PICOCYANOBACTERIA FROM A CLADE OF MARINE *Cyanobium* REVEALED BIOACTIVE POTENTIAL AGAINST MICROALGAE, BACTERIA, AND MARINE INVERTEBRATES

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The production of bioactive compounds either toxic or with pharmacological applications by cyanobacteria is well established. However, picoplanktonic forms within this group of organisms have rarely been studied in this context. In this study, the toxicological potential of picocyanobacteria from a clade of marine *Cyanobium* strains isolated from the Portuguese coast was examined using different biological models. First, strains were identified by applying morphological and molecular approaches and cultured under lab conditions. A crude extract and three fractions reflecting a preliminary segregation of lipophilic metabolites were tested for toxicity with the marine microalga *Nannochloropsis* sp., the bacteria *Pseudomonas* sp., the brine shrimp *Artemia salina,* and fertilized eggs of the sea urchin *Paracentrotus lividus*.
No significant apparent adverse effects were noted against *Artemia salina.* However, significant adverse effects were found in all other assays, with an inhibition of *Nannochloropsis* sp. and *Pseudomonas* sp. growth and marked reduction in *Paracentrotus lividus* larvae length. The results obtained indicated that *Cyanobium* genus may serve as a potential source of interesting bioactive compounds and emphasize the importance of also studying smaller picoplanktonic fractions of marine cyanobacteria.

The photosynthetic prokaryotes cyanobacteria are important components and primary producers of marine, estuarine, and freshwater ecosystems. Among several characteristics that make this group of organisms attractive for scientific studies, the capability to produce unique secondary metabolites is one that is most explored (Nunnery et al., 2010; Tan,
2010). Among those metabolites, toxins were extensively explored due to the deleterious effects they induce on ecosystem equilibrium and human health (Codd et al., 2005; Zurawell et al., 2005; Martins and Vasconcelos, 2009).
Nevertheless, and owing to their ecological and biochemical diversity, cyanobacteria were found to be a prolific source of compounds with potential biotechnological applications, namely in the field of pharmacological agents. In fact, a wide range of secondary metabolites exhibiting pharmacodynamic properties such as antibacterial, antiviral, antifungal, and anticancer has been reported (Berry et al., 2008; Costa et al., 2014).
With respect to marine cyanobacteria, filamentous non-heterocystous genera such as *Lyngbya*, *Moorea*, *Microcoleus*, *Schizothrix*, *Symploca*, and *Trichodesmium* were considered for drug development and identified as promising taxa from which new biologically active compounds can be isolated (Tan, 2007; Engene et al., 2012). However, other picocyanobacteria genera such as *Cyanobium*, *Prochlorococcus*, *Synechococcus*, and *Synechocystis* have been overlooked, probably because these forms do not usually reach sufficiently high densities in environmental conditions and thus successful biomass production is limited to isolation and culturing.
These morphologically simple taxa are well known to occur in open ocean and coastal waters globally (Scanlan et al., 2009; Huang et al., 2012), some of which are major contributors to primary production (Waterbury et al., 1986; Chisholm et al., 1988; Liu et al., 1997).
However, taxonomic classification, and thus identification, is difficult to correctly establish as (1) there are few taxonomic characters present in these small coccoid cyanobacteria (Komarek et al., 1999), (2) there are morphological resemblances that exist between these and other taxa, and (3) there is a lack of experimental support derived from molecular-based approaches to identify picocyanobacteria.

In fact, a number of lineages are recognized to exist among picocyanobacteria genera, which indicates that their taxonomy needs to be revised (Honda et al., 1999; Huang et al., 2012). The biological activity displayed by marine and estuarine picocyanobacteria strains has been previously studied in our lab (Martins et al., 2007, 2008; Frazao et al., 2010; Lopes et al., 2011; Costa et al., 2014) and by other investigators (Hamilton et al., 2014). Since bioactivity effects were predominantly reported for strains of the *Synechocystis* and *Synechococcus* genera, the aim of this study was to assess the potential of novel marine *Cyanobium* strains as producers of bioactive compounds. To achieve this, five isolates were identified by a morphological and genomic approach, complemented with a phylogenetic analysis, and extensively cultured.

The bioactivity of the isolates, which are herein recognized to belong to the same phylotypes, was assessed by a battery of bioassays. Considering the ability of cyanobacteria to produce compounds toxic to coexisting organisms, assays with microalga *Nannochloropsis* sp., brine shrimp *Artemia salina*, and embryos of the sea urchin *Paracentrotus lividus* were performed. Bioassays with the bacteria *Pseudomonas putida* were also conducted in order to determine antibacterial potential.

MATERIALS AND METHODS

Cyanobacteria Isolation, Characterization, and Culture

Five marine cyanobacteria strains belonging to the genus *Cyanobium* were included in this study (Table 1). Strains were obtained from water samples and substrate collected on beaches from the Portuguese coast. For isolation purposes, raw biological materials were inoculated in liquid Z8 medium (Kotai, 1972) supplemented with 25 g/L NaCl and 10 µg/ml vitamin B_{12}, for enrichment. When visible growth was detected in the media, aliquots were transferred and streaked onto solid Z8 medium plates containing 1.2% agar, and supplemented as already described. Single colonies were then selected and two subsamples were made: One was inspected under a microscope, while the other was aseptically streaked onto fresh Z8 medium agar plates.

This procedure was repeated until isolation was achieved and reconfirmed after transferring to liquid medium. The unicyanobacterial isolates obtained were kept in our collection (LEGE Culture Collection), at 19◦C and under a 12:12-h light (approximately 25 µmol/m^2/s photon irradiance): dark cycle, while isolation was carried out at 25◦C under a 14:10-hr light (30–40 µmol/m^2/s photon irradiance): dark cycle.

The morphology of the strains was examined using a BX41 light microscope coupled to a DP72 digital camera (Olympus, Hamburg, Germany), and morphometric measures were performed using image analysis software Cell B (Olympus). The identification of the cyanobacteria was performed according to the traditional classification system of Komárek and

TABLE 1. Strain Information for the *Cyanobium* sp. Isolates Included in This Study

Strain	Collection date	Sampling site[a]	Latitude/longitude coordinates	Isolation source	Morphometry[b]	Accession number
LEGE 06098	16-07-2006	Martinhal	N 37°01′07.30″/W 8°55′36.17″	Green macroalgae, intertidal zone	1.0 ± 0.2 µm	KC469572
LEGE 06134	30-06-2006	Moledo	N 41°50′58.68″/W 8°52′0.18″	*Sabellaria* sp. reef, intertidal zone	0.8 ± 0.1 µm	KC469573
LEGE 06139	26-06-2006	Aguda	N 41°02′58.35″/W 8°39′19.22″	*Mytilus* sp. shell, intertidal zone	0.8 ± 0.1 µm	KC469574
LEGE 07175	02-05-2007	Martinhal	N 37°01′07.30″/W 8°55′36.17″	Seawater, coastal	1.0 ± 0.2 µm	KC469575
LEGE 07186	02-05-2007	Martinhal	N 37°01′07.30″/W 8°55′36.17″	Submerged stone, intertidal pool	1.0 ± 0.1 µm	KC469576

[a]Sites refer to Portuguese beaches.
[b]Mean ± SD, $n = 20$.

Anagnostidis (1999) and compared with criteria of *Bergey's Manual of Systematic Bacteriology*

(Boone et al., 2001). Information for the strains used in this study is shown and summarized in Table 1. For biomass production, strains were grown in Z8 medium supplemented with NaCl at a concentration of 20 g/L (Martins et al. 2005). Cultures were maintained at 25◦C, under a light:dark cycle of 14:10 h, provided by cool white fluorescent tubes. Cells were harvested after one month of growth by centrifugation, frozen, and freeze-dried.

DNA Extraction, PCR, Cloning, and Sequencing

Strains were characterized by molecular analysis of the 16S rRNA gene. Total genomic DNA was extracted from frozen samples using the Purelink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA) following the protocol described for gram-negative bacteria. The primers CYA359F and 1494R (Neilan et al., 1997) were used for amplifying and sequencing the 16S rRNA gene. The polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 94◦C for 4 min, followed by 35 cycles of denaturation at 94◦C for 30 s, annealing at 52◦C for 30 s, and extension at 72◦C for 80 s, and a final extension step at 72◦C for 5 min. All PCR reactions were prepared in a volume of 20 μl containing 1× PCR buffer, 2.5 m*M* $MgCl_2$, 250 m*M* of each deoxynucleotide triphosphate, 10 pmol of each of the primers, 0.5 U of *Taq* DNA polymerase (Bioline, Luckenwalde, Germany) and 10 ng of template DNA. PCR products were separated by electrophoresis on a 1.5% (w:v) agarose gel. Gels were stained with ethidium bromide and photographed under ultraviolet (UV) transillumination.
Amplicons were purified using Cut&Spin DNA Gel Extraction Columns (Grisp, Portugal), according to the manufacturer's instructions. Purified PCR products were then cloned into pGEM-T Easy vector (Promega, Madison, WI), and transformed into OneShot TOP10 chemically competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA) using standard procedures following the manufacturer's instructions (Sambrook and Russell, 2001). Plasmid DNA was isolated using GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO) and sequenced (Macrogen, Inc., Seoul, Korea) using M13 primers. The sequences obtained in the present study were assessed for the existence of chimeras using the DECIPHER program (Wright et al., 2012) and deposited in GenBank under the accession numbers KC469572–KC469576 (see Table 1 for correspondence with strains).

Phylogenetic Analysis

The 16S rRNA gene sequences obtained were aligned with sequences from the best BLASTn hits in GenBank and with available sequences from picocyanobacterial reference strains according to *Bergey's Manual of Systematic Bacteriology* (Boone et al., 2001).
The multiple sequence alignment (using the clustal W algorithm) and phylogenetic analyses were performed using the MEGA 5 software package (Tamura et al., 2011). Following the
Bayesian Information Criterion (Tamura et al., 2011), the T92 + G + I model of nucleotide substitution was selected as the best-fitting model for the datasets. Phylogenetic trees were reconstructed using the maximum likelihood (ML) and maximum parsimony (MP) methods, and, as an indication of nodal support, bootstrap analyses were performed (1000 replicates for each method). Ambiguously aligned regions were omitted ("complete deletion" option) from the analyses. The topologies retrieved from the two methods were then evaluated using TreePuzzle 5.2 (Schmidt et al., 2002), and the best topology was selected according to the results of the test comparisons (one- and two-sided Kishino–Hasegawa test, Shimodaira–Hasegawa test, expected likelihood weights). The branch support values derived from the two analyses were then compared using TreeGraph 2 (Stover and Muller, 2010).

Cyanobacteria Extracts

Freeze-dried cyanobacterial biomass was extracted repeatedly, twice at room temperature and then at 40◦C, with a methanol: dichloromethane (1:2) solution.
After extraction, the solvents were evaporated in vacuo. A portion of the resulting crude extract was fractionated by normal-phase column chromatography in disposable silica (2-g) cartridges

(Phenomenex), using a solvent gradient of increasing polarity. Starting with 100% hexane, to 100% ethyl acetate, to 100% methanol, three fractions (A, B, and C) were obtained. The crude extract and respective fractions were then dissolved in dimethyl sulfoxide (DMSO) at different concentrations (0.1, 1, and 10 mg/ml).

Bioassays

Crude cyanobacterial extract and fractions were tested at final concentrations of 1, 10, and 100 µg/ml, using 96-well plates, except for the sea urchin assay, which was performed in 24- well plates and where concentrations were 0.1, 1, or 10 µg/ml. All assays were run in triplicate and averaged.

Nannochloropsis Growth Inhibition Assay

The marine microalga *Nannochloropsis* sp. LEGE Z-004 was cultured under the same conditions as described for cyanobacterial biomass production. Microalgal cells were inoculated in 200 µl test solution at a 0.1 optical density (OD) (750 nm). Potassium dichromate (4 µg/ml) was used as positive control. After 72 h of incubation, cell growth was estimated by measuring the OD at 750 nm in a microplate reader (Synergy HT, Biotek, Winooski, VT).

Bacterial Growth Inhibition Assay

The bacterial strain *Pseudomonas putida* NB3L (Lage and Bondoso, 2011) was grown in liquid M607 medium in the dark at 25◦C with shaking, until the exponential growth phase was reached, and then was diluted to 0.1 OD (750 nm). A mixture of penicillin (50 U/ml), streptomycin (50 µg/ml), and neomycin (100 µg/ml) was used as positive control. OD at
750 nm was measured after 24 h of incubation.

***Artemia salina* acute toxicity assay**

Acute toxicity to the brine shrimp was screened using *Artemia salina* nauplii, freshly hatched (24 h) from dried cysts in artificial seawater. Aliquots (10 µl) of nauplii solution containing about 15–20 organisms were pipetted into microplate wells containing 200 µl test solutions. Potassium dichromate was used as a positive control as described for the *Nannochloropsis* assay. Plates were incubated at 25◦C, in darkness. After 24 and 48 h, the numbers of dead larvae and total numbers of shrimps per well were determined. Results were expressed as percent mortality.

***Paracentrotus lividus* Embryo–Larval Acute Toxicity Assay**

Sea urchins were collected from rocky beach at Valadares (Porto, North Portugal, (N 41◦ 05_30.37_; W 8◦39_28.40_). Bioassays were performed according to the methods described by Fernandez and Beiras (2001). Briefly, sea urchins were dissected and sperm and eggs pipetted from the gonads. A volume of 10 µl undiluted sperm from one male was added to the egg suspension and carefully stirred to allow fertilization. Twenty fertilized eggs/ml were exposed to 3000 µl test solutions prepared in artificial seawater. After 48 h of incubation at 20◦C in the dark, solutions were fixed in 36–38% formaldehyde. Embryogenesis success was evaluated by formation of plutei larvae and larvae length.

Statistical Analysis

Data were analyzed using general linear models or generalized linear models. The dependent variables were those used to estimate growth (percent viability, OD, larva size).
Treatment, time, and cyanobacterial strain were used as fixed factor. For the analysis of sea urchin assay, a mixed-effects model was employed with nested block design. Normality was tested in the model residuals with the Shapiro–Wilks test. Nonnormal data were transformed using the

Box–Cox function. Then, if the normality test was still negative, a generalized linear model with gamma distribution was employed. In these cases, fit to gamma distribution was tested as the dependent variable (sometimes also transformed with the Box–Cox function). In some of the analyses, clear outliers were omitted from the data set. Post hoc pairwise comparisons were performed with the Tukey's or Dunnett's test. Whenever usingTukey's test, homogeneous subsets of treatment levels were obtained based on significance levels.
Those levels that did not differ significantly among them were classified into the same subset. Homogeneous subsets were not calculated for those factors with low number of levels or few significantly different pairwise comparisons. The criterion for significance was set at $p < .05$. As a measure of standardized effect size, the value of Dunnett's *t* statistic was calculated for each treatment level, averaged over comparisons against all other levels. The software employed was R version 2.15.2 with functions from *base, stats, car, multcomp,* and *nlme* packages.

RESULTS

Identification and Phylogeny of the Isolates

The morphological characterization of the picocyanobacterial isolates showed that cells were mainly spherical with some also being shortly rod-shaped (Figure S1; see Supplementary Material). Following botanical taxonomy (Komárek and Anagnostidis, 1998; Komarek et al., 1999), morphological and ecological features of the organisms enabled us to identify the strains as *Cyanobium* sp. This identificationis also in accordance with the criteria of Bergey's classification (Boone et al., 2001). Taking into account the 16S rRNA gene sequences, the best BLASTn match for all strains was *Cyanobium* sp. NS01 (99% similarity). This strain was previously isolated from central North Sea waters (Fuller et al., 2003).
Regarding their phylogenetic placement among the picocyanobacterial diversity, data demonstrated (Figure 1) that the strains were placedtogether in a major clade that comprised two well-defined subclades: the first containing *Synechococcus* spp. along with a reference strain (WH 8103, cluster 5.1), and the second, *Prochlorococcus marinus* strains. In addition, two small subclades emerged, supported byboth ML and MP methodologies; one of the subclades included other *Cyanobium* sp. strainsfrom our culture collection, and the other comprised three freshwater isolates, including the reference strain PCC 6307 (cluster 1), which is also the holotype for the genus *Cyanobium* (Rippka and Cohen-Bazire, 1983). The other sequences from this large clade belong to strains indistinctly assigned to *Synechococcus* and *Cyanobium* (some of which are reference strains).

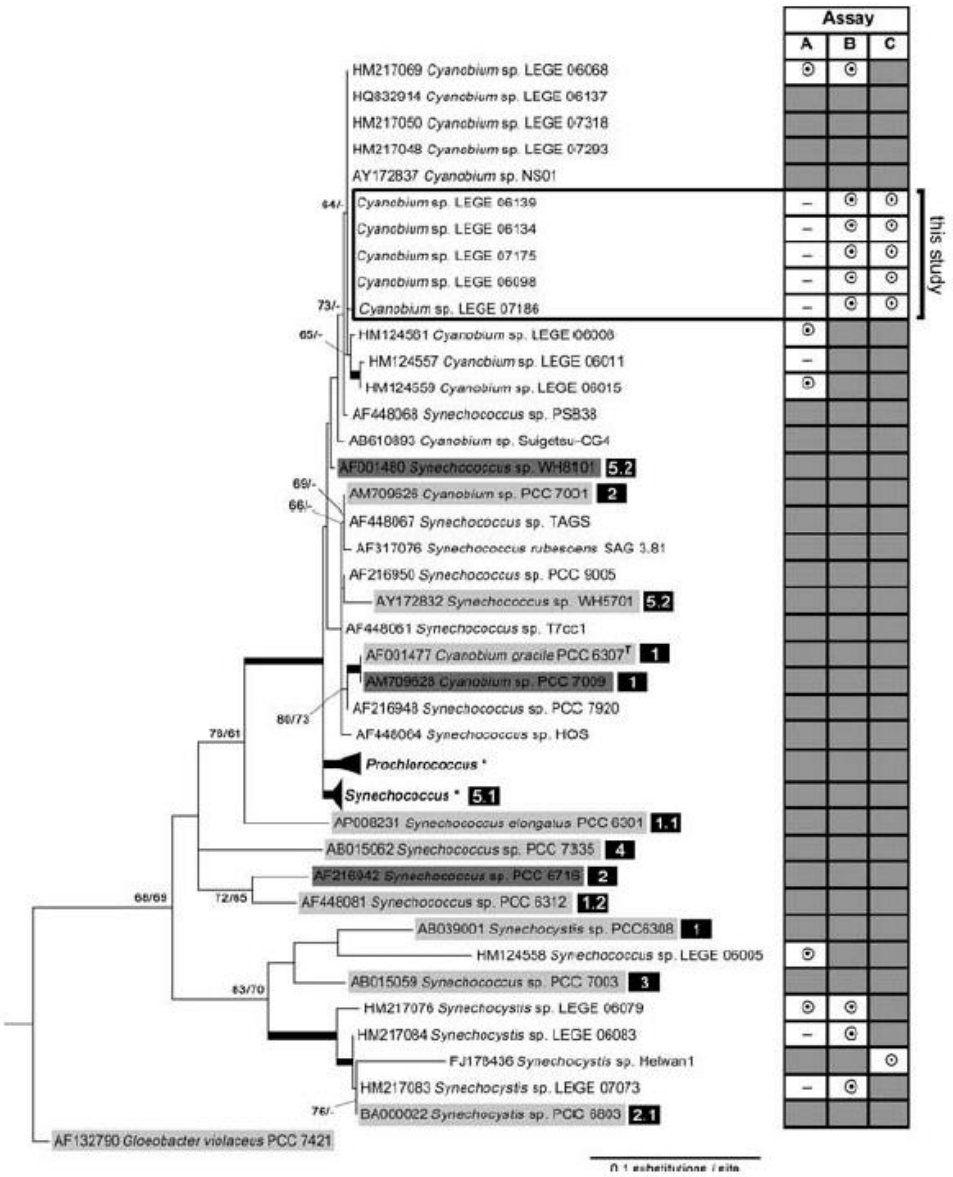

FIGURE 1. Maximum likelihood tree of picocyanobacterial 16S rRNA gene sequences (638 positions) and bioassay results from tested strains in this and other studies studies (A, *Artemia salina*; B, *Pseudomonas* sp.; C, *Paracentrotus lividus*). The nodal support values indicated near internal branches were determined by ML and MP methods, respectively; bootstrap values below 60% were omitted. Thick tree branches indicate ≥80% bootstrap values support. Reference strains are highlighted in light gray and their close relatives in dark gray, while the holotype species for the genus *Cyanobium* is marked with (^{T}). Numbered boxes refer to reference strains' clusters according to Boone et al., 2001. The condensed "*Synechococcus* clade" contains the reference strain WH 8103 (accession number: AF311293), while the "*Prochlorococcus* clade" encloses CCMP1375 (AE017126). The tree was rooted using *Chloroflexus aurantiacus* J-10-fl (CP000909) as outgroup, and removed for clarity.

Bioactivity of Isolates

For the assay with *Nannochloropsis*, a generalized linear model was fitted with treatment (crude extract and fractions) and cyanobacterial strains as fixed factors. The analysis of deviance found all factors significant (treatment, time and strain) (Figure 2). The homogeneous subsets for "treatment" showed level Fraction B at 1μg/ml as the lower viability group. For "strain," no significant differences were found (Figure 2).

In the bacteria assay, a general linear model was fitted with the same formulation as for *Nannochloropsis*. An analysis of variance found all factors significant. A post hoc analysis was performed with Dunnett's test (reference level:

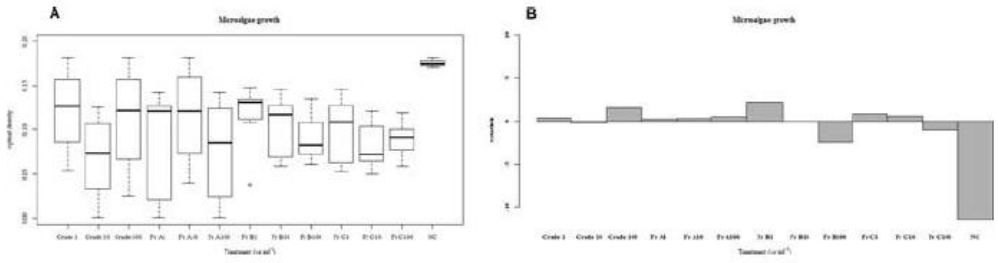

FIGURE 2. Effects of cyanobacterial extracts on *Nannochloropsis* growth. (A) Microalgae growth rate per treatment (cyanobacterial extracts). (B) Averaged Dunnett *t* statistic for the same variable (see Methods section).

negative control). This test demonstrated significant differences with the following levels:
Crude 1 μg/ml, Crude 10 μg/ml, Fr C 1 μg/ml, and Fr C 10 μg/ml. For "strain," no significant differences were found.
In the *Artemia salina* acute toxicological assay, no significant percent mortality was detected and thus no bioactivity was considered expressed (data not shown).
In the *P. lividus* bioassay, a general mixedeffects linear model was fitted using treatments and cyanobacterial strains as fixed factors.
Block (location of replicate groups in the chamber) was used as a random factor. An analysis of variance of this model found all factors significant, treatment and strain (Figure 3). A post hoc Dunnett's test (reference level: negative control) showed significant difference with level Fr
B 10 μg/ml. For "strain" no significant differences were found.

Bioactivity Pattern Among Phylotypes

To better visualize the bioactivity of extracts from diverse picocyanobacteria, a summary next to the phylogenetic tree is provided (Figure 1), with bioassay results obtained in this study compared to those from previous studies (Frazao et al., 2010; Semary and Naby,
2010; Lopes et al., 2011).The same bioactivity pattern was observed for isolates used in our study. Nevertheless, unlike that observed for other LEGE strains that represent closely related phylotypes, no apparent bioactivity was detected for the *A. salina* assay. Among these, only the previously studied *Cyanobium* sp. LEGE 06011 (Frazao et al., 2010) was also not active for this particular assay.

DISCUSSION

The morphological identification attempted here could not be carried out more stringently since the attributes of the strains were not assignable to any species with validly published names (Komárek and Anagnostidis, 1998). Thus, the strains were only identified at the genus level. The picocyanobacteria are recognized as having few characters, and as a result, their classification based on cytomorphological features is difficult (Komarek et al., 1999). Similarly, the 16S rRNA gene sequencing did not enable us to reach a

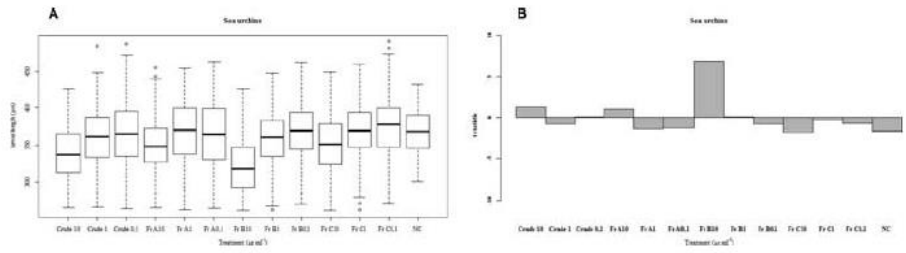

FIGURE 3. Effects of cyanobacterial extracts on *Paracentrotus lividus* larvae length. (A) Embryonic size per treatment (cyanobacterial extracts). (B) Averaged Dunnett *t* statistic for the same variable (see Methods section).

higher taxonomic resolution. The best hits retrieved from GenBank were sequences from *Cyanobium* spp. and *Synechococcus* spp. that, along with the sequences from our strains, resulted in closely related phylotypes (Figure 1).

This seemingly incongruent phylogeny is actually the well-recognized polyphyly of some morphotypes that are presumed to belong to the same cyanobacterial taxon, which is specifically true for several picocyanobacterial taxa (Honda et al., 1999; Robertson et al., 2001). Consequently, if a comparison of the presence or absence of particular aspects (e.g., bioactivity potential of extracts) in different morphologically identified taxa is to be made, this unresolved taxonomy might yield misleading conclusions. For this reason, it is preferred to compare phylotypes. Hence, in this investigation, marine *Cyanobium* strains shown to belong to the same subclade (Figure 1) were used, and their potential as producers of bioactive compounds was tested. The results obtained demonstrated that these *Cyanobium* strains, in general, inhibited microalgae and bacterial growth and induced a decrease in *P. lividus* larvae. When comparing these data with available results from less related phylotypes (Martins et al., 2005, 2007; Frazao et al., 2010) (Figure 1), it becomes evident that the limited number of picocyanobacteria studied to date exhibited activity in the majority of assays in which extracts were tested. The exceptions were two *Synechocystis* sp. strains (LEGE 06083 and LEGE 07073), which did not demonstrate toxicity for *A. salina* assay. Prior to this study, activity toward members of the *Pseudomonas* genus was tested only once for a picocyanobacterium with available 16S rRNA gene sequence.

The strain in question is *Synechocystis* sp. Helwan1 and its lipophilic extract also inhibited growth of this bacterium (Semary and Naby, 2010). The outcome noted from Figure 1 is somewhat biased, since the results presented are just for picocyanobacteria with available 16S rRNA gene sequences. There are other studies on the bioactivity of extracts from picocyanobacteria (Martins et al., 2007, 2008), but the isolates used therein were not characterized by molecular markers (particularly, the 16S rRNA gene sequence). Thus, even if they are assigned to the same taxa as those present on the phylogenetic tree, their placement remains uncertain. Therefore, it should be noted that some of the picocyanobacterial diversity known to show bioactivity is not covered in this presented phylogenetic tree.

With a crude extract and three fractions of different polarity, a preliminary segregation of the lipophilic metabolites in the cells was achieved. Among crude extract and fractions, in general, fraction B was found to be the one with a most pronounced adverse effect.

Fraction B corresponds to the extraction eluted from the silica column with a higher proportion of ethyl acetate corresponding to compounds with an intermediate polarity, which are known to include cyclic lipopeptides or depsipeptides (Luesch et al., 2002; Han et al., 2006).

The diversity of marine cyanobacteria bioactive compounds already identified belongs to such chemical classes of compounds. To cite only a few, the cyclic depsipeptides aurilides, isolated from *Lyngbya majuscule* probably *Moorea producens* (Engene et al., 2012), were shown to be inhibitors of different cancer cell lines (Han et al., 2006) and the cyclic hexapeptides venturamides A and B, isolated from a marine *Oscillatoria* sp., displayed antimalarial activity (Linington et al., 2007). Interestingly, evidence indicated that the crude extract did not produce similar inhibitory effects as faction B. This may be attributed to concentration of the toxic compound. Although the tested concentrations were the same, the compound inducing the effect is, in principle, more concentrated in fraction B than in crude extract. Alternatively, in the crude extract, an interaction of the toxic compound with others may have altered toxicity, consequently resulting in differences between crude extracts and fractions B.

Our results, along with the findings from previous studies, emphasize the importance to also explore smaller forms of cyanobacteria for bioactive and toxicological purposes. The genus *Cyanobium* then presents itself as a source of bioactive compounds with effects on other organisms, namely coexisting organisms. Since this bioactivity is reflected in terms of toxicity, the occurrence of these picocyanobacteria might thus be seen as a threat to ecosystem equilibrium. Inhibition of bacterial growth is, however, a promising result with respect to the pharmacological and biotechnological applications of compounds.

Identifying new sources of bioactive compounds represents the initial step in the process of discovering natural drugs. In this sense, this study represents a contribution to broaden the range of cyanobacteria from which new compounds with relevant bioactivity profiles might be isolated. According to our results, fraction B is promising for the isolation of compounds in the studied strains. Future investigations need to focus on the isolation and identification of compounds, as well as on the evaluation of their bioactive profile.

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