

Dinitrogen-Fixing Cyanobacteria in Microbial Mats of Two Shallow Coral Reef Ecosystems

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Abstract Dinitrogen-fixing organisms in cyanobacterial mats were studied in two shallow coral reef ecosystems: La Reunion Island, southwestern Indian Ocean, Sesoko (Okinawa) Island, and northwestern Pacific Ocean. Rapidly expanding benthic miniblooms, frequently dominated by a

single cyanobacterial taxon, were identified by microscopy and molecular tools. In addition, nitrogenase activity by these blooms was measured in situ. Dinitrogen fixation and its contribution to mat primary production were calculated using $^{15}\text{N}_2$ and ^{13}C methods. Dinitrogen-fixing cyanobacteria from mats in La Reunion and Sesoko showed few differences in taxonomic composition. *Anabaena* sp. among heterocystous and *Hydrocoleum majus* and *Symploca hydnooides* among nonheterocystous cyanobacteria occurred in microbial mats of both sites. *Oscillatoria bonnemaisonii* and *Leptolyngbya* spp. occurred only in La Reunion, whereas *Hydrocoleum coccineum* dominated in Sesoko. Other mats dominated by *Hydrocoleum lyngbyaceum*, *Phormidium laysanense*, and *Trichocoleus tenerriimus* occurred at lower frequencies. The 24-h nitrogenase activity, as measured by acetylene reduction, varied between 11 and 324 nmoles C_2H_2 reduced μg^{-1} Chl *a*. The highest values were achieved by heterocystous *Anabaena* sp. performed mostly during the day. Highest values for nonheterocystous cyanobacteria were achieved by *H. coccineum* mostly during the night. Daily nitrogen fixation varied from nine (*Leptolyngbya*) to 238 nmoles $\text{N}_2 \mu\text{g}^{-1}$ Chl day $^{-1}$ (*H. coccineum*). Primary production rates ranged from 1,321 (*S. hydnooides*) to 9,933 nmoles C μg^{-1} Chl day $^{-1}$ (*H. coccineum*). Dinitrogen fixation satisfied between 5% and 21% of the nitrogen required for primary production.

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Introduction

Biological N_2 fixation is a process unique to prokaryotes occurring commonly in freshwater and marine cyanobacteria. Although energetically demanding, this process provides the organisms with a particular advantage when

growing under N-limited conditions, which are most frequent in marine environments [17, 44]. The rates of nitrogen fixation, their relations to nitrogen concentration levels, and contribution to global oceans have been subject to extensive studies and reviews [e.g., [3, 8, 10, 29, 39]]. Due to oxygen sensitivity of the nitrogenase enzyme, the fixation of carbon and dinitrogen in an oxygen-producing organism needs to be separated either in space or in time [3, 39]. Spatial separation is achieved in heterocystous cyanobacteria which are able to fix dinitrogen during the day, contemporaneous with their energy generation. With some exceptions (e.g., *Trichodesmium*), nonheterocystous cyanobacteria temporarily separate N₂ fixation and oxygenic photosynthesis by fixing dinitrogen at night, using photosynthetic energy as ATP generated during the previous day [32].

Biological N₂ fixation appears to make a major contribution to N supply in coral reef ecosystems, as it has been shown for the Eniwetok Atoll [48], the Great Barrier Reef [31], and for the lagoons of Tikehau Atoll [11] and New Caledonia [10]. Many studies of dinitrogen fixation have dealt with shallow areas of coral reefs [8, 16, 31, 42] including exposed communities of an atoll rim [12]. To our knowledge, none of these studies were performed on identified cyanobacterial populations that support microbial mats.

The main objective of our study is to identify the dominant taxa in cyanobacteria mats and determine their contribution to N₂ fixation in shallow coral reef ecosystems.

For that, (1) we studied two shallow coral reef lagoons (Fig. 1): St. Gilles (La Reunion Island, Western Indian Ocean) and Sesoko (Okinawa Island, Japan, northern Pacific), (2) we combined phenotypic and molecular taxonomy to identify in situ dominant N₂ fixers among benthic cyanobacteria, and (3) we used acetylene reduction, ¹⁵N, and ¹³C methods to measure their nitrogenase activity and carbon production.

Materials and Methods

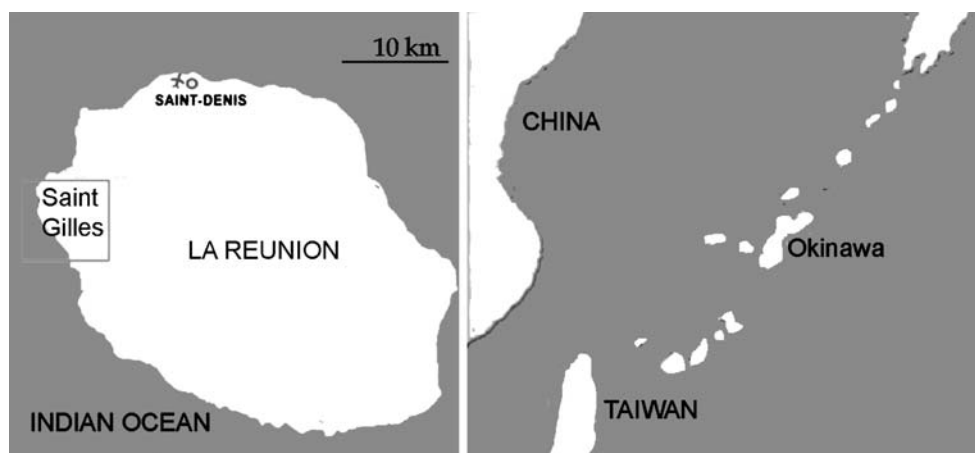
Areas of Study

The present contribution is based on the field research performed in the framework of a Japan–French collaborative program at La Reunion Island (November, 2004 and February, 2007) and on Sesoko Island offshore of Okinawa (July, 2005 and October, 2006). In both sites, we used the facilities of marine stations laboratories.

La Reunion Island is located in the southwestern Indian Ocean (21°7'S, 55°32'E) 700 km east of Madagascar. The climate is tropical with alternating rainy season from November to April (austral summer) and dry season from May to November. The tropical storm season is from November to April. The common reef geomorphology includes an outer slope, outer and inner reef flats, and a back reef. The fringing reefs are discontinuous and narrow (less than 550-m wide), and they cover only 7.3 km². The reef flats are exposed at low water. Coral reef degradation has been monitored since 1985 by measuring physicochemical parameters and the state of benthic communities [14]. The area of our study (station A) is located at La Saline along the southwestern coast of Reunion Island (Fig. 2a). Five transects were made in November, 2004, and February, 2007, from the beach over back reef zone covered with detrital sediment to the inner reef flat, each about 300-m long. No sampling was done on the outer reef flat. The depth of the study area never exceeded 2 m. The position of transects was selected so as to avoid nutrient enrichment due to submarine groundwater discharge, which is located north of the La Saline village [13, 15, 38]. These authors gave percentage coverage of live coral between 20% and 30% and macroalgal cover up to 40% on the reef flat.

Sesoko Island (26°38'N, 27°51'E) is located off the NW coast of Okinawa in the Ryukyu Archipelago, southwestern Japan. The sea around the Ryukyus is under the influence of the warm Kuroshio Current and characterized by the

Figure 1 Geographic location of the studies of dinitrogen fixing cyanobacteria in La Reunion, Indian Ocean, Sesoko–Okinawa, and Pacific Ocean



development of coral reefs from latitudes 24° to 31°N marking their northernmost distribution. The climate is subtropical with a rainy season in early summer from May to the end of June. The tropical storm season is from July to October. Three sampling stations were selected in July, 2005, and October, 2006 (Fig. 2b). Station B (1- to 2-m depth) is located in a moat on the west coast of Sesoko Island behind a well-developed reef crest, located 150–200 m from the shore [24]. Station C (1.5- to 2-m depth) is close to the bridge linking Sesoko to Okinawa, and Station D (2- to 3-m depth) is located next to the pier of Sesoko Station, Tropical Biosphere Research Center of the University of the Ryukyus.

Environmental Parameters and Nutrients

Temperature, salinity, and nutrient concentrations were routinely measured during field research operations. Triplicate subsamples of sea water for nutrient measurement were collected into clean acid-washed 100 mL polyethylene bottles and kept frozen. Nutrients were determined with an autoanalyzer (TRAACS-2000: BRAN+LUBE) according to Hansen and Koroleff [23]. Nitrate was determined by subtracting the values of nitrite from the values of nitrate + nitrite. The detection limits were 0.052 μM for $\text{NO}_3^- + \text{NO}_2^-$, 0.01 μM for NO_2^- , 0.020 μM for NH_4^+ , and 0.020 μM for PO_4^{3-} . Reproducibility (margin of error) of nutrient analysis was $\pm 0.2\%$ for NO_3^- , $\pm 0.5\%$ for NO_2^- , $\pm 1.2\%$ for NH_4^+ , and $\pm 0.8\%$ for PO_4^{3-} .

Distribution of Cyanobacterial Mats

To estimate frequency and coverage of cyanobacterial mats in La Reunion lagoon, five transects, each ca 300-m long

from the beach to the reef flat were examined. The coverage of different morphotypes of cyanobacteria was estimated along each transect. This method of estimation could not be performed for Sesoko where mats were attached to small coral reef pinnacles with irregular distribution. Therefore, we could not calculate mean percent coverage of the mats along transects.

Sampling

Rapidly expanding local miniblooms of cyanobacteria, distinguished by their color and consistency, were documented by underwater photography and sampled using SCUBA diving. Live samples were microscopically analyzed immediately following collection. Samples found to be unicyanobacterial and in the expanding growth phase, were selected for further analyses. Samples were taken at the end of the day in order to optimize night nitrogenase activity, which depends on the light energy received during daylight [10]. Following sampling, one aliquot of the mat was preserved with formaldehyde (4%) for later microscopic analysis; a second aliquot was stored in guanidine thiocyanate for DNA analysis [2], and a third aliquot was used for acetylene reduction assay in triplicate.

Microscopic Analysis

Microscopic analysis was carried out following collection to establish the composition of samples for analyses of carbon and nitrogen metabolism. Detailed microscopy and photomicrography, including morphometric evaluation, has been carried out subsequently on formaldehyde- and guanidine-thiocyanate-fixed samples to aid morphotypic and genotypic characterization of the studied cyanobacteria. Zeiss RA and

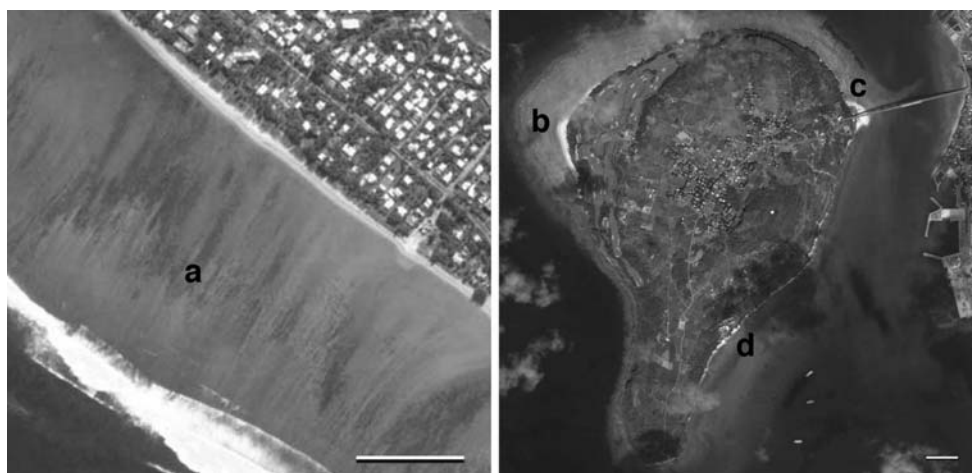


Figure 2 Areas of studies of cyanobacterial dinitrogen fixation in tropical lagoons. Lagoon on La Reunion Island at La Saline (station A) on the southwestern coast of the island. Stations B, C, and D around

the Sesoko Island (Okinawa, Ryukyu Archipelago, southwestern Japan). Scale bars are 200-m long

Universal Microscope (Carl Zeiss, Inc., Oberkochen, Germany) equipped with transmitted light and DIC illumination has been used with a digital photomicrographic camera attachment Moticam 2000 (Motic China, Co, Ltd.).

Morphotypic Characterization

The taxonomic identities of cyanobacteria under study were determined by polyphasic analysis in order to correlate their morphotypic properties (determined by S. Golubic) according to classifications by Geitler [20], Komárek and Anagnostidis [30] and Bergey's Manual [9] with sequences of 16S rRNA gene fragments (performed by K. Palinska). Measurements of cell dimensions were carried out on the basis of photomicrographs and in-scale camera-lucida projections. Measurements were recorded using SigmaScan software (Jandel Scientific, Sausalito, CA) and expressed as means \pm standard deviation of 20 to 50 measurements.

DNA Extraction, PCR Amplification, Sequencing and Phylogenetic Analysis

One milliliter of each population suspended in guanidine thiocyanate was added to 1 ml of TESC (10 mM Tris, 1 mM EDTA, 20 mM NaCl, 2% acetyltrimethylammonium bromide, pH 8.0). After addition of lysozyme (1% final concentration), the samples were incubated at 37 °C for 1 h. After ten cycles of freeze (in liquid nitrogen) and thaw (at 65 °C), 5 μ l proteinase K (100 μ g/ml) and 90 μ l 10% sodium dodecyl sulfate was added, and the samples were incubated at 52 °C overnight. The samples were centrifuged in a microcentrifuge at 12,000 \times *g* for 5 min, and the supernatants were extracted twice with phenol, phenol/chloroform, and chloroform. The DNA was precipitated from the aqueous phase with 0.6 volumes of 2-propanol, washed with 70% ethanol, vacuum dried, and stored in 100 μ l TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Primers PLG1.1 and PLG2.1 described by Nadeau et al. [37] were used for amplification of partial 16S rRNA genes. The reaction volume was 100 μ l and contained: 1 \times REDTaq PCR Buffer, 200 μ M of each deoxynucleotide, 200 μ g BSA, 500 ng of each oligonucleotide primer, 5 U of RED Taq DNA polymerase (Sigma-Aldrich), and 1–10 μ l of DNA extract. After an initial denaturation step (4 min at 94 °C), 31 incubation cycles followed, each consisting of 1 min at 94 °C, 1 min at 52 °C, and 1.5 min (8 min at the last cycle) at 72 °C. The presence of PCR products was detected by standard agarose gel electrophoresis and ethidium bromide staining. Amplification products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

DNA samples were directly sequenced in both directions by a commercial sequencing laboratory. Sequence similar-

ities were calculated online with ClustalW ([25] at <http://www.ebi.ac.uk/clustalw/index.html>). Sequence alignment and phylogeny of 16S rRNA fragments obtained from the populations were carried out using the ARB software version 07.12.17prv and the official database (www.arb-home.de) from February 2005 for small subunit RNA sequences (ssu_jan04_corr_opt.arb). Additionally, cyanobacterial gene sequences available from the GenBank were imported and aligned in the database of the ARB software. These sequences were then aligned with the sequences in the ARB database using the alignment ARB tool. The alignment was corrected manually. The phylogenetic tree was calculated by maximum likelihood, based on long 16S rRNA gene sequences. The sequences were inserted into the preestablished tree using the parsimony ARB tool while maintaining the overall tree topology without changes. The final tree was minimized for simplicity in presentation.

Incubation with Stable Isotopes and Acetylene Reduction

Immediately following sampling, the mats were placed in seawater collected at the same place and depth, enriched with C₂H₂ or ¹⁵N₂ and NaH¹³CO₃, and incubated in situ. Incubation started before the sunset and continued until early morning for dinitrogen fixation of nonheterocystous cyanobacteria. For heterocystous cyanobacteria, the incubation continued until the end of the following day [10].

Nitrogenase Activity Measurements

Nitrogenase activity was estimated by measuring the acetylene reduction rate (ARR). Short (1 h) and long-term (12 h) measurements were performed, using a single injection of acetylene [47]. Pieces of 1-cm² of mat were placed inside 175-mL polycarbonate bottles fitted with a Teflon septum and filled with 125 mL of sea water from the sampling site. Experiments, in triplicate, were started by removing 5 mL of air above the sampled water and injecting the same volume of C₂H₂, followed by swirling for several minutes in the purpose of dissolving injected acetylene. C₂H₂ concentration measurements performed at the beginning of incubations demonstrated that 80% of the added C₂H₂ was dissolved in seawater. Light energy (PAR) was continuously recorded using a miniature light recorder (Alec Electronics Company). Four milliliters of gas mixture were taken using a vacuum tube at the end of the daylight and at the sunrise after swirling the sample for several minutes each time. Ethylene concentrations were measured using a gas chromatograph (Agilent μ GC) calibrated with commercial gas standards. Three replicates were measured from each incubation bottle. The dissolved ethylene concentration was calculated using the Bunsen gas solubility coefficient of 0.08 according to Breitbarth et al. [6] for a

temperature of 28 °C and a salinity of 35 psu. ARR was calculated within each incubation period by subtracting C_2H_4 formed in the preceding period. The biomass of incubated benthic cyanobacteria was calculated from the chlorophyll *a* content. At the end of incubation with C_2H_2 , the mat was dried using Whatman filter paper and frozen for later spectrophotometric analysis of the La Reunion mats and for high performance liquid chromatography (HPLC) analysis of the Sesoko mats. The relative efficiency of nitrogenase activity was expressed as ARR per Chl *a*. Results are also presented as gross dinitrogen fixation rate calculated by using factor 4 in conversion from ARR [36].

Net Dinitrogen Fixation and Primary Production

Simultaneous dinitrogen fixation rate and dissolved inorganic carbon uptake measurements were carried out in duplicate (two incubation bottles) according to Slawyk et al. [43]. For each analysis, a 1-cm² piece of mat was placed inside a 175-ml polycarbonate bottle fitted with a septum. In each bottle, ¹³C-labeled sodium bicarbonate (NaH¹³CO₃; 6 g 250 ml⁻¹ deionized water, 99.9 atomic% ¹³C; Eurisotop) was added in order to obtain 11.5% final enrichment. Subsequently, 0.36 mL of ¹⁵N₂ (99.8 atom %, Shoko Co. Ltd., Tokyo, Japan) was added with a gas-tight syringe to obtain an enrichment of 6.8%. Incubations were terminated by filtration under gentle pressure through a precombusted 47-mm diameter GF/F filter. The total wet weight of the incubated material was determined. An aliquot was dried at 60 °C for particulate organic carbon (POC), particulate organic nitrogen (PON), and isotope analysis, and another aliquot was frozen for HPLC analysis. Before analysis, filters were exposed to HCl fumes for 4 h. Measurements of delta ¹³C, delta ¹⁵N, and POC, PON were done using a mass spectrometer DELTA plus Advantage (ThermoFinnigan Co.) equipped with EA1110 for measurements of POC and PON. Primary production was calculated according to Hama et al. [22] using a time 0 enrichment of 1.089 and an initial DIC concentration of 2,400 μM. Dinitrogen fixation rate was calculated by isotope mass balance as described in Montoya et al. [34] using an N₂ initial concentration of 386.16 μmoles L⁻¹ calculated for a temperature of 24 °C (laboratory temperature) and a salinity of 35 psu, assuming equilibrium with the overlying atmosphere.

Chlorophyll Analysis

Chlorophyll (Chl *a*) analyses were performed by spectrophotometry for La Reunion 2007 samples and by HPLC for La Reunion 2004 and Sesoko samples. Chlorophyll for spectrophotometric analysis was extracted in 10 ml of 100% methanol, and absorption at 665 nm was recorded before and after acidification. Chl *a* concentration was

calculated using the extinction coefficients given by Porra et al. [41].

For HPLC analysis, the pigments were extracted with 95% ethanol. HPLC was carried out with a model LC-10AT_{VP} (Shimadzu, Kyoto, Japan) equipped with a column-temperature controller CTO-10AS_{VP} (Shimadzu) using Waters Symmetry C₈ column (150×4.6 mm; Waters, Milford, Massachusetts). Pigments were eluted at a flow rate of 1.0 mL/min at 25 °C with a programmed binary gradient elution system. The solvents used were methanol–acetonitrile–water (50:25:25) containing 62.5 mM pyridine (solvent A) and methanol–acetonitrile–acetone (20:60:20; solvent B). Separation was performed with a gradient of ratio (A:B, v/v) containing the break points of 0 min (100:0, v/v), 22 min (60:40), 28 min (5:95), 38 min (5:95), and 41 min (100:0). Separated pigments were detected spectrophotometrically with a photodiode array detector (Shimadzu SPD-M10AVP) measuring from 400 to 760 nm. In this paper, we present only chlorophyll *a* data.

Results

The Environments Compared

Temperature, salinity, and nutrient concentrations in the ambient waters were measured during our studies in both compared settings in La Reunion and Sesoko islands and are summarized in Table 1. The total dissolved inorganic nitrogen (DIN=NO₃⁻+NO₂⁻+NH₄⁺) was highest in La Reunion in November 2004 (1.71 μM) and lowest in Sesoko in station B in October 2006 (0.20 μM). The high values in La Reunion were also reflected in the concentration of ammonia (1 μM) and nitrate (0.45 μM) indicating processes associated with eutrophication and coastal pollution. Reduced nitrogen compounds were always low in Sesoko. Dissolved PO₄³⁻ concentrations were low (<0.21 μM) in all stations at all times of study. Water temperature and salinity in both sites were similar during the times of these studies.

Mat-Forming Cyanobacteria

At both sites, benthic cyanobacteria occurred as scattered colonies which formed different types of organo–sedimentary structures on the lagoon floor. These structures differed in appearance and species composition. The species composition analyzed by light microscopy was correlated with the macroscopic appearance of the mat. The most common unicyanobacterial structures occurred as green mats, green tufts, dark colored mats, and globular orange-colored colonies. These were collected, analyzed, and used for determination of carbon and N₂ fixation activities.

Table 1 Temperature, salinity, mean \pm SE of light energy received between 11:00 and 16:00 at the incubation depth, and nutrient concentrations in La Reunion and Sesoko

Place	Temperature (°C)	Salinity	Light energy	NO ₃ ⁻	NO ₂ ⁻	NH ₄ ⁺	DIN	PO ₄ ⁻³
Reunion, Nov. 2004	28.5	32.3	1928 \pm 854	0.45	0.26	1.00	1.71	0.10
Reunion, Feb. 2007	26.3	35.1	1696 \pm 724	0.32	0.13	0.10	0.55	0.21
Sesoko, Jul. 2005	29.4	34.3	2104 \pm 497	0.66	0.04	0.10	0.80	0.07
Sesoko, St B, Oct. 2006	27.6	34.5	1816 \pm 598	0.09	0.001	0.11	0.20	0.14
Sesoko, St D, Oct. 2006	27.3	34.6	1442 \pm 392	0.28	0.04	0.17	0.49	0.04

Values presented are mean \pm SE of light energy ($\mu\text{mol cm}^{-2} \text{s}^{-1}$) and nutrient concentrations (μM)

Percent coverage of cyanobacterial mats on bottom substrates observed at La Reunion in February 2007, are presented in Table 2. Microbial mats were much more abundant in February during the summer rainy season than in November at the end of the dry season. During the study, the bottom consisted 75.4% of sand, 22.5% of coral, and 2.1% of macroalgae. Microbial mats occurred mainly over sand and to lesser extent on other substrates. They were dominated by *Leptolyngbya*, *Oscillatoria*, *Anabaena*, and *Hydrocoleum*. In Sesoko Island, the nature of the bottom consisted of areas with sand, corals, and macroalgae, but the proportions were not measured. In July, 2005, sandy bottom at the station C was covered by mats of *Hydrocoleum* spp. Other microbial mats produced by *Phormidium*, *Anabaena*, *Symploca*, *Oscillatoria*, and *Leptolyngbya*, were exclusively attached to hard substrates. Common species in both sites were: *Anabaena* sp., *Symploca hydnoidea*, and *Hydrocoleum majus*.

The field aspects of dominant microbial mat-forming cyanobacteria are shown in the left columns of Figs. 3 and 4 with their corresponding photomicrographic illustrations in the right column of these figures. The positions of the obtained 16S rRNA gene sequences (412–552 bp) of these organisms are shown in a reconstructed phylogenetic tree of cyanobacteria in Fig. 5.

Anabaena sp.-LC1 is the closest morphotypic identity of this common heterocystous benthic cyanobacterium that forms bright blue–green colonies with upright oriented filaments waving in the current (Fig. 3a). Its trichomes were comprised of torulose cells 7.65 \pm 0.37- μm wide, slightly shorter or longer than wide; heterocysts were slightly larger

Table 2 Percent of coverage \pm SE of cyanobacteria mats in La Reunion in February 2007

Organism	Percent coverage
<i>Anabaena</i> sp. LC1	0.07 \pm 0.04
<i>Leptolyngbya</i> sp. LC2	3.09 \pm 1.30
<i>H. majus</i> RU2007	0.01 \pm 0.01
<i>O. bonnemaisonii</i> LC4	1.50 \pm 0.42

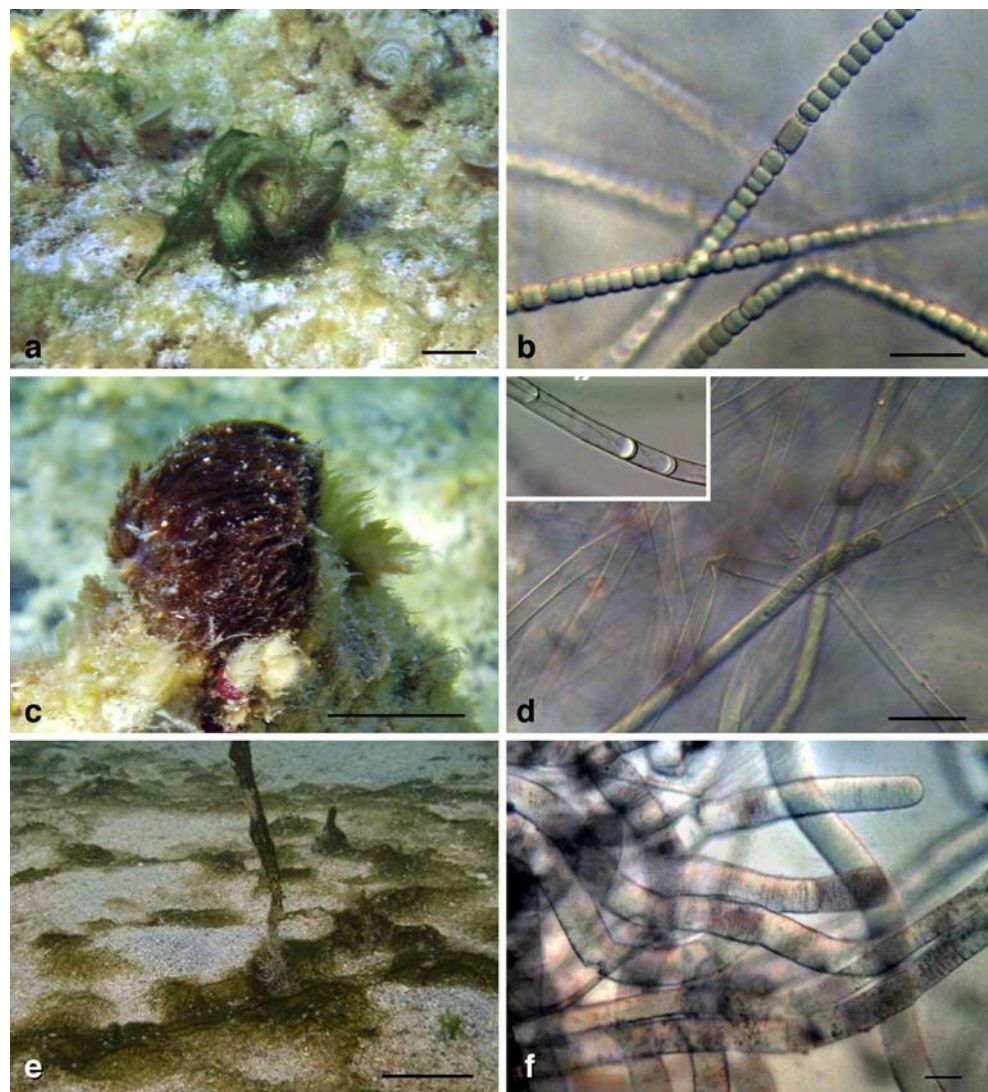
and longer than vegetative cells (Fig. 3b). The 16S rRNA gene sequence (accession no. GQ251521) similarity to GenBank listings of *Anabaena* sp. SSM-00 and *Anabaena* sp. LMECYA 185 show 98% and 96%, respectively (Fig. 5). The organism was present at low density in several transects in the lagoon of La Reunion in November, 2004, and February, 2007, close to the inner reef flat. *Anabaena* sp. was observed in Sesoko in July, 2005 at the station B but not in October, 2006.

S. hydnoidea Kützing ex Gomont formed characteristic dark brown cusp-shaped colonies (Fig. 3c) comprised of bundled, sheathed filaments externally, often in a leaf-like arrangement. Trichomes were 7.14 \pm 1.32- μm wide, not constricted at the cross walls but with slight constriction toward the tips. Apical cells were rounded without calyptra (Fig. 3d). Sheaths were firm, birefringent in polarized light with periodic convex closures (Fig. 3d, insert). The 16S rRNA gene sequence (accession no. GQ251525) matched with a 94% similarity with GenBank listings of *Symploca* PCC8002 and *S. hydnoidea* TK22 population described at Tikehau Lagoon [1] (Fig. 5). The colonies of *S. hydnoidea* were observed in La Reunion lagoon attached to *Acropora* close to the inner reef flat in 2004 and in 2007 as well as in Sesoko at station B in July, 2005.

Oscillatoria bonnemaisonii Crouan ex Gomont identified large unsheathed cyanobacteria forming loose olive–green mats, which dominated the floor of the La Reunion lagoon in February, 2007 (Fig. 3e). Trichomes were unsheathed, motile, and flexuous with tendency of forming loose helical undulations (Fig. 3f). Cells were slightly constricted at the cross walls, 32.8 \pm 2.28- μm wide, and very short (3–6 μm). The 16S rRNA gene sequence (accession no. GQ251524) matched the GenBank sequences listed as *Oscillatoria spongelliae* isolate 513bg and *Oscillatoria corallinae* with similarities of 96% and 95%, respectively (Fig. 5). The organism was observed only during one occasion in La Reunion but then as a dominant cyanobacterium that covered 1.5 \pm 0.4% of the seafloor along the measured transect.

H. majus Holden formed distinct bush-like colonies blue to violet in color, comprised of upward diverging filaments

Figure 3 Cyanobacterial colonies and mats in the lagoons (*left column*) with corresponding microscopic aspects of the organisms (*right column*). **a** Field view of *Anabaena* colony with filaments waving in the current, Sesoko station B, *scale bar* is 5-cm long. **b** Trichomes of *Anabaena* sp.: note the heterocyst in the center, *scale bar* is 20- μ m long. **c** Compact colony of *S. hydroides*, Sesoko station B; note upright leaf-like filament arrangement, *scale bar* is 5-cm long. **d** Filament and empty sheaths of *S. hydroides*. *Insert*: sheath with closures; *scale bar* is 20- μ m long. **e** Loose *Oscillatoria* mats over sand floor in the lagoon, La Reunion station A; *scale bar* is 50-cm long. **f** Intertwined trichomes of *Oscillatoria bonnemaisoni*; *scale bar* is 20- μ m long



(Fig. 4a). Filaments contained one to three trichomes surrounded by a thick, layered, and colorless sheath. Trichomes were straight and rigid, 29.05 ± 1.25 - μ m wide, cells 5.84 ± 1.43 long, not constricted at the crosswalls, abruptly narrowing at the tips, and ending with a distinct apical cell covered with calyptra (Fig. 4b). Cells were dark purple in color due to high concentrations of phycocyanin and phycoerythrin. The 16S rRNA gene sequence (accession no. GQ251521) showed closest similarity at 96% to a sequence listed in GenBank as *Hydrocoleum lyngbyaceum* HBC7 (Fig. 5). Colonies of *H. majus* RU2007 were observed sporadically on *Acropora* in La Reunion in February 2007 as well as a minor component in *H. coccineum* mats of Sesoko.

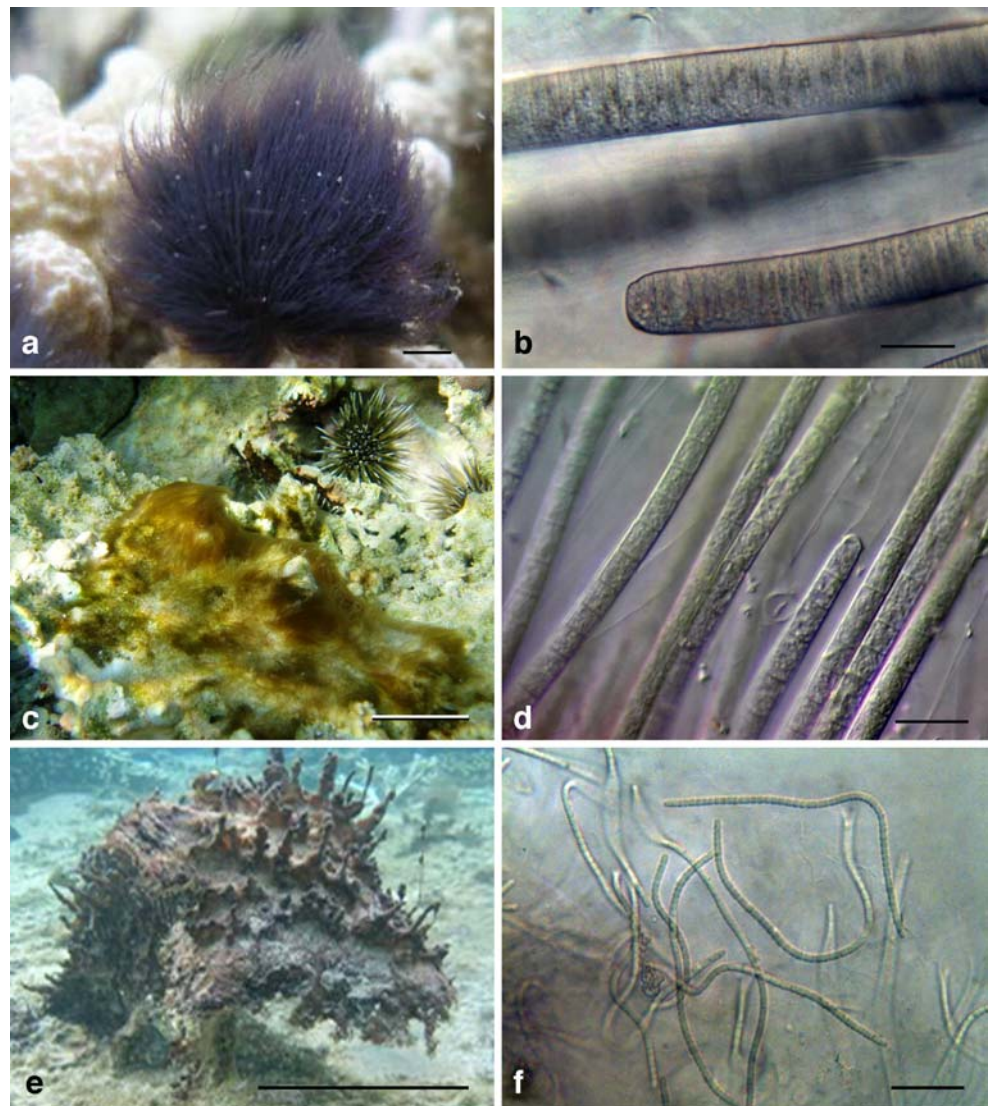
Other *Hydrocoleum* morphotypes corresponding to species *H. coccineum* Gomont, *H. glutinosum* Gomont, and *H. lyngbyaceum* Kützing were observed in 2006 in Sesoko, station B, forming brown-colored mats. The mats covering large areas of the sandy bottom at station C at 2-m depth in

July, 2005, were dominated by *H. coccineum*. They occurred intermixed with small (4- μ m wide) representatives of the genus *Oscillatoria* and *Leptolyngbya*. *H. glutinosum* was present at the same time only on the outer rim of the reef, close to station B at 6-m depth. The sequences of these organisms were not obtained.

Phormidium laysanense Lemmermann formed colonies of varying shape and consistency, from loose veils to upright extending coatings, bright red to pink in color (Fig. 4c). Filaments contained single trichomes within a thin, firm sheath. Cells were 5.8 ± 0.44 - μ m wide and 7.83 ± 1.72 - μ m long. End cell gradually attenuated covered at the tip by a conical or hemispherical calyptra (Fig. 4d). *P. laysanense* was observed in Sesoko at station D in 2005 and 2006, at shallow depths (2–5 m) inside caves of reef pinnacles.

Leptolyngbya spp. represents nonheterocystous cyanobacteria with narrow trichomes, which formed coatings over sand and massive corals (Fig. 4e). The trichomes

Figure 4 Cyanobacterial mats and mat-forming cyanobacteria from La Reunion and Sesoko. **a** Bush-like colony of *H. majus* showing upward diverging multitrichomous filaments detectable by plain eye view; La Reunion station A; scale bar is 1-cm long. **b** Trichomes of *H. majus*: note the distinct calyptrate apical cell; scale bar is 20- μ m long. **c** Orange color mats of *P. laysanense* coating coral rubble, Sesoko station B; scale bar is 20-cm long. **d** Filaments of *P. laysanense* in growth position arranged parallel to each other: note the capitate and calyptrate apical cell and thin firm sheaths containing single trichomes; scale bar is 20- μ m long. **e** Mat of *Leptolyngbya* sp. coating a dead coral. Note the pinnacle-like outgrowth and extensions of the mat, La Reunion station A; scale bar is 50-cm long. **f** *Leptolyngbya* sp. filaments containing single trichomes inside thin firm sheaths. Cells with distinct crosswalls, close to 1- μ m wide and slightly shorter or longer than wide; scale bar is 20- μ m long



were sheathed, comprised of cells 1–1.5- μ m wide, slightly shorter or longer than wide and with distinct cross walls, frequently gliding and abandoning the sheaths (Fig. 4f). Two populations from La Reunion that have been sequenced (accession nos. Gq251522 and GQ251523) cluster closely together (Fig. 5) but had different pigmentation. In La Reunion, in February, 2007, these mats were the second-most common mat formations (next to *O. bonnemaisonii*). Along the measured transect in station A, *Leptolyngbya* mats covered on the average $3.1 \pm 1.3\%$ of the sediment. In the parts of transect between 30 and 80 m from the beach, they covered 5% to 10%. At the end of the day, the mats become partially uplifted by the bubbles of oxygen they produced. Other mat-forming cyanobacteria were observed as minor constituents of benthos. Flat, brown–red mats consisting of *Trichocoleus tenerrimus* were found in 2005 and 2006 at low densities at stations B and D.

Nitrogenase Activity

Nitrogenase activities of dominant heterocystous and non-heterocystous benthic cyanobacteria measured by ethylene production and expressed as ARR as well as N_2 fixation rates (NF) are presented in Table 3 as means \pm standard error of triplicate measurements.

In both sites, *Anabaena* sp.-LC1 reduced C_2H_2 mainly during the daytime. In La Reunion the mean ARR value was 11.3 ± 1.2 nmoles $C_2H_2 \mu g^{-1}$ Chl *a* h^{-1} during the day and 0.45 ± 0.05 nmoles $C_2H_2 \mu g^{-1}$ Chl *a* h^{-1} during the night. In Sesoko Island, *Anabaena* sp. reduced C_2H_2 with a mean ARR value of 32.3 ± 4.2 nmoles μg^{-1} Chl *a* h^{-1} during the daytime and between 0 and 8 nmoles $C_2H_2 \mu g^{-1}$ Chl *a* h^{-1} (ten times lower but variable) during the night.

In contrast to heterocystous cyanobacteria, the mats dominated by *Hydrocoleum glutinosum* and *H. coccineum*, analyzed in Sesoko, showed nitrogenase activity only during

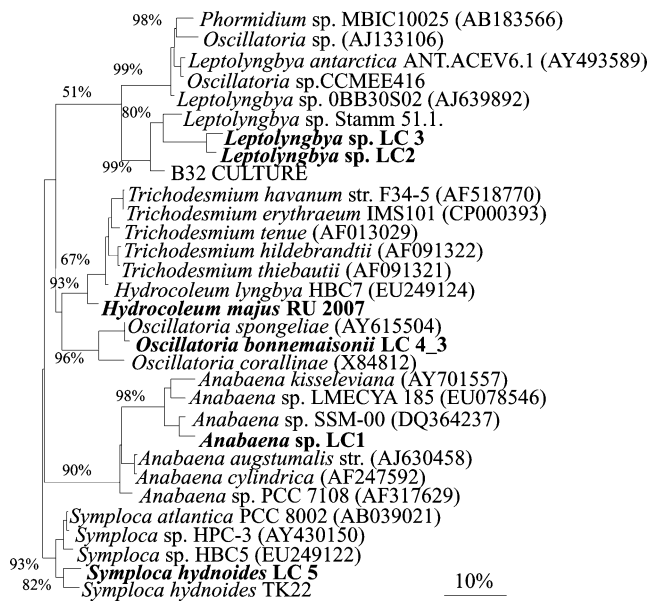


Figure 5 16S rRNA-based phylogenetic reconstruction showing the affiliation of sequences retrieved from six cyanobacterial populations originating from La Reunion lagoon. Accession numbers are indicated in parentheses. The scale bar indicates 10% sequence divergence. Data from in this study are shown in bold

the night. Their mean ARR were 14.5 ± 3.1 and 12.5 ± 4.8 nmoles μg^{-1} Chl *a* h^{-1} , respectively. Mats dominated by *H. lyngbyaceum* showed in Sesoko, nitrogenase activity both during the night and during the day after. In La Reunion, *H. majus* mat was measured only one time at night and showed very low activity: 1.07 ± 0.24 nmoles $\text{C}_2\text{H}_2 \mu\text{g}^{-1}$ Chl *a* h^{-1} .

Mats dominated by nonheterocystous *Leptolyngbya* spp. (LC2, LC3), *O. bonnemaisonii* (LC4), and *S. hydnooides* (LC5) in La Reunion showed nitrogenase activity both during the night and the daylight time. Their mean night ARR were 2.70 ± 0.19 , 1.15 ± 0.21 , and 0.48 nmoles $\text{C}_2\text{H}_2 \mu\text{g}^{-1}$ Chl *a* h^{-1} , respectively. Their mean daylight ARR were 1.62 ± 0.14 for *Leptolyngbya* sp., 1.64 ± 0.47 for *O. bonnemaisonii*, and 0.48 nmoles $\text{C}_2\text{H}_2 \mu\text{g}^{-1}$ Chl *a* h^{-1} for *S. hydnooides*.

In Sesoko, *P. laysanense*, and *T. tenerrimus* showed nitrogenase activity during the night and the daytime. Their mean night ARR were 6.8 ± 0.8 , 1.24 ± 0.11 , and 2.12 ± 0.75 nmoles μg^{-1} Chl *a* h^{-1} , respectively. Their mean daylight ARR was 7.53 ± 0.12 , 0.98 ± 0.14 , and 2.18 ± 1.06 nmoles μg^{-1} Chl *a* h^{-1} , respectively. *S. hydnooides* did not show nitrogenase activity.

Daily gross N_2 fixation rates (Table 3), calculated using a theoretical value of four as discussed in Charpy et al. [10], showed the highest N_2 fixation rates of 101 ± 17 nmoles $\text{N}_2 \mu\text{g}^{-1}$ Chl *a* day^{-1} for *Anabaena* sp. of Sesoko. The rates for the same species in La Reunion were significantly lower: 36 ± 4 nmoles $\text{N}_2 \mu\text{g}^{-1}$ Chl *a* day^{-1} . In Sesoko, *H. coccineum*, and *Hydrocoleum glutinosum* daily N_2 fixation rates were high, 77 ± 33 and 54 ± 16 nmoles, $\text{N}_2 \mu\text{g}^{-1}$ Chl *a* day^{-1} , respectively.

Isotopic Measurements of N_2 Fixation and Primary Production

Dinitrogen fixation rates and primary production rates based on measurements of $^{15}\text{N}_2$ and ^{13}C incorporation,

Table 3 Mean \pm SE of acetylene reduction rate during night and daylight and dinitrogen fixation calculated from ARR and the ratio $\text{C}_2\text{H}_2:\text{N}_2=4$

Organism	ARR _{ni}	ARR _{dl}	ARR _{24h}	NF _{ni}	NF _{dl}	NF _{24h}
La Reunion						
<i>Anabaena</i> sp.	0.45 ± 0.05	11.33 ± 1.17	142.7 ± 16.1	1.9 ± 0.4	34.4 ± 3.9	35.7 ± 4.0
<i>S. hydnooides</i>	0.48	0.48	11.5	1.32	1.55	2.9
<i>Leptolyngbya</i> sp.	2.70 ± 0.19	1.62 ± 0.14	50.8 ± 5.8	7.4 ± 0.5	5.3 ± 0.4	12.7 ± 0.8
<i>H. majus</i>	1.15 ± 0.21	ND	ND	3.2 ± 0.6	ND	ND
<i>O. bonnemaisonii</i>	1.07 ± 0.24	1.64 ± 0.47	34.9 ± 8.0	2.6 ± 0.7	5.3 ± 1.5	8.7 ± 2.0
Sesoko						
<i>Anabaena</i> sp.	0–8	32.29 ± 4.24	324.1 ± 69.2	13.2 ± 6.1	96.9 ± 12.0	101.0 ± 17.3
<i>S. hydnooides</i>	0	ND	0	0	ND	ND
<i>Leptolyngbya</i> sp.	5.99 ± 3.36	0.18 ± 0.20	97.9 ± 107.0	24.5 ± 26.7	0	24.5 ± 26.7
<i>H. coccineum</i>	12.48 ± 4.80	0	306.8 ± 130.3	76.7 ± 32.6	0	76.7 ± 32.6
<i>Hydrocoleum glutinosum</i>	14.54 ± 3.09	0.06 ± 0.03	216.6 ± 65.0	35.7 ± 10.7	0.2 ± 0.1	54.2 ± 16.2
<i>H. lyngbyaceum</i>	6.8 ± 0.8	7.53 ± 0.12	171.1 ± 18.4	20.4 ± 4.8	22.6 ± 0.7	42.8 ± 4.6
<i>P. laysanense</i>	1.24 ± 0.11	0.98 ± 0.14	26.7 ± 1.6	3.71 ± 0.98	2.94 ± 1.27	6.7 ± 0.4
<i>T. tenerrimus</i>	2.12 ± 0.75	2.18 ± 1.06	51.6	6.37 ± 2.08	6.54 ± 1.05	12.9 ± 6.8

Acetylene reduction rates are presented as mean \pm SE (nmoles C_2H_2 reduced μg^{-1} Chl *a* h^{-1}) and dinitrogen fixation was calculated from ARR and the ratio $\text{C}_2\text{H}_2:\text{N}_2=4$ (nmoles N_2 fixed μg^{-1} Chl *a*). ARR acetylene reduction rate, ni night, dl daylight, and NF dinitrogen fixation

were performed in three mats in La Reunion, dominated by *Anabaena* sp., *S. hydnooides*, and *Leptolyngbya* sp. and one mat in Sesoko Island dominated by *H. coccineum*. The results are summarized in Table 4. Daily nitrogen fixation varied from nine (*Leptolyngbya*) to 238 nmoles N₂ μg⁻¹ Chl day⁻¹ (*H. coccineum*). The primary production rates ranged from 1,321 (*S. hydnooides*) to 9,933 nmoles C μg⁻¹ Chl day⁻¹ (*H. coccineum*).

Discussion

Benthic Cyanobacteria in Coral Reef Ecosystems

Mats collected in La Reunion and Sesoko represent a variety of cyanobacterial morphotypes that are active in N₂ fixation. Populations of cyanobacteria dominated by representatives of the form-genera *Anabaena*, *Oscillatoria*, *Phormidium*, *Hydrocoleum*, *Symploca*, and *Leptolyngbya* were present in both geographically remote ecosystems surveyed in the present study. The following dominant morphospecies occurred in both sites: *Anabaena* sp., *P. laysanense*, *H. majus*, and *S. hydnooides*.

The heterocystous cyanobacteria form a monophyletic group [9, 21, 26]. However, for the members of this group, especially genera: *Anabaena*, *Nodularia*, *Aphanizomenon*, and *Cylindrospermopsis*; descriptions of morphotypes are often insufficient to discriminate between them and so are descriptions of strain histories. Genetically closely related strains or strains of similar morphotype often carry different specific or even generic epithets.

The genus *Anabaena* Bory describes a large number of taxa in freshwater plankton including toxic bloom-forming species. Morphologically similar marine taxa are usually classified under the genus *Nodularia* [4, 5]. Diversity and ecological features of *Anabaena* and *Nodularia* from marine benthic habitats are less well known than those from planktonic habitats [46]. The study of Lyra et al. [33] showed that benthic cyanobacteria of the genus *Nodularia*

from the Baltic Sea are nontoxic, without gas vacuoles, able to glide and are genetically more diverse than planktonic *Nodularia*. Marine *Anabaena* strains have already been reported from the Mexican Gulf coast of Texas as fast-growing N-fixing organisms well adapted to warm shallow marine environments [35, 45].

The 16S rRNA gene sequence of *Anabaena* sp.-LC1 identified morphologically in samples from La Reunion shows similarity to other heterocystous cyanobacteria, identified as *Anabaena* (98%) and *Cylindrospermopsis raciborskii* (95%) but only 94% to *Nodularia harveyana*. Such low level of similarity indicates that the organisms we encountered on the reef of La Reunion were not yet represented in GenBank.

O. bonnemaisonii is a large, well-characterized filamentous cyanobacterium reported from marine habitats worldwide [30]. However, the closest match with published sequences identifies a relation to a listing under the name *O. spongelliae*. The basis for the listing identification is not known; however, the original description of that morphotype, *Oscillatoria spongelliae* Schulze 1879, is different from our strain and not even typical for the form-genus *Oscillatoria*. That taxon has been classified by different authors as *Phormidium* and *Borzia*, in addition to *Oscillatoria* [30].

H. majus, the largest in the genus, was observed in mats at La Reunion, as dominant species, and at Sesoko, as subdominant. In the latter case, mats were dominated by *H. coccineum*, *H. glutinosum* and *H. lyngbyaceum*. Species of *Hydrocoleum* (*Blennothrix* in Komárek and Anagnostidis [30]) were reported to be very abundant in Tikehau Atoll lagoon [1], Kabira reef sediments, Ishigaki Island, SW Japan [29], and in the SW lagoon of New Caledonia [10]. They are considered to be among the most common mat-forming cyanobacteria in tropical oceans with the genetic potential to fix N₂, closely related to the species of the planktonic genus *Trichodesmium* [2].

Leptolyngbya sp. was the most abundant cyanobacterium at La Reunion in February 2007, covering 3.1±1.3% of

Table 4 Mean ±SE of dinitrogen fixation calculated from ¹⁵N experiments during the night and 24 h, 24 h primary production, C:N ratio of mats, and the contribution of NF to the total N requirement for PP

Organism	¹⁵ NF _{ni}	¹⁵ NF _{24h}	C ₂ H ₂ :N ₂	PP (%)	C:N	Percentage (%)
La Reunion						
<i>Anabaena</i> sp. LC1	0.7	27.8	5.1	3,291±630	7.4±0.2	6.3
<i>S. hydnooides</i> LC5	8.2±4.1	10.0±4.3	1.2	1,321±431	11.3±3.6	8.6
<i>Leptolyngbya</i> sp. LC2	8.7±2.6	8.8±2.6	5.8	2,709±825	15.3±0.9	5.0
Sesoko						
<i>H. coccineum</i>	288.1	238.5	1.1	9,933	8.8±0.3	21.1

Mean ±SE of dinitrogen fixation was calculated from ¹⁵N experiments. Primary production was measured in nmoles C μg⁻¹ Chl a; C:N ratio (*at:at*) of mats. ¹⁵NF ¹⁵N experiments, *ni* night, *PP* primary production

transect over sandy bottom. However, the morphogenus *Leptolyngbya* is polyphyletic and includes a number of unrelated organisms of simple morphology [9].

Dinitrogen Fixation

The acetylene reduction method does not measure N_2 fixation rates directly; it needs to be calibrated by calculating the $C_2H_2:N_2$ ratio (mol:mol). Multiple studies have shown the conversion ratio of ethylene to N to be highly variable. In coral reef ecosystems, values for benthic mats range from 1.8 to 4.8 in Tuamotu Atoll lagoon [11] and from 0.3 to 4.7 in Kabira reef [29]. In Sesoko and La Reunion, the ratio ranged between 1.1 and 5.8 with an average of 3.3. This value was close to the conversion ratio of 4:1 appropriate for quantification of gross N_2 fixation as measured by C_2H_2 reduction [19, 27, 36].

Paerl et al. [40] observed a decrease in N_2 fixation after an enrichment of $100\ \mu M\ NO_3$. This enrichment is 60, the maximum DIN observed in La Reunion waters. Joye and Lee [28] observed a substantial (80%) and instantaneous reduction in N_2 fixation rates after the addition of inorganic nitrogen ($2\ mM\ NO_3$). However, these authors reported that in some cases, the addition of inorganic nitrogen had no effect on N_2 fixation. The enrichment used here is 1,000 times the maximum DIN concentration in overlying waters. Camacho and de Wit [7] observed that a $400\ \mu M\ NH_4NO_3$ addition had no direct effect onto N_2 fixation rate. All these enrichments are largely higher than DIN concentrations in La Reunion and Sesoko waters.

Dinitrogen fixation in oligotrophic waters of La Reunion in 2004 was observed to continue when the surrounding waters contained $1.71\ \mu M$ nitrate concentration. It is possible that, in such cases, the availability of dissolved nitrogen in the microenvironment immediately surrounding the microorganism was exhausted and its replacement rate deficient. Thus, the concentration of a nutrient measured in the macroenvironment does not necessarily correspond to its availability to the microorganisms.

Dark N_2 fixation by heterocystous cyanobacteria and light N_2 fixation by nonheterocystous cyanobacteria are frequently reported in the literature. Gallon et al. [19] observed that the planktonic heterocystous *Nodularia* can fix N_2 during the night. They consider that cells can sustain N_2 fixation, at least to a limited extent, by metabolic processes that operate in the dark. The nonheterocystous cyanobacterium, *Symploca* PCC 8002, is capable of fixing N_2 aerobically in light. The synthesis of nitrogenase takes place in a minor proportion of consecutively arranged cells [18].

Data on N_2 fixation for coral reef mats are relatively rare and are mainly expressed per square meter of substrate rather than per unit of Chl *a*. Our results related to unit of biomass (Table 5) can be directly compared with the data set of Kayanne et al. [29] and Charpy et al. [10]. ARR in *Anabaena* sp., *H. lyngbyaceum*, *H. cantharidosmum*, and *H. coccineum* were in the same order of magnitude in New Caledonia, Sesoko, and La Reunion but higher than the ARR value reported for *H. cantharidosmum* in Kabira reef.

Dinitrogen Fixation as an Input to the Primary Production in Microbial Mats

To estimate the contribution of dinitrogen fixation to the total nitrogen requirement for the primary production (PP) of four mats, we used the value of PP and C:N ratio from the Table 4. Based on these values, N_2 fixation represents between 5% (*Leptolyngbya* sp.) to 21% (*H. coccineum*) of that required for primary production. This contribution is close to the percentage (19%) given by Charpy et al. [10] for sediment of New Caledonia lagoon and nearly ten times higher than the percentage (2%) given by Charpy-Roubaud et al. [11] for Tikehau atoll lagoon.

Lagoonal Dinitrogen Fixation

The whole lagoonal mat N_2 fixation could be estimated only for La Reunion in February 2007, when we had a

Table 5 Comparison of acetylene reduction rates in coral reef environments

Mats (dominant cyanobacteria)	Place	ARR (nmol $C_2H_2\ \mu g^{-1}\ Chl\ a\ d^{-1}$)	Reference
<i>Nodularia harveyana</i>	New Caledonia	205±12	Charpy et al. [10]
<i>Anabaena</i> sp.	Sesoko	324±69	This study
<i>Anabaena</i> sp.	La Reunion	143±16	This study
<i>H. lyngbyaceum</i>	New Caledonia	123±25	Charpy et al. [10]
	Sesoko	171±18	This study
<i>Hydrocoleum cantharidosmum</i>	Kabira reef	2.4	Kayanne et al. [29]
	New Caledonia (St. MBO)	122±20	Charpy et al. [10]
<i>H. coccineum</i>	Sesoko	307±130	This study

ARR acetylene reduction rates

good estimation of the mat coverage. Using the lagoon bottom percent coverage of the mats and the dinitrogen fixation rate per square centimeter² of mats, we obtained a mat N₂ fixation of 2.16 mg N₂ fixed per square meter of lagoon. This value is inside the large range of dinitrogen fixation rates reported by Charpy-Roubaud et al. [11] for various coral reef ecosystems.

Dinitrogen fixation measurements of microbial mats combined with polyphasic studies of cyanobacterial diversity proved advantageous by achieving identification of the principal phototrophic contributors of benthic diazotrophy. The heterocystous genus *Anabaena* and the non-heterocystous genus *Hydrocoleum* appear to be important benthic dinitrogen fixers in the two investigated coral reef ecosystems. Their N₂ fixation rates are in the same order of magnitude and both heterocystous and nonheterocystous cyanobacteria are capable of light and dark N₂ fixation.

Mat diversity and their role in N₂ fixation in the two geographically distant ecosystems under study appear to be similar. Mats in both ecosystems were also comparable in sea water characteristics, such as light, temperature, salinity and nutrients.

The results suggest global importance of benthic cyanobacterial dinitrogen fixation and call for further investigations of microbial mats in coral reef ecosystems. Our approach in identifying natural populations involved in dinitrogen fixation are required to assess the contribution of benthic cyanobacteria to dinitrogen fixation in relation to their distribution and biodiversity.

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