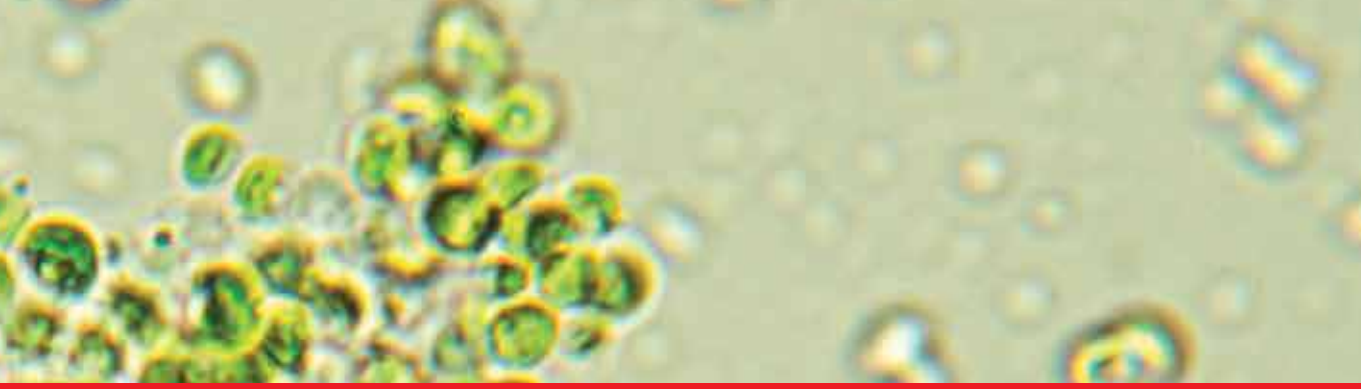

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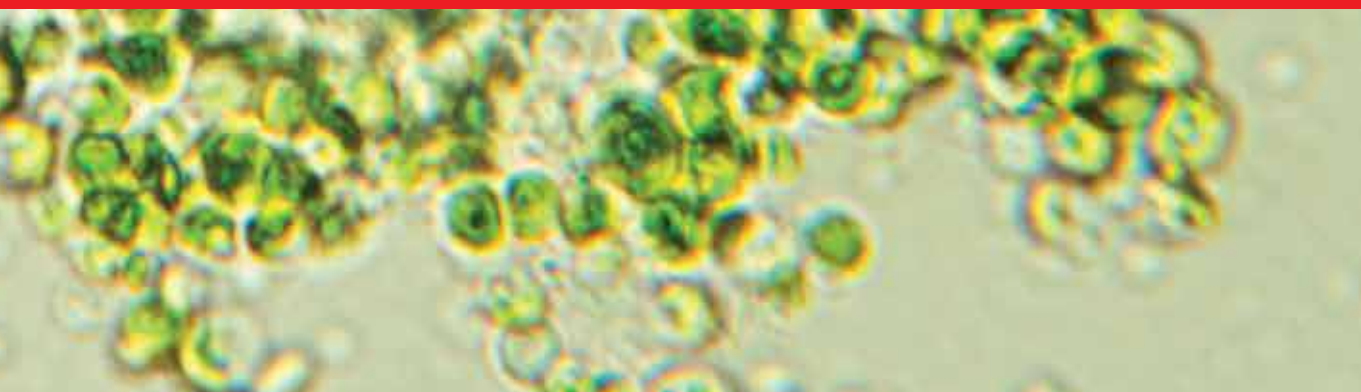
Algae

Organisms for Imminent Biotechnology

Edited by Nooruddin Thajuddin and Dharumadurai Dhanasekaran



ALGAE - ORGANISMS FOR IMMINENT BIOTECHNOLOGY

Edited by **Nooruddin Thajuddin** and **Dharumadurai Dhanasekaran**

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Meet the editors

Dr. Nooruddin Thajuddin is working as Professor and Head at Department of Microbiology, School of Life Sciences and as Dean at Faculty of Science, Engineering and Technology, Bharathidasan University, Tiruchirappalli, India. He has vast experience in microbial taxonomy, isolation, cultivation, harvesting, extraction of valuable products, and expertise in employing molecular tools in the identification and phylogeny of various microorganisms, bioremediation of effluents and bioenergy from Microalgae and Cyanobacteria. He had one-year post–doctoral training on molecular taxonomy and phylogeny of cyanobacteria at the Department of Biology, Rensselaer Polytechnic Institute, Troy, New York, USA, through Dept. of Biotechnology (Govt. of India) overseas fellowship. Recently Department of Biotechnology (Govt. of India) sanctioned a major grant to Prof. N. Thajuddin for the Establishment of National Repository for Freshwater Microalgae & Cyanobacteria. He is a life member of various academic bodies and member in editorial boards in national and international journals. He received Dr. G. S. Venkataraman Memorial Best Scientist Award of National Academy of Biological Sciences for the year 2014.

Dr. Dharumadurai Dhanasekaran is working as an Assistant Professor, Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli, India. He has experience in fields of actinobacteriology and mycology. His current research focus is on actinobacteria, microalgae, fungi and mushroom for animal and human health improvement. He has received the UGC-Raman Post doctoral Fellowship to visit USA, University of New Hampshire, Durham. He is a life member in Mycological Society of India, National Academy of Biological Sciences and member in editorial boards in National, International Journals, Doctoral committee member and Board of study member in Microbiology. As per the reports of Indian Journal of Experimental Biology, 51, 2013, Dr. Dharumadurai Dhanasekaran is rated in second position among the top five institutions in the field of Actinobacteria research in India.

Contents

Foreword

It is with immense pleasure and a sense of satisfaction; I agreed to write the foreword for this book titled "ALGAE: ORGANISMS FOR IMMINENT BIOTECHNOLOGY" edited by N. Thajuddin & D. Dhanasekaran. 'Pleasure', because the editors have themselves contributed substantially to the field of algae and particularly Prof. N. Thajuddin, has pioneered work in certain aspects of algae. 'Satisfaction', because the editors are bringing out this compendium, at the most opportune moment when algal biotechnology has to enter the logarithmic phase from its current lag phase. In my opinion, after the great Industrial, Agricultural and Information technology revolutions, it is now the turn of Biotechnology. The importance of algal biotechnology lies in its ability to minimize the use of dwindling arable and inhabitable land and precious drinking water resources as well as help in mitigating greenhouse effect. Effluents and saline water could be the medium of choice for several useful algae. There cannot be a greener technology than algal biotechnology. I am quite confident that within a decade or two, this technology is going to 'rule the roost'.

The book touches upon a number of important aspects connected to both micro and macro-algae, including their cultivation; carbon sequestration and use as biosensors. I hope workers in the field would be benefitted by the information provided.

I wish them well.

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Preface

Algae are one of the most important groups of photosynthetic organisms in the plant kingdom having both prokaryotic and eukaryotic features (Blue-green algae (=Cyanobacteria), Microalgae and Macroalgae) occur in all aquatic and terrestrial habitats, including polar and other extreme environment where moisture sun light is available. Cyanobacteria along with several other microalgae were treated with contempt as 'scum' since it was believed that these organisms were only of nuscence value-fouling water bodies and causing diseases and toxemia. Ecologists however started giving them respect as primary producers in all aquatic ecosystems, and realized that, without them no animal populations including fishers and prawns could exist. They were, till recently in the oblivion, uncared and unrecognized and have shot into fame and popularity owing to a host of their innate properties that make them ideal organisms for use in various ways to meet our needs and to promise us a bright future. In recent years, worldwide attention is drawn towards these organisms for their possible use in various areas such as food, feed, fuel, fertilizer, medicine, industry, scrubbing the excess atmospheric CO_2 and other gases causing the "green house effects" and combating pollution.

Although, the first use of microalgae by humans, dates back 2000 years to the Chinese, who were used Nostoc to survive during famine. However, algal biotechnology only really began to develop in the middle of the last century. Genetically engineered microalgae & cyanobacteria have been the trend setters for the studies of basic concepts and their applications in technology and industry. No other group of organisms has comparable achievements. Keeping these points in view, we initiated to bring out this volume titled "Algae - Organisms for Imminent Biotechnology" which will be useful source of information on basic and applied aspects of algae for post-graduate students, researchers, scientists, agriculturists, and decision makers.

The book comprises a total of 12 chapters covering various aspects of algae particularly on microalgal biotechnology, bloom dinamics, photobioreactor design and operation of microalgal mass cultivation, algae used as indicator of water quality, microalgal biosensors for ecological monitoring in aquatic environment, carbon capture and storage by microalgae to enhancing CO_2 removal, synthesis and biotechnological potentials of algal nanoparticles, biofilms, silica-based nanovectors, challenges and opportunities in marine algae, genetic identification and mass propagation of economically important seaweeds and seaweeds as source of new bioactive prototypes.

We are very much thankful to the multiple contributors around the world including Brazil, Chile, Finland, Mexico, Malaysia, Portugal, Russia, Republic of Mauritius, Italy, India, Indonesia and Turkey for their excellent articles. We offer our special thanks and appreciation to

Ms. Dajana Pemac, Publishing Process Manager for her encouragement and help in bringing out the book in the present form. We are also grateful to InTech Publishers for their concern, efforts and encouragement in the task of publishing this volume.

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Chapter 1

Microalgae and Cyanobacteria as Green Molecular Factories: Tools and Perspectives

Hector Osorio Urtubia, Lucy Belmar Betanzo and Mónica Vásquez

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/100261

Abstract

Cyanobacteria and eukaryotic microalgae are phototrophic microorganisms capable of producing organic compounds using solar energy. Owing to their fast growth, low cost cultivation, and the diversity of high-value chemical substances produced, they are considered an attractive target to be exploited by the biotechnology industry. While genetic modulation of these organisms has been extensively proved in the laboratory, present-day microalgal industry uses mainly non-transgenic strains. Although some unicellular cyanobacteria can be successfully engineered, many commercial bioproducts are synthesized preferably by eukaryotic microalgae or filamentous cyanobacteria to take advantage of their better-suited natural physiological characteristics. The successful genetic engineering of these microorganisms is not limited to the understanding of the gene expression machinery (e.g., promoters, codon usage, ribosome binding sites), but it must also include other subjects, such as defense mechanisms against the intrusion of foreign DNA. This chapter reviews current strategies in microalgae biotechnology and analyzes the most frequent problems we faced to genetically manipulate these microorganisms, including their transformation and selection methodologies. In summary, we attempt to provide a comprehensive review of the relevant information and tools required for optimal engineering of a photosynthetic microorganism employed in sustainable biotechnology applications.

Keywords: microalgae, cyanobacteria, transgenic strains, biotechnology, transformation

1. Introduction

Ancestors of present-day cyanobacteria developed the oxygenic photosynthesis as far back as 3.6 billion years ago [1], and the primary endosymbiotic event at the origin of all photosynthetic eukaryotes can be traced to 1.8 billion years ago [2]. It is estimated that algae are a group of about 72,500 species worldwide [3], with sizes varying from unicellular to giant multicellular organisms. On contrast, cyanobacteria is a monophyletic bacterial phylum composed by unicellular and multicellular (clusters, filaments, and ramified filaments) forms, including cellular differentiation in some filamentous representatives, and whose morphological complexities do not represent strictly an evolutionary history [4].

Eukaryotic microalgae and cyanobacteria are widely distributed, inhabiting aquatic (from marine to freshwater environments) and terrestrial ecosystems, and even extreme environments [5, 6]. They are responsible for ~50% of global primary production, fixing a substantial amount of carbon dioxide, especially in oligotrophic marine surfaces [5, 7]. In addition, diazotrophic cyanobacteria are major players in global nitrogen fixation [8], and many produce unique secondary metabolites, such as toxins [9].

These photosynthetic microorganisms have been cultivated for a long time as a food source and complementary diet for humans and also for the fish, shrimp, and poultry industries. Microalgae and cyanobacteria naturally produce diverse compounds of commercial interest such as proteins, carbohydrates, lipids, pigments, and alcohols; and some of their subproducts are currently commercialized by the pharmaceutical and cosmetic industries [10, 11]. Current industrial processes based on microalgal biotechnology mainly use non-transgenic strains. However, recent research has improved the genetic modulation in microalgal and cyanobacterial strains, despite the popular skepticism and mistrust surrounding the generation of transgenic strains, in particular, the potential generation of antibiotic resistant microorganisms. These advances have paved the way to obtain phenotypes that can considerably improve the production of high-valued compounds. The genetic transformation systems have been better developed in unicellular cyanobacteria because their amenability to genetic modulation. Nevertheless, progresses in the eukaryotic microalgae modification for the synthesis of several products have been achieved too, pushed by the idea of taking advantage of their natural phenotypic characteristics [12].

This chapter reviews current biotechnology strategies in microalgae and highlights the most frequent constraints for the genetic manipulation of these photosynthetic microorganisms. The emphasis of this analysis is focused on successful experiences that overcame previously described problems.

2. Microalgal and cyanobacterial biotechnology: from natural to transgenic strains

Considering the enormous diversity of microalgal and cyanobacterial species, they represent a unique reservoir of potentially novel commercial compounds. They have been used for the

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production of biomass and extraction of commercially attractive compounds for industries relative to human nutrition and health (e.g., nutraceuticals, sunscreens, pharmacological bioactives), agro- and aqua-culture (e.g., bio-fertilizers, food supplements), and renewable fuels (e.g., fatty acid methyl esters, alcohols, and hydrogen) [10, 11]. Eukaryotic microalgae are interesting due to their cellular storage of compounds (e.g., lipids, starch, and proteins), while cyanobacteria are relevant for the production of unique molecules (e.g., toxins) or molecules that can be secreted (e.g., ethanol, butanol, exopolysaccharides).

Unicellular cyanobacteria rarely produce large quantities of commercially attractive bioproducts. Thus, the genetic manipulation of them is normally oriented to increase the production of biofuels and other chemicals (e.g., [13, 14]). On the other hand, eukaryotic microalgae and filamentous cyanobacteria are more complicated for genetic modification. In the former, the foreign DNA needs high energy to trespass cell walls and membrane compartments; whereas the segregation of mutants is more difficult in multicellular organisms, such as filamentous cyanobacteria. Nonetheless, these organisms are good biotechnology prospects because they naturally accumulate commercially interesting compounds.

2.1. Biofuels

Over past decades, the energy industry has faced the challenge of providing new energy sources, and as new climate policies demand, it has to fulfill the requirement of finding ecologically friendly alternatives [15]. In this context, biofuel production from microalgae biomass is an alternative that captures and sequesters atmospheric carbon dioxide, the main greenhouse gas responsible for the current global warming [16].

In general, biofuels are classified into four main types: biodiesel, bioethanol, biogas, and biohydrogen. Below, we discuss examples of wild-type or genetically modified cyanobacterial and microalgal strains that produce biofuel [17, 18].

2.1.1. Biodiesel

Relative to their size, microalgae can store high amounts of lipids, especially intracellular oils, that can yield biodiesel through chemical (transesterification) and physical (distillation and cracking) conversions [16]. As the lipid composition differs among microalgae species, not all of them are useful for the production of biofuels. Some species of the *Nannochloropsis* genus are one of the better-suited microalgae for biofuel production. They are able to accumulate considerable amounts of intracellular lipids into vesicles (up to 70% of the cell dry weight), especially under stress conditions, and mainly in the form of triglycerides [19, 20]. Other examples of lipid-producing microalgae are *Chlamydomonas reinhardtii*, *Dunaliella salina, Botryococcus braunii*, and various *Chlorella* species [21, 22].

Genetic strategies used to generate strains of microalgae with greater ability to produce lipids include both knockout and overexpression of genes involved in lipid synthesis. These approaches have had diverse results. The overexpression of genes that belong to lipid synthesis pathway (ACCase, KASIII) increased the enzymatic activity, but did not increase the amount

of cellular lipids in different organisms [23]. Other approaches consistent on blocking metabolic pathways that promotes lipid synthesis. In this context, it has been reported that mutant strains of *C. reinhardtii*, where the ADP-glucose pyrophosphorylase or isoamylase genes (starch synthesis) had been disrupted, were able to accumulate higher amounts of lipids [24].

On the other hand, cyanobacteria accumulate lipids in thylakoid membranes at fast growth rate when high levels of photosynthesis are detected, reaching ~40% of dry biomass in unicellular cyanobacteria [25]. Genetic modifications of model cyanobacteria to overproduce, and even excrete, fatty acids have also been demonstrated [26–28]. However, redirecting the carbon metabolisms has detrimental effects, which limit the expected yield in some engineered strains [29]. Modifications of expression of genes related to mitigate the stress in *Synechococcus elongatus* PCC 7942 (e.g., overexpression of ROS-degrading proteins) resulted in the recovery of the fitness [27]. Otherwise, the selection of more resistant strain can be crucial for successful genetic modifications. For example, an engineered strain of *Synechococcus* sp. PCC 7002 yielded high levels of fatty acids without the detrimental effects observed in *S. elongatus* PCC 7942, when subjected to similar genetic modifications [28].

Furthermore, several prokaryotic and eukaryotic organisms naturally produce hydrocarbons (e.g., alkanes or alkenes), a direct fuel compatible with the existing fossil fuel infrastructure [30]. Cyanobacteria produce mostly C17 and/or C15 long-chain alkanes, using fatty acid precursors via two different pathways. One pathway involves two step enzymatic reactions driven by an acyl–acyl carrier protein reductase (AAR) and an aldehyde-deformylating oxygenase (ADO) [31]. The other known pathway produces alkenes through a multidomain protein homologous to type I polyketide synthases (PKS), which convert fatty acyl-ACP to α-olefin via elongation–decarboxylation mechanisms [32]. Molecular engineering attempts to enhance the production of alka(e)nes in unicellular cyanobacteria (e.g., *Synechocystis* sp. PCC6803 [33]), and in filamentous nitrogen-fixing cyanobacterium (*Anabaena* sp. PCC7120 [34]) of these two pathways generated low production of alka(e)nes.

2.1.2. Bioethanol

Like aliphatic hydrocarbon, alcohol-based biofuels can be used directly to power diesel engines. Ethanol is the most common alcohol-based biofuel synthesized via fermentation driven by heterotrophic microorganisms. Microalgae and macroalgae are good sources of carbohydrates (in the form of glucose, starch, and other polysaccharides) and proteins, which are used as raw material to produce bioethanol through various processes of hydrolysis and fermentation by bacteria, yeast, or fungi [35]. Microalgae such as *Chlorella vulgaris, Chlorococcum* sp., and *Chlorococcum littorale* and the cyanobacterium *Arthrospira platensis* (also called *Spirulina*) are frequently used in these processes (e.g., [36]). Fermentation-related genes, genes coding pyruvate decarboxylase (*pdc* gene) and the alcohol dehydrogenase (*adh* gene) from the ethanogenic bacterium *Zymomonas mobilis* have been expressed in *Synechococcus* and *Synechocystis* using native and strong promoters (P*rbcLS* and P*psbAII*) [37, 38]. However, ethanol production was still low compared to the amounts produced by microbial fermentation, probably due to the low tolerance of cyanobacterial systems to ethanol stress [38–40].

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2.1.3. Biogas

An alternative to liquid biofuels is the use of gas, which has some advantages over the use of liquid biofuels such as improved energy conversion efficiency of substrates and lower emission of toxic gases to the environment [41]. Biogas can be produced from different biomass feedstocks, such as dedicated energy crops, algal biomass, food wastes, animal manure, agricultural residues, industrial wastes [42]. Through the use of microalgae, it is possible to generate different types of biogas either by anaerobic fermentation of biomass (methane) or through the reduction of protons due to their hydrogenase activity (hydrogen gas, H_2) [43].

Methane can be produced through anaerobic digestion of microalgae biomass by bacteria and archaea, in a process that involves various reactions (hydrolysis, acidogenic, acetogenesis and methanogenesis). However, the productivity levels correlate directly with the degree of cell wall disruption and with the solubilization levels of the organic cell compounds. Different physical, chemical, and biological pretreatments have been tested for yield optimization [44–46]. Some examples of microalgae in which anaerobic enzymatic digestion have been described are *Scenedesmus* sp. [47], *Rhizoclonium* sp. [48], *C. vulgaris* [49], *S. obliquus*, and *A. maxima* [50].

Biohydrogen is an attractive fuel alternative because its combustion produces no carbon byproducts and it is superior for electricity production by fuel cells. While algae are capable of forming biomass through photosynthesis, oxygen hinders this process by inhibiting the enzyme hydrogenase. It is for this reason that, under anaerobic and dark conditions, microalgae from the genera *Chlamydomonas, Scenedesmus, Lobochlamys*, and *Chlorella* have the ability to produce H_2 by the action of hydrogenase or other enzymes able to metabolize H_2 [51]. The genetic engineering of microalgae to modify the hydrogenase has failed to significantly increase the levels of H_2 produced [52]. Moreover, overexpression of this enzyme in *Chlorella* has only achieved modest increases in H_2 production [53]. Other more successful strategies have been based on the inhibition of photosystem II (PSII) in order to inhibit the production of oxygen [54, 55].

2.2. Microalgal and cyanobacterial biomass and valued-added nutrients

Biomass of microalgae and cyanobacteria has also been harvested to be used as human nutrient supplements, animal feed, and fertilizers for many centuries [10].

Edible microalgae and cyanobacteria include the chlorophyceae *Chlorella* sp. and *Scenedesmus obliquus* and cyanobacteria from the genera *Arthrospira, Nostoc, Spirulina*, and *Aphanizomenon*. Microalgae and cyanobacteria are a good source of protein (including essential amino acids), essentials polyunsaturated fatty acids (PUFA) like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), carbohydrates, and other beneficial compounds for human and animal health, such as vitamins, astaxanthin, lutein, beta-carotene, chlorophyll, phycobiliprotein, and beta-1,3-glucan [56, 57].

Lipid metabolism has been mainly studied in the green microalga *C. reinhardtii* through genomic data and genetic tools. A general overview of these discoveries can be found in several scientific reports [58, 59]. However, the molecular mechanisms involved in regulatory pathways in algae are still poorly understood.

Algal pigments, such as carotenoids, are widely exploited by the food industry for the pigmentation of salmon, chicken, trout, and shrimps; by the nutraceutical industry for their antioxidant and other health benefits; by the cosmetics business for their skincare and anti-aging properties. Algal pigments are also used for the fortification of foods and beverages. The most popular pigments are chlorophyll, beta-carotene, alpha-carotene, lutein, lycopene, zeaxanthin, and astaxanthin. Astaxanthin has surged in the nutraceutical market because its unusual antioxidant capacity and several health benefits [60]. Even though the main supply of astaxanthin used for the pigmentation of salmon is now 95% of synthetic origin, there has been a growing trend toward using natural ingredients. Thus, the demand for natural astaxanthin from the green microalgae *Haematococcus pluvialis* has grown considerably [61].

3. Gene expression and its constraints for microalgae and cyanobacteria molecular cell physiology

The basic genetic construct used to express a specific gene must consider the proper recognition of the DNA structures related to transcription and translation by the host cell, that is, promoters, ribosome binding sites, the codon usage to translate the target gene, and transcription terminators. Therefore, there are many constraints that need to be considered during the planning of genetic modifications.

3.1. Transcriptional control

3.1.1. Promoters

The selection of the right promoter will depend on diverse variables such as the organism to be modified (cyanobacteria, microalgae), the gene expression levels required (constitutive, induced expression, strong expression), or the target DNA that can belong to different cellular compartments in eukaryotes.

In eukaryotes, native promoters have been extensively described for nuclear and organelle transformation. Their main advantage is the correct recognition by the enzymatic transcriptional machinery of the microalgae. Some examples of these promoters are as follows: (i) for *C. reinhardtii*, the 5′untranslated region (5′-UTR) of the RuBisCO small subunit gene *rbc*S2 and heat shock protein 70A gene *hsp*70A [62, 63]; and (ii) for *Nannochloropsis*, the 5′-UTR of violaxanthin/chlorophyll *a*-binding protein (VCP) genes and the ß-tubulin promoter [64–66]. Heterologous promoters have also been used in different types of microalgae such as viral promoters 35S, CaMV35S, SV40, and CMV. One of the advantages of these promoters is that they can be recognized in some microalgal systems such as *C. reinhardtii* [67], *D. salina* [68], *D. bardawil* [69], *C. vulgaris* [70], *Platymonas subcodiformis* [71], *Nannochloropsis* sp. [70], *H. pluvialis* [72], and *Phaeodactylum tricornutum* [73].

For cyanobacteria, there is a set of constitutive and inducible native promoters used in biotechnological studies to express heterologous genes or increase the expression of native

ones. Some examples of these promoters are as follows: (i) the copper-inducible promoter P*petE* [14], (ii) the nitrate-inducible promoter P*nirA* [74], and (iii) the constitutive promoters P*psbA2* and P*rcbL* [13]. Genetic transformations for biotechnological purpose considering inducible promoters have gained a remarkable interest due to the advantage to obtain high yield of biomass before interfere the normal cell function and growth with the expression of the gene(s) of interest. However, efficient controllable promoters in cyanobacteria are scarce [75]. Additionally, the effectiveness of heterologous promoters in cyanobacteria is very low and successful examples using lac-regulated and *tetR*-regulated promoters need to include critical modifications to fit the sequences to the transcriptional machinery of cyanobacteria [76].

3.2. Translational control

3.2.1. Codon usage

Different organisms usually bear particular codon usage patterns. Therefore, when a gene from one species is cloned and expressed in another, some codons might be rare in the new host, leading to poor translation efficiency or starvation for certain amino acids and consequently lose the cell's fitness [77]. Specific variations in codon usage are often cited as one of the major factors impacting protein expression levels. The presence of rare codons that correlate with low levels of their endogenous tRNA species in the host cell can decrease the translation rate of target mRNAs.

Genes in cyanobacteria show a bias in the use of synonymous codons [78–81]. The importance of codon optimization in algal genetic applications also been seen in microalgae such as *C. reinhardtii* [77], *Gonium pectorale* [82], and *Porphyra yezoensis* [83]. In *C. reinhardtii*, codon usage was reported to affect the expression of some foreign genes such as GFP [77]. Sequencing studies and expression profiles of microalgae have clarified these issues, thereby allowing optimization of codon usage at the level of expression in the nucleus [82] and in the chloroplasts [84–87].

3.3. Barriers for transfer foreign DNA into microalgae and cyanobacteria

There are several obstacles to be faced when trying to insert exogenous DNA into eukaryotic and cyanobacterial systems. The first is physical barriers such as cell wall, plasma membrane, nuclear membrane, mitochondrial membrane, and chloroplast membrane.

To overcome these "natural barriers" diverse experimental strategies have been designed, such as the generation of spheroplasts (cells without cell wall) for eukaryotic microalgae by mechanical disruption or enzymatic treatments [88, 89]. However, problems of protoplast viability and low post-transformation recovery have been described in some systems. Other strategies include the application of multipulse or high electrical voltage for the disruption of the algae extremely rigid cell walls (e.g., *Nannochloropsis* sp.) and the entry of exogenous DNA [64–66, 90]. The low number of transformed cells recovered is one of the problems with this type of strategy. This is probably due to the high stress conditions to which the cells are subjected and the difficulty of foreign DNA integration into the genome found in many

eukaryotic systems. Another obstacle is the transient insertion of the exogenous DNA and the eventual loss of the phenotype of interest over time. This is due in part to the lack of understanding of the DNA insertion mechanisms into the cell compartments of some microalgae.

Cyanobacteria have a special envelope formed by the outer membrane, a thick peptidoglycan layer, the s-layer, and frequently an exopolysaccharide EPS envelope [91]. The EPS can represent a high proportion of the total cell dry biomass of environmental or extremophile cyanobacteria (e.g., *Nostoc calcicola*, [92]) and can be a barrier for DNA uptake in cyanobacteria. Several protocols for reducing EPS prior to transformation have been implemented for those cyanobacteria such as: (i) growth conditions under constant agitation, (ii) culture media supplemented with low concentrations of saline sodium citrate (SSC) [93], (iii) pumping the cells out of the sheath using a syringe [94], and (iv) treatment by agitation with concentrated NaCl and several washing steps [95].

In addition, cyanobacteria have mechanisms of defense against exogenous DNA that hinders the transformation process. The majority of filamentous cyanobacteria and some unicellular representatives contain restriction enzymes that can degrade the transferred DNA [96]. Strategies to improve the DNA transfer efficiency include the following: recipient strains with inactivated endonucleases [97]; deletion of the restriction sites from the foreign DNA [98]; or more often, the protection of the foreign DNA by methylation, using specific methylases encoded in helper plasmids, prior to transformation of the cyanobacteria [99–101].

4. Planning and strategies for genetic modifications in microalgae

4.1. Transformation of eukaryotic microalgae

Transformation in eukaryotic systems has several advantages when compared to cyanobacteria. For example, the functional recombinant protein needs, in many cases, the post-translational modifications only developed by the eukaryotic machinery, for example, protein folding assisted by chaperones in chloroplasts or specific protein glycosylation in the endoplasmic reticulum and Golgi apparatus (See [102], and references therein). Furthermore, the organellar machinery (i.e., mitochondria and chloroplast) gene expression resembles that of prokaryotic cells. Thus, genetically modified eukaryotes can produce a versatile range of proteins, including toxic proteins with prospects in cancer therapies [103].

Among examples of successfully transformed microalgae are *C. reinhardtii* [104], *Cyanidioschyzon merolae* [105], *Ostreococcus tauri* [106], and *Nannochloropsis* sp. [64, 90].

The integration of genes for nuclear transformation of microalgae occurs at random locations, with homologous recombination occurring at very low frequency [107]. On the contrary, plastidial transformations are based on homologous recombination similar to what is described for cyanobacteria.

Different strategies for inserting foreign DNA into microalgae have been described (biolistics, electroporation, natural transformation, random mutation) [102, 108]. However, each micro-

algal species has its own morphological, structural, and physiological characteristics, making the development of standard protocols for all species difficult.

4.1.1. Biolistic transformation

Biolistics is one of the most effective methods of transformation which has been mainly used for the insertion of genes into plastids such as chloroplast. This method makes use of DNA-coated heavy metal (mostly gold) microprojectiles and allows transformation of almost any type of cell. Its advantages are its versatility. Different types of DNA constructs can be used on metal particles, and they can transform various types of organelles (nucleus, mitochondria, and chloroplast). Also, high amounts of recombinant proteins can be accumulated into plastids such as chloroplasts, which have high stability due to biochemical differences with the cytoplasm. Among the difficulties is the high cost of the equipment and the inability to express proteins with complex translational modifications. This method has been applied so far in the transformation of many algal species such as the diatoms *Cyclotella cryptica* and *Navicula saprophita* [109], Chlorophyta such as *C. reinhardtii* [110], *Volvox carteri* [111], *Chlorella sorokiniana* [112], *Chlorella ellipsoidea* [113], *Chlorella kessleri* [114], *H. pluvialis* [72], *P. tricornutum* [115], and *G. pectorale* [82].

4.1.2. Electroporation

This technique has been used for many years for the transformation of numerous prokaryotic and eukaryotic cells. The great advantages of this technique are its versatility, simplicity, and high efficiency.

Electroporation conditions are dependent on the type of microalgae and structural characteristics of each one. For example, *Nannochloropsis* microalgae species that have a rigid cell wall need the application of high electrical voltage in order to allow the entry of foreign DNA into the cell nucleus. However, these very demanding conditions have reported low rates of recovery of transformed clones.

This technique has been successfully used in some microalgae such as *C. reinhardtii* [116], *C. merolae* [105], *D. salina* [117], *C. vulgaris* [118], and *Nannochloropsis* sp. [64–66, 90, 119].

4.1.3. Random mutagenesis

Mutation followed by selection of favorable phenotypes has been used for crop plants, and some promising strategies are now beginning to emerge for microalgae. Random mutation strategies applied to microalgae include the use of mutagenic chemicals and radiation. Among the disadvantages of these techniques is the difficulty in identifying the location in the genome of these mutations in the genome and the difficulty of controlling the number of mutational events per experiment. One way to solve these problems is to randomly mutagenize the genome of microalgae using genetic elements such as transposons of random insertion. This

methodology allows for easy recognition of the DNA incorporated using amplification molecular tools [120].

Microalgae that have been transformed with these methods include *Isochrysis affinis galbana* [121], *Nannochloropsis* sp. [122], *C. reinhardtii* [120], *Pavlova lutheri* [123], *Scenedesmus dimorphus* [124], *C. sorokiniana* and *S. obliquus* [125], and *Parietochloris incisa* [126].

4.2. Transformation of cyanobacteria

Unicellular and multicellular cyanobacteria have a varying number of genome copies per cell (variable polyploidy), even between members of the same strain under different physiological conditions through their life cycle [127]. This characteristic complicates considerably the full segregation of genome-targeted mutations. However, the scientific literature shows many examples of genetically modified cyanobacteria, especially unicellular cyanobacteria, mainly through natural transformation, conjugation, and electroporation. DNA bombardment (biolistics) is less frequent, but it has been used [95, 128].

4.2.1. Natural transformation

Some cyanobacteria are naturally competent, being able to be transformed by natural incorporation of foreign DNA from the environment. This ability is a simple and rapid way to introduce exogenous DNA into cyanobacteria and has been commonly used to transform cyanobacterial strains through gene replacement by double crossover homologous recombination, which is attained by the arrangement of two homologous sequences flanking the exogenous DNA of interest. Example of natural transformable model strains are as follows: *S. elongatus* PCC 7942 [129, 130], *Synechococcus* sp. PCC 7002 (or *Agmenellum quadruplicatum*) [131, 132], *Synechocystis* sp. PCC 6803 [133–135], and *Thermosynechococcus elongatus* BP-1 [97, 136, 137].

The exogenous DNA can be a linear or be carried on a plasmid prepared using standard procedures. The cyanobacterial cells with this ability are easily transformed during the logarithmic growth phase; while on stationary growth phase, they show reduced transformation efficiencies [131, 135, 137, 138].

As natural transformation triggers DNA fragmentation [137, 139, 140], this procedure is not recommended for single recombination or intact replicating plasmids. For these, conjugation or electroporation is preferred.

4.2.2. Electroporation

Electroporation is commonly used for transferring plasmids into diverse cyanobacterial cells (see further in [141]). Electroporation promotes single rather than double recombination events. It is worth noting that mutagenic events and events of recombination in nonspecific loci are frequent when electroporation is used to transform cyanobacteria [137, 140].

4.2.3. Conjugation

The most common technique for transforming cyanobacteria has been conjugation. Conjugation is the transfer of plasmid DNA from a donor (commonly *E. coli*) to a recipient cell through direct contact. This technique has been widely used in the filamentous-type strains *Nostoc* PCC 7120 [98], *N. punctiforme* ATCC 29133 [142–144], and *Anabaena variabilis* ATCC 29413 [145], as well as for the unicellular strains *S. elongatus* PCC7942, *Synechococcus* sp. PCC6301 [140], and *Synechocystis* PCC 6803 [146, 147].

Because several cyanobacteria, especially filamentous cyanobacteria, contain endogenous restriction endonucleases, the design of the plasmids must avoid predicted target sites of native restriction enzymes [98], or must be previously methylated to prevent the action of them [99, 100]. The most frequent conjugation procedure typically includes two different *E. coli* strains (e.g., [95, 148]). One carries the conjugal plasmid, and the other the helper plasmid(s) and the cargo plasmid to be transferred to the cyanobacterium. Cargo plasmid contains the DNA information to be transfer into the cyanobacteria and can be either a replicating plasmid or an integrative plasmid. It must also carry an "origin-of-transfer" (oriT) from a fragment of RP4 or relative plasmids, and a *mob* gene encoding a nickase, which recognizes the oriT and provides a single-stranded DNA for transfer [98, 146]. Alternatively, the *mob* gene may also be provided in *trans* in the helper plasmid, which also carries genes encoding methylases to protect the DNA of interest from the mentioned degradation by restriction enzymes.

4.2.4. Plasmids vectors

Plasmids vectors are one of the most common tools for transferring foreign DNA containing the information for the genetic manipulation of cyanobacteria. They can be classified based on the type of transformation into (i) integrative vectors, which modify the genomic information of the target organism by recombination or transposition (*cis*-transgene expression); and (ii) replicative or shuttle vectors, which are plasmids that can replicate themselves with the aim of expressing genes without any alteration to the host's genome (*trans*-transgene expression).

cis-transgene expression, achieved through an integrative vector by double homologous recombination, is the most frequent approach in cyanobacterial research, either for biotechnology or basic science studies. Plasmids introduced by conjugation are circular and a single recombination allows integration of the selective marker. Alternatively, a strategy to select less frequent double recombination events has been implemented based on suicide plasmids [149]. For gene knockout and knockin, researchers prefer these suicide plasmids to ensure the integration of the exogenous DNA and avoid the gene expression from the plasmid (e.g., [150]). The suicide plasmid contains an antibiotic resistance cassette and a *sacB* gene, which encodes levansucrase, an enzyme that converts sucrose to levans (a toxic fructose polymer). Only the antibiotic resistance gene is planned into be integrate in the chromosome by the double homologous recombination, while the *sacB* gene will remain in the plasmid. Double recombinant mutants are selected first using the antibiotic resistance, and then, the survival mutants are placed onto media with 5% sucrose to kill the cells containing the suicide plasmid or the single recombination. The *sacB*-antibiotic resistance cassette is also used to select "markerless"

chromosomal mutations [38]. In this case, the complete *sacB*-antibiotic resistance cassette fragment is integrated into the chromosome and mutants are selected for resistance to the antibiotic. A second transformation of the survival mutants is made with a fragment designed to replace the *sacB*-antibiotic resistance sequence. The survival mutants growing on culture media with sucrose are the ones containing the double transformation, although PCR corroboration is needed.

Gene expression can be enhanced through shuttle plasmids. The recognition of the replication origin by the host cell is essential for shuttle vectors, and in cyanobacteria, there are few studies using this type of plasmids, mainly based on the self-replicating base RSF1010 plasmid [146, 151, 152]. Recently, Taton and colleagues [152] offers an online bioinformatic tool to assembly cyanobacterial plasmids, including self-replicating ones.

4.3. New transformation strategies for microalgae and cyanobacteria

The insertion of exogenous genetic material using organisms such as *Agrobacterium tumefaciens* has also been reported in some microalgae. This strategy allows efficient insertion of genetic material into the nuclear DNA and expression of the respective reporter genes. Among the target organisms, we found species of *Chlamydomonas* [67], *Dunaliella* [69], *Chlorella* [153], *Schizochytrium* [154], *H. pluvialis* [72], and *Nannochloropsis* sp. [70]. Examples of natural transformation have been recently described for diatoms *P. tricornutum* and *Thalassiosira pseudonana*, in which, episomal vectors were inserted by conjugation with *E. coli* [155]. The ability to use such strategies in other microalgae is something that should be evaluated on a case-by-case basis.

The CRISPR/Cas system, a heritable adaptive immunity system [156–158], has been adapted for targeted gene editing in mammalian, plant, fungal, and bacterial hosts [159]. In addition, silencing of multiple genes through a CRISPR-interference (CRISPRi) platform that contains a nuclease-deficient Cas9 has also been reported [160, 161]. The use of the CRISPRi system for gene silencing in *Synechocystis* sp. PCC 6803 was described recently [162]. This technique was able to repress the expression (or at least lower the expression) of several genes in this model cyanobacterium, including genes related to the formation of carbon storage compounds during nitrogen starvation (glycogen and polyhydroxybutyrate) and genes probably involved in long chain alkane production (aldehyde reductases and dehydrogenases) [162]. However, additional studies are needed to evaluate the functionality of these promising systems as tools for genetic engineering in others cyanobacteria and eukaryotic microalgae.

4.4. Selection and reporter markers genes

The use of selectable marker genes is normally required in all experiments that aim to generate stable transgenic algae because only a very low percentage of treated organisms are successfully transformed. Selectable markers are often antibiotic resistance genes, which are dominant markers as they confer a new trait to any transformed target strain of a certain species, independent of the respective genotype.

Numerous genes that confer resistance to various antibiotics and herbicides have been reported in microalgae and cyanobacteria (antibiotics such as zeocin, hygromycin, bleomycin, chloramphenicol, kanamycin, phleomycin and the herbicides sulfounylurea, norflurazon) [163] allowing easy selection of transformed transgenic organisms (dominant selection markers). Among the known disadvantages of such markers is that their sensitivity is specific to each microalgae.

Another class of transformation markers are reporter genes that allow selecting transformant strains based on a particular phenotypic characteristic conferred by this gene. Among these are luminescent, fluorescent, and chromogenic proteins (such as sfCherry, mCherry, GFP, GUS, SHCP, and luciferase) are currently been used to evaluate gene expression levels in cyanobacteria and eukaryotic microalgae [65, 66, 84, 119, 164, 165]. The combination of these gene reporters with optical techniques such as flow cytometry coupled to cell sorting could make this process ecologically friendly and efficient for the selection of promising phenotypes (e.g., higher gene expression of commercially interesting compounds). The use of reporter genes that provide detectability through optical methods has been recently implemented for the selection of successful transformants, especially in eukaryotic microalgae [119, 166].

5. Conclusions and future outlook

Microalgae and cyanobacteria are true sources of different types of natural compounds with applications as diverse as: human food, animal feed, aquaculture, chemicals and pharmaceuticals, pigments, diatomites, fertilizers, wastewater treatment, cosmetics and fuels. Hence, better understanding of their physiological, morphological, biochemical, and molecular characteristics could be profitably applied in biotechnology.

Nowadays, there are a limited but increasing number of transformed microalgae. The technological advances and increasing collection of genomes sequenced, together with the identification and characterization of new molecular elements within the cell (e.g., promoters, codon usage, terminators, plasmids, selection markers, and reporter genes) may reduce unplanned gene expression deficits and achieve a well-developed, photosynthetic cellular platform for the implementation of useful and sustainable applications. Furthermore, the discovery of these new elements allows the development of new transformation strategies and the re-evaluation of genetic elements to study the functionality of key genes.

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Chapter 2

Bloom Dynamics of *Emiliania Huxleyi* (Lohmann) Hay & Mohler, 1967 in the Sea of Marmara: A Review

Muhammet Turkoglu

Additional information is available at the end of the chapter

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Abstract

Coccolithophores have had an effect on global climate change for a few million years. The marine phytoplankton group is responsible for approximately 20% of the total carbon fixation in marine systems. They can form blooms spreading hundreds of thousands of square kilometers, are recognized by elegant coccolith structures formed from calcium carbonate exoskeletons, and are visible from space. Although carbon is transferred to the sediments in organic matter and calcite form by coccolithophores, carbon dioxide (CO_2) is released by the calcification process. Therefore, they have a complex effect on the carbon cycle due to their activity regarding CO_2 production and consumption. This review represents the first attempt to present temporal and vertical distributions of *Emiliania huxleyi* (Lohmann) Hay & Mohler, 1967 (*Ehux*), which is one of the most important species of the coccolithophores in the bloom periods and the interaction of this species with other phytoplankton groups in the Sea of Marmara. This study may also indicate the advance of this species from the Black Sea region through the Sea of Marmara and the Dardanelles under favorable conditions.

Keywords: Phytoplankton, coccolithophore, *Emiliania huxleyi*, bloom dynamics, Sea of Marmara

1. Introduction

The Sea of Marmara is a very important water passage between the Aegean Sea and the Black Sea via the Dardanelles and the Bosphorus. It has two current systems that run in opposite directions. Deep water high in density (38.0 ppt) coming from the Aegean Sea flows into the Black Sea. Surface water low in density (18.0 ppt) coming from the Black Sea flows into the Aegean Sea. Therefore, Marmara is affected by both neighbor systems [1–3].

Coccolithophores are marine haptophyte phytoplankton. They are one of the most important groups of phytoplankton in today's oceans and contain about 300 species. *Emiliania huxleyi* (*Ehux*) is one of the most abundant coccolithophores found globally in all oceans, except polar ones. Although some coccolithophores produce toxins, *Ehux* does not produce any. However, *Ehux* has received considerable attention because of its capacity to produce massive blooms under favorable conditions [4–8]. This species also blooms to excess in the Sea of Marmara as a result of the system's unique character not only in summer periods but also in winter periods due to climate change and eutrophication [1, 9–11].

The coccolithophore *Ehux* has captured the attention of various scientists—especially those working in marine biology, geology, biogeography, and paleoclimatology. As a result of its coccolith structure—its remains can be traced back millions of years—this species carries important hints on climate change and oceanic conditions during geological time periods [12]. It is known that massive blooms of *Ehux* provide a chemical balance between the hydrosphere, geosphere, and atmosphere in the context of the carbon cycle. The blooms are known to exceed tens of thousands of square kilometers in area and can be detected by remote sensing due to its reflective calcite form. In addition to its important role in the global carbon cycle, *Ehux* also plays an important role in the global sulfur cycle [12, 13].

The main phytoplankton groups in the Sea of Marmara are dinoflagellates, diatoms, coccolithophores, and cyanobacteria. Although there are many different phytoplankton species—more than 150 in the Sea of Marmara—the blooms tend to be extremely rich in a single, or only a few, predominant species such as *Ehux* and *Noctiluca scintillans* [9–11, 14]. The Sea of Marmara has a three- or four-phase phytoplankton bloom sequence which changes year to year. Diatoms tend to predominate in March–April, dinoflagellates in April–May, the dramatically colorful blooms of *Ehux* predominate in May–June, and diatoms in July–August [1, 9–11, 14–16].

This study looks at *Ehux* from the unique character of the system in that there are blooms not only in summer but also in winter due to climate change and eutrophication in the Sea of Marmara.

2. Methodology

The methodology adopted by this review study on the bloom dynamics of the coccolithophore *Ehux* in the Sea of Marmara has as its aim to expose both similar and specific bloom behaviors as well as differences from any other system. The bloom dynamics of the key species—not only in summer but also in winter—are discussed in light of their associations with other phytoplankton groups (such as diatoms and silicoflagellates) and physicochemical variables such as temperature, salinity, and nutrients under the unique character of the Sea of Marmara and under the pressure of climate change and eutrophication (**Fig. 1**).

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Fig. 1. Sea of Marmara under pressure from climate change and eutrophication [17].

3. *Emiliania huxleyi* (Lohmann) Hay & Mohler, 1967

3.1. General characters

Ehux does not produce any toxin; Thomas Huxley and Cesare Emiliani pointed this out in their study of marine deep sediment. *Ehux* is a coccolithophore species with a worldwide distribution from tropical waters to subarctic waters. It is one of thousands of photosynthetic phytoplankton that live freely in the euphotic ocean zone, forming the first step of all ocean food webs. *Ehux* is a single-celled phytoplankton covered with coccoliths, which are uniquely ornamented calcite disks [18, 19]. The coccoliths are frequently colorless and transparent, but they are formed of calcite which refracts light very efficiently. *Ehux* emits more carbon dioxide (CO_2) and hence has a greater effect on climate change than other phytoplankton species as a result of calcium carbonate ($CaCO_3$) accumulations in their coccoliths [7, 12, 13].

3.2. Global distribution and abundance

Ehux is the most abundant coccolithophore species found in the oceans. It has a worldwide distribution, the exception being the polar regions. During comprehensive blooms, sometimes covering areas of 100,000 km^2, *Ehux* cell densities can exceed all other phytoplankton densities in the region combined, accounting for 75% or more of the total number of phytoplankton in the region. These massive blooms can be shown through satellite imagery because of the large amount of light backscattered from the ocean water column, which provides a method to assess their biogeochemical importance on both basin and global scales [4–8].

3.3. Climate change and harmful algal blooms

It is predicted that climate change will seriously impact aquatic ecosystems, both freshwater and marine. Together with nutrient pollution, these climatic impacts might bring about increases in the densities and field effects of harmful algal blooms (HABs). Climate change might affect HABs in many ways as a result of increased water temperature, higher CO_2 values, changes in rainfall and salinity, coastal upwelling, rise in sea levels.

As waters warms more than usual due to climate change, HABs will increase both in number and area. HABs usually occur in warm summer periods. Warm waters favor the formation of HABs in a number of ways. For example, nanoplankton and picoplankton species such as the more toxic cyanobacteria and coccolithophores prefer warmer waters. High temperature levels at the surface prevent mixing of the water column, which allows HABs to become thicker and grow faster. It is generally accepted that warm waters favor the proliferation of tiny phytoplankton bloom species and allow them to survive much easier in the surface waters. It is known that algal blooms absorb solar radiation, which makes the water even warmer than usual and facilitates more algal blooms. On the other hand, climate change might lead to more drought seasons, making freshwater saltier. So, marine algal bloom species could spread to freshwater and brackish water ecosystems.

Phytoplankton species need dissolved CO_2 to proliferate. Higher CO_2 levels—first in the air and then water—might lead to rapid phytoplankton increase, particularly picoplanktonic species that float on the surface layer of the water. Moreover, climate change might affect precipitation dynamics, leading to alternating periods of drought and intense storms. The main source of nutrients is rain and river water discharge into aquatic ecosystems, supporting more algal blooms.

In view of current sea level rise, more scientists predict that sea levels will rise by as much as 1 m by 2100. Rising sea levels would create an increase in shallow and coastal waters—perfect conditions for the growth of phytoplankton and other algae. Another important factor is the increase in coastal upwelling events due to climate change. Coastal upwelling is a transport process from ocean deep-layer waters rich in nutrients to surface-layer waters of coastal zones due to the drifting of surface waters by offshore winds. Waters rich in nutrients delivered by upwelling lead to more algal blooms.

On the other hand, it is important to remember that this is a two-way process in which climate change affects HABs and HABs affect climate change. Looked at from the perspective of global excessive *Ehux* blooms, there are two special factors affecting global climate change. The first is that *Ehux* blooms locally act as an important source of $CaCO_3$ and dimethyl sulfide (C_2H_6S), the dense production of which can have a significant impact not only on the properties of the ocean surface mixed layer, but also on global climate change. As with all phytoplankton, *Ehux* primary production through photosynthesis is a sink of CO_2. However, the production of coccoliths through calcification is a source of CO_2. This means that coccolithophores, which include *Ehux*, have the potential to act as a net source of taking CO_2 out of the ocean. Whether they are a net source or sink in terms of CO_2 and how they will react to ocean acidification is not very clear. However, the chemical processes in **Fig. 2** are informative. The second factor is

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that the scattering induced by *Ehux* blooms not only causes more heat and light to be pushed back up into the atmosphere more than usual, but also causes more of the remaining heat to be trapped closer to the ocean surface [20–23].

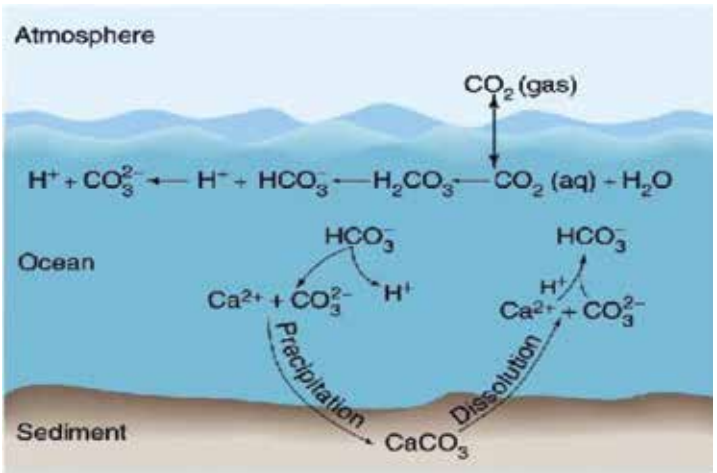

Fig. 2. Carbon cycle between atmosphere and ocean sediment.

4. Bloom dynamics of *Emiliania huxleyi* in the Sea of Marmara

4.1. Effect of temperature, salinity, and CO_2 in the Sea of Marmara

Average annual air temperature anomalies between 1981 and 2014 in Turkey (**Fig. 3**) confirm increased annual average temperature. For example, looking at temperature changes in the last 5 years the average temperature in 2014 (14.9°C) exceeds the average between 1971 and 2010 (13.5°C) (**Figs. 3 and 4**).

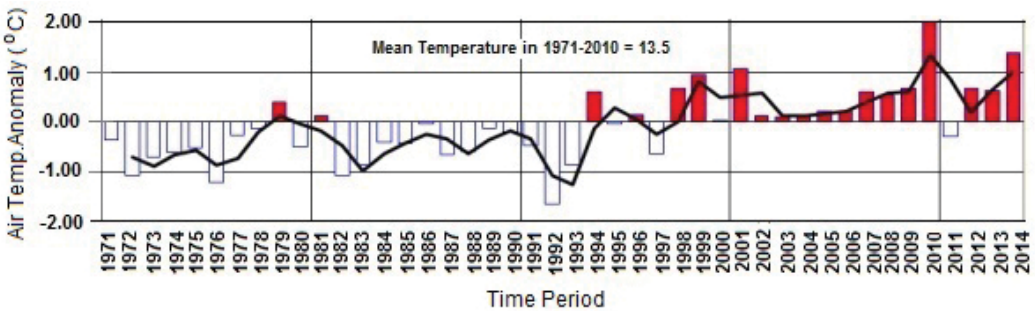

Fig. 3. Average annual air temperature anomalies between 1971 and 2014 in Turkey [24].

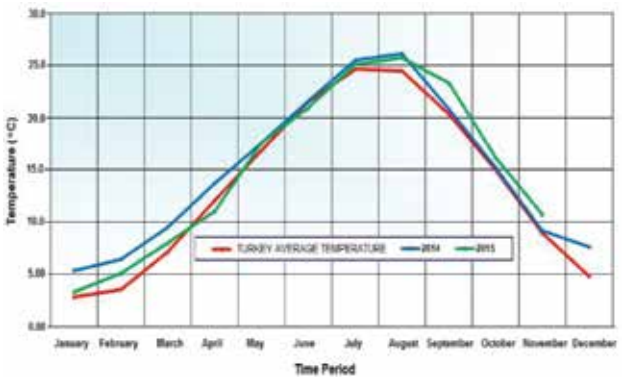

Fig. 4. Monthly average temperature variations in 2014 and 2015, and long-time temperature anomalies in Turkey [24].

The increase in average air temperature also causes an increase in surface water temperature in the Sea of Marmara and Black Sea—as is the case in other Turkish seas (**Fig. 5**). Due to the similar climatic behavior coupled with the effect of Black Sea surface waters via the Bosphorus, sea surface temperature variations in the Sea of Marmara are largely similar to the time series of basin mean winter anomalies of sea surface temperature (SSTA), surface atmosphere temperature (SATA), and the meridional component of surface wind (WA, m/s, dashed lines) for the Black Sea [25].

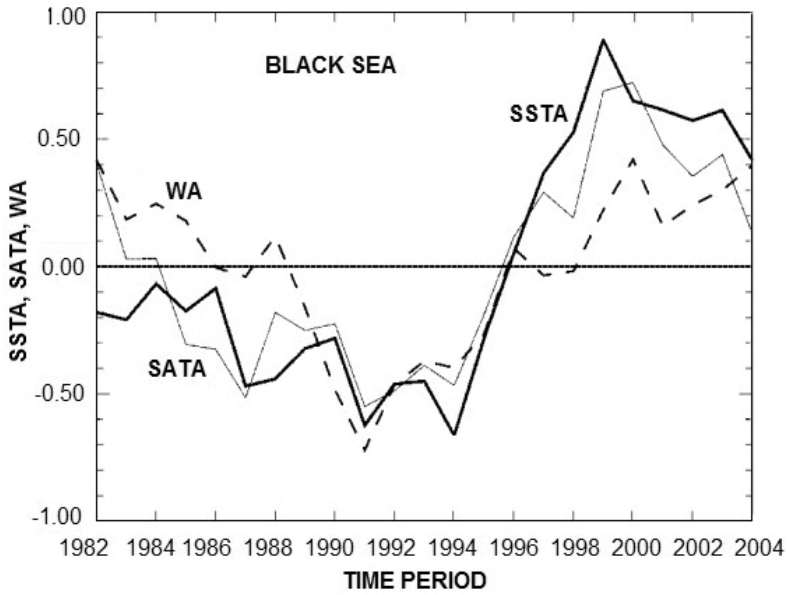

Fig. 5. Time series of basin mean winter anomalies of sea surface temperature (SSTA, °C, bold solid lines), surface atmosphere temperature (SATA, °C, solid lines), and the meridional component of surface wind (WA, m/s, dashed lines) for the Black Sea [25].

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Ehux is one of the most abundant coccolithophores showing a global distribution in all the oceans (apart from the polar oceans), generally in late spring or early summer. This species drifts freely and prefers the surface layers of oceans. *Ehux* blooms occur not only in summer, but also in winter when the temperature does not fall below 10.0°C in the Sea of Marmara [9, 10]. Sorrosa et al. [26] show that low temperature suppresses coccolithophorid growth, induces cell enlargement, and stimulates the intracellular calcification that produces coccoliths. For example, while the coccolithophore *Ehux* tolerates a wide temperature range (10–25°C), another coccolithophore species *Gephyrocapsa oceanica* Kamptner, 1943 tolerates a narrow temperature range (20–25°C) when cell sizes are correlated with temperature in a negative manner [26]. On the other hand, increased global CO_2 emissions (**Fig. 6**) coupled with other favorable factors are behind the massive blooms of *Ehux* in the Sea of Marmara, as is the case in the rest of the world. *Ehux* has received considerable attention since it tends to produce massive blooms under favorable temperature and high CO_2 emission conditions [4–10, 27, 28]. On the other hand, **Fig. 6** illustrates what is claimed to be a causal correlation between CO_2 and temperature[29].

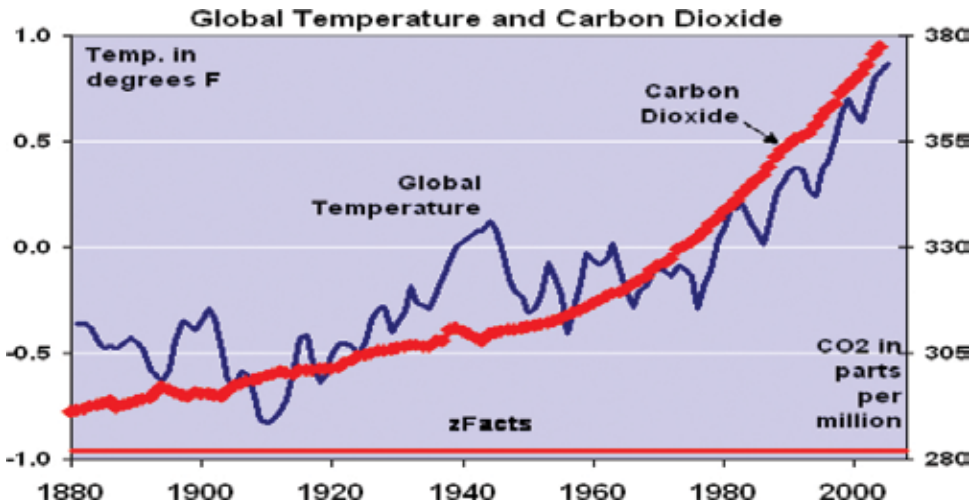

Fig. 6. Worldwide temperature and carbon dioxide anomalies [29].

The Sea of Marmara has two current systems that flow in opposite directions: upper-layer water that originates from less salty Black Sea surface water (18.0 ppt) and lower-layer water that originates from very salty Mediterranean water (39.0 ppt). Therefore, there is stratification in terms of both temperature and salinity during the year. However, the stratification in temperature in summer and winter in the Sea of Marmara is reversed, but the stratification in salinity is not. Hence, surface waters are consistently cold in winter (8–12°C) and hot in summer (20–25°C) irrespective of temperature during the year. When it comes to minimum and maximum temperature variations the Sea of Marmara provides the most favorable conditions for massive *Ehux* blooms during the year. As mentioned earlier, it is known that *Ehux* tolerates a wide temperature range between 10 and 25°C [9, 10, 26].

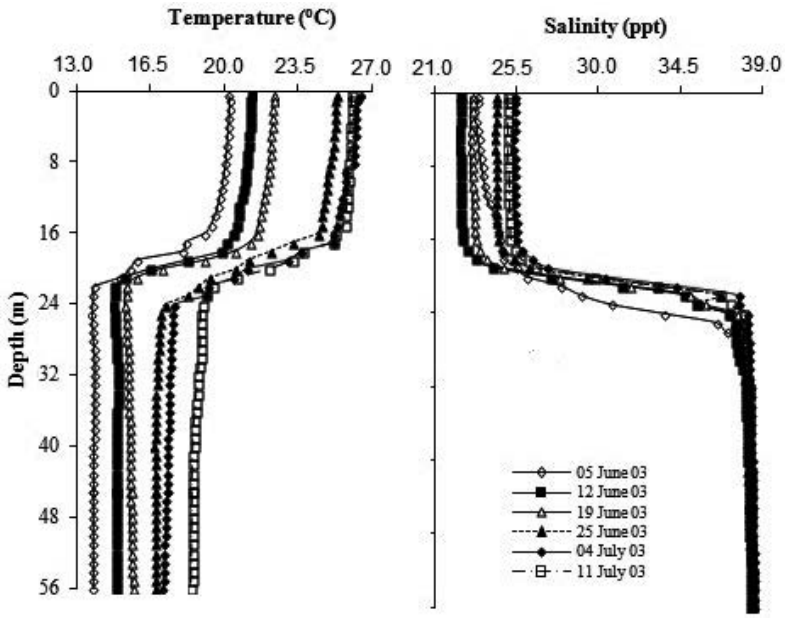

Fig. 7. Vertical distribution of temperature and salinity during the summer 2003 *Ehux* bloom in the Dardanelles [9].

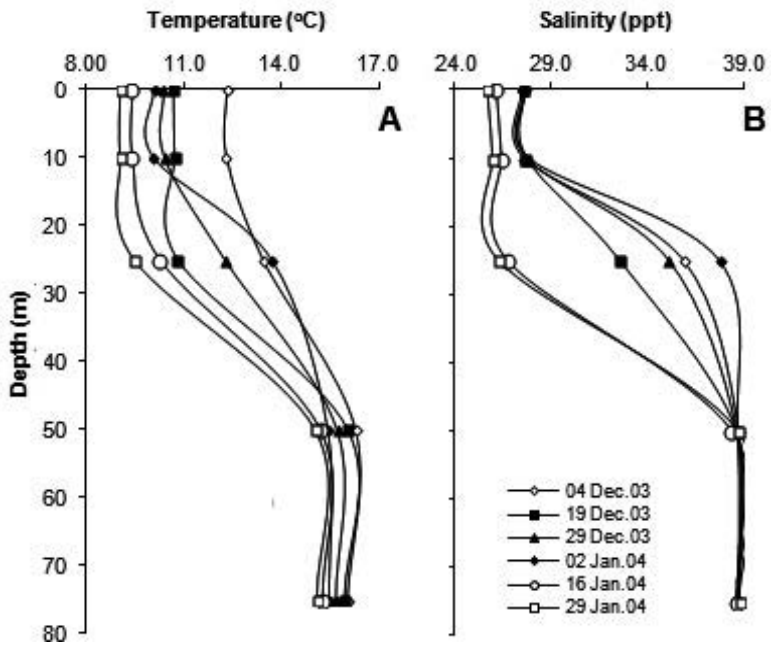

Fig. 8. Vertical profiles of temperature (A) and salinity (B) during the winter 2003/2004 bloom of *Ehux* in the Dardanelles [10].

In the summer *Ehux* bloom period [9], whereas the upper layer (0–15 m) has low salinity values (22.3–25.4 ppt) the much thicker lower layer (25–60 m) has high salinity values (36.5–38.5 ppt). However, temperature variations in both the upper layer (19.1–26.48°C) and lower layer (13.8–18.98°C) were more variable than variations in salinity. The seasonal thermocline and halocline interfaces are clear and form between 15 and 25 m during algal blooms (**Figs. 7 and 8**).

4.2. Effect of nutrient and nutrient ratios on *Emiliania huxleyi* blooms in the Sea of Marmara

There is experimental and natural evidence for the exceptional competitive ability of *Ehux* when the nitrate:phosphate (N:P) ratio is high. In multispecies competition experiments, Riegman et al. [30] found that *Ehux* isolated from the North Sea, along with some diatom species such as *Chaetoceros socialis*, outcompeted other species when the N:P was 100. With an N:P ratio of 1.50, although other species outcompeted *Ehux, Ehux* still maintained a relatively high population in the assay medium. In another study, Riegman et al. [31] demonstrated that *Ehux* had an incredibly high affinity for phosphate under phosphorus stress (N:P=300). Further, Riegman et al. [31] showed that *Ehux* has two cell surface–bound alkaline phosphatase enzymes enabling it to utilize organic phosphate at nanomolar concentrations, supporting the findings of a previous study [32]. Therefore, *Ehux* would be expected to be particularly competitive at low phosphate concentrations and high N:P ratios. Mesocosm studies in a Norwegian fjord support this idea [33, 34].

However, another examination of mesocosm experiments over several years showed that *Ehux* also grows well in mesocosms where the N:P ratio is low [33]. In many experiments the N:P ratio immediately before *Ehux* bloom is lower than the initial ratio. Clearly, there are other environmental factors in addition to high N:P ratios that are critical for *Ehux* blooms. The various findings of these experiments suggest that *Ehux* can still gain an advantage under a wide range of nitrate and phosphate ratios and concentrations in mesocosms even when the nutrient environment is artificially changed.

Turkoglu [9, 10] demonstrated that N:P ratios are significantly lower than the assimilatory optimal of the Redfield ratio (16:1) in the Sea of Marmara during *Ehux* bloom periods not only in summer (**Fig. 9**), but in winter *Ehux* bloom periods as well (**Fig. 10**). On the other hand, it is observed that N:P ratios are lower (<10) and silicate:phosphate (Si:P) ratios are higher (>10) in *Ehux* bloom periods in the upper layer under bloom conditions (**Figs. 9 and 10**). However, due to nitrogen scarcity Si:N ratios are over 1.00 (average: 3.07 ± 2.16) both in the upper layer and in the lower layer (average: 2.89 ± 1.93) during *Ehux* blooms in the Dardanelles, which is a part of the Turkish Straits System (**Figs. 9 and 10**).

On the other hand, vertical profiles of inorganic nutrients in bloom periods show that both nitrogen and phosphate concentrations in the Sea of Marmara are higher (**Figs. 10 and 11**) than any other marine system, even its neighbor the Black Sea [10, 11, 14, 16, 35–39]. As a result of heavy eutrophication, nutrient concentrations in the Sea of Marmara have gradually increased since 1960 (**Fig. 12**).

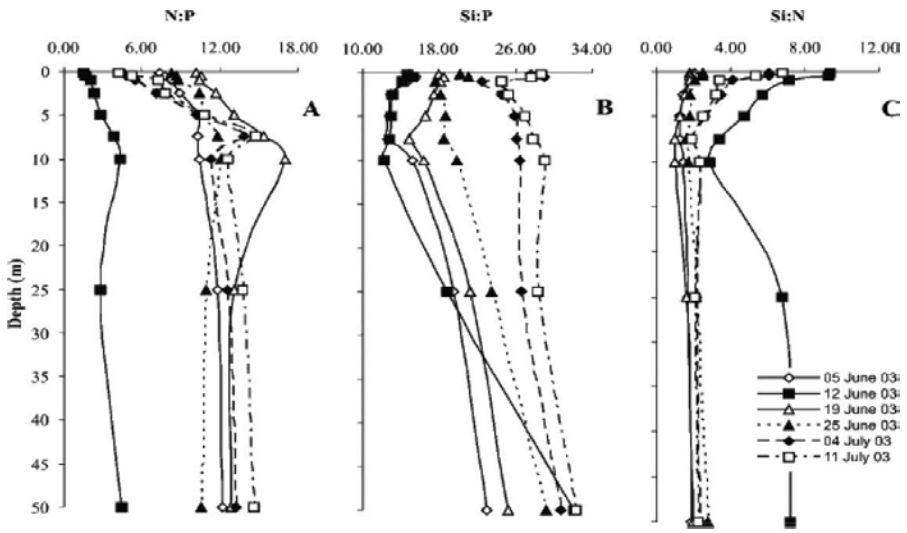

Fig. 9. Vertical distribution of some nitrogen ratios in the winter bloom period in the Sea of Marmara (Dardanelles) [9].

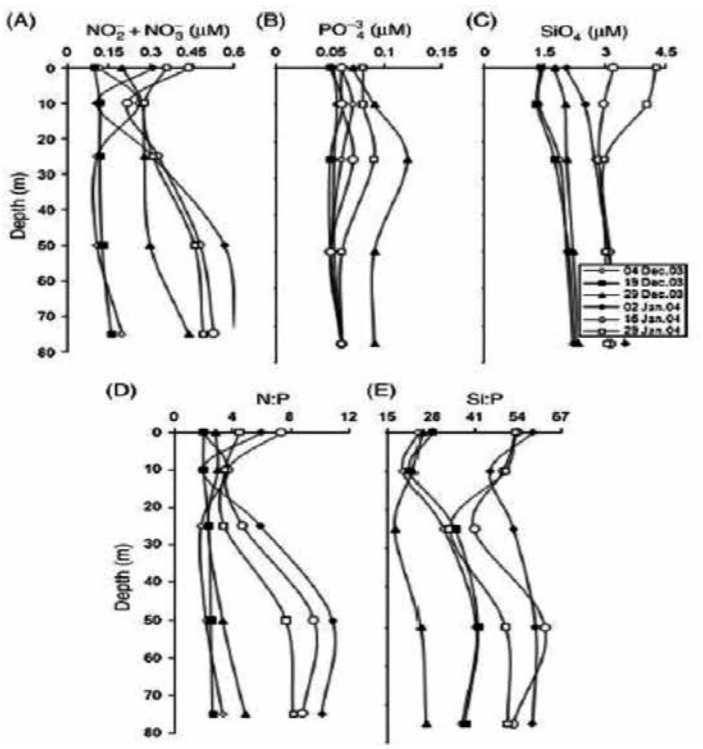

Fig. 10. Vertical distribution of nutrients and nitrogen ratios in the summer bloom period in the Sea of Marmara (Dardanelles) [10].

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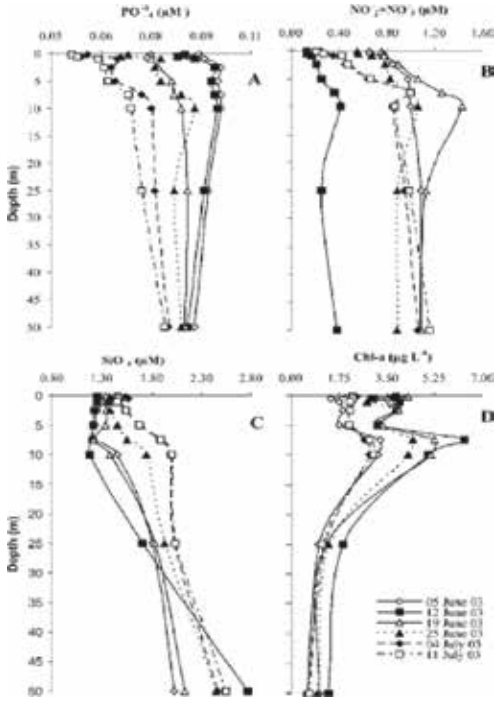

Fig. 11. Vertical distribution of nutrient concentrations in the summer bloom period in the Sea of Marmara (Dardanelles) [9].

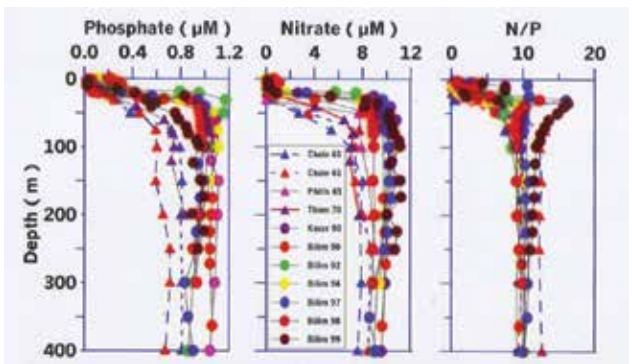

Fig. 12. Vertical variations of phosphate, nitrate, and N:P ratio in the long term in the deep basin of the Sea of Marmara (METU-IMS Data).

In actuality, what triggers *Ehux* blooms in the Sea of Marmara cannot be attributed to nutrient concentration levels or nutrient ratios such as N:P, Si:P, and Si:N on their own. Since this species proliferates under both low/high nutrient concentration levels and low/high N:P ratios, *Ehux* blooms are probably dependent on other phytoplankton blooms such as diatoms. In other words, competition between other phytoplankton groups, especially diatoms, may well be

critical. The concentration of silicates and phosphates in the system plays a major part in competition between *Ehux* and diatoms. *Ehux* blooms follow the blooms of diatoms in different marine systems (9, 10, 37–44).

In *Ehux* bloom periods, one of the probable causes of insufficient nutrient concentrations, especially silicate (<2.00 mm) and phosphate concentrations (<0.05) in the surface layer, is the utilization of nutrients by early diatom blooms. In other words, low phosphate and silicate concentrations favor *Ehux* blooms [6, 8, 33, 45]. In actuality, *Ehux* blooms are not stimulated by low phosphate or low silicate concentrations. *Ehux* predominates in the absence of diatoms due to the low phosphate and silicate concentrations. The proliferation or bloom of diatoms almost stops when the silicate concentration falls below 2.00 μM in marine systems, bringing the competition between diatoms and *Ehux* to an end. Subsequent to the very high diatom density in spring, there begins the excessive bloom of *Ehux* some time in late spring (second half of May), some time in early summer (June and July), and again some time in early/middle winter periods which varies year to year in the Sea of Marmara [9, 10].

Merico et al. [46] showed low N:P ratios in the southeastern Bering Sea during the *Ehux* bloom years 1997–2000 as a result of nitrogen scarcity, not phosphate abundance. This is unlike the Sea of Marmara where phosphate exceeds nitrogen due to domestic waste water rich in phosphate [9, 10]. In fact, in most bloom studies to date, nitrogen levels are lower than phosphate and hence N:P ratios are low. For example, the limiting factor for *Ehux* blooms is generally nitrogen rather than phosphate in the Sea of Marmara. N:P ratios cannot be trusted to reflect nutrient conditions at the initiation of *Ehux* blooms if there is prior preferential phosphate or nitrate utilization by other blooms, especially diatoms in the Sea of Marmara [9, 11].

On the other hand, inorganic N:P ratios may not be good indicators of phosphorus stress if organic forms of nitrogen and phosphorus are available to phytoplankton. Organic forms of nitrogen and phosphorus are used by many phytoplankton and may be important in their nutrition, but data on organic nutrient forms, bioavailability, and species-specific abilities to use them are still limited [47, 48]. *Ehux* is able to use some amino acids, amides, purines, and urea [49]. In the few studies that have examined the uptake of nonnitrate nitrogen during blooms, *Ehux* primarily used NH^{+}_{4} and urea. At the very least, *Ehux* has a superior ability to use regenerated nitrogen. It is perhaps the combined ability of *Ehux* to use nonnitrate nitrogen in addition to its exceptional phosphorus procurement capacity that grants it competitive success in nutrient-depleted waters exposed to high solar radiation such as the Sea of Marmara, which is nitrogen limited [1, 9–11, 14, 16].

4.3. Phytoplankton chlorophyll *a* levels in the Sea of Marmara

Turkoglu (2008) revealed that, in the summer *Ehux* bloom period, chlorophyll *a* concentrations range from 1.5 to 6.5 μg L^{-1} in the upper layer where there are massive *Ehux* blooms (**Fig. 11**). In contrast, Turkoglu [10] showed that, in the *Ehux* winter bloom period, chlorophyll *a* concentrations are lower (min–max:1.23–2.32 μg L^{-1}; average: 1.94 ± 0.43 μg L^{-1}) than summer bloom periods in the upper layer. However, chlorophyll *a* maxima were also observed in the subsurface layer (10 m) due to diatom and other blooms at this depth during the bloom period

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[10, 11]. The system is so productive that the annual average phytoplankton chlorophyll *a* level is 2.78 ± 3.21 μg L^{-1}. On the other hand, chlorophyll *a* levels reach 20.0 μg L^{-1} in some spring and late summer periods (**Fig. 13**) [50, 51].

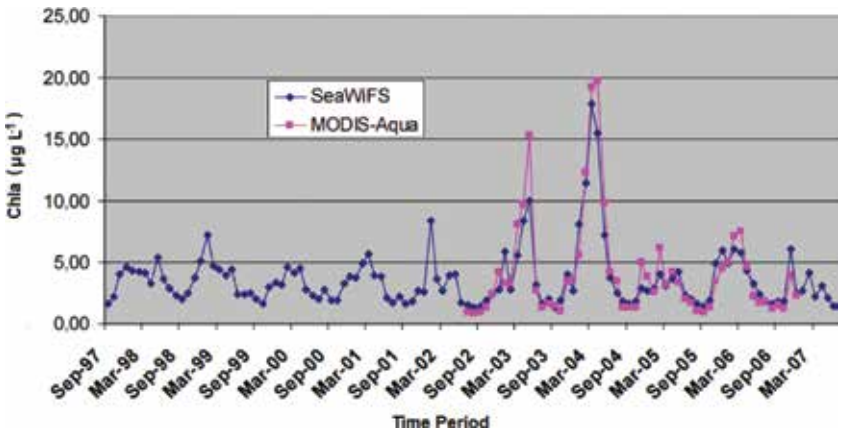

Fig. 13. Variations of MODIS and SeaWiFS chlorophyll a in the Sea of Marmara [50].

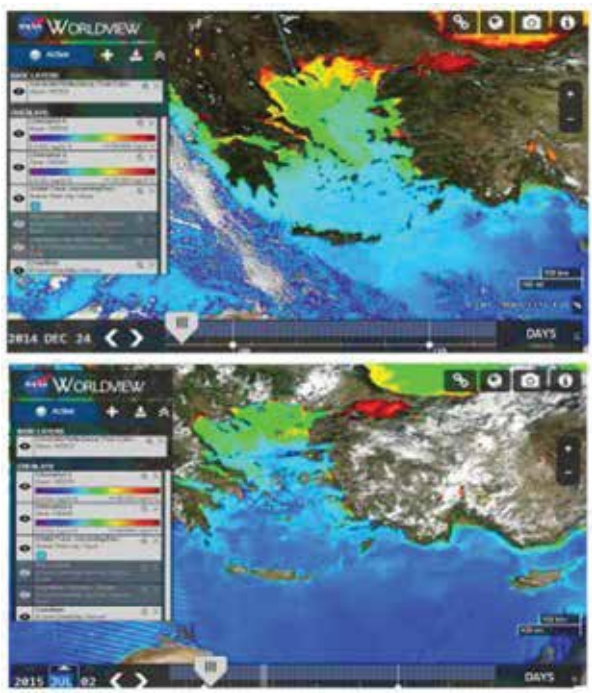

Fig. 14. SeaWiFS satellite images of chlorophyll a concentrations in winter (upper image) and summer (lower image) in the Sea of Marmara [17].

SeaWiFS (Sea-Viewing Wide Field-of-View Sensor) satellite images for chlorophyll *a* concentrations show that high concentrations are found not only in summer but also in winter in the Sea of Marmara (**Fig. 14**).

5. *Emiliania huxleyi* bloom characters of the Sea of Marmara

5.1. Interactions of *Emiliania huxleyi* and other phytoplankton

Various scientists studying phytoplankton taxonomy have listed over 150 different types of phytoplankton in the Sea of Marmara [1, 52]. However, the blooms tend to be extremely rich in a single, or only a few, predominant species. This sea has a three-phase phytoplankton bloom sequence. Diatoms tend to predominate in March, dinoflagellates in April, and the dramatically colorful blooms of *Ehux* predominate in the second half of May. The phytoplankton bloom colors the system with brilliantly coloured swirls of various shades of green in late spring and early summer.

Fig. 15. SeaWiFS true-color satellite images showing the effect of toxins from the Black Sea on the Sea of Marmara and the North Aegean Sea on June 21, 1997 (upper image) and June 16, 1998 (lower image) (SeaWiFS Project, NASA/ Goddard Space Flight Center, and ORBIMAGE).

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Ehux is one of the most abundant coccolithophores found globally in all oceans (except the polar ones) in late spring, early summer, and even in early winter. In the bloom periods, factors such as high surface irradiance, shallow stratification with a mixed layer depth of about 0–20 m, anomalies in salinity and temperature, and low phosphate and silicate concentrations come together to create favourable conditions for *Ehux* blooms in the Sea of Marmara [9–11]. The system is so eutrophic that, following the massive summer bloom of *Ehux* in June and July 2003 [9], a winter bloom was observed between late December 2003 and early January 2004 in the Dardanelles [9–11]. However, the population density of *Ehux* was small in early January 2004 (5.03×10^7 cells L^{-1}) in the superficial layer compared with the summer *Ehux* bloom period (2.55×10^8 cells L^{-1}). In bloom periods, *Ehux* is the dominant species and represents about 90% of the phytoplankton in the Sea of Marmara [9–11]. The relatively strong bloom potential of *Ehux* in winter and in summer period testifies to the fact that the Sea of Marmara is suffering severe hypereutrophication due to the transport of toxins from the Black Sea (**Fig. 15**).

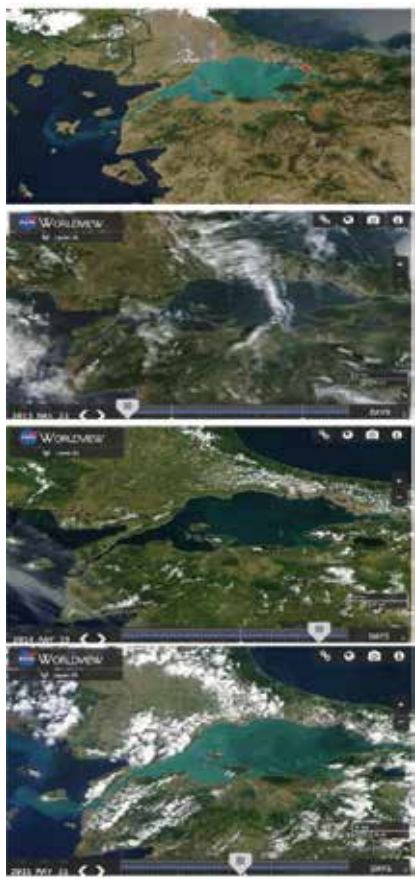

Fig. 16. SeaWiFS true-color satellite images showing algal blooms on different dates (the top image was taken on July 25, 2003) in the Sea of Marmara [17].

The Moderate Resolution Imaging Spectroradiometer (SeaWiFS/MODIS) produced true-color images of the extensive bloom events of *Ehux* on June 25, 2003; May 22, 2013; May 19, 2014; and May 31, 2015 (**Fig. 16**).

The abundance of *Ehux* during bloom periods in the Sea of Marmara is higher [9–11] than previous bloom densities in the entire Atlantic Ocean [27, 53, 54]. It is little surprise then that the albundance of *Ehux* is higher than previous bloom densities in the Aegean Sea [1, 55], the Black Sea [37, 40, 43, 44], and the Bering Sea [46].

MODIS images (**Figs. 15 and 16**) reveal how the Sea of Marmara and hence the Aegean Sea are affected by the biophysicochemical character of the Black Sea. The images exhibit turquoise water discharge flowing from the Black Sea into the Sea of Marmara and then into the North Aegean Sea (**Figs. 15 and 16**). The effect of the *Ehux* bloom in the Sea of Marmara is clearly seen in the North Aegean Sea, demonstrating the influence of the surface current emptying from the Dardanelles. Thus, some biogenic organic matter such as bacterioplankton and phytoplankton in the Black Sea are naturally exported to the Sea of Marmara and then reach the eastern Mediterranean via the Dardanelles (**Fig. 15**).

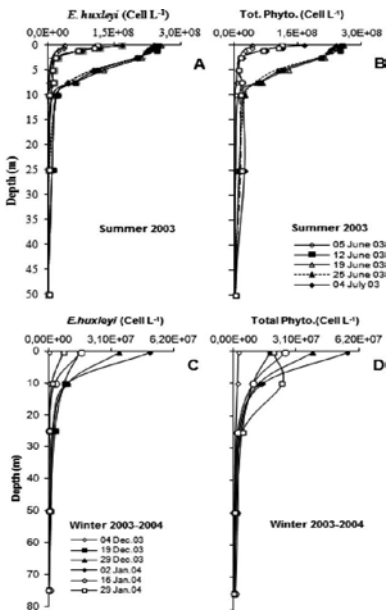

Fig. 17. Vertical variations of *Ehux* and total phytoplankton in the *Ehux* summer and winter bloom periods in the Dardanelles (Sea of Marmara) [1, 9, 10].

The sea level in these bodies of water is in equilibrium—were it not the surface of these seas would be rising or falling. The flow of water into one is counterbalanced by an approximately equal flow of water out of another. The flow of surface water out of the Black Sea and the Sea of Marmara into the Aegean Sea is approximately 600 km^3 y^{-1}. The water flow is balanced by annual freshwater discharge of about 300 km^3 from rivers, especially from the Danube River,

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which discharges into the Black Sea, and by annual saline water input of about 300 km^3 coming from the Mediterranean Sea via the Bosphorus. Black Sea surface-layer water is substantially less saline than Mediterranean water due to the freshwater discharge of rivers [28, 56, 57]. Input of less saline water from the Black Sea and the Sea of Marmara, accompanied by the clouds of coccoliths, is very important to the physicochemical oceanography of the Aegean Sea. The movement of the turquoise water, which stays in the surface layer due to its lower density, can be tracked through the Aegean Sea (**Figs. 15 and 16**).

During both the *Ehux* summer and winter bloom periods the vertical profiles of *Ehux* (**Fig. 17**) reveals that, whereas the cell density of *Ehux* increases from 3.58×10^7 to 2.55×10^8 cells L^{-1} in the surface layer in summer, there is also an increase from 1.60×10^4 to 5.03×10^7 cells L^{-1} in the upper layer. Again, whereas the cell density of *Ehux* exceeds 2.0×10^8 cells L^{-1} in the surface layer in summer, it also exceeds 5.00×10^7 cells L^{-1} in middle winter in the upper layer in winter period. The very high bloom density in the surface layer both in summer and winter dramatically decreases with depth and almost all of the bloom density is lost at a depth of 10 m in both periods [1, 9, 10].

6. Management of HABs and *Emiliania huxleyi* blooms

HABs can be managed in three ways: (1) prevention, (2) mitigation, and (3) control efforts. Prevention involves reducing the incidence and extent of HABs by controlling or decreasing the input of anthropogenic waste water, rich in nutrients and other pollutants, and ballast water, rich in invasive species, before HAB onset. HAB mitigation generally involves monitoring for blooms and their toxins, public communication programs, and the transfer/removal of fish cages from critical areas to less critical regions. HAB control involves a number of methods: biological, chemical, ultrasonic, ozonation, chemical and clay flocculation.

Nature dictates that all organisms are controlled by other organisms. There are many host-specific viruses, predators, parasites, and pathogens involved in the biological control of many HAB species. To date, the control mechanisms on the distribution of *Ehux* blooms are little known; however, there is evidence that viruses are linked to sudden crashes of *Ehux* blooms [58, 59]. Viruses are found in all marine environments and are a very important control mechanism over populations of bacteria and phytoplankton, affecting biodiversity, nutrient, and biogeochemical cycles [60, 61]. Although much work here is related to viruses that infect *Micromonas pusilla* [62–64] and *Heterosigma akashiwo* populations [65], scientific understanding of the effects of viruses on *Ehux* population dynamics is still limited. Some studies have revealed that the number of viruses dramatically increase following the death of massive *Ehux* blooms [66–69, 13]. *Ehux* viruses (EhVs) isolated from some dense *Ehux* blooms [70, 71] have recently been assigned to a new genus, *Coccolithovirus* [72], in the family Phycodnaviridae [71].

Recently, amplified segments of the major capsid protein (MCP) gene from viruses that infect *Ehux* were cloned and sequenced by Schroeder et al. [72] using denaturing gradient gel electrophoresis (DGGE). Schroeder et al. [73] distinguished many virus genotypes—such as

EhV84, EhV86, EhV88, EhV163, EhV201, EhV202, EhV203, EhV205, and EhV207. This led to elimination of an *Ehux* bloom in a mesocosm experiment off western Norway.

7. Conclusion

The overutilization of nutrients by summer and winter diatom blooms immediately before *Ehux* blooms leads to insufficient inorganic nutrients (especially inorganic phosphate) that could lessen the impact of such blooms. Coupled with high irradiance, two stable temperature structures at the surface (due to two thermocline formations in summer and winter that result from two currents that run in opposite directions to each other), and a stable water column in terms of vertical mixing following the establishment of the seasonal thermocline in summer and reverse thermocline (due to the two currents running opposite to each other in winter) are the main characteristics of *Ehux* summer and winter blooms in the Sea of Marmara [9–11, 14, 16], confirming previous studies, especially on summer *Ehux* blooms in the North Sea and northeast Atlantic [6, 8, 45]. It is known that coccolithophore *Ehux* summer blooms follow the dense blooms of diatoms in spring in many different marine systems [37, 38, 40–44, 74]. Furthermore, *Ehux* forms extensive and intensive blooms in many cold coastal and oceanic regions [10, 75].

Because of their potential impact on global carbon and sulfur cycles [41], *Ehux* blooms attract a lot of attention. The environmental factors involved in triggering *Ehux* blooms are incompletely known. Some physicochemical factors—such as strong temperature stratification, high solar radiation, phosphate limitation, and low N:P ratios—and some biological factors—such as reduced grazing ratios and competition between phytoplankton groups—seem to be prerequisites for intense *Ehux* blooms [9, 10, 54]. However, there is conflict about whether the evidence supports the universality of high N:P ratios as a controlling factor for *Ehux* blooms. Almost all researchers studying nutrient ratios in the Sea of Marmara show that the N:P ratios are lower than the Redfield ratio not only during *Ehux* blooms, but also during the rest of the year in the Sea of Marmara [9–11, 14, 16, 39, 51, 57, 76–80].

However, there is general agreement that—in light of the high levels of nutrients, changing nutrient ratios, chlorophyll *a*, and successive algal blooms—the Sea of Marmara is subject to heavy pollution as a consequence of urban and domestic waste water from Istanbul and northwestern Black Sea surface waters where pollutants are discharged by the Danube River.

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Chapter 3

Considerations for Photobioreactor Design and Operation for Mass Cultivation of Microalgae

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Gema Lorena López Lizárraga

Additional information is available at the end of the chapter

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Abstract

Microalgae have great biotechnological potential for production of substances through photosynthesis. Light capture process and electron transportation imply energy losses due to reflection, fluorescence emission, and energy dissipation as heat, giving a maximum theoretical value of 8-9% for microalgae energy capture efficiency and conversion to biomass. For development of full potential of microalgae the knowledge of the light capture process is required. High yields can only be obtained linking photobioreactor design with biological process taking place inside. In massive microalgae cultures, light gradients are generated and this depends on the biomass concentration, cellular types, cells sizes, and pigment content, and also on geometry, hydrodynamic, and light conditions inside the photobioreactor. In the present chapter we explain the relationship between light energy capture process and photobioreactor design and operation conditions, like turbulence, gas exchange, and nutrient requirements. Finally, the productivity and costs are discussed, and the parameters that determine the economic viability of any microalgae culture.

Keywords: photobioreactor design, photosynthesis efficiency, nutrients, mixing, gas exchange

1. Introduction

From a biotechnological point of view, the term microalgae refers to unicellular organisms capable of carrying out oxygenic photosynthesis, they contain Chlorophyll *a* (Chl-a) and/or other similar photosynthetic pigments. This definition takes into account a very large and diverse group of photosynthetic prokaryotic and eukaryotic organisms, capable to catalyze

the process of carbon dioxide (CO_2) fixation and its conversion into organic matter. The number of species of microalgae has been estimated from 1 to 10 million [1, 2], these organisms are ubiquitous and have managed to colonize almost every habitat known on the planet, from the tropic to the poles.

1.1. Advantages of culturing microalgae

Microalgae possess great biotechnological potential for the production of a wide variety of compounds such as polysaccharides, lipids, proteins, carotenoids, and other pigments, vitamins, steroids, among others. Dozens of algal species are used to produce animal feed, human nutrition, cosmetics, and pharmacy industry components. They also find application as wastewater treatment, CO_2 fixation, and greenhouse gas emissions reduction. Microalgae can be used to produce biofuels, hydrocarbons, and hydrogen [3–5]. They can be a clean and renewable energy source because of their high yield and low spatial requirements, if compared to terrestrial plants. Some authors consider microalgae as biodiesel feedstocks for the future [4].

The only natural process that allows the production of biomass using only sunlight as the energy source and CO_2 is photosynthesis. Unicellular photoautotrophic organisms are capable of using sunlight in a more efficient way than superior plants [6]. The advantages of microalgae culture compared to superior plants are listed [2, 6, 7]:

1. Microalgae biological systems are considered the most efficient for solar light capture, and the production of compounds through the photosynthetic process.
2. Whole microalgae biomass can be harvested and used because they lack complex reproductive organs and have no vascular systems.
3. Many algal species produce and accumulate particular compounds of high commercial value can be induced, for example, proteins, carbohydrates, lipids, and pigments.
4. The isolation, genetic selection, and strain studies is relatively easy and less time consuming because microalgae reproduce themselves by simple cellular division and can fulfill their life cycles in just a few hours or days.
5. Microalgae can be cultivated with low inorganic nutrients concentration. These make them of particular interest as a protein source, assuring protein availability in regions of low agriculture productivity due to the lack of water and nutrient poor soils.
6. Systems for biomass production can be adapted or scaled up to different operation levels, allowing later incorporation of these systems to fully automated facilities for large scale production.

1.2. Beginning of mass culture of microalgae

Large scale culture of microalgae began in the early 1960s in Japan with the culture of *Chlorella* [8], this development was followed by the establishments of other facilities, for example in the 1970s it established the culture of *Spirulina* in the Texcoco lake in Mexico city by the Sosa Texcoco Co. [9]. In 1977 Dai Nippon Ink and Chemicals Inc. established a commercial *Spirulina*

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production facility in Thailand and by 1980 there were 46 large scale facilities in Asia that produced more than 1000 Kg of microalgae per month, mainly *Chlorella* [10] and in 1996 near 2000 tons of *Chlorella* were commercialized only in Japan [11]. The commercial production of *Dunaliella salina* as a source of β-carotene was the third microalga of industrial production when Western Biotechnology Ltd. and Betatene Ltd. installed a production facility in Australia in 1986. These were followed by the installation of other production facilities in Israel and in the United States of America [5].

Commercial production success of microalgae in large scale facilities depends on many factors, among which, we can mention the development of large scale culture systems of economic feasibility, and development of these systems has been a gradual process [5].

Productivity of microalgae biomass is affected by factors like photosynthetic pigments efficiency of the in capturing and converting light energy to chemical energy, accumulation of dissolved oxygen produced by photosynthesis, insufficient CO_2 mass transfer rate, depletion of nutrients, and photoinhibition [12, 13].

2. Light capturing process and photosynthetic efficiency

Autotrophic growth of microalgae depends on photosynthesis, which involves light electromagnetic radiation energy absorption and conversion by photosystems I and II (PSI and PSII) into electrochemical potential and chemical energy (NADPH and ATP). Energy is later used in the CO_2 fixation process [14]. Thermodynamic efficiency over the PAR region of systems working with low light regimes (100–300 μmole m^{-2} s^{-1}) can be below 5%, decreasing to 2% under large solar irradiance (>500 μmole m^{-2} s^{-1}). In addition, under outdoor conditions around 95% of the captured total light spectrum energy is converted into heat [15].

In an ideal case, a photobioreactor (PBR) must capture all light available in the environment and transfer it into the culture to be used for biomass production [16]. Although in normal conditions this does not happen [17] because photosystems are exposed to an amount of light energy below or greater than the amount that can be transformed into chemical energy. If biomass concentration is too low, some of the light is transmitted through the culture. Conversely, if biomass concentration is too high, a dark zone appears. Maximal productivity will require the exact condition of full absorption of all light received but without a dark zone in the culture volume. This is called luminostat regime. But maintaining luminostat regime over the year in outdoor conditions has no interest in practice as it cannot be applied in actual operating conditions due to the disconnect between the dynamics of irradiation conditions (below 1 h) and biomass concentration changes (days) [15].

Under sunlight, biomass growth rate is insufficient to compensate for the rapid changes in sunlight intensity. Consequently, light attenuation conditions that are fixed by biomass concentration are never optimal. This is why determining the maximum photosynthetic efficiency and the upper limits of biomass production through photosynthesis has been a central topic of investigation in mass culture of microalgae [18] since its beginning.

2.1. Phothosynthetic efficiency

Only a fraction of the energy of sunlight can be used to build up biomass and derived products. One form of measuring the overall light usage for biomass production is known as photon conversion efficiency (PCE) [19], also called energy conversion efficiency (η) [17, 20]. The conversion of light energy is limited by several factors; some due to the physical nature of light itself and others inherent to the photosynthetic process [19]. First, light must travel from the Sun to the Earth surface, losing one part of the energy just by passing through the atmosphere, for the remaining amount of energy, only the part that has a wavelength between 400-700 nm can be used for photosynthesis because this wavelength can be captured by the photosynthetic pigments and is known as photosynthetic active radiation (PAR).

Solar energy conversion efficiency (η_{solar}) depends on three factors: 1) Light-Harvesting Yield (LHY), 2) Fractional Energy Yield (FEY) of the redox products of PSII, and 3) quantum yield (QY), therefore η_{solar}=LHY·FEY·QY. LHY value depends on the coordinated functioning of the anthena complex, which absorbs light energy through dozens or even hundreds of protein bound pigments, and the oxygen liberating complex of PSII. This last requires Mn as cofactor to split the water molecules and liberate electrons and generate an excitation of the Chlorophyll (P680*) in the reaction centre. LHY has a maximum value of 34% under ideal conditions. The FEY depends on the electron transfer chain of the PSII, from P680* to quinone B (QB) and also has a maximum value of approximately 34%, again in ideal conditions. The QY denotes the probability that P680* formation results in product formation along the main path of redox chemistry and has an accepted value of approximately 0.875 [17].

In the light absorption process and the electronic transport chain (FHY and FEY), most of the energy is lost due to reflection, fluorescence emission, and energy dissipation as heat by photosynthetic pigments, this makes that the PCE or η decreases to a value of 12.6%. Also, the conversion of light energy into biomass diminish even more the energetic yield, making the light energy capture and conversion to biomass efficiency of roughly 8-9% [17, 19, 20]. Important is to highlight that this last value is the theoretical maximum accepted for microalgae. Maximum light energy conversion efficiency and its conversion to biomass in higher plants have an actual value of just 5% [17].

2.2. Light and culture conditions

Exposure of photosynthetic cells to an excessive amount of light could lead to photoinhibition and to a decrease in the growth rate. On the other hand, the self-shading effect between individual cells presented in mass cultures of microalgae causes a productivity decrease, even when the amount of light is sufficient for the population of microalgae in the PBR [21]. The quality of light, which means the light wavelength that is used in photosynthesis by the microalgae cultures, also affects the culture performance [22, 23]. During batch culture, or where light is constant, cells can experience photoinhibition at the beginning of the culture, and the limitation of light when a high cell concentration is reached [24]. This can be avoided using fed batch cultivation or continuous culture mode. For example, Garcia-Cañedo [25] have reported that photosynthetic efficiency is maximum when nutrients like nitrogen are supplied,

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and fed-batch culture mode application promotes a high maximum photosynthetic efficiency, close to the reported maximum theoretical value.

To develop the full potential of photosynthetic organisms, that can be economically feasible and similar to heterotrophic eukaryotic organisms, like yeasts or filamentous fungi, it is required to identify the "bottle neck" of the bioprocess. The growth of microalgae requires appropriate light capture and conversion into biomass, therefore, it required novel PBR designs with geometries not commonly used for heterotrophic organisms in different operation modes that promote higher photosynthetic efficiencies [25, 26].

3. Photobioreactor design

The fundamental principle for photobioreactor design is a high surface area to volume ratio in order to use light energy efficiently, and is a requirement to obtain high values of PCE (**Figure 1**). Higher photosynthetic efficiency can result in higher biomass productivity and concentration, but at much higher cost because of high energy use (mixing, cooling, and embodied energy) and capital cost [27]. PBR design must include a short light path, which can be obtained using different geometries and low level of liquid to minimize the energy used for mixing the culture [19]. At high liquid level, the water column could generate high hydrostatic pressure and require higher energy input for mixing of the culture inside the PBR with air injection.

3.1. Opens systems: open ponds

Normally, microalgae and cyanobacteria large scale mass cultivation is done in shallow open ponds tanks, of circular or raceway type, with solar light. One of the major advantages of using open systems is that they are easy to build, operate, and they have lower costs than closed systems. Even though it has been demonstrated that open pond culture is economically feasible, they still have some disadvantages and limitations, they use light in a very inefficient manner, have evaporation water loses, low CO_2 mass transfer rate from the atmosphere, due to its inefficient mixing mechanisms; open ponds also require a large area of land for the culture due to its shallow depth. Additionally, open systems can be contaminated with predators or fast growing microorganisms like bacteria that can compete with microalgae for nutrients, this is why open systems are only used for organisms that can tolerate extreme conditions [28–30], like high salinity or pH. The scaling up of open ponds culture systems can only be performed by increasing the area, because increasing depth will not increase light penetration leading to lower productivities.

3.2. Closed systems: photobioreactors

To overcome the problems detected in open systems it has been proposed the use of closed photobioreactors (PBRs). The former are more appropriate for strains that cannot tolerate extreme environments or when final product is highly susceptible to degradation or contamination. Closed systems also allow the prevention of contamination, allowing the operation in

culture modes like photoautotrophic, heterotrophic, or mixotrophic. Also, closed systems can obtain up to three times more biomass than open systems, thus reducing harvesting costs [31]. Despite the great advances that have been achieved in the construction and operation of PBRs, its technology is still in development. Around 90% of current biomass production worldwide is obtained in open systems, despite the fact that PBR technologies offer greater potential in terms of productivity, control of culture conditions, and applicability to cultivate various strains [15].

Several closed PBR designs that are in operation are in laboratory, pilot plant levels, and even some have been successfully scaled up to an industrial level. One of this successful closed PBR design is the tubular type, in this the tubes configuration where the culture is hold is one of the main factors affecting productivity of photosynthetic biomass [32]. Tubular PBRs can be built with plastic materials like rigid transparent polyvinyl chloride (PVC), polycarbonate or flexible plastic bags, among other materials. They can be arranged in vertical, horizontal, conical, and inclined form, with degasifying units that allow the removal of the O_2 produced during photosynthesis [30], the tubes can also be arranged in an annular form [19, 33], each of these forms affect the productivity expected in this type of systems.

Appropriate design of vertical tubular PBRs can reduce the culture area and distribute photosynthetic organisms in vertical columns. Vertical reactors can increase the exposure of the organisms to light, and also the contact time between gas and liquid, thus increasing residence time of CO_2 and the efficiency of CO_2 assimilation [13, 28, 34]. Vertical columns can be compact, low cost, easy to operate aseptically [35], and very promising for large scale culture. It has been reported that vertical PBRs, vertical columns, and airlift type PBRs of even 0.19 m of diameter can reach a final biomass concentration and a specific growth rate comparable to values reported for PBRs of smaller diameters [30, 35]. The main disadvantages of vertical systems are light reflection and/or incidence of light at the peak hours of the day when the sun is in the summit due to the incidence angle of light on the PBR surface, also vertical form can generate hydrodynamic and shear stress if the height of the PBR is too large.

Horizontally displayed tubular PBRs are considered appropriate for mass cultivation of microalgae because they possess a large illuminated area and have better usage of light at sun summit. Some have been successfully scaled up to a volume of 4000 L or more [36, 37]. Even though this PBR design generate oxygen accumulation, when used outdoors can present photoinhibition [38]. When scaling up these systems, it must also be considered that increasing the diameter of the tubes will decrease the area to volume ratio, and the increase of the length of the tubes could generate CO_2 and nutrient gradients and oxygen concentration that could rise up to toxic levels [30, 38]. Formerly described designs are combined in inclined tubular PBRs, which have lower hydrodynamic stress and better illumination because the incidence light angle can be adjusted with the inclination of the PBR, also mixing is better than in horizontal tubular PBRs [38].

Flat panel PBRs have also been studied in order to make an efficient use of light for algal biomass production [39, 40]. These PBRs have a large illumination surface and the advantage of high area to volume ratio, and therefore optimum illumination of the cells and low oxygen concentration can be achieved. There are varieties with flat and curved semicircular bottom.

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In this last form, mixing dead zones is avoided and favour biomass accumulation [41, 42]. Although, it is difficult to achieve efficient biomass productivity per area of land using flat panel PBRs. Factors affecting biomass productivity in this type of reactors are the angle, direction of flat panels, and the number of panels per land unit [16], also their scale up requires the addition of compartments and support materials for the PBR [30]. Generally flat panels are displayed in vertical form but they can also be arranged inclined. Examples of different types of PBRs can be observed in **Figure 1**.

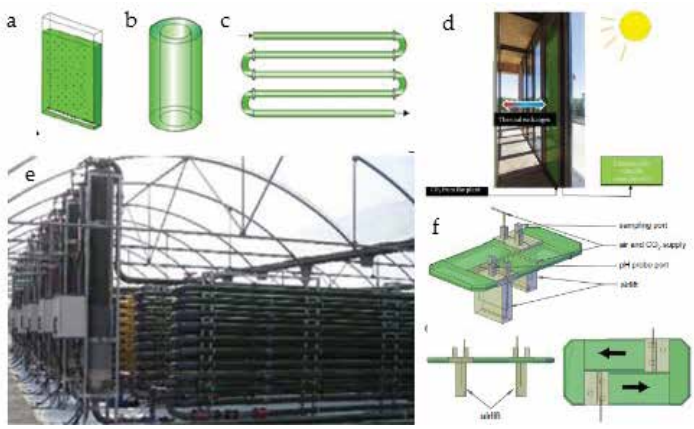

Figure 1. Examples of different photobioreactor designs. Basic designs: a) flat panel, b) vertical tubular, c) horizontal tubular [19], d) Flat panel building integrated PBR [15], e) Mass cultivation 28, 000 Liters PBR in operation in Spain [36], f) Floating horizontal PBR [27].

3.3. Photobioreactor design considerations: power and cost effective designs

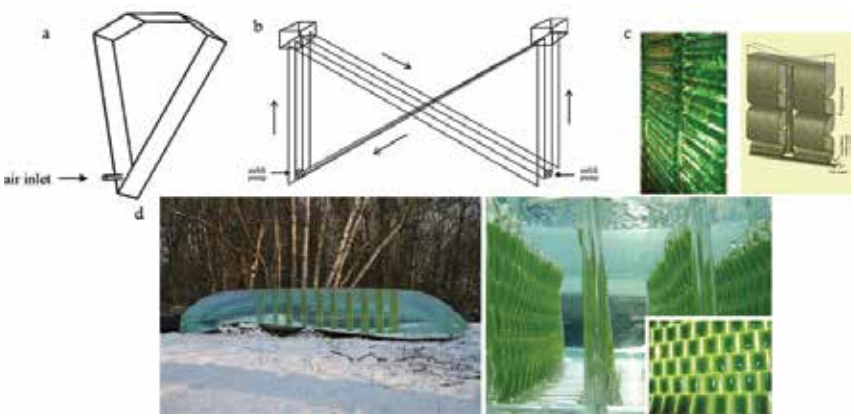

Figure 2. Examples of unconventional photobioreactor designs. a) Conic flat panel; b) alfa form; c) Subitec flat panel PBR; d) Proviron plastic bag PBR. Images taken from [44–49], http://subitec.com/en/flat-panel-airlift-fpa-photobioreactor and http://www.proviron.com/node/39.

Unconventional designs have been proposed, which include alveolar type [43], alpha form (**Figure 2a**) [44], flat panel and tubular conic form (**Figure 2b**) [45, 46], and also spiral tubes [47]. When proposing new design forms it must be considered to combine a high productivity with a low need for auxiliary power [19]. A German enterprise called Subitec [48] has developed a flat panel airlift PBR of 180 L (Patents EP 1 169 428 B1 and EP 1 326959 B1) for outdoors culture (**Figure 2c**), that consists of two fine layers of sealed plastic bags, this design includes baffles to generate vortex that enhance turbulence and mixing of the culture inside the reactor, improving light utilization. This company claims that the energy consumption of their PBR is only 200 W m^{-3}, consumption verified in pilot plant scale. Company Proviron [49], has developed plastic bag PBR in multiple vertical panels of 1 cm thick, this reactor is displayed unrolling a plastic bag film and does not require additional support; Proviron claims that in their design, it is possible to achieve a biomass concentration of 10 g L^{-1} with a low investment cost of only 20 Euros m^{-2}, the most important issue about this design is its low auxiliary power of 2 W m^{-2}, which represents approximately half of the maximum value that allows economic feasibility in Central Europe [19].

More recently, buoyant inexpensive plastic film PBRs has been developed. It consists of two plastic films forming the top and bottom surfaces of the horizontal raceway, sealed to each other and connected to two vertical airlift units. This design combines the advantages of open ponds and closed systems in a cost-effective way and can be used on both water and ground, depending on the end user's particular needs [27].

There is significant incentive to design and operate algal PBRs with high biomass productivity and conversion efficiency. Although many factors affect performance of PBRs, such as the type of PBR, culture media, temperature, pH, microorganism used, CO_2 mass transfer, O_2 accumulation, mixing, light intensity, and light/dark cycles. Among these, the major limiting factors for growth of microalgae are usually light availability and interphase mass transfer [50].

4. Turbulence and light capture

Nutritional and light requirements of photosynthetic microorganisms can be covered in high light path PBRs [42], higher than 0.10 m, if the design and operation characteristics are adequate, for example low mixing time and high axial dispersion. Among the advantages of using high light path PBRs are the decrease on construction cost and in energy expenditure, also, it can contain more liquid quantity in less land area.

In dense microalgae cultures, incident light intensity on the PBR surface (Io) decreases with culture depth, and in a certain depth it reaches an intensity equal to the saturation of photosystems (Is), this is why all the light that penetrates further will be used with maximum efficiency [51]. Grobbelaar [20] suggested that in a PBR with dense culture of microalgae exists a light gradient and several illuminated regions designated as follows: 1) Light limited region, is the deepest part of the PBR, 2) Light saturated region, is the region where all the penetrating light can activate photosynthesis and saturation of photosystems is achieved, 3) Photoinhibi-

tion region, presented in some cases in intense light conditions, and corresponds to the outer part.

4.1. Light gradient determination

The exact dimensions of each region depends on the concentration of biomass, geometry, hydrodynamic, and light conditions of the photobioreactor, this will be influenced by cellular size, forms, and pigments content [24, 52]. The light penetration depth in a photobioreactor can be calculated using the modified Evers model [53] (**Figure 3**):

$$PFD(s)=\frac{PFDin}{\int_{0.5\pi}^{1.5\pi}\cos(\theta+\pi)d\theta}\left[\int_{0.5\pi}^{1.5\pi}\cos(\theta+\pi)exp\left[-a_{chl-a}\cdot\left[chl-a\right]\cdot b\right]d\theta\right]$$

$$b=(r-s)\cdot cos\theta+\left[r^2-(r-s)^2\cdot sen\theta^2\right]^{0.5}$$

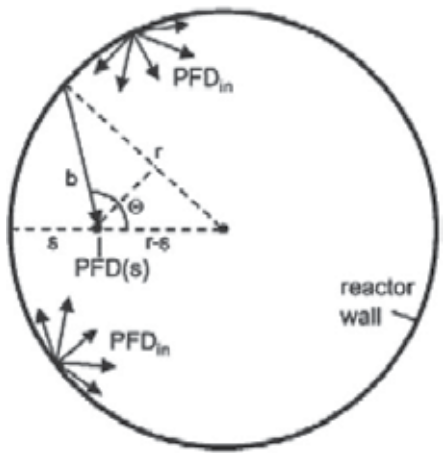

Figure 3. Light gradient calculus illustration according to the modified Evers Model [52, 53].

where PFDin is the photon flux density of the incident light intensity on the photobioreactor surface, PFD(s) photon flux density that saturates the photosystems, $a_{chl\text{-}a}$ wavelength dependent Chl-a specific absorption coefficient, Chl-a concentration, r radial distance, and s distance between the PBR surface and the hypothetical point where light saturation is reached at a certain PFD.

From above, it can be deduced that the microalgae inside a PBR will be moving between the three light regions due to the turbulence generated by aeration. Higher aeration rate generates more turbulence, and higher turbulence could generate faster movement of microalgae between the light regions.

If the nutritional requirements of a mass culture of microalgae are met, and the culture conditions do not limit growth, then a design aimed to create a turbulent flow will be the most important requisite to obtain higher biomass yields [54].

4.2. Biomass production enhancing: turbulence and mixing effects

Turbulence and mixing of cultures have three main effects: 1) prevent microalgae sedimentation, 2) avoid formation of nutritional CO_2 and O_2 gradients, and 3) moving cells through light gradients, where the quantity and quality of light received by cells vary [20].

The turbulence can be measured in two forms, one is using the Reynolds number (Re) [20], and the other form is using the Swirl number (Sn) [55]. From the definition of the Swirl number [56], the average turbulence or liquid movement inside a PBR can be calculated using the following expression:

$$Sn_v = \frac{\int_0^L \int \int_S UVrdSdz}{\int_0^L \int \int_S U^2 rdSdz}$$

where U is the mean axial velocity component; V mean circumferential velocity component; r is the radial distance from z-coordinate; and L is the photobioreactor length [47].

The use of Re is recommendable when the liquid properties and change with time are known, for example viscosity, but it is difficult to determine when the geometry of the photobioreactor is "special", this is with less common geometries or with baffles that help to create more turbulence, in those cases the use of the Sn is recommendable.

If a mass culture of microalgae has good mixing, then the cells will be exposed to a light gradient in their movement through the PBR [52]. Near the reactor irradiated surface, algal radiative exposure is usually adequate or in excess, whereas a dark volume with insufficient light for photosynthesis to occur often resides only a few centimeters or less from the irradiated surface, depending on the cell concentration [50].

Normally photosynthetic systems become saturated with light irradiance values of approximately 200 μmol photon m^{-2} s^{-1}, this value is equivalent to 10% of the maximum light irradiance in the summer of approximately 2000 μmol photon m^{-2} s^{-1}. The saturation of light is considered one of the main limitations of using solar light efficiently [51]. The former has awaken the interest of studying energy light usage with the objective of maximizing photoautotrophic organisms culture productivity [24], through the design of PBRs with geometries that can enhance and make better use of turbulence.

It has been proposed that the control of light irradiance to the culture can be made by giving the necessary amount of light based in cell concentration, aimed to maintain a luminostatic environment inside the PBR. In this sense "specific light uptake rate, qe" has been studied:

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$$q_e = \frac{(E_{in} - E_{out})A}{VxC}$$

where A and V are the surface and volume of the column or columns that contain the culture, C is the cell concentration. E_{in} is the input light energy to the PBR and E_{out} is the outgoing light energy from the PBR, these are quantified calculating the average value of the light intensity measures on 16 points, every $\pi/8$ radians, on the inner and outer surface of the PBR [24].

Although, luminostatic operation is efficient to generate high cell density cultures, it is evident that an amount of light applied to the exterior of a PBR will only be effective if it is combined with good mixing and turbulence (Re ≥ 3300) of the culture inside the PBR. For this reason, a key factor in the design of PBRs is the incorporation of mechanisms to periodically transport or expose cells between light and dark regions of the reactor (mixing-induced light/dark cycles) [50].

One alternative to make better use of light is providing internal illumination with light guides, which can increase the illuminated surface in the same volume of PBR [16] and, therefore, resembles artificial leafs described by Janssen et al. [52]. Solar light is captured by lenses and then transported to the PBR interior with the use of optic fibers [57] or with the usage of light guides of Polymethylmethacrylate (PMMA), which is a plastic that has more light transmittance compared with other plastics, this is why it is considered the ideal material for the construction of light guides. PMMA has a refraction index of 1.49-1.50 for visible light spectrum; much greater than the required 1.415, assuring a total reflection of the light inside the guide when it is surrounded by air, while it limits light reflection in the upper part of the guide [16].

Optical fibers have been previously used to provide internal illumination, better light dispersion, and increase illuminated surface per volume unit of PBR [57–59]. Unfortunately, large quantities of optical fibers are needed to achieve an increase on the illuminated surface to volume ratio in comparison with externally illuminated PBR. Costs and construction considerations for large scale cultivation systems using optical fiber further limited their application [57] because PBRs containing large quantities of optical fibers will not allow achieving good turbulence (Re ≥ 3300) and mixing at a low cost. Multiple light guides of PMMA seem to be more promising. One displayed after the other can increase the illuminated surface in large scale PBR because the effect of self shading inside the PBR, common in flat panel and column systems, will be reduced in internally illuminated systems, therefore, the potential of light capture and usage can be increased, achieving optimal conditions with a more uniform illumination.

Mixing, which governs the movement of the cells between the illuminated and the dark zones, can considerably enhance the productivity for a wide range of operational conditions, as it can create beneficial light fluctuations onto the cells. Mixing induced light/dark (L/D) cycles usually occur at frequencies on the order of 1 Hz or less, which is significantly lower than the minimum frequencies required to produce the flashing light effect (>25 Hz). Nevertheless, it has been demonstrated that photosynthesis can be enhanced by low frequency L/D cycles.

Mixing time decreases with the increase of the superficial gas velocity. Superficial gas velocity not only enhances the mixing and the light–dark cycle of microalgae, but also increases the rate of shear in the reactor, which is harmful. Mixing and the shear stress should be balanced carefully when a suitable superficial gas velocity is adopted. It has been reported that an optimal superficial gas velocity of 8.333 $x10^{-4}$ m s^{-1} for the cultivation of the *Chlorella vulgaris*. Economic analysis has estimated that mixing accounts for 53% of the total costs in some types of PBRs, and one critical challenge to algae biofuel generation was its poor energy balance due to high auxiliary energy requirements for the mixing and the mass transfer [50, 60].

5. Gas exchange, carbon dioxide and oxygen

Aeration rate is a key parameter to improve the growth of microalgae cell. Gas supplied to the culture increases the mass transfer coefficient, avoiding deficiency of CO_2, control the toxic level of dissolved O_2 and the inhibitory level of CO_2, reduce nutrients gradient, avoids cell sedimentation, clumping, fouling, and dead zones [31], can create an optimized light/dark cycle that can enhance the photosynthesis. However, excessive aeration may produce cell damage due to mechanical shear forces in susceptible microalgae. Also a high aeration rate will lead to high running costs. A deep knowledge of the fluid dynamics and the mass transfer is needed for the PBR rational design and optimization. It is necessary to understand the interplay among gas holdup, liquid circulation velocity, mixing, and gas–liquid mass transfer [31, 60].

5.1. Carbon dioxide mass transfer in photobioreactors

CO_2 consumption is proportional to microalgae growth rate; this consumption can be increased by increasing the light irradiation to the culture, but only when light is limiting the photosynthetic process. Because carbon represents approximately half of the dry weight of microalgae biomass, the CO_2 demand for cellular growth will be lower than the maximum demand at low light intensities. For example, the maximum demand of CO_2 in a flat panel PBR considering a maximum radiation of 1000 μmol photon m^{-2} s^{-1}, was a demand of CO_2 for photosynthesis that will require a value of CO_2 specific mass transfer rate (K_La CO_2) of only 4-6 h^{-1} (0.0011-0.0016 s^{-1}), due to the growth rate of microalgae [61]. Therefore, if the gas contains a low concentration of CO_2, it will require a high K_La to satisfy the CO_2 demand during microalgae growth [61]. The former K_La values are very low and most of the designed PBRs under normal operation conditions present a K_La of CO_2 from 10 to 100 times superior to this requirement [30], because CO_2 can be added to the air supplied to the PBR.

Even though from the economic point of view, a high aeration rate will increase costs, it is not recommendable for large scale production PBRs. It is necessary to establish a minimum aeration rate for each culture conditions [61]. An aeration rate of 0.05 vvm is appropriate for cell production, and is recommended for an efficient PBR [60].

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5.2. Avoiding O_2 deleterious effects

Considering a carbon fraction in the biomass of 0.45 and CO_2 as the only carbon source in the medium, it must be provided a minimum of 1.65 g of CO_2 to generate 1 g of microalgae biomass [19]. Additionally, to this stoichiometric aspect, the competitive inhibition of O_2 and CO_2 for the active site of the Rubisco enzyme (Ribulose-1, 5-diphosphate carboxylase oxygenase) must be considered. This is why O_2 removal from the medium is very important. O_2 can accumulate to levels that can be toxic to microalgae. Oxygen concentrations above 35 mg L^{-1} are toxic to most of the microalgae species [31]. In this sense, dissolved O_2 concentration of 100% is equal to 8.6 mg L^{-1}, this means that dissolved O_2 concentrations above 300% of air saturation can be detrimental to algal cells and therefore could reduce productivity [27, 62].

In closed systems like, for example, horizontal tubular PBRs of 5 cm of internal diameter with exponential growth of *Spirulina,* the oxygen concentration can increase up to 70-80 mg L^{-1}, even when there is ventilation every 50 seconds [51]. Oxygen build up, generated by photosynthesis, is a particular serious problem in closed PBRs with high area to volume ratio [31] and can generate a decrease of the biomass productivity of up to 33%, this imposes strong limitation to tubes length in tubular PBRs and scale up of PBRs [51].

To overcome this problem, some authors have proposed the use of degasifiers [63–66] (**Figure 4**), even though, to achieve an efficient gas separation from the liquid, the distance between the entrance and exit to the degasifier unit must be of a magnitude that allows the smallest gas bubbles enough time to separate from the liquid [31]. Another possible solution is the use of low altitude vertical PBR like for example flat panel PBRs [41, 42] or alveolar systems that allows more contact between the liquid and the air [67].

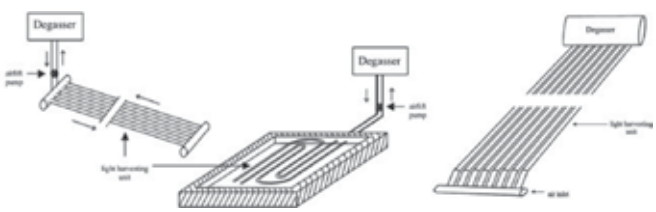

Figure 4. Degasifiers units coupled to horizontal PBRs designs [31].

5.3. CO_2 utilization efficiency

Another aspect that must be considered is CO_2 utilization efficiency. Daily fixated CO_2 as g CO_2 fixated per g^{-1} of injected CO_2 d^{-1} can be calculated with the formula FD = ($FA_{(t+1)}$ − FA_t)*mid^{-1}, where $FA_{(t+1)}$ is the accumulation of CO_2 fixed during t + 1(d), FA_t the accumulation of CO_2 during t(d) and mid(g) the mass in grams of CO_2 injected each day. It has been demonstrated that is possible to fixate even 80% of the introduced CO_2 (FDmax%) in an air current with 0.04% of CO_2 using vertical tubular PBRs connected in serial three stage manner, and up to 40% of the introduced CO_2 in an air current with 12% of CO_2 [28]. Therefore a high aeration rate results in low CO_2 utilization efficiency, indicating that it could be highly

expensive in an industrial facility to use CO_2 supplementation. Aeration conditions must be optimized considering biomass productivity and supplemented CO_2 utilization efficiency.

Despite all the presented alternatives are very ingenious, at this moment there is no universal unit to achieve an optimal degasification, the selected choice will depend on the cultured microalga and the preselected objective of this culture (for example, biomass, pigments, H_2 production, etc.).

6. Nutrients

Nutrient supply like nitrogen and phosphate is another factor of special interest. The dynamics of these nutrients are strongly coupled to each other, and to the metabolic processes present in the PBRs. Exploring the fate of nitrogen and other nutrients through the different biological pathways during cultivation in PBRs is a valuable tool for designing such systems for full scale with an ever growing demand for more efficient nutrient removal systems. For this, knowing the true metabolism of nutrients in PBRs and its effects on algae growth is vital [62]. For example, in nitrogen-deprived culture of *Haematococcus pluvialis* the photosynthetic electron transport chain is heavily damaged due to the significant reduction of cytochrome b6/f complex [68]. To prevent cells being overreduced by photosynthesis, a correct amount of nitrogen needs to be supplied.

6.1. Culture media development considerations

Nutrients are needed to generate biomass and high productivities. Concentration of macronutrients in the medium has a wide range and micronutrients have a narrower range [20]. Minimum requirements for medium composition are obtained from elemental mass balance [19]. Requirements for the development of culture media are enlisted as follows:

- The total salt content is determined by the habitat where the microalga was isolated.
- First, the composition of the major ionic components must be considered.
- Nitrogen sources are mainly nitrate, ammonium, and urea.
- The carbon source could be CO_2, HCO_3^-or organic carbon, like acetate or glucose.
- pH is generally required above 7 for maximum specific growth rate.
- Trace elements and chelated components, and vitamin requirements are considered last [69].

Medium nutrients can be manipulated in order to obtain a different responses from microalgae, for example, nutritional stress can be a strategy for the production of specific compounds like carotenoids and fatty acids that are produced when there is a nitrogen deficiency in the medium [25, 70, 71], low nitrogen conditions promotes the synthesis of these compounds while the synthesis of proteins and nucleic acids is inhibited.

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Also, it must be considered that the fact that some microalgae have the capacity of consuming nutrient in an excessive manner and store them, a phenomena known as "luxury uptake" [20], this type of consumption presents when cells have been exposed to a medium with low concentration of certain nutrients ("starvation") or when cells have the capacity of accumulating nutrients, in this last case a previous starvation is not required [72, 73]. It is important to highlight that luxury uptake is desirable in a strain used in waste water treatment, because it allows the removal of nutrients or contaminants from waste water without generating large quantities of biomass as a secondary product.

6.2. Importance of nutrient in the photosynthetic process

All organisms have minimum optimal and maximum nutrient requirements, and the nutrient level in the media affects the growth rate [20], media usually used on laboratories generally are not suitable to obtain high biomass concentration because high concentration of their components can inhibit growth or precipitate [19]. Fed-batch cultivation mode can be used to maintain adequate nutrient concentrations in the media, even though, very few works have been published in this theme [25, 70–73] and most of these works have been focussed on the fed of organic carbon source [74, 75], but heterotrophic cultivation of microalgae using an organic carbon source is not suitable for all species, also some strains often change their chemical composition under heterotrophic culture [5].

It is important to mention that microalgae productivity can be dependent on nutrients like nitrogen, phosphorous, magnesium, iron, and manganese because these are required during the photosynthethic process. Nitrogen is found in the form of proteins that form antenna complexes (LHC), reaction centers, and the enzymes that participate in the photosynthesis process. Phosphorous is required in phosphate form to store captured light energy as chemical energy in the form of NADPH and ATP. The magnesium in the porphyrin ring of the Chlorophyll molecule, iron is part of the ferredoxin molecule, this later is an electron transporter of the PSI, and last manganese is important because it acts as a cofactor in the oxygen liberator complex, this has the function of liberating electrons from the water molecule [76, 77].

Beside nutrients, there are other factors that can be manipulated in a PBR during its operation like the pH and temperature, but because each of these factors deserves its own review, they are not part of the discussion on the present chapter.

7. Productivity and costs

7.1. Maximum biomass productivity

In PBR design it is important to define the upper limits of light capture efficiency. Maximum biomass productivity has been determined to be 14.31 g dry biomass m^{-2} d^{-1}, considering an average solar irradiation of 1104 μmol photons m^{-2} s^{-1}. But some regions in the world possess

a higher solar irradiation, for example in some regions of the United States of America and Mexico, maximum solar irradiation ranges between 1450 and 2300 μmol photon m^{-2} s^{-1}, this can be equal to productivities as high as 29.81 g dry biomass m^{-2} d^{-1} [20, 78]. Main objective in the industrial-scale deployment of this new technology today is to decrease PBR costs without compromising system performances [15].

7.2. Biomass productivity in different photobioreactors

Reported biomass productivities per unit of land area for different PBR are limited by the sub-optimal conditions of light inside the PBR, this limits the biological photosynthetic efficiency, or even the design is the limiting factor that affects light usage inside of it. Therefore, high yields can only be obtained linking PBR design with the biological process occurring within. PBR efficiency is determined by factors like capture, transportation, distribution, and use of light energy [16], and also by the overall use of other main nutrients like carbon, nitrogen, phosphorous, magnesium, and manganese.

It has been reported for an outdoor cultivation in a facade PBR run on a whole-year basis, an expected yearly production of around 25–30 tons biomass per ha with *Chlorella vulgaris* (i.e. average daily productivity of 7.68 g m^{-2} day^{-1}), which corresponds to around 40–50 tons of CO_2 fixed per year per ha [15].

A cascade type open culture system with high cellular densities has been developed since 1970 and is still in use for *Chlorella* cultivation in the city of Trebon, Czech Republic. It can reach cellular concentrations as high as 10 g L^{-1}, with high growth rates and biomass productivity of even 25 g dry biomass m^{-2} d^{-1}. Despite its high yields, the implemented system at Trebon is very expensive because of the material (glass) used to give the PBR slope, but using other materials a similar system can be built at a significant lower cost. Also, in the geographic location of Trebon, it is only possible to use this PBR during a short period of time during the year because of climate conditions [79].

For economic feasibility of microalgal biorefinery, every cell components of microalgae need to be utilized as much as possible. Continuous or semi-continuous mode of cultivation for a long period helps to improve microalgae cultivation as commercially successful [15].

A semicontinuous system was used to produce *Chlorella* in a lagoon build up with plastic, near the city of Dongara located in Western Australia. This system had an average productivity near to 25 g dry biomass m^{-2} d^{-1}, because of the optimal climate conditions. Unfortunately, technical problems in its scale up led to the closure of this facility [5].

A narrow light path from 1.2-12.5 cm allows reaching cellular concentrations of up to 20 g L^{-1} and a volumetric biomass productivity of 0.25-3.64 g L^{-1} d^{-1} in outdoors cultures operated in fed batch mode. Ironically, the biomass productivity per area of land unit in a PBR displayed horizontally was 25.0-27.8 g m^{-2} d^{-1} [63, 80–8282] and was not superior to the reported for an open pond system, this last with a productivity of 25 g m^{-2} d^{-1} [79]. The same had been observed in alveolar type vertical panels [67]. Volumetric productivity in an inclined PBR, with a light

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path of 1.2-1.3 cm, was only 1.5-1.7 times superior to the one obtained in open cultures of 1 cm of depth [82].

7.3. Economical experiences in previous designs

Based on cost of materials and manufacturing labour extrapolations it has been estimated the capital cost of wall PBR at full production to be $25,000 per hectare. Open ponds ranges approximately from about $10,000 to almost $79,000 per hectare taking into account the costs of the liner and the paddlewheel [27].

Raceway type open ponds are used in Israel, United States of America, China, and other countries. It has been reported that this type of PBR can maintain a cellular concentration of 0.5 g L^{-1} and a productivity of 25 g m^{-2} d^{-1} [63]. Despite their low construction and operation costs, the average cost of these systems is $8-15 U.S. dollars per Kg of dry biomass [82]. The system that is more widely used in large scale facilities (near 1000 L) are the ones that use sterile plastic bags near to 0.5 m of diameter, adapted with an aeration system. These systems require intensive labour and generally present poor mixing. This makes very expensive to produce microalgae biomass. The costs are nearly $50 U.S. dollars per Kg of dry microalgae biomass, for smaller cultures the costs can raise up to $300 and even to $600 U.S. dollars per Kg. Costs are very high and superior to the estimated for the production of *Chlorella*, *Spirulina,* and *Dunaliella*, which are between $9 and $25 dollars per Kg [5].

Productivities and cost are important questions for an algal industry whose economic survival depends on production, and vary accordingly to cultivation methodology. From the perspective of scaling between laboratory to large-sized outdoor facilities, differences might arise in products or co-products expected if cultivation methods are not the same [25, 83].

8. Conclusions

Microalgae autotrophic growth is first limited by the photosynthethic process itself, and by the process of light energy captures and CO_2 conversion into biomass. This is why PBR design must consider this biological process and focus on providing light, CO_2, and other nutrients at a low cost.

Microalgae mass cultures can only be achieved with PBRs designs aimed to improve photosynthetic efficiency in light capture, maintaining at the same time adequate turbulence conditions that can promote cells movement through the different illuminated regions, a high mass transfer rate and high usage efficiency of supplied CO_2, allowing efficient O_2 removal produced by photosynthesis, and avoiding the generation of nutrients gradients; besides supply adequate nutrient quantities in the moment they are required, in order to improve their use by the culture. This can be done using PBRs with a long light path operating in fed-batch cultivation mode, if the design and operation characteristics are adequate.

Therefore, current designs of PBR still can be improved with the objective of lowering costs, increase efficiencies, and maintain high productivities. New PBR materials and different

culture modes need to be investigated and evaluated because responses of specific strains cannot be inferred from other PBRs or culture conditions. Future investigations must consider microalgae as systems and aim to evaluate interactions between photosynthetic efficiency, CO_2 and nutrient assimilation under different culture modes and operation conditions.

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Abbreviations

CO_2	carbon dioxide
PSI	photosystem I
PSII	photosystem II
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ATP	adenosine-5'-triphosphate
PAR	photosynthetic active radiation
PCE	photon conversion efficiency
η	energy conversion efficiency
η_{solar}	solar energy conversion efficiency
LHY	light harvesting yield
FEY	fractional energy yield of the PSII redox products
QY	quantum yield
P680*	excited PSII reaction center
Q_B	quinone B
PBRs	photobioreactors
Io	incident light intensity on the photobioreactor surface
Is	photosystems saturation light intensity
PFDin	photon flux density incident light intensity on the photobioreactor surface

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PFD(s)	photon flux density that saturates the photosystems
a_{chl-a}	wavelength dependent Chlorophyll a specific absorption coefficient
[Chl-a]	Chlorophyll a concentration
r	Photobioreactor radial distance
s	Distance between the photobioreactor surface and the hypothetical point where light saturation is reached at a certain PFD
Re	Reynolds number
Sn	Swirl number
U	Mean axial velocity component
V	Mean circumferential velocity
L	Photobioreactor length
S	cross-sectional surface area
dS	differential cross-sectional surface area
Fv/Fm	Maximum photosynthetic efficiency of dark adapted cells
Fq'/Fm'	Operational photosynthetic efficiency of light adapted cells
q_e	specific light uptake rate
A	photobioreactor superficial area
V	photobioreactor operational volume
C	Cellular concentration
E_{in}	input light energy to the photobioreactor
E_{out}	outgoing light energy of the photobioreactor
PMMA	polymethyl methacrylate
FD	daily CO_2 fixation in g CO_2 fixed per g CO_2 injected per day
$FA_{(t+1)}$	accumulation of CO_2 fixed during t + 1 (d)
FA_t	accumulation of CO_2 during t (d)

mid mass in grams of CO_2 injected each day (g).

FDmax % overall maximum percentage daily CO_2 fixation

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Chapter 4

Algae as an Indicator of Water Quality

Didem Gökçe

Additional information is available at the end of the chapter

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Abstract

The formation of plankton/algae under natural conditions is related to tolerance class (ecological optimum) due to abiotic limiting factors of ecosystem, as well as the biotic interactions among algae. In the ecological niche, the appearance of organisms is affected by anthropogenic and non-anthropogenic environmental factors. Algae composition and temporal variation in abundances are important in determining the trophic level of lakes. Algal communities are sensitive to changes in their habitat, and thus, total biomass of algae and many algae species are used as indicators of water quality. Algae communities give more knowledge on variations in water quality than nutrient or chlorophyll-a values. Water quality is a canonical group of physical, chemical, and biological properties of the given water. Consequently, eutrophication of freshwater is regarded as a water quality which results in the degeneration of the aquatic ecosystem and affects water utilisation. Cyanobacteria has been accepted as a major indicator of eutrophication in freshwater as their blooms are common in waters affected by nutrient concentration. The purpose of this chapter is to assess physical and chemical variables and the role of algal abundance to determine the water quality in the freshwater ecosystems.

Keywords: algae, biomonitoring, indicator, nutrients, spatial-temporal variation, water quality

1. Introduction

Algae are a significant component of biological monitoring programs for assessing water quality. They are eligible to water quality assessment because of their nutrient requirements, rapid reproduction rate and very short life cycle. Algae are important indicators of environment situation since they respond immediately to both qualitative and quantitative composition of species in a wide range of water situations due to alters in water chemistry such as increases in

water pollution based on domestic/industrial wastes and affect the composition of genera that are able to tolerate these situations.

From an ecological and public health perspective; the abundance of nutrients-containing nitrogen (N) and phosphorus (P) that flow into lakes, reservoirs, and the other aquatic ecosystems resulting in eutrophication is of great importance. The N:P ratio identifies which alga genera are dominant, present or absent in these nutrient impacted aquatic ecosystems [1]. Sources of inorganic compounds that contain these elements involve domestic detergents, commercial fertilizers used for agriculture, and runoff along with organic pollution from sewage and livestock waste.

Biological analyses can define possible changes in water quality, as well as the tendencies with times that are reflected in environment variations and the composition of aquatic organisms. Phytoplankton consists of a large diversity of algae with different forms and life history strategies to increase productivity; planktonic genera such as *Microcystis*, *Anabaena*, *Nodularia*, *Planktothrix*, *Aphanizomenon*, *Cylindrospermopsis*, *Trichodesmium*, which have gas vacuoles that help to float, or benthic species (*Lyngbya*, *Phormidium*, *Oscillatoria*, *Schizothrix*) that tend to locate at the sediment [2], and neutrally buoyant algae having a similar density to water such as *Oocystis* and *Chlorella* and species of Dinoflagellates and Euglenophyta migrating freely in the water column [3].

When reservoirs and lakes become more eutrophicated, the diversity of phytoplankton species gradually declines, which leads eventually to Cyanobacteria dominance and toxin production [4].

Phytoplankton communities are sensitive to alterations in their habitats, and thereby, phytoplankton total biomass and many phytoplankton species are utilized as indicators of aquatic habitat qualifications [3, 4]. Phytoplankton/algal communities give more evidences concerning alterations in water quality than nutrient or chlorophyll-a concentration. Water quality is a whole of physical, chemical, and biological properties of the water [5].

It is important to consider that the phytoplankton community changes quickly as a response to changes in water quality. The first reaction on such changes in the water environment is a quantitative change of the phytoplankton community. The amount of algae increases or decreases depending on the type of impacts on the water mass, which is followed by qualitative changes of the phytoplankton community. New species colonize in the lakes and some of the original species may decrease in importance based on local extinction in some cases.

2. Bioindicator systems

According to in Ref. [6], the Baas–Becking hypothesis [7], which explains that 'everything is everywhere—the environment selects', has dominated the view on microbial distribution for decades and has definitely conduced to the prevailing concept that algae are cosmopolitan organisms. While physical and chemical water quality measurements can indicate the level of water degradation, these methods represent only a "*snapshot*" of the current conditions in

aquatic systems by giving only a transient picture of prevailing environmental conditions. The abundance and community patterns of organisms "*in-situ*" reflect precisely the water quality at any point. Organisms can be used to compare relative variations in water quality in terms habitat variability or time [8–12].

Water quality parameters (particular and dissolved nutrients concentrations, suspended solids and/or turbidity, and chlorophyll-a as phytoplankton biomass) are usually detected directly, either by traditional periodical water sample collection and following analysis, or with continuously logging instruments (available for turbidity and chlorophyll) [8]. However, the dilemma is that periodical measurements of parameters that vary significantly with time frames less than the sampling interval will require long time series to detect any change; whereas, continuously logging instruments are expensive to purchase and require regular field working for maintenance. Moreover, as our understanding of relationships between habitat situations and biological communities remains poor, it can be difficult to explain the ecological relevance of detected variability in water quality. For these reasons, numerous studies have proposed to use bioindicators for evaluating changes in water quality, testing various measures of lakes and rivers, and assessing biological organisms [13].

It is important that bioindicators amalgamate ecological conditions over time. The changes in any environmental condition cause variation of bioindicator groups. Theoretically, a bioindicator system should combine a number of specific measures that vary in their effect ranges and response times to altering water quality [14] and which can be quantified during intermittent visits once reference point levels are established. The Ref. [15] defines the term bioindicators as characteristic species or communities, which, by their presence, give information about the surrounding physicochemical properties of ecosystem (water quality) at a specific site.

2.1. Why are bioindicators better than the other methods?

Bioindicators comprise biological processes, species, and communities and are used to assess water quality of the ecosystem and how the water quality alters with time. Variations in the ecosystem are often related to anthropogenic effects or natural stressors. Finally, the use of bioindicators caused a drastic change in classic measure of their ecosystem quality and offers great benefits:

1. Bioindicators add a periodical component corresponding to the life span or residence time of an organism in a specific system by allowing the integration of current, past or future habitat situations. Unfortunately, many physicochemical analyses describe only situations at the time of sampling but have an increasing probability of missing inadvertently pulses of pollutants. Furthermore, contaminants can occur at very low concentrations. On the other hand, the tolerance range of bioindicators obtains a frame of biologically significant pollutant values, no matter how small.

2. Bioindicator indicates indirect biological impacts of pollutants when physicochemical analysis cannot indicate (phosphorus concentration effects on phytoplankton population). Therewith, chemical measurements may not precisely demonstrate a declining in

species diversity or how the growth and reproduction of other species may decline depend on competitive exception.

3. Indirect contaminant effects are complex to explain from chemical and physical analysis in case of bioaccumulation. Other contaminants such as metal accumulate in organisms are found at concentrations to amplify through food webs. Therefore, contaminant values at higher trophic levels may be insufficient to describe physical and chemical analysis.

4. While the use of whole communities (all species' responses within them) can be informative, problems can arise in especially speciose habitats. Moreover, a bioindication signal can be unclear by an excessive number of divergent species' responses (some species may increase while others decrease). This narrowed approach makes monitoring more biologically relevant and cost-effective.

Also, a general dilemma about physicochemical analysis is that they simplify a complicated indigenous response in these species-rich habitats. Due to complexities of environments bioindicators use a representative or collected response to transmit a dynamic condition of the environments.

Communities of algae living in the water bodies provide evidences of the environmental history of the water in two ways: firstly, by differential sensitivities and recovery rates of species to substances in the water; and secondly, by concentration and accumulation of substances in their cells [16].

Algae are known to have very specific requirements for growth and reproduction, and the presence of a characteristic species in a habitat remarks that the given determinant is within the tolerance limits of that species. It is in the sense that the term 'bioindicator' is used.

On the other hand, not all biotic processes, species or communities can serve as successful bioindicators. Physicochemical and biological factors such as substrate, light, temperature, competition vary among habitats. With time, populations develop strategies to maximize growth and reproduction within a specific range of habitat conditions [17]. Bioindicator species indicate effectively the environmental situation cause of their moderate tolerance to fluctuation of ecosystem properties [18–20].

In contrast, rare species (or species assemblages) with narrow tolerances are often too sensitive to habitat alterations, or too sporadically encountered, to demonstrate the general biological response. Bioindicators possess a moderate tolerance to ecosystem variability compared to rare and cosmopolite species. This tolerance affords them sensitivity to indicate ecosystem alterations, yet endurance to withstand some variabilities and show the general biological response.

Bioindicator species differ from key indicator species, while both are useful in revealing information about their habitats. Bioindicators explain the characteristic of habitat through their population abundance of particular responses to the ecosystem. Key indicator species are those species that are essential to an environment. If such species were to disappear, an important of the food web will disappear or undergo shifts in dominant species.

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On the other side, the utilization of bioindicators is not just restricted to a single species with a limited ecological tolerance. Entire communities, involving a wide range of ecological tolerances, can serve as bioindicators and represent multiple sources of data to assess ecosystem situation in a "biotic index" or "multimetric" approach.

3. Water quality monitoring

The studies using phytoplankton and algae for water quality monitoring have revealed that alteration in composition demonstrates not only variations in water quality, but also alterations in physical parameters and biotic relationships. The Ref. [21] asserts that differences in water chemistry may change relative proportions of a few dominant taxa but often have a little effect on general species composition. and growth rates of species taxonomically change under influence of factors regulating composition of algae and population structure, which is closely regulated with water quality.

Algae were indicated as a beneficial tool for evaluating long-term alters in ecosystem such as those related to eutrophication, water management, alters in land use at the scale of watershed, and climate changes. In this sense, algae appear a useful biological indicator because they respond rapidly to alterations in ecosystem situations, thus enabling a quick assessment of water quality [21].

In general utilization, the terms "biomonitoring" and "bioindication" are substitutable; on the other hand, in the scientific sense, these terms have more specific meanings. Bioindicators qualitatively evaluate biological responses to ecological stress (abundance of algal species), while biomonitors quantitatively define a response (reductions in algal chlorophyll content or diversity indicates the presence and severity of water pollution). The term "bioindicator" is used as a unique term to refer to all terms related to the detection of biotic responses to ecological limiting factors. There are some major monitor roles of bioindicators:

1. The ecosystem qualification (physical and/or chemical fluctuations).
2. Ecological processes.
3. Biodiversity [22].

Chemical analysis of water quality such as inorganic nutrients, organic/inorganic pollutants and salinity is descriptive. However, conducting continuous analysis is not useful due to the associated time and particularly cost limitations. Nevertheless, biological measures can show all aspects of water quality with time and give a direct measure of the ecological impact of ecosystem variables. Biomonitoring allows a reliable relatively low-cost way to record conditions over a number of sites [15].

The following properties of the algae make it better suited to biomonitoring than the other biota [16, 23]:

- The algae are autotrophic and placed at the interface between the habitat and biotic components of the food web.

- The algae are mainly sessile; they cannot migrate to avoid pollution so must tolerate or disappear.
- Algal communities are species-rich and each species has its own tolerances.
- All algae have short life cycles and so have a rapid response to change, while the community lives long enough to integrate impacts with time.
- The algae are spatially dense and easy to sample and store.
- The algae are smaller in size than other biota and so are potentially more sensitive to pollution at lower concentrations.

4. Phytoplankton/algal ecology in lake and reservoir management

A vital key to the management of water bodies has been the development of models linking nutrient loading to the reduction of algal biomass to levels acceptable for a particular usage [24]. While phosphorus is described as limiting in most temperate lakes, nitrogen plays a crucial role in forming phytoplankton communities in tropical aquatic ecosystems [25].

Assessments related to phytoplankton are required to involve taxonomic composition, abundance, biomass, and plankton blooms for the ecological classification of waters [26, 27]. Phytoplankton succession and community composition demonstrate habitat situations of the ecosystem and nutrient availability acts an important role [28–30] in structuring that community. Given suitable habitat situations, the main biotic response to nutrient enrichment in aquatic ecosystems is the growth of phytoplankton and aquatic macrophyte.

Algae, in particular phytoplankton, play a fundamental role in lake and reservoir ecosystems, where nutrients are known to limit primary productivity rates. Populations in water bodies reflect the quality of water and affect community structure, biomass and productivity rates [31, 32]. Studies conducted on the relationships between algae and its fluctuating environment have resulted in an understanding of the main driving forces behind temporal and spatial patterns of its existence.

Desmids, Chrysophyceae and diatoms of the genera *Tabellaria* and *Cyclotella* peculiarize the infrequent but diverse plankton of the oligotrophic Caledonian-type lakes; Cyanobacteria (*Anabaena*, *Aphanizomenon*, *Microcystis*) and the diatoms *Asterionella*, *Aulacoseira*, *Fragilaria* and *Stephanodiscus* are demonstrative for more eutrophic levels [33]. Diatoms, Chlorophytes and Cyanobacteria occur right across the width of the spectrum by embracing from ultra-oligotrophy to hyper-eutrophy. Desmids, centric diatoms, even the Chrysophyceae, occupy substantial horizontal blocks. As in single orders, such as, the Oscillatoriales, and the same ostensive genera such as *Planktothrix*, *P. rubescens* has dominance in deep, mesotrophic alpine lakes; and, to *P. agardhii* has dominance in shallow basins in case of increasing nutrient content. Generally, *Chlorella*, *Scenedesmus*, *Ankistrodesmus* and Euglenoids are found in the nutrient-rich and eutrophic lakes. While *Ceratium*, *Microcystis*, *Aulacoseira granulate* in summer in the eutrophic lakes, *Cyclotella* spp. and *Aulacoseira subarctica*in the mesotrophic temperate

lakes in spring or *Dinobryon*, *Uroglena*, *Gemellicystis* and *Sphaerocystis in the oligotrophic lakes have high abundance*.

The abiding challenge is to explain *why* the algae should be distributed in this way. The trophic status of a habitat is dependent on community succession of some species. Phytoplankton succession follows R-C-S strategies both in natural and experimental situations [34]. Experiments completed in mesocosms revealed that the initial community primarily represented by R- and S-strategists (*Planktothrix*, *Cylindrospermopsis* and *Microcystis*) was gradually substituted by C-strategists *Cryptomonas* spp. Chlorococcales in general [35]. The Ref. [4] indicated that the Cyanobacteria, *Planktothrix* overwhelmingly dominated during the dry period and was succeeded by the colonial Cyanobacteria, *Microcystis aeruginosa* in the wet period and the members of Chlorococcales simply co-existed along with these dominant species of Cyanobacteria.

The potential ecological complications of nutrient fortification and disturbance also comprise modifications of the natural phytoplankton community composition, which may in turn alter ecosystem food web and nutrient cycling [17, 36]. If the growth of more readily grazed phytoplankton groups such as diatoms is favored, trophic transfer and nutrient cycling will occur widely in aquatic ecosystem, with enlarged export of the assimilated algae to lake ecosystems. On the other hand, if the nutrient loading favors the phytoplankton functional groups, which may not be readily grazed, such as Dinoflagellates, trophic transfer will be poor and relatively large amounts of unconsumed algal biomass will eventually be found in the sediment.

The Refs. [37, 38] reported that the collected seasonal epipelic diatom samples were correlated with a combination of environmental variables such as temperature, the concentrations of nitrite, and percentage of total organic carbon in the sediment.

The growth rate of phytoplankton hinges on bioavailable phosphorus and nitrogen values in water, light intensity and half saturation constants of nutrients, and light in compliance with the Michaelis–Menten function [39].

On the other hand, in the growth rate, the limitations of each nutrient and light are multiplied instead of the usual way to take the minimum of the three limiting factors. Thus, the growth rate hinges on availability of both nutrients and light at the same time, not just on the minimum factor. Expressed in this way the growth rate is not as sensitive to the limiting factor and inexactness in nutrient relations (**Figure 1**). The nutrient level that can be utilized by algae is calculated by subtracting the nutrients in algae and detritus from the total nutrient value [39].

Before beginning a discussion on plankton indicators, it may be preferable to indicate that some clutters have resulted from two different approaches. On the one side, attempts have been made to characterize plankton by the number of species, regardless of whether these species are presented by few or many individuals. On the other side, we have the search for ecological dominants and concurrent attempts to classify and name communities or associations by their dominant species. Therefore, the plankton of an oligotrophic lake is represented by desmids, which means that it includes many species of desmids but the plankton sample may be dominated by diatoms. This is a very general condition in oligotrophic lakes.

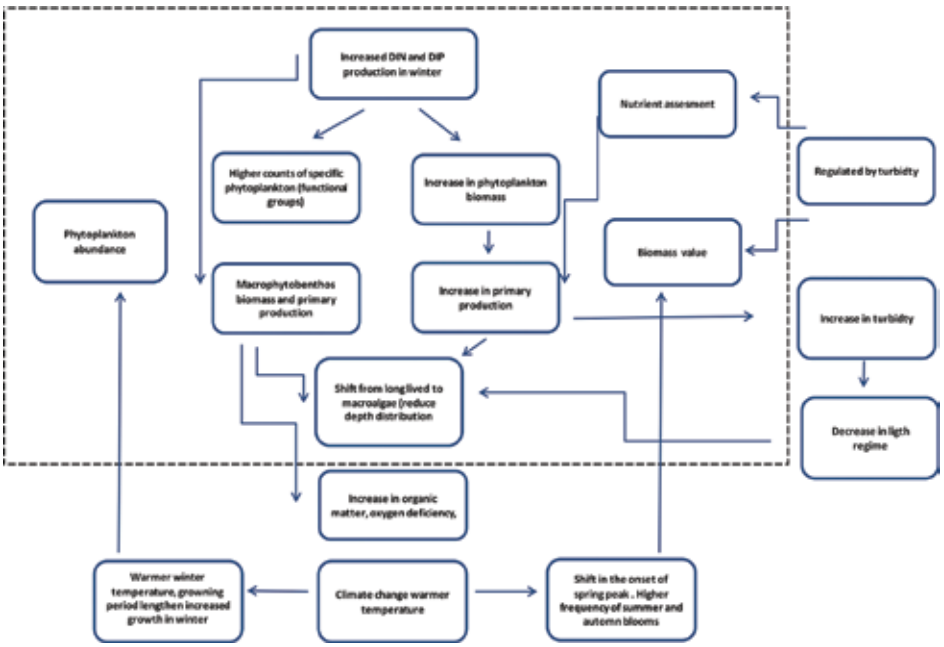

Figure 1. Diagram of the phytoplankton responses to changing nutrient and environmental conditions (according to [36]).

Multivariate methods such as classification and ordination have been used to explore cooperation among ecological factors or sites and to reveal the significance of hierarchy of their variability. These methods aid in making ecological clarification of field data and in creating new hypotheses [31, 40].

5. Indicator values of the algal groups

In general, the water quality is recognized and detected by several physical, chemical, and biotic procedures. The biotic analysis (qualitative and quantitative analyses of phytoplankton communities) is performed in support of the interpretation of the results gained from physical and chemical analysis of the water. The monitoring of phytoplankton and algae is of great significance because the monitoring based solely on physical and chemical analysis is sometimes insufficient. The phytoplankton composition not only demonstrates the certain situation of the waters but also the previous situations of aquatic ecosystem. Phytoplankton demonstrates water quality through changes in its community composition, and distribution, and proportion of sensitive species [41].

Species rarity is of specific significance in total structure of species diversity. Rare species constitute an important component of species richness and are a focus of many ecological theories and disputations [41, 42]. If rare species constitute the largest component of species

richness, they may act an important role as a 'safety net' for community conservation and diversity [42]. Species diversity diminishes to minimum levels when one or a few species are dominant.

The Ref. [36] showed that a number of indicators of nutrient improvement are briefed below. These indicators related to indicators of direct effects:

- Too much growth of phytoplankton in the aquatic ecosystem.
- Perturbation in specific plankton community.
- Too much growth of opportunistic macroalgae on intertidal sediments and rock.
- Too much growth of epiphytic algae, particularly on macrophyte.

Indicators of undirected effects include:

- Oxygen depletion in the water gradually increases. After this effect, phytoplankton blooms start to disappear. This could have lethal and sub-lethal effects on fish and invertebrates.
- Increased turbidity in the water bodies causing to decrease photic zone and shading out macrophytes.
- Reduction of oxygen in surface sediment causing to anoxia. This could have lethal influences on invertebrates which would also effect birds feeding on them.

Chlorophyll-a values characterize a very simple and integrative determinant of the phytoplankton community response to nutrient improvement. An increase in the phytoplankton biomass can be detected as an increase in the chlorophyll-a values (**Table 1**). Chlorophyll-a is a profit parameter of phytoplankton and algae biomass and is arguably the single most responsive indicator of N and P enrichment in the freshwater ecosystem [36].

Variables (indicators)	Threshold	Units	Trophic status
[24]			
TP	<7.9	μg/L	Oligotrophic
	8.0–11.0	μg/L	Oligotrophic to mesotrophic
	12.0–27.0	μg/L	Mesotrophic
	28.0–39.0	μg/L	Mesotrophic to eutrophic
	>40	μg/L	Eutrophic
Chl-a	<2.0	μg/L	Oligotrophic
	2.1–2.9	μg/L	Oligotrophic to mesotrophic
	3.0–6.9	μg/L	Mesotrophic
	7.0–9.9	μg/L	Mesotrophic to eutrophic
	>10	μg/L	Eutrophic

Variables (indicators)	Threshold	Units	Trophic status
SD	>4.5	m	Oligotrophic
	4.5–3.8	m	Oligotrophic to mesotrophic
	3.7–2.4	m	Mesotrophic
	2.3–1.8	m	Mesotrophic to eutrophic
	<1.7	m	Eutrophic
[27]			
TP	<10	μg/L	Oligotrophic
	10–35	μg/L	Mesotrophic
Chl-a	35–100	μg/L	Eutrophic
	<2.5	μg/L	Oligotrophic
	2.5–8.0	μg/L	Mesotrophic
	8.0–25	μg/L	Eutrophic
[5]			
DIN	<6.5	μM	(Good) Oligotrophic
	6.5–9.0	μM	(Fair) Mesotrophic
	9.0–16.0	μM	(Poor) Mesotrophic to eutrophic
	>16.0	μM	(Bad) Eutrophic
	<0.5	μM	(Good) Oligotrophic
DIP	0.5–0.7	μM	(Fair) Mesotrophic
	0.7–1.1	μM	(Poor) Mesotrophic to eutrophic
	>1.1	μM	(Bad) Eutrophic

TP: total phosphorus; Chl-a: chlorophyll-a; SD: Secchi disk; DIN: dissolved inorganic nitrogen; DIP: dissolved inorganic phosphorus.

Table 1. Some indicators for evaluating trophic status based on physical, chemical, and biological parameters.

Generally, two kinds of indices can be recognized. Indices are based on trophic levels of each species. To construct them, the abundance of each species in lakes with different nutrient levels is estimated by taking a trophic score, and in some cases, an indicator value into account [43]. These indices are based on the consideration that along with a gradient in nutrient concentration, each status can be identified by a specific structure of algal community [44].

All these indices are based on data from a number of lakes belonging to a relatively homogenized habitat in order to minimize the effects of biogeographic and climatic properties. These data are used to evaluate the trophic levels and the indicator values of the species, either by weighted averages [45] or using the lake score in a constrained ordination, considering the gradient in nutrient concentrations as the interpretive variable [43].

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Diversity and similarity indices are an approach to estimate biological quality through the structure of the community. Diversity indices estimate the data on abundance within species in a population. The frequency of each species present in the fixed samples is determined according to relative units: (1) occasional, (2) rare, (3) frequent and (4) dominancy. Different indices are used to estimate the community structure:

1. Hurlbert's probability of interspecific encounters (PIE) [46]:

$$PIE = \left(\frac{N}{N-1}\right)\left(1-\sum_{i=1}^{s} p^2{}_i\right)$$

N = the number of individuals in a community,

pi = the fraction of a sample of individuals belonging to species I;

2. Margalef's species richness [47]:

$$D = \frac{S-1}{lnN}$$

S = the number of species in a sample,

N = the number of individuals in a community;

3. Menhinick's diversity [48]:

$$D = \frac{S}{\sqrt{N}}$$

S = the number of species in a sample,

N = the number of individuals in a community;

4. Shannon–Wiener diversity index [49, 50]:

$$H' = -\sum_{i=1}^{s} \frac{n_i}{n} \ln \frac{n_i}{n}$$

N = the number of individuals in a sample from a population,

n_i = the number of individuals in species i from a population;

As shown in **Table 2**, three classes of water quality were defined for the Shannon–Weaver diversity index by [51], who implied that a high H′ value suggested a rich diversity and

therefore a healthier ecosystem (less pollution), whereas a low H′ value suggested poor diversity and thus a less healthy ecosystem (more pollution).

Shannon–Weaver	Class	Condition
>3	I	Clean water quality
1–3	II	Moderate pollution
<1	II	High pollution

Table 2. The water quality classes determined for the Shannon–Weaver diversity index.

5. Pielou's evenness index: The evenness of a community can be represented by Pielou's evenness index [52]:

$$J' = \frac{H'}{H'_{max}}$$

H′ is the number derived from the Shannon diversity index and

H'_{max} is the maximum value of H′

J′ is constrained between 0 and 1. The less variation in communities between the species, J′ is higher.

6. Simpson's diversity index [53]:

$$D = \sum_{i=1}^{s} \frac{n_i(n_i - 1)}{n(n-1)}$$

n = the number of individuals in a sample from a population,

n_i = the number of individuals in species i from a population

7. McNaughton's dominance index [54]:

$$I = \frac{n_1 + n_2}{N} x100$$

N = the number of individuals in a community,

n_1, n_2 = the number of individuals of the two most dominant species in the sample;

8. Algal genus/species pollution index [55], as shown in **Table 3**. The index factors of the algae present are then summed. The top 20 algal species are rated on a scale from 1 to 6 (intolerant to tolerant), and the index is simply calculated by summing up the scores of all related species

present within the sample. Pollution scores of >20 means high organic pollution, scores between 15 and 19 mean probable evidence of organic pollution, and scores of <15 mean no or very low organic pollution and lack of nutrient enrichment.

Algal pollution index	Condition
≤14	Low organic pollution
15–19	Moderate organic pollution
≥20	High organic pollution

Table 3. Algal pollution index assesses the tolerance of algal species to organic pollution and for rating water quality.

9. Jaccard's similarity index is a measure of the similarity between two samples [56]:

$$J = \frac{A}{A + B + C}$$

A = the number of data points shared between the two samples and

B and C = the data points found only in the first and second samples, respectively.

10. Saprobic index (S) [9]:

$$S = \frac{\sum(rh)}{\sum(h)}$$

r = the taxon saprobic rating (1 = oligosaprobic organism, 2 = β-mesosaprobic organism, and 3 = α-mesosaprobic organism),

h = the taxon occurrence rating (1 = occurring incidentally with <100 cells ml^{-1}, 2 = occurring frequently with 100–200 cells ml^{-1}, and 3 = occurring abundantly with >200 cells ml^{-1}; **Table 4**).

Saprobic index	Class	Condition
1–1.5	I	Very slightly contamination
1.5–2.5	II	Moderate contamination
2.5–3.5	II	High contamination
3.5–4	IV	Very high contamination

Table 4. Water quality classes according to saprobic index.

11. Carlson's Trophic State index (TSI) [57]:

Eutrophication is the situation by which lakes are enriched with N, P, and organic compounds, increasing the production of rooted aquatic plants and algae. This is a condition demonstrating a lake's trophic level. This is a measure of the trophic classification of a lake by using several analysis of water quality including: transparency or turbidity (Secchi disk depth), chlorophyll-a values (algal biomass), and total phosphorus concentrations. The TSI ranges from 0 to 100. Oligotrophy is between 0 and 30 TSI, where water is very clear, phosphorus is low, and algae are sparse. Thirty to fifty is a level showing increased in algae due to more available phosphorus (**Table 5**). If the TSI is more than 50, it describes a hypereutrophic habitat. In the Ref. [58] was detected water quality of Akgöl Lagoon was detected as mesotrophic level due to Trophic State index.

TSI	Chl-a (μgL^{-1}) (surface)	SD	TP (μgL^{-1})	Trophic class/water quality
<30	<0.95	>26.2	<6	*Oligotrophy*, clean water, oxygen throughout the year at the bottom of the lake
30–40	0.95–2.6	13.1–26.2	6–12	Bottom of shallower lakes may become anoxic
40–50	2.6–7.3	6.6–13.1	12–24	*Mesotrophy*, water moderately clear most of the summer
50–60	7.3–20	3.3–6.1	24–48	*Eutrophy*, algae and aquatic plant problems possible
60–70	20–56	1.6–3.3	48–96	Cyanobacteria dominate, algal scums and aquatic plant problems
70–80	56–155	0.8–1.6	96–192	*Hypereutrophy*, dense algae and macrophytes, light limited
>80	>155	<0.8	192–384	Algal scums, few aquatic plants

TP: total phosphorus; Chl-a: chlorophyll-a; SD: Secchi disk.

Table 5. Water quality classes according to Trophic State index (TSI).

12. Species number; species number as a simple measure of species richness, in spite of its simplicity, was reported to be a good tool for eutrophication appraisement in the freshwater ecosystem.

13. A similarity index was calculated by using the Euclidean distance algorithm. Two kinds of matrices were used: one considering only species presence–absence data and the other considering both presence–absence and abundance data.

6. Case studies on relationship between algae composition and water quality

The objectives of these studies were: (1) to recognize freshwater properties (physiological, population and community structure) that consistently change along with water quality gradients, and to quantify their direction and size of responses; (2) to recognize the water

quality variables that best prognosticate alters in these prospects; (3) to isolate and calibrate a final set of bioindicators, based on their consistency of response across regions and with due consideration of the practicality of their measurement, which can be monitored as a proxy of water quality in places where direct water quality analyses are not available.

According to the Ref. [59], blooms of *Anabaena circinalis, A. spiroides, and Aphanizomenon flos-aquae of filamentous Cyanobacteria were* coincided with a decrease of the zooplankton abundance in the Yeniçağa Lake. It may be assumed that the appearance of inedible filamentous cyanobacteria results from the eutrophication of the lake, and so there is evidence concerning the eutrophication steps.

Lake trophic condition is typically evaluated by monthly sampling of some kinds of physical and chemical indicators. If changes in species diversity and population abundance occur from either direct or indirect ecological stressors, then changes in biota may be used to assess descriptive alters in the ecosystem. The altitude of lakes will affect the algal diversity depending on oxygen saturation changes. Increases in growth rate and the algal diversity at high altitude lakes will indicate that they can be safe and productive water sources for the future time. The Ref. [60] investigated that the physicochemical properties and planktonic composition of the lakes showed that there was a fast tendency towards 'eutrophism' especially Mogan and Abant lakes. The temporal and spatial variables of situations affecting structure of the plankton composition appeared in the two lakes which were mainly resulting from pre-eutrophication. The Ref. [61] pointed out clearly that water quality monitoring was based on algal community structure. Especially, the improvement of diatom-based pollution indices has become a significant part of water quality monitoring in Turkey.

Furthermore, Refs. [62–64] have indicated that certain indices for the appraisement of eutrophication states in aquatic habitats are not as highly developed as in freshwater ecosystems and are not clearly defined. Plankton size structure can be used as a common taxon-independent tool for the study of community and ecosystem structure in aquatic habitats, in order to evaluate energy flows, biomass and abundance appropriation among different size fractions or different size classes in continuous size range. It is widely indicated that morphometric size has significant implications for the physiology and ecology of species through underlying dynamics that identify specific body size due to metabolic rates and ecological regulation of organism density, which in turn affect coexistence mechanisms.

Due to multiple species forming communities, certain adaptations are shown to limiting environmental factors and biochemical tolerance limitation of individuals of different species in the community constitute such type adaptation. At the other side, there might be species that are each limited by different nutrients (homogenized habitat) [65]. Thus, limitation at the community level is probably dependent upon a combination of mechanisms, from those that cause species to be similarly limited by the same nutrients, and to niche specialization mechanisms that cause species to be differently limited by different nutrients. Due to the effect of ecological factors, these species are considered as indicator species in the habitat according to their tolerance limits. According to Ref. [22], managing an aquatic ecosystem consistent with the ecological requirements of a specific bioindicator may fail to protect rare species with different necessities.

7. Conclusion

Algal communities react rapidly to disturbance of water, for example physicochemical conditions of water or to pollution-affected catchment area. They frequently change their species composition or diversity, which can vary from species-rich to monotonous communities. Because of this characteristic, benthic diatom communities are useful tools in detecting anthropogenic impacts.

The qualitative samples and community measurements of algae are beneficial in order to monitor the aquatic ecosystems. Majority of the interannual differences in temporal dynamics of algae, from oligotrophic to hyper-eutrophic lakes, can be caused by ecological factors, morphometry and climate. There are modest interannual variations that may affect the numbers, relative abundance and occasional dominance variation of the algae in consecutive years. In conclusion, algae are increasingly used to monitor the ecological quality and health of the aquatic ecosystem and also to define the effectiveness of management or restoration programs, or regulatory actions.

Consequently, the total objective of bioindicators is to use a single species, or a specific community to evaluate the quality of an ecosystem and how it alters with time, but this can represent a gross more vulgarization of a complex system. As is in all water management implementation, we must be conscious of its defects. On the other side, the limits of bioindicators are apparently minimized by their advantages. Bioindicators can be employed at a range of scales, from the microhabitat to the ecosystem level, to assess the health of a characteristic ecosystem. They bring together information from the biological and physicochemical structure of all aquatic ecosystems as is in changes of population density, community structure and ecosystem processes. Bioindicator is valuable to reflect biological sustainable concept for water management.

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Chapter 5

Biosensors Based on Micro-algae for Ecological Monitoring of the Aquatic Environment

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Additional information is available at the end of the chapter

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Abstract

This chapter is devoted to research possibilities of using micro-algae as sensor elements for biological sensors of aquatic pollutants. The characteristics of the fluorescence of micro-algae pigments caused by laser light, called laser-induced fluorescence (LIF), were used as informative parameters. It is shown that the functioning of biological sensors is determined by the consistency and concentration of micro-algal cells as well as their internal state, which depends on the temperature, illumination, and chemical composition of the water. The results indicate a significant dependence of the LIF intensities of micro-algae from exposure of heavy metal ions, temperature, and illumination. In text is demonstrated specificity of the reaction of various micro-algae, belonging to different phylum. This fact can be used for identify the phylum of micro-algae in situ, and for the creation the biosensors of ecological monitoring aquatic environment.

Keywords: laser-induced fluorescence, micro algae phylum, ions of heavy metals, depending on the lighting and temperature, biosensors

1. Introduction

Monitoring of aquatic environments requires obtained information in real time, long before the appearance of visible signs of pollution, far exceeding the norms of maximum permissible concentrations (MPC). In recent years, organisms, which have high sensitivity to adverse factors, are widely studied as bio-indicators for water monitoring. Single-celled organisms including

micro-algae of phytoplankton can quickly respond to changes in their habitat due to their small size and high rate of intracellular metabolic processes [1].

Unicellular microorganisms violation of life appears to change their functioning—reversibly or irreversibly. In the latter case usually occur damage cells, typically leading to its destruction. The initial, specific impact of the damaging environmental factor is aimed at very specific molecular structure of the cells. The composition of unicellular organisms mainly includes nucleic acids, proteins, lipids, and polysaccharides. All these components may be the target for of the damaging action of factors of the environment surrounding the cell. Violation of the functions of these structures causes a cascade of events that ends with response of the cell as a whole system. Thus, it is possible to distinguish several stages cellular response to external adverse impact. As a rule, at first, there is a nonspecific reaction, typical for any irritations. For most of the cells, there is a change of cell membrane permeability during the action of metal ions [2] and subsequent activation of intracellular systems aimed at the suppression of stress reactions and stabilization of intracellular metabolism [3]. For example, in the presence of calcium ions at the initial stage is an activation of different intracellular systems: protein kinases, phospholipases, protein biosynthesis systems, phosphodiesterase, cyclic nucleotides, adenylatecyclase, a contractile apparatus of the cell, etc. This is the first, reversible stage when the cell tries to compensate the effect of damaging factors. When the damaging factor is greater or more prolonged exposure, violation of cellular functions occurs. By direct action is the damaging effect of poisons, aimed directly at the cell, such as potassium cyanide, which inhibits cellular respiration. Direct violation of cell activity and its damage can be caused by lack of oxygen, excessively low pH, and low osmotic pressure of the substances that are necessary for the life of cells, ultraviolet or ionizing radiation, and so on.

These properties of unicellular organisms determined the increased interest in using them as a part of sensory systems. Analysis of publications demonstrates the sensitivity of the micro-algae of the phytoplankton to rather a wide spectrum of aquatic pollutants [4], including metal ions [5, 6], herbicides, [7, 8], pesticides, cyanide, methyl parathion (MPt), N′(3,4-dichloro-phenyl)-N, N-dimethyl-urea (DCMU), toxic agents of chemical weapons [9, 10]. For example, in article [11] shows the results of experiments in which the presence of DCMU was detected in a concentration of 6×10^{-7} M, which is less than the MRL for a given substance in the seven times (4.3×10^{-6} M), and also the presence of mercury ions at a concentration of 3×10^{-6} M.

Reduction of photochemical quantum yield of algae under influence of some toxic substances noted in [12]. **Table 1** shows the analysis of the influence of potassium cyanide (KCN), MPt, and DCMU on photochemical quantum yield for certain water area. Here, $A = F_v/F_m$ shows the efficiency of energy use of light during photosynthesis, F_m—the maximal fluorescence, which is caused by intense flashes of inducing light, F_0 is the value of the minimum of chlorophyll fluorescence intensity in the absence of constant illumination, $F_v = F_m - F_0$—variable of fluorescence.

Methods of the phytoplankton investigations are regularly improved. Their development began by capture of the phytoplankton samples with special nets and investigations them under an ordinary microscope, to *in situ* measurements of the phytoplankton condition using

lasers or satellite systems. The electrochemical, optical, laser, radiation, statistical, and other methods of studying the state of the phytoplankton exist are developing.

Sample site	Toxic agent [A ± probable error (%)]		
	KCN	MPT	DCMU
1 Clark Center Recreation Park	22.78 ± 1.63	8.32 ± 0.21	17.71 ± 1.32
2 Melton Hill Hydroelectric Dam	29.85 ± 4.17	7.66 ± 0.90	23.45 ± 4.77
3 Oak Ridge Marina	25.88 ± 0.90	8.58 ± 0.27	12.81 ± 0.81
4 Tennessee River	21.89 ± 0.76	3.28 ± 0.18	14.77 ± 1.81

Table 1. Decrease in photochemical yields of naturally occurring algae in primary-source drinking waters from the Clinch and Tennessee Rivers following exposure to toxic agents.

The fluorescence of pigments contained in the cell caused by the laser light, is one of the most important properties of the single-celled algae, and is called laser-induced fluorescence (LIF). Methods LIF measurements are characterized by high sensitivity, rapidity, and the possibility of analysis of fluorescent signal nonlinear parameters [13, 14]. Furthermore, the fluorescent response of micro-algae cells depends not only on the type and concentration of the culture, but also on the conditions in which they were during the measurement: temperature and light, the presence of dissolved organic and inorganic matter [15]. At present, LIF is one of the main methods used in modern techniques and instruments for the study of condition of the phytoplankton and measuring the concentration of micro-algae [16, 17]. An important feature is the possibility of LIF measurements directly into the environment without damaging the cells.

2. LIF spectra analysis as a method of research of phytoplankton internal conditions

A LIF spectrum depends on the composition of pigments in micro-algae cells, and external environmental conditions which affect occurring therein biochemical reaction. **Table 2** shows the list of the main pigments in algae belonging to different phylum [18].

Phylum	Chlorophylls	Phycobilins	Main carotinoids	Main xanthophylls
Cryptophyta	*a*, *c*2	B-phycoerythrin (545), R-phycocyanin. allophycocyanin	α-, β-, ε-carotin	Alloksantin
Ochrophyta	*a*, *c*1, *c*2, *c*3	Missing	α-, β-, ε- carotin	Fucoxanthin, violaxanthin
Haptophyta	*a*, *c*1, *c*2	Missing	α-, β-carotin	Fucoxanthin
Chlorophyta	*a*, *b*	Missing	α-, β-, ε- carotin	Lutein, violaxanthin

Table 2. Main pigments in algae belonging to different phylum.

Possibility of measurement techniques LIF micro-algae is that under the influence of the exciting laser radiation photosynthetic pigments absorb energy inducing light. Part of the energy emitted as fluorescence, the other part is converted into heat energy and a part is used in photosynthesis. The basic idea of using LIF for investigating the internal state of phytoplankton is that the reduction transferring or storage of light energy in photosynthesis, leads to a change in fluorescence intensity [19]. Competition between the processes of photosynthesis, the heat scattering and fluorescence allows estimating a condition of cells of micro-algae using LIF. **Figure 1** shows a typically diagram of the electronic levels, which shows the main competing processes in the cell of phytoplankton.

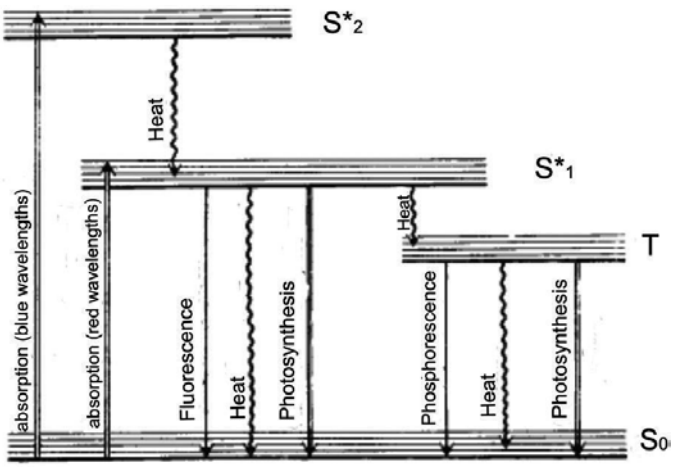

Figure 1. A diagram of the electronic levels in the cell of phytoplankton.

Multiple studies LIF spectra of phytoplankton cells demonstrate that chlorophyll-*a* is the predominant pigment whose the spectral density of the fluorescence is much higher than that of other pigments. So chlorophyll-*b* fluoresces very weakly, due to the fact that transfers the absorbed energy of the exciting light to chlorophyll-*a* with almost 100% efficiency. Other pigments such as phycobilins may have different intensity of LIF, depending on what part of the absorbed energy of the exciting light they transmit to chlorophyll-*a*. The difference among pigments of LIF spectral maxima and the maximum value of the absorption spectra allows using LIF for the study of specific pigments and reactions in which they participate. The level of fluorescence intensity depends on the photochemical reactions that occur in reaction centers (RCs), and especially in the second photosystem (PS2). In essence, PS2 is not only responsible for the absorption of light and the forming of oxygen, but also provide the main part of the fluorescence spectrum of phytoplankton cells. Methods of collect and process data, obtained by measurement of the fluorescence spectra, may be different. Are widely used methods as follows:

- assessing the effectiveness of the photosynthetic apparatus of phytoplankton cells [19];
- analysis of the chlorophyll-*a* fluorescence induction [20];

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- calculating of variable fluorescence of chlorophyll-*a* [21];
- pump-and-probe [22, 23];
- studying the fast repetition rate fluorescence [24, 25].

The spectral density of phytoplankton fluorescence is a weighted average, depending on the relative amount of PS2 systems, inducing of light intensity and condition of chlorophyll-*a* in these complexes [21]. To study the composition of the fluorescent, pigments are exploring wideband fluorescence spectra with high spectral resolution. A monochromatic high-density light is used to increase the resolution of the measuring devices. The LIF spectra of phytoplankton contain spectral components from different pigments that are part of both photosystems, but the largest part of the observed fluorescence is generated by chlorophyll-*a* in PS2 [26]. Therefore, only the measurement of a wide spectrum of LIF allows take account the contribution of all pigments in the spectral density of fluorescence at the wavelength of 680 nm [27, 28].

The dependence of the fluorescence intensity by lighting changes over time is called kinetics of fluorescence. The parameters of chlorophyll-*a* fluorescence kinetics are very informative to characterize the state of the photosynthetic apparatus. This is due to the fact that changes in the state of the photosynthetic apparatus are accompanied by a change in the probability of electronic excitation energy quenching of chlorophyll-*a*, which appears to change intensity of LIF at the light. The study of photosynthesis using the kinetics of fluorescence allows the detection of damage under the influence of anthropogenic pollution, increasing the intensity of the sun and UV radiation, a lack of mineral nutrients, temperature, well before they will find their external manifestation, for example, in reducing the number of cells [26, 29, 30].

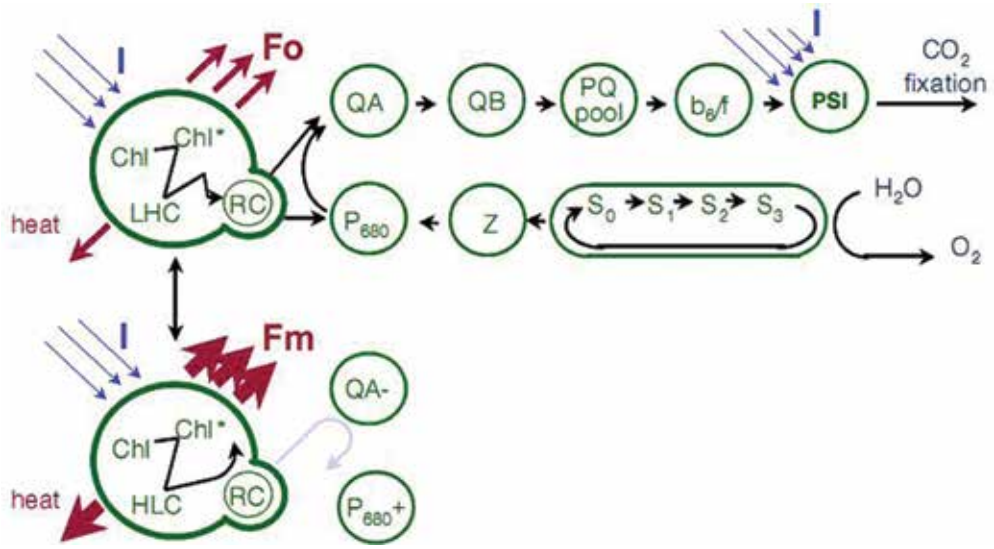

Figure 2. Simplified diagram of energy migration in photosynthesis.

In the state, where the RC of phytoplankton cells are "closed" (quinone acceptor Q_A is in a reduced state), the fluorescence quantum yield of chlorophyll-*a* in the PS2 is considerably greater than for Q_A in the oxidized state. This phenomenon is shown, schematically, in **Figure 2** [28]. Here, in the upper part of the figure shows a diagram of the energy migration in the normal state of PS2, the bottom diagram shows the migration of energy in closed RC with the restored Q_A.

The closing of the centers can be caused by a powerful flash of light, at which quickly restored quinone acceptors Q_A, and the intensity of LIF reaches the maximum level F_m.

The actual increase in illumination conditions or an increase in ambient temperature leads to a reduction of the fluorescence intensity to a value F_m' ($F_m' < F_m$). This can be caused by the processes of adaptation of the photosynthetic apparatus of the cell and damage its internal structures. If the reduction in fluorescence intensity is due to the redistribution of the absorbed light energy in a result of photosynthesis, there is a photochemical quenching. If the reduction in fluorescence intensity caused by an increase in heat transfer or damage cell structures, there is a nonphotochemical quenching.

If measurements are made in real conditions, the difference between the values fluorescence intensity of maximum F_m' and the level of initial F shows the share of PS2 complexes in which the acceptor Q_A was in the oxidized form (up to flashes of light). LIF intensity F_m' is registered under irradiation of phytoplankton by weak flashes of light. In this case, the RC of phytoplankton cells not closed. The difference between the values of F_m' and F_0' shows the share of PS2 complexes in which the acceptor Q_A was in the reduced form.

Changes in fluorescence due to the reaction of the photosynthetic apparatus of the cell are characterized by a coefficient of photochemical quenching (qP) [19] as follows:

$$qP = \frac{F_m^{'} - F}{F_m^{'} - F_0^{'}} \tag{1}$$

The degree of nonphotochemical quenching is characterized by coefficient of nonphotochemical quenching (NPQ) [19] as follows:

$$NPQ = \frac{F_m - F_m^{'}}{F_m^{'}} \tag{2}$$

Quenching coefficients (1) and (2) can be successfully used for determining the presence of chemical substances in the aqueous environment using the cells of micro-algae. Disadvantage of the use of these coefficients is the impossibility during their calculations to separate the contributions of illumination, temperature, and dissolved substances.

Since the F_m is measured with closed RCs, it is best suited for noninvasive study of the characteristics of cells under changing environmental conditions. Therefore, given below

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results of experiments on analysis of the fluorescence spectra of algae, obtained in a saturating laser exposure.

3. Realization of biosensors based on measurement of phytoplankton LIF parameters

At the present time, there are many different methods for determining the concentration of chlorophyll-*a*. Most of them do not allow studies of living phytoplankton cells since include mechanical action on the cells, as well as the use of cytocidal chemical reagents. Bathometric water sampling and subsequent laboratory tests are used at great depths. The procedures for such measurements take much time, and the accuracy is highly dependent on the concentration of phytoplankton and qualification of the personnel. Laboratory tests require the use of chemical reagents, and consequently, quite expensive, moreover, such studies are making changes in the state of phytoplankton cells and usually lead to the death of the samples. Large areas of the oceans and seas are investigated by the color analysis from satellites, but these methods do not work for measurements in coastal waters, rivers, and lakes, the most important in terms of their use in economic activities [16].

Currently, there are several major realizations of biosensors based on measurement of parameters of phytoplankton LIF. Are widely used biosensors, whose action is based on the use of green micro-algae cell suspensions [6]. In the presence of toxicants, fluorescent characteristics of chlorophyll-*a*, which is a part of PS2 photosynthetic apparatus of these algae are changed. The level of micro-algae fluorescence is registered using a fluorometer in the wavelength range 680–690 nm. From the change in fluorescence intensity relative to its initial value (before toxic effects) determine fact of the presence and the concentration of toxicant in water. Using the biosensors based on of micro-algae cell suspensions in research practice are require the search of micro-algae species is most sensitive to the effects of various matters. However, the choice of species is determined not only by the initial fluorescence intensity of cells and its changes in the presence of dissolved substances, but also their availability, and the possibility of long-term preservation in a viable form.

A perspective direction is the development of biological sensors based on immobilized cells of micro-algae placed into a porous, water permeable matrix. Small pore sizes prevent the movement of the cells and their division. Immobilization of cells algae in a porous matrix allows to give to them spatial and temporal stability, to provide convenient storage and use the sensors based on these not only for discrete analysis of toxicants in the samples taken from the water, but also to analyze the presence of toxicants *in situ* using a flow-through systems. Biosensor configurations realized on the basis of the immobilized cells are distinguished of micro-algae culture and the matrix in which they are immobilized.

There are implementation of sensors in which are used as templates a various materials, for example, porous glass [31], silica gel [11], filter paper, coated for hardening Ca-alginate gel [32], PVA cryo gel [33]. These biosensors can be used in flow systems to determine the presence

of heavy metals and herbicides at a flow rate of 60, 120, and 360 ml/h. **Table 3** shows the data from [34] which confirms the prospectivity of such directions of biosensors realization.

Toxicants	Detection limit, g/l (*Chlorella autotrophica*)	Detection limit, g/l (*Thalassiosira weissflogii*)	MPC, g/l
Zn^{2+}	$(6.5 \pm 0.2) \times 10^{-5}$	$(5.2 \pm 0.3) \times 10^{-6}$	5.0×10^{-5}
Hg^{2+}	$(1.4 \pm 0.1) \times 10^{-5}$	$(1.0 \pm 0.1) \times 10^{-5}$	3.4×10^{-5}
Cu^{2+}	$(6.4 \pm 0.1) \times 10^{-6}$	$(1.3 \pm 0.1) \times 10^{-5}$	1.9×10^{-3}
Atrazine ($C_8H_{14}ClN_5$)	$(6.5 \pm 0.2) \times 10^{-5}$	$(1.3 \pm 0.1) \times 10^{-5}$	1.0×10^{-4}
DCMU ($C_9H_{10}Cl_2N_2O$)	$(7.0 \pm 0.3) \times 10^{-6}$	$(7.0 \pm 0.3) \times 10^{-6}$	1.0×10^{-3}
Paraquat ($C_{12}H_{14}N_2$)	$(7.7 \pm 0.4) \times 10^{-5}$	$(7.7 \pm 0.2) \times 10^{-6}$	1.0×10^{-4}

Table 3. The lower detection limit of toxicants when using biosensor in the form of immobilized cells of micro-algae in the flow system.

It should be noted that for the biosensors based on immobilized of micro-algae cells is necessary to provide optimal storage conditions under which a high level of fluorescence signal of immobilized cells is maintained.

4. Influence of environmental conditions on the parameters of micro-algae LIF

Stable operation of biological sensors is determined by the constancy of composition and concentration of micro-algae cells, as well as the stability of their internal state, which depends on the chemical composition of the environment, temperature, illumination, salinity. Under real-life conditions (*in situ*), the current changes in environmental parameters can lead to significant measurement errors. In the [35, 36] indicates that an increase in temperature leads to a nonphotochemical quenching of chlorophyll-*a* LIF. The appearance of temperature quenching processes caused by increased frequency of molecular collisions, which is accompanied by deactivation of the excited levels by nonradiative vibrational relaxation of molecules and decrease in the quantum yield of LIF. The increase in temperature should lead to a change in the spectrum and to decrease the spectral density of chlorophyll-*a* LIF and, consequently, should be taken into account when calculating the concentration of chlorophyll-*a*. This is important in case of monitoring the state of phytoplankton, when the water temperature varies during the day, and the measurements of the vertical distribution profile of phytoplankton, when the difference in temperature between the surface and the depth may reach tens of degrees Celsius.

The article [15] describes the results of experiments obtained by study of the dependence of chlorophyll fluorescence on the temperature and concentration of algae. The experiment demonstrates that by measuring intensity of chlorophyll-*a* LIF, the correct determination of the concentration is only possible in cases where the ambient temperature does not change

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and is equal to the calibration temperature. Otherwise, the error may be very large. For example, **Figure 3** [15] showed that the intensity of the LIF at a temperature of 17°C for a sample of the culture at a concentration of 106 cells/ml (upper curve in **Figure 3**) differs little from the LIF intensity at a temperature of 3°C for a sample at a concentration of 2×10^5 cells/ml (second curve in **Figure 3**). In their turn, the intensity of LIF at 17°C for a sample with a concentration about 2×10^5 cells/ml differs little from that obtained at a temperature of 3°C for a sample with a concentration of 105 cells/ml. Consequently, the relative error in determination of the chlorophyll-*a* concentration, excluding the temperature can reach 2–5 times.

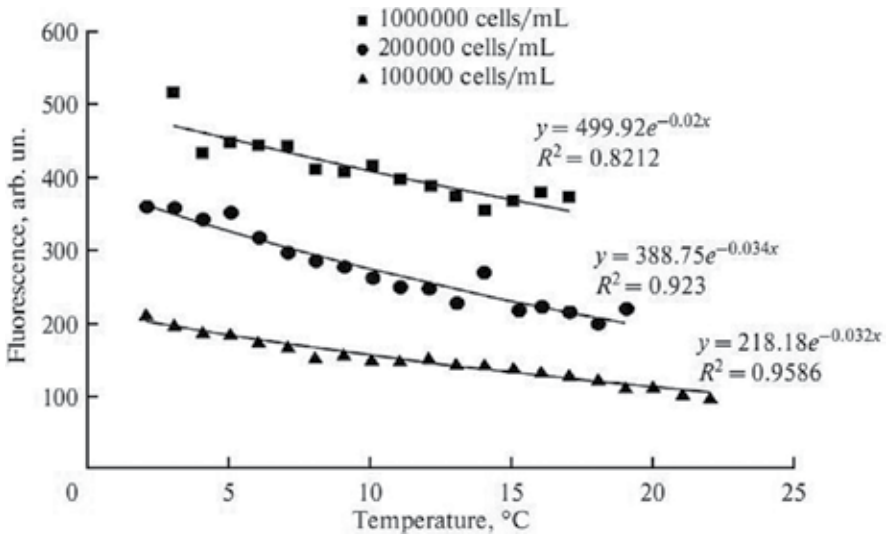

Figure 3. The appearance of the error in determining of the micro-algae concentration.

Increasing light exposure leads to a reduction of fluorescence of phytoplankton cells for two reasons: firstly, due to the increase of energy expended in the cells on photosynthesis, which consequently causes a photochemical quenching [37–39], secondly, due to changes in the structure of chloroplasts containing chlorophyll-*a* [40]. In the article [41], also shows that if a critical value of light exposure saturation occurs, the fluorescence reaches its maximum or minimum level.

To determine the fluorescence intensity dependence on illumination and temperature, it was carried out a series of experiments with the culture of micro-algae from the collection of the Institute of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences. The experimental values of fluorescence intensity dependence on intensity of ambient illumination for the culture of micro-algae *Tisochrysis lutea* are shown in **Figure 4** [15]. The obtained data are well approximated by a straight line whose equation is shown in graph field. Calculated correlation coefficient between the experimental data and approximation line confirms the high degree of coincidence.

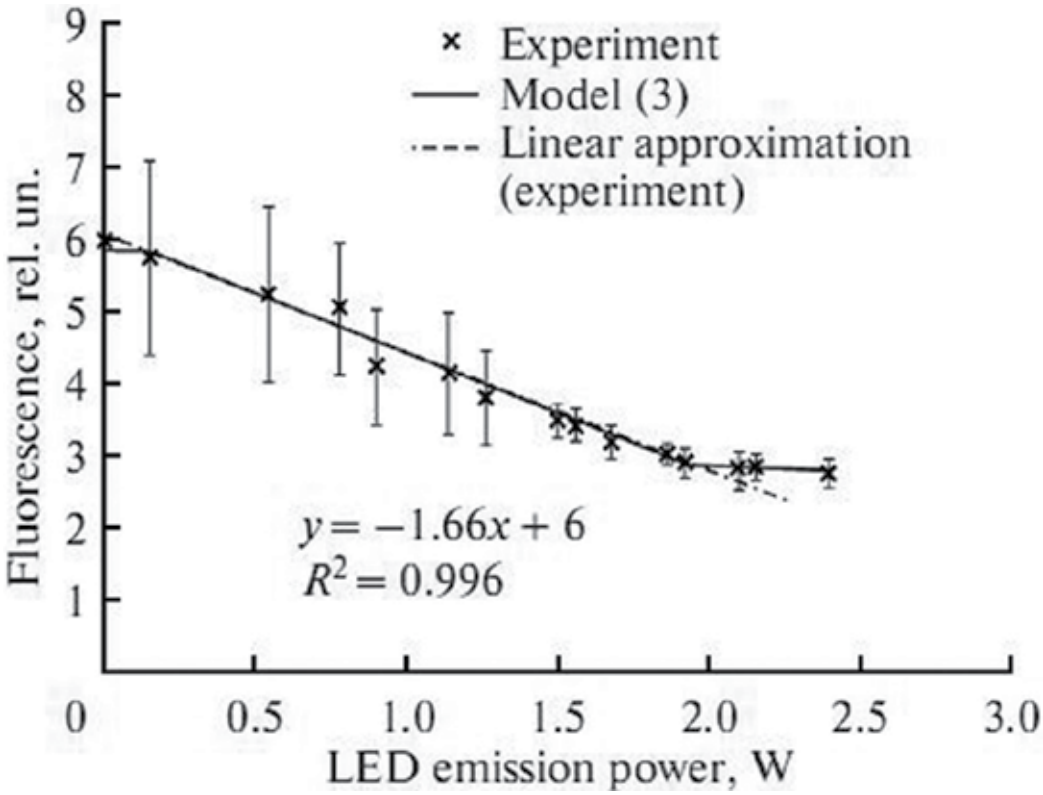

Figure 4. The experimental values of fluorescence intensity dependence on intensity of ambient illumination for the culture of micro-algae *Tisochrysis lutea.*

Since the F_m, for measurements *in vivo* depends on the parameters of the environment, such as illumination $F_m(Q)$ and the temperature $F_m(T)$, it is necessary to carry out the adjustment of the measured value F_m taking into account these modifications. **Figure 4** is clearly seen the presence of two areas of fluorescence saturation: at low and at high illumination, which confirms the conclusions drawn in [40, 42]. Taking into account, these nonlinear effects of saturation of fluorescence, fluorescence dependence on the illumination can be represented by the following system of Eq. (3):

$$F_m(Q) = \begin{cases} f_{max}, & if\ Q \le Q_1 \\ \left(f_{max} + k(Q - Q_1)\right), & if\ Q_1 < Q < Q_2 \\ f_{min}, & if\ Q_2 \le Q \end{cases} \quad (3)$$

where f_{max}—fluorescence intensity measured at a saturating stimulating radiation at a minimum illumination;

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f_{min}—fluorescence intensity measured at a saturating stimulating radiation at maximum illumination;

Q_1—the value of the light level below which the fluorescence intensity is stabilized at the maximum level f_{max};

Q_2—the value of the light level above which the fluorescence intensity is stabilized at the minimum level f_{min};

k—coefficient of proportionality, which is for the linear section of the chart can be calculated by the formula:

$$k = \frac{f_{max} - f_{min}}{Q_1 - Q_2} = \frac{\Delta f}{\Delta Q} \tag{4}$$

Representation of the coefficient k as 4 allows interpreting it as a rate of fluorescence variation of light levels. Determination of the coefficient k can be easily fulfilled by sequential measurements of fluorescence in a sample of selected cultures of micro-algae under different levels of ambient illumination, for each species of phytoplankton. Depending on the species of phytoplankton, values of the critical illuminations Q_1 and Q_2, and the corresponding fluorescence intensity f_{max} and f_{min} will be different. This can be used to identify the species composition of micro-algae in the water samples.

For the graph in **Figure 4**, the coefficient $k = -1.66$; $f_{max} = 5.8$; $f_{min} = 2.7$; $Q_1 = 0.15$ W; $Q_2 = 1.9$ W. The correlation coefficient between the experimental data and approximation line is 0.996, which confirms the high degree of conformity.

To account for the dependence fluorescence intensity by changes in illumination, we introduce a function $s_1(Q)$ as the coupling coefficient between the maximal value of fluorescence intensity the dark, and the value obtained during the lighting changes:

$$s_1(Q) = \frac{f_{max}}{F_m(Q)} \tag{5}$$

By substituting (3)–(5), we obtain an expression for $F_m(Q)$ as a piecewise linear function (6), which determines dependence of the intensity of fluorescence of micro-algae by change in the intensity of illumination:

$$s_1(Q) = \begin{cases} 1, & if\ Q \le Q_1 \\ f_{max} / \left(f_{max} + k\left(Q - Q_1\right)\right), & if\ Q_1 < Q < Q_2 \\ f_{max} / f_{min}, & if Q_2 \le Q \end{cases} \tag{6}$$

In [43], it was shown that dependence of intensity LIF of chlorophyll-*a* by changes of temperature is well approximated by an exponential function (7):

$$F_m(T) = f_0 \cdot e^{a \cdot T} \tag{7}$$

where f_0—the fluorescence intensity, measured at saturated excitation without the temperature quenching (including any normalization);

a—temperature coefficient of the fluorescence;

T—temperature of the environment.

To account for the dependence of fluorescence intensity by change in ambient temperature, we introduce a function $F_m(T)$ as the coupling coefficient between the maximal fluorescence values (at the optimal temperature for the specific culture of micro-algae) and the observed at change of temperature as follows:

$$s_2(T) = \frac{f_0}{F_m(T)} \tag{8}$$

Having substituted (7) at (8), we obtain an expression for $F_m(T)$ as a function (9), which determines dependence of fluorescence intensity of micro-algae from ambient temperature as follows:

$$s_2(T) = e^{a \cdot T} \tag{9}$$

Traditionally [43], when determining the concentration of chlorophyll-*a* by the fluorescence method, assume a linear dependence of fluorescence intensity by chlorophyll concentration. Calculation of concentration may be performed by the formula (10) as follows:

$$C = K \cdot F_m \tag{10}$$

where C—the concentration of chlorophyll-*a*;

K—coefficient of proportionality, determined by the design of the measuring instrument;

F_m—the measured fluorescence intensity.

From [43] implies that the coefficient K has various values depending on the type of micro-algae present in the water sample. In [15] presented data, confirming the dependence fluorescence intensity and the coefficient K from light intensity and temperature. Assuming their influence independent, then the coefficient K can be represented by the function (11) [15]:

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$$K = s_0 \cdot s_1(Q) \cdot s_2(T) \tag{11}$$

where s_0—coefficient of proportionality, depending on the design of a measuring instrument and phytoplankton species;

$s_1(Q)$—function, depending on the illumination;

$s_2(T)$—function, depending on the temperature.

Coefficient K can be interpreted as a function, which characterizes the efficiency of fluorescence of micro-algae cell under a changing of environment parameters. The greater the numerical value of K is, the smaller the fluorescence intensity of cells.

Derived analytical expressions are aimed at accounting of the influence of external factors on the intensity of LIF of micro-algae for reducing the measurement error. Below are the results of studies of the temperature influence and the presence of certain metals ions on the intensity of LIF for the several cultures of micro-algae that belong to different phylum.

5. The dependence of the intensities of micro-algae LIF by temperature stress

Biology, Far Eastern Branch of the Russian Academy of Sciences, allocated into the culture from the Japan Sea, which have a single cells and are characterized by high mobility. Also, the choice of micro-algae has been associated with a variety of their pigment composition (**Table 2**).

Plagioselmis prolonga. Butch (Cryptophyta) strain is PP-02. The average size of the cells is 7.0 ± 0.5 microns in length and 3.5 ± 0.4 microns in width.

Chroomonas salina (Wisl.) Butch (Cryptophyta) strain is CS-92. Cells were 10–14 microns in length, 5–8 microns in width, oval, or ellipsoidal.

Heterosigma akashiwo Hada (Ochrophyta) strain is HA-ZR 11. Cells size 9.7–15.3 microns.

Tisochrysis lutea Bendif Probert (Haptophyta) strain is TL-V 08. Cells size 5.7–7.5 microns, ellipsoidal.

Nannochloris maculata (Chlorophyta) strain is NM-86. The cells are small 2–3 microns in diameter, spherical, rarely—elliptical.

LIF excitation was performed by laser radiation with a wavelength at 442 nm. Time of action of laser radiation on the sample in all cases was 2 s. LIF spectra of phytoplankton at a different temperature were measured by a spectrometer Shamrock 303i company [Andor Technology (USA)] input slit of the monochromator was 100 microns, range of wavelengths was 500–770 nm, the resolution of the diffraction grating was 0.167 nm. Measurement of spectra was carried in the mode of accumulation: the exposure time of the intermediate spectrum was 0.2 s, the

number of accumulations—10, the frequency of measurement of intermediate spectrum was 5 Hz.

Samples of micro-algae cultures were placed in a thermostatic cuvette holder QPOD 2e [Quantum (USA)], which provides the rate of change of temperature 1°C/min. Accuracy of digital meter with an immersed thermocouple was ±0.15°C. Uniform distribution of temperature of the sample was ensured by a magnetic stirrer with rotation speed 300 rev/min.

A sample of micro-algae culture in volume of 1.5 ml by dose was placed into a quartz cuvette in volume 3.5 ml. LIF spectra measurements were performed in increments of changes of temperature 1°C in the range of 20–80°C. For each culture, the measurement procedure was repeated at least three times.

For graphs forming data derived in the experiments were processed as follows:

1. From the LIF spectra were subtracted dark current of the spectrometer.
2. To reduce the noise signal obtained data were subjected to filtration (using a fast Fourier transform) for frequencies above 0.5 Hz.
3. After filtration, the chlorophyll fluorescence intensity in the wavelength range 680–690 nm and Raman scattering (RS) of water were determined. The obtained results were normalized according to the formula (12) as follows:

$$F_S(T) = \frac{I_{\max}(T)}{I_{RS}} \tag{12}$$

where $F_S(T)$—LIF intensity of chlorophyll-*a*, determined from the fluorescence spectrum at a predetermined temperature;

$I_{max}(T)$—maximum LIF intensity in the wavelength range 680–690 nm at a predetermined temperature;

I_{RS}—water Raman intensity at a predetermined temperature.

1. The relative LIF intensity obtained by rationing $F_S(T)$ by formula (13) as follows:

$$F_m(T)_N = \frac{F_S(T)}{F_{max}} \tag{13}$$

where $F_m(T)_N$—LIF relative intensity of chlorophyll-*a* at a predetermined temperature;

F_{max}—maximum LIF intensity of chlorophyll-*a* in the whole temperature range.

Figures 5–7 present the results of measurements of the fluorescence of investigated micro-algae culture in the temperature range 20–80°C. On the graph, each point is the result of averaging $F_m(T)_N$ of three independent experiments. In all this figures, the results of experiments, each performed in triplicate. Error bars on the graph show the standard deviation.

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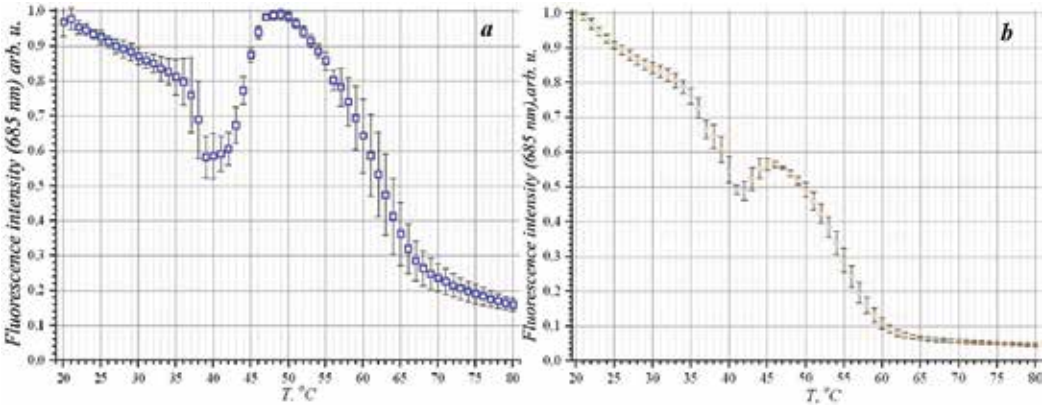

Figure 5. The temperature dependence of the LIF intensity for Cryptomonad: (a) *Chroomonas salina;* (b) *Plagioselmis prolonga*.

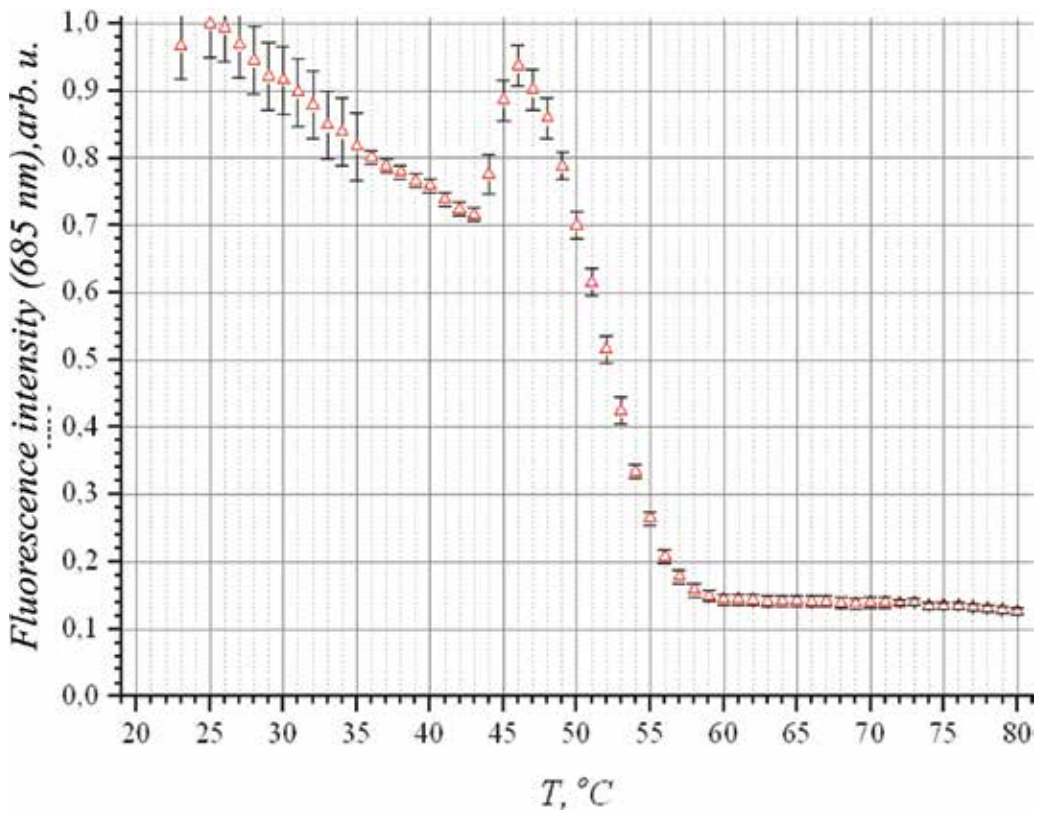

Figure 6. The temperature dependence of the LIF intensity for *Heterosigma akashiwo*.

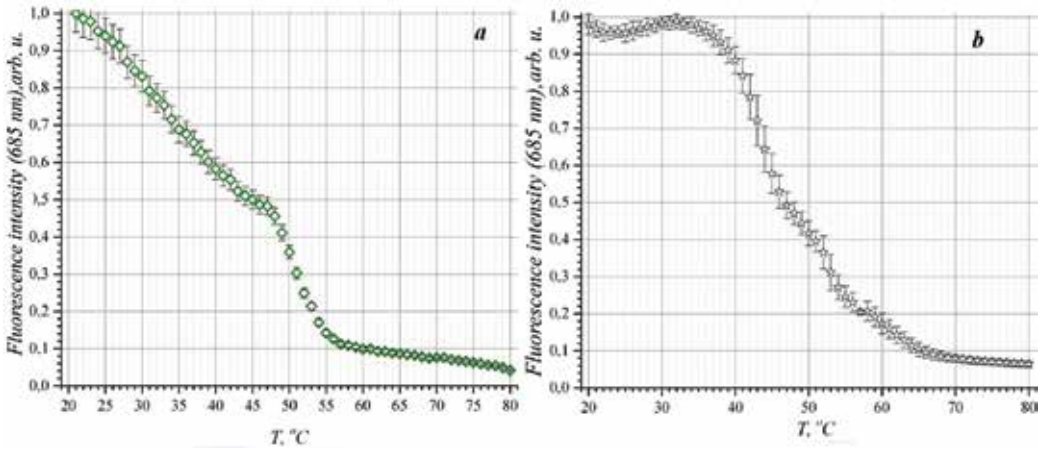

Figure 7. The temperature dependence of the LIF intensity for micro-algae containing no of phycoerythrin: (a) *Tisochrysis lutea;* (b) *Nannochloris maculata*.

Analysis of charts allows selecting specific areas of the LIF intensity, having a different degree of expressiveness for each culture:

1. The range of 20–35°C—there is a monotonic decrease in the fluorescence intensity.
2. The range of 35–42°C—is typical for Cryptomonad which distinctive feature is the presence of phycobilins. For them, in this range, there is accelerate of decrease in fluorescence intensity, whereas for other algae of this effect is not observed.
3. The range of 42–50°C—a rapid increase in fluorescence intensity to the maximum.
4. The range of 50–80°C—monotonic decrease in fluorescence intensity followed by stabilization at the minimum level.

The values of the maximum and minimum temperatures and the corresponding amplitudes of the LIF intensities for the characteristic points of their temperature dependence for the investigated cultures of micro-algae are shown in **Table 4**.

Cultures of micro-algae	T_{min}, °C	$F_n(T_{min})$	T_{max}, °C	$F_n(T_{max})$
Chroomonas salina	40	0.5	49	1.0
Heterosigma akashiwo	43	0.7	46	0.92
Plagioselmis prolonga	41	0.5	46	0.56
Tisochrysis lutea	–	–	–	–
Nannochloris maculata	–	–	–	–

Table 4. The values of the maximum and minimum temperatures and the corresponding amplitudes of the LIF intensities for the characteristic points of their temperature dependence for the investigated cultures of micro-algae.

The absence of characteristic points for cultures *T. lutea* and *N. maculata*, in our opinion, related to the lack of phycobilins.

According to [35, 36], the temperature quenching of fluorescence caused by increase of molecular vibrational energy which results in the deactivation of excited levels and, consequently, to a decrease in the fluorescence quantum yield. In our case, there is in the range of 20–33°C decrease in the fluorescence intensity has the character of nonphotochemical quenching and is caused by the influence of temperature on the chlorophyll-*a* molecules which is as a part of cells of micro-algae. In [41], it is shown that in this temperature range, the experimental data are well approximated by an exponential function (7). The values for the temperature coefficient of the fluorescence (a) for the investigated cultures of micro-algae and the maximum deviation of the experimental data from calculated data by the least squares method (S) are presented in **Table 5**.

Cultures of micro-algae	a	S
Chroomonas salina	−0.0073	0.07
Heterosigma akashiwo	−0.0035	0.12
Plagioselmis prolonga	−0.017	0.015
Tisochrysis lutea	−0.0080	0.09
Nannochloris maculata	−0.01	0.13

Table 5. The temperature coefficient of the fluorescence of micro-algae cultures.

Expressed minimum intensities of fluorescence in the temperature range 35–42°C, probably associated with the beginning of denaturation of protein structure in micro-algae cells, and their subsequent destruction. Thus, for Cryptomonad, there is an infringement in the electron transport chain, because of which is stopped transmission to chlorophyll-*a* the energy absorbed by other pigments. This is well illustrated in **Figure 8** by an increase in phycobilins fluorescence intensity for Cryptomonad cells in the temperature range 35–42°C.

Increase of temperature to 46–50°C leads to intensive degradation membranes and internal components of cells. As a result, a chlorophyll-*a* is released, which, as is known, has fluorescence intensity always higher than that for chlorophyll-*a*, which is associated in the chloroplasts of cells. Therefore, in this temperature range, there is a sufficiently intense of fluorescence increase to a maximum value. Further temperature increase leads to a decrease of fluorescence due to the increase of nonradiative relaxation and chlorophyll-*a* destruction.

Data obtained by us demonstrate a significant depending of LIF intensities of micro-algae on the temperature and its distinction for different cultures of micro-algae. Furthermore, the temperature coefficient, which is introduced by us, determines the change of LIF intensities in the range of actual temperatures of an aqueous environment that can be used as a distinguishing feature for identifying of micro-algae.

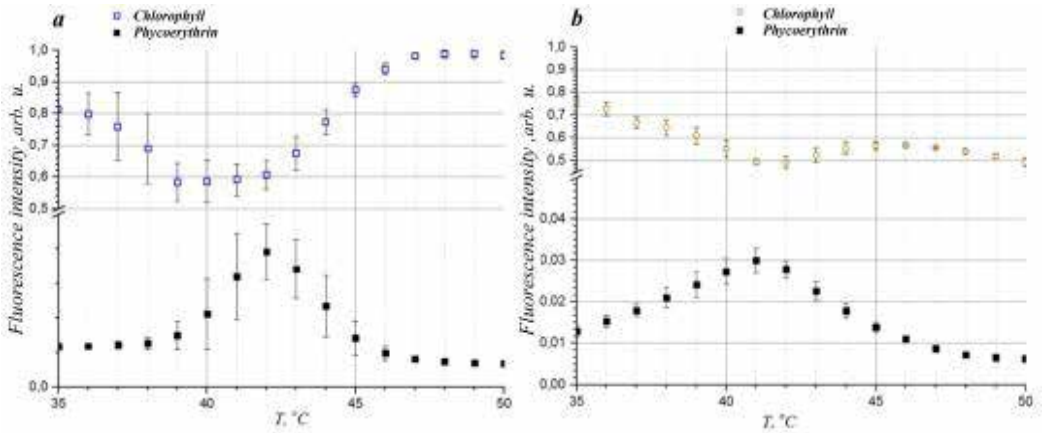

Figure 8. Simultaneous LIF intensities changes of chlorophyll-*a* and phycoerythrin for Cryptomonad cells: (a) *Chroomonas salina*; (b) *Plagioselmis prolonga*.

On the other hand, such researches are subsidiary in solving the main task—the development of biological sensors of aquatic pollutants based on the analysis of micro-algae LIF. Particular interest is the group of parameters characterizing the sensitivity of each species of micro-algae to the presence of specific substances in the environment, such as heavy metal ions. Such investigations allow identifying the suitable species of micro-algae for use as biological indicators of the presence of certain types of pollutants or hazardous organic and inorganic substances.

6. Influence of heavy metal ions on intensities of micro-algae LIF

For the experiments were selected cultures of micro-algae *N. maculata* and *T. lutea* and salts of copper, zinc, and iron(II).

N. maculata is representative of the green algae, which in their turn, is the most extensive department, which incorporates more than 13,000 species. The presence such a great of species diversity suggests that the representatives of green algae can be found in different waters and, therefore, can become convenient indicators of their pollution.

T. lutea is Haptophyte, which has a high resistance to adverse environmental conditions, in particular, to a lack of nutrients. Due to its structure, this type of phytoplankton can be kept in a hostile environment over many months and can be used as a potential basis for creation of chemo sensors [34, 35].

Figure 9 illustrate the temporal variations of fluorescence intensity of chlorophyll-*a* for *N. maculata* at a 680-nm wavelength at the presence in salt solution ions of copper, zinc, and iron(II) at a concentration equal to the MPC.

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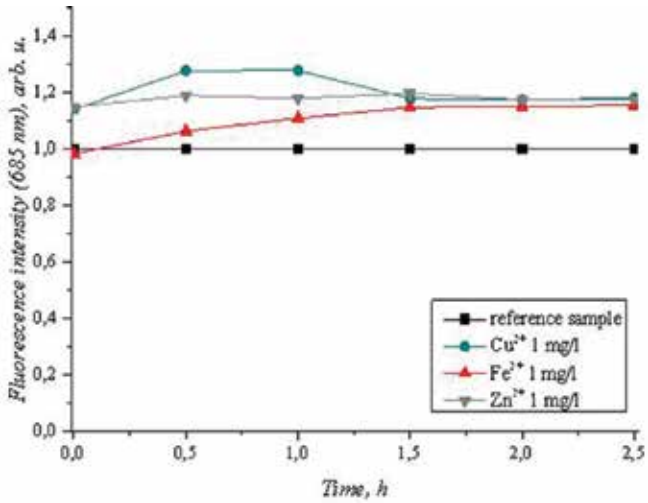

Figure 9. Change of the *Nannochloris maculata* chlorophyll fluorescence intensity under the influence of salts of copper, iron, and zinc at a concentration equal to MPC.

It can be seen that adding of metal salts increases the fluorescence intensity of the micro-algae culture in the first half hour after the addition of salt, and then stabilizes at a constant value. Iron in the time of addition is not strongly influence, unlike of copper and zinc, from which it can be concluded that zinc and copper ions are more toxic for the species of micro-algae.

To define the minimal concentration of metal ions, which influence the photosynthetic apparatus of micro-algae, we have carried out measurements with salt concentrations below

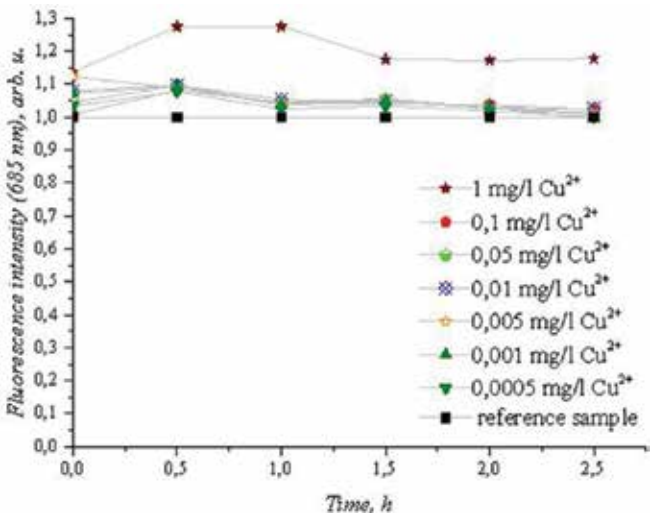

Figure 10. Changing the fluorescence intensity of micro-algae *Nannochloropsis maculata* under the influence of Cu^{2+} at concentrations of copper ions in the solution below the MPC.

the MPC. The **Figures 10** and **11** show the results of the influence of salts of copper and zinc, respectively, at the green micro-algae *N. maculata*.

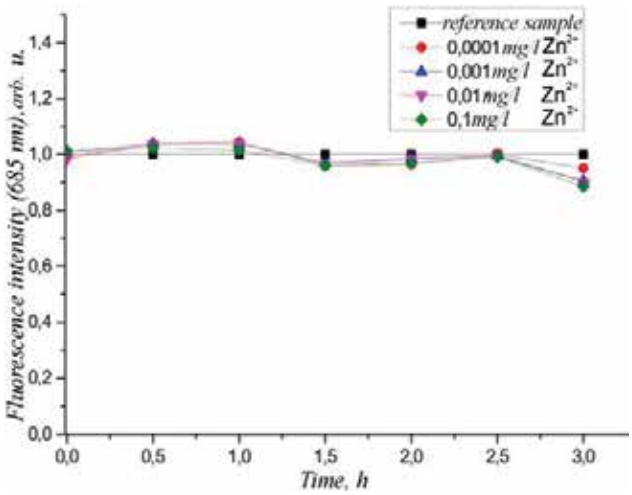

Figure 11. Changing the fluorescence intensity of micro-algae *Nannochloropsis maculata* under the influence of Zn^{2+} at concentrations of zinc ions in the solution below the MPC.

The resulting data from these experiments showed that, after the series of measurements, there is change of concentration of phytoplankton cells in the sample. Increasing concentrations of phytoplankton cells C_{Php} leads to that the relative concentration of the metal ions C_{MI} becomes very small and the effect of exposure of metal ions on phytoplankton not observed. The ratio of metal ion concentration to the concentration of phytoplankton cells can be called the coefficient of sensitivity of phytoplankton to metals (14):

$$s_3(MI) = \frac{C_{MI}}{C_{Php}} \tag{14}$$

From this expression, it follows that the increasing the concentration of phytoplankton in biosensor, for a constant concentration of the metal salt, will reduce the sensor sensitivity, at the same time, reducing the concentration of phytoplankton, on the contrary, increases the sensitivity of the sensor.

For confirmation of that conclusion of experiments were repeated with the culture of micro-algae *T. lutea*, which are resistant to changes in nutrient composition of the environment, therefore for a long time, culture can be regarded as stable and use it for long-term experiments without changing the concentration of each sample. **Figure 12** shows the results of measuring fluorescence intensity of samples with different concentrations of micro-algae *T. lutea* at the fixed copper ion concentration equal to 1 mg/l. The hatched symbols refer to the control sample without the shaded symbols to the sample under the influence of copper ions.

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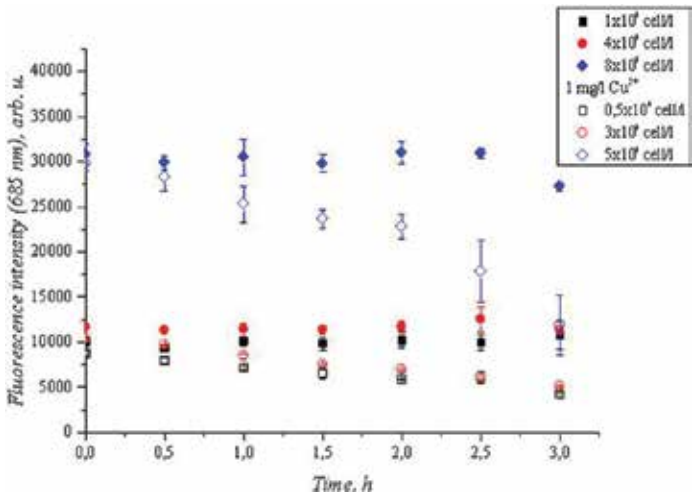

Figure 12. The results of measuring fluorescence intensity of samples with different concentrations of micro-algae *Tisochrysis lutea* at the fixed copper ion concentration equal to 1 mg/l.

Figure 13 shows the results of measuring the intensity of fluorescence of micro-algae *T. lutea* with a combination of variations in the concentrations of copper and phytoplankton in the investigated samples. For convenience of representation, fluorescence intensity of cultures by copper ions is given in the meanings of normed on fluorescence intensity of control cultures

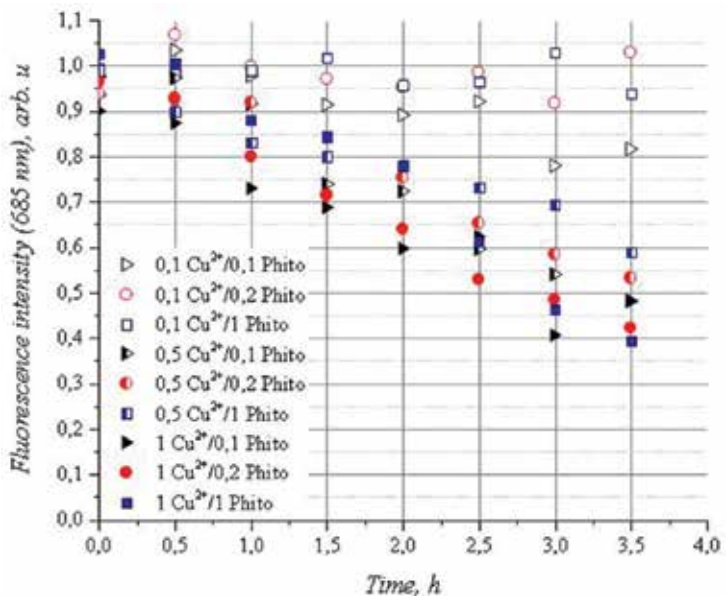

Figure 13. The fluorescence intensity of micro-algae *Tisochrysis lutea* with a combination of variations in the concentrations of copper and phytoplankton in the investigated samples.

In approximating of the data by the equation $y = ux + b$, the u factor in the equation will be equal to the rate of decrease in the fluorescence of the investigated interval of time. The equations for the approximation of all graphs in **Figure 13** are shown in **Table 6**.

$C_{Php}\backslash C_{MI}$	**0.1**	**0.5**	**1**
1	1 – 0.007×	1 – 0.1×	1 – 0.18×
0.2	1 – 0.01×	1 – 0.13×	0.98 – 0.17×
0.1	1 – 0.05×	1 – 0.15×	0.92 – 0.15×

Table 6. The rate of change fluorescence intensity micro-algae *Tisochrysis lutea* with a combination of variations in the concentrations of copper and phytoplankton.

Figure 13 shows that within the first 3 h of exposure copper ions on micro-algae culture, the fluorescence intensity decreases uniformly. If the concentration of phytoplankton is high, the influence of copper ions is small. However, if the copper concentration reaches high values, its effect does not depend on the concentration of micro-algae. This allows us to make an important conclusion that for sensitivity to low concentrations of copper, well below the MPC, are needed culture of micro-algae with a low concentration of cells. This reaction may be among micro-algae, which have both individual and collective metabolism, which increases resistance to stress. However, if the stress reaches a critical value (copper concentration of 1 mg/l in our experiment), the rate of change of intensity fluorescence of chlorophyll increases with increasing concentration of phytoplankton.

In addition to changes in the intensity of fluorescence of algae under the influence of heavy metal salts having spectral changes of fluorescence intensities in the wavelength range from 645 to 740 (**Figure 14**). Peak fluorescence at 740 nm is caused by the presence of chlorophyll belonging to PS1, which, in turn, is not involved in the primary reactions of photosynthesis.

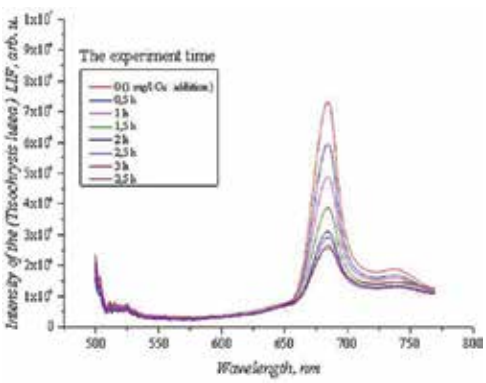

Figure 14. The fluorescence spectra of micro-algae *Tisochrysis lutea* under the influence of copper ions at a concentration of 1 MPC.

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Nevertheless, this photosystem also exposed to heavy metals as reflected on the spectral picture, shown in **Figure 14**.

Figure 14 shows that the change of fluorescence intensity at a wavelength of 685 nm and is significantly faster than the fluorescence at wavelength of 740 nm. This is due to the fact that the PS2 is involved in the primary stage of photosynthesis and the metal ions primarily impact on its fluorescence. **Figures 15** and **16** shows the intensity of fluorescence at wavelengths 685 and 740 nm for the culture of *T. lutea* at a concentration 27.25 × 10^4 cells/ml.

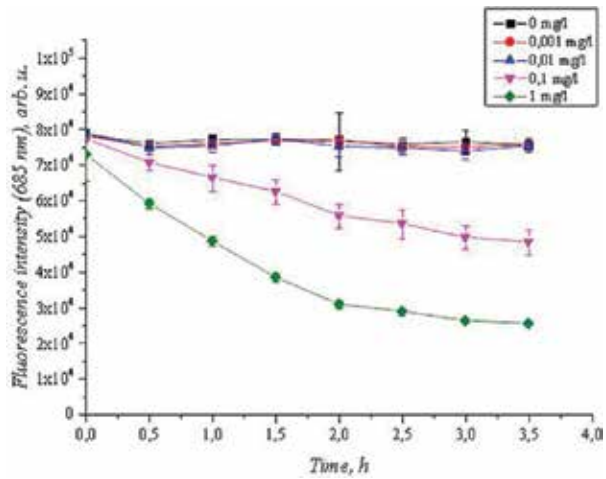

Figure 15. Dependence of the fluorescence intensity of micro-algae *Tisochrysis lutea* at a wavelength of 685 nm on the concentration of copper ions.

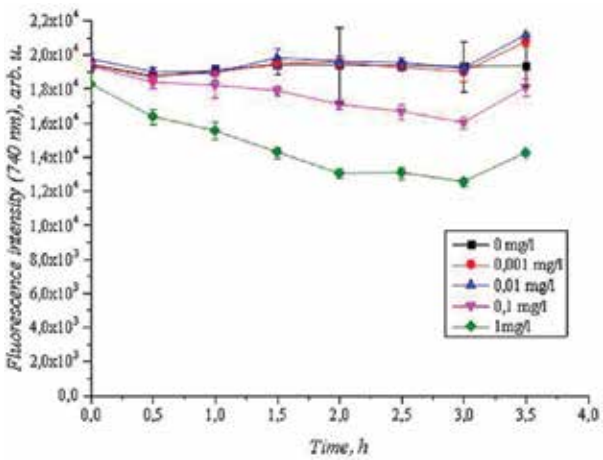

Figure 16. The fluorescence intensity of micro-algae *Tisochrysis lutea* at a wavelength of 740 nm on the concentration of copper ions.

Stabilization of the intensity of fluorescence of chlorophyll-*a* at a wavelength 685 nm in **Figure 15**, with the copper ion concentration of 1 mg/l, occurs earlier than in **Figure 13** because of the difference in the concentration of micro-algae. Increasing the concentration of phytoplankton at high concentrations of copper (1 MPC) accelerates the fall of the fluorescence intensity. It is easily seen that the concentration of micro-algae 27.25×10^4 cells/ml and a copper ion concentration of 1 mg/l, the fluorescence intensity decreases according to the equation $y = 0.93 - 0.28u$, where 0.28 is the rate of change. From this, it can be concluded that for reducing the fluorescence to a stationary state in the given conditions, less time is required (1.5 h) than in the experiments shown in **Figures 12** and **13**.

The ratio of fluorescence intensities at a wavelength 740 nm to the fluorescence intensity at a wavelength 685 nm is a spectral characteristic, reflecting the impact of ions of heavy metals on the whole photosynthetic apparatus (15).

$$S_R = \frac{F_{740}}{F_{685}} \tag{15}$$

where S_R—spectral feature (of the right shoulder of chlorophyll fluorescence);

F_{740}—the chlorophyll fluorescence intensity at a wavelength 740 nm;

F_{685}—the chlorophyll fluorescence intensity at a wavelength 685 nm.

Figure 17 shows the change in S_R under the influence of copper ions within 3 h. The increase in spectral feature, as mentioned above, reflects the fact that the PS2 is more sensitive to metal ions than PS1. Moreover, after the second hour of the experiment, if copper concentrations 0.01 and 0.001 mg/l, also begins to increase spectral feature. This phenomenon requires a thorough and detailed study.

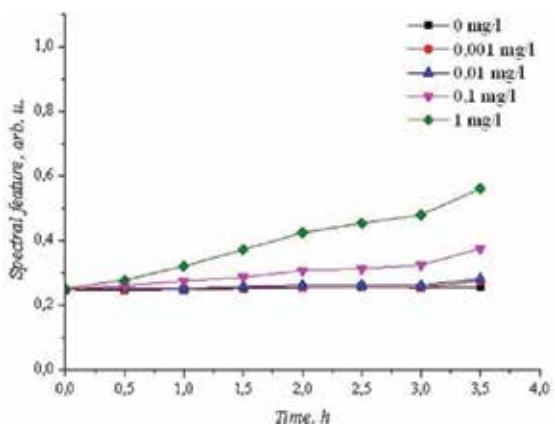

Figure 17. Dependence of the spectral feature of micro-algae *Tisochrysis lutea* from time under the influence of copper ions.

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7. Conclusions

Analysis of the bibliography and the experimental data obtained for the four phyla of micro-algae confirms the dependence of their LIF from the environment parameters such as temperature, light and the presence of metal ions. It demonstrates the importance of analysis of the mutual influence of environment parameters to minimize the error in obtaining the final result. The possibility of practical application of the concept of the proposed measurements and calculation of the chlorophyll-*a* concentration in the composition of phytoplankton cells in view of the impact of environmental parameters. Error of determination of the chlorophyll-*a* concentration at calculation by the proposed method can be reduced by three times as compared to the error of determination of a concentration by classical method.

Obtained experimental data demonstrate the fundamental possibility of micro-algae cultures identification according to the parameters of LIF spectra. In cultures of micro-algae studied wavelength, maximum LIF has a different value in the wavelength range 680–690 nm at 22°C. This allows to using it as informative features for species identification by their fluorescence spectra. In addition, for all the studied cultures of micro-algae, dependence intensity of the chlorophyll-*a* LIF on the environmental temperature is well approximated by an exponential function. At the same time, the value of a temperature coefficient of fluorescence different for the diverse cultures, which also allows to use it as an informative feature to identify species of micro-algae. The obtained reactions of micro-algae for the presence of metal ions demonstrate their specificity and can be used at creation of biosensors ecological monitoring of water environments.

Acknowledgements

Financial support from Russian Science Foundation (Grant No. 14-50-00034) in the part of determine the dependence of the fluorescence parameters of micro-algae from the effects of environmental factors, Program of fundamental researches of FEB RAS "Far East" (Grant No. 0262-2015-0089) in the part of experiments with cultures of micro-algae, and Russian Foundation for Basic Research (Grant No. 16-34-00257) in the part of algae cultivation is gratefully acknowledged.

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Chapter 6

Biotechnology Carbon Capture and Storage by Microalgae to Enhance CO_2 Removal Efficiency in Closed-System Photobioreactor

Dr. Astri Rinanti

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62915

Abstract

The global warming issue caused mainly by carbon dioxide (CO_2) has triggered various efforts to reduce excess amount of CO_2 emitted into the atmosphere. A viable option is to utilize biomass production potential of microalgal consortium in aquatic ecosystem that constantly requires CO_2 to perform photosynthesis. This study aims to provide scientific contributions to the development of environmental studies, particularly of using microalgal consortiums as carbon capture and storage (CCS) agent by engineering their culture conditions. A number of studies analyzing biological reduction of atmospheric CO_2 by using CO_2 absorption capability of terrestrial plants have been facing many difficulties. Compared to various terrestrial plants, microalgae are generally considered photosynthetically more efficient. Exploitation of microalgal capability has numerous advantages, including their faster regeneration time, ability to grow in less space than terrestrial plants, and because the cultivation of microalgae can be done on a small-scale or large-scale operation, under controlled conditions, and is independent to climatic changes. Taking into account long-term advantages, this study is a preliminary study which is expected to be able to provide information and feedback regarding integrated microalgal culture system that may lead to alternative solutions of eco-friendly and sustainable environmental management technology that are capable of mitigating environmental problems caused by CO_2 (as greenhouse gas) emissions. Hence, the results of this research could be implemented by building urban microalgal ponds in efforts to develop sustainable cities in terms of CO_2 emission reduction in urban areas.

Keywords: Microalgae, CO_2 removal, carbon fixation, biomitigation, carbon capture and storage, photobioreactor

1. Introduction

1.1. Carbon dioxide and global warming

Carbon dioxide (CO_2) is the most significant greenhouse gas that contributes to global warming. CO_2 emissions and other combustion products such as NO_x, SO_x, CH_4, and poly aromatic hydrocarbon (PAH) released from industrial, transportation, and residential activities have become environmental problems associated to uncertainty in annual climate prediction. Carbon dioxide is usually emitted freely from industrial processes in an uncontrolled way. In gas-controlled combustion units, CO_2 is a desirable by-product since it provides good indication of a complete combustion [1–3]. At an average concentration of 330 ppm in the atmosphere, CO_2 is harmless to humans because it is colorless, it is odorless, and it will not cause any chemical reactions in human body. At the same time, plants and microalgae will grow better in a CO_2-rich environment [4]. However, recently CO_2 concentration in the troposphere is getting serious attention as CO_2 is categorized as greenhouse gas that is believed to be the cause of global warming effects. Impacts of greenhouse gases are becoming more apparent mainly due to the increase of the earth's surface temperature [5].

CO_2 generated by the combustion of fossil fuels, as in the flue gas from power plants and exhaust gas from cement and steel manufacturing processes, can be captured and sequestered. Currently, the vast majority of large emission sources have CO_2 concentrations of less than 15%, although in some cases, substantially less [6]. However, a small portion (less than 2%) of the fossil fuel-based industrial sources has CO_2 concentrations of up to 95%. These high-concentration emission sources are potential candidates for implementation of CCS concept. However, some estimates predict the costs of non-biological CCS technology deployment to be economically attractive only after the year 2030, making implementation at a large scale unlikely in the near term [7].

1.2. Anthropogenic contribution

The major anthropogenic sources of CO_2 emissions over the last 20 years have resulted from fossil fuel burning, changes in land use, primarily deforestation [8–10] and other industrial processes like oil refineries; cement, lime, and steel production; and coal-fired power plants (13–15% of CO_2 concentration by volume) and natural gas power plant (8–10%). Globally, CO_2 emissions from fossil-fuel use in the year 2000 totaled about 23.5 Gt CO_2/year. Of this, close to 60% was attributed to stationary emission sources. However, not all of these sources are amenable to CO_2 capture and process [11].

Human activities are greatly increasing the atmospheric concentrations of carbon dioxide. The rate of increase in atmospheric CO_2 is reaching approximately 3 billion tons every year [12], mainly due to fossil fuel combustion and deforestation [8–10]. Various reports have mentioned that the atmospheric CO_2 concentrations have increased from around 280 ppm to 368 ppm over the past 200 years [13], contributing up to 50% to the global temperature increase known as greenhouse effects. The global warming causes sea level to rise and various climate anomalies linked to global warming, including floods and droughts [5, 14–15]. Considering

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these high impacts, it is necessary to perform strategic activities which aim at reducing atmospheric CO_2 concentrations. A researcher [16] estimated that anthropogenic contribution to the carbon cycle in the form of CO_2 released into the atmosphere is approximately 9 Gigatons (Gt) per year. Approximately 7.6 Gt of this is from fossil fuels and 1.4 Gt from land-use change. As much as 55% of this carbon is absorbed by natural processes, and the rest up to 4 Gt are deposited in the atmosphere every year.

1.3. History of carbon dioxide biomitigation

Faced with issues of climate change, various efforts have been made by countries to find an appropriate solution. Despite the variety of strategies to reduce CO_2 emissions has been explored, so far there has been no single mitigation technology that can provide the ideal solution. Biological CCS technologies can be used to mitigate carbon emissions that would otherwise be released to the atmosphere. CCS technology incorporates three stages, the first of which is to collect CO_2 from specific emission sources such as industrial related sources and power plants by using certain techniques, the second stage is to transport the CO_2 to a suitable storage or processing location, and the third stage is to process the CO_2 and store it away to prevent it from being released into the atmosphere for a long period of time [6, 8].

Figure 1 illustrates the process by which industrial and transport CO_2 emissions in the air are absorbed by microalgae, thus allowing photosynthesis process to occur. In microalgal cells, CO_2 molecules enter the Calvin-Benson cycle to form sugars. The mechanism in which CO_2 is bonded to the enzyme ribulose-1,5-bisphosphate (RuBP) or ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) is called carbon fixation. Depending on the use of the accumulated biomass, products derived from this process are at best carbon neutral.

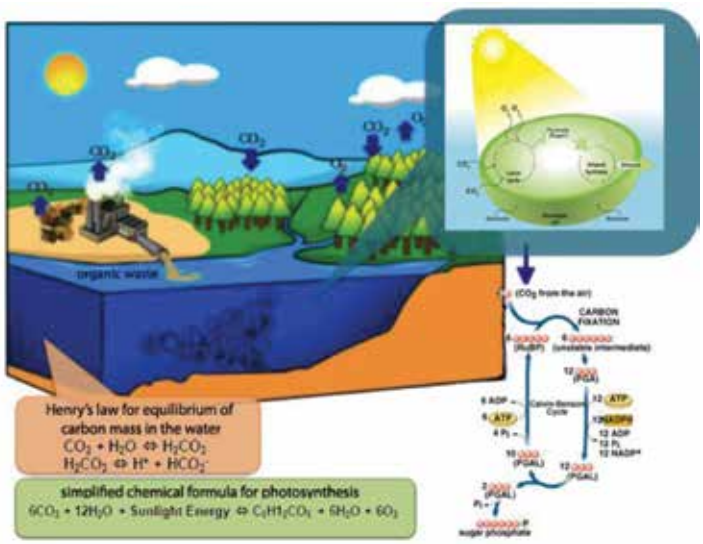

Figure 1. Illustration of atmospheric CO_2 absorption by microalgae in photosynthetic process.

Since microalgae are producers, they have the ability to continuously undertake photosynthesis. One of the primary requirements for photosynthesis is atmospheric CO_2. Microalgae are generally characterized by their relatively high photosynthetic efficiencies compared to various terrestrial plants and have been proposed as an alternative way of reducing carbon dioxide emissions into the atmosphere [15]. As photosynthetic organisms, microalgae are well adapted to capturing ambient CO_2. Growing algae to capture ambient CO_2 will remove carbon dioxide and sequester it in the form of biomass [17]. Thus, microalgae can be exploited as biological CCS agent.

Studies of microalgae as CO_2 catcher and absorber have been conducted in various countries around the world, particularly in terms of adaptation and selection of species that indicates tolerance of high CO_2 concentration and high CO_2 absorption rate. As a photosynthetic organism, microalgae are well adapted to capture ambient CO_2, although the limited CO_2 content is considered inefficient. High CO_2 concentration can hamper photosynthesis process and slow the growth of microalgae and take into account exhaust gas from combustion contains 5–15% CO_2. **Table 1** presents several types of microalgae that play a role in CO_2 bio-mitigation research in a laboratory scale.

Microalgae	Temperature (°C)	Max CO_2 concentration (%, v/v) that removed	P_{volume} ($gL^{-1}day^{-1}$)	Carbon utilization efficiency (%)	Reference
Chlorella sp.	26	Air	0.682	-	[18]
Chlorella sp.	26	2	1.445	58	[18]
Chlorella sp.	26	5	0.899	27	[18]
Chlorella sp.	26	10	0.106	20	[18]
Chlorella sp.	26	15	0.099	16	[18]
Chlorella kessleri	30	18	0.087	-	[19]
Scenedesmus sp.	25	10	0.218	-	[20]
Chlorella vulgaris	25	10	0.105	-	[20]
Botryococcus braunii	25	10	0.027	-	[20]
Scenedesmus sp.	25	Flue gas	0.203	-	[20]
Botryococcus braunii	25	Flue gas	0.077	-	[20]
Chlorella vulgaris	25	Air	0.040	-	[21]
Chlorella vulgaris	25	Air	0.024	-	[21]
Haemotococcus pluvialis	20	16–34	0.076	-	[22]
Scenedesmus obliquus	-	Air	0.009	-	[23]
Scenedesmus obliquus	-	Air	0.016	-	[23]
Chlorella vulgaris	27	15	-	-	[24]
Scenedesmus obliquus	30	18	0.14	-	[19]
Spirulina sp.	30	12	0.22	-	[19]

NA = not available data.

Table 1 Some species of microalgae that play a role in CO_2 bio-mitigation.

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There are many fundamental questions that still need to be answered regarding microalgal growth at elevated CO_2 concentrations. The most critical determination is the maximum amount of CO_2 sequestered from a given concentration of input gas. There was, however, a debate as to the actual amount of CO_2 that can be removed from the input stream. Data presented by [19] suggested that less than 5% of the CO_2 can be removed from a stream containing >1% CO_2 if the cells are only at a low density. However, [25] suggested that as much as 70% uptake from a 2% CO_2 stream could be captured in cyanobacteria. The pH of the media and the tolerance of the organism to high CO_2 concentration will play important roles in the amount taken up. Further results indicate that the maximum amount of CO_2 sequestered from a given concentration of input gas is all of it, while [26] reported that the determination of this limit depends on many factors including the design of the photobioreactor, bubble diameter, and cell density in the culture. The wide range of values necessitates further research into this key component of carbon capture. At both ambient and elevated CO_2 concentrations, there are important issues to consider when growing algae for the purpose of CO_2 capture and high productivity.

2. Biotechnology of Carbon Capture and Storage

CCS technology can be used to mitigate carbon emissions that would otherwise be released to the atmosphere. According to [27], the definition of CCS is as follows: a process consisting of the separation of CO_2 from industrial and energy-related sources, transport to a storage location, and long-term isolation from the atmosphere. Capture of CO_2 can be applied to large point sources. The CO_2 would then be compressed and transported for storage in geological formations, in the ocean, and in mineral carbonates, or for use in industrial processes. According to [6, 8, 27], CCS is a process consisting of the separation of CO_2 from industrial and energy-related sources, transport to a storage location, and long-term isolation from the atmosphere. Capture of CO_2 can be applied to large point sources. The CO_2 would then be compressed and transported for storage in geological formations, in the ocean, and in mineral carbonates, or for use in industrial processes.

Biological systems could potentially make a significant contribution to CCS technology, as they can be deployed in a sustainable and renewable manner [28]. Photosynthetic microbes are an attractive option as agents of biological CCS technology because they have the ability to capture sunlight and use that energy to store carbon in forms useful to humans, such as fuels, food additives, and medicines [4, 29]. Microalgae could be used as a biological capturing agent in CCS technology. However, there are some important limitations that need to be overcome. Transporting CO_2 from a source of carbon dioxide, such as power plant flue gas, into algal cultures to increase CO_2 capture efficiency and productivity could be a challenge [30, 31]. In this study, **the second stage** in the CCS concept is to transport the CO_2 for the purpose of microalgae photosynthesis, while **the third stage** is to store the CO_2 in microalgal biomass in forms useful to humans such as fuels, food additives, and medicines [32].

Efficiently capturing carbon dioxide from an elevated CO_2 source depends on many factors, but one of the most limiting factors at present is the ability of the microalgae to capture and

fix carbon at a proper concentration to avoid acidification of the medium and crash of the culture, relatively high temperatures of CO_2 exhaust gas, and the presence of NO_x and SO_x co-products [31], all of which could inhibit the growth of microalgae. Therefore, selecting microalgal strains tolerant to high CO_2 concentrations, and at the same time able to efficiently mitigate large amounts of CO_2, is essential.

2.1. Biology of microalgae as photosynthetic organisms and CO_2 absorbers

Microalgae are microorganisms that vary greatly in size, ranging from a few micrometers to several meters in length. Many of microalgal strains are single celled (unicellular) whose shapes may be spherical, rod shaped, spiral, and other shapes. Other microalgae exist as aggregates of single identical cells held together after cell division to create larger formation, while other microalgae may include various types of cells that perform specific functions or multicellular cells with enormous size and complex morphology [33].

Physiologically, microalgae are aerobic photosynthetic organisms, thus they all contain chlorophyll and other photosynthetic pigments. Microalgae are most commonly found in aquatic environments, mostly as phytoplankton that serve as food source for other organisms and are the primary producers of organic matters or an important source of oxygen and form the base of the aquatic food chain. As photosynthetic organisms, microalgae are producers of organic carbon compounds, analogous to those produced by terrestrial plants.

Researchers [34] explained that microalgae have a membrane-bound nucleus just like other eukaryotic organisms. Besides eukaryotic cells organelles, microalgal cells also contain starch granules, oil droplets, and vacuoles that are arranged in groups. Each microalgal cell contains one or more chloroplasts that may be ribbon- or disc-shaped like those found in plants. Within chloroplast matrix are found flat vesicles called thylakoids. The membrane of the thylakoid contains chlorophyll and other supplementary pigments as the photochemical reaction sites of photosynthesis. Just like chlorophyll-bearing protozoa, several microalgae possess flagella or cilia, thus they resemble protozoa. Furthermore, [34] also explained that microalgae possess three types of photosynthetic pigments, that is: chlorophylls, carotenoids, and phycobilins that are present in chloroplast. There are five types of chlorophyll called chlorophyll a, b, c, d, and e, which are all green in color. All microalgae contain chlorophyll a. A researcher [35] described that carotenoids are water-insoluble hydrocarbons, which are of two types, that is, carotenes and xanthophylls. Phycobilins or biliproteins are water-soluble protein complexes, which are classified into two types, that is, phycocyanin and phycoerythrin. The brownish color of microalgae results from the dominance of the carotenes and the xanthophylls, whereas the reddish color of microalgae results from the high content of phycobilins.

2.2. Carbon dioxide fixation in photosynthetic process

As stated previously, the second stage in the CCS concept means to transport the CO_2 to a suitable storage or processing location for the sake of microalgal photosynthesis, while the third stage means to store it, as microalgal biomass, in forms useful to humans such as fuels, food additives, and medicines. The occurrence of photosynthesis requires the presence of not

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only chlorophyll but also CO_2 and water (which contains nutrients). The chemical reactions involved in photosynthesis are as follows:

$$(H_2O + NADP^+ \text{ light} \rightarrow NADPH + H^+ + O_2)\ 12x$$

$$(CO_2 + ATP + NADPH \rightarrow CH_2O + NADP^+ + ADP + P)\ 6x$$

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

Research studies that utilize the potential of microalgae as CO_2 absorber have been carried out in various countries, particularly in efforts toward adaptation and selection of microalgae species tolerant to high CO_2 concentrations and high CO_2 absorption rate. Researchers [33] and [19] have reported that the microalgal species *Chlorococcum littorale* could grow under CO_2 concentrations of over 20 *Chlorella* sp. were able to grow up even in aeration containing CO_2 up to 40%, at culture pH as low as 4 [36].

The saturation point of atmospheric CO_2 concentration for the growth of microalgal culture varied from 2% to 5% v/v, which means that above this saturation point, the microalgal cells do not have the ability to absorb additional CO_2 from the atmosphere, hence the dissolved CO_2 is abundant and the equilibrium of carbon ions will not shift toward the carbonate ions. As a result, at high CO_2 concentrations, the pH of the culture was relatively stable [37]. During photosynthesis, free CO_2 is the main inorganic carbon source to use in microalgal cultures. According to [38], absorption of dissolved CO_2 from the water due to photosynthesis will lower the concentration of dissolved CO_2. This reduction will increase the pH since the dissolved CO_2 exists in chemical equilibrium with bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}) in the water. Therefore, the rate of photosynthesis may be limited by a reduction in the amount of carbon, in this case carbon dioxide, changes in the forms of carbon in the water, and pH value. CO_2 uptake rates of microalgal cells could be stimulated by increase in CO_2 concentration in the media, and therefore culture without additional CO_2 would exhibit low CO_2 absorption rates, and although the pH of the culture seemed to increase, the pH never exceeded 8. In our previous study, [39] reported that the media pH varied from 7 to 8 which is considered sufficient for algal culture at laboratory scale.

Atmospheric CO_2 concentration also influences biomass concentration of microalgal culture in a hyperbolic curve response pattern. The accumulation of high biomass over a short time period is desirable and may be essential for making algal culture a viable option for contributing to the energy supply. The highest biomass concentration was achieved at CO_2 concentration of 10% v/v, which was equal to 2.05 g/l in *Chlorella vulgaris* culture and 2.95 g/l in *Ankistrodesmus convolutus* culture. These biomass concentrations correspond to absorptive capacity of 1.91 g CO_2/l/day in *C. vulgaris* culture and 3.41 g CO_2/l/day in *A. convolutus* culture, respectively [40]. An observation using microscope has demonstrated that *Chlorella* cultures grown under high CO_2 concentrations tend to have bigger cell size. Thus, although they had lower cell density when grown under CO_2 concentration of 10% v/v, they produced higher biomass weight. This can be understood as microalgal cells grown under adequate carbon

supply would have a chance to develop better than those grown under limited carbon supply. Therefore, in terms of biomass productivity, the most optimum CO_2 concentration for microalgal culture was found to be 10% v/v [41]. As a result, CO_2 as the primary carbon source for photosynthesis process in microalgae is adequately available, resulting in fast metabolism process and higher cell densities.

Carbon fixation competes with photorespiration because CO_2 and O_2 are both substrates for Rubisco [42, 43]. The oxygenase activity is not desirable as it leads to lose in carbon fixation. Analysis of the natural genetic variation in the kinetic properties of Rubisco from divergent photosynthetic organisms reveals that forms with higher specificity factors have lower maximum catalytic rates of carboxylation per active site, and vice versa (**Figure 2**). This inverse relationship implies that higher specificity factors would increase light-limited photosynthesis, while the associated decrease in catalytic rate would lower the light-saturated rate of photosynthesis. The CO_2 uptake by a crop canopy is determined by a dynamic combination of light-limited and light-saturated photosynthesis. Canopy simulations reveal that 10% more carbon could be assimilated by C3 crops if they were operating with a C4 Rubisco and this advantage would grow as atmospheric CO_2 levels continue to increase [43].

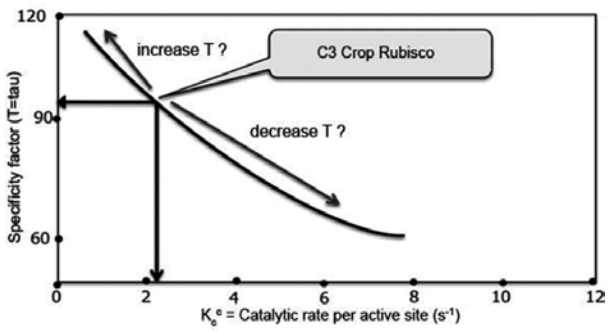

Figure 2. Relationship between Rubisco specifity and catalytic rate per active site [42].

2.3. Calvin-Benson cycle

Transformation of energy from one form to another through certain metabolic pathways corresponds to cellular functions. Enzymes are required for these transformation processes. The main energy production pathway is called cellular respiration, which can be aerobic or anaerobic. Photosynthesis is the major metabolic pathway in which energy is required under aerobic conditions.

Anaerobic pathway or fermentation pathway is a metabolic pathway that occurs in the absence of oxygen. Glycolysis is the first stage of anaerobic pathways. During glycolysis, glucose is broken down into two pyruvate molecules, yielding a net gain of two nicotinamide adenine dinucleotides (NADH) and two adenosine triphosphates (ATPs) [45]. However, the anaerobic reactions do not break down glucose completely to carbon dioxide and water, and the anaerobic pathways produce no more ATP beyond the yield from glycolysis. The final steps

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serve only to generate NAD^+, a coenzyme that is essential for the anaerobic pathway's process [29].

The process of photosynthesis is divided into two parts, the first of which requires the presence of light, also called the "light reactions," that is, the transformation of solar energy that is captured by color pigments (chlorophyll, phycocyanin) into chemical energy in the form of ATP and NADH by releasing oxygen as a byproduct. These reactions take place in an inner membrane system that is called the thylakoid membrane system, and occur in three phases as follows:

1. Pigments absorb sunlight energy and give up electrons.
2. Electron and hydrogen transfers lead to ATP and NADPH formation.
3. The pigments that gave up electrons in the first place get electron replacements.

According to [46], systems that capture solar energy to produce energy molecules (including ATP) are called photosystems. In thylakoid membranes, there are two types of photosystems that excite electrons by two different electron transport systems as follows:

1. Photosystem I: The cyclic pathway of ATP formation.
2. Photosystem II: The non-cyclic pathway of ATP formation. In photosystem II photolysis process occurs, which is a series of reactions that dissociate water molecules into oxygen ions, hydrogen ions, and electrons. Electrons from photosystem II are passed to photosystem I.

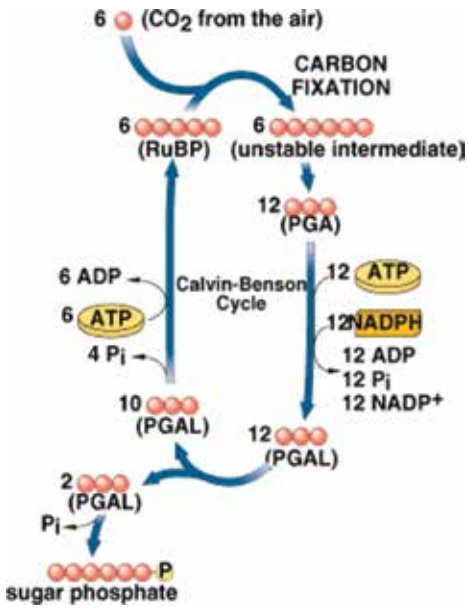

Figure 3. Calvin-Benson cycle [47].

During the first stage of photosynthesis, that is, the "light reactions," sugars have not yet been produced. Sugars are produced during the second stage [43].

The second stage, or the light-independent stage, as it can take place in the absence of light, provided there is sufficient ATP and NADPH to synthesize organic molecules from CO_2 and H_2O, is illustrated in **Figure 3**. The first step is the incorporation of CO_2 molecules into RuBP, catalyzed by Rubisco enzyme, commonly known as carbon fixation, followed by the next step, that is, entering the Calvin cycle, often referred to as the Calvin-Benson cycle, with the end product being organic groups, such as sugars [47, 48, 98].

Much research needs to be carried out to discuss the theoretical limits of photosynthetic efficiency in an effort to determine what can be done to reach these goals. Researcher [33] described that photosynthetic efficiency is the fraction of total solar radiation that is converted into chemical energy during photosynthesis, expressed as the following equation: $2H_2O + CO_2 + \text{energy} \rightarrow CH_2O + H_2O + O_2$

In oxygenic photosynthetic organisms, CO_2 is fixed in the Calvin cycle by Rubisco to increase the efficiency [49]. Substantial losses to photosynthetic efficiency lie between initial transfer reactions of photosynthesis and carbohydrate biosynthesis. Depending on the mechanism utilized to fix carbon and the amount of ATP and NADPH utilized, and assuming total incident radiation including infra-red, the maximal theoretical efficiency at this stage (including light capture and energy transduction) is between 8% and 13% before photorespiration and respiration [42].

Therefore, photosynthetic efficiency is affected by several factors, that is, light intensity, partial pressure of oxygen and CO_2 [33], mass transfer of CO_2 into liquid, temperature, and availability of nutrients [42]. Additionally, the amount of RuBisCO represents an intrinsic limit which determines the rate of carbon fixation (Bar-Even et al., 2010). However, in some cases, photosynthetic efficiency will be different in aquatic versus terrestrial species [13].

2.4. Carbon concentrating mechanisms

Carbon concentrating mechanism (CCM) acts as an enhancer to a higher microalgae growth, and therefore can be used to improve productivity in a photobioreactor [50]. The anhydrase carbonic enzyme expression is related with CCM induction. Carbonic anhydrase (CA) enzyme catalyzes CO_2 and HCO_3^- interconversion. It is a major component in intracellular mobilization from pool HCO_3^- *pool*, by catalyzing CO_2 production for Rubisco enzyme [51]. The role of CCM is mainly to increase CO_2 concentration for Rubisco, which is the enzyme responsible for CO_2 initial fixation.

Photosynthetic microorganisms in water as eukaryote microalgae, cyanobacteria, and photosynthetic bacteria have an ability to utilize CO_2, facilitated by ribulose-1,5 bisphosphate carboxylase/oxygenate enzyme (Rubisco). This Rubisco enzyme serves to capture Ci in the form of CO_2 in darkness (light-independent) through photosynthesis reaction (Calvin cycle) [38, 98]. When pure CO_2 is dissolved, pH drops to below 7, therefore creating acidic condition.

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The dominant Ci species in acids are CO_2 and HCO_3^- [52]. Microalgae have developed various ways to ensure that Rubsico enzyme will accompany CO_2 in a certain CCM through a movement of such inorganic carbon across plasma membrane [30].

For microalgae, carbon dioxide is an important limiting factor that will affect growth and metabolism. An active, continuous supply of carbon dioxide by Rubisco in chloroplast is a requirement during photosynthesis. The carbon dioxide will enter through a CO_2 gas diffusion within medium or through a carbonate conversion. Rubisco only reacts with CO_2, not with bicarbonate or carbonic ion. Therefore, another enzyme, CA, is needed to convert carbonic and bicarbonate ion into CO_2. This enzyme is intracellular or extracellular. CA is utilized to help photosynthesis process of carbonic compounds into biomass. CO_2 in culture medium will reach saturation and will turn into carbonic compounds when it reacts with water. This carbonic compound will be transformed into biomass with the assistance of CA.

Concentration mechanism strategy depends on the forms of carbon in the process. CO_2 to HCO_3^- conversion in aquatic environment highly depends on pH, while basic environment affects HCO_3^- formation. Inside a cell, enzymatic interconversion takes place to transport and concentrate CO_2 in the carbon fixation process, particularly inside chloroplast pyrenoid of green microalgae cell or cyanobacteria carboxysome [98].

3. Microalgal cultivation

The key to the success of culture techniques depends on the suitability between microalgal species being cultured and several environmental factors. Researcher [53] described factors affecting microalgal growth (cultivation) as growth factors. The growth factors are further classified into resource factors and supporting factors (non-resources factors). The resource factors involve resources that are directly utilized by algal cells for their growth, such as nutrient elements, sunlight, and CO_2. While the supporting factors consist of environmental factors affecting metabolism process in microalgal cells, such as temperature and acidity level (pH). Influences of the resource factors on microalgal growth are commonly illustrated by a hyperbolic function that describes saturation phenomenon, in which increasing the availability of resource factors will not be able to increase the growth of microalgae anymore. The saturation phenomenon is further used in microalgal culture assessments in determining optimum conditions to achieve the most efficient productivity level.

Nitrogen and phosphorus are parts of the resource factors. Numerous studies have been conducted to assess the optimum concentrations of both elements to culture microalgae based on their uses. Many previous studies have reported decreased algal cell viability as the result of nutrient deficiencies of various nutrient elements. This is due to the loss of cell's ability to construct functional structures associated with the limited nutrients. Researchers have [54] underlined the importance of biomass productivity to assess economic feasibility of algal culture, while [55] reported the necessity of attaining biomass productivity in line with production of unsaturated fatty acids in algal culture.

3.1. Promising photobioreactor as a closed cultivation system

Researchers [56] suggested that the best result obtained from the microalgal cultivation in terms of productivity depends greatly on the choice of culture systems. Microalgae can be cultured using a wide variety of systems ranging from controlled indoor systems such as closed laboratory to less predictable systems, such as tanks and pools. There are two microalgal culture systems: (1) open ponds and (2) closed photobioreactors [57]. In laboratories, there are three culture techniques that are most routinely adopted including static, semi-continuous, and continuous cultures [58]. Advantage of indoor culture is its high degree of control over environmental factors such as temperature, light intensity, contamination, and nutrition; however, it requires higher cost than outdoor culture. Outdoor culture is less expensive, but it is difficult to control environmental conditions for optimum growth of microalgae, and it is readily contaminated.

The major limiting factor for both open-pond and enclosed photobioreactor operation is water usage. Typically, sites considered the best for algal production have warm temperatures and high average irradiance throughout the year. In locations with these properties, evaporation from open ponds, and gradual heating of photobioreactors become a problem. The solution to both of these problems is to use more water, either to replace the water lost through evaporation or to evaporatively cool the photobioreactor. Total water usage for production processes inflates dramatically, and sometimes the cost and availability of water become necessary to ensure continuous process. Open pond design needs to become more resistant to contamination, and resistant to evaporation. Additionally, a low-cost gas delivery technique needs to be designed if microalgae are to ever capture carbon from power plants.

Laboratory-scale photobioreactors are mainly equipped with fluorescent light or other light source distributions, as performed to other types of bioreactor, for example, bubble column [59, 60], airlift column [61], stirred tank [62], helical tubular [63], conical [64], and torus [65].

Central to microalgae-based carbon capture are photosynthesis processes, where such processes are supposed to take place, how to improve capture efficiency, and how to easily maintain the system. Therefore, photobioreactor needs to be designed as a reaction vessel.

Researchers [26] state that photobioreactor is a device used to provide an optimum condition for microalgae to perform the process of photosynthesis. This is because it is designed to adapt with available lighting, temperature, pH, CO_2, and nutrition. In term of microalgae productivity, the photobioreactor diameter is a critical design element. Researchers [66] state that light intensity directly depends on photobioreactor diameter, while [67] proved that an increase of vertical photobioreactor of 13 ft (3.96 m) does not affect biomass. The photobioreactor specifically designed for this research is made from transparent glass with tube diameter of 15 cm to receive adequate lighting for a high density of microalgae culture. Therefore, photosynthesis efficiency in artificial environment is higher compared to natural environment.

Every photobioreactor known at present has its own advantages and limitations (**Table 2**). However, regardless the selected reactor design, there are similar technical requirements to a maximization of microalgae growth. When designing a photobioreactor, the main objective

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remains to maximize specific growth rate (μ), defined as "an increase of cell mass in culture per time unit per cell mass unit."

Photobioreactor system	Advantages	Disadvantages
Vertical column	A high mass transfer, good mixing with a low shear stress force, potential for multi-scale application, easy to sterilize, ready to use, appropriate for algae immobilization, reducing photo-inhibition and photo-oxidation	Small surface area exposed by light, complex material for its construction, shear stress in microalgae culture, reduced surface area exposed by light during scale up
Plate type	Large surface area exposed by light, appropriate for outdoor culture, appropriate for algae immobilization good lighting path, high biomass productivity, relatively cheap, easy to clean, ready to use, small accumulation of oxygen	Scale u requires many spare parts and supporting material, difficult to control culture temperature, the presence of growth in wall area, possible hydrodynamic stress in some algae species
Tubular	Large surface area exposed by light, appropriate for outdoor culture, high biomass productivity, relatively cheap	The presence of pH, dissolved oxygen, and CO_2 gradients along the pipes, scaling, the presence of growth in wall area, vast land requirement

Table 2. Advantages and limitations of microalgae photobioreactor systems [26].

3.2. Enhancing CO_2 removal efficiency in closed-system photobioreactor

In our previous study, controlling environmental parameters in closed-system photobioreator could improve the ability of microalgae to remove CO_2. Important parameters will be discussed below.

3.2.1. Nitrogen and phosphorus requirement

Nutrients required by microalgae include macronutrients and micronutrients. Elements that belong to macronutrients are C, H, N, P, K, S, Mg, and Ca, while micronutrients include Fe, Cu, Mn, Co, Mo, Bo, Vn, and Si. Specifically, Mn, Fe, Zn, and Vn are required for photosynthesis. Mo, Bo, Co, and Fe for nitrogen metabolism, and Mn, Co, and Cu for other metabolic functions. Of these nutrients, N and P frequently become limiting factor for microalgal growth [68]. Micronutrients act in enzyme systems, oxidation and reduction process in microalgal metabolism, and chlorophyll production. Micronutrients are required to perform various functions during microalgal growth. The most general effect of nutrient deficiency on microalgae is a decrease in protein and photosynthetic pigments and an increase in concentration of carbohydrate and lipid [69, 94].

Nitrogen and phosphorus are the main inorganic nutrients required by microalgae to grow and reproduce. Nitrogen in waters is present as molecular nitrogen (N_2) or as organic nitrogen compounds that have dissociated to inorganic salts such as nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+) [34]. Dissociation of protein and other nitrogenous compounds can result

in pH level increase. Generally, when utilizing nitrogen, microalgae have tendency to gradually and sequentially take up ammonium, nitrate, and nitrite [70].

Microalgae commonly use nitrate as their primary source of nitrogen. Nitrogenous compounds are greatly influenced by the amount of oxygen dissolved in the water. In presence of low oxygen, nitrogen is converted into ammonia (NH_3), whereas high oxygen content encourages conversion of nitrogen into nitrate (NO_3^-). However, under unfavorable environmental conditions, ammonium, or urea may serve as nitrogen sources [71, 72]. Ammonium is generated through dissociation process of ammonium hydroxide. Ammonium hydroxide is a solution of ammonia in water. Researchers [70] reported that the ammonium formation reaction is as follows: $NH_3 + H_2O \Leftrightarrow NH_4OH \Leftrightarrow NH_4^+ + OH^-$. As the forward reaction proceeds, the concentration of ammonium increases, and the medium pH becomes alkaline. Components of organic nitrogen include amino acid (constituent of protein), nucleic acid, enzyme and energy carriers such as chlorophyll, adenosine diphosphate (ADP), and ATP. Nitrogen is required by *Arthrospira* sp. during amino acid formation, cellular growth, and gas vacuole regulation.

Absorbed phosphorus constitutes part of the cell's structural component and contributes in cellular energy conversion processes. Commonly, phosphorus is absorbed by *Arthrospira* sp. in the form of phosphate (PO_4^{2-}). Phosphate is required to synthesize nucleotides, phospholipids, and sugar phosphates [70, 94]. The ratio of N to P in a water body also results in the growth of microalgae with different strain compositions. In a laboratory algal culture, the ratio of nitrogen to phosphorus equal to 30 : 1 was found to be more favorable for diatoms, the ratio of N to P equal to 20 : 1 is more favorable for chlorophyceae, while the ratio of N to P equal to 1 : 1 was more favorable for dinoflagellata [70]. Phosphate uptake requirement is higher when the nitrogen is present as nitrate than ammonium salts. The lowest phosphate concentration for optimal microalgal growth ranges from 0.018 to 0.090 ppm P-PO_4, and the highest concentration ranges from 8.90 to 17.8 ppm P-PO_4 when the nitrogen is present as nitrate and 1.78 ppm P-PO_4 when N occurs as ammonium. For optimum growth of phytoplankton, NO_3^-N should be between 0.9 and 3.5 mg/l, while phosphate should range from 0.09 to 1.80 mg/l [73].

In laboratories, microalgae are cultured in artificial medium containing macronutrients and micronutrients. Compared to heterotrophic organisms, photosynthetic organisms require substantially more metal ions as they act as redox active cofactors in photosynthetic electron transfer. Additionally, many algae are autotrophic for certain vitamins such as vitamin B12 which they must obtain from the environment. Potassium is a nutrient that is required as a cofactor for several enzymes and is involved in protein synthesis and osmotic regulation. Sulfur is an essential constituent of some amino acids, vitamins, and sulfolipids, and is essential for the growth of *Arthrospira* sp. [74]. Sodium plays a role in cellular osmoregulation. However, excessive levels of sodium may cause a reduction in photosynthetic pigments [75, 76]. Micronutrients such as Mg, Ca, Fe, EDTA, Cu, Mn, Mo, B, Co, and Zn are essential nutrients that are required in very small amounts. However, a lack of these essential nutrients may inhibit the growth of phototrophic organisms as the metabolism was disturbed [77]. Magnesium is important for the synthesis of accessory photosynthetic pigments due to its position

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as the center of the molecular structure of chlorophyll. Additionally, magnesium has a key function in the aggregation of ribosomes into functional units and for the formation of catalase. Calcium is required in cellular membrane activities and acts as a catalyst in enzymatic reactions. Iron and the other metals act as cofactors [74].

3.2.2. Light intensity and temperature in photobioreactor

The development of micro-communities of algae is a function of factors regulating the growth of their components. Each species of microalgae has its own unique temperature and light intensity requirements for its maximum growth [78]. Light intensity plays a significant role in electronic excitation in photosystems, thus allowing photosynthesis to occur [79]. Algae can grow in the absence or presence of light. In the absence of light, microalgae can grow heterotropically using limited carbon, such as glucose, as substrate. In this mode of growth, the growth rate is much higher than it can be when microalgae grow in the presence of light and photosynthetically or photoautotrophically. Under optimal conditions, the maximum photoautotrophic growth rate (μ_{max}) is only half of that of heterotrophic bacteria because of major differences in the allocation of cellular resources [80].

During photoautotrophic growth, as much as 30% of the total cellular protein is allocated to the processes of photosynthesis and carbon fixation. Typically, RuBisCO accounts for 10% of total protein content of these cells and the apoproteins in the photosynthetic organelles accounts for up to 20% [79]. Microalgae grown photomixotrophically, where they use not only endogenous but also exogenous carbohydrates as an energy source, show a higher μ_{max} than when grown photoautotrophically, but the cost of the resulting fuel is increased because of the added cost of reduced carbon sources. Additionally, photomixo-trophic growth has many implications for greenhouse gas emissions depending on how the feedstock anticipates the availability of the reduced carbon [54], and how the feedstock was obtained and processed [81].

Photosynthetic rates may increase when microalgae are cultured in a photobioreactor. For the CO_2 fixation and biomass production, optimum light intensity is necessary. Below the optimum light intensity, light becomes the limiting factor for the microalgae productivity, while exposure of cells to long period with high light intensity causes photoinhibition [82]. Researchers [83] also described phenomenon of photoinhibition. Under prolonged irradiation at a supraoptimal level, photosynthetic rates usually decline from their light-saturated values. A further rise in light intensity to above 8000 lux did not make much difference to either the growth rate or the dry weight of the microalgae, suggesting that a light saturation point had been reached. Saturation light intensity roughly varies from 30 to 45 W/m2 (140–210 $\mu E\ m^{-2}\ s^{-1}$) with a good estimation. For example, according to [84], saturation light intensity of *Chlorella* sp. and *Scendesmus* sp. is around 200 $\mu E\ m^{-2}\ s^{-1}$. The ratio of light to dark (or low-intensity light) periods in a cycle is crucial for microalgae productivity [85].

Some experts suggested that while durations of daily light/dark cycles are considered long enough to allow algal cells to adapt to the light/dark cycles, the cycle durations of intermittent illumination caused by stirring the culture are too short to adapt, thus microalgal cells may otherwise adapt to an average light intensity in the reactor [86]. A biomass/chlorophyll ratio which tends to decrease as the atmospheric CO_2 level increases indicates excessive chlorophyll

synthesis and shows the importance of high light intensity to stimulate CO_2 absorption rate and enhance productivity rate of microalgal culture [87]. Some other experts suggested that the increase in photosynthetic efficiency might be associated with time synchronization between photosynthetic dark-light reactions in the cultures. Dark fractions of intermittent illumination are long enough to permit microalgal cells to accomplish the dark reaction process prior to receiving light energy for activation of the next photosynthetic process [53]. Dark period of 10–12 h is the optimum photoperiod for the growth of diatoms. Increase in light intensity of 5000–12,000 lux can enhance growth of diatoms, but the growth decreases when the light intensity exceeds 12,000 lux [80]. Light intensity of 4000–5000 lux is an optimum light intensity range for auxospore formation [39].

When optimum light intensity is maintained during the process, *Arthrospira* will attain optimum level of biomass and optimum growth rate. However, light intensity may also result in photoinhibition and photooxidation [88]. Researchers [78] defined photoinhibition as the decrease in photosynthetic capacity caused by excessive photon flux densities (PFDs), which leads to damage to photosynthetic pigments. The damage occurs when electron energy produces superoxide dismutases which play a role as free radicals in the cells. On the contrary, when the light intensity is not optimum, the growth of *Arthrospira* will become less optimum as the energy produced via the photosynthetic process is limited due to low electron excitation.

Temperature is the most important limiting factor, after light, for culturing algae in both close and open outdoor systems that regulate cellular, morphological, and physiological responses of microalgae [40, 89, 90, 91]. Higher temperatures generally accelerate the metabolic rates of microalgae, whereas low temperatures lead to inhibition of microalgae growth [85]. The optimum growth temperature of most microalgae is in the range of 20–30°C [5]. When the temperature is much lower or much higher than the optimum, specific growth rate of microalgae is reduced [92, 93]. Temperature is not a limiting factor for microalgae in natural waterbody as long as many species can grow in appropriate environmental conditions. However, temperature greatly affects speed of growth and reproduction.

In our previous study, controlling environmental parameters in closed-system photobioreactor could improve the ability of microalgae to remove CO_2. CO_2 removal efficiency was highest when microalgae consortium cultivated in 4000 lux light intensity, periods of light/dark (16/8), and temperature 30°C. Microalgae consortium demonstrated optimum capacity to remove CO_2 at 10% CO_2 supplied. This was evidenced by dry weight of biomass which was 2.5 times higher, CO_2 removal efficiency above 2.5 times higher and the CO_2 utilization efficiency over 5 times higher. In addition, carbon transfer rate also increased. All results were compared with initial condition (2500 lux, light/dark (24/0) and 25°C [39, 41, 94].

3.2.3. Mass transfer

Carbon dioxide mass transfer is one among hydrodynamics variables related to microalgae growth and carbon dioxide reduction effectiveness. CO_2 mass transfer coefficient or $k_La(CO_2)$ in general can demonstrate mass transfer condition occurring in the reactor. k_La (CO_2) value is a hydrodynamic parameters commonly used to assess bioreactor performance in microalgae cultivation process. This process requires an optimum k_La value; higher k_La value indicates a

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better CO_2 mass transfer within microalgae culture. Mass transfer of carbon dioxide from air into the media can be growth limiting in dense microalgal cultures. Transfer of CO_2 from gas to liquid depends on many parameters. Physical parameters such as gas flow rate, CO_2 partial pressure, bubble diameter, and lifetime can have large influences on the rate of transfer [47, 95].

CO_2 transfer efficiency affects CO_2 bio fixation in regard to improving microalgae productivity in a photobioreactor culture system. Mass transfer rate of gas mass transfer in a photobioreactor serves as one of parameters determining microalgae growth and CO_2 gas bio fixation. Therefore, Sparger is installed in the lower part of bioreactor to transform gas with different CO_2 flow rate into a small bubble aiming to improve mass transfer. Sparger is also deliberately installed in the lower part to distribute CO_2. The formed bubbling can increase CO_2 mass transfer while removing O_2 produced during photosynthesis.

The water chemistry also influences the solubility of CO_2 and the transfer capacity. CO_2 can be dissolved in water according to Henry's law and reacts with water to form carbonic acid (H_2CO_3). The equilibrium shifts toward HCO_3^- (bicarbonate) as the pH increases to a neutral range [81, 96]. HCO_3^- is actively transported into microalgae while CO_2 enters the cell by passive diffusion [97]. The pH of the media plays a major role in mass transfer and can drastically alter growth dynamics of the microalgae. Controlling pH by the addition of buffering agents can affect mass transfer of CO_2 and carbon uptake by the microalgae.

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Chapter 7

Algal Nanoparticles: Synthesis and Biotechnological Potentials

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Additional information is available at the end of the chapter

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Abstract

A nanoparticle can be defined as a small object that behaves as a whole unit in terms of its transport and properties. Nanoparticles are sized between 1 and 100 nm in diameter. Nanoparticles can act against the microbes in multiple ways, and the microbes are less likely to develop resistance against nanoparticles because it requires multiple gene mutations. The large surface-to-volume ratio of nanoparticles, their ability to easily interact with other particles, and several other features make them attractive tools in various fields. Nanoparticles are widely used various fields such as electronics, cosmetics, biomedical, and biotechnology. Nanoparticles can be synthesized by physical methods such as attrition, pyrolysis, and using some wet chemical methods. The physical and chemical methods have various drawbacks such as high cost of production, require high energy input and generation of toxic by-products. To overcome this, several biological methods are employed in the synthesis of nanoparticles. The biological methods are generally cost effective, nontoxic, and ecofriendly. This chapter focuses on the methods involved in algal-synthesized nanoparticles and its applications.

Keywords: Nanoparticles, Green synthesis, Antibiotic activity, Antitumor, Antibiofilm

1. Introduction

Nanotechnology is a vibrant and developing area of science, engineering, and technology accomplished at the nanoscale level. The products of nanotechnology are nanoparticles or

nanomaterials (NPs), lying in the range of 10^{-9} m and having dimensions of 1–100 nm. NPs are categorized into three types: natural nanoparticles, incidental nanoparticles, and engineered nanoparticles [1]. The large surface-to-volume ratio of nanoparticles, their ability of easy interaction with other particles, and several other features make them as an attractive tool in various fields. NPs are widely used in electronic, cosmetic, biomedical, and biotechnological applications. The efficient crystallographic and physiochemical properties of NPs make nanotechnology as an excellent area to focus. The synthesis of NPs can be achieved by some physical methods and chemical methods. The traditional and commonly used method for nanoparticles synthesis is wet method. In chemical synthesis, nanoparticles are grown in a liquid medium containing various reactants particularly reducing agents such as sodium borohydride [2], potassium bitartartarate [3], methoxypolyethylene glycol [4], or hydrazine [5]. Some stabilizing agents such as sodium dodecyl benzyl sulfate [5] or polyvinyl pyrrolidone [3] are added to the reaction mixture to prevent the agglomeration of metallic nanoparticles. Most commonly used chemical methods are chemical reduction [6], electrochemical techniques [7], and photochemical reactions in reverse micelles [8]. Commonly used physical methods are attrition and pyrolysis. Attrition involves grinding of the particles by a size-reducing mechanism. The particles are then air-classified, and oxidized nanoparticles are recovered. Pyrolysis involves burning of the precursor by passing them through an orifice at high pressure. The ash obtained is air classified to recover the oxidized nanoparticles [9]. Chemical methods are of low cost for high volume, and their major drawbacks include contamination from precursor chemicals, use of toxic solvents, and generation of hazardous by products, and the demerits of physical methods are low production rate, high cost of production, and high energy consumption [5]. There is need for replacing the toxic ingredients with environmentally safe method for synthesizing NPs. To overcome this, researchers are focusing on employing biological method for the synthesis of nanoparticles. They are generally cost effective, nontoxic, and ecofriendly [10]. So far, several plant extract [11], bacteria [12], fungi [13], enzymes [14], and algae [15] have been used for the synthesis of NPs. To our surprise, an emerging trend of synthesizing NPs using algae is developing in the recent years.

Algae are economically and ecologically important group of photosynthetic organism. They are unicellular or multicellular organisms dwelling in different environment such as freshwater, marine water, or surface of moist rocks [16–18]. Algae are categorized as microalgae (microscopic) and macroalgae (macroscopic). They play a key role in medical, pharmaceutical, agriculture, aquaculture, cosmetics applications. Algae are valuable source for various commercial products such as natural dyes and biofuels [19–22]. Till now, for the biosynthesis of metallic NPs, different group of algae such as Chlorophyceae, Phaeophyceae, Cyanophyceae, Rhodophyceae, and others (diatoms and euglenoids) have been used [23]. The ability of algae to accumulate metals and reduce metal ions makes them the superior contender for the biosynthesis of nanoparticles. Furthermore, algae are relatively convenient and easy to handle, along with several other advantages such as synthesis at low temperature with greater energy efficiency, less toxicity, and risk to the environment. In physical and chemical method, different commercially available surfactants were used as templates and capping agents in NPs synthesis with different morphologies. Removal of the residual components becomes a major issue. Considering this utilization of naturally eco-friendly methods having been developed

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which involves the synthesis of NP using different biological sources which could naturally modify the shape or size of a crystal with superior quality [24].

Among the biological materials, algae are called as —bionanofactories‖ because both the live and dead dried biomasses were used for the synthesis of metallic nanoparticles [25]. Several algae such as *Lyngbya majuscule*, *Spirulina platensis*, and *Chlorella vulgaris* were used as a cost effective method for silver nanoparticles synthesis [26, 27]. The synthesis of silver nanoparticles using *Ulva fasciata* extract as a reducing agent and this nanoparticles inhibited the growth of *Xanthomonas campestris* pv. *malvacearum* [28]. In addition to seaweeds, microalgae such as diatoms (*Navicula atomus* and *Diadesmis gallica*) have the ability to synthesize gold nanoparticles, gold, and silica–gold bionanocomposites [29]. Comparing with other organism such as fungi, yeast, and bacteria, algae is equally an important organism in the synthesis of NPs; therefore, the study of algae-mediated biosynthesis of nanometals can be taken towards a newer branch and it has been termed as phyconanotechnology [10, 23, 30]. Thus, this work explains the potential and beneficial application of algal-mediated synthesized nanoparticles for present and future perspectives.

2. Types of nanoparticles

There are two different types of NPs, inorganic NPs and organic NPs. The inorganic NPs include metal and metal oxides, which are potent antibacterial agents [31] (**Figure 1**). Metal

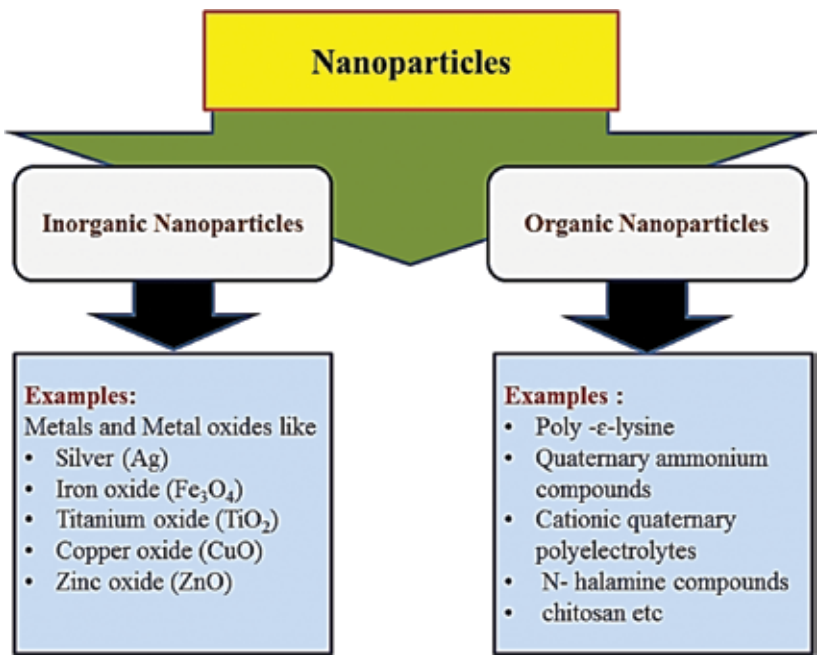

Figure 1. Different types of nanoparticles.

oxide nanoparticles such as silver (Ag), iron oxide (Fe_3O_4), titanium oxide (TiO_2), copper oxide (CuO), and zinc oxide (ZnO) are certain examples of inorganic NPs. Organic NPs includes poly-◎-lysine, quaternary ammonium compounds, cationic quaternary polyelectrolytes, N-halamine compounds, and chitosan . Organic nanoparticles are generally less stable at high temperatures. Due this reason, inorganic nanoparticles are more preferred as antimicrobial polymers [32].

2.1. Inorganic nanoparticles

So far, there are different types of inorganic metals and metal oxide NPs, which have been studied. Some important examples are detailed (**Figure 2**)

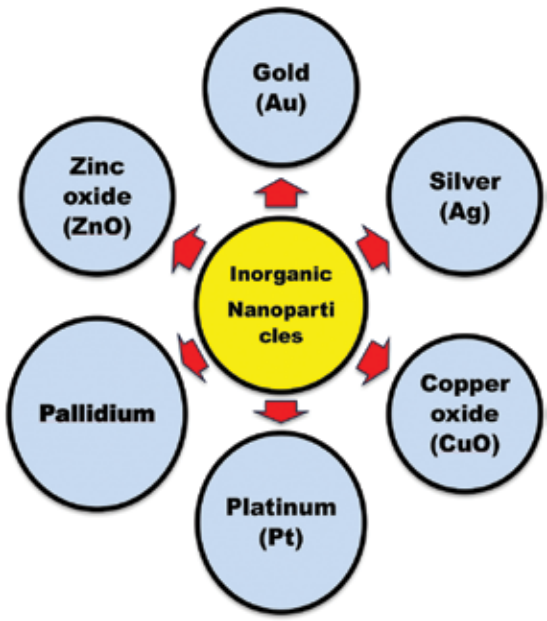

Figure 2. Types of inorganic nanoparticles.

2.1.1. Silver

Silver nanoparticle (AgNP) is the most widely used antimicrobial agent against many bacteria, fungi, and viruses [33]. The antimicrobial activity AgNP was found to be size dependent, and larger particles are less active than smaller one against many pathogens in both *in vitro* and *in vivo* analysis [34–36]. The resistance of bacteria towards antibiotics has made AgNPs more effective than antibiotics [37, 38]. Though there is plenty of research in AgNPs, the actual mode of action of AgNPs is still unclear [39]. In *E. coli*, the AgNPs create holes in the cell wall and increase the membrane permeability, thereby inactivating the cell activity [40, 41]. Some reports revealed that the Ag ions disrupt the protein structure by binding to thiol and amino groups [42]. AgNPs are photocatalytic [43], and they can generate reactive oxygenic species

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(ROS) [44, 45]. AgNPs are effective against both Gram-positive and Gram-negative bacteria [46, 47].

2.1.2. Titanium oxide

Titanium oxide (TiO_2) is found to be effective against both Gram-positive/Gram-negative bacteria, viral, and parasitic infections [48, 49]. They are photocatalytic; their toxicity can be induced by visible light, or UV light, generates ROS [50]. TiO_2 is an effective bactericidal agent and a potent sporicidal agent against wide range of bacteria [51].

2.1.3. Zinc oxide

ZnO nanoparticles (ZnONPs) are another broad spectrum antibacterial agent, based on concentration and size of the NPs, and they are effective against methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and methicillin-resistant *Streptococcus epidermis* (MSSE) [52]. They are of low cost and found to inhibit the growth of a wide range of pathogenic bacteria (*Klebsiella pneumoniae*, *Listeria monocytogenes*, *Salmonella enteritidis*) [53], *S. mutants*, *Lactobacillus* sp., and *E. coli* [53, 54], with less toxicity to human cells. Their UV blocking and anti-biofilm activity makes them as a suitable coating material for medical and other devices, and it is approved by the Food and Drug Administration (FDA) in the treatment of disease and ingredients in food additives [50, 55].

2.1.4. Iron oxide

Iron oxide is generally inactive in their bulk form. Reducing their size to nanoscale makes them a potential antimicrobial agent. Iron oxide nanoparticles-coated surfaces prevent the adhesion and colonization of Gram-positive and Gram-negative bacteria [56].

2.1.5. Gold

As compared to Ag, Au nanoparticles are less effective and lack antimicrobial properties when used alone but found to be effective when used in combination with antibiotics such as ampicillin [57, 58], vancomycin [59], and lysozyme (an antibacterial enzyme) [60]. The Au nanoparticles can also be used in combination with nonantibiotic molecules such as amino substituted pyrimidines [61] and citrate, which induces the generation of ROS and mutations, hence used in cancer therapy [62].

2.1.6. Copper oxide

Despite copper oxide (CuO) nanoparticles are used as antibacterial agents, they are less effective than that of Ag and ZnO. So a comparatively higher concentration is required to get desired results. But some bacteria are more susceptible to CuO than Ag. For example, *E. coli* and *S. aureus* were more sensitive to silver but *B. subtilis* and *B. anthracis* were more sensitive to Cu [63, 64]. The cell wall composition of *B. subtilis* and *B. anthracis* is rich in amine and carboxyl groups, which allow the strong affinity of CuO towards the bacteria [65, 66]. CuO NPs exhibit antibacterial activity by membrane disruption and ROS production [65].

2.1.7. Magnesium oxide

Magnesium oxide (MgO) nanoparticles are efficient antimicrobial agent exhibiting bactericidal activity against both Gram-positive and Gram-negative bacteria, spores and viruses. The MgO NPs can be prepared from available and economical precursors. Along with membrane disruption and ROS generation, it also inhibits the essential enzymes of bacteria [50, 67].

2.1.8. Nitric oxide

Nitric oxide (NO) nanoparticles are highly reactive antibacterial agent. Similar to other nanoparticles, the activity of NO is also size dependent [68, 69]. The mode of inhibition is by the production of reactive nitrogen species (RNS) rather than ROS. They are effective against MRSA and various biofilm forming bacterial species [70, 71].

2.1.9. Aluminium oxide

Aluminium oxide is a mild antibacterial agent effective only at higher concentrations [65]. There mode of inhibition is by pit formation, perforation, and membrane disruption leading to cell death [66].

2.2. Organic nanoparticles

Some of the well-known examples of organic NPs are discussed below (**Figure 3**).

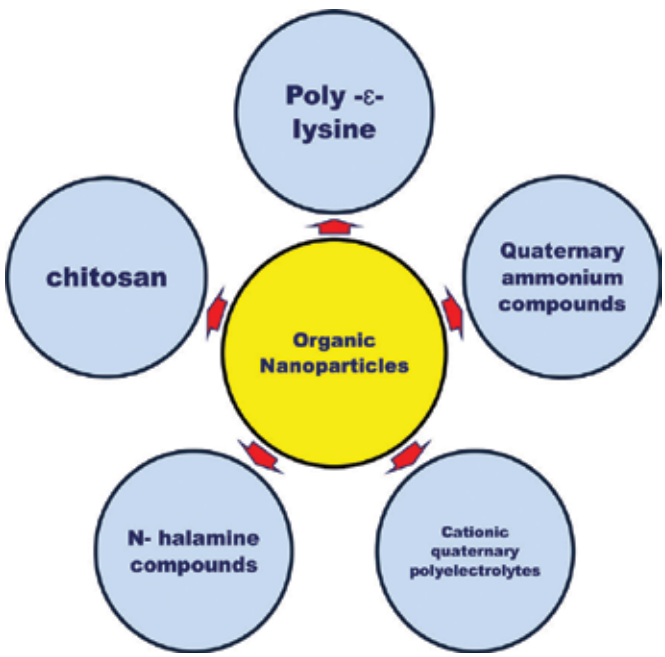

Figure 3. Types of organic nanoparticles.

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2.2.1. Poly-ε-lysine

Poly-ε-lysine, a cationic homopeptide of L-lysine is effective against Gram-positive bacteria and spores of *B. coagulans*, *B. subtilis*, *and B. stearothermophilus* [72].

2.2.2. Quaternary ammonium compounds

Quaternary ammonium compounds are well known disinfectants and their antimicrobial property dependents on the chain length. The positively charged moieties of the compounds are attached to the negatively charged bacterial membrane by weak electrostatic interaction, followed by the insertion of hydrophobic tail of the compound in to the bacterial hydrophobic membrane core leading to the denaturation of structural proteins and enzymes [73].

2.2.3. Cationic quaternary polyelectrolytes

They are synthesized from methacrylic monomers such as 2-(dimethylamino) ethyl methacrylate and majority of them are derivatives of acrylic and methacrylic compounds. These molecules possess a wide range of biological applications due to their structural flexibility through the alteration of hydrophobicity, molecular weight, surface charge and other factors [74].

2.2.4. N-halamine compounds

N-halamine compounds are formed by the halogenation of imide amide or amine groups with one or more nitrogen–halogen covalent bonds. These are high stable compounds releasing free active halogen groups slowly in to the environment leading to the inhibition or inactivation of the microbial cells [75].

2.2.5. Chitosan

Chitosan NPs are biocompatible, nontoxic, and have the ability to act as absorption enhancer. These characteristics make the chitosan nanoparticles as an effective antimicrobial agent with broad spectrum activity against a wide range of bacteria, fungi and viruses. The antibacterial activity of chitosan nanoparticles depends on several factors such as pH and the nature of solvent [76, 77]. The use of chitosan along with metal nanoparticles is not feasible since chitosan reduced the activity of metal nanoparticles such as Zn. It can be used in combination with antibiotics [76, 78]. Even though some studies state that the interaction of cells with chitosan lead to membrane destabilization, followed by lysis and cell death, the detailed mode of action is unclear [79].

2.3. Synthesis of NPs using algae

The abundance and ease of availability of algae make them good and worthwhile sources for the synthesis of metallic nanoparticles [80]. Synthesis of nanoparticles using algae can be performed in three important steps, (i) preparation of algal extract in water or in an organic solvent by heating or boiling it for a certain duration, (ii) preparation of molar solutions of

ionic metallic compounds and (iii) incubation of algal solutions and molar solutions of ionic metallic compounds followed either by continuous stirring or without stirring for a certain duration under controlled conditions [10, 30]. The synthesis of NPs is dose dependent and it is also related to the type of algae used. There are a variety of biomolecules responsible for the reduction of metals which include polysaccharides, peptides, and pigments. Stabilizing and capping the metal nanoparticles in aqueous solutions is done by proteins through amino groups or cysteine residues and sulfated polysaccharides [81]. Synthesis of nanoparticles using algae takes comparatively shorter time period than the other biosynthesizing methods [10, 30]. So far, several seaweeds (*Sargassum wightii* and *Fucus vesiculosus*) have been used for the synthesizing AgNPs of different sizes and shapes [81, 82]. Marine algae are meagerly explored for the synthesis of NPs. *C. vulgaris* has strong binding ability towards tetrachloroaurate ions to form algal-bound gold reducing into Au(O). Approximately 88% of algal-bound gold attained metallic state, and the crystals of gold were accumulated in the inner and outer parts of cell surfaces with tetrahedral, decahedral, and icosahedral structures [83]. *S. platensis* has been for the extracellular synthesis of gold, silver, and Au/Ag bimetallic NPs [26]. Senapati et al. [84] reported the intracellular production of gold nanoparticles using *Tetraselmis kochinensis*. The biomass of the brown alga *F. vesiculosus* was reported for the reduction of Au(III)–Au(O) [82]. In addition to seaweeds, microalgae such as diatoms (*N. atomus* and *D. gallica*) have the ability to synthesize gold nanoparticles, gold, and silica–gold bionanocomposites [15].

2.4. Application of algal-synthesized NPs

The biomedical application of algal-synthesized NPs is significantly becoming more important due to their antibacterial, antifungal, anti-cancer, and wound healing activity. They are given (**Figure 4**).

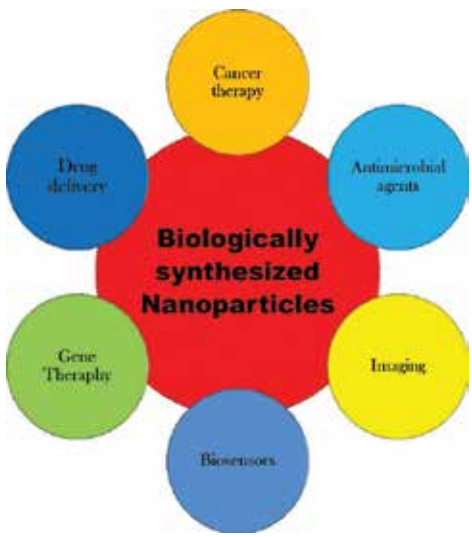

Figure 4. Applications of algal-synthesized nanoparticles.

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2.4.1. Antibacterial activity

Algal-synthesized NPs are known to possess efficient antibacterial activity (**Figure 5**; **Table 1**). Brown alga (*Bifurcaria bifurcate*) is reported for the synthesis of copper oxide nanoparticle (5–45 nm) exhibiting antibacterial activity against *Enterobacter aerogenes* (Gram-negative) and *S. aureus* (Gram-positive) [85]. Gold nanoparticles synthesized using *Galaxaura elongata* (powder or extract) were evaluated for their antibacterial activities which showed better antibacterial effects against *E. coli*, *K. pneumoniae*, MRSA, *S. aureus*, and *Pseudomonas aeruginosa* [86]. In another work, silver chloride (AgCl) NPs synthesized using marine alga *Sargassum plagiophyllum* were analyzed using fluorescence and electron microscopy showed bactericidal activity against *E. coli* [87]. Synthesis of AgNPs using fresh extract and whole cell of microalga *Chlorococcum humicola* inhibited the growth of Gram-negative bacteria *E. coli* (ATCC 1105) [88]. In a recent report, the aqueous extract of a diatom *Amphora*-46 was used for the light-induced biosynthesis of polycrystalline AgNPs, in which fucoxanthin a photosynthetic pigment was responsible for the reduction of Ag ion. Furthermore, the synthesized AgNPs were tested against Gram-positive and Gram-negative bacteria for its antibacterial activity [89].

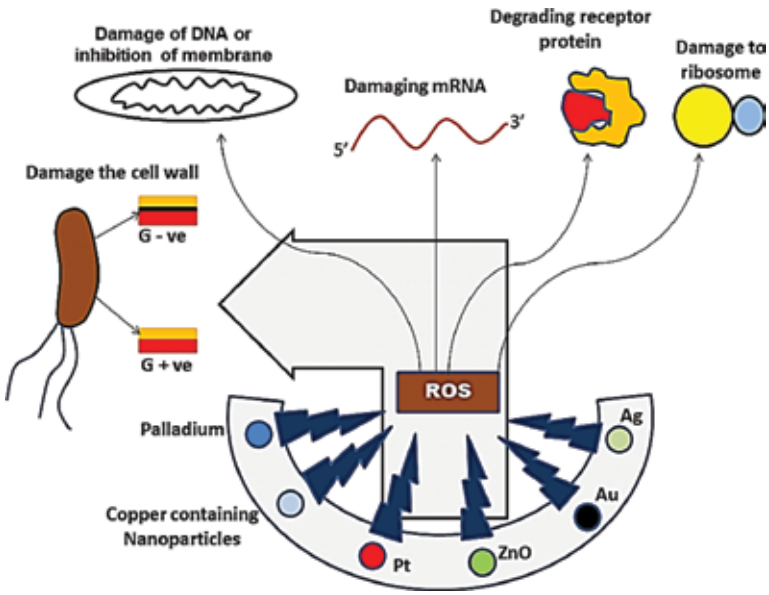

Figure 5. Different nanoparticles and their mode of inhibition against bacteria.

AgNPs synthesized using *Caulerpa racemose*, a marine algae, exhibited antibacterial activity against human pathogens such as *S. aureus* and *Proteus mirabilis* [90]. The cellular metabolites of *Microcoleus* sp. used to synthesize AgNPs, and it enhanced the antibacterial activity of antibiotics against *Proteus vulgaris*, *Salmonella typhi*, *Vibrio cholera*, *Streptococcus* sp., *Bacillus subtilis*, *S. aureus*, and *E. coli* [91]. In a work done by Merin et al. [92], he used marine microalgae *C. calcitrans*, *C. salina*, *I. galbana*, and *T. gracilis* were used for the synthesis of AgNPs and tested the antibacterial activity of AgNPs against *E. coli*, *Klebsiella* sp., *Proteus* sp., and *Pseudomonas*

sp. were tested high inhibitions over the growth of *E. aerogenes*, *S. typhi*, and *P. vulgaris* was exhibited by AgNPs synthesized using seaweed extracts of *Sargassum cinereum* [93]. In addition to antibacterial activity, the nanoparticles synthesized by seaweed extracts do have stabilizing effect on cotton fabrics [94].

Algae	NPs	Size	Shape	Intracellular (IC) or extracellular (EC)	Pathogens	References
Bifurcaria bifurcate	CuO	5–45 nm	Spherical and elongated	IC	*E. aerogenes* *S. aureus*	[85]
Galaxaura elongata	Au	3.85–77.13 nm	Spherical	IC	*E. coli* *K. pneumoniae* MRSA *S. aureus* *P. aeruginosa*	[86]
Sargassum plagiophyllum	AgCl	18–42 nm	Spherical	IC	*E. coli*	[87]
Chlorococcum humicola	Ag	4 and 6 nm	Spherical	IC	*E. coli* (ATCC 1105)	[88]
Amphora-46	Ag	5–70 nm	Spherical	IC		[89]
Caulerpa racemose	Ag	5–25 nm	Spherical and triangle		*S. aureus* and *P. mirabilis*	[90]
Microcoleus sp.	Ag	–	–		*P. vulgaris*, *S. typhi*, *V. cholera*, *Streptococcus* sp., *B. subtilis*, *S. aureus*, *E. coli*	[91]
Ulva fasciata	Ag	28–41 nm	Spherical	IC	*Xanthomonas campestris* pv. *malvacearum*	[96]
Turbinaria conoides	Au	60 nm	Triangle, rectangle & square	IC	*Streptococcus* sp., *B. subtilis* and *K. pneumoniae*	[97]
Padina pavonica	Ag	10–72 nm	Spherical	IC	*Fusarium oxysporum* f. sp. *vas* infectum *Xanthomonas campestris pv. malvacearum*	[99]
Gracilaria dura	Ag	6 nm	Spherical	IC	*B. pumilus* (accession number HQ318731)	[100]
Spirulina platensis	Au	5 nm	–	IC	*B. subtilis* and *S. aureus*	[101]

Table 1. Different types of algal-synthesized NPs and its antibacterial activity.

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The aqueous extract of red marine algae *Gracilaria corticata* as the reducing agent was explored for its antibacterial activity against Gram-positive and Gram-negative bacteria [95]. *U. fasciata*-based AgNPs were synthesized and used to inhibit the growth of *Xanthomonas campestris* pv. *malvacearum* [96]. Another work shows the antibacterial activity of AuNPs synthesized using marine brown algae *Turbinaria conoides*, against *Streptococcus* sp., *B. subtilis*, and *K. pneumoniae* [97]. Ag, Au, and bimetallic alloy Ag–Au nanoparticles were synthesized from marine red alga, *Gracilaria* sp., exhibited good antibacterial activity against Gram-positive bacteria *S. aureus* and Gram-negative bacteria *K. pneumoniae* [98]. Extracellular synthesis of AgNPs from the thallus broth of marine algae *Padina pavonica* (Linn.) inhibited the growth of cotton Fusarium wilts (*Fusarium oxysporum* f. sp. *vasinfectum*) and bacterial leaf blight (*Xanthomonas campestris* pv. *malvacearum*) [99]. Bactericidal activity of AgNPs and nanocomposite material synthesized using agar extracted from the red alga *Gracilaria dura* was tested against *B. pumilus* (accession number HQ318731) [100]. In a work done by Suganya et al. [101] blue green alga *S. platensis* protein mediated synthesis of AuNPs was performed; further, it showed efficient antibacterial activity against Gram-positive bacteria (*B. subtilis* and *S. aureus*) (**Table 2**)

Nanoparticle	Target organism	References
Silver nanoparticles	*S. paratyphi*, *P. aeruginosa*, *S. epidermidis*	[112, 113]
Bismuth oxide aqueous colloidal nanoparticles	*C. albicans*, *S. mutans*	[114, 115]
Nano-oil formulation from *Mentha piperita* L	*Staphylococcus* sp.	[116]
Nano-emulsion (detergent, oil, and water) in combination with cetylpyridinium chloride	*A. baumannii*	[117]
Silver- and gold-incorporated polyurethane, polycaprolactam, polycarbonate, and polymethylmethacrylate	*E. coli*	[118]
Silver nanoparticles in combination with nystatin and chlorhexidine	*C. albicans*, *C. glabrata*	[119]
Silver nanoparticle and 12-methacryloyloxydodecylpyridinium bromide (MDPB)	Dental plaque microcosm biofilms	[120, 121]
Copper	*P. aeruginosa*	[108]
Zinc	*Actinobacillus pleuropneumoniae*, *S. Typhimurium*, *Haemophilus parasuis*, *E. coli*, *S. aureus*, *S. suis*	[122]
Magnetite nanoparticles	*C. albicans*	[56]
Eugenia carryophyllata essential oil stabilized by iron oxide/oleic acid core/shell nanostructures	*S. aureus*	[123, 124]
Zinc and copper oxide nanoparticles	*S. mutans*	[125]
Zerovalent bismuth nanoparticle	*S. mutans*	[114]

Nanoparticle	Target organism	References
Dextran sulfate nanoparticle complex containing ofloxacin and levofloxacin	*P. aeruginosa*	[126]
PEG-stabilized lipid nanoparticles loaded with terpinen-4-ol	*C. albicans*	[127]
Magnesium fluoride nanoparticles	*S. aureus*, *E. coli*	[128–130]
Yttrium fluoride nanoparticles	*S. aureus*, *E. coli*	[131]
Iron oxide/oleic acid in combination with essential oil from *Rosmarinus officinalis*	*C. albicans*, *C. tropicalis*	[132]
Gold nanoparticles and methylene blue	*C. albicans*	[133]
Starch-stabilized silver nanoparticles	*S. aureus*, *P. aeruginosa*	[134]
Iron oxide–oleic acid nanofluid	*S. aureus*	[124]
Quaternary ammonium polyethylenimine nanoparticles	Oral biofilms	[41]
Zinc oxide nanoparticles, chitosan nanoparticles, and combination of both	*E. faecalis*	[135]
Polyurethane nanocomposite	*S. epidermidis*	[136]

Table 2. Antibiofilm activity of different NPs against microbial pathogen.

2.4.2. Antifungal activity

Algal-synthesized NPs were used as efficient antifungal agents. Only countable number of work has been carried out in this aspect. This includes the synthesis AgNPs using the aqueous extract of red seaweed *Gelidiella acerosa* as the reducing agent exhibited antifungal property against *Humicola insolens* (MTCC 4520), *Fusarium dimerum* (MTCC 6583), *Mucor indicus* (MTCC 3318), and *Trichoderma reesei* (MTCC 3929) [102]. In another report, the effect of the algal (*Sargassum longifolium*)-mediated AgNPs against the pathogenic fungi *Aspergillus fumigatus*, *Candida albicans*, and *Fusarium* sp. was determined [103].

2.4.3. Anticancer activity

In a work done by Boca et al. [104] synthesized chitosan-coated silver nano-triangles (Chit-AgNPs) were used as a photothermal agents against a line of human nonsmall lung cancer cells (NCI-H460) [104]. In another work, AgNPs (10 nm) were synthesized using *Sargassum vulgare* and its ability to kill cancerous human myeloblastic leukemic cells HL60 and cervical cancer cells HeLa was tested [105].

2.4.4. Other applications

Algal-synthesized NPs are explored in certain other area of applications, which include the synthesis of spherical palladium nanocrystals via aqueous Na_2 $[PdCl_4]$ solution using the photosynthetic reaction within *C. vulgaris*, which can be used as a material for recycling as a catalyst for the Mizoroki–Heck cross-coupling reaction [106]. The antioxidant potentials of

AgNPs synthesized using *G. corticata* was also determined [95]. In another work, AuNPs were synthesized using the dried biomass of an edible freshwater epilithic red alga, *Lemanea fluviatilis* (L.) C. Ag., as both reductant and stabilizer; further, its antioxidant property was determined using DPPH assay [107].

2.5. Future application of algal-synthesized NPs

2.5.1. Antibiofilm agents

The use of nanoparticles as antibiofilm agents is an emerging area of research. Due to the extensive use and misuse of antibiotics, many of the pathogens acquired resistance toward multiple drugs. As the bacteria are less likely to develop resistance against nanoparticles, they can be used as a promising therapeutic agent against biofilms. Nanoparticles have the ability to penetrate EPS and the cell membranes (**Figure 6**). Silver nanoparticles were found to be more prevalent than the other ones, and they exhibit antibiofilm activity against both Gram-positive and Gram-negative pathogens. In a work done by LewisOscar et al. [108]. Chemical synthesis of CuNPs was performed by one-pot synthesized method and used for biofilm inhibition against *P. aeruginosa* PA14, *P. aeruginosa* ATCC10145 and some clinical isolated of *P. aeruginosa*. Along with the biofilm, CuNPs also weakened the extracellular polymeric substance and cell surface hydrophobicity of *P. aeruginosa*.

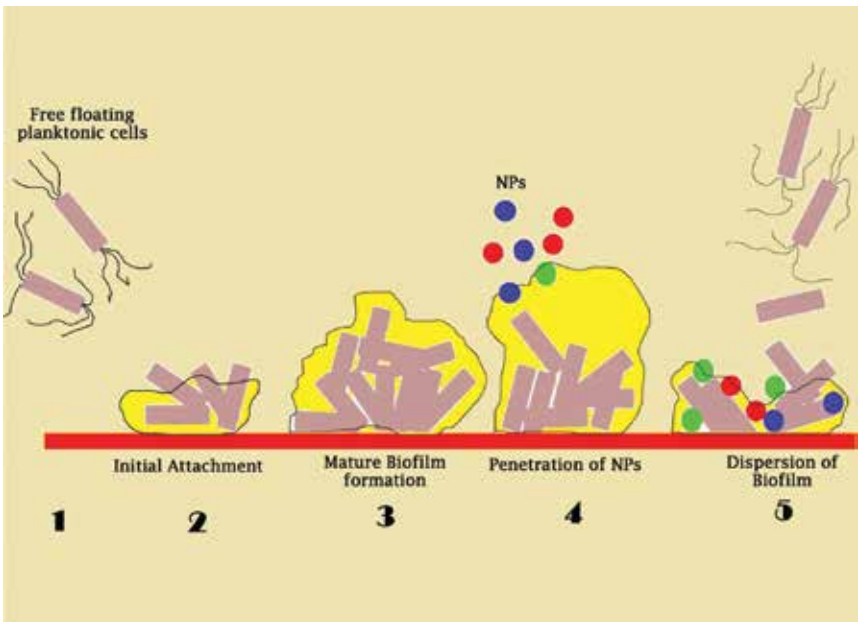

Figure 6. Antibiofilm activity of different nanoparticles.

The zero-valent selenium and tellurium NPs synthesized using *Stenotrophomonas maltophilia* and *Ochrobacterium* sp. were found to be effective against biofilms of *E. coli*, *P. aeruginosa*, and

S. aureus [109]. Similarly, AgNP synthesized from *E. faecalis*, when used in the form of nanocolloids, inhibited the biofilm of multidrug resistant pathogens [110]. Green-synthesized AgNP coated on medical devices inhibited the *S. aureus* biofilms [111]. Some other potential NPs against biofilm of different Gram-negative and Gram-positive bacteria are given below in **Table 2**.

2.5.2. Nanocomposite

The diatom *Stauroneis* sp. was used for the preparation of silicon–germanium nanocomposite, and this method of nanocomposite preparation has great importance for possible future applications due to its accessibility, simplicity, and effectiveness [137].

2.5.3. Lipid nanoparticles

There are possibilities for the production of lipid nanoparticles with the help of lipid-rich marine organisms such as algae, fungi, and bacteria [138]. Lipid nanoparticles can be synthesized from the organisms through heating to liquefy fatty acids; incorporating active agents of pharmacological and cosmetics importance; adding a hot surfactant; and stirring or homogenized under high pressure by ultrasound. These can be used in the production of food stuffs, cosmetics, and medicines [139].

2.5.4. Biosensing

Algal-synthesized NPs can be explored in biosensing applications. Such as, AuNPs has been proved as an important tool for hormone (HCG) detection in pregnant women urine sample [140]. Platinum (Pt) NPs act as a novel biosensor with high sensitivity for the determination of adrenaline for the treatment of allergies, heart attack, asthma, and cardiac surgery [141]. Synthesis of nanoscale Au–Ag alloy prepared using chloroplasts exhibited high electrocatalytic activity for 2-butanone at room temperature which can be developed as a tool for detecting cancer at early stages [142].

2.6. Conclusion

The developing era of nanoscience is a renowned gift for the development of science all over the world. Despite numerous studies conducted over the last decade, there are still considerable gaps in our knowledge about the biotechnological potential of green-synthesized nanoparticles. Furthermore, the precise basis of their antibiotic and antibiofilm activity has yet to be defined. However, the toxicity of nanoparticles to eukaryotic cells is a legitimate concern and still remains uncharacterized. One way of avoiding this potential drawback might be to target green-synthesized nanoparticles to the specific site of an infection so that toxic nanoparticles concentrations are localized. In addition, improvements in the way that green-synthesized nanoparticles are incorporated into medical devices could increase their efficacy and diminish any side effects, but considerable research effort is still required to perfect this technology.

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Chapter 8

Biofilms: An Extra Coat on Macroalgae

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Additional information is available at the end of the chapter

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Abstract

Biofilms are a mixture of complex communities of organisms mostly composed of diverse bacteria that vary depending on the surrounding environmental conditions induced by physical and chemical factors. In biofilms, symbionts play major roles in the relationship among organisms by the production of bioactive molecules involved in quorum sensing signaling. A cohesive structure of a multi-layer of extracellular polymeric substances (EPS) such as polysaccharides and proteins is the base of biofilm structural organization. Biofilms can be found in a variety of habitats, on free-living, on the surface of other organisms or inert surfaces, both in aquatic as well as terrestrial environments.

The importance of macroalgae in marine ecosystems is unquestionable. They are main key players along ocean coastlines, contributing to the overall primary production and providing shelter as well as food to many forms of life which can vary from microbes to large fish and mammals. Macroalgae are intimately associated with a huge microbial community coating their surface. As this microenvironment is very rich both in terms of biodiversity and food availability, life in it is very complex and competitive. The microorganisms, bacteria, archaea, fungi, microalgae like diatoms and protozoa, play fundamental roles in the development, defence and metabolism of the macroalgae. They benefit from the availability of diverse organic carbon sources commonly produced by the algae.

In this chapter, we intend to do a comprehensive revision of the actual state of the art of the biofilm community of macroalgae focusing on biodiversity, role played by both the microbiome and the host in this ecological system and its regulation namely through the quorum sensing. Furthermore, biofilm-related biotechnological applications, their role in macroalgae diseases and their influence in neighbor organisms will be also addressed.

Keywords: Macroalgae, Biofilm, Bacteria, Microalgae, quorum sensing

1. Introduction

Biofilms are complex, highly dynamic, structured ecosystems formed by a community of different microorganisms living attached to inert or living surfaces and embedded in a matrix designated extracellular polymeric substance (EPS). The colonization of a surface begins with EPS production by the initial bacterial colonizers through the formation of weak, reversible bonds called van der Waals forces and production of transparent exopolymer particles and their precursors by macroalgae that set the basis for the first bacterial colonizers settlement [1]. Several other microorganisms come after entering in this very competitive ecosystem where quorum sensing and antibiotic resistance are determinants in the development of the community. Observation of biofilms dates back to the seventeenth century when Antonie van Leeuwenhoek observed bacteria from the plaque biofilm of his teeth under his primitive microscope [2]. However, it was only in the 1940s that the concept of biofilm begun to arise with the works of Heukelekian and Heller [3] and of Zobell [4] whereas the first publication referring to the word biofilm appeared just in 1975 [5]. Due to the invisibility of microbial biofilms and inexistence of adequate methodologies for their study during many years, comprehension of this ecosystem is still scarce. The best studied biofilm systems are the ones associated with human body surfaces and technical surfaces like sensor heads or reverse osmosis membranes of desalination plants [6] and references therein. More recently, increasing attention has been paid to other systems like the epiphytic community on macroalgal surface. Several scientific groups are presently dedicated to the study of these biofilms with a consequent boost in the number of publications (see [7] for the increase in bacteria-macroalgae related publications). Since the description of the algal epiphyte bacterium, *Leucothrix mucor* [8, 9], more than 50 new bacterial genera and species have been described after their isolation from macroalgae [7]. This environment is proving to be prolific for the discovery of novel bacterial taxa. Several reviews on microbiome – macroalgae association have been done demonstrating the importance of microorganisms in this ecosystem [6, 7, 10–15]. The majority is mainly focused on the bacterial component of the microbiome which is the known main key player in these biofilms. Aspects of the importance of virus on macroalgal biofilms have recently appeared [16, 17].

The idea of considering biofilms as an extra "tissue" on the surface of eukaryotic organisms is based on the analogy between these two systems [6, 18]. We can thus consider the existence of an extra coat outside the macroalgal epidermis that gives an extra buffering between the host cells and the surrounding environment. In the biofilm, although cells are genetically different and variable, what does not happen in tissues, they interact functionally through nutritional exchange, communicate through quorum sensing and reproduce.

2. Macroalgal colonization and chemical interactions

Macroalgae inhabit an environment prone to epibiotic colonization (**Figure 1**) due to a constant pressure from the surrounding microbial community [19]. The relationship between bacteria in biofilm and their planktonic counterparts is of 1 to 2 orders of magnitude higher [6, 20]. However, Chan and McManus [21] in their study with *Polysiphonia lanosa* and *Ascophyllum*

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nodosum found 100–10,000 times more bacteria associated with the algae than in the surrounding water. Bacteria on surfaces can reach densities higher than 10^7 cells cm^{-2} [22]. On the alga *Caulerpa racemos*a bacterial densities were of about 20×10^3 cells mm^{-2} and diatom densities of 40 cells mm^{-2} [23] while in *Ulva reticulata* there were 27×10^3 bacterial cells mm^{-2} and 5 diatom cells mm^{-2} [24]. On the kelp *Laminaria hyperborea* microbial cell densities varied between 8.3×10^2 cells cm^{-2} and 1.0×10^7 cells cm^{-2} [25] and on *Fucus vesiculosus*, Wahl et al. [26] reported values for epibacterial density of $7.7 \times 10^6 \pm 2.2 \times 10^6$ cells per cm^2 of algal thallus. In a study of the epiphytic diatom community on macroalgae from Iceland, Totti et al. [27] found diatom abundances between 7 ± 5 and 7524 ± 3491 cells mm^{-2}.

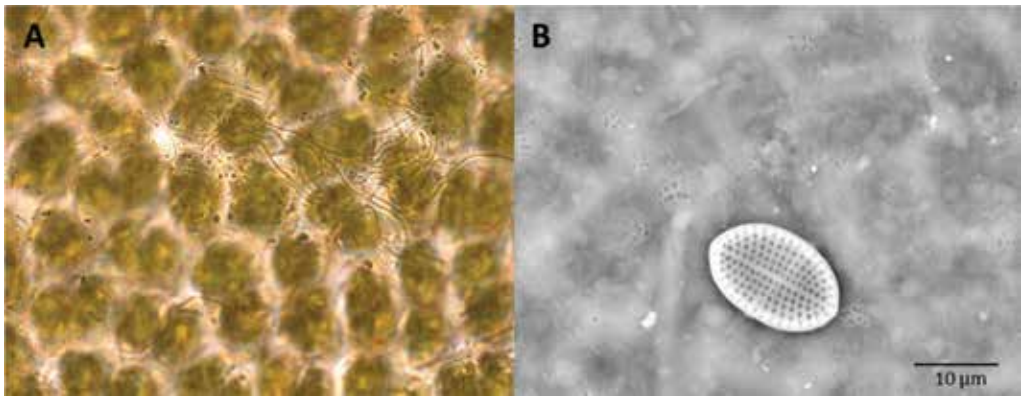

Figure 1. Macroalgal surface colonization of *Porphyra dioica* (A) and *Ulva* (B) by bacteria (A and B) and diatom (B) by light (A) and scanning electron (B) microscopy.

As perceived by these cell density numbers, marine macroalgae are one of the most important eukaryotes that provide excellent conditions for microbial colonization on their surfaces in the marine environment. Several factors are determinant for colonization. These include (1) the microtexturing (size and surface features) of the surface [28], (2) the production by the macroalgae of natural compounds with antifouling properties which include antimicrobials and quorum sensing disruptors [7, 26, 29–32], (3) the production of organic carbon compounds that trigger the chemotactic behavior of bacteria [33–34] and (4) the releasing of certain substrates that fulfill the nutritional needs of the epiphytic microbial community. On the other hand, macroalgae also benefit from the presence of this rich community as their growth and development are somehow dependent essentially on their bacteriome [14, 35].

In the microhabitat of the biofilm and on its interface with the macroalgae, complex chemical interactions occur. Both basibiont (macroalgae) and epibiont (microorganisms in the biofilm) contribute to this myriad of compounds. The macroalgae supply bacteria with oxygen and fixed carbon which is released as extracellular exopolysaccharides such as alginate, cellulose and mannitol [36–38]. Bacteria, through the mineralization of organic compounds released in the biofilm, supply the macroalgae with CO_2, minerals, vitamins and growth factors [39–42]. In the mineralization process, many other molecules are formed which enrich the biofilm microenvironment and contribute to its biomass formation.

Growth factors produced by bacteria are phytohormones and biostimulators of growth and development [7]. Strains isolated from *Ulva mutabilis*, *Roseobacter*, *Sulfitobacter*, and *Halomonas*, associated with strain *Cytophaga* were effective in the development of the *Ulva* gametes into normal thalli due to specific regulator factors (cytokinin-type and auxin-type, respectively) excreted into the environment [43]. Secondary metabolites produced by bacteria in the biofilm are also fundamental for the completion of macroalgal life cycle and spore release and germination. Mixed microbial biofilms were shown to stimulate the rate of settlement of zoospores of the green alga *Enteromorpha* [44] and a positive correlation was observed between the number of zoospores settling and the number of bacteria in the biofilm [45]. Strains of *Vibrio* and *Shewanella* showed stimulation of spore settlement while *Pseudoalteromonas* strains inhibited settlement and also induced paralysis and lysing of *Enteromorpha* zoospores [46]. A similar stimulatory effect was also observed in the zoospore settlement of *Ulva linza* [47]. *Ulva* zoospores have the capacity to sense a range of different bacteria produced N-acyl homoserine lactone (AHL) molecules which interfere with their settlement [48].

Bacteria are fundamental for the growth and morphogenesis of several macroalgae. Provasoli [49] observed that *Ulva* in axenic culture did not develop normal morphology which was recovered after inoculation of bacteria previously isolated from this macroalgae [50]. Similarly, other Chlorophyta, *Monostroma oxyspermu* and *U. linza* that also lose their normal morphology when in axenic cultures could reestablished their normal morphology after culture incubation with bacterial extracts or inoculation with an appropriate bacterial community [47, 51]. Bacteria with morphogenesis-inducing activity were identified to be related to the genera *Flavobacterium, Vibrio, Pseudomonas, Deleya, Escherichia* and Gram-positive cocci [52]. Matsuo et al. [53] identified 40 active strains that were affiliated to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) complex, mainly in a clade comprising *Zobellia uliginosa*. In 2005, Matsuo et al. [54] proved that it was the specific bacterial strain YM2 that produced a secondary metabolite designated thallusin that was responsible for the normal development of ulcacean foliose. But this interaction is not restricted to Chlorophyta. The Rhodophyta *Pyropia yezoensis* (former name "*Porphyra yezoensis*") also needs bacteria to induce normal morphogenesis in its gametophytic phase [55]. Recently, Fukui et al. [56] identified the bacteria that induced normal morphogenesis in this red alga. They are members of *Alfaproteobacteria*, *Gammaproteobacteria* and *Flavobacteria* with special relevance to strains of *Hyphomonas*. Bacteria are, thus, fundamental for algal morphogenesis and life cycle development.

Furthermore, bacteria are also sources of fixed nitrogen and detoxifying compounds [7, 57, 58]. Nitrogen-fixing cyanobacteria are known to provide fixed nitrogen to macroalgae. These include *Calothrix* sp., *Anabaena* sp., and *Phormidium* sp. on *Codium* species [59, 60], *Dichothrix fucicola* on *Sargassum natans* and *Sargassum fluitans* [61, 62] and *Azotobacter* sp. on *Codium fragile* [63].

Another advantage of microbial community in macroalgal biofilms is their ability of scavenging of heavy metal [42, 58] or crude oil [64]. Many bacteria also play a fundamental role in biotransformation and nutrient cycling in the oceans due to the capacity to decompose the macroalgal cell walls [7, 65]. Bacteria, with the appropriate enzymatic machinery, contribute to the decay process of seaweeds [66].

Macroalgal epiphytic colonization is very uneven [67]. Macroalgae inhabiting the same environment or closely related species like *F. vesiculosus* and *Fucus evanescens* can possess very different levels of fouling [68]. These can be justified by different levels of antifouling defence mechanisms. Macroalgae defend themselves from invaders through the mechanical sloughing off of the outermost cell layer [7, 69] and the release of antimicrobials including reactive species of oxygen [7, 70] and bacterial communication blockers, the furanones [71]. Quorum sensing inhibitors and antimicrobial compounds produced by the bacteria are fundamental in the protection against pathogens, herbivores and fouling. These act in conjunction with the compounds produced by the macroalgae.

Macroalgae are a rich source of bioactive compounds against colonizing organisms. They are assisted on this task by the many antimicrobials produced by microorganisms on their biofilms, production that is widespread among bacteria [72–75]. Members of the genera *Pseudomonas, Pseudoalteromonas, Stenotrophomonas, Vibrio, Aeromonas, Shewanella, Streptomyces* and *Bacillus* are common antimicrobial producers from macroalgae [75]. Goecke et al. [7] provide several examples of antimicrobials produced by bacteria associated with macroalgae.

The oxidative burst response is based on the production of large amounts of reactive oxygen species by the macroalgae inducing the death of undesired microorganisms like pathogens and also controlling bacterial growth on algal surfaces. Elicitors of oxidative burst, signals that mediate the activation of cell-based induced defence responses, were recognized to be glycoproteins and glycopeptides, low-molecular weight peptides in the red agarophyte *Gracilaria conferta* [76] and oligomeric degradation products of alginate and bacterial lipopolysaccharides (LPS) in the brown algal kelp *Laminaria digitata* [77, 78]. Reaction to alginate oligosaccharides was also observed in other Laminariales [79]. Furthermore, in *L. digitata* arachidonic acid, linolenic acid and methyl jasmonate were found to be strong triggers of an oxidative burst [80].

Bacteria, once thought to be silent, were discovered to have specific intra- and inter-species signaling mechanism of communication that has been named quorum sensing (QS). They communicate via production of chemical signals with multifunctional activity due to their interacting QS gene regulatory 'modules' which are able to produce several different molecules, from the same or different chemical class that interact in hierarchies [81–83]. These molecules act as gene regulators of the population behavior in food uptake or common defence or escape when the survival of the community is at risk [81, 84]. In addition to communication with other microbes, bacteria also perceive molecules from eukaryotes that are known to be key factors in host-epibiont interaction [15, 83].

QS communication was discovered in the 1990s and proved to fulfill different ecological purposes like the induction of biofilm formation, movement of bacteria and the production of bioluminescence, antibiotic and virulence factors [85–87]. Similar to the QS signals that balance the equilibrium of the community, quorum quenching (QQ) signals are inhibitors of QS and also have impact on biofilm communities.

Halogenated furanones are structural analogues to N-acyl homoserine lactones (AHLs) and interfere with AHL-regulated processes and impair biofilm formation [88–90]. The first

compound with QS disruptor capacity isolated from a marine source, the red alga *Delisea pulchra,* was furanone. It is used by this alga to control surface colonization of marine bacteria [91]. *D. pulchra* has been used as a model organism for understanding the ecological role of secondary metabolites as natural antifoulants [71]. Furanones are produced by the macroalgae and on their surfaces at a concentration where they regulate bacterial colonization and the settlement of epibiota by interfering with the acylated homoserine lactone regulatory system (quorum-sensing pathway) in Gram-negative bacteria and with the alternative AI-2 signaling system in Gram-negative and Gram-positive bacteria [71, 83]. Furthermore, they also interfere with the attack mode of action of bacteria by inhibiting the expression of bacterial exo-enzymes that actively degrade components of the immune system thereby enhancing macroalgal immune response.

Since the discovery of these QS inhibitors, other studies lead to the isolation of more compounds able to block QS signal like the mixture of floridoside, betonicine and isethionic acid, isolated from the red alga *Ahnfeltiopsis flabelliformis*, that inhibited the activity of N-octanoyl-DL-homoserine lactone [92]. Moreover, studies using several macroalgae revealed a strong QS inhibitor produced by *Asparagopsis taxiformis* [93].

The production of QS signals not only affects bacterial responses but also the settlement of the green macroalga *Ulva* that react to AHL signaling of *Vibrio anguillarum* in the selection of surface sites for zoospores attachment [48]. Moreover, the study of the epi- and endobiont bacterial community associated to the macroalgae *Ulva fasciata*, *Ulva lactuca*, *Gracilaria corticata* and *Gracilaria dura* allowed the isolation of several strains of which some Gram-negative strains were able to induce carpospore liberation from *G. dura* by AHLs production.

AHLs also have effect on diatoms in the biofilm matrix as was observed by Yang et al. [94]. 3,4-dibromo-2(5)H-furanone,4-nitropyridine-N-oxide and indole were able to decrease significantly the growth of two marine diatoms, *Cylindrotheca* sp. and *Nitzschia closterium*.

Although biofilms are important for macroalgae biology, they can also bring on detrimental effects as their members compete for nutrients, interfere with gaseous exchange, form a barrier to light that is fundamental for macroalgal photosynthesis and can lead to disease and degradation of algal tissue [95–97].

Similar to humans and plants, macroalgae possess their own defence mechanisms and immunity adaptations developed to survive and coexist with pathogenic or phycocolloids degrader's organisms [18, 98]. It is suggested that, since microbes are more predominant in water than in air, macroalgae evolved to more resistant forms by elimination of more susceptible individuals and resistance of the ones capable of producing chemicals for self-defence [99, 100]. Actually, few are the reports on the study of pathogenic microorganisms in macroalgae. Although, studies on algal diseases have risen due to the increase of the use of algae in food industry, seaweed mariculture and to the consequences of global warming and its impact on ocean temperature [96, 101]. The most studied pathogen-macroalgae systems studied are the host-specificity infection by *Roseobacter* in *Prionitis lanceolata*, that induces the formation of a tumor-like growth in the thallus [102] and the induction of bleaching in the red alga *Delisea pulchra* by *Nautella italica* R11 and *Phaeobacter* sp.LSS9 [16, 101, 103, 104]. Also, studies on

Laminaria religiosa health revealed that *Alteromonas* sp. is a pathogenic strain that allied with abiotic factors induce severe damage and bleaching to the alga [105]. Not only bacteria and fungi threaten macroalgae, but also the pathogenic epiphytic alga *Neosiphonia apiculate* induces reduction of carrageenan production and secondary bacterial infection [106]. This constant fight to survive invasions through the production of chemical compounds like antibacterials led to the isolation of several compounds from marine macroalgae, such as lobophorolide isolated from *Lobophora variegata* with activity against pathogenic and saprophytic fungi [99].

The biofilm community present on macroalgae not only has influence on the host life but also on other eukaryotes living nearby. Effects on sea urchin larval settlement by coralline algae biofilm communities [107] and on *Mytillus edulis* larval settlement by producing attraction or repellent signals to mussels [108] have been observed. These studies reveal the impact of the macroalgae biofilm in the surrounding organisms.

3. The diversity of microbial community on macroalgae

In marine environments, biofilms are mainly formed by bacteria but also by several different eukaryotes such as diatoms, fungi and protozoa [14] in a ratio of 640:4:1 of Bacteria:Diatoms:Flagellates [22].

3.1. Bacterial diversity

Hollants and collaborators in 2013 [12] did an exhaustive analysis of the macroalgae-bacterial diversity compiling information from 55 years and 159 seaweed species (36 green, 72 red, and 51 brown marine macroalgae). They concluded that bacteria associated to macroalgae belong to the phyla *Proteobacteria, Bacteroidetes* (CFB group), *Actinobacteria, Cyanobacteria, Firmicutes, Planctomycetes, Verrucomicrobia, Chloroflexi, Deinococcus-Thermus, Fusobacteria, Tenericutes*, and the candidate division OP11. The dominant groups were *Gammaproteobacteria* with 37% relative abundance in published records, followed by the CFB group (20%), *Alphaproteobacteria* (13%), *Firmicutes* (10%), and *Actinobacteria* (9%). At the order level, *Flavobacteriales* (14%), *Alteromonadales* (12%), *Vibrionales* (10%), *Pseudomonadales* (9%), *Bacillales* (9%), *Actinomycetales* (8%), and *Rhodobacterales* (7%) were the most abundant. On their analysis they also found that all groups have been isolated from the three lineages of macroalgae, Chlorophyta, Rhodophyta and Heterokonthophyta (Phaeophyceae) but differences were observed between them: *Bacteroidetes* and *Alphaproteobacteria* were more associated to green macroalgae while species of *Firmicutes*, *Actinobacteria*, and *Planctomycetes* to red and brown algae. On their study at lower taxonomic level (genus and species), bacterial taxa variability was found in closely related seaweeds. Genera like *Alteromonas*, *Bacillus*, *Flavobacterium*, *Pseudoalteromonas*, *Pseudomonas*, and *Vibrio*, in a total of 33 genera, were frequently associated with the three groups of macroalgae while *Cytophaga*, *Planococcus* and *Tenacibaculum* appear commonly in green and red but not in brown seaweeds.

During many years, the study of macroalgal biofilm diversity was based on organism isolation in pure cultures with their subsequent taxonomic characterization. With the development of

molecular and new generation sequencing techniques, a much more precise and detailed assessment of diversity has been possible. Presently, our knowledge, essentially at bacterial level, has been extended to many groups of organisms known for their difficulty to be brought into culture.

Longford et al. [109], using 16S rRNA gene library analysis, compared the bacterial community of the red macroalga *Delisea pulchra* and the green intertidal alga *Ulva australis*. *D. pulchra* contained 7 phyla and *U. australis* only 4 phyla and the two shared representatives from *Alpha-*, *Delta-* and *Gammaproteobacteria, Planctomycetes* and *Bacteroidetes. A*lpha diversity was relatively high in *D. pulchra* and comparatively lower in *U. australis*. Beta diversity at the species level, the measurement of the turnover of species between two sites in terms of gain or loss of species, was high as no species showed universal distribution between the two macroalgae.

Tujula et al. [110] in a catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and denaturing gradient gel electrophoresis (DGGE) study found that the epiphytic microbial community of *U. australis* was mainly constituted by bacteria (90%) of which 70% were *Alphaproteobacteria* (mainly the *Roseobacter* clade) and 13% *Bacteroidetes*. The 16S rRNA gene clone libraries of *Ulva australis* showed that its biofilm was dominated by bacterial members of *Alphaproteobacteria* and *Bacteroidetes*, especially within the *Rhodobacteriaceae*, *Sphingomonadaceae*, *Flavobacteriaceae* and *Sapropiraceae* families [111]. No consistent species-specific bacterial community was observed between libraries.

The brown alga *Laminaria saccharina*, now classified as *Saccharina latissima*, was studied by Staufenberger et al. [112]. Its bacterial community, as revealed by DGGE and 16S rRNA gene clone libraries, varied in the different parts of the alga (rhizoid, cauloid, meristem and phyloid) and the bacterial phylotypes obtained were affiliated to *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* groups.

The epiphytic bacteria on the macroalga *Chara aspera* was colonized mostly by members of the *Cytophaga-Flavobacteria-Bacteroidetes* group but also by *Betaproteobacteria, Gammaproteobacteria, Planctomycetes* and *Actinomycetes* [113].

Hengst et al. [114] studied the composition and structure of bacterial communities on three macroalage from two coastal areas in the Northern Chile varying in copper concentration in seawater. They found that the bacterial communities' structure was determined by the algal host and time dependent. Significant changes in the bacterial community structure induced by copper were observed in *Ulva* spp. but not in *Scytosiphon lomentaria* and *Lessonia nigrescens*. The phyla encountered in the algal biofilm were *Bacteroidetes, Alfaproteobacteria, Verrucomicrobia, Planctomycetes, and Cyanobacteria. Verrucomicrobia* were exclusively found in polluted sites. The bacterial communities in this study were determined by algal species>temporal changes>copper levels.

In a DGGE and clone libraries study, Lachnit et al. [115] verified that the macroalgae *F. vesiculosus* (brown), *Gracilaria vermiculophylla* (red) and *Ulva intestinalis* (green) living in close proximity showed consistent seasonal differences in their bacterial community at phylum level. However, each macroalgal species possessed a species-specific and temporally adapted epiphytic bacterial community. *F. vesiculosus* harbored *Alphaproteobacteria*, *Bacteroidetes*,

Verrucomicrobia and *Cyanobacteria* in summer while in winter *Cyanobacteria* were not observed and the abundance of *Gammaproteobacteria* increased. In summer, *G. vermiculophylla* possessed mainly *Alphaproteobacteria* and *Bacteroidetes* and in winter the phylum *Deinococcus* was detected. In *U. intestinalis, Alphaproteobacteria* was the major phylum both in winter and summer but also *Gammaproteobacteria* and *Bacteroidetes* were present. Phyla also detected in this study were *Beta-*, *Epsilon-* and *Deltaproteobacteria*, *Planctomycetes*, *Actinobacteria* and OD1-OP11-WS6-TM7. *Octadecabacter arcticus*, *Granulosicoccus antarcticus*, a *Bacteroidetes* strain (EU246795), *Roseibacillus* spp. and *Planctomyces* sp. (EF591887) were the closest related bacterial strains to the operational taxonomic units (OTUs) found on *F. vesiculosus* while *Mesorhizobium* (DQ269119), *Hyphomonadaceae* (EU642858), *Actinobacterium* (DQ289932), *Bacteroidetes* (DQ269100), *Roseobacter* (AY167339), *Cytophaga* (AB015265) and *Bacteroidetes* (DQ269042) were the ones observed in *G. vermiculophylla.*

The bacteriome of the kelp *L. hyperborea* from two sites on the southwestern coast of Norway was studied by DGGE by Bengtsson et al. [25]. They found that *Planctomycetes* and *Alphaproteobacteria* were the most frequent phyla but also *Verrucomicrobia*, *Cyanobacteria*, *Gammaproteobacteria*, *Betaproteobacteria*, and *Bacteroidetes* were detected throughout the year.

The macroalgae *Osmundaria volubilis, Phyllophora crispa*, and *Laminaria rodriguezii*, from the Balearic Islands (western Mediterranean Sea), were found to have their surfaces dominated by bacterial ammonium monooxygenase (*amoA*) genes as determined by quantitative PCR analyses [116]. Comparatively lower levels were found for archaeal counterparts. The ammonium monooxygenase bacteria (AOB) community (15 operational taxonomic units (OTUs)) was mainly composed of members of *Nitrosospira* spp. and of *Nitrosomonas europaea* and the ammonia-oxidizing archaea (AOA, 43 OTUs) showed higher diversity. Trias et al. [116] estimated 6 times higher abundance of AOB comparatively to AOA and that the former accounted for about 1% of the total bacterial community on the algal surfaces.

The effect of temperature on the bacteriome of the brown macroalga *F. vesiculosus* was studied by DGGE and 454 pyrosequencing of the 16S rRNA gene [117]. Of the 21 present phyla, the dominant OUTs found were *Proteobacteria* (~68%) and *Bacteroidetes* (~18%). *Alphaproteobacteria* and *Rodobacteriaceae* were respectively the prevalent class and family in all the temperatures but this family more than doubled in abundance from the lowest to the highest temperature assayed. Temperature did not influence cell density but was responsible for 20% of the variation in the bacterial community composition. Furthermore, Stratil et al. [118] also analyzed, by 454 pyrosequencing of 16S rRNA gene sequences, the effect of salinity on the biofilm of *F. vesiculosus* and observed a significant influence of salinity on bacterial OTU richness and evenness. Alpha diversity, the number of species and the proportion in which each species is represented in the community, was lower at the lowest salinity assayed (5 ‰), in which the more relevant bacterial group was *Betaproteobacteria*. Members of this phylum were absent at the two higher salinities assayed (19 and 25 ‰) where *Gammaproteobacteria* strains dominated. Compared to the colonization of a non-living substrate (stone), *F. vesiculosus* was less colonized by *Cyanobacteria* and microalgal chloroplasts (probably diatoms) reveling antifouling ability against these organisms. Stratil et al. [118] results showed the importance of salinity in the structuring of algal biofilms.

Martin et al. [119] showed that *A. nodosum* biofilm is significantly enriched in macroalgal-polysaccharide-degrading bacteria. Of the cultivable bacterial subpopulation associated with *A. nodosum*, about 25% were algal polysaccharide degraders [119] which belonged to the classes *Flavobacteria* (*Cellulophaga, Maribacter, Algibacter,* and *Zobellia*) and *Gammaproteobacteria* (*Pseudoalteromonas, Vibrio, Cobetia, Shewanella, Colwellia, Marinomonas,* and *Paraglaceciola*). Regarding the total bacterial isolates obtained, the most abundant groups observed were *Bacteroidetes* and *Gammaproteobacteria*. However, phyla like *Planctomycetes* (known to possess enzymatic machinery for macroalgae polysaccharides degradation) and *Cyanobacteria* commonly found on brown algae were not isolated.

Using 16S rRNA gene clone libraries, Wu et al. [120] observed a host-specific but temporally and spatially variable epibacterial community on the surface of the four red macroalgae, *Gracilaria lemaneiformis*, *Gloiopeltis furcata*, *Mazzaella* sp. and *Porphyra yezoensis. Alfa-* and *Gammaproteobacteria and Bacteroidetes* dominated these communities but *Deinococcus-Thermus, Spirochaetes and Epsilonproteobacteria* were also found. The most frequent genera in the four clone libraries were *Pseudoalteromonas* in *Gr. lemaneiformis* and *Gl. furcata*, *Sulfitobacter* in *P. yezoensis* and an undefined cluster within *Deinococcus-Thermus* in *Mazzaella* sp.

The composition of *Porphyra umbilicalis* bacterial community was analyzed by high-throughput pyrosequencing and classified into eight phyla: *Bacteroidetes, Proteobacteria, Planctomycetes, Chloroflexi, Actinobacteria, Deinococcus-Thermus, Firmicutes*, and the candidate division TM7 [121]. In the *Bacteroidetes*, *Sphingobacteria* was the most represented group. A core microbiome which included *Granulosicoccus* (Gammaproteobacteria), *Hyphomonadaceae, Hellea* and *Loktanella* (*Alphaproteobacteria*), *Iamia* (*Actinobacteria*), members of the *Sphingobacteria* (namely *Aureispira, Haliscomenobacter, Lewinella*, *Saprospiraceae* and *Chitinophagaceae*), *Tenacibaculum* (*Flavobacteria*) and *Rhodopirellula* (*Planctomycetes*) was present in *P. umbilicalis*. Richer and more diverse bacterial communities were observed in algae from autumn than in the ones from winter.

Previous studies have shown that planctomycetes are common inhabitants in macroalgal biofilms [111, 115, 122]. In 2014, Bondoso et al. [123] analyzed the *Planctomycetes* communities epiphytic on six different macroalgae (red - *Chondrus crispus*, *Mastocarpus stellatus*, *Porphyra dioica*; brown – *Fucus spiralis*, *Sargassum muticum*; and green – *Ulva* sp.) from two rocky beaches in the North of Portugal. Based on DGGE profiles, the lowest diversity was observed in *F. spiralis* and the highest in *M. stellatus* and *P. dioica* from Porto and each alga revealed a planctomycetes specific community.

In the various studies of bacterial diversity associated to macroalgae, several patterns were observed. Seasonal and geographical (Baltic Sea and North Sea) differences in bacterial communities of *Saccharina latissima* were observed by Staufenberger et al. [112]. Tujula et al. [110] also observed differences between *U. australis* individuals between seasons and from both the same and different tidal pools. This difference was highest in winter. However, they found that there was a constant sub-population present (members of the *Alphaproteobacteria* and the *Bacteroidetes*). On a comparative DGGE study of six macroalgae (*Fucus serratus, F. vesiculosus, L. saccharina, Ulva compressa, Delesseria sanguinea* and *Phycodrys rubens*) from the Baltic and North Seas, Lachnit et al. [124] observed the existence of significant differences

between the epibacterial communities of these algae that differed less between regions than between host species and were more similar on closely related host species. This work suggested that the biofilm communities are controlled by the macroalgae. Lachnit et al. [115] also verified that the macroalgae *F. vesiculosus*, *G. vermiculophylla* and *U. intestinalis* living in close proximity showed seasonal differences in their bacterial community at phylum level which were seasonally consistent. However, each macroalgal species possessed a species-specific and temporally adapted epiphytic bacterial community.

Although changes occur over season, life span and macroalgal thallus parts, specific association seems to exist between bacteria and macroalgae [25, 110, 112]. However, Burke et al. [111] verified that *Ulva australis* individuals co-inhabiting a common environment harbored a unique assemblage of bacterial species and that this community was established based on functional genes and not on the taxonomy of the species [125].

3.2. Viriome studies

Recently, attention started to be paid to the viruses associated with macroalgae [17]. The viriome associated with the red macroalga, *Delisea pulchra* was analyzed and virus-like particles were icosahedral, bacilliform to coiled pleomorphic and bacteriophages. The viruses found suggest an infection role as dsRNA viruses were affiliated to the genus Totivirus and a ssRNA to the order Picornavirales, both known to infect, respectively, plant pathogenic fungi and marine diatoms.

3.3. Fungal diversity

Singh et al. [126] did a comprehensive revision on marine fungi associated with the three groups of seaweeds. Their relationship towards the macroalgae can be of parasitism, saprotrophic nature or symbiosis, being pathogens and parasites the dominant ones [127]. Many macroalgae of the three lineages can harbor a diverse assemblage of marine fungi. Endosymbiotic representatives are members of the genera *Acremonium*, *Alternaria, Arthrinium, Aspergillus, Cladosporium, Fusarium, Geomyces, Penicillium*, and *Phoma* of which Ascomycota and anamorphic fungi are the most common [126]. *Geomyces* species, *Penicillium* sp. and *Metschnikowia australis* were the most common fungi associated with eight macroalgae from Antarctica [128]. Chlorophyta seems to be the macroalgae more densely colonized by fungi but with a lower diversity and Pheophyceae revealed the highest diversity [129]. The marine fungus *Pestalotia* sp. was isolated from the surface of the brown alga *Rosenvingea sp.* [130]. Zhang et al. [131] studied the fungal community associated with four species of red alga, two species of brown alga and two species of green alga, and verified that the brown alga *Sargassum thunbergi*i, and the red alga *G. lemaneiformis* yielded many more cultivable isolates than the other ones (*Rhodomela confervoides, Gelidium amansii, A. flabelliformis, Colpomenia sinuosa, Enteromorpha prolifera* and *Ulva pertusa*). *Penicillium glabrum, Fusarium oxysporum*, and *Alternaria alternata* were also identified in this study.

Porphyra red rot disease caused by *Pythium porphyrae* has important economic impact in countries like Japan and China where this alga is intensively cultivated. Li et al. [132] studied

oomycetes and fungi parasites of marine macroalgae and they found a total of 13 species that are parasites being some obligate pathogens (*Eurychasma dicksonii*, *Eurychasmidium tumefaciens*, *Olpidiopsis porphyrae*, *Petersenia lobata*, *Petersenia palmariae*, *Petersenia pollagaster*, *Pontisma antithamnionis*, *Pontisma feldmannii*, *Pontisma lagenidioides*, *Pythium marinum*, *Pythium porphyrae*, *Sirolpidium andreei* and *Sirolpidium bryopsidis*).

Using 28S rRNA gene PCR-DGGE and real-time PCR analyses, Zuccaro et al. [133] studied the filamentous fungi present in healthy and decaying *Fucus serratus* thalli. They found *Lindra*, *Lulworthia*, *Engyodontium*, *Sigmoidea*/*Corollospora* complex, and *Emericellopsis*/*Acremonium*-like ribotypes. By cultivation approach, *Sigmoidea marina* was the fungus highly isolated. In decaying thalli, the fungal community changed and was composed of members of the Dothideomycetes.

3.4. Algal diversity

The epiphytic microalgal community on macroalgae has been studied by a restricted number of authors [27, 31, 134–138]. This community is mostly dominated by benthic diatoms and some few centric species possessing an attached mode of life. The composition of benthic diatoms on macroalgal biofilms can be modulated by several environmental conditions including nutrients, salinity, light conditions and hydrodynamic regime as well as by biological factors like grazing, adhesive capacity of diatoms and chemical interactions with the host [27] and references therein.

In a study performed by Al-Handal and Wulff [134], of the 50 epiphytic diatoms identified, *Cocconeis* spp., *Entopyla australis* var. *gigantea*, *Grammatophora arctica*, *Licmophora Antarctica* and *Pseudogomphonema kamtschaticum* were the most common taxa detected on the surface of several macroalgae which showed a different behavior as host: Chlorophyta harbored no diatoms; Phaeophytes an higher number; and Rhodophyta species, *Pantoneura plocamioides*, *Delesseria lancifolia* and *Georgiella confluens* were the most colonized macroalgae.

Based on molecular data and/or SEM characteristics, two abundant diatoms epiphytic on the assimilation hairs of the brown macroalga *Chordaria flagelliformis* were identified as *Fragilaria barbararum* and *Fragilaria striatula* [135].

Three macroalgae, the brown alga *Pilayella littoralis*, the red alga *Ceramium gobii*, and the green alga *Cladophora glomerata* were comparatively analyzed regarding their diatom colonization [136]. This was higher in spring and in higher salinity (Baltic Sea comparatively to Bothnian Sea). The green alga harbors lower diatom numbers. Although the brown macroalgae was the one possessing the highest diatom numbers, it presented the lowest community diversity. The highest community diversity was found on *Ceramium*.

Diatoms epiphytic on red macroalgae living under the sea ice showed a species pattern with depth in which *Cocconeis fasciolata* dominated at 10 and 15 m, *Porosira glacialis* at 20 m and *Eunotogramma marginopunctatum* at 25 m [137].

Totti et al. [27] in their study of the epiphytic communities on macroalgae from Iceland detected that erect growth forms of diatoms represented 50% of its community (*Achnanthes* cf. *bre-*

vipes var. *parvula, Tabularia investiens, T. fasciculata, Hyalosira* cf. *delicatula, Gomphoseptatum aestuarii, Pseudogomphonema plinskii*), adenate diatoms 29% (*Cocconeis stauroneiformis, C. scutellum*) and motile forms 21% (*Nitzschia* cf. *amphibia and Navicula perminuta*).

Tanaka [139] studied the adhesive capacity of diatoms and verified no close correlation with cell size, their cell form, motility, and mucus secretion. Also, no macroalgal species specificity existed in diatom colonization which was composed preferentially by *Navicula* sp., *Cocconeis* spp., *Gornphonema* sp., *Nitzschia closterium* and *Synedra tabulate.*

4. Biotechnological potential of macroalgae biofilms

The identification of thousands of microbial species and the increase in knowledge on macroalgal biofilms diversity and functioning lead to the valorization of its diversity with the development of several products in a wide variety of fields. The communities living on the surface of macroalgae benefit from a mutualist relationship with their host. The macroalgae are a reliable source of nutrients and on the other hand epiphytic bacteria and fungi help their hosts by producing bioactive molecules that protect all the community from unwanted invaders [39]. The microorganisms in a biofilm community compete against each other and protect themselves from other surrounding pelagic microorganisms by working together as a team producing different kinds of chemicals such as antifungal, antiprotozoal, anti-settlement and antibiotic molecules [39, 74, 126, 140]. It is the high competition in these communities that induce microorganisms to produce allelochemicals that can be applied in industries such as pharmaceutics, cosmetic industry or even in agriculture [126].

Globally, hundreds of new natural molecules produced by marine organisms are discovered in a temporal pattern wherein the last discovery peak of new metabolites happened 10 years ago [141]. These authors tried to explain this effect with the need of improvement of the techniques used to discover new compounds. The genomic data now available, the high-throughput assays for cytotoxicity in cell-based screening and the automation in nuclear magnetic resonance (NMR) and mass spectroscopy assist the discovery, the accurate identification and validation of new leads to treat human diseases [141]. This review emphasizes the importance of the searching of new compounds from macroalgae and their associated microorganisms. The highest number of bioactive hits found was provided from marine invertebrates, although in the last decades it was discovered that the compounds were actually produced by the associated/symbiotic microorganisms. Even though marine microorganisms provided the highest percentages of bioactive compounds, microorganisms associated to algae are still a minority [141].

Nowadays, the boom of reports that address the search of bioactive compounds produced by macroalgae-associated microorganisms reflects the importance and novelty of the compounds obtained from these sources. Singh et al. [126] described in a review several reports regarding antimicrobial compounds from seaweeds-associated bacteria and fungi published until 2014. Furthermore, they provided details on the bacteria and fungi associated with macroalgae that are producers of bioactive molecules. It is worth mentioning the ecological role of several new

compounds such as haliangicin, korormicin, thallusin or violacein (antifungal, antibiotic, morphogenesis and photosynthetic activities, respectively) in the defence response in macroalgal biofilm [53, 142–144]. In the communities associated with green, brown or red macroalgae, 12% to 50% of strains were able to produce antimicrobial effects in one or more target microorganisms [72, 74, 75, 140]. Remarkable was the discovery of the antidiatom activity produced by 80% of the strains isolated from *Ulva lactuca* against the diatom *Cylindrotheca fusiformis* [145] and 72% of the strains isolated from *Ulva reticula* against the diatom *Nitzschia paleacea* [24].

The most recent publications concerning the biotechnological potential and bioactivity production of microorganisms living in macroalgal biofilms will be referred below.

Two species of *Streptomyces* isolated from the brown macroalgae *Fucus spiralis* and *Cystoseira baccata* allowed the isolation of the following bioactive compounds: daunomycin, cosmomycin B, galtamycin B (antitumor and antibiotic activity); maltophilins (antifungal), and lobophorins (anti-inflammatory and antituberculosis) [146]. Compounds capacity of more than one activity has already been described [141]. Some alkaloids can even show quadra-activity. The study of marine bacteria and fungi from different sources led to the isolation of one bacterial strain (BMA6) from macroalgae with low activity against *Vibrio* sp. P3b [147]. The isolation of 31 Gram-positive and pigmented bacteria from Antarctic macroalgae allowed the selection of five strains phylogenetically related to *Agrococcus*, *Brachybacterium*, *Citricoccus* and *Kocuria*, that showed inhibitory effects, although without broad antibacterial spectrum, in the antagonism assay performed against other resident bacteria [148]. The cytotoxic and antibiotic compound cytochalasin D was, for the first time, isolated from a marine source, an endophytic *Xylaria* sp from the red algae *Bostrychia tenella* [149]. Susilowati et al. [150] isolated from *Sargassum* a bacterial strain with 95% similarity to *Bacillus subtillis* with high levels of inhibition against *Staphylococcus aureus* MRSA and *Staphylococcus epidermidis*. A study on green, red and brown macroalgae endo- and epiphytes revealed that 25% of the isolated epiphytes were able to produce inhibition against *Staphylococcus aureus* (ATCC 25922), *Escherichia coli* (ATCC 25923) and *Candida albicans* (ATCC 90028) [151]. In this study, the red algae were the ones providing more bioactive strains.

Striking is the lack of reports of epiphytic fungi regarding bioactive production, in disparity to bacteria and endophytic fungi. Godinho et al. [128] isolated 148 fungi from the Antarctic macroalgae, *Monostroma hariotii* and *Pyropia endiviifolia*, of which two *Penicilium* strains were able to produce antifungal, antiprotozoal and cytotoxic compounds. Furthermore, 239 fungi were isolated from the same macroalgae and 6 showed between 61 and 96% bioactivity against selected targets, with even better results than the positive control against yellow fever virus [152]. A pseudodeflectusin compound produced by an *Aspergillus pseudodeflectus* associated to *Sargassum fusiform* showed to induce cytotoxicity in stomach and cervix human cancer cell lines [153].

Additional to the pharmacological applications of compounds produced by members of the macroalgal biofilm, another potential application is the usage of anti-settlement compounds produced by some strains in paints used in aquatic environments that will inhibit the adhesion and settlement of algae in the surface of boats and other objects.

Although in this genomic era there is an incredible increase of information about microbial communities, it is foreseen that only 1–5% of the microorganisms are able to be cultivated. However, several advances in the search for genes encoding secondary metabolites biosynthetic pathways by culture-independent methods, like metagenomics analysis and metabolomics, and application of this information in synthetic microbiology is increasing the possibilities to reveal new drugs impossible to discover until now. Unexpectedly, as opposed to what is found in sponge's bioactive studies, few are the genomic searches for genes that encode for polyketide syntethases or nonribosomal peptide synthases in macroalgae associated microorganisms.

The discovery of new compounds and new bioactive producers open us possibilities to fight against emergent and still incurable diseases and provide new clues to the understanding of the ecological role played by the complex macroalgal biofilm communities that live under constant societal and environmental pressures.

5. Conclusion

Macroalgae are protected microniches prone to epibiosis by microorganisms where complex and highly dynamic interactions occur. Firstly colonized by bacteria, many other microorganisms which include microalgae like diatoms, fungi and protozoa constitute these biofilms.

Awareness of the importance of macroalgae and their biofilm has risen recently and, in the last years, we are gaining knowledge on its diversity, especially on the bacteriome, on the multiple functions played by both components of the holobiont, on macroalgal diseases and on the biotechnological potential of these communities. As only a low number of bacteria have been cultivated, we still have a relevant ecological potential to discover in many unknown bacteria. Furthermore, only very recently the world of macroalgal associated viruses started to be revealed. New methodological advances, metagenomics associated with metabolomic/proteomic studies will certainly foster our comprehension of the community structure and functioning of the microbial–macroalgal system. As only a very small part of the more than 35,600 different known species of macroalgae have been studied, we still have a long way to discover the hidden microbial diversity in their biofilms and its biotechnological potential, to understand all potential interactions between algal host and its microbial community, and the regulatory mechanisms in the extra coat of macroalgae.

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Chapter 9

Silica-Based Nanovectors: From Mother Nature to Biomedical Applications

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Abstract

Diatomite is a natural porous silica material of sedimentary origin, formed by remains of diatom skeletons called "frustules." The abundance in many areas of the world and the peculiar physico-chemical properties made diatomite an intriguing material for several applications ranging from food production to pharmaceutics. However, diatomite is a material still rarely used in biomedical applications. In this chapter, the properties of diatom frustules reduced to nanoparticles, with an average diameter less than 350 nm, as potential drug vectors are described. Their biocompatibility, cellular uptake, and capability to transport molecules inside cancer cells are discussed. Preliminary studies of *in vivo* toxicity are also presented.

Keywords: diatomite, porous material, nanovector, bioconjugation, drug delivery

1. Introduction

Nanomedicine is an innovative research field combining nanotechnology and medicine, radically changing the healthcare drug delivery scenario, in particular in the cancer treatment [1]. The application of nanotechnology in the cancer therapy is expected to solve a number of issues associated with conventional therapeutic agents, including lack of targeting capability, nonspecific distribution, systemic toxicity, and low therapeutic index [2, 3].

Nanotechnology has provided the opportunity to get direct access to the cancerous cells selectively with increased drug localization and cellular uptake, making the therapy more patients' compliant, efficient, and painless. Moreover, nano-based systems allow delivery of poor water-soluble molecules (e.g., most of the anticancer drugs) difficult to administer and can also protect the new therapeutics molecules, such as oligonucleotide analogs (e.g., siRNA) from degradation, preserving their therapeutic efficacy while in the blood circulation [3, 4]. Thus, the aim of nanomedicine in cancer therapy is the production of nanoparticles (NPs) able to deliver a drug to a specific site enhancing local drug molecules accumulation and reducing systemic toxicity [5–7]. Different types of organic and inorganic NPs including liposomes, micelles, nanotubes, and porous silicon (PSi) nanostructures have already been investigated for drug delivery purposes [8–13]. In particular, PSi is one of the most inorganic material used in biomedicine due to its unique properties such as high-specific surface area, tunable pores size, biocompatibility, non-toxicity, high loading capability, controllable dissolution kinetic [1, 14, 15]. Moreover, PSi surface can be easily modified using well-known silane and silanol chemistries in order to incorporate gold and magnetic NPs giving to the hybrid complex (i.e., PSi nanocarrier + metallic or magnetic NP) additional diagnostic and therapeutic functions [16].

In recent years, diatomite, a natural porous silica-based material with similar physico-chemical properties to man-made fabricated PSi, has been suggested as feasible alternative to synthetic porous media for biomedical applications [17–20]. Diatomite is a compound of sedimentary origin formed by remains of diatoms deposited on the bottom of seas or lakes [21, 22]. Due to its peculiar properties including highly ordered pore structure, high-specific surface area, modifiable surface chemistry, biocompatibility, non-toxicity, low cost, optical, and photonic properties, diatomite has been used in different applications ranging from optics and photonics to biosensing [23–27]. Despite these properties and the recognition by the Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) for foods and pharmaceuticals production, its use in nanomedicine is still surprisingly undervalued [28]. Recently, diatomite has been explored as microcapsules for oral drug delivery resulting in a non-cytotoxic biomaterial with high potential to improve the bioavailability of loaded oral drugs [20]. To date, there is only one manuscript on the intravenous injection of diatomite microparticles into mice, which investigates the biodistribution and tissue damage of this material. This study demonstrated that none of the animals exhibited any observable abnormalities in the major organs after diatomite injections [29]. Over the last few years, diatomite frustules reduced to nanoparticles have been explored as potential nanocarriers for biomedical applications [30, 31].

In this chapter, the properties of diatomite nanoparticles as non-toxic nanocarriers are described. Different surface functionalization strategies performed in order to transport molecules inside cancer cells and to improve diatomite NPs biocompatibility and cellular internalization are summarized. Preliminary studies of *in vivo* toxicity are also reported.

2. Diatomite nanoparticles (DNPs)

Diatomite is a material of sedimentary origin, formed by siliceous skeleton (called "frustule") of diatoms, unicellular microalgae, deposited on the bottom of seas, or lakes over centuries.

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Due to the presence of diatom frustules with different size (ranging from 2 μm to 2 mm) and shape, diatomite morphology can be very complicated (**Figure 1**) [32, 33].

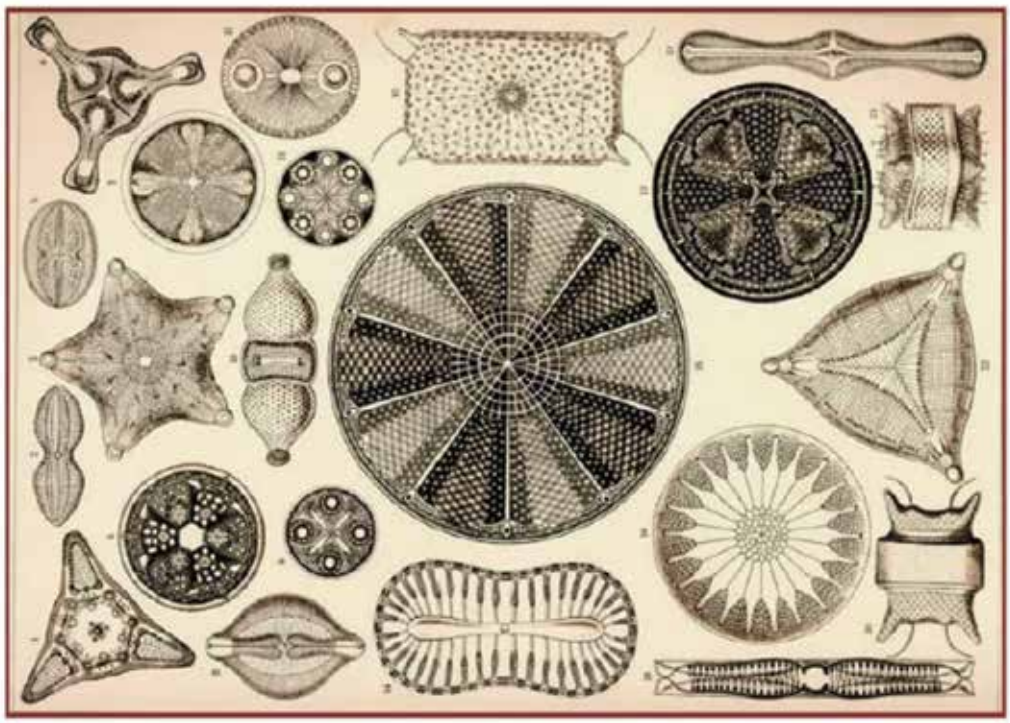

Figure 1. Diatoms by Ernst Haeckel in 'Kunstformen der Natur' 1904 (Reproduced from Ref. [32]).

Diatomite is characterized by highly porous structure with a large specific surface area up to 200 m^2/g [34]. Its fundamental constituent is amorphous silicon dioxide, even if it can contain organic and metallic impurities (e.g., MgO, Al_2O_3, Fe_2O_3) coming from environment [35, 36]. Several processes, including calcination and hot acid treatments, have been developed in order to remove these contaminations from frustules [37, 38].

The abundance in many areas of the world and the peculiar physico-chemical properties (e.g., chemical stability, non-toxicity) made diatomite an intriguing material for several applications ranging from food production to pharmaceutics [39–41].

In recent years, diatom frustules have been investigated as porous platform in several innovative biotechnological applications [42, 43]; the silica surface of diatoms can be easily modified with different functional groups (–SH, $–NH_2$, –COOH,) for the immobilization of biomolecular probes (DNA, proteins, antibodies) using the reactive silanol (Si–OH) groups present on it [44]. Recent works reported the surface chemical modification of a marine diatom with an antibody used as bioprobe; photoluminescence and fluorescence microscopy were used in order to investigate the antibody-antigen molecular recognition [45, 46]. **Figure 2**

shows an image of marine diatom *Coscinodiscus wailesii* functionalized with an antibody after the interaction with the corresponding rhodamine labeled antigen; the dose-response curve of biosensor (i.e., diatom platform + antibody) is also reported [45].

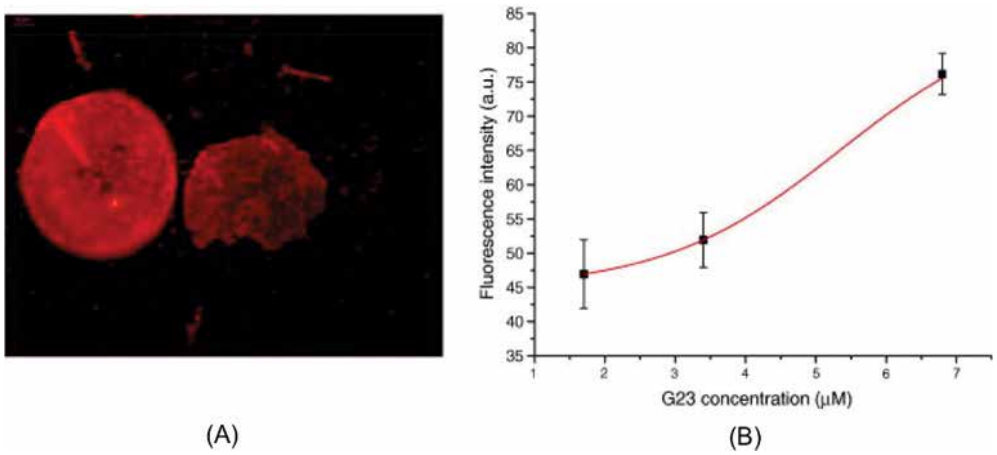

Figure 2. (A) Image of diatom frustules after the interaction between an antibody, covalently linked on them, and the corresponding rhodamine labeled antigen. (B) Fluorescence intensity versus antigen concentration (Reproduced from Ref. [45]).

Although diatomite, produced in tons by mining industry, is a cheaper material compared to diatom frustules, it is still scarcely used in biomedical applications. Recent pioneering papers demonstrated the use of diatomite silica microshells as microcapsules for oral drug delivery [17, 20, 47]. For example, indomethacin, an anti-inflammatory drug poorly soluble in water was explored as model drug in experiments of drug loading and release [18].

More recent works explored the possibility to obtain NPs with a diameter less than 300 nm from micrometric diatomite powder by means of a process based on mechanical crushing, sonication, and filtering [30]. The size reduction is fundamental in drug delivery applications in order to optimize the cellular uptake of particles.

The process for diatomite nanoparticles (DNPs) fabrication is briefly described as follows. About 5 g of diatomite powder was dissolved in 250 ml of absolute ethanol and sonicated for 5 h in order to break up macroscopic aggregates. The dispersion was then filtered through a nylon net filter with pore size of 41 μm (Millipore). In order to remove the organic and inorganic contaminants [34, 36] from natural diatomite, purification treatments were performed: the diatomite dispersion was then centrifuged and the pellet suspended in piranha solution (2 M H_2SO_4, 10% H_2O_2) for 30 min at 80°C. Dispersion was again centrifuged for 30 min at 13,500 rpm, and the supernatant removed. Next, diatomite was washed twice with deionized water. 5.0 M HCl solution was then added to diatomite and incubated over night at 80°C. After HCl incubation, the diatomite dispersion was centrifuged for 30 min and the supernatant removed. The pellet was then washed twice with deionized water in order to remove the excess of HCl.

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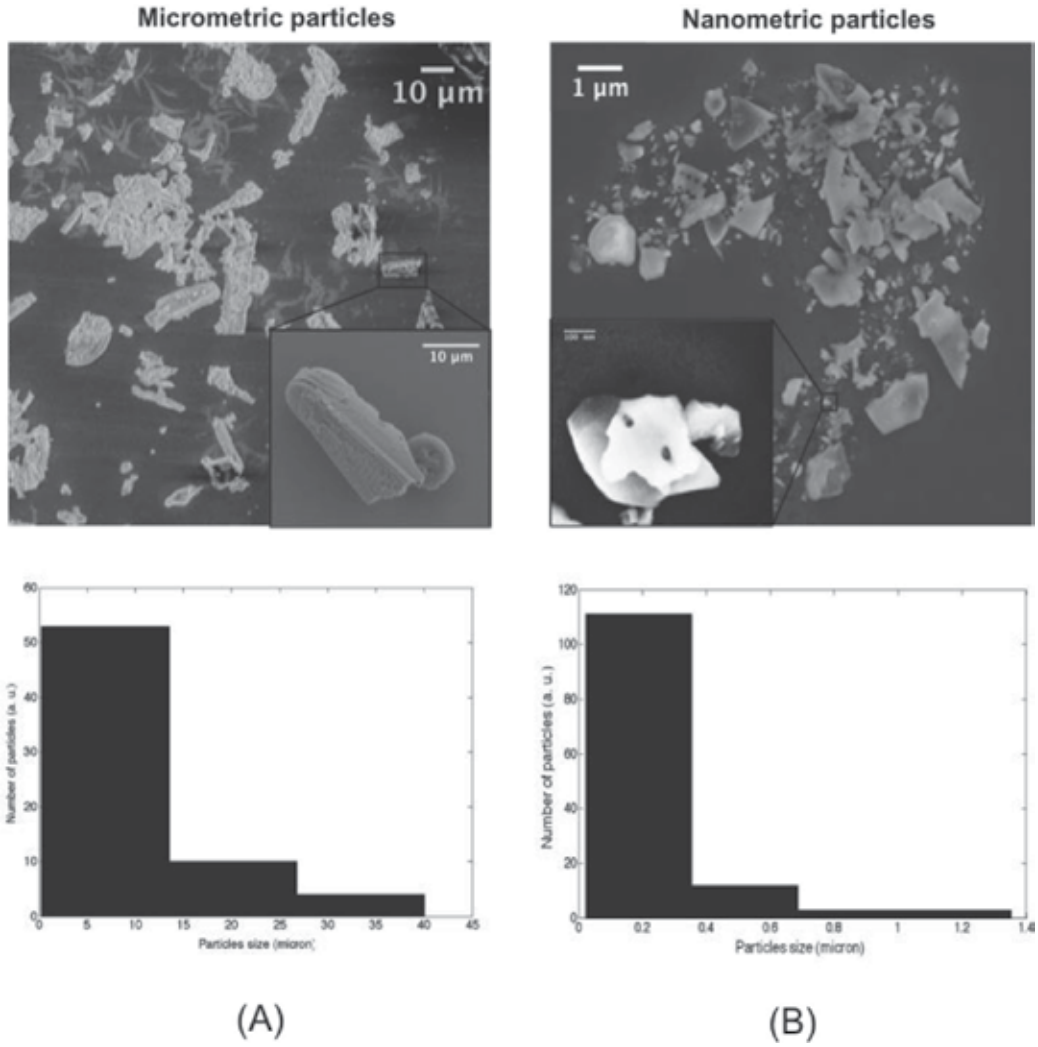

Figure 3. (A) SEM images of diatomite frustules before size reduction treatments and corresponding histogram of particles size distribution. (B) SEM images of diatomite particles after size reduction and corresponding histogram of particles size.

Figure 3 shows the scanning electron microscope (SEM) images of diatomite fragments before (A) and after (B) size reduction. Before treatments, diatomite was composed by fragments with circular, elliptical, elongated, and squared shape. The histogram of particle size showed dimensions distribution ranging from few microns up to about 40 μm with an average maximum size of 7 μm. From these images, it was also possible to appreciate the porous morphology of diatomite useful for drug loading. Morphological characterization, performed after size reduction and purification, revealed the presence of diatomite fragments with different shape and size distribution, ranging from few nanometers to about 1 μm, with an average maximum size of 250 nm. Size of polydispersity can be reduced performing more

filtration steps using filters of 0.45 μm. After nanometric size reduction, it is still possible to observe the porous structure of the NPs with pores of about 40 nm in size.

Dynamic light scattering (DLS) analysis was used in order to investigate size and surface charge of the purified diatomite NPs in water (pH = 7). **Figure 4** shows size and zeta-potential distributions with average values of 220 ± 90 nm and −19 ± 5 mV, respectively. The negative value of zeta potential is due to Si–OH groups present on the surface of DNPs after piranha treatment.

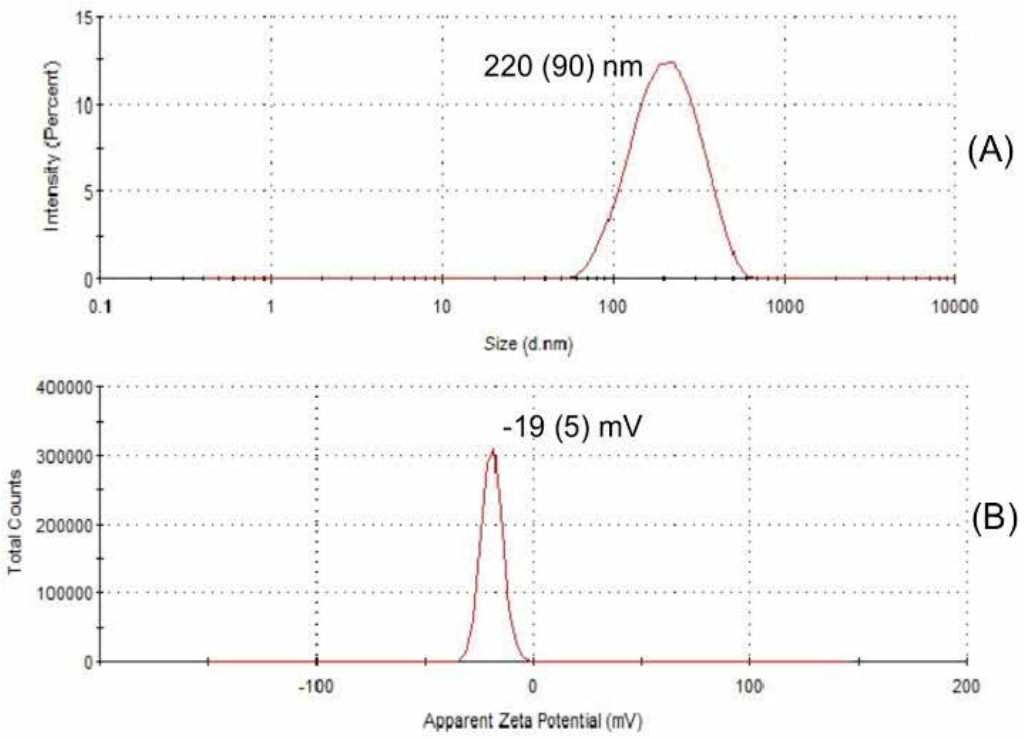

Figure 4. Size (A) and zeta-potential (B) distributions of diatomite nanoparticles in water (pH = 7) (Reproduced from Ref. [30]).

A critical issue for biomedical applications of new drug delivery nanocarriers is the evaluation of their potential toxicity and biocompatibility [17, 48, 49]. *In vitro* cytotoxicity of DNPs can be evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a method based on the reduction of MTT by cellular oxidoreductases of viable cells that yield the formation of crystalline blue formazan. Human lung epidermoid carcinoma (H1355) cells were used as an *in vitro* cell model to test the cytotoxicity of DNPs. H1355 cells were incubated with different concentrations of diatomite NPs for 24, 48, and 72 h. The obtained results are shown in **Figure 5**. H1355 cells exposed to increasing NP concentrations (20, 100, 200 and 300 μg/ml) show an average viability lower than 100%, demonstrating a very low toxicity of NPs and confirming their potentialities as nanovectors in nanomedicine [50, 51].

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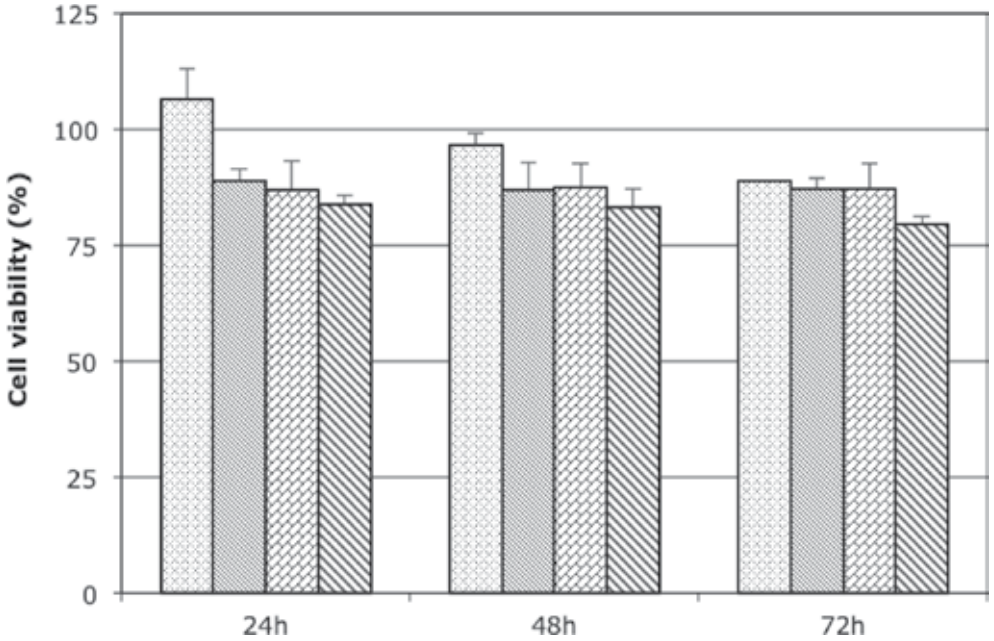

Figure 5. Cytotoxicity assessment of DNPs using MTT assay. Cell viability of H1355 cells treated with 20, 100, 200, and 300 μg/ml of nanoparticles for 24, 48, and 72 h at 37°C. Data represent the mean ± s.d. (n = 3). Cell viability was expressed as the percentage of viable cells compared with cells cultured without NPs as control (100%) (Reproduced from Ref. [31]).

3. siRNA delivery by DNPs inside cancer cells

Small interfering ribonucleic acid (siRNA) is a powerful approach for silencing genes associated with a variety of pathologic conditions, but its systemic delivery is inefficient due to the difficulty to penetrate the cell membrane [52, 53]. siRNA conjugation to nanovectors (e.g., liposomes, gold and magnetic NPs, quantum dots) is one of the possible strategies developed to overcome this challenging problem [54, 55]. DNPs have been shown as potential nanocarriers for siRNA transport inside cancer cells and gene expression silencing [31].

siRNA* (labeled with Dy547), complexed with a poly-D-Arg peptide, was loaded onto DNPs following the functionalization procedure sketched in **Figure 6**. Briefly, (3-aminopropyl)triethoxysilane (APTES)-functionalized diatomite reacts with N-(γ -maleimidobutyryloxy) sulfosuccinimide (NHS) ester (sulfo-GMBS), a water-soluble amine-to-sulfhydryl crosslinker that contains NHS-ester and maleimide reactive groups at opposite ends of a short spacer arm that allow covalent conjugation of amine-(diatomite surface) and sulfhydryl-containing molecules (peptide). A poly-arginine peptide and a nonpolar homopeptide, used as negative control, were used in a molar nitrogen/phosphate ratio of 20:1, previously described as optimal condition to form a stable complex with siRNA [31].

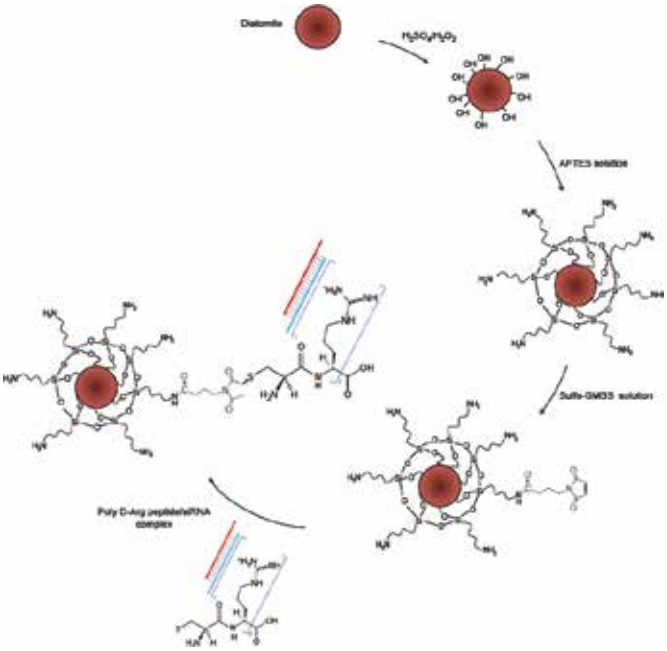

Figure 6. Functionalization scheme of diatomite frustules with labeled siRNA (siRNA*) (Reproduced from Ref. [31]).

In order to study the uptake and intracellular localization of the nanoparticles, H1355 cells (50 × 10^3/coverslip) were incubated with 300 μg/ml of siRNA* modified DNPs (DNPs-siRNA*) for 24 h. A representative confocal microscopy acquisition was reported in **Figure 7**.

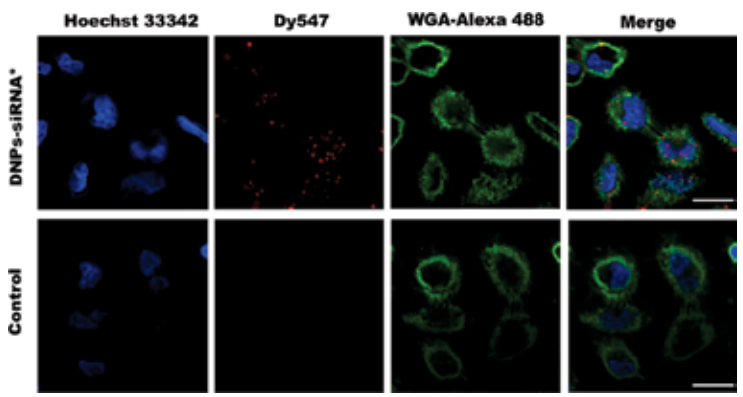

Figure 7. Confocal microscopy on cells treated with siRNA*-modified diatomite nanovectors (first line) and untreated cells as control (second line). Cell nuclei and membranes were stained with Hoechst 33342 and WGA-Alexa Fluor 488, respectively. siRNA was labeled with Dy547. Scale bar corresponds to 20 μm.

Cell nuclei were stained with Hoechst 33342 (blue), whereas WGA-Alexa Fluor 488 (green) and Dy547 (red) were used to stain cell membranes and siRNA, respectively. In **Figure 7**, the

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cytoplasmic localization of DNPs-siRNA* is well evident and it was observed as both spots and diffuse signal [56–59]; no red fluorescence was found inside the nuclei. The efficiency of DNPs-siRNA* internalization was quantified by fluorescence microscopy: counting the number of red fluorescent cells and the total number of cells (determined in bright field), a ratio of about 75% was calculated.

The capability of a siRNA direct toward glyceraldehyde 3-phosphate dehydrogenase (GAPDH), conjugated on DNPs, to determine gene knockdown, was evaluated by Western blot analysis; a scramble (SCR) siRNA was used as negative control. A conventional transfection method (Lipofectamine 2000) was carried out to compare the two siRNA uptake systems [31].

The obtained results are showed in **Figure 8**. The densitometric analysis of the bands (Panel B) shows a decrease in the GAPDH protein expression (Panel A, upper gel) of about 22% (lane 2) after 48 h incubation at 37°C with respect to control and DNPs-SCR-siRNA-treated cells (lane 1 and lane 3, respectively). The analysis of GAPDH expression level when lipofectamine was used is reported in panels C and D; a down-regulation of about 20% is observed.

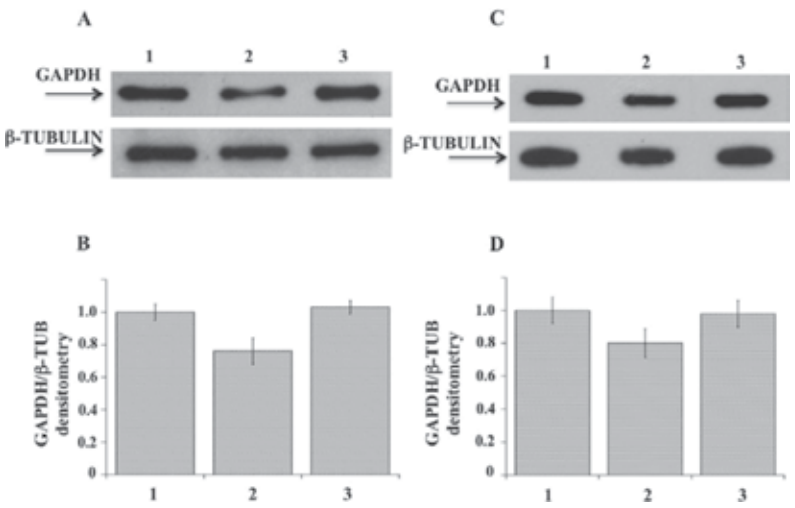

Figure 8. (A) Immunoblotting analysis of GAPDH (upper gel) and β-tubulin (lower gel) protein expression in DNPs-siRNA-treated cells. Lanes: (1) control cells; (2) DNPs-GAPDH-siRNA; (3) DNPs-SCR-siRNA. (B) Densitometric intensity band ratio of GAPDH and β-tubulin used as internal control. The intensities of the bands were expressed in arbitrary units. (C) Immunoblotting analysis of GAPDH (upper gel) and β-tubulin (lower gel) protein expression in lipofectamine-siRNA transfected cells. Lanes: (1) control cells; (2) GAPDH-siRNA; (3) SCR-siRNA. (D) Densitometric intensity band ratio of GAPDH and β-tubulin used as internal control. The intensities of bands were expressed in arbitrary units. Each measurement and Western blot were carried out in triplicate. Error bars indicate the maximum deviation from the mean value of two independent experiments (Reproduced from Ref. [31]).

Nevertheless, the decrease in the protein expression obtained with the two delivery methods was comparable; the use of diatomite as nanovectors allows high stability, biocompatibility, biodegradability, together with high payloads of drugs, selective cell targeting, co-delivery of

molecules functioning with a different mechanism of action (e.g., drugs and siRNA) and controlled release of active compounds at the molecular level.

4. Dual-biofunctionalization of DNPs for enhanced stability, biocompatibility, and cellular internalization in cancer cells

The small size, appropriate aqueous solution stability, biocompatibility, and cellular uptake are the most important characteristics of NPs as drug delivery systems. The PEGylation [i.e., the covalent attachment of poly(ethylene glycol)] of NPs has been frequently used in the design of drug nanocarriers, as a valid functionalization to improve physico-chemical properties of NPs such as the increase of their stability in aqueous medium reducing the nonspecific aggregation and improving biocompatibility, drug loading, and cellular internalization [60–62]. Moreover, an efficient approach to improve the NPs' cellular uptake is to bind them to peptides that can cross the cellular membranes, enhancing their translocation inside the cells [63]. A valid strategy to improve the intracellular drug delivery of nanocarriers is their bioconjugation with cell-penetrating peptide (CPP), due to the CPP property to overcome the

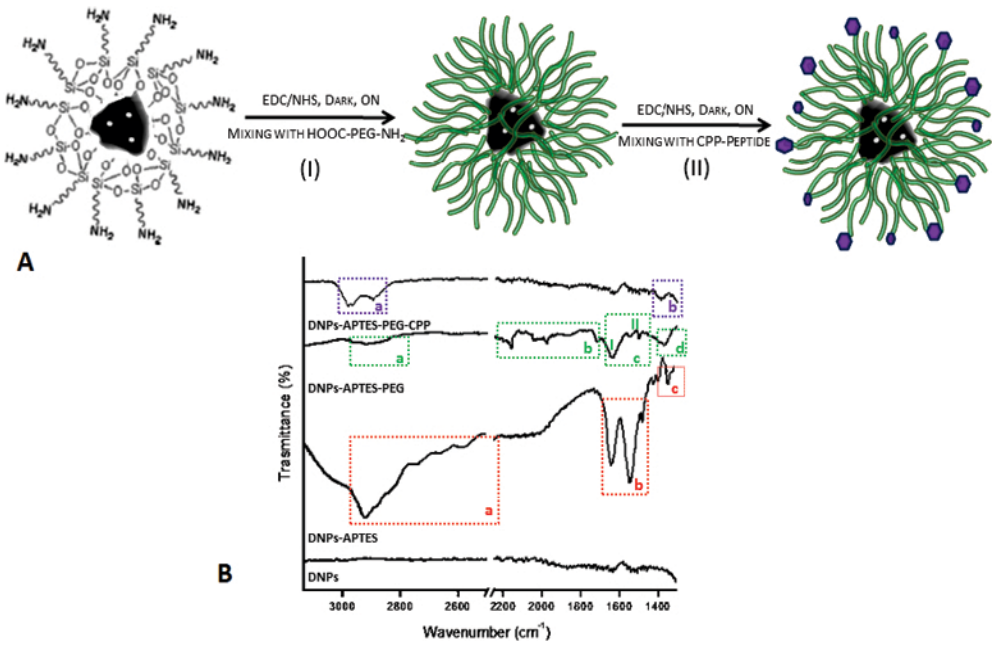

Figure 9. (A) Schematic representation of the DNPs functionalization. Reaction I, the PEGylation of DNPs-APT (I) via EDC/NHS, under stirring overnight (ON) at room temperature. Reaction II, CPP-peptide bioconjugation of DNPs-APT-PEG via EDC/NHS, under stirring ON at room temperature. (B) ATR-FTIR spectra of DNPs before the biofunctionalization, after the silanization process, after the PEGylation, and also after the CPP-peptide bioconjugation. The **a** indicates CH_x stretching vibration, **b** the bending mode of the free NH_2, **c** the C–N stretching, **d** the C–H bending vibrations, and **c-I** and **c-II** N–H bending vibration, and the C–N stretching vibration, respectively.

lipophilic barrier of the cellular membranes and deliver NPs inside the cells [64, 65]. PEGylation and CPP bioconjugation have been used as biofunctionalization strategies to improve the physico-chemical and biological properties of the DNPs, in order to enhance the intracellular uptake in cancer cells and to increase the biocompatibility of APTES modified-DNPs (DNPs-APT) [66]. The decoration of NPs' surface with PEG chains was achieved via covalent bond between the carboxyl groups (–COOH) of PEG molecules and the amino groups ($–NH_2$) of silanized DNPs using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/NHS chemistry (**Figure 9A, I**) [30, 31]. Subsequently, the free amino groups of DNPs-APT-PEG were chemically conjugated with the carboxyl groups of CPP, by EDC/NHS chemistry (**Figure 9A, II**) [67].

The improvement of the NPs' stability in aqueous solutions was confirmed by hydrodynamic diameter, PDI, and surface charge ζ-potential measurements, before and after the DNPs' surface modification. A progressive decrease of the nano-aggregates size from 364 ± 3 nm (DNPs-APT) to 346 ± 4 nm after PEGylation (DNPs-APT-PEG), and to 340 ± 8 nm after CPP-conjugation (DNPs-APT-PEG-CPP), was observed. This result is due to an increase of the DNPs surface repulsion forces of the modified surface (DNPs-bare,–19.2 ± 2.0 mV; DNPs-APT, +19.8 ± 3.0 mV; DNPs-APT-PEG, +35.6 ± 1.5 mV; DNPs-APT-PEG-CPP, +40 ± 2 mV), which can be attributed to the positive charge of PEG-peptide and CPP onto the NPs' surface. The result of DNPs modification was also evaluated by attenuated total reflectance Fourier transform infrared spectroscopy (ATR–FTIR). **Figure 9B** shows the progressive change of DNPs FTIR spectra after each modification step. After the silanization process, the DNPs-APT displayed the typical bands of APTES corresponded to the CH_x stretching at 2941–2570 cm^{-1}, the free NH_2 bending mode at 1630–1470 cm^{-1}, and the C–N stretching at 1385 cm^{-1} [68, 69]. After the PEGylation, the DNPs-APT-PEG showed the stretching bands of the CH_x at 2960–2849 cm^{-1}, the C–H bending vibrations at 2160–1722 cm^{-1}, the amide I band at 1640 cm^{-1} associated with the C=O stretching vibration, the amide II resulted from the N–H bending vibration, and the C–N stretching vibration at 1580 and 1360 cm^{-1}, respectively, thus confirming the covalent binding of the PEG molecules onto the NPs' surface [69]. After incubation with the CPP-peptide, the DNPs-APT-PEG-CPP displayed a band of the CH_x stretching at 2984–2881 cm^{-1}, and the C–N stretching of amide II at 1930 cm^{-1}, confirming the successful of CPP-peptide bioconjugation onto DNPs surface [69, 70].

The hemocompatibility of NPs is of critical importance for their systemic administration as drug delivery systems, in order to avoid serious risks to human health [71, 72]. The effect of modified-DNPs on red blood cells (RBCs) was evaluated studing the % lysed RBCs and their morphology after exposure to the NPs at increasing incubation times (1, 4, 24, 34 and 48 h) and concentrations (25, 50, 100, and 200 μg/ml) [66, 73]. The NPs hemotoxicity was qualitatively determined by naked-eye color evaluation of RBCs' supernatant incubated with modified-DNPs, observing an higher hemotoxicity degree of the DNPs-APT than PEG and CPP modified-DNPs, since the red color intensity of DNPs-APT-RBCs supernatant was closer to the positive control one (water), as shown in **Figure 10A**. The %-hemolysis determined by spectrophotometric analysis of the supernatants after 48 h incubation at the maximum concentration of modified-DNPs (200 μg/mL) was 34% for DNP-APT, 7% for DNP-PEG, and

1.3% for CPP-DNP, demonstrating that the dual-biofunctionalization improved the DNPs hemocompatibility (**Figure 10B**).

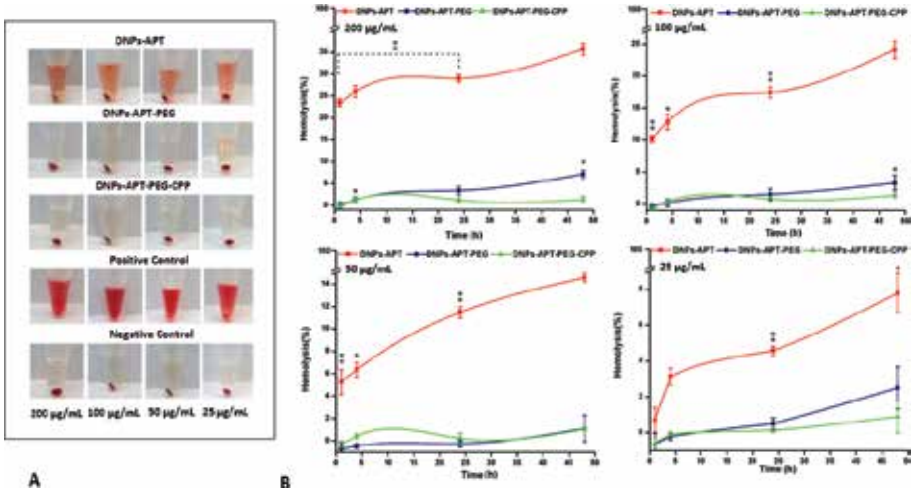

Figure 10. (A) Representative pictures of the RBCs after interaction with the modified-DNPs. The DNPs were incubated with the cells for 48 h and at different concentrations (25, 50, 100, and 200 μg/ml). (B) Hemotoxicity of APT-, PEG-, and CPP-modified DNPs incubated for 48 h at different concentrations (25, 50, 100, and 200 μg/ml) with RBCs, estimated by spectrophotometric methods (577 nm) to analyze the amount of lysed-hemoglobin in the supernatants. The level of significance from negative control was set as probabilities of $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ by ANOVA. Error bars represent s.d. ($n = 3$).

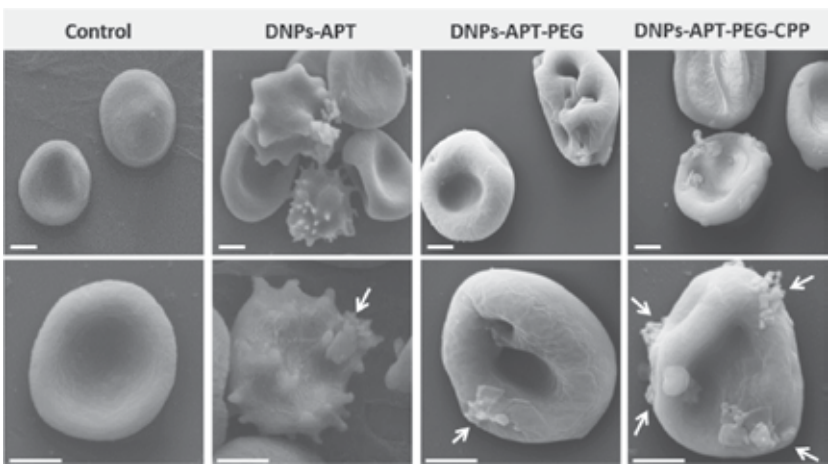

Figure 11. SEM pictures of the RBCs morphological modification after the exposure to the modified DNPs. The modified DNPs (100 μg/ml) were incubated with RBCs for 4 h at room temperature. The DNPs-APT showed the higher toxicity than the PEG and CPP-modified DNPs, resulting in severe morphological changes of cell. The CPP-bioconjugation improved significantly the DNPs–cells membrane interactions, as indicated by white arrows. Scale bars are 3 μm (Reproduced from Ref. [66] with permission from the Royal Society of Chemistry).

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Figure 11 shows SEM characterization of RBCs after exposure to the modified-DNPs (200 μg/ml for 4 h at room temperature). The RBCs, in the presence of DNP-APT completely altered their morphology, changing from the biconcave-like disks to shrinked shape, with consequent hemolysis due to the free positive amine groups on the surface of the NPs, which strongly interact with the negative charge surface of the RBCs. In the case of PEGylated particles, there was a slight change in the RBC's morphology by membrane wrapping around with the appearance of small holes, but without significant hemolysis. The relevant decrease of the DNPs-APT hemotoxicity after PEGylation is due to the improved biocompatibility of the NPs as a result of PEGylation [74, 75]. In the case of DNPs-APT-PEG-CPP, there were no relevant changes observed in the morphology due to the low cytotoxicity of CPP-peptide, which improved the DNPs' biocompatibility [76–78].

Efficient delivery of the nanocarriers to the cells and tissues is another key requirement for drug delivery applications. The CPP-peptide, used to improve the cellular uptake of the DNPs, is a short cationic peptide with intrinsic ability to enter cells and mediate uptake of a wide range of molecular cargos, such as oligonucleotides, small molecules, siRNA, NPs, peptides, and proteins [79–81].

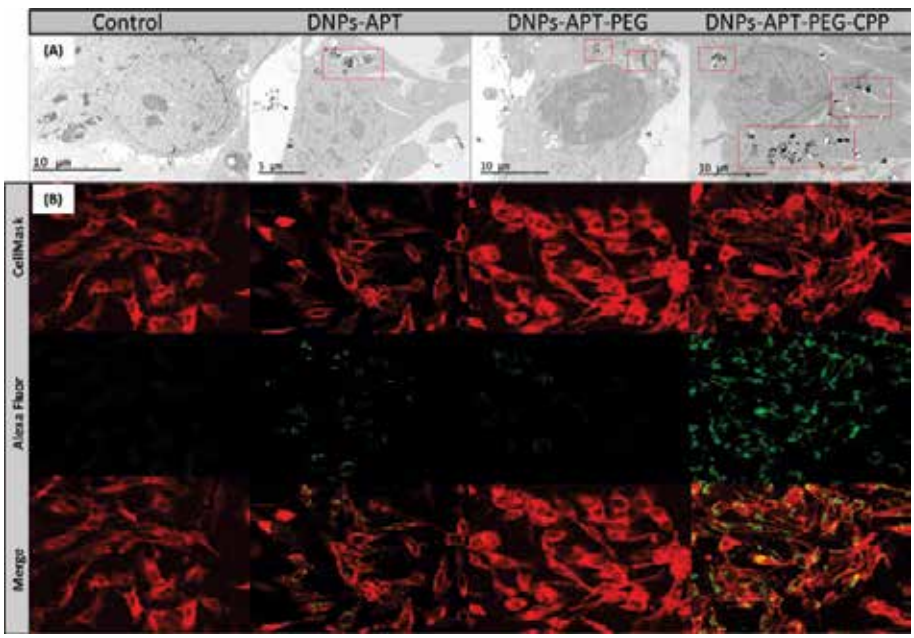

Figure 12. (A) TEM images of MDA-MB-231 cells treated with 50 μg/ml of DNPs-APT, DNPs-APT-PEG, and DNPs-APT-PEG-CPP for 12 h at 37°C. A very small amount of APTES- and PEG-modified DNPs (in dotted boxes) was found inside the cells. In the case of DNPs-APT-PEG-CPP (in dotted boxes), a considerable amount of the NPs was observed inside the cells. Scale bars are 10 μm. (B) Confocal fluorescence microscopy MDA-MB-231 cells treated with 50 μg/ml of APT, APT-PEG, APT-PEG-CPP modified-DNPs for 12 h at 37°C. CellMask® (red) and Alexa Fluor-488® (green) were used to label the cells membrane and the DNPs, respectively. The merge figures are obtained by overlapping the DNPs and the cells membrane images, allowing to determine whether the NPs are located outside (green color) or inside (yellow color) the cells.

The cellular uptake of modified DNPs was evaluated by transmission electron microscope (TEM), after the incubation of MDA-MB 231 breast cancer cells with the NPs (50 μg/mL) for 12 h. In **Figure 12A**, the APTES-modified DNPs were mainly localized in the proximity of the cell membrane, while in the case of the DNPs-APT-PEG, any significant cellular uptake was observed. For the CPP-modified NPs, a considerable amount of DNPs was internalized into the cells with a homogeneous distribution into the cytoplasm and very close to the nucleus. The cellular uptake was also evaluated by confocal fluorescence microscope after DNPs and cellular membrane labeling with Alexa Fluor-488® and CellMask™ Deep Red, respectively (**Figure 12B**). In merged images, for DNPs-APT, the green color is indicative of the presence of DNPs on the cell membrane surface; while for the CPP modified-DNPs, the yellow color, resulting from the co-localization of the green labeled-DNPs and red-stained cancer cell membranes, is representative of NPs located inside the cells. These results confirmed that the CPP bioconjugation is a valid functionalization strategy to increase the cell penetration of DNPs [82, 83].

5. *In vivo* evaluation of DNPs toxicity

In vitro testing is the most common scientific analysis used to determine the effects of NPs toxicity. However, the success of *in vitro* assays is not predictive for promising *in vivo* results; for this reason, *in vivo* evaluation of NPs toxicity is a crucial issue in order to develop safe nanodevices for biomedical applications.

In this context, *Hydra vulgaris* (Cnidaria, Hydrozoa) was used as preliminary *in vivo* model to evaluate diatomite NPs toxicity.

Hydra is a simple multicellular organism at the base of the metazoan evolution. It consists of a tube which is made of two connected epithelial cell layers: the outer ectoderm and inner endoderm, separated by an acellular mesoglea layer (**Figure 13**) [84]. As shown in **Figure 13**, at the top end of the tube, there is the hypostome composed by a mouth surrounded by 6–8 tentacles that contain the most part of stinging cells (cnidocytes or nematocytes) that let *Hydra* to catch its prey [85]. Hydra column has four distinctive sections: the gastric region located between the tentacles and the first (apical) bud; the budding section, which produces the buds; the peduncle, which is located between the lowest bud and basal disc; and the basal disc, which is the foot-like formation [86]. This structural complexity, simpler than vertebrates with central nervous system and specialized organs, but more complex than cultured cells, makes *Hydra* comparable to a living tissue whose cells and distant regions are physiologically connected. It possesses a simple nervous system consisting of a diffuse nerve net throughout the body [87]. *Hydra* typically reproduces asexually resulting in the rapid production of a large number of new organisms that can be cultured in a short period of time. *Hydra* is sensitive to a range of pollutants and has been used as a biological indicator of water pollution [88]. Metal pollutants such as copper, cadmium, and zinc have been tested against different Hydra species, and the relative toxicity based on the median lethal concentration (LC50) for all species was ranked from copper, the most toxic, to cadmium with zinc, the least toxic [88].

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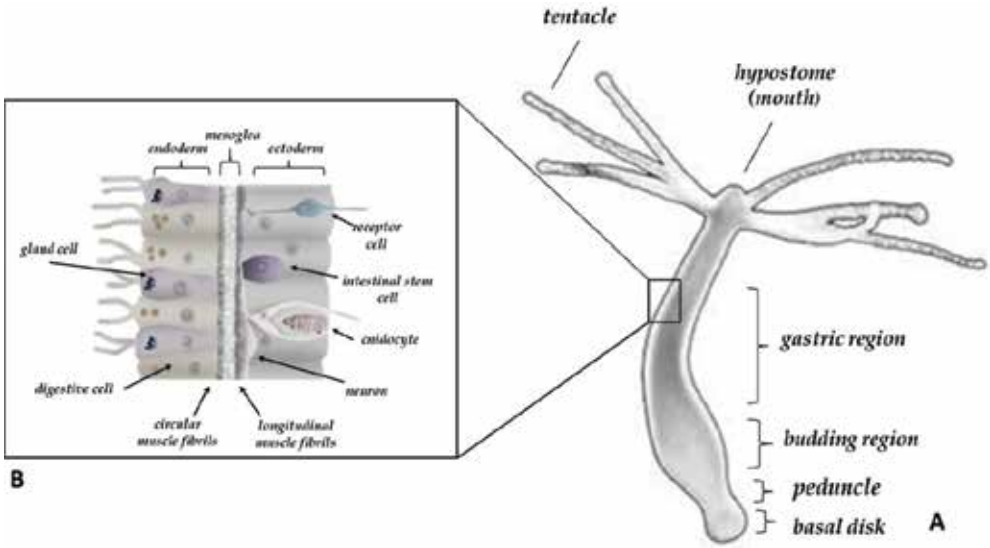

Figure 13. Anatomical structure of *Hydra vulgaris*. The inset shows the bilayer structure characterizing the whole body, from the foot to the tentacles, that is, the ectoderm and endoderm layers separated by the mesoglea. The few specialized cell types differentiated by the interstitial stem cells are shown (neurons, cnidocyte, gland cells).

Hydra has also been used as alternative *in vivo* model, to study the toxicity of different NPs, as well as their uptake and fate [89–91]. Due to a simple tubular body and being diploblastic, an even exposure of the whole animal to NPs by simple soaking is allowed [92, 93]. Several bioassays are available to assess the toxicity of a given substance in terms of acute or sub-lethal toxicity [94]. *Hydra* exposure to different substances may cause alteration of (1) morphological traits and developmental programs, (2) regeneration or pattern formation, and (3) population growth rates.

Toxicity of a substance is conventionally measured in *Hydra* observing changes in the animal morphology following Wilby's classification ranging from score 10 (normal, elongated tentacles and body), 8 (clubbed or bulged tentacles), 6 (shortened tentacles), 5 (tulip phase), 2 (loss of osmoregulation), to 0 (disintegrated) [95]. Scores 10–6 are reversible, sub-lethal indicators, while the tulip phase (score 5 and below) is considered irreversible and used as the endpoint for lethality [92, 95].

Toxicity of diatomite NPs in *Hydra* was investigated monitoring changes in its morphology after exposure to DNPs at increasing incubation times (24, 48 and 72 h) and concentrations (0.5, 1, 1.5, 2.5, 3, 3.5, and 4 mg/mL). *Hydra vulgaris* was asexually cultured in physiological solution by the method of Loomis and Lenhoff with minor modifications [96]. The animals were kept at 18°C and fed three times per week with freshly hatched *Artemia salina* nauplii. All animals were starved 24 h prior to the experiments. For each DNPs concentration, twenty polyps were used [97]. Either control or treated animals were placed into plastic multiwells refreshing the medium every 24 h. The morphophysiological effects of DNPs on *Hydra* were recorded by microscopic examination of each polyp and used to extrapolate the Wilby's score

key. A representative *in vivo* image of *Hydra* after exposure to DNPs is reported in **Figure 14**. Any change in polyp morphology was not observed after exposure to DNP concentrations up 4 mg/mL for 72 h; this result corresponds to score 10 of Wilby's classification.

Figure 14. *In vivo* imaging of Hydra polyps treated with DNPs. Polyp morphology is not affected by the nanoparticle treatment. Scale bar: 1 mm.

These data confirm *in vitro* toxicity results. The transparency of *Hydra* epithelium makes it possible to track and localize fluorescent nanoparticles in the animal body [92–94]. The internalization of DNPs in *Hydra* was evaluated by *in vivo* fluorescence microscopy analysis, after labeling DNPs by Alexa Fluor-488® (DNPs*). Ten living *Hydra* were treated with DNPs* (3.5 mg/mL) up to 72 h. Bright-field and fluorescence images of *Hydra* polyps treated with DNPs* for 24 (C, D) and 72 h (E, F) are reported in **Figure 15**; **Figure 15A, B** shows an untreated animal as control. In all figures, the foot is on the lower part of the panel, while a crown of tentacles surrounds the mouth. The image taken after 24 h (D) shows an intense fluorescence of DNPs, distributed in the whole body and confined to the outer ectoderm. After 72 h (F) of incubation, the DNPs were mainly localized in the inner endodermal cells, due to inter-epithelial migration of free or cell-containing nanoparticles between the two cell layers [92]. These preliminary *in vivo* results are in agreement with the *in vitro* data, confirming that DNPs could be used as safe and biocompatible nanocarrier for long incubation times and up to high concentrations.

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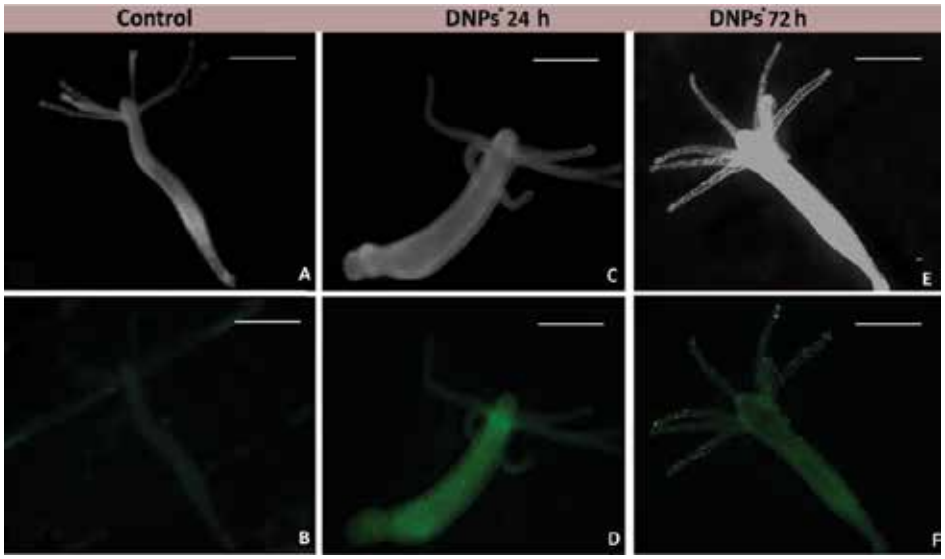

Figure 15. *In vivo* fluorescence imaging of Hydra polyps treated with labeled DNPs (3.5 mg/mL) for different incubation times. Scale bars: 1 mm. The fluorescence pattern appears as rather uniform after 24 h of incubation, while at 72 h it appears as granulates covering most of body regions. They might represent storage vacuoles, as it has been shown for other fluorescent nanoparticles [92, 94].

6. Conclusions and future trends

Diatomite is a fossil material of sedimentary origin formed over centuries by siliceous skeleton of aquatic unicellular microalgae diatoms, with similar physico-chemical properties of man-made fabricated PSi. Due to its ordered pore structure, high surface area, tailorable surface chemistry, high permeability, biocompatibility, non-toxicity, low cost, optical, and photonic properties, diatomite has been exploited as an innovative platform in several biotechnological applications, resulting as a viable and promising cheap alternative to synthetic porous silica. In this chapter, the potentialities of DNPs, with an average size of about 350 nm, as drug nanocarriers were discussed. Preliminary tests of cytotoxicity and cellular uptake demonstrated the biocompatibility of the DNPs and their capability to penetrate inside cancer cells. Different functionalization procedures of diatomite surface for preparation of bioengineered nanovectors were also described. These results, compared with those reported in literature on standard systems, encourage the use of diatomite-based materials as new class of nanostructured drug carriers.

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Chapter 10

Challenges and Opportunities in the Present Era of Marine Algal Applications

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Additional information is available at the end of the chapter

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Abstract

Marine algae are of high importance in their natural habitats and even more now in the world of green technology. The sprouting interest of the scientific community and industries in these organisms is driven by the fast-growing world of modern biotechnology. Genomics, transcriptomics, proteomics, metabolomics and their integration collectively termed here as 'marine algal-omics' have broadened the research horizon in view of enhancing human's life by addressing environmental problems and encouraging novelty in the field of pharmaceuticals among so many more. Their use in the human society dates back to 500 B. C. in China and later across the globe; they are still being used for similar purposes and more today. There is a hiking interest in marine algae and their derivatives—from phycoremediation, food supplements, pharmaceuticals to dyes. Marine algae are currently considered as an emerging panacea for the society. They are being studied in a multitude of arenas. The multi-use of marine algae is enticing and promises to be a boon for industrial applications. Yet, most marine algae face challenges that might variably constrain their commercialisation. This chapter gives an overview of marine algae including all the 'omics' technologies involved in studying marine algae and it explores their multitude applications. It also draws the various successful industries budded around them and presents some of the challenges and opportunities along with future directions.

Keywords: applications, challenges, marine algae, 'marine algal-omics', opportunities

1. Introduction

Algae can be generally categorised into two large sub-groups, namely, microalgae (microscopic) and macroalgae (macroscopic). Although both groups have common traits such as the ability to carry out photosynthesis, they differ in various ways from their size to their phylogeny. Yet, they are of common interest to scientists and industries around the globe as part of biotechnological development and the exploitation of their metabolites of high economic values. Their use as food dates back to 500 B.C. in China and the ninth century A.D. in Chad [1]. Eventually, the purpose of their exploitation has expanded to other avenues. Since the nineteenth century or so, several marine macroalgae have been used as a natural fertiliser in several countries, for instance, *Ascophyllum* spp. in Scotland and *Sargassum* spp. in the Philippines among others [2]. In the late twentieth century, around 1980, the natural blue dye phycocyanin from the microalga *Spirulina* sp. was mainly used as a colourant for ice creams and cosmetics [3]. Over the years, several industries have experienced a spike in the use of marine algae owing to the 'omics' technologies [4]. Akin to other organisms and cellular omics studies, omics studies of marine algae—termed 'marine algal-omics' in this chapter—essentially include genomics, transcriptomics, proteomics, metabolomics and system biology tagged along with miscellaneous ones (e.g., fluxomics) which furthers the understanding of marine algae as a whole system [4].

At the very beginning of the twenty-first century, genomics marked the dawn of a different epoch of biological research providing a blueprint for genetic engineering for the optimisation of productivity of marine algae, reassembling the puzzle of evolution and the discovery of genes of interest coding for biological compounds of high significance. For instance, Stephenson et al. [5] provided an insight of several genes that have the potential to improve the solar conversion efficiency in mass culture of marine algae for biofuel production. Although genomics provide a static view of the capacity of a marine algal cell, the integration of transcriptomics, proteomics, metabolomics and system biology allows the study of its gene expression in response to environmental stresses [6–8], evolution among others. Examples range from the upregulation of gene coding for proteins of several pathways of the microalga *Ostreococcus tauri* in response to the effect of nitrogen deprivation and chemical defence activation in the macroalga *Caulerpa taxifolia* to cell damage [9] to the phylogenetic relationships of bacterial and the macroalgal *Laminaria digitata* enzymes—mannuronan C-5-epimerases in the alginate biosynthesis [10]. The advent of the 'omics' technologies has allowed for the discovery of a consortium of novel molecules that are guiding the nascent industries such as pharmaceuticals, biodiesel and papermaking to promote instances of international interest under the blue economy umbrella.

The Economist Intelligence Unit Limited [11] acknowledges the emergence of several ocean industries including marine biotechnology, e.g., the commercial production of β-carotene from the microalga *Dunaliella salina* [12]. Alongside the sustainability aspect of the industries, the blue annotation to the ocean economy is now gaining momentum worldwide. However, further research is needed to test the potential of the different aspects of this sector including the marine algae component. In such a quest, there are multiple challenges and opportunities

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that need to be addressed. The marine algal large-scale production and its pounding monetary implications of the cost of production represent the major challenges to date [13]. However, as per literature, marine macroalgae are presently more appealing for industrial applications as they are readily accessible, easy to harvest compared to microalgae [2] have lower cost of production [14] and biosynthesise commercially important molecules such as the phycocolloids while remediating environmental stresses [15]. On the other hand, marine microalgae are also stirring interest in other fields such as biodiesel production that could cut several costs if symbiotic microalgae such as *Symbiodinium* spp. are used [16]. The choice of the most appropriate marine algal strain is an option which could be coupled with genetic engineering and commercialisation of by-products to enhance the economic value of marine algal production processes [17].

This chapter describes the taxonomic classification of marine macroalgae and microalgae as well as gives a brief description of their characteristics. It also discusses the phycoremediation technology using marine algae and the study of the metabolites of interest using the 'omics' technologies for their subsequent commercial/industrial applications. In addition, it points out the main challenges that their associated industries are facing and discusses future directions in both the research and commercialisation/industrialisation arenas.

2. Description of marine algae: morphological and genetic characterisation

More than 71% of the world's surface is covered by oceans that serve as habitats to a diversity of marine organisms including marine algal species [18, 19]. Ecologically, marine algae are at the base of most aquatic food chains and are important in biogeochemical cycling and, in addition, serve as habitats for many organisms in aquatic ecosystems [20–22]. Marine algae can be prokaryotic or eukaryotic. They usually inhabit shallow waters and belong to two main sub-groups (**Figure 1**), namely, macroalgae (also commonly known as seaweeds) and microalgae. The macroalgae are macroscopic and consist of three main groups: Chlorophyta (green macroalgae), Rhodophyta (red macroalgae) and Phaeophyta (brown macroalgae) [25]. They are thallophytes (non-vascular plants). They constitute of leaf-like structures known as blades, stem-like structures known as stipe and root-like structures such as rhizoids and holdfasts. In contrast, microalgae are microscopic and are grouped as follows: Cyanophyta (blue–green algae), Pyrrophyta (dinoflagellates), Chrysophyta (diatoms and golden-brown algae) and Chlorophyta (green algae) [23, 24, 26]. Marine algae are taxonomically classified using diverse methods including analyses of their morphological key features and molecular characteristics —pigments (phycocyanin, phycobilins, β-carotene and chlorophyll) [27], genetic molecules, fatty acids distribution, secondary metabolites distribution [28, 29], and diffraction, light scatter and fluorescence parameters (through flow cytometry)[30]. The choice of use or the extent of the use of the different methods is dependent upon the level of difficulty in identifying a particular species.

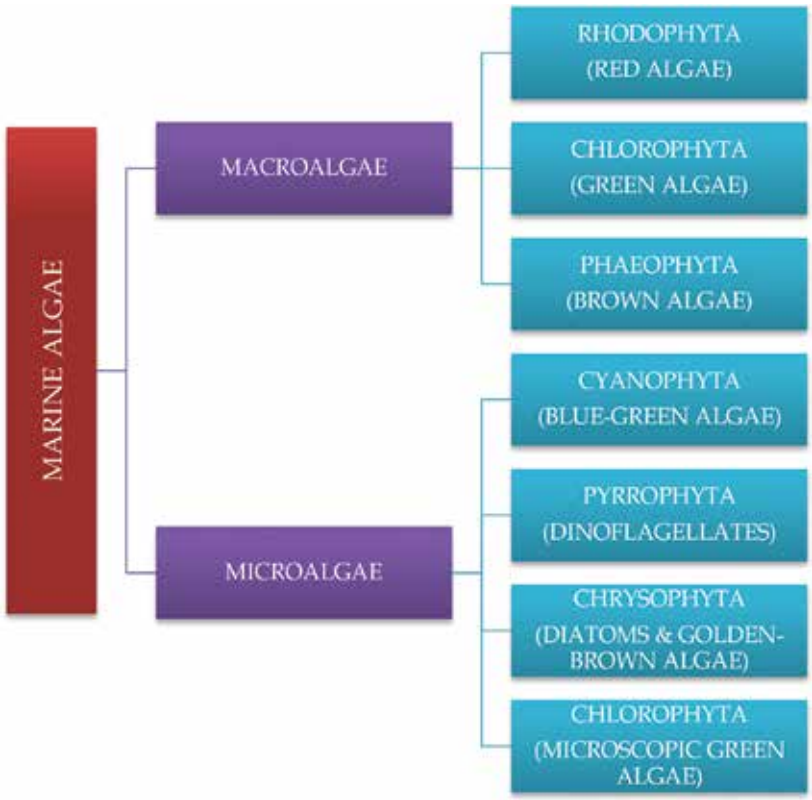

Figure 1. Classification of marine algae—adapted from Richmond and Radesandratana [23] & Oliveira et al. [24].

3. Applying biotechnology tools to explore the hidden properties of marine algae

The 'omics' technologies have revamped biological research and have led the twenty-first century into the post-genomic era that goes beyond the static state of genomics. Organisms being subjected to such high-throughput technologies include marine algae and hence the expression: 'marine algal-omics.' Analogous to other organisms and cellular omics studies, 'marine algal-omics' include essentially genomics, transcriptomics, proteomics, metabolomics and system biology tagged along with miscellaneous ones (e.g., fluxomics) which deepen our understanding of the respective organisms [4]. In this chapter, we use the expression 'marine algal-omics' to illustrate the advanced technologies involved in the study of marine algae.

Marine algae have built up their profiles exponentially in the omics world: their significance as primary producers of the blue planet, their impacts on global productivity as well as biogeochemical cycling are acknowledged [31]. 'Marine algal-omics' provide better understanding of biological system as well as commercially important molecules for marine

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macroalgae and microalgae alike [1, 32–34]. Hitherto, the marine algae had to satisfy a set of criteria before being considered as potential candidates for such endeavours [31], but with the advent of meta-omics, the possibilities now seem endless.

3.1. Genomics

Genomics, the first of the 'omics' technologies, defines an organism's native biosynthetic and metabolic capacities as a potential microbial cell factory, and provides a blueprint for engineering and optimising productivity. Although during the early genomics era studies were focused mainly on bacterial and mammalian organisms for biomedical applications and subsequent improvement to the human health sector, genomics has been a key technology involving several steps (**Figure 2**) in unlocking the biocatalytic potential of marine algae.

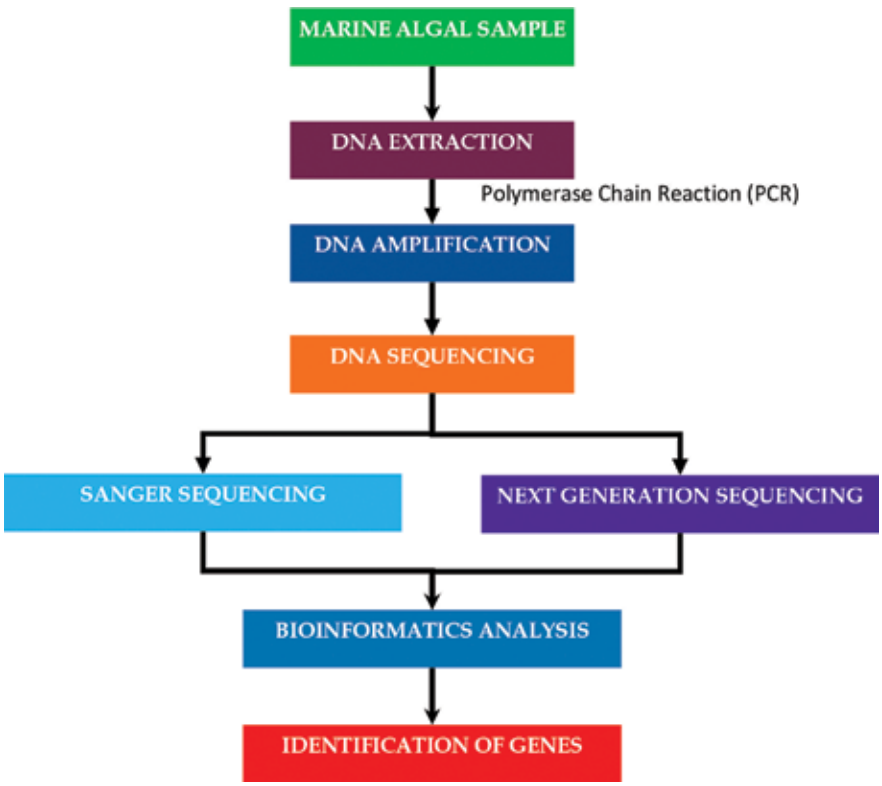

Figure 2. Methodology for genomics analysis—adaptation from Li et al. [35].

The advent of the next-generation sequencing technologies such as sequencing by litigation, pyrosequencing and real-time sequencing has revolutionised the field of genomics [36]. It has helped in the rapid, reliable and accurate sequencing of a number of marine algal species at a comparative cost [31, 37]. Successively, after more than a decade following the human genome project, scientists around the world have sequenced many different organisms including

marine algae [4]. About 8000 organisms' genomes from different kingdoms have been completely sequenced and published with thousands more in the pipeline [Genome OnLine Database (GOLD)]. The first marine alga to be sequenced is *Guillardia theta* that was followed by other marine algae including *Thalassiosira pseudonana, Phaeodactylum tricornutum, Aureococcus anophagefferens, Emiliania huxleyi, Ostreococcus tauri, O. lucimarinus, Micromonas pusilla* strain NOUM17 and *M. pusilla* strain CCMP1545 [37].

Genome sequencing provides a sequence of nucleotides which should be assembled and analysed for gene annotation. It indicates genes encoding proteins and functional RNAs available to the cell along with their associated regulatory elements [38]. Gene sequencing of the microalga *O. tauri* has exposed its very condensed genomes boiling down to a very low number of non-functional genes and allowed the identification of 8166 protein-coding genes in the nuclear genome. The same study also provided an understanding of the metabolic pathways of the pigment biosynthesis and photosynthesis. The absence of the gene coding for the light-harvesting complex proteins associated with photosystem II depicted the difference between *O. tauri* and terrestrial plants as well as other algae [39]. Grossman [31] discussed the importance of the genomic studies of the microalgae (diatoms) *T. pseudonana* and *P. tricornutum* with respect to its biology and nanotechnology related to the biogenesis and organisation of the highly patterned silicified diatom cell wall. The analysis of the genome of *T. pseudonana* unravelled the presence of five genes encoding the silaffin polypeptides most likely associated with the cell wall of the diatom. Stephenson et al. [5] summarised several genes that could be overexpressed or modified in targeted marine algae to improve their solar conversion efficiency in mass culture for biofuel production—some targeted genes are the *psa*A, *psa*B, *psb*A, sedoheptulose-1-7-bisphosphatase (SBP) and transketolase (TK).

Evolution study is another prime element of genomics providing a description of the phylogenetic relationship of all organisms on Earth. The comparative genomics analysis of the genome sequence of the filamentous brown marine macroalga *Ectocarpus siliculosus* and others has allowed the understanding of evolutionary hypothesis of the carbon storage and cell wall biosynthesis metabolic processes [32, 40, 41]. Furthermore, DNA barcoding is one of the most important aspects of taxonomic investigation and the economics of the marine algae because it allows the identification of strains of interest for the study of evolution as well as commercial/industrial applications [42, 43]. DNA barcoding uses molecular markers for characterisation of the marine algae of interest. Other technologies include DNA–DNA hydridisation, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) among others [42]. Examples include the application of AFLP technique to 16 strains of *Alexandrium tamarense* in a phylogenetic analysis [44]; RFLP analysis of 18S ribosomal RNA for identification of five species of marine *Dunaliella* resulting in identification of three species, namely, *D. salina, D. parva* and *D. bardawil* [45]; and RAPD technique for the phylogenetic analysis of the marine maroalga *Porphyra* spp., *Ulva, Sargassum, Ceramium, Hizikia, Chara* and *Gracilaria* [46].

In addition, marine algal genomics encourages the understanding of algae allowing them to serve as model organisms [32]. Subsequently, studies have used several marine algae as model

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organisms such as the microalga *O. tauri* and the macroalga *E. siliculosus* to study cellular and developmental processes, respectively [32, 47, 48].

3.2. Transcriptomics

The term 'transcriptomics' refers to the study of transcriptome, the whole set of transcribed RNAs, at a certain period of development as well as under a specific biological condition. Transcriptomics gives insights into genome expression that lends a view on gene structure, gene expression regulation, gene product function and the dynamics of the genome. Over the years techniques used for transcriptome analysis have evolved from the initial expression sequencing tag (EST) strategy to gene chips, and now the RNA-seq and bioinformatics analysis (**Figure 3**) [50]. Dong and Chen [50] and Morozova et al. [51] provide an in-depth review of transcriptomics techniques.

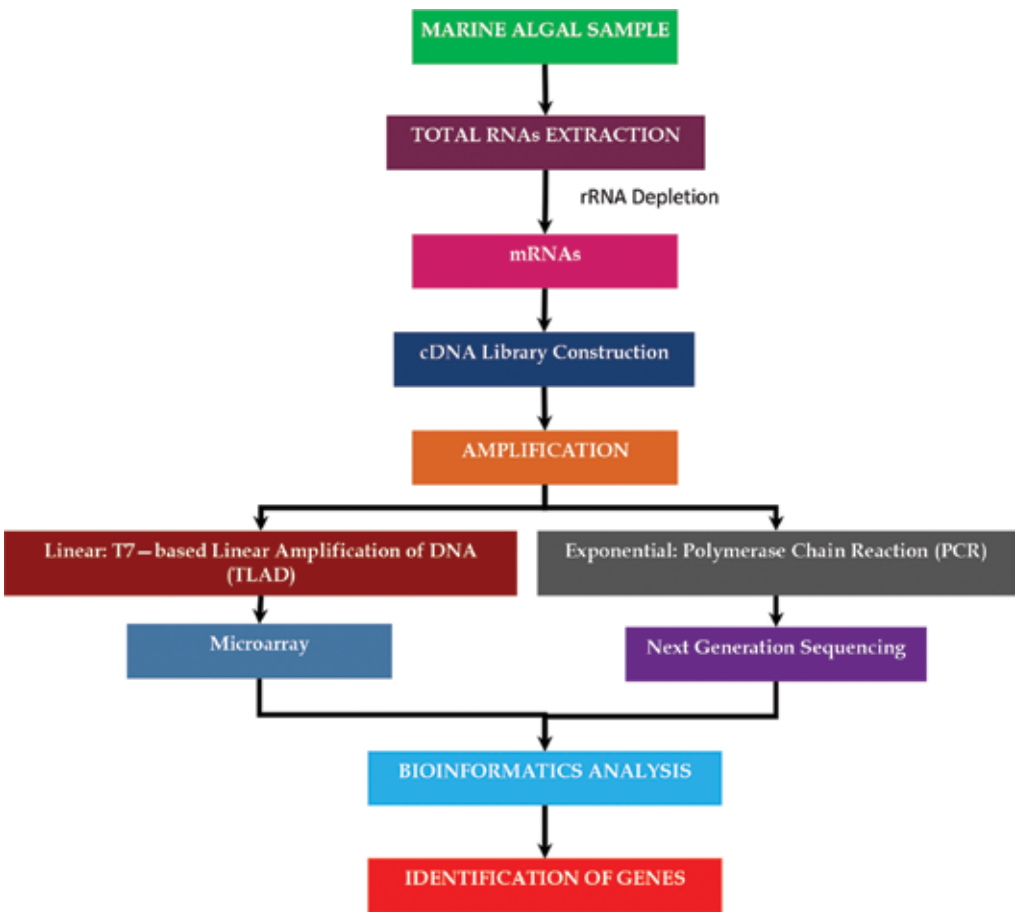

Figure 3. Methodology for transcriptomics analysis—adaptation from Tang et al. [49].

The integration of transcriptomic studies in 'marine algal-omics' assists the elucidation of gene expression in response to environmental stresses [6, 7], evolution [52], biochemical pathways [53] and the characterisation of genes of those biochemical pathways [10]. The relevance of transcriptomics exemplified by the studies herein mentioned ranges from the understanding of overexpression of respective enzymes in a particular biochemical pathway for pertinent applications to that of carbon capture.

The ability of marine algae to produce secondary metabolites in response to an environmental change is well understood. Radical changes are observed at the first gene expression level (i.e., transcription level). Transcriptomics studies on the response to environmental variability include *E. siliculosus*' response to saline environment as well as heavy metal concentrations [6, 54] and *Saccharina japonica*'s (macroalga) response to blue light induction [55] along with the response of microalgae *E. huxleyi* to nitrogen and phosphorus starvation [7] and *P. tricornutum* to iron starvation [56].

E. siliculosus subjected to hyposaline, hypersaline and oxidative stress conditions study revealed consequential alterations at transcription level which further impacted translation, amino acid metabolism, protein turnover, photosynthesis, and protein and nutrient recycling. Many unprecedented reactions such as the upregulation of many unknown genes along with a number of gene coding for chlorophyll *a* and *c* binding proteins were also observed [6]. In response to high copper contaminations, *E. siliculosus* exhibited several changes at transcriptomic level [54]. Ritter et al. [54] reported novel genes specific to brown macroalgae along with the downregulation of gene coding for enzymes related to nitrogen assimilation but an increase in free fatty acids content. A comparative analysis of the gene expression of *S. japonica* under blue light induction revealed a transcriptome reprogramming that resulted in the upregulation of 7808 and the downregulation of 3852 unigenes. The study also provided important points of information for other functional genes identification in kelp [55]. On the other hand, the long serial analysis of gene expression of the marine coccolithophore *E. huxleyi* suggests a strong transcriptomic response to both nitrogen and phosphorus starvation with an upregulation of the respective metabolism [7]. The transcriptomic analysis of *P. tricornutum* under iron starvation indicated the downregulation of photosynthesis, mitochondrial electron transport and nitrate assimilation [56].

Furthermore, the transcriptome analysis of marine macroalgae of economic importance in China covering two groups Rhodophyta and Phaeophyta—3 classes, 11 orders and 19 families —helped to decipher the proteins involved in the ability of these macroalgae to cope with extreme environmental variabilities [57]. The study reported three types of phycobiliproteins in *Gracilaria* spp. and all studied red algal species. Moreover, the study also helped to annotate the whole set of macroalgal C4-pathway genes including genes encoding pyruvate kinase, phosphoenolpyruvate carboxylase and others [57]. Nyvall et al. [10] carried out the first characterisation of a gene involved in the synthesis of alginate in the brown algae,

L. digitata, by means of northern-blot analysis and reverse transcriptase-polymerase chain reaction. The same study established the phylogenetic relationships of the bacterial and brown algal enzymes—mannuronan C-5-epimerases in the alginate biosynthesis.

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3.3. Proteomics

In a broad sense, the term 'proteomics' can be defined as the study of proteins coupled with transcriptomics and genomics for they are complementary [58]. A cell's Proteome – the whole cell protein – is dynamic: proteins extracted and studied at a particular point in time and under certain physiological condition(s) represent the cell's immediate response to its environment, transcriptome alike. While there is a fine line of distinction between the classical and contemporary proteomics, the aim remains the global study of a cell's proteome—protein–protein interaction, protein modifications, protein function and location of proteins among others. Proteomics involves different separation techniques to multiple analyses and different identification tools (**Figure 4**). Graves and Haystead [58] provide an in-depth review of proteomics techniques.

Figure 4. Methodology for proteomics—modified from Graves and Haystead [58].

For the past decade or so, several novel marine algal proteins have been identified by two-dimensional electrophoresis (DE) and mass spectrometry (MS) including proteins from the macroalga *Gracilaria changii* [59], and the microalgae *Dunaliella bardawil* [60] and *Nannochloropsis oculata* [61]. Nevertheless, proteomics does not end with the identification of a particular protein, it also helps to uncover the underlying function of the latter including its role in evolution as well as taxonomic studies and biochemical pathways [8, 61, 62].

Proteomic studies on algae are still relatively limited compared to higher plants. So far, it is the freshwater Microalga – *Chlamydomonas reinhardtii* — that has been thoroughly studied from various omics-angle. *O. tauri* is now being considered as a research model [63].

O. tauri being particularly small with a compact genome of around 13 Mbp makes it a perfect candidate to be used as a model organism to better understand the multiple metabolic pathways involved in a marine microalgae. Nonetheless, there are other marine algae that have been studied for several biochemical pathways such as the urea cycle [62], glycolysis [8] and Calvin-Benson cycle [64] among others.

The shotgun proteomics technique is being profusely applied to the study of marine algal proteome. The first shotgun proteomics analysis of *T. pseudonana* revealed 1928 proteins expressed under optimal growth conditions—out of which 70% were found to be involved in cellular metabolism and 11% in transport of molecules while all the participatory proteins in the urea cycle were unveiled [62].

Le Bihan et al. [8] investigated the effect of nitrogen deprivation on the biosynthetic pathways of *O. tauri* which led to the upregulation of proteins of several pathways including that of glycolysis, carbon storage and phosphate transport while proteins related to nitrogen assimilation in its plastid experienced downregulation. Liska et al. [64] examined the effect of high salinity concentration on the photosynthetic pathway of the halotolerant organism *D. salina* using nanoelectrospray MS combined with MS BLAST and MultiTag. The study revealed the upregulation of gene coding for several enzymes including plasma membrane carbonic anhydrases, Rubisco and other fundamental enzymes of the Calvin-Benson cycle, enzymes for adenosine triphosphate (ATP) and redox energy, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and enzymes in amino acids biosynthesis [64]. High copper concentrations also affect the metabolism of marine algae. Although copper is an essential micronutrient to macroalgae, it can be toxic at high concentrations. Nonetheless, it has been reported that the brown macroalgae *Scytosiphon* spp. are tolerant to high concentration of copper leading to bioaccumulation. In order to decipher this particular genus' tolerance, a proteomic analysis was carried out by Contreras et al. [65] on *S. gracilis*. Twenty-nine proteins were identified including 19 overexpressed and hypothetically involved in copper tolerance.

It is noteworthy that marine macroalgal proteomic studies are relatively uncommon and studies of those under stress are further limited but there are some whose expressed proteome in response to an environmental stress has been explored and these include *Pyropia orbicularis* and *S. gracilis*. López-Cristoffanini et al. [66] probed into the proteome of *P. orbicularis*, a desiccation-tolerant red macroalga, by means of 2-DE and liquid chromatography (LC)–MS/MS analyses. A decline in photosynthetic activity but an increase in the antioxidant activity

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by the upregulation of gene coding for phycobiliproteins and production of proteins such as superoxide dismutase were observed. On the other hand, *S. gracilis* in response to high copper concentration using peptide *de novo* sequencing revealed overexpression of proteins including cytosolic phosphomannomutase and glyceraldehyde-3-phosphate, a chloroplast peroxiredoxin among others [65].

Additionally, proteomic studies are also being carried out to investigate the biosynthesis mechanism of harmful marine algae particularly in relation to human health and safety [67]. Saxitoxin, for instance, is associated with paralytic shellfish poisoning. For a long time, little was known of the biosynthetic pathway of saxitoxin synthesis but studies are now revealing the enzymes implicated [68]. Marine algal toxins are being considered for potential commercial applications such as the use of saxitoxin and tetrodotoxin as an anaesthetic [69] and insights of their production would be an advantage for the industry.

3.4. Metabolomics

Owing to the relationship between genes and proteins, proteomics became the main focus of the post-genomic era. However, being of subordinate relevance to the evaluation of phenotypic responses of organisms, proteomics gradually took the backseat while metabolomics was brought in the limelight [70]. Akin to the definition of the other 'omics' terms, 'metabolomics' refers to the study of the metabolome that is the complete set of metabolites of an organism. The metabolites of an organism can be categorised as primary (fundamental for cell develop-

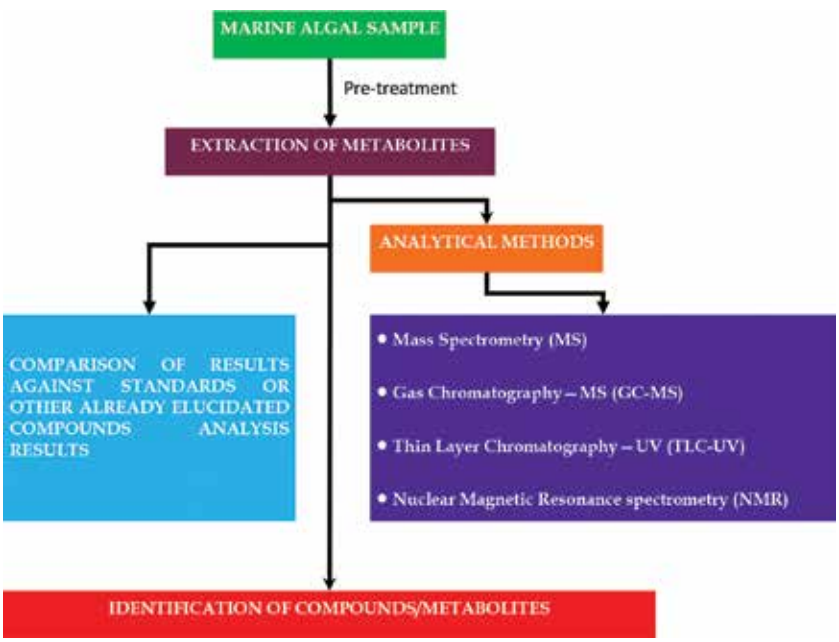

Figure 5. Methodology for metabolomics—adaptation from Verpoorte et al. [70].

ment and are continually produced, e.g., amino acids and polysaccharides) or secondary (produced in response to a stimulus such as an environmental distress, e.g., sterols). Secondary metabolites of marine algae are of major interest but not all can be expressed at all times. Their synthesis must be triggered by a stimulus—mostly physiological and/or environmental. Verpoorte et al. [70] advocated that the prime goal of metabolomics is the qualitative and quantitative analysis of all the metabolites present in an organism. They mentioned five major approaches including high-performance liquid chromatography/thin layer chromatography–ultraviolet (HPLC/TLC–UV), gas chromatography–MS (GC–MS), LC–MS, MS and nuclear magnetic resonance (NMR) spectrometry. Roessner and Bowne [71] summarised metabolomics approaches as follows: target analysis, metabolite profiling, metabolomics and metabolic fingerprinting (**Figure 5**).

As metabolomics depicts the physiological states of any organism including marine algae, the research database of this omics surpasses that of proteomics as well as transcriptomics in the functional genomics arena. The exometabolome and endometabolome of both marine microalgae and macroalgae have been thoroughly studied for multiple purposes including ecology [72], physiological states [73] and applications in multiple sectors such as health [34, 74] and energy [16].

Barofsky et al. [72] explored the exometabolomes of the marine microalgae (diatoms) *Skeletonema marinoi* and *T. pseudonana* to understand the dynamic nature of their ecology and the function of the metabolites they exuded. It was observed that some of the metabolites exuded acted as info-chemicals for the microalgal ecology very much like quorum sensing by bacteria. Furthermore, Vidoudez and Pohnert [73] untangled the general patterns of the metabolism of *S. marinoi* during the different growth (exponential, stationary and declining phase) by analysing its metabolome. Sugar and amino acids metabolisms were reported to be the highest in the exponential phase and accumulating in the night, whereas glucose and glutamate exhibited other different characteristics. The declining phase was characterised by the catabolism-related metabolites and the increase in terpenes as well as putrescine. The studies indicate that investigations dealing with both ecological and physiological aspects of diatoms need to consider the dynamic changing nature of their metabolism.

Barre et al. [75] depicted several analytical methods used to study the marine macroalgal metabolites including coupling MS with GC, coupling MS with HPLC and NMR spectroscopy. Several applications of those analytical methods for the understanding of species like the macroalga *Delisea pulchra* whose level of compounds have been quantified using GC–MS along with the characterisation of the variation in levels of furanones were reviewed. On the other hand, LC/APCI–MS provided opportunities to study the response of the macroalga *C. taxifolia* to cell damage revealing subsequent chemical defence activation [9]. NMR has also been used in metabolic profiling of macroalgae such as *L. digitata* and *Gracilaria conferta* among others [9, 75].

The understanding of metabolisms (e.g., fatty acids) and identification of respective metabolites go beyond the mere comprehension of the marine algal world. Its application as a panacea for the benefits of the society is what is mostly driving the marine algal metabolomics. A concrete example is the quest for cancer remedy. Cancer is the gangrening scourge of the

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modern world and is on the forefront in the research arenas of several organisms including marine algae. Studies on the red macroalga *Callophycus serratus* by Lin et al. [34] and Kubanek et al. [74] provide ounces of hope for such festering health problems. The metabolites of *C. serratus* were explored by 1-dimensional (1D) and 2D NMR along with mass spectroscopic analyses resulting in the identification of four bromophycolides. The bromophycolides presented cytotoxicity towards selected human cancer cell lines. It is noteworthy that one of the bromophycolides exhibited submicromolar activity against human malaria parasite *Plasmodium falciparum* [34]. The *C. serratus* also revealed a bromophycolide that presents cytotoxicity against several human tumour cell lines via specific apoptotic cell death [74]. The plausible applications of marine algal metabolites are ever expanding. Gupta et al. [76] carried out NMR-based metabolome analysis on marine macroalgae—*Ulva lactuca, Gracilaria dura* and *Sargassum tenerrimum*. The metabolic level regulations of choline containing lipids of marine macroalgae, presence of acetate and lactate for all the macroalgal species indicating the existence of fermentative regulatory switching and identification of non-proteinogenic cysteine-oxoforms such as hypotaurine in *U. lactuca*, isethionic acid in *G. dura* and cysteine-sulfinic acid in *S. tenerrimum* were noted. The hypotaurine looks very promising for non-communicable diseases and can be an anti-hypertensive and a hypocholesterolemic agent. It is noteworthy that a limited number of studies have been carried out on the exometabolomes for pertinent application such as therapeutic ones. Kumari et al. [77] investigated the quantitative profiles of targeted hydroxy-oxylipins from 40 macroalgal taxa. The oxylipins extracted from the medium were analysed using HPLC. Even if the study indicated low hydroxyl-oxylipin content, the compound being similar to those of mammalian oxylipins could serve as substitute for the treatment of inflammatory diseases, cancer and atherosclerosis among others.

The metabolome composition is affected by mutations and endogenous as well as exogenous stimuli. There are some metabolites that can even induce perturbations at the transcriptional level and, consequently, modify the proteins' activities. Goulitquer et al. [78] reported on a broad spectrum identification of several metabolites using GC–MS of marine microalgae including *Cocconeis scutellum* and *S. marinoi* as well as targeted studies on diatom metabolism such as fatty acids profiling of *Cylindrotheca closterium* and *Seminavis robusta*.

As is the case with proteomics, the nature and concentration of metabolites vary with respect to environmental stress conditions. An inexhaustive list of such metabolomic studies includes defence response of the macroalga *Gracilaria vermiculophylla* [79] and others. Chen et al. [80], Lee et al. [81], Ye et al. [82] and Satoh et al. [83] provide for more details on metabolomics.

3.5. System biology

System biology is a multidisciplinary field of study with the integration of the 'omics' promoting the holistic approach and condemning the reductionist one [38]. The integration of the 'omics' results is imperative for the appreciation of an organism at the system level. The aim of system biology is to establish a profound understanding of the behaviour of and the interaction between the individual components of the organisms [4]. The basic principle of

system biology is modelling, which unveils the dynamics of the organisms. To date, studies on marine macroalgal and microalgal system biology are very limited.

E. siliculosus has been considered as a system biology model detailing its acclimatisation to environmental variability [84]. One of the issues highlighted in the study was the importance of non-coding RNAs in the regulation of the abiotic stress response mechanisms. Furthermore, proteomics and transcriptomics complement each other as do metabolomics and transcriptomics. Allen et al. [56] described the amalgamation of non-targeted metabolomics and transcriptomic analyses to explore the biochemical pathways of *P. tricornutum* under iron starvation. The study revealed several issues including the downregulation of photosynthesis, mitochondrial electron transport and nitrate intake, but there was a compensation of nitrogen and carbon from protein and carbohydrate breakdown, and adaptations to the biosynthesis of chlorophyll and the metabolism of pigment.

System biology is unravelling the novelty of metabolic capabilities and potential bioproducts of marine algae. Genomics, transcriptomics, proteomics and metabolomics are leading to the discovery of innumerable novel molecules, which are proving to be crucial resources and assets in emerging industries such as nutraceuticals, biofuels, pharmaceuticals and cosmeceuticals based on marine algae.

In this section, we have attempted to address the majority of 'marine algal-omics' research that has been conducted to date. However, it is far from being an exhaustive review. 'Marine algal-omics' have the potential to further develop a whole range of relevant industrial products including commodity and specialty chemicals and enzymes, biopolymers and pigments as well as application to the examination of marine algal bioremediation.

4. Existing and potential applications of marine algae

The array of marine algae and their derivatives are gaining increasing recognition worldwide. In addition to the panoply of ecosystem services that marine microalgae and macroalgae provide, the extensive range of biotechnological exploitation and the subsequent industrial applications of these organisms as biological factories are thoroughly documented. Marine algae are being widely used (**Figure 6**)—both at the molecular and organismal levels—as food [85, 86] and nutraceuticals [87], animal and fish feed [86, 88], biofertiliser [89], bioplastics [90], pharmaceuticals [91], cosmeceuticals [91–93], fluorophores [94], food colourants and textile dyes [95, 96], and biofuels [16] as well as for phycoremediation [97–99] (**Table 1**).

4.1. Phycoremediation

Phycoremediation is the use of algae to destroy or biotransform pollutants to innocuous level [107]. Marine algae are being considered for their multiple advantages. Marine algae can remediate heavy metal contamination [97, 98, 108], contribute to wastewater treatment [99], lower the atmospheric carbon dioxide [14, 15] via photosynthesis [109] and produce biomass for industrial applications [15, 108].

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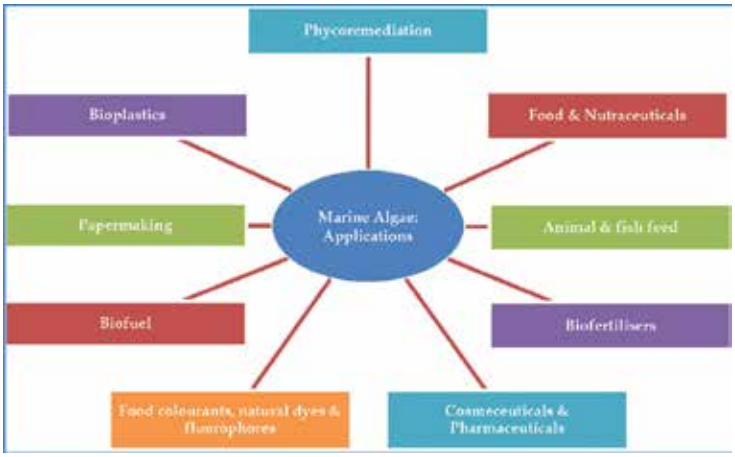

Figure 6. Applications of marine algae.

Carsky and Mbhele [108], Lawton et al. [110] and Imani et al. [98] investigated the potential of several marine algae to be considered for heavy metal contamination remediation. *Sargassum* spp. were found to have good copper biosorption capacity making it a good candidate for effluent treatment processes for copper pollution control purposes. On the other hand, six *Ulva* spp. found in Eastern Australia were studied for bioremediation of land-based aquaculture activities. *Ulva ohnoi* was found to be the most appropriate one as it had high growth rates, tolerance to extreme environment and good ability to use multiple sources of nitrogen [110]. Imani et al. [98] tested the tolerance of *Dunaliella* to mercury, cadmium and lead contamination revealing the ability of *Dunaliella* as a useful biological tool for the elimination of heavy metals in the environment.

Increasing atmospheric carbon dioxide concentration is another major environmental threat environmental threat affecting the balance of nature. Brennan and Owende [111] enumerated three sources of CO_2, namely, the atmosphere, industrial power plants and soluble carbonate. The desirable traits of microalgae, we assume which applies to algae in general, were considered to be the high growth rates, high metabolism of CO_2, high tolerance to SO_x and NO_x, biosynthesis of economically viable products, non-tedious harvesting methods, and tolerance to extreme environmental conditions.

Sydney et al. [112] reported that the freshwater microalga *Botryococcus braunii* had the highest CO_2 fixation rate than the marine *Spirulina platensis* and *Dunaliella tertiolecta*. Nonetheless, a high lipid accumulation was also noted in *D. tertiolecta*—a characteristic coveted in the biodiesel industry [16]. In addition, Moheimani et al. [113] stated that the coccolithophorid microalgae are of prime interest for CO_2 bioremediation as they form $CaCO_3$ scales along with photosynthetic carbon fixation. It was also noted that CO_2 is one of the photosynthetic rate limiting factor for marine phytoplankton. However, they cited studies which have shown that many of the phytoplankton species have evolved a CO_2 concentrating mechanism that allows them to use either CO_2 and/or HCO_3^- such as *E. huxleyi*. Chiu et al. [114] stated that *Chlorella*

sp. is a potential candidate for lipid and biomass productivity. The study also showed that *Chlorella* sp. underwent rapid growth in a high-density culture with CO_2.

Chung et al. [14] mentioned that over half a million tonnes of carbon is removed from the sea yearly within commercially harvested macroalgae (seaweeds). They stated that large-scale seaweed cultivation is attractive owing to their decades-proven, low-cost technologies and the panoply uses of their products. They provided an overview of the Korean Coastal CO_2 Removal Belt which promotes the removal of atmospheric CO_2 via marine forests—approximately 10 tonnes of CO_2 per hectare yearly for the brown macroalga *Ecklonia*.

Kaladharan et al. [15] investigated the carbon sequestration ability of the following marine algae: *G. corticata, S. polycystum, U. lactuca, N. salina* and *Isochrysis galbana*. It was found that *U. lactuca* is more efficient in carbon utilisation than its fellow counterparts. They advocated that large scale mariculture of commercially significant macroalgae can help mitigate the atmospheric CO_2 concentration and, subsequently, provide a consequent amount of biomass to be used as raw material for the phycocolloid industry.

The phycoremediation through marine algae is still at an infancy stage and it is unclear whether marine macroalgae or microalgae will monopolise this sector. So far, taking into consideration the literature cited, it is of opinion that marine macroalgae will be the first monopoly of this sector as it has been for the other sectors since the advent of marine algal industrialisation.

4.2. Food and nutraceuticals

The consumption of marine algae originates from countries such as Japan, China and the Republic of Korea [2, 89] where several genera of marine macroalgae, such as *Porphyra, Laminaria, Saccharina* and *Undaria*, have been used as staple food since prehistoric times [115]. The marine algal metabolites such as carotenoids, fatty acids, polysaccharides, minerals, vitamins and antioxidants have enabled synergistic benefits to humans via direct consumption [86]. The concept of 'nutraceutical' encompasses nutrition and pharmaceutical whereby food products comprise supplementary aforementioned chemical compounds that are beneficial to the human society, especially in the health sector [87, 116]. In quest for a healthy lifestyle, consumers are showing an ever-growing interest in products with health-enhancement capabilities [117]. Consequently, the demand for marine algae as food products and nutraceuticals has increased [2].

The high nutritional value and rich source of phycocolloids of several genera of marine macroalgae have been reported to be used as food. In this chapter, the term 'phycocolloid' refers to three commercially important high molecular weight polysaccharides, namely, alginate, carrageenan and agar. Sulphated galactans—agar and carrageenan—are extracted from Rhodophytes [118], whereas alginate (composed of mannuronic acid and guluronic acid [119]) is extracted from Phaeophytes. They are vastly used in the food industry as cost-effective gelling, viscosifying or thickening agents in ice creams and jellies [85]. The global value of these phycocolloids approximates to US$1 billion [13]. Other economically important phycocolloids extracted from macroalgae are ulvans and fucoidans [101].

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Marine microalgae have also been reported to contain proteins, carbohydrates and lipids in substantial amounts [85], which have been widely utilised as nutritional supplements and health food. *Dunaliella* spp. (Chlorophyceae) have been a rich source of β-carotene [120] that is a red–orange pigment acting as an antioxidant to protect against cancer [119, 121]. Chidambara et al. [122] reported on the significant restoration in antioxidant activities of enzymes such as catalase, superoxide dismutase and peroxidase in rats when treated with *D. salina* powder. Thereupon, *D. salina* has been widely exploited in the nutraceutical industry by companies such as Nature's Plus® and Carlson® for soft gelatine capsules of β-carotene for instance [117].

Polyunsaturated fatty acids (PUFAs) such as omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [85] and omega-6 arachidonic acid (ARA) [89] are sourced from marine microalgae. Presently, focus is being laid on *Nannochloropsis oceanic N. oculata,* and *N. gaditana* for the commercial extraction of EPA and DHA, as tablets and freeze-dried powder by Eco Mundo®. High levels of EPA and DHA in diets have also been linked to reduced occurrence of cardiovascular diseases, rheumatoid arthritis, psoriasis, dementia, depression and Alzheimer's disease [123, 124]. The omega-6 ARA commercially extracted from the marine microalga *Porphyridium* [102] taken as supplement has been reported to enhance cognitive response [125] and coronary flow velocity reserve [126] in elderly persons.

4.3. Animal and fish feed

Strains of marine microalgae with high EPA/DHA levels used as a component of aquaculture and livestock feed [127] have been observed to augment its nutritional value [85, 128], increase the digestion capability, and induce positive growth and reproductive results to a certain extent [128]. Shifting from the conventional use of fish meal, a protein-rich product made from processing remainder fish after human consumption, fish bones and offal, microalgae are now progressively being used as constituents of animal feed. Furthermore, farmed carnivorous fish species fed by a marine microalgae-based diet obtain ample amount carotenoids, lipids, vitamins, proteins and energy for enhanced growth and reproduction [129]. Among cyanobacteria (blue-green algae), species of the genus *Arthrospira*, such as *A. platensis* and *A. maxima*, are mainly used in aquaculture to feed larvae and zooplankton (*Arthrospira* dietary supplement is known as *Spirulina*) [129, 130]. *Arthrospira* spp. also help to protect cultured organisms against pathogens, thereby, reducing the costs associated with the use of antibiotics in aquaculture [131].

The marine microalgae *I. galbana* and *Pavlova lutheri*, rich in DHA, are used as live feed for aquaculture [128, 129], whereas *Dunaliella* spp. are used in the dried form [102]. A mixed diet of marine microalgae *I. galbana, P. lutheri* and *Chaetoceros calcitrans* is commonly favoured to feed the larval, early juvenile and broodstock stages of bivalve molluscs [129]. *I. galbana* and *Diacronema vlkianum* have been obtaining a lot of attention due to their ability to produce long chain PUFAs (DHA and EPA). A diet composed of an amalgam of species is preferred to a diet consisting of a single species for it provides a more balanced nutrition and improves growth rates [130]. Another vital nutritional component for the growth of aquacultured organisms is sterols. The ability to carry out *de novo* synthesis of sterols is usually low or entirely absent in bivalves [119]. In order to overcome this inefficiency, phytosterols such as ergosterol and 7-

dehydroporiferasterol in *Dunaliella* spp. [132], obtained from a mixed diet of microalgae act as supplements in the diet of cultured bivalves. Countries such as Norway, Iceland and United Kingdom have been using the marine macroalgae *Ascophyllum* spp., growing in the eulittoral, zone as seaweed meal to feed animals in coastal areas, while France as well as Iceland have been using *Laminaria* spp.. "Seaweed meal consists of dried seaweed powder containing useful amounts of potassium, magnesium, sodium, phosphorus, calcium, chloride and sulphur, vitamins and trace elements." On the other hand, fresh brown and red macroalgae, such as *Macrocystis pyrifera* and *Gracilaria edulis* respectively, have been used as feed for abalone in Australia, whereas in South Africa the macroalga *Porphyra* has a huge market for abalone feed [2].

4.4. Biofertilisers

Marine macroalgae have long been used as a natural fertiliser on the shore and nearby land: *Ascophyllum* spp. in Scotland, brown algae in certain places in United Kingdom and France, *Sargassum* spp. in the Philippines and green algae in certain parts of Argentina among others [2]. These marine algae were buried into the sand on the beach or nearby land and allowed to rot and form a useful organic fertiliser. Brown algae, namely, species of *Ascophyllum, Ecklonia* and *Fucus*, are commonly used to produce organic compost. This compost is produced by drying and milling the macroalgae, and is sold as soil additives that function both as a biofertiliser and a soil conditioner. Compared to conventional composts, marine macroalgae-based composts provide the following advantages: high nitrogen, high potassium and low phosphorus content, good moisture retention properties, and improvement of soil structure and aeration. Examples include commercially available dried macroalgae Afrikelp® from brown algae *Ecklonia maxima, Ascophyllum* used as soil conditioner [133] and commercially available fertiliser Maerl® derived from red algae *Phymatotithon calcareum* and *Lithothamnion coralliodes* [2].

4.5. Cosmeceuticals and pharmaceuticals

The bioprospecting of marine algae has gained momentum in the development of products for cosmeceuticals and pharmaceuticals. Significant marine algal compounds of the cosmeceutical industry include phlorotannins (also used in the food industry), sulphated polysaccharides, tyrosinase inhibitors and suppressors of matrix metalloproteinase (MMP). The marine algal extracts have recently received bouncy attention especially for their antimicrobial properties and in the treatment of the skin related issues (skin anti-aging, skin whitening and pigmentation reduction) [93]. Examples of such extracts from marine algae include *Arthrospira* (microalga) extract that prevents early skin aging by exerting a tightening effect [93]; *Alaria esculenta* (macroalga) extract that induces a significant decline in the amount of progerin that triggers cellular senescence in skin [134]; the macroalgae *Fucus vesiculosus* and *Turbinaria conoides* that contain polysaccharides such as laminaran, fucoidan and alginate having antioxidative properties [135] as well as properties for skin care and cutaneous disorders either as nutritional supplements or for topical application. *Porphyra umbilicalis* also contains large amounts of Mycosporine-like amino acids (MAAs) that can absorb UV light; therefore, it acts

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as sunscreen [136] in cosmetics. *Sargassum horneri* extract, containing Sargachromanol E., inhibits lipid peroxidation and, thus, has great potential to prevent photo-aging of the skin if applied in cosmetics [93]. It has been reported that some marine macroalgae extracts have the potential to suppress the production of MMP in skin which normally cause the degradation of collagens and elastic fibres, and induce loss of skin elasticity. For instance, phlorotannins (dieckol and eckol) is isolated from *E. stolonifera* and *Corallina pilulifera* methanol extract [92, 137]. Additionally, the marine algae–derived antioxidants can help to maintain the organoleptic properties of cosmetic products by inhibiting lipid oxidation, thus, avoiding changes in appearance, odour and flavour of the cosmetic products [93].

Furthermore, marine algae are the most abundant source of natural polysaccharides—fucoidans, Carrageenans and ulvans. Those polysaccharides are used in some cosmetics as moisturising and thickening agents, rheology modifiers, suspending agents, and hair conditioners among others [93, 138]. Polysaccharides extracted from the macroalgae *S. japonica* and *Chondrus crispus* performed as a better moisturising agent in cosmetics as compared to hydroxy acid (HA) in conventional cosmetics. They also have hydrating, therapeutic as well as moisturising effects whereas *Codium tomentosum* regulates water distribution in the skin; hence, protects the skin from dryness. Marine algal pigments such as carotenoids are also used as natural pigmenting agents in pharmaceutical and cosmetic products [93].

4.6. Food colourants, natural dyes and fluorophores

Marine microalgae have also been explored for and used as a renewable source of natural food colourants and dyes. The bright red pigmentation exhibited by the carotenoids of *Dunaliella* is used as natural food colourants [102, 120]. Betatene® (natural mixed carotenoids), a natural colourant extracted from *D. salina*, has been approved and affirmed safe as a natural food colour for use in various food products and beverages by the United States Food and Drug Administration [120, 139]. Another important carotenoid of the microalgae species *Dunaliella* – which is incorporated in animal feed (poultry) and fish meals as a colour enhancer to allow pigmentation of the flesh, egg yolk and shell of the farmed organisms (fish and prawns) and ornamental fish – is lutein [140].

Microalgae are also sources of phycobiliproteins (phycoerythrin and phycocyanin and allophycocyanin) and chlorophylls that are used as natural dyes in various industries and as food colourants [94, 102]. These proteins are brightly coloured and fluorescent constituents of cyanobacteria and red macroalgae (*Hypnea, Acanthophora, Porteria* and *Sarconema*). Phycoerythrin, extracted from the microalga *Porphyridium cruentum*, is gaining momentum for its application in the food industry as a red pigment [96, 141]. In addition, the natural blue dye phycocyanin, extracted from the microalga *Spirulina* sp., has been used as a food colourant and an edible dye for ice creams since 1980 under the brand 'Lina Blue-A' marketed by the Daipon Ink & Chemicals, Inc. [3]. Owing to the increasing demand for a healthier food additive to enhance colour in the food industry, mass cultivation of the microalgae *Porphyridium, Rhodella* and *Rhodosorus* is practiced for phycobiliprotein extraction [96]. Furthermore, on account of their spectroscopic properties, phycobiliproteins are also used as fluorescent probes with numerous applications in fluorescent immunoassays, flow cytometry, fluorescence

microscopy, fluorescence activated cell sorting and immunodiagnostics to name a few [94, 141, 142].

4.7. Biofuels

Both sub-groups of marine algae have the potential to contribute towards the world's future energy security, at the same time helping to reduce CO_2 emissions and mitigate global climate change impacts as compared to conventional fuels. The biomolecules for the biofuel industry are carbohydrates and lipids for the production of bioethanol and biodiesel respectively. Several strains of marine algae which produce carbohydrates and lipids via photosynthesis have the potential to be exploited as biofuel feedstock [143]. The choice of marine algae as biofuel feedstock is dictated by a number of advantages: relatively rapid growth rate and high productivity compared with other conventional oil crops, high photosynthetic efficiency, great potential for CO_2 fixation, low percentage of lignin and a high content in carbohydrates and lipids (20–50%) [143–147]. Microalgae can provide several types of renewable biofuels, for example, methane — produced by the anaerobic digestion of the algal biomas; biodiesel — derived from microalgal oil; and biohydrogen – produced by photobiologically [16, 144]. Several studies have focused mostly on eukaryotic species such as *Nannocloropsis* sp. because of their relatively higher lipid content [148] and cyanobacteria because of their fast growth rate and higher lipid content [16, 149]. According to Chu [102], microalgae appear to be a promising potential for biodiesel production. The study carried out by Beetul et al. [16] corroborated that argument; the cyanobacterial mats and the symbiotic microalga *Symbiodinium* clade C were found to contain relatively significant amounts of lipids making them good candidates for biodiesel feedstock.

4.8. Papermaking

Delving into the potential applications of marine macroalgae, many authors have reported the use of algae-sourced cellulose in papermaking, a field of application still at an infancy stage. In general, wood-based pulp utilised in the papermaking industry has to undergo a lignin removal process to liberate the cellulose, which is the desired component for producing high quality bleached paper. Lignin is a polymer intercalated between the cellulose fibres in cell walls. Lignified pulp is normally used for low-quality papermaking, for instance, newsprints. Owing to the lack or substantially low amount of lignin in the cell walls of algae [150, 151], the lignin removal process is omitted when using algae [152], making them potential candidates for sustainable and profitable papermaking as long as cultivation methods are cost effective. This can be achieved by mass cultivation of raw material for a fully established algae-based papermaking industry. Seo et al. [150] attempted to use lignin-free red macroalgae *Gelidium corneum* and *G. amansii* to produce printing grade paper. The wild Rhodophytes yielded 8–11% pulp with a brightness of over 80%. The smoothness and opacity of the red macroalgae handsheet, characteristics critical for good quality paper, were high compared to commercial high-quality paper manufactured from wood pulp [150]. Other patented methods to extract cellulose from marine macroalgae for papermaking have also been described by Sakai et al. [152] and You and Park [153].

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4.9. Bioplastics

Bioplastics have been derivative from organic sources such as potatoes, corn, vegetable oil, and most recently from marine algae. Polysaccharides from macroalgae – carrageenan, agar and alginate – can be used to make bioplastics [90]. Marine algae–based bioplastics have the following advantages: no competition with food resources, ease of growth in a wide range of environments, high yield/biomass, cost-effectiveness, address the issue of excessive CO_2 emissions and is environment-friendly [2, 154]. The various types of plastics that are derived from marine algal feedstock include the following: *hybrid plastics*—which are made by adding denatured algal biomass (for example, the filamentous macroalga *Cladophora*) to petroleum-based plastics (like polyurethane and polyethylene) as filler to increase their biodegradability; *cellulose-based plastics*—which are made from cellulose component of the algal biomass left after the extraction of algal oil (about 30% of the total algal biomass); *Poly-lactic acid* (PLA)—which is produced by the bacterial fermentation of algal biomass; and *bio-polyethylene*—which can be derived from the bacterial digestion of algal biomass [2]. Other examples of bioplastics sources include the red macroalga *Eucheuma cottonii* [103] and the microalga *Spirulina dregs* [154] However, these marine algae cannot be easily harvested [104]. Marine algae-based bioplastics are used in the packaging and automobile industries, as catering, gardening and medical products [154].

Macroalgae			
Product	**Level of exploitation**	**Applications**	**Genus**
Biomass	Organism	Food	*Porphyra, Laminaria, Saccharina, Undaria*
		Biofertiliser, organic compost, soil conditioner	*Ascophyllum, Sargassum, Ecklonia, Fucus, Phymatotithon, Lithothamnion*
Vitamins, minerals and trace elements	Organism	Feed	*Ascophyllum, Laminaria, Macrocystis, Gracilaria, Porphyra*
Phycocolloids—alginate	Extract/molecule	Stabilisers and thickeners in food industry, wound dressing, prosthetic devices, matrices to encapsulate and/or release cells and medicine, medical sutures	*Undaria, Laminaria, Ascophyllum, Durvillaea, Ecklonia, Lessonia, Macrocystis, Sargassum*
Phycocolloids—agar	Extract/molecule	Gelling agent in food industry, food gums, thickener in ice creams, excipient in pills, growth media for bacteria culture, biotechnological applications	*Gracilaria, Gracilariopsis, Gellidiella, Pterocladia, Ahnfeltia, Gelidiopsis, Hypnea, Gelidium, Gigartina*
Phycocolloids—carrageenan	Extract/molecule/ algal biomass (for bioplastics)	Food industry, gel formation and coatings in the meat and dairy industries, stabilisers and thickeners in cosmetics, binders in toothpaste	*Chondrus, Eucheuma, Gigartina, Sarcothalia,*

Macroalgae			
Product	**Level of exploitation**	**Applications**	**Genus**
		and tablets, smoothers in feed, paint industry, bioplastic	*Mazzaella, Iridaea, Hypnea, Mastocarpus, Kappaphycus*
Phycobiliproteins	Molecule	Natural dyes in cosmetics, natural food colourants, fluorophores in immunodiagnostics	*Hypnea, Acanthophora, Porteria, Sarconema, Gracilaria*
Cellulose	Extract	Papermaking	*Gelidium*
Carotenoids -β-carotene	Organism/ molecule	Health food, food supplement, feed, natural food colourant	*Dunaliella*
Carotenoids—phytosterols	Organism	Feed	*Dunaliella*
Carotenoids—lutein	Organism/ molecule	Natural colour enhancer in aquaculture	*Dunaliella*
Fatty acids	Organism/ molecule	Health food, food supplement, cosmetics, prevention of disease	*Nannochloropsis, Porphyridium, Odontella, Isochrysis, Phaedactylum*
	Organism	Live feed, mixed algal diets	*Arthrospira, Pavlova Chaetoceros, Diacronema*
Polysaccharides	Molecule/extract	Pharmaceuticals, cosmetics, nutrition	*Porphyridium*
Phycobiliproteins	Molecule	Natural dyes in cosmetics, natural food colourants, fluorophores in immunodiagnostics	*Porphyridium, Arthrospira, Rhodella, Rhodosorus*
Lipids	Organism	Biofuels	*Botryococcus, Chlamydomonas, Nannocloropsis*
	Organism/ molecule	Nanotechnology, optical systems, semiconductor, nanolithography, drug delivery, medical	*Spirulina*

Table 1. Application areas of selected marine macro and microalgal species and derivative compounds (data from: [2, 13, 87, 89, 100–102, 90, 100, 103–106]).

5. Challenges and opportunities in biotechnological applications of marine algae

> 'In the end, the objective of microalgal biotechnology is to make money by selling a product for a higher price than it costs to produce.'—Olaizola [155]

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'Marine algal-omics' studies have given rise to new opportunities and subsequent industrial applications. While the prospects of those organisms are huge, there are multiple challenges that this sector has been facing since its foundation. Olaizola [155] presented one of the major issues that the field of marine algal biotechnology faces and why many marine algal applications have not yet reached the commercial and industrial scale. As a whole, for this chapter's scope, the challenges and prospects are discussed under the following umbrellas: marine algae taxonomic classification, 'marine algal-omics' and applications of marine algae.

5.1. Marine algae taxonomic classification

The taxonomic classification of marine algae has been tedious for the past decades. Morphological identification (using both microscopic and macroscopic features) of algae engenders various limitations such as change in morphology due to environmental factors [156], presence of similar morphotypes, complex cellular structure [157], lack of characteristics morphological features of the organisms as well as time-consuming and expertise-requiring in this field [158]. The ambiguities of identification using morphological keys have been found in most microalgal genera following phylogenetic analyses of ribosomal genes (SSU and ITS rDNA sequences) [159].

Identification of marine algae is highly problematic. In this view, taxonomic classification of marine algae is being reviewed through phylogenetic studies supported by analyses of cell division processes, physiological products (pigments and oils produced) and genetic characterisation [27]. For example, the taxonomy of the *Sargassum* spp. of the Mascarene Islands was revised as the available list of species was often biased using morphological identifications. Using morphological features, specimens previously identified as *S. polycystum* and *S. gaudichaudii* were recommended to be considered superfluous and synonyms of *S. polycystum* [160]. As for marine microalgae, the morphological misidentifications with respect to the genus *Dunaliella* have been spelled out in the past. The taxonomic revision following phylogenetic analyses allowed a better taxonomic classification of the *Dunaliella* spp. [161, 162].

As aforementioned, phylogenetic analyses help in categorising species which are difficult to identify. However, from a taxonomic point of view, DNA sequence information without other corroborating evidence can never be used by itself as an indicator for species delimitation [163]. In conjunction, molecular phylogenetic studies often address such issues. Nonetheless, it is still unclear whether the detected sequence differences may be used for delimitation of different species. This is explained by the progress of lineage sorting [164]. The use of different tools as well as sorting out of all lineages of algae is imperative for the identification of marine algae and further enhances their taxonomic exploration.

5.2. Marine algal-omics

'Marine algal-omics' are subjected to pretty much the same challenges that other microbial 'omics' agonise over. Graves and Haystead [58] put forward that the foremost challenge of proteomic studies is the analysis of low-abundance proteins and further deplored the archaic methods used to study proteins including the dearth of bioinformatics tools for data interpre-

tation. They also advocated that there is an urgency for new algorithms and that the mundane technologies should retreat while other technologies involving large-scale analyses need to be conceived.

On the other hand, transcriptomics experiences challenges of both worlds—genomics and proteomics. Dong and Yan [165] summarised the challenges as being experimental, technological and at the level of data interpretation to address issues like unveiling the regulating targets for each non-protein coding RNA and to decipher the complexity of the transcriptome. Bioinformatics is the resonance factor in all the 'omics'. The advancement in high-throughput technologies of metabolomics is gaining momentum. Nevertheless, there is still an echo of data interpretation difficulties: identification of a panoply of unknown metabolites and transformative agents of metabolism, and data mining [166].

We are certainly past the genomics era; however, it is only recently that marine algal genomics took off. It has multiple challenges with an echo of bioinformatics especially in the context of the acquisition of growing amounts of data. It is further to be noted that significant advances in algal bioinformatics tool development, such as the Algal Functional Annotation Tool [167] and GreenCut2 [168], are therefore unequivocally important drivers in the study of 'omics' in marine algae. 'Marine algal-omics' also represents a major challenge at organismal level for a set of criteria, such as *in vitro* culture, and must be satisfied before being considered for 'omics' studies [31]. Algae, inclusive of marine species, have a failure record in terms of culturing and cultivation. This might be also part of the reasons why development in marine algal genomics is lagging behind in the genomics arena. Interestingly, with the advent of 'meta-omics', such challenges may be circumvented to some degree. The National Human Genome Research Institute [169] defines meta-genomics as being '*the study of a collection of genetic material (genomes) from a mixed community of organisms*'. Meta-genomics can be applied to marine algae that are hard to culture or to isolate from a microbial community. The significance of this technique is tremendous, for instance, meta-genomics could provide a sequence that could be of importance to several industries or even to our understanding of evolution among others. In this context, 'meta-omics' is being considered to study the proteome, transcriptome and metabolome of the marine algae that are hard to be isolated from their microbial community or cultured *in vitro*.

In addition, 'marine algal-omics' encounters pronounced issues with respect to macroalgae and microalgae. Contreras-Porcia and Lopez-Cristoffanini [170] underlined that protein extraction of algae follows no common protocol and pointed out that it is particularly difficult to extract proteins from macroalgae due to their low concentration and the co-extraction of contaminants. In the years to come, the proteomics studies of marine algae are expected to increase by multiple folds taking into account their commercial/industrial implication and the will of many countries to develop their blue economy. Such 'omics' studies are also opening a plethora of opportunities for several algal species in multiple fields such as *D. salina* for carbon capture to address climate change. As mentioned earlier, the marine microalga yields glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Those are of prime importance as they boost the pentose phosphate pathway which is crucial for carbohy-

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drate metabolism that includes the provision of precursors for the biosynthesis of Lipids and sugar derivatives among others [64].

The 'omics' technologies detailed in this review are the most commonly used in 'marine algal-omics.' Conversely, earlier on, we deplored the static nature of the results that they provide. We also brought the perspective of other avenues (e.g., fluxomics) to the attention of the readers for a dynamic distinction in this arena. Many metabolomics studies provide a snapshot of the targeted or untargeted metabolome at a specific time and can be altered at any given time in response to stimuli or different life stages and growth conditions [78]. Metabolomics allows scientists to catch a glimpse of such a dynamic nature which in fact makes the static picture a limitation in itself. The curiosity even more importantly the urgency to discover the function of several metabolic pathways is now taking over the post-genomic era leading to the field of fluxomics, which is the study of metabolites fluxes. Fluxomics is still in its infancy stage and demands more research in this area for an understanding of how the system works.

5.3. Applications of marine algae

As a consequence, only a few species have been able to reach the industrial and commercial level while many marine algae still remain largely untapped as an asset due to an apparent lack of utility as a primary active ingredient [93]. While some advocate for the industrial application of marine macroalgae, others favour the marine microalgae. However, literature shows that both the marine macroalgae and microalgae can be useful to different industries. Nonetheless, marine macroalgae are currently more appealing for industrial application as they are readily accessible and easy to harvest compared to microalgae [2] but also have lower cost of production [14] and biosynthesise commercially important molecules such as the phycocolloids [15]. Marine microalgae, on the other hand, are attracting more attention as a feedstock for biodiesel production and other products such as β-carotene. However, as listed in **Table 2**, marine algal applications face many challenges that need to be addressed prior to commercialisation.

Aspects hindering commercialisation of marine algae	Challenges	References
Biomass production	High cost of production due to significant cost implication with respect to resource supply: water, carbon dioxide and nutrients	[17]
In-sea cultivation	Alterations to biosynthesis of molecules of interest due to spatial and seasonal variations, location and depth	[172]
Biomass harvest	Energy intensive process: centrifugation*	[16, 17, 173]
Oil extraction	Use of petroleum derivatives	[16, 17, 173]

Aspects hindering commercialisation of marine algae	Challenges	References
Algal strain	In-breeding leads to negative alteration of trait of economic importance such as decline in yield and quality	[17, 172, 173]
Algal diseases	Affect the candidature of strain to be considered as a feedstock	[172]
External factors affecting production	Impact of microbial interactions with marine algae on bioactivity	[172]
Land use	Non-arable land hosting marine algal mass production increases proportionately with increase in demand	[147, 173]
Carbon dioxide input	Contribute to high cost of production due to purchased carbon dioxide	[17]
Nutrient supply (especially nitrogen and phosphorus)	Limited nutrient supply limits algal mass production	[17]
Dehydration	Energy intensive	[17, 128]
Water use	Limited by freshwater	[17, 173]
Light	Optimal production limited by shading as well as photo inhibition	[17]

*Applicable to marine microalgae only.

Table 2. Challenges of industrial applications of marine algae.

As a whole, the numerous challenges mentioned (Table 2) can be addressed by genetically enhancing the algal strains, improving the production of high-value products and lowering its cost. Hybridisation of marine macroalgae [172] and genetic engineering [17] of both macroalgae and microalgae are promising tools assuring expression of important traits such as disease resistance and overproduction of specific compounds of interest. The compounds of interest may be targeted molecules as well as by-products. Furthermore, the in-sea culture exercise should be carried out along a standard for cultivation to circumvent issues such as inconsistent depth, environmental changes and others which may impact the production of bioactive compounds and composition [172]. In view of lowering the cost of production of marine algae, the by-products of macroalgae biomass production such as protein, alginates and phenolic compounds should be considered as an integral part of commercialisation to enhance the economic value of marine algal biofuel production process. The nutrients can be recycled while wastewater can also be used to some extent [17].

The last few decades have witnessed several developments in biotechnological applications of marine algae on three scales: research, commercial and industrial. However, the inexhaustive list of challenges mentioned here has hindered the Full-fledged application of marine algae. The challenges are being progressively addressed and opportunities are being generated. It is with confidence that we advocate the determinant role 'marine algal omics' will play in the marine algal economy.

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Chapter 11

Genetic Identification and Mass Propagation of Economically Important Seaweeds

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Additional information is available at the end of the chapter

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Abstract

Seaweeds are a primary source of hydrocolloids, which can be processed into various food additives, cosmetics, and pharmaceuticals. The inability of current commercial seaweed farming projects to meet industrial demands is underscored by a plethora of challenges, which include the lack of high-quality germplasm with the desired cultural characteristics. This chapter describes the current trends in commercial seaweed production and the potential technological advances in production methods and genetic selection strategies, which can be applied to raise the productivity of seaweed farms. Molecular markers have become increasingly relevant to the selection of a diverse range of wild varieties for domestication, and this augurs well for strain identification. The development of high-density linkage maps based on molecular markers offers an avenue for the implementation of molecular breeding strategies based on quantitative trait loci (QTLs). Concurrently, productivity of existing varieties can be enhanced by the analysis of exogenous factors known to affect the growth and survival of tissue-cultured seedlings. The application of photobioreactors for tissue culture is another important development, which will be digressed upon. In addition to this, quality control which focuses on the comparison of chemical and physical qualities of the tissue-cultured and conventional cultivated seaweeds will become increasingly relevant to the development of industry standards for sustainable seaweed production to fulfill the increasing demands of seaweed-related industries.

Keywords: Acclimatization, Genetic marker, Marker-assisted selection (MAS), Photobioreactor, Quantitative trait loci (QTLs), Tissue culture

1. Seaweeds and their economic importance

Seaweeds are marine macroalgae generally found living in oceans and coastal areas throughout the world. The classification of seaweeds has been typically based on their phenotypical features, which include pigmentation and photosynthetic properties. Since the mid-nineteenth century, seaweeds have been empirically distinguished into three main divisions based on their color: red algae (phylum: Rhodophyta) consist of about 6000 species, brown algae (phylum: Ochrophyta) consist of about 1750 species, and green algae (phylum: Chlorophyta) consist of about 1200 species [1]. Until recently, a wide variety of seaweeds and their products have been studied for their industrial, culinary, and renewable energy applications, which include cosmetics, chemistry, paint, medicine, biofuel, etc. [2, 3]. Increasing global demand for seaweed resources and overexploitation of natural seaweeds have highlighted the need for sustainable seaweed cultivation to significantly increase captive production in mariculture systems.

Among all the seaweed-based products, hydrocolloids viz. carrageenans, agar, and alginates continue to be the principal extracts, which received commercial attention through their application in various industries [4, 5]. *Kappaphycus* and *Gracilaria* are the two most important red seaweeds in the world trade market, which have been reported to contribute significant amount (60–80%) of world's carrageenan and agar resources, respectively [3]. Seaweed hydrocolloids are mainly used in food-processing industries as is evident from their applications in health-care products such as carrageenan gel capsules and alginate micro-beads [4]. Apart from hydrocolloids industry, seaweed natural products are also a promising source of biologically active compounds with medical and pharmaceutical applications [6], and nutritional supplements for animals and plants [2, 7].

2. Current limitations of conventional seaweed breeding and alternative solutions

Emerging applications of hydrocolloids in food industry and other hydrocolloid-related industries have led to an enhancement of the economic values of red algae including *Kappaphycus*, *Eucheuma,* and *Gracilaria* species. Production of seaweed biomass has globally increased from 4 million wet tones in 1980 to 20 million wet tones in 2010 [8] with 95.5% of biomass produced from artificial cultivation in mariculture program [3, 9]. The economic viability of the seaweed industry is dependent on the production of high-quality germplasm with the desired traits viz. high growth rates, amenability to treatment with fertilizers and resistance to diseases for incorporation into breeding program. The high variation of morphological features in the wild [10, 11] and the lack of diagnostic morphological characters have resulted in the misidentification of cultivated seaweed strains, which in turn have contributed to a decline in productivity and quality of cultured seaweeds.

Current practices of seaweed cultivation are predominantly based on traditional methods, where seaweeds are exposed to environmental challenges and pathogens [12, 13]. Most of the

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seaweeds are cultivated using seedlings produced by vegetative propagation from cultured germplasm. Through this practice, parasites or pathogens from the harvested seaweeds may be re-introduced and subsequently reduced the productivity of the farm. The other logistical problems faced by conventional seaweed farmers include the identification of appropriate sites for farming, labor intensive tasks such as inspection, disease, and seedling losses resulting from extreme weather conditions and water quality. In order to increase the productivity, modern biotechnology via tissue culture can be considered as one of the best options to overcome the conventional breeding challenges including shortage of raw material for planting and seedlings destruction by epiphytes, subsequently facilitate the propagation of high-quality seaweeds [9, 14, 15].

3. Application of molecular markers in seaweed breeding

The establishment of an effective seaweed breeding programs is founded on the selection of strains of seaweed with desirable cultural characteristics such as high growth rates, high carrageenan content, disease resistance, and accelerated growth in response to supplemental fertilizers. Phenotypical identification methods are currently the standards by which specific seaweed strains are selected. Although invaluable, morphological characterization can be time-consuming and requires a high level of expertise to discriminate key morphological features indicative of the seaweed species. In addition to this, the physical characteristics of seaweeds tend to be variable as they are directly influenced by environmental factors [16]. Most of these seaweeds cannot be distinguished on the basis of one or collection of specimen using morphological characters alone, and an exhaustive taxonomic study is essential before the variety can be identified. For example, high morphological plasticity within the Hawaiian *Eucheuma* seaweeds has led to the misidentification of three introduced eucheumatoid species [17]. A commercially important seaweed species is selected on the basis of the types of biopolymers that they synthesize, where the infrared spectroscopy of their gels has become a measurement to differentiate among genera and species [18]. Nowadays, morphological data have to be complemented with molecular data in order to characterize the desired species of seaweed. Molecular markers are an ideal tool for the classification of cultivated and wild seaweeds independent of their morphological appearance and growth stage. Their application can be extended to marker-assisted selection (MAS) and the development of isogenic strains for the application in current and future propagation programs. The development of molecular markers for germplasm will be of useful for species- and variety-specific identification, plant variety protection and interspecific and intergeneric crosses development for economically important seaweed species.

3.1. Genetic marker for identification of commercially important seaweed species

To date, the application of different genes for the genetic identification of seaweed species is widely carried out, where the targeted DNA regions are the nuclear, plastid, and mitochondrial DNA (mtDNA). Most molecular characterization targets seaweeds with economic value such as *Palmaria palmate* (Dulse), *Porphyra umbilicalis* (Nori), *Gracilaria changii*, *Kappaphycus alvare-*

zii, *K. striatus*, *Eucheuma denticulatum* (carrageenophytes), and many more [19–23]. Currently, large-scale DNA barcoding such as Red Algal Tree of Life Initiatives (RedToL) is analyzing phylogenetic relationship of 471 red seaweeds using two nuclear, four plastid, and two mitochondrial encoded gene markers [24]. China has also conducted a large-scale phylogenomic analysis of marine red algae revealing evolutionary lineages for rhodophytes [25, 26]. A good DNA barcoding locus should have adequate internal variability to enable differentiation at the species level and contain flanking regions that are conserved enough to study routine amplification across highly divergent taxa [27].

Nuclear ribosomal regions, which include sequences of large subunit (28S or LSU), small subunit (18S or SSU), and the intergenic transcribed spacers (ITS1, ITS2), can be served as target sites for molecular markers because the ribosomal DNA (rDNA) genes contain both highly conserved and variable regions that can be used as diagnostic tools for certain organisms [28]. The small subunit (18S) and the large subunit (28S) regions are the most used regions for marker development as they are best suited for inference at high taxonomic levels [29]. However, ITS region is often targeted for intraspecific genetic studies in Chlorophyceae (*Codium fragile*) [30], Phaeophyceae (*Fucus serratus, F. evanescens*) [31], and Rhodophyceae (*Chondrus crispus, Ulva intestianlis, U. compressaa*) [32, 33] on account of its high rate of nucleotide substitution, permitting comparison between relatively diverged taxa [30, 33]. Hu et al. [19] had also used the ITS1 region to study the intraspecific relatedness among 59 *Porphyra yezoensis* (Nori) for selective breeding program of economically important nori crops.

mtDNA has a higher mutation rate that gives rise to variation in its DNA sequence [34]. mtDNA is usually used to analyze the phylogenetic relationships of groups within a species or individuals that are closely related [35]. The gene map of mtDNA of the red alga, *P. purpurea,* is available, where all the different genes have been successfully sequenced [36]. The mtDNA loci, which are generally targeted in seaweed identification, are cytochrome c oxidase subunit 1 (*cox*1), cytochrome oxidase subunit 2–3 intergenic spacer (*cox*2–3 spacer) and cytochrome b (*cytb*) genes [17, 37–42]. Tan et al. [41] had evaluated the effectiveness of three mtDNA markers, *cox*1, *cox*2, and *cox*2–3 spacer in barcoding the two commercial important carrageenophytes, *Kappaphycus* and *Eucheuma* seaweeds, and has determined that the *cox*2–3 spacer DNA marker is more suitable as a barcoding gene because its widespread use. Recently, Lim et al. [42] had found higher species diversity of *Kappaphycus* seaweeds in Southeast Asia (Malaysia, Indonesia, the Philippines, and Vietnam) using the mitochondrial *cox*1 and *cox*2–3 spacer.

Loci derived from the chloroplast genome (cpDNA) can be used for the identification of seaweed species due to the low frequency of structural changes and low sequence evolution rate of cpDNA [43]. The cpDNA loci that are routinely used for seaweed identification are the ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) gene, specifically the large subunit of the RuBisCo (*rbcL*) [41, 44–46] and the RuBisCo spacer [47–50]. In a study conducted by Geraldino et al. [46] molecular phylogeny of 23 specimens of red alga, *Hypnea flexicaulis* from three countries (Korea, Taiwan, and the Philippines), was successfully studied based on the plastid rbcL region. Guillemin et al. [48] utilized the RuBisCo spacer region to identify six species of Gracilariaceae: *G. gracilis, G. conferta, G. dura, G. multipartite, G. vermiculophylla,* and *G. longissima*, which exhibit a high degree of phenotypic similarity.

Identification of commercially important seaweeds based on standard DNA barcodes or single marker amplification has proven to be useful as the phenotypic plasticity of the species can confound traditional taxonomic approaches. **Table 1** showed the summary of nuclear, mitochondrial, and plastid DNA regions that are used for the identification of rhodophyta [51]. Molecular markers are still valuable, despite the increasing popularity of next generation sequencing technologies, where the identity of an unknown seaweed species can be acquired based on a simple polymerase chain reaction amplification and a single sequence read (two sequence reads if both strands are sequenced). Examples of DNA markers used to identify commercially important seaweeds are given in **Table 2** [46, 52–57].

DNA regions	Abbreviation	Size (bp)
Nuclear DNA		
Small subunit ribosomal DNA	*SSU*	~1800
Internal transcribed spacer ribosomal DNA	*ITS*	~650–1100
Large subunit ribosomal DNA	*LSU*	~2700
Plastid DNA		
Photosystem I P700 chlorophyll a apoprotein A1	*psa*A	~1600
Photosystem I P700 chlorophyll a apoprotein A2	*psa*B	1250
Photosystem II thylakoid membrane protein D1	*psb*A	~950
Plastid LSU (23S) domain V	*UPA*	~370
Ribulose-1,5-bisphosphate carboxylase large subunit	*rbcL*	~1350
Mitochondrial DNA		
Cytochrome c oxidase subunit 1 DNA barcode region	*COI*-5P	~664
Cytochrome c oxidase subunit 1 extended fragment	*COI*	~1232
Cytochrome b	*COB*	~940
Cytochrome oxidase subunit 2-3 intergenic spacer	*cox* 2–3	~350–400

Table 1. Summary of nuclear, mitochondrial and plastid DNA regions used for identification in Rhodophyta [51].

DNA markers	Primers	Primer sequences	References
Nuclear DNA			
SSU ribosomal DNA	Forward primer	5′-CAACCTGGTTGATCCTGCCAGT-3′	[52]
	Reverse primer	5′-TGATCCTTCTGCAGGTTCACCTAC-3′	
ITS ribosomal DNA	Forward primer	5′-TCGTAACAAGGTTTCCGTAGG-3′	[53]
	Reverse primer	5′-TTCCTTCCGCTTATTGATATGC-3′	
Mitochondrial DNA			

DNA markers	Primers	Primer sequences	References
*cox*1	COXI43F COXI1549R	5′-TCAACAAATCATAAAGATATTGGWACT-3′ 5′-AGGCATTTCTTCAAANGTATGATA-3′	[46]
*cox*2-3 spacer	Cox2_for Cox3_rev	5′-GTACCWTCTTTDRGRRKDAAATGTGATGC-3′ 5′-GGATCTACWAGATGRAAWGGATGTC-3′	[54]
Plastid DNA			
*rbc*L	F7 R753	5′-AACTCTGTAGTAGAACGNACAAG-3′ 5′-GCTCTTTCATACATATCTTCC-3′	[55, 56]
RuBisCo spacer	Forward primer Reverse primer	5′-TGTGGACCTCTACAAACAGC–3′ 5′-CCCCATAGTTCCCAAT-3′	[57]

Ambiguous nucleotide codes according to IUPAC: K=G/T, R=A/G, W=A/T, D=A/G/T.

Table 2. Examples of DNA markers used for seaweed identification.

3.2. Quantitative trait loci (QTLs) for economically important traits

Quantitative trait locus (QTL) analysis is a statistical method that links two types of information, phenotypic data (trait measurements) and genotypic data (usually molecular markers), in an attempt to explain the genetic basis of variation in complex traits [58]. A wide range of agronomic traits in crop plants, including plant productivity and stress tolerance, are complex traits controlled by QTLs [59]. Identification of these QTLs will facilitate the development of novel varieties of seaweeds via conventional breeding approaches as well as genetic engineering. A (QTL) is a region of DNA (the locus) that correlates with variation in a phenotype, which is designated as the "Quantitative Trait". The QTL contains the genes that encode for the phenotype is tightly linked to the trait over successive generations. Traits of agronomic importance may be controlled by a single gene or in most cases by multiple genes. For example,

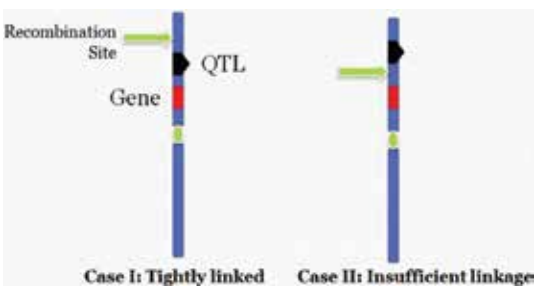

Figure 1. The gene encoding the quantitative trait is designated as a quantitative trait locus (QTL). The genetic markers for this trait can be located with the gene itself; however, in most cases, breeders will utilize the regions flanking the locus to design the markers. This is to ensure that the QTL can be tracked over successive breeding cycles and the markers are tightly linked to the QTL.

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if we bred a disease tolerant seaweed with a seaweed that yields a high amount of carrageenan, we can generally assume that the resultant hybrid will be a high yielding, disease tolerant variety. This is a generalized assumption based on the hypothesis that the traits are discrete (**Figure 1**). Markers are generally selected based on regions that flank the quantitative trait (**Figure 2**) and these can be validated over successive breeding cycles.

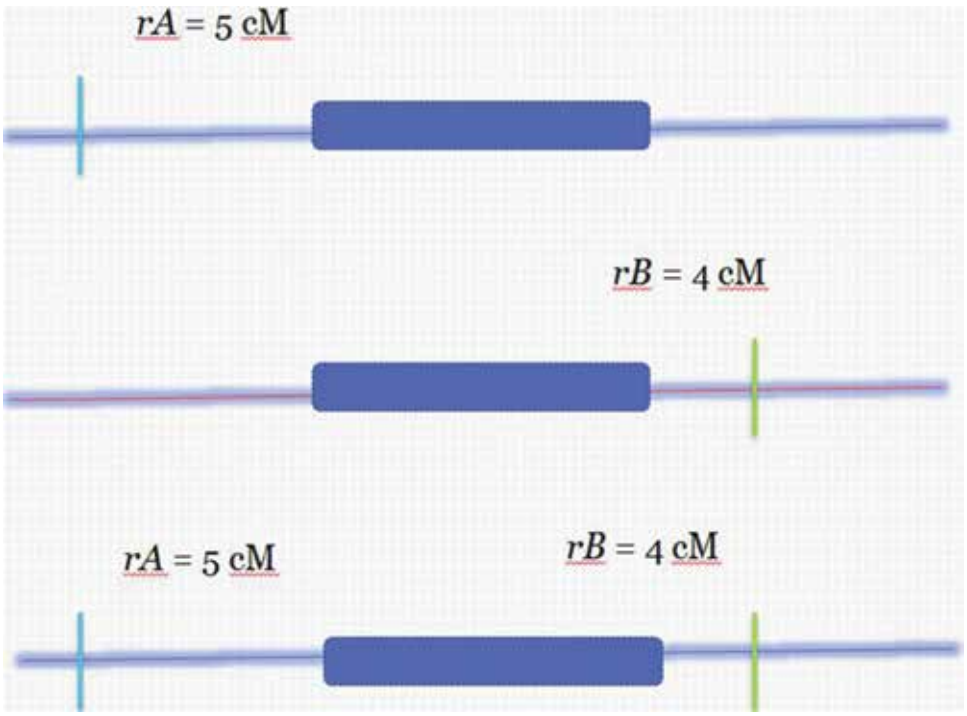

Figure 2. The ideal set of markers for QTL mapping is located on either side of the QTL and at a map distance of 4–10 cM on either side of the QTL. This ensures reliability over several cycles of recombination due to tight linkage.

However, it should be noted that the majority of traits are not discrete. Analysis of hybrids reveals that segregating populations consist of individuals with continuous traits. In the case of seaweeds, one example of this class of QTL can be the diversity of pigmentation observed in variants of *E. denticulatum* and *K. alvarezii*. This can be attributed to the process of indeterminate hybridization in the wild. Continuous traits cannot be analyzed in the same manner as discontinuous traits because a multiplicity of interactions between genes and their regulatory elements determines their phenotypic presentation. Commercial cultivators of seaweeds screen wild populations and select variants with traits such as rapid growth, resistance to diseases, adaptation to different salinity levels, carrageenan yield and type, response to treatment with fertilizers and ability to overcome parasites. All of these traits are controlled by multiple genes. For example, a rapid growth rate will be determined by the genetic proclivity to fix and utilize dissolved carbon dioxide at a higher rate as compared to slow

growing varieties. Tolerance to diseases and parasites may be the result of the ability to secrete antagonistic principles that inhibit pathogens. Traits controlled by multiple genes segregate independently according to Mendel's Laws, and their expression as phenotypes will be affected by environmental factors.

Commercial breeders adopt a range of approaches in order to integrate diverse traits in order to produce an elite strain with all the desired agronomic traits. This approach entails the selection of wild genotypes purely on the basis of their phenotypic characters. This is an ideal strategy to adopt in the case of seaweeds, where no established elite strains exist. Marker-assisted gene pyramiding aims to produce individuals with superior economic traits according to the optimal breeding scheme, which involves selecting a series of favorite target alleles after cross of base populations and pyramiding them into a single genotype [60]. The strategy for pyramiding is depicted in **Figure 3**.

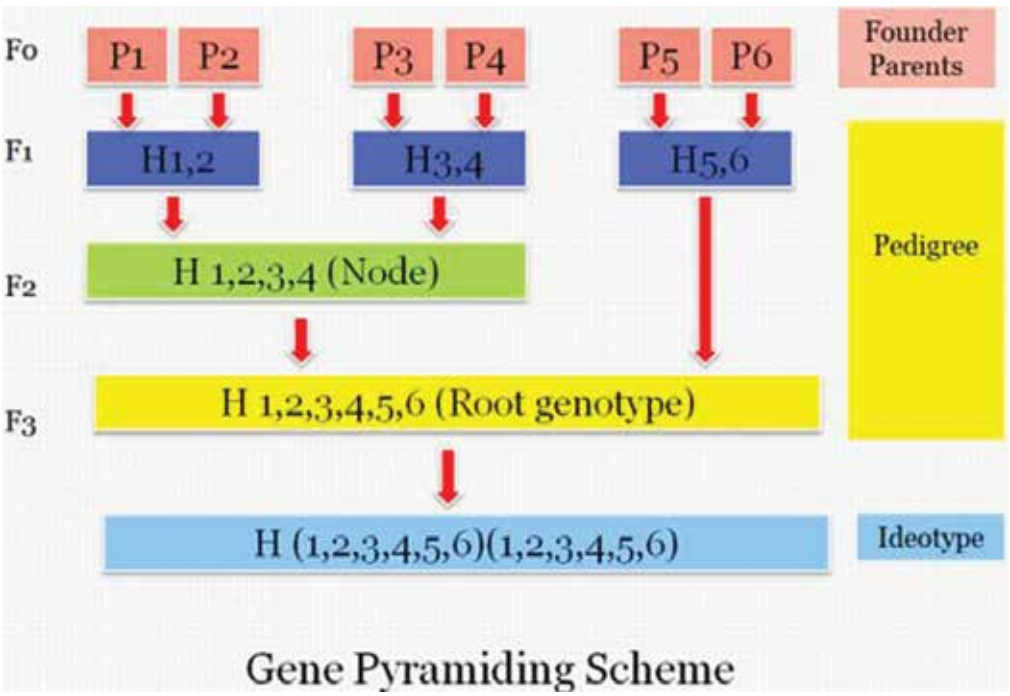

Figure 3. Marker-assisted pyramiding of ideal agronomic traits in seaweeds. In this scheme, six varieties with the desired traits are merged into one ideotype over successive breeding cycles. The loss or integration of QTLs can be assessed at every generation using polymerase chain reaction (PCR)-based molecular markers.

An alternative approach which can be undertaken when a grower has an elite strain, which needs to be supplemented with additional traits is backcrossing with the parental genotype over successive generations. This approach (**Figure 4**) mitigates the likelihood of genes encoding for undesired traits from the wild type from manifesting in the hybrid phenotype.

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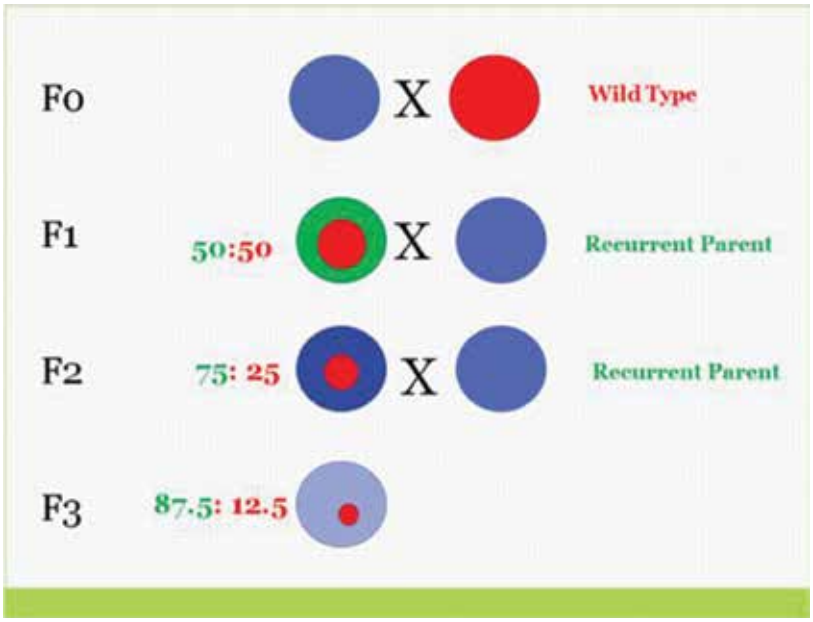

Figure 4. Marker-assisted backcrossing using molecular markers can be applied in circumstances, where it is necessary to improve the elite strain.

The first step in developing a linkage map using QTLs involves information pertaining to phenotypes and their association with specific genotypes [61]. Ideally, molecular breeding should commence with the collection of all the available phenotypes from the wild. The second step will be the identification of specific traits, which are associated with each phenotype; once this has been established, the third step will involve the elucidation of genomic information and its conversion into suitable molecular markers. This can be done using available genomic information or on the basis of expressed sequence tags (ESTs) [62–64]. Seaweeds, unlike terrestrial plants, have unusual breeding cycles [65] and the mechanism need to be established prior to commencing a defined breeding program. New insights into the draft genomes of *Chondrus crispus* [66], *Ectocarpus siliculosus* (Dillwyn) [67] will provide a strong basis for linkage mapping in related species of seaweeds.

Current research work on QTL mapping in seaweeds has focused on commercially cultivated species, which are *Laminaria japonica* [68] in which QTLs for frond length and width were identified using AFLP mapping. Linkage maps for hybrid populations of *Saccharina japonica* X *S. lonigissima* have been developed, and the authors have concluded that the map is not sufficiently dense in order to identify quantitative traits and establish linkage [69]; however, this represents the first step in development of a putative linkage map, which has the potential for transferability to closely related species. Genetic analysis of the marine red algae (*P. yezoensis*) has provided a basis for the identification of genes related to photosynthesis and the fixation of chromogens [70]. Interestingly, the similarity between seaweed genomes has been highlighted in *Laminaria digitata*, in which case a majority of molecular markers could be

applied in *Saccharina japonica* [71]. The first attempt at linking genetic markers with economic traits has been made in the case of the red alga *Pyropia haitanensis* using sequence-related amplified polymorphism (SRAP) markers [72], and specific genes related to stress tolerance have been identified in the model seaweed *Ectocarpus siliculosus* [73] by profiling the gene expression of enzymes that counteract oxidative stress. The mapping of QTLs in seaweeds is currently in its infancy. Unlike commercially cultivate crops such as rice and wheat, the culture of diverse kinds of seaweeds is confined to small geographic regions. The cost of development of QTLs needs to be offset with the returns on investment, which can be realized when the molecular markers have been validated and applied for breeding. The seaweed farming and breeding community urgently needs to identify a universal model seaweed, which can be characterized at the genomic level before a fairly accurate QTL map can be developed.

3.3. Marker-assisted selection (MAS)

Marker-assisted selection is defined as an indirect selection method of an individual with desired traits in a breeding program based on DNA markers [74]. The important of MAS in a seaweed breeding program is to obtain basic genetic knowledge of the chosen commercially important seaweed. Some desired seaweed traits, such as crop yield or phycocolloid content, may be controlled by one gene or a group of genes. Therefore, it is beneficial to develop markers for a range of commercially important seaweed species to provide the foundation needed for MAS in the seaweed breeding program [75].

In seaweed farming, specifically for the phycocolloid industry, the desired traits of seaweed would be disease resistance, suitable carrageenan content, high productivity, and yield. These desired characteristic or traits can be genetically simple, where only one gene is involved. However, most economically important crops tend to have traits that are genetically complex, where it is controlled by many genes (QTL) and the environment [75]. For example, Babu et al. [76] had detected a total of 47 QTLs for drought resistance traits from various plant water stress indicators to increase production and yield of rice in rainfed agriculture ecosystems. To date, there are no reports in the literature of the application of MAS in seaweed breeding program. Recently, Maili et al. [77] had successfully developed eight out of 112 single loci DNA markers to discriminate between varieties of *K. alvarezii*, *K. striatus,* and *E. denticulatum* seaweeds, where the markers could be applied in MAS and hybrid development. In future, application of DNA markers in MAS could be used as a tool that can help seaweed breeders to select more efficiently for desirable traits for the improvement in the culturing method of seaweed.

4. Mass propagation of seaweed seedlings via tissue culture

Repeated vegetative propagation applied in conventional seaweed cultivation was found decreasing genetic variability of seaweeds and subsequently contribute to the decreased in growth rates and yields and increased susceptibility to diseases [78]. Micro-propagation via tissue culture technology has been proposed as an alternative method compared to conven-

tional breeding to resolve the seedling shortage problem and increase the productivity of seaweed raw materials. Micro-propagation is a versatile tool to produce high number of uniform specimens from selected strains with desirable characteristics and increase seed stock production in shorter period of time [79]. However, challenges including lack of optimized protocols to obtain axenic cultures and regeneration of explants have limited the widespread use of tissue culture technology in commercial seaweed production. The efficacy of seaweed tissue culture is depends on the effective manipulation of endogenous (age, source, developmental stage, and physiological state of explants) and exogenous (media composition, light, salinity, pH, and temperature) factors [80, 81]. Current researches have been strategized to improve the culture conditions for mass production of high-quality laboratory seedlings to enhance the overall productivity of seaweed cultivation [3, 9, 14, 15].

4.1. Preparation of axenic cultures

Explants have to be sterilized in order to obtain the axenic cultures for mass propagation in tissue culture [82]. Seaweed samples collected from the wild are associated with a significant level of biological contamination, which is likely to be commensal or symbiotic; therefore, it is necessary to surface sterilize the explants with general disinfectants as well as targeted antibiotics prior to cultivation. Povidone iodine and alcohol are common disinfectants used for surface sterilization as they have a localized activity compared to the narrow spectrum antibiotics with their functionality limited to specific classes of microbes [83]. Surface sterilization of seaweeds is difficult as they lack of thick protective surface, and therefore, sodium hypochlorite and similar agents can easily damage the tissues especially newly regenerated thallus [84]. Prolonged exposure of explants to excessive disinfectants (e.g., more than 5 min in 2% betadine and more than 72 h in 5% antibiotic mixture) was reported causing patches of damaged surface on thallus and explants [85].

4.2. Media composition

Culture media commonly used for rapid propagation of rhodophyte are reported to be Provasoli's enriched seawater (PES) [86], seawater supplemented with von Stosch (VS) solution [87], and seawater enriched with half strength "f medium" (F/2) [88]. The selection of culture media for seaweed propagation is highly dependent on the nutrient level, ambient water, and cultured species. The optimized culture medium for economically important *K. alvarezii* was discovered to be seawater enriched with 50% of PES solution, whereas enrichments with 50% of VS and 50% of F/2 solutions were found not effective for *K. alvarezii* cultures [15]. Besides, *G. changii* cultured in 25% of PES enriched seawater was revealed propagating well and demonstrated promising growth rate [80]. Although some rhodophytes have been reported to grow well in VS and F/2 media [89, 90], the difference in media used may be due to the source of different explants or different genotype of explants. PES medium has low concentration of nutrients, whereas F/2 and VS media have a higher concentration of salts which may interfere with the growth of *K. alvarezii*. The F/2 medium is literally formulated for growing coastal marine algae especially diatoms [88], while the VS medium is developed for culturing and investigating the life cycle of the freshwater red algae *Bangia atropurpurea* [91].

4.3. Plant growth regulators

The addition of plant growth regulators and their role in seaweed tissue culture have been extensively reviewed [92–94]. Cellular competence to plant hormones in cultivated seaweeds is significant only if the cells possess ability to perceive, transduce, and respond to the hormonal signal [95]. The common plant hormones used in seaweed tissue culture are auxins (indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid), cytokinins (benzyadenine, isopentenyladenine, kinetin), and gibberellins (gibberellic acid). The presence of phytoregulators in tissue culture medium is known to be able to stimulate tissue elongation and contributes to the overall plant growth [15]. Generally, auxins are used to increase protein synthesis, induce morphogenesis, and elicit changes in genetic expression of explants [96], while cytokinins are used to stimulate cell division, enhance metabolic activities, and affect cell differentiation in seaweed tissue cultures [97]. Combination of auxins (α-naphthalenacetic acid and phenylacetic acid) and cytokinins (N^6-(2-isopentenyl) adenine and 6-benzylaminopurine) has been reported to induce the highest callus growth in *K. alvarezii* [98], whereas indole-3-acetic acid and 6-benzylaminopurine in their combination have been revealed to stimulate the regeneration process of *K. alvarezii* [15, 79].

4.4. Organic fertilizers and biostimulant

The organic requirements for axenic seaweed culture are remained unclear although additions of organic complexes (coconut milk, yeast, and algal extracts) to increase the growth rates of seaweed tissue have been reported [99]. Three commercially available formulated fertilizers and biostimulant in global market for seaweed cultivation are Acadian marine plant extract powder (AMPEP), Gofar600 (GF), and natural seaweed extract (NSE). AMPEP is extracted from *Ascophyllum nodosum* [100], while GF and NSE are the mixture extracts of brown seaweeds including *A. nodosum, Sargassum* sp., and *Laminaria* sp. in different ratios of concentration [101]. The use of AMPEP was first reported to successfully induce the regeneration of young plants from different varieties of *Kappaphycus* seaweed [100]. Other studies have also highlighted the positive influence of AMPEP application on the growth and health of *K. alvarezii* cultures both in vitro and in the field [101–104]. Brown seaweed extracts as contained in AMPEP have been discovered to potentially activate the natural defense system of *K. alvarezii* against pathogens and ameliorate the negative impacts of exposure to oxidative bursts, which may result in bleaching of thallus [105].

5. Exogenous factors affecting seaweed tissue culture

Studies on optimizing the growth of economically important seaweeds, especially *Kappaphycus* and *Gracilaria* spp., in tissue culture conditions can help to mass propagate these viable species for continuous, steady, and defined production, while circumventing the barriers of seasonality and environmental vagaries in seedlings production. Several protocols for callus induction and thallus regeneration of wide variety of seaweeds are available in the literatures [3, 85, 106]. A number of studies have also reported direct regeneration of micro-propagules

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from the explants of red algae for maintenance and clonal propagation of maricultural stock [15, 78, 98]. Apart from media composition and supplementation of phytoregulators and fertilizers, the abiotic factors determined to have significant effect on the growth of seaweed tissue cultures are reported to be light intensity, aeration activity, salinity, and pH [15, 83]. The daily growth rate (DGR) of seaweeds in tissue culture optimization was measured and calculated as DGR = $[(W_t/W_0)^{1/t} - 1] \times 100\%$, where W_0 is the initial fresh weight, and W_t is the final fresh weight of the seedlings after t days of culture [107].

5.1. Light intensity

Light source is one of the most important parameters to be optimized in seaweed cultivation. The intensity, wavelength, and spectral quality of light, all influence the photosynthetic productivity of algae. Different strains or varieties of seaweed may exhibit different optimum growth range and tolerance to different light resources. *K. alvarezii* strains from Sabah, Malaysia, have been discovered to achieve optimum growth under photon flux density of 75 μmol photons m^{-2} s^{-1} with DGR of 4.3 ± 0.5% day^{-1} [15]. Various forms of *K. alvarezii* and *E. denticulatum* from the Philippines have been reported grew under irradiances of 25–160 μmol photons m^{-2} s^{-1} with optimum growth in 60 μmol photons m^{-2} s^{-1} [98], while two different strains of *E. denticulatum* and *K. striatus* from Southern Japan have been revealed attained highest growth rate under light irradiance of 145 μmol photons m^{-2} s^{-1} [108]. Light intensity in the range of 5–100 μmol photons m^{-2} s^{-1} is commonly used for tissue culture of *K. alvarezii* and other phycocolloid yielding seaweeds [85, 109]. Explants were found responded to higher light intensity by producing more buds and subsequently leading to an increase in biomass. However, further increase of light intensity was found to be a detriment to the growth of seaweeds as this might be due to the effect of photoinhibition [83]. Nitrogen concentration in seaweed tissues was reported influenced by light intensity, where the increase of light intensity beyond a critical level has resulted in the decreased of crude protein content in seaweeds [110].

5.2. Aeration activity

Aeration is an ideal method of energy transfer; whereby, atmospheric carbon dioxide is diffused into the culture medium. Continuous aeration is an important process to provide enough carbon dioxide for carbon fixation in seaweed metabolism. Meanwhile, carbon source can be provided in the organic form such as glycerol yet the addition of glycerol in the culture medium was found to reduce the morphogenetic capacity and totipotency of the explants [111]. Continuous aeration (at 30.0 L h^{-1}) was found to significantly enhance the growth of *K. alvarezii* with an optimum DGR of 4.2 ± 0.3% day^{-1} [15]. Growth of *Gelidium pulchellum*, another red algae, was reported to achieve maximum rate under continuous light with the support of aeration activity [112]. Mixing of the culture can be accomplished by different mechanisms such as aeration, sparging, pumping, mechanical agitation, or a combination of these depending on the scale and type of cultivation systems [15, 113]. Agitation by mixing is considered as the key parameter for the equal distribution of nutrients and cells in the liquid phase [113], and maintenance of their uniform concentration to increase the mass transfer rate [114]. Growth of cultures is normally enhanced under aerated conditions provided the other culture

requirements (light, photoperiod, salinity, pH, temperature) are remained constant [115, 116]. Appropriate aeration activity may increase thallus exposure to light, eliminate nutrient diffusion barriers, improve gas exchange, and facilitate heat transfer to avoid thermal stratification [15].

5.3. Salinity

Salinity is reported to be one of the factors affecting the growth [15, 80] and exerting strong influences on the photosynthetic capacity [117, 118] of the cultured seaweeds. Prolonged exposure to low salinity may induce stress that led to reduced photosynthetic efficiency, inhibited cell division, and subsequently result in stunted growth and declined in growth rate [80]. *K. alvarezii* explants cultivated in the salinity range of 25–35 ppt have been reported to achieve higher DGR of 4.2 ± 0.4 to 4.7 ± 0.5% day^{-1} as compared to those cultivated in salinities of 20 and 40 ppt with DGR of 2.6 ± 0.3 and 2.2 ± 0.4% day^{-1}, respectively [15]. While *Gracilaria* seaweeds have been found growing well in a wide geographical range with salinities from 15 to 60 ppt, their optimal growth performance was still reported in salinities around 30 ppt [119]. *K. alvarezii, E. denticulatum,* and *G. changii* explants were observed to be unhealthy in the exposure to hyposaline conditions (below 20 ppt) with the formation of ice-ice whitening and bleaching throughout the branches leading to fragmentation and completely damage of the branches [80, 120]. Extremely low salinity may cause oxidative stress in which peroxide may be accumulated in the explants and lead to loss of thallus rigidity as observed in *Gracilaria corticata* under exposure to 15 ppt [121]. Moreover, *K. alvarezii* explants treated in hypersaline conditions (above 40 ppt) have also been reported to exhibit lower growth rate as did those treated in hyposaline conditions [15], where the growth metabolism may be sacrificed near the salinity tolerance limits to carry out osmoregulation for survival in a short period of time [122].

5.4. pH

The ordinary seawater is slightly alkaline (pH ~8) with bicarbonate ions (HCO_3^-) constituted about 91% of total dissolve inorganic carbon (DIC), followed by 8% of carbonate ions (CO_3^{2-}), and 1% of dissolved CO_2 [123]. Alterations in seawater pH may vary the equilibrium of carbonate system and change the concentration of inorganic carbon species [124], subsequently affect the growth of seaweeds which depend on the supply of inorganic carbon for photosynthesis. The pH range for normal growth of most seaweed cultures was reported to be 7–9 with optimum growth in between 8.2 and 8.7 [125]. *K. alvarezii* explants were discovered to attain higher growth rates when cultured in the alkaline conditions (pH 7.5 and 9.5) with respective DGR of 5.5 ± 0.7 and 4.7 ± 0.6% day^{-1} as compared to the acidic condition (pH 5.5) with DGR of 1.2 ± 0.4% day^{-1} [15]. The increased of hydrogen ions (H^+) concentration and decreased of photosynthetic carbon source (HCO_3^-) under acidified condition may severely limit the photosynthesis process of explants and reduced their growth rate [124]. Proteins are the primary effector molecules potentially influenced by environmental conditions and associated with the response to various abiotic stresses. Enzymes involved in biological activities are generally respond immediately to the changed of pH and achieve their highest performance under the optimum pH range [125]. Low growth rate of *K. alvarezii* explants in acidic conditions

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may also due to the denaturation of proteins beyond the tolerance limit, which in turn hinders the cellular physiological and biological processes of seaweeds [15]. However, better growth of purple *K. alvarezii* morphotype in slightly acidic condition (pH 6.7) was reported indicating different *Kappaphycus* varieties may respond differently to pH conditions [104].

6. Optimal growth of seaweed micro-propagules in tissue culture and photobioreactor

In order to maximize the growth of micro-propagules and enhance the productivity of seaweed propagation, incorporation of optimized parameters in their combination in tissue culture system (**Figure 5a**) and application of photobioreactor with optimal growth condition (**Figure 5b**) are highly recommended. Maximum DGRs of *K. alvarezii* and *G. changii* have been reported to achieve 5.5 ± 0.7% day^{-1} [15] and 6.6 ± 1.5% day^{-1} [80], respectively, under optimized tissue culture condition. The growth of *K. alvarezii* micro-propagules has further been increased to 6.5 ± 0.2% day^{-1} in a customized airlift photobioreactor with incorporation of optimized growth parameters [15]. These growth rate achievements were found significantly higher than the earlier reports of 3–4% day^{-1} for the growth of *K. alvarezii* in tissue culture [126] and 1–1.5% day^{-1} for the growth of *G. dura* in vertical polythene-tube-column culture [9].

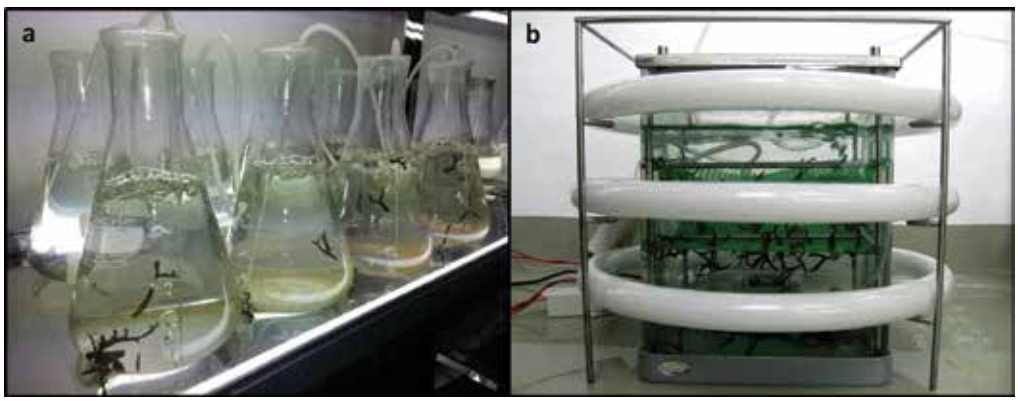

Figure 5. (a) Tissue culture and (b) photobioreactor cultivation of *K. alvarezii* under optimal growth conditions.

Although macroalgal tissue culture is underdeveloped relative to that of land plants, there are more than 85 species of seaweeds from which tissue culture aspects including successful callus formation, plant regeneration, somatic embryogenesis, and thallus development have been reported [92, 127]. Exploitation of seaweed organogenetic potential for the isolation of superior clones has been initiated since the late twentieth century to improve the performance of cultivated species including *Chondrus, Gigartina, Gracilaria,* and *Kappaphycus* [127]. Studies on optimizing the growth of the commercially important seaweeds in tissue culture can help to mass propagate these viable species and open up new opportunities to produce and recover

seaweed products from cell and tissue aggregates in photobioreactor [15]. The development of bioprocess engineering including bioreactor design and identification of strategies for secondary metabolite production can expedite the production of valuable compounds from seaweeds [128] and subsequently derive the maximum benefits from photobioreactor-grown cultures for various industrial applications.

7. Acclimatization of tissue-cultured seedlings prior to farming

While the studies of seaweed tissue culture and micro-propagation have been reported from various literatures, information about acclimatization and successful out-planting of tissue-cultured seedlings are still limited to date. Acclimatization to ex vitro conditions (nursery or glasshouse) is necessary to provide a buffer condition to the seaweed cultures for suitable adaptation before their exposure to the complex open sea environment [9]. Direct planting out of tissue-cultured seaweeds without going through the acclimatization phase may cause stress and shock to the seedlings due to sudden changes in environmental conditions [101]. Therefore, effective acclimatization process is considered to be a key element to enhance the survival rate of tissue-cultured seaweeds after they have been out-planted to the open sea.

Transferring of micro-propagated *K. alvarezii* seedlings from an in vitro flask culture to a partially in vitro tank culture (**Figure 6a**) prior to their acclimatization to outdoor nursery (**Figure 6b**) has been recommended to improve their survival capability and growth performance. Investigation of factors or parameters affecting the DGR of micro-propagated *K. alvarezii* during their acclimatization in outdoor nursery has also been carried out and reported [101]. Through the observation, *K. alvarezii* seedlings were found to achieve optimum growth with DGR of 7.14 ± 0.30% day^{-1} when acclimatized in seawater enriched with mixed-algae fertilizer as formulated in NSE, under daily replenishment of seawater and culture density of 0.40 g L^{-1}. The acclimatization protocol was found to promote faster and healthier growth to

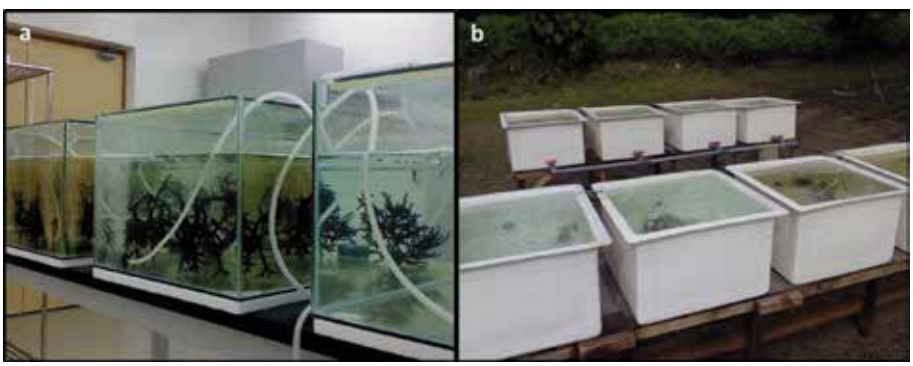

Figure 6. Acclimatization of micro-propagated *K. alvarezii* seedlings in (a) partially in vitro tank culture and (b) outdoor nursery.

K. alvarezii seedlings with DGR of 3.91 ± 0.16% day^{-1} and 83.33 ± 5.77% of survival after they have been out-planted to the open sea.

Furthermore, acclimatization of *G. dura* in outdoor tank culture with continuous aeration and daily replenishment of 2/3 seawater without supplementation of nutrients has been suggested to improve the growth performance of the species prior to their transplantation to open sea [9]. The growth rate of *G. dura* seedlings during their acclimatization phase was reported to achieve 2.25 ± 0.14% day^{-1} as comparable to other outdoor cultures of *Gracilaria* species [129]. Apart from that, the used of perforated polythene bags covered in nylon net bags has been recommended for out-planting of acclimatized seedlings to avoid loss of biomass, which may result from grazing and drifting [9]. The application of floating rafts accompanied with net convening has also been discovered to ease the seeding, maintenance, and harvesting processes of seaweed farming in open sea [130].

8. Quality assessment of tissue-cultured seaweeds

Comparison of the quality between tissue-cultured and conventional cultivated *K. alvarezii* (**Figure 7**) has been reported especially on their growth rate and carrageenan properties [131]. After 60 days of post-cultivation from their first introduction as seedlings in open sea, tissue-cultured *K. alvarezii* has been reported to achieve higher growth rate (6.3 ± 0.1% day^{-1}) as compared to conventional cultivated seaweeds (3.4 ± 0.3% day^{-1}). No epiphytes have been discovered on the tissue-cultured *K. alvarezii,* while the presence of epiphytes and symptoms of "ice-ice" disease were observed on the conventional cultivated seaweeds [101]. From the analyses of their semi-refined carrageenan properties, tissue-cultured *K. alvarezii* was found to produce higher carrageenan yield with significantly better quality in viscosity, gel strength, and sulfate content (**Table 3**) in contrast to conventional cultivated seaweeds [131].

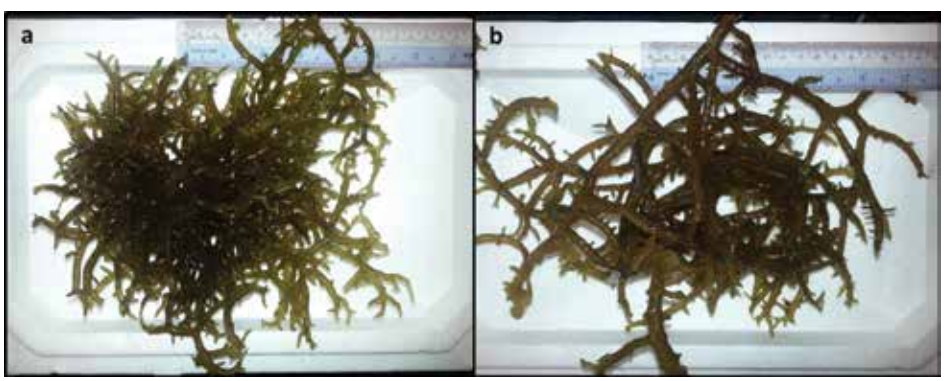

Figure 7. Comparison of growth rate and quality between (a) tissue-cultured and (b) conventional cultivated *K. alvarezii*.

Carrageenan properties	Tissue cultured *K. alvarezii*	Conventional cultivated *K. alvarezii*
Yield (%)	67.3 ± 16.4	51.5 ± 21.0
Viscosity (cP)	1280.0 ± 25.0	87.8 ± 20.9
Gel strength (g cm^{-2})	703.5 ± 14.1	288.3 ± 19.3
Sulphate content (%)	34.2 ± 10.9	7.5 ± 6.7

Table 3. Semi-refined carrageenan properties of tissue cultured and conventional cultivated *Kappaphycus alvarezii* [131].

In terms of other chemical composition, tissue-cultured *K. alvarezii* has been revealed to produce significantly higher total lipids and mineral elements including calcium, magnesium, beryllium, cobalt, copper, lithium, manganese, and zinc against the conventional cultivated seaweeds [132]. Research finding has suggested tissue-cultured seaweeds to be a better food source for consumption and other seaweed-related industries. Bioprocess technology for the production of high-value chemicals such as food additives and biomedicinals from cell and tissue cultures of different macroalgae has been proposed and developed using specially designed photobioreactor [128, 133]. Additionally, new approaches in understanding seaweed physiology, biochemistry, and molecular biology has been anticipated to contribute new insights into human nutrition and enable genetic engineering of favorable agronomic traits to improve the quality and the overall productivity of commercially important seaweeds [134].

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Chapter 12

Seaweeds as Source of New Bioactive Prototypes

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Additional information is available at the end of the chapter

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Abstract

Living organisms endowed with natural benefits have been used for millions of years in the medical practice. Seaweeds have been widely used around the world for the production of agar and food; however, the pharmaceutical industry has drawn attention to the activities of these natural products. In this chapter, we present some bioactive metabolites of the three phyla of seaweed (green, brown, and red algae) along with their potential for drug development.

Keywords: Seaweeds, bioactive compounds, natural products, drug development, bioactivities

1. Introduction

The use of natural resources for medicinal purposes in the treatment and prevention of diseases is one of the oldest practices of mankind. The earliest historical report describing the use of natural derivatives was written and found in Nagpur, India, and is approximately 5000 years old. These records comprise 12 recipes for drug preparation and refer to more than 250 plants [1]. Another historical example is the book written by Emperor Shen Nung circa 2500 BC. This Chinese book describes the use of more than 365 parts of medicinal plants; among these are camphor, the great yellow gentian, ginseng, jimson weed, cinnamon bark, and ephedra [2]. The Ebers Papyrus is one of the oldest and most important medical treatises known in the world. Written in ancient Egypt, it is dated at around 1550 BC and contains more than 700 species of plants and drugs used in therapy, such as pomegranate, castor oil, aloe, senna, garlic, onions, figs, willow, coriander, juniper, and common centaury [3].

The first initiative in the search of natural products of marine origin with pharmacological potential began at a conference in Rhode Island, USA, under the name of "Drugs from the Sea" in 1967. Since this important date, researchers from around the world pledged in search of primary and secondary metabolites of various marine organisms. In 1980, the University of Utah in the USA discovered a toxin derived from cone snail that was able to block a voltage-gated calcium channel. Based on the initial data, a peptide was synthesized and developed by Elan Corporation. The FDA authorized the sale in 2004 of the first drug derived from a marine natural product under the trade name Prialt for the treatment of chronic pain in spinal cord injuries. In 2007, the second drug derived from marine organisms was developed. The isoquinoline derived from the sea squirt *Ecteinascidia turbinate* was approved by the European Union for the treatment of soft tissue sarcoma with the name of Trabectedin / Yondelis [4]. Even today, researchers from around the world are working to isolate, identify, and test promising natural products derived from marine organisms to develop new drugs.

Seaweed is used extensively for development in the industries of cosmetics, fuel production, agar production, and it also serves as animal and human food. However, due to advances in the isolation and structural elucidation of its primary and secondary metabolites, the pharmaceutical industry is turning its attention toward algae. In this section, we describe the natural products derived from seaweed, which have potential for drug development.

2. Marine seaweeds as a source of new bioactive prototypes

2.1. Green seaweeds as a source of new bioactive prototype

The primary metabolites in green seaweeds are more exploited for the development of drugs than the secondary metabolites. Molecules such as peptides, glycolipids, and sulfated polysaccharides have shown interesting results and are in an advanced phase in the drug test. As an example, the depsipeptide Kahalalide F (KF) isolated from the mollusk *Elysia rufescens* that feeds on green seaweeds of the genus *Bryopsis*. For isolation of the peptide, the ethanol extract of animals was chromatographed on a silica flash column, from which the peptide mixture was eluted with EtOAc/MeOH (1:l). A new column using high-performance liquid chromatography (HPLC) on C18 reversed-phase was performed, obtaining the isolation of KF. When measuring their biological activities, KF showed IC_{50} values of 2.5, 0:25, and <1.0 μg/mL against A-549, HT-29, and LOVO cells, respectively. Also observed was interesting antiviral activity against *Herpes simplex* virus type 2 (HSV-2) and antifungal against *Aspergillus oryzae*, *Penicillium* ***chrysogenum*** **(also known as** ***P. notatum***), *Trichophyton mentagrophytes*, *Saccharomyces cerevisiae*, and *Candida albicans* [5]. In 1996, five new depsipeptides (Kahalalide A-E) were isolated from *Elysia rufescens*, which feeds on green seaweed *Bryopsis* sp. [6]. Due to the results obtained previously, the target for the anticancer peptide, KF, has been studied in cultured cells. During the experiment, it was observed that the cells become swollen, due to the formation of large vacuoles, which appeared to be the consequence of changes in lysosomal membranes. Thus, lysosomes are a target for KF action [7]. However, over the years, other mechanisms of action have been proposed. In 2000, KF has also shown activity against human

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prostate cancer xenografts in animal models in vivo [8]. To facilitate the advancement of studies of anticancer activity and toxicity of KF, its synthesis was described [9]. In 2001, a stable parenteral formulation of KF was developed, to be used in early clinical trials [10]. In 2002, preclinical toxicity studies of KF using single- and multiple-dose schedules were done in rats [11]. In 2005, a study was initiated with the objective of determining the maximum tolerated dose, profile of adverse events, and dose-limiting toxicity of KF in patients with androgen refractory prostate cancer. The study concluded that the peptide can be given safely as a one-hour i.v. infusion during five days at a dose of 560 μg/m^2 per day once every three weeks [12]. Also in the Phase I clinical stage, another group found that the maximum tolerated dose was 800 μg/m^2 to patients with advanced solid tumors [13]. Recently, a group evaluated the effect of demographics and pathophysiologically relevant factors on KF pharmacokinetic parameters, however, no clinically relevant covariates were identified [14].

Activity-directed isolation of the n-hexane and dichloromethane fractions from the *Capsosiphon fulvescens* resulted in obtaining three glycolipids (Capsofulvesins A-C) of pharmacological interest. These compounds exhibited IC_{50} values of 53.13 ± 2.83, 51.38 ± 0.90, and 82.54 ± 0.88 μM when measuring AChE inhibitory activity, respectively, and IC_{50} values of >132.28, 114.75 ± 4.13, and 185.55 ± 6.95 μM in BChE assay [15]. A screening for Aldose reductase inhibitors using the ethanol extract of 22 algae was held in South Korea. The green seaweed *Capsosiphon fulvescens* had one of the best results and the fractionation of its extract also resulted in the isolation of Capsofulvesins. Capsofulvesin A and B showed potential rat lens aldose reductase inhibitory activity with the IC_{50} values of 52.53 and 101.92, respectively [16].

Also in primary metabolism, green seaweeds produce sulfated polysaccharides, which are said to ulvans. These molecules have shown interesting immunomodulatory and anticoagulant activities. For example, the sulfated polysaccharides of green seaweed *Enteromorpha prolifera* were used to determine their in vitro and in vivo immunomodulatory activities. In vitro, fractions rich in sulfated polysaccharides increase nitric oxide production and cytokine (TNF-α, IL-6, IL-10, and COX-2) release in Raw 264.7 cells. In vivo, the sulfated polysaccharides increase Con A-induced splenocyte proliferation and IFN-γ and IL-2 secretions [17]. Anticoagulant activity of Sulfated polysaccharides of green seaweed is also disclosed in literature [18].

Among the secondary metabolites, green seaweeds synthesize mainly sterols, alkaloids, and prenylated bromohydroquinones. Historically, the ether extract from green seaweed *Cymopolia barbata* has shown antibiotic and antifungal activities, but no specific compounds were isolated. In 1976, the fractionation was carried out in the same sample, resulting in the isolation of the first seven bromohydroquinones prenylated [19]. In 1987, another eight bromohydroquinones were isolated in the same seaweed, starting the study of biological activities of these substances [20]. A study in north coast of Puerto Rico was conducted with the green seaweed *Cymopolia barbata* leading to the isolation of two bromohydroquinones guided by antimutagenic assay. These compounds were active in inhibiting 2AN mutagenicity toward *Salmonella typhimurium* at doses of 300, 150, and 75 μg/plate [21]. Other prenylated bromohydroquinones have been described over the years [22]. In 2012, the green seaweed *C. barbata* was again collected from Jamaica at Fairy Hill Beach and its extract prepared in dichloromethane:methanol (1:1). The fractionation of the extract resulted in the isolation of two prenylated bromo-

hydroquinones. The anticancer activity of both products was carried out using CCD18 Co, HT29, HepG, and MCF-7 cells. The compound **1** obtained IC_{50} values of 55.65 ± 3.28 and 19.82 ± 0.46 to CCD18 Co and HT29, respectively. Compound **2** did not prove active in all tested cells. Furthermore, the ability of compounds to inhibit the enzyme cytochrome P450 also was evaluated. Compounds **1** and **2** showed an IC_{50} value of 0.93 ± 0.26 and 0.39 ± 0.05 μM, respectively [23].

Alkaloids may be defined as a compound that has nitrogen atom(s) in a cyclic ring. In marine algae, these substances can be classified into: a) Phenylethylamine alkaloids; b) Indole alkaloids; or c) Other alkaloids [24]. In green seaweeds, the indole alkaloids are the main natural products isolated. Caulerpin **(3)** was the first alkaloid isolated from *Caulerpa* genus [25] and its isolation from the substance has shown amazing results. In India, the methanolic from the seaweed *Caulerpa racemosa* was fractionated using column of silica gel (60–120 mesh) and was eluted successively with various percentages of solvent mixtures containing petroleum ether, chloroform, and methanol. The fractions eluted with chloroform-petroleum ether (1: 1) resulted in the isolation of caulerpin, which was used to evaluate the anticorrosion activity using polarization, impedance, and atomic force microscopy assays. A protective layer on the steel surface was observed by electrochemical impedance spectroscopy when treated with caulerpin, demonstrating an anticorrosion effect [26].

In Brazil, caulerpin was used for investigation of their cytotoxicity on Vero cells and antiviral activity against *Herpes simplex* virus type 1 (HSV-1) KOS strain. Caulerpin demonstrated a selectivity index better than the reference drug Acyclovir, with CC_{50} of 1167 μM and EC_{50} of 1.29 μM values. In addition, its mechanism of action was studied on the virus replication cycle. Caulerpin seems to inhibit the alpha and beta phase of replication of HSV-1 virus [27]. Recently, the synthesis of Caulerpin and its analogues has been proposed along its assessment of anti-tuberculosis activity. All compounds exhibited activity against bacillus *Mycobacterium tuberculosis* strain H37Rv. However, Caulerpin demonstrated the best IC_{50} value compared to their analogues and reference drug Rifampin [28].

Steroids are triterpenic compounds having a tetracyclic system; its A, B, and C rings have six carbons while ring D has five carbons. The vast majority of green seaweeds synthesize sterols of 28 and 29 carbons, as an example, with one of the first compounds isolated from *Codium fragile* [29]. Over the years, several sterols were isolated from green seaweeds and have shown interesting biological activities, however, the anticancer activity is the most explored for this type of metabolite. The green seaweed *Tydemania expeditionis* were collected from the Yellow Sea in Weihai, Shandong Province of China. Its partition prepared in EtOAc was subjected to silica gel (200–300 mesh) and eluted with cyclohexane-acetic ether in various proportions. Refractionation using Sephadex LH-20 column resulted in the isolation of four sterols, which have been used to evaluate the anticancer activity in human prostate cancer cells lines (DU145, PC3, and LNCaP). Compound **4** exhibited inhibitory activity against the prostate cancer cells DU145, PC3, and LNCaP with IC_{50} values of 12.38 ± 2.47, 2.14 ± 0.33, and 1.38 ± 0.07 μM, respectively. Compounds **5** showed IC_{50} values of 31.27 ± 1.50, 40.59 ± 3:10, 19.80 ± 3.84 μM. It was noted by researchers that the presence of hydroxyl at C-3 increased the cytotoxic activity of sterols, however, the presence of the hydroxyl in C-24 diminished activity. To investigate if

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the inhibitory activities against prostate cancer cells were due to inhibition of androgen receptor, the binding affinity of sterols was evaluated. Competitive binding assay showed that compound **5** exhibited significant affinity to the androgen receptor with an IC_{50} value of 7.19 ± 0.45 μM, while the compound **4** was inactive [30].

The partition prepared in EtOAc of the green seaweed *Codium iyengarii*, was collected from Karachi cost of Arabian, was subjected to fractionation using silica gel and hexane, chloroform, and methanol at binary mixture or pure, which resulted in the isolation of four sterols. The compound **6** showed the best IC_{50} values when tested against *Corynebacterium diptheriae, Klebsiella pneumonia, Snigella dysentri, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi*, and *Streptococcus pyogenes* microorganisms [31].

Other products found less in green algae also exhibit biological activities described; for example, bromophenols (BPs) found in *Avrainvillea* genre presented inhibitory activity for HMG-CoA reductase enzyme [32].

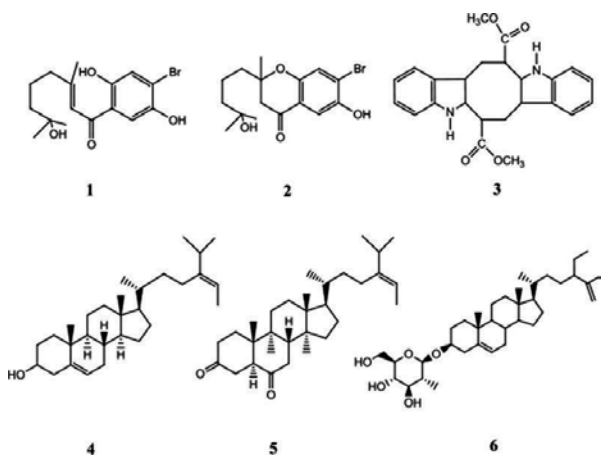

Figure 1. Bioactive compounds isolated from the green seaweeds.

2.2. Brown seaweeds as a source of new bioactive prototypes

Among the primary metabolites of brown seaweeds, molecules as glycolipids and sulfated polysaccharides are used in the search for bioactivities to develop new drugs.

The brown seaweed *Sargassum muticum* was collected in France and its extract prepared using organic solvent chloroform. The chloroform extract was subjected to fractionation using column vacuum chromatography and high pressure liquid chromatography; this resulted in the isolation of galactoglycerolipids with antibacterial activity against *Shewanella putrefaciens* and *Polaribacter irgensii*, and antifungical activity against *Pleurochrysis roscoffensis*, *Exanthe-*

machrysis gayraliae, *Cylindrotheca closterium*, *Navicula jeffreyii*, *Halosphaeriopsis mediosetigera*, *Asteromyces cruciatus*, *Lulworthia uniseptata*, and *Monodictys pelagica* [33].

The brown seaweed *Lobophora variegata* was collected in Mexico and its extract prepared using dichloromethane-methanol (7:3). The extract was dissolved in methanol-water (9:1) and subjected to partitioning using hexane, chloroform, ethyl acetate, and n-butanol. The chloroform fraction was subjected to column chromatography on Sephadex LH20, eluted with hexane-chloroform-methanol (3:2:1), resulting in isolation of three sulfoquinovosyldiacylglycerides. The mixture of sulfoquinovosyldiacylglycerides showed activity against *Entamoeba histolytica*, *Trichomonas vaginalis*, and *Giardia intestinalis* with value of IC_{50} of 3.9 ± 0.03 μg/mL, 8.0 ± 0.42 μg/mL, and 20.9 ± 0.89 μg/mL, respectively [34]. Other activities, such as anticancer and inhibitor of DNA polymerase, have also been described for glycolipids [35, 36].

An experiment performed with fucoidans from the *Laminaria saccharina* was evaluated for its biological activities. Fucoidans from the *L. saccharina* showed the inhibition of neutrophil extravasation into peritoneal cavity in an acute peritonitis rat model at a dose of about 4 mg/kg. Anticoagulant activity was measured as the activated partial thromboplastin time related to the heparin standard. Fucoidans the *L. saccharina* showed an APTT value of 33.0 ± 2 U/mg [37]. In the subsequent article, the mixture fucoidans was fractionated by ion-exchange chromatography that produced two differing fractions. The first fraction consisted of sulfated fucomannoglucuronan and the second fraction consisted mainly of sulfated fucans. The sulfated fucan showed an increased anticoagulant activity with APTT values of 29.2 ± 1.6 [38]. A study in Phase I clinical with fucans from the *Laminaria japonica* investigated orally administered effects on hemostatic parameters in healthy volunteers [39].

Among the secondary metabolites, brown seaweeds synthesize different types of terpenes and phenolic compounds. Among the diterpenes, the brown seaweeds synthesize secondary metabolites with different carbon frameworks including dolabellane, dolastane, prenylated guaiane diterpenes, and meroditerpenes skeletons of interest in drug development.

The brown seaweed, *Dictyota pfaffii*, was collected in Rocas Atoll reef, Brazil, and its extract prepared using the mixture of organic solvents dichloromethane/methanol (7:3). This extract was subjected to silica gel column chromatography eluted with hexane, dichloromethane, ethyl acetate, and methanol at pure or binary mixture. The fraction eluted with dichloromethane pure and dichloromethane/ethyl acetate (9:1) resulting in compound **7**, which was recrystallized from n-hexane and the fraction eluted with dichloromethane/ethyl acetate (6:4) was purified using silica gel column chromatography resulting in compound **8**. Compound **9** was obtained by addition of hydroxyl groups by chemical reaction. The cytotoxicity on Vero cells and HSV-1 antiviral activity of the compounds was evaluated. Compounds **7–9** demonstrate CC_{50} values of 185 ± 5.0, 189 ± 1.2, 184 ± 3.4 μM, and $TCID_{50}$ values of 89 ± 4.5, 87 ± 3.9, 81 ± 4.1%, respectively. Compared to the drug acyclovir, the compounds demonstrated an effective antiviral activity; however, they also showed high cytotoxicity [40]. The mechanism of action of the compound on HSV-1 replication cycle was studied and compound **9** inhibits the initial events in HSV-1 replication and decreases the levels of some early proteins of HSV-1, such as UL-8, RL-1, UL-12, UL-30, and UL-9 [41]. Due to the presence of an interesting antiviral activity, the substance Dolabelladienetriol **(9)** was used to inhibit human immuno-

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deficiency virus (HIV) RT enzyme, with an IC_{50} value of 16.5 ± 4.3 μM. This same compound was also used to assess their ability to inhibit HIV-1 replication in peripheral blood mononuclear cell and macrophages. Compound **9** showed an EC_{50} value of 8.4 μM in PBMC and 1.85 μM in macrophages. Knowing that HIV entry into cells may be mediated by various coreceptor, dolabelladienetriol was used to determine its inhibition of HIV replication at different strains. When used at a concentration of 25 μM, the compound was able to inhibit more than 80% of the replication of all strains of HIV. Tests indicate that the compound **9** can inhibit HIV-1 replication at a posttranscriptional step [42]. Subsequent studies demonstrated that compound **9** blocked the synthesis and integration of HIV-1 provirus and acts as a Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) [43]. In order to develop an antiviral drug, compound **9** was evaluated for their toxicity in vivo. Mice deaths were not observed at any dose during the ten-day period. Significant changes were not observed in the concentrations of urea nitrogen, creatinine, alanine aminotransferase, uric acid, and total protein [44]. In 2014, three news dolabellane-type diterpenoids named dolabelladienols A–C were isolated from the brown seaweed *Dictyoya pffafi*. These compounds have also been shown to inhibit HIV-1 replication in MT-2 cells [45]. The compound Dolabelladienetriol has also proven to be effective in the inhibition of *Leishmania amazonensis* replication in peripheral blood mononuclear cells (IC_{50} = 43.9 μM), even in the presence of factors that exacerbate parasite growth, such as IL-10, TGF-b, and HIV-1 coinfection [46].

The brown seaweed *Canistrocarpus cervicornis* was collected in Rio de Janeiro, Brazil, and its extract prepared using the organic solvent dichloromethane. This extract was subjected to column chromatography resulting in the isolation of two dolastane diterpenes. Both diterpenes have been shown to inhibit the activity of organ crude homogenates and purified Na+K+-ATPase of the kidney and brain. Compound **10** showed the best results [47]. The other two dolastane diterpenes were isolated from brown seaweed *C. cervicornis* and evaluated their cytotoxicity in Vero cells and antiviral activity (HSV-1). The compounds demonstrated a CC_{50} value of 1423 and 706, and the percentage of inhibition of viral replication of 90% and 99% when used 50 μM, respectively. The position of the double bond and the hydroxyl group seems to interfere with the cytotoxicity and antiviral activity of the substances [48]. From the same seaweed, other dalastane diterpene was isolated from the Brazilian coast and showed anticoagulant and antiplatelet effects [49].

The extract prepared in dichloromethane/methanol (1:1) of brown seaweed *Dictyota menstrualis* collected on the Brazilian coast was used to evaluate its antiviral activity (HIV-1). Through isolation guided activity, it was possible to isolate two diterpenes prenylated guaianes. When evaluating the antiviral activity of compounds **19** and **20** by the p24 antigen dosage in the supernatant of PM-1 cells, it was possible to obtain an EC_{50} value of 40 and 70 μM, respectively. Both compounds showed cytotoxicity with values above 200 μM. Seeking to understand the mechanism of action of the substances led to other tests being performed. When treated with 100 μM of the two substances, the synthesis of viral DNA was inhibited. Furthermore, compounds **11** and **12** show IC_{50} values of 10 and 35 μM when evaluating their ability to inhibit the enzyme reverse transcriptase [50]. The results obtained in the previous study investigated the mechanism of action of two diterpenes on the enzyme reverse transcriptase. The kinetic

analyses of the HIV-1 RT demonstrate that both prenylated guaianes have similar mechanisms of inhibition of RNA-dependent DNA-polymerase activity, with the compound **11** being more effective in inhibiting [51]. Subsequently, the compound **11** has also shown antiviral activity against herpes simplex virus type 1 with CC_{50} value of 1000 ± 83 μM and EC_{50} of 1.60 ± 0.08 [52].

The brown seaweed *Padina pavonia* collected from the Red Sea coast in Hurghada, Egypt, was subjected to extraction using methanol 80%, where this extract was partitioned using n-hexane. The n-hexane fraction was chromatographed resulting in the isolation of two xenicane diterpenes, which were used in the assessment of anticancer activity. Compounds **13** and **14** showed IC_{50} values of 13.2 μg/mL and 18.4 μg/mL in H460 cells, respectively, and IC_{50} values greater than 20 μg/mL in HepG2 cells [53].

The extract prepared in dichloromethane of brown seaweed *Stypopodium zonale* was collected on the coast of Tenerife, Spain and fractionated by flash chromatography. The fraction eluted with hexane-ethyl acetate (8:2) resulted in three meroditerpenes. Compound **15** exhibits cytotoxic activity with IC_{50} values of <2.5 μg/mL in HT-29 cells and IC_{50} value of 2.5 μg/mL in H-116 and A549 cells [54]. Meroditerpenes isolated from brown seaweed *Taonia atomaria* also feature an interesting anticancer activity [55]. Meroditerpenes isolated from *Stypopodium zonale* collected on the Brazilian coast showed antiviral activity [56].

Brown seaweeds also synthesize products such as phlorotannins and sesquiterpenes, which can be used in the development of drugs. The brown seaweed *Eisenia bicyclis* was bought in Japan and its extract prepared using the organic solvent Methanol. The extract was partitioned by column system using Diaion HP-2MG. After successive column chromatographic and reversed-phase HPLC, it was possible to obtain the isolation of phlorotannins. Compound **16** exhibits inhibitory activity on glycation and α-amylase enzymes [57], the angiotensin-converting enzyme I inhibitory [58] and inhibits the protein tyrosine phosphatase 1B and α-glucosidase [59].

The brown seaweed *Dictyopteris divaricata* collected at the coast of Qingdao, China, was subjected to extraction using ethanol where this extract was partitioned using ethyl acetate. The EtOAc fraction was chromatographed resulting in the isolation of four sesquiterpenes that were used in the evaluation of anticancer activity. These compounds have shown a moderate anticancer activity, with IC_{50} values above 10 μ against several human cancer cell lines including lung adenocarcinoma (A549), stomach cancer (BGC-823), breast cancer (MCF-7), hepatocellular carcinoma (Bel7402), and colon cancer (HCT-8) cell lines [60]. Six other sesquiterpenes showed a similar IC_{50} value against several human cancer cell lines [61].

2.3. Red seaweeds as a source of new bioactive prototypes

Among the primary metabolites of red seaweeds, some molecules, such as glycolipids and sulfated polysaccharides, are being used in the search for bioactivities in order to develop new drugs.

Activity-guided isolation of red seaweed *Palmaria palmata* resulted in the isolation of ten polar lipids, among them, two sulfoquinovosyldiacylglycerides (SQDGs). The bioactive compounds **25** and **26** were demonstrated nitricoxide inhibitory activity in macrophage RAW264.7 cells

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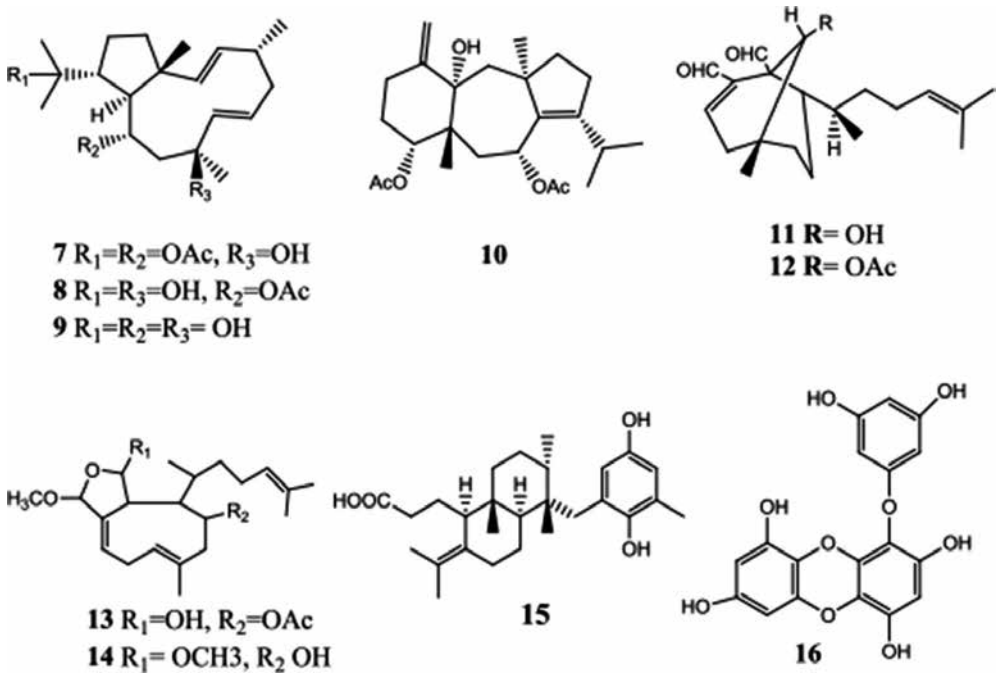

Figure 2. Bioactive compounds isolated from the brown seaweeds.

with IC_{50} values of 36.5 and 11.0 μM. Moreover, the compound **26** also has been shown to inhibit the production of nitric oxide synthase in a dose-dependent manner [62]. Other activities, such as antiviral (HSV-1 and HSV-2) and anticancer, have also been described for glycolipids [63, 64].

Antiviral activity has been a major focus in the study of biological activities of polysaccharides of red algae, because its polysaccharides have shown a low cytotoxicity and high efficiency [65]. The sulfated polysaccharides of red seaweeds *Sphaerococcus coronopifolius* and *Boergeseniella thyoides* collected on the coast of Morocco were used in the investigation of antiviral activity against HSV-1 and HIV-1. Sulfated polysaccharides were capable of inhibiting the HSV-1 on Vero cells with values of EC_{50} of 4.1 and 17.2 μg/mL, respectively. After investigation of the mechanism of action of these substances, sulfated polysaccharides appear to inhibit viral adsorption step of HSV-1. The polysaccharides of *S. coronopifolius* prevents HIV-induced syncytium formation at the lowest concentration tested (12.5 μg/mL), however, the polysaccharides of *B. thyoides* did not demonstrate the same efficiency [66]. Some studies involving the use of carrageenan are in development; for example, a Phase II trial study in the USA has the objective of developing a vaginal gel for reducing the rate of human papilloma virus (HPV) infection [67].

Among the secondary metabolism, red seaweeds synthesize substances of different chemical classes such as terpenes, phenols, and acetogenins. Additionally, species of Rhodophyta are skilled in the incorporation of chlorine and bromine atoms.

The monoterpenes are substances with ten carbons formed by two isoprene units, and can be cyclic or aliphatic (acyclic) [68]. Halogenated monoterpenes are found in genres *Plocamium, Portieria*, and *Ochtodes* [69]. In general, it is believed that in the marine environment, halogenated monoterpenes serve as chemical defense in response to stressor agents, especially herbivores. The production and storage of these metabolites must be related to the survival of algae in the marine environment, and therefore may be potential prototypes for important pharmacological activities [70]. An example of the activity of red algae monoterpenes was described by Chilean researchers from studies with hexane extract of *Plocamium cartilagineum* collected on the coast of Antarctica. The hexane extract was subjected to column chromatography and fractions purified by HPLC, resulting in the isolation of four cyclic halogenated monoterpenes. One of the products **27** showed an intense insecticidal activity against *Heliothis virescens* larvae and moderate activity against *Spodoptera frugiperda*. In the same study, the other monoterpene **28** showed antibacterial activity, resulting in a zone of inhibition of 19.35 mm when used to Gram-negative bacterial strain *Porphyromonas gingivalis*, a major organism responsible for chronic periodontitis [71]. Other activities of monoterpenes belonging to Rhodophyta have been described, for example, to inhibit DNA methyltransferase activity [72], as anticancer [73, 74], and antifungal activities [75].

Sesquiterpenes are natural products with 15 carbons, formed from three isoprene units. The sesquiterpenes are the class of natural products more produced by phylum Rhodophyta, especially by species of *Laurencia*. These secondary metabolites can have various types of carbon skeletons, such as bisabolane, brasilane, chamigrane, cuparane, eudesmane, laurane, and snyderane.

Halogenated sesquiterpenes with bisabolane skeleton are mainly synthesized by species *Laurencia aldingensis* and *Laurencia catarinensis*. Recently, the in vitro production of bisabolanes was developed through genetic engineering techniques and *Saccharomyces cerevisiae* metabolism [76]. This fact aroused the interest of biotechnologists, in view of the possibility of large-scale production. The halogenated bisabolene sesquiterpene **(29)** showed anthelmintic activity against parasitant stage (L4) of *Nippostrongilus brasiliensis* [77].

The chamigrane sesquiterpenes exhibit a *spiro* ring attached to a five-carbon ring. The chamigranes can be divided into: those that contain an epoxide between carbons 5 and 10 [78] and others that do not [79]. The total synthesis of chamigrane sesquiterpene elatol was described, which further stimulated the search for their biological activities [80], taking into account that the elatol is the most potent known natural product with antifouling activity [81, 82]. The elatol **(30)**, isolated from *Laurencia dendroidea*, collected on the northern Rio de Janeiro coast, was tested against the Y strain of *Trypanosoma cruzi*. Elatol showed a dose-dependent effect against the epimastigote, trypomastigote, and amastigote forms, with IC_{50} values of 45.4, 1.38, and 1.01 μM, respectively [83]. The elatol was also obtained from *Laurencia microcladia* and evaluated for their anticancer activity in vitro and in vivo. This substance showed of IC_{50} 1.1 μM in L929 cells and IC_{50} of 10.1 μM in B16F10 cells. It also caused a delay in the transition from the G1/S phase of the cell cycle and induces apoptosis [84].

The cuparanes sesquiterpenes are rarely described in red seaweeds. The great majority of isolates is formed by an aromatic ring attached to a ring structure of five carbon atoms and

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may or may not have double bonds in its interior. In 1996, the synthesis of Cuparene and Cuparenol metabolites has been described from β-cyclogeraniol [85]. Currently, these substances are marketed, which brings a lot of interest as prototypes with biological activities. Two cuparane sesquiterpenes **(31 and 32)** showed good cytotoxicity against two cell types of lung cancer (NSCLC-N6 and A549). This antitumor activity seems to be related to the presence of the phenolic group and the double bond in the five-carbon ring [86].

The eudesmane sesquiterpenes are formed by two rings of six carbons with the isopropyl group at the carbon 7 and a bromine atom at carbon 1. Eudesmane sesquiterpenes also been isolated from brown seaweeds, for example, from *Dictyopteris divaricata* [87]. In red seaweeds, the metabolites 1-bromoselindiene and 9-bromoselindiene **(33 and 34)** were isolated species *Laurencia composita* and demonstrate potent toxicity to brine shrimp [88].

The laurane sesquiterpenes are similar in chemical structure to cuparanes. These molecules have a phenolic group attached to a cyclopentane through carbon 6, where the vast majority has an addition of bromine to carbon 10 or 12. This metabolite class also shows the first examples of iodinated naturally occurring substances [89]. In 2004, a major study was conducted to evaluate the antibacterial activity of secondary metabolites isolated from the genus *Laurencia*. The Dibromohydroxylaurene substance **(35)** showed a minimum inhibitory concentration (MIC) of 1.56 μg/mL against *Streptococcus pyogenes*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* strains [90]. In Greece, 12 sesquiterpenes were isolated from red seaweed *Laurencia microcladia* being investigated for anticancer activity of these metabolites. The 7-hydroxylaurene substance **(36)** showed the best results against K562, MCF7, PC3, HeLa, A431, and CHO cells [91].

The snideranes form the second largest group of sesquiterpenes described for the algae *Laurencia* genre. These sesquiterpenes are widely studied from an ecological point of view since they are constantly being found in the digestive tract of species of mollusc *Aplysia* [92]. Some biological activities have been described for 8-Bromo-10-epi-snyderol substance **(37)**, which showed an IC_{50} value of 2700 and 4000 ng/mL against strains D6 and W2 clones of *Plasmodium falciparum*, respectively [93].

The brasilanes sesquiterpenes were isolated only in the species *Laurencia obtuse* (Hudson) JV Lamouroux, collected on Greek Island Simi [94] and in southern Turkey [95]. Only four brasilanes are known and their biological activities have not been explored.

The diterpenes are composed of 20 carbon atoms, which correspond to four isoprene units. Among the known red seaweeds are more than 20 kinds of diterpene skeletons, with irieane, labdane, and parguerane [96] being the main ones. The first irieane of red seaweeds were isolated in 1975 from the chloroform extract of the genus *Laurencia* and identified by x-ray spectroscopy [97]. In 2010, five populations of an unidentified species of *Laurencia* were collected in Malaysia for chemical research and evaluation of its antibacterial activity. The diterpene 10-acetoxyangasiol **(38)** was isolated and demonstrated an MIC of 250 μg/mL and 100 μg/mL against *Staphylococcus aureus* and *Vibrio cholerae*, respectively [98].

The labdane diterpenes are bicyclic, usually with ramification on carbon 9. Historically, brominated diterpene Ent-13-epiconcinndiol was found in the specie *Chondria tenuissima* [99].

However, today they are found in the genus *Laurencia* in great abundance [100]. Despite already having several isolated molecules, their biological activities have been little explored.

The parguerane diterpenes are formed by a tricyclic structure with six carbons in each cycle. All described skeletons have a standard addition of the bromine atom at carbon 15. The vast majority of pargueranes was isolated from red algae, and has a double bond between carbons 9 and 11 with the addition of a hydroxyl group on carbon 16. In a study from Theuri Island, Japan, the isolation of parguerane-type diterpenes was performed and its anticancer activity was tested in P388 and HeLa cells. The monoacetate parguerane diterpene showed the best results with IC_{50} values of 0.3 and 1.1 μg/mL for HeLa and P388 cells, respectively. The acetoxy group at C2 and bromine at C15 are important for anticancer activity [101].

The triterpenes have 30 carbons in their structure and are derived from six isoprene units [102]. Over 20,000 triterpenoids were isolated and identified in nature, where their structures can be classified into different chemical skeletons, such as squalene, lanostane, dammarane, lupane, oleanane, ursane, and others [103].

The halogenated triterpenoids found in red seaweeds are the type squalene and are known to exhibit excellent anticancer activity. As an example, we can highlight the triterpenoid isolated from red seaweed *Laurencia mariannensis* collected in China. This compound exhibited significant cytotoxic activity against cancer cells P-388, with CC_{50} value of 0.6 μg/mL [104]. In order to obtain products with biological activity, synthesis of some halogenated triterpenes have been proposed, for example, the synthesis of Thyrsiferol **(39)** [105]. Using low concentrations of this product (3 μM), we observed 60% inhibition of Hypoxia-inducible factors-1 in T47D human breast cancer cells. Furthermore, the same natural product has been shown to inhibit the production of messenger RNA of VEGF and GLUT-1 [106].

The acetogenins are derived from the metabolism of fatty acids. The first acetogenin halogenated C15 of red seaweed was isolated from the methanol extract of seaweed *Laurencia glandulifera* [107]. Halogenated aromatic polyketides can be classified as linear or cyclic, where the ring of the cyclic metabolites can range from five to twelve atoms in their structure [108]. On the coast of Spain, *Laurencia marilzae* was collected in the intertidal zone for chemical and biological research evaluation. Its extract was obtained in CH_2Cl_2/MeOH (1: 1) and subjected to column chromatography using Sephadex and HPLC chromatography. The linear acetogenin Adrienyne (Figure 6A) and its isomer were isolated. The biological activity was tested using the A2780 cell line, HBL-100, HeLa, SW1573, T-47D, and WiDr. After the incubation period, the substance showed CC50 greater than 10 μg/mL [108]. Acetogenins cyclic halogenated also have anticancer activity described in the literature [109].

The BPs are substances formed by one or more benzene rings linked to at least one bromine atom. The first BPs isolated from marine organisms were found in red seaweed *Rhodomela larix* [110]. The BPs are known to have various biological activities [111]. The BPs isolated from the polar extract (MeOH:H_2O - 95:5) of *Rhodomela confervoides*, collected off the coast of China were tested against strains of *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli*, and *Pseudomonas aeruginosa*. The ether 2,3-dibromo-4,5-dihydroxybenzyl ether **(41)** showed the

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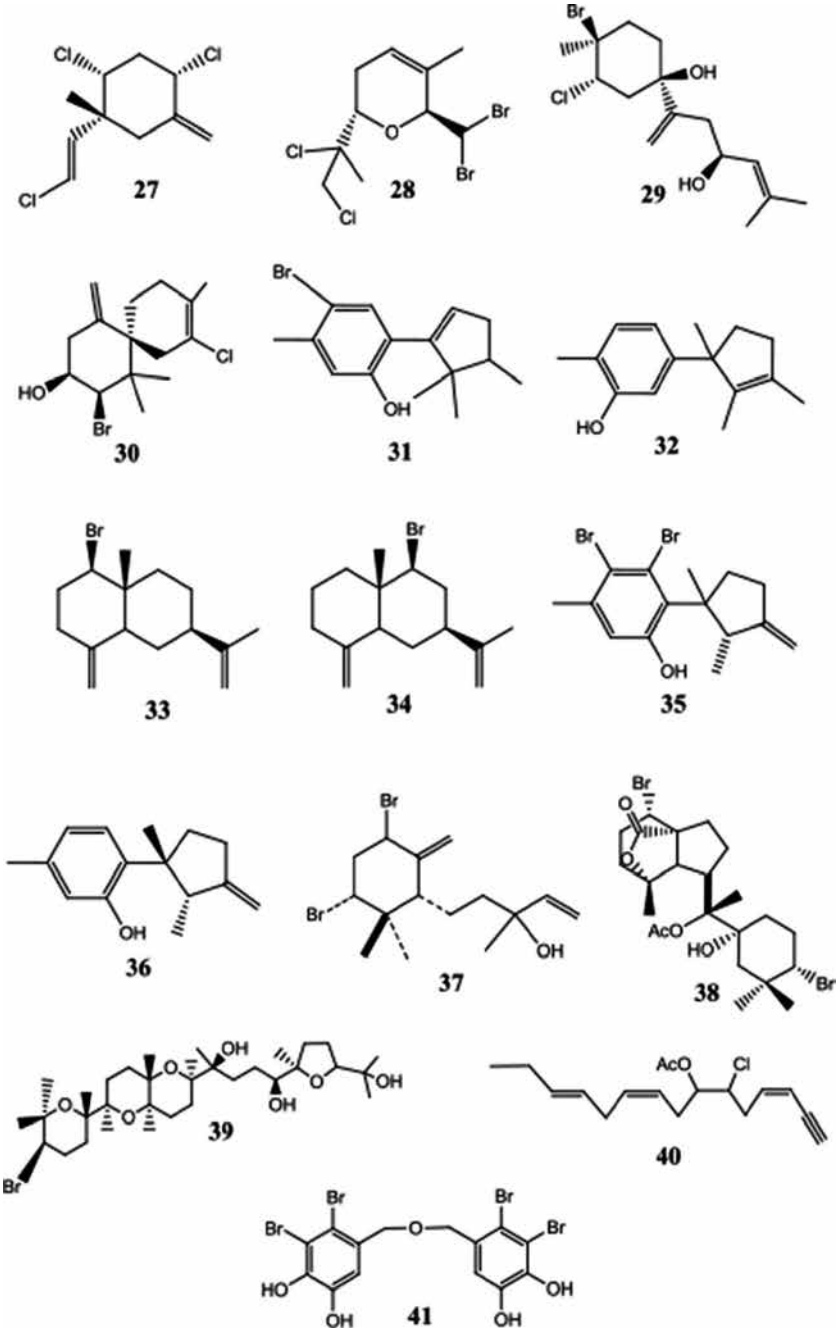

Figure 3. Bioactive compounds isolated from the red seaweeds.

best results [112]. Inhibiting the activity of the enzyme Glucose-6-phosphate dehydrogenase also was described by BPs [113].

3. Conclusion

Based on the work described in this chapter, it is clear that seaweed is endowed with a variety of structurally and chemically diverse metabolites having a broad spectrum of biological activities. Of all natural products presented, KF peptide from green seaweed appears to be the most promising in the development of a new drug, since it has excellent biological activity and a known synthesis pathway. We also believe that Dolabelladienetriol, a dolabellane diterpene isolated from *Dictyota pfaffii*, can be used as an antiviral drug in the future.

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Algae - Organisms for Imminent Biotechnology will be useful source of information on basic and applied aspects of algae for post graduate students, researchers, scientists, agriculturists, and decision makers. The book comprises a total of 12 chapters covering various aspects of algae particularly on microalgal biotechnology, bloom dynamics, photobioreactor design and operation of microalgal mass cultivation, algae used as indicator of water quality, microalgal biosensors for ecological monitoring in aquatic environment, carbon capture and storage by microalgae to enhancing CO_2 removal, synthesis and biotechnological potentials of algal nanoparticles, biofilms, silica-based nanovectors, challenges and opportunities in marine algae, and genetic identification and mass propagation of economically important seaweeds and seaweeds as source of new bioactive prototypes.

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