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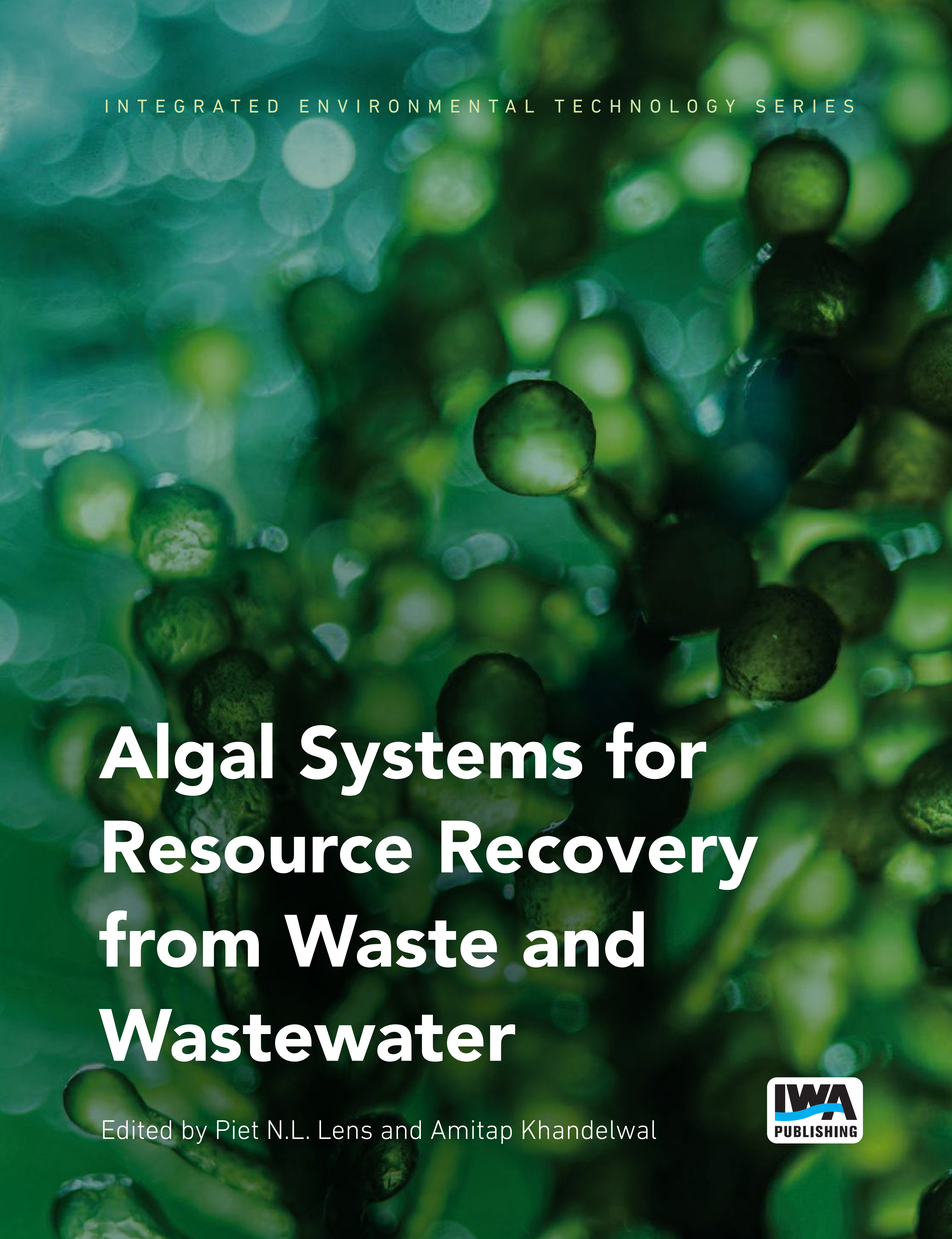

INTEGRATED ENVIRONMENTAL TECHNOLOGY SERIES

Algal Systems for Resource Recovery from Waste and Wastewater

Edited by Piet N.L. Lens and Amitap Khandelwal

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Preface

In the past few decades, our planet has witnessed an unprecedented surge in population, carbon emissions, and the demand for essential resources, particularly energy and water. This exponential growth has come at a cost: a staggering increase in waste and wastewater, presenting formidable challenges to our environment and sustainability. Faced with this urgent dilemma, the imperative to develop innovative technologies for resource recovery has never been more critical. Amidst this challenge, algae, tiny yet extraordinary photosynthetic organisms, have emerged as potent microbiota in the quest for environmental solutions. In this context, this book *Algal Systems for Resource Recovery from Waste and Wastewater* testifies to the pivotal role algae can play in addressing some of the world's most pressing issues.

In recent years, algae-based wastewater treatment has made significant strides. Rigorous research validated the integration of specific algae strains into existing treatment plants, elevating their efficiency. Cutting-edge technologies, such as advanced photobioreactors and real-time monitoring systems, empowered precise control and seamless automation. Symbiotic systems and the dual-purpose utilization of harvested algae for biofuel production bolstered economic viability. Scalable implementations and widespread commercialization swiftly followed successful pilot programs. Ongoing cutting-edge research continues to sharpen the focus on efficiency enhancements, new strain exploration, and integration of other modern technologies such as anaerobic digestion and bioelectrochemical systems, promising an unwavering and sustainable technical solution to the pressing issue of wastewater pollution.

Within this book, we embark on a profound exploration of various algae-based systems, unveiling their transformative potential and transition from laboratory trials to real world solutions. Wastewaters, rich in resources like phosphorus, demand efficient nutrient removal for the development of a circular bioeconomy. Algae-based treatment systems achieve both wastewater clean-up and valuable biomass production. Algae have a unique ability to absorb pollutants or transform them into sustainable bioproducts. Their capacity to convert wastewater into valuable biomass and value-added commodities opens doors to a multitude of applications, ranging from the production of sustainable biofuels to the creation of nutrient-rich animal feed and fertilizers. This book chronicles the remarkable journeys of scientists and researchers from around the globe to unlock the potential of these tiny organisms. It presents the current status, major challenges and recent scientific innovations in algae-based technologies for waste remediation and nutrient recovery.

Authored by experts and researchers at the forefront of algal biology, bioprocess engineering, and environmental science, this comprehensive volume aims to provide an authoritative resource for academics, researchers, industry professionals, and policymakers. Its pages will empower readers with knowledge about the latest advancements, challenges, and breakthroughs in the use of algae for wastewater treatment and energy recovery. Each contributed chapter is presented on a stand-alone basis, so that the reader will find it helpful to consider only the theme of each chapter. There are nevertheless many connections between what may at first seem to be quite different topics. As in all the books of the *Integrated Environmental Technology* series, one of our purposes was to draw out and emphasize these interdisciplinary links. For this reason, a comprehensive index is included to facilitate cross-referencing. We hope that the work described in this book will inspire those working in the field and will encourage those who are beginning to investigate it.

We wish to thank all contributors to this book for their valuable contributions by sharing their expertise in the various chapters. We also thank all past and present co-workers as well as all collaborators who joined in unravelling different areas of the application of algae in environmental technology as described in this book, especially those at National University Ireland Galway and UNESCO-IHE. We would also like to thank all the reviewers who put a lot of effort into improving the quality of this book. In addition, the national and international granting agencies who supported our work on various aspects of algal based pollutant removal and resource recovery over the years are gratefully acknowledged, in particular the Science Foundation Ireland (SFI), who financially supported the open access publication of this book through the SFI Research Professorship Programme *Innovative Energy Technologies for Biofuels, Bioenergy and a Sustainable Irish Bioeconomy* (IETSBIO^3; grant number 15/RP/2763) and the Department of Foreign Affairs (DFA) under the SDG Challenge project *Floating Treatment Wetland* (grant number SFI/21/FIP/SDG/9933). We are also grateful to the editorial team of IWA Publishing, in particular Mark Hammond, Andrew Peart and Katharine Allenby for their help and editorial support in realizing this book.

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Part 1

Process Fundamentals

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Chapter 1

Algal systems for resource recovery from waste and wastewater

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ABSTRACT

This chapter provides an overview of the book. The introduction highlights the need for algal-based technologies in waste management and resource recovery in order to boost the circular bioeconomy globally. The book is divided into four parts, consisting of twelve chapters in total, which provide a detailed description of topics ranging from process fundamentals to up-to-date information on various modern algal-based technologies for waste remediation, nutrient recovery, and simultaneous energy generation. The book is suitable for students, research professionals and policymakers who are working in the domain of environmental engineering/sciences, wastewater treatment and renewable energy.

As a consequence of the swift proliferation of the global economy and population, the availability of water resources for direct human consumption has become insufficient. Forecasts indicate a projected 40% global water deficit by 2030, which gives rise to critical challenges for both society and economic advancement (Kandasamy *et al.*, 2023). This scarcity is primarily attributed to escalating water demands, the contamination of existing water supplies, and a lack of efficient technologies for water recycling. As a result, the imperative of water remediation is bound to assume a central role on the international stage, demanding urgent attention and action.

Historically, wastewater treatment arising from diverse industries has predominantly relied on the implementation of chemical processes such as flocculation, disinfection, oxidation, and neutralization and physical techniques, including grit chamber, floatation, and screening (Chojnacka *et al.*, 2020; Kurniawan *et al.*, 2022). Despite their widespread use, these chemical and physical treatment methodologies remain financially burdensome and generate substantial volumes of slurry or sludge, thereby requiring supplementary treatment steps. Moreover, the wastewater treatment processes are energetically expensive and demand trained staff for the operation of treatment facilities, which are associated with considerable capital costs for infrastructure development (Kandasamy *et al.*, 2023).

Consequently, scientists and researchers are currently exploring alternative approaches for wastewater treatment and nutrient recovery, centering on the utilization of microalgae. These innovative methods hold the promise of providing an environmentally friendly and sustainable means of treating wastewater, potentially enabling the recovery of nutrients as high as 95% (Moradi & Saidi, 2022). Microalgae growing in wastewater can facilitate the production of biomass, which contains valuable components such as carbohydrates, proteins, lipids, and other valuable biomolecules that can be utilized in the production of third-generation biofuels (Shearian Sattari *et al*., 2022). Several modes such as open ponds, photobioreactors, and advanced culture systems, are being considered to foster the cultivation of microalgae, offering diverse and promising pathways for their effective implementation (Kandasamy *et al*., 2023; Khandelwal *et al*., 2023).

The successful cultivation of microalgae in diverse industrial wastewaters, along with the efficacy of the effluent treatment processes, is contingent upon achieving an optimal nutrient load and composition within the wastewater. In instances where the nitrogen:phosphorus (N:P) ratios in the water are reduced, certain strains of *Cladophora* have demonstrated enhanced efficiency in removing nutrients from the environment (Sandani *et al*., 2020). On the other hand, algal families characterized by higher N:P ratios, such as *Pseudanabaena*, exhibit more effective nutrient removal capabilities (Kandasamy *et al*., 2023). Nonetheless, a comprehensive study involving filamentous benthic algae has indicated that for the specific context of municipal wastewater nutrient removal, the optimal N:P ratios should fall within the range of 5:1 to 15:1, 7:1 to 10:1, and 7:1 to 20:1 for *Cladophora, Klebsormidium*, and *Pseudanabaena*, respectively (Valchev & Ribarova, 2022). Generally, the various strains of algae do not respond similarly to different N:P ratios, leading to varying impact on their nutrient removal capabilities.

The shift toward a circular bioeconomy, which emphasizes resource diversification, has provided the impetus for transforming conventional wastewater treatment processes capable of handling various waste streams. The transition has gained momentum, and the increasing enthusiasm can be credited to the dynamic and evolving nature of microalgal-based wastewater treatment solutions. Overcoming critical barriers related to nutrient assimilation and achieving increased microalgae growth rates have rendered microalgae-based wastewater treatment a compelling and powerful alternative to traditional methods (Khan *et al*., 2022). In this context, this book explores the potential applications of algal biomass in wastewater remediation and bioenergy production. The book is divided into the following four parts.

1.1 PROCESS FUNDAMENTALS

This chapter discusses the cultivation of microalgae in wastewater, their metabolic modelling to analyze the growth rate (Chapter 2) and their interaction with bacteria (Chapter 3). To advance sustainable wastewater treatment technology, a comprehensive investigation is proposed, focusing on the symbiotic bacterio-algal relationship (Chapter 3) and the role of quorum sensing signal molecules in shaping the integrated wastewater treatment solution involving both algae and bacterial processes. This segment aims to lay the groundwork for refining algae–bacteria based wastewater treatment methods through various approaches.

These findings are expected to offer valuable insights for promoting sustainable economic and environmental development. Additionally, the utilization of synergistic bacterial–algal wastewater treatment technologies has the potential to contribute toward lowering the carbon emissions (Hena *et al*., 2021). By combining these approaches, the research endeavors to pave the way for more effective and environment-friendly wastewater treatment practices, with the ultimate goal of fostering sustainable development and mitigating environmental impacts.

Furthermore, the basics of macroalgae-based biorefinery are also discussed in detail (Chapter 4), which makes the book suitable for every phycologist. This part majorly focuses on the following three aspects: (1) metabolic modelling of algal growth using waste as substrate, (2) synergistic approach of algae–bacteria for efficient wastewater treatment and selection of key microalgae and bacterial species in wastewater treatment systems, and (3) use of of macroalgae to produce fertilizers, feed (additives), and other value-added products.

1.2 ALGAL-BASED WASTEWATER TREATMENT

Although the use of microalgae for wastewater treatment was proposed in the last century, the technology was not sufficiently efficient and robust to be applied at a commercial scale. Only recent advances in the knowledge of biological systems, the engineering of the reactors and the harvesting and processing of the produced biomass allow the development of the first industrial demonstrations (Acién *et al*., 2016). Facilities of several hectares are already in operation demonstrating the feasibility of this technology (Nguyen *et al*., 2022). However, challenges remain for the further improvement and enlargement of these systems. They are related to (a) the improvement of knowledge and management of the biological system, (b) the development of adequate strategies for the allocation and implementation of large-scale facilities, (c) the definition of optimal operational conditions, including the development of non-assisted systems capable to operate under variable environmental conditions, and (d) the development of adequate routes for biomass valorization (Acién *et al*., 2016).

Large efforts which are being devoted to solving these challenges and thus to making this technology reliable for industrial applications, are detailed in Chapter 5. Furthermore, possibilities and challenges in coculturing methanotrophs with microalgae for wastewater treatment are discussed in Chapter 6. Part 2 summarizes the status, major challenges, and potential contribution of microalgae-related wastewater treatment processes.

1.3 VALORIZATION OF ALGAL BIOMASS BY INTEGRATING WITH DIFFERENT TECHNOLOGIES

Microalgal systems play a crucial role in shifting the perspective of wastewater from being seen as disposable waste to being recognized as a valuable resource capable of yielding new value-added products. This shift toward a more sustainable approach brings together significant environmental and economic potential, endorsing the principles of a circular economy (Amaro *et al*., 2023). Through the production of bioenergy and bioproducts, these systems contribute to the energy–environment nexus, paving the way for a sustainable closed-loop economy (Bele *et al*., 2023).

Given the pressing challenges of global water scarcity and the escalating costs associated with wastewater treatment, numerous research works and government projects have emerged, exploring the application of microalgal systems for wastewater treatment while concurrently extracting valuable biomass resources. Specifically, managing manure poses significant difficulties and expenses for livestock and poultry operations, particularly in cold climate regions (Bele *et al*., 2023). Addressing these challenges necessitates adopting sustainable approaches to nutrient management, reuse, and recycling, which can not only generate additional income for farmers but also enhance agricultural environmental sustainability.

The integration of green innovations, such as algae cultivation, bioelectrochemical systems (BES), and anaerobic digestion emerges as a key strategy to recover nutrients, complete utilization of manure, and make the overall process more sustainable. Part 3 aims to comprehensively explore the potential of integrating microalgae into the growing biogas and wastewater industry along with the potential of BES for simultaneous waste remediation, algae cultivation, and power generation. It seeks to identify opportunities and challenges inherent in this approach and reviews the prospective bioproducts, such as bioelectricity arising from BES (Chapter 7), biogas (Chapter 8), and bioethanol (Chapter 9). Such integration represents a transformative approach that harnesses the vast untapped potential of waste, aligning with the principles of the circular economy and advancing the sustainable development goals.

1.4 ALGAL BIOTECHNOLOGY

The microalgae biorefinery presents a promising and sustainable solution for producing biofuels and a diverse array of bulk chemical products. Extensive efforts have been dedicated to utilizing microalgae biomass in biorefineries to advance sustainable development, primarily due to their abundant bioactive constituents (Okeke *et al*., 2022). Part 4 provides a comprehensive review of potential strategies aimed at enhancing microalgae biorefinery obtaining high-value-added renewable products (Chapters 10 and 11) and optimizing the transformation of microalgae-based technologies into economically viable products (Chapter 12). The focus is on ensuring the long-term viability of the processes, taking into account both economic feasibility and environmental considerations.

Moreover, ongoing research explores the integration of microalgae biorefineries with other eco-friendly alternatives, such as microalgae-based bioplastics, which opens up new possibilities for synergistic applications (Okeke *et al*., 2022). The microalgae biorefining process holds the promise of becoming a key element of green technology, facilitating the biosynthesis of a broad range of valuable biofuels and biochemical products, further reinforcing the outlook for sustainable and environmentally friendly solutions to recover resources from waste and wastewater.

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Chapter 2

Metabolic modelling of microalgae for wastewater treatment

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ABSTRACT

Wastewater treatment using microalgae presents a promising approach for sustainable and efficient removal of pollutants. However, the complexity of metabolic networks involved in microalgae metabolism poses challenges for computational analysis. This chapter explores network reduction methods, specifically focusing on the application of the DRUM (dynamic reduction of unbalanced metabolism) framework, to streamline the modelling of microalgae-based wastewater treatment systems. This chapter describes the general core metabolism of microalgae, reviews methods of metabolic network reduction, and ends it with the application of a case study. The DRUM framework divides the complete metabolic network into subnetworks where the quasi-steady-state assumption (QSSA) holds, reducing the number of state variables and simplifying the kinetic modelling. By calculating the elementary flux modes (EFMs) for each subnetwork, macroscopic reactions are derived, representing the collective behaviour of internal reactions within the subnetworks. To demonstrate the effectiveness of the DRUM framework, a case study based on *Chlorella* sp. microalgae is presented. The study focuses on treating volatile fatty acid waste, a common byproduct of dark fermentation. The reduced metabolic model, obtained using the DRUM framework, accurately captures the dynamics of microalgae growth and medium concentration. This chapter underscores the significance of network reduction methods in optimizing microalgae-based wastewater treatment systems. These reduction methods pave the way for further advancements in the development and optimization of microalgae-based wastewater treatment technologies.

Keywords: microalgae, wastewater, metabolism, modelling, model reduction.

2.1 INTRODUCTION

Microalgae are unicellular organisms capable of growing autotrophically with solar energy through photosynthesis. Some species can also grow heterotrophically by absorbing a source of organic carbon compounds, such as glucose and acetate. They have emerged as a promising solution for wastewater treatment due to their ability to remove pollutants and nutrients while simultaneously producing valuable biomass. Microalgae are also very important organisms in the carbon cycle of the planet, being responsible for 40% of global fixation of carbon. They can be used to produce a variety of products such as proteins, vitamins, cosmetics, feedstock, and food (Barsanti & Gualtieri 2018).

Despite advances made in microalgae research during the past decades, the production of microalgae at industrial scale is still limited. Different bottlenecks explain the limited use of these processes in comparison with their potential, in particular, the economic and environmental profitability must still be improved. In this context, metabolic modeling plays a crucial role in understanding and improving the efficiency of microalgae-based wastewater treatment systems.

The use of mixotrophic growth, provided that the substrate is cheap and with a low environmental impact, is a promising way to increase productivity. This is of key interest if the substrate is a waste, and the process is used at the same time to produce biomass and to treat wastewater. By analyzing the metabolic pathways involved in the utilization of waste substrates, metabolic models can guide the optimization of cultivation conditions and nutrient supplementation strategies to enhance microalgae productivity and pollutant removal efficiency. This approach not only addresses the economic viability of microalgae-based wastewater treatment, but also offers a sustainable solution for waste management (Castillo *et al.*, 2021).

The use of metabolic networks provides a solid foundation to model microalgae growth in complex environments, such as wastewater. However, because of the complexity of these metabolic networks, in particular genome-scale ones, the utilization of methods to reduce the size of metabolic models proves to be essential for optimizing the efficiency of microalgae-based wastewater treatment. By employing metabolic models, it is possible to gain insights into the utilization of waste substrates, guide cultivation conditions, and design nutrient supplementation strategies to enhance microalgae productivity and pollutant removal.

Consideration of cultivation methods and bioreactor design is also essential, even in the case of metabolic models, especially regarding the modeling of the effects of light on the growth. The two most widespread processes for producing microalgae are closed photobioreactors and open raceways (Schade and Meier 2019). Photobioreactors can lead to a higher production output with better resistance to biological contaminants, but the energy input necessary for mixing and cooling strongly penalizes the economic and environmental balances (Tan *et al.*, 2018). The more rustic raceways are a simpler and cheaper way for producing microalgae outdoors. They need less energy input and the functional design is simpler. The drawback is the higher contamination in the culture by grazers, bacteria, viruses, or even other competitive microalgae species (Williams *et al.*, 2010, Mata *et al.*, 2010). Both of these cultivation methods may operate in batch, continuous, or even fed-batch conditions.

In this chapter, we focus on the theoretical approach, using modeling in order to optimize the system's efficiency. Such approaches have proven to be efficient in many different biotechnological applications. In the microalgae field, they are probably even more important to rationally manage the complexity of these nonlinear systems, which are exposed to weather fluctuations, affecting light and temperature. The development of numerical models is thus a prerequisite for understanding and managing these dynamical systems, involving several time scales, and permanently submitted to different perturbations. There is a need to bridge the gap between the detailed metabolic knowledge in the cell, and the necessity for control to keep a limited model complexity. Reducing metabolic models is difficult in a framework of permanent environmental fluctuations, maintaining the cell far from the balanced growth conditions which are generally the rule in metabolic modeling. Going from a metabolic model to a mechanistic model that can support process control is, therefore, a challenging objective. This chapter mentions different approaches to meet this goal.

2.2 MAIN METABOLIC PATHWAYS

In this section, we aim to elucidate the key metabolic pathways associated with microalgae, particularly focusing on relevant literature. The fundamental significance of microalgae lies in their ability to undergo growth through the utilization of photosynthetic energy. This process involves the absorption of carbon dioxide (CO_2) via the photosynthetic pathway, whereby energy derived from photons, whether from artificial or solar light sources, is captured.

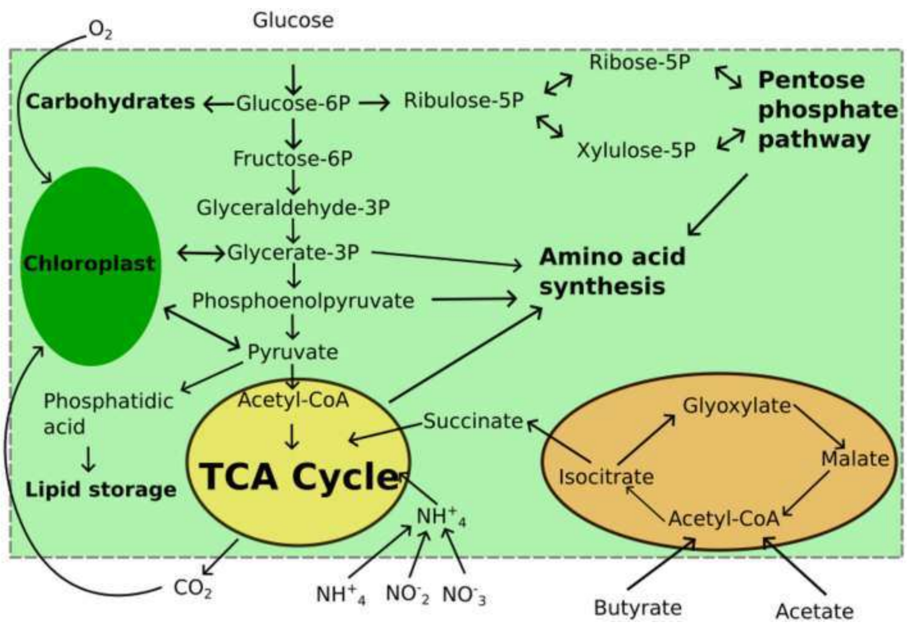

Figure 2.1 Main metabolic pathways of microalgae, demonstrating growth using as carbon substrates: butyrate, acetate, glucose, and CO_2.

A brief summary of the metabolism is important to understand model results and the soundness of these numerical results. Furthermore, a better comprehension also enables modelers to grasp what can be possibly achieved with the model and formulate subsequent steps accordingly. Figures 2.1 and 2.2 provide a simplified representation of the principal metabolic reactions. The eukaryotic nature of microalgae, coupled with their photosynthetic characteristics, leads to a complex compartmentalization of metabolic processes. For example, there is specific production of coenzymes, such as NADPH in different organelles of the cell. This compartmentalization is also dependent on the species.

Not only the knowledge of the reactions taking place is necessary to understand the metabolism, but also computational tools such as flux balance analysis (FBA) can help us comprehend the overall functionality of the metabolism. Here, we will briefly mention the main pathways and their main reactions, although details of how metabolic fluxes operate are species-dependent. While we will briefly touch upon the main pathways and their primary reactions, the specific operation of metabolic reactions is contingent upon the particular microalgal species under consideration.

2.2.1 Photosynthesis

Microalgae exhibiting autotrophic or mixotrophic growth strategies employ carbon dioxide (CO_2) as an inorganic carbon source, while cellular energy is derived from light. Photosynthesis occurs within the chloroplast and encompasses a combination of light-dependent reactions and the Calvin cycle.

In the thylakoid lumen, energy derived from photons is utilized for the synthesis of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), with oxygen (O_2) being produced as a byproduct. Within the Calvin cycle, CO_2 undergoes a reaction with ribulose biphosphate, facilitated by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), resulting in the production of two molecules of glyceraldehyde 3-phosphate (GAP). Subsequently, GAP is transported to the cytosol, where it integrates into other metabolic pathways, such as its reduction to glucose 6-phosphate (glucose 6-P) for carbohydrate production or its oxidation within the tricarboxylic acid (TCA) cycle. Notably, the Calvin cycle remains inactive in the absence of light (Tibocha-Bonilla *et al.*, 2018).

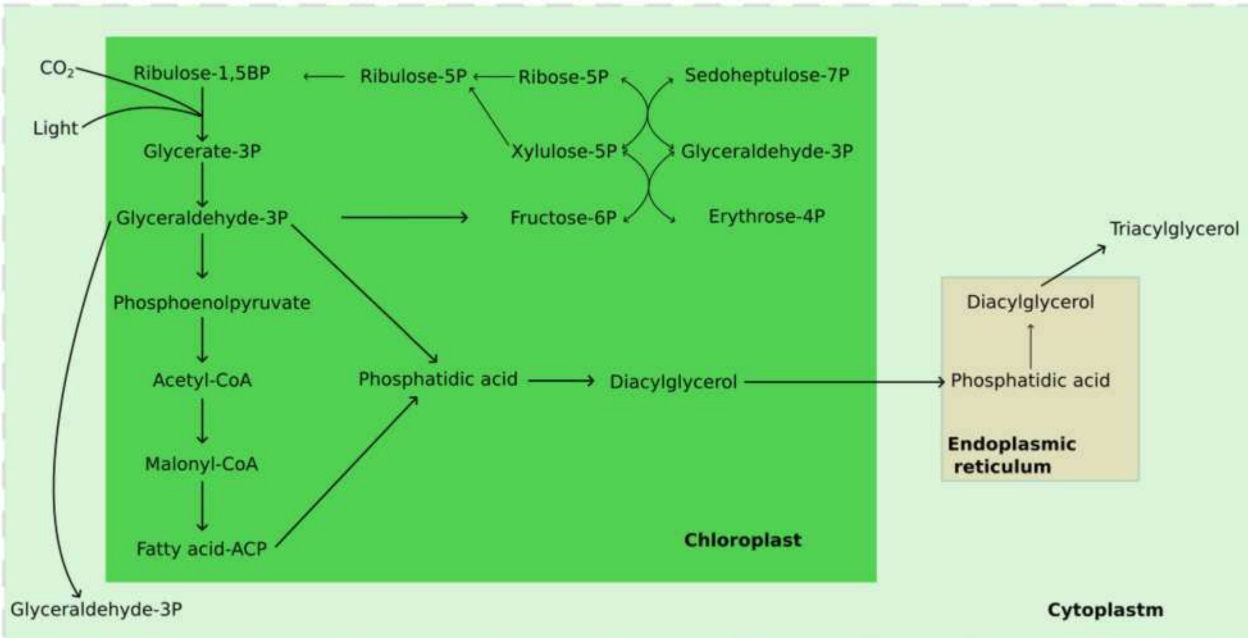

Figure 2.2 Metabolic reactions related to photosynthesis (Calvin cycle and light-dependent reactions) taking place in the chloroplast. Also, simplified pathway for the synthesis of lipids, takes place in the chloroplast and cytoplasm.

2.2.2 Glycolysis and pentose phosphate pathway

Glycolysis, a fundamental metabolic pathway in living organisms, occurs within the cytosol and plays an essential role in energy metabolism. In the case of heterotrophic growth external glucose is absorbed by phosphorylation producing glucose 6-P. Glucose 6-P can be utilized for the synthesis of carbohydrates or directed toward the pentose phosphate pathway (PPP), a parallel metabolic pathway to glycolysis, where it undergoes further transformations leading to the production of pentoses (5-carbon molecules). Most importantly, these pentoses are used for the synthesis of nucleic acid, but they are also used for the synthesis of many other biomass precursors. During the oxidative phase, the PPP produces NADPH.

2.2.3 Tricarboxylic acid cycle

The tricarboxylic acid (TCA) cycle, also known as the citric acid cycle or Krebs cycle, plays a crucial role in energy production and the synthesis of biosynthetic precursors. In the context of autotrophic or mixotrophic growth, the active reactions within the TCA cycle are primarily focused on the generation of biosynthetic precursors, whereas during heterotrophic growth, the emphasis shifts in the direction of energy production. The TCA cycle is essential for its anaplerotic reactions, which replenish intermediary metabolites. Therefore, it is considered a central axis in the core metabolism. Typically, at the entrance of the cycle there is acetyl-CoA that reacts with oxaloacetate-producing citrate. This metabolic pathway leads to the production of NADH, $FADH_2$, and GTP, thereby increasing cellular energy levels. However, there is carbon loss via the excretion of CO_2.

It is worth noting that not all microalgae possess the complete TCA cycle, as the presence of specific enzymes is dependent on the species. Furthermore, depending on certain environmental conditions, bypass variations may also take place. In those cases, for example, we can have pyruvate at the entrance of the cycle, producing oxaloacetate regulated by the enzyme phosphoenol pyruvate carboxylase (Fachet *et al.*, 2020).

2.2.4 Glyoxylate shunt

During the process of heterotrophic growth, microalgae have the capability to utilize acetate and, to a lesser extent, butyrate – because of its lower affinity – as carbon sources through the operation of the glyoxylate shunt. This metabolic pathway occurs within the glyoxysome, a specialized cellular organelle. Here, acetate and butyrate are converted first to acetyl-CoA, although additional enzymatic steps are required for the metabolism of butyrate. Subsequently, acetyl-CoA is used for the synthesis of succinate. Succinate can, then, be integrated into the core metabolism via the TCA cycle. By incorporating succinate into the TCA cycle, microalgae can further utilize the carbon and energy obtained from acetate and butyrate during heterotrophic growth.

2.2.5 Lipid biosynthesis

The synthesis of lipids has been an important topic in microalgae research for its potential use for the production of biofuels. There are three main classes of lipids: triacylglycerol (TAG), phospholipids, and glycolipids. Phospholipids and glycolipids have a higher polarity and form a stable bilayer, as a result, they are the major components of cell membranes and in stable growth conditions they form the majority of a cell's content of lipids. On the other hand, under stress conditions, the majority of cell's lipids are made of TAGs, since they serve as energy reserves. TAGs have a better yield for the production of biofuels and, therefore, are the focus of this section.

There are three main steps in the synthesis of lipids: (1) the production of malonyl-CoA from acetyl-CoA catalyzed by the enzyme ACCase, (2) the elongation of the acyl chain by the fatty acids synthase, both steps occur in the chloroplast, and (c) the formation of TAGs in the endoplasmatic reticulum. This process is illustrated in Figure 2.2. Following TAG synthesis, these lipids are stored in the form of lipid droplets. Furthermore, different specific pathways exist for the production of TAGs, for example acyl-CoA-dependent and -independent pathways. These pathways contribute to the diversification of lipid biosynthesis strategies (Chen & Wang, 2021; Huerlimann & Heimann, 2013).

2.3 GENOME-SCALE METABOLIC MODELS

Genome-scale metabolic models (GEMs) are stoichiometric representations of the complete metabolism of an organism, encompassing the connections between genes, proteins (enzymes), and reactions. Significant advances have been made concerning the mapping of metabolic reactions through the analysis of genomic data (Kim *et al.*, 2017). GEMs are constructed based on whole-genome sequencing, but the process of building a functional metabolic model involves several steps and iterations. Initially, it is necessary to identify functional roles in the genome and link them to enzyme complexes and reactions (Cuevas *et al.*, 2016).

New GEMs are regularly developed and more and more organisms have their proper GEM (Kim *et al.*, 2017). These models are continuously updated and refined, particularly for model organisms such as *Escherichia coli*, as knowledge regarding their genomes and expressed proteins become consolidated (Singh and Lercher, 2020). Experimental validation of metabolic models is crucial in light of this ongoing refinement. One of the first GEMs constructed for cyanobacteria predicted, through FBA, that photorespiration would allow for optimal growth rates (Knoop *et al.*, 2010). Analysis of GEMs helps to gain insights into possible metabolic engineering interventions and substrate allocation (Kim *et al.*, 2017).

Usually, the construction of these models is first focused on the carbon-core metabolic network. Later on, they are refined by accounting for more details, such as improved compartmentalization by including more organelles. Microalgae metabolic models require, at least, the reactions taking place in the chloroplast, cytosol, and mitochondria. GEMs are particularly valuable for identifying targets to modify strains. Despite the increasing availability of high-throughput analytical tools, the gathering and application of proteomic and metabolomic data for metabolic engineering in microalgae purposes remain limited. Currently, most studies focus primarily on lipid production, while other metabolites and pathways receive less attention.

Although genome sequences of numerous microalgal species have been resolved and made publicly available, the information provided by the genome and transcriptome alone offers only a limited view of the cell's metabolic pathways. However, the task of deciphering the nature and function of metabolic pathways in microalgae is challenging due to variations introduced through evolutionary processes. As for other eukaryotic organisms, compartmental complexity and intracellular transport further increase the difficulty in considering all aspects in the model. For these reasons, it is important to remember that current comprehension of the functioning of the cell in different conditions, although vast, will continue to evolve and expand in the coming years.

Figures 2.1 and 2.2 give an overall view of the metabolism of microalgae. They show the network and interconnectedness of some metabolism and how they are connected to the production of biomass. In a GEM, those reactions are all represented as coefficients of the stoichiometric matrix, linking the reactants and products of each reaction. One of the important features of GEMs is the biomass reaction, which describes the composition of the cell and therefore what metabolites and substrates are necessary for the growth of the cell. Therefore, the accurate determination of the macromolecular biomass composition is crucial for achieving accurate flux and growth rate simulations.

2.4 MODELLING METABOLIC NETWORKS

Metabolic networks are chains of reactions taking place inside the cell. The different metabolic pathways keep the cell functioning, for instance, the production of energy via ATP or the synthesis of macromolecules such as DNA, lipids, and proteins. Metabolic network models can be constructed based on the knowledge of biochemical processes, such as photosynthesis or glycolysis, or on the genomic knowledge of the organism (through the use of GEMs), which in general produces more accurate, though more complex models. The level of detail in the model can also be constrained by the objective of its use, and many reactions can be omitted.

In general, simplifications and assumptions to reduce the size of the system are necessary because the large number of states in standard models of metabolic networks makes optimization and control impracticable. In general, it is assumed that the system is in quasy steady state (QSS), known as the quasy steady state approximation (QSSA).

The ordinary differential equations (ODEs) representing the system in a continuous perfectly mixed stirred tank and can be written in the following general form:

$$\frac{\mathrm{d}C}{\mathrm{d}t} = \frac{\mathrm{d}cX}{\mathrm{d}t} = Nv - D.C$$

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \mu X - DX$$

$$\frac{\mathrm{d}P}{\mathrm{d}t} = N_{\mathrm{P}} vX - DP$$

$$\frac{\mathrm{d}S}{\mathrm{d}t} = N_{\mathrm{S}} vX + D(S_{\mathrm{in}} - S)$$

where $C \epsilon \mathbb{R}^{n_c}$, $S \epsilon \mathbb{R}^{n_s}$, $P \epsilon \mathbb{R}^{n_p}$ are concentration vectors of size n_c, n_s, n_p, respectively, representing the number of internal metabolites, substrates, and products. X is the biomass concentration. Substrates, products, and biomass are written as mass per volume of the reactor; $N \epsilon \mathbb{R}^{n_c * n_r}$ is the matrix of stochiometric indices of the reactions in the metabolic network; $v \epsilon \mathbb{R}^{n_r}$ is the vector of the reactions kinetics, giving the rate of all n_r reactions of the network; μ is the growth rate of the microalgae; D is the dilution rate; $S_{\mathrm{in}} \epsilon \mathbb{R}^{n_s}$ is the concentration vector of incoming substrates. This system of equations also describes a batch cultivation process when D equals zero.

When the metabolite concentrations are written per mass/volume of the cell, the ODE is written as:

$$\frac{\mathrm{d}c}{\mathrm{d}t} = Nv - c\mu$$

where c is the concentration of metabolites inside the cell written as a fraction, that is, mass of metabolites per total mass of the cell.

In the QSSA, internal metabolites are assumed to be in steady state, that is, the equilibrium is reached instantaneously, while only the concentration of external metabolites or substrates behaves dynamically. Mathematically, the QSSA is written as:

$$\frac{\mathrm{d}c}{\mathrm{d}t} = 0$$

or

$$Nv - c\mu = 0$$

The term $c\mu$, which describes the dilution of metabolites due to cellular growth is generally ignored because the dynamics of the chemical reactions are considerably greater than the loss of concentration due to the change in the cell mass (Provost & Bastin, 2004). In the end, we have the following equation:

$$Nv = 0$$

The QSSA is a necessary assumption for most frameworks and modeling of metabolic networks. The QSSA cannot always be applied, for example in cases where metabolites accumulate inside the organism, such as in microalgae. Due to dial variations of light intensity, microalgae accumulate different metabolites depending on light availability. Consequently, the classical frameworks face limitations when applied to the modeling of microalgae systems.

Constrained-based modeling techniques considering the QSSA are the most widely used when dealing with metabolic networks, enabling the estimation of intracellular fluxes at different conditions (Tibocha-Bonilla *et al.*, 2018). The two most important techniques are elementary flux modes (EFM) and FBA (Lotz *et al.*, 2014).

2.5 TOOLS FOR STEADY-STATE CONDITIONS

2.5.1 Elementary flux modes

2.5.1.1 Mathematical construction of EFMs

EFMs) are often described as a minimum set of pathways capable of representing the total of the network at the steady state. A flux mode is defined mathematically as a set M:

$$M = \left\{ v \epsilon \mathbb{R}^{n_r} \middle| v = \lambda v^*, \lambda > 0, Nv^* = 0 \right\}$$

where v^* is a vector respecting the steady-state condition $Nv^* = 0$, having a subset $v^{\text{irr}} \geq 0$, corresponding to the irreversible reactions, while the subset v^{rev} corresponding to the subset of reversible reactions has no sign restriction (Schuster *et al.*, 1999).

A representative v^* of M is an EFM if and only if it fulfills the simplicity condition: there is no couple of vectors v', v'' with the following properties:

- v^* is a non-negative linear combination of v' and v''
- v'' and v'' satisfy the conditions to be a flux mode
- v' and v'' contain at least the same number of zero elements as v^*, and at least one of them contains more zero elements than v^*.
- The elements at boundary reactions of v' and v'' have the same sign or one element is a zero (e.g. $v_i' = -1, v_i'' = 0$).

The vectors v satisfying the steady-state equation are necessarily non-negative belonging to the kernel of the stoichiometric matrix N. Therefore, the space generating these vectors is a polyhedral cone in the intersection between the kernel of N and the positive orthant. The vectors v can then be written as a non-negative linear combination of a set of vector e_k which forms the unique convex base of the polyhedral cone.

$$v = \sum \lambda_k e_k; \ \lambda_k \geq 0$$

The vectors e_k forming the convex basis are the EFMs, being the simplest pathway connecting substrates to products at a steady-state condition. The EFMs are useful to deduce macroscopic reactions (MR) or global reactions in the metabolic network. Because of the QSSA, the dynamics of internal metabolites can be ignored, simplifying the dynamic equation of the macroreaction.

As $e_k \in \mathbb{R}^{n_r}$, each position corresponds to a reaction participating in the elementary mode.

The macroreactions are easily deduced by multiplying by zero the components representing the internal reactions between metabolites, then keeping the substrates and products in the remaining reactions. Figure 2.3 has a visual representation of the EFMs for a toy network. For more complex metabolic networks, the determination of EFMs requires much more computational effort, with the number of EFMs increasing exponentially with the size of the network. An efficient algorithm to calculate all EFMs may be necessary to reduce computational time. Also, the existence of reversible reactions in the network might increase the difficulty of determining the set of EFM.

2.5.1.2 Minimal generating sets and EFM reduction

The presence or not of reversible reactions in the metabolic network change the algorithm necessary to compute the set of EFMs. The simplest case is when all reactions are irreversible. In this case, every

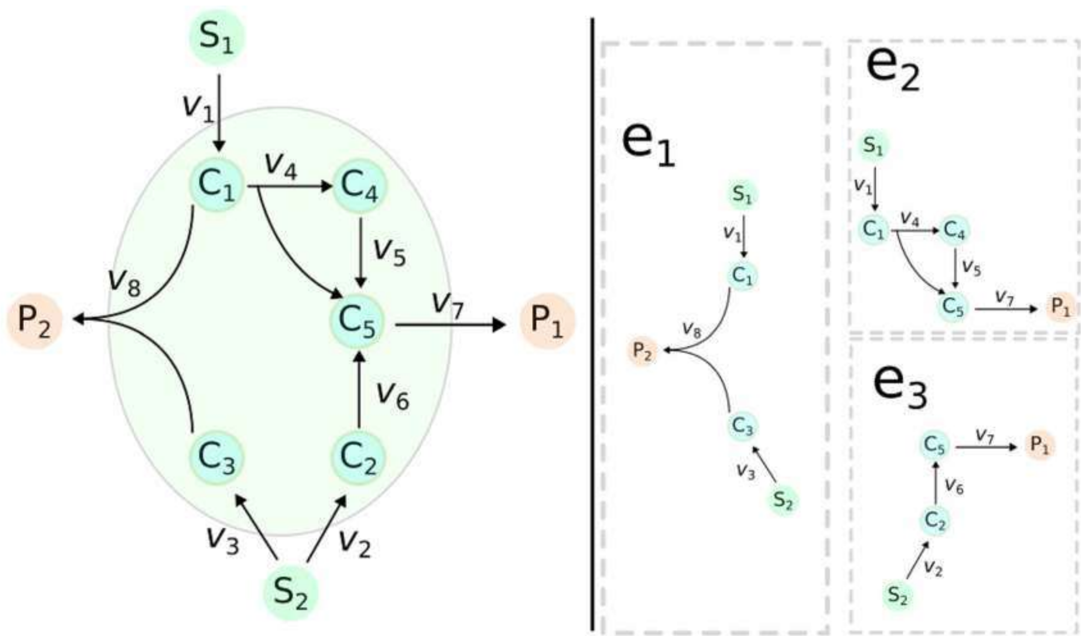

Figure 2.3 Example adapted from Provost and Bastin (2004) of the set of elementary flux modes being calculated for a toy-network. In this case, a linear combination of the three calculated elementary modes can reconstruct all possible steady states of the network.

component of all flux vectors v is non-negative and the cone representing the space of allowed flux vectors at steady state is a pointed cone. A convex cone K is pointed if:

$$K \cap -K = \{0\}$$

If a polyhedral cone is pointed there exists a unique minimal set of generating vectors and the elements of this set are the extreme rays of the cone. These vectors also serve as a complete set of representatives of elementary modes. In summary, when all reactions are irreversible, that is $v = v^{\text{irr}}$, there is a unique minimal generating set (MGS) which is equivalent to the set of EFMs.

There are three cases when reversible reactions are present in the network:

- The system has only irreversible elementary modes. Despite the presence of reversible reactions, no EFM can work in the reversible direction, that is, $\nexists e_k = -e_{k'}$
- The system has irreversible and reversible elementary modes
- The system has only reversible elementary modes.

In the first case, the polyhedral cone is still pointed, the MGS is unique, and it corresponds to the set of EFM. This is not the case anymore for the two remaining cases, where the cone is not pointed. Also, the MGS will not be unique and the set of EFM might be greater than the size of the MGS. Given this, the set of EFM will always be a superset of the MGS.

To understand the difference between the MGS and the EFMs getting deeper into convex analysis is necessary. In convex analysis it is shown that the space generating the solution of a linear homogenous system of equations is a convex polyhedral cone, C. Every point of such a cone is a non-negative combination of fundamental vectors, f, and basis vectors, b,

$$C = \left\{ v : v = \sum n_k f^k + \sum \lambda_m b^m ; n_k, \lambda_m \geq 0 \right\}$$

The fundamental and basis vectors are also called the generating vectors. There is a minimum necessary number of generating vectors to span the cone. The basis vectors are the extreme rays of C for which the negative vector is also contained in C (Schuster *et al.*, 1999). The definition of C here is identical to the minimum set of elementary modes (MEMO) in Röhl and Bockmayr (2019), where every v vector in the steady-state cone is written as a non-negative linear combination:

$$v = \sum \lambda_e e + \sum \lambda_f f$$

where $f \in \left\{ U \cap E_N^{\text{irr}} \right\}, e \in \left\{ U \cap E_N^{\text{rev}} \right\}$, where U is an inclusion-minimal set which is a subset of E_N which is the set of all EFMs for the stoichiometric matrix N.

The basis vector b is then equivalent to the vector e corresponding to the reversible EFMs, while the fundamental vectors f are equivalent to the irreversible set of EFMs. This implies that when there are no reversible EFMs, that is, the cone is pointed, C has no basis vectors. By contrast, when there are only reversible EFMs, C has only basis vectors.

Jevremović and Boley (2013) and Röhl and Bockmayr (2019) provide algorithms to compute the MGS when there are reversible reactions in the metabolic network. Röhl and Bockmayr (2019) rely on a method of splitting reversible reactions, with a minimal number of splits, until no reversible EFM is left creating a pointed cone. While Jevremović and Boley (2013) divide the stoichiometric matrix based on the reversibility or not of the reaction, then they compute the null space of a modified matrix representing the reversible reactions and the MGS of the irreversible subnetwork.

The set of EFMs is in general much larger than the MGS. For example, the carbon metabolism of *E. coli* has 6421 EFMs while only 15 vectors are in the MGS/MEMO. The division of a network into subnetworks, as in the DRUM method (described below), also reduces the number of EFMs. The use of MGS may be another way to reduce the size of the system, though only the number of macroreactions is guaranteed to be reduced compared to the use of EFMs – the number of metabolites might still be the same. Furthermore, it is not guaranteed that the MGS is able to create meaningful macroreactions capable of accurately modeling the network, as it happens with the use of EFMs. The calculation of EFM becomes prohibitive when the metabolic network is too large, but the enumeration of EFMs is still possible by computing only a subset of the EFMs. Many methods have been developed in recent years to facilitate the computation of EFMs.

The method in Kaleta *et al.* (2009) is an example of subsystem analysis. This paper introduces the concept of elementary flux patterns, where instead of giving a stoichiometrical proportion to a reaction, it only considers the index. It means that it only calculates the list of reactions participating in an elementary mode. Oddsdóttir *et al.* (2015) use optimization in metabolic flux analysis to reduce the number of EFMs. The idea is to find the best-fitting EFMs to some measured external flux. There is an algorithm minimizing the difference between the measured flux and the EFMs to reproduce those fluxes.

Tabe-Bordbar and Marashi (2013) couple EFM with FBA. The proposed algorithm removes reactions by FBA, considering a random objective reaction. They select a list of reactions to remove, followed by FBA calculation, if the objective flux is non-zero, then they proceed with the deletion of the reactions. On the other hand, if the flux of the objective reaction is zero, then the reactions are kept. The goal is to find, at least, a subset of the EFMs of a genome-scale metabolic network by reducing the size of the total network. In a recent paper, Maton *et al.* (2022) calculate a reduced set of EFMs based on several steps, including geometrical criteria, optimization techniques, and also external observations to derive macroreactions for the system.

2.5.2 Flux balance analysis

Flux balance analysis (FBA) is one of the most common tools to analyze metabolic networks (Orth *et al.*, 2010). Together with EFMs, they can be used to identify feasible routes in the metabolic network and estimate internal metabolic fluxes based on substrate uptake and excretion rates (Lotz *et al.*, 2014). As in the case of EFMs, FBA also assumes the cell to be at steady state. However, instead of trying to determine the possible set of reactions constructing the steady state, the method consists of the maximization (or minimization) of an objective function:

$$\max Z = c^{\mathrm{T}} v$$

where $c \epsilon \mathbb{R}^{n_r}$ is a vector of weights, indicating how much a certain reaction influences the objective function, and v is the vector of fluxes of metabolic reactions. Besides the constraint of the steady state ($Nv = 0$), it considers boundaries for the vector of reaction fluxes v:

$$l_i \leq v_i \leq u_i$$

where l_i and u_i are the lower and upper boundary, respectively.

One of the most common cases is the maximization of biomass production, in this case c will be a vector containing zeros in every position, except the position for the reaction of biomass.

The system of equations and constraints of FBA leads to a linear programming problem, which can be computed with standard algorithms such as interior-point methods.

2.6 METABOLIC NETWORKS REDUCTION

The increasing size of metabolic networks makes it difficult to apply numerical analysis, especially when considering dynamical aspects. Even in the case of the steady state, computational power becomes limiting. For example, as discussed above, the number of EFMs grows exponentially as the metabolic network increases. As a consequence, calculation of EFMs for genome-scale models even for simple organisms such as *E. coli* may not be possible due to computational limitations. Methods to reduce the size of these genome-scale metabolic models become imperative to analyze steady state and dynamical behavior.

Here, we will briefly mention the current methods used to reduce metabolic networks, with emphasis on genome-scale networks.

Many methods have been released in the literature in recent years regarding the reduction of metabolic networks (Singh and Lercher 2020). They differentiate on the assumptions regarding the network in order to proceed with the model reduction, methods, and goals. While some techniques focus on keeping the same phenotype of the full network, others have a more greedy approach to the reduction, focusing on minimizing the most possible of the network and only keeping some desired reactions or phenotypes.

One of the first techniques used was the consideration of '*Enzymes Subsets*' (Pfeiffer *et al.*, 1999). An enzyme subset is a group of enzymes that work together in a metabolic pathway and can be considered as a unit structure catalyzing a series of reactions. Mathematically, a group of reactions (or enzymes) belongs to an enzyme subset if in all flux vectors v satisfying the steady-state condition, the ratio between the fluxes of the reactions in the enzyme subset, for example $v_n/v_{n'}$, has the same non-zero value and the directions of the reactions are not contradictory. It is possible therefore to reduce the network without losing the original information and capabilities, because it is considered that the enzymes belonging to such a subset are expressed coherently, regulating metabolism in the unit. Nevertheless, the drawback of this method is the limited capability in reducing the total size.

A method to further reduce the metabolic network was later implemented by Tabe-Bordbar and Marashi (2013), called minimal reaction sets. The method consists in solving a mixed-integer linear programming problem, where the objective is to minimize the number of reactions of the network, while still keeping a minimal flux of biomass production. A more recent method *NetworkReducer* has recently been published where the objective is to reduce the network while at the same time keeping certain protected phenotypes, metabolites, and reactions (Erdrich *et al.*, 2015). The algorithm functions in two major steps. First the pruning phase where reactions are iteratively removed until no more reaction can be deleted without breaking protected parts. Second, the compression phase is a loss-free simplification by the lumping of coupled reactions. An improvement of this method was made by Röhl and Bockmayr (2017), by including the minimization of the number of reactions as in Burgard *et al.* (2001). In Küken *et al.* (2021), a method of reduction based on the use of complexes (combination of the species participating in one side of the reaction), where the stoichiometric matrix is written as the product of two matrices, $N = Y.A$, where Y is a matrix having as columns the complexes and metabolites in the rows, A is a matrix having indices of -1, 0 or 1 with reactions represented on the columns and the complexes on the rows. Depending on the structural conditions and the balancing of the complexes, the network is reduced while keeping the phenotype of the original network.

2.6.1 The DRUM framework

DRUM (dynamic reduction of unbalanced metabolism) is a metabolic modeling framework created in order to circumvent the problem of inappropriate use of the QSSA to the whole metabolic network (Baroukh *et al.*, 2014). It was initially developed for organisms which dynamically accumulate and reuse some metabolites, such as microalgae under varying environmental conditions.

The idea of the DRUM framework is to divide the complete metabolic network into subnetworks, in which the QSSA is valid, also reducing the total number of state variables representing the system. After the division of the network, the EFMs are calculated for each subnetwork, generating MR, representing the result of all the internal reactions of the subnetwork with much simpler kinetics. See Figure 2.4 for a representation of the steps required in the DRUM framework.

After the application of the DRUM method, the dynamical equations of the system are reduced to the number of metabolites that are allowed to accumulate, external substrates and products. The form of the system of differential equations is the same as the original one, but the stoichiometric matrix is reduced and modified to represent the new MR deduced from the EFMs. The DRUM method is able

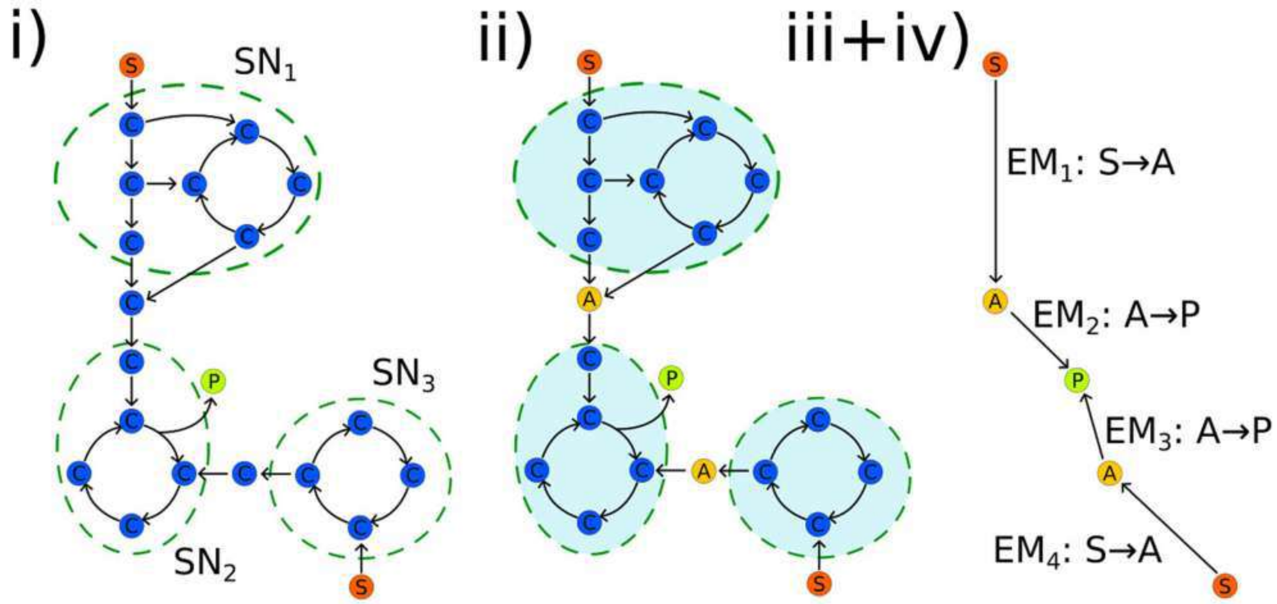

Figure 2.4 Example of application of the DRUM framework to a toy network. Initially, the network has two substrates (orange), one product (green), and 18 internal metabolites (blue). In step (i) the sub-networks are determined. In step (ii) there is the choice of accumulating metabolites connecting the SNs. In step (iii), the elementary flux modes are calculated for each of the SNs. In step (iv), the original network is reduced by the use of the macroreactions. Finally, the number of 18 internal metabolites is reduced to only 2.

to accurately represent empirical data, predicting for example the accumulation of carbohydrates and lipids during the day and its consumption during the night. Despite this, a more objective method to divide the subnetworks still needs to be defined. Finding new ways to split the metabolic network might reduce the size of the system even more, while still being able to predict the accumulation of metabolites.

The DRUM framework is grounded on the key concept and assumption of the different time scales characterizing metabolic reactions. This discrepancy in time scales gives rise to the accumulation of metabolites and consequential modifications in cellular composition. However, due to the present limitations in our knowledge of internal reaction rates, the selection of accumulating metabolites is currently non-deterministic and relies on prior knowledge or subjective preferences concerning the partitioning of the metabolic network. As discussed earlier, taking into consideration cellular compartments, intersection metabolites, and possible simplification strategies employed during the reduction process will influence the choice of accumulating metabolites. Nevertheless, with the advancement of knowledge regarding internal kinetic rates, future developments are expected to provide more rigorous approaches for determining accumulating metabolites. These refined methods will not only enhance the accuracy of phenotype approximation in reduced metabolic models, but also facilitate the evaluation of their proximity to the complete metabolic system.

In the DRUM framework, the metabolic network is represented by the following system of ODEs:

$$\frac{\mathrm{d}M}{\mathrm{d}t} = \frac{\mathrm{d}}{\mathrm{d}t}\begin{pmatrix} S \\ C \\ P \\ B \end{pmatrix} = \begin{pmatrix} N_\mathrm{S} \\ N_\mathrm{C} \\ N_\mathrm{P} \\ N_\mathrm{B} \end{pmatrix}.v(M).B - D.M + D.M_\mathrm{in} = N.v(M).B - DM + DM_\mathrm{in}$$

where M represents the vector of the concentrations of metabolites composed of substrate (S), intracellular metabolites (C), excreted products (P), and biomass (B). M_{in} is the influent concentration of these quantities. The dilution rate of the reactor (ratio of influent flow rate over the reactor volume) is D ($D = 0$ for a batch process). All the concentrations are expressed as total concentrations in the solution. $v \epsilon \mathbb{R}^{n_r}$ is the reaction kinetic vector, while the matrices $N_S \epsilon \mathbb{R}^{n_s \times n_r}$, $N_C \epsilon \mathbb{R}^{n_c \times n_r}$, $N_P \epsilon \mathbb{R}^{n_p \times n_r}$, and $N_B \epsilon \mathbb{R}^{1 \times n_r}$ correspond, respectively, to the stoichiometric matrices of substrates S, products P, intracellular metabolites C and biomass B ($n_s + n_c + n_p + 1 = n_m$).

The DRUM method consists in dividing the metabolic network into k quasi-stationary subnetworks, so the matrix N is rewritten in the following form:

$$N = \left[N_{SN_1}, N_{SN_2}, \ldots, N_{SN_k}\right]$$

where $N_{SN_i} \in \mathbb{R}^{n_m \times n_{SN_i}}$ and $\sum_{i=1}^{k} n_{SN_i} = n_r$. Each sub-network is assumed to be at steady state:

$$\forall i = 1, \ldots, k : N_{SN_i}.v_{SN_i} = 0$$

By considering the steady-state condition, it is possible to calculate the EFMs for each of these N_{SN_i} sub-networks, then construct macroreactions:

$$\forall i = 1, \ldots, k : v_{SN_i} = E_{SN_i} \alpha_{SN_i}, \alpha_{SN_i} \geq 0$$

$$\forall i = 1, \ldots, k : \rightarrow (N_{S_{SN_i}}.E_{SN_i}).S_{SN_i} \rightarrow (N_{P_{SN_i}}.E_{SN_i}).P_{SN_i}$$

where E_{SN_i} is the matrix of EFMs of the sub-network SN_i, and α_{SN_i} the kinetics of the MR described by the reduced stoichiometric matrix.

Following this step, we group all the sub-networks, and considering that only metabolites A are allowed to accumulate. Meaning that other metabolites have simple dynamics and their concentration is directly determined by the A metabolites. We obtain a reduced dynamic model, defined by the new metabolites vector $M' \epsilon \mathbb{R}^{n_m}$, the new stoichiometric matrix $N' \in \mathbb{R}^{n_m \times n_E}$ \$N′ and α the kinetic vector associated with these MR:

$$\frac{dM}{dt} = \frac{d}{dt}\begin{pmatrix} S \\ A \\ P \\ B \end{pmatrix} = \begin{pmatrix} N_S \\ N_A \\ N_P \\ N_B \end{pmatrix}.\alpha.B - D.M + D.M_{\text{in}} = N'.\alpha.B - DM' + DM'_{\text{in}}$$

Furthermore, because of the consideration of the accumulating metabolites, there is a distinction between the dry weight biomass or total biomass (X) and the functional biomass. In this case, experimentally measured biomass is the sum of the functional biomass and the total mass of the accumulating metabolites:

$$X = B + \sum_{i=1}^{k} A_i$$

2.7 CASE STUDY: MICROALGAE CULTIVATION

2.7.1 Introduction: volatile fatty acid

In this section, the DRUM framework will be applied to a case study based on the research paper by Pessi *et al.* (2023). The objective of this case study is to construct a reduced metabolic model for the *Chlorella* sp. microalgae to address the treatment of volatile fatty acid (VFA) waste with the addition of organic carbon substrates, namely glucose and glycerol. The case study explores the concept of integrating wastewater treatment with biofuel production, where the VFA waste from dark fermentation, produced by bacteria, serves as the substrate for microalgae cultivation.

The metabolic network utilized in this study is based on the study by Pessi *et al.* (2023), initially with 188 reactions and 173 metabolites. Noticeably, this metabolic network is relatively small, since it is not a genome-scale metabolic network, but it was constructed based on core known metabolic reactions with some other reactions being included to enhance its coverage of metabolic possibilities. This means that in the case of using GEMs, as mentioned above, it may be harder to apply the DRUM method. Although it would be encouraged to apply it to a GEM since a more representative reduced model would be obtained, and after reduction, the model could be used again for other applications. The composition of VFAs coming from dark fermentation varies depending on the conditions, usually determined by the composition and characteristics of the substrate coming for treatment via dark fermentation. The VFAs present in the waste are primarily butyrate and acetate. Frequently, there is also production of lactate in this kind of process, but since *Chlorella* does not consume lactate, it is not included in the model.

The model encompasses four organic carbon substrates: butyrate (BUT), acetate (ACE), glucose (GLC), and glycerol (GLY), along with one inorganic carbon dioxide (CO_2). Additionally, the choice of the nitrogen source is an important consideration, as it influences the stoichiometric production of biomass. NH_4^+ is known to have a better yield for biomass production compared to other nitrogen sources, assuming the same carbon source.

The inclusion of glucose and glycerol as additional carbon sources in the treatment of wastewater containing VFAs is motivated by an optimization approach. Previous research has highlighted the inhibitory effects of butyrate on algal growth, manifested in both heterotrophic and mixotrophic growth conditions. Additionally, the presence of acetate has been observed to hinder the absorption of butyrate, leading to the occurrence of diauxic growth. Consequently, it is imperative to devise a strategy to mitigate this inhibition and enhance the consumption of butyrate by microalgae. The strategy proposed in this study involves supplementing the wastewater with glucose and glycerol, which serve as readily assimilated carbon sources for the microalgae. This supplementation aims to accelerate algal growth, enabling them to overcome the inhibitory effects of butyrate by attaining a higher biomass concentration that can more efficiently consume the remaining butyrate.

2.7.2 Determination of the subnetworks and accumulating metabolites

Upon determining the substrates to be represented within the model, the subsequent step involves the delineation of subnetworks and the identification of accumulating metabolites within the DRUM framework. This task resides under the discretion of the modeler, who may opt for a straightforward approach by considering cellular compartments. In the present case, the chloroplast and glyoxysome, previously discussed, are designated as initial compartments. Moreover, glyceraldehyde 3-phosphate (GAP) and succinate (SUC) are chosen as accumulating metabolites due to their pivotal role as intersection metabolites, facilitating the interconnection of diverse subnetworks within the model (Figure 2.5).

Succinate assumes paramount importance as an essential precursor, as it possesses the ability to enter the mitochondrial tricarboxylic acid (TCA) cycle, subsequently undergoing conversion to oxaloacetate and eventually participating in the generation of phosphoenolpyruvate. Likewise, GAP emerges as an important precursor, with its synthesis occurring, for example, in the chloroplast during photosynthesis and also in the cytosol during glycolysis, thus serving as a bridging molecule across multiple metabolic pathways.

It is important to bear in mind that these choices are devised to simplify the process of reducing the metabolic network. However, alternative strategies for partitioning may be deemed more appropriate as further knowledge is acquired. For instance, if the incorporation of lipid content in the model becomes essential, it may be prudent to exclude it from the biomass synthesis subnetwork and instead establish a separate subnetwork specifically dedicated to lipid synthesis. Nonetheless, this approach introduces an additional dynamic variable that will have to be simulated and entails the acquisition of more experimental data to calibrate the model.

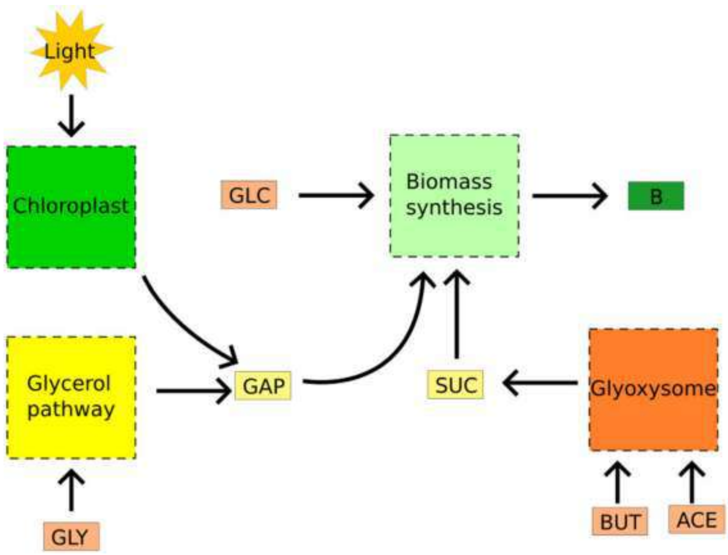

Figure 2.5 Simplified representation of the metabolic network of microalgae *Chlorella* after reduction with the DRUM framework. Sub-networks (squares with dashed lines), substrates (orange rectangles), accumulating metabolites (yellow rectangles), and biomass (green rectangles).

The first determination of subnetworks and selection of accumulating metabolites, by consequence, is able to pre-determine further subnetworks from still non-connected substrates. Given the prior selection of GAP, a subnetwork connecting GAP and glycerol (GLY) is naturally formed. The pathway created is, furthermore, called glycerol pathway. As for the remaining metabolic pathways, such as glycolysis, the TCA cycle, and protein and lipid synthesis, they are amalgamated into a comprehensive global network named here as the biomass synthesis pathway.

As for glucose, we establish a direct linkage to the biomass synthesis pathway, owing to the immediate synthesis of glucose-6P subsequent to glucose uptake by the cellular system. An alternative approach could involve the segregation of the glycolysis pathway, with the 'upper glycolysis' pathway serving as a distinct subnetwork. Such an arrangement would give rise to an elementary flux mode, encompassing a macroreaction that connects glucose (GLC) to glyceraldehyde 3-phosphate (GAP). It is worth noting that the construction of subnetworks may undergo iterations as the modeller refines the composition of the reduced network, ultimately culminating in the selection of a definitive configuration.

2.7.3 Derivation of MR

Following the completion of subnetwork and accumulating metabolite selection, the subsequent phase entails the determination of EFMs to derive the MR. This computational step can be accomplished using numerical tools such as efmtool (Terzer & Stelling, 2008) or COBRA methods (Ebrahim *et al.*, 2013). In situations where multiple EFMs are calculated for a given pair of subnetwork and accumulating metabolite, a selection process becomes necessary. Generally, the optimal approach for selection involves identifying the reaction that has the highest yield for the product. A list of the chosen EFMs and their corresponding MR for each subnetwork and accumulating metabolite/substrate is presented in Table 2.1.

2.7.4 Choice of kinetic model

After the reduction of the metabolic network and the determination of the final set of variables required for simulating the system dynamics, it is necessary to determine the appropriate kinetic models for

Table 2.1 List of the seven elementary flux modes selected to represent MR of the reduced network.

	Subnetwork	Macroscopic Reaction
MR_1	Glyoxysome	$2ACE + 3.5H + 0.5O_2 \rightarrow SUC + 0.5H_2O$
MR_2	Glyoxysome	$BUT + 7H + 1.5O_2 \rightarrow SUC + 5H_2O$
MR_3	Chloroplast	$Light + 3CO_2 + 2H_2O + Pi \rightarrow GAP + 3O_2$
MR_4	Glycerol pathway	$GLY + Pi \rightarrow GAP + H_2O$
MR_5	Biomass synthesis	$4.64GAP + 2.04O_2 + 0.99NO_3 + 0.98H + 0.02SO_4 + 0.01Mg_2 \rightarrow B + 5.39CO_2 + 2.90H_2O + 4.51Pi$
MR_6	Biomass synthesis	$4.90SUC + 5.28O_2 + 0.99NO_3 + 0.12Pi + 10.78H + 0.02SO_4 + 0.01Mg_2 \rightarrow B + 11.07CO_2 + 8.31H_2O$
MR_7	Biomass synthesis	$2.34GLC + 2.14O_2 + 0.99NO_3 + 0.12Pi + 0.98H + 0.02SO_4 + 0.01\ Mg_2 \rightarrow B + 5.49CO_2 + 7.63H_2O$

each MR. The selection of these kinetic models is crucial in ensuring accurate dynamical simulations. Even if the underlining representation of the metabolism is correct, inadequately estimated parameters or ill-fitted kinetic models will result in poor results.

A classical approach to model the kinetics of biochemical reactions is the use of Monod-like functions. In the case at hand, where acetate consumption is the MR of interest, the rate of the reaction increases with the concentration of the substrate, but reaches a maximum rate when the concentration saturates the quantity of enzymes catalyzing the reaction. Thus, the selected function is represented as follows:

$$\alpha_1 = \alpha_{1\max}\frac{ACE}{KS_1 + ACE}$$

As previously mentioned, there is inhibition in the consumption of butyrate coupled with a diauxic effect involving acetate. To model both of these effects, we first consider a Haldane-like function which describes the inhibition, wherein an optimal concentration exists at which the rate of the reaction is maximized, but after this concentration the rate is reduced. The Haldane function is then multiplied by a function that decreases with acetate concentration to account for diauxic growth. Hence, the model for the MR involving butyrate is expressed as follows:

$$\alpha_2 = \alpha_{2\max}\frac{BUT}{BUT + \frac{\alpha_{2\max}}{\beta_2}\left(\frac{BUT}{S_{2\mathrm{opt}}} - 1\right)^2}\frac{k_\mathrm{d}}{(ACE + k_\mathrm{d})}$$

Macroscopic reaction 3 (MR_3 in Table 2.1) describes the autotrophic growth, and as such, it is dependent on light intensity. Numerous functions can be employed to model autotrophic growth, taking into account phenomena such as photoacclimation, which introduces a dependency on past light intensities and necessitates the consideration of an additional dynamical variable. However, one important aspect is the consideration of light absorption due to medium turbidity, which increases with biomass concentration. In this case, light absorption is modeled by the Beer–Lambert equation, where light intensity depends on the light at the top of the reactor $(I)_0$, the extinction coefficient (σ), which depends on biomass concentration, and the depth of the reactor (L). See Martínez *et al.* (2018), for a thorough discussion on modeling light absorption.

$$I = I_0\exp(-X.\sigma.L)$$

$$\sigma = aX^{1-b}$$

$$\alpha_3 = \frac{\alpha_{3\max}}{\sigma.X.L} \ln\left(\frac{k_I + \sigma I_0}{k_I + \sigma I_0 \exp(-\sigma.X.L)}\right)$$

The model for glycerol utilizes a Haldane-like function. However, if only low or moderate concentrations of glycerol are under consideration, a Monod-like function may also be appropriate.

$$\alpha_4 = \alpha_{4\max} \frac{GLY}{GLY + \frac{\alpha_{4\max}}{\beta_4}\left(\frac{GLY}{S_{4\text{opt}}} - 1\right)^2}$$

For internal accumulating metabolites, because of the lack of experimental data of their dynamic concentrations, it is necessary to minimize the number of model parameters to facilitate the calibration process. For these reasons, linear kinetics is used to model the reactions with GAP and SUC. Furthermore, it is important to note that the Monod function is approximated to a linear function in low substrate concentrations, which also justify the choice of a linear equation. It is important to emphasize that, in the context of internal metabolites, the reaction rate relies on the internal concentration rather than the total concentration within the reactor.

$$\alpha_5 = \alpha_{5\max} \cdot \frac{GAP}{X}$$

$$\alpha_6 = \alpha_{6\max} \cdot \frac{SUC}{X}$$

Glucose, like glycerol, is also modeled by a Haldane-like function. Equally, if only low concentrations of glucose are being considered, a Monod-like function would also be fitting.

$$\alpha_7 = \alpha_{7\max} \frac{GLC}{GLC + \frac{\alpha_{7\max}}{\beta_7}\left(\frac{GLC}{S_{7\text{opt}}} - 1\right)^2}$$

Finally, at the end of the process of applying the DRUM framework, we are going to have the following system of seven ODEs when considering a continuous reactor:

$$\frac{\mathrm{d}ACE}{\mathrm{d}t} = -2\alpha_1.B + D(ACE_{\text{in}} - ACE)$$

$$\frac{\mathrm{d}BUT}{\mathrm{d}t} = -\alpha_2.B + D(BUT_{\text{in}} - BUT)$$

$$\frac{\mathrm{d}SUC}{\mathrm{d}t} = (\alpha_1 + \alpha_2 - 4.9\alpha_6)B - D.SUC$$

$$\frac{\mathrm{d}GLY}{\mathrm{d}t} = -\alpha_4.B + D(GLY_{\text{in}} - GLY)$$

$$\frac{\mathrm{d}GLC}{\mathrm{d}t} = -2.34.\alpha_7.B + D(GLC_{\text{in}} - GLC)$$

$$\frac{\mathrm{d}GAP}{\mathrm{d}t} = (\alpha_3 + \alpha_4 - 4.64\alpha_5)B - D.GAP$$

$$\frac{\mathrm{d}B}{\mathrm{d}t} = (\alpha_5 + \alpha_6 + \alpha_7)B - D.B$$

This set of equations can then be used to simulate the system, for process control and optimization.

2.7.5 Model calibration and validation

With the mathematical structure of the model at hand, it is now possible to simulate the dynamics of metabolite and biomass concentrations in the reactor. To achieve this, we need to determine the parameters governing the rates of the MR. The calibration process involves adjusting these parameters to align the model's predictions with experimental data, typically obtained from laboratory experiments.

Several methods can be employed for parameter estimation, and the choice of method depends on the available data and the complexity of the model. Some common approaches include least-squares fitting, maximum likelihood estimation, and Bayesian parameter estimation. In Pessi *et al.* (2023), a combination of methods is used. First, a global optimization method called differential evolution (Storn & Price, 1997) is used to identify initial parameter values and avoid local minima in the objective function. Subsequently, a Markov chain Monte Carlo method is applied to identify parameter values within a range of uncertainty (Foreman-Mackey *et al.*, 2013). Alternatively, if the modeler possesses a good estimation of the parameter range, local optimization with Markov chain Monte Carlo might suffice.

During the calibration process, it is essential to consider the uncertainties in the experimental data and the model structure. The use of statistical tools to quantify parameter uncertainty and confidence intervals can aid in this process. Uncertainty in the experimental data can be included during calibration with the Markov chain Monte Carlo method and the confidence interval for the parameters is also obtained at the end of this process.

Furthermore, to facilitate calibration it is possible to divide the set of parameters to calibrate, following a 'divide and conquer' strategy (Mairet & Bernard, 2019). In the case of our system of equations, it is possible to calibrate the model in multiple ways. For instance, kinetics of glucose, glycerol, and acetate consumption rely solely on their respective concentrations, allowing for separate calibration of the relevant parameters. Only butyrate consumption also depends on the acetate concentration, the relation described by the parameter k_d. The parameters of internal metabolites (GAP and SUC) have to be calibrated with at least one substrate, or even in autotrophic conditions for GAP. Ideally, α_5 and α_6 should be calibrated using data of multiple external substrates.

Once calibration is completed, the model should be tested against other independent experimental data for validation. This step is crucial to assess the model's predictive capability and involves comparing the calibrated model's predictions with additional experimental data that were not used during the calibration phase. Successful validation ensures the reliability of the model and the accuracy of the parameter values.

Following model calibration and validation, it can be used for various simulation scenarios to gain insights into the system's behavior under different conditions. One important aspect is the optimization of the process, where optimal operating conditions can be found.

2.7.6 Example of application: optimization of waste treatment time

When optimizing a process, multiple targets for optimization can be considered, such as minimizing costs, maximizing profits, or enhancing product production. In the context of butyrate inhibition, the model can be used to minimize treatment time, by overcoming the slow consumption of butyrate. The initial concentrations of acetate and butyrate are fixed, as they are the product of dark fermentation. Only the optimal addition of glucose and glycerol is found using numerical optimization techniques. When considering continuous or fed-batch cultivation, the objective function is as follows:

$$\min_{D,y} t_f : S(t_f) \leq \bar{S}$$

Here, $\bar{S}$ is a vector of the regulation threshold for the external substrates, indicating the maximal allowed concentration of external substrates at the end of the process. t_f corresponds to the time of process completion, when all substrate concentrations are below the defined threshold. The ratio y denotes the proportion between glucose and glycerol added, and D is the dilution rate (or flux) for the added substrates.

In the case of a batch system, the optimization problem consists in finding the optimal addition of glucose or glycerol at the beginning of the process. The objective function is formulated as follows:

$$\min_{S_{\mathrm{in}}} t_{\mathrm{f}} : S(t_{\mathrm{f}}) \leq \bar{S}$$

Here, S_{in} is a vector containing the initial concentrations of the added substrates.

Solving these optimization problems employs similar algorithms used during the calibration process since they consist of function minimization. The major difficult arises in case of fed-batch or continuous cultivation, where the optimal D needs to be found. D can be approximated as a function of time ($D = f(t)$), specified by the modeler, with the parameters of this function obtained during optimization. In other words, the problem of optimization of the process would become a calibration for the D function. Otherwise, more advance techniques of optimal control would be necessary.

Finally, the obtained optimal results could be tested experimentally. If experimental results are coherent with the optimal conditions predicted by the model, it validates the model's performance. Usually, some deviations between model prediction and experimental data will be encountered. In such cases, the newly acquired information can be used as a feedback for the model to check if parameters must be re-calibrated or if the structure of the model should be modified.

2.8 CONCLUSION

In this chapter, we reviewed the current state of the art of metabolic modeling for microalgae, exploring various aspects such as metabolic network regulation, reduction methods, and dynamic simulations. A case study on microalgae-based wastewater treatment with a focus on VFAs provided practical insights into the application of these modeling techniques.

The exploration of metabolic network modeling and its application to microalgae-based wastewater treatment and biofuel production has provided valuable insights into the potential of sustainable biotechnology applications. Throughout this chapter, the DRUM framework has been demonstrated as a powerful approach for reducing complex metabolic networks, facilitating the creation of simplified ODEs that describe the dynamics of the system. Following model calibration and validation using experimental data, the resulting model becomes a robust tool for simulating microalgae cultivation processes under various conditions.

Future research in the field should continue to focus on advancing modeling techniques and experimental data collection, in particular of internal metabolites. Additionally, a more in-depth understanding of microalgae metabolic pathways and their interactions with environmental factors, such as temperature, can lead to more accurate models. Additionally, integrating the metabolism of multiple organisms into future models is essential, as it has been demonstrated that the microbial community plays a crucial role in such processes.

While the current method of metabolic network reduction and dynamical simulation shows promising results, modelers must stay attuned to the rapid developments in this field. The continuous advancement in biological understanding and modeling techniques related to computational biology is inevitable. As a result, we can anticipate significant improvements in sustainable biotechnology applications using microalgae.

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Chapter 3

Wastewater treatment using microalgal–bacterial consortia in the photo-activated sludge process

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ABSTRACT

Nitrogen-rich wastewaters (10–400 mg N/L) are produced by municipal, industrial and agricultural wastes, including effluents from anaerobic treatment processes. These represent a risk to the environment due to the high nutrient concentrations (nitrogen and phosphorous), which can cause eutrophication of water bodies, deteriorating the quality of the ecosystems. As a solution, the nitrogen removal capacity of a novel bio-treatment system, the photo-activated sludge (PAS), composed of microalgae and bacteria consortia can be applied. Photobioreactors used for the simultaneous cultivation of microalgae and bacteria under sequencing batch conditions showed that microalgal–bacterial consortia can remove ammonium 50% faster than solely microalgal consortia. The increase in ammonium removal rates is due to the action of nitrifying bacteria, supplied with oxygen produced by the algae. The microalgal–bacterial system offers the possibility of reducing the hydraulic retention time, which can decrease the large area requirements often demanded by algal systems. The SRT is the main parameter to control the efficiency of the technology. The control of the suspended solids concentration, by adjusting the SRT, influences the light penetration within the reactor, which can limit or enhance the oxygen production of the algae. The photo-activated sludge system using microalgal–bacterial consortia is a sustainable treatment option for ammonium-rich wastewaters, providing clean effluents and opening reuse options for the biomass.

3.1 MICROALGAL–BACTERIAL CONSORTIA

3.1.1 Use of microalgal–bacterial consortia in environmental technologies

Microalgae and bacteria co-habit in freshwater, wastewater and marine systems. Symbiosis among aerobic bacteria and microalgae for treatment of wastewater was first reported by Oswald *et al.* (1953) in oxidation ponds. One of the interactions reported is the exchange of oxygen: the oxygen produced by the microalgae, through photosynthesis, is used by aerobic bacteria (heterotrophic and nitrifiers) to oxidize organic matter and ammonium (Figure 3.1). Heterotrophic bacteria produce carbon dioxide through respiration and oxidation of organic matter, which can be taken up as a carbon source by the microalgae. In the case of nitrogen, after nitrate is produced, it can be taken up by microalgae as a source of nitrogen, or further denitrified by bacteria when anoxic conditions are met, usually during dark periods, or dark zones within the reactor. These interactions create a synergistic

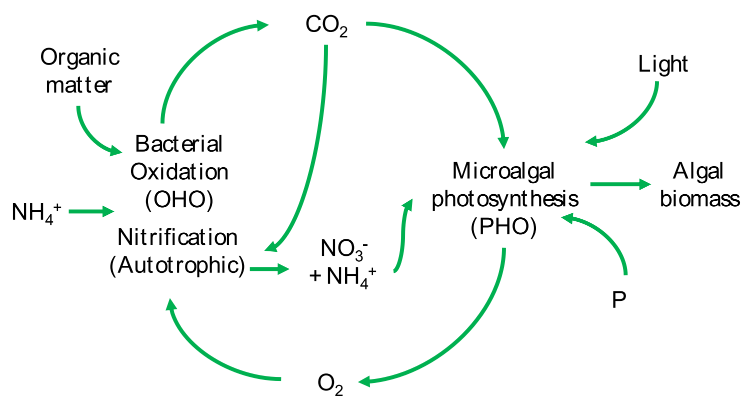

Figure 3.1 Microalgae and bacterial oxidation interactions in microalgal–bacterial consortia. (Source: adapted from Muñoz and Guieysse, 2006). OHO: heterotrophic organisms, PHO: phototrophic organisms and P: phosphorous.

relationship between microalgae, heterotrophs and nitrifiers in which the required oxygen is supplied by microalgae. The aeration supplied by microalgae is defined as photosynthetic oxygenation. The term was first defined by Oswald *et al.* (1953) as '*production of oxygen through the action of light on the chloroplastic tissue of microscopic green plants, growing dispersed in the aqueous medium*'.

The symbiosis occurs in waste stabilization ponds, oxidation ponds and high-rate algae ponds (HRAP). Zhou *et al.* (2006) reported removal of nutrients through nitrification/denitrification in HRAP treating rural domestic wastewater. About 50% of the nitrogen was removed through nitrification/denitrification, followed by algae assimilation and sedimentation. In the case of phosphorus, the main removal mechanisms were through algae assimilation followed by chemical precipitation.

Additional to the removal of nutrients, a consortium of algae and bacteria is able to remove hazardous pollutants, as reviewed by Muñoz and Guieysse (2006). Pollutants such as acetonitrile were found to be removed at a rate of 2300 mg/L/d by a consortium of *Chlorella sorokiniana* and a bacterial consortium suspended in a stirred tank reactor. Safonova *et al.* (2004) reported the removal of different xenobiotic compounds through a consortium of algae and bacteria. They observed different removal efficiencies for phenols (85%), anionic surfactants such as secondary alkane sulfonates (73%), oil spills (96%), copper (62%), nickel (62%), zinc (90%), manganese (70%) and iron (64%). The consortia used consisted of the algal strains *Chlorella* sp., *Scenedesmus obliquus*, *Stichococcus* and *Phormidium* sp. and of bacterial strains such as *Rhodococcus* sp., *Kibdelosporangium aridium* and two other unidentified bacterial strains. The removal mechanisms were the association between the oil-degrading bacteria and the algal strains, the ability of algae to supply oxygen and at the same time the ability of aerobic bacteria to degrade hydrocarbons.

3.1.2 Interactions within microalgal–bacterial consortia

The interactions between algae and bacteria are not limited to the exchange of carbon dioxide and oxygen. On the opposite, the interactions can be either mutualism, parasitism or commensalism (Ramanan *et al.*, 2016). As a result, algae and bacteria are able to change their physiology and metabolism (Ramanan *et al.*, 2016).

There are several studies showing the benefits and negative effects of bacteria and algae when present in consortia (Unnithan *et al.*, 2014). Algae can either promote bacterial growth through the release of organic exudates (Abed *et al.*, 2007), nutrient exchange as result of algal lysis (Unnithan *et al.*, 2014), or decreased algal growth through the release of algicidal substances by bacteria (Fukami *et al.*, 1997) and/or pH fluctuations as a result of the photosynthesis. Kirkwood *et al.* (2006) reported how the production of exudates by cyanobacteria did not completely inhibit bacterial growth, but instead were used as substrate in a consortium of heterotrophic bacteria and cyanobacteria treating pulp and paper wastewater. In addition, the study revealed that the exudates also enhanced the removal of dichloroacetate and at the same time affected the removal of phenolic compounds.

Choi *et al.* (2010) reported the negative effect of cyanobacteria on the nitrification rates in a bioreactor growing only nitrifiers. The presence of algae and cyanobacteria in the autotrophic bioreactor inhibited the maximum nitrification by a factor of 4, however, the ammonium was still efficiently removed (Choi *et al.*, 2010). Other negative effects of microalgae on bacteria are the increase in pH due to the photosynthetic activity and high dissolved oxygen concentration. The fast growth rate of microalgae can create a high density in the culture that led to the increase of dark zones, in which microalgae can perform respiration and diminish the amount of oxygen for bacteria (Muñoz & Guieysse, 2006).

On the contrary, there are also microalgae growth-promoting bacteria. As the name states, these bacteria enhance the growth of microalgae. de Bashan *et al.* (2004) demonstrated how the bacterium *A. brasilense* boosted the growth of *Chlorella sorokiniana*, which lead to an effluent with less nitrogen and phosphorus. Additionally, the consumption of oxygen by the aerobic bacteria helps to prevent oxygen saturation conditions.

The presence of bacteria in microalgal cultures improves the flocculation of suspended algae. Some studies have reported the improvement in the settling characteristics of the biomass in microalgal–bacterial cultures through the formation of granules or aggregates (Gutzeit *et al.*, 2005; Lee *et al.*, 2013; Van Den Hende *et al.*, 2014). The formation of flocs in an algal–bacterial consortium is promoted by the bacterial exopolymers, increasing the aggregation and stabilizing the already existing aggregates, while increasing settleability (Subashchandrabose *et al.*, 2011). Algal–bacterial flocs vary from 50 μm to 1 mm, but the predominant size is between 400 and 800 μm (Gutzeit *et al.*, 2005). Tiron *et al.* (2017) reported the development of granules or as the author calls them 'activated algae flocs', for this already formed algal flocs and the bacterial population already present in the raw dairy wastewater were used as inoculum. The developed activated algae granules had a size between 600 and 2000 μm and a settling velocity of 21.6 (±0.9) m/h (Tiron *et al.*, 2017). Figure 3.2 presents an example of an activated algae granule. This positive effect tackles one of the drawbacks of solely algal systems: efficient biomass harvesting. Tiron *et al.* (2017) show that the formation of the granules was achieved

Figure 3.2 Algae granules containing the algae strains: *Chlorella* sp. and *Phormidium* sp. (Source: Tiron *et al.*, 2017).

in a 1.5 L photobioreactor operated as sequencing batch using diluted pretreated dairy wastewater (15.3–21.8 mg NH_4^+-N/L) with an HRT between 96 and 24 hours.

Despite some of the negative interactions, a consortium of microalgae and bacteria enhances the removal of nutrients and other pollutants. The synergistic relationship provides sturdiness to overcome extreme environmental conditions and fluctuations due to operational changes. The complexity of these interactions needs to be understood in order to maximize the positive effects to develop culture conditions that enhance wastewater treatment.

3.1.3 Nutrient removal by microalgal–bacterial consortia

The main difference between an algal system and a microalgal–bacterial consortium in terms of nitrogen removal is the removal pathway. In algal systems, assimilation into the biomass and ammonium volatilization due to pH fluctuations are the two main removal mechanisms. In microalgal–bacterial consortia these are not the only removal mechanisms, but another important pathway of nitrogen removal is nitrification, as nitrifiers can make use of the oxygen produced by the microalgae (Karya *et al.*, 2013). The exchange of oxygen and carbon dioxide allows the efficient removal of organic matter and nitrogen by heterotrophic and nitrifying bacteria. Furthermore, open and closed photobioreactors contain dark zones in which anoxic conditions allow denitrification by anoxic heterotrophic (denitrifying) bacteria.

Phosphorus can be removed from the water either by chemical or microbiological mechanisms. Like nitrogen, phosphorus is an essential nutrient for microalgae. Phosphorus is taken up by algae preferably in the forms of $H_2PO_4^-$ and HPO_4^{2-} and incorporated into the cell through phosphorylation (transformation into high energy organic compounds) (Martínez *et al.*, 1999). However, there is no clear description in the literature about how the phosphorous removal is achieved in waste stabilization ponds, as the reasons are not well understood (Powell *et al.*, 2008). The chemical mechanism of phosphorus removal is through precipitation. This mechanism depends on the pH and the dissolved oxygen concentration in the bulk liquid. At high pH and dissolved oxygen concentrations, phosphorus will precipitate (Cai *et al.*, 2013). de Bashan and Bashan (2004) presented a review of the different forms of phosphorus precipitation. Usually it can occur at pH higher than 9, depending on the concentrations of the different ions and phosphorus. Due to the fact that phosphorus does not exist in gaseous form (like atmospheric nitrogen which eventually could be fixed by algae) and that it can be easily bound with other ions, it is the most important growth limiting factor in microalgae cultivation, besides light (Grobbelaar, 2008). Phosphorus assimilation is the main biological mechanism of removal in algal systems. Di Termini *et al.* (2011) achieved phosphorus removal between 80 and 90% in outdoor and indoor closed photobioreactors through microalgae assimilation.

Several authors have reported the use of microalgal–bacterial consortia for nutrient (nitrogen and phosphorous) removal from real or synthetic wastewater using different types of photobioreactors (Subashchandrabose *et al.*, 2011; Zhang *et al.*, 2018). The different studies showed nitrogen removal efficiencies were between 100% and 15%, whereas the phosphorous removal efficiencies were between 90% and 31.5% (Subashchandrabose *et al.*, 2011).

The symbiosis between microalgae and bacteria offers a large potential for the treatment of nutrient-rich wastewaters, although some aspects need to be taken into account, as they determine the nutrient removal efficiencies or the nutrient removal pathways. The selection of a particular strain for wastewater treatment is a decisive step when engineering a consortium of microalgae and bacteria. In open ponds, there is a natural selection of the microalgae species, which depends on the organic load of the wastewater, species interactions, seasonal environmental conditions, competition and interactions among the microorganisms present in the culture (Riaño *et al.*, 2012). Natural selection of microalgae within a microalgal–bacterial consortium allows to achieve higher efficiencies as there are no inhibitory effects by the source of the wastewater.

González-Fernández *et al.* (2011) compared the removal efficiency of four ponds using microalgal–bacterial consortia for the treatment of pig slurry. The ponds differed in terms of operational conditions (optimal and real conditions) and source of the slurry (anaerobically digested or fresh). The three reported removal mechanisms were nitrification/denitrification, stripping and biomass uptake. Among these three, the main driving force of removal depended on the substrate source. The NH_4^+-N/COD ratio of the substrates was responsible for the different removal rates and the main removal pathway. The anaerobic digested slurry had a ratio of 0.46 NH_4^+-N/COD, whereas the fresh slurry had a NH_4^+-N/COD ratio of 0.13. Since the organic matter in the anaerobically digested slurry is more recalcitrant, the oxygen is more likely taken up for nitrification, the reason why nitrification rates were higher for ponds fed with anaerobically digested slurry (González-Fernández *et al.*, 2011).

Molinuevo-Salces *et al.* (2010) compared open and close configurations and the results showed that even though ammonium was completely removed, the removal mechanisms were different. In the open configuration the biomass uptake was between 38 and 47%, while 52–29% was nitrified/denitrified. In the closed reactor 10.5% was volatilized and 11.3% nitrified, 41% nitrified/denitrified and 31.3% taken up by algae (Molinuevo-Salces *et al.*, 2010). About 80% of the phosphorous was removed regardless of the configuration.

Ammonium removal through nitrification/denitrification as the main removal mechanism in microalgal–bacterial systems has the advantage of achieving faster removal rates in comparison with solely algal systems, especially for high concentrated effluents from industrial sectors. Wang *et al.* (2015) used microalgal–bacterial consortia to treat anaerobically digested swine manure with ammonium concentrations up to 297 (±29) mg NH_4^+-N/L (value after 3 times dilution) in a sequencing batch photobioreactor (4 days hydraulic retention time), achieving a 90% total nitrogen (TN) removal efficiency, from which 80% was removed through nitritation/denitritation without any external aeration. Furthermore, Manser *et al.* (2016) reported the successful combination of microalgae, ammonium-oxidizing bacteria (AOB) and anammox in a sequencing batch photobioreactor achieving ammonium oxidation to nitrite at a rate of 7.0 mg NH_4^+-N/L/h in the light periods and during the night periods in which anoxic conditions were achieved, about 82% of the nitrite was reduced by anammox bacteria (Table 3.1).

Table 3.1 Nutrient removal using microalgal–bacterial consortia for different types of wastewater and using different types of reactors.

Cyanobacterium/ Microalga	Bacterium	Source of Waste Water	Nutrients and Removal Efficiency	System-Reactor Used
Spirulina platensis	Sulphate-reducing bacteria	Tannery effluent	Sulphate 80% (2000 mg/L)	HRAP
Chlorella vulgaris	*Azospirillum brasilense*	Synthetic wastewater	Ammonia 91% (21 mg/L) Phosphorous 75% (15 mg/L)	Chemostat
Chlorella vulgaris	Wastewater bacteria	Pretreated sewage	DOC 93% (230 mg C/L Nitrogen 15% (78.5 mg/L) Phosphorous 47% (10.8 mg/L)	Photobioreactor pilot-scale
Chlorella vulgaris	*Alcaligenes* sp.	Coke factory wastewater	NH_4^+ 45% (500 mg/L) Phenol 100% (325 mg/L)	Continuous photobioreactor with sludge recirculation

(*Continued*)

Table 3.1 Nutrient removal using microalgal–bacterial consortia for different types of wastewater and using different types of reactors (*Continued*).

Cyanobacterium/ Microalga	Bacterium	Source of Waste Water	Nutrients and Removal Efficiency	System-Reactor Used
Chlorella vulgaris	*A. brasilense*	Synthetic wastewater	Phosphorous 31.5% (50 mg/L) Nitrogen 22% (50 mg/L)	Inverted conical glass bioreactor
Chlorella sorokiniana	Mixed bacterial culture from an activated sludge process	Synthetic wastewater	Phosphorous 86% (15 mg/L) Nitrogen 99% (180 mg/L)	Tubular biofilm photobioreactor
Chlorella sorokiniana	Activated sludge bacteria	Pretreated piggery wastewater	TOC 86% (645 mg/L) Nitrogen 87% (373 mg/L)	Glass bottle
Chlorella sorokiniana	Activated sludge bacteria	Pretreated swine slurry	TOC 9–61% (1247 mg/L) Nitrogen 94–100% (656 mg/L) Phosphorous 70–90% (117 mg/L)	Tubular biofilm photobioreactor
Chlorella sorokiniana	Activated sludge bacteria	Piggery wastewater	TOC 47% (550 mg/L) Phosphorous 54% (19.4 mg/L) NH_4^+ 21% (350 mg/L)	Jacketed glass tank photobioreactor
Euglena viridis	Activated sludge bacteria	Piggery wastewater	TOC 51% (450 mg/L) Phosphorous 53% (19.4 mg/L) NH_4^+ 34% (320 mg/L)	Jacketed glass tank photobioreactor
Microalgae present in tertiary stabilization pond treating domestic wastewater	Bacteria present in tertiary stabilization pond treating domestic wastewater	Piggery wastewater	COD 58.7% (526 mg/L) Total Kjeldahl nitrogen 78% (59 mg/L)	HRAP

Source: Subashchandrabose *et al.* (2011).

3.1.4 Microalgal–bacterial systems and configurations

Algal wastewater treatment systems can be divided into open and clos photobioreactors. According to the reactor geometry, closed photobioreactors can be divided into: (1) vertical columns, (2) tubular reactors and (3) flat panel reactors (Wang *et al.*, 2012). Open reactors can be listed into: (a) waste stabilization ponds (WSP), (b) raceway ponds and (c) HRAP. Figure 3.3 presents a scheme of the three most used photobioreactors for algal cultivations. Currently, open systems are the most used type for wastewater treatment and biomass cultivation using microalgae (Carvalho *et al.*, 2006;

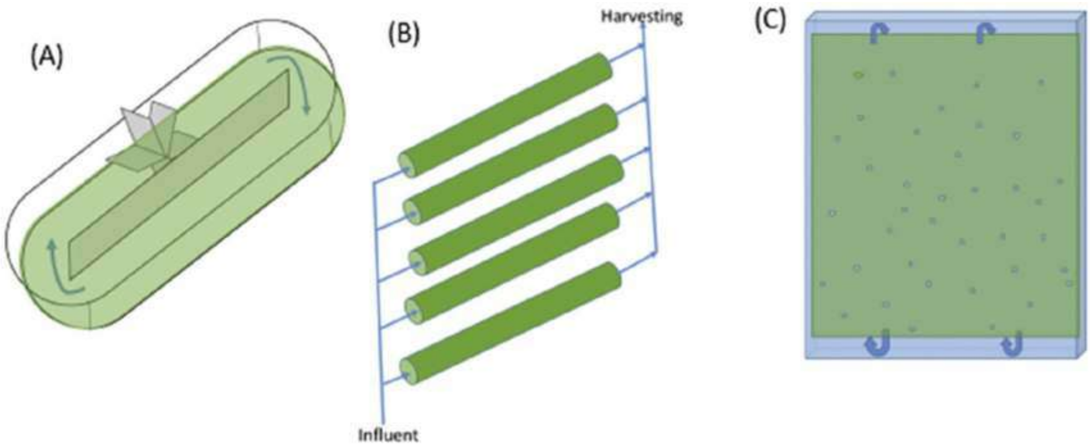

Figure 3.3 The three most used algal system configurations. (a) high-rate algae pond, (b) closed tubular photobioreactor and (c) flat panel airlift reactor. (Source: Wang *et al.*, 2018).

Wang *et al.*, 2012) due to their low investment and maintenance cost and easiness to scale up (Cai *et al.*, 2013). Closed systems are mostly used for sensitive microalgae strains, products vulnerable to microbial degradation or when the harvested biomass is aimed at direct human consumption such as for cosmetics or nutritional supplements (Carvalho *et al.*, 2006). Closed systems have a higher light harvesting, thus biomass production can achieve a higher population density; however, the investment and maintenance costs are higher compared with open systems (Carvalho *et al.*, 2006).

HRAP are the most efficient open systems as they are operated with a higher depth in comparison with the other options. HRAP are raceway-type ponds with depths between 0.2 and 1 m. They can treat up to 35 g BOD/m^2/d compared with 5–10 BOD/m^2/d in waste stabilization ponds (Muñoz & Guieysse, 2006). However, light penetration in such reactors is limited by the depth or solids concentration. Furthermore, open and close systems both require large areas for operation in order to either efficiently remove the contaminants or to achieve high biomass production. Therefore, the reactor selection and the growth medium composition depend on the objective of the system.

3.1.5 Limiting and operational conditions of microalgal–bacterial photobioreactors

There are several factors that can affect the growth of algae and bacteria, especially when using wastewater as growth medium, since there are many substances, compounds and factors to take into account. In open and close photobioreactors there are physical, chemical, biological and operational factors that can limit the growth of microalgae (Borowitzka, 1998). Among those, the parameters that have a strong effect on the efficiency of microalgae and bacteria when treating wastewater are: pH, light intensity, temperature, dissolved carbon dioxide, nutrients, mixing, dilution and algae harvesting (Borowitzka, 1998; Rawat *et al.*, 2011).

In terms of operation, different operational parameters have an effect on the cultivation of microalgae and bacteria separately. Therefore, special attention should be given when combining these two groups of microorganisms. One of the most critical operational parameters is the biomass retention time, which in the case of a consortium can be determined by the influent flow rate and whether there is biomass recirculation. Solid retention time (SRT) and hydraulic retention time (HRT) influence the biomass concentration and the overall productivity of the microalgal–bacterial systems (Valigore *et al.*, 2012). This PhD research study focused on open photobioreactors such as HRAP. For this reason, the implications of some of the factors limiting algal and bacterial growth in high rate open algal ponds are described below.

3.1.5.1 Light

Light is the energy source to perform photosynthesis, allowing microalgae growth. Hence, the uptake efficiency of light is crucial for the productivity of algal biomass and photo-oxygenation. Microalgae can absorb only a fraction of the irradiance, between 400 and 700 nm. This range is called the photosynthetically active radiation (PAR). Open ponds obtain this irradiance from the sun hence, the ponds are shallow in order to allow a maximal light penetration. Height is not the only limitation for the light irradiance, attenuation by the biomass itself is another factor, which can increase when co-cultured with bacteria, and the fact that light can be easily absorbed by other materials or substances (Fernández *et al.*, 2013; Jeon *et al.*, 2005). Dense and concentrated cultures present mutual shading, reducing the light intensity from the illuminated surface to the centre of the reactors, which increase the dark zones and consequently microalgal respiration (Chen *et al.*, 2011; Fernández *et al.*, 2013). Due to this, microalgae are exposed to light/dark zones. For instance, in open ponds except for the upmost thin layer, the irradiance in the pond is below the photo-compensation point for algal growth (Barbosa *et al.*, 2003), as a result of this photosynthetic rates decrease, as well as algal growth. This effect can be compensated by a good mixing which allows the cells to be exposed to a sufficient amount of irradiance (Chen *et al.*, 2011). In open ponds, usually the mixing is provided by a paddle wheel, while aeration is usually applied in closed photobioreactors.

Indoor cultures and closed photobioreactors use other sources of light different from sunlight. For instance, high-pressure sodium lamps, tungsten–halogen lamps, fluorescent tubes and light-emitting diodes (LED lights). Although, these lamps provide a reliable source of energy, the disadvantages are the high power consumption and high operational costs, and they do not contain the full spectrum of light energy (Chen *et al.*, 2011). On the contrary, sunlight is free and holds the full spectrum of light energy.

3.1.5.2 pH

pH is one of the most important parameters in microalgal cultures, as it determines the solubility of carbon dioxide, removal of other nutrients like P and N, and most importantly it affects the metabolism of the microalgae (Becker, 1994). Furthermore, pH fluctuations can inhibit bacterial activity such as autotrophic and heterotrophic bacteria. Fluctuations of pH in microalgae cultures are a consequence of the processes of photosynthesis and respiration during the light and dark periods, respectively. During the day, the pH increases due to the assimilation of CO_2 and the release of OH^-. pH values of up to 10 have been reported after the depletion of NO_3^- and CO_2 (Becker, 1994). Increments of the pH are limited in some cases by the respiration of the different microorganisms. Additionally, nitrogen removal through nitrification has an effect on the pH fluctuations, since the pH decreases during this process due to the release of H^+. Therefore, the addition of ammonium can help to reduce the pH increment (Larsdotter, 2006), making it a good option for pH control in open ponds. Also, the addition of CO_2 can help to control the pH as shown by Park and Craggs (2010).

pH values can affect the growth of microalgae and therefore the removal of nutrients, this can vary for the different strains. Some algae such as *Microcystis aeruginosa* and *Anabena spiroides* have growth limitations and inhibition when exposed to a pH below 6 (Wang *et al.*, 2011). pH fluctuations can also determine the removal of N and P, as higher pH causes ammonium volatilization and phosphorus precipitation. When this occurs faster than the uptake by algae, it leads to algal growth limitation due to the lack of nutrients. Therefore, pH control strategies must be developed in order to avoid possible negative effects caused by drastic pH fluctuations.

In the case of nitrifiers, the growth is suppressed when the pH is not within the 7–8 range (Ekama & Wentzel, 2008a). Nitrification performed by aerobic bacteria release hydrogen ions, reducing the alkalinity of the bulk liquid. Stoichiometrically, for every 1 mg free and saline ammonia (FSA) nitrified, 7.14 mg alkalinity ($CaCO_3$) is consumed (Ekama & Wentzel, 2008a). When alkalinity is lower than 40 mg/L in activated sludge systems, the pH decreases to low values, compromising the nitrification rates and settleability characteristics of the sludge (Ekama & Wentzel, 2008a). In systems working with algae and bacteria, the pH drop by nitrification can be counterbalanced by photosynthetic activity. Also denitrification recovers alkalinity, which occurs under anoxic conditions. In algal–bacterial systems, dark conditions guarantee the absence of oxygen production by algae, instead algae respire releasing CO_2, which helps to decrease the pH. Based on this, it is evident that the balance in terms of alkalinity between microalgae and bacteria is important.

3.1.5.3 Hydraulic retention time

Hydraulic retention time controls the nutrient loading rates, which at the same time will control the productivity and nutrient removal rate of an algae system. In an open pond with well mixed and steady-state conditions, the productivity is governed by the dilution rate and the depth of the pond. The HRT corresponds to the reciprocal of the dilution rate. In algal ponds and HRAP, the HRT is the same as the solids retention time (SRT), since it is not common to recirculate the biomass, as the harvesting of algal biomass is one of the biggest challenges due to their low cell size (Lee *et al.*, 2013). Therefore, in order to achieve complete removal rates of pollutants, it is common practice to operate algal systems at a HRT between 2 and 8 days and depths between 0.2 and 0.5 m (Shilton, 2006). Due to seasonal variations, it is recommended to vary the HRT, as the temperature changes limit or enhance the growth rates.

Furthermore, shorter HRT in algal systems enhance the biomass production (Oswald *et al.*, 1953; Takabe *et al.*, 2016). Valigore *et al.* (2012) compared different HRT (from 8 to 1.4 days) in a microalgal–bacterial culture, concluding that a shorter HRT enhanced the biomass productivity. However, a shorter HRT can decrease the nutrient removal rates in microalgal–bacterial systems, especially when it can promote wash out of the biomass. An optimum HRT enhances nutrient removal by allowing the proper growth of algal–bacterial populations, which will promote faster nitrification rates, especially since the growth rate of nitrifying microorganisms is low, that is $\mu_m = 0.45$ per d at 20°C (Ekama & Wentzel, 2008a). Therefore, the HRT must be chosen depending on the objective, whether the maximization of the biomass production or the treatment of wastewater. Also, it must be taken into account that due to the depth of the HRAP, a longer HRT will result in larger areas, therefore, optimization of this parameter is crucial for algal systems.

3.1.5.4 Solid retention time

When working with a consortium of microalgae and activated sludge bacteria for nutrient and organic matter removal through photo-oxygenation, the sludge retention time plays an important role within the operational parameters. In fact, it is the most fundamental and important decision for the design of activated sludge systems (Ekama & Wentzel, 2008b). Sludge retention time controls the growth of the microorganisms and corresponds to the relation between the volume of the reactor and the waste biomass flow from the reactor. Therefore, the sludge production in activated sludge systems decreases with the increase of the SRT (Ekama & Wentzel, 2008b). On the other hand, for suspended algae systems, the algae biomass production is controlled by the HRT. This parameter controls the biomass concentrations, which will affect the light utilization by microalgae (Lambeert–Beer law).

3.1.5.4.1 Biomass

Figure 3.4 presents the productivity curve for a flat panel reactor for different biomass concentrations and light intensity. The optimal concentration ($C_{x,\mathrm{opt}}$), where the biomass production is at the maximum, will depend on the efficient use of light. This is achieved when the light at the back of the reactor equals the compensation point for microalgae growth. For lower concentrations, the light will pass through the reactor un-used, whereas for higher values, the light will not be able to reach the bottom/back of the photobioreactor (Janssen & Lamers, 2013). Therefore, there is a need for optimum SRT and HRT combinations to achieve a microalgal–bacterial biomass concentration that allows complete nitrification by ensuring sufficient oxygen without biomass wash-out.

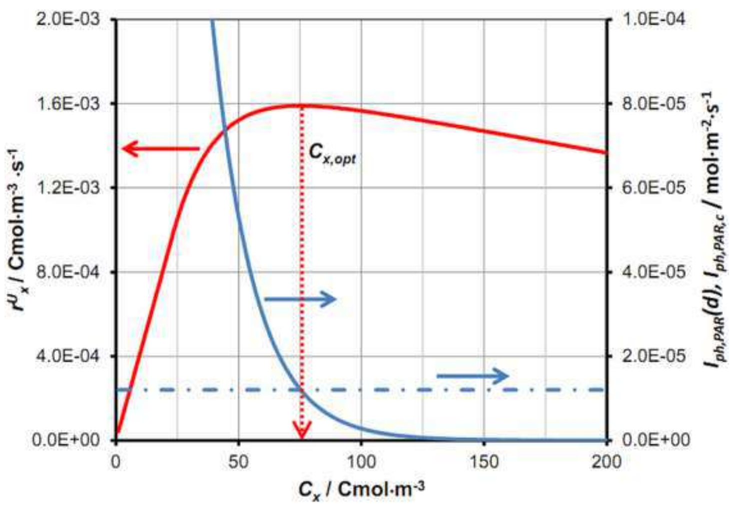

Figure 3.4 Volumetric productivity of a photobioreactor r_x^u as a function of the biomass concentration C_x. Light intensity at the back of the reactor $I_{ph,PAR}$ (d) and the compensation light intensity $I_{ph,\ PARc}$, are also shown. (Source: Janssen and Lamers, 2013).

3.1.5.4.2 Nitrogen removal

Valigore *et al.* (2012) concluded that biomass recycling at a SRT higher than the HRT reduces the wash-out of the microorganisms present in the reactor. Therefore, an appropriate SRT will ensure the successful growth of nitrifiers (slower growing microorganisms in activated sludge) and in addition guarantees light availability for photo-oxygenation. The recommended ranges of SRT values for complete nitrification are divided in two: (1) intermediate, between 10 and 15 days, this range ensures complete nitrification and (2) long sludge age refers to more than 20 days, for which the production of sludge is low with a rather inactive sludge (Ekama & Wentzel, 2008b).

Rada-Ariza *et al.* (2017) showed that the uncoupling of the SRT and HRT is imperative for the development of a steady nitrifying microalgal–bacterial consortium. Furthermore, Arashiro *et al.* (2016) and Rada-Ariza *et al.* (2019) showed the effects of the SRT on the removal mechanism of microalgal–bacterial consortia, still the ammonium removal efficiency was 100% under the different operational conditions tested. In both studies, volumetric and specific ammonium removal rates were higher at shorter SRTs (17 days SRT for Arashiro *et al.* (2016) and 7 days SRT for Rada-Ariza *et al.* (2019)). Furthermore, the ammonium removal mechanisms differ at different durations of the SRT. In Arashiro *et al.* (2016), at a longer SRT of 52 days, ammonium removal by algal uptake represented up to 38% of the total ammonium removal, while it decreased up to 11% at an SRT of 17 days. In both cases, the main ammonium removal mechanism was nitrification/denitrification.

One of the most important operational parameters to control the efficiency and rates of ammonium removal in microalgal–bacterial consortia is the SRT. The SRT controls the amount of solids in the reactor, which will have a high impact on the light penetration used for algal growth and consequently oxygen production. Longer SRTs in activated sludge increase the concentration of endogenous residues, which reduce the active fraction of the biomass and increase the oxygen consumption through respiration of the bacterial biomass (Ekama & Wentzel, 2008b). In addition, longer SRTs increase the solids concentration in the reactor, hence the dark zones within the reactor increase, which will also increase the oxygen consumption by algal respiration. As a result, oxygen is less available for the aerobic processes such as organic carbon oxidation and nitrification, resulting in a shift in the removal mechanism from nitrification to algal uptake. However, if the HRT is not long enough and the ammonium concentration in the influent is high, the efficiency of the system could be hindered and both high concentrations of nitrite and ammonium (partial nitrification and no denitritation) and organic carbon can end up in the effluent.

The uncoupling of the SRT from the HRT permits to select an optimum SRT that allows enough light penetration to maximize the nitrification rates and reduce the solids concentration. This will decrease the endogenous residue by the bacterial biomass, while at the same time increase the growth rate of the nitrifiers (Ekama & Wentzel, 2008a). Decreasing of the SRTs and increasing the ammonium removal rates can help to further decrease the HRT, which would as well offer the possibility to reduce the area requirement of the technology as stated above. However, HRTs shorter than 0.5 days have, to the best of our knowledge, not yet been tested. Therefore, further research studies are required to demonstrate the feasibility of this low HRT. Furthermore, it is imperative to not fall below the SRT_{min} for nitrifiers, since below this value, nitrifiers would be washed out of the system and the system would collapse. Finally, based on the experiments conducted by Arashiro *et al.* (2016) and Rada-Ariza *et al.* (2019), the optimum SRTs for microalgal–bacterial reactors is between 5 and 10 days.

3.1.5.4.3 Biomass retention

The sludge retention time also plays a role in the floc formation, since longer SRT and biomass recirculation enhances the biomass settleability and floc formation (Gutzeit *et al.*, 2005; Medina & Neis, 2007; Valigore *et al.*, 2012). It was reported that settleability of algal–bacterial biomass increased from 13 to 93% when the SRT increases up to 40 days (Valigore *et al.*, 2012). Additionally, Gutzeit *et al.* (2005) achieved during a period of 18 months a flocculent algal–bacterial biomass with excellent sedimentation characteristics, using a SRT between 20 and 25 days. On the contrary, longer SRT promotes algal death due to high solids concentrations, which limits the light penetration and creates higher dark zones increasing the respiration activity (Oswald *et al.*, 1953). Since HRT and SRT can operationally define the removal rate, biomass characteristics and productivity, it is essential to further investigate different conditions of these two in order to define the operational conditions for novel algal–bacterial-based wastewater treatment systems.

3.2 ADVANTAGES OF MICROALGAL–BACTERIAL CONSORTIA FOR AMMONIUM REMOVAL

3.2.1 Advantages on ammonium removal rates

Microalgal–bacterial consortia removed ammonium 50% times faster than in a solely microalgal system, which ultimately increases the efficiency of the system. Rada-Ariza *et al.* (2017) achieved the highest ammonium removal rate and specific ammonium removal rate in comparison with their other studies (Table 3.2). The main removal mechanism that contributed to the increase in the ammonium removal rates was nitrification. Furthermore, other studies have also reported the successful treatment of high strength wastewater using microalgal–bacterial cultures (de Godos *et al.*, 2010; González *et al.*, 2008; Wang *et al.*, 2015; Zhao *et al.*, 2014). The removal rates reported in Table 3.2 based on the research study of Rada-Ariza (2018) are higher than those reported by solely algal cultures treating a diverse range of ammonium concentrations in the influent (Abou-Shanab *et al.*, 2013; Aslan & Kapdan, 2006; Cabanelas *et al.*, 2013). Furthermore, the algae strains used as inoculum were a combination between eukaryotic algae and prokaryotic cyanobacteria (Rada-Ariza *et al.*, 2017). Yet, once the reactors reached steady state, the most predominant algal strain was *Chlorella*. In the literature, it can be found that the most used strains of microalgae for wastewater treatment are *Chlorella* sp. (Cabanelas *et al.*, 2013; Ruiz *et al.*, 2011), *Scenedesmus* sp. (Kim *et al.*, 2013; Park *et al.*, 2010) and *Spirulina* sp. (Olguín, 2003).

The presence of nitrifiers in the microalgal culture increased the volumetric and specific ammonium removal rates. The oxidation of ammonium by nitrifiers is faster than the algal uptake (Arashiro *et al.*, 2016). Therefore, the presence of nitrifiers in the biomass has a strong impact on the removal of ammonium despite they have a low content in the total biomass composition, between 1.8 and 17%

Table 3.2 Volumetric and specific ammonium removal rates of algal bacterial reactors under the different operational conditions tested by Rada-Ariza (2018).

Influent (mg NH_4^+/L)	r_{Am_T} (mgNH_4^+-N/L/h)	k_{Am_T} (mgNH_4^+-N mg/VSS/d)	SRT (d) and HRT (d)	Light intensity (μmol/m²/s)	Reference
297.3	4.16 ± 0.75	1.84 ± 0.12	SRT: 4.2 ± 0.3 HRT: 1	700	Rada-Ariza *et al.* (2017)
23	2.12	0.063 ± 0.009	SRT: 17 HRT: 0.5	25.9	Rada-Ariza *et al.* (2019)
264 ± 10	2.4 ± 0.17	0.033 ± 0.002	SRT: 7 HRT: 4	84 ± 3	Arashiro *et al.* (2016)
45.36 ± 5.52	3.21 ± 0.24	0.063 ± 0.012	SRT: 10 HRT: 1	766.5 ± 154.1	Rada-Ariza (2018)

r_{Am_T}: Volumetric ammonium removal rate; k_{Am_T}: specific ammonium removal rate.

(Arashiro *et al.*, 2016; Rada-Ariza *et al.*, 2017, 2019). Also, the presence of other microorganisms played an important role in the total nitrogen removal. For instance, heterotrophic bacteria not just removed the organic carbon present in the influent, but also removed ammonium for their biomass growth (Arashiro *et al.*, 2016; Rada-Ariza *et al.*, 2019). In addition, during anoxic periods, heterotrophic bacteria, when sufficient organic carbon is present, could denitrify the nitrate or nitrite produced by nitrification (Arashiro *et al.*, 2016; Rada-Ariza *et al.*, 2019).

3.2.2 Operational conditions and area requirement

The ammonium removal rate by a reactor containing just microalgae was 1.84 (±0.66) mg NH_4^+-N/L/h and the specific ammonium removal rate was 0.025 (±0.009) mg NH_4^+-N mg/VSS/d (Rada-Ariza *et al.*, 2017). These values are significantly lower than those for the microalgal–bacterial reactors tested (Table 3.2). Thus, for 100% ammonium removal in the microalgal reactor described in Rada-Ariza *et al.* (2017) and assuming that the volumetric ammonium removal would remain similar, the required HRT would be approximately 6.7 days, assuming all other macronutrients and micronutrients are sufficient. Alcántara *et al.* (2015) calculated that in a microalgae-based system, such as HRAP treating medium-strength domestic water, the necessary HRT would be 7.5 for complete nitrogen and phosphorous removal. Higher nitrogen uptake by algae would result in a higher concentration of solids, which limits the light penetration and thus reduces the growth rate of algae. Noteworthy, HRT values in HRAP could be reduced when carbon dioxide is sparged to avoid inorganic carbon limitation. This can also help as a pH control to maintain an optimum pH. Park and Craggs (2011) obtained ammonium removal efficiencies of up to 83.3% at a HRT of 4 days with CO_2 addition in a high rate algae pond treating an effluent from anaerobic digestion. However, in HARPs with CO_2 supply, the growth of nitrifiers can be enhanced, especially when inorganic carbon is not limiting and in most cases when the HRT is not long enough for nitrifiers to grow (de Godos *et al.*, 2016; Park & Craggs, 2011). The latter occurs in conventional HRAPs where the HRT and the SRT are not uncoupled and therefore the HRT corresponds to the SRT.

The high ammonium removal rates (volumetric and specific) by microalgal–bacterial consortia can further help to reduce the HRT of the system. This can be done by ensuring that the main ammonium removal mechanism within the microalgal–bacterial system is through nitrification. Comparing the oxygen production by algae with the oxygen consumption by nitrification, the yield of oxygen on ammonium consumed is 16.85 gO_2 gNH_4^+-N^{-1} consumed (Mara, 2004). This is significantly higher than the 4.57 gO_2 gNH_4^+-N^{-1} required for complete nitrification (Ekama & Wentzel, 2008a). Therefore, the design of a microalgal–bacterial system should ensure enough oxygen production by algae to support all aerobic processes. Another important condition that should be met is the retention of nitrifiers within the system. Thus, for the cultivation of a microalgal–bacterial consortium in which nitrification is envisioned as the main removal mechanism, there should be an uncoupling between the SRT and the HRT (Rada-Ariza *et al.*, 2017; Valigore *et al.*, 2012).

The possibility of reducing further the HRT by the uncoupling between the SRT and HRT in a microalgal–bacterial system has positive effects on the nitrification process and the objective of microalgae supplying the necessary oxygen to support the aerobic processes. Also, the reduction of the HRT contributes to the reduction of the large area requirements of algal systems. Since microalgae would not be the main removal mechanisms, the limitation of light by solids should be enough to support photo-oxygenation. Therefore, the designing depths of reactors using microalgal–bacterial consortia could be deeper. The microalgal–bacterial system of Rada-Ariza *et al.* (2017) had a surface removal rate of 10.2 g $NH_4^+ - N/m^2/d$, compared with 4.4 g $NH_4^+ - N/m^2/d$ for the microalgal consortia. Comparing these values with the study of Tuantet *et al.* (2014), who achieved a maximum removal rate of 54.1 mg $NH_4^+ - N/L/h$ using urine as growth medium, the surface ammonium removal rate calculated was 6.5 g $NH_4^+ - N/m^2/d$. This value is lower than for microalgal–bacterial systems and also the reactor used for cultivation by Tuantet *et al.* (2014) had a short light path of 5 mm, which avoided any light limitation in the culture.

In practice, HRAP are designed with a HRT between 2 and 8 days and depths between 0.2 and 0.5 m (Shilton, 2006). Using the information reported by Park and Craggs (2011) in a HRAP treating domestic wastewater, the surface removal rate was estimated to be 1.1 g $NH_4^+ - N/m^2/d$, which is considerably lower than the values found in this thesis. In summary, the uncoupling of the HRT and SRT allows to develop a higher settleable biomass. Consequently, both SRT and HRT can be further shortened, which has a positive result on the light limitation by solids and on the nutrient removal rates. As a result, the depth (light path) of the reactors using microalgal–bacterial consortia, in which the main ammonium removal mechanism is through nitrification, can be further decreased, which would help reduce area requirements. Rada-Ariza *et al.* (2017) showed that the area requirements for microalgal–bacterial consortia can be reduced up to 50% in comparison with solely algal systems. Nonetheless, the rates presented in the above study were calculated based on laboratory-scale experiments and more research is required at pilot- and full-scale levels in order to define minimum depths that are able to meet the necessary oxygen production and at the same time maintain the nutrient removal efficiency of the system.

3.2.3 Photo-oxygenation and algal harvesting

Another important advantage of the use of microalgal–bacterial consortia over other technologies are the economic costs. Especially on two aspects: the cost of aeration when comparing this technology with activated sludge and the cost of harvesting when comparing with algal systems. Comparing this technology with activated sludge systems, the oxygen required for nitrification and COD oxidation is fully supported by microalgae (Rada-Ariza *et al.*, 2017, 2019). Operational costs by aeration can represent up to 60–80% (Holenda *et al.*, 2008) of the total operational costs in activated sludge plants. The energy consumption is on average between 0.33 and 0.60 kWh/m^3 in activated sludge plants in the United States (Plappally & Lienhard, 2012), while for HRAP the power consumption for mixing, calculated by Alcántara *et al.* (2015), was 0.023 kWh/m^3. Therefore, the energy needed for removal of ammonium in high strength wastewater using an activated sludge process would be considerably higher when compared with a microalgal–bacterial system.

Another advantage of the microalgal–bacterial systems is the improvement in the settling characteristics of the biomass (Arashiro *et al.*, 2016; Rada-Ariza *et al.*, 2019) when compared with algal systems. The uncoupling of the SRT and HRT, and the operation in sequencing batch creates a selective environment for fast settleable microalgae and furthermore promoted the formation of algal–bacterial aggregates. This positive effect on biomass harvesting by the presence of bacteria in algal systems has been reported by other studies as well (Gutzeit *et al.*, 2005; Park & Craggs, 2011; Van Den Hende, 2014). Furthermore, the increase in settleability reduces the cost of operation in these systems and so no extra energy is required for solids separation, such as centrifugation or dissolved air flotation. In addition, the bioflocculation avoids contamination of the biomass, since no chemicals are needed to promote flocculation (Su *et al.*, 2011).

Several studies found ways to improve this positive effect of algae and bacteria aggregation. Tiron *et al.* (2017) published an approach to develop activated algae granules which have sedimentation velocities of 21.6 (±0.9) m/h and in terms of the separation of the algal biomass from the bulk liquid, the biomass recoveries were up to 99%. Zhang *et al.* (2022a, 2022b) investigated the granulation process of algae/bacteria granules, starting from aerobic granular sludge growing on acetate-based synthetic domestic wastewater. The inoculum aerobic granular sludge size greatly affected the characteristics of the photo-granule and the optimal inoculum aerobic granular sludge size for the start-up of photo-granule process was 0.8–1.4 mm (Zhang *et al.*, 2022a). Furthermore, the granulation process could be accelerated by applying algal–mycelial pellets as nuclei for the rapid development of the symbiotic algal–bacterial granular sludge (Zhang *et al.*, 2022b).

3.3 MICROALGAL–BACTERIAL MODELLING

Modelling of processes in wastewater treatment has the advantage of getting insight into the performance of the technology, evaluation of possible scenarios for upgrading, evaluation of new plant design, support to the decision making related with operational conditions and personal training (van Loosdrecht *et al.*, 2008). Modelling of microalgae systems, more specifically for open ponds, has to take into account several factors, such as light, wind, stripping of ammonia and carbon dioxide, as well as biological and hydrodynamic processes (Gehring *et al.*, 2010). There are several models which focus on different microalgae processes, for instance on the net growth of microalgae (Decostere *et al.*, 2013; Solimeno *et al.*, 2015; Wágner *et al.*, 2016), models dealing with light limitation and photosynthesis rates (Yun & Park, 2003), kinetics of nutrient removal (Kapdan & Aslan, 2008), pigments dynamics and respiration (Bernard, 2011) and dissolved oxygen rates (Kayombo *et al.*, 2000).

In the case of activated sludge, bacteria are mostly modelled by a set of models (ASM1, ASM2 and ASM3, ASM3, ASM2d, ASM3-bio-P) developed by task groups of the International Water Association (IWA) and the metabolic model developed at Delft University of Technology (Gernaey *et al.*, 2004). The activated sludge model no. 1 (ASM1) (Henze, 2000) is considered the reference model. It describes the removal of organic carbon compounds and nitrogen, while consuming oxygen and nitrate as electron acceptors. Additionally, it describes the sludge production and has adopted the chemical oxygen demand (COD) as measurement unit for organic matter (Gernaey *et al.*, 2004). Furthermore, similar to ASM1, ASM3 was developed to correct the deficiencies of the ASM1 model. The main difference of the ASM3 model is the inclusion of the intracellular storage process of readily biodegradable COD, for the slower conversion from readily biodegradable into slowly biodegradable organic matter (Gernaey *et al.*, 2004; van Loosdrecht *et al.*, 2008). Other models include biological phosphorus removal, i.e. ASM2d and the TUDelft model (van Loosdrecht *et al.*, 2008).

As mentioned in previous sections, usually in open ponds that are treating wastewater, not only microalgae play a role in the removal of nutrients and biomass production, but at the same time, heterotrophic and nitrifying bacteria carry out different processes like oxidation of organic matter, nitrification, denitrification and respiration (Figure 3.1). Therefore, they make the system more complex as those microorganisms and their associated parameters and variables should be taken into account. Furthermore, models describing these complex relationships should be based on the microalgae models and activated sludge models. Models describing the relationships of algal–bacterial consortia in open ponds have been reported at first by Buhr and Miller (1983). Their objective was to develop a mathematical model for high-rate algal–bacterial wastewater treatment systems. This model takes into account the algal and bacterial growth, light limitation and solution equilibrium related with the pH and mass balances. The variations of pH, DO and substrate concentrations along the pond length were evaluated under different feed loads and hydraulic residence times. Later on, Gehring *et al.* (2010) developed a model to simulate the processes in a waste stabilization pond. The activated sludge model no. 3 (ASM3) was used as a basis. The new components were the integration of algae biomass and gas transfer processes for oxygen, carbon dioxide and ammonia depending on wind velocity. Furthermore, it had the possibility to model the algae concentrations based on measured chlorophyll-*a*, light intensity and total suspended solids (TSS) measurements (Gehring *et al.*, 2010). However, modelling of nitrification and denitrification was not considered in the simulations carried out by Gehring *et al.* (2010) because the experimental data did not show any nitrification or denitrification rates. Therefore, the model was not evaluated under the two conditions of nitrification and algal growth.

In the literature some models focused on algal–bacterial consortia (Solimeno *et al.*, 2017; van der Steen *et al.*, 2015; Wolf *et al.*, 2007; Zambrano *et al.*, 2016). Solimeno *et al.* (2017) developed the BIO-ALGAE model for suspended microalgal–bacterial biomass, which was an updated version of the algal model proposed by the same author (Solimeno *et al.*, 2015). The model was calibrated and validated, reporting good results on the prediction of biomass characterization. Furthermore, it identified the light factor as one of the most sensitive parameters for microalgal growth. The model takes into account the algal growth on carbon and nutrients, gas transfer to the atmosphere, photorespiration and photoinhibition.

The PHOBIA model was developed by Wolf *et al.* (2007) for microalgal–bacterial biofilms. It includes the modelling of different kinetic mechanisms of phototrophic microorganisms, such as internal polyglucose storage, growth in darkness, photoadaptation and photoinhibition, as well as nitrogen preference (Wolf *et al.*, 2007). These models can serve as a basis for the development of further models whose aim is to explain and describe the microalgae–bacteria symbiosis for their cultivation for wastewater treatment in suspended cultures. For this reason, there is still a need for models calibrated and validated with longer data sets or at different operational conditions treating diverse types of wastewaters.

3.4 INTEGRATION OF PHOTOACTIVATED SLUDGE IN WASTEWATER TREATMENT CONCEPTS

The photo-activated sludge (PAS) system could fit within a holistic approach for wastewater treatment consisting of an anaerobic digester coupled with a microalgal–bacterial photobioreactor (Figure 3.5). The anaerobic digester is used for bioenergy production through a combined heat and power (CHP) system and the high nutrient strength centrate is further treated in a microalgal–bacterial photobioreactor. The biomass produced in the photobioreactor can be returned to the anaerobic digester to increase biogas production by co-digestion with the main waste(water) streams (Wang & Park, 2015). Part of the stabilized solids from the anaerobic digester and the microalgal–bacterial reactor could be used as biosolids for fertilizer replacement, promoting a circular economy within the treatment of wastewater.

At full scale and using sunlight as energy source, it is important to take into account the feeding conditions of the medium. However, this also depends on the final objective of the water reclamation of the treated effluent. For instance, effluents with high concentrations of nitrate, when just nitrification is performed in the microalgal–bacterial system, can support irrigation for crop growth (Taylor *et al.*, 2018). In case that due to the prior treatment there is a lack of micronutrients or other nutrients such as phosphorous, the effluent can be mixed in a certain ratio with the influent from the anaerobic digester to supply all the compounds needed. When the objective of the microalgal–bacterial system is the treatment of the wastewater to negligible ammonium and total nitrogen concentrations, the system should support nitrification and denitrification as shown by Arashiro *et al.* (2016) and Rada-Ariza *et al.* (2019). Then, during a HRT of 1 day, nitrification can be performed during the daylight and denitrification can be supported at night when there is no longer oxygen production. Therefore, it is recommended that the influent is fed during the dark conditions, then some of the oxygen

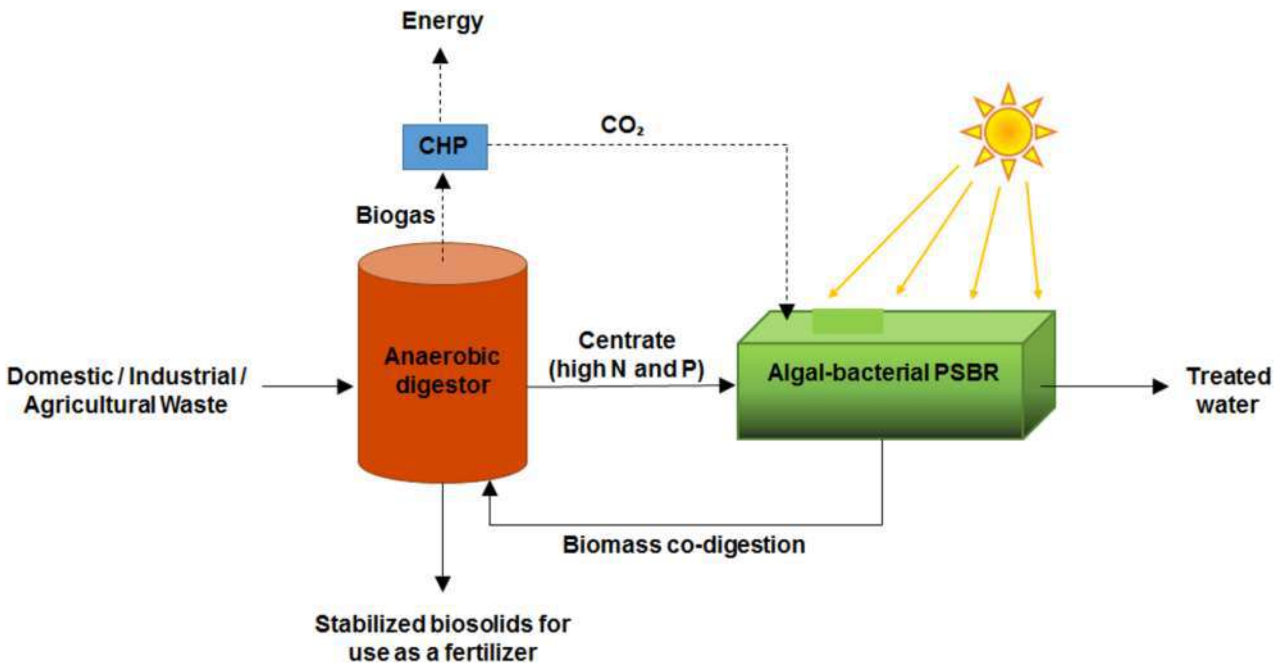

Figure 3.5 Scheme of the proposed holistic approach for treatment of domestic, industrial and agricultural wastes. CHP: combined heat and power system, N: nitrogen and P: phosphorous.

still present from the light phase would be consumed for organic matter oxidation and part of the ammonium would be oxidized or taken up by algae. The rest of the organic matter would be used for denitrification and the remaining ammonium that is not nitrified or taken up in the dark phase would be nitrified in the next light phase.

3.5 CONCLUSIONS

Microalgal–bacterial consortia are able to effectively remove nitrogen at shorter SRTs and HRTs than usually used in algal systems, showing high ammonium removal efficiencies. Furthermore, the co-cultivation of microalgae and bacteria offers advantages such as higher ammonium removal rates through nitrification/denitrification and consequently reduction of the area requirements in the implementation of the technology. Also the development of a bioflocculant algal–bacterial biomass without the addition of chemicals or energy input is an advantage. The symbiosis of microalgae and bacteria has shown promising results not just for nutrient and organic carbon removal, but for the elimination of other pollutants and contaminants from different industries as well (Rawat *et al.*, 2011). This offers new directions for research on microalgal–bacterial consortia. New studies on the co-culturing of different microorganisms for treatment of wastewater have already been reported (Manser *et al.*, 2016; Mukarunyana *et al.*, 2018). This shows the ability of algae to be resilient and adapt to different microbial populations and environments, and can help to further develop microalgal–bacterial consortia as sustainable approach to today's and tomorrow's wastewater problems.

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Chapter 4

Macroalgae biorefinery and its role in achieving a circular economy

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ABSTRACT

Availability of fossil fuels and feedstocks is a major problem currently faced by a variety of sectors thus highlighting the importance of transitioning towards a circular economy. Increased pollution, fossil fuel availability and other adverse effects are just some of the reasons that have prompted a need to find additional resources for fuel. One such feedstock that has shown to be both promising and viable is macroalgae. This chapter focuses on the latest scientific literature related to the development of macroalgae biorefineries, focusing on the different biological processes and how the resulting generated bioproducts can positively impact the global bioeconomy. The fundamental biological processes are explained while also providing details on specific problems the sector currently faces. Potential areas of further development and recent scientific discoveries of a variety of macroalgal species are also discussed.

4.1 INTRODUCTION

The world energy consumption was recorded at 488 EJ (exajoule) in 2005, 580 EJ in 2018 (Kober *et al.*, 2020) and is expected to exceed 650 EJ by 2025; 86% of this can be attributed to fossil fuel energy (Drapcho *et al.*, 2008). These figures indicate a clear overreliance on the use of fossil fuels across many different industries. One such sector which plays a huge role involving this energy consumption is the transportation sector. The current use of high-powered vehicles in the transportation sector makes it difficult to promote decarbonization. For this reason, researchers have focused on the promotion of biofuel usage and production in achieving a more sustainable future involving transportation. According to the International Energy Agency (IEA) (2014), one third of the final energy consumption is associated with transport-related liquid fuels such as petrol and diesel. This clear overreliance on a non-renewable energy source has led to many organizations working towards development of a plan to transition to a mode of cleaner energy consumption. The need for this transition is further highlighted by directives from both world and European environmental agencies in highlighting responsibilities regarding energy admission and consumption. One such directive is the terms of the European Union (EU) Renewable Energy Directive stating a new, legally binding aim for the EU's use of renewable energy for 2030 of at least 32%, with a provision for a potential modification to the higher level in 2023. This objective builds on the 20% renewable energy supply goal for 2020 (EEA, 2023).

To fulfil such directives, the production and utilization of biofuels is of utmost importance. Electrification of transport through battery electric vehicles (BEV) is a viable option for light vehicles and short-distance heavier transport (Forrest *et al.*, 2020), but to decarbonize long-distance heavy vehicles we will need renewable hydrocarbon fuels either in gaseous or liquid form (Gray *et al.*, 2021). Biofuels, such as biogas, biomethanol, bioethanol and biobutanol, are considered an alternative to fossil fuels going forward because they can reduce transport emissions and increase the security of supply (Nigam & Singh, 2011). Another biofuel which has been shown to have potential consists of a hydrogen (H_2) and methane (CH_4) blend known as biohythane (Lay *et al.*, 2020). Biohythane consists of a blend of 70–90% v/v methane and 10–30% v/v hydrogen (Bolzonella *et al.*, 2018). Research has shown that this biofuel exhibits major potential in terms of application in the transport sector. By harnessing this potential, this approach can contribute to decarbonizing and fuelling maritime ferries (Dahlgren *et al.*, 2022), along with specific elements within the broader transportation sector (long-distance haulage, coaches and ships). One source of biomass that is effective in the production of these biofuels (biohythane (Keskin *et al.*, 2019) and biogas (Saqib *et al.*, 2013)) is macroalgae (seaweed). Interest in this area has been constantly growing due to the increase in energy demand as well as the potential shown by microalgae in wastewater treatment (Chapter 5) and biofuel production (Chapter 9).

4.2 MACROALGAE SPECIES

4.2.1 Green algae

One species of green algae considered to holster much potential in terms of energy is *Ulva lactuca* (Figure 4.1a). Utilization of this species is appealing due to its high potential growth rate and high content of carbohydrates. Nutritional composition studies have shown that carbohydrates are the major component of *U. lactuca*, nearing 60% (Rasyid, 2017). Pre-treatment, saccharification, fermentation, and distillation are all steps in the conversion of macroalgae to bioethanol. Korzen *et al.* (2015) demonstrated the use of sonication as a pretreatment method in bioethanol production from *Ulva* sp. Since macroalgae contain low lignin (5.11% according to Allouache *et al.*, 2021), they can be easily depolymerized. Enzymatic hydrolysis of the resultant polysaccharides, followed by the addition of microbes such as *Saccharomyces cerevisiae*, can convert them into ethanol.

Currently, most of the naturally produced *U. lactuca* biomass is an unused resource ending up in a landfill due to the waste problems it poses to beaches and ultimately not being used efficiently for energy conversion. This build-up has also resulted in beach waste problems (Figure 4.1b, d and f) in countries such as Spain (Madejón *et al.*, 2022), Brazil (Harb & Chow, 2022) and Korea (Sunwoo *et al.*, 2017). This highlights a worldwide issue in that there is a need for better utilization for algal bloom waste management. Utilization of seaweed such as *U. lactuca* as a potential source for biofuel production dates back as far as the 'aquatic species programme' that was run in the United States from 1978 to 1996. The conclusion of this study stated that *U. lactuca* usage as a source of energy was not economically feasible (Ryther *et al.*, 1984). While this study may have demonstrated a lack of sustainability going forward, the need to revisit utilizing aquatic energy crops for biofuel production has resurfaced in recent times. Due to issues around climate change and growing opportunities in renewable energy production, traditional biomass availability has plummeted. For this reason, macroalgae are back on the radar due to their offering as an alternative and sustainable resource in terms of production of bioenergy (Lehahn *et al.*, 2016).

U. lactuca growth is commonly found worldwide although the strains vary among regions due to the influence played by different climates. Studies have shown the species has been harvested from shallow coastal areas (Cecchi *et al.*, 1996) or else land-built systems. *Ulva* blooms occur mainly in shallow waters with surplus of nutrients and the decomposition of this alga can produce acidic vapours, which highlights the importance of controlling and cultivating the biomass.

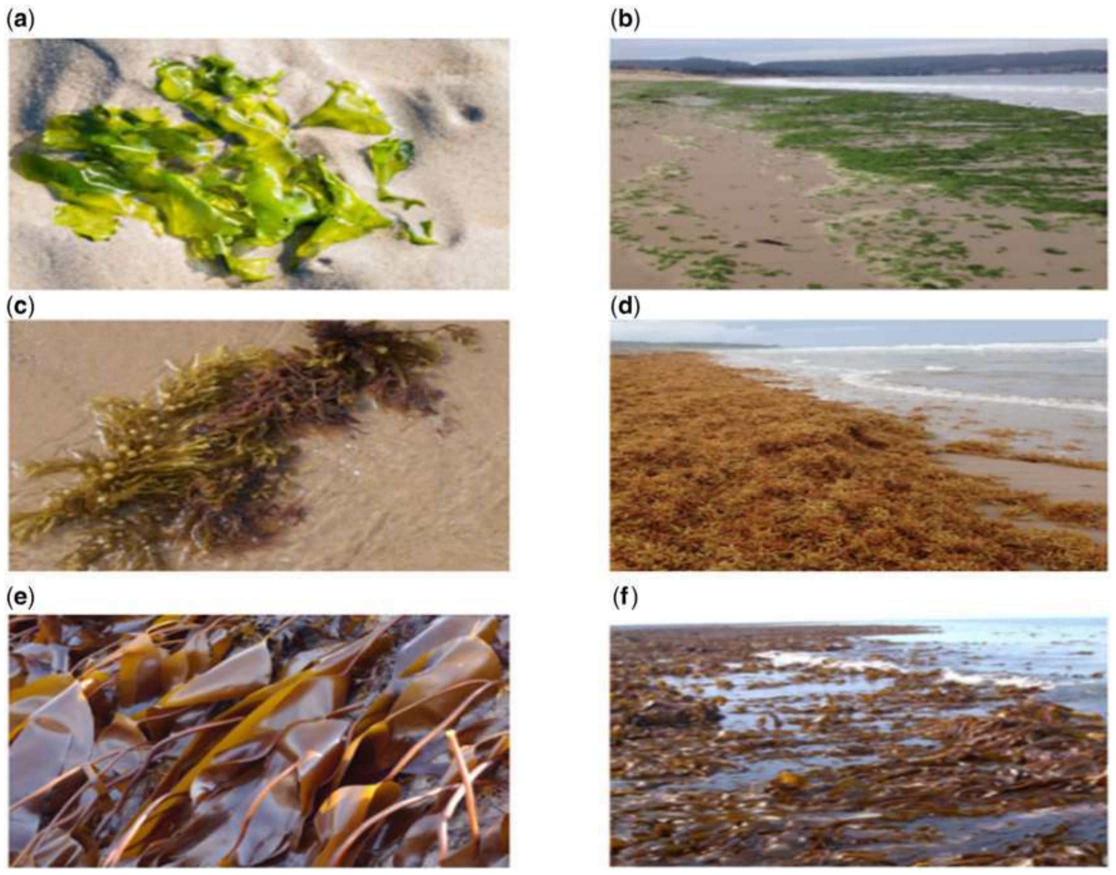

Figure 4.1 Major species of macroalgae with potential for biorefinery applications: (a) *Ulva* sp., (c) *Sargassum* sp., and (e) *Laminaria* sp. as well as their respective blooms (b), (d), and (f).

Growth conditions such as season are the predominant factors which affect the chemical composition of macroalgae (Thorsteinsson *et al.*, 2023). One of the main points of attraction for using *Ulva* sp. in biofuel production is attributed to its high carbohydrate content. This is illustrated by Ortiz *et al.* (2006), who highlighted the total solid carbohydrate content to be close to 60%. This carbohydrate content is predominantly in the form of the complex hydrocolloid ulvan, see Section 4.3. This sulphated polysaccharide is a structural component of the cell wall alongside cellulose (Lahaye & Robic, 2007). The unique chemical properties of ulvan make it an attractive prospect to be used as an active polymer for the pharmaceutical and agricultural sector.

4.2.2 Brown algae

4.2.2.1 Laminaria sp.

Promising macroalgal species used within the bioenergy and bioproducts industry also include *Laminaria* sp. (Figure 4.1e) and *Sargassum* sp. (Figure 4.1c), both of which belong to the brown algae family. Brown macroalgae, referred to as *Phaeophyceae* are the second largest group of macroalgae with over 2000 species identified to date (Guiry, 2023). *Laminaria* contains many structural and functional polysaccharides with compositions as high as 60% (Holdt & Kraan, 2011) as well as its unique alginate composition makes this species an ideal candidate for alginate production. Alongside this, *Laminaria* is also a source of a range of high-value products which are the precursors to biofuels and biochemicals (Bojorges *et al.*, 2022).

To maximize the potential of this species in obtaining these valuable products it is pivotal to select an appropriate pretreatment method. One study demonstrated that hydrothermal treatment is an effective means of improving biohydrogen and methane yield (showing an increase of 26.7%) via two-stage dark fermentation of the species *Laminaria* (Ding *et al.*, 2020). Further appeal in the utilization of this species is illustrated in its wide-ranging polyphenol content featuring both low-weight phenolic acids and sulphated phenolic compounds (Wekre *et al.*, 2022). Phenolics have shown potential in terms of bioactivity features such as acting as an antioxidant, with antidiabetic and anti-cancer properties making them highly desirable for the medical industry (Wekre *et al.*, 2023). One method to extract phenolic compounds from *Laminaria* sp. is an ionic liquid-based extraction which uses three kinds of 1-alkyl-3-methylimidazolium with different cations and anions coupled with ultrasonic treatment (Han *et al.*, 2011). High phenolic compound concentrations have also been proven to function as an inhibiting factor in terms of the digestion process and produce a lower biomethane potential yield (BMP) in brown seaweeds (Hierholtzer *et al.*, 2013). Whereas work conducted on the brown seaweed *Ascophylum nodosum* detailed how seasonal variation during the summer months increases polyphenolic content of the seaweed and in turn adversely affects BMP yield (Tabassum *et al.*, 2016).

4.2.2.2 *Sargassum sp.*

Sargassum sp. (Figure 4.1c) also belongs to the family Phaeophyceae. This macroalgae often floats on the ocean's surface in large quantities which results in the formation of *Sargassum* blooms (Figure 4.1d). Pelagic *Sargassum* blooms, linked to rising sea temperatures and nutrient discharge from the Amazon basin (Thompson *et al.*, 2020), have caused a huge waste management problem for tropical Atlantic countries since 2011 with the costs attributed to beach cleanup rising to US$0.3–1.5 million per kilometre (Rodríguez-Martínez *et al.*, 2023). While this highlights a clear economic problem for the countries affected by more frequent blooming events, it has a detrimental impact in terms of both ecology and human health-related problems. Ecological impacts include the smothering of coral reefs causing fish deaths due to hypoxia and the alteration of pH in coastal waters. One study illustrates this impact on the sea urchin species *Diadema antillarum* (Cabanillas-Terán *et al.*, 2019). The hypoxic conditions generated by the leachates released from the decomposition of *Sargassum* led to reduced taxonomic diversity of the macroalgal food sources. Further findings saw that these changes impacted the trophic characteristics of *D. antillarum*, which highlights the need for this ongoing *Sargassum* problem to be addressed before further impacts into the functioning of coastal ecosystems and alterations in biodiversity arise.

While much attention has been given to the environmental impacts of *Sargassum,* it is highly important to focus on the health hazards it can pose to humans and animals. Following the decomposition of *Sargassum* onshore, large amounts of toxic gases such as hydrogen sulphide (H_2S) and ammonia (NH_3) are produced which is a problem also associated with *U. Lactuca* in both France (Loret *et al.*, 2020) and Ireland (Murphy *et al.*, 2015). Human exposure to such gases can have health consequences such as hypoxic pulmonary, neurological, and cardiovascular lesions. Across an 8-month spell in 2018, it was found that exposure to such toxic gases reached case numbers of 3341 in Guadeloupe and 8061 in Martinique (Resiere *et al.*, 2018). Alongside these health threats, *Sargassum* blooms have also been shown to impact the economy and loss of income due to many of the impacted countries relying heavily on tourism. To combat these ongoing problems government agencies have developed ecological briefs detailing best practices and methods of remediation of the waste generated (Hinds *et al.*, 2016) as well as providing funding to the affected countries (Oxenford *et al.*, 2021).

4.3 BIOMATERIALS AND BIOPRODUCTS FROM MACROALGAE

Macroalgae exhibit many advantages over alternative biofuel feedstocks. Unlike feedstocks used for second-generation biofuels high in lignocellulosic materials, macroalgae are easier to biologically degrade. Subsequently, the digestion of algae may be shown to be cost-effective in comparison to feedstocks derived from lignocellulosic crops. Moreover, macroalgae do not compete with food sources for land usage or irrigation by freshwater (Smith *et al.*, 2010); though they are a significant resource for food in Asian countries (Pereira, 2021). Macroalgae can take advantage of the nutrients present in wastewater and seawater to promote growth. In addition, macroalgae also boast a faster growth rate with higher biomass yields in comparison to other terrestrial plants (Dutta *et al.*, 2014).

Ulva sp. provide a potential in terms of extraction of its high-value product, that is Ulvan, and utilization of the leftover biomass in terms of biofuel production. Figure 4.2 details the possible routes in which *Ulva* sp. may be utilized within a biorefinery concept. Ulvan is a cell wall polysaccharide found in *Ulva* species and its percentage composition in dry-weight biomass shows a variance from species to species of 8–29% (Lahaye and Robic, 2017), and 9–36% (Lakshmi *et al.*, 2020). Ulvan is a value-added product which is used in the pharmaceutical industry as a biomaterial. It can be harvested prior to *Ulva* biomass use in anaerobic processes for biofuel production to boost the overall efficiency and profitability of the process, as ulvan within a reactor is a potential precursor for high sulphide levels that is an inhibiting compound for anaerobic bacteria (Chen *et al.*, 2008). Ulvan is used in hydrogels (Morelli & Chiellini, 2010), membranes and films, and, particularly, in food packaging due to its antioxidant properties (Ganesan *et al.*, 2018). Pharmaceutically it is currently being investigated for anticancer properties although there are thus far no human trials (Kidgell *et al.*, 2019) and similar investigations are being undertaken regarding its immunomodulatory effects (Kidgell *et al.*, 2020).

Ulvan can be extracted in several different ways including acid extraction, combined enzymatic and chemical extraction (Yaich *et al.*, 2017) and Soxhlet extraction (Ben Amor *et al.*, 2021). Acid extraction is particularly cost-effective and eco-friendly as citric acid can be utilized (Manikandan & Lens, 2022a, 2022b). The principle relies on the hydrophobic nature of rhamnose causing ulvan to fold into a neutral pH that will then aggregate in the presence of NaCl allowing for easy removal from the solution (Kidgell *et al.*, 2019).

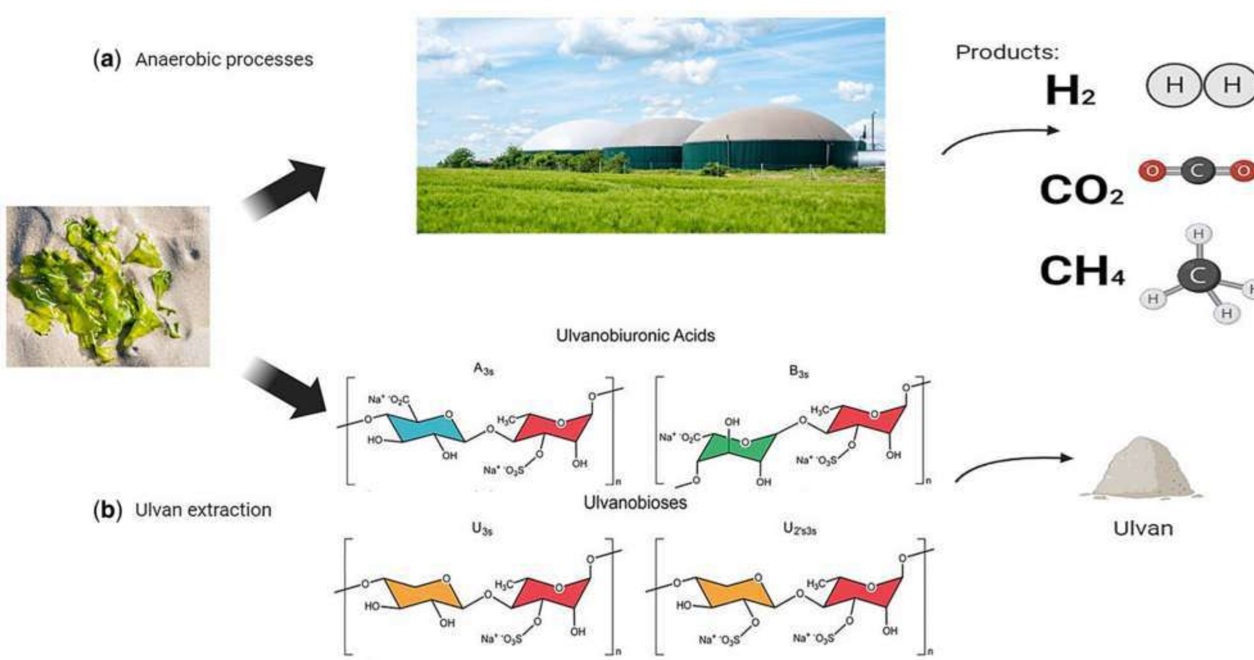

Figure 4.2 Potential biofuel and bioproducts produced from *Ulva* sp. (a) Biofuel production – H_2, CO_2, CH_4 and (b) ulvan – a cell wall polysaccharide utilized within the biopharmaceutical industry.

4.4 BIOFUELS FROM MACROALGAE

The transportation sector is one of the largest and fastest-growing energy consumers in today's world, while also being difficult to fully decarbonize (Papadis & Tsatsaronis, 2020). For this reason, it is pivotal to commit to a future that consists of lowering carbon consumption and increasing sustainable energy. To allow for this transition to occur, it is important to find means of maximizing energy efficiency, discovering renewable energy supplies, and optimizing energy systems from source to end use. Transitioning to a cleaner energy future using biofuel energy will bring inflated costs due to the need for robust investment in research and technological development. For this reason, it is pivotal that innovative and cost-saving technologies are used. The process of anaerobic digestion has been demonstrated to be an effective and feasible way of producing biofuels from the digestion of various feedstocks such as seaweed (Tabassum *et al*., 2017a, 2017b).

Due to the ongoing fuel and climate crisis and efforts being made in reaching a circular economy, there is a newfound importance placed on maximizing bioprocesses to generate multiple products from the same biomass. This is not different to the seaweed industry with growing interest due to its potential use in creation of a variety of bioproducts and biofuels. One of the most promising products in the seafood sector, the commercial seaweed market is anticipated to rise from $15.01 billion in 2021 to $24.92 billion in 2028 at a compound annual growth rate (CAGR) of 7.51% (CBI, 2023). Furthermore, the compound annual growth rate of the industry is estimated at 9.7% for the years 2020–2025 (Mordor Intelligence, 2022).

4.4.1 Biogas

An advantage of utilizing algae is that algal tissue which is used to produce biofuels may potentially be a waste product from other industries. Chemical compounds and components of many algal species are used in food and livestock feed production. The extraction of value-added products from biofuel feedstock creates a sustainable cyclical system, especially considering the potential for leftover biomass after fuel production as fertilizer for crops or substrate for generating other types of biofuels. *Ulva lactuca* has been identified as having potential use to produce biofuels (Bikker *et al*., 2016), such as methane (Bruhn *et al*., 2011) and hydrogen (Dogmaz & Cavas, 2023).

Previous research into the role of *Ulva* sp. in biofuel production suggested that it is not economically viable or sustainable (Ryther *et al*., 1984; Liu *et al*., 2022). Allen *et al*. (2013) indicated that despite a low C:N ratio in *Ulva* sp., if pretreated, this macroalgae can be a suitable source for third-generation biofuel production. *Ulva* sp. is favourable for biofuel production due to its composition being enriched in polysaccharides, starch, and cellulose which are vital components required for microbes to feed on in producing clean biofuels like H_2 (Olsson *et al*., 2020). Table 4.1 details various biomethane yields obtained from a range of seaweeds featuring a variety of pretreatment methods.

4.4.2 Biohydrogen

Biohydrogen production from marine macroalgal biomass is considered a clean energy technology with a high caloric value produced via dark fermentation. In comparison to the complex and extremely variable cell wall of lignocellulosic biomass (Oliva *et al*., 2022), macroalgae features a much simpler carbohydrate cell wall which allows for a variety of biomass pretreatment methods to be applied in enhancing biohydrogen production. Various pretreatment technologies feature physical, chemical, biological, and combinational that allow for the breakdown of algal biomass into simpler compounds and releasing fermentable sugars efficiently. Table 4.2 details various biohydrogen yields obtained from a range of seaweeds featuring a variety of combined pretreatment methods.

Issues surrounding the use of algal biomass in biohydrogen production may be attributed to factors such as its high ammonium, sodium, and sulphate content (Xia *et al*., 2016). This high sulphur content can lead to increased levels of H_2S production, which is a foul smelling, toxic and corrosive harmful gas. Optimization of the carbon to sulphur ratio can overcome the bottleneck that comes with utilizing *Ulva* with a high sulphur content in a dark fermentation process (Allen *et al*., 2014).

Table 4.1 Comparison of biomethane yields and varying pretreatment conditions obtained from *Laminaria, Sargassum* and *Ulva* sp.

Seaweed	Inoculum	Pretreatment	Biomethane Yield	Reference
Laminaria digitata	Digested slurry	Mechanical	282 L CH_4/kg VS	Tabassum *et al.* (2017a, 2017b)
Sargassum fulvellum	Digested slurry	Enzymatic	186.60 mL CH_4/g VS	Farghali *et al.* (2021)
Sargassum fulvellum	Digested slurry	Mechanical	142.91 ± 0.004 mL CH_4/g VS	Yuhendra *et al.* (2021)
Ulva lactuca	Cattle digestate	Biologically	408 ± 20.02 mL CH_4/g VS	Mhatre *et al.* (2019)
Ulva lactuca	Digested slurry	Drying	250 L CH_4/kg VS	Allen *et al.* (2013)

Table 4.2 Comparison of biohydrogen yields and varying pretreatment conditions obtained from *Laminaria, Sargassum and Ulva* sp.

Seaweed	Inoculum	Pretreatment	Biohydrogen Yield	Reference
Laminaria japonica	Seed sludge	Mechanical	71.4 mL H_2/g TS	Shi *et al.* (2011)
Laminaria japonica	Anaerobic sludge	Microwave – acid treatment	28 mL H_2/g TS	Yin and Wang (2018)
Sargassum tennerimum	Rumen fluid	Ultrasonic coupled treatment	86 mL H_2/g COD	Snehya *et al.* (2022)
Sargassum sp.	*C. saccharolyticus* DSM 8903	Mechanical	91.3 ± 3.3 L H_2/kg VS	Costa *et al.* (2015)
Ulva fasciata	Rumen fluid	Surfactant-coupled sonication	91.7 mL H_2/g COD	Snehya *et al.* (2021)
Ulva reticulata	Digested sludge	Surfactant-induced microwave disintegration	54.9 mL H_2/g COD	Kumar *et al.* (2022)

4.4.3 Biohythane

Biohythane – a H_2 and CH_4 blend, is produced in a two-stage fermentation process. The first stage (operated at a low pH and retention time with a corresponding relatively high organic loading rate with inhibited methanogenesis) involves H_2 production controlled by a diverse population of hydrolytic and acidogenic bacteria. The metabolism of hydrogen involves the oxidation of pyruvate to acetyl-CoA by the enzyme pyruvate-ferredoxin oxidoreductase by obligate anaerobes. Hydrogen is then formed due to the reduction of ferredoxin as it undergoes oxidation by the enzyme hydrogenase. Hydrogen can also be formed by facultative anaerobes which oxidize pyruvate to formate and acetyl CoA following the catalysis of the enzyme pyruvate formate lyase (Hallenbeck, 2013). Meanwhile, in the second stage (neutral pH, retention time typically 5 times higher and organic loading rate typically five times lower than that of the first stage), methanogenic archaea control methane generation with the enzyme methyl-coenzyme M reductase (MCR) playing a key role (Ghimire *et al.*, 2017). This role is key to the fact that methanogenic microorganisms have an energy metabolism which is controlled by the reduction of C1 transfer coenzymes, enzymes and activated C1 intermediates.

By combining a dark fermentation reactor alongside an AD reactor in a two-phase process, biohythane can be produced in a cost-effective and environmentally friendly way (Bolzonella *et al.*, 2018). Biohydrogen production via dark fermentation is typically carried out by anaerobic bacteria, such as *Clostridium* spp., *Thermoanaerobacterium* spp., *Enterobacter* and *Bacillus* (Reith *et al.*, 2003). This occurs due to the breakdown of glucose into pyruvate through the glycolytic pathway. The fate of pyruvate is then dependent on the microbes present as the pyruvate formate lyase (PFL) pathway is utilized by facultative anaerobes whereas the pyruvate : ferredoxin oxidoreductase (PFOR) pathway is for strict anaerobic microorganisms (Cao *et al*., 2022). Alongside the presence of a suitable microbial community, environmental conditions such as pH (6.0) (Ding *et al*., 2020), temperature (20°C–45°C) (Qu *et al*., 2022) and HRT of 72 h (Soares *et al*., 2020) are favourable in maintaining bacterial cooperation and in turn enhancing the dark fermentation process for hydrogen production. Meanwhile, biomethane production is produced by microorganisms such as *Methanosarcina barkeri* and *Methanococcus*, which require a more stable temperature and pH as well as less vigorous agitation (Battista *et al*., 2016).

In addition, numerous by-products are formed because of the above biological processes involved in producing gas from macroalgae. Volatile fatty acids (VFAs) are a by-product of hydrogen production via dark fermentation and are a value-added product because of the demand for VFAs in industries such as cosmetics, food, bioenergy, and pharmaceuticals. The most well-known VFA is acetic acid, which is often used in food preservation. Butyric acid may be used in bioenergy production as a precursor in the form of ethyl butyrate or butyl butyrate. Butyric acid is also valued in pharmaceuticals as an intermediate in the production of drugs for the treatment of cancers such as leukaemia and colorectal cancer (Pouillart, 1998).

4.4.4 Bioethanol and biobutanol

4.4.4.1 Acetone–butanol–ethanol fermentation

Numerous studies have been completed on the conversion of lipids from algal species into alcohols by a variety of different methods. This fermentation strategy is known as acetone–butanol–ethanol (ABE) fermentation (Figure 4.3). The strategy usually utilizes *Clostridium* sp. to ferment sugars to form acetone, butanol, and ethanol in a ratio of 3:6:1 (Awang *et al*., 1988). Several different microorganisms from the *Clostridium* genus can be used in ABE fermentation with all having slightly different product distributions, nutrient requirements, and carbon source preferences. Such organisms are from the *Clostridia* species such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium saccharobutylicum*, and *Clostridium saccharoperbutylacetonicum* (Patakova *et al*., 2013).

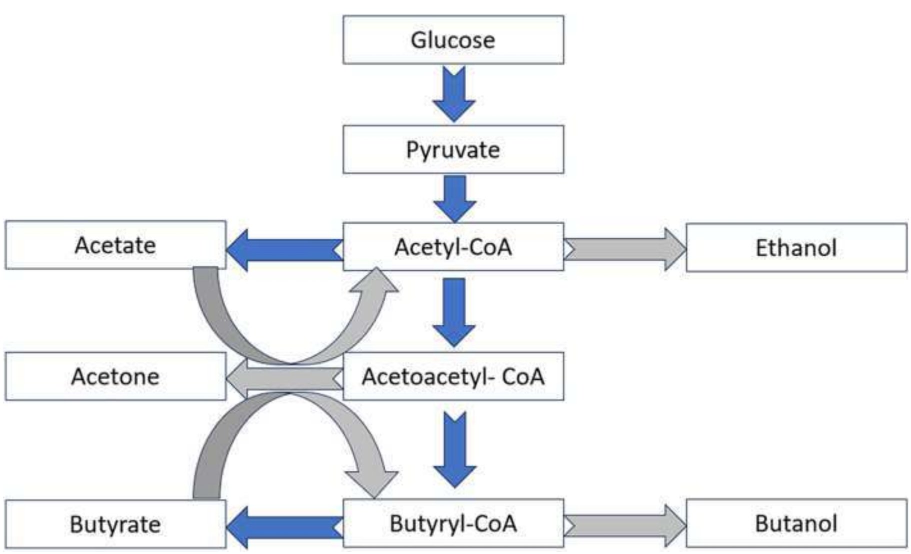

Figure 4.3 Schematic diagram of the metabolic pathway in ABE fermentation – arrows classifying an enzymatic conversion. Notched arrows (⇨), as seen first from glucose to pyruvate, indicate multiple steps shown as one. Arrows in blue represent the steps in acidogenesis, and arrows in grey represent reactions during the solventogenesis phase.

Typically, ABE solvents are produced during two designated time-based phases (Potts *et al.*, 2018). First bacteria produce organic acids such as lactic acid, acetic acid, and butyric acid, which is followed by bacteria converting the acids to their corresponding solvents. In this two-stage process, stage one is known as acidogenesis, while stage two is referred to as the solventogenesis phase. The move from acidogenesis to solventogenesis is triggered as cell growth slows down following the rapid production of acetate and butyrate that occurs during acidogenesis (Amador-Noguez *et al.*, 2011). Typically, a change in pH of the fermentation broth allows metabolism to transition between acidogenesis and solventogenesis (Richter *et al.*, 2016).

4.4.4.2 Biobutanol

Like ethanol, butanol is also typically a biomass-based renewable fuel that can be produced by alcoholic fermentation of a range of different feedstocks with one being macroalgae, as reported by Potts *et al.* (2012). By comparison of carbon structures, butanol (C_4H_9OH) possesses a four-carbon structure whereas methanol (CH_3OH) and ethanol (CH_3CH_2OH) have a one- and two-carbon structure, respectively. A benefit of butanol is its ability to blend with gasoline efficiently while studies have also demonstrated potential of blending with diesel (Yusri *et al.*, 2019). Due to butanol possessing a higher oxygen content than biodiesel, there is a reduction in the amount of soot produced. A further advantage of using butanol over ethanol and petrol blends (Sanap *et al.*, 2023), is related to the fact NO_x emissions can be reduced due to its higher heat evaporation, thus resulting in a lower combustion temperature (Rakopoulos *et al.*, 2010). The main disadvantage centred around the use of butanol is related to its low production rates and end-product toxicity and for this reason often ethanol production was favoured over that of butanol. Nevertheless, thanks to recent advancements in technology and the development of butanol fermentative techniques, the production rates of butanol have been improved. One study indicated that the production cost of butanol from wheat straw stands at $1.37/kg (Wang *et al.*, 2023). Meanwhile, a second study also detailed through a technoeconomic analysis of the production of butanol alongside further biorefinery products from the macroalgae *Ulva rigida* is also economically feasible. Results from the modelling indicated an internal rate of return (IRR) of 37% (Llano *et al.*, 2023).

4.4.4.3 Bioethanol

The utilization of biofuels in the transportation sector is constantly growing. According to preliminary European Environment Agency (EEA) statistics, in 2021, the proportion of renewable energy utilized for transportation in the EU stabilized at 10.2% (EEA, 2021a, 2021b). Two products which have demonstrated their potential for use in this sector are butanol and ethanol. Ethanol is a biomass-based renewable fuel that is commonly produced by the fermentation of sugar from a range of different substrates one being macroalgae (Enquist-Newman *et al.*, 2014). It is often considered an alternative fuel for internal combustion engines (Li *et al.*, 2019). The adoption of ethanol blended fuel (E85, 85% ethanol and 15% fossil fuels) vehicles together with electric and compressed natural gas vehicles is expected to make up 34% of all private vehicle stock by 2050 (Saraf & Shastri, 2023). Meanwhile, methanol has also been shown to be a promising fuel of the future with numerous technoeconomic studies available detailing its potential in decarbonization of the maritime industry (de Fournas & Wei, 2022; Shi *et al.*, 2023). Methanol is produced from coal or petrol-based products (Khalafalla *et al.*, 2020) but in future will be generated from reforming of biomethane or reaction of green hydrogen with biogenic CO_2 (Rinaldi & Visconti, 2023). Thus, in 2023 ethanol production is considered more favourable than methanol in industry due to the higher technology readiness of the decarbonized versions of the fuel; although some concerns are detailed in relation to its sustainability from the use of food crops (Kumar *et al.*, 2023). In this reasoning, bioethanol production from macroalgae offers a promising solution (Aslanbay Guler *et al.*, 2023).

While ethanol has clear benefits for use as an engine fuel, several shortfalls need to be addressed to favour its commercialization at a large scale. Due to ethanol being corrosive, problems can occur to the engine's pipelines. Ethanol is corrosive in three different ways: general corrosion, dry corrosion, and wet corrosion. Ionic impurities such as chloride ions and acetic acid are the main causes of general corrosion. Metals such as magnesium, lead and aluminium are often at risk of chemical attack due to dry corrosion, while wet corrosion is caused by ethanol absorbing moisture from the atmosphere leading to an oxidation of most metals (Jin *et al.*, 2011). One such method to overcome this is the use of an inhibitor such as ascorbyl palmitate that acts in protection against corrosion in C-steel in blended fuel (Deyab, 2016).

4.5 MACROALGAL BIOREFINERIES

4.5.1 Biorefinery concepts

All biorefinery concepts focus thoroughly on the maximum valorization of the algae biomass by the production of target compounds of increased value (see Chapter 10). This can be achieved by selection of the cell content and growth characteristics of macroalgae strains, which are often impacted by environmental growth conditions such as light intensity, growth habitat, seawater salinity and temperature (Biris-Dorhoi *et al.*, 2020). Meanwhile, it is also key to look at stimulating the main target compounds during macroalgae cultivation. In recent times, researchers have laid emphasis on the importance of finding multiple cascading approaches to biorefining different species of macroalgae for multiple product generation (Manikandan & Lens, 2023) (Figure 4.4).

Depending on the type of species used and the manner of cultivation, macroalgae can produce biofuels such as CH_4, CO_2, H_2, ethanol and butanol (see Section 4.4). Macroalgal biomass has several advantages over conventional energy crops. Although macroalgae are typically cultivated in the sea, land cultivation is also viable with a tumbling technique adopted. This sees a steady flow of air injected into the cultivation tank suspending the macroalgae and allowing for agitation (Titlyanov and Titlyanova, 2010). Higher production costs associated with land-based cultivation has resulted in this approach being far less common in comparison to offshore farming (Ghadiryanfar *et al.*, 2016). As of 2019, 97% of the global aquaculture output came from artificial

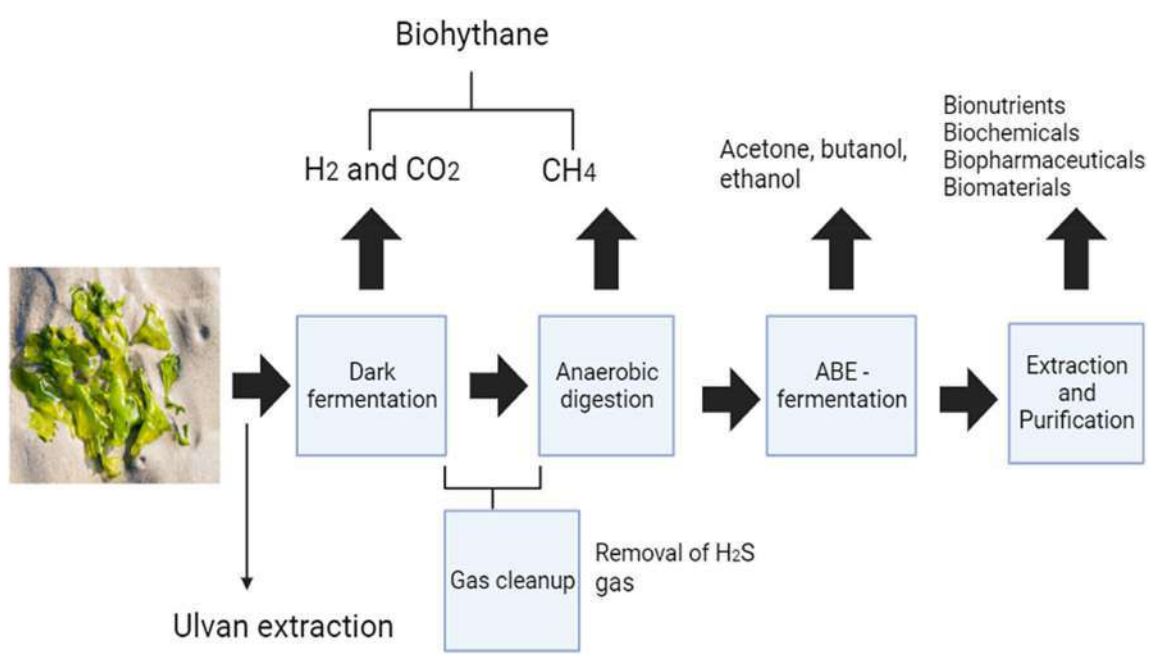

Figure 4.4 Potential biorefinery routes and products achievable utilizing macroalgae as a feedstock.

farming (Zhang *et al.*, 2022) with one study indicating that approximately 20.8 km^2 of the ocean is suitable for farming macroalgae (Liu *et al.*, 2023). As such, a major advantage of macroalgae is that it is cultivated either in the sea or on marginal non-fertile land which leads to a decrease in competition of land for human crop foods (McKennedy & Sherlock, 2015). A second important factor is related to the fact that macroalgae do not need freshwater to grow as seen by their capability to grow in salt water, which is detailed in the impact salinity can play on its morphology (Simon *et al.*, 2022). On the contrary, the major disadvantage which surrounds the use of macroalgae is related to the high expenditure for infrastructure and the energy demand and costs of harvesting (Kostas *et al.*, 2021).

Due to both environmental (Tang *et al.*, 2021) and economic (Steinbruch *et al.*, 2020) benefits associated with the macroalgae biorefinery, it is expected for this industry to grow exponentially going forward. While the benefits and potential for growth in this industry are clear to see due to the increased growth of the sector ($15.01 billion in 2021 to $24.92 billion in 2028 at a CAGR of 7.51%) (Fortune Business Insights, 2021) there are also numerous challenges to overcome. Section 4.5.3 gives a breakdown of the key challenges that must be overcome for the success of the macroalgae biorefinery going forward and its potential for further growth (Figure 4.5).

4.5.2 Key processes

4.5.2.1 Anaerobic digestion

A key concept in the process of achieving the biorefinery concept associated with macroalgae is anaerobic digestion (AD). AD involves a combination of biological processes by which CO_2 and CH_4 are produced by the breakdown of organic matter under anaerobic conditions (Adekunle & Okolie,

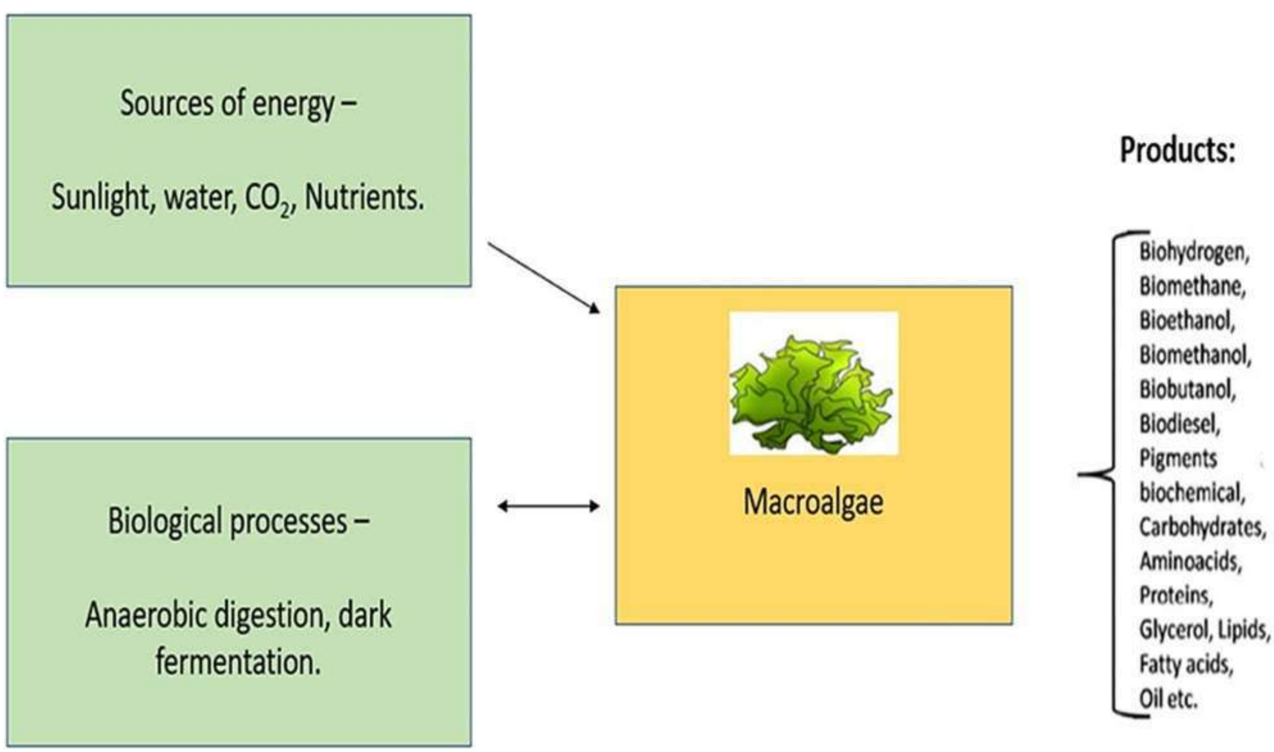

Figure 4.5 Macroalgae products relating to various biorefinery sectors. (Source: Redrawn from Rodionova *et al.*, 2017).

2015). The microbial consortium which acts during AD consists of hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria, and methanogenic archaea. The first step of AD is hydrolysis, during which hydrolytic bacteria break down the substrate, that is, seaweed (macroalgae), into sugars, amino acids, and fatty acids. These compounds are then available to acidogenic bacteria which break down the sugars into VFAs and alcohols during acidogenesis. Following this step, they are converted into acetic acid or H_2 and CO_2 in a process called acetogenesis. The final stage is methanogenesis which involves the production of CH_4 and CO_2 through archaea (Meegoda, *et al*., 2018).

The typical biogas composition is 60% methane, 38% carbon dioxide, and 2% trace gases (Frank-Whittle *et al*., 2014). When methanogenesis is blocked, hydrogen gas can be produced in a process known as dark fermentation (Nath & Das, 2004). Dark fermentation ultimately ends with VFAs and hydrogen production by anaerobic fermentative bacteria as highlighted in Figure 4.6. The microorganisms involved include *Escherichia coli, Enterobacter aerogenes, Citrobacter intermedius, Enterobacter cloacae, Ruminococcus albus, Clostridium beijerinckii,* and *Clostridium paraputrificum* (Koutra *et al*., 2020). Meanwhile, acidogenic fermentation involves maximizing the production of acetate by consuming H_2 to favour the acetogenesis process.

When characterizing AD by its desirable end products it can be broken down into both single-stage and two-stage AD. Two-stage AD offers advantages such as increased energy efficiency, optimal process stability and increased opportunities to control key parameters when compared to one-stage AD (Srisowmeya, *et al*., 2020). An important aspect of both one and two-stage AD is reactor setup. In stage one, the first three phases of AD are carried out, that is hydrolysis, acidogenesis, and acetogenesis. Hydrolytic bacteria hydrolyse the complex organic polymers into monomers while

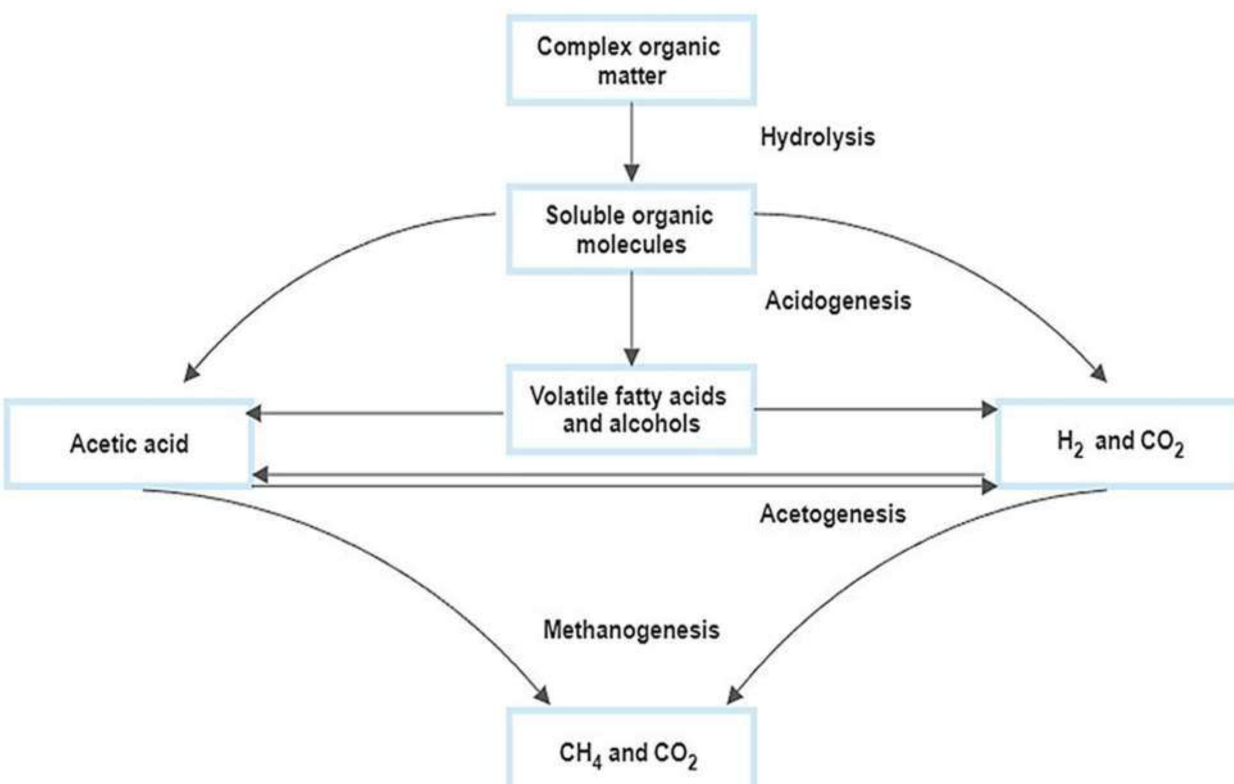

Figure 4.6 Schematic of the processes involved in anaerobic digestion.

acidogens and acetogens convert all the organic acids into acetic acid, H_2 and CO_2. In the second stage of AD, methanogens utilize the products of the first stage to produce CH_4 and CO_2 (Hans *et al.*, 2019). Table 4.3 highlights the stoichiometry involved in each of these four processes.

4.5.2.2 Reactor design

AD and dark fermentation can be carried out in a range of different reactor configurations either as attached or suspended growth systems. Continuously stirred tank reactors (CSTR) (Tabassum *et al.*, 2016)

Table 4.3 Chemical equations involved in anaerobic digestion processes.

Hydrolysis

$$(C_6H_{10}O_4)_n + 2H_2O \rightarrow C_6H_{12}O_6 + O_2$$
Cellulose — Glucose

$$(-RCH(NH_2)COO-)_n + (n-1)H_2O \rightarrow nRCH(NH_2)COOH$$
Protein — Amino acids

$$(H_2COOC(CH_2)_n CH_3)_n \rightarrow n(H_2COOC(CH_2)_n CH_3)$$
Fat — Fatty acids

Acidogenesis

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$
Glucose — Ethanol

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$$
Glucose — Butyrate

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$
Glucose — Butyrate

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH$$
Glucose — Acetate

Acetogenesis

$$CH_3CH_2COO^- + 3H_2O \longleftrightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$$
Propionic — Acetate

$$C_6H_{12}O_6 + 2H_2O \longleftrightarrow 2CH_3COOH + 2CO_2 + 4H_2$$
Glucose — Acetate

$$CH_3CH_2OH + 2H_2O \longleftrightarrow CH_3COO^- + 2H_2 + H^+$$
Ethanol — Acetate

Methanogenesis

$$CH_3CH_2OH \rightarrow CH_4 + 2CO_2$$
Ethanol

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

$$2CH_3CH_2OH + CO_2 \rightarrow CH_4 + 2CH_3COOH$$
Ethanol — Acetate

Source: Adapted from Hans and Kumar (2019).

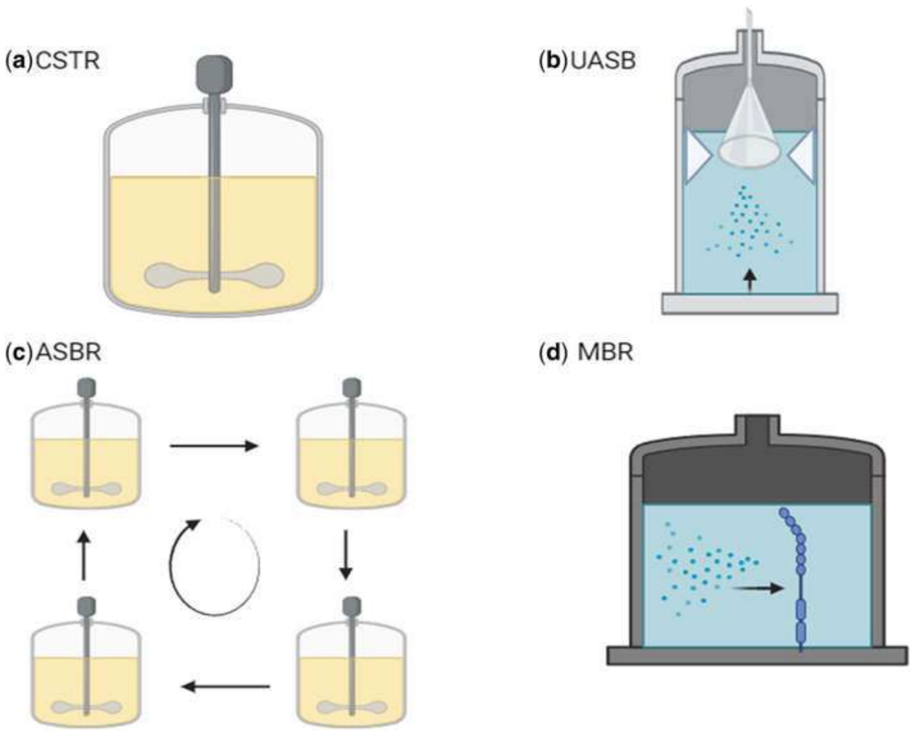

Figure 4.7 Reactor configurations for biogas production. (a) CSTR, (b) UASB, (c) ASBR and (d) MBR.

(Figure 4.7a) and up-flow anaerobic sludge blanket (UASB) (Liu *et al.*, 2013) (Figure 4.7b) reactors are the most performing and used designs. Alternatively, both anaerobic sequencing batch reactor (Figure 4.7c) and membrane bioreactor (MBR) (Figure 4.7d) are also used. CSTRs are frequently used in continuous hydrogen production. In comparison with batch reactors, microbial cultures in a CSTR are evenly suspended in the liquor with lower resistance in mass transfer. The CSTR has continuous input and output of material. The CSTR is well mixed with no dead zones or bypasses in ideal operation with typical total solid content reaching 10% (Thakur *et al.*, 2023).

UASB reactors tend to be used for high strength wastewater rich in COD, the influent wastewater flows from the bottom into the reactor, and it is distributed in an up-flow mode through a blanket of granular sludge. Wastewater flows upward through the blanket and is processed by anaerobic microorganisms. Following this, the treated effluent passes out around the edges of a funnel. UASB reactors offer several benefits such as greater contact surface area, operation at higher OLR, better settleability, enhanced solids retention and efficient solid separation from treated effluent. The sludge blanket is suspended by gravity-settling coupled with the upward flow of the effluent. Dense, spherical, compact biofilms are referred to as granules, with active methanogenic microbial consortia. Nevertheless, the disadvantage of UASB reactors is that the feedstock with high solids content prevents the development of dense granular sludge.

Anaerobic sequencing batch reactors (ASBR) are high-rate liquid digestion systems which rely on the sequential feeding of the reactor followed by mixing and the settling of solids (Figure 4.7c). The operation of this reactor is heavily influenced by factors such as the organic loading rate, temperature, pH, and substrate concentration. An ASBR reactor typically performs more effectively in terms of hydrogen production (hydrogen content $29.2 \pm 8.8\%$) when operated at a hydraulic retention time (HRT) of 24 h (Buitrón & Carvajal, 2010). Alternatively, MBRs (Figure 4.7d) have also been shown to be highly successful in terms of the dark fermentation processes due to their ability to control the biomass concentration (Show *et al.*, 2011), while Kim *et al.* (2006) found hydrogen production to increase with higher glucose concentrations (10–35 g/L). An MBR reactor offers further advantages in terms of improving effluent quality and having a smaller footprint. In choosing this reactor type it is also key to look at the desired HRT as typically membrane reactors are operated at a high volumetric rate thus resulting in much lower HRTs (<24 hrs) while it is also beneficial to keep a lower HRT to avoid membrane fouling (Rahman *et al.*, 2023).

4.5.3 Key challenges of macroalgal biorefineries

A major drawback in terms of the possibilities of utilizing macroalgae in a biorefinery concept is attributed to the scale of cultivation needed to produce enough macroalgae to make a significant impact should natural recurring resources become limited. To meet 1% of the UK's total energy demand it would require an area of cultivation of almost 5440 km^2 which is equal to half of the current global aquaculture production area (Hughes *et al.*, 2013). Needing an area of such size would cause huge problems in terms of the availability of feedstock supply chains as well as species selectivity and suitability. The impact of seasonality on cultivation is also a huge problem due to impacts on the macroalgae's biological composition and thus bioproduct potential (Kostas *et al.*, 2021). Such problems will have noticeable knock-on effects in terms of the scalability and large-scale integration of macroalgal biorefineries. Research to date has focused much on laboratory-scale projects. Further research and development are thus needed in terms of upscaling and overcoming potential challenges. Key challenges to note are in terms of complex licensing regulations (Camarena-Gómez *et al.*, 2022), which vary across the world as well as seasonal issues that can have an impact on the biochemical composition of the macroalgal species produced.

A second key issue that may hinder the progression of macroalgae biorefinery technologies is linked to the extensive costs involved in the removal of water, washing and drying steps that commence post-harvesting in preparation of the biomass. Considerable amounts of fresh water are required to wash the biomass of salts, epiphytes, and sand (Chisti, 2013). This step is also key in preservation of bioreactors as large amounts of salt contained in biomasses are known to cause both corrosive damage in bioprocess infrastructure as well as the bioreactor itself. For this reason, it is pivotal to look at ways of preserving and saving freshwater in terms of the whole process. Research to date has focused on a variety of methods such as closed production facilities and water recycling strategies (Pate *et al.*, 2011). Novel technologies such as using advanced textiles for cultivation of seaweed as trialled off the west coast of Ireland (Taelman *et al.*, 2015) have sought to cut costs associated with macroalgae farming. While early research has indicated that a complete salt-based macroalgal biorefinery concept is viable (Kostas, *et al.*, 2021), further research and development is needed in this area.

To allow for the continued growth and prosperity of the seaweed industry, it is pivotal to create a complete understanding of the optimal integrated bioprocessing pathways for each macroalgae species that is currently being cultivated. With this information, a sustainable and environmentally friendly biorefineries that generate bioproducts and biofuels from macroalgae biomasses can be generated. Going forward, industry and academia need to work together to identify the optimal bioprocessing routes for each species of seaweed utilizing environmental assessment tools for each individual bioprocess to combat the ongoing fossil fuel shortages (Su *et al.*, 2023). Such tools include attributional and consequential life cycle analysis (LCA), exergy and energy-based models. The use of such models allows for quantifiable and clear comparisons of energy yields of different feedstocks and how best to maximize and develop the numerous macroalgal biorefinery pathways.

4.6 CONCLUSION

Macroalgae have potential in terms of a feedstock for biorefinery industries to produce biochemicals and bioenergy while tackling the current issue of depleting petrochemical resources. Due to ongoing research and further scientific breakthroughs, it is expected that the macroalgae biorefinery industry will continue to grow due to the many financial and environmental benefits it possesses. This has been evidenced by the industries increasing compound annual growth rate (CAGR). While its potential for use in the production of biofuels, bioproducts and high-value products is clear, it is also obvious that further research is needed in terms of enhancement and scaling up of biorefinery systems. Problems related to cultivation and biorefinery design must be considered in terms of unlocking the full potential of this industry and the many possible economic and environmental benefits it possesses. With further collaboration between industry and academia, the seaweed biorefinery industry will become more established and continue to contribute to the creation of a low-carbon economy.

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Part 2

Algae-Based Wastewater Treatment

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Chapter 5

Wastewater treatment by microalgae-based processes

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ABSTRACT

This chapter summarizes the status, major challenges and potential contribution of microalgae-related wastewater treatment processes. Although the use of microalgae for wastewater treatment was proposed in the last century, technology was not sufficiently efficient and robust to be applied at a commercial scale. Only recent advances in the knowledge of biological systems, the engineering of reactors and the harvesting and processing of the produced biomass allow the development of the first industrial demonstrations. Facilities of several hectares are already in operation demonstrating the feasibility of this technology. However, challenges remain for the further improvement and enlargement of these systems. They are related to (1) the improvement of knowledge and management of biological systems, (2) the development of adequate strategies for the allocation and implementation of large-scale facilities, (3) the definition of optimal operation conditions including the development of non-assisted systems capable of operating under variable environmental conditions and (4) the development of adequate routes for the valorization of biomass. Much effort is being devoted to solving these challenges and thus making this technology reliable for industrial applications. Once it is achieved, the use of microalgae will be incorporated into the portfolio of available technologies for wastewater treatment. In this respect, no single technology is capable of solving all the scenarios related to wastewater treatment, but microalgae-related processes represent a semi-intensive technology capable of contributing to efficiently treating wastewater while recovering nutrient-energy-water scenarios related to temperate climates with no severe land restrictions. Moreover, the use of microalgae represents a change of paradigm in the field of wastewater treatment because by using this type of microorganisms it is possible to produce valuable biomass at a higher price than the wastewater treatment cost. The potential of microalgae-related wastewater treatment processes is thus highly relevant, and valuable to achieve the sustainable development goals defined by the United Nations.

Keywords: wastewater treatment, microalgae, raceway, nutrients recovery, biomass production, valorization, modelling, advanced control.

5.1 INTRODUCTION

Wastewater treatment is a continuous process due to the increase in population and the enhancement of lifestyle. Additionally, the current scenario of global warming imposes the necessity to reduce the impact of existing processes and to recover resources from waste. Specifically, the sustainable development goals (SDGs) of the United Nations identify 'Clean water and sanitation' as a major objective in addition to others such as 'Climate action', 'Life below water' and 'Life on land' (United Nations, 2015). Thus, to mitigate water and nutrient scarcity the recovery of water, energy and nutrients contained in wastewater is mandatory, and this fact drives the development of new processes alternative to conventional processes based on activated sludge (Muga & Mihelcic, 2008). Moreover, energy saving or energy recovery is also a necessity to mitigate global warming related to the emission of greenhouse gases involved in energy production systems. In this scenario, the use of microalgae emerges as an interesting alternative.

Microalgae naturally occur in conventional wastewater treatment processes, but usually, the presence of this type of microorganisms is disregarded or prevented. However, they can be an interesting partaker for wastewater treatment due to their capacity to produce oxygen (O_2) and fix compounds such as carbon, nitrogen and phosphorous, into valuable biomass. This makes microalgae-based wastewater treatment one of the most promising alternatives to conventional methods (Cano *et al.*, 2022; Lundquist *et al.*, 2010). Thus, wastewater treatment processes based on microalgae were already proposed in the last century but until now only a few examples of large-scale processes exist (Arbib *et al.*, 2022; Craggs *et al.*, 2013; Mehrabadi *et al.*, 2017). The reason for that was the lack of the necessary knowledge of the process and the low capacity of the technology that was used. Recent advances in both fields allow for overpassing these barriers, and the first demonstrators of the technology are already in operation. However, the knowledge of processes and technologies currently utilized must be still improved. Moreover, the gap between the efficiency of the current processes and the theoretical values remains high. Thus, large opportunities exist to improve technology and make it more robust and reliable for its commercial development.

One of the major aspects to be considered when developing wastewater treatment processes based on microalgae is the existence of microalgae and bacteria consortia. This fact is highly relevant because it imposes the necessity to design bioreactors and the overall production system as a function of culture conditions required for each of them, which are frequently different for both microalgae and bacteria (Umamaheswari & Shanthakumar, 2016). In the case of bacteria, the scenario is similar to an activated sludge-based process, which means that organic matter and O_2 concentrations determine the growth of heterotrophic bacteria, whereas other parameters such as nitrogen and total inorganic carbon concentration influence the behaviour of other microorganisms such as nitrifying and denitrifying bacteria. Concerning O_2, to ensure aerobic conditions the dissolved O_2 concentration must be higher than 2 mg/L, under these conditions it can still operate but the maximal performance is achieved when operating at 10 mg/L. The behaviour of bacteria is independent of availability of light and they prefer moderate pH and temperature values; a slightly high temperature and low pH are recommendable. In the case of microalgae, the growth is fully linked to the availability of light and independent of the presence of organic matter, the major nutrients being inorganic carbon, nitrogen and phosphorous. A large capacity of microalgae cells to produce O_2 is remarkable, although they have a low tolerance to high dissolved O_2 concentrations (higher than 20 mg/L) that induces photorespiration phenomena. Regarding pH and temperature, most of the microalgae prefer slightly alkaline pH and moderate temperature. In practice, the major difference between microalgae and bacteria is the necessity of light for microalgae growth. This fact imposes the necessity to use outdoor photobioreactors capturing natural sunlight as the driver of the process. The influence of culture conditions on the behaviour of major types of microorganisms present in microalgae-related wastewater treatment processes has been studied (López Muñoz & Bernard, 2021; Sánchez-Zurano *et al.*, 2022; Solimeno & García, 2019).

The necessity of providing light to microalgae-related wastewater treatment implies the use of specifically designed photobioreactors instead of conventional bioreactors. In this sense, although in some cases the use of artificially illuminated reactors has been proposed, the energy consumption and the required investment costs make it unfeasible for large-scale systems. Thus, the maximum efficiency of the photosynthesis process is 10% of photosynthetically active radiation. To produce 1 kg of microalgae biomass (on average with an energy content of 20 MJ/kg), up to 200 MJ of light are required which is equivalent to 5.6 kWh (Acién *et al.*, 2016). Considering a 100% conversion of electricity from light and an electricity cost of 0.1 €/kWh, it means that a minimum of 0.56 €/kg is needed, but this value increases to 5.6 €/kg when reducing the efficiency of the photosynthesis process to 1%. According to the nutrient content of wastewater, up to 1 kg of biomass can be produced per m^3 of wastewater treated, which means that only the cost of artificial illumination is higher than the cost of wastewater treatment using conventional technologies (0.2 €/m^3). Focusing on outdoor reactors the use of open raceways is the most suitable alternative because of the low cost (below 20 €/m^2 installation cost just considering the construction of raceway ponds, not ancillaries) and energy consumption (below 5 W/m^2) of this type of system (Acién *et al.*, 2016). For a base case of 1 ha, the systems already in operation demonstrate to be feasible for treating up to 5,000 m^3/day and producing 100 ton/year of dry matter, fixing up to 10 ton N/year and 2 ton P/year, while consuming less energy compared to conventional wastewater treatment systems, below 0.2 kWh/m^3 versus up to 0.8 kWh/m^3 for activated sludge systems (Acién Fernández *et al.*, 2017). These figures make the development of microalgae-based wastewater treatment processes very attractive for small- and medium-sized cities.

In this chapter, the major aspects of technologies that are currently utilized are summarized to provide an overview of the current status of the art, and based on that the major challenges to be faced are analysed. Some alternatives to improve the reliability of microalgae-related wastewater treatment processes are then provided. Finally, the relevance of improving and expanding the use of this type of technology is discussed.

5.2 CURRENT STATUS OF MICROALGAE-RELATED WASTEWATER TREATMENT PROCESSES

Although microalgae-related wastewater treatment processes were developed by Oswald in the last century (Oswald & Golueke, 1960), their development has been quite limited, with the capacity of previous processes remaining lower than required for commercial development. Thus, the size of more relevant demonstration facilities was scaled up to a few thousand square metres. However, the basis of these previous processes allows understanding the principles of the process and identifying the major barriers to solve for industrial development (Craggs *et al.*, 2012; Olguín, 2012; Park *et al.*, 2013).

5.2.1 Biology of microalgae–bacteria consortia

When considering the treatment of wastewater using microalgae it is necessary to understand that always a consortium of microalgae and bacteria exists. No specific microalgae strains are utilized; equally as in conventional processes based on activated sludge naturally occurring bacteria are managed. The challenge is to know the most relevant microorganisms involved in the process and their optimal culture conditions to maintain these conditions in the bioreactor, and then favour the development of positive microorganisms versus other competitors. In the case of microalgae–bacteria consortia, the most relevant microorganisms include microalgae and heterotrophic bacteria, in addition to others such as nitrifying or denitrifying bacteria. Microalgae are the microorganisms responsible for O_2 production and inorganic carbon fixation through the photosynthesis process, whereas heterotrophic bacteria are responsible for organic matter removal producing carbon dioxide (CO_2) and consuming O_2. The nexus between both microalgae and heterotrophic bacteria is the O_2; then the O_2 produced by microalgae and consumed by heterotrophic bacteria is ideally equal.

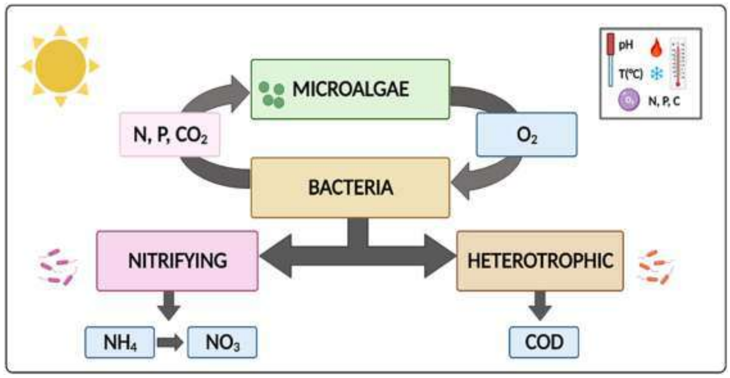


Figure 5.1 Scheme of major phenomena taking place in microalgae–bacteria consortia developing in microalgae-related wastewater treatment processes.

Knowing the phenomena and interactions taking place when managing microalgae–bacteria consortia is critical; a basic scheme of these phenomena is shown in Figure 5.1. To ensure the degradation of biodegradable organic matter a minimum population of heterotrophic bacteria is required according to the load of organic matter to be removed, and then the amount of O_2 required for this process can be calculated. This helps to define the minimum population of microalgae required to produce the necessary O_2 for heterotrophic bacteria by knowing the O_2 production capacity of the microalgae cells. The latter is a function of availability of light, and then the relevance of light for the bioreactor is determined. In addition to this basic phenomenon, other phenomena such as nitrogen and phosphorous solubilization, their release by bacteria and their consumption by both microalgae and bacteria must be considered. Moreover, the presence of other types of microorganisms such as nitrifying and denitrifying bacteria, or phosphorous-related bacteria, could be also important. Finally, the effect of culture conditions such as nutrient concentration, temperature and dissolved O_2 concentration on the performance of all these microorganisms must be taken into account.

Fortunately, there is a large set of knowledge and models already developed to simulate the behaviour of this type of biological systems. Different biological models such as BIO-ALGAE, ABACO and ALBA have been reported, all of them based on the same phenomena described but with some assumptions or differences about the chemistry of the water or phenomena considered (Casagli *et al.*, 2021b; Sánchez-Zurano *et al.*, 2021d; Solimeno *et al.*, 2019). Unfortunately, the diversity of models is large and still a unified model does not exist; however, similar to the activated sludge model it is expected to achieve a unified microalgae sludge model very soon. Related to this objective, recently the use of photo-respirometry methods to evaluate the performance of microalgae–bacteria consortia has been established (Sánchez-Zurano *et al.*, 2020; Sforza *et al.*, 2019). This tool allows a fast and reliable evaluation of the status of the biological system thus helping in the decision-making process for the operation of industrial facilities. Some of these models are already implemented in easy-to-use tools such as simulators, facilitating the analysis of scenarios and comparison of alternatives (Sánchez-Zurano *et al.*, 2021a).

Availability of light is a major parameter in the management of microalgae–bacteria consortia prevailing in wastewater treatment processes. This parameter is resumed in the average irradiance inside the culture, which is a function of solar radiation on the reactor surface and the attenuation of the light by the biomass, which is a function of biomass concentration, the attenuation properties of the biomass and the water depth (Grima *et al.*, 1994). The average irradiance is a key factor in determining the performance of the biological system and thus both the wastewater treatment and biomass production capacities. In this sense, two main scenarios are usually considered. If the objective is to maximize the capacity of wastewater treatment, microalgae are used mainly as O_2

producers, and then high water depths are used (30–40 cm) because enough average irradiance will exist to produce the O_2 required by bacteria to degrade the organic matter (Sutherland *et al.*, 2014). However, in this scenario, the percentage of bacteria in the biomass is high (up to 60%) and then the quality of the produced biomass reduces. Regarding nitrogen removal, the assimilation into biomass only accounted for 57% of the inlet nitrogen under the best conditions because nitrification and volatilization reduced the availability of this element. On the contrary, if the objective is to maximize the recovery of resources from wastewater, the production of microalgae biomass must be prioritized. For that, low water depths must be utilized to enlarge the average irradiance inside the reactor and then the increased growth of microalgae biomass (Morillas-España *et al.*, 2021). In this scenario, the amount of O_2 produced by the microalgae is more than that required by bacteria. Moreover, excess dissolved O_2 concentrations can prevail and the desorption of O_2 by aeration using adequate mass transfer units is necessary. In addition, if an inadequate C/N ratio is present in the wastewater additional inorganic carbon must be provided to allow the microalgae cells to fix the nitrogen and phosphorous present in the wastewater. Under these conditions, the wastewater is used as a nutrient source and the flow of wastewater treated is reduced compared to the first strategy. The operation at short hydraulic retention times presented a more interesting performance with higher biomass productivity (de Godos *et al.*, 2016). The priority and operation mode is very relevant because they determine the quality of the produced biomass in terms of microalgae and bacteria composition. This is very relevant for further applications of biomass and to ensure the accomplishment of regulation in terms of wastewater treatment (Nordio *et al.*, 2023).

5.2.2 Engineering of photobioreactors

Microalgae-related wastewater treatment is performed in raceway reactors, as shown in Figure 5.2. The reasons for that include the low cost and energy consumption of this technology and its well-established technology (Lundquist *et al.*, 2010). However, the design and operation of this type of reactor are far from optimal values, and large improvements are possible. Raceways consist of horizontal surfaces on which a liner is installed, the perimeter of the reactor being defined using concrete blocks or sand barriers. The reactor usually consists of two channels along which the culture is continuously recirculated using a paddlewheel. The water depth is in the range of 20–40 cm,

Figure 5.2 Image of raceway reactors utilized for wastewater treatment at Agramon (Spain) designed and operated by Aqualia.

and the paddlewheel is designed specifically based on these data to ensure adequate efficiency. In general, a length-to-width ratio of 10 : 20 is preferred. In summary, the design and construction of raceway reactors is a hydrodynamic problem, the objective being to minimize the pressure drop along the reactor to minimize the energy consumption into the paddlewheel and to enlarge the size of the reactor. In this sense, the overall size of the raceway ponds is limited because the only input of energy is provided by the paddlewheel and the capacity of this system to provide energy to the liquid requires a maximum water drop of 15 cm between the inlet and outlet of the paddlewheel. For reactors operating at high water depths of 30–40 cm the overall surface can be up to 10,000 m^2, whereas for reactors operating at low water depths of 10–20 cm the overall surface can be up to 5,000 m^2 (Craggs *et al.*, 2012). Traditionally, the design of raceway reactors was performed based on the Manning equation. This equation is accurate mainly for channels, but not for bends and other accessories, then the use of the Bernoulli equation proves a more general and accurate design (Mendoza *et al.*, 2013a).

A critical part of raceway reactors is the bends connecting the channels. This part represents a relevant pressure drop in these systems and thus the number of bends must be minimized, according to the dimensions of the available land. The design of the bends must be carefully optimized, and the installation of 'islands' or baffles facilitating the circulation of the culture is necessary (Sompech *et al.*, 2012). The use of baffles is simple and 50% cheaper, thus it is the recommendable solution (Mendoza *et al.*, 2013a). Concerning the paddlewheel it must be designed according to the water depth. The recommendation is to install systems with a total diameter equal to four times the water depth and include a range of 10–12 paddles (Weissman & Goebel, 1987). The minimization of hydraulic losses in the system allows for the optimization of the performance of the paddlewheel, the challenge being to avoid split velocity between the rotation of the paddlewheel and the liquid velocity. It means that ideally the rotation velocity must be adequate to provide a tangential velocity equal to the liquid velocity. As the recommended liquid velocity is 0.2 m/s it means that rotation velocity must be in the range of 2–5 rpm for water depths ranging from 0.4 to 0.2 m. Commercial systems normally operate at higher rotational velocities, in the range of 6–10 rpm. The design and operation of the paddlewheel largely determine the energy consumption of the system, and inadequate design increases the energy consumption (Mendoza *et al.*, 2013a). The adequate design allows maintaining the energy consumption of this type of reactor in the range of 1–10 W/m^2. This energy consumption can be reduced by using turbines instead of paddlewheels, although the investment cost increases. The low-energy algae reactor developed by Aqualia is based on this concept and allows for a reduction in energy consumption by up to 25% of the initial value (Arbib *et al.*, 2022). Recent advances are being developed thanks to the use of computational fluid dynamic tools such as ANSYS Fluent for the optimal design and operation of raceway reactors (Hreiz *et al.*, 2014; Inostroza *et al.*, 2021).

Whatever the impulsion system, two problems of raceway reactors are (1) the inadequate light regime to which the cells are exposed in this type of reactor and (2) the low mass transfer capacity of these systems. Concerning the light regime, both experimental and computer-assisted analyses of flow patterns in raceway reactors demonstrated that it is laminar, the vertical movement of cells being very scarce (Barceló-Villalobos *et al.*, 2019). Vertical velocity has been estimated to be 0.02 m/s, which imposes average frequencies of light exposition in the order of 10^{-2} Hz, a hundred times lower than that required for integration of light, of 1 Hz. To solve this problem it would be possible to increase the liquid velocity into the channel. However, this will require a large increase in energy consumption which makes it not feasible. Moreover, the increase in liquid velocity only allows for an increase in the frequency of light exposition on a limited amount, up to a maximum of 10^{-1} Hz. Another alternative proposed has been the use of airfoils favouring vertical mixing (Figure 5.3). These airfoils must be carefully designed and installed, a large number of them being required because their effect is limited to short distances (Inostroza *et al.*, 2023). Although promising results have been provided, still no industrial demonstrator of this technology has been developed. Concerning mass transfer, raceway reactors are designed to minimize energy consumption while allowing the exposure of large volumes

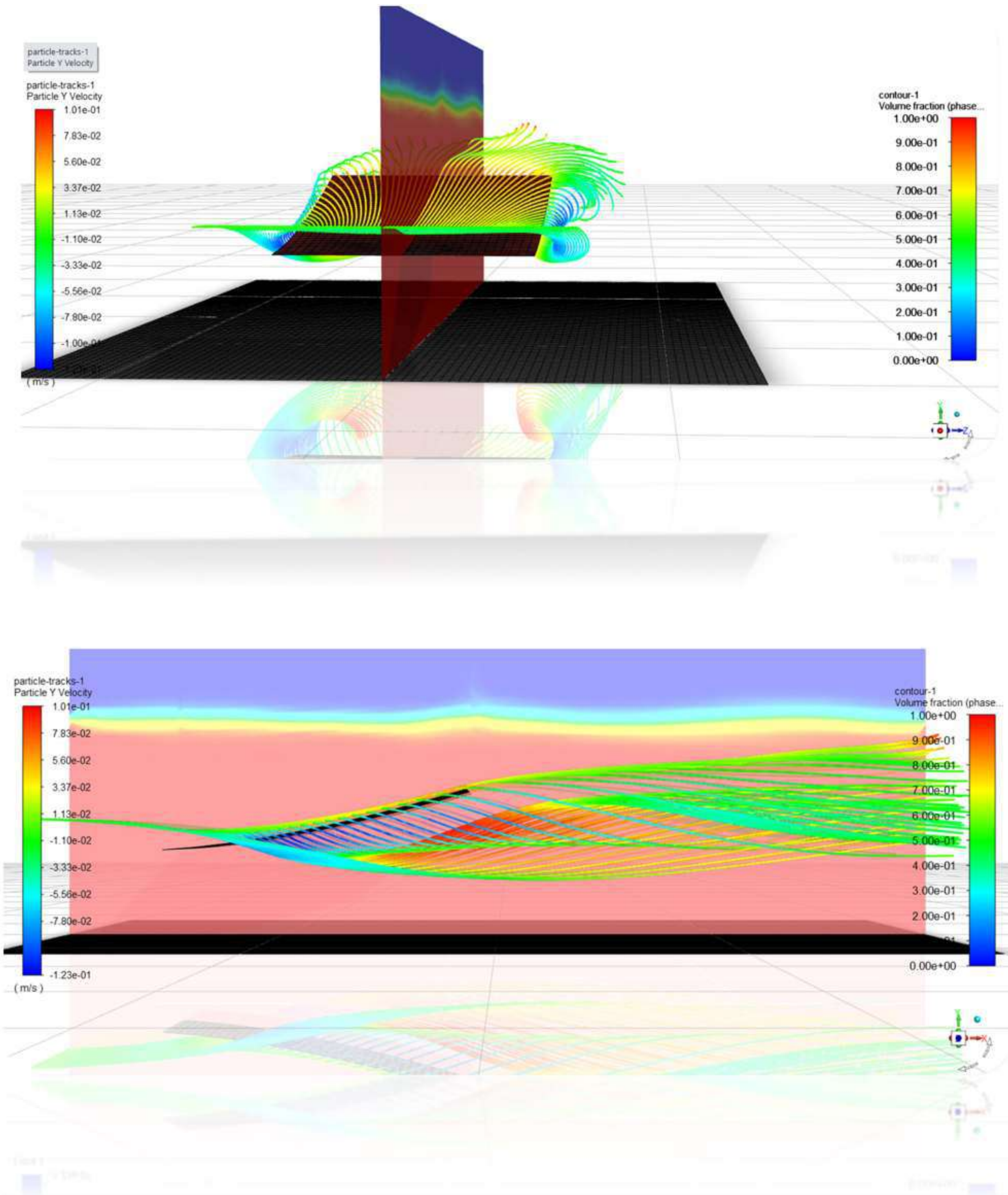


Figure 5.3 Example of baffles that can be used to improve the vertical mixing in raceway reactors to maximize the light utilization efficiency by the microalgae cells.

of culture to the sunlight, the exchange of gases such as CO_2 and O_2 with the atmosphere taking place only through the reactor surface. This exchange is very low due to the scarce mixing between the liquid and air in the channel, thus limiting biomass productivity both by carbon limitation and O_2 oversaturation (Mendoza *et al*., 2013b). Regarding O_2, the dissolved O_2 concentration in a raceway pond long-term operated in south Spain at a large scale exhibited pronounced daily variations (Arbib *et al*., 2017). The differences between the representative days of each season were also noticeable. Maximum values were recorded during June with concentrations up to 32 mg/L at midday, whereas at the same hour of the day during winter considerable lower values were achieved, ≈12 mg/L. The concentration of dissolved O_2 during the no photosynthetically active period (night) also presented seasonal differences with lower concentrations during summer and higher during winter (ranging from 1.1 ± 0.7 to 5.2 ± 1.2 mg O_2/L), and in many cases, the dissolved O_2 attained 0 mg/L during the night. To improve the mass transfer capacity the installation of sumps is recommendable. The sump allows for an increase in the mass transfer capacity and adjusts it to the necessities of the systems, both in terms of CO_2 supply and O_2 removal. Recent advances in this field allow adequate design and operation of these systems according to the final purpose of the reactor and its size, thus facilitating the operation and management of large-scale systems (Barceló-Villalobos *et al*., 2018).

5.2.3 Harvesting and processing of the biomass

One of the major problems related to wastewater treatment involving microalgae is the recovery of biomass for the clarification of water. Microalgae biomass has different and highly variable properties in comparison with bacteria usually involved in activated sludge-related processes. Thus, in the case of microalgae-related processes, the biomass concentration is lower, in the range of 0.35–1.5 g/L, whereas the amount of the solids is also smaller, being in the range of 5–20 μm. In processes involving activated sludge, the concentration of solids is in the range of 1.5–3.0 g/L and the biomass is in flocs of several millimetres in diameter. These characteristics make the separation of solids difficult due to the microalgae biomass. To solve this problem the modification of the characteristics of microalgae cells by the use of coagulants/flocculants has been proposed (Wu *et al*., 2015). However, using these compounds the biomass becomes contaminated, and thus it must be taken into account for further applications of the biomass. Moreover, the dosage of these reactants is highly variable and must be carefully adjusted for each case and continuously, to prevent the exhaust of microalgae biomass and especially the not accomplishment of regulation in terms of content of solids at the end of the wastewater treatment process. Once the biomass is flocculated, the settling properties improve and conventional separation units such as sedimentation or flotation provide adequate results in terms of biomass recovery (higher than 90%) and accomplishment of water discharge criteria. This separation step allows the pre-concentration of the culture to achieve a maximal solid content up to 40 g/L (Arbib *et al*., 2022). The use of membranes for the pre-concentration step has been developed. In this case, non-pressure membranes of micro- and ultrafiltration can be used to pre-concentrate the biomass up to 10 g/L but ensure 100% removal of solids into the supernatant, independently of biomass properties and without the addition of any reactant as coagulant/flocculant (Zhang *et al*., 2010). To complete the harvesting process further dewatering using filtration or centrifugation is mandatory. These unit operations consume more energy and are more expensive than the previous ones, thus their direct use to separate the biomass from the supernatant is not recommendable. Figure 5.4 shows a comparison of different harvesting strategies in terms of (1) biomass concentration at the beginning and end of the process and (2) operation cost/energy consumption. Data clearly show that the two-step process including a pre-concentration plus dewatering step is the best alternative. Although the use of dissolved air flotation and sedimentation is possible, it implies the dosage of flocculants, thus making the process difficult and contaminating the biomass. However, the use of membranes allows for achieving similar results in terms of biomass concentration, while avoiding cost and energy consumption by the use of flocculants. The challenge is to reduce the water content as much as possible to reduce the cost of downstream processes, in some cases including the drying of the

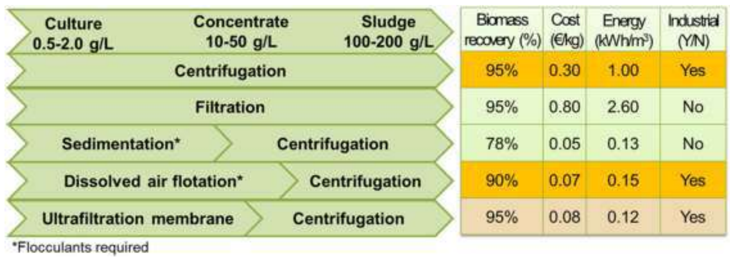


Figure 5.4 Scheme of different harvesting processes and comparison of their performance at a demonstrative scale (greater than 1 m³/h). Data from SABANA EU founded project.

biomass being required. However, to save energy and cost the reduction of the water content must be adjusted to that required for the valorization step. It is thus critical to define the more suitable ones at this stage (Arbib *et al.*, 2022).

Although microalgae biomass has been proposed as potentially useful for a large set of applications, when produced in wastewater some of them are strictly forbidden by the regulation, whereas others are also not recommendable according to the prevention criteria. For example, the regulation completely forbids the use of biomass produced from wastewater for direct human use. This is not the case when using biomass for feed although in this case, the feed industry doesn't use this type of material according to the prevention criteria. The remaining applications are related to materials, agriculture or bioenergy (Acién *et al.*, 2016; Arbib *et al.*, 2022). In the case of materials, no relevant examples of the use of microalgae biomass as a source of polymers or composites exist to date, but technically it would be possible (Arias *et al.*, 2020). Concerning agriculture, microalgae contain relevant contents of nutrients such as nitrogen, phosphorous, iron and magnesium, among others. However, more than the inorganic content the organic molecules present in microalgae biomass have been reported as highly valuable for agriculture. In this respect, the amino acid profile of microalgae contains essential amino acids that have been reported to act as plant growth promoters in different crops (Kapoore *et al.*, 2021). Moreover, microalgae biomass is very rich in phytohormones such as auxin, cytokinins and gibberellins improving the root development of plants, and the growth or the development of fruits in crops (Stirk *et al.*, 2002). Microalgae biomass has also been proposed as a source of biopesticides, thus replacing other chemicals less sustainable and toxic compounds already used to prevent diseases in crops (Costa *et al.*, 2019). Microalgae biomass can be used as a raw material to obtain bioenergy, more specifically biofuels. Although the burning of microalgae biomass to produce heat or electricity is possible, the necessity to dry the biomass for this use strongly reduces the efficiency of the process in terms of energy and cost. The production of biofuels is based on the chemical composition of the microalgae biomass: carbohydrates can be converted into bioethanol, lipids can be converted into biodiesel or the whole biomass can be converted into bio-oil by thermochemical treatment, or into biogas/biomethane through anaerobic digestion (Murthy, 2011). This latter process is the most simple and feasible in wastewater treatment plants. This process allows the production of biomethane from microalgae biomass produced in wastewater treatment processes, yielding up to 0.2 kg CH_4/kg volatile solids (VS) equivalent to 0.6 kWh/m³ of treated wastewater (Arbib *et al.*, 2022).

A techno-economic analysis must be performed to analyse the different alternatives to the application of biomass and to define the most recommendable approach. In this sense, the development of processes capable of proceeding with wet biomass is highly recommendable to avoid the cost and energy consumption of drying processes such as spray-dryers or freeze-dryers. Some applications require the inclusion of cell disruption steps that are usually performed by high-pressure homogenization although the use of ultrasound or milling has been reported as well (Halim *et al.*, 2012). An in-depth analysis of alternatives for the valorization of microalgae biomass produced in wastewater was performed allowing identifying the anaerobic digestion of the biomass to produce biogas or the combination of the production of biofertilizers followed by the production of biogas as the most recommendable (Acién *et al*., 2016). In this sense, FCC Aqualia is performing the production of biomethane from microalgae biomass produced in wastewater treatment plants in Spain. The biomass is harvested using dissolved air flotation up to concentrations of 40 g/L with biomass recovery efficiencies higher than 95%. The biomass sludge is directly fed to an anaerobic digester to produce biogas, which is cleaned-up to biomethane using a patented technology. According to this company, 1 ha of raceway reactor is capable of producing the fuel required for up to 35 cars annually (Arbib *et al*., 2022). This example opens the door for the real production of biofuels from microalgae biomass when integrating algal biomass production with wastewater treatment.

5.3 MAJOR CHALLENGES OF MICROALGAE-RELATED WASTEWATER TREATMENT PROCESSES

To facilitate the development of microalgae-related processes for the treatment of wastewater it is necessary to improve the knowledge and the technologies currently used: to improve their efficiency but especially to improve the robustness of processes. The combination of these advances and the implantation of the first commercial units will target the expansion of this technology and thus the enlargement of its contribution to this field.

5.3.1 Improvement of biological systems

To improve the efficiency of microalgae-related wastewater treatment processes it is mandatory to improve the knowledge and management of microalgae–bacteria consortia. In this sense, large efforts are being devoted to know in detail the microbiological composition of this type of consortia, using huge and valuable information being acquired by omics tools in this respect (Casagli *et al*., 2021a; Clagnan *et al*., 2022; Robles *et al*., 2020; Sánchez-Zurano *et al*., 2021b). Recent advances include the identification of microalgae strains prevailing in this type of biological systems. Data show fast-growing strains such as *Chlorella*, *Scenedesmus* or *Tetradesmus* as prevailing strains, although others such as *Ochromonas*, *Picochlorum*, *Oocystis*, *Dictyosphaerium*, *Poteriospumella* and *Micractinium* can be found as well (Clagnan *et al*., 2022). These strains are well known and they tolerate large variations of culture conditions such as temperature and pH. Moreover, they are strains with high efficiency in terms of light utilization, which explains their higher adaptability to stringent culture conditions. However, changes in microalgae populations are observed due to changes in environmental and operational conditions, still no distinct patterns are being defined. Concerning bacteria, highly variable results have also been obtained according to the environmental and operational conditions. In general, a high proportion of bacteria have been characterized by genera that harbour pathogenic species, for example *Chryseobacterium*, *Aeromonas*, *Brevundimonas*, *Roseomonas* and *Elizabethkingia*. However, as expected, multiple genera are also involved in biodegradation and bioremediation activities, for example *Arenimonas*, *Phenylobacterium*, *Porphyrobacter*, *Gemmatimonas*, *Leptothrix* and *Polymorphobacter* (Clagnan *et al*., 2022). The presence of nitrifying or denitrifying bacteria is highly variable according to the culture conditions imposed, especially how suitable they are for the growth of microalgae cells. The main ammonia-oxidizing bacteria and nitrite-oxidizing bacteria (NOB) families found in wastewater treatment-related processes belong to the family *Chromatiaceae*, which includes the genus *Nitrosococcus*. The family *Nitrosomonadacea*, which includes the genera *Nitrosomonas*, *Nitrosospira* and *Nitrosovibrio* was also found (Sánchez-Zurano *et al*., 2021b). Two common NOBs were detected in these systems *Nitrospiraceae* and *Bradyrhizobiaceae*, within these families, *Nitrospira* and *Nitrobacter* were the main genera detected (Sánchez-Zurano *et al*., 2021b). Finally, in some cases, microalgal grazers and parasitoids are also found, some of them including *Adineta*, *Brachionus* and *Amoeboaphelidium*. *Adineta* and *Brachionus* are known microalgal grazers, whereas *Amoeboaphelidium* is an algal parasitoid. The presence of these predators could negatively affect biomass yield and lead to the collapse of the system (Clagnan *et al*., 2022).

The necessity for a better understanding of biological systems managed in microalgae-related wastewater treatment processes imposed the need of (1) developing fast methods for the characterization of the cultures and (2) strategies to optimize their performance. In the first aspect, the development of the photorespirometric method is a valuable tool for the fast monitoring of the performance of the biological system. A scheme showing the steps involved in this methodology is presented in Figure 5.5. This methodology combines light/dark cycles and the addition of specific nutrients such as acetate or ammonium to differentiate the metabolism of microalgae, heterotrophic and nitrifying bacteria (Rossi *et al.*, 2018; Sánchez-Zurano *et al.*, 2022). Largely different results are obtained when applying this methodology to microalgae–bacteria consortia developed using different wastewater types such as urban wastewater, manure or digestate, but also for the same type of wastewater as a function of operating conditions such as availability of light, residence time or organic load. In the second aspect, it is necessary to develop strategies to face the variations in wastewater composition and environmental conditions found in real systems. In this sense, seasonal and daily variations of the culture conditions are inherent to the use of outdoor systems and they must be taken into account. The variations of wastewater composition, usually in organic matter chemical oxygen demand (COD), ammonium concentration and turbidity are more relevant (Figure 5.6). The increase in organic matter favours the development of bacteria, and the increase in the O_2 demand favours the opposite the development of microalgae and the production of O_2. Thus, the COD load influences the O_2 balance and finally the microalgae to bacteria ratio. Control of the concentration of dissolved O_2 is a key factor to fight these variations. Regarding ammonium, a similar trend is

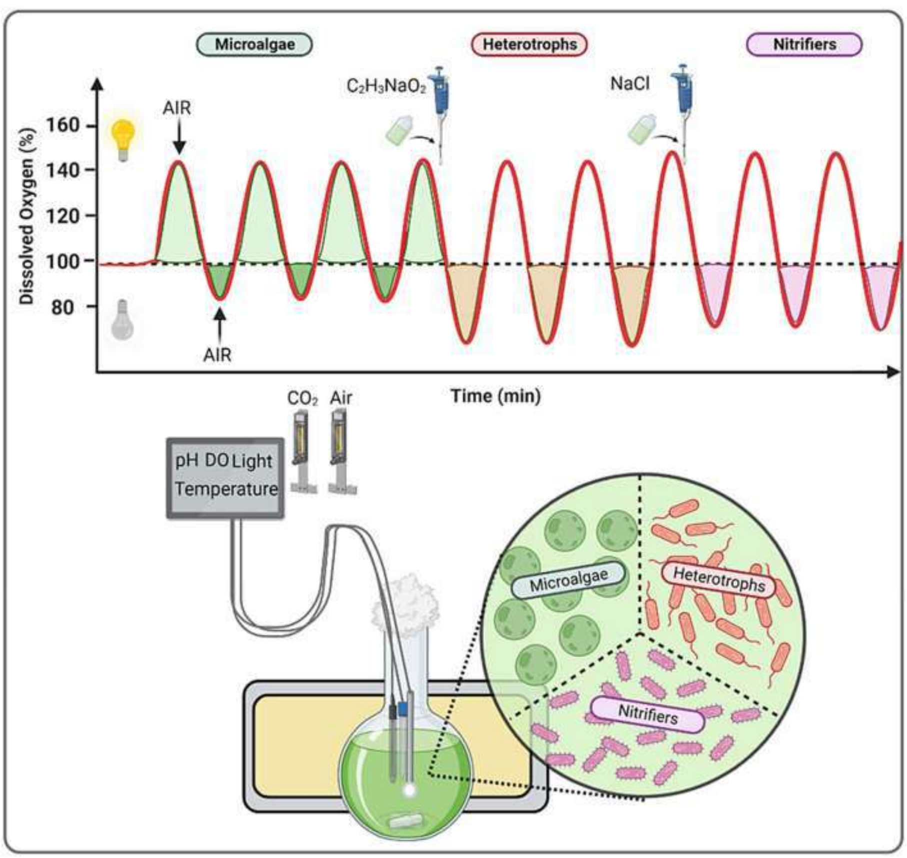


Figure 5.5 Scheme of the photo-respirometric method to evaluate the performance of microalgae–bacteria consortia prevailing in wastewater treatment processes.

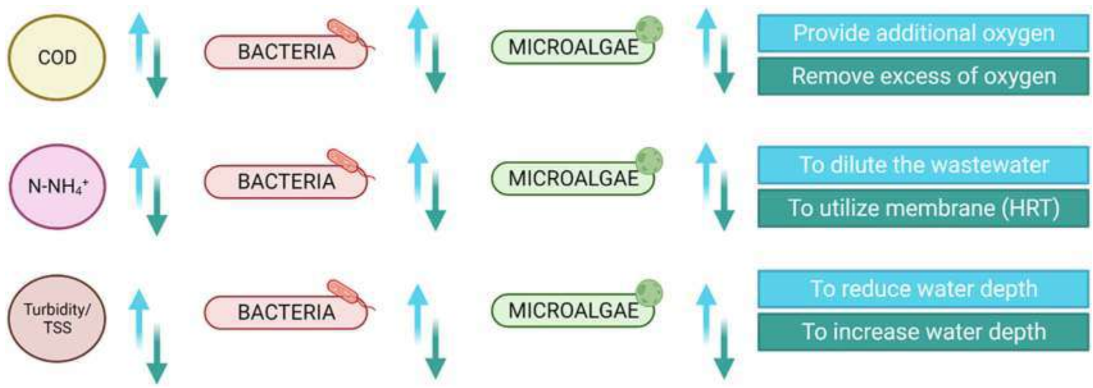


Figure 5.6 Influence of characteristics of wastewater on the performance of microalgae–bacteria consortia and some strategies to mitigate it.

observed, thus the increase in the ammonium concentration favours especially the development of nitrifying bacteria and in some cases damages microalgae cells (Collos & Harrison, 2014). To control these phenomena the load of ammonium must be controlled. Finally, turbidity is a major factor influencing light penetration and microalgae performance. Because no additional filtration systems can be implemented to avoid cost increases the main strategy is to modify the water depth to facilitate the light penetration and the performance of microalgae cells.

Although these general rules allow a better understanding and management of wastewater microalgae-related processes, still much more knowledge about the biology of these systems is required. Reliable models and simulators of the behaviour of these systems are required. Only an in-depth knowledge of biological systems and the development of methods for monitoring and regulating it will allow the development of robust industrial processes.

5.3.2 Allocation and implementation of large-scale facilities

The implementation of microalgae-related wastewater treatment processes imposes the necessity of large surfaces including under optimal conditions because solar radiation is the driver of the process. Previously, up to 10 m^2/pe (population equivalent) was required, but recently this figure has been reduced to 2 m^2/pe (Arbib *et al.*, 2022). That means that a minimum of 1 ha is required for every 5,000 inhabitants. This requirement for large surfaces imposes a challenge in the identification of adequate locations for this type of technology. Factors to be considered in this respect include (1) the topography and nature of the land, (2) the necessity of land movement and landfill management, (3) the land use and conflicts about other uses and (4) the shape of the available plot. Topography and land nature in addition to the necessity of land movement and landfill management imposes serious limitations on the identification of suitable locations for this type of facility. Flat surfaces requiring the minimum of land movement are required otherwise the installation cost can be increased up to 35%. Moreover, the nature of the terrain is critical to support the installation of the reactor, and geological studies are necessary. Concerning land use, this aspect is highly relevant because regulation already defined suitable land for industrial processes, but more relevant than this could be conflicted with other human activities, such as tourism or agriculture. Finally, the shape of the available plot will largely define the size of the reactors and their final arrangement. For example, for a population of 50,000 inhabitants up to 10 ha is required, but due to the restrictions on the geometry of raceway reactors different units of different sizes must be accommodated on it (Figure 5.7). Different scenarios must be studied from a techno-economic point of view as the final step to define the final distribution of the reactors. For large-scale projects the shape of the available land is also relevant; therefore, different design raceway ponds will be needed, from long length

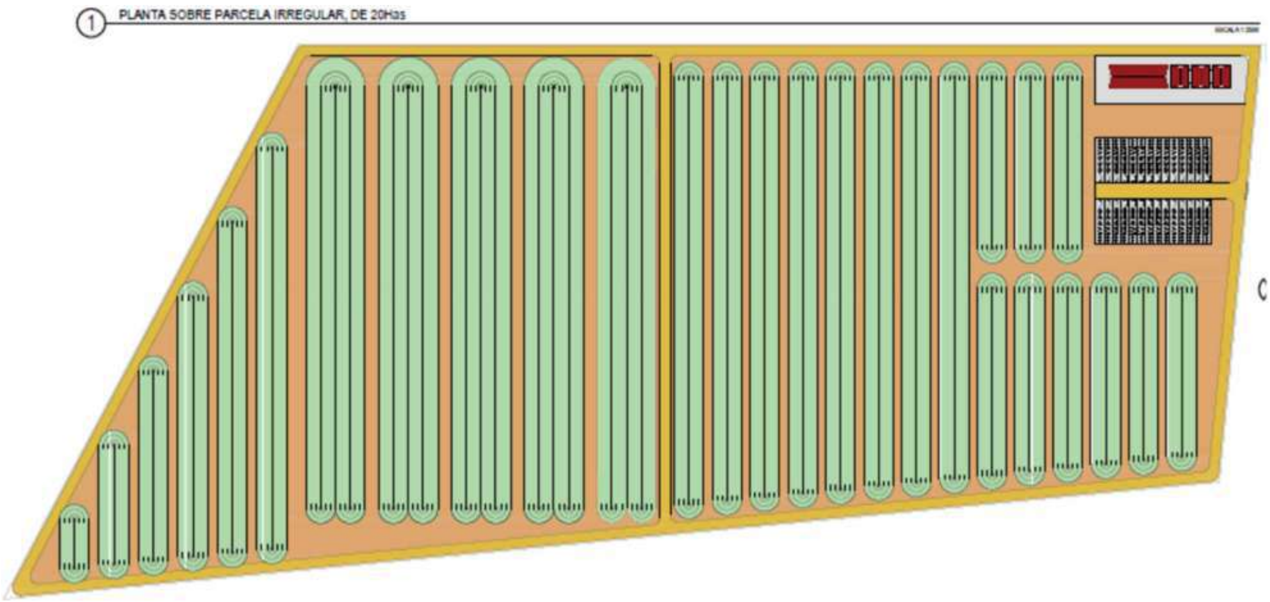


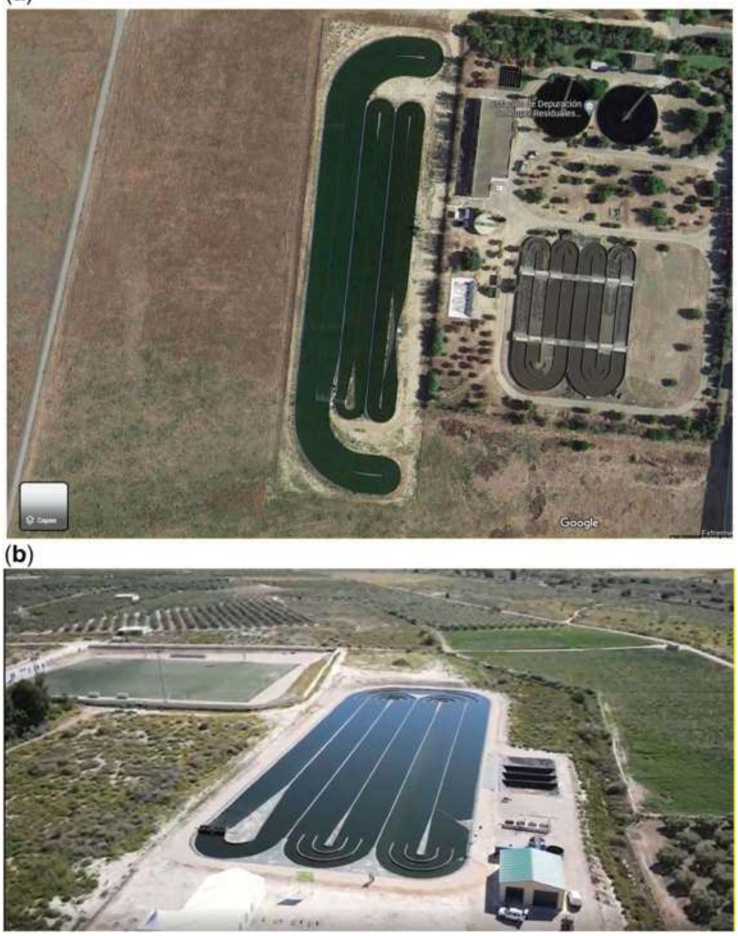
Figure 5.7 Example of distribution of raceway reactors for the installation of a 20 ha facility for wastewater treatment of a city with 100,000 pe, design provided by Aqualia.

two-channel ponds where there is no space limitation, to compact multichannel ones (six channels or more). Figure 5.8 shows real industrial facilities constructed by Aqualia in Merida and Agramon in the framework of the SABANA project.

Construction strategy is another relevant topic. The conventional method consists of the use of reinforced concrete over compacted land and finally covered by the liner (Figure 5.9). This technology is expensive but it is durable and can be shaped in complex ways. As an alternative, the low-cost method is based on land movement, digging the channels, compacting and final lining. Earthen raceways with plastic liners cost little and are easy to build, thus this strategy is less expensive. However, because of the slope required to maintain the wall stability, this design needs more space in comparison with the conventional concrete raceway ponds (Arbib *et al.*, 2022). A third option has been recently developed in which the raceway reactor is made of semi-rigid polyethylene reinforced with a metal. This strategy allows for minimizing the cost of land movement, and avoids the use of concrete while maximizing the use of land. Still, this technology has been only validated at scales up to 1,000 m^2 but it would represent a comfortable option, especially for pilot and demonstration units (Sánchez-Zurano *et al.*, 2021c).

Finally, the design of the reactor and the entire process must be fitted to each case. Similar to other conventional technologies such as the use of activated sludge, according to the capacity and boundary conditions of the process the specific design of the reactor and harvesting/downstream steps must be specifically designed. Concerning the reactor, the water depth is a relevant decision, affecting not only the design and area of the reactor but also to the necessity to incorporate mechanisms for O_2 desorption of inorganic carbon supply. In this sense, the optimal design of sumps allowing improving the mass transfer capacity in raceway reactors has been recently reported (Barceló-Villalobos *et al.*, 2018). Adequate sumps allow for minimizing the energy consumption for O_2 desorption at the same time than maximizing the efficiency of carbon capture when providing CO_2-rich gases as a source of inorganic carbon (Barceló-Villalobos *et al.*, 2022). Moreover, the use of advanced control algorithms allows the optimization of both processes and then the system approaches its theoretical optimal performance (Rodríguez-Torres *et al.*, 2021).

(a)



(b)

Figure 5.8 (a) Merida plant composed of one raceway of 1 ha and two raceways of 0.5 ha based on two-channel design and (b) Agramon plant composed of a 1 ha compact design composed of six channels (Aqualia).

5.3.3 Optimal operation of processes

Microalgae-related wastewater treatment processes are performed outdoors using natural sunlight as an energy driver. Moreover, large surfaces are required on which the cultures are exposed to both daily and seasonally changing environmental conditions, mainly solar radiation and temperature. Besides, wastewater treatment typically faces problems related to disturbances of both flux and quality of wastewater. Thus, defining the optimal conditions of microalgae-related processes for wastewater treatment can be a difficult task, requiring integrating largely different changing variables. To solve this problem different approaches are possible. The most conventional is the development of mathematical models considering the phenomena taking place in the processes, both biological and physical/chemical. This approach provides an adequate description of the most relevant phenomena, allowing the simulation of different scenarios and to take decisions based on the results from simulations (Hoyo *et al.*, 2022; Sánchez-Zurano *et al.*, 2021a). Moreover, the use of weather forecasts allows preventing failures of the systems and to adapt the operational conditions to the most adequate ones (De-Luca *et al.*, 2019; Rodríguez-Miranda *et al.*, 2022). This approach faces the problem of changes in the composition of the biological system or its adaptation to changing environmental conditions. Thus, a continuous evaluation of the performance of the biological system and recalibration of parameters of the biological model are required.

Figure 5.9 Image of raceway reactors. Different construction strategies of raceway reactors for wastewater treatment: (a) use of reinforced concrete, (b) use of dining channels and (c) polyethylene reinforced with a metal.

Alternatively, the use of artificial neural networks is emerging as a promising initiative. Compared to first-principles models, models based on artificial neural networks are faster to run and simpler to re-calibrate on account of their smaller number of parameters and more straightforward formulation. These models allow the simulation of the behaviour of each specific system based on previous data, without an in-depth knowledge of the phenomena taking place. They can infer patterns in the data beyond human comprehension, being especially useful in image or text processing tasks, speech recognition and recommendation management. By providing adequate and enough data, and using adequate algorithms, the neural networks are capable of properly predict the behaviour of the system (Otálora *et al.*, 2021, 2023). Equal to models based on first principles, the models based on neural networks can be fed with weather forecasts to anticipate changes in environmental conditions and to modify the operational parameters to optimize the performance of the systems.

Whatever the operation strategy, based on first-principles models or neural networks, microalgae-related processes are semi-intensive processes much more simple than intensive technologies and require much less supervision and maintenance. The integration of this type of strategy will facilitate the development of fully non-assisted processes, capable of working with the intervention of operators, and always performing under optimal conditions. This fact will facilitate their implantation in small- and medium-sized cities, and especially in rural areas or locations far from large infrastructures required by conventional technologies. In this sense, the challenge is to develop modelling and control frameworks to improve the efficiency, productivity, design and optimization of microalgae-related wastewater treatment processes (Figure 5.10). With this technology, three different objectives could be addressed: (a) maximize biomass production/quality, (b) maximize wastewater treatment capacity or (c) a tradeoff between biomass production and wastewater treatment. According to the selected objective, the process specifications will be imposed by selecting the microalgae strain, biomass quality and required production costs; that is the control requirements for the control optimization problem. Thus, adequate modelling and control approaches are technology solutions that can contribute to better reproducible conditions with competitive market costs by analysing/simulating environmental conditions (solar radiation and ambient temperature), compensating for the permanent non-stationary behaviour of the processes, the presence of disturbances, taking advantage of nutrients provided by wastewater (mainly carbon, nitrogen, O_2 and phosphorous), removing any toxic metabolic products (e.g. CO_2 mitigation) and controlling important internal cellular parameters (e.g. temperature, pH and dissolved O_2) to optimize the biomass production and wastewater treatment (Guzmán *et al.*, 2021).

5.3.4 Develop valuable applications of microalgae biomass

As previously explained, the final use of biomass is highly relevant to the economic reliability of the process. Of all the possible applications those related to agriculture and bioenergy are the most suitable. In the case of agriculture, microalgae biomass can be used as biofertilizers or as biostimulants. The value of microalgae biomass as fertilizer is limited; thus, considering the N and P contents of the biomass and the price of fertilizers currently on the market the maximum price of microalgae biomass for this application is 100 €/ton. This value can be higher if considering microalgae biomass as organic fertilizer authorized for the production of organic foods, thus increasing up to 300 €/ton. However, the value of microalgae biomass as a biostimulant is much higher. Microalgae biomass, including when produced in wastewater, acts as a plant growth promoter favouring the development of plants and fruits, thus improving the production of foods by agriculture in the range of 10–20%, at the same time reducing the regular fertilizers requirement up to 10%, in some cases also reducing the prevalence of diseases and phytopathogens up to 20% (Fernández *et al.*, 2021; Stirk *et al.*, 2013). All these benefits make microalgae biomass a valuable tool for the improvement of food production by conventional agriculture. Companies already selling these products buy dry biomass of *Spirulina* at prices of 5–10 €/kg (dry biomass), which is a minimum of 5,000 €/ton (dry basis). That means that a conservative value for microalgae biomass produced from wastewater can be 1,000 €/ton, much higher than the one for its use as a regular source of N/P or fertilizer.

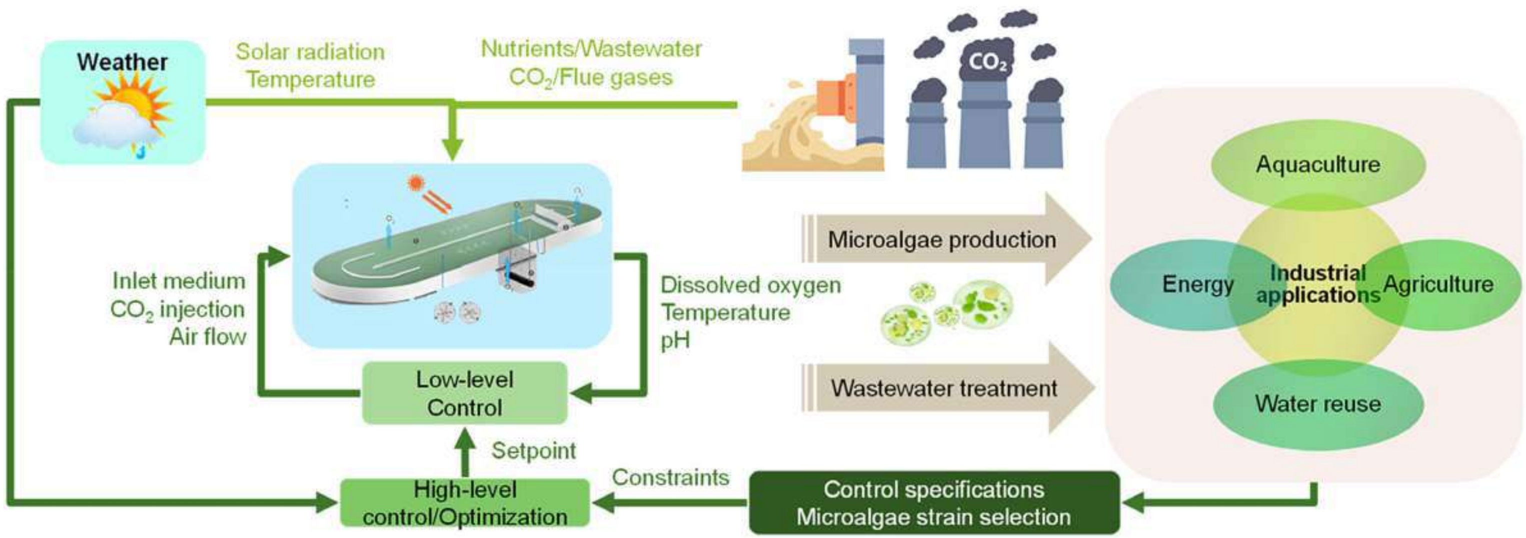


Figure 5.10 Concept of tools for continuous optimization of operating conditions in microalgae-related wastewater treatment processes.

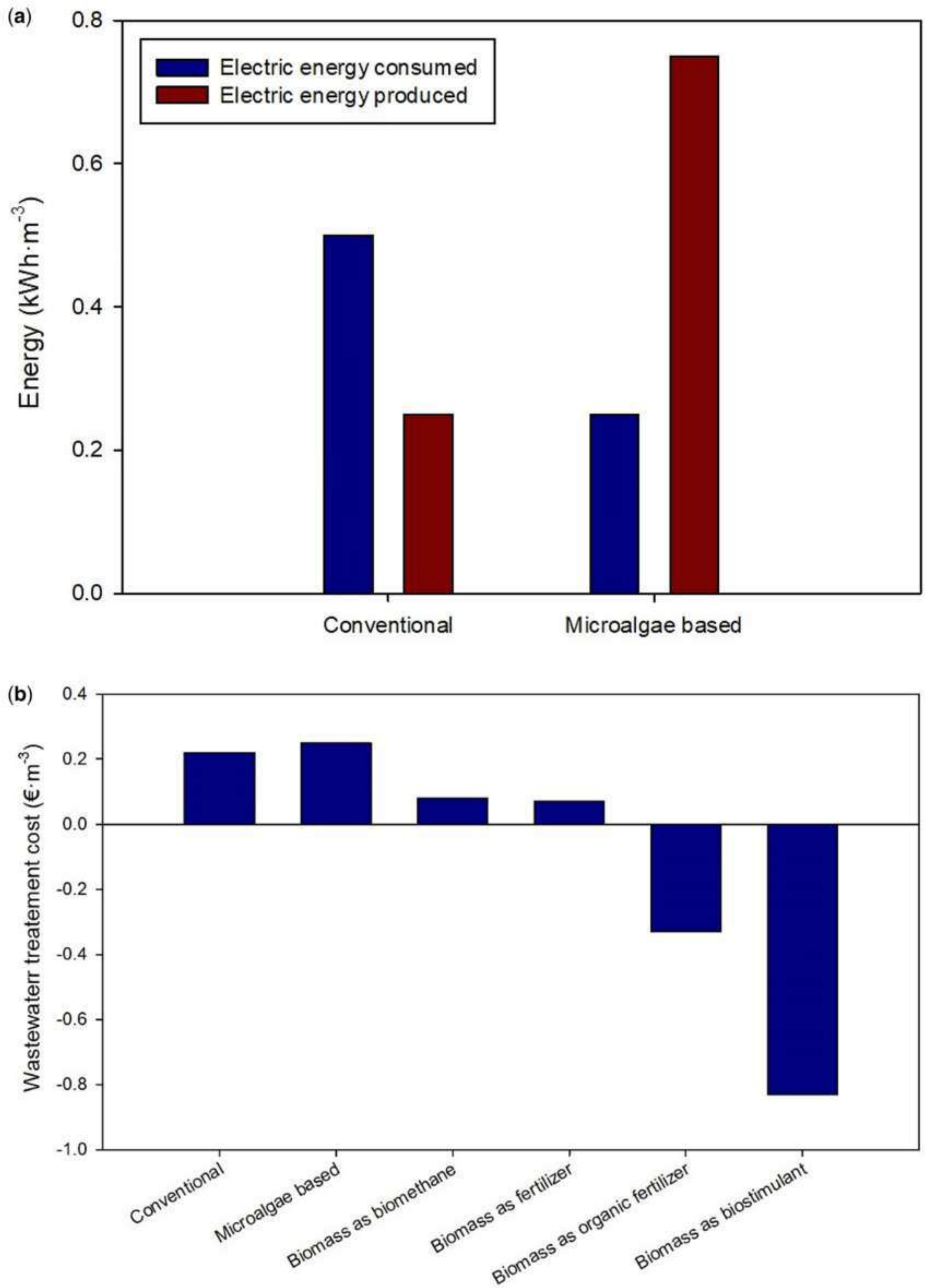


Figure 5.11 Comparison of (a) energy consumption and (b) wastewater treatment cost in different scenarios.

A much more simple application is the production of bioenergy as biomethane. Anaerobic digestion and production of biogas is a well-established technology, already existing in most wastewater treatment plants. The advantage of feeding microalgae to anaerobic reactors is the high methane potential of the biomass in the range of 150–300 L/kg VS depending on the characteristics of the biomass (Posadas *et al.*, 2015; Uggetti *et al.*, 2017). In this sense, FCC Aqualia already demonstrated that energy produced from the anaerobic digestion of microalgae biomass produced in a 1 ha reactor is much higher than the initial energy content of the wastewater, due to the fixation of solar energy by the microalgae cells into the reactor. This energy is released as biomethane during anaerobic digestion, and then the overall process results in a positive energy balance (Figure 5.11). Thus, in conventional wastewater treatment, the energy consumption corresponds to 0.5 kWh/m^3 and the produced biomass when transformed into biogas allows recovering a maximum of 0.25 kWh/m^3. When using microalgae-based processes the energy consumption reduces to half, up to 0.25 kWh/m^3, whereas the amount of biomass increases up to 100 tons/ha/year, equivalent to energy production through anaerobic digestion of 0.75 kWh/m^3. This is a change of paradigm in the wastewater treatment industry because instead of consuming energy, they become energy producers. Moreover, in terms of cost the change of paradigm is more relevant. Thus, the wastewater treatment cost reduces from 0.22 to 0.17 €/m^3 when using microalgae-based processes instead of conventional technologies, mainly due to the reduction of the energy consumption (Figure 5.11). However, if considering the value of microalgae biomass for different applications the net balance can be negative, meaning the value of the biomass compensates the wastewater treatment cost. Thus, if considering the use of biomass for the production of biomethane (0.3 €/kg, 30% conversion) or regular fertilizers (0.1 €/kg, 100% conversion), the wastewater treatment cost reduces to 0.08 and 0.07 €/m^3 respectively, whereas it becomes negative with values up to −0.33 and −0.83 €/m^3 when considering the production of organic fertilizers (0.5 €/kg, 100% conversion) and biostimulants (1.0 €/kg, 100% conversion). This fact opens an avenue for the development of energy and economic positive processes related to wastewater treatment (Arashiro *et al.*, 2018).

5.4 RELEVANCE OF DEVELOPING MICROALGAE-RELATED WASTEWATER TREATMENT PROCESSES

The development of microalgae-related wastewater treatment processes is a non-return way and finally, these processes will be implemented at industrial scale. It will not be a solution suitable for whatever location or scenario, but it will be suitable for certain scenarios. In this section, the relevance of applying microalgae processes for wastewater treatment is analysed.

5.4.1 Improvement of sustainability of wastewater treatment

Microalgae-related processes are one of the most suitable alternatives for wastewater treatment. Data included in this chapter already demonstrate this fact in terms of energy saving (50% of conventional processes), nutrient recovery (up to 90% of those contained in wastewater, equivalent to 10 ton N/ha/year, 2 ton P/ha/year) and production of valuable biomass for agriculture or bioenergy. The European Commission is driving policies to enlarge the sustainability of processes such as 'Green Deal' and 'FarmToFork' programmes in addition to others such as Blue Bioeconomy among others. The United Nations also recommends the development of policies to enlarge the sustainability of human actions, especially related to water management, recovery of nutrients and saving energy. In this sense, microalgae-related wastewater treatment processes demonstrate to be reliable technologies allowing to remove pollutants from wastewater minimizing the release of reusable water, and at the same time recovering nutrients contained in the wastewater as valuable biomass and saving energy or including producing energy as part of the process. The life-cycle assessment of this type of process demonstrates the sustainability of the technology, although the final results are different according to the boundary conditions considered (Arashiro *et al.*, 2018; Colzi Lopes *et al.*, 2018; Garfí *et al.*, 2017).

A favourable life-cycle performance was generally found for microalgae-based systems when displacing conventional energy products (Colzi Lopes *et al*., 2018). Specifically, the potential environmental impact of the conventional wastewater treatment plant was five times higher than that generated by microalgae-based systems. Even when comparing with other technologies such as constructed wetlands, microalgae-based processes showed to be the less-expensive alternative (Garfí *et al*., 2017). On the whole, implementing microalgae-based instead of activated sludge systems increases the sustainability and cost-effectiveness of wastewater treatment in small communities, especially if implemented in warm climate regions and coupled with biofertilizer production (Arashiro *et al*., 2018).

5.4.2 Distributed wastewater treatment

The technology of wastewater treatment is a mature technology capable of offering suitable alternatives for different scenarios, especially for large cities in which the cost of the required infrastructure is well assumed. Moreover, the cost of conventional technologies for wastewater treatment is currently quite reduced due to the continuous improvement of technologies, saving of energy and improvement of control strategies. Thus, both aerobic and anaerobic processes are operating close to optimal conditions under adequate control and supervision. However, these technologies are not cost-effective for medium-small cities, due to excessive cost of infrastructure or inadequate control and supervision. These cities represent the larger fraction of locations that don't accomplish the European Union (EU) regulations. The latest figures for wastewater treatment in Europe show improvements in collection and treatment, even if big differences remain between member states.

It is worth noting that the EU has been working to improve wastewater treatment across member countries through various directives and regulations. The Urban Waste Water Treatment Directive, for instance, sets standards for the collection and treatment of urban wastewater. EU member states have been implementing measures to comply with these standards, but the progress can vary. Microalgae-based technologies offer a feasible solution for small- and medium-sized cities. However, still, the economic feasibility of the process under practical operating conditions must be improved. To enhance the economic feasibility of microalgae-related wastewater treatment processes it is necessary to reduce labour costs, which implies that more automated designs need to be integrated into the design and operation to replace manpower. The selection of materials for the construction of reactors should focus on cheap and durable choices. The energy consumption for aeration, mixing and liquid conveying, microalgae harvesting and dewatering could be further reduced through the optimization of design and operation. Research on these topics will allow the development of robust and reliable technologies for distributed wastewater treatment.

5.4.3 Reuse of effluents in agriculture

Agriculture is a strategic sector for whatever society, the production of more sustainable and healthy foods being a demand of consumers, especially from developed countries. The EU has set forth various goals and initiatives to promote sustainable agriculture as part of its broader commitment to the United Nations' SDGs and the Agenda 2030. In this respect, the EU Commission imposes in the agenda for 2030 a 20% reduction in the use of fertilizers and pesticides, a 20% reduction in the use of land for food production and a 30% improvement in food production capacity. The recycling of water and nutrients contained in wastewater for the production of foods by agriculture is thus mandatory. Microalgae are an interesting option for this purpose because the environmental conditions required for their production are similar to those required for agriculture. Moreover, geographic areas devoted to agriculture are normally full of land, including non-arable land that can be used for microalgae-related processes. Finally, water and biomass obtained as products of the process are suitable for their utilization in food production by agriculture. Thus, as an example, for a population of 200,000 inhabitants (Almeria, Spain), it is estimated that up to 55,000 m^3/day of treated water can be obtained in a 10 ha facility by using microalgae-related processes for wastewater treatment. This amount of water corresponds to the water demand of up to 4,000 ha of tomato crops with a demand of 5,000 m^3/ha/year. This overall surface represents 14% of the overall surface of greenhouses in Almeria, producing 25% of horticulture crops consumed in Europe. Moreover, the overall biomass production capacity corresponding to these processes will represent up to 1,000 tons/year of dry matter, equivalent to 100 ton N/year and 20 ton P/year, allowing partially replace the consumption of mineral fertilizers in greenhouses, up to 10% of the current demand. However, the improvement of the performance of crops is more relevant, which allows increasing the food production by 10% by increasing the efficiency of nutrients uptake by the plants, which allows reducing up to 15% in the supply of mineral fertilizers, which means an overall reduction of 25% of the demand of mineral fertilizers. Thus, microalgae-based processes perfectly fit with the demand of the agriculture sector and consumers to produce and consume products from sustainable agriculture.

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