1 **Cyanobacterial diversity and related sedimentary facies as a function of water flow**

2 **conditions: Example from the Monasterio de Piedra Natural Park (Spain)**

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16 **ABSTRACT**

17 The River Piedra in the Monasterio de Piedra Natural Park (NE Spain) is a modern tufa-

18 depositing river that encompasses various depositional environments that are

19 inhabited by different cyanobacterial populations. Molecular (16S rDNA) and

20 morphological analyses of the cyanobacteria from different facies showed that

21 *Phormidium incrustatum* dominates in the fast-flowing water areas where the mean

22 depositional rate is 1.6 cm/year. Stromatolites in these areas are formed of palisades

23 of hollow calcite tubes (inner diameter of 6.0-7.5 μm, walls 2-12 μm thick) that formed

24 through calcite encrustation around the filaments followed by decay of the trichomes.

25   In contrast, in slow-flowing water areas with lower depositional rates (mean

26   depositional rate of 0.3 cm/yr), *Phormidium aerugineo-caeruleum* is the dominant

27   species. In these areas, randomly oriented calcite tubes (inner diameter of 5-6 μm,

28   walls 3-8 μm thick) formed by calcite encrustation, are found in thin and uneven

29   laminae and as scattered tubes in the loose lime mud and sand-sized carbonate

30   sediment. Although this species did not build laminated deposits, it gave cohesiveness

31   to the loose sediment. In the stepped and low waterfalls, with intermediate deposition

32   rates (mean depositional rate of 0.9 cm/yr), both species of *Phormidium* are found in

33   association with spongy moss and algal boundstones, which is consistent with the

34   variable flow conditions in this setting.

35   The calcite encrustations on the cyanobacteria from different environments

36   exhibit irregular patterns that may be linked to changes in the calcite saturation index.

37   The physicochemical conditions associated with extracellular polymeric substances

38   may be more significant to $CaCO_3$ precipitation in microbial mats in slow-flowing water

39   conditions than in fast-flowing water conditions. These results show that flow

40   conditions may influence the distribution of different cyanobacteria that, in turn, leads

41   to the development of different sedimentary facies and structures in fluvial carbonate

42   systems.

43

44   **Key words:** Cyanobacterial diversity, fluvial tufa facies, varied depositional settings,

45   recent carbonate sedimentation

46

47

1. Introduction

The roles that climatic, hydrological variables, and microbial communities play in the development of the sedimentary record in modern fluvial carbonate systems have been the focus of many studies (e.g., Janssen et al., 1999; Shiraishi et al., 2008; Pedley et al., 2009; Gradziński, 2010; Vázquez-Urbez et al., 2010; Manzo et al., 2012). In fluvial carbonate environments (Arp et al., 2001; Pentecost, 2005; Santos et al., 2010; Beraldi-Campesi et al., 2012) there is typically a high diversity of bacteria that commonly mediate the development of various organosedimentary structures, including stromatolites and oncolites.

Filamentous and coccoid cyanobacteria, which are photosynthetic prokaryotes that can live in a wide range of environments, play a major role in the growth and development of stromatolites, as recognized in both modern (Reid et al., 2000; Arp et al., 2001; Santos et al., 2010; Shiraishi et al., 2010) and fossil examples (Awramik, 1991; Golubić et al., 2000; Riding, 2000). It has been suggested that cyanobacteria have become the most successful mat-building organisms, possibly because they can photosynthesize even under extremely low light conditions (Schopf, 2012). Their role in microbialite formation, however, is not fully understood and the relationship between cyanobacterial communities, environmental conditions, and the resulting structures is poorly known.

Actively forming tufa found in various fluvial environments in the River Piedra (Fig. 1) has been the focus of a thirteen-year study of modern sedimentation. The dominant facies are (1) dense laminated tufa, (2) loose lime mud and sand that commonly lack laminations, and (3) spongy, moss- and alga-bearing tufa that is either coarsely laminated or non-laminated (Arenas et al., 2014). Modern fluvial tufa systems

72   in NE Spain, including those in the River Piedra, are characterized by a high diversity of

73   bacteria with cyanobacteria being dominant (approximately 43%; Beraldi-Campesi et

74   al., 2012). In the River Piedra, cyanobacterial mats are found in variably flow conditions

75   that include fast- and slow-moving water. The different attributes of the structures

76   found in these contrasting flow conditions are, however, unknown. Thus, the main aim

77   of this paper is to compare the cyanobacterial diversity and related sedimentary facies

78   that develop in these contrasting flow conditions. This is achieved by examining the

79   (1) dominant cyanobacterial populations in each of the main depositional

80   environments found in the river, (2) cyanobacterial structures that developed on

81   artificial substrates over a period of 13 years, and (3) calcification of the cyanobacteria.

82   Integration of this information provides an assessment of the relationships between

83   the environmental parameters and the cyanobacterial structures that are evident in

84   the tufa. As far as we are aware, this is the first study in which morphological and

85   phylogenetic (16S rDNA) analyses have been used to determine the relationship

86   between different depositional structures and calcification style of the dominant

87   cyanobacterial constituents, relative to the physical and chemical attributes of the

88   fluvial system. As such, the results have important implications for similar depositional

89   systems throughout the world.

90

91   **2. Geological setting, hydrology and climate**

92   This study, conducted in the Monasterio de Piedra Natural Park (NE Spain),

93   focused on the lower reaches of the northward flowing River Piedra, which is a

94   tributary of the River Ebro (Fig. 1A). From its headwaters to its mouth in the La

95 Tranquera Reservoir, the river flows across Mesozoic and Cenozoic carbonates and

96 siliciclastics (Fig. 1A), and Quaternary tufa deposits.

97 The River Piedra is fed mainly by an aquifer that is located in Lower Jurassic and

98 Upper Cretaceous limestones and dolostones. The most important natural springs,

99 with a flow of ~ 1.4 m^3/s, are located near the village of Cimballa (Fig. 1A). The mean

100 river discharge is ~ 1.06 m^3/s (measured downstream from the Natural Park between

101 October 1999 and September 2012; data from *Confederación Hidrográfica del Ebro*,

102 https://195.55.247.237/saihebro). Water in the River Piedra is of the HCO_3-(SO_4)-Ca

103 type. Downstream of the springs at Cimballa, the water has a conductivity of 503–734

104 µS/cm, alkalinity of 238–350 mg/L, Ca concentrations of 75–112 mg/L, SO_4

105 concentrations of 54–157 mg/L, and pH of 7.7–8.5 (data from analyses from 1999 to

106 2012; Arenas et al., 2014).

107 The climate of the area is continental Mediterranean with strong seasonal

108 contrasts in temperature and precipitation. Between October 1999 and September

109 2012, mean annual air temperature was 13.1°C. Air temperature was highest in July

110 and August (monthly mean values of 21.7–25.0°C), and lowest between December and

111 February (monthly mean values of 2.4–7.0°C). Water temperature ranged from 16.5 to

112 17.7°C in July and August to 9 to 10°C in December and January. During the same

113 period, mean annual rainfall was 397.4 mm (based on data from the La Tranquera and

114 Milmarcos meteorological stations), with maxima in April, May, and October (air

115 temperature and precipitation data from *Agencia Estatal de Meteorología*).

116 Close to the Monasterio de Piedra Natural Park, the gradient of the River Piedra

117 becomes steeper than in upstream reaches. In the park, caves have formed behind the

118 Caprichosa and Cola de Caballo waterfalls that have vertical drops of 15 and 35 m,

119 respectively (Fig. 1B). Other fluvial features are (1) rapids, (2) slow-flowing water areas

120 that have formed upstream of small waterfalls, and (3) small waterfalls along the river

121 (in places damming water upstream) and several stepped waterfalls (5 to 10 m high)

122 that have developed on the riversides.

123 **3. Terminology**

124 Given the diversity of depositional settings in the fluvial system, the term

125 "environment" herein refers to areas that are characterized by distinct physical and

126 biological attributes (e.g., physical flow characteristics, morphological features of the

127 river bed, biota). Facies refer to different types of sediments that are defined by their

128 textural components and their sedimentary structures.

129 The term "microbialite" is used *sensu* Burne and Moore (1987, pp. 241-242) to

130 designate "…organosedimentary deposits that have accreted as a result of a benthic

131 microbial community trapping and binding detrital sediment and/or forming the locus

132 of mineral precipitation". Laminated microbialites that grow on the sediment surface

133 are termed stromatolites (cf., Riding, 1991).

134 A biofilm consists of a microbial community that is embedded in extracellular

135 polymeric substances (EPS) (Rosenberg, 1989; Neu, 1996; Decho, 2010). Typically, the

136 EPS is a hydrogel that allows microbes to attach themselves to substrates while

137 buffering them from the immediate extracellular environment (Decho, 2010).

138 Krumbein et al. (2003, pp. 13) considered that "microbial mats are intimately

139 interwoven microbial communities including laminated, concentric and network like

140 growth patterns, which by their upward directed growth, physical and chemical

141 gradients, barriers and sticky EPS products trap and embed mineral grains, produce

142 new minerals and, ultimately, laminated and spherulitic sedimentary rocks and

143   structures". Microbial mats involve stratification of the microbial populations into

144   several layers. They may therefore be considered as complex biofilms (Stolz, 2000). Arp

145   et al. (2001, 2010) considered that tufa stromatolites result from calcification of

146   cyanobacterial-dominated biofilms. Herein, the term "microbial/cyanobacterial mat"

147   is used in a general sense and refers to microbial/cyanobacterial populations that coat

148   the substrate, independent of the complexity of their internal structure.

149   **4. Materials and methods**

150   *4.1. Sample collection and related parameters*

151   Samples for cyanobacterial analysis were collected in September 2010 from eight

152   sites in the Monasterio de Piedra Natural Park (Fig. 1B). These sites included three

153   different fluvial environments (Figs. 2A, 3A, 4A) with that include facies A, B, and C

154   (Table 1). At each site, samples were taken from the uppermost surface of the

155   deposits. Part of each sample was fixed in 4% formaldehyde for microscopic

156   observation, and two other parts were kept at low temperature during transport to

157   the laboratory before being frozen prior to genetic analyses.

158   Data for each sampled site came from a comprehensive study of the River Piedra

159   that took place between 1999 and 2012. Water velocity and depth were measured

160   every three months (at the end of each season), and various hydrochemical

161   parameters were measured every six months (end of December or beginning of

162   January, and end of June), following the procedures outlined in Arenas et al. (2014).

163   Deposition rates were determined from the sediment that accumulated on the

164   artificial substrates (limestone tablets, 25 x 16 x 2 cm) that had been placed in the river

165   between 1999 and 2012. Sediment thickness on the tablets was measured at the end

166   of March and the end of September, so that deposition rates corresponded to six-

167   month periods (April–September: warm period; October–March: cool period). Once

168   removed, the tablets were cut perpendicular to the accumulation surface, and the six-

169   month intervals were identified on the cross-sections by plotting the successive

170   thickness measurements on the corresponding raw cuts (see procedure details in

171   Vázquez-Urbez et al., 2010 and Arenas et al., 2014). These data include the six-month

172   period (April–September 2010) that is the focus of this study (Tables 1, 2).

173

174   *4.2. Laboratory analyses*

175   The structures and textures of the carbonate deposits that formed on the tablets

176   were documented by thin section and scanning electron microscope (SEM) analyses in

177   the Servicio de Apoyo a la Investigación (SAI) facilities of the University of Zaragoza

178   (Spain) and University of Alberta (Canada). Such analyses provided critical information

179   on the calcification structures, size and shape of crystals, and other components that

180   collectively control the textures of the carbonates. Samples (up to approximately 1.5 x

181   1 x 0.5 cm) were selected for SEM analyses from deposits that corresponded to

182   different six-month periods. The samples were coated with gold or carbon. Common

183   working conditions were 3-5 kV and 150-500 pA. SEM analyses were done on a JEOL

184   JSM 6400 scanning electron microscope (SEM) (JEOL Limited, Tokyo, Japan) and Carl

185   Zeiss MERLIN^{TM} (Carl Zeiss Group, Jena, Germany).

186

187   *4.3. Morphological characterization of cyanobacteria*

188   The morphology of the cyanobacteria in the collected samples were imaged using

189   an Olympus BH2-RFCA photomicroscope equipped with phase-contrast,

190   epifluorescence and video camera systems (Leica DC Camera; Leica Microsystems).

191   Morphological identifications follow Komárek and Anagnostidis (1998, 2005) and

192   Whitton (2011). Their percentage abundance in the samples was evaluated by

193   counting the presence of each species (as cells in a filament or as equal numbers of

194   individual cells) as a percentage of all cells counted.

195

196   *4.4. DNA extraction, amplification of the 16SrRNA gene, cloning and sequencing*

197   Genomic DNA from field samples was extracted following a modification of a

198   technique for isolating DNA from fresh plant tissue that utilizes

199   cetyltrimethylammonium bromide (CTAB), as described by Berrendero et al. (2008).

200   The 16S rRNA gene sequences were amplified from the genomic DNA using primers pA

201   (Edwards et al., 1989) and cyanobacteria-specific B23S (Lepère et al., 2000).

202   Amplifications by polymerase chain reaction (PCR) were performed in a 25-µl reaction

203   volume, following the method of Berrendero et al. (2008), under the conditions

204   described by Gkelis et al. (2005). PCR products were cloned into pGEM-T vectors using

205   the pGEM-T Easy Vector system (Promega), in accordance with the manufacturer's

206   recommendations, and transformed into DH5α chemically competent *Escherichia coli*.

207   Clones were screened for inserts by PCR amplification with the aforementioned

208   primers. Correct-sized amplified products were purified using the Real Clean Spin kit

209   (Real) and sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit in the ABI

210   Prism 3730 Genetic Analyzer (Applied Biosystems), according to the manufacturer's

211   instructions. Sequences were obtained for both strands independently. Clones are

212   named after the sampling site and the number of the selected transformed colony.

213

214   *4.5. Analysis of nucleotide sequence data*

215   Nucleotide sequences obtained from DNA sequencing were compared with

216   information available from the National Center for Biotechnology Information

217   database using the Basic Local Alignment Tool (BLAST)

218   (http://www.ncbi.nlm.nih.gov/BLAST). Taxonomic identity was assigned to sequences

219   based upon the sequence identity matches on BLAST. Each sequence was checked for

220   identification-anomalous 16S rRNA gene sequences with the DECIPHER´s Find

221   Chimeras web tool (Wright et al., 2012;

222   http://decipher.cee.wisc.edu/FindChimeras.html).

223   For the phylogenetic analysis, the 16S rDNA sequences of unicellular and non-

224   heterocystous cyanobacteria most closely related to the sequences obtained and

225   longer than 1000 bp were downloaded from GenBank, where available. These, our

226   own sequences, and that of an outgroup taxon were aligned by MAFFT v. 6 (Katoh and

227   Standley, 2013; http://mafft.cbrc.jp/alignment/server/). Alignments were manually

228   corrected to remove ambiguous sites with the BioEdit program (Hall, 1999).

229   The alignment was submitted to FindModel (http://www.hiv.lanl.gov), which

230   determined that the general time reversible (GTR) model with a gamma distribution of

231   rate variation was the most appropriate model (Tavare, 1986). Phylogenetic trees were

232   generated using the MEGA 6.0 program (Tamura et al., 2013).

233   For maximum likelihood (ML) analysis, the GTR model was selected, assuming a

234   discrete gamma distribution with four categories of site-to-site variability of change

235   with the nearest-neighbor-interchange algorithm. Distances for the neighbor-joining

236   (NJ) tree were estimated by the algorithm of Jukes and Cantor (1989); nucleotide

237   positions containing gaps and missing data were initially retained for all such sites in

238   the analyses, and then excluded as necessary in the pairwise distance estimation

239   (pairwise deletion option). One thousand bootstrap replicates were run to evaluate the

240   relative support of branches in all analyses; bootstrap values greater than 50% were

241   indicated at the nodes of the trees.

242   **5. Depositional environments and sedimentary facies**

243   The fluvial environments in the study area (Figs. 2-4), distinguished by the riverbed

244   geometry, physical flow characteristics (e.g., water velocity, depth), and floral and

245   bacterial associations (Vázquez-Urbez et al., 2010; Arenas et al., 2014), are divided

246   into: (1) fast-flowing water areas (Fig. 2A), (2) slow-flowing water areas (Fig. 3A), (3)

247   stepped waterfalls and small waterfalls, 1 to 10 m high (Fig. 4A), (4) vertical waterfalls

248   (15 to 35 m high) with moss and other macrophytes, and (5) spray and splash areas

249   near the waterfalls. The first three environments, targeted for cyanobacterial analyses

250   (Table 1; Figs. 2-4) because they are the largest and easiest areas to access, include

251   three dominant facies (A, B, and C).

252   • **Facies A** (stromatolites) consists of dense laminated deposits that developed

253   from cyanobacterial mats. Deposits that formed on the tablets were well

254   laminated, with laminae up to 6 mm thick (Fig. 2C, D). Two to five laminae

255   formed over a six-month interval.

256   This facies formed in the gentle- to steep-sloped areas with fast-flowing

257   water (velocity > 90 cm/sec) that lack macrophytes (Fig. 2A). They are, however,

258   covered by well-developed microbial mats that form brown to gray, hard surfaces

259   with smooth to knobby topographies (Fig. 2B). Facies A is also found in zones of

260   strong flow associated with the stepped waterfalls and small waterfalls (Fig. 4),

261   where the corresponding microbial mats are not extensive and give rise to

262   stromatolite interbeds within the dominant macrophyte deposits that constitute

263   facies C.

264   • **Facies B** consists of lime mud, sand-sized carbonate particles, diatoms,

265   macroscopic algae, scattered oncoids and intraclasts, along with uneven

266   interbeds of stromatolites. Deposits on the tablets, characterized by poorly

267   defined or no laminations (Fig. 3D, E), consist of loose sediment that includes

268   very thin and uneven laminae consisting of cyanobacterial calcite that form thin,

269   discontinuous interbeds of stromatolites (Fig. 3D).

270   This facies developed in the slow-flowing water areas (velocity < 80 cm/s)

271   where large patches of soft, greenish to gray microbial mats lie on the sediment

272   (Fig. 3A-C).

273   • **Facies C** is formed of spongy moss and alga boundstones. The deposits on the

274   tablets are formed largely of moss and macroscopic algae (probably *Vaucheria*

275   and *Cladophora*) that are coated by calcite, and include rare, very thin and

276   discontinuous irregular cyanobacterial calcite masses, and form spongy

277   boundstones (Fig. 4C, D).

278   Facies C is dominant around the stepped waterfalls and small waterfalls

279   (Fig. 4A). This environment includes fast- and moderate- to slow-flowing water

280   areas. On the surface, the microbial mats in facies C develop as soft, greenish to

281   gray, poorly calcified patches (Fig. 4B), which are limited in extent by growth of

282   the dominant macrophytes. Centimeter-thick, dense stromatolite layers (Facies

283   A), formed in zones of stronger flow in the waterfalls, may be interbedded with

284   the spongy boundstones (Fig. 4C, D).

285   Textural analysis of the deposits on the tablets showed that they are largely

286   biological substrates (e.g., grasses, mosses, algae and bacteria) that are coated with

287   calcite. Calcite impregnation of biological substrates (e.g., cyanobacterial filaments) is

288   less common. The matrix between these components is usually a heterogeneous mass

289   of calcite crystals, diatoms and tufa fragments, along with extracellular polymeric

290   substances (EPS).

291

292   **6. Results**

293   *6.1. Cyanobacterial phylotypes and corresponding morphotypes*

294   *6.1.1. Phylogenetic assignments*

295   Analysis of the amplified and cloned environmental DNA revealed 56 good-quality

296   sequences of approximately 1250 bp (Fig. 5). Comparison with the GenBank archival

297   database indicated that most belonged to the order Oscillatoriales (Table 3) with

298   several species of *Phormidium*. One sequence showed a high percentage of similarity

299   with the 16S rDNA sequence of the unicellular cyanobacterium *Chamaesiphon*

300   *subglobosus* PCC 7430.

301   The phylogenetic tree, constructed together with 43 cyanobacterial sequences

302   from the public database Gen Bank public domain, showed that the retrieved

303   sequences belong to four distinct clusters, although most of the sequences are in

304   clusters I and III (Fig. 5). Cluster I, which is the largest, is formed of 36 sequences that

305   were found from all sampling sites (Phylotype I). In addition, three sequences from

306   various *Microcoleus* species, and several *P. autumnale* sequences from the database

307   belong to this group. Cluster II consists of two sequences obtained in this study

308   (Phylotype II) and a freshwater strain of cf. *Wilmottia* from Canterbury (New Zealand),

309 which received strong bootstrap support. Cluster III includes 16 sequences from three

310 out of the seven sampling sites (Phylotype III) together with two environmental

311 sequences of *P. aerugineo-caeruleum* from other Spanish rivers (Loza et al., 2013).

312 Phylotype IV, composed by a sequence from this study and other freshwater

313 *Chamaesiphon* sequences from the database, corresponds to a *Chamaesiphon* cluster.

314

315 *6.1.2. Morphological analysis*

316 The morphological identification of the tufa-forming cyanobacteria by optical

317 microscopy (from *in situ* surface samples) showed that most of them are members of

318 the Oscillatoriales group (Fig. 6, Table 4).

319 *Phormidium incrustatum* (Naegeli) Gomont ex Gomont 1892 (Fig. 6A, B) and *P.*

320 *aerugineo-caeruleum* (Gomont) Anagnostidis et Komárek 1988 (Fig. 6C) are the

321 dominant morphospecies. *P. incrustatum* is characterized by simple, cylindrical,

322 isopolar and non-branched filaments that form irregular clusters or dark blue-green

323 colonies with more or less parallel-oriented trichomes. The filaments, with a thin

324 sheath and unconstricted trichomes, have cells 6-8 μm-wide, and attenuated trichome

325 tip with weakly conical terminal cell.

326 *P. aerugineo-caeruleum* (Gomont) (Fig. 6C) is characterized by a dark blue-green

327 thallus or solitary, scattered filaments. The variously curved unbranched filaments are

328 encased by non-lamellated colorless sheaths. Trichomes, which are cylindrical,

329 unconstricted and not attenuated at the ends, are composed of cells 5.5-7.0 μm wide

330 and up to half as long as wide or almost isodiametric. The broadly rounded apical cells

331 lack calyptra. The cell content is granulated.

332   A third morphotype, *Phormidium* sp., is also present in the studied samples. This

333   morphotype is characterized by straight, unconstricted trichomes, 3.5-4.0 μm in

334   diameter, with abruptly narrowing and commonly bent ends. Cell length is typically

335   less than cell width and the apical cells being slightly conical or almost cylindrical and

336   rounded and without calyptra (Fig. 6D, E).

337   Other non-heterocystous filamentous cyanobacteria found in small numbers (≤

338   4%) include *Leptolyngbya truncata* (Lemmermann) Anagnostidis et Komárek 1988 (Fig.

339   6F) and *L. foveolarum* (Rabenhorst ex Gomont) Anagnostidis et Komárek 1988 (Fig. 6G,

340   H). The former has irregularly and feebly curved filaments composed of pale blue-

341   green and non-constricted trichomes that are 0.8-1.3 μm wide (Fig. 6F), whereas the

342   latter has constricted trichomes with cells 1-1.5 μm wide that are not attenuated at

343   the end and have rounded or hemispherical apical cells (Fig. 6G, H). Other species of

344   *Leptolyngbya* (Fig. 6I, J) have non-constricted and straight trichomes formed of

345   approximately isodiametric cells, 2-3 μm wide, with truncated apical cells and

346   enveloped by thin, colorless sheaths.

347   Several species of unicellular cyanobacteria are also present, including

348   *Aphanocapsa* sp., *Aphanothece* sp. and *Chamaesiphon* sp. (Fig. 6K, L). These

349   cyanobacteria were rare in all the samples studied (≤ 3%).

350   Diatoms, including *Cocconeis placentula*, *Gyrosigma obtusatum*, *Bacillaria*

351   *paxillifera*, *Amphora* sp. and *Navicula* sp. are commonly associated with the

352   cyanobacteria (Figs. 7-9).

353

354   *6.1.3. Relationships between cyanobacterial morphotypes, phylotypes, and*

355   *sedimentary facies*

356   In the stromatolites of Facies A, *P. incrustatum* is the dominant morphotype,

357   jointly with phylotype I. On the basis of the dominant morphotype found at these

358   locations and the genetic distance to *P. autumnale* (best BLAST hit), phylotype I was

359   ascribed to *P. incrustatum* (Tables 4, 5).

360   Facies B is dominated by *P. aerugineo-caeruleum* and the corresponding phylotype

361   III. Sites with Facies C and/or a combination of Facies C and A are characterized by

362   populations of *P. incrustatum* and the corresponding phyloptype I. Moreover*, P.*

363   *aerugineo-caeruleum*, and phylotype III, were also found in all those sites with Facies C

364   and/or with Facies C and A, although it was dominant only in site P11 (Tables 4, 5).

365

366   *6.2. Cyanobacterial structures in different environments*

367   Evidence from the deposits that formed on the tablets shows that the

368   cyanobacterial structures found in the fast-flowing areas, the slow-flowing areas, and

369   the stepped waterfalls and small waterfalls vary in accord with the dominant

370   cyanobacterial populations.

371

372   *6.2.1. Stromatolites in fast-flowing water areas*

373   The laminae in the stromatolites of Facies A, which dominates in this environment,

374   are formed largely of subperpendicular tube-shaped calcite bodies (Fig. 7A, B). The

375   tubes resulted from calcite precipitation around filamentous cyanobacteria that were

376   later lost to decay (e.g., as noted and explained by Merz-Preiß and Riding, 1999;

377   Golubić et al., 2008). The inner diameter of the tubes, from 6 to 7.5 μm, corresponds

378   to the diameter of *P. incrustatum*. The wall of these tubes, 2 to 12 μm thick, are

379   formed of calcite crystals, up to 6 μm long, that range in morphology from subhedral

380   to triangular-shaped to rhombohedral (Fig. 7B-E). Less porous fabrics developed in

381   areas where calcite was precipitated around groups of filaments (Fig. 7B). A variety of

382   diatoms and calcified EPS strands, along with morphologically variable calcite crystals

383   are common between the tubes (Fig. 7C, E).

384   *6.2.2. Uneven laminae/stromatolites and scattered cyanobacterial tubes in slow-*

385   *flowing water areas*

386   Facies B, which dominates in this environment, is characterized by laminae

387   formed of thin, discontinuous stromatolites, and isolated calcite tubes. Calcite tubes

388   formed by encrustation around cyanobacterial filaments are isolated (Fig. 8A), form

389   clusters of 4 to 6 tubes, form isolated domes, or develop uneven laminae with

390   randomly oriented tubes (Fig. 8B). In general, these tubes do not form clearly defined

391   laminae. Most of the calcite tubes have an inner diameter of 2.5 to 6.0 μm (most are 5

392   to 6 μm, which corresponds to the diameter of individual *P. aerugineo-caeruleum*

393   cells)*. The walls of the tubes, 3 to 8 μm thick, are formed of morphologically variable

394   calcite crystals (Fig. 8C, D). Varied pennate diatoms occur on and between the calcite

395   tubes (Fig. 8C, D). Other smaller calcite tubes (inner diameter of 1-3 μm), which are

396   scattered throughout, may belong to various species of *Leptolyngbya*.

397   *6.2.3. Stromatolites and irregular cyanobacterial masses formed in stepped waterfalls*

398   *and small waterfalls*

399   The two types of cyanobacterial structures found in this setting are as follows.

400   • Stromatolites (Facies A; Fig. 9A), up to 1.5 cm thick, interbedded with calcite-

401   coated moss and algal deposits (see Fig. 4C). Most are formed of calcite tubes that

402   have an inner diameter of 6-7.5 μm (*cf. P. incrustatum)* and calcite coatings 3-6

403   μm thick, in which crystal sizes and shapes are similar to those formed in fast-

404   flowing water areas devoid of macrophytes (Fig. 9B).

405   • Isolated and unevenly grouped cyanobaterial tubes that form irregular and

406   discontinuous masses (a) among the moss and macroscopic-alga calcite-coated

407   bodies, and (b) in the calcite coatings around the macrophytes. These masses are

408   formed of randomly oriented calcite tubes, uncoated bacterial filaments, diatoms,

409   and EPS strands (Fig. 9C, D). The calcite tubes that formed around the

410   cyanobacterial filaments have an inner diameter of 2.5-6 μm, and walls up to 10

411   μm thick that include numerous diatoms (Fig. 9D). The most abundant calcite

412   tubes, with an inner diameter of 5-6 μm, are assigned to *P. aerugineo-caeruleum.*

413   Some of the small diameter tubes may correspond to *Leptolyngbya* (Fig. 9D).

414   *6.3. Cyanobacterial calcification*

415   *6.3.1. Calcification of P. incrustatum*

416   *P. incrustatum* has been calcified to varying degrees (Fig. 10A-D). Some specimens

417   have small and dispersed groups of calcite crystals on their sheaths (Fig. 10A, B),

418   whereas other sheaths have an uneven calcite coating (Fig. 10C, D). Such coatings are

419   commonly thicker around the base of the filaments (Fig. 10D). The living trichomes

420   commonly vacated their sheaths, leaving behind empty tubes (Fig. 10D).

421   Different degrees of calcification are also evident in Facies A that formed on the

422   tablets that were placed in the areas of fast-flowing water devoid of macrophytes

423   (Figs. 7B-E, 10E-G), and in the stepped waterfalls and small waterfalls (Fig. 9B). In

424   these deposits, the calcite coatings around the filaments are 2 to 12 μm thick. Rare

425   calcified sheaths and filaments are also present (e.g., Fig. 7C). Most cross-sections

426   through the coatings show that there is no consistent pattern in terms of crystal size

427   and shape. In some cases, the coating is < 2 µm thick and formed of irregular,

428   anhedral, $CaCO_3$ nanoparticles (e.g., Fig. 10E). In some of the thicker coatings,

429   however, there is a change from smaller and/or irregular crystals (mainly subhedral) in

430   the inner part, to larger and/or well formed, chiefly rhombohedral and triangular-

431   shaped crystals outwards (Figs 7C-E, 5D, 10F, G).

432   Pennate diatoms and EPS strands remain attached to the filament coatings (Fig.

433   7C, E). Calcified EPS strands between tubes encompass subrhombohedral to anhedral

434   nanoparticles (Fig. 7E).

435   *6.3.2. Calcification in other cyanobacteria*

436   Encrustations around *P. aerugineo-caeruleum* tubes (inner diameters of 5-6 µm),

437   found in Facies B that formed on tablets placed in the areas of slow-flowing water

438   (Figs. 8B-D, 11A, B), are formed of a thin coating (3-4 µm thick) of subhedral to

439   rhombohedral crystals that are generally < 1 µm long (Figs 8C, D, 11A, B). In thicker

440   coatings, the crystals were more varied in size. In general, numerous diatoms and EPS

441   are associated with these tubes (Fig. 8C, D).

442   Calcification of the less common cyanobacteria species is more difficult to

443   ascertain because of their rarity and the fact that they did not form specific structures.

444   A variety of smaller tubes (inner diameter 1-3 µm), which may correspond to various

445   species of *Leptolyngbya,* was found in Facies B and C on the tablets placed in the slow

446   flowing areas and stepped waterfalls and small waterfalls. Such tubes, commonly

447   arranged in small groups (Fig. 9D), are typically formed of a wall (up to 4-5 µm thick)

448   that is formed of subhedral to rhombohedral calcite crystals of varied size (commonly

449   < 1 μm long), but commonly with rhombohedral crystals that increase in size outwards

450   (Fig. 11C, D).

451   **7. Discussion**

452   Molecular studies have shown that cyanobacterial diversity in tufas and

453   stromatolites that developed in freshwater and marine habitats varies in accord with

454   environmental conditions (Janssen et al., 1999; Reid et al., 2000; Arp et al., 2001, 2010;

455   Santos et al., 2010; Shiraishi et al., 2010; Bosak et al., 2012). Bacterial communities in

456   the River Piedra are dominated by cyanobacteria, with the filamentous Oscillatoriales

457   being the most common group, as shown by morphological and phylogenetic analyses.

458   In addition, a unicellular representative was recorded in both microscopic examination

459   and the sequence analysis. These filamentous cyanobacteria are typically preserved as

460   hollow tubes that formed through calcite encrustation around the filaments followed

461   by decay of the soft tissues. There are only rare examples where the filament and/or

462   sheath have been preserved through calcite impregnation (cf. Riding, 1991). Thus,

463   most of the stromatolites in these deposits are formed of laminae that are constructed

464   of hollow tubes. Cyanobacteria with similar styles of preservation are also found in the

465   non-laminated and poorly laminated deposits that form in the slow-flowing water

466   areas (Facies B) and in stepped waterfalls (Facies C). The cyanobacterial structures that

467   developed under slow flow conditions, however, do not form extensive or regularly

468   laminated deposits.

469   In the River Piedra, stromatolites (Facies A) in the fast-flowing water areas are

470   dominated by *P. incrustatum*. In contrast, *P. aerugineo-caeruleum* dominates in areas

471   that are characterized by Facies B that formed under low water velocity conditions.

472   Both species are found in the stepped waterfalls and small waterfalls. In this

473   environment, *P. incrustatum* dominates in zones with fast-flowing and strong vertical

474   water flow, where it constitutes Facies A, whereas *P. aerugineo-caeruleum* dominates

475   in zones with slow- to moderate-flowing water that are dominated by moss and algae

476   (Facies C). Other cyanobacteria (e.g., *Leptolyngbya*) are also present in the areas with

477   slow flowing water.

478
479   *7.1. Flow conditions and variations in cyanobacterial populations*

480   This and previous studies have suggested that the development of sedimentary

481   facies with variable deposition rates in the River Piedra are related to flow conditions,

482   which in turn, control the flora and bacteria and their calcification patterns (Vázquez-

483   Urbez et al., 2010; Arenas et al., 2014). In this river, cyanobacterial populations

484   dominated by *P. incrustatum* thrive in areas with shallow, fast-flowing water where

485   there is intense mechanical CO_2 outgassing, the highest deposition rates, and

486   laminated deposits. In contrast, cyanobacterial populations dominated by *P.*

487   *aerugineo-caeruleum* are found in areas with slow flowing water, where there is less

488   intense mechanical CO_2 outgassing, lower deposition rates, and the development of

489   poorly laminated deposits.

490   CO_2 uptake through photosynthesis is generally considered to be much less than

491   that of physical CO_2 outgassing, especially in high-CO_2 and/or fast flowing water

492   systems (Arp et al., 2001; Chen et al., 2004; Shiraisi et al., 2008, 2010). Arenas et al.

493   (2014) reached a similar conclusion for the River Piedra even though the precise

494   contribution of each type of CO_2 removal was unknown. Pentecost (1975), from

495   calculations based on rates of photosynthetic CO_2 uptake with ^{14}C, argued that up to

496   20% of the calcification in *Rivularia* could be the direct result of photosynthesis. Mass

497   balance estimations in other European karst streams also indicated that in fast flowing,

498   shallow water, cyanobacterial photosynthesis accounts for 10–20% of the total Ca^{2+}

499   loss, with the remaining Ca^{2+} loss being caused by physicochemical precipitation

500   (Shiraishi et al., 2008; Arp et al., 2010; Pentecost and Franke, 2010). In these systems,

501   it therefore appears that photosynthesis exerts less influence on $CaCO_3$ precipitation

502   than the abiotic CO_2 evasion related to flow conditions. These results are consistent

503   with the results obtained in this study.

504   Although carbonate precipitation is not necessarily biologically driven, the

505   cyanobacteria do provide surfaces that are amenable to calcite precipitation

506   (Pentecost and Whitton, 2000). Specifically, it has been argued that various geochemical

507   properties of the surface of the sheath and the colony architecture of *P. incrustatum*

508   actively promotes the early stages of calcification (Pentecost and Whitton, 2000). Thus,

509   differences in the cyanobacteria, resulting from adaptive traits to cope with specific

510   environmental conditions, may explain some of the differences in calcification.

511   Therefore, in fluvial carbonate systems, if all other parameters are equal (e.g., water

512   composition, temperature, insolation), it is the depositional conditions (primarily flow

513   conditions) that control the distribution of the different cyanobacterial populations

514   and, in turn, the $CaCO_3$ precipitation processes that take place in each environment.

515   *7.2. Cyanobacterial structures: calcification, filament orientation, and lamination*

516   Calcification is a characteristic feature of many cyanobacteria, including

517   representatives of the Oscillatoriales such as *Phormidium*, *Lyngbya,* and *Plectonema*

518   (Zavarzin, 2002). It is important to note, however, that other cyanobacteria may

519   remain uncalcified even when exposed to waters with the same degree of $CaCO_3$

520   saturation (Brehm et al., 2006; Golubić et al., 2008). It is possible that species-specific

521   differences may play a role in determining many aspects of calcification, including

522   crystal shape, sheath impregnation, and encrustation around the filaments (Merz-Preiß

523   and Riding, 1999; Merz-Preiß, 2000; Jones and Peng, 2014).

524   The strong influence of depositional conditions on colonization patterns of

525   cyanobacteria in different environments of the River Piedra does not allow definitive

526   resolution of the species-specific attributes. Nevertheless, differences in the degree of

527   calcification associated with *P. incrustatum* (Fig. 10) may have resulted, at least in part,

528   from slight differences in parameters such as the CO_2 outgassing rates, temperature,

529   and Ca concentrations, that contribute to the calcite saturation index (Table 2), as has

530   been suggested by several authors (e.g., Pentecost, 2005; Pedley et al., 2009; Jones

531   and Peng, 2014).

532   Textural attributes of the deposits in the River Piedra indicate that flow conditions

533   exert a strong influence on filament orientation and calcification of the microbial mats.

534   In areas with rapid flow with intense CO_2 outgassing and high calcite saturation indices,

535   filaments of *P. incrustatum* are rapidly coated by $CaCO_3$ and their growth keeps pace

536   with calcification. This produces a tight, rigid structure that is formed largely of coated

537   filaments set subperpendicular to surface that can withstand the fast flow water.

538   Moreover, the dense filamentous mats formed by *P. incrustatum* increase the surface

539   area available for $CaCO_3$ precipitation and thereby increase resistance to water flow

540   (cf., Golubić et al., 2008). In the River Piedra, such rapid calcification may also favour

541   the formation of several laminae over a few months (Fig. 2C) that may, for example, be

542   related to short-term changes in flow rate. Slight variations in any of the

543   environmental parameters that affect the calcite saturation index in the carbonate

544   system may promote the formation of distinctive laminae (Guo and Riding, 1994).

545   Areas of the River Piedra with slow flowing water, where CO_2 outgassing is less

546   intense and $CaCO_3$ precipitation is slower, are characterized by cyanobacterial mats

547   that are dominated by *P. aerugineo-caeruleum*. Uneven laminae formed of randomly

548   oriented and less commonly subperpendicular tubes with open to dense fabrics are

549   interbedded with loose sediment. Features in these beds are consistent with the

550   slower precipitation of $CaCO_3$ around the filaments that is engendered by the slow

551   flowing deeper water that commonly has a lower calcite saturation index than found in

552   the fast flowing water (Table 2). These cyanobacterial mats, dominated by *P.*

553   *aerugineo-caeruleum*, do not form thick or extensively lateral deposits. Although these

554   mats may not build thick laminated deposits, they mediate deposition by trapping and

555   binding sediment to the substrate, thus enhancing cohesiveness of the loose sediment

556   (cf., Golubić et al., 2000; Seong-Joo et al., 2000; Noffke et al., 2003).

557   *7.3. The role of EPS in calcification*

558   EPS play an important role in microbial mat formation (Decho, 2000; Frank and

559   Belfort, 2003). Comparative studies of cyanobacteria have shown that *Phormidium* is

560   the highest producer of EPS (Nicolaus et al., 1999; Di Pippo et al., 2013). The high EPS

561   production associated with *P. incrustatum* in the River Piedra would contribute to the

562   better attachment to surfaces and permit growth in fast-flowing waters.

563   EPS has also been considered essential for providing mineral nucleation sites in

564   tufa-forming microbial mats in karst streams (Pentecost, 1985, 2005). EPS can remain

565   associated with the cell surface as sheaths and/or be released into the surrounding

566   environment (De Philippis and Vincenzini, 2003). EPS mediates $CaCO_3$ precipitation by

567   providing diffusion-limited microenvironments that create alkalinity gradients in

568   response to metabolic processes, and by attracting and binding Ca ions to negatively

569   charged sites (Arp et al., 2010).

570   In addition to variations in some of the parameters that affect the bulk saturation

571   index of water with respect to calcite, changes in calcite saturation levels in the

572   microbially produced EPS around the cells may also contribute to morphological

573   variations evident in calcite that forms around the microbes (e.g., Figs 7C-D, 8C, D, 9B,

574   10E-G, 11). Studies of *Scytonema julianum* encrustations in different geological and

575   geographic areas showed that there was a progressive change in crystal morphology

576   that was probably microbially controlled by microscale changes in the saturation levels

577   that developed in the EPS (Jones and Peng, 2014).

578   The outward increase in crystal size, which is a common feature of the tubes

579   that developed around many of the microbes (particularly *P. incrustatum*) found in the

580   River Piedra, may be due to the fact that the ongoing precipitation of $CaCO_3$ around

581   the microbes will progressively isolate the growth surface from microbial influence

582   (Jones and Peng, 2014). With this model, the larger crystals on the outermost surfaces

583   of the cyanobacterial tubes are probably related to the physiochemical conditions in

584   the surrounding water rather than in the EPS. The innermost part of the thick

585   encrusting layers and all of the thinner encrusting layers around *P. incrustatum*, *P.*

586   *aerugineo-caeruleum* and *Leptolyngbya* (Figs. 8D, 11B-D) are commonly formed of

587   small, irregularly shaped calcite crystals. This may reflect the fact that higher

588   saturation levels are expected around the cell walls (Jiménez-López et al., 2011). This

589   idea also suggests that in slow flowing water, precipitation may be more influenced by

590   metabolic processes associated with the EPS than in fast flowing water.

591

592   **8. Comparison with other carbonate fluvial systems**

593   Stromatolites made of cyanobacterial calcite tubes similar to those found in this

594   study have been described in other modern fluvial tufa systems (e.g., Merz-Preiß and

595   Riding, 1999; Arp et al., 2001, 2010; Golubić et al., 2008). *P. incrustatum* is the

596   dominant cyanobacterium in laminated deposits of some tufa-forming streams in

597   Belgium (Janssen *et* al., 1999), Germany (Merz-Preiß and Riding, 1999; Arp et al., 2001,

598   2010) and Japan (Shiraishi et al., 2010). In those examples, *P. incrustatum* was found in

599   fast-flowing water. Other cyanobacteria are also common in fast-flowing conditions of

600   other tufa systems (Golubić et al., 2008; Arp et al., 2010). For instance, in the Plitvice

601   system Golubić et al. (2008) identified *P. incrustatum*, *Schizothrix fasciculate*, *P.*

602   *favosum*, *P. uncinatum* and *Hydrocoleum homoeotrichum*, and *Rivularia* haematites. In

603   this example, species-specific differences in the degree of calcification and in the shape

604   of the resulting calcium carbonate crystals are apparent (Obenluånneschloss and

605   Schneider, 1991). In the River Piedra environments, similar variations evident between

606   the different environments can be attributed to differences in flow conditions and

607   associated hydrochemical parameters.

608   Most studies of fluvial stromatolites have focused on taxonomic questions,

609   phylogenetic relationships (Arp et al., 2010; Santos et al., 2010; Brinkmann et al.,

610   2015), composition of the stromatolite-forming mats (e.g., Arp et al., 2010), the

611   conditions under which cyanobacteria calcify (Merz-Preiß and Riding, 1999), or the

612   contribution of cyanobacterial photosynthetic CO_2-uptake to calcification (Arp et al.,

613   2010; Shiraishi et al., 2008, 2010). The cyanobacteria most frequently recovered in

614   these studies were Oscillatoriales, although representatives of unicellular genera were

615   also found. In general, however, the depositional structures and calcification of the

616   microbial constituents have not been related to variations in flow conditions or to

617   variations in depositional rates between different fluvial environments.

618   Merz-Preiß and Riding (1999) focused on conditions under which cyanobacteria

619   calcify and concluded that (1) the CO_2 content in water determines if filament

620   encrustation or sheath impregnation takes place, and (2) calcite encrustation is

621   conspicuous when the calcite saturation index > 0.8. They inferred that in fast-flowing

622   streams cyanobacteria utilize CO_2 in photosynthesis, whereas in sluggish water

623   cyanobacteria utilize HCO_3, which leads to sheath impregnation by $CaCO_3$ even where

624   calcite saturation index is only 0.2–0.3. Results from the River Piedra show that calcite

625   encrustation is the dominant process for cyanobacterial calcification in all settings

626   where the mean calcite saturation index is between *ca* 0.6 and 0.9 (Arenas et al.,

627   2014). In addition, data from the River Piedra indicate that variations in the crystal size,

628   crystal shape, and thickness of the encrustation may reflect the variable influence of

629   physicochemical conditions associated with EPS around the cells (cf., Jones and Peng,

630   2014). This may be more important to calcite precipitation in slow-flowing water than

631   in fast-flowing water.

632   This study has shown that the fluvial tufa environment contains morphologically

633   diverse and genetically recognizable cyanobacterial populations with the dominant

634   cyanobacterial communities being dependent on the depositional environmental

635   conditions (primarily water flow conditions). These factors are manifest in the distinct

636   textures and structures found in the associated facies. Therefore, the distinct

637   depositional structures (e.g., arrangement of calcite tubes) and calcification styles

638   (e.g., size and shape of crystals and thickness of encrustations) of the cyanobacterial

639   calcite tubes found in different sedimentary facies should be taken into account in

640   interpreting environmental conditions of ancient carbonate sedimentary systems.

641

9. Conclusions

Analysis of sedimentological attributes and cyanobacterial diversity in the River
Piedra in the Monasterio de Piedra Natural Park has produced the following important
conclusions.

- Morphological and phylogenetic analyses of living bacterial mats showed that the
  distribution of the dominant cyanobacterial species is linked to flow conditions.

- *Phormidium incrustatum* dominates in the fast-flowing water where the mean
  deposition rate is 1.6 cm/year. This taxon is responsible for the formation of
  extensive, thick stromatolites that are formed largely of palisades of calcite tubes
  that formed as a result of calcite encrustation around the living filaments.

- *Phormidium aerugineo-caeruleum* dominates in areas with slow-flowing water,
  where it formed uneven laminae, and calcite tubes that are scattered throughout
  the loose, structureless sediments, that accumulated at 0.3 cm/yr. Although this
  species tends not to form stromatolites, it does contribute to the cohesiveness of
  the loose sediment.

- *P. incrustatum* and *P. aerugineo-caeruleum* are found in stepped waterfalls and
  small waterfalls enviroments where they are associated with the spongy moss
  and algal boundstone.

- The calcite encrustations that form around the cyanobacteria do not typically
  exhibit regular patterns. This may be a reflection of changes in the parameters
  that affect the calcite saturation index. The change in shape and size of the $CaCO_3$
  precipitates may be more strongly influenced by the physicochemical conditions
  in the EPS around the cells than by the chemical characteristics of the river water.

665   These metabolic processes may be more significant to calcite precipitation in

666   slow-flowing water than in fast-flowing water.

667   • In fluvial carbonate systems it is the depositional conditions (primarily physical

668   flow conditions) that controls the cyanobacterial populations and the calcium-

669   carbonate precipitation processes in each environment.

670   • The varying cyanobacterial structures and calcification styles are significant

671   attributes that can be used to assess changes in depositional conditions of

672   ancient carbonate sedimentary systems.

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876 **Fig. 1.** (A) Location of Monasterio de Piedra Natural Park and geological map of the

877 region. (B) Location of studied sites and the main waterfalls along the River Piedra

878 (modified from Arenas et al., 2014).

879 **Fig. 2.** Fast-flowing water environment and corresponding sedimentary facies

880 (stromatolites, Facies A). (A) Field view. (B) Plan view of stromatolite with knobby

881 surface. (C) Cross-section of stromatolite formed on a tablet in fast-flowing water

882 areas. The six-month periods are indicated: "Warm" corresponds to April–

883 September and "Cool" to October–March. (D) Optical microscope image of

884 stromatolite formed on a tablet. Lamination consisting of laminae with tight

885 filamentous cyanobacterial calcite bodies subperpendicular with respect to the

886 growth surface. Note voids formed by aquatic insects and worms.

887 **Fig. 3.** Slow-flowing water environment developed upstream of a small waterfall,

888 (loose lime mud and carbonate sediment, Facies B). (A) Field view. (B, C) Plan views

889 of deposit showing grayish-blue color of the cyanobacterial mat. (D) Cross-section

890 of deposit formed on a tablet; note that lamination is poor and mostly

891 characterized by the presence of thin white laminae. (E) Optical microscope image

892 of deposit formed on a tablet. Typical loose, structureless sediment consisting of

893 micrite with allochemical components (sections of calcite coated algae and

894 macrophytes). Note presence of a micrite mass made of elongated bodies that

895 resemble filamentous cyanobacteria (Cy).

896 **Fig. 4.** Stepped waterfall environment (moss, macroscopic algae, grass and

897 cyanobacterial mats; Facies C). (A) Field view. Tablet, installed for determining

898   deposition rates, is located at the bottom left of the image. (B) Detail of surface in

899   A, showing moss, algae and cyanobacterial mats. (C) Cross-section of deposit

900   formed on a tablet, consisting of moss and macroscopic-alga boundstone (Facies

901   C), associated with a stromatolite deposit at the base (Facies A). (D) Optical

902   microscope image of deposit formed on a tablet. Association of stromatolite

903   (laminae with tight filamentous cyanobacterial bodies, Cy) and boundstone

904   consisting of coated filamentous algae (cross sections, Al (tu)).

905   **Fig. 5.** Phylogenetic tree based on 16S rRNA gene sequences (1,315 positions) obtained

906   by the neighbor-joining method. Bootstrap support from neighbor-joining and

907   maximum likelihood analysis is reported above the nodes (≥50%). I-IV are the

908   clusters in which sequences from this study are included. Sequences from this

909   study are indicated in bold and their associated GenBank accession numbers are

910   listed in Table 3. GenBank accession numbers for database sequences are in

911   parentheses following their name. The scale bar indicates 0.02 mutations per

912   nucleotide position.

913   **Fig. 6.** Light and fluorescence microscopy photomicrographs showing the main

914   cyanobacterial morphotypes identified in the samples. (A, B) *Phormidium*

915   *incrustatum*; (C) *Phormidium aerugineo-caeruleum*; (D, E) *Phormidum* sp.; (F)

916   *Leptolyngbya truncata*; (G, H) *Leptolyngbya foveolarum*; (I, J) *Leptolyngbya* sp.; (K,

917   L) *Chamaesiphon* sp. Scale bars = 20 μm.

918   **Fig. 7.** Scanning electron microscope images of samples selected from stromatolites

919   (Facies A) in which the dominant species is *Phormidium incrustatum*. (A, B) Calcite

920   tubes formed around *P. incrustatum*, with dominant subvertical orientation and

921   different degrees of calcification, with abundant EPS (in A). (C–E) Detail of calcite

922   tubes formed around *P. incrustatum*, with abundant calcified EPS in E. Note the

923   preserved calcified sheath (Sh) and abundant diatoms (Di) in C.

924   **Fig. 8.** Scanning electron microscope images of samples from deposits formed on

925   tablets installed in Facies B. (A) Typical loose, structureless sediment consisting of

926   isolated and clumped calcite crystals, diatoms and cyanobacterial tubes (arrow). (B)

927   Randomly oriented calcite tubes formed around cyanobacteria. (C, D) Calcite tubes,

928   probably formed around *Phormidium aerugineo-caeruleum*, consisting of small

929   calcite crystals (< 1 μm) and attached small diatoms. Note diatoms around the

930   tubes and EPS between tubes in D.

931   **Fig. 9.** Scanning electron microscope images of samples from deposits formed on

932   tablets installed in Facies C. (A) Mostly subvertical calcite tubes around *Phormidium*

933   *incrustatum* in stromatolite formed in a fast-flowing water zone. (B) Detail of

934   calcite tube in A. (C) Calcite crystals associated with EPS and cyanobacterial tubes.

935   (D) Randomly oriented calcite tubes, probably corresponding to *Leptolyngbya* sp.,

936   consisting of thick calcite coatings containing attached diatoms. Note EPS

937   (Extracellular Polymeric Substances) around the tubes. Cy (tu): Cyanobacteria

938   (tubes); Di: Diatoms.

939   **Fig. 10.** (A–D) Optical microscope images of *Phormidium incrustatum* showing different

940   degrees of calcification (samples taken on site from surface living mat). Explanation

941   in the text. (E–G) SEM images of samples selected from deposits formed on tablets

942   in fast-flowing water areas, showing calcite/$CaCO_3$ coatings formed around

943   *Phormidium incrustatum*. (E) Thin coating formed of irregular (anhedral) and small

944    $CaCO_3$ forms. (F, G) Thick coatings showing larger crystals on outer part of coating.

945   **Fig. 11.** Scanning electron microscope images of samples selected from deposits

946   formed on tablets in slow flowing water areas. (A, B) $CaCO_3$ coatings formed

947   around *Phormidium aerugineo-caeruleum.* Note the small and irregular $CaCO_3$

948   forms throughout the coating. (C, D) $CaCO_3$ coatings formed around *Lyptolyngbya*

949   sp. Note in C the smaller size and irregular $CaCO_3$ forms in the interior and size

950   increase of rhombohedra outwards.
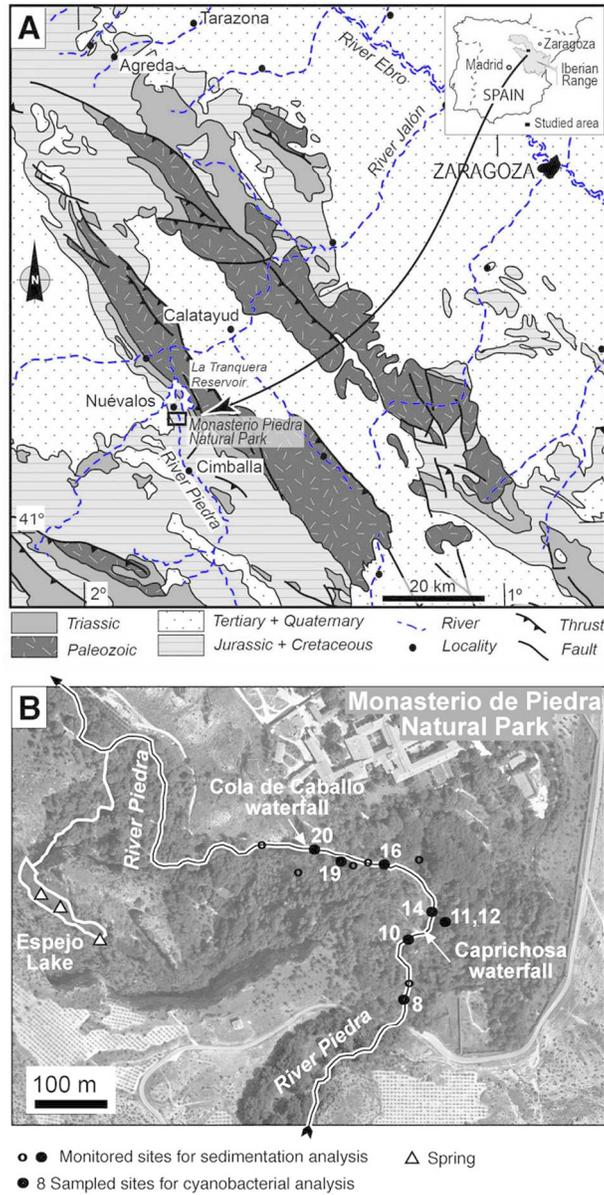
Fig. 1. (A) Location of the Monasterio de Piedra Natural Park and geological map of the region. (B) Location of studied sites, indicating the sites sampled for cyanobacterium analysis, and the main waterfalls along the River Piedra (modified from Arenas et al., 2014).
78x155mm (250 x 250 DPI)

Fig. 2. (H) Stepped waterfall with moss, macroscopic algae, grass and cyanobacterial mats. The tablet installed for controlling deposition rate is located at the bottom left of the image. (I) Detail of surface in H, showing moss, algae and cyanobacterial mats. (J) Plan view of deposit in H showing stromatolite (hard calcified cyanobacterial mat) and calcite coated moss. (K) Cross-section of deposit formed on a tablet in the stepped waterfall shown in H, consisting of moss and macroscopic algal boundstone (facies C), associated with a stromatolite deposit at the base (facies A).

170x191mm (250 x 250 DPI)

Fig. 2. (H) Stepped waterfall with moss, macroscopic algae, grass and cyanobacterial mats. The tablet installed for controlling deposition rate is located at the bottom left of the image. (I) Detail of surface in H, showing moss, algae and cyanobacterial mats. (J) Plan view of deposit in H showing stromatolite (hard calcified cyanobacterial mat) and calcite coated moss. (K) Cross-section of deposit formed on a tablet in the stepped waterfall shown in H, consisting of moss and macroscopic algal boundstone (facies C), associated with a stromatolite deposit at the base (facies A).

170x189mm (250 x 250 DPI)
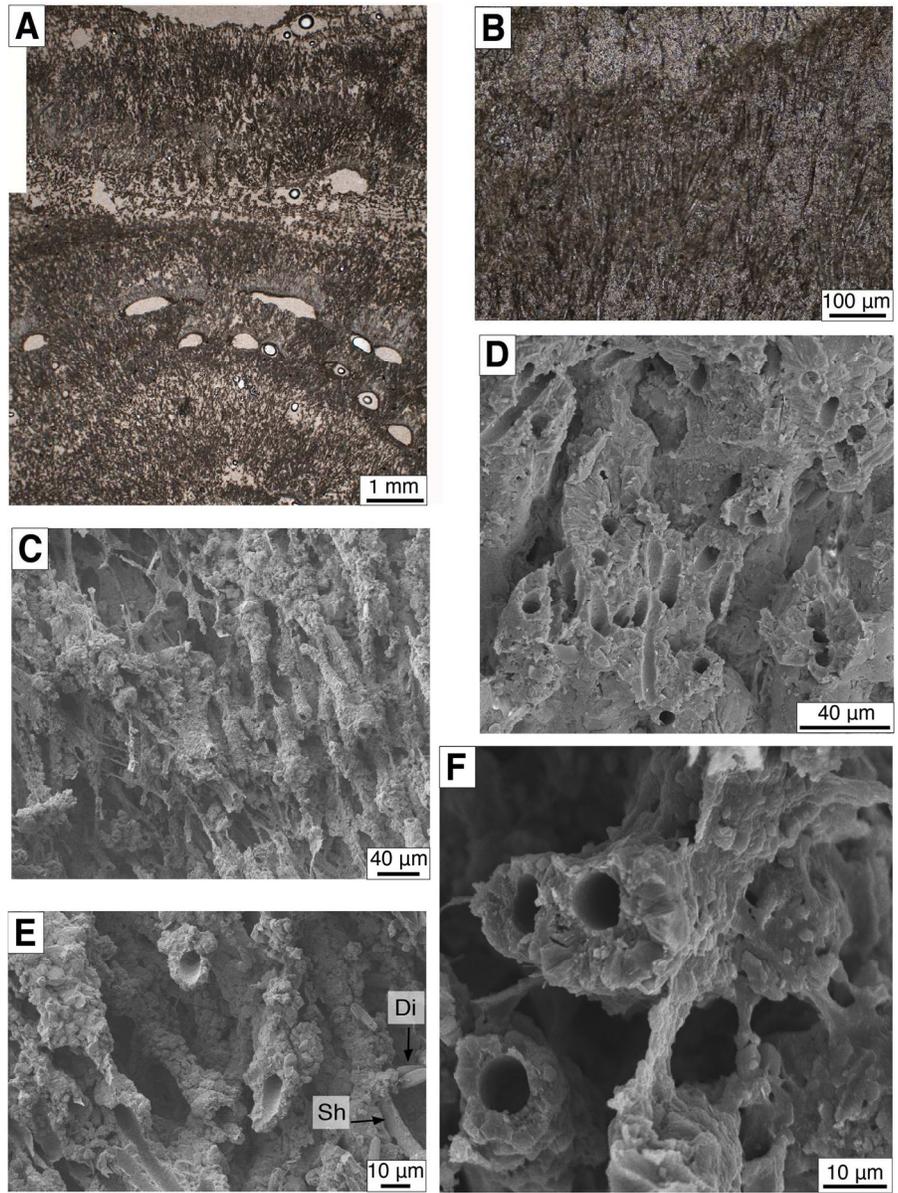
Fig. 3. Optical and scanning electron microscope images of samples selected from stromatolites (Facies A) formed on tablets installed in fast-flowing water areas. (A) Lamination consisting of laminae with tight filamentous cyanobacterial bodies subperpendicular with respect to the growth surface. Note voids from aquatic insects and worms. (B) Detail of filamentous cyanobacterial bodies. (C) and (D) Calcite tubes with dominant subvertical orientation and different degrees of calcification, with abundant EPS in A. (E) and (F) Detail of calcite tubes, with abundant calcified EPS in F. Note the preserved sheath (Sh) and the abundance of diatoms (Di) in E. Inner diameter of tubes in D to F is 6.5-7 µm.

169x228mm (250 x 250 DPI)
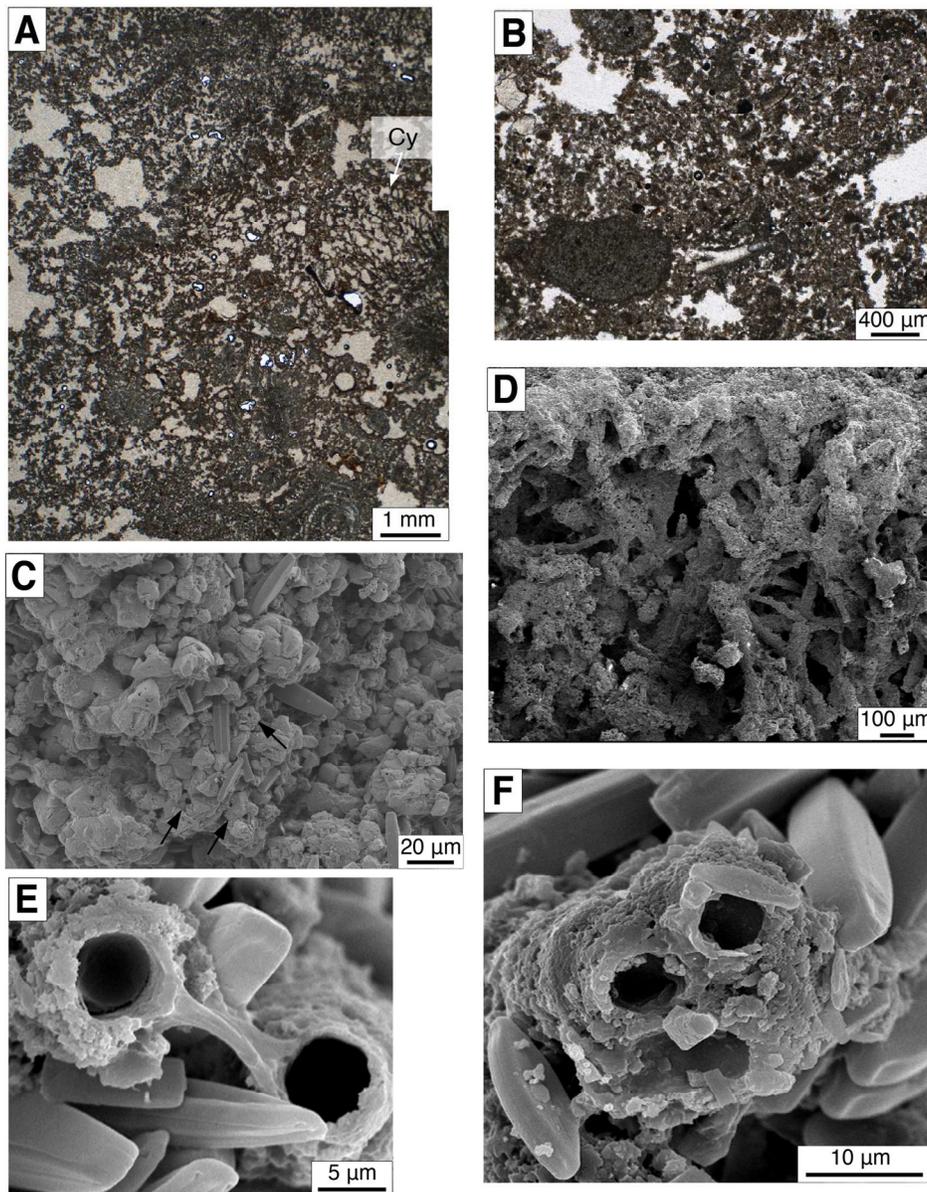
Fig. 4. Optical and scanning electron microscope images of samples selected from deposits formed on tablets installed in slow-flowing water areas (Facies B). (A) and (B) Typical loose, structureless sediment consisting of micrite with allochemical components (sections of calcite coated algae and macrophytes and intraclasts). Note in A the presence of a micrite mass evoking filamentous cyanobacteria (Cy). (C) Detail of texture of facies B: Isolated and clumped calcite crystals, diatoms and cyanobacterial tubes (arrowed). (D) Randomly oriented calcite tubes formed around cyanobacteria. Note that the inner diameter of most tubes is less than that of the tubes in Fig. C and D. (E) and (F) Calcite tubes (inner diameter = 5 µm) consisting of small calcite crystals (1-2 µm) and attached small diatoms. Note EPS in E and diatoms around the tubes. 169x216mm (250 x 250 DPI)
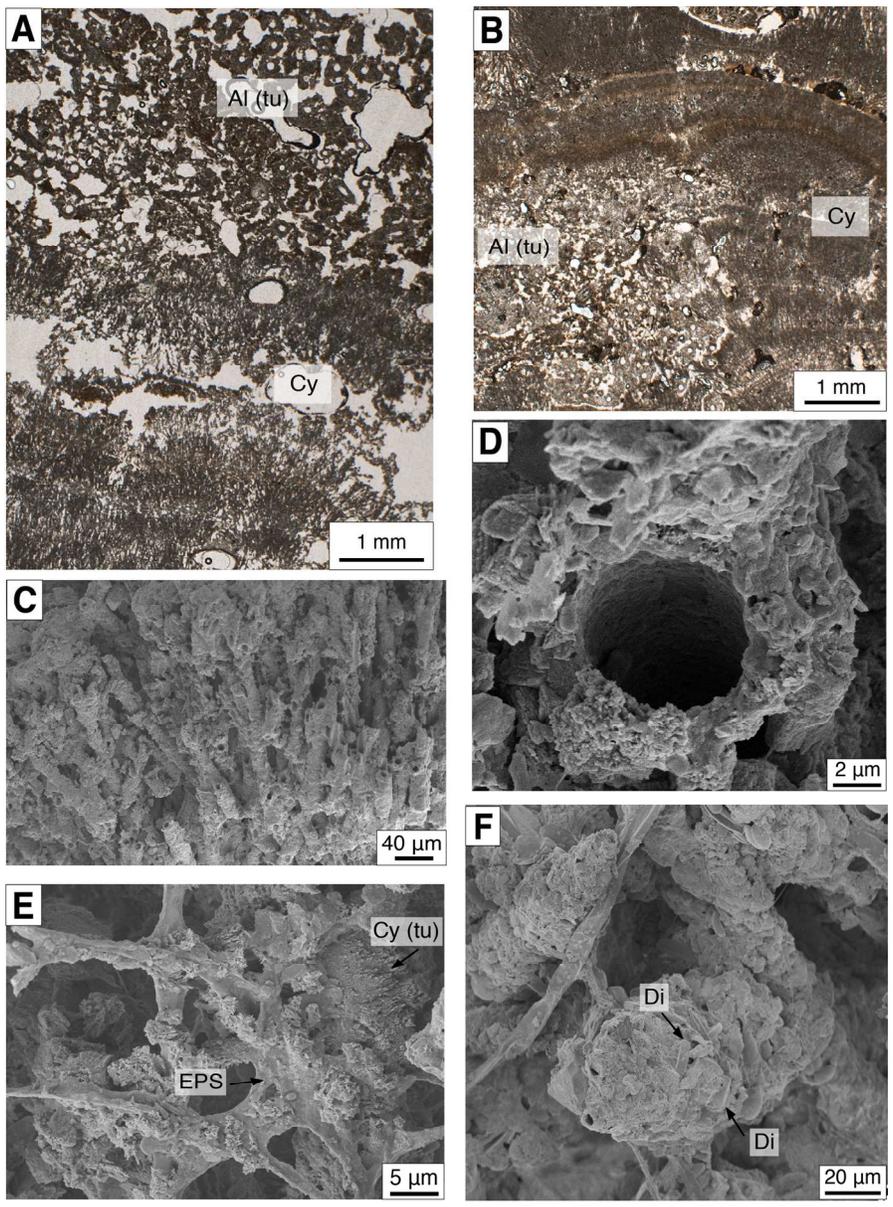
Fig. 5. Optical and scanning electron microscope images of samples selected from deposits formed on tablets installed in stepped waterfall areas (Facies C). (A) and (B) Association of stromatolites (laminae with tight filamentous cyanobacterial bodies) and boundstone consisting of coated filamentous algae (cross sections). (C) Mostly subvertical calcite tubes in stromatolite formed in a fast-flowing water zone. (D) Detail of calcite tube in C (inner diameter = 7 μm). (E) Detail of calcite crystals associated with EPS and cyanobacterial tubes. (F) Randomly oriented calcite tubes (inner diameter = 2.5 μm) consisting of thick calcite coatings containing attached diatoms. Note EPS around the tubes. Al (tu): Algae (tubes); Cy: Cyanobacteria; Cy (tu): Cyanobacteria (tubes); Di: Diatoms; EPS: Extracellular Polymeric Substances. 170x230mm (250 x 250 DPI)
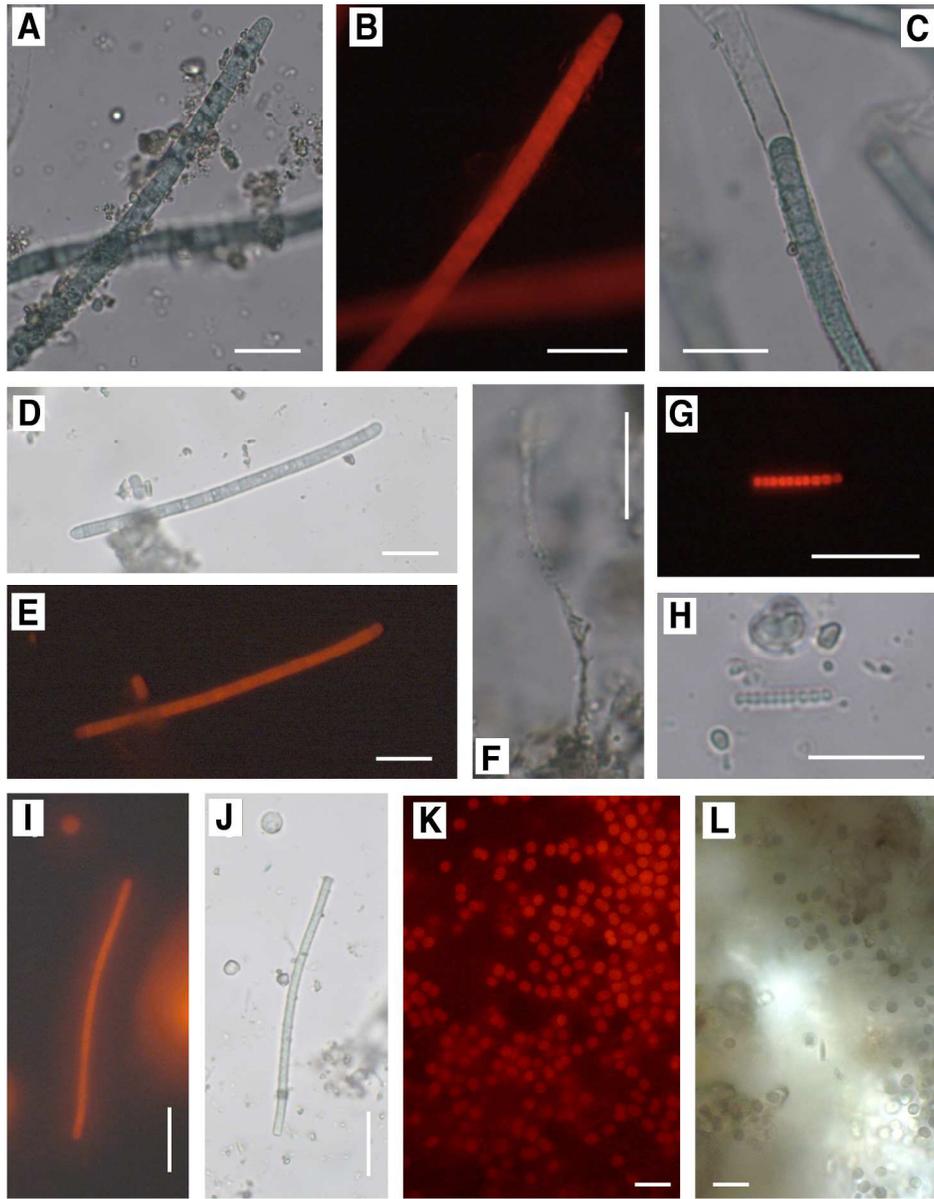
Fig. 6. Light and fluorescence microscopy photomicrographs showing the main cyanobacterial morphotypes identified in the samples. (A) and (B) Phormidium incrustatum; (C) Phormidium aerugineo-caeruleum; (D) and (E) Phormidum sp.; (F) Leptolyngbya truncata; (G) and (H) Leptolyngbya foveolarum; (I) and (J) Leptolyngbya sp.; (K) and (L) Chamaesiphon sp. Solid bars, 20 µm.
187x239mm (299 x 299 DPI)
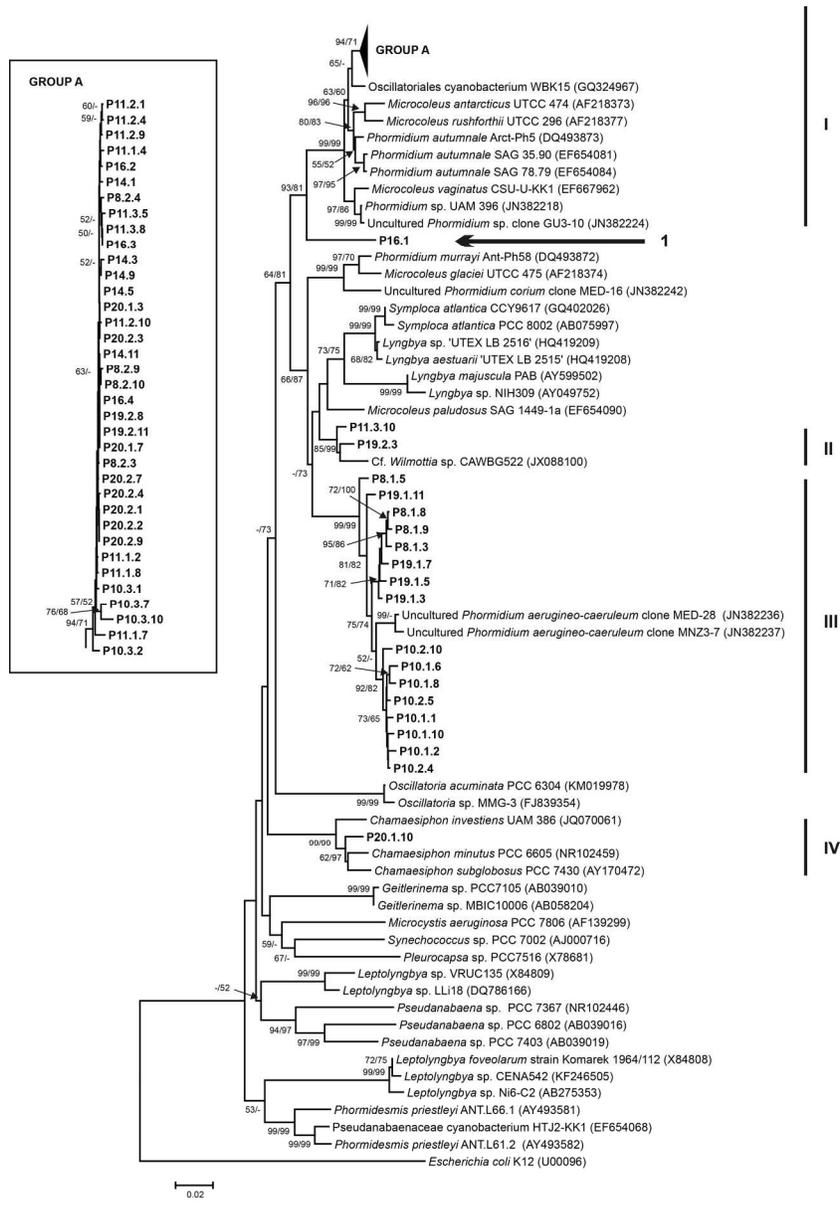
Fig. 7. Phylogenetic tree based on 16S rRNA gene sequences (1,315 positions) obtained by the neighbor-joining method. Bootstrap support from neighbor-joining and maximum likelihood analysis is reported above the nodes (≥50%). Sequences from this study are indicated in bold and their associated GenBank accession numbers are listed in Table 4. GenBank accession numbers for database sequences are in parentheses following their name. The scale bar indicates 0.02 mutations per nucleotide position.
200x287mm (250 x 250 DPI)

Fig. 8. (A) to (D) Optical microscope images of Phormidium incrustatum showing different degrees of calcification (samples taken on site from surface). Explanation in the text. (E) and (F) SEM images of CaCO3 coatings formed around Phormidium incrustatum (samples from tablets). (E) Thin coating formed of irregular and small CaCO3 forms. (F) and (G) Thick coatings showing larger crystals outwards. (H) and (I) SEM images of CaCO3 coatings formed around Phormidium aerugineo-caeruleum (samples from tablets). Note the small and irregular CaCO3 foms through the entire coating. (J) and (K) SEM images of CaCO3 coatings formed around Lyptolyngbya (samples from tablets). Note the smaller size and irregular CaCO3 forms in the interior and size increase of rhombohedra outwards.
167x211mm (250 x 250 DPI)

Fig. 8. (A) to (D) Optical microscope images of Phormidium incrustatum showing different degrees of calcification (samples taken on site from surface). Explanation in the text. (E) and (F) SEM images of CaCO3 coatings formed around Phormidium incrustatum (samples from tablets). (E) Thin coating formed of irregular and small CaCO3 forms. (F) and (G) Thick coatings showing larger crystals outwards. (H) and (I) SEM images of CaCO3 coatings formed around Phormidium aerugineo-caeruleum (samples from tablets). Note the small and irregular CaCO3 foms through the entire coating. (J) and (K) SEM images of CaCO3 coatings formed around Lyptolyngbya (samples from tablets). Note the smaller size and irregular CaCO3 forms in the interior and size increase of rhombohedra outwards.

168x158mm (250 x 250 DPI)

Table 1. Depositional environments, sedimentary facies and deposition rates of the sampled sites (Location of sites in Figure 1B). Water velocity and depth correspond to maximum and minimum values measured at the end of the four seasons from 1999 to 2012. Deposition rates correspond to mean values from 1999 to 2012 for the sampled sites in this study. Warm periods: spring + summer seasons (6 months). Cood periods: autumn + winter seasons (6 months). Data compiled from Arenas et al. (2014).

Depositional environments Sampling sites	Water velocity (cm/s)	Water depth (cm)	Sedimentary facies	Mean deposition rates (mm)		
				Warm periods	Cool periods	Yearly
Areas of fast-flowing water, including steeper stretches along the riverbed, devoid of bryophytes and macrophytes. Sites P14, P16, P20.	90 – 260	7 – 11	A, Stromatolites: dense laminated deposits formed from cyanobacterial mats, preserved as tube-shaped calcite bodies subperpendicular to the substrate.	10.27	5.52	15.79
Areas of slow-flowing to standing water, upstream and downstream of waterfalls and barrages. Site 10-2.	20 - 80	10 – 45	B, Loose, commonly non-laminated deposits: Lime mud, varied mm-cm carbonate grains and macroscopic algae, with interbedded bacterial laminae. Rare oncoids. Boundstones of macrophytes in palustrine conditions.	0.76	1.92	2.68
Stepped waterfalls and small waterfalls (1 – 10 m high) with bryophytes and algae. Sites P8, P10-1, P11, P19.	30 -90	Water film (mm)	C, Mostly spongy tufa: mats of moss, macroscopic filamentous algae, cyanobacteria and herbaceous plants, coated by calcite. Rare and poor lamination and banding. In places, associated with facies A (e.g., P8 and P10-1).	5.97	3.03	9.00

Table 2: Tufa deposition and physical and chemical parameters of water at the sampled sites. Water temperature and velocity measured on site on 24 June and 23 September 2010 (between 10:00 and 12:00h). Hydrochemical parameters correspond to water samples collected on 24 June 2010 (conductivity and pH measured on site). Data compiled from Arenas et al. (2014). Location of sites in Figure 1B.

Sites	Facies	Tufa deposition (mm) April 2000 - Sept 2010	Water Temp (ºC)		Water depth (cm)		Water velocity (cm/s)		Conductivity (μS/cm)	Alkalinity (ppm HCO_3)	Ca (ppm)	pH	log pCO_2	Saturation index (calcite)
			June	Sept	June	Sept	June	Sept						
P8	C + A	3.5	15.5	16.3	9	11	132	43	619	276.2	95.2	8.2	-2.87	0.96
P10-2	B	5.1	15.7	16.4	24	21	42	28	660	267.4	82.4	8.1	-2.73	0.76
P11	C + A	4.8	15.6	16.3	0.5	0.5	89	70	640	260.8	79.5	8.1	-2.77	0.76
P14	A	9.6	15.6	16.4	10	9	259	253	644	261.0	79.6	8.4	-3.04	1.00
P16	A	10.4	15.7	16.5	7	5	172	181	512	261.3	81.5	8.4	-3.06	1.04
P19	C	2.7	16.1	16.7	0.5	0.5	-	-	503	244.2	78,0	8.2	-2.95	0.87
P20	A	10.7	16.0	16.8	8	7	205	203	514	253.2	80.2	8.4	-3.13	1.07

Table 3. Sampling sites, codes, accession numbers and closest relatives of sequences obtained from community DNA of cyanobacterial mats.

Sampling sites	Code	Accesion numbers	Closest GenBank relative	Similarity %
P8	P8.2.3, P8.2.4, P8.2.9, P8.2.10	**KP872586-89**	*Phormidium autumnale* SAG 35.90	98
	P8.1.5	**KP872583**	*Phormidium aerugineo-caeruleum mat MED clone 28*	97
	P8.1.3, P8.1.8, P8.1.9	**KP872582, 84-85**	*Phormidium aerugineo-caeruleum mat MED clone 28*	98
P10-1	P10.3.1, P10.3.2, P10.3.7	**KP872594, KP872635-36**	*Oscillatoriales cyanobacterium* WBK15	99
	P10.3.10	**KP872637**	*Oscillatoriales cyanobacterium* WBK15	98
P10-2	P10.1.1, P10.1.2, P10.1.6, P10.1.8, P10.1.10, P10.2.4, P10.2.5, P10.2.10	**KP872590–91, KP872631, KP872592, KP872632, KP872593, KP872633-34**	*Phormidium aerugineo-caeruleum mat MED clone 28*	98
P11	P11.1.2, P11.1.4, P11.1.7, P11.1.8, P11.2.1, P11.2.4, P11.2.9, P11.2.10, P11.3.5, P11.3.8	**KP872595-99, KP872600-04**	*Phormidium autumnale* SAG 35.90	98
	P11.3.10	**KP872605**	Cf. *Wilmottia sp.* CAWBG522	98
P14	P14.1, P14.3, P14.5, P14.9, P14.11	**KP872606-10**	*Phormidium autumnale* SAG 35.90	98
P16	P16.1	**KP872611**	*Phormidium autumnale* Arct-Ph5	93
	P16.2, P16.3, P16.4	**KP872612-14**	*Phormidium autumnale* SAG 35.90	98
P19	P19.2.8, P19.2.11	**KP872620-21**	*Phormidium autumnale* SAG 35.90	98
	P19.2.3	**KP872619**	Cf. *Wilmottia sp.* CAWBG522	98
	P19.1.11	**KP872618**	*Phormidium aerugineo-caeruleum mat MED clone 28*	97
	P19.1.3 P19.1.5, P19.1.7	**KP872615-17**	*Phormidium aerugineo-caeruleum mat MED clone 28*	98
P20	P20.1.10	**KP872624**	*Chamaesiphon subglobosus* PCC 7430	98
	P20.1.3, P20.1.7, P20.2.1, P20.2.2, P20.2.3, P20.2.4, P20.2.7, P20.2.9	**KP872622-23, KP872625-30**	*Phormidium autumnale* SAG 35.90	98

Table 4. Relative abundance (%) of cyanobacteria (with respect to total cyanobacteria) determined from optical microscope examination at each sampled site in this study. Location of sites in Figure 1B.

Species	Sites							
	P8	**P10-1**	**P10-2**	**P11**	**P14**	**P16**	**P19**	**P20**
P. incrustatum	87	78	10	33	75	77	72	98
P. aerugineo-caeruleum	5	13.5	75	55	0	0	16.5	0
Phormidium sp.	0	1.5	5	5	18.5	16	4.3	0
Leptolyngbya foveolarum	4	1.5	3.5	1.5	1	0	1	0
Leptolyngbya truncata	1	0	0	<1	0	2	1	0
Leptolynbya sp.	<1	1	0	<1	1	1.5	2	0
Aphanocapsa sp.	1.4	1.5	3	<1	1.5	1.5	1.2	0
Aphanothece sp.	1	2	3	2	3	2	2	0
Chamaesiphon sp.	0	<1	<1	1	0	0	0	2

Table 5. Comparison of sedimentary facies, deposition rates, morphotypes and phylotypes at each sampled site. Deposition rates from thickness measurements on tablets from Arenas et al. (2014). Location of sites in Figure 1B.

Sites	Sedimentary facies	Deposition (mm) April 2000-Sept 2000	Mean yearly deposition (mm) April 1999-Sept 2012	Dominant morphotype	Phylotypes
P8	C + A	3.52	7.88	*P. incrustratum*	I, III
P10-1	C + A	4.17	9.33	*P. incrustratum*	I
P10-2	B	5.13	2.68	*P. aerugineo-caeruleum*	II, III
P11	C + A	4.85	10.29	*P. incrustratum*	I, II
				P. aerugineo-caeruleum	
P14	A	9.58	16.02	*P. incrustratum*	I
P16	A	10.38	16.53	*P. incrustratum*	I, V
P19	C	2.74	7.99	*P. incrustratum*	I, II, III
P20	A	10.70	14.80	*P. incrustratum*	I, IV
				Chamaesiphon sp.	