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Cyanobacterial populations that build 'kopara' microbial mats in Rangiroa, Tuamotu Archipelago, French Polynesia

Laurent Richert[1], Stjepko Golubic[2,*], Roland Le Guédès[3], Annie Hervé[3] And Claude Payri[1]

1 Laboratoire d'Ecologie Marine, Université de la Polynésie Française, Papeete, Polynésie Française
2 Biological Science Center, Boston University, 5 Cummington Str., Boston, MA 00215, USA
3 IFREMER, Centre de Nantes, Laboratoire VP/PBA, BP 21105, 44311, Nantes, France

*: Corresponding author : Correspondence to: Stjepko Golubic, Tel.: (617) 353-2432. Fax.: (617) 353-6340. e-mail: golubic@bu.edu

Abstract:

Cyanobacterial populations, the primary producers and builders of 'kopara' microbial mats were studied in four selected ponds along the rim of the Rangiroa Atoll, French Polynesia using a polyphasic approach. Seven isolates were maintained in uni-cyanobacterial cultures, characterized morphotypically and phylogenetically by evaluating sequences of the 16S rRNA gene of about 620 base pairs in length. Cyanobacteria in natural populations were analyzed microscopically, characterized morphotypically, and compared with cultured strains. Three of the isolates were identified in the field samples: *Lyngbya aestuarii*, *Johannesbaptistia pellucida* and *Chroococcus submarinus* were present in the mats only as minor components, whereas the species of *Schizothrix* that dominated the mat community could not be cultured. The sequence of *Johannesbaptistia pellucida* is published for the first time. The phylogenetic and taxonomic relations are discussed on the basis of a reconstructed phylogenetic tree in relation to morphotypic characters. Sequences of Kopara isolates plot separately from those cultured from the lagoon of the neighbouring atoll Tikehau, indicating a narrow niche differentiation of benthic cyanobacterial taxa. The results support the application of a polyphasic approach to characterization, ecology and diversity of cyanobacteria.

Keywords: culture, cyanobacteria, diversity, kopara, microbial ecology, microbial mats, phylogeny, polyphasic approach, 16S rRNA

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LAURENT RICHERT[1], STJEPKO GOLUBIC[2], ROLAND LE GUÉDÈS[3], ANNIE HERVÉ[3] AND CLAUDE PAYRI[1]

[1]*Laboratoire d'Ecologie Marine, Université de la Polynésie Française, Papeete, Polynésie Française*
[2]*Biological Science Center, Boston University, 5 Cummington Str., Boston, MA 00215, USA*
[3]*IFREMER, Centre de Nantes, Laboratoire VP/PBA, BP 21105, 44311, Nantes, France*

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Cyanobacterial populations, the primary producers and builders of 'kopara' microbial mats were studied in four selected ponds along the rim of the Rangiroa Atoll, French Polynesia using a polyphasic approach. Seven isolates were maintained in uni-cyanobacterial cultures, characterized morphotypically and phylogenetically by evaluating sequences of the 16S rRNA gene of about 620 base pairs in length. Cyanobacteria in natural populations were analysed microscopically, characterized morphotypically, and compared with cultured strains. Three of the isolates were identified in the field samples: *Lyngbya aestuarii*, *Johannesbaptistia pellucida* and *Chroococcus submarinus* were present in the mats only as minor components, whereas the species of *Schizothrix* that dominated the mat community could not be cultured. The sequence of *Johannesbaptistia pellucida* is published for the first time. The phylogenetic and taxonomic relations are discussed on the basis of a reconstructed phylogenetic tree in relation to morphotypic characters. Sequences of Kopara isolates plot separately from those cultured from the lagoon of the neighbouring atoll Tikehau, indicating a narrow niche differentiation of benthic cyanobacterial taxa. The results support the application of a polyphasic approach to characterization, ecology and diversity of cyanobacteria.

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Introduction

Microbial mats are stratified communities arranged along steep vertical gradients of light, oxygen, sulphide, Eh and pH, with each stratum dominated by specialized guilds of microorganisms (see Riding & Awramik, 2000; Krumbein *et al.*, 2003). The illuminated surface layers are dominated by microbial oxygenic and anoxigenic phototrophs, separated by a sharp oxic-anoxic boundary (Potts; 1980; Stal *et al.*, 1985; Nicholson *et al.*, 1987; Golubic, 1991), with chemolithotrophs distributed across this boundary (Jørgensen & Gallardo, 1999). Under stress conditions, such as fluctuations in temperature, water supply and salinity, cyanobacteria often remain unchallenged as the principal primary producers of microbial mats (Golubic, 1994; Golubic *et al.*, 2000). Microbial mat communities are encountered world wide in a variety of marine, freshwater and terrestrial environments, including marine subtidal and intertidal ranges (Golubic, 1985; Pearl *et al.*, 2001; Rütters *et al.*, 2002; Abed *et al.*, 2003*a*, *b*), estuaries (Mir *et al.*, 1991), hypersaline ponds (Krumbein *et al.*, 1977), hot springs (Ward *et al.*, 1994; Lopez-Cortes, 1999*a*), and desert soils (Campbell *et al.*, 1989; Garcia-Pichel *et al.*, 2001).

A variety of shallow ponds on land rims of South Pacific atolls offer good conditions for development of thick microbial mats called 'kopara' by cyanobacteria. This system of ponds with variable water supply and a wide range of salinities provides a good model to study environmental preferences and differentiation of microbial communities. The study is relevant to our understanding of similar ancient microbial systems. Possible fossil counterparts of 'kopara'-type mats were recognized in Jurassic strata (Tribovillard *et al.*, 2000) and fossil microbial mats comprise a significant part of the stromatolite fossil record, which dominated early Earth history.

The biogeochemical properties of 'kopara' have been extensively studied with respect to relationships between organic compounds and mineralization processes (Trichet, 1967; Défarge *et al.*, 1985, 1996; Défarge & Trichet, 1990, 1993;

Correspondence to: Stjepko Golubic, Tel.: (617) 353-2432. Fax.: (617) 353-6340. e-mail: golubic@bu.edu

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Trichet & Défarge, 1997; Trichet *et al*., 2001). The role of proteinaceous compounds and amino acid composition in controlling calcification was studied in deeper layers of 'kopara' mats (Gautret & Trichet, 2005) and other microbialites (Gautret *et al*., 2004). The formation of phosphorite deposits in 'kopara' was studied by Jehl & Rougerie (1995) and Rougerie *et al*. (1997). Exopolysaccharides were studied by Rougeaux *et al*. (2001) and recently, in conjunction with the present investigation, by Richert *et al*. (2005). The ultrastructure of accumulated exopolymers (mostly polysaccharides) was studied using Cryoscan scanning electron microscopy (Défarge *et al*., 1996), but only a few studies dealt with the microbial composition of 'kopara' mats using light microscopy (Défarge *et al*., 1994*a*, *b*; Mao Che *et al*., 2001).

This study deals with polyphasic characterization of cyanobacterial populations of 'kopara' microbial mats as the principal primary producers in four selected ponds in the north-western part of the Rangiroa Atoll, by combining field observations, microscopic analysis of natural populations and cultured isolates from these populations. It explores the apparent and cryptic cyanobacterial diversity in a series of tropical ponds. The characterization assesses morphotypic and genotypic properties, the latter based on partial (620 base pair [bp]) sequences of 16S rRNA gene.

Materials and methods

Environmental setting

Rangiroa Atoll in the Tuamotu Archipelago, French Polynesia is the second largest atoll in the world covering 1,763 km^2 (Fig. 1). It consists of a narrow land rim surrounding the central lagoon. The land rim is segmented by shallow channels, locally called 'hoa', into islets called 'motu'. Most 'hoa' are shallow passages, which link the ocean and the lagoon. Along the atoll's rim there are numerous isolated shallow ponds, mostly less than 1 m deep, varying in size from tens to several hundreds of square metres. These ponds originated from the combined action of erosion and sedimentation. Parts of the land rim damaged during major storms produced depressions, which were subsequently closed by accumulating reef sand and gravel sediments. The ponds are fed by rain and, during heavy swells, by seawater from the ocean and from the adjacent lagoon. They support luxurious growth of microbial mats, which contribute to particular organic-rich sediment build-up. The salinity in these ponds is quite variable, ranging from nearly freshwater to hypersaline conditions. Major storms and swells may re-set the salinity to the values prevailing in the ocean and/or lagoon, i.e. between 34 and 36 ‰.

A typical structure of the 'kopara' mat consists of a succession of gelatinous laminae, each a few mm thick, forming layered organic-rich sediment 20–50 cm thick.

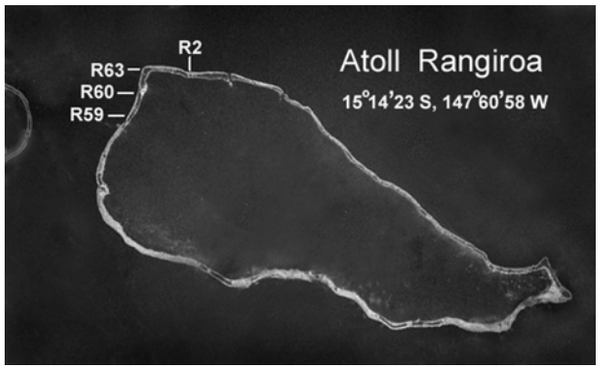


Fig. 1. Rangiroa Atoll, Tuamotu Archipelago, French Polynesia with the locations of the studied 'kopara' ponds. The neighbouring Tikehau Atoll is seen on the left. Modified from the NASA image. Scale bar: 10 km.

The surface layers of 'kopara' are orange, dark blueish-green or greyish-black depending on the prevailing microorganisms, which, in turn, respond to the degree of wetness. Deeper layers are reddish-orange alternating with white layers encrusted with carbonate (Défarge *et al*., 1985, Gautret & Trichet, 2005).

Sampling

The current study explored microbial composition in mats in four ponds located in the northwestern part of the Rangiroa Atoll designated as R2, R59, R60 and R63 (Fig. 1). Pond R2, located on the islet Pavete, was studied earlier by Défarge *et al*. (1994*a*). Mao Che *et al*. (2001) studied ponds R2 and R59 and characterized their physical and chemical properties. The other two ponds, R60 and R63 were studied for the first time. This study was initiated by two field trips, in November 2000 and November 2001. Pond R59 was sampled during both trips and the other three ponds only in 2001.

In each of the ponds studied, several sampling sites were identified and selected by colour, texture and degree of wetness of the mats. The basic environmental parameters (pH, salinity and temperature) were measured and recorded as a part of the sampling procedure. Square pieces of mats (~50 cm^2) were cut, placed in sterile plastic boxes and stored at ambient temperature overnight. They were then stored at 20°C in the dark for 48 h before the isolation procedure. Subsamples were fixed in 5% formaldehyde solution in environmental water and stored in the dark in 5-ml vials for microscopic analysis.

Isolation and maintenance of cultures

A small piece (*c*. 1 g) of mat was excised under sterile conditions and homogenized in autoclaved environmental water; 0.1 ml of the suspension was plated on solid Conway medium (agar 1.3%). The Conway medium is a modified version of the Walne medium (Walne, 1966). In 1 l of natural sea water: $NaNO_3$, 100.00 mg; Na_2EDTA, 45.00 mg; H_3BO_3, 33.60 mg; NaH_2PO_4-$2H_2O$, 26.00 mg; $FeCl_3$-$6H_2O$, 1.28 mg; $MnCl_2$-$4H_2O$, 0.36 mg; trace metal solution, 1 µl; vitamins solution,

50 µl; Trace metal solution: $ZnCl_2$, 1.05 g; $CoCl_2$-$6H_2O$, 1.00 g; $(NH_4)6Mo_7O_{24}$-$4H_2O$, 0.45 g; $CuSO_4$-$5H_2O$, 1.00 g; H_2O, 50 ml; vitamins solution: vit. B1, 400 mg; vit. B12, 20 mg; H_2O, 100 ml. The Petri dishes were incubated at 25°C with permanent artificial lighting (70 µmol m^{-2} s^{-1}) for 1–3 weeks.

During incubation, the cultures were frequently observed under light microscopy for colony picking. The apparently monospecific colonies of cyanobacteria and microalgae were picked using micromanipulation techniques (Rippka, 1988). The small colonies were transferred into liquid medium in plates with 96 wells – each well contained about 200 µl of Conway medium. The plates were incubated under the same conditions as for the Petri dishes.

The emerging colonies were checked for monospecificity using an inverted microscope, subcultured and transferred for maintenance to 30 ml of fresh liquid medium in 50-ml flasks containing 30 ml of double-spiked (2 × conc.) Conway medium at 22°C under cool-white fluorescent light (20 µmol m^{-2} s^{-1}) with a 12:12 h light-dark cycle. No attempt was made to make the isolates axenic. Five ml of the stock cultures were transferred into 25 ml of fresh Conway medium every month and pictures were taken using a Donpisha-TriCCD (Sony) digital camera mounted on a Leitz Diaplan microscope. Aliquots of the stock cultures were fixed in 3% formaldehyde and stored in darkness at room temperature for later microscopic comparative analysis.

Microscopy and morphometry

Light-microscopic analysis of fixed field samples and cultures was carried out by using an AxioStar microscope (Zeiss, Germany) equipped with transmitted light, phase contrast and Differential Interference Contrast (DIC, Nomarski) illumination. Additional information was obtained by autofluorescence using blue and green light excitation. The findings were photodocumented using an AxioCam digital camera attached to the microscope. The measurements were performed with a Zeiss Universal microscope equipped with an in-scale camera lucida. The projections of cell dimensions were scanned and subjected to morphometric analysis using Sigma-Scan measuring software (Jandel Scientific, Sausalito CA). The dimensions are given as mean ± standard deviation (number of measurements). The species dominance is expressed numerically based on percentage coverage in compressed microscopic preparations, assuming the same proportionality in volume. This estimate follows botanical practice as modified for microorganisms (Golubic, 1967).

PCR amplifications

PCR was carried out without DNA extraction to amplify a 16S rRNA gene fragment of about 650 bp in length using two of the cyanobacteria-specific primers designed by Nübel *et al.* (1997), CYA106F and CYA781R corresponding to equivalent positions of the *E. coli* genome. One ml of exponentially growing culture was centrifuged for 3 min at 9,000 *g*, the pellet rinsed twice with 500 µl of H_2O and re-suspended in 500 µl of 1 × PCR buffer (Eurogentec). The samples were stored at 4°C overnight prior to PCR.

The PCR amplifications were performed with a PTC 100 Programmable Thermal Controller (MJ Research Inc., Waltham, USA). Fifty picomoles of each primer, 25 nmol of each deoxynucleoside triphosphate, 5 µl of 10 × PCR buffer (Eurogentec), 0.5 µl of Taq polymerase (Eurogentec), and 2 µl of prepared culture were combined with H_2O to a volume of 50 µl in a 100-µl test tube and overlaid with two drops of mineral oil (Sigma Chemical Inc., Saint Louis, USA). After 10 min at 94°C, 35 cycles were carried out as follows: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The amplification was finished with the tubes kept at 72°C for 6 min before storage at 4°C. The PCR products were purified using the StrataPrep PCR Purification Kit (Stratagene, CA, USA) and sequenced commercially in both directions. The forward and reverse-complementary of the reverse sequences obtained were aligned against each other in order to check the quality of the sequences. The seven consensus sequences were then deposited at EMBL under the accession numbers: AJ621832 to AJ621838.

Phylogenetic affiliations

The sequences were first used for a BLAST search (Altschul *et al.*, 1997) to check whether they were cyanobacterial in origin. They were then added to a text file (graciously provided by Annick Wilmotte) containing 143 complete and 163 partial (> 600 bp) cyanobacterial sequences of the 16S rRNA gene. The 16S rRNA sequences of *E. coli* (J01859), *Bacillus subtilis* (AJ276351), and *Agrobacterium tumefaciens* (D14500) were used as the outgroup. The file was edited with BioEdit v5.0.9 software (Hall, 1999). All these sequences were multiple-aligned using ClustalX v1.81 program (Thompson *et al.*, 1997) prior to construction of the phylogenetic trees with MEGA v2.1 (Kumar *et al.*, 2001). Phylogenetic trees were constructed using neighbour-joining and maximum parsimony methods. The distance matrix was computed using Kimura 2-parameter model for dissimilarity values, followed by the construction of a tree with the neighbour-joining method (Saitou & Nei, 1987); these calculations were subjected to bootstrap analysis with 1,500 replicates. The maximum parsimony calculation was also subjected to bootstrap analysis (500 replicates).

Morphotype identification

The collected and cultured cyanobacteria were characterized morphotypically and identified to the species level, using traditional phycological determination manuals of Gomont (1892), Bornet & Flahault (1886–88), Tilden (1910), Geitler (1932), Kossinskaya (1948), Desikachary (1959) and Umezaki (1961). Generic assignments were made in consultation with newer phycological (Anagnostidis & Komárek, 1985; Komárek & Anagnostidis, 1989, 1999, 2005; Komárek, 1994) and bacteriological systems (Rippka *et al.*, 1979;

Fig. 2. 'Kopara' microbial mat in pond R59 on the NW rim of Rangiroa Atoll. Note the colour changes from the wet centre to the dryer margins of the pond.

Castenholz *et al*., 2001). Distinct populations of species that could not be related to published and named taxa were identified to the genus level with a species designation marked provisionally by a number.

Results

The 'kopara' ponds as microbial habitats

The 'kopara' ponds studied could be divided into two groups on the basis of their prevailing salinity. Ponds R59 (Fig. 2) and R60 are characterized by fluctuating salinity with periodic returns to sea water values, following recharging during storm events. Prolonged calm and dry periods introduce hypersaline conditions in these ponds, whereas they turn brackish in rainy periods. Pond R60 is located in a protected tortuous branch of a 'hoa', connected with the lagoon at high tide. The 'kopara' mat of this pond is submerged by lagoonal water only when the tide is relatively high. During extended periods of exposure, the mat surface dries out, hardens, turns dark grey to black in colour and cracks into polygons. A gradient becomes established with relative moisture increasing from the edge towards the centre of the pond. The wet part of the mat is soft and orange-brown in colour. Pond R59 is larger than pond R60 and is well delimited as it is in an enclosed depression on the land rim of the atoll. It is separated from the ocean by several hundred metres of land with terrestrial vegetation and from the lagoon in places only by a beach ridge comprised of coral rubble. The 'kopara' mat covers a larger surface in this pond and the wet-to-dry gradient is better expressed. The cracks are deeper and the polygons thicker than in pond R60 and the wet parts in the protected areas are bright orange.

In contrast, ponds R2 (Fig. 3) and R63 exhibited consistently low salinity correlated with their isolated position within the land rim of the atoll (Table 1). Défarge *et al*. (1994*b*) noticed a rain-dependent decrease of salinity in pond R2 from 8.9 ‰ in 1983 to 1.0 ‰ in 1992. We measured a salinity of 7.8 ‰ in November 2001. Pond R63 was a well protected depression separated from both ocean and the lagoon and surrounded by land vegetation. It was in part deeper than 1 m and fish were observed to live in this pond. The 'kopara' mat was well expressed at the edges of this pond. Polygon formation and colour distribution was similar to those observed in ponds R59 and R60. Pond R2 was larger than pond R63 and the mat was very well developed. The colour of large areas of the mat surface, however, was different from other ponds: it was green to pale orange in the wet parts. The familiar coloration gradient was here compressed against the shore, showing a narrow orange zone followed by an equally narrow dark zone with polygons along the shoreline (Fig. 3).

Natural populations of cyanobacteria were analysed by light microscopy for species composition and abundance in ponds R2 and R59, which were selected as representative local end members with respect to the observed salinity gradient. Pond R2, located within the atoll's rim maintained very low salinity levels, whereas pond R59 fluctuated from hypo- to hypersaline conditions relative to the surrounding ocean and the nearby lagoon. The samples from all four ponds were used as a source of inocula for culturing.

Field populations of cyanobacteria

The microscopic analysis of field populations focused on actively growing surfaces of the mat,

Fig. 3. Microbial mat zonation at the margin of pond R2. Dried, polygonally cracked mats in the front of the picture are dark (black when wet) due to high concentration of UV-protective scytonemin pigment. The zone with bumpy surface in the centre of the picture is regularly wetted and bright orange in colour due to high concentration of carotenoid pigments. A germinating coconut serves as a scale.

Table 1. Water temperature, salinity and pH in four ponds studied on Rangiroa Atoll.

Pond	R59	R60	R2	R63
Temperature (°C)	33.7–40.0*	31.1	32.0	36.6
Salinity (‰)	12.0–49.0*	27.3	7.8	6.1
pH	9.5–8.3*	8.4	7.7	8.2

*Measured November 2000.

less than 1 cm in depth. Samples in each pond were collected along a gradient of varying water supply from dry toward wet, as identified by surface colour, wetness, consistency and internal texture and by species composition of the mat.

The species composition of the actively growing surface layer of microbial mats was analysed in five sets of samples, two from Pond R2 and three from Pond R59. The results are presented in Table 2. Cell dimensions characterizing particular populations are listed as cell width × length (shorter × longer cell dimension for coccoid species). Species determination was carried out wherever a relationship to published descriptions could be established. The abundance was estimated as% coverage (see Golubic, 1967).

Differences in species composition and dominance were conspicuous within each pond along the gradient of water supply and retention. The change in species composition along this desiccation gradient was related with the observed change in colour and texture of the mat (Fig. 3). Microbial mats along the margins of 'kopara' ponds were often polygonally cracked by desiccation. *Scytonema* cf. *myochrous* (Dillwin) Agardh (Figs 4–6) occurred in ponds with relatively low salinity (R2-4). This is a large cyanobacterium with 25.9 ± 2.1 (16) µm wide falsely branched filaments. Thick, dark brown sheaths with upward diverging layering surround 8–10-µm wide trichomes. Cells are short and compacted in the meristematic apical regions becoming longer and torulose in the older, mature trichome segments. Dry habitats in the ponds with higher salinities (R59-8) were occupied by *Calothrix* sp. (Figs 7, 8) and to a lesser extent by *Lyngbya aestuarii* (Mertens) Liebman (R59-5). Both taxa are protected by thick, layered, scytonemin-stained sheaths. *Lyngbya aestuarii* was successfully transferred into culture (see below).

Microbial mats in the slightly wetter zone inside the peripheral dark rim around the ponds were intensive yellow-orange in colour due to a high concentration of intracellular carotenoid pigments. They were observed in all ponds, but were particularly well developed in Pond R59, where the salinity fluctuated between brackish and hypersaline levels. Scanning electron microscopy (SEM) of critical-point dried preparations of these mats showed that they comprised intertwined filaments of variable diameter (Figs 9–11). Light microscopy revealed that these mats were built by multitrichomous filaments of two species of *Schizothrix*. In pond R59, they were distributed in a mosaical pattern, in which the larger one, identified as *S*, *splendida* (Golubic, 1973) formed cushions up to 5 cm in diameter elevated up to 1 cm above the water level (Figs 12, 13). The depressions around these cushions were overgrown by the smaller species designated here as *Schizothrix* sp. 2 (Fig. 16; Table 2). *S*, *splendida* achieved complete dominance in dryer portions of the mat (samples R59-5, R59-8). A third species, designated here as *Schizothrix* sp. 1 (Figs 14, 15) was dominant only in the semi-dry samples from pond R2. None of the *Schizothrix* species encountered in 'kopara' mats grew in culture.

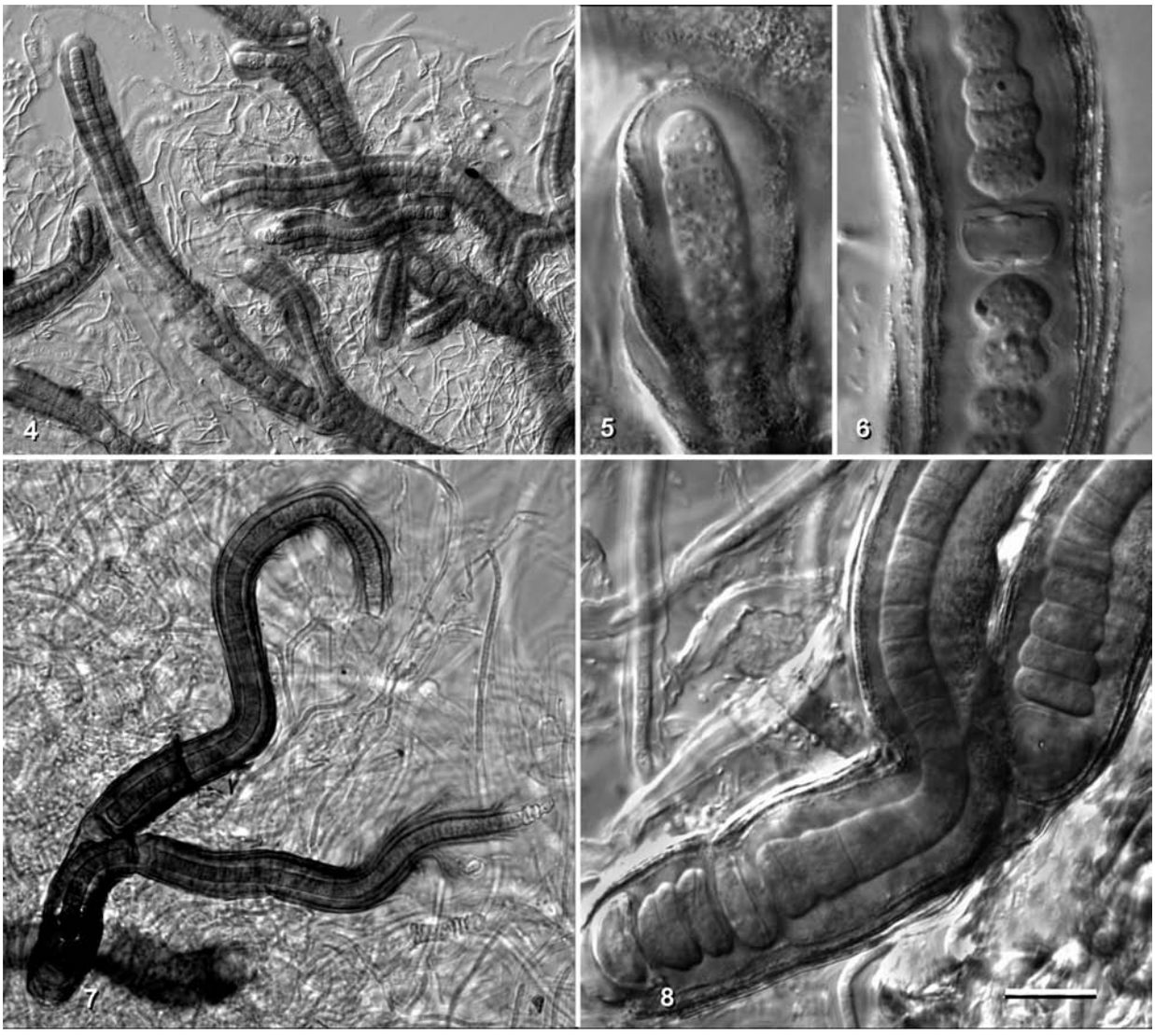
Table 2. Cell dimensions and abundance of cyanobacteria in 'kopara' ponds.

Taxon	Cell width × length (μm)[a]	Abundance in each pond[b]				
		R2-1	R2-4	R59-5	R59-2	R59-8
Schizothrix splendida.	2.03 ± 0.37 (196) × 4.59 ± 0.98 (175)			1	5	5
Schizothrix sp.1	1.35 ± 0.15 (52) × 2.66 ± 0.52 (52)	2				
Schizothrix sp.2	0.88 ± 0.17 (51) × 4.13 ± 0.90 (47)			3	+	+
Scytonema cf. *myochrous*	9.02 ± 0.87 (15) × 5.11 ± 1.02 (35)		2			
Calothrix sp.	5.89 ± 0.98 (7) × 2.61 ± 0.58 (14)				1	2
Lyngbya aestuarii	8.92 ± 0.37 (11) × 1.82 ± 0.44 (11)				1	
Oscillatoria spp.	na	1	2	2	1	1
Leptolyngbya spp.	na	1	1	1	1	1
Spirulina spp.	na	1		2	1	
Johannesbaptistia sp.1	4.93 ± 0.63 (67) × 2.74 ± 0.65 (62)	1	1			
Johannesbaptistia sp.2	3.02 ± 0.36 (227) × 1.06 ± 0.22 (223)		1	1		
Chroococcus sp.1	9.02 ± 3.77 (5) × 12.74 ± 1.21 (5)	1	1	1		
Chroococcus sp.2	6.22 ± 1.70 (14) × 8.08 ± 0.81 (14)	1	1	1		
Chroococcus sp.3	3.92 ± 0.64 (58) × 5.16 ± 0.69 (58)	1	1	1		
Aphanocapsa sp.1	2.42 ± 0.31 (146) × 2.83 ± 0.33 (146)			1	1	
Aphanocapsa sp.2	2.91 ± 0.31 (12) × 2.52 ± 0.42 (12)	1				
Aphanocapsa sp.3	4.70 ± 0.37 (16) × 5.24 ± 0.22 (16)			1		
Aphanothece sp.1	3.86 ± 0.32 (107) × 5.64 ± 0.88 (105)	1				
Aphanothece sp.2	3.82 ± 0.36 (15) × 7.74 ± 1.39 (16)		1			
Aphanothece sp.3	2.12 ± 0.24 (35) × 2.84 ± 0.64 (29)				1	
Gloeocapsa deusta	2.50 ± 0.44 (7) × 3.52 ± 0.41 (7)				+	
Gloeocapsa sp.1	3.24 ± 0.78 (20) × 4.24 ± 0.62 (20)	1				
Gloeothece sp.1	2.95 ± 0.26 (24) × 4.98 ± 0.48 (16)	1				
Gloeothece sp.2	4.58 ± 0.61 (46) × 6.71 ± 1.06 (44)			1		
Xenococcus sp.	na	1				

[a] These dimensions are expressed as mean ± SD (number of measurments); na = not applicable.
[b] Abundance codes: 5 = 75–100% coverage; 4 = 50–75% coverage; 3 = 25–50% coverage; 2 = 10–25% coverage; 1 = < 10% coverage, but frequent; + = rare.

Microbial mats in pond R2, with lower and stable salinity, were significantly more diverse than those in pond R59. The colour ranged from pale orange to blueish-green. Populations of different *Spirulina* species and cyanobacteria with extremely narrow trichomes classified as *Leptolyngbya* spp. alternated in frequency of occurrence without prominent dominance. The group of species listed in Table 2 as *Spirulina* spp. refers to the following identified morphospecies: *S*, *tenerrima* Kützing, *S*, *subtilissima* Kützing, *S*, *subsalsa* Oersted and *S*, *labyrinthiformis* (Meneghini) Gomont. The most common were *S*, *tenerrima* with helices 1.32 ± 0.13 (69) μm wide and 9–10 windings per 10 μm length of helix (Figs 17–21) and *S*, *subtilissima* with helices 2.49 ± 0.2 μm wide and 6–7 windings per 10 μm length. Several sheathed cyanobacteria with narrow trichomes, classified as *Leptolyngbya*, less than 1 μm wide were present in these mats in considerable densities. Some of these morphotypes are characterized by aerotopes (intracellular clusters of gas vesicles; Figs 22, 23), as determined by the use of Differential Interference Contrast (DIC) microscopy. Others have gas vesicle clusters at the tip of their terminal cells (Figs 24–26). These observations need to be confirmed by electron microscopy. Some of these filaments are distinguished by their extremely small cell dimensions (Figs 27–29). The identification as cyanobacteria needed to be confirmed by autofluorescence to distinguish them from flexibacteria that were also present in the mat (e.g. Fig. 30, upper right). Several small filamentous cyanobacteria occurred intermingled in the mat as free trichomes. These could be classified within the genus *Geitlerinema* (*Oscillatoria*, *sensu* Geitler).

Coccoid cyanobacteria commonly occurred interspersed among filamentous members of the mat. They included colonial forms with division in one plane representing different species of *Aphanothece* (Figs 31–33), and those dividing in two or three planes such as *Aphanocapsa* (Figs 34, 35) and *Gloeocapsa* (Fig. 36), At least three different species of *Chroococcus* were observed as minor constituents of the mats, occurring in small clusters (Figs 37–41). These populations were distinct, with hardly any overlap in size (Table 2) and were considered different species. The largest, *Chroococcus* sp.1. (Figs 37, 38), was most likely the source of the cultured strain *C*, cf. *submarinus* strain BM. The two smaller taxa, *Chroococcus* sp. 2 and 3 did not correspond to any known species and may represent new species. Clusters of cells with variable dimensions (Fig. 42) possibly belong to cyanobacteria with multiple fission, such as *Xenococcus* spp.



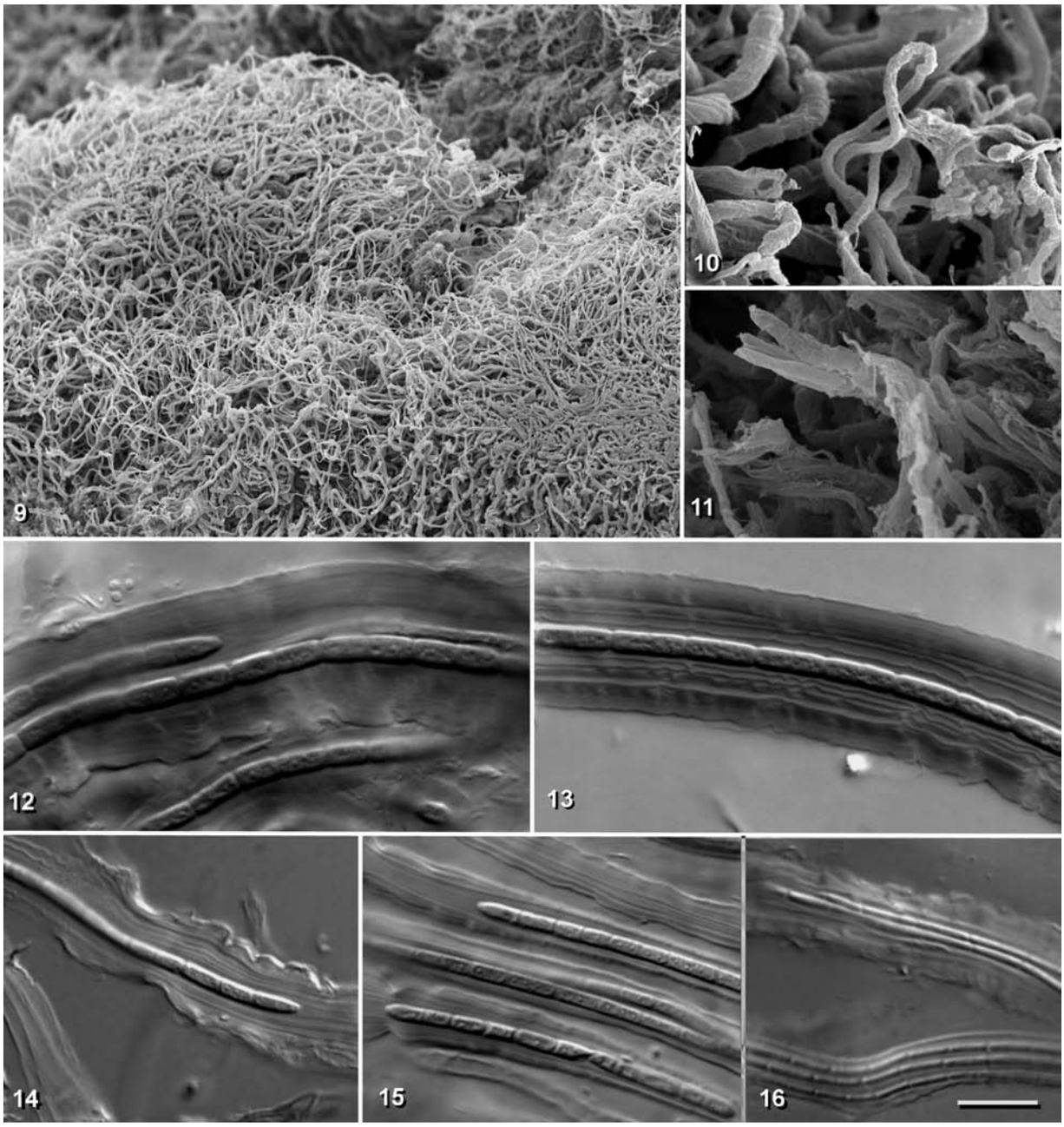
Figs. 4–8. Filamentous, heterocystous (N-fixing), falsely branched cyanobacteria responsible for dark colouration of the mat along the margins of 'kopara' ponds. These relatively large microorganisms are embedded into a matrix of finer filamentous cyanobacteria of *Leptolyngbya* morphotype. Fig. 4. Filaments of *Scytonema* cf. *myochrous* from pond R2, with dark scytonemin pigment invested in the sheaths. Fig. 5. Club-shaped 'meristematic' growth-zone at the tip of a *Scytonema* trichome, characterized by compact stacks of short cells. Fig. 6. Central portion of the filament with torulose trichome cells and a bipolar intercalary heterocyst. Fig. 7. *Calothrix* cf. *pulvinata* from pond R59. Fig. 8. Detail of false branching in *Calothrix* with differentiated basal unipolar heterocysts. Scale bars: 100 μm (Fig. 4), 50 μm (Fig. 7) and 10 μm (Figs 5, 6, 8).

Two different morphotypes of *Johannesbaptistia pellucida* (Dickie) Taylor et Drouet (listed in Table 2 as *Johannesbaptistia* sp. 1 and 2) occur in separate populations with distinctive size ranges (see below). Both morphotypes co-occurred in pond R2, but only the larger one was found in pond R59. One strain of this species has been successfully isolated and grown in culture.

Phylogenetic and morphotypic characterization of cultured isolates

None of the dominant forms of cyanobacteria observed in natural samples grew in our cultures, but most cultured cyanobacteria were identified in natural populations as minor components. The isolation and culturing lead to establishment of seven uni-cyanobacterial cultured strains, three of coccoid and four of filamentous cyanobacteria:

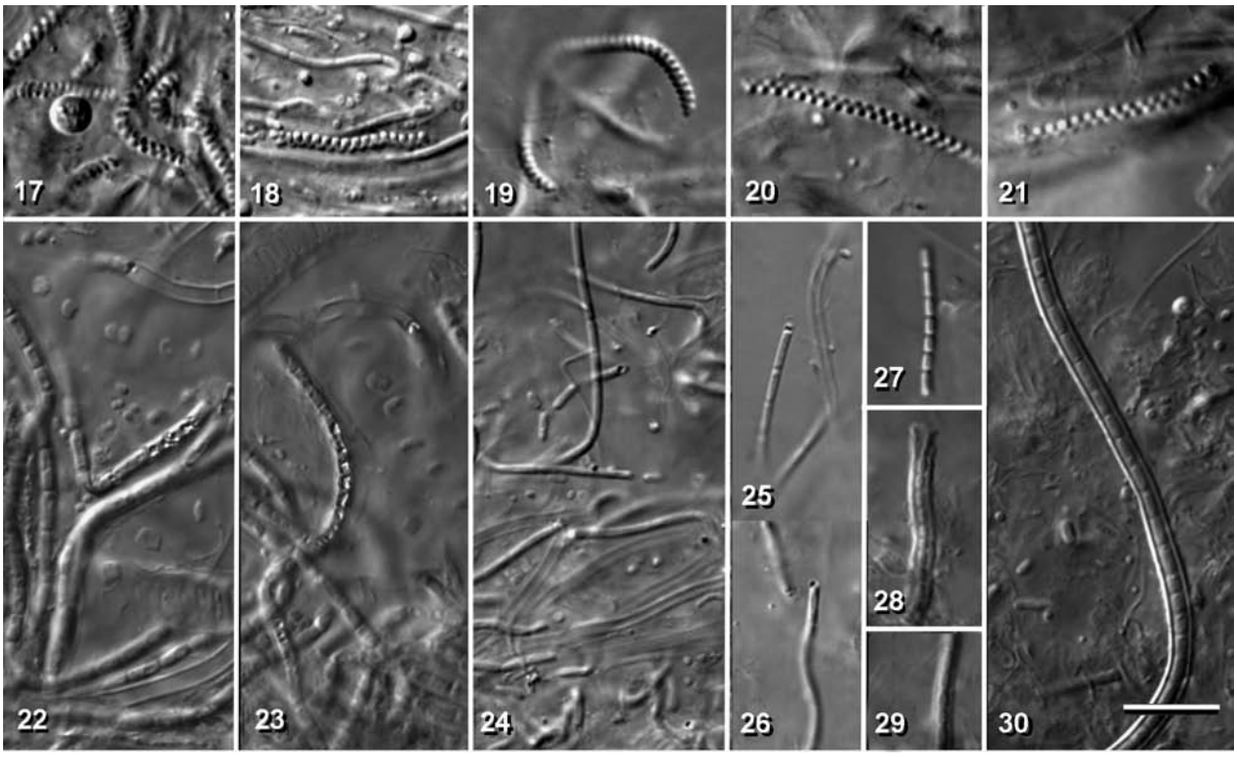
(1) C*hroococcus* cf. *submarinus* (Hansgirg) Kováčik – strain BM;
(2) *Johannesbaptistia pellucida* (Dickie) Taylor et Drouet – strain GC;
(3) *Rhabdoderma* cf. *rubrum* (Ålvik) Komárek et Anagnostidis – strain CH;
(4) Geitlerinema (*Oscillatoria*) sp. – strain FE;



Figs. 9–16. *Schizothrix* species are dominant mat-building cyanobacteria in 'kopara' ponds, comprising most of the biomass of the bumpy brightly orange mats. Fig. 9. SEM of critical point-dried fabric of multitrichomous filaments of *Schizothrix splendida* forming1–2-cm high cushions on surface of mat. Figs 10, 11. Details of fabric. Figs 12–16. DIC (Nomarski contrast) light micrographs. Fig. 12. Multitrichomous filaments of *Schizothrix splendida* from pond R59. Fig. 13. Filament of *Schizothrix splendida* with single trichome and wide textured and birefringent sheath. Fig. 14. Trichome of *Schizothrix* sp.1, from pond R2 with conical terminal cell, surrounded by wavy, externally diffluent sheath. Fig. 15. A bundle of filaments of *Schizothrix* sp.1. Fig. 16. Multitrichomous filament of *Schizothrix* sp.2 from pond R59. Scale bars: 100 μm (Fig. 9), 20 μm (Fig. 11) and 10 μm Figs (10, 12 –16).

(5) *Lyngbya aestuarii* (Mertens) Liebman – strain LY;
(6) *Leptolyngbya* cf. *golenkiniana* (Gomont) Komárek & Anagnostidis – strain FF;
(7) *Pseudophormidium* cf. *battersii* (Gomont) Komárek & Anagnostidis – strain GF.

These organisms were characterized phenotypically by their microscopic morphological properties and phylogenetically by analysing partial, (~620 bp) 16S rRNA sequences. Their phylogenetic affiliation was first assessed through the BLAST search, which related them to complete or partial 16S rRNA gene sequences published in the data banks (Genbank, EMBL, DDBJ). The closest affiliations varied between 99% and less than 92%. The seven sequences obtained were then multiple-aligned with 143 complete and



Figs. 17–30. Small filamentous cyanobacteria distributed within the matrix of the 'kopara' mats. Figs 17–21. *Spirulina tenerrima* occurred in small groups among fine filamentous cyanobacteria. Figs 22, 23. A sheathed *Leptolyngbya*-type cyanobacterium with coarse intracellular gas vesicle clusters. Note the sheathed trichomes on the top of both pictures and the hood-like gas vesicle clusters in the terminal cells (also in Fig. 21, upper right). Figs 24–26. A submicron-sized sheathed *'Leptolyngbya'* morphotype with light-refracting terminal gas vesicle clusters. Figs 27–29. Fine, filamentous *'Leptolyngbya'* forms with shorter, clearly separated cells. Fig. 30. A larger *'Leptolyngbya'* form with thick sheaths, without gas vesicles. Scale bar: 10 μm.

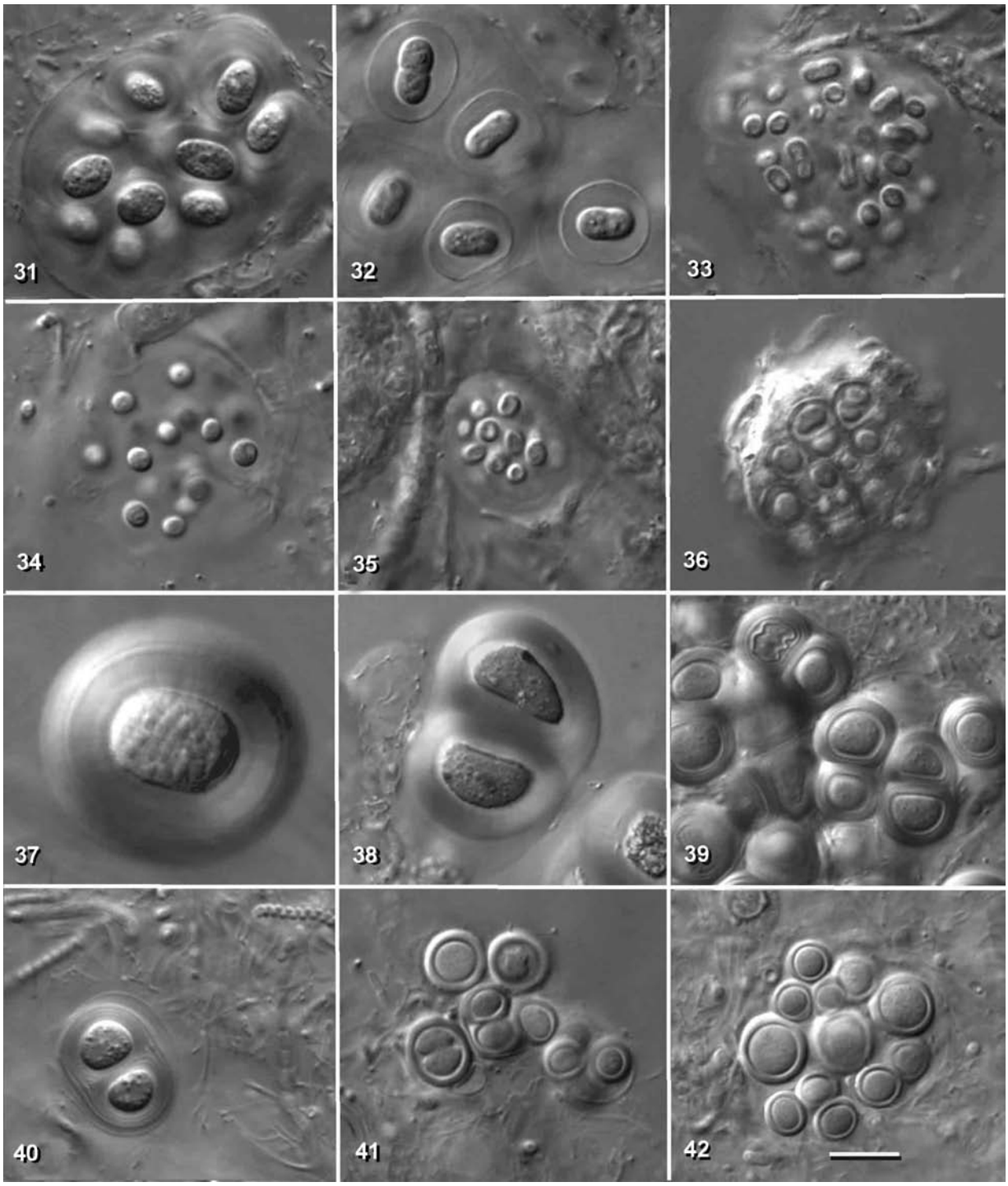
163 partial (>600 bp) known sequences of the cyanobacterial 16S rRNA gene. The interrelationships were expressed by their positions in the constructed phylogenetic trees. The topology of the trees is the same with the two methods used, neighbour-joining and maximum parsimony. Only the distance tree is presented here as a simplified outline in Fig. 43. The isolates are marked by bold lines and lettering.

The total number of nucleotides compared was 600 after elimination of non-informative portions. Fifteen shorter partial (~450 bp) sequences of mat-forming cyanobacteria from the lagoon of the neighbouring atoll Tikehau (Abed *et al*., 2003*b*) were added for comparison (TK, bold).

1. *Chroococcus* cf. *submarinus* strain BM (Figs 44–47) is characterized by cells dividing by cleavage in three planes, surrounded by firm gelatinous envelopes. Cells are isodiametric, sometimes mutually flattened at contact, elongated in the course of the division with shorter to longer dimensions: 7.78 ± 1.09 (59) μm × 10.35 ± 1.28 (59) μm. The cultured strain occupies by its size an intermediate position between the natural populations of *Chroococcus* sp. 1 and 2. (Figs 37, 38; Table 2).

There are at present no 16S rRNA gene sequences available in data banks for the genus *Chroococcus*, although two *Chroococcus* strains are maintained in culture collections, identified as *Chroococcus turgidus* PCC 7946 and *Chroococcus prescottii* Drouet and Daily CCAP 1412/4. The sequence of our *Chroococcus* cf. *submarinus* strain BM plots distant from all published sequences. This strain occupies a position close (8.1% divergence) to *Chroococcus turgidus* PCC 9340 in the phylogenetic tree published in Bergey's manual, i.e. between clusters VII and VIII of Wilmotte & Herdman (2001). The next closest sequence with 8.8% divergence is *Cyanothece* PCC 7424 (isolated by P.A. Roger from a rice field, Senegal), which is currently the PCC reference strain for the *Cyanothece* group, subcluster 1.1.

2. *Johannesbaptistia pellucida* strain GC (Figs 48, 49) is a coccoid cyanobacterium, dividing in one plane forming series of cells, often in pairs after division, embedded into a thick smooth gelatinous sheath. Cells of the cultured strain are wider than long, 10.87 ± 1.94 (122) μm wide and 6.12 ± 2.14 (116) μm long. They are significantly larger than the cells in natural populations (Fig. 50; Table 2). As in the case of *Chroococcus*, there are



Figs. 31–42. Coccoid cyanobacteria in 'kopara' mats. Figs 31–33. Three species of *Aphanothece* distinguished by cell division in one plane and production of extracellular gel. Figs 34, 35. Two species of *Aphanocapsa*, which divide in two or three planes, but form similar colonies held by extracellular gel. Fig. 36. *Gloeocapsa* cf. *deusta* with scytonemin-stained envelopes, Figs 37–41. Different morphotypes of *Chroococcus* with populations covering a wide spectrum of sizes. Fig. 42. Clonal cluster of cells of *Xenococcus* sp. Variable sizes within the same population are characteristic of cyanobacteria with multiple fission. Scale bar: 10 μm.

at present no 16S rRNA gene sequences available in data banks for *Johannesbaptistia*. To our knowledge, there are also no strains of *Johannesbaptistia* available in culture collections. The closest sequence with about 7.5% divergence was *Gloeocapsa* PCC 73106, isolated by R. Rippka from a sphagnum bog in Switzerland and the next closest was *Cyanothece* ATCC 51142 with 8.9% divergence.

3. *Rhabdoderma* cf. *rubrum* strain CH (Fig. 51) exhibited properties between those of coccoid and filamentous cyanobacteria. It is a cyanobacterium

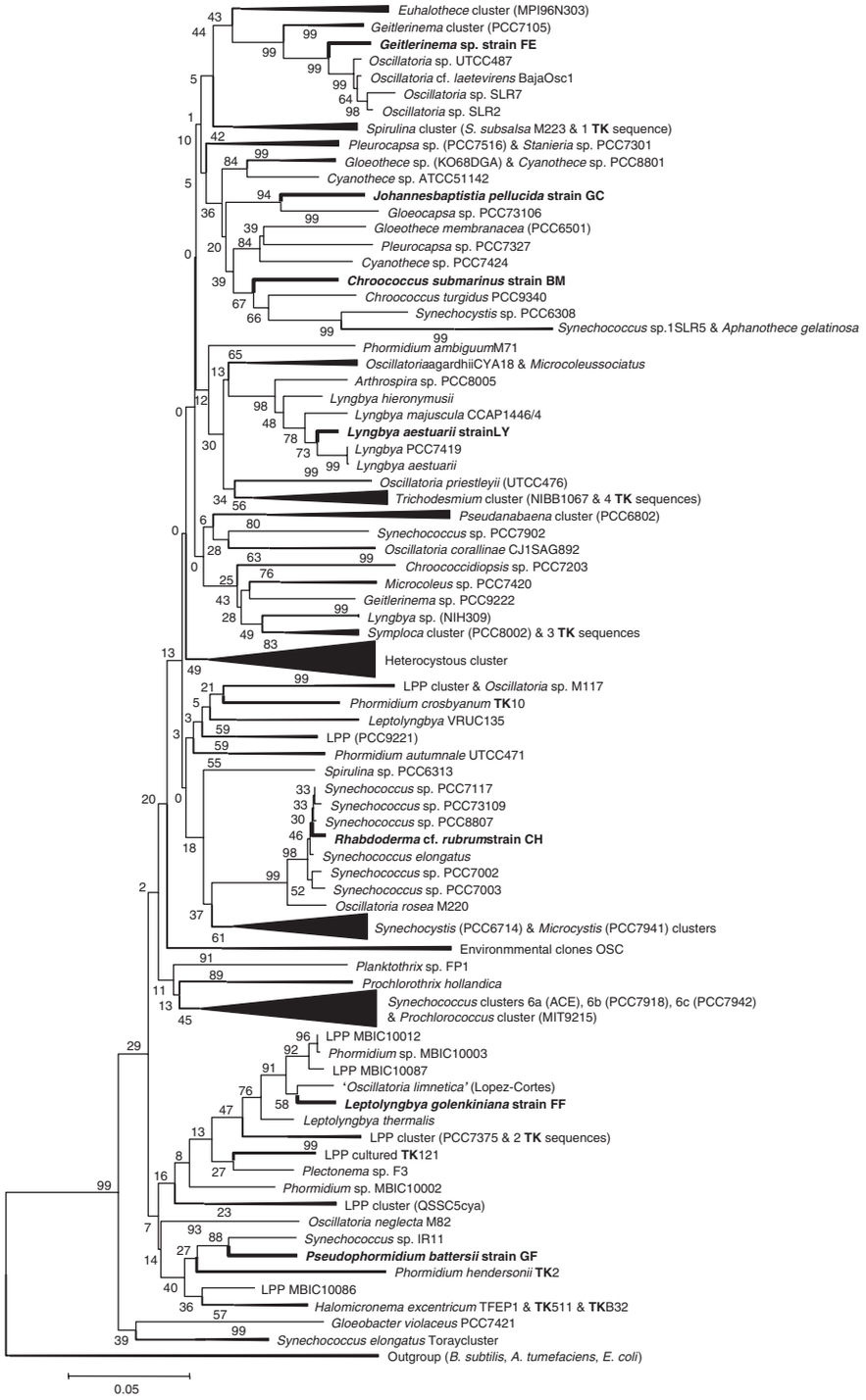
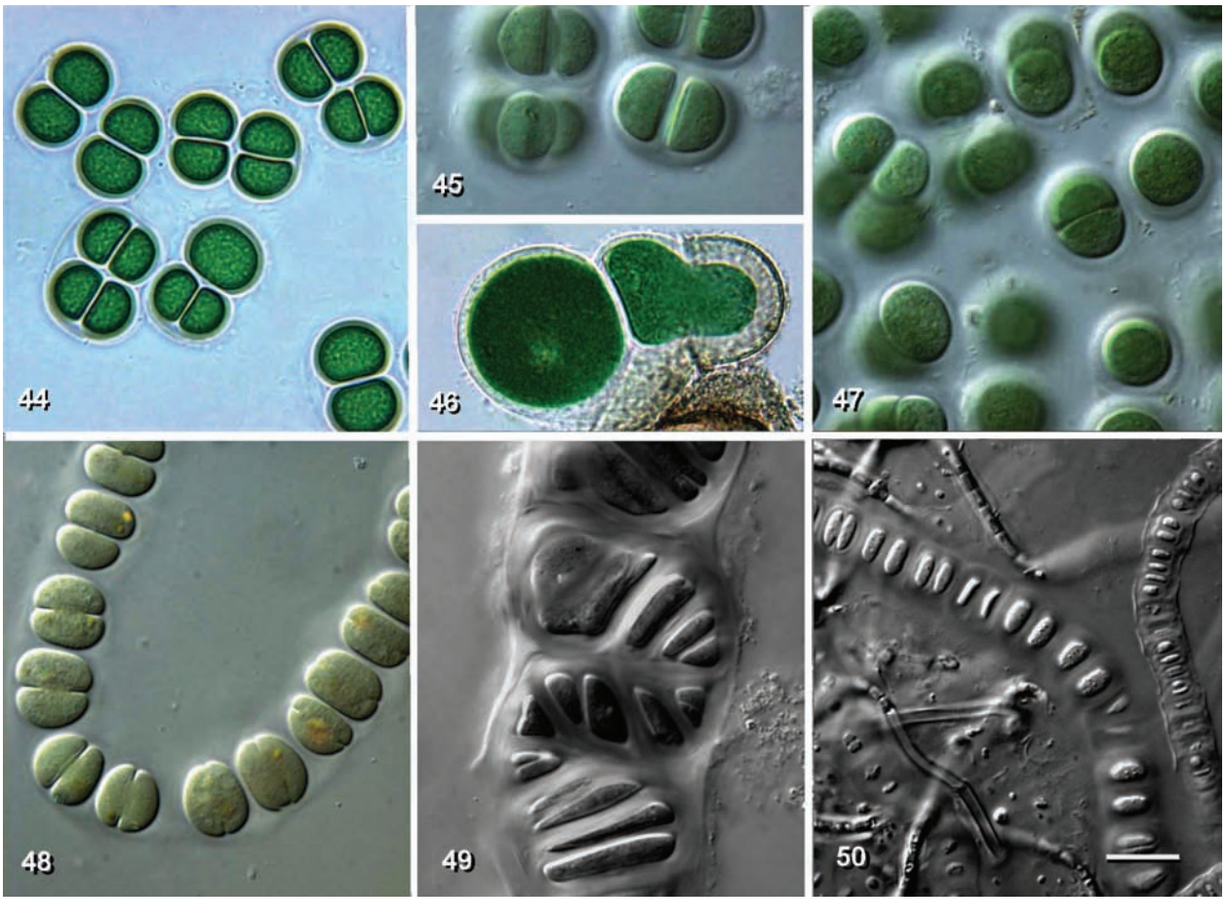


Fig. 43. The positions of 'kopara' mat isolates within a simplified 'closest neighbour' phylogenetic tree for cyanobacteria, bold lines and letters showing the positions of *Geitlerinema* sp. strain FE, *Johannesbaptistia pellucida* strain GC, *Chroococcus submarinus* strain BM, *Lyngbya aestuarii* strain LY, *Rhabdoderma rubrum* strain CH, *Leptolyngbya golenkiniana* strain FF, *Pseudophormidium battersii* strain GF and 15 sequences of Tikehau lagoonal populations (TK). Triangular branches represent condensed clusters. Numbers are bootstrap percentages.

with rod-shaped cells dividing in one plane and forming short series of 4–8 cells, without visible sheath or envelope, with cells 1.54 ± 0.24 (198) μm wide and 2.29 ± 0.90 (221) μm long. The strain has not been observed in natural populations. Morphologically, it is close to *Rhabdoderma rubrum* (Ålvik) Komárek & Anagnostidis, which was also described from cultures isolated from salt-water bodies along the coast of Norway (Komárek & Anagnostidis, 1999, p.115).

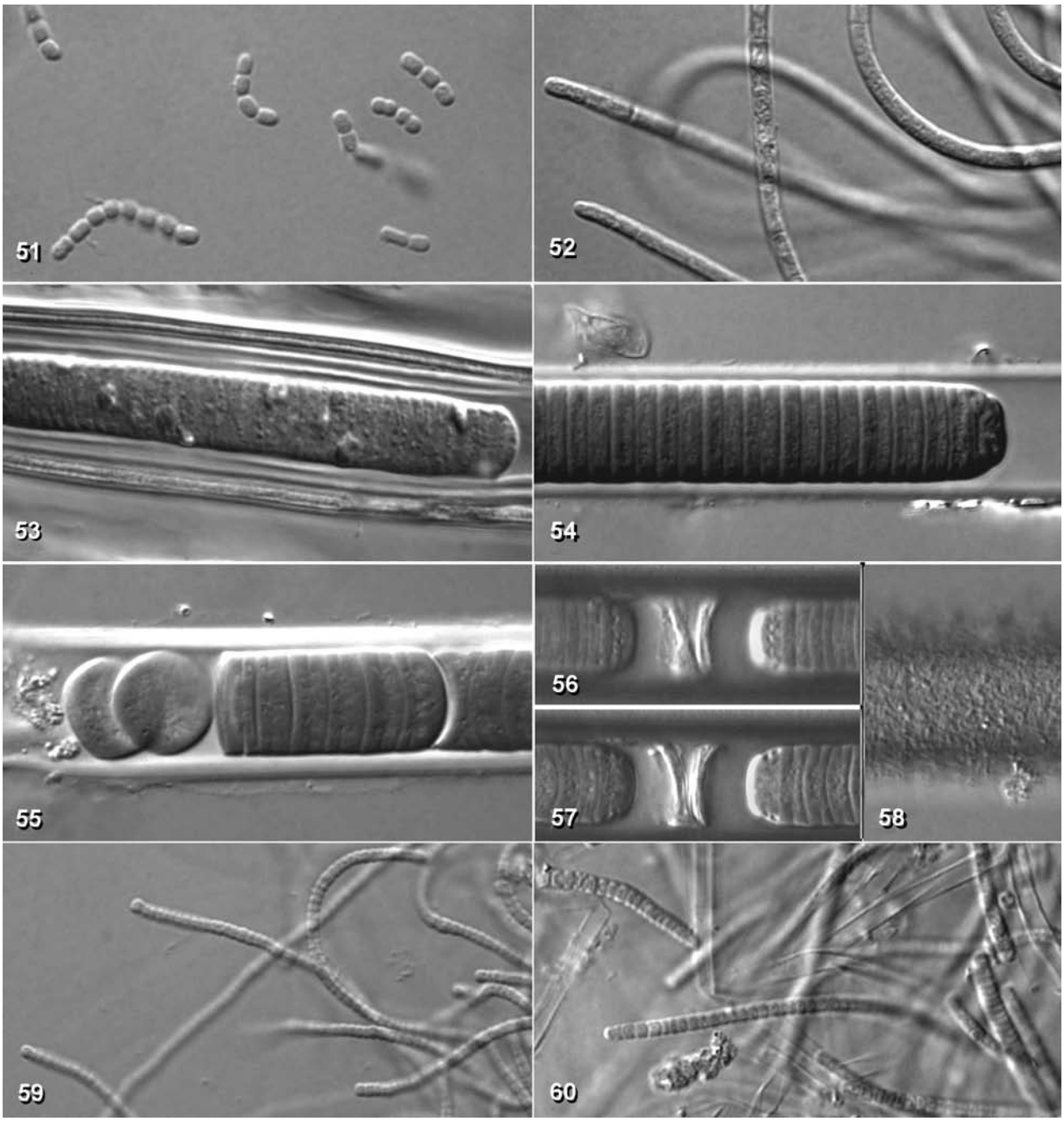
The sequence of *Rhabdoderma* cf. *rubrum* strain CH plots close to several marine *Synechococcus*



Figs. 44–50. Cocoid cyanobacteria isolated in culture and sequenced. Figs 44–47. *Chroococcus submarinus* strain BM in culture. Fig. 44. Culture shows stable rhythmic growth with cells dividing by binary fission and separating after two subsequent divisions. The intermittent production of gelatinous envelopes confines the cells following cleavage. Fig. 45. Culture showing binary fission in three planes forming groups with separation after three subsequent divisions. Fig. 46. Teratologic irregular cell size increase in freshly transferred culture. Fig. 47. Culture with rapidly dividing cells separating after each division. Figs 48–50. *Johannesbaptistia pellucida* in culture and nature. Fig. 48. Cultured strain GC showing stable growth and normal morphology. Cells divide in one plane and remain connected in a series by the intercellular gel. Fig. 49. Teratologic deformation of shape in and cells shifted by crowding, following the transfer into fresh medium. Fig. 50. *Johannesbaptistia* populations observed in nature among other mat microorganisms including *Leptolyngbya* morphotypes with gas vesicle clusters (lower left). Scale bars: 20 μm (Fig. 44), 25 μm (Fig. 46), 10 μm (Figs 45, 47–50).

sequences: within 0.7% divergence of *Synechococcus* PCC 7117 (isolated by A. Neilson from a low salinity brine pond, Port Hedland, Western Australia), at 0.8% divergence of both *Synechococcus* PCC 8807 (isolated by K. Giebler & R. Rippka from a lagoon at Port Gentil, Gabon) and a strain identified as *Synechococcus elongatus*, isolated by A. Lopez-Cortes (1999*a*) from calcareous oncoids in a coastal thermal spring in Bahia Concepcion, Baja California, Mexico (salinity of 2.9% and temperature ranging from 28 to 45°C, pH 7.1). The marine strain of *Synechococcus* PCC 73109 (isolated by C. Van Baalen from sea water at City Island, New York) shows 1.0% divergence with our strain of *Rhabdoderma* cf. *rubrum*. Because of the formation of distinct cell series by this organism and the known polyphyletic nature of the genus *Synechococcus*, we preferred to affiliate this morphotype with the morphogenus *Rhabdoderma* Schmidle & Lauterborn, a designation which may be applicable to other phylogenetically and ecologically close isolates of the same branch.

4. *Geitlerinema (Oscillatoria)* sp. strain FE (Fig. 52) is a motile cyanobacterium with narrow, somewhat rigid trichomes, slightly attenuated and curved at the tip, without sheath. The constrictions at the cross walls are absent or barely noticeable, the cells slightly longer than wide: 2.33 ± 0.31 (121) μm wide and 3.55 ± 1.14 (122) μm long. The end-cell is narrower than trichome cells and rounded. This sequence clusters within 3.1% divergence with *Oscillatoria* cf. *laetevirens* (isolated by T.L. Nadeau and R.W. Castenholz from a hypersaline pond in Guerrero Negro, Baja California, Mexico), 3.2% divergence with the



Figs. 51–60. Filamentous cyanobacteria isolated in culture and sequenced, Fig. 51. *Rhabdoderma* cf. *rubrum* strain CH forms short cell series of 4–8 cells following division in one plane. Fig. 52. *Geitlerinema* sp. strain FE with trichome tips slightly tapered and bent. Figs 53–58. *Lyngbya aestuarii* as observed in nature and culture. Fig. 53. Field population in 'kopara' pond R59 with characteristic layered yellow-brown sheath. Fig. 54. Cultured *Lyngbya aestuarii* strain LY, showing trichome tip with a slightly narrower calyptrate end cell within a firm but thin sheath. Fig. 55. Degraded trichome with cells separating like coins in a stack (left), and a biconcave collapsing necridic cell (right). Figs 56, 57. Two optical sections of a site of trichome fragmentation, which starts with the biconcave collapse of necridic cells, and ends by differentiation of terminal cells at the two ends of separating fragments. Note the formation of a ring of probable gas vesicle clusters around the new terminal cells. Fig. 58. External surface of an old *Lyngbya* sheath degraded by bacteria. Fig. 59. *Leptolyngbya* cf. *golenkiniana* strain FF. Fig. 60. *Pseudophormidium* cf. *battersii* strain GF. Scale bar: 10 μm.

highly salt resistant *Oscillatoria* UTCC 487 (isolated by J.A. Hellebust from potassium mine drainage in Saskatchewan, Canada), and at 3.5% divergence with *Oscillatoria* sp1-SLR2 (isolated by T. Kuritz from hypersaline alkaline lake Qaroun, Egypt). More distantly at 7.0% divergence there is the *Geitlerinema* cluster including the PCC reference strain for a marine species of *Geitlerinema* strain PCC 7105. This identification is compatible with morphological characterization, particularly

with respect to the terminal cell morphology, but the strain FE is smaller than given in the species description of *Oscillatoria laetevirens* (Gomont, 1892).

5. *Lyngbya aestuarii* strain LY (Figs 54–58) is characterized by short-cells, somewhat rigid trichomes within a thick layered and brown-pigmented sheath. Trichomes are slightly constricted at the cross walls and briefly attenuated at the tip with a distinct hourglass-shaped calyptra. Cells are 12.48 ± 1.30 (51) μm wide and 2.51 ± 0.47 (46) μm long. The strain LY sequence is closest to two sequences corresponding to the PCC 7419 strain, which is the PCC type strain of *Lyngbya aestuarii* (isolated by J.B. Waterbury, from a salt marsh, Woods Hole, Massachusetts). The LY sequence clusters at 2.1% divergence with the sequence of *L. aestuarii* PCC 7419 deposited by Nübel *et al*. (1997) and 2.2% divergence with the sequence of the same strain deposited by Ishida *et al*. (2001). It clusters at 3.3% divergence with *Lyngbya majuscula* strain CCAP 1446/4 (isolated by George from a brackish ditch at Great Yarmouth, Norfolk, England), and 4.4% divergence with *Lyngbya hieronymusii* (isolated by W.J. Lee and K.S. Bae from Daecheong reservoirs, Korea). By morphotype, the strain LY is similar to the field population in 'kopara' mats (Fig. 53); it complies with the description and is within the range of variation of *L. aestuarii* as described by Gomont (1892).

6. *Leptolyngbya* cf. *golenkiniana* strain FF (Fig. 59) is characterized by very narrow trichomes without sheaths, non-attenuated at the tip, exhibiting peculiar crooked growth, with sudden angular distortions. Cells are slightly wider than long: 1.28 ± 0.18 (132) μm wide and 1.02 ± 0.26 (184) μm long with clearly expressed constrictions at the cross walls. The end-cell is rounded and larger than others. The sequence clusters at 3.1% divergence with a strain isolated by A. Lopez-Cortes (1999*b*) from an intertidal pond in Mexico under the name '*Oscillatoria limnetica*', 4.1% divergence with LPP strain MBIC10087 (isolated from Pacific Ocean around Japan), 4.4% divergence with *Phormidium* strain MBIC10003 (isolated from equatorial Pacific Ocean), and 4.5% divergence with LPP strain MBIC10012 (isolated from the Pacific Ocean). More distantly at 4.6% divergence there is *Leptolyngbya thermalis* isolated by A. Lopez-Cortes (1999*a*) from a thermal spring in a coastal brackish system (salinity 2.5 to 2.8%), Bahia Concepcion, Mexico.

7. *Pseudophormidium* cf. *battersii* strain GF (Fig. 60) has filaments with thin sheaths, with torulose trichomes with clear constrictions at the cross walls, not attenuated at the tip. Cells are slightly wider than long: 1.63 ± 0.24 (144) μm wide and 1.39 ± 0.40 (294) μm long; the end-cell is conical. The sequence clusters at 5.6% divergence with *Synechococcus* IR11 – sequence deposited by N. Tezuka and M.M. Watanabe in 2001 and the second closest (with 7.9% divergence) is LPP MBIC 10086 – sequence deposited by S. Suda and H. Sekiguchi in 2001.

Discussion

The results of the current study of 'kopara' microbial mats support the polyphasic approach to microbial ecology, which combines culture-dependent and culture-independent methods, and includes morphotypic as well as phylogenetic characterizations (e.g. Abed *et al*., 2002, 2003*a*, *b*). The results illustrate the value as well as the limitations of the culture-dependent approaches, and the need to complement them with culture-independent ones (see Amann *et al*., 1995). The taxa grown in our cultures represented a small subset of those observed in natural populations, from which the cultures were isolated. None of the taxa that were observed dominating natural populations survived in culture, although three out of seven cultured morphotypes could be observed and confidently identified in natural populations. The other four were not observed but are assumed to be present as minor components. Thus, culturing as a selective process is insufficient to characterize microbial diversity, but it does reveal a part of a cryptic diversity, which may be important when environmental conditions change.

The identification of the field populations studied and the isolated strains was carried out to the species level whenever possible. The concept of species in microbiology is currently under scrutiny (Castenholz, 1992); however, a new picture of the speciation process in prokaryotes is gradually emerging (Ward, 1998; Rossello-Mora, 2001; Cohan, 2001, 2002; Konstantinidis & Tiedje, 2005; Gevers *et al*., 2005), which is more consistent with the dynamics of microbial natural populations (Abed *et al*., 2003*a*). For determination of morphotypes, we have used the taxonomic references that are traditional in phycological field studies as the best approximation, and related them to the terminology accompanying published phylogenetic information. Morphotypic characterization was carried out comparatively for natural populations and those isolated and grown in our cultures. Phylogenetic characterization was applied to cultured forms alone and related to the morphotypic properties observed on cultured strains.

Phenotype comparisons of natural populations with cultured isolates

The dark grey colouration (black when wet), which characterizes the mats on dry margins of 'kopara' ponds, originates from the cyanobacterial extracellular pigment scytonemin, which protects the cells from high irradiances of light and UV radiation (Garcia-Pichel & Castenholz, 1991). The pigment is located in thick cyanobacterial polysaccharide envelopes and sheaths. In 'kopara' ponds, dark pigmented envelopes were observed in species of the coccoid *Gloeocapsa* and *Chroococcus*, in non-heterocystous *Lyngbya*, and heterocystous *Scytonema* and *Calothrix* (Figs 4, 7*F*).

Scytonema cf. *myochrous* occurred in pond R2 with low salinity, which is ecologically consistent with the known terrestrial-subaerial habitats of this species. *Calothrix* sp. was found in the ponds with higher salinity and is presumed to be a marine or salt-tolerant organism close to *C*, *pulvinata* (Mertens) Agardh. Neither of these dominant cyanobacteria survived in culture. The traditional phycological systems recognize separate marine and freshwater species of this genus (Gomont, 1892; Geitler, 1932; Komarek & Anagnostidis, 2005). In contrast, Bergey's manual reserves the genus name *Calothrix* exclusively for freshwater isolates, whereas the marine cultured forms of similar morphology are moved to the genus *Rivularia* (Rippka *et al*., 2001), which, in phycological systems, also includes several marine and freshwater species with distinctive morphotypical and ecological properties (Golubic & Campbell, 1981; Whitton, 1987; Obenlüneschloss & Schneider, 1991). The problem remains unresolved as these populations were not phylogenetically evaluated.

Large areas covered by bright orange-yellow microbial mats of *Schizothrix* (Figs 2, 3) were the most conspicuous feature in the 'kopara' ponds (see also Golubic *et al*., 1999, colour plate I. fig. *A*). The dominance of *Schizothrix* species in pond R59 was also observed earlier (Mao Che *et al*., 2001). The presence of small-size multitrichomous cyanobacteria, consistent with the morphological characters of the genus *Schizothrix* was also photodocumented for pond R2 by cryo-scaninng electron microscopy (see Trichet & Défarge, 1997, plate 53). Although consistently present in the ponds, none of the *Schizothrix* species grew in our cultures. In our study, *Schizothrix* species dominated in ponds with elevated salinities. Apparently, the two species in pond R59 achieved a niche differentiation, in which the larger (*S*, *splendida*), occupied minor elevations with better drainage and higher insolation, whereas the smaller (*Schizothrix* sp. 2) preferred lower, water-logged positions in the mat. Only a few species of *Schizothrix* have been described from marine and saline habitats. Gomont (1892) described this genus as containing terrestrial and aquatic, but not completely marine species. Geitler (1932) shared the same opinion, but accepted *S*, *creswellii* Harvey and two species described incompletely by Hansgirg, *S*, *minuta* and *S*, *litoralis* as marine. Since then, *bona fide Schizothrix* species have been observed in both marine and hypersaline environments (Komárek & Anagnostidis, 2005). *Schizothrix gebeleinii* forms subtidal stromatolites in the high-energy marine setting of the Exuma Sound, Bahamas (Golubic & Browne, 1996). *Schizothrix splendida* dominates hypersaline ponds in the Coorong lagoon (Australia) and intertidal mats of Abu Dhabi (United Arab Emirates), the Bahamas and Florida Keys (Golubic, 1973), suggesting a global distribution as has been documented for *Microcoleus chthonoplastes* by Garcia-Pichel *et al*. (1996). *Schizothrix* sp.1, which was observed in the low salinity 'kopara' pond R2, is similar to the marine *S*, *cresswellii* Harvey, but also close to the freshwater *S*, *coriacea* (Kützing) Gomont and *S*, *lacustris* A. Braun.

We have observed in 'kopara' mats a variety of cyanobacteria with single narrow and sheathed trichomes, which could be classified as *Leptolyngbya* Anagnostidis & Komárek, or placed among the narrow species of *Phormidium sensu* Geitler. Mao Che *et al*. (2001) and Défarge *et al*. (1994*a*) reported a small *Phormidium* to be dominant in pond R2, which may correspond to one of the *Leptolyngbya* populations observed by us in the submerged 'kopara' mats (Figs 22–30). However, the identity of these small forms is difficult to establish on purely morphological grounds, in part because of the incomplete nature of early descriptions. Marine *Leptolyngbya (Pormidium)* morphotypes were characterized by Wilmotte (1991) and subsequently sequenced (Wilmotte *et al*., 1992). However, many organisms of similar morphology plotted distant from this group. Forms with narrow trichomes, long cylindrical cells and a terminal gas vesicle cluster (Figs 24–26) are similar to *Phormidium* cf, *crossbyanum* Tilden [= *Leptolyngbya crossbyana* (Tilden) Anagnostidis & Komárek], which forms large microbialites in the lagoon of the neighboring Tikehau Atoll.

Some of the cyanobacteria with narrow trichomes are also characterized by unusually short, torulose cells with clear constrictions at cross walls and occasional formation of false branching. Forms with similar growth habit, but slightly larger, have been cultured by Silva & Pienaar (2000) under the generic designation of *Leptolyngbya*, Two strains of this morphotype

developed in our cultures (Figs 59, 60), which were quite similar to common marine epiphytes, described originally by Gomont as *Plectonema golenkinianum* and *P. battersii* (Gomont, 1892; see also Golubic *et al.*, 1999, pl.2-A). Komárek & Anagnostidis (2005) are uncertain regarding the generic identity of these forms, placing them alternatively either within the genus *Leptolyngbya* or within *Pseudophormidium*, With respect to the 16S rRNA sequences obtained, these two morphotypes plotted distant from one another suggesting distinction above the genus level. For this reason, we have also assigned them to two morphotypically similar but distinct genera. Such designations remain provisional awaiting results of molecular sequencing of a larger number of similar morphotypes (Komárek & Anagnostidis, 2005).

Species of the genus *Oscillatoria* Vaucher with narrow trichomes and elongated cells have recently been classified separately under the name *Geitlerinema* (Anagnostidis, 1989; Castenholz *et al.*, 2001; Komárek & Anagnostidis, 2005). Our cultured strain *Geitlerinema* (*Oscillatoria*) strain FE (Fig. 52) has not been observed in field samples collected from the ponds. Our cultured organism is similar in shape and size to a South African marine isolate of Silva and Pienaar (2000), which they identified as *Geitlerinema lemmermannii* (Wołoszyńska) Anagnostidis, a species that was originally described from a lake in Java. Our isolate is also similar to, but slightly smaller than a hypersaline taxon found in Solar Lake, Sinai and described as *Oscillatoria lacus solaris* (Campbell & Golubic, 1985, fig. 8), currently revised as *Geitlerinema* (Anagnostidis, 1989).

The genus *Rhabdoderma* is placed in the family *Synechococcaceae*, Our isolate is consistent with the description of the genus. When cells are separated, the organism is similar to *Synechococcus elongatus*. Défarge *et al.* (1994*a*) observed in pond R2 a coccoid rod-shaped cyanobacterium, identified by them as *Synechococcus elongatus*. Formation of short chains of 4–8 cells following division has been described in several species of *Synechococcus* (Komárek & Anagnostidis, 1999). This feature occurs regularly in the genera *Rhabdogloea* and *Rhabdoderma*, although in conjunction with extracellular envelope formation, which was not observed in our culture. Silva & Pienaar (2000) cultured a similar marine organism isolated from a *Codium* sp., which they identified as cf. *Borzia* sp.1. We consider the affiliation of our isolates to be closer to coccoid cyanobacteria of the genus *Rhabdoderma* Schmidle & Lauterborn than to *Borzia* Cohn, which was originally described as a filamentous oscillatoriacean cyanobacterium.

Among our seven isolates, the following source populations could be identified in natural settings: *Chroococcus* cf. *submarinus*, *Johannesbaptistia pellucida* and *Lyngbya aestuarii* (Figs 7, 13, 14). These taxa were present in natural populations in substantial numbers, but did not dominate in any of the mats. The isolates could be characterized and identified by their morphology, because their morphotypic expression was maintained in culture with only minor modifications, including some change in dimensions. Apparently the application of simple, low-nutrient media helped to minimize morphological alteration in cultured populations, which is common in standard media for cyanobacteria such as BG11 and ASN-III.

The *Chroococcus* cf. *submarinus* strain BM isolated from Rangiroa ponds retained a morphology typical of the genus, including multiple encapsulation by extracellular gelatinous envelopes (Fig. 44). The strain is slightly smaller than the natural *Chroococcus* sp. 1 population to which it is morphologically closest, and larger than the natural populations of *Chroococcus* spp. 2 and 3.

Cell dimensions are regularly used in determination of cyanobacterial species. Dimensions of cyanobacterial cells in optimally growing natural populations appear to be fairly stable with little intraclonal variability, although interclonal variability is higher within the same basic morphotype (Campbell & Golubic, 1985; Abed *et al.*, 2003*a*). Similarly narrow variabilty was observed in culture as long as the growth conditions were favourable. Minor variation in cell dimensions correlate with changes in cell division rhythm, as well as with the stage in which cells separate from each other (compare Figs 13*A*, *B* and *D*). However, under unfavourable growth conditions, the balance between cell size increase and cell division rhythm is often disturbed, resulting in changes in cell dimensions and proportions and leading to teratogenic cell shape distortions. We observed such teratogenic effects following initial culture transfers of *Chroococcus* cf. *submarinus* strain BM (Fig. 46) and *Johannesbaptistia pellucida* strain GC (Fig. 49), which later stabilized and assumed normal morphology. Similar cell size increases associated with cell deformations, called 'involution forms' were described for *Synechococcus* species in thermal springs and hypersaline habitats. Their frequency increased toward the tolerance limits of these taxa to high temperature and salinity respectively (Komárek & Anagnostidis, 1999, pp. 121–122). Such distortions caused occasional confusion in taxonomic determinations (e.g. Hoff & Frémy, 1933, reviewed by Montoya & Golubic, 1991 and Komárek & Anagnostidis, 1999, p. 91). Dependence of cell size from culture conditions, specifically with changes in illumination and

temperature was tested for cultured marine cyanobacteria in South Africa (Silva & Pienaar, 2000). These studies reported that a marine *Chroococcus* cf. *turgidus* isolate almost doubled in size when transferred from standard medium with low light at 22°C to increased irradiance and 25°C. The effect was reversed when temperature reached 29°C. Burja *et al*. (2002) found size differences when they compared a fresh isolate of *Lyngbya majuscula* from Moorea lagoon (French Polynesia) with *L*, *majuscula* CCAP 1446/4 strain, which was isolated and maintained in culture since 1953.

The growth conditions appear to influence strongly the cell size of *Johannesbaptistia*, The cells of the cultured *Johannesbaptistia pellucida* strain GC are significantly larger than those observed in natural populations of the same species (compare Figs 13*E* and *F* with *G*). Several species of *Johannesbaptistia* have been described in the literature, but subsequently revised to the status of synonyms of a single species *Johannesbaptistia pellucida* (Dickie) Taylor & Drouet (reviewed by Komárek & Anagnostidis, 1999, p. 135), Natural populations of *Johannesbaptistia* in 'kopara' ponds exhibit two distinct size ranges. These different populations, which co-occur in one pond, but do not have intermediate forms, suggest taxonomic distinction. It remains open whether these distinctive morphotypes represent genetically distinct taxa, or whether this taxon is capable of adjusting its cell size to available nutrient conditions, which may account for its unusual size variation. *Johannesbaptistia* is common in tropical coastal ponds and is cosmopolitan in distribution. Défarge *et al*. (1994*a*, *b*) record its presence in the 'kopara' of Moruroa Atoll (French Polynesia), but not for pond R2.

Our cultured strain of *Lyngbya aestuarii* – strain LY (Figs 54–58) – with cells 12.48 ± 1.30 (51) μm wide, was also somewhat larger than the specimens observed in the 'kopara' mat (Fig. 53; Table 2), but well within the range attributed to this species in the literature: (8–) 10–16 (–24) μm (Gomont, 1892). *Lyngbya aestuarii* is a common and often dominant cyanobacterium in intertidal microbial mats world wide (e.g. Golubic, 1985).

Phylogenetic characterization of cyanobacteria cultured from 'kopara' mats

For the construction of phylogenetic trees, it is important to have long sequences and the minimum size required for phylogenetic characterization by bacterial 16S rRNA analysis is more than 1,000 bp (Murray *et al*., 1990). Our goal was to orient our isolates in terms of the phylogenetically closest neighbours as an aid to their characterization and identification. Because our cultures were not axenic, we have resorted to extracting partial sequences of ~600–700 bp, marked by cyanobacterial group-specific primers (Nübel *et al*., 1997). For tree construction, we have compared a set of aligned 600 bp from the known complete and partial sequences after elimination of gaps. The clusters formed on the basis of 16S rRNA sequences appear to have good reliability in distinction of genera, possibly approaching species-level resolution, although published trees are frequently burdened by misidentification of isolates.

In our tree (Fig. 8), we could recognize particular clusters revealed by other phylogenetic studies (Wilmotte, 1994; Turner, 1997; Ishida *et al*., 1997, 2001; Honda *et al*., 1999; Robertson *et al*., 2001; Wilmotte & Herdman, 2001; Abed *et al*., 2003*b*): the cluster of heterocyst-forming cyanobacteria (i.e. the first phylogenetic lineage according to Honda *et al*., 1999), the cluster of *Synechococcus* PCC 6716, PCC 6717, *Synechococcus elongatus* (Toray), proposed by Robertson *et al*. (2001) to be the eighth phylogenetic lineage, as well as seven other clusters as revealed by Honda *et al*. (1999). The small cluster including *Oscillatoria* sp. M-117, *Leptolyngbya boryanum* PCC 73110, *Phormidium* sp. M-99 and *Leptolyngbya foveolarum*, as well as the *Halothece* cluster of extremely halotolerant cyanobacteria as in Garcia-Pichel *et al*. (1998) are all distinguished. In addition, the sequences of the isolates obtained from the lagoon of the Tikehau Atoll, the neighbouring atoll to Rangiroa, maintain the same relations as in the tree presented by Abed *et al*. (2003b). For example, the isolates '*Leptolyngbya*' strains TKB32 and TK511 shared 93.5 and 95% sequence similarity, respectively, with the strain of *Halomicronema excentricum* TFEP-1 (Abed *et al*., 2002), and 95% to each other (Abed *et al*., 2003*b*). In our calculations, the values are 93.2, 94.9 and 95.2%, respectively. The small differences might be due to the algorithms used and the shortness of the sequences (400 bp) but the strong correlations are maintained in our trees.

Thus, our results show that our strains are not closely related to the lagoonal strains of the Tikehau Atoll. In spite of geographic proximity, the ecological differences between the open lagoon and 'kopara' ponds are significant, and have selected for different mat-building organisms. This conclusion is consistent with the results of phenotype determinations.

The phylogenetic tree presented here illustrates the phylogenetic distance between our sequences (GC, BM, FF, GF, FE) and all the previously known ones, and thus represents a new contribution to our knowledge of cyanobacterial diversity. Our *Geitlerinema* sp. strain FE occupied a separate

branch within a cluster of phenotypically similar organisms. The separate positions of the sequences of *Johannesbaptistia pellucida* strain GC and *Chroococcus* cf. *submarinus* strain BM were expected, since no sequences of these morphotypically well-characterized genera were available in the data banks. Our isolate *Lyngbya aestuarii*, characterized by phylogenetic and morphotypic properties, differs slightly from the type strain *L. aestuarii* PCC 7419 in size (diameter 12.5 μm for our strain LY vs. 15–16 μm for PCC 7419) and in 16S rRNA sequence (2.1% divergence). The latter difference could be due to the relatively short length of our sequence (600 bp) biasing the calculation, or it could indicate a micro-evolution on environmental or geographic grounds (tropical vs temperate). A polyphasic study of tropical marine *Lyngbya* species (*L*, *aestuarii* not included) combining phylogenetic clustering of short (605 bp) 16S rRNA gene sequences with phenotypic (morphological and biochemical) properties has recently been published (Thacker & Paul, 2004). The interest in this genus has been generated by the toxic properties of many marine species and massive blooms occurring and threatening coral reefs (Luesch *et al*., 2000).

The cultured organism identified morphotypically as *Rhabdoderma* cf. *rubrum* strain CH (Fig. 51) plotted within cluster 3 of Herdman *et al*. (2001), i.e. within the fifth phylogenetic lineage of Robertson *et al*. (2001) of the polyphyletic form-genus *Synechococcus*. This lineage contains euryhaline *Synechococcus* strains PCC 7002, PCC 7003, PCC 73109, PCC 7117 and *Oscillatoria rosea* M-220, all isolated from marine environments, but capable of growth both in freshwater and marine media. They do not synthesize phycoerythrin. There are at least two documented cases where filamentous and coccoid cyanobacteria appear in genotypically close clusters (Wilmotte & Herdman, 2001). The cell size and proportions, pigmentation and ecology are consistent with the observations on our strain. However, the species *Synechococcus elongatus* is itself considered polyphyletic because it has been used to name the strains in at least three separate and distant lineages. Robertson *et al*. (2001) proposed that only the strains of the sixth phylogenetic lineage of the form-genus *Synechococcus* (cluster 1.1 of Herdman *et al*., 2001) be considered a monophyletic group of the 'real' *Synechococcus*, whereas all *Synechococcus* sequences that fall outside the sixth lineage should be considered misclassified. The *Synechococcus* strains of the cluster containing *Rhabdoderma* cf. *rubrum* strain CH (fifth lineage) is distant from the sixth lineage and is expected to be re-named.

Oscillatoriacean cyanobacteria with narrow trichomes have been classified by the bacteriological system as LPP-group B (Rippka *et al*., 1979), a term which has since been replaced by the form-generic designation of *Leptolyngbya* (Komárek & Anagnostidis, 2005), without any claim of phylogenetic coherence (Castenholz *et al*., 2001). Early work on 16S rRNA sequence analyses has already shown that many small cyanobacteria of similar size and morphology are polyphyletic (Wilmotte & Golubic, 1991; Wilmotte, 1994). Our sequences of cyanobacterial strain with narrow trichomes (strains GF and FF) are separated from the known *Leptolyngbya* sequences. When compared with similar morphotypes in marine environment, our strains GF and FF showed similar cell sizes and arrangements to morphotypes classified in the past as *Plectonema golenkinianum* and *P*, *battersii*, respectively. However, these isolates plotted separately from each other and from other known sequences both by sequence analysis and the BLAST search. This finding appears to support Komárek & Anagnostidis (2005) revision of these forms by placing them in separate genera. *Leptolyngbya* cf, *golenkiniana* (Gomont) Komárek & Anagnostidis strain FF plotted distant from other sequences classified as *Leptolyngbya* and showed 94% similarity with another form isolate with the name '*Oscillatoria limnetica*'. *Pseudophormidium* cf, *battersii* (Gomont) Komárek & Anagnostidis strain GF showed 94% similarity with *Synechococcus* IR11. Thus, better generic assignments for these forms awaits further investigations of similar natural populations using polyphasic approach.

In conclusion, our results are consistent with the hypothesis that isolation and enrichment culturing favour cyanobacteria, which are minor components in natural settings. These may be opportunistic species, which respond to particular nutrient conditions (Abed *et al*., 2003*b*). However, their presence is indicative of a cryptic diversity in natural populations of cyanobacteria, which may be important when environmental conditions change. Our conclusions are in agreement with those derived from other studies applying a polyphasic approach to cyanobacterial populations in extreme (Garcia-Pichel & Belnap, 1996; Garcia-Pichel *et al*., 1998, 2001; Nübel *et al*., 1997, 1999; Abed & Garcia-Pichel, 2001) as well as in normal marine environments (Abed *et al*., 2003*a*, *b*). Such conclusions were firmly supported by studies applying inoculum dilution series in conjunction with genotype determination (Ward *et al*., 1997, 1998; Ramsig *et al*., 2000). There is considerable influence of growth conditions on size and shape of cyanobacteria in culture and in natural settings, which need to be quantified for

the purposes of characterizing cyanobacteria. Identification of cyanobacteria is presently at a stage of orienting the specimens within the spectrum of available phenotypic and genotypic characters.

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