N2 fixation by marine sponge associated cyanobacteria from the Portuguese coast.

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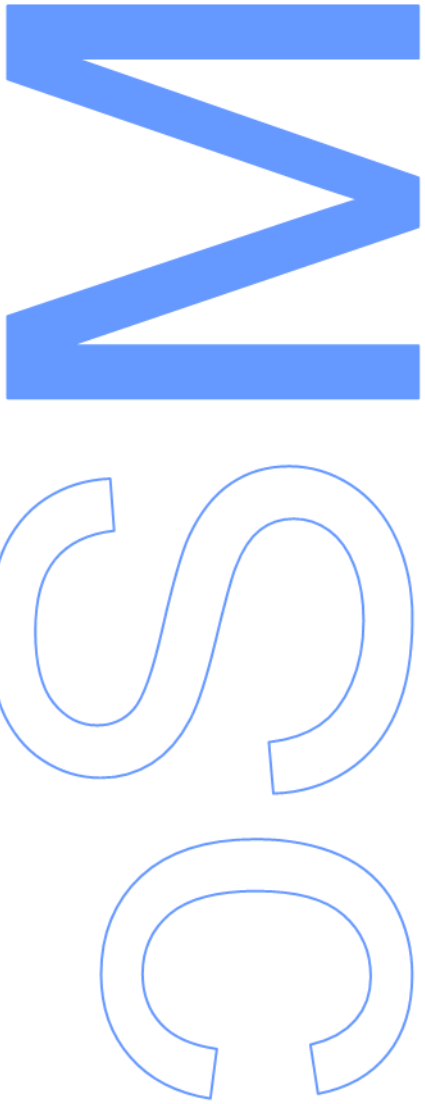

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ABSTRACT

Nitrogen (N_2) integrates the structure of many amino acids and proteins being an essential element of all known life forms. Fixed N_2 is often the limiting nutrient for growth of living organisms. Although N_2 is found in abundance in the atmosphere, it needs to be reduced in order to be taken up by most organisms. Marine biological N_2 fixation is regarded as an important process, especially on N_2 depleted areas, and the main challenge remains in developing reliable techniques to quantify and identify N_2 levels, fixing organisms and fixation rates; a lot of gaps exist on the knowledge regarding N_2 in marine environments. N_2 fixation in marine sponges remains to be clearly understood, several microorganisms have been linked to specific host sponges and whether microbial N_2 fixation is of major ecological significance for sponge's remains uncertain; in this study, we focus on cyanobacteria isolated from different marine sponges of the Portuguese coast analyzing them, in order to assess their capacity to fix nitrogen. Isolates were gradually cultivated in media with decreasing concentrations of combined nitrogen until being transferred to $BG11_0$ medium (NaNO3 free medium). Presence for both nifH and nifk genes was detected in *Pseudophormidium* sp. (LEGE 11386) which was able to grow prolifically in $BG11_0$ medium and was the only isolate to reveal nitrogenase activity, and therefore nitrogen fixation, using the acetylene reduction assay (ARA).

KEY-WORDS: CYANOBACTERIA; MARINE SPONGES; NITROGEN FIXATION; NITROGENASE ACTIVITY; ACETILENE REDUCTION ASSAY; NIF GENES.

RESUMO

O azoto (N_2) integra a estrutura de aminoácidos e proteínas sendo um elemento essencial para todas as formas de vida conhecidas. N_2 fixo é por muitas vezes o nutriente limitante no crescimento de organismos vivos. Apesar de o N_2 ser encontrado em abundancia na atmosfera, é necessário que seja reduzido para poder ser utilizado pela maioria dos organismos. A fixação biológica marinha de N_2 é considerada como um processo importante, especialmente em áreas desprovidas de N_2, e o principal desafio consiste no desenvolvimento de técnicas fiáveis para quantificar e identificar níveis de N_2, organismos fixadores, taxas de fixação, dado que existem muitas lacunas no conhecimento da fixação de N_2 em ambiente marinho. A fixação de N_2 em esponjas marinhas, ainda não esta totalmente percebida, vários organismos têm sido associados a esponjas hóspede específicas, e se a fixação de N_2 é ecologicamente significante para as esponjas é também incerto; este estudo foca as cianobactérias isoladas de diferentes esponjas marinhas da costa Portuguesa analisando-as de forma a avaliar a sua capacidade para fixar N_2. Os isolados foram gradualmente cultivados em meio com concentrações decrescentes de azoto combinado até serem transferidas para meio $BG11_0$ (sem NaNO3). Os genes nifH e nifK foram detectados em *Pseudophormidium* sp. (LEGE 11386) que foi capaz de crescer prolificamente no meio $BG11_0$ e foi o único isolado a revelar actividade da nitrogenase, e portanto fixação de N_2, usando o ensaio de redução de acetileno (ARA).

PALAVRAS-CHAVE: CIANOBACTERIAS; ESPONJAS MARINHAS; FIXAÇÃO DE AZOTO; ATIVIDADE DE NITROGENASE; ENSAIO DE REDUÇÃO DE ACETILENO; GÉNES NIF.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

N_2	Nitrogen
NO3	Nitrate
PO4	Phosphate
rRNA	Ribosomal ribonucleic acid
ARA	Acetylene reduction assay
O2	Oxygen
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
LEGE	Laboratório de Ecotoxicologia, Genómica e Evolução
ML	Maximum-Likelihood

1. Introduction

Theories on the role played by biotic life on the different nutrient cycles have often changed as advance on technological progress provides new tools and understanding. By the beginnings of the XX century Redfield developed the notion of a correlative ratio between nitrate and phosphate (NO3:PO4) discussing the stoichiometry of phytoplankton and seawater, thus achieving a better understanding of marine ecology. Later on, carbon (C) as well as other elements, were introduced in the equation and nowadays it is quite clear that several variables make the mechanisms explained by Redfield's ratio a more complex concept than initially thought (Kuypers et al, 2003).

Nitrogen is an essential element for the support of all life forms as integrates the structure of many amino acids and proteins; it occurs abundantly in the planet's atmosphere but in its molecular form is unusable and it needs to be reduced in order to be taken up for most organisms. Although initially the research on biological dinitrogen (N_2) fixation had other motivational origins, such as the one pursued by agricultural sciences, aiming towards increases on soil fertility in order to maximize crop yields (Belnap, 2001). Considering the fact that available nitrogen is crucial for most life forms we can understand that organisms like diazotrophic (nitrogen fixing) cyanobacteria are present all over Earth ecosystems. In fact, usually, they are the first organisms to colonize areas of exposed rock or soil. Generally cyanobacteria are known as aerobic photoautotroph's and mainly they require water, carbon dioxide, inorganic substances and light since they use photosynthesis as the principal way to obtain metabolic energy. Some species are known to be able to survive and adapt quite well to otherwise adverse conditions such as darkness or reduced oxygen levels and even switch to a heterotrophic nutrition (Fay, 1965). Thanks to these adaptations cyanobacteria usually become quite important assuming the role of base organisms by sustaining balance on the functional processes of the ecosystems and nutrient cycling (Whitton, 1992).

Over the last decade marine biological nitrogen fixation has been regarded as an important process especially on nitrogen depleted areas, the main challenge remains in developing reliable techniques to quantify and identify N_2 levels, fixing organisms and fixation rates in order to assess the magnitude of this process; even today a lot of gaps exist on the knowledge regarding nitrogen in marine environments (Zehr, 2002; Frank et al, 2003).

1.1 Classification of Cyanobacteria

Despite being monophyletic, cyanobacteria are morphologically diverse and regarding this diversity they were grouped into five subsections: Cyanobacteria of subsection I (formerly Chroococcales) and II (Pleurocapsales) are unicellular coccoids, cells from organisms on subsection 1 divide by binary fission, whereas those from subsection 2 undergo multiple fission producing baeocytes. Cyanobacteria classified in subsections 3, 4 and 5 form filaments of variable morphological diversity. Subsection 3 (Oscillatoriales) have exclusively vegetative cells, but in subsection 4 (Nostocales) and 5 (Stigonematales), cells can differentiate into heterocysts that are cells specialized in nitrogen fixation under aerobic conditions, or akinetes that have the function of dormant cells in order to survive adverse environmental conditions. Subsection 5 have complex branching patterns and are included among the most highly developed prokaryotes. Molecular phylogeny has become a powerful tool in clarifying evolutionary pattern and 16S rRNA sequences show that cyanobacteria from subsections 2, 4 and 5 are phylogenetically coherent. In other hand phylogenies reconstructed by using nifH and nifD do not support monophyly of subsection 5; the nifH phylogeny also indicates paraphyly of subsection 2. Furthermore, direct evidence from geologic records is required in order to constrain actual divergence times since molecular phylogenies show only relative timing of diversification events (Tomitani *et al*, 2006).

1.2 Nitrogenase

Diazotrophic organisms have this ability due to the presence and function of an enzyme system known as Nitrogenase. Its activity is crucial in supporting the nitrogen cycle functioning across earth ecosystems; fixed nitrogen is often the limiting nutrient for growth of living organisms (Postgate, 1998). The enzyme system is divided into two distinct proteins: Dinitrogenase, a tetramer formed by two pairs of different subunits, each pair designated as α and β, being the Mo-Fe cofactor an essential component that binds and reduces dinitrogen ($N_2 + 6H^+ + 6e^- \rightarrow 2NH_3$) among other substances such as; acetylene, hydrogen azide, hydrogen cyanide, or nitrous oxide. Acetylene, which is reduced to ethylene, has a particularly importance because both acetylene and ethylene can be detected conveniently and with great accuracy by gas chromatography; the acetylene reduction assay (ARA) is often used to estimate nitrogenase activity (Fay, 1992). Dinitrogenase reductase supplies electrons for the

reduction of N_2, the reaction is highly endergonic, and needs about 12 to 15 mol of ATP per mol of N_2 reduced. Dinitrogenase reductase is also known as Fe protein which functions as electron conduit, one at a time, to dinitrogenase. Dinitrogenase reductase can be reduced by ferredoxin, or flavodoxin under conditions of iron deficiency, and it binds MgATP lowering its potential in 500 mV, allowing the electron transfer to dinitrogenase together with the hydrolysis of MgATP to MgADP and Pi. A single electron is transferred for each 2MgATP hydrolyzed, but the cycle is repeated until the dinitrogenase has accumulated enough electrons to reduce N_2. The overall reaction is $N_2 + 8H^+ + 8e^- \rightarrow 2NH_3+H_2$. Since 2MgATP are used for the transfer of one electron there is at least the requirement for 16MgATP under ideal conditions that translates into 20-30 under normal physiological conditions (Zumft and Mortenson, 1975; Burris, 1991). The product of nitrogen fixation ammonia (NH_3) is mainly assimilated via the glutamine synthetase-glutamate pathway the same as exogenously supplied ammonia. Nitrogen-fixing organisms preferentially assimilate ammonia or other forms of fixed nitrogen (nitrate, urea, amino acids, etc.), and synthesize nitrogenase only when such sources of combined nitrogen are absent; furthermore, the supply of ammonia may even rapidly inhibit nitrogenase activity. Ammonia in all nitrogen-fixing organisms is primarily involved through glutamine synthetase in the regulation at transcriptional level of nitrogenase synthesis (Shanmugam *et al*, 1978 Reich and Boeger, 1989; Fay, 1992).

Atmospheric oxygen has an inhibitory effect on nitrogenase fixation ability; this claim was formulated especially due to the fact that nitrogen fixation was observed in obligate anaerobic and facultative organisms in the absence of oxygen, or at very low concentrations (Parker and Scutt, 1960).

Bergersen, 1965 provided new evidence supporting the initial findings by demonstrating nitrogen fixation as an essentially reductive process in which ammonia is the main product; other works, around that period and earlier, showed that various nitrogen fixing organisms were only able to synthesize nitrogenase under anaerobic or microaerobic conditions while still growing in an aerobic environment if a suitable nitrogen source was provided (Pengra and Wilson, 1938; Hino and Wilson, 1958; Grau and Wilson, 1963; Klucas, 1972). Proof that oxygen inhibits nitrogenase activity was also found on aerobic *Azotobacter* species when Burk, 1930 showed a 10 to 20 fold increase of N_2 fixed per O_2 consumed when partial pressure of O_2 dropped from 21.27 kPa (partial atmospheric O_2 pressure) to 1.01 kPa. Despite the fact that *Azotobacter* spp. was known to rely on oxidative metabolism in support of nitrogen

fixation; the results when plotting nitrogenase activity against decreased O_2 pressure, suggested that fixation rates are suboptimal under ambient atmospheric pressure values (Burk, 1930; Dalton and Postgate, 1969). Furthermore, direct proof on nitrogenase system oxygen sensitivity was found in studies with purified enzyme preparations where destruction when in O_2 presence took place regardless of the anaerobic or aerobic origins (Carnahan *et al*, 1960; Bulen *et al,* 1965; Bulen and LeComte, 1966).

However it was proved later, that various strains can fix N_2 aerobically and over the last years various studies objectified the non-heterocystous cyanobacteria strains that possess the ability to sustain both oxygenic photosynthesis and the O_2 sensitive process of N_2 fixation in the same cell. The efforts aimed towards, not only, the better understanding on the mechanisms that protect nitrogenase from inactivation by atmospheric and photosynthetic O_2, but also at the way in which photosynthesis and respiration generate the energy (ATP) needed to sustain N_2 fixation. In order to meet these high N2 fixation energetic demands (16 ATP per mol of N_2) cyanobacteria have to overcome the incompatibility resulting from being phototrophic organisms (Fay, 1992).

Almost all of non-heterocistous diazotrophic cyanobacteria fix N_2 exclusively under anaerobic conditions, although some types were able to overcome O_2 presence by implementing strategies such as temporal or spatial separation (Bergman *et al*., 1997). A well-known aerobic diazotrophic cyanobacterium *Trichodesmium* spp. actually combines both strategies in order to separate the two antagonistic processes, photosynthesis from N_2 fixation. It is believed that the filamentous non-heterocyst *Trichodesmium* is the most abundant N_2 fixing microorganism in the open ocean; another important feature of these cyanobacteria is its ability to generate gas vacuoles that can regulate buoyancy allowing vertical migration thus optimizing light intensity and accessing nutrients, such as iron and phosphorus, from deeper waters. Other N_2 fixers have as well this ability like diatoms that are in symbiosis with cyanobacteria. Buoyancy and depth control are in fact rare abilities for most diazotrophic organisms, but are very helpful on providing the ideal conditions for nitrogenase activity (Capone *et al*., 2005; LaRoche and Breitbarth, 2005; Berman-Frank *et al*., 2007; Zehr, 2010).

Respiration is crucial not only in rendering the N_2 fixing cyanobacteria cells anoxic but also in providing part of the energy demands of nitrogenase, light is also regarded as an important source of energy for N_2 fixation in cyanobacteria. The importance of light

is proven by the light-response curve recordings of nitrogenase activity; these are similar light-response recordings on photosynthesis. These studies yield important information on the physiological status of the N_2 fixing cyanobacteria and their adaptation to different light conditions and cycle lengths. Light is absorbed by photosynthetic pigments and its quality influences N_2 fixation in cyanobacteria (Staal *et al.,* 2002; Staal *et al.*, 2003).

Studies revealed that strains of *Synechococcus* sp. fix nitrogen continuously under constant light. Nitrogen is fixed mainly during the dark periods when the culture is switched to light/dark cycles (Huang & Chow, 1986). Ambient CO_2 concentration will increase the rate of CO_2 assimilation but consequently decrease nitrogenase activity due to competition for the energy and reducing power required for both reactions (Grobbelaar *et al.,* 1992). Nitrogenase activity of a constant light culture is drastically reduced by CO_2 concentrations higher than 1% which is uncommon among the majority of cyanobacteria. Further studies showed also that *Synechococcus* culture fix nitrogen and take up leucine when adapted to continuous illumination under aerobic conditions. When the arrhythmic culture is entrained by a suitable diurnal Light/Dark or temperature regimen, the culture exhibits a circadian nitrogenase activity and leucine uptake rhythm, but the peaks are about 12 h out of phase compared to the nitrogenase activity peaks. A close relationship between nitrogenase activity and the dark respiration is established in *Synechococcus* sp. Dark respiration increased a few hours before the increase in nitrogenase activity after the establishment of a circadian rhythm. When nitrogenase is repressed by nitrate, an increase in the dark respiration rate corresponding to that observed for the nitrate-free treatment could not be detected, therefore there does not appear to be a fixed relationship between the peaks of the nitrogenase activity and the increase in the respiration rate. The results show that the increase in the dark respiration rate is not directly controlled by the clock because nitrate does not prevent the establishment of the leucine uptake rhythm dynamic (Chen *et al.*, 1989; Chen *et al*., 1991; Grobbelaar *et al*, 1991; Huang *et al*., 1994). Increased respiration is a reaction coupled to nitrogen fixation. Since nitrogenase is O_2 labile, the increase in the respiration rate is suggested to be essential for the protection of the nitrogenase activity in the *Synechococcus* strain (Grobbelaar et al., 1987). Calcium ions are equally important, as its presence in the medium is essential for nitrogenase activity as well as for the increase of the dark respiration rate. The nitrogenase activity is inhibited rapidly if the calcium ions are made unavailable. This inhibition can be completely abolished by a replenishment of

the calcium (Chen *et al*., 1988; Chen *et al*., 1989). By inhibiting the nitrogenase activity and then reversing the inhibition by adding a calcium ion supplement, one nitrogenase activity peak can be selectively delayed for several hours, without affecting the position in time of the subsequent nitrogenase activity peaks (Huang *et al*., 1990). The results indicate that the circadian rhythm for nitrogenase activity is different from an ordinary feedback reaction. *In vivo* requirement for calcium ions is interesting because calcium ions are not required for nitrogenase activity in vitro. When O_2 in the gas phase above the culture is below 1%, the inhibition of the nitrogenase activity is reduced to less than 20% of the control value. The results indicate that calcium ions are essential for nitrogenase activity only when the culture is growing aerobically. It has been suggested that calcium ions might have the function to protect the nitrogenase in the cell from O_2 inactivation (Chen et al., 1988). Studies on the effects of calcium ions on the nif gene expression indicate that the role of extracellular calcium ions is not at a transcription level (Huang & Chow, 1990). The circadian rhythm can also be disrupted and even reset by changing the diurnal Light/Dark phases or by exposing the culture to a pulse of low temperature (Chen *et al*., 1991; Huang *et al*., 1991; Grobbelaar & Huang, 1992).

Nitrogenase complex is encoded by structural nif genes; nifK and nifD that code for subunit α and β and nifH linked to dinitrogenase reductase. However, these are not active until modified by the action of other gene products (like flavodoxin or pyruvate flavodoxin oxidoreductase) are required. The nucleotide sequence for dinitrogenase reductase has been established for each of the structural genes, nifH, vnfH and anfH. This may vary depending on the type of nitrogenases, a major difference is that some nitrogenases carry a third subunit, 6, which is coded for by vnfG and anfG, rather than only two subunits characteristic of other dinitrogenases (Dixon and Kahn, 2004).

Reconciling nitrogen fixation with O_2 presence is also challenging at a genetics expression level as transcriptional regulation of nif genes in diazotrophs is tightly dependent in response to external O_2 concentration. In addition, nitrogenase activity is also subject to posttranslational regulation in some cases. ADP-ribosylation of the Fe protein of nitrogenase by dinitrogenase reductase ADP-ribosyltransferase, inactivates the nitrogenase in response to ammonium and light intensity. Covalent modification of the nitrogenase complex can be reversed by DraG (dinitrogenase reductase activating glycohydrolase), which removes the ADP–ribose moiety under favorable conditions to nitrogen fixation (Halbleib et al., 2000).

Biological N_2 fixation is a particularly problematic to biochemists due to the fact that N_2 reduction requires the collaboration of the two enzymes (dinitrogenase and dinitrogenase reductase), and in addition involves a high and unusual heavy energetic demand operating on a very stable substrate, N_2. The fact that this process, as happens with photosynthesis, is crucial to the survival of life on the planet made its study a center of attention for biochemists during decades, but thanks to the advances on molecular genetics, some advances on the understanding of this process and its complex relations continue to be accomplished.

When in oligotrophic waters where nitrogen levels are scarce, such as coral reefs environments, microorganisms may contribute to the nitrogen budget of their symbiotic host by fixation of atmospheric nitrogen. In the case of marine sponges the first evidence came from a study performed by Wilkinson and Fay, 1979 with measurements of nitrogenase activity in three Red Sea sponges. Activity was estimated using ARA and could be measured only in *Siphonochalina tabernacula* and *Theonella swinhoei*, both of which contained cyanobacteria. In contrast, *Inodes erecta*, which did not contained cyanobacteria, showed no evidence of N_2 fixation. Additionally, nitrogenase activity was higher in illuminated tissue than in the one maintained in dark and did not correlate with the abundance of the heterotrophic bacterial communities in *S. tabernacula* and *T. swinhoei*. These data suggested that nitrogenase activity was due mainly to the presence of cyanobacteria (Wilkinson and Fay, 1979). Supporting these facts, a later study provided more concrete proof of N_2 fixation in sponges by demonstrating incorporation of the stable isotope $15N_2$ into various amino acids in *Callyspongia muricina* (Wilkinson *et al*, 1999). Whether microbial N fixation is of major ecological significance for sponges remains uncertain, but it does appear that its occurrence in sponges might not be limited to cyanobacteria, as other nitrogen-fixing organisms have been isolated form sponges (Shieh and Lin, 1994).

In this study, we focus on cyanobacteria isolated from different sea sponges of the Portuguese coast analyzing them, in order to assess their capacity to fix nitrogen.

2. Materials and methods

2.1 Sample collection and preparation

The cyanobacteria included in this study (Table 1) were isolated from sponges collected during September 2010 and 2011 from various intertidal sites (Figure 1) during the lowest tide hours of the month (under 0.5 m of the sea level) at the Portuguese coast, except *Pseudophormidium* (LEGE 11386) that was collected at a location 600 meters offshore during a 14 meter depth dive. Sponges were identified based on shape, consistency, texture, colour, smell and habitat and spicules morphology and dimensions. All sponge species were confirmed according to Hooper and Van Soest (2002) (Regueiras *et al*, 2013).

1	Praia de S. Bartolomeu do Mar	41°34'25.59"N 8°47'54.81"W	LEGE 10374, LEGE 10375, LEGE 10372
2	Praia das Angeiras	41°15'50.01"N 8°43'37.14"W	LEGE 10371
3	Praia da Memória	41°13'50.96"N 8°43'18.09"W	LEGE 11381, LEGE 11382, LEGE 11383, LEGE 11384,
4	Porto de Leixões (Offshore)	41°11'8.04"N 8°43'8.76"W	LEGE 11386
5	Praia da Aguda	41°02'52.13"N 8°39'13.19"W	LEGE 10377, LEGE 10379
6	Porto Côvo	37°53'33.19"N 8°47'38.25"W	LEGE 10376

Figure 1. Location and identification of the sampling points in Portuguese coast.

Cyanobacterial cultures were maintained using the following media: MN (Rippka, 1998), BG11 (Stainer *et al*, 1071), and Z8 (Kotai, 1972) supplemented with 25 g L^{-1} NaCl. Media were supplemented with B12 vitamin, and when necessary with cycloheximide (Rippka, 1998). The cultures were kept under 14h light (10–30 µmol photons m^{-2} s^{-1})/10h dark cycles at 25 ◦C. Cyanobacterial isolates are deposited at LEGE Culture Collection (Laboratório de Ecotoxicologia, Genómica e Evolução; CIIMAR, Porto, Portugal).

Table 1- Cyanobacterial strains identification and respective source sponge.

Cyanobacterial strain (sampling site)*	Culture Media	LEGE reference	Source Sponge	GenBank accession nº (16S rDNA)
Synechococcus sp. (1)	Z8	LEGE 10372	*Hymeniacidon perlevis*	JQ927346
Synechococcus sp. (1)	Z8	LEGE 10374	*Halichondria panicea*	not in ncbi
Synechococcus sp. (1)	Z8	LEGE 10375	*Halichondria panicea*	JQ927347
Synechococcus sp. (5)	Z8	LEGE 10379	unidentified	not in ncbi
Synechococcus sp. (3)	Z8	LEGE 11381	*Polymastia penicillus*	JQ927352
Synechococcus sp. (3)	Z8	LEGE 11382	*Hymeniacidon perlevis*	JQ927353
Pseudanabaena cf. *curta* (2)	Z8	LEGE 10371	*Hymeniacidon perlevis*	JQ927345
Pseudanabaena sp. (3)	MN	LEGE 11383	*Stelligera rigida*	JQ927354
Nodosilinea cf. nodulosa (4)	Z8	LEGE 10376	*Hymeniacidon perlevis*	JQ927348
Nodosilinea cf. nodulosa (5)	Z8	LEGE 10377	*Halichondria panicea*	JQ927349
Phormidium sp. (3)	MN	LEGE 11384	*Phorbas plumosus*	JQ927355
Pseudophormidium sp. (6)	BG11	LEGE 11386	*Myxilla rosacea*	not in ncbi

***1-** S. Bartolomeu do Mar, **2-** Angeiras, **3-** Memória, **4-** Porto de Leixões, **5-** Aguda, **6-** Porto Côvo (see also Fig. 1).

2.2 DNA extraction, purification, PCRs and sequencing

Cell harvest was achieved by centrifugation and DNA was extracted using the Maxwell® 16 System (Promega Corporation, Madison, WI, USA) following the instructions of the manufacturer.

PCR DNA fragments amplification within the 2 genes (*nifK*, *nifH)* was performed using the oligonucleotide primers listed on Table (2). Amplification of *nifH*, by nested PCR, was performed using the primer pair nifH3/nifH4 followed by the primer pair nifH1/nifH2 (Omoregie *et al,*, 2004), and amplification of *nifK* was achieved using the primer pair nifk01F/nifk3´R (Brito *et al.*, 2012).

PCR was carried out in a thermal cycler MyCycler (Bio-Rad laboratories PCR MyCyclerTM thermal cycler (Bio-Rad laboratories Inc., Hercules, CA, USA) using the methodology described by Tamagnini *et al.*, 1997. The PCR profiles, after a denaturation step of 5 min at 94 ◦C, were the following:

- *nifK* – 35 cycles of 94 ◦C 1 min, 50 ºC 1 min, and 72 ºC for 7 min;
- *nifH* – 30 cycles of 94◦ C 1 min, 57 ºC 1 min, and 72 ºC 1 min;

In all cases a final extension of 7 min at 72 ºC followed.

The PCR products were separated by agarose gel electrophoresis using standard protocols described in Sambrook and Russell (2001).

Isolation of DNA fragments from gels was carried out using the NZYGelpure Kit (NZYtech, Lda. INOVISA, Lisbon, Portugal), according to the manufacturer's instructions. The purified PCR products were cloned into pGEM®-T Easy vector (Promega, Madison, WI, USA), and transformed into *Escherichia coli* DH5α competent cells following the manufacturer's instructions, and the methodology described in Ramos *et al.* (2010). The DNA fragments were sequenced at STAB Vida (Lisbon, Portugal).

Table 2- Target genes and oligonucleotide primers used in this study.

Target Genes	Primers	Sequence 5'→3'	References
nifK	nifk01F	CAAGGTTCTCAAGGTTGTGTG	Brito *et al.* (2012)
	nifk3'R	GGGATGAAGTTGATTTTGCCGTT	
nifH	nifH3	ATRTTRTTNGCNGCRTA	Omoregie *et al.* (2004)
	nifH4	TTYTAYGGNAARGGNGG	
	nifH1	TGYGAYCCNAARGCNGA	
	nifH2	ADNGCCATCATYTCNCC	

2.3 Phylogenetic analysis

In order to integrate the cyanobacteria isolates into a broader phylogenetic context their 16S rRNA gene sequences were compared with the ones currently available in the databases and used to construct phylogenetic trees. Each sequence was independently used as the query in a BLAST search against the non-redundant nucleotide database of the National Centre for Biotechnology Information (NCBI, July 2013) and the closest sequences (ordered by their e-value) were retrieved. Six reference strains (selected according Bergey's Manual of Systematic Bacteriology, 2001) were also included. A multiple alignment encompassing 16S rRNA gene

sequences mentioned above was performed using the ClustalW algorithm, included in the Geneious R6 package (Drummond, Ashton *et al*. 2011), with all the default parameters. To avoid bias in the analysis introduced by the different sizes of the sequences, the alignment was trimmed to a common core of 902 nucleotides. A Maximum-likelihood (ML) (Guindon and Gascuel, 2003) phylogenetic tree was computed from the alignment using the MEGA 5 software (Tamura, Peterson *et al.* 2011), excluding all positions with gaps. Kimura 2-parameter (Kimura 1980) was used as the substitution model, allowing the software to estimate the transition/transversion ratio and the gamma distribution parameter with 5 substitution rate categories and a fraction of invariant sites. For statistical purposes a bootstrap analysis was made considering 1000 pseudo-replicates.

2.4 Nitrogenase activity

For the measurement of the *in vivo* nitrogenase activity by the acetylene reduction assay, the cultures were grown in $BG11_0$-medium (Stainer *et al*, 1071) under 12h light (20-30 µmol photons m^{-2} s^{-1})/12h dark cycles at 25 ◦C for 24h. 10 mL of each culture where transferred to Erlenmeyer flasks with a total volume of 135 ml and incubated with 12,8% C_2H_2 for 24h in sealed flasks. Afterwards, 1ml gas samples were withdrawn to determine the amount of acethylene reduced to ethylene. The measurements were performed using the gas chromatograph Clarus 480 (Perkin Elmer), equiped with a Thermal Conductivity Detector (TDC) and a capillary Porapak N 80/100 column, under the control of theTotalChrom® software (Perkin Elmer). The nitrogenase activity was expressed per chlorophyll *a* and time. Chlorophyll *a* content was determined as described by Meeks and Castenholz, 1971. For positive and negative controls, cells of *Nostoc punctiforme* PCC 73102 grown in $BG11_0$ or $BG11_0$ supplemented with ammonium chloride were used (Oxelfelt et al. 1995).

3. Results and discussion

In this work 12 cyanobacterial strains, previously isolated from marine sponges collected at several sites at the portuguese Atlantic coast and deposited at LEGE culture collection, were studied. To begin with**,** phylogenetic analyses were performed to assess the relative positioning of the isolates. An ML algorithm was applied to a multiple aligment of partial sequences of 16S rRNA gene (902 bp) of our isolates, other cyanobacterial close sequences and reference cyanobacterial strains. The phylogenetic tree revealed 3 distinct clusters A, B, C (Fig. 2) with bootstrap values equal or higher than 73%. **Cluster A** contains unicellular strains belonging to Subsection I (Chroococcales), including 6 of our isolates identified as *Synechococcus*, the reference strain *Cyanobium* sp. PCC 7001, several other isolates from Portuguese waters (LEGE) (Regueiras et al, 2013), as well as marine *Synechococcus* (HOS) and *Cyanobium* (NS01) strains. **Cluster B** contains mainly strains belonging to Subsection III (Oscillatoriales) with the exception of *Synechococcus* sp. PCC 7335 that has been previously reported to group with filamentous cyanobacteria (Wilmotte and Herdman, 2001; Brito *et al*, 2012). This cluster includes the isolates *Phormidium* sp. LEGE 11384 and the two *Nodosilinea* strains (LEGE 10376 and LEGE 10377), and these two strains constitute a sub-cluster together with others recently re-classified as *Nodosilinea*. It is interesting to notice that several *Leptolyngbya* isolated from estuarine Portuguese waters are also part of this cluster. **Cluster C** is more heterogeneous since includes cyanobacterial strains belonging to Subsection III (Oscillatoriales) and Subsection IV (Nostocales), and it contains 3 of our isolates: *Pseudophormidium* sp. LEGE 11386, *Pseudanabaena* cf. *curta* LEGE 10371, and *Pseudanabaena* sp. LEGE 11383. Our *Pseudanabaena* strains group together with three isolates from sponge-coral reefs.

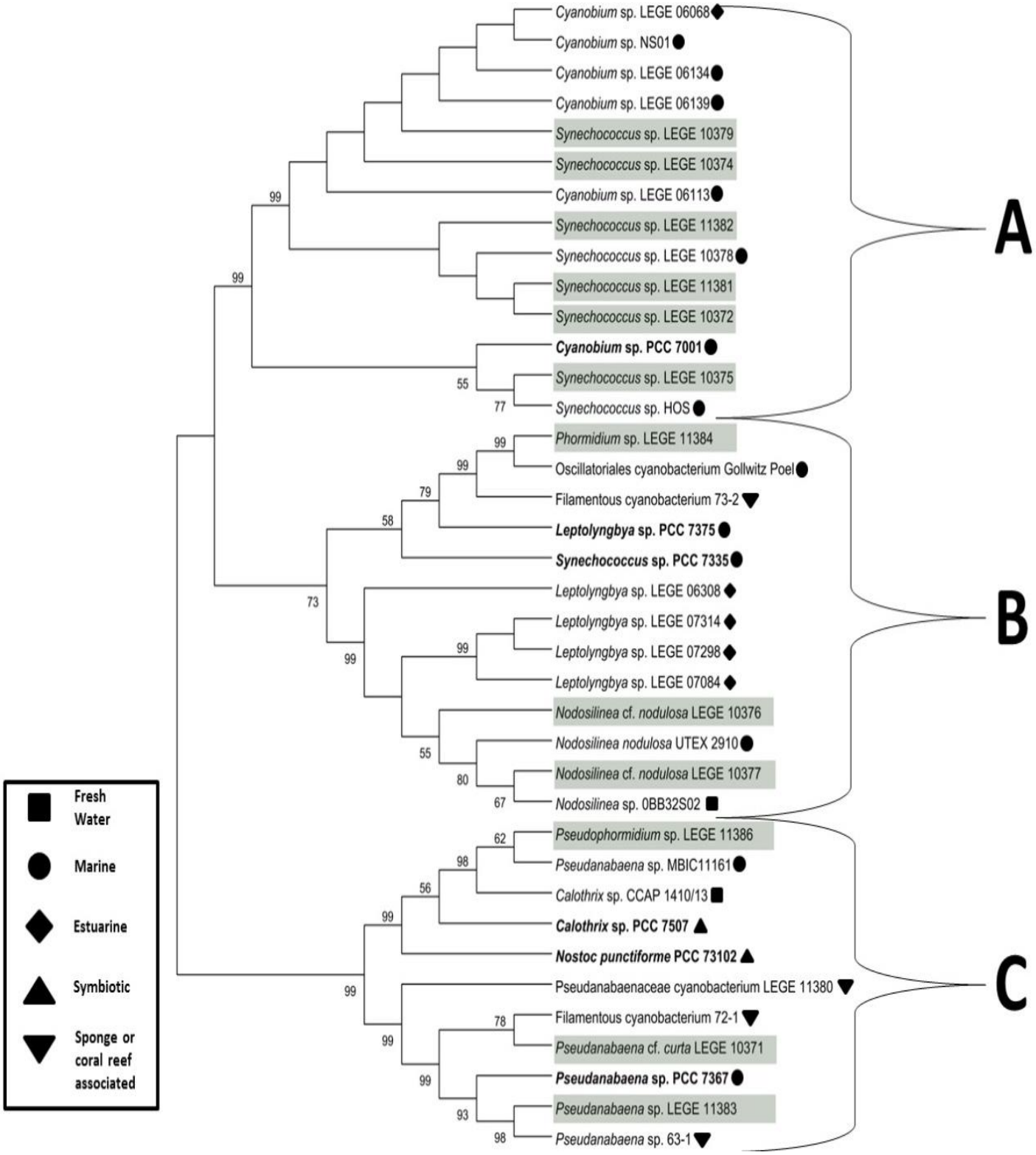

Figure 2. Maximum-Likelihood phylogenetic tree of partial 16S rRNA gene sequences from the sponge isolates, closest sequences (ordered by e-value) and reference cyanobacterial strains. Numbers along branches indicate the percentage of bootstrap support considering 1000 pseudo-replicates: only those equal or higher than 50% are indicated. Cyanobacteria isolated within this study are highlighted in grey, whereas reference strains are indicated in bold.

To evaluate the potential of the cyanobacterial isolates to fix nitrogen, a PCR screening for the presence of genes encoding the dinitrogenase reductase (*nifH*) the dinitrogenase β subunit (*nifK*) was performed. Under the conditions tested: second PCR anneling temperature 57ºC (for more details see Material and Methods), the presence of *nifH* was detected in 3 isolates, namely *Synechococcus* sp. (LEGE 11382), *Pseudoanabaena* sp. (LEGE 11383) and *Pseudophormidium* sp. (LEGE 11386) (Figure 3, lines 9, 10 and 12). Similar results were obtained when the anneling temperature of the second PCR was changed to 55 ºC or 59 ºC (data not shown).

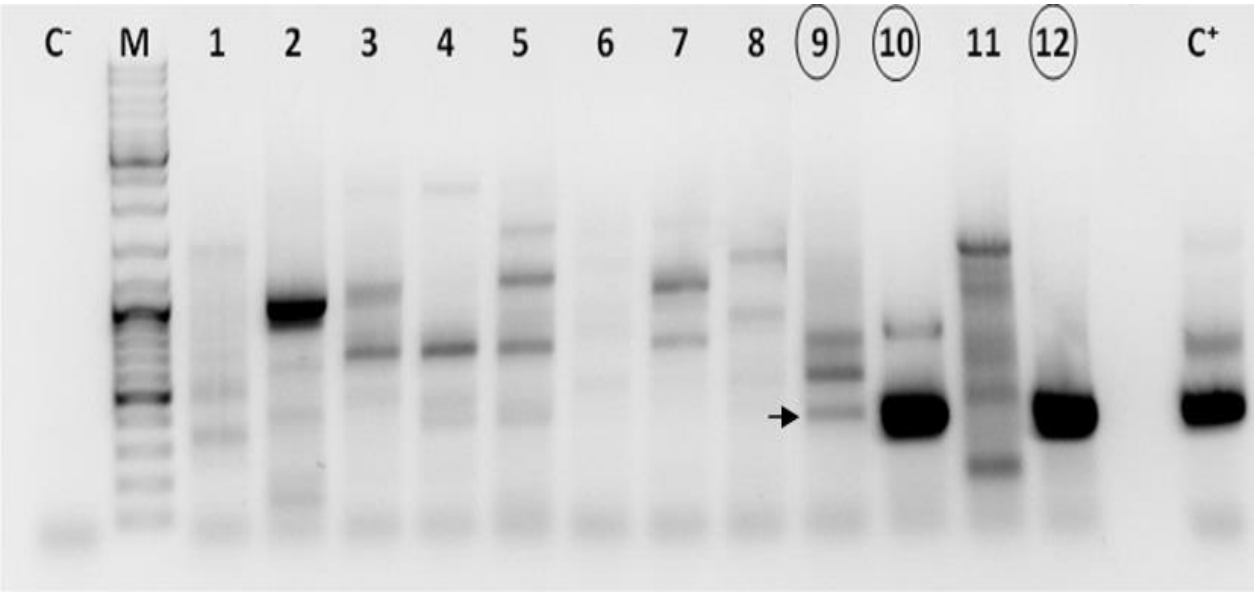

Figure 3 – PCR amplification of a 361 bp DNA fragment within *nifH***,** gene encoding the dinitrogenase reductase and using DNA from the following cyanobacterial isolates: **1-** *Pseudoanabaena* cf. *curta* (LEGE 10371), **2-** *Synechococcus* sp. (LEGE 10374), **3-** *Synechococcus* sp. (LEGE 10375), **4-** *Synechococcus* sp. (LEGE 10372), **5-** *Nodosilinea* cf. *nodulosa* (LEGE 10376), **6-** *Nodosilinea* cf. *nodulosa* (LEGE 10377), **7-** *Synechococcus* sp. (LEGE 10379), **8-** *Synechococcus* sp. (LEGE 11381), **9-** *Synechococcus* sp. (LEGE 11382), **10-** *Pseudoanabaena* sp. (LEGE 11383), **11-** *Phormidium* sp. (LEGE 11384), **12-** *Pseudophormidium* sp. (LEGE 11386). **C⁻-** negative control (DNA replaced by water), **C⁺-** positive control (using DNA from LEGE 06123 – see Ramos *et al.*, 2010), **M-** GeneRuler DNA Ladder Mix (Thermo Scientific).

The presence of nifK was only detected in *Pseudophormidium* sp. (LEGE 11386) (Figure 4, line 12). Similar results were obtained with an annealing temperature of 52ºC and a different primer pair (data not shown). It is important to notice that the primer pairs used to amplify fragments within nifK are more specific than the ones used for nifH.

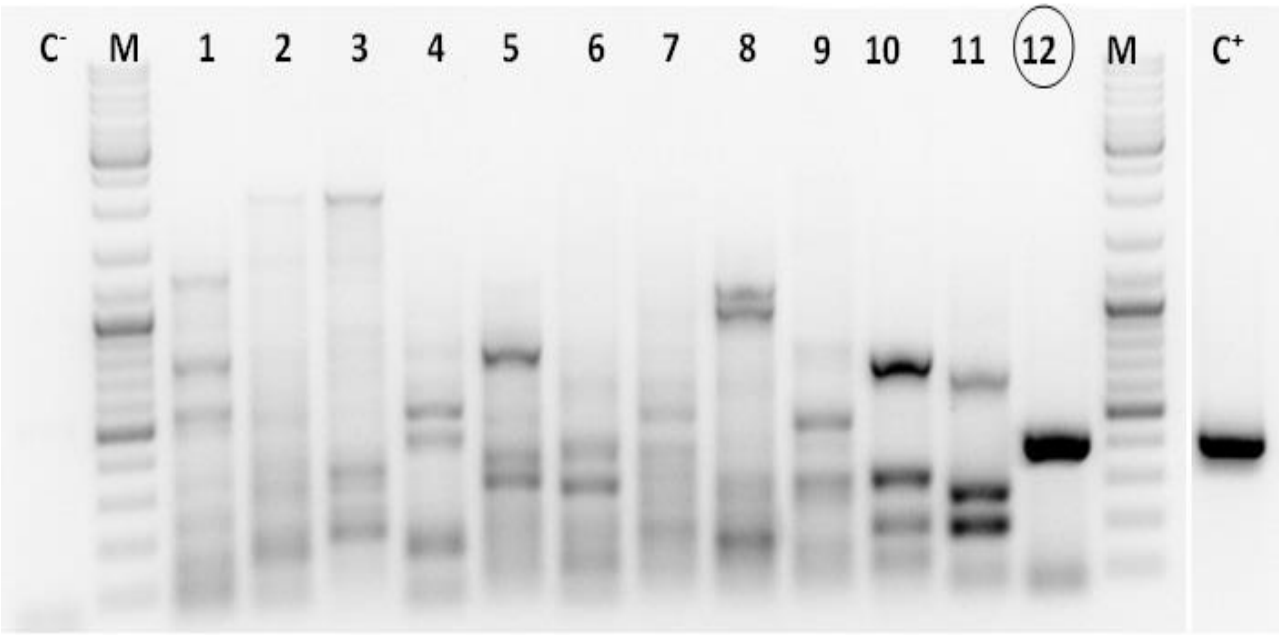

Figure 4 - PCR amplification of a 407 bp DNA fragment within nifK, gene encoding the nitrogenase β subunit, and using DNA from the following cyanobacterial isolates: 1- Pseudoanabaena cf. curta (LEGE 10371), 2- Synechococcus sp. (LEGE 10374), 3- Synechococcus sp. (LEGE 10375), 4- Synechococcus sp. (LEGE 10372), 5- Nodosilinea cf. nodulosa (LEGE 10376), 6- Nodosilinea cf. nodulosa (LEGE 10377), 7- Synechococcus sp. (LEGE 10379), 8- Synechococcus sp. (LEGE 11381), 9- Synechococcus sp. (LEGE 11382), 10- Pseudoanabaena sp. (LEGE 11383), 11- Phormidium sp. (LEGE 11384), 12- Pseudophormidium sp. (LEGE 11386). C-- negative control (DNA replaced by water), C+- positive control (using DNA from LEGE 06123 – see Ramos et al., 2010), M- GeneRuler DNA Ladder Mix (Thermo Scientific).

The *nif* genes sequences were compared against the NCBI database (July 2013) and the results are shown in table 3. These results are not conclusive since there no close relatives in the databases, as it can be perceived by the maximum identity lower than 97%.

Table 3- Molecular analysis using partial sequences of *nifH* and *nifK* of 3 cyanobacterial strains.

Cyanobacterial strain	Gene	Best hits indicated by BLASTn	% Max. Identity
Synechococcus sp. LEGE 11382	*nifH*	Uncultured marine bacterium clone A11-0M-26	96
Pseudanabaena sp. LEGE 11383	*nifH*	Uncultured bacterium clone L2C_20 *Nostoc punctiforme* PCC 73102	90 90
Pseudophormidium sp. LEGE 11386	*nifH*	*Nostoc punctiforme* PCC 73102	90
Pseudophormidium sp. LEGE 11386	*nifK*	*Nodularia sphaerocarpa* PCC 7804	94

Since a negative PCR result does not exclude the presence of the gene, and the presence of the gene does not translate into expression and activity of the enzyme, the cyanobacterial isolates were gradually cultivated in media with decreasing concentrations of combined nitrogen until being transferred to $BG11_0$ medium ($NaNO_3$ free medium). The only culture able to grow prolifically in $BG11_0$ medium was *Pseudophormidium* sp. LEGE 11386, interestingly the only one for which both *nifH* and *nifK* genes were detected. This result strongly indicates that this strain fixes nitrogen. However, it is important to keep in mind that other factors such as growth media specific components and environmental conditions can influence ability of cyanobacteria to grow and/or fix nitrogen, therefore one cannot exclude that *Synechococcus* sp. LEGE 11382, *Pseudanabaena* sp. LEGE 11383 and others can fix nitrogen in symbiosis (Bergman *et al*, 1997).

Table 4- *In vivo* nitrogenase activity assessed using the acetylene reduction assay.

Cyanobacterial Strain	nmol C_2H_4 µg chlorophyll $a^{-1}h^{-1}$
CTL (-)Nostoc punctiforme	**0,17**
CTL (+) Nostoc punctiforme	**7,90**
Synechococcus sp. (LEGE 10372)	**0,00**
Nodosilinea cf.*nodulosa* (LEGE 10376)	**0,23**
Nodosilinea cf. *nodulosa* (LEGE 10377)	**0,65**
Pseudophormidium sp. 1MC (LEGE 11386)	**4,61**

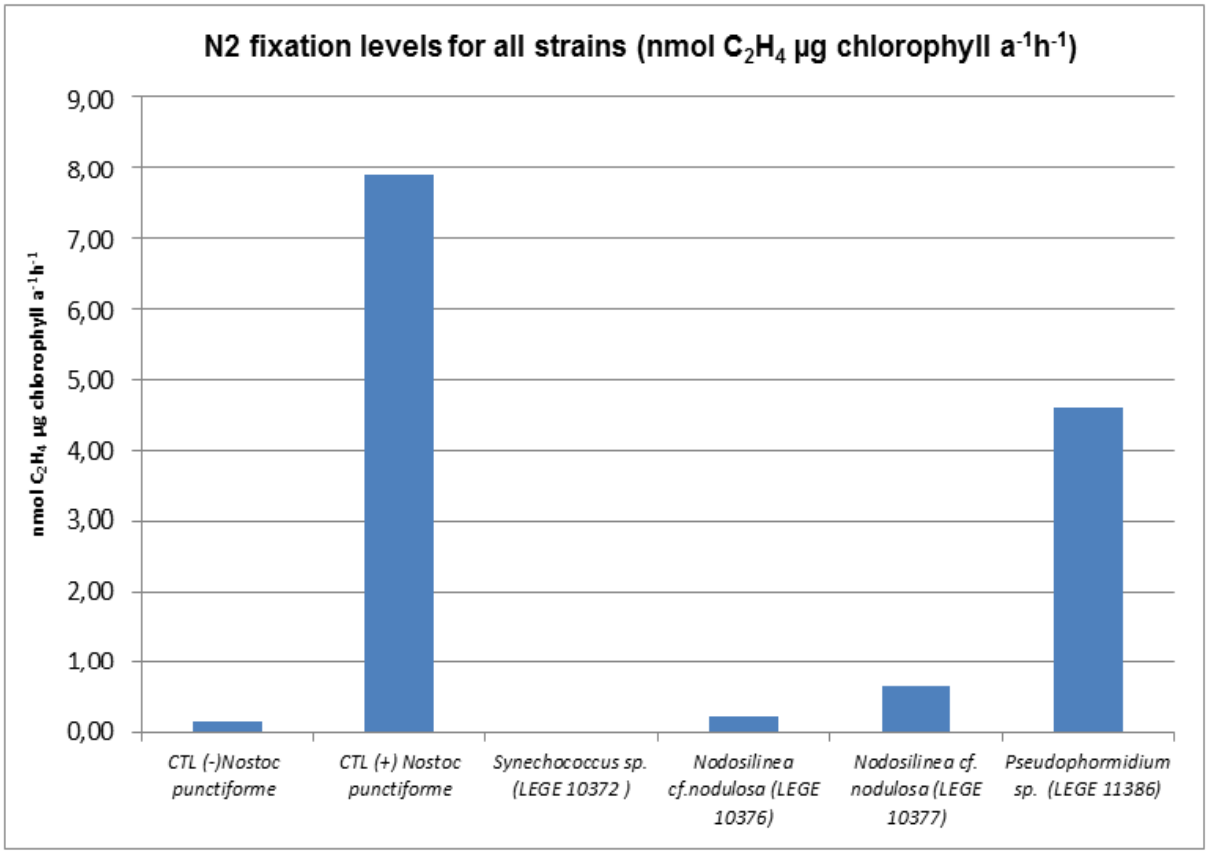

Figure 5 - N_2 fixation levels for all strains expressed in nmol C_2H_4 µg chlorophyll $a^{-1}h^{-1}$.

4. Conclusion

The *in vivo* nitrogenase activity was assessed using the acetylene reduction assay, and again, the only isolate for which a positive result was obtain was *Pseudophormidium* sp. LEGE 11386 (Table 4, Figure 5), inequivocally demonstrating that this isolate is able to fix nitrogen. Moreover, our results are in agreement with previous works that report similar values ranging from 1-30 nmoles of C_2H_4 µg chlorophyll a^{-1} h^{-1} for marine unicellular and filamentous cyanobacterial strains (Berman-Frank et al, 2001; Staal *et al,* 2007).

Pseudophormidium sp. (LEGE 11386) was the only strain obtained from 14 meter depths offshore and in fact proved to be quite more resilient, probably due to natural adaptations in order to survive in adverse environments with low O_2 and light exposure.

Further studies regarding N_2 fixation levels and notably using recently collected sponges/sponge tissues/isolated cyanobacteria are needed to clarify the role of the cyanobionts.

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