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Culture-dependent characterization of cyanobacterial diversity in the intertidal zones of the Portuguese coast: A polyphasic study

Ângela Brito[a,b], Vitor Ramos[a,b,c], Rui Seabra[b,c], Arlete Santos[a,b], Catarina L. Santos[a], Miguel Lopo[a], Sérgio Ferreira[a], António Martins[c], Rita Mota[a,b], Bárbara Frazão[b,c], Rosário Martins[c,d], Vitor Vasconcelos[b,c], Paula Tamagnini[a,b,*]

[a] *IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal*
[b] *Faculdade de Ciências, Departamento de Biologia, Universidade do Porto, Rua do Campo Alegre, Edifício FC4, 4169-007 Porto, Portugal*
[c] *Interdisciplinary Centre of Marine and Environmental Research (CIIMAR/CIMAR), University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal*
[d] *CISA – Centro de Investigação em Saúde e Ambiente, Escola Superior de Tecnologia da Saúde, Instituto Politécnico do Porto, Rua Valente Perfeito 322, 4400-330 Vila Nova de Gaia, Portugal*

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Cyanobacteria are important primary producers, and many are able to fix atmospheric nitrogen playing a key role in the marine environment. However, not much is known about the diversity of cyanobacteria in Portuguese marine waters. This paper describes the diversity of 60 strains isolated from benthic habitats in 9 sites (intertidal zones) on the Portuguese South and West coasts. The strains were characterized by a morphological study (light and electron microscopy) and by a molecular characterization (partial 16S rRNA, *nifH*, *nifK*, *mcyA*, *mcyE*/*ndaF*, *sxtI* genes). The morphological analyses revealed 35 morphotypes (15 genera and 16 species) belonging to 4 cyanobacterial Orders/Subsections. The dominant groups among the isolates were the Oscillatoriales. There is a broad congruence between morphological and molecular assignments. The 16S rRNA gene sequences of 9 strains have less than 97% similarity compared to the sequences in the databases, revealing novel cyanobacterial diversity. Phylogenetic analysis, based on partial 16S rRNA gene sequences showed at least 12 clusters. One-third of the isolates are potential N_2-fixers, as they exhibit heterocysts or the presence of *nif* genes was demonstrated by PCR. Additionally, no conventional freshwater toxins genes were detected by PCR screening.

Introduction

Continental Portugal has an extensive coastline, of about 940 km, facing the North Atlantic Ocean. It is one of the warmest European countries, and its climate is classified as Mediterranean type Csa in the south (C – warm temperate; s – summer dry; a – hot summer) and Csb in the north (C – warm temperate; s – summer dry; b – warm summer), according to the Köppen's scheme [23]. The near-shore wave energy has a strong spatial and seasonal variability [30]. Western and southern coasts are evidently under different wave regimes, with the unsheltered west coast sites experiencing higher wave height and power than southern ones, and a moderate decreasing gradient from north to south can be observed. Wave height and power in the winter are also much higher than in the summer [30], and along the coast it is possible to encounter different topographies and beach morphologies – rocky beaches, beaches with sand and rocks and sandy beaches with dispersed rocks, resulting in diverse levels of exposure to the prevailing wave regimen.

Cyanobacteria are photosynthetic prokaryotes with a wide geographical distribution that are present in a broad spectrum of environmental conditions [49]. Taxonomy of cyanobacteria is a controversial subject, with two prevailing approaches, the "botanical" and "bacterial". The reorganized taxonomic revision based on the botanical code uses morphological, biochemical and molecular characters [19–21]. After the recognition of the prokaryotic features of cyanobacteria, Rippka et al. [36] published a bacteriological taxonomy based on morphological, biochemical and genetic characters of axenic cultures, while Bergey's Manual of Systematic Bacteriology [5] uses a phylogenetic approach primarily based on 16S rRNA sequence comparisons. In summary, cyanobacteria can be classified into five Subsections [5,36] that broadly coincide with Orders of the botanical approach [19–21]. Cyanobacteria belonging to subsections I (Chroococcales) and II (Pleurocapsales) are unicellular, but while the organisms in subsection I divide exclusively by binary fission, the ones from subsection II can also undergo

* Corresponding author at: IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, Rua do Campo Alegre 823, 4150-180 Porto, Portugal. Tel.: +351 226074900; fax: +351 226099157.
E-mail address: pmtamagn@ibmc.up.pt (P. Tamagnini).

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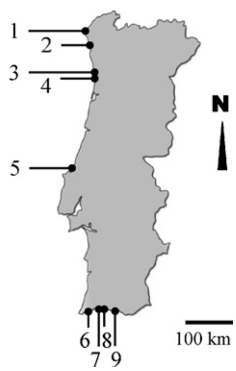
Table 1
Localization and characteristics of the sampling sites.



Sampling site latitude/longitude	Wave exposure	Beach type
1. Moledo do Minho N 41°50′58.68″/W 8°52′0.18″	High	Rocky (Rivermouth)
2. S. Bartolomeu do Mar N 41°34′25.59″/W 8°47′54.81″	High	Sand and rocks
3. Lavadores N 41°07′45.07″/W 8°40′6.88″	High	Sand and rocks
4. Aguda N 41°02′58.35″/W 8° 39′19.22″	High	Sand and rocks
5. Foz do Arelho N 39°25′59.79″/W 9°13′48.99″	High	Sandy (Rivermouth)
6. Martinhal N 37°01′07.30″/W 8°55′36.17″	Intermediate	Sandy
7. Burgau N 37°04′15.84″/W 8°46′34.97″	Intermediate-low	Sand and rocks
8. Luz N 37°05′10.38″/W 8°43′31.35″	Intermediate-low	Rocky
9. Olhos d'Água N 37°05′23.01″/W 8°11′27.66″	Low	Sandy

multiple fission producing small easily dispersible cells called baeocytes. The subsection III (Oscillatoriales) includes the filamentous strains without cell differentiation. Subsections IV (Nostocales) and V (Stigonematales) comprise the filamentous strains that are able to differentiate heterocysts and akinetes. In addition, filaments of cyanobacteria from subsection V are able to divide in multiple planes displaying true branching.

Benthic cyanobacteria grow along the shore on different substrata, mainly in the intertidal zones, as part of complex multi-taxa communities, often forming cohesive mats and biofilms. In these habitats, they are exposed to a range of daily stresses such as nutrient limitation, high UV-radiation and desiccation [1,6]. The successful adaptation to these harsh environments is largely due to their morphological and functional versatility [31]. Cyanobacteria play a major role in the global carbon cycle as important primary producers performing oxygenic photosynthesis, and the diazotrophic taxa are fundamental to the nitrogen cycle, particularly in oceans [18]. In addition to their ecological importance, cyanobacteria are also recognized as being a prolific source of biologically active natural products; some of these compounds are toxic to a wide array of organisms [50]. Nevertheless, little is known about the diversity of these organisms along the Portuguese coast with only a few reports published [e.g. 2,9]. Araújo et al. [2] provided an updated checklist of the benthic marine algae of the northern Portuguese coast, including 26 species of cyanobacteria. However, these authors based their work on new records, literature references and herbarium data but did not isolate or maintain cultures of the observed specimens.

The aim of this work was to identify the cyanobacteria present in the intertidal zones of the Portuguese coast using a polyphasic approach. The isolated specimens are kept at LEGE Culture Collection, and available for further studies. In addition to the assessment of cyanobacterial diversity, a PCR-based screen for putative diazotrophs and toxin-producers was performed to unveil their role(s) in the ecosystem.

Materials and methods

Sampling sites

The sites (Table 1) were selected in order to represent the diversity of the Portuguese coast. Along the coast it is possible to discriminate between rocky beaches (sampling sites 1 and 8), beaches with sand and rocks (sampling sites 2, 3, 4, 7) and sandy beaches with dispersed rocks (sampling sites 5, 6 and 9) (Table 1). Several relevant variables: wave power [30], sea surface temperature [SST [25]], river runoff and other important climatic variables such as air temperature and precipitation (Instituto de Meteorologia, IP, Portugal) were also taken into account. In brief, West coast sampling sites experience generally higher energetic wave regimes, lower overall mean SSTs and mean air temperatures [40], and higher fresh water inputs, both from river runoff and precipitation, than their South coast counterparts.

Cyanobacteria sampling, isolation, and culturing

Biological samples were collected in both summer and autumn of 2006, and spring of 2007. Sample collection was performed by scraping the surface of a wide range of substrates (e.g. seaweeds, rock surfaces, seashells, *Sabellaria alveolata* reefs) present on bare rocks or shallow puddles tidal pools. For the isolate LEGE 06009 see also [9]. In addition, seawater samples from the surf zone were also collected.

Raw biological samples were screened for cyanobacterial specimens using a light microscope (Leica DMLB), and subsequently subjected to liquid culture enrichment, agar plates streaking or micromanipulation. Whenever possible single cells/filaments were isolated and transferred to liquid or solid medium using a Pasteur pipette [34]. When micromanipulation was found unsuitable, aliquots of the enriched liquid cultures were transferred to liquid medium or streaked on agar plates. Sea water samples were filtered with sterile glass fiber filters (GF/C – Ø 47 mm, Whatman), and subsequently placed on liquid medium until a visible colony appeared. Single colonies were picked and streaked on agar plates. New colonies were transferred into liquid medium. Cultures were maintained using the following media: MN [34], $BG11_0$, BG11 [44], and Z8 [22] supplemented with 25 g L^{-1} NaCl, further named Z8 25‰. The media were supplemented with B12 vitamin, and when necessary with cycloheximide or amphothericin B [34]. The cultures were kept under 14 h light (10–30 μmol photons m^{-2} s^{-1})/10 h dark cycles at 25 °C. Cyanobacterial isolates are deposited at LEGE Culture Collection (Laboratório de Ecotoxicologia, Genómica e Evolução; CIIMAR, Porto, Portugal). Additionally, the isolate LEGE 06123 is also deposited at Culture Collection of Algae and Protozoa (CCAP 1425/1), for details on this organism see [33].

Light and transmission electron microscopy (TEM) and morphological identification

Cells were observed using a Leica DMLB microscope (Leica Microsystems GmbH), images were captured with a Leica ICCA Analogue Camera System, and the cells were measured using Qwin