DIAZOTROPHIC GROWTH AND NITROGENASE ACTIVITY OF CYANOBACTERIA FROM THE PATOS LAGOON ESTUARY, SOUTHERN BRAZIL[1]

J. S. YUNES*
A. G. SILVEIRA
M. T. SUZUKI
M. G. CAMARGO
V. R. WERNER**

ABSTRACT

Several samples of cyanobacteria were collected from freshwater, brackish and marine environments of, and near, the Patos Lagoon estuary in Southern Brazil. Laboratory isolation and purification techniques were applied to obtain 25 axenic cultures in medium free of combined nitrogen. From these, 10 cultures were selected and their diazotrophic growth and nitrogenase activity were followed for 30 days of cultures in a mineral freshwater medium (VB-S) and in a saltrwater medium (ASN-III°). Diazotrophic growth and nitrogenase activity were monitored by the chlorophylla content and acetylene reduction rates, respectively.

Cultures of *Nostoc* strain RSA_z 8601, *Calothrix* strain RSA_z 8601, *Nostoc* strain RSJ 8502, *Microchaete* strain RSJ 8501 and *Anabaena* strain RST 8701, all of which are heterocystous, isolated originally from fresh and brackish water, showed an increasing diazotrophic growth. These cultures also presented higher levels of nitrogenase activity at the lag phase of growth. The two non-heterocystous strains, *Synechocystis* RSSM 8801 and *Oscillatoria* RSSM 8801, originally isolated from marine waters, presented a diazotrophic growth comparable to the heterocystous cultures, although no nitrogenase activity was detected throughout the light and dark cycles.

Cultures of *Anabaena* strain RSC 8801, *Mastigocladus* strain RSC 8801, *Lyngbya* strain RSSM 8801 originally isolated from marine and brackish waters grew much more slowly and no nitrogenase activity was detected.

Unidade de Pesquisas em Cianobactéria - Dep. de Química - URG - C. P. 474 - 96201-900 - Rio Grande - RS - Brasil.

* To whom correspondence should be adressed.

** Museu de Ciências Naturais, Fundação Zoobotânica do Rio Grande do Sul, Caixa Postal 1188, CEP 90690-000, Porto Alegre, RS.

These results suggest a division of the 10 cultures in the basis of the growth rate under the conditions tested. It is possible to conclude that such genera (particularly the heterocystous strains studied) from freshwater and brackish environments may be important as potential N_2 fixers for laboratory studies regarding environmental conditions.

KEY WORDS: cyanobacteria, nitrogenase activity, diazotrophic growth.

RESUMO

Diversas amostras de cianobactéria foram coletadas em ambientes de água doce, mixoalina e marinha no estuário da Lagoa dos Patos e próximos a este. Vinte e cinco culturas axênicas, em meios livres de nitrogênio, foram obtidas através de técnicas de isolamento e purificação em laboratório. A partir destas, 10 culturas foram selecionadas e estudadas por 30 dias em seu crescimento diazotrófico, nos meios VB-S (para água doce) e ASN-III° para espécies marinhas, e na atividade da enzima nitrogenase. O crescimento diazotrófico e a atividade da nitrogenase foram medidos pelo conteúdo de clorofila *a* e pela técnica da redução do acetileno, respectivamente.

As culturas de *Nostoc* RSAz8601, *Calothrix* RSA_z8601, *Nostoc* RSJ8502, *Microchaete* RSJ8501 e *Anabaena* RST8701, todas heterocísticas e isoladas originalmente da água doce e mixoalina, demonstraram alto crescimento diazotrófico. Estas culturas também apresentaram altos níveis de atividade da nitrogenase durante a fase de crescimento inicial. Os dois gêneros não-heterocísticos, *Synechocystis* RSSM8801 e *Oscillatoria* RSSM8801, originalmente isolados do meio marinho, apresentaram crescimento diazotrófico comparável ao das culturas heterocísticas, embora nenhuma atividade da nitrogenase tenha sido detectada nos ciclos de luz e escuro.

As culturas *Anabaena* RSC8801, *Mastigocladus* RSC8801 e *Lyngbya* RSSM8801 isoladas dos meios marinhos e mixoalinos cresceram mais lentamente e nenhuma atividade da enzima nitrogenase foi detectada.

Os resultados propõem uma divisão das 10 culturas em relação ao seu crescimento sob as condições de culturas testadas. Foi possível concluir que esses gêneros (particularmente os heterocísticos) originários de águas doces ou mixoalinas são importantes fixadores de N_2 para estudos de laboratório associados às condições de crescimento no meio ambiente.

1 - INTRODUCTION

Cyanobacteria are photosynthetic prokaryotes, many species of which (including unicellular, filamentous non-heterocystous and filamentous heterocystous) are able to fix N_2 (Rippka et al. 1979) and may have an important role in nitrogen cycling in coastal benthic environments (Capone, 1983). The present work aims to study cyanobacteria isolated from the Patos Lagoon (Brazil), an estuary characterized by a rich nutrient supply (Niencheski

and Windom, 1994) and distinct salt gradients (Kantin, 1983). Benthic cyanobacteria have been isolated from the margins of the Patos Lagoon and their ability to fix N_2 studied using the acetylene-reduction technique of Stewart et al. (1967). Although this technique has limitations, it is still a rapid, simple and useful method to assess nitrogenase activity. Investigations were performed in the field and in the laboratory under conditions approximating to those in the lagoon itself. Such an approach can provide useful information on the potential contribution of the cyanobacteria to the environment (Horne and Commins, 1987).

The diazotrophic growth of *Nostoc muscorum* strain RSJ 8501 under conditions of combined nitrogen availability approximating to those in the lagoon (Yunes et al. 1990) has been previously reported. In the present work, this evaluation is extended to other benthic cyanobacterial isolates representing the variety of benthic habitats in the Patos Lagoon and its surrounding areas.

2 - MATERIALS AND METHODS

2.1 - Isolation and culture conditions

The organisms investigated below were sampled from the estuarine margins of the Patos Lagoon and nearby areas, situated in the state of Rio Grande do Sul, Brazil (Fig. 1). Isolates were purified by successive plating on nitrogen-free growth medium. Samples from brackish and fresh waters were grown on liquid cultures of the VB-S mineral medium (Yunes & Melo, 1987) and salt water samples in the ASN-III° mineral medium (Rippka et al., 1979). Strains were identified according to Desikachary (1959), classified following the nomenclature for cyanobacteria proposed by Rippka et al. (1979) and code-registered by the local and year of sampling. The axenic nature of the culture was controlled by microscopic examination. Isolates were grown in 1.8ℓ batch culture at 25°C in either VB-S or ASN-III° media, and 12h light/ 12h dark regime was imposed with a photon flux rate of 107 $\mu E\ m^{-2}\ s^{-1}$ irradiance of the surface of the culture during the light period. Air was filtered through 25% H_2S0_4, 1% $NaHCO_3$ and distilled water, and bubbled into the cultures at a rate of 0.265ℓ/min.

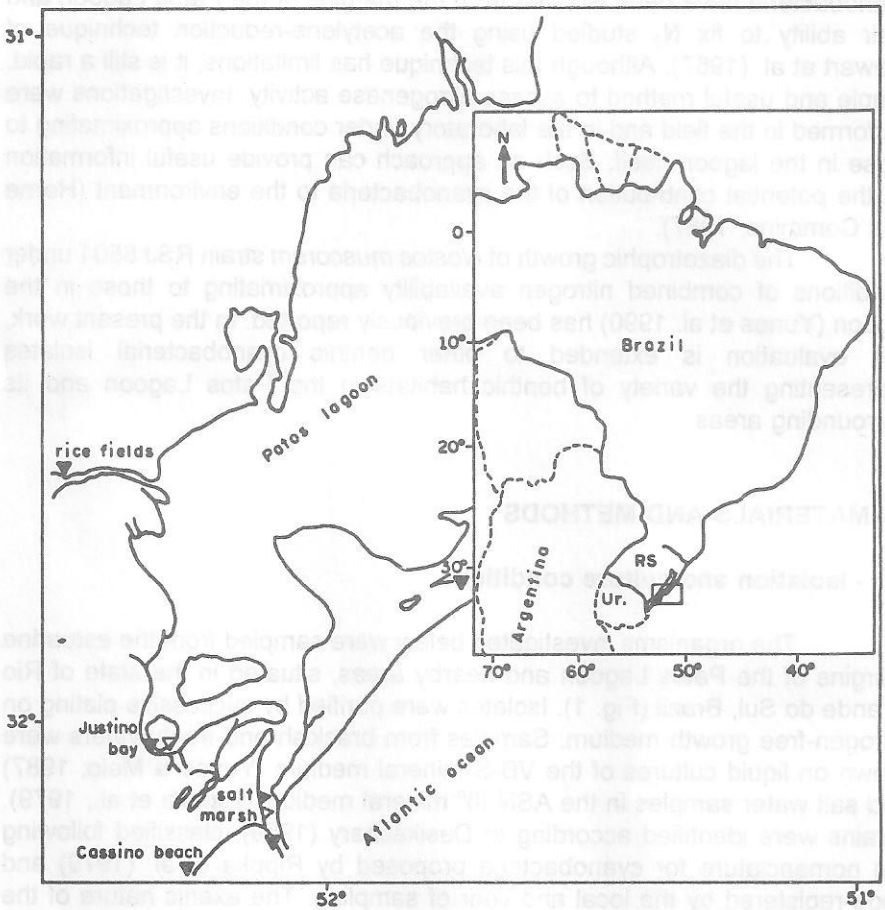

FIGURE 1 - The Patos Lagoon estuarine area and its location in the South of Brazil (inset). The places marked are sampling sites for cyanobacteria (▾) and where in situ acetylene reduction measurements (▽) were performed.

2.2 - Nitrogenase activity

The acetylene reduction assay (Stewart et al. 1967) was used to measure nitrogenase activity. Ten mℓ aliquots of growing cultures were sealed in 20ml vacumtainer tubes (NEW VACUO). Acetylene was injected to a 10% v/v of internal atmosphere and incubated for 1h at 25°C and under

107μE. m^{-1} s^{-1} of irradiation. Ethylene production was measured by injecting 1ml of the gas phase into a gas cromatograph (VARIAN MODEL 2485) with a flame ionization detector and a PORAPAK N COLUMN. All gases were super dried type (WHITE MARTINS). *In situ* nitrogenase activity tests were performed by using inverted funnels sealed with rubber stoppers and placed over cyanobacterial mats. Acetylene was injected through the rubber seals to a 1% v/v of the remaining atmosphere. After 3h of *in situ* incubation, 1ml of the gas phase was sampled into syringes which were then sealed and transferred to the laboratory. Ethylene production was measured as above.

2.3 - Diazotrophic growth

It was followed by the increase in chlorophyll *a*. Ten ml aliquots of culture were collected by centrifugation, resuspended in 5ml of methanol and incubated in the dark at 4°C for 24h. Absorption was recorded at 663mm and 750mm and the chlorophyll *a* calculated with the coefficient of 75 L.g. cm^{-1} (Rieman, 1980).

3 - RESULTS AND DISCUSSION

3.1 - Diazotrophic growth

The diazotrophic growth was different in the ten strains examined (Fig. 2). The most rapid growth (as estimated by chlorophyll *a* concentration) was shown by cultures of *Nostoc* RSA_Z8601 (*Nostoc muscorum*), *Anabaena* RST8701 (*Anabaena verrucosa*), *Nostoc* RSJ8502 (Nostoc *punctiforme*), *Microchaete* RSJ8501 (*Microchaete tenera*) and *Calothrix* RSA_Z8601 (*Calothrix brevissima*). These organisms grew to a density greater than 1.2mg. chlorophyll *a*.ml^{-1} after 30 days of culture period. Most notably, the two Rivulariacean genera, *Microchaete* RSJ8501 and *Calothrix* RSA_Z8601 grew to a density of 3.0mg. chlorophyll *a*.ml $culture^{-1}$ during the same period. Although variable, these cultures tended to enter the log phase of growth after 15 days.

All the above are heterocystous strains isolated from fresh and brackish waters and their ability to grow diazotrophically is not surprising. A high efficiency of growth in a nitrogen-free mineral medium is a common feature of some *Anabaena* and *Nostoc* isolates either from paddy soils (Antarikanonda and Lorenzen, 1982; Chen, 1985) or from marine coastal environments (Gotto et al., 1979).

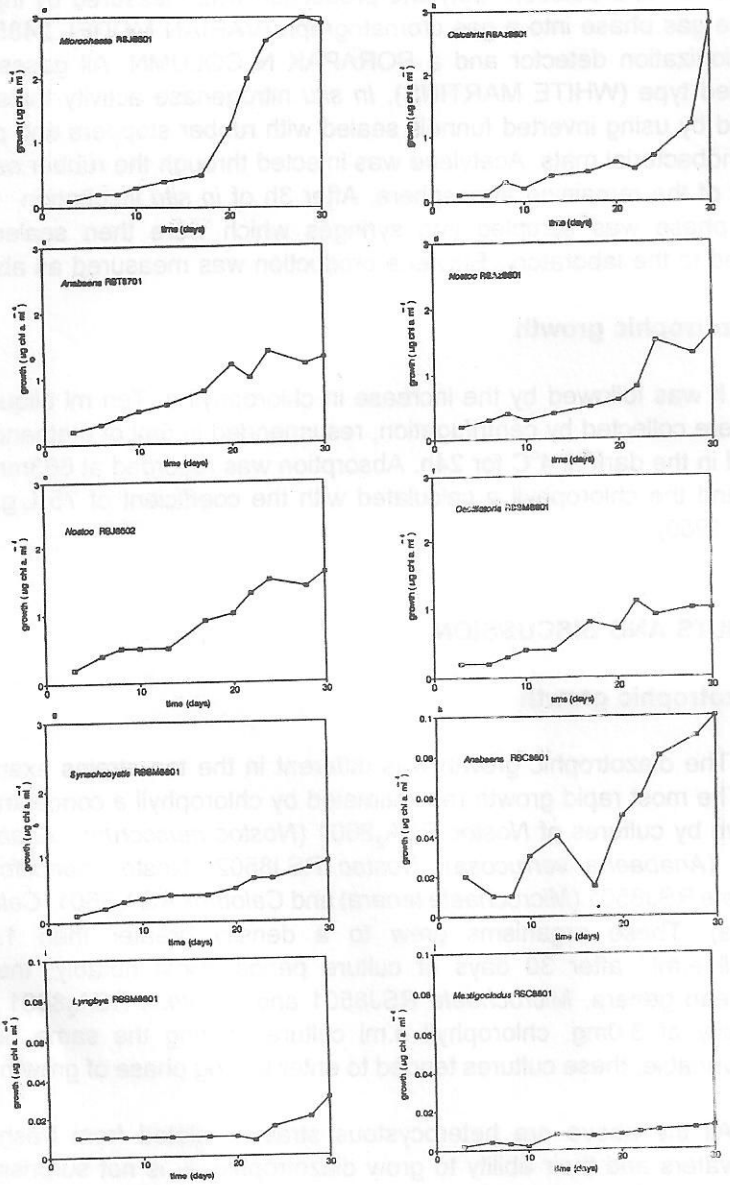

FIGURE 2 - Diazotrophic growth of the ten strains studied in nitrogen-free mineral media during 30 days. Growth was followed as µg chlorophyl-a per ml culture. Note the different scale for h, i and j.

Growth of the two marine non-heterocystous *Oscillatoria* RSSM 8801 and *Synechocystis* RSSM 8801(*Aphanothece stagnina)* strains was slow in comparison to the heterocystous strains. Growth reached 1.0mg chlorophyll *a*:ml culture^{-1} after 30 days and both cultures have not left the lag phase of growth during the test. However, on these cyanobacteria (which lack heterocysts) the ability of *Oscillatoria* species to fix N_2 under light and dark cycles is well documented (Khames et al., 1987) and are important as a nitrogen-fixing organism in oceanic surface waters (Carpenter, 1983). Similarly, *Synechocystis* species are widely distributed in coastal areas and some species can fix N_2 (Rippka and Waterbury, 1977); therefore, the lower diazotrophic growth observed in these strains is possibly related to less favourable growth conditions, e.g. nutrient composition and physical factors. Recently, cultures of *Synechocystis* RSSM 8801 (*Aphanothece stagnina)* have been successfully grown on nitrate added medium (our own observation).

Cultures of *Anabaena* RSC 8801 exhibited an even lower growth than the cultures above. The strains *Lyngbya* RSSM 8801 and *Mastigocladus* RSC8801 (*M.laminosus*) have not shown any ability to grow diazotrophically under the conditions tested. The growth of the marine *Anabaena* RSC8801 compares poorly with that of the freshwater *Anabaena* strain (*Anabaena* RST8701). One reason for this may be the higher temperature required for growth in some marine *Anabaena* species (Gotto et al. 1979). Although the occurrence of *Lyngbya* in estuarine and coastal environments has been frequently reported (Baker, 1987; Bebout et al., 1987), there is little information on its ability to fix N_2 (Stewart, 1968). *Mastigocladus* RSC8801 has been reported to fix N_2 (Fogg, 1951); however, this ability is restricted to temperatures above 45°C (Stevens et al., 1985) and the low temperatures used in the present study may explain the poor growth of *Mastigocladus* RSC8801. In the Patos Lagoon, the water temperatures rarely exceeds 30°C and as such, the importance of diazotrophic growth of this *Mastigocladus* strain may be minor.

3.2 - Nitrogenase activity

Nitrogenase activity (expressed per ml of culture) was detectable in five heterocystous strains only (Fig. 3) and was higher in *Microchaete* RSJ8501, *Nostoc* RSA_Z8601 and *Nostoc* RSJ8502. *Calothrix* RSA_Z8601 and *Anabaena* RST8701, among them, exhibited lower levels of nitrogenase activity supported by the photoautotrophic growth regime. Photosynthesis in the vegetative cells provides sufficient ATP and reductant to sustain nitrogenase activity in heterocysts (Bottomley and Stewart, 1977). Heterocysts are especialized cyanobacterial cells which lack photosystem II and provide

ideal conditions for nitrogenase synthesis and activity (Haselkorn, 1978).

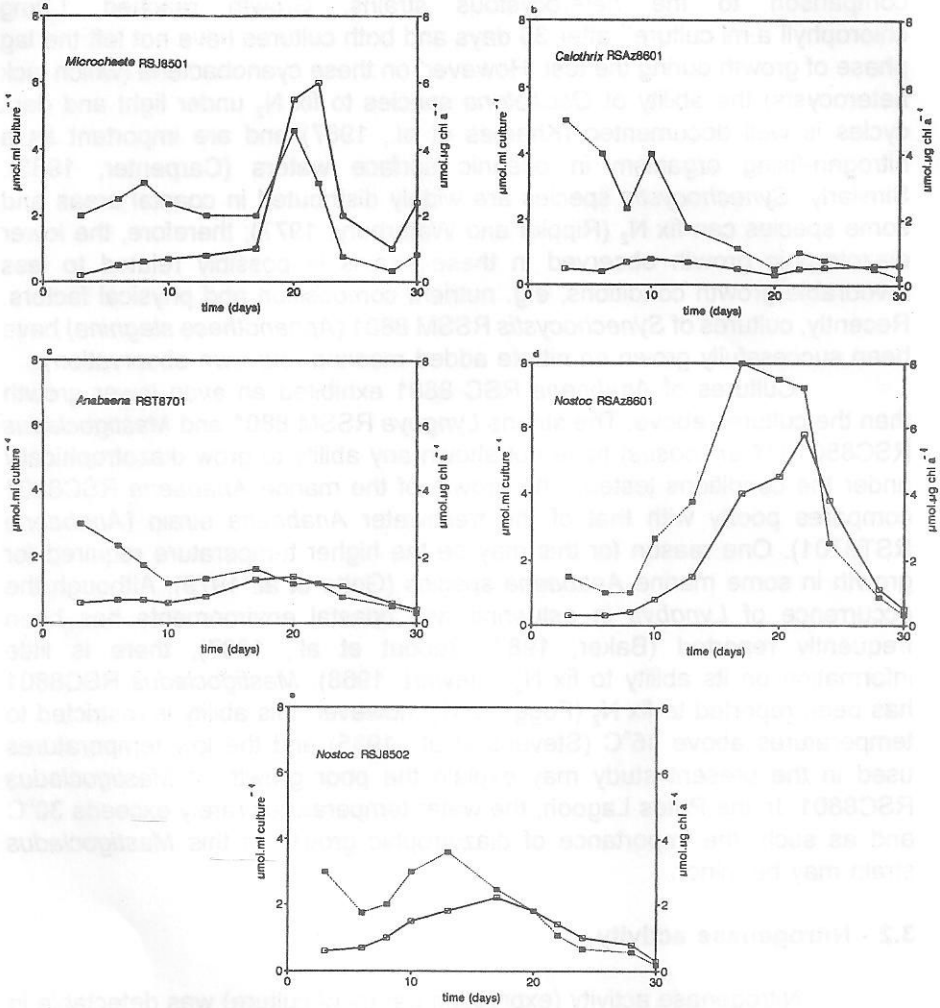

FIGURE 3 - Nitrogenase activity assayed as µmol of acetylene reduced per ml of culture (□) and as a ratio of chlorophyl-a concentration per ml of culture (■) during 30 days of growth.

However, this especialization is not provided in non-heterocystous species. Their nitrogenase and photosynthesis enzymes are restricted to the same vegetative cells and, in this case, their diazotrophic hability is

maintained by several mechanisms (Gallon, 1981). In the other five cultures, nitrogenase activity could not be detected in either the light or the dark phases of the diurnal illumination regime. In the case of *Oscillatoria* RSSM8801 and *Synechocystis* RSSM8801 which had a lower diazotrophic growth (Fig. 2), the lack of detectable nitrogenase activity may be due to inhibition by sampling procedures on insufficient volume of cells. In the other cultures where diazotrophic growth was undetected, a limitation of N_2-fixation by sub-optimum temperatures (*Mastigocladus* RSC 8801 - Stevens et al., 1985) or oxygen inhibition (*Lyngbya* species - Gallon, 1980) may be responsible for the lack of nitrogenase activity.

3.3 - Nitrogenase activity and pigment concentration

In N_2-fixing cultures, the ratio of nitrogenase activity to chlorophyll *a* concentration decreased during the 30 days of culture, except for *Microchaete* RSJ8501 and *Nostoc* RSA_z8601 cultures (Fig. 3). In most cases, a higher ratio was found in the first 10 days of culture, followed by a sharp decrease which led to a steady and lower ratio towards the end of 30 days of culture. The increase observed in *Microchaete* RSJ8501 and *Nostoc* RSA_z8601 nitrogenase activity (as a chlorophyll *a* ratio) until the 20th day of culture is explained by the late start of log phase of growth already mentioned.

If this model of nitrogenase activity is compared to diazotrophic growth, it resembles an opposite feature of the two-phase growth model previously presented (Fig. 2). This may suggest that the higher rates of nitrogenase activity in the first days of cultures promoted sufficient nitrogen reserves to allow higher rates of cell division and pigment synthesis throughout log phase. This correlation has been previously demonstrated for chlorophyll *a* and also phycocyanin synthesis in both unicellular (Gallon et al., 1975) and heterocystous (Thomas, 1972) cyanobacteria. Such processes may also be relevant for growth in the environment.

3. 4 - In situ nitrogenase activity

In situ nitrogenase activity was estimated in cyanobacterial mats of several sites on the Patos Lagoon margins. At most of these sites, a mixture of cyanobacteria species were present. In the brackish and freshwater environments of Justino bay and in the EMBRAPA rice fields, *Nostoc, Anabaena* and *Calothrix* species dominated. In marine habitats (salt marsh), *Oscillatoria, Lyngbya* and other unicellular and filamentous non-heterocystous species were found. Values for acetylene reduction at sites where nitrogenase activity was detected ranged from 0.0423 nmol $C_2H_4 . cm^{-2} . h^{-1}$ to 1.3572 nmol $C_2H_4\ cm^{-2} . h^{-1}$ (Tab. 1). At several sites where *Oscillatoria* and other non-heterocyst species

were dominant, nitrogenase values were lower than 0.01 nmol C_2H_4 cm^{-2} h^{-1}.

TABLE 1 - Nitrogenase activity assayed as nmol C_2H_4 cm^{-2} h^{-1} of acetylene reduced and the predominant genera of cyanobacteria found on the margins of two sites in the Patos Lagoon estuary. The data shown are the average of 5 determinations on each test.

SITE OF ASSAY	nmol C_2H_4 cm^{-2} h^{-1}	PREDOMINANT GENERA
Brown mat on flooded margins, Justino bay.	0.245	*Nostoc, Anabaena* and *Oscillatoria.*
Green mat on wooden surface under water, Justino bay.	0.230	*Calothrix*
Green mat on flooded margins, Justino bay.	1.357	*Nostoc* and *Anabaena*
	0.070	*Nostoc, Anabaena, Oscillatoria* and *Lyngbya.*
Green mat on the upper littoral margins, salt marsh, Cassino beach.	< 0.010	*Oscillatoria* and *Chlorogloea*
	< 0.010	*Oscillatoria, Anabaena*
	0.303	*Microcystis* and *Spirulina*
	< 0.010	*Nostoc* and *Oscillatoria*
		Oscillatoria, Microcystis Anabaena and *Spirulina*

Likewise, previously reported field data on nitrogenase activity in cyanobacterial mats at coastal and estuarine sites (Gotto et al.,1981; Huber, 1986) and salt marshes (Stewart et al., 1978) have been within the same range. There are however a wide range of potential sources of errors in estimating nitrogenase activity in the field; notably temperature (Whiting and Morris, 1986), light and diel variations (Huber, 1986; Bebout et al., 1987), presence of nitrogen salts and oxygen (0_2). Furthermore, the acetylene reduction technique has limitations on accuracy (Dicker and Smith, 1980) and calibration and interpretation (Seitzinger and Gaber, 1987). As such, it would

be unwise to draw firm correlations between *in situ* and laboratory data. However, despite the limitations, nitrogenase activity was recorded in cyanobacterial mats where the studied strains were predominant and it suggests that these strains may play a role as diazotrophs in the estuarine area studied.

4 - CONCLUSION

Ten cyanobacterial strains isolated from the Patos Lagoon estuary can be grouped on the basis of growth rate under Nitrogen-free culture conditions. It is possible to conclude that such heterocystous genera from brackish and freshwater environments may be useful for laboratory studies and potentially important as nitrogen-fixing organisms, in their original habitats.

ACKNOWLEDGEMENTS

This work was supported by research grants and research assistantships awarded by CNPq (Brazil). We wish to thank Dr. Gordon Simpson for his important suggestions and language corrections.

REFERENCES

1. ANTARIKANONDA, P. & LORENZEN, H. 1982. N_2-fixing blue-green algae (cyanobacteria) of high efficiency from paddy soils of Bangkok, Thailand: characterization of species and N_2-fixing capacity in the laboratory. *Arch.Hydrobiol.*(Suppl 63) I (30): 53-70.
2. BAKER, K. K. 1987. Systematics and ecology of *Lyngbya* spp. and associated species (cyanophyta) in a New England salt marsh. *J. Phycol.* 23: 201-208.
3. BEBOUT, B. M.; PAERL, H. W.; CROCKER, K. M. & PRUFERT, L. E. 1987. Diel interactions of oxygenic photosynthesis and N_2- fixation (acetylene reduction) in a marine microbial mat community. *Appl. Environ. Microbiol.*, 53, (10): 2353-2362.
4. BOTTOMLEY, P. J. & STEWART, W. D. P. 1977. ATP and nitrogenase activity in nitrogen-fixing heterocystous blue-green algae. *New phytol.* 79: 625-638.
5. CARPENTER, E. J. 1983. Nitrogen Fixation by marine *Oscillatoria (Trichodesmium)* in the world's ocean. In: CARPENTER, E. J. & CAPONE, D. G. (eds.) *Nitrogen in the Marine Environment.* New York: Acad. Press. p. 65-103.
6. CAPONE, G. D. 1983. Benthic Nitrogen Fixation. In: CARPENTER, E. J. & CAPONE, D. G. (eds.) *Nitrogen in the Marine Environment,* New York: Acad. Press. p. 105-137.
7. CHEN, P. C. 1985. Physiology of nitrogen fixation in two new strains of *Anabaena. L. Naturforsh* ,40_c: 406-408.
8. DESIKACHARY, T. V. 1959. *Cyanophyta.* New Delhi: Indian Council of Agricultural Research. 686p.
9. DICKER, H. J & SMITH, D. W. 1980. Acetylene reduction (nitrogen fixation) in a Delaware, USA, salt marsh. *Mar. Biol.,* 57: 241-250.
10. FOGG, G. E. 1951. Studies on Nitrogen Fixation by Blue-Green. *J.Exptl.Bot.,* 2: 117-120.
11. GALLON, J. R.; KURZ, W.G.W & LA RUE, T.A. 1975. The physiology of nitrogen fixation by *Gloeocapsa* sp. In: STEWART, W. D. P. (ed.) *Nitrogen fixation by free living Organisms.* Cambridge: University Press. p. 159-174.

12. GALLON, J. R. 1980. Nitrogen Fixation by Photoautotrophs. In: STEWART, W. D. P. & GALLON, J. R. (eds). *Nitrogen Fixation.* LONDON: Acad. Press, p. 197-233.
13. GALLON, J. R. 1981. The oxygen sensivity of nitrogenase: a problem for biochemists and microorganisms. *Trends in Biochem. Sci.,* 6: 19- 23.
14. GOTTO, J. W. TABITA, F. R. & VAN BAALEN, C. 1979. Isolation and characterization of a rapidly-growing, marine nitrogen-fixing strains of blue-green algae. *Arch. Microbiol.*, 121: 155-159.
15. GOTTO, J. W.; TABITA, F. R. & VAN BAALEN, C., 1981. Nitrogen Fixation in Intertidal Environments of Texas Gulf Coast. *Estuarine, Coastal and Shelf Sciences,* 12: 231-235.
16. HASELKORN, R. 1978. Heterocysts. *Ann. Rev. Plant. Physiol.,* 29: 319- 344.
17. HORNE, A. J & COMMINS, M. L., 1987. Macronutrient controls on nitrogen-fixation in planktonic cyanobacterial populations. *New Zealand Journal of Marine and Freshwater Research,* 21: 413-423.
18. HUBER, A. L. 1986. Nitrogen fixation by *Nodularia spumigena* Mertens (Cyanobacteriacea). 1. Field Studies and the contribution of blooms to the nitrogen budget of the Peel-Harvey Estuary, Western Australia. *Hydrobiol.,* 131: 193-203.
19. KANTIN, R. 1983. Hydrologie et qualité des eaux de la region sud de la lagune dos Patos (Brésil) et de la plateforme continentale adjacente. Thèse de Doctorat d'État et Sciences. Université de Bordeaux. 185p.
20. KHAMEES, H.; GALLON, J. R.; CHAPLIN, A. E., 1987. The pattern of acetylene reduction by cyanobacteria grown under alternating light and darkness. *Br. Phycol. J.* 22: 55-60.
21. NIENCHESKI, L. F.; WINDOM, H.L. 1994. Nutrient flux and budget in the Patos Lagoon Estuary. The Science of the Total Environment. In press.
22. RIEMANN, B., 1980. A note on the use of methanol as an extraction solvent for chlorophyll-a determination. *Arch. Hydrobiol .Belh. Erg. Limnol.,* 14: 70-78.
23. RIPPKA, R. & WATERBURY, J. B. 1977. The synthesis of nitrogenaseby a non-heterocystous cyanobacteria. *FEMS Microbiol.,* lett. 2: 83-86.
24. RIPPKA, R; DERUELLES, J.; WATERBURY, J. B.; HERDMAN, M. & STANIER, Y. 1979. Generic assignments, strains histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* 111: 1-61.
25. SEITZINGER, S. P. & GARBER, J. H. 1987. Nitrogen fixation and $^{15}N_2$ calibration of the acetylene reduction assay in coastal marine sediments. *Marine Ecology, progress series* 37: 65-73.
26. STEVENS, E. S.; MHETA, V. B.; LANE, L. S. 1985. Heterocyst development and nitrogen fixation by *Mastigocladus laminosus.* In: LUDDEN, P. W. & BURRIS, J. E. (eds.) Nitrogen Fixation and CO_2 metabolism. *Elsevier.* p. 235-245.
27. STEWART, W. D. P.; FITZGERALD, G. P.; BURRIS, J. E. 1967. *In situ* studies on N_2-fixation using the acetylene reduction technique. *Proc. Nat. Acad. Sci.,* U.S.A., 58: 2071-2078.
28. STEWART, W. D. P.; FITZGERALD, G. P. & BURRIS, J. E. 1968. acetylene reduction by nitrogen-fixing blue-green algae. *Arch. Mickrobiol.,* 62: 336-348.
29. STEWART, W. D. P.; SAMPAIO, M. J.; ISHIEI, A. O.; SYLVESTER-BRADLEY, R. 1978. Nitrogen fixation by soil algae of temperate and tropical soils. In: DEBEREINER et al. (eds.) *Limitations and Potential for Biological Nitrogen Fixation in the Tropics.* Plenum Publishing Corporation. p. 41-63.
30. THOMAS, J. 1972. Relationship between age of culture and occurrence of pigments of photosystem II of photosynthesis in heterocystous blue-green alga. *J. Bacteriol.* 110: 92-95.
31. WHITING, G. J.; MORRIS, J. T. 1986. Nitrogen fixation (C_2H_2 reduction) in a salt marsh: its relationship to temperature and an evaluation of an *in situ* chamber technique. *Soil Biol. Biochem.,* 18 (5): 515-521.
32. YUNES, J. S.; MELO, S. R. 1987. Cianobactérias fixadoras de nitrogênio do estuário da Lagoa dos Patos, RS: métodos de cultivo em laboratório. *Neritica,* 2: 159-70.
33. YUNES, J. S.; SUZUKI, M. T.; SILVERA, A. G.; CAMARGO, M.; WERNER, V. R. 1990. Cianobactérias fixadoras de nitrogênio do estuário da Lagoa dos Patos, RS: *Nostoc muscorum. Ciência e Cultura,* 42 (5/6): 375-383.