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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*.

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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-) 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

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50 environment (Dzombak et al., 2006). This compound has been found in some foods and seeds in the
51 amounts above the limit recommended by WHO and FAO (Gernah et al., 2011). The pKa of cyanide
52 is 9.3; it is therefore present largely as HCN at neutral pH values (Eisler and Wiemeyer, 2004). HCN is
53 volatile (boiling point 26°C) and is less dense than air. Hence, cyanide formed by microbial cultures
54 will be rapidly lost to the environment. Cyanide is largely toxic for aerobic cell metabolism as it binds to
55 the mitochondrial cytochrome oxidase a3 enzyme; binding of cyanide to the ferric ion of the
56 cytochrome oxidase a3 inhibits the terminal enzyme in the respiratory chain and halts the electron
57 transport and oxidative phosphorylation, which subsequently leads to intracellular hypoxia (Hall,
58 2007).

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

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

74 Diazotrophic cyanobacteria have been linked with hydrogen cyanide, based on their ability to
75 catabolize it by nitrogenase, an enzyme normally responsible for the reduction of N_2. Nitrogenase can
76 also use hydrogen cyanide instead of its normal substrate, dinitrogen and convert it to methane and
77 ammonia (Gantzer and Maier, 1990). The N_2-fixing cyanobacterium, *Anabaena* is able to biodegrade
78 cyanides and produce CH_4 in batch reactors. Gantzer and Maier (Gantzer and Maier, 1990) showed
79 that *Anabaena* reduced cyanides by nitrogenase to CH_4 and NH_3. The rate for CH_4 production was
80 ten times faster than expected based on literature. However, in these cases, the assumption was
81 made based on induced HCN in batch reactor.

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

88 2. Materials and Methods

89 *2.1 Cyanobacterial Strains*

90 Seventy-eight (78) strains of cyanobacteria, representing five cyanobacteria orders, with
91 different morphological features and a wide variety of habitats and lifestyles, were used in this study
92 (Table S1). Twenty-nine strains were isolated from different freshwaters of Greece (Gkelis et al.,
93 2019), nine of them were isolated from sponges (Konstantinou et al., 2018), whereas the rest 40
94 strains were isolated in this study from various environments (terrestrial caves, coastal areas, thermal
95 springs, brackish and freshwater systems) across Europe, between 2013 and 2018. Strains were
96 isolated on solid growth media using classical microbiological techniques (see (Gkelis et al., 2005,
97 2015)), purified by successive transfers and using antibiotics (such as cycloheximide and ampicillin)
98 as described in Rippka (Rippka, 1988); all strains were derived from a single colony or trichome. The
99 cultures were grown as batch clonal unialgal cultures in BG11, with or without (for the nitrogen-fixing
100 strains) nitrogen, and MN medium (Rippka, 1988). All strains are deposited in the Thessaloniki
101 Aristotle University Microalgae and Cyanobacteria (TAU-MAC) culture collection (Gkelis and Panou,

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102 2016) and maintained at 20 ±1°C or 25 ±1°C (for strains of the genera *Desertifilum* and *Calothrix*) with
103 a light intensity of 25 μmol $m^{-2}s^{-1}$ and with a light/dark cycle of 12:12h.
104

105 *2.2 Cyanogenesis analysis*

106 In order to assess the ability to produce HCN and estimate the relative frequency of cyanogenesis
107 (proportion of cyanogenic versus acyanogenic cyanobacteria strains), we screened each strain for the
108 presence of HCN using the Feigl-Anger assay, which determines the presence or absence of HCN in a
109 sample using a colour change reaction (Gleadow and Møller, 2014). In this assay, the dried paper turns
110 blue following oxidation of the tetra base when it is exposed to the HCN gas that is produced. For the
111 extraction of HCN, we modified the original protocol (Thompson et al., 2016), implemented in plants and
112 includes freeze-thaw, as freeze-thaw is not an efficient method for cell lysis in cyanobacteria (Kim et al.,
113 2009). Cyanobacteria cells were harvested at the exponential growth phase (between 30-45 days of
114 growth) by centrifugation of 1.5 mL culture material. The supernatant was removed and the pellet was
115 dissolved in 0.8 mL of Lysis Buffer (2% w/v Cetyl trimethylammonium bromide, 100 mM Tris-HCl, 1.4 M
116 NaCl, 1% w/v Polyvinylpyrrolidone, 20 mM, Na_2EDTA 0.2% w/v LiCl, pH 8). The same procedure was
117 repeated with the addition of dH_2O instead of Lysis Buffer. A 96-well plate was filled with 80 μL of
118 cyanobacteria isolates extract to each well. We secured Feigl-Anger test paper over the plates,
119 incubated the plates for 1.5 h at 37 °C, and then scored each well for cyanide, which is indicated by a
120 blue colour (Figure S1). A permanent record of the detection paper was made by scanning the paper
121 immediately after exposure because the blue color fades with time. As positive control, 4 cm^2 of leaf
122 tissue of a cyanogenic individual of the plant *Trifolium repens* (Deligiannis et al., 2018) was used.

123 *2.3 Nitrogen fixation capability*

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

130 *2.4 Polyphasic taxonomy*

131 The cyanobacteria isolates, which were positive for the production of HCN, were characterised
132 based on their morphology and their phylogeny as described in Gkelis et al. (Gkelis et al., 2019).
133 Briefly, strains were identified based on their morphology using the taxonomy books by Komárek and
134 Anagnostidis (Anagnostidis and Komarek, 1988; Komarek and Anagnostidis, 1989; Komárek and
135 Anagnostidis, 2008) and Komárek (Komárek, 2013). The phycocyanin operon and the internal
136 genetic spacer (*cpcBA*-IGS), 16S rRNA gene, and the 16S-23S rRNA internal transcribed spacer
137 (ITS) were used to assess the molecular phylogeny of the strains. PCR was carried out on using the
138 primer pairs shown in Table S2 and PCR conditions described in detail in Gkelis et al. (Gkelis et al.,
139 2019). Sequence data were obtained by capillary electrophoresis (GENEWIZ, Takeley, UK). The
140 obtained nucleotide sequences were edited with Unipro UGENE 1.29.0 (Okonechnikov et al., 2012).
141 Nucleotide sequences were deposited in GenBank database of the National Center for Biotechnology
142 Information (NCBI) (Table S3). Sequences were blasted and the closest relative(s) for each
143 sequence were included in the phylogenetic trees. For the phylogenetic analyses, we selected
144 sequences (>1200 and >600 bp, for 16S-23S rRNA and *cpcBA*-IGS, respectively) in order to examine
145 phylogenetic position of our strains. The phylogenetic analyses were conducted with Mega (V7.0)
146 software (Kumar et al., 2016). Multiple sequence alignments were conducted using the CLUSTALW
147 software. All missing data and gaps were excluded from the analysis by choosing the complete
148 deletion option. A consensus phylogenetic tree were constructed using maximum likelihood (ML).
149 The best fitting evolutionary models for the ML analyses were the Tamura 3-parameter + G model for
150 all the targets analysed. Bootstrap replicates (n=1,000) were performed. Phylogeny was also inferred
151 with Bayesian Inference (BI) phylogenetic approach with MrBayes (V3.2.6) software (Ronquist et al.,
152 2012). The general time-reversible (GTR) with gamma distribution of rates and a proportion of

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153 invariable sites evolutionary model was selected by applying PAUP* (V5.0) (Swofford, 2002).
154 Bayesian analysis consisted of two independent Markov Chain Monte Carlo runs, performed by four
155 differentially heated chains of 10 x 10^6 generations and trees were sampled from the chain every
156 1000 generations. All phylogenetic trees were visualized via the FigTree (V1.4.3) software
157 (Rambaut).

158 **3. Results**

159 *3.1 HCN Production*

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

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

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

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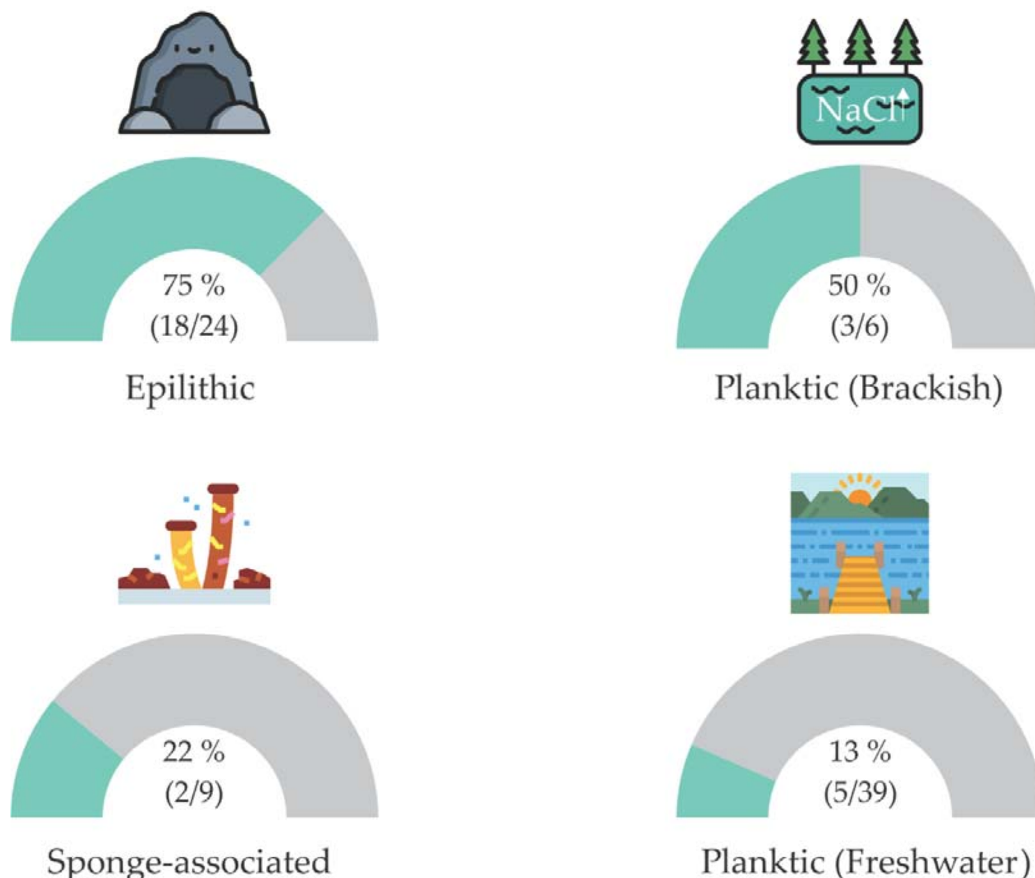
	Cave)		
Radiocystis sp. TAU-MAC 1214	Planktic (Freshwater)	Lake Karla, Greece	This study
Scytonema hyalinum TAU-MAC 2618	Epilithic (Terrestrial Cave)	Grava, Greece	This study
Sphaerospermopsis aphanizomenoides TAU-MAC 1314	Planktic (Freshwater)	Lake Karla, Greece	This study
Synechococcus sp. TAU-MAC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

171



172

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 *Geilteria* sp.
186 sequences, whilst *Alliinostoc* sp. TAU-MAC 3117 was placed separately, outside two uncultured
187 *Alliinostoc* sp. sequences. *Komarekiella* strains, isolated from a show cave in Chios Island in NE

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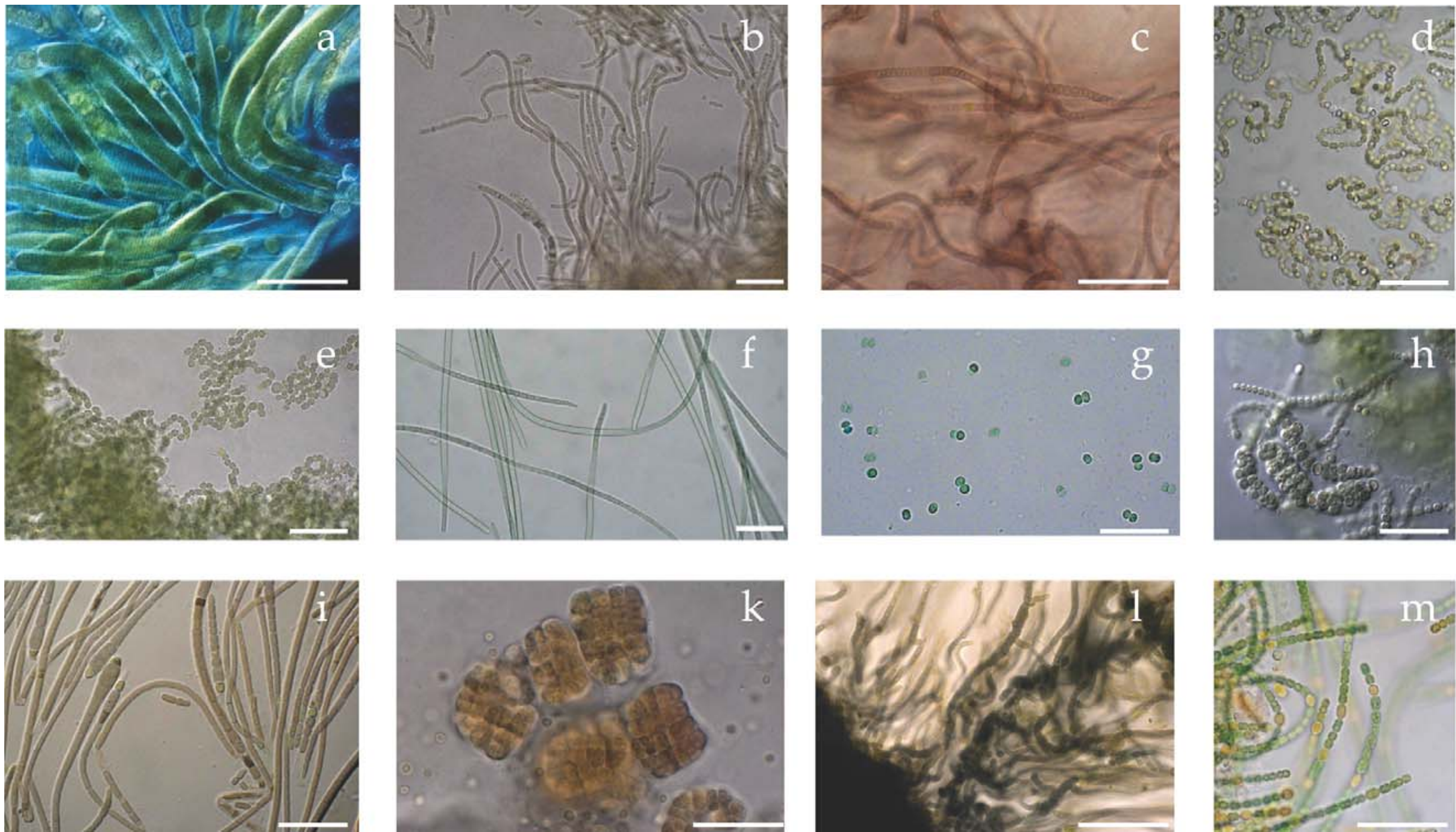
188 Aegean Sea, clustered together, separately of two *Komarekiella atlantica* strains, isolated from a
189 tropical rainforest in Hawaii.

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.



195
196
197 **Figure 2.** Microphotographs of strains, isolated in this study, representing 12 genera of cyanobacteria strains capable of producing HCN. (a) *Gloeotrichia echinulata*
198 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.

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201 .

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

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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 stoicheiometry 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- Caldehyde, imidazole4carboxylic 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_2 and supplemented with Mn^{2+} 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_2 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 nitride 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_2-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

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261 epilithic mats, found to be more capable of HCN production compared to strains from aquatic
262 ecosystems. Since cyanogenesis is a defence mechanism widely distributed in the plant kingdom and
263 present in many major crop species (Thompson et al., 2016; Alberti et al., 2017), our results could
264 possibly imply an unidentified chemical cue, released by different grazers, that triggers cyanobacteria
265 to use HCN as a defence mechanism. Indeed, in environments where cyanobacteria are vulnerable
266 to grazers such as rotifers or ciliates a rapid defence system should be favoured evolutionary (Wolfe,
267 2000). Even though no direct observations of activated chemical systems in unicellular organisms
268 have been made, there are several examples of activated microbial defence reactions, which might
269 serve as conceptual models for such systems (Mazard et al., 2016). One cannot exclude that physical
270 contact with a grazer might stimulate cyanobacteria cells to produce compounds with a potential
271 defensive role. Yang et al. (Yang and Kong, 2012) observed that the cyanobacterium *Microcystis*
272 *aeruginosa* remaining under *Ochromona*s grazer pressure not only created colonies but also
273 increased the amount of produced exopolysaccharides.
274 Cyanobacteria that thrive under extreme and diverse conditions tend to accumulate more
275 compatible solubles such as sucrose, trehalose, glucosylglycerol, and glycine (Soule and
276 Garcia-Pichel, 2019). However glycine accumulation could be toxic for the cyanobacterium and
277 should be moderated (Eisenhut et al., 2007). Castric et al. (Castric, 1983)suggested that
278 cyanogenesis is a response to a build-up of the intracellular glycine concentration. The key primary
279 metabolic enzymes are serine hydroxymethyltransferase and glycine cleavage enzyme which
280 catalyse conversion of serine into glycine and glycine into CO_2 and ammonia, respectively. This could
281 be the link between HCN production and cyanobacteria that pose a non planktic lifestyle.

282 *4.2 Biodiversity*

283 The polyphasic taxonomy applied to the strains of this study revealed taxa known to be part of
284 bloom-forming communities (*Sphaerospermopsis* and *Nodularia),* rock-dwelling communities
285 (*Scytonema* and *Tolypothrix*), and hot spring cyanobacteria mats (*Desertifilum* and *Gloeotrichia*)
286 (Dadheech et al., 2014; Mazur-Marzec et al., 2015; Gkelis et al., 2017; Joanna and Andrzej, 2018).
287 Our results revealed the presence of taxa not previously described from Greek habitats (Gkelis et al.,
288 2016), such as *Allinostoc* (Saraf et al., 2018) and *Oculatella* (Osorio-Santos et al., 2014) and taxa
289 previously described only from the tropical zone like *Komarekiella* and *Kovacikia* (Miscoe et al., 2016;
290 Hentschke et al., 2017). Furthermore, the *Geitleria calcarea* strain isolated in this study, to the best of
291 our knowledge, is the first isolate in world's cyanobacteria culture collections depositories
292 (Friedmann, 1979; Coute, 1989), whilst *Chlorogloea* sp. TAU-MAC 0618 consists the first bacteria
293 isolate from an anchialine type environment.
294 The strains TAU-MAC 0817 and 3417 isolated from two brackish environments belong to
295 species *Nodularia harveyana* and *Nodularia spumigena*, respectively. These strains constitute the
296 first isolates of *Nodularia* strains in Greece, whilst in the Mediterranean there are only five strains of
297 *Nodularia* isolated from Turkey (Akcaalan et al., 2009). The scarce records of *Nodularia* across
298 Mediterranean could be linked with the absence of a high number of brackish environments.
299 Anagnostidis (Anagnostidis, 1968)**,** decades ago, reported the occurrence of *N. spumigena* and *N.*
300 *harveyana* in Greece, (Gkelis et al., 2016). The largest research activity on the genus *Nodularia*
301 occurs on the Baltic Sea, where *Nodularia spumigena* forms highly toxic blooms with significant
302 effects on aquatic and non-aquatic organisms (Sivonen et al., 1989; Finni et al., 2001; Mazur-Marzec
303 et al., 2015). Strain TAU-MAC 1517, isolated from a thermal site in Iceland was classified to the
304 species *Desertifilum tharense* in both phylogenetic and morphological analysis, exhibiting the
305 trichome's "anchored" end, that discriminates it from *Microcoleus* and *Geitlerinema* (Dadheech et al.,
306 2014)**.** *Desertifilum tharense* has been recorded in India, Kenya, Mexico, Greece, Mongolia, and
307 China (González-Resendiz et al., 2019). The presence of *Desertifilum tharense* in Iceland, a different
308 ecological niche, supports the theory that despite its wide ecological span, the genus *Desertifilum*
309 remains genetically stable (Sinetova et al., 2017; González-Resendiz et al., 2019).
310 Several strains of this study, such as *Geitleria calcarea*, *Kovacikia muscicola*, *Komarekiella* sp.,
311 *Scytonema hyalinum*, and *Tolypothrix* sp. were isolated from extensive dark-green coverings
312 dominated by cyanobacteria like *Phormidium*, *Tolypothrix, Scytonema*, and *Geitleria* species.
313 Cyanobacteria are considered the pioneering inhabitants in cave colonization (Joanna and Andrzej,

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314 2018). They prevail in the cave entrances compared to the other microalgae (Mulec and Kosi, 2009)
315 by colonizing various parts of the cave entrances, where biodiversity is the lowest (Vinogradova N. et
316 al., 1998). Cyanobacteria represent the first photosynthetic colonizers on the calcareous surfaces
317 usually thriving both as epiliths and as endoliths (Lamprinou et al., 2009). Lamprinou et al.
318 (Lamprinou et al., 2013) observed predominance of Oscillatoriales group over Chroococcales in
319 caves; our results show that also many Nostocales species form part of the cave communities,
320 several of which, are understudied. For example, the *Komarekiella* sp. strains we isolated here are
321 reported for the first time as cave inhabitants. Hentschke et al. (Hentschke et al., 2017), based on
322 intensive examination of species life cycle, proposed the new genus *Komarekiella* and classified the
323 strains from both Hawaiian and Brazilian rainforests in the single *Komarekiella atlantica* species. Our
324 phylogenetic analysis of 16-23S and *cpcBA*-IGS sequences, as well as the different climatic zone and
325 habitat, suggests that strains TAU-MAC 0117 and 0217, may belong to a new taxon, thus further
326 research is required to describe it. Concerning *Geitleria calcarea* TAU-MAC 0118, as in the original
327 description of the species, the most obvious finding is its apparent inability to produce heterocytes
328 naturally (Coute, 1989). In our phylogenetic analysis, *Geitleria calcarea* TAU-MAC 0118 clustered
329 with the only two available *Geitleria* sequences in GenBank, belonging to a non-cultured *Geitleria*
330 species, derived from a population genetics study.
331 Terrestrial meteoric water mixed with saline groundwater resembles in a two-layered circulation,
332 in estuaries termed as subterranean estuaries or anchialine (meaning near the sea) environments
333 (Moore, 1999). *Chlorogloea* sp. TAU-MAC 0618 consists, to the best of our knowledge, the first
334 bacterium isolate from this type of environments, as the research concerning bacteria, is focused
335 mainly in community analysis, either by metagenomics (Brankovits et al., 2017), or fluorescence
336 microbial profiling analysis (Seymour et al., 2007; Krstulović et al., 2013).
337

338 **5. Conclusions**

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

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

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372

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