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Review

Emerging Technologies for the Discovery of Novel Diversity in Cyanobacteria and Algae and the Elucidation of Their Valuable Metabolites

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Abstract: Until recently, the study of cyanobacteria and microalgae has been hampered by the need to cultivate these organisms to gain insight into their cytomorphology, life cycle and molecular biology. However, various microbial species characterized by thick sheaths of exopolymeric substances were difficult to isolate in culture due to their associated symbiotic bacteria. Other microbes evaded culture. Such challenges have now been overcome by the development of metagenomic techniques that allow direct DNA sequencing from environmental samples, as well as high resolution microscopy techniques that permit direct imaging of environmental samples. The sampling of understudied taxa from extreme environments and of toxic species has been facilitated by specialized robotic equipment. Single-cell sequencing has allowed for the proper characterization of microalgal species and their response to environmental changes. Various strains of cyanobacteria, microalgae and macroalgae have gained renewed interest for their high-value metabolites. This paper provides an overview of the emerging technologies and explains how they are being used to identify such strains and their products for industrial application. Advances in genetic engineering and CRISPR technology have facilitated the production of strains that are more amenable to culture, metabolite extraction, scale-up and application in biorefinery approaches. Emerging analytical techniques are discussed, with the advent of multiomics and its application in this field.

Keywords: algal discovery; ancient algal DNA; biomedicine; human health; integrated systematics; phylogenomics; polyphasic approach; natural products; secondary metabolites; transcriptomics

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1. Introduction

New workflows for cyanobacterial and algal taxonomy have developed rapidly over the past fifteen years. In our own studies, the combination of genetic data with traditional morphological methods has allowed for the analysis and discovery of novel diversity from microbial biofilms colonizing under-investigated environments [1–4]. These biofilm-forming cyanobacteria and microalgae were found to be adapted to survive in specific habitats, such as ancient hypogea and coastal rock pools [3,5,6]. Subsequently, the polyphasic approach to cyanobacterial taxonomy, which considers additional features such as ecology and biogeography, led to the description of new cyanobacterial genera and species [7,8].

Although genetic data have been collected for decades, barcode sequences are still lacking for certain taxonomic groups, such as marine algae, from specific geographical locations, such as the Mediterranean Sea [9]. Genetic barcoding of algal germlings from incubated substrata has recently led to the publication of new records of evasive species and revealed cryptic algal diversity from this region [10–13]. Integrative systematic techniques, which replaced traditional taxonomic methods, and additionally considered factors such as

genetics, ecology and biogeography, also led to the discovery of new marine algal species from the central Mediterranean [14].

Cyanobacteria, microalgae and macroalgae have recently gained increasing importance as a sustainable source of nutrition and valuable metabolites, presenting a potential solution to the issue of food security exacerbated by global population growth. Hence, this review outlines emerging technologies which are currently being applied to the discovery and description of novel diversity, such as state-of-the-art sampling, microscopy and high-throughput DNA sequencing (Figure 1). This review also examines novel secondary metabolites, valued for their applicability in industry and whose structure is elucidated via modern chromatographic, spectroscopic and multiomic techniques.

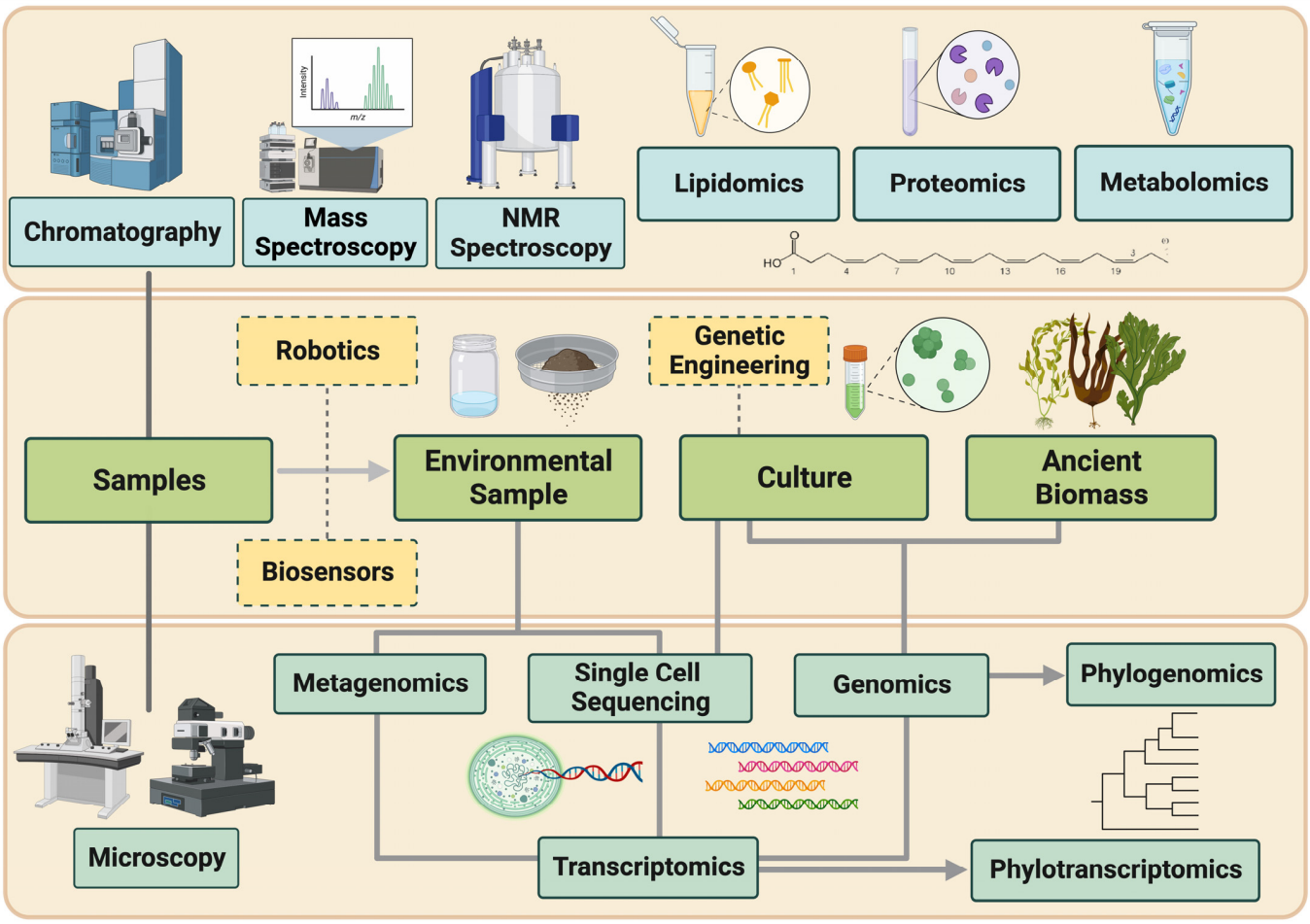

Figure 1. Scheme representing the state-of-the-art, with a focus on emerging techniques that are currently being applied to identify strains of cyanobacteria, microalgae, and macroalgae, as well as their valuable metabolites. Further information is provided in the text.

2. Sampling and Robotics

Recent developments in the field of robotics have enabled a hands-off approach for the sampling of extreme environments and the monitoring of ecological phenomena, including the discovery of new biodiversity and assessment of toxins from cyanobacteria and algae. Examples of these include microorganisms growing in the acidic soils of the Solfatara Crater in Italy [15] and in the acidic waters of the Río Tinto in Spain [16].

Microbial communities perform critical roles in deep-sea habitats, acting as nutrient recyclers and energy providers. Recently, dedicated technologies, including robotic sampling arms and acrylic core samplers have been specifically developed for identifying and characterizing these intricate deep-sea communities. For example, a remotely operated vehicle (ROV) equipped with a robotic arm and a sediment core sampler was employed

to explore an asphalt ecosystem 3000 m deep in the southwestern Atlantic Ocean. This investigation enhanced our understanding of the habitat and its microbial communities, which was previously limited. The samples presented a high diversity of prokaryotes, with the most abundant being members of the Gammaproteobacteria and Crenarchaeota. A group of cyanobacteria, that were previously only recorded in habitats exposed to sunlight was also discovered. The high abundance of cyanobacteria in these samples indicated that they are indigenous to the cold seep environment at the investigated station [17].

In order to transport deep-sea samples, specialized incubators are required. One such container is a pressure-retaining sampler, which is suitable to incubate and transport microbial samples under high pressure (up to 60 MPa). This allows for the sampling of microbes from deep-sea environments up to 6000 m in depth, and prevents sample decompression, which can significantly alter prokaryotic and eukaryotic cell activity, gene expression, and community composition [18]. This ready-to-use sampler was also equipped with advanced features, such as the ability to measure oxygen consumption at high pressures and the use of radio-labeled compounds in accordance with safety standards. This newly developed incubator demonstrated its efficacy in isolating novel barophilic strains from deep-sea environments [18].

Robotic technology is proving to be an invaluable tool for examining algal blooms, which are becoming more frequent worldwide due to climate variability. These blooms present a threat to both the aquatic ecosystem and public health. As a result, autonomous surface vehicles (ASVs) have been designed and constructed for monitoring lakes and reservoirs. To this end, an ASV equipped with a multi-sensor sonde was used to gather water quality data including dissolved oxygen levels, temperature, conductivity, pH, turbidity, and total algae/phycocyanin concentration near the lake's surface. The ASV was also equipped with a bathymetric sonar, enabling the collection of water quality data in relation to bathymetric morphology. Using an ASV and wireless data collection was found to be highly effective in remotely analyzing harmful blooms in freshwater lakes [19].

An environmental sample processor (ESP) was used to conduct real-time surveillance of harmful algae on the Pacific Northwest shelf. The ESP is a durable and cost-effective system that enables in situ sampling of seawater and utilizes molecular probes to detect and analyze target analytes. Additionally, ESP technology can be combined with physicochemical sensors to analyze the conditions in which harmful algal blooms thrive. For example, conductivity–temperature–depth and nutrient sensors can be integrated to the ESP to correlate the relationship between algal bloom toxicity and the nutrients necessary for their growth. An ESP system was incorporated into a moored observatory forsampling and near real-time monitoring of surface waters for toxic *Pseudonitzschia* sp. cells [20].

Harmful algal and cyanobacterial bloom occurrences in Rapa Nui (Easter Island) were investigated, employing a ROV equipped with two laser pointers and a high-resolution video camera recording at 30 fps, with a 1920 × 1080 pixel resolution output. The ROV observations revealed the presence of filamentous biomats enveloping deceased mesophotic reefs. The observed mats consisted of the filamentous cyanobacteria *Lyngbya* and *Pseudanabaena* spp., as well as a brown alga identified as *Ectocarpus* sp., and a green alga belonging to *Cladophora* sp. The observation of healthy reefs in other areas of this island led to the conclusion that the proliferation of these filamentous biomats and the resulting damage of the reefs were associated with anthropogenic effects [21].

3. Microscopy

3.1. Atomic Force Microscopy

Atomic force microscopy (AFM) is a powerful characterization technique, with numerous applications in various biological fields. It can produce nanometer-scale three-dimensional (3D) imaging of cells in their physiological environment, while preserving their function within biological systems. AFM detects interaction forces at a piconewton scale utilizing a sharp scanning probe across the sample's surface. In phycology, AFM is a widely utilized to investigate the morphological characteristics and properties of cyanobacteria and

microalgae [22], as it produces high-quality surface imaging of these cells, allowing for the observation of individual biomolecules without the necessity for fixation and staining [23].

In fact, Novosel et al. (2022) employed AFM to examine how temperature affected *Dunaliella tertiolecta*, *Tetraselmis suecica* and *Cylindrotheca closterium*. The results revealed that all three algae maintained their shape at different temperatures and exhibited no distinctive morphological changes at the cell surface. Studying algal cell morphology was facilitated by AFM, which does not require a vacuum environment for imaging, unlike electron microscopy. This renders it possible to image cells that do not retain their morphology under a vacuum environment [24].

Demir et al. (2021) developed a novel technique using fluidic force microscopy (FluidFM), which combines microfluidics with AFM, to study cell hydrophobicity. The FluidFM utilized a micro-sized channel integrated into the AFM cantilever and connected to a pressure controller creating a closed fluidic system. This system enabled the generation of stable micro-sized bubbles for investigating interactions between these bubbles and the cell surface [25].

AFM was also used to investigate the effects of nanoplastics on *Cylindrotheca closterium* [26]. The imaging revealed that positively charged polystyrene nanoplastics were absorbed through the cell surface membrane, while both positive and negatively charged polystyrene nanoplastics were integrated into the microalgal extracellular polymeric layer (EPS). The EPS provided protection against the nanoplastics by preventing direct contact with the cell membrane. However, the infiltration of nanoplastics into the EPS could pose a substantial threat to microalgae and other taxa [26].

Deniset-Besseau et al. (2021) employed AFM-based infrared spectroscopy (AFM-IR), a novel infrared nanospectroscopy technique, to investigate the organization and formation of lipid body droplets (LBs) in *Parachlorella kessleri*. This study utilized AFM-IR to monitor the production of LBs and their chemical composition in N-starved cells. Triacylglycerol (TAC), the key component of LBs, has an important application in the production of lipid-based biodiesel, a sustainable alternative to fossil fuels [27].

3.2. Cryo-Electron Microscopy

In cryo-electron microscopy (cryo-EM), the sample is rapidly frozen, surrounding it with vitreous ice, enabling the 3D elucidation of biological cells and molecules. Cryo-EM has recently enabled breakthroughs in the 3D analysis of the structure of biomolecules, for instance membrane protein structure. This has led to an exponential increase in the number of small molecules characterized through cryo-EM since it does not necessitate excessive sample manipulation, a large sample size or crystals [28]. It enables ultra-high magnification and the observation of cellular ultrastructure with minimal cell manipulation and preparation. It is also suitable for investigating proteins and their complexes, including those of large molecular weight, without impairing their functional state [29].

One such structure is the cyanobacterial Photosystem I (PSI), a membrane complex that is partly responsible for oxygenic photosynthesis. This photosystem is present in monomeric, trimeric and tetrameric forms in cyanobacteria, which differs from the strictly monomeric form in plants and algae. Cryo-EM was used to analyze the tetrameric form in the cyanobacterium *Chroococcidiopsis* sp. TS-821 for its potential evolutionary, structural and physiological significance. The 3D model reconstruction from 4845 micrographs demonstrated that PSI has two interfaces and is adapted to high light conditions. It might prove to be an intermediate in the evolution of PSI structure in algae and plants [30].

Cryo-EM investigations of *Thermosynechococcus vulcanus* have revealed the phycobilosome's spatial arrangement, consisting of a pentacylindrical core of allophycocyanin and rods containing phycocyanin. This discovery indicated important interactions between linker proteins and chromophores, and the energy transfer pathways from the phycobilisomes to PSI [31]. Cryo-EM was also employed to investigate the phycobilisome with an attached ferredoxin-NADP+oxidoreductase (FNR), which plays an essential role in cyclic

electron transfer in *Synechocystis* sp. PCC 6803. The imaging revealed the phycobilisome's ability for efficient energy transfer within its structure and to PSI [32].

Microcrystal electron diffraction (Micro-ED) is a novel technique that expands the application of cryo-EM by providing high-resolution structural determination of small molecules and proteins, enabling the discovery of potential biomedicines from cyanobacteria and algae [33]. Micro-ED was used in a study conducted to elucidate algal–bacterial symbiotic interactions by characterizing algicidal molecules produced by the marine bacterial symbiont *Phaeobacter inhibens*. The full structural characterization of eight new alkaloids was achieved via the slow evaporation growth of microcrystals on a grid. These algicidal metabolites imply that microbial symbionts can convert precursor molecules into cytotoxins [34].

4. Metagenomics

Metagenomics allows for the reconstruction of (partial) microbial genomes directly from environmental DNA. It has revealed several insights regarding environmental microbe diversity and host microbiomes by eliminating the need for prior cultivation and revealing novel sequences directly from the environment. It has also proven to be advantageous when associating metabolites with genes [35] and when dealing with unusual, difficult-to-culture species, such as microorganisms living in extreme and aphotic environments [36]. Metagenome-assembled genomes (MAGs) from environmental samples have revealed the functional and taxonomic diversity of various microbiota [37].

4.1. Environmental DNA

Metagenomic analyses (Figure 1) have been used to study microbial communities colonizing extreme environments, such as polar lakes. Diverse uncultured polar cyanobacteria have been discovered through metagenomics, providing extensive knowledge regarding the ecology of extremophilic cyanobacteria [35]. Genome-resolved metagenomic methods were employed to study microbial mats in the Arctic, Antarctic and sub-Antarctic regions. Cyanobacteria were found to be the second most abundant microorganism in polar areas. These methods led to the recovery of 37 MAGs, representing 17 different cyanobacterial species. Most MAGs were linked to filamentous taxa of the orders Oscillatoriales and Nostocales. Nostocales are known to dominate the oligotrophic, polar microbial communities due to their nitrogen fixation ability. These results produced new MAGs that were distinct from previously sequenced genomes, demonstrating the significance of genome-resolved metagenomics in understanding cyanobacterial diversity in remote and extreme environments [35].

The metagenomics of phytoplankton from the Red Sea were studied to understand shifts in the microbiome community following an oil spill. Planktonic marine organisms play a crucial role in determining the fate of oil-derived organic pollutants, such as heavy metals in the ocean. Samples were collected for four consecutive days after the accident, to investigate its impact on planktonic organisms including zooplankton and microbiota. The microbial communities were assessed via 16S rRNA gene sequencing and were predominantly composed of *Prochlorococcus* and *Synechococcus* spp. Microzooplankton have been observed to consume crude oil droplets and accumulate toxic oil components within their biomass, resulting in further contamination of the euphotic zone. While oil spills can foster the growth of phytoplankton, the ingestion of oil contaminants serves as the primary conduit for these substances to enter the food chain. Moreover, metagenomic sequencing identified 54 MAGs, 46 of which were attributed to bacteria. A noteworthy rise in bacteria, such as *Alcanivorax* sp., capable of alkaline degradation, was observed. The aftermath of brief oil-spills included detrimental effects on various components of the marine ecosystem, due to imbalances in bacterial and picophytoplankton communities [38].

Metagenomics could provide a valuable approach to investigate environmental cyanobacterial populations, including those responsible for harmful blooms under lentic and lotic environments. Shotgun metagenomic sequencing has been conducted to explore microbial

communities in 12 large rivers across the U.S. Further analysis of cytotoxin-producing cyanobacteria and their seasonal abundance was conducted through amplification and deep sequencing of the microcystin E (mcyE) gene. Multiple microbial communities were identified, with comparable composition and diversity across various locations and times. The most abundant taxa included *Microcystis* and *Planktothrix* spp., which harbored the mcyE gene and produced cytotoxins. These data revealed additional insight into the taxonomic and genotypic diversity of North American rivers, including harmful cyanobacterial species capable of causing toxic blooms [39].

4.2. Microbiomes

Corals are metaorganisms that coexist with microorganisms, such as bacteria and cyanobacteria, in a symbiotic relationship. Microorganisms are primarily responsible for nutrient cycling and the biomineralization of organic materials within the coral reef ecosystem. Metagenomic studies have demonstrated that microorganisms significantly impact the health of coral reef ecosystems [40], and that toxic cyanobacteria can cause coral disease and potential harm to reef ecosystems [41]. Furthermore, metagenomic investigations have identified variations in the coral microbiome in response to environmental change, including pollution [42] and carbon dioxide gradients [43].

Lichens represent another important example of symbiotic associations. Traditionally, they have been thought to involve a fungal partner and one or two phototrophic cyanobacteria or microalgae. Nonetheless, recent metagenomic studies have uncovered complex lichen associations, including both photo and mycobionts, leading to the identification of various new taxa [44].

Recent studies have identified several microbial species within human gut microbiomes that play a critical role in the host's metabolism and overall health. The microbial communities present in the human gut comprise the phyla Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Verrucomicrobia and also Cyanobacteria [45] but genomic sequences from these microbial communities are still being discovered. A large-scale metagenomic assembly was used to reconstruct over 60,000 prokaryotic MAGs from 3810 fecal metagenomes. These served as reference points for 2058 newly identified closely related species. It has been well established that a significant portion of the microbial communities present within the human genome are not represented by cultured isolates. This holds great significance for several research and biotechnological applications [46].

Cyanobacteria are the main source of Beta-N-Methylamino-L-alanine (BMAA) which is one of the neurotoxins associated with amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), and Parkinson dementia complex (PDC). Recently, the presence of BMAA has also been linked to cyanobacteria found in the human intestine. Although intestinal cyanobacteria are typically not abundant, illness and dietary changes may disrupt the balance of intestinal microflora, potentially leading to an overgrowth of cyanobacteria. Thus, investigating the human intestinal microflora further may help identify the cause of neurodegenerative diseases [47]. Nevertheless, additional research is required to assess whether a definitive correlation exists between intestinal cyanobacteria and heightened levels of BMAA in individuals with ALS, AD, and PDC [48].

Alterations in the human gut microbiome have also been linked to chronic liver disease and hepatocellular carcinoma (HCC). The role of the oral microbiome in liver cancer has been examined by analyzing various bacterial 16S rRNA sequences from oral samples of HCC patients. Their analysis revealed 65 genera and 144 species of freshwater, marine, and terrestrial cyanobacteria. Some of these included *Synechococcus*, *Anabaena*, *Anabaenopsis*, *Lyngbya*, *Planktothrix* and *Raphidiopsis* spp., all of which have the potential to produce cytotoxins. This study provided evidence that oral cyanobacteria, which accounted for less than 1% of the overall oral microbiome, may pose a potential risk factor for HCC due to their tumor-promoting effects and their role in dysregulating lipid metabolism [49].

5. Single-Cell Sequencing

Analysis of the genetic material of individual cells (Figure 1), including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) has become possible via single-cell Whole Genome Sequencing (WGS). This technique facilitates the identification and characterization of cellular heterogeneity, providing insights into cellular functions and development. Single-cell sequencing technologies have become essential for describing cell behavior and in recognizing the heterogeneity of gene-expression among individual cells [50].

5.1. Single-Cell Genome Sequencing

Single-cell genomic sequencing has enabled the understanding of both specific and universal mechanisms of environmental adaptation, as well as the coping mechanisms of unicellular organisms [51]. Advances have been made in the isolation of single cyanobacterial cells using a microcapillary pipette under an inverted microscope [52]. Such methods have permitted the genomic sequencing of a unicellular cyanobacterium living in close symbiotic association with marine heterotrophic eukaryotic dinoflagellates of *Ornithocercus magnificus* [52]. This technology has provided new insights into a cryptic lineage of symbiotic cyanobacteria and has generated new data regarding marine microbial ecology [52].

Liu et al. (2022) developed a custom microfluidic device, in conjunction with optical tweezers and an inverted microscope, that facilitated the high-throughput single-cell whole genome sequencing of the filamentous cyanobacterium *Nostoc* sp. CCCryo 231-06.

To maintain cell purity, individual *Nostoc* cells were trapped and placed in separate chambers within the microfluidic device. Each cell was checked for contaminants using the microscope before lysis, amplification, and full genome sequencing. This technique is applicable to other filamentous cyanobacteria and can be used to study extremophiles of interest in astrobiology [53].

Another method that has revealed significant information about microalgae involves the use of fluorescent activated cell sorting. This technique uses a mitotracker and a fluorescent dye to isolate cells. Subsequent single-cell genomics has shed new light on picozoan biology and supports the theory that they lack a plastid and were probably never photosynthetic [54].

5.2. Single-Cell RNA Sequencing

Single-cell RNA sequencing (scRNA-seq) methods measure the gene-expression levels of individual cells. They are effective in the transcriptomic analysis of single cells that are difficult to isolate and cultivate in the lab, thus providing further insight into microbial heterogeneity and cellular processes [55]. This is a potent tool that distinguishes between the gene expression and molecular mechanisms of individual cells, which were previously impossible to understand on bulk microbial populations [56].

An innovative NuGen RNA amplification system was developed for amplifying small amounts of RNA in individual cells. This approach employed both poly(T) and hexamer primers to ensure dependable prokaryotic mRNA amplification. The reliability of this system was confirmed via the newly developed BaSiC RNA-seq technology, which utilizes a micromanipulator and a 10 µm diameter micropipette to isolate single cells of *Synechocystis* sp. This study demonstrated an increased gene-expression heterogeneity in *Synechocystis* sp. cells under nitrogen starvation stress [50].

In another study, *Salmonella enterica* cells were investigated for transcriptional heterogeneity based under varying growth conditions. Bacterial cells were cultivated under different conditions and isolated using a cell-sorting method. The alternate scRNA-seq process, multiple annealing and dC-tailing-based quantitative scRNA-seq (MATQ-seq) [57], was employed for this investigation. The MATQ-seq technique enabled a more efficient acquisition of cDNA from a single cell through the hybridization of primers to internal transcript regions. This allowed for the detection of all transcripts, even those of low abundance, facilitating the robust transcriptomic characterization of single bacterial cells [57].

This method can be applied to other prokaryotes, such as cyanobacteria, as it provides vital transcriptomic reference points.

In another study of the gene expression of low abundance bacteria, researchers developed a hybrid technique of scRNA-seq by combining random displacement amplification sequencing (RamDA-seq) with Cas9-based RNA depletion. A common strain of *Escherichia coli* was harvested and isolated using a single-cell sorting method into 8-well tubes and 96-well plates prefilled with the RamDA cell lysis kit for sequencing. Sequencing libraries were generated from RNA via cDNA amplification from RNA using a library preparation kit. Subsequently, the libraries underwent purification and CRISPR-Cas9-based RNA depletion by introducing a CRISPR-Cas9 reaction-based CRISPRclean Pan-Bacterial rRNA Depletion Kit. This CRISPR-Cas9-based RNA depletion technique enhances the detection of low abundance bacterial RNAs among the captured total RNAs with increased efficiency [58]. This method is also applicable to cyanobacteria.

RNA-seq methods have been used to study unicellular microalgae, such as *Chlamydomonas reinhardtii* to examine gene expression heterogeneity brought about by changes in the environment. Cells were cultured under varying Fe and N concentrations to assess their impact on the cell cycle. RNA-seq analysis of *C. reinhardtii* revealed important stress marker genes expressed by the algal cells. RNA-seq methods can be employed to examine the growth of *Chlamydomonas* in the natural habitat [59].

6. Phylogenomics and Phylotranscriptomics

Phylogenomics has become the method of choice for studying the phylogenetic relationships of cyanobacteria and algae due to its provision of high-throughput data for analysis [60]. Moreover, a stronger classification system for the phylum has been created by implementing a phylogenomic approach to enhance the polyphasic taxonomy for cyanobacteria [61]. This has resulted in the description of new orders and families. Furthermore, recent phylogenomic analysis has elucidated uncertain relationships between different cyanobacterial genera [62]. This approach yields a more comprehensive understanding of the evolutionary history of cyanobacteria.

Phylogenomics has also revealed new insights into diverse morphologically cryptic marine algal taxa [63]. Phylogenies based on plastid genomes have also aided in resolving complex relationships within the red algal order Ceramiales [64]. Additionally, analysis of picozoan genome datasets has led to important discoveries concerning the endosymbiotic origin of plastids [54].

Phylogenomics has provided exciting new insights into the identification of functional genes encoding conserved proteins, which are essential in the production of valuable bioproducts, such as polyunsaturated fatty acids (PUFAs) by *Phaeodactylum tricornutum*. The discovery of such genes promotes a better understanding of microalgal metabolism and potential enhancements for increased yields in algal biorefineries [65].

Recently, the fields of metatranscriptomics and phylotranscriptomics have also gained traction since transcriptomes which are usually smaller than genomes, and thus cheaper to sequence, also provide insight into functional profiles [60]. Phylotranscriptomics constructs phylogenies using DNA sequences obtained from transcriptomes. De novo assembled transcriptomes have been used to investigate gains and losses in metabolic function during algal evolution [66]. Phylotranscriptomic and evolutionary analyses have strongly supported taxonomic relationships for the green algae belonging to the Chaetophorales that are consistent with those from chloroplast genome data [67]. As this field gains momentum, protocols are being published to streamline access to programming and bioinformatics tools for phycologists [68].

7. Ancient Cyanobacteria and Algae

The study of microorganisms, including cyanobacteria and algae, through fossils, archaeological remains and ancient DNA (Figure 1) provides a valuable insight into their history, evolution and interaction with the biosphere.

7.1. Fossils and Archaeological Remains

Chemical and molecular laser mass spectroscopy have been used to investigate both heterotrophic bacterial and cyanobacterial microfossils preserved in rock and gypsum deposits. Biosignatures revealed a well-preserved record of diverse microbes, which proves the viability of this highly efficient technology. In fact, next-generation laser-based mass spectrometers may be applicable for future space exploration of microbial life trapped in Messinian gypsum, providing valuable insights on the search for life on Mars [69].

A catalogue of Pleistocene algal genomes has also been presented that shed light on the different seaweeds used as medicines and food supplements by early humans in Monte Verde, Chile. For this study, a masticated cud formed by a mixture of algae was recovered for DNA extraction. In total, 250 million reads were obtained from 60 brown algae and 24 red algae. The alignment results showed sequences of previously detected algae from this site, including *Macrocystis*, *Sargassum*, *Porphyra*, *Durvillaea* and *Gracilaria* Spp. New algal records such as *Pyropia*, *Ectocarpus* and *Pylaiella* spp., were also revealed, indicating a high diversity of algae preserved for 14,500 years in Monte Verde [70].

7.2. Ancient DNA

Exploring ancient cyanobacterial DNA is crucial for understanding the history of toxicity associated with harmful cyanobacterial blooms. Recent research has recognized the potential for recovering ancient sedimentary DNA (sedaDNA) from both terrestrial soils and aquatic sediments, which can aid in explaining the impact of climate change and human activity on cyanobacterial blooms. Additionally, understanding the ancient history of cyanobacteria provides contextual clues for current-day management strategies [71].

A high-resolution molecular analysis on Lake Tiefer See sediment was conducted to determine the composition, diversity, and abundance of cyanobacterial communities over the past 11,400 years. This study presented the first Holocene record of cyanobacterial DNA, including the toxic genus *Aphanizomenon*. Several high-throughput molecular approaches were employed to explore changes over time, including metagenome sequencing, quantitative polymerase chain reaction assays, amplicon sequence variant analysis, and the detection of cyanobacterial lipid biomarkers. This analysis of ancient DNA recovered important information about the ecosystem, revealing that the most abundant taxa were *Cyanobium* and *Synechococcus*, which formed large blooms. These findings suggested that anthropogenic impacts might have exerted the major influence on the increase of cyanobacterial blooms since the Holocene period [72].

A study regarding the genomics of algal blooms of Anderson Lake in Washington State, USA, yielded a substantial pigment record from the sediment, indicating that a diverse community of toxic algae and cyanobacteria pervaded the lake ecosystem in the past. Using shotgun metagenomic and DNA analysis on sediment samples, this study indicated an increase in cyanobacterial blooms, including the toxic *Dolichospermum* sp. WA102, from the 1950s to 1970s, due to an influx of nutrients from lakeshore farming. These blooms produced toxins that exceeded state recreational water quality guidelines, potentially posing health risks for both humans and animals [73].

A comparable study of sediment cores and sedaDNA from four lakes in New Zealand offered insight into the effects of historical changes on toxic cyanobacterial blooms. The initial Polynesian human settlement in New Zealand occurred around 750 years ago, bringing about several landscape modifications. These intensified during the 1840s following European settlement, leading to the release of fertilizers and the introduction of non-native freshwater species that impacted the native food web. High-throughput sequencing (HTS), metabarcoding analysis and droplet digital polymerase chain reaction (ddPCR), were performed on sediment core samples, to reconstruct cyanobacterial communities over the past 1000 years. These techniques facilitated the amplification of ancient DNA from environmental samples, making them ideal for paleolimnological studies. This study demonstrated that potentially toxic cyanobacteria were not abundant before human migration but increased

in abundance after the first arrival of humans. In addition, a more substantial increase in abundance occurred upon European settlement [71].

8. Metabolomics, Metabolic Profiling and Functional Genomics

Metabolomics (Figure 1) is a technological platform designed for analyzing a specific set of metabolites, providing the capacity to examine the physiology of an organism, organ, or cell. It complements other systems toolboxes, including genomics, transcriptomics and proteomics. Emerging metabolomic techniques utilize high throughput separation methods, such as capillary electrophoresis (CE), gas or liquid chromatography (GC or LC), coupled with compound identification and quantification tools based on mass spectrometry (MS) or nuclear magnetic resonance (NMR) [74]. The goal is to obtain comprehensive data sets for the quantification of cellular metabolites in either relative or absolute terms. Identifying and quantifying specific cellular metabolites through a MS fragment pattern poses a challenge due to the complexity of the metabolome [75].

Genomic analysis through PCR, Sanger sequencing and WGS has revealed the presence of numerous biosynthetic gene clusters (BGCs). Recent studies have extensively screened cyanobacteria for the presence of non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) genes through the methods mentioned above. One such study targeted the halogenase VirX1 found in cyanophage syn10, which infects *Synechococcus* and *Prochlorococcus.* This halogenase was investigated to uncover its natural substrates [76]. Other studies resulted in advances in the screening of cyanobacterial genomes, whether in vivo through PCR or in silico using genome-mining techniques [77].

8.1. Metabolite Target Analysis

This metabolomic approach quantitatively analyzes specific metabolites that are linked by shared reactions or pathways. For instance, cyanobacteria produce bioactive secondary metabolites, including cyclic peptides, polyketides, alkaloids and amino acid derivatives. These compounds exhibit a range of toxicities and are often identified by employing techniques such as high-performance liquid chromatography (HPLC) and NMR spectroscopy coupled with MS identification (Figure 1) [78].

8.2. Metabolite Profiling

Metabolite profiling generates spectra that can be used to identify microorganisms. A standard profiling technique involves utilizing isolated fatty acids, which are converted into methyl esters and then analyzed by GC coupled to the classical flame or MS-based detection methods. Chemotaxonomic markers then allow for bacterial classification via libraries of fatty acid methyl esters. This technology has been utilized to analyze various cyanobacteria, including *Aphanizomenon*, *Microcystis*, *Nostoc*, and *Planktothrix* spp. [79]. In a study focused on the unicellular alga *Chlorella*, alcohol was rapidly mixed with cells from the photoreactor using an outlet valve to ensure rapid metabolic inactivation and enable the profiling of the cyanobacterial metabolome [80].

The discovery of new natural products has also progressed via mass spectrometric metabolic profiling used in combination with genomic analysis. This approach led to the identification of columbamides, a new class of di- and tri-chlorinated acyl amides possessing cannabinomimetic activity. Following cell culture, the cyanobacterial strains *Moorea producens* 3L, *M. producens* JHB and *M. bouillonii* PNG underwent genome sequencing and were analyzed for any detectable biosynthetic pathways. The resulting data were compared with the corresponding metabolomes identified through MS profiling. Genome analysis allowed for the identification of a putative regulatory domain upstream of several previously reported BGCs in *M. producens* 3L and *M. producens* JHB. The *M. bouillonii* PNG genome unveiled a comparable regulatory domain [81].

8.3. Metabolite Fingerprinting

Metabolite fingerprinting aims to quantify intracellular metabolite pools (endometabolome), including unknown metabolites, in a high-throughput and non-targeted manner. In cyanobacteria, metabolic fingerprinting attempts have separated low molecular weight metabolites utilizing CE, GC, or LC often coupled with MS detection and identification. Researchers have evaluated the growth of *Synechocystis* 6803 under varying CO_2 concentrations for both wild-type cells and mutants lacking photorespiratory enzymes [82]. The resulting metabolic fingerprint displayed clear differences depending on the CO_2 concentration and/or genotype, indicating their usefulness for analyzing changes in cyanobacterial metabolism due to different growth conditions or epigenetics [74].

8.4. Metabolite Footprinting

In contrast to the previous technique, metabolite footprinting aims to analyze the content of metabolites secreted or excreted from cells (exo-metabolome) through high throughput stable isotope labeling combined with LC-MS or GC-MS [74]. Cyanobacteria release and take up metabolites, producing a metabolic footprint in culture media. To obtain distinct metabolic profiles of the endo- and exo-metabolomes, modern metabolomic sampling techniques are required to separate suspended cells from the surrounding liquid medium [83]. This was essential to ensure the proper evaluation of metabolite uptake and release by axenic cells of *Synechococcus* sp. PCC 7002, which was found to metabolize compounds such as histidine betaine and g-glutamylphenylalanine, which had not been previously reported as cyanobacterial metabolites. This led to the conclusion that the cyanobacterial strain benefited from the organic nutrients that were released into the medium due to cell lysis following stress, viral attack, or predation [83].

8.5. Flux Analysis

The systematic approach currently being utilized is the evaluation of stable isotopes (e.g. ^{13}C, ^{15}N, ^{18}O) by mathematical modeling through flux analysis (fluxomics) in combination with steady-state metabolomics. This approach enables a comprehensive understanding of cellular metabolic networks and their adaptability in a wide range of organisms under different conditions. ^{13}C-bicarbonate pulse labeling has been employed to identify the primary carbon fixation pathways in *Synechocystis* sp. 6803. Moreover stable isotope labeling has been combined with a modeling approach, using the isotopically non-stationary metabolic flux analysis (INST-MFA). This enabled the analysis of isotopomer patterns, providing a detailed, quantitative description of CO_2 fixation in cells of *Synechocystis* sp. This was fundamental to kinetic modeling of photoautotrophic growth solely dependent on ^{13}C flux measurements evaluated with respect to potential targets for the optimization of photosynthetic carbon fixation to support biotechnological applications [80,84].

9. The OMICS Approach

Genome sequences are crucial for reconstructing metabolic pathways. In contrast to traditional genomics, metagenomics targets entire microbial communities, providing additional insights from DNA data directly from environmental samples [85]. Additionally, metabarcoding targets a specific DNA sequence as a marker for the diversity and phylogenetic mapping of the organisms under investigation. Consequently, cyanobacterial and algal communities can be easily identified and characterized via these approaches [85].

On the other hand, synthetic biology, an omics technology, uses a stepwise approach for designing an organism capable of producing a desired product [86]. The genomics approach can help identify BGCs via bioinformatic genome annotation and offer insights into the potential of various microbial strains to produce desirable compounds [85].

9.1. Proteomics

Proteomics (Figure 1) is a valuable tool for investigating changes in metabolomes of cyanobacteria and algae. This is achieved through analyzing protein expression and post-

translational modifications (PTMs) [87]. Comprehensive understanding of photosynthetic apparatus biogenesis, assembly and dynamics has been attained by scrutinizing the sub-proteomes of the thylakoid membranes and cytoplasm [88]. Isobaric tags for relative and absolute quantification (iTRAQ) are a frequently utilized method for quantitatively analyzing the cyanobacterial proteome [89]. Isobaric reagents effectively bind to primary amine groups in proteins and peptides [90]. PTMs, such as phosphorylation and acetylation, are also analyzed using this approach [91]. Within cyanobacterial cells, acetylation has a significant impact on the regulation of proteins, specifically in terms of protein interaction, stability, enzymatic activity and the localization of proteins [90].

A total of 776 acetylation sites were identified via a global analysis of the lysine acetylome in *Synechocystis* sp. PCC 6803. Acetylated proteins were found to be prevalent in cellular metabolism, particularly within phycobilisome subunits [92]. A phosphoproteomic investigation of *Synechococcus* sp. PCC 7002 revealed a total of 410 sites where phosphorylation occurred on phosphoproteins. It was shown that these sites played a vital role in essential molecular processes, including two-component signal transduction pathways and photosynthesis [93].

9.2. Integrated Omics (Multi-Omics)

To enhance understanding of cyanobacterial cellular physiology and metabolic capabilities, Amer and Baidoo (2021) proposed utilizing multi-omics [94]. However, in environmental biofilms and microbial mats, Proteobacteria and Bacteroidetes tend to adhere to cyanobacteria due to the trophic interactions and the presence of the EPS [3]. Therefore, meta-omics are progressively becoming more important in cyanobacterial research [87].

A study was conducted to examine intracellular responses to the synthesis of the valuable molecule 3-hydroxypropionate-(3-HP) produced by *Synechocystis* sp. PCC 6803 via these proteomic and metabolomic approaches. This study demonstrated that cellular processes were differentially regulated, and that the overexpression of certain transporter genes resulted in the increased production of 3-HP. This systems biology approach is currently being employed for multi-omics investigations of cyanobacteria acting as hosts for biotechnological applications [89,90].

An integrated omics database for *Synechococcus* sp. PCC 7002, called CyanOmics, has been established. This database encompasses the entire genome sequence, along with functional annotation, metabolomics, transcriptomics, and proteomics, all analyzed under variable conditions [95]. Genome-scale metabolic models (GSMMs), which are structural models based on stoichiometry that offer insight into metabolic networks, are being combined with protein information to facilitate for structural bioinformatics analysis from a systems-level perspective [96,97].

Further research has emphasized that cyanobacterial adaptation strategies focus on optimizing light capture and utilization, while making negligible changes to other metabolic pathways. Incorporating the global proteome with the genome sequence played a crucial role in recording these outcomes. Quantitative proteomics has facilitated a comprehensive understanding of the resilience and adaptability of metabolic pathways to diverse environmental triggers, such as those found in *Halomicronema hongdechloris* cells [98].

Specific metabolic pathways in microalgae such as *Chlamydomonas reinhardtii*, have been mapped following exposure to the anti-proliferative drug rapamycin under differing growth conditions. Partially time-resolved unlabeled metabolomic and transcriptomic data have been included into the time-resolved expression and metabolite-based prediction of flux values (TREM-Flux), providing a novel approach to metabolic modeling. This approach facilitates the forecasting of steady-state fluxes that mirror systemic physiological responses to environmental stimuli [99].

Numerous algorithms, tools and databases are currently in development due to the enhanced significance of multi-omics data. Two notable examples are GECKO (GEMs with enzymatic constraints using kinetic and omics data) and REMI (relative expression and metabolomics integrations). These methods are rapidly evolving to incorporate omics data

with metabolic modeling, utilizing advanced statistical approaches such as multivariate data analysis and data depictions via correlation maps [100,101]. Bacterial genomic and metabolomic data have been integrated into the DeepRiPP database to expedite the discovery of novel ribosomally-synthesized post-translationally modified peptides (RiPPs) [102].

Looking forward, incorporating artificial intelligence and machine learning (AI/ML) techniques with systemic biology and omics data sets is essential. These methods can efficiently and precisely produce designer strains of cyanobacteria and algae for biotechnology by generating innovative information that offers novel insights on cellular regulation at varying organizational levels [90].

10. Genetic Engineering

Advances in molecular technologies have established the foundation for genome editing. These techniques encompass three main approaches: zinc-finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats-CRISPR-associated protein (CRISPR-Cas) [103]. ZFNs comprise zinc-finger proteins with the non-specific DNA cleavage domain of the *FokI* restriction endonuclease. The technique activates the DNA damage response pathways by inducing targeted DNA double-strand breaks (DSBs) using ZFN dimers. TALENs function similarly, with the *FokI* cleavage domain combining with DNA-binding domains from transcriptional activator-like effector (TALE) proteins. The TALE array recognizes a single base pair, leading to the activation of the DNA damage response pathway [103].

CRISPR-Cas

Recently, the CRISPR-Cas genome-editing technique has been applied to microalgae as it presents great versatility, adaptability, and ease of use. The type II system, which utilizes Cas9, generates genomic breaks in a sequence-specific manner through recognition tools [104]. *Chlamydomonas reinhardtii* served as the initial model for studying the effects of gene manipulation on the production of bioactive products such as biofuels, biopeptides, pigments, nutraceuticals, and drug leads [104]. The approach involved visually analyzing the phenotypic color of microalgal colonies [105]. In 2016, a study examined the genes responsible for producing green pigment as a demonstration of algal CRISPR transformation [106]. When the genes encoding phytoene synthase 1 (*PSY1*) or the chloroplastic FTSY (*CpFTSY*) were knocked down or disrupted, a visible change in the pigment occurred within the colonies [106,107]. To date, other microalgal species, such as *Nannochloropsis oceanica*, *Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and *Chlorella vulgaris* have also undergone genetic modification via this genome-editing technique [108–111].

The establishment of an inducible CRISPRi gene repression library in *Synechocystis* sp. PCC 6803 has contributed to a greater understanding of the relationship between cyanobacterial genotype and phenotype [112]. This library contains 10,498 clones of the aforementioned species, each featuring Cas9 and a single-guide RNA (sgRNA) that targets a protein-coding open reading frame (ORF) or a non-coding RNA (ncRNA) [112]. The clone quantities within this library are monitored via a barcode. The sgRNA's protospacer region, which is only about 20 nucleotides in size, permits next-generation sequencing (NGS) [112]. The findings revealed pathways employed by cyanobacterial cells during physiological difficulty. When evaluating the necessity of genes using CRISPRi libraries, the gene's position on the operon is given due consideration. To enhance efficiency, viable knockdowns were explored by applying a CRISPRi library coupled with a fluorescence assay [112].

The cyanobacterium *Synechococcus elongatus* PCC 7942 was utilized to identify the critical genes required for cells that were grown under alternating light-dark conditions or continuous light. Fitness was compared under both conditions by screening a transposon library, establishing a crucial link between circadian rhythms and the metabolic processes necessary for proper cell functioning [113].

CRISPR-Cas technology has been utilized to investigate RNA-directed transposition in Tn7-like elements from cyanobacteria. A new type I-D CRISPR-Cas system has been developed that incorporates RNA-guided transposition. The experimental validation in *E. coli* was performed using a transposon system from a desert cyanobacterium, *Myxocorys californica* WJT36-NPBG1.

This study revealed that the newly developed I-D Mc-CRISPR-associated transposon (CAST) system does not necessitate an active Cas6d protein for RNA-directed transposition. Interestingly, naturally fused transposase proteins in the I-D CRISPR-Cas transposon are functional in cut-and-paste transposition. Given the unique features of the I-D system, it presents exceptional prospects for future research in gene editing. This research implemented bioinformatic analysis to enhance current understanding of the evolution of Tn7-like elements. These findings demonstrated extensive swapping of targeting systems among Tn7-like elements in cyanobacteria [114].

11. HiTES and Chemical Biology

A high-throughput elicitor screening (HiTES) approach utilizes a reporter gene within the gene cluster of interest to screen and identify small molecule libraries against the reporter strain. Once identified, the resultant small molecule product can be characterized and the silent/cryptic BGCs regulated [115]. These specific types of BGCs have recently been discovered in bacteria through innovative techniques like NGS and bioinformatic identification [116]. One drawback of relying solely on HiTES is the inability to connect bioactivity with the cryptic metabolite or the BGCs.

Moon and colleagues (2019) introduced Bioactivity-HiTES, a combination approach for identifying cryptic metabolites possessing a desired bioactivity. This involves using an elicitor to activate BGCs, in combination with activity-guided fractionation to isolate and characterize these cryptic metabolites [117]. Cryptic antibiotics were induced in three strains of actinomycetes: *Streptomyces lavendulae*, *Amycolatopsis kerathiniphila* and *Streptomyces hiroshimensis*. Further downstream analysis allowed for the structural elucidation of two cryptic metabolites (taylorflavins A and B), elicited by the β-blocker atenolol in *S. hiroshimensis*, with selective growth-inhibitory activity against Gram-negative bacteria, notably *Escherichia coli* and *Acinetobacter baumannii*. A similar technique employed by Xu et al. (2019) utilizes imaging mass spectroscopy (IMS) to investigate cryptic bacterial metabolomes under various conditions. This study demonstrated the applicability of the HiTES-IMS approach across diverse bacteria and led to the discovery of nine cryptic metabolites with potentially therapeutic bioactivities, including a new inhibitory glycopeptide chemotype activity against a pathogenic virus. The bioactivity-HiTES and HiTES-IMS methods are widely applicable to other microorganisms, including cyanobacteria and microalgae [117,118].

Advances in MS have led to the development of specific techniques, like matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI), and IMS, to enhance the detection and location of secondary metabolites related to the morphology of cyanobacteria and macroalgae [119]. Technological advances have enabled researchers to sample smaller amounts of biological material and bioproducts, thereby allowing for the investigation of microscopic marine species like microalgae. Grindberg and colleagues (2011) utilized a two-pronged method of single-cell genomic sequencing via multiple displacement amplification (MDA) and metagenomic library screening, ultimately obtaining access to the BGC responsible for producing apratoxin A, a promising anti-cancer agent, within the marine cyanobacterium *Moorea bouillonii* [120]. Chemical structures of small molecules are being determined on a nanomolar scale through advanced MS techniques and algorithms that are being utilized in conjunction with MicroCryoProbeTM or flow-cell NMR spectrometers [121]. The MicroCryoProbeTM allows for the examination of environmental and/or non-culturable microorganisms, and biosynthetic studies of slow-growing microbes [122].

Studies of the structure of cyclic peptides are crucial due to their potential use as drugs and biomedicine. Tandem MS is preferred for analyzing complex samples. Mohimani et al. (2011) developed a cyclic peptide sequencing web program and Cycloquest database for identifying the chemical composition of cyclic peptide products and deciphering their unique fragmentation patterns [123]. This research has been evaluated on microorganisms such as *Bacillus subtilis* and is also applicable to other microbes.

12. Microsensors

Optical and electrochemical microsensors, with a sensory tip diameter of just a few microns, can measure microscale gradients with high sensitivity, specificity and selectivity [124]. These sensors provide valuable data about microalgal biofilms and the EPS, composed of polysaccharides, proteins, nucleic acids and lipids. Understanding microalgal biofilms and microbial mats enables the quantification of reaction kinetics and mass balances, as well as the discovery of active metabolic pathways. Key metabolic analytes and biofilm characteristics that significantly fluctuate daily include the pH, dissolved oxygen levels, temperature and redox potential [125].

12.1. Oxygen Sensors

Luminescence-based fiber-optic oxygen microsensors were utilized on a *Chlorella sorokiniana* biofilm to gauge the rate of oxygen uptake at different timepoints, disclosing the corresponding biofilm thickness [126]. These fiber-optic microsensors possess numerous advantages over other electrochemical devices, such as zero oxygen consumption, a lack of sensitivity to interfering agents and a faster response time [127]. In a recent study by Zhong et al. (2019), a D-type plastic optical microsensor was developed to effectively monitor the progression and phenol degradation of a *Chlorella vulgaris* biofilm [128]. The oxygen sensor, which is based on a Clark electrode, measures the catalyzed oxygen reduction reaction at a platinum electrode covered with a membrane. In turn, it is separated from the environment by an oxygen-permeable membrane to reduce fouling [129]. Rincon et al. (2019) employed oxygen microelectrodes to track photosynthetic activity in *Chlorella vulgaris* biofilms [130].

12.2. Temperature Sensors

Heat production in microbial cells and biofilms, along with resulting temperature changes, lead to metabolic alterations [124]. The Bragg grating sensor, a fiber-optic sensor capable of detecting temperature fluctuations with an accuracy of 1.1 °C, was developed to specifically monitor biohydrogen production and temperature changes in biofilms [131]. This fiber-optic sensor allows for measurable dynamics, as it undergoes changes in light scattering properties in response to temperature fluctuations [124]. A study by Boukazia et al. (2021) utilized a thermal excitation sensor to detect biofouling by measuring changes in thermal resistance during the development of the biofilm EPS [132].

12.3. pH Sensors

pH is another key factor in determining the physical and structural properties of biofilms [124]. Babauta et al. (2012) utilized multiple microelectrodes to measure the pH and redox potentials in respiring biofilms of *Geobacter sulfurreducens*. This study aimed to quantitatively assess electron and proton transfer occurring in these types of biofilms [133].

12.4. Biosensors

A biosensor is an analytical device that contains a biorecognition element, such as antibodies, enzymes, nucleic acids, and aptamers, as well as a transducer that converts the recognition element into a measurable signal using optical, electrochemical, thermometric, or piezoelectric technology [134,135]. Biosensors are being used in the discovery of drugs and bioactive compounds [135,136]. Páscoa and colleagues (2023) developed a novel whole-cell biosensor that incorporated reporter genes for signal intensity and stability, a promoter

to modulate Fluc expression, and assay multiplexing to improve efficiency and output. The sensor's selectivity was analyzed using clotrimazole and validated by recognizing fragments of cyanobacterial extracts possessing peroxisome proliferator-activated receptors (PPARs) agonist properties. Multiplex and uniplex screening assays were conducted to determine which PPARs the bioactive extracts were targeting [136].

Another study involved the construction of a surface-enhanced Raman scattering (SERS) biosensor for detecting microcystin-LR, a cyanobacterial biotoxin. Particles with a plasmonic gold nanostar core containing Raman reporter molecules (4-nitrothiophenol) were developed. The core was embedded within a protective silica shell [137].

13. Conclusions

Although the outlined technologies have enabled a better understanding of cyanobacteria and algae, their biodiversity and their valuable metabolites, they are not a panacea, as challenges and limitations prevail.

For instance, metagenomics can provide a snapshot of the genetic diversity within a microbial community, but it might not capture all the organisms present. Certain microbes may be low in abundance or may have genomes that are difficult to sequence, leading to an underrepresentation of specific strains or individual microbial cells within a community. While metagenomics can identify genes and gene families, it may be difficult to accurately assign their functions, as many of these are still unknown or poorly characterized, making it difficult to understand the metabolic capabilities of a community [138]. Therefore, other techniques, such as metatranscriptomics and single-cell genomics, as previously discussed, may be required for finer-scale analysis. Additionally, metagenomics does not reveal the spatial organization of microorganisms within an environment, making high-resolution microscopy techniques particularly useful in this sense. Additionally, digital documentation of the cytomorphology of organisms from environmental samples is essential for their proper classification, especially for organisms that do not survive in culture.

However, microscopy may be hindered by cellular structure, as well as low-contrast cellular components. For AFM observation, immobilizing the cells on a surface could be challenging and may alter their natural state. Interaction forces between the AFM tip and cells could result in soft structure deformation, causing a change in cell shape. While a non-contact mode is an option, it is less precise. The lateral resolution is typically limited to a few nanometers, which may be inadequate to elucidate the finer details of subcellular components. Cell variability may also hinder the attainment of representative images.

The availability of high-quality reference genomes for cyanobacteria and algae is limited compared to more well-studied organisms. In addition, many of their genes have functions that are unknown or poorly characterized, thereby complicating the interpretation of the functional implications of omics data. This could hinder the accuracy of omics analyses, particularly when mapping sequences or identifying genes and pathways. It can also be challenging to accurately identify conserved genes and pathways across different strains when a high genetic diversity may exist within a single cyanobacterial or microalgal species. Differences in growth parameters such as light, temperature, nutrients, and other factors can lead to variations in gene expression, protein profiles, and metabolite levels, making omics data highly dependent on these conditions.

Metabolomic studies may not fully capture all metabolites produced by cyanobacteria and microalgae, as some of these could present in very low concentrations. Analyzing natural samples that contain multiple species is challenging, since it is difficult to attribute specific omics data to individual cyanobacterial or microalgal species, especially when studying microbial communities [90].

Despite progress made in genetic engineering, the manipulation of cyanobacterial and microalgal cells continues to present challenges due to the lack of well-established, standardized genetic tools and resources [139]. Certain strains may possess complex sheaths, cell walls and membranes, which hinder transformation efficiency. The genetics of certain species are still not fully understood, and this can complicate genetic engineering

strategy design. Identifying the appropriate promoters and regulatory elements to control gene expression may not be straightforward. The instability of transgenes may also limit their expression and long-term effectiveness [140].

The use of genetically modified cyanobacteria and microalgae for biotechnological purposes is subject to ethical and regulatory scrutiny, particularly when employed in environmental or industrial applications affecting human health. Transitioning from small-scale laboratory experiments to large-scale industrial production can be difficult, involving challenges related to culture management, bioreactor design, and cost-effectiveness.

Even so, the synthetic biology and genetic engineering of cyanobacteria and algae offer immense potential for biotechnological and industrial application, despite the present obstacles. Many of these issues are currently being addressed through constant research, discovery and innovation. Soon, artificial intelligence might provide effective strategies for deciphering complex datasets and devising solutions to hurdles in the design and implementation of industrial processes.

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