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RIASSUNTO

L'utilizzo intensivo di combustibili fossili ha generato una serie di conseguenze che solo negli ultimi anni si stanno manifestando in tutta la loro gravità. L'aumento della concentrazione di CO_2 nell'atmosfera, rilasciata dalla combustione di carbone, petrolio e gas naturale, ha innescato disequilibri ambientali su scala planetaria. Effetto serra, riscaldamento globale, desertificazione, sono solo alcune delle catastrofi che l'umanità deve affrontare urgentemente. A questo si aggiunge il pericolo di esaurimento di tali combustibili fossili, nonché un quadro socio-politico mondiale molto complesso: la pandemia del Covid prima e la guerra in Ucraina sono solo gli ultimi esempi di eventi che hanno intaccato la disponibilità e l'approvvigionamento delle risorse energetiche, con gravi ripercussioni sulle economie globali. Lo sviluppo di tecnologie per l'utilizzo di fonti energetiche alternative e sostenibili è ormai diventato una esigenza improrogabile, da cui nessun governo o organismo internazionale può sottrarsi.

Energia solare, eolica, nucleare, sono alternative valide ma comunque non esenti da criticità; non ultimo il fattore economico. Abbandonare i combustibili fossili per le energie alternative richiede, infatti, un consistente investimento monetario che non sempre è sostenibile nell'immediato.

Negli ultimi anni la ricerca si sta concentrando sullo sfruttamento della biomassa, e in particolar modo quella derivata dalle microalghe, che si è rivelata particolarmente vantaggiosa rispetto ad altre tipologie di biomassa e più in generale di fonti energetiche rinnovabili.

In questo progetto di dottorato è stata valutata le potenzialità di utilizzo di questi microrganismi fotosintetici nella produzione di bioenergia su ampia scala. La coltivazione delle microalghe è infatti avvenuta in raceway ponds da 80m^2, adottando un approccio da bioraffineria allo scopo di massimizzare l'intero processo. Per tale motivo alla produzione di biocarburanti è stato associato un processo di biorisanamento di acque reflue; così come sono stati valutati approcci operativi differenti, ad esempio implementando l'utilizzo di una membrana di ultrafiltrazione per il recupero della microalga dalla coltura.

Lo scopo principale è stato quello di mettere a punto un sistema produttivo economicamente sostenibile, e i primi risultati ottenuti sono promettenti per eventuali applicazioni future.

ABSTRACT

The intensive use of fossil fuels has generated a series of consequences that have only been manifesting themselves in all their seriousness in recent years. The increase in the concentration of CO_2 in the atmosphere, released by the combustion of coal, oil and natural gas, has triggered environmental imbalances on a planetary scale. Greenhouse effect, global warming, and desertification are just some of the catastrophes that humanity must urgently face. Moreover, the risk of fossil fuel depletion and a very complex global socio-political picture makes the situation even worse. The Covid pandemic and the war in Ukraine are just the latest examples of events that have affected the availability and supply of energy resources, with severe repercussions on global economies. The development of technologies for alternative and sustainable energy sources has become an urgent need from which no government or international body can escape.

Solar, wind and nuclear energy are valid alternatives, but even they are afflicted by some critical issues, among which the economic factor stands out. In fact, abandoning fossil fuels for alternative energies requires a substantial monetary investment which is hardly immediately sustainable.

In recent years, research has been focusing on exploiting biomass, particularly the one derived from microalgae, which has proved to be advantageous compared to other types of biomass and, more generally, to renewable energy sources.

In this PhD project, the potential application of these photosynthetic microorganisms in bioenergy production on a large scale has been evaluated. The microalgae cultivation took place in 80m^2 raceway ponds, adopting a biorefinery approach to maximize the entire process. For this reason, a wastewater bioremediation process was associated with the production of biofuels. Also, different operational procedures have been evaluated, for example, by implementing the use of an ultrafiltration membrane for the recovery of the microalgae from the culture.

The main aim was to develop an economically sustainable production system, and the first results obtained are promising for future applications.

1. INTRODUCTION

1.1. ENERGY AND FUELS

Energy is defined as the ability to do work, and it undoubtedly is the most valuable resource. In fact, every human activity need energy to be accomplished. And the total consumed energy is regarded as an indicator of development, since it is related to increased industrialization, rapid urbanization and economic activity of a country [1].

Primary energy is the energy directly derived from any fuel, with no need for intermediate transformations. For centuries, because of its availability and ease of collection and usage, the most used source of energy has been wooden biomass. With the Industrial Revolution, the first big energy transition happened, and wood started to be gradually substituted by fossil fuels, until the present day, when the global energy mix is dominated by 84,3% by fossil fuels. As shown in figure 1.1, in the beginning, coal was mostly used, soon overtaken by oil and natural gas [2].

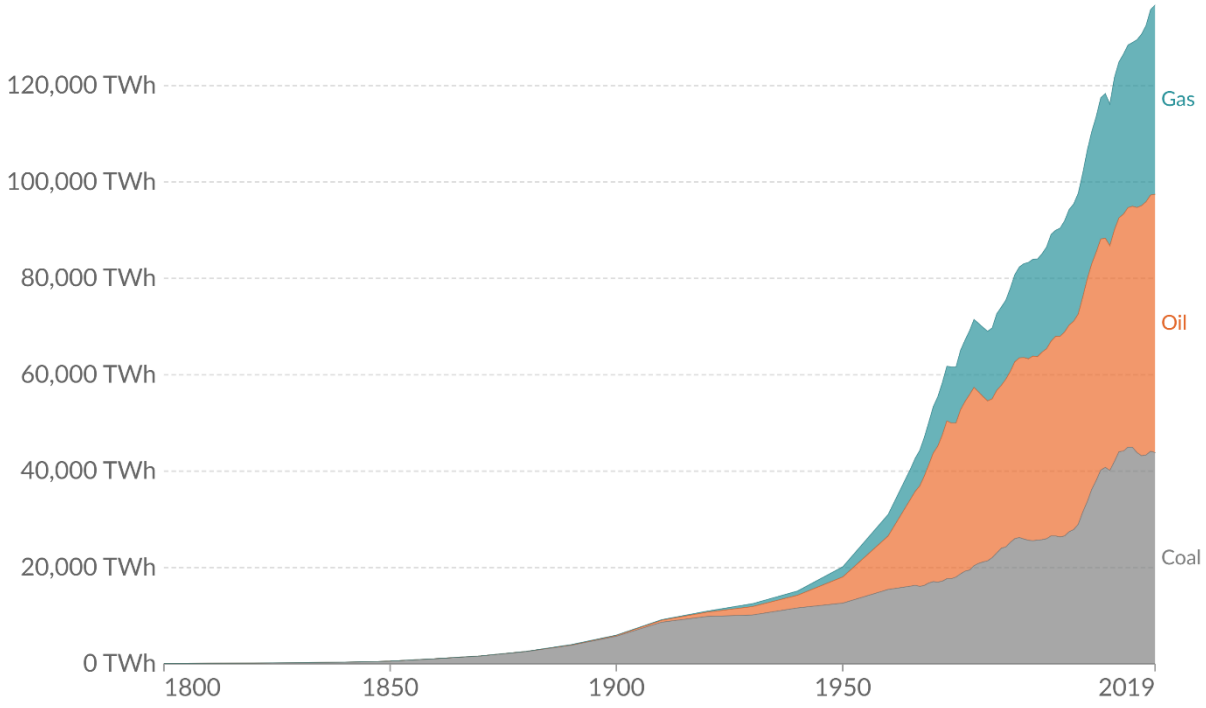


FIGURE 1.1: *Fossil fuels consumption since Industrial Revolution [2]*

There are several reasons why fossil fuels are so massively used: first, they have a high calorific value, providing a discrete amount of energy (between 30 and 50 kJ/g depending on the fuel) at a low cost, also given their large availability, at least so far. Second, the

technologies that employ them, starting from the internal combustion engine designed by Lenoir in 1860, have constantly developed over the decades, becoming today tested and within everyone's reach. Finally, they provide numerous secondary products, from chemicals to plastic materials, that find applications in several technology applications [3].

On the other hand, the massive exploitation of these natural resources has led to a series of negative consequences, that have been difficult to predict as they occurred over a long period of time and are often indirectly interrelated. The Greenhouse Effect, ocean acidification, weather and ecosystems changes, ice sheet melting are only some of the side effects relatable to the release of CO_2 and other harmful gases derived from fossil fuels combustion; not to mention the damage to human health [4]. If before the industrial revolution the levels of CO_2 in the atmosphere fluctuated between 250-290 ppm (parts per million), today this value is around 394,5 ppm, and current estimates predict that it could reach 500 ppm by 2050, affecting our planet in a devastating and irreversible way [5].

These alarming prospects, combined with the progressive depletion of fossil fuels, as they are non-renewable resources, and with the complex global socio-economic framework that often can cause difficulties in supplying, are forcing world governments to adopt alternative energy strategies. In 2015 the Paris Climate Agreement was stipulated, which mandates each nation to reduce the CO_2 emissions intensity by 30–35 % of their Gross Domestic Products (GDP) by 2030 [6]. At the moment, three are the main viable alternative[7]:

1. Nuclear energy: according to the International Energy Agency (IEA), nuclear energy represents the keystone for the energy transition towards a low CO_2 emission system. Currently, there are 452 nuclear power reactors operating in 31 different countries, for an overall energy production equal to 10% of their total requirement [8]. However, this value is much lower than that initially expected in the 1950s and 1960s when there was a boom in government investment in this new technology. Estimates at the time predicted that by 1990 nuclear energy would cover 15% of the world's energy needs. Catastrophic events such as Three Miles Island (1979), Chernobyl (1986), and, more recently, Fukushima (2011) marked a heavy setback in the development of this energy source. To date, the main challenges of nuclear energy are the impact on public opinion, which looks with fear at this technology, the huge economic investment that the start-up of a nuclear power plant requires, and the difficulty in waste management [9]. To address these issues, Small Modular Reactors

(SMRs) are currently under development, with the objective of cutting costs and maximizing the energy produced [10].

2. Carbon Capture and Storage (CCS): this name defines a group of technologies with the purpose to reduce CO_2 emissions in the atmosphere. CCS take place in three steps: separation and purification of CO_2 from fuels, feedstocks and industrial processes; compression and transportation of the recovered CO_2 toward the storage site; and injection of the CO_2 into the geological reservoirs. IEA estimated that this technology may contribute to a 15–20% of global CO_2 emissions reduction by 2050 [11]. This technology is relatively very young, and although it has scientifically demonstrated its effectiveness on a theoretical base, it still requires deepening. For examples, modelling a typical reservoir is not sufficient because every site has its specific geological characteristics that need to be analyzed to perform an optimal CO_2 storage. Still, CSS suffers for capital intensity, inadequate regulatory and legal framework, and absence of efficient mechanisms for carbon markets management, which strongly slow down its application [12].
3. Renewable energy: wind, solar, hydro and biomass are the main renewable energy source, so defined because their usage does not deplete them, or at least they are restored on a human timescale [13]. Renewable resources are projected to cover about 50% of world energy needs by 2050. At the moment, European countries lead for what concerns renewable energy, thanks to a community policy and joint effort of every nation involved. Following there are the US, which lack a federal policy, and the emerging countries like China, India, Korea that need to face the technological delay in order to take advantage of these resources [14].

   Wind is the most exploited renewable source, with a global capacity of about 600 GW/year, followed by solar and hydro energy. However, these sources suffer from intermittency in energy supply due to seasonality and weather conditions, the latter further exacerbated by climate change. For these reasons, their usage must consider the implementation of storage systems associated with the power generator, in order to save all the excess energy produced in peak moments and subsequently distribute it in the stall phases. At the moment different storage solutions are available: batteries, vehicle-to-grid, hydrogen based solutions, all coming up with their own not negligible costs, and also with major drawbacks which make them difficult to realize [15].

1.2. BIOMASS

Among all the renewable energy source, we will now focus our attention on biomass. The term biomass generally refers to any substrate derived from living organisms, both animal and vegetal. In the context of energy production only biomass of plant origin is used. The fuels derived from biomass through chemical or biological processes are called biofuels. Humanity has always used plant biomass to produce energy (for example the simple combustion of wood), but it is only in recent years that it is gaining greater interest in the scientific and technological fields because of the following several advantages.

First, vegetal biomass contains lots of valuable macromolecules, namely lipids, carbohydrates (e.g., starch, hemicellulose, cellulose, and pectin), lignin, proteins, ashes, that are suitable substrates for the production of different kind of biofuels alternative to biomass direct combustion. These compounds are present in vegetal biomass in variable percentages according to different factors, such as the genotypes, and environmental and culture conditions [16]. Second, biomass is not subjected to the intermittency phenomenon typical of other renewable sources, allowing a constant supply of material. Moreover, thanks to the ability of performing photosynthesis, thus fixing atmospheric carbon, vegetal biomass is a fully green fuel, with a low environmental impact. It can be defined a carbon capturing fuel, or at least carbon-neutral, and therefore it could represent a key element for a sustainable circular economy. Currently, the annual production of biomass is around 1800 billion tons [17].

It is possible to identify different feedstock of biomass exploitable to produce biofuel, which are commonly referred to as "generations". Each generation has its own problems and applications.

The “first generation” biomass is the most used to produce biofuels and includes edible crops, such as soybeans, corn, palm, sunflower, safflower, rapeseed, coconut and peanut. It is also the one that presents the critical issues of competition with food production. The applications of these crops for the production of biofuels would lead to a reduction in available food, which consequently would impact the worldwide food economy and supply. Increasing the production of first generation biomass to meet both food and energy purpose would result in a further intensification of cultivation. Intensive agriculture, in turn, leads to over-exploitation of cultivable land, reducing its availability and fertility, and to detrimental impact on natural ecosystems. Furthermore, this type of cultivation requires high quantities

of clean water, worsening problems of water supply especially in those areas suffering from water scarcity [18].

"Second generation" biomass is represented by non-edible biomass and residues from agriculture, such as sugarcane leaves, cassava stem, and forestry feedstocks. This type of biomass overcomes the problem related to food supply but its high cellulose content requires specific pre-treatments to be used in the production of biofuels. This translates into an increase in production costs and therefore in little convenience. For this reason, the use of biomass of this generation is still rather limited, and the related technological and production processes are not well optimized [19].

The "third generation" consists of algal biomass. Particularly microalgae are becoming the focus of several studies and technological applications due to the numerous advantages they present, often overcoming the limitation of the other generations of biomass. First, microalgae have a short harvesting cycle, not being subjected to the seasonal growth and development cycles of higher plants. This means that it is possible to have a constant supply of biomass in a short time. Data reported in the scientific literature states that, despite a theoretical optimal yields of 77 g $m^{-2}day^{-1}$, actual biomass production yields at a large, industrial scale cultivation are around 25 g $m^{-2}day^{-1}$, mainly due to loss of absorbed active radiation [20]. Microalgae do not compete for the use of fertile lands as they are aquatic organisms and their need of fresh water is much lower compared to terrestrial crops. Furthermore, they can grow in a huge variety of water, from the sea water, since many species of microalgae are marine, to different kind of wastewater, from urban to industrial one, carrying out a bioremediation process.

Microalgae are effectively biological laboratories, capable of producing numerous substances of interest not only for the bioenergy sector, but also for other application fields such as nutrition, agriculture, pharmaceuticals, cosmetics. Microalgae are, for example, an excellent source of essential amino acids and natural antioxidants, for which they are usually used as food supplements. They are also rich in polyunsaturated fatty acids and also produce enzymes with bio-stimulating efficacy for agriculture. For this reason, by adopting a so-called biorefinery approach, it could be possible to maximize microalgae and all their by-

product, gaining important economic advantages that will make this type of biomass the most convenient among all the other generations [21].

Microalgae are suitable for genetic improvement processes, always aiming at improving their yields of biomass and molecules of interest. Genetically enhanced biomass is often referred to as the "fourth generation" [22].

1.3. MICROALGAE

Microalgae are unicellular microrganisms present in all ecosystems, from terrestrials to aquatics. The latters can be both marine or freshwater. This huge diffusion is possible because microalgae can tolerate wide range of temperature, salinity, pH [20][23]. At the moment almost 40.000 different species of microalgae have been described, but estimates suggest that this number represent only a small percentage of the total existing [24].

Microalgae is a generic term for both eukaryotic and prokaryotic species. The latter are usually called cyanobacteria [25]. What they both have in common is the ability to carry out photosynthesis, a photochemical process by which, starting from carbon dioxide and water, and using light energy, they are able to produce glucose and molecular oxygen according to the following reaction:

$$6\ CO_2 + 12\ H_2O + Photons \rightarrow C_6H_{12}O_6 + 6\ O_2 + 6\ H_2O$$

The molecular oxygen produced is partly used by the cell to carry out cellular respiration, and partly released into the surrounding environment. It is estimated that 50% of atmospheric oxygen is produced precisely by the micro-algae [23].

The photosynthetic process is divided into two phases: the light phase and the dark phase. The light phase occurs inside the thylakoids, elements contained in the chloroplast, in which the photosynthetic pigments responsible for the absorption of light energy are present. Two main types of pigments are distinguished: chlorophylls and accessory pigments (such as carotenoids and phycobilins), normally associated with proteins in molecular complexes called photosystems. Two photosystems exists, named Photosystem I (PSI) and Photosystem II (PSII), and within them, the light energy is converted into chemical energy providing ATP and NADPH [26].

The dark phase, also known as the Calvin-Benson cycle, occurs within the stroma of the chloroplast. During this phase, the chemical energy previously produced is used to reduce carbon dioxide into glucose for the micro-algal metabolism [26][27].

Due to their ability to perform photosynthesis, micro-algae are defined as autotrophic organisms. However, they can also adopt alternative forms of metabolism.

In fact, microalgae can have a heterotrophic metabolism, which can be performed in dark condition by using alternative carbon sources, such as acetate or bicarbonate, or a mixotrophic metabolism, which combine both of the strategies mentioned above. [25] [27].

Microalgae can have very variable dimensions, reaching up to 50 µm in diameter. The smallest one, instead, is *Ostreococcus tauri* with a diameters of just 0.8 µm [28]. Their morphology is also very diversified, changing both according to the species they belong to and according to the stage of growth in which they are. [39]. To date, the morphologies described are: coccoid, filamentous, flagellate, amoeboid (for their ability to alter the cellular shape) and palmelloid (so defined when the cells are wrapped in a gelatinous matrix). Environmental conditions can change the shape of a micro-alga too. For example *Haematococcus pluvialis* and *Chlamydomonas reinhardtii* can change from the flagellated condition to that of a non-motile cell [29] (Figure 1.2).

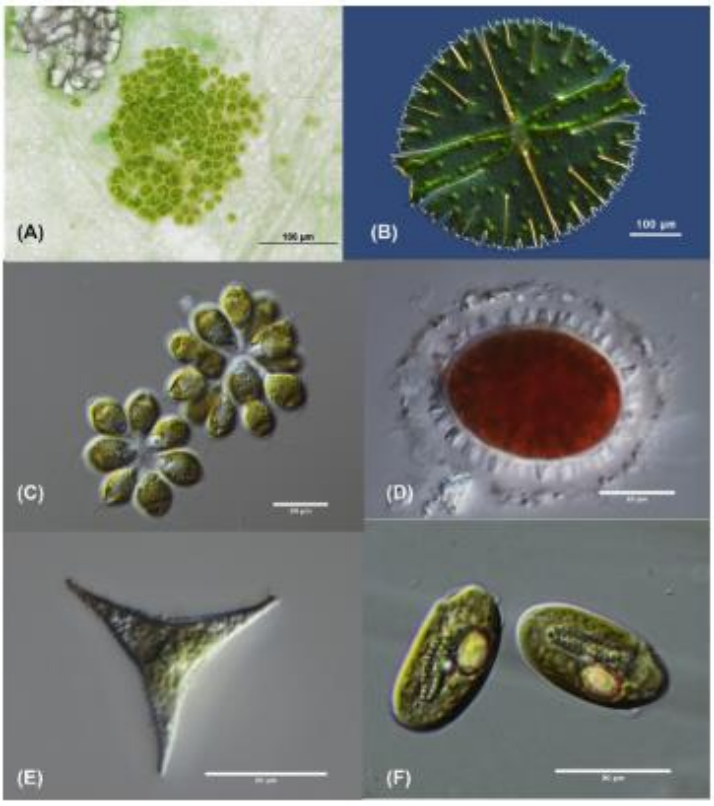


FIGURE 1.2: *Example of different microalgal morphology: A) Colony of Pediastrum sp. B) Micrasterias radiosa C) Synura petersenii D) Chlamydomonas sp E) Pseudogoniochloris tripus F) Cryptomonas sp. [28]*

1.3.1. CLASSIFICATION

Microalgae are a very heterogeneous group of organisms, with very different characteristics; therefore it is quite difficult to carry out an unique classification. The first attempt was proposed in 1989 by Lee, who divided them into four groups. The first group differed from the others as it was made up of prokaryotic micro-algae: *Cyanobacteria* and *Proclorophyta*. The rest included only eukaryotic algae, further subdivided on the basis of the membrane that surrounds the chloroplast, indicating a different evolution of the cell [30].

Among the subsequent classification criteria used, the simplest is based on the analysis of the photosynthetic pigments contained within the cell. However, this modality does not allow an exhaustive organization of all micro-algal species, so it has often been necessary to accompany it with further evaluation criteria such as: the chemical nature of the wall, the reserve organic substances produced, the morphology of the cell and the presence or absence of the flagellum [30] [31].

The classification according to *Hemaiswwarya et al* divides the micro-algae into two domains: prokaryotes and eukaryotes. The prokaryotic domain includes: *Cyanophyta* and *Prochlorophyta*, the latter less abundant than the former; while the eukaryotic one, which is much richer in species, collects nine *phyla*: *Glaucophyta, Rhodophyta, Heterokontophyta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta, Chlorachniophyta* and *Chlorophyta* [31] [32].

Cyanobacteria have been dated about 2.5 billion years ago, while eukaryotic microalgae date back to about 2 billion years ago [33]. There is no information on the birth of prokaryotic micro-algae, but it is thought that they were fundamental for the origin of life on earth, since they determined the accumulation of atmospheric oxygen [34]. On the contrary, the genesis of the eukaryotic domain is thought to be due to a process of primary endosymbiosis between a cyanobacterium and a heterotrophic organism, which led to the formation of the phyla *Rhodophyta* and *Glaucophyta*, characterized by a plastid with a double membrane coating. Subsequently, a further endosymbiosis, defined as secondary, led to the emergence of new algal classes, with different characteristics [30] [35].

Among the various algal *phyla*, the most important in terms of abundance are [36]:

- *Rhodophyta* (red algae), so defined for the red pigmentation due to the presence of phycobiloproteins (chromophore water-soluble protein), *chlorophylls a* and

chlorophylls d. They include both multicellular and filamentous forms, while unicellular forms are less present. Generally, the cells have a spheroidal shape, with a single chloroplast inside delimited by a double layer of membrane, which contains a single pyrenoid that plays an important reserve role. Red algae are found mainly in temperate and tropical regions [37][30];

- *Phaeophyta* (brown algae), thus defined because of their predominantly dark color due to the predominance of fucoxanthin (a carotenoid typical of brown algae) over *chlorophylls a* and *chlorophylls c*. Small filamentous forms fall into this class, but most are marine algae [30] [37];
- *Chlorophyta* (green algae), mostly freshwater, so defined for their bright green pigmentation due to the presence of *chlorophylls a* and *chlorophylls b* inside the chloroplast. They have a high morphological diversity, since they include unicellular, multicellular, filamentous algae, etc. [30][37][28];
- *Heretokontophyta*, among the main classes of this *phylum* we find the *Bacillariophyceae* (also called diatoms), considered the most numerous and usually present in marine phytoplankton or benthos (category of aquatic organisms that live in contact with the bottom). The peculiar element of differentiation of the species belonging to this class is the presence of a cell wall consisting of silicates that forms a shell, called frustule. A frustule consists of valves that are arranged by closing one on the other. Based on the symmetry of the frustule, diatoms are divided into centrales and pennales, respectively if they possess a central or bilateral symmetry [28];
- *Chrysophyta* (golden-brown algae), whose colour derives from the presence in the chloroplast of *chlorophylls a, chlorophylls c1, chlorophylls c2* and fucoxanthin. They generally prefer fresh waters, especially waters with low calcium content. They occur in unicellular or colonial form and are characterized by the presence of two flagella in an apical position to perform the motor function. The phylum includes only two classes: *Crysophyceae* and *Synurophyceae* [30].

1.3.2. CYANOBACTERIA

Cyanobacteria, also known as blue-green algae, are Gram-negative eubacteria capable of carrying out oxygenic photosynthesis. They are simple organisms with disparate morphologies and sizes: it is in fact possible to observe from unicellular forms with a diameter of 0.2 µm to filamentous ones with a length of 200 µm [37]. The modern taxonomy includes about 2000 species of cyanobacteria different in shape and structure, grouped into 150 *genera* and 5 *orders* [34] (Figure 1.3).

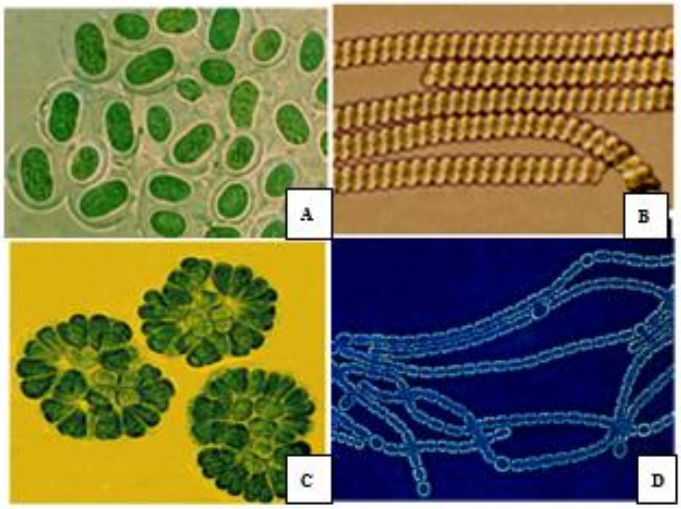


FIGURE 1.3: *Examples of cyanobacteria: A) Gloeothece B) Arthrospira platensis (Spirulina) C) Gomphosphaeria, D) Anabaena [28]*

The photosynthetic pigments present in cyanobacteria are chlorophylls a and phycobiloproteins such as phycocyanin, phycoerythrin and allophycocyanin, which give them the characteristic green-blue color. [33].

In addition to the ability to carry out photosynthesis, some filamentous cyanobacteria, members of the *Nostocales* and *Stigonematales* family, have the nitrogenase enzyme that gives them the ability to fix atmospheric nitrogen (N_2), in a similar way to nitrogen-fixing bacteria, transforming it into ammonia (NH_3), that can then be used to synthesize amino acids and proteins [34] [36]

Cyanobacteria play a fundamental role in the transformations of oxygen, carbon and nitrogen in the aquatic environments in which they live. They are usually found both in freshwater environments such as ponds, streams, springs and wetlands and in marine environments [34].

Cyanobacteria generally reproduce by binary fission, an asexual reproduction mechanism typical of bacteria. There are, however, some particular cases of filamentous cyanobacteria, that show an alternative asexual reproduction mechanism called random fragmentation, by which there is the production of hormogones (groups of cells that detach from the mother cell to form a new colony) [30]. A sexual reproduction has not been found, however alternative mechanisms are possible such as conjugation and transformation that allow the acquisition of external genetic material and therefore cause genetic variability [30].

Cyanobacteria also have the ability to produce cyanotoxins (hence the name cyanobacteria), which can cause damage to zooplankton (animal component of phytoplankton) [38] as well as contaminate drinking water thus becoming a real risk also for human health [39]. Examples of harmful cytotoxins are microcystin, nodularia and cylindrospermopsin, which can cause cytotoxicity, neurotoxicity, skin rashes and gastrointestinal problems [38]. However these cyanotoxins can be used in the pharmaceutical field as they are considered excellent natural cytotoxic compounds with anti-tumor action. An example is the use of Curacin A, deriving from *L. majuculata*, which has a cytotoxic and anti-proliferative action, used in pre-clinical trials for the treatment of breast cancer [38][39].

1.3.3. MICROALGAE

Properly called microalgae are eukaryotic cells equipped with numerous organelles such as chloroplasts, ribosomes, endoplasmic reticulum, etc. The nucleus is surrounded by a double-layered membrane, and inside it we find DNA molecules and nucleoli. The chloroplasts, essential for photosynthesis, are also enveloped by a double-layer membrane that contains the thylakoids, surrounded by a matrix called stroma. The photosynthetic pigments are present in the thylakoids, which differ according to the algal class [30]. The algal cell is then surrounded by a cell wall that prevents possible osmotic shocks and regulate the exchange with the external environment. Its biochemical composition can vary according to the different phylum to which it belongs. Its main components are silicates or calcified, which determine greater or lesser hardness [30], to which are then added for example in the green algae cellulose micro-fibrils and polymers of sulphates and galactans; in the red ones, on the other hand, cellulose, hemicellulose, peptide compounds and glycoproteins are present [29].

Eukaryotic microalgae also reproduce asexually, according to mechanisms such as binary fission, fragmentation and spore production. However, unlike cyanobacteria in some

eukaryotic micro-algal species, such as *Chlamydomonas reinhardtii*, mechanisms of sexual reproduction have been highlighted. These mechanisms are important for the formation of new species capable of resisting environmental changes [40].

1.4. CULTIVATION OF MICROALGAE

1.4.1 FUNDAMENTAL PARAMETERS

Microalgae are ubiquitous microrganisms, present in practically all natural ecosystems. It is possible to cultivate them in suitable systems called bioreactors, both on small laboratory scale or on large industrial scale.

For this purpose, there are several factors to pay attention to in order to ensure optimal growth of biomass: first of all is nutrients supply, then other physical-chemical factors such as light, temperature, pH, salinity, and mixing.

The fundamental nutrient is the one that provides a source of carbon for the alga to perform photosynthesis. As said in the previous paragraph, the most common one is CO_2, which is generally insufflated into the culture. However, given the metabolic versatility of these microorganisms, it is possible to provide them with different carbon compounds, both inorganic (bicarbonate) and organic (glucose, acetate).

In addition to carbon, other essential nutrients are nitrogen (N), phosphorus (P), and sulfur (S), generally in the ratio 18:1:0.99.

Nitrogen, like carbon, can be both inorganic (NO^{3-}, NO^{2-}, NH_4^+) and organic (Urea). However, in the form of ammonia NH_3, it can be harmful.

Phosphorus, on the other hand, is preferred in the form of phosphate; while sulfur is assimilated in the sulfate form, which is then reduced to sulfide in the chloroplast.

Finally, metallic micronutrients such as iron, silicon, magnesium, zinc, copper ... are needed to complete the ideal culture medium for microalgae, since they are essential for enzyme functioning [28].

As for the other parameters, light is essential because by hitting the photosystems, it starts the whole photosynthesis process. It can be both the natural sun light for open cultivations, or an artificial source for laboratory scale: what really matter is the wavelength, that has to

be in the range between 400nm and 700nm, known as Photosynthetic Active Radiations (PAR). Light intensity need to be controlled, too. The optimal range that guarantees the maximum algae growth rate is between 26−400 μmol photons m^{-2} s^{-1} (nevertheless microalgae can bear up to 700 μmol photons m^{-2} s^{-1} ; and some extremophiles species up to 2000 μmol photons m^{-2} s^{-1}). Low light intensity could not provide sufficient energy for the photosynthesis, while higher intensity may cause photoinibition [41].

Photo-scarcity can occur also because of self-shading. The high concentration of culture or the particular geometry of the bioreactor can cause that cells shadow each other, thus reducing their growth rate. For this reason a good mixing system is necessary to keep the microalgae in constant motion, so that every cell can reach the light. If with low laboratory volumes bubbling due to the insufflation of CO_2 is enough, with reactors on an industrial scale it is necessary to provide adequate solutions such as the padle wheele of the race way.

Temperature, pH and salinity are strongly dependent on the microalgal strain. But it is possible to indicate optimal range. Microalgae best grow between 20 and 30°C, but generally they can tolerate lower temperatures. Higher ones, instead, can kill them. Concerning pH, it usually need to be between 6 and 8. Ongoing studies indicates that it should not be underestimated because it can deeply affect biomass productivity. And finally salinity represent a bigger issue especially in case of open cultivation systems because of the phenomenon of evaporation that reduce the water content of the culture, thus increasing the salinity and causing cell stress [42].

1.4.2. OPERATIONAL STRATEGY

A microalgae culture during its growth process goes through four different stages (Figure 1.4).

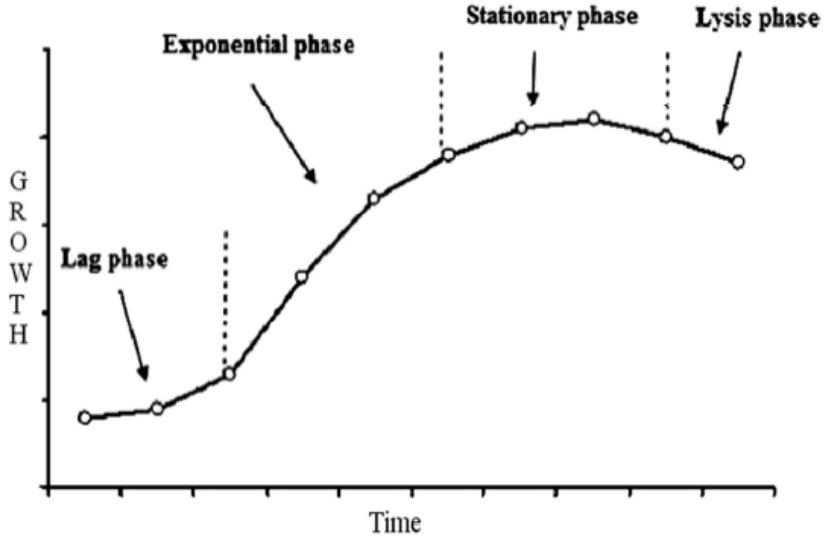


FIGURE 1.4.: *Algae growth phases*

Different operational strategies can be applied to the culture to maximize biomass production.

The first strategy is the *batch*. In this mode, the culture is only initially supplied with the maximum necessary nutrients, which depends on the operational volume. Then a closed system is established in which only the control gases are added. In this way, the microalgae grow to the steady state, which, however, can only last for a short time because of the shortage of nutrients that happens.

A variant of this strategy is the *fed-batch,* in which nutrients are periodically supplied to the crop so that these no longer represent a limiting factor. In this case, the time required to reach the steady state is slightly prolonged. Nevertheless, accumulation phenomena of toxic substances and inhibitory agents become more probable.

Batch cultures are used either for short-term experiments or as an initial step toward the second mode which is the *continuous* one. Switching from batch to continuous mode is possible once the steady state of the culture has been reached.

In this second strategy, a calculated culture volume is periodically removed, and an equal quantity of fresh medium is added. This volumes exchange determines the harvesting of a variable percentage of biomass that depends on the level of productivity achieved; and, consequently, a dilution of the culture. The latter must be less than the doubling time of the microalga so that the culture can return to its maximum concentration between one harvest and the next [43].

1.4.3. LARGE SCALE CULTIVATION

Microalgae can be grown on a large scale in systems that can be divided into two main categories: open and closed. The choice of how to cultivate depends on several factors: the growing medium, the amount of water, the nutrients, the temperature, and the final product to be obtained .

Excluding natural water pools, which can not be exploited for industrial applications, open systems can be simple basins or more complex circular ponds and raceway ponds.

In general, open systems are very advantageous because they are easy to build and manage. Consequently, even economically, they do not require excessive expenditure for their maintenance. On the other hand, in addition to requiring more or less large spaces depending on their size, they do not consent a precise and punctual control of all parameters. In open ponds, for example, the management of light is complex, and the loss of water due to evaporation must be taken in consideration and corrected to avoid dangerous variations in the pH and salinity of the culture. Moreover, since they are constantly in contact with the open air, it is impossible to maintain the sterility of the culture, to the point that often, in these cases, we do not speak of a single strain but of a consortium, both between different microalgae and with bacteria and other microorganisms.

Despite this, these are the most widely used systems on an industrial scale. In fact, in 95% of cases, microalgae cultivation occurs in raceway ponds. These are simple and versatile systems that can be easily built to the desired size. They consist of various channels and curves in which the crop flow thanks to the constant agitation induced by paddlewheels. These prevent the cells from sedimenting on the bottom and guarantee an adequate mass transfer. The raceways are equipped with a sump to further optimize this last process.

The microalgae culture's optimal depth in a raceway pond varies between 15 and 20 cm. In the best possible conditions, it can reach a productivity of 30 g $m^{-2}day^{-1}$.

An evolution of this system is the thin-layer reactor, which operates with a thin film of microalgae not deeper than 2 cm. In this way, the exploitation of light radiation is optimized to obtain higher production yields [44] (Figure 1.5).

FIGURE 1.5: *Raceway pond located in IFAPA Experimental Center, University of Almeria, Spain*

Even closed systems, which more precisely are called photobioreactors, can have different geometries, from flat plates to tubular ones. They are much more complex systems than previously seen, requiring a more significant investment in economic terms. However, they allow more precise control of the various chemical-physical parameters, as well as guarantee the sterility of the crop, never being directly in contact with the external environment. These systems can therefore be used for producing high-value biomass to be used in the food and pharmaceutical sectors.

Flat panels are systems made up of panels of transparent material to ensure the passage of light, placed in such a way as to leave a thickness no greater than 10 cm in which to insert the crop. They can be positioned vertically or tilted to improve the uptake of light radiation.

Flat panels can reach high productivity in terms of biomass (up to 15 g $m^{-2}day^{-1}$); however, they are very complicated to manage, mainly because they suffer from severe fouling problems.

Tubulars are currently the most widely used closed systems. They consist of a system of tubes of transparent material inside which the crop flows, recirculated by pumps. The geometry of a tubular system must be carefully designed according to the production needs and taking into account that an adequate mass transfer must be guaranteed so that in every point of the duct there would always be the right amount of nutrients for the optimal growth of the alga, which in any case does not reach the productivity levels of flat panels [45] (Figure 1.6).

FIGURE 1.6: *Tubular photobioreactor*

1.4.4. HARVESTING

Harvesting is the process of collecting microalgae from the water they have been cultivated. This step is undoubtedly one of the main bottlenecks of the entire microalgae production process since it is costly, time- and energy-consuming. Furthermore, it should not adversely affect biomass quality and integrity.

Several harvesting techniques have been developed, such as filtration, flotation, flocculation, and centrifugation.

Centrifugation is the most commonly utilized method, representing a good compromise between operational time and yields. Depending on the instrument's power, it can concentrate biomass up to a 10^2 factor, still granting a good quality slurry. The main problem is that it requires a high energy amount; thus, the operational cost can considerably rise.

Filtration is a viable alternative. Membranes have a high separation efficiency and can operate in continuous mode for long periods, consuming less energy than a centrifuge. What negatively affects membranes is fouling. High biomass quantity can saturate the membrane, thus blocking the filtration process. Backwashing and pulsed air scouring are good ways to solve the problem, but the turbulence they generate can cause shear stress on the cells.

Flocculation can be seen as more efficient sedimentation. By adding positively charged coagulants, microalgae, that have negative charges on their surface, tend to form lumps that precipitate. The most common flocculant agents are aluminum or iron salt, but recently organic compounds have been studied to replace them. Chitosan has shown an acceptable recovery rate. However, the flocculation process is not as effective as filtration or centrifugation. Thus, flocculation can be used as a pre-treatment since it is cheap.

Once obtained microalgal slurry, a freeze-drying step is necessary to completely remove the water, obtaining a dry powder suitable for every further application. This is another critical step since also a freeze dryer is expensive and energy-consuming. [46,47].

1.5. BIOFUELS

The classification in generations previously seen for biomasses applies equally to biofuels, to indicate from which starting feedstock they are derived. Biofuels can also be distinguished into liquid (bioethanol, biodiesel) and gaseous (biogas, biomethane, biohydrogen).

Bioethanol is considered to have the potential to replace the fossil-derived fuels. Although its energy density of 24 MJ L^{-1} is lower than that of other fuels, bioethanol has a motor octane number of 98 (gasoline has 90), and an oxygen content equal to 37% by weight. The motor octane number indicates the capacity to resist compressions in the engine without detonating. These characteristics mean that this biofuel is way more efficient in combustion engine, and also that it can be mixed to other fuels to enhance combustion and at the same time reducing exhaust emission [48,49]. Bioethanol is produced via fermentation of the monosaccharides, which are derived from starch, lignocellulose, and other kind of polysaccharides through hydrolysis. The major production of bioethanol is from sugarcane, whose yields can reach around 9000 L ha^{-1}, followed by sugar beet (2700 L ha^{-1}), and corn (1300 L ha^{-1}) [19]. Microalgae can be a very convenient alternative source of bioethanol given their high content of carbohydrates. Carbohydrates are the main photosynthetic product and microalgae store them under many different forms in their plastids or as structural component of their cellular wall. Some species of microalgae can produce carbohydrates up to 67% of their biomass [50]. Bioethanol from microalgae can be produced following three different strategies: Dark fermentation, photo-fermentation and fermentation of pretreated microalgal biomass. The latter is the most used because it guarantees better yields than other strategies, which still require extensive research to be optimized. Different types of biomass pretreatment are possible, in order to reduce the long chains of polysaccharides into easily fermentable monosaccharides. The most effective involves sulfuric acid, which allows to recover large quantities of carbohydrates for a final ethanol yield of about 0.202 g g^{-1}[51]. However, this method has a strong environmental impact, so the application of an enzymatic pre-treatment is currently emerging, using enzymes such as cellulase, amylase and pectinase, individually or in combination with each other. The enzymatic pretreatment guarantees, in addition to still advantageous yields (up to 79 g L^{-1} [52]), a more green and eco-sustainable process [53].

Biodiesel is obtained from the lipid fraction of biomass through a simple mono-alcoholic transesterification process. It has an energy density of 33 MJ/L, and a flash point between 100°C–130°C that make it safer than conventional diesel (petrodiesel), which has a flash point of approximately 60°C–80°C. Biodiesel is ecological and non toxic, emitting up to 83% less pollutant than petrodiesel [54]. First and second generation biodiesel are produced from oily biomass such as soybean, corn, oil palm, jatropha and pongamia. These biomasses, beside not having advantageous productive yields (table 1), suffers of all the typical limitations of their generation as mentioned above. Third generation microalgal biomass has instead a lipid content that can rise up to 80% depending on the algal strain and the growth conditions applied [21].

TABLE 1.1: *Comparison of oil content, yield, and biodiesel productivity among different generations of biomass [21]*

Feedstock source	Oil content (% oil by wt. in biomass)	Oil yield (oil in litres/ha/year)	Biodiesel productivity (kg biodiesel/ha/year)
Oil palm	36	5366	4747
Maize	44	172	152
Physic nut	41–59	741	656
Caster	48	1307	1156
Microalgae with low oil content	30	58,700	51,927
Microalgae with medium oil content	50	97,800	86,515
Microalgae with high oil content	70	136,900	121,104

The main obstacle to massive biodiesel production from microalgae is the lipid recovery phase. While the subsequent transesterification step is a simple process with high yields, lipid recovery is more complex. Generally, the extraction takes place using organic solvents, preferably on dry biomass. Biomass harvesting and freeze-drying, in addition to pre-treatments necessary to break up the cells and facilitate extraction, are highly costly procedures. Alternative methods are being developed to simplify these steps and make them more economically advantageous [55,56].

Biogas is obtained by the direct anaerobic digestion of the biomass. It is a mixture of gases than can contains up to the 60% of methane, thus making its energy content almost similar to natural gas. Since microalgae have a high biomass productivity, they are the ideal substrate for this type of process, which result to be almost carbon free because the

microalgae consume CO_2 during photosynthesis, balancing in this way the quantity of carbon released by the biogas. Moreover, biogas from microalgae does not contain highly polluting sulfur compounds, and the aerobic digestion generates a nutrient-rich "digestate" that can find application as a biofertilizer in agriculture [43].

Similar to biogas is syngas, a synthesis gas obtained through a particular hydrothermal process. Hydrothermal is a protocol that works on microalgal biomass slurry in a hot-compressed water medium, therefore avoiding the highly costing dewatering step to recover dry biomass. It can be conducted at different operating conditions, which determine the final products. At low temperatures, less than 200°C, the process is referred to as hydrothermal carbonization (HTC) and predominantly produces a char. At intermediate temperatures of approximately 200–375°C, the process is known as hydrothermal liquefaction (HTL), primarily producing an oil. At the higher end of the temperature range, greater than 375°C, the process is called hydrothermal gasification (HTG), predominantly producing syngas. Syngas has a high content of H_2, CH_4, CO and light hydrocarbons (C2 –C3). The side effect is the productions of a CO_2 amount, together with an high energy request to reach the extreme condition of the process itself [57].

1.6. BIOHYDROGEN

The biofuels described so far can be obtained from biomass of any generation. Biohydrogen, instead, can be obtained only from microalgae.

Hydrogen gas is currently the most attractive energy alternative, the one that seems to provide the most sustainable and green solution. Indeed, it has significant thermodynamic parameters. Its calorific value (122 kJ/g) is, on average, three-fold higher than fossil fuels [58]. Technically, gaseous hydrogen is not an energy source. It is defined as an energy carrier, which means it is not found directly in nature, but it is necessary to generate it first in order to exploit it. Steam reforming and water electrolysis are the most used techniques to produce hydrogen, but they are highly energy demanding. This inevitably makes it necessary to resort to energy solutions that involve fossil fuels, nullifying the green potential of hydrogen. Although there are still problems related to storage and distribution, hydrogen has the great advantage of carbon free-combustion [59,60]. Inside fuel cell devices, it is possible to make hydrogen combustion with oxygen, obtaining only water as a product. To

produce hydrogen in a green way, microorganisms, such as microalgae and bacteria, offer a potentially ideal solution (hence the name biohydrogen) [61].

The microalgal hydrogen production has been characterized mainly in the microalgae *Chlamydomonas reinhardtii*, in which three production pathways have been identified (Figure 1.7). However, the phenomenon is transitory as it occurs only in hypoxic environment that is not common in photosynthetic organisms. Furthermore, in these conditions other metabolic pathways compete for electrons used as substrates by the central enzymes of the process, hydrogenases, decreasing the production yields [62–64].

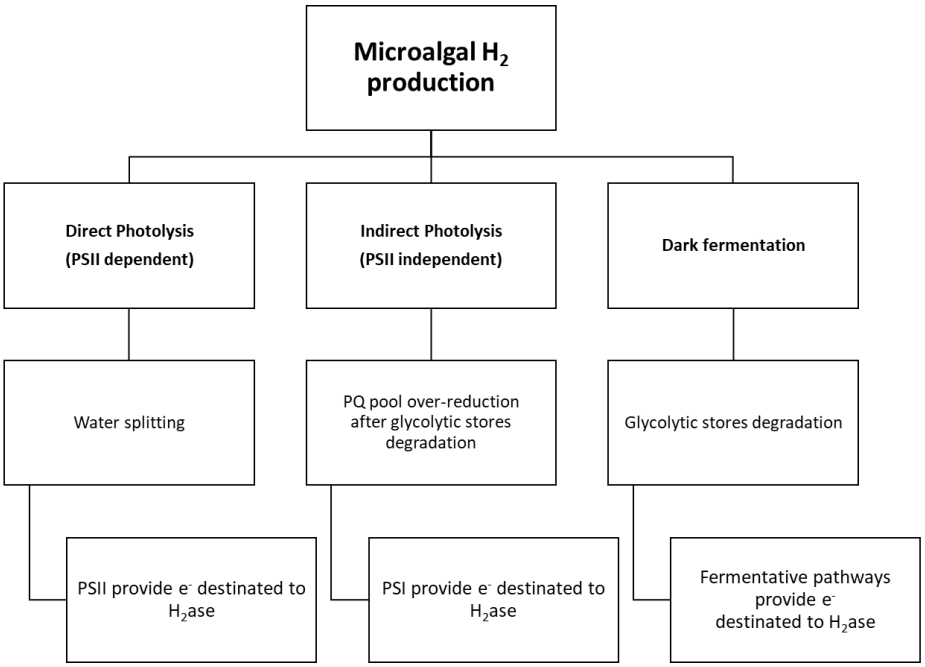


FIGURE 1.7: *Metabolic processes involved in microalgal biohydrogen production*

Direct bio photolysis at the PSII level uses solar energy to break up water molecules. Under aerobic conditions, the resulting protons and electrons are destinated to ferredoxin NADPH oxidoreductase (FNR) to produce NADPH for the subsequent CO_2 fixation. In hypoxic conditions, one of the possibilities is that employing ferredoxin protons and electrons are addressed and rearranged within the enzyme hydrogenase. Although this process contributes about 80% to hydrogen production, it also involves the cogeneration of oxygen which in the long run ends up restoring an aerobic environment [65,66]. An indirect photosynthetic pathway provides electrons from carbohydrate stores, mobilized through a type - II calcium-dependent NADH dehydrogenase (NDA2). This phenomenon is also called non-photochemical reduction of the plastoquinone (PQ) pool and is also referred to as the PSII-

independent production pathway. Through the PQ and PSI, the electrons finally reach the hydrogenase by the means of ferredoxin. In this process, oxygen does not occur at the same time, but other pathways, such as the circular electron flow (CEF) around PSI, negatively impact the yields, significantly lower than the PSII-dependent pathway [67–69]. A fermentation pathway also contributes to hydrogen production, albeit with minimal yields. The process essentially concerns the degradation of starch reserves. The main products include formate, acetate, and ethanol, while hydrogen and CO_2 are mostly marginal products [64,70,71].

The crucial enzymes involved in microalgal hydrogen production are hydrogenases, oxidoreductases having a catalytic cluster containing metal ions, responsible for the following reversible reaction [63,72]:

$$2H^+ + 2e^- \leftrightarrows H_2$$

Two different iron hydrogenases (H_2ases) (Figure 1.8) are known in *Chlamydomonas*: hyd1, which contributes the most, and hyd2. Thanks to a transit peptide, the hydrogenases (about 47-48 kDa) migrate into the chloroplast where they carry out their functions. In the presence of oxygen, however, the catalytic site of these enzymes is irreparably compromised and also gene transcription begins to be inhibited. In a hypoxic environment, the enzymes can be synthesized and the catalytic activity can take place, welcoming the electrons through ferredoxin [73–76].

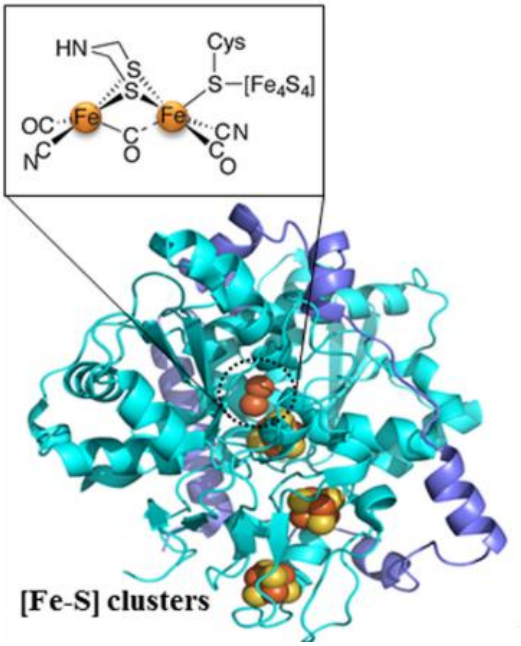


FIGURE 1.8: *Typical [FeFe] Hydrogenase structure [37]*

A decrease in the photosynthetic process and a simultaneous increase in mitochondrial activity are necessary to lower the oxygen levels and increase hydrogen production. In addition, protons and electrons should preferentially divert at the level of the hydrogenase enzyme. The approaches used to improve yields by acting on these aspects were essentially two. Initially, a first attempt was to modulate microalgae culture physiologically by trying to change the growth conditions. The expansion of knowledge leads progressively to targeted strategies of genetic engineering. At a physiological level, the most easily implemented action is to change the composition of the culture medium by depriving it of some macronutrients [77]. This leads to a decrease in the CO_2 fixation rate and the accumulation of reserve substances which are then mobilized later and used to sustain hydrogen production over time. The most promising strategy in this area is sulfur deprivation. It has multiple effects, but the most evident is at the PSII level: its activity decreases due to the partially irreversible damage reported by the D1 protein, which is most PSII protein affected by the altered protein turnover. With saturating light, the culture undergoes an anaerobiosis condition within a few days. This triggers a hydrogen production mainly related to the PSII pathway contribute [78–80]. Nitrogen deprivation also leads to increased hydrogen production. In any case, this dependent PSI production is scarcer and more dilated over time. The lack of nitrogen has a more lethal impact than sulfur on the overall cell physiology [81,82]. To a lesser extent, phosphorus deprivation results in increased hydrogen production but intracellular reserves hinder the achievement of complete deprivation [83].

As for the effect of carbon sources, most of the studies were under heterotrophic conditions using acetate. Minimally, some studies have also evaluated autotrophic productions but with significantly lower yields. In particular, acetate proved to be a good promoter of anoxia in sealed cultures, even under low light intensity and in replete media conditions in *Chlamydomonas* strains [77,84,85]. Interesting results have also been obtained in strains of *Scenedesmus* or *Chlorella* with carbon sources such as glucose, sucrose, or fructose. However, the mechanisms that trigger these hydrogen productions remain to be further clarified [81,86,87].

Acting by modifying the composition of the culture medium can involve various issues at the operational level, such as the transfer of cells to experiment with different sequential culture conditions. Therefore, investigated immobilization systems include fiberglass matrix, alginate film or beads. While these systems entrapping cells also limit oxygen circulation favouring hydrogen production, on the other hand, they pose new challenges in

the design of new bioreactors that are more suitable than those with algae in the liquid phase [88–90]. Closed systems have been the most used among classical bioreactors to monitor the multiple parameters involved, and among theme, flat panels have given the best results regarding hydrogen production [91–93].

However, these strategies do not represent a permanent solution to the transient and low-yield hydrogen production. Furthermore, the application of prolonged cellular stress culminates in cellular suffering or death. Today, there are many molecular tools able to modify the algae metabolism in an efficient and targeted way. To apply these strategies, however, extremely great knowledge of biochemical processes and genetic aspects of the organism under study is required [94]. Initially, the process of random mutagenesis allowed the isolation of algal strains with a more marked tendency to produce hydrogen. Chemo-chromic assays based on substances that change colour with the released gas helped with this purpose. Anyway, the screening phases between mutants risk being too long and expensive [95]. Targeted molecular strategies require a significant initial design phase. Today, numerous omics data and dedicated software greatly facilitate these aspects. The extensively described and studied model algae *Chlamydomonas* proved to represent an excellent starting point for targeted mutants to overcome bottlenecks associated with hydrogen production [94]. One of these is related to the saturation and dissipation of sunlight. Mutants with a truncated light-harvesting antenna (*tla*) in the chloroplast have proved to be less subject to photoinhibition and saturation phenomena, improving hydrogen production [96,97]. Another limitation is the competitive pathways that subtract electrons from the hydrogenase. The *pgrl1* mutant (protein gradient regulation like 1) limited one of the competitive pathways, CEF, producing more hydrogen [98]. Moreover, an O_2 tolerant clostridial [FeFe]-hydrogenase, heterologously expressed in *Chlamydomonas*, showed better enzymatic performance and hydrogen production too [99]. Among the most recent strategies, interesting implications have also been observed using of co-cultures of microalgae and bacteria. Indeed, the reciprocal influence in the metabolism has shown significant results also in the production of hydrogen compared to single mono-cultures [100,101].

Finally, following Table 1.2 reported highest yields in microalgae *C. reinhardtii* hydrogen production:

TABLE 1.2*: Hydrogen production highest yields recorded in microalgae C. reinhardtii* [102])

Strategy	**Parental alga strain**	**Mutant strain**	**Condition**	**Reported H_2 production**	**Estimated average H_2 production rate (mL/L·d)**
Monoculture/Genetic modification/S deprivation	cc124	pgrl5	TAP-S, 60 PPFD	850 mL/L (9 days)	≈94.4
Monoculture/Genetic modification/S deprivation	cc1618	stm6	TAP-S, 100 PPFD	540 mL/L (14 days)	≈38.6
Monoculture/Genetic modification/S deprivation	11/32b	L159I-N230Y	TAP-S, 70 PPFD	504 mL/L (12 days)	≈42
Monoculture/Genetic modification/S deprivation	137c(cc124)	pgrl1	TAP-S, 200 PPFD	≈1.5 mmol/mg chl (5 days)	≈87.4
Monoculture/Genetic modification/S deprivation	cc1618	Stm6Glc401	TAP-S + 1 mM glucose, 450 PPDF	361 mL/L (≈8 days)	≈46
Consortia/*Pseudomonas* sp. /S deprivation	FACHB-265	–	TAP-S, 200 PPFD	170.8 mL/L (13 days)	13.1
Consortia/*Bradirizhobium japonicum*/S deprivation strain	cc849	Transgenic lba strain	TAP-S, 60 PPFD	298.54 µmol/40 mL (14 days)	≈11.95
Consortia/*Bradirizhobium japonicum*/S deprivation strain	cc503	–	TAP-S, 200 PPFD	310 µmol/mg chl (16-days)	≈10.3
Monoculture/S deprivation	137c (cc125)	–	TAP-S	≈155 mL/L (≈4 days)	≈38.75

1.7. AIM OF THE WORK

The rapid growth of the world population makes it essential to produce large quantities of energy. The use of fossil fuels is no longer a sustainable alternative: centuries of coal, oil, and natural gas exploitation have had a devastating environmental impact, causing phenomena such as greenhouse gases, global warming, melting glaciers, ocean acidification, and desertification, seriously jeopardizing the life of our planet. The use of new forms of renewable energy has become an unavoidable necessity. While on the one hand, world governments must commit to implementing sustainable strategies, on the other, the research and technological development sector must continue to develop valid technological alternatives. In this scenario, microalgae represent a promising alternative, given their high productivity and versatility. It is possible to obtain different types of bio and sustainable fuels from microalgae, which can represent an effective alternative to fossil fuels. If, in theory, these processes are pretty simple, they actually come up against costs that are not always advantageous. It is necessary to adopt a biorefinery approach, integrating the production of biofuels with parallel processes that make everything more sustainable.

This PhD project aimed to follow the entire large-scale biofuel production process by introducing improvements that can help increase production yields and lower costs.

Specifically, the work is divided into three main steps: during the cultivation phase, the microalga *Scenedemus almeriensis* was kept in two 80m^2 raceways, one fed with fresh water and fertilizer and the other with wastewater to evaluate its bioremediation effectiveness.

During the biomass harvesting phase, the effectiveness of an ultrafiltration system to increase recovery yields and reduce operating times and costs was evaluated when performed before centrifugation of the biomass,

Finally, in the biofuel production phase, the biomass recovered from the two reactors was analyzed, to compare the production yields, its composition and the potential applications.

2. MATHERIALS AND METHODS

2.1. CULTURE CONDITIONS

Microalgae were cultivated in two 80 m^2 raceways pond located in IFAPA Experimental Center, University of Almeria, Spain during 7 month, from May to November 2022. The culture depth was kept at 15 cm, pH constantly monitored and kept around 8.00 by injecting CO_2 at the occurrence. Irradiance and weather conditions were daily recorded.

The main microalgal strain inoculated was *Scenedesmus almeriensis*. Although, being an open bioreactor, a consortium with bacteria and other microalgae took place. The culture were in continuous mode with a dilution rate of 0,2 day^{-1}.

The first raceway was fed with freshwater and a nutrient medium with the following composition:

- Sodium Nitrate: 0,9 g/L;
- Magnesium Sulfate: 0,18 g/L;
- Potassium Phosphate: 0,14 g/L;
- Microelements solution: 0,02 g/L;

The microelement solution supplies the microalga with the principal metal ions that act as cofactors of the enzymatic complexes [28]

- Boron (B) 0,5% p/p;
- Copper (Cu) 0,30% p/p;
- Iron (Fe) 7,5% p/p;
- Manganese (Mn) 4,0% p/p;
- Molybdenum (Mo) 0,2% p/p;
- Zinc (Zn), 0,5% p/p.

In the second raceway, instead, urban wastewater was added as culture medium for microalgae. Its average composition was as following:

- Nitrates: 0,69 mg/L;
- Phosphate: 41,91 mg/L;
- Ammonia: 185,62 mg/L;
- Chemical Oxygen Demand (COD): 302,39 mg/L.

2.2. DAILY ROUTINE

The daily routine to monitor the well-being of microalgae in the reactors consisted of measuring three different parameters: chlorophyll fluorescence (Fv/Fm), absorbance and dry weight.

Chlorophyll fluorescence (Fv/Fm) was determined with an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic) after keeping the sample for 15 minutes in the dark.

Absorbance was measured using a GENESYS 10S UV–Vis spectrophotometer (Thermo Fisher Scientific, Spain). The selected wavelengths were 680 nm for chlorophyll and 750 nm for cell density.

Microalgae biomass dry weight was calculated by filtering 50 mL aliquots of the culture through Macherey-Nagel glass fiber MN 85/90 and drying it in an oven at 80°C for 24 h. From the dry weight was then calculated the daily concentration of the reactor and its productivity, knowing the dilution rate.

2.3. NUTRIENT ANALYSIS

To observe the bioremediation effect of microalgae on wastewater, nitrate, phosphate, ammonia and chemical oxygen demand (COD) were daily measured on samples of inlet wastewater and outlet collected from the reactor after the filtration of the biomass. The applied protocols, approved by the Spanish Ministry (MAPA. Métodos oficiales de análisis, 1986) were performed with a GENESYS 10S UV–Vis spectrophotometer (Thermo Fisher Scientific, Spain) and chemicals from Panreac Aplichem ITW Reagents (Barcelona, Spain).

2.3.1. NITRATES

In a 50 ml volumetric flask were added

- 500 µl of sample
- 2,5 ml of hydrochloric acid 37%;
- Distilled water up to 50 ml.

Nitrates were quantified measuring the absorbance at 220 nm and 275 nm.

2.3.2. PHOSPHATES

They were determined through the phospho-vanado-molybdate complex method.

In a 25 ml volumetric flask were added:

- 1 ml of sample;
- 1 ml of hydroquinone 0,5% in distilled water;
- 1 ml of sodium sulfite 20% in distilled water;
- 1 ml of ammonium molybdate 5% in sulfuric acid 2,7M;
- Distilled water up to 25 ml.

After 30 minutes of incubation at room temperature, phosphate were quantified by measuring the absorbance at 430 nm.

2.3.3. AMMONIUM

The Nessler reactive method was applied:

In a 25 ml volumetric flask were added:

- 1 ml of sample;
- 1 ml of sodium tartrate 20% in distilled water;
- 1 ml Nessler reactive;
- Distilled water up to 25 ml.

After 15 minutes of incubation at room temperature, ammonium were quantified by measuring the absorbance at 410 nm.

2.3.4. CHEMICAL OXYGEN DEMAND (COD)

It was determined by spectrophotometric measurement using Hach-Lange kits (LCl-400). COD is an indicator of water pollution, measuring the biological matter available and susceptible to oxidation by a strong oxidizing agent in a hot acid environment. In other words, it is the quantity in mg of oxygen necessary to chemically oxidize the polluting substances (organic and inorganic) present in a liter of water.

2.4. HARVESTING

Biomass harvesting was performed in two step.

First, the microalgae culture was submitted to an ultrafiltration membrane (MUF) to raise its concentration, then it was centrifuged to recovery the dry biomass.

The ultrafiltration membrane is a BIO-CEL® BC100F-C25-UP150. It is located in a separate 5000 L tank, connected to the raceways with tubes and submergible pumps (Figure 2.1). To avoid the membrane fouling, a backwash system has been set up, which has been tested with different operating time: 1 minute of backwashing every 15, 30 or 60 minutes. For the same reason, a compressor (Airtech Europe, Luxemburg) was connected to the membrane , insufflating air continuously for the whole duration of the process, in order to keep the pores opened and to supply the microalgae in the tank with fresh air.

Daily, 20% of the volume of the bioreactor was harvested and sent to the membrane's tank were it got concentrated. The filtrate, instead, was returned back to the raceway. The biomass in the tank was monitored with daily measurement of Fv/Fm and dry weight, performed as explained in paragraph 2.2.

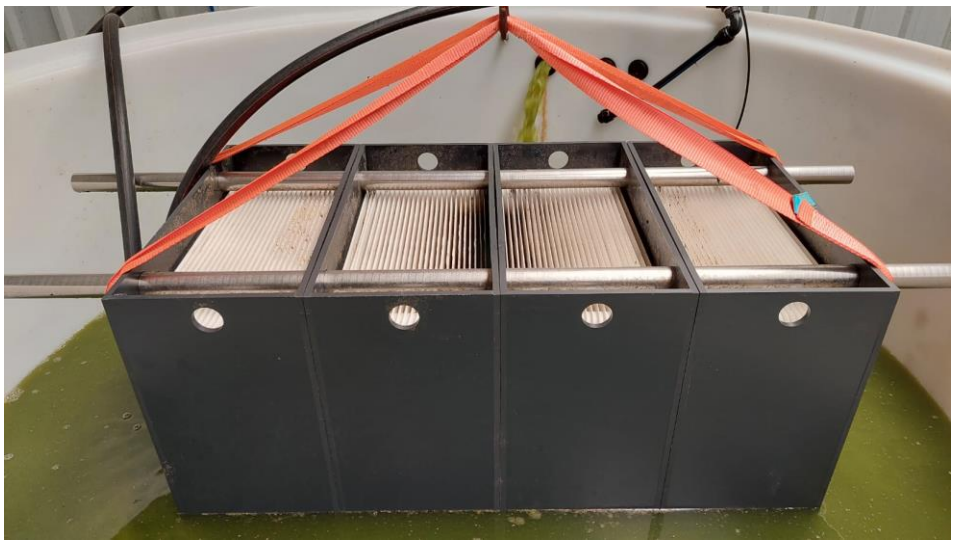


FIGURE 2.1: *Ultrafiltration membrane*

The centrifugation step was performed with a SSD 6-06-007 GEA WESTFALIA Continuous Centrifuge (GEA Group, Westfalia, Germany) (Figure 2.2).

FIGURE 2.2: *Continuous centrifuge*

The centrifuge was connected to the membrane tank, in order to recover the concentrated biomass from ultrafiltration. Working sessions of 1 hour have been performed, setting the entry flow at 1000 L/hour and the pression at 4,2 mbar. The biomass was collected in an external container while the supernatant was redirected to the membrane tank to keep its level almost constant and to recover the amount of biomass that is not centrifuged. Samples of the inlet, supernatant and biomass sludge were taken and suitably diluted when necessary, in order to measure the absorbance at 680 nm and 750 nm and the dry weight.

Three assays were performed, one as a control by directly centrifuging the culture from the raceway without the intermediate filtration step; and the other two after three and seven days from the beginning of the filtration process, when the microalgae in the tank had reached concentrations of 2,32 and 44,9 g/L respectively.

The harvested biomass concentration was determined by dry weight, performed as explained in paragraph 2.2. Finally, an half of the wet biomass was freeze-dried and stored at -20°C for subsequent analyses, while the other half was kept wet and stored at -20°C, to compare it with the dry biomass and thus evaluate the possibility of eliminating the freeze-drying from the entire production process.

2.5. BIOMASS COMPOSITION

For an accurate analysis, the samples collected from both the bioreazctors were lyophilized in order to have less than 10% moisture by weight, and then pulverized. Moisture and ash levels were determined gravimetrically by drying in an oven at 105 °C and after incineration in a muffle furnace at 550 °C, respectively, until constant weight. Total nitrogen (N) was measured by elemental analysis (950 °C furnace) using a Leco N determinator (model FP-528, Leco Corporation, USA) with ultra-high-purity oxygen as the combustion gas and ultra-high-purity helium as the carrier gas. The nitrogen-to-protein conversion factors used to estimate crude protein content were N×6.38 for sodium caseinate and N×4.78 for microalgae. Crude lipid was extracted with a Soxhlet automated system (model 2050, FOSS North America, USA) in 33×80-mm cellulose extraction thimbles (CT33080, Rose Scientific Ltd., Canada) using chloroform/ methanol (2:1 v/v) at 150 °C for 82 min. The final weight of the crude lipid extract was determined gravimetrically after oven-drying (105 °C) for 90 min. Carbohydrate content was estimated by difference.

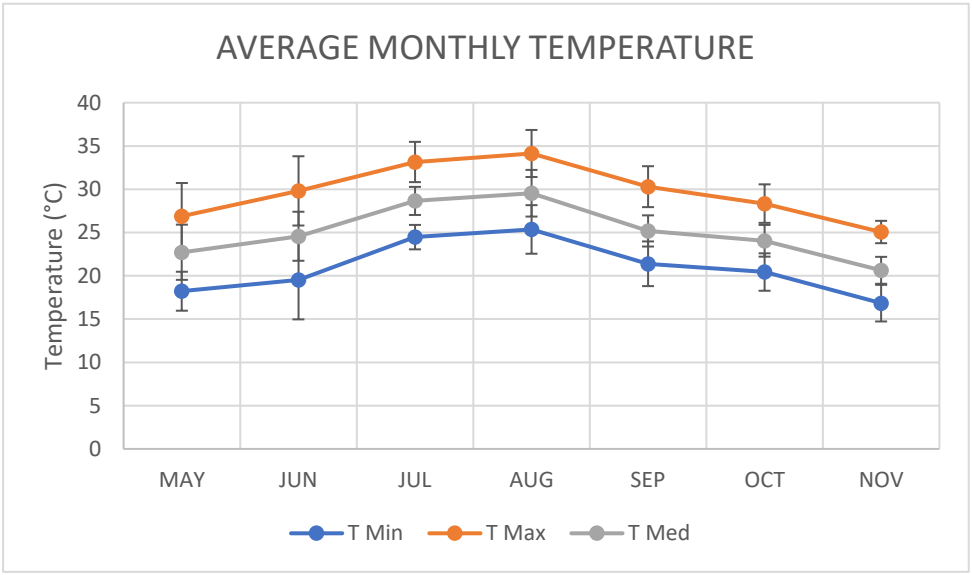
2.6. BIOMASS PYROLYSIS

Syngas and biochar production has been made with a slow pyrolysis into a dedicated furnace. The process was conducted in inert atmosphere by using a flow of 2 ml/min of N_2. The furnace was heated at a rate of 5°C/min, reaching three different maximal temperature, which was 300°C, 400°C and 500°C. The biochar yield was calculated as a percentage of the initial biomass. the syngas produced as a difference, considering the production of bio-oil as negligible [103].

3. RESULTS

3.1. CULTURE CONDITIONS

The climatic monitoring carried out daily during the experimentation period shows the temperature and irradiance monthly trend, in line with the averages relating to the climatic zone of reference (Figure 3.1a e 3.1b).



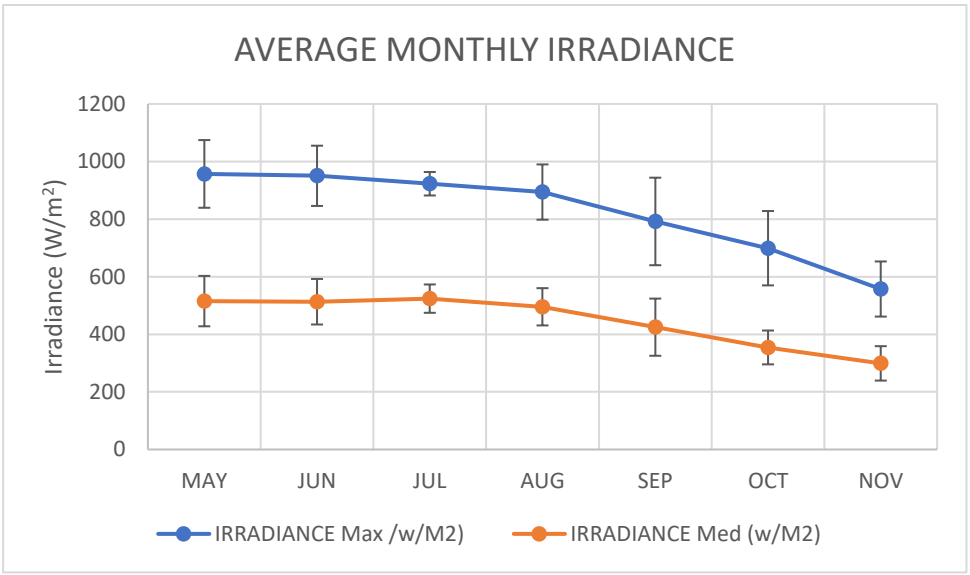


FIGURE 3.1: *Climatic monitoring. A) Average montly temperature; B) Average monthly irradiance*

pH too was constantly monitored and, except for sporadic fluctuation, it has always been maintained at an average value of 8,00 (Figure 3.2)

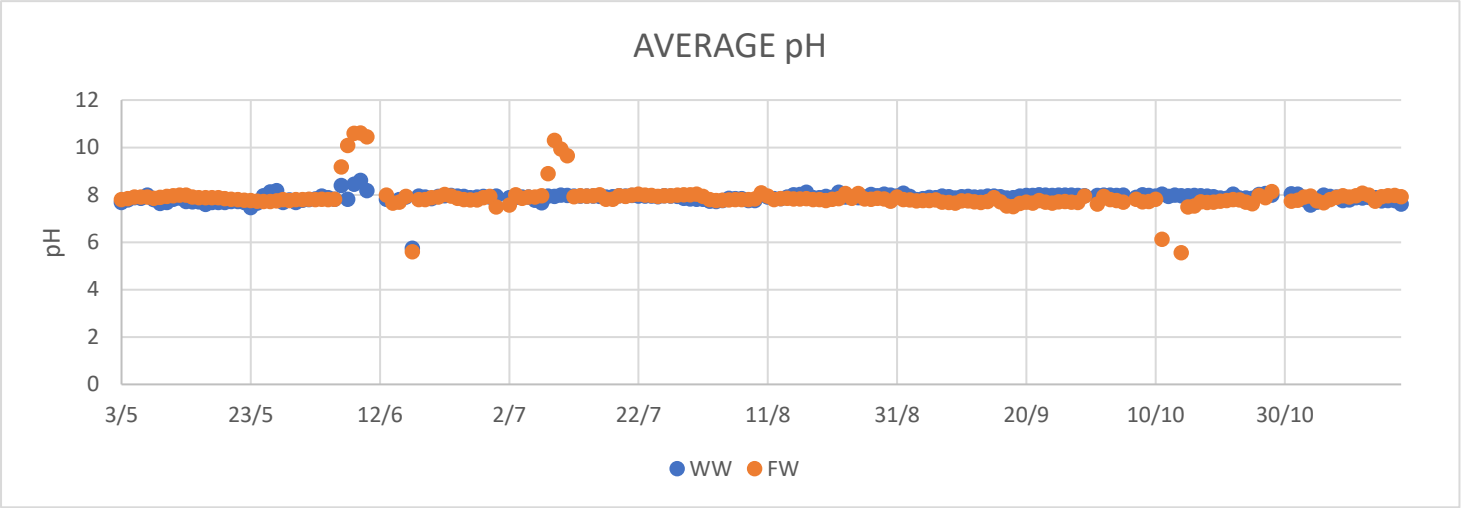


FIGURE 3.2: *Average pH of the two bioreactors*

3.2. DAILY ROUTINE

The Fv/Fm of the cultures has always kept around 0,7 (Figure 3.3), while the microalgae concentration was always almost the double in wastewater than in freshwater (Figure 3.4). As a consequence, the productivity of the wastewater raceway was on average of 26,35 g/m^{2}day compared to the productivity in freshwater that only reached 14,62 g/m^{2}day (Figure 3.5).

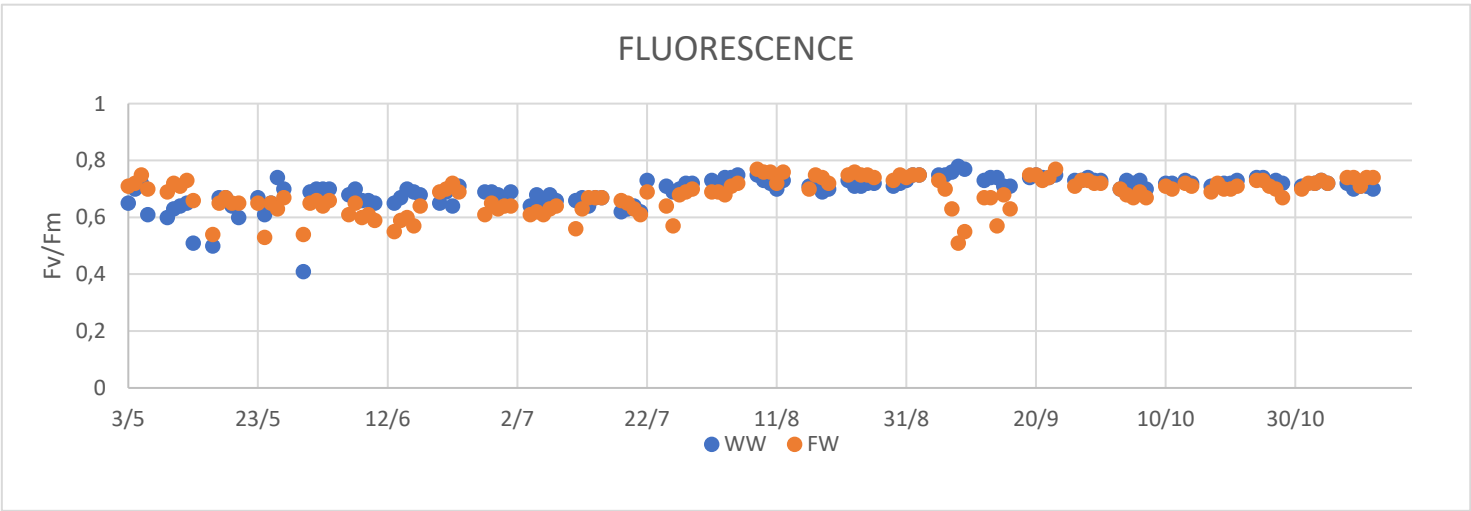


FIGURE 3.3: *Daily chlorophyll fluorescence*

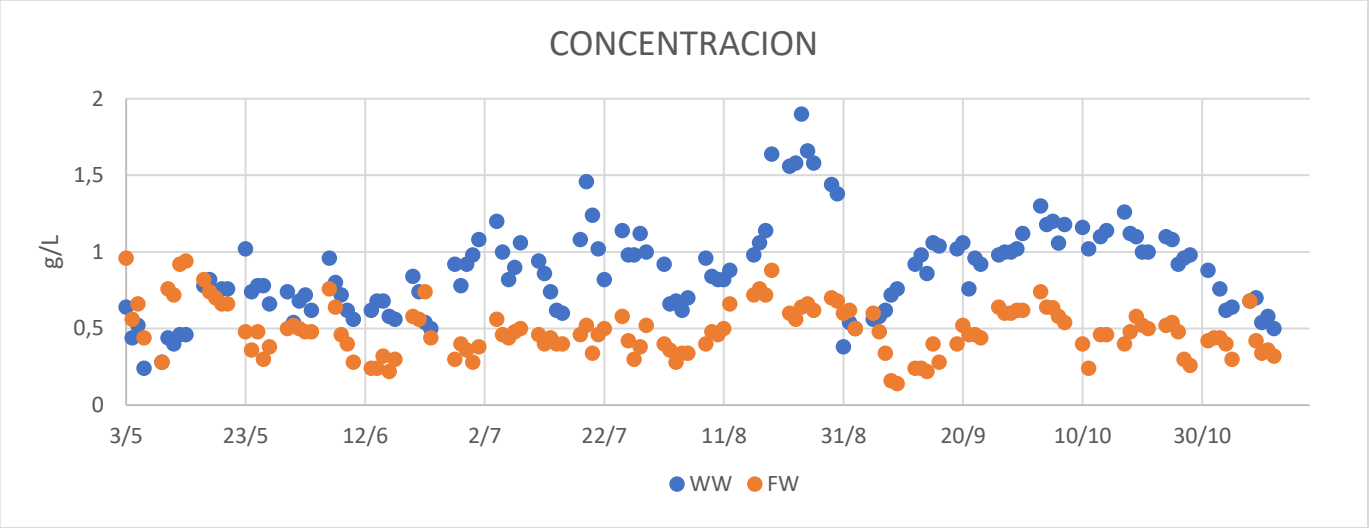


FIGURE 3.4: *Daily reactors concentrations*

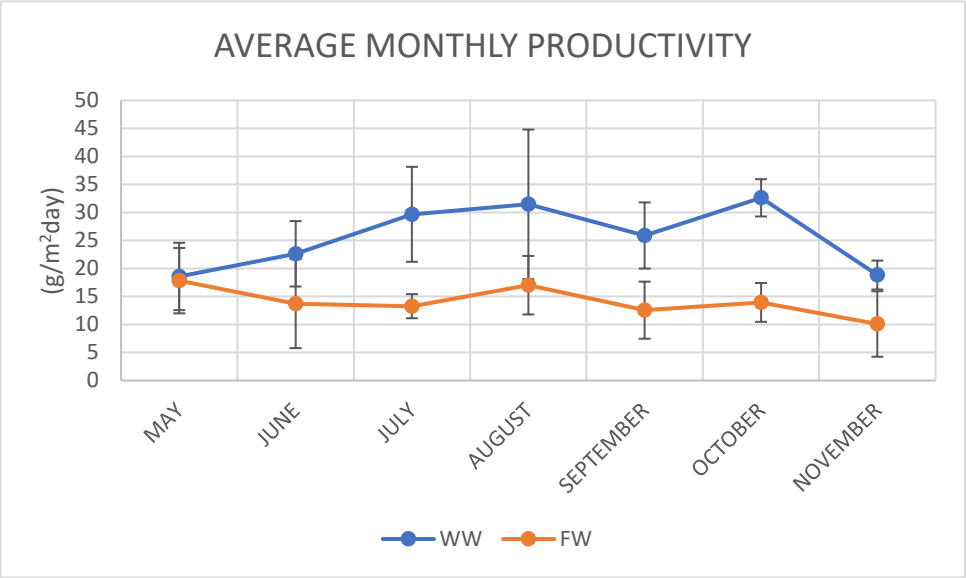


FIGURE 3.5: *Montly average productivity*

3.3. NUTRIENT ANALYSIS

The daily measurement of the nutrients in the inlet wastewater and in the outlet supernatant obtained after the filtration of the culture clearly show the bioremediation performed by the microalgae. During these seven months of monitoring, the content of total nitrogen (Figure 3.6 a), phosphate (Figure 3.6 c) and COD (Figure 3.6 d) was almost always reduced to quantities below the limits imposed by the ministerial decree D.M. 185/2003 of the Italian government for the agricultural uses of water. In the case of nitrogen, the predominant form is that of nitrate. The ammoniacal nitrogen, abundant in the inlet, was always reduced to minimum levels (Figure 3.6 b).

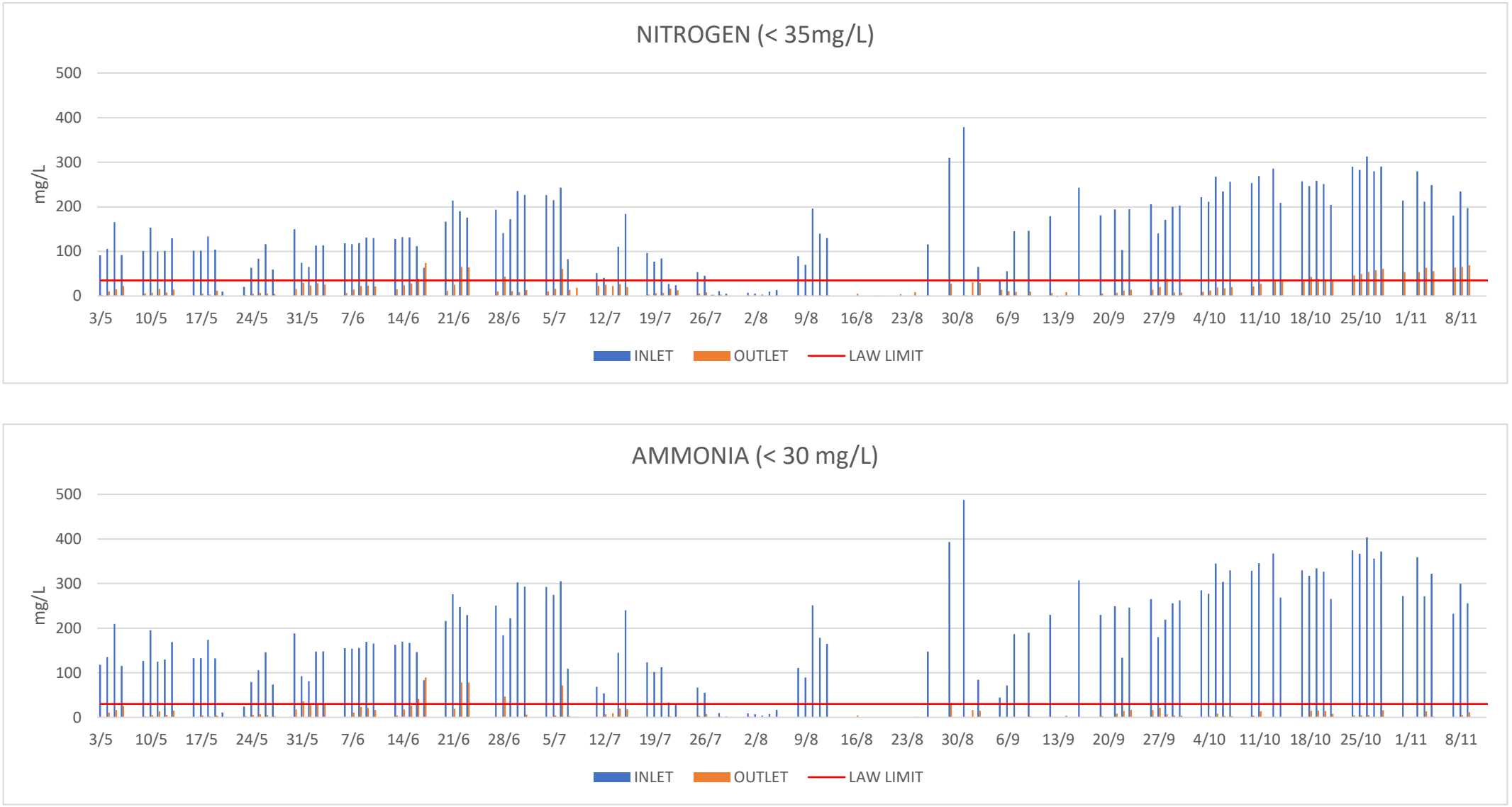


FIGURE 3.6: *a) Total nitrogen content; b) Content of nitrogen in form of ammonia*

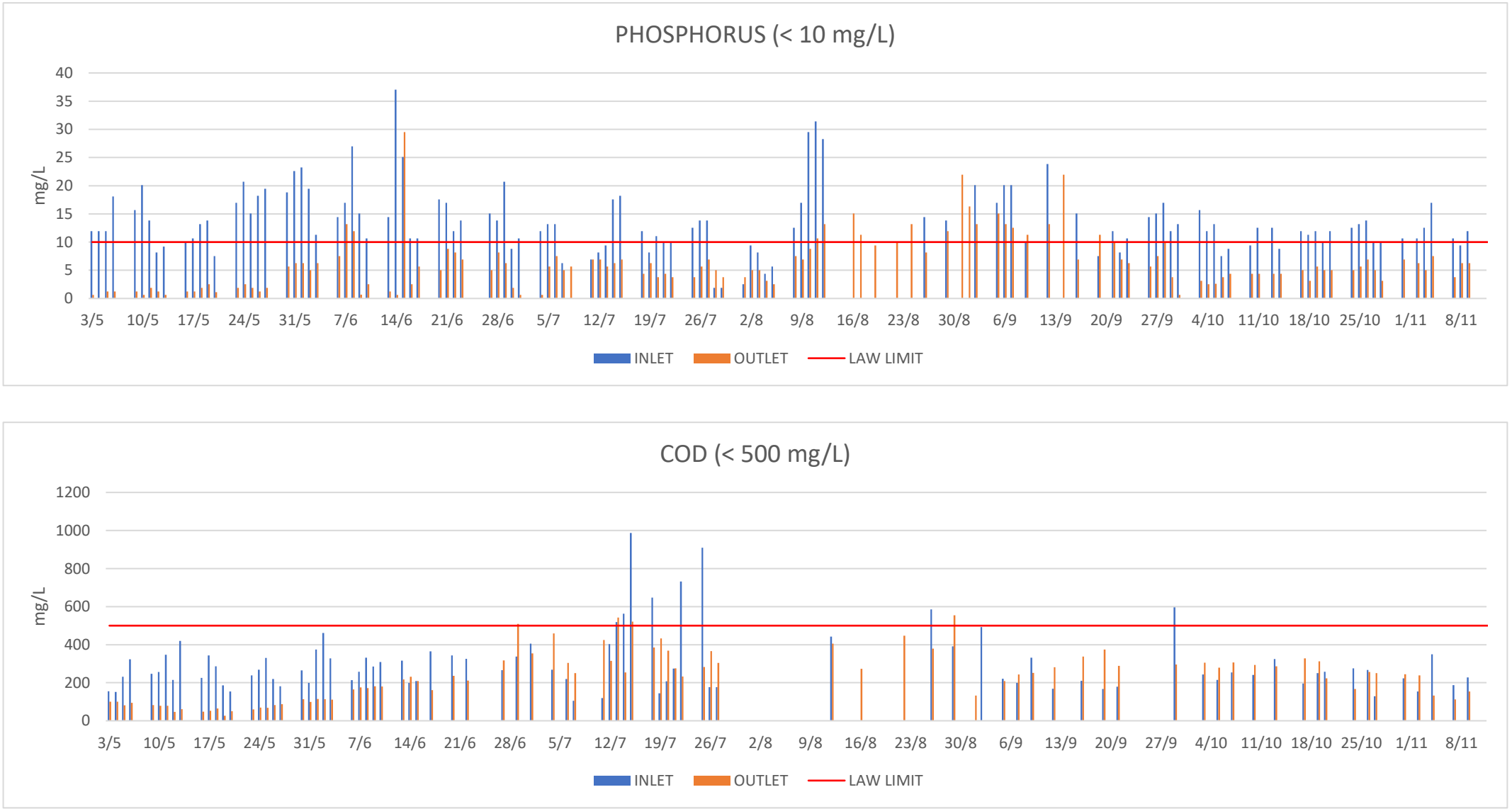


FIGURE 3.6: *c) Phosphorous content; b) Chemical Oxygen Demand*

3.4. HARVESTING

The ultrafiltration membrane shows a great biomass concentration capacity. Different assays conducted over several weeks have demonstrated that it is possible to concentrate the microalgal culture of a factor up to 35 times, reaching values of 12 g/L (Figure 3.7). The optimal backwash setting has proved to be 1 minute every 30 minutes of filtration (Essay 02 and 03), in which a good biomass concentration is obtained with moderate water consumption. 1 minute of backwash every 15 minutes (Essay 01) was rejected because excessive water entry into the membrane tank slows down the centrifugation process. Finally, the 1 minute every 60 seconds option was not considered because it was ineffective in preventing membrane obstruction (data not included). Even if slightly lower than that of the reactor due to the higher concentration of biomass in the tank which reduces the penetration of light, the Fv/Fm value is still in the optimal range, indicating that the alga continues not to undergo particular stress.

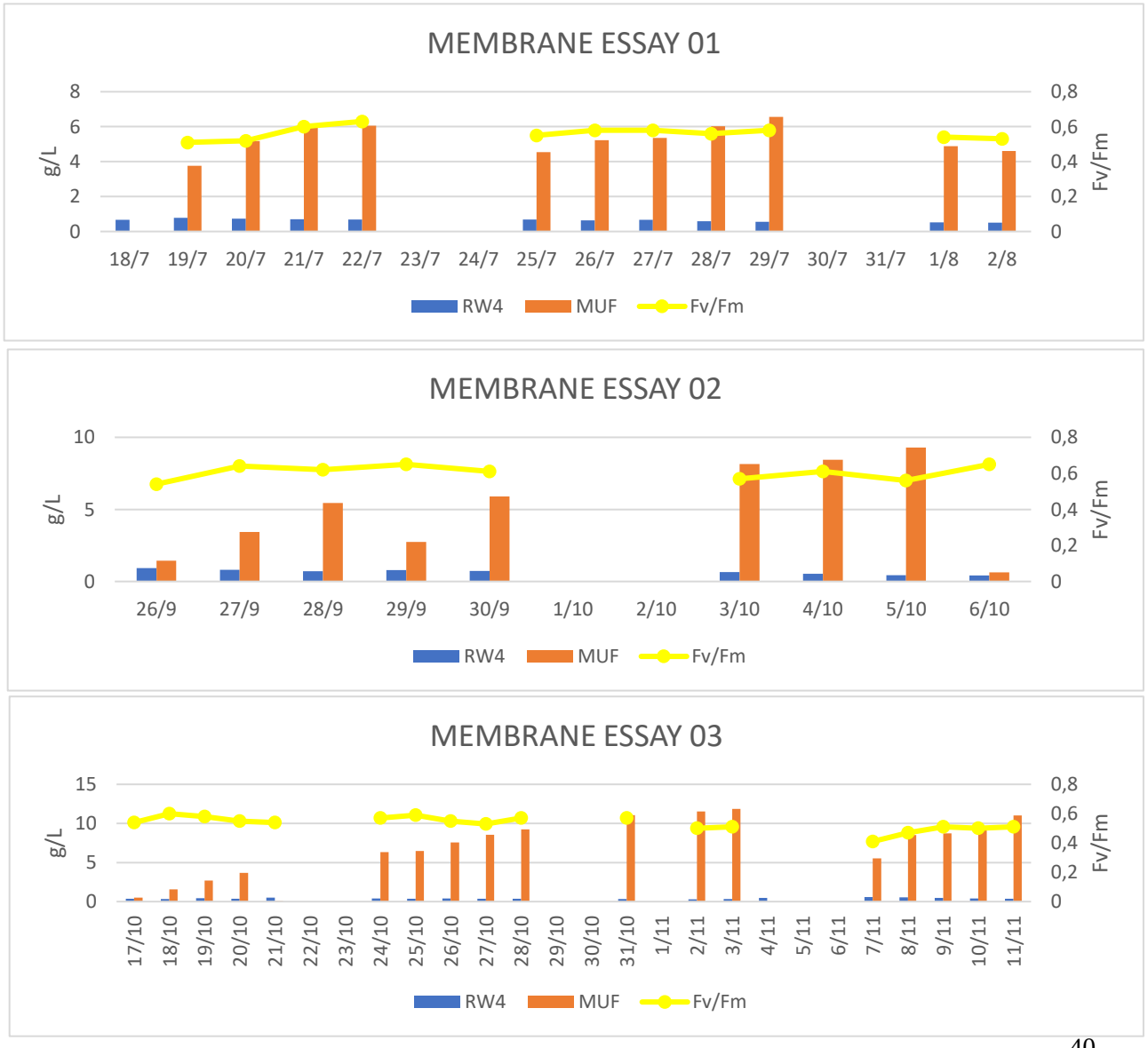


FIGURE 3.7: *Daily monitoring of the ultrafiltration membrane yields: concentration and chlorophyll fluorescence*

After the centrifugation, different biomass volumes were collected (30 L, 50L and 70L) and the yield of the process was calculated in percentage terms with respect to the total input biomass. As can be seen from the graph in figure 3.8, increasing the input quantity, thus its concentration, reduces the ability of the centrifuge to recover biomass.

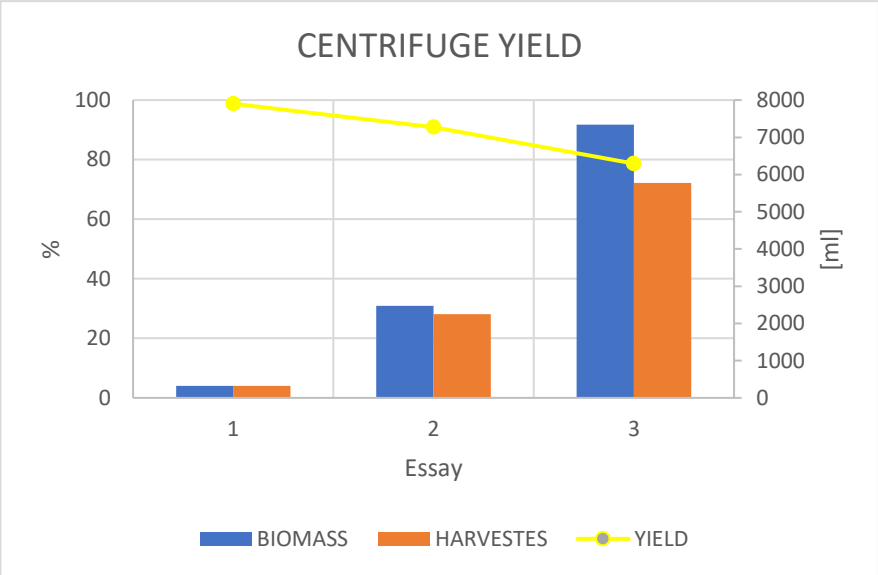


FIGURE 3.8: *Centrifugation yields in percentage and volume of harvested biomass*

However, this data alone could be mischievous, and the concentration of the harvested biomass need to be considered, in order to evaluate the real upcoming of the process. As shown in figure 3.8, in the first experiment, the collected biomass was 32,33 more concentrated than that of the raceway, while in the following essay, this concentration factor rose to 140, 31 and 164,07.

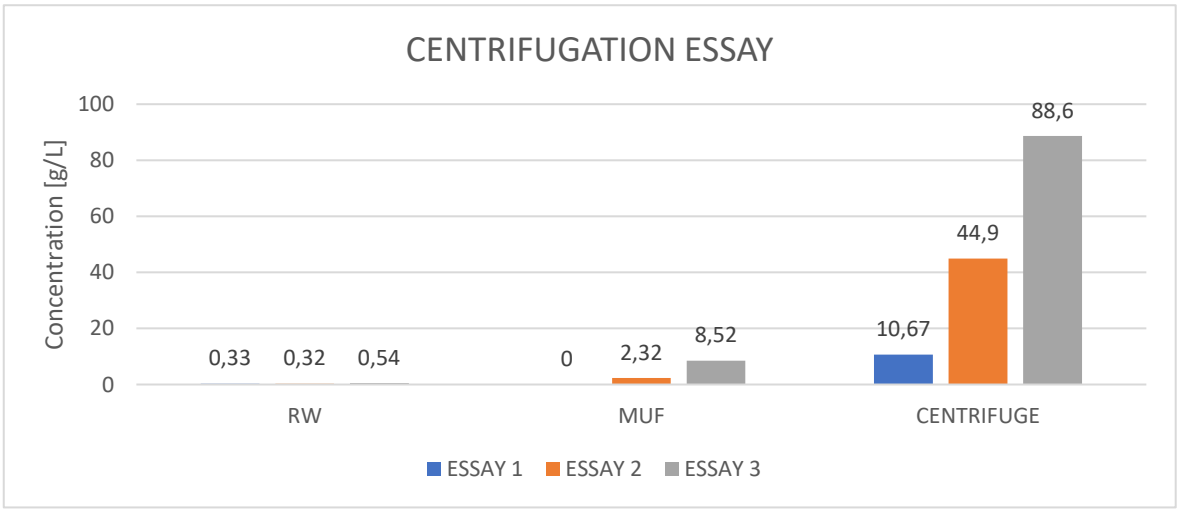


FIGURE 3.9: *Concentration of the biomass at different step of the harvesting process. In the essay 1 the MUF was not implemented*

3.5. BIOMASS COMPOSITION

Figure 3.10 shows the results from the HPLC analysis on the biomasses. Except for proteins, which are a 2,2% lower, the amount of all the component is higher when microalgae are kept in wastewater.

FIGURE 3.10: *Composition of dried microalga biomass samples from freshwater (FW) and wastewater (WW)*

3.6. BIOMASS PYROLYSIS

Figure 3.11 shows the yield of biomass pyrolysis. The main products are biochar and syngas, in variable percentages depending on the final temperature of the process. Higher temperatures give higher amount of syngas. Yields differences between fresh water and wastewater biomass are negligible

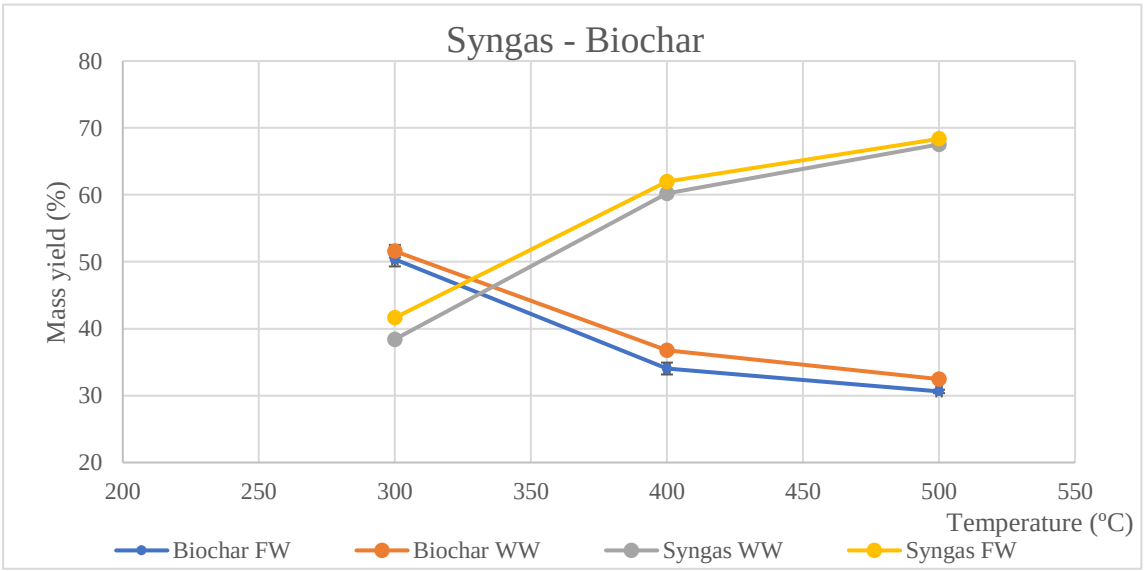


FIGURE 3.11: *Pyrolysis yields of fresh water (FW) and wastewater (WW) biomass*

4. DISCUSSIONS

4.1 PRODUCTION

Different types of photobioreactors available for the cultivation of microalgae on a large scale have been described in the introduction chapter. Indeed the most straightforward system to manage and also the cheapest is that of outdoor cultivation in raceway ponds. Since the microalga efficiency for bioremediation was also tested in this work, the raceway pond undoubtedly was the adequate system to treat large volumes of water.

Being outdoors, however, environmental factors become fundamental to consider. In this case, as Almeria is in a semi-desertic climatic zone with very little rainfall, the main parameters to be monitored were temperature and irradiance. The highest peaks were reached in the summer when maximum temperatures were around 35°C and irradiance 957 W/m^2; seasonal variations were about 10°C in temperature and 400 W/m^2 of irradiance.

These environmental conditions are not always ideal, so choosing the microalgae to use becomes a strategic element for starting optimal cultivation capable of dealing with such conditions.

The microalga used in this work is *Scenedesmus almeriensis*, a species isolated and characterized in 2008 [104]. As an autochthonous microalga, *S. almeriensis* can well tolerate the local environmental conditions and their seasonal variations.

Demonstration of this is the data on the fluorescence of chlorophylls, the Fv/Fm ratio, which throughout the study period always remained in the optimal range between 0.6 and 0.8, both for the microalgae in wastewater and in freshwater. These indicate that despite high temperatures and high light radiation, the microalga has never suffered damages or stress.

The productivity, instead was very different among the two raceways. In the case of freshwater and nutrients, it has been always around the average value of 14,62 g/m^2day, which is compatible with data reported in previous publications [104,105], but is lower than the productivity in wastewater. In this case, in fact, biomass productivity reached an average of 26,35 g/m^2day. These data are perfectly in line with previous publications [106]. Nevertheless, it should be underlined that although the obtained biomass productivity is in line with the literature data, it is still far from the theoretical value for raceways, estimated at 40-70 g/L. For this reason, the production process should be further optimized, for

example, by deeply investigating solar radiation's effect by modifying the culture's height or the agitation conditions.

In addition to higher productivity, *Scenedesmus almeriensis* in wastewater has demonstrated excellent nutrient recovery capacity and, consequently, water bioremediation. Nitrogen, phosphorus and COD present in the inlet have almost always been absorbed by the microalga and reduced to minimum quantities, within the limits established by the ministerial decree D.M. 185/2003, which indicates the conditions for reusing wastewater in agriculture. This capacity strongly depends on the productivity of the bioreactor and, therefore, on the concentration of the microalgae . At peak efficacy, nitrogen and phosphorus reduction was 281.82 mg/L and 20.72 mg/L, respectively.

However, in these seven months of analysis, punctual episodes of nutrients above law limits occurred, independently from the biomass concentration. The composition of the wastewater also needs to be taken into account. In this case, wastewater is recollected from the nearby University of Almeria, thus resulting in higher concentration during periods of greater affluence of people.

Another element to consider is the presence of nitrifying bacteria within the consortium, which inevitably form in the reactor. If, on the one hand, they contribute to the reduction of the incoming ammonium concentration, on the other hand, they release quantities of nitrate, which induce a slight increase in total nitrogen content.

Summing up, *Scenedesmus almeriensis* has demonstrated an excellent bioremediation potential that can be advantageously exploited in broader processes to obtain economic and productive advantages once the culture parameters in the bioreactor are stabilized [106,107].

4.2. HARVESTING

The harvesting step is one of the critical points of the entire microalgae cultivation process. Among the various methods available, centrifugation is the most widely used because it guarantees operational simplicity and good recovery yield, but at the expense of operating costs. Indeed, depending on the initial culture concentration, centrifugation may require a long working time to recover significant quantities of biomass, thus involving a costly investment in energy and operational terms. It is for this reason that harvesting costs can often represent up to about 30% of the total production costs.

In this project, it was thought to reduce these costs by introducing an intermediate ultrafiltration step. This strategy has already been adopted in past works, although only on a small scale [108]. The filtration step, in fact, increases the concentration of the culture to be centrifuged, thus ensuring a higher yield.

The obtained results show that, at the same working time, the higher the concentration entering the centrifuge, the higher the quantity of biomass recovered, which in addition, also had a lower percentage of humidity. It follows that, if setting a quantity of biomass to be collected, the centrifugation times can be lowered when introducing the filtration step.

In this way, the costs of the process can be considerably reduced while maintaining a high yield in terms of biomass for subsequent applications, also thanks to the low energy consumption of the filtration membrane.

Furthermore, since the humidity in the recollected biomass is also lower, the following freeze-drying process can take place more quickly, further reducing production costs.

4.3. BIOMASS COMPOSITION AND APPLICATIONS

It is not possible to precisely establish the percentages of the different fractions of the microalgal biomass. The various components in more or less variable ranges are reported in the literature: proteins 6 - 52%, carbohydrates 5 - 23%, and lipids 7 - 23% [109,110].

This extreme variability depends on the conditions in which the microalgae grow: autotrophic or heterotrophic metabolism and the availability or otherwise of certain nutrients modify the biomass composition. One of the most straightforward solutions to induce an increase in lipid content is the deprivation of nutrients, precisely nitrogenous compounds [41].

Since the cultivation conditions adopted in this process instead provide for a high quantity of nitrogen, especially in the reactor with wastewater, the percentage of lipids of the collected biomass is relatively low, as expected.

From this, it was considered inconvenient to proceed with biodiesel production. This process, although theoretically simple because it consists only of transesterification steps, has medium-high costs, especially in using the reaction catalysts, which would not be repaid

by the amount of biodiesel obtained. Yields, infact, are generally around 80%, and furthermore, it is necessary to evaluate the quality of the product obtained [111].

On the other hand, the percentage of carbohydrates measured bodes well for bioethanol production. The most recent technologies developed for the direct fermentation of biomass significantly reduce costs while still guaranteeing good yields reaching up to 80% [50].

Syngas production through pyrolysis has proven to be a process with high potential. In the face of reduced costs, since the biomass does not require specific treatments before being subjected to pyrolysis, it has shown a yield of 70%, to which must be added the parallel production of biochar, which can be used in agriculture as a biofertilizer [57], representing a further optimization of the performed process.

Finally, tbio -hydrogen production has not been taken into consideration because it requires particular growth conditions, as explained in the introduction of this thesis, which does not reconcile with the process experienced in this project.

5. CONCLUSIONS

Data obtained so far are very valid and show a great potential for a future optimization of the process on large industrial scale.

Integrating a bioremediation process in the microalgae cultivation phase significantly improves yields and lowers costs, effectively eliminating those due to the supply of nutrients to be added to the culture medium.

Furthermore, the water recovered from the bioremediation process represent an additional advantage since it can be reused in microalgal cultivation or also in agriculture.

The choice of an autochthonous strain has proved effective because it maximises the production yields in relation to the site where the process occurs. It would be desirable to adopt this type of precaution, evaluating the biomass to be produced with particular attention to the characteristics of the production site.

The addition of the ultrafiltration step represents a substantial advantage, improving yields in terms of biomass recovery and further reducing costs, in this case, due to the energy consumption of the machinery used.

The potential applications of the biomass obtained must be further verified, but the data collected so far show the effectiveness of the biorefinery approach. The production of biofuels does not remain the only goal, but the applications expand considering the secondary products of the process, as shown in the case of biochar. Indeed the proposed project will need to be expanded, evaluating, on the one hand, the possibility of combining the different production processes, for example, by reusing the residue of bioethanol production as a substrate for pyrolysis.

A further step to effectively complete the work done is a technical-economic analysis, or even better, a life cycle assessment, to precisely establish the advantages obtained from the implementations carried out at the process and evaluate the actual sustainability of the proposed production chain.

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Review

Biohydrogen from Microalgae: Production and Applications

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Abstract: The need to safeguard our planet by reducing carbon dioxide emissions has led to a significant development of research in the field of alternative energy sources. Hydrogen has proved to be the most promising molecule, as a fuel, due to its low environmental impact. Even if various methods already exist for producing hydrogen, most of them are not sustainable. Thus, research focuses on the biological sector, studying microalgae, and other microorganisms' ability to produce this precious molecule in a natural way. In this review, we provide a description of the biochemical and molecular processes for the production of biohydrogen and give a general overview of one of the most interesting technologies in which hydrogen finds application for electricity production: fuel cells.

Keywords: green fuel; biohydrogen; microalgae; *Chlamydomonas reinhardtii*; hydrogenase; fuel cell

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1. Introduction

Nowadays, searching for renewable energy represents one of the major challenges for the global scientific community. Population growth and industrial activities, with consequential high-energy demand, require solutions and proposals in the short-term. It is assumed that the world population could reach 8–10 billion in 2040, while the increase in global energy demand, within the same year, is estimated between 100 and 170 qBtu. However, models cannot accurately predict sudden changes in the global energy situation, as different socio-economic scenarios and political choices modulate each country's response [1,2]. Considering the slow oil, coal, natural gas formation processes, the excessive exploitation of fossil fuels has triggered a drastic and irreversible reserve reduction. Another side aspect of fossil fuel use concerns greenhouse gas (GHG) emissions, particularly carbon dioxide (CO_2), during the combustion reaction [3]. In particular, in the years between 1998 and 2018, CO_2 emissions increased by 48%, making it necessary to implement carbon capture and storage (CCS) technology to limit serious and detrimental effects on climate change [2]. In this worrying scenario, fossil fuels still provide more than 80% of primary energy needs, although the interest in renewable sources, together with their consumption, are steadily increasing [4].

Among renewable options, hydrogen shows the important advantage of CO_2-free combustion, with water as a by-product. Thermodynamic properties, compared to traditional fuels, confirm the interest in its investments in research, although several aspects related to production and storage are still to be mastered. Today, several industrial applications depend on hydrogen, but the most is obtained by techniques, such as steam reforming or electrolysis, not entirely free from the involvement of fossil fuels [5]. Some living organisms, such as microalgae and bacteria, are the basis of processes capable of producing hydrogen in a completely eco-sustainable way. Microalgal hydrogen production is made possible

by biological processes directly or indirectly, depending on sunlight, or by fermentation processes and thermochemical technologies for biomass conversion (Figure 1).

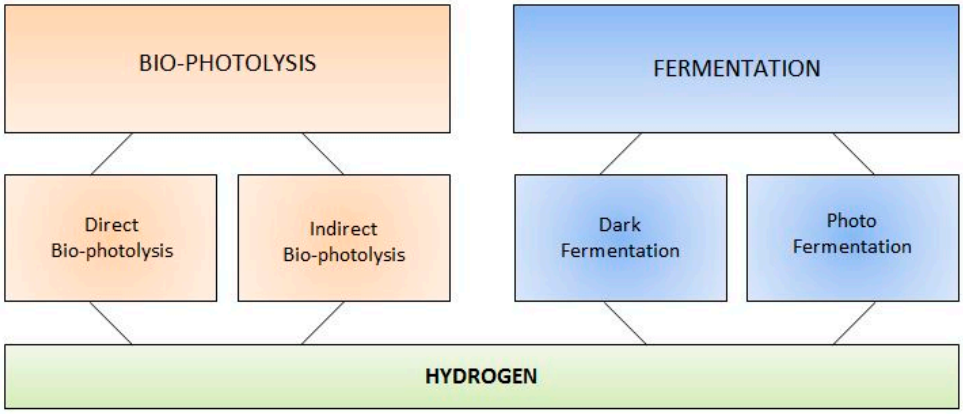

Figure 1. Hydrogen production processes in microalgae.

In recent years, many green systems have focused on algal biomass to obtain energy from living matter. Microalgae store and exploit light energy thanks to the photosynthetic process. To support cell growth and metabolic activities, they use resources widely available in nature, such as water and CO_2. These organisms can concentrate considerable CO_2 amounts and obtain nutrients necessary for growth, even from substrates or waters deriving from industrial waste. Algal cultivation systems, which are simple and relatively inexpensive, ensure advantageous growth rates. Furthermore, they can be set up on infertile territories without competing with agricultural areas that can be exploited for food resources. In particular, the general interest in microalgae has increased significantly in recent decades due to the variety and versatility of the metabolites present in various and numerous species [6].

The present review article is a collection of the most recent evidence on hydrogen production in green microalgae and the efforts to understand and improve the above-mentioned processes in the most widely used species.

2. Hydrogen Metabolism in Green Microalgae: Biophotolysis

Hydrogen metabolism was firstly observed in eukaryotic microalgae in 1939 by German physiologist Hans Gaffron [7]. Due to the oxygen incompatibility, it occurs only transiently in photosynthetic organisms.

2.1. Photosynthetic Electrons and Hydrogenase

The pivotal process of microalgal metabolism consists of oxygenic photosynthesis and complex redox reactions that take place at the level of the thylakoid membranes in chloroplasts through two successive phases. During the first light-depending reactions, ATP and reduced NADH, and NADPH, are generated to be involved in the next dark reactions where the atmospheric CO_2 is fixed by a RuBiSco (ribulose-1,5- bisphosphate carboxylase/oxygenase) enzyme to ultimately generate energy rich-carbohydrate stores. Specifically, during the light phase, an electron transport chain is generated along with photosystems II (PSII) via the plastoquinone (PQ) pool, cytochrome b6f complex (Cyt b6f), and photosystems I (PSI) due to the light energy received as photosystems are associated with light-harvesting complexes I and II (LHC I and LHCII), consisting of numerous photoreceptive pigments. These electrons through PSI leave the electron transport chain and reach the final acceptor ferredoxin (Fd) [8,9].

In anoxic conditions, Fd is able to address electrons to the hydrogenase enzyme. This kind of enzyme catalyzes a reversible reaction in which hydrogen can also be split into electrons and protons:

$$2H^+ + e^- \leftrightarrow H_2 \quad (1)$$

Hydrogenases from the green algae *Chlamydomonas reinhardtii* are the most well-characterized among microalgae. This model organism expresses two different Fe-hydrogenases genes: *HydA1*, the isoform mainly involved under anoxic conditions, and *HydA2* [10,11]. Hydrogenase activity is part of the various responses that microalgal organisms carry out in response to anoxic or stress conditions, and is highly regulated on several levels. After the ribosomal translation in the cytoplasm, the protein is driven to chloroplasts through a transit peptide at the N-terminal end [12]. Assembling of the protein and the catalytic center, H cluster, are required to obtain the functional 47–48 kDa enzyme. *HydEF* and *HydG* genes encode for maturation proteins, crucial for its activation [13]. H cluster is composed of [4Fe4S] unit cysteine-linked to a di-iron unit able to receive electrons via Fd, but easily reversibly inactivated by oxygen [14,15]. Evidence of hydrogen metabolism is reported also in less investigated species, such as *Scenedesmus obliquus* [16,17], *Chlorella fusca* [18], *Chlamydomonas moewusii*, *Lobochlamys culleus* [19], *Chlorococcum littorale* [20], *Tetraselmis subcordiformis* [21].

2.2. Direct and Indirect Biophotolysis

Hydrogenase can receive electrons from different metabolic sources deriving upstream from the biophotolysis of water. At the PSII level, the splitting of water simultaneously produces O_2 and electrons, which, in lighting conditions, reach the Fd and are processed for carbon fixation through the ferredoxin-$NADP^+$ reductase. Under anoxic conditions, oxygen depletion generates a favorable environment for the expression of hydrogenases that start to receive photosynthetic electrons. This process is the direct biophotolysis.

Biophotolysis can also indirectly feed the hydrogen evolving process through the electrons deriving from the breakdown of complex stored carbohydrates, such as starch, which reach Fd and, finally, the hydrogenase. Moreover, the electron load is transferred to the PQ pool via a specific type-II calcium-dependent NADH dehydrogenase (Nda2), and then is transferred to Fd, bypassing PSII without oxygen cogeneration. This process is called indirect biophotolysis [22,23]. Hydrogen evolution is triggered and promoted by modulating different growth strategies, largely experimented on *Chlamydomonas reinhardtii* strains. The most commonly adopted strategy is sulfur deprivation that forces cells to reduce protein synthesis and recover sulfur (S) from protein degradation, such as PSII linked protein D1, impairing the photosystem functions. Exposure to light after dark incubation causes the formation of electrons, but also reactive oxygen species near the defective photosystems with potentially harmful consequences on cell structures. In this context, hydrogenase activity works as a safety valve to preserve structures from oxidative stress, combining electrons with protons to produce hydrogen [24].

3. The Role of Growth Parameters in Hydrogen Production

Acting on cultivation parameters is the simplest way to produce immediate effects on the growth or the specific metabolite production.

Regarding media components, the aforementioned S deprivation gives the expected effects only if preceded by a photosynthetic growth phase, providing all of the necessary elements for growth. More recently, alongside this strategy, which envisages separate growth and a deprivation step with several operational issues, a different modality that involves minimal quantities of S to simulate a condition of starvation has also been tested [25,26]. Moreover, particular attention should be paid to the carbon source used: it has been observed that an increase in starch reserves during the phase preceding S deprivation can be crucial in supporting production. Mixotrophic conditions involving both inorganic and organic carbon sources appear to be preferred. [27,28].

The less studied condition of nitrogen (N) deprivation induces hydrogen production in *Chlamydomonas reinhardtii,* albeit with a delayed effect and lower yields, suggesting a different mechanism in establishing process [29]. In *Scenedesmus obliquus,* N deprivation modulates metabolism towards lipids accumulation to be used as an energy reserve, suggesting that this strategy is not advantageous for the purposes of hydrogen production [30]. In ma-

rine *Chlorella* spp., where S deprivation is inapplicable due to the sulfates-rich presence in seawater, the effect of phosphorus (P) deficiency was evaluated. Similarly, P deprivation was able to establish anaerobic metabolism with a sustained hydrogen photoproduction, even if low-density culture were required to reduce the effect linked to cellular P reservoirs [31]. Less common element deprivations, such as magnesium (Mg), were also tested. Mg-deprived *Chlamydomonas reinhardtii* cells exhibited a longer hydrogen production over time than the same cells in the case of S deprivation. This response may depend on the lesser Mg importance for cellular activities compared to S [32].

Various chemical compounds added in the cell media can affect protons and electrons mobilization inside the cell and, consequently, hydrogen metabolism. The protonophores carbonyl cyanide m-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNF) increase hydrogen production, owing to proton mobilization [33]. The action of other similar decoupling agents, such as 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DMCU) and carbonyl cyanide p-trifluoromethoxy phenylhydrazone (FCCP) allow to stimulate hydrogen production through the PS II independent pathway, although the underlying mechanisms are not deeply understood [34].

Parameter changes to produce hydrogen require several operational steps, especially during cell transfer upon different media. To handle culture more easily, some attempts have demonstrated the feasibility of cell cultivation through different immobilization systems [35]. Most of the attempts have been applied in *Chlamydomonas reinhardtii* through the use of fiberglass matrix, thin alginate films, or, more recently, sodium alginate beads. In particular, the latter study selected beads of few millimiters diameter to further investigate the process in the classical photobioreactors, bringing out the need to adapt bioreactors to new immobilization systems [36–38].

Indeed, photobioreactor design must take into account downstream applications and process needs. Closed systems are used in lab-scale hydrogen production experiments and represent a better alternative to open systems. Growth parameters, especially temperature and pH, are strictly monitored and, at the same time, the collection of the gas produced is less subject to dispersions. Particular importance is attached to the agitation mode and the intensity and quality of the light radiation. Vertical column, tubular, and flat panel, are the most investigated photobioreactor modalities for hydrogen production. The latter seems to be preferred as it provides the highest surface/volume ratio, although it is not a solution to all technical issues, and this area of research still faces numerous challenges [9,39].

4. Genetic Engineering Approaches

4.1. Random Mutagenesis

The approaches described in the previous paragraph act in a transitory way, since it is not possible to manage microalgal culture by applying stress conditions permanently. Indeed, depriving a cell culture of the optimal growth conditions for a long time leads to cellular suffering that can culminate in cell death. In this way, only a discontinuous hydrogen production is possible, alternating stress and recovery phases to avoid permanent damage to the cells. One strategy to overcome this limitation concerns the selection of organisms with a higher aptitude to withstand these stress conditions; another one could be the selection of organisms which are less susceptible to the conditions that usually hinder hydrogen evolution in photosynthetic green microalgae.

Physical and chemical treatments induce transmissible genomic mutations that favor the appearance of new traits in an organism. These treatments are not oriented towards a specific target and it is necessary to take into account an expensive screening phase among the mutants produced to select the ones with characteristics of interest. However, a recent study demonstrated that *Chlamydomonas reinhardtii* mutants obtained via atmospheric and room temperature plasma (ARTP) showed a lighter green coloration, compared to wild types, indicative of lower chlorophyll content. The lower chlorophyll content is associated with better photosynthetic performance (probably due to the improved light transmit-

tance and the consequent increased solar energy conversion efficiency), as confirmed by transcriptomic analyses, with consequent benefits in hydrogen production [40].

4.2. Targeted Mutagenesis

On the other hand, targeted approaches turn out to be more advantageous and several proposals have been provided by genetic engineering tools to increase yields and overcome current limitations.

As already mentioned, Fd receives electrons from the electron transport chain to address them to hydrogenase. Even under optimal conditions for enzyme expression, other assimilatory pathways compete for the reductants impairing the overall yield. Ferredoxin-$NAD(P)^+$ reductase (FNR) represents the main competitor shuttling electrons from Fd towards CO_2 fixation. To bypass this limitation, fusion protein has been designed. Among the most recent attempts, a fusion complex between Fd and hydrogenase in an in vivo culture of *Chlamydomonas reinhardtii* was evaluated. The complex demonstrated higher production rates and greater oxygen tolerance than the sole enzyme [41]. Similar behavior was also observed in the *Chlamydomonas reinhardtii* protein D1 mutant. A double amino acid substitution (L159I – N230Y) gave the mutant several new characteristics, including greater oxygen tolerance than the wild strain [42]. Similarly, *Chlamydomonas reinhardtii* mutants knock out for flavodiiron protein (FDP) showed higher photoproduction of hydrogen than wild types. In this way, it is again demonstrated how by eliminating a competing pathway (for example the FDP-mediated O_2 photoreduction pathway), the electrons are preferentially conveyed towards the production of hydrogen. Furthermore, it has been seen that, even exposure to prolonged light pulses in these mutants do not direct the metabolism towards CO_2 fixation. It is, therefore, demonstrated how genetic engineering approaches together with actions on growth parameters are jointly useful to increase production [43].

LHC complexes exhibit a poor light energy conversion with more than 80% absorbed light energy wasted as fluorescence or heat and not addressed towards hydrogen production. Conversely, a truncated light-harvesting antenna has demonstrated improvements in terms of photoinhibition and light saturation phenomena. Afterward, the same approach has also shown encouraging results for hydrogen production: tla1 CC-4169 *Chlamydomonas reinhardtii* mutant has exhibited to produce until six-time more hydrogen compared to the wild type strain with a light intensity of 350 $\mu E\ m^{-2}\ s^{-1}$ [44,45].

Non-coding RNA molecules, such as microRNA (miRNA) or long non-coding RNA (lncRNA), with regulatory function, are part of the most recent discoveries in microalgae and several studies have already exploited them for innovative approaches. In particular, miRNAs exhibit a regulatory function on the translation process by binding or degrading the messenger RNA and avoiding the corresponding protein synthesis. Transcriptomic studies showed that stressful situations in microalgae lead to an increase in these molecules which reflects the need to obtain immediate responses by the cell [46]. In *Chlamydomonas reinhardtii*, some endogenous miRNAs have overexpressed in S deprivation conditions. These observations led to the design of several artificial miRNAs (amiRNAs) to increase hydrogen yields by stimulating a faster oxygen consumption or repressing *psbA* gene expression that encodes for PSII linked D1 protein [47,48]. Similarly, optogenetic systems have also developed using properly blue light-inducible expression amiRNAs. This gene control system has enhanced hydrogen production, confirming as a most promising tool [49]. Approaches related to genetic engineering require a fine upstream design and considerable resources. Certainly, specific approaches compared to random ones allow for better management of resources. Genome-scale metabolic reconstructions have already been used to direct choices in this sense for many species of prokaryotes and eukaryotes. For algal organisms, a similar tool has been developed using information from the literature. In particular, the AlgaGEM software is configured as a tool capable of defining the primary metabolism of Chlamydomonas reinhardtii and allowing the in silico prediction of any changes in the growth parameters or the engineering of specific metabolic pathways [50].

Engineering strategies generally allow the establishment and introduction of new traits that are advantageous for different research fields. Although they are not easy to implement techniques and require huge resources, new bioinformatics tools to support this area seem to push further to consider this strategy as one of the most promising for the energy sector.

5. Fermentation Processes and Biomass-Applied Technologies

Direct and indirect biophotolysis processes are intrinsically linked to the photosynthetic process and the connected electron transport chain. Together with these two photosynthetic pathways that contribute to the production of hydrogen, another one linked to fermentative metabolism has also been identified. In dark conditions, the enzymatic activity of pyruvate:ferredoxin oxidoreductase (PFR) in *Chlamydomonas reinhardtii* is responsible for the reduction of Fd and the passage of electrons towards hydrogenase. Overall, this pyruvate-dependent hydrogen production acts in ways similar to those observed in bacteria, and though the yield is low, its contribution is not negligible [51]. The accumulation of complex carbohydrates, such as starch, or endogenous substrates, is positively associated with the production of hydrogen, while, it was also observed that exogenous carbon-rich media further stimulate its production in the early anaerobic stages. Several fermentative bacteria use anaerobic processes to transform carbon sources into various by-products, including hydrogen. Processes, such as photo- and dark- fermentation, are commonly exploited and widely investigated in bacteria species, such as *Escherichia coli*, *Clostridium* spp., *Thermococcales* spp., *Rhodobacter* spp., and *Rhodopseudomonas* spp. [52,53]. The consumption of organic substrates, also deriving from waste, by photofermentation, include the transformation into organic acids, alcohols, CO_2,, and H_2 in presence of light, but with a low overall yield of solar energy conversion. In a similar way, but without light, dark fermentation uses various substrates and waste too, leading to the release of different components and gaseous mixtures in which hydrogen is present [46,54]. It has recently been observed that hydrogen production can be increased by up to 60% compared to *Chlamydomonas reinhardtii* monoculture systems, by using co-culture systems with *Escherichia coli*. Growth media glucose-rich are exploited by bacteria that produce acetic acid, which can be used in algal metabolism [55]. Synergistically, different photobiological and fermentative microbial metabolisms may interact and cooperate increasing the hydrogen yields [56].

Microalgae show an enormous biodiversity being present in different habitats, even extreme and hostile to most living organisms, suitably adapting their metabolism. It is therefore possible to modulate the growth conditions to obtain biomass of the desired composition, based on the requirements of the downstream process also using industrial and agricultural processing water and waste. In this perspective, various strategies have been applied by combining bioenergy production and bioremediation approaches [57,58]. Unicellular green alga *Scenedesmus obliquus* managed to biodegrade the phenolic content present in the olive oil mill wastewater. This strategy makes it possible to remedy a problem particularly encountered in the Mediterranean area and, since the biotransformation carried out consumes oxygen, favorable conditions to trigger a concomitant production of hydrogen are also generated [59]. A consortium of microalgae, mainly composed of *Scenedesmus* and *Chlorella* species, grown in pig manure showed good growth, without the addition of external nutrients, and significant fermentative hydrogen production [60,61].

One of the main problems associated to these approaches is the elevated costs in terms of management and purification of the components obtained, which include the presence of several by-products, also toxic. Moreover, although the biomass of the microalgae contains a reduced lignin content compared to other lignocellulosic feedstock previously used for energy purposes, preliminary treatments are often necessary to facilitate the extraction and conversion of the microalgae content. Mechanical, thermal, chemical, or biological treatments are often applied to biomass separately or in combination as a preliminary step [57,58].

6. Fuel Cell

Hydrogen is an energy carrier with a high calorific value of 122 kJ/g, which is about 2.75 folds than fossil fuels, and it is also an environmentally friendly molecule since it only gives water as a by-product of its combustion [62]. For this reason, hydrogen environmental damage ratio has been estimated as 1, compared to that of several hydrocarbon fuels about 20 times higher [8,63]. Hence, hydrogen is considered as the best alternative to fossil fuels, which usage is supposed to be drastically reduced by 2050, according to the 2015 Paris Climate Agreement. Several technologies are currently available for hydrogen production (Figure 2), which can be classified according to the starting material: on the one hand, from fossil fuels, hydrogen can be obtained by thermochemical conversions, such as partial oxidation, autothermal reforming, or steam reforming. On the other hand, exploiting renewable sources, hydrogen is made by water electrolysis or biomass thermochemical conversion [64].

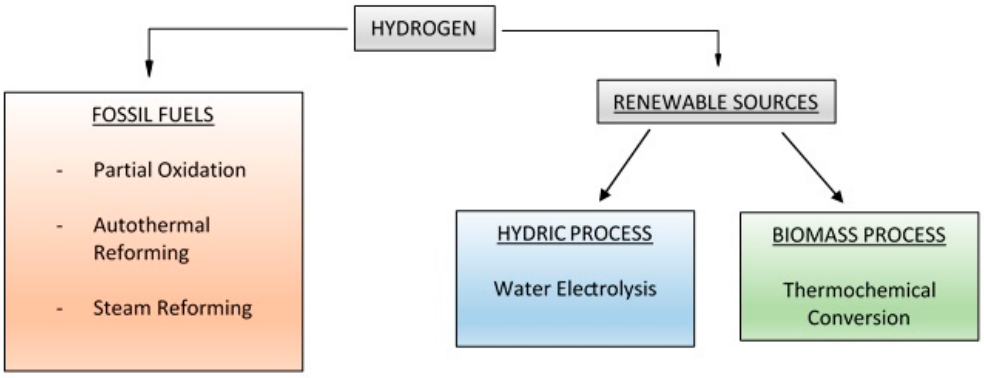

Figure 2. Major hydrogen production technologies.

However, none of these processes is sustainable: they all are energy-intensive process, usually requiring high temperatures. For example, water electrolysis requires temperatures ranging from 20 °C to 100 °C [65], while thermochemical processes can reach 2000 °C [66]. Moreover, fossil thermochemical conversions release high amounts of CO_2 [62]. Theoretically, it could be possible to convert these technologies into green ones by pairing them to mechanism that prevent CO_2 releasing into the atmosphere. This is the CSS approach, according to whom CO_2 can be stored into adequate geological sites, or reutilized for the chemical synthesis of useful products such as CO, urea, methanol, polymer, and carbonates [67]. Nevertheless, CSS approach has high design and operational costs that make his application unfeasible on the long term [68]. To implement a completely green sustainable economy, it is necessary to switch to biohydrogen, the biological hydrogen produced by microorganisms, including microalgae, according to the methods described in the previous paragraphs.

An interesting technology that has raised attention in recent years is that of fuel cells, in which hydrogen is often used as fuel. A fuel cell is an electrochemical technology capable of energy conversion. It is indeed capable to transform the chemical energy of a fuel into electricity [69]. A fuel cell may vary for it architecture, for the kind of fuel or for its catalyst; but it always consists of a few simple main parts (Figure 3): electrodes (an anode and a cathode), electrolytes, and an external circuit [70].

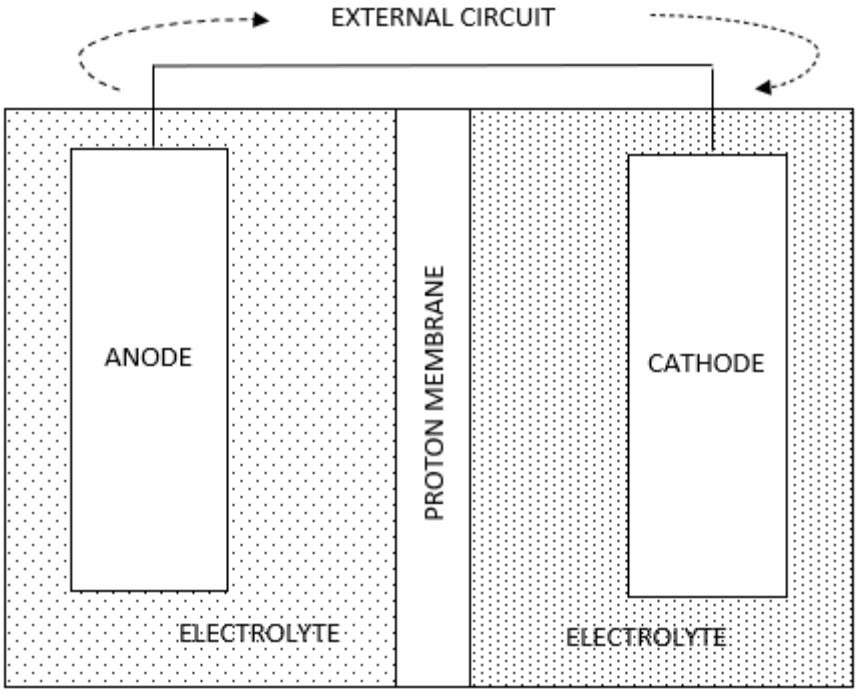

Figure 3. A basic fuel cell.

The reactions that happen in a fuel cell are simple: the fuel (usually hydrogen) at the anode is oxidized; then the electrons, through the circuit, reach the cathode, where (usually) oxygen is reduced to water [71]. Despite the simplicity of the processes, fuel cells currently present numerous criticalities that limit their use on a large scale. Their main problems are durability of the materials and high costs, often both related to the catalyst used. The most commonly used catalyst is platinum, both pure and alloyed, due to its maximum activity and chemical stability. In some cases, platinum group metals (PGMs) have even been used, including palladium, ruthenium, rhodium, iridium, and osmium. However, all of these materials have high costs due to their global scarcity [72]. The second issue of this technology concerns how to get hydrogen to the fuel cell itself. Storage is, at the moment, the main limitation to the development of an effective hydrogen economy. Many technologies are being studied in order to reach the highest volumetric density possible: physical methods, such as high-pressure cylinders for gaseous hydrogen or cryogenic tanks for liquid hydrogen, chemical reaction with metal and alloys, new materials, such as carbonaceous nanostructures for hydrogen absorption [73–75].

For this reason, technological research continues unabated. Based on the various innovative solutions that are proposed, it is possible to give a classification, to the different types of fuel cells currently under development. There are six of them [72]:

1. Proton Exchange Membrane Fuel Cell (PEMFC);
2. Alkaline Fuel Cell (AFC);
3. Phosphoric Acid Fuel Cell (PAFC);
4. Molten Carbonate Fuel Cell (MCFC);
5. Solid Oxide Fuel Cell (SOFC);
6. Microbial Fuel Cell (MFC).

6.1. Proton Exchange Membrane Fuel Cell

In a PEMFC, platinum is substituted by an ion exchange membrane that facilitates ion migration. This membrane is a polymer that usually has negatively charged group in order to let protons to flow toward cathode; but there also exists anion exchange membranes that hold positively charged groups so that the anion can be transported [76]. The most commonly used membrane is that made of perfluorosulfonic acid polymers—commonly

known as Nafion®. It was first commercialized by the DuPont company in the 1960s and it soon received wide acceptance because of its qualities. Nafion® is a robust polymer with high chemical and mechanical resistance, good conductivity, and little water or fuel crossover [70,77]. However, it is a costly material, covering about 20% of the cost of a fuel cell [77], and it is also thermolabile since it works only at a low temperature (50–80 °C) [78]. Alternative polymers are under development, mainly to allow the fuel cell to operate at high temperatures in order to optimize its yield. The most promising materials are currently aromatic-based membranes consisting of aryl rings and polybenzimidazole linkages [77].

6.2. Alkaline Fuel Cell

AFCs work at high pH using anion exchange membranes generally based on poly(olefine), poly(arylene ether), poly(phenylene oxide), poly(phenylene), polysulfone, and poly(ether imide). These fuel cells have lower costs than PEMFCs, and they are more resistant to high temperature. However, their main weak point is intrinsic due to the lower conductivity of OH- compared with protons [79].

6.3. Phosphoric Acid Fuel Cell

Phosphoric acid as an electrolyte in fuel cell consent to elevate the working temperature to high temperature around 220 °C. Therefore, it is possible to connect the PAFC directly to a steam reformer, to easily take up hydrogen from the source. The main flaws of this type of fuel cell are the necessity to use a metal catalyst on the electrode, and the hydrogen source that is not sustainable [80]. Recent studies have evaluated how the yield of PEMFCs improve by doping the membrane with phosphoric acid [81].

6.4. Molten Carbonate Fuel Cell

The electrolyte of this fuel cell is a molten carbonate salt solubilized in a lithium aluminate matrix. It can reach very high working temperatures (650 °C); thus, it is not necessary to connect it to an external hydrogen source because it self-reforms gases, also functioning with different hydrocarbon fuels [82,83].

6.5. Solid Oxide Fuel Cell

It is a high-temperature fuel cell and exists in two different types, the oxygen ion conducting fuel cell, and the proton-conducting one. Both have high-energy conversion efficiency and fuel flexibility, but the high fabrication cost makes them commercially not competitive [84].

6.6. Microbial Fuel Cell

These are the greenest and most sustainable types of fuel cells, and undoubtedly represent the future of energy production. MFCs can be double-chambered, with separated anodes and cathodes, or single-chambered, having the electrodes in the same container [85]. In both cases, they exploit microorganisms and their metabolism to produce the fuel necessary for the fuel cell to function. Most MFCs are mixed, using anode bacterial cultures for hydrogen production and cathode microalgae strains for oxygen supply [86–88]. However, prototypes of fuel cells that work only with algal strains are in development [89,90]. MFCs have significant environmental benefits. Thanks to the biological processes underlying their functioning, they can combine energy production with other functions, such as bioremediation activities [91–93]. It also bypasses the hydrogen storage limitation, since hydrogen is produced and utilized almost at the same time in the anodic chamber. However, this technology is not yet widely applied due to high costs and ineffective yields, which require further study for improvement [86,94–97].

7. Conclusions

In this review, we offered a general summary of the current status of biohydrogen applications. Hydrogen is undoubtedly the future fuel for its green and environmentally

friendly properties. However, its production technology is still based on fossil fuel; thus, carbon releasing. Scientific research is working hard to improve new strategies to reach green and sustainable hydrogen production and exploitation technologies.

Microalgae seem to be an attractive solution to this problem. As previously described, they can produce biological hydrogen without carbon emissions; rather, by fixing it during the process. The limiting factor for large-scale applications of this ability is that of low production yield, and, therefore, scientific research must focus in this direction. Solutions described in this review represent the most promising developments for implementing hydrogen yield.

The extreme versatility of microalgae also consents to combine several applications; thus, multiplying the benefits. The use of microalgae in dedicated fuel cells allows the development of an ecological energy production system, which can be associated with bioremediation advantages. In fact, microalgae can grow even in wastewater, purifying them from heavy metals and other dissolved substances.

Since current wastewater treatment plants present some critical issues concerning GHG emissions [98–100], developing an integrated purification and energy production facility based on microalgae could represent a promising ecological technology for the future.

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Review

Lipids from Microalgae for Cosmetic Applications

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Abstract: In recent years, there has been considerable interest in using microalgal lipids in the food, chemical, pharmaceutical, and cosmetic industries. Several microalgal species can accumulate appreciable lipid quantities and therefore are characterized as oleaginous. In cosmetic formulations, lipids and their derivatives are one of the main ingredients. Different lipid classes are great moisturizing, emollient, and softening agents, work as surfactants and emulsifiers, give consistence to products, are color and fragrance carriers, act as preservatives to maintain products integrity, and can be part of the molecules delivery system. In the past, chemicals have been widely used but today's market and customers' demands are oriented towards natural products. Microalgae are an extraordinary source of lipids and other many bioactive molecules. Scientists' attention to microalgae cultivation for their industrial application is increasing. For the high costs associated, commercialization of microalgae and their products is still not very widespread. The possibility to use biomass for various industrial purposes could make microalgae more economically competitive.

Keywords: lipids; microalgae; cosmetic products; new ingredients

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1. Microalgae

Microalgae are microscopic unicellular organisms with several sizes, structures, and forms [1–3]. The "microalgae" term ties together photosynthetic prokaryotic and eukaryotic microorganisms that grow rapidly and have the ability to live in different aquatic and terrestrial ecosystems. More than 50,000 species of them are known but a large number of microalgae remains still unexplored [4–6]. Microalgae differ from macroalgae commonly referred to as seaweed which are instead macroscopic and multicellular organisms [7].

The general term "algae" is not a taxonomic term, but is a common collective name for all the plant-like organisms which contain chlorophyll a, have oxygenic photosynthesis, and are not specialized land plants. There is no consensus among taxonomists around the world to use one classification system since these groups are revised continuously due to new genetic data. The current microalgal classification considers eight major phyla belonging to four kingdoms, Eubacteria, Protozoa, Chromista, and Plantae. The large majority of microalgae are nested in the Eukaryota domain and distributed in seven main phyla Euglenozoa, Cryptista, Haptophyta, Heterokontophyta, Glaucophyta, Rhodophyta, and Chlorophyta. Cyanobacteria are the

only representative phylum of microalgae in the Prokaryota domain. Despite the low representation in this domain, Cyanobacteria are among the most copious phylum alongside Chlorophyta and Heterokontophyta phyla [8,9]. One of the main characteristics of microalgae is their color determined by the presence of pigments such as chlorophylls, carotenoids, and phycobiliproteins, responsible for green, yellow/orange, and red/blue colors, respectively [7].

Microalgae use solar energy, water, and inorganic nutrients to reduce CO_2 into complex organic compounds. Some of them also have the ability to use some organic carbon, so their cultivation can be performed into three modes: Photoautotrophy, heterotrophy, and mixotrophy [4]. Microalgae also require nitrogen, phosphorus, sulfur as macronutrients, and potassium, iron, magnesium, calcium, and other micronutrients to support physiological activities [10].

Microalgae as photosynthetic organisms and compared to higher plants, have greater annual photon to natural biomass conversion efficiencies and no natural sensibility to seasonality [11]. Their high photosynthesis efficiency tied with their rapid growth and the ability to accumulate a large number of bioproducts within their cells make them a suitable candidate to serve as industrial raw material [12]. Microalgae present an original chemical composition and many substances of high biological value: They can accumulate a high percentage of lipids and they are an unconventional source of proteins and amino acids. They typically have a high carbohydrates content and other valuable compounds such as vitamins, antioxidants, and minerals [13]. The extraordinary diversity and complexity of microalgae are due to the fact that they are one of the oldest life forms on Earth and they have evolved and adapted over billions of years [7]. Microalgae cultivation for human purposes became increasingly important, precisely for their variety. The mass microalgae cultivation started in the early 1960s in Japan with the culture of *Chlorella*. Mexico was the first in the early 1970s to grow and harvest *Spirulina*. The third major marketing area for algae was Australia, with the growth of *Dunaliella salina* for the production of β-carotene. Plants were then subsequently built in the USA, Israel, and India [14]. Today, the largest commercial production of microalgae is mainly located in Asia. *Spirulina*, *Chlorella*, *Haematococcus*, *Dunaliella* are some examples of microalgae that currently are used for commercial purposes [6].

Microalgae are presented as new model organisms for a wide range of biotechnological applications including human and animal nutrition, cosmetics, pharmaceuticals, CO_2 capture, bioenergy production, and nutrient removal from wastewater [15–17]. The most important biological activities of natural products from microalga are antioxidant, anti-angiogenic, cytotoxic, anticancer, anti-obesity, and antimicrobial activities [16]. For these reasons, the mass culture and commercial production of microalgae have strengthened in the last few years but their industrial exploitation is at its early stages [4,6].

2. Lipids

In the last few years, microalgae cultivation has focused on lipids production for commercial applications. Lipids are a diversified selection of compounds for which no internationally agreed definition occurs. Generally, they are chemically heterogeneous substances, insoluble in water, but soluble in non-polar solvents. Lipids all contain either fatty acyl/alkyl, sphingosine or isoprene fractions as their hydrophobic building blocks. According to their hydrophobic or amphipathic characteristics and chemically functional backbones, lipids have been classified into eight categories, fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides, each of these eight lipid categories consists of further lipid classes and subclasses. [18–20]. Lipids are involved in many vital cellular processes. Lipids are the main constituents of biological membranes thanks to their hydrophobic nature, and therefore constitute the physical basis of all living organisms. Another task that lipids accomplish is the storage of surplus energy for later consumption. Finally, lipids also play

a role in extra and intracellular signaling processes, as they transduce signals and amplify regulatory cascades. In plants, lipids are also involved in the photosynthesis processes [21,22]. In addition to their role in the regulation of a variety of physiological processes, lipids are associated with abnormal metabolism in many diseases such as atherosclerosis, diabetes, obesity, Alzheimer's disease, and tumorigenesis [23]. The rapid progress in analytical chemistry techniques, mostly chromatographic separation methods and mass spectrometry identification techniques, alongside the wide knowledge in bioinformatics, led to the development of "lipidomics". Lipidomics is used to describe the complete lipid profiles and networks of cellular lipid metabolism within a cell, tissue or a biological system, and provides a powerful tool to quantify the changes in individual lipids that may help reveal lipid biomarkers in diseases, for example [24,25].

Alongside their biological importance, lipids are exploited for various commercial purposes. Lipids are a major component of food and their demand is increasing in food production and nutritional supplements for human food or animal feed, other important markets for oils are detergents, biofuels, lubricants, hydraulic fluids, inks, paints, and phytochemical compounds [26]. Growing consumer demand is increasingly oriented towards vegetal oils. An important aspect to consider is lipids susceptibility, strongly influenced by numerous factors. Most of all, lipids are prone to oxidation that negatively affects their biological activities. Oxidative stability depends on many intrinsic and extrinsic factors. First, variations in oxidative stability exist among different lipid classes. Oxidative susceptibility of lipids depends largely on the degree of unsaturation of fatty acids and the stereospecific positional distribution of fatty acids in the triacylglycerol (TAG) molecules. Environmental factors to which lipids are exposed during processing and storage may affect their oxidation rate. In addition, the presence of minor components in fats and oils also affects their oxidative stability in both positive and negative manners. Lipid oxidation has detrimental effects on food quality and human health, for example [27]. Therefore, it is necessary to minimize oxidation and improve the oxidative stability of lipid products. The addition of antioxidant or encapsulation strategies are techniques commonly employed for lipid molecules stabilization [28].

Plant-derived lipids are conventionally used to satisfy the high industrial request, particularly lipids derived from oilseeds such as palm, soy, rapeseed, and sunflower oils. The increasing lipid demand raised some questions, among them the reduced availability of cultivable lands and consumption of resources. Microorganisms represent a sustainable alternative to lipids sources. Both prokaryotic and eukaryotic microorganisms are known to produce lipids in different quantities and compositions [29]. When their lipid content exceeds 20% of dry biomass they are classified as "oleaginous". The three different family groups of oleaginous microorganisms are microalgae, fungi (molds and yeast), and bacteria [30–32].

3. Lipids for Cosmetic Uses

Cosmetics have always played an important role in society. They are stable and homogenized mixtures of substances, resulting from an exact combination of active principles, excipients, and additives that are respectively the ingredients responsible for the asserted cosmetic activity, the substances ensuring the desired pharmaceutical dosage form, and the substances added to preserve the product and to improve its organoleptic properties [33]. Cosmetics clean, perfume, protect or modify the appearance of the part of the human body where they are differently applied by rubbing, pouring, and spraying, such as epidermis, hair, nails, lips, external genital organs, teeth, mucous membranes of the oral cavity [34]. Therefore, the applications of cosmetics range from everyday hygiene products such as soap, shampoo, deodorant, and toothpaste to beauty items including perfumes and makeup. Cosmetics production is carefully regulated to ensure consumer safety [35]. Natural compounds have been used for these applications in the past but the cosmetic market progressively increased the use of many synthetic chemicals over time [36]. Mineral oils and waxes are examples of these. They are stable and dermatologically

well-tolerated compounds, used to regulate the viscosity of formulations and for protective and lubricating properties. Mineral oils and waxes are prepared from naturally crude petroleum oil through various refining steps including distillation, extraction, crystallization, and purification to remove potentially toxic substances. Moreover, mineral oils are non-allergenic, are highly stable, and not susceptible to oxidation or rancidity [37]. Although stringent regulations apply, many concerns emerge for the possibility to find hazardous solvents traces in final formulations and consequent risks for human health. Hence, the intention to replace them with vegetable oils, still not fully implemented because chemicals are more competitive economically. Many concerns are emerging for all chemicals: In some cases, safety data are lacking for synthetic ingredients and they might cause hypersensitivity reactions, anaphylactic reactions, lethal poisonings or long time effects to users. Systematic monitoring of these substances can be made by testing their genetic toxicity, phototoxicity, photogenotoxicity, toxicokinetics, and carcinogenicity and new studies might reveal different toxicity data. [38–41]. Furthermore, the daily use of many cosmetic products leads to continuous exposure to different chemicals. As a result, the synergistic interaction of different chemicals and additive action may occur due to the presence of the same ingredients in different products [42]. Due to these reasons, the need to substitute chemicals become increasingly imperative and lead cosmetic industries to incessantly look for innovations. One of the current challenges is finding natural ingredients to achieve customers' requests more and more often oriented by increased awareness about the importance to use quality products and environmentally sustainable [43]. Marine sources and specifically algae represent valid alternatives of new raw materials [34,44–46]. Currently, macroalgae-based cosmetic products are present on the market and they are progressively substituting synthetic equivalent products. These products can contain macroalgal extracts with different bioactive compounds or their purified form. Active substances that lead to macroalgae utilization in cosmetics are extremely different (polysaccharides, proteins, peptides, amino acids, fatty acids, sterols, glycolipids, phospholipids, pigments, phenolic compounds, vitamins) although they are mostly used as thickening and gelling agents. The world of microalgae is still to be explored even if some ingredients derived from them are already on the market [47,48].

Lipids constitute one among the different categories of cosmetic ingredients (Table 1). In addition to lipids of chemical origin, a wide range of vegetable and animal oils and fats can be used as neutral bases and bioactive ingredients but today the lipids and their derivatives in cosmetic formulations are of plant or biotechnological origin. The types of lipids commonly used in cosmetics include triacylglycerides, waxes, ceramides, glycerophospholipids, sterols, hydrogenated, esterified, and oxidized lipids [49–51].

Table 1. Properties and lipid molecules most common in cosmetic products.

Properties	Lipids
Moisturizing and softening properties	Hydrocarbons, fatty acids, fatty alcohols, triacylglycerols, waxes, phospholipids, sterols
Surfactant and emulsifier agents	Phospholipids, glycolipids, lipopeptides, fatty acids
Texturizer agents	Waxes, alkenones, triacylglycerols
Color carriers	Isoprenoids
Fragrance carriers	Essential oils, triacylglycerols
Preservative agents	Glycerolipids, sphingolipids
Active ingredients	Glycerolipids, sphingolipids, sterols, isoprenoids, flavonoids
Molecule delivery	Phospholipids

Lipids perform different functions in cosmetic formulations. They are moisturizing agents that limit water loss through different mechanisms. The first way is occlusion, obtained by placing a waterproof film on the skin to delay water evaporation from the

surface. Substances typically used for these aims are hydrocarbons, fatty acids, fatty alcohols, vegetable waxes, phospholipids, and sterols. Another mechanism is realized by applying humectant substances to the skin surface to attract water. An additional form of moisturization is the use of hydrophilic matrices that forms a physical protective coating over the skin preventing evaporation. Then, photoprotection is considered an indirect form of moisturization using sunscreen ingredients that prevent cellular damage and related dehydration.

Lipids are emollient and softening agents that make the skin smooth and soft. Skin softness and smoothness are due to the capacity of thin oily substances to temporarily deposit between corneocytes making their edges smoother. Skin radiance and luminosity are closely related to the smoothness of the skin surface since they depend on the amount of light reflected by the skin surface improving skin appearance [52].

Lipids are used as surfactants and emulsifiers, to reduce surface tension between the skin's surface and product and to keep water and oil blended in a product. Surfactants reduce surface tension and facilitate the formation of emulsions between liquids of different polarities due to their chemical structure with hydrophobic and hydrophilic moieties. Their amphiphilicity performs detergency, wetting, emulsifying, solubilizing, dispersing, and foaming actions. In cosmetics, polyethylene glycol ethers are the most commonly used traditional commercial surfactants. Today, there is an increased use of biosurfactants, generally classified into glycolipids, lipopeptides, phospholipids, fatty acids, and polymeric compounds according to their chemical structures. The biosurfactants commonly used in cosmetics and personal care products are glycolipids due to their physico-chemical properties, biological activities, biocompatibility, and biodegradability and are used as multifunctional ingredients in the formulation of cosmetics. Sophorolipids, rhamnolipids, and mannosylerythritol lipids are the most known glycolipids with application to cosmetics and pharmaceutical technology [53,54].

Lipids can also function as texturizers, to give consistency to gel-like products to improve spreadability and feel consistency, and as color and fragrance carriers [49]. Color and fragrance are two important characteristics for cosmetics selection among consumers. Pigments are examples of lipid colorful molecules for industrial applications [48]. Instead, essential oils can impart pleasant aromas in different products, besides, they can act as preservatives and active agents and, simultaneously, offer various benefits to the skin [55]. As preservative carriers, lipids can prevent bacteria growth [49]. The use of preservatives in cosmetic formulations is for bacterial contamination risk during use. There are several examples of the use of lipids as part of a preservative system in cosmetics. In the past, lipids have been used as penetration enhancers in cosmetics and recently they have been used as nanoparticles such as solid lipid nanospheres, liposomes, nanosomes, and nanostructured lipid carriers for bioactive molecule delivery [56].

4. Microalgae as Lipids Bio-Factories

Microalgae are a promising platform for a wide range of high-value compound production. The last decade has seen intensive interest in microalgal lipids that can be used in various fields, from food to chemical, pharmaceutical, and cosmetic. Microalgae can produce various types of lipids such as triacylglycerols, phospholipids, glycolipids or phytosterols, used for energy storage, as energy substrates, as structural components of the cell membrane, and for metabolic processes such as signal transduction, transcriptional and translational control, intercellular interactions, secretion, and transfer of vesicles. Typical lipid molecules found in microalgae are common in other high plants but there are some unusual lipids not present in other organisms [57].

Microalgae cultivation processes consist of many important steps to optimize the production of the desired amount of target products (Table 2).

Table 2. Microalgae cultivation process and aims of each stage.

Microalgae Selection	Cultivation	Harvesting	Extraction	Purification
Based on quality and quantity of lipids desired	Balance between biomass production optimization and production costs	Separation of growth medium and biomass	Biomass pre-treatment (for cell wall breaking) and lipid recovery	Removal of impurities
	• Open systems • Closed systems	• Centrifugation • Filtration • Sedimentation • Coagulation • Flocculation • Foam flotation • Electric-based methods	• Mechanical methods • Chemical methods • Physical methods	• Filtration • Chromatographic methods

The first step is the selection of microalgae species. The specific composition and quantity of lipids are species-dependent and common oleaginous microalgae are *Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus* sp., and *Dunaliella* sp. [58–60]. A suitable microalgae selection is crucial for production since cultivation conditions, harvesting, and extraction methods change accordingly to improve production efficiency, yield, and quality of the products [40]. Depending on the species selected and the objectives to be achieved, cultivation conditions may vary.

Currently, there are two primary types of mass-cultivation systems: Closed cultivation systems in photobioreactors and open cultivation systems in raceway ponds. The first allows controlling physical and chemical cultivation conditions such as temperature, pH, aeration, mixing, light intensity, and growth mode. In addition, photobioreactors allow monitoring possible contaminations but the capital, operational, and energetic costs remain significantly high. The second are less complex and require a lower capital investment, indeed microalgae are exposed to full sunlight and cultivated at high concentrations to maximize biomass productivity [11,61].

Microalgae accumulate lipids up to about 70–80% of their biomass weight under favorable conditions. In any case, the oil productivity of microalgae is higher if compared to the most commercially productive plant [59,62–65]. Even if the composition and quantity of lipids are species-dependent, the accumulation of lipids in microalgae can usually be induced by stress conditions. Under harsh environmental conditions, microalgae synthesize and produce various secondary metabolites to preserve their growth, among which lipids that act as an energy-rich carbon storage substrate that enables the cells to survive under transient extreme environmental conditions and additional amounts of carotenoids to alleviate the oxidative damage induced by stress conditions [66]. Although each species of microalgae has different optimal stress conditions for the overproduction of desired metabolites, the main external cultivation conditions affecting lipid productivity are light intensity, temperature, carbon dioxide, nutrient starvation, salinity stress, and metal stress. Stress conditions stimulate lipid production but might inhibit cell growth and induce oxidative damage. According to considerable analyses on technology and economy, a two-stage culture strategy can be an interesting way to enhance lipid productivity. In the first stage, the algae are cultivated under optimum conditions to promptly achieve an optimal cell density, in the second stage, cultivation conditions are changed to trigger the accumulation of lipids [59,63,64,66]. The stress caused by the depletion of the nutrients is a commonly used strategy to trigger the accumulation of energy storage metabolites such as lipids in microalgae [10]. In the literature, there are also several genetic approaches to increase lipid production of microalgae. Molecular approaches include random mutagenesis, genetic engineering including genome editing, and metabolic pathway engineering [59,67,68].

During microalgae cultivation, growth can be followed in several ways such as counting the total cells, through a particle analyzer, measuring the doubling time, and

biomass productivity. When the desired quantity of final product is reached, it is necessary to remove water from the culture media for biomass recovery. This important harvesting process can be performed through different solid-liquid separation techniques such as centrifugation, filtration, sedimentation, coagulation, chemical flocculation, foam flotation, electrical-based methods or a combination of various methods. Harvesting is an important and costly step in the large-scale microalgae cultivation process, which means that their use on an industrial scale is not economically sustainable [40,69].

The following step is the extraction of lipid products. In the literature, several techniques were reported for microalgal lipids extraction, mostly used for lipids recovery for biodiesel production [70]. No method can be considered effective as each microalga species has very specific characteristics. Typical conventional extraction methods include mechanical, chemical, and physical processes. The cell wall breaking is the key factor for the success of the whole process of lipids extraction. The microalgal cell wall composition is variable depending on the species, generally, the cell wall consists of an extracellular polymeric structure composed of polysaccharides, proteoglycans, peptides, proteins, and associated inorganic elements [71]. Solvent extraction methods are common methods for lipids recovery. Extraction with solvents can be improved by several approaches of pre-treatment of the biomass for weakening and breaking of the cell wall, whose effectiveness varies from one microalga to another. Multiple laboratory-scale microalgae cell disruption methods are available. An example of biomass pre-treatment method is grinding that involves mixing the freeze-dried biomass with liquid nitrogen through a ceramic mortar and pestle. Bead vortexing leads to microalgae cell walls breaking by grinding and agitating the cells on a solid surface of glass beads [70]. The expeller press through the application of a high mechanical pressure shatters and breaks the cells. Cells rupture can occur also with osmotic shock, realized whit hyper-osmotic or hypo-osmotic conditions or with thermolysis when the vessels containing algal cells are heated through a water bath [31]. Ultrasonic treatment and electroporation lead either to cells disruption, the first using the energy of high-frequency acoustic waves, the second with an external electric field [72]. The use of microwaves allows the extraction of lipids or other molecules [73]. When microalgal cells are exposed to the microwave, inter- and intramolecular movements are generated in cells with consequent heat generation. Intracellular heating causes water vapor, which disrupts the cells and subsequently opens the cell membrane [73]. In addition, the algicidal treatment exploits the capacity of some bacteria to attack and destroy target algae for the presence of hydrolytic enzymes able to break down the cell wall [70]. These techniques improve product recovery but are also potentially costly steps making biomass pretreatment an obstacle for large-scale production [16]. Various organic solvents or combinations of them have been suggested. Organic solvents are absorbed within the cell wall, and they cause swelling and rupture of the microalgal cell. In this way, the cell contents are available to be separated on the following step. Among organic solvents, a mixture of chloroform-methanol can efficiently extract lipids. The two traditional methods for lipids extraction, the Folch method [74] and Bligh and Dyer method [75], employed the mixture in different volume ratios: The sample is homogenized with the organic solvents and the addition of a saline solution leads to a phase separation and consequently lipids extraction [76–82]. Even though the extraction with chloroform is very effective, large-scale lipid extraction using these methods is excluded for environmental and health risks. Solvents such as ethanol, butanol, hexane, isopropanol, methyl-tert-butyl ether, acetic acid esters, and various combinations of different solvents have been examined, many of these are still harmful. More often, lipids extraction is carried out through a Soxhlet extractor. Soxhlet extraction is performed using solvents at boiling temperature and ambient pressure, and even if it requires a high amount of solvents and a long extraction time, it is capable of providing high yields and does not affect the bioactivity of the extracted molecules. An alternative is represented by green solvents residues, which have good solubilizing properties such as conventional solvents. They are environment-friendly solvents or bio-solvents, derived from natural or

processing of agricultural. Examples are 2-methyltetrahydrofuran, ethyl acetate, ethyl lactate, and cyclopentyl methyl ether [83]. Other environment-friendly technologies over other conventional methods are supercritical fluid extraction [84], generally using carbon dioxide, and extraction with ionic liquids [12] that are considered as green, non-aqueous salt solutions, which contain both anions and cations. Liquid polymers and fluorous solvents are among the emerging green solvents. Liquid polymers are considered non-volatile class-based solvents, such as poly(ethylene glycol), poly(propylene glycol), poly(tetrahydrofuran), and poly(methylphenylsiloxane). The fluorous solvents are colorless, are free-flowing liquid, and have low toxicity. Various types are available, the mainly used are perfluorinated alkanes, perfluorinated dialkyl polyethers, and perfluorinated trialkylamines [70].

After extraction processes, further purification steps can be requested through filtration or chromatographic separation techniques [40].

The lipids of the microalgae can be considered an alternative raw material to classic natural sources for the market and today, different microalgal species are used as lipid bio-factories. Microalgae can produce various lipid categories (which will be illustrated below) with remarkable applications in various industries such as pharmaceuticals, foods and feed, cosmetics, and biofuel production. As previously mentioned, until a few years ago chemical synthesis represented the primary choice for the recovery of substances. Currently, there is a marked return to oils and fats of vegetable and animal origin to replace petroleum-based and synthetic products. The driving forces for this trend are primarily environmental and ecological. Oils and fats are renewable resources and do not cause an increase in CO_2 production. In addition, their biodegradability is less complicated as compared to synthetic analogs.

Most of the industrial lipids, also in the cosmetic sector, come from plants such as sweet almond, avocado, Jojoba, copra, castor, and palm oils. The high use of environmental resources such as arable land and water represent the main problems in the use of vegetable lipids. Animal fats can be used as cosmetic additives. Lanolin, obtained from refined lamb's wool grease, and squalene, obtained from shark liver oil, have been used extensively. Today, animal fats are less used in cosmetic preparations for many reasons related to animals' protection, environmental problems, and microbial contamination derived from animal infections [29].

Therefore, the use of microorganisms represents a valid alternative. Lipids producers microorganisms are bacteria, fungi, and microalgae, among them microalgae are a very interesting source. They have a strong environmental adaptation ability, do not need cultivable land for their growth, require less water than land crops, could accumulate high biomass quantity, and do not have a natural sensibility to seasonality. Microalgae include very different photosynthetic organisms and can accumulate a great variety of lipid molecules and other bioactive compounds in their cells. For these reasons, their use in the cosmetic sector is very interesting. However, production costs are the most significant obstacles that limit the production of microalgal oil in a large scale [85]. Different aquatic microalgae mainly from the genera *Aphanizomenon*, *Arthrospira*, *Chlorella*, *Desmodesmus*, *Dunaliella*, *Haematococcus*, *Nannochloropsis*, *Scenedesmus*, and *Spirulina* are broadly used in cosmetic and cosmeceutical applications [45].

Table 3 summarizes the main lipid categories with useful applications in cosmetics and the related microalgal species from which they are extracted.

Table 3. Microalgal species and related lipid categories extracted.

Lipid Categories	Microalgae Species	References
Triacylglycerols	*Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus* sp., *Dunaliella* sp., *Chlamydomonas* sp.	[32]
Polyunsaturated fatty acids	*Nannochloropsis* sp., *Dunaliella* sp., *Schizochytrium* sp., *Isochrysis* sp., *Tetraselmis* sp.	[86,87]
Sterols	*Diacronema lutheri*, *Tetraselmis* sp., *Nannochloropsis* sp.	[88]
Waxes	*Euglena gracilis*, *Isochrysis* sp.	[89,90]
Carotenoids	*Dunaliella salina*, *Haematococcus pluvialis*, *Chlorella* sp., *Scenedesmus* sp., *Muriellopsis* sp., *Spirulina* sp., *Porphyridium* sp.	[91–93]

5. Microalgal Lipids for Cosmetic Applications

Although most of the literature on microalgae lipids focuses on their use for biofuel production, many other sectors can exploit microalgal lipid properties, for example, food and feed fields. The most commonly used lipids for these sectors are omega-3 and omega-6 lipids, instead, as a renewable energy source a transesterification step is necessary for the production of fatty acid alkyl or methyl esters, commonly named biodiesel [31].

Moreover, cosmetic and pharmaceutical sectors show increasing interest in lipid with functional properties such as polyunsaturated fatty acids (PUFAs), phytosterols, and carotenoids [16,94,95]. In addition, oils are generally used as dermatological delivery agents, formulated into creams or emulsions to provide more uniform, efficacious application, and transport of active agents, as already mentioned.

In the next paragraphs, we shortly analyze the different lipid categories found in microalgae, classified according to the eight classes described in the classification system cited previously [20] and LIPID MAPS Structure Database [96]. Where possible, we describe their functions and properties for industrial application, particularly in the cosmetic field. The articles analyzed in this review were selected using the Pubmed and Google Scholar databases. After a first study of the literature concerning the general use of lipids in cosmetics, we focused our research on the lipids of microalgae. The selected works are those resulting from a research carried out by entering the individual lipid categories as keywords.

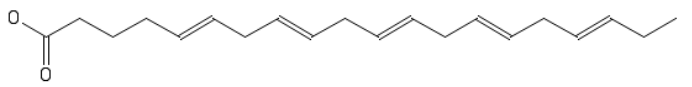
5.1. Fatty Acyls

Fatty acyls are one of the most important categories of biological lipids with a structure that represents the major lipid building block of complex lipids [20]. The category of fatty acyls sees further subdivision into subclasses, including fatty acids and conjugates, eicosanoids, fatty alcohols and esters, etc. In the cosmetic field, the fatty acyls group from oil seeds serve as the principal bio-based surfactant feedstock [97]. Biosurfactants are a diverse group of lipids with amphiphilic character containing both hydrophilic and hydrophobic domains within the molecule. This structural diversity of biosurfactants implies a large variety of biological and physicochemical properties. The main activity of these compounds is to lower interfacial tension allowing, for instance, solubilization of hydrophobic substances in water. Another frequent property of biosurfactants includes low critical micelle concentrations, allowing biosurfactants to exert their function at much lower concentrations than many chemically produced surfactants. Moreover, biosurfactants show prominent bioactivities including antibacterial, antifungal, and anti-tumor effects. Finally, they exhibit low eco-toxicity about excellent biological degradability preventing environmental accumulation [98]. Fatty acids (FA) consist of a hydrocarbon chain with a carboxyl group at one end. In cells, FAs can be free or bound. FA considered essential oily raw materials in cosmetic applications, can be used not only as emulsifiers but also as softeners, detergents, lighteners. They are thin oily substances capable of depositing between the desquamating corneocytes temporarily, making the skin smooth and soft and therefore brighter [52].

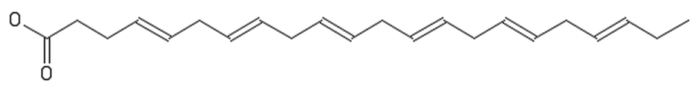
Many kinds of fatty acids can be used as raw materials, such as lauric acid, myristic acid, palmitic acid, and stearic acid. In addition, FAs are skin components as an important player in the maintenance of normal skin barrier function [99].

Microalgae contain a rich profile in PUFAs. They are fatty acids with more than one double bond in the carbon chain and they have many beneficial properties. Microalgae can synthesize members of the PUFAs ω-6 family, including linoleic acid (LA), γ-linolenic acid (GLA), and arachidonic acid (ARA), as well as of the PUFAs ω-3 family, including α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) [62,100–103]. Many microalgae can synthesize the long chain of ω-3 PUFAs, with yields greater than 20% of their total lipids. Marine members of the Thraustochytriacea and Crythecodiniacea families are the microalgae most currently used for the production of algal oil rich in ω-3 and biomass [30]. They are important ingredients for food supplements and feeds for their evident beneficial effects on tissue integrity and health. Microalgae such as *Chlorella vulgaris*, *Arthrospira platensis*, *Haematococcus pluvialis*, *Dunaliella salina* have already been recognized as safe or authorized as additives for humans and animals. Other species that have been studied but that have not yet been marketed are *Scenedesmus almeriensis* and *Nannocholoropsis* sp. [87].

PUFAs have antioxidant and anti-inflammatory properties which prove to be very useful in different cosmetic formulations [104]. They help prevent heart disease [105] and inhibit the growth of cancerous cells [106]. PUFAs have shown positive effects in the treatment and prevention of various diseases including inflammatory ones, atherosclerosis, thrombosis, arthritis, and a variety of cancers. EPA and DHA (Figure 1) are the most valuable ones: They are associated with the reduction of complications in hypertension and show significant hypolipidemic activity, to reduce triglycerides and increase high-density lipoprotein cholesterol [85,107,108]. Furthermore, PUFAs contribute to the growth and performance of the retina [109], brain [110], and reproductive tissues [111]. In addition, PUFAs showed an antiproliferative effect on epithelial and bronchopulmonary cell cultures and improved glycogenesis in diabetic mice [111]. ARA and EPA promote the aggregative and vasoconstrictive action of platelets and the anti-aggregative and vasodilatory effects in the endothelium, as well as showing chemotactic action in neutrophils [100,112]. PUFAs derivates also play important roles. Particularly, the oxidative transformation of PUFAs results in a diverse family of lipid mediators named oxylipins, which play a key role in different metabolic responses, including their function in the resolution of inflammation [113–115]. For these reasons, all these lipids are also important for their biological activity when included in cosmetic formulations [116].



(**a**)



(**b**)

Figure 1. Chemical structures of some PUFAs produced by microalgae: (**a**) Chemical structure of EPA; (**b**) chemical structure of DHA.

Waxes fall into the fatty ester class, among the fatty acyl category [20]. *Euglena gracilis* is a microalga that accumulates a high amount of wax-ester as a by-product of the degradation process of storage polysaccharides, useful today for biofuel production but they could also prove helpful in cosmetics [89,117,118]. For example, waxes are key components in lipsticks since they provide appropriate rigidity, hardness, stability, and

texture to the stick. A variety of waxes are available on the market today for lipsticks. Commonly used waxes are synthetic ingredients derived from petroleum but also mineral waxes derived from shale and animal or plant-derived ingredients are used. These last are often confined to certain parts of the world and their availability could be potentially affected by environmental factors. Alkenones are a family of unique lipids, long-chain ketones, biosynthesized by certain haptophyte microalgae including the *Isochrysis* sp. that are used as structuring agents in some cosmetic formulations in substitution to animal-derived and petroleum-derived waxes. They are a marine-based vegan and renewable ingredient that well answer consumer requests. Alkenones can be produced in many locations, therefore, their availability is not as limited as that of some other waxes. Given their waxy nature and reasonably high melting point, alkenones could represent an attractive class of natural ingredients that may find useful applications in a variety of cosmetic and personal care applications [90].

5.2. Glycerophospholipids

Glycerophospholipids, also known as phospholipids, are present in all organisms, including microalgae, and are key components of the lipid bilayer of cells. They provide a selectively permeable barrier that protects the cell from the outside and helps in the separation of the different intracellular organelles. In addition to their primary role in cellular membrane components, glycerophospholipids can influence cell signal transduction serving as binding sites for proteins and as second messengers or their derivatives [20]. There is also evidence that phospholipids are essential to many important biological processes, such as stress response and photosynthesis [119]. Their structure consists of a glycerol backbone with two hydrophobic acyl tails and a hydrophilic head group linked to the glycerol scaffold through phosphodiester linkage [24]. The major phospholipids are phosphatidylcholine (PC) (Figure 2), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI) [120]. The presence of both hydrophilic head groups and hydrophobic tails confers them amphiphilicity, which makes phospholipids excellent emulsifying agents and therefore can stabilize oil-water emulsions as delivery systems for food, cosmetic ingredients, and drugs [121]. Lecithins are a complex mixture of PC, PE, phosphatidylserine (PS), and PI, combined with various amounts of other substances such as triglycerides, fatty acids, and carbohydrates. They are used in foods as emulsifiers and surfactants to alter viscosity and crystallization properties. Lecithins are also used in the industrial field as an emulsifying agent in fabrics, leather, cosmetics, paints, plastics, and a release agent for concrete and insecticides [26].

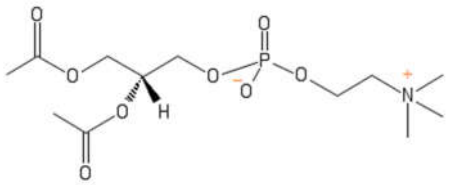


Figure 2. General structure of a PC.

5.3. Glycerolipids

Glycerolipids constitute the lipids category that encompasses all glycerol-containing lipids, except glycerophospholipids, which constitutes a separate category due to their abundance and important roles. The class of glycerolipids is dominated by the mono-, di-, and tri-substituted glycerols. Triacylglycerols (TAGs) are the most well-known glycerolipid category consisting of tri-substituted glycerols. They are responsible for energy storage in the cells [20,122] and their industrial application mainly concerns biofuel production [63,123,124]. Genera of microalgae used for biofuel production are *Chlamydomonas*, *Chlorella*, *Nannochloropsis*, *Synechocystis*, *Tetraselmis*, *Monoraphidium*,

Ostreococcus, *Tisochrysis*, and *Phaeodactylum* [32]. TAGs from vegetable oils are also used in the formulation of bath products, cleansing products, eye makeup, fragrances, foot powders, facial makeup, personal cleanliness, suntan, and other skin products since they work as emollients. TAGs keep the skin's hydration level high since they form an occlusive layer on the skin that reduces the skin's water loss kinetics [42].

Chloroplast-specific glycolipids are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) (Figure 3), and sulfoquinovosyldiacylglycerol (SQDG) that fall into the glycerolipids category. They are characterized by the presence of one or more sugar residues linked to glycerol via a glycosidic linkage, and they are primarily the constituents of thylakoid membranes in plant cells, including microalgae [120,122,125]. These lipids have been identified as possessing a variety of bioactivities, such as antioxidant and antimicrobial associated to the length of their fatty acyl chains, the number and position of the double bonds, the structure of the sugar moiety, and its anomeric configuration. It must be specified that several studies identified glycolipid fractions from algae having an antimicrobial activity even if only a few of these have been able to isolate and characterize the main molecular species responsible for this activity [126].

(**a**) (**b**)

Figure 3. General structure of some glycolipids: (**a**) Chemical structure of a MGDG; (**b**) chemical structure of a DGDG.

5.4. Sphingolipids

Sphingolipids are a composite category of lipid molecules that share a common structural feature, a sphingoid base backbone consisting of an aliphatic amino alcohol group [20]. They play important structural and intracellular roles and take part in extracellular signaling. Moreover, they form specialized micro-domains in plasma membranes involved in different cellular processes, alongside sterols [57].

In the group of sphingolipids, different chemical structures were identified belonging to different sub-classes such as ceramides and glycosphingolipids, also known as cerebrosides. Ceramides are used in several skin care cosmetic products for regulating trans-epidermal water loss and promoting epidermal barrier repair. They are abundant lipids in the stratum corneum produced when barrier damage occurs and which become sphingolipids when glycosylated. Different ceramides have been identified and synthetically duplicated for inclusion in moisturizer formulations renowned by their polar head group structure, as well as by their hydrocarbon chain properties [49,52]. Moreover, these lipids have shown an antimicrobial activity [126]. Sphingolipids are widely distributed metabolites of microalgae but knowledge of these metabolites in microalgae remains poor [57].

5.5. Sterol Lipids

The sterols category includes molecules with different biological functions, and are characterized by the presence of a unique fused ring structure [20]. They constitute an important component of membrane lipids specifically for the optimal maintenance of

membrane fluidity. Sterols present in microalgae are not only free but also in conjugated forms, many of them are unusual in terrestrial higher plants [57].

A great variety of sterols is present in plants and they are also called phytosterols. In the last few years, phytosterols have become particularly interesting for their beneficial health effects. Numerous studies have reported that phytosterols interact with cholesterol absorption through inhibitory mechanisms leading to reduction of low-density lipoprotein cholesterol in the blood. In addition, they protect against nervous system disorders and exhibit anti-oxidative, anti-inflammatory, anti-atherogenicity, and anti-cancer properties [57]. Current industrial sources for phytosterols are tall oil and vegetable oils but microalgae are an alternative source [29,127]. Phytosterols are found in all microalgal species. Recently, *Diacronema lutheri*, *Tetraselmis* sp. and *Nannochloropsis* sp. have been identified as the highest phytosterol producers, but other studies on the phytosterols content are done in different microalgae species and classes [88]. In some studies, phytosterols and the total lipid fraction isolated from microalgae are identified as responsible for an anti-inflammatory activity due to an increase of anti-inflammatory cytokines and decrease of pro-inflammatory cytokines secretion. Therefore, their use in animal feed could control immune responses during inflammation and minimize the use of antibiotics [128,129]. These biological activities make them useful in the cosmetic sector.

5.6. Prenol Lipids

Microalgae are also a source of natural pigments with lipid characteristics, particularly carotenoids. Carotenoids are isoprenoid molecules belonging to the prenol lipids category. The general structure of carotenoids consists of a tetraterpene backbone optionally flanked by terminating rings. These rings are either oxygenated or non-oxygenated and distinguish carotenoids between xanthophylls and carotenes, respectively [20,94]. It is possible to distinguish between primary and secondary carotenoids. The first are a structural and functional part of the photosynthetic system of microalgae, having both a light-harvesting role and also photo-protective role. The second are accumulated in the cytoplasm and have only a photo-protective function [130]. Carotenoids absorb light within a wavelength of 400–550 nm and are the source of the yellow, orange, and red color of microalgae [131]. Carotenoids have a wide range of practical applications. They have various medicinal properties that lead to their use in the pharmaceutical sector (anti-angiogenic, anti-cancer, anti-diabetic, anti-inflammatory, anti-obesity, anti-oxidant properties, cardio-protective, and photo-protective effects) [132]. In cosmetic formulations, carotenoids are used as active ingredients with biological activity for their antioxidant properties and nutritional value to the skin and hair. The chemical structure of carotenoids is responsible for their antioxidant activities. Carotenoids contain long conjugated double bonds in a polyene chain that are responsible for antioxidant activities by quenching singlet oxygen and scavenging radicals to terminate the chain reaction. They are known to play important roles in scavenging reactive oxygen species (ROS) but there is little information regarding their roles in cellular defenses against reactive nitrogen species (RNS) [132]. Fucoxanthin has a strong radical-scavenging activity due to the presence of the unusual double allenic bonds in its structure. Another important carotenoid with strong antioxidant activity is astaxanthin, which acts as a scavenger of various reactive species and exhibits higher levels of antioxidant activity than other carotenoids such as β-carotene, zeaxanthin, and canthaxanthin. Its powerful antioxidant activities result from the unique molecular structure. Astaxanthin contains a conjugated polyene chain at the center and hydroxy and keto moieties on each ionone ring. Astaxanthin shows better biological activity than other antioxidants since it can link with the cell membrane from the inside to the outside. The polyene chain in astaxanthin traps radicals in the cell membrane, while the terminal ring of astaxanthin can scavenge radicals both at the surface and in the interior of the cell membrane [133]. The anti-oxidant activity is of great importance in cosmetic formulations, especially for skincare products. Ultraviolet (UV)-exposure could determine the

accumulation of high levels of ROS. Accumulation of ROS in cells causes cell death and excessive cell death can lead to wrinkling and dryness of the skin. ROS accumulation also plays an important role in photo-aging conditions such as cutaneous inflammation, melanoma, and skin cancer. The skin has naturally occurring antioxidant agents which can block the effects of ROS and suppress cell disruption and damage but these defenses may not provide adequate protection when levels of ROS are very high. Carotenoids can limit lipid peroxidation events by scavenging the ROS formed during photo-oxidative processes. Carotenoids protect humans from UV-light damage and are applied externally via the skin (as a topical treatment) as well as via dietary means. Moreover, they are also used in tan lotions due to their color [131].

Due to their color and nutritional properties, carotenoids are traditionally used in food and feed industries. They are vitamin precursors and their presence in a regular alimentation is fundamental since humans and animals are incapable of synthesizing them [130]. Carotenoids are considered safe natural dyes and are added to a variety of products to enhance their color. The animal feed sector was the highest consumer of carotenoids [132].

Nowadays, most of the carotenoids produced by the industry are chemically synthesized, although a small portion is obtained naturally from plants or algae. Their synthetic production is faster and cheaper since it requests low-cost labor and inexpensive chemicals, does not need living organisms, and there are no harvesting and extraction costs. Unfortunately, synthetic carotenoids are less effective in terms of their health-promoting properties to natural carotenoids and are hence less valuable and desirable as a product. Due to the adverse side effects commonly associated with drug therapy, public interest in recent times has focused on natural products with health-promoting properties as alternatives to conventional drugs. Consumers prefer naturally derived compounds in cosmetic formulations, so there is an increasing global demand for naturally derived carotenoids rather than those synthesized chemically [132].

The main carotenoids of microalgae are β-carotene, lycopene, astaxanthin (Figure 4), zeaxanthin, violaxanthin, and lutein [134], and the most common microalgae commercially interesting for pigment production are *Dunaliella salina*, *Haematococcus pluvialis*, *Chlorella* spp., *Scenedesmu*s spp., *Muriellopsis* spp., *Spirulina* spp., *Porphyridium* spp. [91–93,95,130].

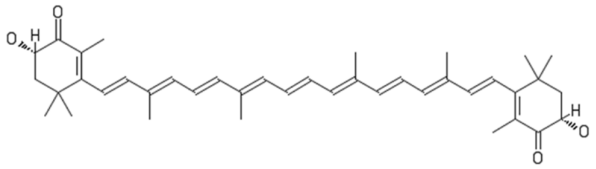


Figure 4. Chemical structure of astaxanthin.

5.7. Polyketides

Flavonoids are important secondary plant metabolites with a variable polyphenolic structure characterized by a heterocyclic oxygen linked to two aromatic rings, which vary in the level of hydrogenation and belonging to the polyketides lipid category [20]. They are widely present in fruits, vegetables, and foods and beverages of plant origin with functions related to various processes ranging from UV protection to signaling and pigmentation [135]. The beneficial health effects of these natural products are known for which they are now considered an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal, and cosmetic applications for their antioxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic properties together with their ability to modulate the key functions of cellular enzymes [135]. Although there are few studies, some scientists have found flavonoids also in microalgae [136–138].

5.8. Saccharolipids

Saccharolipids are another lipid class in the LIPID MAPS Structure Database. The term describes compounds in which fatty acyls are linked directly to a sugar backbone. This group is distinct from the term "glycolipid" that was defined by the International Union of Pure and Applied Chemists (IUPAC) as a lipid in which the fatty acyl portion of the molecule is present in a glycosidic linkage. It is necessary to specify that classification in LIPID MAPS Structure Database does not have a separated glycolipids category but instead places glycosylated lipids in appropriate categories based on their core lipids [20]. In literature, we found few studies that described their presence in microalgae [139,140].

6. Conclusions and Future Perspectives

Microalgae are an interesting source of bioactive compounds with potential industrial applications. Lipids are a wide category of useful substances in many sectors. They are used in the enhancement of the nutritional value of food and animal feed, pharmaceutical industry, biodiesel production, etc. Microalgae lipids are most exploited as feedstock for third-generation biodiesel production. Different from the first and second generations of biodiesel that are mainly produced from edible and non-edible vegetable oils, respectively, biodiesel of the third generation is obtained from microbial oils, such as microalgal oil, representing a powerful alternative to traditional feedstock [141]. The industrial interest for algal lipid is also for the capacity of microalgae to synthesize considerable quantities of PUFAs [100,142]. PUFAs are essential nutrients for humans that lack the requisite enzymes to synthesize some of them. Fish and fish oil are the common sources of long-chain PUFAs but microalgae could be an interesting alternative [143,144]. Unfortunately, the global supply from all the traditional sources of these nutrients is insufficient to satisfy human nutritional requirements. In addition, fish oil is not recommended for vegetarians or people allergic to fish, has an unpleasant odor for some people, and may contain fat-soluble environmental pollutants. For these reasons, microalgae could be an interesting alternative [17,143–145].

Microalgae used in cosmetics is an interesting strategy to increment searching for new natural ingredients from environmentally sustainable biomass. Whole cells can be used to provide a mixture of molecules with useful properties. On the other hand, extracted molecules could also be proposed for targeting specific effects, since whole microalgae biomass is difficult to incorporate in cosmetic formulations [48]. Lipid products are a useful perspective for cosmetic formulations. To date, different lipids extraction methods are exploited but the recovery of algal oils has usually been carried out at a laboratory scale. Thus, it is necessary to test new extraction and purification methods possibly oriented versus green technologies. The selection of an appropriate extraction procedure is a crucial factor. In fact, a biocompatible buffer which does not alter the bioactivity of the extracted molecules needs to be used. Nowadays, conventional extraction techniques involve the use of organic solvents for a long time and also the use of dry biomass as a starting material. New techniques are being developed which do not require the involvement of toxic solvents, reduce the extraction time, improve the extraction yields without affecting the biological activity, and minimize environmental impact [146].

As already mentioned, lipids are used in different industrial sectors and are mostly of animal, plant or biotechnological origin. Recently, there was a return to natural compounds making the industrial market environmentally sustainable. Plants can be exploited for lipids recovery but involve the use of a large number of environmental resources such as arable land and water. In this context, microalgae could show numerous advantages. Microalgae have a strong environmental adaptation ability so they do not compete with terrestrial crops for arable land, they require less water, exhibit a rapid biomass production when in favorable conditions and show high oil contents, higher than land-based oleaginous crops. About the many advantages associated with microbial lipids

production, the possibility to manipulate the genetic machinery of microorganisms to produce the desired lipid molecules with high economic value is most interesting. Alongside lipid production, microalgae could be exploited for the production of various bioactive molecules. The cosmetic sector is always in search of new ingredients for innovative product formulations. The present production of algae extracts in cosmetics is widely dominated by seaweeds and the use of microalgae was relatively secretive until the 2010s when the demand for beauty treatments started to grow significantly. The market for microalgae-based cosmetic products is expected to grow further. In fact, in the last years, there is great interest in cosmeceutic products defined as "cosmetic products with biologically active ingredients claiming medical or drug-like benefits" making thinner the edges between cosmetics and pharmaceutics. In addition to the properties of lipids already used in the cosmetic sector, such as moisturizing agents or surfactants, research has shown that there are numerous lipid categories with interesting biological activities, such as anti-inflammatory and anti-oxidant properties or anti-microbial activity becoming promising molecules to inactivate a wide spectrum of microorganisms [147]. Microalgae adapt well to the new requests of the market.

Despite intensive research efforts, the commercial use of microalgae biomass as a source of useful products is still not economically sustainable due to some problems. Significant obstacles that limit the production of microalgal lipids on a large scale are the restricted lipid synthesis in the microalgal cells and the low growth rate of these organisms, compared to other oleaginous microorganisms such as bacteria [148]. Another question is the monitoring of the different growth parameters of microalgae. Indeed, certain cultivation parameters, such as light intensity, temperature, starvation nutrients or other stress conditions, can affect the final biomass composition profile and specific lipids production. If closed cultivation systems allow monitoring cultivation conditions, the high costs associated make the open cultivation systems more competitive for industrial aims. Furthermore, the processing of microalgae biomass, such as extraction, isolation, and purification steps, is often complex and very expensive [149]. Although there are still numerous issues to be resolved in order to extend the production of lipids from microalgae on a large scale, microalgae are a promising sustainable resource for lipids and many other bioactive molecules production [16,59,64,65]. To make microalgae industrial utilization economically sustainable, alongside the genetic engineering of strains, lipids production from microalgae can be associated with other high-value metabolic products. It is possible to extract hydrophobic compounds and lipids at the same time and then purify them, while hydrophilic compounds such as proteins and sugars can be extracted from the defatted biomass. Furthermore, lipids can be co-products of environmental applications of microalgae, for example, for the treatment of wastewaters [85].

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