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MOLECULAR APPROACHES IN CYANOBACTERIA: FROM DETECTION AND DIVERSITY TO DNA-BASED BIOSENSORS

Elisabete Maria Pinto Valério

Ph.D. in Biology

(Microbiology)

Lisbon

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The work was financially supported by Fundação para a Ciência e a Tecnologia, Programa de Gestão de Recursos Humanos, grant SFRH/BD/8272/2002.

To my most precious treasures: Rodrigo and Maria.

This thesis is based on the following publications:

“Potentiality of molecular methods for cyanobacteria identification, differentiation and traceability”, Elisabete Valério, Lélia Chambel, Sérgio Paulino, Natália Faria, Paulo Pereira, Rogério Tenreiro. *Microbiology* (submitted).

"Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples", Elisabete Valério, Lélia Chambel, Sérgio Paulino, Natália Faria, Paulo Pereira, Rogério Tenreiro. *Aquatic Microbial Ecology* (submitted).

"Molecular characterization of Cylindrospermopsis raciborskii strains isolated from Portuguese freshwaters", E. Valério, P. Pereira, M.L. Saker, S. Franca, R. Tenreiro *Harmful Algae* 4 (2005): 1044-1052.

"Validation of a multiplex PCR for the detection of cylindrospermopsin producing strains", Elisabete Valério, Lélia Chambel, Sérgio Paulino, Natália Faria, Paulo Pereira, Rogério Tenreiro. *Journal of Applied Phycology* (submitted).

"Preliminary studies towards the development of DNA biosensors for detection of cylindrospermopsin - a cyanobacterial toxin", E. Valério, A. Tenreiro and L.M. Abrantes, *Portugaliae Electrochimica Acta* (in press).

E. Valério, declares that was involved on the conception and execution of the experimental work, in the results interpretation and writing of the published or submitted manuscripts.

ACKNOWLEDGMENTS

I would like to thank Professor Rogério Paulo de Andrade Tenreiro and Professor Luisa Maria Abrantes for their tutoring and supervising, for all the scientific support, for providing all the resources necessary to the execution of this work, and for the careful reading of this thesis.

To Susana Franca I acknowledge the warm reception in her group at the Instituto Nacional de Saúde Dr. Ricardo Jorge, and for providing all the conditions and support concerning the cyanobacterial cultures.

I am grateful to Paulo Pereira, for helping me writing this project, for the co-guidance and for all the support in the morphologic and toxicologic analyses.

Special thanks to Sérgio and Natália, for the care, for always being available, and for providing all the necessary informations essential for this work. I must also acknowledge all the other collaborators of the QHME-INSA for always being so helpful.

I am also grateful to Prof. Lélia Chambel, for the co-guidance, for the brainstorming, all our talks and most especially for the friendship.

Thanks also go to Prof. Ana Tenreiro and Ana Viana for the support and help during this studies, and to Prof. Rui Malhó for performing and helping in the fluorescence measurements.

Big thank you to all my laboratories colleagues, from QHME-INSA, ICAT and C8. Special thanks to Sandra Chaves, Virgínia and Ana Mourato for the friendship, support and for always being available to answer my questions.

I would also like to thank to my hosting institutions: Faculdade de Ciências da Universidade de Lisboa and Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA), the financial support of the Fundação para a Ciência e Tecnologia, Programa de Gestão de Recursos Humanos, for my PhD grant attribution (SFRH/BD/8272/2002) and also to Fundação Calouste Gulbenkian for the financial support to my participation on the “6th European Workshop on the Molecular Biology of Cyanobacteria”, Gdansk, Poland, in 2005.

My particular gratitude goes to my family, specially to my mother, for the encouragement and also to my fathers-in-law Otília and Álvaro, for all the help and support throughout this process, especially during this last year.

To my loving children, Rodrigo and Maria, thank you for lighting up even the darkest days, for the unconditional love and beautiful smiles.

And to Tiago for everything…

Abbreviations

4-ATP - 4-Aminothiophenol

ARDRA - Amplified rDNA Restriction Analysis

BLAST - Basic Local Alignment Search Tool

bp - Base pairs

CP - Conducting Polymers

CV - Cyclic Voltammetry

CYL - Cylindrospermopsin

E - Potential

EDC - N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride

ELISA - Enzyme-Linked Immunosorbent Assay

ERIC - Enterobacterial Repetitive Intergenic Consensus

HPLC - High Pressure Liquid Chromatography

I - Current

ITS - Intergenic Transcribed Spacer

LMECYA - Cyanobacteria Culture Collection Estela Sousa e Silva (Laboratório de Microbiologia e Ecotoxicologia do Instituto Nacional de Saúde Dr. Ricardo Jorge).

LTRR - Long Tandemly Repeated Repetitive

MB - Methylene Blue

MCYST - Microcystin

MES - 2-Morpholinoethanesulphonic Acid

NCBI - National Centre for Biotechnology Information

NHS - N-Hydroxysuccinimide

NRPS - Non-ribosomal peptide synthesis

OCP - Open Circuit Potential

PB - Phosphate Buffer

PC-IGS - Non-coding InterGenic Spacer of the PhycoCyanin operon

PCR - Polymerase Chain Reaction

PST - Paralytic Shellfish Toxin

PTy - Polytyramine

RFLPs - Restriction Fragment Length Polymorphisms

SAMs - Self-Assembled Monolayers

SCE - Saturated Calomel Electrode

SDS - Sodium Dodecyl Sulfate

SEM - Scanning Electron Microscopy

SSC -Sodium Chloride + Sodium Citrate

SSR - Short Sequence Repeats

STR - Short Tandem Repeats

STRR - Short Tandemly Repeated Repetitive

SWV - Square Wave Voltammetry

UPGMA - Unweighted Pair Group Method with Arithmetic Average

WHO - World Health Organization

υ - Sweep Rate

Abbreviations of the genera names:

A. - *Anabaena*

Aph. - *Aphanizomenon*

C. - *Cylindrospermopsis*

M. - *Microcystis*

O. - *Oscillatoria*

P. - *Planktothrix*

RESUMO

As cianobactérias dulçaquícolas, colonizam e por vezes dominam as comunidades fitoplanctónicas de reservatórios e de cursos de água em diversos estados tróficos, distribuindo-se quer a níveis superficiais, quer a níveis mais profundos da coluna de água. Algumas espécies de água doce, em condições favoráveis, podem dividir-se rapidamente formando populações com densidades celulares extremamente elevadas, designadas por florescências ou "blooms". Para além das consequências nefastas para o ambiente, resultantes do esgotamento de nutrientes, das variações produzidas nos níveis de oxigénio e das elevadas turvações que conferem à água, entre outros, o desenvolvimento de florescências em massas de água doce superficial está também frequentemente associado à produção de compostos tóxicos para o homem e para os animais. A literatura científica neste campo de investigação tem vindo a desenvolver-se nas últimas duas décadas e os registos de ocorrências de florescências tóxicas em águas doces superficiais têm vindo a aumentar progressivamente, adquirindo um carácter global de distribuição.

Em Portugal, o desenvolvimento de florescências de cianobactérias é um fenómeno comum em águas doces superficiais usadas para agricultura, criação de gado, fins recreativos ou para consumo público. O crescente registo de ocorrências de estirpes tóxicas, produtoras de PSTs, mas sobretudo atribuídas à produção de microcistinas, é igualmente comum e tem vindo a alertar as autoridades de saúde e ambientais para a importância destas ocorrências.

Portugal ainda não estabeleceu um quadro legal que regulamente de forma inequívoca e eficaz o uso e a vigilância de águas contaminadas com este tipo de toxinas. No entanto, a crescente necessidade de utilização de água com origem superficial, para consumo público e para actividades balneares, reforça a necessidade de se monitorizar a qualidade da água e de avaliar a eficiência dos processos de tratamento na remoção de cianobactérias e das várias toxinas potencialmente associadas.

A ocorrência de toxinas associadas a florescências de cianobactérias é imprevisível e pode variar temporal e espacialmente. As variações resultam fundamentalmente de diferenças na densidade e composição das comunidades cianobacterianas florescentes. De facto, as florescências naturais podem ser constituídas por uma única espécie dominante ou ser compostas por uma variedade de espécies. Acresce ainda que diferentes estirpes da mesma espécie podem apresentar diferentes graus de toxicidade ou produzir diferentes tipos, por vezes mais de um tipo, de toxinas. Isto significa que a toxicidade de uma florescência é determinada não só pela densidade, como também pela composição e proporção das estirpes que

a compõem, que podem ser umas mais tóxicas, outras menos tóxicas e outras não tóxicas. Portanto, a monitorização destas ocorrências requer um diagnóstico rápido e seguro que permita determinar em tempo útil situações de risco real ou potencial para a saúde humana.

A correcta identificação das cianobactérias presentes no ambiente aquático é crítica para o diagnóstico e monitorização das ocorrências, já que fornece informação sustentada para a escolha dos ensaios biológicos ou analíticos a ser usados na determinação das toxinas produzidas. No entanto, a abordagem botânica tradicional, baseada na observação microscópica, é muitas vezes problemática, dado que as cianobactérias constituem um grupo intrincado de organismos, frequentemente difíceis de reconhecer apenas através de critérios morfológicos. As maiores dificuldades resultam da ausência de caracteres distintivos facilmente observáveis e das variações que estes podem apresentar em função das condições ambientais ou de crescimento.

Neste trabalho, um conjunto de 124 isolados, compreendendo isolados tóxicos e não tóxicos, mantido na colecção de culturas do LMECYA (Cyanobacteria Culture Collection Estela Sousa e Silva mantida no Laboratório de Microbiologia e Ecotoxicologia do Instituto Nacional de Saúde Dr. Ricardo Jorge), foi sujeito a diversas análises.

Analisaram-se os polimorfismos de pequenas sequências repetitivas de 118 isolados, de modo a avaliar o seu potencial identificativo, diferenciante e de rastreabilidade de estirpes de cianobactérias presentes nas albufeiras. Verificou-se que a análise hierárquica combinada dos perfis “fingerprinting” obtidos com “primers” dirigidos para as sequências STRR e LTRR (específicas de cianobactérias), permite identificar os isolados após o seu posicionamento prévio a nível de ordem por análise morfológica. A confirmação da identificação de representantes de cada “cluster” formado anteriormente foi realizada recorrendo à análise filogenética de um sub-conjunto de 73 isolados, usando dois marcadores moleculares, o gene de rDNA 16S, ubíquo nos procariotas, e o gene que codifica a subunidade γ da RNA polimerase dependente de DNA, apenas presente nas cianobactérias. Este estudo permitiu identificar a nível de espécie 26 dos 73 isolados pela sua posição filogenética, contudo em alguns casos apenas foi possível obter a identificação a nível de género (33 dos 73). Foi também avaliada a congruência destes dois marcadores moleculares, além da congruência da identificação molecular com a morfológica. Foram ainda avaliadas as relações filogenéticas dos vários grupos taxonómicos.

Uma subsequente análise hierárquica combinada dos perfis de M13 e ERIC “fingerprinting” revelou ter potencial diferenciante das várias estirpes e ainda permitiu identificar

estirpes tóxicas residentes nas albufeiras, pelo que estes métodos se revelaram adequados para rastreabilidade de cianobactérias, nomeadamente de estirpes tóxicas presentes nos recursos hídricos.

Construiu-se uma chave de diagnóstico baseada nos perfis de restrição do rDNA 16S com as enzimas AvaII e BanII e ainda com a dimensão dos fragmentos de amplificação do ITS e da sua subsequente restrição com a enzima TaqI, que permite distinguir 15 das 18 espécies de cianobactérias analisadas.

A pesquisa de marcadores moleculares dirigidos para regiões específicas e conservadas dos genes responsáveis pela toxicidade, que possam ser utilizados na detecção das estirpes toxigénicas e na discriminação das toxinas produzidas numa florescência, constitui também um passo fundamental para a implementação destas metodologias no diagnóstico de cianobactérias tóxicas em águas doces superficiais.

Neste trabalho, desenvolveu-se um método de PCR multiplex, dirigido para três locais do "cluster" de genes envolvido na biossíntese das microcistinas, que permite detectar a presença de estirpes produtoras de microcistinas em amostras naturais provenientes de albufeiras, tendo esta análise sido validada nos 124 isolados em estudo e usando os resultados dos perfis toxicológicos dos isolados ou das amostras ambientais obtidos por métodos analíticos como "gold standard" para avaliar a fiabilidade deste método. Outros métodos de PCR, previamente descritos, direccionados para a detecção de estirpes produtoras de cilindrospermopsina foram aplicados a nove isolados Portugueses e subsequentemente validados usando 120 isolados.

Uma vez que não existem padrões analíticos de cilindrospermopsina e também os resultados obtidos nos métodos de PCR se revelaram positivos, seleccionou-se um dos "primers" usados na reacção de PCR como sonda a ser imobilizada numa superfície metálica modificada, com vista a desenvolver um biosensor electroquímico de DNA. A modificação da superfície metálica foi efectuada de duas formas, através de uma matriz polimérica de politiramina e através do uso de monocamadas automontadas de 4-aminotiofenol. O azul de metileno foi o indicador redox seleccionado e revelou ser apropriado para discriminar os processos de imobilização e hibridação da sonda por voltametria cíclica ou por voltametria de onda quadrada.

Palavras chave: Cianobactérias, taxonomia, diversidade, rastreabilidade, marcadores moleculares de toxicidade, biosensores de DNA.

ABSTRACT

In Portugal, the occurrence of cyanobacterial blooms is a common phenomenon of the Portuguese freshwaters, used for human consumption, recreational activities and agriculture. Some cyanobacterial strains of diverse species produce toxins, and as a result, blooms create major threats to animal and human health, tourism, recreation and aquaculture.

In this study a set of 124 isolates, including toxic and non-toxic ones, maintained in the LMECYA culture collection (Cyanobacteria Culture Collection Estela Sousa e Silva at Laboratório de Microbiologia e Ecotoxicologia of Instituto Nacional de Saúde Dr. Ricardo Jorge), were subjected to different analyses. The potential of several molecular targets for identification, differentiation and traceability of cyanobacteria in freshwater reservoirs was evaluated in 120 isolates and enabled the establishment of a PCR method based of the composite analysis of hierarchical clustering of STRR and LTRR fingerprints to perform species identification, after allocation to order level by morphological criteria. Representative isolates of the clusters formed in the previous analysis were selected in order to confirm its identification through its phylogenetic positioning using two molecular markers (16S rRNA and *rpo*C1 genes). The congruence of these two markers was also assessed as well as the congruence between molecular and morphological identification. The composite analysis of hierarchical clustering of M13 and ERIC PCR fingerprints revealed to be useful for strain differentiation and traceability of toxic strains. Moreover, a diagnostic key based on 16S PCR-RFLPs, ITS dimension and ITS-ARDRA has also been constructed and allowed the identification of 15 out of 18 species.

A multiplex PCR for the detection of microcystins producers in freshwaters was developed and validated. Previously described PCR methods for the detection of cylindrospermopsin producing strains have also been applied and validated.

Given the lack of analytical standards for the detection of cylindrospermopsin, a molecular marker for cylindrospermopsin-producing strains, previously investigated, was used for the development of electrochemical DNA biosensors. This approach followed two different metal surface modifications, conducting polymers or self-assembled monolayers, for the immobilization of this molecular marker. Methylene blue reveals to be an appropriate indicator to discriminate the probe immobilization and the hybridization through the characterization of the modified electrodes by cyclic voltammetry or square wave voltammetry.

Keywords: Cyanobacteria, taxonomy, diversity, traceability, toxicity molecular markers, DNA biosensors.

Preface

Why this thesis?

Why this approach?

Aims of the study

Thesis layout

Why this thesis?

An accurate identification of the cyanobacterial species that exist in a given sample of freshwater is of major importance on the reliable diagnosis and monitoring of toxic blooms, since it provides the essential information to take the right choice concerning to the assays, biological or analytical ones, that should be employed in order to determine the type of associated cyanotoxin. The traditional botanic approach, based on the morphological identification by microscopy, can represent sometimes a problem. Cyanobacteria consists on an intricate group of organisms, usually difficult to recognize only through morphologic and citologic criteria. The main difficulty concerns the absence of distinctive characters easily observed and the variations that these can present as a result of the environmental or growth conditions. Therefore, the lack of standardization on culture conditions turns difficult the taxonomic assessment in such a way that influences its morphology, resulting in misidentification of some isolates.

Why this approach?

The last decade has witnessed a tremendous development in the cyanobacterial identification and genomic characterisation and also has been featured by an intensive investigation of toxicity molecular markers using molecular biology techniques.

The search for molecular methods that could provide more simple identification and differentiation tools than the existing ones, as well as the detection of toxicity markers that can be routinely used to early recognise toxic strains in the freshwaters and give authorities the necessary conditions to take all the needed precautions, is the main purposes of this thesis.

Electrochemical DNA-based sensors have been poorly used as a selective method, despite the several advantageous properties exhibited by this tool. They provide a fast, sensitive and specific way for monitoring in real time.

Though it is known that toxic cyanobacterial blooms occur with high frequency in freshwaters in Portugal, there has been a reduced investment in this research field. Moreover, presently there has not been reported an integrated study of cyanobacteria classification and diversity analysis, as well as the research on toxicity markers that could be tested in environmental samples by PCR and biosensors techniques.

Aims of the study

The aims of the present study were to:

1. Re-evaluate the taxonomic classification of the cyanobacterial species isolated in Portugal through the construction of phylogenies of the 16S rRNA and *rpo*C1 genes and assessment of congruence between morphological and molecular identification;

2. Develop identification methods based on PCR amplification to be used in routine monitoring of the water samples in laboratories;

3. Evaluate the inter and intra-specific genomic diversity using fingerprinting methods;

4. Identify toxicity molecular markers to be tested in environmental samples using PCR amplification or through the use of electrochemical DNA-based sensors.

The experimental approach followed to reach these objectives is depicted in Figure 1.

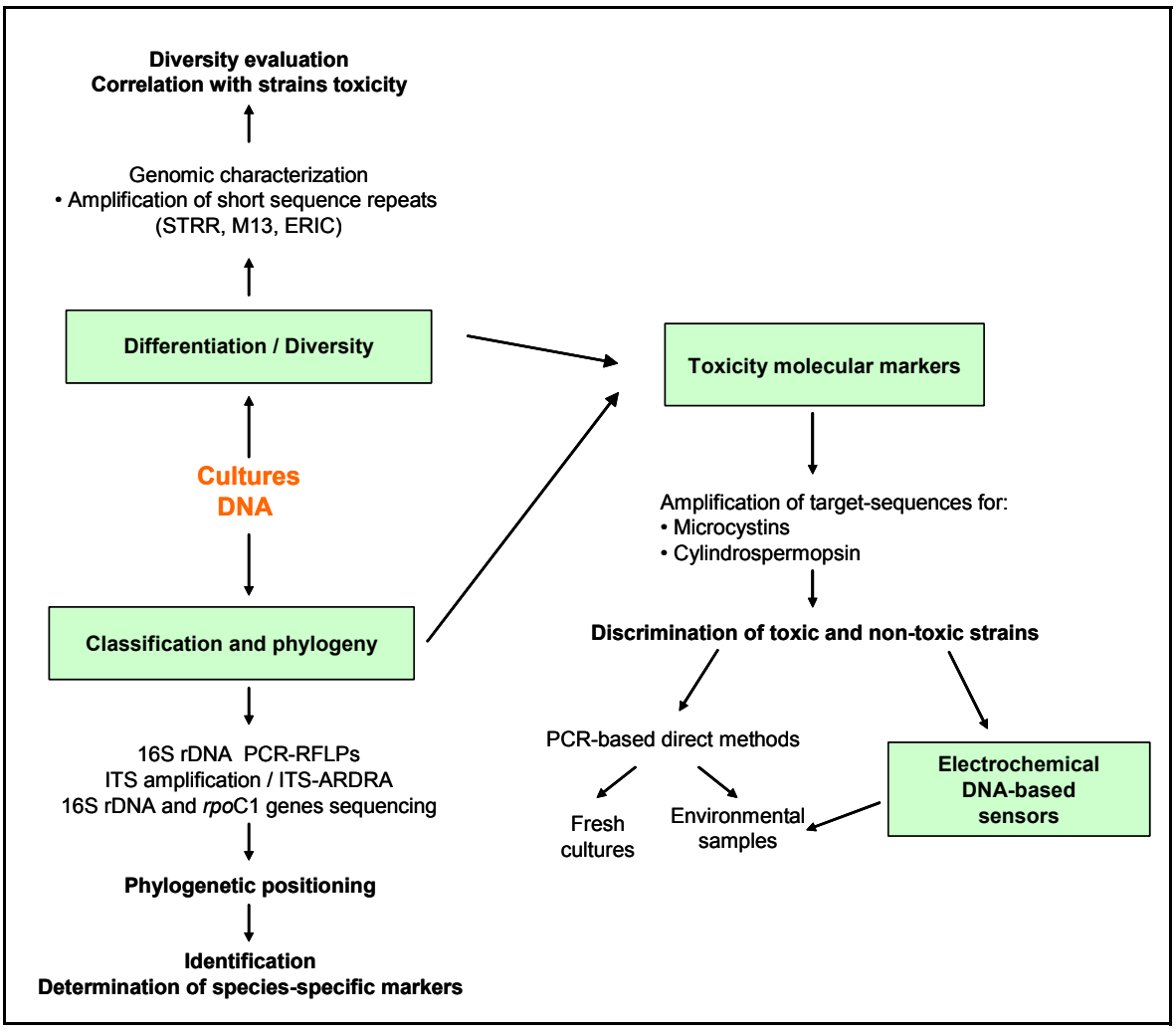


FIGURE 1 Schematic representation of the experimental approach used in this study.

Thesis layout

Due to the high diversity of subjects and methodologies, a conventional structure was not followed in this thesis in order to get a better insight on the fundamental aspects inherent to this work. Thus, the layout has been adopted to reflect all the exploited themes and, in some cases, a chapter could be composed by more than one paper, published or under submission.

This dissertation comprises 6 chapters. Chapter 1 regards an introduction to cyanobacteria with particular emphasis to their general features, taxonomy, blooms occurrences, as well to the state-of-the-art of the molecular approaches related to this work. Chapter 2 involves a description of the isolates, their geographical origin and methods for biomass production and DNA extraction. Chapter 3 describes the characterisation of the isolates using STRR and LTRR PCR fingerprinting for identification purposes, their representatives identification by the phylogenetic positioning using 16S rDNA and *rpo*C1 gene and M13 and ERIC techniques used for strain differentiation and traceability. The fragments obtained after 16S rDNA PCR-RFLPs, ITS amplification and restriction (ITS-ARDRA) are also used to assemble a diagnostic key. On the other hand, Chapter 4 involves the study of suitable toxicity molecular markers, as well as their application using DNA extracts obtained from the lyophilised cells and direct use of the fresh cultures and environmental samples in the PCR reaction without a prior DNA extraction step. In Chapter 5, that deals with electrochemical DNA-based sensors, are described two possible metal surface modifications using conducting polymers or self-assembled monolayers, for the immobilization of a molecular marker for cylindrospermopsin-producing strains and subsequent hybridization, as well as methods used to confirm these processes.

Finally, Chapter 6 summarizes the concluding remarks and future perspectives.

Contents

1

Introduction

The First Plague *(Exodus 7:14-24)*
All of the water in Egypt - right from water already in buckets and jars, to ponds, canals, streams, even the Nile River - turned to blood. Then all of the fish of the river died, causing a terrible stench.

1.1 Cyanobacterial features

Cyanobacteria represent one of the major bacterial phyla, being an ancient group of prokaryotic microorganisms exhibiting the general characteristics of gram-negative bacteria whose fossil register date of 3,5 billion years [1, 2]. Sedimentary rocks deposits, dating from the first thousand million years, in shallow seas and lakes reveal the presence of cyanobacterial colonies. The cyanobacterial colonies called stromatolites appear in rocks as fossilized mushroom shapes (Figure 1.1) and are widely distributed around the globe. These rocks have shown a fossil evidence of a wide range of both filamentous and spherical organisms, many identical in size and shape to present cyanobacteria [3, 4].

FIGURE 1.1 Stromatolites. A) Shark Bay, Australia; B) Section of stromatolite from a saline lake in Innes National Park, Australia (extracted from [5]).

Cyanobacteria constitute an extraordinarily diverse group of prokaryotes which due to their particular features have successfully colonized a wide range of habitats such as fresh, brackish and marine waters, nonacidic hot springs, hypersaline environments, Antarctic soils, rocks, ice and deserts [3, 4, 6, 7]. Only pH seem to restrict the distribution of cyanobacteria, since they tend to prefer neutral or basic conditions and are less common at low pH [6].

They are unique among the prokaryotes in possessing the capacity of oxygenic photosynthesis, being presumably the first oxygen-evolving photosynthetic organisms during the Precambrian era and are thought to be responsible for the transition of the atmosphere of the

Earth from its primordial anaerobic state to the current aerobic condition [7]. Oxygenic photosynthesis can be summarized by the equation:

$$CO_2 + 2H_2O \xrightarrow{h\nu} [CH_2O] + H_2O + O_2 \qquad \textbf{(1.1)}$$

Some cyanobacteria present an alternative system under anaerobic conditions (anoxygenic photosynthesis) resembling sulphur bacteria. The usual oxygenic photosynthesis of cyanobacteria can be altered in an adaptive response to the presence of free sulphide, where photosystem II is inhibited and electrons derived from sulphide enter the photosynthetic electron transport system and result in CO_2 reduction [3, 6]. Hydrogen sulphide serves as the electron donor instead of water [6], giving the equation:

$$2H_2S + CO_2 \longrightarrow [CH_2O] + 2S + H_2O \qquad \textbf{(1.2)}$$

Their morphology ranges from simple unicellular, colonial and multicellular filamentous forms. In Figure 1.2 is depicted a schematic representation of a cyanobacterial vegetative cell. The vegetative cell wall is of Gram-negative type but in some species the peptidoglycan layer is considerably thicker than in other bacteria. This thickness is usually 1-10 nm, but in the case of *Oscillatoria princeps* it reaches 200 nm [8]. Small-diameter pores are present in regular or scattered order in the wall of all cyanobacteria, but the arrangement varies greatly. Many unicellular and filamentous cyanobacteria possess an “envelope” outside the lipopolysaccharide (LPS) “outer membrane”, which is called: sheath, glycocalyx, or capsule, and depending on the consistency, gel, mucilage or slime. The sheaths of cyanobacteria are predominantly polysaccharide, but a part of its weight may be polypeptides, and depending on the species, some types of sugar residues may be involved [8]. The photosynthetic apparatus of a cyanobacteria contains photosystem I and photosystem II, as found in higher plants, chlorophyll *a* and specific accessory pigments, including allophycocyanin, phycocyanin and phycoerythrin. Prochlorophytes are also cyanobacteria that contain chlorophyll *a* and *b*, but, opposing to other cyanobacteria, lack phycobiliproteins [8]. Cyanobacteria possess the ability to use low light intensities effectively, since they are able to produce the accessory pigments needed to adsorb light most efficiently in the habitat in which they are present, providing them a great advantage for the colonisation of their wide range of ecological niches [9, 10]. Phycobiliprotein synthesis is particularly susceptible to environmental influences, especially light quality. The chromatic adaptation is largely attributable to a change in the ratio between phycocyanin and phycoerythrin in the phycobilisomes. The photosynthetic pigments are located in thylakoids

that are free in the cytoplasm near the cell periphery (Figure 1.2). Cell colours vary from blue-green to violet-red due to the chlorophyll *a* masking by the carotenoids and accessory pigments. The pigments are involved in phycobilisomes, which are found in rows on the outer surface of the thylakoids (Figure 1.2) [9].

Cyanobacteria are also able of storing essential nutrients and metabolites within their cytoplasm. Prominent cytoplasmic inclusions such as glycogen and cyanophycin granules (polymers of the amino acids arginine and asparagine), polyphosphate bodies, carboxysomes (containing the primary enzyme for photosynthetic CO_2 fixation, ribulose 1,5-bisphosphate carboxylase-oxygenase: RuBisCO) and gas vacuoles (Figure 1.2) can be observed by electron microscopy [8-10]. The occurrence of fimbriae (pili) is abundant in many cyanobacteria with varying patterns. Some filamentous forms are also able of gliding (sliding) [8, 10].

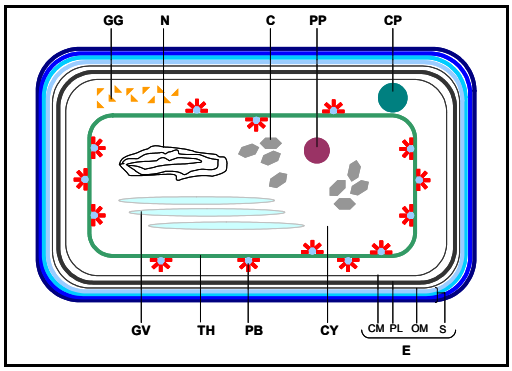


FIGURE 1.2 Schematic diagram of a cyanobacterial vegetative cell. S: external 4-layered cell wall (slime or sheath); OM: outer membrane; PL: peptidoglycan layer; CM: cytoplasmic membrane; CW: cell wall; E: cell envelope; TH: thylakoid; PB: phycobilisome; CY: cytoplasm; GV: gas vesicle; GG: glycogen granules; N: nucleoplasmic region; C: carboxysome; PP: polyphosphate granule; CP: cyanophycin granule (adapted from [8, 10]).

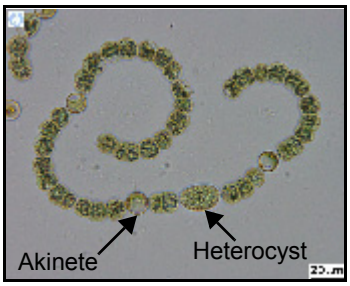
Some strains are able to fix molecular nitrogen (diazotrophy) under anaerobic conditions in differentiated cells, the heterocysts (Figure 1.3), which are cells that are structurally and functionally adapted to protect the nitrogenase complex against oxygen inactivation [7] and where nitrogenase converts N_2 directly into ammonium (NH_4), a form through which nitrogen enters the food chain [9, 10]. This feature allows them to be found also in symbiotic associations with eukaryotic organisms as diverse as lichenized fungi and within the plant kingdom including Bryophyta (mosses, liverworts and hornworts), Pteridophyta (aquatic ferns of the genus *Azolla*), gymnosperms of the family Cycadaceae and angiosperms of the family Gunneraceae [7, 11], to which they provide fixed carbon and nitrogen.

One other type of differentiated cells, that may be present in some species of cyanobacteria, are akinetes (Figure 1.3). These are tick-walled cells that are formed at the end of a

period of growth and survive in a dormant state until conditions are again favourable for its development [6, 10]. Some species, namely planktonic, have also the ability to form gas vesicles allowing them to control their buoyancy and thus manage their position in the water column to where the light intensity is optimal for photosynthesis, beside poise themselves within vertical gradients of physical and chemical factors for nutrient uptake [4, 7, 9, 10].

The cyanobacterial genomes range in mean of DNA base composition from 32 to 71 mol% G + C and from 1.6 to 13.2 Mbp [7, 12, 13].

FIGURE 1.3 Photograph of an *Anabaena* strain (LMECYA 161) showing a heterocyst and akinete (provided by N. Faria, QHME-INSA).



Isolation and obtention of cyanobacterial cultures is laborious, time consuming and sometimes difficult. Isolation by manipulation with glass needles under a dissecting microscope, to recover a single colony or trichome, followed by washing is generally preferred over any method that involves enrichment, since the isolate may then be more easily related to the natural population from the field collection [8]. The obtention of cyanobacterial axenic cultures is in some cases difficult since, although the initial manipulation and washing may seem to be enough, quite often the heterotrophic bacteria remain, specially if the isolate presents a sheath. Besides that, some of the axenic cultures may be nonviable or difficult to preserve without the presence of heterotrophic bacteria. However, the presence of one or more heterotrophic bacteria in the cyanobacterial culture does not obstruct the obtention of information about these isolates, including some physiological data, nucleotide sequences and even fingerprinting patterns, although much biochemical and physiological data cannot be obtained from these cultures [8]. The principal collection of axenic cyanobacteria is the Pasteur Culture Collection of Cyanobacteria (PCC).

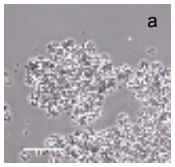
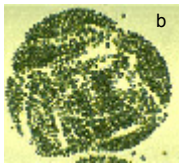
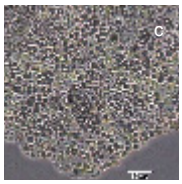
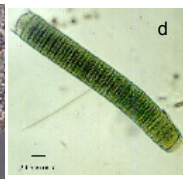
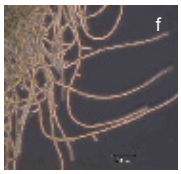
1.2 Taxonomy

For more than 150 years cyanobacteria were considered to be eukaryotic algae, with botanists and phycologists placing them into the Cyanophyceae or Blue-green algae [11]. Thus, initial classifications followed the International Code of Botanical Nomenclature. Traditional techniques for cyanobacteria identification and systematics have relied essentially on the observation of morphological characteristics. The confirmation by molecular methods, that these denominated blue-green algae where in fact photosynthetic bacteria and their transfer from Cyanophyceae to Cyanobacteria was of most importance. Stainer *et al*. [14] proposed that cyanobacterial taxonomy should follow the International Code of Nomenclature of Bacteria, and in 1979, Ripka et al. [15] published a taxonomy of the cyanobacteria that was based on physiological, morphological and some genetic criteria. However, in Oren's 2004 revision [16] of the status of the nomenclature of cyanobacteria under the Bacteriological Code, only 13 names of cyanobacterial species have been proposed and since the corresponding genera name had not been validly published according to the International Code of Nomenclature of Bacteria, all these species names are not valid [16]. Although at the present there has been an attempt to classify cyanobacteria using both Botanical and Bacteriological Code of Nomenclature [11], currently the cyanobacterial names are not covered by the Bacteriological Code [17].

Taxonomically, cyanobacteria are currently divided in six orders [18] which can be differentiated according to the features summarised in Table 1.1.

Although there are references of the existence of about 150 genera and 2000 cyanobacterial species [3, 10], many of the described species are not valid and must thus be combined [6]; moreover, some of the names are synonyms. In order to obtain a list of valid species whose names are distributed in several classification systems, a compilation of the cyanobacterial taxonomy, taking into account the present classifications [11, 16, 18, 19], is presented in Figure 1.4 for the 27 genera and in Annex for the detail of the 251 species names.

TABLE 1.1 Diagnostic features [11] of the cyanobacterial orders defined by Cavalier-Smith [18].

Characteristic	Gloeobacterales (Bergey's subsection I)	Chroococcales (Bergey's subsection I)	Pleurocapsales (Bergey's subsection II)	Oscillatoriales (Bergey's subsection III)	Nostocales (Bergey's subsection IV)	Stigonematales (Bergey's subsection V)
	 a	 b	 c	 d	 e	 f
Cells type	Unicellular	Unicellular or non-filamentous aggregates	Colonial or filamentous	Unbranched linear filaments	Unbranched filaments	Branched filaments
Cells morphology	Spherical, ellipsoidal, rod-shaped	Spherical, ellipsoidal, rod-shaped				
Cell size (μm)	0.5 - 10	0.5 - 30		1 - 100	2 - 15	
Reproduction	Binary fission or budding	Binary fission in one to three planes or budding	Multiple fission (baeocytes)	Binary fission in a single plane	Binary fission in a single plane perpendicularly to the long axis of the trichomes	Binary fission in more than one plane
Heterocysts	-	-	-	-	+	Sometimes present
Akinetes	-	-	-	-	+	Sometimes present
Thylakoids	- (only phycobilisomes)	+	+	+	+	+
Mol% G + C of DNA	35 - 71	31 - 71		33.9 - 67	38.3 - 46.7	41.9 - 44.4
Genome size (Gdal)		1.3 - 5.2		2.14 - 5.19	3.17 - 8.58	3.2 - 5.4

a: *Gloeobacter*; b: *Microcystis*; c: *Pleurocapsa*; d: *Oscillatoria*; e: *Anabaena*; f: *Fischerella* (from [5]).

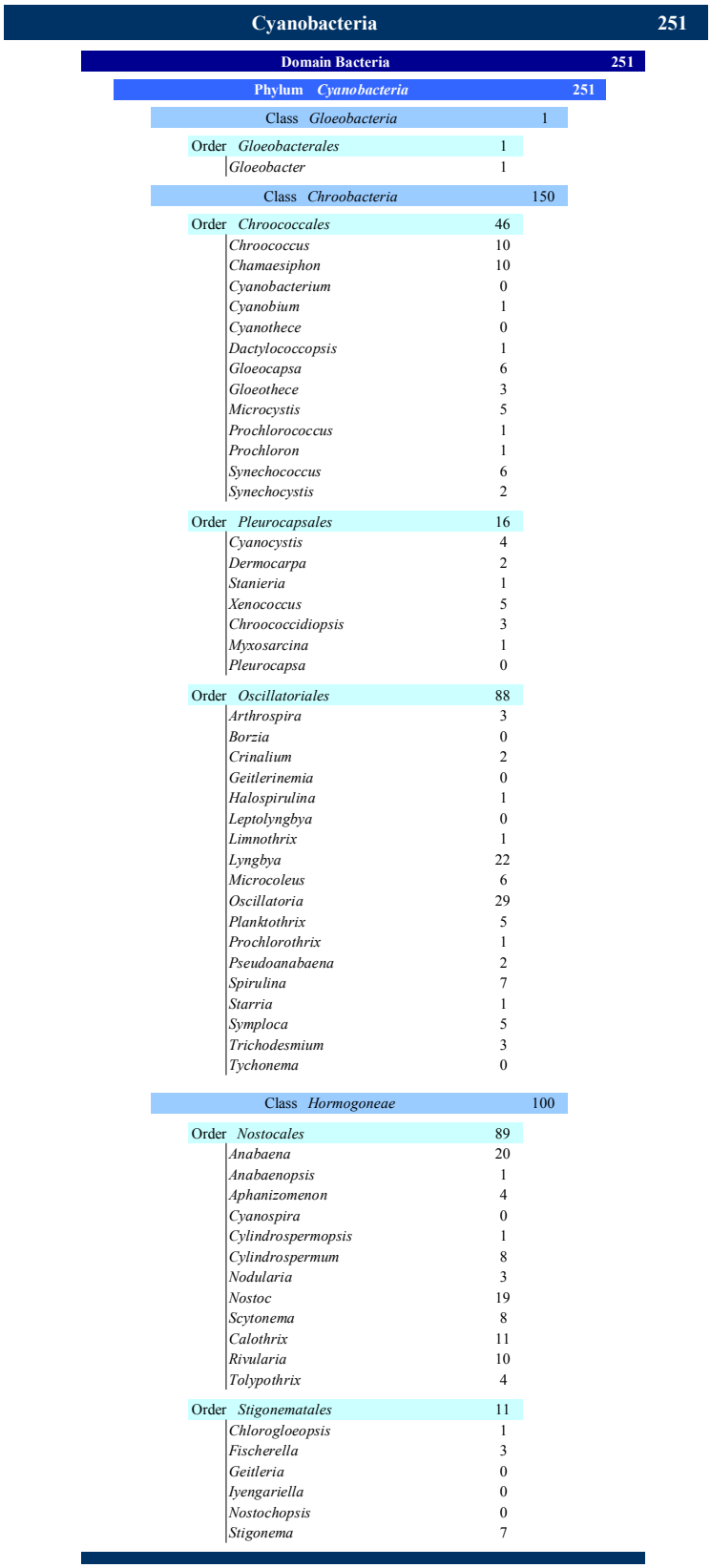


FIGURE 1.4 Taxonomic structure of the cyanobacteria, from domain to genus level. The total number of cyanobacterial species is indicated for each taxonomic level (based on data from [11, 16, 18, 19]).

The traditional observation of the morphological characters, by light microscopy, requires considerable expertise to identify species in this way. Moreover, some diagnosticant features, such as gas vacuoles or akynetes, can vary with environmental or growth conditions and even be lost during cultivation [20, 21]. Although cyanobacteria identification at genus level is easier to be reached, particularly where morphological characteristics are significantly different from other genera, there are some cases where this separation is not clear, namely the delineation of *Microcystis* from *Synechocystis*, *Anabaena* from *Nostoc*, as well as *Anabaena* from *Aphanizomenon* and *Nodularia* [22]. In addition, it is difficult to accurately identify cyanobacteria of the order Oscillatoriales. The identification problems increase further at species level, being of course impossible to differentiate strains through this approach. Sometimes, even for someone with experience, misidentification can be obtained, as occurred with an *Aphanizomenon* strain (LMECYA 31), originally classified as *Aphanizomenon flos-aquae* [23] and later re-classified as *Aph. issatschenkoi* with the help of the 16S rRNA phylogenetic positioning [24]. Given these several issues of the morphological features of cyanobacteria, Komárek and Anagnostidis [25] have estimated that more than 50% of the strains in culture collections have taxonomic names which do not agree with the morphological description of the taxon.

The limitations of phenotypic characters in cyanobacterial identification led to the development of molecular techniques, including DNA base composition, DNA and RNA hybridizations, gene sequences, and PCR fingerprinting methods, for cyanobacteria taxonomy. However, it has been difficult to define taxonomic or phylogenetic relationships within the cyanobacteria because of the scarcity of distinct, consistent characters that support a taxonomic scheme. Compounding the problems of cyanobacterial taxonomy are name changes of some strains, besides the misidentification issue of others. Consequently, an ever-changing classification system and a lack of a consensus phylogeny are the proofs of the unresolved evolutionary relationships among cyanobacteria.

1.3 Approaches to classification

After 1980, with the prospering of molecular methods, particularly the analysis of DNA by hybridization and sequencing, classifications based on the inference of phylogenetic relationships became prevalent.

1.3.1 Phenetic classification

The purpose of phenetic classification is to create clustered groups of strains, established as a hierarchy of species and genera on the basis of their overall similarity or affinity [26, 27]. Members of a species should share high levels of similarity and be more similar to species within their genus than to species of other genera. At every taxonomic level, taxa share common characters and can be circumscribed by a description that distinguishes their members from others at the same level [28]. Phenetic relationships comprise all aspects of the organisms, from molecular structure and physiology to habitat. The phenetic classification is based on the complete organism (genotype and phenotype) as it exists presently and can be considered to indicate evolutionary relationships, but with no reference to the evolutionary pathways or ancestry of the organism [27]. However, it does not have specific evolutionary implications, accepting the possibility that there may be taxa whose phylogenetic relationships can no longer accurately be ascertained, and takes no account of the effects of parallel or convergent evolution upon taxonomic interpretations [26].

Methods involving the capture of data by gel scanning, have been a step towards the phenetic ideal of comparing total bacterial phenotypes and have played an important role in circumscribing specific bacterial groups [28]. Some of these methods will be described in section 1.4.

1.3.2 Phylogenetic classification

An alternative of systematics, which has been widely adopted in the past decade, is the creation of a phylogenetic classification of bacteria based on ancestral relationships [29]. A central outcome of phylogenetic classification is that taxa must be monophyletic. By this, it is meant that all members of any taxon under consideration shall share the same common ancestor. A further requirement is that taxa sharing more recent common ancestry in time [30] are considered to be more closely related to one another than they are to other taxa. However, unless evolutionary change occurs at similar rates in different phylogenetic branches, concordance of phylogenetic and phenetic taxonomic groups cannot always be expected [26, 28]. In fact, phenotypic similarity does not necessary reflect phylogenetic relatedness.

In the case of cyanobacteria, phylogenies that do not just provide the overall shortest tree, but instead allow the incorporation of morphology-based classification schemes, may represent valid compromises, more reflective of true evolutionary relationships.

1.4 Molecular phenetic markers

1.4.1 Restriction fragment length polymorphisms (RFLPs)

The restriction fragment length polymorphisms (RFLPs) detected by restriction of PCR products with endonucleases can provide signature profiles specific to the genus, species, or even strains. Genetic characterization of cyanobacterial strains has been undertaken using restriction fragment length polymorphisms of the 16S rRNA gene (16S PCR-RFLPs) [31, 32], of the intergenic transcribed spacer (ITS): ITS-ARDRA [22, 33-35] and of the intergenic spacer of the genes encoding phycobilisome subunits (cpcBA) [36].

1.4.2 Short-sequence DNA repeats

Repetitive DNA, which occurs in large quantities in eukaryotic cells, has been increasingly identified in prokaryotes [37]. In bacteria, most of the repeated DNA sequences have been shown to belong to the large family of transposable elements or to the bacteriophage family. These multimeric repeats are built from identical units (homogeneous repeats) [poly(A), poly(G), poly(T) or poly(C)], mixed units (heterogeneous repeats), or degenerated repeat sequence motifs. Most of these elements are shorter than 200 bp, designated as mini (15-30 bp) and microsatellite (2-10 bp) DNA, consisting of short sequence repeats (SSR) or short tandem repeats (STR), noncoding, intercistronically located and distributed evenly in genomic molecules [38] and their hypervariability among individuals can provide a distinct DNA fingerprint [38, 39]. Random amplification of polymorphic DNA allows screening for genetic variation without prior knowledge of the DNA sequence. To obtain a DNA fingerprinting profile, the adjacent regions of the SSR are sufficiently conserved to allow the design of PCR primers targeting these surrounding regions, and the existence of polymorphisms in repeat number and/or length is documented by running the obtained amplicons in an agarose gel, producing a banding pattern. Variation in repeat numbers and sequence degeneracy can be explained by DNA recombination between multiple loci consisting of homologous repeat motifs. Furthermore, there is an evidence that the regions adjacent to the SSR loci are susceptible to more frequently occurring mutagenic events [38]. The function of the repetitive sequences, ubiquitously distributed throughout eukaryotic and prokaryotic genomes, is still unclear, as well as their dispersion and maintenance. A postulated mechanism for their propagation and dissemination is as “selfish” DNA by gene conversion [37, 40]. Regardless of how these repetitive sequences are maintained and dispersed, their presence, widespread distribution and highly conservation strongly suggest that they are important to the structure and evolution of genomes and make them methodologically important for DNA fingerprinting,

that can be used as an alternative for the identification of species or strains, as well as for diversity studies.

1.4.2.1 Enterobacterial repetitive intergenic consensus (ERIC)

Characteristic prokaryotic repeats such as the enterobacterial repetitive intergenic consensus (ERIC) sequences, initially named intergenic repeat unit (IRU), were originally observed in *Escherichia coli*, *Salmonella typhimurium* and other members of the family *Enterobacteriaceae* [40]. The ERIC sequence has approximately 126 bp in length, appears to be located in noncoding transcribed regions of the chromosome, in either orientation with respect to transcription, and includes a conserved inverted repeat. The chromosomal locations of the ERIC sequence can vary in different species [37, 40].

ERIC-like repetitive sequence elements have recently been demonstrated throughout other bacterial species [41-45] and were also used for identification and differentiation purposes in cyanobacteria [11, 21, 46, 47]. Usually, the two primers described by Versalovic *et al.* [40] are used in the PCR reaction.

1.4.2.2 M13 DNA

Another universal marker for DNA fingerprinting is the DNA core sequence of phage M13 which contains, in its protein III gene (that encodes for the minor capsid protein of the virion), tandem repeats of a 15 bp motif that can recognize a family of hypervariable minisatellites. They were firstly detected in humans and animals [48] and further in plants [49] and microorganisms [50, 51].

This sequence has been used as a fingerprinting probe for identification and epidemiological studies in filamentous fungi [39], yeasts [52, 53], bacteria [41, 54, 55] and cyanobacteria [46].

1.4.2.3 Short tandemly repeated repetitive (STRR) sequences

In the particular case of cyanobacteria, a distinct family of repetitive sequences, the short tandemly repetitive repeats (STRR) sequences, has been described [56], and a PCR-based fingerprinting method was developed for cyanobacteria using STRR sequences as primers [11]. These sequences consist of three different simple tandemly heptanucleotide sequence repeats (Table 1.2), initially described for *Calothix* species, where the copy number was estimated as about 100 per genome [56].

The STRR sequences have been identified in several other cyanobacterial genera and species, so far mostly in heterocystous cyanobacteria [11, 58-61] but also in some non-hete-

rocystous ones [11]. The specificity of these sequences has made the STRRs useful even for nonaxenic cyanobacterial cultures [59]. In some studies only one primer is used in the PCR reaction [58-60, 62, 63], whereas two primers, directed for the same repetitive motif [11] or for two different motifs [61, 64], are also used in others.

1.4.2.4 Long tandemly repeated repetitive (LTRR) sequence

A 37 bp long tandemly repetitive repeats (LTRR) sequence (Table 1.2) has also been identified in some cyanobacterial species [11, 57, 63]. The LTRR sequence was initially detected in low copy number by hybridization experiments in *Anabaena* strain PCC 7120 [57]. Also in this case, in some studies only one primer is used in the PCR reaction [63] and in other cases two primers directed for the repetitive motif [11] are also used.

1.4.2.5 Highly iterated palindromic (Hip1) sequence

Another interspersed repeated sequence known to be common to many, but not all, cyanobacteria is a 8 bp highly iterated palindromic sequence known as Hip1 (Table 1.2) [65]. Genomic polymorphism analysis employing cyanobacteria specific Hip1 repeats has been used to distinguish species and strains [66-70].

TABLE 1.2 Consensus of the cyanobacteria specific repetitive sequences.

	Consensus sequence	Reference
STRR1	5'- CCCCA(A/G)T - 3'	[56]
STRR2	5'- TT(G/T)GTCA - 3'	[56]
STRR3	5'- CAACAGT - 3'	[56]
LTRR	5'- GTTTTAACTAACAAAAATCCCTATCAGGGATTGAAAG - 3'	[57]
Hip1	5'- GCGATCGC - 3'	[65]

1.5 Phylogenetic markers

The assessment of the phylogeny of organisms through gene sequence analysis has increased dramatically since the advent of PCR and automated sequencing. A number of genes have been used as evolutionary markers for inferring phylogenetic relations and delineation of cyanobacterial taxonomy, being the 16S rRNA gene analysed most extensively [71].

1.5.1 16S rDNA

The 16S rRNA gene has been extensively accepted as the ultimate molecular chronometer because it is universal, structurally and functionally conserved, with a mosaic structure of conserved and more variable regions (reflecting different evolution rates), assumed as not prone to lateral transfer events and because its length allows reliable statistical comparisons and easy sequencing. Nowadays, the vast database of sequences available for this gene makes the finding of closer phylogenetic species relatively easy for each new obtained sequence.

Detailed studies of phylogenetic relationships between cyanobacteria based on 16S rRNA gene sequences have been intensively reported [21, 72-87].

Despite the 16S rRNA molecule contains variable regions, in some cases it may not be divergent enough to give good species separation, although it can be used to establish relationships between genera and well-resolved species [88]. Furthermore, the existence of multiple *rrn* operons within a single genome of some species may complicate the interpretation of sequence data. From the 12 completely sequenced cyanobacterial genomes, a variable number of *rrn* operons, ranging from one to six have been detected, as resumed in Table 1.3 [13].

TABLE 1.3 List of the cyanobacterial genomes completely sequenced until date (adapted from [13]).

Strain	Chromosome size (bp)	number of *rrn* operons
Synechocystis sp. PCC 6803	3,573,470	2
Anabaena sp. PCC 7120	6,413,771	4
Thermosynechococcus elongatus BP-1	2,593,857	1
Gloeobacter violaceus PCC 7421	4,659,019	1
Prochlorococcus marinus SS120	1,751,080	1
Prochlorococcus marinus MED4	1,657,990	1
Prochlorococcus marinus MIT9313	2,410,873	6
Synechococcus sp. WH 8102	2,434,428	2
Synechococcus elongatus PCC 6301	2,696,255	2
Synechococcus sp. CC9311	2,606,748	1
Chlorobium tepidum TLS	2,154,946	6
Rhodopseudomonas palustris CGA009	5,459,213	2

Therefore, presently there has been a more intense study of less-conserved nucleotide sequences of protein-coding genes.

1.5.2 *rpo*C1

The DNA-dependent RNA polymerase of cyanobacteria contains a slightly different arrangement than the RNA polymerase of other bacteria. It contains a dissociable σ factor and a core of five subunits: β', β and two α subunits, characteristic of all bacteria, and an additional 66,000 molecular weight polypeptide called γ, absent from the RNA polymerase of other bacteria [89]. It was first characterised in *Anabaena* sp. PCC 7120 [90] and revealed to be a common feature of the cyanobacterial RNA polymerase.

The cyanobacterial *rpo*C1 gene encodes the γ subunit of RNA polymerase and is thought to exist as a single copy in the genome [95]. The nucleotide sequence divergence between closely related species is much greater for the *rpo*C1 gene than for the 16S rRNA sequences, making *rpo*C1 more appropriate to resolve genus/species level issues than 16S rRNA [91, 92].

Phylogenetic studies using this marker helped to clarify the phylogenetic relationships among cyanobacteria [64], revealed the structure and intraspecific diversity of cyanobacterial communities [64, 84, 91-94] and provided further evidence to the cyanobacterial origin of chloroplasts and the existence of a divergent evolutionary pathway among bacteria [95].

1.5.3 Intergenic transcribed spacer (ITS)

Bacterial rRNA genes are commonly organized in an operon in the order 16S rRNA - 23S rRNA - 5S rRNA, each rRNA gene being separated by an internal transcribed spacer (ITS) region. The amplification of the 16S-23S rRNA internal transcribed spacer (ITS) in cyanobacteria have shown to present different sizes [22, 32, 35, 96] and number [32, 34] and also to be more variable in sequence even within closely related taxonomic groups [87, 97-100] than the 16S rRNA.

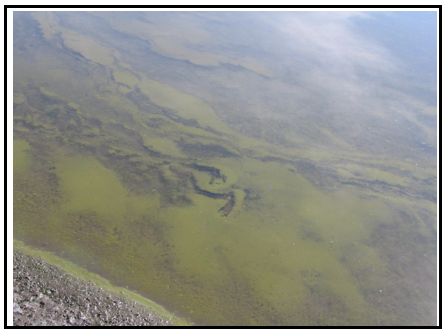
1.5.4 Other phylogenetic markers

In some cases other sequences have also been used for phylogenetic inferences: the non-coding intergenic spacer of the phycocyanin operon (PC-IGS) [80, 98, 101-104]; other DNA-dependent RNA polymerase regions, *rpo*B [85] and *rpo*D [84]; the gene encoding a serine-type of protease with a regulatory role in the differentiation process of heterocysts (*het*R) [104]; nitrogen fixation (*nif*) genes [103, 105, 106]; carbon-fixation-associated gene (RubisCO spacer) (*rbc*LX) [74, 81, 85]; and the subunit B protein of DNA gyrase (*gyr*B) [84].

1.6 Blooms and toxicity

Cyanobacterial cells numbers in water bodies vary seasonally as a consequence of changes in water temperature and irradiance as well as meteorological conditions and nutrient supply. Interactions among phytoplankton organisms in freshwater ecosystems have been detected through changes in the relative abundance of microalgae populations within the phytoplankton communities. In temperate regions, seasonal successions of organisms belonging to different phytoplankton taxa are often observed. Whereas at the beginning of the summer a great variety of microalgae and cyanobacteria usually co-exist in the same water body, towards the end of summer this diversity may drop drastically as the result of the mass development of the cyanobacterial communities (blooms) (Figure 1.5). One of this most known phenomena are the dense blooms of *Trichodesmium erythraeum* that produce a red discoloration of the water and gave the Red Sea its name [6]. Detrimental effects of such cyanobacterial blooms are of major concern nowadays for water managers. They have become an increasing worldwide problem in aquatic habitats such as lakes, rivers, estuaries, oceans and in man-made water storage reservoirs. These occurrences can be partially attributed to the gradual eutrophication of the waterways, exposition to constant sunshine, warmth and nutrients like phosphate, silicate, nitrates, CO_2 and lime [8]. A low ratio between nitrogen and phosphorous concentrations may favour the development of cyanobacterial blooms [4, 9]. Since cyanobacteria possess maximum growth rates at temperatures higher than for green algae and diatoms, the cyanobacterial blooms in temperate water bodies occur mostly during the summer months [8, 9]. However there is an unpredictable nature of cyanobacterial blooms. Although the underlying factors that trigger these phenomena are still poorly understood, the erratic behaviour of blooms, in respect to their occurrence, composition, intensity and persistency, demands careful attention in assessing risks for animal and human health.

FIGURE 1.5 Macroscopic appearance of a bloom from Alvito reservoir (E. Valério, 29/09/2005).



The water quality deterioration produced by cyanobacterial blooms include foul odours and tastes, deoxygenation of bottom waters (hypoxia and anoxia), toxicity, fish kills and food web alterations [107].

Some cyanobacterial strains of diverse species produce toxins, and as a result, blooms create major threats to animal and human health, tourism, recreation and aquaculture. The occurrence of toxic mass populations appears to have a global distribution [108, 109]. The first documented case of a lethal intoxication of livestock after drinking water from a lake heavily populated with cyanobacteria was published in the 1800s [110] and cases recorded since have included sheep, cattle, horses, pigs, dogs, fish, rodents, amphibians, waterfowl, bats, zebras and rhinoceroses [111]. A number of human deaths have been reported through exposure to cyanobacterial toxins through renal dialysis [112] and also implicated in drinking water [113]. Other reported cases of human illness associated with exposure to cyanobacteria are summarized by WHO [114]. These features highlight the importance of implementing regular monitoring programs for cyanobacteria and cyanotoxins in freshwater environments, in order to minimize potential health risks to animal and human populations resulting from exposure through drinking and recreational activities.

Several cyanobacterial toxins have been described and these secondary metabolites present a vast diversity of structures and variants. Most of cyanobacterial secondary metabolites are peptides or possess peptidic substructures [115]. The majority of these oligopeptides are assumed to be synthesised by NRPS (non-ribosomal peptide synthesis, involving peptide synthetases) or NRPS/PKS (involving peptide synthetases and polyketide synthases) hybrid pathways, allowing to reach structures not possible to be obtained by ribosomal peptide synthesis [115]. Hepatotoxins (liver damaging), neurotoxins (nerve damaging), cytotoxins (cell damaging) and toxins responsible for allergenic reactions (dermatotoxins) have been isolated from cyanobacteria [114]. The taxa from which these toxins have already been isolated are summarised in Table 1.4. A single species may contain toxic and non-toxic strains and so identification of a cyanobacterial species by microscopic morphology does not indicate the potential for toxin production. Toxic variations, between and within species of cyanobacteria, are well known from laboratory studies based on isolated cultured strains [116-119] and the number of publications using molecular methods to detect toxic cyanobacteria is rapidly increasing [46, 93, 119-125].

TABLE 1.4 Cyanotoxins detected and correspondent taxa from which have been isolated as well as their primary target in mammals (adapted from [4, 108, 114]).

Toxin	Taxon	Primary target in mammals
Hepatotoxins		
Microcystins (~70 derivatives) (cyclic peptides)	*Microcystis* *Planktothrix* *Oscillatoria* *Nostoc* *Anabaena* *Anabaenopsis* *Hapalosiphon* *Snowella* *Woronichinia*	Liver
Nodularin	*Nodularia spumigena*	Liver
Cytotoxin		
Cylindrospermopsin (alkaloid)	*C. raciborskii* *Umezakia natans* *Aph. ovalisporum* *Raphidiopsis curvata* *Anabaena bergii* *Aphanizomenon flos-aquae* *Lyngbya wollei*	Liver, kidneys, lungs, heart
Dermatotoxins		
Aplysiatoxins	*Lyngbia* *Planktothrix* *Schizothrix*	Skin
Lyngbiatoxin-A	*Lyngbia*	Skin
Neurotoxins		
Anatoxin-a (alkaloid)	*Anabaena* *Oscillatoria* *Cylindrospermum* *Microcystis* *Aphanizomenon* *Planktothrix*	Neuromuscular junction
Anatoxin-a(s) (unique organophosphate)	*Anabaena* *Aphanizomenon*	Neuromuscular junction
Saxitoxin (carbamate alkaloids)	*A. circinalis* *Aphanizomenon* *C. raciborskii* *Lyngbya wollei* *Planktothrix*	Axons

Given their high toxicity, the World Health Organization has placed microcystins (MCYST) on the list of potential health hazards, and has defined a drinking water guideline value of 1µg/L [126].

Although most reports of human intoxication caused by cyanobacteria are due to direct ingestion of contaminated water [111] or use of water containing toxins for dialysis [112], chronic intoxication may occur by ingestion of food contaminated with cyanobacterial toxins. For instance it is known that several cyanobacterial toxins can be accumulated and thus be transferred through the food chain, e.g microcystins can accumulate in mussels [127, 128], crayfish [129] and even plants [130]; PSP toxins can accumulate in cladoceran *Daphnia magna* [131] and freshwater mussels [132]; and cylindrospermopsin in mussels [133].

1.7 Cyanobacteria occurrence in Portuguese freshwater reservoirs

In Portugal, the occurrence of cyanobacterial blooms has been reported since the 30's [134] and is a common phenomenon of the Portuguese freshwaters, lakes and rivers, used for human consumption, recreational activities and agriculture [135, 136]. Since 1972 that a regular monitoring of the phytoplankton [137] and zooplankton [138] has been performed. From the 80's onward that the development of cyanobacterial blooms associated with the eutrophication of several freshwater reservoirs [137, 139-148], lakes [143, 146-152] and rivers [144, 146, 148, 153-155] has been reported.

Toxic cyanobacteria are common in Portuguese freshwaters and are a cause of concern, given that exposure to subacute levels of cyanobacterial toxins through drinking and recreational water might have deleterious effects on human health. Descriptions on human or animal intoxication caused by cyanobacteria in Portugal are scarce. However, in 1991 Oliveira [153] reported an outbreak of intoxication in Alentejo populations presenting headache, vomiting, gastrointestinal perturbations with diarrhoea, that had consumed water from the Guadiana river presenting a senescent bloom dominated by *Aphanizomenon flos-aquae* that has caused fish kills. The symptoms disappeared after ceasing the contact with the contaminated water. There has been also the suspected role of a cyanobacterial bloom in haemodialysis clinic deaths (aluminium principally responsible) [156].

Several cyanotoxins have been found in Portuguese natural lakes, rivers and reservoirs. So far, a wide range of microcystins derivatives and concentrations has been encountered in many Portuguese rivers and reservoirs and on different cyanobacterial strains isolated from natural water samples and maintained under laboratory controlled conditions [136]. Studies of the blooms toxicity have been initiated by Vasconcelos in the 90's [143] and pointed to a high percentage of toxic occurrences, namely associated with hepatotoxins (mostly microcystins) production by *Microcystis aeruginosa* [119, 143-150, 155], although other potentially toxigenic species also occur as resumed in Table 1.5.

The production of PSP (paralytic shellfish poisons) toxins has been detected in *Aphanizomenon issatschenkoi*, *Aph. gracile* and *Aph. flos-aquae* strains isolated from Portuguese freshwater reservoirs [23, 158-160].

Cylindrospermopsin (CYL) has not yet been reported in Portugal, although several *C. raciborskii* strains, a known potential CYL producer, have been isolated from three Portuguese reservoirs and one river [46, 157].

Anatoxin-a has also been detected in isolates from Portuguese freshwaters [161].

TABLE 1.5 Occurrence of potentially toxic species of cyanobacteria in Portugal.

Species	Source	References
Anabaena affinis	Reservoirs	[140, 141]
	River	[153]
Anabaena flos-aquae	Reservoirs	[137, 140, 144-146, 148]
	River	[148, 153]
	Lakes	[144, 145, 148]
Anabaena sp.	Reservoirs	[156]
	Rivers	[155]
Aph. flos-aquae	Reservoirs	[135, 137, 139, 146, 158]
	River	[153, 155]
	Lakes	[151, 152]
Aph. gracile	Reservoir	[159]
Aph. issatschenkoi	Reservoir	[23, 159, 160]
C. raciborskii	3 Reservoirs and 1 river	[46, 157]
M. aeruginosa	Reservoirs	[119, 137, 140, 142-148, 156]
	River	[148, 153-155]
	Lakes	[143-145, 147, 148, 150-152]
Nostoc spp.	Reservoirs	[146, 148]
Oscillatoria spp.	Reservoirs	[139, 156]
	River	[153]
	Lakes	[150]
Raphidiopsis sp.	River	[153]

The complexity of natural bloom communities, which can develop in a rather sudden and unpredictable way and may be formed by a consortium of cyanobacteria producing different amounts of toxins at different rates, with the same bloom-forming species having both toxigenic and non-toxigenic strains, indistinguishable by morphological examination, enlightens the importance of implementing regular monitoring programs for cyanobacteria and cyanotoxins in freshwater environments, in order to minimize potential health risks to animal and human populations resulting from exposure through drinking and recreational activities.

1.8 Technological applications

Cyanobacteria constitute a resource for several applications such as aquaculture, food, feed, fuel, fertilizer, medicine, industry and even in combating pollution [9, 162-164].

Cyanobacteria produce numerous bioactive compounds. Some secondary metabolites are potentially of therapeutical importance, such as antiviral compounds, immunomodulators, inhibitors, or cytostatics [109, 162, 163, 165].

Species of cyanobacteria, namely strains of *Spirulina* (*Arthrospira*) *platensis*, *Nostoc commune* and *Aphanizomenon flos-aquae*, are currently used as food and supplements due to their amounts of proteins (e.g. up to 70% of *Spirulina* dry weight), lipids, chlorophyll, carotenoids, vitamins, minerals, unique pigments and also due to their potential prebiotic effects [6, 162, 163, 166]. *Spirulina* is also used as an aquaculture and animal feed source [162, 163]. As a crop, *Spirulina* has the advantage of growing well in saline ponds in arid environments, places that few other crops could support [6, 10].

Several fine chemicals such as pigments (carotenoids and phycobiliproteins), vitamins (B-complex group and vitamin E) and enzymes with varied applications from cyanobacteria are being commercialized. The pigments are used as natural food colourants, food additives, cosmetics and as enhancers of ornamental fish colour [162, 163]. Isotopically labelled (^{14}C, ^{15}C, ^{3}H and ^{15}N) cyanobacterial metabolites such as sugars, lipids and aminoacids are commercially available [162]. Several endonucleases produced from cyanobacteria are being marketed, e.g. AcyI (*Anabaena cylindrica*), AflI and AflIII (*Anabaena flos-aquae*), AvaI, AvaII and AvrII (*Anabaena variabilis*), MstII (*Microcoleus* sp.) and NspCI (*Nostoc* sp.) [162].

N_2-fixing cyanobacteria are used as biofertilizers, for instance in the rice paddies where nitrogen to support the rice plants comes from fixation by free-living and symbiotic cyanobacteria, besides being also used in other tropical and subtropical agriculture [6, 10, 162, 163]. Reports concerning the advantageous effects of cyanobacteria inoculation on other crops such as barley, oats, tomato, radish, cotton, sugarcane, maize, chili and lettuce have been described [162].

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2

The isolates

Part of the contents of this chapter is presented in the following paper:
"Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters", Valério, E., Pereira, P., Saker, M.L., Franca, S., Tenreiro, R. *Harmful Algae* 4 (2005): 1044-1052.

This chapter summarises general information concerning the isolates used in this thesis, as well as the methods used in cellular biomass production and DNA extraction from non-axenic unicyanobacterial cultures.

All the procedures involving the collection of the environmental samples, cultures isolation, maintenance, propagation in higher volume of culture medium, biomass recovery and lyophilisation, as well the morphological identification of the isolates, have been transmitted to me by the staff of the Laboratório de Microbiologia e Ecotoxicologia at Instituto Nacional de Saúde Dr. Ricardo Jorge (QHME-INSA) and applied at the QHME-INSA facilities. However my real contribution concerning isolates maintenance, identification and biomass recovery was reduced.

All the methods involving molecular biology techniques were performed by me at the Instituto de Ciência Aplicada e Tecnologia (FCUL).

2.1 The isolates under study

This study included 124 isolates listed in Table 2.1, from which 110 are Portuguese isolates from natural blooms in freshwaters, rivers, lagoons, lakes and artisan punctures, isolated since 1996 up to 2005 by the LMECYA staff and maintained in culture in the QHME-INSA laboratory. Seven of the 124 isolates (in boldface at the end of Table 2.1) were gently provided through a collaboration established with Doctor Martin L. Saker (Centro Interdisciplinar de Investigação Marinha e Ambiental, Porto), that supplied a small amount of lyofilised biomass of these isolates.

TABLE 2.1 Isolates used in this work, including their code, identification, geographical origin and isolation date.

Isolate	Final identification (morphological identification)	Origin	Isolation date	Note
LMECYA 1	*Microcystis aeruginosa*	Montargil	10-06-1996	A
LMECYA 2	*M. aeruginosa*	Torrão	05-06-1996	B
LMECYA 3	*M. aeruginosa*	Montargil	10-05-1996	A
LMECYA 7	*M. aeruginosa*	Montargil	27-06-1996	A
LMECYA 8	*M. aeruginosa*	Monte da Rocha	25-09-1996	B
LMECYA 9	*Aphanizomenon gracile* (*Aph. flos-aquae*)	Peneireiro	15-03-1996	E
LMECYA 10	*Aph. flos-aquae*	Torrão	05-06-1996	A
LMECYA 12	*M. aeruginosa*	Monte da Rocha	25-09-1996	A
LMECYA 13	*M. aeruginosa*	Montargil	11-07-1996	A

Isolate	Final identification (morphological identification)	Origin	Isolation date	Note
LMECYA 17	*Anabaena circinalis* (*A. spiroides*)	Montargil	10-05-1996	F
LMECYA 23	*M. aeruginosa*	Magos	22-10-1996	G
LMECYA 29	*M. aeruginosa*	Monte da Rocha	25-09-1996	A
LMECYA 31	*Aph. issatschenkoi*	Montargil	10-06-1996	A
LMECYA 33	*Aph. gracile*	Peneireiro	15-03-1996	A
LMECYA 40	*Aph. gracile*	Crato	02-08-1996	A
LMECYA 50	*M. aeruginosa*	Roxo	03-07-1997	A
LMECYA 53	*M. aeruginosa*	Patudos	13-08-1997	G
LMECYA 55	*M. aeruginosa*	Patudos	13-08-1997	G
LMECYA 56	*M. aeruginosa*	Patudos	13-08-1997	A
LMECYA 57	*M. aeruginosa*	Patudos	13-08-1997	G
LMECYA 59	*M. aeruginosa*	Patudos	13-08-1997	A
LMECYA 64	*Aph. gracile*	Patudos	13-08-1997	A
LMECYA 68	*Synechocystis* sp.	Wastewater stabilisation pound, Marrakesh, Morocco	2000	B
LMECYA 77A	*Aph. flos-aquae*	Montargil	18-11-1999	A
LMECYA 77B	*Aph. flos-aquae*	Montargil	18-11-1999	A
LMECYA 79	*Phormidium* sp. (*Anabaena* sp.)	Magos	24-06-1998	F
LMECYA 81	*M. aeruginosa*	Magos	24-06-1998	B
LMECYA 82	*M. aeruginosa*	Agolada de Baixo	09-07-1998	B
LMECYA 85	*M. aeruginosa*	Agolada de Baixo	15-07-1998	A
LMECYA 87	*M. aeruginosa*	Magos	16-07-1998	A
LMECYA 88	*Aph. flos-aquae*	Montargil	28-11-1999	A
LMECYA 89	*Aph. flos-aquae*	Montargil	18-11-1999	G
LMECYA 91B	*M. aeruginosa*	Montargil	13-11-1999	B
LMECYA 92A	*M. aeruginosa*	Montargil	13-11-1999	G
LMECYA 92B	*M. aeruginosa*	Montargil	13-11-1999	G
LMECYA 92C	*M. aeruginosa*	Montargil	13-11-1999	G
LMECYA 93	*M. aeruginosa*	Montargil	13-11-1999	G
LMECYA 97	*M. aeruginosa*	Montargil	28-11-1999	G
LMECYA 99	*Aph. flos-aquae*	Montargil	18-11-1999	A
LMECYA 103	*M. aeruginosa*	Montargil	18-11-1999	G
LMECYA 106	*M. aeruginosa*	Patudos	15-12-1999	A
LMECYA 107	*M. aeruginosa*	Caia	12-11-1999	G
LMECYA 108	*M. aeruginosa*	Caia	12-11-1999	G
LMECYA 110	*M. aeruginosa*	Montargil	11-03-2000	B
LMECYA 113	*M. aeruginosa*	Montargil	28-10-2000	G

Isolate	Final identification (morphological identification)	Origin	Isolation date	Note
LMECYA 123	*A. circinalis*	Montargil	10-09-2000	A
LMECYA 123A	*A. circinalis*	Montargil	10-09-2000	C
LMECYA 123C	*A. circinalis*	Montargil	10-09-2000	H
LMECYA 125	*Aph. flos-aquae*	Monte Novo	23-10-2000	A
LMECYA 126	*A. circinalis*	Montargil	27-11-2000	B
LMECYA 127	*M. aeruginosa*	Montargil	27-11-2000	G
LMECYA 128	*M. aeruginosa*	Montargil	27-11-2000	G
LMECYA 129	*Aph. flos-aquae*	Montargil	27-11-2000	A
LMECYA 130	*Cylindrospermopsis raciborskii*	Odivelas	24-07-2000	G
LMECYA 132	*C. raciborskii*	Odivelas	24-07-2000	A
LMECYA 134	*C. raciborskii*	Odivelas	24-07-2000	B
LMECYA 135	*C. raciborskii*	Odivelas	24-07-2000	A
LMECYA 136	*M. aeruginosa*	Bravura	11-11-2000	B
LMECYA 137	*M. aeruginosa*	Bravura	11-11-2000	G
LMECYA 140	*Aph. flos-aquae*	Divor	17-07-2001	G
LMECYA 141	*Aph. flos-aquae*	Monte Novo	17-07-2001	A
LMECYA 142	*M. aeruginosa*	Montargil	23-07-2001	B
LMECYA 143A	*Aph. flos-aquae*	Montargil	23-07-2001	G
LMECYA 143B	*Aph. flos-aquae*	Montargil	23-07-2001	G
LMECYA 144	*M. aeruginosa*	Montargil	23-07-2001	G
LMECYA 145	*Limnothrix redekei* (*Oscillatoria* sp.)	Magos	11-09-2001	E
LMECYA 146	*Aph. flos-aquae*	Funcho	14-01-2002	G
LMECYA 147	*M. aeruginosa*	Funcho	30-01-2002	G
LMECYA 148	*Aph. gracile*	Patudos	21-05-2002	A
LMECYA 151	*M. aeruginosa*	Monte da Barca	10-09-2002	B
LMECYA 152	*Planktothrix agardhii*	Magos	07-01-2003	C
LMECYA 153	*P. agardhii*	Enxoé	12-02-2003	C
LMECYA 153A	*P. agardhii*	Enxoé	12-02-2003	H
LMECYA 153B	*P. agardhii*	Enxoé	12-02-2003	H
LMECYA 153C	*P. agardhii*	Enxoé	12-02-2003	H
LMECYA 153E	*P. agardhii*	Enxoé	12-02-2003	D
LMECYA 153F	*P. agardhii*	Enxoé	12-02-2003	C
LMECYA 156	*Synechococcus nidulans*	Serralves	May-2001	G
LMECYA 157	*M. aeruginosa*	Central Park - USA	September-2000	B
LMECYA 159	*M. aeruginosa*	Magos	08-07-2003	B
LMECYA 160	*M. aeruginosa*	Montargil	2001	G
LMECYA 161	*A. spiroides*	Agolada de Baixo	11-07-2003	A

Isolate	Final identification (morphological identification)	Origin	Isolation date	Note
LMECYA 162	*P. pseudoagardhii*	Guadiana River (Mértola)	31-07-2003	C
LMECYA 163	*Aph. issatschenkoi*	Magos	05-09-2003	A
LMECYA 164	*Aph. issatschenkoi*	Magos	05-09-2003	G
LMECYA 165	*A. flos-aquae*	Magos	05-09-2003	C
LMECYA 166	*Aph. issatschenkoi*	Vale Michões reservoir, military camp, Alcochete, Portugal	29-10-2003	A
LMECYA 167	*M. aeruginosa*	Corgas	20-10-2003	G
LMECYA 168	*C. raciborskii*	Odivelas	24-07-2000	G
LMECYA 169	*M. aeruginosa*	Évora (artisan puncture)	August-2003	G
LMECYA 170	*M. aeruginosa*	Magos	05-07-2003	B
LMECYA 171	*M. aeruginosa*	Guadiana River (Mértola)	31-07-2003	G
LMECYA 173	*Phormidium* sp. (*Pseudoanabaena* sp.)	Hydrothermal pond 60ºC (Coimbra)	2003	F
LMECYA 175	*A. circinalis*	Magos	21-05-2004	G
LMECYA 177	*A. planctonica*	Toulica	30-07-2004	A
LMECYA 178	*A. flos-aquae*	Guadiana River (Vitonogales - Spain)	July 1999	A
LMECYA 179	*M. aeruginosa*	Guadiana River (Vitonogales - Spain)	July 1999	G
LMECYA 180	*A. flos-aquae*	Guadiana River (Vitonogales - Spain)	July 1999	C
LMECYA 182	*Anabaena* sp. *(Aph. flos-aquae)*	Guadiana River (Vitonogales - Spain)	July 1999	E
LMECYA 183	*M. aeruginosa*	Guadiana River (Vitonogales - Spain)	2001	G
LMECYA 185	*Anabaena* sp. (*Aph. flos-aquae*)	Guadiana River (Vitonogales - Spain)	July 1999	E
LMECYA 187	*M. aeruginosa*	Guadiana River (Valdelacalzada - Spain)	June-2000	G
LMECYA 188	*M. aeruginosa*	Guadiana River (Vitonogales - Spain)	November-2000	B
LMECYA 190	*Aph. issatschenkoi*	Maranhão	10-09-2004	A
LMECYA 192	*M. aeruginosa*	Campo Grande Lake	19-04-2005	G
LMECYA 193	*M. aeruginosa*	Campo Grande Lake	19-04-2005	G
LMECYA 196	*Aph. gracile*	Enxoé	20-05-2005	G
LMECYA 197	*Aph. gracile*	Enxoé	20-05-2005	G
LMECYA 198	*P. agardhii*	Enxoé	19-05-2005	H
LMECYA 199	*P. agardhii*	Enxoé	19-05-2005	H
LMECYA 203	*P.rubescens*	Beliche	30-06-2005	A
LMECYA 205	*Anabaenopsis circularis*	Lagoa, Nafarros, Sintra	14-07-2005	G
LMECYA 206	*Anabaenopsis circularis*	Lagoa, Nafarros, Sintra	14-07-2005	G

Isolate	Final identification (morphological identification)	Origin	Isolation date	Note
LMECYA 207	*Anabaenopsis circularis*	Lagoa, Nafarros, Sintra	15-07-2005	G
LMECYA 211	*Aph. gracile*	Enxoé	23-07-2005	G
LMECYA 213	*A. circinalis*	Alvito	22-07-2005	B
LMECYA 214	*Phormidium* sp.	Enxoé	23-07-2005	A
LMECYA 238 (AQS)	*C. raciborskii*	Queensland - Australia	NA	G
Caia	*C. raciborskii*	Caia	NA	A
Marau1	*C. raciborskii*	Maranhão	NA	A
4799	*C. raciborskii*	Odivelas	NA	A
4899	*C. raciborskii*	Ardila	NA	G
LJ	*C. raciborskii*	Lake Julius - Australia	NA	G
MS1 (ILC-146)	*Aph. ovalisporum*	Lake Kinneret - Israel	NA	G

NA: Information not available.
A: Morphological identification confirmed by 16S rDNA sequencing.
B: As in A and also confirmed by *rpo*C1 gene sequencing.
C: Morphological identification at genus level and species assignment by 16S rDNA sequencing.
D: As in C and also confirmed by *rpo*C1 gene sequencing.
E: Identification obtained by 16S rDNA sequencing different from the morphological identification.
F: As in E and also confirmed by *rpo*C1 gene sequencing.
G: Morphological identification.
H: Morphological identification at genus level and species assignment by PCR fingerprinting techniques.

The final identification presented in Table 2.1 is a consequence of the different methodologies used to attain that purpose. Of the 124 set, 45.1% (56/124) of the isolates were sequenced, with the phylogenetic identification by 16S rDNA displaying a good congruence with the morphological identification and 13.7% (17/124) were also confirmed by *rpo*C1 gene sequencing. In 6.4% (8/124) of the cases an identification at species level was only possible after 16S rDNA sequencing and one of these cases was also confirmed by *rpo*C1 gene sequencing. In 5.6% (7/124) of the isolates the identification obtained by sequencing of the 16S rDNA gene was different from the previous morphological assignment and for three of these seven isolates the identification was also confirmed by *rpo*C1 gene sequencing. Some isolates (37.9% [47/124]) were only identified by morphological characters. The identification at species level was attained by PCR fingerprinting methods in 4.8% (6/124) of the isolates, whose morphological identification had been only possible at genus level.

Table 2.2 summarises the distribution of the isolates throughout the three cyanobacterial orders to which they belong. So far, members of the remaining three orders have not been isolated at the QHME-INSA facilities and thus are not present in this collection.

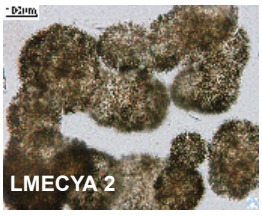
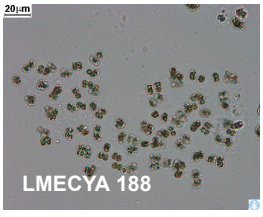
TABLE 2.2 Distribution of the 124 isolates under study through their respective orders and genera.

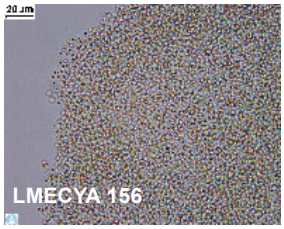
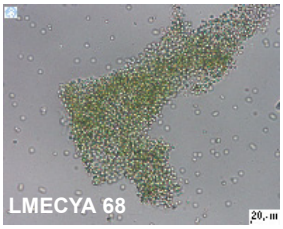
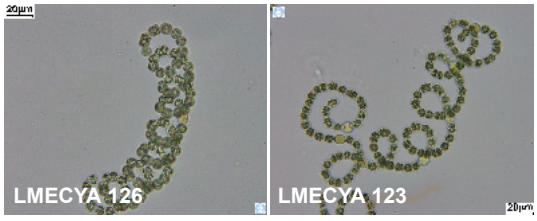
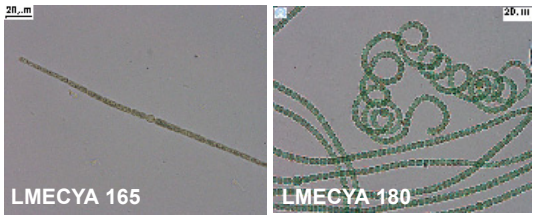
Order	Nº of isolates	Genera	Nº of isolates
Chroococcales	54	*Microcystis*	52
		Synechococcus	1
		Synechocystis	1
Oscillatoriales	15	*Limnothrix*	1
		Phormidium	3
		Planktothrix	11
Nostocales	55	*Anabaena*	14
		Anabaenopsis	3
		Aphanizomenon	27
		Cylindrospermopsis	11

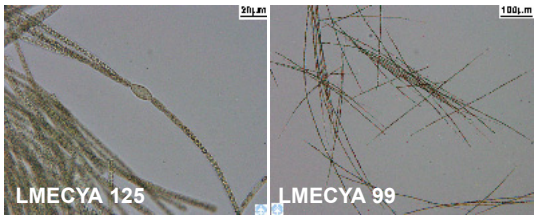
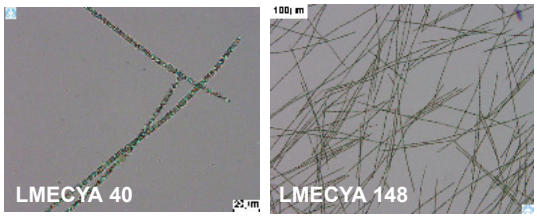
2.2 Species morphological characteristics

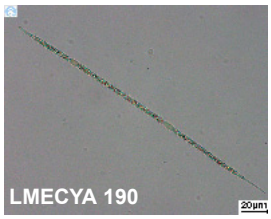
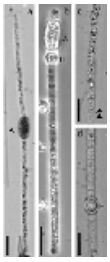
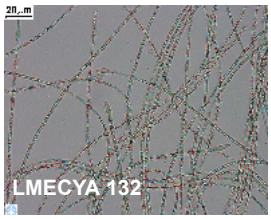
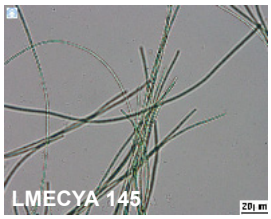
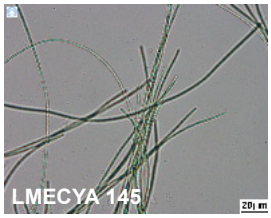
The morphological identification was performed by observation of the cells and filaments using an Olympus BX60 light microscope with a digital camera. The following parameters were analysed to describe the morphology of the studied strains: length and width of vegetative cells, heterocysts and akinetes; morphology of terminal cell; distance between heterocysts and distance between a heterocyst and the nearest akinete; presence or absence of terminal heterocysts, gas vesicles and sheath; shape of filament and its aggregation in colonies [1-7]. The description of the morphological characteristics and illustrations of the species/genera identified in this study are summarised in Table 2.3. All the morphological identification of the isolates was performed by Sérgio Paulino (also responsible for the cultures maintenance) and Natália Faria, from the QHME-INSA.

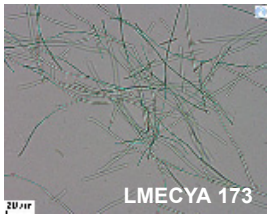
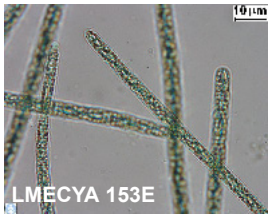
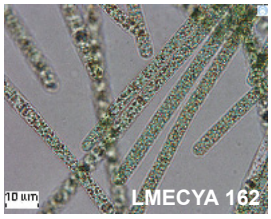
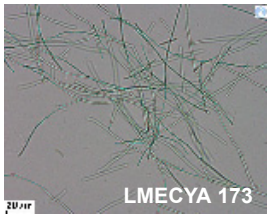
TABLE 2.3 Morphological characteristics of the cyanobacterial species, used to perform their identification.

Species representative photograph [a]	Description
Microcystis aeruginosa (Kützing) Kützing, 1846	
 	Colony forming with spherical, ovate or irregularly lobed cells. Saccate and clathrate colony of numerous spherical cells sparsely distributed within. In some gas vacuoles the cell has a bluish-green colour, granular and conspicuous pseudovacuoles may be present. Colourless mucilage sometimes forms a distinct, narrow margin around the cells. Cell diameter is 4-6 (9.4)* µm. [b]

Species representative photograph [a]	Description
Synechococcus nidulans (Pringsheim 1968) Komárek In Bourrelly, 1970 	Cells solitary, free-floating, short oval to rod-shaped, without mucilage, with more or less homogeneous, pale blue-green content, straight or sometimes slightly sigmoid or arcuate. Filamentous involution cells up to 35 μm long often arise (particularly in cultures). Cell diameter is 1.5-8.5 x 0.4-1.3 (2.2)* μm. [c]
Synechocystis Sauvageau, 1892 	Globular cells, with or without mucilage. Cell diameter ranges from 2.2 to 29 μm. [c]
Anabaena Bory ex Bornet and Flahault, 1888 	Planktonic or attached to most substrates. Trichomes solitary or aggregated in floccose masses or united in a thin mucilaginous stratum; straight, flexous, circinate, spirally coiled or irregularly twisted; metameric in structure; uniformly broad or slightly attenuated; gas vacuoles present in some taxa, absent in others; mucilaginous sheath sometimes present, usually inconspicuous. Vegetative cells spherical, sub-spherical, ellipsoidal, barrel-shaped, quadate or cylindrical; homogeneous or granulose contents, with or without gas vacuoles. Heterocysts intercalary; spherical to cylindrical in shape. Akinetes spherical to cylindrical in shape, adjacent to or developing distant from the heterocysts (para-heterocytic), solitary or in catenate series. [d]
Anabaena circinalis Rabenhorst ex Bornet and Flahault, 1888 	Planktic. Trichomes solitary, widely spirally coiled, 70-120 μm wide, sometimes forming small bundles or several trichomes joined together. The trichomes are mostly without mucilage, or have a very narrow diffluent sheath. Cells barrel-shaped to spherical, 7-9 μm long, 7-8-11 μm wide, with gas vacuoles and blue-green in colour. Heterocysts spherical, as wide as the vegetative cells. Akinetes are elongate oval to short cylindrical, sometimes conical at the ends, 20-28 x 15-21 μm. [b]
Anabaena flos-aquae (Lyngbye) Brébisson In Brébisson and Godey ex Bornet and Flahault, 1888 	Planktic. Trichomes irregularly twisted, spirals of different widths in clusters without mucilage. Cells round, 3.5-7 μm; heterocysts slightly oval, 5-6 μm in diameter, with gas vacuoles; akinetes oval to cylindrical, 2-3 in a row, distant from heterocysts, 15-35 μm long. [b]

Species representative photograph [a]	Description
Anabaena planctonica Brunnthaler,1903 	Planktic. Trichomes solitary, straight, surrounded by a wide mucilaginous sheath. Cells are spherical to barrel-shaped, often shorter than wide, 9-15 μm broad, up to 10 μm long with gas vacuoles. Heterocysts spherical, as wide as the vegetative cells. Akinetes oval to cylindrical, with broadly rounded to bluntly conical ends, sometimes hexagonal, 10-20 μm wide and 15-30 μm long. [b]
Anabaena spiroides Klebahn, 1895 	Planktic. Trichomes solitary, approximately screw-like twisted surrounded by massive mucilage. Cells are barrel-shaped to spherical, 6.5-8 μm wide, with gas vacuoles. Heterocysts round, 6.5-10 μm in diameter. Akinetes widely oval, sometimes slightly curved, distant from heterocysts, solitary or in pairs, 17-21 x 10-14 μm. [b]
Anabaenopsis circularis (West 1909) Miller, 1923 	Planktic. Trichomes spirally twisted, number of spirals per trichome about 1-2 and 4.5-6 μm wide. Cells cylindrical to barrel-shaped, with gas vacuoles, 6.2-10.2 x 3.1-4.3 μm. Terminal heterocysts are spherical to oval, 4.7-6.3 x 4.7-5.9 μm. Akinetes are ellipsoidal, 10-14 x 6.2 μm. [b]
Aphanizomenon flos-aquae (L.) Ralfs ex Bornet and Flahault, 1888 	Planktic. Trichomes in parallel rows, forming macroscopic flakes, 2.5-9 mm, grass-blade or sickle-shaped (on the right). Filaments long, up to 1 mm, straight with cylindrical cells, slightly constricted at cross walls. The end cells are long with plasmatic threads extending throughout the cell. Cells in the middle of the trichomes are 5-7 μm wide and 5-14 μm long. End cells are long, 10-19 μm, and of nearly the same width as the middle cells. Heterocysts cylindrical, 10-14(17)* x 5-6.4 μm, intercalar, 1-2 per trichome, and distant from the akinetes. Long, cylindrical akinetes, 54-82 x 7-10 μm. [b]
Aphanizomenon gracile (Lemmermann 1898) Lemmermann, 1907 	Planktic. Trichomes with elongated cells, slightly constricted at cross walls, with gas vacuoles, 2.6-3 μm wide, 3-6 μm long. Heterocysts oval 4-8 μm long and 3-5 μm wide. Akinetes long cylindrical with rounded ends, distant from the heterocysts, 4-6 μm wide and 8-15 μm long. End cells slightly narrower. [b]

Species representative photograph [a]	Description
Aphanizomenon issatschenkoi (Usacev) Proskina-Lavrenko, 1968 	Planktic. Trichomes with cylindrical cells, not or only slightly constricted at cross walls. Cells 3-4 μm wide, 6-9(15.9)* μm long, pale blue-green to gray-blue. End cells colourless, thin, pointed. Heterocysts cylindrical to oval, 1(2-3)* per trichome, 7-11 x 3-6 μm. Akinetes cylindrical, 1-3 in a row and distant from heterocysts, 9-19 x 4-7 μm. [b]
Aphanizomenon ovalisporum Forti In Huber-Pestalozzi, 1938 [e] 	Trichomes solitary 60–700 μm long with a low incidence of terminal hyaline cells, narrowed slightly toward the ends and had gas vesicle containing cells 2.5–6.9 μm long, 2.4–5.1 μm wide. Heterocysts spherical or ellipsoidal 4.4–10.5 μm long, 4.8–8.2 μm wide. Heterocysts usually located in the middle of the trichome, but also observed near the tip, and connected to neighbour cells by small bridges. Akinetes 16.0–27.8 μm long, 6.0–15.9 μm wide located in the middle of the trichomes. [e]
Cylindrospermopsis raciborskii (Woloszynska 1912) Seenayya and Subba-Raju, 1972 	Planktic. Trichomes solitary straight or slightly curved trichomes with lengths up to 200 μm. Cells are cylindrical, slightly constricted at the cross walls, 2.5-4 μm wide and 2.5-16 μm long. The terminal heterocysts are long and conical with dimensions 5-6 x 2-2.5 μm. Akinetes are elongated oval and situated adjacent to the hetetocyst or the terminal vegetative cell, 2.8-3.3 μm wide and 4.5-11(16) μm long. [b]
Limnothrix redekei (Van Goor 1918) Meffert emend. Suda *et al.*, 2002 	Planktic. Trichome mass is brownish-green to reddish-brown under normal daylight, but the trichomes are gray-blue in colour. Trichomes are solitary, straight or slightly bent, cross walls not constricted and not granulated. Apical cells are rounded, not capitate and have no calyptra. The cells are long cylindrical, containing two large gas vacuoles distributed at the poles of the cells and occasionally in the centre of the cells. The cell width is 1.2-2.5(3.5) μm and length of 6-14 μm and the ratio of cell length to width is about 3:1-7:1. [b, f]
Oscillatoria Vaucher ex Gomont, 1892 	Filamentous; filaments simple, never branched, usually in fine, smooth, layered (but not leathery) strata (mats), microscopic or (later) macroscopic up to several cm in diameter, rarely solitary or in small groups, without sheaths, only in suboptimal (extreme) conditions (or sometimes in cultures) enveloped by thin, colourless, firm sheaths, opened at the apex. Trichomes isopolar! Straight or slightly waved, usually wider than 8 μm (up over 60 μm wide), uniserial, composed of shortly cylindrical or barrel-like (discoid) cells (always shorter than wide, usually several times), constricted or unconstricted at the cross walls, not attenuated or shortly attenuated to the ends, motile under convenient environmental conditions (waving, trembling, oscillation). Cells without aerotopes but with fine granulation (sometimes prominent at the cross walls) or with solitary granules, blue-green, brownish or pinkish, with coiled thylakoids, situated usually irregularly throughout the whole cell content. End cells widely rounded, sometimes capitate or with narrow calyptra. False branching, heterocysts and akinetes absent.[g]

Species representative photograph [a]	Description
Phormidium Kützing ex Gomont, 1892 	Mostly benthic, trichomes cylindrical forming a mat like thallus. Straight undulated or irregularly coiled, not constricted or slightly constricted at the cross-walls. 1-12 μm broad, false branching lacking, capable of forming firm and unlamellated sheath adherent to the trichomes, but dependent on environmental conditions. Motile by gliding, creeping or oscillating motion. Cells more or less iso-diametric or shorter or longer than broad. Without gas vacuoles.[h]
Planktothrix agardhii (Gomont 1892) Anagnostidis and Komárek emend. Suda *et al*., 2002 	Trichomes pale blue-green or yellow-green, solitary, straight or slightly curved, usually attenuated towards the ends and not surrounded by a sheath under conditions favourable for growth. Under unfavourable conditions (e.g. nutrient depletion, heavy contamination by heterotrophic bacteria), trichomes may be surrounded by a gelatinous sheath. Slightly constricted or not constricted at cross walls and not granulated. Gas vacuoles are relatively large and scattered at the periphery of the cells. Apical cells show variable shapes: rounded, tapered, bluntly conical, occasionally capitate, sometimes with a calyptra. Cell width is 2.3-9.38 μm, cell length is 1.3-5.1 μm and the ratio of cell length to width is about 1:3-1:1. [b, f]
Planktothrix pseudoagardhii Suda and Watanabe 2002 In Suda *et al*., 2002 	Trichomes are blue-green or yellow-green, solitary and float. Straight or slightly curved, sometimes attenuated towards the ends and not surrounded by sheath under conditions favourable for growth. Cross walls may be slightly constricted or not constricted and not granulated. Gas vesicles are relatively large and scattered at the periphery of the cells. Apical cells show variable shapes such as rounded, tapered, bluntly conical, occasionally capitate, with and without calyptra. Cell dimensions are 3.0-6.4 μm wide, 1.2-4.2 μm long and the ratio of cell length to width is about 1:3-1:1. [f]
Planktothrix rubescens (D.C. ex Gomont 1892) Anagnostidis and Komárek emend. Suda *et al*., 2002 	Trichomes are reddish purple or reddish brown, straight or slightly curved and attenuated or not towards the ends. Trichomes are solitary and float. Cross walls are slightly constricted or not constricted and not granulated. Gas vacuoles are relatively large and scattered at the periphery of the cells. Apical cells show variable shapes: rounded, tapered, bluntly conical, rarely capitate, with or without calyptra. The cell width is 3.9-9.4 μm, cell length is 1.1-4.9 μm and the ratio of cell length to width is about 1:3-1:1. [f]
Pseudoanabaena Lauterborn, 1915 	Filamentous; trichomes solitary or agglomerated in very fine, mucilaginous mats, straight or slightly waved or arcuated, simple, usually; not very long, without any branching, 0.8-3 μm wide, composed from cylindrical cells, usually with slight constrictions at the distinct cross walls; only in young trichomes are the cross walls thin and unclear, without firm sheaths, sometimes with fine, colourless, diffluent, narrow mucilagenous envelopes (staining); ends of trichomes not attenuated, but the apical cell is sometimes conical; indistinct and facultative motility (trembling). Cells cylindrical, always longer than wide, without aerotopes, but sometimes with solitary granules, or with gas vesicles agglomerated into aerotopes, localized in the ends of cells (in polar positions), thylakoids localized parallely, peripherally; special pore patterns on the cell walls; end cell cylindrical and rounded at the end, or more or less conical up to bluntly or sharply pointed.[g]

a - the photographs were take off and provided by Natália Faria (QHME-INSA).
* - values in parentheses refer to the maximum value measured for that feature.
b - according to [1]; c - according to [2]; d - according to [3]; e - according to [4]; f - according to [5]; g - according to [6]; h - according to [7].

The distribution of the number of the 110 portuguese isolates through their origin is represented in Figure 2.1. As can be observed, most of the isolates came from the South of the Tagus River (Rio Tejo). This is a consequence of the implementation of regular monitoring programs for cyanobacteria and cyanotoxins in freshwater environments, in order to minimize potential health risks to animal and human population resulting from exposure through drinking and recreational activities. The major customers that utilize the services supplied by the QHME-INSA are from the South of the Tagus River, where blooms frequently occur.

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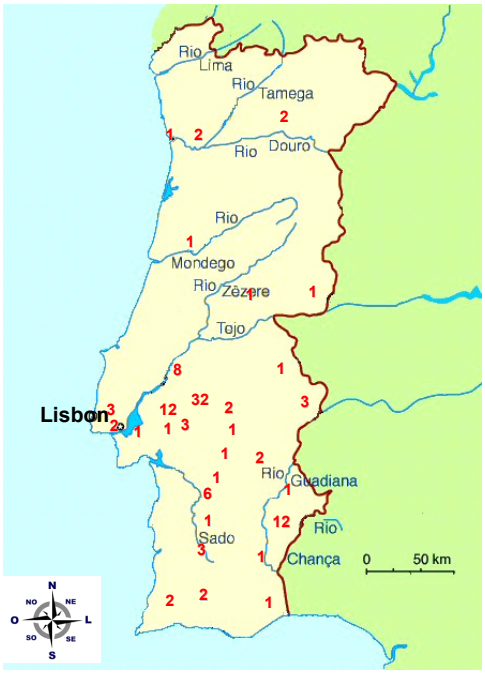

FIGURE 2.1 Map of continental Portugal and location of the isolates origin and respective number recovered from each location.

The isolates distributed themselves in a very disperse way throughout the species under study as can be seen in Figure 2.2. In fact, they correspond to (in decreasing order): 41.94% *Microcystis aeruginosa*; 10.48% *Aphanizomenon flos-aquae*; 8.87% *Cylindrospermopsis raciborskii*; 7.26% *Planktothrix agardhii*; 6.45% *Aph. gracile*; 5.65% *Anabaena circinalis*; 4.03% *Aph. issatschenkoi*; 2.42% *A. flos-aquae* sp.; 2.42% *Anabaenopsis circularis*; 2.42% *Phormidium* sp.; 1.61% *Anabaena* sp.; 0.81% *A. planctonica*; 0.81% *A. spiroides*; 0.81% *Aph. ovalisporum*; 0.81% *Limnothrix redekei*; 0.81% *P. pseudoagardhii*; 0.81% *P. rubescens*; 0.81% *Synechococcus nidulans*, and 0.81% *Synechocystis* sp. It can be verified that about half of the isolates belong to *M. aeruginosa* and *Aph. flos-aquae*, which reflects their predominant appearance in the Portuguese freshwater blooms.

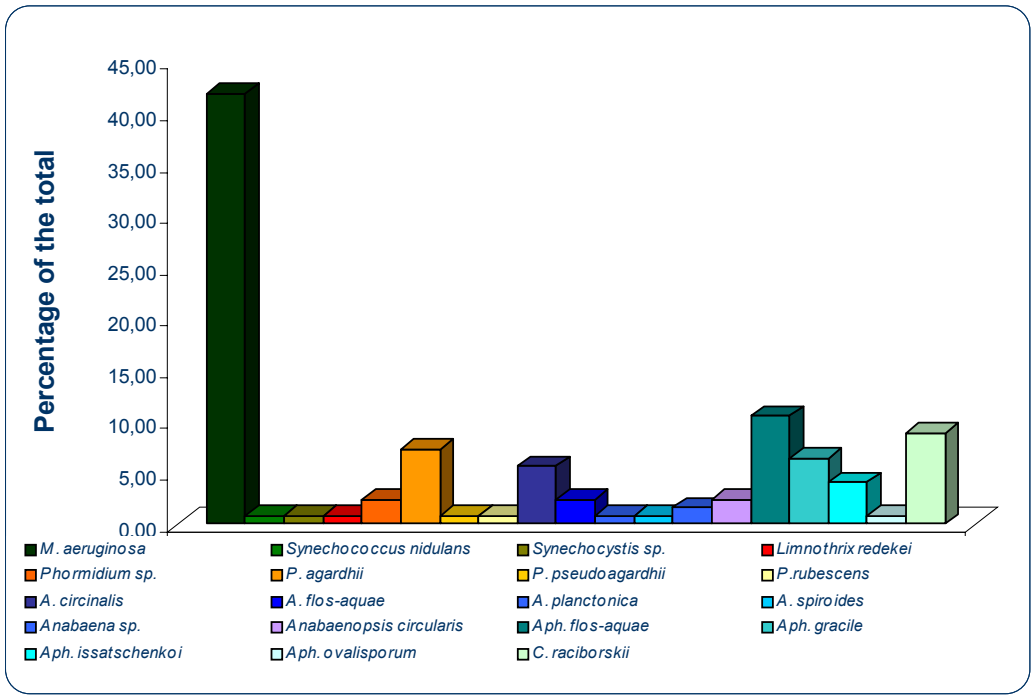

FIGURE 2.2 Distribution of the 124 isolates through their corresponding species (final identification).

2.3 Obtention of cellular biomass

The unicyanobacterial cultures (in non-axenic state) were obtained by repetitive washing of cyanobacterial trichomes in successive drops of sterilized Z8 medium (Table 2.4, [8]). A single filament or colony was transferred to a 50 mL capacity flask containing 15 mL of Z8 medium, as depicted in Figure 2.3A. Stock cultures were maintained at 20 ± 1ºC with a light intensity of 20 ± 4 μmol m^{-2} s^{-1} in a 14:10 h light:dark cycle. For biomass production, stock cultures were transferred to round 6 L glass flasks containing 5 L of Z8 medium, as shown in Figure 2.3B. Mass cultures were maintained with a constant aeration under the light and temperature conditions described above. Lyofilised samples of the isolates for DNA and toxins extraction were obtained as described by Saker *et al.* [9], and illustrated in Figure 2.4. This work has been mostly supported by the QHME-INSA staff.

FIGURE 2.3 A) Cyanobacteria stock culture flasks of the LMECYA collection. B) Propagation of the cyanobacterial cultures in higher culture volume for cellular biomass production.

TABLE 2.4 Formulation of Z8 medium [8].

	Reagent	Quantity
Z8 medium	Solution A	10 mL
	Solution B	10 mL
	Fe-EDTA solution	10 mL
	Gaffron micronutrients	1 mL
	Deionized water to	1L
Stock solution		
Solution A	$MgSO_4.7H_2O$	0.250 g
	$NaNO_3$	0.467 g
	$Ca(NO_3)_2.4H_2O$	0.059 g
Solution B	K_2HPO_4	0.031 g
	Na_2CO_3	0.020 g
Fe-EDTA solution	Add 10 mL solution C and 9.5 mL solution D plus water to 1 L	
Solution C	2.8 g $FeCl_3.6H_2O$ dissolved in 100 mL of HCl 0.1 M	
Solution D	3.9 g EDTA-Na_2 dissolved in 100 mL of NaOH 0.1 M	
Gaffron micronutrients:	$Na_2WO_4.2H_2O$	0.0033 g
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.00880 g
	KBr	0.01200 g
	KI	0.00830 g
	$ZnSO_4.7H_2O$	0.02870 g
	$Cd(NO_3).4H_2O$	0.01550 g
	$Co(NO_3).6H_2O$	0.01460 g
	$CuSO_4.5H_2O$	0.01250 g
	$NiSO(NH_4)SO_4.6H_2O$	0.01980 g
	$Cr(NO_3)_3.9H_2O$	0.04100 g
	V_2O_5	0.00089 g
	$Al_2(SO_4)_3K_2SO_4.24H_2O$	0.04740 g
	H_3BO_3	0.31000 g
	$MnSO_4$	0.22300 g
	Deionized water to	1 L

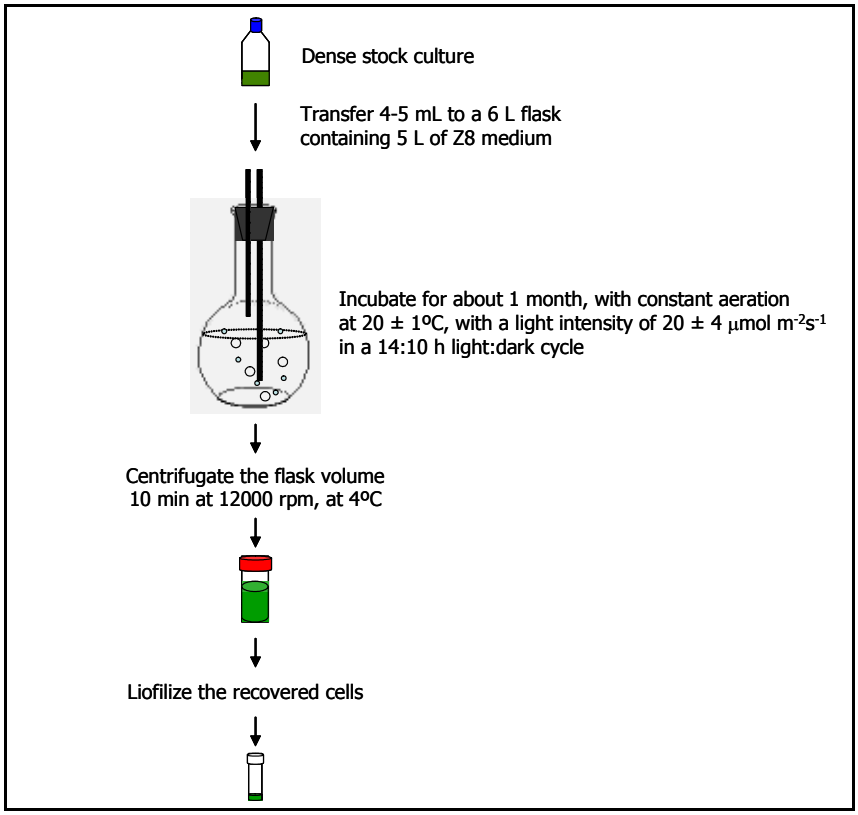

FIGURE 2.4 Scheme of the steps needed to obtain the cyanobacterial biomass for toxin and DNA extraction.

2.4 DNA extraction

The application of most molecular biology techniques is based on a good yield of quality nucleic acids (DNA and/or RNA) from the cultures and/or the samples under study. The extraction yield and DNA fragment size should be appropriate and the extract recovered should be pure enough to allow its use in applications as enzymatic digestion and amplification. In terms of routine monitoring procedures, the extraction protocol should also be simple and appropriate to be quickly applied to cultures presenting the most diverse morphologic characteristics.

The major challenge for DNA isolation of good quality and quantity from cyanobacteria lies in breaking the cell walls, as they are often resistant to traditional DNA extraction procedures. The cellular wall of cyanobacteria is identical to the Gram-negative bacteria but, in some species, the peptidoglican layer may be considerably thicker. This thickness varies between 1-10 nm, being able to reach 20 nm in *Oscillatoria princeps* [10]. Besides, some of these organisms possess additional surface structures (e.g. S-layer, pili or fimbrae, slime, capsule or sheath) [11-14]. These characteristics may be a problem to obtain an effective lysis

of the cyanobacteria, and so reducing the efficiency of recovery of the nucleic acids, since a successful wall lysis is a critical step in the final efficiency.

There are some reports of nucleic extraction methods for cyanobacteria, that include enzymatic digestion using lysozyme [15, 16], addition of detergents (SDS, sarkosyl) [15], mechanical lysis with freeze/defrosting [17] or using glass beads [18]. However, most of these procedures use toxic solvents (e.g. phenol), are exhaustive and time consuming or must be adapted according to the species and/or genera used.

The diverse morphologic characteristics of the cyanobacteria can determine that distinct conditions to reach an excellent lysis would be needed, and so when designing an efficient and simple method to obtain quality nucleic acids, it is best to combine different methods, such as mechanical lysis in the presence of a detergent, such as SDS or sarkosyl.

After the nucleic acids extraction, the next step is their purification. In the case of cyanobacteria, several compounds are concomitantly extracted, for instance polysacharides, polyphenols, or other substances, which are difficult to remove and interfere in the subsequent enzymatic utilisation of the nucleic acids [15]. The application of the mixture phenol/chloroform/isoamilic alcohol (25:24:1) [16, 19] is the method commonly used to remove these contaminants, however this procedure generates toxic waste, creating disposal problems.

At the beginning of this study, the extraction of the nucleic acids was performed using an aliquot of 10 mL of fresh culture, recovered by centrifugation, but the DNA final yield using this procedure was very low. Subsequently, instead of fresh culture, the use of lyophilised biomass was tested, which provided an increase of the final yield. It was also detected that some strains produced nucleases that were released after the extraction and that degraded the extracted DNA. Therefore, an additional step with proteinase K was included to eliminate these nucleases and prevent the DNA degradation.

Procedure for genomic DNA extraction from cyanobacteria

Genomic DNA of cyanobacterial strains was extracted following the method described by Pitcher *et al.* [20], with some modifications, as described by Valério *et al.* [21]. An aliquot of 100 μL of lyophilised culture was suspended in 500 μL of lysis buffer containing 50 mM Tris, 250 mM NaCl, 50 mM EDTA, 0.3% SDS, pH 8, and mechanically broken with glass beads (400-600 μm) by vortex shaking for 2 min. The suspension was incubated for 1 h at 65ºC. After new vortex shaking for 2 min, 1000 μL of GES (5 M guanidium thiocyanate, 100 mM EDTA, 0.5% (v/v) sarkosyl) were added and the suspension was incubated at 4ºC overnight.

After addition of 250 μL of 10 M ammonium acetate and 10 min incubation on ice, nucleic acids were extracted with chloroform:isoamilic alcohol (24:1) and precipitated with one volume of isopropanol. After centrifugation, DNA was washed with ethanol 70% (v/v), ressuspended in TE with 1 mg proteinase K and incubated for 2 h at 50ºC. A second extraction with chloroform:isoamilic alcohol (24:1) was performed and DNA was precipitated with 1/10 volume of sodium acetate 3 M, pH 5.2 and 2.5 volumes of ethanol, centrifuged, washed with ethanol 70% (v/v) and ressuspended in 100 μL of TE.

An amount of 2 μL of the ressuspended nucleic acids was used to check the performance of the extraction by 0.8% agarose gel electrophoresis, as presented in Figure 2.5, generally revealing good extraction of the genomic DNA. All DNA extracts were stored at 4ºC.

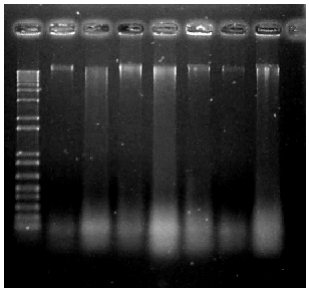

FIGURE 2.5 Agarose gel (0.8%, w/v) electrophoresis (90 V for 1 h) of DNA extracts from some cyanobacteria.

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3

Molecular approaches in identification, differentiation and traceability

This chapter is presented in the following paper:

"Potentiality of molecular methods for cyanobacteria identification, differentiation and traceability", Elisabete Valério, Lélia Chambel, Sérgio Paulino, Natália Faria, Paulo Pereira, Rogério Tenreiro. *Microbiology* (submitted).

Potentiality of molecular methods for cyanobacteria identification, differentiation and traceability

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ABSTRACT

Molecular techniques were applied using DNA from 118 cyanobacterial isolates representative of three orders of cyanobacteria: Chroococcales (54), Oscillatoriales (15) and Nostocales (49), mostly sourced from Portuguese freshwater reservoirs.

The aim of this study was to assess the potential of several molecular targets for identification, differentiation and traceability of cyanobacteria in freshwater reservoirs. The isolates were identified by morphological methods and subsequently characterized by composite hierarchical cluster analysis of STRR and LTRR (short and long tandemly repeated repetitive sequences) PCR fingerprinting profiles for identification purposes. Representative isolates were selected from each cluster and their identification was obtained or confirmed by its phylogenetic positioning using 16S rDNA and *rpoC1* phylogenies. Composite analysis of hierarchical clustering of M13 and ERIC PCR fingerprints revealed to be suitable for strain differentiation and traceability within a reservoir. Species variability was assessed based on their distinctiveness (Simpson Index) and evenness (Shannon-Wiener Index) and a high diversity was observed in all species, having *Planktothrix agardhii* the lowest diversity whereas *Aphanizomenon flos-aquae* presents the highest diversity. The hierarchical clustering of M13 and ERIC fingerprints points out to its possible use in cyanobacterial monitoring, as quality management control.

A diagnostic key based on 16S PCR-RFLPs, ITS amplification and ITS-ARDRA for identification purposes is also presented.

Keywords: Cyanobacteria, identification, diagnostic key, differentiation, molecular methods, traceability

INTRODUCTION

Cyanobacteria are a morphologically diverse group of bacteria ranging from unicellular, colonial and filamentous forms. The latest taxonomic reclassification of cyanobacteria separated them in six orders (Cavalier-Smith, 2002): Gloeobacterales, Chroococcales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematales.

Traditionally, the classification of cyanobacteria has been based on morphological characters such as trichome width, cell size, division planes, shape and arrangement, pigmentation and the presence of characters such as gas vacuoles and a sheath. Beyond the considerable expertise required to identify species by such characters, the subjective judgment by operators can lead to errors, resulting in incorrect assignment of isolates. Komárek & Anagnostidis (1989) have estimated that more than 50% of the strains in culture collections are misidentified. Moreover, some diagnosticant features, such as gas vacuoles or akynetes, can present variations with different environmental or growth conditions and even be lost during cultivation (Rudi *et al*., 1997; Lyra *et al.*, 2001). Such limitations of phenotypic characters highlighted the requirement of more reliable methods and promoted molecular approaches in cyanobacterial taxonomy, including DNA base composition, DNA and RNA hybridizations, genes sequencing, and PCR fingerprinting. As axenic cultures are difficult to obtain, cyanobacterial specific methods not requiring them revealed to be of utmost importance.

Repetitive sequences constitute an important part of the prokaryotic genome. Regardless of their unknown function, and how these are maintained and dispersed, their presence, widespread distribution and high conservation make them methodologically important for DNA fingerprinting and allow their use as an alternative for the identification of species or strains and diversity studies. In the particular case of cyanobacteria, a family of repetitive sequences, the short tandemly repetitive repeats (STRR) sequences, has been described (Mazel *et al*., 1990). These heptanucleotide sequences have been identified in several cyanobacterial genera and species, so far mostly in heterocystous cyanobacteria (Rasmussen & Svenning, 1998; Zheng *et al.*, 1999; Nilsson *et al.*, 2000; Wilson *et al.*, 2000; Teaumroong *et al.*, 2002; Lyra *et al.*, 2005; Prasanna *et al.*, 2006), but also in some non-heterocystous ones (Rasmussen & Svenning, 1998). Furthermore, a 37 bp long tandemly repetitive repeats (LTRR) sequence has also been identified in some cyanobacterial species (Maselpohl *et al*., 1996; Rasmussen & Svenning, 1998; Prasanna *et al.*, 2006). Analysis of STRRs and LTRRs has been described as powerful tools for taxonomic studies (Mazel *et al*., 1990). Moreover, the specificity of these sequences has made the STRR useful even for non-axenic cyanobacterial cultures (Nilsson *et al.*, 2000).

A universal marker for DNA fingerprinting is the oligonucleotide csM13. It has been already tested in a small number of cyanobacteria (Valério *et al.*, 2005), and demonstrated ability to differentiate strains at intra-specific level. On the other hand, techniques based on enterobacterial repetitive intergenic consensus (ERIC) have also been used for identification

and differentiation purposes in some cyanobacteria (Rasmussen & Svenning, 1998; Lyra *et al.*, 2001; Valério *et al.*, 2005; Bruno *et al.*, 2006).

The restriction fragment length polymorphisms (RFLPs) of particular PCR products can provide signature profiles specific to the genus, species, or even strains. Genetic characterization of cyanobacterial strains has been undertaken using restriction fragment length polymorphisms of the 16S rRNA gene (16S PCR-RFLPs) (Lyra *et al.*, 1997) and of the intergenic transcribed spacer region (ITS-ARDRA) (Lu *et al.*, 1997; West & Adams, 1997). Furthermore, the amplification of the 16S-23S rRNA internal transcribed spacer (ITS) that have shown to be polymorphic in length (Rocap *et al.*, 2002; Iteman *et al.*, 2002; Neilan, 2002, Laloui *et al.*, 2002), and number (West & Adams, 1997; Iteman *et al.*, 2002) in cyanobacteria, can also be used has an identification tool.

A sequential polyphasic approach was used in this study. The isolates were identified by observation of their morphological features. A hierarchical analysis with STRR and LTRR PCR fingerprinting patterns was performed and representatives of the clusters obtained were identified by a phylogenetic analysis carried out using two genes, one coding for the small subunit rDNA (16S rRNA gene) and the other for the DNA-dependent RNA polymerase subunit (*rpoC1*). Subsequent characterization of all isolates by M13 and ERIC fingerprints allowed the differentiation of strains, revealing also the traceability potential of these last methods for routine freshwaters monitoring. Furthermore, a diagnostic key was constructed for the identification of cyanobacterial species, based on the use of 16S PCR-RFLPs, ITS dimension and ITS-ARDRA.

METHODS

Cyanobacterial strains

A total of 118 unicyanobacterial non-axenic cultures, belonging to the orders Chroococcales (54), Oscillatoriales (15) and Nostocales (49), mainly isolated from Portuguese freshwater blooms and maintained in the LMECYA (Cyanobacteria Culture Collection Estela Sousa e Silva - INSA) culture collection using Z8 medium (Skulberg & Skulberg, 1990), have been used in this study (Table 1). The isolates are kept under a 14:10 h light:dark cycle (20 ± 4 μmol m^{-2} s^{-1}) at 20 ± 1 ºC. Lyophilized samples of the isolates were obtained as described by Saker *et al*. (2003).

TABLE 1 Cyanobacterial strains used in this study and GenBank accession numbers for 16S rDNA and *rpoC1* sequences.

Taxon and strain designation	Origin	Collection date	GenBank accession no.	
			16S rDNA	*rpoC1*
CHROOCOCCALES				
Microcystis aeruginosa LMECYA 1	Montargil	10-06-1996	EU078482	
M. aeruginosa LMECYA 2	Torrão	05-06-1996	EU078483	EU078443
M. aeruginosa LMECYA 3	Montargil	10-05-1996	EU078484	
M. aeruginosa LMECYA 7	Montargil	27-06-1996	EU078485	
M. aeruginosa LMECYA 8	Monte da Rocha	25-09-1996	EU078486	EU078444
M. aeruginosa LMECYA 12	Monte da Rocha	25-09-1996	EU078487	
M. aeruginosa LMECYA 13	Montargil	11-07-1996	EU078488	
M. aeruginosa LMECYA 23	Magos	22-10-1996		
M. aeruginosa LMECYA 29	Monte da Rocha	25-09-1996	EU078489	
M. aeruginosa LMECYA 50	Roxo	03-07-1997	EU078490	
M. aeruginosa LMECYA 53	Patudos	13-08-1997		
M. aeruginosa LMECYA 55	Patudos	13-08-1997		
M. aeruginosa LMECYA 56	Patudos	13-08-1997	EU078491	
M. aeruginosa LMECYA 57	Patudos	13-08-1997		
M. aeruginosa LMECYA 59	Patudos	13-08-1997	EU078492	
M. aeruginosa LMECYA 81	Magos	24-06-1998	EU078493	EU078454
M. aeruginosa LMECYA 82	Agolada de Baixo	09-07-1998	EU078494	EU078455
M. aeruginosa LMECYA 85	Agolada de Baixo	15-07-1998	EU078495	
M. aeruginosa LMECYA 87	Magos	16-07-1998	EU078496	
M. aeruginosa LMECYA 91B	Montargil	13-11-1999	EU078497	EU078457
M. aeruginosa LMECYA 92A	Montargil	13-11-1999		
M. aeruginosa LMECYA 92B	Montargil	13-11-1999		
M. aeruginosa LMECYA 92C	Montargil	13-11-1999		
M. aeruginosa LMECYA 93	Montargil	13-11-1999		
M. aeruginosa LMECYA 97	Montargil	28-11-1999		
M. aeruginosa LMECYA 103	Montargil	18-11-1999		
M. aeruginosa LMECYA 106	Patudos	15-12-1999	EU078498	
M. aeruginosa LMECYA 107	Caia	12-11-1999		
M. aeruginosa LMECYA 108	Caia	12-11-1999		
M. aeruginosa LMECYA 110	Montargil	11-03-2000	EU078499	EU078458
M. aeruginosa LMECYA 113	Montargil	28-10-2000		
M. aeruginosa LMECYA 127	Montargil	27-11-2000		
M. aeruginosa LMECYA 128	Montargil	27-11-2000		
M. aeruginosa LMECYA 136	Bravura	11-11-2000	EU078500	EU078462
M. aeruginosa LMECYA 137	Bravura	11-11-2000		
M. aeruginosa LMECYA 142	Montargil	23-07-2001	EU078501	EU078463
M. aeruginosa LMECYA 144	Montargil	23-07-2001		
M. aeruginosa LMECYA 147	Funcho	30-01-2002		
M. aeruginosa LMECYA 151	Monte da Barca	10-09-2002	EU078502	EU078466
M. aeruginosa LMECYA 157	Central Park - USA	September-2000	EU078503	EU078468
M. aeruginosa LMECYA 159	Magos	08-07-2003	EU078504	EU078469
M. aeruginosa LMECYA 160	Montargil	2001		
M. aeruginosa LMECYA 167	Corgas	20-10-2003		
M. aeruginosa LMECYA 169	Évora (artisan puncture)	August-03		
M. aeruginosa LMECYA 170	Magos	05-07-2003	EU078505	EU078472

Taxon and strain designation	Origin	Collection date	GenBank accession no.	
			16S rDNA	*rpoC1*
M. aeruginosa LMECYA 171	Guadiana River (Mértola)	31-07-2003		
M. aeruginosa LMECYA 179	Guadiana River (Vitonogales - Spain)	July 1999		
M. aeruginosa LMECYA 183	Guadiana River (Vitonogales - Spain)	2001		
M. aeruginosa LMECYA 187	Guadiana River (Valdelacalzada - Spain)	June-2000		
M. aeruginosa LMECYA 188	Guadiana River (Vitonogales - Spain)	November-2000	EU078506	EU078476
M. aeruginosa LMECYA 192	Campo Grande Lake	19-04-2005		
M. aeruginosa LMECYA 193	Campo Grande Lake	19-04-2005		
Synechococcus nidulans LMECYA 156	Serralves	May-2001	EU078507	
Synechocystis sp. LMECYA 68	wastewater stabilisation pound, Marrakesh, Morocco	2000	EU078508	EU078451
OSCILLATORIALES				
Limnothrix redekei LMECYA 145	Magos	11-09-2001	EU078512	EU078464
Phormidium sp. LMECYA 79	Magos	24-06-1998	EU078509	EU078453
Phormidium sp. LMECYA 173	Hydrothermal pond 60ºC	2003	EU078510	EU078473
Phormidium sp. LMECYA 214	Enxoé	23-07-2005	EU078511	EU078481
Planktothrix agardhii LMECYA 152	Magos	07-01-2003	EU078513	
P. agardhii LMECYA 153	Enxoé	12-02-2003	EU078514	
P. agardhii LMECYA 153A	Enxoé	12-02-2003		
P. agardhii LMECYA 153B	Enxoé	12-02-2003		
P. agardhii LMECYA 153C	Enxoé	12-02-2003		
P. agardhii LMECYA 153E	Enxoé	12-02-2003	EU078515	EU078467
P. agardhii LMECYA 153F	Enxoé	12-02-2003	EU078516	
P. agardhii LMECYA 198	Enxoé	19-05-2005		
P. agardhii LMECYA 199	Enxoé	19-05-2005		
P. pseudoagardhii LMECYA 162	Guadiana River (Mértola)	31-07-2003	EU078517	
P. rubescens LMECYA 203	Beliche	30-06-2005	DQ497635	EU078477
NOSTOCALES				
Anabaena circinalis LMECYA 17	Montargil	10-05-1996	EU078518	EU078447
A. circinalis LMECYA 123	Montargil	10-09-2000	EU078519	
A. circinalis LMECYA 123A	Montargil	10-09-2000	EU078520	
A. circinalis LMECYA 123C	Montargil	10-09-2000		
A. circinalis LMECYA 126	Montargil	27-11-2000	EU078521	EU078460
A. circinalis LMECYA 175	Magos	21-05-2004		
A. circinalis LMECYA 213	Alvito	22-07-2005	EU078526	EU078480
A. flos-aquae LMECYA 165	Magos	05-09-2003	EU078523	EU078471
A. flos-aquae LMECYA 178	Guadiana River (Vitonogales - Spain)	July 1999	EU078527	
A. flos-aquae LMECYA 180	Guadiana River (Vitonogales - Spain)	July 1999	EU078525	
A. planctonica LMECYA 177	Toulica	30-07-2004	EU078522	EU078474
A. spiroides LMECYA 161	Agolada de Baixo	11-07-2003	EU078524	EU078470
Anabaena sp. LMECYA 182	Guadiana River (Vitonogales - Spain)	July 1999	EU078545	EU078475
Anabaena sp. LMECYA 185	Guadiana River (Vitonogales - Spain)	July 1999	EU078546	
Anabaenopsis circularis LMECYA 205	Lagoa, Nafarros, Sintra	14-07-2005	EU078528	EU078478
Anabaenopsis circularis LMECYA 206	Lagoa, Nafarros, Sintra	14-07-2005	EU078529	EU078479
Anabaenopsis circularis LMECYA 207	Lagoa, Nafarros, Sintra	15-07-2005		
Aphanizomenon flos-aquae LMECYA 10	Torrão	05-06-1996	EU078537	EU078446
Aph. flos-aquae LMECYA 77A	Montargil	18-11-1999	EU078538	EU078452
Aph. flos-aquae LMECYA 77B	Montargil	18-11-1999	EU078539	
Aph. flos-aquae LMECYA 88	Montargil	28-11-1999	EU078540	EU078456
Aph. flos-aquae LMECYA 89	Montargil	18-11-1999		

Taxon and strain designation	Origin	Collection date	GenBank accession no.	
			16S rDNA	*rpoC1*
Aph. flos-aquae LMECYA 99	Montargil	18-11-1999	EU078541	
Aph. flos-aquae LMECYA 125	Monte Novo	23-10-2000	EU078542	EU078459
Aph. flos-aquae LMECYA 129	Montargil	27-11-2000	EU078543	
Aph. flos-aquae LMECYA 140	Divor	17-07-2001		
Aph. flos-aquae LMECYA 141	Monte Novo	17-07-2001	EU078544	
Aph. flos-aquae LMECYA 143A	Montargil	23-07-2001		
Aph. flos-aquae LMECYA 143B	Montargil	23-07-2001		
Aph. flos-aquae LMECYA 146	Funcho	14-01-2002		
Aph. gracile LMECYA 9	Peneireiro	15-03-1996	EU078530	EU078445
Aph. gracile LMECYA 33	Peneireiro	15-03-1996	EU078531	
Aph. gracile LMECYA 40	Crato	02-08-1996	AY354194*	EU078449
Aph. gracile LMECYA 64	Patudos	13-08-1997	EU078532	EU078450
Aph. gracile LMECYA 148	Patudos	21-05-2002	EU078533	EU078465
Aph. gracile LMECYA 196	Enxoé	20-05-2005		
Aph. gracile LMECYA 197	Enxoé	20-05-2005		
Aph. gracile LMECYA 211	Enxoé	23-07-2005		
Aph. issatschenkoi LMECYA 31	Montargil	10-06-1996	AY196088*	EU078448
Aph. issatschenkoi LMECYA 163	Magos	05-09-2003	EU078534	
Aph. issatschenkoi LMECYA 164	Magos	05-09-2003		
Aph. issatschenkoi LMECYA 166	Vale Michões reservoir, Alcochete, Portugal	29-10-2003	EU078535	
Aph. issatschenkoi LMECYA 190	Maranhão	10-09-2004	EU078536	
Aph. ovalisporum MS1	Lake Kinneret - Israel	NA		
Cylindrospermopsis raciborskii LMECYA 130	Odivelas	24-07-2000		
C. raciborskii LMECYA 132	Odivelas	24-07-2000	EU078547	
C. raciborskii LMECYA 134	Odivelas	24-07-2000	AY699989	EU078461
C. raciborskii LMECYA 135	Odivelas	24-07-2000	EU078548	
C. raciborskii LMECYA 168	Odivelas	24-07-2000		
C. raciborskii LMECYA 168	Queensland-Australia	NA		

* - sequences obtained by others; NA - information not available.

Morphological identification

The morphology of cells and filaments was studied using an Olympus BX60 light microscope with a digital camera. The following parameters were analyzed: length and width of vegetative cells; morphology of terminal cell; presence or absence of heterocysts, akinetes and gas vesicles; distance between heterocysts and distance between a heterocyst and the nearest akinete and shape of filament and its aggregation in colonies.

DNA extraction from cyanobacteria and heterotrophic bacteria

Genomic DNA of cyanobacterial isolates was extracted according to the method previously described (Valério *et al*., 2005).

For isolation of the heterotrophic bacteria, associated with some cyanobacterial cultures (randomly selected), an aliquot of the supernatant of the LMECYA 64, AQS, LMECYA

79, LMECYA 13 and LMECYA 173 isolates was grown in LBAgar (tryptone 1% w/v, yeast extract 0.05% w/v, NaCl 0.05% w/v and agar 2% w/v) at 22 ºC for three days. The strains obtained were subsequently purified and grown in LB medium (tryptone 1% w/v, yeast extract 0.05% w/v, NaCl 0.05% w/v) overnight at 22 ºC for DNA extraction. Cells were recovered by centrifugation and total DNA extracted by guanidium thiocyanate method, according to Pitcher *et al*. (1989).

PCR fingerprinting and data analysis

STRR PCR fingerprinting was performed using both STRR1F (5'-CCCCARTC-CCCART-3') and STRR3F (5'-CAACAGTCAACAGT-3') primers in the PCR reaction (Wilson *et al.*, 2000). For LTRR PCR fingerprinting both LTRR1 (5'-GGATTTTTGTTAGTTAAAAC-3') and LTRR2 (5'-CTATCAGGGATTGAAAG -3') primers (Rasmussen & Svening, 1998) were used.

M13 PCR fingerprinting was performed using oligonucleotide csM13 (5'-GAGGGT-GGCGGTTCT-3') (Huey & Hall, 1989) as single primer. For ERIC PCR fingerprinting both ERICIR (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGT-GAGCG-3') primers (Versalovic *et al.*, 1991) were used.

The PCR reactions were performed in 50 μl containing 1 X PCR buffer (Invitrogen), 0.4 mM of each of the four dNTPs (Invitrogen), 0.5 μM (for STRR and LTRR fingerprinting) or 1μM (for M13 and ERIC fingerprinting) of each primer, 10-15 ng of genomic DNA, 3 mM (for STRR, LTRR and M13 fingerprinting) or 2 mM (for ERIC fingerprinting) of $MgCl_2$, 1 mg ml^{-1} of BSA and 1 U of *Taq* DNA polymerase (Invitrogen). Amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 95 ºC for 10 min, followed by 35 cycles of 90 s at 95 ºC, 2 min at 56 ºC (for M13 fingerprinting), 40 ºC (for ERIC fingerprinting) or 38 ºC (for STRR and LTRR fingerprinting), and 2 min at 72 ºC, and a final extension step of 5 min at 72 ºC.

The PCR products were electrophoresed in 1.4% (w/v) agarose in TBE 0.5 X buffer at 90 V for 3 h 30 min, using 1 kb plus DNA Ladder (Gibco-BRL) as molecular size marker. After staining with ethidium bromide, the image obtained under UV transillumination was digitalized using Kodak 1D 2.0 system.

To assess reproducibility of the fingerprinting methods, duplicates (above 10%) were randomly selected and analyzed in independent experiments. The similarity between each pair of duplicates was obtained from the analysis based on a dendrogram computed with

Pearson's correlation coefficient and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering, using BioNumerics^{TM} software v4.0 (Applied Maths, Kortrijk, Belgium) (data not shown). The reproducibility value was determined as the average for all pairs of duplicates.

Isolates hierarchical clustering, based on their genomic fingerprinting patterns, was performed with Pearson's correlation coefficient and UPGMA using BioNumerics^{TM} software v4.0 (Applied Maths, Kortrijk, Belgium). The accuracy of each dendrogram to represent the similarity matrix was assessed by the cophenetic correlation coefficient (ρ) that ideally should be between 0.6 and 0.9 (Priest & Austin, 1993). Composite analyses were performed by constructing dendrograms based in the patterns obtained with two different methods. Clusters of isolates were correlated with species allocation, geographical origin and available toxicity data.

The intra-specific diversity of each cyanobacterial species comprising more than seven isolates was evaluated with the indexes of Simpson (Hunter & Gaston, 1988) and Shannon-Wiener (Zar, 1996). Simpson index (D) is based on the number of types and isolates for each type and measures the probability of two non-related strains, taken from the tested population, belonging to two different genomic types. Shannon-Wiener index (J') is an evenness measure, expressing the observed diversity as the proportion of the possible maximum diversity and reflecting the homogeneity/heterogeneity of the distribution of isolates among the genomic types.

PCR amplification and sequencing of 16S rDNA and *rpoC1*

Since universal primers for 16S rDNA direct sequencing are usually designed to be used with axenic cultures and available primers for *rpoC1* amplification and sequencing are highly degenerated, specific primers were selected or designed in order to obtain clean sequences for both genes without the need of a cloning step. Primers used in the amplification and sequencing of the 16S rDNA and *rpoC1* genes are listed in Table 2 and the target sites are presented in Fig. 1 and 2. The PCR reaction was performed in 50 μl mixture containing 1 X PCR buffer (Invitrogen), 0.4 mM of each of the four dNTPs (Invitrogen), 0.25 μM of each of the two primers, 10-15 ng of genomic DNA, 2 mM of $MgCl_2$ (Invitrogen), 0.5 mg ml^{-1} of BSA (Invitrogen) and 1 U of *Taq* DNA polymerase (Invitrogen). Amplification was performed in a T Gradient thermocycler (Biometra) using the following conditions: 10 min of initial denaturation at 95 ºC, followed by 35 cycles at 94 ºC for 45 s, 55 ºC for 45 s and 72 ºC for 1 min. The amplification was completed by holding for 5 min at 72 ºC to allow the complete extension of the PCR product. PCR products were visualized by ethidium bromide staining after electro-

phoresis in a 1% (w/v) agarose gel and their sizes were estimated by comparison with 1 kb plus DNA ladder molecular marker (Gibco-BRL). The amplification reaction products were purified with a Jet Quick-PCR Purification kit (Genomed) as described by the manufacturer. The purified PCR products were sequenced in both directions using an automated ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) with dye terminators, using standard protocols and the cyanobacterial specific primers used for amplification, as well as primers CYAN16S (5'-ATACCCCWGTAGTCCTAGC-3'; Valério *et al.*, 2005) and CYAN16SR2 (5'-GCTAGGAC-TACWGGGGTAT -3'; this study) for the 16S rDNA sequencing.

TABLE 2 Primers used for 16S rDNA, *rpoC1* and ITS region amplification and for 16S rDNA and *rpoC1* sequencing.

Gene	Primer	Sequence (5' to 3')*	Amplicon size (nt)	Target site	Reference or source	Observation
16S rRNA	CYAN106F	Fw:CGGACGGGTGAGTAACGCGTGA			Nübel et al., 1997	
	CYAN16SR2	Rv:GCTAGGACTACWGGGGTAT	630	738-765†	This study	
	CYAN16S	Fw: ATACCCCWGTAGTCCTAGC			Valério et al., 2005	
	CYAN16SR	Rv: GCAATTACTAGCGATTCCTCC	1260		Valério et al., 2005	
rpoC1	RPOC1M-F1	Fw:GGAAATGGATGGGTTATTCTGC	580	61-82‡	This study	Specific for *Microcystis*
	RPOC1M-R1	Rv:TAAAACCATCCATTCTGCCTC		624-643‡	This study	Specific for *Microcystis*
	RPOC1-F2	Fw:ACTCTGAARCCAGAAATGGA		145-164§	This study	Except for *Microcystis*
	RPOC1-R2	Rv:AARTTRTCAATTACCCGCA	555	683-701§	This study	Except for *Microcystis*
	RPOC1-R3	Rv:TGCTTACCTTCAATAATGTC	880	1006-1025§	This study	Except for *Microcystis*
ITS region	ITSCYAN-F	Fw: CTGGTTCRAGTCCAGGAT		236¦	This study	
	ITSCYAN-R	Rv: TGCAGTTKTCAAGGTTCT	variable	225#	This study	

* - Fw: Forward primer; Rv: reverse primer; † - 16S rRNA of *Synechocystis* PCC 6803 (NC000911); ‡ - *rpoC1* sequence of *M. aeruginosa* PCC 7806 (AY425000); § - *rpoC1* sequence of *Synechocystis* sp. PCC 6803 (BA000022); ¦ - at 5' end of 16S rRNA of *Synechococcus* sp. WH7803 (CT971583); # - at 5' end of 23S rRNA of *Synechococcus* sp. WH7803 (CT971583).

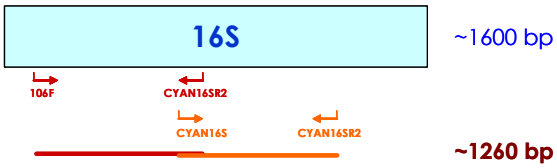

FIGURE 1 16S rDNA gene primers target site.

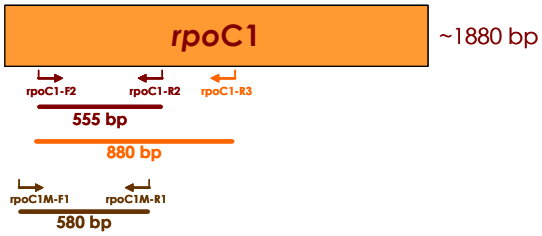

FIGURE 2 *rpoC1* gene primers target site.

Phylogenetic analysis and identification at species level

An alignment of 1182 or 1203 nucleotides of the 16S rRNA gene, as identified in the figures legends, from 71 LMECYA strains was used. In the case of *rpoC1*, an alignment of 452 nucleotides of 38 strains was used in the phylogeny. Alignments were made with ClustalX version 1.83 and visually corrected. Phylogenetic trees were constructed based on a Bayesian approach using MrBayes software version 3.0b4 (Huelsenbeck & Ronquist, 2001). Each analysis consisted of 2000000 generations from a random starting tree and four Markov chains (with default heating values) sampled every 100 generations. The first 8000 sampled trees were discarded resulting in a set of 12000 analysed trees sampled after stationary. The eubacterial *Escherichia coli* sequence was used as outgroup.

The BLAST tool of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) was used to find homologous and other close sequences to be included in the phylogenies.

The *rpoC1* and 16S rDNA sequences have been deposited in the GenBank database under the accession numbers EU078443 to EU078480 (*rpoC1*) and AY699989, DQ497635 and EU078482 to EU078548 (16S rDNA).

In the case that the phylogenetic positioning of an isolate retrieved an identification at species level, this prevailed; if the phylogenetic positioning only allowed the genus determination, then its identification at species level was based on the morphology. Moreover, if no identification was possible through the phylogenetic studies, then the identification at genus or species level was achieved by analysis of the morphological features.

16S rDNA and ITS analysis

To select the most suitable restriction endonucleases to provide an easy identifiable 16S rDNA restriction profile, a theoretical study was performed with NEBcutter v2.0 software (Vincze *et al*., 2003), using cyanobacterial 16S rDNA sequences available in databases, resulting in the selection of *Ava*II and *Ban*II enzymes.

For 16S PCR-RFLPs, the obtained amplicon of 1260 bp (amplified with CYAN106F and CYAN16SR primers) (10 μl, approximately 1 μg) was digested without further purification with *Ava*II (BioLabs) and *Ban*II (Takara) restriction endonucleases in separated reactions, according to manufacturer's instructions.

The restriction products were electrophoresed in 2% (w/v) agarose in TBE 0.5 X buffer at 90 V for 2 h, using 1 kb plus DNA Ladder (Gibco-BRL) as molecular size marker. After staining with ethidium bromide, the image obtained under UV transillumination was digitalized using Kodak 1D 2.0 system.

The ITS region was amplified using DNA of non-axenic cultured and two cyanobacterial specific primers designed in this study (Table 2). The PCR reactions were performed in 50 μl containing 1 X PCR buffer (Invitrogen), 0.4 mM of each of the four dNTPs (Invitrogen), 0.5 μM of each primer, 10-15 ng of genomic DNA, 2.5 mM of $MgCl_2$, 0.5 mg ml^{-1} of BSA and 1 U of *Taq* DNA polymerase (Invitrogen). Amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 95 ºC for 6 min, followed by 35 cycles of 45 s at 95 ºC, 45 s at 52 ºC, and 1 min at 72 ºC, and a final extension step of 5 min at 72 ºC.

Also in this case, a theoretical study was performed with NEBcutter v2.0 software (Vincze *et al*., 2003), using cyanobacterial ITS sequences available in databases, resulting in the selection of *Taq*I enzyme.

For ITS-ARDRA, amplified DNA (10 μl, approximately 1 μg) was digested without further purification with *Taq*I restriction endonuclease (Fermentas) according to manufacturer's instructions.

The ITS amplification products were electrophoresed in 1.2% (w/v) agarose for 90 V for 1 h 30 min, and the ITS-ARDRA profiles in 2% (w/v) agarose in TBE 0.5 X buffer at 90 V for 2 h, using 1 kb plus DNA Ladder (Gibco-BRL) as molecular size marker. After staining with ethidium bromide, the image obtained under UV transillumination was digitalized using Kodak 1D 2.0 system.

RESULTS

Morphological identification

Identification of isolates at species or genus level was previously assessed by their morphology. Out of the 118 set, 99 isolates where identified at species level and the remaining only at genus level (19 out of 118). The results obtained revealed that the cyanobacterial species present in Portuguese freshwaters, most of which responsible for bloom formation, are

diverse, and belong to three of the six cyanobacterial orders: Chroococcales, Oscillatoriales and Nostocales. Of the 118 isolates under study, 45.8% belong to Chroococcales, 12.7% to Oscillatoriales and 41.5% to Nostocales. The species predominantly found were *Microcystis aeruginosa* (44%) and *Aphanizomenon flos-aquae* (11%).

Assessment of PCR fingerprinting reliability

The average reproducibility of the tested fingerprinting techniques, estimated by the similarity average value for all pairs of duplicates was 92%, representing a good reproducibility, since the Pearson's correlation coefficient used is very sensitive to the band intensity leading to lower the final similarity values. In all cases the cophenetic value (ρ) is above 0.85, which corresponds to a good representation of the original data.

In order to evaluate the influence of the uni-cyanobacterial cultures that are maintained non axenic, the PCR fingerprinting methods to be tested (STRR, LTRR, M13 and ERIC) were applied to a set of cultures and their respective cultivable bacteria. From the same culture (Fig. 3 underlined group of lanes), cyanobacteria (in bold) and cultivable heterotrophic bacteria displayed M13 (Fig. 3c) and ERIC (Fig. 3d) PCR fingerprints with very few co-migrating fragments (white boxes). However, LMECYA 79 and LMECYA 173 isolates displayed identical fingerprinting profiles with each technique, despite the fact that their correspondent cultivable bacteria present different M13 and ERIC profiles from each other but with some co-migrating fragments with the cyanobacteria (Fig. 3, lanes 8 to 11).

On the other hand, STRR and LTRR PCR fingerprinting (Fig. 3a and b) showed specificity for cyanobacteria, since no amplification was obtained for the cultivable bacteria.

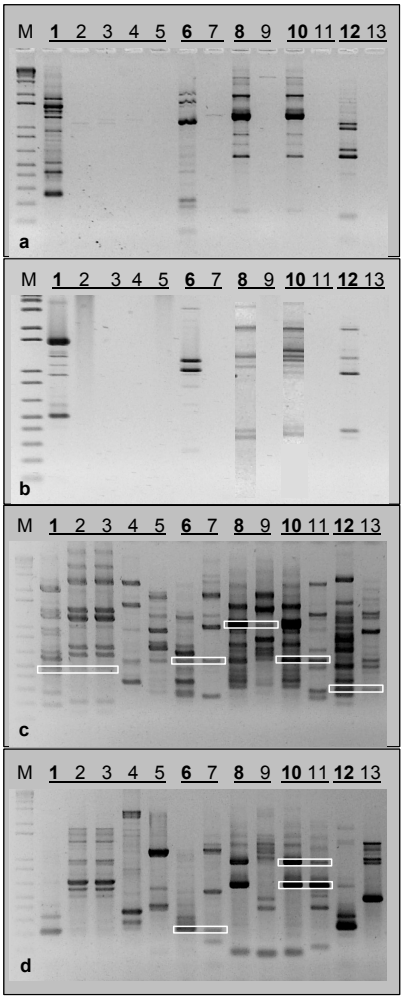

FIGURE 3 Fingerprinting profiles of cyanobacteria and cultivable bacteria from each non axenic culture. (a) STRR PCR-Fingerprinting; (b) LTRR PCR-Fingerprinting; (c) M13 PCR-Fingerprinting; (d) ERIC PCR-Fingerprinting. (1) LMECYA 64; (2) LMECYA 64 heterotrophic bacteria #1; (3) LMECYA 64 heterotrophic bacteria #2; (4) LMECYA 64 heterotrophic bacteria #3; (5) LMECYA 64 heterotrophic bacteria #4; (6) AQS; (7) AQS heterotrophic bacteria #1; (8) LMECYA 79; (9) LMECYA 79 heterotrophic bacteria #1; (10) LMECYA 173; (11) LMECYA 173 heterotrophic bacteria #1; (12) LMECYA 13; (13) LMECYA 13 heterotrophic bacteria #1. The white boxes mark the common fragments.

STRR and LTRR PCR fingerprinting

A composite analysis of hierarchical clustering of STRR and LTRR patterns was performed with all isolates (data not shown) and subsequently for each order as presented in Fig. 4. It can be observed that the isolates grouped themselves according to their genomic similarity. To confirm the species identification, a set of representative isolates (marked with bold italics in Fig. 4), including members of different clusters and members of the same cluster, was selected and their identification was obtained or confirmed by phylogenetic positioning using 16S rDNA and *rpoC1* phylogenies.

The identification presented in Fig. 4 resumes the final identification of the isolates obtained by the 16S rDNA sequencing (that in some cases was also confirmed by the *rpoC1* sequencing, as presented below). It can be observed that the majority of the clusters corresponds to different species, showing the clusters taxonomic potential of STRR and LTRR fingerprints.

The three species (two of them composed by only one isolate) belonging to the order Chroococcales (Fig. 4a) are intermixed and could no be separated according to their STRR and LTRR fingerprinting patterns. In fact, the major group of isolates represents *Microcystis aeruginosa* with very diverse profiles and the isolate of *Synechocystis* sp. has a higher similarity to *M. aeruginosa* isolates than some *M. aeruginosa* isolates between them. *Synechococcus nidulans* has the lower similarity with all the isolates together with one isolate of *M. aeruginosa*. The delineation of the *Microcystis aeruginosa* isolates was possible with respect to the geographical origin of each isolate, and also their toxicological profile. The majority of the isolates sourced from the same reservoir grouped together. In a dendrogram of all *Microcystis aeruginosa* isolates (data not shown), three major clusters were formed, one comprising five isolates being two toxic and three non-toxic, the second cluster including 22 toxic isolates and two non-toxic and the third one containing 20 non-toxic isolates and two toxic.

Fingerprinting patterns of isolates belonging to the order Oscillatoriales (Fig. 4b) led to the formation of two clusters and four single member clusters at 40% similarity, while nine major clusters and four single member clusters were observed at 50% similarity for isolates of the order Nostocales (Fig. 4c). In both cases almost all the clusters correspond to different species, except for *Anabaena circinalis* and *Aphanizomenon gracile* which formed two distinct clusters (genotypes).

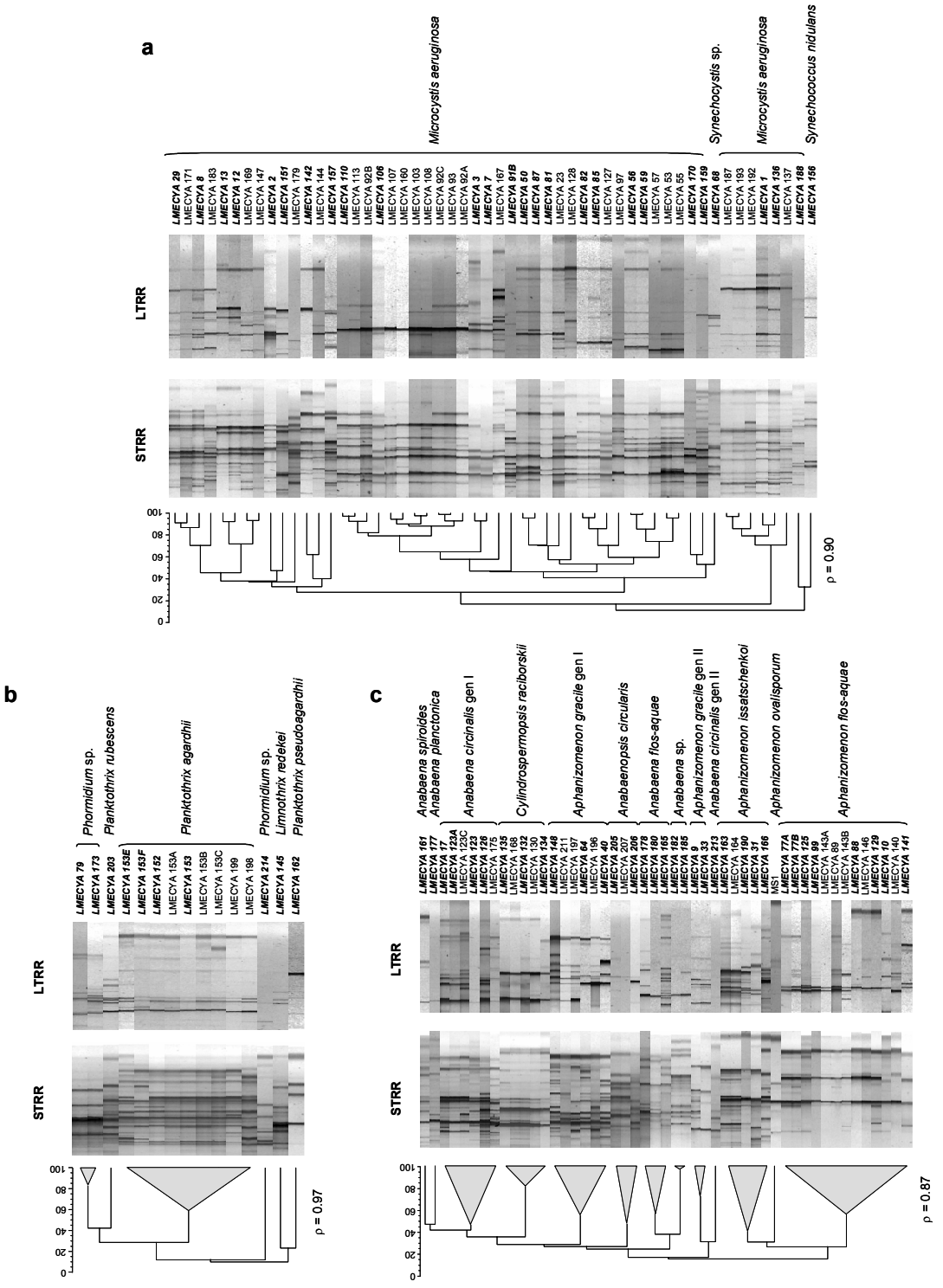

FIGURE 4 Dendrograms obtained by composite hierarchical analysis of STRR and LTRR fingerprinting profiles using Pearson's correlation coefficient and UPGMA clustering method for the orders used in the study: (a) Chroococcales; (b) Oscillatoriales and (c) Nostocales. The cophenetic correlations (ρ) of the dendrograms are discriminated in each case. The scale corresponds to global percentage of similarity. Gen: genotype. A “weight of 2” was attributed to the STRR patterns, and a “weight of 1” was ascribed to the LTRR patterns.

Phylogenetic analysis of 16S rDNA

Given the high number of 16S rDNA sequences to be used, a separated phylogeny was constructed for each order (Fig. 5 to 7). From each phylogeny, representative sequences were then selected to perform a global 16S rDNA phylogeny including the six cyanobacterial orders (Fig. 8).

The phylogenetic tree presented in Fig. 5 corresponds to the partial 16S rDNA phylogeny of the order Chroococcales. This phylogeny confirmed the identification of the 27 sequenced isolates only at genus level. In fact, although the genus *Microcystis* forms a monophyletic cluster, all the five species present a high homology and thus can not be distinguished. The remaining genera are also monophyletic, except *Synechococcus*, where an isolate of *Prochlorococcus marinus* is grouped within the cluster.

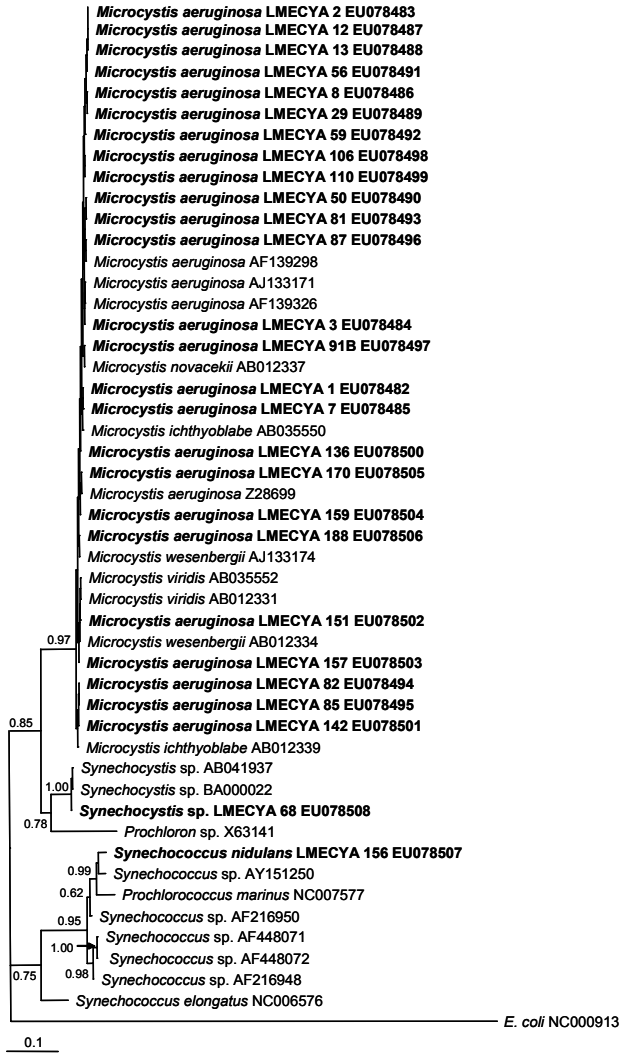

FIGURE 5 MrBayes tree of the species belonging to the Chroococcales order using 1203 nt of the 16S rDNA sequences in the alignment. The posterior probabilities values, above 50, are indicated to provide branch support. Bar: 10% sequence divergence. GenBank accession numbers are indicated after species designation (names in bold-face correspond to sequences determined in this study).

The partial 16S rDNA phylogeny of the order Oscillatoriales is depicted in Fig. 6 and allowed the identification at species level of seven sequenced isolates (out of 10), four of these (LMECYA 152, 153, 153E e 153F) as *Planktothrix agardhii*, one (LMECYA 203) as *Planktothrix rubescens*, one (LMECYA 162) as *Planktothrix pseudoagardhii* and one (LMECYA 145) as *Limnothrix redekei*. The three remaining isolates (LMECYA 79, 173 and 214) were grouped in a cluster containing sequences of *Phormidium* and *Leptolyngbya* isolates. However, taking into account their morphological characteristics, these isolates were identified as *Phormidium* sp..

The distribution of *Oscillatoria* and *Lyngbya* isolates in several distinct clades also reveals the polyphyletic nature of these genera.

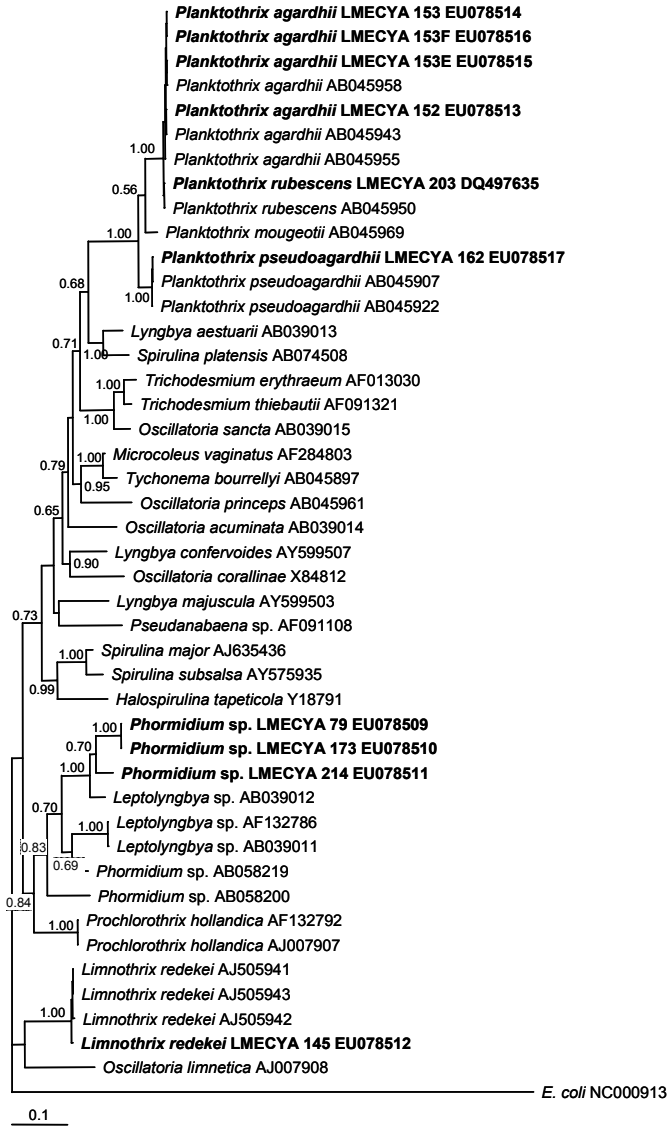

FIGURE 6 MrBayes tree of the species belonging to the Oscillatoriales order using 1182 nt of the 16S rDNA sequences in the alignment. The posterior probabilities values, above 50, are indicated to provide branch support. Bar: 10% sequence divergence. GenBank accession numbers are indicated after species designation (names in bold-face correspond to sequences determined in this study).

The partial 16S rDNA phylogeny concerning the order Nostocales (Fig. 7) allowed the identification of 21 sequenced isolates (out of 34), with isolates LMECYA 17, 123, 123A, 126 and 213 belonging to *Anabaena circinalis*, isolate LMECYA 161 to *Anabaena spiroides*, isolate LMECYA 10 to *Aphanizomenon flos-aquae*, isolates LMECYA 40, 64 and 148 to *Aphanizomenon gracile*, isolates LMECYA 205 and 206 to the genus *Anabaenopsis*, isolates LMECYA 31, 163, 166 and 190 to *Aphanizomenon issatschenkoi*, isolates LMECYA 132, 134 and 135 to *Cylindrospermopsis raciborskii*, and isolates LMECYA 165 and 180 belonging to *Anabaena flos-aquae*.

The isolate LMECYA 177 groups in a cluster where several *Anabaena* species are present. This phylogenetic positioning does not allow an identification of this isolate; however, its morphological features are similar to those presented by *Anabaena planctonica*, which is also present in the cluster. This is also the case of isolates 77A, 77B, 88, 99, 125, 129 and 141 grouped in a cluster with mainly *Aphanizomenon flos-aquae* but also an *Aphanizomenon gracile*, however the morphological features of these isolates are typical of *Aphanizomenon flos-aquae*. Isolates LMECYA 9 and 33 grouped in a cluster with *Anabaena flos-aquae*, *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* sequences, however the morphological features of these isolates are typical of *Aphanizomenon gracile*.

The isolates LMECYA 178, 182 and 185 did not group with any species or genus, however their morphology is congruent with *Anabaena*, being LMECYA 178 identical to *Anabaena flos-aquae*.

This phylogeny also showed that *Anabaena* and *Aphanizomenon* do not form monophyletic clusters, with isolates of both genera intermixed along the tree.

The global 16S rDNA phylogeny of representative species of the six cyanobacterial orders is illustrated in Fig. 8. It shows that the Nostocales form a monophyletic cluster, except for the sole *Scytonema* sequence presented, as well as the Stigonematales having a monophyletic clade, containing the genera *Chlorogloeopsis*, *Fischerella* and *Nostochopsis*, except the unique *Stigonema* sequence here presented. The order Pleurocapsales forms a monophyletic cluster, whereas the orders Chroococcales and Oscillatoriales are separated in two distinct clades.

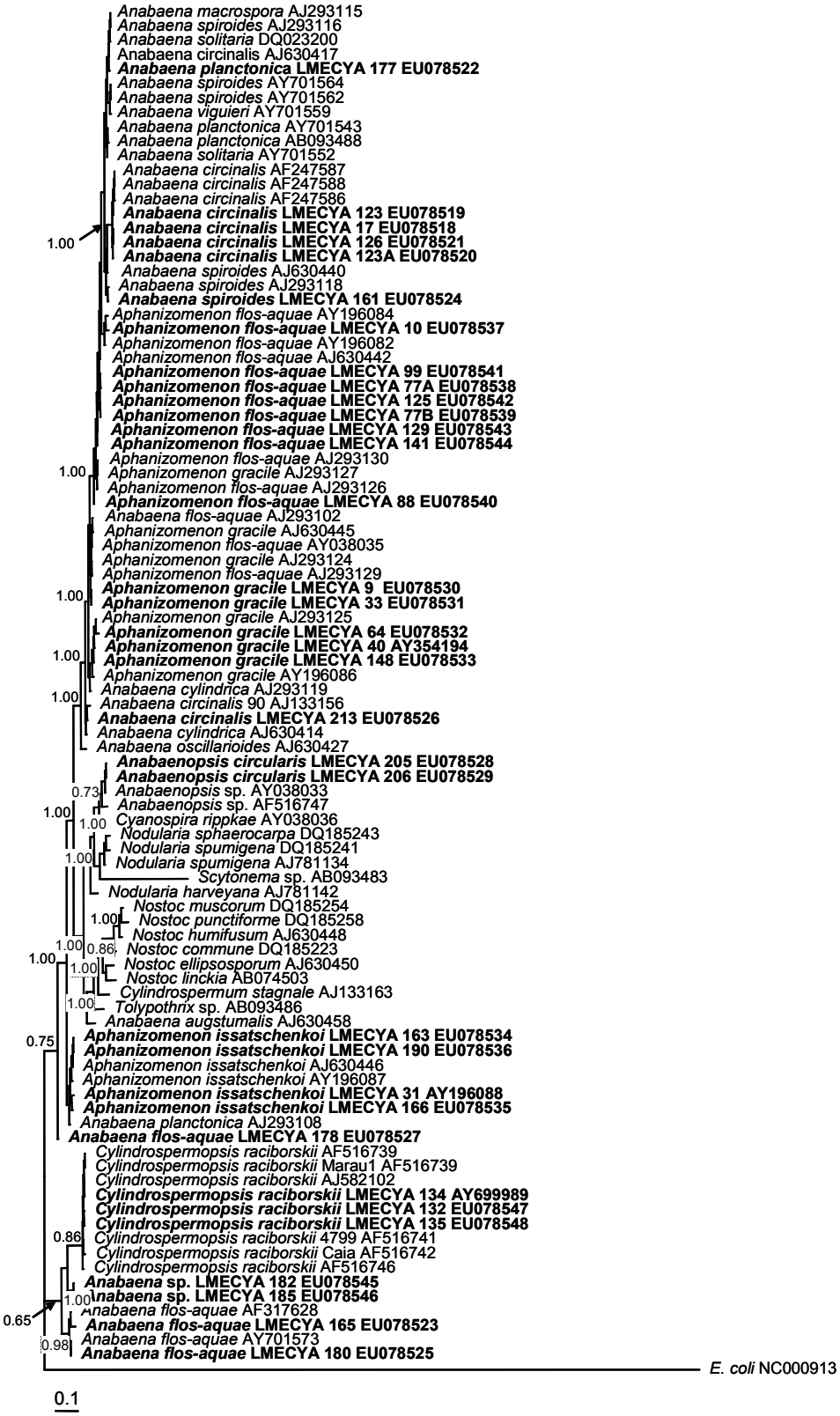

FIGURE 7 MrBayes tree of the species belonging to the Nostocales order using 1203 nt of the 16S rDNA sequences in the alignment. The posterior probabilities values, above 50, are indicated to provide branch support. Bar: 10% sequence divergence. GenBank accession numbers are indicated after species designation (names in bold-face correspond to sequences determined in this study).

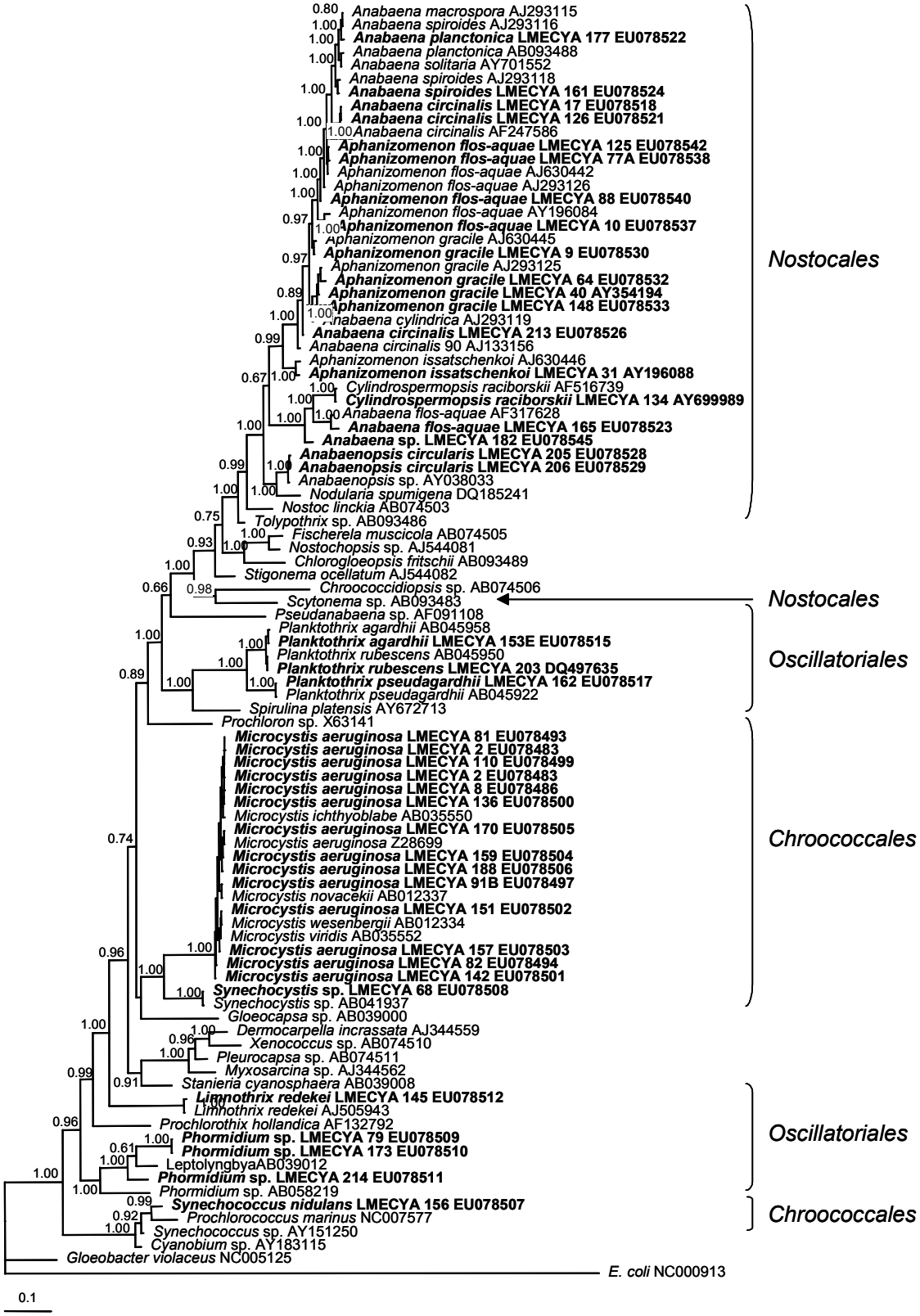

FIGURE 8 Global MrBayes tree of representative species using 1182 nt of the 16S rDNA sequences in the alignment. The posterior probabilities values, above 50, are indicated to provide branch support. Bar: 10% sequence divergence. GenBank accession numbers are indicated after species designation (names in bold-face correspond to sequences determined in this study).

Phylogenetic analysis of *rpoC1*

The *rpoC1* gene based phylogeny presented in Fig. 9 allowed the identification of six of the 38 sequenced isolates. Two of them (LMECYA 17 and 126) belong to *Anabaena circinalis*, one (LMECYA 134) to *Cylindrospermopsis raciborskii*, one (LMECYA 153E) to *Planktothrix agardhii* and one (LMECYA 68) to *Synechocystis* sp.. All these identification were congruent with those obtained by 16S rDNA sequencing.

Isolates LMECYA 9, 40, 64 and 148, identified both morphologically and by 16S rDNA sequencing as *Aphanizomenon gracile*, clustered in two distinct groups, neither of them closely related with the available *Aphanizomenon gracile rpo*C1 sequence.

Phormidium sp. isolates (LMECYA 79 and 173) grouped in a cluster containing another *Phormidium* sequence but also *Leptolyngbya* and *Synechococcus* sequences.

Some isolates did not group with a particular species, due to the low number of *rpo*C1 sequences available.

The results obtained here seem to point out to possible lateral gene transfer of this protein-coding gene in *Anabaena* and *Aphanizomenon* species, since there are isolates from different species presenting a high similarity of this gene.

In this phylogeny, the Nostocales formed two clusters, opposing to a major one obtained by 16S rDNA, and the Oscillatoriales were also separated in two distinct clades equivalent to the 16S rDNA ones, whereas the Chroococcales presented a monophyletic cluster except for the *Prochloron* and two *Synechococcus* spp. sequences that where distributed in others clusters.

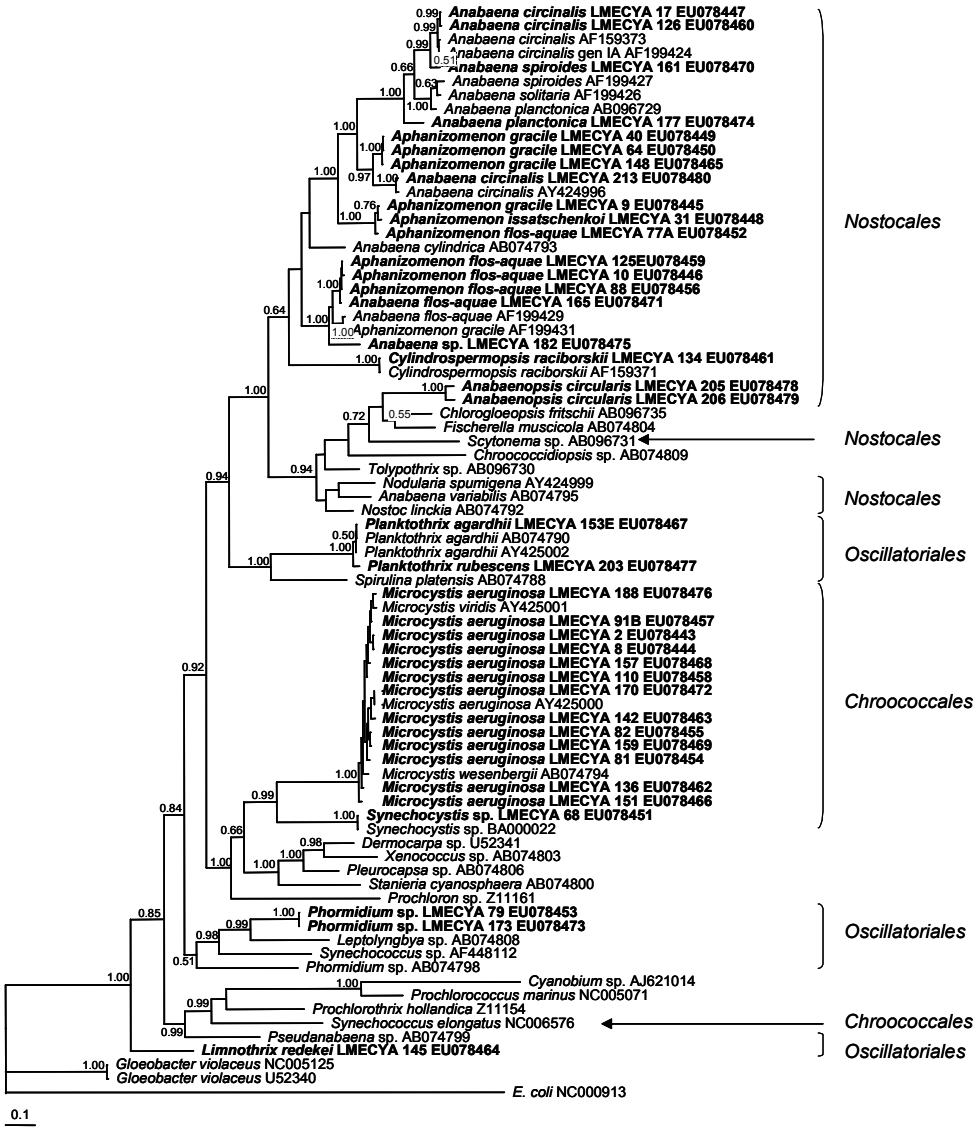

FIGURE 9 MrBayes tree of the *rpoC1* sequences using 452 nt of in the alignment. The posterior probabilities values, above 50, are indicated to provide branch support. Bar: 10% sequence divergence. GenBank accession numbers are indicated after species designation (names in bold-face correspond to sequences determined in this study).

Strain differentiation and traceability

Representative M13 and ERIC PCR fingerprinting profiles of the genomic types of the species comprising more than five isolates are presented in Fig. 10. As observed, the richness and diversity of the DNA banding patterns allowed the discrimination of all distinct isolates and the detection of identical ones.

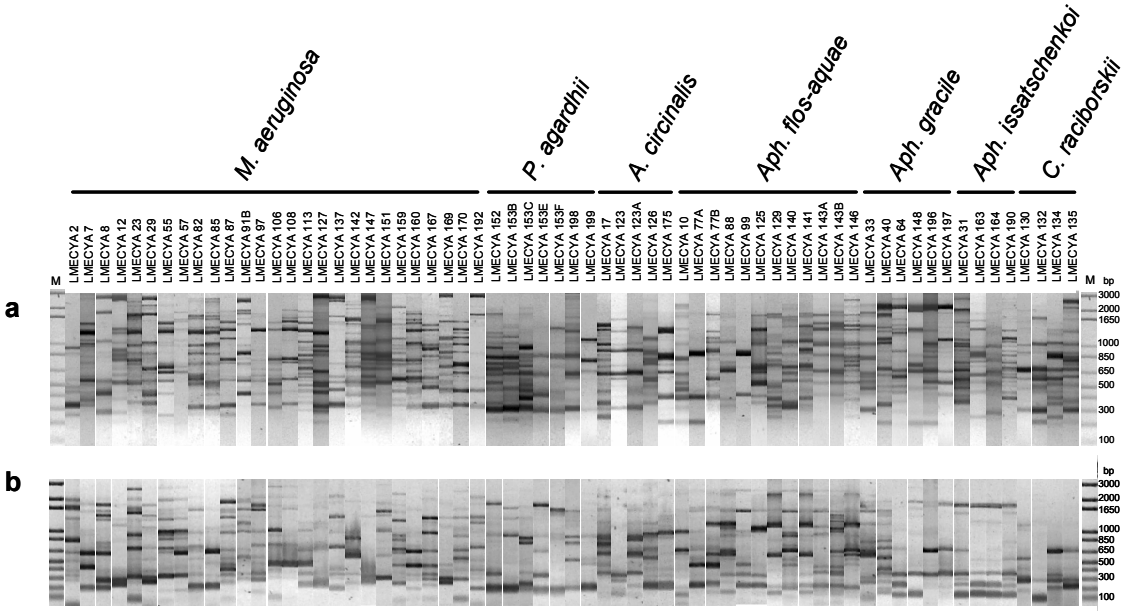

FIGURE 10 Representative M13 (a) and ERIC (b) PCR-fingerprinting profiles of the cyanobacterial species comprising more than five isolates. M: 1 kb plus molecular ladder.

Individual dendrograms were constructed for each of the seven species (data not shown). Based in the M13 and ERIC PCR fingerprints of all the *Microcystis aeruginosa* isolates evaluated by a composite analysis of hierarchical clustering, a cut-off level of 75% similarity was established for definition of a genomic type. Table 3 presents the percentage of types and values of Simpson and Shannon-Wiener diversity indexes (for the species with more than seven isolates). The lowest diversity values were obtained for *Planktothrix agardhii*, where four of the nine isolates have the same genomic type. The other four species, whose diversity indexes were determined, revealed higher diversity values, with *Aphanizomenon flos-aquae* presenting the highest one.

TABLE 3 Genomic diversity of cyanobacterial species.

Species	% Types *	D †	J' ‡
M. aeruginosa	59.6 (32/52)	0.96	0.93
P. agardhii	66.7 (6/9)	0.83	0.88
A. circinalis	85.7 (6/7)	0.95	0.98
Aph. flos-aquae	92.3 (12/13)	0.99	0.99
Aph. gracile	75.0 (6/8)	0.93	0.97
Aph. issatschenkoi	80.0 (4/5)	-	-
C. raciborskii	80.0 (4/5)	-	-

* % Types: (number of types/number of isolates) x 100; † D: Simpson diversity index; ‡ J': Shannon-Wiener diversity index.

To test the traceability potential of these fingerprints, a dendrogram comprising *Microcystis aeruginosa* isolates, recovered from Montargil reservoir at different dates, was constructed (Fig. 11). It is verified that the three major clusters and one single member cluster defined are related with the microcystins ability production of the isolates, identified by NT (non-toxic) and T (toxic). A dashed line at 92% similarity allowed the detection of two isolates, LMECYA 92C and LMECYA 110, recovered in different years, which should be assumed as representing the same strain.

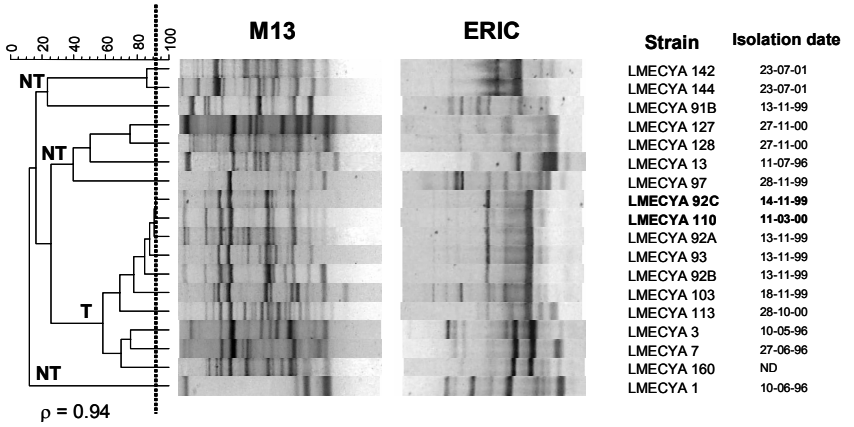

FIGURE 11 Traceability potential of composite hierarchical analysis of M13 and ERIC fingerprints of *Microcystis aeruginosa* sourced from Montargil reservoir in different dates. NT: non-toxic; T: toxic.

Diagnostic key for cyanobacteria identification

In order to reach a fast and reliable identification of the isolates, PCR amplification and subsequent restriction of 16S rDNA and ITS region were tested.

The dimensions of the fragments obtained after 16S rDNA digestion are summarized in Table 4. After restriction with *Ava*II four out of the 18 species are identified, *Limnothrix redekei* (630 bp, double band), *Cylindrospermopsis raciborskii* (1100 and 160 bp bands), *Phormidium* sp. (900 and 360 bp bands) and *Synechocystis* sp. (960 and 300 bp bands). For the remaining species, seven present a 1260 bp band (no restriction) and the others present a profile of an 810 and 450 bp bands. A further restriction with *Ban*II enables the identification of more seven out of the 14 species not identified previously. However, *Microcystis aeruginosa*, *Anabaena circinalis*, *Anabaena flos-aquae*, *Anabaena plactonica*, *Anabaena spiroides*, *Aphanizomenon flos-aquae* and *Aphanizomenon gracile* can not be distinguished, since they all present a profile with two bands of 815 and 445 bp.

The ITS amplicons, obtained with the cyanobacterial specific primers designed in this study, retrieved the amplification fragments also summarized in Table 4. The number of the

amplified fragments varied between one and three, and the size ranged from 180 and 930 bp. Most of the species included in Nostocales presented a 300 bp fragment. The range of ITS sizes for members of the genus *Microcystis* overlapped in some cases with *Anabaena* and *Aphanizomenon*. Some amplification profiles can also provide an identification, as is the case of *Synechocystis* sp. (170 bp band), *Limnothrix redekei* (350 and 250 bp bands), *Anabaena flos-aquae* (420 and 300 bp bands), *Anabaenopsis circularis* (380 bp band), *Aphanizomenon ovalisporum* (315 bp band) and *Cylindrospermopsis raciborskii* (300 and 195 bp). Moreover, a 220 bp fragment was always obtained for the *Planktothrix* spp. isolates.

The ITS-ARDRA fragments obtained after restriction of the ITS with *Taq*I enzyme are resumed in Table 4. Some of the isolates present a profile that can be used for identification. The genotypic pattern produced using this restriction enzyme can provide a species identification marker, for instance, *Planktothrix pseudoagardhii* that presented a distinct profile from the other two *Planktothrix* species, *Planktothrix agardhii*, difficult to distinguish from the former by morphological characteristics, and *Planktothrix rubescens* that can be identified by its cells dimensions and brownish color.

TABLE 4 16S PCR-RFLPs fragments generated with *Ava*I and *Ban*II endonucleases, ITS amplicons dimension and ITS-ARDRA fragments generated with *Taq*I.

	16S PCR-RFLPs fragments (bp)		**ITS amplicons (bp)**	**ITS-ARDRA fragments (bp)**
	***Ava*II**	***Ban*II**		
Chroococcales				
Microcystis aeruginosa	1260	815; 445	Type 1: 930; 300; 180 Type 2: 470; 300 Type 3: 300; 180 Type 4: 300 Type 5: 180	ND* ND ND 190; 110 180
Synechococcus nidulans	1260	715; 430; 115	300	190; 110
Synechocystis sp.	960; 300	715; 350; 195	170	170
Oscillatoriales				
Limnothrix redekei	630; 630	715; 445; 100	350; 250	ND
Planktothrix agardhii	1260	665; 420; 175	220	120; 100
Planktothrix pseudoagardhii	1260	815; 320; 125	220	100; 90; 30
Planktothrix rubescens	1260	640; 490; 130	220	120; 100
Phormidium sp.	900; 360	670; 420; 170	220	115; 105
Nostocales				
Anabaena circinalis	810; 450	815; 445	300	180; 100; 20
Anabaena flos-aquae	1260	815; 445	420; 300	ND
Anabaena planctonica	810; 450	815; 445	300	160; 95; 45
Anabaena spiroides	810; 450	815; 445	300	160; 95; 45
Anabaenopsis circularis	810; 450	1260	380	190; 110; 80
Aphanizomenon flos-aquae	810; 450	815; 445	300	160; 95; 45
Aphanizomenon gracile	810; 450	815; 445	300	170; 110; 20
Aphanizomenon issatschenkoi	810; 450	815; 360; 85	300	160; 80; 60
Aphanizomenon ovalisporum	1260	715; 445; 100	315	225; 90
Cylindrospermopsis raciborskii	1100; 160	715; 430; 115	300; 195	ND

*ND - not determined

Based on the 16S PCR-RFLP, ITS amplification and ITS-ARDRA a diagnostic key was constructed (Fig. 12) allowing the identification of 15 out of the 18 species analysed. Only *Anabaena planctonica*, *Anabaena spiroides* and *Aphanizomenon flos-aquae* could not be distinguished.

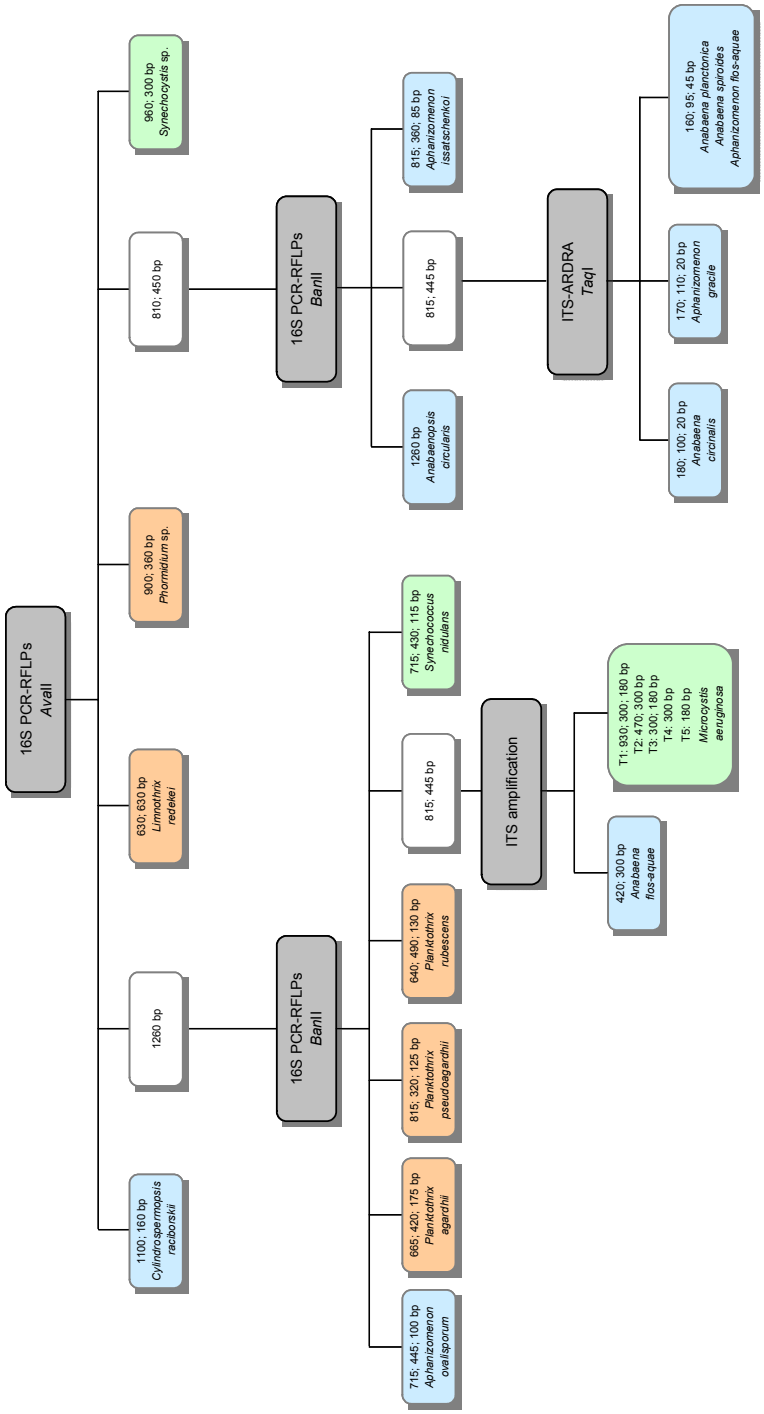

FIGURE 12 Diagnostic key based on 16S PCR-RFLPs with *Ava*II and *Ban*II, ITS amplification and ITS-ARDRA with *Taq*I, for cyanobacteria identification purposes. Green: Chroococcales; orange: Oscillatoriales; and blue: Nostocales.

DISCUSSION

In this study, several molecular techniques were used to examine the level of genetic diversity of 118 cyanobacterial isolates for taxonomic purposes.

All PCR fingerprinting methods displayed good reproducibility and the inter and intra-specific variability, observed in all species analyzed, revealed to be appropriate for assessment of genomic relationships, isolates differentiation and traceability, in spite of the non-axenic character of the cyanobacterial cultures. STRR fingerprints were obtained in a larger number of non-heterocystous cyanobacteria than described so far (Rasmussen & Svenning, 1998) and the LTRR PCR fingerprinting was also tested in a large number of species. Beyond some differentiation ability, the specificity of the STRR and LTRR fingerprinting patterns revealed identification potential. In fact, all species, from Nostocales and Oscillatoriales, could be discriminated by the composite hierarchical analysis of the STRR and LTRR fingerprints, when only species/genera of the same order were analysed. A good correlation was found between morphological identification and genomic clustering, for most species. In the case of *Anabaena circinalis* two clusters were formed, corresponding to two genotypes whose 16S rDNA gene presents 96.5% similarity between them, opposing to the 99.9% similarity displayed within the isolates of the same genotype. A similar finding was observed for *Aphanizomenon gracile* isolates, that were also divided in two clusters displaying 98% 16S rDNA similarity between them and 99-100% within the clusters. This method reveals to reflect the identification obtained by 16S rRNA sequencing, and thus can be used as an identification tool, decreasing the 16S rDNA gene sequencing effort, often necessary to determine/confirm the morphological identification of a cyanobacterial strain.

The genus *Microcystis* forms a monophyletic group distant from the other genera in both 16S rDNA and *rpoC1* phylogenies. All five *Microcystis* species presented a high homology (>98.5% with both genes) preventing their discrimination. This has also been verified by Otsuka *et al.* (2001), that proposed the unification of all five species, with *Microcystis aeruginosa* having nomenclatural priority. However, despite the high sequences homology and also the high DNA-DNA reassociation levels (Kondo *et al.*, 2000) presented among these species, their maintenance as distinct taxa may be justified by their distinct morphological features.

The phylogenies using both molecular markers show that the genera *Synechococcus* and *Synechocystis* form distinct clades. In the case of the *Synechococcus* genus there is evidence that it should be revised and reclassified into different genera, as also described by

Robertson *et al*. (2001), since for instance the species *Prochlorococcus marinus* is among other members of this genus.

Regarding the order Oscillatoriales, we also verified that the *Planktothrix* genus forms a monophyletic cluster more distant from the other species of this order in all the presented phylogenies. It is also evident that the genus *Oscillatoria*, that has been suffering revisions in the last years, still needs major revisions since it is not monophyletic, being its species distributed along the 16S rDNA phylogenetic tree. The genus *Lyngbya* also revealed to be polyphyletic, justifying its taxonomic revision. This might be related with the fact that the traditional criteria used for classification of the Oscillatoriales at genus level predominantly rely on the characteristics of external sheaths and colony formation, rather than on cellular features (Marquardt & Palinska, 2007). Other 16S rDNA analysis have already demonstrated the polyphyletic origin of members of the orders Chroococcales (Litvaitis, 2002; Seo & Yokota, 2003) and Oscillatoriales (Ishida *et al*., 2001; Litvaitis, 2002).

We can verify that the order Nostocales is not monophyletic in both phylogenies and that their members are closely related to the ones from Stigonematales. *Anabaena* and *Aphanizomenon* isolates were genetically heterogeneous and intermixed in both genes trees, confirming previous results obtained with other strains (Lyra *et al*., 2001; Gugger *et al*., 2002; Rajaniemi *et al*., 2005). Furthermore, the possible presence of incorrect database species-indexed sequences may also distort the inferred conclusions. *Cylindrospermopsis raciborskii* forms a monophyletic group, more distant from the other species of the Nostocales with both markers.

The validity of the presented phylogenetic analyses was enhanced by using mainly sequences from well characterized and reliably identified strains of validated species, such as sequences from completely sequenced genomes; sequences from the strains used by Suda *et al*. (2002), for the taxonomic revision of the genus *Planktothrix*; *Limnothrix redekei* sequences determined by Gkelis *et al.* (2005) and *Microcystis* sequences determined by Otsuka *et al*. (2001).

The identification of genomic types by analysis of M13 and ERIC fingerprints allowed the assessment of the intra-specific variability for the representative species of this study. This analysis was based on diversity indices as well as geographical origin of isolates to detect possible clonal relationships. *Planktothrix agardhii*, where eight of the nine isolates were obtained from the same reservoir, presents the lowest diversity values, whereas *Aphanizomenon flos-aquae* displayed the highest diversity, although eight of the 13 isolates have been collected from the same reservoir. For *Anabaena circinalis*, even though five of the seven iso-

lates have been obtained from the same reservoir, a high diversity value was also found. Although both indices revealed a high diversity in all species, the Shannon-Wiener index was usually higher than the Simpson index. For *Aphanizomenon flos-aquae*, both indices presented the same value (0.99) showing that almost all the isolates are unique. Furthermore, the Shannon index enabled the detection of cases where a high number of isolates of the same type were present (the lowest J' values), which was the case of *Planktothrix agardhii* and *Microcystis aeruginosa*.

The composite analysis of the hierarchical clustering of M13 and ERIC fingerprints also revealed to be suitable for strain differentiation and detection of isolates representing the same strain, further providing a powerful tool for traceability purposes within a freshwater reservoir. In fact, it was possible to detect a two-years resident toxic strain in Montargil reservoir, highlighting the potential of this traceability approach to fulfil the goal of ensuring water safety. Besides, as already observed for *Cylindrospermopsis raciborskii* isolates (Valério *et al.*, 2005), M13 and ERIC PCR fingerprinting are useful for ecological studies and evaluation of populations when high resolution is needed.

An expedite method to identify cyanobacterial isolates was developed through the construction of a diagnostic key based on the amplification of the 16S rDNA gene and further digestion with one or two restriction endonucleases. For the cases where no identification could still be obtained, the amplification or even further restriction of the ITS region with *TaqI* provided the identification of most species under study. This diagnostic key provides a faster, easier and reliable method to perform cyanobacteria identification, without the need of an expert experience to perform a morphological identification, thus avoiding a misidentification of the isolates, as frequently happens (Komárek & Anagnostidis, 1989). As an example of the application potentiality of this method, if the key here presented had been applied to the 118 isolates included in this study 84.7% of them (100 out of 118) would have been immediately identified at species level.

ACKNOWLEDGEMENTS

Elisabete Valério is recipient of Fundação para a Ciência e a Tecnologia grant SFRH/BD/8272/2002.

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4

Toxicity Molecular Markers

The content of this chapter is presented in the following papers:

"Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples", Elisabete Valério, Lélia Chambel, Sérgio Paulino, Natália Faria, Paulo Pereira, Rogério Tenreiro. *Aquatic Microbial Ecology* (submitted).

"Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters", E. Valério, P. Pereira, M.L. Saker, S. Franca, R. Tenreiro
Harmful Algae 4 (2005): 1044-1052.

"Validation of a multiplex PCR for the detection of cylindrospermopsin producing strains", Elisabete Valério, Lélia Chambel, Sérgio Paulino, Natália Faria, Paulo Pereira, Rogério Tenreiro. *Journal of Applied Phycology* (submitted).

Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples

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Abstract

The aim of this study was to develop a PCR-based method of gene-directed multiplex PCR to rapidly identify microcystins producing cyanobacteria, regardless of their taxa, that could be applied in routine freshwater monitoring. A multiplex PCR that simultaneously amplifies *mcy*A-cd, *mcy*AB and *mcy*B fragments of the microcystin gene cluster was validated with DNA from 124 cyanobacterial strains and applied in 37 environmental samples. The toxicological status of the strains was assessed by high-performance liquid chromatography (HPLC) and used as the "gold standard" for the evaluation of multiplex *mcy* genes-based PCR, where a sensitivity of 92.3% and a specificity of 100% have been obtained. For the environmental samples, a rapid protocol for their use in PCR has been developed and, by using ELISA results as "gold standard" for the presence of microcystins in these samples, a sensitivity of 80% and a specificity of 100% were achieved, showing that this multiplex PCR test is a rapid, reliable and economical way of assessing the microcystin producing potential of cyanobacteria in freshwaters, regardless of their taxa or microcystins variant produced.

Keywords: Cyanobacteria; microcystins; multiplex PCR; environmental samples; blooms.

1. Introduction

Cyanobacteria are able to produce a wide range of secondary metabolites including toxins. Of the known cyanotoxins produced, microcystins are the predominant toxins in freshwaters worldwide. This group of hepatotoxins poses a significant threat to human and animal health and has been implicated in acute poisoning incidents involving the death of animals (Matsunaga et al., 1999) and humans (Azevedo et al., 2002). Furthermore, the chronic ingestion of sublethal doses has been associated to hepatocellular carcinoma (Yu et al., 2001).

Microcystins target the liver by specific binding to the organic anion transport system in hepatocyte cell membranes, inhibiting eukaryotic serine/threonine protein phosphatases type 1 and type 2A. This effect results in an excessive phosphorylation of cytoskeletal filaments, which triggers apoptosis of hepatocytes (Batista et al., 2003). The World Health Organization

has placed microcystin on the list of potential health hazards, and has defined a drinking water guideline value of 1 µg/L (WHO, 1998).

Microcystins are cyclic peptides sharing the common structure of Adda- D-Glu-Mdha-D-Ala-L-X-D-MeAsp-L-Z, where X and Z are variable L-amino acids, and Adda is the unusual C_{20} amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methylaspartic acid, and Mdha is N-methyl-dehydroalanine (Sivonen and Jones, 1999). About 70 different variants of microcystins have been described (Codd et al., 2005).

Microcystins are produced nonribosomally via a thio-template mechanism, by a multienzyme complex consisting of peptide synthetases (PS), polyketide synthases (PKS), and tailoring enzymes. The gene cluster for microcystin biosynthesis, containing approximately 55 kb, has been identified and sequenced in the three phylogenetic distantly related species, *Microcystis aeruginosa* PCC 7806 (Tillet at al., 2000), *Planktothrix agardhii* CYA 126 (Christiansen et al., 2003) and *Anabaena* sp. strain 90 (Rouhiainen et al., 2004) (Fig. 1).

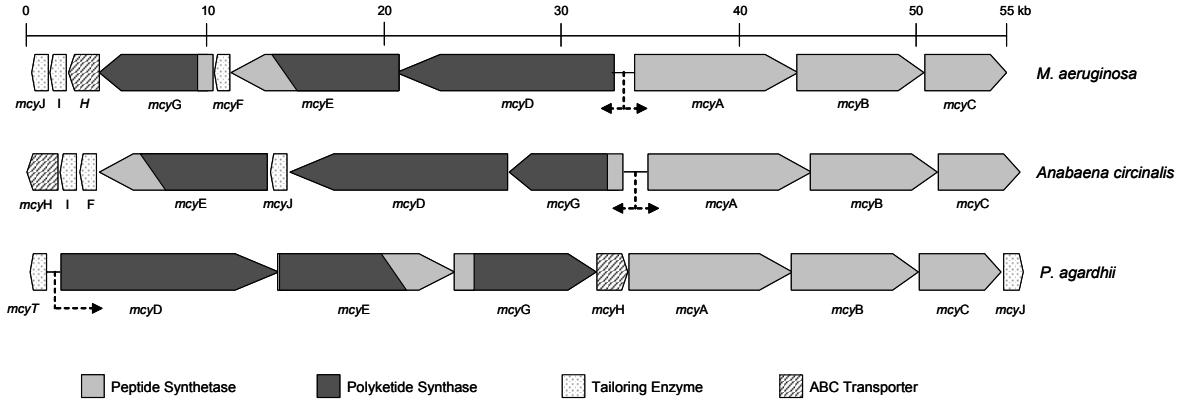

FIGURE 1 Gene clusters coding for the biosynthesis of microcystin in *Microcystis aeruginosa* PCC 7806, *Anabaena circinalis* strain 90 and *Planktothrix agardhii* CYA 126 (adapted from Dittmann and Börner, 2005).

It consists of nine to ten open reading frames (ORFs), six of them with a mixed nonribosomal peptide synthetases / polyketide synthase nature (*mcy*A to *mcy*E and *mcy*G) and three to four smaller ORFs with putative precursor and tailoring functions (*mcy*F and *mcy*H to *mcy*J). The two PKS modules of McyG and McyE, together with the bimodular PKS McyD, are responsible for the synthesis of the unique Adda moiety of microcystins (Tillet et al., 2000; Kaebernick et al., 2002).

The organization of this gene cluster seems unique within each genus (Fig. 1). In *Microcystis* and *Anabaena*, the genes are transcribed from a central bidirectional promoter region, whereas in *Planktothrix* all *mcy* gene except *mcy*T seem to be transcribed unidirec-

tionally from a promoter located upstream of gene *mcy*D. Although the multienzyme components encoded by the different genera are highly similar, the arrangement of their genes clearly differs between *Anabaena* and genera *Microcystis* and *Planktothrix*. Only the *mcy*A-C arrangement seems fairly conserved among toxic strains of the different genera.

The sequence of the gene cluster for microcystin biosynthesis provided the tool to search for specific molecular targets that could be used to discriminate microcystins producing from non-producing cyanobacteria. Several amplified fragments of *mcy* genes have been proposed as molecular markers to detect microcystins production in field samples and in cultured strains. Usually, the amplification of one fragment of one single gene is considered (Baker et al., 2002; Bittencourt-Oliveira, 2003; Hisbergues et al., 2003; Kurmayer et al., 2003; Kurmayer et al., 2004; Kurmayer and Kutzenberger, 2003; Neilan et al., 1999; Pan et al., 2002; Tillet et al., 2001; Vaitomaa et al., 2003). In some cases, however, the separated amplification of more than one fragment of different genes has also been used (Mbedi et al., 2005; Kurmayer et al., 2005; Mikalsen et al., 2003; Ouahid et al., 2005; Rantala et al. 2004). Nevertheless, some of the existing primers, when deduced from only one genus, fail to amplify *mcy* sequences from other genera (Kurmayer et al., 2003; Vaitomaa et al., 2003).

In this work, different PCR methods directed towards specific regions of the *mcy* gene cluster were applied to a collection of cultured cyanobacteria and environmental samples, in order to discriminate microcystins-producing from non-producing strains and develop a rapid and reliable PCR test to detect microcystin producers.

2. Materials and Methods

2.1 Cyanobacterial strains

A total of 124 cyanobacterial strains, representative of three orders of cyanobacteria were used in this study: *Chroococcales* (54), *Oscillatoriales* (15), and *Nostocales* (55) (Table 1). Unicyanobacterial non-axenic cultures were grown and maintained in the LMECYA culture collection using Z8 medium (Skulberg and Skulberg, 1990). The strains were grown under a 14:10 h light:dark cycle (20 ± 4 μmol m^{-2} s^{-1}) at 20 ± 1 ºC. Their taxonomic classification was based on microscopic observation of distinctive morphological characteristics such as: type and form of the colonies, form and dimensions of the vegetative cells, morphology of heterocysts and akinetes when present, as well as their relative position within the colony (Baker 1991, 1992; Bourrelly, 1970; Komárek and Anagnostidis, 1986). The identification of representative strains of each species was also confirmed by 16S rDNA gene sequencing.

TABLE 1 Cyanobacterial species used in this study

Species	Number of isolates
Anabaena circinalis	7
Anabaena flos-aquae	3
Anabaena planctonica	1
Anabaena spiroides	1
Anabaena spp.	2
Anabaenopsis circularis	3
Aphanizomenon flos-aquae	13
Aphanizomenon gracile	8
Aphanizomenon issatschenkoi	5
Aphanizomenon ovalisporum	1
Cylindrospermopsis raciborskii	11
Limnothrix redekei	1
Microcystis aeruginosa	52
Oscillatoria spp.	3
Planktothrix agardhii	9
Planktothrix pseudoagardhii	1
Planktothrix rubescens	1
Synechococcus nidulans	1
Synechocystis sp.	1
Total	124

2.2 Environmental water samples

Water samples (1L) were collected from six freshwater reservoirs in the south Portugal: Alqueva, Alvito, Enxoé, Monte Novo, Odivelas and Roxo from May to December of 2005 and from April to July 2006. Collections were carried out at surface and/or down the water column, from a depth-integrated water samples made up of 3 individual sub-samples taken from the surface to 1 and 3 times the Secchi-disk depth, to collect samples representative of the theoretical euphotic zone (Preisendorfer, 1986). The samples were transported to the laboratory on ice chest. Within 24 hours of collection, sample aliquots (100 mL) were preserved in Lugol's solution and sedimented in the laboratory. A 5 to 25 mL aliquot of each sample was examined in an Utermöhl chamber under an inverted microscope (Olympus CK40) for phytoplankton identification and cell counting.

Competitive ELISA was performed for microcystins analysis using a commercially available plate kit for detection of microcystins in water (EnviroGard® Microcystins Plate Test Kit, Strategic Diagnostics Inc., Newark, USA).

2.3 Extraction and HPLC analysis of microcystins

Microcystins were extracted from cultured lyophilized material using the procedure described by Krishnamurthy et al. (1986), with slight modifications. Briefly, the cells (50 mg) were extracted in 5 mL of butanol-methanol-water solution (5:20:75, v/v/v), for one hour at room temperature by constant magnetic stirring. After homogenization, the cell suspensions were centrifuged and the cell pellets were re-extracted. Supernatants from both extractions were combined and concentrated in Speed Vac (AESI 1000, Savant, NY, USA). The concentrated extracts were subjected to a C18 Sep-Pak solid phase extraction cartridge (Millipore), previously conditioned with 10 mL of methanol 100% and washed with 20 mL of deionized water. The toxin containing fraction was eluted with methanol 80% (v/v) and filtered through a 0.45 µm membrane prior to HPLC analysis.

Reverse phase HPLC was carried out with a Shimadzu HPLC pump (model LC-6A) connected with a silica based reverse phase C18 column (Hypersil ODS 5 µm, 150 x 4.6 mm, Supelco Inc., Bellefonte USA). A 0.05 M phosphate buffer (pH 3) in methanol (42/58, v/v) was used as mobile phase, with a flow rate of 1 mL/min. The UV detection was performed at 238 nm with a photodiode array detector (Shimadzu SPD M10Avp). The absorbance spectrum was scanned between 200 and 300 nm. The presence of microcystins-RR -YR and -LR was confirmed by comparing peak retention times and absorbance spectra in the extracts with those of the standards purchased from SIGMA chemicals (St. Louis, MO, USA).

2.4 Cyanobacterial DNA extraction

Genomic DNA of cyanobacterial strains was extracted from lyophilized material as previously described (Valério et al., 2005).

2.5 Preparation of environmental samples for PCR

The environmental samples were treated differently depending on the presence or not of a dense bloom. In the cases were dense blooms were collected, an aliquot of 500 µL of sample was pelleted in a microcentrifuge at 14000 rpm for 10 min and the supernatant was removed. The cells were washed in 500 µL of sterile Milli-Q water (Millipore), and then pelleted again as above. After removing the supernatant, the cells were ressuspended in 500 µL

of ultra pure water (Gibco) and sonicated in ultrasounds for 5 min. When no dense bloom was observed, the samples were subjected to the same treatment with some modifications: an aliquot of 2 mL of sample was pelleted, and after washing the cells were resuspended in 150 μL of ultra pure water (Gibco).

2.6 PCR amplification of mcyA-cd, mcyAB and mcyB gene fragments

Primers used for PCR amplification of *mcy* gene fragments are shown in Table 2. PCR amplification of each *mcy* gene fragment was performed independently in a 50 μL reaction mixture containing: 1 X PCR buffer (Invitrogen), 0.4 mM of each dNTP (Invitrogen), 0.5 μM of each pair of primers, 10-15 ng of genomic DNA, 3 mM $MgCl_2$ (Invitrogen), 0.5 mg/mL of BSA (Invitrogen) and 1U of *Taq* DNA polymerase (Invitrogen). The amplification of *mcy*A-cd was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 95 ºC for 6 min, followed by 35 cycles of 45 s at 94 ºC, 45 s at 55 ºC and 1 min at 72 ºC and a final extension step of 5 min at 72 ºC.

The amplification of each *mcy*AB and *mcy*B gene fragments was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 95 ºC for 6 min, followed by 35 cycles of 1 min at 95 ºC, 1 min at 50 ºC and 1 min at 72 ºC and a final extension step of 5 min at 72 ºC.

The PCR products were electrophoresed in 1.2% agarose in TBE 0.5 X buffer at 90 V for 2 h, using 1 kb plus DNA Ladder (Invitrogen) as molecular size marker. After staining with ethidium bromide, the image obtained under UV transillumination was digitalized using Kodak 1D 2.0 system.

TABLE 2 Primers used for mcy genes and 16S rDNA amplification

Primer	Target gene	Sequence (5' to 3')	Amplified product (bp)	Reference
*mcy*A-cd 1F	*mcy*A-cd[a]	AAAATTAAAAGCCGTATCAAA	300	Hisbergues et al., 2003
*mcy*A-cd 1R	*mcy*A-cd[a]	AAAAGTGTTTTATTAGCGGCTCAT		Hisbergues et al., 2003
135-F	*mcy*AB	GACTTATAGCCATCTCATCT	541	Mikalsen et al., 2003
676-R	*mcy*AB	TTGACGCTCTGTTTGTAA		Mikalsen et al., 2003
2156-F	*mcy*B	ATCACTTCAATCTAACGACT	955	Mikalsen et al., 2003
3111-R	*mcy*B	AGTTGCTGCTGTAAGAAA		Mikalsen et al., 2003
CYAN106F	16S rDNA	CGGACGGGTGAGTAACGCGTGA	1200	Nübel et al., 1997
CYAN16SR	16S rDNA	GCAATTACTAGCGATTCCTCC		Valério et al., 2005

[a] These primers amplify a region of the condensation domain (cd) of mcyA gene.

2.7 mcy gene fragments multiplex PCR

The multiplex PCR for the simultaneous amplification of *mcy*A-cd, *mcy*AB and *mcy*B gene fragments was performed in a 50 μL reaction mixture containing: 1 X PCR buffer (Invitrogen), 0.4 mM of each dNTP (Invitrogen), 0.2 μM of each of the six *mcy* primers, 0.05 μM of the two 16S rDNA primers (see Table 2), 10-15 ng of genomic DNA (or 5 μl of environmental sample prepared as above), 2.5 mM $MgCl_2$ (Invitrogen), 0.5 mg/mL of BSA (Invitrogen) and 1U of *Taq* DNA polymerase (Invitrogen). Amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 94 ºC for 6 min, followed by 35 cycles of 30 s at 94 ºC, 30 s at 50 ºC and 1 min at 72 ºC and a final extension step of 5 min at 72 ºC.

The PCR products were electrophoresed and the image was obtained as above.

2.8 Data analysis

Microcystins detection by HPLC was used as the "gold standard" for the assessment of the diagnostic ability of *mcy* genes-based PCR detection of microcystin-producing strains of the culture collection. In the case of the environmental samples, the evaluation of the presence of microcystins detected by ELISA was used as the "gold standard". The sensitivity, specificity, and predictive values of individual PCR and multiplex PCR were also assessed (Campbell and Machin, 1990).

3. Results

3.1 Assessment of toxicity by HPLC

The ability to produce microcystins was detected by HPLC in 26 of the 124 strains analyzed. Within the 26 microcystin producers, 25 belong to *Microcystis aeruginosa* (Chroococcales) and the other toxic strain belongs to *Planktothrix rubescens* (Oscillatoriales). Table 3 shows the toxin profiles and concentrations detected in those 26 toxic strains. The amounts and types of toxins produced were diverse among strains. The concentrations of these microcystin variants ranged between 0.20 to 1.52 μg mg^{-1} DW for microcystin-YR, 0.21 to 9.20 μg mg^{-1} DW for microcystin-LR and 0.08 to 3.43 μg mg^{-1} DW for microcystin-RR. Most strains (11 out of 26) produced more than one microcystin variant. Microcystin-LR was the major toxin in 15 strains, being the only microcystin detected in eight of these strains. Microcystin-RR, in turn, was detected in 16 strains, being the major toxin in nine of these strains and the only microcystin variant present in five strains. Microcystin-YR, on the other hand, was detected in eight strains, but never as the single variant. [D-Asp^3]MCYST-RR was only detected in the P. rubescens strain at a level of 3.15 μg mg^{-1} DW.

Besides microcystin-LR, -RR, -YR and [D-Asp3]MCYST-RR, other peaks were detected in HPLC chromatograms of some cyanobacterial isolates, showing the typical absorbance spectrum of microcystins, with a maximum absorbance at 238-240 nm (data not shown). These compounds could not be assigned to known microcystin variants due to the lack of analytical standards, but their presence was detected in a considerable number of toxic isolates (12 out of 26).

TABLE 3 Microcystins contents detected by HPLC in the 26 cyanobacterial toxic isolates

Isolate	**Microcystins concentration ($\mu g\ mg^{-1}$ DW)**			
	MCYST-YR	**MCYST-LR**	**MCYST-RR**	**Others**
M. aeruginosa				
LMECYA 2	-	0.71	0.08	a
LMECYA 3	-	3.45	0.20	a
LMECYA 7	-	4.90	0.15	-
LMECYA 53	0.68	1.58	0.95	-
LMECYA 55	0.37	2.05	1.86	-
LMECYA 56	0.80	0.81	0.61	-
LMECYA 57	0.21	1.25	0.13	-
LMECYA 59	0.67	0.78	1.11	-
LMECYA 87	-	-	0.33	-
LMECYA 92A	-	6.90	-	a
LMECYA 92B	-	0.33	-	-
LMECYA 92C	-	1.24	-	-
LMECYA 93	-	6.59	-	a
LMECYA 103	-	-	2.53	a
LMECYA 106	-	1.61	-	a
LMECYA 107	-	-	2.54	a
LMECYA 108	-	-	2.41	a
LMECYA 110	-	9.20	-	-
LMECYA 113	-	3.02	-	-
LMECYA 151	0.82	0.71	0.75	a
LMECYA 159	0.20	-	1.12	-
LMECYA 160	-	-	2.54	a
LMECYA 167	-	-	-	a
LMECYA 170	-	0.21	-	a
LMECYA 179	1.52	1.01	3.43	-
P. rubescens				
LMECYA 203	-	-	-	3.15 [D-Asp^3]MCYST-RR

a - Suspected but not quantified.

3.2 PCR amplification of individual mcyA-cd, mcyAB and mcyB fragments

PCR amplifications of each *mcy*A-cd, *mcy*AB and *mcy*B gene fragment were performed in separate reactions for 66 isolates, including 10 toxic and 56 non-toxic (data not shown).

The *mcy*A-cd gene fragment PCR resulted in the amplification of a single 300 bp product in 17 out of the 66 isolates tested. Comparing these results with those presented in Table 3, only 10 of these 17 isolates having the *mcy*A-cd gene fragment correspond to microcystins producers. In the case of the *mcy*AB gene fragment, the amplification of a single 541 bp product was obtained for 15 of the 66 isolates tested. From these 15 isolates, however, only 10 correspond to microcystins producers. Finally, PCR directed for *mcy*B gene fragment resulted in the amplification of a single 955 bp product for 16 of the 66 isolates tested, only nine of those *mcy*B$^+$ isolates being detected as microcystins producers by HPLC. Additionally, isolate LMECYA 167, negative for *mcy*B amplification, is a microcystin producer, according to HPLC analysis (Table 3).

The *mcy*B fragment amplified from five non-toxic isolates, LMECYA 10 (*Aphanizomenon flos-aquae*), LMECYA 31 (*Aphanizomenon issatschenkoi*), LMECYA 40 (*Aphanizomenon gracile*), AQS (*Cylindrospermopsis raciborskii*) and LMECYA 153A (*Planktothrix agardhii*) was sequenced. Sequencing and nucleotide Blast analysis of the amplified fragments showed a homology of 94%, 99%, 99%, 99% and 93% respectively, with the *Microcystis aeruginosa* K-139 *mcy*B gene for microcystin synthetase (GenBank accession number: AB019578).

Prior to the multiplex PCR development we have also tested another *mcy* molecular marker: *mcy*E, experienced by others and seemed to display good results with different toxic species (Mankiewicz-Boczek et al., 2006, Rantala et al., 2004). The *mcy*E fragment PCR amplification was carried out as described previously (Rantala et al., 2004) with DNA from seven isolates, including three toxic (LMECYA 2, 7 and 167) and four non-toxic (LMECYA 40, 81 127 and 177) (data not shown). Beyond all toxic isolates, amplification of the *mcy*E fragment was also observed in two of the non-toxic isolates (LMECYA 81 and 177), pointing to the unsuitableness of this marker.

3.3 Evaluation of the multiplex PCR

The suitability of the multiplex PCR was tested with DNA from 124 cyanobacterial isolates, including the 66 isolates previously submitted to independent PCR amplifications of *mcy* genes.

For 20 non-toxic isolates, the multiplex PCR resulted in the amplification of one or two *mcy* fragments tested (Fig. 2A). The *mcy*A-cd fragment (300 bp) was amplified in all these 20 isolates including one *Anabaena planctonica* (LMECYA 177), one *A. circinalis* (LMECYA 175), three *Aphanizomenon flos-aquae* (LMECYA 77A, 88 and 89), two *Aph. issatschenkoi* (LMECYA 31 and 166), two *Aph. gracile* (LMECYA 40 and 64), two *Cylindrospermopsis raciborskii* (LMECYA 134 and Marau 1), one *Limnothrix redekei* (LMECYA 145), two *Microcystis aeruginosa* (LMECYA 144 and 183), and three *P. agardhii* (LMECYA 152, 153E and 153F) isolates. The simultaneous amplification of the *mcy*A-cd and *mcy*AB gene fragments (300 and 541 bp) was obtained in three of these 20 non-toxic isolates, namely, in one *Anabaena circinalis* (LMECYA 123) and in two *M. aeruginosa* (LMECYA 91B and 137). For the remaining 78 non-toxic isolates, the PCR resulted only in the amplification of a single 1200 bp product correspondent to the 16S rDNA (represented by the LMECYA 127 profile in Fig. 2A).

In the multiplex PCR assay, a positive result is only considered when all the three fragments are amplified. The simultaneous amplification of all the *mcy* gene fragments tested (*mcy*A-cd, *mcy*AB and *mcy*B) was only obtained for microcystin producing isolates (Fig. 2B). However, two false negatives were observed. These false negatives correspond to a toxic *M. aeruginosa* isolate (LMECYA 167), which failed to amplify the *mcy*B fragment, and to a toxic *P. rubescens* isolate (LMECYA 203), where only the *mcy*A-cd fragment was amplified. Table 4 summarizes these results by showing the sensitivity, specificity, and predictive values obtained for the individual PCRs and multiplex PCR in the screening of microcystin producing isolates. As shown, 100% sensitivity (no false-negatives) was observed for individual *mcy*A-cd and *mcy*AB PCRs, whereas 100% specificity (no false-positives) was only obtained for multiplex PCR. Regarding prediction ability, high similar predictive values were obtained in all methods for the negative test, whereas only the multiplex PCR showed 100% predictiveness for the positive test. So, the diagnosis based on the simultaneous detection of the three *mcy* markers by multiplex PCR showed the highest specificity and predictive values, associated with high sensitivity and good correlation with HPLC results.

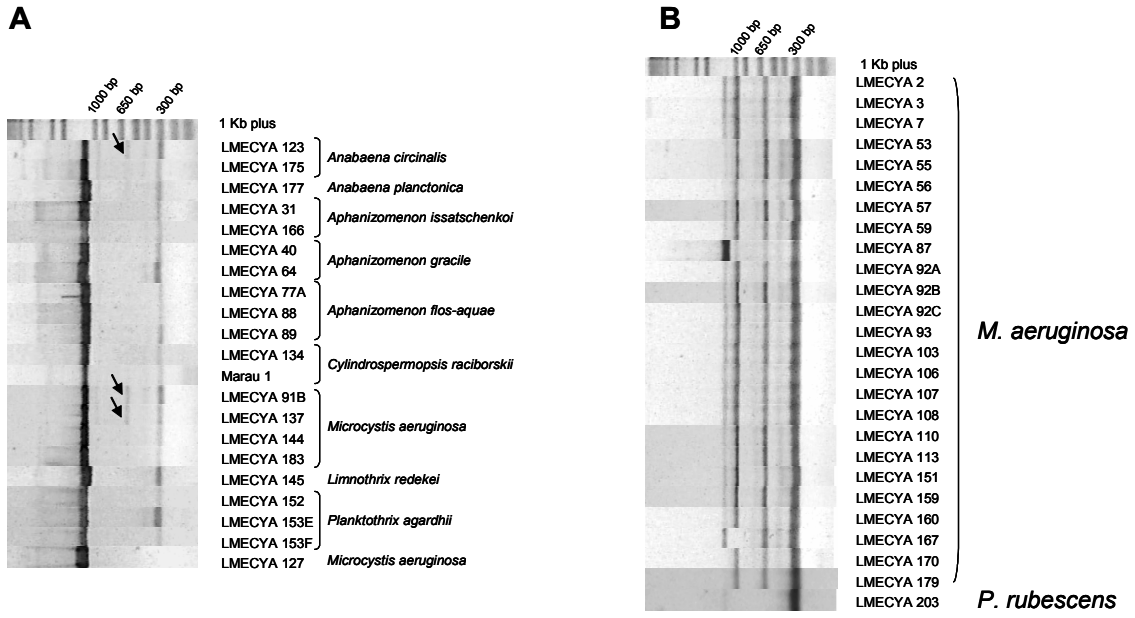

FIGURE 2 (A) Results obtained by multiplex PCR assay for the simultaneous amplification of *mcy*A-cd, *mcy*AB and *mcy*B in 21 non-toxic isolates. (B) Multiplex PCR assay results of the 26 toxic isolates. The amplification of a cyanobacterial 16S rDNA gene fragment was also included as a PCR reaction control. Marker: 1 kb plus DNA Ladder.

TABLE 4 Reliability of *mcy*-based PCR methods tested for the diagnosis of microcystin-producing isolates. HPLC analysis of microcystins was used as the "gold standard".

Parameter	No. of isolates based on mcyA-cd PCR	No. of isolates based on mcyAB PCR	No. of isolates based on mcyB PCR	No. of isolates based on multiplex PCR
True positive	10 (15.2)[c]	10 (15.2)	9 (13.6)	24 (19.4)
False positive	7 (10.6)	5 (7.6)	7 (10.6)	0 (0)
True negative	49 (74.2)	51 (77.3)	49 (74.2)	98 (79.0)
False negative	0 (0)	0 (0)	1 (1.5)	2 (1.6)
Sensitivity	100.0	100.0	90.0	92.3
Specificity	87.5	91.1	87.5	100.0
PPV[a]	58.8	66.7	56.3	100.0
NPV[b]	100.0	100.0	98.0	98.0
Number of tested isolates	66	66	66	124

[a]PPV, predictive value of positive test; [b]NPV, predictive value of negative test. [c]Values in parentheses refer to percentages of the total of tested isolates.

3.4 Application of the multiplex PCR to environmental samples

Prior to the application to environmental samples, multiplex PCR was tested with 2 μL of five well grown fresh cultures, three toxic and two non toxic, without previous DNA extraction (data not shown). The results were identical to those obtained using culture DNA in the PCR reaction.

The multiplex PCR method developed was applied to 10 environmental samples. In the initial trial experiments (data not shown), PCR amplification was attempted with unwashed cell suspensions. The obtained profiles were in some cases diffuse and in others no PCR product was observed, even for the 16S rDNA control. These results could be due to the presence of nucleases that cause degradation of DNA; agents that inhibit the DNA polymerase activity, e.g., humic acids, which are the most commonly reported group of inhibitors in environmental samples, and oils; insufficient or excess (in the case of the fresh blooms) DNA template; or lack of access to the sample DNA. However, the method described above for the environmental samples preparation for PCR is simple and quick and has proven to be effective with fresh bloom material, when most cells are intact.

To assess the toxicity of environmental samples an ELISA kit was used. This toxicity test enables determination of microcystins over the range 0.1-1.6 μg/L of microcystin-LR and its equivalents. Of the 37 environmental samples tested, 10 were positive for microcystins and three also exceeded the drinking water guideline value of 1 μg/L. Samples Odivelas s3 and Odivelas s4 refer to a Microcystis spp. bloom prevalent during two months and that was accumulated at one side of the reservoir. The samples collected from the same reservoir and made up of 3 sub-samples only presented 0.5 μg/L of microcystins (Odivelas c4), whereas the Odivelas s4 sample (the dense fresh bloom) presented 222.2 μg/L of microcystins.

The ELISA results were corroborated by the multiplex PCR reaction in almost all the samples (illustrated in Fig. 3). The 16S rDNA fragment was always amplified (no false negatives) and congruent with the presence of cyanobacteria in all collected samples. In the Odivelas c3 sample *mcy*A-cd (300 bp) and *mcy*B (955 bp) fragments were amplified, but since the *mcy*AB fragment was not detected we considered this sample as negative, which is in agreement with the ELISA results. The reliability of the multiplex PCR has been evaluated for the environmental samples and 80% sensitivity and 100% specificity were obtained. Regarding predictive values of the multiplex PCR, 93.1% and 100% were obtained for the negative and positive tests, respectively. So, the diagnosis based on the simultaneous detection of the three *mcy* markers in the multiplex PCR reaction also showed high specificity and predictive values and displayed good correlation with the ELISA results, for environmental samples.

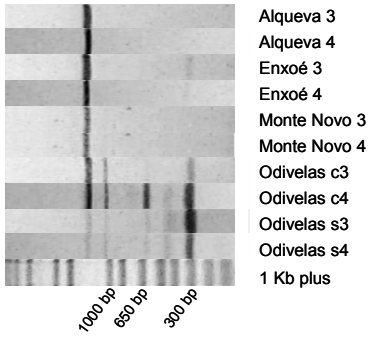

FIGURE 3 Representative results of the *mcy* multiplex PCR obtained in environmental samples. Marker: 1 kb plus DNA Ladder.

4. Discussion

In order to develop a rapid and more reliable PCR test to detect microcystin producers, we search in the literature for suitable primers to discriminate microcystins-producing from non-producing isolates. First we selected primers that have been tested by others and displayed good results with different toxic species. This was the case for the *mcy*A-cd fragment tested by Hisbergues et al. (2003) and Rantala et al. (2004) in *Microcystis*, *Anabaena* and *Planktothrix*. Thereafter, we also included primers for *mcy*AB and *mcy*B gene fragments, which had only been tested for *Microcystis*, but showed good results regardless of the microcystin variants produced (Mikalsen et al., 2003). Our first approach in order to discriminate microcystin-producing from non-producing isolates was to test the amplification of these three different markers individually, in three separated PCR reactions. We expected that at least one of those markers could perform this differentiation within our cyanobacteria collection. PCR directed towards *mcy*A-cd or *mcy*AB genes resulted systematically in the amplification of fragments with the correspondent expected size for all the toxic isolates. Thus, these *mcy* markers showed a good sensitivity towards microcystin producers, regardless of their toxin contents and profiles. Unexpectedly, however, the same amplicons were also obtained in a significant number of non-toxic isolates: seven for *mcy*A-cd, five for *mcy*AB and seven for *mcy*B. These false positives reflected the poor specificity of the markers towards microcystin producers, when used alone.

A small number of *Microcystis* spp. (Mikalsen et al., 2003; Tillet et al., 2001) and *Planktothrix* spp. (Kurmayer et al., 2004) strains have been shown to contain microcystins biosynthetic genes, although lacking detectable microcystins. The reason why those strains do not synthesize microcystins is unclear. It has been hypothesized that the sporadic distribution of

microcystin producers, either between or within genera of cyanobacteria, is the result of repeatedly losing the ability to produce microcystin in the course of evolution (Rantala et al., 2004). This fact has to be considered when describing a molecular test for the discrimination of microcystins producers from non producers. If only one *mcy* fragment is used to assess the microcystin production potential, false positives can then be expected.

This led us to design a multiplex PCR in which the three *mcy* markers must be simultaneously amplified in order to give a positive result. In this design we have also included the amplification of a 16S rDNA gene fragment, using primers specific for cyanobacteria (Nübel et al., 1997; Valério et al., 2005). This allowed us to confirm that the DNA used as a template in the PCR had a cyanobacterial origin, and at the same time acted as a PCR control in the negative results. Different species, from different genera and within different orders, were included in this study to assess and validate the potential use of this PCR method in identifying toxigenic cyanobacteria regardless of their taxa. Moreover, the observed diversity in toxin profiles and contents among the toxic isolates made them suitable to test the effectiveness of PCR methods to differentiate toxic from non-toxic isolates, regardless of the microcystins variants being produced. By using this multiplex PCR we have only obtained the simultaneous amplification of the three *mcy* fragments in microcystins producing isolates, regardless of the microcystins variants being produced. No false positives were obtained and the specificity of the method increased to 100%. Moreover, the inclusion of a 16S rDNA control fragment made it easier to decide on the veracity of a negative result.

The molecular method applied was also highly sensitive (92.3%), however, two false negatives were obtained. One of those false negatives corresponded to a *M. aeruginosa* isolate (LMECYA 167) which failed to amplify the *mcy*B gene fragment either by individual PCR or by multiplex PCR. Contrarily to all the other toxic isolates, the HPLC chromatograms of this isolates showed no peaks with retention times close to the standards microcystin-LR, -RR and -YR. The analysis only showed one major unidentified peak deduced to be a member of the microcystin family by its UV spectra. Given the PCR results, we started to question this assumption. So, we decided to apply this isolate to a commercial ELISA kit for the detection of microcystins in water (Envirogard, Strategic Diagnostics Inc, Newark, USA). The results obtained by ELISA for this isolate were comparable with those obtained for other well established toxic isolates (data not shown). Since no other peaks were observed in the HPLC chromatogram that could explain the detection of microcystins by ELISA, we concluded that the compound was in fact a microcystin variant. The reason why the isolate LMECYA 167 failed to amplify the *mcy*B gene fragment remains unclear. We can only speculate that it might have

suffered a mutation in the annealing site of the *mcy*B primers and this would, in turn, result in the absence of PCR amplification.

The other false negative was obtained with a *Planktothrix rubescens* isolate (LMECYA 203), where only the *mcy*A-cd fragment was amplified. The lack of amplification of the other two fragments might be due to mutations within the *mcy* gene cluster which prevented the annealing of the primers used for *mcy*AB and *mcy*B amplification. In fact, Christiansen et al. (2006) showed the wide presence of insertions and deletions in the *mcy* gene clusters of *Planktothrix rubescens* strains isolated from different regions. Although the sequence of the *mcy* gene cluster from our isolate was not analyzed, it is reasonable to assume that similar mutations might have occurred in this isolate.

Despite the small number of false negatives, the multiplex PCR, showed a high specificity and sensitivity towards microcystin producing isolates.

Toxicity testing by available methods (chemical assay, mouse bioassay, protein synthesis inhibition assay) can require high concentrations of cells and toxins and can take up to a week to obtain the results. At this time blooms are already established and even perfectly visible to public, increasing the risk to exposure to the toxins. Since an indication of the toxicity in early stages could minimize these problems and given the lack of toxin analytical standards and the resource limitations on the wide-scale of analytical methods, this rapid multiplex PCR-based low-cost technique shows a clear potential as an alternative method for the regular screening of microcystins producers and potentially bloom forming in routine freshwater monitoring. The results obtained with the environmental samples showed that this method works well with these samples and the sample preparation described makes it an easy-to-use method to be applied in freshwaters monitoring, detecting potential toxigenic cyanobacteria in early stages of bloom formation, and therefore acts as an effective alert to possible health risks.

Acknowledgements

Elisabete Valério thanks the financial support (Grant SFRH/BD/8272/2002) by Fundação para a Ciência e a Tecnologia.

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Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters

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Received 5 August 2004; received in revised form 28 February 2005; accepted 10 March 2005

Abstract

Cylindrospermopsis raciborskii is a toxic bloom forming cyanobacteria that is a common component of the phytoplankton assemblage in temperate freshwaters, as well as in temperate climates. This species is of major concern in public health, due to its known ability to produce toxins, including cylindrospermopsin and paralytic shellfish poisoning toxin (PSP).

In this study, M13 PCR fingerprinting, ERIC PCR fingerprinting and amplification of the internal transcribed spacer (ITS) region were used to characterize nine cultured strains of *C. raciborskii*, sourced from several freshwater lakes and rivers in Portugal, and two other Australian. Strains belonging to other taxa including *Microcystis aeruginosa*, *Aphanizomenon* spp., *Planktothrix agardhii* and *Oscillatoria neglecta* were also analysed to evaluate the taxonomical potential of the fingerprinting methods.

Data obtained from genomic fingerprinting were used to perform hierarchical cluster analysis and demonstrated ability to differentiate strains at intra-specific level. However, the high level of variability prevents their use as an identification tool. ITS amplification displayed intra-specific polymorphism both in number and length of the obtained amplicons, but revealed itself as a good method for strain clustering. The unsuccessful amplification of peptide synthetase (PS) and polyketide synthase (PKS) genes pointed to the inability of Portuguese *C. raciborskii* strains to produce cylindrospermopsin. HPLC analysis further confirmed this lack of toxicity, since negative results were obtained for cylindrospermopsin and PSP toxins.

Keywords: *Cylindrospermopsis raciborskii*; ERIC PCR fingerprinting; ITS; M13 PCR fingerprinting; *PS* and *PKS* genes; PSP toxins

1. Introduction

Cylindrospermopsis raciborskii is a well-known toxic bloom-forming cyanobacteria, originally described as a species of tropical origin (Woloszynska, 1912), but increasingly found across a wide range of

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doi:10.1016/j.hal.2005.03.002

latitudes. This species is of major concern from a water quality and public health perspective, due to its known ability to produce toxins, including the potent hepatotoxic alkaloid cylindrospermopsin (Hawkins et al., 1985; Li et al., 2001; Saker and Neilan, 2001; Fastner et al., 2003), and the highly toxic paralytic shellfish poisons (PSP) (Lagos et al., 1999). Suspected causation of human sickness by cylindrospermopsin (Hayman, 1992) and cattle mortality associated with *C. raciborskii* (Saker et al., 1999) has, so far, been restricted to Australia. However, the recent reports of *C. raciborskii* from many temperate countries have highlighted the invasive nature of this species, spreading worldwide.

Saker et al. (2003) recently reported the occurrence of *C. raciborskii* in several Portuguese water bodies used for potable and recreational purposes. Although tests for the presence of cylindrospermopsin and PSP were negative, a number of strains grown in pure culture showed atypical toxicity by mouse bioassay, suggesting that a new type of toxin should be present. While the chemical structure of the causative compound(s) was not determined, this report drew attention to the need for a comprehensive research on the genetic variability of Portuguese *C. raciborskii* and pointed to the need for an adequate monitoring of this cyanobacterium in freshwater reservoirs.

So far, several molecular methods have been used to characterize *C. raciborskii*, including the genetic analysis of the 16S rRNA (Chonudomkul et al., 2004; Saker and Neilan, 2001) and *rpoC1* (Wilson et al., 2000) genes, which display a considerable consensus within the species. On the contrary, other genomic targets such as *Hip1* (Saker and Neilan, 2001; Smith et al., 1998), STRR sequences (Fergusson et al., 2000; Wilson et al., 2000), *nifH* (Dyble et al., 2002) and PC-IGS (Baker et al., 2001; Dyble et al., 2002) showed significant variations among different strains of *C. raciborskii*.

The M13 PCR fingerprinting has never been used in cyanobacteria, although its use in other bacteria (Grif et al., 1998) and yeasts (Valério et al., 2002) pointed to its potential as an identification tool. On the other hand, techniques based on highly repetitive sequences such as enterobacterial repetitive intergenic consensus (ERIC) have already been used for differentiation of some cyanobacterial genera showing different profiles for all the strains tested (Lyra et al., 2001). The amplification of the internal transcribed spacers (ITS) of rDNA operons from planktonic heterocystous cyanobacteria (Iteman et al., 2002) also showed that these regions displayed size variability.

In the search for molecular tools able to differentiate the toxic from non-toxic strains of *C. raciborskii*, nine cultured strains of this species, sourced from several Portuguese freshwater lakes and rivers, were characterized by using M13 and ERIC fingerprinting and amplification of ITS region, techniques that have not been applied yet to this particular species. The Portuguese isolates were compared with two Australian ones and strains belonging to other taxa, including *Microcystis aeruginosa*, *Aphanizomenon* spp., *Planktothrix agardhii* and *Oscillatoria neglecta*, were also analysed in order to evaluate the taxonomical potential of these molecular methods.

The ability of *C. raciborskii* to produce cylindrospermopsin was evaluated by the amplification of the polyketide synthase (PKS) and peptide synthetase (PS) genes. Detection of cylindrospermopsin and PSP toxins was performed by HPLC analysis.

2. Materials and methods

2.1. Strains

The strains used in this study are listed in Table 1, including their code, origin and references to toxicity assessment. Lyophilised samples of the isolates were obtained as described by Saker et al. (2003). All strains were identified morphologically at species level. All *C. raciborskii* strains shared common morphological traits and presented straight trichome morphology, as described for this species.

2.2. DNA extraction

Genomic DNA of cyanobacterial strains was extracted following the method described by Pitcher et al. (1989), with some modifications. Briefly, an aliquot of 100 μl of lyophilised culture was suspended in a lysis buffer containing 50 mM Tris, 250 mM NaCl, 50 mM EDTA, 0.3% SDS, pH 8 and mechanically broken with glass beads (400–600 μm) by vortex shaking for 2 min. The suspension was incubated for 1 h at 65 °C. After new vortex shaking for 2 min, 1000 μl of GES (5 M guanidium thiocyanate, 100 mM

Table 1
Cyanobacterial strains used in this study

Species	Strain no.	Origin	Toxicity	
			PS gene/*PKS* gene[a]	CYL/PSP[b]
Cylindrospermopsis raciborskii	4899	Ardila	−/−	−/−
	Caia	Caia	−/−	−/−
	J5	Maranhão	−/−	−/−
	Marau 1	Maranhão	−/−	−/−
	4799	Odivelas	−/−	−/−
	LMECYA 134	Odivelas	−/−	−/−
	LMECYA 132	Portalegre	−/−	−/−
	LMECYA 135	Portalegre	−/−	−/−
	LMECYA 168	Portalegre	−/−	−/−
	AQS	Queensland (Australia)	+/+	+/−
	LJ	Lake Julius (Australia)	−/−	−/−
Aphanizomenon issatschenkoi	LMECYA 31	Montargil	NT[c]	NT/−
Aphanizomenon ovalisporum	MS1	Israel	+/+	+/−
Microcystis aeruginosa	LMECYA 7	Montargil	NT	NT
Planktothrix agardhii	LMECYA 152	Magos	NT	NT/−
	LMECYA 153	Enxoé	NT	NT/−
	LMECYA 153B	Enxoé	NT	NT/−
Oscillatoria neglecta	LMECYA 173	Hydrothermal pond	NT	NT/−

[a] Results from the PCR detection of *PS* (peptide synthetase) and *PKS* (polyketide synthase) genes.
[b] Results from the HPLC detection of toxins: CYL, cylyndrospermopsin; PSP, paralytic shellfish poisons.
[c] NT, not tested.

EDTA, 0.5% (v/v) sarcosil) were added and the suspension was incubated at 4 °C overnight. After addition of 250 μl of 10 M ammonium acetate and 10 min incubation on ice, nucleic acids were extracted with chloroform:isoamilic alcohol (24:1) and precipitated with one volume of isopropanol. After centrifugation, DNA was washed with ethanol 70% (v/v), ressuspended in TE with 1 mg proteinase K and incubated for 2 h at 50 °C. A second extraction with chloroform:isoamilic alcohol (24:1) was performed and DNA was precipitated with 1/10 volume of sodium acetate 3 M, pH 5.2 and 2.5 volumes of ethanol, centrifuged, washed with ethanol 70% (v/v) and ressuspended in 100 μl of TE.

2.3. ITS amplification

The PCR reaction was performed in 50 μl containing 1× PCR buffer, 0.4 mM of each of the four dNTPs, 50 μM of PS1490 (5′-TGCGGCTGGATCCCCTCC-TT-3′) and PL132 (5′-CCGGGTTTCCCATTGG-3′) universal primers (Normand et al., 1996), 10–15 ng of genomic DNA, 2 mM $MgCl_2$, 1 mg ml^{-1} of BSA and 1 U of *Taq* DNA polymerase. Amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 95 °C for 6 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C and a final extension step of 5 min at 72 °C.

The PCR products were electrophoresed in 1.4% agarose in TBE 0.5× at 90 V for 3 h 30 min, using 1 Kb plus DNA Ladder (Invitrogen) as molecular size marker. After staining with ethidium bromide, image was obtained under UV transillumination and digitalized using the Kodak 1D 2.0 system. Gel images were analysed with BioNumerics software (Applied Maths, Kortrijk, Belgium) and strain clustering was performed with Pearson's correlation coefficient and UPGMA method (Sneath and Sokal, 1973). Strain clusters were correlated with both morphological species allocation and available data on toxicity. Species identification was confirmed by 16S rDNA sequencing of a representative from each cluster.

2.4. 16S rDNA PCR amplification

PCR amplification of complete 16S rDNA gene was performed using the universal primers PA (5′-AGAGTTTGATCCTGGCTCAG-3′) and PH (5′-AAGGAGGTGATCCAGCCGCA-3′) (Massol-Deya et al.,

1995). The PCR reaction was performed in 50 μl containing 1× PCR buffer (Invitrogen), 0.2 mM of each of the four dNTPs (Invitrogen), 25 μM of each primer, 10–15 ng of genomic DNA, 2 mM $MgCl_2$, 0.5 mg ml^{-1} of BSA and 1 U of *Taq* DNA polymerase (Invitrogen). Amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C and a final extension step of 3 min at 72 °C. The amplification reaction products were purified with a JETquick spin column (Genomed, Germany) and sequenced on a Beckman-Coulter automated DNA sequencer (model CEQ-2000) with dye terminators, using standard protocols and the cyanobacterial specific primers CYAN16S (5′-ATA CCC CWG TAG TCC TAG C-3′) and CYAN16SR (5′-GCA ATT ACT AGC GAT TCC TCC-3′) designed in this study. The first primer recognition site is located in positions 738–765 and the other primer recognition site is located in positions 1281–1302 of the 16S rRNA operon of *Synechocystis* PCC 6803 (GenBank accession no. NC000911). The confirmation of the identification was performed by using the BLAST tool of the Nacional Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

2.5. PCR fingerprinting

M13 PCR fingerprinting was performed using the single primer M13 (5′-GAGGGTGGCGGTTCT-3′) (Meyer et al., 1993). For ERIC PCR fingerprinting both ERICIR (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) primers (Versalovic et al., 1991) were used.

The PCR reactions were performed in 50 μl containing 1× PCR buffer (Invitrogen), 0.4 mM of each of the four dNTPs (Invitrogen), 100 μM of each primer, 10–15 ng of genomic DNA, 3 mM (for M13 fingerprinting) or 2 mM (for ERIC fingerprinting) $MgCl_2$, 1 mg ml^{-1} of BSA and 1 U of *Taq* DNA polymerase (Invitrogen). Amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of 90 s at 95 °C, 2 min at 56 °C (for M13 fingerprinting) or 40 °C (for ERIC fingerprinting) and 2 min at 72 °C and a final extension step of 5 min at 72 °C.

The PCR products were electrophoresed in 1.4% agarose gel in TBE 0.5× at 90 V for 3 h 30 min, with 1 Kb plus DNA Ladder as size marker. Gel images were obtained as described above.

DNA banding patterns obtained with both fingerprinting methods were analysed using the BioNumerics software (Applied Maths, Kortrijk, Belgium). Similarities among strains were estimated using Pearson's correlation coefficient and clustering was based on the UPGMA method.

2.6. PS gene and PKS gene amplification

For the *PS* gene amplification, the PCR reaction was performed in 50 μl containing 1× PCR buffer, 0.4 mM of each of the four dNTPs, 50 μM of PS M13 (5′-GGCAAATTGTGATAGCCACGAGC-3′) and PS M14 (5′-GATGGAACATCGCTCACTGGTG-3′) primers (Schembri et al., 2001), 10–15 ng of genomic DNA, 2.5 mM $MgCl_2$, 1 mg ml^{-1} of BSA and 1 U of *Taq* DNA polymerase. Amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 95 °C for 6 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C and a final extension step of 5 min at 72 °C. For the *PKS* gene amplification, the PCR reaction was performed as above, using PKS M4 (5′-GAAGCTCTGGAATCCGGTAA-3′) and PKS M5 (5′-AATCCTTACGGGATCCGGTGC-3′) primers (Schembri et al., 2001). The PCR products were electrophoresed in 0.8% agarose in TBE 0.5× at 90 V for 2 h, with 1 Kb plus DNA Ladder as size marker.

To confirm that the PCR amplicons corresponded to the *PS* and *PKS* genes, the amplification reaction products were purified with a JETquick spin column (Genomed, Germany) and sequenced on a Beckman-Coulter automated DNA sequencer (model CEQ-2000) with dye terminators, using standard protocols with the same primers used for the amplification. Sequences were analysed with the BLAST tool of the Nacional Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

2.7. Toxin analysis by HPLC

Analysis of *C. raciborskii* for the presence of cylindrospermopsin was performed by reverse phase

HPLC and mass spectroscopy according to Eaglesham et al. (1999).

Presence of PSP toxins was assessed by HPLC-FLD according to the method of Oshima (1995).

3. Results

3.1. Molecular identification

The set of primers used to amplify the ITS regions of the 11 Portuguese isolates of *C. raciborskii* strains revealed polymorphisms both in number and length of the obtained amplicons (Fig. 1). The number of amplified amplicons varied between two and six, and the size ranged from 375 to 875 bp. Since the primer PS1490 has the recognition site located in position 1469–1489 of the 16S rRNA gene of *Synechocystis* PCC 6803 (GenBank accession no. NC000911) and the primer PL132 has the recognition site located in position 262–278 of the 23S rRNA gene of this strain, the amplified fragments correspond to the length of the ITS plus a total of ca. 300 bp from the adjacent genes and therefore represent ITS regions of length 70–575 bp. As already described before (Iteman et al., 2002), in all the profiles there are less intensive bands, some with more than 875 bp and others of intermediate size between 375 and 875 bp, which are probably heteroduplexes formed during the PCR amplification by reannealing of single strands of the different ITS species.

Hierarchic analysis of the ITS patterns led to the formation of two major clusters at 45% similarity. The first cluster included strains that presented an almost identical ITS banding pattern, whereas the other cluster presented some ITS heterogeneity. From the first cluster, three strains were already confirmed as *C. raciborskii* isolates (Neilan et al., 2003) by 16S rDNA sequencing: Caia (GenBank accession no. AF516742), 4799 (GenBank accession no. AF516741) and Marau 1 (GenBank accession no. AF516739). A representative from the other cluster was selected for 16S rDNA sequencing and its identification as *C. raciborskii* was confirmed by BLAST homology search (LMECYA 134, GenBank accession no. AY699989).

3.2. Strain differentiation by PCR fingerprinting

Both M13 and ERIC PCR fingerprinting methods produced variable DNA banding patterns that allowed discrimination of all *C. raciborskii* strains (Fig. 1). Strains J5 and Marau 1, both sourced from Maranhão reservoir, presented some differences in their patterns and grouped in separated sub-clusters. Similar differences were also observed between strains 4799 and LMECYA 134, both sourced from Odivelas reservoir, and between LMECYA 132, LMECYA 135 and LMECYA 168, also sourced from the same reservoir (Portalegre). The two Australian strains grouped in a sub-cluster at 68.6% similarity and displayed a fingerprint profile distinct from the Portuguese strains.

To assess reproducibility of the fingerprinting methods, duplicates derived from different experiments, where the entire procedure was repeated, were used for dendrogram construction (data not shown). The average similarity between these duplicates was 93.5%, pointing to a good reproducibility of the techniques, since the band intensity may influence the final similarity values when Pearson's correlation coefficient is used.

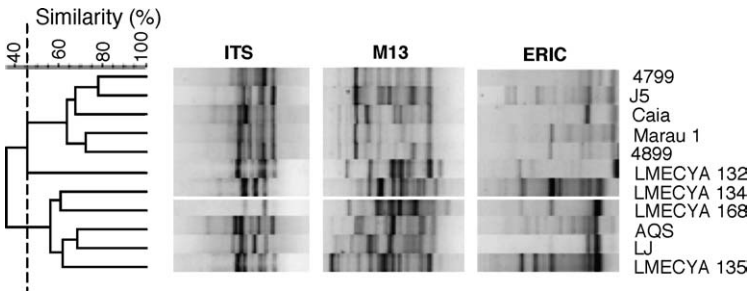

Fig. 1. Dendrogram obtained from the combined analysis of the ITS profiles and M13 and ERIC PCR fingerprinting data of *Cylindrospermopsis raciborskii* strains, using Pearson's correlation coefficient and UPGMA agglomeration method.

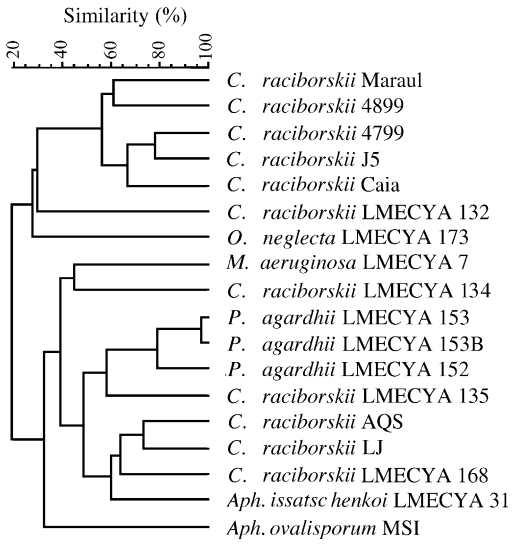

Fig. 2. Dendrogram obtained from the combined analysis of M13 and ERIC PCR fingerprinting data of all the strains listed in Table 1, using Pearson's correlation coefficient and UPGMA agglomeration method.

To evaluate the taxonomical potential of molecular methods, strains belonging to other taxa including *M. aeruginosa*, *Aphanizomenon* spp., *P. agardhii* and *O. neglecta* were also analysed. In the dendrogram obtained with the combined analysis of M13 and ERIC fingerprints for all species (Fig. 2), almost all strains were interspersed along the dendrogram and no grouping was observed at species level. Only isolates from *P. agardhii* formed a coherent sub-cluster according to the species. In this cluster, isolates LMECYA 153 and LMECYA 153B sourced from the same reservoir presented an overall similarity of 97.5% (higher than the reproducibility level) and should be assumed as members of the same strain. The other *P. agardhii* strain (LMECYA 152), with a different origin, only presented an overall similarity of 82% with the previous two isolates.

3.3. Assessment of toxicity

The *PS* and *PKS* genes, implied in the cylindrospermopsin production, were amplified by PCR. The strain MS1 (*A. ovalisporum*) was used as a positive control, since it is an already described cylindrospermopsin-producing isolate with both *PS* and *PKS* genes sequenced (GenBank accession no.: PS, AAM33468; PKS, AAM33470). From the 11 isolates of *C. raciborskii*, only two presented amplification of both *PS* and *PKS* genes. The amplicons obtained for strain AQS presented the expected size, 650 bp for peptide synthetase gene and 800 bp for polyketide synthase gene (as obtained with MS1 control strain). The *C. raciborskii* AQS amplicons were sequenced and the nucleotide sequences have been deposited in the GenBank database under the following GenBank accession numbers: AY693995 (*PS* gene) and AY693996 (*PKS* gene).

Analysis of the primary amino acid sequence deduced from each of the amplified products revealed that both putative *PS* and *PKS* genes displayed strongest similarity (up to 97% amino acid identity) with PS and PKS proteins from *A. ovalisporum* MS1 and *A. bergii* 283A (Fig. 3), strains already described as cylindrospermopsin producers (Banker et al., 1997; Schembri et al., 2001, respectively). For strain LMECYA 134, two amplification products of unexpected size were observed. Sequencing and nucleotide Blast analysis confirmed the unspecific nature of these amplification products. Beyond confirming the results obtained above for cylindrospermopsin, HPLC analysis also revealed that none of the tested strains produced PSP toxins (Table 1).

4. Discussion

In this study, the level of genetic diversity in nine Portuguese *C. raciborskii* isolates was examined. Hierarchical strain clustering based on ITS amplification profiles, followed by selection of cluster representatives, showed to be an appropriate strategy to reduce the 16S rDNA sequencing effort necessary to confirm the morphological identification of cyanobacterial strains. As already described for other cyanobacterial species (Iteman et al., 2002), polymorphisms both in number and length of the ITS amplicons were also observed in Portuguese *C. raciborskii* strains.

Both fingerprinting methods used in the study were found to be reproducible and displayed intra-specific variability in all the species analysed. Similar ERIC results have been observed by Lyra et al. (2001) in other cyanobacterial genera, thus confirming the differentiation potential of this method. Although

(A)

```
AQS              WHQQTRTSVQGVRTLQFCAVSFDFSCHEIFSTLCLGGILVLVPEAVRQNPFALAEF
C. raciborskii   ------------------------------------------------------------
Aph. ovalisporum ------------------------------------------------------------
Anab. bergii     -L----P-------------------------------------------------------

AQS              ISQQKIEKLFLPVIALLQLAEAVNGNKSTSLALCEVITTGEQMQITPAVANLFQKT
C. raciborskii   ------------------------------------------------------------
Aph. ovalisporum ------------------------------------------------------------
Anab. bergii     ------------------------------------------------------------

AQS              GAMLHNHYGATEFQDATTHTLKGNPEGWPTLVPVGRPLHNVQVYILDEAQQPVPL
C. raciborskii   ------------------------------------------------------------
Aph. ovalisporum --I-----------------N-------------------------------V
Anab. bergii     --I-----------------N-------------------------------V
```

(B)

```
AQS              IGIGGSNYKSLMIENRSRIGKTDLYELSGTDVSVAAGRISYVLGLMGPSFVIDTA
C. raciborskii   ------------------------------------------------------------
Aph. ovalisporum ------------------------------------------------------------
Anab. bergii     ------------------------D---------------I-------------

AQS              CSSSLVSVHQACQSLRQRECDLALAGGVGLLIDPDEMIGLSQGGMLAPDGSCK
C. raciborskii   ------------------------------------------------------------
Aph. ovalisporum ------------------------------------------------------------
Anab. bergii     ------------------------------------------------------C--
```

Fig. 3. (A) Amino acid alignment of the *Cylindrospermopsis raciborskii* (AQS) PS sequence with homologous sequences present in GenBank database, *C. raciborskii* ATWT 205 (GenBank accession no. AAF76934), *Aphanizomenon ovalisporum* MS1 (GenBank accession no. AAM33468) and *Anabaena bergii* 283 A (GenBank accession no. AAG38957). (B) Amino acid alignment of the *C. raciborskii* (AQS) PKS sequence with homologous sequences present in GenBank database, *C. raciborskii* CYPO 20B (GenBank accession no. AAG38959), *A. ovalisporum* MS1 (GenBank accession no. AAM33470) and *A. bergii* 283 A (GenBank accession no. AAG38958). Dashed lines indicate residues identical to those of the *C. raciborskii* AQS sequence.

M13 fingerprinting has already been used with success for the identification of other organisms at species level (Grif et al., 1998; Valério et al., 2002), in the cyanobacterial species here analysed only discriminatory potential at strain level is observed as no hierarchic cutting level could be defined to produce only mono-specific clusters. Our results reveal genetic heterogeneity among Portuguese *C. raciborskii* strains and demonstrate that this variation also exists within a single cyanobacterial population from the same reservoir.

By applying a polyphasic approach, the evaluation and potential application of these fingerprinting methods was validated. Since both display high strain discriminatory potential they cannot be used as an identification tool, although they reveal to be appropriate for strain differentiation and assessment of genomic relationships. As a consequence, M13 and ERIC PCR fingerprinting are useful for ecological studies and evaluation of populations when high resolution is needed.

The *PS* and *PKS* genes code for enzymes involved in secondary metabolite biosynthesis by cyanobacteria. As described by Schembri et al. (2001), simultaneous presence of both genes seems to be associated with the ability to produce cylindrospermopsin. In the analysed set of Portuguese *C. raciborskii* strains no toxin production was observed, according to HPLC data. When PCR detection of *PS* and *PKS* genes was compared with assessed toxicity, the expected amplicons were only found for a positive Australian strain. Beyond giving support to the described non-toxic nature of European *C. raciborskii* strains (Fastner et al., 2003), these results confirm that

this two-gene directed PCR method is a powerful, easy to perform and rapid approach to evaluate the cylindrospermopsin-related toxicity of cyanobacterial strains.

5. Conclusion

C. raciborskii strains isolated from the Portuguese freshwaters were shown to be genomically heterogeneous and revealed a non-toxic behaviour. *PS* and *PKS* genes of the Australian *C. raciborskii* AQS strain code for putative proteins displaying 97–100% of amino acid identity with those from other strains of *C. raciborskii*, *A. ovalisporum* and *A. bergii*.

16S rDNA sequencing of cluster representatives selected from ITS analysis is an appropriate strategy to confirm the morphological identification of cyanobacterial strains. M13 and ERIC PCR fingerprinting are appropriate methods for strain differentiation and assessment of genomic relationships.

Acknowledgements

The authors thank G.K. Eaglesham for the HPLC determination of cylindrospermopsin. Elisabete Valério thanks the financial support (Grant SFRH/BD/8272/2002) by Fundação para a Ciência e a Tecnologia.

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Validation of a multiplex PCR for the detection of cylindrospermopsin producing strains

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Abstract

Aims: Validation of a PCR multiplex to monitor cylindrospermopsin producing strains in freshwaters, using a wider taxonomic range of cyanobacteria.

Methods and Results: A previously described multiplex PCR was applied to 120 isolates belonging to 10 genera (18 species) of the orders Chroococcales, Oscillatoriales and Nostocales. The repetitive concomitant amplification of both target genes was only obtained for isolate AQS (Cylindrospermopsis raciborskii) and MS1 (Aphanizomenon ovalisporum), the unique cylindrospermopsin producers included in the study. Although some unspecific amplicons were observed in several isolates, most of them differed in size from the expected ones. In a non-toxic Synechococcus nidulans strain, one of the fragments with the expected size was obtained, but its sequence displayed only 23.5% similarity with the one from AQS strain.

The detection limit was found to be 100 pg of cyanobacterial dry weight biomass.

Conclusions: These results confirm the high specificity of this multiplex PCR and point out to the possibility of its application in routine monitoring.

Significance and impact study: Cylindrospermopsin detection by HPLC and PS/PKS gene detection by multiplex PCR showed to be fully correlated, reinforcing the potential of gene-directed PCR as a tool for toxicity assessment of cyanobacteria.

Keywords: Cylindrospermopsin, cyanobacteria, method validation, multiplex PCR.

INTRODUCTION

Cylindrospermopsin (CYL) is a potent alkaloid, with a molecular weight of 415 and consisting of a tricyclic guanidine moiety combined with hydroxymethyluracil. This toxin is hepatotoxic *in vivo* and also a known general cytotoxin that blocks protein synthesis, being the first clinical symptoms kidneys and liver failure (WHO 2003). The LD_{50} of pure CYL after intraperitoneal administration to mice is 2.1 mg kg^{-1} over 24h and 0.2 mg kg^{-1} over 6 days (Ohtani *et*

al. 1992). There are also evidences of its carcinogenic potential in mice (Falconer and Humpage, 2001).

CYL and its analogues, deoxy-cylindrospermopsin and 7-epi-cylindrospermopsin, are known to be produced by some cyanobacterial species, namely, *Cylindrospermopsis raciborskii* (Hawkins *et al.* 1985; Ohtani *et al.* 1992; Li *et al.* 2001a), *Umezakia natans* (Harada *et al.* 1994), *Aphanizomenon ovalisporum* (Banker *et al.* 1997), *Raphidiopsis curvata* (Li *et al.* 2001b), *Anabaena bergii* (Schembri *et al.* 2001), and more recently *Aphanizomenon flos-aquae* (Preuβel *et al.* 2006) and *Lyngbya wollei* (Seifert *et al.* 2007).

Genes responsible for the biosynthesis of cylindrospermopsin were described by Shalev-Alon *et al.* (2002) for an *Aphanizomenon ovalisporum* strain. Schembri *et al.* (2001) had already described the presence of polyketide synthases (PKS) and peptide synthetases (PS) genes in *C. raciborskii* isolates and demonstrated a direct link between the presence of these two genes and the ability of those isolates to produce cylindrospermopsin. The location of these last two fragment genes in the gene cluster described by Shalev-Alon *et al.* (2002) is represented in Figure 1.

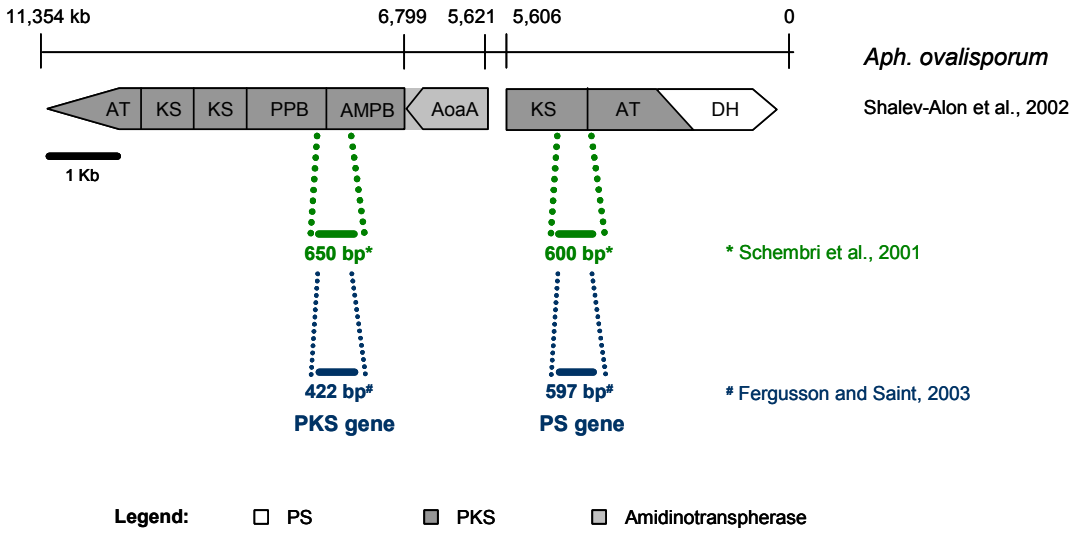

FIGURE 1 Gene cluster involved in the cylindrospermopsin biosynthesis of *Aph. ovalisporum* (Shalev-Alon *et al.* 2002) and identification of the PS and PKS gene fragments within this cluster, described by Schembri *et al.*, 2001 and Fergusson and Saint, 2003.

The use of these two markers was tested in nine cultured isolates of *C. raciborskii*, isolated from different freshwater lakes and rivers in Portugal (Valério *et al.* 2005), displaying concordant results.

In this study, we applied the multiplex PCR described by Fergusson and Saint (2003) to 120 cultured isolates, in order to validate this PCR method and evaluate its potential to be used in the routine monitoring for the presence of cylindrospermopsin producing isolates in freshwater reservoirs.

MATERIALS AND METHODS

Isolates and DNA extraction

A total of 120 unicyanobacterial cultures were isolated from blooms and water samples of Portuguese freshwaters. After isolation they were grown and maintained in the LMECYA culture collection using Z8 medium (Skulberg & Skulberg, 1990). The isolates were grown under a 14:10h light:dark cycle (15.93-24.98 μmol m^{-2} s^{-1}) at 20 ± 1 ºC. Their genomic DNA was extracted according to a method previously described (Valério *et al.* 2005).

Multiplex PCR and detection limit

The PCR reactions were performed in 50 μL reaction mixtures, containing 1 X PCR buffer, 0.4 mM of each dNTP, 0.25-1.00 μM of each primer, 1 ng of genomic DNA, 2.5 mM $MgCl_2$, 0.5 mg mL^{-1} of BSA and 1U of *Taq* DNA polymerase.

The multiplex PCR for the simultaneous amplification of PS and PKS genes, as well as the amplification of a *C. raciborskii* control fragment, was performed according to the conditions described by Fergusson and Saint (2003). The amplification was performed in a T Gradient thermocycler, consisting of an initial denaturation step at 95 ºC for 6 min, followed by 35 cycles of 30 s at 94 ºC, 30 s at 50 ºC and 1 min at 72 ºC and a final extension step of 5 min at 72 ºC.

The results obtained were correlated with toxicity available data.

The detection limit of the PS and PKS amplification was also evaluated. The DNA was extracted from 2 mg of lyophilized biomass, with a final yield of 300 ng. A series of DNA dilutions were subsequently used in each PCR reaction. PS gene amplification was done using both PS M13 (5'-GGCAAATTGTGATAGCCACGAGC-3') and PS M14 (5'-GATGGAACATCGCTCACTGGTG-3') primers and PKS gene amplification was done using both PKS M4 (5'-GAAGCTCTGGAATCCGGTAA-3') and PKS M5 (5'-AATCCTTACGGGATCCGGTGC-3') primers, according to the conditions described by Schembri *et al.* (2001).

Toxin analysis by HPLC

Analysis for the presence of cylindrospermopsin was performed by reverse phase HPLC and mass spectroscopy according to Eaglesham *et al.* (1999).

PS and PKS genes sequencing

To confirm that the PCR amplicons corresponded to the PS and PKS genes, the amplification reaction products were purified with a Jet Quick-PCR Purification kit (Genomed) as described by the manufacturer. The purified PCR products were sequenced in both directions using an automated Beckman-Coulter DNA sequencer (model CEQ-2000) with dye terminators and standard protocols, with the same primers used for the amplification. Sequences were analysed with the BLAST tool of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

RESULTS

In this study, the multiplex PCR described by Fergusson and Saint (2003) was applied to 120 cultured isolates belonging to three orders of cyanobacteria - Chroococcales, Oscillatoriales and Nostocales, enclosing 10 genera (18 species). Two cylindrospermopsin producing isolates, AQS (*C. raciborskii*) and MS1 (*Aph. ovalisporum*), were included as positive controls.

As presented in Figure 2, the repetitive concomitant amplification of PS and PKS genes was only obtained with the cylindrospermopsin producing isolates (AQS and MS1), although some unspecific amplicons were observed in several isolates, most of them differing in size from the expected ones.

In a non-toxic *Synechococcus nidulans* strain (LMECYA 156), one of the fragments presented a similar size to the PS gene. However, its sequence displayed only 23.5% similarity with the one from AQS toxic strain.

Amplification of both genes was achieved with DNA quantities from 15 fg to 0.75 ng. The lower limit corresponds to approximately 100 pg of cyanobacterial dry weight biomass (Figure 3). DNA quantities above 0.75 ng seem to inhibit the PCR reaction.

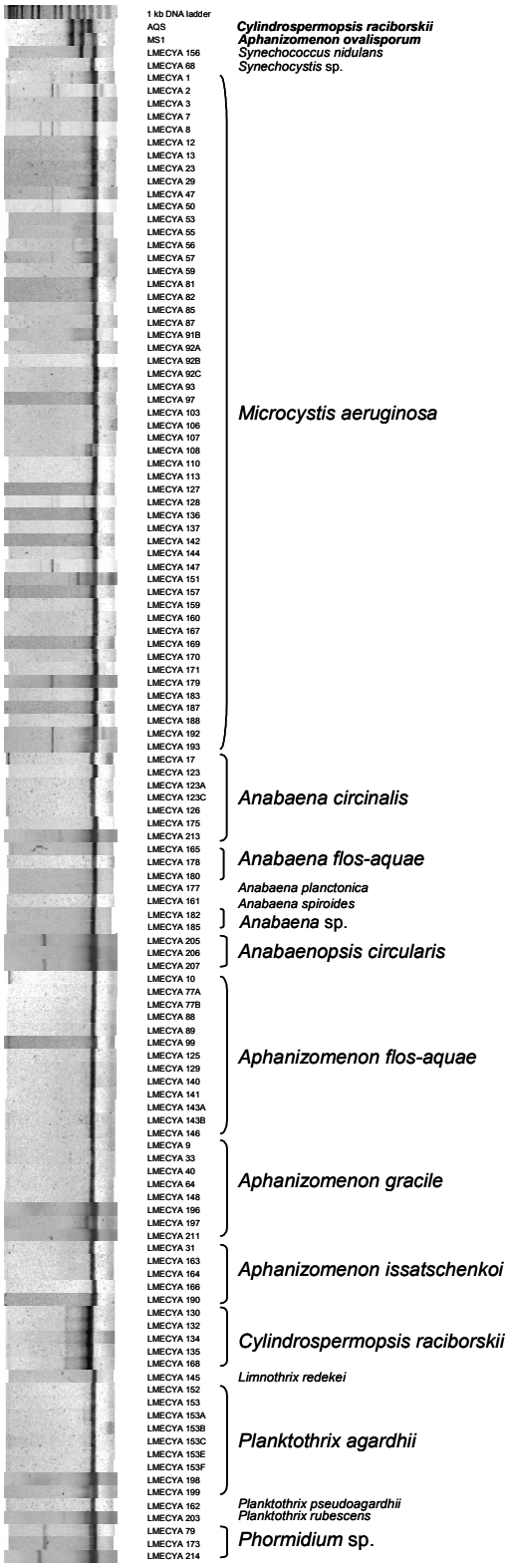

FIGURE 2 Results obtained by multiplex PCR assay for the simultaneous amplification of PS and PKS cylindrospermopsin genes in 120 isolates. An internal control fragment for *C. raciborskii* isolates detection is also included in the PCR reaction.

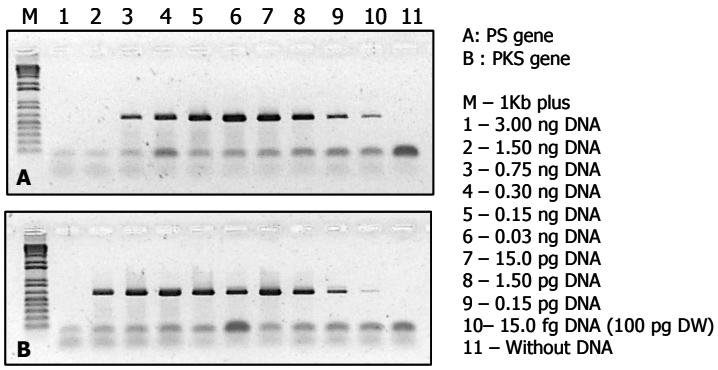

FIGURE 3 Detection limits of PS and PKS genes amplified using decreasing amounts of DNA in the PCR reaction.

DISCUSSION

In this study, we have applied the multiplex PCR described by Fergusson and Saint (2003) to 120 cultured isolates comprising different taxa, in order to validate this PCR method and evaluate its potential in the routine monitoring for the presence of cylindrospermopsin producing strains in freshwater reservoirs.

The amplification of gene fragments with the expected size was only obtained in the two toxic isolates used as control of the PCR. The occurrence of the false positive for a strain of *Synechococcus nidulans*, as well as the unspecific smaller-sized amplicons observed for other isolates, seem to be an indication of the existence of other possible sites where the primers might anneal. As a consequence, only the concomitant occurrence of both amplicons should be considered as a true positive result.

This sensitive multiplex PCR gave a detection limit as low as 15 fg of DNA (correspondent to 100 pg of cyanobacterial dry weight biomass), which indicates that this method is suitable in environmental samples that might contain a very low number of copies of target DNA.

These results confirm the high specificity and detection limit of this molecular method and point out to the possibility of its application in routine monitoring. However, until now, in Portugal there was no detection of cylindrospermopsin in freshwater reservoirs, and so the application of this method to environmental samples has not been possible.

ACKNOWLEDGEMENTS

The authors thank G.K. Eaglesham and J. Fastner for the determination of cylindrospermopsin.

Elisabete Valério thanks the financial support (PhD Grant SFRH/BD/8272/2002) to Fundação para a Ciência e a Tecnologia.

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SUPPLEMENTARY DATA

Isolates ability to produce several toxins

Taxon and isolate designation	MCYST production	PSTs production	CYL production
Chroococcales			
Microcystis aeruginosa LMECYA 1	Neg	ND	ND
M. aeruginosa LMECYA 2	Pos	ND	Neg
M. aeruginosa LMECYA 3	Pos	ND	ND
M. aeruginosa LMECYA 7	Pos	ND	ND
M. aeruginosa LMECYA 8	Neg	ND	ND
M. aeruginosa LMECYA 12	Neg	ND	Neg
M. aeruginosa LMECYA 13	Neg	ND	ND
M. aeruginosa LMECYA 23	Neg	ND	ND
M. aeruginosa LMECYA 29	Neg	ND	ND
M. aeruginosa LMECYA 50	Neg	ND	ND
M. aeruginosa LMECYA 53	Pos	ND	ND
M. aeruginosa LMECYA 55	Pos	ND	ND
M. aeruginosa LMECYA 56	Pos	ND	ND
M. aeruginosa LMECYA 57	Pos	ND	ND
M. aeruginosa LMECYA 59	Pos	ND	ND
M. aeruginosa LMECYA 81	Neg	ND	ND
M. aeruginosa LMECYA 82	Neg	ND	ND
M. aeruginosa LMECYA 85	Neg	ND	ND
M. aeruginosa LMECYA 87	Pos	ND	ND
M. aeruginosa LMECYA 91B	Neg	ND	ND
M. aeruginosa LMECYA 92A	Pos	ND	ND
M. aeruginosa LMECYA 92B	Pos	ND	ND
M. aeruginosa LMECYA 92C	Pos	ND	ND
M. aeruginosa LMECYA 93	Pos	ND	ND
M. aeruginosa LMECYA 97	Neg	ND	ND
M. aeruginosa LMECYA 103	Pos	ND	ND
M. aeruginosa LMECYA 106	Pos	ND	ND
M. aeruginosa LMECYA 107	Pos	ND	ND
M. aeruginosa LMECYA 108	Pos	ND	ND

Taxon and isolate designation	MCYST production	PSTs production	CYL production
M. aeruginosa LMECYA 110	Pos	ND	ND
M. aeruginosa LMECYA 113	Pos	ND	ND
M. aeruginosa LMECYA 127	Neg	ND	ND
M. aeruginosa LMECYA 128	Neg	ND	ND
M. aeruginosa LMECYA 136	Neg	ND	Neg
M. aeruginosa LMECYA 137	Neg	ND	ND
M. aeruginosa LMECYA 142	Neg	ND	ND
M. aeruginosa LMECYA 144	Neg	ND	ND
M. aeruginosa LMECYA 147	Neg	ND	ND
M. aeruginosa LMECYA 151	Pos	ND	ND
M. aeruginosa LMECYA 157	Neg	ND	ND
M. aeruginosa LMECYA 159	Pos	ND	ND
M. aeruginosa LMECYA 160	Pos	ND	ND
M. aeruginosa LMECYA 167	Pos	ND	ND
M. aeruginosa LMECYA 169	Neg	ND	ND
M. aeruginosa LMECYA 170	Pos	ND	ND
M. aeruginosa LMECYA 171	Neg	ND	ND
M. aeruginosa LMECYA 179	Pos	ND	ND
M. aeruginosa LMECYA 183	Neg	ND	ND
M. aeruginosa LMECYA 187	Neg	ND	ND
M. aeruginosa LMECYA 188	Neg	ND	ND
M. aeruginosa LMECYA 192	Neg	ND	ND
M. aeruginosa LMECYA 193	Neg	ND	ND
Synechococcus nidulans LMECYA 156	Neg	ND	ND
Synechocystis sp. LMECYA 68	Neg	ND	ND
Oscillatoriales	ND	ND	ND
Limnothrix redekei LMECYA 145	Neg	Neg	ND
Phormidium sp. LMECYA 79	Neg	Neg	ND
Phormidium sp. LMECYA 173	Neg	Neg	ND
Phormidium sp. LMECYA 214	Neg	Neg	ND
Planktothrix agardhii LMECYA 152	Neg	Neg	ND
P. agardhii LMECYA 153	Neg	Neg	ND
P. agardhii LMECYA 153A	Neg	Neg	ND
P. agardhii LMECYA 153B	Neg	Neg	ND
P. agardhii LMECYA 153C	Neg	Neg	ND

Taxon and isolate designation	MCYST production	PSTs production	CYL production
P. agardhii LMECYA 153E	Neg	Neg	ND
P. agardhii LMECYA 153F	Neg	ND	ND
P. agardhii LMECYA 198	Neg	Neg	ND
P. agardhii LMECYA 199	Neg	Neg	ND
P. pseudoagardhii LMECYA 162	Neg	Neg	ND
P. rubescens LMECYA 203	Pos	ND	ND
Nostocales	ND	ND	ND
Anabaena circinalis LMECYA 17	Neg	Neg	ND
A. circinalis LMECYA 123	Neg	ND	ND
A. circinalis LMECYA 123A	Neg	Neg	ND
A. circinalis LMECYA 123C	Neg	ND	ND
A. circinalis LMECYA 126	Neg	Neg	ND
A. circinalis LMECYA 175	Neg	Neg	ND
A. circinalis LMECYA 213	Neg	Neg	ND
A. planctonica LMECYA 177	Neg	Neg	ND
A. flos-aquae LMECYA 165	Neg	Neg	ND
A. flos-aquae LMECYA 178	Neg	Neg	ND
A. flos-aquae LMECYA 180	Neg	Neg	ND
A. spiroides LMECYA 161	Neg	Neg	ND
Anabaena sp. LMECYA 182	Neg	Neg	ND
Anabaena sp. LMECYA 185	Neg	Neg	ND
Anabaenopsis circularis LMECYA 205	Neg	Neg	ND
Anabaenopsis circularis LMECYA 206	ND	Neg	ND
Anabaenopsis circularis LMECYA 207	Neg	ND	ND
Aphanizomenon gracile LMECYA 9	Neg	Neg	ND
Aph. gracile LMECYA 33	Neg	Neg	ND
Aph. gracile LMECYA 40	Neg	Pos	ND
Aph. gracile LMECYA 64	Neg	Neg	ND
Aph. gracile LMECYA 148	Neg	Pos	ND
Aph. gracile LMECYA 196	Neg	Neg	ND
Aph. gracile LMECYA 197	Neg	Neg	ND
Aph. gracile LMECYA 211	Neg	Pos	ND
Aph. issatschenkoi LMECYA 31	Neg	Pos	ND
Aph. issatschenkoi LMECYA 163	Neg	Neg	ND
Aph. issatschenkoi LMECYA 164	Neg	Neg	ND

Taxon and isolate designation	MCYST production	PSTs production	CYL production
Aph. issatschenkoi LMECYA 166	Neg	Neg	ND
Aph. issatschenkoi LMECYA 190	Neg	Neg	ND
Aph. flos-aquae LMECYA 10	Neg	Neg	ND
Aph. flos-aquae LMECYA 77A	Neg	Neg	ND
Aph. flos-aquae LMECYA 77B	Neg	Neg	ND
Aph. flos-aquae LMECYA 88	Neg	Neg	ND
Aph. flos-aquae LMECYA 89	Neg	Neg	ND
Aph. flos-aquae LMECYA 99	Neg	Neg	ND
Aph. flos-aquae LMECYA 125	Neg	ND	ND
Aph. flos-aquae LMECYA 129	Neg	Neg	ND
Aph. flos-aquae LMECYA 140	Neg	Neg	ND
Aph. flos-aquae LMECYA 141	Neg	Neg	ND
Aph. flos-aquae LMECYA 143A	Neg	Neg	ND
Aph. flos-aquae LMECYA 143B	Neg	Neg	ND
Aph. flos-aquae LMECYA 146	Neg	Neg	ND
Aph. ovalisporum MS1 (ILC-146)	ND	ND	Pos
Cylindrospermopsis raciborskii LMECYA 130	Neg	ND	Neg
C. raciborskii LMECYA 132	Neg	Neg	Neg
C. raciborskii LMECYA 134	Neg	Neg	Neg
C. raciborskii LMECYA 135	Neg	Neg	Neg
C. raciborskii LMECYA 168	Neg	Neg	Neg
C. raciborskii LMECYA 238 (AQS)	Neg	ND	Pos
C. raciborskii Caia	ND	ND	Neg
C. raciborskii Marau1	ND	ND	Neg
C. raciborskii 4799	ND	ND	Neg
C. raciborskii 4899	ND	ND	Neg
C. raciborskii LJ	ND	ND	Neg

MCYST: microcystins; PST: paralytic shellfish toxins; CYL: cylindrospermopsin; NA: not available; ND: not determined.
All these results were determined and supplied by the QHME-INSA staff.

5

Electrochemical DNA-based sensors

Part of the content of this chapter is presented in the following paper:
"Preliminary studies towards the development of DNA biosensors for detection of cylindrospermopsin - a cyanobacterial toxin", E. Valério, A. Tenreiro and L.M. Abrantes, *Portugaliae Electrochimica Acta* (in press).

5.1 Introduction

5.1.1 Biosensors

The development of biosensors offer simplified analysis for a range of biomedical and industrial applications; therefore this multidisciplinary field is in continuous development for the last couple of decades [1]. A biosensor is an analytical device that combines the specificity of a biological recognition element (interacting with the target analyte) with a physical transducer that translates the biorecognition event into a signal proportional to target analyte concentration as illustrated in Figure 5.1 [2, 3].

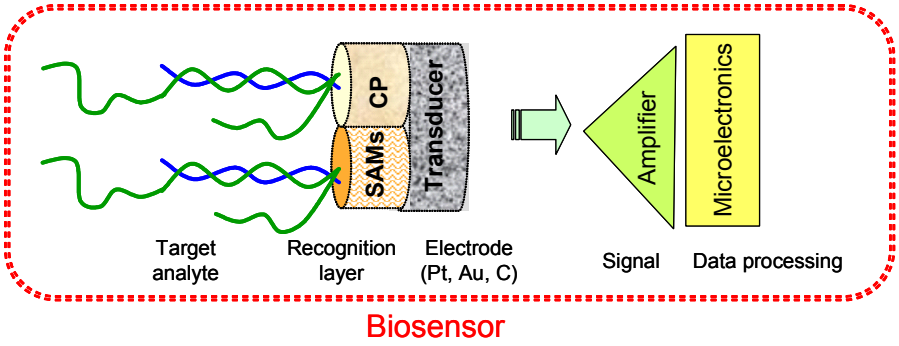

FIGURE 5.1 Biosensor scheme (Adapted from [3]). SAMS - Self Assembled Monolayers; CP - Conducting Polymers.

Common transducing elements, include optical, electrochemical, piezoelectric, electrical or thermal devices, that generate light, current, frequency, conductivity or calorimetry signals, respectively. Through signal processing, this interaction is converted into values that relate to the concentration or activity of the analyte in the vicinity of the device, which should be representative of the levels in the bulk sample. There are two types of biosensors, depending on the nature of the recognition event. Bioaffinity devices rely on the selective binding of the target analyte to a surface-confined ligand partner (e.g. antibody, oligonucleotide). In contrast, in biocatalytic devices, an immobilized enzyme is used for the recognition of the target substrate [3, 4].

Electrochemical DNA-based sensors

The concept of "*the electrochemical DNA hybridization biosensor*" was first introduced in 1993 by Millan and Mikkelsen [5]. Hybridization biosensors rely on the immobilization of a single-stranded (ss-DNA) oligonucleotide (*probe*) (Figure 5.2) on a transducer surface to selectively recognize its complementary (*target*) DNA sequence via hybridization. The DNA duplex formed (Figure 5.2) at the electrode surface is known as a hybrid [3, 6, 7]. This event is

then converted into an analytical signal by the transducer, which can be electrochemical (amperometric or potentiometric), gravimetric, optical (e.g. surface plasmon resonance-based) or electrical device [6].

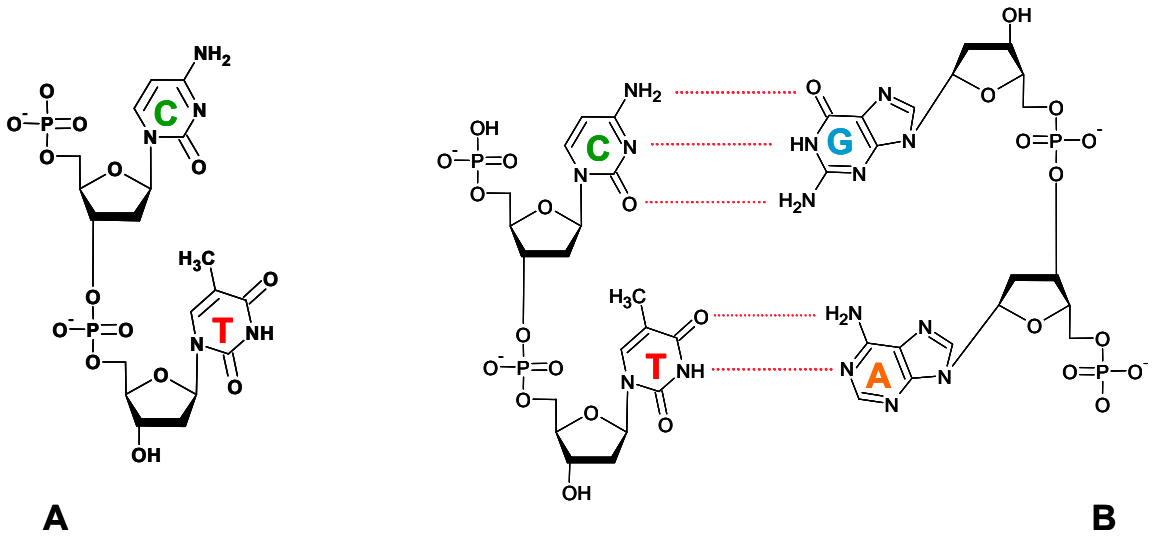

FIGURE 5.2 (A) Single stranded (ss-DNA) and (B) double-stranded DNA (ds-DNA) or duplex (adapted from [8]). C - Cytosine; T - Thymine; G - Guanine; A - Adenine.

DNA biosensors are of considerable interest due to their remarkable promise for obtaining sequence-specific information in a faster, simpler and economical manner compared to traditional hybridization assays. Electrochemical biosensors have been attracting increased attention due to its superior properties since they are very sensible, even in turbid solutions and enable fast, simple and low-cost detection, and may be miniaturized into portable devices [6, 9]. It is also a technology with reduced requirements for sample pre-treatment and large sample volume, and can be used by someone without prior laboratory skills [4].

5.1.2 Electrode surface modification

5.1.2.1 Conducting Polymers

Polymers offer a lot of advantages for sensor technologies: they are relatively low cost materials, requiring quite simple synthesis, can be deposited on various types of substrates and the wide choice of their molecular structures and the possibility to build in side-chains, charged or neutral particles on its surface region, enables films to be produced with various physical and chemical properties including sensing behaviour.

The deposition of polymer films can be achieved by several processes. The electrochemical polymerisation allows the synthesis and deposition of conducting and semicon-

ducting polymers onto surfaces of given substrates, including C, Au, and Pt, from monomer containing solutions [10-13]. This method involves the oxidation of the monomers units to form a radical cation followed by coupling of the oxidised monomer species leading to the formation of di-cations, then the oxidation of the dimmer couples with more oxidised monomer leading to the growth of the polymer chain. The advantage of this method, regarding for example the chemical synthesis, which can be carried out at room temperature, is its simplicity and reproducibility, along with the possibility of controlling the films thickness and morphology by monitoring of the potential or current applied to the working electrode. The electrochemical approaches for the preparation of conducting polymers include galvanostatic (constant current), potentiostatic (constant potential) or potentiodynamic (cyclic voltammetry) methods [10-12].

Electronically conducting polymers (ECP) exhibit intrinsic electronic conductivity. Their structure contains a one-dimensional organic backbone based on the alternation of single and double bonds (π-conjugated system), which enables a superorbital to be formed for electronic conduction. Development of a variety of polymer films with different conductive properties, like polyacetylene, polypyrrole, polyaniline, polythiophene, polyphenol and polytyramine and their copolymers was demonstrated over the past decades [10-12]. The importance of these materials was recognised by the award of the Chemistry Nobel Prize in 2000 [14, 15].

A number of different applications of conductive and semiconductive polymers have been exploited, including their use in electrochromic, electroluminescent and optoelectric devices, biomedical and environmental sensors, corrosion protection and antistatic coating among others [11, 16-18].

One of the most studied applications of ECP is their use in biosensors, particularly in electrochemical sensors. This is due to the above mentioned easy deposition onto electrodes combined with their large surface area and to the ability of transferring the electric charge produced by the biological recognition process to the electronic circuit into a useful analytical signal in an efficient way [10, 11].

Tyramine (Ty) (Figure 5.3) was chosen as a monomer because of its pendant amine group, which can be used for the attachment of organic molecules or biomolecules of interest [19].

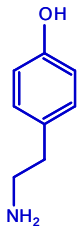

FIGURE 5.3 Tyramine structural formula.

The oxidation of Ty is described as similar to electrochemical polymerisation of other phenols [19]. The mechanism that leads to polytyramine formation has been recently reported [19-21]. The use of acidic aqueous medium to polymerise the polytyramine (PTy) films allows the formation of thicker films than those obtained from neutral solutions, with improved conductivity properties [20, 21]. Polytyramine presents one primary aliphatic amine per tyramine moiety. The attachment of organic molecules or biomolecules of interest can be for instance obtained by the covalent attachment through a carboxamide or a phosphoramidate bond [20]. For this reason, PTy has been employed in immobilization studies of enzymes [22-28], oligonucleotides (ODN) [20, 29, 30] and antibodies [31].

5.1.2.2 Self-Assembled Monolayers

Self-Assembled Monolayers (SAMs) provide another route for the modification of the electrode surface. This simple method provides the obtention of highly organized and packed monolayers that form spontaneously by chemisorption of functionalized molecules at a metal surface, such as gold, platinum, silver and mercury, and do not require the establishment of anaerobic or anhydrous conditions, nor vacuum [32].

The molecules that form the SAMs consist of a *surface headgroup* (e.g. SH), which displays a high affinity for the metal surface, and so constitutes the driving force for the film formation; a *spacer* – that is usually an alkane chain of variable length, or an aromatic group, that strongly affects the intermolecular separation, molecular orientation and the degree of order in the film; and a *terminal functionality* (e.g. COOH, NH_2, OH), as represented in Figure 5.4.

The mechanism of self-assembly is described as occurring in two stages. Initially, the functionalized molecules adsorb horizontally onto metal substrate. Subsequently, they lift up and organise themselves laterally, mostly by intermolecular Van der Waals interactions to form the vertically oriented layer [32, 33].

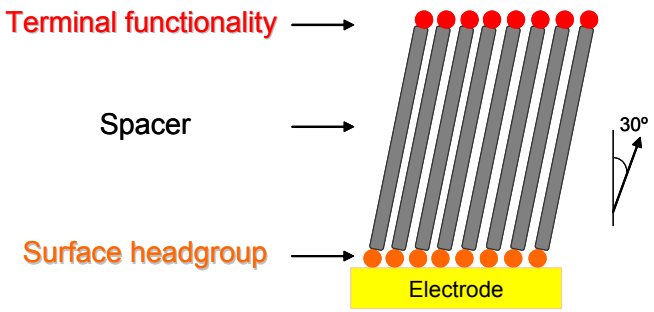

FIGURE 5.4 Schematic representation of an organized monolayer on a gold electrode, displaying its principal components.

The self assembly method is relatively insensitive to the choice of solvent, as long as the adsorbate is dissolved at millimolar or micromolar level. Ethanol is the solvent most commonly used due to its low toxicity [32].

Metal-sulphur bonds can be formed stably between molecules containing thiols, disulfides and sulfides molecules and the metal electrode surface [32, 33], being gold the most common substrate used. For the monolayer obtention an organic-free metal surface is preferred, however it is not absolutely necessary because the Au-S bond has a high strength, of about 167.36 kJ mol^{-1} [32, 34]. Monolayers of alkanethiols on gold have been the most studied system; in the majority of the cases, the thickness of the SAM is linearly related to the chain length of the thiol used [32].

SAMs preparation using very dilute solutions provides the formation of more ordered monolayers, whereas higher concentrations and long adsorption time favor multilayers formation [35]. Usually in the most common metal substrate, Au (111), alkanethiols form a $\left(\sqrt{3} \times \sqrt{3}\right)R\,30^{o}$ structure hexagonal lattice with an average spacing of 5 Å between the alkane chains.

The electrochemical reductive desorption of thiols, disulfides and sulfides is one of the most common methods used to characterise the modified substrates with SAMs and it is usually observed at negative potentials in very basic solutions (e.g. NaOH or KOH 0.5 M), where they desorb quantitatively, equation 6.1:

$$M \mid S(CH_2)_nX + 1e^- \longrightarrow M \mid + S(CH_2)_nX^- \qquad \textbf{(6.1)}$$

M is the metallic electrode [32, 36].

The area of the desorption peak, corrected for charging current, is relatively independent of the chain length for pure alkanethiols. However the peak potential is dependent of the spacer nature and length, mostly because of the adsorbates intermolecular interactions. For instance as the alkane chains length increases, the desorption peak potential becomes more negative [37]. The monolayer coverage (Γ) at the electrode surface is evaluated using equation 6.2:

$$\Gamma = \frac{Q}{\mathbf{n}FA} \qquad \textbf{(6.2)}$$

where *Q* is the charge in coulombs, **n** is the number of electrons transferred in the reaction (n = 1, according to equation 6.1), *F* is Faraday's constant (96485 C mol^{-1}), and *A* is the electrode area [32, 34].

SAMs provide several features that make them attractive for various applications. Their reproducibility, ease of preparation, flexibility, organisation and molecular control are the most representative characteristics of these organic layers. Due to their versatility, the terminal group of SAMs can be chemically tailored in order to achieve the desired property of the modified surface. One example is the attachment of biomolecules to the specific SAMs end groups towards the development of biosensors. One of the advantages of using SAMs to immobilize biomolecules is their low non-faradic (capacitive) currents [33].

SAMs of aromatic thiols have also been used since they may offer advantages over pure alkanethiols. Although they form, in general, a more disorganised layer, leading to different packing structures, they are expected to have strong interactions between the spacer molecules [33, 38]. The benzene ring is expected to reduce the molecular flexibility, and also to provide higher electrical conductance due to the delocalize electrons of the ring [33, 38, 39].

4-Aminothiophenol (4-ATP) (Figure 5.5) is one aromatic thiol that has already been used for the self-assembly method over platinum [40], silver [41] and gold electrodes [39, 42], with the purpose of covalently attach different substances, such as peptide [43], colloidal Au [44, 45] and IgG [41] to the terminal amines function of these monolayers.

In this work, SAMs of 4-aminothiophenol were prepared as an alternative method to CP for the metal surface modification to immobilize the cylindrospermopsin ss-DNA probe.

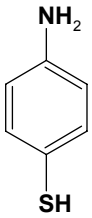

FIGURE 5.5 4-aminothiophenol structural formula.

5.1.3 Immobilization of oligonucleotides

The immobilization of the nucleic acid probe onto the transducer surface plays an important role in the overall performance of DNA biosensors. In a DNA biosensor the probe frequently consists of an oligonucleotide of about 15 to 50 bases long [9]. The immobilization step should lead to a well defined probe orientation, accessible to the target to ensure high reactivity [3, 46]. Various methods can be used to attach the DNA probe to the surface, including simple adsorption, crosslinking, encapsulation, avidin-biotin complexation and covalent attachment to functional groups of the modified electrode [3, 4, 7, 9]. Adsorption is the most simple method to immobilize oligonucleotides (ODN) on solids, however those could be released from the surface during the washing steps, or due to hybridization, since the targets may remove the probes from the surface [20].

The achievement of high sensitivity and selectivity also requires minimisation of non-specific adsorption and the stability of immobilization [46]. Furthermore, to obtain the immobilized probes presenting structural flexibility and conformational changes, allowing a higher efficiency of the hybridization reaction, it is desirable to attach a nucleic acid probe covalently to the modified electrode surface through a linker bound to one of the 5’ or 3’ ends of the chain. The covalent attachment also avoids the leaking of the ODN from the transducer surface to the solution [7, 20].

Water soluble N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and N-Hydroxysuccinimide (NHS) can be used as activating reagents of the 5'-phosphate group of a ss-DNA probe and promote its covalent attachment to primary amino groups of the electrode surface by the formation of a phosphoramidate linkage, as illustrated in Figure 5.6 [7, 47-49].

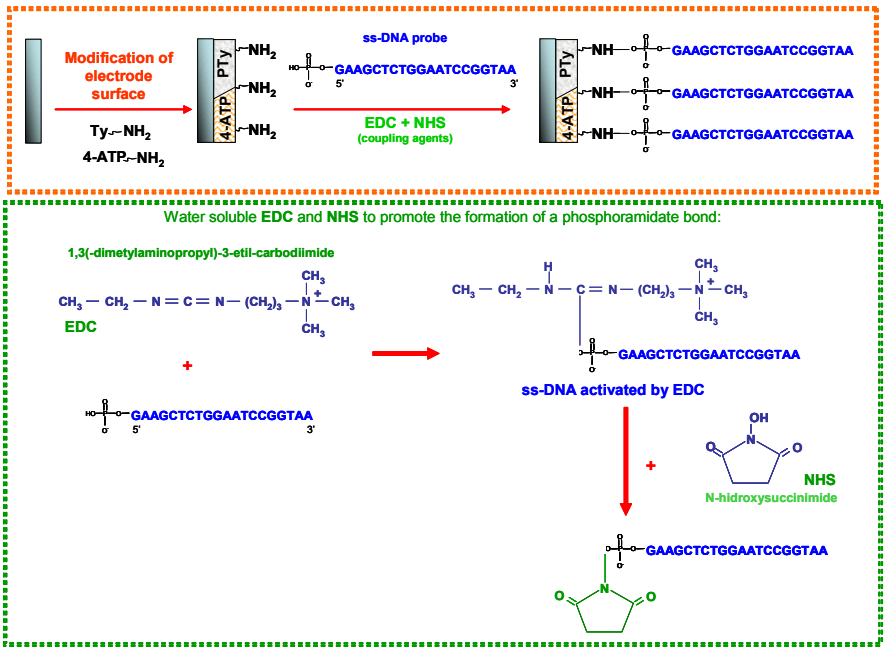

FIGURE 5.6 EDC-mediated with NHS assistance bounding of ss-DNA probe to amine groups of support materials.

5.1.4 Detection of hybridization

The electrochemical detection of the hybridization process is mainly based on the differences in the electrochemical behaviour of compounds that interact in a different way with ss-DNA and double-stranded DNA (ds-DNA). A variety of small molecules may interact reversibly with DNA either through intercalation or electrostatic binding in well-defined binding sites, as depicted in Figure 5.7. For instance, the methylene blue (MB), an electroactive redox indicator which displays a blue colour in the oxidised form and is colourless in its reduced one, is widely used. The MB can establish electrostatic interactions to the negatively charged DNA sugar phosphate backbone [50, 51]. Heterocyclic dyes, such as ethidium, anthracyclines, phenothiazines, and acridine derivatives, bind through intercalation with the planar, aromatic group stacked between base pairs [5-7, 52]. Some square planar and octahedral transition metal complexes can also associate reversibly with ds-DNA through interaction with the major groove (Figure 5.7) [5, 8].

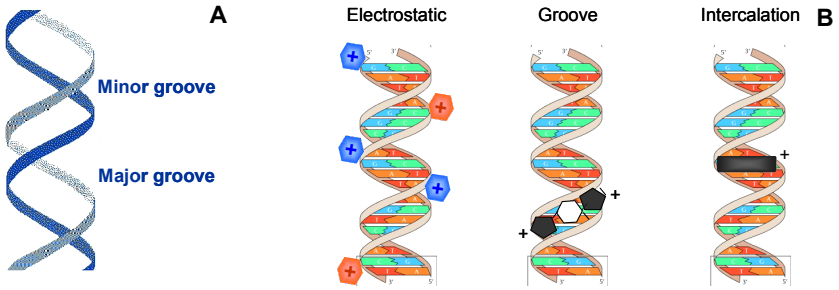

FIGURE 5.7 DNA structure and DNA interactions. (A) Illustration of DNA major and minor groove (adapted from [8]); (B) possible binding of small molecules to DNA.

Electrochemical detection of DNA hybridization usually involves monitoring the current response under controlled potential conditions. This can be evaluated by the changes in the oxidation or reduction peak current of the redox indicator (which selectively binds with ds-DNA or ss-DNA) or of electroactive DNA bases (e.g. guanine or adenine). Also, the detection of the electrochemical signal of the substrate after hybridization with an enzyme-tagged probe is commonly used, as well as the monitoring of the electrochemical signal of a metal nanoparticle probe attached after hybridization with the target [6].

The conditions required for hybridization events (e.g. ionic strength, temperature, presence of accelerators) need to be optimized both for solution-based hybridization assays and also for biosensor design [3]. Surface chemistry and density of probe strand on the surface play a key role on the biosensor performance; the crucial issue with this last factor is that probes should not be too close together to start repelling each other and hence repelling incoming target DNA. It is known that the distance between probe strands where electrostatic repulsion becomes significant depend on the ionic strength of the hybridization solution [9].

An alternative route for the detection of hybridization relies on the fluorescence measurements. The PicoGreen® reagent, Figure 5.8 [53], is a fluorochrome described as an ultrasensitive fluorescent nucleic acid stain, that selectively binds to ds-DNA [53-55] by intercalation in the DNA minor-groove (Figure 5.7) [53], and allows the detection of as little as 25 pg mL^{-1} of ds-DNA and has a minimize fluorescence contribution of RNA and ss-DNA (Figure 5.9). It has an excitation maximum at 480 nm and an emission peak at 520 nm (Figure 5.9). When bound to ds-DNA, fluorescence enhancement of PicoGreen® is exceptionally high; and little background occurs since the unbounded dye has virtually no fluorescence [53-55].

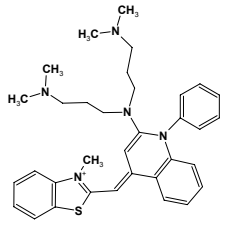

FIGURE 5.8 PicoGreen® structural formula (adapted from [53]).

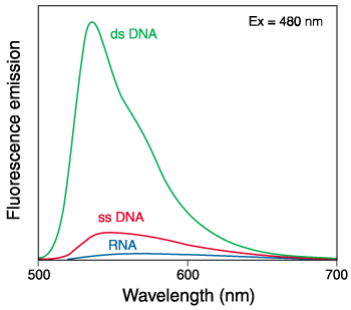

FIGURE 5.9 Fluorescence spectra of the PicoGreen® reagent upon binding to ds-DNA, ss-DNA and RNA (from [54]).

In this work, the covalent immobilization of a molecular marker for cylindrospermopsin producing strains (PKSM4, [56]), bearing a 5'-phosphate modification, to a polytyramine film and to self-assembled monolayers of 4-aminothiophenol has been performed. Subsequent hybridization assays with complementary and non-complementary sequences were carried out as presented in Figure 5.10. Several methods were used to confirm the immobilization and hybridization processes. These included the monitoring of the open circuit potential with time, characterization of the modified electrodes by cyclic voltammetry and square wave voltammetry, using methylene blue as electroactive indicator and also the fluorescence quantification, after staining with PicoGreen® reagent.

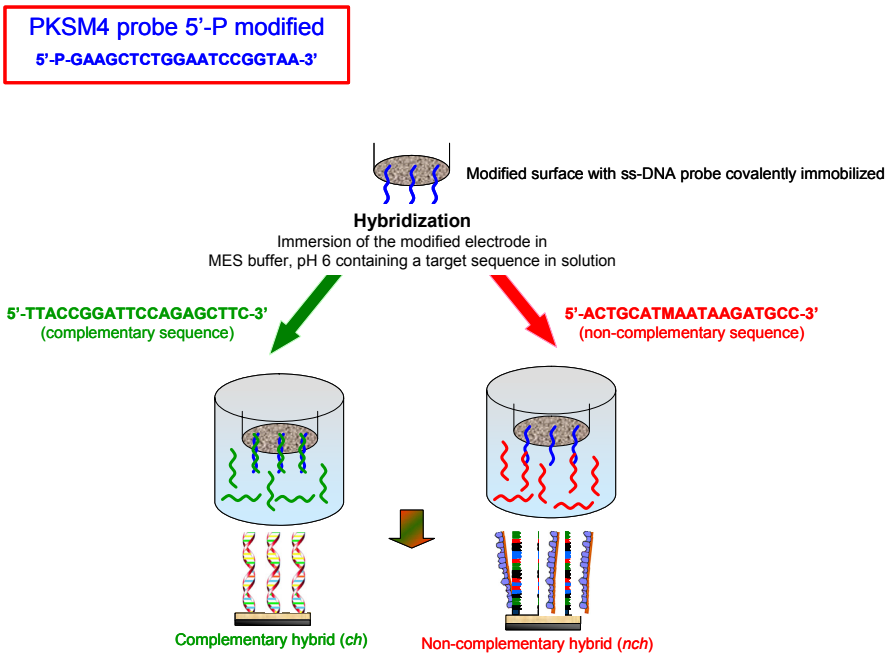

FIGURE 5.10 Scheme of the biosensor use in the detection of cyanobacteria containing sequences coding for cylindrospermopsin.

5.2 Experimental details

All the solutions for the electrochemical studies, were prepared from ultra-pure water, obtained from a Millipore purification system (nominal resistivity 18.2 MΩ cm at 25 ºC) and deoxygenated directly in the cell with a stream of nitrogen (purity > 99.99997%) for at least 30 min before use. Unless specified, all experiments were performed at room temperature.

The following chemicals, used in this research, were from analytical grade and utilised without further purification:

- Sulphuric acid (H_2SO_4, *BDH AnalaR*)
- Hydrogen peroxide 30% (H_2O_2, *Fluka*)
- Ethanol absolute (CH_3CH_2OH, *Panreac*)
- Dipotassium hydrogen orthophosphate (Na_2HPO_4, *Merck*)
- Potassium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$, *Merck*)
- 4-hydroxyphenethylamine (Tyramine, $C_8H_{11}NO$, *Sigma*)
- 4-aminothiophenol ($H_2NC_6H_4SH$, *Aldrich*)
- 2-morpholinoethanesulphonic acid (MES, $C_6H_{13}NO_4S.H_2O$, *Fluka*)
- N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, $C_8H_{17}N_3.HCl$, *Sigma-Aldrich*)
- N-Hydroxysuccinimide (NHS, $C_4H_5NO_3$, *Fluka*)
- SSC buffer, pH 7.0 (20x: 3 mol dm^{-3} sodium chloride + 0.3 mol dm^{-3} sodium citrate, *Invitrogen*)
- 3,9-bisdimethyl-aminophenazithionium chloride (methylene blue, $C_{16}H_{18}ClN_3S$, *Merck*)
- 2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl, $C_4H_{11}NO_3.HCl$, *Duchefa*)
- Ethylenediaminetetraacetic acid disodium salt (EDTA, $C_{10}H_{14}N_2O_2Na_2$, *Duchefa*)
- PicoGreen® (*Invitrogen*).

Oligonucleotides, PCR grade were purchased from Invitrogen Life Technologies:

- Probe modified with 5’ phosphate (PKSM4-5’P): 5’-P-GAAGCTCTGGAATCCGGTAA-3’
- Complementary sequence (PKSM4comp): 5’-TTACCGGATTCCAGAGCTTC-3’
- Non-complementary sequence A (DSR737F): 5’-ACTGCATMAATAAGATGCC-3'
- Non-complementary sequence B (DSR756R): 5’-GGCATCTTATTKATGCAGT-3'

Stock solutions of the various oligonucleotides were prepared with autoclaved Milli-Q water and kept frozen.

5.2.1 Polymer based sensors

Experiments were carried out in the three-electrode compartment glass cell shown in Figure 5.11, with a Pt foil counter electrode (A = 2 cm^2) and a saturated calomel reference electrode (SCE).

FIGURE 5.11 Photograph of the electrochemical cell.

Prior to all measurements, the glassware and cells were soaked overnight in concentrated chromosulphuric solution, followed by rinsing with purified water.

The working electrode consisted of a Pt disk, 0.196 cm^2 geometrical area, embedded in a Teflon holder and sealed with ceramic resin (Pattex) as can be seen in Figure 5.12.

FIGURE 5.12 Photograph of Pt working electrode and electrode support.

Before each assay, the working electrode was hand-polished with successively finer grades of 5, 1 and 0.3 μm alumina suspension (Al_2O_3, Buehler) until a mirror-finishing surface has been generated followed by consecutive potential cycling, from the hydrogen evolution region to the oxygen evolution domain, -0.250 to +1.050 V *vs.* SCE, in 0.1 mol dm^{-3} H_2SO_4 aqueous solutions to remove possible oxide layer which blocks electron transfer at the electrode interface. The resultant cyclic voltammogram (CV) is depicted in Figure 5.13. The experimental data shows four different regions; from cathodic to anodic potentials: H_A,

corresponding to oxidation of adsorbed hydrogens, Q_A, oxides formation; in the reverse cycle, Q_C, corresponds to the reduction of the oxides and H_C indicates the hydrogen adsorption [57].

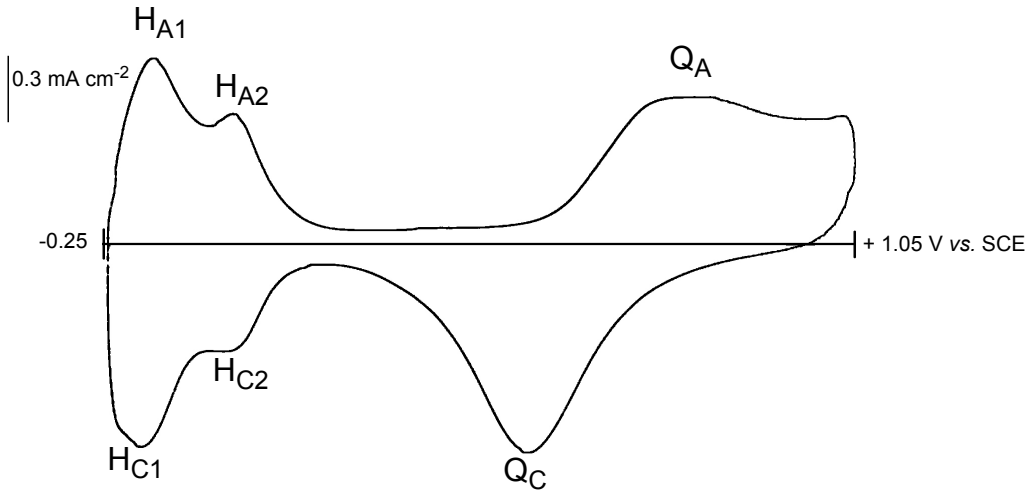

FIGURE 5.13 Cyclic voltammogram of Pt electrode in 0.1 mol dm^{-3} H_2SO_4 at 0.1 V s^{-1}. Regions of oxide formation (Q_A) and reduction (Q_C) and of hydrogen adsorption (H_C) and its oxidation (H_A) are indicated.

5.2.2 Electrochemical Techniques

Cyclic voltammetry (CV) is one of the most versatile electroanalytical technique for the study of electroactive species since it allows to probe the mechanisms of redox and transport properties of a system in solution over a wide potential range. In the present work, this technique is employed to carry out the polymerisation and also to characterise the resulting films and the immobilization and hybridization processes.

Voltammetric measurements use a three electrode electrochemical cell. The *working electrode* is where the electrochemical phenomena being investigated takes place and may be considered an interface at which the mechanism of charge transfer changes between electronic (movement of electrons) and ionic (movements of ions) occurs. The second electrode is the *reference electrode* that is taken as the reference standard against which the potentials of the other electrodes present in the cell can be measured. The third electrode is the *counter* or *auxiliary electrode* which serves to carry the current flowing through the cell, and at the surface of which no processes of interest occur. To support the current generated at the working electrode, the surface area of the auxiliary electrode must be equal, or larger than that of the working electrode. The *electrolyte* is the medium through which charge transfer can take place by the movement of ions [58, 59, 61].

The potential applied to the working electrode is scanned linearly, in a triangular waveform, from an initial value (E_i) to a predetermined limit ($E_{\lambda 1}$) (known as the *switching potential*) where the direction of the scan is reversed as shown in Figure 5.14. The scan rate, is reflected by the slope. Single or multiple cycles can be used [58-61].

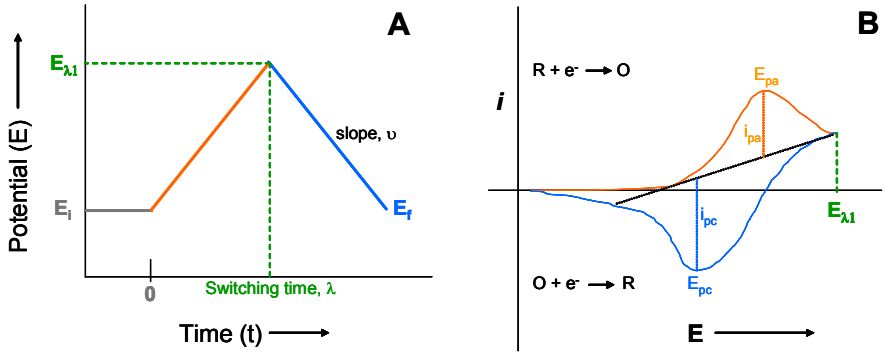

FIGURE 5.14 (A) Triangular potential waveform for cyclic voltammetry (adapted from [58][59]); (B) Resulting cyclic voltammogram (adapted from [58]).

During the potential sweep, the potentiostat measures the faradic current at the working electrode resultant from the applied potential. At the start of the experiment, where the bulk solution contains only one form (e.g. the reduced) of the redox couple, so that at potentials lower than the redox potential (initial potentials) there is no conversion of reduced (R) to the oxidised (O) form. As shown Figure 5.14, B, the forward scan produces a current peak for any analytes that can be oxidised through the range of potential scan. Current will increase as the potential reaches the oxidation potential of the analyte, but then falls off as the concentration of the analyte is depleted close to the electrode surface. As the applied potential is reversed, it will reach a potential that will reduce the product formed in the first oxidation reaction and produce a current of reverse polarity from the forward scan. This reduction peak will usually have a similar shape to the oxidation peak. The current recorded during a cyclic voltammetric experiment gives rise to a peak-shaped response [58-61]. The peaks present in a voltammogram correspond to a particular electroactive reactant in the electrolyte solution, and the height of a peak is proportional to the concentration of the reactant [61]. As a result, information about the redox potential and electrochemical reaction rates of the compounds is obtained.

The important parameters of a cyclic voltammogram are the magnitudes of the anodic and cathodic peak current (i_{pa}, i_{pc}), and the anodic and cathodic peak potential (E_{pa}, E_{pc}) illustrated in Figure 5.14 [58-61].

A redox couple in which both species rapidly exchange electrons with the working electrode is termed an *electrochemically reversible couple*. The number of electrons transferred in the electrode reaction (*n*) for a reversible couple can be determined from the separation between the peak potentials [58-61], equation 6.3.

$$\Delta E_p = E_{pa} - E_{pc} \cong \frac{0.059}{n} \text{ at } 25^{\circ} C \qquad \textbf{(6.3)}$$

Thus, for a *reversible system*, E_p is independent of scan rate (υ), and i_p is proportional to $\upsilon^{1/2}$ and also directly proportional to concentration. The values of i_{pa} and i_{pc} should be identical for a simple reversible couple [58-61], equation 6.4.

$$\frac{i_{pa}}{i_{pc}} = 1 \qquad \textbf{(6.4)}$$

However, peak ratio can be influenced by chemical reactions coupled to the electrode process.

Electrochemical irreversibility is caused by slow electron exchange of the redox species with the working electrode. Moreover, electrochemical irreversibility is characterised by a separation of peak potentials greater than indicated by equation 6.3 [61].

Electrochemical studies were performed with a standard potentiostat - Wenking ST 72 (Bank Elektronik), a voltage scan generator - Wenking Model VSG 72, and a XY recorder - Servogor 790 (Goerz), as presented in Figure 5.15.

FIGURE 5.15 Photograph of the equipment used in the electrochemical studies.

The open circuit potential (OCP) (also referred to as the equilibrium potential, or the rest potential), is the potential were no current or potential is being applied to the working electrode. To perform this measurements only a cell with two electrodes, the working and the reference electrodes, is required. The observed potential variations are referent to modifications at the electrode surface being the process diffusion controlled. Open circuit potential has attracted little attention so far [62], although such measurements are very simple, they are able to provide important informations on the system under study, representing a suitable tool for evaluating surface processes, adsorption of species, corrosion and redox capacitance discharge [62, 63]. In this work, this technique is employed to follow the immobilization and hybridization processes.

Square Wave Voltammetry (SWV) is a sensitive pulse voltammetry technique which can be used for qualitative and quantitative purposes [64]. The analysis of the characteristic parameters of this technique allows the evaluation of the kinetic and mechanisms involved in the process under study [64, 65].

In square-wave voltammetry, a constant pulse potential amplitude is applied during a τ period of time utilising a square modulation of the forward (positive) and reverse (negative) potential pulses on a staircase wave form as shown in Figure 5.16.

The current is measured at the end of each pulse, where potential has been steady and so the capacitance current is low. The positive (if) and negative (ir) currents are then subtracted (difference current). The difference between the forward and reverse pulse is plotted as a function of the applied staircase potential. The major component of this difference current is the faradic current, which flows due to an oxidation or reduction at the electrode surface [61, 65]. The resultant is a peak rather than voltammetric waves. Voltammograms can be scanned anodically and cathodically depending on the species of interest and their characteristic redox potential window.

Square wave voltammetry experiments were conducted with an Electrochemical Workstation CH 420 (CH Instruments, Inc).

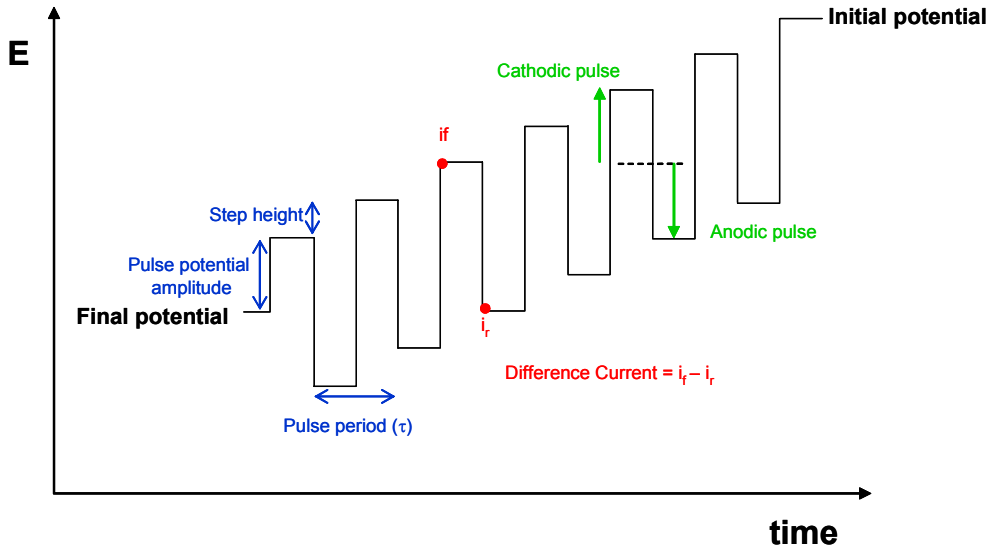

FIGURE 5.16 The potential-time waveform for square wave voltammetry (adapted from [61, 64, 65]).

5.2.3 Microscopic techniques

5.2.3.1 Scanning electron microscopy

The first Scanning Electron Microscope (SEM) was developed and described in 1942 by Zworykin *et al.* [66]. The SEM instrumentation consists primarily of four systems [67], as illustrated in Figure 5.17:

An *illumination/imaging system* that produces the electron beam and directs it onto sample. The *information system* includes the data released by the sample during electron bombardment and detectors which discriminate among and analyse these information signals. A *display system* which consists of one or two cathode-ray tubes for observing and photographing the region of interest. And finally, a *vacuum system* for the remotion of gases from the microscope column which would otherwise interfere with high-resolution imaging.

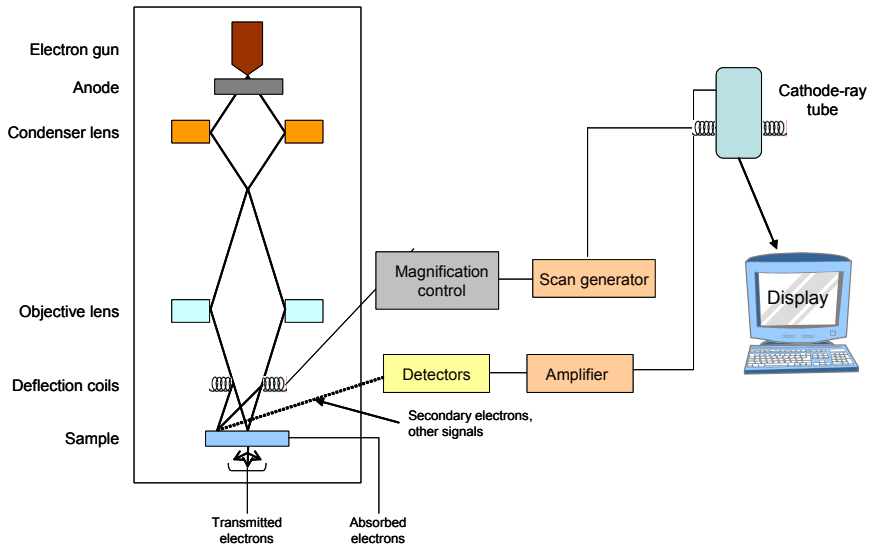

FIGURE 5.17 Schematic illustration of a SEM systems.

The SEM is a microscope that uses a beam of electrons, thermionically emitted from a filament in vacuum, which are accelerated towards an anode. That beam follows a vertical path through the column of the microscope and is collimated by electromagnetic condenser lenses, focused by an objective lens, and scanned across the surface of the sample by electromagnetic deflection coils. Data signals result from interaction between the bombarding electrons and the atoms of the sample. Regardless of their nature, data signals arise from either *elastic* or *inelastic* collisions of beam (primary) electrons with the atoms of a sample. *Elastic* collisions will produce backscattered electrons (BSE), which provide topographic information. *Inelastic* collisions deposit energy within the sample, which then returns to the ground state by releasing distinct quanta of energy in the form of secondary electrons (SE), X-rays, light photons (cathodoluminescence), and nonradiative transitions as phonon (heat) production [66, 67] (Figure 5.18).

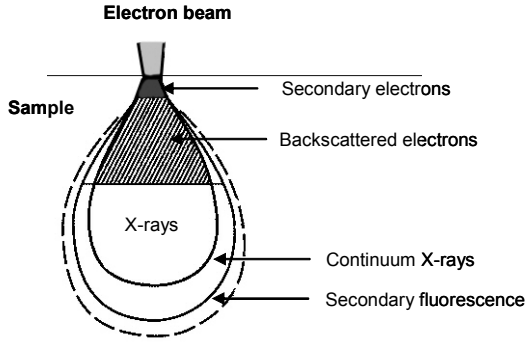

FIGURE 5.18 Relative volume of emission for the different radiations (some micrometers depth).

Imaging is usually obtained using secondary electrons for the best resolution of fine surface topographical features. Alternatively, imaging with backscattered electrons improves the topographical information, due to the relatively high energy of the backscattered electrons signal. Qualitative and quantitative chemical analysis information can also be obtained using an energy dispersive x-ray spectrometer coupled with the SEM. Most SEMs have sample chambers with limited dimensions, and the sample must be fixed to a stage holder for orientation and manipulation within the chamber. Conductive adhesives with low vapour pressure or mechanical devices are used to mount the sample. The sample can be moved in the X, Y, and Z directions, tilted, and rotated. Enhanced resolution (around 2.5 or 1.0 nm) in turn permits higher magnification without loss of detail [67-69]. Another characteristic of SEM concerns the ability to maintain focus across a field of view regardless of surface roughness (depth of field), therefore keeping the three-dimensional appearance of textured surfaces. The combination of high resolution, an extensive magnification range, and high depth of field makes the SEM appropriate for the study of surfaces [67, 68].

For SEM analysis of a surface, it requires the sample to be conductive in order to drain the adsorbed electrons. Examination of uncoated, nonconductive samples as polytyramine, in the SEM is difficult because the sample behaves like insulators. The best method for increasing the secondary electron yield and improving image quality over the entire magnification range of the SEM is to deposit a conductive uniform and thin film, e.g. gold, over the sample surface. These thin films greatly improve the point-to-point resolution of a given sample without suppressing fine surface features [67].

In this work, this technique is employed to observe the PTy surface after its polymerisation and after immersion in buffer solutions at two different temperatures (room temperature and 50 ºC). The instrumentation consisted on an analytical SEM (Hitachi S-2400) with tungsten filament, using 25kV acceleration tension, 20,000x magnification, and a sputter coater of gold (Polaron) as depicted in Figure 5.19.

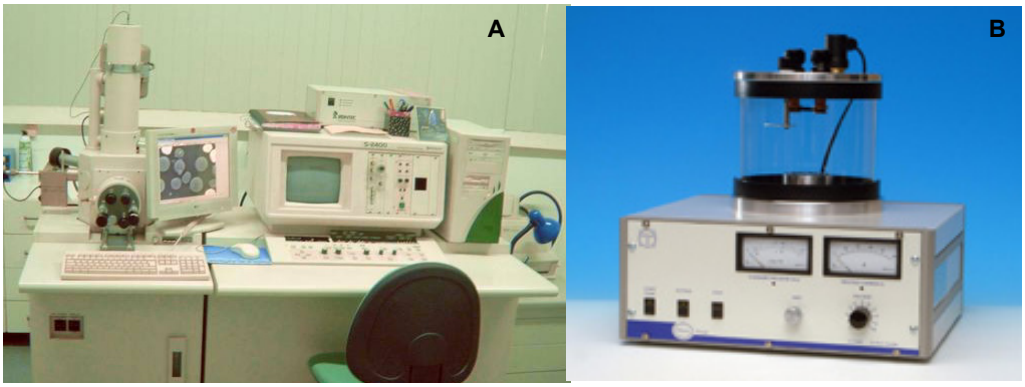

FIGURE 5.19 Photograph of the SEM equipment and sputter coater for the Au deposition, used for the imaging of PTy films. (A) SEM apparatus; (B) sputter coater.

5.2.4 Spectrophotometric techniques

5.2.4.1 Fluorescence

Fluorescence is the result of a three-phase process that occurs in certain molecules (usually polyaromatic hydrocarbons or heterocycles) called fluorophores or fluorescent dyes. The process responsible for the fluorescence of fluorescent probes and other fluorophores can be schematically illustrated by the classical Jablonski diagram, first proposed by the Polish physicist Alexander Jablonski in 1935 [54, 65], which describes the absorption and emission of light as shown in Figure 5.20.

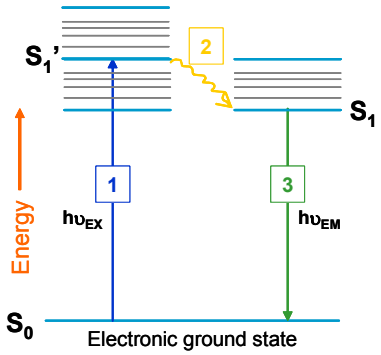

FIGURE 5.20 Jablonski diagram illustrating the processes involve in the creation of an excited electronic singlet state by optical absorption and subsequent emission of florescence (adapted from [54, 65]).

Phase 1: Excitation

Prior to excitation, the electronic configuration of the molecule is described as being in the ground state. A photon of energy $h\upsilon_{EX}$, usually of short wavelengths, is supplied by an

external source such as an incandescent lamp or a laser and absorbed by the fluorophore, creating an excited electronic singlet state (S_1').

$$S_0 + h\upsilon_{EX} \text{ ---> } S_1'$$

Phase 2: Excited-State Lifetime

The excited state subsists for a finite time (typically 1-10 nanoseconds). During this time, the fluorophore undergoes conformational changes and is also subject to a multitude of possible interactions with its molecular environment. These processes have two important consequences. First, the energy of S_1' is partially dissipated, yielding a relaxed singlet excited state (S_1) from which fluorescence emission originates. Second, not all the molecules initially excited by absorption (Phase 1) return to the ground state (S_0) by fluorescence emission. The fluorescence quantum yield (equation 6.3), defined as the ratio of the number of fluorescence photons emitted (Phase 3) to the number of fluorescence photons absorbed (Phase 1), gives a measure of the efficiency of the fluorescence process. The maximum fluorescence quantum yield is 1.0 (100%) where every photon absorbed results in a photon emitted [54].

$$\Phi = \frac{\text{\# photons emitted}}{\text{\# photons absorbed}} \qquad \textbf{(6.5)}$$

Phase 3: Fluorescence Emission

A photon of energy $h\upsilon_{EM}$ is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is lower, and therefore of longer wavelength, than the excitation photon $h\upsilon_{EX}$.

$$S_1 \text{ ---> } S_0 + h\upsilon$$

$h\upsilon$ is a generic term for photon energy where: h = Planck's constant and υ = frequency of light. (The specific frequencies of exciting and emitted light are dependent on the particular system).

Fluorescence microscopes use incident illumination (epi-fluorescence) to deliver light of a defined wavelength to the specimen; the excitation source is usually a xenon / mercury lamp and the choice of wavelengths is done by attached filters. In laser confocal microscopes the light source is a laser emitting at defined wavelengths; through variable pinhole apertures, the light detected is confined to the focal plane [70].

In the present work, this technique is employed to measure the fluorescence of the different modified electrodes after staining with PicoGreen® dye. The setup consisted of an Olympus IX-50 inverted microscope using a 20X (0.75NA) Plan Apo objective, Ludl BioPoint filter wheels and a PCO Sensicam cool CCD. The camera has a frame transfer architecture and requires no shutter. Integrated control of filter wheel and image acquisition is achieved by Image-Pro Plus 4.0 and Scope-Pro 3.1 (Media Cybernetics) as depicted in Figure 5.21. Settings for image acquisition (camera exposure time, filters, time interval and storing modes) are determined by custom-made macros. Fluorescence intensity were measured using Image-Pro Plus 5.0 software (Media Cybernetics, Leiden, Netherlands).

FIGURE 5.21 Photograph of the inverted microscope used in the fluorescence measurements.

5.2.5 Self-assembled monolayers based sensors

The electrochemical experiments were conducted in a single-compartment Teflon electrochemical cell as presented in Figure 5.22. A three electrode system was used. A saturated calomel electrode (SCE) and a platinum wire served as the reference and counter electrode, respectively. The working electrode (gold slide) was mounted at the bottom of the cell using an O-ring, which defines a geometric area of 0.57 cm^2. All experiments were carried out at room temperature and all solutions were deoxygenated with N_2 (purity 99.99997%) for at least 1 h before use.

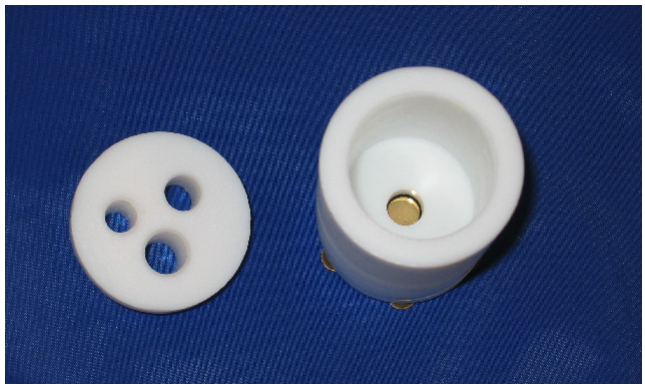

FIGURE 5.22 Photograph of the electrochemical cell.

Gold film (200 nm) on glass (1.1 x 1.1 cm^2) with a pre-layer of chromium (2-4 nm), as can be seen in Figure 5.23, purchased from Arrandee^{TM}, was used as substrate for SAMs preparation and will be further referred as Au slide.

Prior to use, the Au slide was cleaned by immersion in piranha solution (3:1 H_2SO_4 / 30% H_2O_2) for a few minutes to remove contaminants [71]. The piranha solution is a strong oxidant and should be handled with caution. Afterwards, the plate was rinsed thoroughly, with alternating Millipore water and ethanol, for removal of possible gold oxide, and dried with a nitrogen stream.

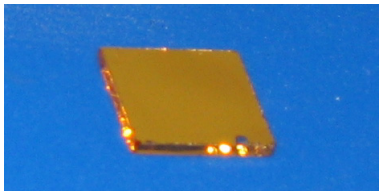

FIGURE 5.23 Photograph of the gold slide.

After cleaning, the Au was annealed in the cold part of a Bunsen-burner flame to yield atomically flat terraces with a predominant (111) crystallographic orientation, cooled in air and immersed in the ethanol solution of 0.001 mol dm^{-3} 4-ATP for about 1 h and 20 h at room temperature (20 ± 2 ºC). The prepared SAM was thoroughly rinsed with ethanol and Millipore water and then dried under a stream of nitrogen.

The roughness factor of these substrates (1.2) has been determined previously [72] and agrees with the reported value for similar thin Au film electrodes.

5.3 Results and discussion

5.3.1 Sensors based on Polytyramine modified electrodes

5.3.1.1 Electropolymerisation and characterisation

In this study, the potentiodynamic polymerisation of tyramine was carried out under the conditions previously developed in the Interfacial Electrochemistry group and described by Tenreiro *et al*. [21]. The polytyramine (PTy) films were grown on Pt electrode, from acidic aqueous solution of 0.1 mol dm^{-3} H_2SO_4 containing 0.025 mol dm^{-3} tyramine. The polymers were synthesised potentiodynamically by cycling the potential between –0.100 and +1.050 V *vs*. SCE for 25 potential cycles at 0.050 V s^{-1} (designated PTy25/50). Before the first potential scan, the initial potential (-0.100 V) was applied for 120 s. This procedure prevents the process to be limited by mass transfer, since it provides time for the Ty monomers to reach the electrode surface, completely covering it and being ready to be oxidise during the first potential scan.

In Figure 5.24 it is represented the current responses of a bare Pt electrode in 0.1 mol dm^{-3} H_2SO_4 in the absence and in the presence of tyramine monomer. Tyramine oxidation currents are detectable at about 0.8 V being a maximum obtained at ca. 1 V. However, no crossing of the currents, corresponding to nucleation of a new fase at the electrode surface is observed. It can also be noted that the cathodic peak, correlative to the platinum oxide reduction, observed in the supporting electrolyte solution (H_2SO_4) is no longer detected in the reverse potential sweep of the Ty containing solution, this is an indication that there should be a deposit at the electrode surface.

The cyclic voltammograms obtained on a Pt electrode during tyramine electropolymerisation are illustrated in Figure 5.25. The recorded data are identical to the already described in the literature [21]: in the first oxidation cycle, tyramine oxidation currents are detectable at about 0.8 V; during the subsequent cycles, a gradual decrease of the anodic current is observed for the first 13 cycles, then the current starts to increase slowly until the 22^{nd} cycle and finally it remains approximately constant, indicating that the electrode is not passivated; throughout the reverse potential sweeps, there are no observation of the appearance of a cathodic peak corresponding to the polymer reduction. A similar behaviour have already been reported for PTy electrodeposition synthesised in solutions containing higher concentration of monomer [19, 20, 22, 30], in neutral solutions and carried out by scanning the potential until more positive potential limits [19, 22].

The electrosynthesised films were washed with Milli-Q water and subsequently discharged by applying a potential of -0.100 V *vs*. SCE for 300 s to assure the egress to the solution of species present in the polymer film and thus to prevent their possible interference during subsequent assays.

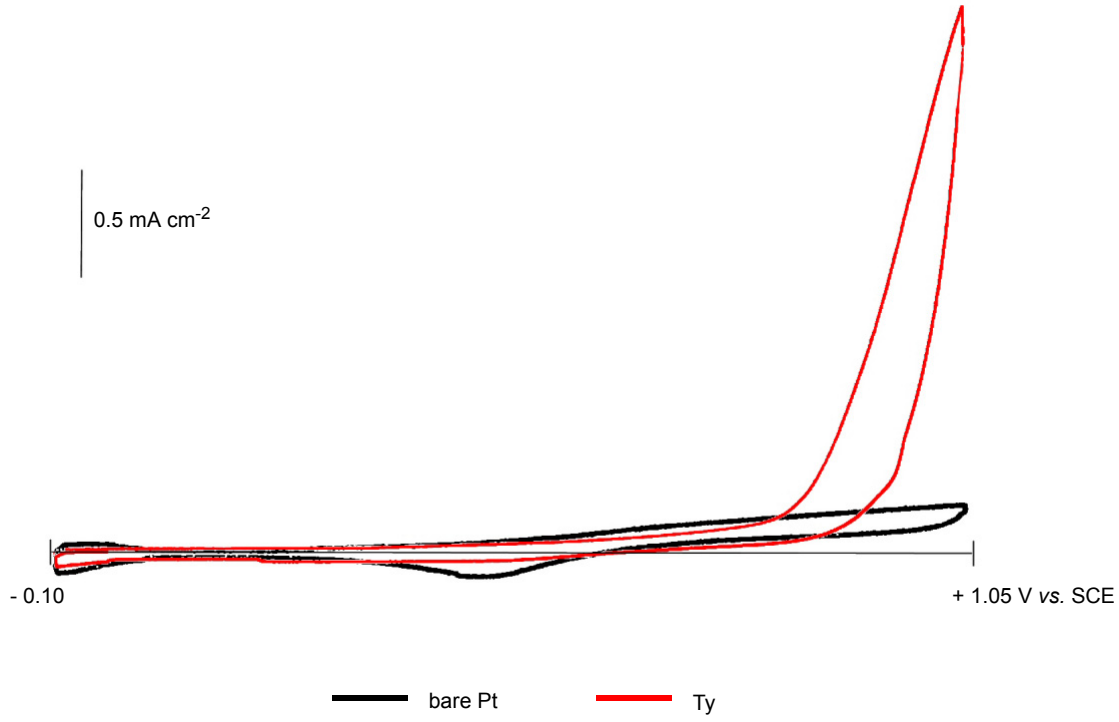

FIGURE 5.24 Cyclic voltammograms of bare Pt in 0.1 mol dm^{-3} H_2SO_4 without (black) and with Ty monomer (red); υ = 0.05 V s^{-1}.

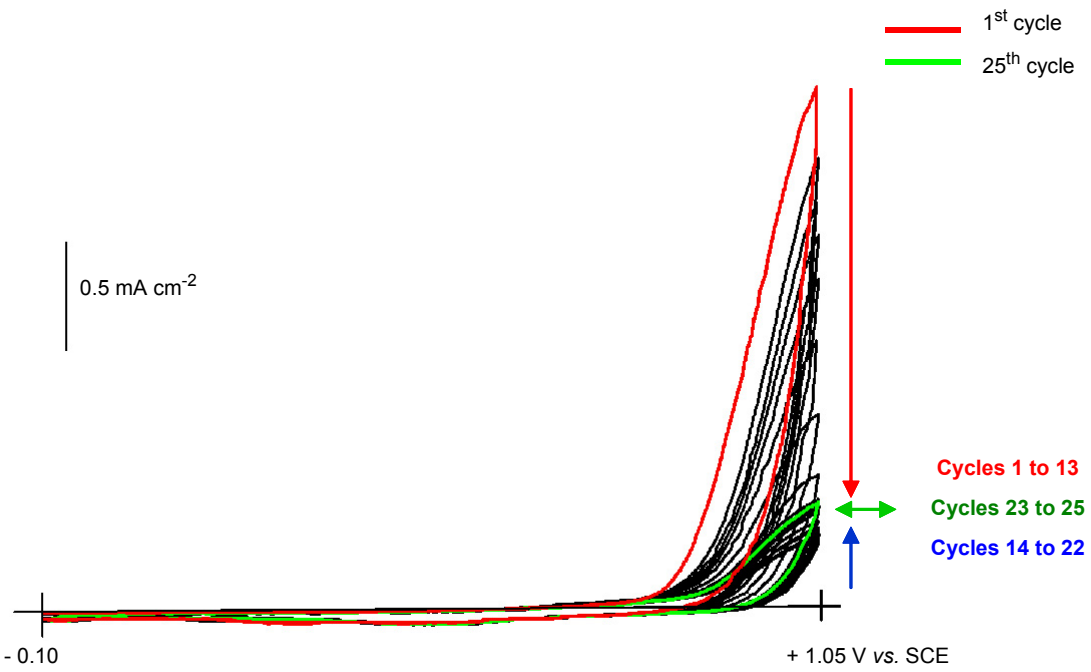

FIGURE 5.25 Cyclic voltammogram for the potentiodynamic growth of PTy (25 potential cycles from -0.10 to 1.05 V) on Pt electrode from 0.025 mol dm^{-3} Ty in 0.1 mol dm^{-3} H_2SO_4; υ = 0.05 V s^{-1}.

The PTy films were morphologically characterised by scanning electron microscopy. The image, Figure 5.26, show that homogeneous deposits have been obtained.

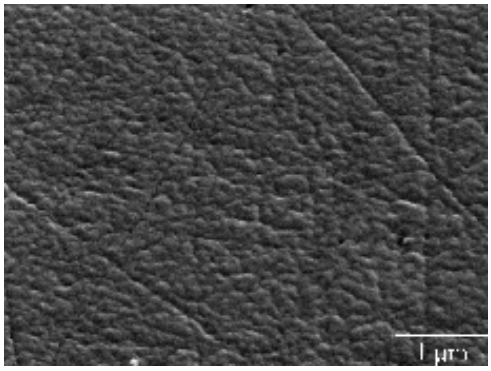

FIGURE 5.26 SEM photomicrograph of a PTy 25/50 film using 20,000x magnification.

The electrochemical characterisation of the PTy modified electrodes has been performed in 0.1 mol dm^{-3} H_2SO_4 (Figure 5.27) and also in 0.1 mol dm^{-3} phosphate buffer (PB) pH 7, a suitable medium for the investigation of biological species (Figure 5.28), where for comparison the bare Pt electrode response is also indicated. The voltammograms are feature-

less and denotes the PTy poor conducting properties, as expected from the electropolymerisation data and also from previous studies [19, 22],.

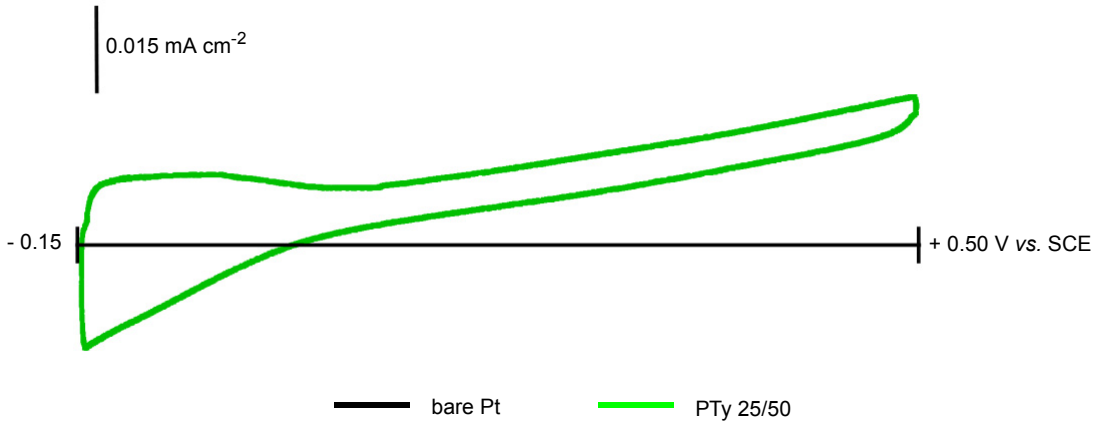

FIGURE 5.27 Cyclic voltammogram of the redox behaviour of PTy 25/50 in 0.1 mol dm^{-3} H_2SO_4; υ = 0.05 V s^{-1}.

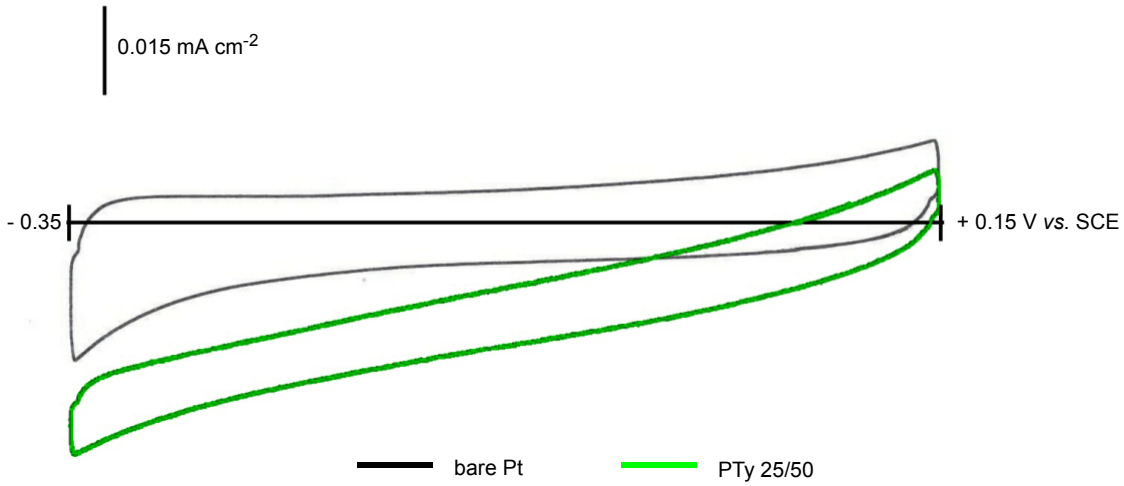

FIGURE 5.28 Cyclic voltammogram of the redox behaviour of PTy 25/50 (green) in 0.1 mol dm^{-3} PB, pH 7; bare Pt electrode characterisation (gray); υ = 0.05 V s^{-1}.

5.3.1.2 Detection of ss-DNA probe immobilization

PTy films present a high density of amino groups (one NH_2 per monomer unit). The attachment of ss-DNA probes was performed via a phosphoramidate bond as represented in Figure 5.6. This covalent link is performed by reaction of a 5'-phosphate modified ODN probe (PKSM4-5'P) to the amines of PTy films in the presence of EDC and NHS activating agents.

The use of buffers based on inorganic or organic salts for the covalent attachment of the probe is limited because of the possible interference of buffers cations and anions with the

reaction under study. It has been shown that common buffer media like phosphate, acetate and citrate interfere with EDC activation, and thus should be avoided [73]. However, zwitterionic buffers, like MES (2-morpholinoethanesulphonic acid) (Figure 5.29), are an appropriate alternative because of the low interference with EDC activation and biological processes [74].

The ss-DNA probe immobilization, using EDC/NHS coupling agents, was carried out in 0.01 mol dm^{-3} MES pH 6 buffer solution.

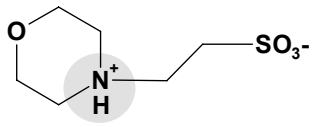

FIGURE 5.29 Schematic representation of MES [75].

Buffered solutions of MES containing EDC, NHS and PKSM4-5'P probe were prepared immediately before use. PTy modified electrodes were immersed in this solution at room temperature. Prior to the voltammetric characterisation, the films were thoroughly washed with Milli-Q water to remove coupling agents and non-covalently bound oligonucleotides.

An expedite method to detect the immobilization process is the monitorisation of the open circuit potential (OCP) evolution with time. This measurement provides an indication on the feasibility of the processes leading to the effective binding between 5' phosphate ss-DNA probe (activated by EDC and NHS) and the PTy amino terminal groups.

To define the time of immersion and appropriate concentrations of coupling agents and probe to be used in the immobilization process, three different situations were tested (Table 5.1), and the OCP evolution with time has been registered as presented in Figure 5.30. The observed OCP variation suggests an alteration in the electrode interface, which can be due to the settling of covalent bonds. The initial OCP of the PTy 25/50 films is approximately + 0.430 V *vs*. SCE. A rapid initial negative shift of OCP is verified; after 3600 s the variation is much less pronounced, although a steady state is not reached even for longer immobilisation times (data not shown). Therefore it was decided to conduct the experiments during 7200 s.

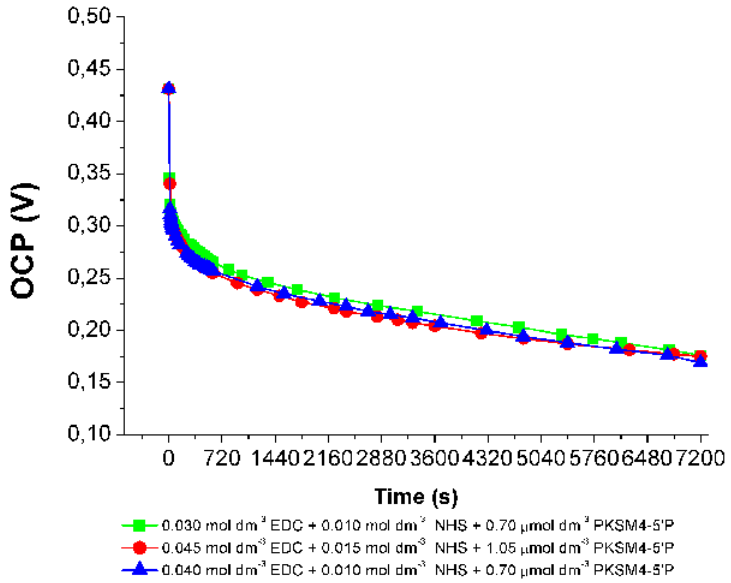

FIGURE 5.30 Evolution of the open circuit potential in time of PTy 25/50 modified electrodes after their immersion in 0.01 mol dm^{-3} MES buffer pH 6 containing different concentrations of EDC, NHS (coupling agents) and PKSM4-5'P probe.

Although a great difference between the three situations can not be denoted by direct observation of Figure 5.30, the potential evolution (ΔOCP) presents some differences as summarised in Table 5.1. Among the three solutions tested, the one that provided slightly higher OCP variation is the medium containing 0.04 mol dm^{-3} EDC, 0.01 mol dm^{-3} NHS and 0.7 μmol dm^{-3} PKSM4-5'P and therefore the following immobilization processes were performed under this conditions.

TABLE 5.1 ΔOCP of PTy 25/50 films observed with coupling agents and probe concentrations variation in 0.01 mol dm^{-3} MES buffer pH 6.

Concentrations tested	ΔOCP (V)
0.030 mol dm^{-3} EDC + 0.010 mol dm^{-3} NHS + 0.70 μmol dm^{-3} PKSM4-5'P	0.255
0.450 mol dm^{-3} EDC + 0.015 mol dm^{-3} NHS + 1.05 μmol dm^{-3} PKSM4-5'P	0.256
0.040 mol dm^{-3} EDC + 0.010 mol dm^{-3} NHS + 0.7 μmol dm^{-3} PKSM4-5'P	0.262

The OCP evolution with time was also monitorised for PTy films immersed only in 0.01 mol dm^{-3} MES buffer solution pH 6, and in 0.01 mol dm^{-3} MES buffer pH 6 containing 0.04 mol dm^{-3} EDC and 0.01 mol dm^{-3} NHS coupling agents, and compared to the response when the

probe is also present in solution. The results are displayed in Figure 5.31 and the corresponding OCP in Table 5.2. As can be seen in Figure 5.31, during the immobilization process, a rapid initial negative shift of OCP is verified, indicative of an immediate reaction; the presence of the activating agents (EDC and NHS) and the probe give rise to stronger OCP modifications (0.262 V *vs*. 0.150 V in its absence), suggesting an increase of negative charges (from the ss-DNA) at the surface of the polymeric layer. Notwithstanding, it must be noticed that the alteration in the potential appears to be mainly due to the presence of the coupling agents, calling for the employment of an independent monitoring method.

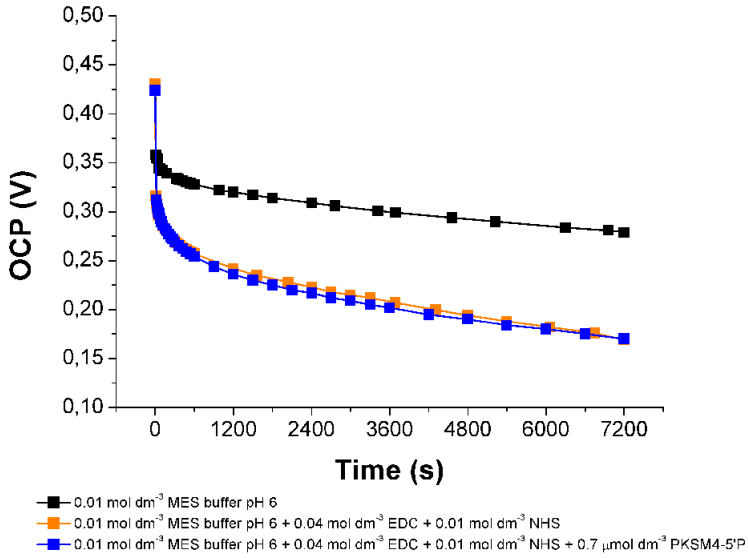

FIGURE 5.31 Evolution of the open circuit potential in time of PTy 25/50 modified electrodes after their immersion in different MES buffered solutions.

TABLE 5.2 ΔOCP of PTy 25/50 films observed in 0.01 mol dm^{-3} MES buffer pH 6, with and without coupling agents and ss-DNA probe.

Medium	ΔOCP (V)
0.01 mol dm^{-3} MES buffer pH 6	0.150
0.01 mol dm^{-3} MES buffer pH 6 + 0.040 mol dm^{-3} EDC + 0.010 mol dm^{-3} NHS	0.254
0.01 mol dm^{-3} MES buffer pH 6 + 0.040 mol dm^{-3} EDC + 0.010 mol dm^{-3} NHS + 0.7 μmol dm^{-3} PKSM4-5'P	0.262

5.3.1.3 Hybridization

Hybridization experiments were performed on ss-DNA probe covalently attached to the PTy matrix. The tests consisted on soaking these probe-modified electrodes in a solution containing 0.7 μmol dm^{-3} of the complementary (complementary hybrid formation) or non-complementary sequence (non-complementary hybrid formation) in 2x SSC (sodium chloride + sodium citrate) buffer pH 7, for 7200 s at room temperature.

UltraPure™ 20x SSC is a solution formulated for use in nucleic acid hybridizations. Ionic strength is one of the parameters that influences the hybridization results, namely with a non-complementary sequence. In order to provide the most stringent conditions and thus establish the more suitable conditions to perform the hybridization studies at room temperature, simulations using different buffer concentrations: 1x, 2x, 3x, 4x and 5x SSC (ionic strength) (Table 5.3) were executed using AutoDimer (v1) program [76]. At room temperature (22 ºC), the condition that provided highest ΔG value, corresponding to the lowest probability of bounding between the immobilized probe and the non-complementary sequence, was the 2x SSC buffer solution and therefore this was the medium chosen for the hybridization process.

TABLE 5.3 Influence of the ionic strength on the hybridization results with a non-complementary sequence.

Temperature	Ionic strength	Non-complementary sequence (DSR737F)
22 ºC	1x SSC	ΔG = -13.35 kJ/mol
	2x SSC	ΔG = -9.66 kJ/mol
	3x SSC	ΔG = -15.81 kJ/mol
	4x SSC	ΔG = -11.25 kJ/mol
	5x SSC	ΔG = -16.99 kJ/mol

The evolution of the open circuit potential with time was also registered as a first approach to monitorise the hybridization process (Figure 5.32; Table 5.4). After immersion in each media, the initial OCP of the PTy 25/50 films with the immobilized probe is approximately +0.315 V *vs*. SCE and in similarity with previous immobilization studies, an abrupt initial negative shift is observed. The potential decreases continuously and an about constant potential plateau is reached after about 1440 s. A clearly noticeable change in the OCP value is only obtained for the hybridization in presence of the complementary sequence, indicating that this

method can be used to distinguish between the presence of a complementary from non-complementary sequence.

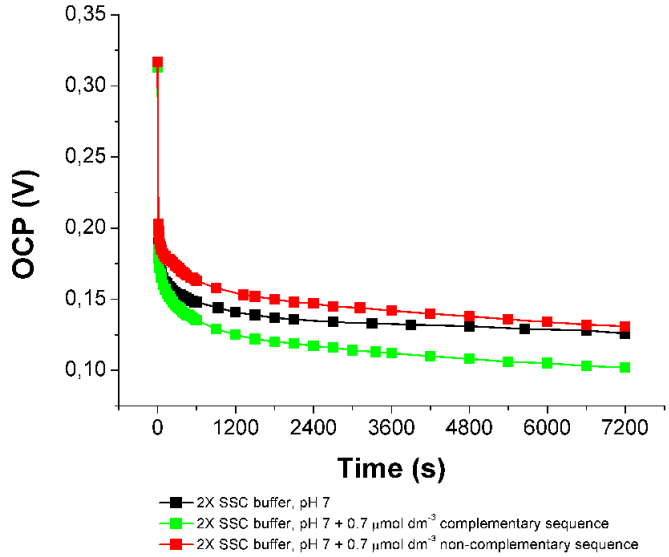

FIGURE 5.32 Evolution of the open circuit potential in time of PTy25/50-ss-DNA modified electrodes after their immersion in 2x SSC buffer pH 7; and in the presence of 0.7 μmol dm-3 complementary or non-complementary sequences.

TABLE 5.4 ΔOCP of PTy 25/50 films with immobilized probe observed in 2x SSC buffer pH 7, and in the presence of a complementary (cs) or non-complementary (nc) sequence.

Medium	ΔOCP (V)
2x SSC buffer, pH 7	0.189
2x SSC buffer, pH 7 + PKSM4 (cs)	0.211
2x SSC buffer, pH 7 + DSR737F (nc)	0.186

5.3.1.4 Detection by cyclic voltammetry

After the above described immobilization and hybridization procedures, cyclic voltammetry was used to characterise the modified electrodes in a solution containing a redox indicator. Based on the previous discussion (6.1.4), the electroactive redox indicator selected was methylene blue (MB). All the MB solutions were used immediately after being prepared.

Since the solution prepared contains mostly MB in the oxidised form, the voltammetric scan has been performed from the most positive potential limit to the most negative limit, allowing to analyse the indicator reduction reaction.

The cyclic voltammogram of MB characterisation in phosphate buffer, pH 7 at bare Pt electrode from 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7 at υ = 0.05 V s^{-1} of unstirred solu-

tion is presented in Figure 5.33. The obtained redox response of MB (blue) is compared to the PB alone (gray). The electrode reaction follows a process of two electrons and one proton at pH 7 [77-79]. A pair of redox peaks with the anodic peak potential (A) at -0.190 V and the cathodic peaks potential at 0 V (B) and -0.215 V (C), with a 0.026 V peak separation is observed. In Figure 5.33 can be verified that the currents ratio is grater than one, suggesting the presence of a quasi-reversible system.

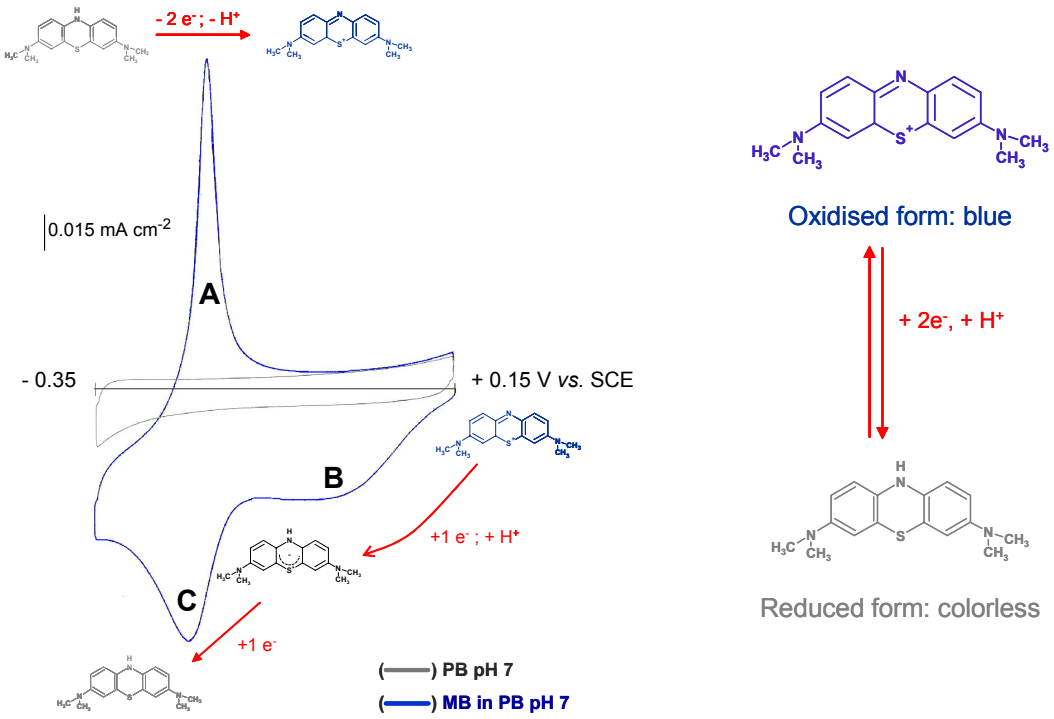

FIGURE 5.33 Cyclic voltammogram of MB (blue) characterisation and PB (grey) on bare Pt electrode from 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7; υ = 0.05 V s^{-1}.

Several parameters have been investigated in order to have the best electrochemical conditions, as shown in Figure 5.33, for the observation of MB redox processes. These included the influence of solution stirring, as shown in Figure 5.34. As it can be observed, the stirring of the solution does not markedly increase the peaks height and thus all the following studies were performed with unstirred solutions.

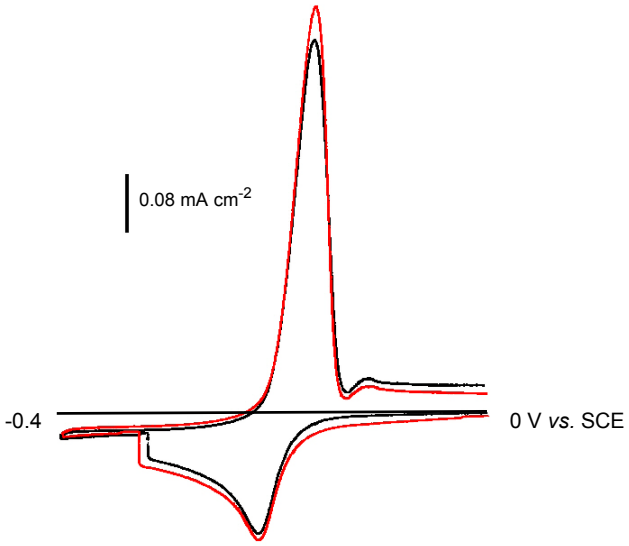

FIGURE 5.34 Cyclic voltammograms of MB characterisation on bare Pt electrode from 1 mmol dm^{-3} MB in 0.1 mol dm^{-3} PB with (red) and without stirring (black), pH 7; υ = 0.05 V s^{-1}.

Also the influence of the MB concentration has been tested, three MB concentrations have been investigated as presented in Figure 5.35. It can be verified that at 1 mmol dm^{-3} a peak (appointed with a *) corresponding to MB adsorption is observed. It is known that the methylene blue cation (MB^+) adsorbs ubiquitously at biological and inorganic surfaces, e.g. gold [77], mercury [78] and platinum [79] and therefore, the MB concentration selected was 250 μmol dm^{-3}, since the small peak due to adsorption is no longer observed and also it provides more defined redox peaks than 100 μmol dm^{-3}.

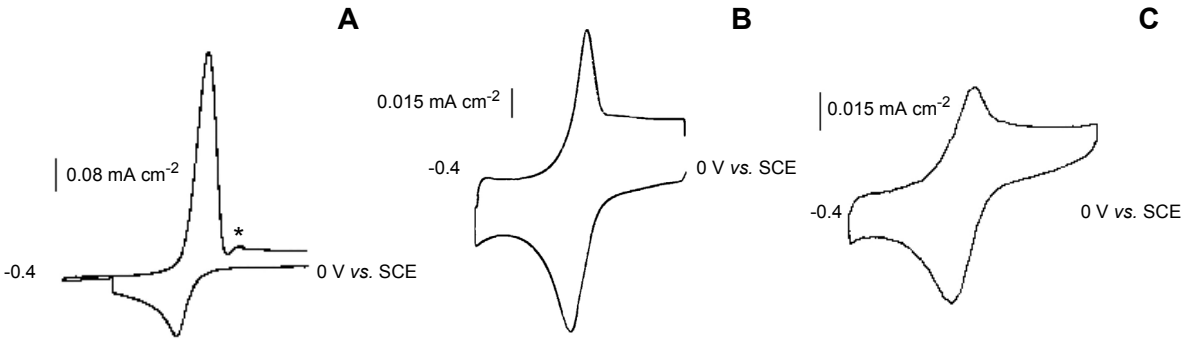

FIGURE 5.35 Cyclic voltammograms of MB characterisation on bare Pt electrode from 1 mmol dm^{-3} (A); 250 μmol dm^{-3} (B) and 100 μmol dm^{-3} (C) MB in PB, pH 7; υ = 0.05 V s^{-1}.

The selection of the potential range to be used in the voltammetric characterisation has also been performed as exhibited in Figure 5.36, where a positive potential limit of +0.150 V *vs*. SCE and a negative potential limit of -0.350 V *vs*. SCE have been chosen.

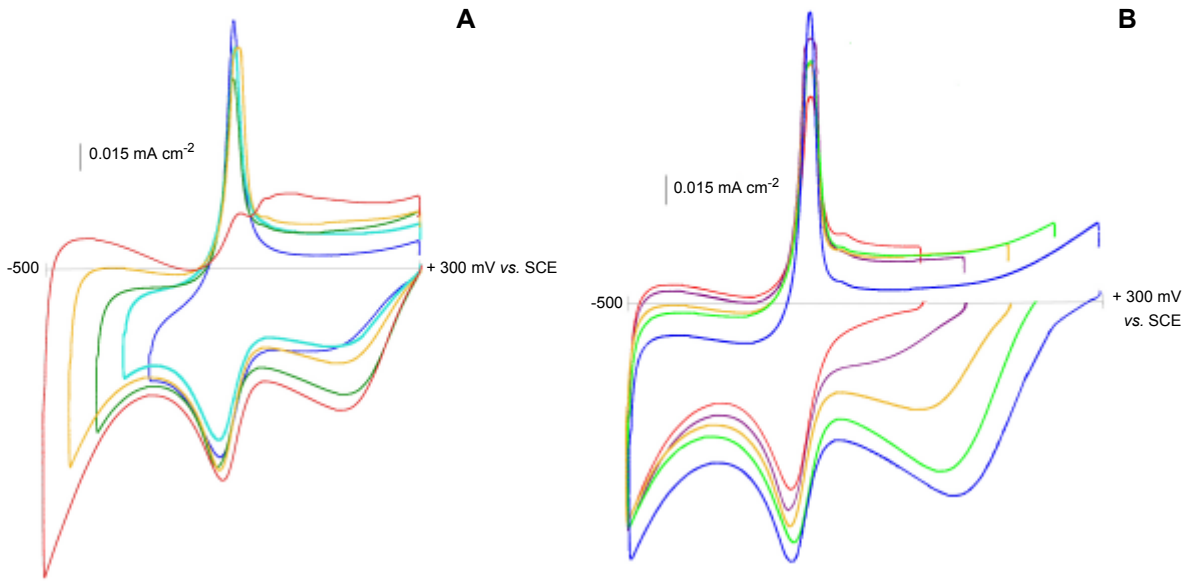

FIGURE 5.36 Cyclic voltammograms of MB characterisation on bare Pt electrode from 250 μmol dm^{-3} MB in PB, pH 7; υ = 0.05 V s^{-1}. (A) Selection of more negative potential limit: -0.350 (blue); -0.400 (light blue); -0.450 (green); -0.500 (orange) and -0.550 V *vs*. SCE (red). (B) Selection of the more positive potential limit: 0 (red); 0.075 (violet); 0.150 (orange); 0.225 (green) and 0.300 V *vs*. SCE (blue).

The effect of the sweep rate on the redox processes of MB is shown in Figure 5.37A, being the representation of the anodic and cathodic peak current densities as function of $\nu^{1/2}$ presented in Figure 5.37B, which allows to verify that the currents increase linearly with $\nu^{1/2}$. This behaviour, together with the ΔEp of 0.026 V (Figure 5.33), are consistent with a quasi-reversible system, also reported by Willner *et al.* [80].

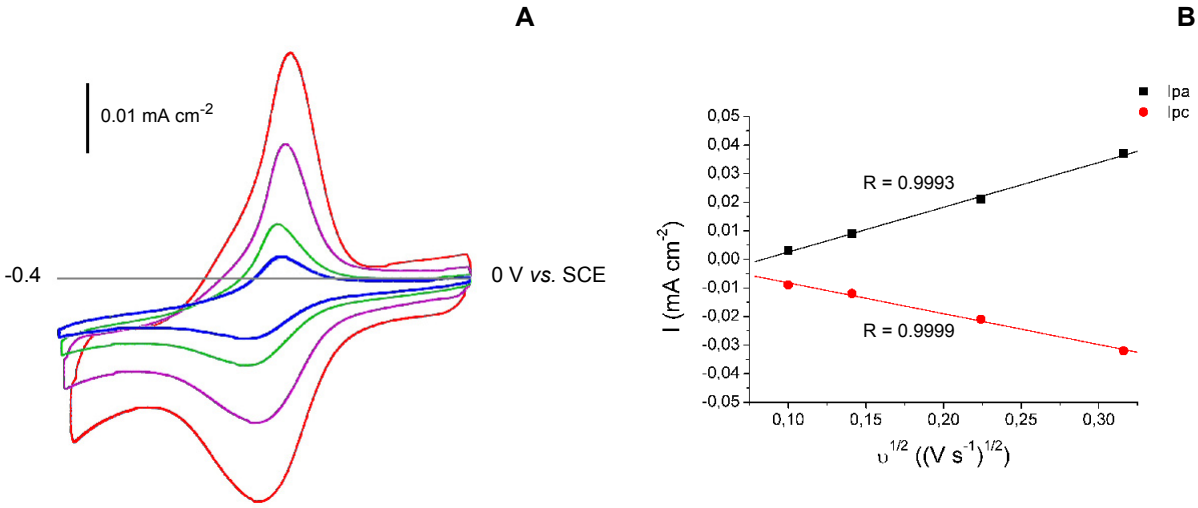

FIGURE 5.37 (A) Cyclic voltammograms of MB characterisation on bare Pt electrode from 100 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB pH 7 at different sweep rates: υ = 0.01 (blue); 0.02 (green); 0.05 (violet) and 0.1 V s^{-1} (red). (B) Graphical representation of the peaks current density as function of $\nu^{1/2}$ for the methylene blue system.

After the hybridization procedure, followed by careful washing, the modified electrodes have been characterised in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7 at υ = 0.100 V s^{-1}, as illustrated in Figure 5.38 and the voltammetric features are given in Table 5.5. Compared to the pristine PTy, an increase of cathodic peak current densities are observed after the immobilization of the ss-DNA probe and the hybridization with complementary or non-complementary sequence; however, the last present a small difference. This behaviour can be due to the establishment of some bounds between the immobilized probe and the sequence chosen as non-complementary, leading to an accumulation of DNA at the surface of the modified electrode and giving rise to a current increase when this electrode is characterised in the MB solution.

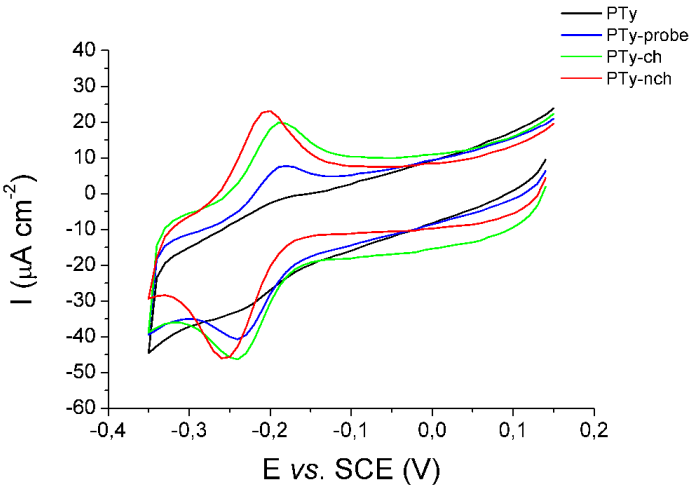

FIGURE 5.38 Cyclic voltammogram of modified electrodes characterisation in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7; υ = 0.100 V s^{-1}. PTy: polytyramine film; PTy-probe: PTy + immobilized ss-DNA probe; PTy-ch: PTy + probe hybridized with complementary sequence; PTy-nch: PTy + probe hybridized with non-complementary sequence.

It must be noted that all the modified electrodes present a MB redox peak separation slightly higher then the one (Table 5.5) of a reversible process (ΔE_p ~ 0.030 V) [61], which is in agreement with the fact of MB being a quasi-reversible system. When compared to the response recorded for MB (Figure 5.33) which presents an anodic peak of -0.190 V and the cathodic peak (B) of -0.215 V, the modified electrodes characterisation resumed in Table 5.5, present a shift to more negative values of the peak potentials, indicating that is more difficult to reduce the MB with increasing concentration of DNA at the electrode surface, besides confirming the electrode modifications.

TABLE 5.5 Values of E_{pa}, E_{pc}, ΔE_p, and I_{pc} obtained from the voltammetric characterisation of the PTy modified films.

Modified electrode	E_{pa} (V)	E_{pc} (V)	ΔE_p (V)	I_{pc} (μA cm^{-2})
PTy	-0.190	-0.229	0.039	31.8
PTy + ss-DNA probe	-0.181	-0.237	0.056	40.5
PTy + probe hybridized with non-complementary sequence A	-0.204	-0.256	0.052	45.7
PTy + probe hybridized with complementary sequence	-0.186	-0.243	0.057	46.3

The temperature is one of the parameters that can be increased to rise the specificity (stringency) of hybridization experiments avoiding the bound between the non-complementary sequence and the immobilized probe [81]. Thus, simulations using different temperatures: 22, 30, 40, 50 and 60 ºC, using 2x SSC buffer concentration, (Table 5.6) were performed using AutoDimer (v1) program [76]. From Table 5.6, it can be verified that the temperature increase provides highest ΔG values, corresponding to less interactions between the non-complementary sequence and the probe and therefore the PTy matrix thermal stability, was evaluated at 50 ºC (one of the higher temperatures tested).

TABLE 5.6 Influence of the temperature on the hybridization results with a non-complementary sequence.

Ionic strength	Temperature	Non-complementary sequence (DSR737F)
2x SSC	22 ºC	ΔG = -9.66 kJ/mol
	30 ºC	ΔG = -9.16 kJ/mol
	40 ºC	ΔG = -8.49 kJ/mol
	50 ºC	ΔG = -7.82 kJ/mol
	60 ºC	ΔG = -7.15 kJ/mol

PTy films were subjected to 7200 s immersion in 0.01 mol dm^{-3} MES buffer pH 6, at 50 ºC. The PTy matrix after these procedure did not revealed great modification when characterised in PB, however, the films characterisation in MB solutions provided an indication of the films alteration since there was an increase of the peak currents, as shown in Figure 5.39. To verify if the redox behaviour observed was related with an alteration of the PTy films structure, the films were morphologically characterised using SEM. The SEM photomicrographs of the synthesized PTy films are presented in Figure 5.39.

The immersion of PTy film in MES buffer at room temperature (RT) does not seem to modify the polymeric surface since there is not a great difference between the photographs related with these situations. However, it can be noted that after the treatment at 50ºC the PTy morphology seems to present some alterations (possible holes), which can explain the current increase in the voltammetric characterisation. Given this result, the use of higher temperatures to perform hybridization procedures is not a viable option.

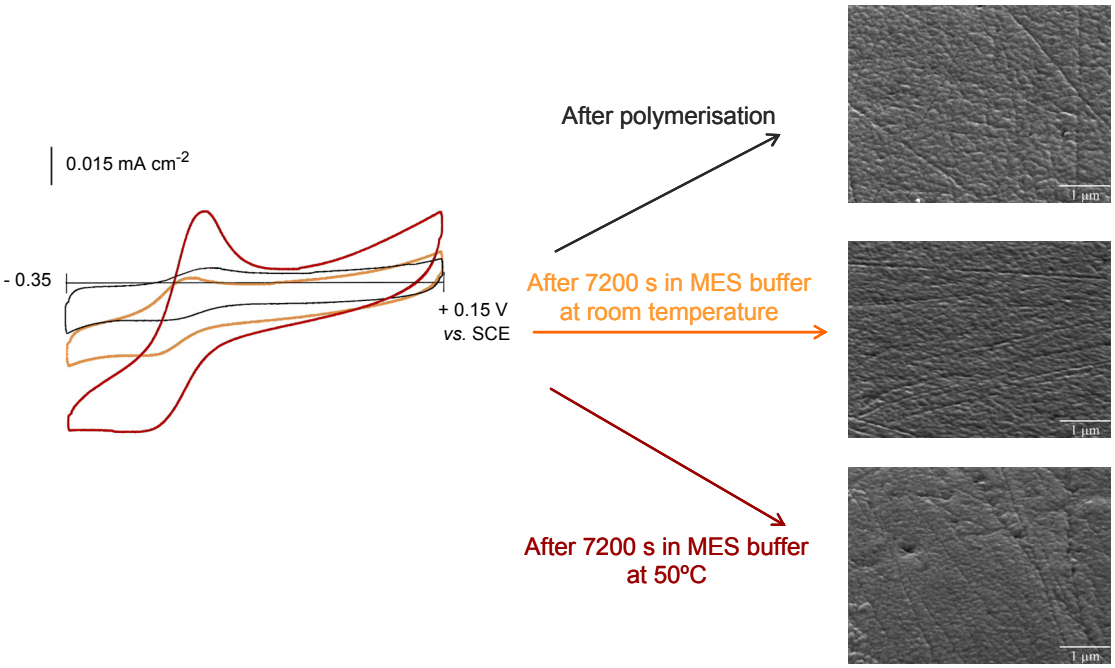

FIGURE 5.39 Cyclic voltammograms of PTy 25/50 characterisation in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7; υ = 0.05 V s^{-1}. After polymerisation (black) after 7200 s incubation in MES buffer at room temperature (orange) and after 7200 s incubation in MES buffer at 50ºC (wine) (left side). SEM microphotographs of PTy films at magnifications of 20,000X, after polymerisation, and after 7200s immersion in 0.01 mol dm^{-3} MES buffer pH 6 at RT and 50ºC (right side).

5.3.1.5 Detection by square wave voltammetry (SWV)

The SWV technique was also used to characterise the modified electrodes in MB solutions. Detection of the electrode modification was monitorised by measuring the cathodic peak current of MB with SWV. Because the capacitive or charging current component, due to the electrical charging of electrode double layer, is largely eliminated, an increase of the signal to noise ratio is obtained [65] and so it should provide a sensitivity increase, as already mentioned before. It is known that the optimum pulse amplitude is about 0.050/*n* V, where *n* is the number of electrons in the redox reaction [65]. Thus, since in the methylene blue conversion there are two electrons involved, the pulse amplitude selected was 0.025 V.

In order to select the more appropriate frequency value to be used in the SWV, several frequency values were tested for PTy25/50 modified electrodes. The results obtained are displayed in Figure 5.40 and allowed to selected a frequency of 5 Hz, since it provided the obtention of a sharper peak with higher current than the other frequencies tested.

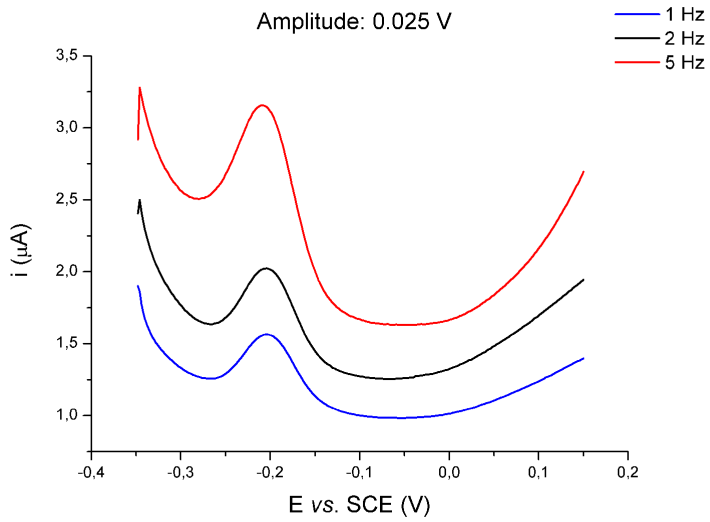

FIGURE 5.40 Square wave voltammetry of PTy25/50 at several frequency values in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7. Other SWV parameters: 0.002 V step, 0.025 V pulse amplitude.

Taking to account the results above mentioned, the parameters selected to characterise the modified electrodes were 0.002 V step, 0.025 V pulse amplitude, 5 Hz frequency and the initial potential was +0.150 V, which results are presented in Figure 5.41. It can be observed that the probe immobilization originates a marked increase of the MB cathodic peak current, which upon its hybridization with a complementary sequence results in an even more noticeable current increase. The differences observed by SWV are much more evident than the ones obtained by CV, allowing a more clear distinction of the PTy25/50 films modifications.

Figure 5.41 shows the results of the hybridization tests with two non-complementary sequences (A - yellow and B -red), both displaying higher cathodic peaks than the one obtained for the electrode with the complementary hybrid (green). Since it is already reported that more positive potentials increase MB adsorption [82], a less positive potential (-0.050 V) was tested as the initial potential for the SWV characterisation of the modified electrode with non-complementary hybrid A (A1 - orange). However, as it can be seen in Figure 5.41, not much difference has been obtained, when compared to the peak observed using an initial potential of +0.150 V.

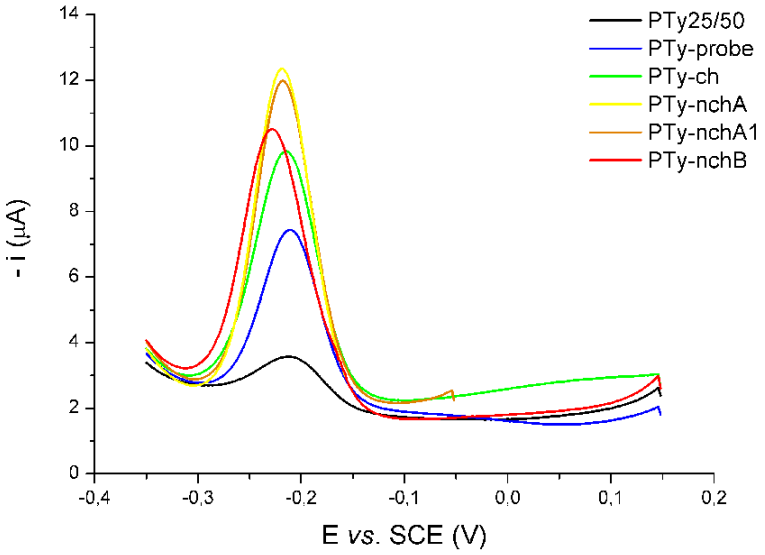

FIGURE 5.41 Square wave voltammetry of PTy (black); PTy + probe (blue); PTy + probe with complementary sequence (ch - green) and PTy + probe with non-complementary sequence (nch - yellow, orange and red) in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7. SWV parameters: 0.002 V step, 0.025 V pulse height, and 5 Hz frequency. The initial potential was 0.15 V.

Since in the literature there are authors that performed an anodic scan [83] to characterise modified electrodes in MB solutions, this approach has also been tested here, as shown in Figure 5.42. Unexpectedly, the results are not identical to the ones obtained when the cathodic scan was performed for the non-complementary hybrids. A possible explanation to this fact is that, unlike methylene blue (the reduced form), leucomethylene blue (the oxidised form of the redox coupe) adsorbs perpendicularly [84] to the surface what may difficult this species reduction.

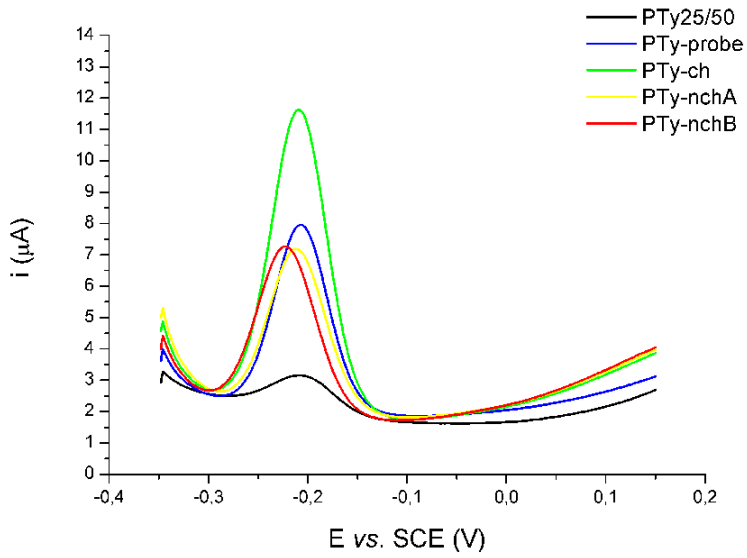

FIGURE 5.42 Square wave voltammetry of PTy (black); PTy + probe (blue); PTy + probe with complementary sequence (ch - green) and PTy + probe with non-complementary sequence (nch - yellow and red) in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7. SWV parameters: 0.002 V step, 0.025 V pulse height, and 5 Hz frequency. The initial potential was -0.35 V.

5.3.1.6 Fluorescence indication of hybridization

An independent analysis of the hybridization process with a complementary sequence, as well as the detection possible interactions between the immobilized probe and the non-complementary sequence mentioned above has been attempted by fluorescence. Presenting different affinity for ss-DNA and ds-DNA, PicoGreen® reagent can be used to distinguish between the immobilized probe and its hybridization with a complementary sequence (maximum quantum yield) and a non-complementary sequence, that in the case of establishment of some bounds with the immobilized probe, will present an intermediary fluorescence.

Immediately before use, a 200-fold dilution of PicoGreen® reagent was prepared in TE buffer pH 7.5 (0.01mol dm^{-3} Tris-HCl + 0.001 mol dm^{-3} EDTA) in a plastic container, since the reagent may adsorb to glass surfaces. The modified electrodes were stained (by immersion) for 10 min with DNA dye ligand PicoGreen® protected from light by covering it with aluminium foil. Thereafter, the electrodes were observed using an inverted microscope and the fluorescence measurements correspond to fluorescence intensity at an excitation wavelength of 485 nm with 530 nm emission. Fluorescence intensity were determined using Image-Pro Plus 5.0 software.

The fluorescence (λ = 530 nm) of modified electrodes was measured, Figure 5.43, after staining with PicoGreen® solution. Thus after the hybridization with the non-complemen-

tary or with the complementary sequences, the fluorescence signals increased by 13%, and 32% respectively, in comparison to the signal of the immobilized probe, that was used as the control. The results confirm the hybridization of the immobilized probe with a complementary sequence but also support that the non-complementary sequence and the probe establish some interactions.

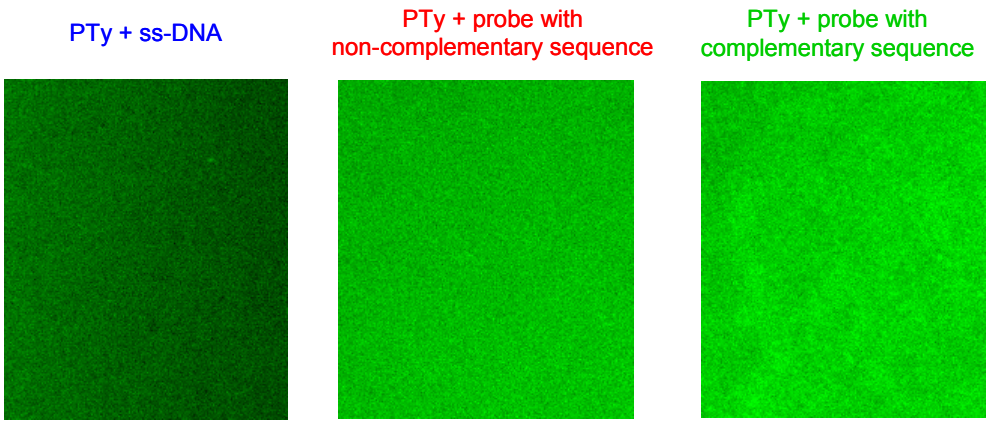

FIGURE 5.43 Fluorescence images and quantification at 530 nm of modified electrodes, after staining with PicoGreen®.

5.3.2 Sensors based on gold modified by self-assembled layers of 4-aminothiophenol

5.3.2.1 Characterization of 4-ATP SAMs

In this study, two 4-ATP monolayers, one of 1 h adsorption time and the other of 20 h have been characterized by electrochemical reductive desorption in 0.1 mol dm^{-3} NaOH solution at sweep rate, ν = 0.020 V s^{-1}, from 0 V to -1.200 V *vs*. SCE. The cyclic voltammograms corresponding to the first cycle of the reductive desorption of 4-ATP SAMs are depicted in Figure 5.44. The electrochemical reductive desorption is one of the most used methodologies to characterise modified surfaces with SAMs [32, 36, 85]. Usually, the SAM reductive desorption from gold (111), assigned to the break of the sulphur-gold (111) bond, a one electron process, as referred previously (equation 6.1), gives rise to one sharp cathodic peak. In the 4-ATP SAM two main cathodic peaks were obtained, as shown in Figure 6.5. The peak A, occurring at -0.580 V and the peak B at -0.800 V for the 1 h 4-ATP monolayer and -0.810 V for 20 h. It can be observed that both peaks increase with time of adsorption. The amount of adsorbed thiols was quantified through the sum of the charges involved in the two reductive desorption peaks using equation 6.2. From the data represented in Figure 5.44, a 2.8 x 10^{-10} mol cm^{-2} surface concentration was obtained for the 1 h SAM and 4.4 x 10^{-10} mol cm^{-2} for the 20 h SAM. The surface coverage value of the 20 h SAM is about half of the theoretical geometri-

cally retrieved for a close-packed monolayer of alkanethiols, estimated as 7.8 x 10^{-10} for Au (111) [32]. This difference can be explained by the larger dimension of the benzene ring compared to the linear chain of an alkanethiol, which should lead to a lower surface coverage for an aromatic monolayer.

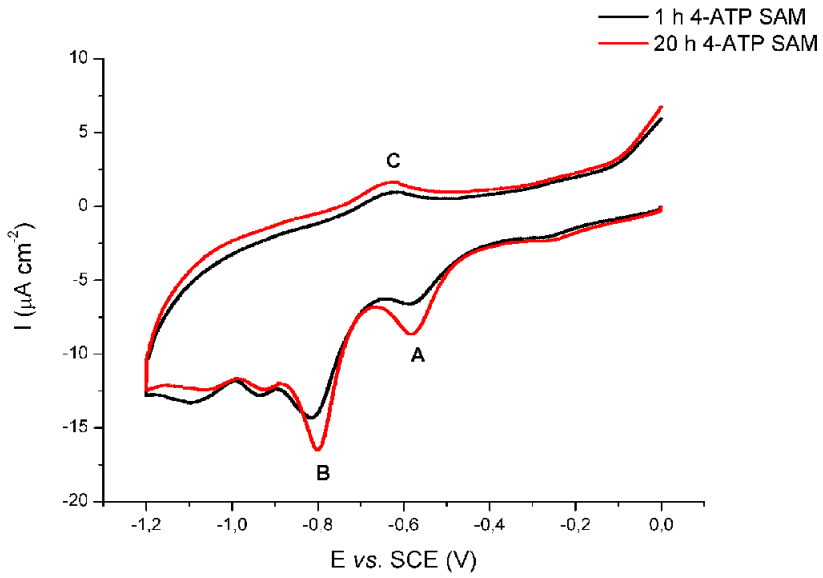

FIGURE 5.44 Cyclic voltammograms of the reductive desorption of 4-ATP SAMs in 0.1 mol dm^{-3} NaOH at 0.020 V s^{-1}.

The existence of two desorption waves for 4-ATP SAMs has been previously observed by Batz *et al.* [39] in a different supporting electrolyte, where the peaks presented a 0.40 V separation, a much higher value than the one presented here (0.28 V). They have speculated that the more positive wave is associated with a phase transitions and the more negative corresponds to desorption. However, in this case, the sum of the charges associated with both peaks are indicative that both should be associated with the monolayer desorption. Therefore, the presence of two cathodic peaks could be explained by the co-existence of two different domains that desorb at distinct potentials. This suggestion is supported by the molecularly resolved STM images of 4-ATP SAMs of Arima *et al.* [42], showing some resolved rows of 4-ATP molecules within what resembled disordered surroundings. The confirmation of the processes associated with 4-ATP desorption, and therefore the amount of the amine groups available for DNA immobilization on the surface, need to be clarified by complementary electrochemical experiments.

It can also be noted that in both voltammograms, at more negative potentials, after the main desorption peak, one much smaller peak also occurs (-0.930 V). This third peak is prob-

ably related with the monolayer desorption from more energetic sites with other gold crystallinities such as (110), also present in a smaller content after the flame annealing process as verified in other SAMs reductive desorption studies [37, 86].

On the reverse sweep, an anodic peak (C) is observed at -0.625 V for the 1 h SAM and at -0.635 V for the 20 h SAM, which is related with the re-adsoption of the desorbed thiolates [86].

5.3.2.2 Detection of immobilization and hybridization

The modified electrodes characterisation was performed in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} phosphate buffer, pH 7 solutions at sweep rate, υ = 0.05 V s^{-1} as previously described.

The cyclic voltammogram of the bare Au slide in PB solution with and without MB is presented in Figure 5.45. In the MB containing electrolyte, a pair of redox peaks with the anodic peak potential at -0.185 V and the cathodic peak potential at -0.230 V and the peak-to-peak separation of 0.045 V are observed. The cathodic peak presents a less defined shape, probably due to some adsorption of the methylene blue [77].

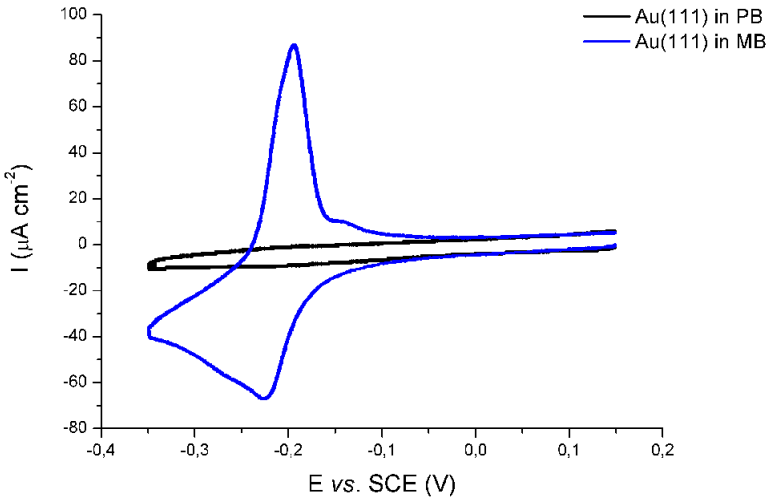

FIGURE 5.45 Cyclic voltammogram of bare Au electrode in 0.1 mol dm^{-3} PB, pH 7 solution with 250 μmol dm^{-3} MB (blue) and without MB (black); υ = 0.05 V s^{-1}.

The electrochemical characterisation of the bare gold electrode and of the 4-ATP SAMs modified electrodes in methylene blue containing electrolytes are shown in Figure 5.46 and the corresponding voltammetric parameters are summarised in Table 5.7.

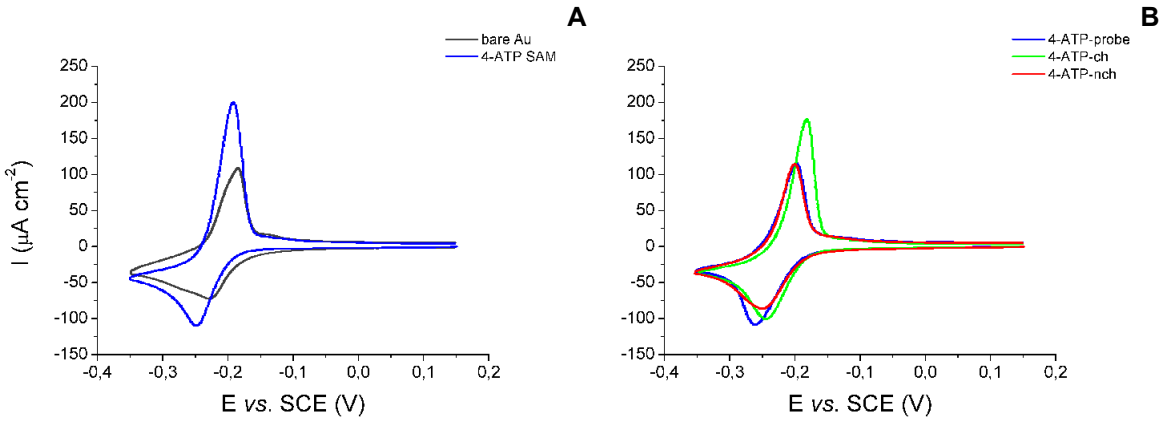

FIGURE 5.46 Cyclic voltammogram of the 4-ATP SAMs modified electrodes characterisation in 250 μmol dm^{-3} MB in 0.1 mol dm^{-3} PB, pH 7; υ = 0.05 V s^{-1}.

TABLE 5.7 Values of E_{pa}, E_{pc}, ΔE_p, I_{pc} and I_{pa} obtained from the voltammetric characterisation (Figure 5.46) of the modified electrodes.

Modified electrode	E_{pa} (V)	E_{pc} (V)	ΔE_p (V)	I_{pc} (μA cm^{-2})	I_{pa} (μA cm^{-2})
bare gold	-0.185	-0.230	0.045	72.3	109.1
4-ATP SAM	-0.192	-0.250	0.058	110.5	199.9
4-ATP SAM + probe	-0.197	-0.260	0.063	107.9	115.8
4-ATP SAM + probe hybridized with-non-complementary sequence A	-0.200	-0.250	0.050	86.3	114.5
4-ATP SAM + probe hybridized with complementary sequence	-0.182	-0.245	0.063	100.0	176.3

It is worth noting the great increase in the current peaks of MB on the 4-ATP SAM regarding to the redox behaviour observed for a bare gold electrode (Figure 5.46A). This phenomenon can be explained by the catalytic reduction of the methylene blue by the "aromatic SAM" NH_2 terminal group, probably through the formation of a complex, involving both species, similar to the one presented in Figure 5.47 [87, 88].

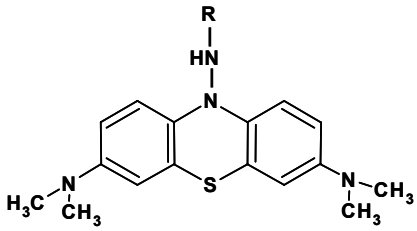

FIGURE 5.47 Type of complex established between methylene blue and amine groups (adapted from [88]).

After the probe immobilization the terminal amines are no longer available for the interaction with MB and therefore a decrease of the peaks current relative to the 4-ATP SAM alone is observed (Figure 5.46B), being however higher then the bare gold, probably due to the negative charge accumulation, as a result of the DNA presence that interacts electrostatically with MB^+ in solution. The modified electrode obtained after the probe hybridization with a complementary sequence (*SAM-ch*) displays an increase in the peaks currents relative to the probe alone, what is in agreement with the further increase of more negative charges at the electrode surface. On the modified electrode with the non-complementary hybrid (*SAM-nch*) the MB presents peak currents closer to the ones obtained for the electrode with the immobilized probe, which suggests that in this case, the non-complementary sequence does not remain, in significant amount, at the electrode surface after washing.

The MB redox process of all the modified electrodes presents a peak separation slightly higher than the expected for a reversible process (ΔE_p ~ 0.030 V), similar to what was observed for the PTy modified electrodes, which is in agreement with the fact the MB conversion is a quasi-reversible system. Also, from Table 5.7, it can be denoted a general shift of the redox peaks towards slightly more negative potentials relative to the bare gold, confirming the surface modification with SAM and DNA.

5.4 Conclusions

The data collected in this study demonstrates that PTy is a suitable matrix for the covalent immobilization of ss-DNA probes with a 5'-phosphate modification.

Methylene blue is an appropriate indicator to discriminate the probe immobilization and the hybridization through the characterization of the modified electrodes by cyclic voltammetry or square wave voltammetry. Immobilization of a cylindrospermopsin probe in PTy modified electrodes and subsequent hybridization have been well succeeded as revealed by the cyclic voltammetry and square wave voltammetry techniques, where a current density increase was obtained upon the immobilization and more after the hybridization process with both methods, in methylene blue containing electrolytes. The use of square wave voltammetry allows a better discrimination of the involved process and thus it is a more appropriate technique for the characterisation of the systems considered in the present work.

An independent method, based on fluorescence of the modified electrodes after staining with a PicoGreen® solution, also confirmed the hybridization of the covalently immobilized probe.

This work demonstrates that 4-ATP forms monolayers on gold and that available terminal NH_2 groups are suitable for the covalent immobilization of ss-DNA probe with a 5´-phosphate modification.

4-aminothiophenol monolayers have a less ordered structure than the ones obtained for alkanethiols as indicated by the presence of two desorption peaks. The aromatic amines in the monolayer promote methylene blue conversion regarding the bare gold electrode.

The cyclic voltammetric characterisation of methylene blue containing electrolytes allowed to confirm the well succeed cylindrospermopsin probe immobilization and further hybridization. The data collected with this metal surface modification shows that the use of 4-ATP aromatic SAMs enhances the signals obtained by CV, when compared to the ones registered for the PTy films. These observations can be due to the fact that this type of substrate modification increased the number of terminal amine groups that remain available for the probe immobilization than the ones obtained with polytyramine films, and also the presence of the benzene ring adjacent to the amine functionality of this monolayers may promote the signal amplification due to the delocalized π electrons in the ring.

The results obtained with the SAMs pave way for the improvement and possible application of this biosensor in a sample containing total DNA extracted from a cylindrospermopsin-producing cyanobacterial strain.

5.5 References

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6

Concluding remarks and perspectives

The global distribution of toxic cyanobacterial blooms has brought out attention to the serious implications of this worldwide problem. Nowadays, there has been a great effort in genome sequencing and analysis of several cyanobacteria, 12 cyanobacterial genomes being so far completely sequenced. The analysis of these genomes, and of other ongoing sequencing projects, may provide new insights regarding biological, toxicological and evolutionary mechanisms. These sequences can be a very useful tool for the search of new phylogenetic or toxicological molecular markers.

The phylogenetic study here presented shows that the phylogenies provided by the 16S rRNA or *rpo*C1 genes do not totally reflect the observed interspecific and even intergeneric morphological differences, namely between *Anabaena* and *Aphanizomenon*. It could be useful to complement the information here contained by sequencing genes encoding the major light-harvesting accessory pigment proteins, particularly the phycocyanin (PC) operon (*cpc*), including the intergenic spacer (IGS) between *cpc*B and *cpc*A and the corresponding flanking regions (*cpc*BA-IGS), and the *rbc*LX (RubisCO) loci, in a multigenic approach for the assessment of a phylogenetic tree that better reflect the phenotypic characteristics of this prokaryotic phylum. The analysis should also evaluate the congruence of the phylogenies obtained from the single genes and from the concatenated sequences.

The fingerprinting methods applied in this work reveal a great inter and intraspecific diversity, which reflects the difficulties of performing cyanobacteria identification. Nevertheless, the establishment of a fingerprinting method based on polymorphisms of STRR and LTRR cyanobacterial sequences for identification purposes, within a previously determined order, provides a faster and easier method, without the need of an expert experience to perform cyanobacterial morphological identification. Since the isolates belonging to Chroococcales could no be distinguished by these methods, perhaps the application of another molecular method, e.g. amplification of Hip1 (highly iterated palindromic sequences) could be tested in order to evaluate its taxonomic potential within this order. The traceability potential of the composite hierarchical clustering of the M13 and ERIC fingerprints also provides a useful tool for the routine freshwaters monitoring. Moreover, all these methods have been applied and validated in non-axenic cultures, which are known to be difficult to obtain.

The development of a PCR multiplex method, directed to three different loci of the microcystins genes cluster, constitutes a step forward in the implementation of molecular methods to detect microcystins producing strains in routine analytical laboratories dealing with freshwaters quality. Since the PCR methods here tested provide only a qualitative analysis of the samples, their future development by real time PCR will increase their potential towards

quantitative analysis.

The search for PST, namely saxitoxin, molecular markers is currently under course by the group of Doctor Brett Neilan, and so there is a continuous search for alternative molecular methods to detect cyanobacterial toxins and thus provide an expedite answer about the water quality.

The search of a quantitative method to detect toxic cyanobacteria was here tested though the use of a DNA-based sensor. Given the promising results of the validated PCR methods for the detection of cylindrospermopsin producing strains and also the lack of analytical standards of this toxin, the covalent immobilization of a molecular marker for cylindrospermopsin producing strains (PKSM4), with a 5'-phosphate modification, to a metallic modified surface has been performed. Subsequent hybridization assays with complementary and non-complementary sequences were carried out.

As for the development of electrochemical DNA-based sensors, the preliminary studies presented here, namely with the PKS probe immobilized in 4-ATP aromatic SAMs, point out to a possible further improvement and application of this biosensor. However, it is necessary to test the immobilization and hybridization of the two cylindrospermopsin primers (PKS and PS). Moreover, testing this biosensor in spiked samples containing total DNA extracted from a cylindrospermopsin-producing cyanobacterial strain is also needed.

Annex

Domain Bacteria 251

Phylum *Cyanobacteria* 251

- Class *Gloeobacteria* 1
  - Order *Gloeobacterales* 1
    - *Gloeobacter* 1
      - *violaceus* B

- Class *Chroobacteria* 150
  - Order *Chroococcales* 46
    - *Chroococcus* 10
      - *cohaerens* A
      - *dispersus* A
      - *giganteus* A
      - *limneticus* A
      - *minor* A
      - *minutus* A
      - *pallidus* A
      - *schizodermaticus* A
      - *turgidus* A
      - *varius* A
    - *Chamaesiphon* 10
      - *britannicus* A
      - *confervicolus* A
      - *fuscus* A
      - *geitleri* A
      - *incrustans* A
      - *minutus* A
      - *polonicus* A
      - *polymorphus* A
      - *pseudopolymorphus* A
      - *rostafinskii* A
    - *Cyanobacterium* 0 D
    - *Cyanobium* 1 D
      - *gracile* B
    - *Cyanothece* 0 B
    - *Dactylococcopsis* 1
      - *salina* B
    - *Gloeocapsa* 6
      - *alpina* A
      - *crepidinum* A
      - *magma* A
      - *polydermatica* A
      - *punctata* A
      - *sanguinea* A
    - *Gloeothece* 3
      - *confluens* A
      - *linearis* A
      - *rupestris* A
    - *Microcystis* 5 D
      - *aeruginosa* A, B
      - *wesenbergii* A
      - *ichthyoblabe* A
      - *novacekii* A
      - *viridis* A
    - *Prochlorococcus* 1 D
      - *marinus* E
    - *Prochloron* 1 D
      - *didemni* E
    - *Synechococcus* 6
      - *aeruginosus* A
      - *bacillaris* A
      - *cedrorum* B
      - *capitatus* A
      - *elongatus* A
      - *lividus* B
    - *Synechocystis* 2
      - *aquatilis* A
      - *endobiotica* A
  - Order *Pleurocapsales* 16
    - *Cyanocystis* 4
      - *aquae-dulcis* A
      - *hemisphaerica* A
      - *leibleiniae* A
      - *olivacea* A
    - *Dermocarpella* 2
      - *incrassata* B
      - *prasinia* A
    - *Stanieria* 1
      - *sphaerica* A
    - *Xenococcus* 5
      - *chousboei* B
      - *kerneri* A
      - *pyriformis* A
      - *rivularis* A
      - *schoesboei* A
    - *Chroococcidiopsis* 3
      - *chroococcoides* A
      - *fluviatilis* A
      - *thermalis* B
    - *Myxosarcina* 1
      - *concinna* B
    - *Pleurocapsa* 0 A, B
  - Order *Oscillatoriales* 88
    - *Arthrospira* 3
      - *jenneri* B
      - *maxima* B
      - *platensis* B

- Class *Hormogoneae* 100
  - Order *Nostocales* 89
    - *Anabaena* 20
      - *aequalis* A
      - *affinis* A
      - *augstumalis* A
      - *catenula* A
      - *circinalis* A
      - *cylindrica* A, B
      - *delicatula* A
      - *elliptica* A
      - *flos-aquae* A
      - *inaequalis* A
      - *macrospora* A
      - *oscillarioides* A
      - *planctonica* A
      - *solitaria* A
      - *sphaerica* B
      - *spiroides* A
      - *torulosa* A
      - *variabilis* A
      - *verrucosa* A
      - *viguieri* A
    - *Anabaenopsis* 1
      - *elenkinii* A, B
    - *Aphanizomenon* 4
      - *aphanizomenoides* A
      - *flos-aquae* A, B
      - *gracile* A
      - *issatschenkoi* A
    - *Cyanospira* 0
    - *Cylindrospermopsis* 1
      - *raciborskii* B
    - *Cylindrospermum* 8
      - *alatosporum* A
      - *catenatum* A
      - *licheniforme* A
      - *majus* A, B
      - *marchicum* A
      - *minutissimum* A
      - *muscicola* A
      - *stagnale* A, B
    - *Nodularia* 3
      - *harveyana* A, B
      - *spumigena* A, B
      - *sphaerocarpa*
    - *Nostoc* 19
      - *carneum* A
      - *coeruleum* A
      - *commune* A, B
      - *cuticulare* A
      - *ellipsosporum* A
      - *humifusum* A
      - *linckia* A
      - *macrosporum* A
      - *microscopicum* A
      - *muscorum* A, B
      - *paludosum* A
      - *parmelioides* A
      - *piscinale* A
      - *pruniforme* A
      - *punctiforme* A, B
      - *rivulare* A
      - *sphaericum* A
      - *spongiaeforme* A
      - *verrucosum* A
    - *Scytonema* 8
      - *alatum* A
      - *crispum* A
      - *crustaceum* A
      - *hofmanni* A, B
      - *julianum* A
      - *mirabile* A
      - *myochrous* A
      - *ocellatum* A
    - *Calothrix* 11
      - *aeruginea* A
      - *braunii* A
      - *confervicola* A
      - *contarenii* A
      - *crustacea* A
      - *epiphytica* A
      - *fusca* A
      - *parietina* A
      - *pulvinata* A
      - *scopulorum* A
      - *stagnalis* A
    - *Rivularia* 10
      - *atra* A
      - *australis* A
      - *beccariana* A
      - *biasolettiana* A
      - *bullata* A
      - *dura* A
      - *haematites* A
      - *minutula* A
      - *nitida* A
      - *polyotis* A

Borzia	0	**B**
Crinalium	2	**E**
endophyticum		**A**
epipsammum		**A, B**
Geitlerinemia	0	**B**
Halospirulina	1	
Halospirulina tapeticola		**E**
Leptolyngbya	0	**B**
Limnothrix	1	**D**
redekei		**D**
Lyngbya	22	
aerugineo-coerulea		**A**
aestuarii		**A**
confervoides		**A, B**
contorta		**A**
diguetii		**A**
epiphytica		**A**
gracilis		**A**
kuetzingii		**A**
lagerheimii		**A**
limnetica		**A**
lutea		**A**
lutescens		**A**
maior		**A**
majuscula		**A**
martensiana		**A**
ochracea		**A**
rivularianum		**A**
semiplena		**A**
sordida		**A**
spirulinoides		**A**
vandenberghenii		**A**
versicolor		**A**
Microcoleus	6	
acutirostris		**A**
chthonoplastes		**A, B**
lacustris		**A, B**
subtorulosus		**A**
tenerrimus		**A**
vaginatus		**A**
Oscillatoria	29	
acuminata		**B**
agardhii		**B**
amoena		**A**
amphibia		**A**
anguina		**A**
angusta		**A**
brevis		**A**
chalybea		**A**
corallinae		**A**
cortiana		**A**
formosa		**A**
granulata		**A**
hamelii		**A**
irrigua		**A**
laetevirens		**A**
limnetica		**A**
limosa		**A**
margaritifera		**A**
nigra		**A**
nigro-viridis		**A, B**
princeps		**A, B**
prolifera		**A, B**
sancta		**A, B**
splendida		**A**
subbrevis		**A**
subtilissima		**A**
subuliformis		**A**
tenuis		**A, B**
terebriformis		**A**
Planktothrix	5	**D**
agardhii		**C**
mougeotii		**C**
pseudoagardhii		**D**
raciborskii		**C**
rubescens		**C**
Prochlorothrix	1	
hollandica		**D**
Pseudoanabaena	2	
catenata		**A**
tenuis		**B**
Spirulina	7	
labyrinthiformis		**A**
laxissima		**A**
major		**A, B**
meneghiniana		**A**
nordstedtii		**A**
subsalsa		**A**
subtilissima		**A**
Starria	1	
zimbabwe'nsis		**B**
Symploca	5	
atlantica		**A**
dubia		**A**
hydnoides		**A**
muralis		**A**
muscorum		**A**
Trichodesmium	3	
erythraeum		**A**
lacustre		**A**
thiebautii		**A**
Tychonema	0	**D**
Tolypothrix	4	
byssoidea		**A**
distorta		**A**
lanata		**A**
tenuis		**A, B**
Order *Stigonematales*	11	
Chlorogloeopsis	1	
fritschii		**B**
Fischerella	3	
laminosus		**A**
muscicola		**A**
thermalis		**B**
Geitleria	0	**B**
Iyengariella	0	**B**
Nostochopsis	0	**A**
Stigonema	7	
hormoides		**A**
informe		**A**
mamillosum		**A**
minutum		**A, B**
ocellatum		**A**
panniforme		**A**
tomentosum		**A**

Legend:

A: From the "A Coded List of Freshwater Algae of the British Isles 2003" [Whitton BA, John DM, Kelly MG, Haworth EY (2003) A Coded List of Freshwater Algae of the British Isles. Second Edition. World-wide Web electronic publication]; **B:** "Bergey´s 2001" [Castenholz, R. W. (**2001**) Phylum BX. Cyanobacteria. In *Bergey´s Manual of Systematic Bacteriology.* D. R. Boone, Castenholz, R.W. (Ed). Springer, New York, 2001, pp. 473-599.]; **C:** Validly published according to the International Code of Botanical Nomenclature (ICBN); **D:** Not validly published according to the International Code of Nomenclature of Bacteria (ICNB); **E:** Species presents in the lists of validly published names of prokaryotes [J.P. Euzéby: List of Prokaryotic names with Standing in Nomenclature].

NOTE: To simplify the diagram only the specific epithet was indicated for each species.

Glossary

Akinete - A thick-walled “resting” cell or spore that can survive harsh conditions and functions as an asexual resting stage, that differentiates from a vegetative cell.

Autotroph - Any organism that obtains carbon directly from carbon dioxide ($C0_2$).

Axenic - Literally "without strangers”. A system in which all biological populations are defined, such as a pure culture.

Baeocyte - small cell formed internally by multiple fissions in parent cells.

Benthic - Occurring in the depths of an aquatic environment.

Bloom - Visible or nuisance algal or cyanobacterial growth often associated with nutrient-rich conditions.

Calyptra - Thickened or enlarged tip of a cyanobacterial filament.

Concatamer - A DNA molecule that contains two or more identical linear sequences covalently linked in tandem.

Cross walls - Transverse cell walls.

Diazotrophy - ability to fix nitrogen.

Enzyme-linked immunosorbent assay (ELISA) - Immunoassay that uses specific antibodies to detect antigens or antibodies. The antibody-containing complexes are visualized through an enzyme coupled to the antibody. Addition of substrate to the enzyme-antibody-antigen complex results in a coloured product.

Filament - A chain of cells in which cell separation does not include an intercalating sheath, although a linear sheath may be present.

Gas vacuoles - Sometimes synonymous with gas vesicles, also refers to a group of gas vesicles.

Gas vesicles - Cylindrical structures used to increase cell buoyancy.

Gram stain - Differential stain that divides bacteria into two groups, Gram-positive and Gram-negative, based on the ability to retain crystal violet when decolorized with an organic solvent such as ethanol. The cell wall of Gram-positive bacteria consists chiefly of peptidoglycan and lacks the outer membrane of Gram-negative cells.

Heterocyst - A specialized thick-walled cell involved in N_2 fixation; differentiates from vegetative cell.

Heteroduplex DNA - A segment on a double stranded DNA molecule in which the two DNA strands are of different origin and thus do not have perfectly complementary nucleotide sequences.

Hybridization - Natural formation or artificial construction of a duplex nucleic acid molecule by complementary base pairing between two nucleic acid strands derived from different sources.

Intercalation - Certain chemicals that are large flat molecules are capable of slipping between the base pairs of the DNA helix. This mode of interacting with DNA is known as intercalation.

Mismatched base pair - A pair of nucleotides that do not hydrogen bond correctly and thus are not complementary.

Monophyletic - It refers to any group of organisms that includes the most recent common ancestor of all those organisms and all the descendants of that common ancestor.

Mucilage - Gelatinous secretions and exudates.

Nucleic acids - Macromolecules composed of a sugar-phosphate backbone and four nucleotide bases. The two types of nucleic acids in living systems are DNA and RNA. DNA contains the bases adenine, cytosine, guanine and thymine. RNA contains the bases adenine, cytosine, guanine and uracil.

Oligonucleotide - Short nucleic acid chain, either obtained from an organism or synthesized chemically.

Peptidoglycan - The macromolecule that is used to build the rigid structure that surrounds the cell walls of bacteria. Peptidoglycan is composed of N-acetylglucosamine, N-acetylmuramic acid, and a few amino acids. Also called murein.

Photosynthetic - Organisms that use sunlight as an energy source to synthesize carbohydrates from carbon dioxide and water.

Photosynthesis - Process of using light energy to synthesize carbohydrates from carbon dioxide.

Phylogenetic - Ordering of species into higher *taxa* and the construction of evolutionary trees based on evolutionary (genetic) relationships.

Phytoplankton - Floating or swimming microscopic algae and cyanobacteria.

Planktonic - Occurring in open waters, near the surface of an aquatic environment.

Polyphyletic - A group of organisms descended from more than one ancestor, may be more closely related to other organisms outside of the taxonomic group.

Polymerase chain reaction - A technique for amplifying any specific segment of DNA in vitro. A piece of DNA is used as the template. Specific DNA primers, one for each strand, are used to initiate DNA synthesis and DNA polymerase from a bacterium that grows at 72ºC are used to synthesize the DNA. The DNA is synthesized by cycling the reaction through three temperatures. 94-95ºC is used to denature double-stranded DNA, a temperature between 40ºC and 55ºC is used to anneal the primers to the template and 72ºC is used for DNA synthesis. This cycle can be repeated 20 to 35 times so that a significant quantity of DNA can be synthesized.

Primer - A small stretch of linked nucleotides. In the case of DNA synthesis, this small stretch consists of ribonucleotides that DNA polymerase can attach deoxyribonucleotides to.

Probe - A molecule that is tagged in some manner so that it can be easily detected and is used to identify a target of interest. For example, a gene of interest can be immobilized and used to detect the complementary gene of interest.

Prokaryote - Organism lacking a unit membrane-bound nucleus and other organelles, usually having its DNA in a single circular molecule.

Restriction - The process by which DNA is cut at a specific sequence by an enzyme known as a restriction endonuclease.

Restriction endonucleases or restriction enzymes - Proteins produced by many different species of bacteria that recognize foreign DNA sequences and cleave the foreign DNA on both strands at a DNA sequence specific for each restriction enzyme. Bacterial restriction systems are a primitive immune system in that they protect bacteria from foreign DNA coming into the cytoplasm. There are three types of restriction enzymes (Type I, II, and III). The three categories are based on the number of proteins that make up the enzyme, the cofactor requirements, if the proteins form a complex and the nature of the sequence that is recognized by the enzyme. Commercially useful restriction endonucleases (Type II) recognize and cleave the short specific sequence of DNA.

Restriction fragment length polymorphism (RFLP) - Method to identify differences between similar genes from different organisms. Digestion of genes with restriction endonucleases followed by separation of the resulting fragments by gel electrophoresis yields banding patterns that are characteristic of the individual gene.

Sheath - Tubular structure formed around a chain of cells or around a bundle of filaments.

Species - In microbiology, a collection of closely related strains sufficiently different from all other strains to be recognized as a distinct unit.

Strain - Population of cells all descended from a single pure isolate.

Symbiosis - Living together in intimate association of two dissimilar organisms. The interactions between the organisms can be commensal or mutualistic.

Taxon (plural, taxa) - A group into which related organisms are classified.

Taxonomy - Study of scientific classification and nomenclature.

Trichome - A filament of a cyanobacteria. Row of cells which have remained attached to one another following successive cell divisions.

Vegetative cells - Cells that grow and divide by binary or multiple fission, involve also in nutrition, or asexual reproduction, but not sexual reproduction.

16S rRNA - Large polynucleotide (about 1,500 bases) that functions as a part of the small subunit of the ribosome of prokaryotes and from whose sequence evolutionary information can be obtained; the eukaryotic counterpart is 18S rRNA.