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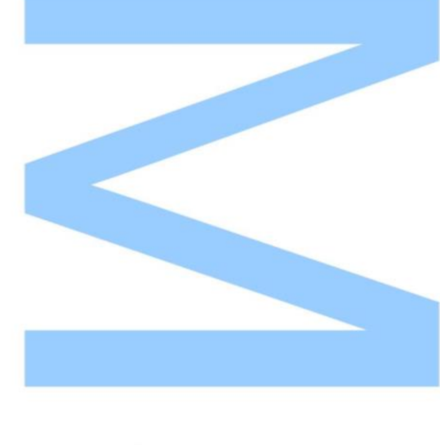
Diversity, toxicity and biotechnological potential of subaerial cyanobacteria



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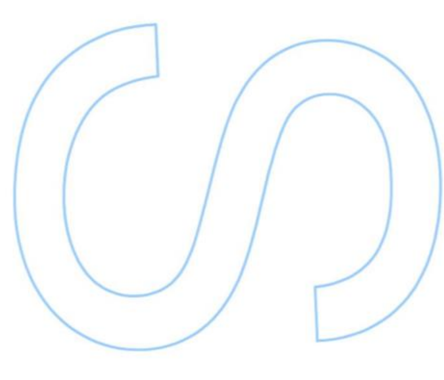

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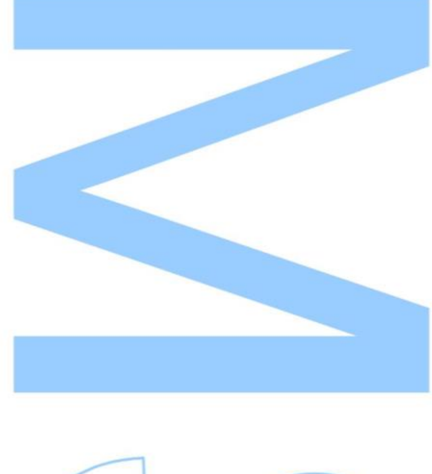

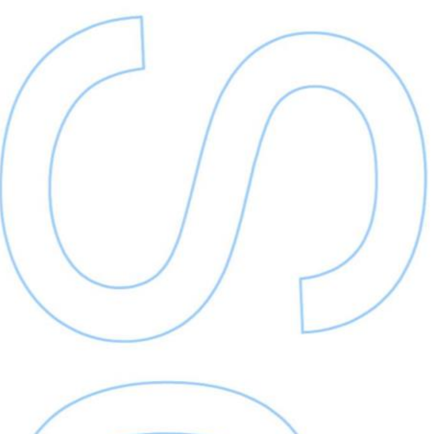

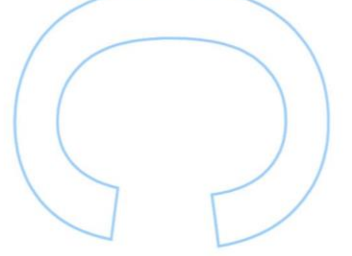

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Resumo

Cianobactérias subaéreas são microorganismos que habitam ambientes que podem ser considerados extremos, estão geralmente em contacto direto com o ar e o seu acesso á água está altamente dependente do clima de uma certo local, devido a isso elas estão mais suscetíveis a dessecação, elas podem estar sob luz solar direta experienciando altos níveis de radiação UV e porque elas não estão submergidas, a turvidez da água, que bloqueia parte da radiação é uma camada de proteção que elas não têm ao contrário das cianobactérias aquáticas. Em vez disso, elas têm outros mecanismos que as ajudam a sobreviver nesses tipos de ambientes, elas conseguem produzir vários metabolitos secundários para combater tipos específicos de perigos característicos de ambientes terrestres. Uma vez que esses metabolitos secundários são moléculas bioactivas que podem ter potenciais usos biotecnológicos, e considerando a diferença de condições ambientais entre ambientes aquáticos e terrestres, as cianobactérias subaéreas podem ser consideradas um bio-recurso inexplorado que pode ser fonte de um diferente conjunto de compostos bioativos, alguns dos quais podem ser tóxicos para os humanos. Nessa perspectiva, tapetes de cianobactérias terrestres foram colhidos ao longo da região hidrográfica 2 de Portugal, no norte do pais, e diversas estirpes foram isoladas usando os meios Z8 e $BG11_0$. A diversidade das estirpes isoladas foi determinada através da sua identificação usando uma abordagem polifásica, que consistiu na observação morfológica das estirpes e da amplificação do gene do 16S rRNA para cada uma, e usando as sequências *consensus* do gene do 16S rRNA uma árvore filógenética de *maximum likelihood* foi construida para avaliar as suas semelhanças relativamente a outras estirpes e entre elas. O seu potencial biotecnológico foi determinado através da examinação da presença dos genes PKS e NRPS através de amplicação por PCR, e revelou que o gene NRPS tinha uma alta prevalência nas amostras ambientais colhidas e nos isolados obtidos e que o gene PKS tinha muito pouca presença nas amostras ambientais. Para avaliar a sua capacidade de produzir cianotoxinas, uma amplificação por PCR foi feita usando vários primers especifícos que amplificavam genes envolvidos na sintese de cianotoxinas, e apesar de não ter sido detectada a presença de nenhum gene de cyanotoxinas em nenhum dos isolados rastreados, foi descoberto que a presença do gene *sxtl*, envolvido na sintese da saxitoxina, era muito alta nas amostras ambientais terrestres, e que genes envolvidos na sintese de microcistinas, nodularinas e cilindrospermopsinas foram também detetados, revelando que os tapetes terrestres podem possuir cianobactérias produtoras de cianotoxinas.

Palavras-chave: Cianobactérias, ambientes terrestres, biorecursos, cianotoxinas, compostos bioativos, potencial, biotecnologia.

Abstract

Subaerial cyanobacteria are microorganisms inhabiting environments that can be considered extreme, they are generally in direct contact with air and their access to water is highly dependent on the weather of a certain location, because of that they are more prone to desiccation, they can be under direct sunlight experiencing high levels of UV radiation and because they are not submerged, water turbidity, that blocks part of the radiation is a layer of protection that they don't have unlike aquatic cyanobacteria. Instead, they have other mechanisms that help them survive in these types of environments, they can produce several secondary metabolites to combat specific types of hazards characteristic of terrestrial environments. Since those secondary metabolites are bioactive molecules that can have potential biotechnological usage, and considering the difference of environmental conditions between aquatic and terrestrial environments, subaerial cyanobacteria can be considered an untapped bioresource that can be the source of a different array of bioactive compounds, some of which may also be toxic to humans. In that perspective, cyanobacterial terrestrial mats were collected along Portugal's hydrographic region 2, in the north of the country, and several strains were isolated using Z8 and $BG11_0$ media. The isolated strains diversity was determined through their identification them using a polyphasic approach, which consisted of morphological observation of the strains and the amplification of the 16S rRNA gene for each one, and using consensus sequences of the 16S rRNA gene a maximum likelihood phylogenetic tree was built to assess their similarities relatively to other strains and to each other. Their biotechnological potential was determined by examining the presence of the PKS and NRPS genes through PCR amplification, and it revealed that the NRPS gene had a high prevalence both in the environmental samples collected and the isolates obtained and that the PKS gene had very little presence in the environmental samples. To access their capacity to produce cyanotoxins, a PCR amplification was made using several specific primers that targeted genes involved in the synthesis of cyanotoxins, and although it was not detected the presence of any cyanotoxin genes in any of the isolates screened for, it was discovered that the presence of *sxtI* gene, involved in saxitoxin synthesis, was very high in terrestrial environmental samples, and that genes involved in the synthesis of microcystin, nodularin, and cylindrospermopsin were also detected, revealing that terrestrial mats can host cyanotoxin-producing cyanobacteria.

Keywords: Cyanobacteria, terrestrial environments, bioresources, cyanotoxins, bioactive compounds, potential, biotechnology.

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List of abbreviations

BLASTn	Basic Local Alignment Search Tool for nucleotides
CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
ddH20	Double Distilled Water
dNTPs	Deoxynucleotides
eDNA	Environmental ribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EPSs	Extracellular polymeric substances
gDNA	Genomic deoxyribonucleic acid
GPS	Global Positioning System
LC-MS	Liquid chromatography-mass spectrometry
LEGEcc	Blue Biotechnology and Ecotoxicology Culture Collection
MAAs	Mycosporine-like amino acids
mBRCs	Microbial biological resource centers
MEGA7	Molecular Evolutionary Genetics Analysis Version 7.0
$MgCl_2$	Magnesium dichloride
ML	Maximum likelihood
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NRPSs	Non-ribosomal polypeptide synthetases
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PKSs	Polyketide synthases

1. <u>Introduction:</u>

1.1. Framework:

Cyanobacteria, previously known as blue-green algae due to the fact that most cyanobacteria produce phycocyanin pigments that give them a blueish color, belong to a group of gram-negative photosynthetic bacteria whose fossil records, the stromatolites, indicate that they date back to approximately 3.5 billion years ago (Awramik *et al.,* 1983), at a stage in which oxygen was first beginning to develop on planet earth. They are believed to be some of the first organisms to appear on our planet and have accompanied its development throughout many of its stages, including the continental drift and the formation of the continents and oceans as we know them today (Santucci, 2005; Paerl *et al.*, 2000). In that sense, given how much the planet has changed since those 3.5 billion years ago, a number factors, namely UV radiation, and other abiotic factors during that period have in a way contributed to their evolution and to the development of defense mechanisms against the environmental stresses they faced in a wide variety of environments (Garcia-Pichel, 1998).

Their resilience has contributed to their survival and worldwide presence even in the most extreme habitats (Paerl *et al.*, 2000). As a result, they are virtually present anywhere on the planet. They can occur in freshwater environments, like rivers and lakes, and higher salinity environments, like brackish waters, salt waters at sea, and can even endure extreme salinities in salterns (Tkavc *et al.,* 2010). Cyanobacteria can also be found in many terrestrial environments present in biocrust communities on top of other organisms (Singh *et al.,* 2017), on rocks, soil and man-made infrastructures (Vázquez-Nion *et al.,* 2016) and they can also survive in other extreme environments like hot springs (Subudhi *et al.,* 2018), in hot and arid deserts (Alwathnani & Johansen, 2011), cold deserts (Vincent, 2007) or survive in low to no sunlight irradiation conditions like in caves (Vasiliki, 2015).

Their ability to resist a broad range of environmental conditions can be attributed to their metabolic plasticity and to their ability to produce secondary metabolites which can aid them in their survival in a particular environment (Paul *et al*, 1999; Paerl *et al.*, 2000). They have a remarkable ability to survive in extreme conditions where many other microorganisms would perish, for example, some strains have a huge tolerance to low or to high temperature (Schmidt *et al.,* 2011; Alwathnani & Johansen, 2011), to high salinity (Tkavc *et al.,* 2010), to low and high pH values (Lopez-Archilla et al., 2004; González-Toril et al., 2003), to desiccation (Potts, 1999) and to exposure to high UV radiation or even in lack of sunlight irradiation (Singh *et al.,* 2017; Lamprinou *et al.*, 2015). So, considering the variety of extreme environments throughout our planet and each of

their specific set of harmful/stressful conditions, the microorganisms that are able to inhabit them are more likely to have unusual ways of surviving in them, and are thus considered a good source for finding new bioactive compounds/substances that might have potential for biotechnological uses (Harvey, 2000).

In this work the object of study were subaerial cyanobacteria, which according to Schlichting (1975) can be defined as being subaerial organisms inhabiting any object above the soil, litter or water surface, meaning those directly in contact with air, although some authors include endolithic forms, which refers to those inhabiting partly on or inside the surface, as also being subaerial cyanobacterial (Pentecost & Whitton, 2012).

Subaerial environments can be considered extreme environments to cyanobacterial mainly due to stress factors like temperature, lack or excess of UV radiation and desiccation (Pentecost & Whitton, 2012). Because subaerial cyanobacteria are not submerged in water, and are generally in direct contact with air and exposed to sun radiation their temperature can rise quickly and they are susceptive to lose water quickly and to have to endure long periods of dryness depending on the weather (Pentecost & Whitton, 2012). One particular characteristic that allows subaerial cyanobacteria to survive in an environment with such a low contact with water is their ability to produce extracellular polymeric substances (EPS) which keeps the mats together by working like a glue and prevents loss of water, EPS are produced by most subaerial filamentous cyanobacteria, because of that they are the first colonizers of the terrestrial mats especially in dry locations (Garcia-Pichel & Wojciechowski, 2009). Their relative wetness varies along the year due to seasonal variations in precipitation and temperature, and it can be classified as mesic, for surfaces that remain wet for long periods, and xeric, for surfaces which are rarely wet (Fletcher, 1973). Temperature is another stress that subaerial cyanobacteria have to endure, they experience much wider temperature changes than cyanobacteria in aquatic habitats. (Pentecost & Whitton, 2012). Relative humidity, temperature and intensity of UV irradiation are all factors that are determined by the weather of a geographical location. Each region of the planet has its own climacteric conditions, and the north of Portugal can be classified as a region with a temperate climate (Kottek, 2006). Nutrient availability can limit the growth of cyanobacteria in terrestrial environments where major nutrients like nitrogen (N) can be in deficiency, but heterocystous cyanobacteria have the ability to fix N_2 (Wolk *et al.,* 1994), acting as N_2 providers to other microorganisms in terrestrial mats (Belnap, 2002).

Although subaerial cyanobacteria can - much in the same way as aquatic cyanobacteria - produce bioactive secondary metabolites with potential biotechnological usefulness (Lamprinou *et al.*, 2015; Martins *et al.,* 2008), they are still understudied when

in comparison with their aquatic counterparts and are pretty much an unexplored resource with the potential to yield new bioactive compounds, especially in Portugal. The secondary metabolites that cyanobacteria produce are products that rarely have a role in their primary metabolism, growth or reproduction but cyanobacteria have evolved to somehow benefit from their production, depending on the type of habitat (Paul *et al*, 1999). Cyanobacteria strains may produce specific secondary metabolites that help them survive and resist biotic or abiotic stresses present in a particular environment. For example, it has been shown that subaerial cyanobacteria from caves, which are dark and nutrient-limited environments, have antibacterial activity against human pathogenic bacteria (Vasiliki, 2015). This happens probably as a response to hinder the growth of other microorganisms and give themselves a competitive advantage over the limited resources in caves. It has also been shown that subaerial cyanobacteria present in building rooftops and trees are able to produce mycosporine-like amino acids (MAAs), that are a UV absorbing compounds that act as a defense mechanism against high UV radiation (Singh *et al.,* 2017). Also, most subaerial cyanobacteria have a thick sheath or extracellular matrix that can protect them from desiccation, which can be particularly helpful in terrestrial environments (Potts, 1999). The secondary metabolites besides being beneficial to their survival, have shown potential for biotechnological applications (De la Coba *et al.*, 2009).

During the last decades, cyanobacteria have gained a lot of attention as great sources of bioactive compounds with potential for pharmacological and biotechnological applications, such as antiviral activity (Lopes *et al.,* 2011), anti-carcinogenic (Leão *et al.,* 2013), anti-microbial (Martins *et al.,* 2008), anti-obesity (Castro *et al.,* 2016) and many others. Because they are considered a reliable object of study for the discovery of new drugs and other potentially useful compounds many institutions recognize their importance and make an effort to catalog and organize their cultures in microbial biological resource centers (mBRCs) (Janssens *et al.*, 2010). These are culture collections that are managed to ensure their preservation and to provide publically access to the strains and to their related information. LEGE's culture collection (LEGE CC) hosted at CIIMAR is a good example of a cyanobacterial biological resource center and it hosts over 380 strains comprising of 46 genera and several of those strains have already shown the capability or potential to produce several bioactive compounds, some of those are toxins (Ramos *et al.*, 2018). LEGE CC strains are derived from many environments, 93% of those are from aquatic environments (2% hypersaline, 46% marine, 11% brackish and 34% freshwater), 3% from terrestrial environments and 4%

with unknown origin. They are also producers of secondary metabolites, such as microcystin, that have toxic effects on human beings (Saker *et al.,* 2005).

1.2. Cyanobacterial secondary metabolite machinery: NRPS and PKS

The synthesis of secondary metabolites in cyanobacteria can occur by deciphering the genetic code on the ribosome or it can happen non-ribosomally on a protein template, via polyketide synthases (PKSs) or non-ribosomal polypeptide synthetase (NRPS) (Shih *et al.*, 2013). They can also be synthetized via a pathway that combines both types. In this case they are called hybrid PKS-NRPS (Fisch, 2013). An example of this is the case of the compound trichloroleucine that is a direct precursor of barbamide, which is the final product in the assembly chain, that displays molluscicidal activity and is produced by a marine strain of cyanobacteria (Chang *et al.,* 2002).

PKSs and NRPSs are big multifunctional protein complexes that have a modular organization, where each module carries all the essential information for recognition, activation and modification of one substrate into the growing peptide chain (Fisch, 2013). Each module can be divided into different domains, each responsible for a specific biochemical reaction. The structure of the final product being assembled depends on the number of those modules and on their organization within each enzyme, so each enzyme is responsible for the production of only one specific type of peptide (Schwarzer & Marahiel, 2001).

Benthic filamentous cyanobacteria are generally a greater source of secondary metabolites than unicellular bacteria (Tidgewell *et al*, 2010). This is partly due to filamentous and colonial cyanobacteria apparently having larger genomes making them more likely to accommodate PKS and NRPS pathways (Shih *et al.,* 2013). Despite that, smaller sized unicellular cyanobacteria also have the potential to equally produce potentially useful natural compounds, for example, Cyanobium sp. – a small unicellular picocyanobacterium has the potential to produce hierridin B, which is a compound that shows antitumoral activity (Leão *et al.,* 2013). By using molecular methods, in combination with chemical methods like the LC-MS analysis, we can use the NRPS and PKS genes as a method to assess the potential of newly isolated strains to produce bioactive secondary metabolites (Brito *et at.*, 2015)

Most cyanotoxins are synthetized by both of NRPS and PKS complexes, such is the case of, for example, the toxin cylindrospermopsin (Kellman *et al.,* 2006).

NRPS are mega enzymes that function as protein templates that direct the formation of compounds from monomers to molecules, responsible for assembling the non-ribosomal peptides (NRP), derived from the secondary metabolism of mostly microorganisms, in a process that has no need for ribosomes and messenger RNA

(mRNA) (Kastin, 2013). Their production can be attributed to determined gene clusters that encode a particular NRPS that is responsible for assembling only one type of peptide.

It is via this assemblage mechanism that many useful bioactive compounds and toxins are produced in cyanobacteria. For example, it is via NRPS and PKS that both the toxins microcystin and nodularin, which are very similar in structure, are produced (Jungblut & Neilan, 2006). Other secondary metabolites produced non-ribosomally are for example the immunosuppressant cyclosporine and antibiotics such as gramicidin S, tyrocidin A, and surfactins (Kleinkauf & Von Döhren, 1996).

1.3. Polyphasic approach

The classical approach for the identification of cyanobacteria is based solely on their phenotypical characters, mainly based on their morphological characters and it was widely used before several technological advances took place, namely the introduction of electron microscopy and very particularly molecular methods, and since those advances its taxonomical system has been revised several times (Komarek, 2014). But the identification of cyanobacteria based only in morphological characters will not lead to a proper classification, because their shape may vary a lot, they can be simple unicellular organisms or multicellular types that form different types of thallus. Traditional classification would group them together in accordance with their phenotypical similarities but molecular analyses indicate that morphologically similar strains can be phylogenetically distant, which is the case of the cryptic groups described by Komarek (2014).

In fact, the study of phenotypical and molecular characteristics has revealed a few points (Komarek, 2016) that are: that the location of the thylakoids in the cell is somewhat in agreement with clusters derived from molecular sequencing, that the coccoids morphotypes are heterogeneous, and that different groups of unicellular and colonial strains are more related to some clusters of filamentous bacteria than to each other.

According to Komarek (2014) a polyphasic approach should be used to identify cyanobacteria in which molecular sequencing should be the primary method for cyanobacterial identification while being combined with other criteria, like morphological or ecological observations, chosen depending on the nature of the samples and taking into consideration which criteria would prove more helpful in the identification. This molecular approach is based on the sequencing of the 16S rRNA gene, that despite being a much conserved gene, allowing to compare groups of very different organisms,

it also has 9 variable zones that allow distinction inside the same group of organisms and is thus used to distinguish cyanobacteria into the proper taxonomic groups, at least at the genus level (Komarek, 2016).

In subaerial cyanobacteria, taxonomical identification is normally difficult because they generally have a simple morphology, so there is a special emphasis in using a molecular approach which should be employed for the sake of determining whether there is more diversity than indicated by classical taxonomy (Komarek, 2016).

1.4. Cyanotoxins

Many genera of cyanobacteria are known to produce a wide variety of toxic secondary metabolites known as Cyanotoxins, being a major concern for public health (Van Apeldoorn *et al.,* 2017). They are usually associated with harmful algal blooms in aquatic environments where cyanobacteria grow very rapidly due to eutrophication while at the same time producing cyanotoxins. This phenomenon usually is attributed to anthropogenic causes, such as nutrient pollution (Heisler *et al.,* 2008) and is also associated with the global temperatures increase that seem to favor their growth (Paul *et al.,* 2008). Molecular methods based on the detection of the gene involved in their production can be employed for monitoring their presence in the environment as an early warning signal (Moreira *et al.,* 2014), but that does not necessarily mean the cyanotoxin is being produced. The presence of the cyanotoxins in the environment should be complementarily quantified through chemical analytical or immunological methods. The table 1 summarizes the cyanotoxins screened for in this study:

Table 1. Summary of cyanotoxins screened in this study.

Structure	Cyanotoxin	Primary target on mammals
Cyclic peptides	Microcystins	Liver
	Nodularin	Liver
Alkaloids	Saxitoxin	Nervous system
	Anatoxin	Nervous system
	Cylindrospermopsin	Liver

They also have shown to have toxic effects on other animals (e.g Puerto *et al.,* 2011) and plants (Freitas *et al.,* 2015).

1.5. Aim of the study

Subaerial cyanobacteria are an understudied group when compared to aquatic cyanobacteria, which inhabit in ecological conditions that can be considered extreme. This exploratory work was conducted with the aim to isolate, identify and asses their diversity of in the north of Portugal, by mean of a culture-dependent, polyphasic approach. At the same time, I aimed to evaluate the biotechnological potential of subaerial cyanobacteria by screening for PKS and NRPS, and to check their ability for

producing cyanotoxins. In both cases, a PCR-based approach was followed by using the DNA from the environmental samples (eDNA) and the isolates (gDNA).

2. Methodology:

2.1. Sampling

2.1.1. Sampling locations

Two areas in the north of Portugal were selected to collect the samples, both in close proximity to freshwaters bodies, less than 300 meters from a body of water. One was an urban park called "Parque da Cidade" located in Porto and the other was Portugal's Hydrographic Region 2 (RH2- Região Hidrográfica 2 in Portuguese), both locations situated in the north of Portugal. Portugal's climate in the north is classified as Csb according to Kottek *et al.* (2006), meaning it has a warm temperate climate with a dry and warm summer.

"Parque da Cidade" has an area of 83 hectares and is located near the coast in the north of Portugal, it has 3 lakes connected via underground pipes and their water is used to feed the irrigation system of the park. The samples were collected from several surfaces along the pathways throughout the park and in proximity to the 3 lakes. "Parque da Cidade" is located just a few kilometers south of the Leça river, whose hydrographic basin incorporates the RH2 region.

Figure 1. Parque da Cidade satellite aerial view marked with the sampling points (1-8)

The figure 1 shows the satellite aerial view of the park with the sampling points marked on it.

The RH2 region has an area of 3 400 km^2 and comprises mainly of 3 hydrographic sub-basins of 3 rivers and their affluents, they are the Cávado, Ave and Leça sub-basins, the region also comprises of the basins of smaller coastal streams along the coast of the region. The samples were collected on several surfaces but always in close proximity to rivers. The figure 2 shows the area and delimitation of the RH2 region.

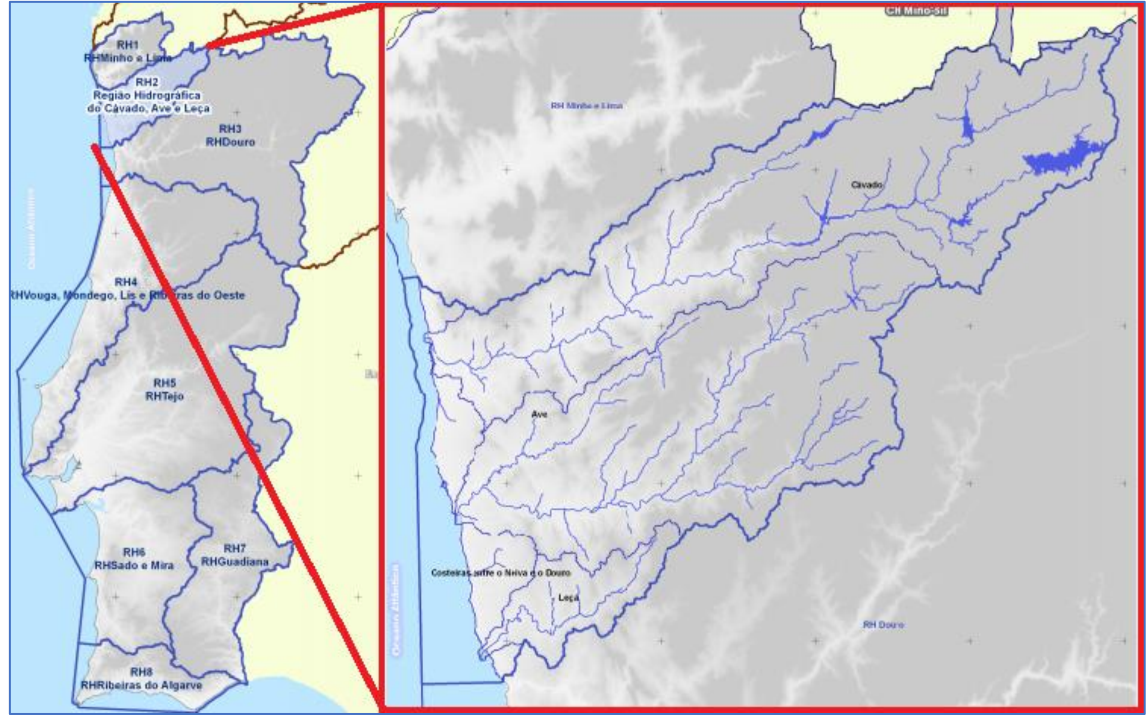

Figure 2. Hydrographic region 2 sampling area.

2.1.2. Sample retrieval

The samples were collected on the 4th of January 2018, during winter, in “Parque da Cidade” where 8 samples were retrieved, and in the RH2 region on the days 21st March and 4th of April 2018, when 11 and 9 samples were retrieved, respectively. The samplings were performed during an atypical winter/early spring season, when precipitation in the north of Portugal has hit historical records. Overall, 28 samples were collected in both sampling locations. The samples were collected during a seasonal period of the year which has the highest annual precipitation and the lowest annual temperatures of the year. The sampling days, especially on the 4th of January, were rainy with only mild raining on the days 21 of March and 4 of April, as such the majority of the surfaces chosen to collect the samples were well wet which facilitated the sampling process.

The samples were collected from several types of surfaces and ranged from several types of microbial communities, such as lithophilous communities inhabiting in rocks and soil (wet or near puddles) or inhabiting in cement or paint from man-made infrastructures, epixylous communities in wooden tables in a park, corticolous communities on the bark of a tree and epimetallous communities on the top of an iron faucet with continuously running water near a fountain, but the majority were lithophilous communities collected from granitic rocks either in nature or used in man-made infrastructures, and according to their relative humidity they can be classified as mesic, but mostly during winter because of higher rates of precipitation, nevertheless the samples collected were well wet and had a moist texture (with the exception of samples ENV001 and ENV002 that had a drier texture than the rest). The table 2 describes the characteristics of each sampling location. Some of those locations were chosen as sampling locations because they are frequently used by people. Indeed, the sampling sites include parks and freshwater beaches (for example, the park tables and a water fountain have been sampled).

Table 2. Description and coordinates of sampling locations.

Location	Coordinates	Site/ Sample code	Description	Sampling date (dd.mm.yyyy)
Parque da Cidade	41.170673, -8.683185	ENV 001	Granitic rock; wall	04.01.2018
Parque da Cidade	41.169606, -8.681526	ENV 002	Xistic rock; wall	04.01.2018
Parque da Cidade	41.168846, -8.680552	ENV 003	Water puddle/soil	04.01.2018
Parque da Cidade	41.168099, -8.679720	ENV 004	Soil	04.01.2018
Parque da Cidade	41.167030, -8.679810	ENV 005	Granitic rock; wall	04.01.2018
Parque da Cidade	41.167554, -8.673021	ENV 006	Granitic rock	04.01.2018
Parque da Cidade	41.167554, -8.673021	ENV 007	Granitic rock	04.01.2018
Parque da Cidade	41.169001, -8.675250	ENV 008	Paint/ wall	04.01.2018
Alto Rabagão’s dam	41.728973, -7.870867	ENV 011	Granitic rock	21.03.2018
Alto Rabagão’s dam	41.728949, -7.870855	ENV 012	Granitic rock	21.03.2018
Caniçada’s dam	41.675939, -8.183609	ENV 013	Granitic rock; fontain	21.03.2018
Parque dos Moinhos	41.635672, -8.137095	ENV 014	Granitic rock; wall	21.03.2018
Praia Fluvial da Esperança	41.577053, -8.167206	ENV 015	Granitic rock; near river	21.03.2018
Praia Fluvial da Esperança	41.577083, -8.166437	ENV 016	Fountain/ iron	21.03.2018
Park near Queimadela’s dam	41.504593, -8.161730	ENV 017	Wooden table	21.03.2018
Travassos	41.405603, -8.207057	ENV 018	Granitic rock near water drainage	21.03.2018
Travassos	41.405345, -8.206949	ENV 019	soil	21.03.2018
Travassos	41.404152, -8.207644	ENV 020	Granitic wall near water drainage	21.03.2018

Park near Queimadela's dam	41.504693, -8.161734	ENV 035	soil	21.03.2018
Barcelos	41.527236, -8.622714	ENV 049	Granitic rock; very close to river	04.04.2018
Barcelos	41.527064, -8.622775	ENV 050	Granitic rock; stairs	04.04.2018
Penide's dam	41.550096, -8.538160	ENV 051	Granitic rock; wall	04.04.2018
Praia Fluvial de Merelim	41.593770, -8.464680	ENV 052	Tree (Quercus)	04.04.2018
Praia Fluvial dos Moinhos	41.654854, -8.399625	ENV 053	Wall; cement	04.04.2018
Praia Fluvial da Navarra	41.613448, -8.385260	ENV 054	Granitic rock; inside windmill	04.04.2018
Praia Fluvial da Navarra	41.613448, -8.385260	ENV 055	Granitic rock; wall inside windmill	04.04.2018
Park near river Ave	41.529815, -8.273271	ENV 056	Granitic rock; tank/fountain	04.04.2018
Santo Tirso's park	41.355048, -8.457896	ENV 057	Cement; ground	04.04.2018

2.1.3. Sample collection process

The itinerary plan was made in Google Maps and GPS coordinates were used to reach each chosen sampling location.

The samples were collected with a stainless steel spatula or knife which was used to scrape the cyanobacterial mats off of rocks into a 50mL Falcon tube, and whenever necessary a Pasteur pipette was used to help collect the mats from more humid surfaces that would sometimes be partially submerged in small puddles by collecting small cyanobacterial mat portions that were scraped off into the more wet parts of the surface. After collecting a sample, the Falcon tube was properly labeled with a code which served to identify the location from where each sample came from and a correspondence was made between each code and sample location characteristics, then the falcon tube was temporarily stored in a thermal box for transportation to the lab.

During the sample collection procedure, disposable nitrile gloves were used and the collection materials (spatula and knife) were always sterilized with alcohol (ethanol at 70%) and cleaned before and after each sample collection, all in an effort to avoid cross-contamination between samples.

One of the samples was lyophilized, after it was observed in the microscope it had a big community of cyanobacteria, to allow in the future to look for substances with potential biotechnology uses. That particular sample (ENV55) was collected inside an abandoned water mill, on the margin of river Cávado.

2.2. Sample processing: culturing and isolation

At the laboratory, the sample processing procedure was performed in aseptic conditions. It consisted in distributing a small portion of biomass from each collected

environmental sample in 4 types of media (solid Z8, solid $BG11_0$, liquid Z8 and $BG11_0$) and in two 1,5µL Eppendorf tubes, one for the microscopic observation of the environmental samples and the other for extracting the sample environmental DNA (eDNA) which was used for molecular screening. The rest of the environmental biomass was left inside the falcon tube and stored at a temperature of -20C° as backup.

The raw environmental samples were observed through light microscopy using a Leica DMLB microscope (Wetzlar, Germany) to check for cyanobacterial presence and their predominance in each sample. Each sample was cultured in two liquid enrichment media, $BG11_0$ (Andersen, 2005) and Z8 (Kotai, 1972), and were allowed to grow freely, some of them were attempted to isolate via micromanipulation at a later date. The solid $BG11_0$ and Z8 mediums in petri dish agar plates, with an agarose concentration of 1.2%, were the primary method used to isolate the environmental samples. All the media for the raw environmental samples were prepared with cycloheximide, at a concentration of 0.025%, to prevent the growth of eukaryotic microorganisms.

To isolate in solid media a bit of biomass from the environmental sample were placed and spread along an agar plate using a streaking technique, that consisted in making a series of strokes in the agar plate in which the last series of strokes had a more diluted concentration of microbial biomass than the first series. Strokes were made with the help of an inoculation loop that was sterilized in an infrared loop sterilizer after each series of strokes were made. Eventually, isolated colonies appeared in the more diluted strokes and were picked up with the help of a disposable surgeon's blade or an inoculation loop to be placed in a new agar plate containing the same medium they were originally picked up from, and the process was repeated if deemed necessary. When two isolated colonies with a different macroscopic appearance appeared on the same agar plate, they were picked up and placed into two new separate agar plates. Colonies though to be isolated were picked up from the agar plate and inoculated in liquid media inside an Erlenmeyer flask where they were allowed to grow, after further confirmation of a monoculture in the flask, an aliquot was collected for extracting the genomic DNA (gDNA) of the isolated strain. All cultures were kept in LEGE's isolation room at a temperature of 20°C, under artificial light with a period of 14h light/10h dark and a light intensity of (12 mol photons m^{-2} s^{-1}). Strain isolates will be deposited at LEGE Culture Collection at CIIMAR (Porto, Portugal).

2.3. Morphological observation

After isolation, the strains were observed and characterized according to their morphotypes using a Leica DMLB microscope (Leica Microsystems GmbH, Wetzlar, Germany), and their microphotographs were captured with a Leica ICCA Camera

System at magnifications of 400x and 1000x, using the Qwin Leica software (Leica Microsystems GmbH) the microphotographs were properly processed. Morphometric characteristics of each strain were measured directly of the microscopic preparation (using an aliquot from the liquid culture medium) at a magnification of 1000x, length and width or diameter were the characters measured 20 times in different individuals of each strain.

2.4. Molecular methods

2.4.1. DNA extraction

Two methodologies were used to extract DNA. For eDNA extraction of raw environmental samples retrieved directly from the field, the DNeasy powersoil kit (QIAGEN, Netherlands) commercial kit was used due to being more suitable for the DNA extraction of mucilaginous subaerial cyanobacteria samples which are soil-like samples that may contain a lot of sediment and various kinds of debris. The extraction procedure was followed accordingly to the protocol provided by the manufacturer of the kit. For instance, the Vortex Genie 2 (MoBio laboratories, USA) was used and set at maximum vortex speed for a period of 10-15 minutes to properly mix and prepare the environmental samples for extraction.

For the gDNA extraction of both the isolated strains and the strains from LEGEcc (serving as positive controls for the molecular screenings), their respective cyanobacterial biomass, collected from the cultures was harvested by centrifugation. The biomass would be centrifuged at 10000 *X* g for 10 minutes and if after that a pellet was not observed the G-force would be increased to 16000 *X* g and the biomass was centrifuged again 10 minutes. After a pellet formed in the 1.5µL Eppendorf tube the liquid medium present in it would be removed and discarded with the aid of a micropipette, replacing it with ddH_20 water. The biomass was then stored at -20°C for the DNA extraction to be performed at a later date. After the cells were harvested, the DNA extraction of the isolated strains and of the positive controls strains was performed using the Purelink Genomic DNA Mini Kit (Invitrogen, USA), and the protocol for extracting DNA from gram-negative bacterial cell was followed according to the manufacturer instructions.

Finally, all the extracted DNA was stored in 1,5µL Eppendorf tubes in a freezer at a temperature of -20°C.

After completing the DNA extraction procedure an electrophoresis was always performed afterward to confirm that the DNA was indeed successfully extracted. Agarose gel (Ultrapure^{tm} Agarose, Invitrogen, USA) at 1% concentration was prepared using a

Tris-Acetate EDTA buffer solution (TAE Ultrapure^{tm}, Invitrogen, USA) at 1x (40mM Tris-acetate and 1mM EDTA), to stain the gel 2µL of SYBRsafe (Invitrogen, USA) was used. Five microliters of extracted DNA mixed with 0.5µL of loading buffer was loaded into the gel, 1µL of molecular marker (1Kb Plus DNA Ladder) was loaded. The gel ran at a voltage of 90V during 45 minutes and was visualized and photographed in the transilluminator Molecular Imager® GEL DOC^{TM} with the software Image Lab^{TM}(USA).

2.4.2. PCR screening

a) Primers:

To check if the isolated strains and environmental samples had cyanobacteria with the potential to produce secondary metabolites, several genes were targeted and amplified using specific primers through the method of Polymerase Chain Reaction (PCR) with the aim to screen for their presence. Most of the genes targeted belong to gene clusters related to the production of cyanotoxins (toxicity potential screening), and others were genes from the NRPS and PKS gene clusters that are responsible for encoding non-ribosomal peptides and polyketide peptides that are able to produce potential bioactive metabolites (biotechnological potential screening). Prior to all other screenings, the isolates and environmental samples were also screened for the presence of the 16S gene by using the cyanobacterial group-specific primer set CYA106F/CYA781R (Nübel *et al.*, 1996). This amplification was used to check if there was any cyanobacterial DNA in the environmental samples and isolates in order to validate the extraction.

The primers used are listed in table 3 along with their target genes and their target groups. The primers used for the toxicity screening were the PKDF/PKDR (Ouahid et al. 2005), the HEPF/HEPR primers which were used to target a domain that is located in both the *mcyE* and *ndaF* genes to detect potential microcystin and nodularin producing strains (Jungblut and Neilan, 2006), the SxtI682F/sxtI877R (Lopes *et al.,* 2012), the anaC-genF/anaC-genR (Rantala-Ylien, 2011), the cylnamR/ cylnamR (Mihali et al. 2008) and the CYTLATF/CYTLATR primers which were used to target the amidinotransferase (AMT) gene whose presence in a cyanotoxin gene cluster is unique to Cylindrospermopsin producing cyanobacteria (Kellman *et al.,* 2006). The primers used for the assessment of biotechnological potential were the DKF/DKR and MTF2/MTR targeting the PKS and NRPS genes, respectively (Moffit et al. 2001; Neilan et al. 1999).

Table 3. Target genes and their respective primers, target groups, primer sequences, amplified fragment size and positive controls.

Target gene	Primer pair	Target group	Primer sequence (5'_3')	size (bp)	Positive control	References
mcyD	PKDF1; PKDR1	microcystin producers	GACGCTCAAATGATGAAAC GCAACCGATAAAAACTCCC	657	*Microcystis aeruginosa* LEGE 91339	Ouahid et al. 2005
mcyE / ndaF	HEPF; HEPR	Microcystin and nodularin producers	TTTGGGGTTAACTTTTTTGGGCATAGTC AATTCTTGAGGCTGTAAATCGGGTTT	472	*Microcystis aeruginosa* LEGE 91339	Jungblut and Neilan, 2006
sxtI	SxtI682F; sxtI877R	Saxitoxin producers	GGATCTCAAAGAAGATGGCA GCCAAACGCAGTACCACTT	195	*Aphanizomenon gracile* LMECYA40	Lopes *et al.,* 2012
anaC	anaC-genF; anaC-genR	Anatoxin producers	TCTGGTATTCAGTCCCCTCTAT CCCAATAGCCTGTCATCAA	366	*Anabaena sp.* LEGE X-002	Rantala-Ylien, 2011
cyrJ	cynsulF; cylnamR	Cylindrospermopsin producers	ACTTCTCTCCTTTCCCTATC GAGTGAAAATGCGTAGAACTTG	586	*Cylindrospermopsis raciborskii* LEGE 97047	Mihali et al. 2008
AMT	CYTLATF; CYTLATR	Cylindrospermopsin producers	ATTGTAAATAGCTGGAATGAGTGG TTAGGGAAGTAATCTTCACAG	1105	*Cylindrospermopsis raciborskii* LEGE 97047	Kellman *et al.,* 2006
PKS	DKF; DKR	Polyketide producers	GTGCCGGTNCC(A/G)TGNG(T/C)(T/C)TC GCGATGGA(T/C)CCNCA(A/G)CA(A/G)(C/A)G	650-700	*Microcystis aeruginosa* LEGE 91339	Moffit et al. 2001
NRPS	MTF2; MTR	Non-ribosomal peptide producers	GCNGG(C/T)GG(C/T)GCNTA(C/T)GTNCC CCNCG(AGT)AT(TC)TTNAC(T/C)TG	~1000	*Microcystis aeruginosa* LEGE 91339	Neilan et al. 1999
16S	CYA106F; CYA781R	Cyanobacteria, plastids	CGG ACG GGT GAG TAA CGC GTG A GAC TAC TGG GGT ATC TAA TCC CAT T	675	*Microcystis aeruginosa* LEGE 91339	Nübel et al. 1996

b) PCR amplification:

Each pair of primers listed in table 3 were used in the PCR reactions, the components and concentrations per reaction of 20 µL were: 1x GoTaq buffer, 2.5 mM for $MgCl_2$, 1 µM for each primer, 0.5 mM for the dNTP mix, 0.5 U for GoTaqR Flexi DNA polymerase, the final volume of each reaction was 20 µL and 1 µL of that volume was the DNA template. The volumes per reaction are listed in table 4.

Table 4. Mastermix preparation volumes for each component of the PCR reaction.

Components	**Volume per reaction (1x)**
Molecular biology water	7.9
5x Buffer	4 µL
$MgCl_2$	2 µL
Forward primer	2 µL
Reverse primer	2 µL
Deoxynucleotides (dNTP's)	2 µL
Taq Polymerase	0.1
DNA template	1 µL
Total:	**20 µL**

The PCR reactions were made using a Biometra T-Professional Standard Gradient Thermocycler (Germany) and the PCR conditions for each pair of primers are described in table 5.

Table 5. PCR conditions for each primer set.

Primer pair	PCR reaction					References
	Initial Denaturation	PCR cycles			Final extension	
PKDF1; PKDR1	94 °C 5 min	35 cycles			72 °C 7 min	Ouahid et al. 2005
		95 °C 60 s	54 °C 30 s	72 °C 1 min		
HEPF; HEPR	92 °C 2 min	35 cycles			72 °C 5 min	Jungblut and Neilan, 2006
		92 °C 20 s	52 °C 30 s	72 °C 1 min		
SxtI682F; sxtI877R	94 °C 3 min	35 cycles			72 °C 7 min	Lopes *et al.,* 2012
		94 °C 10 s	52 °C 20 s	72 °C 1 min		
anaC-genF; anaC-genR	94 °C 2 min	25 cycles			72 °C 5 min	Rantala-Ylien 2011
		94 °C 30 s	50-60°C 30 s	72 °C 30 s		
cynsulF; cylnamR	94 °C 3 min	30 cycles			72 °C 7 min	Mihali et al. 2008
		94 °C 10 s	55-65°C 20 s	72 °C 1-3min		
CYTLATF; CYTLATR	94 °C 3 min	30 cycles			72 °C 7 min	Kellman *et al.,* 2006
		94 °C 10 s	50-55°C 20 s	72 °C 1 min		
DKF; DKR	94 °C 2 min	30 cycles			72 °C 7 min	Moffit et al. 2001
		94 °C 5 s	65 °C 10 s	72 °C 20 s		
MTF2; MTR	94 °C 2 min	35 cycles			72 °C 7 min	Neilan et al. 1999
		93 °C 10 s	51 °C 20 s	72 °C 1 min		
CYA106F; CYA781R	94 °C 5 min	35 cycles			72 °C 7 min	Nübel et al. 1996
		94 °C 1 min	60 °C 1 min	72 °C 1 min		

After PCR amplification, all PCR products would be analyzed and visualized via electrophoresis in an agarose gel ((Ultrapure^{tm} Agarose, Invitrogen, USA) at a concentration of 1.5% which was stained with 2µL of SYBRsafe (Invitrogen, USA). The selected voltage and running time was 90V and 45 minutes. The agarose gel was visualized and photographed in the transilluminator Molecular Imager® GEL DOC^{TM} with the software Image Lab^{TM}(USA), and the presence or absence of the amplified target gene was observed for each environmental sample and for each isolate by looking at the position of the positive controls in the gel and by using the molecular markers to check if a fragment was present (or absent) in the expected position according to its amplified molecular size.

The strains used as positive controls for the molecular screenings were retrieved from the LEGE Culture Collection. They have been previously confirmed by sequencing to have the target genes, making them suitable to be used as positive controls.

2.4.3. Sequencing

Environmental samples that exhibited positive PCR results (i.e presence of the gene) for each cyanotoxin target gene were selected and sequenced.

The 16S rRNA gene was amplified and sequenced for all the isolates obtained, in order to identify the cyanobacteria and thus assess the diversity of the samples.

In this case, for sequencing purposes, the PCR preparation involved triplicating the number of reactions for each environmental sample (or isolate), in order to have enough amplified product to enable sequencing. So the final volume of PCR product per sample (and isolate) to be loaded into the 1.5% agarose gel was 60 µL. The primers and PCR conditions used were the same used for the screening and are listed in table 5. The electrophoresis ran at 90V for 60 minutes, then the amplified fragments were observed in the CSMICRODOC system (Cleaver scientific, UK) transilluminator coupled with a Canon PowerShot G9 camera. Then, bands with the expected size were excised from the gel and collected to be purified using the Nztech - genes & enzymes (NZYGelpure, Portugal) purification kit, following the manufacturer's instructions. The check the efficacy of the DNA purification, an electrophoresis (90V; 45 minutes) ran in a 1% agarose gel, and the purified DNA was mixed with loading buffer, corresponding to a tenth of the total loaded DNA, with the aid of a micropipette. All the purified PCR products and the respetive pair of primers were sent to GATC Biotech (Germany) to be sequenced.

2.5. Sequence analysis

The forward and reverse sequences (i.e. 5' and 3') obtained from the same PCR product were examined in the bioinformatic software Geneious (v.8) and were assembled together (de novo assembly), their chromatograms were analyzed to check the quality of the sequences and to determine if further sequences were needed to form a consensus sequence (i.g. if the quality was bad on either one of the forward or reverse sequences). The sequences were usually trimmed at the extremities due to bad quality. Then, the consensus sequences of each strain isolate were compared with the sequences in the GenBank® database using the BLAST®n (Basic Local Alignment Search Tool for nucleotides) tool available in the NCBI (National Center for Biotechnology Information) and compared with other cyanobacterial sequences in the GenBank® database to check for similarities and to help in their identification.

2.6. Phylogenetic analyses

To assess the relative position of our isolated strains relatively to each other and other reference strains a phylogenetic 16S rRNA gene-based tree was built based on the Maximum Likelihood method using the software MEGA7 (Molecular Evolutionary

Genetics Analysis Version 7.0). First, all the sequences were aligned using the algorithm ClustalW (Kumar et al, 2016) and then visually inspected. This multiple sequences alignment consisted of (1) the 16S rRNA consensus sequences of each strain isolate obtained in this study; (2) the best hit sequences of each isolates' sequence obtained in this study (the isolates sequences were previously compared with the sequences in the GenBank® database using the Blast® tool; if the best hit was an unidentified cyanobacterium then a second sequence belonging to the closest identified cyanobacteria was also retrieved and used in the construction of the tree); and (3) 11 reference strains collected from LEGE's Cyanotype database and from GenBank, in order to obtain a reliable representation of the diversity of the cyanobacteria. The model of substitution has been chosen according to the AICc criteria. Thus, the phylogenetic tree was built using the model GTR+G+I and with all the positions with a coverage of less than 97% site coverage removed. The labels in the phylogenetic tree were edited using the Inkscape software (V. 0.92; free software).

3. Results and Discussion

Although isolations attempts through micromanipulation were made using the liquid culture mediums containing the environmental samples those did not yield any success due to the fact that the strains exhibited poor growth in the liquid enrichment. Due to time constraints and deadlines the idea of any further attempts at micromanipulation was abandoned. So both the Z8 and $BG11_0$ liquid mediums holding each sample served only as a sort of a backup in laboratory and the raw sample was allowed to grow, this most likely changed the community and the relative amount of certain strains of cyanobacteria in the sample because some strains are more capable of growing in those mediums than others, such is the case of opportunistic strains that can easily dominate in these mediums. Instead, all of the strains were isolated through solid media on agar plates.

Following Komarek and co-authors' (2014) criteria for identification of cyanobacteria, all isolates were properly identified using a polyphasic approach based mainly on molecular methods (amplification and sequencing of the 16S rRNA gene) combined with the strain morphological characteristics, 7 isolates could be identified to the taxonomic level of species, 13 isolated were identified to their genus and 5 were identified to their order. A few exceptions happened:

- For the isolate JPS1 no molecular data has been obtained due to the lack of biomass available in the liquid culture to properly perform a DNA extraction, so

that isolate was not identified. Only its morphological characteristics were able to be determined.

- For the isolates JPS13 and JPS26, it was only possible to obtain 1 sequence out of each one, corresponding for both strains to the reverse sequence (primer CYA785R). Blasting those sequences and comparing them with those in GenBank database along with their microscopical observation and characterization would allow us to conclude that they most likely belong to the genera *Wilmottia* and *Timaviella*, respectively. For all the other isolated strains I have obtained 2 or more sequences that were assembled together to form a consensus sequence that was used to build the phylogenetic tree. Since there was only 1 sequence for the isolates JPS13 and JPS26 it was not possible to produce a consensus sequence, consequently they were not included in the phylogenetic tree.

3.1. Morphological characterization and strain identification

A total of 26 strains were isolated, characterized according to their morphological features (morphotypes) and their morphometric characteristics (i.e cell diameter or cell length and width) were measured under light microscopy. Morphological observations revealed 17 different cyanobacterial morphotypes (figure 3) and three main types of morphologies were distinguished as well, the filamentous cyanobacteria with heterocysts, filamentous cyanobacteria without heterocysts (non-heterocytous) and unicellular cyanobacteria (table 6).

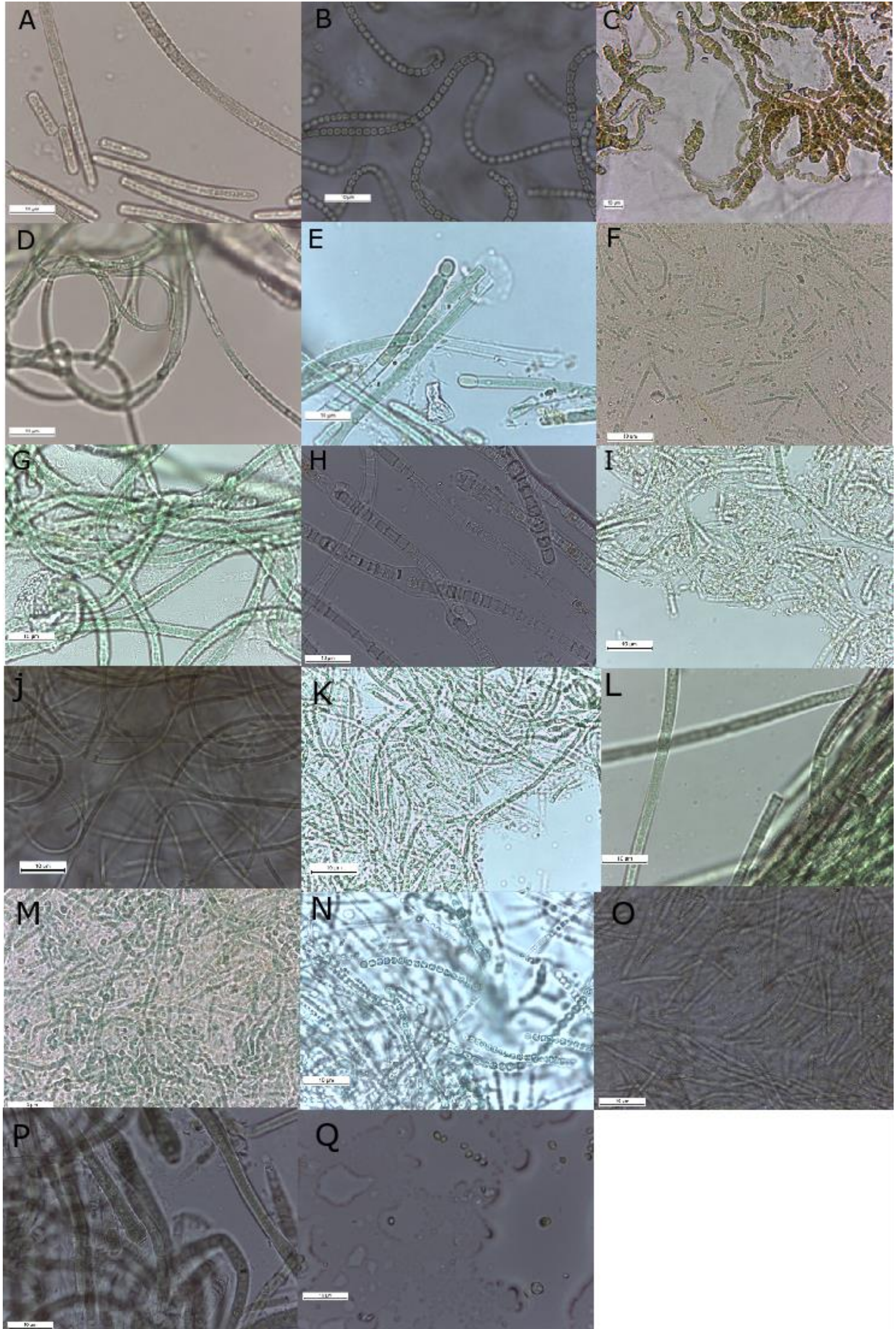

Figure 3. Diversity of different morphotypes among strains; (image C at 400x magnification, all others at 1000x); A- *Microcoleus* sp.; B- *Nostoc* sp.; C- *Roholtiella edaphica;* D- *Timaviella* sp.; E- *Tolypothrix* sp.; F- *Nodosilinea epilithica;* G- *Wilmottia* sp.; H- Tolypothrichaceae; I- Leptolyngbyaceae; J- *Leptolyngbya* sp. 1; K- *Leptolyngbya* sp. 2; L- *Tychonema* sp.; M- Synechococcales sp. 1; N- Nostocales; O- Synechococcales sp. 2; P- *Macrochaete* sp.

; Q- unidentified unicellular strain; Scale Bar= 10 μm.

Table 6. Isolated strains, their respective identification (Komarek *et al.*, 2014) with each correspondent morphotypes represented in figure 3 and their classification according to: 1- Unicellular, 2- heterocytous filamentous 3- non-heterocytous filamentous; the colors indicate: green- strains isolated using Z8 medium; blue- Strains isolated using $BG11_0$ medium.

Isolated	ID	Order	Morphotype (represented in figure 3)	Nº isolados obtidos	Sampling location
JPS001	No molecular info	n.i	Q-1	1,72 ± 0,31	ENV001; Parque da Cidade
JPS002	*Microcoleus vaginatus*	Oscillatoriales	A-2	3	ENV003; ENV 4 Parque da Cidade
JPS004	*Nostoc* sp.	Nostocales	B-3	1	ENV003; Parque da Cidade
JPS005	*Roholtiella edaphica*	Nostocales	C-3	1	ENV004; Parque da Cidade
JPS007	*Timaviella circinata*	Synechococcales	D-2	1	ENV005; Parque da Cidade
JPS008	*Timaviella* sp.	Synechococcales	D-2	3	ENV005; env 6;Parque da Cidade; ENV054; Praia Fluvial da Navarra #
JPS009	*Tolypothrix* sp.	Nostocales	E-3	1	ENV005; Parque da Cidade
JPS010	*Nodosilinea epilithica*	Synechococcales	F-2	2	ENV006; Parque da Cidade
JPS013	*Wilmottia* sp. #	Oscillatoriales	G-2	2	ENV006; Parque da Cidade; ENV016; Praia Fluvial da Esperança
JPS014	unidentified Tolypothrichaceae	Nostocales	H-3	1	ENV006; Parque da Cidade
JPS015	unidentified Leptolyngbyaceae	Synechococcales	I-2	1	ENV007; Parque da Cidade
JPS016	*Leptolyngbya* sp. 1	Synechococcales	J-2	2	ENV011; ENV012; Alto Rabagão's dam
JPS017	*Leptolyngbya* sp. 2	Synechococcales	K-2	0,89 ± 0,10 x 0,83 ± 0,14	ENV012; Alto Rabagão's dam
JPS018	*Tychonema* sp.	Oscillatoriales	L-2	1,82 ± 0,27 x 2,38± 0,25	ENV013; Caniçada's dam
JPS019	unidentified Synechococcales sp. 1	Synechococcales	M-2	0,89 ± 0,11 x 1,11 ± 0,12	ENV014; Parque dos Moinhos
JPS021	*Tychonema* sp.	Oscillatoriales	L-2	1,75 ± 0,34 x 2,36 ± 0,22	ENV018; Travassos
JPS022	*Tychonema* sp.	Oscillatoriales	L-2	1,62 ± 0,27 x 2,58 ± 0,18	ENV020; Travassos
JPS023	unidentified Nostocales	Nostocales	N-3	1,39 ± 0,28 x 1,51 ± 0,23	ENV020; Travassos
JPS024	unidentified Synechococcales sp. 2	Synechococcales	O-2	1,57 ± 0,38 x 0,91 ± 0,14	ENV035; Park near

					Queimadela's dam
JPS025	*Macrochaete* sp.	Nostocales	P-3	2,82 ± 0,41 x 2,29 ± 0,32	ENV049; Barcelos

based on a singleton only

It was observed during the identification process, when comparing the sequences of the isolates from this study with their more similar sequences from GenBank, that most of the obtained strains are similar to other terrestrial strains from other studies that examined cyanobacterial diversity in extreme habitats. For example, our JPS002 and JPS003 isolates, which are very similar to each other, both revealed to be have high similarity with an uncultured cyanobacterium (acc.nbr: KC463588) (figure 6. and Annex A) from soil crust from a study conducted in south of Africa (Dojani *et al.,* 2015), JPS007 and JPS008 show a 99% similarity with an uncultured cyanobacterium (acc.nbr: HQ188993) in the dry valleys of the high Himalayas and Antarctica (Schmidt *et al.,* 2011)

3.2. Isolation and diversity analysis

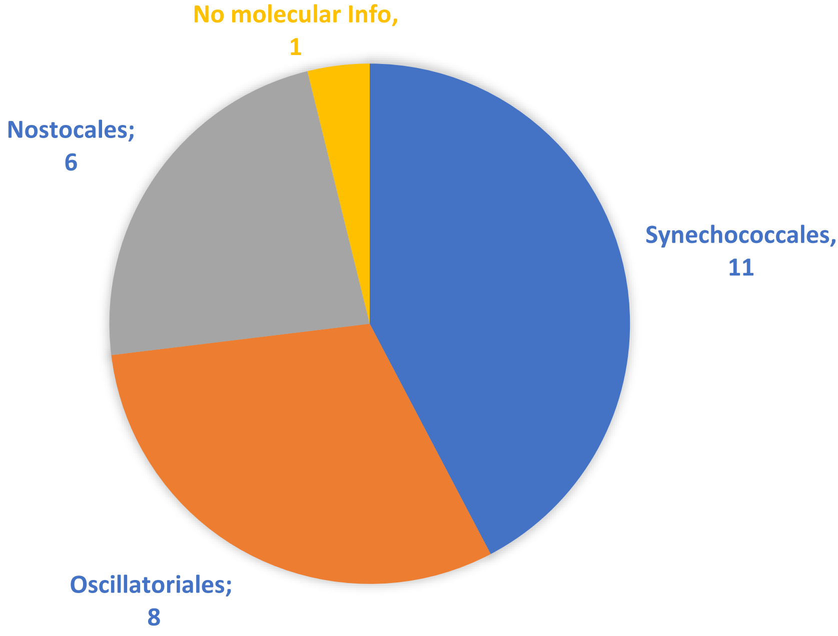

Figure 4. Number of strains isolated per -order level.

In total 26 strains were isolated from both the urban park “Parque da Cidade” and from the hydrographic region “RH2” both situated in the north of Portugal. From these, 15 of the isolates obtained have an origin in “Parque da Cidade” and 11 have an origin in the “RH2” region. The figure 4 shows the number of strains obtained per -order:

Eleven strains belong to the order Synechococcales, which is an order that can have over 70 genera with unicellular (including colonial forms) and filamentous types, and it is a group that is not defined as monophyletic (Komarek *et al.,* 2014).

Eight strains isolated belonged to the Oscillatoriales order, which is a group that has the morphological characteristics of not having true-branching, heterocysts or akinetes, and with cells shorter than wide.

Six strains belonged to the order Nostocales, which is an order of filamentous cyanobacteria that can have very diversified thalli and have specialized cells such as heterocysts and akinetes (Komarek *et al.*, 2014). The heterocysts are responsible for fixating atmospheric nitrogen, which means that in environments deprived of nitrogen, for example in the $BG11_0$ medium, the cyanobacteria that possess those cells have an advantage over other strains are not able to fix N_2. In that sense, in such conditions heterocytous cyanobacteria are able to outcompete them and outgrow other colonies that might have formed in other conditions (Pentecost & Whitton, 2012).

Of the 26 isolated strains, 21 were isolated using the Z8 solid medium while 5 were isolated using the $BG11_0$ solid medium. All of the strains isolated using the $BG11_0$ solid medium belonged to the Nostocales order, something that was to be expected because it is in accordance with the fact that they fix dinitrogen. Although there are some unicellular or non-heterocytous cyanobacteria that are also capable to fix N_2 (Berrendero et al., 2016), none was isolated in this work. The low number of strains successfully isolated by using the $BG11_0$ solid medium, in comparison with the number of strains isolated from the Z8 solid medium, can be attributed to their slow growth and colony formation in that media, fact that was observed during the isolation process. Sometimes no growth would occur at all, or the growth would be so minimal that no isolated colonies would appear, thus not allowing for the isolation process to proceed for those particular plates. In conclusion, the strains in the Z8 medium grew faster and yielded more isolates (mostly from the Synechococcales and Oscillatoriales order, only 1 Nostocales strain) than in the $BG11_0$ medium. However, as said, with $BG11_0$ it was possible to obtain new diversity that was not possible with Z8.

It is to note that while the most predominant strain of cyanobacteria present in the environmental sample – and that were observed under the microscope would possibly be isolated, this was not certain due to the ubiquity and opportunistic behavior of certain strains. This is a major point that possibly determined the diversity of the isolates obtained, which was performed following a culture-dependent approach. Still, in most cases the predominant strain in the environmental sample would be the one isolated. For example, two *Microcoleus vaginatus* strains were isolated from the sample ENV003, whichwas dominated by *Microcoleus* strains (figure 5), although during the isolation process two isolated colonies with different macroscopic characteristics appeared in the agar plate (at the time assumed to be different strains). They were

separated into two Petri dishes in order to be isolated: both yielded the same strain belonging to the genus that dominated the environmental sample.

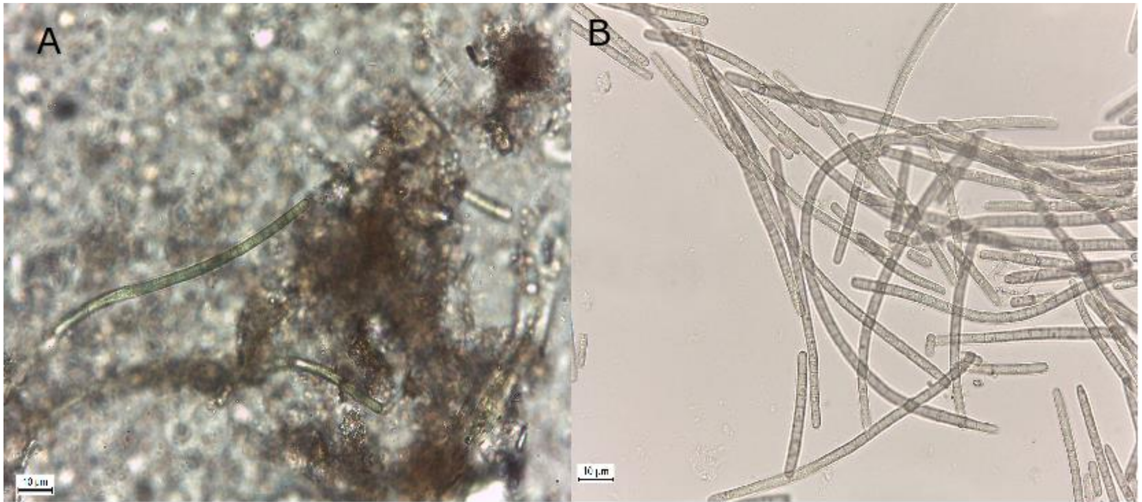

Figure 5. A- ENV003 environmental sample showing Microcoleus spp. B- Microscopic preparation of biomass collected from the ENV003 agar plate, during the later stages of isolation, showing the Microcoleus vaginatus strain. Scale bar= 10 µm.

Almost all the isolated strains are filamentous cyanobacteria, which are capable of producing an exopolysaccharide (EPS) matrix that promotes the stabilization of the mats and helps maintain favorable conditions (preventing the loss of water) that allow colonization of other microorganisms (Mager & Thomas, 2011). Filamentous strains similar to *Microcoleus* traditionally recognized as EPS producers, are the group of organisms to first colonize a dry terrestrial-like environment (Garcia-Pichel & Wojciechowski, 2009). Only one non-filamentous unicellular strain strain was isolated (I was not able to identify it).

A higher number of non-heterocystous strains were isolated from the Z8 but this might be due to the faster growth occurring in this medium, which seemed to favor the growth of non-heterocystous strains. Still, 6 heterocystous strains were isolated, and although they were less prominent than the non-heterocystous strains they are known to play an important role as nitrogen contributors in terrestrial environments (e.g biocrusts) by fixing dinitrogen (Belnap, 2002).

The characteristics of a terrestrial surface (e.g texture, slope, chemical composition) may determine the cyanobacteria strain or groups that are able to attach on it, successfully colonize and dominate it (Pentecost & Whitton, 2012). The table 6 gives an insight into the type of surface and the strain isolated, and it is noted that all the strains belonging to the genus *Timaviella* (figure 3-D) were isolated from a biocrust on top of granitic surfaces, from two distanced locations correspondent to sites ENV005 and ENV006 (same location) and to ENV054.

3.3. Phylogenetic results

In order to verify the relative positioning of the isolates obtained, a Maximum likelihood (ML) tree was constructed with the sequences from the isolates, their Best Hits and some reference strains.

The phylogenetic tree consisted of 12 clusters (figure 6), which were defined by being monophyletic groups with a bootstrap value of 70% or higher and had to include at least 1 isolate strain or 1 reference strain. An exception was made to the Cluster D which had a bootstrap value lower than 50% but was defined as a cluster because it is a monophyletic group that includes the isolate strain JPS004 (*Nostoc* sp.) and a matching reference strain (*Nostoc punctiforme*), and also no other bootstrap values justified the definition of another clade, that would include either of these trains. For the most part, the species or genus attributed to the isolates match with the reference strains they are clustered with. However, some isolates are not clustered with any reference strain being only matched by another strain (Best hit) with a high bootstrap value support.

Clade A (Figure 6) comprises of 3 *Tolypothrix* strains and one *Kryptousia* strain, all belonging to the Tolypothrichaceae family, it includes: the isolate JPS009 that has been identified to the genus level only *Tolypothrix* sp. the Best hit strain *Tolypothrix* UAM 357 and 2 reference strains, *Tolypothrix distorta* ACOI 731 and the *Kryptousia microlepis* CENA343 strain, which is placed more distantly in relation the previous strains. The genus *Kryptousia* despite being morphologically similar to the *Tolypothrix* has been distinguished from it through molecular methods (Alvarenga et al., 2017). Still they are closely related, as it is shown in the phylogenetic tree (figure 6). The clade B includes the isolate obtained in this study (JPS014) – an unidentified Tolypothrichaceae and two Best Hit strains, an unidentified cyanobacteria (acc.nbr: KC463244) and the strain *Hassallia cf. pseudoramosissima* ACSSI 158 (Tolypothrichaceae family), although no reference strain is present. This clade is supported by a high bootstrap value of 99%. The clade C (figure 6) includes the isolated strain (JPS005) identified as *Roholtiella edaphica*, its respective Best hit *Roholtiella edaphica* AR5 strain and the reference strain *Roholtiella edaphica* CCALA 1063. Clade D (figure 6) includes the isolated strain (JPS004), the Best hit strains, *Nostoc commune* NTC and an unidentified cyanobacterium (acc.nbr: JX255093) and the reference strain *Nostoc punctiforme* PCC 73102. With a low bootstrap value (<50 %) this clade was only defined to illustrate the close proximity of the isolate JPS004 (Annex A) to the reference strain according to the topology of the tree, relatively to the other strains, and they have shown a 97% of similarity by blasting them. So, according to molecular data only we can say that they belong to the same genus (Kim et al., 2014; Yarza et al., 2014), but not the same species

(Stackebrandt *et al.,*2006). Clade E (figure 6) includes the isolate strain (JPS023), and the Best hit Nostoc sp. NQAIF313, the clade does not integrate a reference strain but is supported by a high bootstrap value of 99%. Strain JPS023 has morphological characteristics (table 6 and figure 3) that fit those of *nostoc* spp (Komarek, 2013). However, since it is placed distantly from the "true" clade of the genus *Nostoc* - which comprsises *Nostoc punctiforme* PCC 73102 (Komárek et al, 2014) – the strain JPS023 is classified as an unidentified Nostocales (figure 6).

Clade F (figure 6) also does not include a reference strain being only supported by a high bootstrap value of 100% as well, it includes the isolated strain (JPS025) identified as *Macrochaete* sp. and the respective best hits that are an unidentified cyanobacterium (acc.nbr: JN020217) and *Macrochaete santannae* CCALA 1093. Clade G (figure 6) includes the isolated strain (JPS020) identified as *Wilmottia* sp., and 2 Best hit strains, an unidentified cyanobacterium (acc. nbr: LC103289) and *Wilmottia stricta* 16PC, and the reference strain *Wilmottia murrayi* CCALA 843 that is the more distanced strain from the clade. Clade H was subdivided into 2 clades (figure 6). Clade H1 included 3 isolated strains (JPS018, JPS021 and JPS022) all identified as belonging to the genus *Tychonema*, and 4 Best hit strains: *Tychonema* sp. LEGE 07216, *Phormidium autumnale* VUW11, *Phormidium autumnale* VUW2 and an unidentified Oscillatoriales (acc.nbr: KR002123), and the reference strain *Tychonema bornetii* NIVA CYA 60 that was more closely related to the isolate JPS021. In a particular study (Shams et al., 2015) it is possible to observe how the *Tychonema* genus is closely related to the *Phormidium autumnale* strains and how they are separated in different clusters which was not observed in this work (figure 6): the JPS022 and JPS018 *Tychonema* strains are more closely related to 2 *Phormidium autumnale* strains than to each other. So, Subclade H1 can be further subdivided into 3 groups: The first including the strains *Tychonema* sp. JPS021, *Tychonema* sp. LEGE 07216 and the reference strain *Tychonema bornetii* NIVA CYA 60 and supported by a bootstrap value of 91% supporting this group, the second including the strains *Tychonema* sp. JPS018 and *Phormidium autumnale* VUW11 strains but with a bootstrap value of 96% supporting this group, and the third including *Tychonema* sp. JPS022, *Phormidium autumnale* VUW and the unidentified Oscillatoriales strain. But most importantly, all the *Tychonema* sp. strains isolated in this work are integrated in a subcluster with a matching reference strain that belongs to the same genus. Subclade H2 (figure 6) was integrated by 3 isolates strains identified as *Microcoleus vaginatus*, this subclade although it did not have a reference strain it was backed up by a high bootstrap value of 99%, and was integrated by the Best hit strains, *Phormidium cf. autumnale* JR6, *Oscillatoria nigro-viridis* PCC 7112, *Tychonema* sp. SAG

23.89, *Microcoleus vaginatus* PUPCCC 120, the unidentified cyanobacterium HL201307-70 (acc.nbr: KU515188) and HL201307 (acc.nbr: KU515125). Overall, clade H shows how related the genera *Tychonema*, *Phormidium*, *Microcoleus* and *Oscillatoria* are, which is illustrated the topologies and bootstrap values and by the fact that strains with different genus. Clade I (figure 6) can be subdivided into 2 subclades and it is supported by a bootstrap value of 95%. The clade I1 is comprised mostly of strains belonging to the *Timaviella* genus. It comprises 2 isolate strains (JPS008 and JPS012) identified as *Timaviella* sp. and one isolate strain (JPS007) identified as *Timaviella circinata*, and the Best hit strains *Timaviella circinata* GR4T, *Timaviella* sp. Us-6-3 and the unidentified strains B10912H (acc. nbr: FM175896) and B10912H (acc. nbr: HQ188993), this subclade is supported by a bootstrap value of 100%. The subclade I2 comprises 3 strains belonging to the Nostocales, Synechococcales and Oscillatoriales indicating that they are phylogenetically close. The isolated strain is only identified has a Synechococcales and it indicates a close relation to a *Nostoc punctiforme* MACC-287 strain (forming a small group supported by a bootstrap value of 100%), and to an unidentified Oscillatoriales (acc. nbr: FJ788926). Clade J (figure 6) is comprised mostly of *Leptolyngbya* strains, it includes 2 isolated strains (JPS016 and JPS017) identified as *Leptolyngbya*, 1 unidentified Leptolyngbyaceae isolated strain, the reference strain *Leptolyngbya boryana* AM M-101, it also includes the strains *Plectolyngbya hodgsonii*, *Leptolyngbya* sp. CENA377, *Leptolyngbya* sp. NIES-3755, *Plectonema* sp. SAG 38.90 an unidentified cyanobacterium (acc.nbr: JQ770050) and another one (acc.nbr: HQ755632). Clade K (figure 6) comprised of a *Limnothrix redekei* NIVA CYA 277/1 strain and two reference strains, the *Pseudanabaena* sp. PCC 7367 strain and the *Pseudanabaena* sp. PCC 6802. Clade L (figure 6) comprised of two isolate strains (JPS010 and JPS011) identified as *Nodosilinea epilithica* that are very closely related, an *Halomicronema excentricum* TFEP1 strain, *Leptolyngbya* sp. PCC 7375 strain, a *nodosilinea nodulosa* UTEX 2910 strain and an unidentified Pseudanabaenaceae strain DPG1-KK5.

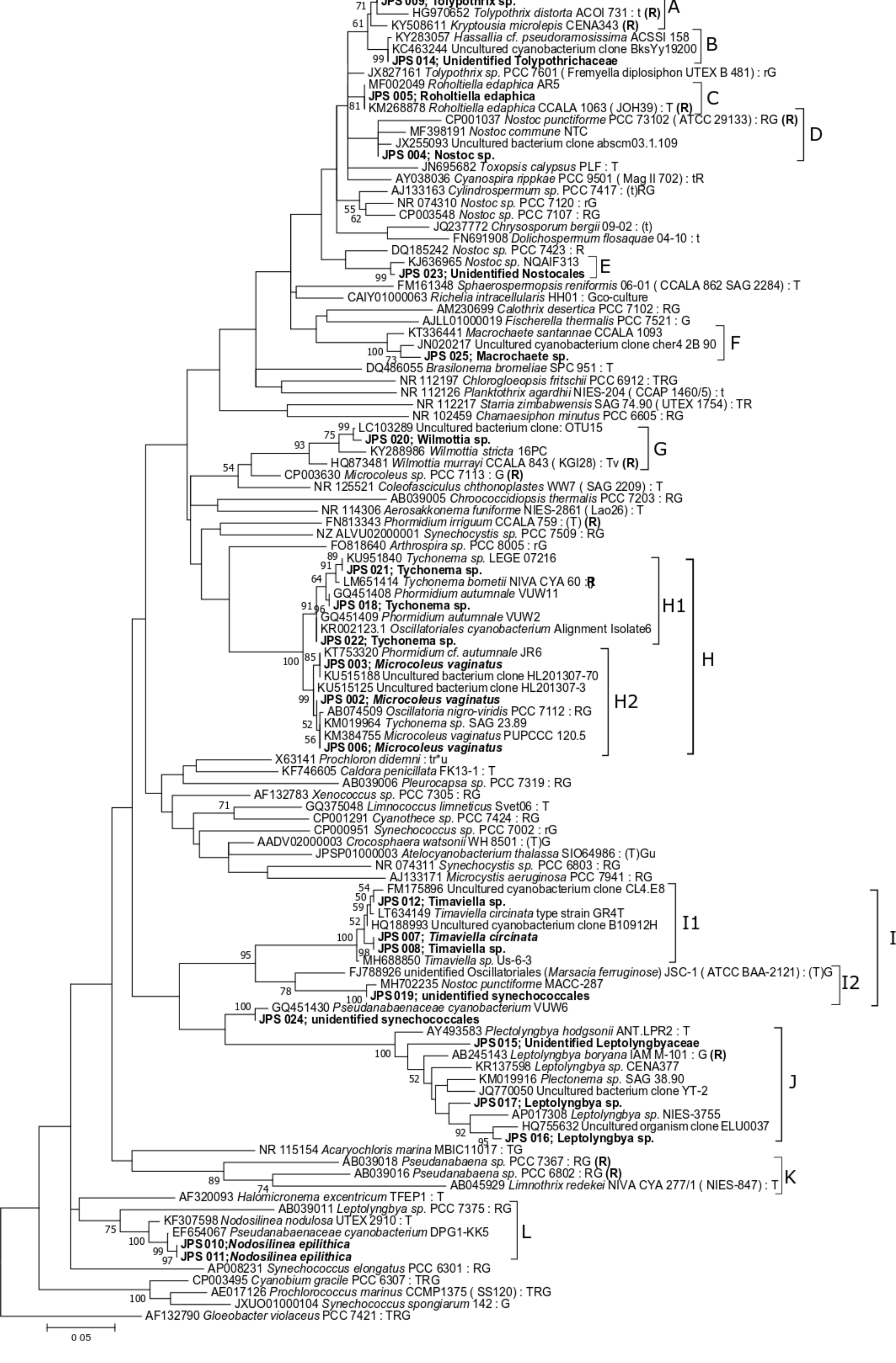

Figure 6. Maximum likelihood (ML) phylogenetic tree based on partial 16S rRNA gene sequence. Isolated strains from this study are in bold and have the JPS code. Reference strains are marked with an **(R).** Only bootstrap values >50% are indicated below the nodes.

3.4. Toxicity screening

In the table 7 the data relative to the presence of toxin producing genes in presented.

Table 7. Environmental samples screening for the presence of genes involved in cyanotoxin production; n.i: no information obtained for this samples; target genes and pairs of primers: *mcyD*- PKDF1/PKDR1, *mcyE*- HEPF/HEPR, *sxtI*- SxtI682F/SxtI877, *anaC*- anaC-genF/anaCgenR, *cyrJ*- cynsulF/cylnamR, AMT- CYTLATF/CYTLATR.

Location	Samples	***mcyD*** PKDF1/PKDR1	***mcyE*** HEPF/HEPR	***SxtI*** SxtI682F/SxtI877R	***anaC*** anaC-genF anaC-genR	***cyrJ*** cynsulF/ cylnamR	AMT CYTLATF/ CYTLATR
Parque da Cidade	ENV 001	n.i	n.i	n.i	n.i	n.i	n.i
Parque da Cidade	ENV 002	n.i	n.i	n.i	n.i	n.i	n.i
Parque da Cidade	ENV 003	-	-	+	-	-	+
Parque da Cidade	ENV 004	+	-	+	-	-	+
Parque da Cidade	ENV 005	+	+	+	-	+	+
Parque da Cidade	ENV 006	-	-	+	-	-	+
Parque da Cidade	ENV 007	-	-	+	-	-	+
Parque da Cidade	ENV 008	-	+	+	-	-	+
Alto Rabagão's dam	ENV 011	-	-	+	-	-	-
Alto Rabagão's dam	ENV 012	-	+	-	-	-	-
Caniçada's dam	ENV 013	+	-	-	-	-	-
Parque dos Moinhos	ENV 014	n.i	n.i	n.i	n.i	n.i	n.i
Praia Fluvial da Esperança	ENV 015	n.i	n.i	n.i	n.i	n.i	n.i
Praia Fluvial da Esperança	ENV 016	-	-	+	-	-	-
Park near Queimadala's dam	ENV 017	-	-	+	-	-	-
Park near Queimadala's dam	ENV 018	-	-	-	-	-	-
Travassos	ENV 019	n.i	n.i	n.i	n.i	n.i	n.i
Travassos	ENV 020	-	-	-	-	-	-
Travassos	ENV 035	-	+	-	-	-	-
Barcelos	ENV 049	-	+	-	-	-	-
Barcelos	ENV 050	-	+	+	-	-	-
Penide's dam	ENV 051	-	+	+	-	-	-
Praia Fluvial de Merelim	ENV 052	-	-	+	-	-	-
Praia Fluvial dos Moinhos	ENV 053	-	-	+	-	-	-
Praia Fluvial da Navarra	ENV 054	-	-	+	-	-	-
Praia Fluvial da Navarra	ENV 055	-	-	+	-	-	-
Park near river Ave	ENV 056	-	-	-	-	-	-
Santo Tirso's park	ENV 057	+	-	-	-	-	-
Positive controls		LEGE 91339	LEGE 91339	LMECYA 39	LEGE X-002	LEGE 97047	LEGE 97047

The molecular screening of each environmental sample gave me preliminary results and indicated if the target gene I was trying to amplify was present or absent in the environmental sample. If the environmental sample showed absence of the target gene, for example for a cyanotoxin, I would know in advance that it would not be possible to isolate a strain that had the potential to produce that specific cyanotoxin. On the other hand, the presence of the target gene observed in the environmental sample did not meant that we would be able to isolate the organism that possessed it in its genome. Furthermore, these preliminary PCR results also served to monitor for the presence of the genes involved in cyanotoxin production in the terrestrial environment and to assess their biotechnological potential in a bioprospecting perspective.

The results of the PCR-based screening for the presence of genes involved in the production of toxins show that the genes associated with the production of several cyanotoxins, more specifically, associated with the production of microcystin, saxitoxin and cylindrospermopsin may be present in subaerial mats present in surfaces ranging from soil, rock, wood and iron. However, since false positives may occur it is highly advisable to sequencing amplicons with the expected size (Kurmayer *et al.,* 2017) in order to validate PCR results. The gene *anaC* associated with the production of anatoxin did not show the presence in any of the samples, similarly, the *cyrJ* gene associated with the production of cylindrospermopsin only seemed to be present in one of the samples from “Parque da Cidade”. Most of the sites sampled show at least the presence of one gene associated the production of cyanotoxins (only 3 sites showed no presence of cyanotoxin genes, and 5 sites without data to be presented), and the gene *sxtI* associated with the production of saxitoxin was the most frequent and widespread among the cyanotoxin gene present in the samples (15 samples showed positive results for the presence of *sxtI*). The *mcyD* and *mcyE* genes were also quite widespread through the study area. These results obtained only reveal the presence of the genes in terrestrial environments and not the actual presence of cyanotoxins in the environment, which should be complemented with analytical or biochemical assays (Kurmayer *et al.,* 2017).

Figure 7. Some examples of sampling sites whose samples showed positive results for the presence of cyanotoxins.

In figure 7 we can see a few places that showed positive PCR results for the toxicity screening, these were places inside anthropogenic agglomerations near places usually frequented by people, inside small towns, parks or in freshwater beach facilities. For example, image ENV006 from a park comprised of 3 lakes inside it, "Parque da Cidade", shows the sampling location of sample006 that showed positive results for the gene *sxtI*, in fact, most of the samples collected in "Parque da Cidade" show the presence of the gene *sxtI*. A previous study on the lakes of this park have shown that the lakes had concentrations of microcystin-LR (MC-LR) with values ranging from 0.20 µg MC-LR eq/L and 10.2 µg MC-LR eq/L (Morais *et al.,* 2014), since the irrigation system of the park is being fed with water from the lakes that are in a eutrophic state with a high concentration of cyanobacterial cells, it is plausible to assume that some strains present in the lake are reaching several parts of the park. Comparing the strains identified in that study with the isolates obtained in the present study, no isolates from the terrestrial mats seemed to match the strains identified from the lakes, which can be due to the fact that the planktonic strains identified in that study are not able to properly colonize the terrestrial environments, examining the benthic mats present in the lakes might reveal a cyanobacterial diversity more similar to the terrestrial mats. Still, the genes *mcyD* and *mcyE* responsible for the production of MC were present in terrestrial mats in the park. Considering the park, it is highly frequented by people, contact with cyanotoxins derived from subaerial cyanobacteria it's a possibility but unlikely to be in high enough concentrations to cause even any minor adverse effects. People can possibly enter in

contact with terrestrial cyanotoxins by interacting with fountains (ENV013 and ENV016), sitting in park tables (ENV017), walking on stairs containing cyanobacterial mats and contaminating their shoes (ENV050) or by touching a wall (ENV053). All these places were sampled and showed the presence of genes associated with the production of cyanotoxins but they do not represent any danger to public health because the concentration of cyanotoxins should be very low and also contamination via dermal contact is not as dangerous as contamination via oral ingestion. The potential presence of cyanotoxins in terrestrial biofilms or mats, present for example in the soil, represents a bigger danger to animals who might be attracted to eating them, effects of terrestrial cyanotoxins were investigated before with but with no conclusive results (McGorum *et al.,* 2015).

All of the isolates screened for potential toxicity did not show presence for any of the cyanotoxin genes screened. Even though some isolated strains belong to genera that are known for being cyanotoxin producers, for example, the isolate *Tolypothrix* sp. JPS009 a genus known to have strains capable of producing microcystins (Aboal *et al.,* 2005) the results indicate that should not be able to produce them. For the isolates JPS 25 (*Macrochaete* sp.), JPS 24 (Synechococcales), JPS 16 (*Leptolyngbya* sp.*),* JPS 13 (*Wilmottia* sp.*),* JPS 5 (*Roholtiella edaphica), JPS004 (Nostoc* sp.) and JPS001 (unidentified cyanobacterium) no information regarding their potential toxicity was obtained, so the presence of cyanotoxin genes is uncertain.

The environmental samples represented in table 8 correspond to some positive PCR results, and they were selected to be sequenced. When it was possible the sequences for each sample would be assembled together to form the correspondent consensus sequence that would be Blasted to obtain the most similar sequence in the GenBank database corresponding to an identified strain.

Table 8. Potential toxic environmental samples sent for sequencing; n.c – no consensus, Blast results in annex B.

Target gene	Environmental sample	Location	Best hit	Similarity
cyrJ	ENV_011	Alto Rabagão's dam	n.c	n.c
mcyD	ENV_004	Parque da Cidade	n.c	n.c
	ENV_013	Caniçada's dam	n.c	n.c
mcyE	ENV_005	Parque da Cidade	*Nostoc* sp. (acc.nbr:KC699835)	94%
	ENV_012	Alto Rabagão's dam	*Nostoc* sp. (acc.nbr:KC699835)	94%
sxtI	ENV_006	Parque da Cidade	*Nostoc* sp. (acc.nbr: CP026681.1)	97%

For the genes *cyrJ* and *mcyD* no consensus was obtained for the environmental samples selected due to bad quality of the sequences, so they could not be used to validate the PCR results (annex B). For the *mcyE* gene and the *sxtI* gene, with a similarity

of 94% and 97% respectively (annex B), the environmental samples shown that some strains similar to *Nostoc* spp. were present.

3.5. Biotechnological potential analysis

In table 9, the results for the screening of PKS and NRPS genes in the environmental samples and isolates are presented.

Table 9. PKS and NRPS results for the environmental samples and isolates

Location	Environmental Samples	PKS DKF/DKR	NRPS MTF2/MTR	Isolates ID	Location	Isolates	NRPS MTF2/MTR
Parque da Cidade	ENV 001	n.i	n.i				
Parque da Cidade	ENV 002	n.i	n.i				
Parque da Cidade	ENV 003	-	+	No molecular info	ENV001; Parque da cidade	JPS 1	n.i
Parque da Cidade	ENV 004	-	+	*Microcoleus vaginatus*	ENV003; Parque da cidade	JPS 2	-
Parque da Cidade	ENV 005	-	+	*Microcoleus vaginatus*	ENV003; Parque da cidade	JPS 3	-
Parque da Cidade	ENV 006	-	+	*Nostoc* sp.	ENV003; Parque da cidade	JPS 4	-
Parque da Cidade	ENV 007	-	+	*Roholtiella edaphica*	ENV004; Parque da cidade	JPS 5	-
Parque da Cidade	ENV 008	-	+	*Microcoleus vaginatus*	ENV004; Parque da cidade	JPS 6	+
Alto Rabagão's dam	ENV 011	-	+	*Timaviella circinata*	ENV005; Parque da cidade	JPS 7	-
Alto Rabagão's dam	ENV 012	-	+	*Timaviella* sp.	ENV005; Parque da cidade	JPS 8	-
Caniçada's dam	ENV 013	-	+	*Tolypothrix* sp.	ENV005; Parque da cidade	JPS 9	+
Parque dos Moinhos	ENV 014	n.i	n.i	*Nodosilinea epilithica*	ENV006; Parque da cidade	JPS 10	-
Praia Fluvial da Esperança	ENV 015	n.i	n.i	*Nodosilinea epilithica*	ENV006; Parque da cidade	JPS 11	-
Praia Fluvial da Esperança	ENV 016	-	+	*Timaviella* sp.	ENV006; Parque da cidade	JPS 12	+
Park near Queimadala's dam	ENV 017	-	+	*Wilmottia* sp. #	ENV006; Parque da cidade	JPS 13	-
Park near Queimadala's dam	ENV 018	-	+	unidentified Tolypothrichaceae	ENV006; Parque da cidade	JPS 14	-
Travassos	ENV 019	n.i	n.i	unidentified Leptolyngbyaceae	ENV007; Parque da cidade	JPS 15	-
Travassos	ENV 020	-	+	*Leptolyngbya* sp. 1	ENV011; Alto Rabagão's dam	JPS 16	-
Travassos	ENV 035	-	+	*Leptolyngbya* sp. 2	ENV012; Alto Rabagão's dam	JPS 17	-

Barcelos	ENV 049	-	+	*Tychonema* sp.	ENV013; Caniçada's dam	JPS 18	+
Barcelos	ENV 050	-	+	unidentified Synechococcales sp. 1	ENV014; Parque dos Moinhos	JPS 19	+
Penide's dam	ENV 051	-	+	*Wilmottia* sp.	ENV016; Praia Fluvial da Esperança	JPS 20	+
Praia Fluvial de Merelim	ENV 052	-	-	*Tychonema* sp.	ENV018; Travassos	JPS 21	-
Praia Fluvial dos Moinhos	ENV 053	-	-	*Tychonema* sp.	ENV020; Travassos	JPS 22	+
Praia Fluvial da Navarra	ENV 054	-	+	unidentified Nostocales	ENV020; Travassos	JPS 23	+
Praia Fluvial da Navarra	ENV 055	-	-	unidentified Synechococcales sp. 2	ENV035; Park near Queimadela's dam	JPS 24	+
Park near river Ave	ENV 056	+	+	*Macrochaete* sp.	ENV049; Barcelos	JPS 25	-
Santo Tirso's park	ENV 057	+	+	*Timaviella* sp. #	ENV054; Praia Fluvial da Navarra	JPS 26	-
Positive controls		LEGE 91339	LEGE 91339	Positive control			LEGE 91339

based on a singleton only

Overall, most of the stains isolated that were screened through PCR for the NRPS gene have revealed that the majority of the samples have strains which have those genes present in them, and 9 isolates also showed the presence of that gene. On the other hand, PKS gene only had 2 samples which showed positive results. Once again, these results should be confirmed by sequencing in the future (something that will allow to assess the potential chemodiversity, as well).

4. Conclusion

In this study it was revealed the diversity of subaerial cyanobacterial isolates obtained from several types of terrestrial surfaces. The culture-dependent approach yielded 26 isolated strains which exhibited a total of 17 different morphotypes belonging to three taxonomic orders. Non-heterocytous strains were the most prevalent and belonged to the orders Synechococcales and Oscillatoriales, the less predominant forms were the Nostocales. The phylogenetic distribution of the isolates was in accordance with the morphological classification, and the isolates could be grouped in according to their phylogenetically affinity in a heterocystous group corresponding to the Nostocales order, and then a non-heterocystous group corresponding to the Oscillatoriales or the Synechococcales order.

The PCR-based screenings for potential toxicity that were performed allowed us to check that, much like planktonic and benthic aquatic cyanobacteria, the terrestrial mats included strains that also have the potential to produce several cyanotoxins. Although the mere presence of the gene in the environment does not mean the

production of the cyanotoxin because it's expression is determined by several factors, the molecular screening allowed us to see that they are present in terrestrial biofilms and mats along the RH2 region in the north of Portugal in quite a widespread manner, and where saxitoxin producing strains were more frequent in terrestrial environments of the study area, as we observed by targeting the *sxtI* gene. Although they revealed to be capable to produce cyanotoxins it is very unlikely that subaerial cyanotoxin-producing cyanobacteria will cause public health-related problems due to the lack of contact people have with them and to the fact that it is practically impossible to reach high enough concentrations to cause adverse effects on people, but analyzing their presence in terrestrial habitats can give some insight to their ecological functions and to why cyanobacteria produce them. In terms of biotechnological potential, they showed a high presence of the NRPS gene in the environmental samples only indicating that they are potentially a good source of bioactive compounds that are produced via a non-ribosomal pathway.

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6. Annexes

Annex A (Blast results of the isolated strains):

Isolate ID	Isolate	Consensus size	Best hits	Query cover	E-value	Identity	Ascension nbr
No molecular info	JPS_001	-	sem extração	-	-	-	-
Microcoleus vaginatus	JPS_002	649	Uncultured bacterium clone HL201307-3 16S ribosomal RNA gene, partial sequence	100%	0	99%	KU515125
			Tychonema sp. SAG 23.89 16S ribosomal RNA gene, partial sequence	100%	0	99%	KM019964
Microcoleus vaginatus	JPS_003	606	Uncultured bacterium clone HL201307-70 16S ribosomal RNA gene, partial sequence	100%	0	100%	KU515188
			Phormidium cf. autumnale JR6 16S ribosomal RNA gene, partial sequence	100%	0	100%	KT753320
Nostoc sp.	JPS_004	610	Uncultured bacterium clone abscm03.1.109 16S ribosomal RNA gene, partial sequence	100%	0	99%	JX255093
			Nostoc commune NTC 16S ribosomal RNA gene, partial sequence	100%	0	99%	MF398191
			REFERENCE STRAIN: Nostoc punctiforme PCC 73102, complete genome	100%	0	97%	CP001037
Roholtiella edaphica	JPS_005	572	Roholtiella edaphica AR5 16S ribosomal RNA gene, partial sequence	100%	0	100%	MF002049
Microcoleus vaginatus	JPS_006	632	Microcoleus vaginatus PUPCCC 120.5 16S ribosomal RNA gene, partial sequence	100%	0	100%	KM384755
Timaviella circinata	JPS_007	633	Uncultured cyanobacterium clone B10912H 16S ribosomal RNA gene, partial sequence	100%	0	99%	HQ188993
			Timaviella sp. Us-6-3 16S ribosomal RNA gene, partial sequence	100%	0	99%	MH688850
Timaviella sp.	JPS_008	619	Uncultured cyanobacterium clone B10912H 16S ribosomal RNA gene, partial sequence	100%	0	99%	HQ188993
			Timaviella sp. Us-6-3 16S ribosomal RNA gene, partial sequence	100%	0	99%	MH688850
Tolypothrix sp.	JPS_009	626	Tolypothrix sp. UAM 357 16S ribosomal RNA gene, partial sequence	100%	0	99%	HM751846
Nodosilinea epilithica	JPS_010	608	Pseudanabaenaceae cyanobacterium DPG1-KK5 16S ribosomal RNA gene, partial sequence; and 16S-23S ribosomal RNA intergenic spacer, tRNA-Ile and tRNA-Ala genes, complete sequence	100%	0	99%	EF654067
Nodosilinea epilithica	JPS_011	623	Pseudanabaenaceae cyanobacterium DPG1-KK5 16S ribosomal RNA gene, partial sequence; and 16S-23S ribosomal RNA intergenic spacer, tRNA-Ile	100%	0	99%	EF654067

			and tRNA-Ala genes, complete sequence				
Timaviella sp.	JPS_012; best hit	629	Uncultured cyanobacterium partial 16S rRNA gene, clone CL4.E8	100%	0	99%	FM175896
			Timaviella circinata GR4 partial 16S rRNA gene, type strain GR4T	100%	0	99%	LT634149
Wilmottia sp. #	JPS_013	-	-	-	-	-	-
unidentified Tolypothrichaceae	JPS_014	619	Uncultured cyanobacterium clone BksYy19200 16S ribosomal RNA gene, partial sequence	100%	0	99%	KC463244
			Hassallia cf. pseudoramosissima ACSSI 158 16S ribosomal RNA gene, partial sequence	100%	0	99%	KY283057
unidentified Leptolyngbyaceae	JPS_015	662	Uncultured bacterium clone YT-2 16S ribosomal RNA gene, partial sequence	100%	0	96%	JQ770050
			Plectonema sp. SAG 38.90 16S ribosomal RNA gene, partial sequence	100%	0	95%	KM019916
Leptolyngbya sp. 1	JPS_016	599	Uncultured organism clone ELU0037-T187-S-NIPCRAMgANb_000372 small subunit ribosomal RNA gene, partial sequence	100%	0	98%	HQ755632
			Leptolyngbya sp. NIES-3755 DNA, complete genome	100%	0	96%	AP017308
			Leptolyngbya sp. CENA377 16S ribosomal RNA gene, partial sequence	100%	0	95%	KR137598
Leptolyngbya sp. 2	JPS_017	619	Plectonema sp. SAG 38.90 16S ribosomal RNA gene, partial sequence	100%	0	97%	KM019916
Tychonema sp.	JPS_018	566	Phormidium autumnale VUW11 16S ribosomal RNA gene, partial sequence	100%	0	100%	GQ451408
unidentified Synechococcales sp. 1	JPS_019	569	Nostoc punctiforme MACC-287 16S ribosomal RNA gene, partial sequence	100%	0	99%	MH702235
Wilmottia sp.	JPS_020	616	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: OTU15	100%	0	99%	LC103289
			Wilmottia stricta 16PC 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence	100%	0	97%	KY288986
Tychonema sp.	JPS_021	637	Tychonema sp. LEGE 07216 16S ribosomal RNA gene, partial sequence	100%	0	100%	KU951840
Tychonema sp.	JPS_022	653	Oscillatoriales cyanobacterium Alignment_Isolate6 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, partial sequence	100%	0	99%	KR002123
			Phormidium autumnale VUW2 16S ribosomal RNA gene, partial sequence	100%	0	99%	GQ451409
unidentified Nostocales	JPS_023	442	Nostoc sp. NQAIF313 16S ribosomal RNA gene, partial sequence	100%	0	98%	KJ636965
unidentified Synechococcales sp. 2	JPS_024	629	Pseudanabaenaceae cyanobacterium VUW6 16S ribosomal RNA gene, partial sequence	100%	0	99%	GQ451430
Macrochaete sp.	JPS_025	626	Uncultured cyanobacterium clone cher4_2B_90 small subunit ribosomal RNA gene, partial sequence	100%	0	98%	JN020217
			Macrochaete santannae CCALA 1093 clone operon 2 16S ribosomal	100%	0	96%	KT336441

			RNA gene, partial sequence; 16S-23S ribosomal RNA intergenic spacer, tRNA-Ile and tRNA-Ala genes, complete sequence; and 23S ribosomal RNA gene, partial sequence				
Timaviella sp. #	JPS_026	-	-	-	-	-	-

based on a singleton only

Annex B (Blast results of the environmental samples for the cyanotoxin genes):

Toxins	Environmental sample	Consensus sequence size	Best hits	Query cover	E-value	Identity	Accession nbr
CYN	ENV_011	None	All sequences bad	-	-	-	-
mcyD	ENV_004	None	All sequences bad	-	-	-	-
	ENV_013	None	All sequences bad	-	-	-	-
mcyE	ENV_005	430	Nostoc sp. 152 microcystin synthetase gene cluster, complete sequence; and hypothetical protein gene, partial cds	100%	0%	94%	KC699835.1
	ENV_012	396	Nostoc sp. 152 microcystin synthetase gene cluster, complete sequence; and hypothetical protein gene, partial cds	100%	2,00E-172	94%	KC699835.2
sxt	ENV_006	140	Uncultured bacterium clone contig49114 genomic sequence	100%	4,00E-61	99%	KP445187.1
			Nostoc sp. 'Peltigera membranacea cyanobiont' N6 chromosome, complete genome	100%	2,00E-58	97%	CP026681.1
			Aphanizomenon gracile partial sxtI gene for O-carbamoyltransferase, strain NIVA-CYA 655, isolate AB2008/16	100%	4,00E-24	80%	HG917839.1