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Nitrogen fixation in coral reef environments

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Abstract. Coral reefs are sites of high nitrogen fixation activity where marine cyanobacteria are the major contributors. In coral reefs cyanobacteria can be found in very diverse environments: water column, sandy bottoms, on coral rubbles as endolithic and epilithic forms, or forming microbial mats. The purpose of the present research is to evaluate N_2 fixation rates in different environments (sandy bottom, coral rubble and cyanobacteria mats) and their contribution to the net primary production. Two different fringing coral reef sites were studied: Sesoko at Okinawa, Japan and La Reunion at the Indian Ocean. N_2 fixation and primary production rates were measured using ^{13}C and ^{15}N fixation techniques. Daily N_2 fixation in sandy bottom was about 3 nanomoles N/µg Chl-*a*, with near 6% contribution to the primary production. In coral rubbles it ranged from 0.5 to 7 nanomoles N/µg Chl-*a* with 2.4 to 28% contribution to primary production, while in cyanobacteria mats it varied between 0.2 to 242 nanomoles N/µg Chl-*a* with contributions of 2.8 to 42% to the primary production. Differences in N_2 fixation rates between daytime and nighttime indicated the presence of both heterocystous possessing and non-heterocystous possessing cyanobacteria.

Key words: N_2 fixation, Cyanobacteria, Endolithic algae.

Introduction

Biological nitrogen 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 (Staal et al. 2001). Due to oxygen sensitivity of the nitrogenase enzyme, the fixation of carbon and nitrogen in an oxygen-producing organism needs to be separated either in space or in time (Berman-Frank et al. 2003). Spatial separation is achieved in heterocystous cyanobacteria which are able to fix nitrogen during the day, contemporaneous with their energy generation. Non-heterocystous cyanobacteria temporally separate nitrogen fixation and oxygenic photosynthesis by fixing nitrogen at night, using photosynthetic energy generated during the previous day (Lundgren et al. 2003). Biological nitrogen fixation appears to make a major contribution to N supply in coral reef ecosystems, as has been shown for the Eniwetok Atoll (Webb et al. 1975), the Great Barrier Reef (Larkum et al. 1988), and for the lagoons of Tikehau Atoll (Charpy-Roubaud et al. 2001) and New Caledonia (Charpy et al. 2007). Many studies on N_2 fixation have dealt with shallow coral reefs (Larkum et al. 1988; Shashar et al. 1994, Charpy-Roubaud and Larkum 2005), however they did not identified N_2 fixers in coral rubbles. In the present study, we measured ^{15}N and ^{13}C uptake in benthic cyanobacterial populations associated to coral rubbles as endolithic or epilithic forms, in sandy bottoms, and forming microbial mats in shallow coral reef lagoons at St. Gilles (La Reunion Island, Western Indian Ocean) and Sesoko (Okinawa Island, Japan).

The objectives were to identify *in situ* dominant nitrogen fixers among benthic cyanobacteria, to measure their nitrogen fixation rates and the percentage of new production achieved throughout the N_2 fixation. Moreover we compared the results obtained from two geographically remote sites of Indian Ocean and Pacific Ocean respectively.

Material and Methods

The present study is based on the field research carried out at La Reunion Island in November, 2004 and in February, 2007 and at Sesoko Island of Okinawa in July, 2005 and October, 2006. La Reunion Island is located in the South-Western Indian Ocean (21°7'S, 55°32'E), 700 km east of Madagascar.

The common reef geomorphology includes an outer slope, outer and inner reef flats and a back reef. The fringing reefs are discontinuous and narrow covering only 7.3 km^2. The reef flats are exposed at low tide. The studied area was located at La Saline along the southwestern coast of Reunion Island. The depth of the study area never exceeded 2m. Live coral coverage varied between 20 and 30% and macroalgal cover reached 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, South-Western Japan. The sea around the Ryukyus is under the influence of the warm Kuroshio Current and characterized by the development of coral reefs from latitudes 24° to 31°N, marking their northernmost distribution. Two sampling stations were selected in July, 2005 and October 2006, Station B (1 to 2m depth) located in a moat on the west coast of Sesoko Island behind a well-developed reef crest, located 150–200 m from the shore, and Station C (2 to 3m depth) located next to the pier of Sesoko Station, Tropical Biosphere Research Center of the University of the Ryukyus. Reefs around Sesoko Island are actually in a post bleaching phase with less than 10% of living coral coverage, 50% of sandy bottom, 30% of coral rubbles and the rest covered by macroalgae and turf algae (Casareto, unpublished data).

Temperature and light were monitored over the duration of the surveys using in situ sensors (MDS-MkV/T and MDS-MkV/L, Alec electronics). Salinity and nutrient concentrations were routinely measured during field studies. Triplicate sub-samples 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 (1999). Nitrate was determined by subtracting the values of nitrite from nitrate + nitrite. The detection limits were 0.052 µM for NO_3 + NO_2, 0.01 µM for NO_2, 0.020 µM for NH_4 and 0.020 µM for PO_4. Reproducibility of nutrient analysis was ± 0.5% .

Sampling of coral rubbles, sand and cyanobacteria mats were done using snorkeling or scuba diving, and documented by underwater photography. Samples were gentle placed into sterilized tubes or plastic bags, kept in cool box and brought back to the laboratory for sub-sampling and treatments. In the laboratory aliquots of the materials were placed in small flasks and kept with formaldehyde (4%) for later microscopic analysis and taxonomic identification. Other aliquots were used in incubation experiments for N_2 fixation and primary production measurements. For coral rubbles, two small branches of similar size were placed into the incubation bottle which was filled with seawater collected simultaneously at the same sampling point (incubation of epilithic plus endolithic algae). Other two branches of similar size were previously gentle brushed to remove the epilithic community and place in another incubation bottle also filled with seawater of the sampling point (incubation of endolithic algae). Aliquots of 10g of sand were placed into the incubation bottles which were filled with seawater from the same sampling point. Aliquots of approximately $1cm^2$ of cyanobacteria mats were placed into the incubation bottles and filled with seawater from the same sampling point. Control incubations using only seawater from the sampling points were carried out. All incubation bottles were enriched with ^{15}N and ^{13}C, and incubated *in situ*. Incubation started before the sunset and continued until early morning (12h incubations) for N_2 fixation of non-heterocystous cyanobacteria. For heterocystous cyanobacteria the incubation continued until the end of the following day (Charpy et al. 2007).

N_2 fixation rate and dissolved inorganic carbon uptake measurements were carried out according to Slawyk et al. (1977): Samples placed into polycarbonateincubation bottles of 180 mL fitted with a septum, were enriched with 0.36 mL of ^{13}C-labelled sodium bicarbonate ($NaH^{13}CO_3$ – 100mg in 10 mL of deionized water – 99.9% ^{13}C) to obtain an enrichment of 11.5%. Subsequently, 0.36 mL of $^{15}N_2$ (99.8 atom %, Shoko Co. Ltd, Tokyo, JAPAN) was added with a gas-tight syringe to obtain an enrichment of 6.8%. For mats, incubations were terminated by filtration under gentle pressure through a precombusted 47-mm diameter GF/F filter, acidified by HCl fumes and dried. For coral rubbles and sand, small subsamples were mashed on mortars and treated with HCl to remove the carbonates, and posterior filtrated onto precombusted 47-mm diameter GF/F filter. Filters were dried on 60°C for POC, PON, and isotope analysis. Similar subsamples without acidification treatment were prepared for HPLC analysis. Measurements of delta ^{13}C, delta ^{15}N, and POC, PON were done using a mass spectrometer DELTA plus Advantage (Thermofinigan Co.) equipped with EA1110 for measurements of POC and PON. Primary production was calculated according to Hama et al. (1993) and N_2 fixation rate was calculated by isotope mass balance as described in Montoya et al. (1996). 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 optical density at 665 nm was recorded before and after acidification. Chl-*a* concentration was calculated using the extinction coefficients given by Porra et al. (1989). For HPLC analysis, the pigments were

extracted with 95% ethanol. HPLC was carried out with a model LC-10AT$_{VP}$ (Shimadzu, Kyoto, Japan). Pigments were eluted at a flow rate of 1.0 mL/min at 25°C with a programmed binary gradient elution system. Separated pigments were detected spectrophotometrically with a photodiode array detector (Shimadzu SPD-M10AVP), measuring from 400 to 760 nm.

Results and discussion

Environmental parameters

Temperature, salinity and nutrient concentrations in the ambient water of studied sites at La Reunion and Sesoko islands are summarized in Table 1. The total dissolved inorganic nitrogen (DIN) was highest in La Reunion in November 2004 (1.71 µM) and lowest in Sesoko in Station B in October 2006 (0.20 µM). In La Reunion, high values of ammonia and nitrate indicate some degree of eutrophication and coastal pollution. Reduced nitrogen compounds were always low in Sesoko. Dissolved PO_4 concentrations were low (<0.21 µM) in all stations during all the study period. Water temperature and salinity in both sites were comparable during the study period and so was the photon flux.

Table 1. Environmental parameters

Place	T °C	Salinity	Light intensity	Nutrients (µM)				
			(µmol cm^{-2} sec^{-1})	NO_3	NO_2	NH_4	DIN	PO_4
La Reunion								
Nov. 2004	28.5	32.3	1928 ± 854	0.45	0.26	1	1.71	0.1
Feb. 2007	26.3	35.1	1696 ± 724	0.32	0.13	0.1	0.55	0.21
Sesoko								
St. B July 2005	29.4	34.3	2104 ± 497	0.66	0.04	0.1	0.8	0.07
St. B Oct. 2006	27.6	34.5	1816 ± 598	0.09	0	0.11	0.20	0.14
St. D Oct. 2006	27.3	34.6	1442 ± 392	0.28	0.04	0.17	0.49	0.04

Taxonomy

Algae associated to coral rubbles were studied microscopically after gentle brushing of rubbles previously fixed using formaldehyde. For the endolithes gentle acidification treatment was done. Algae associated with sand or cyanobacteria forming mats were studied under microscope from formaldehyde fixed samples. The epilithic algae were composed by a wide spectrum of algae including chlorophytes, phaeophytes, rhodophytes and bacillariophytes. Cyanobacteria of the genus *Lyngbya, Oscillatoria* and *Phormidium* were the main as epilithic in La Reunion and *Calothrix, Gardnerula* (both possessing heterocysts), *Hydrocoleum* and *Lyngbya* in Sesoko. Endolithic algae were composed by chlorophytes (mainly *Gomontia* and *Ostreobium*) and *Hyella* (cf.) *caespitosa*, *Plectonema* (cf.) *terabrans*, *Mastigocoleus testarumin* and *Scytonema* (cf.) *conchophillum* (the last two species with heterocysts). Same species of cyanobacteria and a high diversity of epilithic diatoms were found in sandy bottoms at both sites. Among the cyanobacterial mats the main identified species in La Reunion were *Nodularia* (with heterocysts*)*, *Oscillatoria* and *Leptolyngbya* at La Reunion and *Hydrocoleum coccineum*, *Nodularia harveyana* (with heterocysts) and *Phormidium laysanense* at Sesoko.

N_2 fixation rates in coral rubble

N_2 fixation rates in coral rubbles from La Reunion and Sesoko at different studied seasons are shown in Table 2. At La Reunion, rates for the incubations with only the endolithic alga during the first 12h (dark period) varied between 0.4 to 0.9 nanomoles N per µg Chl-*a*, and were 2.8 to 6.8 nanomoles N per µg Chl-*a* during 24h incubation. This shows that N_2 fixation was performed mainly during the 12h light period, indicating that heterocysts possessing cyanobacteria were the main components of endolithic algae. N_2 fixation rates for rubble incubations with endolithic plus epilithic algae varied from 0.33 to 2.7 nanomoles N per µg Chl-*a* during 12h (dark) and 0.45 to 4 N per µg Chl-*a* during 24h of incubations. These results show that the contribution of epilithic algae to the total N_2 fixation in rubbles is not so important if compared with rates obtained for only endolithes. Moreover in November 2004 N_2 fixation rate during 24h for endolithic plus epilithic algae was lower than that during 12h dark period, indicating that during day time there is a loose of N_2 fixed during the night. For the overall data of coral rubble in La Reunion Island it seems that N_2 fixation raters are higher in February (summer) than that of November (dry season). At Sesoko, N_2 fixation rates of endolithic alga during the first 12h (dark) incubations varied between 0.6 to 3.6 nanomoles N per µg Chl-*a* and 1.4 to 7.2 nanomoles N per µg Chl-*a* during 24h. Higher values of N_2 fixation during 24h incubations indicate that during light period N_2 was fixed and therefore this shows the presence of heterocyst possessing cyanobacteria. Rates for incubation with endolithic plus epilithic varied from 1.9 to 5.5 nanomoles N per µg Chl-*a* during the 12h dark period and 0.8 to 1.4 nanomoles N per µg Chl-*a* during 24h. These results show that in incubations with both epilithic plus endolithic algae fixed N_2 during the night was lost during the light period. For the overall data at Sesoko it seems that N_2 fixation rates by coral rubbles are higher in October (post summer season) than that of May (spring).

Table 2. Primary production, N_2 fixation rates and the contribution of N_2 fixation to net primary production of coral rubbles at La Reunion and Sesoko Islands. En: Endolithic algae; Ep: Epilitic algae

Site	Organism Rubble	Primary Production (nmoles C µg Chl-a^{-1}day^{-1})	N_2 fixation 12h (dark) (nmoles N µg Chl-a^{-1} time^{-1})	N_2 fixation 24h (nmoles N µg Chl-a^{-1} time^{-1})	C/N	Organic carbon prod. by N_2 fixation (nmoles C µg Chl-a^{-1}day^{-1})	Contribution of N_2 fix. to Primary Production (%)
La Reunion (Feb. 2007)	En	194.7 ± 53.8	0.9 ± 0.1	6.8 ± 2.3	6.9 ± 0.3	46.6 ± 13.7	23.8 ± 0.5
	Ep + En	191.91 ± 34.1	0.33 ± 0.2	4.0 ± 0.4	7.9 ± 0.8	32.0 ± 6.8	16.6 ± 0.6
La Reunion (Nov. 2004)	En	399.4 ± 300.1	0.4 ± 0.2	2.8 ± 1.3	21.6±11.7	76.1 ± 55.5	28.1 ± 11.7
	Ep + En	210.62 ± 42.5	2.7 ± 1.8	0.45 ± 0.18	11.6±6.1	5.5 ± 3.5	2.4 ± 1.1 n = 12
Sesoko (May 2007)	En	356.2 ± 271.1	0.6 ± 0.4	1.4 ± 0.52	9.0 ± 2.4	12.6 ± 6.8	3.5 ± 0.2
	Ep + En	134.6 ± 55.2	1.9 ± 0.4	1.43 ± 0.5	9.7 ± 0.6	13.8 ± 5.2	10.3 ± 0.3
Sesoko (Oct. 2007)	En	366.6 ± 6.1	3.6 ± 0.6	7.17 ± 0.9	8.5 ± 0.5	60.9 ± 5.2	16.62
	Ep + En	176.2 ± 84.2	5.5 ± 3.8	0.77 ± 0.4	9.6 ± 1.1	7.4 ± 9.7	4.19 n = 12

N_2 fixation rates of cyanobacteria mats

N_2 fixation rates of cyanobacrteria mats are shown in Table 3. Among the three species of cyanobacteria forming mats *Nodularia* showed high fixation rates (112 nanonoles N per µg Chl-*a*) during 24 hours compared with rates at dark period indicating the efficiency of the heterocyst possessing cyanobacteria. The other two identified species forming mats, *Oscillatoria* and *Leptolyngbia* showed almost no N_2 fixation activity during the day time. In Sesoko *Hydrocoleum coccineum* showed the higher rates of N_2 among all the studied cyanobacterial mats, being 288 nanomoles N per µg Chl-*a* during the night and 242 nanomoles N per µg Chl-*a* during 24h.While these species do not posses heterocysts and the N_2 fixation activity was observed mainly during the night period, these rates were one order of magnitude higher if compared with other mats from Sesoko or two times to two orders of magnitude higher when compare with fixation rates of mats at La Reunion Island

N_2 fixation rates of sandy bottom

N_2 fixation measurements of sand were performed only at Sesoko Island. Rates are shown in Table 4. During the dark period, rates were 0.35 nanomoles N per µg Chl-*a* and 2.8 nanomoles N per µg Chl-*a* in 24 h. These rates are comparable to the lower rates observed for coral rubbles at La Reunion during November.

Table 3. Primary production, N_2 fixation rates and the contribution of N_2 fixation for the net primary production of cyanobacteria mats at La Reunion and Sesoko Islands

Site	Organism	Primary Production (nmoles C µg Chl-a^{-1}day^{-1})	N_2 fixation 12h (dark) (nmoles N µg Chl-a^{-1}time^{-1})	N_2 fixation 24h (nmoles N µg Chl-a^{-1}time^{-1})	C/N	Organic carbon production by N_2 fixation (nmoles C µg Chl-a^{-1}day^{-1})	Contribution of N_2 fixation to Primary Production (%)
La Reunion (2004, 2007)	*Nodularia**	2359.9	0.63	112.2	8.8	987.0	41.8
	Oscilatoria	2063.3	7.80	4.2	13.6	57.0	2.8
	Leptolyngbia	14.0	0.64	0.2	12.6	2.9	20.7
Sesoko (Stn. C)	*Hydrocoleum coccineum*	9125.6	288.1	242.0	10	2419.5	26.5
(Stn. B)	*Nodularia harveyana**	3884.9	0.7	28.5	16	464.7	12
(Stn. B)	*Phormidium laysanense*	6289.3	28.9	27.1	23	629.2	10

*posess heterocysts

Table 4. Primary production, N_2 fixation rates and the contribution of N_2 fixation for the net primary production of sandy bottom at Sesoko Island

Site and 0rganism	Primary Production (nmoles C µg Chl-a^{-1}day^{-1})	N_2 fixation 12h (dark) (nmoles N µg Chl-a^{-1}day^{-1})	N_2 fixation 24h (nmoles N µg Chl-a^{-1}day^{-1})	C/N	Organic carbon production by N_2 fixation (nmoles C µg Chl-a^{-1} day^{-1})	Contribution of N_2 fixation to Primary Production (%)
Sesoko Sandy bottom	545.4 ± 118.2	0.35 ± 0.3	2.85 ± 2.0	10.9 ± 1.6	31.6 ± 25.2	5.7 ± 3.9 n = 6

Contribution of N_2 fixation to primary production:
On the bases of measured C/N ratios of all the studies substrata (see tables 2, 3 and 4) it is possible to calculate the amount of organic carbon which can be fixed as new production on the bases of the fixed atmospheric nitrogen. Further, using the measured primary production rates, it is possible to estimate the contribution of new production to the total primary production of the studied substrata. For coral rubble from La Reunion this contribution varied from 24% to 28% for endolithic algae and from 2.4% to 17% for epilithic plus endolithic algae. At Sesoko the contribution of N_2 fixation to primary production of coral rubbles was lower and varied between 3.5% to 17% for endolithic algae and 4% to 10% in case of epilithic plus endolithic algae. In case of cyanobacteria mats, the contribution of N_2 fixation to the total primary production varied from 2.8% to 42% at La Reunion and from 12 to 28.5 at Sesoko. In sandy bottom, the contribution of new production to the total primary production was 5.7%

Table 5. N_2 fixation rates during night and 24h of coral rubble, cyanobacteria mats and sandy bottom in comparable units of mg N m^{-2} $time^{-1}$

Type of Sediment	Location	N_2 fixation 12h (dark) mg N m^{-2} $time^{-1}$	N_2 fixation 24h mg N m^{-2} $time^{-1}$
Coral gravel	Sesoko	1.45 ± 0.84	2.37 ± 1.93
	La Reunion	0.57 ± 0.37	2.07 ± 1.2
Cyanobacteria mat	Sesoko	64.14 ± 3.05	94.81 ± 7.42
	La Reunion	27.12 ± 7.32	96.98 ± 2.28
Sand	Sesoko	0.20 ± 0.17	3.08 ± 1.79

Conclusions
Biological N_2 fixation was found in all the studied substrata. The presence of heterocysts possessing cyanobacteria was important among the endolithic algae and showed higher efficiencies of N_2 fixation and important contribution to primary production (up to 28%). Highest N_2 fixation was observed in cyanobacterial mats. In particular *Hydrocoleum coccineum* (Sesoko) was the highest among the non-heterocysts cyanobacteria and *Nodularia* (La Reunion) among heterocysts possessing ones. The contribution to primary production was up to 34%. In sandy bottom N_2 fixation rates was similar to that of coral gravel at the same location and season (Sesoko, May), but this fixation represented less than 6% of required N for the primary production.

Table 5 shows a summary of the results of N_2 fixation rates for the three studies substrata in terms of mg of fixed N_2 per m^2. These results show that N_2 fixation rates of cyanobacteria mats are order of magnitude higher than that of coral rubbles or sandy bottom. However, considering the covered area in the whole reef, the contribution of coral gravel and sandy bottom might be the highest. In the present study all cyanobacteria mats were found to be of small size and covered less than 1% of the total reef area.

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