Universidade de Aveiro
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Departamento de Química

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Biodiversidade e Potencial Biotecnológico de Cianobactérias da região de El Jadida, em Marrocos

Biodiversity and Biotechnological Potential of Cyanobacteria from the region of El Jadida, Morocco

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, ramo Biotecnologia Alimentar, realizada sob a orientação científica da Doutora Luísa A. S. Serafim, Professora auxiliar do Departamento de Química da Universidade de Aveiro, do Doutor Guilherme Scotta Hentschke, bolseiro de investigação no CIIMAR e do Doutor Vitor Vasconcelos, Professor Catedrático da Faculdade de Ciências da Universidade do Porto.

Todo o projeto foi desenvolvido em parceria com o CIIMAR, no âmbito dos projetos EBB e EMERTOX.

o júri

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"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota."

(Madre Teresa de Calcutá)

palavras-chave Cianobactérias, Marrocos, Diversidade, Cianotoxinas, Potencial Biotecnológico

resumo

Marrocos, por ser um local alvo de poucos estudos, representa uma fonte com excelente potencial para a pesquisa e descoberta de novas espécies de cianobactérias com interesse biotecnológico. Tendo isso em conta, este trabalho teve como principal objetivo conhecer a diversidade de cianobactérias terrestres, marinhas e de água doce da região de El Jadida, em Marrocos. Para tal, procedeu-se ao isolamento de estirpes a partir das amostras recolhidas na região de El Jadida. Posteriormente as estirpes isoladas foram caracterizadas morfologicamente, onde foram registadas as suas principais características, tais como, cor, forma e dimensões. De seguida procedeu-se à sequenciação do gene 16S rRNA de 11 estirpes isoladas, com essa informação conheceu-se o género dos isolados e posteriormente foi contruída uma árvore filogenética. Para avaliar o potencial tóxico dos isolados, através de PCR fez-se a deteção dos genes responsáveis pela produção das principais cianotoxinas (cilindrospermopsina, saxitoxina, anatoxina, microcistinas e nodularinas). Por fim, uma das estirpes isoladas, a estirpe 1, foi selecionada para a realização de três ensaios biotecnológicos: antioxidante, anti-obesidade e anti-inflamatório. Relativamente aos resultados experimentais, foram isoladas 15 estirpes de cianobactérias, sendo 11 filamentosas e 4 cocoides. Das 11 estirpes sequenciadas, existe a possibilidade de algumas pertencerem a novos géneros de cianobactérias, ainda por descrever. Das estirpes isoladas, 3 demonstraram potencial tóxico, sendo que em 1 delas foram detetados os genes responsáveis pela produção de cilindrospermopsina e em 2 delas foram detetados os genes responsáveis pela produção de nodularinas. Relativamente aos ensaios biotecnológicos, a estirpe 1 apresentou atividade pro-oxidante e não se verificou um comportamento anti-obesidade, nem atividade anti-inflamatória.

Todo o projeto foi desenvolvido em parceria com o CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental), no âmbito dos projetos EBB (European Marine Biological Resource Centre Biobank) e EMERTOX (Emergent Marine Toxins in the North Atlantic and the Mediterranean).

keywords Cyanobacteria, Morocco, Diversity, Cyanotoxins, Biotechnological Potential

abstract

Morocco has been a target of few studies despite its excellent potential for research and discovery of new species of cyanobacteria with biotechnological interest. Taking this into account, this work had as the main objective of to know the diversity of terrestrial, marine and freshwater cyanobacteria in the region of El Jadida, in Morocco. In this way, strains were isolated from samples collected in the El Jadida region. Subsequently, the isolated strains were morphologically characterized, where their main characteristics were analysed, such as color, shape, and dimensions. Then, the 16S rRNA gene of 11 isolated strains was sequenced, with that information the genus of the isolates was known and later a phylogenetic tree was built. In order to assess the toxic potential of the isolates, PCR detected the genes responsible for the production of the main cyanotoxins (cylindrospermopsin, saxitoxin, anatoxin, microcystins and nodularins). Finally, one of the isolated strains, strain 1, was selected to carry out three biotechnological tests: antioxidant, anti-obesity and anti-inflammatory. Regarding the experimental results, 15 strains of cyanobacteria were isolated, 11 filamentous and 4 cocoids. Of the 11 strains sequenced, there is a possibility that some of them are new genera of cyanobacteria, yet to be described. Of the isolated strains, 3 demonstrated toxic potential, and in 1 of them the genes responsible for the production of cylinderspermopsin were detected and in 2 of them the genes responsible for the production of nodularins were detected. Regarding biotechnological tests, strain 1 showed pro-oxidant activity and there was no anti-obesity behavior or anti-inflammatory activity.

The entire project was developed in partnership with CIIMAR (Interdisciplinary Center for Marine and Environmental Research), under the EBB (European Marine Biological Resource Center Biobank) and EMERTOX (Emergent Marine Toxins in the North Atlantic and the Mediterranean) projects.

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List of Abbreviations

AC – Anhydrase Carbonic

BBE - Blue Biotechnology Ecotoxicology

BLASTn - Basic Local Alignment Research Tool for nucleotides

CCM - CO_2 concentration mechanism

CIIMAR – Interdisciplinary Centre of Marine and Environmental Research

C:N – Carbon/nitrogen ratio

DMSO - Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

dNTPs - Deoxynucleotides

DPF - Day post fertilization

EBB - European Marine Biological Resource Center Biobank

gDNA – genomic deoxyribonucleic acid

IC – Inorganic carbon

LPS – Lipopolysaccharide

LEGE-CC - Blue Biotechnology and Ecotoxicology Culture Collection

MTT - 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide

NBT - Nitro blue tetrazolium

NCBI - National Center for Biotechnology Information

PCR - Polymerase Chain Reaction

PTU - 1-phenyl-2-thiourea

REV – Resveratrol

RuBisCo - Ribulose-1,5-bisphosphate carboxylase oxygenase

UV - Ultraviolet radiation

WHO - World Health Organization

16S rDNA - 16S ribosomal DNA

16S rRNA – 16S ribosomal RNA

1. Introduction

The great differentiation of habitats and the tropical and subtropical ecological diversity have led to a higher speciation rate in these areas. With regard to cyanobacteria, many species have not yet been identified in these regions, and, consequently, were poorly studied. In this context, Morocco with its subtropical climate has an excellent potential for the discovery of new isolates of cyanobacteria with biotechnological interest. The present project was developed in partnership with CIIMAR (Interdisciplinary Center for Marine and Environmental Research), within the scope of the EBB (European Marine Biological Resource Center Biobank) and EMERTOX (Emergent Marine Toxins in the North Atlantic and the Mediterranean) projects.

The general objective of the project was to increase the knowledge about the diversity of terrestrial, marine and freshwater cyanobacteria in the El Jadida region in Morocco. Several samples were collected *in situ* and a polyphasic approach was used to identify genera and species. This approach included morphological studies of the population of nature and isolated strains, and molecular analysis including 16S rRNA gene. Finally, the potential for biotechnological exploitation was performed through the analysis of genes responsible for the production of toxins and using three different bioassays: antioxidant, anti-obesity and anti-inflammatory.

This work also had some more specific objectives, such as:

1) to isolate strains from the sampled communities;

2) to identify taxonomically the isolated strains;

3) to describe new genera and species of cyanobacteria;

4) to evaluate the biotechnological potential of the isolated strains.

2. Literature Review

2.1. Cyanobacteria

2.1.1. Biological characteristics

Cyanobacteria, also known as blue-green algae, are the oldest photosynthetic organisms on Earth [1]. Their name derives from the Greek prefix "*cyanos*" and means "blue" [2]. They are a group of gram-negative, prokaryotic organisms [3], belonging to the Bacteria domain [4] that appeared in the coastal waters of primitive continents approximately 3,500 million years ago. Taking into account their ability to perform photosynthesis with oxygen release, it is believed that cyanobacteria originated the atmosphere as we know today [2]. These organisms were extremely important in the appearance and formation of stromatolites [4]. These structures are preserved in various parts of the World and are still formed today in some areas of Australia, Bahamas, Mexico and other places [2].

Cyanobacteria are photoautotrophic aerobic microorganisms that only need water, carbon dioxide, inorganic matter and light to perform photosynthesis, thus acquiring the energy necessary for their own metabolism [4]. They were the pioneer organisms of oxygenic photosynthesis, a characteristic later assimilated by other microorganisms, such as plants and algae, by endosymbiosis with cyanobacteria, thus resulting in the formation of chloroplasts [5]. In addition, due to photosynthesis, they contribute significantly to the fixation of atmospheric carbon [6].

Due to their cellular organization, cyanobacteria are characterized as prokaryotic microorganisms, biochemically very similar to bacteria, but performing photosynthesis like algae. However, they are distinguished from algae by several characteristics, including the predominance of a bluish green color [4]. This bluish coloration is caused by the pigment phycocyanin and other photosynthetic pigments such as chlorophyll-a, phycoerythrins, and phycocyanins. Chlorophyll-a is a universal pigment for aerobic photosynthesis, in addition to a series of accessory and protective pigments. Some species may also have chlorophyll-b. The proportion of these pigments can be changed to increase the absorption of light in the visible spectrum [2].

Likewise, cyanobacteria have carotenoids that can also be found in eukaryotic algae, but others present exclusively in cyanobacteria, such as glycosidic carotenoids.

Carotenoids mainly serve as protection for high light intensities, acting as antioxidants, diverting excess electron flow to prevent damage to photosystems. On the other hand, some carotenoids can perform protective functions at low temperatures or act as accessory pigments for chlorophyll-a [2].

Cyanobacteria have some parameters in common, both with other bacteria and eukaryotic algae, thus giving them unique characteristics that allows them to tolerate adverse environments with a high adaptive capacity [2]. They are halotolerant organisms, which are able to thrive in environments with high concentrations of salt, as they have specific mechanisms that regulate the osmotic circulation intracellularly [7]. They are the only known photoautotrophic organisms bearing mechanisms that allow for the fixation of atmospheric nitrogen, which can occur in the absence of this element or in environments with excess phosphorus [2].

Cyanobacteria are the only group of organisms capable of producing oxygen, reducing carbon dioxide and fixing nitrogen under aerobic conditions, thus playing a relevant role in nitrogen and carbon cycles [8]. The fixation of atmospheric molecular nitrogen occurs in differentiated cells called heterocysts and represents an adaptative advantage for cyanobacteria. This process is essential for the biological processes of the whole ecosystem since the molecular nitrogen is reduced to ammonium and consequently made available for consumption by plants and animals [9].

More than 14 genera of cyanobacteria, including *Anabaena*, *Anabaenopsis*, *Calothrix*, *Nostoc*, *Aphanocapsa*, *Cyanothece*, *Chroococcidiopsis*, *Microcoleus*, *Gloeobacter*, *Oscillatoria*, *Synechococcus*, *Microcystis* and *Arthrospira* ("*Spirulina platensis*") are known for their ability to produce hydrogen under various culture conditions and is considered a promising source of alternative energy [10]. The hydrogen production can result from the reversible activity of the enzyme hydrogenase, which as two subunits and is present in the thylakoid membrane of filamentous cyanobacterial heterocysts [12]. Also, under nitrogen limitation, hydrogen can be formed as a by-product of the action of another enzyme, nitrogenase. Nitrogenase in the heterocysts of filamentous cyanobacteria when they grow under conditions of low concentration of nitrogen and absence of oxygen.

Cyanobacteria are able to switch between different modes of metabolism. All species are able to perform oxygenic photosynthesis, but some can switch to anoxygenic

photosynthesis using sulfide as an electron donor. Under anoxic conditions and in the dark, cyanobacteria usually ferment organic matter [11].

Photosynthetic microorganisms, including cyanobacteria, are able to adapt and grow under a wide range of CO_2 concentrations [12]. 3.5 billion years ago, during the early years of their existence, cyanobacteria were subjected to a changing gaseous environment, where CO_2 decreased and O_2 increased. This imposed an evolutionary pressure to develop strategies for the efficient acquisition of inorganic carbon to perform photosynthesis. Consequently, they developed a very effective CO_2 concentration mechanism (CCM) [13]. This mechanism allows to capture CO_2 and bicarbonate as a source of inorganic carbon (IC) and increase the intracellular CO_2 level [14]. The adaptation process is mediated by a series of changes, at various cellular levels, including the modulation of the expression of genes involved in the CCM [12].

In general, cyanobacterial CCM has two main components - the IC uptake system and carboxysomes. IC capture systems have two CO_2 capture systems and three HCO^{3-} transport systems. CO_2 capture systems, located on the thylakoid membrane, convert cytosolic CO_2 into HCO_3^-. In carboxysomes, Anhydrase Carbonica (AC) catalyzes HCO^{3-} in CO_2 [15]. There are two classes of ACs: type α and type β. Type β ACs are directly related to carboxysomes (the main CO_2 fixation site in cyanobacteria), while type α ACs mainly act as receptors for external environmental stimuli, allowing the microorganism to respond quickly to possible extracellular changes that may occur [16].

In recent years, there has been a rapid increase in the understanding of the reactions and genes involved in cyanobacterial CCM. CCM is perhaps the most effective CO_2 fixation mechanism present in photosynthetic organisms, allowing CO_2 to be concentrated up to 1000 times more around the active site of RuBisCO (enzyme responsible for carbon fixation) [13].

Due to their physiological and ecological characteristics, cyanobacteria are competitively superior to other phytoplankton organisms [2]. Reproduction is asexual and occurs through cell division, through the production of baeocytes or exocytes (formerly called endospores and exospores, respectively), by fragmentation of the trichome or by the formation of mobile trichome fragments, the hormogones [4].

2.1.2. Morphology

Cyanobacteria are morphologically diverse, presenting themselves in multiple forms, including unicellular (Figure 1-A) and filamentous (Figure 1-B) [1].

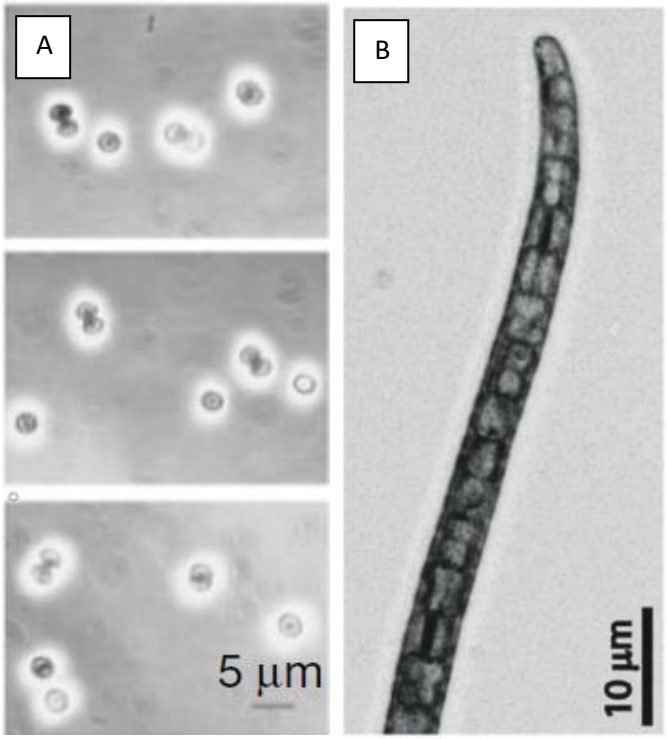

*Figure 1 – **(A)** unicellular cyanobacteria, Synechocystis sp.* [119]*; **(B)** – filamentous cyanobacteria, Kamptonema formosa [17]*.

Unicellular genera can exist in the form of single, benthic, aggregated cells, while planktonic filamentous genera can be thin or thicker, single, with or without sheath [11].

Some filamentous species have modified vegetative cells: heterocysts and akinetes [2]. Heterocysts develop from vegetative cells and can appear isolated, in pairs or several in a row [17], playing an important role in fixing atmospheric nitrogen [2]. They have a distinct pigmentation and contain an extra cell envelope that is composed of two chemically different layers located outside the outer membrane, one consisting of glycolipids and the other of polysaccharides. A distinct characteristic of heterocysts is the presence of differentiated structures located at their poles [18].

The akinetes are modified resistance cells, differentiated by the formation of a thin multilayered envelope [16] whose function is the storage of reserve substances [2]. Their shape (spherical, ellipsoidal, cylindrical), size, position within the trichome,

characteristics of the cell wall, color and mode of germination, are genetically acquired characteristics and have been used as critical factors for the distinction of genera and species [17]. Several environmental factors have been identified as capable of triggering the formation of akinetes, such as the intensity of light, the quality of light (the formation of akinetes is inhibited by blue light), thermal shock, frequency of temperature fluctuations and quantity of nutrients such as such as phosphate and the carbon/nitrogen ratio (C:N) [19].

Cyanobacteria contain three different membranes: the outer, the plasma and the thylakoid. Each of them performs different functions [20]. Cyanobacteria are classified as being gram-negative taking into account the characteristics of the cell wall. However they have a thicker membrane than most other bacteria [4] that can measure from 1 to 10 nm in thickness [21]. The membrane plays a protective role against various external agents, such as, drying out, antibacterial agents or even protozoa [4].

Cyanobacteria can be covered by exopolysaccharides, which is called mucilage or firm sheath [2], which give them the ability to adhere to solid surfaces [4].

Most planktonic cyanobacteria produce intracellular air vesicles, also known as aerotopes or aerocysts (<300 nm) [2], delimited by a protein membrane [4], whose function is to regulate the level of fluctuation of organisms. This membrane allows these organisms to be in ideal conditions for their reproduction, such as controlling the level of exposure to light, preventing sedimentation, among others [2].

Even without propellant structures such as flagella, some species or genera have the ability to slide due to the extrusion of mucilage or the activity of special structures such as pili type 4 or locomotive structures similar to cilia [4], thus enabling distribution of organisms in different types of sediments [7].

Some filamentous cyanobacteria may have branches, called true branches, characterized by the presence of branching cells in contact with three different neighboring cells, exhibiting the highest level of complexity and morphological differentiation in cyanobacteria [22]. Three main types of true branches can be distinguished, namely, 'T', 'V' and 'Y'. The T-branch is formed by an almost perpendicular side branch originated by the change of the plane of division from transversal to longitudinal. The V-branch (Figure 2) consists of a dichotomous or pseudo dichotomic bifurcation caused by a change in the division plane at or near the tip of the trichome. Y-

branching arises from the displacement of an intermediate cell from the branching point by the meristematic growth around it. False branching can also be seen in some genera [22].

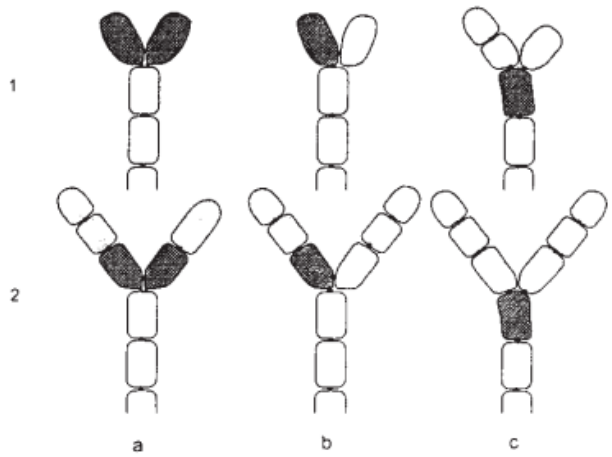

Figure 2 - Schematic representation of the V-branch [113].

2.1.3. Ecological characteristics

The dispersion of cyanobacteria can be accelerated by human activities, animal migration or winds [19], so, cyanobacteria can be found in almost all environments on the Earth's surface [23]. During their long evolution, cyanobacteria have differentiated and developed mechanisms to adapt to a wide range of environmental factors [24] and for this reason, cyanobacteria can survive in a wide variety of habitats [1], from aquatic environments (fresh, brackish and marine), terrestrial (soil, associated with lichens and leaf surface) and different extreme aquatic and terrestrial environments such as hot springs, salt ponds or deserts) [25]. When in aquatic environments, cyanobacteria are divided into two major ecological groups: planktonic cyanobacteria, which float freely in the water column, and benthic cyanobacteria, which adhere to submerged solid surfaces, such as sediment, rocks, stones, algae and aquatic plants [26].

Cyanobacteria are unique among all prokaryotes for their efficiency in forming symbiotic relationships with a wide and varied range of hosts, such as corals, diatoms, dinoflagellates, seagrass, and sponges. This ability results from their efficient photosynthetic system, allowing them to continue this activity even in low light levels, which is common in most symbiotic relationships [26]. As photosynthetic cyanobacteria,

light is one of the crucial parameters for its monitoring, so, these organisms are able to detect several wavelengths and many have photoreceptors, capable of regulating vital cellular processes, such as growth, phototaxis, cell aggregation and photosynthesis [27].

It is important to note that, although they develop in the most diverse environments, the most favorable for the growth of cyanobacteria are fresh water, that is, neutral-alkaline waters (pH: 6-9), temperature between 15 to 30ºC with a high concentration of nutrients such as nitrogen and phosphorus [4]. On the other hand, when subjected to extreme environmental conditions, such as long periods of drought, high ultraviolet (UV) radiation [2], high salt concentration, etc., some species of cyanobacteria produce a mucilaginous sheath that allows them to survive and helps the formation of blooms [7].

2.1.4. Blooms

When subjected to certain environmental conditions, some cyanobacteria have the ability to cause blooms [2]. Generally these blooms are considered toxic due to their potential to produce a wide variety of neurotoxic, hepatotoxic and tumor-inducing secondary metabolites that can cause problems for human and animal health [28].

The formation of blooms depends on a wide range of physical, chemical and biological factors [29]. The increase on the temperature of the earth's surface [30], heavy rain or long periods of drought [31], are factors that favor the growth of flower-forming cyanobacteria, but may also change the composition of the communities of cyanobacteria [30]. Blooms may also be caused by discharges of untreated or improperly treated sewage [31]. As a consequence, cyanobacteria form large clusters where bacteria and other organisms may be strongly cohesive, thus making it difficult to obtain pure cultures [7]. Due to the production of exopolysaccharides, filamentous and some coccoid cyanobacteria are responsible for the sediment trapping and bonding [32].

Dense blooms of cyanobacteria have the potential to significantly alter the functioning of the ecosystem [19], however, there is no established number of cells from which a bloom can be considered formed [29]. There may be a decrease in CO_2 dissolved in surface waters, increasing pH, to values above 9 or even 10 [14]. Also, blooms can decrease water transparency, decrease oxygen concentration and result on the production

of toxins [33], which affect the color, smell, taste and appearance of the waters [2]. These blooms are mainly produced by some genera, such as, *Anabaena*, *Aphanizomenon*, *Nodularia*, *Microcystis*, *Cylindrospermopsis* and *Oscillatoria* [34].

There is evidence that the frequency and magnitude of algal blooms in aquatic environments are increasing worldwide [35]. However, there are some differences between blooms in temperate waters and in tropical waters. Countries in which the temperate climate predominates, cyanobacteria dominate the waters in the summer period. The turbulence of the waters and the absence of light with the arrival of winter, can cause the replacement of cyanobacteria by diatoms. In countries where the tropical climate predominates, the changes between seasons are not drastic enough to cause the replacement of cyanobacteria by other species. Thus, cyanobacteria remain in the waters for most of the year, leading to problems associated with the high amount of biomass, thus increasing the levels of toxins and consequently causing health risks [31].

2.1.5. Toxins

Blooms can release cyanotoxins in very high concentrations, to the point of posing a threat to human health, algae, zooplankton and fish, also having a negative impact on water quality and the functioning of the ecosystem itself [30]. Cyanotoxins can cause liver, digestive and neurological diseases when ingested by waterfowl, livestock, and humans [14]. In the latter, problems can arise from skin exposure or accidental ingestion [36]. Pets, domestic animals and wild animals are also affected by exposure to cyanotoxins, with deaths reported annually [37].

The production of cyanotoxins is described in at least 20 genera of cyanobacteria, mostly belonging to Chroococcales and Nostocales orders and very few to Stigonematales [38]. Cyanotoxins are classified as endotoxins, they can be produced at any stage of cell growth and their release only occurs when the cell breaks. When released to external medium, they can dissolve in water and depending on the characteristics of the medium, such as pH and temperature, they may reside for a long time (from days to months) [39].

According to the mechanism of action in the human body, cyanotoxins are classified as hepatotoxins (for example, microcystin, nodularin), neurotoxins (for example, anatoxin-a, homanatoxin, saxitoxin), cytotoxins (for example

cylindrospermopsin), dermatotoxins (for example, lyngbyatoxin-a) and irritating toxins (for example lipopolysaccharides) [40].

Hepatotoxins damages the liver and can cause poisoning, a feeling of weakness, anorexia, swelling of the mucous membranes, vomiting, leading to death within a few hours or a few days after the initial exposure to the toxin [41]. Some neurotoxins block the sodium and calcium channels, thus inhibiting the conduction of the nervous impulse, which prevents the release of the neurotransmitter and, consequently, causes muscle paralysis [29]. Cytotoxins are inhibitors of protein synthesis, capable of affecting many types of cells, but above all the kidneys and liver of mammals. In more severe cases of intoxication, it can cause generalized cell necrosis in the kidneys, liver, spleen, lung or intestine [29]. Dermatoxins are associated with irritations of the skin, mucosa and gastrointestinal intoxications [41].

Among the various groups listed above, microcystins (belonging to the class of hepatotoxins) (Figure 3) are the most frequent cyanotoxins. Microcystins can be produced by several species of the genera *Microcystis*, *Anabaena*, *Anabaenopsis*, *Nostoc* and *Planktothrix* [39]. There are some reports on the negative effects of microcystins on marine ecosystems, with particular concern for the possibility of transferring this cyanotoxin along the aquatic food chain. Some authors have verified the presence of microcystins in many aquatic animals, such as fish, snails, gastropods and bivalves [39].

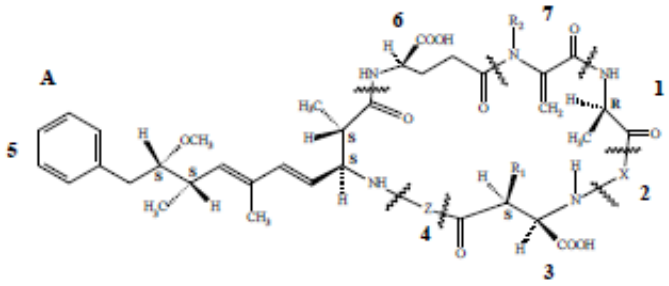

Figure 3 - Structure of microcystins [31].

Microcystins have with several harmful effects, whether in humans or animals. In humans, they can cause gastrointestinal disorders, atypical pneumonia, headache or nausea [39], in other animals they can act negatively at the level of hepatocytes [29]. Due

to cyanotoxins, the World Health Organization (WHO) has listed cyanobacteria as an emerging health problem, although it does not consider them as emerging pathogens [40]. According to Article 40 of Chapter VI of Ordinance No. 2914, of December 12, 2011, issued and in force in Brazil, “When the density of cyanobacteria exceeds 20,000 cells/ml, analysis of cyanotoxins in water must be performed, at the capture point, with weekly frequency ” [42].

2.2. Taxonomy

The main objectives of the taxonomy are to discover, describe and classify the diversity of living organisms [43]. Systematic (taxonomic) classification is the only method that allows recognizing, understanding, and organizing the diversity of organisms. However, the methods used must be constantly updated and adapted according to the introduction of new methodologies and investigations, being necessary to be in accordance with the progress and development of modern science. On the other hand, taxonomy must always take into account all the relevant knowledge that has been proven in previous investigations [44].

Cyanobacteria are one of the most diverse phyla morphologically and, in fact, their morphological characteristics play an important role in their taxonomic classification [45]. Cyanobacteria share characteristics metabolically similar to eukaryotic algae and were initially classified as algae according to the Botanical Code, after they discovery by Edmondson in Lake Washington in 1955. In 1977, they were described for the first time by optical microscopy. The inclusion of cyanobacteria in the taxonomic classification of bacteria was proposed in 1978 by Stanier, and over time the bacterial taxonomic names came into conflict with the botanical nomenclature [46]. The coexistence of two different nomenclature codes, Botanical and Bacteriological, made the classification of cyanobacteria even more complex. Finally, in 1979 the criteria for their morphological classification were established [47].

The traditional classification system for cyanobacteria was based mainly on morphological characteristics due to their morphological diversity [44], such as cell dimensions, shape, color, type of branch, sheath characteristics and cell content [48]. Recently, with the introduction of both electron microscopy and molecular analysis resulted in some modifications on the taxonomic criteria for cyanobacteria [44]. It was found that taxonomy cannot be based only on the morphological characters of the strains, since not all genotypes were correctly identified [45]. The construction of evolutionary relationships, as evidenced in genomic studies in cyanobacteria, showed that evolutionary patterns do not necessarily correspond to morphological characters [49]. Cyanobacteria show the phenomenon of “cryptic species”, which means that organisms potentially belonging to the same species from the morphological point of view, but are genetically distinct [47]. Similar morphotypes can differ widely in physiology, ecology, and

phylogeny. However, different morphotypes, mainly colonial forms, can be almost genetically identical. In addition, strains grown and maintained for long periods under artificial laboratory conditions, may exhibit unusual or slightly distorted characteristics. Due to these situations, these organisms can no longer be identified through microscopy [50].

To overcome these problems, prokaryotic species are currently characterized using a polyphasic approach [43]. This approach relies in a characterization of microorganisms based on their phenotypic characteristics, such as morphology, cell differentiation or ultrastructure, physiology, ecology and mainly genetic profile, such as 16S rRNA gene sequences among other molecular markers [50]. DNA and RNA sequencing is considered the most reliable approach to phylogenetic inference. Since in 1990, Woese proposed the 16S rRNA gene as a universal marker for all bacteria, it become the most used gene in phylogenetic reconstructions [48]. The modern taxonomy should include data from the 16S rRNA gene sequence that can be complemented by the internal 16S-23S rRNA (ITS) spacer [45].

To assess the similarity of the RNA ribosomal gene sequence, taxonomists invented a method for creating evolutionary trees [43]. A simplified phylogenetic tree that shows the relationship between the orders and some families of cyanobacteria is shown in Figure 4 [51].

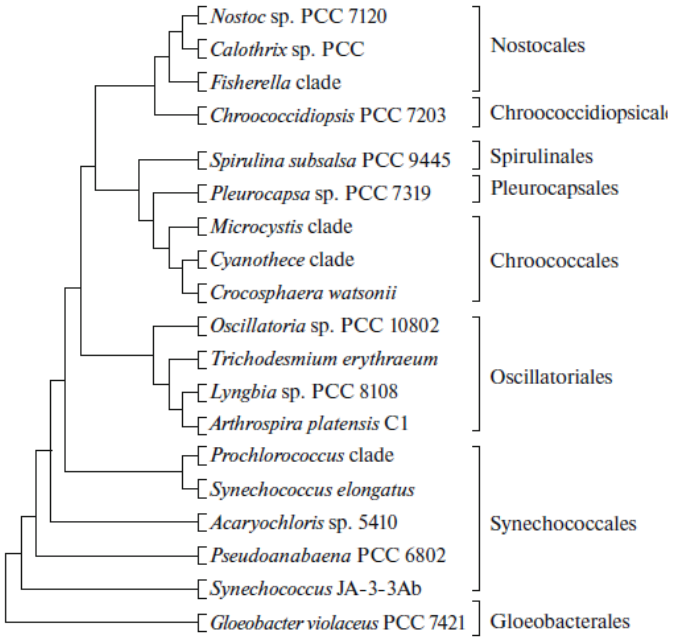

Figure 4 - Taxonomic and phylogenetic relationships between representatives of the phylum of cyanobacteria [51].

The molecular approach must be the basis for the identification of organisms, however, it should be complemented with the morphological and ecological characteristics of the cyanobacteria genotypes [52]. Consequently, it is essential to find the relationships, correlations, interactions and coincidences between genetic analysis and morphological and ecological markers [44].

Taxonomically, cyanobacteria comprise approximately 150 genera and 2000 species [28]. They are classified into five subsections (Table 1): subsections I and II include unicellular cyanobacteria and subsections III to V include filamentous cyanobacteria [5].

Table 1 - Morphological classification of cyanobacteria [53].

	Subsection	Order	Genera
Non-heterocysted	I	**Chroococcales**	*Chamaesiphon, Chroococcus, Cyanobacterium, Cyanobium, Cyanothece, Dactylococcopsis, Gloeobacter, Gloeocapsa, Gloeothece, Microcystis, Prochlorococcus, Prochloron, Synechococcus, Synechocystis*
	II	**Pleurocapsales**	
		Subgroup I	*Cyanocystis, Dermocarpella, Stanieria, Xenococcus*
		Subgroup II	*Chroococcidiopsis, Myxosarcina, Pleurocapsa, Hyella, Solentia*
	III	**Oscillatoriales**	*Arthrospira, Borzia, Crinalium, Geitlerinema, Leptolyngbya, Limnothrix, Lyngbya, Microcoleus, Oscillatoria, Planktothrix, Prochlorothrix, Pseudanabaena, Spirulina, Starria, Symploca, Trichodesmium, Tychonema*
Heterocysted	IV	**Nostocales**	
		Subgroup I	*Anabaena, Anabaenopsis, Aphanizomenon, Cyanospira, Cylindrospermopsis, Cylindrospermum, Nodularia, Nostoc, Scytonema*
		Subgroup II	*Calothrix, Rivularia, Tolypothrix*
	V	**Stigonematales**	*Chlorogloeopsis, Fischerella, Geitleria, Iyengariella, Nostochopsis, Stigonema*

The different subsections of cyanobacteria can be distinguished based on their morphological and physiological characteristics. Cyanobacteria of subsection I reproduce by binary fission or budding, while the cyanobacteria of subsection II reproduce by rapid division from a mother cell into smaller spherical cells called “baeocysts”. Reproduction of these taxa occurs through the rupture of the filament (trichome) and the formation of short, mobile filaments, called hormogones. While the cyanobacteria of subsection III grow uniseriate (a single cell width) and unidirectional filaments in a single plane, the organisms in subsections IV and V have the ability to form differentiated cells, such as metabolically specialized heterocysts for nitrogen fixation and resting cells , the so-called akinetes. The taxa belonging to subsection V are the only cyanobacteria that grow multiseriate filaments (variable cell width) and in several planes exhibit 'true' branching, which should not be confused with 'false' branching [5].

The Chroococcales order comprises 11 families and about 90 genera, including unicellular and colonial forms [54]. Organisms of this order are found in both aquatic and terrestrial environments [48]. Generally, organisms belonging to this order are isopolar and have one, two or three cell division plans. Therefore, this resource is one of the most important for identifying Chroococcales families. In addition to cell division plans, the correct observation of the shapes and sizes of cells and colonies, the presence of aerotopes, the characteristics of the mucilaginous envelope and the type of environment are fundamental to characterize the different groups [54].

The organisms of the order Pleurocapsales are closely related to the order Chroococcales. They exhibit irregular cell division, specific formations of pseudofilamentous or pseudoparenchymal stems and various types of polarized cells. The genus *Pleurocapsa* includes almost 40 species, capable of reproducing all over the world. Few genera of Pleurocapsales have been sequenced due to their inability to grow under laboratory conditions. Thus, true biodiversity within the order must be re-examined using field studies and molecular data [48].

The Oscillatoriales order has been significantly expanded since its description and now contains 47 genera, well characterized by the type of sheath and characteristics of the trichomes. The order Oscillatoriales contains filamentous taxa with a mainly fasciculate, radial or irregular tilacoid arrangement. As with other cyanobacteria, most oscillators are polyphyletic, that is, not all organisms have a common ancestor [48]. In

terms of molecular data, the order Oscillatoriales is particularly poorly studied and that of cyanobacteria is the one that falls under section III, with filamentous members that never form heterocysts [55].

Nostocales represent a rich and diverse lineage of cyanobacterial species, characterized by the ability (mandatory or not) to produce specialized cells, mainly heterocysts and acinets. Although many members of this clade are difficult to fully assess due to difficulties in culture or sequencing, modern approaches are developing new methods to elucidate phylogenetic relationships. In addition, many strains have thick and abundant sheaths, requiring additional steps to ensure that sequencing will be successful. Although this strain represents one of the most cyanobacterial strains rich in morphological characters, there are probably not enough unique morphologies to truly differentiate this diverse strain [48]. Nostocales are widely distributed and found in terrestrial or aquatic, polar or tropical environments. They are usually involved in symbiotic associations. It is also known that many Nostocales produce a wide variety of bioactive compounds, which can be potent neuro, hepato and dermatotoxins [48].

Among cyanobacteria, Stigonematales are the least studied group [56]. The order Stigonematales is composed of filamentous nitrogen fixing cyanobacteria that exhibit true branching. Species of this order generally do not have a dominant ecological role [56] and can be present all over the world, in fresh waters and soils, but most species are restricted to tropical / subtropical and thermophilic habitats [38]. They exhibit a high degree of morphological complexity [22].

2.3. Biotechnological Potential of Cyanobacteria

The concept of biotechnology comprises the application of science and technology to living organisms, as well as parts, products or models, to obtain compounds of interest, services or knowledge. Due to their very diverse morphology and genetics [57], that can result in a wide range of potential biotechnological applications, cyanobacteria have gained, especially in recent years, high attention [2]. Cyanobacteria represent a unique source of small molecules, since these organisms are believed to be the oldest organisms on Earth and, therefore, may represent chemical factories with highly evolved machinery for the production of metabolites [58]. In addition, they present rapid cell growth, basic nutritional needs [59], are environmentally sustainable, have high yield rates, thus presenting great economic advantages [60]. The fact that they only need light, carbon dioxide, water and minimal nutrients to grow, automatically eliminates the costs spent on carbon sources and complex growth media [1].

Many intra and extracellularly secondary metabolites and bioactive compounds are produced by cyanobacteria in order to survive in extreme environmental conditions, such as toxins, vitamins, enzymes, pigments or amino acids [3]. The production of these compounds allows cyanobacteria to be commercially used in the most diverse areas [11]. Furthermore, several bioactive compounds produced by cyanobacteria have the advantages of self-degradation and are less toxic than conventional chemicals, thus contributing to the interest of their use. [3].

For example, some marine cyanobacteria are valuable sources of vitamins and they are being used for the large-scale production of vitamins such as vitamins B and E. *Spirulina Arthrospira* is known to be a rich source of vitamin B12, beta-carotene, thiamine, and riboflavin, for this reason, it is commercially available in diverse forms (powder, granules, tablets, or capsules) [61]. The most industrially important enzymes produced by cyanobacteria include proteases, amylases, and phosphatases. Proteases are predominantly used for food processing industries; alpha-amylases are used in the starch industries and acid phosphatases and phosphatases are widely used as markers in disease diagnostics [62]. Phycobiliproteins and carotenoids are two important cyanobacterial pigments, widely used in bioindustry and have high commercial value. The most important carotenoids accumulated by cyanobacteria are beta-carotene, zeaxanthin, nostoxanthin, echinenone, and canthaxanthin and these pigments are commonly used as

food colorants, food additives, and supplements for human and animal feeds [63]. Cyanobacterial biomass has been used with good results (better immune response, fertility, appearance, or weight gain) as a feed additive for cows, horses, pigs, poultry, and even dogs and cats. Cyanobacteria of the genus *Spirulina*, is also used as feed in the aquaculture of mollusks, crustaceans (shrimp) and fish, because it has been proven to provide aquaculture with adequate levels of both protein and unsaturated fatty acids [60].

Some of the compounds previously mentioned possess antibacterial, antifungal, antiviral, anticancer, antispasmodic, algaecide, immunosuppressant [11], anti-inflammatory, antioxidant [64] and anti-obesity potential [65]. In the last few years, many techniques/assays have been developed that allow the research of the biotechnological potential of secondary compounds produced by cyanobacteria. To respond to the emerging need to obtain natural anti-inflammatory compounds, with or without fewer side effects than non-steroidal anti-inflammatory drugs, extracts and isolated compounds from cyanobacteria have been assessed *in vitro* using the RAW 264.7 macrophages. Among the various compounds found, carotenoids are particularly interesting and have demonstrated the potential to fight the inflammatory process, suppressing nitric oxide (NO) production by lipopolysaccharide-stimulated macrophage cells (RAW 264.7) [64]. Cyanobacteria also produce numerous biologically active molecules such as polyunsaturated fatty acids, phycobiliproteins, β-carotene, provitamins and phenolic compounds which are known to exhibit antioxidant activity [66]. Free radical scavengers and antioxidants can reduce lipid peroxidation and the generation of ROS. To assess the antioxidant potential of the extracts/compounds, they are assessed against the radical anion superoxide ($O_2^{\bullet-}$) assay [64].

Recent researches demonstrated that many natural products, including secondary metabolites produced by cyanobacteria possess anti-obesity activities [65]. So, in order to investigate the potential of cyanobacterial compounds with anti-obesity potential, was developed the zebrafish Nile red assay, a method for visually identify non-toxic molecular effectors of fat metabolism using a live transparent vertebrate [67]. Among the various known compounds, it has been proven that pheophorbide A and pheophytin A (two products of chlorophyll A degradation) have lipid reducing activity [68].

In addition to being an excellent source of secondary metabolites and bioactive compounds, cyanobacteria can also be used to overcome some environmental issues, such

as, H_2 production [60], biofuels production [69], soils fertility [70], heavy metals accumulation and wastewater treatments [1], among others.

The photobiological production of H_2 by microorganisms is of great interest, as it ensures a renewable energy carrier, using only resources widely found in nature: solar energy and water. *Cyanothece* sp. ATCC 51142, a unicellular, diazotrophic cyanobacterium has showed the capacity to generate high levels of hydrogen under aerobic conditions. It was also reported that hydrogen production in this strain is mediated by an efficient nitrogenase system, which can be manipulated to transform solar energy into hydrogen at higher rates, when compared to other hydrogen-producing photosynthetic organisms. In cyanobacteria, two natural pathways for H_2 production can be used: H_2-production as a by-product during nitrogen fixation by nitrogenases, and H_2-production directly by bidirectional hydrogenase [60].

Fermentative pathways are attractive for biofuel synthesis as many lead to the formation of reduced compounds with high heat of combustion [71]. Cyanobacteria are capable of secreting glucose and sucrose, and these simple sugars by anaerobic fermentation, under dark conditions, produce ethanol [59]. These short chain alcohols can be used as a source to produce fuels [71] so, if ethanol can be obtained directly from the culture media, the process may dispend less money and energy, than traditional biofuel processes [60].

The use of photosynthetic cyanobacteria to directly convert carbon dioxide into biofuels has also become an area of emerging interest, since the photosynthetic system of cyanobacteria is capable of fixing carbon dioxide in a reduced form, being therefore "biosynthetic machines" ideal for the sustainable production of various chemicals and biofuels [1]. Considering that cyanobacteria are the most efficient biological producer of oil on the planet, producing biodiesel from cyanobacteria is more advantageous since, converting oil into biodiesel consumes less energy than methods for conversion to other fuels. So, these organisms may in the future be used in the production of biofuels, consequently leading to a decrease in the use of fossil fuels [60]; due to their natural diversity and capacity to grow in a large variety of environments and conditions, even those unfit for agriculture, could be exploited for biofuel production (solving the potential conflict between the use of land for food or for biofuel production); and exists the

possibility of transport costs reduction, since the biofuel production can be done near the location of fuel use [69].

Cyanobacteria also play an important role in maintaining and increasing soil fertility, consequently increasing the growth and yield of plantations, thus acting as a natural biofertilizer [70]. It is also in soils that occurs the accumulation of heavy metals released by natural sources (volcanoes or fires) and anthropogenic activities (mining, burning fuels, etc.); this is a serious environmental problem, since heavy metals are persistent and accumulate in the environment [72]. Cyanobacteria are a promising tool for wastewater treatment and has the ability to remove heavy metals, for example, *Spirulina indica*, *Spirulina maxima*, and *Spirulina platensis* showed the capacity to remove Nickel and Zinc from a sample of wastewater [1]. It has also been described that cyanobacteria can be used for bioremediation. According to Gahlout et al. (2017) bioremediation is "a pollution control technology that uses biological systems to catalyze de degradation or transformation of various toxic chemicals to less armful forms" and is very efficient to reduce environmental pollution. It is described in the literature that cyanobacteria belonging to the genera *Anabaena*, *Nostoc*, *Calothrix*, *Plectonema*, *Gloeothece*, *Aphanocapsa*, *Tolypothrix* and *Rivularia*, showed a good amount of chromium biosorption efficiency. Chromium is one of the toxic heavy metals which are discharged into the environment from various industries such as leather tanning, electroplating, metal finishing, chromate preparation, wood preservation and manufacture of dyes and pigments. Bearing this in mind, this ability of cyanobacteria is an asset for reducing contaminants in the environment [70].

Even though cyanobacteria possess a great potential in the most diverse areas, there are some obstacles that need to be evaluated before moving to the industrial scale. The success of using cyanobacteria depends on lowering costs and increasing efficiency in various aspects such as, strain improvement, photosynthetic capacity, and harvesting and isolation of the compounds of interest [69].

3. El Jadida, Morocco

El Jadida (Figure 5) is located on the northwest coast of Morocco, next to the Atlantic Ocean (33° 14'N 8° 30'W) [73].

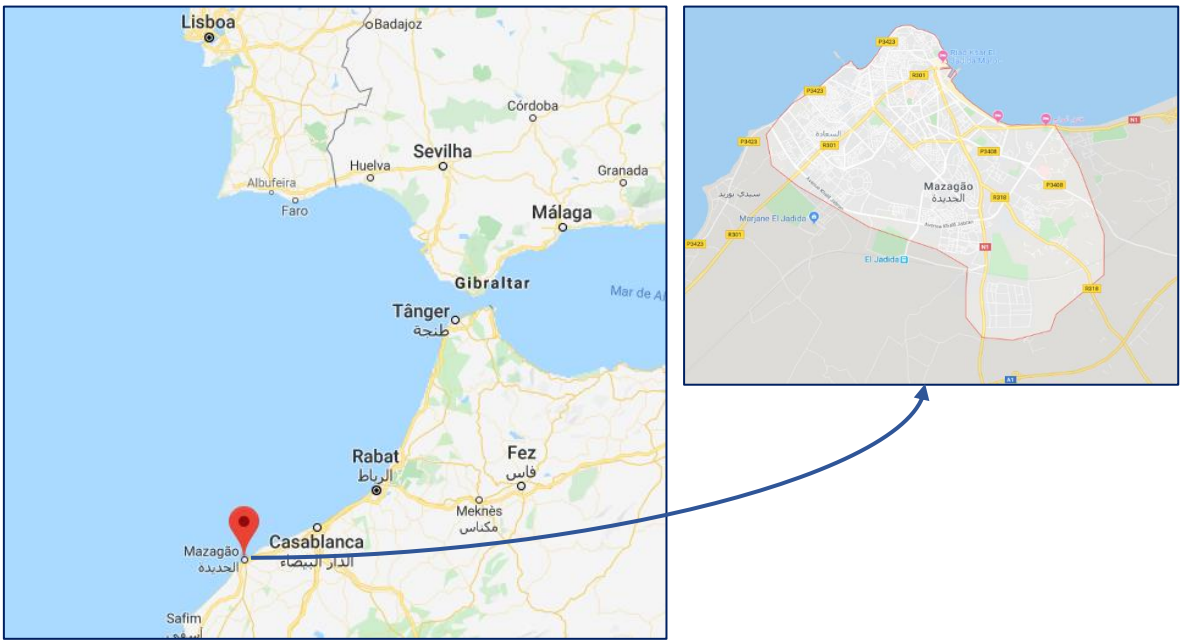

Figure 5 - El Jadida region, Morocco.

In Morocco, the proliferation of toxic cyanobacteria is common in some reservoirs of drinking water or water for recreational purposes, lakes, rivers and natural ponds [74]. Several studies have been carried out in many Moroccan reservoirs that have shown that their rapid eutrophication is due to high amounts of nutrients and the climatic conditions characteristic of the region [74]. Climatic data for 2018 show that the climate in El Jadida is hot and is classified as a semi-arid climate. The average temperature is 17.4 ºC and the rainfall is 372 nm [75]. The coast of the Morocco region has a special economic, social and ecological interest, due to the fact that it presents a high biodiversity of algae [76].

Although it is known that cyanobacteria appear in Morocco in some water reservoirs, studies on toxicology and toxinology of these potentially dangerous algae are still underdeveloped in this region [77]. Studies in northern Mediterranean countries are more common, mainly in France, Greece, Italy, Portugal and Spain [77]. A study was carried out that concluded that, in Morocco, about 18 of the 26 lakes and reservoirs used

to supply water for human consumption, contained at least one species of potentially toxic cyanobacteria [78].

4. Materials and Methods

4.1. Sampling

The samples analyzed in the present work were collected between July 8 and 18, 2019, in El Jadida, region of Morocco, in terrestrial, marine, and freshwater environments, in the framework of EMERTOX project. In order to cover different locations and substrates, 32 samples were collected in different types of environment. The collection points are shown in Figure 6.

Figure 6 - Sample collection points.

Using a spatula, scraps of biofilms were made on rocks, soils, and walls. The collected samples were placed in culture medium and transported to the laboratory, where they remained at a controlled temperature of 25ºC ± 2ºC

4.2. Culture Media

The culture media used during this work were: liquid Z8 medium, liquid Z8 medium with 25 g/L sea salt, solid Z8 medium with 1.5% concentration agarose and solid Z8 medium with 1.5% concentration agarose with 25 g/L sea salt. The Z8 culture medium is a very complete medium and rich in necessary nutrients, commonly used for the growth and maintenance of cyanobacterial cultures [79].

To proceed to the media preparation, all glass material was sterilized and/or sterile plastic material was used; sterile (autoclaved) or deionized water was used; A, B, Fe-EDTA and micronutrient solutions were autoclaved separately; medium was prepared in the flow chamber, under aseptic conditions; solutions were at room temperature when added (to avoid precipitation); and, at the end, stock solutions were kept in the refrigerator.

The solutions were added to the water according to the following concentrations: Solution A: 10 ml/l; Solution B: 10 ml/l; Fe-EDTA solution: 10 ml/l; Micronutrient Solution: 1 ml/l.

The composition of A, Fe-EDTA and micronutrients stock solutions, are described in Tables 2, 3 and 4.

Table 2 - Composition of Solution A.

Reagent	Code	g/l	g/500 ml
$NaNO_3$	N4	46.7	23.35
$Ca(NO_3)_2.4H_2O$	C1	5.9	2.95
$MgSO_4.7H_2O$	M2	2.5	1.25
K_2HPO_4	K1	3.1	1.55
Na_2CO_3	N3	2.1	1.05

Table 3 - Composition Fe-EDTA (keep in the dark)

Reagent	ml/l	800 ml
$FeCl_3$*	10	8 ml
EDTA-Na**	9.5	7.6 ml

Table 4 - Composition of the micronutrient solution

Reagent	ml/l	ml/500 ml
1 a 12	10	5
13 e 14	100	50

The composition of the base solutions is described in Tables 5, 6 and 7.

*Table 5 - *Solution Composition $FeCl_3$*

Reagent	Code	100 ml	200 ml
$FeCl_3.6H_2O$	F1	2.8 g	4.16 g
HCl (0.1 N)	H2	100 ml	200 ml

*Table 6 - **Solution Composition EDTA-Na*

Reagent	Code	100 ml	200 ml
EDTA	E1	3,9 g	7,8 g
NaOH (0.1 N)	N5	100 ml	200 ml

Table 7 - Composition of Solutions 1 to 14

Reagent	Code	g/l	g/100 ml
1- $Na_2WO_4.2H_2O$	N11	0,33	0,033
2- $(NH_4)_6Mo_7O_{24}.2H_2O$	M4	0,88	0,088
3- KBr	K2	1,2	0,12
4- KI	K3	0,83	0,083
5- $ZnSO_4.7H_2O$	Z1	2,87	0,287
6- $Cd(NO_3).4H_2O$	C2	1,55	0,155
7- $Co(NO_3)_2.6H_2O$	C4	1,46	0,146
8- $CuSO_4.5H_2O$	C6	1,25	0,125
9- $NiSO_4(NH_4)2SO_4.6H_2O$	N6	1,98	0,198
10- $Cr(NO_3)_3.9H_2O$	C5	0,41	0,041
11- V_2O_5	V1	0,089	0,0089
12- $Al_2(SO_4)_3K_2SO_4.24H_2O$	A3	4,74	0,474
13- H_3BO_3	H1	3,1	0,31
14- $MnSO_4.4H_2O$	M3	2,23	0,223

To proceed to solid Z8 medium preparation, a sterile distilled or deionized water that was autoclaved with 1-1.5 g agar per 100 ml of water (1-1.5% w/v) was used; the solutions was at room temperature when added (to avoid precipitation) and the temperature of the water with agar should not drop below 55-60ºC (to prevent the agar from polymerizing); medium was prepared in the flow chamber, under aseptic conditions; and, at the end, stock solutions were kept in the refrigerator.

The solutions were added to the water according to the following concentrations: Solution A: 10 ml/L; Solution B: 10 ml/L; Fe-EDTA solution: 10 ml/L; Micronutrient Solution: 1 ml/L. The stock solutions were the same as that of the liquid Z8 medium (see above).

After adding the stock solutions, flasks were gently shaked to avoid the formation of air bubbles; plates were spread, semi-opened, in the flow chamber, the medium was distributed over the plates and allowed to solidify (approximately 20 min); plates were closed, inverted and sealed with parafilm; the set of plates was kept in the refrigerator until further use.

To prepare the salt water Z8 medium (25 ‰), was proceed as before, either for liquid Z8 or solid Z8; before distilled or deionized water was autoclaved, was added 25 g NaCl/l; after cooling, was added the vitamin B12 (final concentration, 10 μg/L); The stock solution (1000x concentrated) was thawed and, in the flow chamber (aseptic conditions), the vitamin was added to the medium through filtration-sterilization (0.2 μm).

4.3. Strains Isolation

The isolation process had as main objective the separation and inoculation of only one organism per flask with culture medium. For the isolation of strains, two techniques were used: micromanipulation (also called capillary or fishing selection) and plating.

Micromanipulation [80] was carried out with the aid of Pasteur pipettes under an inverted optical microscope (LEICA). A drop of a sample (which contained several different strains) and a few drops of culture medium were placed on a sterile slide. Under the inverted microscope, the organism to be isolated was collected preferably from a cleaner area. Using a flame-drawn Pasteur pipette, the tip of the flame was broken according to the size of the selected organism, the microorganism of interest was transferred to a new drop with clean culture medium in the same slide. The procedure was repeated until only the microorganism of interest was obtained on the drop (Figure 7).

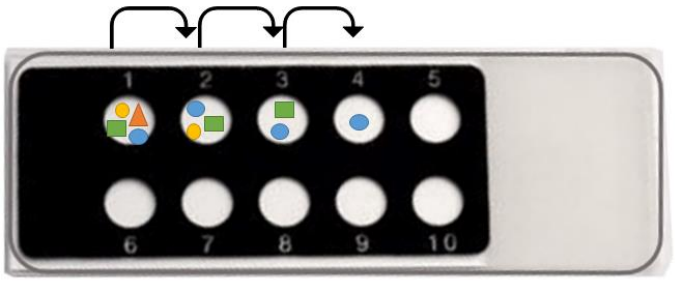

Figure 7 - Scheme of micromanipulation technique. (1) Drop with several individuals; (4) Drop with only the individual that will be inoculated in culture medium.

Then, with the aid of a stretched Pasteur pipette and with the broken tip, the isolated organism was transferred and placed in a flask with culture medium (section 3.2). Since the isolated organism could not grow, either due to its fragility, but also due to its loss during the entire process, several attempts to isolate the same strain for different flasks with culture medium were made. The flasks were kept under controlled conditions of light (14h light, 10h dark) and temperature controlled at 25ºC ± 2ºC.

Plating technique (Figure 8) consisted of placing a few drops of the sample on Petri dishes with solid culture medium in a laminar flow chamber. Then, the drops were spread over the Petri dish with the aid of a previously sterilized inoculation loop.

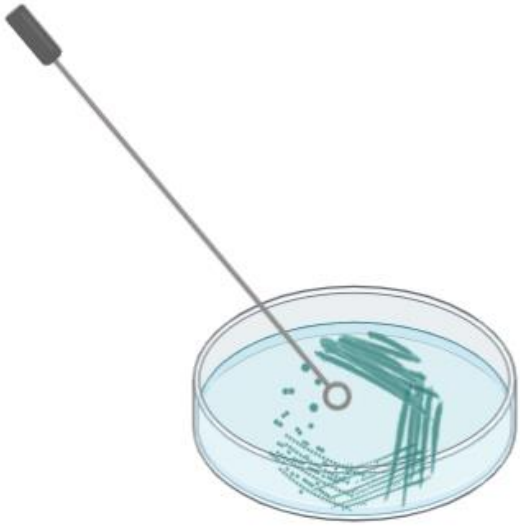

Figure 8 - Plating technique.

After drying the inoculum in the culture medium, the plates were closed with Parafilm and the face of the plate with the medium was turned upwards to avoid contamination due to water vapor that sometimes forms inside the plate. The Petri plates were kept under controlled conditions of light (14h light, 10h dark) and temperature controlled at 25ºC ± 2ºC.

The growth of the cultures was being controlled through observation under an optical microscope (Leica DMLB) over time. As biomass growth was observed, cultures were either picked or micromanipulated again into other flasks or Petri dishes until total isolation of the desired organism was obtained. All stages of isolation and maintenance of the strains (peaking every 60 days) were made with sterile material and autoclaved culture media, in a laminar flow chamber previously sterilized with UV light.

4.4. Biodiversity Study

After isolating the strains, they were identified through morphological analysis, genomic DNA extraction, sequencing, and phylogeny of the 16S rRNA gene.

4.4.1. Morphological assessment

For morphological studies (figure 9), in a laminar flow chamber, a small portion of each isolate was transferred to a separate slide and observed under a microscope. Morphological characters such as color, shape, dimensions (length, width or diameter) were observed and photographed using an optical microscope (Leica DMLB) together with a camera (Leica ICC50 HD), using specialized software, Leica Qwin Color (Leitz, Germany), and a 1000x magnification.

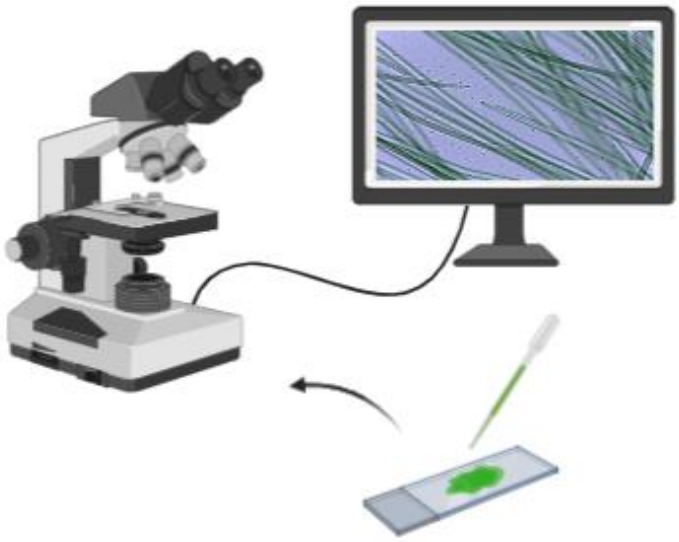

Figure 9 – Scheme of morphological assessment

4.4.2. DNA extraction

The first step to extract the gDNA was collect a small amount of biomass to 1.5 mL Eppendorf tubes, in aseptic conditions (in a flow chamber). Then, tubes were centrifuged at 10000 g for 1 min, to concentrate the biomass in the bottom of Eppendorf tubes and easily remove the excess liquid. Once this step was complete, the tubes with biomass were stored at -20 ºC for later extraction of gDNA. The genomic DNA of each

isolated strain was extracted using a commercial DNA extraction kit for gram negative bacteria (Purelink Genomic DNA Mini Kit, Invitrogen, USA), always following the instructions in the kit. DNA extraction was proven by electrophoresis on 1% (w/v) agarose gel (UltrapureTM Agarose, Invitrogen, USA), using a buffer solution. About 2 µl of SYBR Safe dye (Invitrogen, USA) and 2 µl of the 1 Kb Plus DNA Ladder marker (Invitrogen, USA) (Figure 10) were added to the agarose gel. The gel was run at 85V for 20 minutes and visualized through a transilluminator coupled to an image acquisition system (Molecular Imager® GEL O CTM XR with Image LabTM Software, USA).

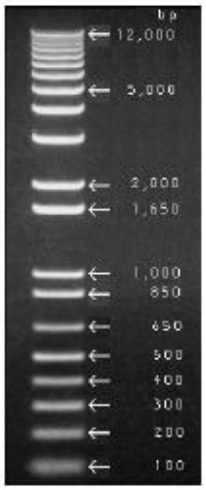

Figure 10 – Ladder marker 1Kb plus (100 bp to 12 Kb)

All reagents mentioned in Section 4.4 and 4.5 were obtained from Promega (Madison WI, USA).

4.4.3. PCR amplification

The 16S rRNA gene was used for the molecular identification of isolates. For the amplification of the 16S rRNA gene by PCR (Polymerase Chain Reaction) the primers used are described in Table 8.

Table 8 - Primer pairs used for amplification of the 16S rRNA gene

Target gene	Primer	Primer sequence (5'- 3')	Target Group	Size (bp)	Reference
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	Cyanobacteria	754	[81]
	781R	GACTACWGGGGTATCTAATC			[82]
	1494R	TACGGCTACCTTGTTACGAC		1135	[82]
	359F	GGGGAATYTTCCGCAATGGG			[81]

The PCR reactions contained 6.9 µl of H_2O, 4 µl of GoTaq Flexi Buffer, 2 µl of $MgCl_2$, 1.5 µl of dNTP's mix, 2 µl of each primer (Forward and Reverse), 0.1 µl of GoTaq Flexi DNA polymerase and 1 µl of genomic DNA. Subsequently, a Biometra thermal cycler (Biometra TProfessional Standard Gradient Thermocycler, Germany) was used as described in Table 9.

Table 9 - PCR conditions used for the primer pair used to amplify the 16S rRNA gene.

<table>
<tr><th rowspan="2">Target gene</th><th rowspan="2">Primer Pair</th><th colspan="4">PCR reaction</th><th rowspan="2">Reference</th></tr>
<tr><th>Initial Denaturation</th><th colspan="3">Annealing</th><th>Final extension</th></tr>
<tr><td rowspan="2">16S rRNA</td><td>27F/ 359F</td><td rowspan="2">95ºC
2 mn</td><td colspan="3">35 cycles</td><td rowspan="2">72 ºC
5 mn</td><td rowspan="2">[81]</td></tr>
<tr><td>781R/ 1494R</td><td>95ºC
2 mn</td><td>55ºC
45 s</td><td>72ºC
1 mn</td></tr>
</table>

Each PCR product obtained was analyzed by electrophoresis on 1.5% (w/v) agarose gel (UltrapureTM Agarose, Invitrogen, USA), using a buffer solution. About 2 µl of SYBR Safe dye (Invitrogen, USA) were added to the agarose gel. The gel was run at 85V for 20 minutes and visualized through a transilluminator coupled to an image acquisition system (Molecular Imager® GEL O CTM XR with Image LabTM Software, USA).

4.4.4. Sequencing

For sequencing, only the samples that exhibited positive PCR results were selected. In order to have enough amplified product to enable sequencing, the PCR preparation was made in duplicate (final volume of PCR product per sample loaded into the 1.5% agarose gel was 40 µL). The gel was run at 90 V for 60 minutes, then the amplified fragments was observed in the CS MICRODOC system (Cleaver scientific, UK) transilluminator. The bands were excised from the gel and purified using the Nztech – genes & enzymes (NZYGelpure, Portugal) purification kit. Lastly, a mixture with 2.5 µL of H_2O, 2.5 µL of each primer (27F and 781R or 359F and 1494R) and 5 µL of each purified PCR product was sent to GATC Biotech (Germany) to be sequenced.

4.4.5. Sequence alignment and phylogenetic analyzes

The Geneious bioinformatics software (v.8) [83] was used to examine the forward and reverse sequences from the 16S rRNA gene. The sequences were assembled (*de novo* assembly), analyzing the chromatograms in order to assess the quality of the sequences. The sequences were always cut off at the ends as these areas had poor quality. Then, after obtaining the consensus sequences for each isolated strain, these were compared with sequences present in GenBank®, using BLAST®n (Basic Local Alignment Research Tool for nucleotides) [84] available at NCBI (National Center for Biotechnology Information). For each isolated strain, the strains with the highest percentage of similarity were noted. Subsequently, to study the phylogenetic relationship between the isolated strains, reference strains, and the strains obtained at NCBI, all these sequences were aligned through the CIPRES Science Gateway v.3.3 resource, using the ClustalW tool [85]. Then, the alignment was introduced in the MEGA 10.1 program [86], in which the evolutionary model used was GTR+G+I (automatically selected by MEGA). Subsequently, a phylogenetic tree was built and edited using the FigTree software (v.1.4.4). The robustness of Maximum Likelihood (ML) trees was estimated by bootstrap percentages, using 1000 replications in MEGA 10.1.

4.5. Toxin-producing Potential

For the research of the toxin-producing potential of the isolated strains, several PCR's were performed to detect the presence of the genes responsible for the production of the main cyanotoxins (cylindrospermopsin, saxitoxin, anatoxin, microcystins and nodularins).

The Table 10 indicates the specific cyanotoxin genes and their coding functions:

Table 10 - Cyanotoxins, specific genes and respective coding functions.

Cyanotoxins	Specific genes	Coding functions
Cylindrospermopsin	cyrJ	Cylindrospermopsin biosynthesis enzyme
Saxitoxin	sxtI	Saxitoxin biosynthesis enzyme
Anatoxin	anaC	Anatoxin synthase enzyme
Nodularins	ndaF	Nodularine synthase enzyme
Microcystins	mcyA	Non-ribosomal enzyme peptide synthase

In each PCR the respective pair of primers and positive controls were used. The information is summarized in Table 11.

Table 11 - Target gene, respective primers, amplified fragment size and PCR conditions.

Target gene	Primer Pair	Primer sequence (5'-3')	Size (bp)	Positive controls*	PCR conditions				Reference
					Denaturation	Annealing			Extension	
cyrJ	cynsulF cylnamR	ACTTCTCTCCTTTCCCTATC GAGTGAAAATGCGTAGAACTTG	586	Strain LEGE 97047	94 ºC 3 mn	30 cycles 94ºC 10s	65ºC 20s	72ºC 60s	72ºC 7 mn	[87]
sxtI	SXTI682F SXTI877R	GGATCTCAAAGAAGATGGCA GCCAAACGCAGTACCACTT	200	Strain LMECYA 040	94ºC 3 mn	35 cycles 94ºC 10s	52ºC 20s	72ºC 60s	72ºC 7 mn	[88]
anaC	anaC-genF anaC-genR	TCTGGTATTCAGTCCCCTCTAT CCCAATAGCCTGTCATCAA	366	Strain LEGE X-002	94ºC 2 mn	35 cycles 94ºC 30s	58ºC 30s	72ºC 30s	72ºC 5 mn	[89]
ndaF	HEPF HEPR	TTTGGGGTTAACTTTTTTGGCCATAGTC AATTCTTGAGGCTGTAAATCGGGTTT	472	Strain LEGE 00063	92ºC 2 mn	40 cycles 92ºC 20s	52ºC 30s	72ºC 60s	72ºC 5 mn	[90]
mcyA	mcyA-Cd1F mcyA-Cd1R	AAAATTAAAAGCCGTATCAAA AAAAGTGTTTTATTAGCGGCTCAT	297	Strain LEGE 00063	92ºC 2 mn	35 cycles 92ºC 20s	56ºC 30s	72ºC 60s	72ºC 5 mn	[91]

*- LEGE 97047 – DNA of *Cylindrospermopsis raciborskii*; LMECYA 040 – DNA of *Aphanizomenon gracile*; LEGE X-022 – DNA of *Anabaena* sp; LEGE 00063 - DNA of *Microcystis aeruginosa*.

4.6. Biotechnological Activity Assays

For the assessment of biotechnological potential, the toxin-producing potential of all isolated strains was evaluated, with the detection of the genes responsible for the production of the main cyanotoxins (cylindrospermopsin, saxitoxin, anatoxin, microcystins and nodularins). Subsequently, strain 1 was selected for three biotechnological assays (anti-obesity, antioxidant and anti-inflammatory).

4.6.1. Cyanobacteria cultivation and extraction

Strain 1 was grown in liquid Z8 medium, to be subsequently tested for biotechnological potential. For that, Strain 1 was inoculated in 500 mL of Z8 medium and kept under controlled conditions of light (14h light, 10h dark) and temperature controlled at 25ºC ± 2ºC. After approximately two months, when a high concentration of biomass was obtained, the culture was collected by filtration. The collected biomass was stored at -80ºC for later lyophilization. To proceed with lyophilization, the biomass was inserted into the freeze dryer (Telstar LyoQuest) for 7 days, at a temperature of -48ºC, under reduced pressure (0.1 mbar) and later stored at -20ºC until proceeding with the extract. To obtain the extract, the freeze-dried biomass was destroyed with the help of a porcelain pestle and a mortar and transferred to an Erlenmeyer. Then, 50 ml of methanol were added to the Erlenmeyer and the flask was placed in an ultrasound bath for 5 minutes. Subsequently, the supernatant was filtered, using a vacuum filtration system. The extract was concentrated in a round flask on a rotary evaporator at 30ºC, under controlled pressure (Figure 11). Then the extract was filtered and transferred to a 20 ml vial. Finally, the solvent was evaporated on a rotary evaporator and left in high vacuum until completely dry. The dry extract was dissolved at a concentration of 10 mg/mL in DMSO and stored at -20 ºC.

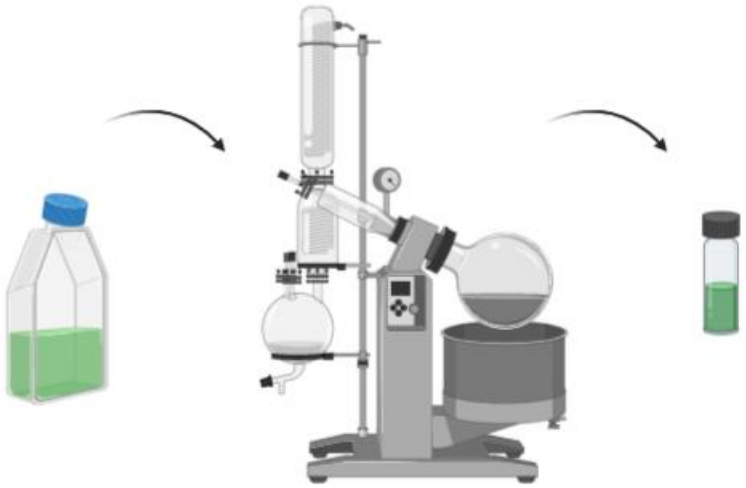

Figure 11 - Procedure to obtain the cyanobacterial extract using a rotatory evaporator

4.6.2. Zebrafish Nile red fat metabolism assay

Lipid reducing activity was analyzed by the zebrafish Nile red fat metabolism assay as described by Freitas et al. [92]. In brief, zebrafish embryos were raised from 1-day post fertilization (DPF) on in egg water (60 µg mL^{-1} marine sea salt) with 200 µM 1-phenyl-2-thiourea (PTU) to inhibit pigmentation. From 3 to 5 DPF, zebrafish larvae were exposed to cyanobacterial fractions at a final concentration of 10 µg mL^{-1} with daily renewal of water and fractions in a 48-well plate with a density of 6 – 8 larvae/well (n = 6 – 8). A solvent control (0.1% DMSO) and positive control (REV, resveratrol, 50 µM) were included in the assay. Neutral lipids were stained with 10 ng mL^{-1} Nile red overnight. The larvae were anesthetized with tricaine (MS-222, 0.03%) for 5 min before imaging on a fluorescence microscope Leica DM6000 FS (Leica Microsystems™, Wetzlar, Germany). Finally, fluorescence intensity was quantified in individual zebrafish larvae using the software Image Processing and Analysis in Java (ImageJ v1.53).

4.6.3. Superoxide anion radical scavenging assay

Serial dilutions of test extracts/compounds were prepared in buffer and 50 µL of each dilution was transferred to a 96 wells plate, and mixed with the same volume of a 166 µM NADH solution and 150 µL of NBT (43 µM). The reaction was started by the addiction of 50 µL of PMS (2.7 µM). The scavenging activity of the test extracts/compounds was determined by monitoring their effect on the reduction of NBT induced by $O_2^{\bullet-}$, using a microplate reader working in kinetic function, at room

temperature, for 2 minutes, at 562 nm. Positive controls were included for all the essays. All reagents and samples dilutions were dissolved in phosphate buffer (19 mM, pH 7.4).

4.6.4. Anti-inflammatory potential

The anti-inflammatory potential of the cyanobacteria extract was assessed through their capacity to reduce NO produced by RAW 264.7 macrophages upon lipopolysaccharide (LPS) stimulation, as described in a paper published by Lopes, G. et al. in 2020 [64]. Briefly, after a 2h pre-treatment with the extract serial dilutions (or vehicle), RAW 264.7 cells were stimulated with LPS (1 μg/mL) and further incubated for 22 h. The effect of the extracts on NO produced by RAW 264.7 cells was also evaluated in the absence of LPS in order to cross-out the direct effect of the extracts on cell stimulation and measure the levels of basal NO produced by untreated cells. After the incubation period, NO was measured in the culture medium through a Griess reaction. Briefly, 75 μL of Griess reagent (sulfanilamide 10 mg/mL and ethylenediamine 1 mg/mL, prepared in 2% H_3PO_4) (Sigma-Aldrich, St. Louis, USA) was mixed with 75 μL cell supernatant and incubated in the dark for 10 min. The absorbance of the reaction product was determined at 562 nm. Results were expressed as the percentage of NO vs. the untreated control.

As described in Lopes, G. et al. in 2020, the cytotoxicity of the extracts was monitored through the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay consisted of the reduction of the yellow MTT to insoluble purple formazan crystals by dehydrogenizing metabolically active cells. After the incubation period of 24 h, 100 μL of MTT solution (0.5 mg/mL), was added to each well and incubated at 37ºC for 45 min. The absorbance of the colored product was determined at 515 nm using a Synergy HT Multi-Detection microplate reader (Biotek, Germany). Cytotoxicity was expressed as the percentage of cell viability vs. the control (0.25% DMSO) [64].

Data were analyzed by using GraphPad PRISM software (GraphPad software, San Diego, CA, USA) (version 9.0 for Windows) and one-way analysis of variance (ANOVA).

5. Results and Discussion

5.1. Ecology and Morphology of Isolated Strains

Cyanobacteria exhibit versatile physiology and wide ecological tolerance, this characteristics contribute to their competitive success in a large range of environments [93]. The isolated strains were collected in two different types of environment (marine and fresh water), different types of habitat and showed different visual characteristics at macroscopic scale. From the various environmental samples collected, 15 strains were isolated, 11 filamentous and 4 coccoid. After doing a microscopic analysis (Figure 12), it was possible to observe that the strains were very different morphologically.

The information about ecology and the morphologic description of isolated strains is summarized in Table 12.

Table 12 - Morphologic description and ecology of each isolated strains.

	Morphology				Ecology	
Strain	**Macroscopic observation**	**Type**	**Microscopic description**	**Dimensions**	**Site description**	**Environment**
1	Green Biofilm	Filamentous	Blue-green color, thin sheath, slightly tapering at the ends, straight or slightly curved trichomes, slightly tangled, rectangular cells, homogeneous, or slightly granular cell content.	3-4 µm in diameter (filament)	Water of the Cistern	Freshwater
2	Green Biofilm	Filamentous	Blue-green color, thin sheath, practically colorless, slightly tapering at the ends, straight or slightly curved trichomes, isopolar, solitary, slightly constricted trichomes, rectangular cells, homogeneous, or slightly granular cell content	4 µm in diameter (filament)	Water of the Cistern	Freshwater
3	Green Biofilm	Filamentous	Blue-green color, thin sheath, practically colorless, isopolar and straight trichomes, without irregularities, rounded apex, solitary or slightly tangled filaments, constricted trichomes, cells longer than wide, homogeneous, or slightly granular cell content	2-3 µm in diameter (filament)	Water of the Cistern	Freshwater

4	Green Biofilm	Coccoid	Blue-green color, rounded or elongated cells, irregularly grouped, microscopic, without aerotopes, homogeneous cell content.	3-4 µm in diameter, 5-6 µm in length	Water of the Cistern	Freshwater
5	Thick, sandy mat, dark green	Filamentous	Greenish color, dense colonies, trichomes tangled together, cells longer than large.	1-2 µm in diameter (filament)	Rocky limestone shore	Marine
6	Brown cyanobacteria	Coccoid	Purplish color, dense colonies, greenish cells, spherical or oval cells, without aerotopes, homogeneous cell content.	2-3.5 µm in diameter	Walls of the Cistern	Freshwater
7	Brown Tuft	Filamentous	Blue-green color, dense colonies, solitary or tangled trichomes, straight, apical cell slightly rounded, cells with similar diameter and width.	1.3-1.4 µm in diameter (filament)	*Sabellaria* Reef, Pond	Marine
8	Green Biofilm	Filamentous	Brownish color, dense clusters, blue-green and straight trichomes, curling together, apical cell slightly rounded, cells with similar diameter and width.	1.6-1.8 µm in diameter (filament)	Patella sp. On dead *Sabellaria*	Marine
9	Brown cyanobacteria	Coccoid	Brownish color, irregular colonies, cells arranged irregularly, densely aggregated, rounded or elongated cells, with greenish color and homogeneous cell content.	3-4 µm in diameter, 5-6 µm in length	Walls of the Cistern	Freshwater

10	Brown cyanobacteria	Filamentous	Purplish color, colonies densely aggregated, trichomes joined in the center of the colony, straight or slightly curved trichomes, slightly rounded apical cell, cells with similar diameter and width.	2-4 µm in diameter (filament)	Walls of the Cistern	Freshwater
11	Green Biofilm	Filamentous	Brownish color, aggregated trichomes, tangled together, rounded apical cell, cells with similar diameter and width.	1-2 µm in diameter (filament)	*Patella* sp. On dead *Sabellaria*	Marine
12	Bryophyta puff	Filamentous	Greenish color, aggregated trichomes, rolled together, apical cell slightly rounded, cells with similar diameter and width.	1-2 µm in diameter (filament)	Rocky limestone shore	Marine
13	Pink and black biofilms	Filamentous	Purplish color, trichomes tangled together, curved, long, cells with similar diameter and width.	1-3 µm in diameter (filament)	Rock, Thin Biofilm	Marine
14	Black Biofilm	Filamentous	Greenish color, solitary trichomes or in small aggregates, straight, curved or slightly wavy, long, randomly arranged, rounded apical cell, without aerotopes, homogeneous cell content.	2-4 µm in diameter (filament)	*Sabellaria* Reef, Walls	Marine
15	Green Biofilm	Coccoid	Blue-green color, rounded cells, irregularly arranged and densely aggregated, without aerotopes, homogeneous cell content.	3.6-4 µm in diameter, 5.5 µm in length	Water of the Cistern	Freshwater

Scale: 20 µm ●———●

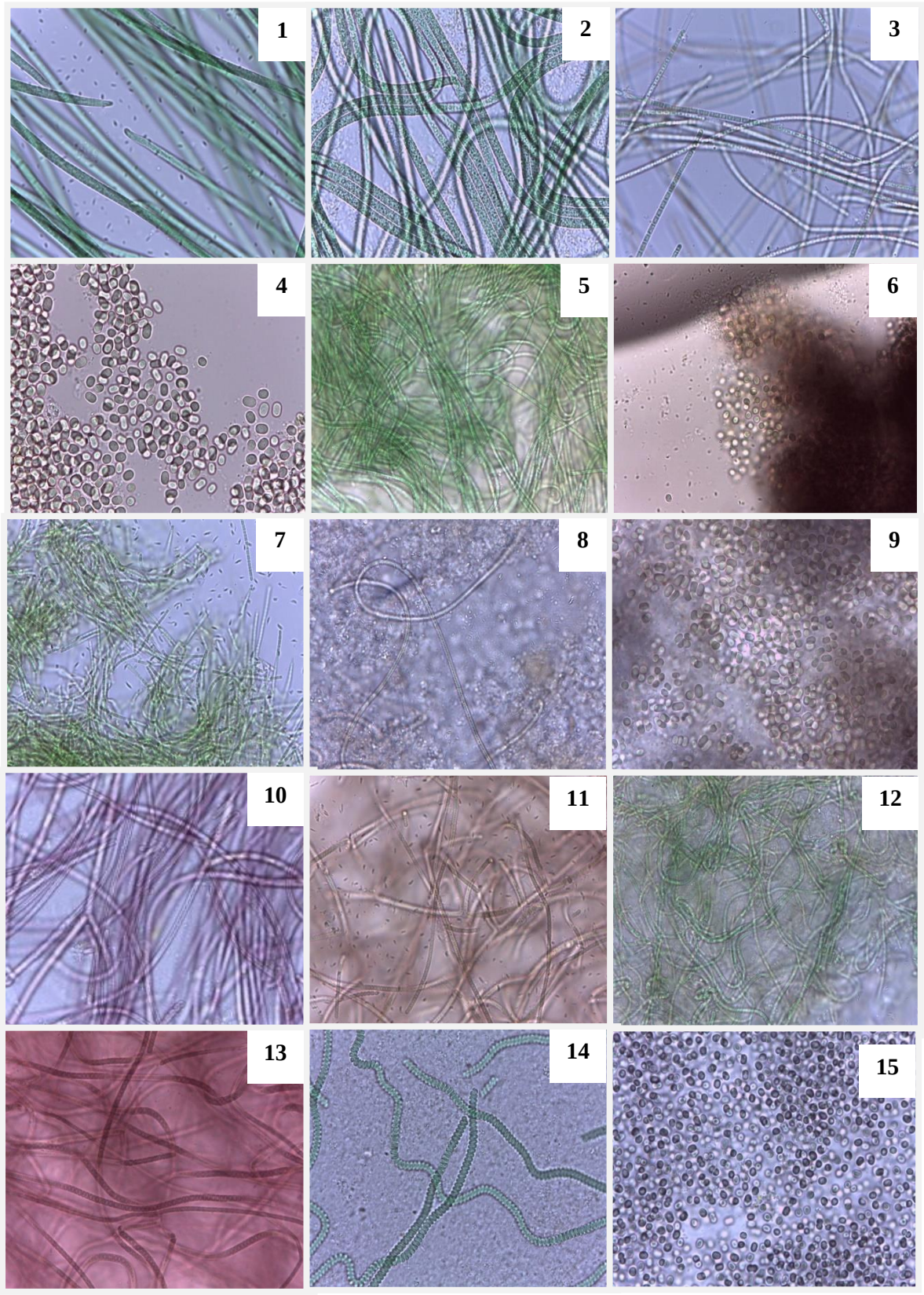

Figure 12 - Optical microscope views of isolated strains. (x1000)

Analyzing the information in Table 12, is possible to state that 8 freshwater strains and 7 marine strains were isolated. Strains 1, 2, 3, 4 and 15 were collected in the same place, in the same environment and present the same visual description. However, according to Figure 12, it is possible to observe that these strains are very different morphologically, thus demonstrating that it is possible for different strains to coexist and survive in the same environment. Strains 6, 9 and 10 were collected from the walls of the cistern, all having a brownish appearance. The rest of them were collected from rocks or other marine species.

5.2. Phylogenetic Analysis

The identification of the cyanobacteria isolates by a molecular approach was based in the 16S rRNA gene sequences. This procedure was performed for 11 of the 15 isolated strains. It was not possible to obtain the sequence of 16S rRNA gene of the strains 7, 8, 13 and 15 because when analyzing these sequences, it was possible to verify that they were of poor quality and there was no time to repeat the entire extraction and sequencing process. Each sequence obtained was compared with sequences present in GenBank and only the genera that showed the highest % ID were considered. The % of ID describes how similar the query sequence is to the target sequence. This information is shown in Table 13.

Table 13 - Top hit Molecular ID Blast of each isolated strain.

Strain	Molecular ID (top cyano hit) Blast	% ID	Seq Length bp
1	*Phormidium* sp. YACCYB597	99.68	1250
2	*Laspinema thermale* HK S3	98.92	1694
3	*Lyngbya* sp. CENA128	97.00	1300
4	*Cyanothece* sp. WH 8904	94.08	710
5	*Phormidium* sp. LEGE 11384	99.85	1806
6	*Gloeothece* sp. KO11DG	93.1	710
7	-	-	-
8	-	-	-
9	Cyanobacteria bacterium CHAB4018	94.85	1010
10	*Calenema singularis* LEGE 06188	98.39	1916
11	Oscillatoriales cyanobacterium LEGE 10370	97.24	1399
12	*Phormidium* sp. MBIC10003	98.24	1901
13	-	-	-
14	*Spirulina* sp. LEGE 11439	97.77	1301
15	-	-	-

Analyzing Table 13, it is possible to verify that strain 1 exhibited 99.68% similarity with the sequence of *Phormidium* sp. YACCYB597. Strain 2 exhibited 98.92% similarity with *Laspinema thermale* HK S3, isolated from thermal springs in Iran [94]. Strain 3, which has 97% similarity to *Lyngbya* sp. CENA128 isolated from Salto Grande Reservoir in Brazil [95], strain 4 has 94.08% similarity to *Cyanothece* sp. WH 8904 isolated in Aloha (Hawaii), strain 5 has 99.85% similarity to *Phormidium* sp. LEGE 11384 isolated from a marine sponge in a Matosinhos beach, Portugal [96], strain 6 has 93.1% similarity to *Gloeothece* sp. KO11DG isolated from the surface of sand in Sungei Buloh Natl, a Park in Singapore [97], strain 9 has 94.85% similarity to Cyanobacteria bacterium CHAB4018, strain 10 has 98.39% similarity to *Calenema singularis* LEGE 06188 isolated from coastal sea water in Lavadores beach (Portugal) [98], strain 11 has 97.24% similarity to Oscillatoriales cyanobacterium LEGE 10370 isolated from a marine sponge in 'Praia da Memória' in Portugal [96], strain 12 has 98.24% similarity to *Phormidium* sp. MBIC10003 and strain 14 which has 97.77% similarity to *Spirulina* sp. LEGE 11439 isolated from the diving spot in front of the fort 'Castelo do Queijo' in Portugal [96].

Isolated strains showed values between 93.1–99.85% of similarity compared to sequences available in the databases, which are values within the recommended threshold values for bacterial species (98–99%) or genera (94.5–95%) demarcation [99], [100]. Strain 4, 6 and 9 showed a percentage of similarity <95%, which may be indicative that these strains belong to a new genus.

The sequences of the BLAST results from the isolates of this study were integrated into the phylogenetic tree shown in Figure 13.

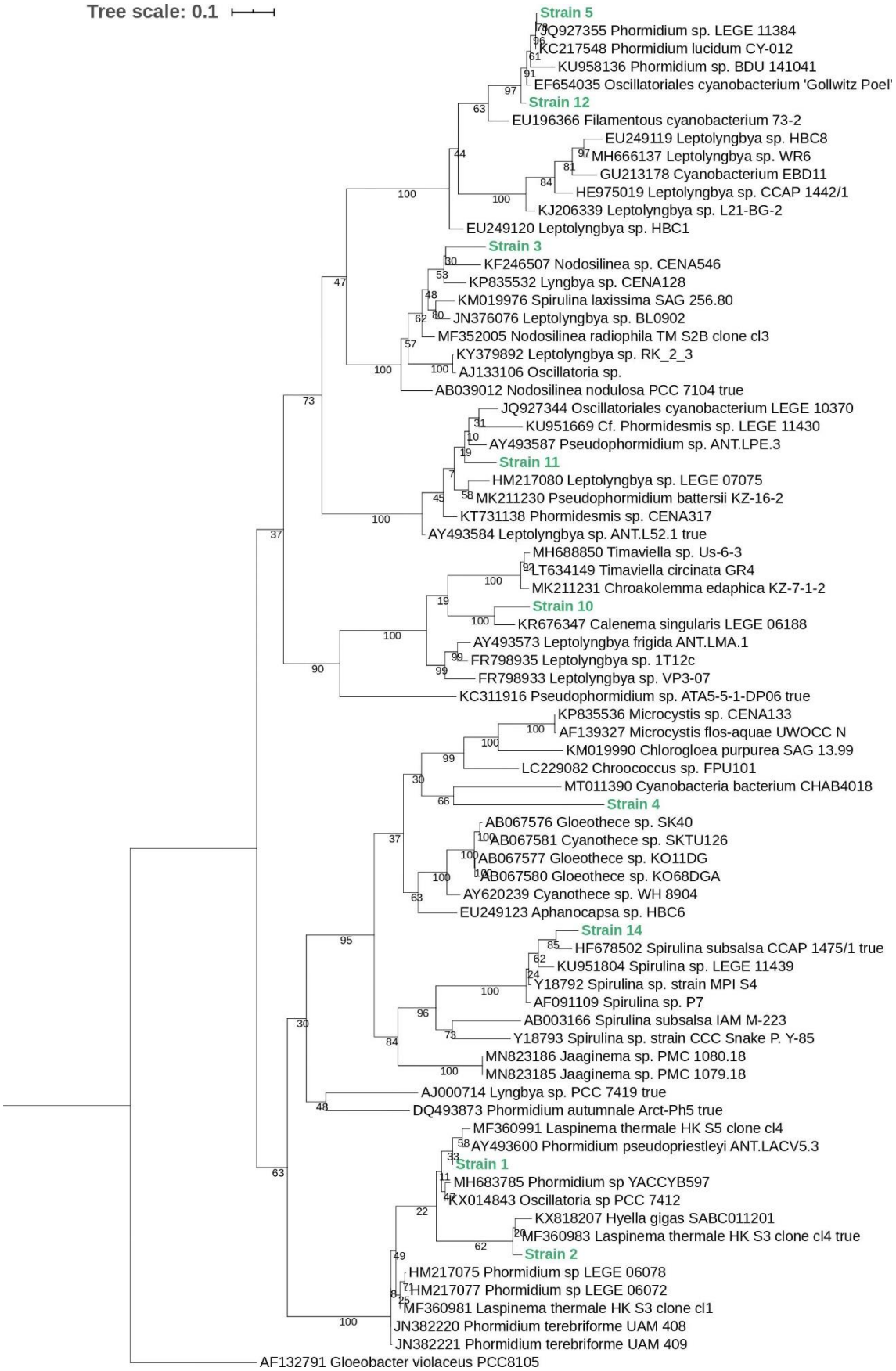

Figure 13 - Phylogenetic distribution based on the 16S rRNA gene.

Of the 15 strains isolated, it was only possible to deepen the molecular study of 11 strains. The phylogenetic tree shown in Figure 12 contains the sequences of the 16S rRNA gene from 9 of the isolates, together with sequences that were taken from the genBank. Although the 16S rRNA gene from strains 6 and 9 were also sequenced, these strains were not included in this tree, since the sequences were too short and would compromise the reliability of the phylogenetic tree obtained.

Analyzing the tree, it is possible to observe that strain 1 and 2 are included in a clade belonging to the genus *Laspinema* (MF360983 *Laspinema thermale* HK S3 clone cl4), presenting a bootstrap of 100. It is very likely that strain 1 and 2 belongs to the genus *Laspinema* since they are morphologically very identical. Strain 3 is inserted in the clade of the genus *Nodosilinea* (AB039012 *Nodosilinea nodulosa* PCC 7104), presenting a bootstrap of 100. Morphologically strain 3 is very similar to the genus *Nodosilinea*. Strain 4 is close to cyanobacteria bacterium yet to be identified (MT011392 Cyanobacteria bacterium CHAB4018). It is worth nothing that strain 4, since it does not belong to any previously published clade, probably is a new genus. Strains 5 and 12 are included in a clade without reference strains. For this reason, there is a great possibility that these two isolated strains are new genera. We can also conclude that these two isolates possibly belong to the same genus, since the clade where they are located has a bootstrap value of 97. Strain 10 is inserted in the clade of the genus *Calenema* (HQ832918 *Calenema singularis* LEGE 06188), presenting a bootstrap of 100. It is very likely that strain 10 belongs to the genus *Calenema* since they are very morphologically identical. Strain 11 is inserted in the clade of the genus *Leptolyngbya* (AY493584 *Leptolyngbya* sp. ANT.L52.1), presenting a bootstrap of 100. It is probably that strain 11 belongs to the genus *Leptolynbya* since they are very morphologically identical. Strain 14 is inserted in the clade of the genus *Spirulina* (HF678502 *Spirulina subsalsa* CCAP 1475/1), presenting a bootstrap of 96. According to the morphology of strain 14, we can verify that it closely resembles the characteristic morphology of cyanobacteria belonging to the genus *Spirulina*.

In summary, 5 different genera (*Laspinema*, *Nodosilinea*, *Calenema*, *Leptolyngbya* and *Spirulina*) and 3 different orders (Oscillatoriales, Synechococcales and Spirulinales) were found, being that *Laspinema* and *Leptolyngbya* belong to the order Oscillatoriales; *Nodosilinea* and *Calenema* belong to the order Synechococcales; and

Spirulina belongs to the order Spirulinales. Besides that, there is a high possibility that strains 4, 5 and 12 are new genera of cyanobacteria.

5.3. Toxin-producing Potential

The genes responsible to produce the main cyanotoxins (cylindrospermopsin, saxitoxin, anatoxin, nodularin and microcystin) were searched by PCR. The results are summarized in Table 14, where "+" stands for the presence of band in the respective gene and "-" corresponds to the absence of band in the respective gene.

Table 14 - Results of cyanotoxin genes PCR's.

Strain	cyrJ	sxtI	anaC	ndaF	mcyA
1	-	-	-	-	-
2	-	-	-	+	-
3	-	-	-	-	-
4	-	-	-	-	-
5	-	-	-	-	-
6	-	-	-	-	-
7	-	-	-	-	-
8	-	-	-	-	-
9	-	-	-	-	-
10	-	-	-	+	-
11	-	-	-	-	-
12	-	-	-	-	-
13	-	-	-	-	-
14	+	-	-	-	-

According to Table 14, it is possible to verify that only two of the isolates showed a positive result in the detection of the genes responsible for the production of cyanotoxins. Cylindrospermopsin gene was detected in strain 14 and nodularin genes were possible detected in strains 2 and 10. For the remaining cyanotoxins (saxitoxin, anatoxin and microcystin) the result was negative for all the isolated strains.

The use of molecular techniques has enabled the development of methods for identifying potentially toxic cyanobacteria [101]. The use of molecular methods to research the production of toxins allows its rapid detection with low cost and sensitivity

according to the nucleic acid sequences. In addition, these techniques do not require the cultivation of the organisms under study [102]. However it's important to know that genetically capable of producing toxin, does not mean that the toxin is being produced [101].

According to Table 15, it is possible to verify that only the strain 14 showed a positive result in detection of the cyrJ gene. The presence of these genes is useful in monitoring the potential for cylindrospermopsin production in conjunction with other biophysical factors, however, the presence of cyrJ is not indicative of active production of the toxin. Have been reported that Cylindrospermopsin is mainly produced by *Cylindrospermopsis*, *Aphanizomenon*, *Umezakia*, *Anabaena* (now *Dolichospermum*), *Lyngbya*, and *Raphidiopsis* [103]. Since strain 14 correspond to the genus *Spirulina*, we can verify that this genus is not included in the main group responsible to produce cylindrospermopsin. Until today there is no scientific evidence to prove that the genus *Spirulina* has the capacity to produce cylindrospermopsin, so, the result obtained in this work for strain 14 would have to be confirmed later, by sequencing the band. The genus *Spirulina* is known for the presence of Lipopolysaccharides (toxin dermatotoxic, and responsible for the impairment of immune and detoxification system, irritation, and allergic effects) [104]. Strains 2 and 10 presented a faith band in detection of ndaF gene. Nodularins are mainly produced by *Nodularia spumigena* and *Nodularia sphaerocarpa* [105]. Strains 2 and 10 are not included in the known group of genera that produce nodularins, since strain 2 possibly belongs to *Laspinema* genus and strain 10 possibly belongs to *Calenema* genus. There are no published articles that prove that *Laspinema* and *Calenema* have the capacity to produce neither nodularins, nor other cyanotoxins, so, this result obtained for strains 2 and 10 would have to be confirmed later as well.

None of the isolated strains showed a positive result in detection of sxtI, anaC and mcyA gene. Saxitoxin is mainly produced by *Cylindrospermopsis*, *Anabaena* e *Aphanizomenons* [106]. The sxtI-F/sxtI-R primer pair is used to amplify a 1669 bp fragment of the sxtI gene involved in STX biosynthesis [107]. Anatoxin is mainly produced by *Anabaena*, *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Raphidiopsis*, *Arthrospira*, *Cylindrospermum*, *Phormidium*, *Nostoc* and *Oscillatoria* [108]. Microcystin is produced by *Microcystis*, *Anabaena*, *Planktothrix* (*Oscillatoria*), *Hapalosiphon* and *Nostoc* [109]. Positive samples for the mcyA gene fragment indicate a high probability of producing microcystin [91]. Strains of microcystin-producing cyanobacteria have a

cluster of microcystin synthase (mcy) genes crucial for the production of the toxin. When that cluster is not present or contains an incomplete copy, strains generally do not produce that toxin [101]. This confirms the need and importance of researching several genes in the mcy cluster to infer the possible presence of the toxin. Previous studies have reported that variations in parameters such as light, temperature, pH and nutrients can influence microcystin production [102], which may be a reason why none of the isolated strains have shown positive results for this cyanotoxin.

5.4. Biotechnological Activity Assays

5.4.1. Lipid reducing activity in zebrafish larvae

The cyanobacterial extract of strain 1 was screened for lipid reducing activity using the zebrafish Nile red fat metabolism assay. Resveratrol (REV) was used as a positive control at a final concentration of 50 µM and 0.1% dimethyl sulfoxide (DMSO) was used as solvent control. Toxicity and malformations were evaluated on zebrafish larvae exposed to these two compounds considering general toxicity (death after 24 h or 48 h) and malformations of larval morphological features. No such adverse effects were observed for both compounds (did not cause any observable toxicity or malformations toward the zebrafish larvae). The Figure 14 represents the quantification of lipid-reducing activity in the zebrafish Nile red fat metabolism assay after exposure over 48 h.

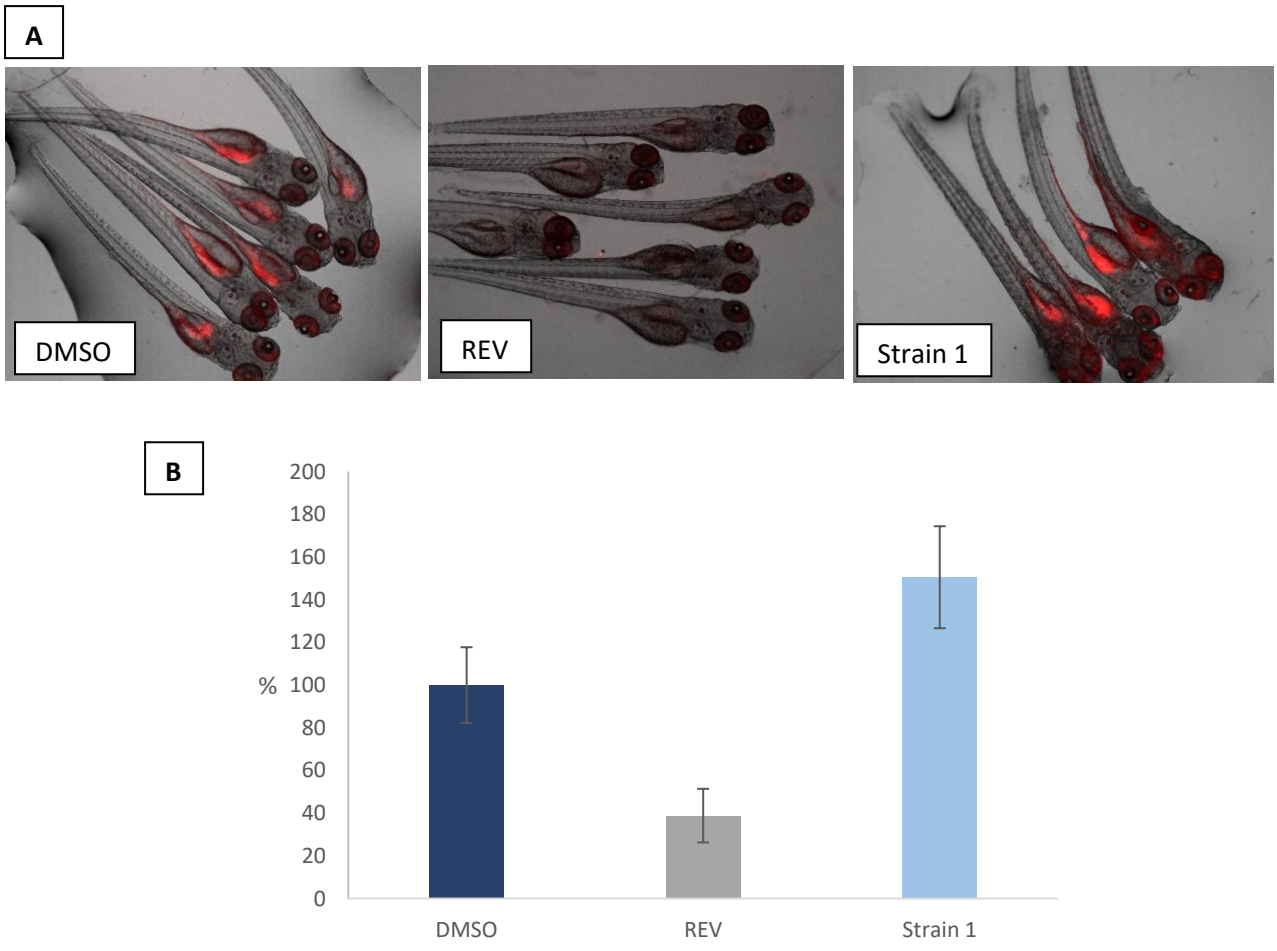

Figure 14 - Bioactivity screening using the zebrafish Nile red fat metabolism. ***(A)*** *Representative images of zebrafish larvae (overlay of brightfield picture and red fluorescence channel). Solvent control was 0.1% dimethyl sulfoxide (DMSO), and positive control was 50 µM resveratrol (REV). Strong fluorescence signal is present in zebrafish larvae from the solvent control around the yolk sac and stomach/intestine. Cyanobacterial extract of Strain 1 increased the Nile red staining.* ***(B)*** *Quantification of lipid-reducing activity in the zebrafish Nile red fat metabolism assay after exposure over 48 h. Values are expressed as mean fluorescence intensity (MFI) relative to the DMSO group and are derived from six to eight individual larvae per treatment group.*

Observing the Figure 14, it is possible to verify that the positive control (REV) reduced the Nile red lipid staining, as described in literature [67], and the cyanobacterial extract of strain 1 increased the Nile red staining, when compared to solvent control (DMSO). With these results, we can see that cyanobacterial extract of Strain 1 empowered the formation of lipids around the yolk sac and stomach, so, the extract was not effective on the reduction of the lipids in zebrafish embryos.

Recent studies demonstrated that many natural products, such as secondary metabolites from plants, cyanobacteria, fungi, and phytoplankton, possess anti-obesity activities [65]. The zebrafish Nile red fat metabolism assay is appropriate to detect anti-obesity activities and is gaining popularity between researchers, because zebrafish belongs to the category of small whole animal assays, which have a higher physiological relevance compared to the traditionally used cellular in vitro assays [67]. Also, zebrafish larvae present a small size, high fecundity, transparency, availability of genetic tools (transgenics and mutants) [110], and were demonstrated to respond to known lipid regulator drugs similarly as humans [65]. The yolk sac is maternally derived and represents the sole source of energy for the embryo and larva during early zebrafish development. Significantly, the yolk sac is a quantifiably finite source of energy that is largely consumed during the first week of larval development. These characteristics give the yolk sac distinct advantages for assaying changes in organismal lipid metabolism [67].

The zebrafish Nile red fat metabolism assay was already successfully used to identify structurally modified polyphenolic compounds with lipid-reducing activity. A study made in 2018, screened, for their lipid reducing activity and toxicity, a library of 88 polyphenol derivates belonging to several chemical families, namely, xanthone derivatives, flavones, curcumins, coumarins, benzophenones, and biaryl ethers derivatives. 38 polyphenols showed strong lipid reducing activity, but the toxicity analysis revealed that only 18 of them did not show any toxicity *in vitro* or *in vivo* [111]. Another study published in 2019, screened a library of cyanobacterial fractions for lipid reducing activity using the zebrafish Nile red fat metabolism assay. From the 263 analyzed fractions, a total of 15 fractions showed the capacity to reduce neutral lipids in zebrafish larvae *in vivo*. The characterization of the most promising fractions with lipid reducing activity led to the identification of the compounds eryloside T, pheophorbide A and phaeophytin A. This fractions belonged to strains LEGE 07175, LEGE 00246, LEGE

07172, and LEGE 07173, that correspond to *Cyanobium* sp., *Dolichospermum* sp., *Synechococcus* sp. and *Synechocystis salina*, respectively [68].

5.4.2. Superoxide anion radical scavenging assay

The antioxidant potential of the cyanobacterial extract of train 1 was determined by superoxide anion radical ($O_2^{\bullet -}$) scavenging assay, as previously described. Various concentrations of cyanobacterial extract were tested, between 0.10 mg/ml and 1.66 mg/ml. The test results are showed in Figure 15.

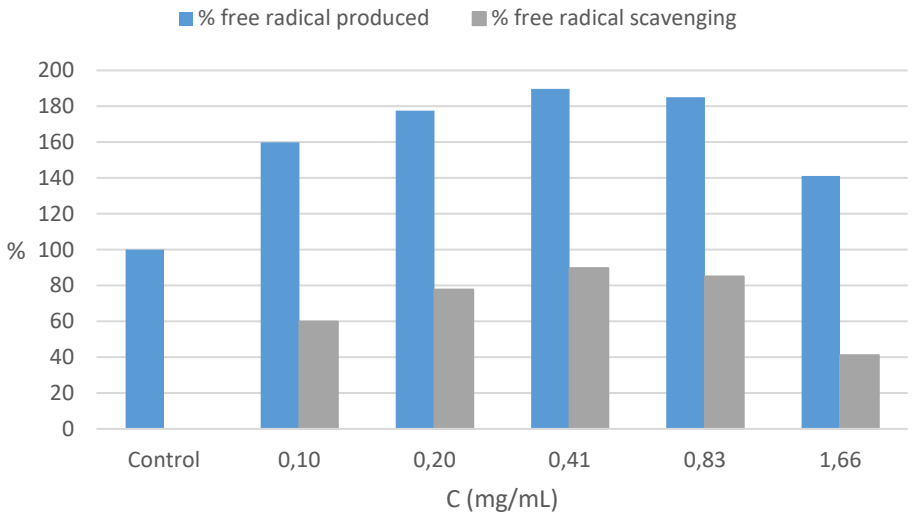

Figure 15 - Antioxidant activity and percentage of free radical sequestration activity

By observing Figure 15, it is possible to verify that the cyanobacterial extract referring to strain 1 stimulated the production of free radicals, as it presented percentage values higher than the values presented by the control. To understand whether the cyanobacterial extract concentration influenced the percentage of free radical formation, a second test was performed with lower concentrations (between 0.01 and 0.16 mg/mL) and the results are showed in Figure 16.

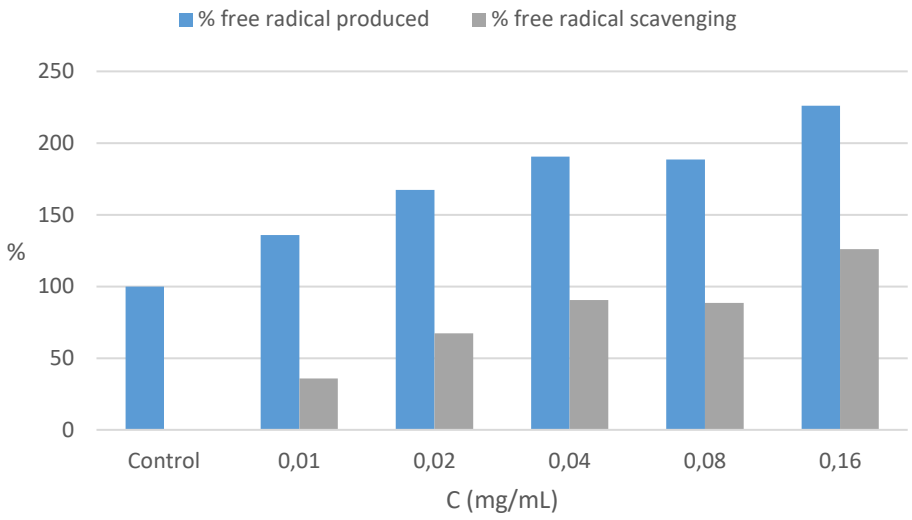

Figure 16 - Antioxidant activity and percentage of free radical sequestration activity

According to Figure 16, it is possible to verify the same behavior shown in Figure 15. The percentage of free radical produced by the cyanobacterial extract of strain 1 is higher than the percentage of free radical present in control. So, the extract of strain 1 probably induced the formation of free radical, demonstrating a pro-oxidant behavior.

The superoxide ($O_2^{\bullet -}$) is a by-product of oxygenic respiration and represents an important role in physiological processes performed by organisms [64]. Free radicals play a major role in the progression of a wide range of pathological disturbances and it can be scavenged by the supplementation of antioxidants to food or to the biological system [66]. Its accumulation can lead to a deregulation of redox homeostasis, production of others reactive oxygen species (ROSs), being correlated with a great amount of diseases. For this reason, is growing in importance the screening of molecules of natural extracts able to scavenge $O_2^{\bullet -}$, and subsequently able to regulate the oxidant activity [64].

Strain 1 belongs to the genus *Laspinema* and have been reported the antioxidant activity of this genus, which contradicts the behavior presented in this assay. It may be due to the fact that they are different strains of *Laspinema* or possibly the extract contains in its composition compounds that favor this behavior [112]. A study published by Bavini et al. (2018), selected 8 cyanobacteria strains, which included *Phormidium* sp. LEGE 05292, *Synechocystis salina* LEGE 06099, *Nodosilinea nodulosa* LEGE 06102, *Cyanobium* sp. LEGE 06113, *Synechocystis salina* LEGE 06155, *Oscillatoriales* LEGE

07167, *Cyanobium* sp. LEGE 07175 and *Tychonema* sp. LEGE 07196, to evaluate the antioxidant potential assessing the superoxide radical ($O_2^{\bullet -}$) scavenging assay. Results showed that *Phormidium* sp. LEGE 05292 was the most interesting cyanobacteria extract scavenging the $O_2^{\bullet -}$ [113]. Alfeus et al. also reported the antioxidant activity of 8 strains of cyanobacteria: LEGE 06113, LEGE 07175, LEGE 06099, LEGE 07167, LEGE 06102, LEGE 06194, LEGE 06108 and LEGE 06173. These include *Cyanobium*, *Leptolyngbya*, *Nodosilinea*, *Pseudoanabaena* and *Synechocystis*. After assessment the superoxide radical scavenging activity assay, a higher antioxidant potential was registered from strains *Leptolyngbya mycoidea* LEGE 06108, *Synechocystis salina* LEGE 06099, *Lusitaniella coriacea* LEGE 07167 and *Cyanobium* sp. LEGE 07175, with 49.7%, 49.57, 42.77% and 47.39%, respectively, at 10 µg/mL [114]. Lopes et al. (2020) verified that the antioxidant potentials of the different cyanobacteria extracts were determined by evaluating their capacities to scavenge the physiologic radical $O_2^{\bullet -}$ using an in vitro cell-free assay. The results showed that acetone extracts were more effective than ethanol towards radical scavenging. The ethanol extract from *Cuspidothrix issatschenkoi* LEGE 03282 was the most effective, reaching IC_{50} at the lowest concentration tested (0.728 mg/mL). Acetone extracts were much more interesting in terms of radical scavenging, with the species *Cuspidothrix issatschenkoi* LEGE 03282 and *Nodosilinea antarctica* LEGE 13457, presenting the lowest IC_{50} values (0.286 and 0.319 mg/mL, respectively), followed by *Alkalinema aff. pantanalense* LEGE 15481 (0.382 mg/mL) [64].

5.4.3. Anti-inflammatory potential

Inflammation is normal protective response of human body to injury and infection, in which reactive species are produced [115]. NO (nitric oxide) represents one of the main molecules involved in the inflammatory response process, but it also acts on vasodilation and as a neurotransmitter [64]. Although this molecule is produced at basal levels, this scenario does not occur when pathogens interfere. NO starts to be produced by macrophages in higher amounts by inducible nitrous oxide synthase (iNOS). The excessive production of NO is related to processes of carcinogenesis, organ dysfunction, tissue damage or septic shock [115].

The anti-inflammatory potential of cyanobacteria extract of strain 1 was explored through the analysis of their effect on the levels of NO produced by RAW 264.7 macrophages upon LPS stimulation.

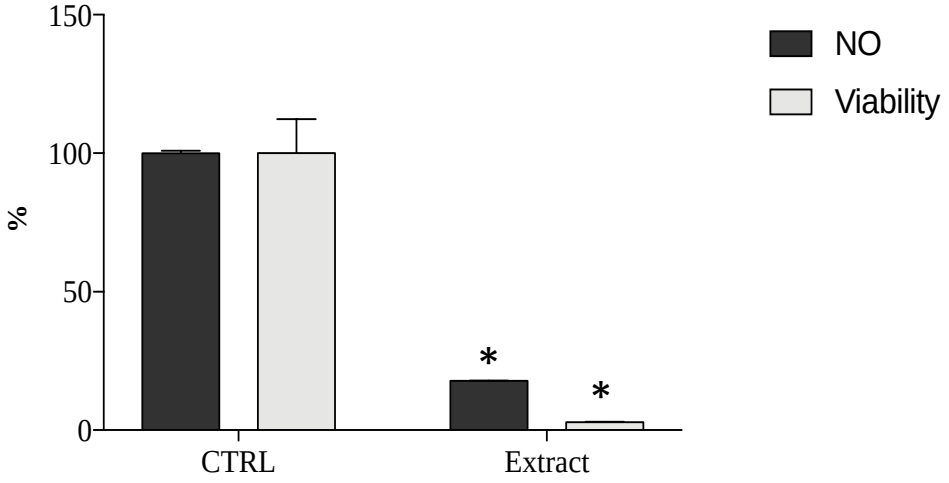

*Figure 17 - Nitric oxide (NO) production by RAW 264.7 cells in the presence of cyanobacterial extract of Strain 1 after stimulation with lipopolysaccharide (LPS). Results are expressed as % of NO relative to the control stimulated with LPS. Results are expressed as the mean ± SD of three independent assays, performed in duplicate. *p < 0.05 (t-test).*

Figure 17 shows that the cyanobacterial extract of strain 1 induced a reduction in NO levels, when compared to control. However, it cannot be considered as anti-inflammatory activity, since the extract was toxic and there was cell death (which can be seen by the viability bars in the graph). In short, there was less nitric oxide production because a significant percentage of cells were dead.

The antioxidant potential of the extracts was described as being able to influence the inflammatory activity, through their capacity to scavenge reactive species [64]. So, considering this association between antioxidant activity and inflammatory potential, this result could be expected when assessing the superoxide assay, in which the cyanobacterial extract was verified to have a pro-oxidant character. Strain 1 belongs to the genus *Laspinema*, and it has recently been described that this genus has a biosynthetic cluster with the ability to produce toxic compounds, giving it a cytotoxic potential. Which confirms the results presented in this essay [112].

In recent years, many researchers have dedicated themselves to researching natural extracts and isolated compounds capable of sequestering free radicals and thus regulating anti-inflammatory activity. A wide variety of seaweed was reported to be an excellent source of beneficial bioactive secondary metabolites for human health [115]. Until now, only few studies have proved the anti-inflammatory potential of cyanobacteria extracts [64]. Gomes Ferreira et al. worked with 13 distinct strains of cyanobacteria, and 117 different fractions were tested in RAW 264.7 cells for testing anti-inflammatory activity. Results showed that fractions of strains *Synechocystis* sp. LEGE 07211, *cyanobacterium* LEGE 07212 and *Limnothrix* sp. LEGE 00237 presented strong potential in anti-inflammatory activity and *Nostoc* sp. LEGE 06077 had a pro-inflammatory activity. *Phormidium* sp. LEGE 06363 presented 3 fractions with strong anti-inflammatory activity and 1 fraction with pro-inflammatory activity [116]. It was previously described that monogalactosyldiacylglycerol (MGDG), an isolated compound of the cyanobacterial strain *Phormidium* sp., acts positively in the inflammatory response induced in human articular cells, without damaging the cell viability [117].

Lopes et al. (2020) explored the anti-inflammatory potential of cyanobacteria extracts through the analysis of their effect on the levels of NO produced by RAW 264.7 macrophages upon LPS stimulation. The study revealed that *N. antarctica* LEGE 13457, *A. pantanalense* LEGE15481 and *Leptolyngbya*-like sp. LEGE 13412 reduced the nitric oxide (NO) in RAW 264.7 cells, revealing anti-inflammatory potential. The decrease in NO levels observed could be correlated with the presence of individual compounds in the extracts, or to a synergism between the whole pigments [64].

Characterization and identification of the compounds in the cyanobacteria extracts can be done by HPLC, comparing retention time with the standard compounds and by co-

injection while quantification is done by comparing peak areas of identified compounds in the samples with those of the standard compounds [118].

6. Conclusions

The present work constituted a preliminary contribution for the study of the biodiversity and bioactive potential of cyanobacteria from El Jadida, Marocco. From the collected samples, 15 cyanobacteria strains were isolated (11 filamentous and 4 coccoid). Evaluating the morphological characteristics, it was possible to verify that the isolates were very different from each other, presenting different colors, types, and dimensions. It was possible to sequence the 16S rRNA gene of 11 isolates and a phylogenetic tree was built with 9 of these sequences. The phylogenetic analysis allowed to know the diversity of the isolated strains, being found 5 genera and 3 different orders of cyanobacteria. The phylogenetic tree also revealed the possibility that strains 4, 5 and 12 belong to new genera, which contributes to enrich the diversity of cyanobacteria. Three isolated strains showed a toxic potential, since the genes responsible for the production of cylindrospermopsin were detected in strain 14 and the genes responsible for the production of nodularins were detected in strains 2 and 10. Strain 1 showed no lipid reducing effect, since the extract induced the formation of lipids around the yolk sac and stomach in zebra fish embryos; demonstrated a pro-oxidant activity, since the extract stimulated the formation of free radicals; and showed no anti-inflammatory effect in RAW 264.7 cells, since it causes cell death.

7. Future work

Some could be done in the future in order deepened the knowledge about the strains analysed in the present work. So, in the future it could be interesting to include the isolated strains in the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). Also, do additional work to sequence the 16S rRNA gene of strains that was not possible to sequence in the course of this work. Also, it would be necessary to formally describe the new genera found, through a polyphasic approach (phylogeny, morphology, ecology, and secondary metabolites). It is also important to verify that the isolated strains that showed toxic potential, are truly cyanotoxin producers or not, and if this is confirmed, the production/quantification of cyanotoxins could be determined through High Performance Liquid Chromatography (HPLC) and Gas chromatography–mass spectrometry (GC-MS). It would also be necessary to subject all isolates to the same biotechnological assays that strain 1 was subjected to. If any of the cyanobacterial extracts tested shows any biotechnological activity, the compounds present in the extract that were responsible for the activity must be individually identified. Also, in case of any promising biological activity test, the cultivation conditions for large-scale growth of that strain should be studied.

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9. Appendix

9.1. Appendix 1 - Consensus sequences

The sequences that follow refer to the amplified sequences, through the 16S rRNA gene, for determining the genera/species of the isolates. All sequences are in the 5'→ 3' direction.

Strain 1

GCAAGTCGAACGGTGAACCCTCGGGTTCATAGTGGCGGACGGGTGAGTAACACGTGAGAATCTGCCTC
CAGGTCGGGGACAACAGCGGGAAACTGCTGCTAATACCCGATGTGCCTAAGGGTGAAAGATTAATTGC
CTGGAGATGAGCTCGCGTCCGATTAGCTAGTTGGTAGAGTAAAAGCCTACCAAGGCTCCGATCGGTAG
CTGGTCTGAGAGGATGAGCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAAGACCGCGTGGGGGATGAAGGCTCTTGGG
TTGTAAACCCCTTTTCTCAGGGAAGAAGAACTGACGGTACCTGAGGAATCAGCATCGGCTAACTCCGTG
CCAGCAGCCGCGGTAAGACGGAGGATGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTTCGTAGG
TGGCTGTTCAAGTCTGCCGTTAAAGACCGGGGCTTAACTCCGGAAAAACTGTGGAAACTGAACAGCTA
GAGTATGGTAGGGGTAAGGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCG
GTGGCGAAGGCGCCTTACTGGGCCATAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAAAGGGAT
TAGATACCCCTGTAGTCCTAGCTGTAAACGATGGATACTAGGTGTTGCCCGTATCGACCCGGGCAGTGC
CGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGGCTT
GACATGTCCAGAATCTCGGGGAAACCTGAGAGTGCCTTCGGGAGCTGGAACACAGGTGGTGCATGGCT
GTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTCCTTAGTTGCC
ATCATTAAGTTGGGCACTTTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAG
TCAGCATGCCCCTTACGTCCTGGGCTACACACGTACTACAATGGGAAGGACAGAGGGTAGCAAGCGCG
CGAGTGCAAGCCAATCCCATAAACCTTTCCTCAGTTCAGATTGCAGGCTGCAACTCGCCTGCATGAAGG
CGGAATCGCTAGTAATCGCAGGT

Strain 2

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CTTAGTTGCCATCATTAAGTTGGGCACTTTAGGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGN
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Strain 3

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Strain 4

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Strain 5

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Strain 6

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Strain 9

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Strain 10

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ACC

Strain 11

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Strain 12

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Strain 14

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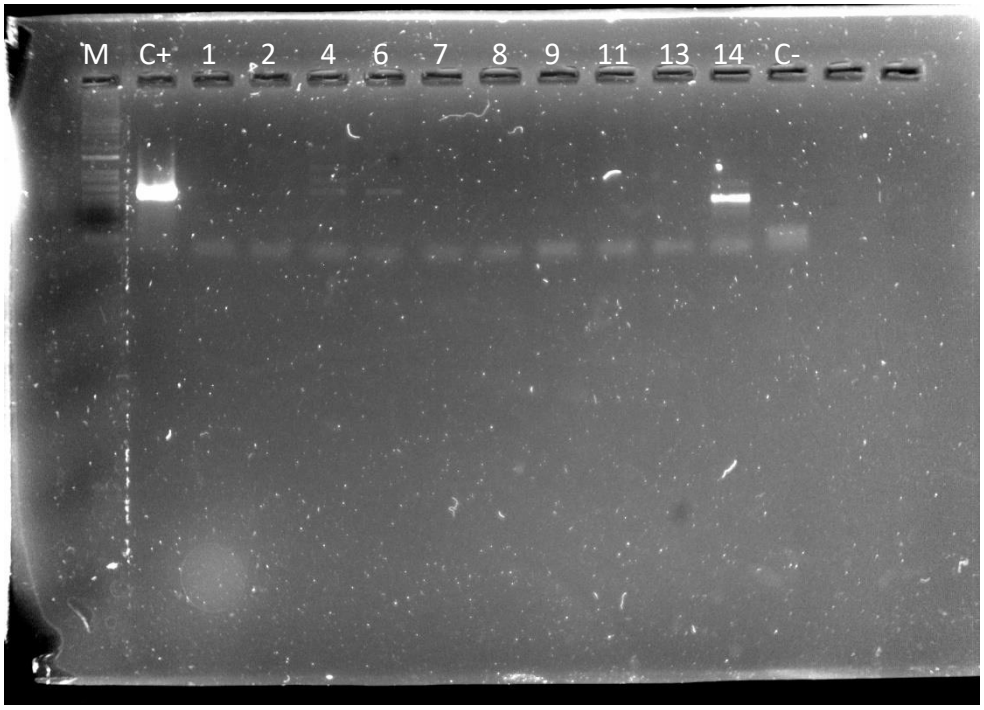
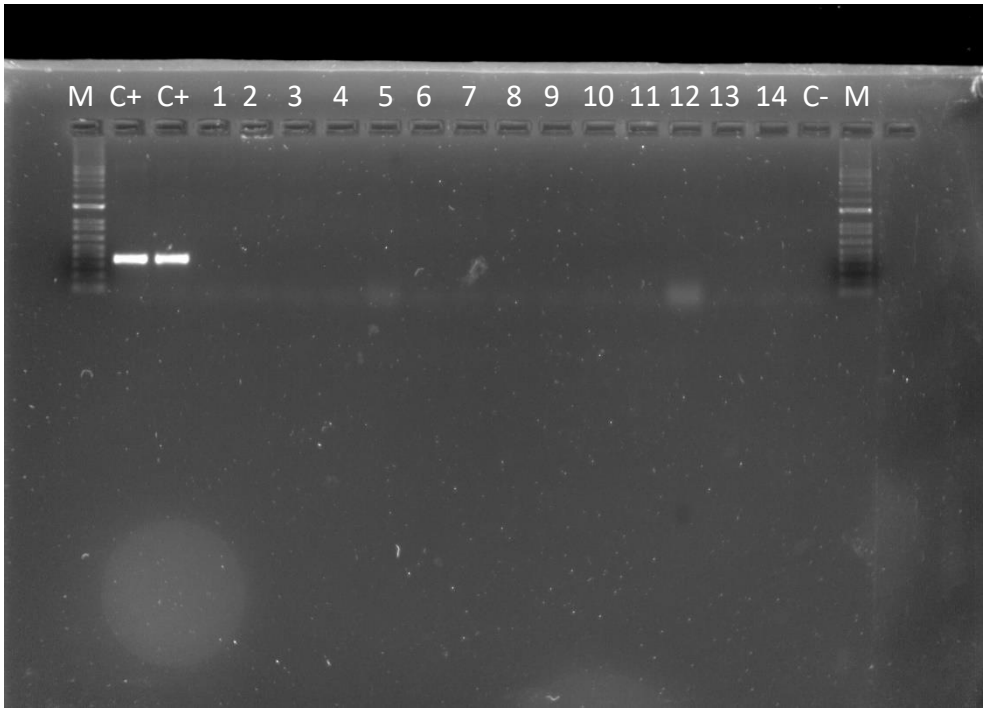
9.2. Appendix 2 - Cyanotoxin genes PCR's

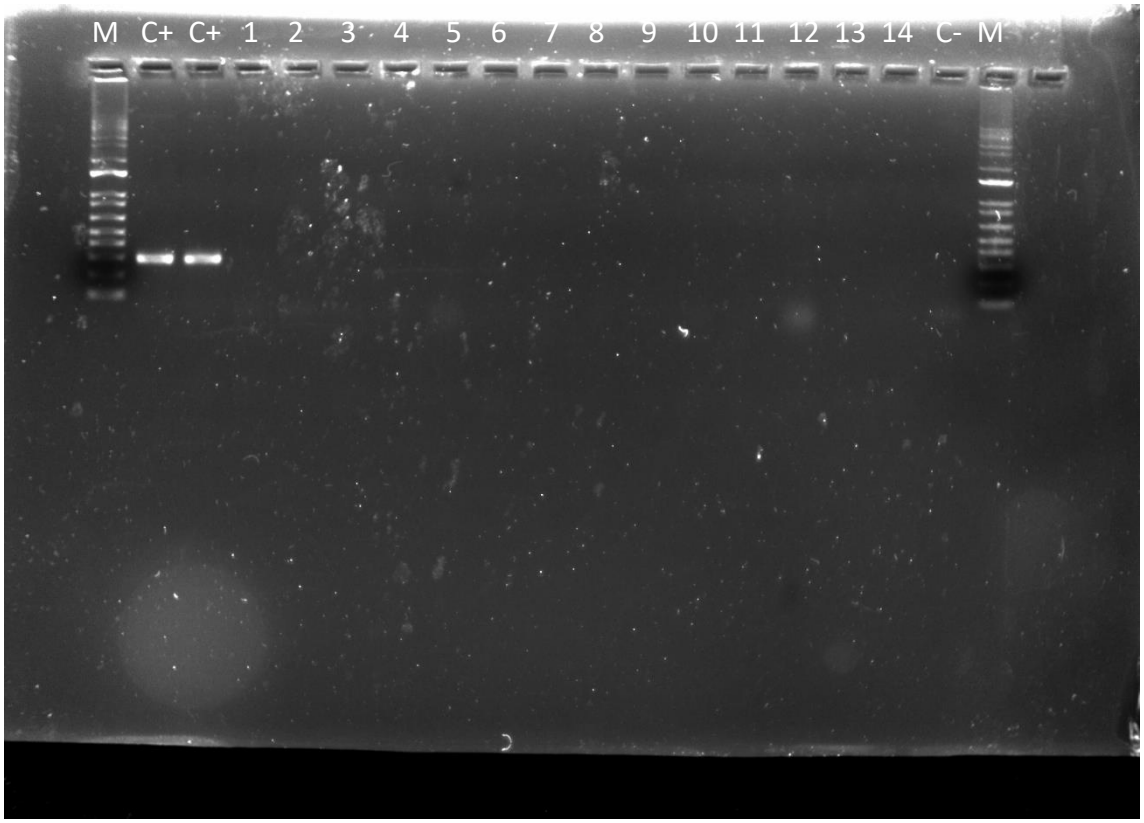
9.2.1. Cylindrospermopsin



9.2.2. Anatoxin



9.2.3. Microcystin



9.3. Appendix 3 - Lyophilization and extraction yield

As previously mentioned, strain 1 was subjected to three different biotechnological activity assays, it was first necessary to proceed with its lyophilization and extraction. In order to calculate the yield, both from the lyophilization process and from the extraction process, the initial and final values of the product were recorded. These values are shown in Table 16.

Table 15 - Values of biomass (before and after lyophilization and extraction) and yield percentage

	Lyophilization	**Extraction**
Initial Biomass	18.8786 g	0.2164 g
Final Biomass	0.2164 g	0.0219 g
Yield	1.15%	10.12%

Both lyophilization and extraction yields showed low values (1.15 and 10.12%, respectively), however, taking into account that there was practically no product loss during the procedures, these yield values are close to the maximum values that could be obtained.