

Article

# Cyano-assassins: Widespread cyanogenic production from cyanobacteria

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**Abstract:** Cyanobacteria have been linked with hydrogen cyanide, based on their ability to catabolize it by the nitrogenase enzyme, as a part of nitrogen fixation. Nitrogenase can also use hydrogen cyanide instead of its normal substrate, dinitrogen and convert it to methane and ammonia. In this study, we tested whether cyanobacteria are able, not only to reduce, but also to produce HCN. The production of HCN was examined in 78 cyanobacteria strains from all five principal sections of cyanobacteria, both non-heterocytous and heterocytous, representing a variety of lifestyles and habitats. Twenty-eight (28) strains were found positive for HCN production, with universal representation amongst 22 cyanobacterial planktic and epilithic genera inhabiting freshwater, brackish, marine (including sponges), and terrestrial (including anchialine) habitats. The HCN production could be linked with nitrogen fixation, as all of HCN producing strains are considered capable of fixing nitrogen. Epilithic lifestyle, where cyanobacteria are more vulnerable to a number of grazers and accumulate more glycine, had the largest percentage (75%) of HCN-producing cyanobacteria compared to strains from aquatic ecosystems. Further, we demonstrate the isolation and characterisation of taxa like *Geitleria calcarea* and *Kovacikia muscicola*, for which no strain existed and *Chlorogloea* sp. TAU-MAC 0618 which is, to the best of our knowledge, the first bacterium isolate from anchialine ecosystems. Our results highlight the complexity of cyanobacteria secondary metabolism, as well as the diversity of cyanobacteria in underexplored habitats, providing a missing study material for this type of environments.

**Keywords:** secondary metabolism; hydrogen cyanide; molecular phylogeny; epilithic lifestyle; defense mechanism; *Geitleria calcarea*.

## 1. Introduction

Cyanobacteria are ubiquitous photosynthetic prokaryotes, found literally in any illuminated environment and unexpectedly in some dark subsurface ones (Hubalek et al., 2016; Puente-Sánchez et al., 2018). This phylum predominated Earth, when the environment was still reductive ca. 1.3 billion years prior to the great oxidation event (Gumsley et al., 2017). Cyanobacteria often produce secondary metabolites, in response to biotic or abiotic stress in the surrounding environment, providing protection and aiding in survival over other species (Gupta et al., 2013; Singh et al., 2016).

Cyanobacteria are considered to be the ancestors of chloroplasts genome (Allen, 2015). For example, plants have probably obtained gene copies implicated in a variety of biosynthetic pathways through early horizontal gene transfer from proteobacteria and cyanobacteria (Timmis et al., 2004). A number of secondary metabolites (e.g. many terpenoids, quinolizidine alkaloids, piperidine alkaloid coniine) are produced completely or partly in chloroplasts and/or mitochondria (Wink, 2003, 2008). Moreover, there is an indication that many enzymes and pathways are common in plant and cyanobacteria secondary metabolite production (Chen et al., 2016; Nielsen et al., 2016).

The term “cyanide” is used loosely and refers to both the cyanide anion (CN<sup>-</sup>) and undissociated hydrogen cyanide (HCN) (Knowles and Bunch, 1986). Cyanides are present in various environmental elements such as water, soil, food and biological materials like blood urine and saliva at the levels of micrograms per litre to milligrams per litre (Dzombak et al., 2006; Barceloux, 2009). Considering the presence of cyanide in various parts of the inanimate environment and biota as well as their toxicity, there is no doubt on increasing demand for information on their prevalence in the elements of the

environment (Dzombak et al., 2006). This compound has been found in some foods and seeds in the amounts above the limit recommended by WHO and FAO (Gernah et al., 2011). The pKa of cyanide is 9.3; it is therefore present largely as HCN at neutral pH values (Eisler and Wiemeyer, 2004). HCN is volatile (boiling point 26°C) and is less dense than air. Hence, cyanide formed by microbial cultures will be rapidly lost to the environment. Cyanide is largely toxic for aerobic cell metabolism as it binds to the mitochondrial cytochrome oxidase a3 enzyme; binding of cyanide to the ferric ion of the cytochrome oxidase a3 inhibits the terminal enzyme in the respiratory chain and halts the electron transport and oxidative phosphorylation, which subsequently leads to intracellular hypoxia (Hall, 2007).

Despite its toxicity, cyanide is a natural compound synthesized by a variety of organisms, including bacteria, fungi, plants, and animals, in which cyanogenesis may serve as defensive or offensive mechanism (Luque-Almagro et al., 2016). The HCN synthase required for bacterial cyanogenesis is expressed during transition from exponential to stationary phase of growth under oxygen limitation in response to the FNR-like anaerobic regulator ANR (Luque-Almagro et al., 2016). On the other hand, many microorganisms have evolved enzymatic pathways for cyanide degradation, transformation, or tolerance, and many of them are even able to use cyanide as a nitrogen source for growth (Luque-Almagro et al., 2016; Kumar et al., 2017; Park et al., 2017).

To date the green alga *Chlorella vulgaris* and the cyanobacteria *Anacystis nidulans* (= *Asterocapsa nidulans*), *Plectonema borganum*, and *Nostoc muscorum*, are the only photosynthetic micro-organisms known to be cyanogenic (Pistorius et al., 1979; Vennesland, 1981). However, it is likely, given the nature of the cyanogenic pathways involved, that many more of these micro-organisms to be cyanide producers. Photosynthetic micro-organisms synthesize cyanide from a wide range of metabolites by at least two distinct systems (Vennesland et al., 1982) (i) the amino acid oxidase-peroxidase system and (ii) the glyoxylic oxime system.

Diazotrophic cyanobacteria have been linked with hydrogen cyanide, based on their ability to catabolize it by nitrogenase, an enzyme normally responsible for the reduction of N<sub>2</sub>. Nitrogenase can also use hydrogen cyanide instead of its normal substrate, dinitrogen and convert it to methane and ammonia (Gantzer and Maier, 1990). The N<sub>2</sub>-fixing cyanobacterium, *Anabaena* is able to biodegrade cyanides and produce CH<sub>4</sub> in batch reactors. Gantzer and Maier (Gantzer and Maier, 1990) showed that *Anabaena* reduced cyanides by nitrogenase to CH<sub>4</sub> and NH<sub>3</sub>. The rate for CH<sub>4</sub> production was ten times faster than expected based on literature. However, in these cases, the assumption was made based on induced HCN in batch reactor.

In this study, we tested whether cyanobacteria are able, not only to reduce, but also to produce HCN, broadening our knowledge on the biosynthesis of this unique molecule. We examined HCN production in representative genera from all five of the principal sections of cyanobacteria, in both non-heterocytous and heterocytous species, representing a variety of lifestyles and habitats. We also correlate the production of HCN with epilithic lifestyle as the main hypothesis for the HCN production from cyanobacteria.

## 2. Materials and Methods

### 2.1 Cyanobacterial Strains

Seventy-eight (78) strains of cyanobacteria, representing five cyanobacteria orders, with different morphological features and a wide variety of habitats and lifestyles, were used in this study (Table S1). Twenty-nine strains were isolated from different freshwaters of Greece (Gkelis et al., 2019), nine of them were isolated from sponges (Konstantinou et al., 2018), whereas the rest 40 strains were isolated in this study from various environments (terrestrial caves, coastal areas, thermal springs, brackish and freshwater systems) across Europe, between 2013 and 2018. Strains were isolated on solid growth media using classical microbiological techniques (see (Gkelis et al., 2005, 2015)), purified by successive transfers and using antibiotics (such as cycloheximide and ampicillin) as described in Rippka (Rippka, 1988); all strains were derived from a single colony or trichome. The cultures were grown as batch clonal unialgal cultures in BG11, with or without (for the nitrogen-fixing strains) nitrogen, and MN medium (Rippka, 1988). All strains are deposited in the Thessaloniki Aristotle University Microalgae and Cyanobacteria (TAU-MAC) culture collection (Gkelis and Panou,

2016) and maintained at  $20 \pm 1^\circ\text{C}$  or  $25 \pm 1^\circ\text{C}$  (for strains of the genera *Desertifilum* and *Calothrix*) with a light intensity of  $25 \mu\text{mol m}^{-2}\text{s}^{-1}$  and with a light/dark cycle of 12:12h.

## 2.2 Cyanogenesis analysis

In order to assess the ability to produce HCN and estimate the relative frequency of cyanogenesis (proportion of cyanogenic versus acyanogenic cyanobacteria strains), we screened each strain for the presence of HCN using the Feigl-Anger assay, which determines the presence or absence of HCN in a sample using a colour change reaction (Gleadow and Møller, 2014). In this assay, the dried paper turns blue following oxidation of the tetra base when it is exposed to the HCN gas that is produced. For the extraction of HCN, we modified the original protocol (Thompson et al., 2016), implemented in plants and includes freeze-thaw, as freeze-thaw is not an efficient method for cell lysis in cyanobacteria (Kim et al., 2009). Cyanobacteria cells were harvested at the exponential growth phase (between 30-45 days of growth) by centrifugation of 1.5 mL culture material. The supernatant was removed and the pellet was dissolved in 0.8 mL of Lysis Buffer (2% w/v Cetyl trimethylammonium bromide, 100 mM Tris-HCl, 1.4 M NaCl, 1% w/v Polyvinylpyrrolidone, 20 mM,  $\text{Na}_2\text{EDTA}$  0.2% w/v LiCl, pH 8). The same procedure was repeated with the addition of  $\text{dH}_2\text{O}$  instead of Lysis Buffer. A 96-well plate was filled with 80  $\mu\text{L}$  of cyanobacteria isolates extract to each well. We secured Feigl-Anger test paper over the plates, incubated the plates for 1.5 h at  $37^\circ\text{C}$ , and then scored each well for cyanide, which is indicated by a blue colour (Figure S1). A permanent record of the detection paper was made by scanning the paper immediately after exposure because the blue color fades with time. As positive control, 4  $\text{cm}^2$  of leaf tissue of a cyanogenic individual of the plant *Trifolium repens* (Deligiannis et al., 2018) was used.

## 2.3 Nitrogen fixation capability

Cyanobacteria strains were also evaluated for the ability to perform nitrogen fixation by PCR targeting one gene fragment (*nifH*) of the nitrogenase gene cluster (Table S2). The reactions were performed according to Panou et al. (Panou et al., 2018). Thermal cycling was carried out using an Eppendorf MasterCycler Pro (Eppendorf). PCR products were separated by 1.5% (w/v) agarose gel in 1X TAE buffer. The gels were stained with Midori Green Advanced (NIPPON Genetics Europe GmbH) and photographed under UV transillumination.

## 2.4 Polyphasic taxonomy

The cyanobacteria isolates, which were positive for the production of HCN, were characterised based on their morphology and their phylogeny as described in Gkelis et al. (Gkelis et al., 2019). Briefly, strains were identified based on their morphology using the taxonomy books by Komárek and Anagnostidis (Anagnostidis and Komarek, 1988; Komarek and Anagnostidis, 1989; Komárek and Anagnostidis, 2008) and Komárek (Komárek, 2013). The phycocyanin operon and the internal genetic spacer (*cpcBA*-IGS), 16S rRNA gene, and the 16S-23S rRNA internal transcribed spacer (ITS) were used to assess the molecular phylogeny of the strains. PCR was carried out on using the primer pairs shown in Table S2 and PCR conditions described in detail in Gkelis et al. (Gkelis et al., 2019). Sequence data were obtained by capillary electrophoresis (GENEWIZ, Takeley, UK). The obtained nucleotide sequences were edited with Unipro UGENE 1.29.0 (Okonechnikov et al., 2012). Nucleotide sequences were deposited in GenBank database of the National Center for Biotechnology Information (NCBI) (Table S3). Sequences were blasted and the closest relative(s) for each sequence were included in the phylogenetic trees. For the phylogenetic analyses, we selected sequences (>1200 and >600 bp, for 16S-23S rRNA and *cpcBA*-IGS, respectively) in order to examine phylogenetic position of our strains. The phylogenetic analyses were conducted with Mega (V7.0) software (Kumar et al., 2016). Multiple sequence alignments were conducted using the CLUSTALW software. All missing data and gaps were excluded from the analysis by choosing the complete deletion option. A consensus phylogenetic tree were constructed using maximum likelihood (ML). The best fitting evolutionary models for the ML analyses were the Tamura 3-parameter + G model for all the targets analysed. Bootstrap replicates ( $n=1,000$ ) were performed. Phylogeny was also inferred with Bayesian Inference (BI) phylogenetic approach with MrBayes (V3.2.6) software (Ronquist et al., 2012). The general time-reversible (GTR) with gamma distribution of rates and a proportion of

invariable sites evolutionary model was selected by applying PAUP\* (V5.0) (Swofford, 2002). Bayesian analysis consisted of two independent Markov Chain Monte Carlo runs, performed by four differentially heated chains of  $10 \times 10^6$  generations and trees were sampled from the chain every 1000 generations. All phylogenetic trees were visualized via the FigTree (V1.4.3) software (Rambaut).

### 3. Results

#### 3.1 HCN Production

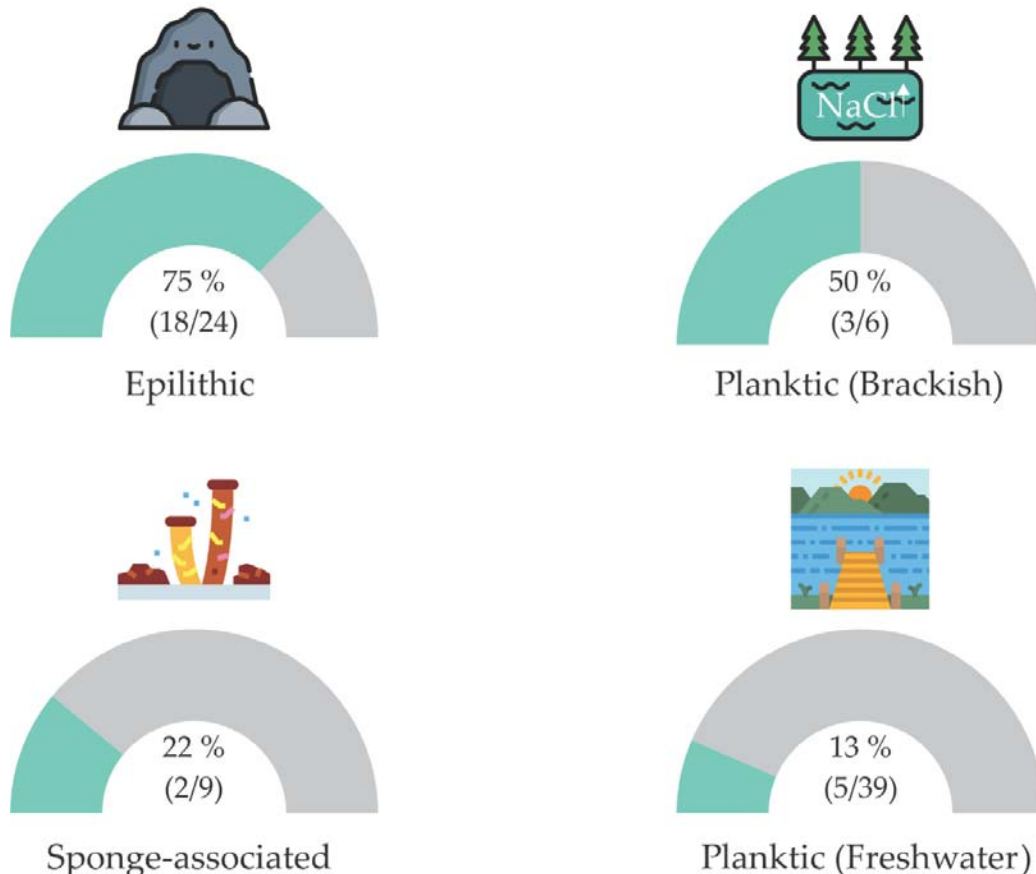
Twenty-eight (28) cyanobacteria strains (Table 1) were found positive for HCN production (Figure S1). Cyanobacteria strains, with different lifestyles, were found capable of producing HCN (Figure 1). Strains, isolated from mats in rocks, were found to be positive in HCN production with a relative frequency of 75 %. Three strains (50 % percentage) from brackish environments were found positive for the production of HCN, whilst cyanide was found to be present also in sponge-associated cyanobacteria, even though in low percentage (only 22% of the sponge symbiotic cyanobacteria tested). The habitat, with the lowest number of HCN-producing isolates, was freshwater ecosystems (Table 1, Figure 1), where only the 13 % of freshwater cyanobacteria were positive for HCN production.

**Table 1.** Cyanobacteria strains of TAU-MAC collection positive for HCN production, their origin, habitat, and lifestyle. Table S3 contains the complete list of strains tested for HCN production.

Strain	Lifestyle (Habitat)	Origin	Reference
<i>Aliinostoc</i> sp. TAU-MAC 3117	Planktic (Brackish)	Lake Pikrolimni, Greece	This study
<i>Calothrix thermalis</i> TAU-MAC 1117	Epilithic (Thermal Spring)	Mývatn, Iceland	This study
<i>Chlorogloeopsis fritschii</i> TAU-MAC 0599	Planktic (Freshwater)	Lake Mikri Prespa, Greece	(Gkelis et al., 2019)
<i>Chlorogloea</i> sp. TAU-MAC 0618	Epilithic (Anchialine Cave)	Túnel de la Atlántida, Spain	This study
<i>Cyanobacterium stanieri</i> TAU-MAC 3217	Planktic (Brackish)	Kalochori lagoon, Greece	This study
<i>Desertifilum tharense</i> TAU-MAC 1517	Epilithic (Thermal Spring)	Mývatn, Iceland	This study
<i>Geitleria calcarea</i> TAU-MAC 0118	Epilithic (Terrestrial Cave)	Anthropograva, Greece	This study
<i>Gloeotrichia echinulata</i> TAU-MAC 3718	Planktic (Freshwater)	Lake Peipsi, Estonia	This study
<i>Jaaginema</i> sp. TAU-MAC 0110	Planktic (Freshwater)	Lake Volvi, Greece	(Gkelis et al., 2019)
<i>Komarekiella</i> sp. TAU-MAC 0117	Epilithic (Terrestrial Cave)	Agio Galas, Greece	This study
<i>Komarekiella</i> sp. TAU-MAC 0217	Epilithic (Terrestrial Cave)	Agio Galas, Greece	This study
<i>Kovacicikia muscicola</i> TAU-MAC 0518	Epilithic (Terrestrial Cave)	Perama, Greece	This study
<i>Leptothoe spongobia</i> TAU-MAC 1115	Sponge-associated	Kassandra, Greece	(Konstantinou et al., 2018, 2019)
<i>Myxosarcina</i> sp. TAU-MAC 3418	Epilithic (Marine Coastal Rock)	Afytos, Greece	This study
<i>Nodularia harveyana</i> TAU-MAC 0817	Planktic (Brackish)	Lake Prikrolimni, Greece	This study
<i>Nodularia spumigena</i> TAU-MAC 3417	Planktic (Brackish)	Kalochori lagoon, Greece	This study
<i>Nostoc muscorum</i> TAU-MAC 1518	Epilithic (Terrestrial Cave)	Perama, Greece	This study
<i>Oculatella</i> sp. TAU-MAC 3318	Epilithic (Terrestrial Cave)	Perama, Greece	This study
<i>Phormidium</i> sp. TAU-MAC 0417	Epilithic (Terrestrial)	Olympon, Greece	This study

<i>Radiocystis</i> sp. TAU-MAC 1214	Cave) Planktic (Freshwater)	Lake Karla, Greece	This study
<i>Scytonema hyalinum</i> TAU-MAC 2618	Epilithic (Terrestrial Cave)	Grava, Greece	This study
<i>Sphaerospermopsis aphanizomenoides</i> TAU-MAC 1314	Planktic (Freshwater)	Lake Karla, Greece	This study
<i>Synechococcus</i> sp. TAU-MAC 0815	Sponge-associated	Kassandra, Greece	(Konstantinou et al., 2018)
<i>Tolypothrix</i> sp. TAU-MAC 2518	Epilithic (Terrestrial Cave)	Grava Cave, Greece	This study

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173 **Figure 1.** Cyanogenic relative frequency of cyanobacteria strains amongst different lifestyles presented with  
174 Gauge charts.

### 175 3.2 Polyphasic Taxonomy

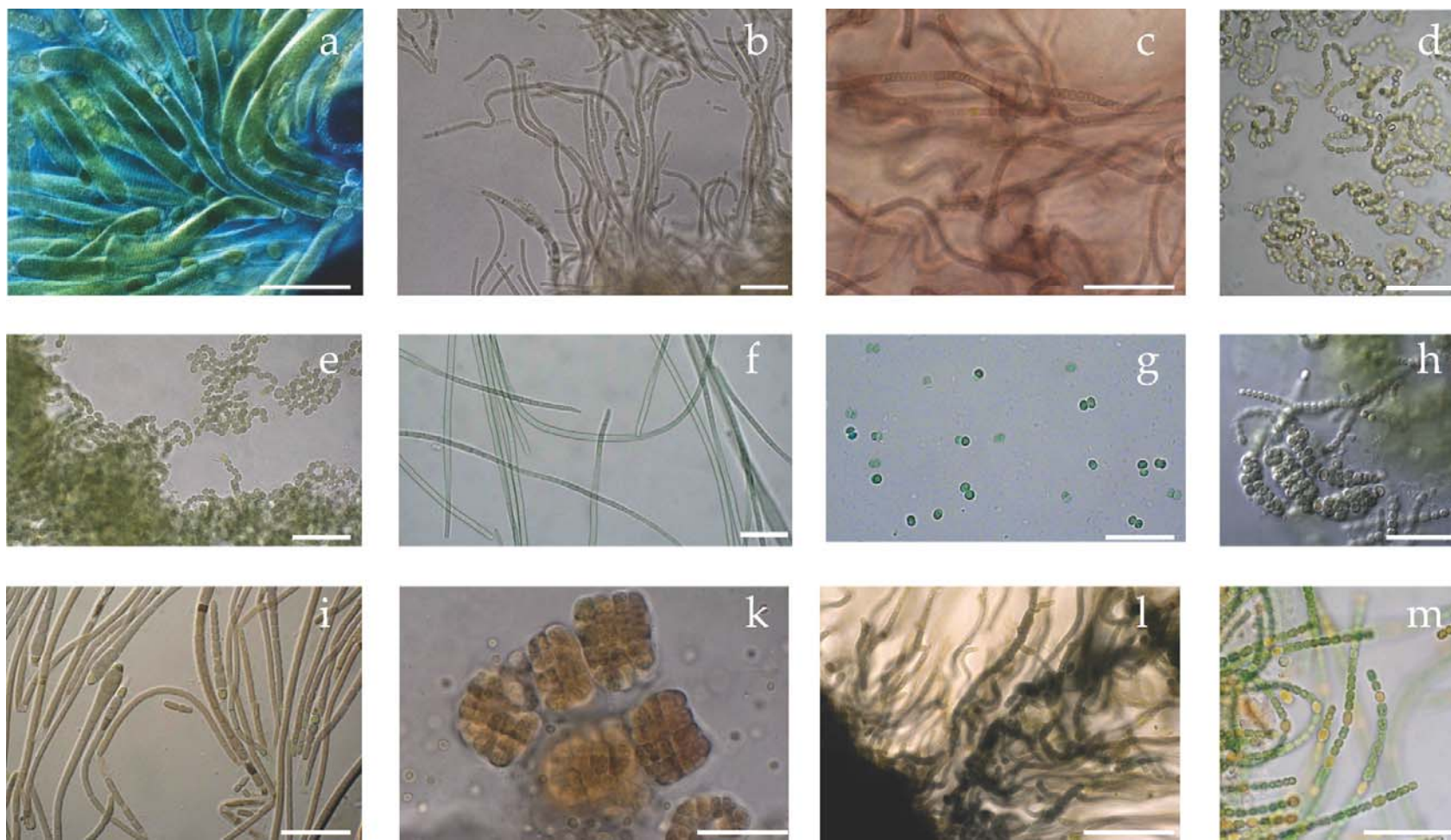
176 According to the combined morphology (Figure 2) and the phylogeny based on 16S-23S  
177 ribosomal RNA (rRNA) and *cpcBA*-internal genetic spacer (IGS) regions (Figure 3), the strains,  
178 isolated in this study, positive for HCN production were classified into 18 genera and 20 taxa  
179 belonging to Chroococcales, Synechococcales, Oscillatoriales, Nostocales, and Pleurocapsales.  
180 Nine strains were identified to the genus level (*Allinostoc*, *Chloroglea*, *Oculatella*, *Komarekiella*,  
181 *Myxosarcina*, *Phormidium*, *Radiocystis*, and *Tolypothrix*), whereas the rest were identified up to the  
182 species level.

183 Strains isolated from terrestrial caves formed clades with no more than two sequences, due to  
184 limited available sequences for cyanobacteria derived from these type of environments (Figure 3).  
185 Specifically, *Geitleria calcarea* TAU-MAC 0118 clustered with two uncultured *Geitleria* sp.  
186 sequences, whilst *Allinostoc* sp. TAU-MAC 3117 was placed separately, outside two uncultured  
187 *Allinostoc* sp. sequences. *Komarekiella* strains, isolated from a show cave in Chios Island in NE

188 Aegean Sea, clustered together, separately of two *Komarekiella atlantica* strains, isolated from a  
189 tropical rainforest in Hawaii.

### 190 3.3 *NifH* Amplification

191 The *nifH* gene fragment was amplified (Figure S2) in 53 of the total 78 (67 %) cyanobacteria  
192 strains tested for HCN production (Figure S2). The *nifH* was present in all HCN-producing  
193 cyanobacteria strains, as well as in 25 more strains. All strains, classified to Nostocales order, carried  
194 the *nifH* gene fragment, whilst *nifH* was also amplified in non-heterocytous genera.



**Figure 2.** Microphotographs of strains, isolated in this study, representing 12 genera of cyanobacteria strains capable of producing HCN. (a) *Gloeotrichia echinulata* TAU-MAC 3718; (b) *Kovacikia muscicola* TAU-MAC 0518; (c) *Oculatella* sp. TAU-MAC 3318; (d) *Aliinostoc* sp. TAU-MAC 3117; (e) *Nostoc muscorum* TAU-MAC 1518; (f)

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199 *Desertifilum tharense* TAU-MAC 1517; (g) *Radiocystis* sp. TAU-MAC 1214; (h) *Komarekiella* sp. TAU-MAC 0117; (i) *Calothrix thermalis* TAU-MAC 1117; (k) *Myxosarcina*  
 200 sp. TAU-MAC 3418; (l) *Geitleria calcarea* TAU-MAC 0118; (m) *Nodularia harveyana* TAU-MAC 0817. Scale bar = 10 µm.



**Figure 3.** Phylogenetic tree based on 16S-23S rRNA and cpcBA-IGS sequences of HCN-producing cyanobacteria strains, isolated and described in this study. The phylogenetic tree was reconstructed using the Maximum-Likelihood (ML) and the Bayesian Inference (BI) analyses. ML topology is demonstrated. Numbers above branches indicate the bootstrap value (as percentages of 1,000 replications) for ML method and the posterior probabilities for BI method, respectively. Strains of the present study are indicated in bold. Bar represents 0.020 nucleotide substitutions per site.

## 208 4. Discussion

### 209 4.1 Cyanogenic Cyanobacteria

210 In this study we demonstrate the production of cyanide from 28 cyanobacterial strains classified  
211 to 22 planktic and epilithic genera inhabiting freshwater, brackish, marine, terrestrial, and  
212 sponge-associated habitats. HCN production is stimulated mainly from histidine (Pistorius and Voss,  
213 1977) and other aromatic amino acids is a general reaction catalysed by D-amino acid and L-amino  
214 acid oxidoreductases. The stoichiometry of this process was investigated by Gewitz et al. (Gewitz et  
215 al., 1980) using snake venom L-amino acid oxidase and horseradish. Under optimal conditions, this  
216 system converted 72% of the added histidine into cyanide. Other products of the reaction were  
217 imidazole- Carbaldehyde, imidazole-4-carboxylic acid, CO, ammonia, water and imidazole acetic acid.  
218 The amount of CO, produced equalled the quantity of histidine oxidized and the sum of the ammonia  
219 plus cyanide formed. The cyanide production pathway is conserved in different type of organisms and  
220 it mainly contains an L-amino acid and its oxidase (Knowles and Bunch, 1986).

221 The production of cyanide by algae was first demonstrated by Gewitz et al. (Gewitz et al., 1976b)  
222 in 1976 in the green-alga *Chlorella vulgaris*. They showed that HCN is formed in small amounts when  
223 extracts were illuminated in the presence of O<sub>2</sub> and supplemented with Mn<sup>2+</sup> ions and peroxidase. In  
224 their study, a large number of amino acids were tested as possible precursors of HCN and D-Histidine  
225 was found to be the best promotor of cyanogenesis. Other aromatic amino acids could also promote  
226 cyanide formation (Gewitz et al., 1976a). These experiments also showed, that even though extracts  
227 of *Chlorella vulgaris* released HCN from amygdalin, a plant cyanogen, the HCN produced under  
228 oxidative conditions was not formed in this way (Gewitz et al., 1976b). Interestingly, the New Zealand  
229 spinach plant has a similar system for producing HCN (Gewitz et al., 1976a), as well as it was able to  
230 form cyanogenic glucosides in the grana. Further studies revealed details of the mechanism, used by  
231 the extracts of *Chlorella vulgaris* to convert histidine to HCN. Pistorius et al. (Pistorius and Voss,  
232 1977) showed that a soluble protein, plus a component of the particulate fraction of extracts, were  
233 necessary for cyanogenesis. The soluble protein was found to be a D-amino acid oxidase, a  
234 flavoprotein, partially purified by Pistorius and Voss (Knowles and Bunch, 1986).

235 The production of cyanide in cyanobacteria has been reported only once in *Anacystis nidulans*  
236 (= *Asterocapsa nidulans*), *Plectonema borganum*, and *Nostoc muscorum* 40 years ago (Pistorius et  
237 al., 1979; Vennesland, 1981). Pistorius et al. (Pistorius et al., 1979) reported that histidine could  
238 stimulate HCN production. Larger quantities of HCN were produced if peroxidase, or certain redox  
239 metals, were also present suggesting that either the amino acid oxidase is located in the outer part of  
240 the cells, or the imino acid intermediate is excreted (Vennesland, 1981). Not surprisingly, the cells  
241 carried an L-amino acid oxidase (Pistorius and Voss, 1980). This enzyme has two subunits, each of  
242 49,000 molecular weight, and contains one molecule of FAD per molecule of enzyme. It acts only on  
243 basic amino acids. Histidine is oxidized at a much slower rate. It is inhibited by divalent cations and  
244 orthophenanthroline. This latter observation implied a requirement for a metal ion, like zinc  
245 (Vennesland, 1981). In *A. nidulans* the L-amino acid oxidase has been reported to be associated with  
246 photosystem I (Pistorius and Voss, 1982).

247 All HCN-producing strains had the *nifH* gene, indicating that are capable of N<sub>2</sub> fixation, and thus  
248 suggesting that their ability to fix nitrogen could be linked to the production and simultaneously  
249 reduction of HCN, although this needs to be confirmed. Nitrogen fixation has been linked with cyanide  
250 not only in living organisms (Knowles and Bunch, 1986), but also through synthetic methods for  
251 terminal nitrile functionalization via conversion to the rare methoxymethyl imido unit (Curley et al.,  
252 2011). The ability of strains to fix nitrogen and produce cyanide seems to be not strictly related with  
253 heterocyte formation, as *nifH* gene fragment was also present in non-heterocytous cyanobacteria  
254 strains that produce nitrogen. Non-heterocytous cyanobacteria can fix nitrogen either in dark  
255 (Bergman et al., 2006) or in light combined with mechanisms for protecting the O<sub>2</sub>-labile nitrogenase  
256 (Berrendero et al., 2016). HCN can inhibit a wide range of metabolic processes (Knowles and Bunch,  
257 1986), but the most pertinent effect in photosynthetic micro-organisms seems to be the inhibition of  
258 the reduced form of nitrate reductase (Lorimer et al., 1974).

259 An interesting relation was noticed concerning the relative frequency of HCN production  
260 amongst different cyanobacteria lifestyles. In the present study, cyanobacteria strains, isolated from

epilithic mats, found to be more capable of HCN production compared to strains from aquatic ecosystems. Since cyanogenesis is a defence mechanism widely distributed in the plant kingdom and present in many major crop species (Thompson et al., 2016; Alberti et al., 2017), our results could possibly imply an unidentified chemical cue, released by different grazers, that triggers cyanobacteria to use HCN as a defence mechanism. Indeed, in environments where cyanobacteria are vulnerable to grazers such as rotifers or ciliates a rapid defence system should be favoured evolutionary (Wolfe, 2000). Even though no direct observations of activated chemical systems in unicellular organisms have been made, there are several examples of activated microbial defence reactions, which might serve as conceptual models for such systems (Mazard et al., 2016). One cannot exclude that physical contact with a grazer might stimulate cyanobacteria cells to produce compounds with a potential defensive role. Yang et al. (Yang and Kong, 2012) observed that the cyanobacterium *Microcystis aeruginosa* remaining under *Ochromonas* grazer pressure not only created colonies but also increased the amount of produced exopolysaccharides.

Cyanobacteria that thrive under extreme and diverse conditions tend to accumulate more compatible solubles such as sucrose, trehalose, glucosylglycerol, and glycine (Soule and Garcia-Pichel, 2019). However glycine accumulation could be toxic for the cyanobacterium and should be moderated (Eisenhut et al., 2007). Castric et al. (Castric, 1983) suggested that cyanogenesis is a response to a build-up of the intracellular glycine concentration. The key primary metabolic enzymes are serine hydroxymethyltransferase and glycine cleavage enzyme which catalyse conversion of serine into glycine and glycine into CO<sub>2</sub> and ammonia, respectively. This could be the link between HCN production and cyanobacteria that pose a non planktic lifestyle.

## 4.2 Biodiversity

The polyphasic taxonomy applied to the strains of this study revealed taxa known to be part of bloom-forming communities (*Sphaerospermopsis* and *Nodularia*), rock-dwelling communities (*Scytonema* and *Tolypothrix*), and hot spring cyanobacteria mats (*Desertifilum* and *Gloeotrichia*) (Dadheech et al., 2014; Mazur-Marzec et al., 2015; Gkelis et al., 2017; Joanna and Andrzej, 2018). Our results revealed the presence of taxa not previously described from Greek habitats (Gkelis et al., 2016), such as *Allinostoc* (Saraf et al., 2018) and *Oculatella* (Osorio-Santos et al., 2014) and taxa previously described only from the tropical zone like *Komarekiella* and *Kovacikia* (Miscoe et al., 2016; Hentschke et al., 2017). Furthermore, the *Geitleria calcarea* strain isolated in this study, to the best of our knowledge, is the first isolate in world's cyanobacteria culture collections depositories (Friedmann, 1979; Coute, 1989), whilst *Chlorogloea* sp. TAU-MAC 0618 consists the first bacteria isolate from an anchialine type environment.

The strains TAU-MAC 0817 and 3417 isolated from two brackish environments belong to species *Nodularia harveyana* and *Nodularia spumigena*, respectively. These strains constitute the first isolates of *Nodularia* strains in Greece, whilst in the Mediterranean there are only five strains of *Nodularia* isolated from Turkey (Akcaalan et al., 2009). The scarce records of *Nodularia* across Mediterranean could be linked with the absence of a high number of brackish environments. Anagnostidis (Anagnostidis, 1968), decades ago, reported the occurrence of *N. spumigena* and *N. harveyana* in Greece, (Gkelis et al., 2016). The largest research activity on the genus *Nodularia* occurs on the Baltic Sea, where *Nodularia spumigena* forms highly toxic blooms with significant effects on aquatic and non-aquatic organisms (Sivonen et al., 1989; Finni et al., 2001; Mazur-Marzec et al., 2015). Strain TAU-MAC 1517, isolated from a thermal site in Iceland was classified to the species *Desertifilum tharense* in both phylogenetic and morphological analysis, exhibiting the trichome's "anchored" end, that discriminates it from *Microcoleus* and *Geitlerinema* (Dadheech et al., 2014). *Desertifilum tharense* has been recorded in India, Kenya, Mexico, Greece, Mongolia, and China (González-Resendiz et al., 2019). The presence of *Desertifilum tharense* in Iceland, a different ecological niche, supports the theory that despite its wide ecological span, the genus *Desertifilum* remains genetically stable (Sinetova et al., 2017; González-Resendiz et al., 2019).

Several strains of this study, such as *Geitleria calcarea*, *Kovacikia muscicola*, *Komarekiella* sp., *Scytonema hyalinum*, and *Tolypothrix* sp. were isolated from extensive dark-green coverings dominated by cyanobacteria like *Phormidium*, *Tolypothrix*, *Scytonema*, and *Geitleria* species. Cyanobacteria are considered the pioneering inhabitants in cave colonization (Joanna and Andrzej,

2018). They prevail in the cave entrances compared to the other microalgae (Mulec and Kosi, 2009) by colonizing various parts of the cave entrances, where biodiversity is the lowest (Vinogradova N. et al., 1998). Cyanobacteria represent the first photosynthetic colonizers on the calcareous surfaces usually thriving both as epiliths and as endoliths (Lamprinou et al., 2009). Lamprinou et al. (Lamprinou et al., 2013) observed predominance of Oscillatoriales group over Chroococcales in caves; our results show that also many Nostocales species form part of the cave communities, several of which, are understudied. For example, the *Komarekiella* sp. strains we isolated here are reported for the first time as cave inhabitants. Hentschke et al. (Hentschke et al., 2017), based on intensive examination of species life cycle, proposed the new genus *Komarekiella* and classified the strains from both Hawaiian and Brazilian rainforests in the single *Komarekiella atlantica* species. Our phylogenetic analysis of 16-23S and *cpcBA*-IGS sequences, as well as the different climatic zone and habitat, suggests that strains TAU-MAC 0117 and 0217, may belong to a new taxon, thus further research is required to describe it. Concerning *Geitleria calcarea* TAU-MAC 0118, as in the original description of the species, the most obvious finding is its apparent inability to produce heterocytes naturally (Coute, 1989). In our phylogenetic analysis, *Geitleria calcarea* TAU-MAC 0118 clustered with the only two available *Geitleria* sequences in GenBank, belonging to a non-cultured *Geitleria* species, derived from a population genetics study.

Terrestrial meteoric water mixed with saline groundwater resembles in a two-layered circulation, in estuaries termed as subterranean estuaries or anchialine (meaning near the sea) environments (Moore, 1999). *Chlorogloea* sp. TAU-MAC 0618 consists, to the best of our knowledge, the first bacterium isolate from this type of environments, as the research concerning bacteria, is focused mainly in community analysis, either by metagenomics (Brankovits et al., 2017), or fluorescence microbial profiling analysis (Seymour et al., 2007; Krstulović et al., 2013).

## 5. Conclusions

The analysis of cyanobacteria strains from various environments revealed a high degree of biodiversity, deserving further research, whilst molecular data from strains may provide new information for cyanobacteria diversity, such as the isolation and characterisation of species like *Geitleria calcarea* and *Kovackia muscicola* that provided a missing study material for underexplored cyanobacteria. We demonstrated the production of HCN by cyanobacteria spanning a wide taxonomic range across different habitats and lifestyles. The high percentage of epilithic cyanobacteria producing HCN suggest that it may be also used as defence mechanism, therefore exploiting cyanide production very differently compared to other cyanogenic microbes. All HCN-producing cyanobacteria carried the *nifH* gene fragment highlighting the complex mechanisms between nitrogen fixation and HCN production. The widespread cyanide production we report here calls for further research to investigate the significance cyanide metabolism has in the cycling of carbon and nitrogen, especially as plants, and probably cyanobacteria, both produce and catabolize cyanide.

**Supplementary Materials:** Table S1: Complete list of TAU-MAC cyanobacteria strains used in this study, along with their description reference and the result of HCN Production. Plate position refers to Fig. S1 and strain number refers to Fig. S2. Strain number column refer to the number assigned to each strain for the PCR amplification of *nifH* gene fragment, Table S2: PCR primers used the phylogenetic analysis of cyanobacteria strains of TAU-MAC culture collection. Table S3: GenBank accession numbers for TAU-MAC strains used in the phylogenetic analysis **Figure S1:** Feigl-Anger Papers of the HCN producing strains. Blue dot is indicating the production of HCN. Plate position per strain refers to table S3. Con indicates positive control - *Trifolium repens*, Figure S2: PCR amplification of *nifH* gene fragment in the 78 TAU-MAC cyanobacteria strains tested for HCN production. Sample numbers refer to Table S3; + and - indicate positive and negative control, respectively; L indicates DNA ladder.

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