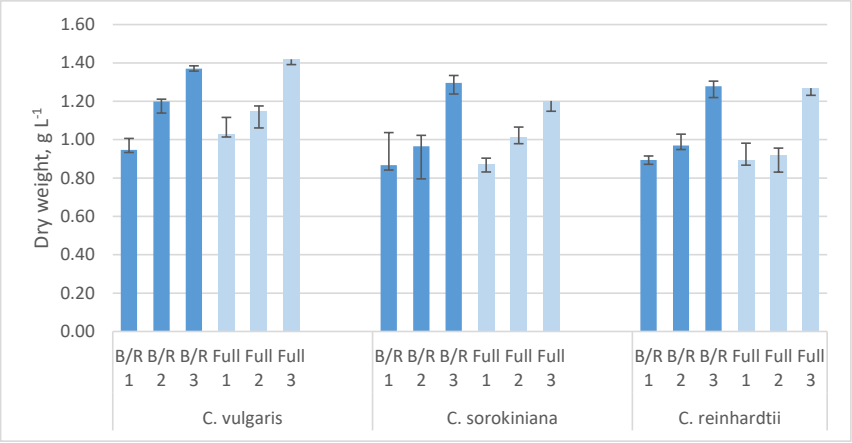
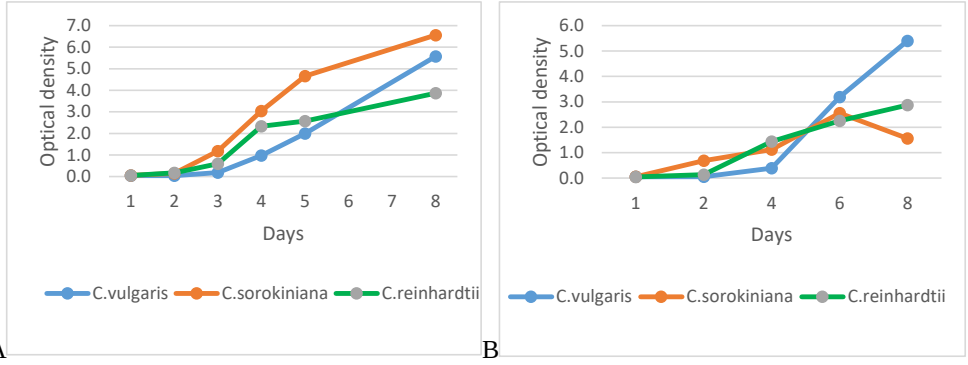


Figure 3.20. Maximum biomass production of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* at blue/red (B/R) and full spectrum (Full) at various light intensities. Error bars indicate standard deviation (n=3).

3.7. Effect of carbon dioxide

Increased CO_2 levels compared to CO_2 content in the atmosphere have been reported to increase the growth rate and productivity of microalgae. To test the maximum CO_2 tolerance of selected microalgae, growth tests with different CO_2 concentrations were performed in the laboratory. Growth curves of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* at 5% and 10% CO_2 are shown in Figure 3.21. A and 3.21. B, respectively. All microalgae species exhibited slower growth at the beginning of cultivation at 10% CO_2 compared to 5% CO_2 mix. The observed longer lag phase is most likely due to the need for acclimatization to the new growth conditions with a higher CO_2 concentration.



A B

Figure 3.21. Growth of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* in 5% (A) and 10% (B) CO_2 mix with air measured as optical density at 750 nm.

While all cultures showed similar growth cures at 5% CO_2, limited growth of *C. sorokiniana* and *C. reinhardtii* was observed at 10% CO_2 supply. A significant decrease in the culture density of *C. sorokiniana* was observed after day 6.

Biomass productivity was measured at the end of cultivation as a cell dry weight. Maximum biomass yield of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* at CO_2 supply of 5% reached 2.0, 3.1 and 3.2 g L^{-1} respectively (Figure 3.22.). The most productive species was *C. reinhardtii* with a comparable biomass yield to *C. sorokiniana*; however, *C. vulgaris* showed the lowest biomass productivity among all three species tested.

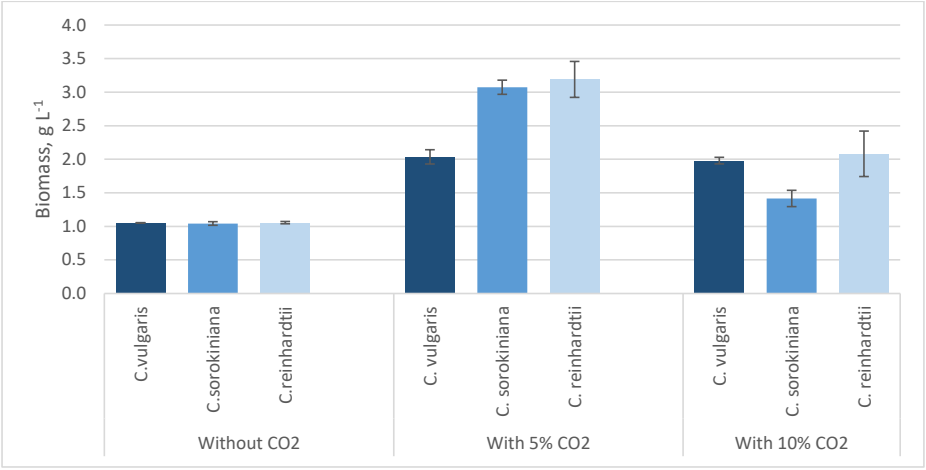


Figure 3.22. Comparison of microalgae biomass yield at different CO_2 sparging rates.

CO_2 greatly enhanced biomass productivity compared to cultivation without extra CO_2 bubbling. *C. vulgaris* doubled biomass at 5% CO_2 supply compared to cultivation with ambient CO_2 level. Moreover, *C. sorokiniana* and *C. reinhardtii* tripled productivity at 5% CO_2 supply reaching 3.1 and 3.2 g L^{-1}, respectively. When the CO_2 level in the CO_2/air mix was increased to 10%, the biomass yield of *C. sorokiniana* and *C. reinhardtii* decreased substantially; however, an increase in CO_2 rate did not significantly change the productivity of *C. vulgaris* (Figure 3.22.). These results indicate that *C. vulgaris* might have higher resistance to high CO_2 concentrations than the other two species tested. At the end of cultivation tests, most cultures were at the exponential growth stage indicating that the selected cultivation time was too short to reach the stationary phase, therefore the cultivation time must be extended to assess the real potential of the species and reach the maximum biomass productivity.

It is generally known that microalgae require higher CO_2 content than naturally present in the atmosphere for a fast growth rate and high biomass productivity [209]. The results from cultivation tests with different CO_2 rates show that indeed an increase in CO_2 rate to 5% resulted in significantly higher biomass yield of all three microalgae tested – *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii*. Furthermore, too high levels of CO_2 can inhibit certain microalgae growth. Air mix with 10% CO_2 content decreased *C. sorokiniana* and *C. reinhardtii* growth rate and biomass productivity, whereas the productivity of *C. vulgaris* was not affected indicating the potential of this microalgal strain to tolerate higher CO_2 levels and might be especially useful to uptake CO_2 from flue gases which usually have high CO_2 content.

3.8. Digestate as a growth medium

Pretreatment with centrifugation and filtration

Digestate was obtained from the Agro Iecava biogas plant to determine its suitability as a low-cost nutrient source for microalgae growth. The raw liquid fraction of agricultural digestate was not suitable for microalgae cultivation due to very high total solids and optical density which visually appeared as black opaque liquid. Dilution of liquid digestate is commonly applied to increase the suitability of digestate for microalgae cultivation; however, other pretreatment methods were tested in the current study to increase the overall feasibility. Centrifugation and filtration were applied as initial pretreatment methods to improve the properties of digestate. The amount of suspended solids in a raw liquid digestate was 9 g L^{-1}. Various pretreatment methods greatly improved digestate suitability for microalgae cultivation. The amount of suspended solids was greatly reduced in pretreated digestate compared to raw digestate (Table 3.2.). The amount of suspended solids, COD, total nitrogen, total phosphorus, nitrate nitrogen, ammonia nitrogen and turbidity varied based on the pretreatment method applied. Filtration as pretreatment was more effective in the reduction of all parameters tested compared to centrifugation (Table 3.2). Filter centrifugation decreased nitrogen and COD more effectively compared to centrifugation and filtration. However, phosphorus and ammonia nitrogen content were higher than with the other two pretreatment methods. Filtration through a 1.6 μm microfiber filter further decreased the solids; however, was considered not a viable option for large-scale digestate pretreatment.

Table 3.2.

Chemical composition of digestate after various pretreatment methods. NA – not available.

Parameter	Unit	Raw liquid digestate	Centrifugation	Filtration	Filter centrifugation
Suspended solids	mg L^{-1}	9080	2450	1700	NA
COD	mg L^{-1}	NA	23210	9580	3630
Total N	mg L^{-1}	NA	11770	6780	6180
Total P	mg L^{-1}	NA	319	157	602
Nitrate N	mg L^{-1}	NA	<0.07	<0.07	<0.3
Ammonia N	mg L^{-1}	NA	3080	2460	3360
Turbidity	mg L^{-1}	NA	NA	7840	NA
Optical density	NA	10.68	NA	NA	NA

Cultivation in pretreated digestate

Pretreated digestate was subsequently tested as a growth medium for microalgae cultivation. Although the amount of solids was greatly reduced by the pretreatment, high optical density was still an obstacle, therefore dilution was used to reduce the optical density. During the first round of experiments, microalgae *C. vulgaris* was cultivated in (1) centrifuged and (2) filtered digestate diluted to 1%, 3% and 5% with distilled water. *C. vulgaris* exhibited better growth in filtered than in only centrifuged digestate at a concentration of 3%; however, no significant difference in growth rate was observed in the case of 1% digestate (Figure 3.23.). The highest culture density was reached at 1% digestate regardless of the pretreatment method applied and was close to that obtained with BG-11 medium showing very promising results. When 3% digestate was applied, cultivation of *C. vulgaris* without filtration step resulted in a very slow growth. Whereas 3% digestate after the pretreatment with filtration provided a good growth medium for *C. vulgaris*.

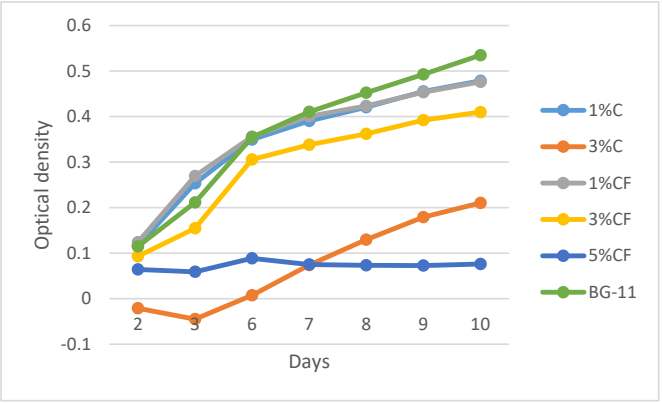


Figure 3.23. Cultivation of *C. vulgaris* in 1, 3 and 5% digestate processed using various pre-treatment methods (C = centrifugation, CF = filtration).

No growth of microalgae was observed when a 5% dilution was applied. This could possibly be due to the optical density of the more concentrated digestate being too high, which can also be observed visually (Figure3.24.) thus limiting the access of light to the microalgae cells.

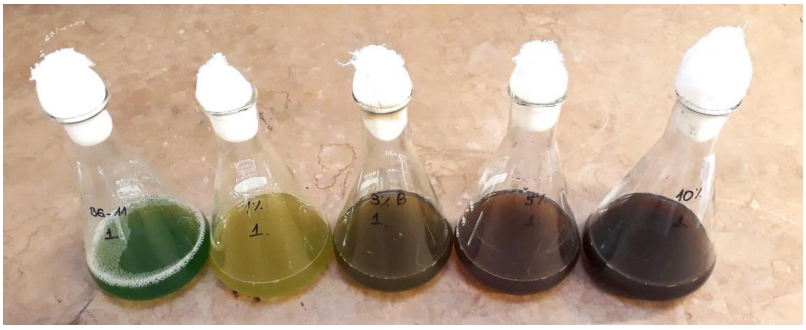


Figure 3.24. *C. vulgaris* cultivation in various dilutions of digestate as a growth medium.

At the end of the growth test biomass yield was calculated. Although the highest cell density was reached with 1% digestate, the highest biomass production was observed with 3% digestate after the filtration pretreatment (Figure 3.25.). However, due to the relatively high standard deviation of replicates, this result must be perceived with caution.

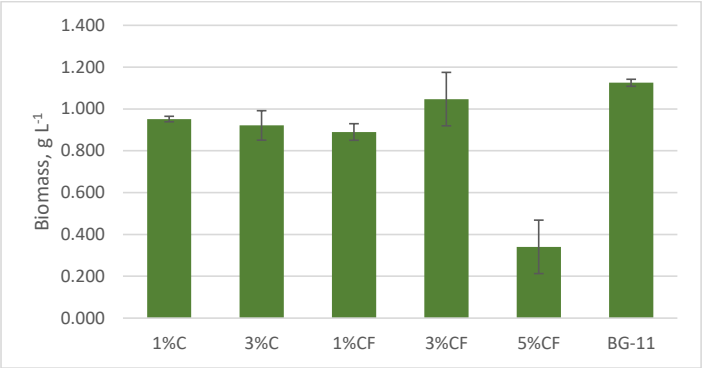


Figure 3.25. Biomass yield of *C. vulgaris* cultivated in 1%, 3% and 5% pretreated digestate. C- centrifuged, CF - filtration. Error bars represent Standard deviation (n=3).

Nutrient removal by microorganisms was calculated at the end of cultivation to evaluate the potential of microalgae for digestate treatment. Removal rates of centrifugation pretreated digestate of two concentrations are shown in Table 3.3. Generally, higher removal rates were achieved with 1% digestate presumably due to lower load of nutrients. COD removal was not effective, resulting in 13 and 19% reduction from 1% and 3% digestate, respectively. Microalgae are known to release extracellular organic substances during growth contributing to increasing COD level, therefore its removal often is not effective with microalgae.

Table 3.3.

Nutrient removal of pretreated (centrifugation) digestate during microalgae growth at 1% and 3% dilution rate.

	100% digestate	1%	After cultivation	Removal rate, %	3%	After cultivation	Removal rate, %
Suspended solids	2450	24.5	-	-	73.5	-	-
COD	23210	232.1	202	13.0	696.3	565	18.9
Total N	11770	117.7	24.5	79.2	353.1	72.4	79.5
Total P	319	3.19	0.411	87.1	9.57	3.21	66.5
NO_3-N	<0.07	N	12.9	NA	N	14.4	NA
NH_4-N	3080	30.8	7.6	75.3	92.4	42.3	54.2

Nutrient removal of filtration pretreated digestate at 1, 3 and 5% dilution is shown in Table 3.4. Higher removal of total P and NH_4-N were observed with 1% digestate compared to 3% dilution. However, total N removal was more efficient from 3% digestate. Considerably lower

nutrient removal was detected in 5% dilution associated with low growth of microalgae in this dilution probably due to the limited light availability. Digestate contains different microorganisms creating a complex symbiotic system. Cultivation of mixotrophic microorganisms is required for digestate treatment because digestate after anaerobic digestion still contains biodegradable organic compounds. Organic matter is assimilated by aerobic bacteria whereas inorganic carbon is used in photosynthesis by microalgae. Heterotrophic and mixotrophic microalgae can also contribute to the assimilation of organic matter. On the other hand, carbon dioxide produced by bacteria is consumed by algae promoting growth and nutrient removal. Microalgae produce oxygen that is used by aerobic bacteria. Consequently, the bacteria-microalgae consortium works as an efficient digestate nutrient and pollution removal system.

Table 3.4.

Nutrient removal of pretreated (filtration) digestate during microalgae growth at 1%, 3% and 5% digestate.

	100%	1%	After cultivation	Removal rate, %	3%	After cultivation	Removal rate, %	5%	After cultivation	Removal rate, %
Suspended solids	1700	17	-	-	51	-	-	85	-	-
COD	9580	95.8	109	-13.8	287.4	292	-1.6	479	411	14.2
Total N	6780	67.8	20.4	69.9	203.4	50.8	75.0	339	199.4	41.2
Total P	157	1.57	0.212	86.5	4.71	1.46	69.0	7.85	4.26	45.7
NO_3-N	<0.07	N	12.7	NA	N	12.6	NA	N	11.8	NA
NH_4-N	2460	24.6	7.6	69.1	73.8	30	59.3	123	86	30.1

The use of liquid digestate as a nutrient source for microalgae cultivation is not straightforward. Some very promising results were achieved at some tests; however, digestate is “alive” containing a variety of microorganisms which interact with microalgae creating a very complex system. Moreover, both digestate content of nutrients and contaminants and microorganisms are changing based on the feedstock of anaerobic digestion, temperature, inoculum and other anaerobic digestion parameters. Our results demonstrate that centrifugation followed by filtration of digestate was the best method for digestate pretreatment prior to microalgae cultivation as it reduced the presence of total solids in the digestate and the highest growth rate of *C. vulgaris* was observed in filtered digestate. However, this solution is not viable when considering large-scale microalgae cultivation. Therefore, filter centrifugation was tested allowing fast large volume digestate filtration and resulting in improved digestate properties.

Although microalgae could grow in pretreated diluted digestate, biomass yield was significantly lower than that of control media. This might suggest that some limiting factors are present in digestate. The high optical density of digestate may imply a reduced light availability caused by light-limited conditions. Therefore, a pretreatment method to reduce optical density was tested.

Pretreatment with activated carbon

A new batch of digestate was obtained from Agro Iecava biogas plant for activated carbon pre-treatment. Chemical characterization of digestate prior to treatment was performed and is reported in Table 3.5. The total solids content of raw liquid digestate reached 23 g L^{-1} resulting in extremely high turbidity (7840 mg L^{-1}) which suggests that there may be an inhibitory effect on photosynthetic potential and low light availability to microalgae cells. Furthermore, exceptionally high optical density (OD 13) was recorded resulting in nearly black opaque liquid (Figure 3.26 A). Organic material and humic substances present in digestate are most likely responsible for the characteristic dark color. It is clear from a visual inspection that no microalgae would grow in this thick black digestate without a very high dilution rate.

Table 3.5.

Characterization of the liquid fraction of raw agricultural digestate and after pretreatment with 3 g L^{-1} and 40 g L^{-1} activated carbon with adsorption time 10 minutes (TS – total solids, SS – suspended solids, VS – volatile solids, DS – dissolved solids, TN – total nitrogen).

Parameter	Unit	Raw liquid digestate	Pretreated	
			3 g L^{-1}	40 g L^{-1}
TS	g L^{-1}	22.9	NA	NA
SS	g L^{-1}	5.1	NA	NA
VS	g L^{-1}	4.25	NA	NA
DS	-	17.83	NA	NA
OD	-	13.03	3.06	2.81
pH	-	8.17	NA	NA
Turbidity	mg L^{-1}	7840	NA	NA
COD	mg L^{-1}	6840	6540	4960
TN	mg L^{-1}	5950	NA	NA
NH_4-N	mg L^{-1}	3600	3000	2667
NO_3-N	mg L^{-1}	47.5	NA	NA
PO_4-P	mg L^{-1}	490	338	278.4

Nitrogen and phosphorus are primary nutrients required for microalgae growth and usually are abundant in liquid digestate [283]. In particular, agricultural digestate is rich in nitrogen when compared to other wastewater streams [230]. Indeed, 5950 and 490 mg L^{-1} total nitrogen and phosphorus, respectively were detected in the current study. Chemical analysis showed that most of the nitrogen in digestate was in a form of ammonium (NH_4-N) as pointed out in other studies [237], [284]. Although ammonium is a preferred source of nitrogen for most microalgae [285], high total ammonia nitrogen may inhibit microalgae growth [265], [283]. No other reports were found stating such a high value of ammonia nitrogen concentration as in this study (3600 mg L^{-1}). Phosphorus content (490 mg L^{-1} PO_4-P) was comparable to or higher than that referred to in other studies [239], [285]. The reported values of COD content in anaerobic

digestion effluents are commonly higher than found in other wastewater streams [230] however, exceptionally high COD (6840 mg L^{-1}) was found in the current study indicating excessive load of organic matter. Uggetti et al. reported COD 210 mg L^{-1} in anaerobic digestate [285], 1980 mg L^{-1} was reported in digestate from livestock waste [286], 2661 mg L^{-1} in anaerobic digested municipal wastewater [231], and 3402 mg L^{-1} in anaerobic digested piggery wastewater [287]. Digestate was slightly alkaline as commonly reported [283] with a pH of 8.17 therefore being at the optimal range for most freshwater microalgae species [283].

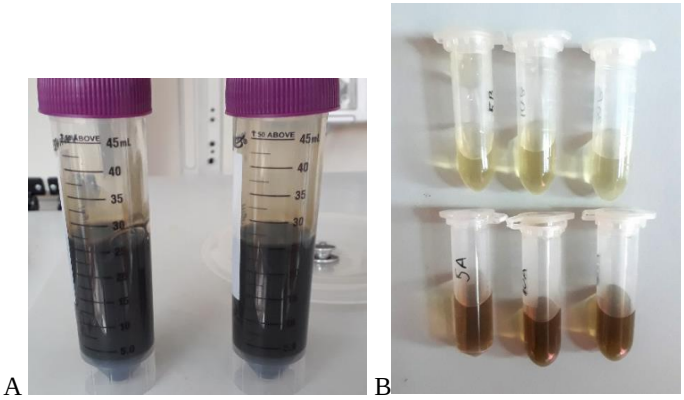


Figure 3.26. The appearance of a raw (undiluted, untreated) liquid fraction of digestate (A) and after the pretreatment with activated carbon at two different concentrations (B).

Typical effluent from anaerobic digestion is known to have high nutrient concentrations [284]; however, generally, all parameters measured in this study were higher than those reported in the literature [283] indicating a very dense and highly concentrated digestate. The nutrient content of raw digestate was significantly higher than recommended for microalgae cultivation. Furthermore, dark color and high turbidity make algae cultivation in raw liquid digestate impossible.

Effect of activated carbon adsorption on OD rate of digestate

Activated carbon holds great potential as an efficient low-cost method to reduce turbidity, optical density and harsh chemicals in digestate due to the high capacity of adsorbing various substances. Although activated carbon has been applied for municipal wastewater treatment, it is a novel pretreatment method for digestate, and its actual potential is still unknown. The initial OD of raw liquid digestate was 13 indicating that light penetration in a raw liquid digestate is not sufficient for microalgae growth. Activated carbon pretreatment was applied to raw liquid digestate in order to reduce the optical density. Activated carbon concentrations from 3 to 40 g L^{-1} were applied at various adsorption durations ranging from 5 to 180 minutes (Figure 3.27.). The highest OD reduction rate of 78% was achieved after 10 minutes of adsorption at 40 g L^{-1} and of 77% at 40 g L^{-1} with 5 minutes, 3 g L^{-1} with 10 minutes and 40 g L^{-1} with 30 minutes of adsorption time (Table 3.6). Contrary, the lowest OD reduction rate was observed after 30 minutes at 3 g L^{-1} and after 180 minutes at 3 g L^{-1} activated carbon concentration showing 64 and 65% reduction, respectively.

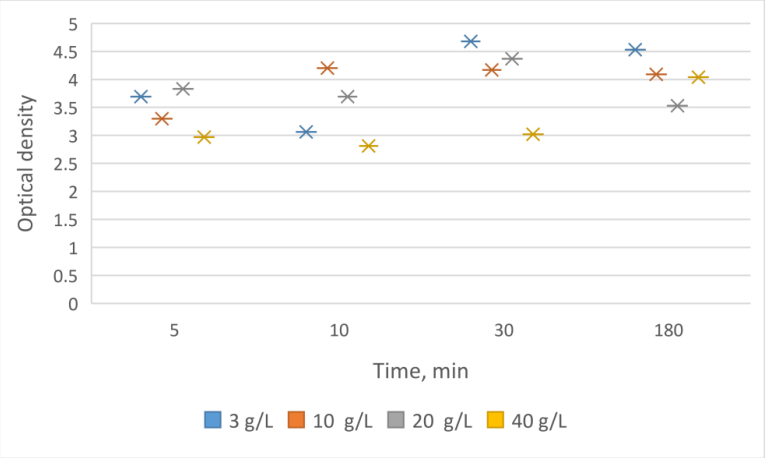


Figure 3.27. Optical density of pretreated digestate based on the activated carbon concentration and adsorption time.

Along with the reduction of OD, the concentration of some nutrients and COD was decreased as well (Table 3.5.). Ammonia concentration at activated carbon concentration of 3 and 40 g L^{-1} was reduced by 16% and 26%, respectively. COD concentration decreased from 6840 mg L^{-1} in raw liquid digestate to 6540 and 4960 at activated carbon concentration of 3 and 40 g L^{-1}, respectively. Adsorption on activated carbon also affected phosphorus content in digestate resulting in a decrease by 31 and 43% in 3 g L^{-1} and 40 g L^{-1} concentration, respectively. Results show up to a 78% reduction of optical density (OD 2.81 was reached), suggesting that activated carbon pretreatment is a highly promising tool to reduce the OD in agricultural digestate to the tolerable level for microalgae in a short time. Since the reduction of OD was similar at 3 g L^{-1} and 40 g L^{-1} activated carbon concentration and keeping the pretreatment costs down, a concentration of 3 g L^{-1} was selected for digestate pretreatment for growth tests.

Table 3.6.

The OD reduction rate of the digestate (%) after activated carbon (AC) treatment with different concentrations and contact times.

AC concentration, g L^{-1}	Adsorption time, min	OD ↓ %	Adsorption time, min	OD ↓ %	Adsorption time, min	OD ↓ %	Adsorption time, min	OD ↓ %
3	5	72	10	77	30	64	180	65
10		75		68		68		69
20		71		72		66		73
40		77		78		77		69

Microalgae culturing in pretreated liquid digestate

To test the activated carbon pretreated digestate as a nutrient source for microalgae cultivation, microalgae growth tests in pretreated and raw liquid digestate were run in parallel for 14 days in a batch cultivation mode in 1.5 L photobioreactors. In PBR 1 and PBR 2 untreated but diluted to an OD of 0.1 digestate was used, in PBR 3 and PBR 4 digestate pretreated with 3 g L^{-1} activated carbon and diluted to 0.1 OD was applied. Microalgae growth rate in untreated and pretreated digestate is reported in Figure 3.28. as cell count (A), biomass productivity (B) and OD (C).

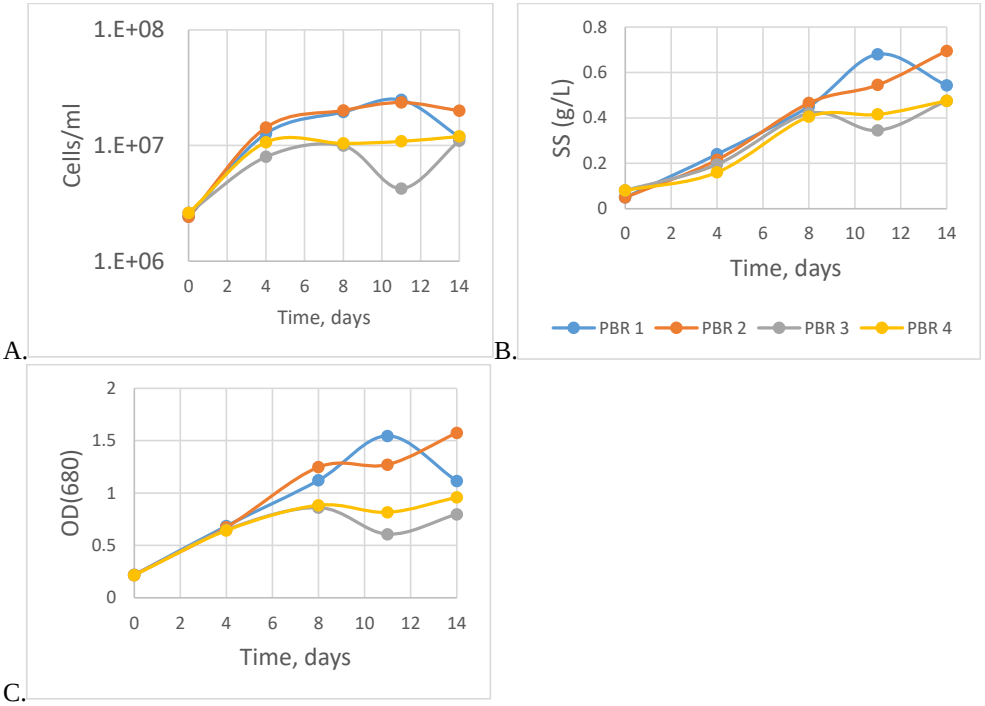


Figure 3.28. The microalgae growth rate in untreated (PBR 1 and 2) and pretreated (PBR 3 and 4) digestate reported as cell count (A), biomass productivity (B) and OD (C).

The number of microalgae cells increased during the cultivation showing exponential growth till day 4, thereafter the growth slowed down in all PBRs (Figure 3.28.A). The maximum number of cells was reached on day 11 in untreated PBRs and on day 14 in pretreated PBRs. Specific growth rate μ was 1.15, 1.19, 1.14 and1.15 d^{-1} in PBR 1, 2, 3, and 4, respectively showing very similar growth in pretreated and untreated digestate. Initial OD in all photobioreactors was 0.2 and increased up to 1.1, 1.6, 0.8 and 1 in PBR 1, 2, 3, and 4, respectively (Figure 3.28. C). The microalgae biomass productivity (dry weight) was calculated from the total suspended solids. Biomass yield increased steadily in all PBRs till day 8 and then started to fluctuate (Figure 3.28. B). Biomass productivity reached 0.69 g L^{-1} in untreated PBRs and 0.48 g L^{-1} in pretreated PBRs. The growth indicators used demonstrate moderate microalgae growth in all PBRs suggesting that some factors might have limited the growth of

cultures in PBRs to reach the maximum productivity. Microalgae biomass yield in untreated diluted digestate was slightly higher compared to pretreated digestate. This could possibly be explained by the higher nutrient load in pretreated PBRs due to the larger initial amount of digestate applied. As seen from chemical analysis, the agricultural digestate used is very high in nutrients therefore some chemicals might be in excess, leading to the suppression of microalgae growth.

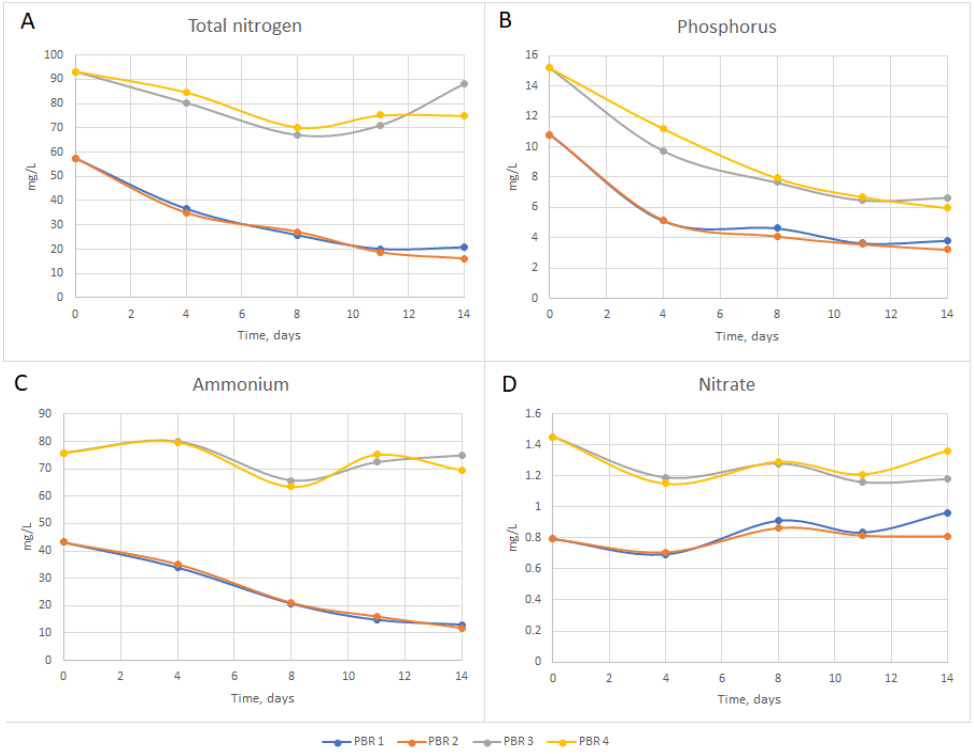


Figure 3.29. Removal of total nitrogen (A), phosphorus (B), ammonium (C) and nitrate (D) in pretreated and untreated PBRs.

Excessive ammonia concentration is frequently cited in the literature as the possible explanation of algal growth inhibition; however, the actual threshold of ammonia tolerance seems to be species dependent. Initial ammonium concentrations were 43 mg L^{-1} and 76 mg L^{-1} NH_4-N in untreated and pretreated PBRs, respectively, which might have an inhibitory effect on the microalgae population growth as reported in a study by Uggetti et al. [285] where an increase in ammonia concentration from 9 to 34 mg L^{-1} resulted in 77% reduction in growth rate in a mixed microalgal culture dominated by *Scenedesmus* sp. Moreover, in another study, *Scenedesmus* sp. showed no growth inhibition up to 100 ppm ammonium whereas values over 200 ppm resulted in a severe decrease in cell density [286]. However, other studies reported microalgae tolerance to much higher ammonium concentrations. Ammonium of 178 mg L^{-1} was not toxic to *Chlorella* sp. and was completely removed from anaerobically digested dairy manure within 21-day cultivation [237]. Resistance to NH_4-N of 1600 mg L^{-1} was shown by a

microalgal consortium dominated by *Chlorella* sp. [265]. Another study tested initial concentrations of ammonium ranging from 20 – 1500 mg L^{-1} and observed no inhibition of *C. sorokiniana* growth at any of the concentrations applied [241]. Moreover, the higher the ammonium concentration the better *C. sorokiniana* growth, thus the highest biomass accumulation was observed with initial ammonium at 1500 mg L^{-1} which is much higher than the ammonium concentration in the growth medium in our study. It seems that ammonium tolerance is not only species-specific but also depends on other factors such as cultivation conditions and acclimatization to high ammonium conditions.

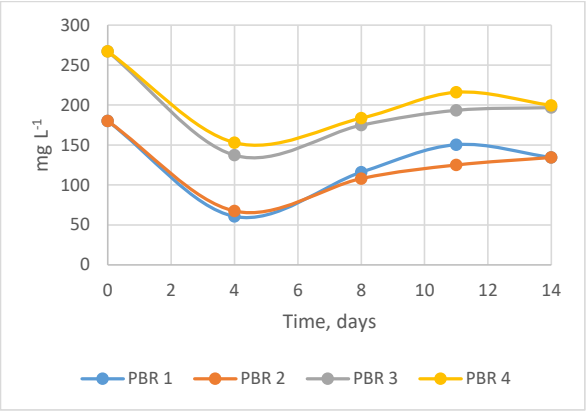


Figure 3.30. Removal of COD in pretreated (PBR 3 and 4) and untreated (PBR 1 and 2) PBRs.

The initial level of nutrients was higher in pretreated PBRs (PBR 3 and 4) than in untreated PBRs (PBR 1 and 2) due to the lower dilution rate. Total nitrogen removal rate was similar in all PBRs until around day 8 when the level of nitrogen started to rise in pretreated PBRs but continued to decrease in untreated PBRs (Figure 3.29.A). Level of phosphorus exhibited decreasing trend throughout the cultivation in all PBRs indicating a good ability of *Chlorella* sp. to remove phosphorus (Figure 3.29.B). Similar to nitrogen content in pretreated PBRs, fluctuations of ammonia nitrogen (NH_4-N) and nitrates (NO_3-N) content were also observed (Figure 3.29.C and 3.29.D, respectively). Fluctuations in nitrogen removal and accumulation are an indication of complicated chemical processes taking place inside PBRs due to interactions between microalgae and bacteria that are not fully understood today. The observed changes in ammonium and nitrate content in PBRs could be due to nitrification processes taking place in PBRs. During nitrification processes, ammonia (NH_4^+) is oxidized to nitrate (NO_3) by nitrifying bacteria. These bacteria could be present in growth medium coming from naturally occurring microorganisms in digestate affecting chemical processes in PBRs.

The level of COD was successfully reduced till day 4 in all PBRs (Figure 3.30.) reaching a reduction rate of 64% in untreated PBRs and 46% in pretreated PBRs. However, COD started to increase thereafter in all PBRs. This phenomenon is well known in wastewater treatment with microalgae due to the fact that microalgae release organic compounds during the growth contributing to the increase of COD [288]. On average 72%, 73% and 70% of total nitrogen, ammonium and phosphorus, respectively were removed in untreated PBR2 (Figure 3.31.).

Nutrient removal rates in PBR1 were similar but slightly lower; 64%, 70% and 65% for total nitrogen, ammonium and phosphorus, respectively.

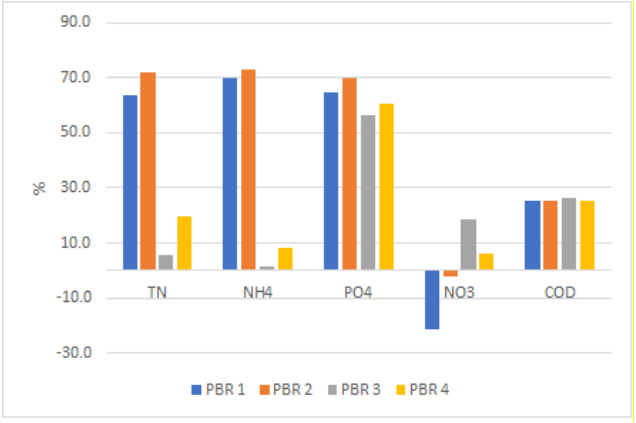


Figure 3.31. Nutrient removal rate (%) at the end of the growth tests.

However, nutrient removal rates varied considerably between replicates of pretreated digestate. While total nitrogen removal till day 8 was 28% in PBR3 and 26% in PBR4 being similar to removal rates in untreated PBRs (26% and 27% in PBR1 and PBR2, respectively), at the end of cultivation nitrogen removal was just 6% of initial level due to the subsequent increase in nitrogen content in pretreated PBRs. The level of phosphorus was slightly higher in untreated PBRs (on average 67%) than in pretreated PBRs (on average 59%) resulting in an efficient removal rate. On the other hand, just 1.3% of ammonium was removed in PBR 3 and 8% in PBR 4 due to considerable fluctuation in ammonium level during the growth test. Removal of nitrates was slightly higher in pretreated PBRs. Nitrate level reduced initially in untreated PBRs followed by fluctuations during the cultivation and resulted in a negative removal rate at the end of cultivation due to the accumulation of nitrates. The nitrate level of PBR1 at the end of the cultivation increased by 21% compared to the initial level. A slight increase was detected also in PBR2 (2.3%). Contrary, nitrate removal in pretreated PBRs was detected, resulting in 19% and 6% removal in PBR 3 and 4, respectively. The observed fluctuations in nitrates, ammonia nitrogen and total nitrogen are most likely a result of the chemical conversion of various forms of nitrogen in the digestate performed by various microorganisms as explained before. In conclusion, the nutrient removal was efficient from both untreated and activated carbon pretreated digestate till day 8, then fluctuations started in the removal of various nitrogen forms indicating that microorganism interaction might not be in balance. It matches with the growth rate indicators where fluctuations and a decrease of growth rate in some PBRs were observed from day 8 indicating some growth limiting factors are in place.

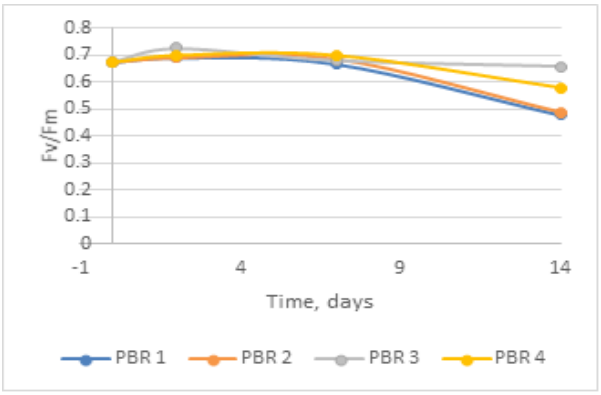


Figure 3.32. Photosystem II performance expressed as *Fv/Fm* ratio in pre-treated and untreated PBRs.

PAM fluorometry was used to evaluate the effect of digestate on the growth of microalgae. PAM measurements showed that the *Fv/Fm* was high (0.67) at the beginning of cultivation in all the reactors indicating good environmental conditions and no stress from the substrate (Figure 3.32.). High *Fv/Fm* values were observed during day 2 and 7 (> 0.6); a small decrease was observed only at the end of the assay, more evident in PBR1 and 2, highlighting that activated carbon pretreated digestate is a suitable medium for microalgae growth.

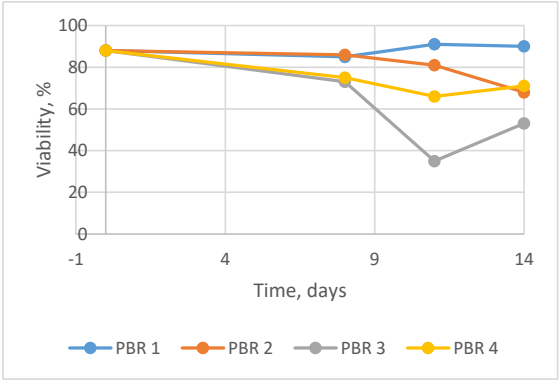


Figure 3.33. Viability (the percentage of live cells) of microalgae cultures during the growth test in pretreated and untreated PBRs.

The viability of microalgae during cultivation was determined by the assessment of the dead/live cell ratio after dyeing cells with Sytox nucleic acid dye. The viability of microalgae cultures was 88% at the beginning of the cultivation (Figure 3.33). Cell viability stayed high during the whole cultivation period in PBR1 and PBR2 with untreated diluted digestate; however, a decrease in culture viability was observed in PBRs with pretreated digestate. All essential nutrients are still present in the growth medium at the end of cultivation therefore, the lack of nutrients cannot be the reason for the observed increased cell death rate in the pretreated PBRs. The highest drop of viability was observed in the PBR3 at the second part of the cultivation when the percentage of live cells dropped to 35% but increased again up to 53% at

the end of the cultivation. PBR3 and 4 are replicates with identical cultivation conditions and nutrient concentrations; however, because the microalgal community is not axenic, other microorganisms, such as bacteria are most likely present in the cultivation medium taking part in biological processes within the PBR. Moreover, rotifer activity was observed in all PBRs at a low level; however, the highest number of rotifers was recorded in PBR3 corresponding to the highest decrease in the cell density observed on day 11 leading to the possible cause of decreased vitality observed.

Activated carbon was used successfully to decrease the optical density of high-strength agricultural liquid digestate. Results show that activated carbon is a valuable novel technique to increase the potential usefulness of dark and dense agricultural digestate for microalgae cultivation by reducing the optical density thus increasing the light transmission into microalgal cultures. Microalgae culture was able to remove nutrients in pretreated as well as untreated diluted digestate. Higher biomass productivity was observed in highly diluted untreated digestate suggesting that some inhibitory effects of pretreated digestate might be present. The higher initial nutrient rate in pretreated PBRs due to the low dilution of digestate applied could have led to an excessive load of some nutrients. Moreover, some other substances that can negatively affect microalgae growth and nutrient consumption might be present in the digestate; whereas, a higher dilution rate has minimized their effect in the untreated PBR1 and 2. Although PAM measurements did not show any inhibitory effects of growth medium on the photosystem performance, toxic mechanisms could have affected other metabolic pathways. Further growth tests with microalgae in pretreated digestate should be carried out to determine the correct dilution rate in order to fully evaluate the applicability of activated carbon pretreated digestate as a growth medium.

3.9. Microalgae growth test in pilot raceway ponds

Created SMORP cultivation system with the greenhouse was constructed and integrated into the Agro Iecava biogas plant using side-products from the biogas plant, namely liquid digestate and flue gases. The Microalgae cultivation test was conducted at the end of April 2021 and lasted for 16 consecutive days.

Probes with temperature, pH, and PAR sensors were installed and used to record the cultivation conditions. The temperature inside the pond, in the greenhouse and outdoors was recorded. Light intensity in PAR at the water level was recorded. The temperature of flue gas pumped inside the pond was also recorded. Samples for nutrient removal and biomass analysis were collected every 3 days. Samples were analyzed for nutrient content, suspended solids and optical density. Analysis of total nitrogen, total phosphorus, ammonia nitrogen, nitrates and chemical oxygen demand was performed in an external laboratory, other analyses were done in the RTU Biosystems laboratory.

Microalgal strain *Chlorella sorokinana* was selected for initial tests in pilot race-ways ponds due to its resistance to high light intensity and based on laboratory scale tests showing its flexibility as the experiments were taking place in springtime when natural light intensity is close to its maximum, but temperature is highly variable with a wide range of fluctuations.

Monitoring the cultivation conditions

Outdoor microalgae cultivation is heavily dependent on weather, which in turn varies according to location and season. Spring conditions are usually dynamic with fluctuating temperatures being not ideal for microalgae cultivation. Indeed, the microalgae cultivation test in the SMORP pilot was challenging due to unstable and variable weather conditions that are characteristic of the spring season in Latvian climate conditions. However, it was possible to evaluate the performance of selected microalga in suboptimal conditions. After the inoculation of the raceway pond, probes with temperature, pH, and PAR sensors were used to record the cultivation conditions.

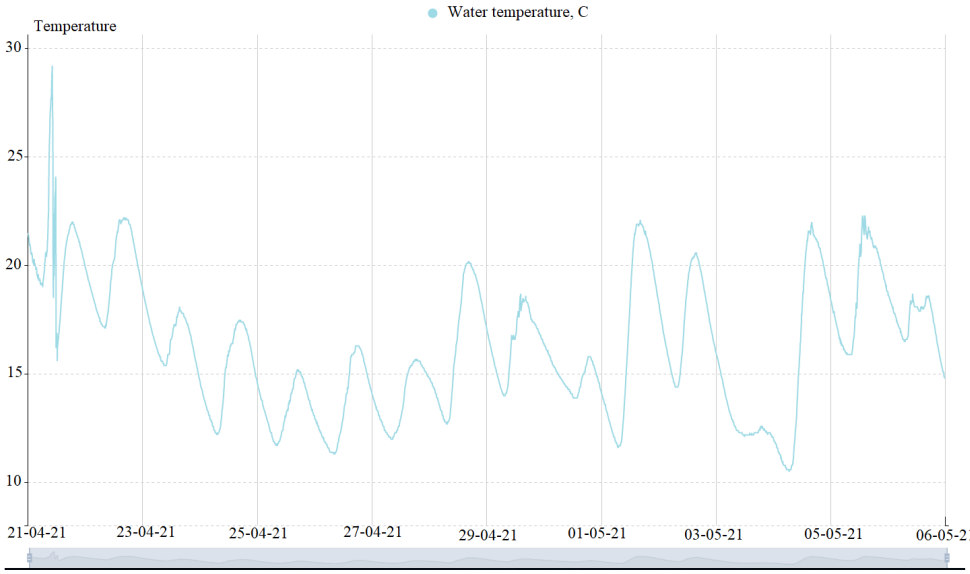


Figure 3.34. Pond water temperature during the biomass cultivation test.

Daytime temperatures can vary greatly from day to day, and the difference between day and night temperatures can be very high. Indeed, recorded fluctuations in temperature during microalgae cultivation were high. Temperature was monitored continuously inside the cultivation pond, furthermore, air temperature in the greenhouse and outside was also recorded. The water temperature in the microalgae cultivation pond during the biomass cultivation is shown in Figure 3.34. The average daytime temperature during the cultivation ranged from around +15 to 22 °C. The highest water temperature recorded was around +22 °C during the day, whereas the lowest daytime temperature was recorded on May 3rd when the pond temperature reached only +12 °C. During the nighttime water temperature dropped considerably which was expected due to the low air temperature outside and was generally between 10 and 16 °C.

Pond temperature was directly influenced by the outdoor temperature. Fluctuations in pond water temperature depending on the temperature outdoors and the temperature in the greenhouse are shown in Figure 3.35. The lowest air temperature outside recorded during the

growth test was around +2 °C during the night. Outdoors nighttime temperature stayed just a couple of degrees above zero for most of the cultivation period. In the last decade of April temperature was 3.2 °C lower than average normally (1981. – 2010.) and 4.1 °C lower than normal 1991 – 2020 [289] which affected the microalgae growth. During the coldest nights, the pond temperature did not drop lower than + 10 °C showing the contribution of greenhouse to keep the temperature at a tolerable level for microalgae during cooler environmental conditions. The greenhouse could ensure around 10 degrees higher temperature than the temperature outside.

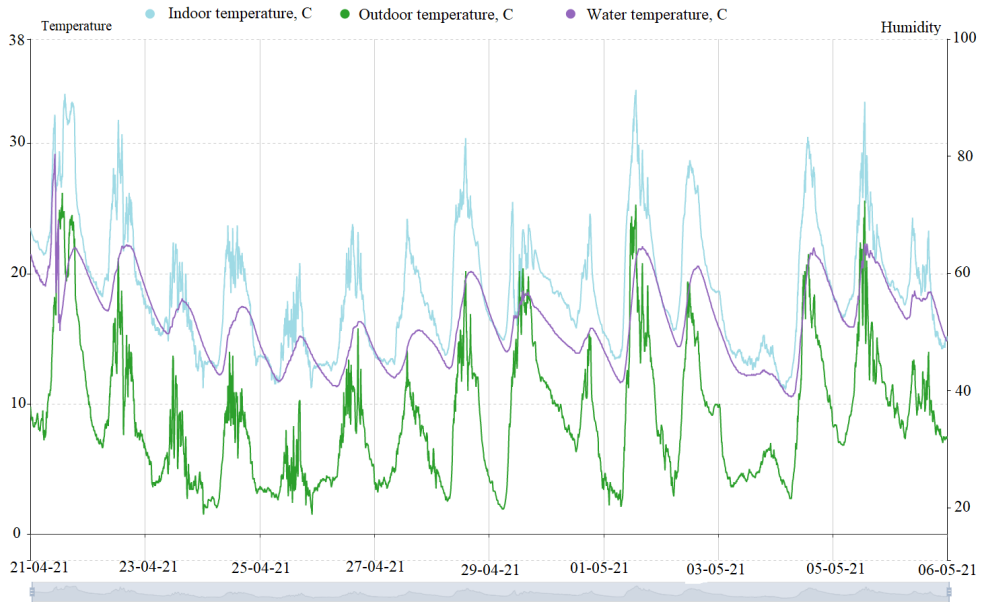


Figure 3.35. Outdoor temperature, indoor temperature at the greenhouse and pond water temperature during the biomass cultivation test. Green line – Outdoor temperature, purple line – Temperature inside the pond, blue line – Temperature inside the greenhouse

The contribution of heat of flue gas is negligible in the present flow rate. Variation in flue gas temperature is shown in Figure 3.36. Flue gas temperature varied due to the changes in outdoor temperature because transfer pipes of the flue gas are located outside of the greenhouse with the purpose of cooling down flue gases coming from the biogas motor room. The temperature of pure flue gases was 522 °C; however, while travelling up the chimney and through the pipes, the temperature decreased considerably. Moreover, after mixing with air the temperature of flue gases reaching the pond was a maximum of 45 °C.

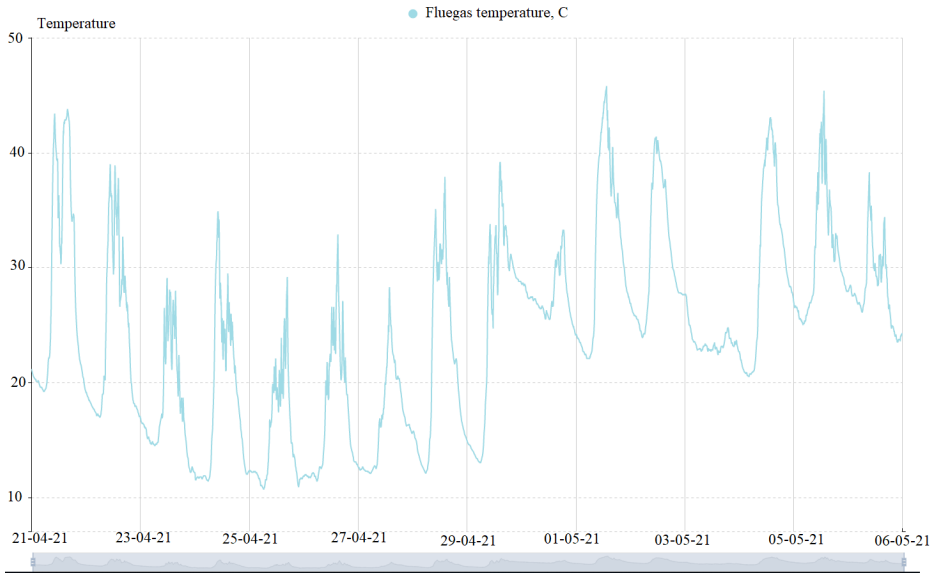


Figure 3.36. Flue gas temperature during the initial biomass cultivation test.

Natural light intensity fluctuation recorded during the biomass cultivation test is shown in Figure 3.37. Maximum light intensity during the daylight hours generally was in a range from 600 to 900 µmol m^{-2} s^{-1} in the middle of the day in mostly clear days with no or very low cloud cover. Significantly lower light intensity was observed on overcast days, for example on April 30th maximum light intensity reached just 300 µmol m^{-2} s^{-1} but light intensity didn't exceed 130 µmol m^{-2} s^{-1} on May 3rd. High light intensity might be causing photodamage because temperatures were generally lower than optimum. The received light might be excessive in low temperature conditions.

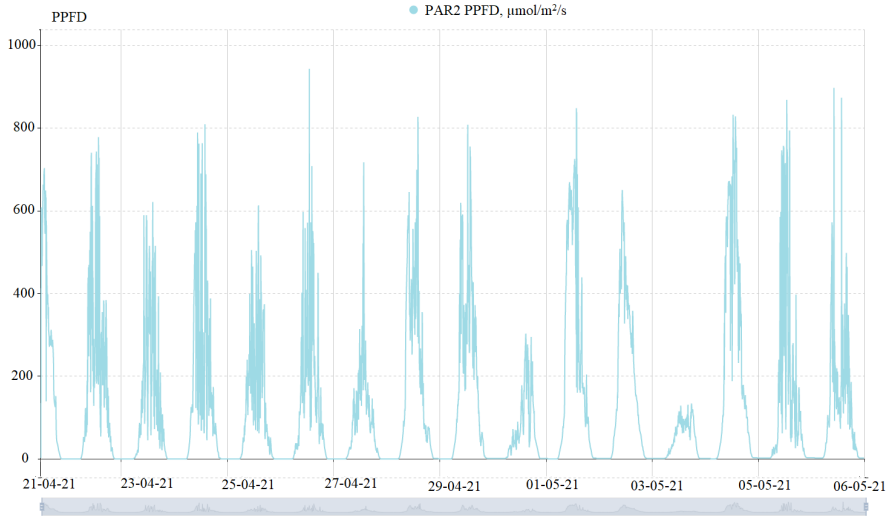


Figure 3.37. Light intensity (PAR) at the water level during the biomass cultivation test.

pH of the cultivation pond fluctuated according to the day/night cycle and is shown in Figure 3.38. The pH of microalgal cultures rises gradually during the day due to the uptake of carbon by microalgae cells and decreases again during the nighttime. pH at the beginning of cultivation was 7.5 and increased in the first few days constantly due to the algae growth and consumption of CO_2. Thereafter pH fluctuation levelled out and was around 9 for the last days of the cultivation. The contribution of CO_2 from flue gases is difficult to evaluate because it was not possible to measure the actual amount of CO_2 entering the ponds in the present setup conditions. The amount of CO_2 in pure flue gases before the mixing with air was 14%, however, the actual volume of CO_2 entering the microalgae pond should be measured. Moreover, higher input of flue gas might be required to lower the pH and increase microalgae productivity.

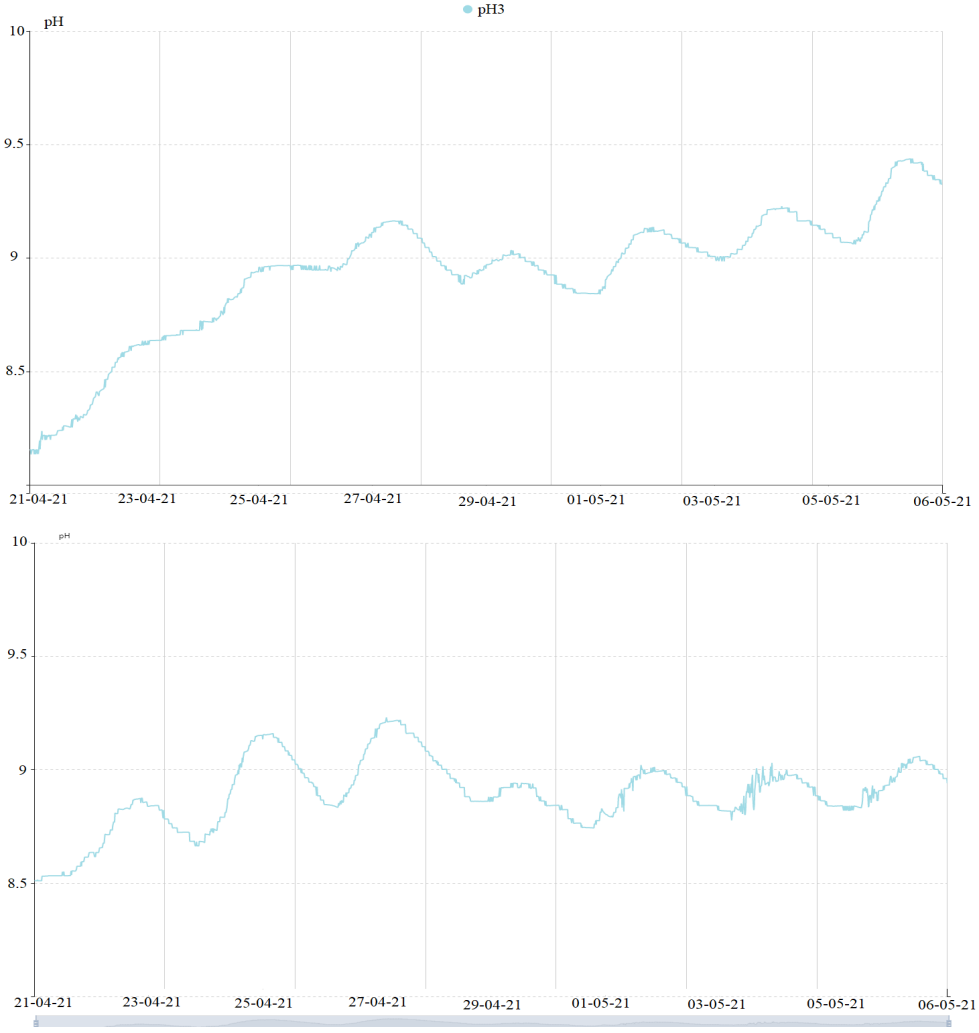


Figure 3.38. the pH of the microalgal pond during the biomass cultivation test.

Microalgae growth and nutrient removal

A liquid fraction of agricultural digestate collected from the Agro Iecava biogas plant was pretreated by filter centrifugation to remove excess solids prior to application to the microalgae pond. Pretreated digestate was analysed for the content of solids, COD and nutrients. The results of the chemical analysis of digestate are shown in Table 3.7. The total nitrogen content of digestate was high – exceeding 6000 mg L^{-1}. More than half of the total nitrogen was in the form of ammonia nitrogen (3360 mg L^{-1}). The content of nitrates was negligible (< 0.3 mg L^{-1}). COD 36300 mg L^{-1} was observed indicating a very high load of organic content. Digestate was diluted with tap water in order to decrease the nutrient load and lower the optical density and turbidity. Tap water was also analysed before the inoculation of ponds showing very low levels of nutrients and contaminants (Table 3.7). The nutrient content in diluted digestate as used for microalgae cultivation is shown in the last column of Table 3.7. (Growth medium).

Table 3.7.

Chemical analysis of pretreated digestate and water used for dilution.

	Units	Tap water	Digestate	Growth medium
Total nitrogen	mg L^{-1}	0.235	6180	12.7
Total phosphorus	mg L^{-1}	0.011	602	1.21
Ammonia nitrogen, $N\text{-}NH_4$	mg L^{-1}	<0.3	3360	8.4
Nitrates, $N\text{-}NO_3$	mg L^{-1}	<6	< 0.3	<0.3
Chemical oxygen demand, COD	mg L^{-1}	0.114	36300	56

Samples from the pond were taken every 3 days to monitor microalgae growth and removal of nutrients from the growth medium. Additional parameters were recorded on days of sampling with a portable multiparameter reader including total dissolved solids, turbidity, and conductivity, as well as temperature and pH (Table 3.8.). The cultivation pond was inspected also visually and can be seen in Figure 3.39.

Table 3.8.

Additional parameters of the cultivation pond during the cultivation test measured with a multiparameter probe reader.

Parameter	Unit	Date				
		21.04. Day 1	23.04. Day 3	27.04. Day 7	30.04*. Day 10	06.05. Day 16
pH	-	7.57	8.77	9.15	8.64	8.85
t	°C	16.3	16.5	12.8	NA	18.3
Total dissolved solids	ppm	285	364	210	NA	222
Turbidity	FNU	46.9	43	29	NA	14.1
Conductivity	µS cm^{-1}	573	727	420	NA	444

*on 30.04. measurements were not done (except pH) due to the flat battery of the reader.

After inoculation of the pond with *Chlorella sorokiniana*, the culture exhibited slower growth at the beginning but showed exponential growth from day 3 to day 7 (Figure 3.40). Culture density started to decrease after day 7 indicating that some limiting factors were present. Thereafter culture density continued to decrease till the end of the 16-day cultivation. Several factors might have impacted the culture growth during the cultivation experiment. Some of the environmental conditions were not optimal during the cultivation period. For most of the cultivation pond temperature was well below the optimum temperature of the species. The cultivation test was conducted during the springtime when the outside temperature fluctuates greatly. Sun in the springtime can be quite strong heating the greenhouse during the day but temperatures can decrease close to zero at night. Microalgae were able to grow in highly changing environmental conditions with fluctuating temperatures. The pond temperature decreased to only +12 °C on day 7 and can be considered as one of the possible explanations for decreasing growth on the following days. Exceptionally low productivity of *C. sorokiniana* has been reported in suboptimal temperatures [290]. It is also very likely that suboptimal temperature decreased the light energy requirements and therefore the maximum spring irradiance was excessive leading to photoinhibition.

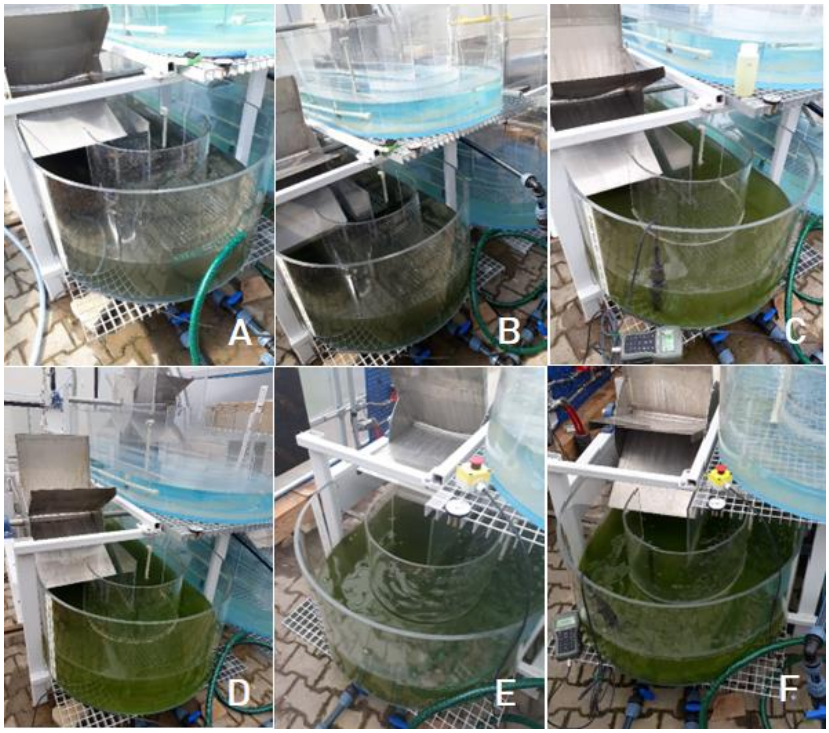


Figure 3.39. A. with digestate Day 1 (Apr.21), B. With digestate + microalgae Day 1., C. Day 3 (Apr.23). D. Day 7 (Apr.27), E. Day 10 (Apr.30), F. Day 16 (May 6).

The optical density of *C. sorokiniana* culture during test cultivation is shown in Figure 3.40. The highest density was reached on day 7, and then a sharp decrease was observed. The same can be seen with biomass yield which was halved on day 10 compared to day 7 and continued to decrease thereafter (Figure 3.41.).

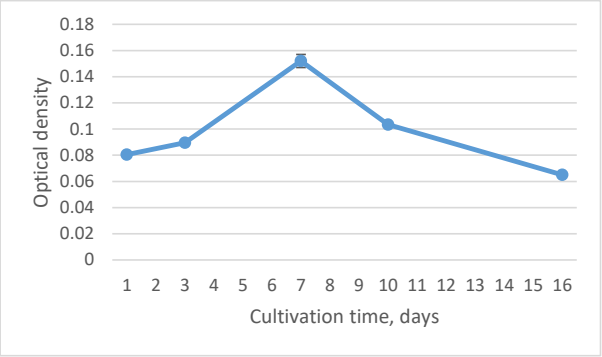


Figure 3.40. *C. sorokiniana* culture density during cultivation test in SMORP pilot ponds. Error bars indicate Standard deviation (n=2).

Although the growth rate of microalgae during the cultivation test was not among the highest reported, it must be considered that cultivation conditions were not optimal for *C. sorokiniana* during the initial trial due to unexpectedly low temperatures. The decrease in growth rate observed after day 7 might be due to several reasons including limited nutrients and light availability, relatively high pH, or some other factors. The addition of a higher flow rate of flue gases could contribute to lowering the pH. Additionally switching on the heating system might be useful when temperatures drop below the optimum but was not used in this trial.

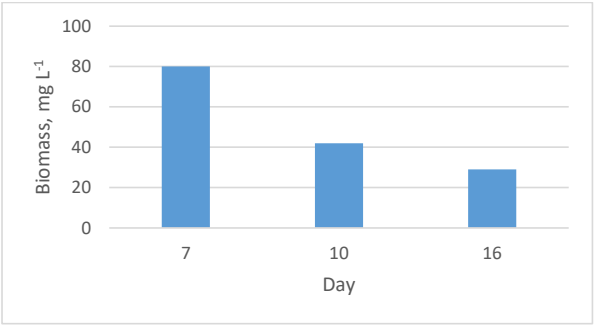


Figure 3.41. Biomass production during the cultivation test.

Although a relatively low growth rate was reached during the cultivation test, the nutrient removal rate seems very promising. During the first three days removal of total nitrogen, total phosphorus and ammonia was negligible most probably due to the adaptation of microalgae to the new growing conditions (Figure 3.42.). Nutrient removal increased considerably after the initial lag phase. Ammonia concentration increased slightly again at the last stage of cultivation.

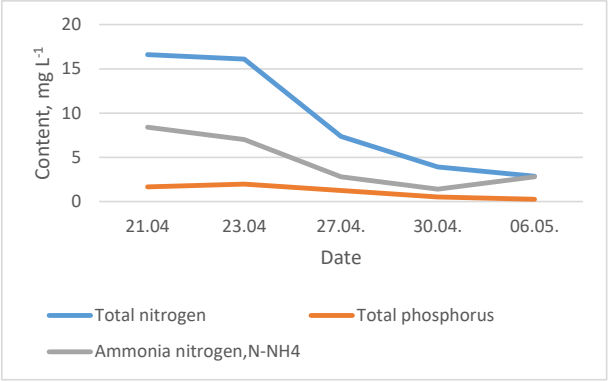


Figure 3.42. Removal of total nitrogen, total phosphorus and ammonia nitrogen by *C. sorokiniana* during 16-day cultivation.

The relationship between COD in wastewater and microalgae growth is complex and influenced by various factors, including COD concentration and microalgae species [291]. Generally, it is known that microalgae can remove COD from wastewater during growth. However, since microalgae are releasing organic compounds during cultivation, the actual COD in cultivation media might be rising. COD during the cultivation of *C. sorokiniana* was increasing (Figure 3.43).

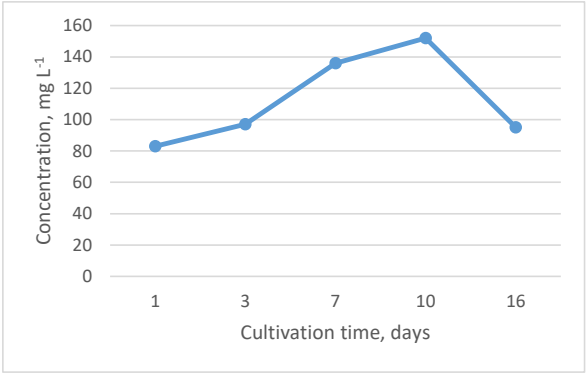


Figure 3.43. Chemical oxygen demand during the cultivation of *C. sorokiniana*.

High nutrient removal efficiency was reached at the end of the cultivation (Table 3.9). In total 83% of nitrogen, 85% of phosphorus, and 83% of ammonia nitrogen were removed from the growth medium during the cultivation of *C. sorokiniana*. The total nitrogen concentration of 2.86 mg L^{-1} was achieved corresponding to national legislation regarding requirements for treatment of wastewaters [292]. In agglomerations with less than 100'000 inhabitants, 15 mg L^{-1} of total nitrogen is the allowance for wastewaters, whereas the allowance of 10 mg L^{-1} of total nitrogen in agglomerations exceeding 100'000 inhabitants. Regarding phosphorus, 2 mg L^{-1} is allowed in agglomerations with less than 100'000 inhabitants, and 1 mg L^{-1} in agglomerations exceeding 100'000 inhabitants. 0.25 mg L^{-1} phosphorus was left in the growth

medium after digestate treatment with microalgae. It can be seen that digestate treatment with microalgae could meet the regulations at the present setup.

Table 3.9.

Nutrient removal from growth medium during *C. sorokiniana* cultivation.

Parameter	Initial level in the pond, mg L^{-1}	Removal, mg L^{-1}	Removal rate, %
Total nitrogen	16.6	13.74	82.8
Total phosphorus	1.67	1.416	84.8
Ammonia nitrogen, $N\text{-}NH_4$	8.4	7	83.3
Nitrates, $N\text{-}NO_3$	<0.3	NA	NA
Chemical oxygen demand, COD	83	-12	-14.5

The current developed technology seems promising regarding digestate treatment in the Latvian climate in suboptimal cultivation conditions. However, it must be taken into account that a high dilution rate of digestate was used for the application as a growth medium due to high optical density. The application of activated carbon adsorption as a digestate pretreatment method was shown to be a very promising technology for OD reduction; however, it must still be developed to be used for digestate treatment at a large scale, therefore it was not used for the initial trial in the novel cultivation system. A higher microalgae growth rate and consequently higher nutrient uptake could be expected in activated carbon pretreated digestate. Furthermore, the selected low-temperature tolerant strain *C. vulgaris* 211-11j must be tested under the current weather conditions, which is likely to lead to higher biomass productivity. Future work includes the cultivation of other selected candidate species in novel raceway ponds, evaluating biomass productivity and digestate treatment efficiency at different seasons.

CONCLUSIONS

The thesis addressed key aspects of energy sustainability and environmental protection challenges. It proposed a novel integration of microalgae cultivation technology within biogas plants, aiming to enhance microalgae biomass production while simultaneously achieving CO_2 sequestration and nutrient recycling. The research successfully developed and patented a microalgae cultivation system optimized for colder climates, identified suitable microalgae strains, and demonstrated the feasibility of coupling the system with existing biogas operations.

The thesis presents a comprehensive framework for integrating the novel microalgae cultivation system into existing biogas plant operations. This integration has the potential to enhance biomass security, reduce transportation costs, and provide an innovative approach to managing digestate overproduction. The findings offer significant contributions to the fields of renewable energy and circular economy, proposing an innovative approach to leveraging waste streams for energy generation. The study highlights the potential of microalgae as a sustainable resource, not only for biogas production but also for the generation of valuable by-products. Despite facing challenges such as scale-up complexity and climate dependency, the research opens up opportunities and viable solutions for enhancing the sustainability of biogas plants. Therefore, the thesis contributes valuable insights and tools for advancing the bioeconomy towards a more sustainable and circular model.

More specifically in connection to Block 1 and Block 2 of the research framework, the following key results were identified:

1. A novel system designed for microalgae cultivation in colder climates has been developed and patented. This system overcomes the limitations of traditional cultivation systems, offering a promising solution for year-round biomass production in regions with challenging climates, such as Latvia.
2. Microalgae strains suitable for the Latvian climate were identified. *C. vulgaris* 211-11j, *C. sorokiniana* 211-8k and *C. reinhardtii* 11-32b are promising strains for outdoor cultivation in the Latvian climate conditions. These strains show potential for high biomass production using agricultural digestate, marking a step forward in developing efficient microalgae-based bioenergy solutions.
3. *C. vulgaris* 211-11j was identified as a potential low-temperature strain for winter biomass production in Latvian climate conditions.
4. Various environmental and cultivation conditions were shown to highly affect the microalgae biomass production, the optimal conditions mostly being species-specific.
5. The optimal CO_2 concentration required for maximum growth was shown to be species-specific. An increased CO_2 concentration of 5% leads to increased biomass of all studied microalgae offering a potential tool for biosequestration of CO_2 from biogas production flue gas.
6. The research demonstrated the potential effective use of agricultural digestate and flue gases from biogas plants as low-cost nutrient and carbon sources for microalgae

growth. This approach reduces the operational costs associated with microalgae cultivation and contributes to nutrient recycling and greenhouse gas mitigation.

7. *C. sorokiniana* can effectively remove nutrients from digestate in outdoor conditions performing digestate treatment and meeting effluent standards for discharge for nitrogen and phosphorus.

The findings from the thesis open several avenues for further research, particularly in the areas of optimizing the system for diverse environmental conditions, setting the ground for exploring the economic feasibility of large-scale implementation, and exploring the range of value-added products from microalgae biomass. Additionally, this work lays a foundation for practical applications, encouraging biogas plant operators to consider the integration of microalgae cultivation into their operations as a viable strategy for sustainable growth.

This work contributes significantly to the transition towards a more sustainable and resilient bioeconomy, highlighting the essential role of innovative technologies in transforming waste into wealth.

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Novel Stacked Modular Open Raceway Ponds for Microalgae Biomass Cultivation in Biogas Plants: Preliminary Design and Modelling

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***Abstract* – Microalgae hold great potential as a source for renewable energy due to their high photosynthetic efficiency, high growth rates and independence from fertile agricultural lands. However, large-scale cultivation systems of microalgae biomass are still not economically viable mainly due to the difficulties with maintaining optimum growth conditions of microalgae in open pond systems and high costs of biomass cultivation and harvesting. Here we propose the Novel Stacked Modular Open Raceway Ponds (SMORPs) system for microalgae biomass cultivation to be integrated in biogas production plant. The proposed technological solution will eliminate the drawbacks of current microalgae cultivation technologies, mainly, will reduce the land use, improve lighting conditions and reduce the cost of cultivation as a result of the application of waste products from biogas production, i.e. anaerobic digestion effluent and flue gas. In this study we propose the initial design of the SMORP concept and a microalgae biomass kinetic model as a simple approach to screen microalgae strains potentially applicable for large-scale ponds. The developed tool is also useful to evaluate the potential benefit of additional artificial LED light sources and to assess the maximum biomass growth rate with minimal light intensity.**

***Keywords* – Biogas; Chlorella spp.; effect of light intensity; kinetic model; microalgae; open raceway pond**

1. INTRODUCTION

The use of microalgae as a promising renewable energy source has been growing within the last decade due to the specific quality and characteristics of microalgae [1], [2] and the capability to cope with climate change from CO_2 anthropogenic emissions.

Microalgae are photosynthetic organisms able to fix solar energy and carbon dioxide into biomass and oxygen production, one of their main characteristics is good adaptability to new growing conditions [3], [4]. Due to their high CO_2 fixation rate, microalgae can grow well under high level of CO_2 making them a beneficial interface acting like a bio-filter for the treatment of exhaust gases and flue gas emissions from thermal and industrial plants [1]. The photosynthetic process of microalgae is higher in efficiency than in terrestrial plants [5], [6], moreover, in comparison with land-based feedstock, microalgae present several other key

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advantages compared to the terrestrial biomass like 5–10 faster growing time and higher biomass production rate [7], [8]. In addition, microalgae cultivation can be placed in unproductive and/or remote areas avoiding competition with food crops [9].

Microalgae farming is also providing an overall environmental benefit to remove macropollutants and nutrients (e.g. N and P) [10]–[12] in different environments. In fact, different outputs from several wastewater treatment systems can be used as nutrient sources such as: domestic water, industrial water, municipal water [13] and the liquid fraction of a biogas digestate (i.e. centrate) [10], [14], [15]. Within the use of these nutrient streams there is the possibility to have even a double-fold advantage in terms of formation of algae/bacteria consortia [16].

The nutrient supply is a key aspect in microalgae farming. Various species of microalgae vary in their need for nutrients. However, the requirements for essential nutrients are similar for most microalgae species and include macro nutrients (i.e. C, N and P), as well as K and Fe. Large amounts of nutrients are required for large scale cultivation of microalgae. It has been estimated that for a production of 100 t of microalgae biomass approximately 200 t of CO_2, 5 t of nitrogen and 1 t of phosphorus are needed [17].

As mentioned, biogas centrate can be used as a nutrient feedstock; however, the centrate use can present relevant disadvantages. In fact, the liquid phase of digestate is characterized by high turbidity and ammonia content [18]. Turbidity caused by dissolved and suspended material has been considered as a major drawback of digestate [19]. This suspended matter causes light scattering and absorbance limiting the availability of light to microalgal cultures. Ammonia inhibition is another major drawback of digestate as a nutrient source. Ammonium concentration in digestate from agricultural waste typically ranges between 500 and 1500 mg NH_4^+ L^{-1} [20]. High ammonium concentrations of more than 1000 mg L^{-1} can lead to inhibitory effects of microalgae growth [21]. Ammonia content can be reduced by diluting the digestate. Adaptation of microalgae to high ammonium concentrations is likely to occur [20].

A large number of microalgae species present a higher lipid production than conventional crops [20]. This is addressing the use of the microalgal biomass to the production of biofuel and in particularly biodiesel [1] strengthening the role of microalgae as a potential substrate to reduce the food-versus-fuel dilemma [22], [23].

The biomass transformation processes can also involve other types of transformation pathways such as thermochemical, biochemical and photosynthetic microbial fuel cell thereby creating an opportunity for a flexible and viable biorefinery concept with a large fuel portfolio (i.e. syngas, bio-oil, bioethanol, biogas/biomethane and biohydrogen) and energy final transformation [24], [25].

However, there are several concerns about the overall feasibility and viability of a full-scale-based microalgae farming system both from technical and economic perspectives for several reasons. One of the main obstacles is the difficulty to achieve proper regulation and optimization of the microalgae cultivation system, particularly in relation to several interrelated input parameters and [1] potential limiting factors such as light and temperature [10].

Specifically for biodiesel, several studies report that despite the efforts made, for the industrial production it is not yet economic viable, especially due to the high cost of biomass cultivation and harvesting [26]. The study from Husesemann et al. [27] identifies the minimal productivity of 30 g/m^2-day as an economically viable threshold for open pond cultivation.

Looking towards biogas production through wet anaerobic digestion of microalgae biomass into methane [28], the recovered energy makes the overall process more viable if the potential use of the digestate as a fertilizer or biostimulant is considered [10], [29].

The potential mass transfer of CO_2 excess from the industrial process to the algae ponds through a simple sparging system using porous material is a beneficial aspect related to the implementation of a microalgae-based system, nevertheless it should be considered that the CO_2 absorption in an open pond only has an efficiency of 10–20 % [30].

Several studies show that rising CO_2 concentration in algal growth medium have enhanced algal productivity, however, too high CO_2 concentrations inhibit algae growth [31]. It has been noted that carbon supply is a major factor limiting the biomass production in raceway ponds [32]. Flue gases with CO_2 concentrations ranging from 5 % to 15 % (v/v) have been successfully introduced directly into ponds [33]. Although SO_x and NO_x are known as toxic compounds for microalgae [34], it has also been observed that SO_x and NO_x impurities in flue gases have no negative effect on microalgae cultures [35]. It has been speculated that high-rate algal ponds need a supply of at least 5 % (v/v) CO_2 to maintain high growth rates [30]. It has been estimated that the cost of pure CO_2 constitutes from 8 to 27 % of the total biomass production costs [36].

Nowadays microalgae cultivation technology in pilots and/or on a pre-industrial scale is focused on open or closed systems [8], [15]. The first ones are systems directly exposed to the atmosphere. The commonly used types are open raceway ponds (ORWPs) [37], [38] and High Rate Algal Pond (HRAP) used for wastewater treatment [39]. ORWPs have a relatively low cost of construction, installation and maintenance and a simpler operational system [8]. The disadvantages of ORWPs are mostly connected to system contamination with unwanted algae species, evaporation (that need to be balanced) and sometimes the lack of an automatized growing control system [40]. ORWPs also require a large land area. Moreover, the biomass concentration is relatively low [8] quantifiable in 10–25 g dry matter of algae biomass/m^2 [8] and the low surface to volume ratio (i.e. 5–10 m^{-1}) is a limiting factor for the productivity [41].

The second type of microalgae cultivation technology is based on closed systems also called photo-bioreactors (PBR). They can have different shapes: tubular reactor, flat plate reactor and pyramidal [42]. The typical most common types are in the shape of tubular, flat-tank, bubble column and serpentine [8]. The main pros of PBRs are the control of algae growth – which leads to high productivity of algal biomass – and the optimization and control of the culture system conditions, in fact avoiding the contamination with other algae species [43], [44]. The study from Jankowska *et al*. (2017) presents biomass concentration in the range of 20–100 g dry matter of algae biomass per day per m^2 [8]. Biomass production rates with PBRs are considered higher than ORWPs, a realistic figure can today be estimated as 60–70 tons ha^{-1} yr^{-1} [45].

Cultivation systems can also be classified according to the use of the artificial light sources or natural light from the outdoor environment. In contrast to open ponds, closed reactors are oriented towards mono-species algal culture and a control system for optimization of nutrients, temperature, CO_2 and pH, resulting in higher productivity per equal system volume and unit of area. PBRs can have very high concentrations due the higher surface-to-volume ratio compared with ORWPs [46]. Nevertheless, PBRs present a higher initial cost than ORWPs and are very dependent on the optimal selection of a specific microalgae strain for cultivation [46].

Looking towards the minimization of operational costs, energy consumption together with the maximization of GHG savings necessary for viable investment in microalgae production,

ORWP technology has a lower energy demand compared to PBRs and a lower complexity of the optimization system and harvesting system [8].

In order to solve aspects related to economic viability (e.g. reduction of the energy cost in the plant management and operational system), a microalgae-based cultivation system can be better considered in terms of an integrated and/or side-stream process concept applicable to different wastewater systems including biogas. This can in fact more beneficially contribute to reduce the energy cost in the overall plant management and operational system [10].

Several ORWP pilot projects have already been realized [12], [15], [47] in terms of finding optimal synergies among the use of CO_2 flue gases from biogas combustion in CHP unit, the use of digestate and excess heat.

Nevertheless, none of these projects considered the possibility to develop Stacked Modular Open Raceway Ponds (SMORP) for microalgae growing as a novel hybrid technology which tries to take the best advantages from the two types of existing microalgae cultivation systems. The proposed novel technology is based on open raceway ponds (ORWPs) for the cultivation of microalgae. However, with an improved mixing system, CO_2 absorption system, lighting system, modular design and use of transparent material, the proposed technology has significant advantages over the currently available ones.

In fact, the current research and studies in the field have shown major problems related to the regulation of optimal microalgae growing conditions as well extensive land use for the ORWPs. Thanks to the combined (sunlight and artificial) lighting system with LEDs, it would be possible to optimize the diurnal and annual lighting cycle. Moreover, having the proper light wavelength (e.g. research has shown that using LEDs with red and blue light ratio 50:50 has a beneficial effect on the microalgae growth) would increase biomass production by 16 %. Modular and stacked cultivation pond design gives growth media a proper area-to-volume ratio (and micro-algae concentration) and reduces the amount of used land space by 40 %.

Thus, there is a key research question if it is possible to improve ORWPs systems to higher productivity while keeping the low cost of investment as a main advantage. The main challenge is the development of mass microalgae cultivation with lower energy requirements, thus further improving the GHGs balance and the whole LCA of the system [41].

Two levels of investigation are required for a successful cultivation of microalgae in outdoor raceway ponds. It is necessary (1) to perform the screening of algae strains and estimate the optimal cultivation conditions at laboratory scale to determine the potential strains and (2) to assess their performance in outdoor cultivation ponds. Productivity rates in open ponds are commonly lower compared to productivity achieved at a laboratory scale. Therefore, it is important to validate the performance of selected strains in outdoor pilot-scale conditions depending on specific identified variable of optimization like temperature [48]–[51], light [52]–[55], nutrients, and CO_2 supply.

The overall focus of this research is the finalization of an integrated microalgal culturing pilot system coupled with a biogas plant. The novelty of the present study consists of the presentation of the preliminary design of an ORWPs system using the proposed SMORP concept namely Stacked Modular Open Raceway Ponds. The overall research aims to evaluate the feasibility of applying a sort of microalgal-based biofilter process as a treatment and management method for the liquid digestate and flue gases from the CHP unit in biogas plant in Latvian climate conditions. A biomass growth model capable of assessing the effects of the light intensity on the specific growing rate and biomass concentration is also proposed. Based on laboratory tests the provided model is applied to a specifically selected microalgae stream under constant light and temperature conditions in order to be further used as a screening method to select algae species.

The paper will explain in the section related to the applied research method the main steps related to: the design of the novel Staked Modular Open Race Pond (SMORP), the selection of the specific material for the pond, the laboratory stand and the measurements of the microalgae biomass in laboratory conditions, the initial selection of the algae strain, and the adopted kinetic model.

2. METHOD

The applied research method is based on three main parts:

- Cultivation pilot stand design;
- Execution of laboratory tests depending on a single factor affecting microalgae growth rate;
- Definition of a simple biomass predicting microalgae kinetic model depending on two species-specific and two physical parameters.

2.1. Pilot Design

The overall proposed technological scheme, related to the SMORP pilot project to be realized, would enable a biogas operator to produce energy and/or biomass creating benefit from the management of waste product(s) and emissions (i.e. digestate and CO_2). At the same time, the pilot concept presented in Fig. 1 would be beneficial as a solution for the issue of digestate storage and transport.

The overall scheme should be through a system integrated into an existing biogas plant for which a microalgae-based system and its harvesting can be considered as a side-stream processing module. This solution will, in fact, create a valuable interface to transform the main environmental drawbacks from the anaerobic digestion related to the management and disposal of the digested biomass (digestate) and CO_2 reduction from the exhaust gas use (see Fig. 1) and overall a closed-loop technological system.

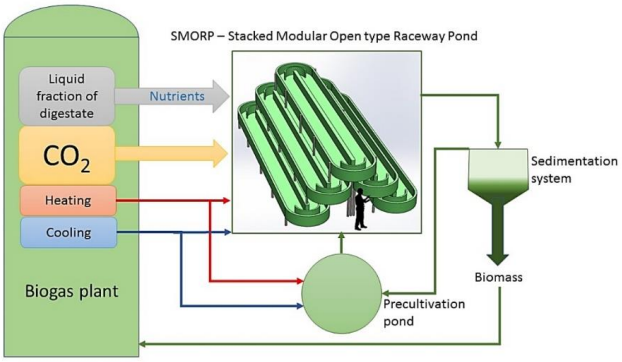


Fig. 1. Integrated concept of the Stacked Modular Open Raceway Ponds (SMORP) in biogas plant.

The pilot is based on a novel technological solution of Staked Modular Open Raceway Ponds (SMORP). The main aim addressed is to provide benefits towards: the reduction of

land use (a drawback in all ORWP cultivations), the light limitation (due to the lack of light penetration at the bottom part and from lateral surfaces of the conventional ORWPs), and the higher investment costs of the PBR in respect to the ORWPs. The pilot is thus representing an opportunity for an "hybrid" optimized system among the state-of-art ORWPs and PBRs [56]. In fact, the typical material (i.e. acrylic) normally used for a photobioreactor would be used within an ORWP system. The proposed concept takes into account a combined sunlight and artificial lighting system with a low power consuming LEDs and a proper light wavelength in order to balance the light variation and shadow made by the upper ponds, in turn compensating with a higher biomass yield as presented in Fig. 3.

The design, the operation and monitoring of the pilot SMORP module was supported by the latest best practices for microalgae cultivation as explained within the project *EnAlgae* [45] and from the technological solutions according to Chisti [46] and Yadala [57] widely used in commercial production of algal biomass.

The main characteristics of commercial ORWPs are: elliptical shape, depth of 15–30 cm, velocity of 15–30 cm/s maintained with paddle wheels, areas among 100–1000 m^2 and length (L) to width (W) ratio ≥10 [41].

For the pilot, the single modular pond presents an oblong shape shallow pond having L/W equal to 2 (i.e. L = 2 m, W = 1 m), an area of 3.6 m^2, a height (H) of 50 cm (considering 40 cm of culture depth) have been defined for the proposed SMORP pilot (see Fig. 2). Some studies have shown that a higher L/W ratio (L/W ≤ 11) is better in terms of flow dynamics of the system [46], [57]. However, one of the prime objectives of the proposed pilot concept is to evaluate the overall effectiveness of a "stacked modularity" of the open pond system consisting of a number of ponds with a comparatively low L/W ratio for a better mechanical resistance of the structure.

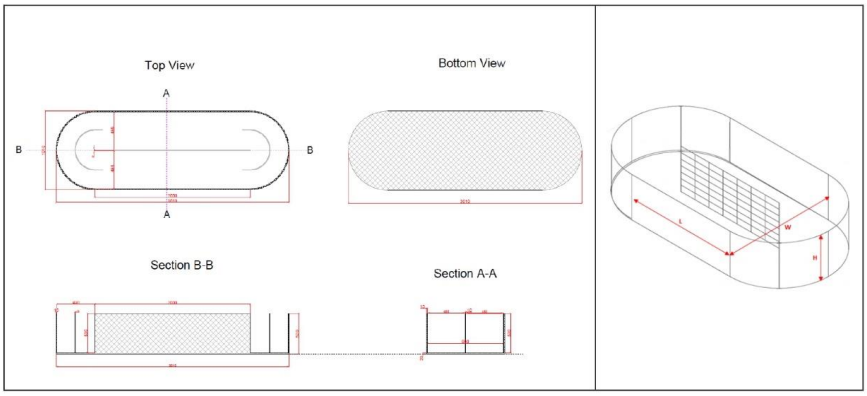


Fig. 2. SMORP single pond shape.

SMORP system is designed with a CO_2 sparging system and energy efficient LED lighting system to balance the energy requirements for the artificial light with a higher biomass production per single unit of used land.

Due to its unique configuration, a transparent material has been selected (acrylic) for construction of SMORP ponds, hence, increasing light penetration through the system.

In Fig. 3 is the proposed process flow diagram for one pond of SMORP system.

The main components of the identified technological scheme are reported below:

- *Liquid Digestate as Nutrient Source*: digestate discharge from the biogas plant is stored in a continuously stirred holding tank. The digestate is fed to the pond by automatically controlled peristaltic pump. Feeding volume is affiliated with the outcomes of laboratory experiments and characteristics of digestate. Critical characteristics of digestate such as pH, ORP (Oxidation Reduction Potential), Turbidity, Temperature will be continuously monitored and integrated with pump operation;
- *Flue Gas as Carbon Source*: flue gas emitted from biogas cogeneration unit will be used as a carbon source for growth of biomass. Gas is fed to the system through microporous tubular diffusers installed at the bottom of each pond. The effect of mixing of flue gas with ambient air on growth of biomass will also be tested by the system;
- *Mixing Mechanism of Pond Culture*: adequate mixing is necessary to maintain culture flow in suspension maintaining homogeneity and most importantly removing dissolved oxygen produced by photosynthesis. Mixing will be performed using a paddle wheel consisting of flat blades. Since the power consumption is greatly affected by the intensity of mixing, it is necessary to maintain the minimum turbulence required in terms of energy efficiency of the system;

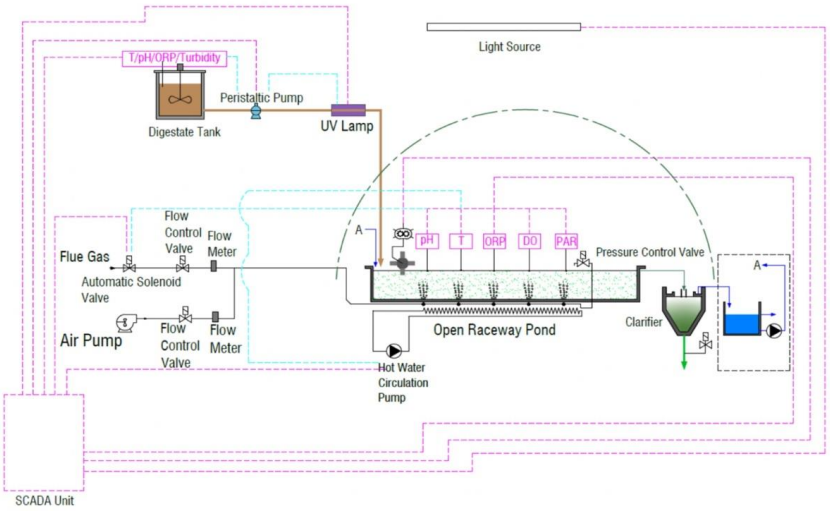


Fig. 3. SMORP technological scheme.

- *Light Source*: energy efficient LED lights are installed into the pilot allowing the maximum irradiation throughout SMORP configuration;
- *Monitoring of Key Parameters*: sensors are planned to be installed in the pond to measure critical parameters which affect growth of microalgae such as pH, PAR (Photosynthetic Active Radiation), ORP, Temperature, DO (Dissolved Oxygen). All signals will be synchronized with a SCADA system which is remotely accessible.

Nutrient detection sensors (i.e. NH_4^+, NO_3, K, Cl) will be installed in a second stage;
– *Green House*: the function of the greenhouse is to protect the cultivation site to external weather conditions and to reach the optimal temperature for the microalgae during the wintertime.

2.2. Laboratory Stand and Measurements of Biomass

In order to have a better optimization of the performance of the microalgae growth rate in the pilot SMORP cultivation and to better estimate the effect of several external parameters (i.e. light intensity, temperature, nutrient supply, dissolved CO_2 and O_2) [1], [55] specific laboratory tests were performed. At this stage of the research these tasks were executed by counting microalgal cells in the culture using a microscope with the Neubauer hemocytometer. Cell counting was done in the centre square of the hemocytometer following a standard procedure [58]. Cell density was calculated according to the Eq. (1):

$$cells / ml = \frac{average\ number\ of\ cell\ per\ square\ dilution - dilution\ factor}{Volume\ of\ square\left[ml\right]} \tag{1}$$

The selected microalga (i.e. *Chlorella vulgaris* strain 211-11j) obtained from the SAG Culture collection of algae at Göttingen University was maintained in a typical liquid BG-11 growth media at room temperature in low light conditions and hand mixed daily to avoid settling of cells. Sub-culturing was done approximately once per month to keep the algae culture growing and in healthy condition.

For light intensity, test algae were grown in batch cultures at +24 °C on an orbital shaker (DOS-10L, Elmi) at 150 rpm for 10 days. *C. vulgaris* cultures were cultivated in 500 ml Erlenmeyer flasks containing 200 ml BG-11 medium at pH 7.4 under a photoperiod of 16:8h (light/dark) providing no additional CO_2. Natural white LED lights were used, and light intensity was set to 50, 100, 200 or 400 µmol photons $m^{-2}s^{-1}$. The initial concentration of *C. vulgaris* cultures was ~2 × 10^6 cells/ml. Daily growth rate was measured by counting cells with Neubauer hemocytometer.

The selected light intensity to finalize the kinetic model was 50 µmol photons $m^{-2}s^{-1}$ with a maximum growing rate (μ_{max}) equal to 0.25 day^{-1}. This value was selected because highest growth rate of *C.vulgaris* in light intensity test was observed under this light intensity.

2.3. Kinetic Models

Process modelling is required as a key aspect to evaluate the performance of a microalgae cultivation technology for the explanation of growth kinetics. Several kinetic models described microalgae growth as descriptive and explanatory models. Explanatory models are mainly made to assess causal relationship or the fundamental system dynamics. Empirical model normally represents this category and are developed supported by a regression analysis of experimental data. Kinetic models can depend on single or multiple factors directly affecting the microalgae growth (i.e. light intensity, nutrient availability, dissolved CO_2 concentration, temperature, and dissolved oxygen concentration) [1].

The kinetic models are focused to evaluate the trends of the six microalgae growth phases: lag phase, exponential phase, linear phase, declining growth phase, stationary phase, and death phase [1]. In the lag phase the presence of non-available biomass defers the real growing prior the exponential phase in which cells grow according to an exponential trend [59], [60]. In this time step, light intensity and nutrients are not representing constraints for microalgae

growth. In the linear growth phase, microalgae growth decreases until the rapid decline to the death phase normally explainable with lack of nutrients, uncomfortable heat, negative effect of pH, or contamination. Normally growth kinetic models are defined as directly linked with specific nutrient concentration.

In order to find favourable microalgae strains for culturing it would be useful to create a simple and flexible screening tool for testing several microalgae strains before the outdoor conditions in cultivation ponds. In this direction, it has been found that biomass growth models present two key common aspects: the assessment of the effect of the light attenuation and the evaluation of the biomass growth depending on both incident and absorbed light. Beer-Lambert's Law (see Eq. (2)) is a well-known method in which the main affecting parameter is the light intensity that declines over the depth of cultivation ponds.

Regarding the relationship between biomass growth and incident or absorbed light, most models employ multifactor regression models implemented in rather complex tools hardly usable as screening tools. Due to this criticality, a biomass growth model depending on measurable species-specific model input parameters namely: the specific growth rate function of light intensity, and the biomass light absorption coefficient is proposed in this study.

For this specific aim, the growth is assessed by the light attenuation in agreement with Beer-Lambert's Law [27]. In fact, Beer-Lambert's Law describes an exponential decrease of the light intensity, $I(z)$, as a function of light penetration depth z.

The model takes into account two physical and two species-specific biological inputs: incident light intensity, culture depth, and the biomass light absorption coefficient and the specific growth rate as a function of light intensity.

$$I(z) = I_o \cdot e^{-k_a B z}\,, \tag{2}$$

where

I_O Incident light intensity at the bioreactor or pond surface, μmol photons $m^{-2}s^{-1}$;
B Biomass concentration, g/L;
k_a Biomass light absorption coefficient, $g/L^{-1}m^{-1}$; assumed equals to 64.7 from [27];
z Depth of light penetration, m.

Due to the increase of the microalgae concentration B with increasing pond depth, the effect of the light attenuation is reinforced over time, according to the general formula expressed in Eq. (3) [27]:

$$\mu = \mu_{max} \cdot f(I)\,, \tag{3}$$

where

μ Specific growth rate, day^{-1};
μ_{max} Maximum specific growth rate, day^{-1};
$f(I)$ Dimensionless function dependent on the light intensity species-specific and experimentally determined.

For the proposed kinetic model, the empirical model of Steele [27], [61] has been considered in terms of light-limitation and photoinhibition. This method is widely used and is able to describe the effects of light-limitation towards the ratio I/I_{opt} and photoinhibition using an exponential expression like expressed in Eq. (4):

$$\mu = \mu_{max} \frac{I}{I_{opt}} e^{\left(1-\frac{I}{I_{opt}}\right)}, \quad (4)$$

where

μ Specific growth rate, day^{-1};

μ_{max} Maximum specific growth rate, day^{-1};

I Light intensity, μmol photons m^{-2}s^{-1};

I_{opt} I at maximum specific growth rate μ_{max}, μmol photons m^{-2}s^{-1}.

During the exponential growing phase, the algal cells grow and divide with an exponential behaviour just before the linear growing phase occurring when growth slows down due to light limitation effect, or nutrients or inhibitors become a constraint. During this phase the specific growth rate (μ) in response to light intensity (I) will increase and the biomass concentration during time interval Δt will be accordingly adjusted to [27]:

$$B(t+\Delta t) = B(t) \cdot e^{\mu \Delta t}, \quad (5)$$

where

B Biomass concentration, g/L;

Δt Time step, day;

μ Specific growth rate, day^{-1}.

Once biomass light absorption coefficient (k_a, defined in Eq. (2)) and the correlation among specific growth rate (μ) and light intensity (I; Eq. (3)) are defined for a specific microalgae species a ''step-by-step'' increase in biomass concentration as a function of time can be calculated using Eq. (4). The effect from temperature is not considered (i.e. constant temperature), nevertheless incident light intensity (I_0) and the culture depth (d) must be assumed. The algorithm proposed in Fig. 4 has been developed for and implemented in an excel visual basic platform.

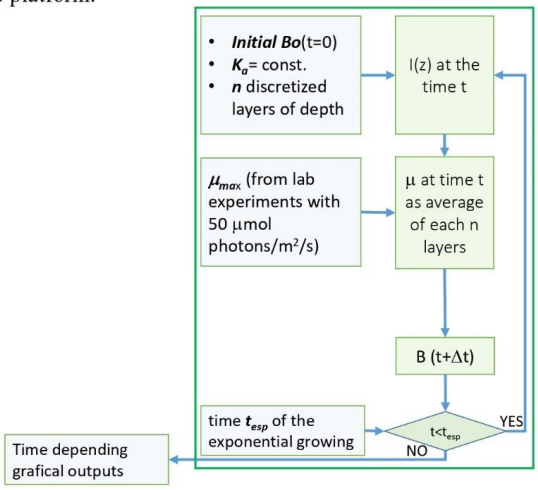


Fig. 4. Light-limitation and photo-inhibition kinetic model algorithm readapted from [25].

The algorithm is explained as follows:

- Selection of an initial biomass concentration at an initial time t_0;
- Discretization of the culture volume into n equalized parallel volume layers orthogonal to I_o, I at the midpoint of each of the n culture volume layers;
- Calculation of μ in each of the n culture volume layers;
- Calculation of the biomass concentration in each of the n culture volume layers during time interval Δt;
- Calculation of the new biomass concentration $B(t+\Delta t)$ in the entire culture;
- Averaging the biomass concentrations of all n culture volume layers, recalculate the previous steps till the desired time set for the exponential growth (t_{esp}).

3. RESULTS AND DISCUSSION

3.1. Selection of the Microalgae

The selection of the specific microalgae for ORWPs or PBRs is site specific. Nevertheless, from studies reporting both pilot and already industrial cultivation, the selection of microalgae strain is defined with a screening method focused on specific attributes of the cultivated microorganisms. These are: growth characteristics, lipid contents, C/N ratio or key factors like final end use of the microalgae, adaptability to the growing conditions, potential growth rate and productivity depending on abiotic effective parameters (i.e. light, pH, temperature, nutrient supply, type and composition of injected flue gases, simplicity of harvesting).

The report of the *EnAlgae* project [47] presents best-case practices of pilot plants utilizing among the others: *Chlorella spp., Scendesmus spp., Nannochlopsis spp., Phaeodactylum spp., Chlamydomonas spp*. The study of Marazzi *et al*. [10] is highlighting that the most cultivated algae in ORWPs i.e. (*Dunaliella salina, Arthospira sp.* and *Clorella spp.* [10]) are those that can be grown in extreme and aggressive environments. A pilot project similar to the one proposed in this research supports a cultivation pilot plant mainly using *Chlorella spp*. and *Scendesmus spp*. Similarly, the extensive use of *Chlorella spp.* for both OPWPs and PBRs is also highlighted in Lee *et al*. [1].

Independently from the theoretical section of the algae strain, there is a need to have monoculture stocked in laboratory conditions to both have a stock culture for further tests in laboratory and to inoculate the cultivation ponds in a scaled-up system. In literature there are several findings about *Chlorella* spp. growing tests in laboratory conditions like in [1], [62].

For this reason, *Chlorella vulgaris* has been selected in the research described in this paper.

3.2. Study of the Material

Usually glass, fiber glass, PVC, Polyethylene (PE), Polycarbonate (PC), HDPE polymer, Plexiglas or acrylic have been used as the basic material for construction of PBRs [63]. Nowadays plastic materials are used more than glass due to characteristics of lower costs, higher light transmission, and facility of transportation, lower maintenance, and resistance to exposure to chemical compounds, durability and better mechanical properties. Among the others, Linear Low Density Polyethylene (LLDPE), High Density polyethylene (HDPE), together with fiberglass, polypropylene, polyethylene, ABS can be also an appropriate material. Nevertheless, if compared with acrylic material, the opaqueness of HDPE could be still considered an inhibiting factor for light penetration. For this reason, acrylic material has

been selected for the pilot SMORP cultivation technology. Acrylic material is also used in several pilot stands [64] with the capability to be easily shaped for rounded geometry. For this reason, such materials have been selected for the SMORP concept as a promising solution. In this way, according to the proposed SMORP concept, the effect of natural light can be maximized.

3.3. Kinetic Models of the Light-Dependent Photosynthetic Activity and Biomass for the Selected Microalgae

From the application of the calculation routine according to the proposal algorithm implemented in *Microsoft Excel* visual basic and presented in the section 2.3, it has been assumed a number of layer equals to 30 with 1 cm thickness with an assumed calculation interval of $\Delta t = 0.1$ day.

Fig. 5 and Fig. 6 present the first results of the model based on the laboratory test for *Chlorella vulgaris* implementing Eqs. (2–4).

Specifically in Fig. 5 the results from the assessment of the light intensity changes taking into account the Beer-Lambert's Law are presented. For the determination of the model outputs, authors made the following specific assumptions:

- Initial biomass concentration at a time t_0 (B_0) equal to 0.1 g/L (as optical density), according to available information from literature [62], [65];
- Incident light intensity on surface equal to 50 μmoles photons/m^2/sec, according to performed laboratory tests, in order to avoid photo-inhibition effects;
- Light absorption coefficient k_a equal to 64.7 $(g/L)^{-1}m^{-1}$, empirically found for *Chlorealla* spp. in the study of Hausemann *et al.* [27];
- Type of microalgae: *Chlorella vulgaris* 211-11j.

The trend presented in Fig. 5 from the implementation of the Beer-Lambert formula is showing that light is attenuated through absorption and scattering depending on light path length and cell concentration similarly like reported in the paper of Yun *at al.* [66]. According to the authors, this can be explained taking into account an average photon flux density within a volume-averaged value of the depth dependent photon flux density.

Fig. 6 shows the variation of the growth rate (μ) according to Steel's formula simulating both light-limitation and photo-inhibition over the depth of the ponds.

For determination of the graphical outputs in Fig. 7(a, b) authors made the following main assumptions:

- Maximum growing rate (μ_{max}) from the performed laboratory tests = 0.25 day^{-1};
- Optimal incident light intensity at the maximum growth rate (μ_{max}) from the laboratory test = 50 μmoles photons/m^2/sec;
- Light absorption coefficient k_a = 64.7 $(g/L)^{-1}$ m^{-1} (from Hausemann *et al.* [27]);
- Exponential growth (t_{esp}) = 8 days;
- Maximum depth if the theoretical ponds equal to 30 cm;
- Type of microalgae: *Chlorella vulgaris* 211-11j.

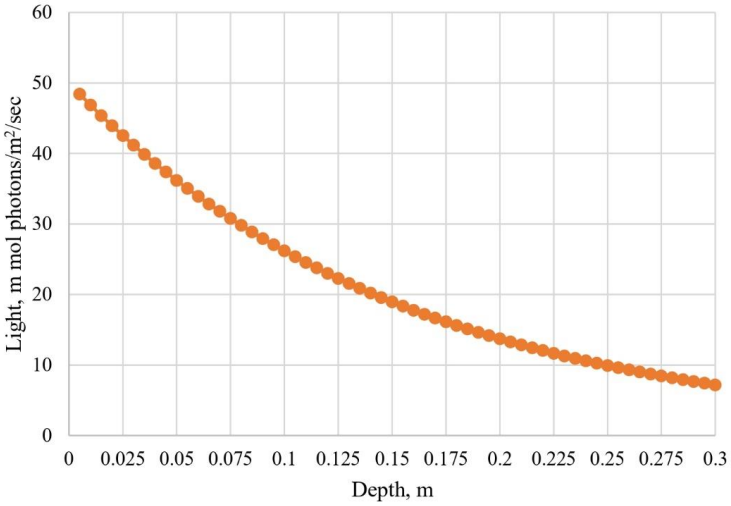


Fig. 5. Light-limitation effects according to Beer-Lambert's Law [27].

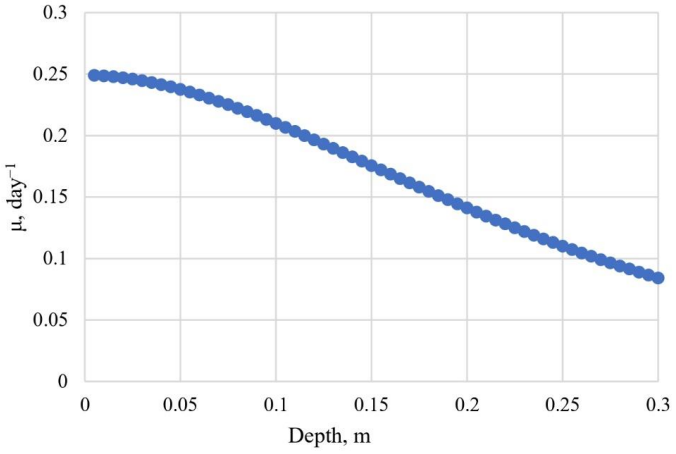
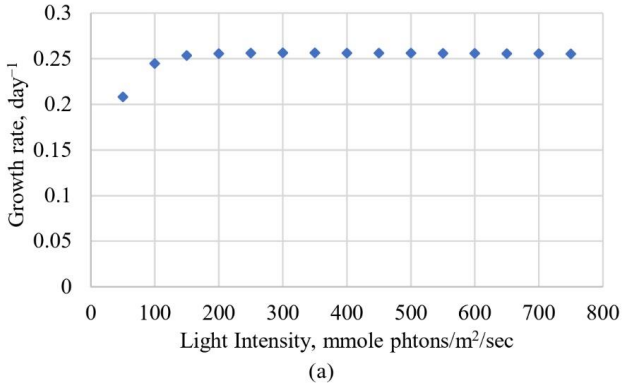


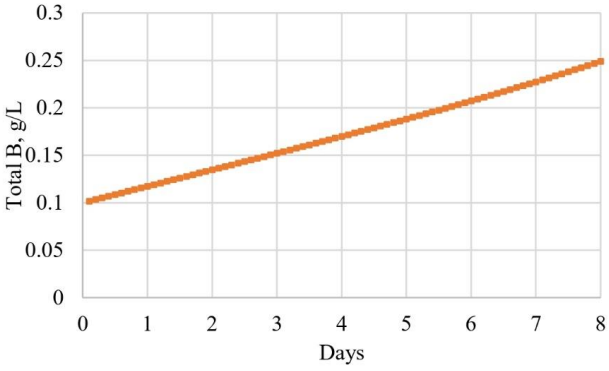
Fig. 6. Calculation of the growth rate depending on z (depth) and light intensity according to Steel's formula [58].

From the implementation of Steel formula (Eq. (4)) Fig. 6 explains well the prevalence of the effects of light-limitation (from the ratio I/I_{op}) rather than photo-inhibition.

Fig. 7(a) shows the effect of theoretical incident light intensity on the growth rate of *Chlorella vulgaris* under white LED light. It could be seen that together with the increase of the light intensity a curve-linear behaviour is followed reaching a maximum around 0.25 day^{-1} in correspondence of an optimal saturating light of about 150 μmoles photons/m^2/sec. In the model at this stage the biomass losses during the dark respiration period are not taken into account. These results are similar to those presented by Haussmann [27] specifically addressed to the valuation of the growth rate of *Chlorella* strain except for the maximum growth rate obtained (i.e. 4.7 day^{-1}) against 0.25 day^{-1} obtained by the kinetic using the laboratory output. This obtained value is in any case more in line with results reported by the study of Daliry [62] and Lee [1] where values of growth rates for *Chlorella* spp. in the range of 0.9 and 2.9 day^{-1} are reported. The reason of a decreased output of the μ_{max} could be related to the underestimated assessment of the cell growth made with the hemocytometer.



(a)



(b)

Fig. 7. (a) Calculation of the growth rate changes for different incident light intensities; (b) biomass concentration during the exponential growth.

The output from Fig. 7(a) shows how the developed tool implementing the kinetic model can be used for finding the maximum biomass growing rate (and thus concentration) with the minimal light intensity, for this specific case equal to 150 μmoles photons/m^2/sec.

Fig. 7(b) shows the concentration increase over time as output of the implemented kinetic model from Beer-Lambert and Steele empirical equation. The model is successfully predicting the overall biomass increase due to the effect of the light intensity during the exponential phase of the microalgae growth.

At this stage more attention should be addressed towards decreasing the biomass production rate caused during the dark respiration period.

It is remarkable to highlight that the idea of the model is more focused to provide a fast and consistent screening method for selecting microalgae strains in order to further assess the overall productivity in pilot or scaled-up ponds. This means that the forecasted behaviour will decline in outdoor conditions, due to counterbalancing effects such as: weather events, human errors, contamination from other microalgae species, bacteria, or predators.

The importance of the proposed model is linked with the optimization of the pond design and operational phases. In fact, the physical parameters implemented in the model – like the depth of the pond – can be easily changed in order to assess the overall effect on the microalgae either concentrations or productivity.

Further improvement of the model can be focused on predicting the performance of ponds in two operative modes (i.e. semi-continuous or continuous), allowing the assessment of the optimal dilution rate for biomass productivity.

The refining of the proposed kinetic, could be further proposed taking into account testing the effect of wavelength type for *Chlorella* stream to be optimal, studies have shown that growth increases in the blue wavelengths [62]. Thus, additional experimental test validation would be needed to increase the reliability of predictions also potentially including other physical conditions like temperature and growing media type.

Nevertheless, from findings of several research studies it is highlighted that models considering multiple effective parameters deal with the complexity of the causal relationship and mechanisms affecting the modelled system sometimes making difficult to validate the model in large scale.

Further improvement for kinetic model development should be addressed to specific factors including nutrient and CO_2 supply, pH, temperature and aeration to better design the operational phase.

4. CONCLUSIONS

The presented research is providing the results of the preliminary steps for the design of a novel type of OWRP in order to provide a feasible solution to bottlenecks for the implementation of microalgae technology based on conventional open ponds and photobioreactors. The definition of Staked Modular Open Raceway Ponds (SMORP) should be beneficial towards the creation of an opportunity for “hybrid” systems taking the lower investment and ease of operability and maintenance from the OWRPs and the lower land use and improved harvest of the light from the PBRs. This aspect is reflected on a combination of the current state-of-the-art PBR technologies with the best ORWP practices. Within this idea, the proposal of a pilot concept integrating the use of transparent material (i.e. optimization of the light penetration) with novel geometry of the open ponds (i.e. a staked system is supposed to save up to 40 % of land use) integrated within an optimized artificial LED lighting system.

The pilot concept proposed is focused on the use of microalgae cultivation within wastewater management specifically, the digestate from biogas plant. The proposed pilot has been designed as a solution to the environmental drawbacks related to the management and disposal of digestate from biogas plants in fact using microalgae as an innovative type of biofilter as CO_2 sink and interface for nutrient recirculation.

At this research stage the preliminary design of the ponds and the technological system together with the selection of both the type of material for ponds and the type of microalgae have been defined. Specifically, acrylic material and *Chlorella vulgaris* have been selected from the performed literature review.

Moreover, a microalgae biomass kinetic model implemented in Excel Visual Basic platform was carried out as a simple approach to screen microalgae strains potentially applicable for open raceway ponds. The model has used Beer-Lambert's Law as growth behaviour depending on the light attenuation due to increased amount of biomass over time, and then calculating the specific growth rate in discretized culture volume slices that receive declining light intensities due to attenuation. In fact, this represents a predicting model depending on two species-specific (i.e. biomass growth rate and light absorption) and two physical (i.e. incident light intensity and culture depth light parameters) able to evaluate the effect of the light-limitation and photo-inhibition. The Steel empirical model has been selected to describe these effects using the ratio I/I_{opt} for light-limitation and an exponential expression for photoinhibition.

The preliminary outputs of the kinetic model were defined considering laboratory tests made at the Biosystem's laboratory of RTU Institute of Energy Systems and Environment using: *Chlorella vulgaris* strain 211-11j, artificial white LED of 50 µmol photons $m^{-2}s^{-1}$, a temperature of +24 °C. The selected light intensity to finalize the kinetic model was 50 µmol photons $m^{-2}s^{-1}$ with a maximum growth rate (μ_{max}) equal to 0.25 day^{-1}.

The developed tool is also beneficial to evaluate the potential benefit of additional artificial LED light sources and in order to achieve the maximum biomass growing rate (and thus concentration) with the minimal light intensity.

It is highlighted that the proposed model needs to be more consistently validated both at the laboratory and further at the scale of the pilot pond.

Further improvement should be addressed to: better characterization of the light absorption coefficient for the selected microalgae, validation of the model on other microalgae species prior to being used for continuous or semi-continuous cultures, refining of the kinetic model (sensitivity analysis and effect of lateral light), introduction of the effect of other variables (e.g. temperature, effects of CO_2 supply and nutrients uptake), daily or seasonal changes of light and temperature to better predict biomass productivity in outdoor conditions.

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Potential of *Chlorella* Species as Feedstock for Bioenergy Production: A Review

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***Abstract* – Selection of appropriate microalgae strain for cultivation is essential for overall success of large-scale biomass production under particular environmental and climate conditions. In addition to fast growth rate and biomass productivity, the species ability to grow in wastewater must also be considered to increase the economic feasibility of microalgae for bioenergy purposes. Furthermore, the content of bioactive compounds in a strain must be taken into account to further increase the viability by integration of biorefinery concept. *Chlorella* spp. are among the most studied microalgal species. The present review attempts to unfold the potential of species of the genus *Chlorella* for bioenergy production integrating applicability for wastewater treatment and production of high added-value compounds. Several key features potentially make *Chlorella* spp. highly beneficial for bioenergy production. Fast growth rate, low nutritional requirements, low sensitivity to contamination, adaptation to fluctuating environments, ability to grow in photoautotrophic, heterotrophic and mixotrophic conditions make *Chlorella* spp. highly useful for outdoor cultivation coupled with wastewater treatment. *Chlorella* is a source of multiple bioactive compounds. Most promising high-value products are chlorophylls, lutein, β-carotene and lipids. Here we demonstrate that although many *Chlorella* spp. show similar characteristics, some substantial differences in growth and response to environmental factors exist.**

***Keywords* – Biomass; biorefinery; microalgae; wastewater treatment**

1. INTRODUCTION

Microalgae are regarded as a promising sustainable energy source due to their fast growth rate, high productivity and ability to accumulate large quantities of lipids [1]. Microalgae biomass has vast applicability, it can be converted to various types of renewable bioenergy, e.g. biogas, biodiesel, biomethane, biohydrogen, bioethanol. Moreover, microalgae biomass and high added-value compounds extracted from the biomass can be used in food industry, medicine, textile industry, feed, aquaculture, agriculture and cosmetology [2]. Several studies have been conducted on the potential and economic feasibility of large-scale microalgae cultivation for bioenergy production [3]–[5]. However, most studies have concluded that economic viability of bioenergy production from microalgae biomass is still an ambitious goal and vast improvements must be implemented before the stage of a commercial low-cost microalgae biomass production. Currently large-scale biomass production is not viable mainly due to high production costs and low productivity of microalgae strains. Lately studies have been focusing on possible solutions to decrease production costs at the same time increasing the efficiency of biomass yield. Optimisation of cultivation conditions must be attained to increase the productivity of microalgae

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mass cultures. Furthermore, effective and low-cost cultivation technology is essential to increase overall biomass productivity. Open ponds and various types of closed photobioreactors have been utilized for microalgae cultivation with certain advantages and disadvantages. Although no consensus has been reached on the most suitable type of cultivation vessel for large-scale cultures, some researchers have proposed that open pond cultivation is more commercially viable for bioenergy production [2], [3], [6], [7].

Lately several novel strategies have been proposed to increase the efficiency and eliminate the costs of microalgae cultivation. Use of wastewater as a nutrient source for microalgae growth and biorefinery are two of the most promising strategies suggested [8], [9]. Many studies have been conducted recently to test microalgae growth in various wastewaters in search for low-cost nutrients. Microalgae cultivation in wastewater offers the possibility of low-cost biomass production at the same time treating wastewater. Furthermore, integrated extraction of high added-value co-products from microalgae biomass is a more sustainable and economical approach to microalgae biomass utilization. Additionally, integration of a cultivation system close to the combustion power plant can benefit from the use of flue gas as a source of CO_2 for increased microalgae growth.

Selection of an appropriate microalgae strain is a crucial factor for high productivity under the selected environment and for the overall success of large-scale biomass production. There are over 70 000 species of microalgae, many of them have not been characterized [10]. Moreover, only a very small fraction of all species has been used in studies of biomass and bioenergy production. The ideal strain for large-scale outdoor biomass production must have the following characteristics: fast growth rate, wide temperature tolerance, high competitiveness, limited nutrient requirements, high CO_2 uptake, tolerance to shear force and to various contaminants in flue gas (e.g. NO_x, SO_x) and wastewater (e.g. heavy metals, ammonium), adaptation ability to fluctuating environmental conditions (light, pH, etc.) and source high-value co-products [1], [11]. Extensive research has been carried out focusing on selection of microalgal strains that can be cultivated for large-scale biomass yield. Among microalgal strains, various *Chlorella* species have been studied extensively. Green microalga *Chlorella vulgaris* has received much attention and is probably the most studied microalga.

This review attempts to investigate the potential of *Chlorella* species for large-scale microalgae cultivation for bioenergy production, with an emphasis on investigation of the capacity for biorefinery and the use of wastewater streams for cultivation of potential species to increase the economic feasibility of microalgal biofuels.

2. CHARACTERIZATION OF GENUS *CHLORELLA*

2.1. General description of Chlorella

Chlorella is a genus of small, single-celled green algae belonging to the division *Chlorophyta*. *Chlorella* cells are non-motile without flagella in a size of 2 to 10 μm in diameter [12]. *Chlorella* spp. have been widely studied since early 1950ies when the first large-scale cultivation was set up in USA for biodiesel production; however, commercial cultivation started in 1961. in Japan, where *Chlorella* was grown as a source of protein for food and feed [13]. *Chlorella* spp. are microalgae with high commercial importance [14] and *C. vulgaris* is one of a few microalgae cultivated commercially for food and feed [13]. *Chlorella* is a cosmopolitan genus with its species found in diverse water habitats including freshwater, marine and wastewater [13], [15]. Species can grow well in a wide temperature range that makes them particularly useful for various applications in outdoor conditions.

Research shows that *Chlorella* spp. can withstand temperatures from 5° C to 42° C [16], [17]. Key characteristics of selected *Chlorella* spp. are shown in Table A1 in the Annex.

Although *Chlorella* has low nutritional requirements [13], the species can withstand high nutrient concentration [18] that may be advantageous for cultivation in high strength wastewater thereby increasing its competitiveness over other species of microorganisms particularly in outdoor cultivation. Other characteristics such as fast growth rate, low sensitivity to contamination and unfavourable environments [19] also make *Chlorella* favourable for wastewater treatment and cultivation in open ponds under fluctuating environmental conditions.

Genus *Chlorella* has a simple life cycle. Reproduction is exclusively asexual by cell division, most often producing four to eight daughter cells [20]. Cells have thick resistant walls with glucosamine as a main wall component [20], [21]. It has its advantages and disadvantages. Robust, non-flagellated cells make *Chlorella* shear resistant beneficial for cultivation in bioreactors where cells are less likely to be damaged by mixing. In contrast, large flagellated cells like those of *Dunaliella* spp. are shear sensitive and are more prone to damage during mixing and pumping in bioreactors [18]. However, looking from a biorefinery point of view, the hard resistant cell walls of *Chlorella* are a major drawback as they require pre-treatment for efficient extraction of bio-compounds increasing the extraction time and costs.

2.2. Taxonomy

Classification of *Chlorella* is not straightforward and species cannot be identified based on morphological features alone [13]. More than 100 microalgae from various habitats have been historically assigned to genus *Chlorella* [22]. Classification of *Chlorella* remains a challenge even today. Although, the genus *Chlorella* has undergone extensive changes in recent years, reorganization of the genus is not complete, several new suggestions for rearrangements of the genus have been proposed [14], [20], [23]. More powerful methods than morphological features are required for the identification of species. Novel, more sensitive identification techniques, such as molecular phylogeny and bioinformatics have been introduced making classification more reliable. Use of molecular markers has revealed that many species formerly assigned to *Chlorella* in fact belong to different lineages of green microalgae [24]. Based on biochemical, physiological, ultrastructural characters and molecular tools, Huss suggested only four species to be kept in the genus *Chlorella*, namely, *C. vulgaris*, *C. lobophora*, *C. sorokiniana* and *C. kessleri* [20]. However, another research on taxonomy of *Chlorella* suggests that five "true" *Chlorella* species exist, namely *C. vulgaris*, *C. lobophora*, *C. sorokiniana*, *C. heiozoae* and *C. variabilis* [23]. *Chlorella kessleri* has been reclassified as *Parachlorella kessleri* [22]. Most strains formerly identified as *C. pyrenoidosa* have been reclassified as other strains of the genus *Chlorella* and other taxa [14]. In the study of Kessler and Huss, several strains of *C. pyrenoidosa* from UTEX collection have been tested with biochemical and physiological markers and it was found that most of the strains belong to different strains of *Chlorella* such as *C. vulgaris*, *C. sorokiniana* and *C. fusca* var. *vacuolate* [25]. *C. pyrenoidosa* is no longer a valid species and most of the strains formerly assigned as *C. pyrenoidosa* have now been reclassified. Champenois suggested that *Coelastrella vacuolate* is the current valid name for this *C. pyrenoidosa* [14]. Just a few studies are available on *C. lobophora* [26], [27], therefore, more research is required to assess the potential of *C. lobophora* for bioenergy production.

In the current review we focus on the most frequently studied *Chlorella* species, although some species have been suggested for reclassification into different genus such as *Parachlorella* and *Auxenochlorella*.

2.3. High-Value Products

Lately, microalgal biorefinery is receiving increasing interest. A commercial potential of microalgae biomass is still an untapped resource. Microalgae are a source of bioproducts such as pigments, proteins, lipids, carbohydrates, vitamins and antioxidants with high commercial value. The extraction of these co-products is essential to improving the economic feasibility of microalgal bioenergy. Microalgae biorefinery concept is a new approach for better utilization of biomass potential, achieve higher viability and sustainability of bioenergy and move towards "zero waste" production in a circular economy framework. Biorefinery results in a cost-effective simultaneous production of bioenergy and various valuable bioproducts. Moreover, besides economic benefit biorefinery also minimizes the environmental impact with the more efficient use of resources and reduction of greenhouse gas emissions.

Studies indicate that *Chlorella* biomass has a wide range of potential applications in pharmaceutical, nutraceutical, agricultural and cosmetics industries [28], [29]. *Chlorella* is a source of many high value compounds nevertheless; it has most often been exploited as a protein source. Depending on the culture conditions, *C. vulgaris* contains up to 58 % proteins and *C. pyrenoidosa* 57 % that are between the highest rates of green microalgae [30]. Due to its high protein content *C. vulgaris* is used in health food industry and aquaculture [31]. It has been reported that *C. vulgaris* contains 37 % starch [32] that can be useful for bio-ethanol production. Sulphur deficiency has been shown to increase starch content in cells that is followed by lipid accumulation in *Chlorella* species [33]. Many high value products are secondary metabolites that are biochemical compounds involved in adaptation of microalgae to changing environmental conditions. Examples of secondary metabolites are carotenoids, phycobiliproteins, phenolic compounds, alkaloids and lignin [34]. Synthesis of some secondary metabolites increases under stress conditions such as oxidative, osmotic or nutrient stress. Therefore, stress conditions must be induced to increase the production of these biochemicals.

Chlorella is a source of high value pigments such as lutein, astaxanthin and β-carotene; vitamins, especially vitamin B complex, ascorbic acid, α-tocopherol and several other bioactive compounds [28]. Othman tested 6 green freshwater microalgae and found that *C. vulgaris* had the highest total carotenoid and β-carotene content, 81 µg/g DW and 18 µg/g DW, respectively [35]. Moreover, highest lutein content (69 µg/g DW) was found in *Chlorella fusca*. Although lutein traditionally is extracted from marigold flowers, the production of lutein is hampered by seasonal availability of marigold flowers. Microalgae can contain a considerable amount of lutein [36]–[38] and can be harvested all year round. *Chlorella* has shown good potential as a lutein source. However, lutein content is dependent on cultivation conditions, therefore, lutein extraction rate reported is highly variable. D'Este was able to extract 0.69 mg/g DW lutein from *C. vulgaris* [37]. Wei achieved 1.98 mg/g DW lutein content in *C. protothecoides* [39], but Chen reached 5.88 mg/g DW lutein with two-stage heterotrophic culture of *C. sorokiniana* [38]. Lutein content in *C. minutissima* reached 8.24 mg/g DW in Dineshkumar's study [40], but the highest extraction reported was 10.4 ± 5.5 mg/g DW in *C. vulgaris* in McClure's study using photoautotrophic cultivation mode [41]. In contrast, very low lutein was reported in Othman's study. *C. vulgaris* was found to produce 63 µg/g DW lutein but *C. fusca* 69 µg/g [35]. Although sustainable and economically viable lutein production still needs extensive research, lutein production rate of

microalgae is reported to be 3 to 6 times higher than that of marigold flowers [42] and the results achieved so far are promising.

Recently, emphasis has been placed for the search of new bioactive compounds in microalgae with antibacterial, antifungal and anticancer activities. *Chlorella* spp. contain valuable bioactive peptides with antioxidant, anti-inflammatory and anticancer properties [43]. For example, *C. pyrenoidosa* contains polypeptide that exhibits antitumor activity [44]. This field is very promising, but requires more studies. Another example of bioactive compound with potentially high commercial interest is β-1,3-glucan – a polysaccharide best known for its immunostimulatory activity [28].

In agriculture *Chlorella* biomass has been applied as a bio-fertilizer and as a feedstock for animals. Algae biomass have been incorporated as a dietary supplement in farm animal, fish and poultry feed. For example, the addition of *Chlorella* biomass to poultry feed has showed improved growth, immune response and gut microflora [28].

2.4. Wastewater Treatment

Large-scale microalgae cultivation requires considerable amount of water and nutrients that makes up a large part of the cultivation costs. On the other hand, large volumes of wastewater in food and processing industries are generated containing valuable micro- and macro elements that can be used for microalgae cultivation. The use of wastewater as a low-cost nutrient source is one of the strategies proposed to reduce biomass production costs and increase the feasibility of low-cost bioenergy [45]. Wastewaters are complex mixtures with a variable composition depending on their origin. Generally, wastewater streams contain organic, inorganic and man-made compounds [46]. Microalgae are known to remove nutrients and heavy metals from wastewaters to the level that meets the requirements for discharge.

Wastewater use has multiple advantages on microalgae cultivation: (1) it is a source of nutrients for microalgae growth, (2) it provides a sustainable water source, and (3) it is a source of organic carbon for heterotrophic and mixotrophic growth [47]. The main environmental issue of microalgae cultivation – the need for enormous amounts of freshwater thus could be mitigated, moreover, it reduces expenses of nutrients required for microalgae cultivation.

Simultaneous nutrient removal and biomass production requires microalgae species to survive in specific conditions and reach high biomass yield. Species for wastewater treatment must exhibit good pollutant removal capacity mainly ammonium, nitrogen, phosphorus and heavy metals under specific environmental conditions. Due to large quantities of organic carbon in wastewaters, microalgae with heterotrophic metabolism are beneficial. It has been demonstrated that *Chlorella* spp. are capable to grow in autotrophic, heterotrophic and mixotrophic conditions [48]. In contrast to photoautotrophy that use solar energy and carbon dioxide, in heterotrophic metabolism microorganisms can utilize organic compounds from the environment as a source both for energy and carbon [47]. Simultaneous use of carbon dioxide and organic carbon, known as mixotrophy, can more efficiently utilize the available light and organic nutrients form wastewater thus potentially enhancing microalgae growth. Recently many studies have been aiming at optimizing heterotrophic and mixotrophic cultivation to overcome the limitations of autotrophic growth such as light deficiency. Several studies have shown higher efficiency in nutrient removal and biomass production in mixotrophic and heterotrophic cultivation mode compared to photoautotrophic conditions [49]–[51]. When cultivated in wastewater *Chlorella* is able to switch from phototrophic to heterotrophic or mixotrophic growth. Glucose is found to be the preferred source of carbon for *Chlorella* species [52]. Mixotrophy with glucose has resulted in a higher growth rate than

autotrophic or heterotrophic cultivation [50]. Some studies have shown that mixotrophic cultivation is the most efficient [51] while others demonstrated better growth in heterotrophic cultivation [49].

Species competitiveness is another important consideration for assessment of species suitability for cultivation in wastewater. Wastewater contains biological contaminants such as bacteria and protozoa, therefore robust and fast-growing microalgae that can outcompete other species are crucial for cultivation in wastewater. Wastewater treatment requires fast and efficient pollutant removal in a possibly shortest period of time therefore, in addition to fast growth rate the potential algal strain must also be tolerant to weather fluctuations and high nutrient concentrations. *Chlorella* spp. are natural inhabitants of wastewater ponds [15], [53] and can survive in various wastewater streams showing great potential to adapt to various environmental conditions [15], [54]–[56]. Oberholster demonstrated that a combination of *C. vulgaris* and *C. protothecoides* is effective in nutrient removal from wastewater stabilization ponds (75 % total phosphorus and 43 % total nitrogen removal) and *Chlorella* spp. stayed dominant after inoculation of ponds, moreover other microalgae species coexisted with *Chlorella* spp. in treatment ponds [57].

Chlorella spp. are found to be between predominant strains in wastewater ponds. Exploring waste stabilization ponds Palmer found that the most abundant and frequent genera were *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Euglena*, *Chlamydomonas*, *Oscillatoria*, *Micractinium* and *Golenkinia* [58]. Furthermore, Palmer published another study with the results of an extensive research covering 165 studies and reported that *Chlorella* is between the top eight pollutant-tolerant genera [53]. Moreover, screening top 17 strains with the best performance in wastewaters collected locally from natural freshwater habitats and wastewater, Zhou found that 60 % belongs to *Chlorella* spp. [15] demonstrating superiority of *Chlorella* over other microalgae strains and indicating its potential for wastewater treatment. Ayre studied microalgal consortium in anaerobic digestate of piggery effluent with high ammonia content and found that *Chlorella* was dominant species at all ammonium concentrations [59]. Moreover, the consortium was able to grow in 800 and 1600 mg NH_4^+-N L^{-1} showing superior resistance to high ammonium concentrations than other microalgae species. *Chlorella* spp. have been used in numerous studies and have shown good nitrogen and phosphorus removal rates. Generally, *Chlorella* spp. can remove 23 %–100 % nitrogen while phosphorus removal efficiency is 20 %–100 % [45]. However, not all microalgae strains can grow in wastewater. Caporngo observed lower growth and nutrient removal level of *Nannochloropsis ocultata* compared to *C. vulgaris* and *C. kessleri* [55]. Alvarez-Diaz found that *Neochloris oleoabundans* did not grow in wastewater [60]. According to Caporngo freshwater microalgae are preferable to wastewater cultivation than marine algae [55]. However, Chinnasamy observed that also marine algal species exhibit good growth in some wastewater (e.g. carpet mill effluent) without salt addition [61].

2.5. Biomass Yield and Lipid Production

Chlorella spp. are among the fastest growing microalgae, often reported being superior to other species [1], [62]. However, because growth rate is highly dependent on cultivation system and growth conditions, reported values are very wide making comparison between studies difficult. Growth rates and biomass productivity of *Chlorella* spp. are summarized in Table A2 in the Annex. Li compared biomass productivity of various *Chlorella* species and observed the highest productivity for *C. kessleri* UTEX 398 (2.01 g TVSS/L), followed by *C. protothecoides* strains UTEX 25 and UTEX 256 [48]. The lowest productivity (0.38 g TVSS/L) was observed for *Chlorella fusca* var. *vacuolata*, the species that is no longer assigned to genus *Chlorella* and reclassified as *Coelastrella vacuolate*. In the same study two

strains of *C. sorokiniana* (UTEX 1230 and UTEX 2805) exhibited biomass productivity of 0.70 and 0.76, respectively.

Chlorella species are capable of accumulating significant amounts of lipids, generally under stress conditions; furthermore, several strains are producing a fatty acid profile suitable for biodiesel production [2], [63], [64]. Lipid content in *Chlorella* spp. under normal growth conditions is generally around 20 %, higher lipid content has been reported in *C. minutissima* (31 %) but lower in *C. protothecoides* (11 %) [65]. However, by adjusting the growth conditions lipid content can reach >50 % [2], [65]. Nitrogen limitation is an effective strategy to increase lipid content in all *Chlorella* strains [65]. Reported lipid concentration in *C. vulgaris* ranges from 5 % to 58 % (DW) and lipid productivity from 11 to 40 mg L d^{-1} [34]. Such a wide range of values could be explained with various growth conditions used in different studies.

Illman compared the growth and lipid production of five strains of *Chlorella*, *C. vulgaris*, *C. emersonii*, *C. protothecoides*, *C. sorokiniana* and marine strain *C. minutissima* [65]. Under nitrogen deficiency conditions the growth rate decreased in all strains except *C. minutissima* which remained in the same level. Highest lipid content was achieved in *C. emersonii* (63 %), *C. minutissima* (57 %) and *C. vulgaris* (40 %). Microalgae *C. vulgaris* and *C. emersonii* are promising species because of high growth rates that stay relatively high also under N limitation condition coupled with good lipid productivity. *C. minutissima* showed no decrease in growth rate under N limitation conditions and high lipid content. *C. emersonii* and *C. minutissima* show high lipid content in optimal growth conditions, 29 ± 2.5 % and 31 ± 3.2 %, respectively. Although, reported lipid productivity of *Chlorella* spp. is variable, high lipid content achieved in some studies are suggesting that high lipid concentration in *Chlorella* can be reached, however, optimization is required.

3. CHARACTERIZATION OF *CHLORELLA* SPECIES

3.1. Chlorella vulgaris

C. vulgaris is a type species of genus *Chlorella* and the most widely studied algae of the genus. *C. vulgaris* has spherical, non-motile single cells with a cell size from 2 to 10 μm in diameter [2]. *C. vulgaris* is a freshwater species and is known as one of the fastest growing microalgae strains with a doubling time of 16 h in photoautotrophic conditions [65]. *C. vulgaris* has rigid cell wall mainly composed of a chitosan-like layer, cellulose, hemicellulose, proteins, lipids and minerals [66]. Cells have a single chloroplast. *C. vulgaris* can accumulate starch or lipids under unfavourable conditions stored in cytoplasm or chloroplast [2], [65]. Reproduction is asexual by autosporulation. Most commonly four daughter cells are formed. *C. vulgaris* has a remarkable ability to withstand a wide range of temperatures, especially low temperatures. It has been demonstrated that *C. vulgaris* can withstand 5 °C and still do slow but continuous growth [16]. However, cells are not resistant to high temperature, already at 30 °C considerable decrease in cell viability has been observed [67]. Optimal temperature of the species is between 25 °C and 28 °C [67], [68].

Although a more alkaline medium is generally thought to be optimal for *C. vulgaris* growth [69], other studies have found that neutral pH (pH 7) leads to a higher growth rate [70]. While a vast number of studies on *C. vulgaris* have been performed, data reported can significantly vary. For instance, it has been reported that *C. vulgaris* contains 42 %–58 % total proteins, but lipid content under optimal growth conditions varies between 5 % and 40 % of dry weight (DW) [2]. The observed wide range of values reported most likely originates from various growth conditions

applied in different studies. Lipid content under normal growth conditions of *C. vulgaris* is around 20 % [65]; however, during stress conditions, normal biochemical composition of cells changes, and an increase in lipids and decrease in proteins is often observed. Application of stress such as nitrogen starvation, can increase lipid content up to 58 % [2] and lipids are mainly in the form of triacylglycerols (TAG). For example, dos Santos observed total lipids 19.6 % DW and 27.7 % TAG under optimal growth conditions [66]. Lipid content increased to 25.4 % DW after nitrogen starvation was applied, moreover, TAG content increased to 41.3 %. Lower percentage of PUFAs was also observed under nitrogen starvation mode compared to optimal growth conditions being more suitable for biodiesel production [66].

C. vulgaris is a source of bioactive compounds with commercial value that could be used for a biorefinery approach. *C. vulgaris* is rich in proteins, carbohydrates, lipids, pigments, minerals and vitamins [2]. Therefore, it has vast applicability in various fields such as human food, animal feed, cosmetology and medicine. Cells contain significant amounts of chlorophyll. Their content in *C. vulgaris* cells can reach up to 1–2 % DW [2]. Cells also contain significant amounts of carotenoids, such as β-carotene, astaxanthin and lutein that have multiple therapeutic properties. Lately many studies have focused on optimization of pigment extraction and increase in pigment content [37], [41]. *C. vulgaris* biomass has been used as a biofertilizer with good results [28].

C. vulgaris has demonstrated high potential for wastewater treatment. Rapid growth and high nutrient removal have been shown in various wastewater streams such as urban [55], industrial [61] and agricultural wastewater [56], [71]. *C. vulgaris* has shown some remarkably high ammonia nitrogen and total nitrogen removal rates over 96 % and total phosphorus removal 69 to 98 % in various wastewaters [55], [56], [71], [72]. Efficiency of heavy metal removal depends on the species, *C. vulgaris* has shown good removal of cadmium, copper and zinc [73].

3.2. Chlorella sorokiniana

C. sorokiniana is the most heat and high light resistant species in the genus *Chlorella* [74]. Species can tolerate temperatures up to 42 °C [17], [75]; however, optimal growth temperatures seem to depend on a combination of several biotic and abiotic factors, as reported optimal temperatures vary across studies and are in range from 30 °C to 40 °C [74], [76], [77]. Still, the most frequently reported optimal temperatures are 36 °C–38 °C [78]–[80]. The performance under extreme environmental conditions was demonstrated by Morita et al., who observed good photosynthetic productivity even at 46.5 °C that was coupled with high light intensity [77]. The common growth temperature which is optimal for some other microalgae strains is not suitable for *C. sorokiniana*. Cuaresma Franko found that temperature below 20 °C had an inhibitory effect on microalga growth [79]. *C. sorokiniana* can withstand not only high temperatures but also high intensity light up to 2500 μmol m^{-2} s^{-1}[74]. Testing five different light intensities of 100, 200, 400, 600 and 750 μmol m^{-2} s^{-1}, Tan found that at 750 μmol m^{-2} s^{-1} resulted in the best growth, indicating higher light requirements than other common microalgae species [31].

Considering tolerance to high temperature and light intensity *C. sorokiniana* can be a good candidate strain for biomass production in outdoor cultivation systems in regions with high insolation. Open ponds tend to reach high temperatures and light intensity especially during mid-day [81], often exceeding the optimum temperature of the strain particularly in the upper layer of the water. Therefore, a heat resistant strain is preferred in these conditions. Temperature has an impact also on lipid productivity. Li observed that highest lipid content of *C. sorokiniana* was reached at 30 °C (37 %), however the highest lipid yield at 37 °C [80].

C. sorokiniana has substantial tolerance to high nutrient concentrations in wastewater and is able to remove up to 99 % of nitrogen and phosphorus depending on the initial concentration [82]. Microalga has exhibited good capability of ammonium removal under extreme temperature and light conditions [74]. Kim [49] found that *C. sorokiniana* exhibited the best growth rate and nutrient removal while cultivated under heterotrophic conditions with glucose as a carbon source compared to autotrophic and mixotrophic conditions. Moreover, the growth rate was more than two-fold higher for heterotrophic cultures than autotrophic [49]. However, Li found that mixotrophy resulted in considerably higher biomass concentration, growth rate and lipid productivity than either heterotrophic or mixotrophic cultivation [83]. Rosenberg reported a rapid nine-hour heterotrophic doubling time [84], while Rai found a remarkable doubling time of 2 h 9 min under mixotrophic conditions [70].

3.3. Chlorella protothecoides (Auxenochlorella protothecoides)

C. protothecoides is a robust, fast growing species able to grow in various wastewaters [57], [72]. The species has received most attention regarding biomass production under various cultivation modes, specifically mixotrophic and heterotrophic conditions to increase lipid production. Furthermore, extraction of value-added compounds, mainly pigments, has been a focus of *C. protothecoides* cultivation [85], [86]. This species is a valuable source of bioactive compounds. Particular attention has been paid to extraction of pigments. High concentrations of carotenoids and chlorophylls have been found under phototrophic and mixotrophic growth modes. Higher cellular accumulation of pigments has been observed at phototrophic mode, however concentration per unit volume was higher under mixotrophic growth [85]. Salt and light stress are known to induce the carotenogenesis process in *C. protothecoides* allowing accumulation of astaxanthin and lutein/zeaxanthin [85]. *C. protothecoides* CS41 had the highest biomass yield and lutein content when seven Chlorella strains were compared (3 strains of C. pyrenoidosa, three strains of C. vulgaris and one strain of *C. protothecoides*) under heterotrophic conditions using glucose as a carbon source [87].

Li was able to achieve 48.7 % lipid content in heterotrophic conditions in 750 L bioreactor [88]. In the same study, successful scale-up was demonstrated, heterotrophic culture density reached 15.5 g L^{-1} in 5 L, 12.8 g L^{-1} in 750 L, and 14.2 g L^{-1} in 11 000 L bioreactors. Shi succeeded to reach remarkable 48 g L^{-1} biomass yield in a 3.7 L fermenter and 45.8 g L^{-1} in upscaled 30 L fermenter [89]. Moreover, lipid content reached 57.8 % in batch and 55.2 % in fed-batch culture of heterotrophic *C. protothecoides* grown on glucose [63].

Studies indicate that *C. protothecoides* can grow in different wastewaters with similar performance and is resistant to high chemical content. Microalga showed good performance in raw, untreated urban wastewaters exhibiting high growth rate and efficient removal of N and P [90]. Results showed that endogenous bacterial contamination did not limit algal growth rate. *C. protothecoides* demonstrates a high growth rate and efficient removal of NH4+-N also from various anaerobic digestion effluents [72]. An additional benefit of this species is the significantly faster settling of cells compared to *C. vulgaris* that is particularly important for biomass harvesting [72].

3.4. Chlorella kessleri (Parachlorella kessleri)

C. kessleri cells are larger than *C. vulgaris* [55] that might be advantage for biomass harvesting. *C. kessleri* has been studied for its potential for biodiesel production and extraction of high value products. TAG accumulation in *C. kessleri* is induced by high light intensity, hyperosmosis and nutrient limitation. Hayashi showed that low temperature could

also stimulate TAG accumulation but only for a limited time [91]. Moreover, the same study demonstrated very high TAG accumulation up to 48.5 % in *C. kessleri* cells due to synergetic effects of hyperosmosis, nutrient-limitation, increased light intensity and low temperature. A notable 54.7 % total fatty acid content was achieved in mixotrophic cultivation with 300 mmol L^{-1} glucose under nitrate depletion conditions that was about 5-hold increase compared to autotrophic cultures [92]. Some high value bioactive compounds have been extracted from *C. kessleri* biomass. Although lutein content in *C. kessleri* cells is not significant, the strain is a natural source of astaxanthin. Soares reported nearly 23 mg g^{-1} astaxanthin in photoautotrophic cultivation conditions [93]. Encouraging results have been achieve for its application in wastewater treatment. Biomass production of *C. kessleri* in wastewater is reported to be comparable to that of *C. vulgaris* [55]. *C. kessleri* has showed more tolerance to some pollutants, like chromium, copper and herbicide than other microalgae species [92]. It was capable to remove 94 % of chemical oxygen demand and 96 % of NH4+-N and P from aquaculture wastewater just in 3 days and was superior to Scenedesmus spp. and *C. vulgaris* [94]. Likewise, *C. kessleri* has demonstrated high uptake of N and P also in urban wastewater showing more than 96 % and 99 % removal, respectively [55].

3.5. Chlorella minutissima

C. minutissima is a high CO_2-tolerant microalga with easy cultivation and fast growth [64]. It has small unicellular spherical cells from 2 μm to 4 μm in diameter when grown in synthetic medium and larger cells, from 2 μm to 8 μm in medium with organic carbon [95]. *C. minutissima* is tolerant to pollution and fluctuating environmental conditions [95]. It can grow at exceptionally wide pH range from 4 to 10, although growth at pH 4–5 is strictly constrained. The optimum growth has been observed at pH 7 [95]. Tolerance to a wide pH range is especially valuable in open raceway pond cultivation where control of environmental parameters is not always straightforward. Moreover, another advantage for outdoor cultivation is dominance over other microorganisms diminishing the risk of contamination with fungi, bacteria and other algae [95].

It has been noted that *C. minutissima* has a fatty acid profile desirable for biodiesel production [64]. Moreover, nitrogen starvation is an effective method for enhancement of total lipid and TAG content in *C. minutissima* [64]. Tang found that neither light source nor intensity or photoperiod had a significant effect on fatty acid methyl esters (FAME) content [64]. Lipid content can reach 57 % when cultivated in low nitrogen medium [65].

C. minutissima can grow in photoautotrophic, heterotrophic and mixotrophic conditions, however, Bhatnagar found that growth in heterotrophic conditions was significantly lower than that of autotrophic whatever the carbon source was used [95]. *C. minutissima* can utilize several carbon sources, such as glycerol, glucose, succinate, molasses and press mud [96]. According to Bhatnagar, glucose is the preferred carbon source for mixotrophic growth and resulted in synergistic growth in the presence of light [95]. Other study proposed that glycerin is the optimal carbon source however, glucose was not tested in this study [97]. *C. minutissima* shows halotolerance up to 3 % NaCl suggesting potential application in treating municipal wastewaters that are often characterized by high sodium content [95]. Bhatnagar demonstrated that *C. minutissima* exhibits better growth on diluted wastewater (up to 75 %) compared to synthetic BG-11 medium [95]. Moreover, 50 % wastewater supported 146 % better growth than BG-11 medium indicating high potential of this microalga for wastewater treatment

4. CONCLUSION

Data on the growth rate and productivity of microalgae reported in the literature varies extensively. The observed dispersion of data is mainly due to cultivation conditions of the microalgae. Growth rate, biomass productivity and lipid content of a microalgal species are parameters particularly difficult to compare across studies as they depend highly on cultivation conditions such as light intensity, temperature, photoperiod, cultivation mode (batch, semi-batch, continuous), metabolic conditions (phototrophic, heterotrophic or mixotrophic growth), scale of the cultivation, growth media and nutrients used (synthetic growth media, wastewater etc.). All these parameters make comparison of various experiments and microalgae strains difficult. Every experiment is carried out in unique conditions and are generally not comparable across studies. Thus, it is of great importance to compare different microalgal strains in one study under the same culturing conditions. There are not enough studies comparing several productive microalgae strains simultaneously to get comprehensive comparable results for the selection of the most promising strains. Another aspect to consider is the degree of variation between strains of the same species. Specific strains have been isolated from different habitats under various environmental conditions and can therefore exhibit different responses to various conditions.

Cosmopolitan species of the genus *Chlorella* can be found in diverse habitats throughout the world. A number of key features potentially make *Chlorella spp.* highly beneficial for large-scale biomass production. Fast growth rate, low nutritional requirements, low sensitivity to contamination and flexibility to fluctuating environments, ability to grow in autotrophic, heterotrophic and mixotrophic conditions make *Chlorella spp.* highly useful for outdoor cultivation coupled with wastewater treatment. Results of the present study demonstrate that *Chlorella spp.* are suitable feedstock for bioenergy production. Some studies have reported very high growth rates of various Chlorella species supporting the goal of high biomass yield. *C. vulgaris* is often receiving the highest rating among microalgae strains in terms of growth rate, resistance to pollution and lipid productivity, moreover it can withstand wide temperature range showing its usefulness in outdoor conditions. Results indicate that *C. vulgaris* is not only a widespread model organism but holds real potential for bioenergy production. *C. sorokiniana* shows potential at locations with warmer climates and high insolation due to its resistance to high temperature and light intensity. Several studies have shown that *Chlorella* species are natural inhabitants of wastewater ponds indicating their potential in wastewater treatment. Indeed, all *Chlorella* species studied are suitable for cultivation in various wastewater streams showing high nutrient removal rates and resistance to contaminants. However, the suitable dilution rate of a stronger wastewater must be obtained to exclude the inhibitory effect of excessive ammonium level. Furthermore, *Chlorella* is a source of many bioactive compounds with commercial value that can be co-extracted to further increase the viability of microalgal bioenergy. The most promising value-added products are chlorophylls, lutein, β-carotene and lipids. The drawback for biorefinery is a thick resistant cell wall that makes downstream processing of algal biomass difficult. On the other hand, a thick cell wall makes *Chlorella* shear resistant and is an advantage for cultivation in bioreactors. Numerous studies show that *Chlorella* species hold great potential for a large-scale biomass production, however optimization of cultivation conditions is of primary importance to achieve high biomass yield and increase the content of high value compounds

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ANNEX

TABLE A1. KEY PARAMETERS OF SELECTED *CHLORELLA* SPECIES

Species	Growth conditions				Light, opt. µmol /m²/s	Optimum CO_2 absorption	Cultivation type	Application in wastewater treatment	High-value compounds	Reference
	Temp. opt.	Temp. min.	Temp. max.	pH opt.						
C. vulgaris	25–28	5	28–30	7–10	150–750	4–15 %	Phototrophic Heterotrophic Mixotrophic	Urban Industrial Agricultural Municipal	Chlorophyll Lutein, β-1,3-glucan	[12], [14], [15], [47], [48], [56], [68], [1], [17], [41], [55], [57], [61], [69]–[71]
C. sorokiniana	28–40	20	38–42	6–7.5	100–2500	5 %	Phototrophic Heterotrophic Mixotrophic	Synthetic Municipal Agricultural	Chlorophyll Lutein	[31], [60], [74], [76], [78]–[80], [100], [101], [77], [82], [102]–[104]
C. protothecoides	25–30	NA	28 - 32	NA	30–150	NA	Phototrophic Heterotrophic Mixotrophic	Industrial Brewery waste Municipal	Chlorophyll Astaxanthin β-carotene Lutein	[39], [57], [61], [85], [86], [89], [105], [106]
C. kessleri	26–30	NA	34–36	NA	70–150	18 %	Phototrophic Heterotrophic Mixotrophic	Urban Aquaculture	Astaxanthin	[1], [20], [55], [91]–[94]
C. minutissima	25–30	NA	32	7	350	NA	Phototrophic heterotrophic Mixotrophic	Municipal	Lipids	[17], [64], [95]–[97] [107]

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TABLE A2. GROWTH RATE, BIOMASS YIELD AND LIPID CONTENT OF *CHLORELLA* SPECIES

Species	Max specific growth rate, μ_{max} d^{-1}	Biomass yield, g L^{-1}	Biomass productivity, g L^{-1} d^{-1}			Lipid content, %/ Lipid productivity mg L^{-1} d^{-1}	Reference
			Phototrophic	Heterotrophic	Mixotrophic		
C. vulgaris	0.293–1.457	0.4–20	0.02–4.64	0.105	2–5	5–58 %/7.5–132.4	[1], [15], [31], [62], [65], [66], [108]–[110]
C. sorokiniana	0.397–1.60	25–37.6	0.18–4.35	0.7–12.2	0.7–1.98	24–31.5 %/49.4–94.8	[15], [31], [38], [48], [80], [111]
C. kessleri	1.27	4.46–13	NA	NA	2.01	48.5–54.67 %/3.3–7110	[48], [91], [92], [112]
C. protothecoides	0.33–0.92	12.73–51.2	0.27	0.88–6.6	1.2–1.31	44.3–57.8 %/77.7–2120	[15], [48], [63], [65], [86]–[89]
C. minutissima	0.43	NA	0.143	0.76–1.78	0.76	5–15 %	[48], [64], [65], [95], [97]

NA – data not available.

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EFFECT OF LIGHT INTENSITY ON THE GROWTH OF THREE MICROALGAE IN LABORATORY BATCH CULTURES

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ABSTRACT: Microalgae are regarded as promising feedstock for bioenergy production due to their fast growth rate, high productivity and low nutritional requirements. However, large-scale biomass production for bioenergy currently is not economically viable due to low productivity and high cultivation costs of microalgal biomass. Optimal growing conditions for specific strains must be set up to increase the microalgae productivity. Therefore, in present study we focus on finding the optimal light intensity conditions for three microalgae cultivation intended for large-scale biomass production in outdoor raceway ponds supplemented with artificial LED illumination. We tested light intensity in a range of 30 to 200 μmol m^{-2} s^{-1}. The impact of light intensity on the growth and biomass production of three microalgae strains was assessed by measurements of optical density and dry cell weight. Our study shows that light intensity of 30 and 50 μmol m^{-2} s^{-1} is too low to maintain high growth rate of microalgae strains studied. Our results suggest that *C. vulgaris* has lower light requirements comparing to *C. sorokiniana* and *C. reinhardtii*. The highest biomass yield was achieved at light intensity of 150 μmol m^{-2} s^{-1} for *C. vulgaris* and at 200 μmol m^{-2} s^{-1} for *C. sorokiniana* and *C. reinhardtii*.
Keywords: microalgae, biomass, bioenergy, light intensity.

1 INTRODUCTION

The increasing prices and environmental impact of fossil fuels have prompted scientific community to look for an alternative energy source. Microalgae are regarded as a promising feedstock for bioenergy production due to their fast growth rate, high productivity and low nutritional requirements. Moreover, microalgae cultivation does not compete with food production as opposed to first generation feedstock [1]. Furthermore, there are other evident advantages of microalgae over other types of feedstock: (1) all year round production, (2) growth in low quality water, (3) high CO_2 fixation rate, (4) no arable land needed for cultivation and (5) production of wide range of added value products [1]–[3]. Despite these potential benefits of microalgae for bioenergy production with respect to other types of biomass, several obstacles for large-scale microalgae cultivation exist. One of the greatest drawbacks is the low productivity of outdoor microalgae cultures compared to lab-scale cultures. Laboratory conditions are very different from constantly changing outdoor environment. A great effort is being made to increase the productivity and to lower the cost of microalgae biomass production to rise the economic viability of microalgal biofuels. Establishment of optimal growth conditions is crucial for the overall success of microalgae bioenergy [4].

Light is the main requirement for photoautotrophic microalgae growth since it is an energy source for photosynthesis reactions and has a direct impact on biomass production. Consequently, light availability is critical to maintain high microalgae growth. Energy of light is used to convert carbon dioxide into sugars, the building blocks for biomass [5].

Cell growth of photoautotrophic microalgae is strongly affected by light intensity. Light availability can be divided in three categories: light limitation, light saturation and light inhibition. Increased photosynthesis rate is generally observed with increasing light intensity leading to higher growth rate. Therefore, an increase in light intensity enhances biomass production. However, algal growth is increasing up to a certain threshold; the maximum algal productivity is achieved at the specific light level known as the light saturation point [6]. Beyond this level a decline in algal productivity is observed. Excess light is damaging cells, slowing down photosynthesis and leading to photo-inhibition which results in a reduction of microalgal growth [7], [8]. Prolonged high irradiation can lead to growth inhibition. When light intensity is below the saturation point, algal growth is limited by light. Too low irradiance will slow down photosynthesis and reduce the biomass yield, creating a condition known as light limitation [9]. Microalgae species have various light requirements and optimal light intensity vary from strain to strain [10], hence optimal light conditions and light saturation point will depend on algal strain used. Optimal irradiation for specific algal strain must be determined in order to reach the maximum growth and the highest biomass yield.

Although there is no consensus on the most suitable cultivation vessel for microalgae biomass production, open raceway ponds are cheapest and more sustainable large-scale cultivation technology for commercially viable microalgae bioenergy production [11]. Even though closed photobioreactors offer more flexibility in terms of the control of the culture conditions and higher biomass productivity per volume [12], high construction and maintenance costs limit their use as a large-scale biomass production technology.

In addition to irradiation, the amount of light received by algal cells depends also on the depth of the cultivation pond, density of the culture and mixing efficiency [13]. Successful bioreactor for microalgae cultivation requires high surface-to-volume ratio to promote the light penetration inside the culture. Therefore, open raceway ponds are commonly of a shallow construction to facilitate the availability of light. Even then, light intensity varies considerably inside a pond, being the highest at the surface and decreasing towards the bottom of the pond. At the top layer of the culture light intensity is high and areal productivity of algae is high [14]. As the density of algal culture is increasing during the cultivation less light can penetrate the deeper layers of the pond. Consequently, at the lower levels of the pond algal growth is limited due to reduced light availability and light scattering caused by shading effect of the top

layer [6]. Moreover, algae near the surface are often exposed to excessive light while cells close to the bottom of the pond can experience severe light limitation conditions. Photo-inhibition can occur during the hours of strongest irradiance, generally around midday. Commonly, algae experience extremely diverse lighting conditions in an open pond system, ranging from light deficiency to photo-inhibition. Good penetration and uniform distribution of light in a pond is essential to avoid photo-inhibition or light limitation and to maintain high growth rates and can be provided by means of appropriate design and efficient mixing.

Light limitation is a key limiting factor of large-scale microalgae cultures [15]. Sunlight is a free and abundant energy source for photosynthetic organisms however, it has some disadvantages like daily and seasonal fluctuation in irradiance level and day/night cycles [16]. Such limitations can be overcome by supplementing with artificial lighting. Addition of artificial lighting in outdoor cultivation systems can enhance algae growth during the conditions of light limitation. Low light conditions in an outdoor environment can occur on rainy days or overcast days with a high cloud cover. Moreover, during the suboptimal light hours, e.g. during winter months, supplementation of available daylight hours with artificial illumination will enhance efficient use of available light. Consequently, efficient and cost-effective illumination is of particular importance to rise the economic feasibility of microalgal biofuels.

Light-emitting diode (LED) lights have several advantages over other types of commonly applied lights. LEDs produce light with a narrow emission peak therefore specific sections of the photosynthetically active radiation (PAR) can be tested. Due to their small size LEDs can be conveniently integrated in any cultivation vessel; and any type of lighting configuration can be easily achieved [17]. Another advantage of LEDs is a dimming option making an adjustment of required light intensity simple and allowing the establishment of light intensity gradients resembling natural light-dark cycles.

Based on the identified research gaps the goal of the present study is to find the optimal light intensity conditions of three microalgal species in laboratory batch cultures that will be applied afterwards in outdoor raceway ponds supplemented by artificial LED lighting to maintain high biomass productivity even in suboptimal environmental light conditions.

2 MATERIALS AND METHODS

2.1 Microalgae cultures

In order to find the optimal illumination conditions, various light intensities were tested on potential candidate strains for large-scale biomass production. Green microalgae *Chlorella vulgaris* 211-11j, *Chlorella sorokiniana* 211-8k and *Chlamydomonas reinhardtii* 11-32b were obtained from SAG Culture collection of algae at Göttingen University, Germany. *C. vulgaris* and *C. sorokiniana* were maintained in a sterile liquid BG-11 growth medium, *C. reinhardtii* was maintained in a TAP medium. All cultures were cultivated in Erlenmeyer flasks with baffles and 0.2 μm PTFE membrane screw caps (Duran, Germany) at 22 °C in low light conditions in shaker-incubator at 150 rpm. Sub-culturing in a new growth media was done approximately once per month to keep the algae cultures growing and in a healthy condition.

2.2 Light intensity tests

Effect of light intensity on microalgae growth and biomass production was assessed under irradiance from 30 to 200 μmol photons m^{-2} s^{-1}. Specifically, light intensity of 30, 50, 100, 150 and 200 μmol photons m^{-2} s^{-1} was applied. Natural white (4000 K) linear 10W LED lights (V-TAC Samsung) were manually installed inside an incubator (Friocell Eco line, MMM group, Germany). Specific light intensity was achieved by adjusting the number of LED lights and their distance from culturing flasks. Installed light intensity was measured with light meter (Testo, Germany). Microalgae *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* were cultivated in 500 ml Erlenmeyer flasks with cotton plugs containing 200 ml BG-11 or TAP medium. Aeration was provided with ambient air using orbital shaker (Elmi, Latvia) at 150 rpm. Algae were grown at constant temperature of 24 °C under photoperiod of 16:8 h (light/dark) for 10 days under batch cultivation mode. The initial optical density (OD) of all cultures was approx. 0.05. Initial pH of BG-11 medium was 7.5-7.7 and 7 for TAP medium.

Cultures were sampled daily for growth rate evaluation by optical density measurements with UV/VIS spectrophotometer (Thermo Scientific, USA) at 750 nm. Moreover, daily pH readings were collected manually with a pH meter (Hanna, USA) to monitor the microalgae growth and to evaluate the effect of non-controlled pH on the growth of microalgae.

Experiments were conducted in two rounds. In the first setup light intensity of 30, 50, 100 and 150 μmol m^{-2} s^{-1} was tested: *C. vulgaris* and *C. sorokiniana* were cultivated in BG-11 medium while *C. reinhardtii* in a TAP medium. Slow growth rate of *Chlorella* species was detected compared to *C. reinhardtii*. Moreover, a sharp rise in pH during the cultivation was observed that might have affected the biomass productivity of *Chlorella* spp. In the second round *Chlorella* species were cultivated in a TAP medium in order to understand if a change of medium can improve the growth rate of *Chlorella* spp. In the second round light intensity was also adjusted and was set to 50, 100, 150 and 200 μmol m^{-2} s^{-1}.

2.3 Biomass production evaluation

All tests were conducted in triplicate. Biomass yield was determined at the end of the batch cultivation for all cultures based on the dry cell weight. Dry weight was measured by the following procedure: 50 ml of homogeneous microalgal culture was collected from the culture flask and centrifuged at 10`000 rpm for 10 minutes at a room temperature in pre-weighted centrifugation tubes. Liquid part was discarded and tubes were dried at 80 °C in oven until constant weigh, cooled to room temperature and weighted. Dry weight was calculated by subtracting initial tube mass from the mass of tubes with the dry biomass. Maximum biomass yield was calculated as grams of biomass per litre (g L^{-1}).

Specific growth rate (μ, d^{-1}) was calculated according to the equation (1);

$$\mu = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (1)$$

where N_2 and N_1 are culture optical densities at the

time t_2 and t_1, respectively.

3 RESULTS AND DISSCUSSION

Three green microalgae *C. vulgaris* 211-11j, *C. sorokiniana* 211-8k and *C. reinhardtii* 11-32b were selected as potential candidate strains for biomass production in outdoor raceway ponds with additional artificial illumination to enhance biomass production. Microalgae were selected based on a literature research as fast-growing species with a good adaptability to changing outdoor environment. In order to find optimal illumination conditions growth rate and biomass production were evaluated at five different light intensities: 30, 50, 100, 150, 200 µmol m^{-2} s^{-1}. When *Chlorella* species were cultivated in BG-11 medium that is commonly used for *Chlorella* spp. cultivation, species exhibited low growth rate compared to *C. reinhardtii* and linear growth curve was observed for all light intensities tested. Consequently, cultures did not reach a stationary phase during the 10-day batch cultivation (Fig. 1. A and B).

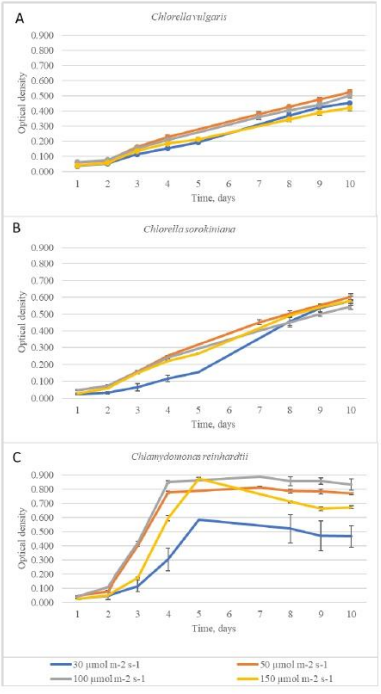

Figure 1: Growth curves of *C. vulgaris* (A), *C. sorokiniana* (B) and *C. reinhardtii* (C) at 30, 50, 100 and 150 µmol m^{-2} s^{-1} cultivated in BG-11 medium (*Chlorella* spp.) and TAP (*C. reinhardtii*). Each dot represents the average of three replicates, error bars indicate standard deviation.

On the other hand, *C. reinhradtii* cultivated in TAP medium showed high growth rate and reached maximum culture density at the day 4 to day 5 of the cultivation (Fig 1. C). The uncommon growth curve of *Chlorella* spp. suggested that cultures are not under optimal growth conditions and some limiting factor exists inhibiting the growth. Furthermore, daily pH measurements revealed high pH of *Chlorella* spp. in BG-11 media that reached pH 11 at the end of the cultivation (Fig. 2). pH of *C. reinhardtii* cultures did not exceed pH 8.48.

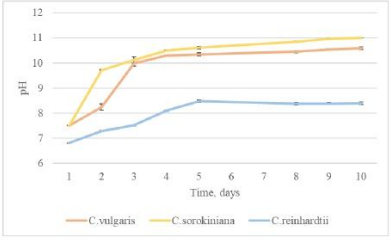

Figure 2: pH range during the cultivation of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* under illumination of 150 µmol m^{-2} s^{-1}.

The slow growth rate of *Chlorella* species observed suggested that cultivation conditions must be improved therefore, a second round of experiments was carried out. pH during the first setup cultivation exceeded optimum pH range of both *C. vulgaris* and *C. sorokiniana*. *C. vulgaris* has a wide optimum pH range of approx. 7 to 10 but for *C. sorokiniana* optimum is approx. pH 6 to 7.5 (Ievina and Romagnoli *In press*). Therefore, actual pH during the cultivation was significantly higher than optimum that might have affected the growth rate. During the subsequent round of experiments all species including *Chlorella* spp. were cultivated in a TAP medium. Furthermore, first set of experiments showed that irradiation of 30 µmol m^{-2} s^{-1} was too low for high biomass production for all species therefore it was excluded from the second set of experiments, moreover, maximum irradiation was extended to 200 µmol m^{-2} s^{-1}, accordingly microalgae were cultivated at 50, 100, 150 and 200 µmol m^{-2} s^{-1}. Other cultivation parameters were not changed. Growth curves of three microalgae during second set of experiments are shown in Figure 3. *C. vulgaris* and *C. sorokiniana* exhibited much higher growth rate in TAP compared to BG-11 medium suggesting that TAP is more suitable medium for fast biomass accumulation of *Chlorella* species.

All strains exhibited an exponential growth starting from the second day. The lower growth rate at the start of the cultivation could be explained by adaptation of the cultures to the new growing conditions. *C. vulgaris* reached maximum culture density at 100 µmol m^{-2} s^{-1} light intensity (OD 2.3). *C. sorokiniana* exhibited comparable growth rate at 100, 150 and 200 µmol m^{-2} s^{-1} (OD 2.3, 2.4 and 2.4, respectively). Growth was slightly lower at 50 µmol m^{-2} s^{-1} light intensity (OD 2.1). *C. reinhardtii* exhibited lower biomass density at OD 750 nm than other two species and reached OD 1.28 at 200 µmol m^{-2} s^{-1}. Lowest growth rate was detected at 50 µmol

m^{-2} s^{-1} for *C. sorokiniana* and *C. reinhardtii* and at 150 and 200 µmol m^{-2} s^{-1} for *C. vulgaris*. After day 5 to 6 growth rate started to slow down supposedly due to scarcity of nutrients. Growth of *C. sorokiniana* started to decrease after the day 4 but the growth curve did not show flat stationary phase and was still growing slowly suggesting that also other factors than lack of nutrients could have affected the growth kinetics. The increased culture density at the end of the cultivation period could have limited the light availability to cells due to the cell shading effect leading to the light limitation conditions.

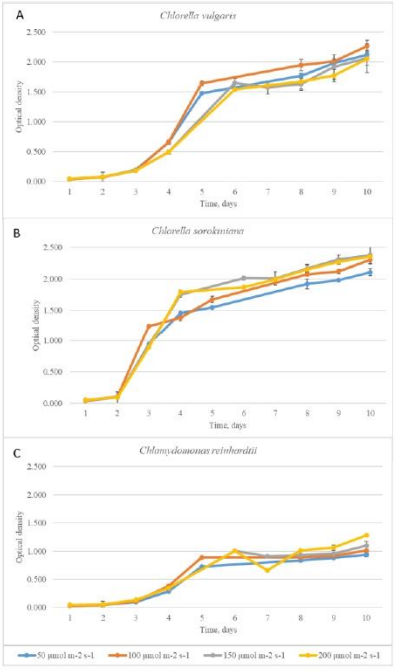

Figure 3: Growth curves of *C. vulgaris* (A), *C. sorokiniana* (B) and *C. reinhardtii* (C) at 50, 100, 150 and 200 µmol m^{-2} s^{-1} cultivated in TAP medium. Each dot represents the average of three replicates, error bars indicate standard deviation.

Yan et al. [19] described a novel strategy for improving light availability in high density cultures suggesting that higher light intensity must be provided to microalgae cultures as the culture density is increasing during the cultivation. He recommended to use 800 µmol m^{-2} s^{-1} at the beginning of cultivation and increase the light intensity to 1600 µmol m^{-2} s^{-1} at the final stage of cultivation. However, the proposed strategy could be applied only if cultivation is performed in a batch mode limiting its usefulness for large-scale biomass production.

Maximum biomass yield (g L^{-1}) was calculated from the dry weight of biomass at the end of the 10-day batch cultivation (Figure 4). Biomass yield was comparable among all the microalgae strains studied. Although *C. reinhardtii* exhibited significantly lower optical density at 750 nm compared to other microalgae strains, biomass production was comparable to other strains. Biomass increased with the increasing light intensity from 50 to 150 µmol m^{-2} s^{-1} for *C. vulgaris* and *C. reinhardtii* and to 200 µmol m^{-2} s^{-1} for *C. sorokiniana*. Highest biomass yield was recorded for *C. sorokiniana* at light intensity of 200 µmol m^{-2} s^{-1} (1.13 g L^{-1}). *C. vulgaris* and *C. reinhardtii* produced most biomass when cultivated at 150 µmol m^{-2} s^{-1}, 1.05 and 1.06 g L^{-1}, respectively. *C. reinhardtii* under illumination of 200 µmol m^{-2} s^{-1} resulted in 1.03 g L^{-1}. Lowest biomass yield was recorded at light intensity of 50 µmol m^{-2} s^{-1} for all three microalgae strains studied, 0.75 g L^{-1} for *C. reinhardtii* and *C. sorokiniana* and 0.82 g L^{-1} for *C. vulgaris*. Our results are indicating that 50 µmol m^{-2} s^{-1} is too low irradiance for high biomass yield.

Results of the biomass production are suggesting that optimal light intensity for *C. vulgaris* and *C. reinhardtii* is around 150 µmol m^{-2} s^{-1} while higher light intensity (approx. 200 µmol m^{-2} s^{-1}) is more suitable for *C. sorokiniana*. It is not surprising as *C. sorokiniana* is known to be a high light intensity tolerant alga [20] consequently optimal light requirements are higher than those of other microalgae. *C. vulgaris* biomass decreased at 200 µmol m^{-2} s^{-1} indicating that this light intensity might be too high and photo-inhibition process might have been initiated during the cultivation at 200 µmol m^{-2} s^{-1}.

Optimal light intensity for *C. vulgaris* reported in the literature varies widely from 62.5 µmol m^{-2} s^{-1} [21] and 80 µmol m^{-2} s^{-1} [22] to 2000 µmol m^{-2} s^{-1} [19]. However, most often light intensity around 200 µmol m^{-2} s^{-1} is proposed [10], [23]. He et al. found that light intensity of 400 µmol m^{-2} s^{-1} leads to degradation of chlorophyll, decrease of protein and carbohydrate content in cells, and an increase in lipid content [23]. Yan et al. observed that much higher light intensities are needed for maximum biomass production of *C. vulgaris* in synthetic wastewater [19]. 2000 µmol m^{-2} s^{-1} was found to be optimal for high growth rate and efficient nutrient removal from wastewater. However, microalgae were cultivated under red light, moreover composition of synthetic high-strength wastewater could have affected the light requirements.

Reported variance in optimal light intensity stresses the importance of the impact of other cultivation parameters on the light requirements. Therefore, optimal light intensity must be determined for the specific cultivation conditions under interest.

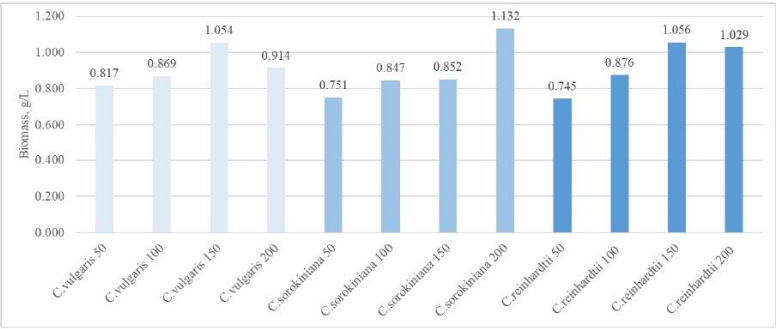

Figure 4: Maximum biomass yield of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* under various light intensities 50 – 200 µmol m^{-2} s^{-1}. Number next to the species name indicates light intensity (µmol m^{-2} s^{-1}).

Specific growth rate (µ, d^{-1}) was calculated for each species at the specific light intensity (Fig. 5). All species exhibited comparable growth rate varying from 0.467 to 0.552 d^{-1}. Highest growth rate was observed for *C. vulgaris* at light intensity 100 µmol m^{-2} s^{-1} (0.552 d^{-1}), followed by *C. vulgaris* at 50 and *C. sorokiniana* at 200 µmol m^{-2} s^{-1}, 0.540 and 0.535 d^{-1}, respectively.

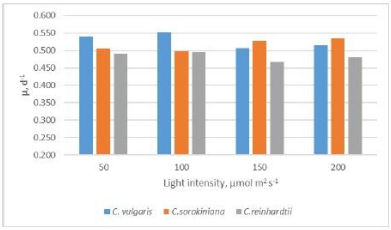

Figure 5: Specific growth rate (µ, d^{-1}) of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* under various light intensities.

pH was measured daily during the cultivation period. Since no additional CO_2 was provided pH reached very high values during the first set of experiments in BG-11 medium. pH rose out of the optimum range of *C. vulgaris* and *C. sorokiniana* thus could have contributed to the slow growth observed in BG-11 medium. During the second set of experiments when all microalgae strains were cultivated in TAP medium pH did not increase more than 8.4; not exceeding the optimum pH range of species (Fig. 6). pH was showing steep rise during the first days of cultivation, rising from 7 to about 8.4. pH is an indication of culture condition, a rise in pH shows growth of microalgae. pH is increasing due to the uptake of carbon by microalgae during the day [24]. pH of cultures was leveling out at the day 4 to 6 and staying constant at the second part of the cultivation period.

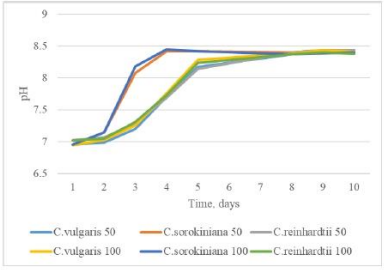

Figure 6: pH of *C. vulgaris*, *C. sorokiniana* and *C. reinhardtii* during the cultivation under 50 and 100 µmol m^{-2} s^{-1} light intensity. Number next to the species name indicates light intensity (µmol m^{-2} s^{-1}).

4 CONCLUSIONS

The current study was carried out to investigate the effect of light intensity on the growth rate and biomass production of three microalgae strains intended for cultivation in outdoor raceway ponds supplemented with artificial LED illumination. The study has shown the impact of light availability on the growth of microalgae. Light intensity has a major role in biomass production. All species tested exhibited similar growth rate and biomass productivity under selected light intensities and the specific cultivation conditions. However, light intensity of 30 and 50 µmol m^{-2} s^{-1} is too low to maintain high growth rate for microalgae strains studied. Our results are suggesting that *C. vulgaris* has lower light requirements comparing to *C. sorokiniana* and *C. reinhardtii*. The highest biomass yield was produced at light intensity of 150 µmol m^{-2} s^{-1} for *C. vulgaris* and at 200 µmol m^{-2} s^{-1} for *C. sorokiniana* and *C. reinhardtii*.

Moreover, we observed that the choice of growth medium has a large effect on microalgae growth. Cultivation in TAP medium resulted in higher growth rate of *C. vulgaris* and *C. sorokiniana* compared to BG-

11 medium.

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APPLICATION OF ACTIVATED CARBON TREATED AGRICULTURAL DIGESTATE FOR MICROALGAE CULTIVATION

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ABSTRACT: Activated carbon pre-treatment was applied to anaerobic digestion effluent in order to reduce the optical density. Up to 78% reduction of OD was achieved suggesting activated carbon pre-treatment a promising tool to increase the light transmission of agricultural digestate. The pre-treated digestate was applied for microalgae cultivation as a source of nutrients and compared to the untreated diluted digestate. Microalgae growth was similar at the first period of cultivation in pre-treated and diluted untreated digestate. However, at the other part of the cultivation higher growth rate and biomass accumulation in untreated digestate was observed. We discuss the possibility of too high nutrient load in pre-treated digestate that may have limited the microalgae growth. PAM measurements showed no stress from the substrate highlighting that activated carbon pre-treated digestate is a suitable medium for microalgae growth. Moreover, vitality of cultures in pre-treated PBRs dropped at the end of the cultivation suggesting some inhibitory effect may be present. Results of current study demonstrate that activated carbon holds a great potential for agricultural digestate pre-treatment to decrease the optical density.
Keywords: microalgae, wastewater, digestate, biomass, adsorbent.

1 INTRODUCTION

Although recognized as a promising source of sustainable energy already decades ago, microalgae have attracted more interest recently due to governmental policies and subsidies. Bioenergy from microalgae is also supported through the Renewable energy directive (EU 2018/2001) promoting the use of algae to achieve EU climate and energy targets. Due to the increased number of studies carried out in the last decade, scientific knowledge on microalgae culturing is continuously growing showing the enormous potential of microalgae biomass both for bioenergy production (i.e. biogas, biodiesel, bioethanol etc.), wastewater treatment and extraction of high value products thus supporting bioeconomy goals. Microalgae are classified as third-generation feedstock and have evident advantages over other types of feedstock, e.g. (1) all year round production, (2) fast growth rate, (3) growth in low quality water, (4) high fixation of CO_2, (5) no competition with food production, (6) no arable land needed for cultivation and (7) production of wide range of bioproducts [1]–[3]. Despite these potential benefits, high cultivation costs of microalgae biomass are limiting a commercial use of microalgae as a feedstock for bioenergy production. Several strategies have been proposed to overcome the economic challenges of microalgae biomass production. The incorporation of wastewater streams as a source of nutrients into microalgae cultivation system is a promising approach recently being studied intensively. Application of biogas anaerobic digestion effluent is one of the possible strategies to decrease the cultivation costs to ensure a cost-effective biomass production.

The number of biogas plants has been steadily growing over the last decade reaching 18 943 biogas plants throughout the Europe by the end of 2019 [4]. The largest portion of biogas plants is working on agricultural residues. Continuous increase in biogas production inevitably leads to an increased digestate load therefore, overproduction of digestate potentially triggering eutrophication is becoming a serious issue. Although classical use of digestate is fertilization of agricultural land, alternative valorization routes of digestate have recently been suggested. Liquid fraction of digestate contains high concentrations of nutrients, such as nitrogen, phosphorus, potassium and others [5] that are in a plant-available nutrient form, therefore liquid fraction of digestate can be an excellent source of nutrients for microalgae cultivation at a minimal cost. Although composition of digestate varies considerably depending mainly on the feedstock used, generally, nutrient level and composition of diluted digestate is favorable for microalgae cultivation [6]. Consequently, the integration of microalgae biomass cultivation system on biogas production site becomes a reasonable solution providing potential advantages [7]. Cultivation of microalgae in liquid digestate combines low-cost microalgae biomass production with wastewater treatment, leading to a substantial reduction of costs by performing these two tasks simultaneously. Microalgae are known to remove nitrogen, phosphorus and toxic substances, such as heavy metals, from various wastewaters [8] therefore can be used as a biological treatment system. Although liquid digestate presents a great potential as a nutrient source there are several drawbacks associated with it. Agricultural digestate compared to other wastewater streams is characterized by very high nutrient load, high turbidity and optical density (OD), and lower carbon levels that can negatively affect microalgae. Some studies have reported microalgae growth inhibition due to the excessively high OD and ammonia. High optical density caused by humic substances and organic matter, which creates the characteristic dark color, is one of the main disadvantages of the digestate [9]. High optical density decreases the light transmission into microalgae culture and reduces the amount of light available for photosynthesis. Being the most important energy source for photosynthesis light availability is crucial to sustaining high microalgae growth. Therefore, light limitation due to the high OD can lead to severely reduced biomass yield. Moreover, liquid fraction of digestate is often characterized by high content of suspended solids leading to high turbidity and further reducing the available light for microalgae growth.

Current studies suggest the dilution of digestate for cultivation of microalgae to decrease the OD and the

effect of inhibitors [6], [10]. Although this approach has shown a positive effect on microalgae growth, a large volume of freshwater is needed to decrease the OD and to dilute the inhibitors to a tolerable level for microalgae; on average 10 to 30-fold dilution is being reported most frequently [10]. High dilution rate requires large volume of freshwater resources limiting the economic and environmental benefit of such a system. Moreover, high dilution rate limits the volume of digestate that can be fed into microalgae ponds for treatment, requiring a much larger area of cultivation ponds, longer time to treat the digestate and thus higher energy demand. Decreasing the amount of freshwater is a key aspect to build a sustainable and economically viable system for digestate application for microalgae cultivation. Therefore, the removal of potential growth inhibitors by pre-treatment of digestate may be a sought-after solution. The main challenge is to find an effective and low-cost pre-treatment method however, only few pre-treatment methods have been described so far [9], [11] indicating a lack of studies on available solutions. Activated carbon holds a great potential as an efficient low-cost method to reduce turbidity, optical density and harsh chemicals in digestate due to the high capacity of adsorbing various substances. Activated carbon works as an adsorbent providing a large surface area where contaminants may be adsorbed providing the opportunity to reduce the optical density of digestate to a desirable level for microalgae and facilitate microalgae growth. Activated carbon may be produced from residual materials resulting in a low-cost and environmentally sound solution [11]. Although activated carbon has been applied for municipal wastewater treatment, it is a novel pre-treatment method for digestate, and its actual potential is still unknown. It has shown some very promising results in reduction of OD of digestate coming from a piggery farm in Italy [11].

The current study deals with a reduction of OD of high-strength agricultural digestate by pre-treating the liquid fraction of digestate with activated carbon. The effect of activated carbon pre-treated digestate on microalgae growth was assessed by carrying out microalgae growth tests in pre-treated digestate and compared to untreated diluted digestate.

2 MATERIALS AND METHODS

2.1 Microalgae cultures

Microalgae culture containing mainly *Chlorella* sp. was obtained from Istituto Spallanzani (Rivolta d'Adda, CR, Italy) and have been acclimated to growing in digestate by culturing in diluted liquid digestate for approximately two weeks before growth tests in photobioreactors. The culture was cultivated at room temperature under white LED lights (12h/12h light/dark cycle) on a magnetic stirrer.

2.2 Digestate characterization

Chemical composition, pH, turbidity, optical density and content of solids were analyzed in a liquid agricultural digestate collected from Iecava biogas plant in Latvia and the level of potential inhibitors was assessed. Raw liquid digestate was centrifuged at 10 000 rpm to decrease the amount of solids and kept at +4 °C before the use. Level of total nitrogen, phosphate, nitrate, ammonium, chemical oxygen demand (COD), turbidity and OD were then determined in the liquid digestate. Commercial cuvette test kits (Hach Lange, Germany) were used for spectrophotometric quantification of phosphate (PO_4-P), total nitrogen (TN), ammonium (total ammonia nitrogen, NH_4-N), nitrate (NO_3-N), chemical oxygen demand (COD) using DR3900 spectrophotometer (Hach Lange, Germany) according to Hach standard methods. All samples were filtered through 0.45 µm filter before the analysis of TN, NH_4-N, NO_3-N, PO_4-P and COD. Turbidity was determined spectrophotometerically with DR3900 spectrophotometer by using pre-set turbidity parameters. OD was measured at 680 nm using spectrophotometer.

Total solids, suspended solids and volatile solids in digestate were analyzed according to the standard methods [12].

2.3 Liquid digestate pre-treatment

An activated carbon pre-treatment was used to reduce the OD of liquid digestate by performing adsorption on activated carbon (Chemviron, UK), applying several activated carbon concentrations and various adsorption durations. Activated carbon concentrations of 3, 10, 20 and 40 g per liter were used. Liquid digestate was incubated with activated carbon on a rotary shaker at 200 rpm for 5, 10, 30 and 180 minutes, and then centrifuged at 13 000 rpm to remove activated carbon particles. The OD was measured after the pre-treatment and OD reduction rate was calculated. The best performing activated carbon concentration and adsorption time combination was then selected for microalgae growth tests based on the most efficient OD reduction.

2.4 Setup of microalgae growth tests

The effect of activated carbon pre-treated digestate on microalgae growth was assessed by performing growth tests. Tests were carried out in parallel with a raw untreated and activated carbon pre-treated digestate as a growth medium in four plexiglass column photobioreactors (PBRs) with 10 cm diameter and working volume 1.5 L (IDEA Bioprocess Technology Srls, Italy) (Figure 1).

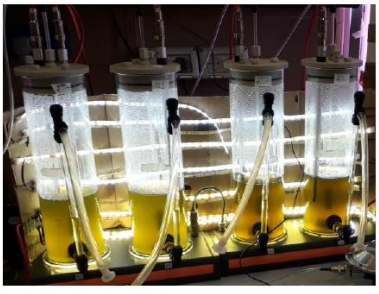

Figure 1: Experimental setup with photobioreactors at the beginning of growth tests.

The untreated digestate was diluted down to OD 0.1 corresponding to 1% dilution with distilled water to increase the light availability and decrease the nutrient load. Digestate pre-treated with activated carbon 3 g/L and adsorption time of 10 minutes was then diluted to OD 0.1 to match the OD of the untreated digestate.

Microalgae cultivation was performed with two replicates for each condition; in PBR 1 and PBR 2 untreated diluted digestate was used, in PBR 3 and PBR 4 – activated carbon pre-treated digestate. PBRs were inoculated with microalgae culture dominated by *Chlorella* sp. previously acclimated in diluted untreated digestate for approximately two weeks. The initial OD of algal culture was 0.1 in all PBRs. PBRs were mixed with magnetic stirrers at 250 rpm. pH was controlled automatically by CO_2 injection in the system when the pH moved out of the set range. Optimal pH was set between 7 and 7.8. Lighting was provided by white LED lights under 12h/12h light/dark cycle at an average light intensity of 50 µmol m^{-2} s^{-1}. The cultivation was carried out in batch cultivation mode at room temperature of approximately 24 °C for 14 days.

2.5 Nutrient removal and biomass accumulation

The initial nutrient concentration, OD, pH and COD were determined in PBRs and thereafter were monitored regularly during cultivation. Phosphate (PO_4-P), total nitrogen (TN), ammonium (total ammonia nitrogen, NH_4-N), nitrate (NO_3-N), chemical oxygen demand (COD) were determined with Hach Lange DR 3900 spectrophotometer as described before under digestate characterization section. Samples were analyzed in two replicates from each PBR. Initial nutrient concentration in treated and un-treated PBRs varied due to different dilution rates of digestate. Nutrient concentrations in PBR 3 and 4 (pre-treated) were higher than those of PBR 1 and 2 (untreated). Nutrient removal rates were subsequently calculated and compared between treated and untreated digestate. Microalgae biomass accumulation was measured based on OD, cell counts and suspended solids content. Microalgal cell counts were carried out using hemocytometer (Marienfeld, Germany) and an optical microscope 40X (B 350, Optika, Italy). Cell counts were performed every three days during cultivation to evaluate the growth of microalgae, changes in species composition and presence of potential predators. *Chlorella* spp., *Scenedesmus* spp. or other species were identified according to their morphological characteristics and counted. Microalgae productivity in each PBR was calculated based on the determination of cell dry weight. Optical density was measured by DR3900 Hach Lange spectrophotometer at a wavelength of 680 nm. Specific growth rate (µ, d^{-1}) was calculated according to the equation (1), where N_2 and N_1 are culture optical densities at the t_2 and t_1, respectively.

$$\mu = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (1)$$

Furthermore, a viability test was performed to assess the condition of microalgal cultures during the cultivation test. Nucleic acid stain Sytox (Thermo Fisher Scientific) was used according to the manufacturer's instructions to determine the viability of cells using a Zeiss fluorescence microscope Axio 170 Scope HBO 50 at the wavelength of 450–490 nm. Sytox can only penetrate the damaged cell walls of dead microalgae which can be detected by their bright green fluorescence. Living cells are red due to the autofluorescence of chlorophyll [13]. 1 mL of each microalgal suspension was centrifuged for 10 minutes at 10 000 rpm. The supernatant was discharged while the biomass was resuspended in 1 mL of distilled water. Finally, Sytox label was added (0,5 µL) for the staining process and the samples were kept in the dark for ten minutes prior the observation. For each sample, an average of 300 cells were counted, assessing the proportion between dead and living cells.

2.6 Photosynthetic efficiency

Photosynthetic performance measured with pulse amplitude modulated (PAM) fluorometry was used to evaluate the effect of digestate on the growth of microalgae. PAM is a valuable technique to evaluate the physiological stress of microalgae caused by potential toxicity of the growth medium that can be seen from photosystem II performance [14]. Microalgal culture samples were collected from all bioreactors and diluted to OD 0.1 at 680 nm. Samples were kept in the dark for 20 min. Algal cultures from bioreactor were compared with algae grown in a synthetic medium. PHYTO-PAM-II (Heinz Walz, Germany) was used for the analysis.

F_v/F_m represent the maximum photochemical quantum yield of photosystem II (equation 2), where F_o is minimum fluorescence level excited by very low intensity of measuring light, F_m is a maximum fluorescence level elicited by a pulse of saturating light.

$$\frac{Fv}{Fm} = \frac{(Fm - Fo)}{Fm} \quad (2)$$

Moreover, different groups of photosynthetic organisms can be detected with PAM readings as differentiation of 4 different pigment types and therefore detection of green algae, cyanobacteria, diatoms/dinoflagellates and phytoerythrin containing organisms can be made.

3 RESULTS AND DISSCUSSION

3.1 Characterization of liquid digestate

Chemical composition of raw liquid digestate is reported in Table 1. Total solids content of raw liquid digestate reached 23 g/L leading to extremely high turbidity (7840 mg/L) suggesting potential inhibitory effect on photosynthetic potential and low light availability to cells. Furthermore, exceptionally high optical density (OD 13) was recorded resulting in nearly black opaque liquid (Figure 2). Organic material and humic substances present in digestate are most likely responsible for the characteristic dark color. It is clear from a visual inspection that no microalgae would grow in this thick black digestate without a very high dilution rate.

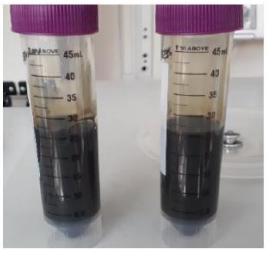

Figure 2: The appearance of raw (undiluted, untreated) liquid fraction of digestate.

Table I: Characterization of liquid fraction of raw agricultural digestate and after pre-treatment with 3 g/L and 40 g/L activated carbon with adsorption time 10 minutes.

Parameter	Unit	Raw liquid digestate	Pre-treated	
			3 g/L	40 g/L
TS	g/L	22.9	NA	NA
SS	g/L	5.1	NA	NA
VS	g/L	4.25	NA	NA
DS		17.83	NA	NA
OD		13.03	3.06	2.81
pH		8.17	NA	NA
Turbidity	mg/L	7840	NA	NA
COD	mg/L	6840	6540	4960
N_{tot}	mg/L	5950	NA	NA
NH_4-N	mg/L	3600	3000	2667
NO_3-N	mg/L	47.5	NA	NA
PO_4-P	mg/L	490	338	278.4

Nitrogen and phosphorus are primary nutrients required for microalgae growth and usually are abundant in liquid digestate [15]. In particular, agricultural digestate is rich in nitrogen when compared to other wastewater streams [8]. Indeed, 5950 and 490 mg/L total nitrogen and phosphors, respectively were detected in the current study. Chemical analysis showed that most of the nitrogen in digestate was in a form of ammonium (NH_4-N) as pointed out in other studies [16], [17]. Although ammonium is a preferred source of nitrogen for most microalgae [18] high total ammonia nitrogen may inhibit microalgae growth [15], [19]. As in this study, a high value of ammonia nitrogen concentration (3600 mg/L) has not been found reported elsewhere. Phosphorus content (490 mg/L PO_4-P) was comparable to or higher than that referred in other studies [11], [18]. The reported values of COD content in anaerobic digestion effluents are commonly higher than found in other wastewater streams [8] however, exceptionally high COD (6840 mg/L) was found in the current study indicating excessive load of organic matter. Uggetti et al. reported COD 210 mg/L in anaerobic digestate [18], 1980 mg/L was reported in digestate from livestock waste [20], 2661 mg/L in anaerobic digested municipal wastewater [6], 3402 mg/L in anaerobic digested piggery wastewater [21]. Digestate was slightly alkaline as commonly reported [15] with the pH of 8.17 being at the optimal range for most freshwater microalgae species [15]. Typical effluent from anaerobic digestion is known to have high nutrient concentrations [17] however, generally, all parameters measured in this study were higher than reported in the literature [15] indicating very rich, highly concentrated digestate. Nutrient content of raw digestate was significantly higher than recommended for microalgae cultivation. Furthermore, dark color and high turbidity make algae cultivation in raw liquid digestate impossible.

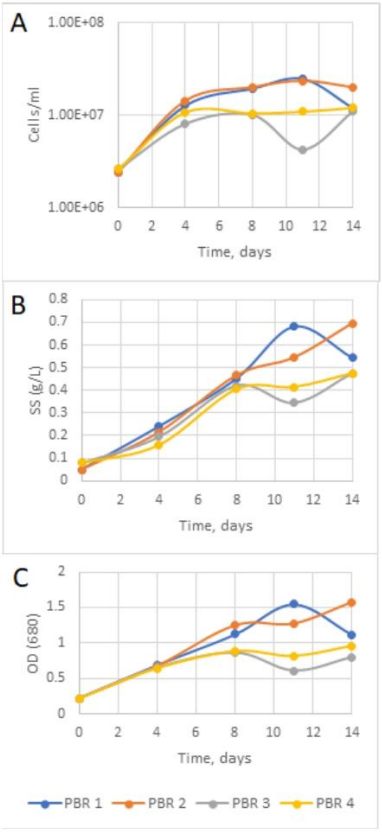

Figure 3: Microalgae growth rate in untreated (PBR 1 and 2) and pre-treated (PBR 3 and 4) digestate reported as cell count (A), biomass productivity (B) and OD (C).

3.2 Effect of activated carbon adsorption on OD rate of digestate

The initial OD of raw liquid digestate was 13 suggesting that light penetration in a raw liquid digestate is not sufficient for microalgae growth. Activated carbon pre-treatment was applied to raw liquid digestate in order to reduce the OD. Activated carbon concentrations from 3 to 40 g/L were applied at various adsorption durations ranging from 5 to 180 minutes. Highest OD reduction rate of 78% was achieved after 10 minutes of adsorption at 40 g/L and of 77% at 40 g/L with 5 minutes, 3 g/L with 10 minutes and 40 g/L with 30 minutes of adsorption time (Table 2). Lowest OD reduction rate was observed after 30 minutes at 3 g/L and after 180 minutes at 3 g/L activated carbon concentration showing 64 and 65%

Table II: OD reduction rate of digestate (%) after activated carbon with different concentration and contact time.

Activated carbon concentration	Adsorption time, min	OD reduction %	Adsorption time, min	OD reduction %	Adsorption time, min	OD reduction %	Adsorption time, min	OD reduction %
3 g/L	5	72	10	77	30	64	180	65
10 g/L	5	75	10	68	30	68	180	69
20 g/L	5	71	10	72	30	66	180	73
40 g/L	5	77	10	78	30	77	180	69

reduction, respectively. Along with the reduction of OD concentration of some nutrients and COD was decreased as well (Table 1). Ammonia concentration at activated carbon concentration of 3 and 40 g/L was reduced by 16% and 26%, respectively. COD concentration decreased from 6840 mg/L in raw liquid digestate to 6540 and 4960 at activated carbon concentration of 3 and 40 g/L, respectively. Adsorption on activated carbon affected also phosphorus content in digestate resulting in a decrease by 31 and 43% in 3 g/L and 40 g/L concentration, respectively. Results show up 78% reduction of optical density (OD 2.81 was reached), suggesting that activated carbon pre-treatment is a highly promising tool to reduce the OD in agricultural digestate to the tolerable level for microalgae in a short time. Due to the fact that reduction of OD was similar at 3 g/L and 40 g/L activated carbon concentration and keeping the pre-treatment costs down, concentration of 3 g/L was selected for digestate pre-treatment for growth tests.

3.3 Microalgae culturing in pre-treated liquid digestate

Microalgae growth tests with pre-treated and raw liquid digestate as a growth medium were run in parallel for 14 days in a batch cultivation mode in 1.5 L photobioreactors. In PBR 1 and PBR 2 untreated but diluted to OD 0.1 digestate was used, in PBR 3 and PBR 4 digestate pre-treated with 3 g/L activated carbon and diluted to 0.1 OD was applied. Microalgae growth rate in untreated and pre-treated digestate is reported in Figure 3 as cell count (A), biomass productivity (B) and OD (C). Number of microalgae cells increased during the cultivation showing exponential growth only till day 4, thereafter the growth slowed down in all PBRs (Figure 3A). The maximum number of cells was reached at the day 11 in untreated PBRs and at the day 14 in pre-treated PBRs. Specific growth rate μ was 1.15, 1.19, 1.14 and

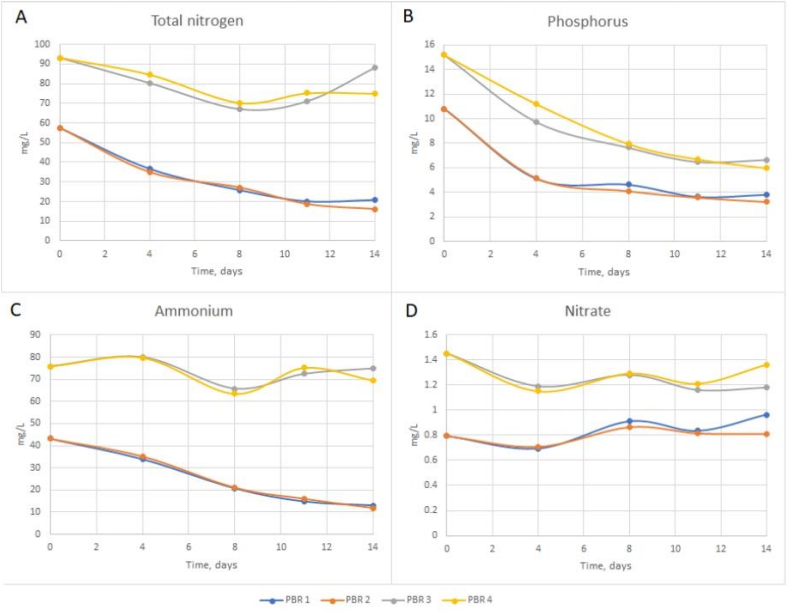

Figure 4: Removal of total nitrogen (A), phosphorus (B), ammonium (C) and nitrate (D) in pre-treated and untreated PBRs.

1.15 d^{-1} in PBR 1, 2, 3, and 4, respectively showing very similar growth in pre-treated and untreated digestate. Initial OD in all photobioreactors was 0.2 and increased up to 1.1, 1.6, 0.8 and 1 in PBR 1, 2, 3, and 4, respectively (Figure 3 C). The microalgae biomass productivity (dry weight) was calculated from the total suspended solids. Biomass yield increased steadily in all PBRs till day 8 and then started to fluctuate (Figure 3 B). Biomass productivity reached 0.69 g/L in untreated PBRs and 0.48 g/L in pre-treated PBRs. The growth indicators used demonstrate moderate microalgae growth in all PBRs suggesting that some factors might have limited the growth of cultures in PBRs to reach the maximum growth. Microalgae biomass yield in untreated diluted digestate was slightly higher comparing to pre-treated digestate. This could possibly be explained by the higher nutrient load in pre-treated PBRs due to larger initial amount of digestate applied. As seen from chemical analysis the agricultural digestate used is very high in nutrients therefore some chemicals might be in excess, leading to the suppression of microalgae growth.

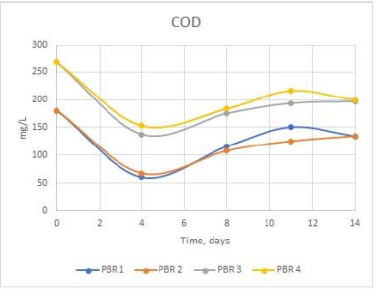

Figure 5: Removal of COD in pre-treated (PBR 3 and 4) and untreated (PBR 1 and 2) PBRs.

Excessive ammonia concentration is frequently cited in literature as the possible explanation of algal growth inhibition however, the actual threshold of ammonia tolerance seems to be species dependent. Initial ammonium concentration in untreated PBRs were 43 mg/L and 76 mg/l NH_4-N in pre-treated which might have an inhibitory effect on the microalgae population growth as reported in study by Uggetti et al. [18] where an increase in ammonia concentration from 9 to 34 mg/L resulted in 77% reduction in growth rate in a mixed microalgal culture dominated by *Scenedesmus* sp. Moreover, in other study *Scenedesmus* sp. showed no growth inhibition up to 100 ppm ammonium whereas values over 200 ppm resulted in severe decrease in cell density [20]. However, other studies reported microalgae tolerance to much higher ammonium concentrations. Ammonium of 178 mg/L was not toxic to *Chlorella* sp. and was completely removed from anaerobic digested dairy manure within a 21-day cultivation [16]. Microalgal consortium dominated by *Chlorella* sp. could resist ammonium concentration up 1600 mg/L NH_4-N [19]. Another study tested initial concentrations of ammonium ranging from 20 – 1500 mg/L and observed no inhibition of *Chlorella sorokiniana* growth at any of concentrations applied [10]. Moreover, the higher ammonium concentration the better the *C. sorokiniana* growth, thus the highest biomass accumulation was observed with initial ammonium at 1500 mg/L which is much higher than the ammonium concentration in the growth medium in our study. It seems that ammonium tolerance is not only species-specific but also depends on other factors such as cultivation conditions and acclimatization to high ammonium conditions.

Initial level of all nutrients was higher in pre-treated PBRs (PBR 3 and 4) than in untreated PBRs (PBR 1 and 2) due to higher volume of digestate applied. Total nitrogen removal rate was similar in all PBRs until around day 8 when level of nitrogen started to rise in pre-treated PBRs but continued to decrease in untreated PBRs (Figure 4A). Level of phosphorus exhibited decreasing trend throughout the cultivation in all PBRs indicating a good ability of *Chlorella* sp. to remove phosphorus (Figure 4B). Fluctuations in nitrogen content in pre-treated PBRs were also observed with ammonia nitrogen (NH_4-N) and nitrates (NO_3-N) content (Figure 4C and 4D, respectively). Fluctuations in nitrogen removal and accumulation are an indication of complicated chemical processes taking place inside PBRs due to interactions between microalgae and bacteria that are not fully understood. The observed changes in ammonium and nitrate content in PBRs could be due to nitrification processes taking place in PBRs. During nitrification processes ammonia (NH_4^+) is oxidized to nitrate (NO_3) by nitrifying bacteria. These bacteria could be present in growth medium coming from naturally occurring microorganisms in digestate affecting chemical processes in PBRs.

Level of COD was successfully reduced till day 4 in all PBRs (Figure 5). COD was reduced by 64% in the first 4 days in untreated PBRs and by 46% in pre-treated PBRs. However, COD started to increase thereafter in all PBRs. This phenomenon is well known in wastewater treatment with microalgae due to the fact that microalgae release organic compounds during the growth contributing to the increase of COD [22]. On average 72%, 73% and 70% of total nitrogen, ammonium and phosphorus, respectively was removed in untreated PBR 2 (Figure 6). Nutrient removal rates in PBR 1 were similar but slightly lower; 64%, 70% and 65% for total nitrogen, ammonium and phosphorus, respectively.

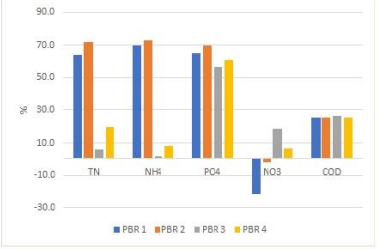

Figure 6: Nutrient removal rate (%) at the end of the growth test.

However, nutrient removal rates varied considerably between replicates in PBRs with pre-treated digestate. Just 6% of total nitrogen, 1.3% of ammonium and 56% of

phosphorus was removed in PBR 3 and 20%, 8% and 61% of total nitrogen, ammonium and phosphorus, respectively was removed in the PBR 4. On the other hand, removal of nitrates was higher in pre-treated PBRs. Accumulation of nitrates was observed in untreated PBR 1 reaching 21% increase in nitrate level at the end of the cultivation. Slight increase was detected also in PBR 2 (2.3%). However, nitrate removal in pre-treated PBRs was detected, resulting in 19% and 6% removal in PBR 3 and 4, respectively.

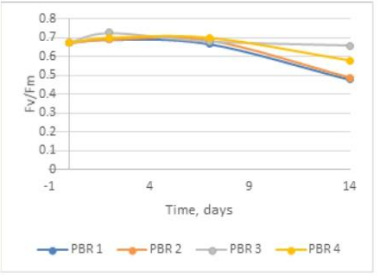

Figure 7: Photosystem II performance expressed as *Fv/Fm* ratio in pre-treated and untreated PBRs.

PAM measurements showed that at the beginning of the test the *Fv/Fm* was high 0.67 in all the reactors indicating good environmental conditions and no stress from the substrate (Figure 7). High *Fv/Fm* values were observed during day 2 and 7 (> 0.6); a small decrease was observed only at the end of the assay, more evident in PBR 1 and 2, highlighting that activated carbon pre-treated digestate is a suitable medium for microalgae growth.

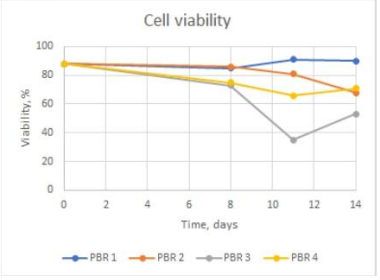

Figure 8: Viability (the percentage of live cells) of microalgae cultures during the growth test in pre-treated and untreated PBRs.

Vitality of microalgae during cultivation was determined by the assessment of dead/live cell ratio after dyeing cells with Sytox nucleic acid dye. Vitality of microalgae cultures were 88% at the beginning of the cultivation. Cell vitality stayed high during the whole cultivation period in PBR 1 and PBR 2 with untreated diluted digestate however, decrease in culture viability was observed in PBRs with pre-treated digestate (Figure 8). All essential nutrients are still present in growth medium at the end of cultivation therefore, the lack of nutrients cannot be the reason for the observed increased cell death rate in the pre-treated PBRs. The highest drop of viability was observed in the PBR 3 at the second part of the cultivation when percentage of live cells dropped to 35% but increased again up to 53% at the end of the cultivation. PBR 3 and 4 are replicates with identical cultivation conditions and nutrient concentrations however, because microalgal community is not sterile other microorganisms, such as bacteria might be present in the cultivation medium taking part in biological processes within the PBR. Moreover, rotifer activity was observed in all PBRs at low level however, highest number of rotifers was recorded in PBR 3 corresponding to the highest decrease in the cell density observed on day 11 leading to the possible cause of decreased vitality observed.

4 CONCLUSIONS

Activated carbon was used successfully to decrease the optical density of high strength agricultural liquid digestate. Results show that activated carbon is a valuable novel technique to increase the potential usefulness of dark high strength agricultural digestate for microalgae cultivation by reducing the optical density thus increasing the light transmission into microalgal cultures. Microalgae culture was able to remove nutrients in pre-treated as well as untreated diluted digestate. Higher biomass productivity was observed in highly diluted untreated digestate suggesting that some inhibitory effects might be present in pre-treated digestate. Higher initial nutrient rate in pre-treated PBRs due to the low dilution of digestate applied could have led to excessive load of some nutrients. Moreover, some other substances that can negatively affect microalgae growth and nutrient consumption might be present in the digestate, however higher dilution rate has minimized their effect in the untreated PBR 1 and 2. Although PAM measurements did not show any inhibitory effects of growth medium on the photosystem performance, toxic mechanisms could have affected other metabolic pathways. Further growth tests with microalgae in pre-treated digestate should be carried out to determine the correct dilution rate in order to fully evaluate the applicability of pre-treated digestate as a growth medium.

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Growth Kinetic Model for Microalgae Cultivation in Open Raceway Ponds: A System Dynamics Tool

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Abstract **– Microalgae culture has the potential to play an essential role in the application of circular economy principles. Microalgae cultivation allows utilizing industrial side-waste streams while ensuring biomass for a wide range of applications in the industrial sector. Specifically, cultivation in outdoor open raceway ponds are a preferred solution due to low costs, ease of operation and large-scale application. However, the economic viability of the cultivation system largely depends on the amount of biomass produced, the technology implemented and the microalgae species and strains. For this purpose, screening of numerous physical, chemical, and environmental factors affecting microalgae growth must be performed before implementing large-scale microalgae cultivation systems. Furthermore, to obtain the highest biomass yield, the design and operating parameters for open raceway pond cultivation must be investigated in depth. Therefore, this study proposes a kinetic growth model for microalgae cultivation in open raceway ponds based on System Dynamics modelling approach. The proposed model aims at overcoming the major problems of existing growth evaluation tools such as separate assessment of different parameters, high complexity, time consumption and other challenges. The proposed system dynamics model proves to be a simple yet powerful tool for modelling the behaviour of algae biomass in an open raceway pond.**

Keywords **– Biogas; causal loop diagram; computational fluid dynamics; microalgae; modelling; system dynamics**

1. INTRODUCTION

Microalgae are a bioresource with a versatile role in the natural environment that maintains the balance of biochemical flows of nutrients (i.e., N, P) [1]. However, anthropogenic activities undermine the natural balance of biochemical flows, leading people to consider promising the use of microalgae-based wastewater remediation technologies or side-stream flows (e.g., digestate from biogas plan) [2].

The use of microalgae application for side-stream waste and waste treatment was discovered more than 50 years ago [1] became a widely used sustainable application in the last decade [3]. The goal is to reach an engineered microalgae growing system acting as bio-filter for both nutrient recirculation and CO_2 fixation in the microalgae biomass [4]. In fact,

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autotrophic microalgae use carbon dioxide as the primary substrate in the photosynthesis process to fix it in biomass. Once a waste carbon source is coupled with the microalgae cultivation process, the waste carbon can be converted into valuable microalgae biomass, becoming a promising source of value-added bioactive for food and nutrition [5], [6]. Nevertheless, the economic payback on microalgae cultivation systems still largely depends on the amount of the algal biomass produced and on the efficiency of the growing systems [5]. In this direction, using models to predict algae biomass productivity and initially screen potentially usable microalgae plays an essential economic role when designing and operating such a system. Since microbiology growth kinetics is a complex biochemical process, these models rely on cellular growth kinetic models developed over decades with the help of laboratory experiments.

However, when scaling the laboratory systems to large scale algae productions, these models have certain limitations primarily associated with validating experimental results in the pilot pre-industrial scale. The researchers use different mathematical tools for growth kinetic modelling implementing complex differential equation solvers [5]. Although these tools are reliable, they are rather complex to use, and the implementation of a specific kinetic model for specific microalgae growing conditions is time-consuming. This aspect is paramount when scaling up from laboratory to large-scale cultivation. Hence there is a growing need for developing a user-friendly growth kinetic model. This study aims to develop a kinetic growth model for microalgae cultivation in open raceway ponds using a System Dynamics approach and overcome current models' limitations.

2. MICROALGAE GROWTH MODELLING

2.1. Microalgae Growth Characteristics

Microalgae are considered fast-growing unicellular species. If the conditions are optimal, these cells continuously increase their population by duplicating. Microalgae biomass is mainly produced by photosynthesis, which utilizes inorganic substances (including CO_2). When compared to other photosynthetic organisms such as terrestrial plants, microalgae highlight the following growth characteristics [4]: higher growth rate and shorter reproduction time, higher photosynthetic efficiency, less space required, and no need for soil substrate to grow on.

Similarly, with most other microorganisms, microalgae typically follow a general growth dynamic behaviour, which has five phases in the case of microalgae [7]. The first is the lag phase which occurs when an algae culture is moved from a plate to the liquid growth media. The growth of culture is slow due to the lack of physiological response of cell metabolism to growth, such as producing enzymes required for cellular division. After the microalgae are acclimatized to their environment if follows an exponential growth. The growth rate relies on the accessibility of nutrients, light and many other factors. The growth declines when the limit or unbalance of physical and chemical factors such as light, pH, Carbon is reached. During the stationary phase the growth rate is equal to zero. This occurs when the algal cells are not growing anymore or the number of cells growing equals the number of cells dying at a unit time. The last phase is the decline, during which algae cells die due to various factors such as nutrient depletion, cellular ageing, toxicity, the effect of temperature and pH. In a batch culture death phase usually occurs when all the nutrients are consumed. At this phase, cell density rapidly decreases as culture collapses.

The effectiveness of microalgae cultivation relies on the characteristics of several environmental, physical, and biological factors and the design of the pond. The main

parameters used are concentration of algae, light, temperature, pH, salinity, concentrations of nutrients, and toxic elements [8]. The following sections will present the main key empirical equations further implemented in the developed System Dynamics model.

2.1.1. Concentration of Microalgae

When designing a cultivation system for microalgae, the aim is to create conditions that allow maintaining the exponential phase for a longer period. Hence describing the mathematical relationship of the biomass concentration (C_b) over time is very important. The kinetic equations proposed in the developed model in this study are based on the exponential growth of biomass in time [9]. The change of biomass at a given time is a function of the biomass specific growth rate (μ), which can be expressed as:

$$\mu = \frac{1}{C} \cdot \frac{dC}{dt}, \tag{1}$$

where

C Microalgae concentration at a given time t, expressed as g l^{-1};
μ Specific growth rate, d^{-1} or hr^{-1}.

The biomass productivity (P_b) (g l^{-1} per day or g l^{-1} per hour), which is the amount of biomass generated after a specific time interval, it can be expressed as:

$$P_b = \mu \cdot C_b = \frac{dC}{dt}, \tag{2}$$

where C_b is initial biomass concentration, g l^{-1}.

The optimal growth rate is reached when the microorganism grows unrestrained by nutrient limits or the accumulation of waste products. The growth rate of the number of cells, or dry weight, per unit of time during the exponential growth is proportional to the number of cells present in the culture at the start of any time unit (C_0). Hence, by integrating the Eq. (2) from $t = 0$ to $t = t$, the microalgae concentration (C_t) after a Δt is expressed as:

$$C_t = C_0 \cdot e^{\mu \cdot \Delta t}, \tag{3}$$

and the growth rate (μ) is expressed as:

$$\mu = \frac{\ln\left(\frac{C_t}{C_0}\right)}{\Delta t} = \frac{\ln(C_t) - \ln(C_0)}{\Delta t}, \tag{4}$$

where

C_0 Initial biomass concentration, g l^{-1};
C_t Biomass concentration at time t, g l^{-1};
Δt Time duration.

However, some cells die in a microalgae culture due to cellular ageing, even if the conditions are ideal. Hence the cumulative biomass productivity is described as follows:

$$P_b = (\mu - m) \cdot C_b, \tag{5}$$

where

m Mortality rate, d^{-1} or hr^{-1};
P_b Biomass productivity, g $l^{-1}d^{-1}$ or g $l^{-1}hr^{-1}$;
C_b Biomass concentration, g l^{-1}.

The mortality rate is typically constant when the growth conditions are not inhibited. The availability of nutrients and light, together with reactor's parameters, play a key role in microalgae kinetics (i.e. residence time, air to liquid mass transfer rate, medium homogeneity).

2.1.2. Nutrients

Most of the sustainable algae cultivation applications in open raceway pond systems utilise wastewater as a nutrient source. The wastewater generally contains high concentrations of nutrients; hence nutrient starvation is not a problem when wastewater is used in microalgae cultivation systems.

For algae growth, nitrogen is a key nutrient. In microalgae the nitrogen amount of algal dry matter is about 7 % (w/w). The increase in nitrogen level until a certain threshold leads to a more outstanding production of cells and proteins and greater chlorophyll synthesis. The lack of nitrogen leads to a significant reduction in lipids polyunsaturated fatty acids) with a direct effect on the photosynthetic and cellular activities [10]. Another important nutrient for microalgae growth is phosphorus and accounts for 1 % (w/w) of the dry algae biomass. Phosphorus is involved in several metabolic pathways and cellular regulations of microalgae. Unfortunately, in most algal cultivation systems, phosphorus is the limiting nutrient. Phosphorus also plays a vital role in lipid accumulation in microalgae cells, which is the most value-added product in microalgae applications [10]–[12].

2.1.3. Light

In photosynthetic cultures such as microalgae and phytoplankton, the cells use light energy for cellular maintenance and new biomass production [13]. Therefore, biomass productivity and cell growth rates are directly related to the light energy available. Light energy can be natural (solar) or from artificial systems in fluorescent tubing around or inside the reactor. Novel systems embed light sources inside the culture media in the form of LED or fibre optics. The design of lighting for a microalgae cultivation system must consider several different factors, such as optical path, optical depth (which characterizes the degree of transparency of the medium), and the illuminated surface ratio by volume culture [10], [14].

2.2. Growth Kinetic Models

Kinetic model aims to describe how the microalgae population behaves in a particular environment over a given time. Such models express the relationship of parameters, which affect the growth rate of microalgae. There is a large variety of kinetic models for understanding the growth of microalgae in natural habitats. Mainly they can be divided into two types of models: descriptive and explanatory. Explanatory models are mainly developed to explain the cause or fundamental systematic behaviour. Most of these models have complex structures and maths, but to a certain extent can be simplified. On the other hand, descriptive models are developed to predict system performance and not to explain mechanisms. This category covers the most empirically developed kinetic models [15].

2.2.1. Single Substrate Models

The growth of microalgae in aquatic environments depends on nutrients' availability, such as Nitrogen, Phosphorus, and Carbon under light-saturating conditions. Often kinetic growth models are expressed according to a single concentration of nutrients. The most popular formula is Monod equation [16]–[18], which is expressed below:

$$\mu = \mu_{max} \cdot \frac{S}{k_s + S}, \tag{6}$$

where

μ Specific growth rate, d^{-1} or hr^{-1};
μ_{max} Maximum specific growth rate, d^{-1} or hr^{-1};
S Concentration of the substrate, g/l;
k_s Half-saturation constant, g/l.

The maximum specific growth rate is the maximum growth rate a certain microalgae strain resembles under ideal nutrient and light conditions. Half saturation constant (k_s) in microbiology refers to the substrate concentration required to reach the growth rate at half of the maximum specific growth rate. The Monod model is suitable to describe growth in low and modest nutrient concentrations but is limited in describing the inhibition of microalgae growth due to high nutrient levels.

2.2.2. Kinetic Model Attenuation Models

A specific level of light, called saturated level of light, is required for microalgae to reach the maximum growth rate. If the intensity of light exceeds the level of saturation well, light inhibits growth. On the other hand, if the light intensity is below the level of saturation, light limits the growth [19].

When cultivating microalgae in conventional open raceway ponds, the light is received only from the top surface of the pond. When the light photons penetrate the pond, the intensity decreases due to absorption by the algae cells which the light passes. This is called light attenuation. Also, the incident light could reflect away from the thick top layer [20].

Light-based kinetic models' growth rates are related to the incident light intensity with two parameters: intensity and saturation constant. When the light intensity incident is less than saturation constant, the growth according to first-order kinetics is limited by light. When light intensity incidents become higher than saturations constant, growth is independent of light saturation and a specific growth rate. This is case of the Steele equation [15], [21], which fits the photo-inhibition effect and can be expressed with the Eq. (7), considering I_{opt} as the optimum light intensity for the specific microalgae strain.

$$\mu = \mu_{max} \frac{I}{I_{opt}} e^{\left(1 - \frac{I}{I_{opt}}\right)} \tag{7}$$

In Steele equation, once the light intensity reaches I_{opt}, the growth rate will be at its maximum value, and any change in light intensity will result in a lower growth rate [15].

The parameter "I" mentioned in this model is a luminous intensity defined as the light irradiance received by an algae cell. This term is doubtful as there is no practical way to measure the amount of light irradiance received by each algae cell.

The Beer-Lambert law [22] expresses the formula to calculate light intensity at a given depth of a microalgae raceway pond accounting for the gradual decay of light [23], described as:

$$I_z = I_0 e^{-k_a B z}, \tag{8}$$

where

I_z Local light intensity received by algal cells at z depth, μmol m^{-2} s^{-1};
Z Depth, m;
I_0 Light intensity at the external surface of the pond, μmol m^{-2} s^{-1};
k_a Biomass light absorption (extinction) coefficient, m^2 kg^{-1};
B Algal concentration, kg m^{-3}.

Unit (μmol m^{-2} s^{-1}) is typically used for Photosynthetically Active Radiation (PAR). It is a measure of the energy absorbed in plants from solar radiation by chlorophyll A and B. It is, in fact, a measure of the energy effectively available for photosynthesis. The biomass light absorption (extinction) coefficient (k_a) is a measure of how strongly a chemical species or material absorbs light at a specific wavelength. It is the inherent property of the particular chemical species, which depends on their chemical composition and structure [23].

2.2.3. Growth Kinetic Model Considering Multiple Factors

In the natural environment, the limitation is more common than in commercial cultivation systems to limit both nutrients and light on the growth of microalgae, which is also called co-limitation. Co-limitation models can be arranged according to two distinctive models, thresholds, and multiplicative models [15].

The threshold model, also known as the minimum law, relies on the assumption that the most limited resource affects the total rate of growth. The final mathematical expression is therefore identical to the growth models that take one factor into account. The threshold model can be expressed as:

$$\mu = \mu_{max,min}[f(x_1), f(x_2), f(x_3), \ldots, f(x_i)], \tag{9}$$

where $\mu_{max,min}$ is the maximum growth rate for the most limited resource and $f(x_i)$ is the function of multiple limited resources and light intensity.

For example, when considering outdoor open raceway cultivation utilising side-waste stream rich of nutrients, the light becomes the limiting factor that affects the growth rate. Hence when modelling the growth rate, light limit μmax for the maximum growth rate should be considered. The cultivation systems, mostly photobioreactors, which utilise secondary treated wastewater, are often limited in phosphorus. Therefore, when modelling those systems, the maximum growth rate should be changed.

On the other hand, the multiplicative model assumes that all resources contribute equally to the growth of microalgae. In the study of [24] a multiplicative model is presented for microalgae growth, considering multiple environmental factors such as light intensity, temperature, nutrients and pH. In such model all resources will simultaneously influence the overall growth rate as described in Eq. (10):

$$\mu = \mu_{max}[f(x_1), f(x_2), f(x_3), \ldots, f(x_i)], \tag{10}$$

where μ_{max} is the cumulative maximum specific growth rate.

Such models frequently describe nitrogen, carbon dioxide and light co-limitation. The Monod formula is the most common expression of individual growth factors in multiplicative models [15].

Hence, the growth rate of microalgae is disturbed by several limiting resources and environmental factors. Though there are several models developed, most of them are limited to laboratory-scale systems. The most significant setback of existing models is that their use for subsequent applications is limited.

3. METHODOLOGY

3.1. Goal and System Boundaries

The goal of this research aims to create a transparent and straightforward yet reliable microalgae growth kinetics model with consideration of different parameters. Specifically, the model is developed for microalgae cultivation in an outdoor open raceway pond. For modelling purposes, the hypothetical system boundaries are assumed to include Microalgae cultivation in the pond, solar irradiation, nutrient and CO_2 supply (see Fig. 1). The mass and energy transfer are considered only between the identified sub-systems within system boundaries.

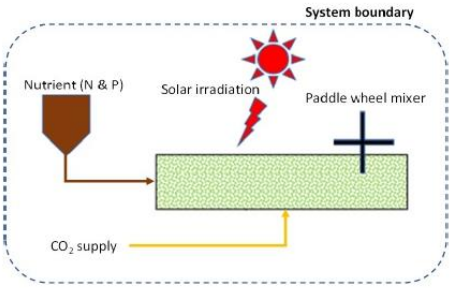

Fig. 1. Open raceway pond as a biological system.

Microalgae cultivation in any outdoor open raceway pond can distinguish 3 phases: fill, react and discharge (see Fig. 2).

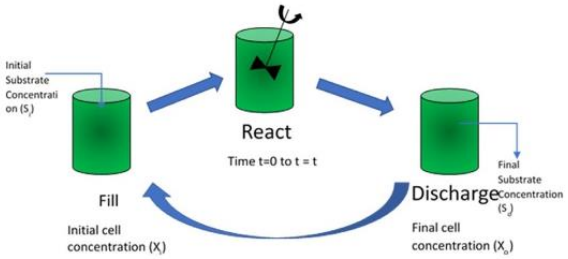

Fig. 2. Operational phases of a discontinuous batch algae reactor.

Initially, a small amount of algae population is present in the reactor, and the pond is filled with nutrients. This is assumed to be the fill phase. Then the suspension is continuously mixed

by the paddlewheel. During that time, microalgae present in the reactor consume the nutrients and the carbon present in their aquatic environment and increase their population. This process is happening in the *react* phase. At the end of the reaction, the algae suspension is removed from the pond. This is the *discharge* phase.

Some of the algae could recycle back to the reactor for the next cycle. However, this study's growth model is focused on the microalgae population's dynamic behaviour during the react phase. Thus, fill and discharge phases and recycling of microalgae after discharge is not considered in this study.

3.2. Assumptions and Limitation

According to the definition of system boundaries following assumptions are made when building and running the model:

1. The microalgae culture in the outdoor open raceway pond is a monoculture strain. There is no interaction between other microorganisms such as bacteria;
2. There is no effect from evaporation during the running phase. The mass inside the system is treated as conserved (closed system);
3. The only causes affecting the growth rate of microalgae is temperature, substrate available in the reactor and the light received by the microalgae;
4. The light available on the surface of the pond (I_o) is constant during the time period. Day-night or dark-light cycles are not considered;
5. The open raceway pond is treated as an ideal continuously stirred tank reactor where microalgae concentration through the pond is homogeneous;
6. Light reflection is neglected.

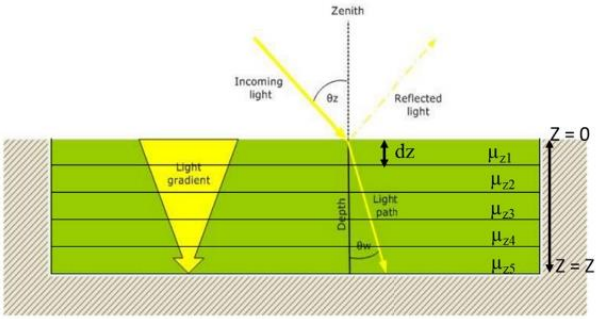

Fig. 3. Dividing the raceway pond into layers along with its depth.

The model is built according to the assumption that the microalgae cells' light (I) depends on the culture depth (z). For this purpose, algae concentration in each predefined equally distributed layer of the pond (dz) is considered in the model structure as shown in Fig. 3. This allows considering a continuous growth rate change at different depths of the pond.

By modelling the growth rate at each layer of the pond (dz) it is possible to screen out the optimum height of the open raceway pond or, the maximum biomass that must be maintained in the pond to have a considerable light penetration for efficient growth of microalgae. Ideally, the dz values should be infinitely small for smoother integration of model calculation for algae growth in the whole pond. However, within the limits of the study the pond is divided into eight depth layers.

3.3. System Dynamics Modelling

System Dynamics modelling is selected to create the microalgae growth kinetic model. System Dynamics is a scientific approach, originally developed by Jay W. Forrester in the mid-1950s. This approach is an integrated part of system thinking which studies whole system by its components, and interactions within these components. System Dynamics is generally used to address the problems arising from complex systems, characterized by an underlying causal structure of accumulation processes that causes lags, feedback, non-linearity and uncertainty [25]. System Dynamics has been demonstrated to be a consistent approach to simulate the behaviour of microbiological systems such as activated sludge wastewater treatment plants [26]. For this reason, has been selected to create the model proposed in

Within the application of System Dynamics a Causal loop diagram (CLD) for the microalgae growth kinetic mode has been developed (see Fig. 4).

The microalgae growth kinetic model based on the CLD is built with components defined in System Dynamics approach as stocks and flows. A stock is a part of the system that accumulates the effects of other variables over time and flows represent the rate at which the stock is changing at any given time [25]. Even though an outdoor open raceway pond is a complex biological system, using System Dynamics, it can be characterized and modelled by implementing a relatively small number of stocks & flow that generate the chain of the causal relationships. The results analysis using System Dynamics modelling allows one to analyse the dynamic behaviour of algae concentration depending on different factors. The software used for the creation of the model was *Stella Architect* ©.

4. RESULTS

4.1. Casual Loop Diagram (CLD)

As mentioned in section 2, the effectiveness of microalgae cultivation mainly depends on a specific parameter, namely: concentration of algae, light, temperature, pH, salinity, concentrations of nutrients, and toxic elements.

According to the co-limitation approach of the factors influencing microalgae growth in the pond, the identification of the main feedback loops for the model was based on the kinetics model as described in section 2 are represented in the CLD presented in Fig. 4.

The implemented kinetics to develop the CLD for the SD algae growth model are shown in Fig. 4. The CLD includes five feedback loops which mostly relay about the effects on the growth rate of the microalgae. The kinetic equations proposed in the model follow an exponential biomass growth in time relationship. During the exponential growth phase, the algal cells grow and divide exponentially before the linear growing phase occurs when growth slows down due to light limitation effect, or nutrients or inhibitors become a constraint. During this phase the specific growth rate (μ) can be defined according to Eq. (1). From this equation would be possible to calculate the overall microalgae population by the biomass productivity defined in Eq. (2). This effect is represented with the reinforcing loop R1: the higher the growth rate, the higher the microalgae population's increase.

According to the Steele growth model, as defined in Eq. (7), the light intensity (I) will increase the biomass concentration during a time interval Δt. Therefore, for this equation, when the growth rate reaches its maximum value, any change in light intensity will result in a lower growth rate.

It should be considered that light intensity changes over time due to the depth of the pond. This explains (balancing loop B1) in terms of the more the biomass will grow, the more the

layers in the bottom part of the ponds will have less light harvestable This behaviour is explained by the kinetics proposed Beer-Lambert law in Eq. (8).

The balancing loop B2 relies on the well-known feedback about the death and birth of living organisms: within the proposed model, the more microalgae are in the growing cultivation pond, and the more is the number of microorganisms dying due to ageing.

The CLD considers the effects of other relevant feedbacks loops. The first is the effect of the nutrient supply to the ponds in terms of N, P and C (i.e., nutrient yield in the CLD of Fig. 7). The kinetics that describe this behaviour are those proposed in Eq. (6) following the formula of Monod. For Monod equation, the maximum specific growth rate is the maximum growth rate a certain microalgae strain resembles under ideal nutrient and light conditions. As reported in section 2, the key parameters are the half-saturation constant (k_s), the concentration of the substrate and the maximum specific growth rate (μ_{max}). The balancing loop B3 affects the growth rate, and thus the microalgae population explains that the more nutrient is injected into the growing media, the more substrate concentration in the algae pond will increase, further affecting the available amount of nutrient for the microalgae population.

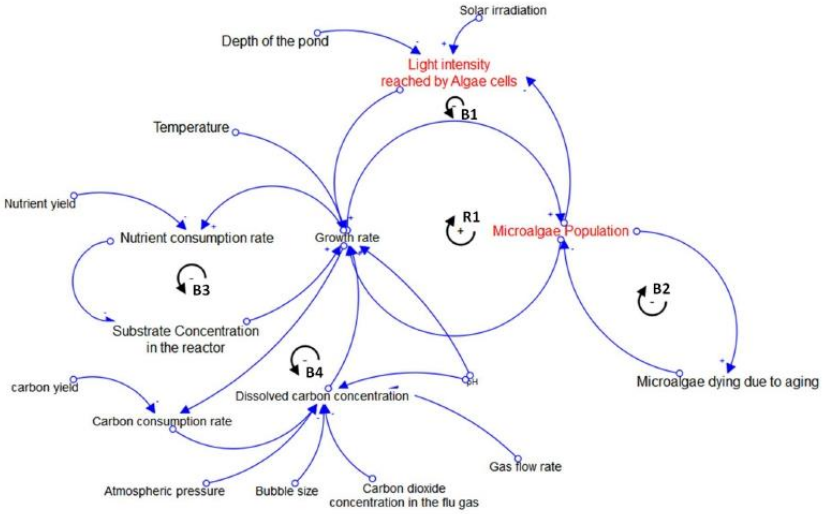

Fig. 4. CLD for algae population behaviour in an open raceway pond.

The last presented feedback loop in the proposed CLD is related to the amount of dissolved Carbon concentration in the growing pond relied upon the pH level in the growing media. One of the underlying parameters which affect the growth is the pH. This parameter has a direct effect on microalgae cell metabolism and biomass formation. Even though each microalgae strain has a narrow range of optimum pH, the growth of most microalgae species is generally known to thrive at neutral pH [14]. The pH value of culture medium largely depends on the concentration of dissolved CO_2 and its derivative compounds (i.e., CO_3^{2-} – carbonate, HCO^{3-} – bicarbonate). Algae consume carbon dioxide during photosynthesis and, at optimum pH, the bicarbonate present in the medium is converted back to CO_2 by the action of the algae enzyme carbonic anhydrase with the release of hydroxyl ions, which tends to increase pH [27]. Therefore, the injection of CO_2 should be carefully monitored and

controlled if used as a source of CO_2 in the system so that the pH of the medium is maintained at an optimum level, guarantying efficient photosynthesis and growth of microalgae. Moreover, the dissolved carbon concentration is also depending on other external factors like CO_2 flow rate, effective bubbling system (i.e., increasing the active surface of CO_2 molecule bubbled into the system), the retention time, the atmospheric pressure and temperature, which directly affect the solubility of the CO_2 in the media. At this stage of the model development, these aspects have not been integrated into the numerical simulations

Based on the CLD the microalgae growth kinetic model is built with *Stella Architect* © software and shown in Annex 1. The implemented stocks and flows in microalgae growth kinetic model are based on a multiplicative co-limitation model expressed as:

$$\mu = \mu_{max}[f(T) \cdot f(N) \cdot f(P) \cdot f(C) \cdot f(I)], \tag{11}$$

where

μ Specific growth rate, d^{-1};
μ_{max} Maximum specific growth rate, d^{-1};
$f(T)$ Growth function for temperature;
$f(N)$ Growth function for N as the substrate;
$f(C)$ Growth function for C as the substrate;
$f(P)$ Growth function for P as the substrate;
$f(I)$ Growth function considering light attenuation.

The functions $f(x_i)$ are adapted from Steele, Monod and Haario growth models [7], [15] and can be further elaborated as:

$$\mu = \mu_{max}\left[\theta^{T-T_{ref}} \cdot \frac{S_N}{k_{(s,N)} + S_N} \cdot \frac{S_P}{k_{(s,P)} + S_P} \cdot \frac{S_C}{k_{(s,C)} + S_C} \cdot \frac{I}{I_0} e^{\left(1-\frac{I}{I_0}\right)}\right], \tag{12}$$

where

S_N, S_P, S_C Concentration of substrates in the culture media, $g\ l^{-1}$;
$k_{(s,N)}$, $k_{(s,P)}$, $k_{(s,C)}$ Half saturation constants of substrates, $g\ l^{-1}$;
θ Temperature coefficient;
T Culture temperature, °C;
T_{ref} Reference temperature where growth rate is maximum, °C;
I_o Average solar irradiance received on the surface of the pond, $\mu mol/(m^2\ s)$;
I Average solar irradiance received by the algae cells at a certain depth, $\mu mol/(m^2\ s)$.

Nevertheless, in the SD model developed with *Stella Architect* ©, the multiple effects on kinetic models have not been fully explored moreover, even though still represented in the given model proposed in Annex. Based on the developed model, the effect of temperature, pH, and changes of the dissolved Carbon have not been considered.

All the constants associated with the equations which are required to build the model previously described are listed in Table 1. In this study, the above model is used to simulate the growth of algae strain *Chlorella vulgaris* at each defined depths of an open raceway pond.

TABLE 1. INPUT DATA FOR MODEL FOR THE SD MODEL

Parameter	Unit	Value	Source
μ_{max}	d^{-1}	0.248	[28]
θ	unitless	1.003	[15]
T_{ref}	Celsius	25	[15]
T	Celsius	20	[15]
$k_{s\,(P)}$	mg l^{-1}	0.001	[15]
$k_{s\,(N)}$	mg l^{-1}	0.0023	[15]
$k_{s\,(C)}$	mg l^{-1}	0.0046	[15]
Yield (P)	g of DW/ g of P	0.002	[15]
Yield (N)	g of dry weight of microalgae/ g of N	0.015	[15]
Yield (C)	g of dry weight of microalgae/ g of C	0.057	[15]
Death rate	d^{-1}	0.06	[29]
S_P	mg l^{-1}	10	[4]
S_N	mg l^{-1}	40	[4]
S_C	mg l^{-1}	1000	[4]
I_o	µmol m^{-2} s^{-1}	100	[4]
z	cm	40	[4]
dz	cm	5	[4]
Surface area of the pond	m^2	2.78	[4]
Initial algae concentration (B_0)	g l^{-1}	0.05	[4]

The biomass productivity in an open raceway pond is expressed as the amount of dry weight produced per cycle [30]. The microalgae biomass productivity calculated for each equally distributed layer can be calculated using Eq. (13):

$$P_t = (B_t - B_0) \cdot A \cdot dz \cdot 10^3 , \tag{13}$$

where

P_t Biomass productivity after time t, g;
B_t Final biomass concentration in the relevant layer, g/l;
B_0 Initial biomass concentration, g/l;
A Surface area of the pond, m^2;
dz Depth of each equally distributed layer, m.

4.2. Analysis of the Growth Based on Culture Depths

In this section are presented the results assuming for the simulation a ten-day microalgae growth period. The results about the analysis of the concentrations of *Chlorella vulgaris* at each layer under different initial biomass concentrations (B_0) (i.e., 0.01 g/l, 0.02 g/l, 0.04 g/l, 0.05 g/l, 0.06 g/l, 0.08 g/l and 0.1 g/l) are al reported in Annex 2.

Fig. 5 shows the trends at different culture depths considering an initial biomass concentration very diluted B_0 = 0.01 g/l. It can be noticed that the microalgae culture in all eight considered layers increases over the ten days. The increment of biomass follows the same exponential relationship until 6 days where the biomass concentration of all the layers

reaches slightly higher than 0.015 g/l. After that, the growth rates of each layer start to deviate slightly.

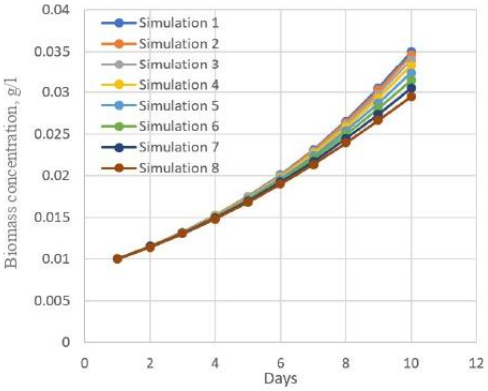

Fig. 5. Microalgae concentration at each layer at the initial concentration of B_0 = 0.01 g/l. Considering simulations related to different culture depth expressed in cm from the surface, i.e. z = 0, in specific 5 cm (simulation 1), 10 cm (simulation 2), 15 cm (simulation 3), 20 cm (simulation 4), 20 cm (simulation 4), 25 cm (simulation 5), 30 cm (simulation 6), 35 cm (simulation 7), 40 cm (simulation 8).

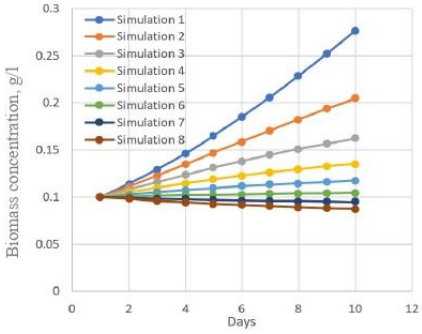

Fig. 6. Microalgae concentration at each layer at the initial concentration of B_0 = 0.1 g/l. Considering simulations related to different culture depth expressed in cm from the surface, i.e. z = 0, in specific 5 cm (simulation 1), 10 cm (simulation 2), 15 cm (simulation 3), 20 cm (simulation 4), 20 cm (simulation 4), 25 cm (simulation 5), 30 cm (simulation 6), 35 cm (simulation 7), 40 cm (simulation 8).

When it comes to very high initial algae concentrations (B_0 = 0.1 g/l), a rapid change of growth behaviour can be observed (Fig. 6). Algae present in the bottom layer starts to die due to a lack of available light. Meantime the top layer's growth rate slightly changes its behaviour into a linear function, suggesting that the top layer's growth rate becomes to follow zero-order kinetics.

The results in Annex 2 shows that when the B_0 reaches 0.08 g/l there is no change of biomass after the depth reaches 30 cm. At that point, the growth rate equals to the death rate. Hence for more concentrated cultures, which is a common approach in open raceway pond

cultivation, the optimum pond depth is around 30 cm. The overall biomass concentration after 10 days is calculated and plotted referring to the depth of the pond (see Fig. 7).

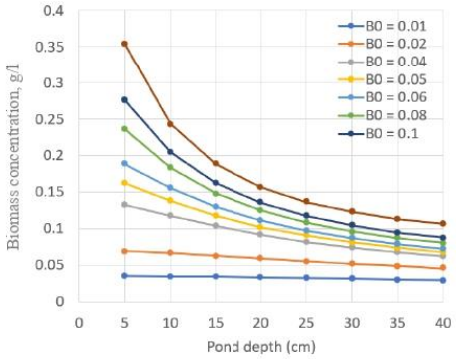

Fig. 7. Final biomass concentration as a function of pond depth.

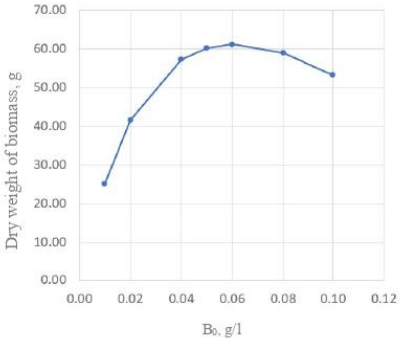

Fig. 8. Biomass productivity and the initial culture concentration relationship.

The model results suggest a similar trend described in other hypothetical models: the biomass decreases with pond depth due to light attenuation. However, for a very diluted initial biomass, the final concentrations are similar at each pond depth. When the initial culture concentration is higher, the concentration is higher at all pond depths.

4.3. Biomass Productivity

The cumulative biomass productivity, which is the total biomass produced during a particular time is calculated by summing biomass productivity in all the layers. By using the model results, the biomass productivity after ten days is calculated for the dimensions (see Table 2).

TABLE 2. MODEL OUTPUT FOR BIOMASS PRODUCTIVITY

B_0	Biomass productivity at each layer, g								Cumulative yield, g
	5 cm	10 cm	15 cm	20 cm	25 cm	30 cm	35 cm	40 cm	
0.01	4.86	4.81	4.72	4.62	4.49	4.37	4.23	4.10	25.08
0.02	9.61	9.23	8.73	8.20	7.68	7.20	6.76	6.36	41.52
0.04	18.46	16.40	14.39	12.72	11.37	10.29	9.40	8.66	57.21
0.05	22.47	19.20	16.38	14.22	12.56	11.27	10.24	9.41	60.15
0.06	26.19	21.59	18.02	15.43	13.52	12.08	10.95	10.05	61.10
0.08	32.79	25.44	20.57	17.33	15.06	13.40	12.13	11.15	58.91
0.10	38.39	28.43	22.54	18.82	16.31	14.51	13.18	12.16	53.16

The relationship between biomass productivity and the initial culture concentration from the obtained results is plotted in Fig. 8.

For low initial concentrations, Biomass productivity also is low. The productivity increases with the increase in initial concentration B_0 value up to 0.06. The biomass productivity decreases for higher values of initial biomass concentration, showing a non-linear relationship between biomass productivity and the initial culture concentration.

4.4. Validation

The kinetic models can use a function of one factor or multiple factors. Most of the models found on literature are based on a single parameter, either a substrate or light. The validation of such models is performed by saturating the system with the rest of the factors, except the variable factor. Models that consider the interactions or links between different factors include several parameters. Models that are multi-nutrient are often complex in form. Often such models have trouble in overfitting because many parameters are involved and are best applied in a wide range of environmental and nutrient conditions, with a small set of assumptions, to minimize this inherent problem [15], [31].

For this reason, the validation of the model based on the results of pilot testing stand, have not been yet realized at this stage of the research. As well as the validation of the SD model structure has not been fully investigated, even though the kinetics are in line with the ones reported in literature [4], [22].

5. CONCLUSIONS

Having precise information on microalgae biomass growth is important when designing and operating an open raceway pond. In this study, a tool for predicting the microalgae growth based on System Dynamics approach is developed. The created microalgae growth kinetic model represents a user-friendly approach for screening different factors affecting microalgae growth in outdoor open ponds.

The developed model is implemented in a straightforward manner rather than a complex simulation tool; however, the study is limited to the assumption that the pond's biomass concentration is homogeneous. The homogeneity is maintained by turbulent mixing of the pond culture with a paddlewheel mixer. In practice, the biomass is not homogeneous, and is rather concentrated at different zones. This effect can be simulated with the help of the particle tracing tool in Computational Fluid Dynamic model. Hence, it is recommended to integrate the developed System Dynamic model with a Computational Fluid Dynamic model

for a more precise growth dynamic model. The System Dynamics model can also be further developed by including several additional causal loops, which are assumed to be constant in the current model, such as pH and gas solubility.

The current microalgae growth kinetic model results suggest that the growth of microalgae in outdoor open raceway ponds is largely affected by the light attenuation, which depends on pond depth and the initial biomass concentration. For dilute initial biomass concentrations (B_0 = 0.01 g/l) there), no significant effect from light attenuation, which results resulting in a rate at different depths. However, when the initial biomass concentration increases, light attenuation at each pond depth becomes an influential factor for algae growth. The analysis of biomass concentration after period of ten days in relation to pond depth showed that the optimum culture depth for an open raceway pond is about 30–35 cm. According to these results, more than 35 cm deep ponds will not provide any advantage for outdoor cultivation.

After ten days, the cumulative biomass yield analysis with different initial biomass concentrations suggests that an initial concentration of 0.06 g/l has the highest biomass yield. The lower initial concentrations would require more time as the growth rate is relatively proportional to the population and higher initial concentrations would have a lower yield as high biomass concentrations lead to higher light attenuation resulting in a low growth rate at the bottom of the pond. The constants related to the mathematical model are obtained from different literature, showing imprecise results for different environmental or climatic conditions.

As mentioned at this stage the validation has not been proposed at this stage of research.

In summary, the main conclusion is that natural light can be the most limiting factor affecting microalgae growth rate in open raceway ponds. As classical open raceway ponds only receive light from the top surface, the maximum biomass that can be maintained in the culture is very low due to light attenuation. Thus, it is required to remove the culture more frequently. This proves the urgency to develop cultivation systems, where the open ponds are made with transparent materials providing higher exposure to solar irradiation. It is recommended to validate the developed model in a pilot case study.

ACKNOWLEDGMENT

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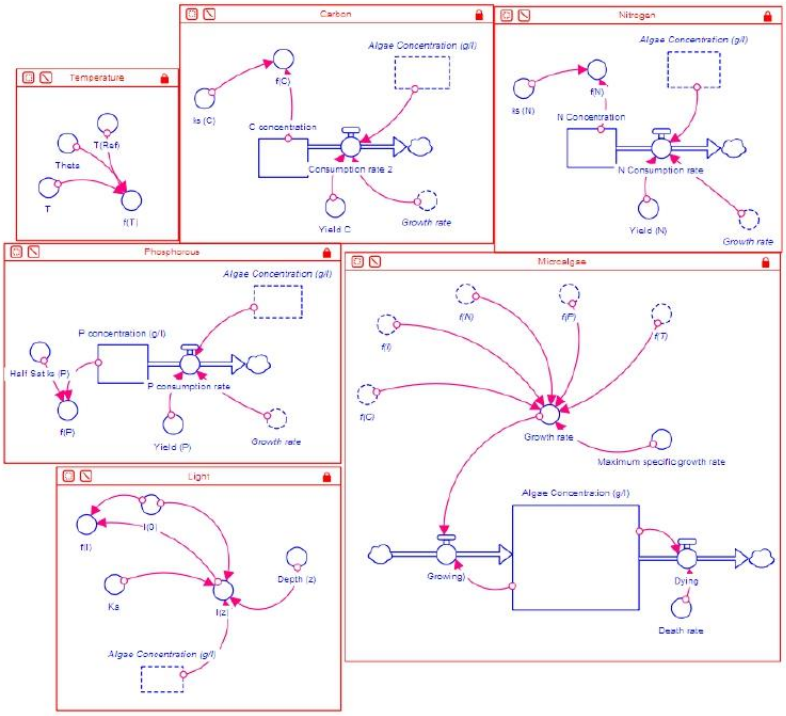
ANNEX 1. THE GROWTH KINETIC SD MODEL DEVELOPED IN *STELLA ARCHITECT* ©



ANNEX 2. MODEL RESULT FOR DIFFERENT INITIAL BIOMASS CONCENTRATIONS IN BETWEEN TWO EXTREME INITIAL VALUES

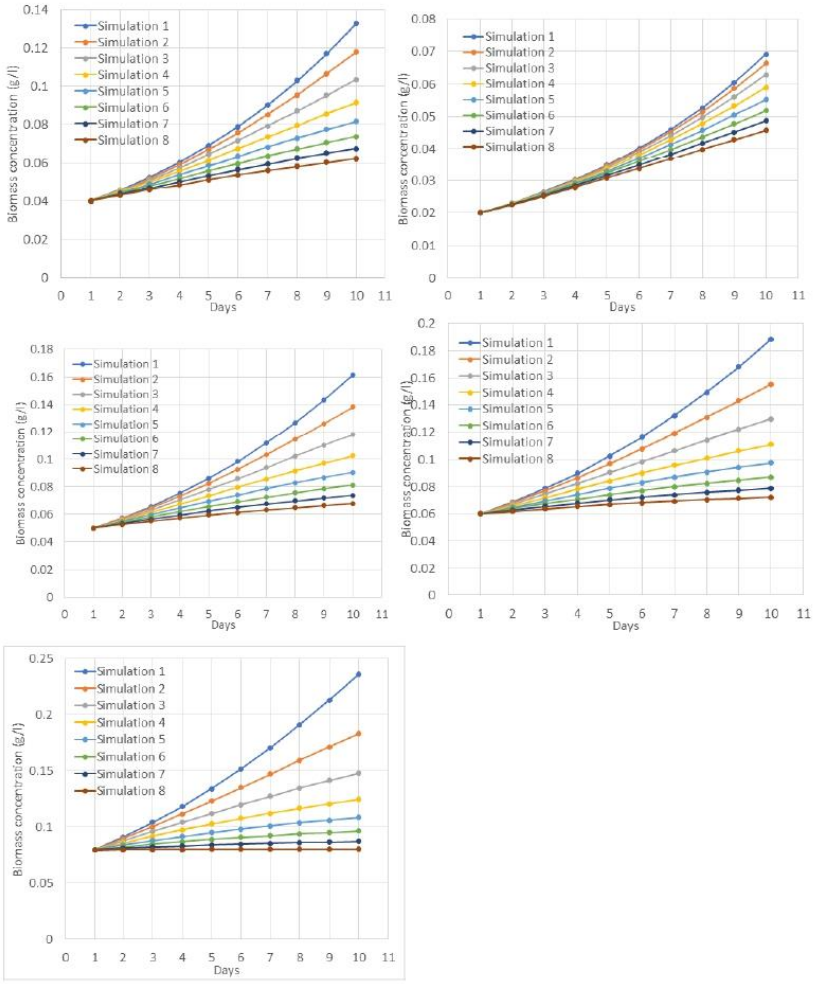


TABLE 1A. MODEL RESULT FOR DIFFERENT INITIAL BIOMASS CONCENTRATIONS IN BETWEEN TWO EXTREME INITIAL VALUES CONSIDERING THE SIMULATIONS FROM TABLE 2; SIMULATIONS RELATED TO DIFFERENT CULTURE DEPTH

Simulation Nr.	Culture depth [cm from surface, $z = 0$]
1	5
2	10
3	15
4	20
5	25
6	30
7	35
8	40

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RESEARCH

Microalga *Chlorella vulgaris* 211/11j as a promising strain for low temperature climate

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Abstract
Microalgae are regarded as a promising source of renewable energy and high-value compounds. To date most large-scale microalgae cultivation is located in hot low-latitude regions; however, high temperature and high solar intensity create a risk of overheating during summer days. Here we explore the option of microalgae cultivation in low temperature regions. The impact of low temperature on microalgae growth is rarely considered in studied species and strains. To assess the optimum temperature and to test the ability to grow in low temperatures, we tested the effect of environmental temperatures ranging from 8 to 32 °C on the growth of the green microalga *Chlorella vulgaris* 211/11j. This strain was selected as a candidate strain for outdoor cultivation after an extensive literature review. The results indicate that *C. vulgaris* 211/11j has a lower optimal growth temperature than some other strains of *C. vulgaris* and exhibits optimal growth over a wide temperature range from 20 to 28 °C. Moreover, we demonstrate that the strain can grow successfully at low environmental temperatures that are below the optimum of this strain. Therefore, we suggest *C. vulgaris* 211/11j as a potential strain for cultivation in outdoor open ponds exposed to changing environmental temperatures in cooler climate regions.

Keywords Microalgae cultivation · Microalgae biomass · *Chlorella vulgaris* · Chlorophyceae · Low temperature · Raceway pond

Introduction

Microalgae biomass is a promising feedstock for biofuels, including biodiesel, biogas, biomethane, biohydrogen and bioethanol (Siddiki et al. 2022). Moreover, microalgae are known to contain a variety of compounds with high commercial interest in food, feed, cosmetics, agriculture and health industries (Koutra et al. 2018; Fernández et al. 2021). Some valuable bioactive compounds from microalgae are proteins, polysaccharides, pigments, lipids and vitamins (Chew et al. 2017). Microalgae from the genus *Chlorella* are considered an alternative source of high-value pigments such as lutein, astaxanthin and β-carotene; vitamins, especially vitamin B complex, ascorbic acid, and α-tocopherol (Ievina & Romagnoli 2020). Commercial production of microalgae requires cultivation on a large scale with a high throughput. However, large-scale biomass production is currently limited due to low biomass productivity largely associated with suboptimal cultivation conditions and high costs (Chu 2017; Sun et al. 2020). Increasing the economic feasibility of microalgae biomass production is a major goal requiring careful consideration of crucial factors affecting microalgae cultivation. Success in microalgae cultivation depends heavily on setting up optimal growth conditions.

Light source and intensity, temperature, pH, nutrients, CO_2 availability, and mixing are essential parameters affecting microalgae growth and must be carefully considered when planning cultivation. Under laboratory conditions, nearly all factors can be managed and controlled, whereas many factors have high variability, are hard to control or are impossible to control under outdoor cultivation conditions. Open raceway ponds are regarded as promising cultivation systems for cost-effective large-scale microalgae cultivation (Kumar et al. 2015). Factors affecting microalgae growth in a raceway pond are shown by means of a Causal Loop Diagram in a study by Romagnoli et al. 2021), including highly variable factors not present in laboratory conditions, such as depth of the pond, solar irradiation intensity and fluctuating temperature.

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Temperature is one of the most crucial factors affecting microalgal productivity because of its direct influence on the photosynthesis rate. Moreover, temperature has a strong effect on chemical reactions within cells, e.g., the uptake of nutrients and CO_2 (Singh & Singh 2015). It has been demonstrated that increasing water temperature enhances algae growth until a certain limit is reached (Ras et al. 2013). When the temperature exceeds or does not reach the optimal range for a species, algal growth is decreased or even inhibited (Park et al. 2011). In addition, cell size, biochemical composition and nutrient requirements are affected by temperature. It has been observed that cell size decreases under high temperature (Peter & Sommer 2013; Skau et al. 2017). Moreover, decreasing or increasing temperature beyond optimum affects biochemical processes in algae cells, e.g., changes in lipid synthesis and composition, and starch and protein content have been observed (Juneja et al. 2013). Furthermore, CO_2 solubility is affected by the temperature in a pond, decreasing as the water temperature increases (Park et al. 2011; Bhola et al. 2014).

Insolation, depth of the pond and evaporation rate are other factors influencing culture temperature. The low depth of the culture in shallow raceway pond makes cultures particularly vulnerable to changing environmental temperatures. The shallower the depth of the culture, the more evident the effect of temperature extremes (Benemann & Tillett 1987). Moreover, changes in external temperatures have a rapid and immediate impact on pond temperature due to the low volume of the culture. Water loss due to evaporation is considerable in open ponds and can reach up to 1 cm a day (Benemann et al. 1982). Consequently, the water level must be constantly monitored and controlled to maintain the required culture depth.

Temperature in a natural environment is in constant change fluctuating both diurnal and seasonally and is highly dependent on the geographical location of cultivation ponds. High fluctuation of temperature in an open environment requires cultivation of algae strains with a broad optimum temperature range (Goldman 1977). Different microalgal strains are accustomed to various temperatures. Some algae exhibit a narrow optimum temperature range, and therefore are not suitable for environments with highly fluctuating temperatures. Others can withstand a wide temperature range (Borowitzka & Moheimani 2013). It is also essential to determine temperature tolerance of the strain. Selected species might have outstanding performance and high productivity at optimal temperatures but may exhibit very low productivity just a few degrees outside the optimum, thus considerably limiting their potential for outdoor cultivation. Therefore, resistance to low or high temperatures is of high importance for microalgae strains in outdoor cultures.

To date most large-scale microalgae cultivation is located in hot low-latitude regions such as Israel, Australia and the southern USA (Borowitzka 2018). In Europe, the top 3 countries in number of microalgae production units are Germany, Spain and Italy (Araújo et al. 2021). However, high temperature and high solar intensity create a risk for overheating during summer months. On the other hand, microalgae cultivation during winter might be more challenging at higher latitudes due to the lower temperature, shorter days, larger declination angle of the sun and increased cloud cover (Kenny & Flynn 2017). Lower productivity of microalgae cultivated in outdoor ponds during the winter season is observed compared to the summer (Lammers et al. 2017). In Northern European conditions, winters are usually long, and cold temperatures are common. Most microalgae prefer moderate to high temperatures with an optimum of approximately 27 – 30 °C (Dolganyuk et al. 2020), however are capable to grow in a wider temperature range from 15 to 30 °C (Singh & Singh 2015). Although optimum temperature for various microalgae has been studied extensively in the literature, the impact of low temperature on microalgae growth is rarely considered. To overcome issues experienced in hot climates and explore the option of outdoor microalgae cultivation in cooler environments, in the present study, we focus on search of the potential microalgae strain with a good resistance to low temperature suitable for outdoor cultivation in Northern European conditions. We determine the optimal temperature range for the selected candidate strain, as well as its ability to grow below the optimum temperatures.

Materials and methods

Microalgae strain and cultivation conditions

The freshwater microalga *Chlorella vulgaris* was selected after an extensive literature review as one of the most promising species for large-scale outdoor cultivation for biofuels and high-value compounds due to its high growth rate, flexibility in cultivation conditions, and capability to absorb high CO_2 concentrations (Wang et al. 2008; Brennan & Owende 2010). *Chlorella vulgaris* strain 211-11j was selected due to its northern origin in Sweden and a report on its cold tolerance (Gong & Bassi 2017). The strain was obtained from the SAG Culture Collection of Algae at Göttingen University, Germany. It was maintained in liquid BG-11 growth medium (Allen 1968) in Erlenmeyer flasks with baffles and 0.2 µm PTFE membrane screw caps (Duran, Germany) at room temperature in low light conditions and hand mixed daily to avoid settling of cells. Subculturing was performed approx. once per month to keep the algae culture growing and in a healthy condition.

To assess the influence of temperature on *C. vulgaris* growth, cells were grown in batch cultures at 8, 12, 16, 20,

24, 28 and 32 °C for 10 days. Illumination was provided with natural white (4000 K) LED lights (V-TAC, Samsung) with a light intensity of 50 µmol photons m^{-2} s^{-1} and a photoperiod of 16:8 h (light/dark). Light intensity was measured with a light meter (Testo, Germany). Cultures were cultivated in 500 mL Erlenmeyer flasks containing 200 mL BG-11 medium with an initial pH of 7.5. Aeration was provided with ambient air (CO_2 concentration ~0.038%) using an orbital shaker (Elmi, Latvia) at 150 rpm. The initial concentration of *C. vulgaris* cultures was approx. 2×10^6 cells mL^{-1}. Daily growth rate was measured by counting cells with an improved Neubauer hemocytometer.

All tests were performed in a benchtop incubated shaker (JeioTech 3075R, Korea) or refrigerated incubator (Friocell Eco line, MMM group, Germany) with manually installed LED lights. All tests were conducted in triplicate.

Microalgal kinetics

Microalgal cell density and dry biomass were measured to characterize the growth rate and biomass productivity. Cell density was determined by daily counting of microalgal cells in each culture flask using an improved Neubauer hemocytometer.

The mean value of triplicates was subsequently calculated for each cultivation temperature, and the standard deviation was determined. The maximum cell density at the end of batch test was also expressed as a percentage for easier comparison between various cultivation temperatures, and 100% was attributed to the maximum cell density reached among all the temperatures tested.

The specific growth rate (µ) based on cell density was calculated according to Eq. (1):

$$\mu = \frac{lnN_2 - lnN_1}{T_2 - T_1} \quad (1)$$

where N_1 is the initial microalgae culture density (cells mL^{-1}) at the beginning of cultivation (T_1) and N_2 is the culture density at the end of cultivation (T_2).

A two-tailed t-test was performed to evaluate the significance of cultivation temperature.

The dry weight of the cultures was determined by vacuum filtering 200 mL of the culture through preweighted 110 mm glass microfiber filters with a pore size of 1.2 µm at the end of the each batch test (Whatman GF/C). Filters were dried in an oven at 80 °C until constant weight and weighed. Dry weight (g L^{-1}) was calculated by subtracting the initial filter mass from the mass of filters with the biomass.

Biomass productivity (g $L^{-1}day^{-1}$) was calculated according to equation (2),

$$P = \frac{X_2 - X_1}{T_2 - T_1} \quad (2)$$

where X_2 is the biomass dry weight (g L^{-1}) at the end of cultivation (T_2) and X_1 is the dry weight of the initial culture at time T_1. Productivity was also expressed as a percentage, and 100% was attributed to the highest productivity reached. Dry cell weight was calculated to further understand the effect of temperature on *C. vulgaris* biomass yield.

Results

Chlorella vulgaris 211-11j was grown in batch cultures at temperatures ranging from 8 to 32 °C. Cultures grew at all temperatures tested except at 32 °C (Fig. 1). The growth of microalgae was very limited at 32 °C (µ = 0.024 day^{-1}) with very little cell division (Table 1). The highest specific growth rate of 0.224 day^{-1} was observed at 20 and 24 °C. The results on the specific growth rate must be perceived with caution due to microalgae growth pattern which was more linear than exponential at some temperatures and some time points. Microalgal cell density increased with increasing the cultivation temperature from 8 to 20 °C. Growth started to decrease at temperatures exceeding 24 °C. The concentration of cells at 20 and 24 °C was comparable at the end of the 10-day cultivation, 1.501×10^7 and 1.495×10^7 cells mL^{-1}, respectively. Moreover, the t-test revealed no significant differences between these cultivation temperatures at the 95% confidence level ($p = 0.878$). Furthermore, the growth of microalgae was slow at 8 °C with the specific growth rate of 0.157 day^{-1}.

Although the highest cell number was observed at 20 and 24 °C, the highest biomass accumulation (dry weight, g L^{-1}) was achieved when cultures were grown at 28 °C, 0.228 g L^{-1} (Fig. 2) and the dry weight of the algae at 20 and 24 °C was 0.208 and 0.210 g L^{-1}, respectively. Algae grown at 8 °C and 32 °C had comparable dry weight, 0.130 and 0.136 g L^{-1}, respectively, whereas cell density was much higher for cultures under 8 °C, 8.24×10^6 cells mL^{-1}. In contrast, the number of cells in the 32 °C cultures was 2.48×10^6 cells mL^{-1} (Table 1).

Cultures grown at 28 °C had the highest biomass productivity per day (g L^{-1} day^{-1}) of 0.025 (Table 1). Biomass productivity at 24 and 20 °C was 92.5 and 91.1% of the maximum productivity observed at 28 °C (Fig. 3). However, the productivity of cultures cultivated at 12 and 16 °C reached 80.7 and 85.4% of the maximum productivity, respectively. Low accumulated biomass was observed at 8 °C, reaching just 57% of the maximum productivity.

The fastest initial growth was observed at 28 and 24 °C, reaching the highest growth rate on day 2 and day 3, respectively, followed by cultures at 16 °C (Fig. 4). The

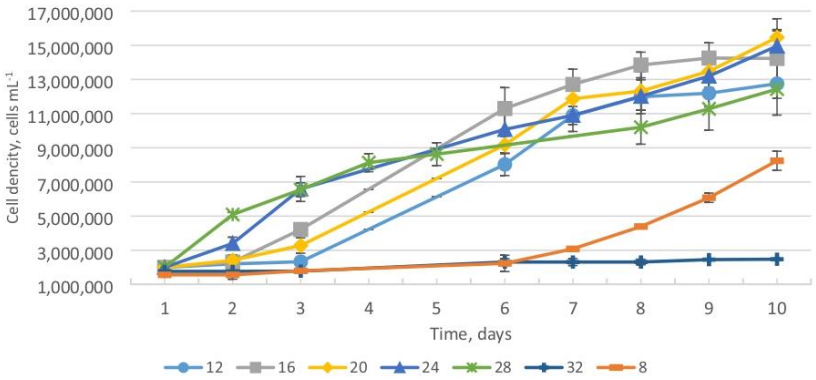

Fig. 1 *Chlorella vulgaris* culture cell density and growth pattern at various temperatures. Error bars indicate standard deviation (n=3)

Table 1 Effect of cultivation temperature on *Chlorella vulgaris* growth kinetics and productivity. Standard deviation of three replicates is shown in brackets where applicable

Cultivation Temperature, °C	Cell density, cells mL^{-1} (± SD)	μ, day^{-1}	Dry weight, g L^{-1} (± SD)	Productivity, g L^{-1} day^{-1}	Cell weight, $x10^{-11}$ g
8	8.24 x 10^6 (± 5.59 x 10^5)	0.157	0.130 (±0.003)	0.014	1.58
12	1.28 x 10^7 (± 1.84 x 10^6)	0.206	0.184 (±0.004)	0.020	1.43
16	1.42 x 10^7 (± 5.57 x 10^5)	0.218	0.194 (±0.006)	0.022	1.37
20	1.50 x 10^7 (±1.09 x 10^6)	0.224	0.208 (±0.006)	0.023	1.38
24	1.50 x 10^7 (±9.67 x 10^5)	0.224	0.210 (±0.001)	0.023	1.41
28	1.2 x 10^7 (±5.41 x 10^5)	0.203	0.228 (±0.009)	0.025	1.83
32	2.48 x 10^6 (±2.36 x 10^4)	0.024	0.136 (±0.002)	0.015	5.47

slowest initial growth was noted at 8 °C. This culture did not reach the stationary growth phase after the 10-day cultivation and the growth rate continued to increase. Due to the longer acclimation phase at the beginning of cultivation, cultures at low temperatures might require cultivation longer than 10 days to reach the stationary phase.

Although cell density was higher at 20 and 24 °C, higher biomass productivity was observed in cultures cultivated at 28 °C that might be attributed to the smaller size of the cells at 20 and 24 °C. Indeed, the calculation of cell weight of dry biomass showed that cell weight was higher at 28 °C than at 20 or 24 °C. The highest cell weight was of microalgae cultivated at 32 °C whereas the lowest was observed at 16, 20 and 24 °C, indicating that cells of *C. vulgaris* 211-11j were larger at high temperatures compared to average cultivation temperatures. An increase in cell weight was observed again in lower temperatures (12 and 8 °C).

Discussion

The maximum biomass yield of *C. vulgaris* 211/11j was observed at 28 °C, therefore we suggest that this temperature is optimal for cultivation for this strain in the given experimental setup. Furthermore, temperatures from 20 to 28 °C can be considered the optimal range for cultivation of this strain as no significant difference in productivity was observed. The data reported in the literature on the optimal cultivation temperature of *C. vulgaris* vary widely, generally

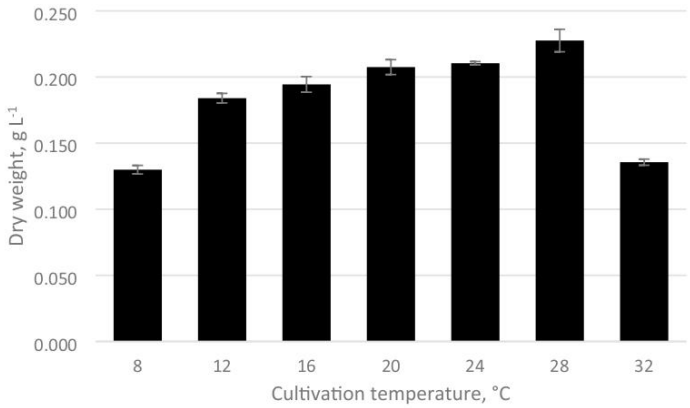

Fig. 2 Biomass yield of *Chlorella vulgaris* at different cultivation temperatures at the end of the cultivation. Error bars indicate standard deviation (n=3)

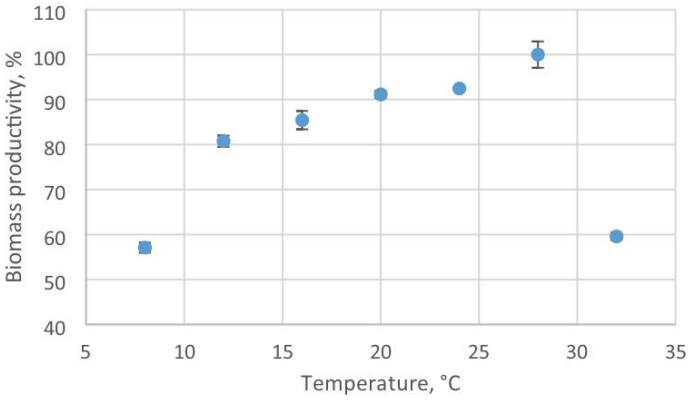

Fig. 3 *Chlorella vulgaris* biomass productivity at different cultivation temperatures expressed in percentage of maximum productivity. Error bars indicate standard deviation (n=3)

ranging from 25 to 32 °C (Mayo 1997; Converti et al. 2009; Barghbani et al. 2012; Gonçalves et al. 2016; Serra-Maia et al. 2016). The findings of this research are consistent with those of a study by Serra-Maia et al. (2016), who observed the highest growth rate (number of cells) at 25 °C when cells were grown at 20, 25, 28 and 30 °C. Similar to the present study, they reported no significant differences between the growth rates at 24 and 28 °C. Moreover, the growth rate at 25 °C only slightly increased when compared to that at 20 °C. Similarly, they observed a decrease in the growth rate from 25 to 28 °C. However, Barghbani et al. (2012) reported 30 ±2 °C as optimum temperature when testing *C. vulgaris* growth at 20, 25, 30 and 35 °C. The observed differences are most likely due to the different strain of *C. vulgaris* used in their study. Microalgae were obtained from Shahriar river near the city of Tehran (Iran), therefore this strain could be more adjusted to hot climates than the strain used in the present study. Some other studies have reported higher optimum temperatures than those observed in the present study. For example, Chinnasamy et al. (2009) reported optimal growth at 30 °C at elevated CO_2 level (6%); however the *C. vulgaris* stain used (ARC1) was originally isolated from an oxidation pond system at Delhi (India) and this is most likely responsible for the higher optimum growth temperature observed. *C. vulgaris* strain 211-11j is rarely studied; only one report was found analysing the optimal growth conditions (Maxwell et al. 1994) and is discussed below.

We observed that the *C. vulgaris* 211-11j growth based on cell density was higher below the optimum rather than above

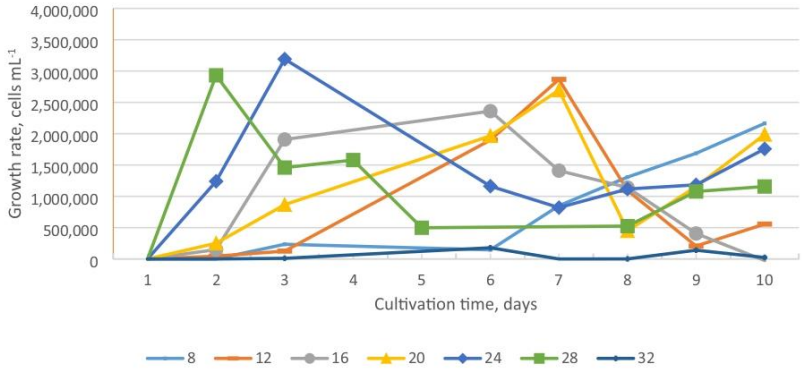

Fig. 4 Growth rate of *Chlorella vulgaris* per day at different cultivation temperatures

the optimum temperature. An increase in temperature by just four degrees above 28 °C resulted in a more than eightfold decrease in the growth rate. At the same time, the growth rate below the optimum decreased gradually. The characteristic of algae that the lethal temperature is only a few degrees above the optimum temperature is well known (Borowitzka 2013) and has been reported often (Kessler 1985; Benemann & Tillett 1987; Converti et al. 2009).

It seems that temperature has a strong effect on the cell weight of this algal strain. Generally, not all microalgae show a positive correlation between cell size and temperature. In this study, the maximum biomass yield observed at 28 °C was due to an increased size of the cells, rather than the number of cells. This finding suggests that *C. vulgaris* cells tend to be larger in size at high temperatures but are not actively dividing. The largest cells were observed at high cultivation temperatures (28 and 32 °C) with a maximum weight at 32 °C. Our finding is in agreement with Dai et al. (2022) who reported that the cell size of *Chlorella pyrenoidosa* was increased under high culture temperature. On the other hand, other studies have reported that cell size decreased at high temperature in various microalgae (Peter & Sommer 2013; Skau et al. 2017). These reports confirm that the response of cell weight to temperature is species specific.

While there are many studies assessing the optimum and maximum growth temperature for *C. vulgaris*, only few studies considering low temperatures can be found. In the present study, we tested the growth of *C. vulgaris* at low temperatures (16, 12 and 8 °C). While the growth rate decreased by nearly 43% at 8 °C, compared to the maximum productivity at 28 °C, productivity was still near 85% and 81% of the maximum at 16 °C and 12 °C, respectively, showing good ability of this strain to grow in a moderate temperatures and substantial resistance to low temperature. The results are encouraging for the cultivation of this strain in higher latitude regions where fluctuations in diurnal temperatures even during summer may be high.

Slower cell growth at the beginning of culturing at lower temperatures may be attributed to acclimation to the new growing conditions. Cultures cultivated at lower temperatures require longer adaptation time to new extreme growth conditions than cultures cultivated at more optimal temperatures. This is especially significant at 8 °C cultivation when cultures started to grow only after 6 days. Although microalgae cultures cultivated at 8 °C did not result in high biomass accumulation at the end of the cultivation, cells were actively dividing, and growth rate was increasing quickly after the long adaptation phase. These results are very promising, showing that cultures cultivated at 8 °C can reach a good growth after the low-temperature acclimation.

Moreover, this strain exhibits a lower optimum cultivation temperature than some other *C. vulgaris* strains showing some advantage over other strains for outdoor cultivation in cooler climates and therefore could be selected as a candidate stain for biomass production in Northern Europe. The results of the present study are consistent with the findings of Maxwell et al. (1994), who reported that this strain of *C. vulgaris* was able to grow at 5 °C. The authors demonstrated that *C. vulgaris* is capable of acclimation to low temperatures by adjusting the photosynthesis apparatus and exhibits a similar pattern to high light acclimation. In another study, it was demonstrated that this strain of *C. vulgaris* could be successfully used for lutein production at low temperatures (Gong & Bassi

2017) suggesting the potential application of harvested biomass supporting the biorefinery concept.

Conclusions

The growth of *C. vulgaris* is substantially affected by cultivation temperature. Our study suggests that *C. vulgaris* strain 211/11j has a great advantage in colder climates. The optimal temperature range for biomass production was 20 to 28 °C, with maximum biomass productivity reached at 28 °C. We demonstrate that *C. vulgaris* 211/11j has a wide optimum temperature range that is also lower than that of other *C. vulgaris* strains reported in the literature suggesting that this strain prefer cooler environment. Moreover, wide optimum temperature range is suitable for highly variable outdoor conditions and confirms the flexibility of this strain.

We demonstrate that *C. vulgaris* 211-11j can grow effectively in moderate temperatures and exhibits good resistance to low temperature. Tolerance to low temperature makes *C. vulgaris* 211-11j a potential candidate for the production of biomass under cooler weather conditions. The obtained results are encouraging for the cultivation of this strain in higher latitude regions however, further studies of the potential of this strain are required.

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Author contributions Both authors contributed to the study design. BI performed laboratory tests and data analysis. Both authors interpreted the data. BI wrote the manuscript. FR critically revised the manuscript. Both authors have approved the final document.

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Data availability Data will be available from the corresponding author on request.

Declarations

Conflicts of interest The authors declare no competing interests.

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Adjustment of light spectral quality for enhanced microalgae growth

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Abstract

Emerging research suggests that targeting specific spectral regions of visible light can result in superior biomass yields compared to conventional white light illumination. Although recent studies have begun to address the influence of the light spectrum on microalgae, our understanding remains limited. The present study unravels the underlying factors of the preferred spectrum for microalgae, including the role of pigment content and the significance of cultivation conditions. While the blue and red fractions of the light spectrum have frequently been proposed as optimal, several investigations indicate that a balanced combination of narrow wavelengths is essential to attain maximum growth. Different response of microalgae to the light spectrum demonstrates a species–specific behavior. Moreover, we demonstrate the major role of weakly absorbed wavelengths in maximum biomass yield. Given the high potential of spectral manipulation for microalgae growth enhancement, it becomes of utmost importance to the economic viability of microalgae mass culturing.

Keywords: microalgae, light-emitting diode, wavelength, light spectrum, light quality

1. Introduction

Microalgae biomass represents a promising resource for bioenergy and a wide array of high-value compounds with substantial commercial potential. Nevertheless, the limited productivity and high cultivation expenses associated with microalgae have restricted the availability of bioactive compounds on the market, and bioenergy remains a distant goal in terms of commercial viability. To address these challenges, optimizing growth conditions plays a pivotal role. Employing light spectra tailored to the specific requirements of individual microalgal species presents a novel and highly promising approach to augmenting microalgae growth rates and, consequently, biomass productivity.

Microalgae constitute a pivotal component of the global bioeconomy, offering extensive promise across various domains. Notably, microalgae biomass has already served as a valuable source for pharmaceuticals, cosmetics, food, and animal feed (Fernández et al., 2021). Furthermore, the investigation of microalgae-derived biofuels has been a long-standing area of research interest. In recent times, an array of emerging applications for microalgae biomass utilization has come to the fore, encompassing innovative concepts such as wastewater treatment and CO_2 biosequestration (Bauer et al., 2021; Salama et al., 2017; Singh and Ahluwalia, 2013). Additionally, microalgae exhibit potential in the production of agriculture-related products, including biofertilizers, biostimulants, and biopesticides (Fernández et al., 2021). However, despite the manifold possibilities offered by microalgae biomass, the realization of large-scale microalgae production currently faces significant constraints, primarily attributed to low yields and high production costs (Rajesh Banu et al., 2020). To overcome these limitations, considerable efforts have recently been focused on enhancing the economic viability of microalgae cultivation. These efforts include optimizing bioreactor design, fine-tuning

cultivation conditions, such as temperature and light regimes, and exploring alternative nutrient sources.

Undoubtedly, light represents one of the paramount factors in microalgae cultivation, constituting a multifaceted system encompassing light intensity, duration, and spectral composition, all of which are indispensable for optimal growth. Optimum light conditions are pivotal in ensuring high photosynthetic rates, consequently facilitating the expeditious accumulation of biomass. The significance of light availability and quality cannot be overstated, as they have been identified as the primary determinants limiting the productivity of large-scale microalgae cultures (Masojídek et al., 2021). Microalgae cultivation can harness both natural sunlight and artificial light sources. Undeniably, sunlight stands out as the most cost-effective illumination option, owing to its abundance, renewability, and zero cost. Nevertheless, artificial lighting offers distinct advantages by affording finer control over critical parameters. In addition to regulation of light intensity and photoperiod, artificial lighting permits the precise manipulation of spectral quality, a feature that significantly impacts algal growth and productivity (Vadiveloo et al., 2015). While research into the influence of light's spectral composition on microalgae growth has lagged behind other light-related factors, such as light intensity, it has garnered increasing attention over the past decade. Several studies have demonstrated that customizing the incident light spectrum delivered to algal cultures can substantially enhance microalgae growth rates. Thus, the definition of an appropriate spectral range assumes paramount importance in achieving economic viability of large-scale microalgae cultivation.

While manipulating light wavelengths holds promise as a strategy for enhancing microalgae growth, it remains an area of incomplete understanding. Published studies investigating the effects of distinct wavelengths on microalgae have yielded inconsistent and often contradictory results, leading to a significant knowledge gap concerning the impact of different light wavelengths on microalgae growth and biomass production. In this study, authors endeavor to shed light on this perplexing issue, recognizing the absence of a comprehensive review dedicated to the influence of light wavelengths on microalgae growth in the existing literature.

The objective of this study is to bridge this gap by providing an in-depth exploration of the nature of light, elucidating the mechanisms by which microalgae harness light, and delving into the pivotal role of pigments in light absorption. Subsequently, we comprehensively review the effects of various light wavelengths on microalgae photosynthetic efficiency and biomass production. Additionally, we consider the potential of manipulating light spectral composition as a promising strategy for enhancing biomass production, thus contributing to a more profound understanding of this crucial aspect of microalgae cultivation. No guidance on the selection of suitable light spectrum could be found.

A literature search was carried out using Scopus database with a time interval of the last 10 years (2012 – 2022). Keywords used were: microalgae, wavelengths, light quality, and light spectrum. Selected papers were checked for conformity by reviewing the titles and keywords. Inconsistent papers were excluded.

2. Underlying concepts

In this section, we elucidate the essential definitions and fundamental concepts of the effects of light types on microalgae photosynthetic efficiency and biomass production. We delve into various research topics on how the light captured by microalgae could affect the characteristics of algae, offering a comprehensive examination and discussion of these underlying processes.

Light capturing by microalgae

In order to explore the possibilities offered by the visible light spectrum, the process of photosynthesis must be understood. When light passes through the atmosphere light spectral composition changes (Carruthers et al., 2001). Short wavelengths ultra-violet light (200 – 400 nm) is absorbed by ozone, whereas long wavelength infrared is absorbed by water vapor and carbon dioxide (Carruthers et al., 2001). Only a fraction of solar radiation reaches the earth's surface and an even smaller fraction can be used by photosynthetic organisms. Light with wavelengths between 400 nm and 700 nm is called photosynthetically active radiation (PAR) and is used by microalgae and higher plants for light conversion processes in photosynthesis. Even within the PAR range, microalgae utilize only a fraction of solar radiation due to the presence of ineffective portion of the PAR spectrum (Blanken et al., 2013), consequently not all wavelengths of PAR are equally absorbed and utilized by microalgae (Moheimani and Parlevliet, 2013). It is considered that the absorption spectrum depends on the pigment composition of microalgal species; therefore, the efficiency of the use of specific wavelengths of radiation depends on the pigment profile of species.

Photosynthetic pigments

Light energy is absorbed by pigments in microalgae cells and converted to chemical energy during photosynthesis. All photosynthetic organisms contain pigments to harvest light energy, however, the composition of pigments is different in various plants and microalgae. Each pigment has a unique absorption spectrum characterized by specific wavelengths of light it absorbs. Most photosynthetic organisms contain various pigments, allowing energy absorption from a wide spectral range. This distinctive set of light-harvesting pigments in a photosystem of different microalgae groups is responsible for the light spectral range they can absorb. Therefore, it is believed that pigments in microalgae play a crucial role in determining the light spectrum requirements. Consequently, it is important to identify the function and role of specific pigment groups and their absorption spectrum.

Three major classes of pigments in microalgae are chlorophylls, carotenoids and phycobiliproteins (Pagels et al., 2020). Chlorophylls (Chl) are green pigments and the most important class in photosynthesis (Kommareddy and Anderson, 2003). Other pigments, called accessory pigments, absorb light energy and deliver it to the Chl *a* for photosynthesis extending the absorption range (Zigmantas et al., 2002). The absorption maximum of chlorophylls is in the red and blue part of the light spectrum. Chl *a* is the main pigment directly involved in photosynthesis being present in all photosynthetic algae (Allakhverdiev et al., 2016; Moheimani and Parlevliet, 2013). Other chlorophylls in microalgae such as *b*, *c*, *d*, *e* and *f* are species-specific (Kommareddy and Anderson, 2003; Zigmantas et al., 2002) and have slightly different absorption spectra with the major absorption bands in blue, blue-green (450–475 nm) and red (630–675 nm) wavelengths (Figure 1). Moreover, Chl *b* is found exclusively in green algae, whereas Chl *c* is found only in some microalgae groups such as red algae, diatoms and dinoflagellates (Christaki et al., 2015). Chl *c* absorbs strongly in the blue light range (Schulze et al. 2014). However, Chl *d* and *f* are exclusive to some cyanobacteria and are far-red absorbing chlorophylls (Allakhverdiev et al., 2016). The maximum in the absorption spectrum of Chl *d* and Chl *f* is shifted towards longer wavelengths compared to Chl *a* (Allakhverdiev et al., 2016). Therefore, cyanobacteria containing Chl *d* or Chl *f* are capable of harvesting far-red (near infrared) light at wavelengths >700 nm (Averina et al., 2018). Still, Chl *a* represents the only chlorophyll in most cyanobacteria while in rare cases other chlorophylls are additionally produced.

Due to chlorophylls, the light absorption of most microalgae is highest in the blue (approx. 460–490 nm) and red (approx. 630–700 nm) part of the light spectrum. While the middle part (490-570) of the PAR range containing mainly green light is hardly covered and is

known as “green gap” (Hintz et al., 2021). Nevertheless, this middle section is used by accessory pigments carotenoids and phycobiliproteins.

Phycobiliproteins - phycocyanin (blue pigment), allophycocyanin (light blue), and phycoerythrin (red) are a major accessory pigment group found in cyanobacteria and red algae (Nwoba et al., 2019). Phycocyanin is primarily found in cyanobacteria, while phycoerythrin is a major pigment of most red algae. In green microalgae, phycobiliproteins are absent (Schulze et al., 2014). Phycobiliproteins have characteristic fluorescent color and provide additional light absorption in the blue-green, green, yellow, and orange part of spectrum with an absorption between 480–660 nm (Glazer, 1994).

Carotenoids are red, yellow and orange pigments that constitute another large group of accessory pigments whose main role is protection against excessive irradiance (Masojidek et al., 2004). Carotenoids absorb in violet, blue and green regions with an absorption spectrum around 400 – 550 nm (Zigmantas et al., 2002). Comprising two large groups: carotenes and xanthophylls carotenoids are a widespread class of pigments. β-carotene is one of the most important carotenoids in microalgae serving as an accessory pigment in photosynthesis (Torregrosa-Crespo et al., 2018). β-carotene absorbs light in a range between 400 – 500 nm with a peak of absorption around 450 nm (Kommareddy and Anderson, 2003). Xanthophylls comprise a wide group of oxygenated derivates of carotenes including lutein, violaxanthin, zeaxanthin and fucoxanthin (Niyogi et al., 1997). They function as accessory light-harvesting pigments and have crucial structural and functional roles in algae. One of the most known functions of xanthophylls is to provide photooxidative protection against photodamage (Polle et al., 2003). The absorption spectrum of some major carotenoids is shown in Figure 2. Both lutein and zeaxanthin absorb blue light (400 – 475), however, zeaxanthin is more effective at absorbing blue-green light at 500 nm and slightly above (Krinsky, 2002). In some microalgae, carotenoid content could be similar to or even larger than that of chlorophyll, e.g. in *Bacillariophyceae* and *Dinophyceae* (Moheimani and Parlevliet, 2013). Consequently, some classes of microalgae depend to a higher degree on the carotenoids to capture light.

Major pigments in microalgae and the corresponding absorption range are compiled in Table 1. Microalgae are an exceptionally diverse group of organisms from different evolutionary lineages. The most important algal groups in terms of abundance are green algae (*Chlorophyceae),* diatoms (*Bacillariophyceae*), cyanobacteria or blue-green algae *(Cyanophyceae)* and golden algae (*Chrysophyceae*) (Khan et al., 2009).

In addition to composition also pigment quantitative content in microalgae is crucial since it affects the light spectrum requirements. Chl *a* and Chl *b* are dominant pigments in green algae, giving the characteristic green color. Various carotenoids including β-carotene and several xanthophylls (e.g. astaxanthin, canthaxanthin, lutein and zeaxanthin) are also present in green algae (Barkia et al., 2019; Tomaselli, 2004). The composition of pigments in different microalgae groups is shown in Table 2. The main pigments found in red algae are phycobiliproteins (phycoerythrin and phycocyanin), Chl *a* and *d*; also various carotenes and xanthophylls are present (Barkia et al., 2019; Tomaselli, 2004). Interestingly, phycobiliproteins are the major light-harvesting pigments in red algae (Kim et al., 2019). Diatoms contain Chl *a* and Chl *c*, xanthophylls and carotenes but lack phycobiliproteins (Kuczynska et al., 2015). Moreover, fucoxanthin is thought to be responsible for the golden brown color of diatoms resulting from its dominance over Chl *a* and *c* (Sahoo and Seckbach, 2015). Although cyanobacteria contain also Chl *a,* phycobiliproteins are major pigments in cyanobacteria present in larger quantities than chlorophyll usually masking the chlorophyll pigmentation (Tomaselli, 2004). The presence of phycobiliproteins results in the characteristic blue-green color of cyanobacteria. Generally, algae with a high concentration of carotenoids appear yellow to brown. Those with a high concentration of phycocyanin appear blue but those with a high concentration of phycoerythrin appear red.

LED lights for microalgae growth

Due to their various beneficial characteristics, light-emitting diode (LED) lights are quickly gaining popularity as grow lamps over traditional lighting sources. Compared to fluorescent lamps, LEDs offer several advantages. In addition to lower heat dissipation and, therefore lower energy consumption, they also have a longer lifetime, are mercury-free and have a narrow emission spectrum (Baer et al., 2016). LED-based lighting is more energy efficient than conventional technologies and enables better control of crucial parameters in microalgae cultivation. However, the greatest advantage of LED lighting is the ability to produce different colored light, allowing modification to specific needs. Not only do LEDs have the potential to sustain a good growth, but also to improve it more than the capacity of conventional lights. The narrow band LEDs emission maximum can be matched with the desired light absorption spectrum of the species, offering a powerful tool for increasing microalgae growth. The advances in the field of lighting and the development of LED technology make the exploitation of specific wavelengths of the light spectrum much easier, enabling the studies on the effect of various wavelengths to be more effortless, precise, and widespread. Moreover, an increasing number of LED-based studies makes them more comparable.

2. The effect of narrow wavelengths on microalgae

Although it has been widely accepted that light quality has a considerable effect on biomass formation it is still poorly understood. Reports on the effects of the light spectrum on microalgae have increased significantly in recent years due to the advent of LED technology. Published studies generally compare the effect of different monochromatic lights with a narrow emission spectrum with each other and white light as a control. In this section we analyze the effect of single monochromatic light on microalgae growth and processes on morphological, physiological and genetic level.

Impact of red light on microalgae growth

Red light (approx. 600 – 700 nm) is considered the most efficient wavelength based on the measurement of the quantum requirement for photosynthesis (Blanken et al., 2013), thus, there are claims that red light is the most suitable for microalgae growth. Indeed, various studies report that red light enhances the growth of various microalgae species compared to white light. Monochromatic red light was found to be optimal for *Chlorella vulgaris* growth when testing different monochromatic lights (Chang et al., 2022; Ge et al., 2013; Kendirlioglu and Kadri Cetin, 2017; Yan et al., 2013). Furthermore, red light was shown to enhance biomass production also in other microalgae species: green algae *Chlorella pyrenoidosa* (Chu et al., 2021) and *Dunaliella salina* (Pereira and Otero, 2019), red alga *Galdieria sulphuraria* (Baer et al., 2016) and cyanobacteria *Microcystis aeruginosa* (Tan et al., 2020). However, inconsistent or contradictory results regarding the effect of red light on microalgae have been reported. Some reports indicate that monochromatic red light is not suitable for high biomass production (de Mooij et al., 2016; Mohsenpour et al., 2012). Likewise, a lower photosynthetic rate in several crop plants grown under sole red light has been reported (Hogewoning et al., 2010).

In addition to its role in photosynthesis, it is known that red light participates in growth regulation and development of higher plants. Studies demonstrate that plants grown under monochromatic red light do not develop normally having abnormal symptoms including stretched, elongated appearance and large and thin leaves (Kaiser et al., 2019) indicating that red light alone may not be sufficient to sustain normal growth. Red light was shown to influence also flowering in plants (Runkle and Heins, 2001). Mechanisms of action of red light in microalgae have not been fully understood (Schulze et al., 2014); however, it has been shown in several studies that red light escalates cell division in microalgae resulting in smaller cells in algae cultivated under monochromatic red light (Kim et al., 2014).

In addition, far-red light (700 – 800nm) is not considered involved in photosynthesis but has a major impact on growth. It is widely reported that far-red light plays a role in flowering, fruit development and biomass production in terrestrial plants (Runkle and Heins, 2001; Schulze et al., 2014). It was demonstrated that supplementation of far-red light (740 nm) to blue-red light to *C. vulgaris* cultures could be responsible for the observed increase in growth rate (Kula et al., 2014). Moreover, authors showed that far-red light changes chemical composition such as lipids, carotenoids and chlorophyll content in *C. vulgaris*. Likewise, the supplementation of white light with far-red light resulted in increased growth of green halotolerant microalga *Dunaliela bardawil* (Sanchez-Saavedra et al., 1996). Nevertheless, reduced maximal cell density, chlorophyll concentration and increased carotenoid content were also observed in this study. It seems that red light has a significant role in microalgae growth regulation and development thus more likely is required for normal functioning of cells.

Impact of blue light on microalgae growth

Blue light (approx. 400 – 500 nm) is considered equally effective as red light at driving photosynthesis. Because blue wavelengths have a rather high energy content, a relatively low intensity of blue light is thought to be required for functioning of photosynthesis in terrestrial plants and green microalgae. Besides the central role in photosynthesis, blue light is also involved in several physiological processes in cells and is known to affect metabolic pathways (Schulze et al., 2014). Blue part of the light spectrum is involved in enzyme activation and regulation of gene transcription (Ruyters, 1984). Moreover, blue light photoreceptors upregulate the genes involved in pigment biosynthesis (McGee et al., 2020). Similar to red light, the cell size of microalgae is influenced by the application of blue wavelengths. Blue light receptors are thought to control the start of cell division inhibiting the division in small cells. Thus, the delay in cell division leads to an increased cell size commonly observed in microalgae cultivated under sole blue light. Consequently, in contrast to red light, the average cell size is larger under blue light compared to white light. This effect has been observed in several microalgae species e.g. *Chlamydomonas reinhardtii* (Wagner et al., 2016), *C. vulgaris* (Izadpanah et al., 2018; Kim et al., 2014), *Chlorella kessleri* (Koc et al., 2013) and *Chlorella sorokiniana* (Izadpanah et al., 2018).

In contrast to red light, blue light usually suppresses extension growth in terrestrial plants, therefore plants grown with blue light are usually shorter with smaller, thicker and darker leaves compared to plants grown without the blue light. While the underlying mechanisms of monochromatic red or blue light effects are not known in microalgae, the lack of both blue or red light in the illumination of tomato plants was shown to affect negatively plant development (Izzo et al., 2020). While blue light alone is not sufficient for normal plant development, in a blue light-containing irradiance a higher biomass production and photosynthetic capacity of terrestrial plants is generally observed (Hogewoning et al., 2010).

Moreover, blue light is involved in light-related regulatory processes, such as phototropism, photomorphogenesis, stomatal opening and leaf photosynthetic functioning in land plants (Hogewoning et al., 2010). Similar mechanisms have been shown to be in place in microalgae. Blue light was required for photoprotection and acclimation to high light intensities in the marine diatom *Phaeodactylum tricornutum* (Schellenberger Costa et al., 2013).

Controversial studies have been published on the effect of blue light on the growth rate of microalgae. Increased growth rate under monochromatic blue light was reported for *Chlorella ellipsoidea* (Baidya et al., 2021), *C. vulgaris* (Atta et al., 2013; Kang et al., 2015), *Chlorella pyrenoidosa* (Asuthkar et al., 2016), *Nannochloropsis* sp.(Das et al., 2011; Teo et al., 2014), *Tetraselmis* sp. (Teo et al., 2014), *Chlorella* sp. (Kang et al., 2015) and *Scenedesmus* sp. (Kang et al., 2015). In contrast, blue light yielded poor productivity of *C. vulgaris* in Yan's study (Yan et al., 2013). Moreover, cultivation under monochromatic blue LEDs resulted in the lowest growth rate in another green alga *Picochlorum* sp. compared to red, green or white (Paper et al., 2022). The

contrasting results demonstrate that the role of blue light is complex and most likely other factors are responsible for the results obtained.

Significant differences in sensitivity to blue light have been observed among higher plant species (Snowden et al., 2016). It has been speculated that response to blue light is species-specific, some plant species being highly sensitive to blue light fraction, whereas others have intermediate or low sensitivity (Bugbee, 2016). If his is also true in microalgae, it could at least partly explain the contradictory results reported.

Impact of green light on microalgae growth

Perhaps the most puzzling is the role of green light in microalgae. Although main absorption is taking place in the red and blue parts of the spectrum, there are ongoing debates on the role of green light in photosynthesis. The green part of the visible light spectrum roughly spreading between 500 and 600 nm has been perceived inconsistently. Traditionally very limited use of green wavebands in the process of photosynthesis have been considered since chlorophyll does not absorb in this part of the spectrum (Wagner et al., 2016) and are thought not to promote growth in higher plants (Johkan et al., 2012). Consequently, green wavebands are often considered unsuitable for microalgae growth if applied without additional light sources (Schulze et al., 2014). It has been argued that green microalgae cannot use yellow and green light effectively due to the lack of phycobiliproteins (Schulze et al., 2014). However, there is strong evidence that green light takes part in photosynthesis and regulation of physiological processes in plants (Nishio, 2000; Smith et al., 2017).

There is only a limited number of studies on the effect of green light on microalgae since green wavebands are often excluded from the light quality studies possibly due to the above-mentioned reasons. Nevertheless, the existing studies demonstrate that sole green light cannot sustain the growth of most algae (McGee et al., 2020; Vadiveloo et al., 2015). However, there is an evidence that some species of microalgae are able to utilize the green part of the spectrum efficiently and grow well under green illumination (Baidya et al., 2021; Coward et al., 2016; Latsos et al., 2021; Lee et al., 2019; McGee et al., 2020; Paper et al., 2022). A relatively high growth rate of green microalga *Ettlia* sp. was achieved under green light showing that green wavebands could support *Ettlia* sp. growth; although the growth rate was lower than that under white light (Lee et al., 2019). The same study revealed that the application of a green light might provide other advantages, e.g., better competitive ability. It was shown that *Ettlia* sp. was able to out-compete *C. vulgaris* under white + green LEDs without losing productivity. Supplementation of white light with the green part of the spectrum could be potentially used as a tool to prevent invasion by other microalgae in mass cultures of *Ettlia* sp. This is an interesting finding and should be further studied.

Probably the most important consideration is that green light is thought to increase the efficiency of sunlight use in deeper parts of a leaf and in dense canopies since the upper layers of the leaf preferentially absorb red and blue lights (Smith et al., 2017). It could be argued that the same effect might be true for dense microalgae cultures in open ponds or photobioreactors. Indeed, light penetration into water is greatly affected by the absorption and scattering processes within the water. Not only intensity but also the color of the light changes greatly with depth (Kirk, 1994). Upper layers of cells close to the top of the water surface absorb most of the red light, while blue light can penetrate deeper layers of water. However, weakly absorbed wavelengths, such as green, have high scattering and low absorbance coefficients (Mattos et al., 2015). Therefore, as culture density and water depth increase, red and blue wavelengths become less available to the algae than green wavelengths (Schulze et al., 2014). Consequently, green light penetrates deeper and can therefore be absorbed by algae cells at lower pond levels leading to higher photosynthetic efficiency in high-density cultures. Although there are not enough studies, a few reports found, suggest that green light indeed is beneficial in high-density

cultures. Mattos et al. (Mattos et al., 2015) observed increased biomass production of the green alga *Scenedesmus bijuga* dense cultures (2.9 g/L) under green light of compared to cultivation under monochromatic red light. Also, de Mooij et al. (de Mooij et al., 2016) demonstrated that weakly absorbed yellow light resulted in the highest biomass productivity in high-density *C. reinhardtii* cultures compared to deep red, orange red or blue light. These results demonstrate that the ability to use green light is a competitive advantage for high biomass production, especially under high-density cultures and long light pathways (Ooms et al., 2017; Paper et al., 2022).

3. Underlying factors of the preferred light spectrum

The role of pigment composition

Pigment composition of the light-harvesting complexes may provide information on the light requirements of microalgae within a taxonomic group (Schulze et al., 2014). Kim et al. (Kim et al., 2019) showed that different parts of the light spectrum yielded the highest biomass productivity for each algae belonging to different microalgae lineages. *Pavlova lutheri* (golden brown alga) exhibited the highest biomass productivity when cultured at blue light, *C. vulgaris* (green alga) at red and *Porphyridium cruentum* (red alga) at green light. A spectral matching strategy has been proposed to increase the light utilization efficiency (Schulze et al., 2014). The photosynthetic efficiency depends on a fraction of photons absorbed from the available light energy. Consequently, to reach the maximum efficiency, all photons emitted from a light source should be absorbed by pigments of the photosynthetic reaction centre of microalgae. Spectral matching aims to match a light source spectrum with the absorption spectra of certain pigments of microalgae species to achieve efficient light utilization and therefore higher biomass yield.

Green algae contain Chl *a* and Chl *b* as major light harvesting pigments therefore the absorption is the highest at red and blue wavelengths similar to terrestrial plants. Consequently, green microalgae utilize red and blue light with high efficiency. Golden brown algae are characterized by the presence of fucoxanthin in high concentration, which is responsible for their golden to brown color. Fucoxanthin absorbs light in approx. 450 – 550 nm (Bricaud et al., 2004) corresponding to the blue-green part of the light spectrum, leading to better utilization of green light than, for example, green algae can. Golden brown algae also contain Chl *a*, Chl *c*, and some carotenoids that contribute to the absorption of blue light. Similarly, diatoms can utilize green light due to the presence of fucoxanthin which together with Chl *a* and Chl *c* is a major constituent of light-harvesting pigments in diatoms (Kuczynska et al., 2015).

Furthermore, red pigment phycoerythrin is a major light-harvesting pigment in red algae responsible for the absorption in green part of the light spectrum. Phycoerythrin was shown to be the major phycobiliprotein in *Porphyridium purpureum* accounting for more than 80% of phycobiliproteins (Guihéneuf and Stengel, 2015). Phycobiliproteins are higher in concentration in many red algae and cyanobacteria than chlorophyll, therefore they can generally utilize green light better than green algae (McGee et al., 2020). Moreover, phycobiliproteins are also at a higher level than carotenoids, such as β-carotene or zeaxanthin in red algae. Consequently, a higher portion of green compared to blue light is required for optimal light utilization and higher biomass productivity of red algae (Baer et al., 2016). On the other hand, blue pigment phycocyanin was shown to be the major phycobiliprotein in another red alga *Galdieria sulphuraria* resulting in the need of the red light to reach the maximum growth rate (Baer et al., 2016). Interestingly, although the maximum biomass was reached at pure red light corresponding to the absorption maximum of phycocyanin, blue fraction was required for the highest phycobiliprotein concentration in this species.

Cyanobacteria lacking Chl *b*, use Chl *a* and phycobiliproteins to harvest light energy. Phycocyanin is usually high in concentration giving cyanobacteria the characteristic blue-green color. Therefore, cyanobacteria are able to utilize mostly red, yellow, and green light. Blue light is used less efficiently in cyanobacteria probably because of the absence of Chl *b*. Moreover, a higher diversity of carotenoids in eukaryotic microalgae (Schulze et al., 2014) leads to a better utilization of blue light than in cyanobacteria. Lately, Luimstra et al. (Luimstra et al., 2018) offered an alternative explanation that blue light is absorbed with the same efficiency in cyanobacteria as red light, however it is used much less effectively for photosynthesis and growth. Consequently, when selecting a spectral range, two parameters must be considered: (1) the preferentially absorbed wavelengths and (2) the utilization efficiency of these wavelengths by cells. These findings were supported by other research (Tan et al., 2020) where in co-cultures of green alga *Chlorella pyrenoidosa* and cyanobacterium *Microcystis aeruginosa*, the latter was more successful in red light and white light, while *C. pyrenoidosa* dominated under blue light. This was further supported by another study where red light promoted the growth of cyanobacteria, but blue and green light were more successful for green algae and diatoms, respectively (Xu et al., 2021). The impact of the light source color on the growth of different microalgae species in a natural microalgae community was studied by Xu et al. (Xu et al., 2021) showing that a change in illumination color induced a shift of the dominant species.

Reports across different microalgae taxonomic groups confirm that the preferred spectrum is species-specific, similar to other light related parameters such as light intensity (Ievina and Romagnoli, 2020). Even from the same taxonomic group not all algae prefer the same light spectrum. Two red algae *Galdieria sulphuraria* and *Porphyridium purpureum* exhibited different light spectrum requirements in a study by Baer et al. (Baer et al., 2016). Likewise, two green algae *Kirchneriella aperta* and *Brachiomonas submarina* showed different results when cultivated under the same light conditions (McGee et al., 2020). It has been noted that the optimal spectral composition for a single species is not transferable to other microalgae because microalgal species respond to various wavelengths differently. Therefore, light spectrum requirements must be addressed to a specific species of interest.

Can monochromatic light promote microalgae growth?

Although many reports have stated that certain microalgae grow best under specific monochromatic wavelength, it should be noted that the vast majority of studies assessing light spectrum effects on microalgae growth have used only single wavelength (such as red, blue or green) and compared with other monochromatic and white lights. Consequently, a mix of different narrow wavebands has not been evaluated and could therefore lead to misconceptions. Consequently, studies claiming a single wavelength as optimal should be viewed cautiously when a mix of different wavelengths has not been tested. For example, although the application of monochromatic blue light sustained good growth and biomass production of *Botryococcus braunii,* the maximum growth was obtained with three-color mixed LEDs (red-green-blue) (Okumura et al., 2014). Moreover, this growth rate was significantly higher than that of two-color mixed LEDs. Studies conducted using two or more wavelengths simultaneously, clearly indicate that a mix of different colors enhances microalgae production rate (Baer et al., 2016; Chen et al., 2015; Kim et al., 2019; Okumura et al., 2014; Ra et al., 2018; Zhang et al., 2017).

Most studies have focused on red or blue spectral regions, either using monochromatic red or blue light as a sole source of illumination or a combination of both. Illumination with red and blue light is known to be beneficial for the cultivation of several higher plant species (Wagner et al., 2016). The effect of different wavelengths has been studied most in green microalgae (Appendix Table 1).

When a mix of red and blue wavelengths was applied it was superior to either red or blue alone (Kim et al., 2013; Kuwahara et al., 2011; Ra et al., 2018). Ra et al. reported that mixed red and blue (1:1) LEDs resulted in higher biomass accumulation than monochromatic exposure to either red or blue, or fluorescent white light in four marine microalgae species from different evolutionary lineages (green, diatom, *Haptophyta*) (Ra et al., 2018). Likewise, a mix of red and blue resulted in significantly higher growth rates than red or blue alone in green alga *C. reinhardtii* (Kuwahara et al., 2011). Moreover, Kim and colleagues reported that a mix of red and blue light resulted in a 50% increase in *Scenedesmus* sp. production rate compared to a single wavelength, irrespective of ratio and was also higher than that under white light (Kim et al., 2013). Contrary, monochromatic red light yielded maximum biomass of red alga *Galdieria sulphuraria* indicating that most probably high content of phycocyanin is responsible for red wavelengths requirement (Baer et al., 2016). Undoubtedly, blue and red wavelengths are both essential for the photosynthesis of green microalgae and must be crucial to sustaining optimal growth and supporting metabolic functions. Whereas, for red algae and cyanobacteria a higher fraction of red light or monochromatic red illumination might be required depending on the pigment profile of the species.

Ratio of wavelengths applied also plays a significant role but is even less studied than a mix of wavelengths. It seems that high red to a lower percentage of blue light is required for optimal microalgae growth. The red+blue ratio of 0.7 was the most effective in higher plants resulting in appropriate plant development (Piovene et al., 2015). This suggests that similar red and blue ratios may also be suitable for green algae due to closely related plastids of microalgae and terrestrial plants in terms of structure, metabolism and biochemical composition. Indeed, supplementation of red light with the blue part of the spectrum (75/25%) resulted in enhanced biomass productivity and carotenogenesis in *Dunaliella salina* as opposed to monochromatic red light (Fu et al., 2013). Several studies have concluded that blue and red wavelengths are promising for *C. reinhardtii* artificial lighting (Wagner et al., 2016). It was demonstrated in a study by Baer (Baer et al., 2016) that although monochromatic lights alone could support *C. reinhardtii* growth, the addition of small fraction of blue and green wavelengths increased biomass productivity significantly. The best performance of *C. reinhardtii* was reached under red+blue+green illumination with a ratio of 80/10/10%, respectively.

Although blue light is generally required for normal plant development, several studies demonstrate that plant growth decreases by increasing the fraction of blue photons above 5 to 10% (Bugbee, 2016). Similar results were also reported in microalgae. de Mooij et al. (de Mooij et al., 2016) demonstrated that blue light in small quantities (3.5%) is essential for mass cultures of *Chlamydomonas reinhardtii* possibly due to the role of blue light as a trigger for metabolic regulatory mechanisms. Moreover, although red light plays a major role in red algae containing high concentration of phycoerythrin, the significant role of blue light fraction was demonstrated in red microalgae *Porphyridium purpureum*. Similar to green microalgae, a decrease in growth with an increasing fraction of blue light above 20% was observed (Baer et al., 2016).

Studies using a mixture of narrow wavelengths in different ratios are indeed scarce, but much needed to better understand the role of specific fractions of light in microalgal biomass production.

The role of weakly absorbed wavelengths

Although pigment content and composition in particular microalgae have an important role in the determination of the preferred light spectrum, it seems it is not the only prerequisite for maximum growth and biomass production. Although spectral matching seems promising, it cannot always explain the spectrum requirements within a taxonomic group sharing a similar pigment composition. This could be because wavelengths of the highest absorption peak of pigments do not always match the preferred wavelength for optimal growth. There are other

pigment functions besides photosynthesis, therefore photosynthetic efficiency is not the only prerequisite for a higher growth rate under certain wavelengths.

The controversial results reported in scientific literature confirm that the spectrum of applied light has a complex effect on microalgae growth. Moreover, the effect of a particular light spectrum is usually difficult to predict due to the complex interaction of many different responses (Hogewoning et al., 2010; Olle and Viršile, 2013). There is growing evidence that other wavelengths than red and blue profoundly affect microalgae cultivation (Baer et al., 2016; Kianianmomeni and Hallmann, 2014). Although previously thought to be "ineffective" or "not required" parts of the spectrum, they are now gaining attention and are frequently called "weakly absorbed wavelengths" usually referring to green and yellow regions of the visible light. It has been suggested that weakly absorbed wavelengths are not required for photosynthesis and should be avoided (Vadiveloo et al., 2015). However, several recent studies have confirmed that regulatory effects of weakly absorbed wavelengths are essential for optimal microalgae growth (Baer et al., 2016; Kianianmomeni and Hallmann, 2014; McGee et al., 2020; Schulze et al., 2014). The regulatory role of green and blue lights, stimulating other biochemical processes besides photosynthesis, was suggested by some authors (Baer et al., 2016; McGee et al., 2020). Moreover, although previously thought to be weakly absorbed, studies show that between 50 and 90% of green light is absorbed in higher plants, in comparison, absorption of blue and red light ranges from 80 to 95% (Terashima et al., 2009).

Although sole green light generally is insufficient for optimal plant and microalgae growth, combined with other parts of the PAR range such as red and blue, it has shown some important physiological effects in higher plants (Dutta Gupta, 2017; Olle and Viršile, 2013). Supplementation of red and blue LEDs with green light at 24% stimulated lettuce growth, while an increase to 51% caused a decrease in growth (Kim et al., 2004) suggesting a similar mode of action to blue light as discussed previously. It is most likely that similar mechanisms are in place in microalgae. Indeed, several studies reported the role of weakly absorbed wavelengths in photosynthesis as well as in metabolic functions that resulted in several physiological and morphological responses (Hultberg et al., 2014; Kim et al., 2013; McGee et al., 2020). The role of green wavelengths in maximizing microalgae biomass production was demonstrated by Baer and coworkers (Baer et al., 2016). A higher yield of *Chlamydomonas reinhardtii* was achieved when red and blue light was supplemented with 10% of green light, than red and blue (90/10%) lights alone indicating that green light can promote the growth of microalgae. Consequently, it was suggested that strongly absorbed lights, such as red and blue, should be supplemented with green light for higher productivity. The same was suggested by other authors (de Mooij et al., 2016; Mattos et al., 2015; Wagner et al., 2016).

The role of cultivation conditions

In natural ecosystems, microalgae are exposed to constantly changing environmental conditions, such as temperature, light intensity, as well as fluctuations in the light spectral composition and therefore have developed various adaptation mechanisms (Michel-Rodriguez et al., 2021). Subsequently, microalgae are able to adjust their pigment profile as as a response to changes in the ambient light color and is known as chromatic adaptation (Sanfilippo et al., 2019). This mechanism allows microalgae to optimize photosynthetic light harvesting according to the available light. Changes in light spectrum and intensity are detected by photoreceptors, which trigger signal transduction cascades that generate physiological responses (Kianianmomeni and Hallmann, 2014). Understanding how different microalgae respond to specific light spectrum conditions could improve cultivation conditions and maximize biomass production. Although most experiments are performed in a batch mode, a few studies report growth in continuous mode contributing greatly to the understanding of the adaptation

mechanisms. Over a 28-day period, Hintz et al. (Hintz et al., 2021) showed that species are able to acclimate to available wavelengths; however, changes in the available light spectrum affect primary production and microalgae community composition.

Various studies reported different optimal light spectral ranges for the same species. For example, maximum growth rate for *C. vulgaris* was reported under red (Chang et al., 2022; Ge et al., 2013; Kendirlioglu and Kadri Cetin, 2017; Yan et al., 2013), blue (Atta et al., 2013; Kang et al., 2015; Mohsenpour et al., 2012; Zhong et al., 2018), yellow (Barghbani et al., 2012; Hultberg et al., 2014) or white light (Blair et al., 2014; Khalili et al., 2015; Rendón, 2013) (See Supplementary Table S1). For *Chlamydomonas reinhardtii* a combination of red, green and blue (80/10/10) (Baer et al., 2016) and yellow and blue (de Mooij et al., 2016) have been suggested. Especially for green algae. The highest diversity of optimum light conditions is reported for *C. vulgaris* also being the most studied microalgae regarding light spectrum requirements.

The controversial results reported on the optimum spectrum for the same species clearly indicate the profound influence of other factors. Among them, cultivation conditions probably have the greatest influence on the selection of spectral composition. Indeed, the greatest interaction was shown with light intensity, illumination duration and culture density.

Illumination such as light-dark cycle, the length of photoperiod and light intensity affect the growth of microalgae and biomass composition. Among them light intensity was shown to have a major interaction with light spectral needs of microalgae. The spectral range preference depending on the applied light intensity was illustrated in several reports (de Mooij et al., 2016; Lee et al., 2019; McGee et al., 2020; Mouget et al., 2005; Paper et al., 2022; Schellenberger Costa et al., 2013). Moreover, it was suggested that each light intensity has an optimum wavelength (Fettah et al., 2022). This could at least partly explain the vast variety of different results reported as the applied light intensity varies widely in reported studies.

It was suggested by de Mooij to use the spectral composition of wavelengths that minimizes light absorption at high light conditions because microalgae photosynthesis is inefficient at high light intensities since microalgae absorb more light energy than can be used (de Mooij et al., 2016). It was shown that weakly absorbed yellow light is used at high efficiency in high light conditions (1500 µmol m^{-2} s^{1}) in high-density *Chlamydomonas reinhardtii* cultures (de Mooij et al., 2016). Moreover, due to higher energy content, blue light is more likely to induce photoinhibition at high light intensity therefore is not suggested under high irradiation conditions. Another study applying weakly absorbed green light supports this idea showing that under high light conditions (250 µmol m^{-2} s^{-1}) green LEDs outperformed red light for biomass productivity in *Chlorella vulgaris* (Mohsenpour et al., 2012). Consequently, weakly absorbed green and yellow wavelengths can achieve the highest productivity at high light intensity due to their lower specific absorption rates and therefore could be beneficial in high-intensity high-density green microalgae cultures. On the other hand, blue light might be beneficial in low light conditions as demonstrated by (Mouget et al., 2005),(Zhong et al., 2018) and (Lee et al., 2019). It has been argued that blue light is absorbed more efficiently and activates photosystem II more effectively than red light at low light intensities below the photosaturation limit, resulting in more efficient conversion of light into biomass (Zhong et al., 2018).

As mentioned previously, culture density greatly affects the preferred light spectrum. Wavelengths that are weakly absorbed may play a major role in photosynthesis in dense cultures where all photons are finally absorbed. Indeed, it was shown that green and yellow lights outperformed strongly absorbed wavelengths in dense *Scenedesmus bijuga* and *Chlamydomonas reihardtii* cultures due to deeper penetration and were able to efficiently drive photosynthesis (de Mooij et al., 2016; Mattos et al., 2015). Blue and red photons are used less

efficiently and are more likely to be dissipated as heat. Contrary, in low density cultures strongly absorbed blue light resulted in higher photosynthesis rate at low light intensity.

It can be concluded that cultivation conditions and a type of cultivation vessel by means of culture depth and density must be considered when selecting the light spectral range for a specific species.

Recently, a novel strategy for enhanced growth was proposed by alternating blue and red light at different growth stages due to the impact of these lights on the cell size as discussed in the section 2 (Kim et al., 2014). Cultures were irradiated with blue light first to increase the average cell size followed by inducing higher division rate by exposure to red light resulting in increase in cell number and the overall productivity. By application of this strategy, biomass of *C. vulgaris* was increased by 20% compared to the control under white light. The proposed strategy seems promising however, more studies are required.

Other effects of the tailored spectral composition

It must be taken into account that the spectral composition significantly affects not only the growth rate of microalgae but also metabolic processes in cells changing the biochemical composition. Particular wavelengths of light can induce or suppress the formation of specific compounds in cells, such as pigments (Baidya et al., 2021; Coward et al., 2016), lipids (Atta et al., 2013), proteins (Gatamaneni Loganathan et al., 2020) and polysaccharides (Markou and Nerantzis, 2013). Therefore, it is recommended to investigate the distinct light spectral composition required for the specific algae depending on the intended application of microalgae biomass.

4. Major findings, current challenges, and future directions

Reports on the effects of the light spectrum have increased significantly in recent years due to the advent of LED technology. LED lights are especially suitable for tailored lighting for microalgae cultivation due to their ability to emit very narrow bands of wavelengths producing a single-color illumination.

This study has underlined the importance of spectral quality in microalgae cultivation for maximum biomass productivity. Various studies show that microalgae growth rate could be significantly enhanced by customizing the incident light spectrum transmitted to algal cultures. Ensuring an efficient utilization of light offers a powerful tool for superior biomass production. On the other hand, application of incorrect wavelengths can lead to suppressed microalgae growth and reduce biomass yield. The effect of narrow wavelengths on microalgae is still poorly understood despite a growing number of studies addressing this issue lately. However, it is clear that light spectral quality has a profound effect on microalgae growth, biochemistry and biomass production.

The effect of single monochromatic lights has been extensively studied in various microalgae species however, contrary results are often reported showing a complicated nature of light spectrum. The application of blue light generally shows improved growth of many microalgae species, most probably due to the direct impact on photosynthetic efficiency. Blue and red irradiation is frequently reported as the most suitable light driving photosynthesis due to the corresponding absorption maximum of major light-harvesting pigments. Although many reports state that certain single wavelengths are optimal for particular microalgae species, a balanced mix of various wavelengths is most likely required for optimal growth and biomass production. A combination of different single wavelengths is rarely studied leading to lack of

data, also pointed out by other authors (Ramanna et al., 2017; Vadiveloo et al., 2015). Consequently, more research on the effect of a combination of various wavelengths at different ratios is highly required to define the optimum lighting conditions and take a full advantage of the powerful spectral adjustment strategy.

Different response of microalgae to light spectrum demonstrates a species–specific behavior, therefore, light requirements must be studied for the species of interest. A pigment profile of species provides information on the light requirements of microalgae within a taxonomic group. Generally, red algae can utilize green light better than green algae due to the presence of phycoerythrin. The same is true for cyanobacteria. However, in species of red algae where phycocyanin is the major phycobiliprotein, a larger fraction of red light is needed. Cyanobacteria is also known to contain high amounts of phycocyanin, therefore red light was shown to be beneficial to cyanobacteria cultivation. However, blue light is used less efficiently by cyanobacteria than by green algae since many cyanobacteria have lost Chl *b*. Generally, green algae can utilize blue light more efficiently than other algae. On the other hand, green algae have a reduced ability to utilize yellow and green wavelengths due to a lack of phycobiliproteins covering the absorption in green wavelengths.

Although strongly absorbed blue and red lights are most likely required for normal functioning of photosynthetic apparatus of most microalgae, our study highlighted the importance of other regions of the spectrum in microalgae culturing. Although previously thought inefficient and not required, most recent studies show that weakly absorbed wavelengths, such as green and yellow, are utilized by photosynthetic organisms and are essential for microalgae growth. Consequently, it has been suggested that strongly absorbed lights, such as red and blue, should be supplemented with green light for higher productivity, as shown in some reports. Moreover, a higher proportion of green–yellow wavelengths might be especially beneficial for high-density microalgae cultures and microalgae grown in photobioreactors with a long light path because of more efficient absorption of weakly absorbed wavelengths.

Contradictory results on the preferred spectral range, especially for green microalgae, are commonly reported indicating that the light spectrum has a much more complex impact on physiology and morphology in microalgae than other light parameters such as light intensity or photoperiod. It is true that other factors affecting microalgae growth, such as light intensity and photoperiod, greatly affect the preferred light spectrum and are most probably responsible for the observed inconsistencies. Light intensity is closely interacting with spectral quality and has a major effect on the preferred spectral range therefore should be adjusted accordingly. It was shown that weakly absorbed green and yellow light are especially useful at high light conditions, whereas strongly absorbed red and blue wavelengths will be more suited for low light conditions. We can conclude that several aspects work together in determining the spectrum requirements for the species: pigment composition and quantity within cells, environmental factors such as light intensity, temperature, nutrients and experimental setup such as cultivation vessel, culture depth, mixing and culture density are the main contributors. Results of the current study show that tailor-made solution of spectral range setup is required for distinct microalgal species to reach maximum growth rate and biomass productivity.

Although the number of reports on spectral quality has increased in the last decade, studies using a combination of various narrow wavelengths are very limited however highly required to contribute to the understanding of interaction of different wavelengths and their

role in achieving maximum biomass yield. More studies using a combination of various wavelengths are urgently needed to fully understand the complex effect of various wavelengths on microalgae biomass production. Moreover, the correct ratio of various wavelengths is of high importance as shown in a few existing studies. Given the high potential of wavelength manipulation for microalgae growth enhancement, it becomes of utmost importance to the economic viability of microalgae mass culturing.

5. Conclusions

The application of tailored spectral range enhances microalgae growth compared to white light offering a powerful tool for boosting biomass production. This study highlights the underlying factors of the preferred spectrum for microalgae, including pigment content and the role of cultivation conditions. We demonstrate the major role of weakly absorbed wavelengths in maximum biomass yield. Although various reports state certain single wavelengths as optimal for particular microalgae, a balanced mix of various wavelengths is most likely required for optimal growth of most microalgae. A combination of various narrow wavelengths requires further study to define optimum spectral conditions.

Tables

Table 1. Major pigments in microalgae and range of absorption (Carvalho et al. 2011, Guiheneuf and Stengel 2015, Glazer 1994, Krinsky 2002, Allakhverdiev et al. 2016).

Pigment group	Pigment	Pigment color	Absorption range nm	Corresponding absorption color
Chlorophylls	Chlorophyll a	Blue green	380 - 450, 600 – 670 (663, 430 peak)	Blue, red
	Chlorophyll b	Brilliant green	410 – 480, 600 – 685 (642, 453 peak)	Blue, red
	Chlorophyll c	Yellow-green	450	Blue
	Chlorophyll d	Brilliant/forest green	700-750	Far-red
	Chlorophyll f	Emerald green	700-750	Far-red
Carotenoids	β-carotene	Red-orange	400 – 500 (454 peak)	Blue, blue-green
	Xanthophylls	Yellow	400-540	Violet and blue-green
Phycobiliproteins	Phycocyanin	Blue	600 – 640	Orange-red
	Phycoerythrin	Red	480 - 570	Blue-green, yellow
	Allophycocyanin	Blue	620 – 660	Orange-red

Table 2. Pigment content in some microalgae groups (Barkia et al. 2019, Tomaselli Chapter 1, in Richmond 2004., Guiheneuf and Stengel 2015, Kuczynska et al. 2015, The algae world 1st ed. 2015, Allakhverdiev et al. 2016, Schulze tal. 2014, Christaki et al. 2015, Chapter 14 in Handbook of marine microalgae, Averina et al. Chapter)

Algae group	Green algae *Chlorophyta*	Cyanobacteria *Cyanophyta*	Red algae *Rhodophyta*	Golden algae and diatoms *Chrysophyta*
Pigments they contain	Chl *a* Chl *b*	Chl *a* Chl *d* Chl *f*	Chl *a* Chl *c* Chl *d*	Chl *a* Chl *c*
	Absent	Phycobiliproteins (phycocyanin, phycoerythrin)	Phycobiliproteins (phycoerythrin, phycocyanin)	Absent
	Carotenes	Carotenes	Carotenes	Carotenes
	Xanthophylls (astaxanthin, violaxanthin, neoxanthin, loroxanthin, canthaxanthin, lutein, zeaxanthin)	Xanthophylls (Zeaxanthin)	Xanthophylls (Zeaxanthin)	Xanthophylls (Fucoxanthin, zeaxanthin))
Preferred spectrum	Blue and red	Red, yellow, and green light	Blue, Green	Blue and green

based on pigment profile		Blue light less efficiently		

Figures

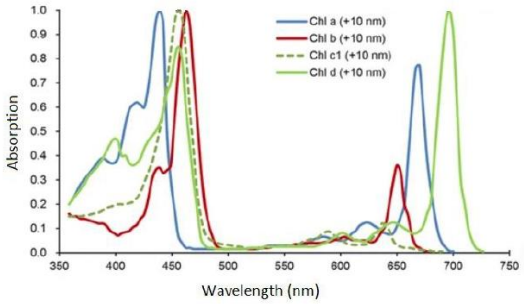

Figure 1. Absorption of major chlorophylls. (Kume et al., 2018)

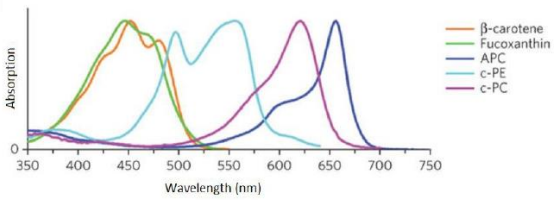

Figure 2. Absorption spectra of major phycobilins and carotenoids. APC - allophycocyanin; c-PC - phycoyanin; c-PE - phycoerythrin. (Croce and Van Amerongen, 2014)

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